A molecular cytogenetic investigation of secondary abnormalities and clonal evolution in *ETV6-RUNX1* positive acute lymphoblastic leukaemia

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Declaration

I certify that no part of the material documented in this thesis has previously been submitted for a degree or other qualification in this or any other university. I declare that this thesis represents my own unaided work, carried out by myself, except where it is acknowledged otherwise in the thesis text.

Halima Al-Shehhi

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Dedication

This thesis is dedicated to the soul of my father who encouraged me to be the best I can be. I feel he is always with me supporting and guiding.

My incredibly wonderful mother has always believed and continues to believe in me. Her prayerful support, encouragement, and constant love have sustained me throughout my life.

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Abstract

The chromosomal translocation, t(12;21)(p13;q22), resulting in *ETV6-RUNX1* fusion gene, is the most common chromosomal abnormality in childhood B-cell precursor acute lymphoblastic leukaemia (BCP-ALL). It acts as an initiating factor which arises *in utero* but is unable to generate clinical leukaemia. Hence considerable research has been devoted to characterising the additional abnormalities that are acquired postnatally for the leukaemogenic transformation. This project investigated the spectrum of secondary abnormalities and clonal evolution in *ETV6-RUNX1* ALL using fluorescence in situ hybridization, multiplex ligation- dependent probe amplification, single nucleotide polymorphism arrays and copy number real time polymerase chain reaction. Abnormalities affecting transcriptional co-regulators (*ETV6*, *TBL1XR1* and *BTG1*) were much more common in *ETV6-RUNX1* ALL compared to other ALL cases (52% v 10%; 13% v 0.69%; 15% v 3%, respectively); while alterations affecting the *IKZF1* gene, which is involved in B-cell lymphocyte development pathway, were less prevalent (3% v 18%).

Interestingly, we have identified and characterised two new abnormalities which both target the der(12)t(12;21) chromosome and the *RUNX1-ETV6* fusion gene in 12% of *ETV6-RUNX1* cases. The first abnormality was a deletion of der(12)t(12;21) (8%) occurring on either sides of the translocation breakpoint, but never both sides simultaneously, and occurred in 100% of *ETV6-RUNX1* positive cells. The second abnormality was the duplication of *RUNX1-ETV6* fusion gene (4%) and occurred in an average of 55% of *ETV6-RUNX1* positive cells as a result of the formation of der(12) [der(12)(21qter → 21q22.12::12p13.2-12p12.3::12p12.3 → 12qter). Detailed investigation of these abnormalities identified potential tumor suppressor genes (*LRP6* and *BCL2L14*) and a role for the RUNX1-ETV6 fusion protein.

Although *ETV6-RUNX1* patients respond well to current therapy, some relapses do occur and the identification of prognostic biomarkers is required. No potential relapse driving genes were defined; however, *CDKN2A/B* losses showed around three fold increase in the relapse cases as compared to non-relapse cases. Analysis of 9 matched diagnostic-relapse samples revealed a high degree of clonal relatedness between the two points but also evidence of low level sub-clones at diagnosis emerging as the major clone at relapse.

Finally, the spectrum of secondary abnormalities in atypical *ETV6-RUNX1* cases (i.e. younger/older patients and Down syndrome) was different with respect to the genetic diversity, nature and frequencies of alterations targeting the following genes – *BTG1*, *RB1* and *CDKN2A/B*. Hence, considering the eight MLPA genes including *ETV6, CDKN2A/B, PAX5, BTG1, RB1, EBF1, IKZF1* and *PAR1*, infant cases showed fewer alterations compared to non-infant cases (0.67 v 1.3 alterations per case) and *CDKN2A/B* seemed not to be contributing in their leukaemogenic development. Both *BTG1* and *RB1* losses are commoner in adolescents and young adults (AYA) group as compared to those under the age of 10 years (33% and 14% v 21% and 7%, respectively).

In conclusion, this study has catalogued the frequency of key secondary abnormalities in *ETV6-RUNX1* ALL and identified a number of genes whose role in the pathogenesis of this subtype warrants further investigation.

List of abbreviations

ABL1	**The tyrosine kinase Abelson**
aCGH	**Array comparative genomic hybridisation**
ALL	**Acute lymphoblastic leukaemia**
BAC	**Bacterial artificial chromosomes**
BCP-ALL	**Precursor B-cell ALL**
BM	**Bone marrow**
bp	**Base pairs**
CNAs	**Copy number alterations**
CNN-LOH	**Copy number neutral loss of heterozygosity**
CNS	**Central nervous system**
CR	**Complete remission**
CRD	**Common region of deletion**
C_T	**Cycle threshold**
der(12)t(12;21)	**Derived chromosome 12**
der(21)t(12;21)	**Derived chromosome 21**
DGV	**Database of Genomic Variants**
DMC	**Differentially methylated CpG**
DS	**Down syndrome**
dup(Xq)	**Duplicated Xq**
EFS	**Event-free survival**

ETS	**E-26 transforming specific**
ETV6	**The *ETS- type variant 6***
FISH	**Fluorescent in situ hybridization**
GC	**Glucocorticoids**
HD	**Heterodimerization domain**
HeH	**High hyperdiploidy**
HLH	**Helix–loop–helix domain**
HR	**High-risk**
HSC	**Haematopoietic stem cells**
IGH	**The immunoglobulin heavy chain**
IR	**Intermediate risk**
MLL	**Mixed lineage leukaemia**
MLPA	**Multiplex ligation dependent probe amplification**
MRD	**Minimal residual disease**
O/N	**Overnight**
PAR1	**The pseudo-autosomal region**
PCR	**Polymerase chain reaction**
Ph	**Philadelphia chromosome**
RT	**Room temperature**
RUNX1	**Runt- related transcription factor 1**
SNP	**Single Nucleotide Polymorphism**
SR	**Standard risk**

T-ALL	**T cell acute lymphoblatic leukaemia**
TSG	**Tumour suppressor genes**
ΔC_T	**C_T difference**
$\Delta\Delta C_T$	**Comparative C_T**

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Chapter 1. Introduction

1.1 Cancer

1.1.1 Overview

Cancer is a heterogeneous collection of diseases that result from uncontrolled growth and invasion of cells owing to the presence of aberrant genes involved in various cellular processes including proliferation and apoptosis. Although germline genetics plays an important role in driving this abnormal growth and its spread either locally or to distant organs, environmental factors are considered as the mainstay of this genetic deregulation. More than 200 different types of cancer have been described and they differed in their genetic complexities and clinical pictures. Thus, cancer is one of the biggest fears in the worldwide public (CRUK, 2011).

1.1.2 Incidence

Cancer incidence in the developed countries has continued to increase. More than 1 in 3 people will develop cancer in the UK during their life; in spite of an increase awareness of the determinants of cancer and the reduction of the risky lifestyle factors (CRUK, 2011). A plausible explanation for this increase might be due to the increase in lifespan which increases the risk of exposure to environmental factors resulting in accumulating genetic aberrations responsible for developing cancer. Generally, males are at a higher risk of developing cancer as compared to females. Both sexes display different incidences and mortality rates of different types of cancer that also vary across the world. Males tend to develop prostate, lung and colorectal cancers, while breast, cervical, lung, colorectal cancers are the most common types in females. It is worth noting that cancer can develop at any age with higher risk in older age compared to children, adolescent and young adults (up to the age of 24 years) who make up less than 1% of cancers (CRUK, 2011).

1.1.3 Multistep development of cancer

The normal biological cell cycle is tightly controlled to ensure the homeostasis of cell numbers and the maintenance of tissue architecture. Transformation of normal cells to malignant cells is a multistep process in which these normal cells acquire certain characteristics including both genetic instability and inflammation that give them

specific capabilities which facilitate neoplastic formation (Hanahan and Weinberg, 2011). Thus the genome instability generates random genetic errors/ mutations, while the inflammation results from the innate immune cells that are normally responsible for fighting infections and healing wounds and released different growth/ survival factors. There are eight acquired capabilities constituting the hallmarks of cancer development namely: 1) production of their own extracellular growth factors (sustainability of the proliferative signalling), 2) insensitivity to anti-growth signals (uncontrolled division of cells), 3) resistance to apoptosis (further proliferation of cells), 4) limitless replicative potential (uncontrolled cell division by maintaining the telomere length), 5) sustained angiogenesis (source of required oxygen and nutrients), 6) tissue invasion and metastasis (abnormal interaction of cancer cells to the adjacent cells and extracellular matrix), 7) deregulation of cellular energetics (reprogramming of cellular metabolism) and 8) avoidance of immune destruction (invasion of the immune system). It is worth noting that these hallmarks are common for all different types of malignancies, but the acquisitioned distinct mechanisms and timing, within the cancer type itself and across all types of cancer, are crucial in the diversification of these neoplasms (Hanahan and Weinberg, 2011).

Understanding the principles of the different hallmarks of cancer sheds light on the development of new targeted therapy (Hanahan and Weinberg, 2011). Thus, each drug is specific to a particular molecular target that is involved in one way or another in the activation of certain capabilities, which are important for the biology of tumours, and accordingly, less non-specific toxicity would result. However, owing to the presence of other parallel pathways regulating that specific capability, these drugs were observed to have transient clinical response ending with relapses due to the partial shut down of that hallmark capability.

1.1.4 Genetic determinants of cancer

There are two classes of genes that are fundamental in cancer development when they are altered: oncogenes and tumour suppressor genes (TSG). Multiple environmental exposures to mutagenic factors (ultraviolet light, ionizing radiation and other chemical mutagens) and inherited cancer syndromes with heterozygous mutation of one allele

(vulnerable for further mutations in the other allele) are predisposing factors resulting in various genetic alterations.

Oncogenes are genes that activate cell transformation. They arise from proto-oncogenes, whose normal function is to regulate cell growth and differentiation, via activating mutations and/or increased expression. Examples of these genes are *RAS, c-MYC* or other chimeric genes *BCR-ABL1* whose activations promote tumourgenesis (Meyer and Penn, 2008; Goldman, 2010; Pylayeva-Gupta *et al.*, 2011). On the other hand, TSG encode for proteins that prevent a cell from progressing towards cancer in response to cellular stresses or DNA damage (Sherr, 2004). These genes can be altered by either inactivating mutations (recessive in nature) or deletions resulting in the deregulation of the cell growth. The two-hit hypothesis of TSG inactivation was proposed by Knudsen that arose of out his interest in retinoblastoma. He hypothesized that in order for the cell to become malignant, both alleles of the TSG must be mutated (Knudson, 1971). This hypothesis sheds light on one of the most important genetic determinants of cancer and helped in the identification of other several TSG. However, not all TSG follow the two hit hypothesis. In some cases loss of only a single copy of TSG results in haplo-insufficiency which can promote cancer as seen in *CDKN1B* gene (Le Toriellec *et al.*, 2008).

Other genetic alterations which inactivate DNA repair mechanisms are also important in neoplastic transformation. Inability to repair damaged DNA will lead to the generation of various mutations that resulted from errors in the base excision repair, homologous recombination and mismatch repair. In addition, gene expression can be affected by epigenetic alterations without any alterations in its genetic sequence and this can be achieved by different mechanisms including: DNA methylation and post translational histone modification. There is a growing body of literature that indicates the importance of epigenetic mechanisms as critical events in tumorigenesis (Jones and Laird, 1999; Jones and Baylin, 2002; Baylin and Jones, 2011).

1.2 Leukaemia development

Haematopoiesis is a highly complex process that is characterised by a hierarchical, stepwise development, which results in the ongoing generation of the cellular constituents of the blood and lymphoid organs. This process provides all blood cell types

which originate from haematopoietic stem cells (HSCs) that can proliferate and maintain the stem cell pool. There are two main subgroups of blood cells: the myeloid compartment, which comprises the macrophages, monocytes, granulocytes, mast cells, erythrocytes and thrombocytes, and the lymphoid compartment, which comprises natural killer cells, T-lymphocytes and B- lymphocytes (Figure 1.1).

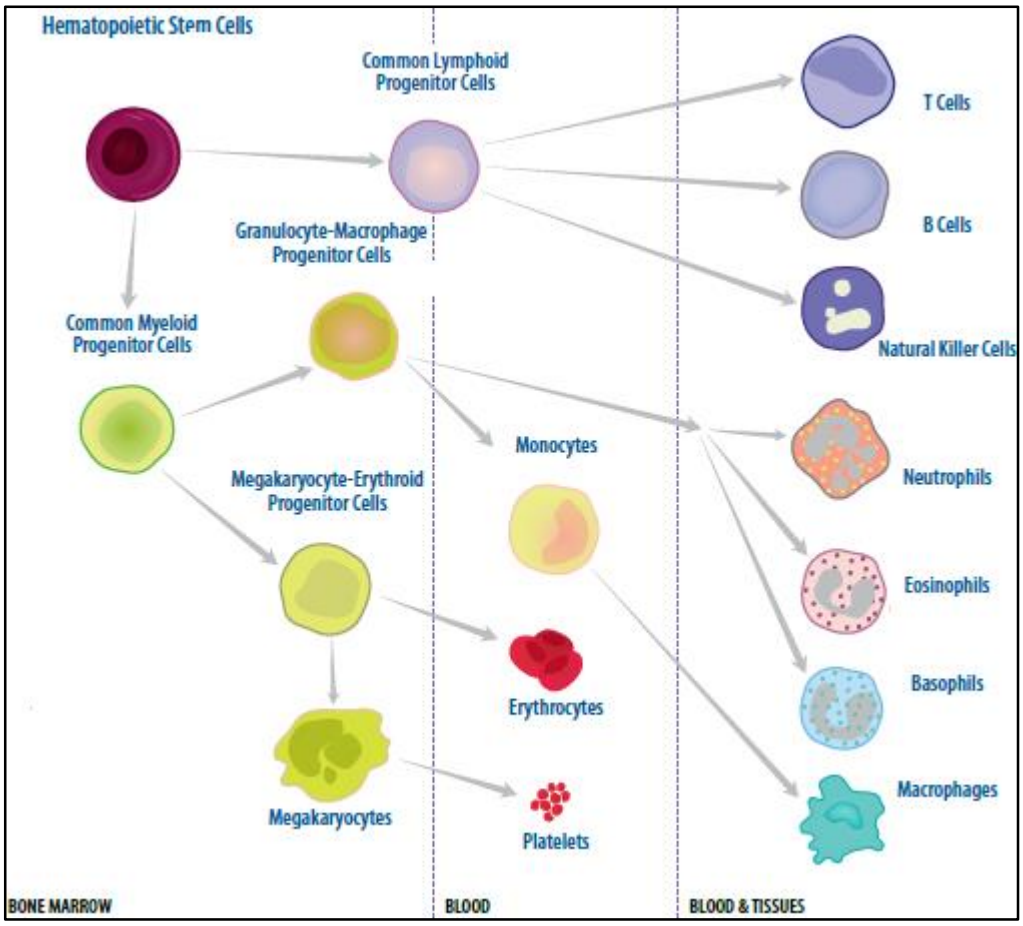

Figure 1.1 Diagrammatic representation of haematopoiesis. Pluripotent stem cell in the bone marrow gives rise via cell division and differentiation to circulating peripheral blood cells. Figure was taken from http://www.bdbiosciences.com/documents/cd_marker_handbook.pdf.

These cell types are responsible for different vital functions including gaseous exchange, clotting and the immune system. Specifically, lymphocytes are responsible for the adaptive immunity, whereas, the myelocytes are involved in diverse roles such as innate and adaptive immunity as well as blood coagulation. Different surface expression markers aid in the specification of different cell types at different stages of differentiation (McKenzie, 1996). This developmental process is tightly regulated by cellular interaction, the micro-environment of the bone marrow (BM), several regulatory glycoproteins and haematopoietic growth factors. Thus, when deregulated,

this process ultimately can result in one of a variety of haematological neoplasms: leukaemia, lymphomas, myeloproliferative and myelodysplastic disorders originating from lymphoid or myeloid compartments. This project will focus on the leukaemia derived from the lymphoid component and accordingly, understanding the regulatory networks, required for maintenance of normal haematopoiesis, is critically needed to uncover their implications for the leukaemic transformation. Leukaemia is a term constituted of the two Greek words; leukos for "white" and haima for "blood". In leukaemia, uncontrolled proliferation of leukaemic blasts that accumulate in BM and peripheral blood (PB) leading to a decrease in normal blood cell production. It is important to note that the precise molecular mechanisms required for the sustainability of normal haematopoiesis remain to be fully elucidated, although some of them have already been defined including numerous genes and cellular networks (cell cycle regulators and the PI3-kinase signalling pathway, transforming growth factor-β, Wnt, Hedgehog, and Notch) (Warr *et al.*, 2011). Recently, the vital role of epigenetic histone modifications has been recognised in the maintenance of HSCs (Wang *et al.*, 2013). This will yield significant insight into the systems biology that will differentiate between the normal HSCs and the leukaemic stem cells enabling the design of targeted therapy that overcome the aberrant function of these regulators.

1.2.1 Epidemiology

Leukaemia is the 12th most commonly diagnosed cancer in UK, representing around 3% of all new cases. It is more common in males than in females, giving incidence rates of 16 males or 11 females per 100,000 per year in 2010. In addition, it is strongly correlated with age with an average of 62% of the diagnosed cases occurring in those aged 65 years and above (Figure 1.2) (CRUK, 2011).

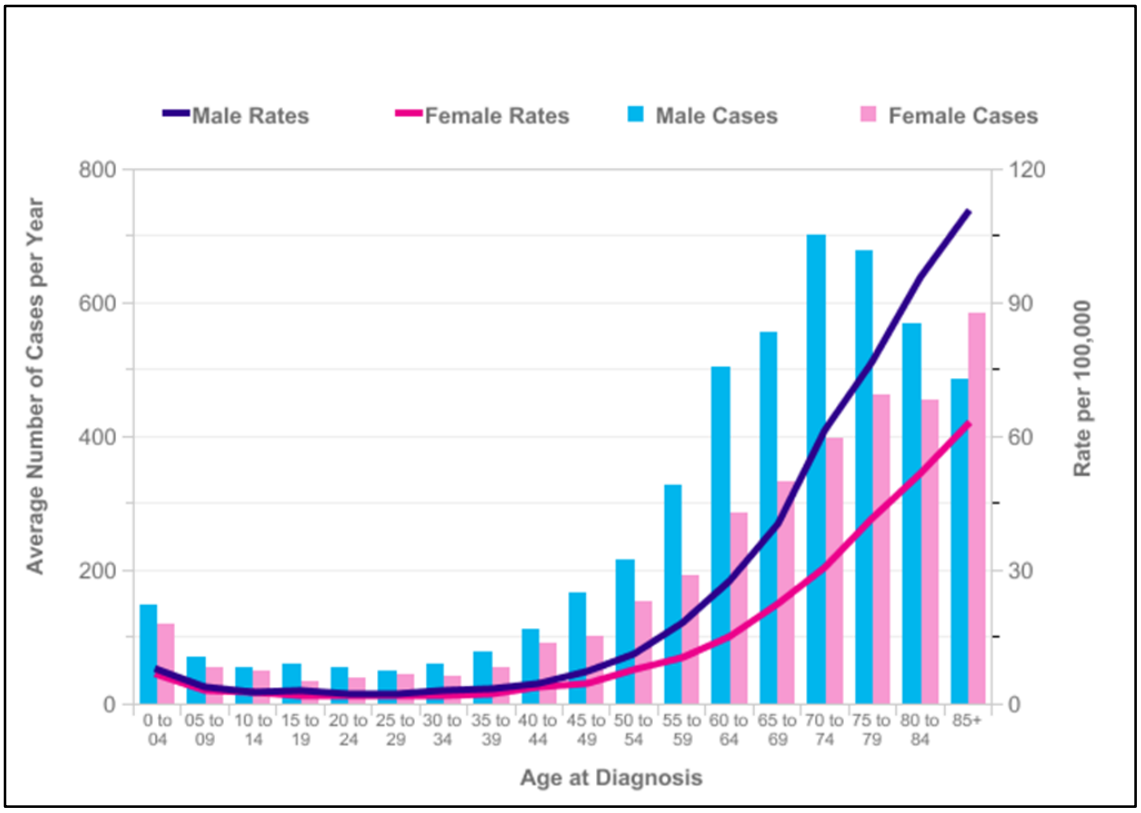

Figure 1.2 Graph showing leukaemia incidence rates in relation to sex and age per 100,000 population in UK in 2010. Figure was taken from (CRUK, 2011).

1.2.2 Classification and Diagnosis

Leukaemia is classified into four major categories depending on the particular cell type affected (myeloid or lymphoid) as well as the rate of cell growth (acute or chronic). They are acute lymphoblastic leukaemia, chronic lymphoblastic leukaemia, acute myeloid leukaemia and chronic myeloid leukaemia. These specific subgroups differ substantially in terms of morphology, cytogenetics features and the characteristic cell surface markers including the cluster differentiation (CD) antigen system, indicating diverse underlying aetiologies. There is evidence that understanding the genetic mutations associated with individual subtypes might uncover the responsible causal mechanisms for the leukaemic transformation.

The complete medical history and physical examination are pivotal in establishing the differential diagnosis. Essential diagnostic investigations include complete blood count, BM aspiration/biopsy, cerebral spinal fluid analysis, lymph nodes biopsy, imaging procedures and other complementary tests related to liver/ kidney/genetics.

Although molecular genetic tests are vital tools in refining the diagnosis, immunophenotyping is considered important to provide a prompt diagnosis. The immunophenotyping involves either flow cytometry or immunohistochemistry techniques.

1.2.3 Clinical overview

Acute leukaemia is characterised by a fast progression in which the immature leukaemic cells accumulate resulting in low levels of mature blood cell types. Early initiation of treatment is critically required due to the fast progression of the disease resulting in the extreme enrichment of malignant cells leading to infiltration into the blood and other organs including liver, spleen and lymph nodes. There are variations in the clinical picture of this disease in each individual but generally the symptoms and signs may include: recurrent infections, anaemia, bleeding, bruising, loss of appetite, loss of weight and enlarged lymph nodes/liver/spleen. In addition, they may include headaches, vomiting, confusion, seizures related to the meningeal spread, sores in the eye or skin or bone and swollen testicles owing to neoplastic infiltrations.

However, in the chronic type, mainly found in older patients, the leukaemic cells accumulate over a long period that usually takes months to years and patients are often monitored for evidence of substantial disease progression before being treated. They are usually asymptomatic or show non- specific signs/ symptoms such as anorexia, weight loss and fatigue.

This Chapter focuses on a subtype of acute lymphoblastic leukaemia (ALL) and the following section will further elaborate on this entity of leukaemia.

1.3 Acute lymphoblastic leukaemia

Acute lymphoblastic leukaemia (ALL) is a heterogeneous disease entity with a variety of genetic aberrations in the leukaemic blasts impeding important functions in cellular growth, differentiation, and death (apoptosis), thus contributing to a wide range of clinical presentations and outcomes. ALL is the most common type of leukaemia in children representing around four-fifths of all diagnosed childhood leukaemia in United Kingdom (CRUK, 2011). Generally, the incidence peaks between the ages of (2-5) years, decreasing to become a much rarer disease of adulthood, with 5 per million cases per

year in ages 20 to 59 years with a two fold increase in ages 60 to 79 years (Juliusson *et al.*, 2010).

1.3.1 Aetiology

The exact cause of ALL remains unresolved in the majority of cases. However, combinations of genetic and environmental factors required for malignant transformation are well established in a small proportion of cases. Patients with Down syndrome (DS) and disorders with excessive chromosomal fragility (Bloom's syndrome, ataxia-telangiectasia, Nijmegen breakage syndrome and Fanconi anemia) are at higher risk of developing ALL compared with the general population, however, they contribute to less than 5% of total ALL cases (Pui *et al.*, 2008; Reaman and Smith, 2011). DS is the most frequent genetic syndrome encountered in childhood ALL with 1 out of 100 DS cases developing ALL and this corresponds to a 10-20 fold increase in risk compared to non-DS cases. In addition, ionizing radiation, prior chemotherapy, high birth weight (> 4,000 g), parental occupation, maternal reproductive history (maternal age more than 35 years), parental tobacco or alcohol use and maternal diet are considered to be risk factors for childhood ALL.

Furthermore, infection was found to play a role in the observed worldwide variation of childhood leukaemia incidences. British investigators have postulated two parallel infection-based hypotheses: Kinlen's population-mixing hypothesis (Kinlen, 1988) and Greaves' delayed-infection hypothesis (Greaves, 2005; Greaves, 2006). These hypotheses originated from the observations of a peak age of development of childhood ALL of 2–5 years, an increased prevalence of the disease with industrialisation, and the occasional clustering of childhood leukaemia cases. Kinlen predicted that low immunity individuals exposed to a common but unidentified infection were at increased risk of developing ALL. Hence, this can give rise to localized epidemics and so-called leukaemia clusters. Greaves' hypothesis is based on a minimal two-hit model and suggests that delayed exposure to common infections predisposes the immune system of these individuals to aberrant or pathological responses resulting in increased lymphoid-cell proliferation (Figure 1.3). The delayed exposure is considered as a second hit that would require prior existence of pre-leukaemic clone (first hit) for the leukaemic transformation.

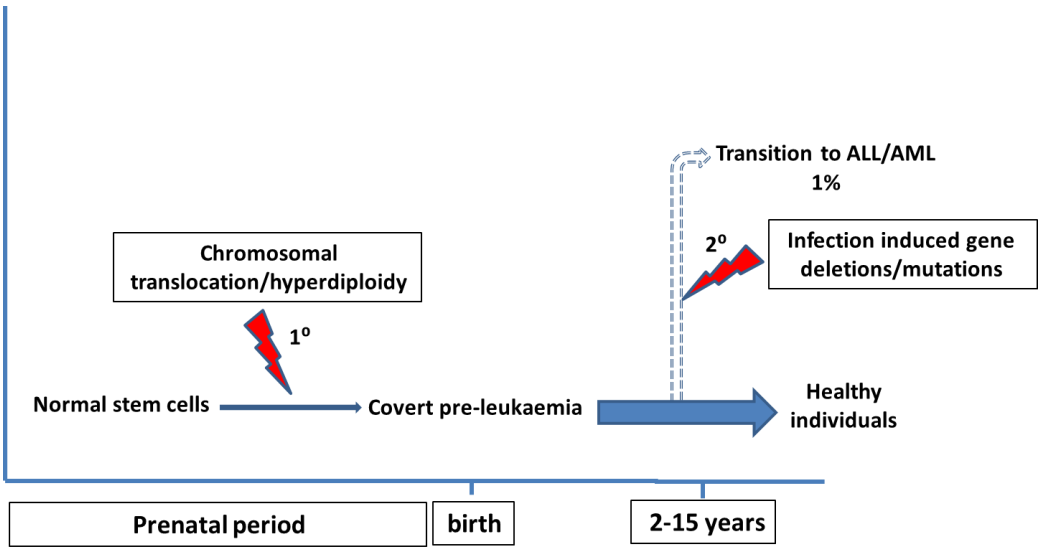

Figure 1.3 A two-step model for the natural history of acute lymphoblastic leukaemia. Figure adapted from (Greaves, 2005).

Interestingly, previous studies examining the contribution of single nucleotide polymorphisms (SNPs) to childhood leukaemia development reported possible role of SNPs targeting genes involved in the folate metabolism and xenobiotic transport/metabolism in increasing the leukaemia risk (Chokkalingam and Buffler, 2008; Koppen et al., 2010). However, recent genome- wide association studies of large populations of leukaemic and non-leukaemic individuals through analysis of SNPs identified other SNPs, known to be important in lymphoid development/differentiation, targeting *IKZF1* (7p12.2) (odds ratio (OR) = 1.69, P = 1.20 x 10^{-19}), *ARID5B* (10q21.2) (OR = 1.65, P = 6.69 x 10^{-19}), and *CEBPE* (14q11.2) (OR = 1.34, P = 2.88 x 10^{-7}) (Papaemmanuil et al., 2009; Trevino et al., 2009). These SNPs were found to be positively correlated with the development of leukaemia, while those SNPs involved in folate and xenobiotic metabolism were not reported to have a significant difference between the cases and controls in one of these studies (Trevino et al., 2009). Furthermore, the SNP targeting *ARID5B* was found to be a characteristic feature distinguishing BCP-ALL high hyperdiploid from other subtypes (p= 3.84 X 10^{-6}). Recently, an additional comprehensive association study, which combined both the local study with other published genome-wide association studies, provided an evidence of additional novel loci located at both 10p12.2 (*PIP4K2A*) (OR = 1.23; P = 2.30 × 10^{-9}) and 10p14 (*GATA3*) (OR = 1.31; P = 8.62 × 10^{-12}) with the SNP affecting *PIP4K2A* was specifically associated with high hyperdiploid BCP-ALL (p= 2.60 x 10^{-7}). Interestingly, the SNP located within *GATA3* was found to be associated with a specific subtype of ALL known ''Philadelphia

like'' ($P = 1.05 \times 10^{-8}$). This subtype is enriched for *CRLF2* rearrangements, *IKZF1* deletions and *JAK* mutations (Perez-Andreu et al., 2013).

1.3.2 Natural history of leukaemia

Molecular studies have contributed to identify the prenatal origin of some translocations and hyperdiploid event in some childhood leukaemic cases and these abnormalities were considered as initiating events (first hits) owing to the variable latency period before the overt leukaemia. The identical (monozygotic monochorionic) twin studies were the most important evidence of *in utero* initiation of leukaemia. There were several twin studies which confirmed the presence of identical primary rearrangement breakpoints (e.g. *MLL* rearrangements, t(12;21)) in both twins in several twin pairs, even those with different timing of leukaemia development. The plausible explanation is that the initiating events arose from a single clone in one twin and was transferred via shared placenta to the other twin (Ford *et al.*, 1993; Greaves *et al.*, 2003; Greaves and Wiemels, 2003; Reaman and Smith, 2011). Recently, comprehensive whole genome sequencing was performed on two pairs of monozygotic monochorionic twins, one with *ETV6-RUNX1* positive and one *ETV6-RUNX1* negative BCP-ALL, respectively, to further elucidate the developmental timing of different somatic alterations. This study focused on twins with *ETV6-RUNX1* fusion gene and showed conclusively that this was the only lesion common to both individuals and therefore must have been the initiating event (Ma *et al.*, 2013).

Furthermore, additional studies utilising the Guthrie cards or neonatal blood spots, which were collected at birth, confirmed the prenatal origin of some childhood leukaemia-specific fusion genes (e.g. *ETV6-RUNX1*, *RUNX1-RUNX1T1*) and hyperdiploidy (Gale *et al.*, 1997; Maia *et al.*, 2004; Greaves, 2005). In addition, some T-cell ALL associated abnormalities targeting T-cell-receptor loci and *NOTCH1* were also identified in both archived neonatal blood spots (Guthrie cards) and the monozygotic monochorionic twin studies with identical breakpoints presented at overt leukaemia (Ford *et al.*, 1997; Fasching *et al.*, 2000; Fischer *et al.*, 2007; Eguchi-Ishimae *et al.*, 2008).

The *ETV6-RUNX1* fusion gene was detected in 1% of the healthy newborn babies, a frequency 100 times greater than the incidence of *ETV6-RUNX1* positive BCP-ALL indicating (a) the requirement of the acquisition of additional abnormalities for the

leukaemogenic transformation; and (b) that most patients do not acquire these additional hits and therefore they do not develop ALL (Mori *et al.*, 2002; Zuna *et al.*, 2011). It is worth noting that the oldest case reported previously with positive Guthrie card test for *ETV6-RUNX1* fusion gene was aged 14 years at the time of clinical leukaemia indicating prolonged latency period (Wiemels *et al.*, 1999). Although researchers have reported an equal prevalence of *ETV6-RUNX1*–positive cells in both healthy newborn and adults (Olsen *et al.*, 2006), the observed cell level of 10^{-3} to 10^{-4} in neonatal blood does not persist throughout adult life. Instead it has been found to be much lower reaching a cell level of 10^{-5} to 10^{-6} which may explain the lower incidence of this abnormality in adult ALL compared to childhood ALL.

1.3.3 Classification and Diagnosis

The classification and accurate diagnosis of ALL involves a stepwise process, which has improved over the past 20 years with the development of new techniques. It is classified according to morphologic, immunologic and cytogenetic features of leukaemic lymphoblast derived from PB smear, BM aspirate or tissue biopsy specimens. It was initially classified solely using the French-American-British morphological classification system that is important in distinguishing between ALL and the leukaemic phase arising from Burkitt lymphoma and this contributes to major therapeutic implications (Bennett *et al.*, 1976; Bennett *et al.*, 1981; Bennett *et al.*, 1985). Later, subsequent immunological classification followed and it was useful to some extent in risk stratification (Kersey *et al.*, 1975; Bene *et al.*, 1995).

The most recent classification system is the 2008 World health organization (WHO) that uses morphologic, immunophenotypic, genetic, and clinical features to define distinct diagnoses within ALL (Table 1-1) (Vardiman *et al.*, 2009). Thus, ALL is classified into three major subgroups: acute leukaemias of ambiguous lineage (rare, do not fit into a defined entity), B lymphoblastic leukaemia/lymphoma and T lymphoblastic leukaemia/lymphoma with the vast majority of ALL cases in children is of B cell type, comprising nearly 85%. To note, lymphoblastic lymphoma can be differentiated from leukaemia when a mass is present based on the percentage of lymphoblast in BM (< 25%). B lymphoblastic leukaemia is further sub-classified based on the recurrent genetic abnormalities. Although several specific recurrent chromosome aberrations and gene

mutations also occur in T cell- ALL, at present they are not used to delineate separate entities within this ALL lineage in this current classification system (Swerdlow, 2008; Vardiman et al., 2009). This approach has been adapted to improve the accurate assessment of patient outcome.

Acute leukaemias of ambiguous lineage
Acute undifferentiated leukaemia
Mixed phenotype acute leukaemia with t(9;22)(q34;q11.2); *BCR-ABL1*
Mixed phenotype acute leukaemia with t(v;11q23); *MLL* rearranged
Mixed phenotype acute leukaemia, B-myeloid, NOS
Mixed phenotype acute leukaemia, T-myeloid, NOS
Provisional entity natural killer cell lymphoblastic leukaemia/lymphoma
B lymphoblastic leukaemia/lymphoma
B lymphoblastic leukaemia/lymphoma, NOS
B lymphoblastic leukaemia/lymphoma with recurrent genetic abnormalities:
B lymphoblastic leukaemia/lymphoma with recurrent genetic abnormalities with t(9;22)(q34;q11.2); *BCR-ABL 1*
B lymphoblastic leukaemia/lymphoma with recurrent genetic abnormalities with t(v;11q23); *MLL* rearranged
B lymphoblastic leukaemia/lymphoma with recurrent genetic abnormalities with t(12;21)(p13;q22); *ETV6-RUNX1*
B lymphoblastic leukaemia/lymphoma with recurrent genetic abnormalities with hyperdiploidy
B lymphoblastic leukaemia/lymphoma with recurrent genetic abnormalities with hypodiploidy
B lymphoblastic leukaemia/lymphoma with recurrent genetic abnormalities with t(5;14)(q31;q32); *IL3-IGH*
B lymphoblastic leukaemia/lymphoma with recurrent genetic abnormalities with t(1;19)(q23;p13.3); *TCF3-PBX1*
T lymphoblastic leukaemia/lymphoma

Table 1-1 The 2008 WHO classification of acute lymphoblastic leukaemia. NOS: not otherwise specified. Information was extracted from (Vardiman *et al.*, 2009).

There are different diagnostic investigations that are required to precisely refine the evolving diagnosis (Crisan, 2010). The BM aspiration and biopsy evaluations, which provide the morphological appearance, are useful in identification of the working differential diagnosis. More detailed phenotypic characterisation of cells is obtained by the flow cytometry and/or immunohistochemistry analysis to determine the specific lineage involved. The cytogenetic/molecular cytogenetic tests are widely used as important tools to further refine the final diagnosis. Thus, karyotype analysis provides information about gross chromosomal abnormalities, while molecular cytogenetic techniques including fluorescence in situ hybridisation (FISH) are important to give information about the presence or absence of specific lesions e.g. deletions, duplications, translocations and inversions. In addition, other molecular tests including

polymerase chain reaction (PCR) are used to detect specific abnormalities e.g. gene fusions.

1.3.4 Clinical presentation

ALL is thought to originate as a result of various important somatic genetic lesions in the differentiating progenitor cells that are committed to either the T-cell or B-cell pathway, giving rise to the different subtypes of ALL. These mutations impart the capacity for clonal expansion of a transformed haematopoietic cell or arrest the differentiation at an early stage in lymphoid cell development.

The clinical presentation of this disease results from the leukaemic blasts replacing normal marrow cells which in turn causes a marked decrease in the production of normal blood cells. This eventually leads to varying degrees of anaemia, thrombocytopenia, neutropenia and suppression of normal residual haematopoiesis which results in fatigue, bleeding, fever and infections. Concomitantly, these blasts can also infiltrate extramedullary tissues such as, e.g., liver, spleen, lymph nodes and the central nervous system (CNS) resulting in painful enlargement which may compromise normal organ function; hepato- and splenomegaly. These symptoms and signs are considered to be common in ALL patients in general.

In addition, bone pains and arthritis may be presenting features of B- cell type of ALL, while T cell-ALL may present with respiratory distress owing to the possible presence of a mediastinal mass. There are other rare symptoms involving the CNS including headache, vomiting, altered mental status which are more common in T-cell ALL than B-cell type. Painless testicular enlargement, usually unilateral, may be presenting feature but it is rare.

1.3.5 Treatment

There are four available standard modalities of treatment for ALL including: chemotherapy, radiation therapy, stem cell transplant and targeted therapy. Treatment typically consists of three phases: remission-induction, intensification and maintenance treatments along with CNS prophylaxis. Patients with mature B cell ALL (Burkitt leukaemia) are usually treated with short-term intensive chemotherapy like other lymphomas. Firstly, remission-induction treatment aims to kill more than 99% of the

initial burden of leukaemic cells and to restore rapidly normal haematopoiesis with associated normal performance status. This phase of treatment typically includes a glucocorticoid agent (preferably dexamethasone) as a choice of treatment along with other agents including vincristine and L- asparaginase drugs. Secondly, intensification treatment is used to eradicate drug- resistant residual leukaemic cells, thus reduces the risk of relapse. Basically, it resembles the induction phase but with augmented doses of different combinations of drugs such as glucocorticoid, L- asparaginase, vincristine and 6-mercaptopurine drugs. Various clinical trials follow different intensification regimens to assess their effectiveness on certain subtypes of leukaemia. Lastly, maintenance treatment is less intensive regimen which is applied to ALL patients except those with mature B- ALL. Some clinical trials such as Medical Research Council ALL97/99 (Mitchell *et al.*, 2010) or United Kingdom (UK) ALL2003 (Qureshi *et al.*, 2010) extended this phase of treatment to 3 years in boys owing to the increase risk of testicular relapses. However, most current protocols apply same duration of therapy in both genders because the gender seems not to be a prognostic factor with improved therapy. In this phase of treatment, drugs such as glucocorticoid, methotrexate and 6-mercaptopurine may be administered in different combinations and timing intervals.

Furthermore, CNS infiltration is a feature of ALL and can be present at diagnosis or cause relapse. Thus, CNS- directed treatment is important in order to control the CNS disease especially in those with high risk features including a T-cell immunophenotype, hyperleucocytosis, high-risk genetic abnormalities and presence of leukaemic cells in cerebrospinal fluid. There are different CNS-directed therapy options including intrathecal chemotherapy, systemic chemotherapy and cranial irradiation. The intrathecal chemotherapy consists of different combinations of methotrexate, cytarabine and glucocorticoid drugs, whereas the systemically administered drugs include methotrexate, L-asparaginase and glucocorticoid drugs. The combination of intrathecal and systemic chemotherapy is needed for controlling the CNS disease along with decreasing the risk of BM or other extramedullary relapses, respectively (Richards *et al.*, 2013). However, the cranial irradiation may be reserved only for patients with refractory CNS disease or as salvage therapy for patients with CNS relapse in some current clinical trials owing to its adverse side effects.

Risk assessment is based on different factors generated by the clinical and laboratory features at diagnosis including: age, WCC, early response to treatment (ER), minimal residual disease (MRD) at the end of induction and genetics (Pui, 2012; Moorman *et al.*, 2013). Currently, most studies stratify patients into three risk groups with similar but not identical risk criteria. For instance, ALL97/99 or UKALL2003 trials divided patients based on a combination of National Cancer Institute (NCI) criteria, cytogenetic and early response to induction therapy. Firstly, patients were classified based on age and WCC following NCI criteria: thus patients aged 1-9 years with a WCC < 50 $x10^9$/L at initial presentation are stratified as standard NCI risk, whereas those with ≥ 10 years or a WCC ≥ 50 $x10^9$/L were considered as high NCI risk category. Genetic abnormalities play an important role in the risk stratification; hence those with high risk cytogenetic (see section 1.4) are treated on the most intensive regimen irrespective of the NCI risk. The identification of those with high or low MRD levels is crucial in the treatment stratification. Hence those with high MRD level get more intensive therapy, while those with low MRD gets less therapy. Accordingly, risk stratification will spare low risk patients from unneeded toxicity. It is noteworthy that risk stratification is still not sufficient to identify all patients destined to relapse; hence most relapses arise in those with low or intermediate risk group (Moricke *et al.*, 2008; Mitchell *et al.*, 2009; Pui, 2012). However, the level of MRD seems to be a promising risk factor for which it is incorporated in the current risk stratification systems (Pui, 2012; Vora *et al.*, 2013).

In addition, haematopoietic stem-cell transplantation is one of the treatment modality that is reserved for high risk patients who have refractory leukaemia (especially T-cell ALL), high MRD level and early haematological relapses. There are two types of grafts including autologous (from the patient) and allogeneic (from matched related or unrelated) transplants. The autologous type carries less risk of infection and graft-versus- host disease owing to the rapid recovery of the immune function and the cells originated from the same individual, respectively. The allogeneic matched related donor is recommended over unrelated one in order to decrease the risk of developing graft-versus- host disease that adversely impacts on the transplant outcome. However, the matched unrelated transplant may be reserved for those with no available matched related donor. In general, the outcome of children with high risk BCP-ALL can be

improved by the application of the allogeneic transplant from either matched related or unrelated donor (Fagioli *et al.*, 2013).

All these modalities cause late effects/ complications which result in increased rates of morbidity and mortality. There are several adverse effects including: secondary neoplasms, many endocrine diseases, growth impairment, neurocognitive dysfunction and neurotoxic effects. In addition, development of chronic medical conditions like cardiac, musculoskeletal, gastrointestinal disorders and other chronic illnesses can arise from such treatment. These therapies have the potential to disrupt the social development in survivors, thus lower rates of marriages and achievement at schools/colleges were recorded.

Recently, new drugs targeting specific genetic alterations/ pathways have been developed and they often have lesser side effects as compared to the conventional therapy. In the USA, clinicians and researchers are collaborating in a project known as TARGET (Therapeutically Applicable Research to Generate Effective Treatments; www.target.gov) which aims to identify alterations in genes/ pathways related to high risk ALL using advancing technologies (e.g. high-throughput genomics and sequencing). Accordingly, these alterations may act as novel targets for therapy. An example of successful targeted therapy is tyrosine kinase inhibitors including imatinib and its analogues which target the *BCR-ABL1* fusion gene. This fusion gene is generated as a primary product of the translocation t(9;22)(q34;q11) in both acute lymphoblastic/ chronic myeloid leukaemia and is responsible for the tyrosine kinase pathway activation (see section 1.4.1.2) (Druker *et al.*, 2006). In addition, the tyrosine kinase inhibitors may be beneficial in treating patients with activated kinase signatures among the newly identified *BCR-ABL1*-like group especially those with *ABL1* translocations (e.g. the *NUP214-ABL1* fusion) (see section 1.4.1.2) (Harrison, 2013).

1.3.6 Outcome

The treatment advances in ALL have resulted in dramatic improvement of the survival rates in children with 5- year event free survival (EFS) and overall survival (OS) of 87·2% and 91·5% with 8.8% 5- year cumulative risk of relapse (Figure 1.4) (Vora et al., 2013). However, adult ALL showed lower survival rates with OS of approximately 40% and two

fold increase in the survival of those with Ph- negative ALL patients (Figure 1.5) (Goldstone et al., 2008).

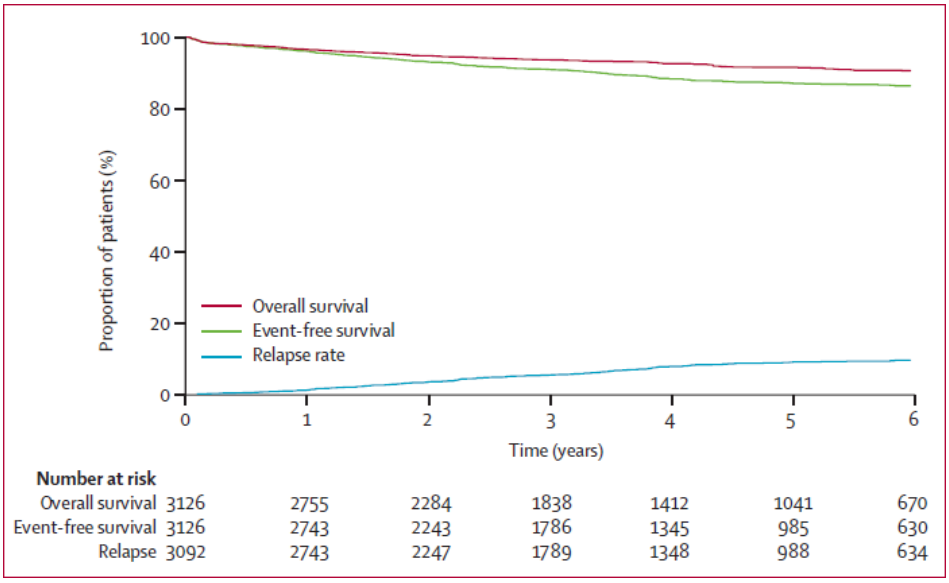

Figure 1.4 Survival and relapse rates of the overall trial of UKALL2003 of children and young adults ALL. Overall survival and event free survival: time from diagnosis to death/ last contact follow up or adverse events, respectively. Figure was taken from (Vora *et al.*, 2013).

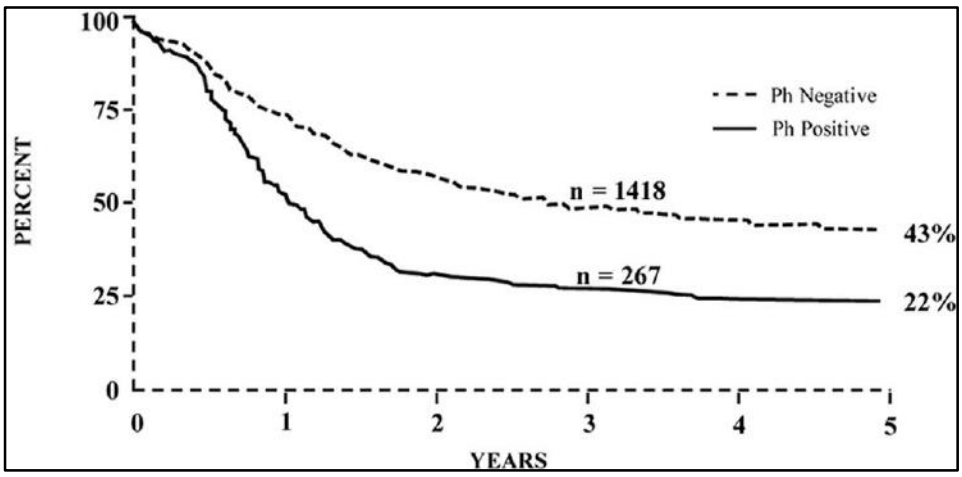

Figure 1.5 Overall survival rates at 4 years of the overall trial of UKALL XII/ECOG 2993 in Ph-negative and Ph- positive adult ALL (no imatinib treatment. The OS rate at 4 years of Ph-positive adult ALL (with imatinib treatment) is 38% as compared to 22% in those who did not receive imatinib (Ph- positive adult ALL (no imatinib treatment) (Fielding *et al.*, 2014). Figure was taken from (Goldstone *et al.*, 2008).

Both age and WCC are important risk factors in the determination of NCI risk groups (see section 1.3.5). Furthermore, cytogenetic plays an important role in the risk stratification of ALL patients (see section 1.4.3). There are other factors that may affect the outcome including: early treatment response, MRD level, immunophenotype, sex, CNS disease and obesity (Table 1-2).

Risk factors	Estimate of risk
Age	>10 years do worse than <10 years
WCC	>50 $\times 10^9$/L do worse than <50 $\times 10^9$/L
MRD risk	Positive MRD do worse than negative MRD
Immunophenotype	T-cell do worse than B-cell

Table 1-2 Generic estimates of acute lymphoblastic leukaemia risk based on several risk factors. WCC white cell count, MRD minimal residual disease.

Early treatment response is considered as an important measure to stratify patients into appropriate treatment allocations based on the percentage of blasts during the induction period at specific days that varied between different protocols. Thus, patients with > 25% blasts are classified as slow early treatment responders who constitute a high risk group. However, this factor lacks the prognostic value, hence 12% (35/285) of those patients with remission-induction failure (the persistence of the leukaemic blasts in BM or an extramedullary site at the end of the induction therapy) had rapid clearance of leukaemic blasts at around day 15 from the treatment initiation (Schrappe *et al.*, 2012).

Importantly, monitoring of MRD is an essential factor in the determination of the relapse risk and its predictive value is dependent on the measuring technique used, time point at measurement and therapy applied before and after the measurement (Vora *et al.*, 2013). MRD level can be obtained using either PCR detection of immunoglobulin and T-cell receptor gene rearrangements or flow cytometry of aberrant immunophenotypes. Although the PCR techniques are highly sensitive, combination of both techniques may be useful in order to reduce the false negative results in either technique. Patients with MRD negative have a favourable outcome compared to those who fail to achieve this immunologic/ molecular remission (Table 1-3) (Stow *et al.*, 2010; Vora *et al.*, 2013).

MRD risk	Relapse %	Death in remission %	Event free survival %
Low	4.0 (2.4-5.6)	1.5 (0.7-2.3)	94.2 (92.4-96.0)
High	15.0 (12.3-17.7)	4.5 (3.1-5.9)	79.8 (76.9-82.7)

Table 1-3 Event rates at 5 years (95% CI) within MRD risk groups treated on UKALL2003 trial. Data was extracted from (Vora *et al.*, 2013).

Almost half of the non-relapsed death cases in induction/ remission, for instance in UKALL2003, were pertaining to the therapy related toxicity (Vora *et al.*, 2013). Dexamethasone might be the contributor for this toxicity owing to increased susceptibility of patients to infections and osteonecrosis with associated negative impact on the quality of life (Hurwitz *et al.*, 2000; McNeer and Nachman, 2010; Vora *et al.*, 2013). In addition, L- asparaginase has potential toxic effects including pancreatitis, hypersensitivity reactions, thrombosis/ haemorrhage and hepatic injury. Hence, the identification of patients with low relapse risk is a pivotal management plan in order to reduce their exposure to the toxic effects of intensive therapy without compromising the survival rates and has been accomplished in some studies (Seibel *et al.*, 2008; Matloub *et al.*, 2011; Vora *et al.*, 2013). In addition, targeted therapy might contribute further to decrease the toxicity by replacing some toxic drugs in conventional chemotherapy.

The outcome of the patients with first relapse ALL is heterogeneous based on the received treatment protocols (Roy *et al.*, 2005; Parker *et al.*, 2010; Eckert *et al.*, 2013; Kako *et al.*, 2013). There are several factors contributing to the prediction of survival after first relapse including: duration of the first complete remission and immunophenotype (Table 1-4). The site of first relapse has also been associated with survival rates after relapse but not consistently.

Furthermore, the interventions received by patients with first relapse are contributing factors in the prediction of second relapse, thus those who are treated by salvage chemotherapy alone, high risk patients in particular, have increasing susceptibility for second relapses with resultant lower survival rates. In addition, matched allogeneic stem cell transplant was found to improve the outcome of patients, adults in particular, when offered in second complete remission (Kako *et al.*, 2013).

Risk factors	Estimate of survival after 1st relapse
Duration of CR1	Shorter CR has lower survival rates
Immunophenotype	T-cell has lower survival rates than B- cell

Table 1-4 Generic estimates of survival after first relapse.

1.4 Genetics of acute lymphoblastic leukaemia

The genetic changes found in haematopoietic malignancies serve as hallmarks for the leukaemic subtypes and provide important diagnostic and prognostic information. Genetic changes in ALL may be of numerical or structural type such as translocations, inversions and deletions. In addition, sequence mutations and methylation alterations occur. Here in this section, an overview will be given around the most prominent alterations found in both B and T cell ALL.

1.4.1 B-cell acute lymphoblastic leukaemia

The genetic subtypes in BCP-ALL can be divided into five main groups: (1) aneuploidy subgroups characterised by the gain or loss of multiple non-random chromosomes; (2) chromosomal translocations which result in the formation of fusion genes that disrupt transcription factors or activate oncogenes; (3) copy number alterations; (4) sequence mutations and (5) methylation alterations. Although these genetic subtypes can be seen in all age groups, the overall distribution of these alterations differs and the structural rearrangements/ aneuploidy subgroups are clear examples (Figure 1.6). In addition, these alterations affect different genes at variable stages of cell development (Figure 1.7). The majority of the chromosomal abnormalities including t(12;21)(p13;q22), t(9;22)(q34.1;q11.2), *MLL* rearrangements, t(1;19)(q23;p13.3), High hyperdiploidy and Hypodiploidy are primary, while the remaining aberrations are secondary abnormalities.

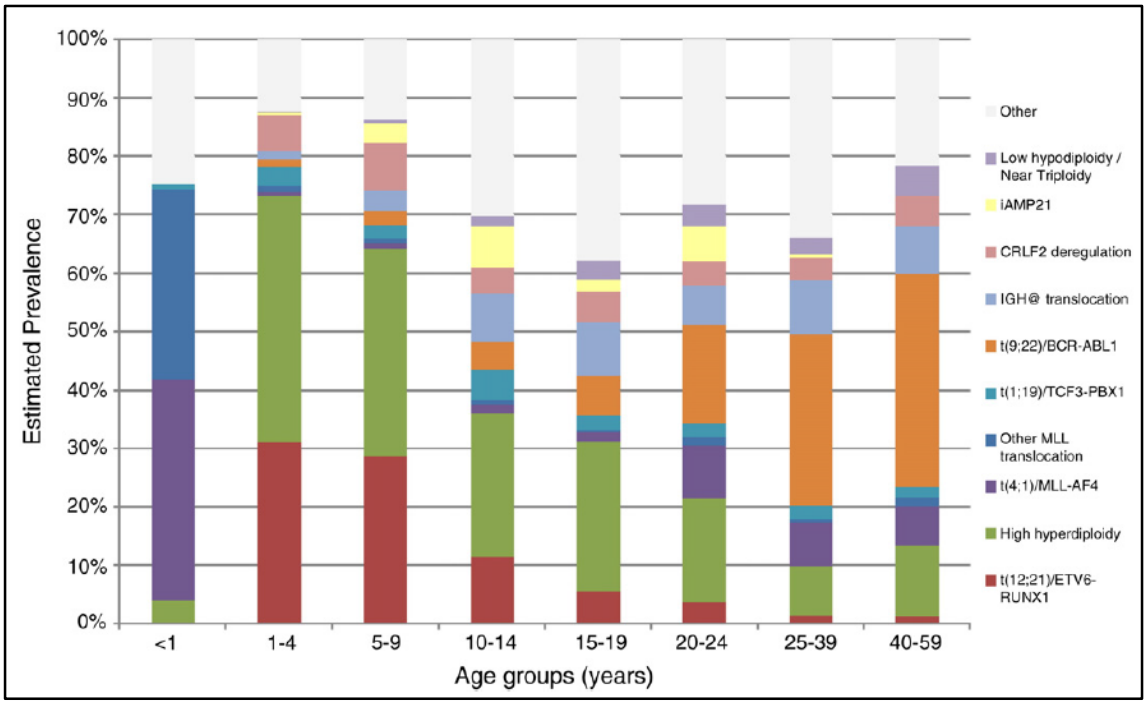

Figure 1.6 The estimated prevalence of different selected chromosomal abnormalities according to the age at diagnosis. Figure was taken from (Moorman, 2012).

1.4.1.1 Numerical chromosomal abnormalities

High hyperdiploidy (HeH) is defined as having a total modal number of 51-65 chromosomes present in the leukaemic cells (Paulsson and Johansson, 2009) with a variable definition of the upper limit as 61 (Forestier *et al.*, 2008a), 65 (Moorman et al., 2003; Sharathkumar et al., 2008), and 67 (Raimondi et al., 1996; Heerema et al., 2007). HeH BCP-ALL is the most common genetic abnormality that is characterised cytogenetically by a non-random gain of eight chromosomes (X, 4, 6, 10, 14, 17, 18, and 21) (Moorman, 2012). Interestingly, around 33% of HeH cases have several mutations affecting *FLT3*, *NRAS*, *KRAS* and *PTPN11* genes and thus in turn implies the possible development of novel therapies targeting the *RTK-RAS* signalling pathway (Case *et al.*, 2008; Paulsson *et al.*, 2008).

Hypodiploidy is characterised by a modal number of ≤ 45 chromosomes. It is subdivided into three distinct subgroups based on chromosome number, other cytogenetic features and clinical parameters: near-haploidy (<30 chromosomes), low hypodiploidy (30-39 chromosomes) and high hypodiploidy (40-45 chromosomes) (Harrison et al., 2004; Moorman, 2012). To note, these subgroups have variable distributions among different age groups, thus near-haploidy is more common in younger children as compared to low hypodiploidy subtype that occurs in older children, adolescents and adults. In addition, the pattern of the chromosome loss is non-random but it varies between near-haploidy and low hypodiploidy. Near-haploidy is characterised by the frequent retention of few chromosomes including: sex chromosomes and chromosomes 10, 14, 18 and 21, while low hypodiploidy typically exhibits loss of chromosomes 3, 7, 15, 16 and 17 with associated retention of chromosomes 1, 6, 11 and 18. Whole genome and exome sequencing studies found that near haploidy group is characterised by an increase number of alterations activating RAS signalling pathways (e.g. *NF1, NRAS* and *KRAS*) (71%) and the lymphoid transcription factor gene *IKZF3* (13%), while the low hypodiploidy group harbours alterations affecting *TP53* (~90%), that are commonly germline in almost half of children, *IKZF2* (53%) and *RB1* (41%) genes (Holmfeldt *et al.*, 2013). Both subgroups have a shared characteristic of activating Ras and phosphoinositide 3-kinase (PI3K) signalling pathways for which further research is

required to assess the efficacy of PI3K inhibitors as targeted therapy. Interestingly, both near-haploidy and low hypodiploidy subgroups showed a phenomenon of doubling-up of the existing clone, that arises from clonal evolution, and resulted in a modal chromosome number of high hyperdiploid (50-56) or high hyperdiploid (60-65) and near triploid (66-78) ranges, respectively, based upon the pre-existing number of chromosomes (Charrin et al., 2004; Moorman, 2012). It is worth noting that all disomic or trisomic chromosomes in the apparent high hyperdiploid or near triploid, respectively, are of uniparental origin unlike the true high hyperdiploid or near triploid which proves the origin of the doubled up clone. Hence, the misclassification of these cases is an important clinical issue that required adhered caution because they differ in terms of outcome and treatment allocation (see section 1.4.3), thus the identification of the uniparental isodisomy using high- resolution genomic profiling techniques can be used to reliably distinguish these subgroups.

1.4.1.2 Chromosomal translocations/rearrangements

The translocation t(12;21)(p13;q22) is the most common translocation in childhood BCP-ALL and results in the formation of the chimeric fusion gene *ETV6-RUNX1* (see section 1.5).

The translocation t(9;22)(q34.1;q11.2) produces the functional fusion gene *BCR-ABL1*, by fusing the 3' segment of the tyrosine kinase Abelson (*ABL1*) oncogene at 9q34 to the 5' segment of the breakpoint cluster region (*BCR*) gene at 22q11. This reciprocal translocation results in the formation of the derived chromosome 22, known as the Philadelphia chromosome (Ph) and was first identified in CML (Rowley, 1973). There are three differently sized transcripts encoding: p190, p210 and p230 (rare) owing to the heterogeneous breakpoints at the *BCR* locus and all these protein products result in activation of the tyrosine kinase.

Translocations involving the mixed lineage leukaemia gene (*MLL;* known as *KMT2A*), which is located at 11q23, create fusion genes with different partner genes (Szczepanski *et al.*, 2010; Meyer *et al.*, 2013). *MLL* rearrangements are also frequently seen in AML, in particular therapy- related AML (Slany, 2009) as well as in some T-ALL cases. The 5'portion of the *MLL* gene fuses to the 3' portion of the partner gene on the derivative chromosome 11 which is thought to exert the leukaemogenic effect. By far the most

common *MLL* translocations in ALL are t(4;11)(q21;q23)/*MLL-AFF1* (*MLL-AF4*), that frequently occurs in infants/ adults and is characterised by CD10 negative cells (very early immunophenotype) and t(11;19)(q23;p13.3)/ *MLL-MLLT1* (*MLL-ENL),* which is common in infants (Moorman, 2012). There are other well defined chromosomal translocations involving *MLL* that are found in all age groups but at lower incidence rates including: t(9;11)(p21;q23), t(6;11)(q27;q23) and t(10;11)(p12;q23). The fusion partners in these translocations are *MLLT3 (AF9), MLLT4 (AF6)* and *MLLT10* (*AF10)*, respectively (Secker-Walker et al., 1998). The *MLL* protein is responsible for the regulation of the HOX genes and contains several functional domains including the SET domain. The SET domain enhances the methylation of lysine 4 of histone 3 (H3K4) which mediates chromatin modification involved in epigenetic transcriptional activity (Milne *et al.*, 2002; Nakamura *et al.*, 2002). MLL rearranged proteins lose the SET domain (Hess, 2004) and hence recruit another histone methyltransferase, namely DOT1L. DOT1L aberrantly methylates histone 3 lysine 79 (H3K79) (Feng *et al.*, 2002; Steger *et al.*, 2008) which results in overexpression of the *HOX* genes which, in turn, drives leukaemogenesis (Okada *et al.*, 2005) .

The translocation t(1;19)(q23;p13.3) juxtaposes the trans-activation domain of the transcription factor 3 gene *TCF3* (*E2A*), located at chromosomal band (19p13), with the majority of the *PBX1* gene (homeobox *HOX*), positioned at 1q23, to form the *TCF3-PBX1* fusion gene (Hunger et al., 1991). It is worth noting that some cases (5%) showed different gene fusions (Privitera *et al.*, 1992; Barber *et al.*, 2007). The t(1;19) occurs either as a balanced translocation t(1;19) or unbalanced form where only der(19)t(1;19)(q23;p13.3) is present. A variant of the t(1;19) translocation exists at an estimated rate of 0.1% and results in the fusion of the *HLF* gene, located at 17q22, to *TCF3* as a consequence of t(17;19)(q23;p13) translocation.

Chromosomal translocations involving the immunoglobulin heavy chain (*IGH*) locus (Table 1-7), at 14q32 chromosomal region, define common subgroups of B-cell mature leukaemia and lymphoma but also occur in BCP-ALL (Moorman *et al.*, 2012; Russell *et al.*, 2014). *IGH* translocations result in deregulation of oncogene expression because of their juxtaposition to *IGH* transcriptional enhancers (Chapiro *et al.*, 2013). Recent advanced molecular studies have identified several novel *IGH* translocations involving important genes involved in cytokine receptors, transcription factors, apoptosis, cell

cycle progression and signalling adapter molecules/ miRNAs. The most common partner genes are *CRLF2* and the *CEBP* family members; however other recurrent genes were reported including *MYC, BCL2, ID4, EPOR* and *CCND1* and ongoing research studies are still uncovering new partners. Thus identification of these oncogenic genes will shed light on the pathogenic mechanisms and may result in the development and rational use of targeted therapies.

Deregulated expression of CRLF2 (Table 1-7) is driven by juxtaposition to the *IGH@* enhancer or *P2RY8* promoter which arise from either a cryptic chromosomal translocation: t(X;14)(p22;q32)/ t(Y;14)(p11;q32), or a cryptic interstitial deletion within the pseudo-autosomal region (PAR1): del(X)(p22.33p22.33)/del(Y)(p11.32p11.32), respectively (Russell *et al.*, 2009). In addition, rare activating mutations have also been reported (Russell *et al.*, 2009; Chapiro *et al.*, 2010; Hertzberg *et al.*, 2010). Interestingly, CRLF2 overexpression was found to arise in the absence of these rearrangements or the rare activating mutations (Russell *et al.*, 2009; Chapiro *et al.*, 2010; Hertzberg *et al.*, 2010). The resulting CRLF2 overexpression activates the JAK-STAT pathway that is responsible for the sustained proliferation and transformation of the primary B-cell progenitors. Aberrant CRLF2 expression in children results from a 5:1 relative proportion of *P2RY8-CRLF2:IGH@-CRLF2* cases, while a 1:2 relative proportion of *P2RY8-CRLF2*:IGH@-*CRLF2* cases has been reported among adults. The prevalence of deregulated CRLF2 expression among DS ALL patients is much higher, occurring in more than 50% cases with a predominant P2RY8-CRLF2 type of deregulation (Kearney *et al.*, 2009; Mullighan *et al.*, 2009a; Hertzberg *et al.*, 2010; Moorman *et al.*, 2012).

Intrachromosomal amplification of chromosome 21 (iAMP21) (Table 1-7) is characterised by multiple regions of inversion, deletion, duplication and amplification affecting the whole long arm of chromosome 21 resulting in 3 or more extra copies of the *RUNX1* gene, however, the target gene in iAMP21 pathogenesis has not yet been determined (Harrison *et al.*, 2013; Li *et al.*, 2014). Although the amplicon size and degree of amplification varied considerably between patients, a common 5.1 Mb region of amplification (CRA) was identified spanning the chromosomal regions 21q22.11 to 21q22.12, which encompasses the *RUNX1* gene. This was accompanied by a <1 Mb common region of deletion (CRD) close to the telomere in 80% of patients.

A newly identified genetic subgroup namely *BCR-ABL1*- like constitutes up to 10%-15% of childhood BCP-ALL cases. This novel subgroup displays a gene expression signature similar to that seen in *BCR-ABL* positive cases and it often has *IKZF1* alterations (either deletion/ sequence mutations) (Den Boer *et al.*, 2009; Roberts *et al.*, 2012). Half of the *BCR-ABL1*- like cases exhibit *CRLF2* rearrangements and 50% of those rearranged *CRLF2* cases have concomitant *JAK1/2* mutations which are responsible for the activation of the tyrosine kinase pathway. The remaining half of the non-*CRLF2* rearranged cases harboured a wide range of rearrangements which generate fusion genes capable of activating cytokine receptor/ tyrosine kinases signalling pathways. These rearrangements include *NUP214-ABL1, ETV6-ABL1, EBF1-PDGFRB* and *IGH-EPOR* (Den Boer et al., 2009; Roberts et al., 2012).

1.4.1.3 Secondary copy number alterations

Generally, CNAs can be classified into potential drivers, potential neutral/non-functional passengers, and physiological CNAs (Bateman *et al.*, 2010). Neutral passengers are defined as non-recurrent lesions in gene poor regions or are targeting genes with no functional relation to lymphocyte biology or leukaemia (Lilljebjorn *et al.*, 2007; Mullighan *et al.*, 2007; Strefford *et al.*, 2007; Tsuzuki *et al.*, 2007; Bateman *et al.*, 2010). Examples of physiological CNAs include gene deletions in IGH or TCR loci which are associated with the developmental rearrangements of these loci and resulted in the diversification of antigen receptors in the lymphoid lineages (Mullighan *et al.*, 2007). The vast majority of genomic studies have focussed on the recurrent potential drivers which are observed in previous genomic screens or in close proximity to functional genes involved in leukaemogenesis or B-cell regulation (Lilljebjorn *et al.*, 2007; Mullighan *et al.*, 2007; Strefford *et al.*, 2007; Tsuzuki *et al.*, 2007). There are more than 50 recurrent CNAs which target a single gene or few genes involved in various cellular processes and responsible for the leukaemic transformation/ progression (Figure 1.7) (Mullighan, 2012). In normal haematopoiesis, multiple transcription factors are tightly regulating the development of the B-lymphoid lineage from haematopoietic stem cells. Thus, alterations affecting *PAX5, EBF1* and *IKZF1* transcription factor genes can result in direct B-cell developmental arrest at pro- to pre-B stage of maturation which is an essential component of BCP-ALL. These alterations can occur as CNA, sequence mutations and rearrangements and are present in over 60% of BCP-ALL cases. Subsequently, other co-

operating events are needed for further progression of the disease targeting various key pathways (Figure 1.7). Generally, the number of gene losses exceeds the number of copy number gains by almost 2:1 with a mean of 6.46 alterations per case (Mullighan *et al.*, 2007). The genetic diversity level varies between BCP-ALL subtypes, with *MLL* translocations tending to have a stable genome with an estimated mean of less than 1 CNA per case (Mullighan, 2009). However, the translocations t(12;21) and t(9;22) have an increased number of alterations with over 6 alterations per patient. Furthermore, the distribution of individual genetic lesions and their associations to different cytogenetic subtypes differ (Table 1-5).

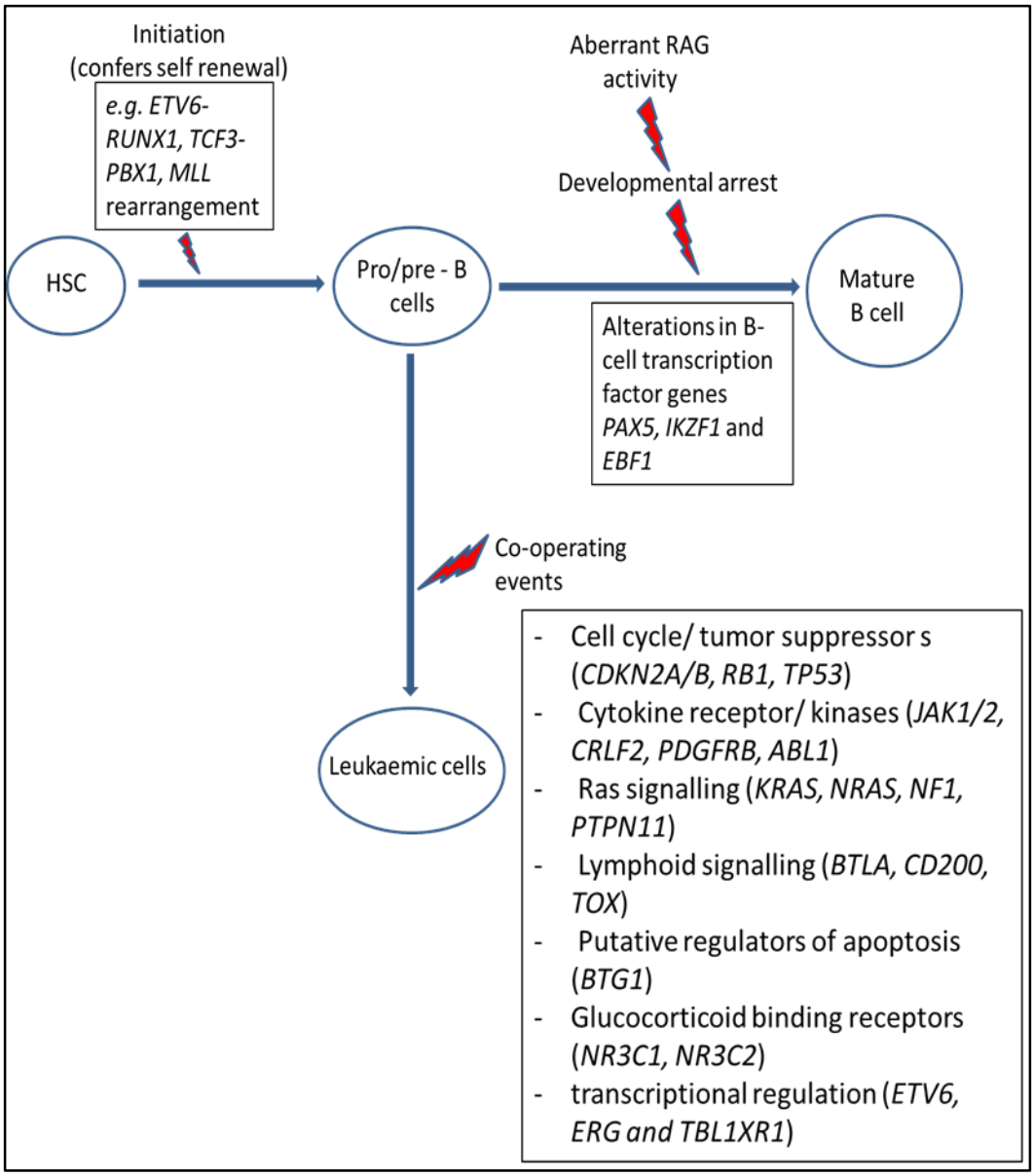

Figure 1.7 Diagrammatic representation of the role of the genetic alterations in the leukaemic development and progression. Acquisition of the primary genetic lesions occurs early leading to aberrant self-renewal. Block in maturation at pro- to pre-B cell stage of maturation as a result of aberrations of B cell transcription genes. Subsequent cooperating genetic alterations responsible for various cellular processes will be acquired by these cells, resulting in leukaemic transformation. Figure adapted from (Mullighan, 2012).

Abnormality	Frequency (total)%*	Cytogenetic subgroup association	Frequency in subgroup%
CDKN2A/B	34	Common	34
PAX5	30	Common	30
ETV6	27	*ETV6-RUNX1*	70
IKZF1	9	BCR-ABL1	85
CD200/BTLA	7	*ETV6-RUNX1*	15
BTG1	7	*ETV6-RUNX1*	13
RB1	5	*iAMP21* Low hypodiploidy	39‡ 41¥
NR3C1	5	*ETV6-RUNX1*	6
EBF1	4	BCR-ABL1 like	38#
NR3C2	3	*ETV6-RUNX1*	9
TBL1XR1	3	*ETV6-RUNX1*	13
RAG1/2	2	*ETV6-RUNX1*	14

Table 1-5 Frequencies of the recurrent gene deletions in BCP-ALL cases with their cytogenetic subgroups' associations. *percentages calculated out of the total number of BCP-ALL cases, all percentages were extracted from (Mullighan *et al.*, 2007) apart from ‡, ¥ and # which were taken from (Schwab et al., 2013), (Holmfeldt *et al.*, 2013) and (Tijchon *et al.*, 2013), respectively.

Most of deletions (44%) are focal (sized less than 1 Mb) encompassing a single gene and specific losses including *IKZF1, CDKN2A/B, RAG1/2* and *BTG1* are postulated to be driven from the aberrant activity of the RAG recombinase which is encoded by recombination-activating genes 1 and 2 (*RAG1* and *RAG2*) (Mullighan and Downing, 2009a; Kuiper and Waanders, 2014; Papaemmanuil *et al.*, 2014). The normal RAG recombinase activity is tightly controlled and responsible for the wide diversity in the antigen receptors during the early stages of lymphocyte development. It is worth noting that the aberrant RAG mediated recombination constitutes a driving mechanism in *ETV6-RUNX1* positive BCP-ALL, in particular, owing to its enrichment of *RAG1/2* alterations as compared to other ALL subtypes (14% v 0.69%) (Mullighan *et al.*, 2007). This RAG activity can be evidenced by the presence of the recombination signal sequences (RSSs) (which bind the RAG complex) and RSS- like motifs (small but highly conserved segments) which were determined in 12% and 40% of the alterations in *ETV6-RUNX1* cases, respectively (Kuiper and Waanders, 2014; Papaemmanuil *et al.*, 2014). In addition, the non-templated sequences were one of the features of RAG mediated mechanism presenting at 70% of the breakpoints in *ETV6-RUNX1* and resulted from terminal deoxynucleotidyl transferase (TdT) activity. Furthermore, these RAG mediated rearrangements are

enriched in active promoters/ enhancers especially those with RSS- like motifs and are repetitive in space/ time.

1.4.1.4 Sequence mutations

Although sequence mutations play an important role in leukaemogenesis, they are less frequent than CNAs with a mean of 1 sequence mutation (range 0-4) per case versus 8.36 CNA alterations (range 0-86) per patient (Zhang *et al.*, 2011; Mullighan, 2012; Mullighan, 2013). These mutations target different genes which are involved in different signalling pathways including B- cell development/ differentiation, Ras signalling, cytokine receptor/ kinase (JAK/STAT), *TP53/RB1* tumour suppression and epigenetic regulators. The identification of these mutations sheds light on the mechanism of leukaemic transformation and progression that arise from the possible interactions between different pathways/ genes alterations. Alterations in the transcription factor genes implicated in lymphoid differentiation (e.g. *PAX5, IKZF1 and EBF1*) are coupled with other mutations involved in cell survival and proliferation (Ras, cytokine and kinase signalling pathways). Alterations in these two common pathways are subsequently followed by further acquisition of genetic defects targeting transcriptional co-regulators and tumor suppressor genes (*TBL1XR1, BTG1* and *CREBBP, CDKN2A/B, RB1* and *TP53*). These mutations can be either activating or inactivating type and their frequencies differ among different ALL subtypes (Table 1-6). For instance, the activating *JAK* mutations involving *JAK1* and *JAK2* are associated with *CRLF2* rearranged cases and mediate the activation of JAK/STAT pathway and are important alterations in almost half of *CRLF2* rearranged *BCR-ABL1*-like cases (see section 1.4.1.2). In addition, *IKZF1* mutations which directly impair the B-cell lymphoid development are often seen in one third of *BCR-ABL1*- like cases.

Signalling Pathway	Genes	Type of mutations	%*	Cytogenetic subtype association	%*
Ras	*NRAS, KRAS, PTPN11* and *NF1*	Activating	35	Near haploidy High hyperdiploidy	71 33
Transcriptional regulation of B-cell development/differentiation	PAX5	Inactivating	10¶	Common	NA
	IKZF1		10¶	*BCR-ABL 1* positive	85¥
JAK/STAT	*JAK1* and *JAK2*	Activating	11¶	*CRLF2* rearranged *BCR-ABL* 1 like cases	57
	ABL1, PDGFRB and *EPOR*			*BCR-ABL 1* like	50
TP53/RB1 tumour suppression	*TP53*	Inactivating	4¶	Low hypodiploid	90

Table 1-6 Recurrent mutations of genes/ pathways in BCP-ALL cases. * frequencies obtained from (Case *et al.*, 2008), (Zhang *et al.*, 2011), (Mullighan, 2012), (Paulsson et al., 2008) and (Holmfeldt *et al.*, 2013), NA: not available, ¥ includes deletions, ¶ frequencies estimated in a biased cohort of high risk BCP-ALL.

At relapse, somatic mutations may be acquired as new lesions or lost similar to CNAs (Mullighan, 2012). RAS signalling pathway (*NRAS, KRAS, PTPN11* and *NF1*) and B cell development (*PAX5*) mutations are found to be lost at relapse in spite of their initial detection at presentation. However, some other mutations affecting CREBBP and CREB binding protein are found at a low level or absent at diagnosis but are enriched at relapse (20%) especially those with high hyperdiploid relapses. *CREBBP* mutations are related to drug resistance owing to the disruption of the normal transcription response to glucocorticoids (Inthal *et al.*, 2012). Given the fact that enriched histone acetyltransferase (HAT) domains are responsible for these mutations, introduction of histone deacetylase inhibitors as a targeted therapy might be of an option. In addition, inactivating *TP53* mutations are enriched in the relapsed cases with 54% of these mutations are acquired at relapse and carry independent poor prognostic factors (Hof *et al.*, 2011).

1.4.1.5 DNA methylation

Epigenetics has emerged as a critical regulator of cell function that is responsible for leukaemogenic progression. This can be achieved by different mechanisms including: DNA methylation and post translational histone modification. The former mechanism targets the cytosine preceding guanine (CpG) nucleotides that are located in regions rich in CG known as CpG islands (CGI) and which normally have a lower rate of methylated CpG sites compared to the whole genome (Nordlund *et al.*, 2013). Overall, differentially methylated CpG (DMC) sites across all genetic subtypes were not shown to target cellular pathways, instead all DMC signatures were enriched for important genes involved in various biological functions in cancer development and progression. Furthermore, approximately 15% of DMC signatures were correlated with expression of the involved genes (Nordlund *et al.*, 2013); hypermethylation of CpG promoters resulted in gene silencing and subsequent neoplastic transformation (tumour suppressor genes). The global decrease in methylation (hypomethylation) that occurs in gene body regions is associated with genomic instability and chromosomal rearrangements.

1.4.2 T-cell acute lymphoblastic leukaemia

T-cell acute lymphoblastic leukaemia constitutes 15 and 25% of total cases of childhood and adult ALL, respectively, and it is more common in older age (Pui, 2004). It is a heterogeneous disease with up to 50% of these cases having an abnormal karyotype with associated frequent translocations (Meijerink, 2010). In general, there are two types of mutations: type A which defines four distinct genetic subgroups and type B mutations which are common and can occur in any subgroup. Type A mutations are characterised by the mutual exclusive rearrangements including *TLX3 (HOX11L2), TLX1 (HOX11)*, *TAL/LMO* complex and *HOXA* abnormalities which have distinct gene expression signatures corresponding to a specific level of T cell development in each subgroup. On the other hand, type B mutations target different cellular processes resulting in cell cycle defects and activation of multiple pathways and they include *CDKN2A/B, NOTCH1, FLT3, BCL11B, PTPN2, BHF6, NRAS* and *WT1* alterations. Interestingly, a fifth distinct subgroup was unmasked by the gene expression profiles namely: an early T-cell precursor that is characterised by immature T cells with no characteristic chromosomal abnormality (Figure 1.8). Generally, both *TAL/LMO* and *TLX1* subgroups are characterised by a good outcome, while those patients in *TLX3*, *HOXA* or ETP-ALL have an adverse outcome (Meijerink, 2010).

