

Importance of different growth factors in oestrogen receptor-negative breast cancer

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Abstract

The aims of this study were firstly, to investigate the role of growth hormones: insulin-like growth factor (IGF-I), epidermal growth factor (EGF) and heregulin-1 (HRG) on the anchorage-independent survival and migration of oestrogen non-responsive breast cancer cells. Secondly, the effects of inhibition of the type I IGF receptor (IGF-IR), EGF receptor (EGFR) and human epidermal growth factor receptor 2 (HER-2) were studied. Thirdly, the expression of IGF-IR, EGFR, HER-2, TFF1 and TFF3 was assessed in oestrogen receptor-negative breast tumours and involved lymph node metastases, and associations with biomarkers of cell survival and proliferation analysed.

IGFs and EGFs contribute to the growth, survival and metastasis of malignant cells and inhibitors of IGF-IR and EGFR are in clinical trials. IGF and EGF signal transduction pathways are proposed to be important in oestrogen non-responsive, HER-2 positive and triple-negative breast cancers. The effects of IGF and EGF on anchorage-independent cell survival and cell migration have not been investigated thoroughly.

An *in vitro* model of anoikis was developed for triple-negative MDA-MB-231, Hs578T and HER-2 positive SK-BR-3 breast cancer cells. For the first time, an anti-anoikis effect of IGF-1 was demonstrated in triple-negative breast cancer cells. The survival effect of IGF-1 was shown to be *via* activation of the IGF-IR. EGF and HRG were protective against anoikis in oestrogen receptor-negative breast cancer cells. The protective effect of EGF was mediated through EGFR. IGF-1, EGF and HRG demonstrated powerful migratory effects on oestrogen non-responsive breast cancer cells and the inhibition of IGF-IR and EGFR in these cells reduced the cell migration. The combination of IGF-1 and EGF induced more cell migration than either ligand alone. IGF-IR, EGFR and HER-2 were expressed in oestrogen receptor-negative breast primary tumours, and expression of IGF-IR and EGFR was associated with biomarkers of cell survival and proliferation. Expression of TFF3 was detected at higher levels than TFF1 in both *in situ* and invasive breast tumours and was associated significantly with expression of HER-2 in invasive breast tumours.

Overall, these results demonstrated the importance of different growth factors alone and in combination in oestrogen non-responsive breast cancer. Further investigation of the potential of targeting the metastatic progression of oestrogen non-responsive breast cancer *via* the IGF and EGF signal transduction pathways is merited.

Dedication

This thesis is dedicated to

My wonderful and lovely

Mother and father

And my

Fiancé

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Abbreviations

| | |
|-------------|--|
| ADAM | A Disintegrin and Metalloprotease |
| ADCC | Anti-tumour Dependent Cell-mediated Cytotoxicity |
| APAF | Apoptosis Protease Activating Factor |
| AR | Amphiregulin |
| BCA | Bicinchoninic Acid |
| BL 1 and 2 | Basal-like (1, 2) |
| BMF | Bcl-2 Modifying Factor |
| BRCA (1, 2) | Breast Cancer Associated (susceptibility gene 1 and 2) |
| BTC | Betacellulin |
| cDNA | Complementary DNA |
| CK 5/6 | Cytokeratin 5/6 |
| DAB | Diaminobenzidine |
| DAPI | 4',6-Diamidino-2-Phenylindole, Dihydrochloride |
| DCC-CS | Dextran-Coated Charcoal-treated Calf Serum |
| DCIS | Ductal Carcinoma <i>In Situ</i> |
| DISC | Death-Inducing Signalling Complex |
| DMEM | Dulbecco's Modified Eagle's Medium |
| DMSO | Dimethyl Sulphoxide |
| DNA | Deoxy ribo Nucleic Acid |
| DPX | Distrene Phthalein Xylol |
| EDTA | ThyleneDiamine Tetra Acetic Acid |
| EGF | Epidermal Growth Factor |

| | |
|--------|---|
| EGFR | Epidermal Growth Factor Receptor |
| EMT | Epithelial Mesenchymal Transition |
| EPG | Epigen |
| EPR | Epiregulin |
| ER | Oestrogen Receptor |
| ErbBs | Epidermal growth factor receptor kinases |
| ERK | Extracellular signal Regulated Kinase |
| FADD | Fas Associated Death Domain |
| FAK | Focal Adhesion Kinase |
| Fas L | Fas Ligand |
| FBS | Foetal Bovine Serum |
| FGF | Fibroblast Growth Factor |
| FLIP | FLICE Inhibitory Protein |
| GAPDH | Glyceraldehyde 3-Phosphate Dehydrogenase |
| Grb2 | Growth factor receptor-bound protein 2 |
| HB-EGF | Heparin Binding EGF |
| HEPES | N-2-hydroxyethylpiperazine-N'-2- ethanesulfonic acid |
| HER-2 | Human Epidermal growth factor Receptor 2 |
| HER-3 | Human Epidermal growth factor Receptor 3 |
| HER-4 | Human Epidermal growth factor Receptor 4 |
| HGF | Hepatocyte Growth Factor |
| IDC | Invasive Ductal Carcinoma |
| IGF-1 | Insulin-like Growth Factor 1 |
| IGF-2 | Insulin-like Growth Factor 2 |

| | |
|------------------|--|
| IGFBP (1-6) | Insulin-Like Growth Factor Binding Protein (1-6) |
| IGF-IR | Type I IGF Receptor |
| IGF-IIR | Type II IGF Receptor |
| IgG | Immunoglobulin |
| IR | Insulin Receptor |
| IRS 1, 2 | Insulin Receptor Substrate 1, 2 |
| ITF | Intestinal Trefoil Factor |
| JAK | Janus Kinase |
| LCIS | Lobular Carcinoma <i>In Situ</i> |
| M | Mesenchymal |
| mAb | Monoclonal Antibody |
| MAPK | Mitogen Activated Protein Kinase |
| ML | Mesenchymal-stem like |
| MMPs | Matrix Metalloproteinases |
| MTOR | Mammalian Target of Rapamycin |
| MTORC2 | Mammalian Target of Rapamycin Complex 2 |
| NRG (1-4) | Neuregulin (1-4) |
| OMM | Outer Mitochondrial Membrane |
| PARP | Poly (ADP) Ribose Polymerase |
| PDK1 | Phosphoinositide Dependent Kinase-1 |
| PDVF | Polyvinylidene difluoride |
| PgR | Progesterone Receptor |
| PI3K | Phosphatidylinositol-3-Kinase |
| PIP ₂ | Phosphatidyl Inositol 4, 5-bisphosphate |
| PIP ₃ | Phosphatidyl Inositol 3, 4, 5-triphosphate |
| PKC | Protein Kinase C |

| | |
|---------------|---|
| PLC γ | Protein kinase C activation Gamma |
| PMSF | Phenylmethylsulfonyl Flouride |
| Poly-HEMA | Poly 2 Hydroxyl Ethyl Methyl Acrylate |
| RIPA | Radio Immuno Precipitation Assay |
| RNA | Ribo Nucleic Acid |
| SDS | Sodium Dodecyl Sulphate |
| SDW | Sterile Distilled Water |
| SFM | Serum Free Medium |
| Shc | Src homology 2 domain |
| siRNA | Small Interfering RNA |
| SOS | Son Of Sevenless |
| STAT | Signal Transducer and Activators of Transcription |
| TBS | Tris-Buffered Saline |
| TBST | Tris-Buffered Saline/Tween 20 |
| TEMED | NNN'N' Tetramethylethylenediamine |
| TFF | Trefoil Factor Family |
| TKI | Tyrosine Kinase Inhibitor |
| TMA | Tissue Micro Array |
| TNF- α | Tumour Necrosis Factor-Alpha |

Chapter 1. Introduction

1.1 Histology of the normal breast

In the human, there is a single pair of mammary glands, one on the right and one on left side of the body situated anatomically over the pectoral muscles of the chest wall and suspended from the anterior chest wall with the help of the fibrous strands called cooper ligaments. Each mammary gland consists of one conical elevated area called the nipple which has small pores of 0.5 mm in diameter. The nipple is surrounded by moderately thin skin called areola which has more melanin than other parts of the breast. The structure of the breast is the same in males and females before puberty. After puberty the structure of female breast undergoes drastic changes due to an increase in the circulatory concentrations of ovarian hormones. This increases growth and development of the ductal-lobular system and the tendency of adipose tissue to accumulate in inter-lobular stroma of the breast (Luis Carlos Junqueira 2003).

Each human breast consists of 15 to 25 lobes that encircle the nipple and are separated from each other by inter-lobar connective tissue. Each lobe comprises numerous smaller lobes, called lobules or terminal duct lobular units which are open to the nipple through their own lactiferous ducts that transport milk from the lobules to the nipple (Figure 1.1 A). The ducts are lined by two layers: an inner columnar or cuboidal epithelial layer and an outer contractile myoepithelial cell layer (Figure 1.1 C) (Robbins 2010).

Throughout the menstrual cycle, the normal breast undergoes morphological changes. During the premenstrual phase, when oestrogen reaches to its peak level, the size of ductal epithelial cells increases and they become more columnar in shape. This growth is followed by swelling of the connective tissue and the breast become slightly larger than it is in the rest of the cycle (Robbins 2010). The structure and function of the normal breast change dramatically throughout pregnancy and lactation. These variations stem from the synergic actions of the ovarian hormones, particularly oestrogen, progesterone, prolactin and human placental lactogen. The breast increases in size due to growth and proliferation of the lactiferous ducts and conversion of the blunt-ended lobules into secretory spherical alveoli. During lactation, the alveoli produces milk,

whereas with termination of breast-feeding, the majority of the alveoli degenerate and cells are eliminated through apoptosis.

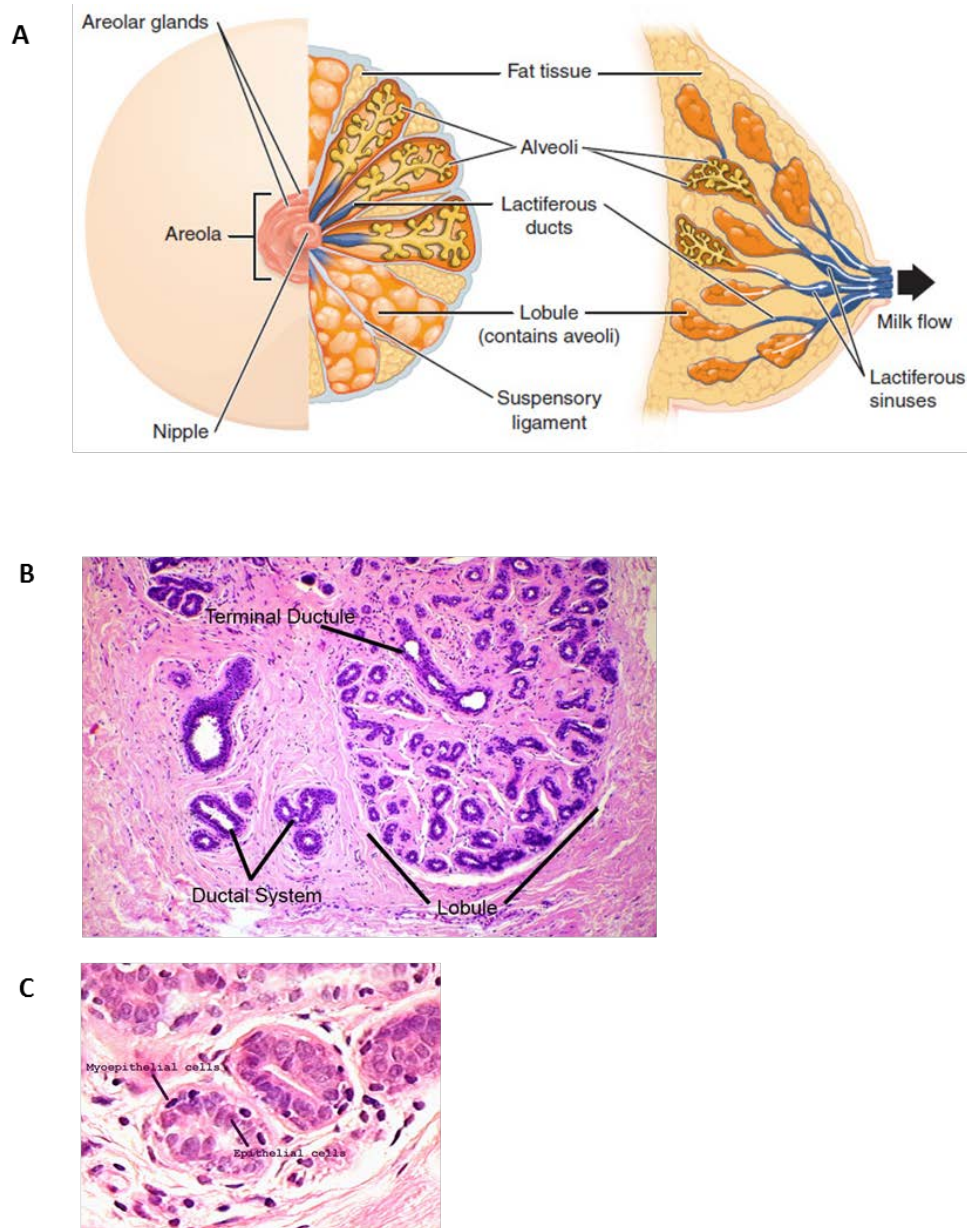


Figure 1.1: Structure of the human breast. The diagram represent that mammary gland contains numerous of lobes which formed a several smaller tubule-alveolar units called lobules. The lobes open separately to the nipple through lactiferous ducts that slightly widen to form lactiferous sinuses. Connective tissues called suspensory ligaments supports the breasts and connect the breast tissues to the dermis of the overlying skin **(A)**. Haematoxylin and eosin stained section shows that the each lobule divided into several terminal ductules **(B)**. The terminal ductules are lined by an inner columnar epithelium and an outer contractile myoepithelial cell layer **(C)**.

1.2 Breast cancer

1.2.1 Epidemiology of breast cancer

Breast cancer is one of the most common cancers diagnosed amongst women and is the leading cause of female mortality worldwide. It is estimated that over 1.7 million women were diagnosed with breast cancer and more than 520,000 cases succumb in 2012 (GLOBOCON 2012). Breast cancer incidence and mortality rates are variable globally. The highest incidence rates are in developed countries, whereas lower rates are found in under-developed or developing countries (Figure 1.2) (Boyle and Ferlay 2005). It has been reported that, in recent years, the rate of breast cancer mortality has declined noticeably in developed countries (Jemal, Center et al. 2010). This decline could be due to an increase in awareness, improvements in treatment, more accurate and early diagnosis which includes implementation of screening programmes with mammography, development of adjuvant therapies and reduction in the use of hormonal replacement therapy (Glass, Lacey et al. 2007).

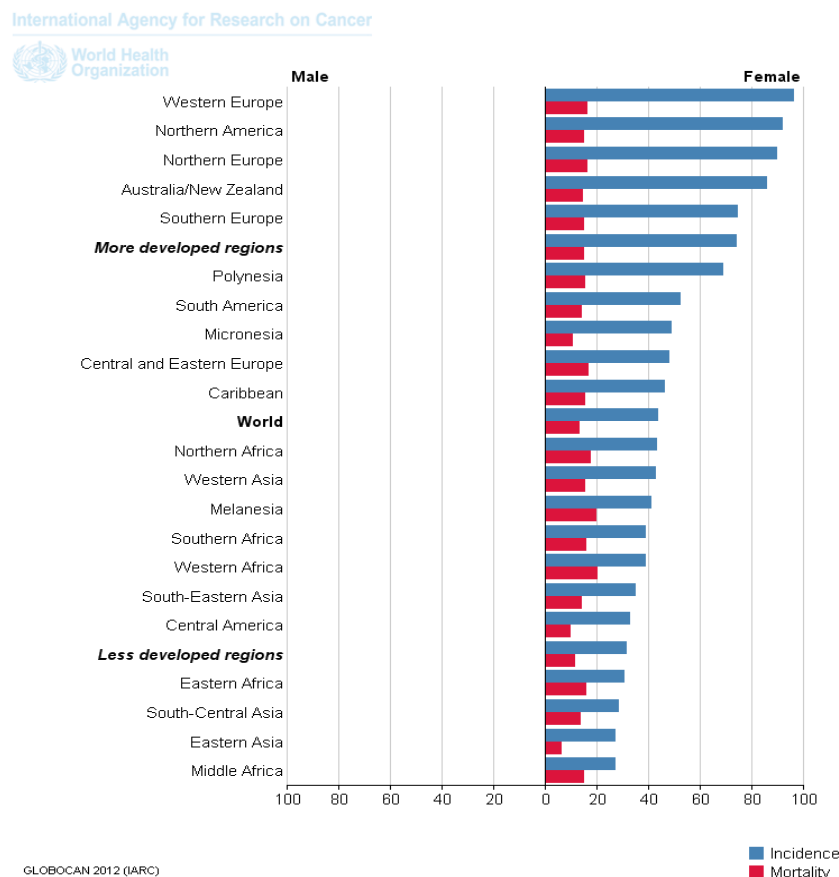


Figure 1.2: Incident and mortality rates of breast cancers worldwide in 2012. Adapted from GLOBOCON 2012.

In the UK, breast cancer is the commonest female malignancy. It is estimated that the lifetime risk of developing breast cancer is 1 in 8 for women (CRUKa 2012). The incidence of breast cancer in women has increased by 6% in the last ten years but the mortality rates have fallen by approximately a fifth in UK. In 2010, 49,961 new cases of breast cancer were diagnosed, which equals 136 women each day. Almost 1,200 death cases of breast cancer were amongst women under age of 50 (Jemal, Center et al. 2010).

1.2.2 Risk factors of breast cancer

Epidemiologic studies have identified many risk factors that might be associated with developing breast cancer. Gender is one of the most important risk factors for breast cancer. The majority of people who develop this disease are women. Breast cancer risk is highly related to age. The risk of developing breast cancer is higher with increasing age. It is estimated that more than 75% of women who are diagnosed with breast cancer are above age 50 (Key, T. J. 2001)

Women with early age at menarche, late menopause and delayed first pregnancy are more prone to develop breast cancer. It is estimated that the risk of developing breast cancer decreases by about 5% with every one year delay in menarche and increases by 3% for each year's delay in menopause. Other variable menstrual factors, such as length or irregularity of the cycle do not have an influence on risk of breast cancer (CRUKa 2012, Key, T. J. 2001).

Childbearing at early ages around 20 years or younger protects a woman against breast cancer. There is evidence that the risk of breast cancer increases by 3% for every one year delay in pregnancy. The risk of breast cancer in women, who breastfeed for longer duration is lower, compared to those who had shorter duration or who never breastfed (Key, T. J. 2001).

There is an association between serum concentrations of endogenous hormones and risk of developing breast cancer. The higher the level of hormones, the higher the risk of breast cancer is. The risk of breast cancer is two times higher in postmenopausal women who have higher levels of oestrogen compared to those with a lower level of this hormone (Key, Appleby et al. 2002). The risk of breast cancer is relatively higher in women who are exposed to exogenous hormones including oral contraceptives and hormonal replacement therapy. The risk of breast cancer increases in current

contraceptive users by 24%, but this rate abates after cessation (Key, Verkasalo et al. 2001, Gierisch, Coeytaux et al. 2013). Hormonal replacement therapy increases the risk of breast cancer by 66% in women who are current users of hormonal replacement therapy. It has been reported that the risk is higher for users of combined oestrogen and progesterone therapy compared to oestrogen alone (Chlebowski, Manson et al. 2013). The majority of breast cancers are sporadic and only small percentage are caused by inherited mutations. Women who carry breast cancer associated susceptibility gene (1, 2) BRCA1 and BRCA2 mutant genes have a 45-56% increased risk of developing breast cancer by the age of 70 (Antoniou, Pharoah et al. 2003). Several studies on familial history of breast cancer show that the risk is approximately double in women with affected first-degree relatives, compared to those with no family background. However, over 85% of women with breast cancer do not have any hereditary predisposition, and more than 85% of women who have a familial history do not develop breast cancer (Key, T. J. 2001, The Lancet 2001).

Epidemiological studies have shown that life style and environmental factors such as bodyweight, physical activity, consumption of alcohol or dietary intake have significant impact on breast cancer incidence. There is a strong association between consumption of alcohol and chance of developing breast cancer. Obesity is another risk factor for breast cancer. In postmenopausal women obesity is one of the risk factors that increases the chance of breast cancer development due to the endogenous synthesis of oestrogen hormone in fatty tissues (Key, Verkasalo et al. 2001).

1.3 Different histological types of breast cancer

The majority of malignant breast tumours are adenocarcinomas which arise from the epithelial lining of the mammary lobular and ductal system (Dreyer, Vandenbroucke et al. 2013). Breast tumours can be categorized as non-invasive or invasive carcinomas. Based on the histological morphology, both non-invasive and invasive carcinomas of the breast are further classified into ductal or lobular carcinomas.

1.3.1 Non-invasive carcinoma (carcinoma in situ)

If the malignant cells stay within the myoepithelial cells of the ductal or lobular system and do not invade the adjacent stroma, they are referred as non-

invasive or *in situ* carcinomas. There is a chance of developing invasive breast carcinoma from non-invasive lesions if they are not treated. In general, non-invasive carcinoma can be divided into two subgroups: ductal carcinoma *in situ* (DCIS) and lobular carcinoma *in situ* (LCIS) (Fabbri, Carcangiu et al. 2008).

1.3.1.1 Ductal carcinoma *in situ* (DCIS)

Ductal carcinoma *in situ* is reported as a heterogeneous lesion which is known as the most common type of non-invasive malignancy (Holland, Peterse et al. 1994, Fabbri, Carcangiu et al. 2008). Ductal carcinoma *in situ* can be classified on the basis of architectural characteristics into different types of comedo, cribriform, solid, papillary and micropapillary (Figure 1.3). According to the cytological pattern, it can be classified as a low, intermediate and high grade tumour. Comedo ductal carcinoma *in situ* is made up of large cells with irregular shapes and contains a high level of central necrosis and large numbers of the mitotic figures. Ductal carcinoma *in situ* comedo type is referred as a high grade tumour due to its poor differentiation and has a more aggressive nature (Figure 1.3 A). If the tumour cells have a solid pattern sheet in appearance, they are called as solid ductal carcinoma *in situ* (Figure 1.3 C), whereas, if they have space in between the cells, they are referred to as cribriform ductal carcinoma *in situ* (Figure 1.3 B) and if they form a finger-like structure, they are termed micropapillary ductal carcinoma *in situ* (Figure 1.3 D, E) (Vinay Kumar 2004, Fabbri, Carcangiu et al. 2008).

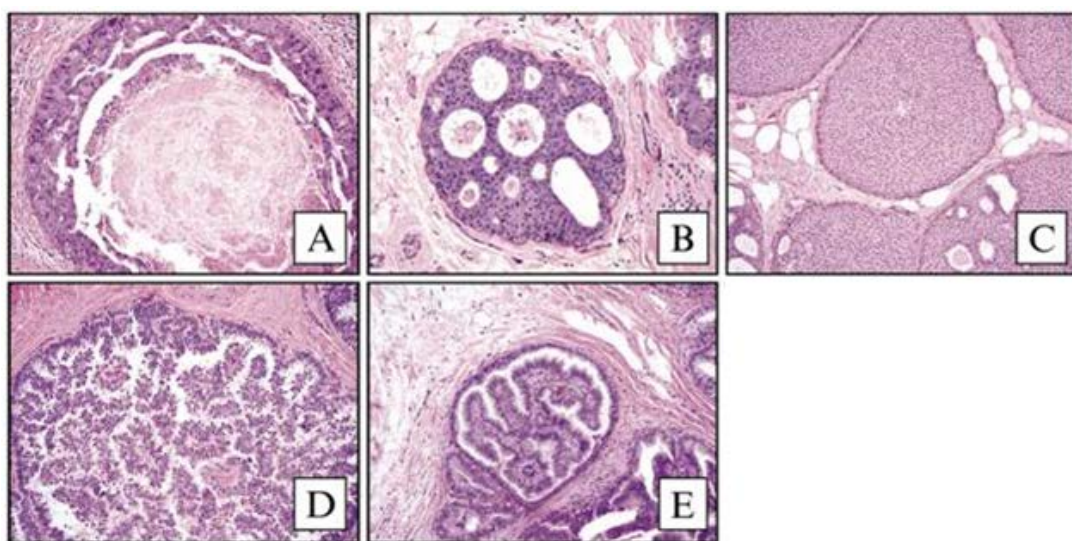


Figure 1.3: Histology of different types of ductal carcinoma *in situ*. High-grade comedo (A), low-grade cribriform (B), low-grade solid (C), low-grade micropapillary (D), low-grade papillary (E).

1.3.1.2 Lobular carcinoma in situ (LCIS)

Lobular carcinoma *in situ* is a growth and division of malignant cells within the lobules which has not yet penetrated into the myoepithelial cells. Women with this type of carcinoma have an increased chance of developing invasive carcinoma. In lobular carcinoma *in situ*, affected lobules are enlarged and filled with small uniform cohesive cells that contain small round nuclei (Figure 1.4). Usually this type of carcinoma is oestrogen receptor and progesterone receptor positive (Vinay Kumar 2004, Fabbri, Carcangiu et al. 2008).

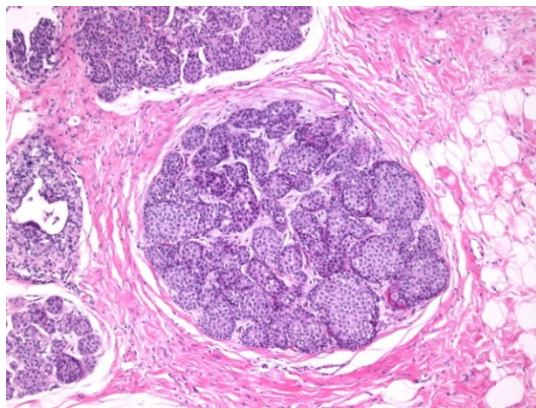


Figure 1.4: Histology of lobular carcinoma *in situ*. The photo micrograph illustrates the growth of tumour cells inside the lobules without infiltration to outside the lobular wall.

1.3.2 Invasive carcinoma

When cancerous epithelial tumours break through the myoepithelial cells and invade into the surrounding normal stroma it is referred as invasive carcinoma. Invasive carcinoma can be subdivided into invasive ductal carcinoma and invasive lobular carcinoma (Vinay Kumar 2004, Fabbri, Carcangiu et al. 2008).

1.3.2.1 Invasive ductal carcinoma (IDC)

Invasive ductal carcinoma (IDC) of no specific type is the most prevalent type of breast cancer, and comprises 70% of all invasive breast carcinomas (Dreyer, Vandorpe et al. 2013). The tumours do not have any particular macroscopic characteristics to achieve a more specific histological classification. They arise from cells within the breast lobular and ductal system followed by penetration of myoepithelial cell layer and spread into the surrounding connective and fatty tissues (Figure 1.5). There is a chance that they will metastasise to different parts of the body via the lymphatic system or bloodstream. About 80% of

invasive ductal carcinomas are oestrogen receptor positive and around 15 to 30% express HER-2 (Vinay Kumar 2004, Fabbri, Carcangiu et al. 2008).

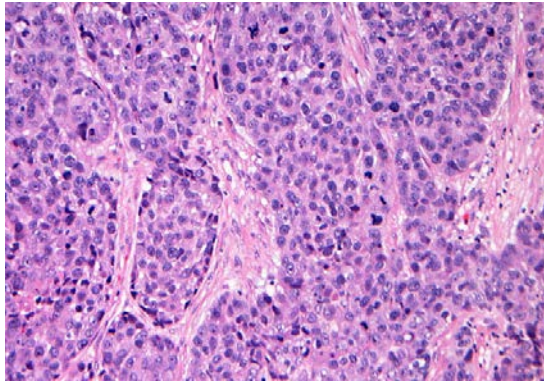


Figure 1.5: Histology of invasive ductal carcinoma. The image illustrates the infiltration of cancerous cells outside of the duct through the stroma.

1.3.2.2 Invasive lobular carcinoma (ILC)

Invasive lobular carcinoma is the second most prevalent malignancy after invasive ductal carcinoma. Morphologically, invasive lobular carcinoma cells have an unclear tumour margin. Their nuclei appear to be round or irregular ovoid (Figure 1.6). Approximately 70 to 90% of invasive lobular carcinomas are oestrogen and progesterone receptor positive (Vinay Kumar 2004, Fabbri, Carcangiu et al. 2008).

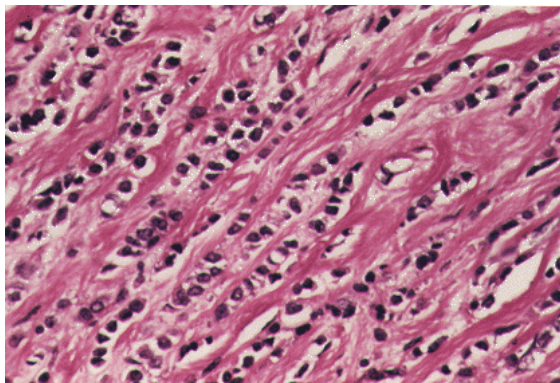


Figure 1.6: Histology of invasive lobular carcinoma. Tumour cells can appear as single file linear pattern in fibrous stroma.

1.4 Molecular classification of breast cancer

Breast cancer is a heterogeneous disease and exhibits diverse clinical and morphological characteristics. Molecular profiling of breast cancer based on the expression of the genes, has classified breast cancer into two main groups of oestrogen receptor-positive and oestrogen receptor-negative tumours. It can be sub-classified further into five different subtypes: luminal A, luminal B, HER-2 positive, triple-negative or basal-like tumours and normal-like breast tumours (Figure 1.7) (Perou, Sorlie et al. 2000, Rakha, Reis-Filho et al. 2008). In addition, in more recent studies, a new subtype has been identified which is called the “claudin-low” subtype (Malhotra, Zhao et al. 2010, Prat, Parker et al. 2010). Another recent study reported the 10 novel subgroups of breast cancer. In this study, breast cancer classification was based on the cluster analysis of genome and transcriptome expression (Curtis, Shah et al. 2012) and identified further molecular subgroups of breast cancer which are proposed to specify the therapeutic intervention for each breast cancer subtypes.

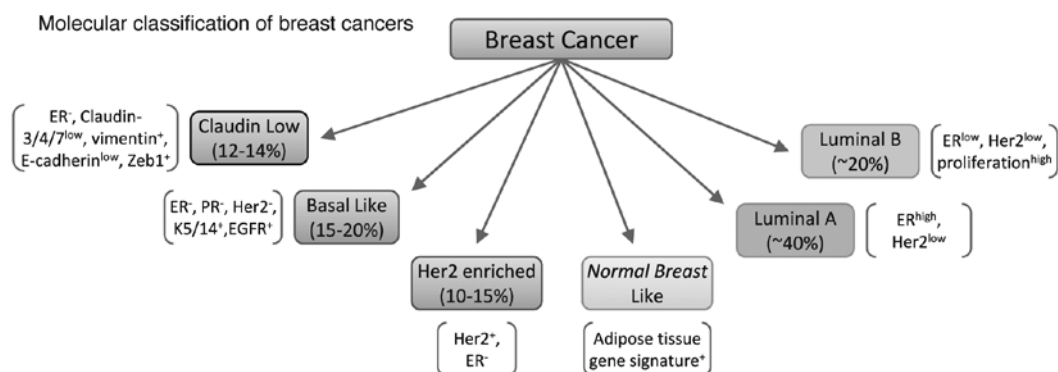


Figure 1.7: Molecular classification of breast cancer. This classification is on the basis of cDNA expression microarray analysis. Taken from (Malhotra, Zhao et al. 2010).

Luminal A tumours express an oestrogen receptor-related gene cluster, have a low proliferation rate, low expression level of Ki-67 protein and are negative for expression of HER-2 (Rakha, Reis-Filho et al. 2008). Luminal A tumours have the best prognosis. Tumours of the luminal B subtypes also express an oestrogen receptor-related gene cluster but have high proliferation rate, as have high level of expression of Ki-67 and are positive for expression of HER-2. HER-2 enriched subtype does not express the oestrogen receptor-related gene cluster, belongs to the luminal groups, but overexpresses HER-2. HER-2 overexpression in tumour cells is identified as poor prognosis (Eroles, Bosch et

al. 2012). Basal-like tumours do not express luminal or HER-2 related genes, but express cytokeratin 5 and 6 (CK 5/6) and epidermal growth factor receptor (EGFR) (Nielsen, Hsu et al. 2004). They are categorised as triple-negative tumour subtype, as they do not express oestrogen receptor, progesterone receptors or HER-2. Triple-negative tumours and basal-like tumours are categorised as one group, due to many common characteristics shared between them. Data from immunohistochemical analysis, molecular features and prognosis suggests that all triple-negative tumours are not necessarily basal-like tumours or *vice versa*. A discordance of up to 30% has been reported and according to microarray-based expression analysis, up to 15-45% in basal-like tumours express one of the oestrogen or progesterone receptors or HER-2 (Rakha, Reis-Filho et al. 2008, O'Brien, Cole et al. 2010)

Although relatively fewer patients have triple-negative breast cancer, it has the highest rate of mortality, particularly in younger women less than 50 years of age. Compared to other molecular subtypes, triple-negative breast cancer is more aggressive and more prevalent among black African American or British black women (Bowen, Duffy et al. 2008). This can be deduced from the observation that the risk of recurrence is more frequent during the first and third years and that the mortality rate is highest in the first five years of therapy. Recently, researches based on gene expression profiling, classified triple-negative breast cancer in to six different subtypes; two basal-like BL1 and BL2 subtypes, mesenchymal, M and mesenchymal-stem like subtype ML, an immunomodulatory and lastly a luminal androgen receptor-positive subgroup (Chen, Li et al. 2012). The mainstay of triple-negative breast cancer treatment is conventional chemotherapy; therefore, identification of therapeutic target for these tumours is a priority.

Based on the gene expression analysis, in year 2007, a new claudin-low subgroup of tumours was identified (Herschkowitz, Simin et al. 2007). The majority of claudin-low tumours are identified as poor prognosis. On hierarchical clustering, claudin-low subtype is located close to the basal-like tumours, as they share expression profiles of some characteristic genes including low expression of oestrogen or progesterone receptors or HER-2 and luminal epithelial genes. Claudin-low tumours are also characterized by high expression of epithelial to mesenchymal transition markers, cancer stem cell-like features

and immune response genes (Prat, Parker et al. 2010). Histologically, claudin-low tumours are high grade infiltrating ductal carcinomas with high proportion of metaplastic differentiation (Prat, Parker et al. 2010, Eroles, Bosch et al. 2012).

Normal-like breast tumours express high level of gene characteristics of non-epithelial cells such as adipose cells and express low level of luminal epithelial cell genes. Significantly these breast cancer subgroups have the best prognosis (Gluz, Liedtke et al. 2009).

1.5 Treatment options for breast cancer

1.5.1 *Surgical treatment for early breast cancer*

Surgery is the main treatment for breast cancer when the tumour is localized to the breast and local lymph nodes. The primary tumour is removed either by wide local excision (lumpectomy) or mastectomy. However, on some occasions prior to the lumpectomy, chemotherapy is administered as neoadjuvant treatment to shrink larger tumours. The status of regional axillary lymph node is assessed by sentinel lymph node biopsy or lymph node dissection (MD 2009). Surgery is followed by systemic therapies called adjuvant therapies which aim to eliminate any residual malignant cells. These adjuvant therapies are: radiotherapy, adjuvant endocrine therapy, molecular targeted therapies such as trastuzumab and cytotoxic therapy.

1.5.2 *Radiotherapy*

Radiation therapy is one of the adjuvant treatments for patients who have undergone a lumpectomy or mastectomy operation. Radiotherapy involves the application of high energy X-rays or other types of irradiation to the chest wall or to the lymph node draining region. Post-mastectomy radiotherapy can decrease the risk of loco-regional recurrence in the affected breast through removing microscopic disease that remains in the breast after mastectomy. It helps in increasing the overall survival rate in patients with high risk breast cancer (Vilarino-Varela, Chin et al. 2009). It has been reported that oestrogen receptor-positive patients benefit more from radiotherapy than oestrogen receptor-negative and triple-negative breast cancer patients (NCI Cancer Bulletin).

1.5.3 *Systematic treatment of breast cancer*

The aim of systemic treatment is to eradicate metastatic disease. Chemotherapy and hormonal therapy and other targeted therapies are different types of systemic therapies. These systemic treatments can be applied to patient either as neoadjuvant therapy which is given prior to the surgery or as adjuvant therapy which is given to patient after the loco-regional surgery. Based on the biological subtypes of breast cancer, chemotherapy can be beneficial. Chemotherapeutic drugs prevent cell growth, cell division and induce cell death. The most common class of chemotherapy drugs used in breast cancer are the anthracyclines such as doxorubicin and epirubicin and the taxanes, docetaxal and paclitaxel. Chemotherapy regimens include one or a combination of these

classes of drugs. Oestrogen receptor negative or HER-2 positive patients benefit more from cytotoxic chemotherapy than other subgroups (Dixon 2012).

1.5.4 Endocrine therapy

Endocrine therapy has made an important impact in improving the overall survival for patients whose tumours express the oestrogen and or the progesterone receptor. The decision about which endocrine therapy is implemented is affected by the menopausal status of the patients and stage of the disease (Johnston and Yeo 2014). The majority of endocrine agents either block the circulating oestrogen production by aromatase inhibition or inhibit oestrogen action at the cellular level by the competitive inhibition of oestrogen binding to its receptor (Buzdar and Hortobagyi 1998, Ellis and Ma 2008, Osborne and Schiff 2011).

1.5.4.1 Tamoxifen

The non-steroidal anti-oestrogen drug tamoxifen is widely used for all the stages of breast cancer. Tamoxifen shows effectiveness in both premenopausal and postmenopausal patients. It improves disease-free survival and overall survival rates of hormone receptor-positive breast cancer patients. Tamoxifen given for 5 years as adjuvant therapy to patients reduces significantly breast cancer relapse and improves overall survival. Based on the positive effect of 5 years usage of adjuvant endocrine treatments along with the knowledge that many relapses occur 5 years after initial surgery, different clinical trials studies reported an advantage of continuing the endocrine therapy for more than 5 years (Johnston and Yeo 2014). Tamoxifen binds to oestrogen receptor, induces conformational changes in the receptor and subsequently inhibits access to one of the co-activator interaction surfaces which in turn prevents oestrogen-regulated gene transcription and cellular proliferation (Johnston and Yeo 2014).

1.5.4.2 Aromatase inhibitors

Aromatase inhibitors improve outcomes either as an alternative to tamoxifen or in a sequential order after initial tamoxifen (Johnston and Yeo 2014). In postmenopausal women, ovarian oestrogen production stops due to complete loss of primordial follicles in the ovaries. However, oestrogens are produced from peripheral tissue like fat, muscles, or even breast tumours themselves

(Dowsett, Folkard et al. 2005). Biosynthesis of oestrogen from androgen is catalysed by a cytochrome P450 enzyme complex called aromatase.

(Brueggemeier, Hackett et al. 2005). Aromatase inhibitors decrease the level of circulating oestrogen by more than 80% in postmenopausal women (Geisler, Helle et al. 2008).

Aromatase inhibitors such as non-steroidal inhibitors: anastrozole and letrozole and steroidal inhibitors: such as exemestane have been approved clinically as endocrine therapy in postmenopausal patients who had failed in anti-oestrogen therapy or in multiple hormonal therapies. Clinical studies have reported that these aromatase inhibitors have more potential in postmenopausal women with metastatic breast cancer than tamoxifen in both the neo-adjuvant and adjuvant settings (Brueggemeier, Hackett et al. 2005). Studies suggest that letrozole might be the most efficient aromatase inhibitor particularly in endocrine-responsive postmenopausal women who have advanced or metastatic breast cancer in terms of significant overall response and reduction in times of disease progression. Furthermore, a recent study addressed the superior effect of combination of anastrozole and fulvestrant compared to anastrozole as an individual drug in treatment of postmenopausal oestrogen positive metastatic breast cancer patients (Mehta, Barlow et al. 2012).

1.5.5 Other Molecular targeted therapy

Molecular targeted therapy in breast cancer has significant impact on improvement and further treatment of breast cancer. Molecular-targeted therapy involves targeting the key molecules that are playing important roles in cellular processes which promote cancer progression such as cell growth, survival, migration, invasion and metastasis without any harm to other normal cells. Monoclonal antibodies and tyrosine kinase inhibitors are two types of targeted therapies. Targeted agents that have been approved for treatment of breast cancer include tyrosine kinase inhibitor lapatinib and monoclonal antibody trastuzumab, which are anti-HER-2 inhibitors and target the HER-2 receptor, and bevacizumab which is an inhibitor of vascular endothelial growth factor (VEGF). There are several other targeted agents that are under preclinical and clinical trials such as anti-EGFR which targets EGFR receptor, anti-IGF-IR that targets type I IGF receptor or agents that interfere with the important signal transduction pathways: PI3K/AKT/mTOR and Ras/MAPK pathways. Another

type of targeted therapy is using the PARP inhibitors that are being studied for treatment of triple-negative subtypes of breast cancer (Munagala, Aqil et al. 2011).

As mentioned previously, the only available systematic treatment for triple-negative or basal-like breast tumours is conventional chemotherapy, due to their resistance to endocrine treatments. There are several compounds available for triple-negative breast cancer such as taxanes, platinating agents, and poly (ADP-ribose) polymerase inhibitors (PARP). Combination therapy of cytotoxic drugs such as: paclitaxel, doxorubicin and cyclophosphamide seem to be beneficial for triple-negative or even HER-2-positive patients (Schneider, Winer et al. 2008). Triple-negative or basal-like breast cancer shares various clinico-pathological features with BRCA1 and BRCA2 mutation in breast cancer, which followed by dysregulation in DNA repair mechanism. Poly (ADP ribose) polymerase (PARP) is a molecule involved in several cellular processes including non-homologous DNA repair mechanisms. It has been hypothesized that poly (ADP ribose) polymerase inhibitors either alone or in combination with chemotherapy or DNA damaging agents such as platinum drugs can result in cell death and could be beneficial as therapeutic agents in treatment of triple negative or basal-like breast cancer (Schneider, Winer et al. 2008, Gluz, Liedtke et al. 2009, Anders, Winer et al. 2010, O'Shaughnessy, Osborne et al. 2011). A clinical trial that evaluated the role of epidermal growth factor receptor inhibitors in triple-negative metastatic breast cancer suggested that patients are more responsive to anti-EGFR monoclonal antibody cetuximab in combination with carboplatin than cetuximab alone (Schneider, Winer et al. 2008). Other evidence reported the beneficial effect of combination of lapatinib and rapamycin in triple-negative breast cancer, suggesting that combination of EGFR and HER-2 inhibitor lapatinib with mTOR inhibitor rapamycin can increase the cell death in some of the triple-negative breast cancer cell lines (Liu, Yacoub et al. 2011).

Some limited clinical data show sensitivity of triple-negative tumours to platinum-based chemotherapy. The rate of response to platinum-based chemotherapy was increased in triple-negative breast cancer. However, patients with an early stage of triple-negative breast cancer had worse survival rate while an improved survival was observed in patients with advanced triple

negative breast cancer (Sirohi, Arnedos et al. 2008). Therefore, there is an urgent need of new therapy for hormone receptor-negative patients.

1.6 Prognostic factors in breast cancer

Prognostic factors are the informative markers that can predict the outcome and course of the disease. They provide the valuable information which can be helpful in treatment decisions particularly in patients with early stage of cancer.

1.6.1 Hormonal receptor status

Expression of oestrogen receptor (ER) and progesterone receptor (PgR) in breast cancer is one of the important well-established predictive markers in response to therapies. Oestrogen receptor and the oncogene ErbB-2/human epidermal growth factor receptor-2 (HER-2) play important roles in the development and progression of breast cancer (Gluz, Liedtke et al. 2009). The majority of breast tumours, around 80%, are hormone receptor-positive. Positive oestrogen receptor expression is more likely in tumours of postmenopausal women than in tumours of premenopausal women. Successful oestrogen targeted therapies such as the anti-oestrogen tamoxifen or aromatase inhibitors have been developed. These endocrine therapies are valuable for the treatment of breast cancer patients whose breast tumour cells express oestrogen and progesterone receptor in the nucleus. Tumours with HER-2 expression are treated with trastuzumab, which is an anti-HER-2 monoclonal antibody (Reis-Filho and Tutt 2008).

1.6.2 Nodal status

Among prognostic factors in breast cancer, nodal status is one of the most important risk factors. The number of involved nodes is crucial for prognosis and treatment decisions. Patient survival is associated with the number and the level of the involved lymph node (Dixon 2012). Patients who have four or more nodes involved have a worse prognosis and are at higher risk of relapse, while those patients who have fewer involved nodes are characterised as being in an intermediate risk group. (Fitzgibbons, Page et al. 2000).

1.6.3 Tumour size

The size of the tumour is another important prognostic factor. Patients with a tumour larger than 2 cm have an intermediate prognosis, while those with tumours of less than 1 cm in diameter have better prognosis (Dixon 2012).

1.6.4 Histological grade

There are different systems for evaluating the grade of breast cancer. Bloom and Richardson is one of the most common grading systems (Bloom and Richardson 1957). The grading is based on the three morphologic features of tumours: the degree of tubular formation, tumour mitotic activity and nuclear polymorphism of tumour cells. Based on this, tumours are classified into three grades of 1, 2, and 3; 1 is low grade, 2 is intermediate and 3 is referred to as high grade tumours. Low grade tumours tend to be less aggressive than high grade tumours.

1.7 Importance of metastasis in breast cancer

Metastasis at distant sites is the main cause of fatality in breast cancer patients, regardless of the breast cancer type. Despite the considerable progress in early diagnosis by mammographic screening and the implementation of systemic adjuvant therapies which have improved the survival rate of breast cancer patients, recurrence after surgery in breast cancer is continuous (Rugo 2008). Metastasis is a multiple step process which takes place through detachment of the cancer cells from the primary tumour, invasion through the myoepithelium and travel into blood or lymphatic stream and subsequently invade to other organs (Rabbani and Mazar 2007, Nguyen, Bos et al. 2009). Development of cancer first involves the formation of the premalignant lesions before formation of invasive tumours. The development of lesions might be due to genetic alterations which lead to monoclonal expansion of the tumour or it can be by environmental factors which results in polyclonal expansion of the tumour cells. Continuous modification in genetic alteration of premalignant lesions can trigger malignant clonal cells to produce the primary tumours. Only small numbers of cells in primary tumours have high potential to metastasis. Shift from non-motile epithelial-like state to the mesenchymal-like migratory state followed by loss of the cell-cell adhesion makes the metastatic tumours cells invade to other organs (Rabbani and Mazar 2007).

Distant metastasis is one of the most frequent types of recurrence in breast cancer patients. Breast cancer forms as a local disease, but it can metastasize to the lymph nodes and any other distant organs. On the basis of the evidence in literature, metastasis to regional lymph nodes occurs in one third of breast cancer patients. The major sites of distant metastasis in breast cancer at first

recurrence are bone, lung or thorax, liver and the central nervous system. Bone is the most preferred site of metastasis in breast cancer (Figure 1.8). According to the prospective study in Strathfield Breast Cancer Centre, the five years disease-free survival rate in patients with local relapse was 41%, in patients with regional recurrence was 20% and in patients with distant metastasis was 13%. Moreover the survival rate was varied in patients with different metastatic sites, for instance: the five years overall-survival rate in patients with bone metastasis was 16%, in lung metastasis patients, 12% and in patients who had liver metastasis was 0%, which suggests that patients who had first bone metastasis had better prognosis compared to those group who had first visceral metastases (Rabbani and Mazar 2007). According to many published studies, breast cancer patients with loco-regional recurrences are more likely to develop distant metastasis in 10 years as compared to those patients who had *de novo* second primary neoplasm.

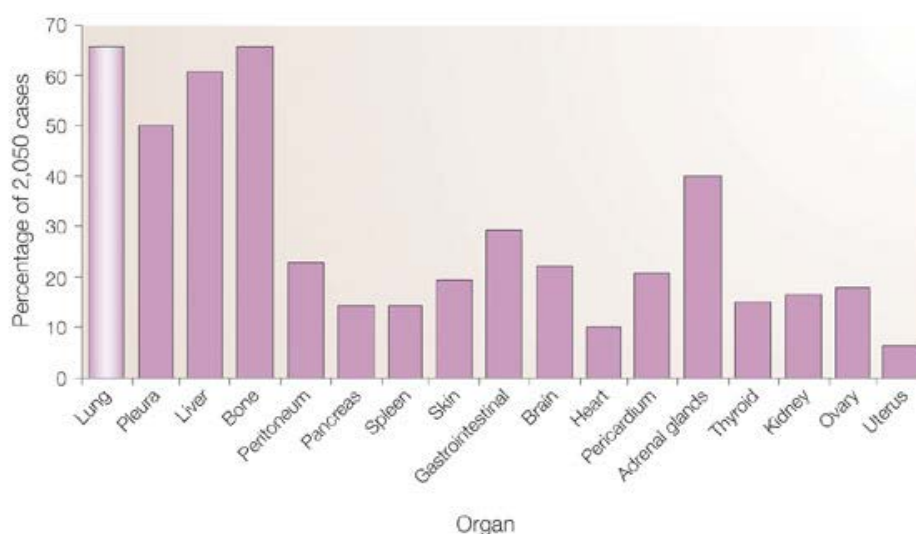


Figure 1.8: Most frequent sites of distant metastasis in breast cancer patients at autopsy. Primary breast cancer cells travel through the blood stream spread into various sites, most preferably they metastasize to lung, bone and liver. Adapted from Nature Reviews (Weigelt, Peterse et al. 2005).

According to many studies, the earliest peak of recurrence risk is between 2 to 3 years after the surgical treatment of primary disease in both oestrogen receptor-positive and negative breast cancer. It has been observed that after early diagnosis, the recurrence rate was higher in oestrogen receptor-negative tumours. However, according to Saphner *et al*, the chance of recurrence

irrespective to the oestrogen receptor status and chemotherapy treatment is higher in postmenopausal patients compare to premenopausal patients (Saphner, Tormey et al. 1996). Involvement of axillary lymph nodes and size of tumour has association with the risk of distant metastases. Patients who are positive for lymph node and have larger primary tumours are more prone to distant metastases. Despite this observation, it has been reported that in about one-third of patients with metastatic breast cancer, tumours have not invaded into lymph nodes, whereas in another cohort of patients with tumours positive to axillary lymph node, there was no evidence of metastases 10 years after initial treatment (Weigelt, Peterse et al. 2005). Moreover, circulating tumour cells has been reported to be found in bone marrow of both lymph node positive and lymph node negative breast cancer patients (Rabbani and Mazar 2007). Increase in the age of patient (above 40 years), larger tumour size, positivity of the axillary lymph nodes (>2), higher histopathological grade of the tumour, tumour stage ($\geq T_3$), invasion of lymphovascular system, poorly differentiated ductal carcinoma *in situ* and unknown oestrogen receptor status are the main independent risk factors for early breast cancer recurrence (Rabbani and Mazar 2007, Rugo 2008).