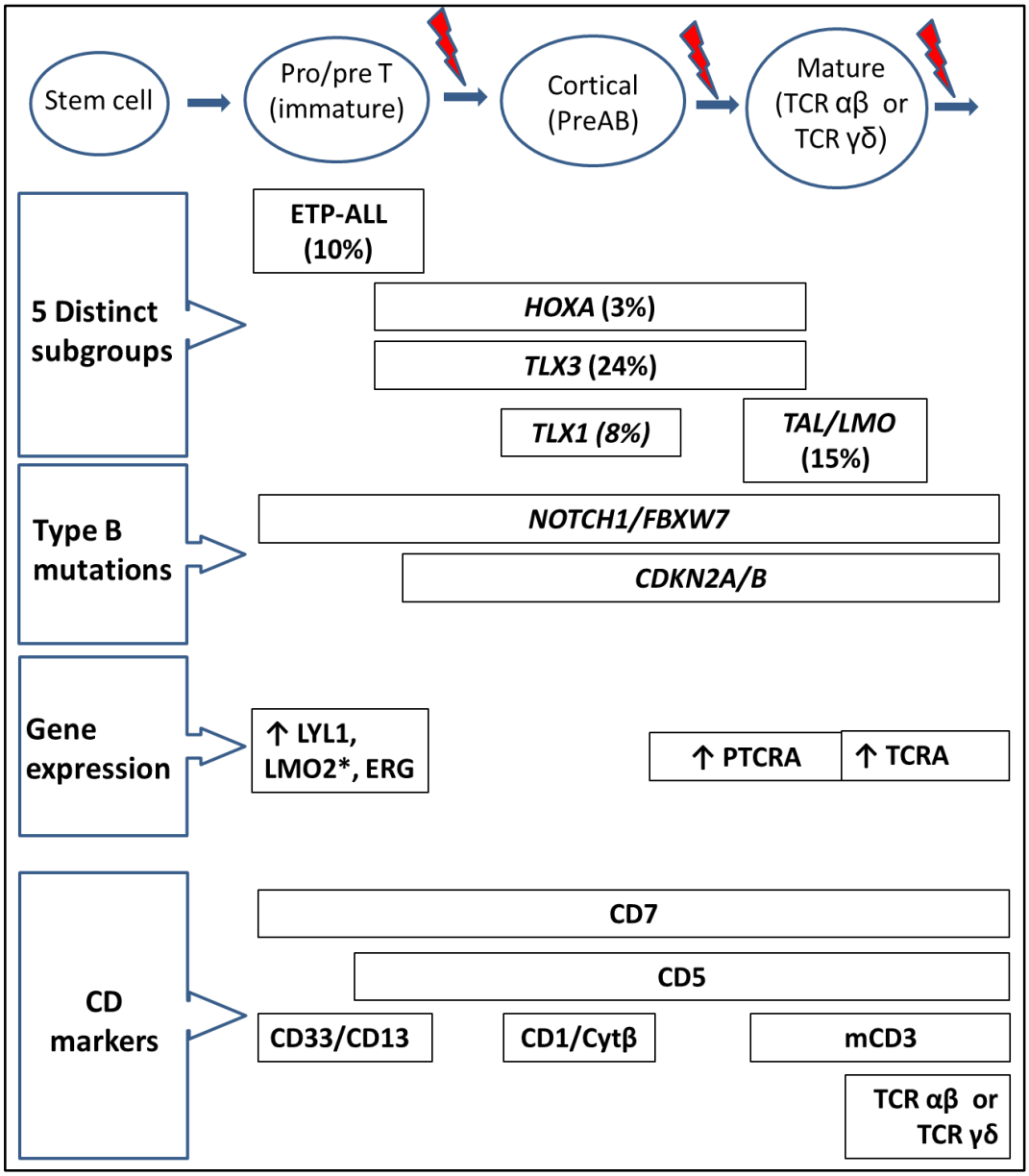

Figure 1.8 Diagrammatic representation of T-cell ALL distinct subgroups (frequency %) in relation to their T-cell developmental stage (based on EGIL or TCR classification systems). Five subgroups: ETP-ALL (early T-cell precursors ALL), *HOXA, TLX3, TLX1 and TAL/LMO*; *NOTCH1/FBXW7* mutations can present in any subgroup; CDKN2A/B alterations occurs rarely in the immature stage; ETP-ALL is characterised by high expression of LYL1, LMO2 and ERG; the less and more mature stage is characterised by high expression of PTCRA (pre T- cell receptor A) and TCRA (T-cell receptor A), respectively; * high LMO2 expression can occur in LMO2-rearranged cases; CD7 occur across the T-cell developmental stages; CD5 is weakly expressed in the immature stages; CD33/CD13 (aberrant myeloid markers) occurs in ETP-ALL; CD1/Cytb are expressed in the cortical stages; mCD3 is expressed in the mature stages; TCR αβ or TCR γδ are expressed in the more mature cells. Information was extracted from (Meijerink, 2010).

1.4.3 The prognostic effect of different genetic alterations

A large and growing body of studies have focussed on the genetic abnormalities that constitute the hallmarks of this disease owing to their importance in understanding the leukaemogenesis and establishing the diagnosis/ classification (e.g. WHO classification) and treatment stratification. This in turn has led to the recognition of their clinical significance and as such they are incorporated into routine clinical practice. It is essential to identify good and poor risk genetic subtypes that will aid in directing therapy (Table 1-7). The outcome of those with poor risk cytogenetics can be improved by more intensive therapy, while those with good risk cytogenetics can be treated with less intensive treatment to enable the reduction in the toxicity related deaths. Although these genetic alterations convey similar prognostic relevance in either children or older patients (Figure 1.9), however, older patients usually exhibit lower survival rates (Figure 1.10).

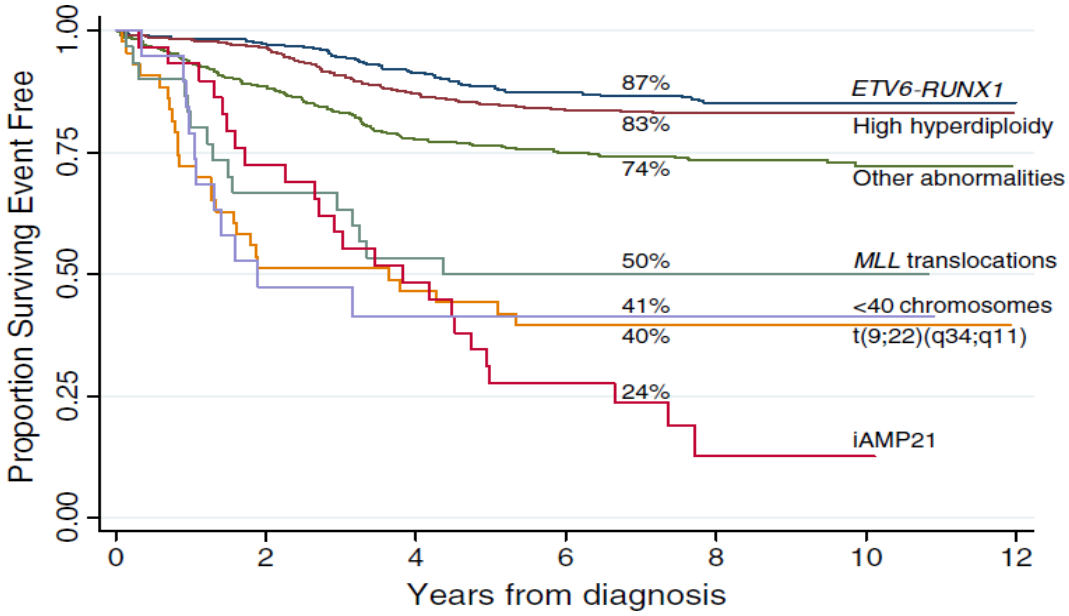

Figure 1.9 Event free survival of childhood BCP-ALL in relation to the primary genetic subgroups. Percentages are event free survival at 7 years after a median follow up time of 8.2 years. Figure was taken from (Moorman, 2012).

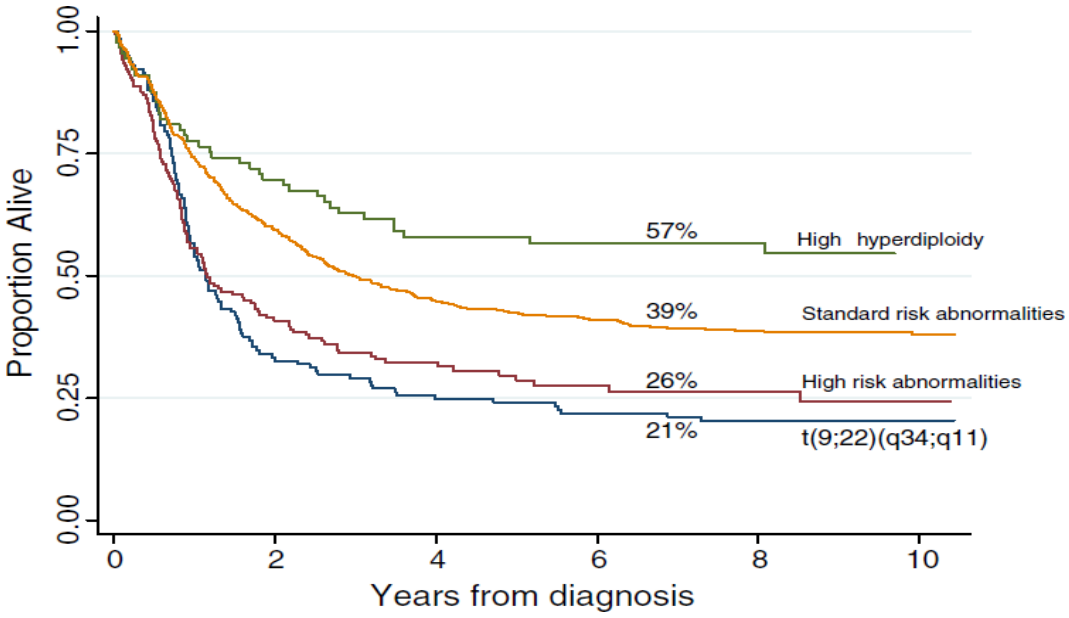

Figure 1.10 Overall survival of adult BCP-ALL by primary genetic subtypes. Percentages are overall survival at 7 years after a median follow up time of 8.2 years. Cases were classified as follows: high hyperdiploidy , t(9;22)(q34;q11), high risk abnormalities [t(4;11)(q21;q23), IGH@ translocations, CRLF2 rearrangements, low hypodiploidy/near-triploidy and complex karyotype], standard risk abnormalities [all other karyotypes]. Figure was taken from (Moorman, 2012).

Numerical chromosomal abnormalities	Modal chromosome number	Incidence		Result of the aberration	Prognostic significance	
		children	adults		children	adults
High hyperdiploidy	51-65/67 chromosomes	35%	10%	Poorly understood	Excellent	Significantly improved survival
Low hypodiploidy Near-haploidy	30-39 chromosomes <30 chromosomes	5–6%	2%	Poorly understood	Very poor	
Chromosomal translocations/rearrangements	**Genes**					
t(12;21)(p13;q22)	*ETV6-RUNX1*	15-35%	4%	Interferes with expression of HOX genes in lymphopoiesis	Favourable	Good
t(9;22)(q34;q11)	*BCR-ABL1*	2–3%	20-40%	Activation of mitogenic signalling, attenuation of apoptosis, altered cell adhesion	Very poor unless treated with imatinib or other tyrosine kinase inhibitor	
11q23 rearrangements	*MLL* gene with different partner genes	Infants 70-80%; Children 2-3%	6%	Alter the pattern of HOX gene expression	Very poor	
t(1;19)(q23;p13.3)/ der(19)t(1;19)(q23;p13.3)	*TCF3-PBX1*	5–6%	4%	Interferes with haematopoietic-lymphoid differentiation	intermediate/good	

Numerical chromosomal abnormalities	Modal chromosome number	Incidence		Result of the aberration	Prognostic significance	
		children	adults		children	adults
IGH@ translocations	*IGH* with different partner genes	5%		Deregulated oncogene expression via its juxtaposition to the IGH@ enhancer	Unkown	Poor
CRLF2 overexpression	*CRLF2*	5%	5%	Activates the JAK-STAT pathway	Intermediate/poor	Poor
iAMP21	Unidentified	2-3%	NA	Unknown	Unfavourable	NA

Table 1-7 Clinically relevant genetic aberration subgroups in B-cell precursor acute lymphoblastic leukaemia patients. NA: no adult cases identified. Data extracted from (Moorman *et al.*, 2010a; Szczepanski *et al.*, 2010; Pui *et al.*, 2011; Moorman, 2012).

ETV6-RUNX1 (see section 1.5.7) and HeH patients are assigned as good prognostic markers. The identification of the risk factors within HeH patients is still to be defined, in spite of good outcome, because 15% of patients do relapse. The Children's Oncology Group consider the presence of the triple trisomy (simultaneous gain of chromosomes 4, 10 and 17) to be a favourable factor, while a UK study found that only trisomy 18 conveys a better indicator of good outcome (Moorman, 2012). Recently, it was reported that trisomy 4 was associated with good outcome in Ph- negative HeH patients treated on UKALLXII/ECOG2993 trial, while patients with trisomy 20 showed inferior outcome (Chilton *et al.*, 2013). However, trisomy 5 had an adverse impact in Ph-positive HeH patients.

The poor risk cytogenetic subgroups constitute the following: t(9;22)/ *BCR-ABL1*, *MLL* rearrangements, near haploidy (< 30 chromosomes), Low hypodiploidy (30-39 chromosomes), iAMP21 and t(17;19)/*E2A-HLF* (Moorman *et al.*, 2010b).

Patients with *BCR-ABL1* fusion gene are assigned to the high risk category and receive more intensive therapy. In addition, the use of tyrosine kinase inhibitors (imatinib, dasatinib, nilotinib) along with intensive chemotherapy is beginning to show promising results in children and adolescents. Thus, paediatric cases that were treated with imatinib had a 4 year disease free survival rate of 75·2% (61·0—84·9) versus 55·9% (36·1—71·7) in those who received only conventional chemotherapy (p=0.06) in the per-protocol analysis (adjusted for minimal residual disease) (Biondi *et al.*, 2012). Furthermore, the improvement of outcome in the imatinib treated adult patients as compared to those who did not receive imatinib was observed with a 4 year OS, EFS and RFS rates of 38% v 22% (p=0.0003), 33% v 18% (p=0.0001) and 50% v 33% (p=0.0003), respectively (Fielding *et al.*, 2014).

In one study, 70-80% of infant cases exhibited *MLL* rearrangements with half of them showing t(4;11) that is characterised by poor outcome in all age groups, whereas the remaining translocations showed insignificant poor outcome in adults (Moorman, 2012). The promising results of the *in vitro* and *in vivo* effects of DOT1L inhibitors (EPZ004777) against *MLL* rearranged cells have offered the basis of the targeted therapy in high risk patients (Daigle *et al.*, 2011).

Children with t(1;19) were associated with a poor outcome prior to the intensive modern protocols but currently on contemporary protocols this rearrangement was shown to have an intermediate/good outcome (Moorman, 2012). On the other hand, the prognostic effect of this rearrangement in adults is still debatable which is probably due to variations in treatment.

The prognostic relevance of CRLF2 overexpression in children has conflicting results with the disparity was attributed to the different protocols studied. One study reported poor outcome (relapse free survival (RFS) at 4 years 35% versus 71%) of high risk patients with CRLF2 overexpression treated on COG P9906 trial (Harvey *et al.*, 2010). In addition, non-high risk patients with *P2RY8-CRLF2* treated on AEIOP-BFM-2000 were also shown to have an adverse outcome (EFS: 28% versus 83% at 6 years) (Cario *et al.*, 2010). In contrast, a similar outcome was found between patients with CRLF2 deregulation and patients in the intermediate cytogenetic risk group in children treated on ALL97 (OS at 5 years 81% versus 85%) (Ensor *et al.*, 2011). To date, there have been few reports on the prognostic relevance of *CRLF2* deregulation in adults, although one study has reported it as being associated with an adverse clinical outcome: EFS <25% at 5 years (Moorman *et al.*, 2012).

iAMP21 was not used previously to guide therapy and patients with this rearrangement were treated as standard risk patients. However, the 3-fold increase in the relapse risk and the dismal outcome experienced by iAMP21 patients compared to those who did not have this abnormality (EFS (29% v 78%), OS (71% v 87%) at 5 years) (Moorman *et al.*, 2007) warranted recruiting patients to the high risk arm in the following trials. Accordingly, marked improvement in outcome was observed as evidenced in UKALL2003 treated patients, thus at 5 years the EFS and OS increased to 78% and 89%, respectively, with reduction in the relapse risk from 70% to 16% (Moorman *et al.*, 2013). In agreement with these findings, iAMP21 patients treated in contemporary Children's Oncology Group also showed inferior outcome (a 4 year EFS and OS of 73% and 88%), but with better survival rates than the previous report owing to possible differences in the treatment protocols (Heerema *et al.*, 2013).

BCR-ABL1- like group is a high risk group which confers a dismal outcome and the introduction of the tyrosine kinase inhibitors have shown promising results especially

those cases with *NUP214-ABL1* and *EBF1-PDGFRB* fusion genes (Roberts *et al.*, 2012; Lengline *et al.*, 2013; Weston *et al.*, 2013).

1.5 t(12;21)(p13;q22)/*ETV6-RUNX1*

1.5.1 Overview

The translocation, t(12;21)(p13;q22), which generates the *ETV6–RUNX1* fusion gene, is the most common chromosomal translocation in BCP-ALL (Golub *et al.*, 1995; Romana *et al.*, 1996; Moorman *et al.*, 2010b). It is invisible by conventional cytogenetic analysis as the banding pattern and the size of the translocated regions between chromosomes 12 and 21 are identical (Heim and Mitelman, 2009). Thus, FISH was responsible for its discovery when investigating patients with deletions in the short arm of chromosome 12 (Romana *et al.*, 1994). The translocation results in the fusion of the ETS- type variant 6 (*ETV6*) gene (previously known as *TEL*-translocated ETS leukaemia gene), located at 12p13, with the *RUNX1* gene (runt- related transcription factor 1, previously known as *AML1*- acute myeloid leukaemia 1), located at 21q22, to create the chimeric fusion gene *ETV6-RUNX1* on the derived chromosome 21 (der(21)t(12;21)) (Figure 1.11) (Golub *et al.*, 1995; Romana *et al.*, 1995a). *ETV6-RUNX1* is postulated to be responsible for the expansion of a candidate preleukaemic stem cell population that has an early B lineage phenotype (CD34+CD38–CD19+) and persists in the bone marrow for several years (Hong *et al.*, 2008). Hence, this fusion gene is the first transforming event arising prenatally (see section 1.3.2), but it is unable to generate overt disease by itself and additional secondary genetic events are required to drive leukaemogenesis (Mori *et al.*, 2002; Greaves and Wiemels, 2003; Zuna *et al.*, 2011; Ma *et al.*, 2013).

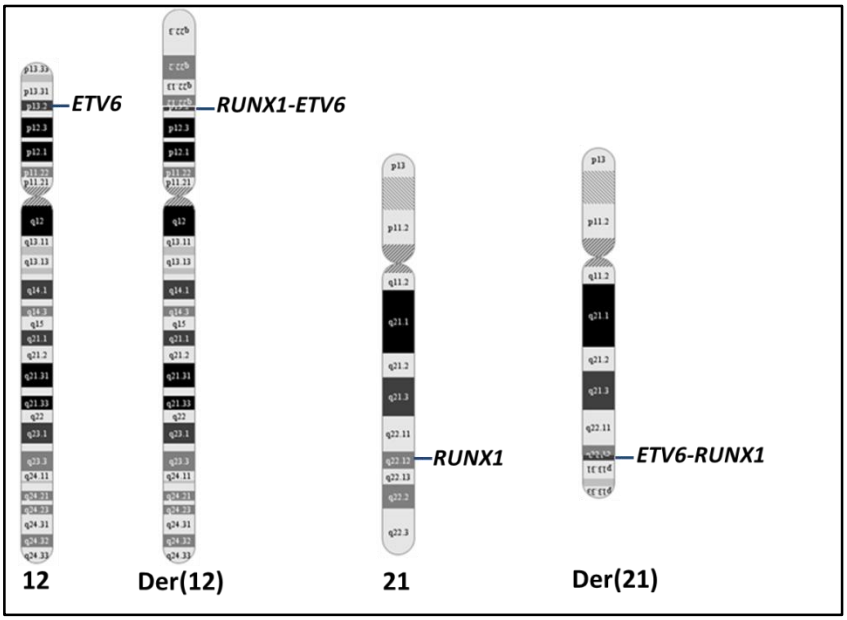

Figure 1.11 Diagrammatic representation of the t(12;21)(p13;q22. Locations of the *ETV6-RUNX1* and its reciprocal *RUNX1-ETV6* fusion genes on der(21)t(12;21) and der(12)t(12;21), respectively.

1.5.2 Epidemiology/ Clinical characteristics

ETV6-RUNX1 is found in ~25% of BCP-ALL cases, with a peak incidence between the ages of 3–6 years of children with BCP-ALL (Golub *et al.*, 1995; Romana *et al.*, 1996; Moorman *et al.*, 2010b). In contrast, this translocation is relatively rare in adults (older than 15 years) constituting (1-4%) of BCP-ALL cases (Aguiar *et al.*, 1996; Raynaud *et al.*, 1996; Kwong and Wong, 1997; Jabber Al-Obaidi *et al.*, 2002). The *ETV6-RUNX1* fusion gene originates in the prenatal period as evidenced from the neonatal blood spots or Guthrie cards (Mori *et al.*, 2002; Greaves and Wiemels, 2003; Zuna *et al.*, 2011; Ma *et al.*, 2013). However, the fusion gene is unable to generate clinical leukaemia and additional genetic alterations are needed for the leukaemic transformation (see section 1.3.2). *ETV6-RUNX1* patients are usually characterised by younger age, low WCC levels with associated immunophenotypic expression of CD10+ and aberrant myeloid markers CD13/CD33 (Shurtleff *et al.*, 1995).

1.5.3 Structure and function of ETV6 and RUNX1 genes

The *ETV6* gene, consists of eight exons, encodes an ETS (E-26 transforming specific) family transcription factor and it is located at 11,802,788-12,044,558 bp (according to Ensembl version 62) on chromosome 12p13.1. *ETV6* gene was first cloned in 1994 as a

novel fusion partner to the *PDGFRb* locus in cases of chronic myelomonocytic leukaemia with t(5;12)(q33;p13) (Golub *et al.*, 1994) and has since been found rearranged with a number of tyrosine kinase genes, including *ABL1* and *JAK2* (Papadopoulos *et al.*, 1995; Lacronique *et al.*, 1997).

The encoded protein contains two functional domains: an N-terminal pointed (PNT) domain (also known as helix–loop–helix (HLH) domain) that is involved in protein-protein interactions with itself and other proteins (Golub *et al.*, 1994; McLean *et al.*, 1996; Jousset *et al.*, 1997), and a C-terminalDNA-binding domain (Ets domain) (Figure 1.12). The latter domain is homologous to all Ets proteins, recognizes a purine-rich GGAA/T core motif within promoters and enhancers of various genes (Golub *et al.*, 1994; Graves and Petersen, 1998) and participates in protein–protein interactions (Bassuk and Leiden, 1995; Batchelor *et al.*, 1998; Kim *et al.*, 1999). *ETV6* has a role in both angiogenesis and hematopoiesis and acts as a transcriptional repressor (Sawinska and Ladon, 2004).

Core binding factor (CBF) is a heterodimeric transcription factor that binds to the core element of many enhancers and promoters. *RUNX1*, located at position 36,160,098-37,357,047 bp (according to Ensembl v.62) on chromosome 21q22, encodes the alpha subunit of CBF that, together with the non-DNA-binding β subunit (CBFβ), forms a heterodimeric transcription factor. *RUNX1* was first identified through its fusion with the *ETO* gene in t(8;21)(q22;q22)-associated AML (Miyoshi *et al.*, 1991; Erickson *et al.*, 1992; Nisson *et al.*, 1992; Miyoshi *et al.*, 1993). RUNX1 proteins contain two functional domains: the N-terminal runt homology domain (RHD) and the C terminal transcription activation domain (TAD) (Figure 1.12). The former domain is required for binding to DNA and to the heterodimerization domain (HD) of CBFβ, whereas, TAD confers both increased DNA-binding affinity and stability to RUNX1 (Ogawa *et al.*, 1993; Wang *et al.*, 1993).

This gene is thought to be involved in the development of normal haematopoiesis by associating with transcriptional cofactors, repressors, and other DNA-binding transcription factors in a promoter context-dependent fashion (Licht, 2001; Durst and Hiebert, 2004). Chromosomal translocations involving this gene are well-documented and have been associated with several types of myeloid and lymphoid leukaemia.

1.5.4 Structure and mechanism of pathogenesis of the fusion gene

The chimeric fusion gene *ETV6-RUNX1* consists of the NH2-terminal part of the ETV6 protein including the dimerization PNT domain and all known functional regions of RUNX1, including its RUNT DNA binding domain (Figure 1.12) (Golub *et al.*, 1996; Berger, 1997; Greaves and Wiemels, 2003). In this respect, ETV6-RUNX1 fusion protein is structurally distinct from other RUNX1 fusion proteins, which retain only the aminoterminal RUNT domain and not the C-terminal regulatory sequences of RUNX1. The chromosomal breakpoints at ETV6 occur almost exclusively within the 15 kb intron 5, whereas the breakpoint cluster region (BCR) of RUNX1 includes the large 155 kb intron 1 or the 5.5 kb intron 2 (Greaves and Wiemels, 2003). Hence, variant transcripts of the fusion gene have been observed in the same patient on some occasions (Satake *et al.*, 1997; Codrington *et al.*, 2000; Tiensiwakul, 2004; Kustanovich *et al.*, 2006). However, the pathogenic effects of these variants are not yet fully understood.

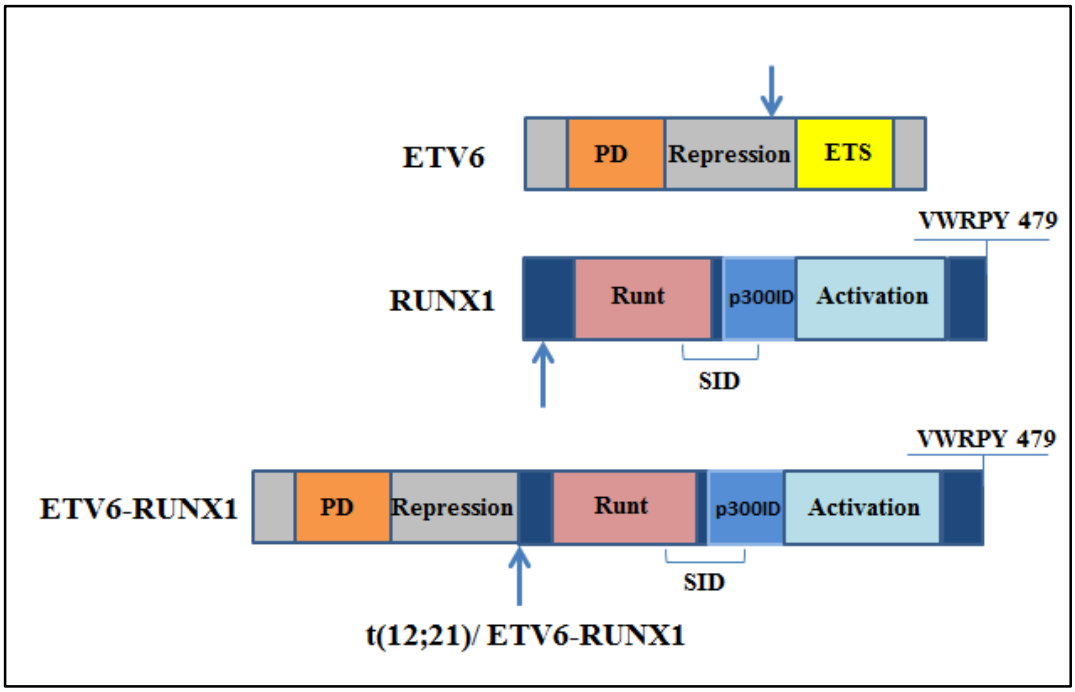

Figure 1.12 Diagrammatic representation of the structures of ETV6, RUNX1 and ETV6-RUNX1 proteins ETV6 protein composed of: an N-terminal pointed (PD) domain, central repression domain (repression) and a C-terminal DNA-binding domain (ETS domain). RUNX1 protein contains: the N-terminal runt homology domain (Runt), mSin3A interaction domain (SID) (which interacts with the p300 HAT (p300 ID)), the C terminal transcription activation domain (Activation) with attached motif namely Carboxy-terminal VWRPY to enable its binding to DNA. Arrows indicate the breakpoints in both ETV6 and RUNX1 in t(12;21) to form the ETV6-RUNX1 fusion protein. Figure was adapted from (Zelent et al., 2004).

The HLH and co-repressor domains of ETV6 and the RHD domain of RUNX1 are all required by ETV6-RUNX1 to alter haematopoiesis (Morrow *et al.*, 2007). The chimeric protein retains both HLH and TA structures which increase its affinity to proteins/co-repressors such as: N-CoR (component of the nuclear receptor corepressor complex with histone deacetylase activity), mSin3 and subsequent complex stability. Accordingly, ETV6-RUNX1–N-CoR or–mSin3 complexes influence the transcriptional activity of other genes including RUNX1 target genes (Figure 1.13) (Hiebert *et al.*, 1996; Fenrick *et al.*, 1999; Guidez *et al.*, 2000). Recently, a comprehensive study was carried out to globally investigate either direct or indirect impact of this fusion protein in a murine cell line that lacks any secondary abnormalities using collective tests of chromatin immunoprecipitation, mRNA expression and differential stable isotope labelling of proteins (Linka *et al.*, 2013). It has been proposed that the promoter regions of this chimeric gene are significantly different than those related to the wild type RUNX1 proteins, but still mediated by the Runt DNA-binding domain, and concomitant suppression of the mRNA transcription results in the downregulation of multiple genes targeting several cellular and biological processes, importantly SMAD-dependent TGF-β pathway resulting in impairment of the signal transduction. These findings further support a previous study (Ford et al., 2009) postulating that the selective expansion of these clones expressing ETV6-RUNX1 might be mediated in part by the inhibitory impact of ETV6-RUNX1 expression on the response to the transforming growth factor β (*TGF-β*). This inhibitory effect is facilitated through the binding of the chimeric gene to a principal signalling target of *TGF-β* (smad3). *TGF-β* is believed to function as a potent tumor suppressor via its Smad dependent inhibitory impact on *c-Myc* and its activation of p15INK4b/p15 and p21/p27KIP1 (Siegel and Massague, 2003; Bierie and Moses, 2006).

The erythropoietin receptor (*EPOR*) gene is expressed as a direct function of the ETV6-RUNX1 fusion protein, thus provides cell survival signals that may also contribute critically to persistence of covert pre-malignant clones in children (Inthal *et al.*, 2008; Torrano *et al.*, 2011). Furthermore, *ETV6-RUNX1* fusion gene has been shown to impair apoptosis through down-regulation of the microRNAs: miRNA-494 and miRNA-320a which in turn increases survivin expression (Diakos *et al.*, 2010). Downregulation of ETV6-RUNX1 expression has led to the inactivation of the phosphoinositide 3-kinase/AKT/mammalian target of rapamycin (mTOR) pathway (Fuka et al., 2011; Fuka et

al., 2012; Tijchon et al., 2013) which endorses the notion of being aberrantly activated in ETV6-RUNX1 BCP-ALL as suggested by different gene expression studies (Yeoh et al., 2002; Ross et al., 2003; Andersson et al., 2005; Gandemer et al., 2007). The mTOR pathway is known to be involved in various cellular processes including proliferation, protein synthesis and apoptosis.

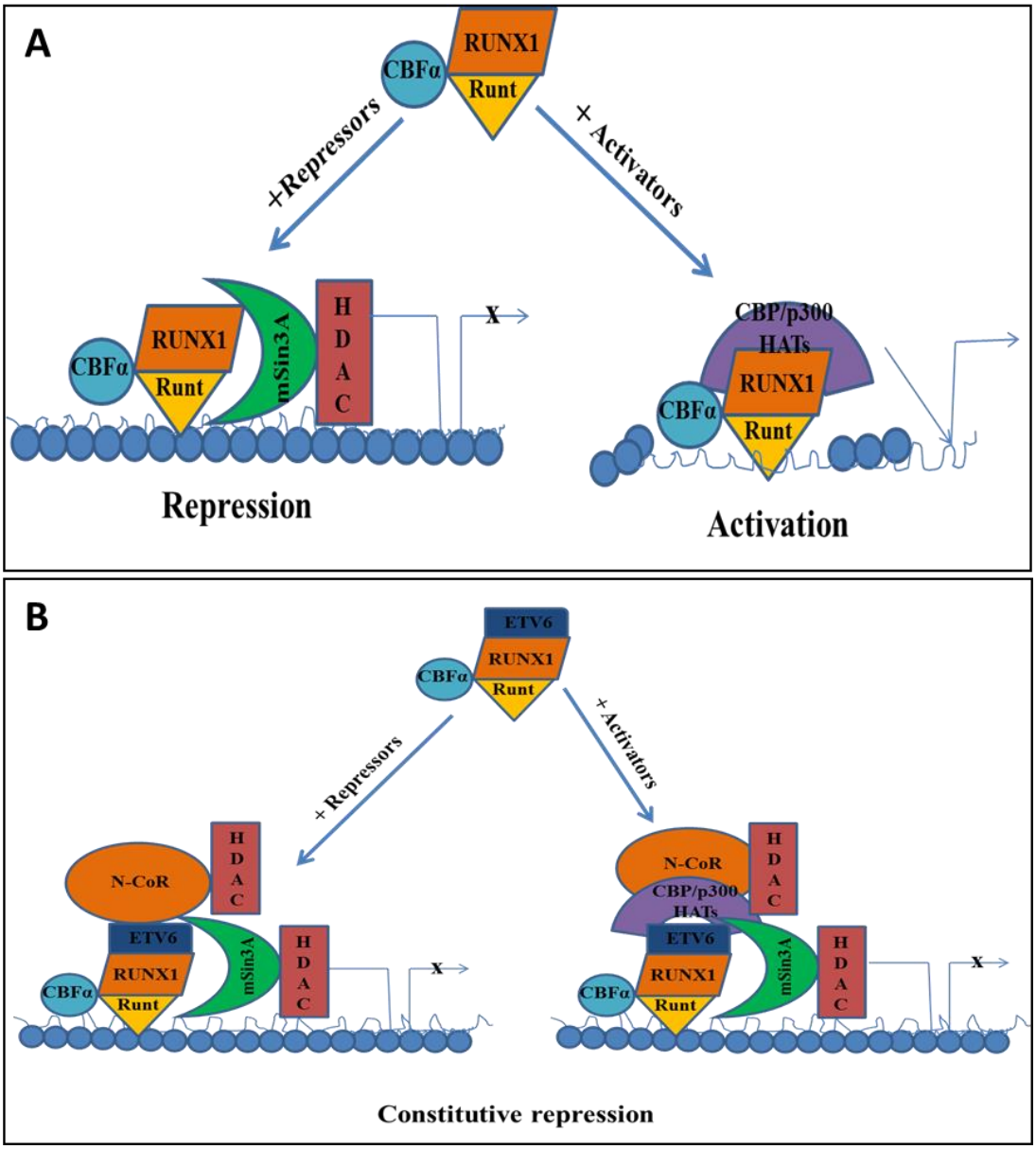

Figure 1.13 Diagrammatic representation of the hypothesized molecular mechanism of ETV6-RUNX1 protein. A. The activating and repressing function of RUNX1 protein is determined by its recruitment to p300 and mSin3A/HDAC, respectively. B. ETV6-RUNX1 fusion protein acts as a repressor complex via its interaction with N-CoR/ mSin3A co-repressors and this interaction is facilitated by the HLH (PD) structures of ETV6 protein. p300 may be capable to interact with the fusion protein but without exerting its activating function as seen in A. Figure was adapted from (Zelent et al., 2004).

1.5.5 Secondary genetic abnormalities

ETV6-RUNX1 requires secondary genetic aberrations to transform a pre-leukaemic clone into a clinical leukaemia. The translocation t(12;21) is mutually exclusive with other well-known BCP-ALL associated genetic abnormalities such as high hyperdiploidy (>50 chromosomes) and other rearrangements including *BCR-ABL1* and *TCF3-PBX1* fusions,

and *MLL* rearrangements (Cayuela et al., 1996; Fears et al., 1996; Borkhardt et al., 1997; Raimondi et al., 1997; Rubnitz et al., 1997; Eguchi-Ishimae et al., 1998; Raynaud et al., 1999; Andreasson et al., 2000; Tsang et al., 2001; Uckun et al., 2001; Attarbaschi et al., 2004). Although co-existence of t(12;21) and high hyperdiploidy has been reported, it is extremely rare and it is likely to reflect a *ETV6-RUNX1* sub-clone with an unusual number of extra chromosomes rather than true coexistence (Moorman et al., 2010b). Two groups reported a significant association between near- tetraploidy (80 or more chromosomes) and *ETV6-RUNX1* which represents the doubling-up of a pseudodiploid *ETV6-RUNX1* clone (Attarbaschi et al., 2006; Raimondi et al., 2006). There are other secondary alterations associated with this ALL subtype including: copy number alterations (CNAs), sequence mutations and DNA methylation.

1.5.5.1 Copy number alterations (CNAs)

Recurrent CNAs target various pathways such as lymphoid development, tumor suppression, cell cycle, apoptosis, lymphoid signalling, nuclear hormone response and drug responsiveness (Table 1-8) (Kuiper *et al.*, 2007; Lilljebjorn *et al.*, 2007; Mullighan *et al.*, 2007; Tsuzuki *et al.*, 2007; Kawamata *et al.*, 2008; Mullighan *et al.*, 2008a; Parker *et al.*, 2008; Lilljebjorn *et al.*, 2010). It is worth noting that alterations affecting nuclear hormone response, involved in transcriptional repression or glucocorticoid/mineralocorticoid sensitivity, include *TBL1XR1* and *NR3C1*/*NR3C2*, respectively and are characteristic features of *ETV6-RUNX1* as compared to other BCP-ALL subtypes (13% v 0.69%, 6% v 2%, 9% v 0.69%, respectively) (Mullighan *et al.*, 2007; Lilljebjorn *et al.*, 2010). They present in 24% of *ETV6-RUNX1* cases, thus this pathway is targeted equally as the B- cell development pathway which involves lesions in *PAX5, EBF1* and *TCF4*.

Overall, 79% of *ETV6-RUNX1* patients exhibit at least one genetic alteration with the majority (74%) of them displaying a unique pattern among individual patients with a mean of 3.5 CNA per case (range 0-13), emphasising the characteristic genetic diversity (Mullighan *et al.*, 2007; Lilljebjorn *et al.*, 2010). The most common abnormalities include gene deletions that represent 74-82% of these aberrations, while the remaining lesions are gains.

Focal deletions (sized less than 1Mb) encompass one or two genes and constitute 40% of the total gene deletions. These focal lesions most commonly affect *ETV6, CDKN2A/B*, *PAX5, BTG1, BMF, CD200/ BTLA, TBL1XR1, NR3C1, NR3C2* and *EBF1* and they differ in their frequencies within this ALL subgroup (Table 1-8).

The remaining (60%) deletion cases compromise large regions (sized more than 1Mb) of different chromosomes including 12p, 6q, 9p, 11q and 13q which are present in 39%, 13%, 10%, 10% and 10% of *ETV6-RUNX1* cases, respectively.

On the other hand, most of the gains (98%) affect larger regions (sized more than 1Mb) or the whole chromosome. The most common alterations include duplicated chromosome Xq, gain of chromosomes 21, der(21)t(12;21), 10 or 16 (Table 1-8) (Lilljebjorn *et al.*, 2010).

Abnormality	Cytoband	Function	Frequency%
Deletions			
ETV6	12p13	Transcriptional repressor	59
CDKN2A/B	9p21	Cell cycle regulator	22
PAX5	9p13.2	B-cell differentiation	20
RAG1/2	11p13	Activation of immunoglobulin V-D-J recombination	14
CD200/BTLA	3q13.2	Immune system or during B-cell differentiation	13
TBL1XR1	3q26.32	Nuclear hormone receptor transcriptional repression via the SMRT/N-CoR complex	13
BTG1	12q21.33	Co-activator of cell differentiation	13
NR3C1	5q31.3	Nuclear hormone receptors binding glucocorticoids	13
NR3C2	4q31.23	Nuclear hormone receptors binding mineralocorticoid/ glucocorticoids	11
EBF1	5q34	Transcriptional activator	11
FHIT	3p14.2	Induction of apoptosis	8.5
TOX	8q12.1	T-cell development regulator	8.5
BMF	15q15.1	Anti- or pro-apoptotic regulators	6
Abnormality	**Genes**	**Pathogenesis**	**Frequency%**
Gains			
dup(Xq)	*SPANXB, HMGB3, FAM50A, HTATSF1* and *RAP2C*	Undetermined	16
der(21)t(12;21)	Multiple	Increase potency of *ETV6-RUNX1* fusion gene	12
21		Undetermined	12
10			9
16			9

Table 1-8 Common recurrent copy number alterations in *ETV6-RUNX1* patients. Gene functions were obtained from gene cards, frequencies were extracted from (Mullighan *et al.*, 2007; Lilljebjorn *et al.*, 2010).

The most prevalent alteration is *ETV6* deletion which occurs in two thirds of *ETV6-RUNX1* cases. This gene acts as a transcriptional repressor (see section 1.5.3). It has been proposed that heterodimerization between ETV6 and ETV6-RUNX1 proteins hampers the function of the fusion protein, thus loss of the non-rearranged *ETV6* would increase the ratio of ETV6-RUNX1 over normal ETV6 proteins which in turn increases the potency of the fusion protein (McLean *et al.*, 1996).

Duplicated chromosome Xq (dup(Xq)) is predominately found in males and the testis-specific gene *SPANXB* was the only gene showing a high and uniform overexpression,

irrespective of gender and presence of dup(Xq) in *ETV6-RUNX1*. Thus, the *SPANXB* gene might be considered as a target of this duplication and it is normally involved in spermatogenesis (Lilljebjorn *et al.*, 2007; Lilljebjorn *et al.*, 2010).

Interestingly, there is a strong associated co-occurrence between trisomy 10 and trisomy 16 ($p<0.001$) which was explained by their tendency to occur in cases with acquired random chromosome gains ($p<0.001$) (Lilljebjorn *et al.*, 2010). The acquisition of gained chromosomes may arise sequentially or through a single abnormal mitosis.

1.5.5.2 Sequence mutations

Whole genome and exome sequencing have enabled better understanding of leukaemogenic pathogenesis by identifying different somatic mutations which might be clinically important. Previous sequencing studies have revealed distinct patterns of somatic mutations in different signalling pathways including B- cell development/ differentiation, Ras signalling, cytokine receptor/ kinase (JAK/STAT), *TP53/RB1* tumour suppression and epigenetic regulators (see section 1.4.1.4) (Zhang *et al.*, 2011; Lilljebjorn *et al.*, 2012; Roberts *et al.*, 2012; Holmfeldt *et al.*, 2013; Mullighan, 2013; Papaemmanuil *et al.*, 2014). The frequency and nature of these mutations vary across different ALL subtypes. Although previous sequencing studies have focussed on high risk ALL groups (Zhang *et al.*, 2011; Roberts *et al.*, 2012; Holmfeldt *et al.*, 2013; Mullighan, 2013), some other studies were conducted on standard risk groups including *ETV6-RUNX1* subtype (Lilljebjorn *et al.*, 2012; Jaffe *et al.*, 2013; Papaemmanuil *et al.*, 2014). A low number of recurrent coding-region and kinase mutations was found in *ETV6-RUNX1* cases, unlike high risk subtypes of ALL.

Of the commonly deleted genes in *ETV6-RUNX1*, there are five genes including *ETV6, BTG1, TBL1XR1, PAX5* and *CDKN2A* which are targeted by deletions or inactivating somatic mutations (Papaemmanuil *et al.*, 2014). Furthermore, *ATF7IP*, *MGA* and *STAG2* genes undergo different mutational processes (deletions, inter-chromosomal rearrangements and inactivating mutations) which highlight possible roles in leukaemogenesis. These genes play important roles in mediating heterochromatin formation/ transcriptional repression, regulating the expression of Max network and T-box family target genes (e.g. *MYC*) and being a component of the cohesion complex, respectively.

Interestingly, there are other significant recurrent activating mutations in *ETV6-RUNX1* cases encompassing important genes namely: *NRAS, KRAS and SAE1* that each occurs in 6% of *ETV6-RUNX1* cases (Papaemmanuil *et al.*, 2014). In addition, activating mutation of WHSC1 (NSD2) was found in *ETV6-RUNX1* cases and constituted 14% of cases (Jaffe *et al.*, 2013). NSD2 protein acts as histone methyltransferase that is involved in transcription regulation. Other mutations target *FLT3, MCAM* and *RUNX1T1* genes at lower rates in *ETV6-RUNX1* cases and they might be of a particular role owing to their occurrence in different types of cancer (Lilljebjorn et al., 2012).

1.5.5.3 Epigenetics

Generally, each ALL subtype exhibits one unique methylation signature which distinguishes it from other ALL subtypes (Busche *et al.*, 2013; Nordlund *et al.*, 2013). In these two studies, there were 2114 differentially methylated CpG sites affecting 156 genes which are unique to *ETV6-RUNX1*. Of these genes, several hypomethylated genes with consistent upregulation in expression targeting *DSC3, EPOR, FUCA1, HLA-DPB1, TCFL5, NRN1, IGF2BP1, BEST3, SOX11* and *TMED6* are suggested as potential candidate targets owing to their roles in leukaemogenesis. In addition, *SPSB1* was the only gene that was characterised by hypermethylation with concordant downregulation in *ETV6-RUNX1* cases, but it is not known to be related to leukaemia.

Of these candidate hypomethylated genes, EPOR expression is already described as a direct function of *ETV6-RUNX1* fusion gene and it is responsible for cell survival (see section 1.5.4) (Inthal *et al.*, 2008; Torrano *et al.*, 2011). High expression of IGF2BP1 plays a role in elevating MYC expression levels, this in turn resulting in the enhancement of tumour proliferation (Kobel *et al.*, 2007). Furthermore, down-regulation of TCFL5 was reported to hamper the cell viability of human colorectal cancer cell lines (Sillars-Hardebol *et al.*, 2012).

It is worth noting that epigenetic modifications can extend to involve the microRNA genes and there are significant hypermethylated miRNA genes including miRNA-320a, miRNA-494 and miRNA-200c which are identified in *ETV6-RUNX1*. Subsequent down-regulation of both miRNA-320a and miRNA-494 results in enhancement of the antiapoptotic signalling pathway via their inhibition of survivin (see section 1.5.4) (Diakos *et al.*, 2010). miRNA-200c acts as a strong tumour suppressor gene which

modulates the expression of BMI1 (Shimono *et al.*, 2009). BMI1 plays an essential role as a regulator of stem cell self-renewal.

1.5.6 Clonal heterogeneity

The clonal heterogeneity with a high degree of genetic variability is one of the driving mechanisms of tumour development and progression. The resultant genetic heterogeneity is a product of the non-linear (branching) evolution of malignant clones, driven by the Darwinian natural selection process, that can extend to include the epigenetic alterations (Marusyk and Polyak, 2010). This genetic diversity aids further proliferation, migration, invasion and metastasis of the malignant cells enabling them to adapt to any hostile environment. In spite of the existence of this heterogeneity in many cancer types including leukaemia, breast, colon, prostate cancer and other various types of malignancies (Maley *et al.*, 2006; Campbell *et al.*, 2010; Park *et al.*, 2010), the exact biology of this important genetic entity still to be resolved. *ETV6-RUNX1* BCP-ALL subtype is one of the clear examples of the presence of the clonal heterogeneity (Mullighan *et al.*, 2008b; Lilljebjorn *et al.*, 2010). Furthermore, treatment may influence the genetic diversity of subclones owing to their different responses to chemotherapeutic agents leading to expansion of resisting clones along with extinction of more sensitive clones. The resistant clones acquire further different mutations that undergo through selection process, similar to the initial diagnosis, and constitute the basis of relapse. In addition, the relapsed leukaemia has been suggested to emerge as being unrelated to the initial diagnostic clones in small number of cases (6%) using solely the whole genomic studies (Figure 1.14) (Mullighan et al., 2008b).

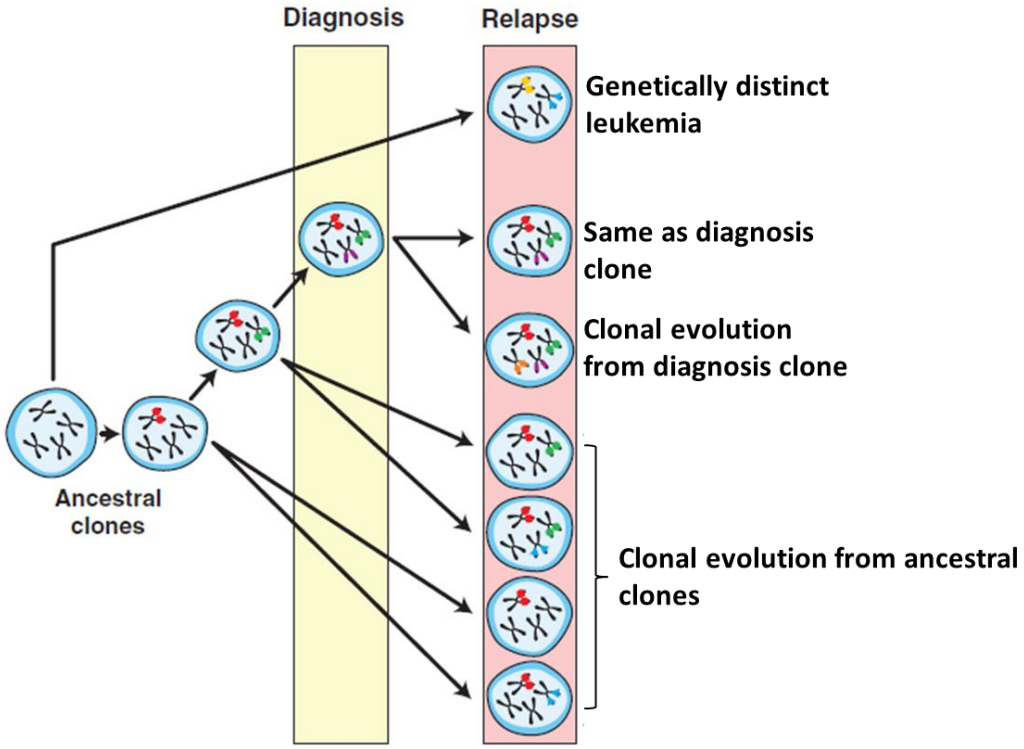

Figure 1.14 Clonal origins of relapsed acute lymphoblastic leukaemia. The majority of relapse cases originate either from the diagnosis (42%), acquiring additional genetic lesions or from an ancestral clone (52%) in which the relapse clone acquires new lesions while retaining some but not all of the lesions found in the diagnostic sample. In only a minority of ALL cases (6%), the relapse clone represents the emergence of a genetically distinct and thus unrelated second leukaemia. Figure was taken from (Mullighan *et al.*, 2008b).

1.5.7 Prognostic impact of ETV6-RUNX1 and its associated genetic alteration

Soon after the identification of *ETV6-RUNX1* fusion gene, it was reported that patients with this fusion had an excellent prognosis (Shurtleff et al., 1995). Since then, numerous studies have investigated several risk factors in this ALL subtype generating inconsistent outcome data owing to different initial risk stratification criteria with subsequent dose intensity treatment. Thus, a high incidence rate of relapses (20%) was initially reported and almost half of ALL relapse cases which occurred ≥ 5 years from diagnosis (late relapses) harboured *ETV6-RUNX1* (Harbott *et al.*, 1997; Forestier *et al.*, 2008a). However, another childhood trial namely UKALLXI indicated that *ETV6-RUNX1* had no effect on outcome (Hann et al., 2001). On modern protocols, the presence of the *ETV6-RUNX1* gene fusion correlates with an excellent outcome that can be explained by the introduction of higher doses of asparaginase in their treatment protocols (Table 1-9) (Loh *et al.*, 2006; Rubnitz *et al.*, 2008; Pui *et*

al., 2009; Conter *et al.*, 2010; Moorman *et al.*, 2010b). *ETV6-RUNX1* positive lymphoblasts are highly sensitive to asparaginase *in vitro* (Ramakers-van Woerden et al., 2000). A Lower rate of relapses (13%) was recorded in *ETV6-RUNX1* patients who were treated on UKALL97/99 after a median follow-up time of 8.2 years (Moorman *et al.*, 2010b). *ETV6-RUNX1* relapses occurred mostly late (> 6 months after end of treatment) (Harbott *et al.*, 1997; Forestier *et al.*, 2008a)

Study	Years	No. cases	Age	EFS at 5 years	Survival at 5 years
Total Therapy XV study (Pui *et al.*, 2009)	2000-2007	96	1-18	98%	99%
AIEOP-BFM ALL 2000 (Conter *et al.*, 2010)	2000-2006	762	1-18	95%*	NA
UKALL97/99 (Moorman *et al.*, 2010b)	1997-2002	368	1-18	89%	96%
DFCI ALL Consortium Protocol 95-01 (Loh *et al.*, 2006)	1996-2000	77	1-18	89%	97%
POG ALinC 16 (Rubnitz *et al.*, 2008)	1995-1998	244	1.4-19.7	86%	NA

Table 1-9 Survival rates of *ETV6-RUNX1* patients from selected modern clinical trials. EFS event free survival rate, OS overall survival rate, * EFS was estimated for those with MRD standard risk (n=440), while those with MRD intermediate (n=312) or high risk (n=10), EFS was estimated to be 82% or 55%, respectively.

Turning to the secondary abnormalities that usually accompanied this ALL subtype; it is worth noting that the prognostic impact of these alterations is still debatable. Several studies have been conducted to investigate this issue but small patient cohorts who were treated with different treatment protocols hampered precise evaluation (Attarbaschi *et al.*, 2004; Stams *et al.*, 2006). For example, investigating the prognostic effect of the abnormalities involving normal chromosome 12 or 21 and der(21)t(12;21) was the main focus in most studies and conflicting data was generated (Table 1-10) (Attarbaschi *et al.*, 2004; Stams *et al.*, 2006; Peter *et al.*, 2009).

Study	Comments
(Attarbaschi *et al.*, 2004)	The non-rearranged *ETV6* allele influence adversely the outcome
(Stams *et al.*, 2006)	The subgroups with either an additional *ETV6/RUNX1* fusion gene or without any secondary aberration involving the *ETV6* and *RUNX1* genes did worse in terms of disease-free survival
(Peter *et al.*, 2009)	High frequencies of additional aberrations of *ETV6* or *RUNX1* genes, with the exception of *ETV6* deletions in relapsed than in diagnostic samples

Table 1-10 Conflicting results of the prognostic effect of the abnormalities involving *ETV6* or *RUNX1* genes from selected study groups.

1.6 Summary and aims

The translocation, t(12;21)(p13;q22)/*ETV6-RUNX1*, is a transforming event and responsible for the generation and maintenance of a pre-leukaemic clone (Hong *et al.*, 2008). The high rate detection in the cord blood of healthy neonates, the high discordance rate for BCP-ALL among monozygotic twins and the latency period indicate that postnatal genetic alterations are needed to promote the leukaemic transformation (Wiemels *et al.*, 1999; Mori *et al.*, 2002). Typically this cytogenetic subgroup is found in 25% of BCP-ALL cases, with a peak incidence between the ages of 3–6 years (Golub *et al.*, 1995; Romana *et al.*, 1995a). This cytogenetic subgroup is usually associated with favourable outcome but relapses do occur at a rate of 13% (Moorman *et al.*, 2010b). Interestingly, ETV6-RUNX1 fusions transcripts are rarely found in the adolescent/adults, infants and DS.

The primary objective of this project was to investigate the secondary abnormalities and clonal evolution in *ETV6-RUNX1* positive acute lymphoblastic leukaemia. The strategies for achieving this were:

1. To quantify copy number of interesting genes associated with *ETV6-RUNX1* BCP-ALL and to identify their incidence in different UK ALL trials utilising Multiplex ligation-dependent probe amplification (MLPA).

2. To identify and characterise additional genetic abnormalities in *ETV6-RUNX1* BCP-ALL involving the derivative 12 der(12)t(12;21).

3. To evaluate the potential relevance of specific gene copy number alterations in the relapse cases and to back-track FISH some of the important genetic lesions from relapse to diagnosis and vice versa.
4. To investigate infants, young adults, those with Down syndrome that might constitute atypical group of patients with unique spectrum of abnormalities.

Chapter 2. Materials and Methods

2.1 General chemicals and Equipment

All details and specifications are standard unless stated otherwise. General lab chemicals and equipment were purchased from Scientific Laboratory Supplies (Nottingham, UK), VWR (Lutterworth, Leicestershire, UK) or Fisher Scientific (Loughborough, Leicestershire, UK), Sigma-Aldrich Company Ltd (Poole, Dorset, UK) and STARLAB (UK), Ltd.

2.2 Patient Cohorts and Material

A total of 423 patients included in this study were confirmed cases of *ETV6-RUNX1* positive BCP-ALL identified by either fluorescence in situ hybridisation (FISH) or quantitative PCR (qPCR) and treated using either UKALL97/99 (April 1997- June 2002) (n=147) for age group 1-18 years (Vora et al., 2006) or UKALL2003 (October 2003- July 2011) (n=271) including children and young adults with age range of 1-25 years. To note, the young adults were added in September 2006 up to the age of 20 years and then the upper age limit increased to 25 years in June 2008 (Qureshi et al., 2010). As per National Cancer Institute (NCI) criteria: patients were classified as standard risk if they were aged 1-9 years with a WCC < 50 $x10^9$/L at initial presentation, whereas those of >= 10 years or a WCC >= 50 $x10^9$/L were considered as high risk. Further cases were added from those treated on UKALLXII protocol (n=5). This trial recruited patients in the age range 15- 55 years registered between 1993 and 2006 (Rowe et al., 2005). In addition, treating clinicians obtained approval from the local ethical committee and written informed consent was given by patients or parents/ guardians on entry to the clinical trials. The demographic and clinical details are described in the relevant results chapters. This study used fixed cell suspensions (see section 2.5) and DNA (see section 2.6), prepared from diagnostic bone marrow material of each patient sample.

2.3 Cytogenetic analysis

The identification of the chromosomal abnormalities is pivotal in the diagnosis and risk stratification of acute lymphoblastic leukaemia. Standard karyotyping has a resolution of 5-10 Mb. However, the low mitotic index and the poor chromosomal morphology represent obstacles in the chromosomal analysis of the leukaemic cells. In addition, karyotyping is labour-intensive and requires culturing of cells.

The samples and the relevant karyotype data were provided by the UK Cancer Cytogenetics Group (UKCCG) laboratories. Karyotypes were reviewed by the cytogeneticists of the Leukaemia Research Cytogenetics Group (LRCG). The Clinical Trial Service Unit and Epidemiological Studies Unit (CTSU) supplied patient details as previously described (Harrison *et al.*, 2001; Moorman *et al.*, 2010b). The final karyotypes, accompanied by clinical and patient details were entered to the LRCG database enabling the storage, retrieval and analysis of data.

2.4 Cell lines

All cell lines, kind gifts of different group members, were received as growing cells in suspension and were maintained in culture in RPM1-1640 growth medium, HEPES-modified, supplemented with 2 mM L-Gln and 10% FCS. Further processing was done to produce fixed cells (see section 2.5) and DNA (see section 2.6) to be used for confirming specific deletions by FISH and as positive controls for real time qPCR experiments, respectively. Specific features of the cell lines are described in more detail in the relevant results chapters. Cell lines used in this study included:

1) Human BCP-ALL REH cell line which was derived from the peripheral blood of a 15-year-old female patient in relapse, positive for t(12;21) translocation and expressing the *ETV6-RUNX1* fusion gene (Rosenfeld *et al.*, 1977; Matsuo and Drexler, 1998).

2) The KG1 cell line was established from the bone marrow of a 59-year-old Caucasian male with erythroleukaemia that evolved into AML (Koeffler and Golde, 1978; Koeffler and Golde, 1980).

3) The Kasumi-1 cell line was derived from the peripheral blood of a 7 year old boy with AML in 2nd relapse after bone marrow transplantation, positive for t(8;21)(q22;q22) rearrangement and expressing RUNX1-RUNX1T1 (AML1-ETO) (Asou *et al.*, 1991).

2.5 Harvesting cells for FISH

Cells in suspension were treated with 0.075M potassium chloride (KCl) in a tube, incubated for 15 minutes at 37 °C, then centrifuged at 1500 rpm for 7 min. Supernatant

was then discarded, and pellet re-suspended. Fresh 3:1 Methanol: Acetic Acid fixative was added a few drops at a time with constant mixing to avoid clumping and further fixative added to 7ml. The fixative washing steps were repeated and the sample was stored in at -20 °C.

2.6 DNA extraction

DNA was extracted from either viable or fixed cells using the Qiagen DNeasy kit (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. Concentrated DNA stocks were stored at -80°C. The extractions were performed by members of the LRCG team including myself. It is worth noting that the DNA extracted from the viable cells is preferable than those extracted from fixed cells especially for the use of single nucleotide polymorphism (SNP) genotyping (see section 2.10).

2.7 DNA quantification using the NanoDrop-1000

DNA concentration and purity were measured using the Nanodrop spectrophotometer (Thermo Scientific) by determining the amount of light that a sample absorbs. It is able to measure DNA concentration in a sample volume of 1 µl. DNA efficiently absorbs UV light, with an absorption maximum at 260 nm. Thus, contaminants such as proteins and carbohydrates impact negatively on the purity of DNA by affecting the absorbance rate owing to their different absorption maximums. The amount of the contaminants is measured using the optical density (OD) of the sample at wavelengths of 260 and 280 nm, and reports a nucleic acid concentration as well as an OD260 / OD280 ratio. The optimum working concentrations differ between different techniques such as multiplex ligation- dependent probe amplification (MLPA), SNP genotyping and qPCR. In MLPA and qPCR, an optimum concentrations of 20ng/µl (4-100ng/µl acceptable) and 5ng/µL are required, respectively, while in SNP arrays, 750ng of genomic DNA at a concentration of 50ng/µl should be provided. However, in general, the 260/280 ratio should be ~1.8 and a ratio <1.5 indicates protein contamination.

2.8 Fluorescence *in situ* hybridisation (FISH)

FISH provides a powerful molecular cytogenetic technique that has higher resolution compared to the classic cytogenetic banding techniques (interphase FISH > 20 kb,

metaphase FISH > 100 kb v standard karyotyping (5-10 Mb) (see section 2.3). It has the ability to specifically target genomic regions of interest in either dividing or non-dividing cells as well as *in situ* tissue preparations. It is based on the principle that the denaturation of the fluorescently labelled DNA probe and the complementary target DNA sequence allows the annealing of complementary DNA sequences (Figure 2.1). Thus, the target DNA can be visualized using a fluorescence microscope. This assay is useful in wide range of applications including enumeration of chromosomes and detection of different chromosomal rearrangements (e.g. translocations, deletions, inversion) that are below the resolution of conventional cytogenetic methods (i.e. g-banding). Furthermore, it enables the use of the panel of disease- specific FISH probes for risk stratification/ therapeutic management, quantitation of minimal residual disease, detection of cytogenetic remission/ relapse, monitoring cross-sex bone marrow transplantation patients for engraftment status (chimerism). It allows identification of chromosome aberrations irrespective of the cell cycle stage. In addition, it has the advantage of maintaining tissue architecture; however, the nuclear truncation artefact and overlapping cells can make the analysis difficult.

There are different types of FISH probes that are designed for specific regions of a chromosome. For the purpose of this project, three different DNA probe types were used: 1) locus specific, 2) subtelomeric and 3) Alpha satellite (centromeric) probes. These probes are described in the relevant results chapters. Locus specific probes can identify reciprocal translocations, gene deletions, amplifications and chromosome inversions and in this study, most of these probes were derived from BAC clones. In contrast, the subtelomeric and centromeric probes were ordered as predesigned commercially available probes (Vysis and Cytocell, respectively). The subtelomeric probes target the most distal unique sequences of a chromosome and they are useful for chromosome enumeration and integrity, while the centromeric probes recognize a highly repetitive alpha-satellite DNA sequence located at the centromere of each chromosome.

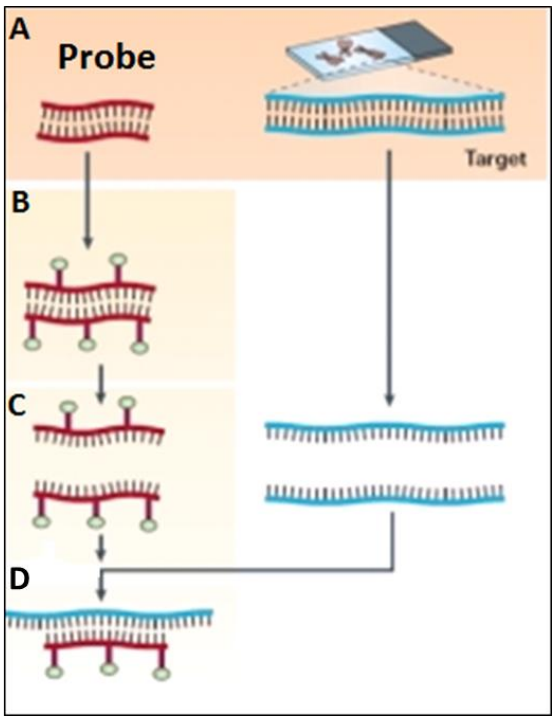

Figure 2.1 Principles of Fluorescence *in situ* hybridisation (FISH). A. a DNA probe and a target sequence are basic elements of FISH, B. The probe is composed of nucleotides that have been directly modified to contain fluorophores (white circles), C. Denaturation occurs in both the labelled probe and the target sequence, D. Annealing of complementary DNA sequences. Figure was taken from (O'Connor, 2008).

2.8.1 Bacterial artificial chromosome (BAC) clones selection

Bacterial artificial chromosome (BAC) clones were mainly used in this study as they produced strong FISH signals. Fosmid probes were occasionally used. BAC clones are larger than fosmid clones and they contain inserts of approximately 100 kb, whereas fosmid clones have inserts of up to 40 kb in size. These locus specific clones were selected using both Ensembl and Human Genome Browser Gateway (UCSC) genome browsers. They were purchased from Roswell Park Cancer Institute (New York, USA) and BACPAC resources (California, USA) and were received as bacterial stabs that were stored at 4°C. Some clones were already available in house and stored as glycerol stocks at -80°C.

2.8.2 Culturing BAC clones

Bacteria from the bacterial stabs or glycerol stocks were streaked using a sterile inoculation loop onto an agar plate (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, 1.5% (w/v) agar in deionised water) containing 12.5µg/ml chloramphenicol.

These plates were incubated overnight at 37°C. The antibiotics were important to prevent loss of the plasmid carrying the target DNA or the selection of faster- growing mutants. The following day, a single colony was incubated in 200ml of growth medium (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl) supplemented with 12.5µg/ml chloramphenicol and was kept overnight in a shaking incubator at 37°C (C24 Incubator Shaker – New Jersey, USA).

2.8.3 DNA extraction

DNA was extracted from the cell culture using Nucleobond Xtra Midi kit (Clontech laboratories, Inc., California, USA) according to manufacturer's instructions. The resultant DNA was stored at -20°C after they were quantified in terms of concentrations and purity using the NanoDrop-1000 as previously described in section 2.7. The concentration of the DNA should be at least 57ng/µl for probe labelling procedures.

2.8.4 Nick Translation (probe labelling)

Direct labelling of probes was carried out to introduce labelled nucleotides into the probe sequence and to decrease the size of the probe DNA to an optimum range of 100-500 base pairs (bp) to yield good signals. The preferred method used was Nick Translation (Abbott Diagnostics, Illinois, USA) and the procedures were followed according to the manufacturer's instructions. The labelled probes were stored at -20°C. The following steps were followed according to a previously published protocol with slight modifications highlighted below (Schwab and Harrison, 2011).

2.8.5 Slide Preparation/ Hybridisation

Both cell pellets and slides were washed in fresh fixative (3:1 Methanol: Acetic acid), then the cell suspension was dropped onto the slide. For good results, the cells should appear grey and flat with no overlapping between cells but at a sufficient high density for easy scoring on high power. The cells were allowed to age by placing slides onto a hot plate at 60°C for 10-15 minutes. The commercial probes and hybridisation buffer (Cytocell) were combined in a 1:10 dilution, while Home-grown probes were diluted in a 1:1 ratio and added to the aged slide. The slides were covered by coverslips which were sealed with rubber cement to prevent the cells drying out and slides were

transferred to a temperature controlled hotplate (Hybrite, Abbott Diagnostics, Illinois, USA) at 72°C for 5 minutes (commercial probes) or 75°C for 5 minutes (home-grown probes) to co-denature the probe and DNA, followed by hybridisation at 37°C O/N.

2.8.6 Post-hybridisation washes/ Counterstaining

Post-hybridisation washing is the process of stringent cleaning aimed at removing background signals which result from the creation of weak bonds from the non-specifically bound probe during hybridisation. The slides were soaked in 20X SSC solution (3M NaCl, 300mM trisodium citrate – Invitrogen) diluted 1:10 in deionised water in order to remove the coverslips. The slides were then soaked in Wash 1 solution (2% (v/v) 20X SSC, 0.3% (v/v) NP40 in deionised water) for 2 minutes at 72°C. Then they were soaked in Wash 2 solution (10% (v/v) 20X SSC, 0.1% NP40 in deionised water) for a further 2 minutes at room temperature and they were removed from the wash and air dried. Vectashield mounting medium, containing 4', 6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Peterborough, UK), was applied to the slides as a counterstain to enable visualization of the signals.

2.8.7 Analysis

Slides were examined using an Axioplan fluorescence microscope (Karl Zeiss, Germany) or Olympus BX61 equipped with appropriate filters and Cytovision software (Leica Microsystems, UK). However, automated capture was carried out in cases with poor FISH signal intensities. The automated capture allows the user to select any area in the slide with good density of cells, thus it results in the optimization of the scanned area allowing the classification and scoring of cells automatically.

2.8.8 Establishment of the cut-off values and algorithm strategy for FISH review

It is vital to ensure the accuracy of the generated results, thus cut-off values for each probe set were determined in order to reduce the impact of false positive results. In this study, three bone marrow samples from normal individuals were hybridised with each individual probe and a minimum of 100 nuclei were scored. The false positive rate for each probe in each normal control was calculated by dividing the number of cells displaying a non-normal pattern by the total number of cells analysed. The cut-off level

is the mean false-positive rate plus 3x standard deviations. This was determined for each probe set, apart from the mapping deletion probes described in Chapter 4.

For all FISH tests, a minimum of 50-100 interphase cells were scored (by myself) and, with the exception of deletion mapping, were independently checked by a colleague. Checking for deletion mapping was carried out only for probes delineating the boundaries of the deletion. The different cut off values and algorithms used for each individual probe set are described in the relevant chapters.

2.9 Multiplex Ligation- Dependent Probe Amplification (MLPA)

2.9.1 Overview

Multiplex Ligation- dependent Probe Amplification is a polymerase chain reaction (PCR) based method for detecting copy number changes in DNA sequences down to the level of a single nucleotide. Unlike standard multiplex PCR, MLPA is able to detect copy number changes of multiple targets (more than 40 different small (50 bp) DNA sequences) with a single primer pair in a single reaction. MLPA shows high concordance with FISH (Coll-Mulet *et al.*, 2008; Schwab *et al.*, 2010). The advantage is that it is a low cost simple technique that has a higher throughput than FISH. MLPA can also identify focal genetic aberrations which are below the resolution of FISH. In this study, the SALSA MLPA kit P335 *IKZF1* (MRC Holland, The Netherlands) was used to determine the copy number of those genes most frequently deleted in BCP-ALL: *ETV6, BTG1, PAX5*, *CDKN2A/B, IKZF1, RB1, EBF1* and genes within the pseudoautosomal region (*PAR1*): *CRLF2, CSF2RA and IL3RA* as described previously (Schwab *et al.*, 2010). Those cases with potential *IKZF1* deletions were further investigated using the P202 *IKZF1* kit that provided more extensive coverage of this gene and its adjacent regions. It was used to confirm deletions in cases with single probe deletions detected by the P335 kit. Genomic DNA from healthy donors was used as control samples.

2.9.2 Experimental set-up

The MLPA probes, composed of two separate nucleotide sequences each with either forward (Y) or reverse (X) primer sequence attached, were hybridized to the target sequences after denaturation (Figure 2.2, Table 2-1). It is worth noting that the forward

primer is fluorescently labelled so that the amplicons can be visualized using capillary electrophoresis. In addition, one of the probes contains a stuffer sequence that varies in length so that a range of up to 50 targets can be amplified and separated by size in a single experiment. The hybridized MLPA probes undergo ligation and only ligated probes are exponentially amplified during a PCR reaction that uses a single PCR primer pair, enabling the identification of copy number changes of multiple targets. The amount of ligated product represents the number of target sequences in the sample. The steps were followed according to the manufacturer's instructions.

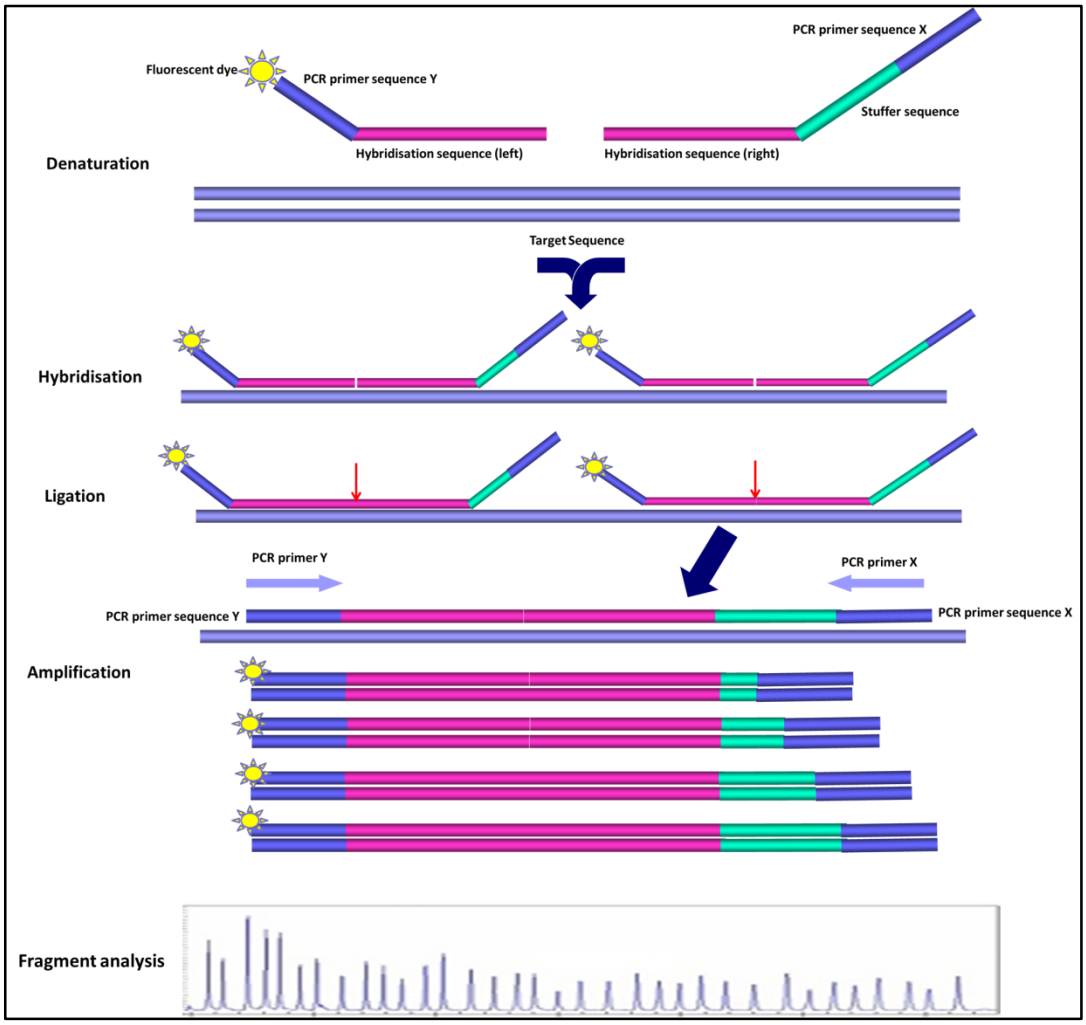

Figure 2.2 Principles of MLPA techniques. Four main steps were followed including: Denaturation, hybridization, ligation, amplification and fragment analysis. Figure was adapted from www.mrc-holland.com.

Stage	Temperature	Time
Denaturation	98°C¶	5 min¶
	98°C*	40 min*
Hybridization	95°C	1 min
	60°C	16 hr.
Ligation	54°C	15 min
	98°C	5 min
	4°C	Overnight
Before the addition of Polymerase Master Mix	60°C	7 min
Amplification (35 cycles)	95°C	30 sec
	60°C	30 sec
	72°C	60 sec
	72°C	20 min
	4°C	unlimited

Table 2-1 Different stages of the MLPA procedure. ¶ for viable cell extractions * for fixed cell pellet extractions, min minutes, sec seconds, hr. hours.

2.9.3 Analysis

Data were analysed using GeneMarker V1.85 analysis software (SoftGenetics, USA). Relative copy number was obtained after normalisation of peaks against controls. Probe ratios between 0.75 and 1.3 were considered to be within the normal range, with 2 being the normal copy number, while values below 0.75 or above 1.3 indicated loss or gain of genetic material, respectively, and corresponded to copy numbers of 1 and 3, respectively. A value below 0.25 indicated biallelic loss which represented a copy number of 0. At least two neighbouring probes within a gene were required to be deleted for the loss to be deemed valid, however, some exceptions did exist (i.e. isolated *ETV6* exon 8 deletions) and they were further investigated in section 4.4.3.1.

2.10 Single nucleotide polymorphism (SNP) genotyping

2.10.1 Overview

Single nucleotide polymorphism (SNP) genotyping is used for the detection of copy number abnormalities, loss of heterozygosity (LOH) and allele specific analyses at the genome level using probes that are hybridised to a chip. The probes are designed to detect SNPs and copy number variants (CNVs) that exist within the general population (1000 Genomes Project, etc.). SNPs are genetic variations at the single nucleotide level

within the genome that appear in a significant proportion (more than 1%) of the population, while CNVs are regions of chromosomes at the level of a particular gene that vary in copy number in humans. There are many factors that are playing roles in the success of this method and they include high quality DNA, the availability of matched reference samples, optimal normalization of the raw microarray data and the computational algorithm (Mullighan, 2011).

This study used a platform with one of the highest resolution levels to detect these genetic variants, namely the Affymetrix SNP 6.0 array. This platform is a variation of DNA microarray that has a high level coverage of 90.5% of 3400 copy number variants and a total of 946,000 non-polymorphic copy number probes, thus providing a higher resolution for the detection of chromosomal aberrations.

2.10.2 Principles of the technique

SNP 6.0 arrays were processed offsite by AROS Applied Biotechnology (Aarhus, Denmark) according to the manufacturer's instructions (Affymetrix). For each sample, approximately 750 ng DNA was used. DNA was either extracted from viable or fixed cells (refer to section 2.6). In brief, total genomic DNA was digested with both Nsp I and Sty I restriction enzymes and adaptors were ligated to the DNA fragments which facilitated their recognition by a generic primer (Figure 2.3). Amplification of the adaptor-ligated DNA fragments followed and the PCR amplification products were combined and purified. Finally, the library was hybridized to probes, which had been fragmented and labelled, for matching allele. A comprehensive genotyping profile was generated based on the pattern of probe: DNA hybridisation enabling the determination of heterozygosity and homozygosity for each allele.

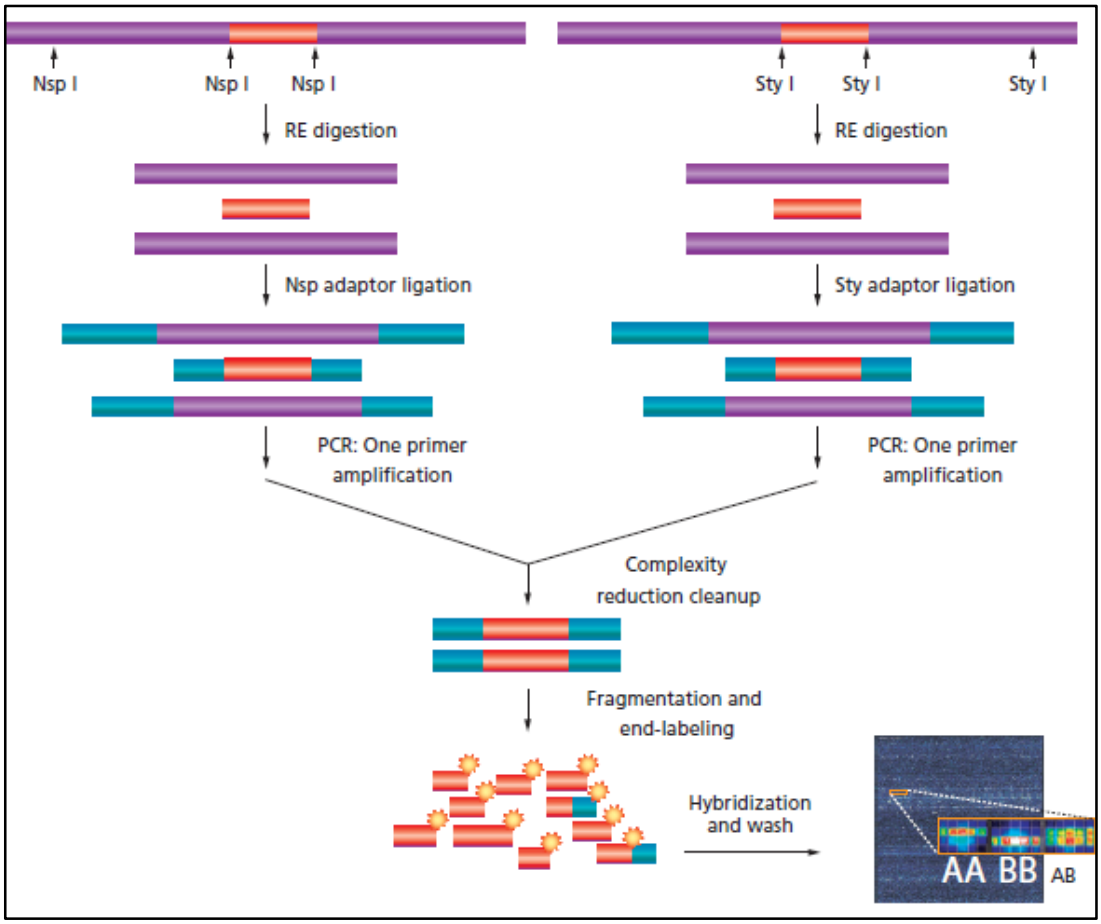

Figure 2.3 Overview on the SNP 6 array processing which involved mainly the following steps: Digestion of sample DNA using both Nsp I and Sty I restriction enzymes, ligation of the fragmented DNA to adaptors to be recognized by a generic primer, amplification of the adaptor-ligated DNA fragments, purification, fragmentation/labelling and hybridization to probes for matching alleles. Figure was taken from www.affymetrix.com.

2.10.3 Analysis

Raw array-generated data in the form of .CEL files were received from AROS. Affymetrix CEL files were analysed for initial quality control, followed by Affymetrix Birdseed algorithm, Version 2.0 to generate SNP genotype calls on all samples using Affymetrix Genotyping Console software (version 4.1.1.834). All samples passed the Affymetrix recommended contrast quality control and SNP call rate threshold. Copy number, allele ratio, and allele specific copy number data analysis were performed. HapMap270 data was used as a reference model for LOH analysis due to unavailability of matched germline DNA. Detected regions of CNA which mapped to regions of known genetic variation were identified by the software and were excluded from further analysis.

2.11 Real-time polymerase chain reaction (qPCR) using TaqMan Copy Number

Assays

2.11.1 Overview

The TaqMan Copy Number Assays are characterised as a fast and simple method for obtaining specific copy number data within the human genome. TaqMan Copy Number Assays are run simultaneously with a TaqMan Copy Number Reference Assay in a duplex qPCR. The Reference Assay detects a sequence that is known to exist in two copies in the genome (for example, *RNase P* (14q11.2) - the standard reference and *TERT* (5p15.33) - an alternative reference assay). Each TaqMan Copy Number Assay contains: two unlabelled primers for amplifying the target sequence of interest and one TaqMan Minor Groove Binder (MGB) probe for detecting the target sequence of interest. The MGB probe includes both 6-carboxyfluorescein (FAM) reporter dye and a nonfluorescent quencher (NFQ) with a MGB. However, the reference assay probe contains VIC reporter dye and 6-carboxytetramethylrhodamine (TAMRA) quencher. As heterogeneous leukaemic samples were tested in this study, the reference DNA sequence might not be present in normal copy number; therefore two different reference assays were used. A more detailed description of the probes used is given in the relevant chapters (5 and 6).

2.11.2 Experiment set-up

Reactions were run on an Applied Biosystems Real-Time PCR System according to the manufacturer's instructions. DNA was either extracted from viable or fixed cells (section 2.6). Each sample was diluted to 5 ng/ul using Nuclease-free water (NFW). Applied Biosystems recommends running the following samples on each plate: genomic DNA (gDNA) samples with unknown target copy number, a sample that does not contain a DNA template (NTC), a calibrator sample- which was known to have two copies of the target sequence- and a positive control sample. The NTC sample shows the background fluorescence and allows for the detection of contamination. It is strongly recommended to use four replicates for each gDNA sample in order to generate the reliable copy number calls. Standard 96-well plate was used with a total volume of 20ul in each well that constituted of TaqMan Genotyping Master Mix, TaqMan copy number target,

reference assays, nuclease-free water and gDNA. The target, reference probes and Genotyping Master Mix were all supplied from Applied Biosystem.