The combination of systemic therapy with localized treatments such as surgery and radiotherapy has been shown to be effective on treatment of the distant metastasis. Radiotherapy after mastectomy reduces both local recurrence and overall distant metastasis. Adjuvant chemotherapy and endocrine therapy improve distant disease-free survival and overall survival of breast cancer patients. Adjuvant endocrine therapy with tamoxifen has proven to have good impact on treatment of hormone-sensitive postmenopausal breast cancer patients. Aromatase inhibitors as initial adjuvant therapy are more effective in treatment of breast cancer patients than tamoxifen, in terms of reduction in the risk of relapse. Amongst aromatase inhibitors, letrozole is reported to be a more effective drug in reducing the risk of distant metastasis than anastrozole after two years of therapy (Rabbani and Mazar 2007).

Identification and characterization of circulating tumour cells (CTC) provides new information regarding cancer dissemination. CTC count is a novel potential quantitative and qualitative biological tool to understand the metastatic process in breast cancer patients (Bidard, Fehm et al. 2013). Cristofanilli *et al* (2004)

addressed CTC count as an independent prognostic factor for metastatic breast cancer patients with progression-free and overall survival (Cristofanilli, Budd et al. 2004).

One of the possibilities to identify the patients who are at higher risk of developing metastases is gene expression profiling. Studies reported the overexpression of diverse sets of genes which have important effects on promoting the metastasis of breast cancer to the bone (Rodenhiser, Andrews et al. 2011). Next generation sequencing could facilitate identification of patients who might be at risk of developing metastatic breast cancer and could have an effective role in individualized treatment decisions. Next generation sequencing could sequence the whole genome of a tumour and detect the mutations in tumour DNA or RNA (Rodenhiser, Andrews et al. 2011).

1.8 *In vitro* assays with which to study metastatic potential

1.8.1 *Cell survival*

Survival of normal mammary epithelial cells depends on their attachment to the extracellular matrix (Davison, Durbin et al. 2013). Adhesion of cells to extracellular matrix involves interaction of integrin receptors, plasma membrane bound receptors with extracellular matrix components which initiate different signal transduction that mediates cellular differentiation, proliferation and migration. Appropriate adhesion of cells to extracellular matrix molecules is essential to determine the correct location of cells (Kim, Koo et al. 2012, Taddei, Giannoni et al. 2012). Frisch and Francis (1994) found that loss or inappropriate attachment of cells to extracellular matrix caused apoptosis. They named this particular type of programmed cell death as anoikis which means 'homelessness' in Greek (Frisch and Francis 1994, Simpson, Anyiwe et al. 2008, Taddei, Giannoni et al. 2012). Detached epithelial cells are eliminated through anoikis process to inhibit their reattachment to the new incorrect matrix, hence preventing dysplastic growth and proliferation of the cells (Kim, Koo et al. 2012, Taddei, Giannoni et al. 2012). Anoikis has a significant role in the physiological induction of apoptosis for mediating tissue-haemostatic and developmental processes (Frisch and Screaton 2001, Grossmann 2002, Kim, Koo et al. 2012) Resistance of tumour cells against anoikis is one of the significant characteristics toward cancer progression and metastasis, because cancer cells may survive without attachment to the extracellular matrix while

travelling through the blood or lymphatic vessels. Understanding the molecular mechanisms that regulate anoikis resistance in cancer cells may lead towards the novel therapeutic approaches to prevent cancer progression and tumour dissemination (Davison, Durbin et al. 2013).

Anoikis is initiated through two pathways: The intrinsic pathway, as a result of mitochondrial permeabilization and the extrinsic pathway induced by the cell surface death receptor (Figure 1.9) (Kim, Koo et al. 2012). Dysregulation in either pathway can trigger malignant cells, resistance to anoikis process. In the death receptor (extrinsic) pathway, ligation of extracellular death ligands such as Fas ligand (Fas L) or tumour necrosis factor- α (TNF- α) and their transmembrane receptors, Fas and TNFR results in development of the death-inducing signalling complex (DISC) as it is illustrated in Figure 1.9. Cell detachments trigger upregulation of FasL and Fas receptor and downregulation of FLIP which is an antagonist of caspase-8. This results in activation of caspase-8 through Fas-associated death domain (FADD). The activation of caspase-8 induce activation of caspase-7 and subsequently caspase-3 which results in cleavage of cellular substrate and cell death. Blockage in signalling pathway of death receptor inhibits activity of death ligands to induce cell death, therefore leads to resistance to anoikis (Taddei, Giannoni et al. 2012, Simpson, Anyiwe et al. 2008).

In the intrinsic pathway, caspase activation occurs as a result of mitochondrial permeabilization (Figure 1.9). Loss of cell attachment to the extracellular matrix activates proapoptotic family such as the BH3-only proteins Bid, Bad, Puma, Noxa, Bim, as well as Bok, Bax and Bak) which in turn deactivate the antiapoptotic proteins Bcl-2, Bcl-xL, Mcl-1. This process leads to translocation of Bax and Bak monomer from cytosol to outer mitochondrial membrane (OMM) and formation of oligomers, which create a channel that makes the mitochondrial membrane permeable. Due to perturbation of outer mitochondrial membrane, cytochrome C is released and forms the apoptosome with caspase 9 and apoptosis protease activating factor (APAF), Subsequently caspase 3 activates (Kim, Koo et al. 2012). Bcl-2 as an antiapoptotic protein binds to proapoptotic proteins Bax and Bad, inhibits their assembly and prevents formation of the pores in the mitochondrial membrane, hence prevents induction of apoptosis. In addition, BH-3 only proteins contribute to promote the apoptosis

by competition with Bcl-2 for binding to apoptotic activators. Loss of cellular adhesion prevents both extracellular signal regulated kinase (ERK) and phosphoinositide-3-kinase (PI3K)/AKT pathway which mediate the phosphorylation of Bim hence inhibit the Bim proteasomal degradation and sequestration by dynein cytoskeletal complexes, as a result, increase in accumulation of cytoplasmic Bim. Activation of Bim induces oligomerization of Bax/Bak on outer mitochondrial membrane (OMM) and promotes execution of the apoptotic process. Furthermore, upon loss of cellular adhesion, Bcl-2 modifying factor (BMF) is released from the myosin V motor complex and deactivates antiapoptotic Bcl-2, which promotes anoikis (Kim, Koo et al. 2012). Suppression of BMF activity could results in anoikis resistance (Reginato, Mills et al. 2003).

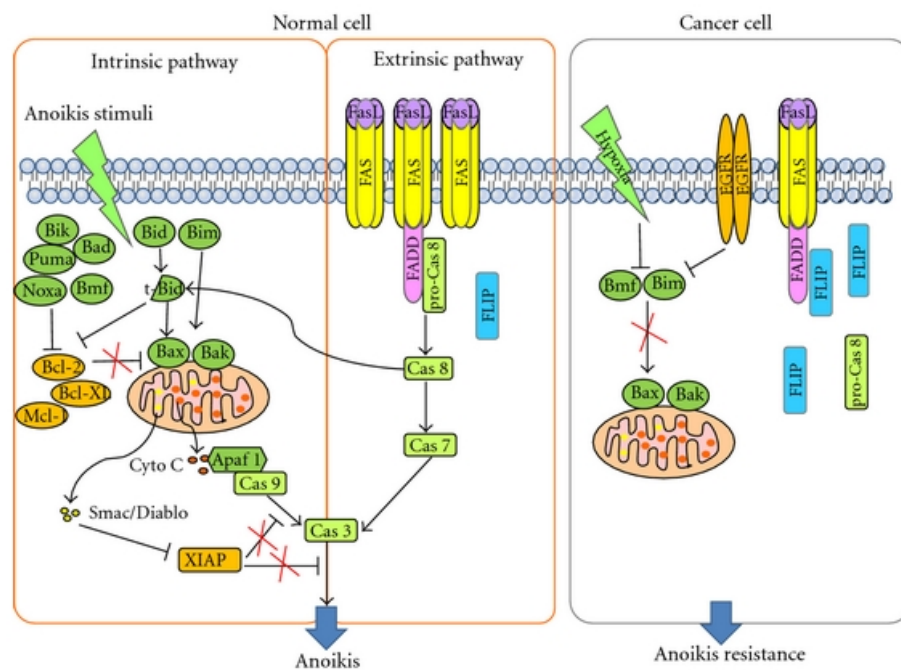


Figure 1.9: Caspase activation of anoikis pathways. Lack or inappropriate attachment of normal cells to the extracellular matrix induces anoikis through activation of intrinsic and extrinsic pathways. The extrinsic pathway is activated through increased expression of FAS and Fas ligand (FasL) and decreased expression of FLIP (FLICE inhibitory protein) which triggers subsequently the activation of caspase 8 (FLICE), caspase 7 and caspase 3, respectively. In intrinsic pathway, loss of cell adhesion induces the activation of proapoptotic Bcl-2 family proteins such as Bid, Puma, Bad, Noxa, Bmf, Bid, Bax and Bak, which neutralise the activation of antiapoptotic Bcl-2 molecules such as Bcl-2, Bcl-xL, Mcl-1, and makes the mitochondrial membrane permeabilize through assembly of Bax/Bak oligomers. These events promote the mitochondrial cytochrome c release and formation of apoptosome, followed by activation of caspase 9 and subsequently caspase 3. Conversely, in cancer cells increased in FLIP expression leads to inhibition of the extrinsic pathway and expression of some oncogenes such as EGFR and hypoxia decrease Bim and Bmf expression, hence prevents mitochondrial permeabilization and therefore causes cancer cells to be resistant to anoikis. Adapted from (Kim, Koo et al. 2012)

Anoikis prevents epithelial cells from colonising elsewhere other than the tissue in which they originated. Integrins are major regulators of cell-matrix adherence by transmission of signals from extracellular matrix ligands to the intracellular signal transduction pathways. Several studies have reported the impact of different types of integrins on cell survival in both normal and malignant cells (Giancotti 2000, Alahari, Reddig et al. 2002, Taddei, Giannoni et al. 2012). When tumour cells acquire resistance to anoikis, the expression pattern of integrins is altered. One of the important players in anoikis resistance is a signal transduction molecule, focal adhesion kinase (FAK) that mediates signal transmission through integrins in cells. FAK transmits the cell-matrix adhesion-induced survival signals through binding to the cytoplasmic tail of integrin and undergoes autophosphorylation which subsequently activates PI3K and AKT signal transduction pathway (Nagaprashantha, Vartak et al. 2012). It has been demonstrated that knocking down FAK by siRNA in anoikis-resistant pancreatic tumour cells increases their anoikis (Duxbury, Ito et al. 2004, Nagaprashantha, Vartak et al. 2012)

Epithelial cancer cells that have undergone epithelial-mesenchymal transition (EMT) acquire resistance to anoikis. EMT is a process which is essential for tissue development and may occur in during tissue restitution and carcinogenesis. E-cadherin is an important protein in mediation of normal epithelial phenotype, its expression is lost during EMT process. Numerous *in vitro* and *in vivo* studies have been reported the importance of E-cadherin loss in metastasis (Hajra and Fearon 2002). Derksen, Liu *et al* (2006) has demonstrated in a mouse model that knock down of E-cadherin and p53 in mammary epithelial cells triggers tumour formation, resistance to anoikis and metastasis. Initiation of EMT also increase in expression of growth factors such as epidermal growth factors (EGF), fibroblast growth factor (FGF) and hepatocyte growth factor (HGF) which in turns enhance the activation of downstream survival cascades PI3K, MAPK and mTOR (Thiery 2002, Thiery and Sleeman 2006, Nagaprashantha, Vartak et al. 2012). Induction of molecular alterations during EMT increases the cancerous cells potential to gain anoikis resistance.

Increase in expression of growth factor receptors such as epidermal growth factor receptor (EGFR) or their signal transduction molecules is reported to be

related to tumour progression through blockade of the apoptosis pathway (Sharma, Bell et al. 2007). In normal condition, cell detachment from the extracellular matrix induces decreased expression of EGFR and increased expression of Bim which is an essential component in anoikis, whereas Reginato *et al* (2003) illustrated in human mammary epithelial cells MCF-10A cells that when EGFR is overexpressed, it mediates ERK pathway activation which in turns blocks the anoikis process through suppression of Bim. In addition, in another study overexpression of HER-2 was shown to suppress Bim expression through activation of MAPK pathway which subsequently conferring anoikis insensitivity (Haenssen, Caldwell et al. 2010). Among growth factors, insulin-like growth factor (IGF-1) is identified as master regulator of cell survival that induces activation of AKT in the process of cell detachment. Inhibition of anoikis by type I IGF receptor in human epithelial prostate cancer cell line LNCaP and mouse embryo fibroblasts has been reported (Kim, Koo et al. 2012). Furthermore, it has been demonstrated that blocking the IGF-1 receptor signalling pathway can increase the anoikis in metastatic variant of MDA-MB-435 breast cancer cells and decrease the population of circulating tumour cells in the blood of tumour-bearing mice (Sachdev, Zhang et al. 2009).

1.8.2 Cell migration

Cell migration is a multistep process involved in embryonic morphogenesis, branching morphogenesis, development of the nervous system, tissue restitution, regeneration and immune cell trafficking. Migration plays a significant role in many pathological processes including development of diseases such as: cancer, rheumatoid arthritis, vascular disease, atherosclerosis and many others. Cell motility was first observed in early 1675 by van Leeuwenhoek who observed cell movement across his microscope slides (Ananthakrishnan R, Ehrlicher A et al. 2007).

As mentioned before, a key step in metastasis is invasion into the extracellular matrix, migration through the circulatory system and dissemination into distant organs (Schroeder, Heller et al. 2012). Regulation of cell adhesion and de-adhesion is essential in cell migration and metastasis. There have been difficulties in clarification between migration and invasion terms in experimental cell biology. Migration is cellular movement on a substrate such as the extracellular matrix, without any disruption of the tissue, which leads to a

change in the location of cells within tissues or between organs. (Kramer, Walzl et al. 2013). Invasion involves break down of tissue barriers such as extracellular matrix, followed by diffusion into underlying tissues. Invasion requires adhesion, proteolysis of matrix components and migration towards different sites. To migrate through matrix, cells need to modify their shape and interact with the surrounding extracellular matrix. Migration is an essential component of invasion; cells are not capable to invade without migration, but they can travel to different sites without invasion (Kramer, Walzl et al. 2013).

Cells have different modes of motility. Several *in vitro* and *in vivo* studies reported various pattern of tumour cells infiltration into adjacent tissue matrix. Tumour cells either disseminate as individual cells which is called as single cell migration, or they can grow in clusters, as files or as solid cell strands or sheets, sometimes called collective migration (Figure 1.10) (Friedl and Wolf 2003). Tumour cells may migrate as single cells and collective migration. Malignant cells of patients with leukemia, lymphoma and the majority of solid stromal tumours such as sarcomas migrate as single cells, whereas epithelial tumours tend to migrate by collective migration. In general, less well differentiated tumour cells migrate as individual cells (Friedl and Wolf 2003).

Single cell migrations have different appearance depending on the cell type, integrin involvement, cytoskeletal structure and protease production. It can be subdivided into amoeboid and mesenchymal migration. Amoeboid migration is observed in certain types of cancer cells, in embryonic cells and leukocytes. In this type of migration, cells move as round and ellipsoid forms without contribution of focal adhesion molecules and cellular attachment. This type of movement is also called as blebby migration because cells move with the help of the propulsive blebs. Cancer cells tend to detach and disseminate from primary tumours at early stages by amoeboid migration. In leukemias, lymphomas and small-cell lung carcinoma this type of migration is reported most frequently (Rintoul and Sethi 2001, Friedl and Wolf 2003). Mesenchymal migration comprises participation of focal adhesion to extracellular matrix, cytoskeletal contractility and cells move as elongated fibroblast-like needle-shaped cell bodies. In this type of movement, high traction force is exerted at both cell poles. Highly dedifferentiated tumour cells that have undergone epithelial-mesenchymal transition move by mesenchymal migration mode for

their movement. Collective migration is described as the movement of groups of cells as a functional unit through the extracellular matrix while they are adhered physically to each other. Collective migration has been observed in highly differentiated tumours such as: epithelial prostate cancer, lobular breast carcinoma and large-cell lung cancer (Friedl and Wolf 2003). Hence, collective movement of cancerous cells can be an initial step towards invasion and metastasis. Dissemination of cancer cells from the primary tumour can be either through amoeboid or mesenchymal movement or by collective migration. It has been hypothesized that various environmental factors play role in different types of tumour-cell motility behaviours, hence participate in invasion (Friedl and Wolf 2003, Bravo-Cordero, Hodgson et al. 2012). For instance; interaction of tumour cells with different growth factors such as epidermal growth factors (EGF), insulin-like growth factor (IGF-1) and chemoattractants can regulate the type of tumour cells motility. Many of these motility enhancing chemokines and growth factors can regulate the migration through transmission of pre-migratory signals through phosphatidyl inositol 3-kinase (PI3K), RAC and RHO signal transduction.

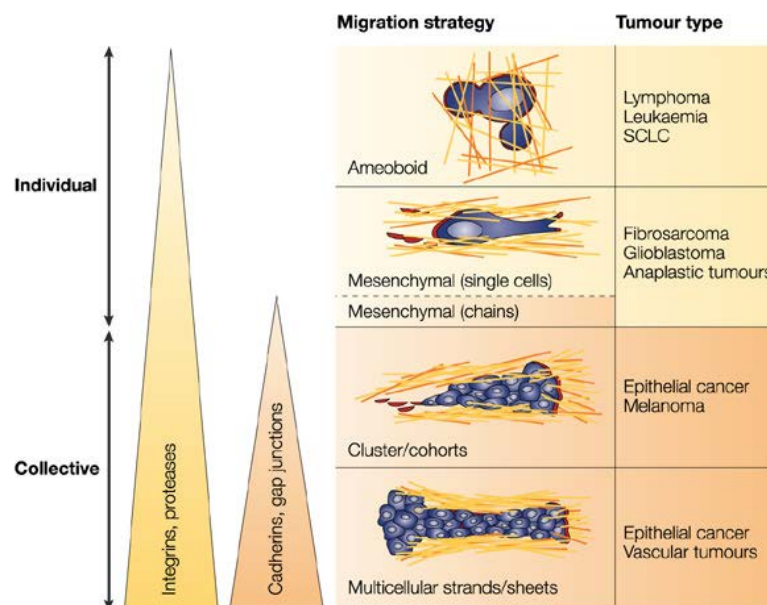


Figure 1.10: Different modes of cell motility. Various molecules (shown in triangles) can regulate individual or collective migration of tumour cells. There is an increase in cell-matrix interactions by integrin and matrix-degrading protease from single cell to collective cell migration. Specific characteristics of collective migration are cell-cell adhesion via cadherins and other adhesion receptors, communication between cells through gap junctions. Amoeboid individual migration occurs in leukemias, lymphomas and small-cell lung cancers (SCLC), whereas sarcomas and glioblastoma tumours have been shown to move by mesenchymal type of individual migration for their movement. Highly differentiated epithelial cancer cells such as breast, colon, prostate cancer and melanoma cells are observed to move by collective

migration. Some epithelial cancer and vascular tumour cells that tend to move in multicellular strands or sheets. Taken from (Friedl and Wolf 2003)

Cell migration is thought to be a continuous cyclical process (Ridley, Schwartz et al. 2003). It can be divided into three phases: protrusion of the leading edge, assembly of the leading edge and disassembly of the cell body at the rear of the cell. The cell is pulled forward through contraction generated by cytoskeleton network. The protrusion occurs normally through polymerisation of actin. The leading edge of the motile cell can be morphologically large, needle-shape filopodia or extensive flat lamellipodia. These changes in morphology of the cells are controlled by small GTPase family proteins such as Rac1, RhoA and Cdc42. The protrusions can be supported by focal adhesion molecules and integrins which provide attachment of the cells to the extracellular matrix or to neighbouring cells through transmembrane receptors that are connected to the actin cytoskeleton. These attachments act as the starting point for forward migration of the cell, which leads to dismantling of the cell attachment at the rear to allow it to detach. The final step in movement of the cells is translocation of the cell body and the rear side of the cell. The acto-myosin network produces a contractile force by sliding of myosin motors and actin filaments in the cell body and rear of the cell which pushes the cell forward (Figure 1.11)

(Ananthakrishnan R, Ehrlicher A et al. 2007). Integrins are the main transmembrane receptors in migration promotion in different cells. The activate migration-related signaling molecules has been described to act as 'feet' for the migrating cells by maintaining the adhesion to the extracellular matrix or other adjacent cells and by connecting through adaptors to the actin filaments inside the cells (Ridley, Schwartz et al. 2003).

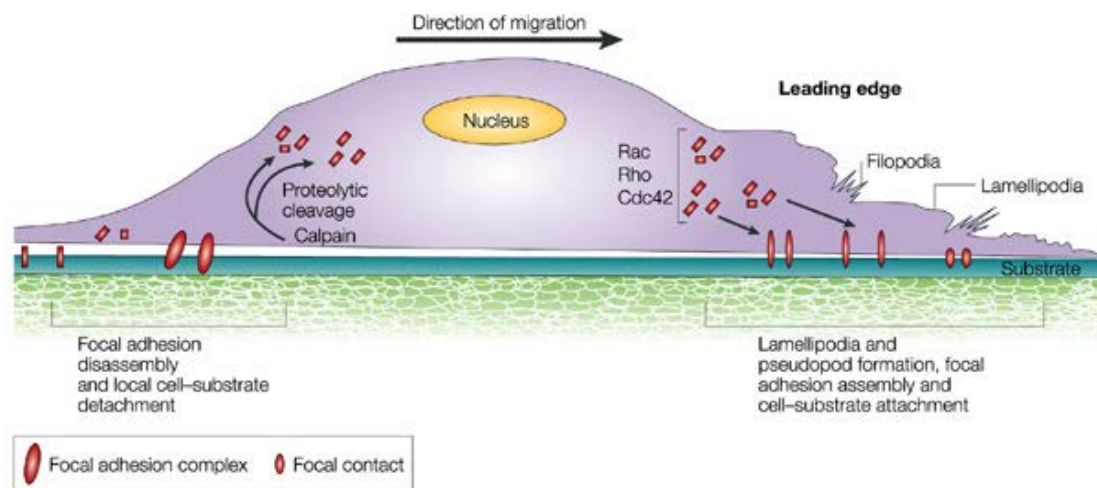


Figure 1.11: Diagram of cell migration. Cell migration depends on the dynamic interaction of cell-substrate adhesion at the leading edge of the cells in co-operation with the cell-substrate disassembly at the rear. Focal adhesion complexes facilitate the adhesive bond between the integrin receptor and intracellular actin cytoskeleton. To migrate, cells need to modify their shape which is often called polarization in response to migratory agents by developing the filopodia or lamellipodia structures at the leading edge. The morphological modification of cells is control by small GTPases family proteins such as Rac1, RhoA, Cdc42. At the leading edge of the cells, lamellipodia stabilised by adhering to the underlying substrate through formation of focal adhesion complex attachment which is called as 'focal contacts' as illustrated in the figure. Development of focal adhesions near the leading edge of the cells facilitates the traction which is necessary for pushing the cells forward. This is followed by disassembly of focal adhesion and detachment of cell-substrate at the rear of the cell and allows the tail retraction. Adapted from (Frame, Fincham et al. 2002).

1.9 Insulin-like growth factor family

1.9.1 IGF ligands and IGF receptors

Insulin-like growth factors (IGFs) are the main regulators in post-natal and adolescent growth and energy metabolism. There are large bodies of evidence that support a significant role of insulin-like growth factors and their signaling networks in cancer progression (Pollak 2008, Sachdev, Zhang et al. 2009, Davison, de Blacquiere et al. 2011, Pollak 2012).

The insulin-like growth factor system comprises three ligands; insulin-like growth factor 1 (IGF-1), insulin-like growth factor 2 (IGF-2) and insulin, three transmembrane receptors namely, type I insulin-like growth factor receptor (IGF-IR), type II insulin-like growth factor (IGF-IIR) and insulin receptor (IR), six binding proteins (IGFBP-1 to IGFBP-6) and binding-protein proteases that control the availability of ligands (Firth and Baxter 2002, Renehan, Frystyk et al. 2006). IGF ligands transduce their signals via two transmembrane receptors the type I IGF receptor and the insulin receptor. The two ligands IGF-1 and IGF-2

have higher binding affinity for the type I IGF receptor, whereas insulin has a higher binding affinity for the insulin receptor. Binding of IGF ligands to the extracellular domains of their receptors is followed by conformational changes in the receptors which induces phosphorylation of the receptors and in turn, enables phosphorylation of downstream adaptor molecules (Pollak 2012, Davison, de Blacquiere et al. 2011).

The main site of IGFs expression is in liver. These growth factors regulate cellular growth, differentiation and transformation through autocrine, paracrine and endocrine manner. Insulin is responsible for glucose metabolism. It is expressed in pancreatic β -cells and its secretion is regulated acutely by the level of circulating glucose. Insulin can travel to the neoplastic tissues through circulation (Pollak 2008, Pollak 2012). IGFs action is regulated by insulin-like growth factor binding proteins (IGFBPs). IGFBPs have relatively high binding affinity for both IGF-1 and IGF-2 and moderate the interaction of IGF with the IGF-IR and hence control the bioactivity of these growth factors. Amongst IGFBPs, IGFBP-3 is the main binding protein for IGF-1. One speculation is that expression of high affinity IGFBPs can lead to increase in the concentration of IGF ligands in tumour microenvironment. Ligands can be released from the complex via the IGFBP protease family (Gunter, Hoover et al. 2009).

Insulin and type I insulin-like growth factor receptor family are expressed on both normal and neoplastic tissues (Law, Habibi et al. 2008, Cox, Gleave et al. 2009, Badzio, Wynes et al. 2010, Kim, Kim et al. 2012). Both types of these tyrosine kinase membrane receptors have a heterotetrameric structure and have two extracellular α -chains and two intracellular β -chains that contain the tyrosine kinase domain (Pollak 2008). In contrast, IGF-IIR does not have catalytic activity; it does not participate in downstream signal transduction. IGF-IIR regulates the level of extracellular IGF-1 and IGF-2 via receptor-mediated endocytosis, which leads to lysosomal degradation of IGF-1 and IGF-2 (Zha and Lackner 2010). The insulin receptor comprises two different isoforms, IR-A and IR-B, due to the presence of a 12 amino acid sequence at the carboxyl terminus of the α -chain in IR-B; IR-A lacks the corresponding sequence. This variation stems from differences in splicing of exon 11 (Siddle 2011). Isoform B has much higher affinity for insulin than IGF-1 and IGF-2 whereas the differences in affinities of these ligands for isoform A is less marked. It has been reported that

isoform A is expressed in tumours cells (Belfiore, Frasca et al. 2009). Heterodimerization of both insulin receptor and type I IGF receptor arises from the dimerization of half receptors to create six potential different receptors (Figure 1.12).

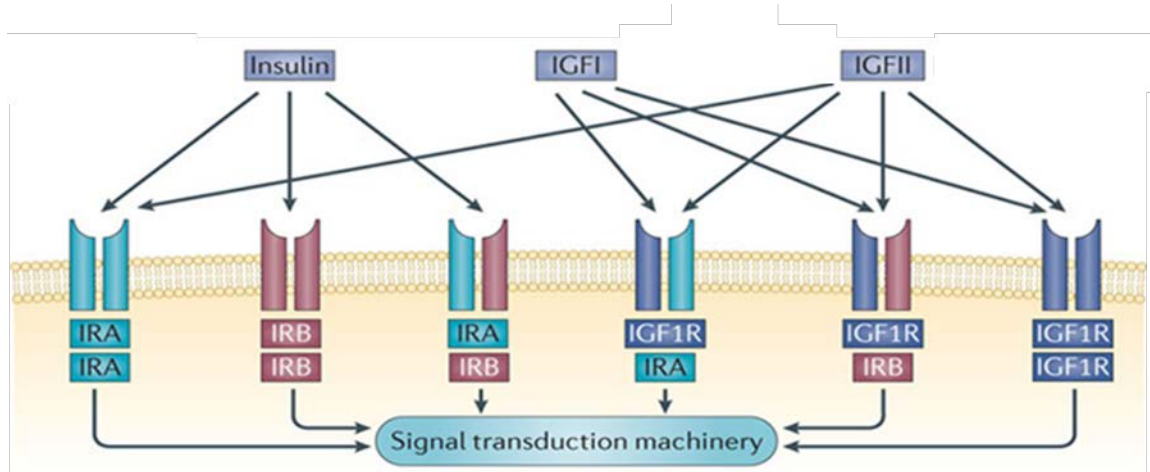


Figure 1.12: The insulin and insulin-like growth factor receptor family. The insulin receptor exists as two isoforms: IR-A and IR-B. The half receptors can dimerize and generate six different types of hybrid receptors with different binding affinities for their ligands. Insulin binds with high affinity to IR and lower affinity with IGF-IR. IGF-I binds to IGF-IR and IR-A and hybrid receptors with higher affinity compared to IR. IGF-II binds IGF-IR, IR-A and a hybrid receptor. Adapted from nature review (Pollak 2012).

1.9.2 Activation of IGF pathway

Binding of IGF-1 to IGF-IR induces autophosphorylation of β subunit tyrosine kinase, followed by phosphorylation of three different tyrosine residue on IGF-IR; Y1131, Y1135 and Y1136 (Foulstone, Prince et al. 2005, Zha and Lackner 2010). Phosphorylated IGF-IR recruits and activates the signalling adaptor proteins, such as insulin receptor substrates (IRS-1, IRS-2) and Src homology 2 domain containing transforming protein (Shc) to the cell membrane which are phosphorylated in turn (Kuijjer, Peterse et al. 2013). Recruitment of IRS-1 is proposed to be essential for mitogenic signalling, while IRS-2 recruitment is involved in response to cellular motility (Byron, Horwitz et al. 2006). Upon activation of IRS-1 by IGF-IR, phosphorylated IRS-1 leads to activation of downstream signalling cascade through binding to the p85 regulatory subunit of phosphatidylinositol-3-kinase (PI3K). The PI3K is a dimeric enzyme that phosphorylates phosphatidylinositol 4, 5-biphosphate (PIP₂) to phosphatidylinositol 3, 4, 5-triphosphate (PIP₃). Akt undergoes phosphorylation and activation by phosphoinositide-dependent kinase-1 (PDK1), which

phosphorylates Akt on Thr 308 and by mammalian target of rapamycin (mTORC2) complex, which phosphorylates Akt on Ser 473 (Guertin and Sabatini 2005). Activation of Akt leads to an increase in cellular proliferation and survival and regulation of glucose metabolism (Figure 1.13), (Zha and Lackner 2010). Through recruitment of growth factor receptor-bound protein 2 (Grb2) and son-of-sevenless (SOS) by Shc Ras-Raf-ERk pathway activates, which subsequently leads to cellular proliferation.

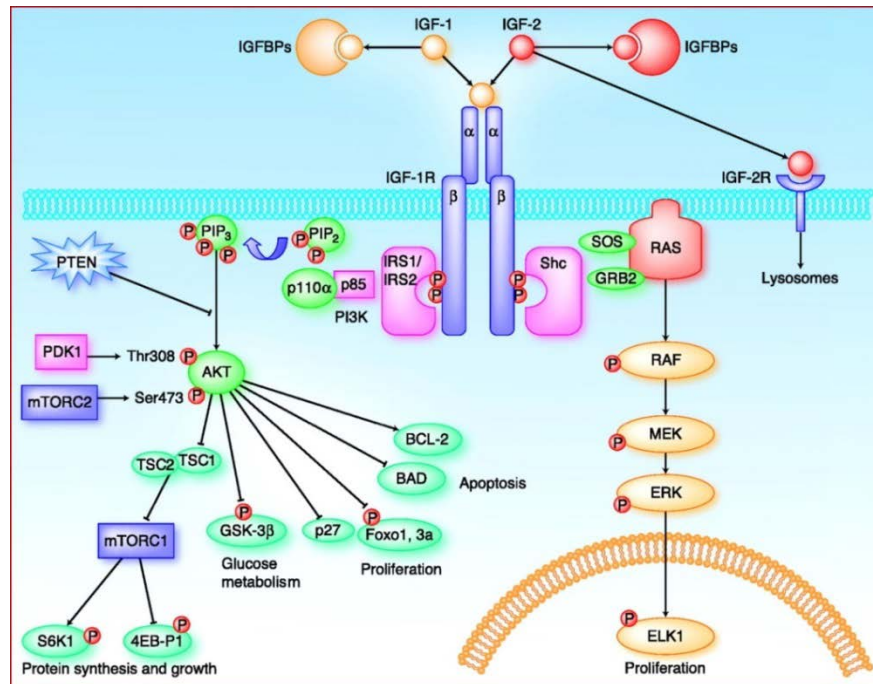


Figure 1.13: Activation of IGF downstream signal transduction pathway. Binding of IGF to type I IGF receptor induces autophosphorylation of the receptor and subsequent phosphorylation of downstream adaptor proteins such as IRS-1, IRS-2 and Shc. This event leads to activation of two main downstream signaling cascades: Ras mitogen-activated protein kinase (MAPK) pathway, which is responsible for cellular proliferation and phosphatidylinositol 3-kinase (PI3K) Akt pathway, which is important in cell survival. IGF binding proteins (IGFBPs) modify the bioavailability of both IGF ligands. Extracellular levels of IGF-1 are controlled by IGFBPs, whereas, IGF-2 availability is controlled by both IGFBPs and IGF-IIR. Upon binding IGF-1 and IGF-2 to IGF-IIR, this receptor sequesters the IGF-2 for lysosomal degradation. Adapted from (Zha and Lackner 2010).

1.9.3 IGF action in breast cancer

A large and growing body of literature has established the significant involvement of the insulin-like growth factor system in tumourigenesis, proliferation, survival and migration of tumour cells (Pollak 2008, Sachdev, Zhang et al. 2009, Davison, de Blacquiére et al. 2011, Pollak 2012). The importance of signal transduction through IGF-IR in malignant transformation, tumour cell survival and proliferation in many different cancers particularly

breast cancer has been demonstrated (Cohen, Baker et al. 2005, Davison, de Blacquiére et al. 2011, Pollak 2012). There is a strong correlation between overexpression of IGF-IR and the early stage of breast cancer development. The risk for development of many cancers including breast cancer is higher in those with highest levels of circulating IGF-1 and low levels of IGFBP3 (Hankinson, Willett et al. 1998, Schernhammer, Holly et al. 2005, Renehan, Harvie et al. 2006). The strong correlation between plasma levels of IGF-1 and high mammographic density is reported and that IGF-1 might be one of the risk factor in breast cancer development by having effect on enhancement of breast density (Diorio, Pollak et al. 2005). Mitogenic effects of both insulin-like growth factors (IGF-1 and IGF-2) has been reported in stimulation of breast cancer cell proliferation (Laban, Bustin et al. 2003).

Numerous studies have demonstrated the relationship between oestrogen and IGF activity in hormone-responsive breast cancer and regulatory role of oestrogens in the expression of key components of the IGF signal transduction pathway such as IGF-2, IGF-IR, IRS-1 and IRS-2 (Stewart, Johnson et al. 1990, Schiff, Massarweh et al. 2004, Bernard, Legay et al. 2006). Moreover, co-expression of IGF-IR and oestrogen receptor is also reported in breast tumours (Surmacz, E et al 2000). Oestrogen may be responsible towards enhancing the responsiveness of oestrogen receptor-positive breast cancer cells to IGFs. It is also speculated that one of the possibilities in induction of breast cancer cell migration by IGFs in oestrogen responsive breast cancer cells might be due to participation of oestrogen in tumour progression. (Stewart, Johnson et al. 1990, Bernard, Legay et al. 2006, de Blaquiére, May et al. 2009).

There is a large body of literatures showing IGF signal transduction pathway as promising therapeutic tool in various oestrogen-responsive subtypes of breast cancer. However, the importance of IGF signal transduction pathway in oestrogen non-responsive breast cancer subgroups, particularly triple-negative breast cancer cells is not well established (Gluz, Liedtke et al. 2009). Several studies failed to demonstrate a response of oestrogen non-responsive breast cancer cells, mainly triple-negative breast cancer cells to IGFs. For instance; in 2001, Bartucci *et al* were not able to demonstrate a mitogenic response of triple-negative breast cancer cell line MDA-MB-231 to IGFs, but have shown a mitogenic effect of IGFs in the oestrogen-responsive breast cancer cell line

MCF-7. Furthermore, in 1999, Jackson *et al* could not illustrate the effect of IGFs on proliferation of MDA-MB468 IRS-1 transfected triple-negative breast cancer cell (Jackson and Yee 1999).

In contrast, some other studies demonstrated the presence of IGF-IR in basal-like breast cancer. In one study, the IGF gene signature was illustrated in most of the oestrogen receptor-negative breast tumours (Creighton, Casa *et al.* 2008). Law *et al* (2008) showed the presence IGF-IR and phosphorylated IGF-IR in different subtypes of breast tumours including triple-negative breast tumours. In *in vitro* part of the same study, growth of triple-negative breast cancer cell line SUM149 was shown to be inhibited with the inhibitor of IGF-IR and the insulin receptor BMS-536924 (Law, Habibi *et al.* 2008). In a recent study, Davison *et al* (2011) illustrated the potential effect of IGF signaling pathway on proliferation and survival of oestrogen non-responsive breast cancer cells (Davison, de Blacquiere *et al.* 2011). The findings provided promising evidence for further investigation of IGF signal transduction pathway as a therapeutic target in treatment of triple-negative breast cancer patients.

1.9.4 Targeting strategies

IGF-IR targeting strategies have progressed over the past few decades and reached to the clinical trials for treating various malignancies. Targeted strategies consist of decrease in the bioactivity or concentration level of ligands or inhibition in receptor activity with receptor-specific antibodies or small-molecule tyrosine kinase inhibitors (Figure 1.14).

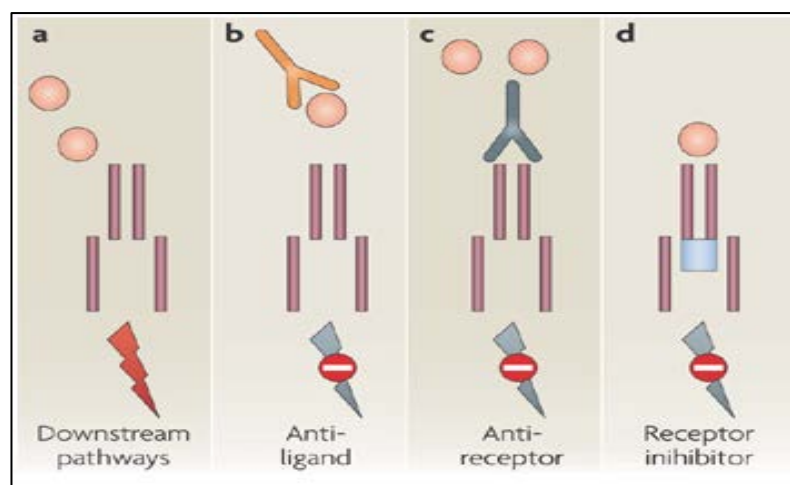


Figure 1.14: Targeting strategies for inhibition of IGF signal transduction pathway. Induction of downstream signalling activation through binding of insulin and insulin-like growth factors to their receptors (a). This event can be inhibited either with specific antibodies against

ligands **(b)**, which prevent ligands bind to their receptors, using receptor-specific antibodies **(c)** which is followed by blocking the IGF-1 receptor or IGF-IR/IR hybrids activity, but not effective against IR alone. Small molecule inhibitor that block the tyrosine kinase activity and is more effective against all IR-IGF-1R family members **(d)**. Taken from (Pollak 2008).

There are many monoclonal therapeutic antibodies against IGF-IR which have been studied preclinically and evaluated in clinical trials. Although these receptor-specific antibodies share several similarities, they can be differentiated by characteristic of the immunoglobulin (IgG) subclass and serum half-life. Some of the antibodies which are currently in clinical trials have shown the promising results particularly in combination with other chemotherapeutic drugs (Kurzrock, Patnaik et al. 2010, McCaffery, Tudor et al. 2011, Pollak 2012). Figitumumab, Pfizer (CP-751, 871), a fully humanized IgG2 monoclonal antibody against the type I IGF receptor was developed and has been evaluated in several clinical trials. In phase II clinical trials, figitumumab in combination with chemotherapeutic drugs exhibited very promising outcome. The response rate and progression-free survival of patients with advanced non-small cell lung cancer increased (Karp, Pollak et al. 2009). However, there were some disappointing phase III trials on figitumumab, which showed lack of efficacy of the antibody. Identification of predictive biomarkers is essential to define a subset of patients that may benefit from figitumumab therapy (Jassem, Langer et al. 2010, Reidy, Vakiani et al. 2010, Pollak 2012).

Administration of IGF-IR monoclonal antibodies, despite sparing insulin receptor, results in serious metabolic toxicity such as hyper-glycemia or hyper-insulinemia. This event is due to elevation in the level of growth hormone and IGF-1 as a result of blockade in IGF-1 receptor. Nonetheless, increase in IGF-1 level due to receptor blockade has no detrimental effect, but it is the high level of growth hormone which causes peripheral insulin resistance which leads to the metabolic cytotoxicity. However, these metabolic adverse events can be treated by adding metformin in the regime of the patients.

Another widely investigated strategy in preclinical trial experiments is use of tyrosine kinase inhibitors. Inhibition of IGF-IR activity alone reveals some moderate effects on preventing the growth of tumours but it is important to bring the involvement of insulin receptor into the consideration (Rodon, DeSantos et al. 2008, Pollak 2012). Targeting both IGF-IR and insulin receptor has been

more potential antineoplastic, when the insulin receptor expresses in tumour cells (Pollak 2008, Rodon, DeSantos et al. 2008).

BMS-754807, a potent and reversible small molecule tyrosine kinase inhibitor of the type I IGF receptor and the insulin receptor is currently being studied in several clinical trials. BMS-754807 inhibits effectively the growth of a wide variety of human tumour types *in vitro*, including breast, lung, colon and gastric. In a triple-negative tumour xenograft model, BMS-754807 inhibited the growth of tumour grafts as monotherapy, and resulted in complete tumour regression when combined with cytotoxic agents (Litzenburger, Creighton et al. 2011). In another *in vitro* study, BMS-754807 in combination with letrozole or tamoxifen showed anti-proliferative effects on oestrogen receptor-positive breast cancer cells by enhancing the activity of the hormonal agents (Mezi, Todi et al. 2012).

Based on several clinical trials, combination therapies are of importance in cancer treatment (Pollak 2008, Pollak 2012). For instance, there is evidence for the role of IGF-IR and the insulin receptors tyrosine kinase inhibitors in resistance of cancerous cells to rapamycin and its analogues; hence combining these drugs with inhibitors is of interest. Likewise, there are several substantial evidences which have reported the resistance of cells to EGF receptor family targeted therapies due to cross activity of IGF-IR-mediated signalling, hence there is a interest in co-targeting both receptor families by simultaneous inhibition of both receptor families (Lu, Zi et al. 2001, Buck, Eyzaguirre et al. 2008, Guix, Faber et al. 2008).

1.10 Epidermal growth factor family

1.10.1 EGF ligands and EGF receptors

The human epidermal growth factor receptor tyrosine kinases (ErbBs) and their ligands play important roles in mediating cellular proliferation, differentiation, survival and migration. Moreover, their crucial role in regulation of cancer cell growth, angiogenesis and metastasis has been reported (Kataoka 2009, Sasaki, Hiroki et al. 2013, Tebbutt, Pedersen et al. 2013).

The human epidermal growth factor receptors family are expressed in different tissues of epithelial, mesenchymal and neuronal lineage. They belong to subclass I of the receptor tyrosine kinase super-family and comprises four receptors; EGFR (HER-1 or ErbB-1), HER-2 (neu or ErbB-2), HER-3 (ErbB-3)

and HER-4 (ErbB-4). All members of the ErbB family receptor have analogous structure and consist of extra-cellular ligand-binding domain, a single hydrophobic transmembrane site and an intracellular cytoplasmic tyrosine kinase domain. On the basis of binding to ErbB receptors, EGF ligands can be divided into three groups: the first group consists of epidermal growth factor (EGF), transforming growth factor- α (TGF- α), amphiregulin (AR) and epigen (EPG), which bind specifically to EGFR; the second group is composed of betacellulin (BTC), heparin binding EGF (HB-EGF) and epiregulin (EPR) which bind to both EGFR and ErbB-4. Neuregulins belong to the third group which forms two subgroups depending on their ability to bind to ErbB-3 and ErbB-4 (NRG-1 and NRG-2) or bind only to ErbB-4 (NRG-3 and NRG-4) (Figure 1.15) (Yarden and Pines 2012). ErbB-2 is not capable of binding to any of the known EGF ligands; however, it is activated through hetero-dimerization with other ligand-bound receptors, and known as a preferred co-receptor for hetero-dimerization with other receptors (Foley, Nickerson et al. 2010). In addition, ErbB-3 lacks tyrosine activity and is only capable of transducing signals by hetero-dimerization with the other receptors. Although, it has been recently reported that HER-3 can bind to ATP and demonstrate low level of tyrosine activity (Shi, Telesco et al. 2010). Hence, the activity of both the HER-2 and HER-3 is based on their hetero-dimerization with other family members.

The EGF family of growth factors are derived from transmembrane precursors. Their availability and receptor activation is being regulated through ectodomain shedding (Hynes and Lane 2005, Kataoka 2009). This process is catalysed by cell surface proteases which belong to the metalloproteinase family, particularly a disintegrin and metalloprotease ADAM family and matrix metalloproteinases (MMPs). EGF ligands are cleaved by these proteases in the process of ectodomain shedding which leads to release of soluble form of EGF ligands in order to facilitate their binding to HER family members. Among these proteases involved in ectodomain shedding process, ADAM9, ADAM10, ADAM15 and ADAM17 have been linked with shedding between EGF ligands in many tumour cells. An association of EGFR activation and high level of ADAM17 has been reported in primary breast tumours (Hynes and Lane 2005, Kataoka 2009, Eccles SA 2011).

Ligand binding to ErbB receptors induces both homo-dimerization and hetero-dimerization of receptors which leads to phosphorylation of tyrosine kinase residues within cytoplasmic tail and subsequently triggers activation of downstream signalling pathways, including the two main RAS-ERK and PI3K-Akt pathways (Hynes and McDonald 2009, Tebbutt, Pedersen et al. 2013). Abnormal activity of tyrosine kinase receptor family member leads to unregulated growth and tumourigenesis in various tumour cells including breast, lung, head and neck, brain and colon tumours as well as melanoma (Yarden and Pines 2012).

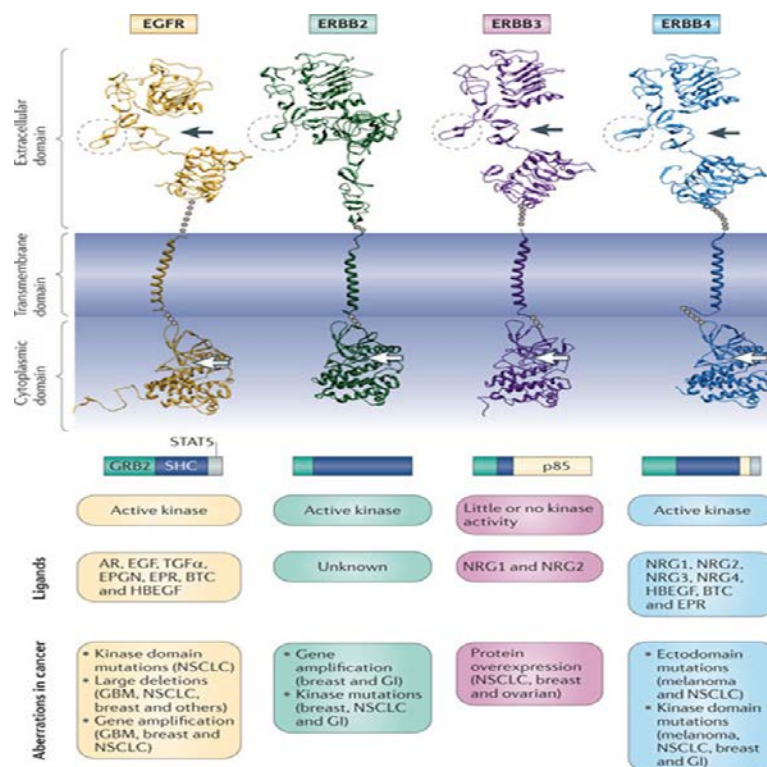


Figure 1.15: Structure and function of ErbB family receptors. The four receptors and crystal structure of their three major domains are illustrated. The structures of four receptors are similar and comprise extracellular ligand-binding domain, transmembrane domain and intracellular tyrosine kinase domain. Dashed circles indicate the dimerization loops; black arrows represent the ligand-binding clefts. Of note, ErbB2 does not have ligand binding clefts and white arrows indicate the ATP binding clefts. The coloured rectangles below the receptor structures indicate specificities of the docking site, the specific ligands for each receptors and the abnormalities which have important roles in cancer development and progression. Taken from (Yarden and Pines 2012).

1.10.2 Activation of EGFR family signal transduction pathways

Phosphorylated EGFR acts as a docking site for direct binding of adaptor proteins Grb2 and Sos or through adaptor molecule Src homology 2 (Shc). As mentioned also in previous section (1.9.2), this interaction makes conformational rearrangement in Sos, which leads to activation of Ras, Raf which induces

subsequently phosphorylation of mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinases 1 and 2 (Scaletti and Baselga 2006). Activated MAPK enters the nucleus for further activation of specific transcription factors important in cellular proliferation process (Figure 1.16).

Additional targets of HER-2 and HER-3 signalling are Phospholipase C-gamma, protein kinase C activation (PLCγ-PKC) and Janus Kinase-Signal Transducer and Activators of Transcription Factor (JAK-STAT) pathways. PLCγ binds directly to the activated EGFR and in turn activate PKC, which subsequently activate c-Jun and MAPK, which are important for cell proliferation (Patterson RL 2005).

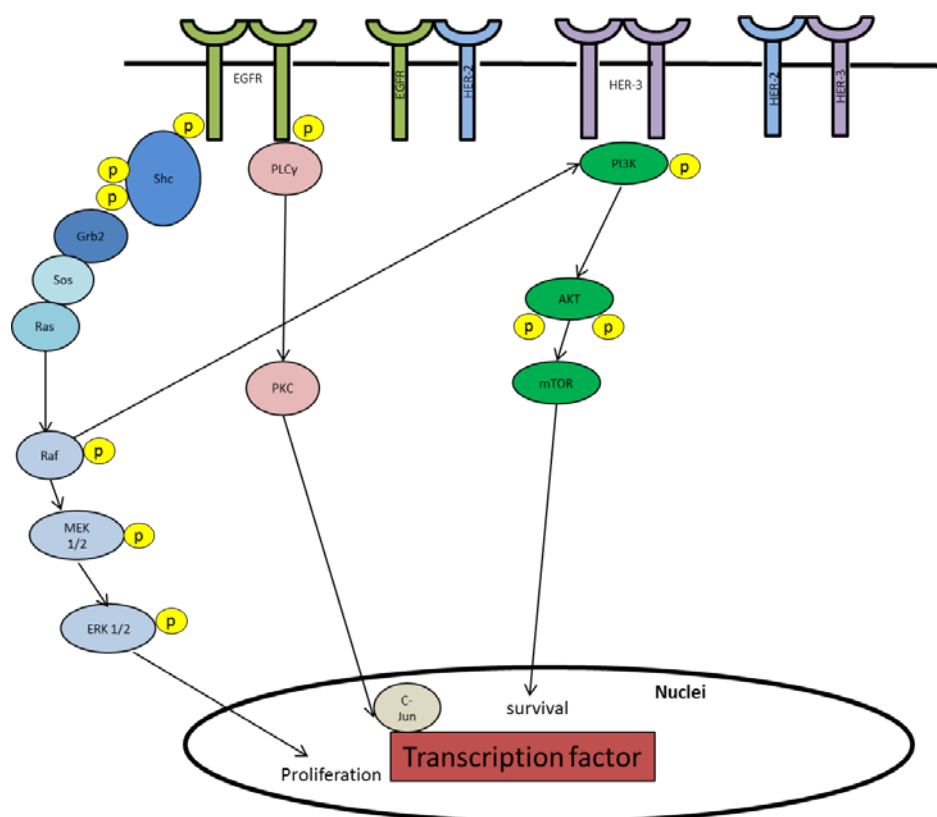


Figure 1.16: ErbB signalling pathway. Ligand binding to ErbB receptors induces the activation of the receptor which is followed by receptor homo-dimerization or hetero-dimerization and subsequent phosphorylation and activation of the tyrosine kinase residue and recruitment of numerous adaptor proteins to specific docking sites of the receptor. Activation of the both PI3K-AKT and MAPK pathways results in activation of genes responsible for cell survival and proliferation.

1.10.3 Role of EGF receptor in breast cancer

Inappropriate regulation in ErbB signal transduction pathway activity may result in uncontrolled growth stimulation and tumourigenesis in many cancers, including breast cancer. A diversity of molecular mechanisms such as overexpression of the ErbB receptors due to gene amplification, mutation

activation of the receptors or activation of receptors through autocrine and paracrine ligand binding may explain their contributions (Eccles 2011)

Overexpression of all four ErbB receptors is observed in breast cancer. Their expression level can be ranked in the order ErbB2>EGFR>ErbB3>ErbB4. Overexpression of the ligands such as NRGs in breast cancer has also been reported. Combined measurement of EPG and NRG4 reported to be strong predictors for relapse-free interval and overall survival in breast cancer (McIntyre, Blackburn et al. 2010). Recent gene expression analyses and functional studies have elucidated the significant role of EGFR in progression of particular subtypes of breast cancer (Foley, Nickerson et al. 2010). High expression of amplified or non-amplified EGFR has been observed in basal-like breast cancer which is a subgroup of triple-negative breast cancer (Sutton, Han et al. 2010). Expression of TGF α , NRG2 β and expression of ADAM and MMP proteases is significantly higher in oestrogen receptor-negative tumours than oestrogen receptor-positive tumours (Foley, Nickerson et al. 2010). Similarly, in basal-like tumours, there is a correlation between expression of EGFR and expression of TGF α and ADAM17 (Kenny 2007). A Recent study reported the ability of an inhibitor of ADAM17 to block the invasion and migration in several triple-negative breast cancer cell lines (Giricz, Calvo et al. 2013).

Overexpression of EGFR has been reported in 67% of triple-negative breast cancer patients who carry a BRCA1 mutated gene (Burga, Hu et al. 2011). These findings suggest that EGFR inhibitors could be partially effective in preventing the progression of these tumours in triple-negative breast cancer patients with BRCA1 mutation. However, to date, clinical trials failed to show responses in unselected groups of triple-negative breast cancer patients (Burga, Hu et al. 2011).

1.10.4 EGFR targeting strategies

Over the past 20 years, various efforts have been made to develop therapeutic agents that target EGFR and HER-2 and these novel therapeutic options have shown results toward treatment of several human malignancies (Tebbutt, Pedersen et al. 2013, Yewale, Baradia et al. 2013).

The most promising tools in clinical trials for targeting EGFR are monoclonal antibodies (mAbs) and small-molecule tyrosine kinase inhibitors (TKIs).

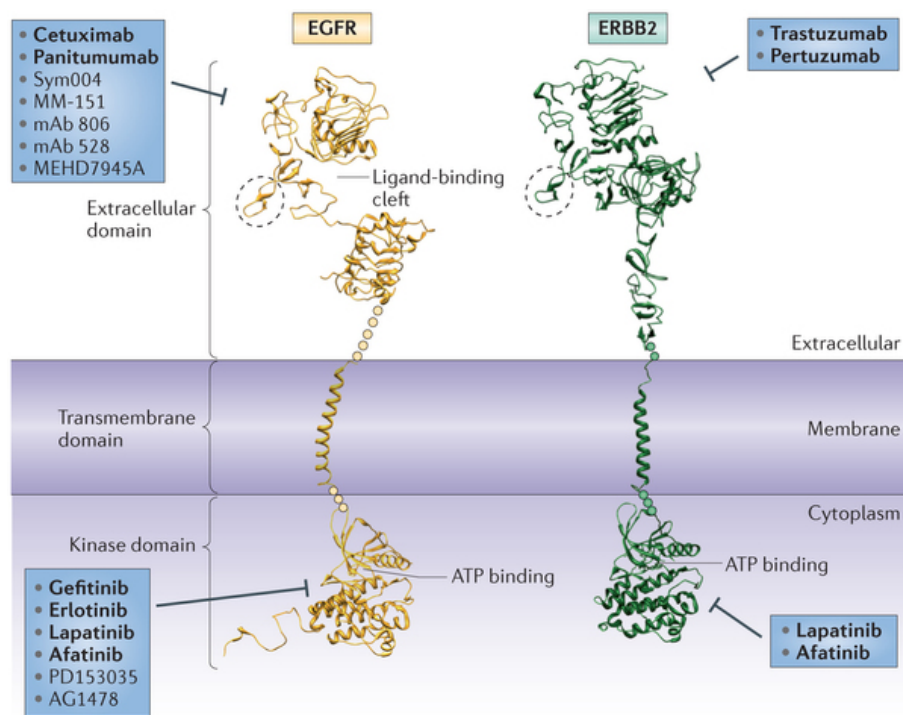
Monoclonal antibodies interact with extracellular domain of EGFR and obscure the ligand-binding site, therefore inhibit the ligand-binding to EGFR and prevent further EGFR tyrosine kinase activity. The potential mechanisms for action of mAbs are not understood clearly. In general, Monoclonal antibodies can stimulate the immunologically mediated mechanism such as anti-tumour-dependent cellular toxicity (ADCC) and complement-mediated cytotoxicity (Mellstedt 2003). They can induce receptor internalization and in this way inhibit ligand-induced autophosphorylation of EGFR (Kataoka 2009). Small-molecule tyrosine kinase inhibitors (TKIs) reversibly block the adenosine 5' triphosphate (ATP) interaction with the intracellular catalytic domain of EGFR tyrosine kinase; hence they prevent the autophosphorylation of EGFR and further downstream signalling (Yewale, Baradia et al. 2013).

Anti-EGFR monoclonal antibodies exclusively identify EGF receptors due to their high selectivity for the receptor. Monoclonal antibodies to EGFR are cetuximab, panitumumab (Figure 1.16). Cetuximab is the most frequent anti-EGFR antibody in use in clinical trials. It is a chimeric IgG1 antibody which causes the EGFR internalisation and stimulates the activation of ADCC and complement-mediated cytotoxicity. Currently, cetuximab and panitumumab are used as anti-EGFR mAbs for treatment of metastatic head and neck and colorectal cancers (Yewale, Baradia et al. 2013).

A recent study on triple-negative breast cancer cells showed that the combination of cetuximab and panitumumab had no cooperative effect on triple-negative tumour cells and did not improve receptor degradation due to overlapping of their antigenic epitopes. Combination of non-competitive antibodies to EGFR has shown to be effective in inhibition in growth of both *in vitro* and *in vivo* triple-negative tumour cells by degradation of EGFR (Ferraro, Gaborit et al. 2013). Consequently, in current clinical trials, combination of two non-competitive anti-EGFR mAbs has been used on both squamous cell carcinoma of head and neck and metastatic colorectal cancer. The combination of two non-competitive antibodies showed better inhibitory effect on tumour growth than antibodies with the overlapping epitopes (Koefoed, Steinaa et al. 2011).

HER-2 has no specific tyrosine kinase inhibitor. Afatinib and lapatinib are dual tyrosine kinase inhibitors that target both EGFR and HER-2. Trastuzumab and pertuzumab are two antibodies that target HER-2. Since HER-2 has no ligand, antibodies that targeted this receptor can block the tyrosine kinase activity through inhibition of receptor dimerization, although the exact mechanism underlying the HER-2 targeted antibodies are not known (Tebbutt, Pedersen et al. 2013). Trastuzumab has shown clinical advantage, particularly in combination with other antibodies such as pertuzumab and cytotoxic drugs for treatment of HER-2 positive metastatic breast cancer (Baselga, Cortés et al. 2012). It is a humanized anti-HER-2 monoclonal antibody that can inhibit the function of HER-2 through inducing the receptor internalization and degradation. Trastuzumab induces ADCC regulated by natural killer (NK) cells. Based on recent studies, combination of anti-HER-2 antibodies with the ability to identify different epitopes has more potential in inhibition of ligand-mediated invasion compared to single agent (Borriello, Laccetti et al. 2011).

Various tyrosine kinase inhibitors with different level of selectivity have been developed. The first generation of EGFR tyrosine kinase inhibitors, are erlotinib and gefitinib which inhibit EGFR tyrosine kinase activity (Figure 1.17) (Tebbutt, Pedersen et al. 2013). Both of these inhibitors have been reported to shown little efficacy when they are used as single agent in breast cancer, even in triple-negative breast cancer patients with high levels of EGFR (Eccles 2011). Therefore, currently in clinical trials these EGFR tyrosine kinase inhibitors are used in combination with other cytotoxic or molecularly-targeted agents. As mentioned above, lapatinib that inhibits both tyrosine kinase activity of EGFR and HER-2, induces the down regulation of both MAPK and PI3K-AKT signalling pathways. Lapatinib has been also used in combination with other cytotoxic and molecularly-targeted agents such as trastuzumab (Eccles 2011). In phase III clinical trials, combination of lapatinib with trastuzumab has shown to be effective towards improvement of progression-free survival in breast cancer patients and also decrease the risk of disease progression in patients who have failed to trastuzumab alone (Eccles 2011).



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Figure 1.17: Therapeutic targeting strategies in EGFR and HER-2. Therapeutic monoclonal antibodies of both EGFR and HER-2 target the extracellular domain of the receptors. Tyrosine kinase inhibitors block tyrosine kinase domain. The clinically approved drugs are illustrated in bold. Afatinib is a recent clinically approved drug by the US Food and Drug Administration in 2013 (Tebbutt, Pedersen et al. 2013).

1.11 Trefoil factor family of proteins

1.11.1 The trefoil protein structure

The mammalian trefoil factor family (TFF) are the secreted proteins that have a unique domain called a trefoil domain which contains a three loop structure. There are three mammalian trefoil proteins: TFF1 (pS2, PNR-2), TFF2 (hsp, spasmodic poly-peptide, SP) and TFF3 (hITF, intestinal trefoil factor). The trefoil domain is 42-43 amino acids long. It has an absolutely conserved sequence of six cysteine residues. That helps to stabilise the unique compact three-loop structures through formation of three intra-molecular disulphide bonds. The nucleotide sequences that encode TFF1, TFF2, and TFF3 have been identified in different species of human, porcine, rat, chimpanzee, murine and canine. The alignment of these three proteins orthologous are shown in Figure 1.18 (Thim and May 2005).

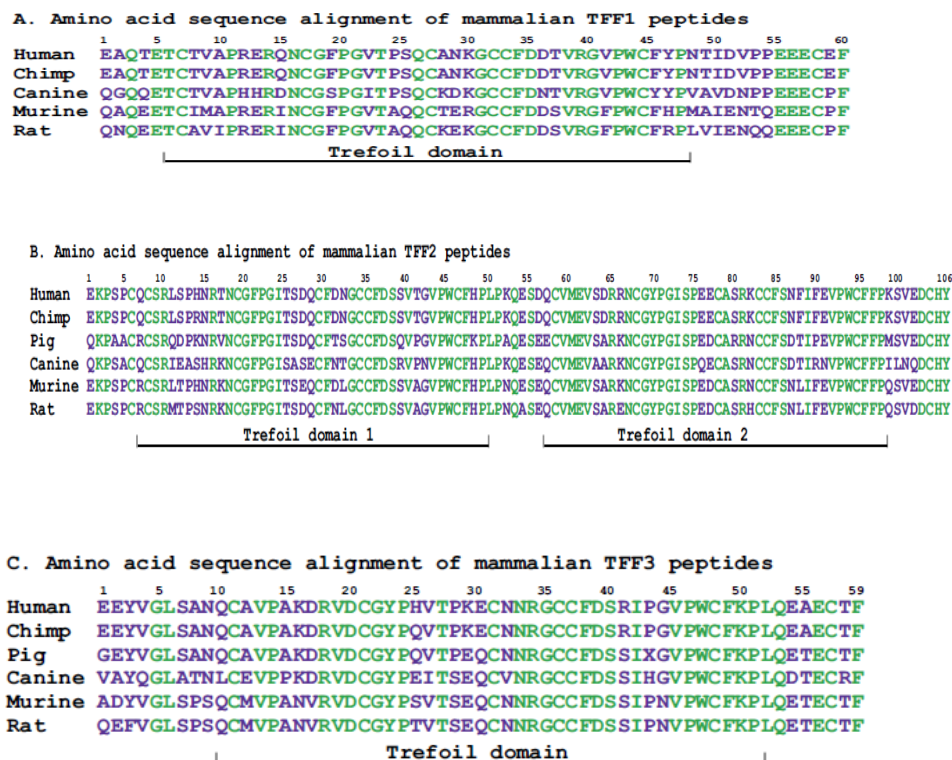


Figure 1.18: Amino acid sequence alignments of mammalian trefoil proteins in different species. The green coloured residues are conserved between the orthologous, and the purple coloured residues are with substitution. *Adapted from* (Thim and May 2005).

The main site of trefoil factor expression is in the epithelial cells of gastrointestinal mucosa. TFF1 is synthesised mainly in the stomach, TFF2 in the stomach and duodenum, while TFF3 is expressed in the goblet cells of small and large intestines (May, Semple et al. 2004, Westley, Griffin et al. 2005).

TFF1 is 60 amino acids long and has a single trefoil domain, TFF2 has 106 amino acids residues and contains two trefoil domains and TFF3 is 59 amino acids long and has a single trefoil domain (Thim and May 2005). Both TFF1 and TFF3 have an extra cysteine residue near the carboxy-terminus that facilitates the formation of homodimers or heterodimers with other proteins (Figure 1.19). Recombinant hTFF1 and hTFF3 can be produced as monomers and as disulphide-linked dimers (Figure 1.20). It has been reported that monomeric TFF2 can stimulate cell migration, whereas, monomeric TFF1 compared to dimeric TFF1, has noticeably lower bioactivity (Prest, May et al. 2002). Further study demonstrated that, dimerization might be necessary for TFF1 to induce cell migration, whereas, TFF3 has shown to be able to stimulate the cell migration without dimerization in case of mutation (Taupin and Podolsky 2003). Overall dimeric forms of both TFF1 and TFF3 compares to the monomeric forms have more efficacies in protection of gastrointestinal tissues (Marchbank, Westley et al. 1998).

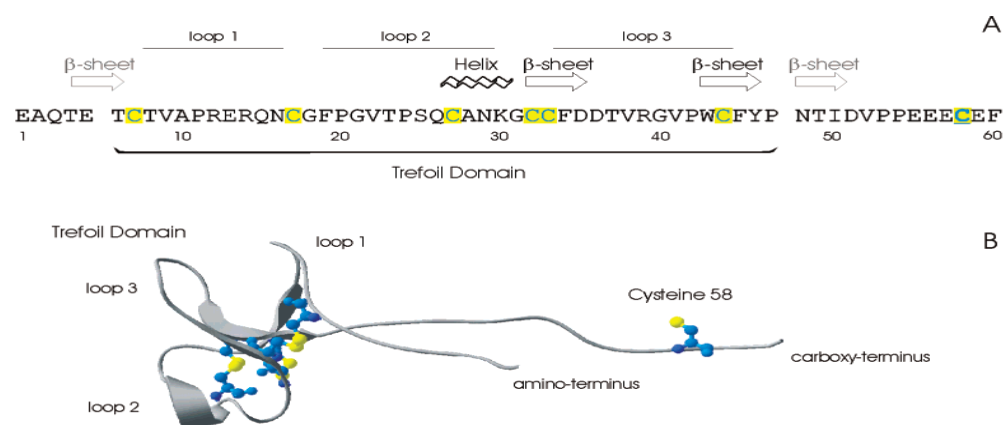


Figure 1.19: Structure of TFF1. (A) Amino acid sequence for TFF1 shows the trefoil domain. (B) Location and orientation of conserved cysteine residues which form intra-molecular disulphide bonds. *Taken from citation* Westley, Griffin et al. 2005).

Based on the high resolution nuclear magnetic resonance studies of monomeric and dimeric forms of trefoil proteins, the compact structure of trefoil proteins can show the secondary structure. The secondary structure might have role in TFFs resistance to the protease degradation and keep them functional in the gastrointestinal luminal surface (Taupin and Podolsky 2003). Comparison between isoelectric points of human TFF1 and TFF3 monomers demonstrates that both of the monomers are acidic. TFF1 with the isoelectric point value of 3.94 is more acidic than the TFF3 with isoelectric point value of 4.75. Both TFF1 and TFF3 contain acidic charged residues in their N- and C-termini. TFF1 has greater number of acidic residues in termini, outside of the trefoil domain, while TFF3 has more charged residues inside the trefoil domain of which most of them are basic residues (May, Church et al. 2003).

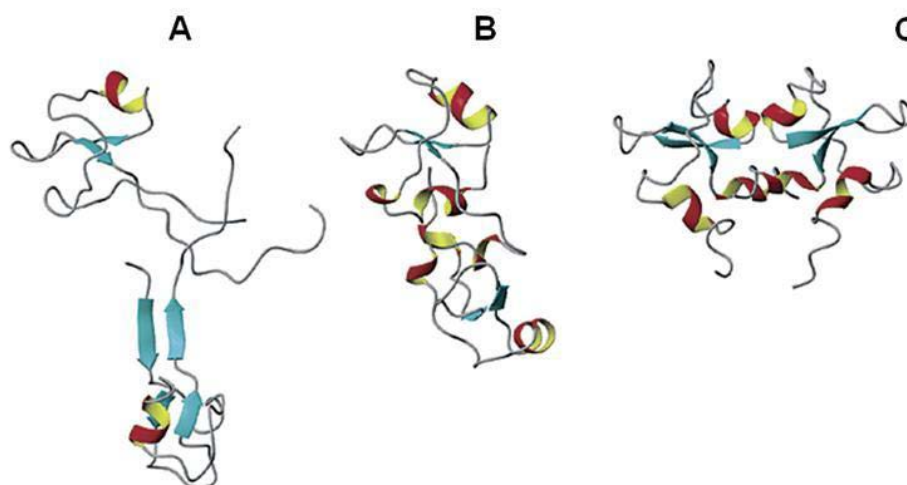


Figure 1.20: Trefoil proteins dimerization. Ribbon illustration of (A) TFF1 homodimer, (B) TFF2 homodimer and (C) TFF3 homodimer. Adapted from (Muskett, May et al. 2003)

1.11.2 The function of trefoil proteins

The exact role of trefoil proteins in tumourigenesis is controversial. Different biological roles have been assigned to trefoil proteins including tumour suppressive and tumour promoting role. Trefoil proteins have an important role in protection of the gastrointestinal mucosa layer against the damage and also subsequent repair through motogenic and anti-apoptotic activity; hence they help in formation of a protective barrier during the process of restitution. In response to injury, trefoil proteins production and secretion are increased and they act in an autocrine fashion to stimulate the migration of epithelial cells over

the basement membrane to the site of the injury (Figure 1.21). The precise signalling pathways which mediate the effects of trefoil proteins and determine the function of putative trefoil protein receptors have not been clarified. If malignant transformation occurs in epithelial cells, then the motogenic and anti-apoptotic properties of trefoil proteins may lead to migration, invasion and metastases of the malignant cells (Taupin and Podolsky 2003).

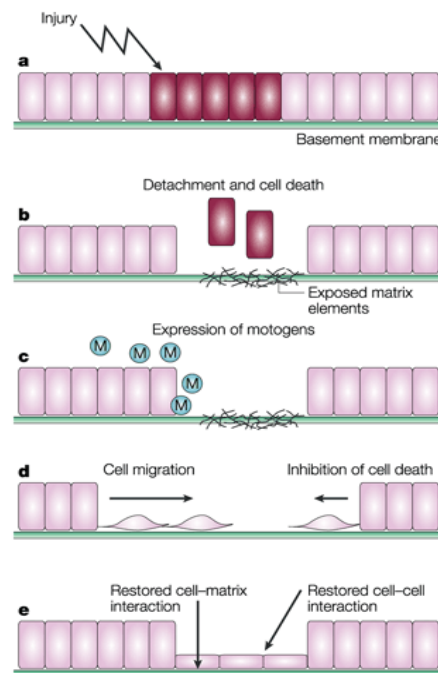


Figure 1.21: A schematic illustration of restitution. Local injury occurs to the mucosal epithelial cells on the basement membrane **(a)** that leads to cell detachment and death **(b)** which is followed by exposure of matrix elements and rapid expression of (motogens; M) from the matrix and adjacent cells **(c)** that helps to speed the repair, and subsequently leads to migration of epithelial cells and inhibition of cell death **(d)**. Restoration between cells and their matrix **(e)**. Taken from citation (Taupin and Podolsky 2003).

It has been reported that trefoil proteins act as a tumour suppressor by inhibition of the cell proliferation in gastric cancer which was confirmed through loss of approximately 50% of TFF1 expression in gastric carcinomas (Lefebvre, Chenard et al. 1996). Similarly, a recent study in gastric cancer reported the decrease in the TFF1 expression followed by increase in expression of TFF3 in gastric tumours, which indicate TFF3 as a marker of poor prognosis in this cancer (Im, Yoo et al. 2013). TFF1-null mice demonstrated hyperplasia and dysplasia in the stomach, which was followed by intra-epithelial and intra-mucosal carcinoma. This evidence can support the hypothesis that TFF1 act as

gastric tumour suppressor genes (Buache, Etique et al. 2011). Trefoil proteins overexpression has a tumour progressive effect through stimulation of the migration and invasion of tumour cells. Hence it has been proposed that they are involved in tumour dissemination (Prest, May et al. 2002). The inconsistencies about the function of trefoil proteins can provide an explanation why trefoil protein expression, in particular TFF1, exerts a beneficial effect to normal cells by having an influence on mucosal protection and wound repair during local damage and subsequently undesirable effects to cancerous cells by exhibiting their role as motogenic, pro invasive and pro angiogenic factors. Inappropriate expression of TFF1 may contribute to tumourigenesis due to its anti-apoptotic and angiogenic characteristics (Emami, Rodrigues et al. 2004).

Over expression of trefoil proteins is reported in several important human solid tumours such as: breast, lung, colon, and prostate cancer. An investigation into TFF1 function in gastrointestinal cells demonstrated that, TFF1 has anti-proliferative and anti-apoptotic roles (Bossenmeyer-Pourri , Kannan et al. 2002). TFF1 activates two cellular responses: firstly, it reduces the gastrointestinal cellular proliferation through delaying the G1-S phase cell phase transition by keeping the cells inside G1 phase of the cell cycle and subsequently postpones their entry into S phase, secondly, it protects cells from apoptosis that can occur by chemical, anchorage free or Bad-induced apoptosis which was demonstrated by reduction in the activities of caspase-3, -6, -8 and -9 (Bossenmeyer-Pourri , Kannan et al. 2002). Collectively, these results elucidate evidence in support of both an anti-proliferative and an anti-apoptotic role for TFF1 in cancerous cells (Bossenmeyer-Pourri , Kannan et al. 2002). Over expression of TFF3 has been shown to enhance the cell survival and migration, also it showed to increase the anchorage-independent growth of the oestrogen receptor-positive breast carcinoma (Kannan, Kang et al. 2010).

1.11.3 Trefoil proteins in breast cancer

It is known that breast cancer cells are influenced by the hormonal levels in human body. Significant numbers of human breast cancers depend on oestrogen level. Research has been directed towards identification of the genes which are affected by the level of oestrogen in tumour cells as potential markers of response to hormonal therapy and novel therapeutic targets. Studies show that amongst the three trefoil proteins, TFF1 and TFF3 are expressed at high

levels in breast tumour cells while TFF2 is not generally expressed (Poulsom, Hanby et al. 1997). Both TFF1 and TFF3, particularly TFF1 are oestrogen-responsive genes that have been identified most frequently in breast tumour cells (Masiakowski, Breathnach et al. 1982, May and Westley 1997, May and Westley 1997). In contrast to the reduction in expression of TFF1 in gastric tumours, TFF1 expression is not reduced in breast tumours and its expression in breast cancer cells is under the control of oestrogen. In the very first line of evidence, It has been demonstrated that oestrogen increases the concentration of TFF1 mRNA up to 100-fold (May and Westley 1988).

The TFF1 promoter has been reported to be regulated also by other molecules apart from oestrogen such as human growth factor (hGH), insulin-like growth factor 1 (IGF-1), transforming growth factor beta (TGF- β), epidermal growth factor (EGF) and fibroblast growth factor (FGF) (Baus-Loncar and Giraud 2005). In contrast to screen-detected tumours in which TFF1 expression is associated positively with oestrogen receptor expression, in interval breast cancers there is discrepancy between oestrogen receptor and TFF1 expression. In this particular type of breast cancer levels of TFF1 expression are noticeably higher, whereas levels of expression of oestrogen receptor is lower (Crosier, Scott et al. 2001). This observation suggests that, in interval breast cancer TFF1 is likely to be expressed independently from oestrogen receptor and TFF1 expression might be more related to the Ki67 proliferation marker of proliferation as interval cancers are characterized by high level of Ki-67, hence this might lead to the possibility that cell division and migration might be responsible in rapid development of tumours.

An association between HER-2 and TFF3 expression has been reported in breast cancer cell lines. In a microarray analysis of pooled mRNA from three HER-2 negative and three HER-2 positive breast cancer cell lines, abundant TFF3 mRNA has been identified in the HER-2 positive breast cancer cell line samples. This observation was validated by northern transfer analysis which showed that there is an expression of TFF3 in two out of the three cell lines which are positive for HER-2 (Wilson, Roberts et al. 2002). However, further analysis did not demonstrate an association between HER-2 and TFF3 expression in primary breast tumour cells. The study did not consider the oestrogen receptor status in the cell lines. In 2013, Yue *et al* reported the high

expression of TFF3 in HER-2 positive breast cancer patients and demonstrated that silencing HER-2 in HER-2 positive SK-BR-3 breast cancer cells, induced downregulation of TFF3 in this cell line (Yue, Xiang et al. 2013).

One group has suggested that TFF1 has oncogenic functions in breast cancer cells. They reported that overexpression of TFF1 increases the anchorage-independent growth in MCF-7 cells which is followed by an increase in cell migration and invasion. The same group reported that overexpression of TFF1 results in increasing the MCF-7 cell number; this was achieved by increased cell proliferation and decreased cell survival. Inhibition of TFF1 functions with a polyclonal antibody, caused inhibition of viability of mammary carcinoma cells both in vitro and in xenograft models. These findings have to be reconciled with very high endogenous expression of TFF1 in MCF-7 cells. On the basis of their results, this group proposed that antagonism of the function of TFF1 has potential therapeutic value for TFF1-positive mammary carcinoma (Amiry, Kong et al. 2009).

In contrast, in a more recent study, the oncogenic property of TFF1 in human breast cancer cells was tested and a negative effect of TFF1 on the development of mammary tumours has demonstrated clearly (Buache, Etique et al. 2011). The data support the conclusion that expression of TFF1 leads to migration and invasion in the mammary epithelial cell lines but that the overexpression of TFF1 has no effect on tumourigenicity or anchorage dependent or independent proliferation of breast cancer cells. Endogenous knockdown of TFF1 in oestrogen-responsive breast malignant cells decreased the cellular invasion and motility. Interestingly knockdown of endogenous TFF1 decreases the formation or progression of tumours in the *in vivo* mice model. Whereas, deficiency in the expression level of TFF1 had result in the development of mammary tumour in nude mice. Collectively, these observations can show the positive effect of TFF1 against tumourigenicity in breast tumours (Buache, Etique et al. 2011).

The non-steroidal anti-oestrogen tamoxifen is used as a treatment in premenopausal patients with advanced breast cancer. In breast cancer cell lines, expression of TFF1 is induced weakly by tamoxifen, and anti-oestrogens prevent the large increase in TFF1 concentrations that is induced by oestrogen

(May and Westley 1986, Prest, May et al. 2002). Prest *et al* (2002) suggested that the motility of breast cancer cells can be stimulated by oestrogen through induction of TFF1, and that the efficacy of hormonal therapies may result in part from their ability to reduce TFF1 expression and as a result breast cancer cell migration. Both TFF1 monomer and dimer stimulate the migration of breast cancer cells, but the dimer has more capability in stimulating the migration of breast cancer cells. There are variety of hormones and growth factors which activate and bind to their receptors through cross-linking with monomer receptors. Thus it might be possible that TFF1 dimer is more potential in migration of breast cancer cells through a bivalent dimeric receptor (Prest, May et al. 2002). Additionally, another study showed the role of TFF3 in regulating the anti-oestrogen resistance breast carcinoma and that overexpression of TFF3 induce the oestrogen independent growth in breast cancer cells and subsequently promotes the resistance of cells to anti-oestrogen therapies such as tamoxifen or fulvestrant (Kannan, Kang et al. 2010).

The presence of metastatic cancer in axillary lymph nodes is one of the significant prognostic factors for predicting the recurrence of the disease. According to gene microarray-based study, TFF1 is one of the genes which are identified as a useful molecular marker for detection of metastatic breast cancer. In addition, measurement of the level of TFF1 over expression in axillary lymph nodes which contain metastatic breast cancer led to the speculation that TFF1 is also a potentially informative prognostic marker for detection of micro metastatic breast cancer (Mikhitarian, Gillanders et al. 2005). In a recent immunohistochemical study on role of TFF3 in oestrogen receptor-positive breast tumours, TFF3 expression was observed in both normal breast epithelia and malignant tumours and there was a strong correlation between expression of TFF3 and the presence of axillary lymph nodes, which can suggest the possible involvement of TFF3 in invasion and metastasis of breast cancer (Ahmed, Griffiths et al. 2012).

Hypothesis:

Ligands of insulin-like growth factor and epidermal growth factor receptors family promote metastasis in oestrogen receptor-negative breast cancers.

Aim:

The specific aims for the research planned to address the hypothesis are:

To elucidate the effect of growth factors on cell survival and migration of oestrogen receptor-negative breast cancer.

1. To analyse the biological roles of IGFs and EGFs on oestrogen receptor-negative breast cancer cells by:
 - i) Protection of cells from anoikis
 - ii) Stimulation of cell migration
2. To investigate the expression of IGF-IR, EGFR, HER-2 and TFF1 and TFF3 in oestrogen receptor-negative breast cancer by immunohistochemistry and analyse their association with biomarkers of cell survival and growth.
3. To test the effectiveness of drugs that inhibit the IGF and EGF signal transduction pathways in:
 - i) Inhibition of cell survival protective effect
 - ii) Inhibition of the migratory effects

Chapter 2. Materials and Methods

2.1 Cell culture

2.1.1 Cell lines

The human breast cancer cell lines and their characteristics investigated in this study are listed in (Table 2.1). There are three oestrogen responsive breast cancer cell lines; MCF-7, BT-474, EFM-19 and five oestrogen non-responsive breast cancer cells lines; SK-BR-3, HCC1419, HCC2218 which are HER-2 positive and Hs578T, MDA-MB 231 which are triple-negative breast cancer cell lines.

| Cell line | Origin | ER α | PgR | HER-2 | Morphology | Growth medium | References |
|-------------------|--|-------------|-----|-------|------------------|--|--|
| MCF-7 | Human breast adenocarcinoma pleural effusion | + | + | – | Epithelial cells | DMEM, 10% FBS, 1 μgml^{-1} insulin | Engel, et al., 1978 Soule, et al., 1973 and |
| BT-474 | Primary human breast adenocarcinoma | + | + | + | Epithelial cells | DMEM, 10% FBS, 1 μgml^{-1} insulin | Simon, et al., 1984 and Clayton, et al., 1997 |
| EFM-19 | Human breast adenocarcinoma pleural effusion | + | + | + | Epithelial cells | DMEM, 10% FBS, 1 μgml^{-1} insulin | Simon, et al., 1984 and Clayton, et al., 1997 |
| SK-BR-3 | Human breast adenocarcinoma pleural effusion | – | – | + | Epithelial cells | DMEM, 10% FBS, 1 μgml^{-1} insulin | Engel, et al., 1978 |
| HCC-1419 | Human breast invasive ductal carcinoma | – | – | + | Epithelial cells | RPMI 1640, 10% FBS, 1 μgml^{-1} insulin | Gazdar AF, et al, 1994 |
| HCC-2218 | Primary human breast ductal carcinoma | – | – | + | Epithelial cells | RPMI 1640, 10% FBS, 1 μgml^{-1} insulin | Gazdar AF, et al, 1998 |
| Hs578T | Primary human breast carcinoma | – | – | – | Epithelial cells | DMEM, 5% FBS, 1 μgml^{-1} insulin | Engel, et al., 1978 and Hackett, et al., 1977 |
| MDA-MB-231 | Human breast adenocarcinoma pleural effusion | – | – | – | Epithelial cells | DMEM, 5% FBS, 1 μgml^{-1} insulin | Engel, et al., 1978; Cailleau, et al., 1978 |

Table 2.1: Human breast cancer cell lines characteristics. Cell line origins, receptor status, morphology, growth medium, sources and references.

2.1.2 Routine cell culture

The cell lines MCF-7, BT-474, EFM-19, SK-BR-3, HCC-1419, MDA-MB-231 and Hs578T cells were cultured routinely as adherent monolayer and HCC-2218 were cultured as suspension in tissue culture flasks (Corning) in Dulbecco's Modified Eagle's Medium (DMEM) with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) modification (sigma) and 10% foetal bovine serum (FBS), supplemented with 1 $\mu\text{g/ml}$ insulin (Sigma). Cells were maintained at 37°C in a humidified 5% CO₂-saturated atmosphere.

Cells were grown in culture medium to approximately 70-80% confluence or as necessary, medium was removed and cells were washed with warm phosphate buffer saline (PBS) before being detached by incubation in a trypsin-EDTA solution (0.17% trypsin and 0.07% Na₄EDTA in PBS w/v) (Sigma). All cell lines apart from EFM-19 cells were detached using a 1:3 x trypsin-EDTA solution diluted in PBS. EFM-19 cells were detached using a 1 x trypsin-EDTA solution. HCC2218 cells were grown in suspension. Cells were collected by centrifugation at 1,200 rpm for 5 minutes (Beckman Allegra X-12 R, rotor SX 4750) and resuspended in fresh medium prior to reseeding into fresh flask. MCF-7 and BT-474 cells were typically reseeded at 1:6 to 1:9 dilution, SK-BR-3, HCC-1419 and HCC-2218 cells at 1:3 to 1:6 dilution and MDA-MB-231 and Hs578T cells at 1:10 to 1:20 dilution. Medium was changed 24 hours after seeding and subsequently every 72 hours at minimum.

Long term storage of the cells was in 70% DMEM, 20% FBS and 10% of dimethyl sulfoxide (DMSO) in 0.5 ml aliquots in liquid nitrogen.

Cell lines were checked regularly by E. C. Matheson using a MycoAlert kit (Lonza Biologics, Slough, UK) to confirm the absence of Mycoplasma contamination.

2.1.3 Preparation of dextran-coated charcoal stripped serum (DCCS)

Dextran-coated charcoal treated serum was prepared by addition of 20 g of acid washed and neutralized charcoal (Sigma) and 0.2 g of dextran T70 (Pharmacia) in a 250 ml of centrifuge bottle (Beckman) and mixed thoroughly with 250 ml of deionised water. The suspension was allowed to stand at room temperature for 10 minutes then centrifuged at 7,000 rpm (Beckman Avanti J-26 XP centrifuge, JA 16.250 rotor) at 4°C for 15 minutes. The supernatant was discarded and pellet was resuspended in 250 ml of deionized water and allowed to stand for 10 minutes. The suspension was centrifuged again at 7,000 rpm for 15 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in 200 ml of newborn calf serum (Invitrogen). The suspension was transferred to a 500 ml glass conical flask and agitated in a shaking water bath at 55°C for 40 minutes. Treated serum was transferred to a fresh 250 ml centrifuge bottles and centrifuged at 10,000 rpm (JA 16.250 rotor) for 30 minutes at 4°C. The supernatant were collected and transferred to the new centrifuge bottles and

again centrifuged at 10,000 rpm for 30 minutes at 4°C. The supernatant were collected and filtered by 0.45 µm filters under tissue culture hood and stored at -20°C.

2.1.4 Withdrawal of cells from the effect of steroid hormones and growth factors

Cells were withdrawn from the effects of growth factors and steroid hormones in maintenance medium by incubation in phenol red-free DMEM (Sigma) and 10% of DCCS. Normal growth medium was removed from the cells, cells were washed with PBS and the medium was replaced with DCCS medium. Medium was changed daily during withdrawal period, which was at least two days for experiments requiring withdrawal from IGFs and EGFs, one day to two days, depending on the types of the experiments.

2.1.5 Stimulation assay

Cells were grown to 70% confluence, detached from tissue culture flasks by incubation of trypsin-EDTA solution and pelleted as described in section 2.1.2. Pellets were resuspended in the maintenance medium and were counted using haemocytometer. Cells were seeded in 12 well plates at a density of 10×10^4 cells/well for MDA-MB231 and Hs578T cells, and 20×10^4 cells/well for SKBR-3 cells and left to grow for 24-48 hours to allow attachment. Medium were aspirated with pulled glass Pasteur pipette and wells were washed with 1 ml of PBS. The medium were replaced with 1 ml of DCCS medium daily and cells were cultured for 48 hours. After 48 hours of withdrawal, cells were washed once with PBS and incubated in in phenol red-free DMEM and 10% DCC-CS alone or supplemented with various concentration of IGF-1 or EGF. Cells were incubated for 15 minutes prior to protein extraction by radioimmunoprecipitation assay (RIPA) buffer plus inhibitors. Aliquots of protein were measured for the levels of total and phosphorylated proteins in IGF and EGF signal transduction pathways by Western transfer as described in section 2.3.2.

2.1.6 Preparation of poly (2-hydroxylethylmethacrylate) coated plates

Poly (2-hydroxylethylmethacrylate) (Sigma) was dissolved in 97% ethanol at a concentration of 10 mg/ml and to avoid precipitation, the solution stored at 37°C. Tissue culture plates were coated with poly-HEMA solution under the aseptic condition in a tissue culture hood. Twelve well plates were coated with 0.5 ml per well poly-HEMA solution. After coating plates were incubated at 37°C

with gentle agitation until dry. The same procedure was repeated using the same volumes of poly-HEMA solution. Wells were then washed with 1 ml of sterile PBS and stored at 4°C.

2.1.7 Anoikis assay

Cells were grown to 70% confluence, detached from tissue culture flask by incubation with a trypsin-EDTA and recovered as described in section 2.1.2. Cells were counted with haemocytometer and diluted to the required density in the experimental medium. For experiments to test the protective effect of IGF-1 and EGF, cells were resuspended in phenol red-free DMEM medium without serum (SFM) supplemented with IGF-1 concentration of 0.5 to 50 ng/ml and EGF at concentration of 0.1 to 50 ng/ml in 0.01% bovine serum albumin (BSA) or with 0.01% BSA alone. For 12 well plates, the cell suspension was diluted to a density of 15×10^4 to 20×10^4 cells/ml and 2 ml of cell suspension were added to each well. MDA-MB-231 cells incubated for 24 hours prior to protein extraction with RIPA buffer plus inhibitors as described in Section 2.2.1, or processed for analysis with the flow cytometer (ImageStream X Mack II-Amnis) as described in Section 2.6. Hs578T and SK-BR-3 cells were incubated for 6 hours prior to protein extraction.

For experiments designed to investigate the effects of monoclonal antibody against the type I IGF receptor CP-751, 871 or dual tyrosine kinase inhibitor BMS-754807, on anti-anoikis effect of IGF-1 in SFM, cells were grown to approximately 70% confluence, following detachment and recovery as described in section 2.1.2, cells were re-suspended in SFM and counted using a haemocytometer. The cell suspension was diluted to a density of a 70×10^4 cells/ml in SFM supplemented with experimental inhibitors or the solvent, and cells were incubated for 30 minutes. The cell suspension then diluted to a density of 10×10^4 cells/ml in the SFM in the absence or presence of CP751, 871 at concentration ranging from 1-10 μ g/ml or BMS-754807 at concentration ranging from 1 to 10 μ M, and in the absence or presence of IGF-1 at concentration of 50 ng/ml. Cells were seeded into a fresh 12 well Poly-HEMA coated plate at density of 20×10^4 cells/well in 2 ml of experimental medium and incubated for different periods of time, depending on the cell lines under investigation as described above, prior to protein extraction with RIPA buffer plus inhibitors as described in section 2.2.1.

For experiment designed to investigate the effect of antibody against type I IGF receptor CP-751871, on anti-anoikis effect of IGF-1 in MDA-MB-231 cells, cells were cultured into two T 25 cm² tissue culture flasks and incubated in the DCCS medium supplemented with CP-751, 871 or IgG₂ antibody (Sigma 15404) at concentration of 10 µg/ml for 24 hours. Cells were detached and recovered as described in section 2.1.2 and were re-suspended in DCCS medium and counted in a haemocytometer. Cells were added into poly-HEMA coated plate at a density of 20 x 10⁴ cells/well and incubated for 24 hours, prior to protein extraction.

For experiments designed to investigate the effect of EGFR inhibitor gefitinib or dual tyrosine kinase inhibitor lapatinib on anti-anoikis effect of EGF, following cell detachment and recovery, cells were re-suspended in SFM and counted in a haemocytometer. The cell suspension was diluted to a density of a 70 X 10⁴ cells/ml in SFM supplemented with experimental inhibitors or the solvent, and cells were incubated for 30 minutes. The cell suspension then diluted to a density of 10 x 10⁴ cells/ml in the SFM in the absence or presence of experimental inhibitors at concentration ranging from 1- 10 µM and in the absence or presence of EGF at concentration of 50 ng/ml. Cells were seeded into a fresh 12 well poly-HEMA coated plate at density of 20 x 10⁴ cells/well in 2 ml of experimental medium and incubated for different periods of time, depending on the cell lines under investigation as described above, prior to protein extraction with RIPA buffer plus inhibitors as described in section 2.2.1.

2.2 Protein extraction and quantification

2.2.1 Cell lysates and protein extraction in RIPA buffer

For protein extraction from attached cells, wells were washed with cold PBS and then lysed with radioimmunoprecipitation (RIPA) buffer which contains 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40 (v/v) and 0.25% sodium deoxycholate (w/v). Protease and phosphatase inhibitors were added to give final concentrations of 1 µg/ml Aprotinin, 1 µg/ml Pepstatin, 1 µg/ml leupeptin, 2mM Orthovanadate, 2mM sodium Fluoride, 2 mM phenyl methyl sulphonyl fluoride (PMSF). For 12 well plate 80 to 120 µl per well of RIPA buffer plus inhibitors was used per well. For 6 well plates 200 to 250 µl of RIPA buffer plus inhibitors per well was used. For tissue culture T75cm² flask 1 ml of RIPA buffer plus inhibitors were used per flask. After addition of lysis buffer, cells

were kept on the ice with gentle agitation for 30 minutes. The cell lysates were collected to Eppendorf tubes and clarified from debris by centrifugation at 1400 rpm (Hettich Mikro 22R) for 10 minutes. The supernatants were transferred to the freshly labelled tubes and stored in -20°C.

For protein extraction from non-attached cells cultured in poly-HEMA coated plates, the cells and medium were collected into the 15 ml falcon tubes (Greiner). Each well was washed with 1 ml of cold PBS by pipetting gently over the surface of the well to maximise the cell collection and added to the cell suspension. Cell suspension was centrifuged at 2,500 rpm (Beckman Allegra X-12R SX 4750 rotor) for 5 minutes at room temperature. The supernatant was removed and cells were lysed with 50 to 70 µl of RIPA buffer plus inhibitors.

2.2.2 Measurement of protein concentration by bicinchoninic acid (BCA) protein assay

Protein concentration of cell lysates were measured with a bicinchoninic acid assay (BCA: Pierce), which is a colorimetric assay for quantification and identification of total proteins. This method is the Biuret reaction in which Cu^{+2} ions in the BCA reagent are reduced to Cu^{+} . The purple colour produced as a result of the chelating of two molecules of BCA with one molecule of Cu^{+} ion, which show the strong absorbance at 562 nm. Aliquots of 0.5 µl of lysates were diluted in 4.5 µl of deionized water. Protein standards are ranging from concentration of 0, 0.025, 0.05, 0.1, 0.2, 0.5, 1, 2 mg/ml were prepared from bovine serum albumin (Sigma) in RIPA buffer that had been diluted in 1 in 10 dilution of sterile deionised water. BCA working reagent contained 50 parts of reagent A and 1 part of reagent B. five µl of each known standard solutions and each known samples were mixed with 95 µl of the BCA working solution and incubated at 37°C for 30 minutes and kept in ice to stop the reactions and avoid further development of the colour. The optical density of the samples was measured with spectrophotometer (Beckman DU 640) at the 562 nm wavelength, the protein concentration of unknown samples were calculated on the basis of BSA standard curve.

2.3 Western transfer analysis

2.3.1 Protein gel electrophoresis

Protein samples were separated by sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis. The acrylamide to bisacrylamide ratios were 200:1 for the separating gel and 20:1 for the stacking gel. The separating gels contained 12% acrylamide, 0.37 M Tris-HCl pH 8.8 and 0.1% sodium dodecyl sulphates (SDS), 0.05% ammonium persulphate (w/v) and 0.1% N, N, N', N'-tetramethylethylenediamine (TEMED) (v/v). The stacking gel contained 3% acrylamide, 125 mM Tris-HCl pH 6.8 and 0.1% SDS (w/v), 0.1% of ammonium persulphate (w/v) and 0.5% TEMED. Gels were cast in vertical slab minigel apparatus (Hoeffer) and allowed to polymerise, 45 minutes for the separating gels and for the stacking gel 15 to 20 minutes. Separating gels of 20% acrylamide and stacking gel containing 5% of acrylamide were used for trefoil protein analysis because of their small molecular mass.

Samples aliquots containing equal amount of protein were mixed with 10 µl aliquots of 0.125 M Tris-HCl, 25 mM Na₂ EDTA, 4% SDS (w/v), 20% glycerol, 0.01% bromophenol blue and 10% β-mercaptoethanol, pH 6.8 (2xSDS mix) and kept for boiling for at least 10 minutes in 95°C heat block. Full range molecular weight marker (Amersham Rainbow markers) was loaded in a separate lane alongside with the protein samples in order to estimate the molecular mass of the analysed proteins. The gels were electrophoresed in 0.38 M glycine, 0.05 M Tris-HCl pH 8.5, and 0.1% SDS running buffer at a constant current of 10 mA per gel.

2.3.2 Western transfer

Proteins were transferred from the gel to Westran 0.45 µM nitrocellulose membrane (VWR) or Westran 0.2 µM polyvinylidene difluoride (PVDF) membrane using semi-dry transfer apparatus (S&S CarboGlas, Peqlab PerfectBlue). The transfer sandwich comprised of two pieces of 3MM filter papers were soaked in 0.3 M Tris-HCl pH 10.4, 20% methanol (anode 1) and placed on the glass plate of the apparatus. Another piece of filter paper soaked in 25 mM Tris-HCl pH 10.4, 20% methanol (anode 2) was placed on top of the two sheets. A sheet of PDVF membrane that had been pre-wet in methanol and equilibrated in anode buffer 2 or a sheet of nitrocellulose membrane that pre-wet in sterile distilled water and equilibrated in anode 2 buffer was placed on top of

this assembly. The gel was placed on top of the membrane and covered with the other remaining three sheets of 3MM filter paper soaked in 25 mM Tris-HCl, 40 mM 6-amino-n-hexanoic acid pH 9.4, 20% methanol (cathode solution). Proteins were transferred from the gel into the membrane with current of 100 mA for 10 to 45 minutes dependent upon the molecular mass of the protein. The membranes were dried overnight.