The experiment setup was performed using ViiATM 7 Real-Time PCR System (ViiA™ 7 Software v1.X) and the Absolute Quantitation method was chosen to capture the CT data from the duplex PCR run. The qPCR cycles were defined as detailed in Table 2-2.

Stage	Temperature	Time
Hold	95 °C	10 min
Cycle (40 Cycles)	95 °C	15 sec
	60 °C	60 sec

Table 2-2 **Stages of qPCR using TaqMan Copy Number Assays.**

2.11.3 Principles of the technique

During the PCR reaction, the genomic DNA template was denatured and each set of assay primers annealed to its specific target sequences (Figure 2.4). Similarly, each TaqMan probe annealed specifically to the complementary sequence which was been generated by the forward and reverse primers. When the target sequence was present, the probe hybridized to the target sequence; thus during each round of PCR, the DNA polymerase cleaved the probes that were hybridized at the 5' end where the reporter dye is attached. This resulted in separation of the quencher and reporter dyes leading to an increase in the level of fluorescence as the reporter became unquenched. However, when the target sequence was deleted, the probe did not hybridize and the reporter dye remained quenched and therefore failed to emit a fluorescent signal. Accumulation of the different PCR products resulting from the amplification of both the target and reference sequences during each cycle was detected by the real time PCR machine and they are represented by FAM and VIC signals, respectively. The relative levels of the two reporter dyes, as represented by cycle threshold (CT) values, are indicative of different starting amount of DNA.

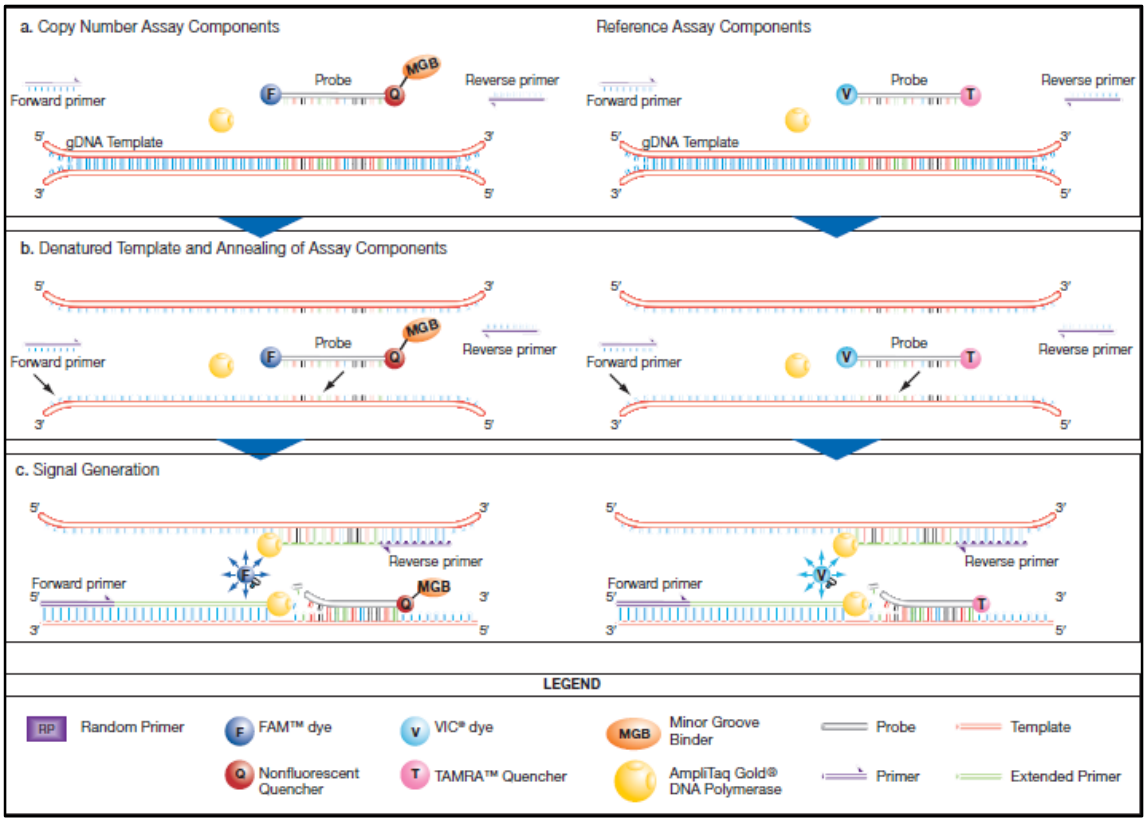

Figure 2.4 Principles of qPCR techniques for the detection of target (left) and reference (right) DNA sequences in a duplex reaction. a. Components of the copy number and reference assays (assay primers and TaqMan probe), b. Denaturation of the DNA template followed by annealing of the assay components, c. DNA polymerase cleaves the probes that are hybridized at the 5' end where the reporter dye is attached, resulting in signal generation. The figure was taken from www.appliedbiosystem.com.

2.11.4 Analysis

ViiA™ 7 Real-Time PCR System was used to analyse the real time PCR data and the Absolute Quantitation method was used to capture the C_T data from the duplex PCR run. The C_T values were determined as the fractional cycle number where the level of fluorescence increased above the baseline threshold. Of note, the threshold for C_T determination was set during the exponential amplification phase, in which all reagents were still in excess. The baseline threshold should be set in the initial cycles of PCR where there is little change in fluorescence signal but should not overlap with the area in which the amplification signal begins to rise above background (Figure 2.5). It is worth noting that both the threshold and baseline should be the same for each specific target probe assay in multiple experiments. Applied Biosystems CopyCaller Software imported and analysed real-time PCR result files from copy number assay experiments that used

absolute quantitation settings to determine the copy number of the target in each sample. This Software analysed copy number data with or without a calibrator sample using the comparative cycle threshold ($\Delta\Delta C_T$) method that determined the relative quantitation (RQ) (Figure 2.6). The method measured the C_T difference (ΔC_T) between target (FAM) and reference (VIC) sequences in all replicates of the test samples. The mean ΔC_T ($\mu\ \Delta C_T$) value of the test samples was then compared to the $\mu\ \Delta C_T$ value of the calibrator sample to give the $\Delta\Delta C_T$ value. The copy number of the target sequence in a test sample was calculated by the software to be the copy number of the target sequence in the calibrator sample multiplied by the relative quantity (RQ) which was calculated from the $\Delta\Delta C_T$ value.

Quality metric assignment, namely confidence and absolute z-score metrics, were taken into consideration. The former metric is the probability that the assigned copy number is the correct copy number relative to any of the other possible assigned copy numbers for the group of analysed samples. However, the absolute z-score metric is defined as the absolute value of the number of standard deviations separating the replicate mean ΔC_T of a sample from the mean subdistribution of the assigned copy number. A specific algorithm was generated in Chapter 5 pertaining to the accuracy of data based on their quality metrics.

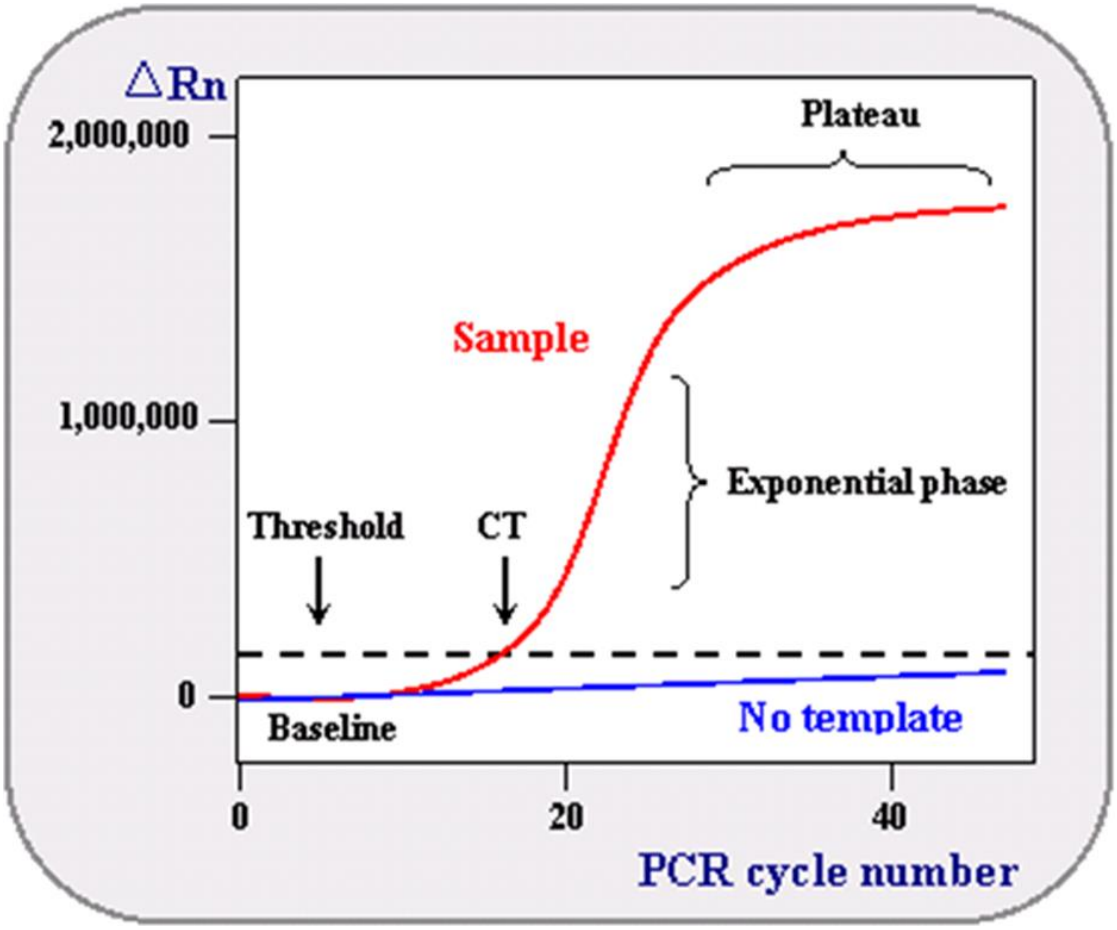

Figure 2.5 Representative plot of real time quantitative polymerase chain reaction. Two phases are shown in the plot: amplification and plateau phases, baseline is set in the initial cycles of PCR where there is little change in fluorescence signal, threshold line is located at the base of the amplification phase, CT values represent the intersection between the amplification curve and threshold line. ΔRn is an increment of fluorescent signal at each time point. The ΔRn values (Y- axis) are plotted versus the cycle number (X-axis). Figure was taken from http://www.ncbi.nlm.nih.gov/projects/genome/probe/doc/TechQPCR.shtml.

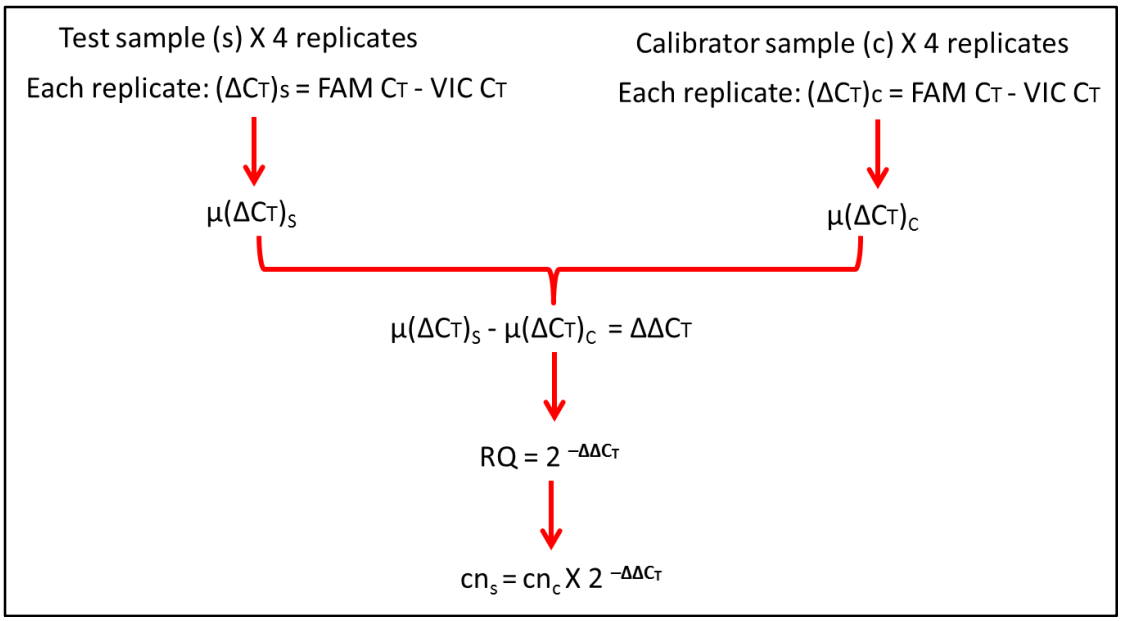

Figure 2.6 Flow chart describing the comparative cycle threshold ($\Delta\Delta C_T$) method calculation of the target copy number in a test sample. s: test sample, c: calibrator sample, C_T value: cycle threshold, Δ : difference, μ: mean, RQ: relative quantity, cns, cnc: copy number of the target sequence in a test and calibrator samples, respectively.

2.12 Statistical Analyses

Statistical analysis was performed using intercooled STATA, version 11.0 (StataCorp, USA), particularly Wilcoxon Rank Sum for non-parametric assays. Comparisons between categorical groups and subgroups were assessed using the Chi-squared using appropriate variations (e.g. Fisher's exact test) when required. As this study was hypothesis generating rather than hypothesis testing/validating no formal p value adjustment was used but all tests were conducted using a 1% level of significance to account for multiple testing. t-test was used to determine if the means of two groups were significantly different from each other.

Chapter 3. Spectrum of key secondary abnormalities in *ETV6-RUNX1* positive BCP-ALL

3.1 Introduction

Robust functional studies have demonstrated that *ETV6-RUNX1* fusion gene by itself is unable to generate leukaemogenic transformation. Rather, it acts as an initiating factor which aids in the generation and maintenance of the preleukaemic stem cells as suggested from xenograft modelling (Bernardin *et al.*, 2002; Tsuzuki *et al.*, 2004; Fischer *et al.*, 2005; Sabaawy *et al.*, 2006; Hong *et al.*, 2008; Schindler *et al.*, 2009). The need for the acquisition of the postnatal genetic alterations is further indicated by the number of healthy neonates carrying this fusion who do not develop overt leukaemia, the low concordance rate (~ 5-10%) for BCP-ALL among monozygotic monochorionic twins and the variable length of the latency period to clinically detectable leukaemia (Wiemels *et al.*, 1999; Mori *et al.*, 2002). A large and growing body of genomic studies on *ETV6-RUNX1* have identified recurrent genetic aberrations involved in B cell development/ differentiation, cell cycle regulation, nuclear hormone receptor transcriptional regulation, glucocorticoid receptor signalling, drug resistance and regulation of apoptosis including *BTG1, PAX5, EBF1, ETV6, CDKN2A/B, TBL1XR1, NR3C1, NR3C2* and *BMF, CD200/BTLA* (Forestier *et al.*, 2007; Lilljebjorn *et al.*, 2007; Mullighan *et al.*, 2007; Kawamata *et al.*, 2008; Mullighan *et al.*, 2008b; Parker *et al.*, 2008; Lilljebjorn *et al.*, 2010). Thus the interaction of these alterations is needed for the leukaemic development and progression (see section 1.4.1.4). Previous genomic studies have focused on the overall frequencies, common breakpoints of these genetic alterations and their associations with different primary genetic groups. However, the distribution of these aberrations among different *ETV6-RUNX1* subgroups in terms of demographic and clinical features was lacking. In addition, it was important to shed light on comparing this cytogenetic group with other BCP-ALL subtypes in terms of the frequency of CNA and their characteristic features including type and extent of deletions along with associated other gene alterations. These important elements still have to be determined in order to point out to the possible driving leukaemogenic factors in *ETV6-RUNX1* positive cases.

3.2 Aims and Objectives

This study aims to identify the frequency and type of the CNA along with their distinct clinical and genetic features in *ETV6-RUNX1* BCP-ALL targeting genes involved in B cell

development/ differentiation and cell cycle regulation including: *ETV6, PAX5, CDKN2A/B, BTG1, RB1, IKZF1, EBF1* and genes within the pseudoautosomal region 1 (PAR1) of the sex chromosomes. The potential relevance of these alterations to *ETV6-RUNX1* subgroups as compared to other BCP-ALL is to be assessed accordingly.

The main objectives in this study are to:

1- Screen a large cohort of *ETV6-RUNX1* patients for the genes mentioned earlier using an accurate and reliable high throughput method: multiplex ligation-dependent probe amplification (MLPA).

2- Describe the incidence and clinical features of the different gene alterations in the *ETV6-RUNX1* overall cohort.

3- Compare the CNA profile observed in *ETV6-RUNX1* cases with other BCP-ALL.

3.3 Patients and Methods

Patients included in this study were confirmed cases of *ETV6-RUNX1* positive BCP-ALL and treated using either UKALL97/99 or UKALL2003 (see section 2.2). National Cancer Institution (NCI) criteria were followed to assess the risk (see section 1.3.5). The diagnostic cell pellets were utilised for DNA extraction, for those patients with no available DNA, using the QIAGEN DNeasy tissue and blood kit according to the manufacturer's instructions (section 2.6). Comprehensive MLPA screening was carried out on a representative cohort of BCP-ALL (n=1019), including *ETV6-RUNX1* cases (n=397), by the Leukaemia Research Cytogenetic Group (LRCG) team, including myself, using the SALSA MLPA kit P335 *IKZF1* and for certain cases, the P202 *IKZF1* kit was used (see section 2.9) (Schwab *et al.*, 2013). Intercooled STATA, version 11.0 was used for the statistical analysis (see section 2.12).

3.4 Results

3.4.1 Cohort Description

A total of 397 cases were found to have *ETV6-RUNX1* positive BCP-ALL. They had a median age of 4 years with few infants (before their second birthday) (5%) and older children (aged 10 years and above) (9%). In addition, there were few cases with an age

of 15 years and more (1.5%). To note, the majority of cases (75%) were classified as standard NCI risk and there was a reduction in the incidence of relapses from UKALL97/99 to UKALL2003 (13.5% to 3.7%). Many of these relapses were late relapses (> 6 months of end of treatment) (~70%).

3.4.2 Cytogenetic abnormalities

The majority of patients had an abnormal karyotype (n= 238/397, 60%), whilst the remainder either had a normal (n=66/397, 17%), or a failed cytogenetic result (less than 20 normal cells were regarded as a fail) (n=90/397, 23%) or data was unavailable (n=3/397, 0.5%). It is worth noting that the cytogenetics failure rate was high in support of previously reported rate (Harrison *et al.*, 2005) (26%) which might be explained by the lower mitotic index seen in *ETV6-RUNX1* cases. Of these cases with successful cytogenetics (n=304), the most prevalent abnormalities were: 12p loss (31%), gain of chromosome 21 (15%) and add/del 6q (11%). Other visible abnormalities which occurred at a lower rate included: add/del 11q (6%), add/del 9p (5%) and near tetraploidy (4%) (Table 3-1) (Appendix A).

FISH studies were helpful in distinguishing between gain 21 and gain der(21)t(12;21) owing to the higher resolution of FISH. Thus, 35 out of 46 cases with visible gain 21 had FISH results and 23 cases (66%) showed concordant FISH and cytogenetic results, while the remaining 12 cases revealed either gain der(21)t(12;21) (n=11, 31%) or no evidence of neither gain 21 nor gain der(21)t(12;21) (n=1, 3%). Among 23 concordant cases, three of them exhibited an additional gain der(21)t(12;21); thus four copies of chromosome 21 in total. In the overall cohort, among the remaining cases with no visible gain 21 and with available FISH data (n= 302), gain 21 was found in further 36 cases (12%).

On the other hand, four out of 5 cases with cytogenetically visible gain der(21)t(12;21) had FISH and MLPA data and all cases showed concordant cytogenetic, MLPA and FISH results. Overall, 41 out of 333 remaining cases with no visible gain der(21)t(12;21) and with available FISH data showed gain der(21)t(12;21). Among 42 cases, 13 cases had cytogenetically visible gain 21 but FISH and MLPA *ETV6* profiles indicated gain der(12)t(12;21), while 26 cases had concordant FISH and MLPA results but not cytogenetically visible. The remaining three cases showed gain der(21)t(12;21) by FISH but not by MLPA owing to either low proportion of the abnormal clones (13% and 6% of

the total cells) (n=2) or an artefact in either MLPA or FISH data (n=1). The MLPA profile of gain der(21)t(12;21) can be identified by the presence of both gain in the copy number of *ETV6* exons 1-5 and normal copy number of *ETV6* exon 8.

Taking together, in the overall cohort with available FISH studies (n=337), a total of 52 cases (17%) exhibited gain 21, 38 cases (11%) had gain der(21)t(12;21) and further 7 cases (2%) harboured both alterations.

Abnormality	**No. (%)**
Total no. of successfully tested cases	304 (100)
add/del (12)p	94 (31)
Gain 21	46 (15)
add/del (6)q	33 (11)
add/del (11)q	18 (6)
del/add (9)p	14 (5)
Near tetraploidy	11 (4)
Gain 16	9 (3)
del/add (13)q	8 (3)
del/add (3)q	7 (2)
add(16)(p)/(q)	6 (2)
del(5)q	6 (2)
Gain der(21)t(12;21)	5 (2)
Gain 10, 16	5 (2)
Gain 10	4 (1)

Table 3-1 Cytogenetic abnormalities and their frequencies in *ETV6-RUNX1* cohort. add: added material, del: deleted material, near tetraploidy: 80 or more chromosomes.

3.4.3 Copy number alterations/Rearrangements in ETV6-RUNX1 BCP-ALL

Overall, 77% of *ETV6-RUNX1* cases (n=307) exhibited an alteration of at least one of the eight genes in the MLPA kit: 151 (38%) patients had one, 115 (29%) had two, 41 (10%) had three or more losses (Table 3-2, Table 3-3, Table 3-4 and Appendix A). There were a total number of 508 alterations with an average number of 1.26 abnormalities per case. The most prevalent gene deletion was *ETV6* that constituted 52% of the cases. Moreover, *PAX5*, *CDKN2A/B* and *BTG1* alterations were the common deletions that represented 23%, 23% and 15%, respectively. However, the remaining alterations including *RB1*, *EBF1*, *IKZF1* and *PAR1* occurred at lower frequencies (8%, 4%, 3% and 1%, respectively). There was no significant association between these alterations and other clinical/

demographic features including: sex, age, WCC, relapses and other gene deletions. However, two thirds of the *ETV6* deleted cases had WCC >10X 10^9/L (p=0.02). In addition, *CDKN2A/B* and *BTG1* losses were absent in those less than 2 years old as compared to older patients (p=0.02). Furthermore, half of *IKZF1* deletions were assigned to the high risk category (p=0.02).

	Cohort	Gene deletions							
		ETV6	***CDKN2A/B***	***PAX5***	***BTG1***	***RB1***	***EBF1***	***IKZF1***	***PAR1***
N. of cases	397 (100)	206 (100)	91 (100)	91 (100)	60 (100)	30 (100)	14 (100)	12 (100)	4 (100)
Sex (M:F)	216:181	103:103	48:43	43:48	29:31	15:15	7:7	4:8	4:0
ratio	1.19	1	1.11	0.9	0.94	1	1	0.5	4
Age (years)									
Median	4	4	4	3	4.5	5	5	7	8
1	21 (5)	7 (3)	0 (0)	5 (5)	0 (0)	1 (3)	1 (7)	0 (0)	0 (0)
2-9	342 (86)	185 (90)	82 (90)	82 (90)	51 (85)	24 (80)	11 (79)	9 (75)	3 (75)
10+	34 (9)	14 (7)	9 (10)	4 (4)	9 (15)	5 (17)	2 (14)	3 (25)	1 (25)
WCC‡ (X 10^9/L)									
Median	11.6	14.7	14.3	15	10.5	12.4	8.9	14.1	22.7
<10	178 (45)	79 (38)	32 (35)	35 (38)	28 (47)	12 (40)	7 (50)	6 (50)	2 (50)
10-49.9	147 (37)	82 (40)	37 (41)	33 (36)	21 (35)	15 (50)	6 (43)	2 (17)	1 (25)
≥50	72 (18)	45 (22)	22 (24)	23 (25)	11 (18)	3 (10)	1 (7)	4 (33)	1 (25)
NCI Risk¥									
Standard	296 (75)	151 (73)	62 (68)	64 (70)	42 (70)	23 (77)	11 (79)	6 (50)	2 (50)
High	101 (25)	55 (27)	29 (32)	27 (30)	18 (30)	7 (23)	3 (21)	6 (50)	2 (50)

Table 3-2 Number and percentages (%) of *ETV6-RUNX1* BCP-ALL patients with different gene deletions stratified by sex, age and white cell count and NCI risk status. all p values >0.01 (not significant), thus not included in the table, ‡ white cell count, ¥ National Cancer Institute (standard risk: aged 1-9 years with a WCC < 50 x10^9/L at initial presentation, high risk category: ≥10 years and a WCC ≥ 50 x10^9/L.

	Cohort	Gene deletions			
		ETV6	***CDKN2A/B***	***PAX5***	***BTG1***
No. of cases	397	206	91	91	60
ALL97/99	126 (100)	74 (100)	30 (100)	34 (100)	26 (100)
No. Relapses	17 (13)	11 (15)	8 (27)	3 (9)	4 (15)
Early*	6 (35)	3 (27)	4 (50)	0	0
Late‡	11 (65)	8 (73)	4 (50)	3 (100)	4 (100)
ALL2003	271 (100)	132 (100)	61 (100)	57 (100)	34 (100)
No. Relapses	10 (4)	1 (1)	1 (2)	1 (2)	0 (0)
Early*	3 (30)	1 (100)	0	0	0
Late‡	7 (70)	0	1 (100)	1 (100)	0

Table 3-3 Correlation between different gene deletions and types of relapses in ETV6-RUNX1 patients treated on ALL97/99 and ALL2003. all p values >0.01 (not significant), thus not included in the table, * within 18 months of the diagnosis until 6 months from the end of treatment (male: 3 years, female: 2 years), ‡ after 6 months off treatment, no reported relapses in *EBF1*, *IKZF1*, *RB1* deleted cases and only one PAR1 rearranged case was relapsed, thus not included in this table.

	Cohort	Gene deletions							
		ETV6	***CDKN2A/B***	***PAX5***	***BTG1***	***RB1***	***EBF1***	***IKZF1***	***PAR1***
N. of cases	397 (100)	206 (100)	91 (100)	91 (100)	60 (100)	30 (100)	14 (100)	12 (100)	4 (100)
Deletions									
ETV6	206 (52)								
CDKN2A/B	91 (23)	54 (26)							
PAX5	91 (23)	47 (23)	21 (23)						
BTG1	60 (15)	33 (16)	14 (15)	12 (13)					
RB1	30 (8)	16 (8)	3 (3)	6 (7)	4 (7)				
EBF1	14 (4)	10 (5)	1 (1)	4 (4)	4 (7)	0 (0)			
IKZF1	12 (3)	8 (4)	1 (1)	5 (5)	4 (7)	2 (7)	0 (0)		
PAR1	4 (1)	1 (0.5)	1 (1)	2 (2)	2 (3)	2 (7)	0 (0)	1 (8)	

Table 3-4 Correlation among different gene deletions within *ETV6-RUNX1* BCP-ALL. all p values >0.01 (not significant), thus not included in the table.

3.4.3.1 Copy number alterations in B- cell differentiation genes

A. PAX5

PAX5 deletions were one of the common B- cell differentiation gene alterations within *ETV6-RUNX1* positive BCP-ALL and occurred in 23% of cases (n=91). All deleted cases harboured monoallelic deletions with the majority of them (38%) being restricted to exons 2-6, followed by losses affecting exons 1-10, exons 1-6, exons 2-5 which represented 18%, 16% and 12%, respectively. The remaining cases exhibited different breakpoints at lower incidences (Figure 3.1).

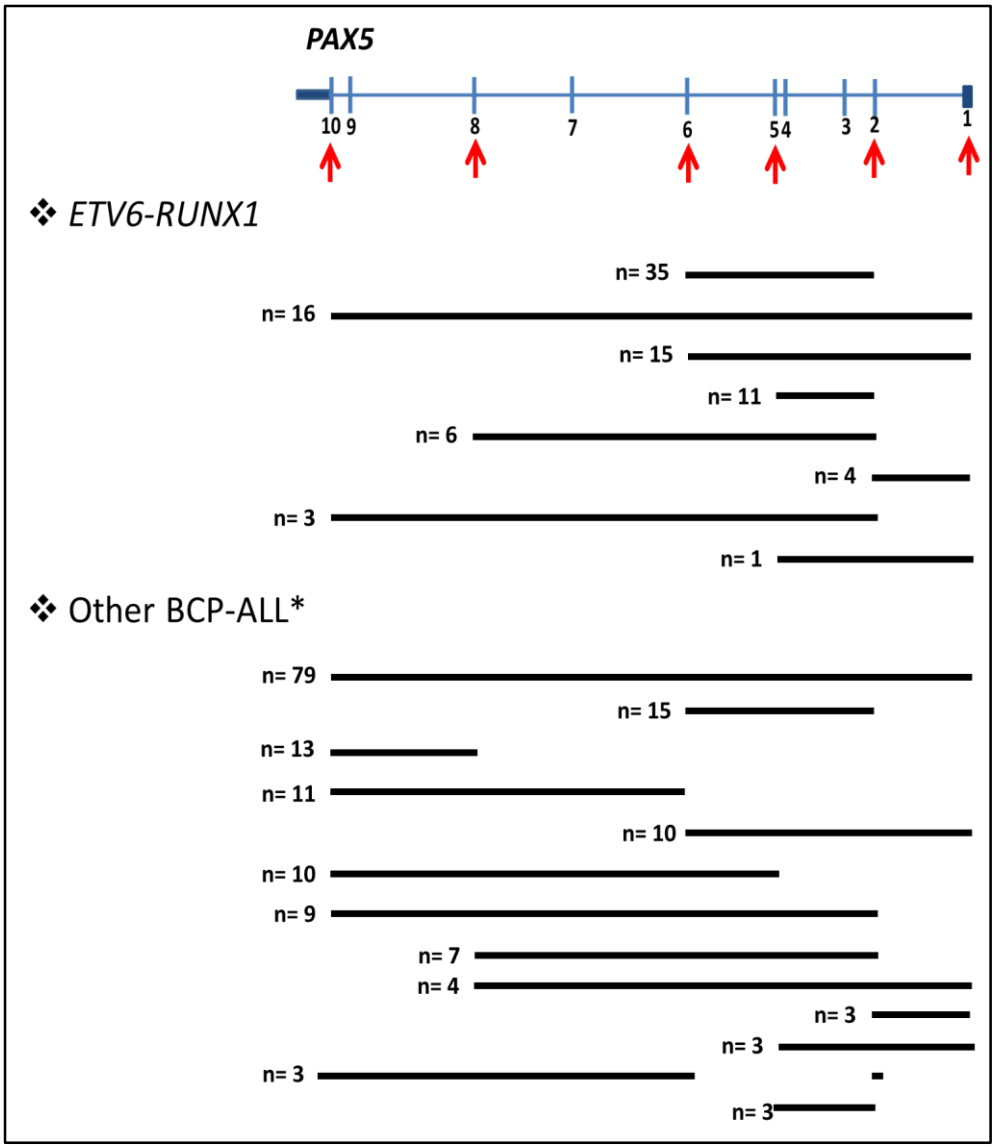

Figure 3.1 Comparison of *PAX5* deletion breakpoints between *ETV6-RUNX1* and other BCP-ALL cases. *PAX5* gene (10 exons) is displayed with the six MLPA probes used from the SALSA MLPA kit P335 *IKZF1* (Red arrows) that covered *PAX5* exons 1, 2, 5, 6, 8 and 10; *PAX5* deletion breakpoints are highly variable; *PAX5* exons (2-6) losses constituted the predominant deletion in *ETV6-RUNX1*, while entire gene loss is the commonest in other BCP-ALL; the total number of *PAX5* losses in *ETV6-RUNX1* and other BCP-ALL cases (91 and 178 cases); * other miscellaneous losses presented at lower frequencies (1% each) including: exon 1, exons 1, 6-10, exons 1, 8-10, exons 2, 6-8, exons 2-5, 8-10, exons 2-6, 10, exons 5-8 and exons 6-8; solid lines are heterozygous losses.

PAX5 losses occur in all age groups but with varying proportions and a median age of 3 years. There was no difference in terms of sex, WCC and NCI risk groups distributions within *ETV6-RUNX1* patients (Table 3-2). Furthermore, there were no associations between *PAX5* losses and other gene deletions tested in this study (Table 3-4) in spite of their high co-occurrence rate with *CDKN2A/B* losses (23%, n=21). Out of these 21 cases, patients had failed (n=6, 29%) or normal cytogenetics (n=1, 5%) or abnormal

karyotype with no 9p abnormality (n=5, 24%) or with 9p abnormality (n=9, 43%). Overall, a total of 10 (16%) out of 61 *PAX5* deleted cases with successful cytogenetic data showed visible abnormalities affecting 9p with nine of them showed concurrent *CDKN2A/B* loss (90%). There were different structural abnormalities observed including: dicentric chromosomes affecting chromosome 9 (dic(9)p) (n=7, 11%), partial deletion 9p (del(9)p) (n=1, 2%) and additional material on 9p (add(9)p) (n=2, 3%). Among those cases with dicentric chromosomes, 5 cases showed dic(9;12) (71%) and one case each exhibited either dic(9;18) (14%) or dic(6;9) (14%). Interestingly, all dic(9;12) and dic(9;18) patients showed whole *PAX5* gene loss except the case with dic(6;9) which showed loss of *PAX5* gene exons (2-6), however, all of these dicentric chromosomes (n=7) had concurrent *CDKN2A/B* deletions. In addition, the entire loss of *ETV6* gene (n=4) or its telomeric exons (1-5) (n=1) were observed in all dic(9;12) cases (n=5) along with concurrent deletions of both *PAX5* and *CDKN2A/B*.

B. EBF1

The *EBF1* gene consists of 16 exons and the SALSA MLPA kit P335 *IKZF1* contains four probes covering *EBF1* exons 1, 10, 14 and 16. *EBF1* deletions were considered as rare alterations that occurred in BCP-ALL patients and only 4% (n=14) of *ETV6-RUNX1* patients harboured these aberrations. The majority of the cases showed loss of the entire *EBF1* gene (loss of all probes) (n=12) while the remaining two cases had deletions restricted to exons (1-10) and all deletions were monoallelic. These losses were not reported to be associated with any of the demographic or clinical features (Table 3-2). Patients with these losses were predominately allocated in the standard NCI risk group (n=11, 79%) and this might explain the observed good outcome with no reported relapses in either treatment protocols. In total, 11 out of 14 deleted cases had successful cytogenetic results with only one of them showing visible deletion at the long arm of chromosome 5 (5q).

C. IKZF1

IKZF1 deletions occurred in around 3% (n=12) of *ETV6-RUNX1* cases. The extent of *IKZF1* deletion sizes varied with the most frequent ones either restricted to exons (2-3) (n=5, 42%) or exons (4-8) (n=3, 25%), while the remaining cases occurred at lower frequencies:

exons (2-7) (n=1, 8%), exons (2-8) (n=1, 8%), exon (1) (n=1, 8%) or the entire gene (n=1, 8%) (Figure 3.2). All deleted cases showed monoallelic deletions. Furthermore, all deletions were further validated by another detailed *IKZF1* MLPA kit (P202) with a higher probe density that confirmed these deletions. This kit is composed of 18 probes covering *IKZF1* gene: six of them (exons 1-2 and 5-8) are similar to those in P335 kit while one probe overlaps with one of the probes contained in P335 kit (exon 3). However, the remaining probes (n=11) encompass *IKZF1* exons 1 (n=2), exon 2 (n=1), exon 3 (n=2), exon 4 (n=2), exon 5 (n=1), exon 6 (n=1), exon 7 (n=1) and exon 8 (n=1) in order to confirm the deletion of single probes obtained from the P335 *IKZF1* kit. In addition, there are additional probes on either side of the *IKZF1* gene (3' and 5' regions) which have 2 and 1 probes, respectively, to aid in the estimation of the extent of deletion on 7p. Overall, the results were consistent using both kits and the new kit added the advantage of confirming the case with a single probe loss targeting exon 1. Furthermore, it estimated the extent of deletions in three cases with either loss of the entire gene (n=1) or deletions of exons (4-8) (n=1) or exons (2-8) (n=1). Thus, the deletion of the former case was shown to involve both flanking breakpoints of *IKZF1* gene indicative of extensive deletion on 7p that was not detected cytogenetically owing to failed results. However, the remaining two cases exhibited deletions extending to the 3' region of the gene and none of them were identified cytogenetically owing to possible smaller deletion (below the cytogenetic resolution) and failed results, respectively. Confirmatory FISH, using fosmid probe specific for the *IKZF1* gene (40 kb), was available for only one case with deletion restricted to exons (2-7) and was shown to harbour the deletion as a major clone (59%). *IKZF1* losses cases occurred mainly in the age group (2-9) years with a median age of 7 years (Table 3-2). Although half of *IKZF1* deleted cases were in the high NCI risk within this ALL subtype, none of them relapsed. There were no associations with other gene deletions (Table 3-4). Among 9 cases with successful cytogenetic, none had a visible cytogenetic abnormality involving the short arm of chromosome 7 (7p).

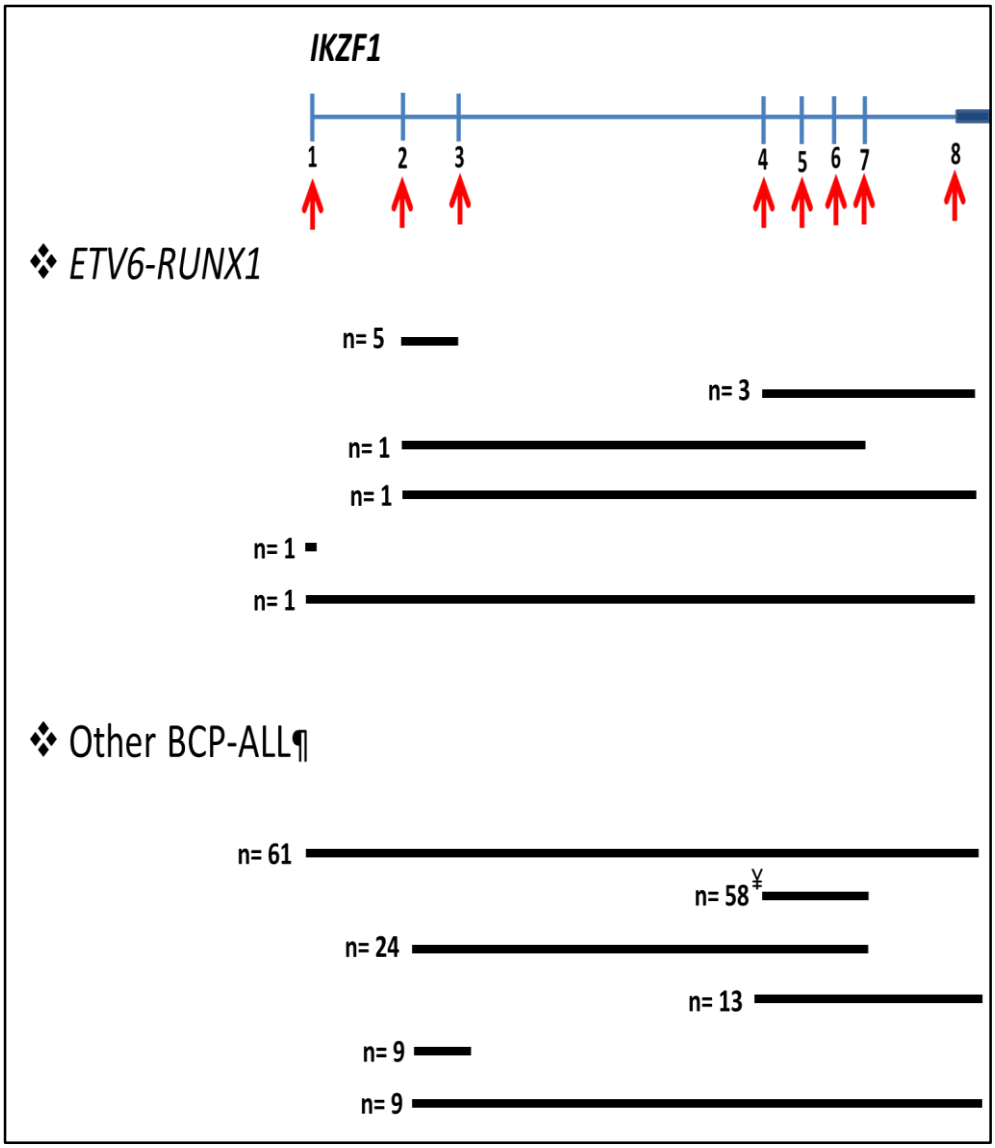

Figure 3.2 Comparison of *IKZF1* deletion breakpoints between *ETV6-RUNX1* and other BCP-ALL cases. *IKZF1* gene (8 exons) is displayed with the eight MLPA probes used from the SALSA MLPA kit P335 *IKZF1* (Red arrows) that covered *IKZF1* exons 1, 2, 3, 4, 5, 6, 7 and 8; *IKZF1* deletion breakpoints are highly variable; *IKZF1* exons (2-3) and exons (4-8) losses constituted the predominant *IKZF1* deletion types in *ETV6-RUNX1*, while the entire gene loss and exons (4-7) were the commonest in other BCP-ALL; ¥ the classic deletion with IK6 isoform production; the total number of *IKZF1* losses in *ETV6-RUNX1* and other BCP-ALL cases (12 and 187 cases); ¶ other miscellaneous losses presented at lower frequencies (1% each) including: exon 1, exons 1-3, exons 1, 4-7, exons 1-4, exons 1-5, exons 3, exons 3-5, 7-8, exons 3-8, exons 4,6-7, exons 4-5 and exons 4-5,7; solid lines are heterozygous losses.

3.4.3.2 Copy number alterations in cell cycle control genes

D. CDKN2A/B

The MLPA kit contains three probes covering *CDKN2A* (exons 1 and 4) and *CDKN2B* (exon 2). *CDKN2A/B* losses were one of the common deletions representing around 23% (n=91) of *ETV6-RUNX1*. The majority of deleted cases encompassed the entire *CDKN2A/B* genes (n=87, 96%), while the remaining four cases harboured deletions restricted to either *CDKN2A* (n=2, 2%) or *CDKN2B* (n=2, 2%). *CDKN2A/B* losses were either monoallelic (n=72, 79%) or biallelic (n=19, 21%). They occurred in all age groups, except younger children <2 years old who lacked any reported case, at varying incidences with a median age of 4 years (Table 3-2). There was no significant association between these aberrations and other demographic and clinical features. However, 27% of the deleted cases treated on UKALL97/99 at presentation relapsed, while only 2% of deleted cases that received more advanced treatment (UKALL2003) relapsed (Table 3-3). Furthermore, there were no associations between *CDKN2A/B* losses and other gene deletions tested in this study (Table 3-4) apart from the previously described high co-occurrence rate with *PAX5* losses in 23% (see section 3.4.3.1 A.). Among 67 deleted *CDKN2A/B* cases with successful cytogenetics, 25% (n=17) showed visible 9p alterations including add(9p) (n=4, 6%), del(9p) (n=5, 7%) and dicentric chromosomes affecting chromosome 9 (dic(9p)) (n= 8, 12%). 6 out of 8 cases with dic(9p) showed dic(9;12) (75%) and one case each exhibited either dic(9;18) (13%) or dic(6;9) (13%). A total of 8 cases (47%) with visible 9p abnormalities did not show concurrent *PAX5* loss because of possible distal breakpoints that were not encompassing *PAX5* gene.

E. RB1

There were five MLPA probes encompassing *RB1* exons 6, 14, 19, 24 and 26 on the MLPA P335 kit. In total, 30 cases (8%) showed loss of *RB1*. The size of *RB1* deletions differed to either affecting the entire gene (n=22) or restricted to exons (19-26) (n=6) and various deletion extents existed but at lower frequencies including monoallelic loss of exons (14-26) (n=1) or biallelic deletion of exons (19-26) with associated monoallelic loss of exons (6-14) (n=1). *RB1* deletions were either monoallelic or biallelic. The majority of these cases were allocated in the age group (2-9) years with a median age of 5 years (Table

3-2). There was no single relapse case reported in these patients and this might be explained by the fact that most cases (77%) were categorised as standard NCI risk. In total, 10 out of 24 cases (42%) with successful cytogenetic results were shown to harbour visible cytogenetic abnormalities including deletions of the long arm of chromosome 13 (del(13q)) (n=4) and loss of the entire chromosome 13 (n=3), while the remaining cases (n=3) exhibited either add(13q) (n=1) or translocations with different partners (n=2).

3.4.3.3 Copy number alterations in transcription regulating genes

F. ETV6

Deletions of *ETV6* were the most prevalent alterations which represented 52% (n=206) of *ETV6-RUNX1* cases. The most common deletion involved the entire gene (n=149, 72%), exons (2-8) (n=18, 9%), exons (1-5) with associated biallelic exon 8 loss (n=9, 4%), exons (1-5) (n=8, 4%) and other miscellaneous deletions (Figure 3.3). Among these losses, either monoallelic (n= 195) or biallelic deletions (n=11) did occur with the later type restricted to exon 1 (n=2, 18%) or exon 8 (n=9, 82%) and accompanied by monoallelic losses of other exons of *ETV6*. These losses predominated in the age group (2-9) years (90%) with a median age of 4 years (Table 3-2). It is worth noting that around quarter of the cases were classified as high NCI risk which was attributed to their tendency to have a high WCC. There were no associations found between *ETV6* aberrations and other gene losses investigated in this study (Table 3-4).

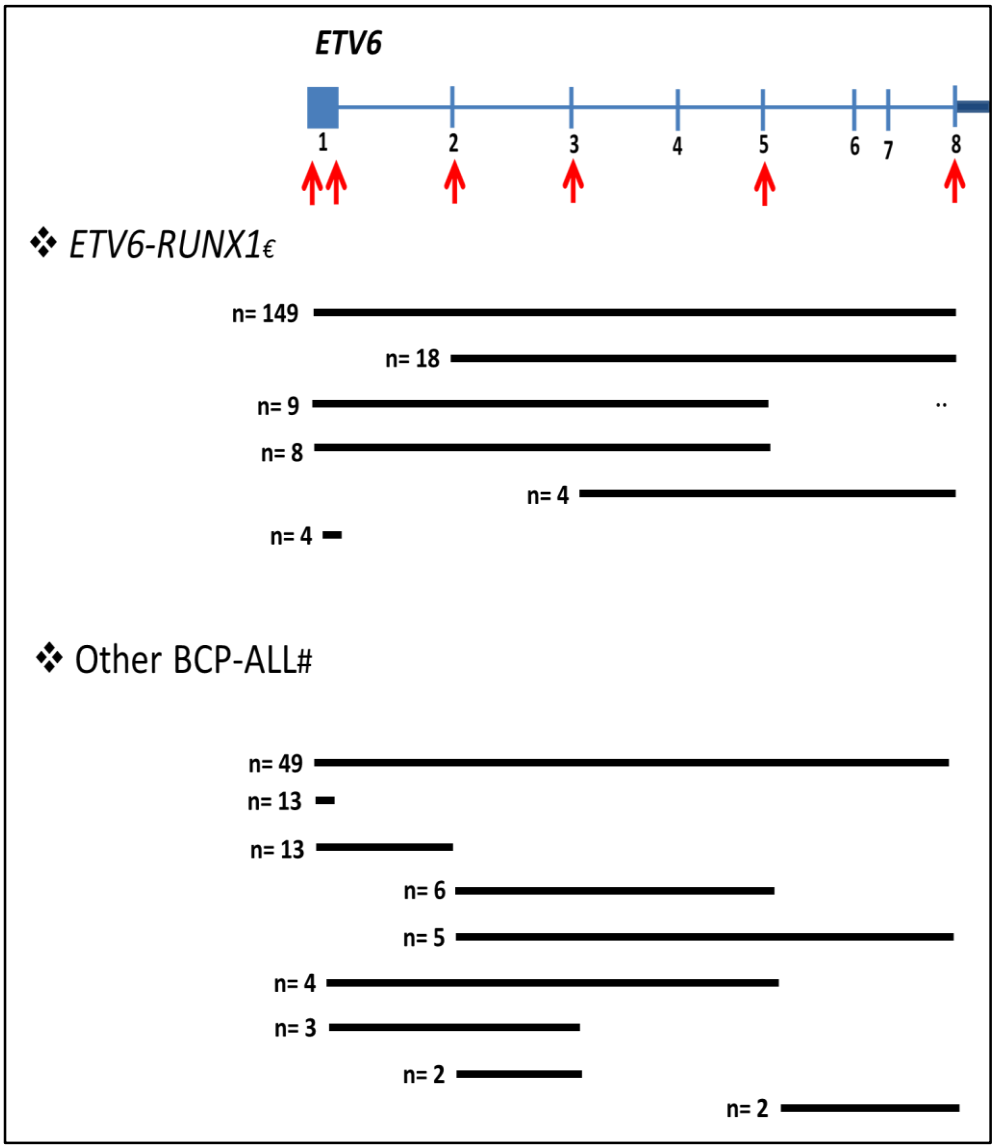

Figure 3.3 Comparison of *ETV6* deletion breakpoints between *ETV6-RUNX1* and other BCP-ALL cases. *ETV6* gene (8 exons) is displayed with the six MLPA probes used from the SALSA MLPA kit P335 IKZF1 (Red arrows) that covered *ETV6* exons 1 (two probes), 2, 3, 5 and 8; the entire gene loss is the commonest in both *ETV6-RUNX1* and other BCP-ALL; the total number of *ETV6* losses in *ETV6-RUNX1* and other BCP-ALL cases (206 and 103 cases); € other miscellaneous losses presented at lower frequencies (1% or less each) including: exons 3-5, exons 5-8, exon 8 with biallelic exon 1, biallelic exons 5-8, exons 1-2, exons 1-2, 8, exons 2-5, exons 3, 8, exons 2-5 with biallelic exon 8 and exons 2-8 with biallelic exon 1; # other miscellaneous losses presented at lower frequencies (1% or less each) including: exons 1, 3-5, exons 1, 3-8, exons 1-2, 5, exons 3,8, exons 3-5 and exons 3-8. Solid lines are heterozygous losses, while dashed lines are homozygous.

In total, 76 out of 161 *ETV6* deleted cases (47%) with successful cytogenetic analysis were observed to harbour visible cytogenetic abnormalities of either the entire chromosome 12 (n=1, 0.6%) or its short arm (12p) (n=75, 47%). The most frequent alterations among 76 cases were both deletion of 12p (n=37, 23%) and additional

material on chromosome 12p (add(12p)) (n=25, 16%). In addition, dicentric chromosomes were detected in 12 cases (16%) including: dic(9;12) (n=6, 50%), dic(12;13) (n=2, 17%), dic(8;12) (n=1, 8%), dic(12;20) (n=1, 8%), dic(12;15) (n=1, 8%) and dic(12;19) (n=1, 8%). They resulted in either the entire loss of *ETV6* gene (n=11, 92%) or its telomeric exons (1-5) (n=1, 8%); the latter case harboured dic(9;12).

FISH studies were available on 175 (85%) of *ETV6* deleted cases and 76% (n=137) of cases had concordant FISH and MLPA results. However, the remaining 38 cases (22%) showed deletion by MLPA but not by FISH owing to the small region of deletions that were below FISH resolution; occurring intragenic in the gap between the *ETV6* BA probes used.

Among the non-deleted *ETV6* cases by MLPA (n=190) in the overall cohort, 161 cases (85%) had successful FISH studies and a total of 42 cases showed *ETV6* deletions which were missed by the MLPA detection threshold criteria or sensitivity (see section 2.9.3) (Appendix B). Among 42 cases, 9 cases (21%) exhibited lower *ETV6* MLPA probe ratios, below the MLPA detection threshold, varying between 0.76-0.89 and FISH studies showed *ETV6* deletions in proportions of 26% or more (range: 26%-84%). However, 30 cases (71%) harboured *ETV6* deletions by FISH only, owing to either a low proportion of deleted clones (25% or less of the total cells, below the MLPA resolution) (n=13, 31%) or being masked by gain of der(21)t(12;21) resulting in normalizing the copy number of *ETV6* exons (1-5) in MLPA in 17 cases (40%). The remaining 3 cases (7%) showed artefacts in either MLPA or FISH studies.

In the overall cohort (n=397), using MLPA and FISH, *ETV6* loss represented around 62% (n=245) of *ETV6-RUNX1* cases.

G. BTG1

The MLPA P335 *IKZF1* kit contains four probes for *BTG1* gene (consists of two exons); two of them cover exons 1 and 2, while the remaining two probes target the 3' region of the gene that has no other genes involved but represents a highly conserved promoter region of the *BTG1* gene. *BTG1* deletions constituted around 15% (n=60) of *ETV6-RUNX1* cases. The most frequent deletions were restricted to the probes covering exon 2 and the adjacent 3' end region with retention of exon 1 (n=53, 88%) and all of them were monoallelic losses except four cases which were biallelic. In addition, there

were a further 6 cases (10%) that showed retentions of both exon 1 and 2 with deletions restricted to the 3' end regions, however, the remaining case (2%) exhibited a mixture of the above deletion extents resulting in both biallelic deletion of 3' end region and monoallelic deletion of exon 2 with retention of exon 1. The majority of *BTG1* losses (85%) were in the age group (2-9) years with a median age of 4.5 years and none of the infant patients exhibited *BTG1* deletions (Table 3-2). There was no significant difference in the prevalence of these deletions and other demographic or clinical categories. There was no association between the occurrence of these deletions and other gene losses (Table 3-4). To note, 2 out of 44 cases (5%), with successful cytogenetic results, showed visible cytogenetic abnormalities of the long arm of chromosome 12 including: deleted 12q [del(12)(q)].

3.4.3.4 Rearrangements of Pseudoautosomal region 1 (PAR1)

Pseudoautosomal region 1 (PAR1) encompassed different genes including *CRLF2*, *CSF2RA*, *IL3RA* and *P2RY8* genes. These genes were each covered by a single MLPA probe which targets *CRLF2* exon 4, *CSF2RA* exon 10*, IL3RA* exon 1 and *P2RY8* exon 2. The PAR1 rearrangements were identified by deletion of both *CSF2RA* /*IL3RA* genes with retention of both *CRLF2* and *P2RY8* genes, thus resulting in the formation of the fusion gene *P2RY8-CRLF2* (Russell *et al.*, 2009). In total, only 4 *ETV6-RUNX1* positive cases showed this rearrangement with a median age of 8 years (ages: 17, 8, 7 and 3 years old) (Table 3-2). These rearrangements were not associated with any gene losses in this ALL subtype (Table 3-4).

3.4.4 Comparison between ETV6-RUNX1 positive and other BCP-ALL subtypes.

It is essential to understand the unique pathobiology of *ETV6-RUNX1*, thus the identification of potential differences in demographic, clinical and genetic characteristics is vital. This can be achieved by comparing the *ETV6-RUNX1* dataset with other BCP-ALL subtypes (Table 3-5) that utilised the data generated from the comprehensive MLPA screening on a representative cohort of BCP-ALL and other available cytogenetic and FISH data. There was a three fold increase in the number of patients aged 10 years and older in other BCP-ALL patients as compared to *ETV6-RUNX1* ($p<0.0001$). Otherwise, there were no differences in terms of sex or WCC distribution.

Three quarters of the *ETV6-RUNX1* cases were classified as a standard risk category which might explain why the relapse rate was half that seen in other BCP-ALL subtypes in either treatment protocols (p<0.0001) (Table 3-6). It is worth noting that late relapses constituted around two third of the relapse cases in *ETV6-RUNX1* as compared to approximately one third in other BCP-ALL (p<0.0001).

	Cytogenetic subgroups		
	ETV6-RUNX1	**Other BCP-ALL**	***P-value***
No. of cases	397 (100)	1019 (100)	
Age (years)			
Median	4	5	
1	21 (5)	80 (8)	
2-9	342 (86)	643 (63)	<0.0001*
10+	34 (9)	296 (29)	
Sex (M:F)	216:181	551:468	0.9
Ratio	1.19	1.18	
WCC (X 10^9/L)			
Median	11.6	12.3	
<10	178 (45)	442 (43)	
10-49.9	147 (37)	378 (37)	0.8
≥50	72 (18)	199 (20)	
NCI Risk Group			
Standard	296 (75)	577 (57)	<0.0001*
High	101 (25)	442 (43)	

Table 3-5 Comparison of the demographics and clinical features between *ETV6-RUNX1* positive and other BCP ALL patients. * statistically significant, WCC: white cell count, NCI: National Cancer Institute (standard risk: aged 1-9 years with a WCC < 50 x10^9/L at initial presentation, high risk category: ≥10 years and a WCC ≥ 50 x10^9/L).

	Cytogenetic subgroups		
	ETV6-RUNX1 **(n= 397)**	**Other BCP-ALL (n= 1019)**	**P-value**
ALL97/99‡ (n=498)	126 (100)	372 (100)	
No. of Relapses	17 (13)	97 (26)	0.004*
Early	6 (35)	62 (64)	0.003*
Late	11 (65)	35 (36)	
ALL2003¥ (n=918)	271 (100)	647 (100)	
No. of Relapses	10 (4)	78 (12)	<0.0001*
Early	3 (30)	61 (78)	<0.0001*
Late	7 (70)	17 (22)	

Table 3-6 Comparison of the incidences and type of relapses between *ETV6-RUNX1* positive and other BCP ALL patients in UKALL97/99 and UKALL2003. * statistically significant, ‡ after a median follow-up time of 9.2 years, ¥ after 5 years follow-up time (ALL2003 cohort is not a representative cohort, had many relapses that were attributed to the availability of material).

Overall, the *ETV6-RUNX1* group is highly heterogeneous and when considering the eight genes screened for by MLPA had an average of 1.26 CNA per case compared to 0.92 alterations per case among other BCP-ALL (p<0.0001) (Table 3-7). Around 77% of *ETV6-RUNX1* cases harboured an alteration of at least one of these genes, while approximately half of other BCP-ALL cases showed normal MLPA profiles in all genes. Thus, the *ETV6-RUNX1* group was found to have increasing number of gene deletions compared to other BCP-ALL subtypes, especially those exhibiting one or two alterations (67% v 41%) (Figure 3.4). It is worth noting that NCI risk had no effect on the increasing tendency to acquire multiple alterations in *ETV6-RUNX1* as compared to other BCP-ALL cases (Figure 3.5). Thus, only 34% of high risk other BCP-ALL showed any aberrations as compared to ~ 60% of standard risk other BCP-ALL (p<0.0001). *ETV6* and *BTG1* losses are five times more common in *ETV6-RUNX1* as compared to other BCP-ALL (p<0.0001), while alterations affecting the *IKZF1* gene were less prevalent (3% v 18%) (p<0.0001). It is worth noting that although the cytogenetically visible 9p alterations were slightly more common in other BCP-ALL as compared to *ETV6-RUNX1* (10% v 5%), both *CDKN2A/B* and *PAX5* deletions were common alterations in both groups with *PAX5* aberrations being slightly

more prevalent (23% v 17%) among *ETV6-RUNX1* cases as compared to other BCP-ALL subtypes (p=0.02).

Total cohort	Cytogenetic subgroups		P-value
	ETV6-RUNX1 n= 397 (100)	Other BCP-ALL n= 1019 (100)	
Deletions			
ETV6	206 (52)	103 (10)	<0.0001*
9p	14 (5)#	138 (10)¥	NA
CDKN2A/B	91 (23)	300 (29)	0.02
PAX5	91 (23)	178 (17)	0.02
BTG1	60 (15)	34 (3)	<0.0001*
6q	33 (11)#	44 (3)¥	NA
RB1	30 (8)	67 (7)	0.5
11q	18 (6)#	36 (3)¥	NA
EBF1	14 (4)	17 (2)	0.03
IKZF1	12 (3)	187 (18)	<0.0001*
PAR1	4 (1)	78 (8)	<0.0001*
Average CNA	1.26	0.92	<0.0001*

Table 3-7 Comparison of the incidences of different genetic alterations between *ETV6-RUNX1* positive and other BCP ALL patients. * statistically significant, # percentages were calculated from 304 cases with successful cytogenetic, ¥: data was extracted from (Moorman *et al.*, 2010b), NA not available.

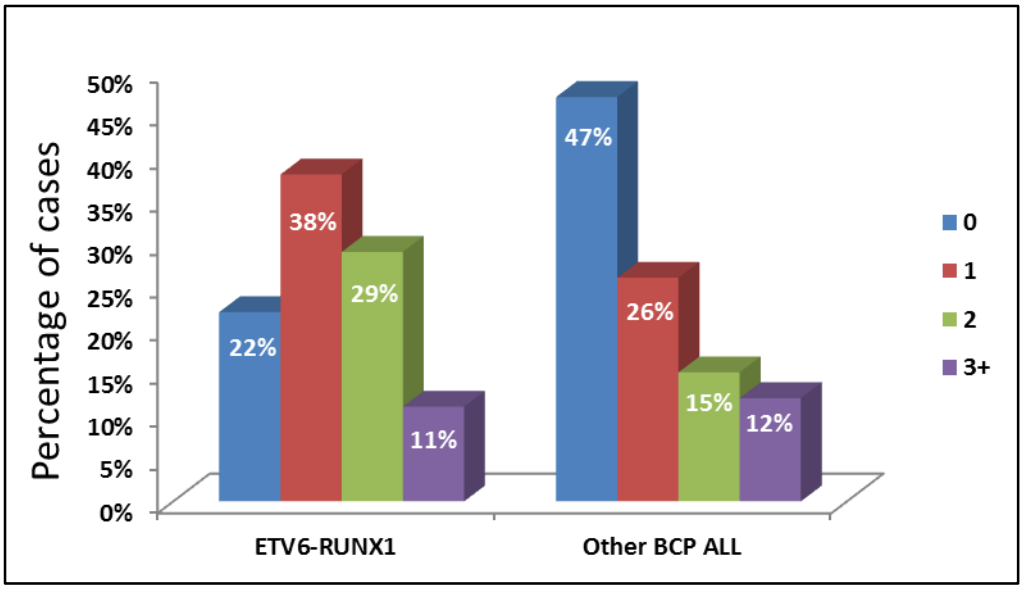

Figure 3.4 Bar chart showing the percentage of patients with increasing numbers of CNA within *ETV6-RUNX1* and other BCP ALL subtypes.

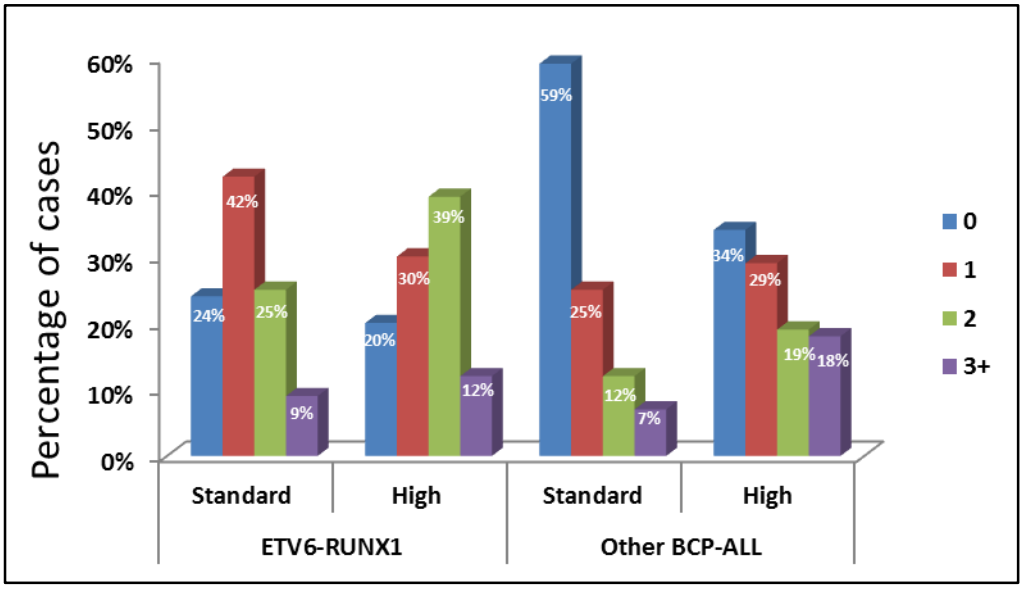

Figure 3.5 Bar chart showing the percentage of patients with increasing numbers of CNA within *ETV6-RUNX1* and other BCP ALL subtypes based on National Cancer Institute categories (High and Standard risk).

PAX5 losses showed variant breakpoints with around 60% of *ETV6-RUNX1* cases exhibited deletions retaining exon 1 with variable extents including the losses restricted to exons 2-6 that constituted 38% of the deleted cases. However, the majority of the deleted cases in other BCP-ALL targeted the whole gene (n=79, 44%) and the remaining deleted cases showed various breakpoints at lower frequencies (Figure 3.1). Unlike *ETV6-RUNX1* cases, other BCP-ALL cases exhibited *PAX5* deletions that frequently involved exon 1 (p=0.006). In addition, intragenic amplifications of *PAX5* affecting exons 2 and/or 5, with a copy number range of (5-9) were detected in other BCP-ALL subtypes (n=11, 1%) but none were found in *ETV6-RUNX1* cases.

The extent of *IKZF1* deletions was highly variable with the most frequent ones in *ETV6-RUNX1* restricted to exons (2-3) (n=5, 42%) which were less prevalent in other BCP-ALL (5%, respectively) (p<0.0001). However, 33% and 31% of *IKZF1* deleted cases in other BCP-ALL exhibited losses encompassing the entire gene and exons (4-7), respectively. *IKZF1* deletions targeting exons (4-7) seemed to occur less frequently in *ETV6-RUNX1* (p=0.02) (Figure 3.2).

Although the entire loss of *ETV6* gene constituted the commonest deletion type in both *ETV6-RUNX1* and other BCP-ALL cases (72% and 48%, respectively), they were associated to *ETV6-RUNX1* cases, in particular (p<0.0001). In addition, other BCP-ALL cases harboured frequent *ETV6 l*osses restricted to exon 1 or exons (1-2) which represented a quarter of the deleted cases, while these types of deletions occurred less frequently in *ETV6-RUNX1* cases (2%) (p<0.0001) (Figure 3.3).

BTG1 losses were mainly characterised by the deletions encompassing exon 2 and the 3' region with retention of exon 1 in both *ETV6-RUNX1* (88%) (See section 3.4.3.3 G.) and BCP-ALL (82%) cases. It is worth noting that exon 1 was retained in *ETV6-RUNX1* group, while around 15% of deleted cases in other BCP-ALL had lost exon 1 as a whole gene deletion either restricted to the gene itself (without the involvement of the probes covering the 3' region) (3%) or extended further to the adjacent 3' region (12%).

3.5 Discussion

The present study highlighted the spectrum of genetic abnormalities in a total of 397 *ETV6-RUNX1* cases that have been treated on UK ALL trials. The potential relevance of

these alterations to this cytogenetic subgroup was assessed by comparing them with a total of 1019 of other BCP-ALL cases (Schwab *et al.*, 2013). An MLPA approach was chosen owing to its ability to generate accurate and reliable data with high throughput (Schwab et al., 2010) using a single kit (P335 *IKZF1*) that targets those genes recurrently involved in BCP-ALL (Lilljebjorn *et al.*, 2007; Mullighan *et al.*, 2007; Kawamata *et al.*, 2008; Mullighan *et al.*, 2008b). These gene aberrations have been shown to be responsible for BCP-ALL development due to their significant biological roles in B cell development/ differentiation (*PAX5, IKZF1, EBF1, ETV6* and *BTG1*), cell cycle regulation (*RB1* and *CDKN2A/B*) and those genes encompassing the PAR1 region (*CRLF2, CSF2RA* and *IL3RA*).

Overall, *ETV6-RUNX1* cases showed a tight demographic profile with three quarters of patients assigned to the standard risk category. Considering the eight genes tested by MLPA, there was a noticeable increase in the number of aberrations irrespective of NCI risk with a mean of 1.26 alterations per case compared to 0.92 abnormalities per case in other BCP-ALL cases. It is worth noting that this observed increase in the number of alterations in *ETV6-RUNX1* might be a reflection of the manner in which these genes were selected for inclusion in the kit, i.e. based on the prevalence of deletions in previous patient cohorts which comprised 25% *ETV6-RUNX1* positive cases (Lilljebjorn *et al.*, 2007; Mullighan *et al.*, 2007; Kawamata *et al.*, 2008; Mullighan *et al.*, 2008b). However, previous genomic studies showed an overall increase of deletions in *ETV6-RUNX1* as compared to other BCP-ALL subgroups (6 v 3.1 losses per case, respectively) (Mullighan *et al.*, 2007). The plausible explanation might be that *ETV6-RUNX1* fusion gene has a positive impact on the persistence and expansion of the pre-leukaemic cells through its inhibitory effects on *TGF*-β (Siegel and Massague, 2003; Bierie and Moses, 2006), miRNA-494 and miRNA-320a (Diakos *et al.*, 2010), and activating effects on EPOR (Inthal *et al.*, 2008; Torrano *et al.*, 2011) and mTOR pathway (Fuka *et al.*, 2011; Fuka *et al.*, 2012; Tijchon *et al.*, 2013) (see section 1.5.4). This clonal expansion in turn increases their susceptibility to acquire further aberrations/ mutations owing to the overwhelming proliferative stress.

Furthermore, the enrichment of focal deletions rather than amplifications might be attributed to the undergoing aberrant RAG recombinase activity which represents a driving mechanism in *ETV6-RUNX1*, in particular (see section 1.4.1.3) (Mullighan and Downing, 2009a; Kuiper and Waanders, 2014; Papaemmanuil *et al.*, 2014).

ETV6-RUNX1 patients exhibited a wide range of genetic abnormalities facilitating leukaemic transformation and progression. The *PAX5* transcriptional factor gene aberrations seem to represent the main driver in blocking the lymphoid differentiation rather than *IKZF1* alterations in *ETV6-RUNX1*, unlike other BCP-ALL group that showed enrichment of both these alterations (23%, 3% v 17%, 18%, respectively). It is worth noting that *IKZF1* losses tended to occur at an older age, thus the rarity of these losses in *ETV6-RUNX1* might be a reflection of the three fold reduction in the number of patients aged 10 years and older as compared to other BCP-ALL patients (9% v 29%, respectively) rather than differences in the biology/ evolution. In addition, the lower frequency of *IKZF1* losses, which are linked to overall poor outcome (Mullighan *et al.*, 2009b), might explain the favourable outcome among *ETV6-RUNX1* compared to other BCP-ALL cases.

Further proliferation and survival of these undifferentiated cells usually require further alterations like activating kinase/ cytokine receptor (e.g. *CRLF2*) genes (e.g. PAR1 rearrangements). However, *ETV6-RUNX1* cases harboured PAR1 alterations less frequently than patients with other BCP-ALL (1% v 8%, respectively). The frequency of *CRLF2* rearrangements is related to older age similar to *IKZF1* losses. Hence, alternative aberrations including those targeting *EPOR*, which are enriched in *ETV6-RUNX1,* may play a role in the survival properties of these cells (see section 1.5.4) (Inthal *et al.*, 2008; Torrano *et al.*, 2011).

The alterations implicated in lymphoid differentiation and proliferation are coupled with further aberrations necessary for altering different cellular processes including transcriptional regulators/ co-regulators (e.g. *ETV6* and *BTG1*) and cell cycle control genes (e.g. *CDKN2A/B*). Both *ETV6* and *BTG1* losses were associated with *ETV6-RUNX1* and constituted 63% and 15% of cases compared to 10% and 3%, respectively, of other BCP-ALL group, however *CDKN2A/B* were common in both groups (23% and 29%, respectively), in agreement with previous studies (Mullighan *et al.*, 2007; Lilljebjorn *et al.*, 2010). Although *ETV6-RUNX1* had a unique distribution of genetic abnormalities compared to those with other BCP-ALL, there were no distinct subgroups separating those with or without the deletions in terms of demographic or clinical features which may be due to a high level of similarity among *ETV6-RUNX1* patients.

Recently, survival analysis was carried out on part of the present cohort of *ETV6-RUNX1* patients who were treated on UKALL97/99 and concluded a lack of impact of these additional abnormalities targeting the eight genes on outcome (Enshaei *et al.*, 2013). In contrast to these findings, high levels of *IKZF1* and *BTG1* deletions were previously reported among relapse cases as compared to the newly diagnosed cases in BCP-ALL (39.3% and 14.3% vs 8.9% and 6.7%), respectively (Hogan *et al.*, 2011).

The *ETV6* gene acts as a transcriptional repressor involved in haematopoiesis and angiogenesis (see section 1.5.3). Whole gene deletion was the predominant type of loss in either group; however other deletion breakpoints existed at lower incidences. *ETV6* losses had conflicting outcome results among *ETV6-RUNX1* cases, some reports showed lack of impact on outcome (Barbany *et al.*, 2012; Enshaei *et al.*, 2013), while other study groups suggested a good prognostic value (Stams *et al.*, 2006; Peter *et al.*, 2009; Ko *et al.*, 2011). On the other hand, only one study indicated an adverse effect on outcome (Attarbaschi *et al.*, 2004), which agrees well with other studies which postulated that the heterodimerization between the retained *ETV6* and *ETV6-RUNX1* fusion gene hampers the potency of the fusion protein (McLean *et al.*, 1996; Lilljebjorn *et al.*, 2010). Thus, *ETV6* loss of function co-operated with *ETV6-RUNX1* fusion gene resulting in the progression of leukaemogenesis (Morrow *et al.*, 2007; Tijchon *et al.*, 2013). The plausible explanation in the differences in outcome might be due to different treatment protocols used or the presence of other factors which might be able to influence outcome in an independent manner.

The *BTG1* gene plays an integral role in differentiation, proliferation, cell survival and glucocorticoid responsiveness. It is worth noting that *BTG1* alterations are contributing factors for leukaemia development through focal deletions and inactivating mutations (Papaemmanuil *et al.*, 2014). In the present study, the majority of *BTG1* losses (88%) in the *ETV6-RUNX1* group, similar to other BCP-ALL, retained exon 1 and extended from exon 2 (last exon) to involve the adjacent 3'end region of the gene that is a highly conserved promoter region, in agreement with previous studies (Waanders *et al.*, 2012). This finding concurs accords with the shared mechanism of abberant RAG activity that is responsible for the generation of these losses similar to that seen in *IKZF1* deletions and the enrichment of this mechanism among *ETV6-RUNX1* might explain the association of these deletions to this cytogenetic subgroup. Deletions targeting exon 2

usually result in the generation of truncated BTG1 transcrpits of no functional use (van Galen *et al.*, 2010). In the present study, all *ETV6-RUNX1* relapse cases with *BTG1* loss were late in UKALL97/99 (Table 3-3). Furthermore, none of the *BTG1* deleted cases was relapsed in UKALL2003 which might be because the follow up in UKALL2003 is currently quite short resulting in underestimation of the true relapse incidence rate. These findings suggest an alternative effect of *BTG1* loss other than glucocorticoid resistance which one would expect to result in early relapses due to poor therapy response. The effect of *BTG1* alterations is not well understood and the different glucocorticoid responses of different cell line models harbouring *BTG1* deletions might depend on the cellular and genetic context in which these lesions occur.

The *IKZF1* gene is an important transcriptional factor involved in the regulation of the lymphocyte differentiation. In the present study, the extent of *IKZF1* deletions was variable and interestingly it differed between *ETV6-RUNX1* and other BCP-ALL groups in which the classic *IKZF1* deletions (exons 4-7) are common in other BCP-ALL than in *ETV6-RUNX1* (31% v 0%), respectively (p=0.02). However, *ETV6-RUNX1* had losses restricted to exons (2-3) (42%) which were less frequent in other BCP-ALL (5%) (p=<0.0001). Thus, these differences in the architecture of these deletions might contribute to the generation of several alternatively spliced variants. These variants are usually transcribed to encode different isoforms that vary in the number of N-terminal zinc finger motifs which determine the DNA binding properties. The non DNA binding isoforms, that lack sufficient number of N-terminal zinc fingers, function as dominant negative factors and their overexpression result in leukaemogenic transformation (Iacobucci *et al.*, 2008; Iacobucci *et al.*, 2009). Thus, the extent of deletions will alter the expression of different isoforms. Deletions of exons (4-7) resulted in the formation of non-DNA binding isoform (Ik6) due to the inability to encode the N-terminal DNA binding domain, while losses targeting exon 2 would inhibit the protein translation owing to compromising the start translation site (Iacobucci *et al.*, 2008; Iacobucci *et al.*, 2009). These deletions are more likely to have similar effects as the whole gene deletion except those losses restricted to the non-coding exon 1 which might not have an impact on IKZF1 function. It is worth noting that none of the *ETV6-RUNX1 IKZF1* deleted cases relapsed.

The most important limitation of this study lies in the fact that this screening focused on a selected number of genes, assuming that other recurrent genes, identified by previous genomic studies, are infrequent. Chapter 5 looked at a wider range of genes in the diagnostic- relapse pairs. Furthermore, the resolution of the MLPA technique used would underestimate those microdeletions that were present in <30% of cells. Although the FISH studies would identify abnormal clones present at low levels, they are more likely to miss genetic rearrangements sized <20kb or <100kb using interphase or metaphase FISH, respectively. Although the incidence of *ETV6* losses by FISH (53%) was similar to that by MLPA (52%), 21% of the cases had discordant results. Accordingly, a combination of both techniques is ideal in estimating the true incidence of a specific abnormality.

In conclusion, these studies have confirmed that the *ETV6-RUNX1* group is unique with tight demographic profile. *ETV6* and *BTG1* losses were more common compared to other ALL cases, while alterations affecting the *IKZF1* gene, which is involved in lymphoctye development pathway, were much less prevalent and did not appear related to poor outcome. However, among the genes investigated, there were neither distinct CNA profiles nor were there any obvious subtypes defined by these CNA. These findings extend those of smaller previous studies and confirm the findings of studies which have been conducted concurrently with this one by other groups. Collectively, these observations have broadened our understanding of the key differences discriminating *ETV6-RUNX1* with other BCP-ALL subtypes.

Chapter 4. Secondary abnormalities involving the der(12)t(12;21) in *ETV6-RUNX1* positive BCP-ALL

4.1 Introduction

ETV6-RUNX1 positive pre-leukaemic cells need secondary abnormalities for their conversion into leukaemic blasts. *ETV6* deletions have been implicated as important secondary events which constitute around two thirds of *ETV6-RUNX1* cases (Mullighan *et al.*, 2007; Lilljebjorn *et al.*, 2010). There are other frequent alterations including +21, +der(21)t(12;21), +10, +16 and deleted (6q) which represent 12%, 12%, 9%, 9% and 20%, respectively, of *ETV6-RUNX1* cases. Considerable research has been devoted to characterising the additional abnormalities involving der(21)t(12;21)/*ETV6-RUNX1* and normal chromosomes 21 or 12 , which are believed to contribute to leukaemogenesis. For the purpose of evaluating the prognostic impact of these abnormalities, comprehensive FISH screening for *ETV6* deletions, +21 and +der(21)t(12;21) was performed on 247 *ETV6-RUNX1* positive BCP-ALL cases by other LRCG members using novel dual colour (DC) break-apart (BA) probes targeting either the *ETV6* or the *RUNX1* loci (Enshaei *et al.*, 2013). Interestingly, the study revealed additional unexpected abnormalities suggesting der(12)t(12;21) involvements in a total of 30 out of 247 cases. The observed signal patterns were suggestive of either an interstitial deletion (n=20) or a duplication of the der(12)t(12;21) (n=10) (Figure 4.1).

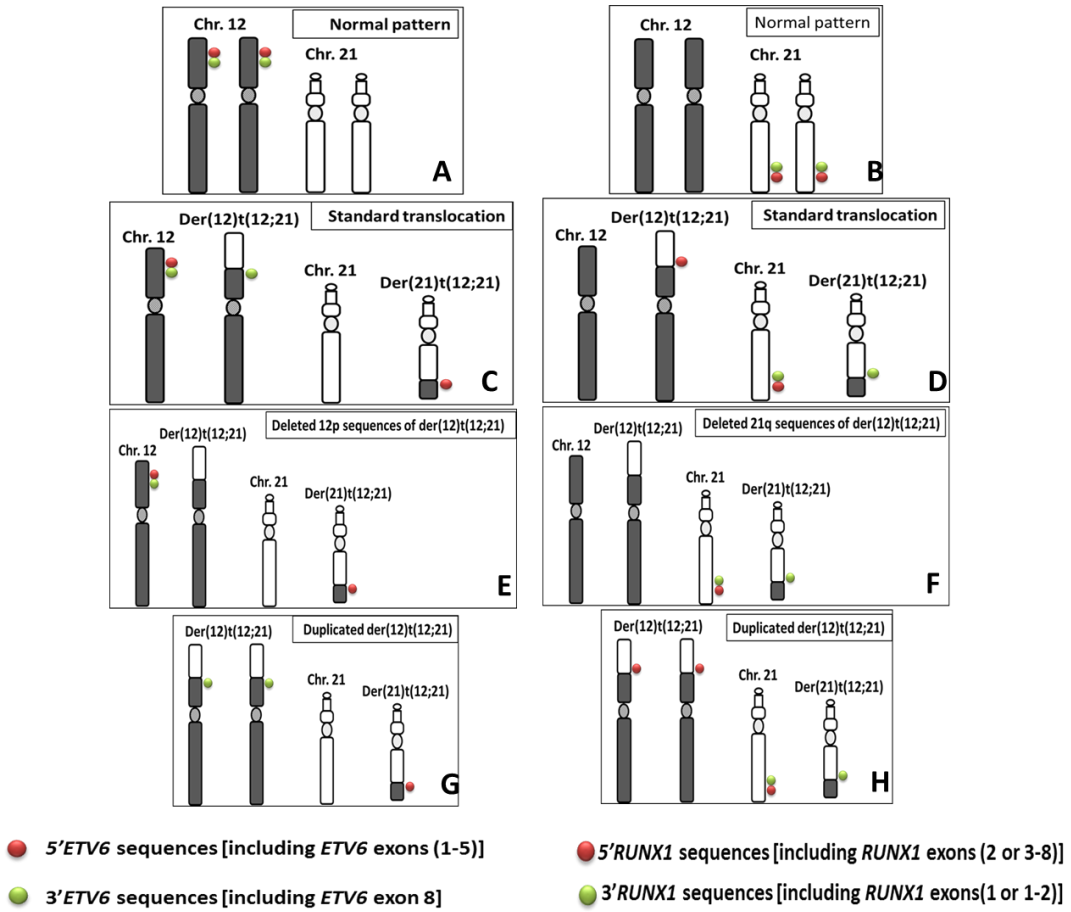

Figure 4.1 Ideograms show the different signal patterns given by *ETV6* (left) and *RUNX1* (right) dual color (DC) breakapart (BA) probes: in a normal [0R 0G 2F] (A), [0R 0G 2F] (B), a standard t(12;21) [1R 1G 1F] (C), [1R 1G 1F] (D), unexpected observed signal patterns of: [1R 0G 1F] suggesting a deleted 12p sequences (*3'ETV6* sequences) of der(12)t(12;21) (E), [0R 1G 1F] suggesting a deleted 21q sequences (*5'RUNX1* sequences) of der(12)t(12;21) (F), collective signal patterns of [1R 2G 0F] (G) and [1G 2R 1F] (H) suggesting duplicated der(12)t(12;21).

4.2 Aims and Objectives

The aim of this Chapter is to investigate incidental findings of earlier FISH screening by the LRCG group. Subsequent identification of the genes responsible in driving the development of the leukaemia would underscore our understanding of the evolution of this cytogenetic subtype.