PVDF membranes were pre-wet in methanol and fix in 0.2% glutaraldehyde for 45 minutes at room temperature on an orbital shaker. The nitrocellulose or PVDF membranes were washed thrice with 20mM Tris-HCl pH 7.6 and 137 mM NaCl containing 1% Tween 20 (TBS-Tween) each time for 5 minutes and blocked in 5% skimmed milk in TBST for 60 minutes at room temperature. The membranes were washed thrice each time in 5 minutes in TBST solution and were incubated with primary antibodies in 5% milk- TBST overnight at 4°C. Membranes were rinse with TBS-Tween and washed a further three times for 5 minutes in TBS-Tween. Membranes were then incubated in 50 ml tubes with horse-radish peroxide (HRP) conjugated secondary antibodies diluted in 5% milk-TBS-Tween for 60 minutes at room temperature. Membranes were washed thrice for 5 minutes with TBS-Tween and then in a final wash with 1 x TBS before development.

For filter development and visualise the proteins the membranes were incubated with Supersignal West Dura extended duration substrate chemiluminescent solutions Luminol/Enhancer and Peroxide buffer in 1:1 ratio for 5 min and exposed to X-ray film (Fuji SuperRX). The films were developed using the development machine. Exposure times ranged from 1 second to overnight.

The antibodies used for Western transfer analysis are listed in Table 2.2.

| Antibody | Source | Company | Catalogue number |
|--|--------|-----------------|------------------|
| AKT | Rabbit | Cell Signalling | 9227 |
| phospho-AKT (Ser 473) | Rabbit | Cell Signalling | 4060 |
| EGFR | Rabbit | Cell Signalling | 4267F |
| phospho-EGFR | Rabbit | Cell Signalling | 3777S |
| ERK 1 and 2 | Rabbit | Cell Signalling | 2933 |
| phospho-ERK 1 and 2 (MAPK)* (Thr 202/Tyr 204) | Rabbit | Cell Signalling | 9102 |
| GAPDH (HRP-conjugated) | Rabbit | Santa Cruz | Sc-25778 HRP |
| HER-2 | Rabbit | Cell Signalling | 4290P |
| phospho-HER-2 | Rabbit | Cell Signalling | 2243S |
| HER-3 | Rabbit | Santa Cruz | Sc-285 |
| phospho-HER-3 | Rabbit | Cell signalling | 4784p |
| IGF-IR | Rabbit | Cell Signalling | 9323 |
| phospho-IGF-IR/IR (Tyr 1135/1136) | Rabbit | Cell Signalling | 3027 |
| Cleaved PARP (Asp 214) | Rabbit | Cell Signalling | 9541 |

Table 2.2: List of antibodies. Source, manufacturers of the primary antibodies used in Western transfer analysis. *All the figure references to “MAPK” in the results chapters refer specifically to ERK 1 and 2.

2.4 *In vitro* wounding assay

Cells were plated at 2.5×10^5 cells/well in 24-well plates, and incubated for 24 hours to allow them to attach. The 24-well plates in this assay had been scored by two parallel lines on the underside of the plate across the middle of the each well. After 24 hours of incubation cells were withdrawn and incubated for another 24 hours. The cell monolayer in each well was wounded with a p20-200 Gilson pipette tip through centre of the well perpendicular to the scored lines. Cells were washed once with phenol red-free DMEM, incubated with phenol red-free DMEM and 0.01% BSA for 2 hours. After 2 hours of incubation, cells were washed once with phenol red-free DMEM and medium replaced with the

phenol red-free DMEM and 0.01% of BSA in the presence and absence of growth factors in triplicate. Images were captured at x100 magnification with digital camera from each wound after 0, 2, 4, 6 hours of time points. Image J software was used to make a 20 measurements of the width of each wound at the site of each score line and the mean width was calculated. The migration of the cells at each edge was calculated by subtraction of wound width at a time point from the wound width at the start of the experiment and then division of the width by 2. The mean movement of the cells is then calculated from the 2 points in 3 wells for each condition.

2.5 Immunofluorescence

Glass coverslips (22 x 22mm) were immersed into a petridish containing 100% ethanol to sterilise. The sterilized coverslips were placed in 6-well plate by using the needle and forceps. Cells were plated at a density of $4-6 \times 10^5$ in per well in 2 ml in 6 well plates. Cells were incubated for 24 to 48 hours to allow the cells to attach. Cells were washed with PBS and fixed in methanol precooled to -20°C or paraformaldehyde at room temperature for 20 minutes. After fixation, cells were washed twice with PBS each time for 20 minutes. Cells were blocked in 5% goat serum, 1 x PBS and 0.3% Triton X-100 for 1 hour at room temperature. Cells were incubated with primary antibodies at overnight at 4°C (Table 2.3). The next day, coverslip were washed thrice with PBS, each time for 15 minutes and incubated with secondary antibody (Alexa fluor 488 conjugated anti-mouse IgG, A-11001, Invitrogen) at a dilution of 1:1000 for 1 to 2 hours at room temperature. The plates were covered with foil due to light sensitivity of the fluorophores. After incubation, cells were washed with PBS thrice each for 15 minutes whilst covered with foil. Phalloidin (Alexa fluor 555) which stains the cytoskeleton by interaction with F-actin was added 15 minutes prior to addition of the mounting medium DAPI. Mounting medium DAPI (H-1200, Vector laboratories UK) which stain the DNA in the cell nuclei was added to the glass slides before the coverslips were inverted into the glass slides. The cells were examined under the confocal microscope (Zeiss LSM).

| Antibody | Source | Manufacturer | Dilution |
|-------------|--------|--------------------------|----------|
| TFF1 | Mouse | Raised in our laboratory | 1 : 10 |
| TFF3 | Mouse | Raised in our laboratory | 1 : 20 |

Table 2.3: List of antibodies. Source, manufacturer and dilution of the primary antibodies used for immunofluorescence.

2.6 Imagestream Flow cytometry

To evaluate cell death in non-attached condition, cells that had undergone anoikis as described in section 2.1.7 were collected by centrifugation at 2500 rpm for 5 minutes. The cells were incubated in 1% formaldehyde for 20 minutes at room temperature, permeabilized in 0.3% saponin for 1 hour at room temperature and blocked with 5% goat serum and 0.3% saponin for another 1 hour at room temperature. Cells were washed with 500 µl of PBS and stained with anti-caspase-3 antibody and Draq5TM (Biostatus, UK) dye, which stains the double- stranded DNA in the nuclei overnight at 4°C (Table 2.4). After overnight incubation cells were washed with PBS and centrifuged at 400 g for 10 minutes and re-suspended in 60 µl of PBS. Images from a total of 1,000 cells from each sample were collected with the ImageStreamX (Amnis Crop.) and analysed with IDEAS 4.0 Software. The intensity of caspase-3 staining for all cells which is sum of the pixel values for each individual cell was displayed in a histogram. The threshold for positivity was determined using visual inspection of the corresponding images. All cells above this threshold were gated as being caspase-3 positive.

| Antibody | Source | Manufacturer | Dilution |
|---|--------|-----------------|----------|
| Cleaved Caspase-3 (Asp 175) Alexa ®488 | Rabbit | Cell signalling | 1 : 100 |

Table 2.4: List of antibodies. Source, manufacturer and dilution of antibodies used for flow cytometry.

2.7 Expression of IGF-IR, EGFR, HER-2 and trefoil proteins in breast cancer tissue

2.7.1 Patients and samples

Ethical permission for the work described in this thesis was obtained from the Joint Newcastle Health Hospitals and University of Newcastle upon Tyne Ethical Committee. Formalin-fixed, paraffin-embedded tissue blocks from 93 breast cancer patients were included in this study. All patients were diagnosed with breast cancer at the Pathology Department of the Royal Victoria Infirmary of the Newcastle Hospitals NHS Trust between 2000 to 2005. Ninety-two of the samples were surgical resection specimens and one was core biopsy specimen. The lesions investigated included 37 *in situ* breast carcinomas, 79 invasive tumours. In addition 7 samples of normal breast tissues distant from tumours and lymph nodes tumour deposits from 26 patients were studied.

2.7.2 Construction of tissue microarray (TMA)

The original haematoxylin and eosin (H&E) stained sections of breast tissue were reviewed with the related formalin-fixed, paraffin-embedded tissue blocks that represent normal breast tissue, *in situ* carcinoma and invasive breast tumours. Duplicate cores of 1 millimetre in diameter size were punched from the donor block and inserted into the recipient block with Galileo TMA CK 3500 machine. Each TMA block contained 84 cores of breast lesions, a core of mouse liver was included to allow the orientation of each block, a core of normal human liver served as reference for IGF-IR immuno-reaction, a core of normal human kidney for EGFR immuno-reactivity, a core of breast tumour known to be HER-2 positive for HER-2 immuno-reactivity, a core of normal human stomach served as reference for TFF1 immuno-reaction and a core of normal human ileum serve as reference for TFF3 immuno-reaction (Figure 2.1). The TMA blocks were incubated for three cycles of heating in a 37°C incubator for one hour and cooling down to room temperature for 30 minutes to congeal the tissue cores inserted into the paraffin blocks. Duplicate blocks were prepared from each group of cases. Section of 4 µm thick were prepared from each TMA block, loaded on superfrost glass slides and incubated at 37°C for at least two hours.

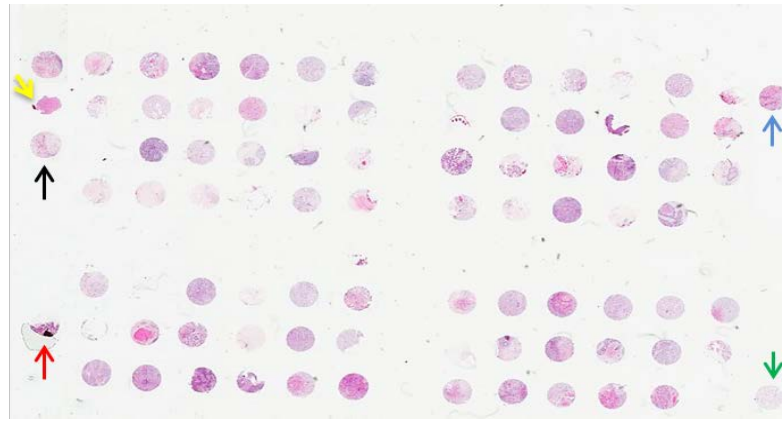


Figure 2.1: Construction of tissue microarray block. One millimetre cores from 84 oestrogens receptor-negative breast tumours were selected and inserted into the recipient TMA block. A core from mouse liver (top left) included to allow the correct orientation of the TMA block, core from HER-2 positive breast tumour (yellow arrow), core from normal human liver (black arrow), normal human kidney (blue arrow), normal stomach (red arrow) and normal ileum (green arrow) provided references for the HER-2, IGF-IR, EGFR, TFF1 and TFF3 immuno-reactivity. A 4 μ m sections stained with haematoxylin and eosin is shown. Magnification is x20.

2.7.3 Haematoxylin and eosin staining

TMA sections was de waxed in xylene for 5 minutes and rehydrated through graded alcohols of absolute, 95% and 70% ethanol and rinsed in tap water. The sections were stained with Harris haematoxylin for 5 minutes, rinsed in running tap water and the colour of the stain was cleared by dipping the slide twice in acid alcohol followed by a rinse in tap water. The sections were then stained with eosin for 30 seconds, washed in running water, dehydrated by dipping in graded alcohol, clear in xylene and mounted with DPX.

2.7.4 Immunohistochemistry

The expression of IGF-IR, EGFR, HER-2, TFF1 and TFF3 proteins in different breast lesions and the association between expression of these proteins and expression of other key markers of proliferation and apoptosis was analysed in the breast samples included in the constructed tissue microarray. The antibodies, their source and the concentrations optimised of immunohistochemistry are shown in Table 2.5.

| Antibody | Source | Manufacturer | Dilution | Incubation time | Catalogue number |
|--------------------------|--------|--------------------------|----------|----------------------------|------------------|
| IGF-IR | Rabbit | Cell signalling | 1:2400 | Overnight at 4 °C | 3027S |
| EGFR | Rabbit | Cell signalling | 1:50 | Overnight at 4 °C | 4267S |
| TFF1 | Mouse | Raised in our laboratory | 1:10 | 60 min at room temperature | - |
| TFF3 | Mouse | Raised in our laboratory | 1:20 | 60 min at room temperature | - |
| HER-2 | Rabbit | Cell signalling | 1:400 | Overnight at 4 °C | 2165S |
| Ki-67 | Mouse | Dako | 1:400 | 60 min at room temperature | M7240 |
| Cleaved-caspase-3 | Rabbit | Cell signalling | 1:300 | Overnight at 4 °C | 9661S |

Table 2.5: List of antibodies. Source, manufacturer, dilution and targets of the antibodies used for immunohistochemistry.

After deparafinization and rehydration as described in section 2.8.3, TMA sections were incubated in 3% hydrogen peroxide for 10 minutes to block the endogenous peroxidase activity and then washed in running tap water for 5 minutes. Antigen retrieval was in 10mM citrate buffer, pH 6.0 with either using a pressure cooker (Decloaking chamber, Biocare medical) at 125°C for 45 minutes. Following antigen retrieval the tissues were left to cool down for 10 minutes and washed 2 x 5 minutes in Tris buffered saline pH 7.6 contain 1% Tween 20 (TBS/Tween) solution. The tissues were incubated with appropriate mouse or rabbit primary antibodies diluted in TBS appropriated duration (Table 1.2). The sections were rinsed in TBS/Tween for 2 x 5 minutes while shaking at room temperature. The sections reacted with rabbit antibody were probed with horse radish peroxidise (HRP-Polymer) for 30 minutes and rinsed in TBS/Tween for 2 x 5 minutes. Whereas, sections reacted with mouse antibodies were incubated with the Universal Probes (X-Cell Plus Polymer HRP detection kit) for 30 minutes, and washed two times in TBST/Tween for 5 minutes. All sections were rinsed in running tap water. For development of the

colour, 3, 3' Diaminobenzidine (DAB) solution was added to the tissues for 5 minutes. The sections were rinsed in running water and counterstained in Gill II haematoxylin for 10 seconds. Sections were washed with water, dipped in Scott tap water to clear the colour and dehydrated through graded alcohols, cleared in xylene and mounted in distrene phthalein xylol (DPX).

2.7.5 Evaluation of immunohistochemistry

2.7.5.1 Evaluation of IGF-IR, EGFR and HER-2 immuno-reactivity

Thousand cells were assessed in each case. The intensity of immuno-reaction in each cell was scored as 0, 1, 3 and 10 corresponding to negative, weak, intermediate and strong membranous IGF-IR, EGFR and HER-2 expression respectively. For each case, the number of negative, weak, moderately-positive and strongly-positive immuno-reactive cells was counted. The final score is obtained with the following equation: 1 x percentage of weakly stained cells + 3 x percentage of moderately stained cells + 10 x percentage of strongly stained cells. A total score between 0 and 1000 was obtained where 0 occurs when all cells are negative and 1000 occurs when all cells are strongly-positive (Figure 2.2). The final score for each tumour was the mean values of duplicate cores (Ahmed, Griffiths et al. 2012).

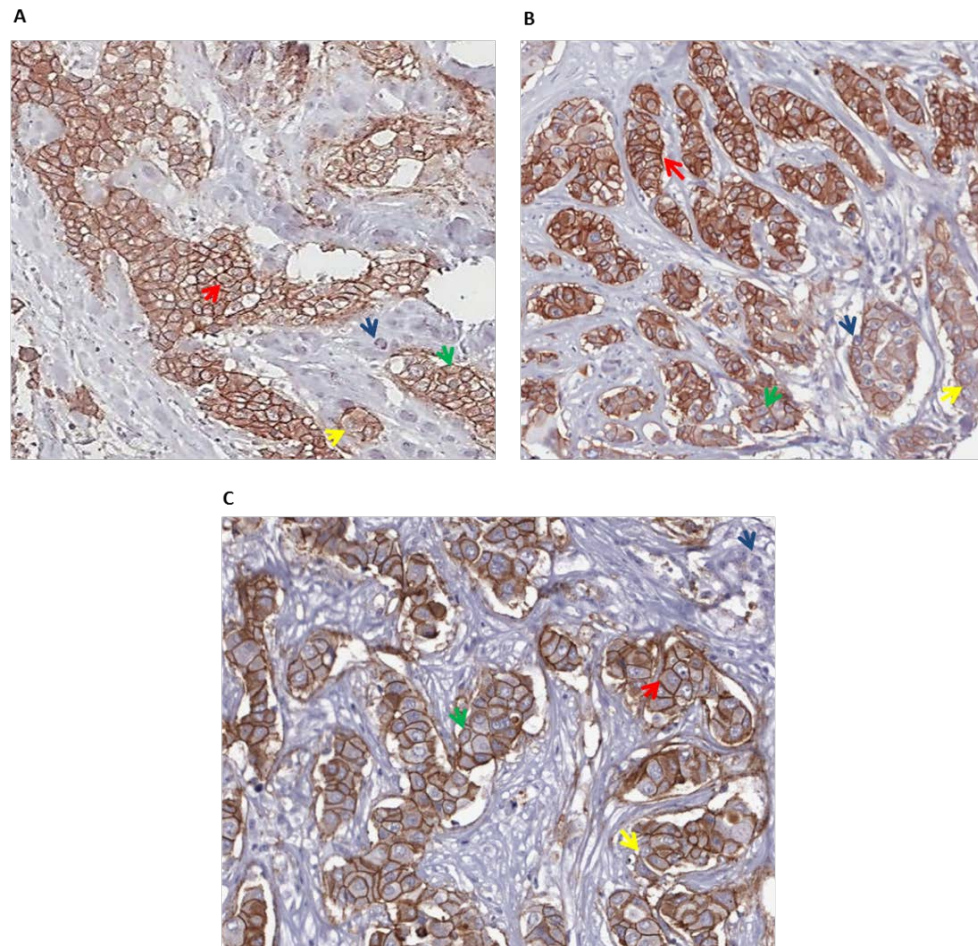


Figure 2.2: Intensity of immuno-reaction to IGF-IR, EGFR and HER-2 in breast cancer cells. Sections of breast cancer were tested for expression of IGF-IR (A) EGFR (B) and HER-2 (C) by immunohistochemistry. Tumour cells were negative (blue arrows), weakly (yellow arrows), moderately (green arrows) or strongly (red arrows) immuno-reactive. Magnification is x200.

2.7.5.2 Evaluation of TFF1, TFF3, Ki-67 and cleaved caspase-3 immuno-reactivity

The immunohistochemical expression of TFF1, TFF3, proliferation marker Ki-67 and apoptotic marker cleaved caspase-3 in breast cancer tissue was assessed without knowledge of IGF-IR, EGFR or HER-2 expression. Duplicate cores with an average of 1000 tumour cells were analysed for each tumour. The immuno-reactivity of the proteins was evaluated on the basis of the histoscore that was described in previous section (Section 2.7.5.1). For TFF1, TFF3 and cleaved caspase-3 cytoplasmic immuno-reactivity was evaluated. For Ki-67 nuclear immuno-reactivity was evaluated. The final score for each tumour was the mean values of duplicate cores.

2.7.6 Statistical analysis

All statistical analyses used the SPSS 21 software package (SPSS, Inc, Illinois, Chicago, USA). Duplicate samples were tested for each case and the mean histoscores were used for the statistical analyses. The data were analysed by non-parametric tests in which the cases are ranked according to the expression level of the tested protein and the mean ranks were used for the statistical analyses. The association between expression of the biomarkers and various clinical and pathological variables was analysed. Correlations between two continuous variables was tested with Pearson Rank Correlation test while correlation between a continuous variable and a categorical variable was tested with Spearman`s rho Correlation test. The distribution of expression of each biomarker amongst two independent groups was compared with the Mann-Whitney test and amongst more than two groups with the Kruskal-Wallis test. Wilcoxon signed rank test was used for paired variables to compare the expression of the biomarkers in invasive tumours. The frequency of occurrences between two variables was evaluated by Crosstabs in descriptive statistics.

Chapter 3. The effect of insulin-like growth factor-1 on anoikis and migration of oestrogen receptor-negative breast cancer cells

3.1 Introduction

Survival of mammary epithelial cells is affected by their attachment to the neighbouring epithelial and myoepithelial cells which secrete basement membrane proteins. Attachment of cells to the extracellular matrix is mediated by transmembrane cell surface receptors called integrins and activates important components of downstream cell survival signals namely, Ras mitogen activated protein kinase (MAPK) pathway and phosphatidylinositol 3-kinase (PI3K) AKT pathway (Kim, Koo et al. 2012, Taddei, Giannoni et al. 2012). Inappropriate attachment or loss of adhesion of the cells to extracellular matrix leads to cell death. This particular type of cell death is called anoikis (Frisch and Francis 1994). Anoikis is caspase-dependant cell death, as cell detachment triggers activation of caspase-8, which in turn activates caspase-7 and subsequently caspase-3 that result in cleavage of its substrates and cell death (Taddei, Giannoni et al. 2012, Simpson, Anyiwe et al. 2008).

An *in vitro* assay to measure anoikis was first introduced by Folkman and Moscona (1978). In 1994, Frisch *et al* described the induction of anoikis by culturing the epithelial cells in plates coated with the inert hydrogel substance called poly-HEMA, which prevents cells attachment (Frisch and Francis 1994). Nuclear fragmentations and blebbing of the membrane are features that occur during anoikis. In normal conditions, detached epithelial cells are eliminated through anoikis to inhibit their reattachment to the new incorrect matrix, hence preventing dysplastic growth and proliferation of the cells (Kim, Koo et al. 2012, Taddei, Giannoni et al. 2012). Anoikis has a significant role in the physiological induction of cell death to mediate tissue haemostasis and developmental processes (Frisch and Screaton 2001, Grossmann 2002, Kim, Koo et al. 2012). The protective role of IGF-1 against staurosporine-induced cell death in triple-negative breast cancer cells has been reported (Davison, de Blacquiére et al. 2011), but the role of IGF-1 on anoikis in oestrogen receptor-negative breast cancer cells has not been reported before.

Breakdown of anoikis contributes to neoplasia and eventually tumour metastasis. Resistance of tumour cells to anoikis is one of the main characteristics of malignancy development in which cells tend to survive in anchorage independent conditions and gain the ability to migrate to distant sites throughout the body (Valentinis, Reiss et al. 1998, Kim, Koo et al. 2012). Metastasis which is dissemination of cancer cells from the primary site to distant organs is the initial step in migration and invasion of cancerous cells into surrounding tissues (Yamaguchi, Wyckoff et al. 2005). Cancerous cells move as a group or single cells, in the form of an elongated or an amoeboid shape (Brooks, Lomax-Browne et al. 2010). Tumour cells use different mechanisms of migration which are similar to the migration mechanism that occur in normal, non-neoplastic cells and spread within the tissues (Karp, Pollak et al. 2009). Invasive carcinoma cells gain a migratory phenotype that is associated with involvement of various genes products associated with cell motility. The malignant cells respond to signals from the microenvironment that cause tumour invasion (Wang, Goswami et al. 2004). Identification of molecules involved in migration of cancerous cells could be important in anti-metastasis targeted therapy. IGF-1 has been reported to play an important role in metastasis of oestrogen receptor-negative MDA-MB-435 breast cancer cells (Tang, Su et al. 2008).

The aims in this chapter are to investigate the effect of IGF-1 on anoikis in oestrogen non-responsive MDA-MB-231, Hs578T, and SK-BR-3 breast cancer cells. The responsiveness of components of the IGF signal transduction pathway to IGF-1 was tested in a panel of oestrogen non-responsive breast cancer cell lines (Table 3.1). The role of the type I IGF receptor in IGF-1 mediated protection from anoikis was investigated with specific inhibitor of the type I IGF receptor. In addition, the effect of IGF-1 on migration of oestrogen receptor-negative breast cancer cells was studied and the inhibitory role of an antibody against IGF-IR and a dual IGF-IR and insulin receptor tyrosine kinase inhibitor on migration of the cells were evaluated.

| Cell lines | ER status | PgR status | HER-2 status | IGF-IR status |
|------------|-----------|------------|--------------|---------------|
| MCF-7 | + | + | - | + |
| BT-474 | + | + | + | + |
| EFM-19 | + | + | + | + |
| SK-BR-3 | - | - | + | + |
| Hs578T | - | - | - | + |
| MDA-MB-231 | - | - | - | + |
| HCC-2218 | - | - | + | + |
| HCC-1419 | - | - | + | + |

Table 3.1: Characteristics of different breast cancer cell lines.

3.2 Results

3.2.1 Expression of the type I IGF receptor in different breast cancer cell subtypes

The response of cells to stimulation by IGF-1 is required expression of the type I IGF receptor family member and downstream signal transduction proteins in the cells. IGF ligands transduce their signals through type I IGF receptor.

Interaction of IGF-1 with the type I IGF receptor is considered as the main mechanism in activation of IGF downstream signal transduction pathway. This event leads to activation of two main downstream signalling cascades: Ras mitogen activated protein kinase (MAPK) pathway and phosphatidylinositol 3-kinase (PI3K) and AKT pathway which play important roles in mediating the cell survival and proliferation.

To evaluate whether IGF signal transduction pathways might be important in oestrogen receptor-negative breast cancer cells, the expression of the type I IGF receptor was compared in oestrogen-responsive breast cancer cells.

The relative expression of the type I IGF receptor was evaluated in oestrogen-responsive MCF-7, BT-474, EFM-19 and oestrogen non-responsive SK-BR-3, Hs578T, MDA-MB-231, HCC-2218 and HCC-1419 breast cancer cell lines by western transfer analysis. Breast cancer cell lines were cultured in maintenance medium, their proteins extracted and analysed for the relative expression of the type I IGF receptor by western transfer analysis (Figure 3.1).

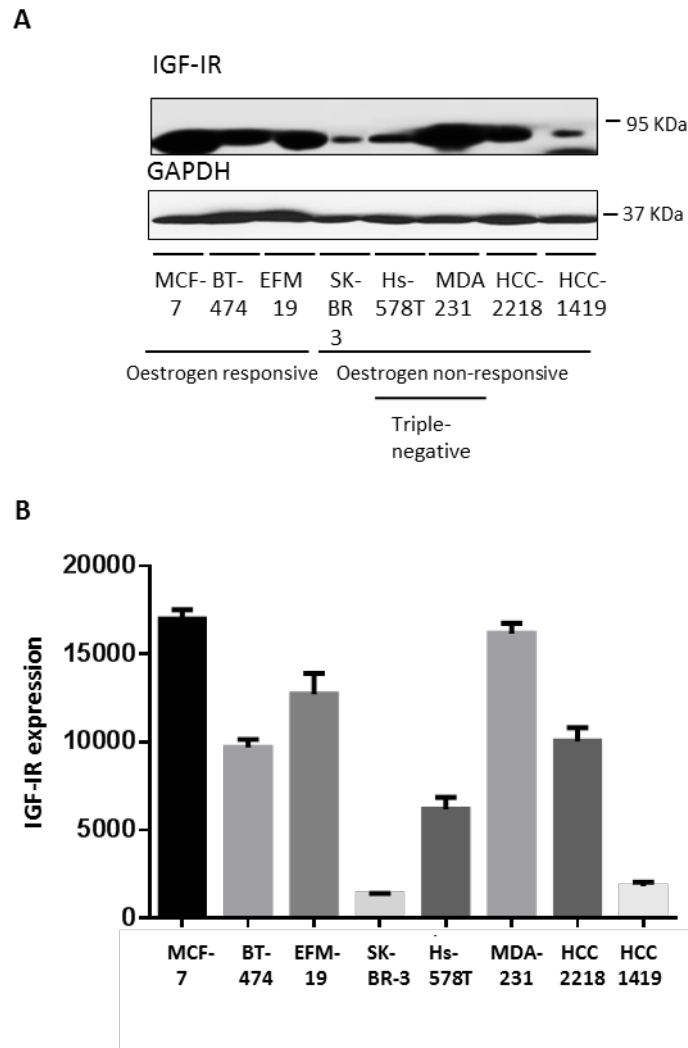


Figure 3.1: Expression of type I IGF receptor in different types of breast cancer cell line. MCF-7, BT-474, EFM-19, SK-BR-3, Hs578T, MDA-MB-231 (MDA-231), HCC-2218 and HCC-1419 cells were cultured to approximately 80% confluence and their proteins extracted with 1 ml of RIPA buffer per 75 cm² tissue culture flask as described in Material and Methods Section. Aliquots containing 20 µg of extracted proteins were electrophoresed on a 12% denaturing polyacrylamide gel and transferred to a nitrocellulose membrane at 100 mA for 45 minutes. Membrane was incubated with IGF-IR antibody (1:1000 dilution) and GAPDH (1:20,000 dilution) **(A)**. Densitometric scanning of X-ray films determined the amount of each protein by analysis with LabWork 4.0 software. The mean \pm SEM after normalisation to GAPDH expression are shown **(B)**.

The type I IGF receptor was expressed at relatively high levels in oestrogen receptor-positive breast cancer cells. Amongst oestrogen-responsive breast cancer cells, MCF-7 cells expressed the highest level of type I IGF receptor and BT-474 cells expressed the lowest level of type I IGF receptor. EFM-19 cells expressed relatively high levels of type I IGF receptor, at a lower level than MCF-7 cells and at a higher level than BT-474 cells. Amongst oestrogen non-responsive cells, triple-negative MDA-MB-231 cells expressed high levels of type I IGF receptor. Hs578T cells expressed the type I IGF receptor at much

lower levels than MDA-MB-231 cells. Amongst HER-2 positive breast cancer cells, SK-BR-3 and HCC-1419 cells expressed relatively low levels of type I IGF receptor which suggests the possibility that these cells might be less responsive to IGF-1 compared to other cell lines. HCC-2218 expressed relatively high levels of the type I IGF receptor. The results demonstrate the considerable variation in expression of the type I IGF receptor in oestrogen receptor-negative breast cancer cell lines.

3.2.2 Induction of anoikis in oestrogen non-responsive breast cancer cells

A robust method was required to create the conditions under which cells would be induced to undergo anoikis, in order to investigate the effect of IGF-1 on anoikis. Anoikis was induced by coating the tissue culture plates with poly (2-hydroxyethyl methacrylate) (poly-HEMA), which is an inert hydrogel that inhibit the cell attachment. Cells undergo morphological changes during the anoikis process. Non-attached cells are morphologically round with the appearance of membrane blebbing, whereas attached cells are morphologically flattened polygonal shapes and proliferate as a monolayer sheets (Figure 3.2).

3.2.2.1 Induction of anoikis in triple-negative breast cancer cells

MDA-MB-231 and Hs578T cells were seeded in poly-HEMA coated plates and cultured in serum-free medium for different lengths of time up to 24 hours. Proteins were extracted after each particular time and the extent of cell death measured by analysing caspase substrate and marker cleaved PARP (Figure 3.2).

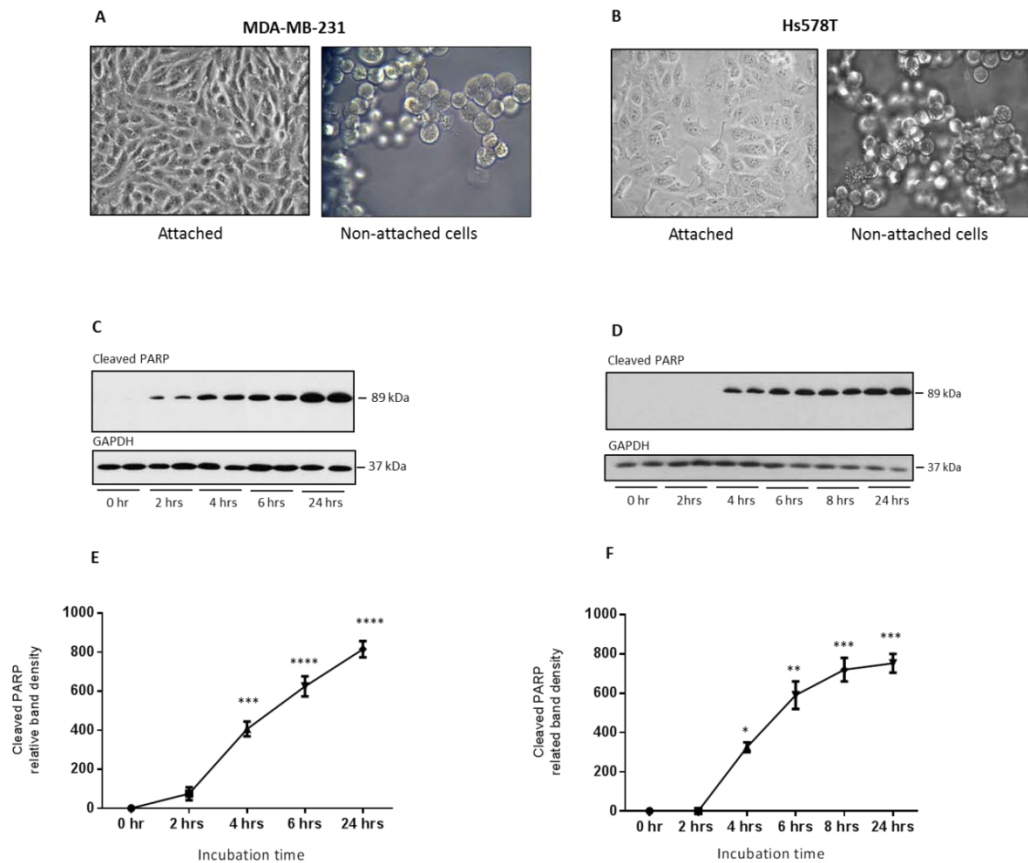


Figure 3.2: Induction of anoikis in triple-negative breast cancer cell lines. MDA-MB-231 (A, C, E) and Hs578T (B, D, F) cells were cultured in phenol red free Dulbecco's Modified Eagle's medium (DMEM) without serum (serum-free medium) in non-attached condition. Photographs of cells in both attached and non-attached condition are shown. Cells (A, B) were seeded in 12-well poly-HEMA coated plates at the density of 20×10^4 cells/well in 2 ml of serum-free medium and cultured for various length of time up to 24 hours. Cells were lysed with 50 μ l of RIPA buffer after each specific time point and proteins were extracted. Aliquots of 10 μ l protein were electrophoresed on 12% denaturing polyacrylamide gels and transferred to nitrocellulose membrane at 100 mA for 45 minutes. This followed by incubating the membrane with cleaved PARP antibody (1:2000 dilution) and GAPDH (1:20,000 dilution). The illustrated figures are representative of bands from one experiment in triplicate that have been replicated at least twice (C, D). Densitometric scanning of X-ray films determined the amount of each protein by analysis with LabWork 4.0 software. The mean \pm SEM after normalisation to GAPDH expression are shown (E, F). Asterisks indicates the amount of cleaved PARP was statistically higher than in the original cells (One way ANOVA, * $p < 0.05$, ** $p < 0.001$, *** $p = 0.0001$, **** $p < 0.0001$).

In MDA-MB-231 cells, cleaved PARP was detected after 2 hours of incubation in non-attached conditions in serum-free medium. Greater amounts of cleaved PARP were gradually detected as the incubation time increased. In Hs578T cells, cleaved PARP was detected first after incubation for 4 hours and greater amounts of cleaved PARP were observed after 24 hours. Based on the results, both MDA-MB-231 and Hs578T cells appear to undergo detectable levels of anoikis following culture in non-attached conditions in serum-free medium for 24 hours.

3.2.2.2 Induction of anoikis in HER-2 receptor positive breast cancer cells

To induce anoikis, SK-BR-3 cells were cultured in non-attached conditions in serum-free medium. Cells were lysed after 0, 2, 4, 6 and 24 hours incubation. The results shown in Figure 3.3 demonstrate that cleaved PARP was detected after 4 hours of incubation and maximum amounts of cleaved PARP were detected at 6 hours of incubation and remained the same up to 24 hours of incubation.

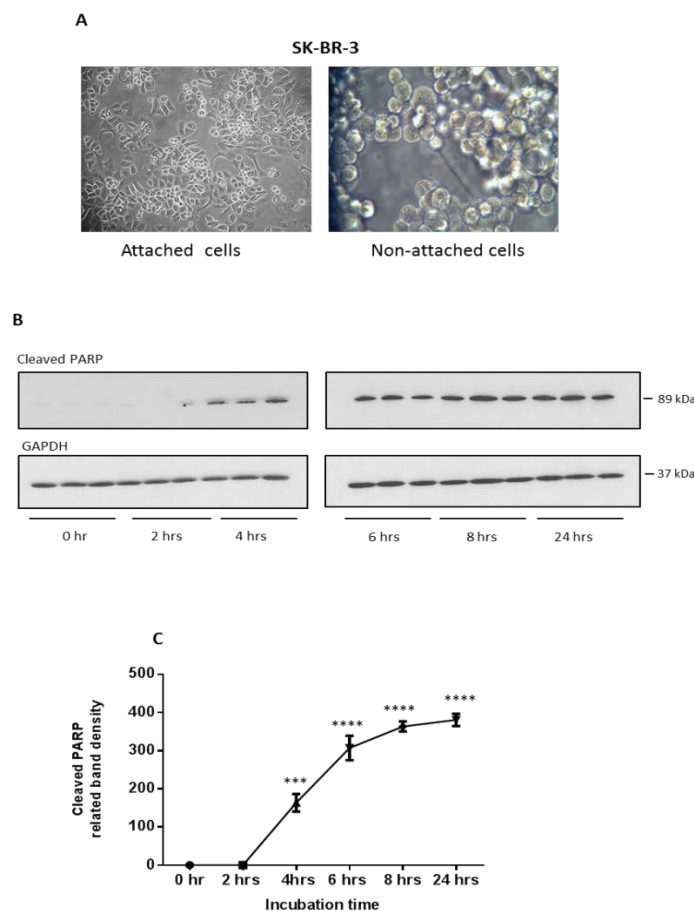


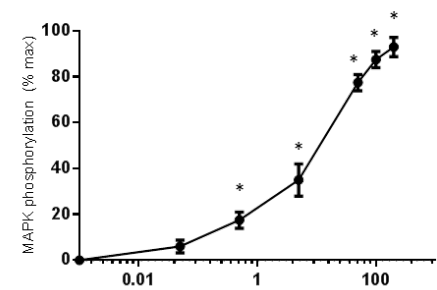
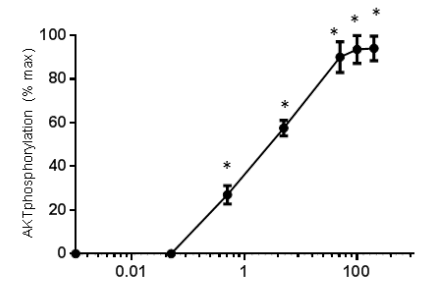
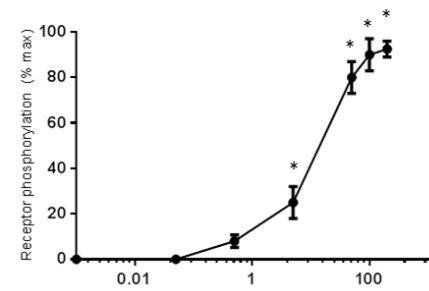
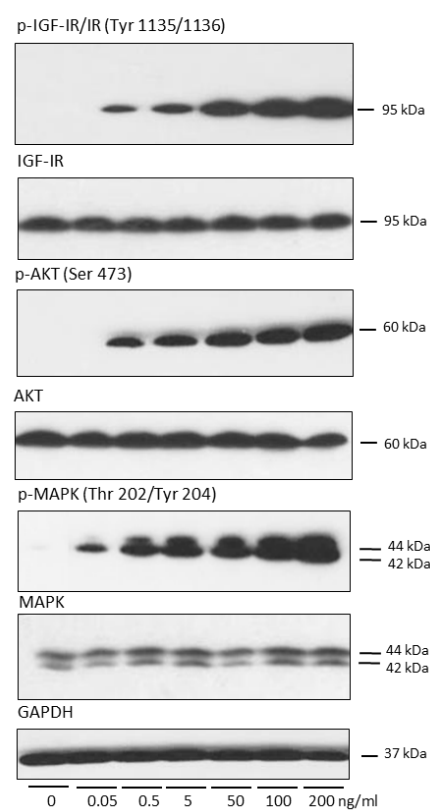
Figure 3.3: Induction of anoikis in HER-2 positive SK-BR-3 breast cancer cell line. SK-BR-3 cells (A) were cultured in phenol red free Dulbecco's Modified Eagle's medium (DMEM) without serum (serum-free medium) in non-attached condition. Photographs of cells in both attached and non-attached condition is shown (A). Cells were seeded in 12-well poly-HEMA coated plates at the density of 20×10^4 cells/well in 2 ml of serum-free medium and cultured for various length of time up to 24 hours. Cells were lysed with 50 μ l of RIPA buffer after each specific time point and proteins were extracted. Aliquots of 10 μ l protein were electrophoresed on 12% denaturing polyacrylamide gels and transferred to nitrocellulose membrane at 100 mA for 45 minutes. This followed by incubating the membrane with cleaved PARP antibody (1:2000 dilutions) and GAPDH (1:20,000 dilutions). The illustrated figures are representative of bands from one experiment in triplicate that have been replicated at least twice (B). Densitometric scanning of X-ray films determined the amount of each protein by analysis with LabWork 4.0 software. The mean \pm SEM after normalisation to GAPDH expression are shown (C). Asterisks indicates the amount of cleaved PARP was statistically higher than in the original cells (One way ANOVA, ***p=0.0001, ****p<0.0001).

3.2.3 The mechanism of IGF-1 signal transduction pathway

3.2.3.1 Activation of proteins involved in the IGF signal transduction pathway by different concentrations of IGF-1 in triple-negative breast cancer cells

To establish whether IGF downstream signal transduction pathways were activated in triple-negative MDA-MB-231 and Hs578T cells, the effect of stimulation with IGF-1 on the IGF signal transduction pathway was investigated. Cells were withdrawn from the effect of IGFs for two days by culture in medium containing dextran-coated charcoal treated serum (DCCS). The activation by phosphorylation of IGF signal transduction pathway components was determined by stimulation with various concentrations of IGF-1 up to 200 ng/ml (Figure 3.4).

A MDA-MB-231



B Hs578T

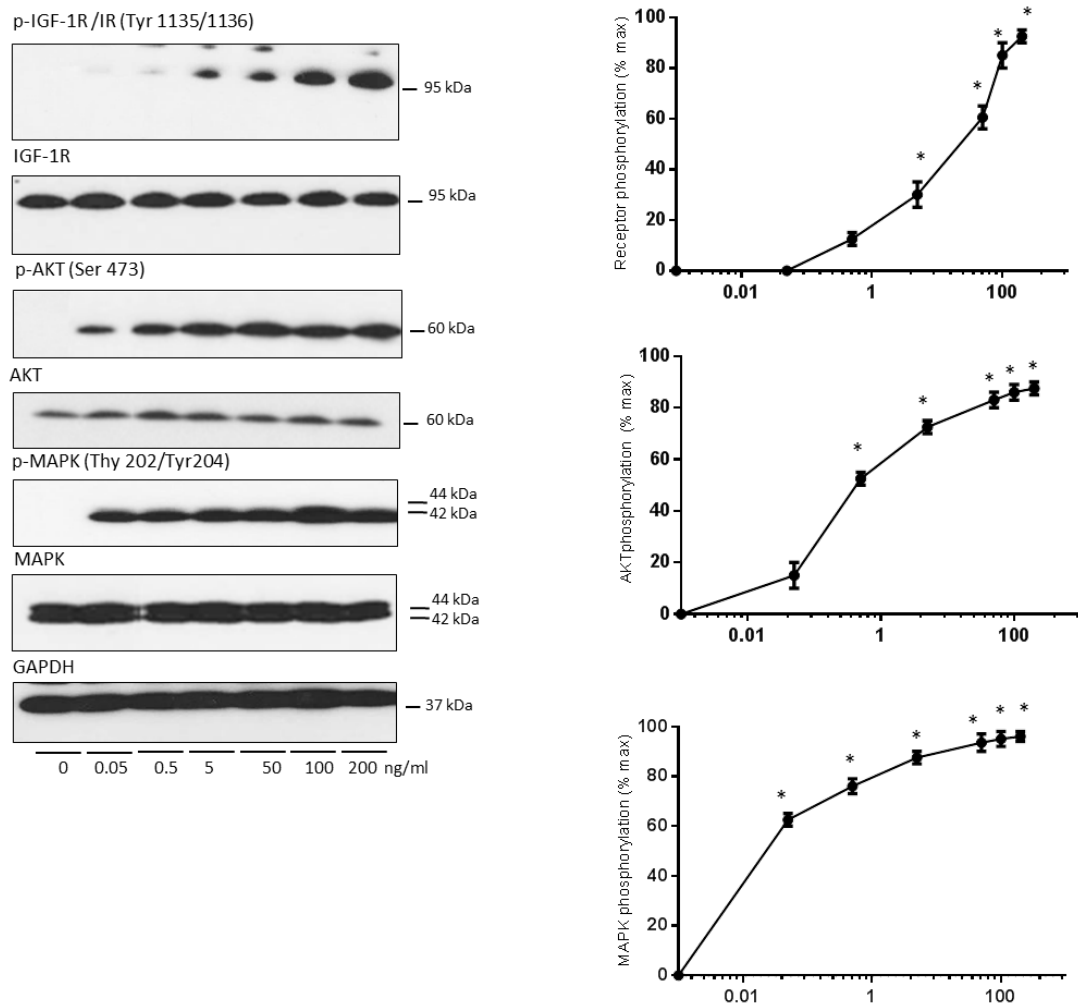


Figure 3.4 Activation of proteins involved in the IGF-1 downstream signal transduction in MDA-MB-231 and Hs578T cells. MDA-MB-231 (A) and Hs578T (B) were seeded at 10×10^4 cells/well in 1 ml of maintenance medium. Cells allowed to attach by incubation for 24 hours. Cells were removed from the maintenance medium, washed with 0.5 ml of PBS and medium were replaced with 1 ml of DCCS medium every day for two days. Medium was replaced with 1 ml of DCCS which was supplemented with IGF-1 at different concentration of 0, 0.05, 0.5, 5, 50, 100, 200 ng/ml for 15 minutes. After 15 minutes of incubation, cells were lysed with 50 μ l per well of RIPA buffer. Aliquots of 10 μ g protein were electrophoresed on 12% denaturing polyacrylamide gels, followed by transformation to nitrocellulose membrane for 45 minutes at 100 mA. Membrane were incubated with IGF-IR (1:1000 dilution), phosphorylated IGF-IR (1:1000 dilution for MDA-MB-231 cells and 1:500 dilution for Hs578T cells), AKT (1:5000 dilution), phosphorylated AKT (1:5000 dilution), MAPK (1:10,000 dilution for MDA-MB-231 and 1:20,000 dilution for Hs578T), phosphorylated MAPK (1:20,000 dilution) and GAPDH (1:20,000 dilution). Illustrated results represent one experiment in triplicate. Densitometric scanning of X-ray films determined the amount of each protein by analysis with LabWork 4.0 software. The mean \pm SEM after normalisation to GAPDH expression are shown. Asterisks denotes statistically significant increase in the phosphorylation of IGF-IR, AKT and MAPK with increasing IGF-1 concentration (One way ANOVA, * $p < 0.05$).

Stimulation with IGF-1 induced a concentration dependent increase in phosphorylation of the IGF-IR which was detected at 0.5 ng/ml in MDA-MB-231 and Hs578T cells (long exposures, not shown). IGF-1 did not affect the expression level of total IGF-IR after 15 minutes of stimulation. In MDA-MB-231 cells, no phosphorylated AKT was observed in the absence of IGF-1. In response to IGF-1 stimulation, Akt phosphorylation was detected at IGF-1 concentration of 0.5 ng/ml and increased in a concentration dependent manner with increasing the concentrations of IGF-1. In Hs578T cells, no phosphorylated AKT was detected in the absence of IGF-1, AKT phosphorylation was observed at 0.05 ng/ml IGF-1 concentration and higher. Stimulation with IGF-1 did not alter the expression level of total AKT in either of the cell lines. Phosphorylation of MAPK was not detected in the absence of IGF-1 in either of MDA-MB-231 or Hs578T cells. In MDA-MB-231 cells, MAPK phosphorylation was detected at IGF-1 concentrations of 0.05 ng/ml and higher, and increased with increasing IGF-1 concentration. In Hs578T cells, phosphorylation of MAPK at only 42 kDa was detected at 0.05 ng/ml IGF-1 concentration and higher. The amount of total MAPK did not change following IGF-1 stimulation.

The response of triple-negative MDA-MB-231 and Hs578T cells to stimulation with IGF-1 resulted in activation of IGF signal transduction pathway, which confirms that IGF-mediated signalling is functional in these cells. This was demonstrated by phosphorylation of downstream signalling components AKT and MAPK after treatment with certain concentration of IGF-1.

3.2.3.2 *Activation of downstream effector proteins by IGF-1 in IGF-1 signal transduction pathway in HER-2 positive SK-BR-3 breast cancer cells*

To evaluate whether IGF downstream signal transduction pathway is functional despite the very low level of IGF-IR in HER-2 positive breast cancer cell line, SK-BR-3 cells were cultured in dextran-coated charcoal treated serum (DCCS) medium to withdrawn from the effect of IGFs. The phosphorylation of IGF signal transduction components was determined by stimulation with various concentrations of IGF-1 up to 200 ng/ml (Figure 3.5). Aliquots of proteins were analysed by western transfer for phosphorylation of IGF-IR, AKT, MAPK (Figure 3.5).