The main objectives in this Chapter are:

1. Characterisation of the deleted der(12)t(12;21) and determination of the size of the deletions and the important genes affected.

2. Confirmation of the duplicated *RUNX1-ETV6* chimeric gene and identification of the responsible mechanism behind its formation and its effect on leukaemogenesis.

3. Investigate whether these two abnormalities which lead to copy number alterations affecting *ETV6* can be detected using the MLPA P335 kit which has seven probes spanning *ETV6* exons (1-8).

4.3 Patients and Methods

4.3.1 Patient cohort

The cohort included in the first part of the study were those treated using ALL97/99 protocol, while the second part, which investigated the third aim, involved patients who were registered in both trials ALL97/99 and UKALL2003 (see section 2.2). A total of 49 patients with suitable material were included in this Chapter. Most of the data included in this Chapter was recently published (Al-Shehhi *et al.*, 2013).

4.3.2 Methods

4.3.2.1 FISH

All FISH studies were performed as described in section 2.8. Different probe sets were applied to accomplish the following objectives:

A. Mapping der(12)t(12;21) deletion

Deletion mapping of der(12)t(12;21) was performed using two different probe sets, depending on the region of the deletion and comprised differentially labelled target and control probes (Appendix C).

Using the *ETV6* BA probe, a signal pattern of 1R 0G 1F was suggestive of a deletion of *3'ETV6* sequences on der(12)t(12;21) (Figure 4.1 E.). Therefore, these cases were screened using a series of target probes mapping along 12p (12p11.21- 12p13.2). For each FISH test, the *5'RUNX1* probe (21q22.12) was used as the control probe (labelled

red) combined with a target probe (labelled green). In a cell with a standard t(12;21) translocation, one would observe a signal pattern of 1R 1G 1F indicating a normal chromosome 21, normal chromosome 12 and intact der(12)t(12;21), respectively (Figure 4.2 B). Thus in each test, a signal patterns of 2R 1G 0F or 2R 0G 0F would suggest the *3'ETV6* sequence deletions at der(12)t(12;21) with or without intact non- rearranged *ETV6*, respectively (Figure 4.2 C).

On the other hand, those with suggestive deleted *5'RUNX1* sequences (21q sequences) of der(12)t(12;21) indicated by the unexpected signal pattern of 0R 1G 1F using *RUNX1* BA probe (Figure 4.1 F.), target probes mapping along 21q from 21q22.12-21q22.3 were serially applied. For each FISH test, *3'ETV6* probe (12p13.2) was used as the control probe (labelled green) combined with a target probe (labelled red). In a cell with a standard t(12;21) translocation, a signal pattern of 1R 1G 1F is expected indicating a normal chromosome 21, normal chromosome 12 and intact der(12)t(12;21), respectively (Figure 4.2 B). Thus in case of cells with deleted *5'RUNX1* sequences at der(12)t(12;21) with associated intact or deleted non-rearranged *ETV6*, one would observe a signal pattern of 1R 2G 0F or 1R 1G 0F, respectively (Figure 4.2 D).

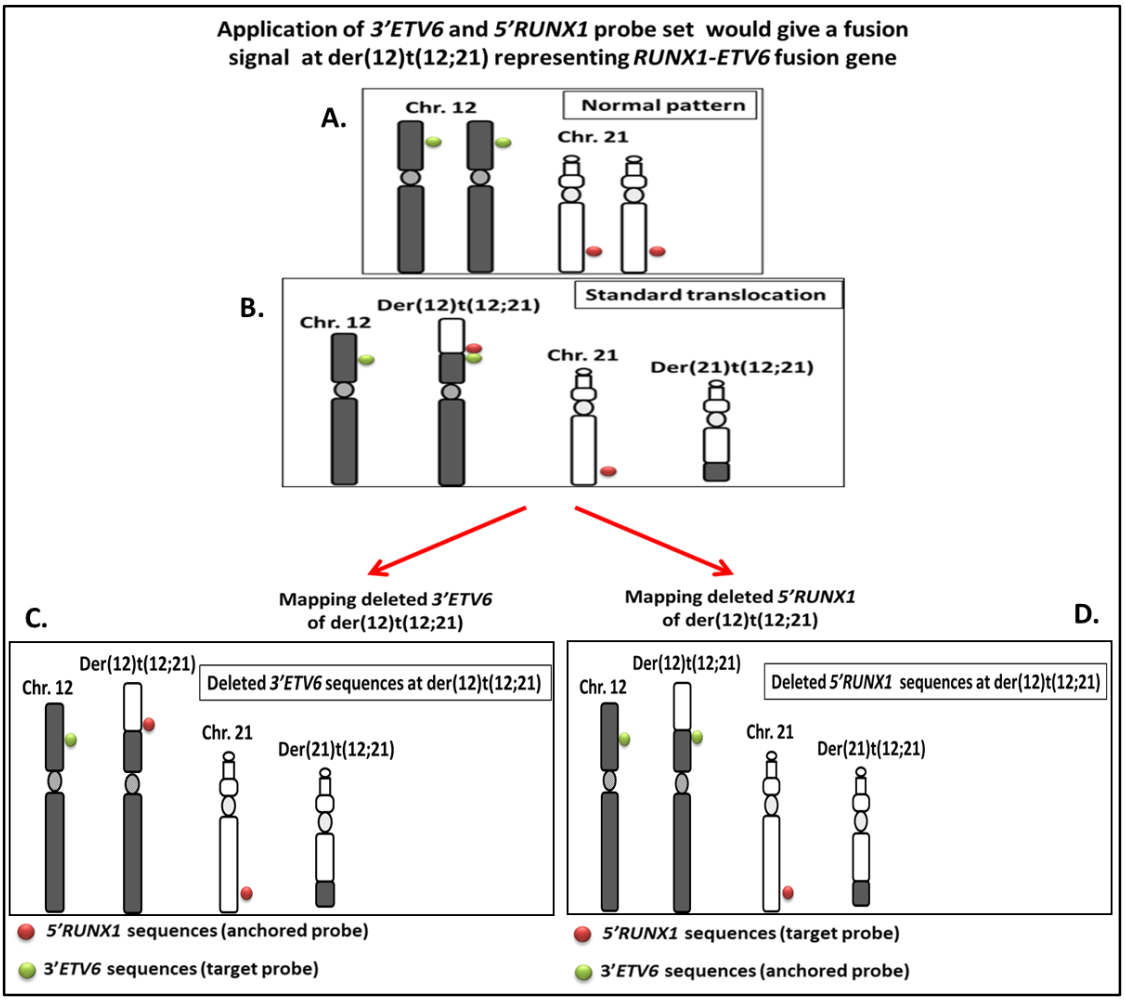

Figure 4.2 Ideograms show the signal patterns generated from the mapping deletion probes targeting either *3'ETV6* or *5'RUNX1* sequences of der(12)t(12;21): in a normal [2R 2G 0F] (A), a standard t(12;21) [1R 1G 1F] (B), a deleted *3'ETV6* region [2R 1G 0F] (C) and a deleted *5'RUNX1* region of der(12)t(12;21) cells [1R 2G 0F] (D).

B. Confirmation of the origin of deleted 12p sequences

In order to confirm the origin of deleted 12p sequences, three colour FISH was carried out using home-grown probes which comprised of the control probe (*5'RUNX1* probe) (labelled red), a known intact *3'ETV6* probe (labelled green) and a known deleted *3'ETV6* target probe (labelled gold). In a standard translocation, a signal pattern of 1R 1F (green-gold) 1F (green-red-gold) would be observed representing the normal chromosome 21, normal chromosome 12 and intact der(12)t(12;21), respectively (Figure 4.3 B). Thus, a signal pattern of 1R 1F (green-gold) 1F (red-green) would indicate the presence of a normal chromosome 21, normal chromosome 12 and deletion of the gold probe from the der(12)t(12;21), respectively (Figure 4.3 C).

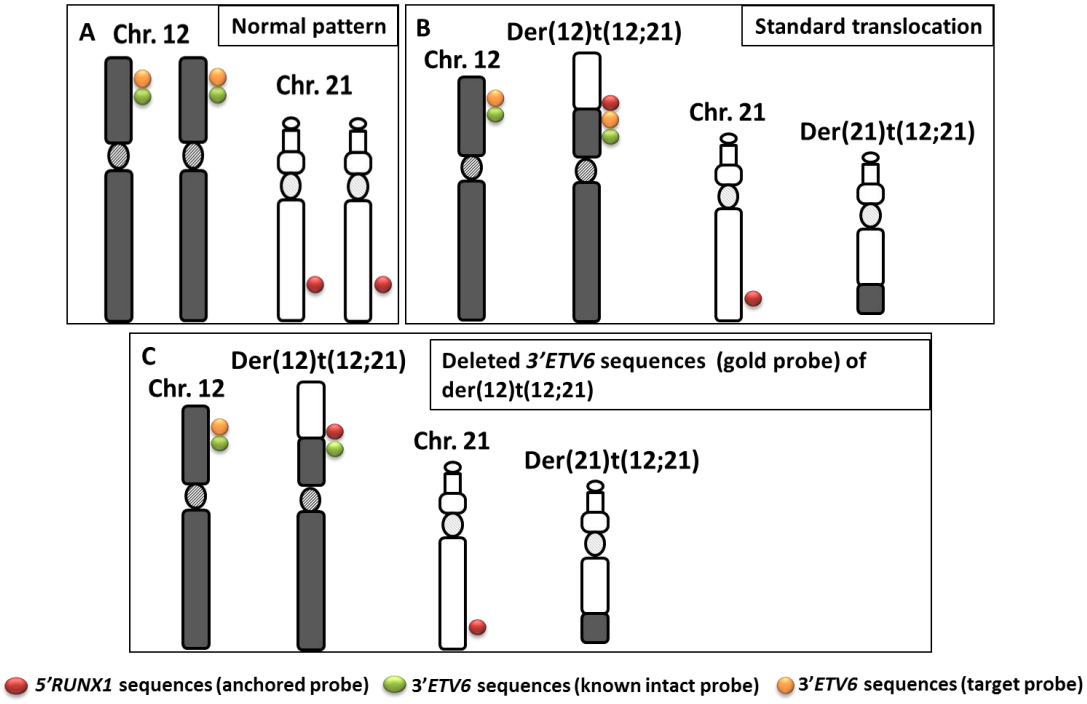

Figure 4.3 Ideograms of the home grown probes used in the three colour FISH: A. a normal signal pattern [2R 0G 2F (green-gold)], B. a standard t(12;21) with signal pattern of [1R 1F (green-gold) 1F (green-gold-red)] and C. deleted *3'ETV6* sequences (gold probe) from der(12)t(12;21) indicated by the signal pattern of [1R 1F (green-gold) 1F (green-red)].

C. Investigations of a possible duplicated der(12)t(12;21)

In order to investigate those patients with the unexpected signal patterns of 1R 2G 0F and 1G 2R 1F, using *ETV6* and *RUNX1* BA probes, respectively, (Figure 4.1 G-H), suggestive of duplicated *RUNX1-ETV6* fusion gene on der(12)t(12;21), a home grown dual colour single fusion (DC SF) probe was applied. The DC SF probe was comprised of the *5'RUNX1* probe (labelled red) and *3'ETV6* probe (labelled green). In a cell with a standard t(12;21) translocation, a signal pattern of 1R 1G 1F would be observed indicating a normal chromosome 21, a normal chromosome 12 and an intact der(12)t(12;21), respectively (Figure 4.2 B). Thus, in each FISH test, a signal pattern of 1R 0G 2F, showing an extra fusion signal with associated loss of the green signal was sought (Figure 4.4).

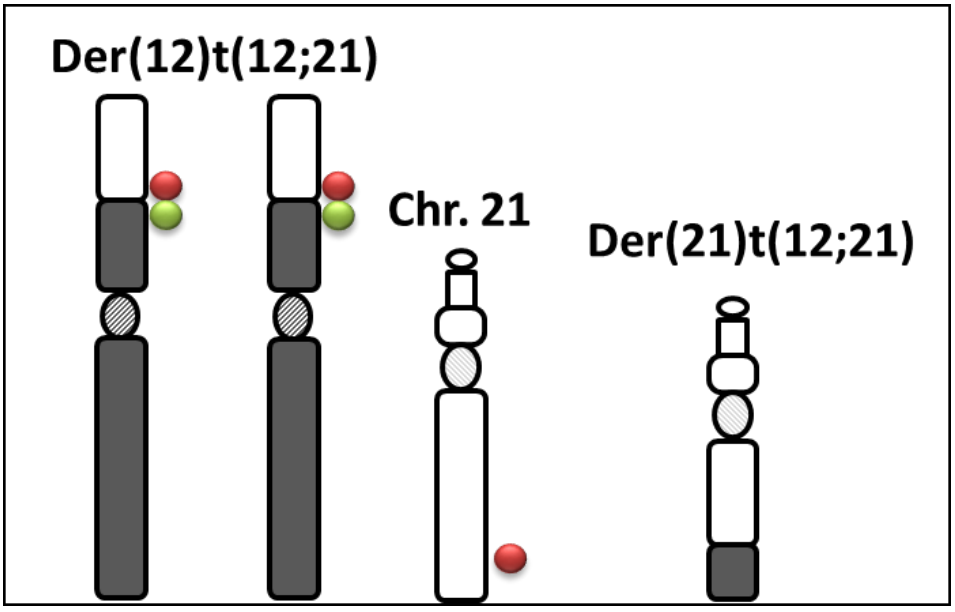

Figure 4.4 The expected signal pattern of the suggested duplicated *RUNX1-ETV6* fusion gene using *5'RUNX1* (labelled red)/*3'ETV6* (labelled green) DS SF probe set. A signal pattern of 1R 0G 2F is shown.

To confirm the number of chromosomes 12 and to assess the genetic stability by obtaining the relative number of chromosome 12 centromeres and 12p subtelomeres in those cases with duplicated *RUNX1-ETV6* fusion gene, FISH using a commercial probe set was carried out. The probe set consisted of centromeric 12 (CEP 12) probe (labelled red, Cytocell) and subtelomeric 12p (subtel 12p) probe (labelled green, Vysis). This probe

set would give a signal pattern of 2R 2G 0F in either normal or a standard t(12;21) cells. Thus, the number of the centromeres and the subtelomeres was investigated.

D. Investigation of cases with isolated ETV6 exon 8 deletions

In order to identify further cases with deleted *3'ETV6* sequences of der(12)t(12;21), cases with isolated deletions of *ETV6* exon 8 (*3'ETV6* region) by MLPA (see section 4.3.2.2), whether mono or biallelic losses, were further investigated by FISH studies. Firstly, application of *ETV6* and *RUNX1* DC BA probes was carried out to uncover the possible abnormalities responsible for the isolated *ETV6* exon 8 losses (e.g. deletions of either the non-rearranged *ETV6* or *3'ETV6* sequences of der(12)t(2;21) (Figure 4.1). If the observed signal patterns suggested that both *ETV6* alleles were intact using *ETV6* DC BA probe, a home grown fosmid probe targeting *ETV6* exon 8 (labelled green) was used as a target probe to identify smaller deletions. This fosmid probe was combined with the *5'ETV6* probe (labelled red) (Figure 4.5), thus, this probe set was considered similar to *ETV6* DC BA, but it was able to identify smaller deletions undetectable by the ordinary *ETV6* DC BA probe.

In order to define the deletion sizes in those new cases with *3'ETV6* sequences of der(12)t(12;21), FISH mapping was done (see section 4.3.2.1 A.).

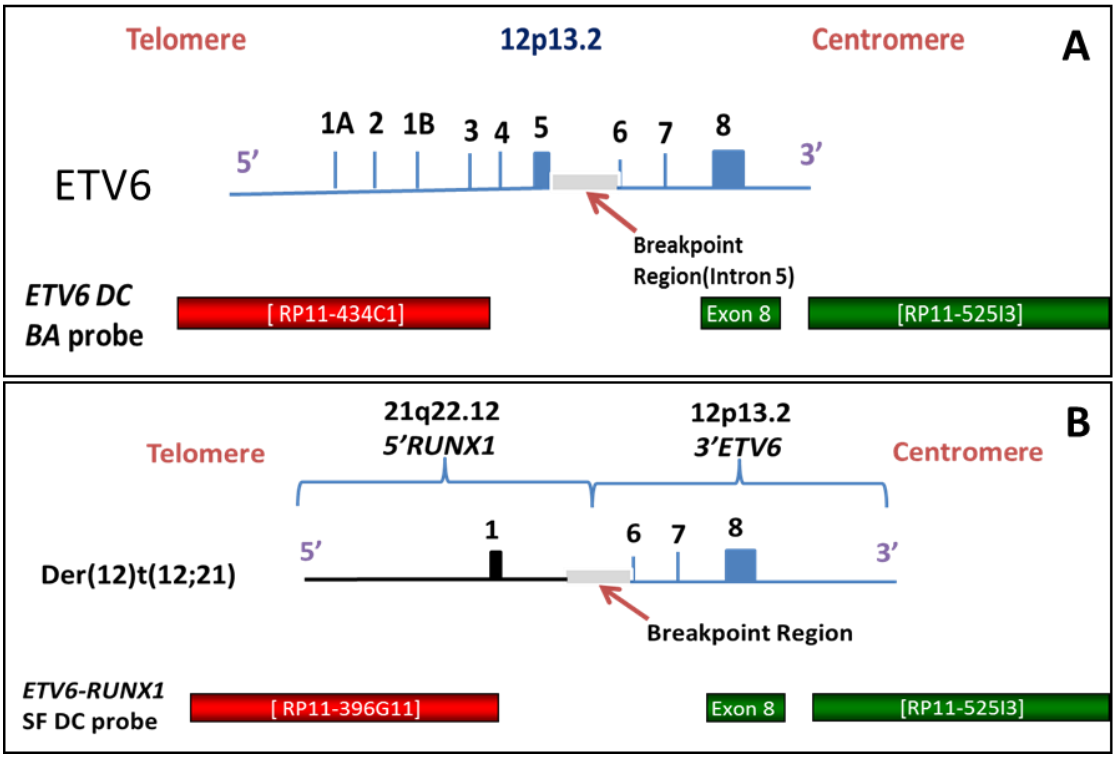

Figure 4.5 The locations of *ETV6* exon 8 [*3' ETV6] probes* with either *5'ETV6* or *5'RUNX1* probes. A. *ETV6* gene at 12p13.2, composed of 8 exons in which the t(12;21) breakpoint occurred within intron 5; Locations of the green and red probes used in the *ETV6* DC BA probe (RP11-525I3 or *ETV6* exon 8 - RP11-434C1), B. The breakpoint at der(12)t(12;21); locations of the green and red probes used in the 3'*ETV6-5'RUNX1* SF DC probe (RP11-525I3 or *ETV6* exon 8 - RP11-396G11).

E. Investigations of cases with isolated ETV6 exons 1-5 deletions

To identify further cases with duplicated *RUNX1-ETV6* fusion gene, cases with MLPA *ETV6* profile of isolated exons 1-5 losses were further investigated by FISH using both BA probes targeting *ETV6* and *RUNX1* loci (Figure 4.1). In case of suspected duplication, further application of *5'RUNX1/3'ETV6* DC SF probe was carried out (see section 4.3.2.1. C.).

4.3.2.2 MLPA

Genomic DNA from nine diagnostic bone marrow samples was extracted using standard procedures. In one case, the DNA was extracted from the same fixed cell pellet as used for FISH (see section 2.6). DNA was analysed using the SALSA MLPA kit P335 (MRC Holland, Amsterdam, The Netherlands). Data was analysed using GeneMarker V1.85 analysis software (SoftGenetics) (see section 2.9).

4.3.2.3 SNP6

Five DNA samples were hybridized to Affymetrix Genome-Wide Human SNP Array 6.0 at AROS Applied Biotechnology (Aarhus, Denmark) according to the manufacturer's instructions (Affymetrix) (see section 2.10).

4.4 Results

4.4.1 Characterisation of the deletion of der(12)t(12;21)

A total of 18 patient samples with indirect evidence for a deletion of der(12)t(12;21) were mapped using FISH. Firstly, they were divided into three groups and investigated separately because they differed in terms of the status of non-rearranged *ETV6* and whether the deleted sequences affected the *3'ETV6* or *5'RUNX1* part of the fusion gene. Groups A and B were both characterised by loss of *3'ETV6* and its adjacent chromosome 12 sequences centromeric of the breakpoint but had either an intact (n=10) or deleted (n=5) non-rearranged *ETV6*, respectively. However, the third group (C) (n=3) consisted of patients with deletion of *5'RUNX1* and contiguous chromosome 21 sequences telomeric of the breakpoint.

4.4.1.1 Deleted 3'ETV6 sequences from der(12)t(12;21)

In order to determine the proximal breakpoint and hence the size of the deletion of the *3'ETV6* region deletions, probes mapping along 12p from 12p11.21-12p13.2 were serially applied to all 15 samples. The deletion was fully mapped in these cases except for patients 3052 (Group A) and 4358 (Group B) where a lack of material prevented completion of all required tests (Figure 4.6). Among 13 deletions fully mapped, the median size of the deletion was 3.24 Mb with a range of 0.35-13.37Mb. A minimum region of deletion, defined by patient 3990 (Group B), was observed spanning 0.35Mb and comprising two genes: *LRP6* and *BCL2L14*. Although patients 3052 (group A) and 4112 (group B) showed evidence of variant t(12;21) translocations involving an additional chromosome, the FISH patterns were consistent with a deletion of *3'ETV6* from the der(12)t(12;21) (Appendix E).

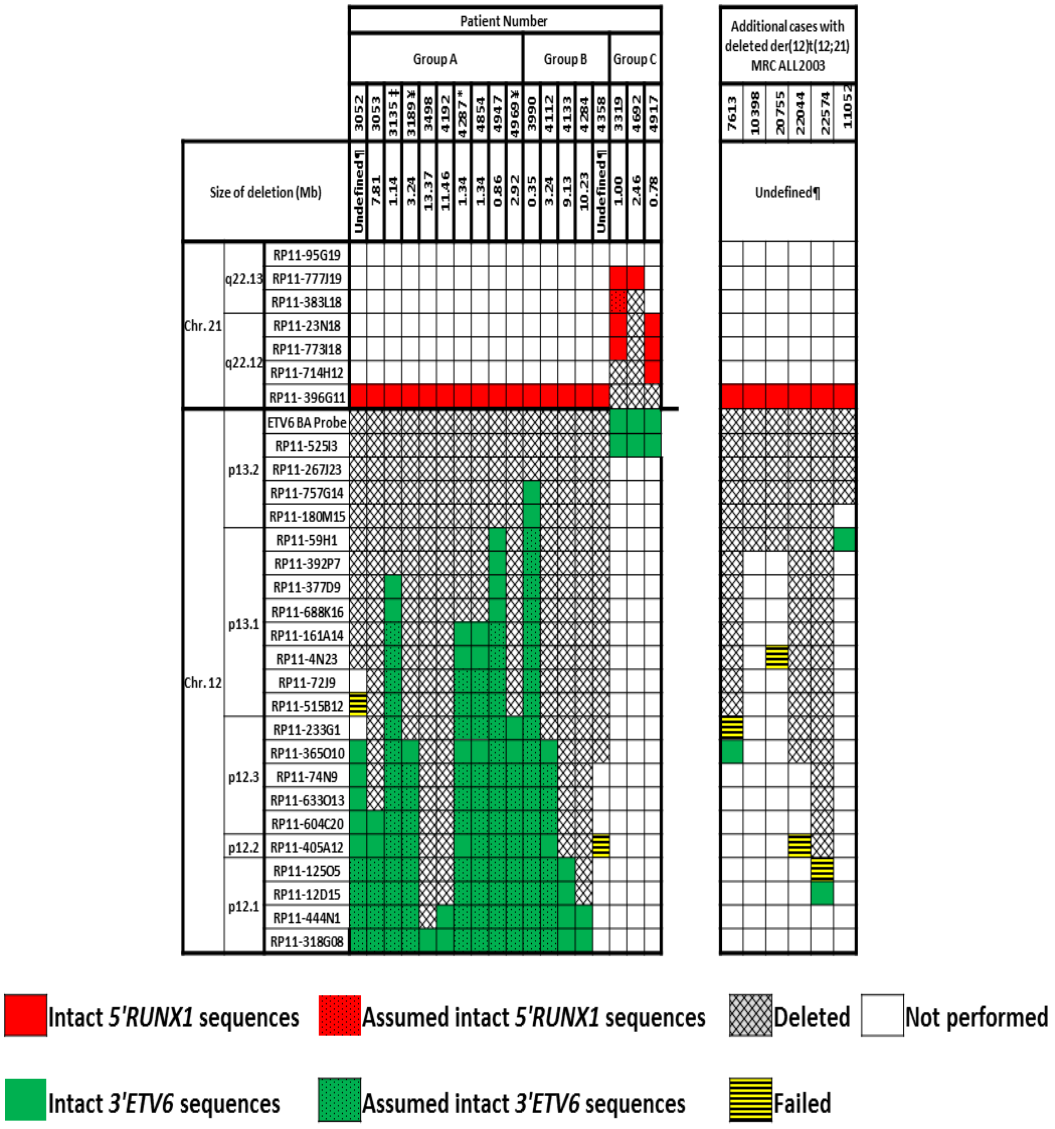

Figure 4.6 Deletion mapping of der(12)t(12;21) cases within *ETV6-RUNX1* positive BCP-ALL. Left: Group A: deletion of *3'ETV6* sequences from der(12)t(12;21) plus intact non-rearranged *ETV6*, Group B: deletion of *3'ETV6* sequences from der(12)t(12;21) plus deletion of non-rearranged *ETV6,* Group C: deletion of *5'RUNX1* sequences from der(12)t(12;21). Right: Additional cases of deleted der(12)t(12;21) which were uncovered by their *ETV6* MLPA profile with further FISH tests. Note: Groups A & B: Deletion starts at 12.02 Mb (within *ETV6* intron 5), Group C: Deletion starts at 36.26 Mb (within *RUNX1* intron 1), the size determined using data from ENSEMBL version no.60, ¶ owing to insufficient material, Other complementary tests were done ‡MLPA, SNP6 and three colour FISH, ¥ MLPA and SNP6, * Three colour FISH.

Three colour FISH was attempted in four cases (3135, 4287, 4854 and 4947) to confirm that the deletion was from the der(12)t(12;21) rather than the normal chromosome 12. It was successful in patients 3135 and 4287 (group A), where it showed a signal pattern of 1R 1F (green-gold) 1F(green-red) confirming that the target probe was deleted from the der(12)t(12;21) rather than from the non-rearranged chromosome 12 (Figure 4.7) (Appendix E).

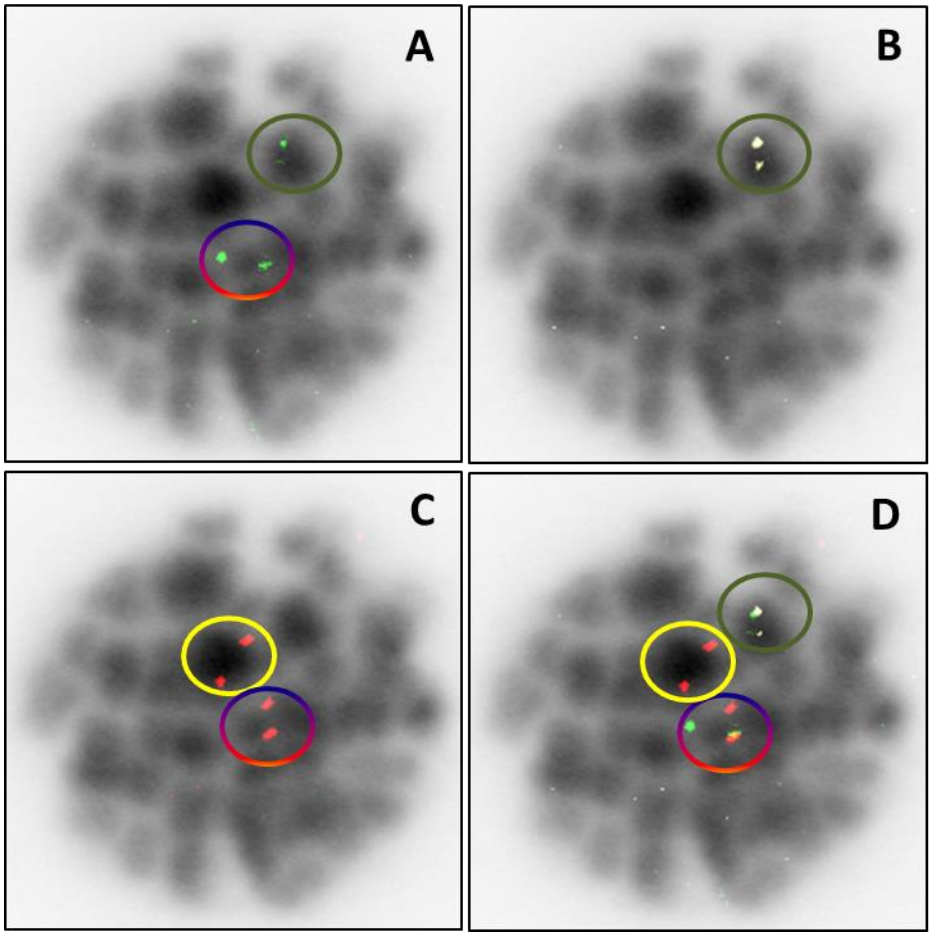

Figure 4.7 Confirmation of the origin of the der(12)t(12;21) deletion using three colour FISH. A-D: shows serial application of the green (A), gold (B) and red(C) probes in a single metaphase in patient no. 3135 showing a signal pattern of 1R 1F(green-gold) 1F(green-red) confirming that the gold (target) probe was deleted from the der(12)t(12;21) (D) [der(12)t(12;21), normal chromosome 12 and normal chromosome 21 indicated by (red-blue), green and yellow circles, respectively.

MLPA was performed on four patients (3135, 3189, 4969 and 3066). The MLPA *ETV6* profiles of patients 3135, 3189 and 4969 (Group A) revealed monoallelic deletion of *ETV6* exon 8 but retention of the remaining exons. However, patient no. 3066 (Group B) showed biallelic deletion of exon 8 with associated monoallelic deletion of exons 1-5 (Appendix A); consistent with loss of the non-rearranged *ETV6*.

The size of the deleted regions in all three patients 3135, 3189 and 4969 was identified by SNP6 (Figure 4.8), which was concordant with FISH, ranging from 1.16 Mb- 3.07 Mb. In addition, the genomic profiles varied between these patients and no distinct pattern of abnormalities was identified due to the small number of cases (Appendix F).

To conclude, FISH, MLPA and SNP array data were concordant for the presence of deletions on der(12)t(12;21), which resulted in loss of *3'ETV6* sequences and contiguous centromeric sequences.

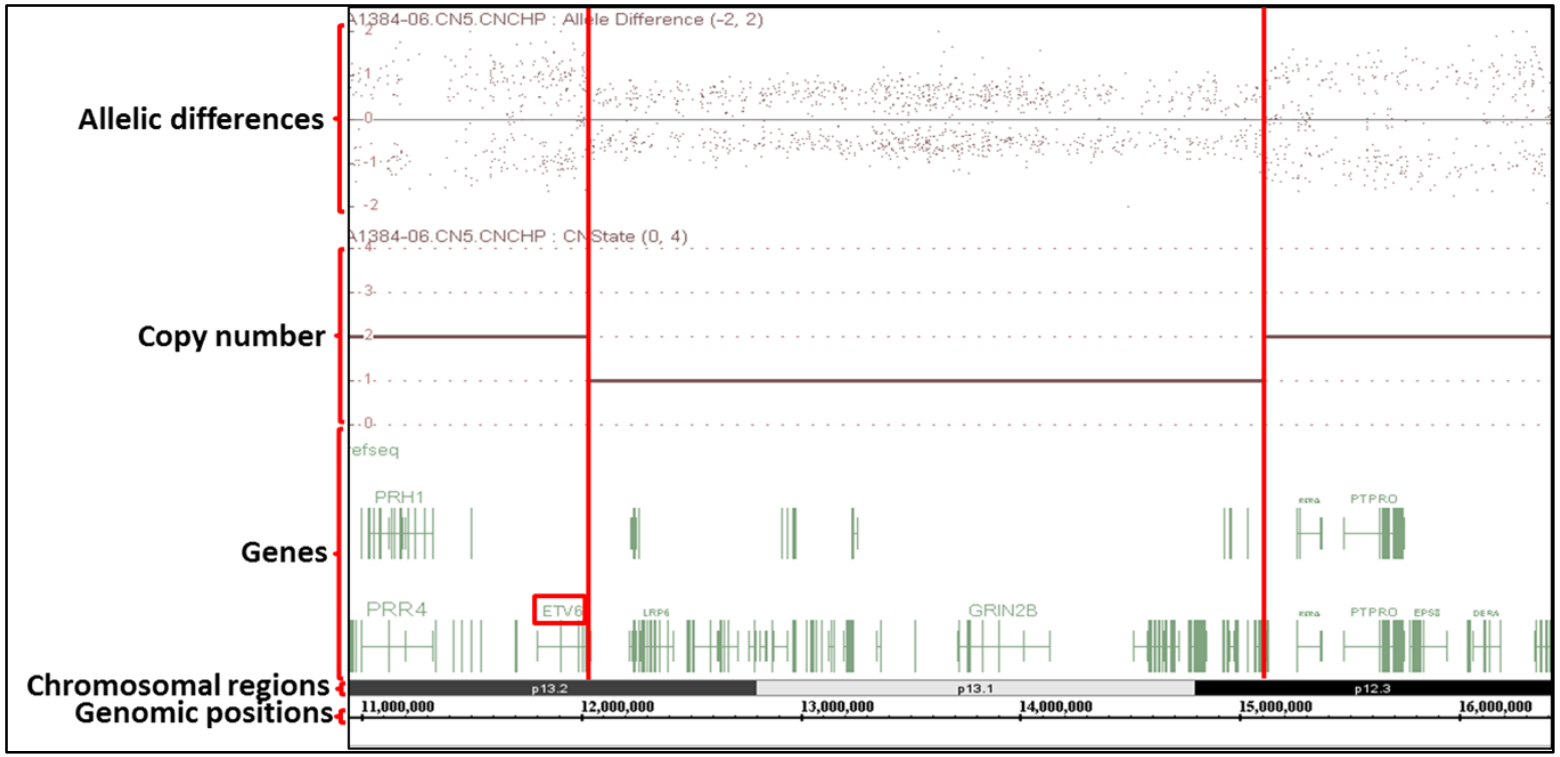

Figure 4.8 SNP6 based genomic profile of chromosome 12 in patient 3189. SNP array showed loss (12)(12.04[p13.2]-15.11[p12.3]), delineated by two red lines, the deletion starts within *ETV6* (enclosed in red square) intron 5 (t(12;21) breakpoint) with centromeric extension encompassing different important genes.

4.4.1.2 Deleted 5'RUNX1 sequences of der(12)t(12;21)

In order to determine the distal breakpoint and hence the size of the deletion of *5'RUNX1* sequences, FISH was performed on three patients using different deletion probe sets. FISH studies revealed deletions with a median size of 1.00Mb (range 0.78-2.46 Mb) (Figure 4.6). The Common region of deletion (CRD) did not encompass any known genes and was defined by patient 4917. Although patient 3319 showed evidence of variant t(12;21) translocations involving an additional chromosome, the FISH patterns were consistent with a deletion of *5'RUNX1* from the der(12)t(12;21) (Appendix E). No further material was available in order to investigate these cases using MLPA and SNP6.

Interestingly, all patients with either types of deleted der(12)t(12;21), exhibited the deleted clone in all *ETV6-RUNX1* positive cells indicating that the deletions occurred at the same time as the translocation (Appendix E).

4.4.2 Gain of RUNX1-ETV6 fusion gene on a der(12)(21qter→21q22.12::12p13.2-12p12.3::12p12.3→12qter)

FISH experiments were performed on seven cases, using the DC SF probe to investigate the origin of the extra der(12)t(12;21) signals. They confirmed two copies of the *RUNX1-ETV6* fusion in all cases suggested by the signal pattern of 1R 0G 2F in an average of 55% of *ETV6-RUNX1* positive cells. Thus, these signal patterns were often seen in conjunction with the standard abnormal signal patterns (1R 1G 1F) indicating that this abnormality represented a secondary event. In addition, metaphases from patient 3726 showed that the two fusion signals were on separate chromosomes (Figure 4.9, Table 4-1 and Appendix E).

Further FISH, using commercial probe set consisting of CEP 12 and subtel 12p probes, revealed the presence of only two chromosome 12 centromeres and the loss of one 12p subtelomeric region in all cases in comparable proportions as the duplicated clone detected earlier using the DC SF probe. This FISH test was applied in order to prove the assumption of the associated loss of normal chromosome 12 and to assess the genetic stability of each patient by comparing the number of chromosome 12 centromeres and telomeres (Table 4-1, Appendix E).

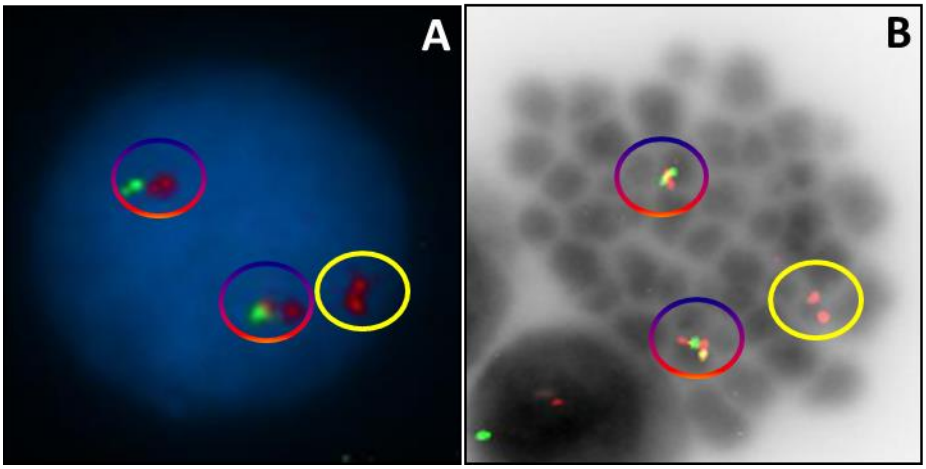

Figure 4.9 The observed signal pattern of 1R 0G 2F in patient no. 3726 with suggested duplicated *RUNX1-ETV6* fusion gene using the DC SF probe seen in: an interphase (A) and a metaphase cells (B).

I.D.	*ETV6* DC BA 1R 2G 0F %*	*RUNX1 DC* BA 2R 1G 1F %*	*5'RUNX1/3'ETV6* DC SF 1R 0G 2F %*	2X CEP12/ 1X Subtel 12p
3726¥	30%	33%	38%	21%
4281¥	100%	100%	61%	100%
3472‡	17%	12%	NA	NA
4040‡	31%	18%	NA	NA
4637‡	100%	100%	92%	100%
3130	62%	61%	74%	77%
4136	92%	96%	94%	87%
4560	76%	62%	69%	60%
4678	100%	100%	92%	100%
4934	81%	94%	NA	NA

Table 4-1 Detailed FISH studies of *ETV6-RUNX1*- Positive BCP- ALL Patients with duplicated *RUNX1-ETV6* fusion gene. DC: dual colour, BA: break-apart, DC SF: dual colour single fusion, CEP: centromeric probe, Subtel: subtelomeric probe, * percentages calculated relative to the *ETV6-RUNX1* positive cells, ¥ MLPA, SNP6 done, ‡ MLPA done, NA: not performed owing to lack of material.

MLPA on five patients (4281, 4637, 4040, 3726 and 3472) showed monoallelic deletions of *ETV6* exons 1-5 but retention of exon 8. However, the latter two patients showed lower borderline ratios of exons 1-5 owing to low proportion of the abnormal clone constituting 30% and 17% of the *ETV6-RUNX1* positive cells, respectively (Table 4-1). In addition, MLPA revealed a normal copy number of *BTG1* gene, located at the long arm of chromosome 12 (12q), in three cases but the remaining two cases had monoallelic loss that encompassed exon 2 and the nearby 3'region with retention of exon 1 (Appendix A).

SNP array analysis of the two analysed cases (4281 and 3726) revealed the presence of two chromosomes 12 with copy number neutral loss of heterozygosity (CNN-LOH) spanning a variable region of the short arm of chromosome 12 in both patients including: 12(12.02[p13.2]-19.87[p12.3]) and 12(12.02[p13.2]-22.91[p12.1]), respectively. The latter abnormality was clearly shown in patient 4281 as it constituted the major clone, unlike in patient 3726. In addition, three copies of a part of the long arm of chromosome 21 21(36.39[q22.12] → 48.10[qter]) and one copy of the subtelomeric region of 12p del(12)(0.19[pter]→19.87[p12.3]) were detected (Figure 4.10, Appendix F).

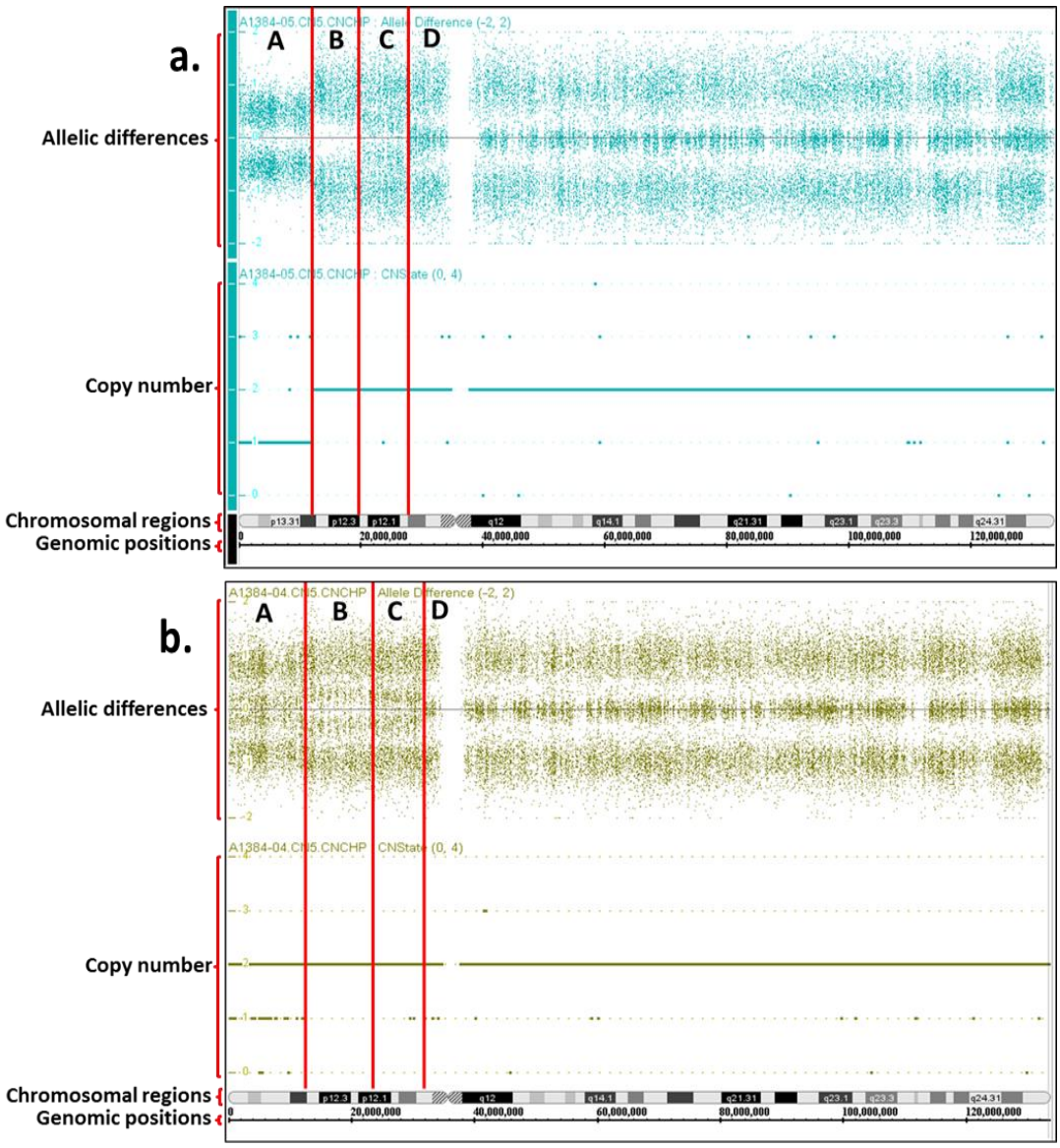

Figure 4.10 SNP6 based genomic profile of chromosome 12 in two patients with duplicated *RUNX1-ETV6* fusion gene. a. patient 4281 showed del(12)(0.19[pter]→19.70[p12.3]) (Area A) with adjacent three different patterns of heterozygosity defined by areas B, C and D respectively; starting from the copy number neutral loss of heterozygosity (CNN-LOH) (12)(12.02[p13.2]-19.87[p12.3]), complex heterozygosity with neutral copy number (12)(19.87[p12.3]-28.06[p11.22) followed by normal heterozygous chromosome 12(28.06[p11.22]-133.85[qter]), respectively, b. patient number 3726 exhibited similar pattern like in patient 4281 but less clear owing to low proportion of the clone harbouring the duplicated *RUNX1-ETV6* fusion gene. The centromeric breakpoints of area B and C differ between both patients. The complex heterozygosity in area C might be suggestive of multiple independent mitotic recombination events in the same patient.

To summarise, FISH, MLPA and SNP array findings confirmed initial observations of the presence of an extra *RUNX1-ETV6* fusion gene which could not be due to a simple gain of the der(12)t(12;21) and loss of the normal chromosome 12. Instead these data pointed to a secondary event which involves mitotic recombination between the normal chromosome 12 and the der(12)t(12;21), resulting in the formation of a der(12)(21qter→21q22.12::12p13.2-12p12.3::12p12.3→12qter) (Figure 4.11). This derived chromosome consists of a copy of the der(12)t(12;21) from the 21q telomere, including the *RUNX1-ETV6* fusion site, to 12p13.2 and thereafter material from the other chromosome 12 that was not involved in the original t(12;21) translocation. The mitotic recombination point not only differed between the two patients but the area of complex CNN LOH (Area C) observed in both patients suggests the presence of multiple independent mitotic recombination events in the same patient. Collectively, these rearrangements result in the loss of the non-rearranged *ETV6* allele with the contiguous telomeric sequences, duplication of the reciprocal *RUNX1-ETV6* fusion gene, as well as the CNN-LOH of a region on the short arm of chromosome 12.

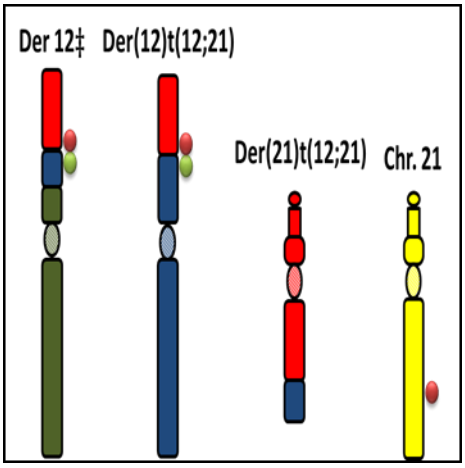

Figure 4.11 Identification and proposed mechanism of the duplicated *RUNX1-ETV6* reciprocal fusion gene formation. This mechanism corresponded to the observed signal pattern obtained by *RUNX1-ETV6* DC SF probe. ‡: der(12)(21qter → 21q22.12::12p13.2-12p12.3::12p12.3 → 12qter.

4.4.3 Detection of additional cases with either deletion or gain of the der(12)t(12;21) based on their MLPA ETV6 profile

A total of 397 *ETV6-RUNX1* cases with available MLPA data, including 74 patients who have been included in the comprehensive FISH screening, were reviewed for the presence of suggestive MLPA *ETV6* profiles of the *3' ETV6* sequence deletions or

duplications of *RUNX1-ETV6* fusion gene. Cases with the MLPA profile of isolated monoallelic or biallelic deletion of *ETV6* exon 8 were investigated for deleted *3'ETV6* sequences of der(12)t(12;21). However, the MLPA *ETV6* profile of normal copy number of exon 8 with monoallelic deletion of exons 1-5 were studied for the possible presence of the duplicated *RUNX1-ETV6* fusion gene.

4.4.3.1 MLPA ETV6 profile of 3'ETV6 sequences deletions of der(12)t(12;21)

A total of 36 (9%) cases with isolated exon 8 deletions, excluding the 4 cases who had FISH suggesting deleted der(12)t(12;21) (section 4.4.1), were observed within the *ETV6-RUNX1* cohort (Appendix A). 20 out of 36 cases were investigated by FISH using *ETV6* and *RUNX1* DC BA probes. The FISH studies revealed different scenarios of FISH signal patterns that indicated the presence of different abnormalities (Figure 4.12).

Among these 20 cases, there were 6 cases (30%) with *3'ETV6* deletion of der(12)t(12;21) (Figure 4.12 A-C), two of them had non-rearranged *ETV6* loss. A further 6 cases (30%) exhibited gain der(21)t(12;21) and loss of the non-rearranged *ETV6* gene (Figure 4.12 D). However, the isolated *ETV6* exon 8 deletions in the remaining 8 cases could not be explained despite the application of both BA probes. Among these 8 cases, there were two patients with biallelic deletion of *ETV6* exon 8 who harboured gain der(21)t(12;21) with associated loss of the non-rearranged *ETV6* (Figure 4.12 E).These two cases are more likely to harbour a microdeletion targeting *ETV6* exon 8 at der(12)t(12;21), undetectable by the *ETV6* BA probe, rather than independent deletion of the non-rearranged *ETV6* owing to the 100% involvement of *ETV6-RUNX1* positive cells with this abnormal clone. Two additional cases with monoallelic deletion of exons 1-5 and biallelic loss of exon 8 did not show a deletion at der(12)t(12;21) using the *ETV6* BA probe but loss of the non-rearranged *ETV6* in all *ETV6-RUNX1* positive cells suggesting that the loss of the other allele of exon 8 originated from der(12)t(12;21) (Figure 4.12 G). On the other hand, the remaining four cases exhibited monoallelic deletion of *ETV6* exon 8, however, the *ETV6* BA probe did not show any evidence of deletion at either non-rearranged *ETV6* or der(12)t(12;21) (Figure 4.12 F). Further FISH studies using a probe targeting specifically *ETV6* exon 8 were successful in one of the four cases (see section 4.3.2.1 D.). The FISH study revealed that the deletion originated from the non-rearranged chromosome 12 rather than der(12)t(12;21).

FISH mapping was performed on the six cases with evidence of *3'ETV6* deletion of der(12)t(12;21) by BA probes. However, the determination of the deletion sizes was not completed in all cases owing to insufficient material (Figure 4.6, Appendix G).

To sum up, among 20 cases with isolated MLPA *ETV6* exon 8 losses, there was a total of 10 cases (50%) with deletion of *3'ETV6* sequences of der(12)t(12;21) by MLPA and FISH results. The remaining cases (n=10) of the isolated *ETV6* exon 8 losses represented other abnormalities in seven cases including: gain der(21)t(12;21) with associated loss of the non-rearranged *ETV6* (n=6, 30%) and deleted *3'ETV6* sequences (including exon 8) of the non-rearranged *ETV6* (n=1, 5%). However, the remaining three cases were failed to identify the origin of the deletion due to insufficient material.

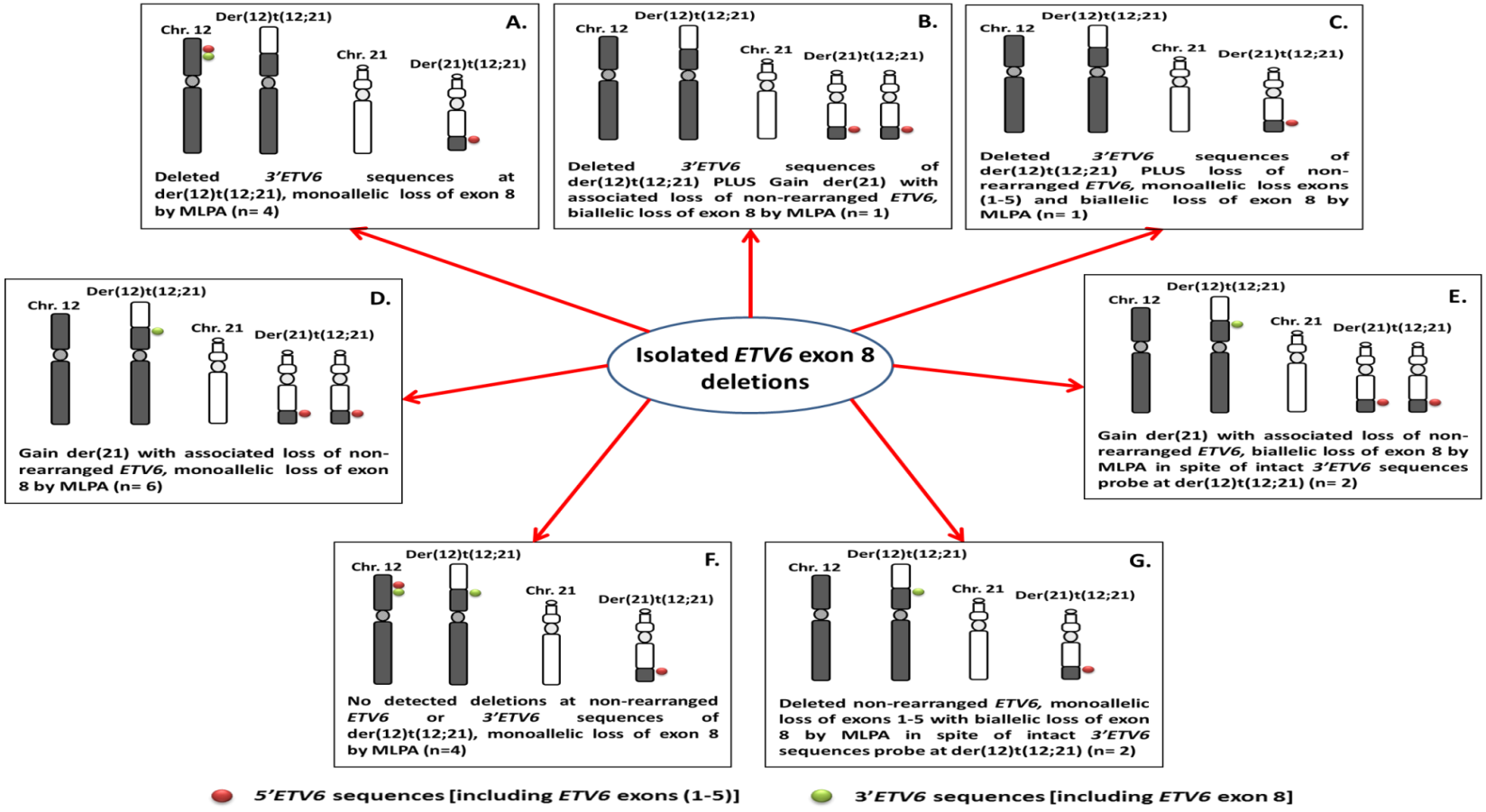

Figure 4.12 Different resultant signal patterns obtained from the application of *ETV6* DC BA probe in 20 *ETV6-RUNX1* patients with isolated *ETV6* exon 8 deletions. The FISH results were correlated individually with other FISH data generated by *RUNX1* DC BA and *TEL/AML1* ES, where available, as well as with the MLPA *ETV6* profile.

4.4.3.2 MLPA ETV6 profile of Gain of RUNX1-ETV6 fusion gene

Overall, 5 out of 397 cases (1%), excluding the two cases who had FISH suggesting duplicated *RUNX1-ETV6* fusion gene (section 4.4.2), were identified to have retention of normal copy number of exon 8 with monoallelic deletion of exons 1-5 by MLPA. Unfortunately, no further material was available to investigate these cases, thus no confirmed cases were obtained (Appendix G).

4.5 Discussion

The *ETV6-RUNX1* fusion gene alone is insufficient to initiate clinical leukaemia. Accordingly, further investigations are required in order to identify and characterise additional abnormalities that might contribute to leukaemogenesis. Previous screening, using BA probes targeting the *ETV6* and *RUNX1* loci, observed abnormal signal patterns suggestive of deletions or duplications targeting the *RUNX1-ETV6* fusion gene located on der(12)t(12;21). The present study has confirmed these novel alterations using several complementary methods including FISH, MLPA and SNP6 arrays. FISH was used for the confirmation of the deletions, followed by mapping the deletions and ascertaining the origin of deletion. Further FISH tests were performed on cases with duplicated der(12) to confirm and understand the responsible mechanism. MLPA and SNP arrays further validated these findings and identified the likely mechanism of the duplication. There were no differences in terms of demographic and clinical features between groups with these abnormalities and all patients showed good outcome.

Deleted der(12)t(12;21) was detected in 8% (n=20) *ETV6-RUNX1* cases, targeting either side of the breakpoint but no reported cases with deletion spanning the breakpoint itself. No previous reports have identified either of these abnormalities The deletion of *3'ETV6* sequences at der(12)t(12;21) was more frequent and larger as compared to the *5'RUNX1* deletions. Deleted 5'*RUNX1* sequences have been inferred previously, suggested by loss of the small extra signal using the commercial *TEL-AML* ES probe (Jabber Al-Obaidi *et al.*, 2002; Martineau *et al.*, 2005; Rothman *et al.*, 2005; Konn *et al.*, 2010). However, these were subclonal. The deleted der(12)t(12;21) in this present study most likely occurred at the same time as the translocation, as evidenced by both the 100% involvement of *ETV6-RUNX1* positive cells and the consistent deletion boundaries

with the translocation breakpoint. In contrast, loss of the extra signal was shown to be present in subclones suggesting that the two abnormalities do not overlap completely. The plausible explanation for the failure in identifying these deletions was the lack of the routine use of probes directly localised to der(12)t(12;21). In addition, genomic copy number studies may mistakenly identify deletions of *3'ETV6* sequences at der(12)t(12;21) as intragenic deletions of the non-rearranged *ETV6* allele.

These deletions join a list of submicroscopic deletions that accompany primary chromosomal rearrangements in leukaemia including t(9;22), inv(16), t(8;21) and *MLL* gene rearrangements (Rothman *et al.*, 2005). For instance, der(9)t(9;22) deletions, involving either *5'ABL1, 3'BCR* or both, are detected in 9-33% of CML patients who showed unfavourable outcome especially those with 5'*ABL* losses treated on different treatment protocols (Vaz de Campos *et al.*, 2007). However, the prognostic value of der(9)t(9;22) deletions lacked significance in patients treated uniformly on imatinib (Castagnetti *et al.*, 2010; Huh *et al.*, 2011). Like der(12)t(12;21), the 3'*BCR* deleted region is smaller than *5'ABL* deletions in der(9)t(9;22) (Vaz de Campos *et al.*, 2007). Furthermore, there are often deletions of 5'*MYH11* (16p13) or 3'*CBFB* which are detected in ~20% (Tirado *et al.*, 2010) and 8% (Kelly *et al.*, 2005) of AML with inv(16), respectively. There are also reported deletions restricted to *5'RUNX1T1* sequences that constitute 9% of t(8;21) cases (Godon *et al.*, 2002). Similarly, *MLL* rearrangements are associated with 3' *MLL* deletions representing 20–25% of *MLL* rearranged cases (Konig *et al.*, 2002). It has been suggested that the loss of one or more tumour suppressor genes may promote leukaemogenic progression through a secondary event or directly. Furthermore, it was hypothesized that patients with these deletions may be more prone to genetic instability favoring subsequent additional genetic alterations. In contrast to these suggested mechanisms, there was no observed increase in the frequency of additional abnormalities during disease progression (Vaz de Campos *et al.*, 2007).

It has been hypothesized that the nature of sequences flanking translocation breakpoint regions might play a role. High density flanking Alu repeats, known to facilitate illegitimate recombination, are distributed in non-random patterns within the vicinity of deletion breakpoints giving rise to a possible deletion mechanism (Kolomietz *et al.*, 2001; Kolomietz *et al.*, 2002). The findings of this present study suggested concomitant occurrence of der(12) deletions and the translocation as seen in der(9) deletions in

t(9;22) patients, hence, the deletions must have occurred after the breakage of the chromosomes and prior to the illegitimate fusion of the chromosomes. Consequently, the deletions will not be under selective pressure, and hence not likely to be drivers. However, we cannot rule out a bystander effect.

The shared characteristic among all der(12) deletions was the loss of the reciprocal *RUNX1-ETV6* fusion gene that consists of the last three exons of *ETV6*, which contains the ETS domain, and the non-functional exon 1 or exons 1-2 of *RUNX1*. This is in agreement with a previous study in which the expression of RUNX1-ETV6 was found concomitantly with the ETV6-RUNX1 fusion products in only 76% of t(12;21) positive ALL cases (Stams *et al.*, 2005). It was hypothesized that the *RUNX1-ETV6* fusion gene would act through the function of this isolated ETS domain and may behave in a similar manner to the non-rearranged *ETV6* when this gene is deleted.

Duplicated *RUNX1-ETV6* fusion gene was identified in 4% (n=10) *ETV6-RUNX1* cases and occurred subclonally in *ETV6-RUNX1* positive cells. Hence, it may be considered as a secondary abnormality, however, its role in leukaemogenesis needs to be determined. Initially it was thought to be a simple gain of the der(12)t(12;21) or the result of an independent translocation, as suggested in previous studies based upon observations of extra ES signals (Ma *et al.*, 2001; Jalali *et al.*, 2003; Martineau *et al.*, 2005; Rothman *et al.*, 2005). However, SNP arrays revealed a layer of complexity in the formation of this aberration, which may have arisen through mitotic recombination between the normal chromosome 12 and the der(12)t(12;21) yielding der(12)(21qter →21q22.12::12p13.2-12p12.3::12p12.3→12qter). This is an entirely novel chromosome abnormality not reported previously, to the best of my knowledge. This abnormal chromosome resulted in duplicated regions of chromosome 21q and chromosome 12p as well as CNN LOH of regions of 12p but not CNN LOH of 12q as would be assumed by simple gain of der(12)t(12;21) and loss of the normal chromosome 12.

In one previous study, it was postulated that RUNX1-ETV6 high expression was an independent prognostic factor in *ETV6-RUNX1* which confers worse outcome, but no available patient details were provided to assess the possible mechanisms behind this high expression (Stams *et al.*, 2005). In addition, the latter study has described the lack of relationship between this expression and drug resistance which indicates that the

effect of RUNX1-ETV6 might result in cell regrowth rather than drug toxicity related pathways (Stams *et al.*, 2005). It is worth noting that the discrepant clinical outcome between patients in the present study, who had an excellent outcome, and those in the Stams paper is likely to be due to advances in treatment rather than evidence that the two groups are necessarily distinct. Clearly the der(12)(21qter →21q22.12::12p13.2-12p12.3::12p12.3→12qter) results in other events which could also be contributing to leukaemogenesis via the unmasking of cancer driver mutations either by the inactivation of tumour-suppressor genes or the activation of oncogenes.

There are several candidate genes on chromosome 12p within the CRD which could be acting as tumour suppressor genes, such as *LRP6, BCL2L14, DUSP16, CREBL2* and *CDKN1B* (Kiyokawa *et al.*, 1996; Guo *et al.*, 2001; Masuda *et al.*, 2001). Interestingly, these genes were among those genes targeted in the CNN LOH region. These genes have been reported in many cancer types, apart from leukaemia, including: lung, breast and prostate. *BCL2L14* encodes a protein that is belonged to BCL2 protein family which forms hetero- or homodimers and it may act as anti or pro-apoptotic regulator (Guo *et al.*, 2001). LRP6 protein belongs to the low density lipoprotein receptor gene family and is involved in the regulation of different cellular activities including cell differentiation, proliferation and migration through its interaction with the Wnt/beta-catenin pathway. DUSP16 is a member of the dual specificity protein phosphatase that is involved in the inactivation of target kinase pathways including the c-Jun amino-terminal (JNK) and extracellular signal-regulated (ERK) kinases. *CREBL2* encodes a protein responsible in the regulation of *CREB1* transcriptional activity required in the adipose cells differentiation and cell cycle regulation. *CDKN1B* is involved in the regulation of the cell cycle progression resulting in G1 arrest in particular. No somatic mutations have been reported in these genes (Montpetit *et al.*, 2004; Papaemmanuil *et al.*, 2014), thus loss of one allele with resultant haploinsufficiency is a possible mechanism of their leukaemogenic effects. Recently, a comprehensive evaluation of the genomic copy number alterations in a large number of *ETV6-RUNX1* relapse cases revealed significant association of *CDKN1B* loss with a shorter remission duration and lower rates of event free survival of 42% at ten years compared to 81% in those without the loss (Bokemeyer *et al.*, 2013), however, this prognostic significance is lost in multivariate analysis. On the other hand, this gene is considered as a possible candidate for the prognostic

stratification of AML across all cytogenetic subgroups, hence its low expression resulted in good outcome, while the high expression carried a worse prognosis (Haferlach *et al.*, 2011; Haferlach *et al.*, 2012). Thus, these genes may act as tumour suppressor genes that might contribute to the leukaemogenic progression in those patients with der(12)t(12;21) abnormalities.

Isolated *ETV6* exon 8 losses were previously either not considered as real losses (Schwab *et al.*, 2010) or counted as non-rearranged *ETV6* deletion (Krentz *et al.*, 2013). However, the occurrence of these isolated deletions in *3'ETV6* sequence deletions at der(12)t(12;21) (see section 4.4.1.1) highlighted the necessity to further investigate 20 isolated exon 8 deleted cases. In addition, investigations of possible cases of duplicated *RUNX1-ETV6* fusion gene were attempted but none of the cases had sufficient material.

Further cases with deleted *3'ETV6* sequences of der(12)t(12;21) were identified in around 50% (n=10) of cases with isolated *ETV6* exon 8 losses by MLPA. Among the remaining 10 cases, 6 cases exhibited deleted non-rearranged *ETV6* with gain of der(21)t(12;21) which explained the isolated *ETV6* exon 8 loss, while one case showed deleted non-rearranged *ETV6*. However, the origin of the deletion could not be determined in the remaining three cases. In my view, the isolated *ETV6* exon 8 loss should not be overlooked but needs to be complemented with FISH studies and interpreted with caution as they may indicate deletions of der(12)t(12;21) and not just standard *ETV6* deletions from the untranslocated chromosome 12.

It is now well established the *ETV6-RUNX1* ALL harbours several additional abnormalities including these two additional aberrations targeting the *RUNX1-ETV6* fusion gene that is located on the der(12)t(12;21) resulting either in its deletion or duplication. Considerable research studies have focussed on the primary products of the chromosomal translocations but the reciprocal fusion gene alterations have been overlooked. Both abnormalities affect the reciprocal RUNX1-ETV6 fusion product, which may contribute to leukaemogenesis by either eliminating or amplifying its expression. However, other consequences such as loss of tumour suppressor genes and amplification of oncogenes may also play a role. In conclusion, this study has defined novel abnormalities in *ETV6-RUNX1* BCP-ALL which affect der(12)t(12;21) and may implicate new genes in leukaemogenesis.

Chapter 5. Investigations of relapse related genes in *ETV6-RUNX1* positive BCP-ALL

5.1 Introduction

Despite the overall favourable outcome of patients with *ETV6–RUNX1* (Pui, 2004; Moorman *et al.*, 2010b), up to 13% of cases still experience relapses which occur usually after cessation of treatment and occasionally many years later. Thus, identification of the CNAs responsible for these relapses at the time of diagnosis is necessary to develop targeted therapy as a rational strategy to improve outcome. Paired genomic analysis of the diagnosis and relapse samples in BCP-ALL pointed to the possible implications of genes involved in cell cycle regulation, B cell development, glucocorticoid receptor signalling and drug resistance, including *IKZF1, CDKN2A/B, ETV6, EBF1, NR3C1, NR3C2, BTG1, TBL1XR1 and CD200/BTLA* (Mullighan *et al.*, 2008b). Most of these alterations were retained at relapse, while some aberrations were more often acquired at relapse including *IKZF1*, *EBF1* and *NR3C1* (Mullighan *et al.*, 2008b; Kawamata *et al.*, 2009). In addition, a notable increase in the number of CNA was found from the diagnosis to relapse with number of deletions exceeding gains (Mullighan *et al.*, 2008b). It has been suggested that the relapse clone can derive from either major or minor subclones detected at diagnosis regardless of time to relapse (Ford *et al.*, 2001; Konrad *et al.*, 2003; Mullighan *et al.*, 2008b). In addition, a *de novo* origin from a premalignant stem cell was also suggested as indicated by the significant differences between the diagnostic and the relapse clones (Mullighan *et al.*, 2008b). These genomic studies focussed on the overall cohort of BCP-ALL without giving much attention to the relapse specific characteristics in the individual cytogenetic subgroups including *ETV6-RUNX1*. In addition, these studies are potentially more likely to miss alterations presenting at low level clones. Newly diagnosed *ETV6-RUNX1 cases* were shown to harbour an increased number of alterations with an average of 6.68 aberrations per case and *ETV6* losses constituted the most prevalent deletions (70%) (Mullighan *et al.*, 2007). Therefore, a detailed analysis of a series of diagnosis-relapse pairs to examine clonal heterogeneity and evolution using several techniques is needed. The cytogenetics and FISH data were already available and MLPA P335 *IKZF1* results were provided by Chapter 3. Accordingly, MLPA was done on the relapse samples and an extensive literature review was carried out to identify informative genes to be tested further (Table 5-1). The final list of genes was chosen based on their frequencies and relationship to relapses either by their functions or poor treatment response in any type of cancer. Since the glucocorticoid

drugs are integral components in the treatment, those genes involved in glucocorticoid resistance were chosen. *CD200/BTLA, TBL1XR1, NR3C2* and *NR3C1* genes constitute the top highest ranking, and a frequency of >10% was used as a cut off value. However, *FHIT* and *TOX* genes were not selected because of their low frequencies and they were not involved in glucocorticoid responsiveness.

TBL1XR1 is required for nuclear hormone receptor transcriptional repression via the SMRT/N-CoR complex and deletion has been reported to be associated with relapses among *ETV6-RUNX1* positive BCP-ALL (Parker *et al.*, 2008), although further validation was recommended. *NR3C1* and *NR3C2* genes are involved in the glucocorticoid and mineralocorticoid binding receptors, respectively and their loss contributed to poor treatment response (Mullighan *et al.*, 2008b)*;* however*, CD200/BTLA* genes play roles in establishment functions in the immune system.

Although *BMF* losses occur at a lower frequency, it represents an integral component in the anti-or pro- apoptotic regulation and plays an important role in glucocorticoid-mediated induction of apoptosis (Pinon *et al.*, 2008; Ploner *et al.*, 2008). Accordingly, *BMF* screening was considered in the above list of genes.

It is important to investigate the potential relevance of these CNAs to the *ETV6-RUNX1* relapse cases and to identify their associations with other relapse specific demographic or clinical features.

Abnormality	Cytoband	Function	Frequency% in *ETV6-RUNX1*	Frequency% in other BCP-ALL
CD200/BTLA	3q13.2	Immune system or during B-cell differentiation	13	4
TBL1XR1	3q26.32	Nuclear hormone receptor transcriptional repression via the SMRT/N-CoR complex	13	0.69
NR3C1	5q31.3	Nuclear hormone receptors binding glucocorticoids	13	2
NR3C2	4q31.23	Nuclear hormone receptors binding mineralocorticoid/ glucocorticoids	11	0.69
FHIT	3p14.2	Induction of apoptosis	8.5	3
TOX	8q12.1	T-cell development regulator	8.5	4
BMF	15q15.1	Anti- or pro-apoptotic regulators	6	2

Table 5-1 Literature search of other important gene deletions in *ETV6-RUNX1* cases. Gene functions were obtained from gene cards, frequencies were extracted from (Mullighan *et al.*, 2007; Lilljebjorn *et al.*, 2010).

5.2 Aims and Objectives

ETV6-RUNX1 fusion gene by itself is unable to drive the pre-leukaemic cells to overt leukaemia, thus additional abnormalities are required for the leukaemic transformation, progression and emergence of relapse. This Chapter aims to describe the genetic landscape at diagnosis of patients who did and did not go on to relapse and also describe the landscape at relapse for the former group in order to assess the potential relevance of these genetic alterations to the evolution of relapses.

The main objectives in this study are:

1. Screen the diagnostic samples of *ETV6-RUNX1* patients who did and did not go on to relapse for gene deletions targeting *TBL1XR1, BMF, NR3C1, NR3C2* and *CD200/BTLA* using both real - time copy number qPCR and FISH.
2. Utilisation of the available MLPA (generated in Chapter 3) and cytogenetics data to gain further insight on the genetic landscape of the relapse and non-relapse cases at diagnosis.
3. Evaluate the potential relevance of the copy number alterations of these genes in the relapse cases.
4. Comparison of the genetic landscape of the relapse cases using matched presentation and relapse samples with BM involvement based on the cytogenetic, FISH and molecular data by back-tracking FISH some of the important genetic lesions from relapse to diagnosis (and vice versa).

5.3 Patients and Methods

5.3.1 Patient cohort

This study comprised of a group of 18 relapse cases and a further 16 cases, who had no history of relapse, within *ETV6-RUNX1* positive BCP-ALL. The comparison group was chosen on the basis of being typical patients with no history of adverse events, any Down syndrome and age range of (2-9) years. All patients were treated on ALL97/99 at initial presentation, except one relapsed case that was treated on ALL2003. At relapse, seven out of 18 cases were treated according to the ALLR3 protocol, while the remaining 11 cases were treated off protocol (Appendix J).

There was a variable availability of materials in the relapse cases. A total of 13 out of 18 relapse cases had both diagnostic samples of DNA and fixed cells, while the remaining five cases had just DNA. At relapse, half of the cases (n=9) did not have any materials and the remaining half (n=9) had either both DNA and fixed cells (n=3) or only fixed cells (n=5) or just DNA (n=1) (Appendix J). However, the amount and quality of the relapsed samples (fixed cells in particular) were not ideal, so they were reserved for important tests needed to confirm the genetic lesions presented at diagnostic samples.

A total of 14 out of 16 non-relapse cases had DNA and fixed cells, whereas the remaining two patients had DNA only (Appendix J).

5.3.2 Real-time polymerase chain reaction (PCR) using TaqMan Copy Number Assays

One target probe was chosen for each gene apart from *TBL1XR1* and *NR3C2* which were previously known to have variable deletion breakpoints; hence two target probes were selected. In addition, no target probe assay for *CD200* was selected because *BTLA* gene deletion usually co-occurrs with *CD200* (Lilljebjorn *et al.*, 2010). Two reference probe assays were used for each patient in separate reactions: *RNase P* and *TERT* (see section 2.11). All seven target probe assays were tested initially on normal controls to ensure efficiency. In each PCR run, the following samples were included: calibrator (mixture of different normal DNA; promega), positive control (monoallelic or/and biallelic deletion), non-template control (No DNA) and patient samples.

5.3.2.1 Selection of the positive gene deletion controls

All positive controls chosen for this study were detailed in Appendix K. The chosen genes are detailed in Table 5-2.

Genes	location	Controls*	Techniques‡	References¥
NR3C1	5q31.3	REH, 9467 (L707)	FISH, qPCR	Sanger cancer genome project
NR3C2	4q31.23	KG1	FISH, qPCR	Sanger cancer genome project
BMF	15q15.1	KASUMI-1	FISH, qPCR	Sanger cancer genome project
TBL1XR1	3q26.32	4037, 4281	FISH, qPCR, SNP	NA
BTLA	3q13.2	9859	qPCR, SNP	NA

Table 5-2 Genes tested and the positive controls used. * cell lines or patient number used as positive controls, ‡ Techniques used to identify and confirm the deletion and performed by LRCG members including myself, ¥ positive cell lines were selected based on previous SNP profiles obtained from Sanger cancer genome project, NA: not applicable.

5.3.2.2 Sensitivity assay of the Real-time polymerase chain reaction (PCR) using TaqMan Copy Number Assays

The KG1 cell line, which used as a positive *NR3C2* deletion control, was chosen to test the sensitivity of this technique. The sensitivity was tested using serial dilution assays, with increasing proportions (0, 5, 10, 20, 25, 30, 40, 60, 80 and 100%) of the KG1 cell line titrated against decreasing proportions (100, 95, 90, 80, 75, 70, 60, 40, 20 and 0%) of normal DNA (Promega). The quantitative PCR was carried out using both *5'NR3C2* and *RNase P* as target and reference probe assays respectively. Based upon the first sensitivity assay results, a second assay was performed to estimate the approximate cut off value, for which the following serial dilutions included: 0, 10, 30, 50, 55, 60, 65, 70, 80 and 100%.

5.3.2.3 Cohort screening

Patients were screened using the six target gene probe assays targeting: *NR3C1, 5'NR3C2, TBL1XR1, 5'TBL1XR1, BTLA* and *BMF*. Each target assay was combined with either reference assays separately as duplex qPCR reactions in individual patients in order to ensure the copy number status of each target. An intact gene would give a predicted copy number of 2 using both reference assays. Discrepant predicted copy number estimates necessitated repetition of some tests, especially those with low quality metrics. Borderline discrepancies between the calculated copy numbers were

not considered as discordant if an existing low proportion of the abnormal clone was detected by FISH. Data must be interpreted with caution and it needs to be correlated with available cytogenetic, FISH, MLPA and genomic data. In addition, karyotypes were reviewed for any gain or loss of either whole or part of chromosome 14 and 5 that might encompass the reference genes. *RNase P* gene status can be predicted from *IGH* gene status, located at 14q, by looking at the *IGH* BA FISH test results, whereas, possible *TERT* gene status can be obtained by viewing the MLPA profile of both *EBF1* gene and other reference genes, located at 5p and 5q, included in the MLPA P335 *IKZF1* kit.

5.3.3 Fluorescence in situ hybridisation (FISH)

At the start of FISH study, the efficiency and the cut off values of all seven home grown probes targeting different genes [*NR3C2* (n=2), *NR3C1* (n=1), *BMF* (n=1), *BTLA* (n=1), *CD200* (n=1) and *TBL1XR1* (n=1)] were determined (section 2.8.8). These cut off values are given in Appendix D. Each probe set contained both target (labelled green) and control (labelled red) probes. The target was considered to be deleted if a signal pattern of one or zero green signal was observed. The detailed information on the individual probe set used is shown in Appendix C and the locations of both the probe assays and the target BAC or fosmid clones in relation to the gene is demonstrated in Figure 5.1.

Initial screening, with all six home grown probes targeting different genes, was applied to the diagnostic samples of all patients in each group. To note, any discrepancies existing between the qPCR and FISH data, owing to possible different mapping regions of each probe, were further investigated by FISH, using other specific probes that encompass the target qPCR assay used. These specific probes were targeting *5'NR3C2* and *3'* or *5' TBL1XR1*. For all FISH tests, a minimum of 100 interphase cells were scored, apart from a few cases with limited material in which a minimum of 50 interphase cells were analysed. Furthermore, an algorithm for FISH review was generated in order to alleviate bias (Table 5-3). Individual evaluation of each case followed a specific algorithm that was generated in this study to ascertain the status of each gene tested based on both results FISH and qPCR (Appendix I). For the back-tracking FISH, there were additional FISH probes used targeting *PAX5* exons 2&7, *ETV6* exons 1&2, *BTG1* and *CDKN2A/B* (Appendix C, Appendix D).

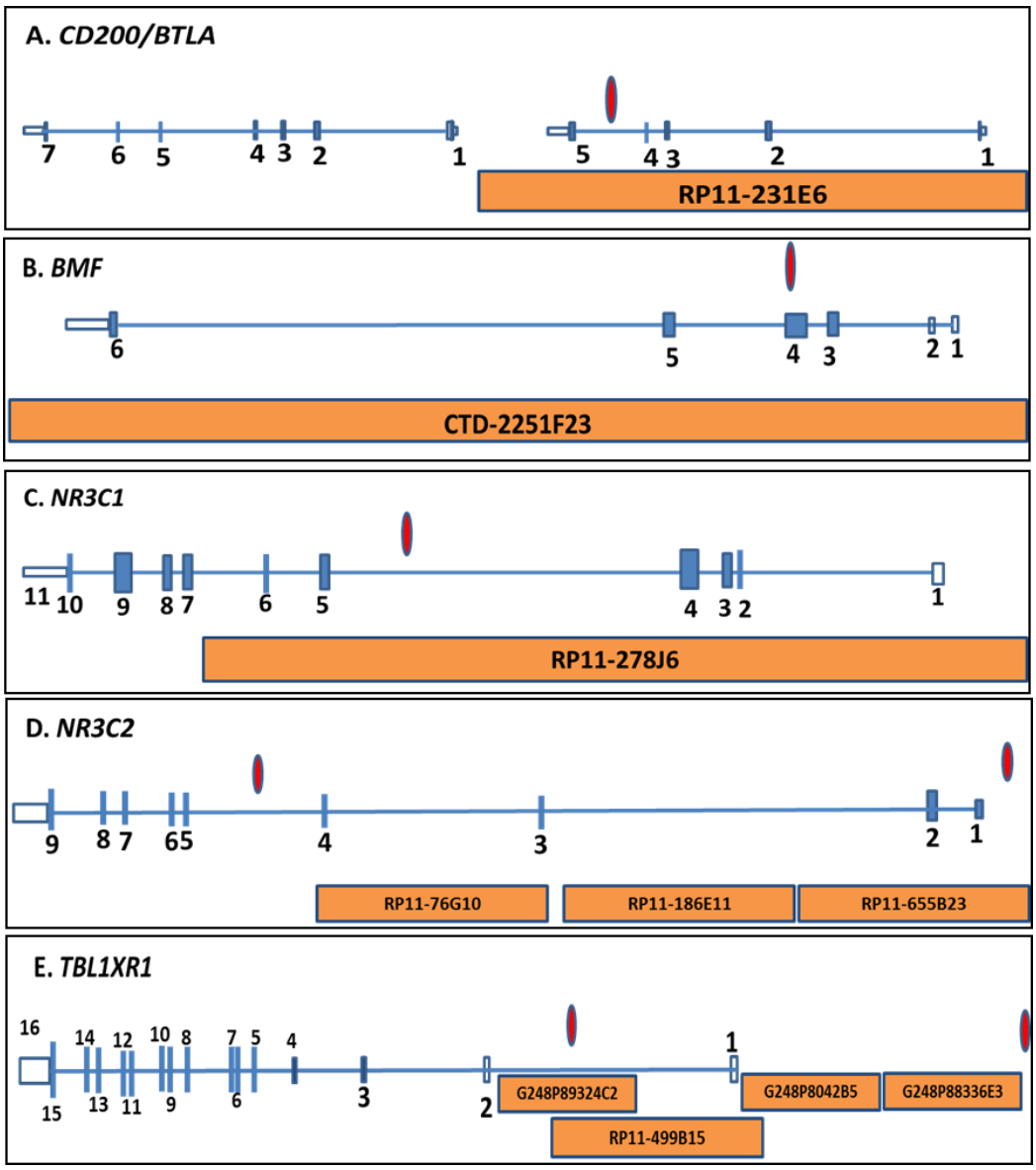

Figure 5.1 The location of both the different target probe assays and the FISH probes in relation to different genes investigated in this study. The probe assay is denoted in a red coloured shape and the BAC or Fosmid clones are shown in orange colour. The genes investigated included: A. *BTLA*, B. *BMF*, C. *NR3C1*, D. *NR3C2* and E. *TBL1XR1*.

% deleted clone (X)	Decision
MFPR + 4 * SD < X =< 30	Accept with no review
X < MFPR + 4 * SD	Accept with review 50 cells
MFPR + 3* SD > X > MFPR + 2 * SD	Accept with review 100 cells
X < MFPR + 2 * SD	Reject

Table 5-3 FISH review algorithm. MFPR: mean false positive rate, SD: Standard Deviation.

5.3.4 Multiplex ligation dependent probe amplification (MLPA)

Analysis of the available MLPA data, using the SALSA MLPA kit P335 *IKZF1,* which were generated in Chapter 3, was carried out to further extend the genetic landscape (section 2.9).

5.4 Results

5.4.1 Cohort Description

A total number of 18 patients with relapsed *ETV6-RUNX1* BCP-ALL were tested in this study (Table 5-4) with blast purity at initial presentation averaging 94%, whereas at relapse the data was lacking for most cases except four cases with 0% (isolated CNS relapse), 35%, 70% and 95%. Time to first relapse from diagnosis ranged from less than 6 months to 6 years 2 months (Appendix A and Appendix J).

A further comparison group of 16 patients (Table 5-4), with no history of relapse, was investigated similarly to evaluate the potential relevance of the copy number gene alterations to the relapse cases. The blast purity averaged around 92% (Appendix A and Appendix J).

	ETV6-RUNX1 positive BCP-ALL		
	Relapse group	**Non- relapse group**	**P-value**
No. of cases	18 (100)	16 (100)	
Age (years)			
Median	5	4.5	
1	1 (6)	0 (0)	
2-9	16 (88)	16 (100)	NA‡
≥10	1 (6)	0 (0)	
Sex (M:F)	10:08	08:08	0.75
Ratio	1.25	1.00	
WCC (X 109/L)			
Median	35.2	6.9	
<10	4 (22)	10 (63)	
10-49.9	8 (44)	1 (6)	0.02
>= 50	6 (33)	5 (31)	
NCI Risk Group			
Standard	11 (61)	11 (69)	NA‡
High	7 (39)	5 (31)	
No. Relapses			
Early	6 (33)	NA	NA
Late	12 (67)	NA	
Location of relapses			
BM	10 (56)	NA	
CNS	4 (22)	NA	NA
Both	4 (22)	NA	
Outcome after first relapse			
Complete remission	14 (78)	NA*	NA
Death	4 (22)	NA*	

Table 5-4 The demographic and clinical features of the relapse and the non-relapse groups within *ETV6-RUNX1 BCP-ALL.* WCC: white cell count, NCI: National Cancer Institute, BM: bone marrow, CNS: central nervous system, Early relapse: within 18 months of the diagnosis until 6 months from the end of treatment (male: 3 years, female: 2 years), Late relapse: after 6 months off treatment, NA: not applicable, * all non-relapse cases had complete remission until the submission of this thesis, ‡ all non-relapse cases were chosen to be from 2-9 years.

5.4.1.1 Cytogenetic abnormalities

Three quarters (n=10, 77%) of 13 relapse cases with successful cytogenetic results showed abnormal karyotypes in their diagnostic samples. The most prominent cytogenetic alterations included deleted or additional material at 12p (del/add(12)(p)) (n=3, 23%), deleted additional material at 9p (del/add(9p)) (n=2, 15%), gain

chromosome 21 (+21) (n=2, 15%), gain der(21)t(12;21) (+der(21)t(12;21)) (n=1, 8%) and deleted 6q (del(6)(q)) (n=1, 8%) (Appendix A).

Further estimation of the frequency of both +21 and +der(21)t(12;21) was carried out on the available FISH studies targeting either *ETV6* or *RUNX1* loci (n=14 relapse cases). Among 14 cases, one case showed gain of chromosome 21 (+21) (7%), a further one case exhibited gain der(21)t(12;21) (+der(21)t(12;21)) (7%), while one additional case harboured both alterations (7%).

A total of 12 cases (86%) out of the 14 non-relapse cases with successful cytogenetic results showed abnormal karyotypes. They exhibited del/add(12)(p) (n=4, 29%), del/add(9p) (n=2, 14%), del(11q) (n=2, 14%), +21 (n=1, 7%), del(6q) (n=1, 7%) and gain chromosome X (+X) (n=1, 7%) (Appendix A).

Similarly, the frequency of both +21 and +der(21)t(12;21) was further estimated utilising the available FISH studies targeting either *ETV6* or *RUNX1* loci (n=14 non-relapse cases). Among 14 cases, three cases showed gain of chromosome 21 (+21) (21%) but none of the cases exhibited +der(21)t(12;21)).

5.4.2 Real-time polymerase chain reaction (PCR) using TaqMan Copy Number Assays

5.4.2.1 Sensitivity assay of the Real-time polymerase chain reaction (PCR) using TaqMan Copy Number Assays

The first sensitivity assay revealed that the calculated copy number for the target gene decreased in relation to increasing proportion of cell line DNA (Table 5-5 and Figure 5.2). Furthermore, the dilutions 60% and 80% were found to have a copy number of 1, whereas the remaining dilutions less than 60% were showing normal copy number. Based upon the first sensitivity assay results, a second sensitivity assay was performed to further refine cut off value. The monoallelic deletion with a copy number of 1 was not detected when it presented in a proportion of 50% or less of cells (Table 5-6).

Serial dilutions	CN Calculated	CN Predicted	Confidence	|Z-Score|
KG1-05	2.05	2	0.98	0.16
KG1-10	1.94	2	0.99	0.02
KG1-20	1.96	2	0.99	0.02
KG1-25	2.01	2	0.98	0.09
KG1-30	1.82	2	0.99	0.16
KG1-40	1.77	2	0.99	0.34
KG1-60	1.46	1	< 0.50	2.2
KG1-80	1.27	1	0.65	1.39
KG1-100	0.87	1	> 0.99	0.04

Table 5-5 The first sensitivity detection test of monoallelic deletion of *NR3C2* gene in KG1 cell line. Confidence and z-score indicated the quality metrics of the PCR run. KG1: Positive control of *NR3C2* monoallelic deletion, CN: copy number, Confidence: the probability that the assigned copy number is the correct copy number relative to any of the other possible assigned copy numbers for the group of analysed samples, |Z-Score|: absolute value of the number of standard deviations separating the replicate mean ΔCT of a sample from the mean subdistribution of the assigned copy number. Predicted CN of 1 is monoallelic deletion, while 2 is normal copy number, for algorithm to evaluate the data: see Appendix I.

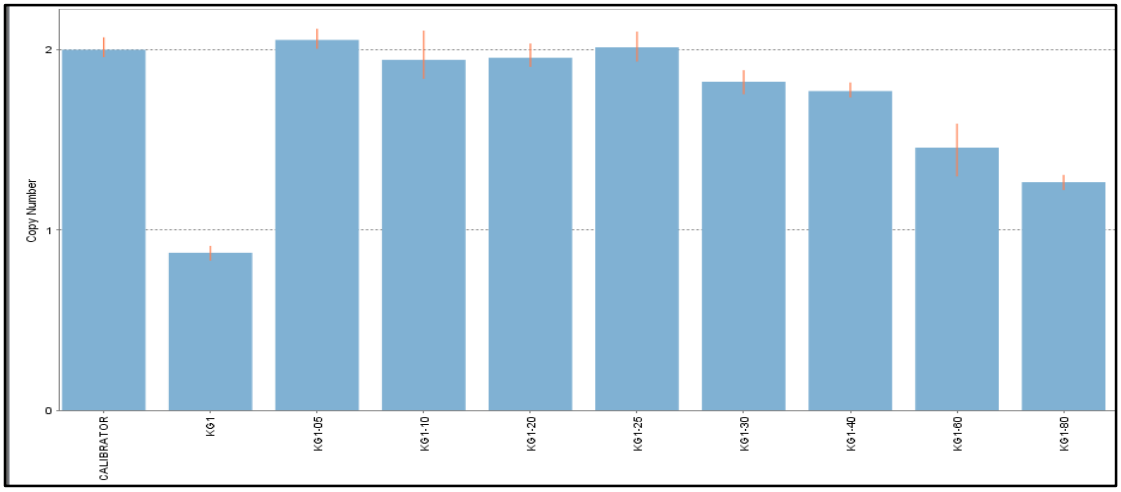

Figure 5.2 Histogram showing the decrease in the copy number with increasing proportion of cell line DNA. The samples are on the x-axis whereas the y-axis represents the copy number of the target gene.

Serial dilutions	CN Calculated	CN Predicted	Confidence	|Z-Score|
KG1-10	2.24	2	0.94	0.24
KG1-30	1.93	2	0.58	0.04
KG1-50	1.57	2	< 0.50	1.41
KG1-55	1.48	1	< 0.50	0.5
KG1-60	1.44	1	< 0.50	0.35
KG1-65	1.36	1	< 0.50	0.08
KG1-70	1.36	1	< 0.50	0.09
KG1-80	1.12	1	0.99	0.37
KG1-100	0.98	1	> 0.99	0.02

Table 5-6 The second sensitivity assay for the detection of monoallelic deletion of *NR3C2* gene in KG1 cell line.

5.4.2.2 Cohort screening

A total of 18 diagnostic samples of cases that went on to relapse were investigated successfully using the six different target probes. Overall, 61% (n=11) of the relapse cases did not harbour any gene deletions, while the remaining cases (n=7, 39%) exhibited a total number of nine aberrations with a mean of 0.5 alterations per sample. Eight patients (44%) had one alteration and the remaining case (6%) had three. *TBL1XR1* and *NR3C2* losses were the most frequent alterations constituting 22% (n=4) and 17% (n=3) of the relapse cases, respectively. *TBL1XR1* deletions were restricted either to the intragenic probe (n=2, 50%), or to the *5'TBL1XR1* probe (n=1, 25%) or encompassed both probes (n=1, 25%). In addition, two out of 17 cases (12%) with successful results showed *BTLA* deletions. It is noteworthy that all deletions were monoallelic. There were no deletions detected in *NR3C1* and *BMF* genes (Table 5-7).

Overall, eight (50%) out of 16 non-relapse cases, which were successfully screened using six different target probes, did not exhibit any gene deletions. The remaining half of the cases harboured a total of 10 aberrations, thus there was an average of 0.63 alterations per case. A total of six patients (38%) had one alteration and a further two patients (19%) exhibited two alterations. These deletions were frequently affecting *TBL1XR1* (n=5, 31%) and *NR3C2* (n=3, 19%), whereas the remaining two deletions targeted *NR3C1* (n=1, 6%) and *BMF* (n=1, 6%). Four (80%) out of five *TBL1XR1* deleted cases compromised the probe covering the *5'TBL1XR1* area and the remaining deletion (20%) extended further

to affect both the intragenic and 5’ region. There were all monoallelic deletions with no *BTLA* deletions detected (Table 5-7).

Reg.ID	*NR3C1*		*NR3C2*		*5'NR3C2*		*TBL1XR1*		*5'TBL1XR1*		*BTLA*		*BMF*		Collective No. of CNA
	q PCR	FISH (%)¥	q PCR	FISH (%)¥	q PCR	FISH (%)¥	q PCR	FISH (%)¥	q PCR	FISH (%)¥	q PCR	FISH (%)¥	q PCR	FISH (%)¥	
Relapse Group															
2712	N	N	NA	N	N	NA	N	5	N	NA	F	F	N	N	1
2774	N	NA	NA	NA	N	NA	N	NA	N	NA	N	NA	N	NA	0
2850	N	N	NA	48	D	NA	D	NA	N	NA	D	NA	N	F	3
3098	N	NA	NA	NA	N	NA	N	NA	N	NA	N	NA	N	NA	0
3181*	N	N	NA	N	N	NA	N	N	N	NA	N	N	N	N	0
3359	N	NA	NA	NA	D	NA	N	N	N	NA	N	NA	N	NA	1
3431	N	NA	NA	NA	D	NA	N	NA	N	NA	N	NA	N	NA	1
3562	N	F	NA	N	N	NA	N	N	N	NA	N	F	N	N	0
3588	N	NA	NA	NA	N	NA	N	NA	N	NA	N	NA	N	NA	0
3700	N	N	NA	N	N	NA	N	N	N	NA	D	100	N	N	1
3703*	N	NA	NA	F	N	NA	N	N	N	NA	N	NA	N	NA	0
3833	N	NA	NA	F	N	NA	N	N	N	NA	N	NA	N	NA	0
4036*	N	N	NA	N	N	NA	N	N	N	NA	N	N	N	N	0
4044	N	N	NA	N	N	NA	N	N	D	80	N	N	N	N	1
4902*	N	N	NA	N	N	NA	N	N	N	NA	N	N	N	N	0
5062*	N	F	NA	F	N	NA	N	F	N	NA	N	F	N	F	0
5597*	N	N	NA	N	N	NA	D	100	D	NA	N	F	N	N	1
10875*‡	N	N	NA	N	N	NA	D	44	N	NA	N	N	N	N	1
Non- Relapse Group															

Reg.ID	*NR3C1*		*NR3C2*		*5'NR3C2*		*TBL1XR1*		*5'TBL1XR1*		*BTLA*		*BMF*		Collective No. of CNA
	q PCR	FISH (%)¥	q PCR	FISH (%)¥	q PCR	FISH (%)¥	q PCR	FISH (%)¥	q PCR	FISH (%)¥	q PCR	FISH (%)¥	q PCR	FISH (%)¥	
2897	N	N	NA	N	N	NA	N	N	N	NA	N	NA	N	N	0
3026	D	100	NA	N	N	NA	N	N	N	NA	N	46	N	N	2
3711	N	N	NA	N	N	NA	N	F	N	NA	N	N	N	N	0
3761	N	NA	NA	N	D	NA	N	F	D	NA	N	NA	N	N	2
4011	N	N	NA	N	N	NA	N	N	N	NA	N	N	N	N	0
4288	N	N	NA	N	N	NA	N	N	N	NA	N	N	N	N	0
4439	N	N	NA	N	N	NA	N	N	N	NA	N	NA	N	N	0
4536	N	N	NA	N	D	73	N	N	N	NA	N	N	N	N	1
4569	N	NA	NA	NA	N	NA	N	NA	N	NA	N	NA	N	NA	0
4609	N	N	NA	N	N	NA	N	N	D	65	N	N	N	N	1
4995	N	N	NA	N	N	NA	N	N	D	100	N	F	N	N	1
5647	N	N	NA	N	N	NA	N	N	N	NA	N	N	N	N	0
5659	N	N	NA	F	N	NA	N	N	N	NA	N	N	N	N	0
5721	N	NA	NA	NA	D	NA	D	NA	D	NA	N	NA	N	NA	2
5807	N	NA	NA	N	N	NA	N	F	N	NA	N	NA	D	F	1
5914	N	N	NA	N	N	NA	N	N	D	81	N	N	N	N	1

Table 5-7 FISH and q PCR data of the relapse and the non-relapse cases . * treated on ALLR3 at relapse, ‡ treated on UKALL2003 at presentation, ¥ percentages are relative to *ETV6-RUNX1* positive cells, F failed, D deleted, N normal, NA not available.

5.4.3 Fluorescence in situ hybridisation (FISH)

A total of 13 out of 18 diagnostic samples from the relapse cohort were tested using all six screening FISH probes with additional specific probes, whenever needed. Seven samples (54%) showed normal FISH studies for all genes tested. The remaining six samples (46%) exhibited a total of 6 alterations affecting different genes including: *TBL1XR1* (4 out of 12 successful tests, 33%), *NR3C2* (1 out of 10 successful tests, 10%) and *BTLA* (1 out of 4 successful tests, 25%) with a mean of 0.46 alterations per sample at diagnosis. To note, the four *TBL1XR1* deletions were shown to encompass either the intragenic probe (n=3) or the 5' probe (n=1) of the gene. Overall, these monoallelic deletions were identified to be secondary events that constituted an average of 60% of *ETV6-RUNX1* positive cells. The deleted clones represented as major clones in three cases, whereas the remaining three cases harboured deletions that occurred at a proportion less than 50%. Both *NR3C1* and *BMF* genes were shown to be intact in all ten samples with successful results (Table 5-7).

Overall, a total of 13 out of 16 non-relapse cases were successfully screened using six screening FISH probes with additional specific probes, whenever needed. Around 62% of samples (n=8) did not show any gene deletions, whereas the remaining five cases (38%) exhibited a total of six different gene deletions yielding an average of 0.46 CNA per individual. These losses targeted the following genes: *TBL1XR1* (3 out of 11 successful tests, 27%), *NR3C1* (1 out of 11 successful tests, 9%), *NR3C2* (1 out of 12 successful tests, 8%) and *BTLA* (1 out of 10 successful tests, 10%). To note, *TBL1XR1* deletions were restricted to the 5' probe. All deletions occurred at subclonal levels with an average of 80% (range: 46%-100%) of *ETV6-RUNX1* positive cells. Overall, the deletions were monoallelic except two cases with biallelic deletions targeting the 5' part of either *TBL1XR1* or *NR3C2* genes. *BMF* deletions were not detected in this cohort (Table 5-7).

5.4.4 Consistency between qPCR and FISH data

It is worth noting that 10 out of 19 deletions, detected by qPCR, had FISH studies and all these deletions (n=10) had concordant qPCR and FISH results.

On the other hand, a further two deletions were detected by FISH only but not with qPCR, owing to the low proportion of the deleted clones that constituted 5% (*TBL1XR1*, 2712) and 46% (*CD200/BTLA*, 3026) of *ETV6-RUNX1* positive cells, respectively (below qPCR resolution).

5.4.5 Multiplex ligation dependent probe amplification (MLPA)

All 34 cases were successfully screened by MLPA at diagnosis (Appendix A).

Among the 18 relapse cases, 17% (n=3) of the diagnostic samples did not harbour any gene deletions. The remaining 15 cases (83%) exhibited a total of 26 CNAs giving a mean of 1.4 alterations per case. One third of patients (n=6) had one, 7 (39%) had two and two (11%) had three alterations. The most frequent losses targeted *ETV6* (n=11, 61%) and *CDKN2A/B* (n=8, 44%), while the remaining deletions affected *BTG1* (n=4, 22%) and *PAX5* (n=3, 17%).

A total of 15 (94%) out of 16 non-relapse patients, who were successfully screened, showed an alteration of at least one of these genes with a total of 25 aberrations. The average number of alterations was 1.6 aberrations per case with 63% of patients (n=10) had two alterations, while the remaining five cases exhibited one alteration (31%). These deletions targeted *ETV6* (n=14, 88%), *PAX5* (n=4, 25%), *BTG1* (n=2, 13%), *CDKN2A/B* (n=2, 13%), *RB1* (n=2, 13%) and *EBF1* (n=1, 6%).

5.4.6 Comparisons between the relapse and non-relapse cohorts

As expected, around two thirds of the non-relapse patients had WCC<$10x10^9$/L compared to 22% of the relapse cases. Based on this small number of cases, there were no noticeable genetic differences between the relapse and non-relapse groups considering the eight genes tested by MLPA. There was a comparable number of deletions (n=26 and n=25) in the relapse and non-relapse groups with a similar mean of 1.4 and 1.6 alterations per case, respectively (p=0.7). It is worth noting that none of the alterations were particularly associated with relapse, however, *CDKN2A/B* losses constituted 44% of the relapse cases compared to 13% of the non-relapse cases (p=0.06). Both groups showed frequent losses targeting *ETV6* (61% v 88%), *TBL1XR1* (28% v 31%), *PAX5* (17% v 25%), *BTG1* (22% v 13%), *NR3C2* (17% v 19%) and *CD200/BTLA* (12% v 6%)

genes (Table 5-8). *TBL1XR1* losses showed variable breakpoints targeting the gene itself or restricted to the 5' region of the gene or both in both groups (Figure 5.3).

	***ETV6-RUNX1* positive BCP-ALL**		
	Relapse group	**Non- relapse group**	**P-value**
No. of cases (%)	**18 (100)**	**16 (100)**	
Deletions			
12p	3 (17)	4 (25)	0.7
ETV6	11 (61)	14 (88)	0.1
9p	2 (11)	2 (13)	1.0
CDKN2A/B	8 (44)	2 (13)	0.06
PAX5	3 (17)	4 (25)	0.7
TBL1XR1	5 (28)	5 (31)	1.0
BTG1	4 (22)	2 (13)	0.7
NR3C2	3 (17)	3 (19)	1.0
BTLA	2 (12)*	1 (6)	1.0
Gain 21	2 (11)	1 (6)	1.0
6q	1 (6)	1 (6)	1.0
NR3C1	0	1 (6)	0.5
BMF	0	1 (6)	0.5
EBF1	0	1 (6)	0.5
11q	0	2 (13)	0.2
Gain X	0	1 (6)	0.5
RB1	0	2 (13)	0.2

Table 5-8 The genetic landscape of the diagnostic samples of the relapse and non-relapse cases. No *IKZF1* or *PAR1* rearrangements in either group, * percentage was calculated from the total number of patients tested successfully (n=17).

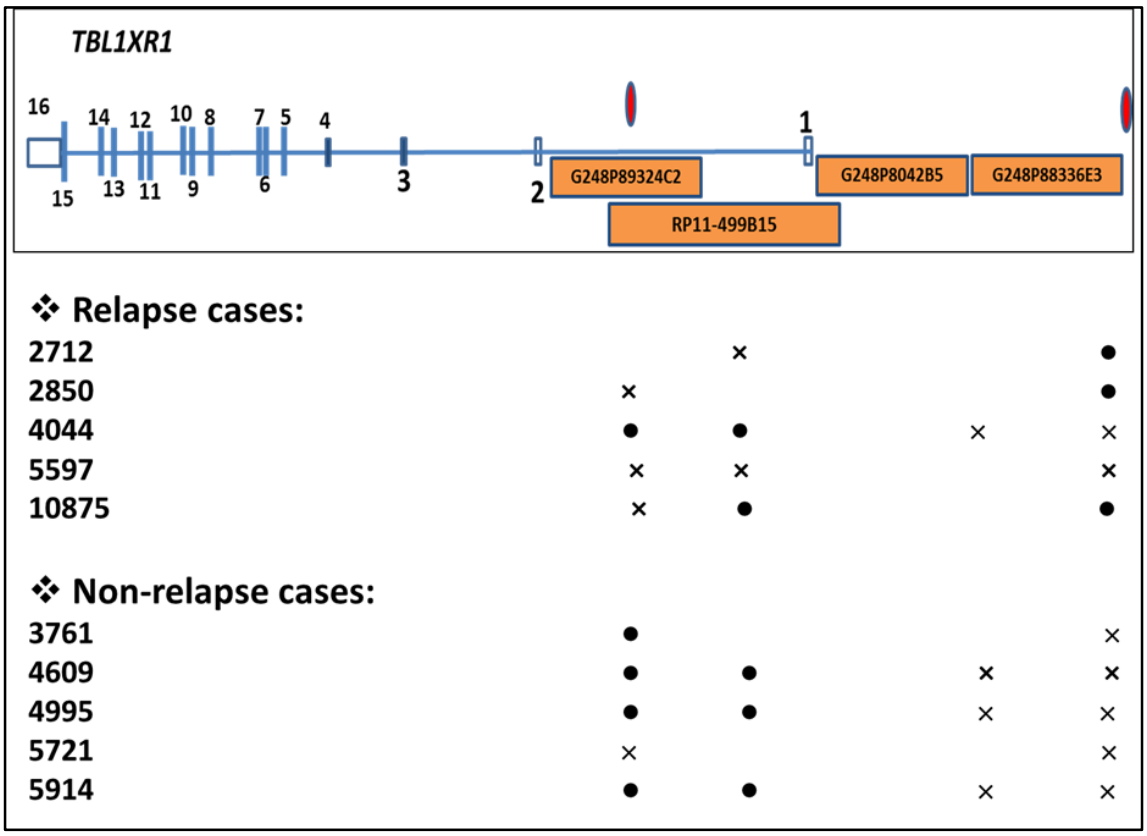

Figure 5.3 Different *TBL1XR1* breakpoints in the relapse and non-relapse cases. The non-relapse cases harboured *TBL1XR1* losses restricted to the 5' region in 80% of deleted cases, while the *TBL1XR1* deletions in the relapse cases encompassed the gene itself with variable breakpoints in 80% of the deleted cases, ● intact, × deleted.

5.4.7 The clonal evolution of the relapsed clones

A total of nine presentation-relapse matched pairs, with BM involvement, were backtracked by FISH for some of the important genetic lesions from relapse to diagnosis (Table 5-9). Overall, 6 (67%) out of 9 cases showed the same abnormalities at diagnosis and relapse, while the remaining three cases (33%) exhibited either loss (n=1) or acquisition (n=2) of at least one genetic abnormality.

Among those cases with similar abnormalities (n=6), the dynamics of progression at both time points were determined in four cases. A total of 3 out 4 cases had one of the abnormalities at presentation regressed at relapse but the rest of the abnormalities maintained at the same level relative to *ETV6-RUNX1* positive cells from diagnosis to relapse. The remaining case showed a static profile of abnormalities at both time points.

On the other hand, among the remaining three out of 9 cases, the dynamics of progression at both time points were determined in two cases. One of the cases had acquired an aberration at relapse which was detected molecularly and not confirmed by FISH, while the rest of the abnormalities showed reverse pattern of progression. The remaining case had lost one of the abnormalities at relapse.

Furthermore, the latter two patients exhibited further two alterations which had different pattern of evolution. One of the patients showed reverse pattern of progression, in which one of the aberration expanded at relapse, while the other aberration regressed. However, the other patient had similar pattern of progression in which both of them regressed at relapse, whereas the rest of aberrations showed almost similar levels at both time points.

Although the dynamics of progression was not identified in the remaining three out of 9 cases, two of the cases showed same genetic abnormalities at both time points. However, the remaining case had acquired one genetic lesion at relapse but the rest of aberrations presented in both time points.

Patient No.	Diagnosis			Relapse			Pattern of Evolution
	Cytogenetic	FISH	Molecular	Cytogenetic	FISH	Molecular	
2712	N	*TBL1XR1* [5%]	N	F	*TBL1XR1* [0%]	NA	A+B+C >>>> B+C
		Gain 21 [7%]	NA		Gain 21 [4%]	NA	
		ETV6 exons 1&2 [64%]	Yes		*ETV6* exons 1&2 [73%]	NA	
2850	add(9)(p13)	*CDKN2A/B* [NA]	Yes	NA	*CDKN2A/B* [46%]	NA	D>>>>D Rest: Cannot be determined
	del(12)(p11)	*ETV6* exons 3-5 [NA]	Yes		*ETV6* exons 3-5 [27%]	NA	
		BTG1 [NA]	Yes		*BTG1* [43%]	NA	
		NR3C2 [48%]	Yes		*NR3C2* [35%]	NA	
		TBL1XR1 [NA]	Yes		*TBL1XR1* [27%]	NA	
		CD200/BTLA [NA]	Yes		*CD200/BTLA* [68%]	NA	
3431	N	*ETV6* exons 2-8 [NA]	Yes	NA	*ETV6* exons 2-8 [26%]	NA	Cannot be determined
		BTG1 [NA]	Yes		*BTG1* [72%]	NA	
		NR3C2 [NA]	Yes		*NR3C2* [70%]	NA	
3700	N	*ETV6* [96%]	Yes	NA	*ETV6* [99%]	NA	A+B >>>> A+b
		BTLA [100%]	Yes		*BTLA* [41%]	NA	
3703	Comp.	*ETV6* [74%]	Yes	N	*ETV6* [89%]	NA	A+B* >>>> A+b*
		BTG1 [NA]	Yes		*BTG1* [2%]	NA	
4036	Comp.	*CDKN2A/B* [100%]	Yes	del(9)(p21p21)	*CDKN2A/B* [100%]	Yes	A+B+C >>>> A+B+C
		ETV6 [100%]	Yes	add(12)(p1)	*ETV6* [100%]	Yes	
		BTG1 [100%]	Yes		*BTG1* [91%]	Yes	
4902	N	*PAX5* exons 2-7 [86%]	Yes	Normal	*PAX5* exons 2-7 [100%]	Yes	A+B >>>> A+b

Patient No.	Diagnosis			Relapse			Pattern of Evolution
	Cytogenetic	FISH	Molecular	Cytogenetic	FISH	Molecular	
		ETV6 [98%]	Yes		*ETV6* [78%]	Yes	
10875	N	Gain der(21) [5%]	No	Gain 21	Gain der(21) [51%]	Yes	a+B >>>> A+b+C
		TBL1XR1 [48%]	Yes		*TBL1XR1* [17%]	No	
		CDKN2A/B [0%]	No		*CDKN2A/B* [F]	Yes	
3181	del(12)(p)	*ETV6* exon 8 [26%]	Yes	Failed	*ETV6* exon 8 [NA]	Yes	Cannot be determined Only acquisition of C
		PAX5 exons 1-2 [NA]	Yes		*PAX5* exons 1-2 [NA]	Yes	
		CSF2RA, IL3RA [N]	N		*CSF2RA, IL3RA* [NA]	Yes	

Table 5-9 Cytogenetics, FISH and Molecular data of the nine matched diagnosis/relapse cases. NA: Not performed, N: normal, F: Failed, Comp. complex, * the pattern of evolution can be perceived from the MLPA data (Abnormality B detected by MLPA at presentation but it occurred in only 2% of *ETV6-RUNX1* positive cells (below the MLPA threshold detection), the percentages shown represent the proportion of the abnormal clone relative to *ETV6-RUNX1* positive cells. A, B, C, D, E and F denote to any aberration in general but taking into consideration the existent number of aberrations. A>>>>a, a>>>>A, A>>>>A indicates regression, expansion, maintenance at the same level of the abnormal clone from diagnosis to relapse, respectively. If the difference between percentages at both time points ≤15%, so the clone level was maintained, if the diagnostic clone level was more than the relapse clone in >15%, thus the diagnostic clone regressed and vice versa.

5.5 Discussion

This Chapter employed the data generated from the copy number qPCR, FISH, cytogenetic and MLPA data on 18 *ETV6-RUNX1* relapse cases. The cohort was tested for selected genes known to be associated with *ETV6-RUNX1* BCP-ALL and to some extent implicated in relapse (Mullighan *et al.*, 2007; Mullighan *et al.*, 2008b; Parker *et al.*, 2008; Lilljebjorn *et al.*, 2010). The potential relevance of these alterations to the emergence of relapses was evaluated by extending the investigations to other *ETV6-RUNX1* patients who did not go onto relapse. The decision made around the choice of techniques was somehow difficult. The ideal way of investigating these patients is to carry out a technique with higher resolution and good sensitivity e.g. SNP arrays that can provide both the copy number and the heterozygosity profiles at a genomic level. However, limited availability of good quality DNA prohibited this technique. Thus, specific genes were selected to screen the chosen cohort and MLPA was initially chosen as the second alternative method that enables detection of copy number alterations of up to 50 different genomic DNA. As compared to array studies, it represents a low cost and simple alternative method to array-based techniques for many routine applications. Unfortunately, there was no predesigned MLPA kit which containing the genes of interest (*TBL1XR1, BMF, NR3C1, NR3C2* and *CD200/BTLA*). Designing a custom MLPA kit was attempted but failed due to poor coverage of all genes selected. As a result, both TaqMan copy number real-time qPCR and FISH were chosen as screening techniques. Based on the sensitivity assays, qPCR technique was able to detect monoallelic deletions when present in a proportion of cells greater than 50%, although lower proportions were still detected but with borderline values and low quality metrics. However, all FISH probe sets used were able to detect deleted clones presented in a proportion as low as 4%. Although there was a probability of missing deletions either not covered by the selected FISH probes or smaller than the probe used. It is worth noting that both techniques were laborious with high consumption of material; hence, the number of probes was restricted to the most common known deletions.

It would be more informative if non-relapse cases were matched in terms of the demographic information, e.g. age, sex and WCC, but the availability of materials hampered this approach, however, they were comparable. Surprisingly, there were no

differences found between both group of patients in terms of number of deletions and the genes involved. This study showed that *CDKN2A/B* losses constituted 44% of the relapse cases compared to 13% of the non-relapse group, in agreement with previous studies that reported higher frequencies of these losses in relapsed BCP-ALL cases (Maloney *et al.*, 1999; Graf Einsiedel *et al.*, 2002; Irving *et al.*, 2005a; Mullighan *et al.*, 2008b; Yang *et al.*, 2008). In contrast to our findings, similar frequencies of *CDKN2A/B* losses between the relapse and newly diagnosed BCP-ALL cases have been recently identified (35.7% v 33.9%, respectively) (Hogan *et al.*, 2011). In line with the latter study, the proportions of the *CDKN2A/B* losses in the diagnostic and relapse samples of *ETV6-RUNX1* relapse cases, in particular, were almost similar (33% v 25%) (Kuster *et al.*, 2011) and corresponded to the overall incidence of these losses in non-relapse *ETV6-RUNX1* cases (22%) (Lilljebjorn *et al.*, 2010). *CDKN2A/B* genes have previously been identified as tumour suppressor genes that were deregulated by several mechanisms in ALL including: focal deletions, DNA hypermethylation and down-regulation occurring either in the same or different patients (Hogan *et al.*, 2011). The enrichment of these losses in both diagnosis and relapse indicates that they are needed in both the initial leukaemogenic transformation and emergence of relapse and are not necessarily relapse-specific alterations. It is worth noting that a positive correlation was observed between *CDKN2A/B* losses and adverse risk factors (e.g. high peripheral blast count, WCC and older age at relapse) in the relapse samples of *ETV6-RUNX1* cases (Bokemeyer *et al.*, 2013), however, *CDKN2A/B* deletions lacked any prognostic value in BCP-ALL and *ETV6-RUNX1* cases in particular.

Although *BTG1* and *CD200/BTLA* are slightly higher in the relapse cases as compared to non-relapse cases (22% v 13%, 12% v 6%, respectively), they were not statistically significant. In agreement with these findings, one previous study found enrichment of these alterations in the *ETV6-RUNX1* relapse cases as compared to the non-relapse cases (21% v 13%, 36% v 15%) (Kuster *et al.*, 2011). *BTG1* losses were previously reported to be associated with drug resistance with emergence of early relapses (defined as within 18 months of the diagnosis until 6 months from the end of treatment). Drug resistance has been postulated to be due to the reduction in the expression of the glucocorticoid receptor that negatively impacts on the glucocorticoid sensitivity (van Galen *et al.*, 2010). This present study showed that all *BTG1* deleted relapse cases were late (after 6 months

off treatment) and thus unlikely to be driven by drug resistance related aberrations. Hence, other genetic alterations might be responsible for these late relapses rather than *BTG1* deletions.

Both relapse and non-relapse groups displayed similar frequencies of common losses in their diagnostic samples targeting *ETV6, PAX5*, *TBL1XR1* and *NR3C2* (61% v 88%, 17% v 25%, 28% v 31%, 17% v 19%, respectively) which suggest the necessity of these alterations in the initiation of leukaemia as well as relapse development. In concordance with previous studies that identified both deletions of the *ETV6* and *PAX5* genes, transcription factors required for B cell development, to represent common alterations in both relapse and non-relapse *ETV6-RUNX1* cases (57% v 70%, 21% v 28%, respectively) (Kuster *et al.*, 2011). However, higher frequencies of *TBL1XR1* losses were observed in *ETV6-RUNX*1 relapse cases compared to non-relapse cases (29% v 13%) (Kuster *et al.*, 2011), but the proportion of these losses in relapse cases was consistent to our findings. On the other hand, *NR3C2* losses were enriched in the relapse samples rather than the diagnostic samples of the relapse cases (14% v 0%) (Kuster *et al.*, 2011), however, *NR3C2* losses constituted 11% of the non-relapse cases comparable to that seen in the relapse samples (11%).

This study showed that *TBL1XR1* losses exhibited variable breakpoints in both groups; relapse group harboured deletions of the gene itself in 80% of the deleted cases, while non-relapse cases showed frequent deletions restricted to the 5' region of the gene. It has previously been reported that the reduction in expression was similar regardless of the deletion extent (Parker *et al.*, 2008). Interestingly, the relationship between *TBL1XR1* deletions and the disruption of the capacity of SMRT/N-CoR to bind receptor molecules has been described resulting in inappropriate control of gene expression through the RAR and THR signalling pathways (Parker *et al.*, 2008). Taken together, *TBL1XR1* deletions might not be relapse-specific alterations, though they seem to constitute an essential element in the development of *ETV6-RUNX1* leukaemia.

None of the relapse cases in this study showed deletions of *NR3C1* and *BMF.* This could be due the detection methods that were designed to detect the common region of deletion or reflect the small number of patients tested. It has been previously reported that *NR3C1* alterations almost exclusively prevail at relapse (Kuster *et al.*, 2011), this

might explain their absence in the diagnostic samples of the relapse cases in the present study. However, *BMF* losses were previously observed to be preserved at relapse with an incidence rate higher than that seen in non-relapse cases in *ETV6-RUNX1* (16.6% v 2.8%). These genes were reported to be involved in glucocorticoid resistance in *ETV6-RUNX1* owing to their known functions in glucocorticoid mediated apoptosis resulting in poor treatment response (Kuster *et al.*, 2011). The dexamethasone resistance was attributed to their negative impact on the expression of the glucocorticoid receptor and the glucocorticoid induced regulation of the pro- and anti-apoptotic *BCL2* pathways, respectively. The present study is in agreement with previous studies showing the frequency of the somatic mutation of the *NR3C1* involved in glucocorticoid receptor expression is reported to be rare (Hillmann et al., 2000; Irving et al., 2005b; Tissing et al., 2005).

Individual patients with BM relapses were investigated by back-tracking FISH for the important genetic lesions from relapse to diagnosis in order to identify the possible origin of the relapse clone. Overall investigations confirmed that all genetic aberrations in relapse samples, irrespective of time to relapse, were able to be identified in the diagnostic samples as a minor or major clone, except two patients (10875, 3181) who showed acquisition of one alteration each. It is worth noting that both two acquired alterations were detected molecularly at relapse with no confirmed FISH studies, thus technical artefacts might play a factor or they might be present in small diagnostic compartments in which a large number of cells is needed to be screened for their detection. In addition, possible origin from ancestral clones might be another explanation. Interestingly, subclonal diversity at diagnosis had been observed in these data as evidenced by FISH, hence this might contribute to the variability of the intra-clonal origins of relapse, in line with previous reports (Mullighan *et al.*, 2008b; Kuster *et al.*, 2011; van Delft *et al.*, 2011). Taken together, the relapse clone is most probably derived from either a major or a minor clone present in the diagnostic sample, irrespective of time to relapse. In addition, *de novo* ALL arising from persisting pre-leukaemic clones harbouring *ETV6-RUNX1* seems less likely since this kind of evolution would result in the acquisition of distinct genetic aberrations that are not identified in the diagnostic samples (Ford *et al.*, 2001; Konrad *et al.*, 2003; van Delft *et al.*, 2011).

In conclusion, frequent *CDKN2A/B* losses in the relapse cases might be indicative of being a responsible factor in initiating the leukaemia in both first and recurrent diseases. Otherwise, no other potential copy number alterations were implicated in contributing to relapse biology. A high degree of clonal relatedness between the two points (diagnosis and relapse) was observed with an evidence of low level sub-clones at diagnosis emerging as the major clone at relapse. This is in agreement with Mullighan who postulated that alterations in the majority of relapse cases had a strong relationship with the diagnostic clone, whereas unrelated leukaemia was found in very low proportion (6%) (Mullighan *et al.*, 2008b).

Chapter 6. The genetic landscape of patients with *ETV6-RUNX1* positive BCP-ALL and an atypical demographic and clinical profile

6.1 Introduction

ETV6-RUNX1 BCP-ALL is characterised by tight demographic features in which the majority of cases are aged between 3-6 years and mostly assigned to the NCI standard risk group which, in part, explains the favourable outcome (Golub *et al.*, 1995; Romana *et al.*, 1996; Moorman *et al.*, 2010b). *ETV6-RUNX1* fusion gene is known to arise *in utero* as an initiating factor but is unable to cause overt leukaemia (Andreasson *et al.*, 2001). A latency period is needed for the additional genetic alterations to accumulate and aid in leukaemic transformation (Wiemels *et al.*, 1999; Mori *et al.*, 2002; Greaves and Wiemels, 2003; Hong *et al.*, 2008; Ma *et al.*, 2013). The latency period is usually broad with a typical peak between the ages of 3–6 years. However, in infant (before their second birthday) cases, this latency period is shorter and is therefore presumably due to accelerated emergence of full blown leukaemia. On the other hand, a prolonged latent period occurs in the older age (adolescent/young adults) (AYA) at around 3.4% incidence rate (Jabber Al-Obaidi *et al.*, 2002; Moorman *et al.*, 2010b). Infants and adults are usually characterised by the predominance of other cytogenetic subtypes including *MLL* rearrangements and *BCR-ABL1* BCP-ALL, respectively, which confer poor outcome (Moorman, 2012). In addition, Down syndrome (DS) group is a common genetic syndrome that is associated with a high incidence of different types of leukaemia including acute lymphoblastic leukaemia (20- fold increase) (Lange, 2000). Thus DS ALL accounts for around 1-3% of all childhood ALL (Whitlock, 2006; Forestier *et al.*, 2008b; Malinge *et al.*, 2009) and is characterised by lower incidences of both favourable (e.g. t(12;21) and high hyperdiploidy) and unfavourable (e.g. t(9;22), *MLL* rearrangements and hypodiploidy) cytogenetic subtypes. *ETV6-RUNX1* constitutes 10-15% of DS ALL as compared to 25% of non-DS ALL patients (Buitenkamp *et al.*, 2012; Buitenkamp *et al.*, 2013a; Patrick *et al.*, 2014). However, DS ALL is enriched with two genetic entities that are considered the hallmark of this disease including *JAK2* mutations and *CRLF2* overexpression that are seen in 19% and 62% of cases, respectively (Kearney *et al.*, 2009; Russell *et al.*, 2009; Hertzberg *et al.*, 2010). Taking into consideration the emphasis on the main bulk of *ETV6-RUNX1* cases, not much attention has been paid to the atypical cases (infants, AYA and DS). Thus, the identification of specific markers unique to each subgroup could be useful in delineating the critical events responsible for the leukaemogenic progression.

6.2 Aims

The aim of this Chapter is to assess whether or not the spectrum of secondary abnormalities in atypical *ETV6-RUNX1* patients are unique and informative about that subtype and *ETV6-RUNX1* as a whole.

The main objectives in this chapter are to:

1- Utilise the MLPA (Chapter 3) and cytogenetic data for characterising the genetic landscape with respect to cell cycle regulation, B cell development and differentiation of these atypical groups including: AYA (n=36), infants (n=21) and DS (n=13).

2- Screen these patients for different gene deletions involving *TBL1XR1, BMF, NR3C1, NR3C2* and *CD200/BTLA* genes that are involved in the nuclear hormone receptor transcriptional regulation, glucocorticoid receptor signalling, drug resistance and regulation of apoptosis using copy number q PCR and FISH

3- Investigate the genomic profiles of four Down syndrome cases using SNP arrays in order to uncover new genetic markers.

4- Compare the genetic profiles of different subgroups (infant v non-infant, AYA v non-AYA and DS v non-DS) in order to assess the potential relevance of specific genetic markers to any of the subgroups.

6.3 Patients and Methods

6.3.1 Patient cohort

A total of 73 cases of different subgroups within *ETV6-RUNX1*-positive BCP-ALL was studied including different atypical groups namely: infants (before their second birthday) (n=21), adolescents/ young adults (AYA) (age of 10-51 years) (n=39), Down syndrome (DS) (n=13). Each subgroup was compared with non-infant (n=381), non-AYA (n=363) and non-DS (n=389). There were a variable number of investigated cases in each subgroup (Table 6-1). Patients were treated on ALL97, UKALL2003 or UKALLXII at initial presentation (see section 2.2).

Subgroup	Total cases	Cytogenetic			MLPA			q PCR*			FISH¥			DNA	Fixed cells
		Successful	Failed	NA	Successful	Failed	NA	Successful	Failed	NA	Successful	Failed	NA		
Infants	21	16	5	0	21	0	0	12	0	9	10	0	11	12	10
AYA	39	33	6	0	39	0	0	13	0	26	9	0	30	13	9
DS¶	13	9	4	0	12	1	0	9	0	4	4	0	9	9	4

Table 6-1 Different atypical subgroups of patients included in this study with the number of cases investigated in each based on the availability of material. AYA: adolescents/ young adults, DS: Down syndrome, the availability of materials was assessed before the start of FISH and q PCR screening, ¶ among the 13 cases; four cases were investigated using SNP6 arrays, NA not done, * there were failed and NA tests within a case pertaining to different target genes [Infants: *BTLA* (2 NA, 1Failed), AYA: *BMF* (3 NA, 1 Failed), *NR3C2* (1 Failed), *NR3C1* (2 Failed), *BTLA* (3 NA), DS: *NR3C1* (2 Failed), *NR3C2* (3 Failed), *BTLA* (1 Failed, 5 NA), *TBL1XR1* (1 NA), BMF (5 NA)], ¥ there were failed and NA tests within a case pertaining to different target genes [Infants: *BTLA* (1 Failed), *NR3C1* (1 failed), AYA: *NR3C1* (1 NA), *NR3C2* (2 NA), *BMF* (1 NA), *BTLA* (1 NA), DS: *NR3C2* (2 NA), *NR3C1* (1 NA), *BMF* (1NA), *BTLA* (1 NA)].

6.3.2 Methods

The techniques used to study these patients were similar to those in Chapter 5; utilising the MLPA (using the SALSA MLPA kit P335) (see section 2.9) and cytogenetic data, followed by the FISH and q PCR screening of specific genes (*NR3C1*, *NR3C2*, *TBL1XR1*, *BMF* and *CD200/BTLA*) using the same positive gene deletion controls (see section 5.3.2 and 5.3.3). To note, the q PCR sensitivity assay was not repeated in this study because it was already described in Chapter 5 (see section 5.3.2.2) Four DS DNA samples were hybridized to Affymetrix Genome-Wide Human SNP Array 6.0 (see section 2.10).

6.4 Results

6.4.1 Infant group

6.4.1.1 Cohort Description

A total of 21 infant cases were investigated; two of them were also Down syndrome cases and one was an early relapse case. They were compared with 381 non-infant cases who were aged from 2-50 years with a median age of 4 years (Table 6-2 and Appendix A). There were no differences in the sex distribution between both subgroups. 81% of the infants cases had WCC >10X 10^9/L with a median of 25 X 10^9/L, while around half of the non-infant cases had WCC <10 X 10^9/L (p=0.02).

	ETV6-RUNX1 subgroups		
	infants	**Non-infants**	**P-value**
No. of cases	21 (100)	381 (100)	
Sex (M:F)	13:8	206:175	0.5
Ratio	1.6	1.2	
WCC (X 109/L)			
Median	25	10.6	
<10	4 (19)	179 (47)	
10-49.9	10 (48)	137 (36)	0.02
≥50	7 (33)	65 (17)	

Table 6-2 Comparison between the infant and non-infant cases within *ETV6-RUNX1* cases in terms of sex and WCC. WCC white cell count, M male, F female, significant P-value if <0.01.

6.4.1.2 The genetic comparison between the infants and non-infant subgroups

The level of the genetic diversity among both infants and non-infants groups was estimated using the available data on eight genes including: *ETV6, BTG1, PAX5, CDKN2A/B, RB1, EBF1, IKZF1* and *PAR1* in infant and non-infant groups (n=21, n=380, respectively). Infant cases harboured fewer aberrations (n=14) with a mean of 0.67 alterations per case, while non-infant cases had a total of 507 alterations with an average of 1.3 aberrations per case (p=0.001). A total of 8 infant patients (38%) had no alterations in any of the eight genes tested, while 12 patients (57%) had one alteration and only a single case (5%) showed two alterations. On the other hand, 79% of non-infant cases showed alterations in at least one of the selected genes (n=299): a total of 142 (37%), 115 (30%) and 42 (11%) patients had one, two and three or more aberrations (Appendix A and Appendix L).

Deletions at 12p were the most common abnormality in infants and non-infants subgroups constituting comparable proportions of 25% and 31%, respectively (Table 6-3, Appendix A and Appendix L). 12p deletions were reflected by the increase incidence of *ETV6* losses detected by MLPA (33% and 53% in infants and non-infants subgroups, respectively). To note, all *ETV6* deleted cases in infant cases affected the entire gene similar to that seen in non-infant cases who harboured whole *ETV6* gene deletions in 72% of cases. *CDKN2A/B* losses were commonly found in non-infant cases compared to

the infant cases (24% v 0%, p=0.006). *BTG1* and *TBL1XR1* losses constituted 17% and 25%, respectively, of the non-infant cases, while none of these deletions were observed in infant cases (p=0.03 and 0.05, respectively). It is worth noting that *NR3C2*, *CD200/BTLA* and 6q losses occurred in both groups at variable proportions. In addition, gain chromosome 21 and gain der(21)t(12;21) were seen in both groups as estimated from the available cytogenetic and FISH data.

Around 60% of *PAX5* deletions in infant cases showed mostly intragenic losses restricted to exons 2-6 (n=3) that were also considered as the commonest type of deletions in non-infant subgroup (35%).

	ETV6-RUNX1 subgroups		
	Infants	***Non- infants***	***P-value***
No. of cases	21 (100)	381 (100)	
Deletions			
12p	4/16 (25)	91/292 (31)	0.8
ETV6	7/21 (33)	201/380 (53)	0.08
9p	0/16 (0)	15/292 (5)	1.0
CDKN2A/B	0/21 (0)	93/380 (24)	0.006*
PAX5	5/21 (24)	88/380 (23)	1.0
NR3C2	1/14 (7)	10/51 (20)	0.4
CD200/BTLA	1/14 (7)	7/47 (15)	0.7
6q	1/16 (6)	34/292 (12)	1.0
RB1	1/21 (5)	32/380 (8)	1.0
EBF1	1/21 (5)	13/380 (3)	0.5
BTG1	0/21 (0)	64/380 (17)	0.03
TBL1XR1	0/14 (0)	13/51 (25)	0.05
11q	0/16 (0)	18/292 (6)	0.6
NR3C1	0/14 (0)	3/50 (6)	1.0
BMF	0/14 (0)	2/50 (4)	1.0
IKZF1	0/21 (0)	12/380 (3)	1.0
13q	0/16 (0)	8/292 (3)	1.0
PAR1	0/21 (0)	4/380 (1)	1.0
Gains			
+10	2/16 (11)	7/292 (2)	0.07
+16	1/16 (6)	13/292 (4)	0.5
+21¥	1/21 (5)	58/366 (16)	0.2
+Der(21)t(12;21)¥	0/21 (0)	47/363 (13)	0.09
Near tetraploidy	0/16 (0)	11/292 (4)	1.0

Table 6-3 Genetic comparison between the infant and non-infant cases within *ETV6-RUNX1* cases. * P value of ≤ 0.01 is statistically significant. ¥ Frequency was estimated from the cases with successful cytogenetic or FISH studies.

6.4.2 Adolescents and young adults (AYA) group

6.4.2.1 Cohort Description

A total of 39 AYA cases were investigated; two of them were also Down syndrome cases. The majority of patients (n= 28, 72%) were aged from 10-14 years, whereas the remaining cases were distributed in the age groups of 15-17 (n= 7, 18%) and 21-50 years (n=4, 10%) with a median age at diagnosis of 12 years. These cases were compared with 363 non-AYA cases who were aged from 1-9 years with a median age of 4 years (Table 6-4, Appendix A). There were no differences in the sex distribution between both subgroups. Two thirds of the AYA cases had WCC <10 X 10^9/L with a median of 6 X 10^9/L, while half of the non-AYA cases had WCC >10 X 10^9/L (p=0.002).

	***ETV6-RUNX1* subgroups**		
	AYA	***Non-AYA***	***P-value***
No. of cases	39 (100)	363 (100)	
Sex (M:F)	20:19	199:164	0.7
Ratio	1.05	1.2	
WCC (X 109/L)			
Median	6.2	12.5	
<10	26 (66)	157 (43)	
10-49.9	8 (21)	139 (38)	0.02
≥50	5 (13)	67 (18)	

Table 6-4 Comparison between the AYA and non-AYA cases within *ETV6-RUNX1* cases in terms of sex and WCC. P value of ≤0.01 is statistically significant, WCC white cell count.

6.4.2.2 The genetic comparison between the AYA and non-AYA subgroups

Considering those cases with available data on the eight MLPA genes in AYA and non-AYA groups (n=39, n=362, respectively), both AYA and non-AYA cases exhibited a similar level of genetic diversity (p=0.1). 74% of AYA cases (n=29) exhibited at least one alteration in one of the genes with a total of 60 alterations, averaging 1.5 deletions per case (range: 0-4). Similarly, a total of 282 non- AYA cases (78%) harboured a total of 461 aberrations with a mean of 1.3 alterations per case (range: 0-5). A higher frequency of AYA cases tended to harbour 3 or more deletions compared to non-AYA cases (23% v 9%, respectively, p=0.02). However, a similar proportion of each AYA and non-AYA

subgroups (28% and 29%, respectively) exhibited two alterations (Appendix A, Table 5-7 and Appendix L).

Deletions at 12p were the most frequent cytogenetic abnormalities in both subgroups (24% and 32%) and accordingly *ETV6* losses, detected by MLPA, constituted 41% and 53% in AYA and non-AYA subgroups, respectively (Table 6-5, Appendix A and Appendix L). Interestingly, *BTG1*, *RB1* losses and near tetraploidy (80 or more chromosomes) were enriched in the AYA compared to the non-AYA subgroup (33% v 14%, p=0.002), (21% v 7%, p=0.003) and (15% v 2%, p=0.003), respectively). *BTG1* deletions commonly affected exon 2 with variable extensions to the 3' region in the non-AYA subgroup as compared to the AYA subgroup (92% v 62%, p=0.01). However, AYA cases showed the entire loss of the gene in 23% (n=3) and none of the non-AYA cases exhibited this type of loss (p=0.007). On the other hand, *RB1* losses commonly encompassed the entire gene in 88% of the deleted cases in non-AYA subgroup compared to the AYA subgroup (37%) (p=0.01). *CD200/BTLA* losses occurred in 36% of AYA cases, while they constituted 8% of the non-AYA subgroup (p=0.03). Although 9p deletions occurred in both groups at lower frequencies (9% and 4%), *CDKN2A/B* and *PAX5* were common alterations in both subgroups (AYA and non-AYA subgroups) (28% and 15% v 23% and 24%, respectively).

	ETV6-RUNX1 subgroups		
	AYA	***Non- AYA***	***P-value***
No. of cases	39 (100)	363 (100)	
Deletions			
12p	8/33 (24)	87/275 (32)	0.4
ETV6	16/39 (41)	192/362 (53)	0.2
CD200/BTLA	4/11 (36)	4/50 (8)	0.03
BTG1	13/39 (33)	51/362 (14)	0.002*
9p	3/33 (9)	12/275 (4)	0.2
CDKN2A/B	11/39 (28)	82/362 (23)	0.4
PAX5	6/39 (15)	87/362 (24)	0.2
NR3C2	3/12 (25)	8/53 (15)	0.4
TBL1XR1	3/13 (23)	10/52 (19)	0.7
6q	7/33 (21)	28/275 (10)	0.06
RB1	8/39 (21)	25/362 (7)	0.003*
NR3C1	1/11 (9)	2/53 (4)	0.4
11q	3/33 (9)	15/275 (5)	0.4
BMF	1/12 (8)	1/52 (2)	0.3
IKZF1	3/39 (8)	9/362 (2)	0.1
EBF1	2/39 (5)	12/362 (3)	0.6
PAR1	1/39 (3)	3/362 (1)	0.3
Gains			
near tetraploidy	5/33 (15)	6/275 (2)	0.003*
+Der(21)t(12;21)¥	5/37 (14)	42/350 (12)	0.8
+21¥	4/37 (11)	55/350 (16)	0.6
+10	1/33 (3)	8/275 (3)	1.0
+16	1/33 (3)	13/275 (5)	1.0

Table 6-5 Genetic comparison between the AYA and non-AYA cases within *ETV6-RUNX1* cases.
* P value of ≤ 0.01 is statistically significant. ¥ Frequency was estimated from the cases with successful cytogenetic or FISH studies.

6.4.3 Down syndrome group

6.4.3.1 Cohort Description

A total cohort of 13 DS cases were studied and the majority of them were in the age group of 2-9 years with a median age of 3 years at diagnosis (range: 1-15). They were compared to non-DS cases (n=389) that had a wide range of age (1-50) years with a median age of 6 years (Table 6-6, Appendix A). There were no differences in the distribution of WCC levels and sex between both subgroups. Among DS cases treated on ALL2003 at initial presentation (n=8), there was a single relapse case which occurred > 6 months after the end of treatment.

	***ETV6-RUNX1* subgroups**		
	DS	***Non-DS***	***P-value***
No. of cases	13 (100)	389 (100)	
Age (years)			
Median	3	4	
1	2 (15)	19 (5)	
2-9	9 (69)	333 (86)	0.09
10+	2 (15)	37 (10)	
Sex (M:F)	8:5	211:178	0.8
Ratio	1.6	1.2	
WCC (X 109/L)			
Median	14.9	11.1	
<10	4 (31)	179 (46)	
10-49.9	5 (38)	142 (37)	0.3
≥50	4 (31)	68 (17)	

Table 6-6 Comparison between DS and non-DS cases within *ETV6-RUNX1* cases in terms of sex and WCC. P value of ≤0.01 is statistically significant, WCC white cell count.

6.4.3.2 The genetic comparison between DS and non-DS subgroups

DS cases exhibited slightly fewer number of alterations (n=11), considering the eight MLPA genes, with an average of 0.92 alterations per case (range: 0-2) compared to the non-DS cases that harboured a total of 510 aberrations with a mean of 1.3 alterations per case (range: 0-5) (p=0.04) (Appendix A, Table 5-7 and Appendix L). There was no association of any gene alterations to any of the subgroups (Table 6-7, Appendix A, Table 5-7 and Appendix L). *PAX5* losses were the most common gene deletions in DS cases (42%), while *ETV6* deletions represented the commonest type of alterations in the non-

DS cases (53%). *NR3C1* losses constituted 18% of DS cases, while they represented 8% of the non-DS cases (p=0.07).

	ETV6-RUNX1 subgroups		
	DS	***Non- DS***	***P-value***
No. of cases	13 (100)	389 (100)	
Deletions			
12p	2/9 (22)	93/299 (31)	0.7
ETV6	3/12 (25)	205/389 (53)	0.08
9p	0/9 (0)	15/299 (5)	1.0
PAX5	5/12 (42)	88/389 (23)	0.2
CDKN2A/B	2/12 (17)	91/389 (23)	0.7
NR3C1	2/11 (18)	1/53 (2)	0.07
NR3C2	1/10 (10)	10/55 (18)	1.0
BTG1	1/12 (8)	63/389 (16)	0.7
6q	0/9 (0)	35/299 (12)	0.6
RB1	0/12 (0)	33/389 (8)	0.6
11q	0/9 (0)	18/299 (6)	1.0
EBF1	0/12 (0)	14/389 (4)	1.0
13q	0/9 (0)	8/299 (3)	1.0
IKZF1	0/12 (0)	12/389 (3)	1.0
PAR1	0/12 (0)	4/389 (1)	1.0
TBL1XR1	0/10 (0)	13/55 (24)	0.2
BMF	0/9 (0)	2/55 (4)	1.0
CD200/BTLA	0 /7 (0)	8/54 (15)	0.6
Gains			
+21¥	0/13 (0)	59/374 (16)	0.2
+Der(21)t(12;21)¥	0/13 (0)	47/374 (13)	0.4
+16	0/9 (0)	14/299 (5)	1.0
near tetraploidy	0/9 (0)	11/299 (4)	1.0
+10	0/9 (0)	9/299 (3)	1.0

Table 6-7 Genetic comparison between the DS and non-DS cases within *ETV6-RUNX1* cases. * P value of ≤ 0.01 is statistically significant. ¥ Frequency was estimated from the cases with successful cytogenetic or FISH studies.

6.4.3.3 The genomic profile of Down syndrome ETV6-RUNX1 cases

Among the four DS cases that were successfully investigated by SNP6 arrays (3428, 3472, 3684 and 21820), there were a total of 23 deletions including mainly focal losses (<1Mb) (n=17) and three copy number gains sized >1Mb (Figure 6.1). Overall, all four patients were found to exhibit different genomic profiles with multiple aberrations averaging 6.5 alterations per case. Thus, patient 3428 had 14, patient 3472 had 6, patient 3684 had 4

and patient 21820 had one alteration. The previously identified known CNAs were observed in different proportions in the four cases and these losses targeted the following regions: 3p14.2 (*FHIT,* n=1), 6q (n=1), 9p13.2 (*PAX5,* n=2*)*, 9p21.3 (*CDKN2A/B,* n=1), 11q (n=1), 12p (n=2), 12q21.33 (*BTG1,* n=1) and 20p12.2 (*C20orf94,* n=1) (Figure 6.2). In addition, gain of chromosome Xq was identified in two patients but with slightly different breakpoints. *CDKN2A/B*, *PAX5* and *BTG1* losses, detected initially by MLPA, were confirmed by SNP6 arrays. On the other hand, none of the patients harboured deletions encompassing *NR3C1, NR3C2, TBL1XR1, CD200/BTLA* and *BMF* genes. Interestingly, patient 3428, harboured loss of chromosome 18q (18)(70.00[q22.3]-78.08[q23]). However, patient 3472 showed copy number neutral Loss of heterozygosity [CNN LOH] at chromosome 18 (18)(72.42[q22.3]-74.33[q23]) which clearly overlapped with the former region of loss which contained some important genes involved in transcriptional regulation including *ZNF407*, *TSHZ1* and *ZNF516* (Figure 6.3, Appendix F).

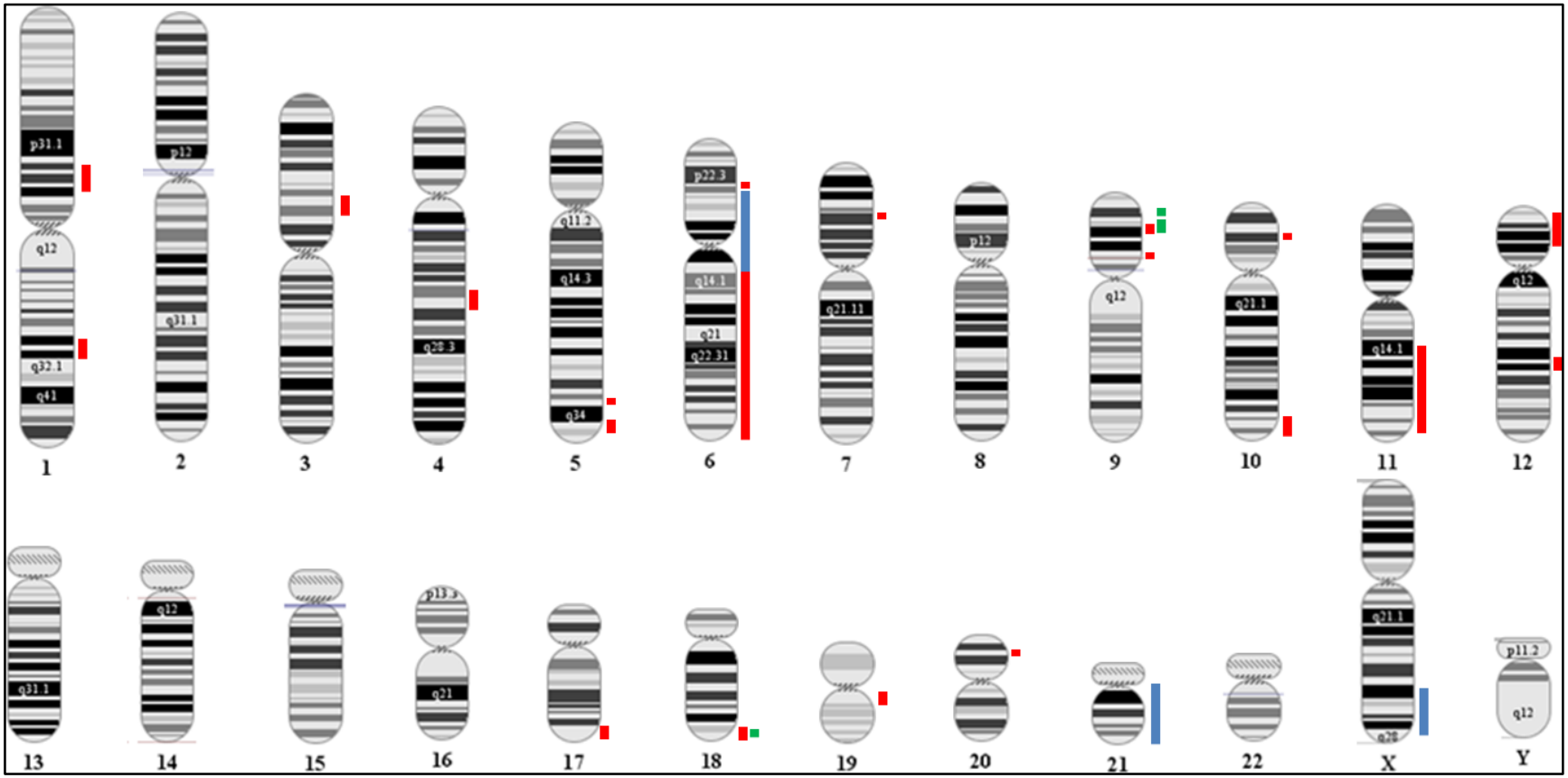

Figure 6.1 Ideogram represent the alterations detected by SNP6 arrays in the four Down syndrome cases. The only recurrent alterations (at least in two patients) included gain of chromosome Xq and deleted 9p targeting *PAX5* gene (occurred in two patients each); Deletions (red lines), gains (blue lines) and CNN LOH (green lines).

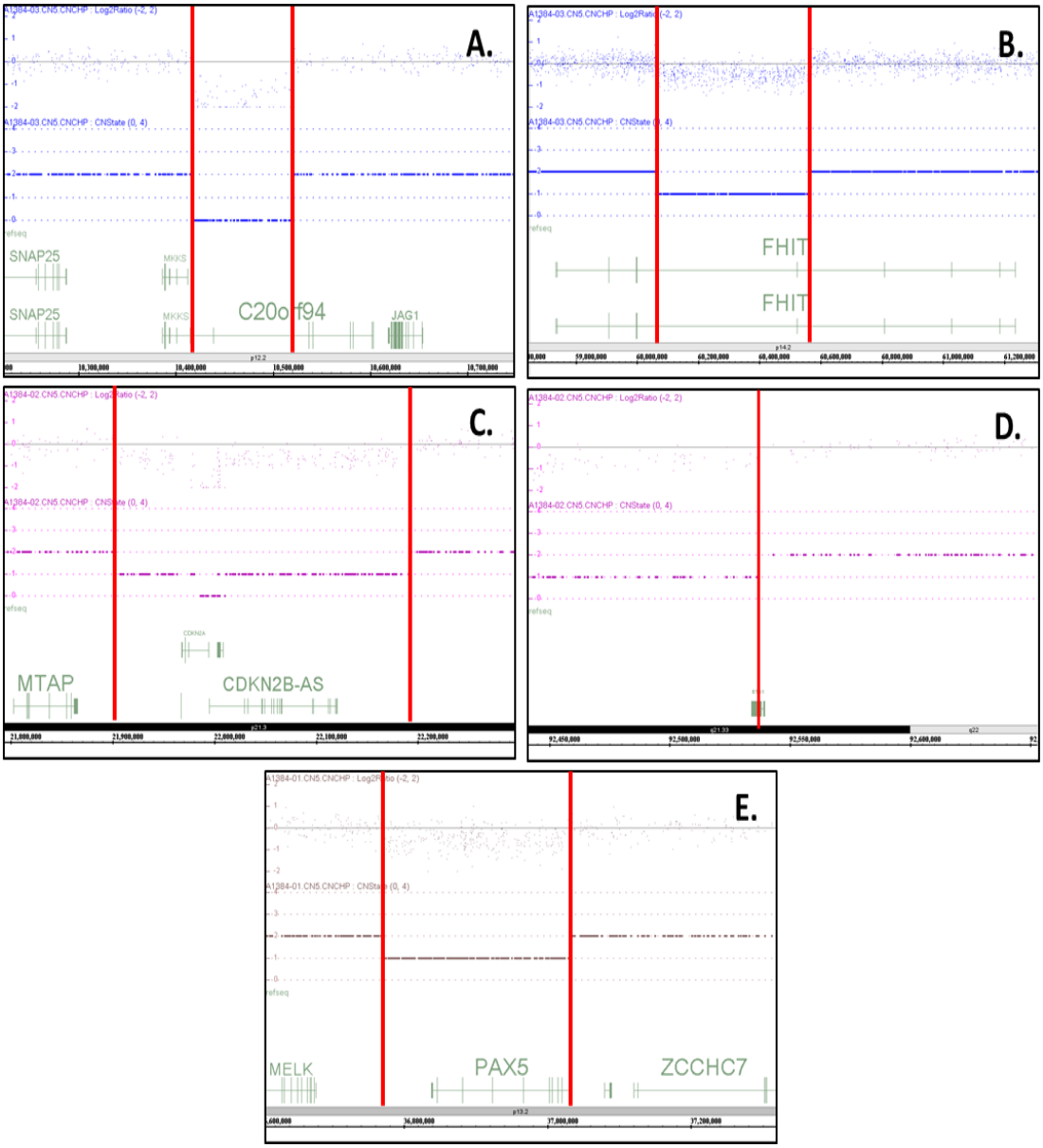

Figure 6.2 Log2 ratio and copy number plots of focal deletions detected by SNP6 arrays in the four Down syndrome cases. Biallelic deletion of C20orf94 (A) and monoallelic deletion of FHIT (B) in patient 3428, Two independent deletions of *CDKN2A/B* [monoallelic and biallelic deletions] (C) and monoallelic deletion of *BTG1* (D) in patient 3472, Monoallelic deletion of *PAX5* in patient 3684 (E). Among these genes, only *PAX5* deletion was identified in two patients 3684 and 21820.

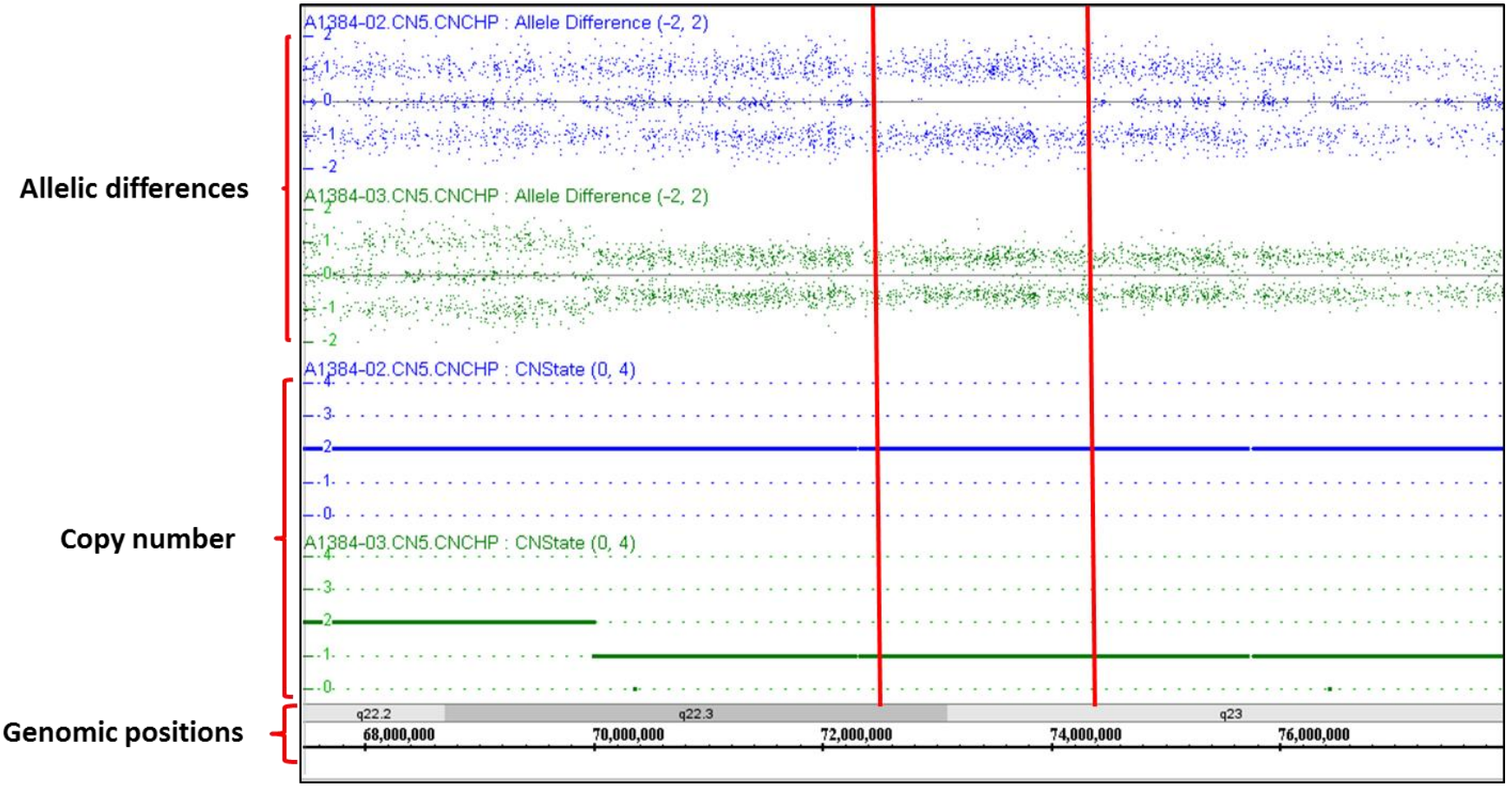

Figure 6.3 SNP 6.0 profile for chromosome 18 from patient no. 3472 (blue) and 3428 (green) showing overlapped loss of heterozygosity at chromosome 18q LOH(18)(72.42[q22.3]-74.33[q23]) either with a copy number neutral or a copy number of 1, respectively (red lines).

6.5 Discussion

ETV6-RUNX1 BCP-ALL has been characterised by a narrow range of demographic and clinical features; however, outlier subgroups do exist including infants, DS and AYA. This study has investigated a total of 21, 13 and 39 cases, respectively, to assess whether or not the spectrum of secondary abnormalities in these patients are unique and distinguish them from *ETV6-RUNX1* as a whole. To the best of my knowledge, this is the first study that has substantially increased our knowledge about these unusual subgroups. Thus, the frequencies of selected gene alterations that are known to be frequent in *ETV6-RUNX1* were estimated by different cytogenetic and molecular techniques in these atypical subgroups and each subgroup was compared with other *ETV6-RUNX1* subgroups.

As Expected, this study showed that the infant subgroup (n=21) was more likely to have high WCC with 80% of cases having a WCC >10 X 10^9/L as compared to half of the non-infant cases (n=381) and this may reflect the aggressiveness of this disease in this subgroup of patients. However, based on selected number of genes incorporated in this study, infant cases displayed fewer alterations as compared to those diagnosed at their second birthday or older (0.67 v 1.3 alterations per case, respectively). Thus, this striking finding might indicate that mutations rather than CNA are the genetic determinants in infant subgroup or other unique CNA, undiscovered by previous genomic studies that were more probably focussed on the main bulk of typical cases, might play a role. In agreement with these findings, a previous genomic study investigated four *ETV6-RUNX1* infant cases aged less than 1 year showed decreased number of CNAs along with increased number of the copy number neutral (CNN) regions compared to older children and those aged 10 years or older with a mean of 2.75 v 5 v 11.7 alterations per case, respectively (Emerenciano *et al.*, 2009). In parallel with these findings, infant *MLL* leukaemia also showed reduced CNAs but increased CNN-LOH regions, however, *MLL* rearrangements are thought to be sufficient by themselves to drive the leukaemogenic processes unlike *ETV6-RUNX1* fusion gene (Bardini *et al.*, 2010; Bardini *et al.*, 2011; Dobbins *et al.*, 2013). Thus, the characteristic stable genome in infants might be attributed to their age group rather than the genetic subtype.

ETV6 and *PAX5* losses were encountered in both infants and non- infants (33% and 24% v 53% and 23%, respectively) indicating that these alterations are necessary genetic lesions that complement with each other for leukaemic transformation and progression (Tijchon *et al.*, 2013).

Furthermore, lack of *CDKN2A/B* alterations and the lower incidence of *RB1* losses (5%) in infants might suggest that cell cycle control pathway is not crucial in their disease progression or other genetic lesions might directly or indirectly affect this pathway. Hence, there is scope for investigating a number of *ETV6-RUNX1* infant cases using whole genome or exome sequencing that enable the discovery of possible gene mutations especially in the CNN loss of heterozygosity (CNN LOH) identified regions as well as uncovering new alterations that might be responsible for the accelerated leukaemic conversion.

Unlike the infant subgroup, the AYA subgroup tended to have low WCC with a median of 6 X 10^9/L as compared to those aged less than 10 years. Nevertheless, they are still assigned to the high risk category owing to their older age. There was no difference in the degree of genetic diversity in AYA subgroup as compared to those aged less than 10 years (1.5 v 1.3 alterations per case) but they differed in the nature and frequencies of alterations. Enrichment of *BTG1* losses in the AYA subgroup (33%, p=0.002) might contribute to relapses in this subgroup of patients owing to the possible drug resistance related to *BTG1* losses. *BTG1* is known to regulate glucocorticoid receptor- dependent transcriptional response, thus its loss would result in glucocorticoid resistance (van Galen *et al.*, 2010). However, another report postulated that *BTG1* loss effect is cellular dependent owing to the pleiotropic treatment responses in cell lines harbouring these losses (Waanders *et al.*, 2012). It is worth noting that the poor outcome encountered in those patients with NCI high risk might be attributed to the increase frequency of *BTG1* losses rather than age *per se*. In addition, *RB1* deletions were frequent in this subgroup (21%) which further underscore the previously reported association between *BTG1* losses and other deletions targeting *RB1*, *EBF1* and *ETV6* that are usually needed for the leukaemia development and they were shown to develop in multiple subclones with different deletion sizes (Waanders *et al.*, 2012). Furthermore, the frequency of 6q deletions was slightly increased among the AYA subgroup compared to the non-AYA (21% v 10%) and it is worth noting that *FOXO3* is a target gene of common 6q deletions

(Bakker *et al.*, 2004). Interestingly, *BTG1* was reported to act as a downstream target of *FOXO3* as identified from DNA microscreens (Bakker *et al.*, 2007), hence disruption of both was suggested to constitute a combined mechanism leading to leukaemia development in ALL. The classic *BTG1* loss commonly affects the second exon with a variable involvement of the 3' region along with the retention of the first exon among *ETV6-RUNX1* cases. Interestingly, 23% of *BTG1* deletions in the AYA group showed entire loss of the gene rather than the typical distinctive pattern in spite of no concomitant occurrence of whole gene loss of *ETV6* that would indicate possible monosomy 12. It is noteworthy that many of the different deletions generate truncated proteins that consist of the open reading frame with consequent loss of two conserved C-terminal protein interaction domains but they are highly unstable with no essential function (Waanders *et al.*, 2012). Hence these losses might act as whole allele deletion with reduced expression and subsequent loss of function.

Furthermore, near tetraploidy was significantly associated with AYA subgroup (15% v 2%, p=0.003) and this aneuploidy is usually a good prognostic factor (Attarbaschi *et al.*, 2006; Raimondi *et al.*, 2006). *CD200/BTLA* alterations seemed to be frequent in AYA subgroup and they might be needed for leukaemic development in this group of patients.

DS cases seemed to have similar demographic and clinical features in terms of age, sex and WCC distribution compared to non-DS cases (n=389). However, this subgroup showed decreased number of alterations with a mean of 0.92 v 1.3 alterations per case in non-DS cases which might be attributed to the selected genes that could be less frequent in this subgroup. In support of this explanation, the genomic profiles of the four cases showed a mean number of deletions of 5.75 losses per case. Another possible explanation might be the fact that DS cases already have gain 21 which may drive the leukaemogenic process (Roy *et al.*, 2012). Although *BTG1* losses were frequent among *ETV6-RUNX1* as a whole, DS seemed to harbour fewer *BTG1* losses (8%) in this study, which disagrees with a previous report that postulated a high frequency among DS ALL (Lundin *et al.*, 2012). This is in agreement with a recent report that did not confirm a high incidence of these losses in a large series of DS ALL (Buitenkamp *et al.*, 2013b).

A previous report evidenced the associated good outcome of *ETV6-RUNX1* DS ALL (Buitenkamp *et al.*, 2013a) as compared to non-*ETV6-RUNX1* DS ALL patients who were

shown to have inferior outcome. The plausible explanation of the differences in outcome might be attributed to the enrichment of J*AK2* mutations and *CRLF2* overexpression with associated *IKZF1* losses (Kearney *et al.*, 2009) in non-*ETV6-RUNX1* DS ALL. None of the *ETV6-RUNX1* DS ALL exhibited these aberrations which might suggest that they are mutually exclusive.

It is worth noting that all four DS cases, analysed with SNP array, showed distinct genomic profiles. The only striking finding was the 18q region alteration that contains important genes including *ZNF407*, *TSHZ1* and *ZNF516,* which are zinc finger proteins playing roles in transcriptional regulations. These genes were found in the overlapping region common to two patients harbouring either monoallelic loss or CNN LOH. This may indicate the possibility of having mutations in the other alleles, consistent with the two-hit hypothesis of TSG inactivation (Knudson, 1971). To the best of my knowledge, there are no previously reported alterations in this region in *ETV6-RUNX1* as a whole which further necessitate investigations to gain further insight in this region in terms of their roles in leukaemia development and their prognostic impact.

The limitation of this study lies in the selection of the techniques that rely on a selected number of genes found to be prevalent in the previous genomic studies in the bulk of *ETV6-RUNX1* cases. Since the aim of the project was to look at the spectrum of secondary abnormalities in atypical patients are unique and informative about that subtype and *ETV6-RUNX1* as a whole, genomic studies might be appropriate to spot these differences. However, the availability of good quality material and the relatively large number of cases to be studied were the main reasons against utilising SNP arrays. The frequencies of *CD200/BTLA*, *TBL1XR1* and *NR3C2* losses tended to be higher in AYA compared to the previously reported incidences among the bulk of *ETV6-RUNX1* cases (36%, 23% and 25% v 13%, 12% and 9%, respectively) (Lilljebjorn *et al.*, 2007; Mullighan *et al.*, 2007; Kawamata *et al.*, 2008; Lilljebjorn *et al.*, 2010). On the other hand, *NR3C1* losses occurred in 18% of DS cases, a proportion that was higher than the previously reported lower incidence (6%) among *ETV6-RUNX1* cases. Hence, further validation of these findings should be carried out by investigating a large number of atypical patients.

In conclusion, the atypical groups showed diversity in terms of the nature and frequencies of CNAs which might suggest different pathogenic processes responsible for

the evolution of leukaemia. When considering the eight MLPA genes, both infants and DS subgroups showed less number of CNAs with a mean of 0.67 and 0.92 aberrations per case as compared to non-infants and non-DS groups which exhibited each an average of 1.3 alterations per case, respectively. However, the AYA subgroup displayed a similar diversity of alterations as compared to those aged less than 10 years (1.5 v 1.3 alterations per case, respectively). *CDKN2A/B* losses were common in older children and AYA subgroups as compared to infants (24% v 0%, p=0.006). In addition, *BTG1, RB1* losses and near tetraploidy are commoner in the AYA group as compared to those under the age of 10 years (33% v 14%, 21% v 7% and 15% v 2%, respectively). On the other hand, there were no genetic aberrations distinguishing DS *ETV6-RUNX1* subgroup from other *ETV6-RUNX1* but they are definitely different from non-*ETV6-RUNX1* DS subgroup sue *CRLF2* rearrangements that are enriched in the latter subgroup. Further collaboration is needed to expand the number of cases to validate the associations with co-operating alterations using whole genome analysis.

Chapter 7. Summary and Discussion

7.1 Overview

B-precursor acute lymphoblastic leukaemia (BCP-ALL) is the most common childhood malignancy, characterised by a wide range of primary chromosomal abnormalities that provide important diagnostic and prognostic information. The chromosomal translocation, t(12;21)(p13;q22), resulting in the *ETV6-RUNX1* fusion gene, is the most common chromosomal abnormality in childhood BCP-ALL (Golub *et al.*, 1995; Romana *et al.*, 1995b; Moorman *et al.*, 2010b). *ETV6-RUNX1* fusion gene is by itself unable to generate clinical leukaemia (Andreasson *et al.*, 2001), indicating the necessity for additional co-operating lesions for leukaemic transformation (Wiemels *et al.*, 1999; Mori *et al.*, 2002; Greaves and Wiemels, 2003; Hong *et al.*, 2008; Ma *et al.*, 2013).