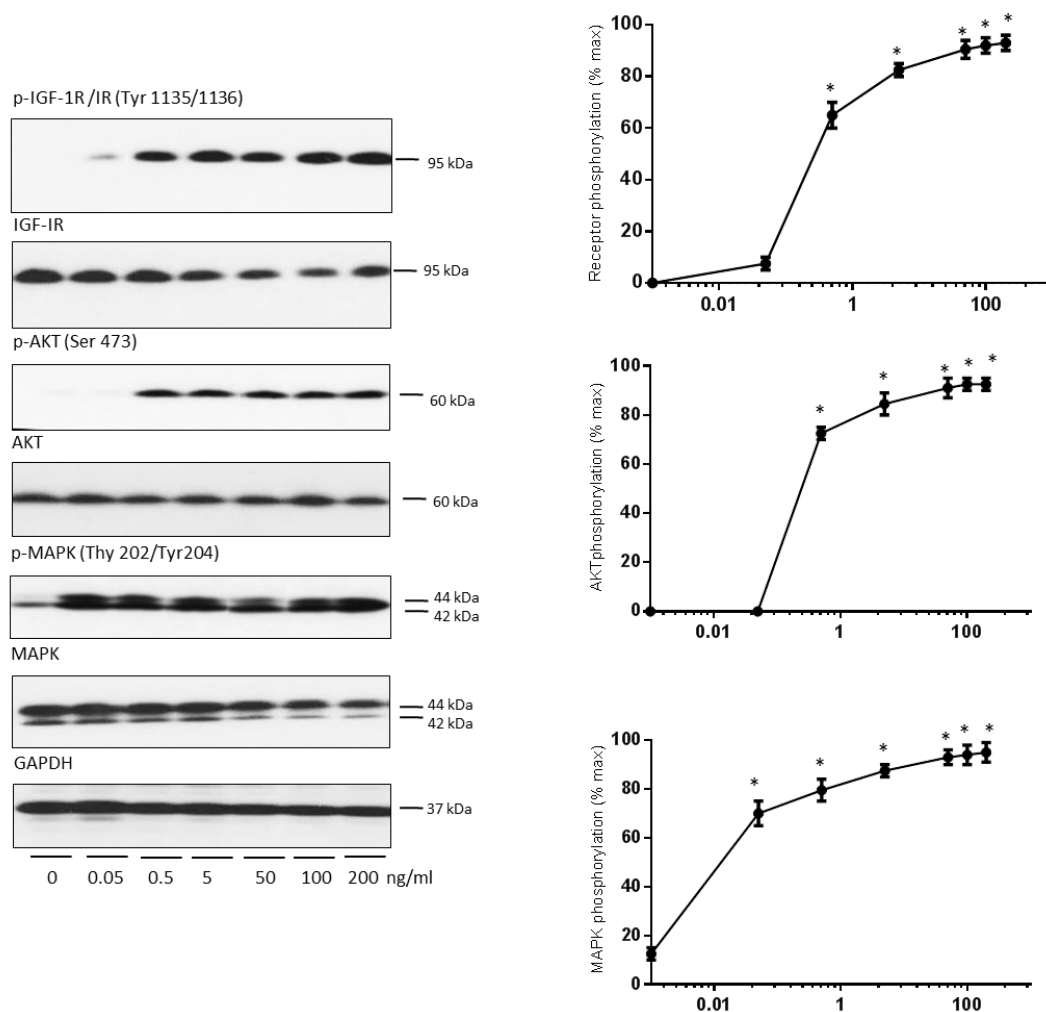


Figure 3.5: Response of SK-BR-3 to stimulation by IGF-1. SK-BR-3 cells were seeded at density of 20×10^4 cell/well in 1 ml of maintenance medium and incubated for 48 hours for proper attachment. Cells were removed from medium and were replaced with 1 ml of DCCS medium daily for two days. Cells were washed with 0.5 ml of PBS and treated with 1 ml DCCS supplemented with IGF-1 at different concentration of 0, 0.05, 5, 50, 100, 200 ng/ml for 15 minutes. After 15 minutes of incubation, cells were lysed with 50 μ l per well of RIPA buffer. Aliquots of 10 μ g of protein were analysed by electrophoresis in 12% denaturing polyacrylamide gel and transferred to nitrocellulose membrane for 45 minutes at 100 mA. Membranes were incubated with IGF-IR antibody (1:1000 dilution), phosphorylated IGF-IR (1:2000 dilution), AKT (1:10,000 dilution), phosphorylated AKT (1:15000 dilution), MAPK (1:20,000 dilution), phosphorylated MAPK (1:20,000 dilution) and GAPDH (1:20,000 dilution). Representative results are shown from an experiment in triplicate. Densitometric scanning of X-ray films determined the amount of each protein by analysis with LabWork 4.0 software. The mean \pm SEM after normalisation to GAPDH expression are shown. Asterisks denotes statistically significant increase in the phosphorylation of IGF-IR, AKT and MAPK with increasing IGF-1 concentration (One way ANOVA, * $p < 0.05$).

In SK-BR-3 cells, stimulation with IGF-1 induced an increase in phosphorylation of IGF-IR in a concentration dependent manner which detected at 0.05 ng/ml. There was no AKT phosphorylation in the absence of IGF-1. AKT phosphorylation in response to IGF-1 in a concentration dependent manner was

detected at IGF-1 concentration of 0.5 ng/ml and higher. The amount of total AKT did not change following IGF-1 stimulation. Phosphorylated MAPK was detected in the absence of IGF-1 at a low level and increases by stimulation with the different concentrations of IGF-1. Data showed that increased in the concentration of IGF-1, did not alter total IGF-IR, total AKT and total MAPK expression level.

Stimulation with IGF-1 in SK-BR-3 cells, suggests that IGF-1 activates the IGF signal transduction pathways. Phosphorylation of downstream protein MAPK was observed after treatment with lower concentrations of IGF-1 than phosphorylation of IGF-IR. This difference might be due to amplification of the signals transduced downstream, which may participate in different thresholds for detection of the activated proteins.

3.2.4 Protective effect of IGF-1 against anoikis in oestrogen receptor-negative breast cancer cells

The effect of IGF-1 on the survival of oestrogen receptor-negative MDA-MB-231, Hs578T and SK-BR-3 breast cancer cells in non-attached condition was evaluated by measurement of cleavage of PARP and activation of caspase-3 as measures of anoikis.

3.2.4.1 Effect of IGF-1 on anoikis in non-attached condition in triple-negative MDA-MB-231 and Hs578T breast cancer cells

The effects of IGF-1 on survival of triple-negative breast cancer cells, MDA-MB-231 and Hs578T in non-attached conditions in serum-free medium were assessed. Cells were incubated in the presence and absence of 50 ng/ml of IGF-1 in serum-free medium in non-attached condition for up to 24 hours. Proteins were extracted after incubation for 0, 6, 8 and 24 hours and level of PARP cleavage was measured by western transfer analysis (Figure 3.6).

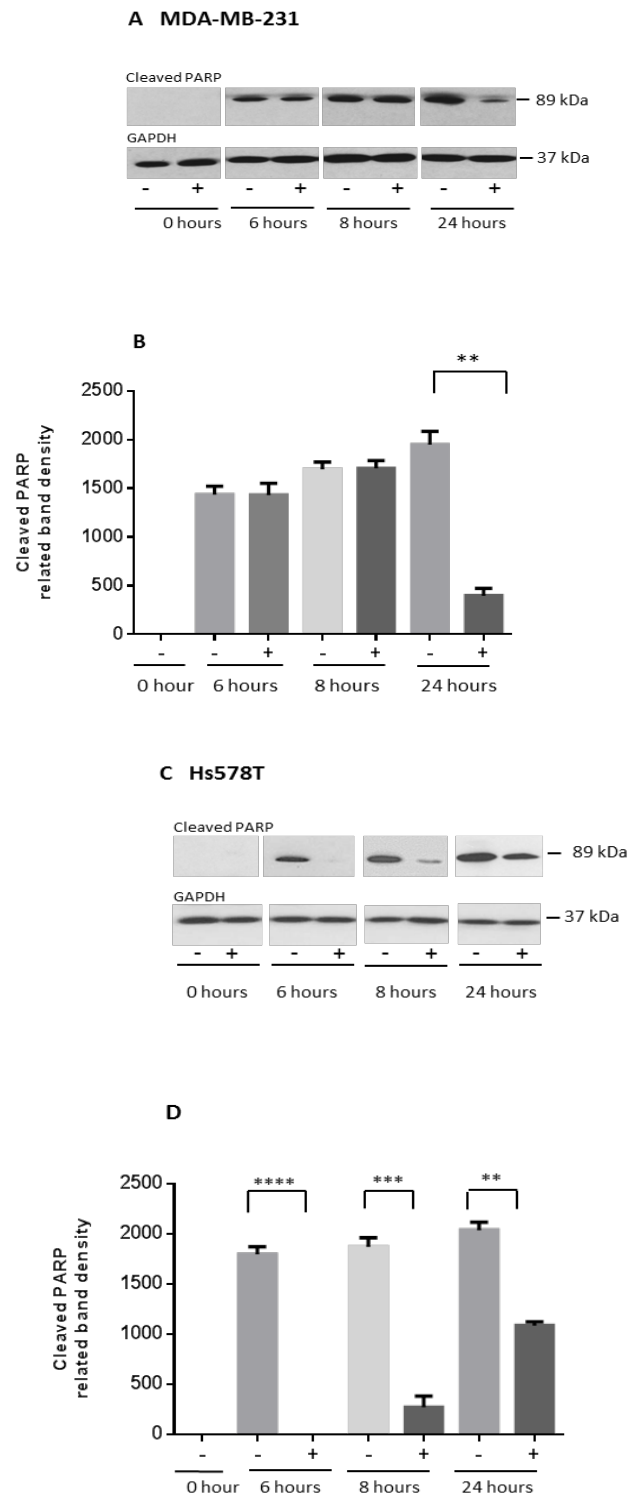


Figure 3.6: Effect of IGF-1 on survival of triple-negative breast cancer cell lines. MDA-MB-231 (**A**) and Hs578T (**C**) cells were seeded in 12 well poly-HEMA coated plates at density of 20×10^4 cells/well in 2 ml of serum-free medium and incubated in the presence and absence of 50 ng/ml of IGF-1. Proteins were extracted by 50 μ l per well of RIPA buffer after incubation for 0, 6, 8 and 24 hours. Aliquots of 10 μ g protein were analysed by electrophoresis on denaturing 12% polyacrylamide gel and transferred for 45 minutes at 100 mA. Membranes were incubated with antibody against cleaved PARP (1:2000 dilution) and GAPDH (1:20,000 dilution). Illustrated images are representative of cleaved PARP from a triplicate experiment that have been repeated at least three times. Labwork 4.0 software used for densitometric analysis of the X-ray films and measurement were normalised with GAPDH (**B**, **D**). Column shows mean \pm SEM, Asterisks denotes statistically significant less cleaved PARP in the presence of IGF-1 compared with in the absence of IGF-1 (One way ANOVA, ** $p < 0.001$, *** $p = 0.0001$, **** $p < 0.0001$).

Cleaved PARP was significantly reduced in MDA-MB-231 cells after incubation with 50 ng/ml of IGF-1 in non-attached condition for 24 hours. In Hs578T cells, PARP cleavage was inhibited completely after incubation for 6 hours and the inhibition was apparent after 8 and 24 hours of incubation. The reduction in cleaved PARP detected in the presence of IGF-1 compared with in the absence of IGF-1 supports the hypothesis that IGF-1 inhibits anoikis in triple-negative MDA-MB-231 and Hs578T cells.

3.2.4.2 *Effect of IGF-1 on anoikis in HER-2 positive SK-BR-3 breast cancer cells*

The protective effect of IGF-1 against cell death in non-attached condition was investigated on HER-2 positive SK-BR-3 cells. Cells were incubated with 50 ng/ml of IGF-1 in serum-free medium at different time points up to 24 hours. Proteins were extracted after 0, 6, 8 and 24 hours and the level of cell death were assessed by western transfer analysis (Figure 3.7).

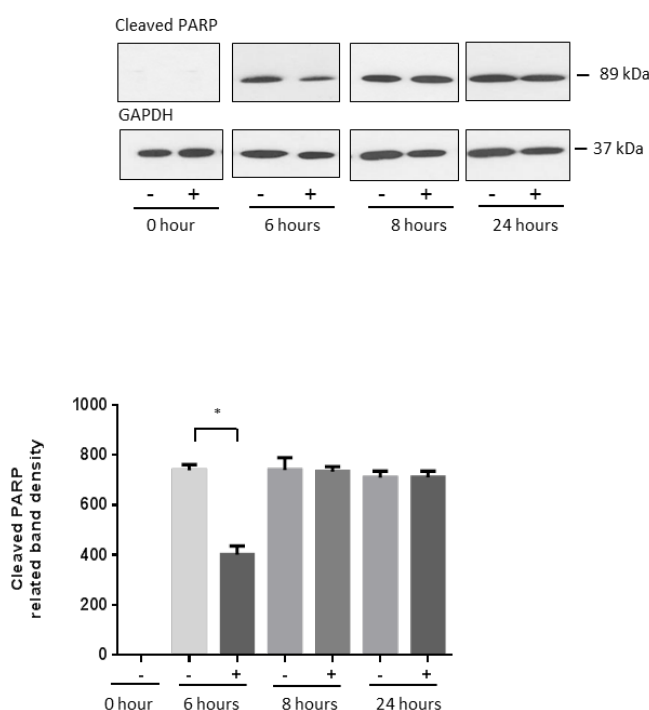


Figure 3.7: Effect of IGF-1 against cell death on HER-2 positive SK-BR-3 breast cancer cells. SK-BR-3 cells were seeded in 12 well poly-HEMA coated plates at density of 20×10^4 cells/well in 2 ml of serum-free medium and incubated in the presence and absence of 50 ng/ml of IGF-1. Proteins were extracted by 50 μ l per well of RIPA buffer after incubation for 0, 6, 8 and 24 hours. Aliquots of 10 μ g protein were analysed by electrophoresis on denaturing 12% polyacrylamide gel and transferred for 45 minutes at 100 mA. Membranes were incubated with antibody against cleaved PARP (1:2000 dilution) and GAPDH (1:20,000 dilution). Illustrated images are representative of cleaved PARP from a triplicate experiment that have been repeated at least three times. Labwork 4.0 software used for densitometric analysis of the X-ray films and measurement were normalised with GAPDH. Asterisks denotes statistically significant reduction in cleaved PARP in the presence of IGF-1 (One way ANOVA, * $p < 0.05$).

Incubation of SK-BR-3 cells with 50 ng/ml IGF-1 induced statistically significant reduction in the level of PARP cleavage after 6 hours of incubation, but there was no reduction in the level of cleaved PARP after incubation for 8 or 24 hours. Based on the result, low level of inhibition of anoikis by IGF-1 suggest that IGF-1 has minor protective effect against anoikis in SK-BR-3 cells.

3.2.4.3 *Effect of IGF-1 on activation of caspase-3 in MDA-MB-231 cells in non-attached conditions*

Protective effect of IGF-1 against anoikis in non-attached cells was determined by measurement of PARP cleavage. To investigate whether IGF-1 also has effect on reduction of caspase-3 activity in order to confirm the survival effect of IGF-1 in anoikis, MDA-MB-231 cells cultured in non-attached condition in serum-free medium for 24 hours in the presence and absence of IGF-1. Cells were collected and incubated with FITC-labelled cleaved caspase-3 antibody and Draq 5 nuclei stain overnight and the amount of cleaved caspase-3 was analysed by flow cytometry (Figure 3.8).

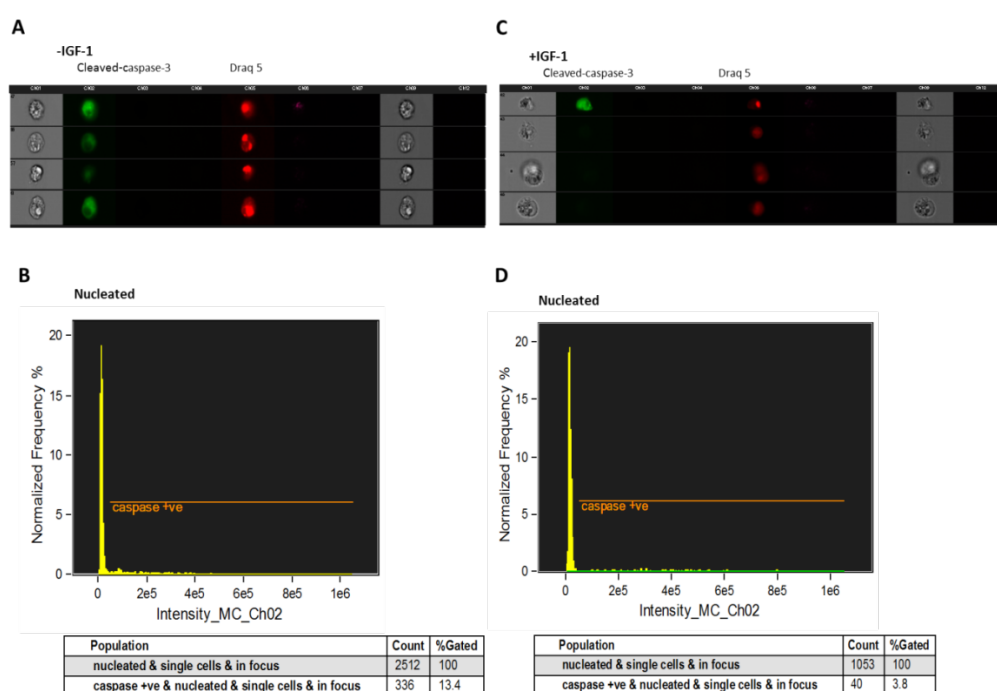
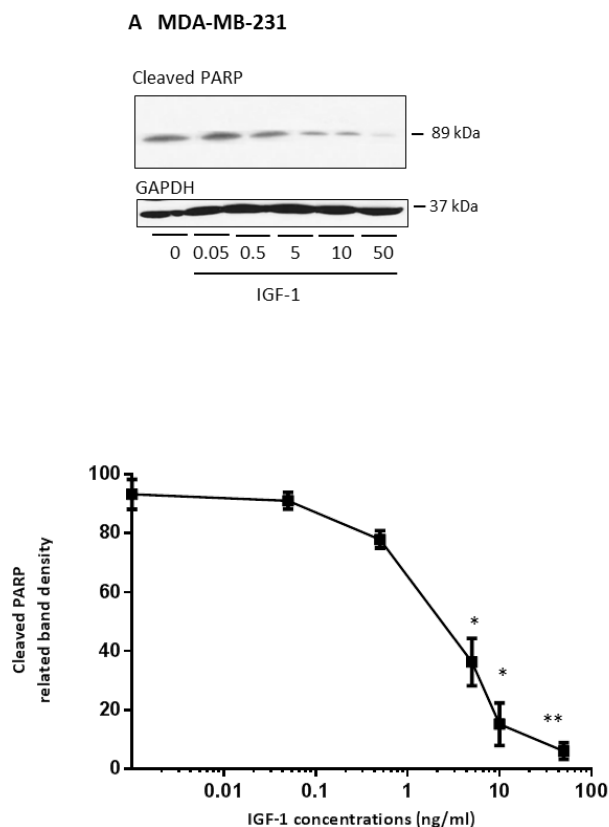


Figure 3.8: The effect of IGF-1 on activation of caspase-3 in non-attached condition. MDA-MB-231 cells were seeded into 12 well poly-HEMA coated plates in 2 ml of serum-free emdium at density of 20×10^4 cells/well and cultured in the presence and absence of 50 ng/ml IGF-1. After 24 hours of incubation, cells were collected, washed with PBS, fixed, permeabilised and blocked. Cells were incubated with FITC-labelled antibodies against cleaved caspase-3 and Draq 5 (nuclei stain) overnight at 4°C. The fluorescence of the cells was measured by Imagestream X (Amnis Crop.) (A, C) and analysed with IDEAS 4.0 Software (B, D). The excitation was at 488 nm and 642 nm and cells were gated for the positivity of cleaved caspase-3 intensity as described in Materials and Method Section. The percentage of the activated cleaved caspase-3 positive cells for an experiment was in triplicate.

Incubation of MDA-MB-231 cells for 24 hours in non-attached condition in serum-free medium, induced caspase-3 activation. The percentage of activated caspase-3 in the presence of IGF-1 was reduced by more than three fold (Figure 3.8), which supports the conclusion of the previous experiment that IGF-1 is protective against anoikis in MDA-MB-231 cells.

3.2.4.4 *Effect of various concentrations of IGF-1 on anoikis in MDA-MB-231 and Hs578T cells*

MDA-MB-231 and Hs578T cells were cultured in non-attached condition in serum-free medium and incubated with different concentration of IGF-1 up to 50 ng/ml. Proteins were extracted from MDA-MB-231 cells after 24 hours and from Hs578T after 6 hours of incubation. To evaluate the relative amount of cleaved PARP, aliquots of extracted proteins were analysed by western transfer analysis (Figure 3.9).



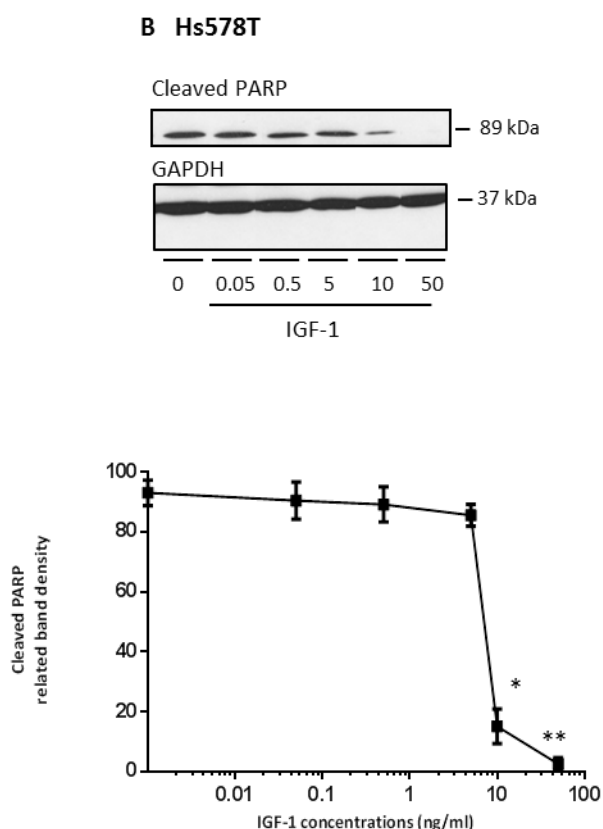


Figure 3.9 The effect of various concentrations of IGF-1 on survival of triple-negative breast cancer cells. MDA-MB-231 (A) and Hs578T (B) cells were seeded in 12 well poly-HEMA coated plates at density of 20×10^4 cells/well in 2 ml of serum-free medium, and incubated with different concentrations of IGF-1 up to 50 ng/ml. Proteins were extracted after incubation of 24 hours from MDA-MB-231 and incubation of 6 hours from Hs578T cells with 50 μ l per well of RIPA buffer. Aliquots of 10 μ l protein were analysed by electrophoresis in 12% denatured gel and transferred for 45 minutes at 100 mA. Membranes were incubated with antibody against cleaved PARP (1:2000 dilution) and GAPDH (1:20,000 dilution). Illustrated images are representative of cleaved PARP from a triplicate experiment that have been repeated at least twice. Labwork 4.0 software used for densitometric analysis of the X-ray films and measurement were normalised with GAPDH. Lines shows mean \pm SEM. Asterisks denote the statistically significant less cleaved PARP in the presence of IGF-1 compared with in the absence of IGF-1 (One way ANOVA, * $p < 0.05$, ** $p < 0.001$).

In both MDA-MB-231 and Hs578T cells, PARP cleavage was induced by culturing the cells in non-attached conditions in serum-free medium. In the presence of IGF-1, the amount of cleaved PARP was reduced in a concentration dependent fashion in both MDA-MB-231 and Hs578T cells. In MDA-MB-231 cells, there appeared to be a reduction in the amount of cleaved PARP in the presence of IGF-1 at a concentration of 0.5 ng/ml compared to its absence, but the difference was not statistically significant ($p=0.255$). A statistically significant reduction was observed at IGF-1 concentration of 5 ng/ml or higher ($p=0.019$). There was a positive correlation between the reduction of cleaved PARP and increasing the concentration of IGF-1. In Hs578T cells, there

was a statistically significant reduction in the amount of cleaved PARP at IGF-1 concentration of 10 ng/ml ($p=0.012$), followed by complete inhibition of cleaved PARP at IGF-1 concentration of 50 ng/ml.

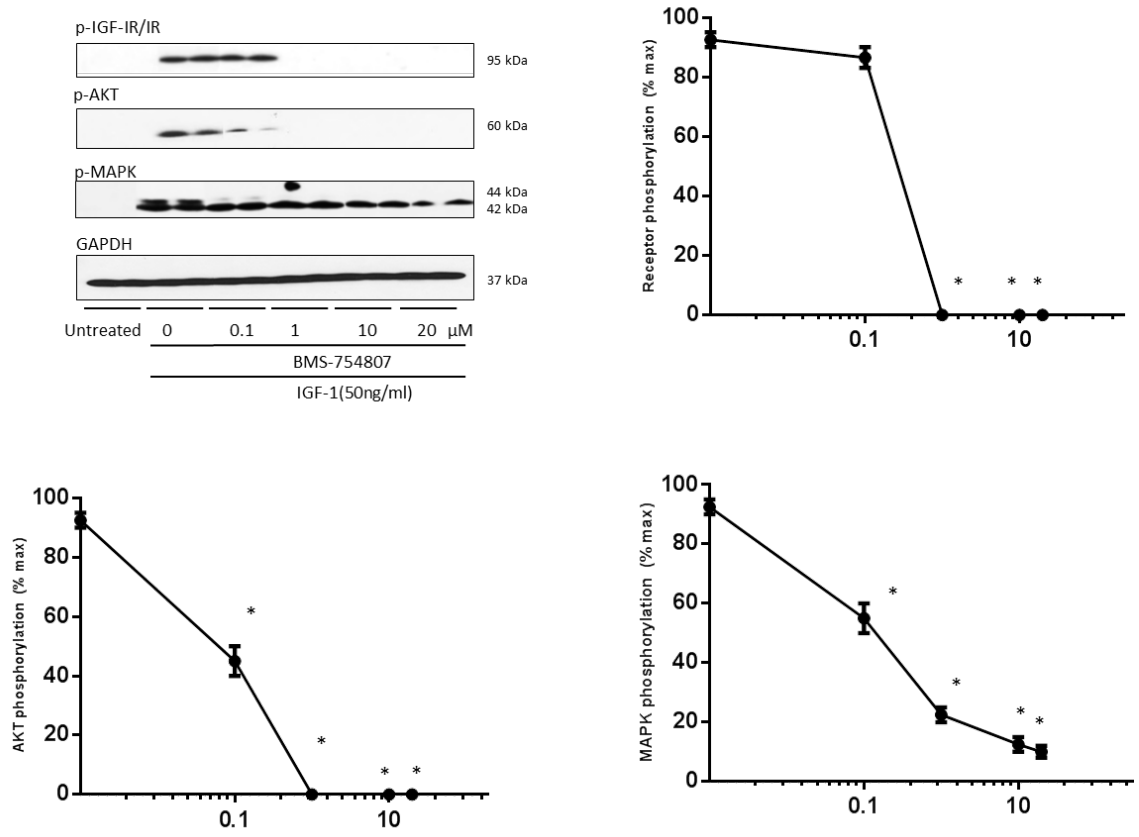
IGF-1 demonstrated a powerful survival effect on both triple-negative breast cancer cells. In Hs578T cells, the maximal reduction of cleaved PARP was greater than in MDA-MB-231 cells at the tested IGF-1 concentrations. Anoikis was completely inhibited in Hs578T cells at IGF-1 concentration of 50 ng/ml. These results demonstrated that MDA-MB-231 cells are more sensitive to anoikis than Hs578T cells and Hs578T cells are more responsive to IGF-1 compared to MDA-MB-231 cells. The concentration dependent reduction in PARP cleavage in the presence of IGF-1 in MDA-MB-231 and Hs578T cells can suggest that the survival effect of IGF-1 is transmitted through the type I IGF receptor.

3.2.5 The effect of dual tyrosine kinase inhibitor of IGF-IR and the insulin receptor (BMS-754807) on activation of IGF-IR and the insulin receptor in triple-negative breast cancer cell lines

BMS-754807 is a small molecule tyrosine kinase inhibitor of both IGF-IR and the insulin receptor (IR). It binds to both IGF-IR and the insulin receptor and blocks their phosphorylation and activation. The effectiveness of dual tyrosine kinase inhibitor, BMS-754807 was evaluated on survival effect of IGF-1.

To investigate the effectiveness of BMS-754807 and establish the concentration at which the maximum inhibition occurs in the cells in which IGF-1 had a protective effect, MDA-MB-231 and Hs578T cells were cultured in attached condition in full maintenance medium and incubated with different concentrations of BMS-754807 up to 20 μ M for 30 minutes. This incubation was followed by 15 minutes stimulation of the cells with 50 ng/ml of IGF-1. Proteins were extracted and aliquots containing 10 μ g of protein were analysed by western transfer for phosphorylated IGF-IR/IR, AKT and MAPK (Figure 3.10).

A MDA-MB-231



B Hs578T

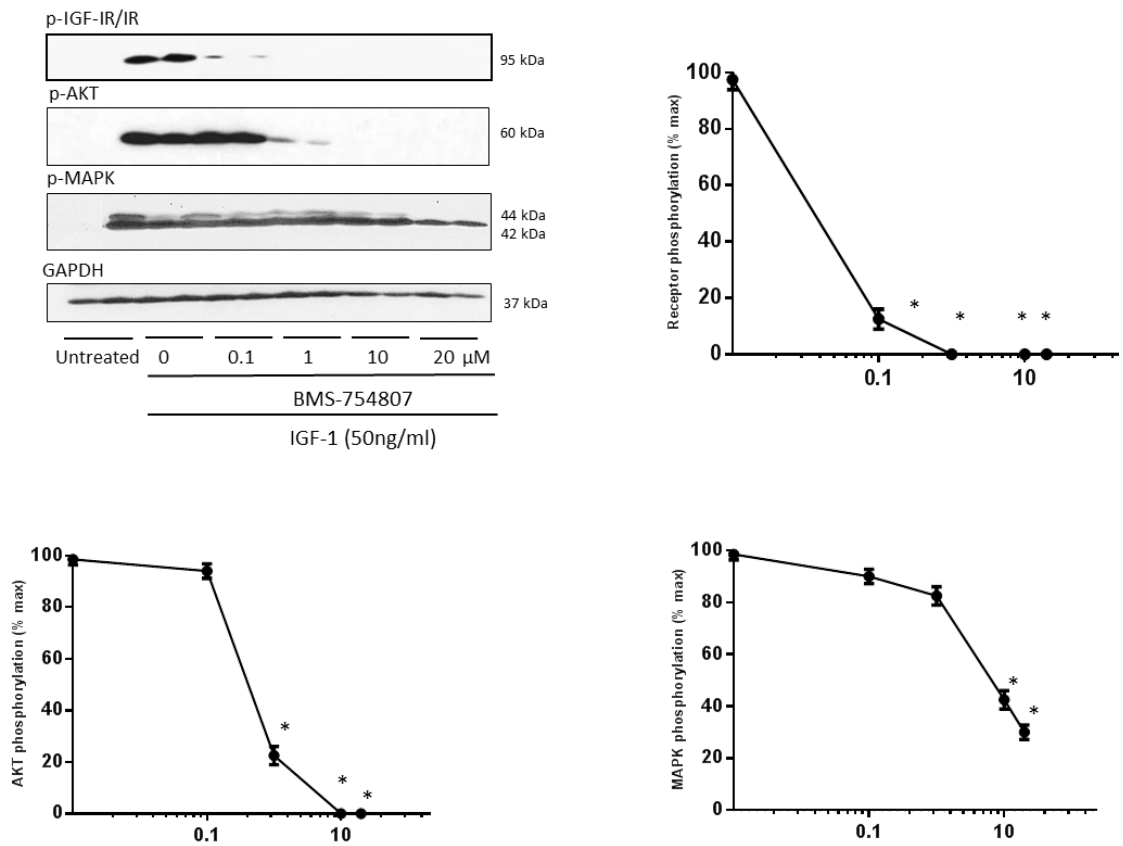


Figure 3.10: The effect of BMS-754807 on inhibition of IGF-IR and the insulin receptor activity in MDA-MB-231 and Hs578T cells. MDA-MB-231 and Hs578T cells were seeded into 12 well tissue culture plates at density of 10×10^4 cells/well in 1 ml of maintenance medium. Cells were incubated for 24 hours to allow proper attachment. Medium was aspirated and cells were washed with 0.5 ml of PBS. Medium was replaced daily with 1 ml/well of DCCS medium for two days. After two days of incubation, medium was replaced with DCCS supplemented with BMS-754807 at final concentrations of 0, 0.1, 1, 10, 20 μ M and cells were incubated with the antibody for 30 minutes. After 30 minutes of treatment, medium was replaced with the DCCS supplemented with 50 ng/ml of IGF-1 and cells were stimulated for 15 minutes. Proteins were extracted with 50 μ l of RIPA buffer. Aliquots of 10 μ g proteins were electrophoresed on denaturing 12 % polyacrylamide gels and transferred into nitrocellulose membrane for 45 minutes at 100 mA. Membranes were incubated with phosphorylated IGF-IR (1:1000 dilution for MDA-MB-231 cells and 1:500 dilution for Hs578T cells), phosphorylated AKT (1:5000 dilution), phosphorylated MAPK (1:20,000 dilution) and GAPDH (1:20,000 dilution). Representative bands are from duplicate experiment that has been repeated at least twice. Lines shows mean \pm SEM, Asterisks represent the statistically significant reduction in phosphorylation of IGF-IR/IR, AKT and MAPK in the presence of BMS-754807 compared with in the absence of BMS-754807 (One way ANOVA, * $p < 0.05$).

In MDA-MB-231 cells, complete inhibition of IGF-IR phosphorylation stimulated by 50 ng/ml of IGF-1 was detected at concentrations of 1 μ M of BMS-754807 and above. Whereas, in Hs578T cells treatment with BMS-754807 following stimulation with 50 ng/ml of IGF-1, induced reduction in phosphorylation of IGF-IR from 0.1 μ M concentration and followed by complete inhibition of phosphorylation at 1 μ M concentrations and higher. In MDA-MB-231 cells, reduction in phosphorylation of AKT was detected at 0.1 μ M concentrations of BMS-754807 and followed by complete inhibition of AKT phosphorylation at higher concentrations. In Hs578T cells, phosphorylation of AKT reduced 1 μ M and fully inhibited at higher concentrations of 10 μ M and higher. In MDA-MB-231 cells, decrease in phosphorylation of MAPK was detected at concentration of 0.1 μ M and higher. In Hs578T cells, BMS-754807 reduced the phosphorylation of MAPK at concentration of 10 μ M and higher. Complete inhibition of MAPK phosphorylation at highest concentration was not achieved in either MDA-MB-231 or Hs578T cells.

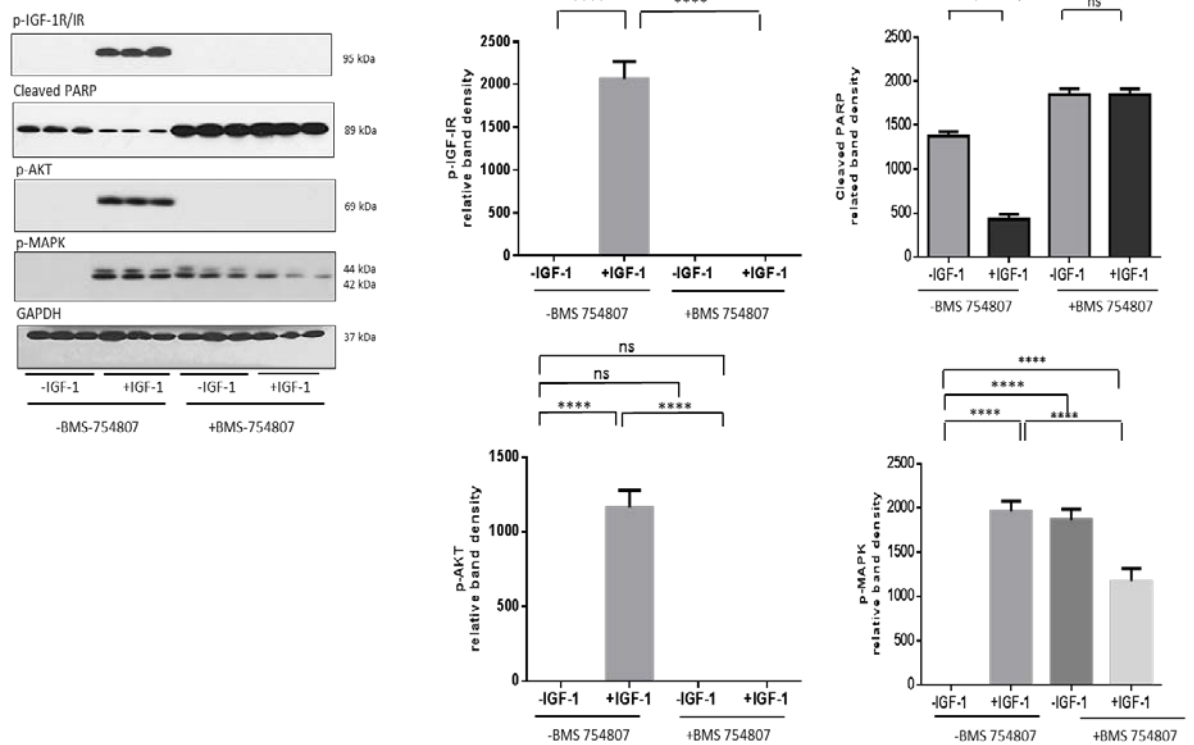
Treatment of MDA-MB-231 and Hs578T cells stimulated by IGF-1 with dual tyrosine kinase inhibitor against IGF-IR and the insulin receptor resulted in full inhibition of phosphorylation of IGF-IR and the insulin receptor and AKT at BMS-754807 concentration of 1 μ M. There was a potential inhibition of IGF-1 stimulated phosphorylation of MAPK in both triple-negative cell lines.

3.2.6 Dual tyrosine kinase inhibitor of IGF-IR and the insulin receptor abrogates the anti-anoikis effect of IGF-1

The effect of dual tyrosine kinase inhibitor of IGF-IR and insulin receptor in preventing the survival effect of IGF-1 was investigated.

MDA-MB-231 and Hs578T cells were cultured in non-attached conditions in serum-free medium in the presence and absence of BMS-754807, and in the presence and absence of IGF-1. Proteins were extracted after 24 hours in MDA-MB-231 cells and after 6 hours in Hs 578T cells. Aliquots containing 10 µg of protein were analysed by western transfer for phosphorylation of IGF-IR, cleaved PARP and phosphorylation of downstream key proteins AKT and MAPK (Figure 3.11).

A MDA-MB-231



B Hs578T

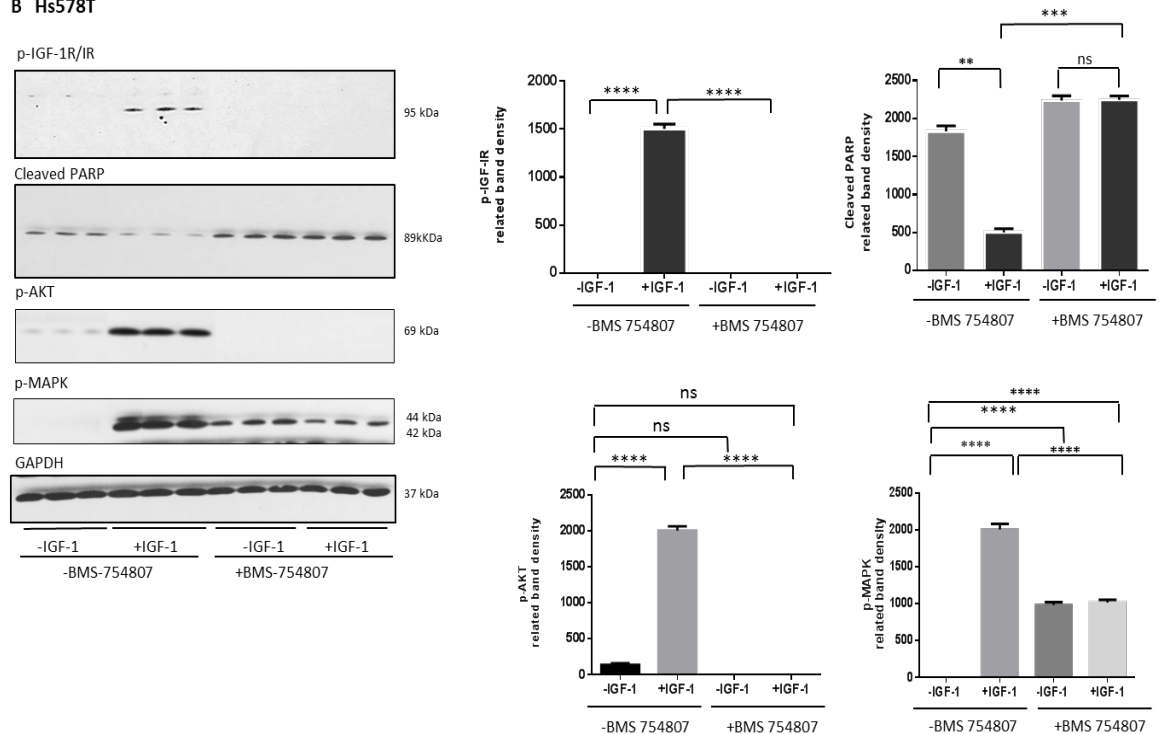


Figure 3.11: The effect of dual tyrosine kinase inhibitor of IGF-IR and the insulin receptor against anti-apoptosis effect of IGF-1 on triple-negative breast cancer cell lines. MDA-MB-231 and Hs578T cells were seeded into 12 well poly-HEMA coated plates at 20×10^4 cells/well in 2 ml of serum-free medium, in the presence of 1 μ M of BMS-754807 or vehicle (DMSO) for MDA-MB-231 cells or Hs578T cells in the presence and absence of 50 ng/ml of IGF-1. Proteins were extracted after incubation of 24 hours in MDA-MB-231 cells and incubation of 6 hours in Hs578T cells with 50 μ l per well of RIPA buffer. Aliquots of 10 μ g proteins were analysed by electrophoresis in denaturing 12% polyacrylamide gels and transferred for 45 minutes at 100 mA. Membranes were incubated with antibody against phosphorylated IGF-IR (1:1000 dilution for MDA-MB-231 cells and 1:500 for HS578T cells), cleaved PARP (1:2000 dilution), phosphorylated AKT (1:5000 dilution for MDA-MB-231 cells and 1:10,000 dilution for Hs578T cells), phosphorylated MAPK (1:20,000 dilution) and GAPDH (1:20,000 dilution). Illustrated images are representative of cleaved PARP from a triplicate experiment that have been repeated at least three times. Labwork 4.0 software used for densitometric analysis of the X-ray films and measurement were normalised with GAPDH. Error bars are \pm SEM, Asterisks denotes statistically significant increase in the amount of cleaved PARP in the presence of BMS-754807 compared in the absence of BMS-754807 in MDA-MB231 and Hs578T cells (One way ANOVA, ***p=0.0001, ****p<0.0001).

In MDA-MB-231, stimulation with IGF-1 in the absence of BMS-754807 induced the strong IGF-IR phosphorylation. In the presence of 1 μ M BMS-754807, no phosphorylation of IGF-IR was detected in response to stimulation with IGF-1, compared with in the absence of BMS-754807. In the presence of 50 ng/ml IGF-1 anoikis was significantly inhibited. The degree of anoikis in serum-free medium was markedly different in the presence of BMS-754807 compared with the absence of BMS-754807. The protective effect of IGF-1 against anoikis was reduced significantly in the presence of BMS-754807 which was demonstrated

by significant increase in the amount of cleaved PARP in the presence of BMS-54807 compared with in the absence of BMS-754807 ($p=0.0001$).

The increase in the phosphorylation of AKT in response to stimulation with IGF-1 was completely inhibited in the presence of BMS-754807. Although IGF-1 induced phosphorylation of MAPK was not inhibited completely in the presence of BMS-754807, there was a significant reduction in the phosphorylation of MAPK.

In Hs578T cells, stimulation with IGF-1 in the absence of BMS-754807 induced phosphorylation of IGF-IR, whereas in the presence of 1 μ M BMS-754807, IGF-1 stimulated IGF-IR phosphorylation was not observed. The level of anoikis in serum-free medium was slightly higher in the presence of BMS-754807 compared with in the absence of BMS-754807. In the presence of 50 ng/ml IGF-1 anoikis was inhibited. The anti-anoikis effect of IGF-1 was reduced significantly in the presence of BMS-754807 ($p<0.0001$). This was demonstrated by significant increase in the amount of cleaved PARP.

The increase in the phosphorylation of AKT in response to IGF-1 was inhibited completely in the presence of BMS-754807, whereas increased in the amount of MAPK in the presence of IGF-1 was significantly reduced in the presence of BMS-754807.

Based on the showed results, the complete inhibition of activated AKT and significant reduction in the activated MAPK suggest that dual tyrosine kinase inhibitor might be effective in inhibition of survival effect of IGF-1.

3.2.7 Effect of CP-751, 871 on inhibition of type-1 IGF-IR by IGF-1 on triple negative breast cancer cells

CP-751, 871 (Pfizer) is a fully humanised IgG₂ monoclonal antibody against the type I IGF receptor. CP-751, 871 binds to the type I IGF receptor but not to the insulin receptor and inhibits the activity of the receptor through internalization of the type I IGF receptor and reduction in level of the receptor.

To determine the appropriate concentration for inhibition of type I IGF receptor, different concentrations of antibody were incubated in triple-negative MDA-MB-231 and Hs578T breast cancer cells.

MDA-MB-231 and Hs578T cells were cultured in attached condition in full maintenance medium and incubated with various concentrations of monoclonal antibody CP-751, 871 up to 20 µg/ml for 24 hours in MDA-MB-231 and for 6 hours in Hs578T cells. After incubation with the antibody, both cell lines were stimulated with the 50 ng/ml of IGF-1 for 15 minutes. Proteins were extracted after 15 minutes of stimulation and analysed for phosphorylation of IGF-IR by western transfer (Figure 3.12).

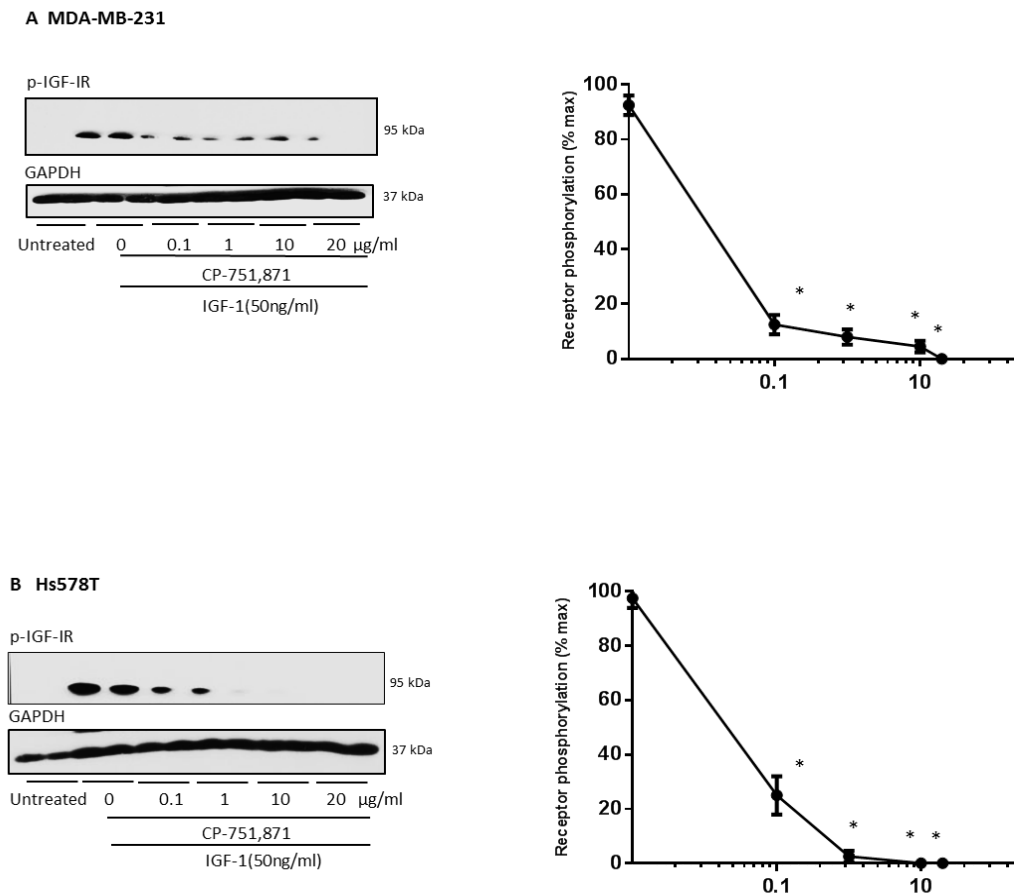


Figure 3.12: Effect of CP-751, 871 on activation of type I IGF receptors in MDA-MB-231 and Hs578T cells. MDA-MB-231 and Hs578T cells were seeded into 12 well tissue culture plates at density of 10×10^4 cells/well in 1 ml of maintenance medium. Cells were incubated for 24 hours to allow attachment. Medium was aspirated and cells were washed with 0.5 ml of PBS. Medium was replaced daily with 1 ml/well of DCCS medium for two days. After two days of incubation, medium was replaced with DCCS supplemented with CP-751, 871 antibody at final concentrations of 0, 0.1, 1, 10, 20 µg/ml and cells were incubated with the antibody for 24 hours (MDA-MB-231 cells) and 6 hours (Hs578T cells). After treatment of cells with various concentrations of antibody, medium was replaced with the DCCS supplemented with 50 ng/ml of IGF-1 and cells were stimulated for 15 minutes. Proteins were extracted with 50 µl of RIPA buffer. Aliquots of 10 µg proteins were electrophoresed on a denaturing 12 % polyacrylamide gel and transferred into nitrocellulose membrane for 45 minutes at 100 mA. Membranes were incubated with phosphorylated IGF-IR (1:1000 dilution for MDA-MB-231 cells and 1:500 dilution for Hs578T cells) and GAPDH (1:20,000 dilution). Representative images are from duplicate experiments are shown. Labwork 4.0 software used for densitometric analysis of the X-ray films and measurement were normalised with GAPDH. Lines shows mean \pm SEM, Asterisks represent the statistically significant reduction in phosphorylation of IGF-IR in the presence of CP-751, 871 compared with in the absence of CP-751, 871 (One way ANOVA, * $p < 0.05$).