The nature and frequencies of additional genetic alterations differs among different cytogenetic subtypes which might, in part, explain their leukaemic development, clinical phenotype and subsequent impact on outcome. Thus, exploring the distinct genetic landscape of *ETV6-RUNX1* compared to other BCP-ALL subtypes was necessary to gain further understanding of the relevant driving genetic lesions in *ETV6-RUNX1* as shown in Chapter 3.

Considerable research has demonstrated the importance of the *ETV6-RUNX1* fusion gene in the expansion and the maintenance of preleukaemic stem cells, thus allowing vulnerable acquisition of multiple co-operating lesions responsible for full blown leukaemia (Hong *et al.*, 2008). However, not much attention has been paid to the leukaemogenic effects of the product of the reciprocal *RUNX1-ETV6* chimeric gene. Nevertheless, high expression of *RUNX1-ETV6* protein was reported to be an independent adverse factor that more likely arose from cell regrowth rather than drug resistance (Stams *et al.*, 2005). Chapter 4 began as a reflection of the abnormal findings observed in a previous comprehensive FISH screening that aimed to detect and assess the prognostic impact of *ETV6* deletions, gain of chromosome 21 and gain of der(21)t(12;21) (Enshaei *et al.*, 2013). These abnormal findings suggested the possibility of *RUNX1-ETV6* alterations with either their deletions or duplications contributing to the leukaemogenic progression. Thus further confirmation and postulation of the possible

mechanisms arising from *RUNX1-ETV6* chimeric gene alterations were reported in Chapter 4.

ETV6-RUNX1 BCP-ALL is characterised by a good outcome; however, relapses occur at a rate of 13-20% (Moorman *et al.*, 2010b). Previous genomic studies of relapsed BCP-ALL revealed an increase in the number of aberrations, mainly deletions, from diagnosis to relapse (Mullighan *et al.*, 2008b). Thus, Chapter 5 focussed on utilising cytogenetic, FISH and MLPA data that covered a selection of genes that are recurrently deleted in BCP-ALL, including *CDKN2A/B, PAX5*, *ETV6*, *IKZF1* and *BTG1*. Further screening for the presence of other possible relapse related genes targeting *TBL1XR1, BMF, NR3C1/2* and *CD200/BTLA* was carried out in the diagnostic samples of *ETV6-RUNX1* relapses using both FISH and qPCR. These genes are involved in cell cycle regulation, B cell development and glucocorticoid receptor signalling and drug resistance. To assess the potential relevance of these alterations in the relapse cases, a comparison group of non-relapse cases was investigated in a similar manner. To test the hypothesis that all genetic aberrations detected at relapse were clonally related to the diagnostic samples and did not arise as a secondary leukaemia, back-tracking by FISH for all detected genetic lesions from relapse to diagnosis was performed.

It is worth noting that the majority of genomic studies of *ETV6-RUNX1* cases have focussed on typical cases and little is known about the genetic makeup of rare cases including DS, infants and AYA. These cases are commonly affected by other genetic entities involving CRLF2 overexpression/ *JAK2* mutations (Kearney *et al.*, 2009; Mullighan *et al.*, 2009a; Russell *et al.*, 2009), *MLL* rearrangements and Philadelphia chromosome which confer poor outcome (Moorman, 2012). Thus, Chapter 6 aimed to assess whether or not the spectrum of secondary abnormalities in atypical *ETV6-RUNX1* patients were unique in terms of their frequencies and diversity compared to other *ETV6-RUNX1* patients. This was accomplished by utilising all available cytogenetic, FISH and MLPA data with additional investigations including FISH, q PCR and SNP array (where available) covering all important genes.

7.2 What is the overall spectrum of secondary abnormalities in *ETV6-RUNX1* positive cases and is it the same as other subtypes of ALL?

Previous genomic studies have focused on overall frequencies and the common breakpoints of the genetic alterations in *ETV6-RUNX1* cases (Mullighan *et al.*, 2007; Lilljebjorn *et al.*, 2010). However, little is known regarding the distribution and association of these aberrations among different *ETV6-RUNX1* subgroups. In addition, it is important to shed light on the possible driving leukaemogenic factors in *ETV6-RUNX1* positive cases compared to other BCP-ALL subtypes. Thus, the study of the genetic differences may help to understand the mechanism of leukaemic development in both groups.

7.2.1 ETV6-RUNX1 inherently favours a higher degree of genetic diversity

ETV6-RUNX1 cases displayed a higher diversity of alterations, deletions in particular, as compared to other BCP-ALL subtypes evidenced from our data (1.28 v 0.95 alterations per case) as well as other previous genomic studies (6 v 3.1 deletions per case) (Mullighan *et al.*, 2007). Nevertheless, the increase in the number of alterations might be an artefact given the fact that *ETV6-RUNX1* positive cases constitute 25% of the overall BCP-ALL cohort.

Despite this genetic diversity, *ETV6-RUNX1* patients showed a good outcome, indicating possibly a lack of impact of the increased number of aberrations on prognosis, as reported recently (Enshaei *et al.*, 2013). In support of these findings, Chapter 5 showed that there were no differences in the mean number of alterations per case between the relapse and non-relapse cases on analysis of the eight significant genes (2 v 2.25, respectively) (see section 5.4.6). The *ETV6-RUNX1* fusion gene has inherent abilities to favour the expansion of the pre-leukaemic cells, thus increases their susceptibility to acquire further aberrations/ mutations owing to the overwhelming proliferative stress (see section 1.5.4). The *ETV6-RUNX1* fusion gene has a positive impact on the persistence and expansion of the pre-leukaemic cells through its inhibitory effects on *TGF-β* (Siegel and Massague, 2003; Bierie and Moses, 2006), miRNA-494 and miRNA-320a (Diakos *et al.*, 2010), and activating effects on *EPOR* (Inthal *et al.*, 2008; Torrano *et al.*, 2011) and mTOR pathway (Fuka *et al.*, 2011; Fuka *et al.*, 2012; Tijchon *et al.*, 2013).

7.2.2 The characteristic genetic alterations associated with ETV6-RUNX1

This study showed that the common genetic deletions associated with *ETV6-RUNX1* are those targeting *ETV6* (52%), *BTG1* (15%), *CDKN2A/B* (23%) and *PAX5* (23%) consistent with previous studies (Mullighan *et al.*, 2007; Lilljebjorn *et al.*, 2010). The latter two deletions occurred commonly in other BCP-ALL cases suggesting their essential roles in leukaemia development in all groups. However, *BTG1* and *ETV6* losses were enriched in *ETV6-RUNX1*, which suggests that these genetic abnormalities play a collaborative role in the development of this BCP-ALL subtype. Higher frequencies of *TBL1XR1* and *NR3C2* deletions were seen in typical *ETV6-RUNX1* patients in this study, constituting 31% and 19% of cases, respectively, which were more frequent than previously reported in the overall cohort (Lilljebjorn *et al.*, 2010). However, other BCP-ALL cases seemed to harbour fewer deletions targeting *TBL1XR1* and *NR3C2* genes (0.69% each). Thus, disruption of both pathways affecting B- cell development and nuclear hormone response constitutes the main components of pathogenesis in *ETV6-RUNX1*, each occurring in around one quarter (Lilljebjorn *et al.*, 2010). On the other hand, *ETV6-RUNX1* cases exhibited less frequently *IKZF1* and *PAR1* alterations; while these aberrations were associated with other BCP-ALL subgroups. This might explain the favourable outcome seen in *ETV6-RUNX1* as *IKZF1* losses are associated with a poor outcome (Mullighan *et al.*, 2009b). It is worth noting that the rarity of *IKZF1* losses in this ALL group might be explained by their tendency to occur at an older age (>10 years) which constituted just 9% of *ETV6-RUNX1* cases.

7.2.3 Aberrant RAG activity drives the most frequent focal deletions in ETV6-RUNX1 positive cases

It is worth noting that *ETV6*, *BTG1*, *TBL1XR1*, *IKZF1* and *CDKN2A/B* deletions are generated by aberrant RAG recombinase activity (Mullighan and Downing, 2009b; Kuiper and Waanders, 2014; Papaemmanuil *et al.*, 2014). This aberrant mechanism represents a driving mechanism in *ETV6-RUNX1*, in particular (see section 1.4.1.3). The RAG activity can be evidenced by the presence of the recombination signal sequences (RSSs) (which bind the RAG complex) and RSS- like motifs (small but highly conserved segments) which were determined in 12% and 40% of the alterations in *ETV6-RUNX1* cases, respectively (Kuiper and Waanders, 2014; Papaemmanuil *et al.*, 2014). In addition,

the non-templated sequences were one of the features of a RAG mediated mechanism presenting at 70% of the breakpoints in *ETV6-RUNX1* and resulted from terminal deoxynucleotidyl transferase (TdT) activity. Furthermore, these RAG mediated rearrangements are enriched in active promoters/ enhancers especially those with RSS-like motifs and are repetitive in space/ time (Kuiper and Waanders, 2014; Papaemmanuil *et al.*, 2014).

7.2.4 The proposed functional effect of ETV6 losses and gain of der(12)t(12;21)

ETV6 plays an important role as a transcriptional repressor involved in haematopoiesis and angiogenesis (see section 1.5.3). It has been postulated that *ETV6* heterodimerizes with the *ETV6-RUNX1* fusion gene, resulting in reduction of its potency (McLean *et al.*, 1996; Morrow *et al.*, 2007; Lilljebjorn *et al.*, 2010; Tijchon *et al.*, 2013). Thus the fusion gene would drive leukaemogenesis as a result of *ETV6* loss. Accordingly, the duplication of der(21)t(12;21) would increase the ratio of ETV6-RUNX1 protein over ETV6 protein, resulting in further aggravation of the disease. It is worth noting that ETV6 showed lower transcript expression in *ETV6-RUNX1* positive cases irrespective of the deletion status of *ETV6*. Thus *ETV6* may also be silenced by inactivating point mutations or hypermethylation (Stams *et al.*, 2005). If so, these alterations would likely have a negative effect on outcome, based on their pathogenic sequelae. However, there is no evidence to suggest a prognostic effect possibly due to effective modern treatment (see section 1.5.7) (Attarbaschi *et al.*, 2004; Stams *et al.*, 2006; Peter *et al.*, 2009; Ko *et al.*, 2011; Barbany *et al.*, 2012; Enshaei *et al.*, 2013).

7.2.5 Possible contribution of BTG1 losses on the outcome of NCI high risk patients

The *BTG1* gene plays an integral role in differentiation, proliferation, cell survival and glucocorticoid responsiveness. In a previous study, *BTG1* losses occurred among *ETV6-RUNX1* patients and were particulary enriched in older patients (> 10 years old) (33% v 11%). Thus, the observed poor outcome of NCI high risk patients with *ETV6-RUNX1* (OS of 85% and 86% versus 97% and 97% in ALL97 and ALL99, respectively) (Enshaei *et al.*, 2013) may be driven by the higher incidences of *BTG1* losses rather than age *per se*. In agreement with these findings, *BTG1* losses were reported to be significantly higher among relapse patients with BCP-ALL (Hogan *et al.*, 2011) which might be attributed to

poor therapy respone related to the glucocorticoid resistance. *BTG1* gene promotes glucocorticoid induced apoptosis through the regulation of the glucortiocoid receptor expression, thus *BTG1* loss renders leukaemic cells refractory to glucorticoid treatment (van Galen *et al.*, 2010).

7.2.6 PAX5 deletions have different breakpoints in ETV6-RUNX1 and other BCP-ALL

Although *PAX5* gene deletions were common across cytogenetic subgroups including *ETV6-RUNX1* (see Figure 3.1), the architecture of the deletion breakpoints differed between *ETV6-RUNX1* cases and other BCP-ALL subtypes. *PAX5* is an integral component in the initiation of early B-cell development, known as the guardian of B-cell identity and regulator of terminal B-cell differentiation (Familiades *et al.*, 2009; Tijchon *et al.*, 2013). The resultant expression of these deletions is dependent on the extent of exons involved (Mullighan *et al.*, 2007; Familiades *et al.*, 2009). The predominant intragenic *PAX5* deletions in this study in *ETV6-RUNX1* positive patients retained exon 1 and had a variable pattern of exon deletion (60%). It has been reported previously that deletions retaining exon 1 resulted in the generation of mutant alleles with altered transcriptional activity owing to the disruption of either the DNA- binding domain or the transactivation domain (Mullighan *et al.*, 2007; Familiades *et al.*, 2009).

On the other hand, other BCP-ALL cases exhibited *PAX5* whole gene loss (44%), resulting in reduced expression of the normal PAX5 protein. Hence, their negative impact would occur in the later stages of differentiation, when both *PAX5* alleles are required. Furthermore, *PAX5* amplification of exons 2 or 5 has not yet been reported among *ETV6-RUNX1* patients, although it represents 1% of other BCP-ALL cases, resulting in a mutant allele. *PAX5* alterations do not generally have an impact on outcome in BCP-ALL patients and in *ETV6-RUNX1* positive cases in particular (Mullighan and Downing, 2009a; Enshaei *et al.*, 2013). However, patients with *PAX5* mutant alleles tended to have a higher estimated cumulative relapse rate at 3 years as compared to those with deleted alleles (48% v 22%) (Familiades *et al.*, 2009). Taking into consideration that the mutant allele is frequent in *ETV6-RUNX1* positive cases, there was no observed increase in the number of relapses as compared to other BCP-ALL in the present study. Thus, the prognostic impact of the two types of deletions needs to be validated in another trial group.

7.2.7 Lack of the classic IKZF1 exons 4-7 deletions among ETV6-RUNX1 positive cases

The rarity of *IKZF1* losses and the lack of the classic exons 4-7 deletions among *ETV6-RUNX1* patients in the present study may explain the disparity in the prognostic value of *IKZF1* deletions compared to other BCP-ALL. Although the common deletions found in both groups are more likely to have similar effects as the whole allele deletion, none of the *IKZF1* deleted *ETV6-RUNX1* cases relapsed. Thus, deletions targeting exons 4-7 with the formation of non-DNA binding isoform (Ik6) (Iacobucci *et al.*, 2008; Iacobucci *et al.*, 2009) may have additional significance in the emergence of relapse compared to other abnormal variants of IKZF1.

In conclusion, data from this thesis supports the hypothesis that *ETV6-RUNX1* represents a distinct group that differs in terms of the nature of genetic abnormalities from other BCP-ALL groups. These differences may explain the different biological and genetic features and clinical responses of both groups.

7.3 Methodologies for detecting secondary abnormalities

The identification of secondary abnormalities in *ETV6-RUNX1* is crucial to understand the mechanism of leukaemic transformation and progression of the disease. These abnormalities may be present in low proportions of cells at initial presentation but can emerge as major clones at relapse, indicating their possible requirement in disease recurrence. Thus, it is important to employ techniques with the highest detection rate. In addition, it is necessary to utilize different techniques in order to resolve the discrepant results which may occur, for instance, in estimating the frequencies of gain of chromosome 21, gain of der(12)t(12;21) and *ETV6* deletions. Furthermore, the high cost of SNP arrays and the inadequacy of suitable material necessitates the development of other screening techniques to detect common aberrations with important biological roles.

7.3.1 Cytogenetics versus FISH

Gain of chromosome 21 and gain of der(21)t(12;21) are common abnormalities in *ETV6-RUNX1* positive cases and their effects on prognosis are debatable. These alterations

may be detected cytogenetically; however, the apparently visible gain of chromosome 21 may be mistaken for gain of der(21)t(12;21). Cytogenetic analysis is an important component of diagnosis and risk stratification of ALL, nevertheless, the characteristic low mitotic index and poor chromosome morphology of *ETV6-RUNX1* positive cases, as evidenced from the higher failure rate of the cytogenetic studies (23%), negatively affect the comprehensive assessment of these chromosomal abnormalities by cytogenetic analyses. Thus, FISH studies were warranted owing to their specificity and sensitivity in distinguishing between these alterations (see section 3.4.2), which differ in biological functions and possibly outcome. Gain of der(21)t(12;21) might be more pathogenic resulting from the presence of additional *ETV6-RUNX1* fusion genes. Stams postulated an associated worse event free survival rate for the gain of der(21)t(12;21) as compared to those with other additional abnormalities involving *ETV6* or *RUNX1* genes (4 years EFS of 60% v 79%) (Stams *et al.*, 2006), consistent with a previous study (Martineau *et al.*, 2005). FISH is a molecular cytogenetic technique that is easy to use at a higher resolution compared to the classic cytogenetic banding techniques (see section 2.8). It allows the identification of the chromosomal aberrations irrespective of the cell cycle stage, and thus it overcomes the issue of low mitotic index and poor morphology.

7.3.2 FISH versus MLPA

ETV6 deletions are considered the most prevalent alteration in *ETV6-RUNX1* positive cases and their frequencies in this study were estimated by MLPA and FISH. However, the discrepancy in the frequency of *ETV6* deletions between FISH and MLPA data drew our attention to the necessity for the application of both techniques in order to refine the estimated frequency. Although MLPA is able to detect smaller deletions below FISH resolution, larger deletions, detectable by FISH, can be missed if only present in a low proportion (below MLPA sensitivity) (see section 3.4.3.3F.). However, the correlation between MLPA and FISH data pertaining to *ETV6* losses, in particular, is a contributing factor in considering the need for a clear algorithm for "calling" deletions using MLPA. The MLPA data presented in this project was based on the requirement of the loss of at least two neighbouring probes within a gene for the deletion to be deemed valid, and thus not considering the isolated *ETV6* exon 8 losses as real deletions. Accordingly, the incidence of existing secondary abnormalities, including the deletion of either the whole

ETV6 gene or *3'ETV6* sequences of der(12)t(12;21) that result in an isolated *ETV6* exon 8 deletion can be underestimated (see section 4.4.3.1).

7.3.3 SNP arrays versus other methods (MLPA, FISH, qPCR)

A considerable number of genomic studies of *ETV6-RUNX1* positive BCP-ALL have identified certain prevalent alterations that may be screened by other techniques, including FISH, MLPA and qPCR. The main reasons for advocating these screening techniques instead of SNP arrays include the difficulty in obtaining high quality DNA, matched normal reference samples, optimal normalization of the raw microarray data, and the computational algorithm (Mullighan, 2011). In addition, SNP arrays are unable to detect aberrations occurring in low proportions of cells (25% or less of the total cells is below SNP resolution). Nevertheless, the characteristic variable deletion breakpoints in some genes, including *TBL1XR1, PAX5* and *IKZF1*, necessitate the need for good coverage across the whole gene that can be ideally provided by SNP arrays. MLPA kits provide a high intensity of probes for *IKZF1* and *PAX5,* although *TBL1XR1* does not have any predesigned kits for detection of the deletion. Hence, copy number qPCR techniques were chosen in both Chapters 5 and 6 to screen for *TBL1XR1* and other gene losses. However, these techniques require large amounts of DNA owing to the need to run four replicates of the same reaction for each sample and target probe, using two different reference probes in separate reactions. At least one or two probes were chosen for each target, which in turn increases the likelihood of detecting deletions with different breakpoints. In addition, the application of FISH probes was useful in confirming or uncovering further losses.

7.3.4 Detection of sequence mutations

In one study, whole genome sequencing of *ETV6-RUNX1* positive cases revealed a low mutational burden compared to CNA (Papaemmanuil *et al.*, 2014). Activating mutations affecting genes involved in the Ras signalling pathway (*NRAS, KRAS* and *SAE1*) were considered significantly recurrent in *ETV6-RUNX1* positive BCP-ALL. To the best of my knowledge, it remains unknown whether these mutations constitute a distinct subgroup of clinical importance; however, mutational studies of these genes in a large cohort of *ETV6-RUNX1* cases, or relapse cases in particular, might be an appropriate approach.

Furthermore, the lower frequency of CNA in infants with *ETV6-RUNX1* suggests that the application of exome sequencing in a large series of infants would possibly identify aggressive mutations responsible for the accelerated course of this disease.

In conclusion, the application of screening methods including FISH, MLPA and qPCR techniques have uncovered the most common abnormalities in *ETV6-RUNX1* positive BCP-ALL. The combination of these techniques improves the accuracy of estimating the frequencies of these alterations.

7.4 Does *RUNX1-ETV6* play a role in the development of *ETV6-RUNX1* ALL?

7.4.1 Abnormalities of RUNX1/ETV6 fusion gene

Chapter 4 addressed novel abnormalities affecting the reciprocal products of the translocation t(12;21), specifically the *RUNX1-ETV6* fusion gene, located on the der(12)t(12;21), which might contribute to leukaemogenic progression during the course of the disease.

The reciprocal products of the translocation t(12;21)(p13;q22), and the *RUNX1-ETV6* fusion gene in particular, may undergo different rearrangements resulting from possible illegitimate recombination events, owing to the enrichment of Alu repeats flanking the translocation breakpoint. Thus, these multiple recombination events may be responsible for either the deletion of der(12)t(12;21) at either side of the breakpoint (see section 4.4.1), or the generation of the complex der(12) with a duplicated *RUNX1-ETV6* chimeric fusion gene (see section 4.4.2).

7.4.2 Pathogenesis of deleted der(12)t(12;21)

The der(12)t(12;21) deletions most likely occurred *in utero* at the same time as the translocation, indicated by their presence in all *ETV6-RUNX1* positive cells and the consistent deletion boundaries of the translocation breakpoint. It is worth noting that deletions of the *3'ETV6* sequences were larger and more common than *5'RUNX1* losses of der(12)t(12;21) and encompassed different potential tumour suppressor genes. Despite the prenatal origin of these deletions, no accelerated leukaemic transformation occurred, indicating their inability to drive leukaemogenesis directly. However, bearing in mind the heterogeneous nature of the deletion breakpoints, one can expect a variable

phenotypic effect because of the lack of selective pressure. Thus, loss of the tumour suppressor genes may variably influence acquisition of additional abnormalities based on the extent of the deletions and the affected genes. Both *LRP6* and *BCL2L14* genes, found in the minimum region of deletion, are involved in different cellular activities, including: cell differentiation, proliferation and apoptosis, and their losses are also encountered in different types of cancer.

On the other hand, the lack of *RUNX1-ETV6* fusion gene formation is considered a shared characteristic of these deletions. This effect might be a contributing factor in disease progression because the *ETV6* ETS domain will be lost in cases with *3'ETV6* sequence deletions. It has been previously hypothesized that this might possibly be equivalent to the consequence of the deleted non-rearranged *ETV6* (Stams *et al.*, 2005). Although the *RUNX1-ETV6* fusion gene was lacking in cases with *5'RUNX1* sequence losses, the ETS domain remained intact, possibly resulting in a reduced leukaemogenic effect than in cases with *3'ETV6* sequence loss. Thus, it would be interesting to firstly elucidate the leukaemogenic potential of the RUNX1-ETV6 chimeric protein with and without ETV6-RUNX1 protein by retroviral transduction of either both fusion genes or *RUNX1-ETV6* alone, respectively, in a haematopoietic cell line. Secondly, the evaluation of the consequences of both *3'ETV6* and *5'RUNX1* sequence deletions at der(12)t(12;21) could be compared by knocking down the ETS domain.

7.4.3 Previously reported similar deletions in ETV6-RUNX1

To the best of my knowledge, no previous studies have identified these novel alterations as the probes localized specifically to der(12)t(12;21) are not in routine use. Another possibility is that they may be mistaken for deletions of the non-rearranged *ETV6* allele detected by molecular and genomic studies. However, *5'RUNX1* sequence losses have previously been referred to as the loss of the small extra red signal when using the commercial TEL-AML ES probe (Jabber Al-Obaidi *et al.*, 2002; Martineau *et al.*, 2005; Rothman *et al.*, 2005; Konn *et al.*, 2010). The latter finding was shown to occur in subclones rather than in major clones, suggesting they are different alterations or that the resolution of FISH for the detection of the small *RUNX1* FISH signal on der(12)t(12;21) is too low.

7.4.4 Previously reported related deletions in other BCP-ALL subtypes

Other primary chromosomal abnormalities, particularly t(9;22)(q34;q11.2); inv(16)(p13.1;q22), t(8;21)(q22;q22) and the *MLL* gene rearrangements (Rothman *et al.*, 2005), have been reported to be accompanied by deletions affecting the reciprocal products. The biological effects and impact on the outcome of these deletions is not yet known, apart from der(9)t(9;22) deletions which have been shown to lack prognostic value in the imatinib era but not in the pre-imatinib era (Castagnetti *et al.*, 2010; Huh *et al.*, 2011). It is worth noting that all der(12)t(12;21) deleted patients had a favourable outcome up to the time of thesis submission. However, the small sample size warrants further evaluation of these deletions, which can be accomplished by prospective screening, in order to validate their prognostic value. In addition, it has been postulated that variant rearrangements of these chromosomal abnormalities are more prone to such deletions, as seen in der(9)t(9;22) losses (Reid *et al.*, 2003). In agreement with this notion, our findings have shown that 17% of patients with deletions at der(12)t(12;21) had variant t(12;21) involving an additional chromosome.

7.4.5 Pathogenesis of the complex der(12) [der(12)(21qter →21q22.12::12p13.2-12p12.3::12p12.3→12qter)]

High expression of the RUNX1-ETV6 fusion protein may arise from duplication of the *RUNX1-ETV6* fusion gene on the complex der(12) [der(12)(21qter →21q22.12::12p13.2-12p12.3::12p12.3→12qter)] (see section 4.4.2). The duplicated *RUNX1-ETV6* fusion gene indicates further progression of the disease as it occurred in only a proportion of *ETV6-RUNX1* positive cells, as a result of possible illegitimate mitotic recombination between the normal chromosome 12 and the der(12)t(12;21). Several possible driving genetic events resulted from this complex rearrangement, including loss of the non-rearranged *ETV6* allele with the contiguous telomeric sequences, duplication of the reciprocal *RUNX1-ETV6* fusion gene, and the CNN-LOH of a region on the short arm of chromosome 12. *ETV6* losses are well recognised alterations in *ETV6-RUNX1* positive cases and have a negative impact on the transcriptional activity required for B-cell development. Other genes were affected in both regions of the deleted telomeric 12p and CNN-LOH region of centromeric 12p, but it is difficult to identify the target genes. Interestingly, however, *LRP6*, *BCL2L14, DUSP16, CREBL2 and CDKN1B* genes, implicated

in der(12)t(12;21) losses, were encompassed within the CNN-LOH region. As there are no reported mutations in these genes from whole exome analyses (Lilljebjorn *et al.*, 2012; Papaemmanuil *et al.*, 2014), haploinsufficiency may be responsible for driving leukaemogenesis owing to their possible roles as tumour suppressor genes (see section 4.5). A syngeneic murine model would be a good approach to assess alternately the leukaemogenic effect of haploinsufficiency of these losses by monoallelic conditional inactivation of each gene.

Furthermore, the additional *RUNX1-ETV6* fusion genes suggested the presence of an extra ETS domain that may compete with the intact non-rearranged *ETV6*, owing to their similar binding sites that might result in disruption of the ETV6 and RUNX1 transcriptional process. It is worth noting that the non-rearranged *ETV6* was deleted in all cases with a complex der(12), hence the ETS domain is likely to be an essential player in leukaemogenic progression. In agreement with this hypothesis, high expression of RUNX1-ETV6 proteins was postulated to confer a poor outcome (Stams *et al.*, 2005), which was attributed to cell regrowth rather than drug toxicity related pathways. However, all patients with this alteration experienced a good outcome up to the point of thesis submission. This discrepant clinical outcome may be due to optimal treatment rather than coming from distinct subgroups. Nevertheless, it would be interesting to measure the expression levels of RUNX1-ETV6 proteins in either patient samples or cell lines harbouring a complex der(12) in order to demonstrate the presence of high expression. Prospective screening for this alteration would be an ideal approach to increase the number of cases for comprehensive evaluation of the prognostic significance.

7.4.6 Importance of the reciprocal products of different translocations in BCP-ALL

As most reciprocal products are not consistently expressed with the primary products in different types of cancer, they are not considered as vital for leukaemic transformation (Rego and Pandolfi, 2002). Thus, the *RUNX1-ETV6* fusion gene might play an essential role in leukaemogenesis owing to the consistent expression of both products in 76% of *ETV6-RUNX1* cases (Stams *et al.*, 2005), similar to translocations t(9;22) and t(15;17) (Sanders *et al.*, 2011). The important roles of the reciprocal products in leukaemogenic development have been postulated in the latter two chromosomal abnormalities, while

the importance of the reciprocal products of t(4;11) is still debatable. It was suggested that AF4-MLL is an integral element that is expressed in around 80% of t(4;11) cases (Kowarz *et al.*, 2007) and contributes to leukaemogenic progression (Bursen *et al.*, 2004; Bursen *et al.*, 2010; Wilkinson *et al.*, 2013). However, another study revealed the lack of influence of this reciprocal fusion gene on the leukaemogenic process (Kumar *et al.*, 2011). These contradictory results might be attributed to the different methods used; the latter study (Kumar *et al.*, 2011) utilized AF4-MLL siRNA for the knockdown of the AF4-MLL protein, while a more recent study has confirmed the inefficient knockdown of this protein by AF4-MLL siRNA (Wilkinson *et al.*, 2013).

To sum up, the *RUNX1-ETV6* fusion gene might be of importance in the development of *ETV6-RUNX1* BCP-ALL because of the presence of the ETS domain, which may be responsible for the disruption of transcriptional activity. In addition, alterations targeting this fusion gene may contribute to further progression of the disease. Thus, acknowledging the importance of the *ETV6-RUNX1* fusion gene in initiation of the disease and the possibility of its interaction with the RUNX1-ETV6 fusion gene highlights the therapeutic potential of inhibiting these fusion genes.

7.5 What are the mechanisms of relapse in *ETV6-RUNX1* ALL?

Relapse BCP-ALL is considered heterogeneous in terms of its biological and clinical features. The clinical response to various modalities of treatment varies with different cytogenetic subtypes. The *ETV6-RUNX1* subtype is one of the good risk cytogenetic subgroups that is characterized by favourable prognosis, although some relapses do occur (13%) (Moorman *et al.*, 2010b; Enshaei *et al.*, 2013). Two thirds of relapses occurred late in contrast to other BCP-ALL subtypes that were enriched with early relapses (64%), suggesting that *ETV6-RUNX1* relapses might be attributed to alternate mechanisms of leukaemia development, rather than drug resistance. Considerable genomic studies on newly diagnosed *ETV6-RUNX1* positive cases have revealed additional genetic aberrations that are responsible for leukaemic transformation. These alterations target multiple genes involved in B-lymphocyte development/ differentiation, cell cycle regulation, transcriptional repression and glucocorticoid receptor signalling/ drug resistance (Mullighan et al., 2007; Kawamata et al., 2008; Lilljebjorn et al., 2010).

7.5.1 What are the key genetic drivers?

This project revealed the frequent occurrence of *CDKN2A/B* losses in the diagnostic samples of the relapse cases (44%) in agreement with previous studies (Maloney *et al.*, 1999; Graf Einsiedel *et al.*, 2002; Irving *et al.*, 2005a; Mullighan *et al.*, 2008b; Yang *et al.*, 2008). Similar frequencies were seen between relapse and non-relapse cases in other studies in BCP-ALL (Hogan et al., 2011) and *ETV6-RUNX1* positive BCP-ALL (Kuster et al., 2011). *CDKN2A/B* is an important element in cell cycle regulation and acts as a tumour suppressor gene. It is worth noting that at least three alternatively spliced variants encoding different proteins exist, two of them act as inhibitors of CDK4 and CDK6 kinases. These kinases normally bind to cyclin D, resulting in the formation of the protein complex that phosphorylates the RB protein. *RB1* acts as a transcriptional repressor of *E2F1* target genes and interaction of both *RB1* and *E2F1* assist in cell cycle regulation. Thus, the resultant phosphorylated and inactive form of RB1 will be unable to interact and repress the transcription activity of E2F1 proteins, yielding an increase in cell proliferation (Sherr, 2001; Naqsh e Zahra *et al.*, 2013; Studniak *et al.*, 2013). The remaining isoform variant (p14-ARF) is structurally different and acts through MDM2 inhibition resulting in activation of the *TP53/RB* pathway (Sherr, 2001). *MDM2* is an important negative regulator of the *TP53/RB* pathway. Thus, loss of CDKN2A function would impact negatively on the cell cycle control pathway, yielding further proliferation and survival of cells. Thus, the plausible explanation of the higher frequency of *CDKN2A/B* alterations in *ETV6-RUNX1* positive relapse cases may be attributed to their essential role in leukaemic transformation of the relapse clone rather than drug resistance, which is in agreement with enrichment of late relapses observed in this study. Interestingly, *ETV6-RUNX1* was found to induce MDM2 overexpression through binding to its promoter yielding subsequent inactivation of the TP53 pathway (Kaindl *et al.*, 2014). As alluded to above, the *TP53* pathway may be a potential target for targeted therapy which would aim to inhibit those proteins responsible for TP53 pathway inactivation, including MDM2 overexpression. The *in vitro* exposure of Nutlin-3 was found to act as an analogue of P53 and resulted in reduction of MDM2 expression with subsequent P53 pathway activation (Kaindl *et al.*, 2014).

Furthermore, this study showed that *BTG1* alterations were seen in 22% of *ETV6-RUNX1* positive relapse cases compared to 13% of the non-relapse cases (p=0.7), consistent

with a previous study (Kuster et al., 2011). However, *BTG1* alterations are usually linked to drug resistance with the emergence of early relapses resulting from their negative impact on glucocorticoid sensitivity (van Galen et al., 2010). It is worth noting that all *BTG1* deleted *ETV6-RUNX1* positive relapses occurred late in this study and thus were unlikely to be driven by drug resistance related aberrations.

TBL1XR1 deletions occurred in almost similar proportions in *ETV6-RUNX1* positive relapse and non-relapse cases, in disagreement with a previous study that postulated their possible association with relapse (Parker et al., 2008). *TBL1XR1* acts as a transcriptional regulator that binds to the co-repressors of nuclear hormone receptors involved in regulation of reproduction, differentiation and homeostasis. *TBL1XR1* deletions are characterised by highly variable breakpoints that may have prognostic significance (Parker et al., 2008). The relapse cases in this study showed intragenic deletions, while non-relapse cases had deletions affecting the 5' region of the gene; despite this, the possible involvement of exon 1 was not ruled out. In agreement with these findings, previous genomic studies of *ETV6-RUNX1* positive relapse cases showed intragenic involvement of *TBL1XR1* rather than the 5' region (22% v 11%) (Kuster et al., 2011). It is worth noting that the common region of deletion of the 5' region was reported to contain LOC339845 (Parker et al., 2008), which shares similar features to ASS, a gene that has been reported to be involved in chronic myeloid leukaemia (Bacher *et al.*, 2005). Interestingly, a similar reduction in TBL1XR1 expression was seen with both deletion types. Thus the biological impact of TBL1XR1 would be expected to be similar in all deleted cases, resulting in disruption of the capacity of SMRT/N-CoR complex to bind receptor molecules, yielding inappropriate control of gene expression through the RAR and THR signalling pathways (Parker et al., 2008). In addition, disruption of other pathways, including NF-Kβ (Perissi *et al.*, 2004) and Wnt- β-catenin mediated transcription (Li and Wang, 2008), has been postulated to be affected. Further evaluation of the prognostic impact of different *TBL1XR1* deletions is recommended in a larger series of *ETV6-RUNX1* positive patients using FISH and qPCR, to allow further evaluation of the functional consequences of the different deletions and determine whether the generation of truncated proteins may play a role in the progression of disease.

This study showed that there are no specific genetic lesions responsible for relapse emergence, in agreement with several studies conducted on matched diagnosis-relapse pairs in ALL (Staal *et al.*, 2003; Beesley *et al.*, 2005; Bhojwani *et al.*, 2006; Mullighan *et al.*, 2008b; Staal *et al.*, 2010). Nevertheless, these studies have highlighted the possible involvement of several pathways in nearly half of their cohort including: cell cycle regulation, B-cell signalling and proliferation.

7.5.2 Possible mechanisms behind reduction in relapse rate

On contemporary protocols, the relapse rate of *ETV6-RUNX1* positive patients has declined, as seen, for instance, in ALL97/99 and UKALL2003 (13% v 4%), owing to the administration of higher doses of asparaginase, vincristine and dexamethasone. *In vitro* drug sensitivity studies have revealed that *ETV6-RUNX1* positive leukaemic cells are more sensitive to these anti-cancer agents, although the mechanism behind this sensitivity is not yet clear (Ramakers-van Woerden et al., 2000; Krishna Narla et al., 2001). However, it has been postulated that the expression of apoptosis-regulating genes, which aid apoptosis-induced chemotherapy, may have a role. Interestingly, *in vitro* studies of *ETV6-RUNX1* positive leukaemic cells indicated high expression of Fas proteins along with low expression of BCL2, which possibly renders these leukaemic cells more sensitive to the apoptosis inducing effects of the specific drugs (Krishna Narla et al., 2001). Fas proteins act as pro-apoptotic factors that belong to the tumour necrosis factor receptor family, while BCL2 proteins have anti-apoptotic functions.

Furthermore, *ETV6-RUNX1* positive lymphoblasts have the ability to accumulate lower amounts of methotrexate polyglutamates (Whitehead et al., 2001), thus *ETV6-RUNX1* positive patients have benefited from the administration of higher doses of methotrexate. Taken together, the inter-individual variability of the clinical response to treatment might be explained by the pharmacokinetic and pharmacogenomics features of the leukaemic cells in any one patient sample.

7.5.3 Possible targeted therapy to avoid toxicity-related chemotherapy

Given the fact that intensive therapy, comprising higher doses of asparaginase, vincristine and dexamethasone, has potential toxic effects (see section 1.3.6), targeted therapy should be considered. It seems that the inherent characteristic of *ETV6-RUNX1*

to favour proliferation and survival of the pre-leukaemic cells may in part constitute the main mechanism of relapse emergence. Several experiments have demonstrated the functional effect of the *ETV6-RUNX1* fusion gene in activation of different pathways related to proliferation (e.g. EPOR/JAK/STAT, mTOR) and inactivation of apoptosis-induced pathways (e.g. TGF-β, TP53, pro-apoptotic microRNAs) (see section 1.5.4). Thus, blocking the binding capacity of this fusion gene could be tested in a murine model in order to assess the subsequent reduction in leukaemia growth rate and to possibly design a suitable inhibitor drug. In my view, targeting important pathways individually, including the mTOR pathway may not be appropriate because other cell signalling pathways may compensate for their inhibition or activation. However, targeting a particular pathway may be of use in the era of personalised medicine which aims to tailor therapy to a patient's genetic architecture.

In conclusion, there are no specific relapse-predicting biomarkers in *ETV6-RUNX1* positive relapses. The characteristic enrichment of late rather than early relapses may be due to the nature of the ancestral *ETV6-RUNX1* positive clones to have a long latency period in order to acquire new genetic lesions or expand existing abnormal clones that emerge as relapse clones. The higher doses of asparaginase are positively associated with the favourable outcome of *ETV6-RUNX1* BCP-ALL, owing to the higher *in vitro* sensitivity of *ETV6-RUNX1* positive cells to this anti-cancer agent. Nevertheless, designing inhibitors against the *ETV6-RUNX1* fusion gene may be of clinical benefit in order to reduce toxicity related chemotherapy.

7.6 Is there anything to be learnt from studying outliers with *ETV6-RUNX1* ALL?

7.6.1 The demographic features of ETV6-RUNX1 ALL are very tight

Unlike other cytogenetic subgroups, *ETV6-RUNX1* positive patients are characterised by very specific demographic features; patients are mostly aged from 3-6 years with two thirds of cases in the standard NCI risk category. These features may in part justify the characteristic favourable outcome (Golub *et al.*, 1995; Romana *et al.*, 1996; Moorman *et al.*, 2010b). Nevertheless, there are subsets of patients who rarely harbour the *ETV6-RUNX1* fusion gene, including infants, DS and AYA subgroups; considered as atypical groups. Given the fact that the *ETV6-RUNX1* fusion gene has been a poor initiator of leukaemia in multiple murine models, a latency period is required for the accumulation

of those additional abnormalities which are necessary for further progression of the disease. A large and growing body of literature has underscored the presence of genomic aberrations in the majority of *ETV6-RUNX1* cases. However, it is not yet known whether the spectrum of secondary abnormalities in these atypical *ETV6-RUNX1* positive patients is unique and informative for each subgroup. This study has highlighted the possibility of differences in the nature and frequencies of genetic alterations among these different *ETV6-RUNX1* positive subgroups. Thus these differences may uncover the different biological features responsible for the development of leukaemia at different time points and possibly a different clinical course of the disease.

7.6.2 What happens outside this scenario - is it clinically relevant?

7.6.2.1 Age

The rarity of infants may indicate that the *ETV6-RUNX1* fusion gene exhibits a benign course of disease as compared, for instance, to *MLL* rearrangements. However, one should consider the possible reasons behind the accelerated emergence of leukaemic transformation in this small number of cases. For example, high levels of pre-existing pre-leukaemic cells bearing *ETV6-RUNX1*, the increase in inherent abilities to develop leukaemia or the acquisition of aggressive alterations/mutations might play a role.

Infants with *ETV6-RUNX1* positive BCP-ALL showed less genetic diversity in this study, which could be expected with the shorter latency period. Nevertheless, masked somatic mutations cannot be ruled out as an increase in CNN-LOH regions in infants in this ALL subtype has been postulated (Emerenciano *et al.*, 2009). It is worth noting that the stable genome in this group of patients might be related to the age group rather than the cytogenetic subtype, since infant *MLL* rearranged ALL is also characterized by fewer genetic imbalances (Bardini *et al.*, 2010; Bardini *et al.*, 2011; Dobbins *et al.*, 2013).

Despite fewer alterations in infants, this study has shown that *ETV6* and *PAX5* alterations still constitute the most common genetic abnormalities in infant *ETV6-RUNX1* BCP-ALL, as seen in *ETV6-RUNX1* leukaemia as a whole, which suggested their essential role in leukaemic transformation. In addition, although *CDKN2A/B* losses were common in *ETV6-RUNX1* positive cases in general, they seemed to play no role in leukaemia development in the infant subgroup. Furthermore, despite the strong association of

BTG1 losses and *ETV6-RUNX1*, leukaemic progression in the infant subgroup did not require *BTG1* losses compared to non-infant cases. The overall findings in this subgroup necessitate further investigations that utilize genomic sequencing in order to comprehensively investigate the driving mechanisms.

The development of clinical leukaemia may be delayed up to an older age in a minority of *ETV6-RUNX1* carriers. One plausible explanation is that these patients require a much longer time to accumulate additional abnormalities because of possible low pre-existing, pre-leukaemic cell levels bearing *ETV6-RUNX1*. In agreement with this notion, the observed pre-leukaemic cell level of 10^{-3} to 10^{-4} in neonatal blood has been found to decrease to a cell level of 10^{-5} to 10^{-6} in adult life (Olsen et al., 2006). Unsurprisingly, the AYA subgroup accumulated multiple alterations in this study, which may be explained by the longer latency period but at a mean level comparable to that seen in non-AYA cases. However, the distribution of alterations differed among both subgroups. Our findings showed that *BTG1*, *RB1* losses and near tetraploidy were significantly associated with the AYA subgroup and constituted higher frequencies compared to that seen in non-AYA subtypes.

It is worth noting that the frequencies of *RB1* losses were similar in both adult and paediatric BCP-ALL cases in a previous study (Okamoto *et al.*, 2010), which might suggest that *RB1* deletions are of particular importance in leukaemic development of the AYA subgroup with *ETV6-RUNX1*. As described in section 7.5.1, the *RB1* gene is a negative regulator of the cell cycle and acts as a tumour suppressor gene involved in other cancer types including retinoblastoma, osteogenic sarcoma and bladder cancer (Jehanne *et al.*, 2014; Kansara and Thomas, 2014). Thus, disruption of the *RB1/TP53* pathway is more likely in this subgroup of patients resulting in increased cell survival and proliferation. *RB1* losses occurring in retinoblastoma usually follow the classic two-hit hypothesis of tumour suppressor gene inactivation in which both alleles of the tumour suppressor gene must be mutated (Knudson, 1971). Given that the majority of *RB1* deletions in this study are heterozygous and the mutational rate of *RB1* is low (2%) (Papaemmanuil *et al.*, 2014) in *ETV6-RUNX1* positive patients, haploinsufficiency might play a role in this subset of patients. Our findings showed that the AYA subgroup encountered focal losses restricted to exons 19-26 rather than the entire gene, unlike the non-AYA subgroup. The functional consequences of these focal deletions are still not yet defined, however, it

has been postulated that they generate truncated proteins with altered functions in association with deletions of *LPAR6* (Schwab *et al.*, 2013). *LPAR6* is located within the *RB1* gene and encodes G-protein-coupled receptors that preferentially respond to adenosine and uridine nucleotides. These receptors are involved in a diverse range of physiological and pathological events including neurotransmission, neoangiogenesis, fibrosis and cancer (Sokolov *et al.*, 2013). Therefore, it is important to assess the expression and function of different *RB1* deletions and the consequent *LPAR6* loss and to shed light on their prognostic role in leukaemic transformation and progression.

Furthermore, in the present study, 6q deletions were found in 21% of the AYA cases compared to 10% in non-AYA cases. In addition, 43% of cases with 6q deletions exhibited concurrent *BTG1* losses. This is in agreement with a previous study which reported that both *BTG1* and 6q deletions were more common in adult compared to paediatric BCP-ALL cases (Okamoto *et al.*, 2010). The *FOXO3* gene has been postulated to be the target of 6q deletions owing to its lower expression in ALL cell lines compared to normal cell lines, suggesting a role for this gene in disease progression (Okamoto *et al.*, 2010). *FOXO3* is a transcriptional activator involved in apoptosis and *BTG1* is a downstream target of *FOXO3*, resulting in a possible synergistic mechanism which negatively affects cell proliferation. Disruption of *FOXO3* transcriptional activity results from the AKT-dependent phosphorylation of *FOXO3* that aids in the exportation of FOXO3 to the cytoplasm through the enhancement of the FOXO3/14-3-3 interaction.

Hence, the driving mechanisms responsible for leukaemic conversion in AYA seems to rely on aberrations commonly found in association with *ETV6-RUNX1*, which are enriched in the AYA subgroup, suggesting different pathogenic processes.

7.6.2.2 DS ALL

DS ALL is characterized by enrichment of *JAK2* mutations and *CRLF2* overexpression, seen in 19% and 62% of cases, respectively (Kearney *et al.*, 2009; Russell *et al.*, 2009; Hertzberg *et al.*, 2010). However, this study has shown that *ETV6-RUNX1* DS ALL seems to be mutually exclusive of CRLF2 overexpression, which points to the possible existence of other alterations responsible for the development of disease in this subgroup.

This study has shown that *ETV6-RUNX1* non-DS BCP-ALL cases frequently harbour abnormal karyotypes (79%) compared to *ETV6-RUNX1* DS BCP-ALL cases (56%) similar to the picture seen in DS ALL and non-DS ALL in general (Lundin *et al.*, 2014). Nevertheless, the degree of genetic diversity in *ETV6-RUNX1* DS ALL was comparable to that seen in *ETV6-RUNX1* as a whole, based on the genomic profiles of four DS cases. In addition, previous gene expression studies revealed clustering of two *ETV6-RUNX1* DS ALL cases with *ETV6-RUNX1* non-DS ALL cases (Hertzberg *et al.*, 2010).

Although both groups displayed a similar degree of genetic diversity, the role of constitutional trisomy 21 opens a fascinating layer of complexity. It is worth noting that constitutional trisomy 21 may play a role in the development of leukaemia in DS ALL but the mechanism is not yet clear. However, its involvement in disruption of foetal liver haematopoiesis has previously been postulated (Roy *et al.*, 2012). Furthermore, a recent report demonstrated the influence of triplication of a 21q22 region on B cell proliferation in both *in vivo* and *in vitro* experiments through the overexpression of genes marked with H3K27me3 including *HMGN1* gene (a nucleosome remodelling protein) (Lane *et al.*, 2014). In agreement with the previous report, a recent report have postulated the differential enrichment of the H3K4me3 between the trisomy 21 discordant monozygotic twins suggesting its possible role in the epigenetic modifications in DS (Letourneau et al., 2014). HMGN1 acts as a modulator of gene expression through its involvement in histone modification (Postnikov and Bustin, 2010; Zhu and Hansen, 2010).

Human chromosome 21 contains around 33.7 Mb and encompasses 364 genes, 31 antisense transcripts and 5 different miRNAs (Malinge *et al.*, 2009). The DS critical region was identified by the genotype-phenotype correlation of children with partial trisomy 21 suspected of having DS, narrowing the list of genes with possible implications in the disease. Nevertheless, the functional influences of these genes are still not yet characterised. There are some genes in this region that are considered potentially important including *ERG, RUNX1*, *GABPA* and *ETS2* (Malinge *et al.*, 2009). It is worth noting that RUNX1 and other HSA21 proteins including DYRK1A and BRWD1 were reported to be involved in the epigenetic modification (Huang *et al.*, 2003; Canzonetta *et al.*, 2008; Bakshi *et al.*, 2010).

RUNX1 is a transcriptional factor involved in leukaemia, often as result of loss of function mutations (Izraeli, 2004); however, the leukaemogenic effect of three copies of *RUNX1* is still not yet known. It has been hypothesized previously that trisomy 21 may express different relative levels of RUNX1 isoforms that might in turn aid tumour development (Malinge *et al.*, 2009). On the other hand, the remaining candidate genes (*ERG*, *GABPA* and *ETS2)* encode ETS transcription factors involved in megakaryocytic differentiation. It is worth noting that these ETS transcription factors have been observed in different types of cancer, apart from *GABPA.* Nevertheless, *GABPA* and *ERG* are thought to contribute in leukaemic transformation of DS ALL through their involvement in either B-lymphocyte development or the pathogenesis of pre-B-ALL, respectively (Malinge *et al.*, 2009).

Given the fact that a small proportion of DS ALL exhibited *ETV6-RUNX1* and that they lack the classic genetic features of DS ALL (CRLF2 overexpression and *JAK2* mutations) (Buitenkamp *et al.*, 2012; Buitenkamp *et al.*, 2013a; Patrick *et al.*, 2014), one may expect that this subset of patients might constitute a sporadic form of DS ALL or just be a distinct form of DS-ALL, like we see in ALL in general.

ETV6-RUNX1 DS ALL cases were previously reported to experience a good outcome (Buitenkamp et al., 2013a) as compared to non-*ETV6-RUNX1* DS ALL patients, who were shown to have inferior outcome. The adverse outcome in the latter group was attributed to enrichment of *CRLF2* rearrangements and *JAK2* mutations. It is worth noting that DS was considered to be an independent adverse prognostic factor in standard risk patients based on multivariate analysis, but this finding was not repeated in a cohort of high risk patients (Malinge *et al.*, 2009). This implies the importance of maintaining the intensity of therapy in all DS ALL cases is important to achieve a comparable outcome to non- DS ALL patients. Therefore, the reduction in chemotherapy in DS ALL patients might be a crucial decision to be taken, however, given the possibility that *ETV6-RUNX1* DS ALL might be a biologically different subgroup; a trial with treatment modification should be considered.

To sum up, the observed differences in the nature and frequency of CNA among atypical subgroups (i.e. AYA, infants and DS) underscore possible differences in their biological

development. Thus, consideration of these biological differences might aid clinical management and improve outcomes through the adjustment of treatment regimes.

7.7 Overall summary of thesis

The translocation t(12;21)(p13;q22)/*ETV6-RUNX1*, is found in 25% of childhood BCP-ALL (Golub et al., 1995; Romana et al., 1996; Moorman et al., 2010). It is a transforming event and is responsible for the generation and maintenance of a pre-leukaemic clone (Andreasson et al., 2001). The high detection rate in the cord blood of healthy neonates, the high concordance rate among monozygotic twins and the latency period to clinically detectable leukaemia all indicate that postnatal genetic alterations are needed to promote the transformation into overt leukaemia (Wiemels et al., 1999; Mori et al., 2002; Greaves and Wiemels, 2003; Hong et al., 2008; Ma et al., 2013). Despite the characteristic favourable outcome in *ETV6-RUNX1*, some relapses do occur. *ETV6-RUNX1* is less common among AYA, infants and DS patients. This project investigated the spectrum of secondary abnormalities and clonal evolution in *ETV6-RUNX1* ALL using FISH, MLPA, SNP arrays and copy number qPCR.

The *ETV6-RUNX1* group is highly heterogeneous and, when considering the eight genes screened for by MLPA, had an average of 1.28 CNA per case compared to 0.95 alterations per case among other BCP-ALL. *ETV6* and *BTG1* losses were much more common compared to other ALL cases (52% v 10%; 15% v 3%, respectively). An alteration affecting the *IKZF1* gene, which is involved in B-cell development pathway, was less prevalent (3% v 18%).

Novel abnormalities affecting the *RUNX1-ETV6* reciprocal fusion gene, either by deletion or duplication, may contribute to leukaemogenesis by either eliminating or amplifying RUNX1-ETV6 expression, loss of tumour suppressor genes (e.g. *LRP6* and *BCL2L14*) or amplification of oncogenes.

Patients with *CDKN2A/B* deletions showed a higher frequency (44%) in the relapse cases (p=0.06). A high degree of clonal relatedness was observed between diagnosis and relapse. Low level sub-clones at diagnosis were seen emerging as the major clone at relapse.

Atypical groups (i.e. AYA, infants and DS) showed diversity in terms of the nature and frequencies of CNA. The infant group showed fewer CNA compared to non-infants. *CDKN2A/B* deletions are common in older children and AYA groups compared to infants (24% v 0%, p=0.006). *BTG1* and *RB1* losses are commoner in AYA group as compared to non-AYA (33% and 14% v 21% and 7%, respectively).

In conclusion, this study enhances our understanding of the spectrum of secondary abnormalities in *ETV6-RUNX1* BCP-ALL as a whole and other unusual subgroups. The detailed analysis of a series of diagnosis-relapse pairs in *ETV6-RUNX1* positive cases has provided valuable insight into the clonal evolution of relapse driving markers.

7.8 Future publications

A number of publications are to be written:

1- A Review of *ETV6-RUNX1* BCP-ALL including different aspects of the disease in terms of epidemiology, clinical characteristics, structure/ function of *ETV6, RUNX1*, both reciprocal products, genetic landscape (alterations in copy number, sequences, DNA methylation), clonal heterogeneity and emergence of relapse and the prognostic significance of specific genetic abnormalities.

2- A Research paper on the detailed analysis of a series of diagnosis-relapse pairs in *ETV6-RUNX1* positive cases to examine clonal heterogeneity and evolution using several techniques.

3- A Research paper on the potential genetic differences between different atypical subgroups of *ETV6-RUNX1* BCP-ALL, their influence on outcome and any suggested therapeutic modifications in any specific subgroup.

Chapter 8. References

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Chapter 9. Appendices

Appendix A. Individual demographic, clinical and different gene copy number alterations information of patients with ETV6-RUNX1 BCP-ALL.

Reg. ID	Age€/ Sex	Karyotype	MLPA							
			BTG1	***CDKN2A/B***	***EBF1***	***ETV6***	***IKAROS***	***PAR1***	***PAX5***	***RB1***
2615	4/M	46,XY,del(12)(p11p13),inc	e 2-3'	N	WG	WG	N	N	e 2-6	N
2712*	5/M	Failed	N	N	N	N	N	N	N	N
2760	6/F	Failed	N	WG	N	N	N	N	N	N
2774*	10/F	47,XX,+5,del(9)(p21),del(9)(p23)	N	WG	N	N	N	N	WG	N
2850*	7/F	46,XX,?t(3;16)(p21;p13),del(12)(p11)/ 45,idem,-X,add(9)(p?13)/ 46,XX	e 2-3'	WG	N	e 3-5	N	N	N	N
2874	6/F	46,XX,add(3)(p21),del(6)(q12q23),del(12)(p13),add(12)(p11)/ 46,XX	N	N	N	WG	N	N	N	N
2880	7/M	Failed	3'	N	N	N	e 4-8	R	e 2-5	e 19-26
2892	8/M	47,XY,t(12;21)(p13;q21),+der(21)t(12;21)	N	N	N	N	N	N	N	N
2897‡	3/F	46,XX,t(2;11)(p?21;p14)/ 46,XX	e 2-3'	N	N	WG	N	N	N	N
3026‡	5/F	48,XX,+X,t(3;20)(q2?7;q1?),del(12)(p13)t(12;15)(p1;q1?),t(12;21)(p13;q22),+21/ 48,idem,add(9)(p?)/ 46,XX	N	N	N	WG	N	N	N	N
3058	5/M	46,XY,del(6)(q21q25),add(12)(p13)/ 46,XY	N	N	N	N	N	N	N	N
3066	1/M	47,XY,+10/ 46,XY	N	N	N	mono e 1-5, bi e 8	N	N	N	N
3098*	5/M	46,XY,t(12;21)(p13;q22)/ 46,XY	N	WG	N	N	N	N	N	N
3119Ψ	6/F	46,XX,add(12)(p)/ 46,XX	N	N	N	e 8¥	N	N	N	N
3135Ψ	9/M	46,XY,der(9)del(9)(p21)del(9)(q34),del(11)(q?13q?23),t(12;21)(p13;q22),der(20)t(8;20)(q13;?p)/	e 2-3'	WG	N	e 8¥	N	N	N	N

Reg. ID	Age€/ Sex	Karyotype	MLPA							
			BTG1	*CDKN2A/B*	*EBF1*	*ETV6*	*IKAROS*	*PAR1*	*PAX5*	*RB1*
		46,XY								
3136	2/M	46,XY,del(12)(p11.2p13)	N	N	N	N	N	N	N	N
3142	5/M	Failed	N	WG	N	N	N	N	WG	N
3181* Ψ	5/F	46,XX,?del(12)(p)/ 47,XX,+mar/ 46,XX46,XX	N	N	N	e 8¥	N	N	e 1-2	N
3189Ψ	1/F	46,XX	N	N	N	e 8¥	N	N	N	N
3190	3/F	Failed	N	N	N	WG	N	N	e 1-6	WG
3194Ψ	4/M	51,X,+X,-Y,+9,del(12)(p12),t(12;21)(p13;q22),+18,+21,+21,+der(21)t(12;21)/ 46,XY	N	N	N	e 8¥	N	N	N	N
3208	6/F	Failed	N	N	N	N	WG	N	N	N
3217	5/M	Failed	N	N	N	e 2-8	N	N	N	N
3250	4/M	46,XY	e 2-3'	WG	N	e 1-5	N	N	N	N
3269Ψ	3/M	46,XY,der(14;21)(q10;q10)t(12;21)(p13;q22),ider(21)t(12;21)(p13;q22)/ 46,idem,del(13)(q14)/ 46,XY	N	N	N	e 8¥	N	N	N	WG
3296	3/F	46,XX	e 2-3'	N	N	WG	N	N	N	N
3301	3/M	47,XY,add(12)(p1?3),+21/ 46,XY	N	N	N	N	N	N	e 1-2	N
3304	6/M	49,XY,+X,+4,+10,dic(12;13)(p11;p11),i(17)(q10),+21/ 46,XY	N	N	N	WG	e 2-7	N	N	N
3359* Ψ	5/F	47,XX,add(12)(p1),+21	N	N	N	e 8¥	N	N	N	N

Reg. ID	Age€/ Sex	Karyotype	MLPA							
			BTG1	*CDKN2A/B*	*EBF1*	*ETV6*	*IKAROS*	*PAR1*	*PAX5*	*RB1*
3409	2/F	46,XX,del(12)(p11p13)/ 49,idem,+10,+16,+21,inc/ 50,XX,+10,del(12)(p11p13),+16,+19,+20/ 46,XX	N	N	N	WG	N	N	N	N
3418¥	5/M	47,XY,+21c	N	N	N	N	N	N	WG	N
3428¥	9/M	47,XY,?r(6)(::p?25q2?1::),-10,add(12)(p1?3),add(18)(q23),+21c,+r	N	N	N	WG	N	N	N	N
3431*	3/M	46,XY	e 2-3'	N	N	e 2-8	N	N	N	N
3453	10/F	46,X,add(X)(q2?8),del(8)(p11),del(11)(q23),del(12)(p13)[cp]/ 46,XX	N	WG	N	WG	e 2-3	N	WG	N
3472¥	2/F	Failed	e 2-3'	WG	N	N	N	N	N	N
3516	8/F	45,X,-X/ 46,XX	N	N	N	WG	N	N	N	N
3521	6/M	47,XY,+21/ 48,XY,+X,+21/ 46,XY	e 2-3'	N	N	N	N	N	e 2-6	N
3532	3/F	47,XX,t(8;12)(p10;p10),del(11)(q?14q2?5),+21/ 46,XX	e 2-3'	N	N	N	N	N	N	N
3562*	1/M	46,XY,der(10)t(10;12)(p11;q2?),ider(12)(p11)t(10;12)(p11;q2?),t(12;21)(p13;q22)/46,XY	N	N	N	WG	N	N	N	N
3588*	6/F	47,XX,del(6)(q?15q2?1),+21/ 46,XX	N	N	N	e 2-8	N	N	N	N
3602Ψ	11/F	46,XX,del(11)(q23),der(12)t(12;21)(p13;q22)ins(12;?)(q2?2;?),der(21)t(12;21)(p13;q22),+21,inc	N	N	N	e 8¥	N	N	N	e 19-26
3648	3/F	46,XX,der(2)t(2;21)(q2?4;q2?),del(12)(p11p13),t(12;21)(p13;q22)	3'	N	N	WG	N	N	N	N
3671Ψ	6/F	48,XX,+16,+21/ 46,XX	N	N	N	e 8¥	N	N	N	N
3672	4/F	46,XX	N	N	N	WG	N	N	N	N
3684¥	1/F	47,XX,+21c	N	N	N	N	N	N	e 2-	N

Reg. ID	Age€/ Sex	Karyotype	MLPA							
			BTG1	*CDKN2A/B*	*EBF1*	*ETV6*	*IKAROS*	*PAR1*	*PAX5*	*RB1*
									10	
3700*	4/M	46,XY	N	N	N	WG	N	N	N	N
3703*	2/M	46,XY,t(12;13)(p13;q14)/ 47,XY,+10/ 46,XY,-8,+10,t(12;13)(p13;q14)/ 46,XY,t(3;12)(q21;p11)/ 51,XY,+8,+10,+14,+16,+21	e 2-3'	N	N	WG	N	N	N	N
3711‡	3/M	46,XY,del(6)(q2?1q2?3),del(12)(p13)[cp]/ 46,XY	N	N	N	WG	N	N	N	N
3726	4/M	46,XY,add(11)(q2?2)/ 46,XY	N	WG	N	N	N	N	N	N
3728	3/F	47,XX,del(12)(p),+21/ 46,XX	e 2-3'	N	N	WG	N	N	N	N
3738	3/M	Failed	N	N	N	N	N	N	N	N
3748	3/M	48,XY,add(9)(p?),?del(12)(p1?p1?),+16,+21/ 46,XY	N	WG	N	N	N	N	WG	N
3750	4/M	46,XY	N	N	e 1-10	WG	N	N	N	N
3761‡	5/M	46,XY	N	N	N	WG	N	N	WG	N
3778	3/F	46,XX,del(9)(q2),der(13)t(X;13)(p1;q),dup(17)(q)	N	N	N	N	N	N	N	N
3787	4/F	45,XX,der(12;17)(q10;q10)/ 46,XX	N	N	N	WG	N	N	e 2-6	N
3801	2/M	46,XY,del(12)(p1?p1?)/ 46,XY	N	N	N	WG	N	N	N	N
3805	4/M	46,XY,der(1)t(1;?11)(q;q),der(6)ins(6;?1)(q;q),del(11)(q?21)/ 46,XY	N	N	N	WG	N	N	N	N
3833*	4/F	Failed	N	WG	N	WG	N	N	N	N
3861	3/F	Failed	N	N	N	WG	N	N	N	N
3878	7/M	46,XY,t(3;9)(p22;q3?4),del(5)(q?31),?del(11)(q?22q?23),del(13)(q22q32),inc	N	N	N	WG	N	N	N	N
3913	7/F	45,XX,der(12;22)(q10;q10)/ 46,XX	e 2-3'	N	N	WG	e 2-3	N	e 2-6	N

Reg. ID	Age€/ Sex	Karyotype	MLPA							
			BTG1	***CDKN2A/B***	***EBF1***	***ETV6***	***IKAROS***	***PAR1***	***PAX5***	***RB1***
3997	2/M	46,XY,t(10;13)(q22;q3?),add(12)(p1?),del(12)(p1?p1?)/ 46,XY	e 2-3'	N	N	WG	N	N	e 2-6	N
4011‡	3/F	45,X,-X,dup(1)(?p),dup(4)(?p),der(12)t(12;14)(p;q),t(12;21)(p13;q22),-13,del(18)(q),+del(18)(?q),der(19)t(13;19)(q;?p),der(20)t(10;20)(?q;q)/ 46,XX	N	N	N	WG	N	N	e 1-6	N
4013	4/F	44-46,XX,add(11)(q23),del(11)(q2?2),add(12)(p?),+mar[cp]/ 46,XX	N	N	N	N	N	N	N	N
4036*	6/M	46,XY,?der(7)t(7;19)(q1;p1),?der(11)t(7;11)(q1;q1),t(12;21)(p13;q22),?der(19)t(11;19)(q1;p1)/ 46,XY	e 2-3'	WG	N	WG	N	N	N	N
4040	4/F	46,XX,del(12)(p1?2),inc/ 46,XX,inc	e 2-3'	WG	N	e 1-5	N	N	e 2-6	N
4044*	3/F	45,X,-X,t(4;15)(q21;q?14)/ 46,XX	N	WG	N	WG	N	N	N	N
4094	2/M	46,XY,add(4)(q?31),del(13)(q14)/ 46,XY	N	N	N	N	N	N	e 2-6	WG
4153	3/M	Failed	N	N	N	WG	N	N	e 2-5	N
4202	5/M	Failed	e 2-3'	WG	N	N	N	N	N	N
4250	3/M	Failed	N	N	N	WG	N	N	N	N
4253	2/M	44-46,XY,der(1;12)(?p10;?q10),del(3)(q2?),del(11)(p11.2),-17,add(19)(q13.?3)[cp]/46,XY	N	WG	N	WG	N	N	N	N
4281	4/M	46,XY,add(6)(q1?)[14]/ 46,XY	N	WG	N	e 1-5	N	N	N	N
4288‡	6/M	45,XY,-13/ 46,XY	N	N	N	WG	N	N	N	WG

Reg. ID	Age€/ Sex	Karyotype	MLPA							
			BTG1	*CDKN2A/B*	*EBF1*	*ETV6*	*IKAROS*	*PAR1*	*PAX5*	*RB1*
4323	7/M	46,XY,?del(8)(p1?),del(12)(p1?)	3'	N	N	WG	N	N	N	N
4373	2/M	45,XY,dic(12;13)(p1?;p1?),?add(9)(p?)/ 46,XY	N	N	N	WG	N	N	e 2-6	N
4425Ψ	5/M	Failed	N	WG	N	e 8¥	N	N	e 2-6	N
4439‡	9/M	46,XY,del(9)(p?2),add(12)(p1?),add(18)(q?)/ 46,XY	N	N	N	WG	N	N	N	N
4524Ψ	1/M	46,XY	N	N	N	bi e 8¥	N	N	N	e 19-26
4528	3/F	46,XX,del(6)(q),del(12)(p)/ 46,XX	e 2-3'	N	N	e 2-8	N	N	N	N
4536‡	4/M	46,XY,der(12)t(12;21)(p13;q22),der(15)t(X;15)(q?;q2?)	N	N	N	WG	N	N	N	N
4541	3/M	46,XY	e 2-3'	N	N	e 2-8	e 2-3	N	N	N
4554	2/M	45,XY,del(12)(p)/ 45,XY,dic(9;12)(p1;p1)	N	N	N	N	N	N	N	N
4569‡	7/F	46,XX,del(11)(q13),t(12;17)(q24;q21)/ 46,XX	N	WG	N	N	N	N	e 1-6	N
4598	3/M	46,XY	N	N	N	N	N	N	N	WG
4609‡	4/F	46,XX,t(12;21;9;18)(p13;q22;p1;q2)ins(18;5)(q?2;q?q?)t(5;11)(q?;q2),dup(21)(q?)	N	N	N	N	N	N	N	N
4616	3/M	46,XY,der(1)t(1;14)(q2?;q1?),add(3)(p2?),del(6)(q1?q2?),der(7)t(1;7)(q2?;p?1),-12,del(13)(q1?q?3),+mar1,+mar2/ 47,idem,+mar/ 46,XY	N	N	N	N	N	N	e 2-8	WG
4620	3/M	Failed	N	N	N	N	N	N	N	N
4637	4/F	46,XX	N	N	N	e 1-5	N	N	e 2-6	N
4675	11/F	80-82<4n>,XX,-X,-X,-2,-6,del(6)(q?),-11,-12,-13[cp]/ 46,XX	N	N	N	N	N	N	N	N

Reg. ID	Age€/ Sex	Karyotype	MLPA							
			BTG1	*CDKN2A/B*	*EBF1*	*ETV6*	*IKAROS*	*PAR1*	*PAX5*	*RB1*
4688	5/M	47,XY,t(13;17)?(q12;p11),+21/ 46,XX	N	N	N	N	N	N	N	N
4698	4/M	46,XY/ 45,XY,dic(9;12)(?)	N	WG	N	WG	N	N	WG	N
4707Ψ	3/M	94-96,inc	N	N	N	e 8¥	N	N	N	N
4731	3/M	46,XY	N	N	WG	WG	N	N	N	N
4739	4/F	46,XX,t(9;18;12;21)ins(18;5)(9qter>9p1::21q22>qter;18pter>q2::5q31.3>q12::9p1>pter;12qter>12p13::18q2>qter;21pter>21q22::12p13>pter)t(5;11)(5pter>q12::11q2>qter;11pter>11q2::5q31.3>qter),del(12)(p13p13)	N	N	N	e 1	N	N	N	N
4758Ψ	3/M	47,XX,del(12)(p11),+21/ 48,idem,+16/ 46,XY	e 2-3'	N	N	e 8¥	N	N	N	N
4778	5/M	46,XY,add(12)(p13)/ 46,XY	N	N	N	WG	N	N	N	N
4875	3/M	47,XY,+21/ 47,idem,add(5)(q?3)/ 48,idem,+10/ 46,XY	N	N	N	e 2-8	N	N	N	N
4894	5/F	47,XX,+21/ 46,XX	N	N	N	mono e 1-5, bi e 8	N	N	N	N
4902*	3/M	Failed	N	N	N	WG	N	N	e 2-7	N
4905¥	3/F	47,XX,del(12)(p1?),+21c/ 47,XX,+21c	f	f	f	f	f	f	f	f
4932	6/F	Failed	e 2-3'	N	N	WG	N	N	N	N
4936	4/M	46,XY,dic(9;12)(p1?3;p11.2)/ 46,XY	N	WG	N	WG	N	N	WG	N
4944	3/F	46,XX,?add(12)(p1?),?add(13)(p),inc	N	WG	N	WG	N	N	N	N
4969Ψ	6/M	46,XY,del(9)(p2?),+21/ 46,XY	N	WG	N	e 8¥	N	N	N	N
4995‡	8/F	44,X,-X,dic(8;12)(p1?;p1?),inv(9)(p11q13)c/ 46,XX,inv(9)(p11q13)c	N	N	WG	WG	N	N	N	N

Reg. ID	Age€/ Sex	Karyotype	MLPA							
			BTG1	***CDKN2A/B***	***EBF1***	***ETV6***	***IKAROS***	***PAR1***	***PAX5***	***RB1***
5038	2/F	46,XX,t(2;12;7)(q?11;p?12;p?11)/ 46,XX	N	N	N	N	N	N	e 2-6	N
5053	1/M	47,XY,del(6)q(1q2),+21,inc[cp]/ 46,XY	N	N	N	N	N	N	e 2-6	N
5062*	2/F	Failed	N	WG	N	N	N	N	N	N
5068	5/F	Failed	e 2-3'	N	WG	e 2-8	N	N	N	N
5072	4/M	Failed	N	N	N	WG	N	N	e 2-6	N
5597*	6/M	46,XY,der(2,5,15)t(2;15)(q?3;q1?)ins(5;2)(q13;q?2q?3),t(12;21;8)(p13;q22;q11)/ 46,idem,-der(2)t(2;15)(q?3;q1?)ins(5;2)(q13;q?2q?3),+t(2;6)(p1?;q1?3)del(2)(p1?p2?)t(2;15)(q?3;q1?)ins(5;2)(q13;q?2q?3),-6 /46,XY	N	WG	N	WG	N	N	N	N
5628	1/F	Failed	N	N	N	WG	N	N	N	N
5647‡	5/F	46,XX	N	WG	N	e 3-8	N	N	N	N
5659‡	3/F	Failed	N	N	N	WG	N	N	e 2-10	N
5721‡	3/M	45-46,XY,add(11)(q23),add(12)(p?),inc/ 46,XY	e 2-3'	N	N	WG	N	N	N	N
5796	4/M	46,XY,del(12)(p12),inv(12)(p11.2q22)	N	WG	N	WG	N	N	N	N
5802	11/F	45,X,-X,t(11;12;21)(p11;p13;q22)/ 45,idem,del(3)(p21)/ 46,XX	N	N	e 1-10	WG	N	N	N	N
5807‡	5/M	47,XY,-12,-13,-15,+16,-17,+21,+mar1,+mar2,+mar3[10]	N	N	N	WG	N	N	N	WG
5848	2/F	Failed	N	N	N	N	N	N	e 1-6	N
5849	3/F	Failed	N	N	N	WG	N	N	N	N
5859Ψ	5/M	47,XY,+21/ 46,XY	N	WG	N	e 8¥	N	N	N	N
5874	14/F	46,XX,add(12)(p11)/ 46,XX	N	WG	N	WG	N	N	N	N

Reg. ID	Age€/ Sex	Karyotype	MLPA							
			BTG1	*CDKN2A/B*	*EBF1*	*ETV6*	*IKAROS*	*PAR1*	*PAX5*	*RB1*
5884	5/F	del(11)(p11p15),add(12)(p1),inc	N	N	N	N	N	N	e 2-5	N
5914‡	4/M	Failed	N	N	N	WG	N	N	N	N
7038¥	13/M	47,XY,+21c	N	N	N	N	N	N	N	N
7149	7/F	Fail	N	N	N	N	N	N	N	N
7239	5/M	46,XY	N	N	N	N	N	N	N	N
7258	3/M	45,XY,dic(12;20)(p11;p1),?t(12;21)(p13;q22)/ 45,idem,-11,+mar/ 46,XY	N	N	WG	WG	N	N	N	N
7292	3/M	46,XY,der(1)t(1;21)(p36;q22),add(9)(p22),del(12)(p13p13),der(12)?(1pter->1p36::21q22::12p13::12p11->12p13::12q15->12qter),der(21)t(12;21)(p13;q22)	N	N	N	WG	N	N	N	N
7361	5/M	46,XY,?t(12;21)(p13;q22),add(16)(p13),inc/ 47,idem,del(12)(p11p13),+21,inc	e 2-3'	N	WG	WG	N	N	N	N
7551	4/M	46,XY,add(12)(p1),?t(12;21)(p13;q22),inc	e 2-3'	N	N	WG	N	N	N	N
7552	3/F	46,XX,add(12)(p1),?t(12;21)(p13;q22),inc/ 47,idem,+21,inc	N	N	N	N	N	N	N	N
7571	2/M	46,XY,add(12)(p1),?t(12;21)(p13;q22)	N	N	N	WG	N	N	N	N
7613Ψ	3/M	46,XY	N	N	N	e 8¥	N	N	N	N
7646	2/F	Failed	N	N	N	WG	N	N	N	N
7780	11/M	46,XY,add(3)(q2),add(12)(p1),t(12;21)(p13q22),add(16)(?q2),add(17)(q2),inc[cp]	N	WG	N	WG	N	N	N	N
7809	14/M	70~85,XXYY,?del(6)(q1),inc[cp]/ 46XY	N	N	N	N	N	N	N	N
8153	2/M	Failed	N	WG	N	N	N	N	e 2-5	N
8161Ψ	13/M	46,XY,del(8)(q13q22),add(12)(p13),add(15)(q?2	e 2-3'	N	N	e 8¥	N	N	N	N

Reg. ID	Age€/ Sex	Karyotype	MLPA							
			BTG1	*CDKN2A/B*	*EBF1*	*ETV6*	*IKAROS*	*PAR1*	*PAX5*	*RB1*
		6)								
8444	5/M	Failed	e 2-3'	WG	N	N	N	N	N	N
8517	4/M	46,XY,add(4)(p1?5),?i(21)(q10),inc/ 46,XY	N	N	N	N	N	N	N	N
8906¥	3/F	47,XX,t(4;4)(q10;q10),+21c/47,XX,+21c	N	N	N	N	N	N	N	N
9034	4/M	46,XY,del(6)(q?2),del(12)(p?1),inc[cp]/ 46,XY	N	WG	N	N	N	N	N	N
9050	7/F	46,X,-X,-6,add(12)(p?),+16,+r[cp]/ 46,XX	N	N	N	WG	N	N	N	WG
9148	12/M	46,XY,add(1)(p?36),add(2)(p?23)/ 47,idem,+X,del(6)(q?14),t(8;13)(p?22;q?13)	N	N	N	e 3-8	e 2-3	N	N	WG
9264	5/M	47,XY,-11,+der(21)t(12;21)(p12;q22),+mar/ 46,XY	N	N	N	N	N	N	N	N
9265	6/F	78-90<4n>,XXX,-X,-3,add(3)(q2),+4,+6,add(6)(q2)x2,-9,-11,-12,-15,+20[cp]	N	N	N	N	N	N	N	N
9378	6/F	46,XX,der(11)del(11)(p11)t(11;?15)(q2?3;q1),add(12)(p1),-15,+mar/ 46,XX	N	N	N	WG	N	N	N	WG
9381	1/M	46,Y,i(X)(q10)	N	N	N	N	N	N	N	N
9412	2/M	46,XY,add(9)(q34)/ 46,XY	N	N	N	WG	N	N	e 2-6	N
9424	2/M	46,XY	N	N	N	WG	N	N	N	N
9426	8/F	46,XX	N	N	N	e 3-8	N	N	N	N
9429	3/M	46,XY,del(6)(q13q23)/ 46,XY	e 2-3'	N	N	WG	N	N	N	N
9516	3/M	?46,del(11)(q2),inc	N	N	N	N	N	N	N	N
9533	4/M	45,XY,der(12)t(12;13)(p1;q1),?der(12;21)t(12;21)(p13;q22)del(21)(q22q22),-13[cp]/46,XY	N	N	N	WG	N	N	N	N

Reg. ID	Age€/ Sex	Karyotype	MLPA							
			BTG1	*CDKN2A/B*	*EBF1*	*ETV6*	*IKAROS*	*PAR1*	*PAX5*	*RB1*
9535	4/M	46,XY,del(6)(q1q2),?t(12;21)(p13;q22)/ 46,idem,add(12)(p13)/ 46,XY	N	N	N	WG	N	N	N	N
9849	2/M	46,XY,del(8)(p?21)/ 47,idem,+mar/ 46,XY	N	WG	N	e 2-8	N	N	N	N
9855	3/M	Failed	N	N	N	N	N	N	N	N
9867	3/M	Failed	e 2-3'	N	N	N	N	N	N	N
9868	4/M	46,XY	N	N	N	WG	N	N	N	N
9873	8/M	46,XY,idic(21)(p1)/ 46,idem,add(12)(p1)/ 46,XY	N	WG	N	N	N	N	N	N
9879¥	15/M	47,XY,+21c	N	N	N	WG	N	N	N	N
10036	3/F	Failed	3'	N	N	N	N	N	e 2-6	N
10060	3/M	46,XY	N	N	N	e 2-8	N	N	N	N
10065	5/F	46,X,-X,del(6)(q13q23),add(8)(p1),del(11)(q22q25)[cp] / 46,XX	N	N	N	N	N	N	N	N
10073	2/F	Failed	N	N	N	WG	N	N	e 2-5	N
10076	5/M	Failed	N	N	N	WG	N	N	N	N
10083	2/F	46,XX,add(6)(q11),dic(9;18)(p1?3;p11),+21/ 46,XX	N	WG	N	N	N	N	WG	N
10086 Ψ	4/M	49,XY,t(3;15)(q27;q1?5),+10,del(12)(p11p13),+16,add(18)(q2),+21	N	N	N	e 8¥	N	N	N	N
10170	6/F	46,XX,?t(12;21)(p13;q22),del(22)(q11q12)/ 47,idem,-X,+16,+21/ 48,idem,-X,+10,+16,+21	e 2-3'	N	N	N	N	N	N	N
10216	17/M	67~76,+3,+4,+6,+9,+12,+14,+16,+17,+18,+21,inc / 46,XY	e 2-3'	N	N	N	N	R	N	e 14-26
10252	8/M	48,XY,?add(6)(q1),del(12)(p1),+21,+mar/ 46,XY	N	N	N	e 8¥	N	N	N	N

Reg. ID	Age€/ Sex	Karyotype	MLPA							
			BTG1	*CDKN2A/B*	*EBF1*	*ETV6*	*IKAROS*	*PAR1*	*PAX5*	*RB1*
Ψ										
10253	3/F	Failed	N	N	N	WG	N	N	e 1-6	N
10259	2/F	45,XX,-8,der(12)t(8;12)(q11.2;p11.2)/ 46,XX	N	N	N	WG	N	N	e 2-6	N
10267	1/M	46,XY,t(3;6)(q29;q1?3),del(12)(p1?1p13)/ 46,XY	N	N	N	N	N	N	N	N
10282	15/M	46,XY,del(6)(q13q25),-18,+mar/ 46,idem,t(2;12)(q33;p13)/ 86~92,idemx2[cp]/ 46,XY	N	N	N	N	N	N	N	N
10296	3/M	46,XY,add(12)(p13),inc[cp]/ 46,XY	N	N	N	N	N	N	N	N
10325	8/F	Failed	N	WG	N	WG	N	N	N	N
10348	6/F	Failed	N	N	N	WG	N	N	N	N
10353	5/M	Failed	N	N	N	bi e 5-8	N	N	N	N
10397	4/M	47,XY,+21,inc[cp]/ 46,XY	N	N	N	N	N	N	N	N
10398 Ψ	5/M	43-45,XY,del(1)(p3),-2,add(6)(q2),-14,add(16)(p1),-22,+mar1,+mar2,inc[cp]/46,XY	N	WG	N	e 8¥	N	N	N	N
10411	4/M	46,XY	N	WG	N	N	N	N	N	N
10472	12/F	46,XX,del(6)(q1?),add(19)(p13)/ 46,XX	N	N	N	WG	N	N	N	N
10505	3/M	46,XY	N	N	N	e 5-8	N	N	N	N
10514	4/F	46,XX,add(3)(q2?1),add(12)(p10)	N	N	N	WG	N	N	N	N
10572	3/M	46,XY,t(1;12)(q3?2;p13),del(6)(q21)/ 46,XY	N	N	N	WG	N	N	N	N
10590	4/M	46,XY	e 2-3'	N	N	e 3-5	N	N	e 2-6	N
10628	5/F	46,XX,del(3)(p13p26),del(6)(q1q2),add(10)(p13)	N	WG	N	e 1-2	N	N	N	N
10688	2/M	47,XY,+21/ 48,XY,+10,+21	N	WG	N	N	N	N	N	N

Reg. ID	Age€/ Sex	Karyotype	MLPA							
			BTG1	*CDKN2A/B*	*EBF1*	*ETV6*	*IKAROS*	*PAR1*	*PAX5*	*RB1*
10690	2/M	46,XY,inv(7)(p13q22)c/ 47,idem,+21	N	N	N	N	N	N	N	N
10694	5/M	49~51,XY,+?5,?add(6)(q?),+?14,+16,+20,+21,inc[cp]/94~97,XXYY,+4,?add(6)(q?),+16,+21,+21,inc[cp]/46,XY	N	N	N	N	N	N	N	WG
10826	3/M	46,XY,del(3)(q2?6),add(14)(q?24),add(18)(p11)/46,XY	N	WG	N	e 3,8	N	N	N	N
10874	6/F	46,XX,del(12)(p13)/46,XX	N	N	N	N	N	N	N	N
10875*	5/M	Failed	N	N	N	N	N	N	N	N
10892	2/M	46,XY	N	N	N	WG	N	N	N	N
10921	3/F	Failed	N	WG	N	N	N	N	N	N
10928	2/F	Failed	N	N	N	N	N	N	N	N
10956	3/M	46~47,XY,?add(12)(p1),?t(12;21)(p13;q22),?+21[cp]	N	N	N	WG	N	N	N	N
10960	9/F	Not Done	N	WG	N	WG	N	N	N	N
11001	6/F	Failed	N	WG	N	N	N	N	N	N
11052 Ψ	6/M	Failed	N	N	N	bi e 8¥	N	N	N	N
11054	3/M	Failed	N	N	N	N	N	N	N	N
11106	5/F	47~51,XX,-2,add(3)(p21),-6,+10,+16,+21,+r,+mar1,+mar2,inc[cp]	N	N	N	N	N	N	N	WG

Reg. ID	Age€/ Sex	Karyotype	MLPA							
			BTG1	***CDKN2A/B***	***EBF1***	***ETV6***	***IKAROS***	***PAR1***	***PAX5***	***RB1***
11110	4/F	46,XX,-5,der(7)t(7;12)(q22;q15),der(8)t(8;12)(p11.2;p11.2),add(9)(p12),der(12)del(12)(q13q24.3)ins(12;?)(q13;?),der(12)t(12;21)(p13;q22)del(12)(q12),add(13)(q34),add(16)(p13.2),add(20)(q13.3),+mar[cp]/ 46,XX	e 2-3'	WG	N	WG	N	N	N	N
11142	12/M	Failed	N	N	N	e 3-5	N	N	N	N
11157	3/M	48,XY,t(1;13;19)(q21;q1;q13),add(2)(q3),?t(12;21)(p13;q22),?del(12)(p13p13),+21,+mar,inc[cp]/ 46,XY	N	N	N	e 2-8	N	N	N	N
11182	7/F	46,X,-X,?t(12;21)(p13;q22)/ 46,XX	N	N	N	N	N	N	N	N
11183	6/M	46,XY,?add(12)(p1)/ 46,XY	N	WG	N	WG	N	N	N	N
11311	4/F	Failed	N	WG	N	WG	N	N	e 1-6	N
11312	4/F	46,XX	e 2-3'	N	N	WG	N	N	N	WG
11385	2/M	46,XY	N	*CDKN2B*	N	e 1	N	N	N	N
11468	4/F	45,XX,dic(12;15)(p11;p1)/ 46,idem,+16/ 45,XX,dic(8;12)(p1;p11)/ 46,XX	e 2-3'	N	N	WG	N	N	N	N
11541	2/F	46,XX	N	*CDKN2A*	N	e 2-8	N	N	e 2-8	N
11542	10/F	Failed	3'	N	WG	N	N	N	e 1-6	N
11545	2/F	Failed	N	N	N	WG	N	N	N	N
11558	7/M	49,XY,+16,+21,+21/ 46,XY	N	N	N	N	N	N	N	N
11562 Ψ	5/F	46,XX	N	N	N	e 8¥	N	N	N	N
11567	1/F	Failed	N	N	N	WG	N	N	e 2-6	N

Reg. ID	Age€/ Sex	Karyotype	MLPA							
			BTG1	*CDKN2A/B*	*EBF1*	*ETV6*	*IKAROS*	*PAR1*	*PAX5*	*RB1*
11576	3/M	47,XY,+21/ 46,XY	N	WG	N	N	N	N	N	N
11636 Ψ	4/F	46~48,XX,der(6)t(6;12)(q12;q11.2)ins(6;?)(q12;?),del(7)(q32q35),del(12)(p13),der(12)add(12)(p11.2)add(12)(q13)t(12;21)(p13;q22),del(15)(q13q26),der(21)t(12;21)(p13;q22),+der(21)t(12;21)[cp]	N	N	N	e 8¥	N	N	N	N
11638	1/M	Failed	N	N	WG	N	N	N	N	N
11675	2/M	46,XY	N	WG	N	N	N	N	N	N
11680	3/M	46,XY	N	N	N	N	N	R	e 1-6	N
11707	6/F	47~48,XX,der(6)add(6)(p2?2)del(6)(q?13q?21),+21,+mar[cp]/ 46,XX	N	N	N	N	e 4-8	N	N	N
11710	3/M	Failed	N	N	N	N	N	N	e 2-6	WG
11763	2/F	46,XX	N	N	N	N	N	N	N	N
11773	5/M	Failed	N	N	N	WG	N	N	N	WG
11777	7/F	Failed	e 2-3'	N	N	N	e 2-8	N	N	N
11783	1/M	47,XY,+10,add(12)(p12)/ 46,XY	N	N	N	WG	N	N	N	N
11833	5/F	Failed	N	N	N	N	N	N	N	N
11852	2/M	46,XY,-12,add(19)(?p13.1),+mar/ 46,XY	N	N	N	N	N	N	e 2-6	N
11854	4/F	46,XX	N	N	N	WG	N	N	N	N
11855	4/M	Failed	N	N	N	N	N	N	e 1-2	N
11908	12/F	46~47,+2,del(3)(q21q27),t(3;12)(p21;q24.1),?8,del(9)(p13p24),del(12)(q22q24.1),t(12;21;13)(p13;q22;q14)[cp]/ 46,XX	e 2-3'	WG	N	N	N	N	N	N
11931	2/F	?,<4n>,add(6)(q?),add(12)(p?),add(14)(q?),inc	N	N	N	N	N	N	N	N

Reg. ID	Age€/ Sex	Karyotype	MLPA							
			BTG1	***CDKN2A/B***	***EBF1***	***ETV6***	***IKAROS***	***PAR1***	***PAX5***	***RB1***
11962	3/F	46,XX,del(12)(p1?1),add(19)(p13.3)/ 46,XX	N	N	WG	WG	N	N	e 2-6	N
12018	1/M	46~47,X,-Y,-10,-12,add(12)(p11.2),-14,-15,+16,+21,+1~5mar[cp]/ 46,XY	N	N	N	N	N	N	N	N
12050	11/F	45~46,X,-X,+mar[cp]/ 46,XX	N	N	N	N	N	N	N	e 19-26
12086	3/M	46,XY,del(9)(p21p?21),t(12;21)(p13;q22)/ 46,XY	N	WG	N	N	N	N	N	N
12127	4/F	46,XX	N	N	N	e 2-8	N	N	N	N
12133	2/M	46,XY,t(2;7)(p11.2;q36)/46,XY	N	N	N	WG	N	N	e 1-6	N
12173	3/M	46,Y,add(X)(q1),t(1;14)(q21;p1),der(6)add(6)(p1)add(6)(q1),?t(12;21)(p13;q22),add(22)(p1)/?,?idemx2,inc/46,XY	N	N	N	N	N	N	N	N
12244	2/F	46,XX	N	N	N	N	N	N	N	N
12246	4/M	46,XY	N	N	N	N	N	N	N	N
12249	3/M	79~84,inc[cp]/ 46,XY	N	N	N	N	N	N	N	N
12251	5/M	46,XY,?t(12;21)(p13;q22),del(12)(p11p13),inc	N	WG	N	WG	N	N	N	N
12287	2/M	Failed	N	N	N	e 2-8	N	N	N	N
12290 ψ	3/F	Failed	e 2-3'	N	N	e 8¥	N	N	N	N
12348	5/F	Failed	N	N	N	WG	N	N	N	N
12351	5/M	45~46,XY,-Y,?del(4)(p15),idic(21)(q10)[cp]/ 46,XY	N	N	N	N	N	N	N	N
12358	1/F	46,XX,?del(4)(q?q?),-10,add(10)(p?),+mar	N	N	N	N	N	N	WG	N
12446	7/F	Failed	N	N	N	mono e 2-5, bi e 8	N	N	N	N

Reg. ID	Age€/ Sex	Karyotype	MLPA							
			BTG1	*CDKN2A/B*	*EBF1*	*ETV6*	*IKAROS*	*PAR1*	*PAX5*	*RB1*
12455	2/M	Not done	N	N	N	WG	N	N	N	N
12457	2/F	46,XX	N	WG	N	N	N	N	N	N
12529	3/F	46,XX	N	N	N	mono e 1-5, bi e 8	N	N	e 2-6	N
12531	2/F	46,XX	N	N	N	WG	N	N	N	WG
12725	12/M	84~86,add(6)(q?),inc/46,XY	N	N	N	N	N	N	N	N
12733	4/F	Failed	N	N	N	N	N	N	N	N
12737	3/M	Failed	N	WG	N	N	N	N	N	N
12795	3/M	47,XY,+add(21)(?p10)/46,XY	N	WG	N	WG	N	N	N	N
12797	6/F	46,XX,t(11;12;21)(q13;p13;q22)[/46,idem,del(6)(q21)	N	WG	N	WG	N	N	N	N
12819	2/F	46,XX,add(5)(p1?5),add(9)(p11),add(12)(p13),idic(21)(p11)/46,XX	N	N	N	N	N	N	N	N
12846	8/M	47,XY,+21,add(21)(p1)x2/48,idem,+18/46,XY	e 2-3'	N	N	N	N	N	N	N
12901	3/F	46,XX,del(12)(p1?1-2p1?3)/46,XX	N	N	N	WG	N	N	e 2-6	N
19598	2/M	45,XX,?dic(6;15)(q11;p1),add(11)(p15),del(13)(q1?3q33)/46,XY	N	N	N	N	N	N	e 2-6	WG
19647	3/M	Failed	N	N	N	WG	N	N	N	N
19685	3/F	45,XX,dic(6;9)(q13;p13),add(16)(p13)/46,XX	N	WG	N	mono e 1-5, bi e 8	N	N	e 2-8	N

Reg. ID	Age€/ Sex	Karyotype	MLPA							
			BTG1	*CDKN2A/B*	*EBF1*	*ETV6*	*IKAROS*	*PAR1*	*PAX5*	*RB1*
19687	3/F	46,XY,-9,der(12)add(12)(p11.2)add(12)(q24.1),add(15)(q21),+mar/46,XY	N	N	N	mono e 1-5, bi e 8	N	N	N	N
19688	2/M	46,XY,del(11)(q22)/46,XY,idem,rea(14)	N	N	N	N	N	N	N	N
19735	4/M	Failed	N	N	N	WG	N	N	e 1-6	N
19795	5/M	46,XY,add(11)(q13),i(21)(q10)/46,XY	N	N	N	N	N	N	N	N
19801	3/M	Failed	N	N	N	N	N	N	N	N
19851	1/M	Failed	N	N	N	WG	N	N	N	N
19993	7/F	?96,XXXX,t(12;21)(p13;q22)x2,+21,inc/46,XX	N	N	N	N	N	N	N	N
20123	1/F	46,XX,?del(9)(q34q34),?t(12;21)(p13;q22)	N	N	N	N	N	N	N	N
20164	4/M	46,XY,-10,ins(12;?)(q13;??),+mar/45,idem,dic(9;12)(p1;p1)/46,XY	N	WG	N	e 1-5	N	N	WG	N
20215	1/M	46,XY,add(12)(p11),-13,+?21/46,XY	N	N	N	WG	N	N	N	N
20220	1/F	46,XX	N	N	N	N	N	N	N	N
20223	6/M	48,XY,+21,+mar[cp]/46,XY	N	N	N	WG	N	N	N	N
20311	10/M	46,XY,add(3)(q25),del(6)(q21q27),add(17)(p11.2),add(19)(p13)/46,XY	3'	N	N	N	N	N	N	N
20383¥	1/M	47,XY,del(7)(q22q34),add(20)(q11),+21c/47,XY,+21c	N	N	N	N	N	N	N	N
20385	2/M	Failed	N	N	N	N	N	N	e 2-6	N
20394	3/F	46,XX	e 2-3'	N	N	N	N	N	N	N
20395	4/M	45,XY,dic(9;12)(p13;p13)[8]/46,idem,+mar/46,XY	N	WG	N	WG	N	N	N	N

Reg. ID	Age€/ Sex	Karyotype	MLPA							
			BTG1	*CDKN2A/B*	*EBF1*	*ETV6*	*IKAROS*	*PAR1*	*PAX5*	*RB1*
20406	5/F	46,XX	N	N	N	e 3-8	N	N	e 2-8	N
20410¥	3/M	Failed	N	WG	N	N	N	N	e 2-6	N
20453	5/F	46,XX,der(12)t(12;?15)(p?13;?q?)/46,XX	e 2-3'	N	N	WG	N	N	e 2-6	N
20519	2/F	Failed	N	N	N	N	N	N	e 2-6	N
20521	3/F	46,XX,add(12)(p1?),?t(12;21)(p13;q22),+mar,inc [cp]/45,XX,der(8;12)(q10;q10),?t(12;21)(p13;q22)/46,XX	N	N	N	WG	N	N	N	N
20571	8/F	45,X,-X,?del(12)(p1?p13),?t(12;21)(p13;q22),inc/46,XX	N	WG	N	e 2-8	N	N	N	N
20620	9/M	46,XY	N	N	N	N	N	N	N	N
20624	8/M	47,XY,add(11)(q2),add(12)(p1),?t(12;21)(p13;q22),add(19)(p1),add(20)(q13),+der(21)t(12;21)[cp]/46,XY	N	N	N	N	N	N	N	N
20626	3/F	46,XX,del(12)(p12)	N	N	N	WG	N	N	e 2-6	N
20627	2/M	46,XY	N	WG	N	N	N	N	N	N
20634	2/M	46,XY,del(12)(p12p13),?t(12;21)(p13;q22)/46,XY	N	N	N	WG	N	N	N	e 19-26
20694	2/M	46,XY	N	N	N	WG	N	N	N	N
20723	6/M	46,XY,der(2)?t(2;?)(?p11.2;?),?dup(21)(q22)[cp]/46,XY	N	WG	N	N	N	N	e 1-6	N
20733	3/M	46,XY,?t(12;21)(p13;q22)/?46,idem,add(19)(p13)	N	N	N	N	N	N	N	N
20739	3/F	Failed	e 2-3'	N	N	bi e 1, mono e 8	N	N	N	N

Reg. ID	Age€/ Sex	Karyotype	MLPA							
			BTG1	*CDKN2A/B*	*EBF1*	*ETV6*	*IKAROS*	*PAR1*	*PAX5*	*RB1*
20747	3/M	46,XY,del(6)(q?13q?21)/46,XY	N	N	N	WG	N	N	N	N
20749	5/F	46,XX	N	N	N	e 1-2, 8	e 2-3	N	N	N
20751	3/F	45,X,-X/46,XX	N	N	N	e 5-8	N	N	N	N
20752	6/F	46,XX	N	N	N	e 5-8	N	N	N	N
20754¥	5/M	46,XY,der(14;21)(q10;q10),+21c	N	N	N	WG	N	N	WG	N
20755	4/M	Failed	N	N	N	mono e 1-5, bi e 8	N	N	N	N
20760	10/M	87,XYY,-X,-1,-3,+5,-6,-9,+10,-14,-15,inc[cp]/46,XY	N	N	N	N	N	N	N	N
20768 Ψ	1/M	46~47,XY,+21,?add(22)(q1)[cp]	N	N	N	e 8¥	N	N	N	N
20770	4/F	46,XX,?t(12;21)(p13;q22)/45,X,-X,add(12)(p1),?t(12;21)(p13;q22)	N	N	N	N	N	N	N	N
20776	7/F	46,XX,del(5)(q1?5q3?3),del(12)(p1?3)/46,XX	N	WG	N	WG	N	N	N	N
20878	6/F	46,XX,del(5)(q13q23),add(16)(p12),-17,add(21)(p11),+add(21)(p11)/46,XX	e 2-3'	N	N	WG	N	N	e 2-10	N
20951	5/F	46,XX	N	N	N	N	N	N	e 1-6	N
21190	12/F	Failed	mono e 2, bi 3'	WG	N	WG	N	N	N	N
21200 Ψ	6/F	48,XX,del(5)(q2?2q3?5),+10,+21	N	N	WG	bi e 8¥	N	N	N	N

Reg. ID	Age€/ Sex	Karyotype	MLPA							
			BTG1	***CDKN2A/B***	***EBF1***	***ETV6***	***IKAROS***	***PAR1***	***PAX5***	***RB1***
21250	9/F	47,XX,?del(12)(p13p13),?t(12;21)(p13;q22),?+21 /46,XX	N	*CDKN2A*	N	e 2-8	N	N	N	N
21316	3/F	46,XX,del(12)(p11p13)/46,XX	N	N	N	mono e 2-8, bi e 1	e 1	N	e 2-8	N
21335	3/M	46,XY,del(4)(p12),i(6)(p10),add(11)(p11),?add(1 2)(p1?2),-15,+mar[cp]/46,XY	N	N	N	N	N	N	N	N
21340	3/M	46,XY	N	N	N	WG	N	N	N	N
21367	3/F	Failed	N	N	N	e 2-5	N	N	e 2-5	N
21390	4/F	Failed	e 2-3'	N	N	N	N	N	N	N
21398	6/F	72-74,XX,-X,-6,+10,+14,+17,+4mar[cp]/46,XX	N	N	N	N	N	N	WG	N
21430	4/M	Failed	N	N	N	N	N	N	N	N
21435	2/M	46,XY,add(12)(p1),-14,-14,+2mar/46,XY	N	N	N	N	N	N	N	N
21436	12/M	Failed	e 2-3'	WG	N	WG	N	N	N	N
21559	3/M	46,XY	N	N	N	mono e 1-5, bi e 8	N	N	N	N
21571	4/M	?85~92,XXYY,?t(12;21)(p13;q22)x2,inc[cp]/46,XY	N	N	N	e 2-8	N	N	N	N
21606	4/M	46,XY,t(4;12)(q21;p13)/46,XY	N	N	N	N	N	N	N	N
21618	4/M	46,XY,add(2)(p1),add(7)(p1),add(12)(p1)/46,ide m,t(3;10)(q21;q26)/46,XY	N	N	N	WG	N	N	e 2-6	N
21667	3/M	46,XY,del(11)(q23),i(21)(q10)[cp]/46,XY	N	WG	N	N	N	N	N	N

Reg. ID	Age€/ Sex	Karyotype	MLPA							
			BTG1	*CDKN2A/B*	*EBF1*	*ETV6*	*IKAROS*	*PAR1*	*PAX5*	*RB1*
21709	7/M	47,XY,?add(7)(q3?2),-12,?del(13)(q?2q?3),+der(21)t(12;21)(p13;q22),+mar/46,XY	e 2-3'	N	N	N	N	N	N	N
21732	5/F	Failed	N	WG	N	e 1	N	N	N	WG
21741	3/M	91~92,XXYY,add(4)(p12)x2,add(5)(q31),-6,del(6)(q1q2)x2,-9,add(12)(p1?)x2,add(12)(p13)x2,-14,-17,+1~4mar[cp]/46,XY	N	N	N	e 1-5	N	N	N	N
21797	2/F	Failed	N	WG	N	WG	N	N	N	N
21820¥	2/M	Failed	N	N	N	N	N	N	e 2-8	N
21870	7/M	46,XY,del(12)(p12)/46,XY	N	N	N	WG	N	N	N	N
21871	8/M	46,XY,add(1)(p36),t(1;12)(p34;p13),add(2)(p?),add(6)(q1),add(22)(q13),inc[cp]/46,XY	N	N	WG	N	N	N	N	N
21910	3/F	47,XX,+21/46,XX	N	N	N	N	N	N	e 2-6	N
21926	2/F	46,XX,inc	N	WG	N	WG	N	N	N	N
21991	8/M	46,XY,add(6)(q1),?t(12;21)(p13;q22)/46,XY,?t(12;21)(p13;q22)	N	WG	N	e 1	N	R	N	N
22010	3/F	45,XX,dic(12;19)(p11;q13),add(13)(q?14)/46,XX	N	N	N	WG	N	N	N	WG
22021	7/F	85~91,XX,-X,-X,+6,+6,add(6)(q1)x2,del(6)(q1q2)x2,-8,-12,add(12)(p1),-13,-14,-15,-16,+21,+21,+1~3mar[cp]/46,XX	N	N	N	mono e 1-5, bi e 8	N	N	N	WG
22029	4/F	44,X,-X,del(11)(q22q23),der(12)t(12;16)(p13;q13),-	N	N	N	WG	N	N	N	N

Reg. ID	Age€/ Sex	Karyotype	MLPA							
			BTG1	*CDKN2A/B*	*EBF1*	*ETV6*	*IKAROS*	*PAR1*	*PAX5*	*RB1*
		16,add(21)(p12)/46,XX								
22044 ψ	1/F	Failed	N	N	N	e 8¥	N	N	e 2-6	N
22047	3/F	Failed	N	N	N	N	N	N	e 1-2	N
22089	11/F	Failed	N	N	N	N	N	N	N	N
22107	4/F	Failed	N	WG	N	WG	N	N	N	N
22115	4/M	46,XY,inv(12)(p13q13)/46,XY	N	N	N	N	N	N	e 2-5	N
22131	12/F	46,XX	N	N	N	WG	N	N	N	mono e 6-14, bi e 19-26
22164	2/F	46,XX,del(12)(p?11.2)/46,idem,del(12)(p?11.2)/46,XX	N	N	N	WG	N	N	N	N
22195	4/F	46,XX,der(12)t(12;14)(p13;q2?),der(14)t(14;21)(q2?;q2?),der(14)t(14;21)(q2?;q2?),+der(14)t(14;?)(q2?;?),-15,?der(21)t(12;21)(p13;q22),-22,+der(?)t(?;14)(?;q2?)/46,XX	N	N	N	N	N	N	e 2-5	N
22196	2/F	46,XX	N	N	N	WG	N	N	N	N
22236	5/M	47,XY,del(12)(p12),+21/46,XY	N	N	N	WG	N	N	e 2-6	N
22240	15/M	46,XY,add(11)(q22)/46,XY	N	N	N	N	N	N	N	N
22247	6/F	46,XX,del(12)(p?13)/92,XXXX,del(12)(p?13)x2/46,XX	N	N	N	WG	N	N	N	N
22264	17/F	46,XX	N	WG	N	e 1-5	N	N	N	N

Reg. ID	Age€/ Sex	Karyotype	MLPA							
			BTG1	***CDKN2A/B***	***EBF1***	***ETV6***	***IKAROS***	***PAR1***	***PAX5***	***RB1***
22268	9/F	45,X,-X,add(9)(q?22),del(12)(p11)	e 2-3'	*CDKN2B*	N	e 2-8	N	N	N	N
22270	10/M	46,XX	e 2-3'	N	N	N	N	N	N	N
22289	4/M	Failed	N	N	N	e 1-5	N	N	e 2-5	N
22324	13/M	91<4n>,XXYY,add(3)(q?),del(4)(q2),del(5)(q22q31),-6,-7,?+8,-9,+?10,+?10,-11,add(12)(p11),?del(12)(p11p13)x2,-13,-14,+?16,-17,-20,+22,+2mar	e 2-3'	N	N	bi e 8¥	N	N	N	N
22344	9/M	46,XY	N	WG	N	WG	N	N	N	N
22372	5/F	Failed	e 2-3'	WG	N	WG	N	N	N	WG
22384	8/F	Failed	N	WG	N	N	N	N	N	N
22403	2/F	46,XX	N	WG	N	N	N	N	N	e 19-26
22419	3/F	45,X,-X,add(2)(p1),add(3)(p1),t(14;18)(q1;p1),-15,-17,-20,+3mar,inc/46,XX	N	N	N	N	N	N	N	N
22467	5/F	46,XX,add(16)(q2?1)[10]/46,XX	e 2-3'	N	N	N	N	N	N	N
22561	3/M	46,X,-Y,add(12)(p?12),add(12)(q?24),+mar	N	WG	N	e 2-8	N	N	N	N
22562	4/M	46,XY	N	N	N	N	N	N	N	N
22565	3/F	44,X,-X,dic(9;12)(p1?1;p1?1)/46,XX	N	WG	N	WG	N	N	WG	N
22574 Ψ	4/F	46,X,-X,-4,del(11)(q23q25),add(12)(p11),+21,+mar/46,XX	e 2-3'	N	N	e 8¥	N	N	N	N
22581	2/M	46,XY,t(2;5)(p11.2;q33),add(12)(p13)/46,XY	N	N	N	WG	N	N	N	N
22639 Ψ	3/M	46,Y,add(X)(p2),inc	N	N	N	e 8¥	N	N	e 2-5	N

Reg. ID	Age€/ Sex	Karyotype	MLPA							
			BTG1	*CDKN2A/B*	*EBF1*	*ETV6*	*IKAROS*	*PAR1*	*PAX5*	*RB1*
22668	3/F	46,XX	N	N	N	mono e 1-5, bi e 8	N	N	e 1-6	N
22721	11/F	48~49,XX,del(1)(q21),add(2)(p?1),?add(11)(p1),-12,-14,-17,+?18,+21,+21,+2mar,inc[cp]/46,XX	N	N	N	N	N	N	N	N
22752	4/M	Failed	N	N	N	N	N	N	e 1-6	N
22758	3/M	46,XY,inc	N	N	N	N	N	N	N	N
22778	4/F	46,XX,add(9)(p21),add(12)(p13),add(14)(q11.2)[cp]/46,XX	N	WG	N	WG	N	N	N	N
22809	3/F	46,XX,add(9)(q34)/46,XX	N	N	N	N	N	N	N	N
22817	3/M	46,XY,del(12)(p11.2),?inv(12)(p1q21~23)/46,XY	N	N	N	WG	N	N	N	N
22974	3/F	46,XX	N	N	N	WG	N	N	N	N
23001	4/M	46,XY	e 2-3'	N	N	N	N	N	N	N
23013	15/F	46,XX,del(12)(p12p13)/46,XX	N	N	N	WG	e 4-8	N	e 1-5	N
23017	4/F	46,XX,add(12)(p11.2)/46,XX	N	N	N	N	N	N	e 1-6	N
23084	4/M	46,XY,add(6)(q21),t(10;17)(p13;q11.2),-12,der(20)t(12;20)(q13;q13.3),+mar/46,XY	N	N	N	N	N	N	N	N
23087	13/M	46,XY,t(3;16)(p21;p13)[cp]/46,XY	N	N	N	N	N	N	N	N
23120	5/F	45,X,-X,t(8;12)(p21;p13),add(15)(q24)[cp]/46,XX	N	N	N	N	N	N	N	N
23121	4/F	46,XX,add(12)(p13)/46,XX	N	N	N	WG	N	N	N	N
23125	2/M	Failed	N	WG	N	WG	N	N	WG	N
23128	3/M	46,XY	N	WG	N	N	N	N	N	N

Reg. ID	Age€/ Sex	Karyotype	MLPA							
			BTG1	***CDKN2A/B***	***EBF1***	***ETV6***	***IKAROS***	***PAR1***	***PAX5***	***RB1***
23138	3/M	46,XY,dic(9;12)(p1;p1),der(13;14)(q10;q10)/45,XY,der(13;14)(q10;q10)	N	WG	N	WG	N	N	WG	N
23148	2/M	38~47,X,-Y,add(1)(p3?),-2,?del(6)(q2?1),?add(7)(p?),-11,-13,-15,+21,+1~9mar,inc[cp]	N	N	N	N	N	N	N	N
23198	5/F	46,XX,t(4;13)(p1?3;q14),del(12)(p1),-20,add(21)(q1),-21,+3mar/46,XY	N	N	N	WG	N	N	N	WG
23240	6/F	46~47,XX,add(12)(p1),add(19)(p13),add(20)(p1),+21,inc[cp]/46,XX	N	WG	WG	WG	N	N	e 2-5	N
23275 ψ	2/F	Failed	N	N	N	e 8¥	N	N	e 2-6	N
24247	14/F	45,X,-X,del(12)(p11),add(17)(p13)/46,XX	N	WG	N	WG	N	N	N	N
24347¥	2/F	Failed	N	N	N	N	N	N	N	N

Adolecents/ young adults (added from Chapter 6) not included in Chapter 3										
Reg. ID	Age /Sex	Karyotype	MLPA							
			BTG1	***CDKN2A/B***	***EBF1***	***ETV6***	***IKAROS***	***PAR 1***	***PAX5***	***RB1***
7597	21/M	46,XY	N	WG	N	N	N	N	e 2-5	e 19-26

1927	27/F	96-97<4n>,XX,-X,-X,+5,del(6)(q?),add(9)(p22), del(12)(?p13),+13,i(17)(q10),+18,+18, +22[cp] / 46,XX	WG	N	N	WG	N	N	N	N
3367	24/F	45,X,-X,der(3,9,12,21)t(3;12;21)(q1?;p13;q22)t(3;9)(q2?;q1?) ,del(6)(q1?)[2]45,idem, -del(6),+der(6) del(6)(q1?)rdup(6)(pter>q1?)	e 2-3'	WG	N	N	N	N	N	N
1780	15/M	46,XY	WG	N	N	N	N	N	N	WG
3936	50/M	>70,inc/ 46,XY	WG	N	N	WG	N	N	e 2-10	WG

Note: € in years, ¥ : Down syndrome cases, *: The relapse cases studied in Chapter 5, ‡: The non-relapse group studied in Chapter 5, Ψ: isolated ETV6 exon 8 not included in the total number of non-rearranged ETV6 deletions, N: Normal, R: rearrangement, e: exons, WG: whole gene, 3': 3' end region, mono: monoallelic deletion, bi: biallelic deletion.

Appendix B. ETV6 deletions detected by FISH but masked by MLPA detection threshold criteria and sensitivity.

Reg I.D	FISH	*ETV6*_Ex01A	*ETV6*_Ex01B	*ETV6*_Ex02	*ETV6*_Ex03	*ETV6*_Ex05	*ETV6*_Ex08
3269	Del *ETV6*+ gain der(21) (2 copies) (36%)	1.364	1.348	1.244	1.316	1.292	0.746
3359	Del *ETV6*+ gain der(21) (1 copy) (93%)	1.011	0.945	1.026	0.959	0.925	0.529
3472	Del *ETV6* (16%)	0.893	0.929	0.945	0.977	0.478	1.069
3602	Del *ETV6*+ gain der(21) (1 copy) (55%)	0.981	1.016	0.992	1.018	1.012	0.605
3684	Del *ETV6* (7%)	0.974	0.993	0.917	0.975	0.901	0.945
3726	Del *ETV6* (26%)	0.876	0.859	0.892	0.864	0.906	0.978
4013	Del *ETV6* (37%)	0.979	0.961	0.94	0.908	0.961	0.937
4554	Del *ETV6* (6%)	0.997	0.96	0.954	0.949	0.972	0.987
4616	Del *ETV6* (13%)	0.947	0.901	0.91	1.02	0.804	0.928
4620	Del *ETV6* (66%)	0.81	0.83	0.791	0.862	0.823	0.853
4707	Del *ETV6*+ gain der(21) (1 copy) (35%)	1.05	0.999	1.008	1.082	0.861	0.611
4758	Del *ETV6*+ gain der(21) (1 copy) (92%)	1.046	1.075	0.956	1.015	0.954	0.584
5038	Del *ETV6* (16%)	0.924	0.953	0.858	0.93	0.914	0.899
7149	Del *ETV6* (30%)	0.81	0.888	0.878	0.907	0.852	0.733
7552	Del *ETV6* (54%)	0.85	0.98	0.948	0.958	0.864	1.073
9873	Del *ETV6*+ gain der(21) (1 copy) (29%)	0.986	1.068	0.929	0.955	0.815	0.802
	Del *ETV6* (23%)						
10083	Del *ETV6*+ gain der(21) (1 copy) (39%)	1.053	1.173	1.162	0.865	1.005	0.923
10252	Del *ETV6*+ gain der(21) (1 copy) (93%)	0.882	0.917	0.92	0.979	0.895	0.636
11001	Del *ETV6* (6%)	0.98	0.928	0.917	0.933	0.909	0.921
11052	Del *ETV6*+ gain der(21) (1 copy) (14%)	0.928	0.984	0.896	0.977	0.956	0.353
11636	Del *ETV6*+ gain der(21) (1 copy) (90%)	1.03	0.958	0.936	0.939	0.95	0.566
11675	Del *ETV6* (11%)	0.945	1.014	0.612	0.868	0.944	0.948
11707	Del *ETV6*+ gain der(21) (1 copy) (8%)	1.353	1.313	1.292	1.252	1.309	0.98

Reg I.D	FISH	*ETV6*_Ex01A	*ETV6*_Ex01B	*ETV6*_Ex02	*ETV6*_Ex03	*ETV6*_Ex05	*ETV6*_Ex08
11833	Del *ETV6* (18%)	0.878	0.945	0.516	0.838	0.911	0.865
12018	Del *ETV6* (11%)	0.981	0.947	1.015	0.903	1.003	0.85
12244	Del *ETV6*+ gain der(21) (1 copy) (5%)	1.03	1.024	0.884	0.939	0.88	1.109
12737	Del *ETV6*+ gain der(21) (1 copy) (51%)	0.943	0.959	0.929	0.959	1.051	0.715
	Del *ETV6* (15%)						
19688	Del *ETV6* (51%)	0.87	0.949	0.941	0.834	0.973	0.941
20311	Del *ETV6* (5%)	0.971	0.867	0.808	0.927	0.952	0.917
20519	Del *ETV6* (26%)	0.811	0.771	0.824	0.765	0.7	0.816
20624	Del *ETV6*+ gain der(21) (1 copy) (64%)	0.987	0.998	0.958	0.877	1.106	0.729
20627	Del *ETV6* (19%)	0.915	0.95	0.755	0.756	0.827	0.882
	Del *ETV6*+ gain der(21) (1 copy) (18%)						
20733	Del *ETV6*+ gain der(21) (1 copy) (8%)	1	1.021	1	1.03	1.008	1.093
20770	Del *ETV6* (85%)	0.884	0.927	0.91	0.883	0.898	0.792
20951	Del *ETV6* (25%)	0.954	0.983	0.997	0.978	1.007	0.967
21200	Del *ETV6* (14%)	1.105	1.114	1.118	1.074	1.149	0.203
	Del *ETV6*+ gain der(21) (1 copy) (13%)						
21335	Del *ETV6* (12%)	0.905	0.948	0.965	1	1.009	0.947
21390	Del *ETV6* (28%)	0.818	0.838	0.743	0.851	0.75	0.842
21435	Del *ETV6* (66%)	0.795	0.787	0.769	0.809	0.837	0.772
23017	Del *ETV6* (84%)	0.832	0.827	0.776	0.762	0.837	0.781
23084	Del *ETV6* (18%)	0.955	1.004	1.059	0.947	0.928	0.818
23275	Del *ETV6* (16%)	0.952	0.988	0.951	0.931	0.936	0.623

Note: MLPA probe ratios: Probe ratios between 0.75 and 1.3 were considered to be within the normal range, with 2 being the normal copy number, while values below 0.75 or above 1.3 indicated loss or gain of genetic material, respectively, and corresponded to copy numbers of 1 and 3, respectively. A value below 0.25 indicated biallelic loss which represented a copy number of 0.

Appendix C. The detailed list of FISH clones used in this study.

Clone name	Br.	Version no.	Start breakpoint	End breakpoint	Chromosome position	Genes
RP11-525I3	E	60	12086278	12183936	12p13.2	
RP11-434C1	E	46	11529145	11739576	12p13.2	*ETV6*
RP11-267J23	E	60	12183937	12374586	12p13.2	*BCL2L14, LRP6*
RP11-757G14	E	60	12374587	12516582	12p13.2	*LOH12CR3*
RP11-180M15	E	60	12724970	12880857	12p13.2-12p13.1	*CREBL2, GPR19, CDKN1B, APOLD1*
RP11-59H1	E	60	12880858	13000779	12p13.1	*hsa-mir-613, DDX47*
RP11-392P7	E	60	13000780	13160327	12p13.1	*GPRC5A/D, HEBP1, hsa-mir-614*
RP11-377D9	E/ U	60/feb 2009	13160328	13251317	12p13.1	
RP11-688K16	E	60	13251318	13363944	12p13.1	*GSG1, EMP1*
RP11-161A14	E/ U	60/feb 2009	13363945	13539444	12p13.1	*C12orf36*
RP11-4N23	E	60	13614529	13701029	12p13.1	
RP11-72J9	E	60	14245642	14339661	12p13.1	
RP11-515B12	E	60	14449642	14613718	12p13.1	*ATF7IP*
RP11-233G1	E	60	14944108	15122278	12p12.3	*WBP11, C12orf60/69, ART4, MGP, ERP27, ARHGDIB*
RP11-365O10	E	37	15259830	15381854	12p12.3	
RP11-74N9	E	37	17093825	17169824	12p12.3	
RP11-633O13	E/ U	60/feb2009	17889133	18071123	12p12.3	
RP11-604C20	E	60	19830459	19957265	12p12.3	

Clone name	Br.	Version no.	Start breakpoint	End breakpoint	Chromosome position	Genes
RP11-405A12	E	41	19976170	20140548	12p12.2	
RP11-125O5	E	41	21153764	21303649	12p12.1	*SLCO1B1*
RP11-12D15	E	41	22250546	22369579	12p12.1	*ST8SIA1*
RP11-444N1	E	41	23483119	23668225	12p12.1	*SOX5*
RP11-318G08	E	41	25394082	25557296	12p12.1	*IFLTD1(part of)*
RP11-396G11	E	45	35424632	35624246	21q22.12	*AML1ba (part of)*
RP11-714H12	E/U	60/Feb 2009	37040715/37039716	37206890/37207890	21q22.12	*hsa-mir-802*
RP11-773I18	E/U	60/Feb 2009	37255989/37254990	37426099/37427099	21q22.12	*SETD4*
RP11-23N18	E/U	60/Feb 2009	37651721/37650722	37833845/37834845	21q22.12- 21q22.13	*MORC3, CHAF1B*
RP11-383L18	E/U	60/Feb 2009	38188159/38187160	38389773/38390773	21q22.13	*HLCS*
RP11-777J19	E/U	60/Feb 2009	38720388/38719389	38886895/38887895	21q22.13	*DYRK1A*
WI2-2188G13	U	39845	12039458	12076941	12p13.2	*ETV6 exon 8*
WI2-3280E3 *	U	Feb-09	176833554	176876728	3q26.32	*3' TBL1XR1 part*
RP11-499P15	E	71	176836521	176920665	3q26.32	TBL1XR1
WI2-3156C10*	U	Feb-09	176918625	176960907	3q26.32	*5'TBL1XR1 area*
WI2-2979I5*	U	Feb-09	176966617	177011487	3q26.32	
RP11-484E7	E	45	76248984	76475216	3p12.3	*TBL1XR1 Control*
Cep 3					Centromeric chromosome 3	
RP11-278J6	E	50	142640980	142798050	5q31.3	*NR3C1*

Clone name	Br.	Version no.	Start breakpoint	End breakpoint	Chromosome position	Genes
RP11-49C20	E	45	40469944	40650640	5p13.1	*NR3C1 Control*
RP11-76G10	E	50	149237494	149397852	4q31.23	*NR3C2*
RP11-186E11	E	50	149420652	149539282	4q31.24	
RP11-655B23*	E	68.37	149318833	149500077	4q31.23	*5' NR3C2 part*
RP11-572O17	U	Mar 2006	1595349	1784573	4p16.3	*NR3C2 Control*
RP11-231E6	E	68.37	112099169	112225162	3q13.2	*BTLA*
RP11-90K6 ¥	E	68.37	112021325	112051638	3q13.2	*CD200*
RP11-54L6	E	68.37	18526330	18628552	3p24.3	*BTLA control*
CTD-2251F23	U	Feb-09	40329778	40452050	15q15.1	*BMF*
Cep 15					Centromeric chromosome 15	*BMF Control*
RP11-149I2	E	71	21911259	22010413	9p21.3	*CDKN2A*
WI2-3139G6	U	Feb-09	36981616	37016002	9p13.2	*PAX5 exon 2*
WI2-1118A2	U	Feb-09	36937043	36977242	9p13.2	*PAX5 exon 7*
CEP 9					Centromeric chromosome 9	*PAX5,CDKN2A/B* control
WI2-1903N19	U	Feb-09	11862785	11903576	12p13.2	*ETV6 exon 2*
WI2-1132J19	U	Feb-09	11811608	11848801	12p13.2	*ETV6 exon 1*
RP11-24B21	E	71	92378756	92536690	12q21.33	*BTG1*
Cep 12					Centromeric chromosome 12	*BTG1 Control*

Note: *: These specific probes were only utilised for discrepant cases and not in the initial screening, ¥: Withdrawn because of suboptimal signals. Br. Genome browser, U UCSC, E Ensembl.

Appendix D. Cut off values of the FISH probes used in the whole PhD project.

Probe set	Gene	Cut off value
Mapping der(12)t(12;21) deletion	Multiple genes	5%
RUNX1-ETV6 DC SF	*RUNX1, ETV6*	6%
ETV6 DC BA	*ETV6*	4%
RUNX1 DC BA	*RUNX1*	4%
CEP12-subtel 12p	*NA*	2%
WI2-2188G13 (G248P86604D7)[*ETV6* exon 8] /RP11-434C1 [*5'ETV6*]	*ETV6 exon 8*	4%
WI2-2188G13 (G248P86604D7)[*ETV6* exon 8] /RP11-396G11 [*5'RUNX1*]		4%
WI2-3280E3 (G248P89324C2)/CEP3	*TBL1XR1*	2%
RP11-499B15/RP11-484E7 or CEP3		4%
WI2-3156C10 (G248P8042B5)/CEP3		2%
WI2-2979I5 (G248P88336E3)/CEP3		2%
RP11-278J6/RP11-49C20	*NR3C1*	2%
RP11-76G10/RP11-572O17	*NR3C2*	2%
RP11-186E11/RP11-572O17		4%
RP11-655B23/RP11-572O17		2%
RP11-231E6/RP11-54L6 or CEP 3	*BTLA*	0%
CTD-2251F23/Cep 15	*BMF*	2%
RP11-24B21/CEP12	*BTG1*	2%
RP11-149I2/CEP 9	*CDKN2A/B*	4%
G248P800846D3/CEP9	*PAX5 exon 2*	2%
G248P82998A1/CEP9	*PAX5 exon 7*	2%
WI2-1903N19 (G248P86897G10)/CEP12	*ETV6 exon 2*	4%
WI2-1132J19 (G248P84985E10)/CEP12	*ETV6 exon 1*	2%

Note: cut off value calculated as the mean false positive rate (MFPR) X 3 standard deviation.

Appendix E. Detailed FISH, MLPA and SNP6 results of patients with deleted and duplicated der(12)t(12;21).

Group A		
Patient No.	**Investigations**	**Results**
3052	TEL/AML1	NA
	ETV6 DC BA	1R 0G 1F [96%], 0R 0G 2F [4%]
	RUNX1 DC BA	1R 1G 1F [95%], 0R 0G 2F [4%]
	Mapping Deletion	
	RP11-4N23	2R 1G 0F [89%], 2R 2G 0F [11%]
	RP11-365O10	2R 2G 0F [80%], 2R 1G 0F [4%]
3053	TEL/AML1	NA
	ETV6 DC BA	1R 0G 1F [98%]
	RUNX1 DC BA	1R 1G 1F [91%], 0R 0G 2F [5%]
	Mapping Deletion	
	RP11-633O13	2R 1G 0F [94%], 2R 2G 0F [6%]
	RP11-604C20	1R 1G 1F [93%], 2R 2G 0F [7%]
3135	TEL/AML1	1R 1G 1F [58%]
	ETV6 DC BA	1R 0G 1F [90%], 0R 0G 2F [6%]
	RUNX1 DC BA	1R 1G 1F [91%], 0R 0G 2F [4%]
	Mapping Deletion	
	RP11-392P7	2R 1G 0F [90%], 2R 2G 0F [7%]
	RP11-377D9	1R 1G 1F [89%], 2R 1G 1F [7%], 2R 2G 0F [4%]
	RP11-396G11/RP11-4N23/ RP11- 180M15	1R 1F(green/gold) 1F (green/red) [93%], 2R 2F(green/gold)[7%]
3189	TEL/AML1	NA
	ETV6 DC BA	1R 0G 1F [89%], 0R 0G 2F [9%]
	RUNX1 DC BA	1R 1G 2F [85%], 0R 0G 2F [9%]
	Mapping Deletion	
	RP11-233G1	3R 1G 0F [83%], 2R 2G 0F [17%]
	RP11-365O10	2R 1G 1F [80%], 1R 1G 1F [5%], 2R 2G 0F [15%]
3498	TEL/AML1 ES	2R 1G 1F [62%], 1R 1G 1F [23%]
	ETV6 DC BA	1R 0G 1F [94%], 0R 0G 2F [5%]
	RUNX1 DC BA	1R 1G 1F [81%], 0R 0G 2F [8%]
	Mapping Deletion	
	RP11-444N1	2R 1G 0F [85%], 2R 2G 0F [15%]
	RP11-318G08	1R 1G 1F [89%], 2R 2G 0F [11%]
4192	TEL/AML1	NA
	ETV6 DC BA	1R 0G 1F [94%], 0R 0G 2F [4%]
	RUNX1 DC BA	1R 1G 2F [74%], 1R 1G 1F [19%], 0R 0G 2F [4%]
	Mapping Deletion	
	RP11-12D15	3R 1G 0F [67%], 2R 1G 0F [24%], 2R 2G 0F [9%]
	RP11-444N1	2R 1G 1F [62%], 1R 1G 1F [31%], 2R 2G 0F [7%]
4287	TEL/AML1	NA

Group A		
Patient No.	**Investigations**	**Results**
	ETV6 DC BA	1R 0G 1F [94%], 0R 0G 2F [3%]
	RUNX1 DC BA	1R 1G 1F [86%], 0R 0G 2F [11%]
	Mapping Deletion	
	RP11-688K16	2R 1G 0F [92%], 2R 2G 0F [8%]
	RP11-161A14	1R 1G 1F [84%], 2R 2G 0F [16%]
	RP11-396G11/RP11-4N23/ RP11- 180M15	1R 1F(green/gold) 1F (green/red) [92%], 2R 2F(green/gold)[8%]
4854	TEL/AML1	NA
	ETV6 DC BA	1R 0G 1F [87%], 0R 0G 2F [9%]
	RUNX1 DC BA	1R 1G 1F [86%], 0R 0G 2F [11%]
	Mapping Deletion	
	RP11-688K16	2R 1G 0F [84%], 2R 2G 0F [16%]
	RP11-161A14	1R 1G 1F [90%], 2R 2G 0F [10%]
4947	TEL/AML1	NA
	ETV6 DC BA	1R 0G 1F [89%], 2R 0G 1F [9%]
	RUNX1 DC BA	1R 1G 2F [88%], 0R 0G 2F [6%]
	Mapping Deletion	
	RP11-180M15	3R 1G 0F [84%], 2R 2G 0F [16%]
	RP11-59H1	2R 1G 1F [87%], 2R 2G 0F [13%]
4969	TEL/AML1	NA
	ETV6 DC BA	2R 0G 1F [73%], 3R 0G 1F [12%], 4R 0G 1F [7%], 0R 0G 2F [6%]
	RUNX1 DC BA	1R 2G 1F [74%], 2R 2G 1F [20%]
	Mapping Deletion	
	RP11-515B12	2R1G0F [89%], 2R2G0F [6%], 3R1G0F [5%]
	RP11-233G1	1R1G1F [82%], 2R2G0F [10%], 2R1G1F [8%]

Group B		
Patient No.	**Invesigations**	**Results**
3990	TEL/AML1	NA
	ETV6 DC BA	1R 0G 0F [72%], 0R 0G 2F [23%]
	RUNX1 DC BA	1R 1G 1F [69%], 0R 0G 2F [27%]
	Mapping Deletion	
	RP11-267J23	2R 0G 0F [90%], 2R 2G 0F [10%]
	RP11-757G14	1R 0G 1F [68%], 2R 2G 0F [32%]
4112	TEL/AML1	NA
	ETV6 DC BA	1R 0G 0F [63%], 1R 0G 1F [23%], 0R 0G 2F [7%]
	RUNX1 DC BA	1R 1G 1F [89%], 0R 0G 2F [5%]
	Mapping Deletion	
	RP11-233G1	2R 1G 0F [57%], 2R 0G 0F [28%], 2R 2G 0F [15%]

Group B		
Patient No.	**Invesigations**	**Results**
	RP11-365O10	2R 2G 0F [94%]
4133	TEL/AML1	NA
	ETV6 DC BA	1R 0G 0F [97%]
	RUNX1 DC BA	1R 1G 1F [93%], 0R 0G 2F [6%]
	Mapping Deletion	
	RP11-405A12	2R 1G 0F [94%], 2R 2G 0F [6%]
	RP11-125O5	1R 1G 1F [90%], 2R 2G 0F [10%]
4284	TEL/AML1	NA
	ETV6 DC BA	1R 0G 1F [71%], 1R 1G 0F [9%], 0R 0G 2F [14%]
	RUNX1 DC BA	1R 1G 1F [77%], 0R 0G 2F [21%]
	Mapping Deletion	
	RP11-12D15	2R 1G 0F [95%], 2R 2G 0F [11%]
	RP11-444N1	1R 1G 1F [86%], 2R 2G 0F [10%]
4358	TEL/AML1	NA
	ETV6 DC BA	1R 0G 1F [68%], 1R 1G 0F [9%], 0R 0G 2F [22%]
	RUNX1 DC BA	1R 1G 1F [79%], 0R 0G 2F [16%]
	Mapping Deletion	
	RP11-365O10	2R 1G 0F [89%], 2R 2G 0F [11%]
	RP11-405A12	Failed
3066	TEL/AML1	NA
	ETV6 DC BA	1R 0G 0F [77%], 0R 0G 2F [17%]
	RUNX1 DC BA	1R 1G 1F [75%], 0R 0G 2F [20%]

Group C		
Patient No.	**Investigations**	**Results**
3319	TEL/AML1	NA
	ETV6 DC BA	1R 1G 0F [93%], 0R 0G 2F [4%]
	RUNX1 DC BA	0R 1G 1F [93%], 0R 0G 2F [4%]
	Mapping Deletion	
	RP11-714H12	1R 1G 0F [89%], 2R 2G 0F [11%]
	RP11-773I18	2R 1G 0F [90%], 2R 2G 0F [10%]
4692	TEL/AML1	NA
	ETV6 DC BA	1R 1G 1F [75%], 1R 1G 0F [4%], 0R 0G 2F [17%]
	RUNX1 DC BA	0R 1G 1F [82%], 0R 1G 2F [4%], 0R 0G 2F [10%]
	Mapping Deletion	
	RP11-383L18	1R 2G 0F [83%], 2R 2G 0F [13%]
	RP11-777J19	1R 1G 1F [79%], 2R 2G 0F [19%]
4917	TEL/AML1	NA
	ETV6 DC BA	1R 1G 1F [67%], 1R 1G 0F [29%]
	RUNX1 DC BA	0R 1G 2F [91%], 0R 0G 2F [7%]

Group C		
Patient No.	**Investigations**	**Results**
	Mapping Deletion	
	RP11- 396G11	2R 2G 0F [50%], 2R 1G 0F [20%], 1R 2G 0F [5%]
	RP11-714H12	2R 1G 1F [50%], 2R 0G 1F [30%], 1R 1G 1F [11%], 2R 2G 0F [9%]
Ψ 3277	TEL/AML1	NA
	ETV6 DC BA	1R 1G 1F [30%], 0R 0G 2F [68%]
	RUNX1 DC BA	0R 1G 1F [89%], 1R 1G 1F [4%], 0R 0G 2F [6%]

Group D		
Patient No.	**Investigations**	**Results**
3130	TEL/AML1	NA
	ETV6 DC BA	1R 2G 0F [56%], 1R 1G 1F [34%], 1R 1G 0F [5%]
	RUNX1 DC BA	2R 1G 1F [58%], 1R 1G 1F [37%], 0R 0G 2F [4%]
	RP11-525I3/RP11-396G11 DC SF	1R 0G 2F [67%], 1R 1G 1F [24%], 0R 0G 2F [9%], 1R 0G 1F [7%]
	CEP12/Subtel12p	2R 1G 0F [69%], 2R 2G 0F [31%]
3726	TEL/AML1	2R 1G 1F [73%]
	ETV6 DC BA	1R 1G 1F [60%], 1R 2G 0F [26%], 0R 0G 2F [10%]
	RUNX1 DC BA	1R 1G 1F [59%], 2R 1G 1F [29%], 0R 0G 2F [11%]
	RP11-525I3/RP11-396G11 DC SF	1R 1G 1F [49%], 1R 0G 2F [30%], 2R 2G 0F [12%]
	CEP12/Subtel12p	2R 1G 0F [79%], 2R 2G 0F [20%]
4136	TEL/AML1	NA
	ETV6 DC BA	1R 2G 0F [84%], 1R 1G 1F [7%], 0R 0G 2F [4%]
	RUNX1 DC BA	2R 1G 1F [78%], 0R 0G 2F [13%]
	RP11-525I3/RP11-396G11 DC SF	1R 0G 2F [61%], 2R 0G 2F [24%], 1R 1G 1F [19%], 0R 0G 2F [10%], 2R 1G 1F [9%], 2R 2G 0F [12%]
	CEP12/Subtel12p	2R 1G 0F [79%], 2R 2G 0F [20%]
4281	TEL/AML1	NA
	ETV6 DC BA	1R 2G 0F [92%], 0R 0G 2F [5%]
	RUNX1 DC BA	2R 1G 1F [91%], 0R 0G 2F [5%]
	RP11-525I3/RP11-396G11 DC SF	1R 0G 2F [43%], 2R 1G 1F [27%], 1R 1G 1F [7%], 2R 2G 0F [19%]
	CEP12/Subtel12p	2R 1G 0F [90%], 2R 2G 0F [8%]
4560	TEL/AML1	NA
	ETV6 DC BA	1R 2G 0F [63%], 1R 1G 1F [20%], 1R 1G 0F [14%]
	RUNX1 DC BA	2R 1G 1F [60%], 1R 1G 1F [37%]
	RP11-525I3/RP11-	1R 0G 2F [59%], 1R 1G 1F [26%], 0R 0G 2F [9%],

Group D		
Patient No.	**Investigations**	**Results**
	396G11 DC SF	1R 0G 1F [6%]
	CEP12/Subtel12p	2R 1G 0F [51%], 2R 2G 0F [49%]
4637	TEL/AML1	NA
	ETV6 DC BA	1R 2G 0F [87%], 0R 0G 2F [11%]
	RUNX1 DC BA	2R 1G 1F [95%], 0R 0G 2F [4%]
	RP11-525I3/RP11-396G11 DC SF	1R 0G 2F [81%], 1R 1G 1F [7%], 0R 0G 2F [6%], 2R 2G 0F [8%]
	CEP12/Subtel12p	2R 1G 0F [88%], 2R 2G 0F [11%]
4678	TEL/AML1	NA
	ETV6 DC BA	1R 2G 0F [84%], 0R 0G 2F [11%]
	RUNX1 DC BA	2R 1G 1F [92%], 0R 0G 2F [6%]
	RP11-525I3/RP11-396G11 DC SF	1R 0G 2F [77%], 0R 0G 2F [11%], 1R 1G 1F [7%], 2R 2G 0F [6%]
	CEP12/Subtel12p	2R 1G 0F [82%], 2R 2G 0F [17%]
3472	TEL/AML1 ES	3R 1G 1F [41%], 2R 1G 1F [30%], 2R 0G 1F [13%], 3R 2G 0F [10%]
	ETV6 DC BA	1R 1G 1F [78%], 1R 2G 0F [16%], 0R 0G 2F [5%]
	RUNX1 DC BA	1R 1G 2F [84%], 2R 1G 2F [11%]
4040	TEL/AML1	NA
	ETV6 DC BA	1R 1G 0F [54%], 1R 2G 0F [24%], 0R 0G 2F [21%]
	RUNX1 DC BA	1R 1G 1F [65%], 2R 1G 1F [14%], 0R 0G 2F [20%]
4934	TEL/AML1	NA
	ETV6 DC BA	2R 2G 0F [65%], 2R 1G 0F [15%], 2R 1G 1F [6%], 0R 0G 2F [4%]
	RUNX1 DC BA	2R 2G 1F [83%], 1R 1G 1F [5%], 0R 0G 2F [5%]

Note: Group A: deletion of *3'ETV6* sequences from der(12)t(12;21) plus intact non-rearranged *ETV6*, Group B: deletion of *3'ETV6* sequences from der(12)t(12;21) plus deletion of non-rearranged *ETV6*, Group C: deletion of *5'RUNX1* sequences from der(12)t(12;21) and Group D: duplication of der(12)t(12;21) fusion gene, Ψ this patient showing discrepant FISH results which cannot be clarified due to unavailable material.

Appendix F. Detailed SNP6 data on deleted, duplicated der(12)t(12;21) and Down syndrome within ETV6-RUNX1 patients.

Reg ID	Abnormality	Size (kb)	GENES
3135‡	loss (1)(19.45[q31.3]-19.47[q31.3])	181	No genes
	gain (4)(19.09[q35.2]-19.10[q35.2])	111	Multiple genes
	loss (7)(14.20[q34]-14.25[q34])	397	Multiple genes
	gain (8)(69.91[q13.2]-146.30[q24.3])	58020	Multiple genes
	loss (9)(14.66[p22.3]-24.18[p21.3])	7854	Multiple genes
	loss (10)(97.80[q24.1]-98.07[q24.1])	264	Multiple genes
	loss (11)(85.41[q14.1]-117.10[q23.3])	26299	Multiple genes
	loss (12)(12.02[p13.2]-13.18[p13.1])	1004	Multiple genes
	loss (12)(92.28[q21.33]-92.51[q21.33])	232	*3' BTG1*
3189‡	LOH (12)(12.04[p13.2]-15.11[p12.3])	3068	Multiple genes
	gain (21)(11.08[p11.1]-48.10[q22.3])	36446	Multiple genes
3428*	loss (1)(19.08[q31.2]-19.14[q31.2])	618	No genes
	loss (3)(60.07[p14.2]-60.56[p14.2])	493	*FHIT*
	loss (4)(108.46[q25]-108.62[q25])	169	*PAPSS1*
	loss (5)(157.50[q33.3]-157.62[q33.3])	121	No genes
	loss (6)(24.99[p22.3]-29.48[p22.1])	4490	Multiple genes
	gain (6)(31.08[p21.33]-74.59[q13])	43510	Multiple genes
	loss (6)(78.73[q14.1]-170.98[q27])	92250	Multiple genes
	loss (7)(33.13[p14.3]-33.56[p14.3])	427	*RP9,BBS9*
	loss (10)(21.68[p12.31]- 22.27[p12.31])	596	Multiple genes
	loss (10)(120.91[q26.11]-135.51[q26.3])	14597	Multiple genes
	loss (12)(20.45[p12.2]-20.73[p12.2])	279	*PDE3A*
	loss (12)(0.15[p13.33]-16.39[p12.3])	16240	Multiple genes
	loss (17)(73.90[q25.1]-74.42[q25.1])	518	Multiple genes
	loss (18)(70.00[q22.3]-78.02[q23])	8015	Multiple genes
	loss (19)(33.40[q13.11]-33.62[q13.11])	221	*GPATCH1,RHPN2,* *C19orf40, CCDC123*
	loss (20)(10.42[p12.2]-10.52[p12.2])	101	*C20orf94*
	gain (21)(10.74[q11.2]-48.10[q22.3])	32000	Multiple genes
3472*	loss (1)(90.10[p22.2]-90.25[p22.2])	151	*LRRC8C*
	loss (5)(16.96[q35.1]-18.05[q35.3])	1090	Multiple genes
	loss (9)(21.90[p21.3]-22.19[p21.3])	289	*C9orf53,CDKN2A/B*

Reg ID	Abnormality	Size (kb)	GENES
	loss (12)(25.40[p12.1]-25.54[p12.1])	133	*5' KRAS*
	loss (12)(92.28[q21.33]-92.54[q21.33])	258	*BTG1*
	CNN LOH (18)(72.42[q22.3]-74.33[q23])	1910	Multiple genes
	gain(X)(12.98[q26.1]-15.49[q28])	2510	Multiple genes
	gain(21)(10.74[q11.2]-48.10[q22.3])	32960	Multiple genes
3684*	loss (9)(36.77[p13.2]-37.03[q13.2])	260	*PAX5*
	loss (11)(85.77[q14.2]-13.49[q25])	49180	Multiple genes
	loss (X)(91.16[q21.31]-91.29[q21.31])	134	*PCDH11X*
	gain(X)(12.31[q25]-15.52[q28])	3210	Multiple genes
	gain(21)(15.20[q11.2]-48.10[q22.3])	32890	Multiple genes
	CNN LOH (9)(11.10[p23]- [12.36[p23])	1260	No genes
3726¶	loss (4)(149.35[q31.23]-149.90[q31.23])	558	*NR3C2*
	loss (7)(142.10[q34]-142.47[q34])	376	*MTRNR2L6,PRSS1*
	gain (8)(64.33[q12.3]-146.30[q24.3])	81970	Multiple genes
	loss (9)(0.844[p24.3]-24.02[p21.3])	23176	Multiple genes
	gain (9)(136.72[q34.2]-136.91[q34.2])	187	Multiple genes
	LOH (11)(81.85[q14.1]-134.94[q25])	53090	Multiple genes
	loss (12)(0.87[p13.33]-9.36[p13.31])	8490	*PZP,A2M,C12ORF33, KLRG1*
	gain (12)(41.66[q12]-41.89[q12])	225	*PDZRN4*
	gain (21)(36.72[q22.12]-47.90[q22.3])	11180	No genes
	LOH (X)(100.34[q22.1]-154.15[q28])	53810	Multiple genes
4281¶Ψ	loss (3)(176.91[q26.32]-177.17[q26.32])	256	*TBL1XR1*
	loss (4)(149.34[q31.23]-149.90[q31.23])	563	*NR3C2*
	loss (6)(6.83[p25.1]-7.10[p25.1])	271	No genes
	LOH (6)(81.56[q14.1]-170.98[q27])	89	Multiple genes
	loss (9)(18.33[p22.2]-22.46[p21.3])	3882	Multiple genes
	gain (11)(134.81[q25]-134.94[q25])	137	No genes
	LOH (12)(0.19[p13.33]-8.36[p13.31])	8172	Multiple genes
	loss (12)(8.59[p13.31]-12.02[p13.2])	3415	Multiple genes
	loss (14)(61.85[q23.1]-62.23[q23.2])	381	*PRKCH,HIF1A*
	gain (14)(69.61[q24.1]-71.57[q24.2])	1830	Multiple genes
	gain (21)(36.39[q22.12]-48.10[q22.3])	11676	Multiple genes
	LOH (X)(2.70[p22.33]-155.23[q28])	87792	Multiple genes
4969‡	loss (2)(22.19[q36.1]-22.21[q36.1])	208	No genes
	loss (3)(11.21[q13.2]-11.22[q13.2])	156	*BTLA/CD200*
	LOH (6)(90.35[q15]-170.98[q27])	76969	Multiple genes
	Loss (9)(0.21[p24.3]-26.08[p21.2])	25465	Multiple genes
	gain (12)(0.15[p13.33]-12.03[p13.2])	11727	multiple genes
	loss (12)(12.03[p13.2]-14.58[p13.1])	2551	multiple genes

Reg ID	Abnormality	Size (kb)	GENES
	LOH (15)(41.38[q15.1]-41.50[q15.1])	121	*INO80,EXD1*
	gain (21)(10.74[p11.2]-36.42[q22.12])	25477	Multiple genes
	LOH (X)(27.04[p22.33]-87.88[q21.31])	60840	Multiple genes
9859Ψ	Loss (1)(49.94[p33]-50.00[p33])	53	*AGBL4*
	Loss (1)(231.01[q42.2]-231.54[q42.2])	525	Multiple genes
	Loss (1)(37.63[p34.3]-37.69[p34.3])	55	No genes
	Gain (3)(126.72[q21.3]-126.94[q21.3])	217	*PLXNA1*
	Loss (3)(112.13[q13.2]-112.22[q13.2])	88	*BTLA*
	Loss (6)(143.19[q24.2]-143.25[q24.2])	58	*HIVEP2*
	Loss (7)(30.33[p14.3]-30.38[q14.3])	48	*ZNRF2*
	Loss (7)(50.36[p12.2]-50.47[p12.2])	109	*IKZF1*
	Gain (10)(131.50[q26.3]-131.55[q26.3])	49	*MGMT*
	Gain (11)(77.42[q14.1]-77.78[q14.1])	361	*RSF1, c11orf67, INTS4*
	Loss (12)(91.98[q21.33]-92.54[q21.33])	554	*3'BTG1*
	Loss (12)(11.73[p13.2]-11.79[p13.2])	58	No genes
	Loss (12)(65.58[q14.3]-65.61[q14.3])	30	*LEMD3*
	Loss (13)(60.55[q21.2]-60.59[q21.2])	43	*DIAPH3*
	Loss (15)(35.67[q14]-35.83[q14])	167	*ATPBD4*
	Gain (16)(8.13[p13.2]-8.16[q13.2])	28	No genes
	Loss (17)(36.95[q12]-36.99[q12])	39	*PIP4K2B, CWC25*
	Gain (21)(48.06[q22.3]-48.10[q22.3])	37	*PRMT2*
	Loss (21)(26.84[q21.3]-26.86[q21.3])	22	No genes
	Loss (X)(44.07[p11.3]-44.71[p11.3])	641	*EFHC2, FUNDC1*
21820*	loss (9)(36.88[p13.2]-37.03[q13.2])	153	*PAX5*
	gain (21)(15.10[q11.2]-48.10[q22.3])	37360	Multiple genes
	CNN LOH (9)(20.18[p21.3]- [22.13[p21.3])	1950	*CDKN2A/B*

Note: patients with : ‡: deleted der(12)t(12;21), ¶: duplicated *RUNX1-ETV6* fuson, *: Down syndrome , Ψ: positive control used for *TBL1XR1* and *BTG1*, respectively.

Appendix G. Detailed FISH results of additional cases with suggested MLPA ETV6 profile of deleted 3'ETV6 of der(12)t(12;21) and duplicated RUNX1-ETV6 fusion gene.

Deleted *3'ETV6* of der(12)t(12;21)		
Patient No.	**Investigations**	**Results**
7613	TEL/AML1	2R 1G 2F [41%]
	ETV6 DC BA	2R 0G 1F [52%], 0R 0G 2F [43%], 1R 0G 1F [5%]
	RUNX1 DC BA	0R 0G 2F [38%], 1R 2G 1F [33%], 1R 1G 1F [25%]
	Mapping Deletion	
	RP11-515B12	2R 1G 0F [54%], 2R 2G 0F [24%], 2R 0G 0F [20%]
	RP11-365O10	1R 1G 1F [64%], 2R 2G 0F [32%]
10398	TEL/AML1 ES	2R 1G 1F [28%]
	ETV6 DC BA	0R 0G 2F [66%], 1R 0G 1F [26%], 0R 0G 1F [7%]
	RUNX1 DC BA	0R 0G 2F [57%], 1R 1G 1F [35%]
	Mapping Deletion	
	RP11-59H1	2R 2G 0F [72%], 2R 1G 0F [21%]
	RP11-4N23	NA
22044	TEL/AML1 DC DF	2R 1G 1F [69%]
	ETV6 DC BA	0R 0G 2F [46%], 1R 0G 1F [43%], 0R 0G 1F [7%]
	RUNX1 DC BA	1R 1G 2F [36%], 1R 1G 1F [30%], 0R 0G 2F [24%], 0R 1G 2F [6%]
	Mapping Deletion	
	RP11-365O10	3R 1G 0F [70%], 2R 2G 0F [24%], 2R 1G 0F [6%]
	RP11-405A12	F
22574	TEL/AML1 ES	2R 1G 2F [61%]
	ETV6 DC BA	0R 0G 2F [47%], 2R 0G 1F [39%], 1R 0G 1F [8%]
	RUNX1 DC BA	1R 2G 1F [53%], 0R 0G 2F [29%], 1R 1G 1F [12%]
	Mapping Deletion	
	RP11-405A12	2R 1G 0F [62%], 2R 2G 0F [36%]
	RP11-12D15	1R 1G 1F [70%], 2R 2G 0F [24%], 0R 1G 1F [6%]
11052	TEL/AML1 ES	2R 1G 1F [54%], 1R 1G 1F [21%]
	TEL(so)/AML1(sg) DC DF	1R 2G 1F [82%]
	ETV6 DC BA	1R 0G 1F [74%], 2R 0G 0F [15%], 0R 0G 2F [7%]
	RUNX1 DC BA	1R 1G 1F [81%], 1R 2G 1F [15%]
	Mapping Deletion	
	RP11-757G14	2R 0G 0F [50%], 3R 0G 0F [18%], 2R 1G 0F [18%], 3R 1G 0F [9%], 1R 1G 0F [5%]
	RP11-59H1	2R 2G 0F [78%], 3R 2G 0F [11%], 4R 2G 0F [7%]
20755	TEL/AML1	NA
	ETV6 DC BA	1R 0G 1F [79%], 0R 0G 2F [7%]
	RUNX1 DC BA	1R 1G 1F [79%], 0R 0G 2F [21%]
	Mapping Deletion	
	RP11-59H1	2R 1G 0F [78%], 2R 2G 0F [18%]

	RP11-4N23	F
4894	TEL/AML1	NA
	ETV6 DC BA	1R 1G 0F [96%]
	RUNX1 DC BA	1R 1G 2F [97%]
12529	TEL/AML1 ES	2R 0G 1F [61%]
	ETV6 DC BA	1R 1G 0F [69%], 0R 0G 2F [24%], 1R 1G 1F [7%]
	RUNX1 DC BA	1R 1G 1F [47%], 0R 0G 2F [33%], 1R 0G 1F [18%]
21200	TEL/AML1 DC DF	1R 1G 2F [95%]
	ETV6 DC BA	1R 1G 1F [48%], 1R 1G 0F [15%], 2R 1G 0F [13%], 0R 0G 2F [9%], 1R 0G 2F [7%]
	RUNX1 DC BA	1R 2G 1F [60%], 1R 1G 1F [21%], 0R 0G 2F [6%]
22324	TEL/AML1 ES	4R 0G 2F [55%], 3R 0G 2F [17%]
	ETV6 DC BA	2R 1G 1F [60%], 1R 1G 1F [32%]
	RUNX1 DC BA	F
Deleted non-rearranged *ETV6*		
Patient No.	**Investigations**	**Results**
3181	TEL/AML1	Not done
	ETV6 DC BA	1R 1G 1F [90%]
	RUNX1 DC BA	1R 1G 1F [94%]
	Exon 8/RP11-434C1	Failed
	Exon 8/RP11-396G11	1R 1G 1F [52%], 1R 0G 1F [24%], 2R 2G 0F [12%], 2R 1G 1F [8%]
Gain der(21)t(12;21) PLUS loss non-rearranged *ETV6*		
Patient No.	**Investigations**	**Results**
3269	TEL/AML1	Not done
	ETV6 DC BA	3R 1G 0F [36%], 2R 1G 1F [16%], 0R 0G 2F [38%]
	RUNX1 DC BA	1R 3G 1F [32%], 1R 2G 1F [18%], 0R 0G 2F [31%]
3359	TEL/AML1	Not done
	ETV6 DC BA	2R 1G 0F [93%]
	RUNX1 DC BA	1R 2G 1F [97%]
3602	TEL/AML1	Not done
	ETV6 DC BA	2R 1G 0F [55%]
	RUNX1 DC BA	1R 2G 1F [60%]
4707	TEL/AML1	Not done
	ETV6 DC BA	4R 2G 0F [53%], 2R 1G 0F [35%]
	RUNX1 DC BA	1R 2G 1F, 2R 2G 2F, 2R 4G 2F
4758	TEL/AML1	Not done
	ETV6 DC BA	2R 1G 0F [92%]
	RUNX1 DC BA	1R 2G 2F [80%], 1R 1G 2F [16%]
11636	TEL/AML1 DC DF	0R 1G 3F

	ETV6 DC BA	2R 1G 0F [90%], 1R 1G 0F [8%]
	RUNX1 DC BA	Failed
Gain der(21)t(12;21) PLUS loss non-rearranged *ETV6*		
Patient No.	**Investigations**	**Results**
5859	TEL/AML1	Not done
	ETV6 DC BA	1F 1R 1G [88%], 0R 0G 2F [11%]
	RUNX1 DC BA	1R 1G 2F[44%], 1R 0G 2F [18%], 0R 1G 2F [15%], 0R 0G 2F [11%]
	Exon 8/RP11-396G11	Failed
11562	TEL/AML1 ES	2R 1G 1F [25%]
	ETV6 DC BA	0R 0G 2F [54%], 0R 1G 1F [28%], 1R 1G 1F [19%]
	RUNX1 DC BA	0R 0G 2F [45%], 1R 0G 1F [29%], 1R 1G 1F [27%]
	Exon 8/RP11-434C1	Not done
	Exon 8/RP11-396G11	2R 2G 0F [45%], 2R 1G 0F [49%]
12290	TEL/AML1 ES	2R 1G 2F [71%]
	ETV6 DC BA	2R 1G 1F [68%], 0R 0G 2F [24%], 1R 1G 1F [8%]
	RUNX1 DC BA	1R 2G 1F [56%], 0R 0G 2F [28%], 1R 1G 1F [8%]
	Exon 8/RP11-396G11	Failed
Suggested duplicated *RUNX1-ETV6* fusion gene		
Patient No.	**Invesigations**	**Results**
3250	TEL/AML1	NA
	ETV6 DC BA	NA
	RUNX1 DC BA	NA
20164	TEL/AML1 ES	2R 1G 1F [54%], 2R 0G 1F [17%], 1R 1G 1F [6%], 2R 2G 0F [21%]
	ETV6 DC BA	1R 1G 1F [29%], 2R 1G 0F [11%], 0R 0G 1F [5%], 0R 1G 2F [5%], 1R 1G 0F [5%], 0R 0G 2F [41%]
	RUNX1 DC BA	NA
21741	TEL/AML1 DC DF	3R 3G 2F [86%]
	ETV6 DC BA	NA
	RUNX1 DC BA	NA
22264	TEL/AML1 ES	3R 0G 1F [80%]
	ETV6 DC BA	F
	RUNX1 DC BA	F
22289	TEL/AML1 ES	2R 0G 1F [78%], 1R 0G 1F [8%], 2R 2G 0F [12%]
	ETV6 DC BA	NA
	RUNX1 DC BA	NA

Note: Group A: deletion of *3'ETV6* sequences from der(12)t(12;21) , Group B: Microdeletion of der(12)t(12;21), Group D: suggested unconfirmed duplication of der(12)t(12;21) fusion gene, NA: not done owing to lack of material, sg: spectrum green, so: spectrum orange.

Appendix H. Detailed information of the target probe assays used in this part of study.

probes	locations	Probe I.D	Genomic position	Amplicon length	Cytoband
TBL1XR1	*5'TBL1XR1*	Hs05887117_cn	Chr.3:177096119	93	3q26.32
	within intron 1	Hs06694058_cn	Chr.3:176844786	99	3q26.32
NR3C1	within intron 4	Hs06057433_cn	Chr.5:142715774	80	5q31.3
NR3C2	*5'NR3C2*	Hs05970766_cn	Chr.4:149490099	110	4q31.23
	within intron 4	Hs04830188_cn*	Chr.4:149103738	109	4q31.23
BTLA	Within Intron 4	Hs04722014_cn¥	Chr.3:112186825	86	3q13.2
BMF	Within Exon 4	Hs02711690_cn	Chr.15:40398104	86	15q15.1

Note: The genomic positions were based on NCBI build 37. *: This probe was withdrawn from the study (non- robust assay, in which the amplification plot was noisy), ¥: showing lower efficiency than the remaining assays but within acceptable range.

Appendix I. Algorithim used for evalution of individual cases in terms of q PCR and FISH results.

Concordant TERT and RNaseP	**Confidance**	**Z-score**	**Evidence by FISH**	**Status**
Yes	>= 0.95	< 2.65	Yes	Accept
Yes	>= 0.95	> 2.65	Yes	Repeat
Yes	< 0.95	Any value	Yes	Repeat
Yes	>= 0.95	< 2.65	No	Accept+Further FISH
Yes	>= 0.95	> 2.65	No	Repeat+Further FISH
Yes	< 0.95 - > 0.50	Any value	No	Repeat+Further FISH
Yes	< 0.50	Any value	No	Reject
No	>= 0.95	< 2.65	Yes	Accept
No	>= 0.95	> 2.65	Yes	Repeat
No	< 0.95	Any value	Yes	Repeat
No	>= 0.95	< 2.65	No	Accept+Further FISH
No	>= 0.95	> 2.65	No	Repeat+Further FISH
No	< 0.95	< 2.65	No	Repeat+Further FISH
No	< 0.95	> 2.65	No	Reject

Appendix J. Details clinical features of the relapse and the non-relapse cases studies in Chapter 5

Reg.ID	Time to 1st relapse/months[€]	Site at 1st relapse	% blasts at diagnosis	% blasts at 1st relapse	Outcome	Material availability
2712	21	BM	87	NA	died 1.5 months after 2nd relapse	Diag. DNA,Fixed cells Rel. Fixed cells
2774	34	CNS	84	NA	Complete remission after 1st relapse	Diag. DNA Rel. NA
2850	34	BM+ CNS	70	NA	Complete remission after 1st relapse	Diag. DNA, Fixed cells Rel. Fixed cells
2897	n.a.	n.a.	99	n.a.	Complete remission	DNA, Fixed cells
3026	n.a.	n.a.	91	n.a.	Complete remission	DNA, Fixed cells
3098	9	BM	95	NA	BMT after 1st relapse	Diag. DNA Rel. NA
3181*	74	BM	99	70	Complete remission after 1st relapse	Diag. DNA, Fixed cells Rel. DNA
3359	39.5	BM	95	NA	BMT after 1st relapse	Diag. DNA Rel. NA
3431	51	BM+ CNS	99	NA	BMT after 1st relapse	Diag. DNA Rel. Fixed cells
3562	5.5	CNS	99	NA	died after 1st relapse	Diag. DNA, Fixed cells Rel. NA
3588	42.5	BM	89	NA	BMT after 1st	Diag. DNA

Reg.ID	Time to 1st relapse/months€	Site at 1st relapse	% blasts at diagnosis	% blasts at 1st relapse	Outcome	Material availability
					relapse	Rel. NA
3700	46.5	BM+ CNS	88	NA	BMT after 1st relapse	Diag. DNA, Fixed cells Rel. Fixed cells
3703*	49.5	BM+ CNS	99	NA	BMT after 1st relapse	Diag. DNA, Fixed cells Rel. Fixed cells
3711	n.a.	n.a.	79	n.a.	Complete remission	DNA, Fixed cells
3761	n.a.	n.a.	99	n.a.	Complete remission	DNA, Fixed cells
3833	35.5	BM	100	NA	BMT after 1st relapse	Diag. DNA, Fixed cells Rel. NA
4011	n.a.	n.a.	98	n.a.	Complete remission	DNA, Fixed cells
4036*	51.5	BM	100	95	BMT after 1st relapse	Diag. DNA, Fixed cells Rel. DNA, Fixed cells
4044	30	BM	96	NA	BMT after 1st relapse	Diag. DNA, Fixed cells Rel. NA
4288	n.a.	n.a.	49	n.a.	Complete remission	DNA, Fixed cells
4439	n.a.	n.a.	99	n.a.	Complete remission	DNA, Fixed cells
4536	n.a.	n.a.	98	n.a.	Complete remission	DNA, Fixed cells
4569	n.a.	n.a.	95	n.a.	Complete remission	DNA

Reg.ID	Time to 1st relapse/months€	Site at 1st relapse	% blasts at diagnosis	% blasts at 1st relapse	Outcome	Material availability
4609	n.a.	n.a.	97	n.a.	Complete remission	DNA, Fixed cells
4902*	52	BM	99	NA	BMT after 1st relapse	Diag. DNA, Fixed cells Rel. DNA, Fixed cells
4995	n.a.	n.a.	91	n.a.	Complete remission	DNA, Fixed cells
5062*	27	CNS	95	35	Died after 3 years from 1st relapse	Diag. DNA, Fixed cells Rel. NA
5597*	24	CNS	95	0	BMT after 1st relapse	Diag. DNA, Fixed cells Rel. NA
5647	n.a.	n.a.	94	n.a.	Complete remission	DNA, Fixed cells
5659	n.a.	n.a.	96	n.a.	Complete remission	DNA, Fixed cells
5721	n.a.	n.a.	NA	n.a.	NA	DNA
5807	n.a.	n.a.	NA	n.a.	NA	DNA, Fixed cells
5914	n.a.	n.a.	NA	n.a.	NA	DNA, Fixed cells
10875‡	46	BM	95	NA	Died after BMT	Diag. DNA, Fixed cells Rel. DNA, Fixed cells

Note: * treated on ALLR3 at relapse, ‡ treated on UKALL2003 at presentation, ¥ percentages are relative to *ETV6-RUNX1* positive cells, n.a. not applicable, NA not available, BM bone marrow, CNS central nervous system, BMT bone marrow transplantation, € Early relapse: from diagnosis to <6 months from end of treatment, Late: >6 months from end of treatment.

Appendix K. Detailed copy number q PCR results of the positive controls used in screening of different gene deletions.

Sample Name	Target	Reference	CN Calculated	CN Predicted	Confidence	|Z-Score|	FISH
L707¥	*NR3C1*	RNAase P	0.07	0			2R 0G 0F [93%], 2R 2G 0F [7%]
	NR3C1	TERT	0.83	1			
REH*	*NR3C1*	RNAase P	1.13	1	> 0.99	0.2	2R 2G 0F [100%]
	NR3C1	TERT	0.99	1	> 0.99	0.7	
KG-1	*5'NR3C2*	RNase P	0.98	1	> 0.99	0.04	2R 1G 0F [96%], 3R 2G 0F [3%]
KG1	*5'NR3C2*	TERT	0.73	1	> 0.99	0.04	
KASUMI	*BMF*	RNase P	1.2	1	> 0.99	1.05	2R 1G 0F [95%], 2R 2G 0F [3%]
KASUMI	*BMF*	TERT	0.83	1	> 0.99	0	
4037	*TBL1XR1*	RNase P	0.82	1	> 0.99	0	1R 2G 0F [90%], 2R 2G 0F [9%]
4037	*TBL1XR1*	TERT	1.09	1	> 0.99	0.08	
4281	*5'TBL1XR1*	RNase P	0.97	1	> 0.99	0.02	NA
4281	*5'TBL1XR1*	TERT	1.01	1	> 0.99	0.37	
9859¥	*BTLA*	RNase P	0.57	1	> 0.99	1.84	
9859	*BTLA*	TERT	0.52	0			

Note: *: one of the green signals was diminished, NA: Not performed. ¥: these positive controls, with documented biallelic deletion either by FISH or SNP6, showed discrepant predicted copy number of 1 and 0 by q PCR but still both calculated copy number are almost similar approaching 0.