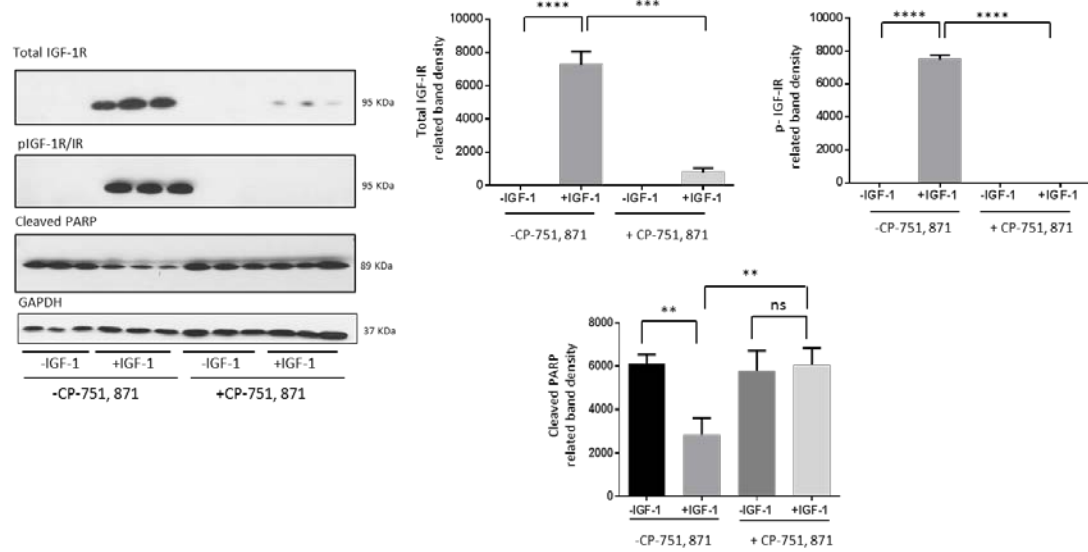
In MDA-MB-231 cells phosphorylation of IGF-IR was reduced at a concentration of 0.1 µg/ml of CP-751, 871 and complete inhibition of phosphorylation stimulated with 50 ng/ml of IGF-1 was detected at concentrations of 20 µg/ml. In Hs578T cells treatment with the antibody following stimulation with 50 ng/ml of IGF-1 induced a concentration dependent decrease in the phosphorylation of IGF-IR, detected from 0.1 µg concentration and complete inhibition was detected at concentrations of 10 µg and higher.

3.2.8 Inhibition of type I IGF receptor abrogate the protective effect of IGF-1 against cell death by CP-751, 871 inhibitor in anoikis

To determine whether the survival effect of IGF-1 is through type I IGF receptor in anoikis condition, the effect of IGF-1 in anoikis was tested in the presence and absence of 20 µg/ml and 10 µg/ml concentration of CP 751, 871 from the previously optimised concentrations in MDA-MB-231 and Hs578T cells respectively.

MDA-MB-231 and Hs578T cells were seeded into poly-HEMA coated plates in serum-free medium and treated with the presence and absence of CP-751, 871 and in the presence and absence of the IGF-1. Proteins were extracted after 24 hours from MDA-MB-231 cells and after 6 hours from Hs578T cells. Aliquots of proteins were analysed for phosphorylated IGF-IR and cleaved PARP by western transfer (Figure 3.13).

A MDA-MB-231



B Hs578T

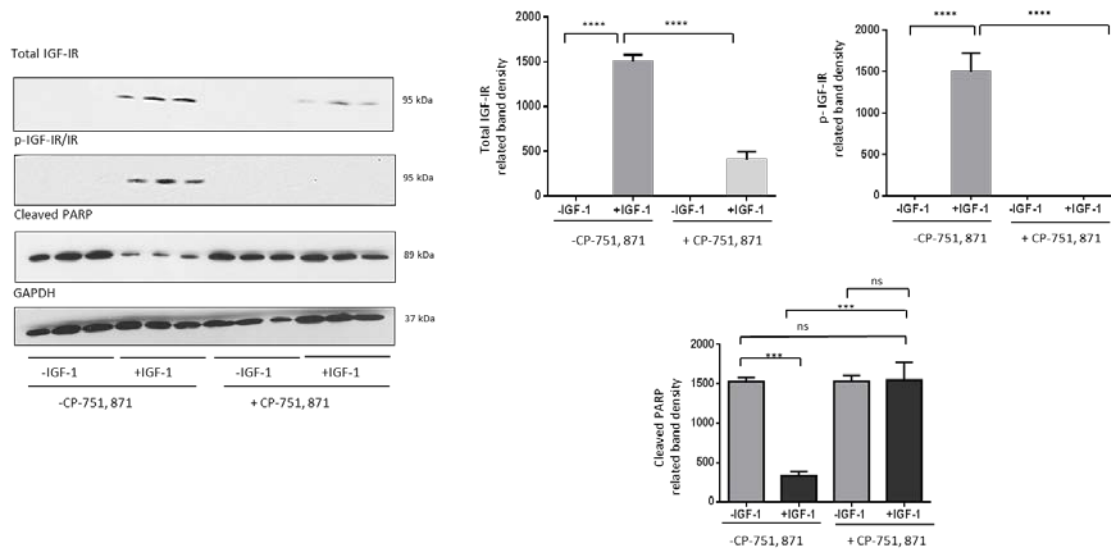


Figure 3.13: The effect of CP-751, 871 on the anti-anoikis effect of IGF-1 in triple-negative breast cancer cell lines. MDA-MB-231 cells first were cultured in two 25 cm² tissue culture flasks and incubated for 24 hours in the presence of 20 µg/ml CP-751, 871 antibody or vehicle IgG₂. After 24 hours of treatment, cells were seeded into poly-HEMA coated plates at 20 X 10⁴ cells/well in 2 ml of serum-free medium, in the presence and absence of CP-751, 871 or IgG₂, and in the presence and absence of 50 ng/ml of IGF-1 and incubated for 24 hours. Hs578T cells were seeded into 12 well poly-HEMA coated plates at density of 20 X 10⁴ cells/well in 2 ml of serum-free medium and incubated in the presence and absence of 10 µg/ml of CP-751, 871 or IgG₂ and in the presence and absence of 50 ng/ml IGF-1. Proteins were extracted after incubation of 24 hours in MDA-MB-231 and after incubation of 6 hours in Hs578T cells with 50 µl per well of RIPA buffer plus inhibitors. Aliquots of 10 µl protein were analysed by

electrophoresis in 12% denatured gel and transferred for 45 minutes at 100 mA. Membranes were incubated with antibody against total IGF-IR (1:1000 dilution), phosphorylated IGF-IR (1:1000 dilution for MDA-MB-231 cells and 1:500 dilution for Hs578T cells), cleaved PARP (1:2000 dilution) and GAPDH (1:20,000 dilution). Illustrated images are representative of cleaved PARP from a triplicate experiment that have been repeated at least three times. Labwork 4.0 software used for densitometric analysis of the X-ray films and measurement were normalised with GAPDH. Error bars are \pm SEM, Asterisks denotes that cleaved PARP significantly increased in the presence of CP-751, 871 compared with in the absence of CP-751, 871 in both cell lines (One way ANOVA, ** $p < 0.001$, *** $p = 0.0001$, **** $p < 0.0001$).

In presence of 20 $\mu\text{g/ml}$ CP-751, 871 the amount of total IGF-IR was reduced in both MDA-MB-231 and Hs578T cells. The amount of cleaved PARP that was induced in each cell lines after incubation in non-attached condition in serum-free medium was reduced by more than 60-70% in the presence of 50 ng/ml IGF-1. In each cell line, in the present of IGF-1, there was an increase in the amount of cleaved PARP which was statistically significant in the presence of CP-751, 871 compared to the absence of CP-751, 871. In MDA-MB-231 cells the amount of cleaved PARP in the presence of IGF-1 was increased by almost two fold in the presence of CP-751, 871 compared to the absence of CP-751, 871. In Hs578T cells the increase in cleaved PARP was almost more than two fold in the presence of CP-751,871 compared to the absence of CP-751, 871.

In conclusion, CP-751,871 inhibited completely the protective effect of IGF-1 against anoikis in both cells lines, which supports the hypothesis that blocking the type I IGF receptor can inhibit the survival effect of IGF-1 and that IGF-1 extents its effects through the type I IGF receptor.

3.2.9 Effect of dual tyrosine kinase inhibitor on anoikis in full maintenance medium

In this chapter the preceding results demonstrate that firstly, anoikis can be induced in triple-negative breast cancer cells when cultured in non-attached conditions in serum-free medium and secondly, IGF-1 has a powerful survival effect under these conditions. In patients, tumor cells are not present in serum-free medium, but they are exposed to growth factors as well as to other serum components. Therefore, to evaluate whether BMS-754807 potentiates the cell death in full maintenance medium in non-attached cells, MDA-MB-231 cells were cultured in non-attached conditions in full maintenance medium. Effects of IGF-1 were inhibited with the dual tyrosine kinase inhibitor of both IGF-IR and the insulin receptor BMS-754807. Proteins were extracted after 24 hours of incubation and analysed for phosphorylation of IGF-IR and cleaved PARP by western transfer (Figure 3.14).

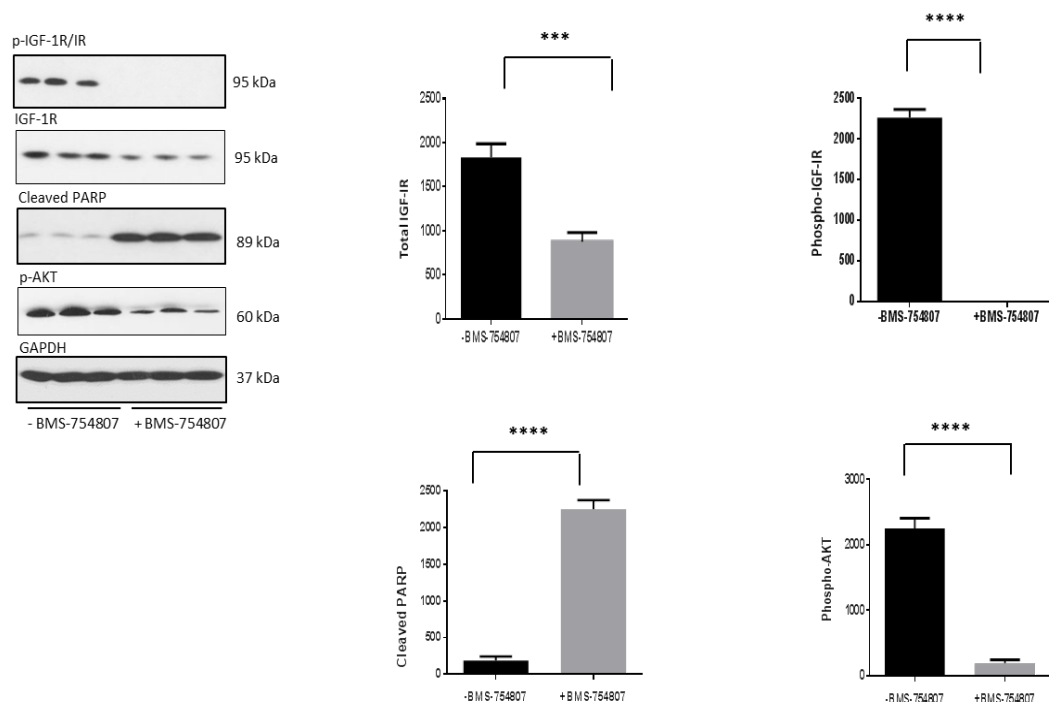


Figure 3.14: The effect of dual tyrosine kinase inhibitor BMS-754807 on anoikis in MDA-MB-231 cells cultured in full maintenance medium. MDA-MB-231 cells were cultured into 12 well poly-HMEA coated plates at density of 20×10^4 cells/well in 2 ml of full maintenance medium in the presence of 1 μ M BMS-754807 or vehicle (DMSO). Proteins were extracted after 24 hours with 50 μ l of RIPA buffer. Aliquots of 10 μ g proteins were analysed by electrophoresis in denaturing 12% polyacrylamide gel and transferred for 45 minutes at 100 mA. Membranes were incubated with antibody against total IGF-IR (1:1000 dilution), phosphorylated IGF-IR (1:1000 dilution), cleaved PARP (1:2000 dilution) and GAPDH (1:20,000 dilution). Illustrated images are representative of cleaved PARP from a triplicate experiment that have been repeated at least twice. Labwork 4.0 software used for densitometric analysis of the X-ray films and measurement were normalised with GAPDH. Error bars are \pm SEM, Asterisks denotes

there was a statistically significant decrease in total level of IGF-IR in the presence of BMS-754807. Asterisks denotes that there was a significant inhibition in IGF-IR phosphorylation, significant decrease in AKT phosphorylation and significant increase in the amount of cleaved PARP in the presence of BMS-754807 compared with in the absence of BMS-754807 in MDA-MB-231 cells in full maintenance medium (One way ANOVA, ***p=0.0001, ****p<0.0001).

There was a reduction in the amount of total IGF-IR following incubation with BMS-754807 for 24 hours. Phosphorylation of IGF-IR was completely inhibited in the presence of BMS-754807 compared with in the absence of BMS-754807. Relatively small amounts of cleaved PARP were detected after 24 hours incubation of cells in full maintenance medium in non-attached conditions. The amount of the cleaved PARP increased significantly by almost 10 fold in the presence of 1 μ M BMS-754807 compared with in the absence of BMS-754807. AKT phosphorylation was decreased significantly in the presence of BMS-754807.

The increase in the level of cleaved PARP and decrease in the level of AKT in the presence of BMS-754807 despite the presence of 10% foetal calf serum in the medium, supports the hypothesis that IGFs play a more important role against anoikis than any other serum components in MDA-MB-231 cells. The results suggest that IGF signalling pathway is important in these triple-negative breast cancer cells.

3.2.10 Effect of IGF-1 on migration of oestrogen receptor-negative breast cancer cells

Increase in cell motility is one of the factors associated with development of malignancy and metastasis. The effect of IGF-1 on migration of oestrogen receptor-negative breast cancer cells was evaluated using a wound healing assay.

3.2.10.1 Effect of IGF-1 on migration of triple-negative MDA-MB-231 and Hs578T breast cancer cells

Both triple-negative MDA-MB-231 and Hs578T cells were plated at high density in 24 well plates which had been scored on their under surface with two parallel lines. Cells were incubated for 24 hours to allow confluent monolayer formation. Cells were washed and withdrawn from the effects of hormones in phenol red-free DMEM medium supplemented with dextran-coated charcoal-treated new born calf serum (DCC-CS) for 16 hours. Cells monolayers were wounded with a sterile pipette tip through the monolayer, washed with phenol free-red DMEM to remove any cell debris or detached cells and incubated in 0.01% BSA and phenol free-red DMEM for 2 hours. After 2 hours of incubation, the first images were taken (time 0). Cells were incubated in the presence or absence of IGF-1 for up to 6 hours and images were captured every two hours. Each condition was in triplicate. The cell migration was evaluated by measuring the distance of the edge of the wound towards the centre of the wound by using Image J software (Figure 3.15). The width of each wound was measured 20 times and the mean of the measurement was calculated.

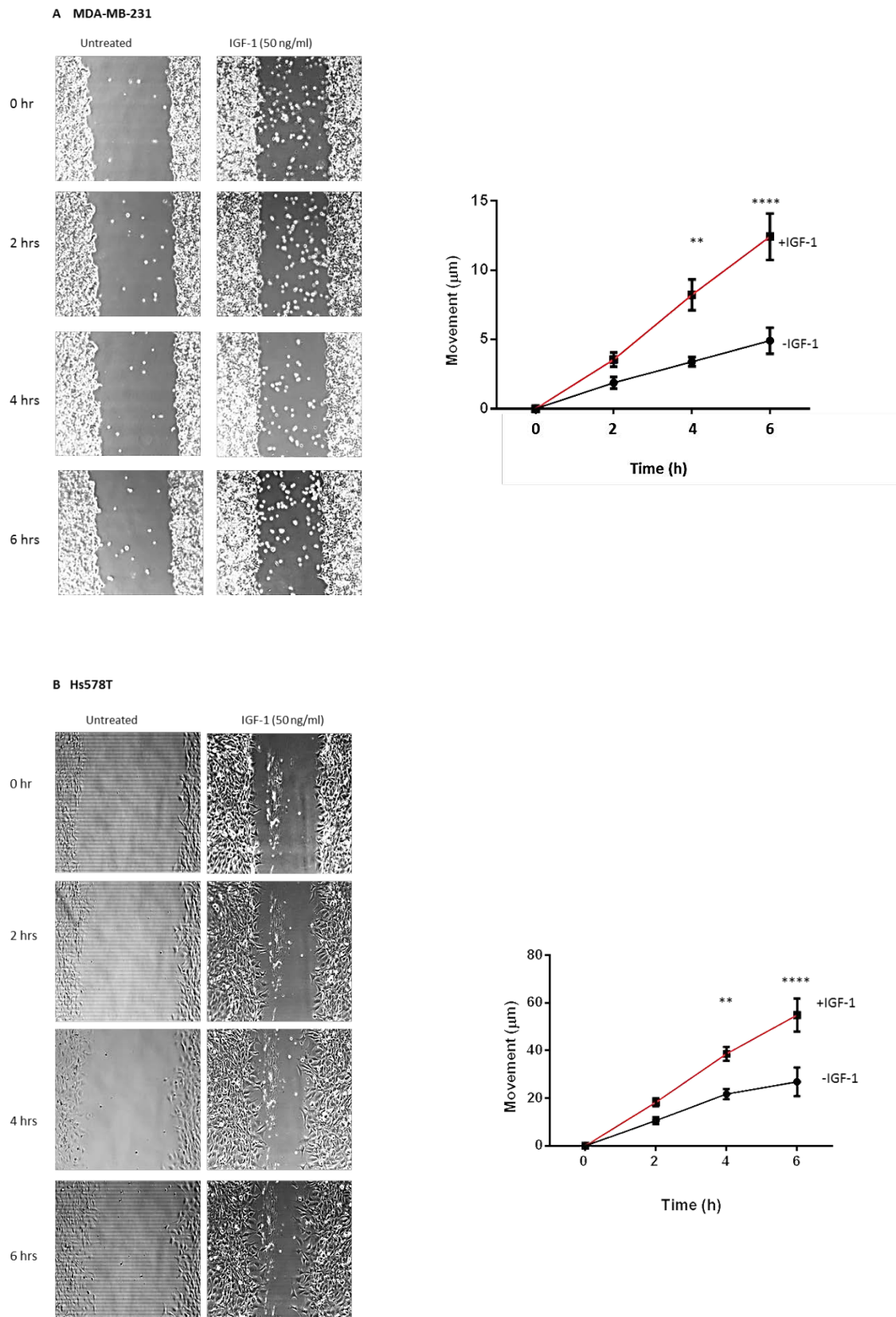


Figure 3.15: Effect of IGF-1 on migration of triple-negative breast cancer cells. MDA-MB-231 (A) and Hs578T (B) cells were seeded at density of 40×10^4 cells/well into 24 well plates that had scored on the under surface with two parallel lines across the middle of each well. Cells were withdrawn in phenol free DMEM medium supplemented with 10% charcoal-treated new born calf serum as described in Materials and Methods Section. The monolayers were wounded with a sterile 20-200 μl Gilson pipette tip through the centre of the well, perpendicular to the scored lines. Wounded cells were washed twice with 0.5 ml of phenol red-free DMEM and

incubated in phenol red-free DMEM supplemented with 0.01% of BSA for two hours. After two hours of incubation, cells were washed again and incubated with phenol red-free DMEM medium and 0.01% BSA alone () or supplemented with 50 ng/ml of IGF-1 (■) for various times up to 6 hours. Images were captured at the beginning of the experiment (time 0) and after each two hours of treatment up to 6 hours. Three separate measurements in three different wells were made for each treatment condition. The distance between the wounds was measured with Image J Software programme. The mean \pm SEM are shown. Statistically significant increases in the cell migration in the presence of IGF-1 compared to the absence of IGF-1 in both MDA-MB-231 and Hs578T cells are shown (one-way ANOVA, Asterisks ** $p < 0.001$, **** $p < 0.0001$).

As illustrated in the Figure 3.15, in both MDA-MB-231 and Hs578T cells, there was a statistically significant increase in the migration of the cells at 4 and 6 hours of incubation in the presence of IGF-1 as compare with in the absence of the IGF-1. IGF-1 enhanced the migration of MDA-MB-231 and Hs578T cells two fold and three fold respectively. On the basis of the demonstrated results, IGF-1 showed a potent migratory effect on both triple-negative breast cancer cells.

3.2.10.2 Effect of IGF-1 on migration of HER-2 positive SK-BR-3 breast cancer cells

The effect of IGF-1 on migration of HER-2 positive SK-BR-3 breast cancer cells was evaluated with the *in vitro* wounding assay as described in detail in section 3.3.1. Wounded monolayers of SK-BR-3 cells incubated in the presence and absence of 50 ng/ml IGF-1 for 0, 2, 4 and 6 hours and at each incubation time images of the cells were captured. Each condition was in triplicate. The width of the each wound was measured 20 times and the average of them was calculated. The cell migration was evaluated by measuring the distance of the edge of the wound towards the centre of the wound by using Image J software (Figure 3.16).

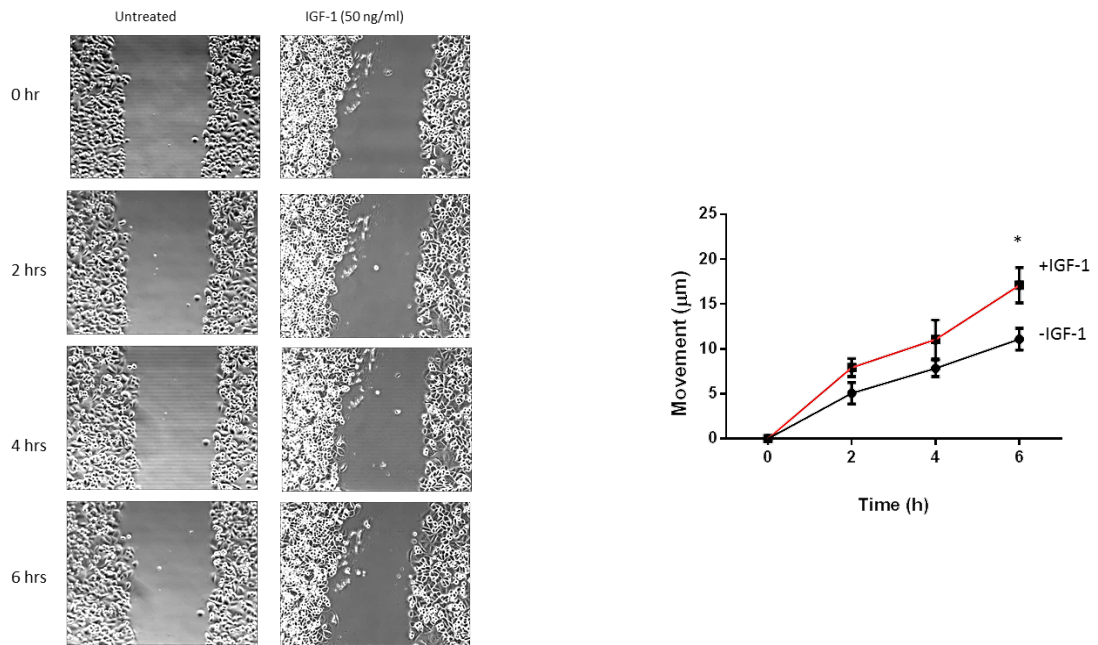


Figure 3.16: Effect of IGF-1 on migration of SK-BR-3 cells. SK-BR-3 cells were seeded at density of 40×10^4 cells/well into 24 well plates that had scored on the under surface with two parallel lines across the middle of each wells. Cells were withdrawn in phenol free DMEM medium supplemented with 10% charcoal-treated new born calf serum as described in Materials and Methods section. Monolayer cells were wounded with a sterile 20-200 µl Gilson pipette tip through the centre of the well, perpendicular to the scored lines. Wounded cells were washed twice with 0.5 ml of phenol red-free DMEM and incubated in phenol red-free DMEM supplemented with 0.01 % of BSA for two hours. After two hours of incubation, cells were washed again and incubated with phenol red-free DMEM medium and 0.01% BSA alone () or supplemented with 50 ng/ ml of IGF-1 (■) for various time points up to 6 hours. Image were captured at the beginning of the experiment (time 0) and after each two hours of treatment up to 6 hours. Three separate measurements in three different wells were made for each treatment condition. The distance between the wounds was measured using Image J software programme. The mean \pm SEM are shown. Asterisks denotes a statistically significant increase in the cell migration in the presence of IGF-1 compared to the absence of IGF-1 in SK-BR-3 cells (One way ANOVA, * $p < 0.05$).

The effect of IGF-1 on migration of SK-BR-3 cells are illustrated in Figure 3.16. IGF-1 stimulated the migration of the cells. There were no statistically significant movement after 2 and 4 hours of incubation but statistically significant movement was observed in the presence of IGF-1 after 6 hours of incubation. The increase in the motility of SK-BR-3 cells in the presence of IGF-1 suggest that IGF-1 plays a role in inducing the migration of this cell line.

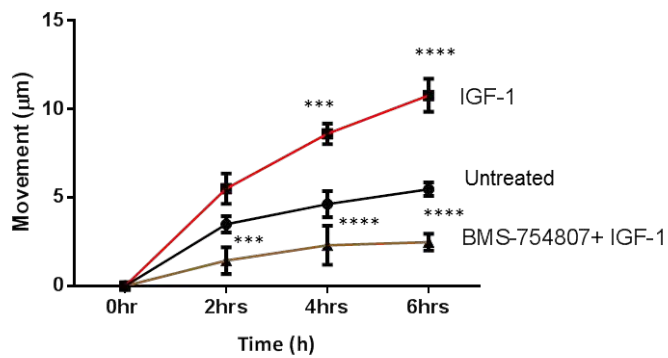
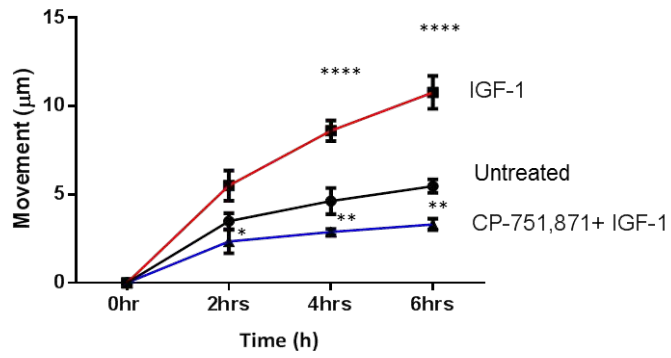
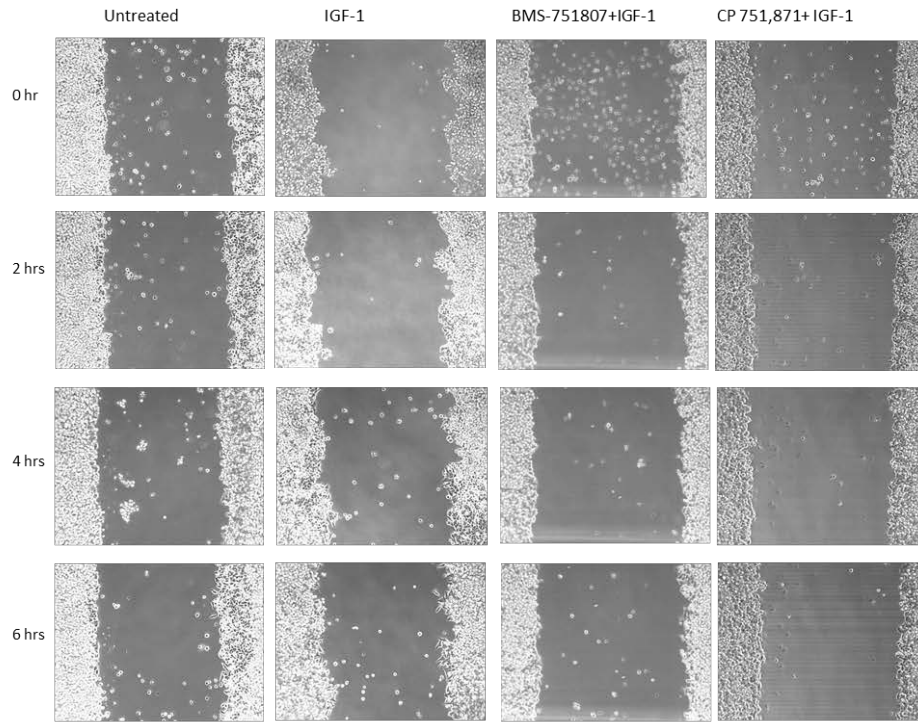
3.2.11 Effect of antibody against IGF-IR and a dual tyrosine kinase inhibitor of IGF-IR and the insulin receptor on migration of oestrogen-receptor negative breast cancer cells

In the previous sections the powerful effect of IGF-1 on migration of oestrogen receptor-negative breast cancer cells was evaluated. The ability of the type I IGF receptor antibody CP-751, 871 or the dual tyrosine kinase inhibitor BMS-754807 to inhibit the migratory effect of IGF-1 was investigated with wound healing assay used in oestrogen receptor-negative cell lines, as described in Section 3.3.1.

3.2.11.1 Effect of an antibody against IGF-IR and dual tyrosine kinase inhibitor of IGF-IR and the insulin receptor on migration of triple-negative MDA-MB-231 and Hs578T breast cancer cells

Wounded MDA-MB-231 and Hs578T cells were incubated in the presence and absence of 50 ng/ml IGF-1 and in the presence and absence of 10 µg CP-751, 871 or 1 µM BMS-754807 at 0, 2, 4 and 6 hours (Figure 3.17).

A MDA-MB-231



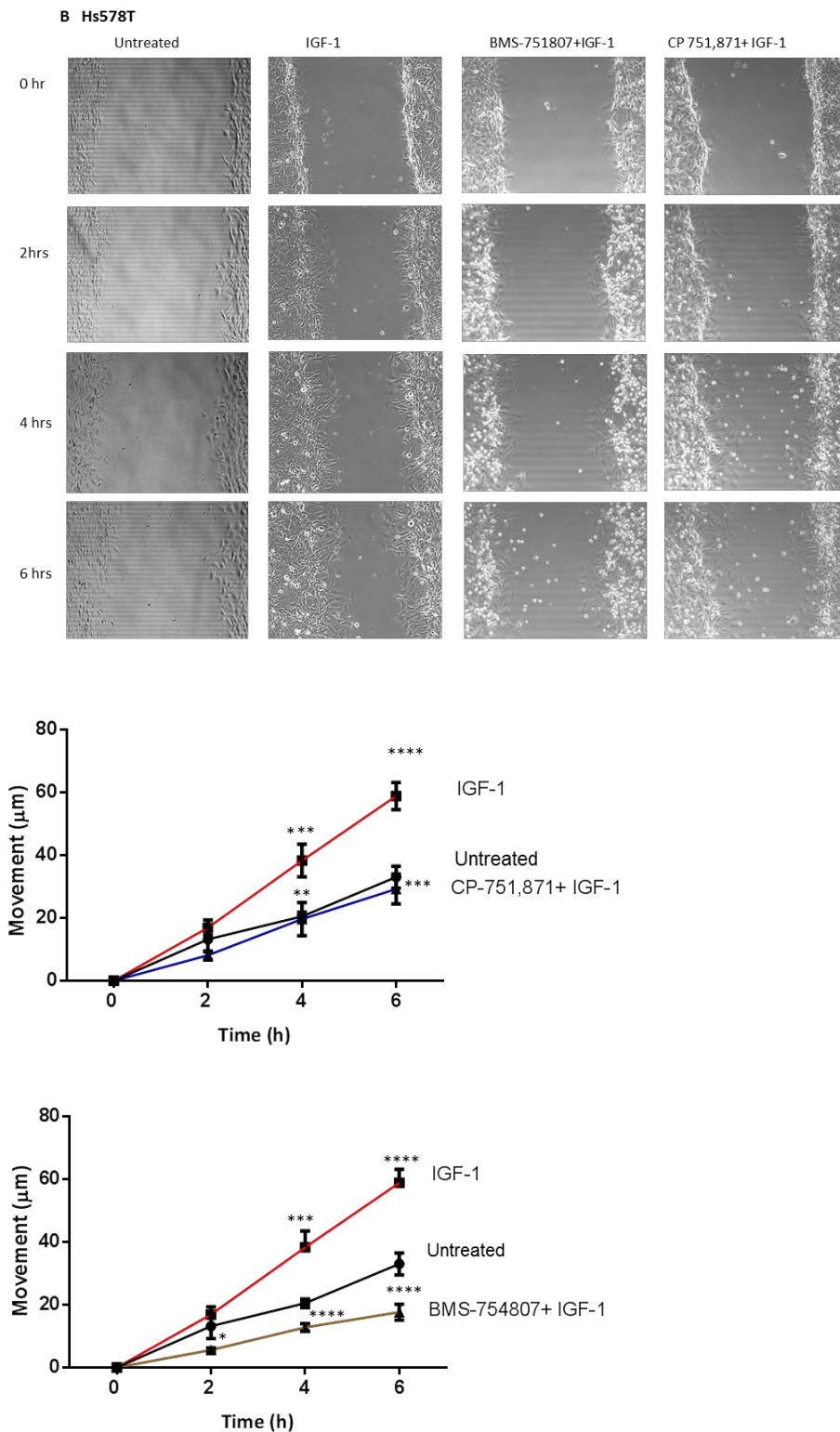


Figure 3.17: Effect of antibody against IGF-IR and dual tyrosine kinase inhibitor of IGF-IR and the insulin receptor against migration of triple-negative breast cancer. MDA-MB-231 (A) and Hs 578T (B) cells were seeded at density of 40×10^4 cells/well into 24 well plates that had scored on the under surface with two parallel lines across the middle of each wells. Cells were withdrawn in phenol free DMEM medium supplemented with 10% charcoal-treated new born calf serum as described in Materials and Methods Section. Monolayer cells were wounded with a sterile 20-200 μ l Gilson pipette tip through the centre of the well, perpendicular to the scored lines. Wounded cells were washed twice with 0.5 ml of phenol red-free DMEM and incubated in phenol red-free DMEM supplemented with 0.01% of BSA for two hours. After two hours of incubation, cells were washed again and incubated with phenol red-free DMEM

medium and 0.01% BSA alone () or supplemented with 50 ng/ml of IGF-1 (-■-), or in the presence of 1 μ M BMS-754807 and 50 ng/ml IGF-1 (), or in the presence of 10 μ g/ml CP-751807 and 50 ng/ml IGF-1 (-▲-) for various time up to 6 hours. Images were captured at the beginning of the experiment (time 0) and after each two hours of treatment up to 6 hours. Three separate measurements in three different wells were made for each treatment condition. The distance between the wounds was measured using Image J software programme. The mean \pm SEM are shown. Asterisks denotes that there was statistically significant reduction in the migration of cells in the presence of CP-751,871 compared to its absence and in the presence of BMS-754807 compared with in the absence of BMS-754807 in both MDA-MB-231 and Hs578T cells (One way ANOVA, * p <0.05, ** P <0.001, *** p =0.0001, **** P <0.0001).

As illustrated in the Figure 3.17, there was a statistically significant increase at 4 and 6 hours of incubation in migration of both MDA-MB-231 and Hs578T cells in the presence of IGF-1 as previously observed. In the presence of CP-751, 871 cell movements significantly decreased in both cell lines. In MDA-MD-231 cells, in the presence of CP-751,871 cell movement that was stimulated with IGF-1 significantly decreased two fold after 2 and 4 hours of incubation and almost three fold after 6 hours. In Hs578T cells, CP-751,871 reduced the migratory effect of IGF-1 almost two fold after 2 and 4 hours of incubation and two and half fold after 6 hours. In the presence of BMS-754807, the motility of the MDA-MB-231 cells in the presence of IGF-1 was significantly reduced two fold after incubation for 2 hours and gradually decreased five fold after 6 hours of incubation. The migration of Hs578T cell in response to IGF-1 was significantly decreased two fold after 2 hours of incubation with BMS-754807 and almost three and half fold after 6 hours incubation.

The results demonstrated the effectiveness of both inhibitors on inhibition the migratory effect of IGF-1 in both triple-negative breast cancer cell lines, and that dual tyrosine kinase inhibitor has more inhibitory effect on migratory effect of IGF-1 compared to the type I IGF receptor antibody in both triple-negative breast cancer cells.

3.2.11.2 Effect of antibody against IGF-IR and dual tyrosine kinase inhibitor of IGF-IR and the insulin receptor on migration of HER-2 positive SK-BR-3 breast cancer cells

The ability of antibody against type I IGF receptor CP-751, 871 and a dual tyrosine kinase inhibitor BMS-754807 to inhibit the migration of SK-BR-3 stimulated with IGF-1 was tested in the *in vitro* wounding assay. Wounded SK-BR-3 cells were incubated with the presence and absence of the IGF-1 and the two inhibitors for different times up to 6 hours (Figure 3.18).

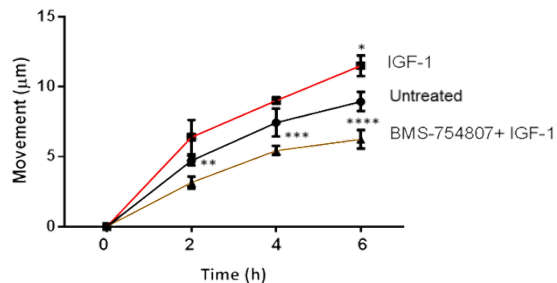
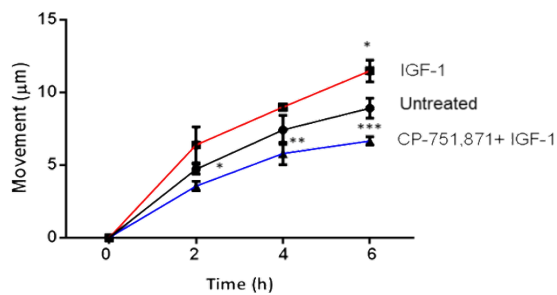
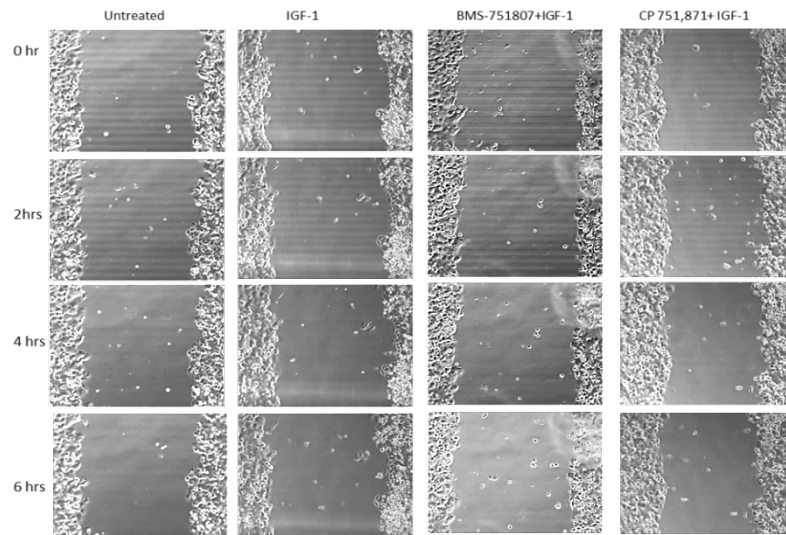


Figure 3.18: Effect of antibody against IGF-IR and dual tyrosine kinase inhibitor on SK-BR-3 cells. SK-BR-3 cells were seeded at density of 40×10^4 cells/well into 24 well plates that had scored on the under surface with two parallel lines across the middle of each wells. Cells were withdrawn in phenol red-free DMEM medium supplemented with 10% charcoal-treated new born calf serum as described in Materials and Methods section. Monolayer cells were wounded with a sterile 20-200 μ l Gilson pipette tip through the centre of the well, perpendicular to the scored lines. Wounded cells were washed twice with 0.5 ml of phenol red-free DMEM and incubated in phenol red-free DMEM supplemented with 0.01 % of BSA for two hours. After two hours of incubation, cells were washed again and incubated with phenol red-free DMEM medium and 0.01% BSA alone () or supplemented with 50 ng/ml of IGF-1 (■), or in the presence of 1 μ M BMS-754807 and 50 ng/ml IGF-1 (), or in the presence of 10 μ g/ml CP-751807 and 50 ng/ml IGF-1 (▲) for various time points up to 6 hours. Image were captured at the beginning of the experiment (time 0) and after each two hours of treatment up to 6 hours. Three separate measurements in three different wells were made for each treatment condition. The distance between the wounds was measured using Image J software programme. The mean \pm SEM are shown. Asterisks denotes that there was statistically significant reduction in the migration of cells in the presence of CP-751,871 compared to its absence and in the presence of BMS-754807 compared with in the absence of BMS-754807 in SK-BR-3 cells. (One way ANOVA, ** $p < 0.001$, *** $p = 0.0001$, **** $P < 0.0001$).

In response to the IGF-1, SK-BR-3 cells migrated more than in the absence of IGF-1 after 6 hours incubation ($p=0.035$). Cell motility stimulated with IGF-1 was significantly reduced in the presence of CP-751,871 two fold after each period of incubation. Treatment of the cells with BMS-754807 significantly reduced the migration of the cells stimulated with IGF-1 one and half fold after 4 hours and two and half after 6 hours of incubation.

3.3 Discussion

The ability to evade anoikis and increase in cell motility is important in the progression of malignant epithelial tumours and contributes to tumour metastasis. Anoikis resistance or survival of cells without attachment to the extracellular matrix is essential in enhancement of metastatic potential. Understanding the molecular mechanisms involved in anoikis resistance and migration of cancerous cells would be a requirement in development of potential therapeutic approaches.

In this chapter, the effect of IGF-1 on survival and migration of oestrogen receptor-negative breast cancer cells was investigated. Effect of IGF-1 on cell survival of oestrogen receptor-negative breast cancer cells cultured in non-attached condition was evaluated. The key components of IGF signal transduction pathways were functional in all tested oestrogen receptor-negative MDA-MB-231, Hs578T and SK-BR-3 breast cancer cell lines, as they were activated in response to stimulation with IGF-1 in a concentration-dependant manner.

Valentinis *et al* (1999) reported that in mouse embryonic fibroblasts, overexpression of the type I IGF receptor in the presence of IGF-1 induced anoikis resistance which was related to both the presence of type I IGF receptor and the concentration of IGF-1 (Valentinis, Reiss *et al.* 1998). Another study reported suppression of anoikis in the presence of IGF-1 via IGF-IR in human epithelial prostate cancer cell line LNCaP by culturing the cells in matrix-deprived condition (Kim, Koo *et al.* 2012). There are few studies on the effect of IGFs against anoikis in breast cancer cells. In 2005, Ravid *et al* reported loss of reduction in number of viable MCF-7 cells assessed by mitochondrial activity with an MTT assay, after culturing the cells in non-attached condition in serum-free medium in the presence of IGF-1 (Ravid, Maor *et al.* 2005). Sachdev *et al* (2009) reported that blocking the type I IGF receptor signalling pathway can increase the cell death, as measured by trypan blue exclusion in a metastatic variant of MDA-MB-435 breast cancer cells in non-attached conditions (Sachdev, Zhang *et al.* 2009).

In 2011, Davison *et al* illustrated the potential effect of IGF signalling pathway on survival of oestrogen non-responsive breast cancer cells by using

staurosporine-induced cell death (Davison, de Blacquiere et al. 2011). The effect of IGF-1 on anoikis of oestrogen-receptor negative breast cancer cells were not studied before.

The present study has shown that MDA-MB-231, Hs578T and SK-BR-3 cells underwent anoikis after culture in poly-HEMA coated plates. Stimulation with IGF-1 powerfully prevented anoikis in non-attached MDA-MB-231 and Hs578T cells cultured in serum-free medium, as this was demonstrated by reduction in the amount of cleaved PARP and cleaved caspase-3 in MDA-MB-231 cells and inhibition of PARP cleavage in Hs578T. These results are in agreement with the previous finding that IGF-1 has an important role on survival of lung cancer cells in anchorage-dependent condition (Sachdev, Zhang et al. 2009) and support the supposition that IGF-1 has important role on survival of triple-negative breast cancer cells (Davison, de Blacquiere et al. 2011).

IGF-1 had minor effects on suppression of anoikis in HER-2 positive SK-BR-3 breast cancer cells. A concentration-dependent effect of IGF-1 was observed in both MDA-MB-231 and Hs578T cells. There was a variation in potential effect of IGF-1 in suppression of anoikis in between cell lines. The protective effect was greater in any tested concentration of IGF-1 in Hs578T cells than MDA-MB-231 cells, with complete inhibition of anoikis in Hs578T cells. This variation might be due to the different level of sensitivity towards anoikis in the cell lines, as MDA-MB-231 appeared to be more susceptible and Hs578T cells more resistant. Another explanation can be due to requirement of various concentrations of IGF-1 in order to activate survival pathways, such as PI3K and AKT pathway in different cell lines. For instance here, MDA-MB-231 cells required higher concentrations of IGF-1 in order to activate PI3K and AKT pathway compared to Hs578T cells.

Dual tyrosine kinase inhibitor of type I IGF receptor and the insulin receptor BMS-754807 effectively inhibited the survival effect of IGF-1 in both MDA-MB-231 and Hs578T cells, followed by complete inhibition of the activation of AKT in both cell lines, also BMS-754807 showed inhibitory effect on MAPK activation in both cell lines as significant reduction in the activation of MAPK was observed. This is in agreement with the previous study on effective inhibition of BMS-754807 on phosphorylation of IGF-IR, AKT and moderate inhibition of

MAPK phosphorylation in MCF-7 cells (Mezi, Todi et al. 2012). Disruption in type I IGF receptor has been reported to make lung cancer cells more susceptible to anoikis, as greater loss of cell viability was observed using trypan blue exclusion (Sachdev, Zhang et al. 2009). Disruption in the activity of the type I IGF receptor through inhibitory antibody reduced the survival effect of IGF-1 by increasing the cell death in both MDA-MB-231 and Hs578T cells. Both tested inhibitors demonstrated inhibition of the survival effect of IGF-1 in both triple-negative cell lines; however the ability of the tyrosine kinase inhibitor to induce cell death and inhibit the PI3K and AKT pathway is a desirable characteristic.

The importance of the activity of type I IGF receptor and the insulin receptor in non-attached condition was shown in MDA-MB-231 cells by increase in the cell death detected in the presence of dual tyrosine kinase inhibitor of type I IGF receptor and insulin receptor, despite the presence of serum in the culture medium. Enhancement in the activation of IGF signal transduction pathway was not affected by other serum components. Inhibition of type I IGF receptor by CP-751, 871 antibody did not increase the anoikis in MDA-MB-231 cells cultured in maintenance medium (data not shown), indicating that inhibition of both type I IGF receptor and the insulin receptor might have greater importance in cell survival in non-attached condition.

In the second part of this chapter, the role of IGF-1 on migration of oestrogen-receptor negative breast cancer cell lines was investigated in a monolayer wound-healing migration assay. Several studies addressed the implication of IGF-1 in migration of different malignant cells. Some literature reports the interaction of tumor cells with IGF-1 which results in mediating the tumor cells motility behavior (Friedl and Wolf 2003, Bravo-Cordero, Hodgson et al. 2012). In 2010, Metalli *et al* reported that IGF-1 induced migration of bladder cancer cells through activation of type I IGF receptor as measured the migration of cells by wound healing assay. Tang et al in 2008 reported the migratory effect of IGF-1 on migration MDA-MB-435 breast cancer cells by using the migration track assay (Tang, Su et al. 2008). It has been reported that IGF-1 induce migration of oestrogen receptor-positive MCF-7 cells and oestrogen receptor-negative MDA-MB-231 cells by using modified Boyden chamber (Bartucci, Morelli et al. 2001). Doerr *et al* in 1996 demonstrated that inhibition of IGF-IR using the

monoclonal IGF-IR inhibitory antibody α IR-3 can prevent the migration of MCF-7 and MDA-MB-231 breast cancer cells (Doerr and Jones 1996).

In the present study, IGF-1 has been shown to stimulate migration of all oestrogen receptor-negative MDA-MB-231, Hs578T and SK-BR-3 breast cancer cells; as evidenced by reduction in distance between the measured wounds. The increase in migration stimulated by IGF-1 in MDA-MB-231, Hs578T and SK-BR-3 cells is 6 μ m, 30 μ m and 3 μ m respectively, which is comparable to the distances reported in other studies (Jackson, Zhang et al. 2001, Mezi, Todi et al. 2012). This is agreed with previous studies that IGF-1 induces migration in oestrogen receptor-negative MDA-MB-435 breast cancer cells (Tang, Su et al. 2008). The migratory effect of IGF-1 was found to be higher in MDA-MB-231 and Hs578T than in SK-BR-3 cells.

Inhibition of type I IGF receptor reported to significantly reduce the metastasis of MDA-MB-435 breast cancer cells, also shown to decrease the number of circulating tumour cells (Sachdev, Zhang et al. 2009). In the same study, disruption of type I IGF receptor investigated on invasion of lung cancer cells using Boyden chamber assay, and showed that disruption of type I IGF receptor inhibits the invasion of cells. Here, the current study shown that migratory effect of IGF-1 was inhibited by treatment of cells with antibody against type I IGF receptor CP-751,871, which is consistent with the previous findings that abrogation of type I IGF receptor inhibits the migration of breast cancer cells (Doerr and Jones 1996, Sachdev, Zhang et al. 2009). The growth inhibitory effects of dual tyrosine kinase inhibitor BMS-754807 on growth of triple-negative breast cancer tumourgrafts were reported (Jackson, Zhang et al. 2001). In the present study, dual tyrosine kinase inhibitor BMS-754807 showed potent inhibition against migratory effect of IGF-1. The significance of the inhibitory effect of BMS-754807 was slightly greater than the significance of the inhibitory effect of the CP-751, 871 antibody in all three cell lines tested after 6 hours of migration ($p < 0.0001$ and $p = 0.0001$, respectively).

In conclusion, the findings in this chapter demonstrate that IGF signal transduction pathway activation plays a significant role in survival of triple-negative breast cancer cells in anchorage-independent condition and that, IGF-1 is important in stimulate the increase in migration of oestrogen receptor-

negative breast cancer cells. Thereby, IGF-1 might contribute to the metastatic potential of breast cancer cells by participation in promotion of migration and survival of oestrogen receptor-negative breast cancer cells and that, targeting the IGF signal transduction pathway could provide valuable information for the treatment of metastatic oestrogen receptor-negative breast cancer.

Chapter 4. Immunohistochemical expression of insulin-like growth factor receptor (IGF-IR)

4.1 Introduction

As breast tissue grows, it undergoes several physiological and morphological changes cyclically under hormonal regulation. Hormones are considered as the key determinants for the majority of benign and malignant lesions in the breast (Guinebretiere, Menet et al. 2005). Well-known breast carcinoma prognostic factors such as tumour size, histological grade, presence of involved axillary lymph nodes, and hormonal receptor status play important roles in the identification of high risk patients with poor prognosis who might benefit from more aggressive therapies. These recognized prognostic factors are not sufficient for final treatment decisions; hence identification of biomarkers can facilitate prediction of the response of tumours to different therapies and improve the individualized treatment decision.

Various clinical and experimental studies addressed the important role of IGF-IR in breast cancer development and progression (Cohen, Baker et al. 2005, Davison, de Blacquiere et al. 2011, Pollak 2012). Other studies reported deregulation of the IGF signal transduction pathway in different human cancers including breast cancer and increased resistance of breast cancer cells to chemotherapy and radiotherapy associated with overexpression and activation of the type I IGF receptor (Lu, Zi et al. 2001, Morgillo, Woo et al. 2006, Sachdev and Yee 2006). It has been reported that IGF-IR function is associated with the action of oestrogen receptor in hormone-responsive breast cancer cells (Stewart, Johnson et al. 1990, Surmacz 2000). In an immunohistochemical study, activation of IGF-IR has been reported as a prognostic factor for breast cancer and that its activation was shown to be correlated with poor survival outcome in different breast cancer subtypes (Law, Habibi et al. 2008).

The results of the *in vitro* studies described in chapter 3 demonstrate an effect of IGF-1 on survival and migration of oestrogen receptor-negative breast cancer cells. The expression of the type I IGF receptor in different subtypes of breast carcinoma and its association with proliferation marker, apoptotic marker and various clinico-pathological criteria in oestrogen receptor-negative breast tumours has not been studied before. This chapter is focused on the analysis of type I IGF receptor expression, and the possible correlations between IGF-IR

expression and expression of a proliferation marker Ki-67, an apoptotic marker cleaved caspase-3 in oestrogen receptor-negative breast tumours and in involved lymph nodes by immunohistochemistry.

4.2 Patients demographic characteristics

Oestrogen receptor-negative breast samples were selected without further bias from patients diagnosed with breast cancer at the Newcastle NHS trust, Royal Victoria Infirmary breast clinic during 2000-2005. The samples consist of 92 surgical resection specimens. From these, we studied 7 samples of normal breast tissue. In addition, the cases investigated included 37 *in situ* breast carcinomas, 79 invasive breast carcinomas and 26 axillary lymph nodes harbouring metastatic breast tumour cells.

4.3 Results

4.3.1 Optimisation of the anti-IGF-IR antibody concentration

To select the optimal dilution for the anti-IGF-IR antibody suitable for immunohistochemistry, three different concentrations of the anti-IGF-IR β antibody (cell signalling #3027L) were tested. At a dilution of 1 in 1200, the anti-IGF-IR antibody gave strong and specific membranous immuno-reaction with minimal background (Figure 4.1 C). The immuno-reaction was weaker at a dilution of 1 in 1800 (Figure 4.1 B) and much weaker at dilution of 1 in 2400 (Figure 4.1 A). An appropriate IGF-IR immuno-reaction was obtained with 1 in 1200 concentration.

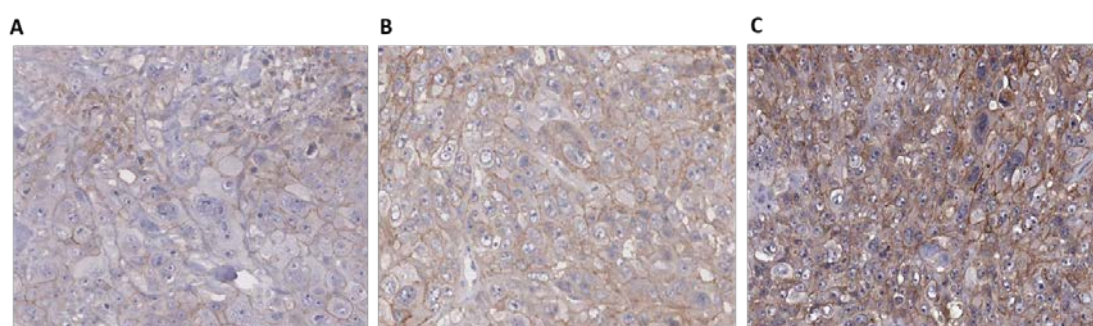


Figure 4.1: Optimisation of immunohistochemistry with the anti-IGF-IR antibody. A breast tumour that expresses IGF-IR was analysed by immunohistochemistry with anti-IGF-IR antibody at a dilution of 1 in 2400 (A), 1 in 1800 (B) and 1 in 1200 (C). Strong membranous immunoreaction was detected at 1 in 1200 dilution. Magnification is x200.

4.3.2 Expression of IGF-IR in normal breast tissue

Expression of IGF-IR was investigated in 7 cases of normal breast tissue by immunohistochemistry. Expression of IGF-IR was detected in all 7 cases. The mean histoscore was 137 and median histoscore was 160. The immuno-reaction was strongly membranous (Figure 4.2).

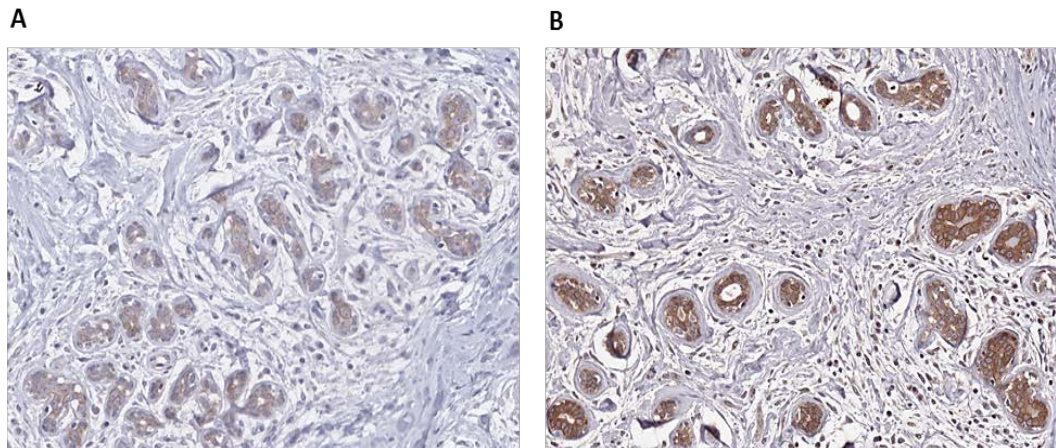


Figure 4.2: Expression of IGF-IR in normal breast tissue. Sections of normal breast tissue that have a histoscore of 125 (**A**) and 330 (**B**) are shown. Membranous immuno-reaction of IGF-IR was detected in epithelial cells that line the acini. Original magnification is x200.

4.3.3 Expression of IGF-IR in *in situ* carcinoma of the breast

Expression of IGF-IR was detected in 32 lesions out of 37 *in situ* ductal carcinoma cases (86%). IGF-IR expression was membranous (Figure 4.3). The majority of the positive tumour cells expressed IGF-IR at a weak level and the mean histoscore was 110.95 and median histoscore was 72. However, 4 tumours were strongly positive for expression of IGF-IR with a maximum histoscore of 706.

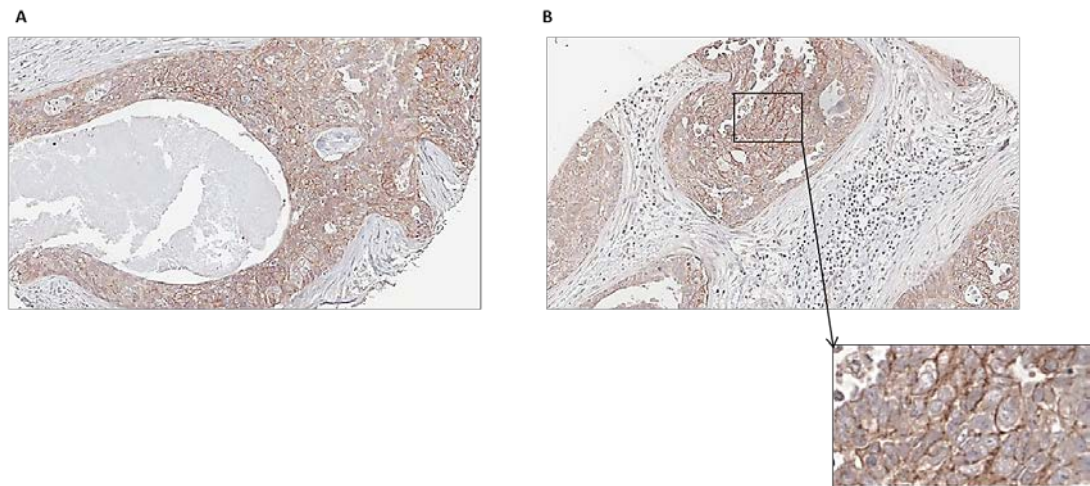


Figure 4.3: Expression of IGF-IR in *in situ* breast carcinomas. Section of (A) comedo and (B) solid type of ductal carcinoma *in situ* with histoscores of 706 and 368, respectively were analysed by immunohistochemistry for expression of IGF-IR. The inset shows *in situ* neoplastic cells. Magnification is x200 for inset and x20 for the images.

4.3.4 Expression of IGF-IR in invasive carcinoma of the breast

All invasive breast tumours included in the study were analysed for IGF-IR expression in duplicate cores. The consistency of expression between the duplicate cores was tested. There was a strong correlation between IGF-IR expression measured in the duplicate cores (Pearson correlation, $p=0.001$). There is no significant difference in the expression levels in the duplicate cores (Wilcoxon ranked test $p=0.843$, Figure 4.4). This finding supports the consistency of the results, thereby validity of evaluating biomarkers expression in one millimetre cores of tissue. The mean values were used for the further analyses.

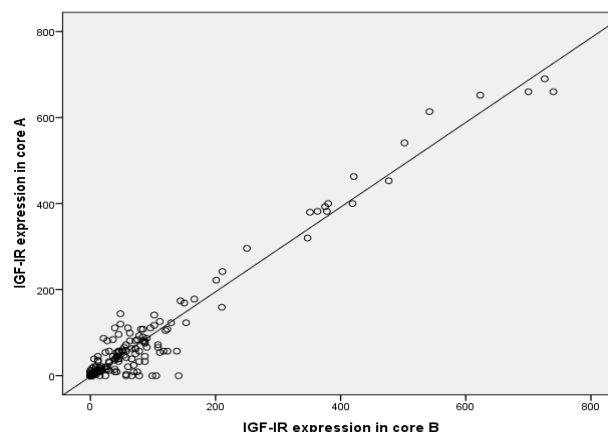


Figure 4.4: Expression of IGF-IR in the duplicate cores of invasive tumours. Two cores (A and B) of one millimetre in diameter were taken from each tumour for the construction of TMA blocks. IGF-IR immuno-reactive sections were analysed for IGF-IR independently. There was a strong correlation of IGF-IR expression between the two cores tested in each tumour (Pearson correlation $p=0.001$).

In the invasive lesions investigated, IGF-IR immuno-reaction was demonstrated in 73 tumours out of 79 invasive carcinomas (92%). The immuno-reaction for IGF-IR was membranous (Figure 4.5) and most of the IGF-IR positive tumours showed weak expression of IGF-IR with a mean histoscore of 94 and median histoscore of 42. However, 7 tumours strongly expressed IGF-IR with maximum histoscore of 630. IGF-IR immuno-reaction was not detected in 6 tumours.

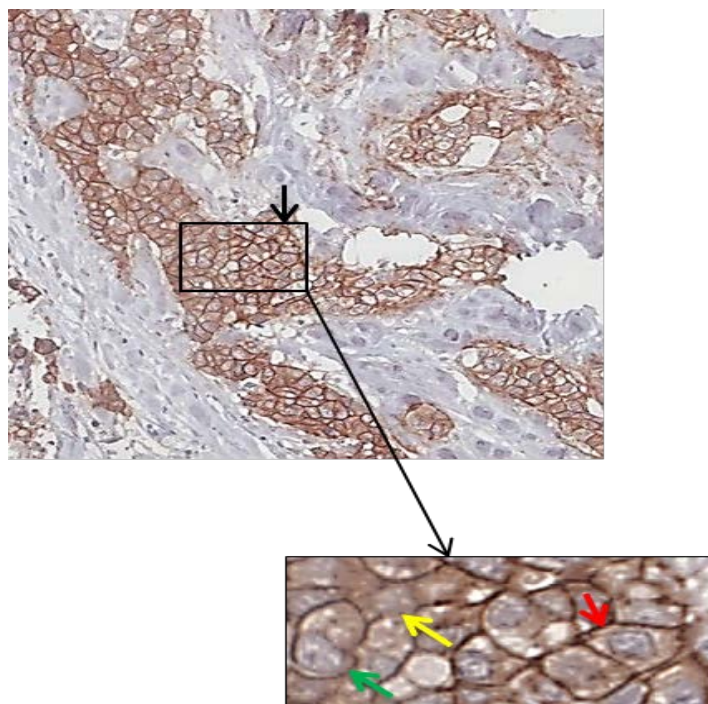


Figure 4.5: Expression of IGF-IR in invasive breast cancer. Sections of invasive breast carcinomas with histoscore of 630 were tested for IGF-IR expression by immunohistochemistry. Specific membranous immuno-reaction was demonstrated in the neoplastic cells (black arrow). Yellow arrow indicates the malignant epithelial cells with weakly positive, green arrow moderately positive and red arrow strongly positive for expression of IGF-IR. Specific membranous immuno-reaction was demonstrated. Magnification is x40 and x200 for inset.

4.3.5 IGF-IR expression and axillary lymph node metastasis

To analyse the role of IGF-IR in further progression of breast cancer, IGF-IR expression was analysed in both primary tumours and corresponding metastatic lymph nodes deposits from same patient. Accurate histological assessment of the axillary lymph node was available for 26 cases. Out of 26 cases, 3 cases were free of tumour cells, while metastatic deposits were detected in 23 cases. There was a significant correlation between the expression of IGF-IR in the primary tumour cells and tumour cells with metastatic lymph node deposits (Pearson correlation, $p=0.001$, Figure 4.6). The expression of IGF-IR was found

to be higher in primary tumours (mean histoscore=207) than their metastatic counterparts (mean histoscore=143). The association of IGF-IR expression with number of involved lymph nodes were tested. There was no significant correlation between expression of IGF-IR and the number of axillary lymph node metastasis (Spearman's rho correlation, $p=0.130$).

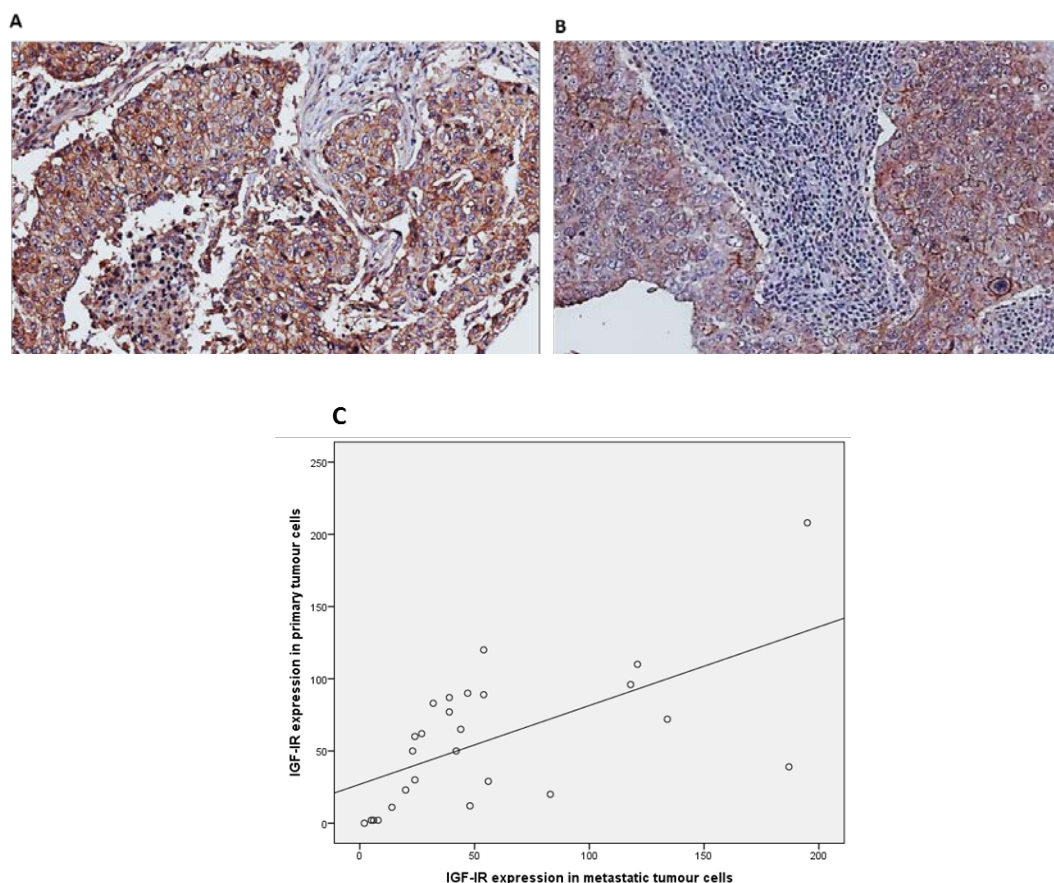


Figure 4.6: IGF-IR expression in primary and metastatic breast tumour cells in lymph nodes. IGF-IR expression was analysed by immunohistochemistry in both primary (A) and metastatic (B) tumour cells of the same patients in 26 tumours. There was a strong significant association between IGF-IR expression in primary and metastatic tumour cells ($p=0.001$) (C). Magnification is x200.

4.3.6 Expression of IGF-IR in different types of breast lesions

IGF-IR expression was demonstrated in normal breast tissues and different types of breast lesions. IGF-IR expression was detected in the epithelial cells of majority of the lesions and in all normal tissues (Figure 4.7). Amongst different types of breast lesions, IGF-IR is mostly expressed in invasive breast tumours.

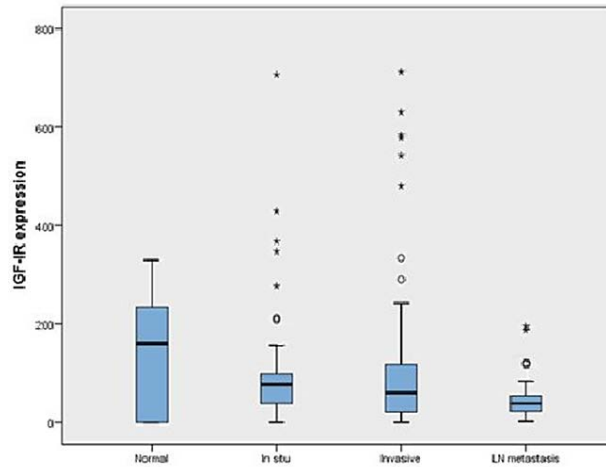


Figure 4.7: Level of expression of IGF-IR in different breast lesions. The expression of IGF-IR in normal breast tissues, *in situ* carcinomas, invasive carcinomas and metastatic breast tumour cells (LN metastasis). The horizontal bars represent the median range of the data, the boxes represent the 50th percentiles, whiskers represent the range of data, the circles represent the outlier values and the asterisks refer to extreme values.

4.3.7 IGF-IR expression and patient's age

The age of the women investigated ranged from 33 to 97 years. The mean age was 59.4 and the median was 58.5 years. There was no significant correlation between expression of IGF-IR and the age of patients (Pearson's rho correlation, $p=0.625$, Figure 4.8).

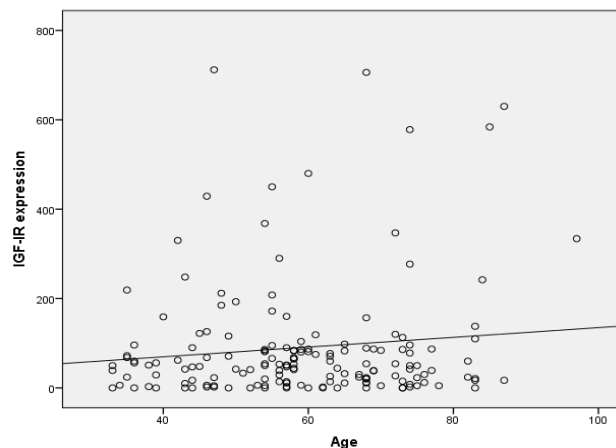


Figure 4.8: Association of IGF-IR expression with patient age. IGF-IR expression is shown against patient's age.

4.3.8 IGF-IR expression and the tumour size and grade

The association between IGF-IR expression and tumour grade and size was investigated. The tumour size ranged from 14 mm to 90 mm. the mean size was 27 mm and the median was 23 mm. The expression level of IGF-IR showed no

association with size of the tumour (Pearson's rho correlation, $p=0.376$). Histological grade of the invasive tumours were assessed by Elston and Ellis's modification of Bloom and Richardson grading method. Twelve tumours of the investigated tumours were moderately differentiated (grade 2) and 67 tumours were poorly differentiated (grade 3). IGF-IR expression was not associated with the tumour grade (Spearman's rho correlation, $p=0.515$). There was no significant differences in the expression level of IGF-IR in lower grade and high grade tumours (Mann-Whitney test, $p=0.448$, Figure 4.9).

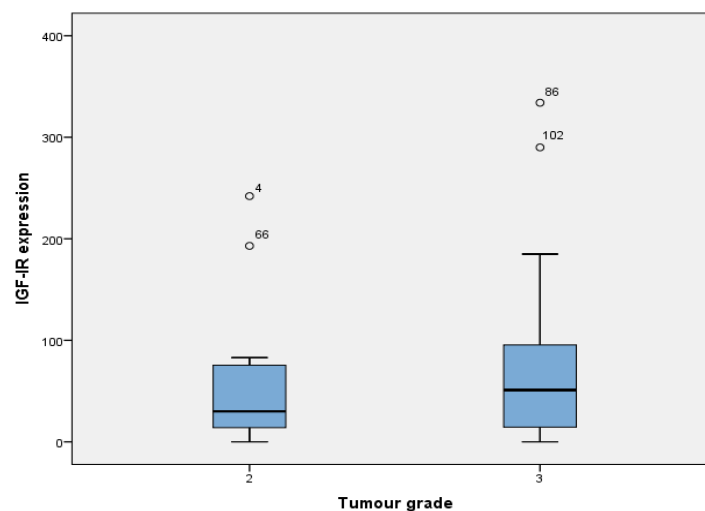


Figure 4.9: Association of IGF-IR expression with tumour grade. The expression of IGF-IR is shown against the histological grades of invasive breast cancer. The horizontal bars represent the median values, the boxes represent the 50th percentiles, the whiskers represent the range of data, the dots refer to outlier values and the star refers to extreme values.

4.3.9 Association between expression of IGF-IR and expression of Ki-67 in malignant breast tumours

It has been reported that IGF-I stimulates the proliferation of oestrogen non-responsive breast cancer cells and that positive stimulation is mediated by IGF-IR *in vitro* (Davison, de Blacquiere et al. 2011). To investigate the possibility that IGF-IR expression is associated with proliferation of breast cancer cells, the expression of Ki-67 was measured in *in situ* and invasive breast carcinoma and in tumour cells with axillary lymph node metastatic deposits by immunohistochemistry. Correlation between IGF-IR expression and expression of proliferation marker Ki-67 was tested. The statistical analysis was tested on 142 cases which include *in situ* carcinomas, invasive carcinomas and tumour cells with lymph node metastases and there was a significant correlation between the expression of IGF-IR and Ki-67 expression in all the breast lesions

analysed (Pearson's rho correlation $p=0.028$). Further analysis evaluated in which particular tumour type of breast lesions the association was apparent.

4.3.9.1 Association between expression of IGF-IR and expression of Ki-67 in *in situ* breast carcinoma

Ki-67 expression was investigated in 37 *in situ* breast carcinomas, of which its expression was detected in 28 tumours with mean histoscore of 82.5 and median histoscore of 54. Overall, Ki-67 was expressed at weak to moderate levels in *in situ* breast carcinomas. Ki-67 immuno-reaction was nuclear (Figure 4.10).

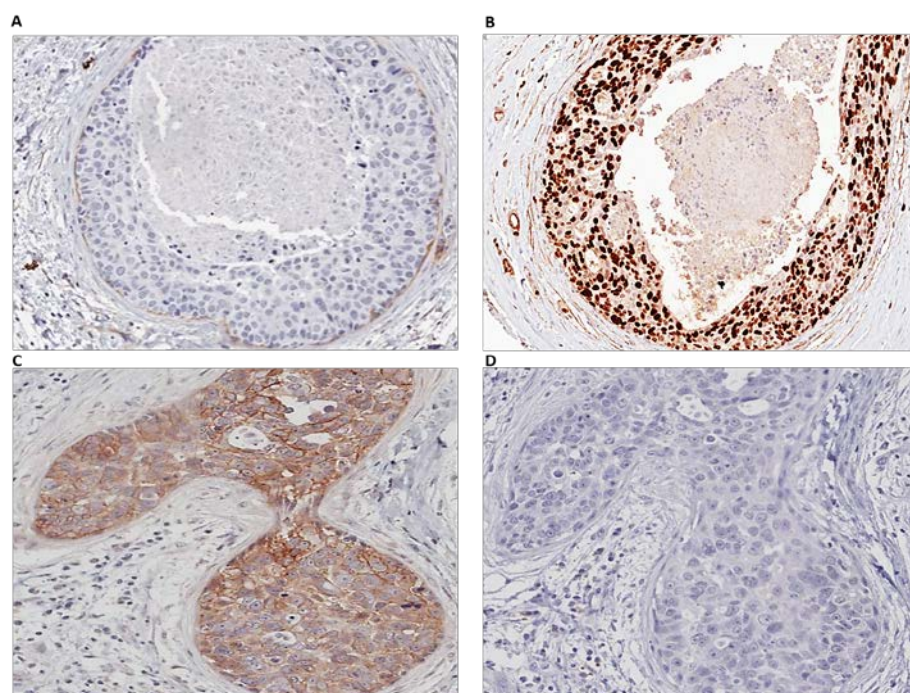


Figure 4.10: IGF-IR expression and expression of Ki-67 in *in situ* breast carcinoma. Sections from comedo type (A, B) and solid type (C, D) of *in situ* breast carcinomas were tested for IGF-IR (A, C) and Ki-67 (B, D) expression by immunohistochemistry. Magnification is x200.

The association between expression of IGF-IR and Ki-67 was investigated in the 37 *in situ* carcinomas. There was no correlation between expression of IGF-IR and Ki-67 expression in *in situ* breast tumours (Pearson's rho correlation, $p=0.838$, Figure 4.11).

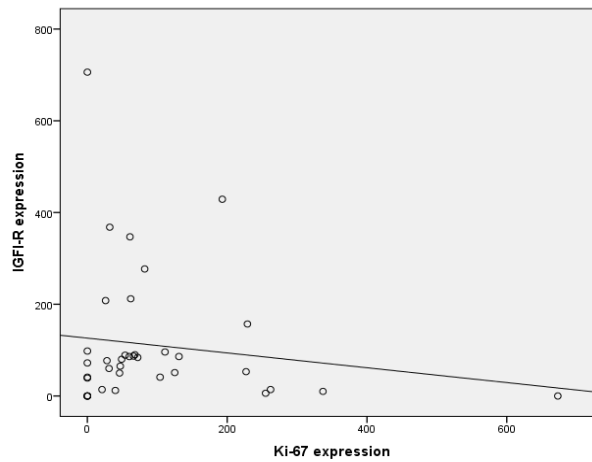


Figure 4.11: Association of IGF-IR expression and expression of Ki-67 in *in situ* breast carcinoma. IGF-IR expression and Ki-67 expression were compared in 37 *in situ* breast carcinomas. No significant association was detected between IGF-IR expression and expression of Ki-67 ($p=0.838$).

4.3.9.2 Association between expression of IGF-IR and expression of Ki-67 in invasive breast carcinoma

Ki-67 expression was investigated in 79 invasive breast tumour, of which 77 tumours were positive for expression of Ki-67 with mean histoscore of 140 and median histoscore of 88. The majority of the positive tumour cells were moderately immuno-reactive. Correlation between IGF-IR expression and expression of Ki-67 was investigated in these invasive tumours (Figure 4.12).

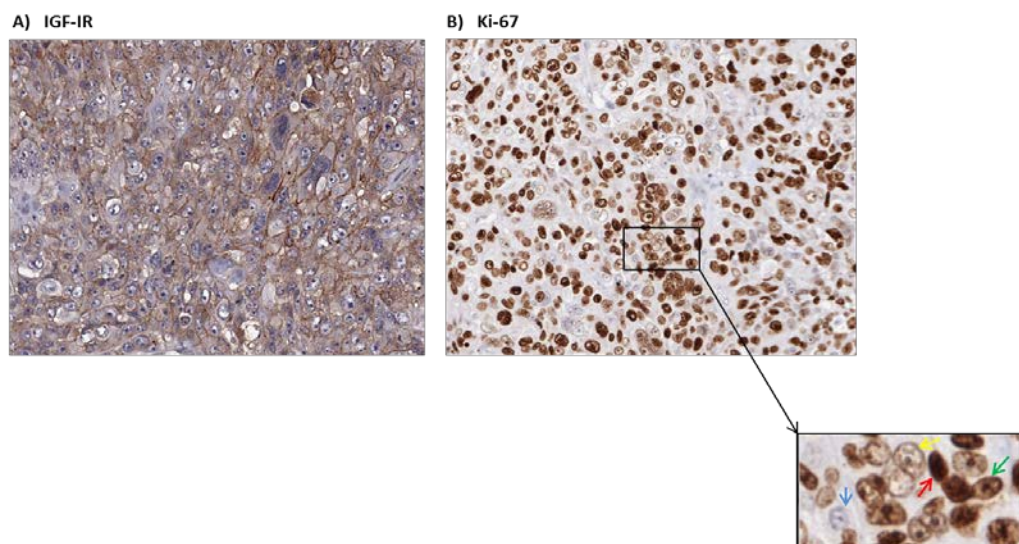


Figure 4.12: IGF-IR expression and expression of Ki-67 in invasive breast cancer. Sections from invasive breast carcinomas were tested for IGF-IR (A) and Ki-67 (B) expression by immunohistochemistry. Specific nuclei immuno-reaction was demonstrated. Arrows indicates the malignant epithelial cells with negative for expression of Ki-67 (blue arrow), weakly positive (yellow arrow), moderately positive (green arrow) and strongly positive (red arrow) are indicated. Magnification is x20 and for inset is x200.

There was a significant correlation between expression of IGF-IR and Ki-67 expression in 79 invasive breast tumours (Pearson's rho correlation, $p=0.005$, Figure 4.13), which supports the contention that IGF-IR is important for the proliferation of oestrogen receptor-negative breast tumour cells.

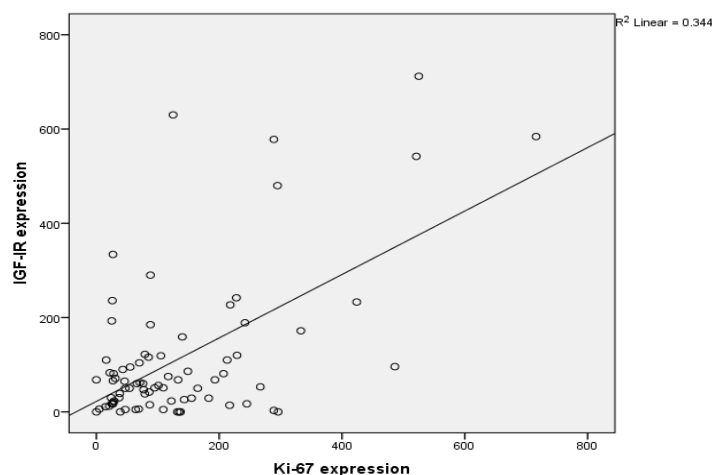


Figure 4.13: Association of IGF-IR expression and expression of Ki-67 in invasive breast cancer. IGF-IR histoscores are shown against Ki-67 histoscores of 79 invasive breast cancers. Expression of IGF-IR was significantly associated with expression of Ki-67 (Pearson correlation, $p=0.005$). The r^2 value is 0.344.

4.3.9.3 Association between expression of IGF-IR and expression of Ki-67 in breast tumour cells with involved lymph nodes

Expression of Ki-67 was evaluated in 26 breast tumour cells with involved lymph nodes. Out of 26 tumour cells 25 expressed Ki-67 with a mean histoscore of 111 and a median histoscore of 74 (Figure 4.14).

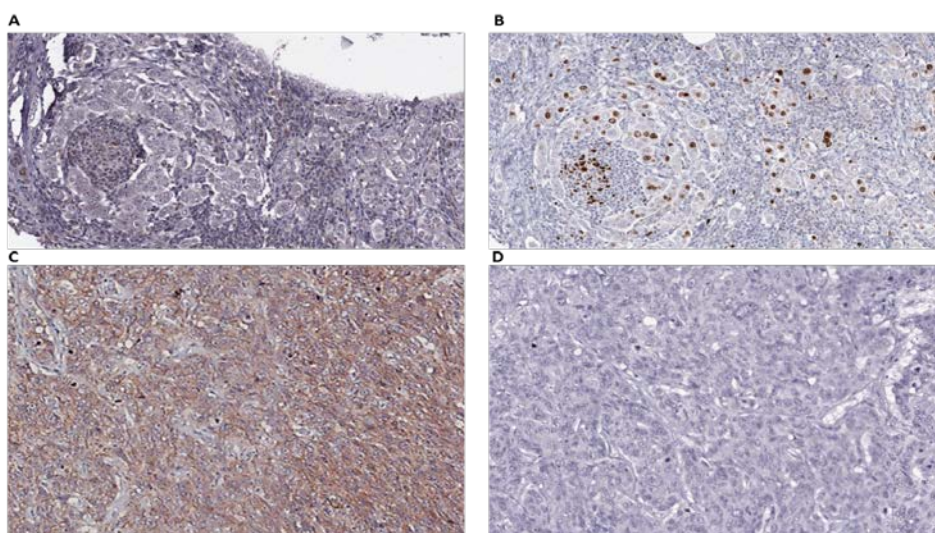


Figure 4.14: IGF-IR expression and expression of Ki-67 in lymph node involved breast tumours. Expression of IGF-IR (A, C) and Ki-67 (B, D) was analysed in breast tumours with axillary lymph node deposits by immunohistochemistry. Magnification is x40.

The association between IGF-IR expression and expression of Ki-67 was evaluated in breast tumours cells in the involved axillary nodes. No significant association was found between expression of IGF-IR and Ki-67 in the primary tumours with involved tumour cells in lymph nodes (Pearson's rho correlation, $p=0.354$, Figure 4.15).

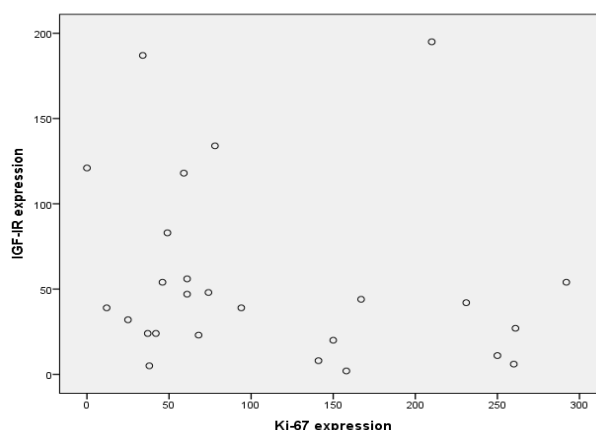


Figure 4.15: Association of IGF-IR expression and expression of Ki-67 in primary breast tumours with involved tumour cells in lymph nodes. IGF-IR expression was compared with expression of Ki-67 in 26 breast tumour cells with involved lymph nodes. There was no association between IGF-IR expression and expression of Ki-67 in breast tumour cells with involved lymph nodes ($p=0.592$).

4.3.10 IGF-IR and expression of cleaved caspase-3

It has been reported that survival effect of IGF-1 in oestrogen non-responsive breast cancer cells is mediated through IGF-IR *in vitro* (Davison, de Blacquiére et al. 2011). In addition, in the previous findings of the current study, the survival effect of IGF-1 on cell death induced by lack of cell-matrix interaction and its mediation through IGF-IR in oestrogen non-responsive breast cancer cells was evaluated. Caspase-3 is a main executor of apoptosis. The association between IGF-IR expression and expression of cleaved caspase-3 was tested. The expression of cleaved caspase-3 was measured in 79 invasive breast tumours. The immuno-reactivity of cleaved-caspase-3 was cytoplasmic (Figure 4.16).

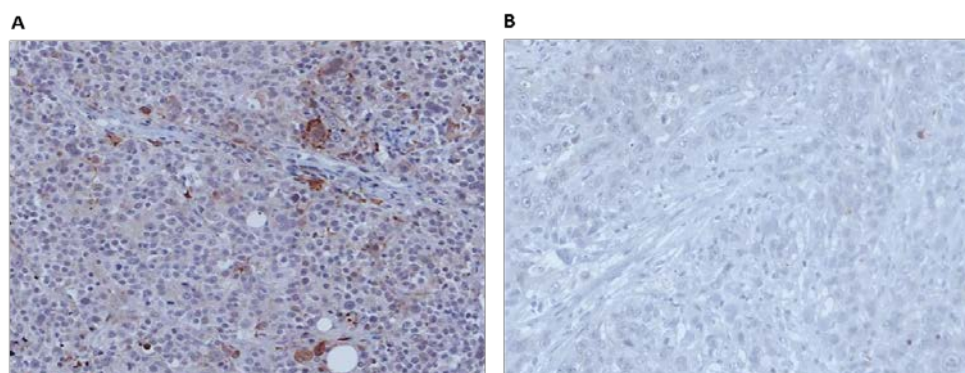


Figure 4.16: Cleaved caspase-3 expression in invasive breast tumours. Expression of cleaved caspase-3 was analysed by immunohistochemistry in invasive tumours. Examples for positive (A) and negative (B) cytoplasmic immuno-reaction were demonstrated. Magnification is x200.

Amongst the 73 IGF-IR positive tumours, 66 were negative for cleaved caspase-3 and only 7 were positive for cleaved-caspase-3. All of the six IGF-IR negative tumours were negative for cleaved caspase-3 (cross tabulation). However, cleaved caspase-3 positive tumours expressed low level of IGF-IR. There was no significant differences between expression levels of IGF-IR and cleaved-caspase-3 (Mann-Whitney test, $p=0.427$, Figure 4.17).

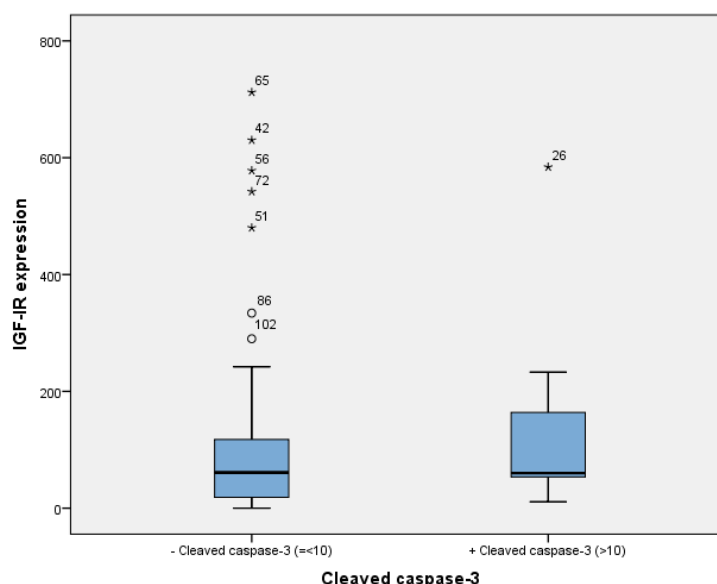


Figure 4.17: Association of IGF-IR and cleaved caspase-3 in invasive breast tumours. The tumours were dichotomised for negative and positive caspase-3 expression at a histoscore of 10. Sixty six tumours out of 73 IGF-IR positive tumours were negative for cleaved caspae-3 and 7 tumours were positive for cleaved caspase-3. The cut off for positive cleaved caspase-3 histoscore was >10. The whiskers represent the range of data, the boxes represent the 50th percentiles, the horizontal bars represent the median values, the dots refer to outlier values and the stars refer to extreme values.

4.4 Discussion

In this study, immunohistochemical expression of IGF-IR in normal breast tissues and different types of breast carcinomas: *in situ*, invasive and breast tumour cells in involved lymph nodes were investigated. In addition, the association between IGF-IR expression with different prognostic factors was studied. Immunohistochemical expression of IGF-IR was detected in all 7 cases of normal breast tissues, 86% of *in situ* breast carcinoma with mean histoscore of 110.95 and 92% of invasive breast cancer with mean histoscore of 94.

The role of IGF-IR in mediating the invasion and metastasis of breast cancer has been reported (Kucab and Dunn 2003). The possibility of a relationship between IGF-IR expression and number of lymph node metastasis was investigated in the present study. There was no significant association between expression of IGF-IR and the number of axillary lymph node metastasis ($p=0.130$). Significant correlation between expression of IGF-IR in primary tumours and in the metastatic cells in lymph nodes was detected ($p=0.001$). However, the expression of IGF-IR was found to be higher in primary tumours than their metastatic counterparts, which is in agreement with the previous reports that expression of IGF-IR was more frequent in primary breast tumours than lymph node metastasis (Koda, Sulkowski et al. 2003).

There was no association between IGF-IR expression and age of patients ($p=0.732$). Expression of IGF-IR showed no correlation with tumour size or grade ($p>0.05$). This finding agrees with a previous study in a cohort of oestrogen receptor-positive and negative breast tumours, that expression of IGF-IR is not associated with tumour size or grade (Koda, Sulkowski et al. 2003).

In 2011, Davison *et al* reported the role of IGF-1 in stimulating the proliferation of triple-negative breast cancer cells which was mediated through IGF-IR *in vitro* (Davison, de Blacquiere et al. 2011). In the current study, association of IGF-IR expression with expression of the proliferation marker Ki-67 was investigated. Expression of IGF-IR showed an association with expression of Ki-67 in invasive breast tumours (Pearson' rho correlation, $p=0.005$), which supports the contention that IGF-IR plays a significant role in mediating the proliferation of oestrogen receptor-negative breast cancer cells. Similarly,

expression of IGF-IR was investigated in *in situ* and metastatic breast tumour cells, but no significant correlation was found.

The role of IGF-1 in stimulating the survival of triple-negative breast cancer cells mediated through IGF-IR has been demonstrated (Davison, de Blacchiere et al. 2011). In addition, the mediation of oestrogen receptor-negative breast cancer cells survival by IGF-IR was demonstrated previously in chapter 3. Therefore, the association between expression of IGF-IR and cleaved-caspase-3 expression as an apoptotic marker in oestrogen receptor-negative breast tumours was investigated. The majority of IGF-IR positive tumours were negative for the expression of cleaved caspase-3. However, 7 tumours were positive for expression of both cleaved caspase-3 and IGF-IR. This can support the contention that IGF-IR is maintaining the survival of oestrogen receptor-negative breast cancer.

In conclusion, expression of IGF-IR was detected in the majority of oestrogen receptor-negative breast tumours. The significant association between expression of IGF-IR with Ki-67 and inversely with cleaved caspase-3 agrees with the contention that IGF-IR plays an important role in proliferation and survival of oestrogen receptor-negative breast tumour cells.

Chapter 5. The effect of epidermal growth factors on anoikis and migration of oestrogen receptor-negative breast cancer cells

5.1 Introduction

Members of the human epidermal growth factor receptor tyrosine kinase family, EGFR/HER-1, HER-2, HER-3 and HER-4 are activated by their respective ligands which induce receptor dimerization and leads to autophosphorylation of tyrosine residues on the receptor cytoplasmic domains. Subsequently multiple downstream signalling pathways are activated including the Ras-MAPK and PI3K-AKT pathways that are important in mediating cell survival and proliferation (Hynes and McDonald 2009, Tebbutt, Pedersen et al. 2013).

HER-2 is not capable of binding to any of the known EGF ligands; however, it is activated through hetero-dimerization with other ligand-bound receptors and is involved in increases in the cell survival, motility and proliferation of tumour cells (Moasser 2007). HER-3 lacks tyrosine autophosphorylation activity, hence it is only capable of transducing signals through hetero-dimerization with other family member receptors, namely: EGFR, HER-2 and HER-4 (Moasser 2007). Hetero-dimerization of HER-2 and HER-3 is reported as a potent activator of PI3K-Akt signalling, which plays an oncogenic role in proliferation of breast tumour cells (Lee-Hoeflich, Crocker et al. 2008).

In 2010, Chiu *et al* reported that in invasive breast carcinoma, decrease in survival of breast cancer patients is associated with HER-3 overexpression (Chiu, Masoudi et al. 2010). Several studies reported the frequent overexpression of EGFR and HER-2 in breast cancer and their significant roles in tumour growth, proliferation and migration (Garcia, Vizoso et al. 2003, Hirsch, Shen et al. 2006, Memon, Sorensen et al. 2006). Expression of heregulin in 30% of breast tumours and its contribution to proliferation, motility and metastasis of breast cancer cells has been reported (Hijazi, Thompson et al. 2000, Tsai, Shamon-Taylor et al. 2003).

In breast cancer, heregulin contribution in tumour progression and induced growth in an anchorage-dependent and independent manner has been reported to be independent from oestrogen (Lupu, Cardillo et al. 1996).

To our knowledge, no study has reported on the effect of EGF and heregulin-1 (HRG) on survival in anchorage-independent and monolayer migration of oestrogen receptor-negative breast cancer cells. Hence, in this chapter, the effect of EGF and heregulin on survival and migration of oestrogen receptor-negative breast cancer cell lines: MDA-MB-231, Hs578T, and SK-BR-3 cells were investigated. The role of EGFR and HER-2 in EGF and heregulin-1 mediated protection from anoikis and stimulated migration of cells has been investigated further with gefitinib an inhibitor against EGF receptor and lapatinib a dual tyrosine kinase inhibitor of EGFR and HER-2.

5.2 Result

5.2.1 Expression of EGFR, HER-2, HER-3 in different subtypes of breast cancer cells

The relative expression of EGFR, HER-2 and HER-3 was investigated in oestrogen-responsive MCF-7, BT-474, EFM-19 and oestrogen non-responsive SK-BR-3, Hs578T, MDA-MB-231, HCC-1419 and HCC-2218 breast cancer cell lines. Cell lines were cultured in maintenance medium and extracted proteins were analysed for EGFR, HER-2 and HER-3 expression by western transfer analysis (Figure 5.1).

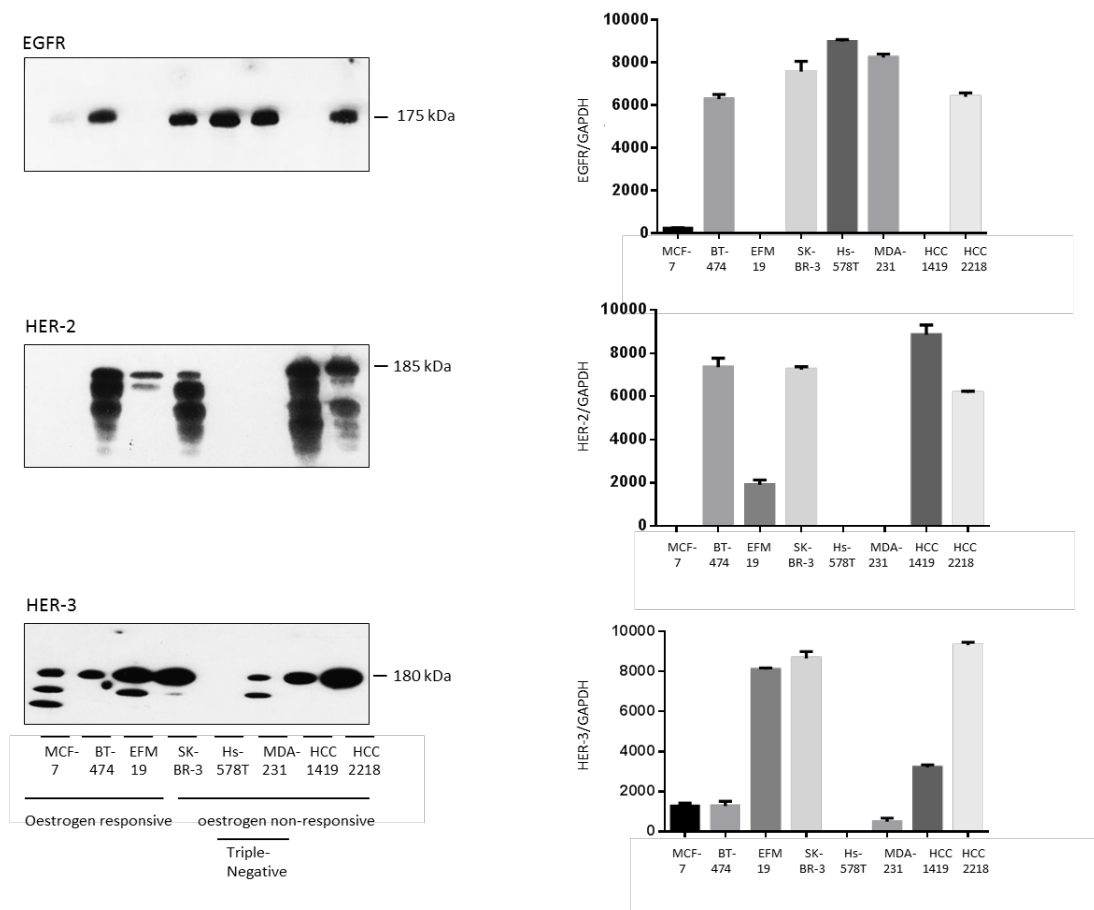


Figure 5.1: ErbB receptor expression in different types of breast cancer cells. MCF-7, BT-474, EFM-19, SK-BR-3, Hs578T, MDA-MB-231 (MDA-231), HCC-1419 and HCC-2218 cells were cultured to approximately 80% confluence and their proteins extracted with 1 ml of RIPA buffer per 75 cm² tissue culture flask as described in the Materials and Methods Section. Aliquots of 20 µg of extracted proteins were electrophoresed on a denaturing 12% gel and transferred to nitrocellulose membrane at 100 mA for 45 minutes. Membranes were incubated with EGFR antibody (1:10,000 dilution), HER-2 (1:2000 dilution), HER-3 (1:2000 dilution), developed with SuperSignal and exposed to X-ray film. Densitometric scanning of X-ray films determined the amount of each protein by analysis with LabWork 4.0 software. The mean + SEM after normalisation to GAPDH expression are shown.