Appendix L. FISH and q PCR data of the atypical cases studies in Chapter 6

Reg.ID	*NR3C1*		*NR3C2*		*5'NR3C2*		*TBL1XR1*		*5'TBL1XR1*		*BTLA*		*BMF*		Collective No. of CNA
	q PCR	FISH (%)¥	q PCR	FISH (%)¥	q PCR	FISH(%)¥	q PCR	FISH (%)¥	q PCR	FISH(%)¥	q PCR	FISH (%)¥	q PCR	FISH (%)¥	
Infant Group															
9381	N	N	NA	N	N	NA	N	N	N	NA	N	N	N	N	0
10267	N	N	NA	N	N	NA	N	N	N	NA	N	N	N	N	0
11638	N	N	NA	N	N	NA	N	N	N	NA	D	47	N	N	1
11783	N	N	NA	N	N	NA	N	N	N	NA	NA	N	NA	N	0
12018	N	N	NA	N	N	NA	N	N	N	NA	F	N	N	N	0
12358	N	N	NA	N	N	NA	N	N	N	NA	N	N	N	N	0
19851	N	NA	NA	NA	N	NA	N	NA	N	NA	N	NA	N	NA	0
20123	N	N	NA	N	D	28	N	N	N	NA	N	N	N	N	1
20215	N	N	NA	N	N	NA	N	N	N	NA	N	N	N	N	0
20768	N	NA	NA	NA	N	NA	N	NA	N	NA	N	NA	N	NA	0
Adult Group															
1780	N	N	NA	N	N	NA	N	N	N	NA	N	N	N	N	0
1927*	U	N	NA	N	D	NA	D	N	U	NA	D	N	U	N	0
3367	N	N	NA	N	N	NA	N	N	N	NA	N	N	N	N	0
3936*	N	N	NA	N	D	NA	N	N	D	NA	D	N	D	N	0
7597	N	NA	NA	NA	N	NA	N	NA	N	NA	D	NA	N	NA	1
10216*	N	N	NA	N	D	NA	D	N	N	NA	D	N	N	N	0
22240	N	NA	NA	NA	N	NA	N	NA	N	NA	N	NA	N	NA	0
22264	N	NA	NA	NA	N	NA	N	NA	N	NA	N	NA	N	NA	0

Reg.ID	*NR3C1*		*NR3C2*		*5'NR3C2*		*TBL1XR1*		*5'TBL1XR1*		*BTLA*		*BMF*		Collective No. of CNA
	q PCR	FISH (%)¥	q PCR	FISH (%)¥	q PCR	FISH(%)¥	q PCR	FISH (%)¥	q PCR	FISH(%)¥	q PCR	FISH (%)¥	q PCR	FISH (%)¥	
23013	N	N	NA	N	N	NA	N	N	N	NA	N	N	N	N	0
24247	U	N	NA	N	N	NA	NA	N	NA	NA	NA	N	NA	N	0
Down syndrome Group															
3418	N	NA	NA	NA	N	NA	N	NA	N	NA	U	NA	N	NA	0
4905	F	NA	NA	NA	F	NA	NA	NA	NA	NA	NA	NA	NA	NA	0
7038	D	N	NA	NA	U	NA	NA	N	NA	NA	NA	NA	NA	NA	1
8906	U	NA	NA	NA	N	NA	NA	NA	NA	NA	NA	NA	NA	NA	0
9879	N	NA	NA	NA	N	NA	N	N	N	NA	NA	N	NA	N	0
20383	N	N	NA	N	N	NA	N	N	N	NA	N	N	N	N	0
20410	N	NA	NA	NA	D	NA	N	NA	N	NA	N	NA	N	NA	1
20754	D	NA	NA	NA	U	NA	NA	NA	NA	NA	NA	NA	NA	NA	1
24347	N	N	NA	N	N	NA	N	N	N	NA	N	N	N	N	0

Note: * showing false positive q PCR results owing to the presence of either near tetraploid or near triploidy suggested by cytogenetics and FISH, NA: not performed, U: undetermined due to intradiscrepant copy number by q PCR, D: deleted, N: normal, F: failed, ¥: percentages calculated as relative to *ETV6-RUNX1* positive cells.

Chapter 10. Publications

Abnormalities of the der(12)t(12;21) in *ETV6-RUNX1* Acute Lymphoblastic Leukemia

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ETV6-RUNX1 fusion [t(12;21)(p13;q22)] occurs in 25% of childhood B-cell precursor acute lymphoblastic leukemia (BCP-ALL) and is associated with a favorable outcome. Additional abnormalities involving der(21)t(12;21) and nonrearranged chromosome 12 are well characterized but aberrations involving the der(12)t(12;21) have rarely been described. Herein, we describe two novel abnormalities affecting the der(12)t(12;21): a deletion (20/247, 8%) and duplication (10/247, 4%). All 30 patients were under 10 years of age, had a median white blood count of 12.4×10^9/L and 19.2×10^9/L, respectively, with a good outcome. Deletions of der(12)t(12;21) on both sides of the breakpoint were confirmed and mapped: centromeric (12p11.21-12p13.2) and telomeric (21q22.12-21q22.3). The size of these deletions extended from 0.4–13.4 to 0.8–2.5 Mb, respectively. The centromeric deletion encompassed the following genes: *LRP6, BCL2L14, DUSP16, CREBL2,* and *CDKN1B*. We postulate that this deletion occurs at the same time as the translocation because it was present in all *ETV6–RUNX1*-positive cells. A second abnormality representing duplication of the reciprocal *RUNX1–ETV6* fusion gene was a secondary event, which we hypothesize arose through mitotic recombination errors. This led to the formation of the following chromosome: der(12)(21qter→21q22.12::12p13.2-12p12.3::12p12.3→12qter). Both abnormalities affect the reciprocal *RUNX1–ETV6* fusion product which could either eliminate or amplify its expression and thus contribute to leukemogenesis. However, other consequences such as haploinsufficiency of tumor suppressor genes and amplification of oncogenes could also be driving forces behind these aberrations. In conclusion, this study has defined novel abnormalities in *ETV6–RUNX1* BCP-ALL, which implicate new genes involved in leukemogenesis.

INTRODUCTION

The translocation, t(12;21)(p13;q22), which generates the *ETV6–RUNX1* fusion gene, is the most common chromosomal abnormality in B-precursor childhood ALL (BCP-ALL). It is found in ~25% cases, with a peak incidence between the ages of 3–6 years (Golub et al., 1995; Romana et al., 1995). Patients with *ETV6–RUNX1* generally have a favorable outcome (Pui, 2004; Moorman et al., 2010). Although this translocation is believed to constitute the first transforming event arising in utero, alone it is unable to generate overt disease (Greaves and Wiemels, 2003). Therefore, additional secondary genetic events are required for leukemogenesis. For instance, deletion of the nonrearranged *ETV6* allele has been implicated as an important secondary event, which occurs in up to 60% of cases (Attarbaschi et al., 2004; Stams et al., 2006). Furthermore, gain of chromosome 21 (+21) and duplication of the derivative chromosome 21 (+der(21)t(12;21)) are frequently observed in both presentation and relapsed *ETV6–RUNX1* BCP-ALL (Attarbaschi et al., 2004; Alvarez et al., 2005; Martineau et al., 2005; Stams et al., 2006; Forestier et al., 2007; Tsuzuki et al., 2007). Recently, genome-wide copy number alteration analyses of *ETV6–RUNX1* BCP-ALL, using high-resolution single-nucleotide polymorphism (SNP) arrays and comparative genomic hybridization arrays, have revealed additional gene deletions targeting various pathways, such as B-cell development and differentiation, tumor suppression, cell-cycle control, apoptosis, drug responsiveness, and nuclear hormone response (Kuiper et al., 2007; Lilljebjorn et al., 2007; Mullighan et al., 2007; Tsuzuki et al., 2007;

Additional Supporting Information may be found in the online version of this article.

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Kawamata et al., 2008; Mullighan et al., 2008; Parker et al., 2008; Lilljebjorn et al., 2010).

In a standard t(12;21) translocation, the reciprocal *RUNX1–ETV6* gene fusion is formed on the der(12)t(12;21). Expression of *RUNX1–ETV6* is found concomitantly with *ETV6–RUNX1* in only 76% of t(12;21)-positive ALL cases (Stams et al., 2005). The factors which govern the expression of *RUNX1–ETV6* and its effect on leukemogenesis are ill-defined. We screened 247 patients with *ETV6–RUNX1* using fluorescence in situ hybridization (FISH) probes covering both the *ETV6* and the *RUNX1* loci and observed a series of novel abnormalities targeting der(12)t(12;21). Further characterization of these abnormalities revealed novel additional abnormalities in *ETV6–RUNX1* BCP-ALL, targeting the reciprocal *RUNX1–ETV6* fusion gene.

MATERIALS AND METHODS

Patients in this study were diagnosed with *ETV6–RUNX1* BCP-ALL by standard criteria and were registered to the UK Medical Research Council (MRC) treatment trial, ALL97 (Moorman et al., 2010). This trial included children aged from 1 to 18 years. Approval from the local ethical committee and written informed consent from patients or guardians were obtained by participating centers on entry to the clinical trial (Moorman et al., 2010). This present study used fixed cell suspensions and DNA from pretreatment diagnostic bone marrow samples, which were obtained from the member laboratories of the United Kingdom Cancer Cytogenetics Group (UKCCG). Karyotypes were collected and reviewed centrally by the Leukaemia Research Cytogenetics Group (Harrison et al., 2001). The Clinical Trial Service Unit and Epidemiological Studies Unit (CTSU, University of Oxford) supplied patient details.

Fluorescence In Situ Hybridization

All patients in the study had been found to harbor *ETV6–RUNX1* fusion by FISH using a TEL-AML1 probe (Abbott Diagnostics, Maidenhead, UK) or by reverse transcriptase polymerase chain reaction (Moorman et al., 2010). Initial screening was performed using dual color (DC) break-apart (BA) probes, targeting either the *ETV6* locus (DakoCytomation, Denmark) or the *RUNX1* locus (centromeric—RP11-272A03 and telomeric—RP11-396G11, Sanger Institute, UK). In a standard t(12;21) translocation, the *ETV6* DC BA probe has a signal pattern of one red, one green, and one fusion (1R 1G 1F), indicating the presence of der(21)t(12;21), der(12)t(12;21), and normal chromosome 12, respectively. Similarly, the expected signal pattern with the *RUNX1* DC BA probe is 1R 1G 1F, representing der(12)t(12;21), der(21)t(12;21), and normal chromosome 21, respectively. Deletion mapping was performed using two different probe sets, depending on the region of the deletion and comprised differentially labeled target and anchor probes (Supporting Information Table S1). The anchor probe was either RP11-396G11 (labeled red) or RP11-525I3 (labeled green) depending on whether we were mapping deletion of chromosome 12 or chromosome 21 sequences, respectively (Fig. 1). The target was considered to be deleted if a signal pattern of either 2R 1G 0F or 1R 2G 0F was observed, respectively. Three-color FISH was performed to confirm that the deletion involved the der(12)t(12;21) rather than the normal chromosome 12. The home-grown probes comprised a control probe (RP11-396G11) (labeled red), a known intact *3′ETV6* probe (RP11-4N23) (labeled green), and the target probe (RP11-180M15) (labeled gold). In a standard translocation, a signal pattern of 1R 1F (green-gold) 1F (green-red-gold) would be observed, representing the normal chromosome 21, normal chromosome 12, and intact der(12)t(12;21), respectively (Figs. 2E–2F). In this test, the signal pattern of 1R 1F (green-gold) 1F (red-green) indicated the presence of a normal chromosome 21, normal chromosome 12, and deletion of the gold probe from the der(12)t(12;21), respectively. To investigate the origin of the extra der(12)t(12;21) signals observed with the *ETV6* DC BA and *RUNX1* DC BA probes, a home-grown DC single fusion (SF) probe was designed. This probe comprised the *5′RUNX1* probe (RP11-396G11) (labeled red) and *3′ETV6* probe (RP11-525I3) (labeled green) (Figs. 3A–3B and Supporting Information Table S1). The signal pattern, 1R 0G 2F, indicated an extra fusion signal with associated loss of the green signal, corresponding to the duplication of the reciprocal chimeric gene *RUNX1–ETV6* with loss of *3′ETV6* sequences from the normal chromosome 12. A commercial probe set consisting of centromere 12 (CEP 12, labeled red, Cytocell, Cambridge, UK) and subtelomere 12p (subtel12p, labeled green, Abbott Diagnostics, Maidenhead, UK) was used to enumerate the chromosome 12

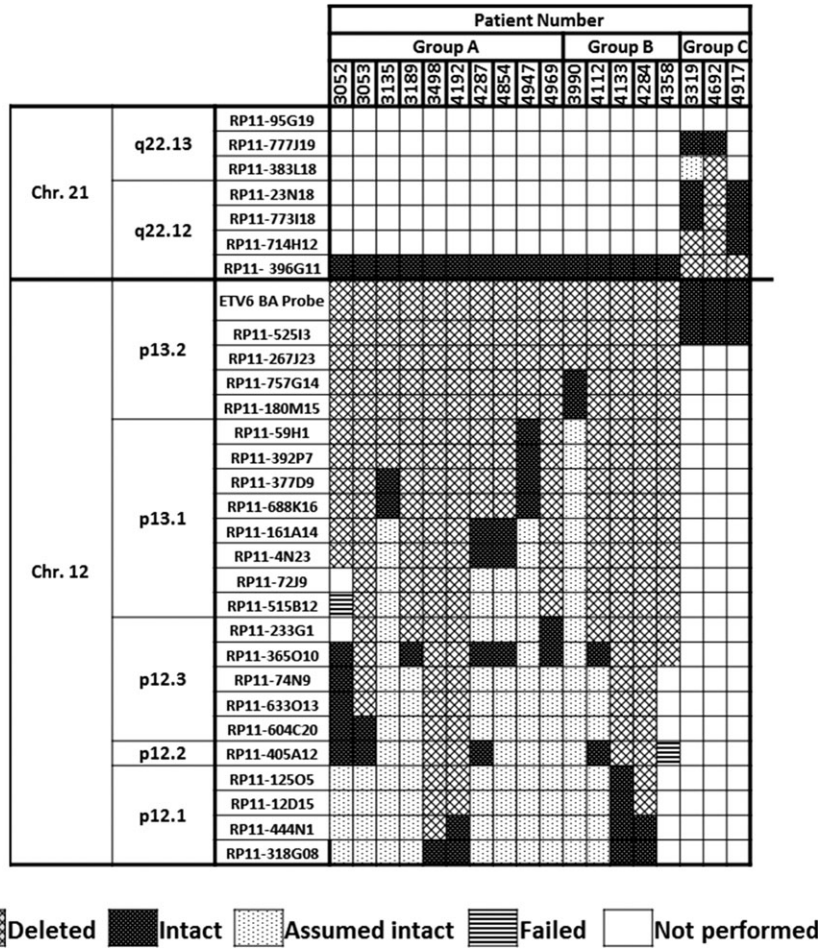

Figure 1. Details of FISH mapping of der(12)t(12;21) deletions in 18 cases with *ETV6–RUNX1* BCP-ALL. Group A: deletion of *3′ETV6* sequences from der(12)t(12;21) plus intact nonrearranged *ETV6*; Group B: deletion of *3′ETV6* sequences from der(12)t(12;21) plus deletion of nonrearranged *ETV6*; Group C: deletion of *5′RUNX1* sequences from der(12)t(12;21).

centromeres and telomeres. For all FISH tests, a minimum of 50–100 interphase cells were scored and, with the exception of deletion mapping, were independently checked. Individual probe-specific cutoff levels were calculated using the mean false-positive rate derived from five control samples plus three times the standard deviation, with the exception of probes for deletion mapping where a generic cutoff value of 4% was used.

Multiplex Ligation-Dependent Probe Amplification

Genomic DNA from six diagnostic bone marrow samples (patients no. 3135, 3189, 4969, 3726, 4281, and 4637) was extracted using standard procedures. In one case, the DNA was extracted from the same fixed cell pellet as used for FISH; this DNA was isolated using the Qiagen DNeasy tissue and blood kit according to the manufacturer's instructions. We have previously demonstrated that these samples provide an effective source of DNA for multiplex ligation-dependent probe amplification (MLPA) when used in conjunction with control DNA prepared in the same manner (Schwab et al., 2010). Genomic DNA from healthy donors was obtained for use as control samples. DNA was analyzed using the SALSA MLPA kit P335 (MRC Holland, Amsterdam, The Netherlands) as described previously (Schwab et al., 2010). This kit includes probes for *IKZF1* (eight probes), *CDKN2A/B* (three probes), *PAX5* (six probes), *EBF1* (four probes), *ETV6* (six probes), *BTG1* (four probes), *RB1* (five probes), and the *PAR1* region: *CRLF2*, *CSF2RA*, and *IL3RA* (one probe each). Data were analyzed using GeneMarker V1.85 analysis software (SoftGenetics).

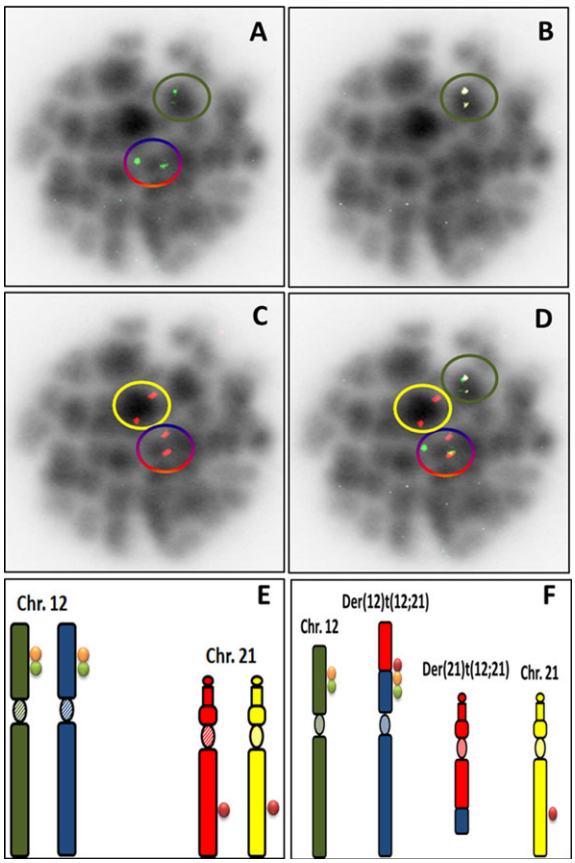

Figure 2. Three-color FISH confirmation of the origin of the der(12)t(12;21) deletion. (A–C) Serial application of the green (A), gold (B), and red (C) probes to a single metaphase from patient 3135, showing a signal pattern of 1R 1F(green-gold) 1F(green-red), confirming that the gold (target) probe was deleted from the der(12)t(12;21) rather than the nonrearranged chromosome 12. (D) The same metaphase highlighting the normal nonrearranged chromosome 12 (green), normal chromosome 21 (yellow), and the der(12)t(12;21) (red-blue); (E and F) Ideograms indicating the positions of the three probes in a normal and a standard t(12;21) cell, respectively. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

SNP Arrays

DNA samples (patients no. 3135, 3189, 4969, 3726, and 4281) were hybridized to Affymetrix Genome-Wide Human SNP Array 6.0 by AROS Applied Biotechnology (Aarhus, Denmark) according to the manufacturer's instructions. Affymetrix CEL files were analyzed using Affymetrix Genotyping Console software (version 4.0) for initial quality control, followed by use of the Affymetrix Birdseed algorithm (version 2.0) to generate SNP genotype calls on all samples. All samples passed the Affymetrix recommended contrast quality control and SNP call rates threshold. Copy number, allele ratio, and allele-specific copy number data analysis were performed on CEL files using Affymetrix Genotyping Console software (version 4.1.1.834). To locate segments with copy number changes, genomic segmentation algorithm of Affymetrix Genotyping Console was used.

RESULTS

A total of 247 *ETV6–RUNX1* BCP-ALL cases were screened using BA probes, targeting the *ETV6* and *RUNX1* loci. Unexpected signal patterns which indicated the involvement of der(12)t(12;21) were observed. First, a group of patients (16/247, 6%) showed a signal pattern of either 1R 0G 1F ($n = 10$, Group A) or 1R 0G 0F ($n = 6$, Group B) using the *ETV6* DC BA probe, indicating the deletion of *3′ETV6* sequences from the der(12)t(12;21) with or without intact nonrearranged *ETV6*, respectively (Table 1, Fig. 1, and Supporting Information Table 2). The *RUNX1* DC BA probe uncovered another group of patients ($n = 4$, 2%, Group C), showing a deletion of the *5′RUNX1* sequences from the der(12)t(12;21) as indicated by a signal pattern of 0R 1G 1F (Table 1, Fig. 1, and Supporting Information Table 2). In all these patients, these abnormal signal patterns were present in 100% of fusion-positive cells, indicating that the deletion occurred at the same time as the translocation (Supporting Information Table 2). Overall, deletions of the nonrearranged *ETV6* allele were observed among 165 (67%) successfully tested cases. Deletions of der(12) were more common among patients with an intact versus deleted nonrearranged *ETV6*: 11/80 (14%) versus 9/165 (5%), $P = 0.026$. This cohort of 20 patients with a deleted der(12) comprised 14 males and 6 females, who were all aged <10 years and had a median white blood cell count (WCC) of 12.4×10^9/L. All 20 patients remain in first complete remission after a median follow-up time of 9.2 years.

Second, a group of 10 patients (4%, Group D) showed abnormal signal patterns of 1R 2G 0F and 2R 1G 1F using both *ETV6* DC BA and *RUNX1* DC BA, respectively, which suggested a duplication of the *RUNX1–ETV6* fusion gene (Table 2 and Supporting Information Table 2). These signal patterns were often found in conjunction with the standard abnormal signal pattern (1R 1G 1F), indicating that this abnormality represented a secondary event. This cohort of patients comprised six females and four males, who were all aged <10 years, had a median WCC of 19.2×10^9/L, and were all in continuing complete remission more than 8 years after diagnosis.

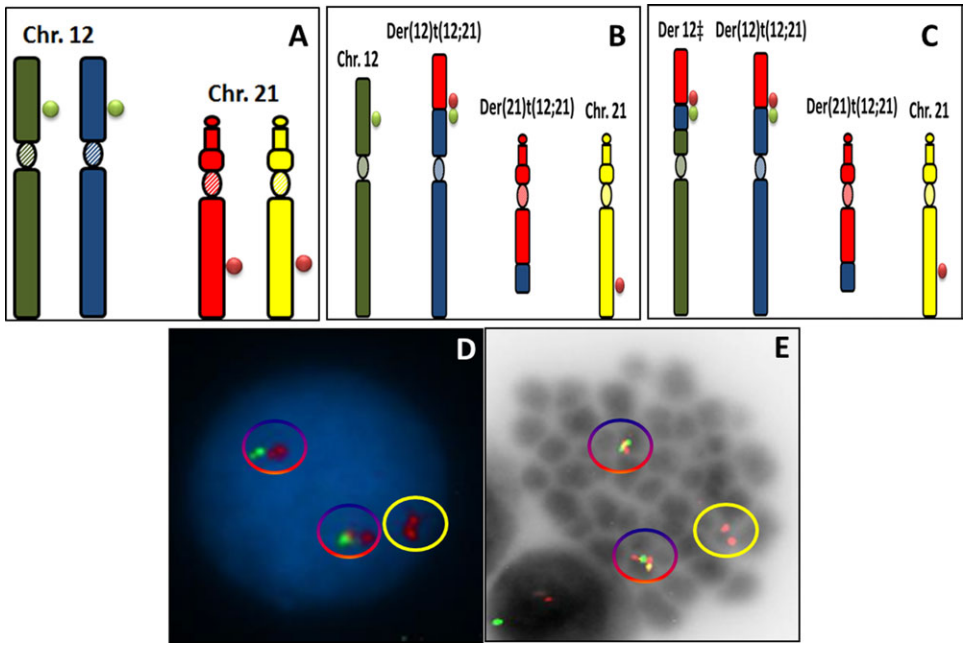

Figure 3. Identification and proposed mechanism for the formation of der(12)(21qter→21q22.12::12p13.2-12p12.3::12p12.3→12qter) (A and B) Ideograms showing the positions of the probes used in *ETV6–RUNX1* DC SF probe in a normal and standard t(12;21) cell, respectively; (C) An ideogram representing the proposed mechanism behind the der(12)(21qter→21q22.12::12p13.2-12p12.3::12p12.3→12qter) through mitotic recombination. (D and E) An interphase and a metaphase cell, respectively, from patient 3726 hybridized with the *ETV6–RUNX1* DC SF probe. The signal pattern of 1R 0G 2F indicates the presence of a normal chromosome 21 (yellow), and a der(12)(21qter→21q22.12::12p13.2-12p12.3::12p12.3→12qter) (red-blue) which harbors a duplicated *RUNX1–ETV6* fusion gene. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE 1. Demographic and Genetic Characteristics of *ETV6–RUNX1*-positive BCP-ALL Patients With der(12)t(12;21) Deletion

Patient number	Sex/age (years)	Karyotype	Range of deletion (Mb)	Size of deletion (Mb)[a]	Other tests
Group A: Deletion of 3′ETV6 sequences from der(12)t(12;21) plus intact nonrearranged ETV6					
3052	F/2	Fail	12.02–max(15.26)[b]	Undefined	NA
3053	F/2	46,XX	12.02–19.83	7.81	NA
3135	M/9	46,XY,der(9)del(9)(p21)del(9)(q34), del(11)(q13q23)t(12;21) (p13;q22),der(20)t(8;20)(q13;p?)	12.02–13.16	1.14	Three-color FISH, MLPA, SNP6
3189	F/1	46,XX	12.02–15.26	3.24	MLPA, SNP6
3498	M/5	Fail	12.02–25.39	13.37	NA
4192	F/3	47,XX,del(11)(q13),del(12)(p?),+21	12.02–23.48	11.46	NA
4287	M/4	46,XY	12.02–13.36	1.34	Three-color FISH
4854	M/2	46,XY,add(2)(p11),del(12)(p11),−22,+2–3mars[cp]	12.02–13.36	1.34	NA
4947	M/3	48–50,XY,add(8)(p2?),+10,t(12;15)(p1?;p1?),+21[cp]	12.02–12.88	0.86	NA
4969	M/6	46,XY,del(9)(p2?),+21	12.02–14.94	2.92	MLPA, SNP6
Group B: Deletion of 3′ETV6 sequences from der(12)t(12;21) plus deletion of nonrearranged ETV6					
3990	M/4	46,XY	12.02–12.37	0.35	NA
4112	M/8	46,XY,add(9)(p21),del(12)(p12)	12.02–15.26	3.24	NA
4133	M/2	46,XY	12.02–21.15	9.13	NA
4284	M/4	46,XY,del(12)(p1?)	12.02–22.25	10.23	NA
4358	M/2	46–47,XY,del(12)(p?)[cp]	12.02–23.36+[b]	Undefined	NA
3066	M/1	47,XY,+10	NA	NA	NA
Group C: Deletion of 5′RUNX1 sequences from der(12)t(12;21)					
3319	F/8	45,X,-X	36.26–37.26	1.00	NA
4692	M/2	46,XY,add(16)(q22)	36.26–38.72	2.46	NA
4917	F/2	47,XX,+21	36.26–37.04	0.78	NA
3277	M/5	46,XY,add(11)(p15),add(12)(p12), del(12)(p12),del(21)(q21)	NA	NA	NA

Note: Groups A and B: Deletion starts at 12.02 Mb (within *ETV6* intron 5); Group C: Deletion starts at 36.26 Mb (within *RUNX1* intron 1); NA: not performed owing to lack of material.

[a]Size determined using data from ENSEMBL version no.60

[b]A lack of material prevented the deletion being fully mapped.

TABLE 2. Demographic and Cytogenetic Features of *ETV6–RUNX1*-positive BCP-ALL Patients with der(12)(21qter→21q22.12::12p13.2–12p12.3::12p12.3→12qter)

Patient number	Age (years)/sex	Karyotype	*ETV6* DC BA	*RUNX1 DC* BA	5′*RUNX1*/3′*ETV6* DC SF	CEP12/Subtel 12p	Other tests
3130	4/F	46,XX,del(6)(q?),der(12),inc	1R 1G 1F [34%], 1R 2G 0F [56%]	1R 1G 1F [37%], 2R 1G 1F [58%]	1R 1G 1F [24%], 1R 0G 2F [67%]	2R 1G 0F [69%], 2R 2G 0F [31%]	NA
3726	4/M	46,XY,add(11)(q22)	1R 1G 1F [60%], 1R 2G 0F [26%]	1R 1G 1F [59%], 2R 1G 1F [29%]	1R 1G 1F [49%], 1R 0G 2F [30%]	2R 1G 0F [18%], 2R 2G 0F [81%]	SNP6, MLPA
4136	2/F	46,XX	1R 1G 1F [7%], 1R 2G 0F [84%]	2R 1G 1F [78%]	1R 0G 2F [61%], 2R 0G 2F [24%]	2R 1G 0F [79%], 2R 2G 0F [20%]	NA
4281	4/M	46,XY,add(6)(q1?)	1R 2G 0F [92%]	2R 1G 1F [91%]	2R 1G 1F [27%], 1R 0G 2F [43%]	2R 1G 0F [90%], 2R 2G 0F [8%]	SNP6, MLPA
4560	2/F	46,XY	1R 1G 1F [20%], 1R 2G 0F [63%]	1R 1G 1F [37%], 2R 1G 1F [60%]	1R 1G 1F [26%], 1R 0G 2F [59%]	2R 1G 0F [51%], 2R 2G 0F [49%]	NA
4637	4/F	46,XX	1R 2G 0F [87%]	2R 1G 1F [95%]	1R 1G 1F [7%], 1R 0G 2F [81%]	2R 1G 0F [91%], 2R 2G 0F [11%]	MLPA
4678	7/M	46,XY,der(6)t(3;6)(q2?;q2?),del(9)(p1p2), der(12)t(12;21)(p13;q22), t(12;21)(p13;q22)	1R 2G 0F [84%]	2R 1G 1F [92%]	1R 1G 1F [7%], 1R 0G 2F [77%]	2R 1G 0F [82%], 2R 2G 0F [17%]	NA
3472	2/F	47,XX,+21c	1R 1G 1F [78%], 1R 2G 0F [16%]	1R 1G 2F [84%], 2R 1G 2F [11%]	NA	NA	NA
4040	4/F	46,XX,del(12)(p12),inc	1R 1G 0F [54%], 1R 2G 0F [24%]	1R 1G 1F [65%], 2R 1G 1F [14%]	NA	NA	NA
4934	4/F	47,XX,der(12)t(12;21)(p13;q22), t(12;21)(p13;q22), der(20)t(X;20)(q?;q?), +der(21)t(12;21)(p13;q22)	2R 1G 0F [15%], 2R 2G 0F [65%]	1R 1G 1F [5%], 2R 2G 1F [83%]	NA	NA	NA

Abbreviations: DC, dual color; BA, break apart; DC SF, dual-color single fusion; CEP, centromeric probe; Subtel, subtelomeric Probe; NA, not performed owing to lack of material.

Characterization of the Deletion of der(12)t(12;21)

A total of 18 out 20 patients with indirect evidence for a deletion of der(12)t(12;21) were selected on the basis of availability of material. They were divided into three groups and investigated separately according to the status of nonrearranged *ETV6* and whether the deleted sequences affected the *3′ETV6* or *5′RUNX1* part of the fusion gene. Groups A and B were both characterized by loss of *3′ETV6* and additional chromosome 12 sequences centromeric of the breakpoint with either an intact ($n = 10$) or deleted ($n = 5$) nonrearranged *ETV6*, respectively. Although the third group (C) ($n = 3$) consisted of patients with deletion of *5′RUNX1* and additional chromosome 21 sequences telomeric of the breakpoint.

The FISH, MLPA, and SNP array data were concordant for the presence of deletions on der(12)t(12;21), which resulted in loss of *3′ETV6* and contiguous centromeric sequences. FISH probes mapping along 12p from 12p11.21-12p13.2 (Supporting Information Table 1) were serially applied to all 15 samples to map the extent of the deletion (Table 1 and Fig. 1). The deletion was fully mapped in all cases except patients 3052 (Group A) and 4358 (Group B), where a lack of material prevented completion of all required tests. Among 13 deletions, fully mapped, including three cases analyzed by SNP array (3135, 3189, and 4969), the median size of the deletion was 3.24 Mb with a range of 0.35–13.37 Mb. A common region of deletion (CRD), defined by patient 3990 (Group B), was observed spanning 0.35 Mb and comprising two genes: *LRP6* and *BCL2L14*. Although patients 3052 (Group A) and 4112 (Group B) showed evidence of variant t(12;21) translocations involving an additional chromosome, the FISH patterns were consistent with a deletion of *3′ETV6* from the der(12)t(12;21) (Supporting Information Table 2).

Three-color FISH was attempted in four cases (3135, 4287, 4854, and 4947). It was successful in patients 3135 and 4287 (Group A), where it showed a signal pattern of 1R 1F(green-gold) 1F(green-red), confirming that the target probe was deleted from the der(12)t(12;21) rather than from the nonrearranged chromosome 12 (Figs. 2A–2D and Supporting Information Table 2). In addition, MLPA in three patients (3135, 3189, and 4969) showed a monoallelic deletion of *ETV6* exon 8 with retention of exons 1–5, a pattern which is consistent with deletion from the der(12)t(12;21) rather than the normal chromosome.

The third group (C) comprised patients with loss of *5′RUNX1* and chromosome 21 sequences from the der(12)t(12;21). FISH mapping of the three patients using different deletion probe sets along 21q from 21q22.12-21q22.3 (Supporting Information Table 1) revealed deletions with a median size of 1.00 Mb (range, 0.78–2.46 Mb) (Table 1, Fig. 1, and Supporting Information Table 2). No subsequent MLPA and SNP array tests were possible owing to a lack of material. The CRD defined by patient 4917 did not include any known genes. Although patient 3319 showed evidence of a variant t(12;21) translocations involving an additional chromosome, the FISH patterns were consistent with a deletion of *5′RUNX1* from the der(12)t(12;21) (Supporting Information Table 2).

Gain of *RUNX1–ETV6* Fusion Gene on a der(12)(21qter→21q22.12::12p13.2-12p12.3::12p12.3→12qter)

FISH, MLPA, and SNP array findings confirmed our initial observation of the gain of the *RUNX1–ETV6* fusion gene of patients in Group D (Table 2). FISH experiments performed on seven patients, using the DC SF probe, confirmed two copies of the *RUNX1–ETV6* fusion in all cases in an average of 55% of *ETV6–RUNX1*-positive cells (Table 2 and Fig. 3D). Metaphases from patient 3726 showed that the two fusion signals were on separate chromosomes (Fig. 3E). Further FISH revealed the presence of only two chromosomes 12 centromeres and the loss of one 12p subtelomeric region in all seven cases (Table 2 and Supporting Information Table S2). MLPA in two cases (4281 and 4637) revealed a single copy of *ETV6* exons 1–5 but two copies of *ETV6* exon 8, a pattern consistent with two copies of the *RUNX1–ETV6* fusion and no normal *ETV6* allele. Although a third patient (3726) did not show the same copy number profile of *ETV6* exons by MLPA, FISH indicated that this abnormality was present only in 18% cells which is below the detection threshold of 30% for MLPA (Schwab et al., 2010). In all three cases, MLPA revealed a normal copy number for the *BTG1* gene, consistent with the FISH data and indicated that only two chromosomes 12 were present. SNP array analysis of two cases (4281 and 3726) revealed the presence of two chromosomes 12 with copy number neutral loss of heterozygosity

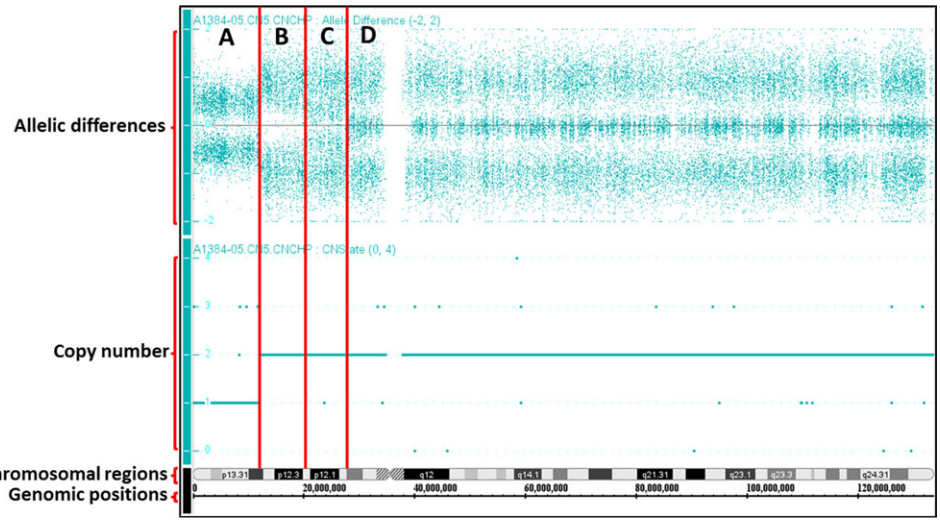

Figure 4. SNP 6.0 profile for chromosome 12 from a patient with der(12)(21qter→21q22.12::12p13.2-12p12.3::12p12.3→12qter). SNP array analysis of patient 4281 showed four regions on chromosome 12: an area of deletion (Area A) from the telomere to 12p12.3 (19.7 Mb) followed by three regions with different patterns of heterozygosity: (B) copy number neutral loss of heterozygosity (CNN-LOH) (12)(12.02[p13.2]-19.87[p12.3]); (C) complex CNN-LOH (12)(19.87[p12.3]-28.06[p11.22]); (D) normal heterozygous chromosome 12(28.06[p11.22]-133.85[qter]). It is not possible to discern whether areas B and C represent distinct or overlapping lesions. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(CNN-LOH), spanning a region of the short arm of chromosome 12 12(12.02[p13.2]-19.87[p12.3]). This abnormality was clearly evident in patient 4281 as it constituted the major clone, unlike patient 3726, where it represented only a subpopulation of cells. In addition, three copies of a part of the long arm of chromosome 21 21(36.39[q22.12]→48.10[qter]) and one copy of the subtelomeric region of 12p del(12)(0.19[pter]→19.87[p12.3]) were detected.

Taken together, these data indicate the presence of an extra *RUNX1–ETV6* fusion gene which could not have arisen from a simple gain of der(12)t(12;21) and loss of the normal chromosome 12. Instead, these data pointed to a secondary event which involved mitotic recombination between the normal chromosome 12 and the der(12)t(12;21), resulting in the formation of der(12)(21qter→21q22.12::12p13.2-12p12.3::12p12.3→12qter) (Fig. 3C). This derived chromosome consisted of one copy of the der(12)t(12;21) from the 21q telomere, including the *RUNX1–ETV6* fusion site, up to 12p12.3 and thereafter, material from the other chromosome 12 that had not been involved in the original t(12;21) translocation. The mitotic recombination point not only differed between the two patients, but also the area of complex CNN-LOH (Area C) observed in patient 4281 (Fig. 4) suggested the presence of multiple independent mitotic recombination events in the same patient. Collectively, these rearrangements resulted in the loss of the nonrearranged *ETV6* allele with the contiguous telomeric sequences, duplication of the reciprocal *RUNX1–ETV6* fusion gene, as well as the CNN-LOH of a region on the short arm of chromosome 12.

DISCUSSION

The *ETV6–RUNX1* fusion gene alone is insufficient to initiate overt leukemia. Accordingly, additional abnormalities are required to contribute to leukemogenesis. Here, we characterize two novel abnormalities which target the *RUNX1–ETV6* fusion gene, either by deletion or by duplication, which were identified using FISH probes, targeting the *ETV6* and *RUNX1* loci. (1) A deleted der(12)t(12;21) was detected in 8% *ETV6–RUNX1*-positive cases and (2) a complex der(12) including a duplication of the *RUNX1–ETV6* fusion gene was identified in 4% *ETV6–RUNX1*-positive cases. The demographic and clinical features of these 30 patients were unremarkable and none of these patients had relapsed or died. However, it should be noted that only 13% of *ETV6–RUNX1*-positive patients treated on ALL97 suffered a relapse (Moorman et al., 2010).

In this study, deletions involving chromosome 12 material from the der(12)t(12;21) were more prevalent than deletion of chromosome 21 material. Neither deletion has been reported previously. However, we and other researchers referred to deletions of 5′*RUNX1* sequences while using the commercial TEL-AML1 extra signal

probe, observing loss of the extra signal (Jabber Al-Obaidi et al., 2002; Martineau et al., 2005; Rothman et al., 2005; Konn et al., 2010). However, as this loss was almost always present in subclones it is unlikely that there is much, if any, overlap between the two groups. The failure of the previous studies to identify this deletion is probably owing to the fact that the majority of commercially available probes do not include probes that would localize to the der(12) in the presence of a translocation. Interestingly, genomic copy number profiling may erroneously identify deletions of chromosome 12 sequences from the der(12)t(12;21) as intragenic deletions of the nonrearranged *ETV6* allele which represent a different entity.

These deletions join a list of submicroscopic deletions that accompany primary chromosomal rearrangements in leukemia including t(9;22)(q34;q11), inv(16)(p13q22), t(8;21)(q22;q22), and rearrangements of the *MLL* gene (Rothman et al., 2005). For example, der(9)t(9;22) deletions, involving either 5′*ABL1*, 3′*BCR*, or both, are detected in 9–33% of CML patients with consequent worse prognosis especially in those with 5′*ABL* losses (Sinclair et al., 2000; Vaz de Campos et al., 2007). Interestingly, the extent of deletion of der(9)t(9;22) is comparable to our findings with smaller 3′*BCR* deletions (<0.5 Mb) than 5′*ABL*-deleted regions (mean size, 2.80 Mb) (Sinclair et al., 2000; Storlazzi et al., 2002; Douet-Guilbert et al., 2006). In addition, deletions of 5′*MYH11* (16p13) or 3′*CBFB* are detected in ~20% (Tirado et al., 2010) and 8% (Kelly et al., 2005) of AML with inv(16), respectively. Similarly, there are deletions of the region 5′*RUNX1T1*, representing 9% of t(8;21) with no occurrence of 3′*RUNX1* losses (Godon et al., 2002). Approximately, 20–25% of *MLL* rearrangements are associated with 3′*MLL* deletions (Konig et al., 2002). Little is known about the time at which these deletions arise and the mechanisms involved in their origin. However, the nature of the sequences that flank translocation breakpoint regions may play a role. Nonrandom distribution of high-density flanking Alu repeats, which are known to facilitate illegitimate recombination, within the vicinity of deletion breakpoints is considered to be one possible mechanism giving rise to deletion (Kolomietz et al., 2001, 2002). Unlike der(9)t(9;22) deletions, the prognostic impact of the submicroscopic deletions that accompany other rearrangements remains to be determined. The molecular mechanisms responsible for the poor prognosis associated with der(9)t(9;22) deletions are obscure although it has been proposed that the loss of one or more tumor suppressor genes may induce more aggressive disease through a secondary event or haploinsufficiency. Furthermore, deletions may result in genetic instability which predisposes to subsequent additional genetic alterations or to intronic deletions within *BCR–ABL1* itself. However, no increase in the frequency of secondary cytogenetic abnormalities during disease progression has been observed in patients with deletions (Vaz de Campos et al., 2007).

We have hypothesized that der(12)t(12;21) deletions, like der(9)t(9;22) deletions, occur at the same time as the translocation and are a by-product of the translocation event itself. This is evidenced by the fact that the deletion is present in 100% fusion-positive cells and that the boundaries of the deletion appear identical to the translocation breakpoint. Hence, the deletion must have occurred after the breakage of the chromosomes and prior to the illegitimate fusion of the chromosomes. Therefore, there is no selection pressure solely for the deletion. Hence, any leukemic effect will not only be a bystander effect but will also vary from patient to patient. There are several candidate genes on 12p within the CRD which may be acting as tumor suppressor genes in this scenario, such as *LRP6*, *BCL2L14*, *DUSP16*, *CREBL2*, and *CDKN1B* (Kiyokawa et al., 1996; Guo et al., 2001; Masuda et al., 2001). However, no somatic mutations or altered expression has been reported for these genes in BCP-ALL (Montpetit et al., 2004). Deletion of chromosome 21 material from the der(12) was rare and this CRD did not include any known genes. The one consistent feature of all der(12) deletions was loss of the reciprocal fusion gene—*RUNX1–ETV6*. This fusion product consists of the ETS domain from the *ETV6* portion, whereas the *RUNX1* portion does not contain a functional domain. It has been suggested that *RUNX1–ETV6* is likely to have a function in *ETV6–RUNX1* ALL through the action of this isolated ETS domain and it may behave in a similar manner to wild-type *ETV6* where the nonrearranged allele has been deleted (Stams et al., 2005). Cases with der(12) deletions will not be able to express the reciprocal fusion product; hence, any functional consequence of *RUNX1–ETV6* would be abrogated in these cases. Stams et al. (2005) have shown that ~25% *ETV6–RUNX1*-positive ALL do not express any *RUNX1–ETV6* mRNA. Der(12) deletions are one

mechanism by which expression of *RUNX1–ETV6* could be switched off.

Previous FISH studies based on observations using the TEL-AML1 ES probes have suggested that duplication of the reciprocal product is owing to a simple gain of the der(12)t(12;21) or the result of an independent translocation (Ma et al., 2001; Jalali et al., 2003; Martineau et al., 2005; Rothman et al., 2005). However, our investigations have shown that the resulting derived chromosome 12 is more complex and results in duplicated regions of 21q and 12p as well as CNN-LOH of regions of 12p but not 12q as would be assumed from gain of der(12)t(12;21) and loss of the normal chromosome 12. It is known that a proportion of *ETV6–RUNX1* cases exhibit high expression of the reciprocal product, *RUNX1–ETV6*. Indeed, it has been shown to be an independent prognostic factor which confers a poor outcome (Stams et al., 2005). It is logical to postulate that duplication of *RUNX1–ETV6* may lead to its overexpression. Unfortunately, we were unable to confirm this hypothesis owing to a lack of material. The difference in survival between our patients, who had an excellent outcome and those mentioned in the article by Stams et al. (2005), is likely to be due to the advances in treatment rather than evidence that the two groups are distinct. Clearly, the der(12) (21qter→21q22.12::12p13.2-12p12.3::12p12.3→12qter) results in other events which may also be contributing to leukemogenesis via the unmasking of cancer driver mutations, either by the inactivation of tumor-suppressor genes or by the activation of oncogenes. There are multiple genes encompassed within this region including *LRP6*, *BCL2L14*, *DUSP16*, *CREBL2*, and *CDKN1B*. In addition, the loss of nonrearranged *ETV6* allele may be one of the driving forces of the leukemogenic process in this group of patients. Further functional studies will be required to address these questions.

It is now well established that *ETV6–RUNX1*-positive ALL harbors several additional abnormalities. In this study, we have characterized two additional aberrations which, although indicated previously, had not been fully characterized. Both abnormalities affect the *RUNX1–ETV6* fusion product located on der(12)t(12;21), resulting either in its deletion or in its duplication. Neither abnormality was associated with a poor outcome within this cohort. This is likely to be a reflection of the highly efficient therapy that is available for *ETV6–RUNX1*-positive patients, which has resulted in a very low relapse rate (Moorman et al., 2010), rather than an indication that the abnormalities do not influence the development of the disease. Generally, most research on chromosomal translocations is focussed on the primary product and *ETV6–RUNX1* ALL is no exception which explains the reason why these abnormalities have remained uncharacterized until now. However, evidence from the studies of the t(4;11) translocation suggest that the reciprocal product, *AFF1-MLL*, may play a more important biological role than the previously imagined (Kowarz et al., 2007). Our data suggest that the same may be true for *RUNX1–ETV6*. Further research into the functional consequences of altered expression of *RUNX1–ETV6* in patients with *ETV6–RUNX1* ALL will be required to establish its role in leukemogenesis.

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Genes commonly deleted in childhood B-cell precursor acute lymphoblastic leukemia: association with cytogenetics and clinical features

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ABSTRACT

In childhood B-cell precursor acute lymphoblastic leukemia, cytogenetics is important in diagnosis and as an indicator of response to therapy, thus playing a key role in risk stratification of patients for treatment. Little is known of the relationship between different cytogenetic subtypes in B-cell precursor acute lymphoblastic leukemia and the recently reported copy number abnormalities affecting significant leukemia associated genes. In a consecutive series of 1427 childhood B-cell precursor acute lymphoblastic leukemia patients, we have determined the incidence and type of copy number abnormalities using multiplex ligation-dependent probe amplification. We have shown strong links between certain deletions and cytogenetic subtypes, including the novel association between *RB1* deletions and intrachromosomal amplification of chromosome 21. In this study, we characterized the different copy number abnormalities and show heterogeneity of *PAX5* and *IKZF1* deletions and the recurrent nature of *RB1* deletions. Whole gene losses are often indicative of larger deletions, visible by conventional cytogenetics. An increased number of copy number abnormalities is associated with NCI high risk, specifically deletions of *IKZF1* and *CDKN2A/B*, which occur more frequently among these patients. *IKZF1* deletions and rearrangements of *CRLF2* among patients with undefined karyotypes may point to the poor risk *BCR-ABL1*-like group. In conclusion, this study has demonstrated in a large representative cohort of children with B-cell precursor acute lymphoblastic leukemia that the pattern of copy number abnormalities is highly variable according to the primary genetic abnormality.

Introduction

The cytogenetics of B-cell precursor acute lymphoblastic leukemia (BCP-ALL) is well documented, with specific chromosomal abnormalities used in risk stratification of patients for treatment.[1,2] Genomic studies have shown that copy number abnormalities (CNA) of genes involved in B-lymphocyte development and differentiation, cell cycle control and those of significance in hematopoiesis are common in BCP-ALL.[3-5] Notable deletions include *PAX5, IKZF1* (*Ikaros*),[6-11] and genes within the pseudoautosomal region (PAR1) of the sex chromosomes, resulting in the *P2RY8-CRLF2* gene fusion and overexpression of *CRLF2*.[12] Here there is particular interest in *IKZF1* and *CRLF2* in relation to outcome and their role as molecular targets for therapy. *IKZF1* deletions have been associated with a poor prognosis in BCP-ALL,[6-10] while the risk relating to *CRLF2* has been variable and dependent on other features.[13-15] Nevertheless, thus far these diverse findings have not led to any treatment changes. Studies have focused on small or selected cohorts and analyses have often been carried out independently from other genetic changes. Thus their accurate incidence, relationship to each other, and the major cytogenetic subgroups still have to be determined in order to understand their true clinical relevance.

Recently, we demonstrated that multiplex ligation-dependent probe amplification (MLPA) provided an accurate and reliable high throughput method to screen for CNA of the significant genes in BCP-ALL.[16] In this study, we screened a cohort of 1427 childhood BCP-ALL patients from two consecutive treatment trials using the same MLPA approach. We report the frequency and type of CNA involving these genes, their associations with established chromosomal abnormalities, and other clinical features.

Design and Methods

Patients in this study were diagnosed with BCP-ALL and registered on UK treatment trials UKALL97/99 (April 1997-June 2002) for children aged 1-18 years[17] and UKALL2003 (October 2003-July 2011) for children aged 1-25 years.[18] Clinical details were provided by the Clinical Trial Service Unit (CTSU), Oxford, UK. All participating centers obtained local ethical committee approval and written informed consent from patients, parents or guardians in accordance with the Declaration of Helsinki. Risk was assessed using National Cancer Institution (NCI) criteria.

Patients were classified into eight cytogenetic subgroups according to the presence of the following chromosomal abnormalities: 1) t(12;21)(p13;q22)/*ETV6-RUNX1* fusion; 2) high hyperdiploidy (51-65

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chromosomes); 3) translocations involving 11q23/MLL rearrangements; 4) t(9;22)(q34;q11)/*BCR-ABL1* fusion; 5) intrachromosomal amplification of chromosome 21 (iAMP21);[19] 6) t(1;19)(q23;p13)/*TCF3-PBX1*; 7) other abnormal (absence of abnormalities in subgroups 1-7 above); and 8) normal karyotype. Patients were classified into good, intermediate, and poor cytogenetic risk groups according to previously published data.[20]

DNA obtained from the presentation bone marrow sample was used to determine the copy number of *IKZF1, CDKN2A/B, PAX5, EBF1, ETV6, BTG1, RB1*, and genes within PAR1: *CRLF2, CSF2RA, IL3RA*, using the SALSA MLPA kit P335 *IKZF1* (MRC Holland, The Netherlands), as previously described.[16] In those patients entered on UKALL2003, deletions of *IKZF1* and *RB1* were confirmed and further characterized by the P202 *IKZF1* and the P047 *RB1* SALSA MLPA kits, respectively.

Deletions of genes within the PAR1 region identified by MLPA were confirmed as *P2RY8-CRLF2* or unbalanced *IGH@-CRLF2* translocations by interphase fluorescence *in situ* hybridization (FISH) as previously reported.[12]

Statistical analysis was carried out using Intercooled STATA v. 12.0 (StataCorp, USA), particularly Wilcoxon Rank Sum for non-parametric assays and χ^2 for comparison of categorical variables.

Results

CNA among the entire cohort

In total, 1427 patients were included in this study. There was no difference between these patients and other trial participants with respect to sex, age, central nervous system (CNS) disease or NCI risk group. Tested patients were more likely to have a white blood cell (WBC) count over 10 x 10^9/L reflecting the increased possibility of surplus available material (*Online Supplementary Table S1*).

Incidences of CNA for genes tested by MLPA are given in Table 1. Overall, 59% of patients showed an abnormality of at least one of these genes: 433 (30%) patients had one, 254 (18%) had two, 131 (9%) had three and 28 (2%) had four or more deletions. Overall, deletions of *CDKN2A/B* and *ETV6* were the most frequent, while *EBF1* deletions were rare. Patients classified as NCI high risk were significantly more likely to have a greater number of deletions compared to those classified as NCI standard risk ($P<0.001$) (Figure 1).

Table 1 shows the distribution of abnormalities in relation to demographic and clinical features. The cohort comprised 665 (46%) females and 762 (54%) males. There was no shift in the gender balance within each subgroup according to the defined CNA. The median age of the cohort was five years (range 1-23) with 24% of patients being 10 years or older. Patients with *IKZF1* and *CDKN2A/B* deletions were significantly older. The median age for *IKZF1* and *CDKN2A/B* deletions was seven years (P <0.0001) and six years (P <0.0001), respectively, with 39% and 33%, respectively, of deletions occurring in patients aged 10 years old or older. The incidence of these deletions increased with age (Table 1), a trend that continued into adulthood as shown by the incorporation of MLPA data from the UK adult ALL treatment trial, UKALLXII[21] (Figure 2). There was a peak in incidence of *ETV6* deletions in children aged 2-4 years (*data not shown*), explained by their strong association with *ETV6-RUNX1*, which has a peak incidence in this age group.[22] There was no significant change in incidence linked to age for dele-

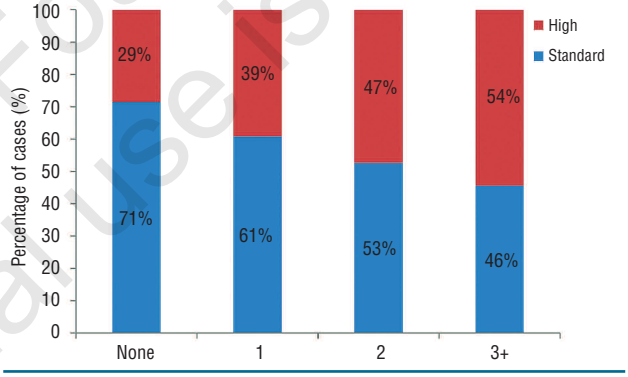

Figure 1. Bar chart showing the percentage of patients within NCI high and standard risk groups with increasing numbers of CNA.

Table 1. Association of copy number abnormalities with demographics and WCC.

	Cohort	Genes with CNA/chromosomal region																	
		IKZF1		*PAX5*		*RB1*		*CDKN2A/B*		*ETV6*		*EBF1*		*BTG1*		*PAR1*[1]	
N. of cases	1427	196	14%	272	19%	92	6%	395	28%	312	22%	30	2%	89	6%	63	4%
Age (years)																	
Median	5	7*		5		7		6*		4		6		6		5	
1-4	704	55	8%	135	19%	30	4%	152	22%	168	24%	10	1%	35	5%	29	4%
5-9	386	64	17%	66	17%	33	9%	115	30%	82	21%	14	4%	29	8%	26	7%
10-14	240	54	23%	53	22%	23	10%	93	39%	40	17%	6	3%	19	8%	5	2%
15-24	97	23	24%	18	19%	6	6%	35	36%	22	23%			6	7%	3	3%
Gender M:F (ratio)	762:665 (1.15)	105:91 (1.15)		146:126 (1.15)		50:42 (1.19)		214: 181 (1.18)		160:152 (1.05)		15:15 (1.0)		46:43 (1.07)		28:35 (0.8)	
WCC (x10^9/L)																	
Median	11.8	21.6*		21.3*		11.7		21.1*		13.1		13.9		14.8		22	
<10	633	65	10%	97	15%	36	6%	131	21%	130	21%	12	2%	37	6%	22	3%
10-49.9	527	69	13%	92	17%	42	8%	150	28%	118	22%	9	2%	32	6%	26	5%
≥50	267	62	23%	83	31%	14	5%	114	43%	64	24%	9	3%	20	7%	15	6%

CNA: copy number abnormalities; [1]*this group includes* P2RY8-CRLF2 *and unbalanced* IGH@-CRLF2 *translocations;* *P<*0.0001.*

tions of the other genes tested, including *ETV6* deletions in *ETV6-RUNX1* negative patients.

The median WBC count of the cohort was 11.8 x 10^9/L with 44% of patients having a count of less than 10 x 10^9/L. Patients with *IKZF1, PAX5* or *CDKN2A/B* deletions were more likely to have a WCC of over 50 x 10^9/L (each $P<0.001$) (Table 1). The association of these genes to age and WBC count meant that there was a significantly higher incidence of patients with *IKZF1, PAX5* and/or *CDKN2A/B* deletions classified as NCI high risk compared to other patients (each $P<0.001$) (Table 2).

The frequency of each cytogenetic subgroup among 1351 patients with a successful cytogenetic result is shown in Table 3. Patients with *ETV6-RUNX1*, high hyperdiploidy and those classified as 'other abnormal' comprised the most common subgroups at incidences of 28%, 30% and 24%, respectively. Patients positive for *ETV6-RUNX1* showed the highest number of CNA overall, followed by those in the other abnormal group. In contrast, CNA occurred at a lower than expected level in high hyperdiploid patients. The incidence of CNA was also low in the subgroup with MLL rearrangements and higher in the other poor-risk subgroups: *BCR-ABL1* positive and iAMP21. The increasing numbers of CNA in each cytogenetic group are shown in Figure 3, while the incidences and distribution of the individual CNA within each cytogenetic group are shown in Figure 4.

Among *ETV6-RUNX1* positive patients, in addition to a high incidence of wild-type *ETV6* deletions, *CDKN2A/B* and *PAX5* were each deleted in 22% of these patients, while the incidence of *IKZF1* deletions was low. Although *BTG1* deletions were rare throughout the cohort (n=87,

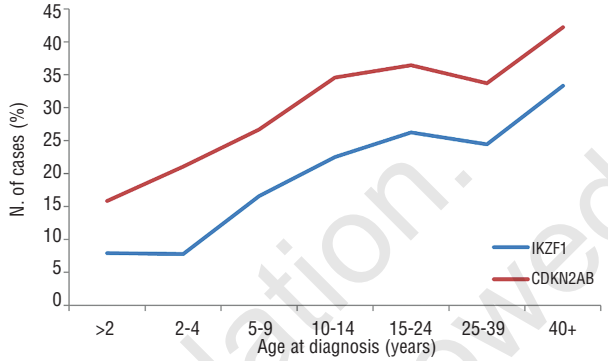

Figure 2. Graphs showing the proportion of cases with ***IKZF1*** and ***CDKN2A/B*** deletions according to age among ***BCR-ABL1*** negative patients. As it is known that there is a strong association between ***IKZF1*** deletions and ***BCR-ABL1*** positive ALL[7] and that the incidence of ***BCR-ABL1*** positive ALL increases with age[22], childhood ***BCR-ABL1*** positive patients were excluded from this age association analysis of ***IKZF1*** and ***CDKN2A/B*** for direct comparison with the MLPA tested adult ***BCR-ABL1*** negative series[21].

Table 2. Association of Copy Number Abnormalities with NCI and Cytogenetic Risk Group.

	Cohort		Genes with CNA															
	IKZF1		*PAX5*		*RB1*		*CDKN2A/B*		*ETV6*		*EBF1*		*BTG1*		*PAR1*[1]			
N. of cases	1351		177	13%	255	19%	89	7%	371	27%	301	22%	27	2%	87	6%	56	4%
NCI Risk Group																		
Standard	838	62%	71	8%	124	15%	53	6%	171	20%	191	23%	18	2%	51	6%	39	5%
High	513	38%	106	21%	131	26%	36	7%	200	39%	110	21%	9	2%	36	7%	17	3%
Cyto Risk Group																		
Good	787	58%	46	6%	101	13%	39	5%	153	19%	231	29%	14	2%	59	7%	10	1%
Intermediate	441	33%	96	22%	121	27%	23	5%	173	39%	57	13%	7	<1%	21	5%	32	7%
Poor	123	9%	35	28%	32	26%	27	22%	45	37%	13	11%	6	5%	7	6%	14	11%

Cyto, cytogenetics; [1]this group includes P2RY8-CRLF2 *and unbalanced* IGH@-CRLF2 *translocations.*

Table 3. Incidences of CNA according to cytogenetic subgroups.

Cytogenetic subgroup	Cases in each group		*IKZF1*		*PAX5*		*RB1*		*CDKN2A/B*		*ETV6*		*EBF1*		*BTG1*		*PAR1*[1]		Total CNA	
	N.	%[2]	N.	%[3]	N.	%[3]	N.	%[3]	N.	%[3]	N.	%[3]	N.	%[3]	N.	%[3]	N.	%[3]	N.	%[4]
Total	1351[5]		177	13%	255	19%	89	7%	371	27%	301	22%	27	2%	87	6%	56	4%	1334	
ETV6-RUNX1	379	28%	11*	3%	83	22%	29	8%	84	22%	203*	54%	14	4%	57*	15%	3*	<1%	484	6%
HeH	408	30%	35*	9%	18*	4%	10*	3%	65*	16%	28*	7%	0		2*	<1%	7	2%	165	12%
MLL rearranged	28	2%	2	8%	2	8%	0		8	31%	1	4%	0		0		0		13	1%
BCR-ABL1	33	2%	21*	64%	15*	45%	3	9%	16[5]	48%	1	3%	2	6%	1	3%	1	3%	60	5%
iAMP21	33	2%	7	21%	4	12%	13*	39%	4	12%	7	21%	3	9%	1	3%	10*	30%	49	4%
t(1;19)/*TCF3-PBX1*	44	3%	3	7%	8	18%	7	16%	10	23%	2	5%	1	2%	0		1	2%	32	2%
Other abnormal	324	24%	77*	24%	115*	36%	23	7%	153*	47%	51	16%	5	2%	3	1%	33*	10%	460	34%
Normal	102	8%	20	20%	9	9%	4	4%	22	22%	8*	8%	1	<1%	6	6%	1	1%	71	5%

[1]This group includes P2RY8-CRLF2 *and unbalanced* IGH@-CRLF2 *translocations; [2]Calculated from total cohort with a successful cytogenetic result; [3]Calculated as total number of patients in this specific cytogenetic subgroup with this CNA; [4]Calculated from total number of abnormalities, which is greater than the total number of patients; [5]Patients with a successful cytogenetics result only; *P<0.0001; [5]P=0.005.*

6%), they were frequently associated with *ETV6-RUNX1* (15%) ($P<0.0001$). There was an association between *BCR-ABL1* and *IKZF1* with 64% having a deletion of *IKZF1*. Deletions of *PAX5* and *CDKN2A/B* were also high in this subgroup at incidences of 45% and 48%, respectively.

Among iAMP21 patients, *RB1* deletions and *P2RY8-CRLF2* were observed at incidences (39% and 30%, respectively) significantly higher than expected ($P<0.0001$). They showed the second highest frequency of *ETV6* deletions. In the *TCF3-PBX1* subgroup, there was a high incidence of deletions of *PAX5* and *CDKN2A/B*. In the group classified as 'other abnormal', deletions of *CDKN2A/B*, *PAX5* and *IKZF1*, as well as *P2RY8-CRLF2*, were seen at higher than expected frequencies.

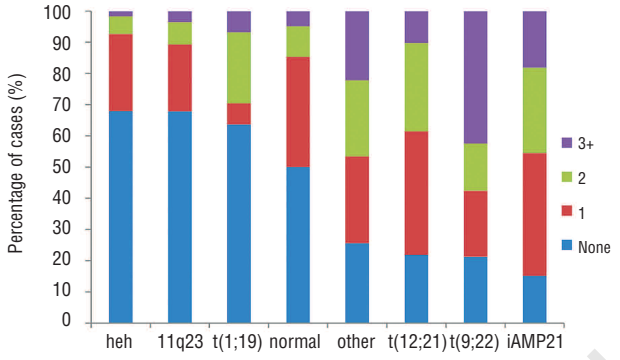

Figure 3. Bar chart showing the proportion of patients within each cytogenetic subgroup with increasing numbers of CNA.

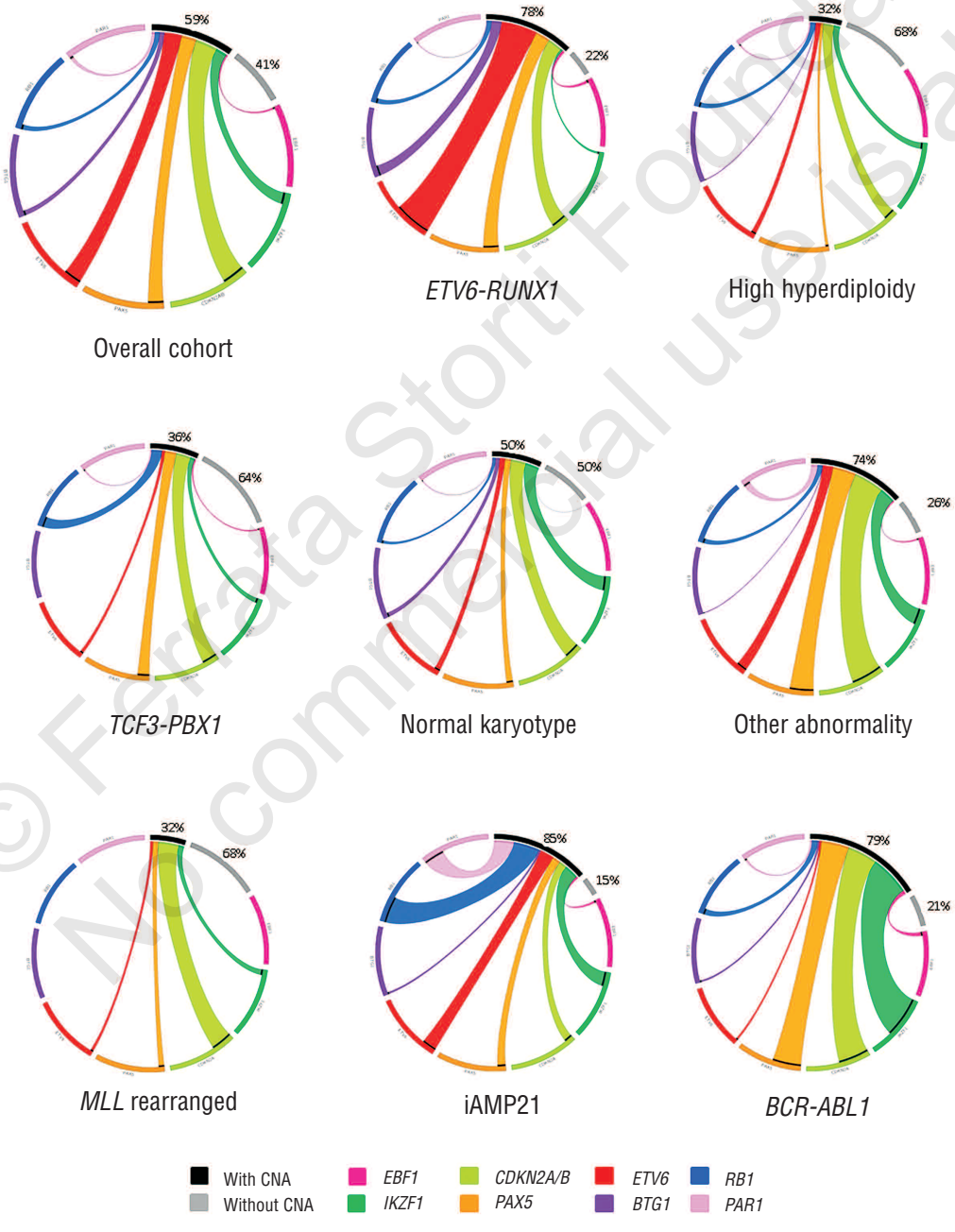

Figure 4. Circos plots showing the relative incidence and distribution of CNA within the overall cohort and the individual cytogenetic groups. The proportions of cases with and without CNA are indicated in black and gray, respectively, around the perimeter and the ribbons representing the individual CNA are color coded. The width of the ribbons reflects the frequency of each CNA.

Individual abnormalities

A range of interesting observations were made in relation to the individual abnormalities. IKZF1: deletions of *IKZF1* were present in 14% (n=196) of the cohort. The size of the deletion varied between patients (*Online Supplementary Figure S1*). The most frequent deletions involved either the whole gene (n=60) or were restricted to exons 4-7 (n=61). Other deletions occurred at lower frequencies: exons 2-3 (n=15), exons 2-7 (n=20), exons 4-8 (n=13), and miscellaneous deletions (n=27). Five patients had biallelic deletions; each showed a different pattern of loss, ranging from biallelic deletion of all exons to a subset of exons. No significant association was seen between the pattern of exon loss and cytogenetic subgroup, age or WBC count. Although unlikely to be significant, the biallelic deletions did not occur in association with any of the major cytogenetic groups. Whole gene deletions were associated with visible cytogenetic abnormalities of the short arm of chromosome 7 in 36 of 60 cases including: monosomy 7 (n=13), i(7)(q10) (n=7), dicentric chromosomes (dic) (n=6), balanced and unbalanced translocations involving chromosome 7 (n=10).

PAX5 and *CDKN2A/B*: a total of 272 (19%) patients showed heterogeneous CNA of *PAX5*. The majority of patients had deletions including exon 1 involving the entire or part of the gene (n=141). The remaining patients had partial deletions excluding exon 1 (n=121) or intragenic amplifications of exons 2 or 5 (n=10), as previously illustrated.[16]

Deletions of *CDKN2A/B* represented the most frequent abnormality in the cohort (n=395), of which 143 showed visible cytogenetic abnormalities of the short arm of chromosome 9 (9p), 212 showed no 9p abnormality, while 40 failed cytogenetic analysis. Among those cases with a visible 9p abnormality, 103 showed concurrent CNA of *PAX5*. A further 59 patients with CNA of both genes had no visible 9p abnormality (n=41) or failed cytogenetic results (n=18). The types of cytogenetic abnormalities involving chromosome 9 associated with these CNA are shown in *Online Supplementary Table S2*. Dicentric chromosomes involving chromosome 9 were shown in 43 patients: dic(7;9) (n=7), dic(9;12) (n=16) and dic(9;20) (n=20). Interestingly, they showed heterogeneous exon loss from PAX5, ranging from the entire gene to loss of the telomeric exons (not including exon 1) and they variably included deletions of *CDKN2A/B*.

ETV6: *ETV6* was frequently deleted throughout the entire cohort (n=312). As expected, *ETV6* deletions were frequent among *ETV6-RUNX1* positive patients (n=203). FISH results were available on 186 of these *ETV6-RUNX1* positive cases. In 154 (83%), the results by FISH and MLPA were concordant. Among the remaining 32 cases, deletions were found by MLPA but not FISH, indicating the presence of focal deletions below the resolution of FISH. Conversely, 40 cases with *ETV6-RUNX1* showed an *ETV6* deletion by FISH only. These either represented small populations of cells containing the deletions (<25% of nuclei) below the level at which MLPA would be expected to detect loss, or deletion of the wild-type *ETV6* allele with an associated gain of the derivative chromosome 21, thus producing a normal copy number for the *ETV6* exons covered by the MLPA probes. These observations highlight the previously described advantages and disadvantages of these two techniques.[16]

In total, 109 *ETV6-RUNX1* negative cases showed loss of *ETV6*; among 86 of these cases with FISH results available, 52 showed loss of the entire *ETV6* by both FISH and MLPA, while 34 cases showed small intragenic deletions by MLPA which were below the resolution of FISH.

RB1: deletions of *RB1* were present in 92 cases. These were of two types: 1) loss of the entire gene (n=60); 2) focal deletions, including exons 19-26 (n=28) (probes for these exons are included in the MLPA kit). An additional 4 cases had biallelic deletions of exons 19-26 as well as monoallelic loss of the remainder of the gene. Further studies using the P047-*RB1* MLPA kit on 65 cases confirmed whole gene loss in 40 cases tested. These studies indicated that the deletions extended into the adjacent genes: *ITM2B* and *RCBTB2* in all 40 cases, including *DLEU* in 38 of them. The relative location of these genes is shown in *Online Supplementary Figure S2A*. These results showed that *RB1* loss in these patients is part of a larger deletion targeting several genes.

Further characterization of those cases with focal deletions, using the *P047-RB1* MLPA kit with a higher probe density, showed the precise location of the deletion breakpoint to be between exons 17 and 18 in 19 of 21 cases tested, while in the 2 remaining cases the breakpoints were located between exons 16 and 17 and exons 18 and 19. In 8 of 21 cases with this focal deletion, the 5' breakpoint included deletion of the *RCBTB2* gene. The 4 patients with both deletions showed different patterns of exon loss: 2 showed monoallelic loss of *ITM2B, RCBTB2* and *DLEU*. The other 2 showed normal copy number for *ITM2B* and *DLEU* with the biallelic loss extending to *RCBTB2* in one case and monoallelic loss of this gene in the other. These results are illustrated in *Online Supplementary Figure S2B*.

From cytogenetic analysis, 52 cases in the entire cohort showed 13q abnormalities although only 52% (n=27) of these were associated with an *RB1* deletion.

CRLF2 *gene rearrangements*: deletions within PAR1 were detected by MLPA in 4% (n=63) of the cohort, of which 54 cases had fixed cells available for FISH investigations. The *P2RY8-CRLF2* fusion was confirmed by FISH in 49 cases, while the remaining 5 cases were found to be *IGH@-CRLF2* translocations with associated deletion within the PAR1 region. FISH testing for the presence of *IGH@* rearrangements had been carried out on 57% (n=807) of the entire cohort. Twelve cases, with a normal result by MLPA, were shown to have balanced *IGH@-CRLF2* translocations by FISH. Collectively, these patients were described as *CRLF2* rearranged, accounting for 5.3% of the cohort.

P2RY8-CRLF2 was most common among the iAMP21 subgroup (30%) and patients classified as 'other abnormal' (10%). Although *P2RY8-CRLF2* was rare in association with other cytogenetic subtypes, it was found in all subtypes in this series except the MLL rearranged group. The positive cases included 3 *ETV6-RUNX1* positive patients and one each of *TCF3-PBX1* and *BCR-ABL1*.

Among these patients with *CRLF2* rearrangements, 48% also showed loss of *IKZF1*. However, *IKZF1* deletions were more significantly associated with *IGH@-CRLF2* than *P2RY8-CRLF2*, occurring in 82% and 37%, respectively (P=0.001). *IGH@-CRLF2* patients were more likely to be classified as NCI high risk than *P2RY8-CRLF2* (65% and 29%, respectively, P=0.008). However, this result did not translate into a correlation between *IKZF1* deletion status and risk among *CRLF2* rearranged patients,

as 56% of high risk and 42% of standard risk *CRLF2* rearranged patients also had *IKZF1* deletions (*P*=0.22).

Discussion

In this study, we present the findings from a detailed retrospective analysis of CNA in significant genes involved in B-cell development, cell cycle control and hematopoiesis among a large consecutive series of pediatric BCP-ALL patients treated on UK ALL treatment trials. Although 59% of patients showed an abnormality of at least one of these genes, 41% showed none. The number of CNA occurring simultaneously in the same patient was low. Thus, these observations, in association with cytogenetic data, confirm that the genomic profiles of childhood BCP-ALL are not generally complex. Although the involvement of other genes not covered by the MLPA kit cannot be ruled out, data from SNP arrays have shown the incidence of other recurrent sub-microscopic abnormalities to be infrequent.[3-5] From MLPA studies, it is not possible to gain information on the temporal order in which these events arose in terms of karyotypic evolution or identify which abnormalities were the potential 'drivers' of leukemogenesis. However, it was possible to examine associations between these abnormalities and demographic and clinical features, as well as with cytogenetics.

In relation to the individual abnormalities, *IKZF1* showed heterogeneity in the size of the deletion, as previously demonstrated.[3] The majority of patients either showed deletion of the entire gene, often seen as a visible cytogenetic change involving 7p, or restricted to exons 4-7. A range of other deletion types were also observed of which a small number were biallelic. No significant association was found between the pattern of exon loss and cytogenetic subtype. *IKZF1* is transcribed in several isoforms as a result of alternative splicing, essentially altering the expression of exons 3 to 5 that encode the N-terminal DNA-binding domain. Deletions of exons 4-7 result in expression of a dominant negative IKZF1 isoform, Ik6, that lacks the N-terminal DNA binding zinc finger and shows oncogenic activity.[11,23] Deletion of exon 2, which harbors the translational start site, will inhibit protein translation. Loss of exon 8 will have an effect on dimerization of *IKZF1*. Thus, deletions involving these exons are likely to have the same impact as whole gene deletions. Loss of the non-coding exon 1 only is likely to be of no significance. Accurate characterization of these heterogeneous deletions of *IKZF1* is important before we can begin to understand their prognostic relevance.

The extent of *PAX5* deletions was variable, ranging from whole gene loss to loss of the telomeric exons, confirming previous SNP data[3] and our earlier observations based on FISH.[24] Those with deletions of the entire or part of the gene including exon 1 are predicted to result in reduced PAX5 expression. Those with partial deletions not involving exon 1 are predicted to express a mutant allele.[25] Ten patients showed intragenic amplifications of exons 2 or 5. These amplifications have been previously reported as a rare occurrence and are similarly predicted to express mutant alleles.[25] In a number of cases, *PAX5* deletions occurred as the result of dicentric chromosomes involving chromosome 9. Interestingly, among those cases with dic(9;12), 4 were associated with *ETV6-RUNX1* fusion, while the remaining 5 were classified as 'other abnormal'. Although the number of cases was small, there was a distinct pattern of exon loss between the two groups of dic(9;12) cases. The other abnormal cases showed loss of *ETV6* exons 1-2 and *PAX5* exons 5-10. This finding was consistent with previously published data in which this abnormality was associated with expression of an *ETV6-PAX5* fusion protein.[26] The dicentric chromosomes associated with *ETV6-RUNX1* showed larger deletions of ETV6 and loss of the entire *PAX5*, indicating that these dic(9;12) translocations do not result in *ETV6-PAX5* fusion.

Deletions of *CDKN2A/B* represented the most frequent abnormality in the cohort, which were often associated with visible abnormalities of 9p and concurrent loss of *PAX5*. Interestingly, 8 of the 10 cases showing intragenic amplification of *PAX5* also showed deletion of *CDKN2A/B*.

RB1 deletions were homogeneous compared to other deletions, being restricted to two types: 1) those including the entire gene, as well as the adjacent genes: *ITM2B, RCBTB2* and *DLEU*; and 2) focal deletions including exons 18-26 in all but one case. They were occasionally biallelic with different sized deletions on the two alleles. Some of the larger deletions were visible at the cytogenetic level. Deletions of 13q have been associated with increased risk of relapse.[20] However, this study showed that only approximately 50% of visible 13q abnormalities were associated with an *RB1* deletion, indicating that in at least some of these cases *RB1* is not the target of the deletion. RB1 deletions of exons 18-27 have been previously reported in lymphoma.[27] The molecular consequence of this recurring deletion is still not well understood. Expression of a truncated protein with altered function or deletion of *LPAR6/P2RY5*, located within *RB1*, may be one of the consequences.

With the exception of patients with *MLL* rearrangements, the presence of *CRLF2-P2RY8* has now been reported in association with all cytogenetic subtypes. In this study, we identified a small number of cases among patients with *ETV6-RUNX1*, *TCF3-PBX1* and *BCR-ABL1*; the latter two have not been previously reported. We showed *CRLF2* rearrangements to be present in 5.3% of the cohort. Several groups have reported a strong association between CRLF2 overexpression and *IKZF1* alterations.[13,14,28-30] Although this study is restricted to the detection of *CRLF2* rearrangements, with the exception of rare mutations of the gene,[31] without measure of expression, we confirmed this association. We also confirmed that *IGH@-CRLF2* occurred at a higher incidence in NCI high-risk patients, while *P2RY8-CRLF2* was seen at a higher frequency in NCI standard risk.

We have previously reported the high incidence of *P2RY8-CRLF2* among iAMP21 patients.[32] We confirmed an increased incidence of *ETV6* deletions as previously reported;[33] however, the frequent occurrence of *RB1* deletions in these patients is shown here for the first time.

There was some correlation between the distribution of CNA and patient age. Patients with *IKZF1* and *CDKN2A/B* deletions were older and their incidence increased with age. Patients with *IKZF1*, *PAX5* and *CDKN2A/B* deletions had significantly higher WBC count than patients with the other CNA. These associations with older age and higher WBC count explain why these deletions occur at a higher frequency in the NCI high-risk group, that is defined by age and WBC count. Previous studies have been inconclusive as to the prognostic relevance of *CDKN2A/B* in both childhood and adult ALL.[34]

The association with NCI risk defined here, coupled with observations that patients with *CDKN2A/B* deletions have a shorter time to relapse than other relapsed patients,[35,36] suggests that further studies are warranted in order to clarify the prognostic relevance of *CDKN2A/B* deletions.

The frequency of each cytogenetic subgroup among this patient cohort was the same as that previously reported by us for a single UK childhood ALL treatment trial, ALL97/99.[15,20] We showed that the incidence of CNA varied according to cytogenetic subtype, although the number did not correlate with the cytogenetic risk. For example, the good-risk cytogenetic groups (*ETV6-RUNX1* and high hyperdiploidy) as well as the poor risk (*BCR-ABL1* and *MLL* rearranged) showed a high and low number of CNA, respectively.

The high number of CNA in the *ETV6-RUNX1* positive group suggests that CNA rather than point mutations may be the drivers of leukemia in this subgroup, as other studies have shown *ETV6-RUNX1* positive leukemia to harbor a modest number of point mutations.[37] Interestingly, although *BTG1* deletions were rare, they were most often associated with *ETV6-RUNX1*, as previously reported.[38] As *BTG1* has been reported to be associated with glucocortoid receptor autoinduction,[39] these patients require follow up to determine whether these deletions affect their overall survival.

The lower than expected level of deletions in high hyperdiploid patients is unlikely to be an artefact of analyzing CNA in the context of ploidy change, as neither the genes tested nor the reference probes in the MLPA kit are located on chromosomes commonly gained in high hyperdiploidy. Point mutations have been found at an increased level in this subgroup, indicating that disease progression in high hyperdiploid patients may be driven by mutations rather than deletions, at least among the genes tested.[40] Although the incidence of CNA was also low in patients in the poor-risk subgroup with *MLL* rearrangements, this finding might be expected from the known potency of this abnormality as a driver of leukemogenesis.[41] From these observations, it is evident that future studies assessing the prognostic value of these CNA must include cytogenetic data in order to gain a clear picture of their association with outcome.

As previously shown, there was a strong association between *BCR-ABL1* fusion and *IKZF1* deletions.[7] Deletions of *PAX5* and *CDKN2A/B* were also high in the *BCR-ABL1* positive group. In the group classified as 'other abnormal', deletions of *CDKN2A/B*, *PAX5* and *IKZF1* were also frequent. The striking similarity in CNA profiles between these two groups is clearly shown in Figure 4. The poor-risk group described as *BCR-ABL1*-like,[6,41] defined as sharing the same gene expression profile as well as the same poor risk as *BCR-ABL1* positive patients, is most likely to be found among those patients with 'other abnormal' karyotypes as no distinctive karyotypic features have yet been described to define them. Thus the occurrence of deletions of *CDKN2A/B*, *PAX5* and *IKZF1*, as well as deregulated *CRLF2*, among patients in this 'other abnormal' group may provide a pointer to the BCR-ABL1-like subgroup, as indicated by others.[42]

This study represents the largest trial-based screen for abnormalities in selected genes of significance in the development of BCP-ALL. It has confirmed findings of previous studies of associations between copy number abnormalities and particular cytogenetic subgroups. It has shown the heterogeneous nature of deletions such as *PAX5* and *IKZF1* and the recurrent nature of *RB1* deletions. The association of *IKZF1* and *CDKN2A/B* deletions with NCI high risk is of interest, and within the 'other abnormal' cytogenetic group, the presence of these deletions and/or rearrangements of *CRLF2* may point to the poor-risk *BCR-ABL1* like group.

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