Amongst oestrogen-responsive cells, MCF-7 cells express low levels of EGFR and HER-3, and no HER-2 expression was detected in this cell line. BT-474 cells express relatively high levels of EGFR and HER-2 and low levels of HER-3. In EFM-19 cells, HER-2 and HER-3 were detected but no EGFR expression was observed. Amongst oestrogen non-responsive cells, SK-BR-3 and HCC2218 cells expressed high levels of EGFR, HER-2 and HER-3. These results suggest that these two cell lines might be highly responsive to all EGF ligands. In contrast, HCC1419 cells do not express EGFR but do express HER-2 and HER-3 which suggests that they might be responsive to heregulin but not EGF. Triple-negative breast cancer cells, MDA-MB-231 and Hs578T cells

express relatively high levels of EGFR but no HER-2, and HER-3 expression was only observed at a low level in MDA-MB-231 cells.

Based on these results, the relatively high expression of EGFR in oestrogen non-responsive cells suggests that the EGF signal transduction pathway might be important in mediating the cell survival and migration of this subtype of breast cancer.

5.2.2 Activation of downstream effector proteins by EGF and heregulin-1 in EGF signal transduction pathways in SK-BR-3 cells.

To investigate the ability of EGF ligands to activate EGF downstream signal transduction pathway in SK-BR-3 cells which express high level of EGFR, HER-2 and HER-3, cells were withdrawn from the effects of EGF by culture in dextran-coated charcoal treated serum (DCCS) medium for two days. Cells treated with 50 ng/ml EGF or 50 ng/ml heregulin-1 for 15 minutes, proteins were extracted and lysed. Aliquots of proteins were analysed for phosphorylation of EGFR, HER-2, HER-3, AKT, and MAPK by western transfer (Figure 5.2).

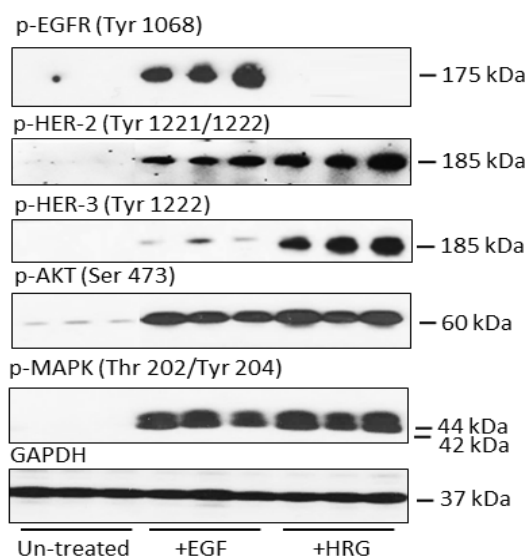


Figure 5.2: Activation of proteins involved in the EGF downstream signal transduction pathway in SK-BR-3 cells. SK-BR-3 cells were seeded both at 20×10^4 cells/well in 1 ml of maintenance medium. Cells were allowed to attach by incubation for 24 hours. Cells were removed from the maintenance medium, washed with 0.5 ml of PBS and medium were replaced with 1 ml of DCCS medium every day for two days. Medium was replaced with 1 ml of DCCS supplemented with 50 ng/ml EGF or 50 ng/ml heregulin-1(HRG) for 15 minutes. After 15 minutes of incubation, cells were lysed with 50 μ l per well of RIPA buffer. Aliquots of 10 μ g protein were electrophoresed on 12% denaturing polyacrylamide gel and transferred to nitrocellulose membrane for 45 minutes at 100 mA. Membranes were incubated with antibodies against phosphorylated EGFR (1:20,000 dilution), phosphorylated HER-2 (1:2000 dilution), phosphorylated HER-3 (1:2000 dilution), phosphorylated AKT (1:10,000 dilution), phosphorylated MAPK (1:20,000 dilution) and GAPDH (1:20,000 dilution). Representative results are shown are from one experiment in triplicate.

Reasonable levels of EGFR and HER-2 phosphorylation was stimulated by EGF in SK-BR-3 cells as these cells express high level of EGFR and HER-2. Incubation of cells with EGF induced some phosphorylation of HER-3 which suggests that EGF-induced activation of EGFR has formed heterodimers with HER-3, because EGF is not a HER-3 ligand. There was a detectable activation of AKT and MAPK after EGF stimulation which agrees with the contention that activation of EGFR homodimers and EGFR and HER-2 heterodimers induce activation of downstream proteins and suggest that EGFR has preference to form heterodimers with HER-2 than HER-3.

Incubation of SK-BR-3 cells with heregulin-1 did not stimulate phosphorylation of EGFR which is consistent with the inability of the ligand to bind to EGFR. Reasonable amount of HER-2 and HER-3 phosphorylation was stimulated by heregulin-1 which suggests that activated HER-3 formed heterodimers with HER-2 but not EGFR, as no EGFR was activated. Heregulin-1 stimulated detectable AKT and MAPK activation which supports the supposition that activated HER-2-HER-3 heterodimer was sufficient to activate PI3K/AKT and MAPK pathways.

Taken these data together, stimulation of SK-BR-3 cells with EGF resulted in detectable homo-dimerization of EGFR-EGFR and hetero-dimerization of EGFR-HER-2 and some EGFR-HER-3, subsequently activated downstream signalling molecules. Heregulin-1 induced activation of HER-2-HER-3 heterodimers, followed by activation of AKT and MAPK at high level.

5.2.3 Effect of EGF and heregulin-1 on anoikis in HER-2 positive SK-BR-3 cells

Based on the results of previous experiments, EGF and heregulin-1 showed noticeable effect on activation of downstream effector molecules in part through hetero-dimerization of receptors. To investigate if EGF and heregulin-1 have protective effects against cell death, SK-BR-3 cells were cultured in non-attached conditions in serum-free medium in the presence of 50 ng/ml EGF or 50 ng/ml heregulin-1 for different length of time up to 24 hours. Proteins were extracted after each incubation time and analysed by western transfer for presence of cleaved PARP (Figure 5.3).

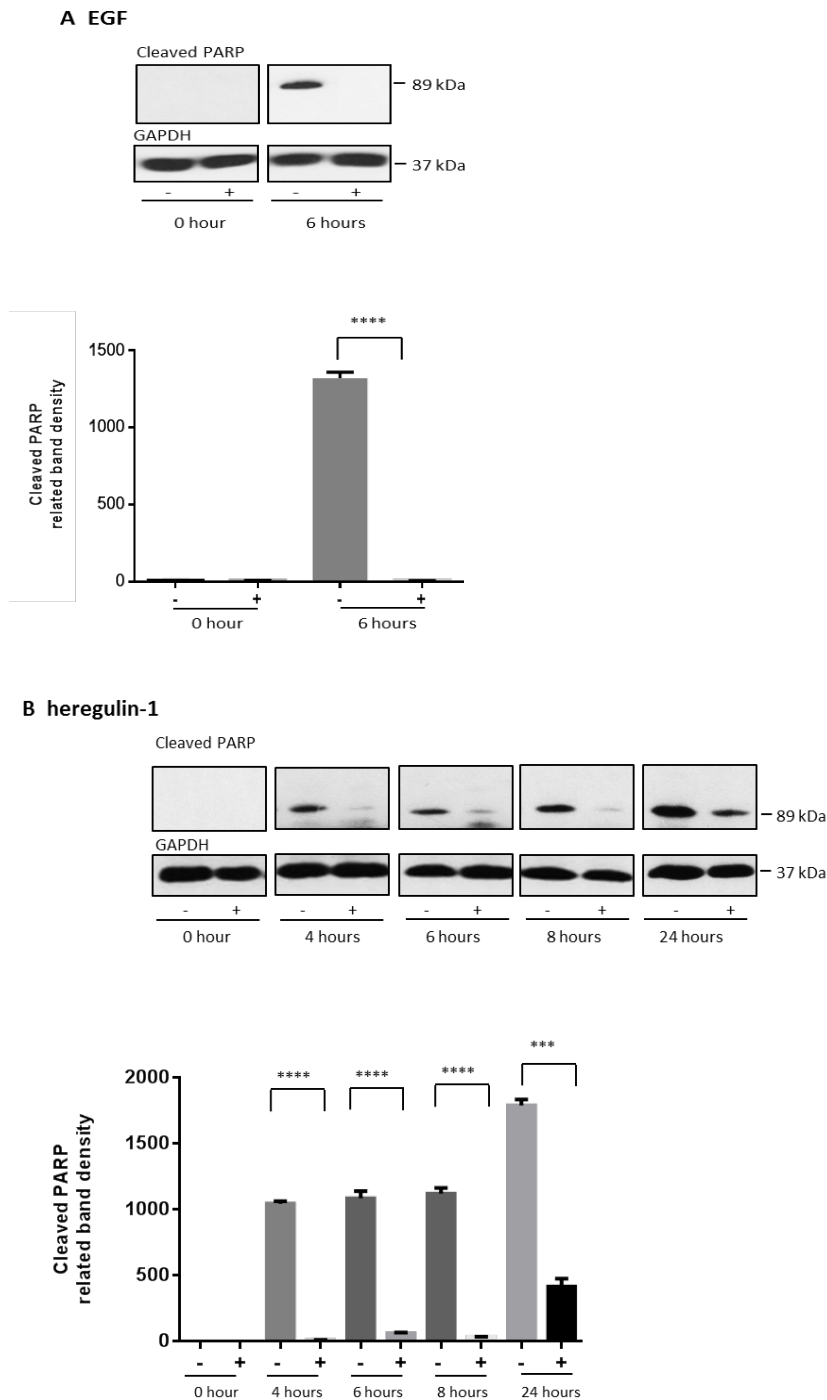


Figure 5.3: Effect of EGF and heregulin-1 on survival of SK-BR-3 cells. SK-BR-3 cells were cultured in phenol red-free Dulbecco's Modified Eagle's medium (DMEM) without serum (serum-free medium) in non-attached condition. Cells were seeded in 12-well poly-HEMA coated plates at the density of 20×10^4 cells/well in 2 ml of serum-free medium, in the presence and absence of 50 ng/ml EGF (**A**) or heregulin-1 (**B**) and cultured for various length of time up to 24 hours. Proteins were extracted and lysed with 50 μ l of RIPA buffer after each specific time point. Aliquots of 10 μ l protein were electrophoresed on 12% denatured gels and transferred to nitrocellulose membrane at 100 mA for 45 minutes. The membranes were incubated with cleaved PARP antibody (1:2000 dilution) and GAPDH (1:20,000 dilution). The illustrated figures are representative of bands from one experiment in triplicate that have been replicated at least two times. Densitometric scanning of X-ray films determined the amount of each protein by analysis with LabWork 4.0 software. The mean \pm SEM after normalisation to GAPDH expression are shown. Asterisks denotes statistically significantly less cleaved PARP in the present of EGF and heregulin-1 than in the absence of EGF and heregulin-1 (One way ANOVA, *** $p=0.0001$, **** $p<0.0001$).

Based on the results, in the presence of 50 ng/ml EGF, PARP cleavage was inhibited completely after 6 hours in SK-BR-3 cells. Additionally, incubation of SK-BR-3 cells with 50 ng/ml heregulin-1 induced almost complete inhibition in cleaved PARP after 4, 6, 8 hours and a statistically significant reduction after 24 hours.

In SK-BR-3 cells both EGF and heregulin-1 showed powerful survival effects. The reduction in cleaved PARP observed in the present of EGF and heregulin-1 supports the contention that these two ligands protect the SK-BR-3 cells against anoikis. These findings agree with previous observation that stimulation of SK-BR-3 cells with EGF and heregulin-1 was induced noticeable activation of PI3K and AKT pathway through activation of EGFR-EGFR homodimer, EGFR-HER-2 and HER-2-HER-3 heterodimers respectively.

5.2.4 Activation of downstream effector proteins in EGF signal transduction pathway by different concentrations of EGF in HER-2 positive SK-BR-3 breast cancer cells

To evaluate other concentrations of EGF on EGF downstream signal transduction pathway in SK-BR-3 cells, cells were cultured in DCCS medium for 48 hours. This was followed by stimulation of cells with various concentrations of EGF up to 100 ng/ml for 15 minutes. Aliquots of proteins were analysed by western transfer for phosphorylation of EGFR, AKT and MAPK (Figure 5.4).

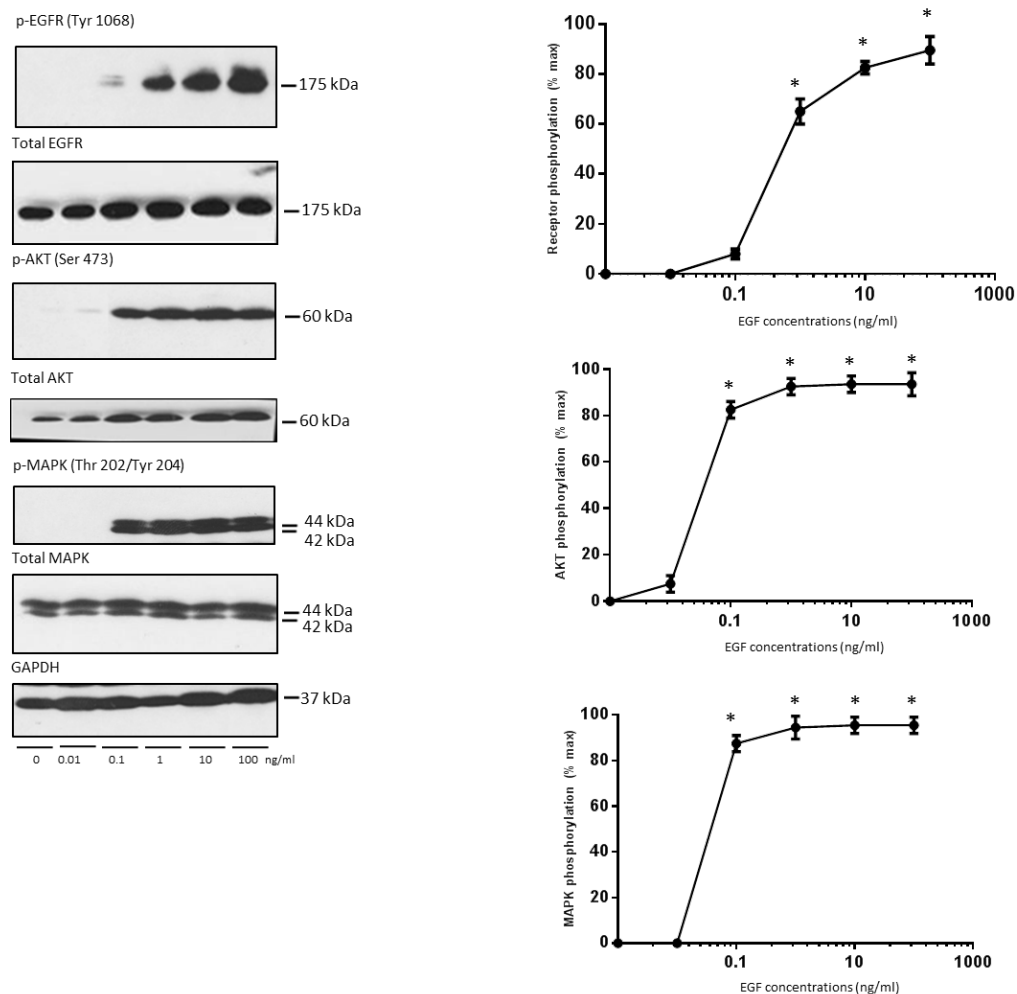


Figure 5.4: Response of SKBR-3 to stimulation by EGF. Cells were seeded at a density of 20×10^4 cells/well in 1 ml of maintenance medium and incubated for 48 hours for proper attachment. After 48 hours of incubation maintenance medium were replaced with 1 ml of DCCS medium daily for two days. Cells were washed with 0.5 ml of PBs and treated with 1ml DCCS supplemented with EGF at different concentration of 0, 0.01, 0.1, 1, 10, 100 ng/ml for 15 minutes. After 15 minutes of stimulation, cells were lysed with 50 μ l per well of RIPA buffer. Aliquots of 10 μ g of protein were analysed by electrophoresis in a 12% denaturing polyacrylamide gel and transferred to nitrocellulose membrane for 45 minutes at 100 mA. Membranes were incubated with EGFR antibody (1:10,000 dilution), phosphorylated EGFR (1:20,000 dilution), AKT (1:10,000 dilution), phosphorylated AKT (1:15,000 dilution), MAPK (1:20,000 dilution), phosphorylated MAPK (1:20,000 dilution) and GAPDH (1:20,000 dilution). Representative results are shown from an experiment in triplicate. Densitometric scanning of X-ray films determined the amount of each protein by analysis with LabWork 4.0 software. The mean \pm SEM after normalisation to GAPDH expression are shown. Asterisks denotes statistically significant increase in the phosphorylation of EGFR, AKT and MAPK with increasing EGF concentration (One way ANOVA, * $p < 0.05$).

EGF induced a concentration dependent increase in the phosphorylation of EGFR that was detected from 0.1 ng/ml in SK-BR-3 cells. No phosphorylation of AKT or MAPK was detected in the absence of EGF. AKT phosphorylation was detected at concentration of 0.01 ng/ml and higher, whereas MAPK

phosphorylation was detected after stimulation with EGF at a concentration of 0.1 ng/ml and higher. The amount of total AKT or MAPK did not change.

Stimulation of SK-BR-3 cells with various concentrations of EGF resulted in activation of EGFR mediated signal transduction pathways. Phosphorylation of downstream protein AKT was detected after stimulation with lower concentrations of EGF than phosphorylation of the EGFR. This difference in apparent sensitivity might be due to amplification of the signal downstream of the receptors.

5.2.5 Effect of different EGF concentrations on anoikis in SK-BR-3 cells

In previous experiment (section 5.2.3) protective effect of EGF at concentration of 50 ng/ml on anoikis of SK-BR-3 cells were shown. In this section the concentration dependence of protective effect of EGF against cell death in SK-BR-3 cells was investigated.

SK-BR-3 cells were cultured in non-attached conditions in serum-free medium in the presence of different concentrations of EGF up to 50 ng/ml. Proteins were extracted after incubation for 6 hours and analysed by western transfer to determine the amount of cleaved PARP (Figure 5.5).

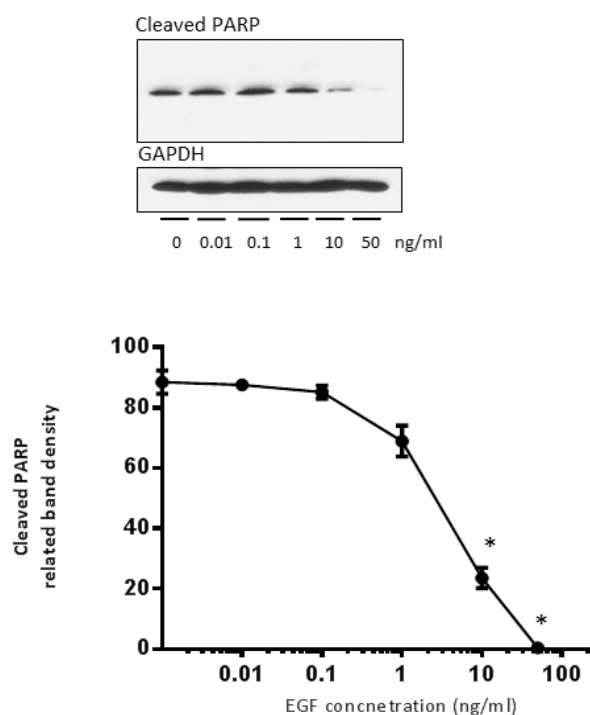


Figure 5.5: Effect of various concentrations of EGF on anoikis in SK-BR-3 cells. SK-BR-3 cells were seeded in 12 well poly-HEMA coated plates at density of 20×10^4 cells/well in 2 ml of serum-free medium, and incubated with different concentrations of EGF up to 50 ng/ ml. Proteins were extracted after incubation of 6 hours from with 50 μ l per well of RIPA buffer. Aliquots of 10 μ l protein were analysed by electrophoresis in 12% denatured gel and transferred for 45 minutes at 100 mA. Membranes were incubated with antibody against cleaved PARP (1:2000 dilution) and GAPDH (1:20,000 dilution). Illustrated images are representative of cleaved PARP from a triplicate experiment that have been repeated at least twice. Labwork 4.0 software used for densitometric analysis of the X-ray films and measurement were normalised with GAPDH. Error bars show mean \pm SEM, Asterisks represent the statistically significant less cleaved PARP in the presence of EGF compared with in the absence of EGF (One way ANOVA, $p^* < 0.05$).

The amount of cleaved PARP detected was reduced in the presence of EGF in a concentration-dependent manner in SK-BR-3 cells. There was a reduction in the amount of cleaved PARP in the presence of EGF at 1 ng/ml compared to its absence, which was not statistically significant. A statistically significant reduction in cleaved PARP was observed at EGF concentration of 10 ng/ml and higher, followed by complete inhibition of cleaved PARP at 50 ng/ml ($p = 0.034$).

EGF had a powerful survival effect in SK-BR-3 cells. The concentration dependence of the protective effect of EGF against anoikis in SK-BR-3 cells can support the contention that the survival effect of EGF is transmitted through the EGF receptor. The protective effect of EGF which was detected from 1 ng/ml is consistent with the previous observation that activation of the EGFR was stimulated at 1 ng/ml concentration of EGF.

5.2.6 Activation of downstream effector proteins by EGF and heregulin-1 in EGF signal transduction pathways in Hs578T cells.

To investigate the activation of EGF downstream signal transduction pathway in Hs578T breast cancer cells, which express high levels of EGFR but no HER-2 or HER-3, cells were withdrawn from the effects of EGF by culture in dextran-coated charcoal treated serum (DCCS) medium for two days. Cells treated with 50 ng/ml EGF or 50 ng/ml heregulin-1 for 15 minutes and proteins were extracted and lysed. Aliquots of proteins were analysed for phosphorylation of EGFR, AKT, and MAPK by western transfer (Figure 5.6).

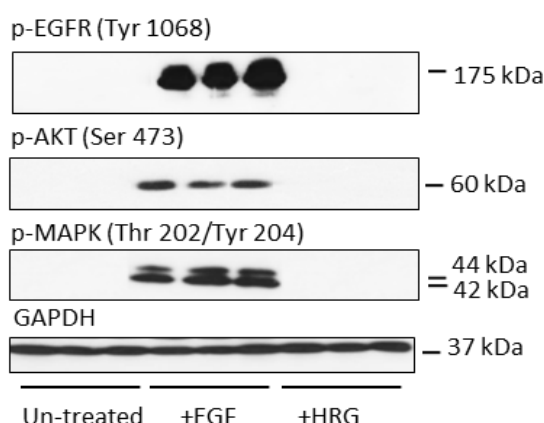


Figure 5.6: Activation of proteins involved in the EGF downstream signal transduction pathway in Hs578T cells. Hs578T cells were seeded both at 10×10^4 cells/ well in 1 ml of maintenance medium. Cells were allowed to attach by incubating them for 24 hours. Cells were removed from the maintenance medium, washed with 0.5 ml of PBS and medium were replaced with 1 ml of DCCS medium every day for two days. Medium was replaced with 1 ml of DCCS which is supplemented with 50 ng/ml EGF or 50 ng/ml heregulin-1 (HRG) for 15 minutes. After 15 minutes of incubation, cells were lysed with 50 μ l per well of RIPA buffer. Aliquots of 10 μ g protein were electrophoresed on 12% denaturing polyacrylamide gel, followed by transfer to nitrocellulose membrane for 45 minutes at 100 mA. Membranes were incubated with phosphorylated EGFR (1:20,000 dilution), phosphorylated AKT (1:10,000 dilution), phosphorylated MAPK (1:20,000 dilution) and GAPDH (1:20,000 dilution). Representative results are shown are from one experiment in triplicate.

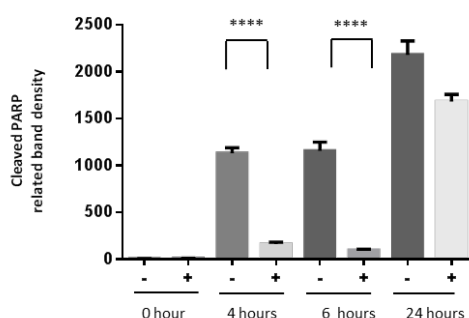
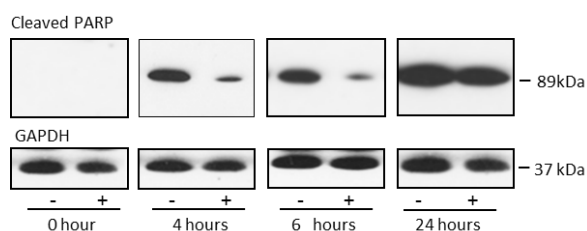
Stimulation of Hs578T cells with 50 ng/ml EGF induced phosphorylation of EGFR. Reasonable amount of AKT and MAPK phosphorylation was detected in the presence of EGF, which suggest that EGF signal transduction pathway is functional in this cell line. Incubation of MDA-MB-231 cells with heregulin-1 did not stimulate phosphorylation of EGFR, which is consistent with the inability of the ligand to bind and activate EGFR. Moreover, heregulin-1 was unable to activate the downstream signal transduction proteins AKT and MAPK due to lack of the availability of its receptor.

In conclusion, stimulation of Hs578T cells with EGF but not heregulin-1 resulted in activation of EGFR mediated signalling pathways. This was demonstrated by activation of both PI3K-AKT and MAPK pathway after treatment with 50 ng/ml of EGF.

5.2.7 Effect of EGF and heregulin-1 on anoikis of Hs578T cells

The effect of EGF and HRG on PARP cleavage induced in Hs578T cells by growth as non-attached cells was investigated. Hs578T cells were cultured in non-attached conditions in serum-free medium in the presence and absence of 50 ng/ml EGF or 50 ng/ml heregulin-1 for up to 24 hours. Proteins were extracted after 0, 4, 6, 8 and 24 hours and the degree of PARP cleavage was assessed by western transfer analysis (Figure 5.7).

A EGF



B HRG

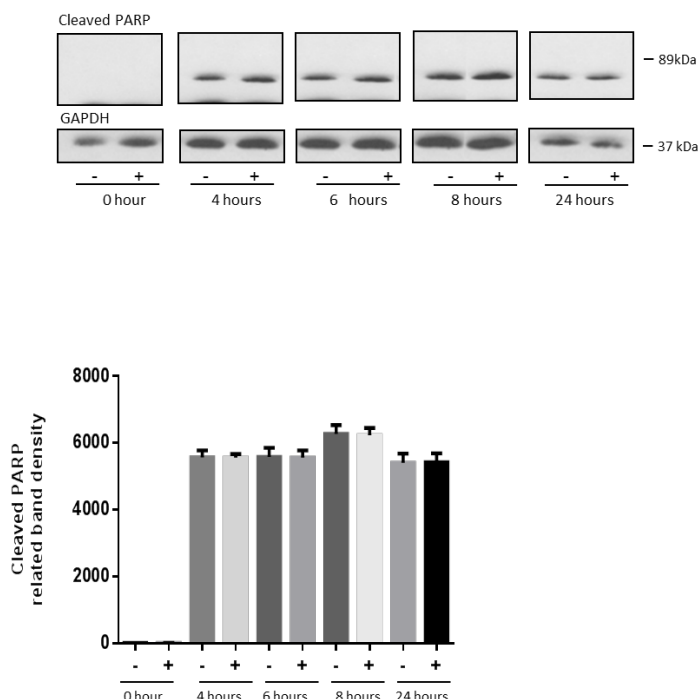


Figure 5.7: Effect of EGF and heregulin-1 on survival of Hs578T cells. Hs578T cells were cultured in phenol red-free Dulbecco's Modified Eagle's medium (DMEM) without serum (serum-free medium) in non-attached condition. Cells were seeded in 12-well poly-HEMA coated plates at the density of 20×10^4 cells/ well in 2 ml of serum-free medium, in the presence and absence of 50 ng/ml EGF (**A**) or heregulin-1 (HRG) (**B**) and cultured for various length of time up to 24 hours. Proteins was extracted and lysed with 50 μ l of RIPA buffer after each specific time point. Aliquots of 10 μ l protein were electrophoresed on 12% denatured gels and transferred to nitrocellulose membrane at 100 mA for 45 minutes. The membranes were incubated with cleaved PARP antibody (1:2000 dilution) and GAPDH (1:20,000 dilution). The illustrated figures are representative of bands from one experiment in triplicate that have been replicated at least two times. Densitometric scanning of X-ray films determined the amount of each protein by analysis with LabWork 4.0 software. The mean \pm SEM after normalisation to GAPDH expression are shown. Asterisks denotes statistically significantly less cleaved PARP in the present of EGF than in the absence of EGF in Hs578T cells (One way ANOVA, **** $p < 0.0001$).

In Hs578T cells, cleaved PARP was reduced markedly in the presence of 50 ng/ml EGF compared with in the absence of EGF after 4 and 6 hours (Figure 5.7 A). As shown in Figure 5.7 B, incubation of Hs578T cells with 50 ng/ml heregulin-1 did not induce reduction in the amount of cleaved PARP at any incubation time.

Based on these results, reduction in cleaved PARP that observed in the presence of EGF in Hs578T cells can support the hypothesis that EGF inhibits anoikis in this cell line. Treatment of cells with 50 ng/ml heregulin-1 did not show any protective effect on survival of Hs578T cells, which is consistent with the previous finding that heregulin-1 does not stimulate the activation of

downstream signal transduction proteins AKT and MAPK pathways in Hs578T cells.

5.2.8 Activation of proteins involved in the EGF signal transduction pathway by different EGF concentrations in Hs578T cells

Although EGF at concentration of 50 ng/ml provided powerful protection against anoikis in Hs578T cells, the effect of various concentrations of EGF on downstream signal transduction pathway was tested on Hs578T cells, to investigate the effect of other EGF concentrations on activation of downstream signal transduction pathways.

Hs578T cells were withdrawn from the effects of EGF by culture in dextran-coated charcoal treated serum (DCCS) medium for two days. Cells treated with EGF at various concentrations up to 100 ng/ml for 15 minutes and proteins were extracted and lysed. Aliquots of protein were analysed for phosphorylation of the EGFR, AKT, and MAPK by western transfer (Figure 5.8).

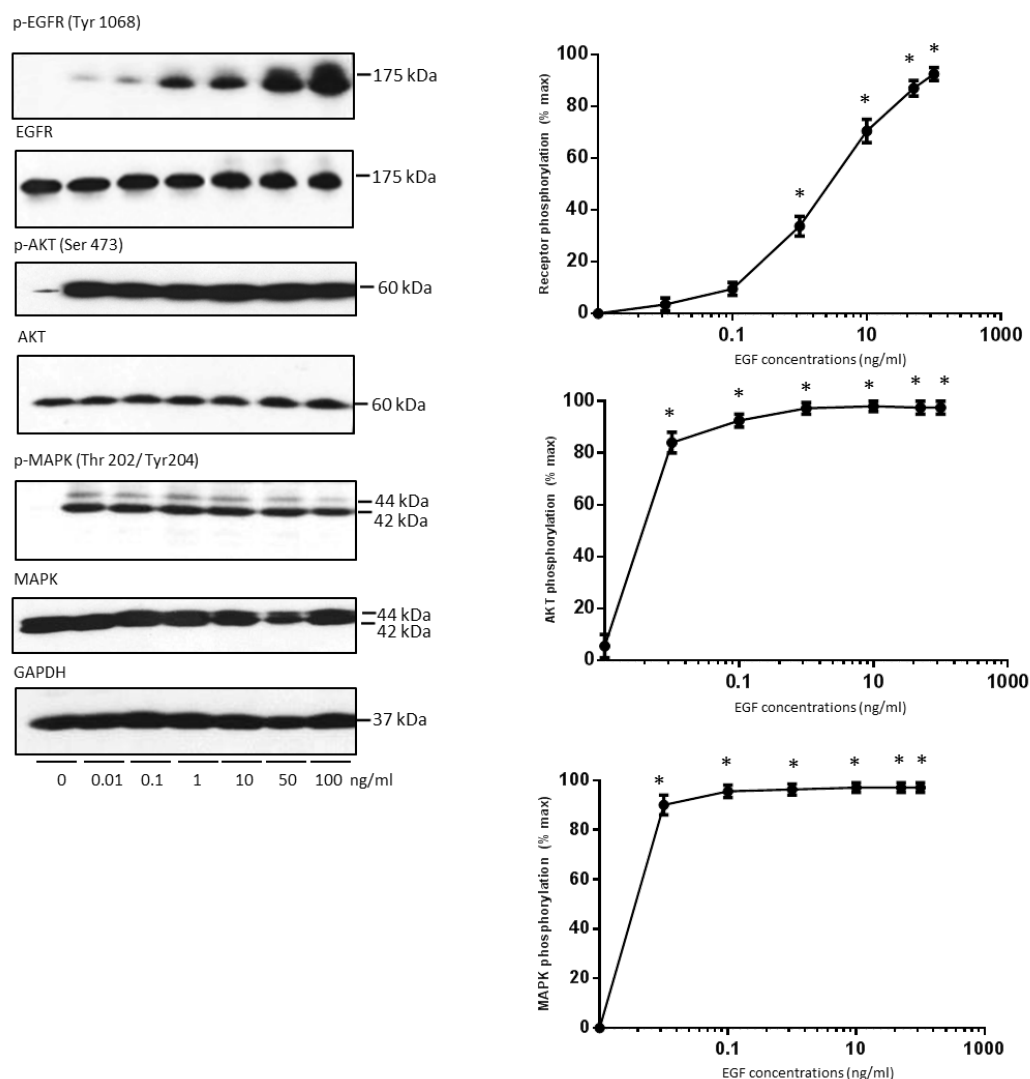


Figure 5.8: Activation of proteins involved in the EGF downstream signal transduction pathway in Hs578T cells. Hs578T cells were seeded both at 10×10^4 cells/ well in 1 ml of maintenance medium. Cells were allowed to attach by incubation for 24 hours. Cells were removed from the maintenance medium, washed with 0.5 ml of PBS and medium were replaced with 1 ml of DCCS medium every day for two days. Medium was replaced with 1 ml of DCCS which is supplemented with EGF at different concentrations of 0, 0.01, 1, 10, 50, 100 ng/ml for 15 minutes. After 15 minutes of incubation, cells were lysed with 50 μ l per well of RIPA buffer plus inhibitors. Aliquots of 10 μ g protein were electrophoresed on 12% denaturing polyacrylamide gel, followed by transferring to nitrocellulose membrane for 45 minutes at 100 mA. Membranes were incubated with EGFR (1:20,000 dilution), phosphorylated EGFR (1:20,000 dilution), AKT (1:5000 dilution), phosphorylated AKT (1:5000 dilution), MAPK (1:10,000 dilution), phosphorylated MAPK (1:10,000 dilution) and GAPDH (1:20,000 dilution). Representative results are shown are from one experiment in triplicate. The amount of the phosphorylated receptor was determined by densitometric scanning of the X-ray films followed by analysis with Labwork 4.0 software. The mean \pm SEM after normalisation to GAPDH expression are shown. Asterisks denotes statistically significant increase in the phosphorylation of EGFR, AKT and MAPK with increasing EGF concentration (One way ANOVA, * $p < 0.05$).

Stimulation with EGF induced a concentration dependent increase in phosphorylation of EGFR, detected from 0.01 ng/ml in Hs578T cells. The expression of EGFR was not altered following EGF stimulation. AKT phosphorylation was detected in the absence of EGF at very low levels and increased following stimulation with 0.01 ng/ml EGF or higher. Phosphorylation of MAPK was not observed in the absence of EGF and phosphorylation was detected after stimulation with EGF at concentration of 0.01 ng/ml and higher. Stimulation of triple-negative Hs578T cells with EGF resulted in activation of EGFR mediated signal transduction pathways. Phosphorylation of downstream effector proteins was detected after stimulation with lower concentrations of EGF than phosphorylation of the EGFR. One of the reasons for this might be amplification of the signal downstream of the receptor. Activation of EGFR pathways with low concentrations of EGF suggests that the Hs578T cell line is particularly sensitive to EGF. This sensitivity can be due to high level of EGFR expression. These results support the contention that cells with higher level of receptor expression are more sensitive for the activation of downstream effector proteins than cells with lower expression level of receptors.

5.2.9 Effect of different EGF concentrations on anoikis of Hs578T cells

To investigate the concentration dependence of the protective effect of EGF against anoikis in Hs578T cells, cells were cultured in non-attached conditions in serum-free medium in the presence and absence of 50 ng/ml EGF for up to 24 hours. Proteins were extracted after 6 hours of incubation and the degree of PARP cleavage was assessed by western transfer analysis. The cell death was evaluated by measuring the level of PARP cleavage using western transfer analysis (Figure 5.9).

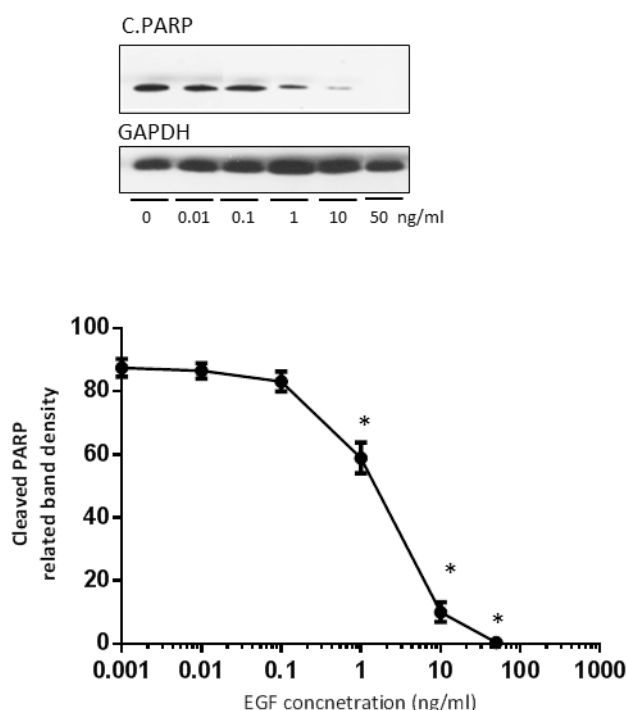


Figure 5.9: Effect of various concentrations of EGF on anoikis in Hs578T cells. Hs578T cells were seeded in 12 well poly-HEMA coated plates at density of 20×10^4 cells/ well in 2 ml of serum-free medium, and incubated with different concentrations of EGF up to 50 ng/ml. Proteins were extracted after incubation of 6 hours with 50 μ l per well of RIPA buffer. Aliquots of 10 μ l protein were analysed by electrophoresis in 12% denatured gel and transferred for 45 minutes at 100 mA. Membranes were incubated with antibody against cleaved PARP (1:2000 dilution) and GAPDH (1:20,000 dilution). Illustrated images are representative of cleaved PARP from a triplicate experiment that have been repeated at least two times. Labwork 4.0 software used for densitometric analysis of the X-ray films and measurement were normalised with GAPDH. Error bars show mean \pm SEM, Asterisks represent that statistically significant less cleaved PARP in the presence of EGF compared with in the absence of EGF (One way ANOVA, $p^* < 0.05$).

The amount of cleaved PARP detected was reduced in the presence of EGF in a concentration-dependent manner in Hs578T cells. There was a reduction in the amount of cleaved PARP in the presence of EGF at 1 ng/ml compared with in its absence, which was statistically significant. There was a positive association between the level of reduction in cleaved PARP and the increase in the concentration of EGF followed by complete inhibition of cleaved PARP at 50 ng/ml concentration.

Based on the results, EGF had a powerful survival effect on Hs578T cells and that the protective effect of EGF was detected at concentration of 1 ng/ml. This agrees with the previous finding that activation of EGFR in Hs578T cells was induced with EGF at concentrations of 1 ng/ml. The concentration dependent

reduction in PARP cleavage in the presence of EGF in Hs578T cells suggests that the survival effect of EGF is transmitted through the EGF receptor.

5.2.10 Activation of downstream effector proteins in EGF signal transduction pathways by EGF and heregulin-1 in MDA-MB-231 cells

To investigate the activation of EGF downstream signal transduction pathway in MDA-MB-231 breast cancer cells which express high levels of EGFR and low levels of HER3 but not HER-2, cells were withdrawn from the effects of EGFs by culture in dextran-coated charcoal treated calf serum (DCCS) medium for two days. Cells were treated with 50 ng/ml EGF or 50 ng/ml heregulin-1 for 15 minutes, cells were lysed and proteins were extracted. Aliquots of proteins were analysed for phosphorylation of EGFR, HER-3, AKT, and MAPK by western transfer (Figure 5.10).

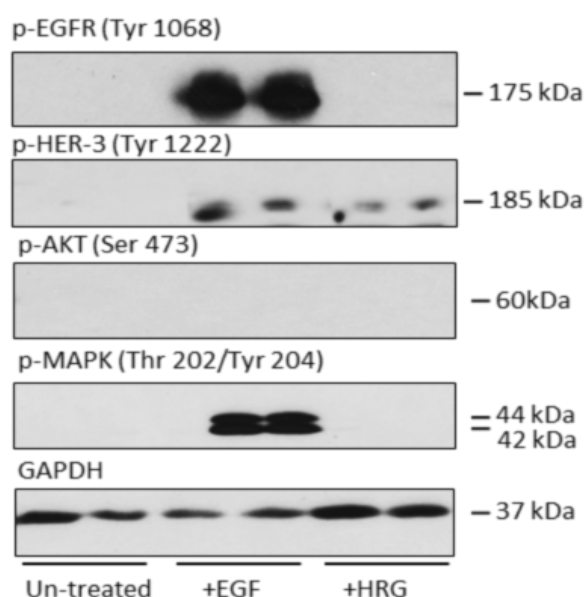


Figure 5.10: Activation of proteins involved in the EGF downstream signal transduction pathway in MDA-MB-231 cells. MDA-MB-231 cells were seeded at 10×10^4 cells/well in 1 ml of maintenance medium. Cells were allowed to attach by incubation for 24 hours. The maintenance medium was removed and the cells were washed with 0.5 ml of PBS and the medium replaced with 1 ml of DCCS medium every day for two days. Medium was replaced with 1 ml of DCCS supplemented with 50 ng/ml EGF or 50 ng/ml heregulin-1 (HRG) for 15 minutes. After 15 minutes of incubation, cells were lysed with 50 μ l per well of RIPA buffer. Aliquots of 10 μ g protein were electrophoresed on a 12% denaturing polyacrylamide gel, followed by transfer to nitrocellulose membrane for 45 minutes at 100 mA. Membranes were incubated with phosphorylated EGFR (1:20,000 dilution), phosphorylated HER-3 (1:2000 dilution), phosphorylated AKT (1:1000 dilution), phosphorylated MAPK (1:10,000 dilution) and GAPDH (1:20,000 dilution). Results shown are from one experiment in duplicate.

Dramatic phosphorylation of EGFR was stimulated with 50 ng/ml of EGF as might have been expected, because MDA-MB-231 cells express high level of EGFR (Figure 5.1). Incubation with EGF stimulated some phosphorylation of HER-3 suggests that EGFR activated by EGF has formed heterodimers with HER-3 as EGF does not interact with HER-3. There was no detectable activation of AKT after EGF stimulation which agrees with the supposition that phosphorylated EGFR does not activate the PI3K and AKT pathway. In contrast, dramatic activation of MAPK phosphorylation was evident after 15 minutes of incubation with EGF.

Incubation of MDA-MB-231 cells with heregulin-1 did not stimulate detectable phosphorylation of EGFR. A small amount of HER-3 phosphorylation was stimulated but phosphorylation of EGFR consequent to heterodimer formation with activated HER-3 was not detected. The amount of phosphorylated HER-3 was not sufficient to activate detectable levels of phosphorylated AKT or MAPK.

In conclusion, neither ligand was able to activate the PI3 kinase pathway in MDA-MB-231 cells. EGF but not heregulin-1 activated the MAPK pathway.

5.2.11 Effect of EGF and heregulin-1 on anoikis of MDA-MB-231 cells

The effect of EGF and heregulin-1 on survival of triple-negative MDA-MB-231 breast cancer cells was investigated. MDA-MB-231 cells were cultured in non-attached conditions in serum-free medium in the presence and absence of 50 ng/ml EGF or 50 ng/ml heregulin-1 separately for up to 24 hours. Proteins were extracted after 0, 4, 6, 8 and 24 hours and the degree of PARP cleavage was assessed by western transfer analysis (Figure 5.11).

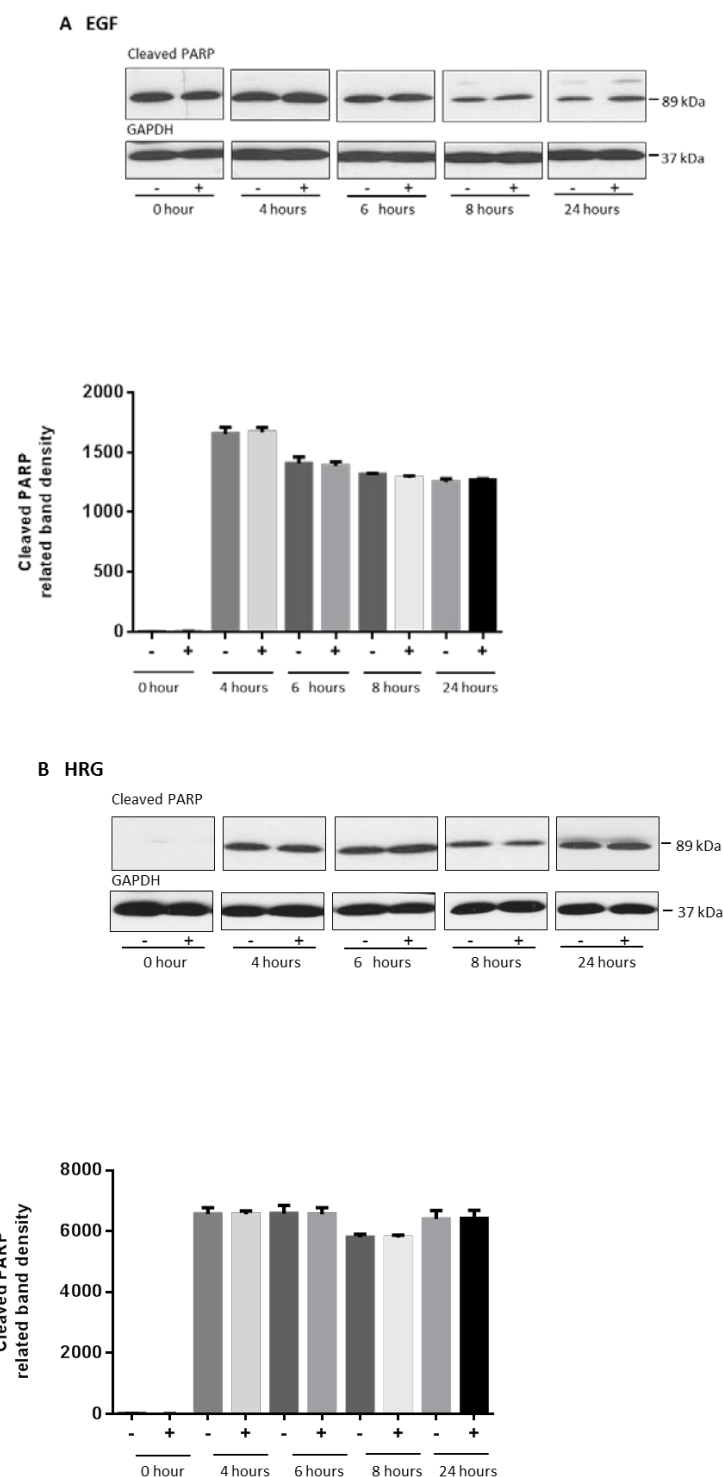


Figure 5.11: Effect of EGF and heregulin-1 on survival of MDA-MB-231 cells. MDA-MB-231 cells were cultured in phenol red free Dulbecco's Modified Eagle's medium (DMEM) without serum (serum-free medium) in non-attached condition. Cells were seeded in 12-well poly-HEMA coated plates at the density of 20×10^4 cells/ well in 2 ml of serum-free medium, in the presence and absence of 50 ng/ml EGF (**A**) or 50 ng/ml heregulin (HRG) (**B**) and cultured for various length of time up to 24 hours. Protein was extracted and lysed with 50 μ l of RIPA buffer after each specific time point. Aliquots of 10 μ l protein were electrophoresed on 12% denatured gels and transferred to nitrocellulose membrane at 100 mA for 45 minutes. The membranes were incubated with cleaved PARP antibody (1:2000 dilution) and GAPDH (1:20,000 dilution). The illustrated images are representative of bands from one experiment in triplicate that have been replicated at least twice. Densitometric scanning of X-ray films determined the amount of each protein by analysis with LabWork 4.0 software. The mean \pm SEM after normalisation to GAPDH expression are shown.

As shown in the Figure 5.11, there was no reduction in the amount of cleaved PARP in presence of 50 ng/ml EGF at any incubation time in MDA-MB-231 cells. Similarly, incubation of MDA-MB-231 cells with 50 ng/ml heregulin-1 did not reduce the amount of cleaved PARP at any incubation time. Based on these results, EGF does not have any protective effect against anoikis on MDA-MB-231 despite the expression of EGFR in this cell line. The absence of a protective effect of EGF despite the strong activation of the MAPK pathway indicates that the MAPK pathway is not involved in protection of breast cancer cells from anoikis. These results are consistent with the AKT pathway being involved in protection of cells from anoikis, because AKT phosphorylation was not induced in response to stimulation with EGF, which had no survival effect in this cell line. The inability of heregulin-1 to protect MDA-MB-231 cells against anoikis is consistent with its inability to activate either the PI3 kinase or MAPK pathways.

5.2.12 Activation of proteins involved in the EGF signal transduction pathway by different concentrations of EGF in triple-negative MDA-MB-231 breast cancer cells

To investigate whether other concentrations of EGF might be able to activate the PI3 kinase pathway in MDA-MB-231 and potentially have a role in protection of cells against anoikis, MDA-MB-231 cells were withdrawn from the effects of EGF by culture in dextran-coated charcoal treated calf serum (DCCS) medium for two days. Cells treated with EGF at various concentrations up to 100 ng/ml for 15 minutes, cells were lysed and proteins were extracted. Aliquots of proteins were analysed for phosphorylation of the EGFR, AKT, and MAPK by western transfer (Figure 5.12).

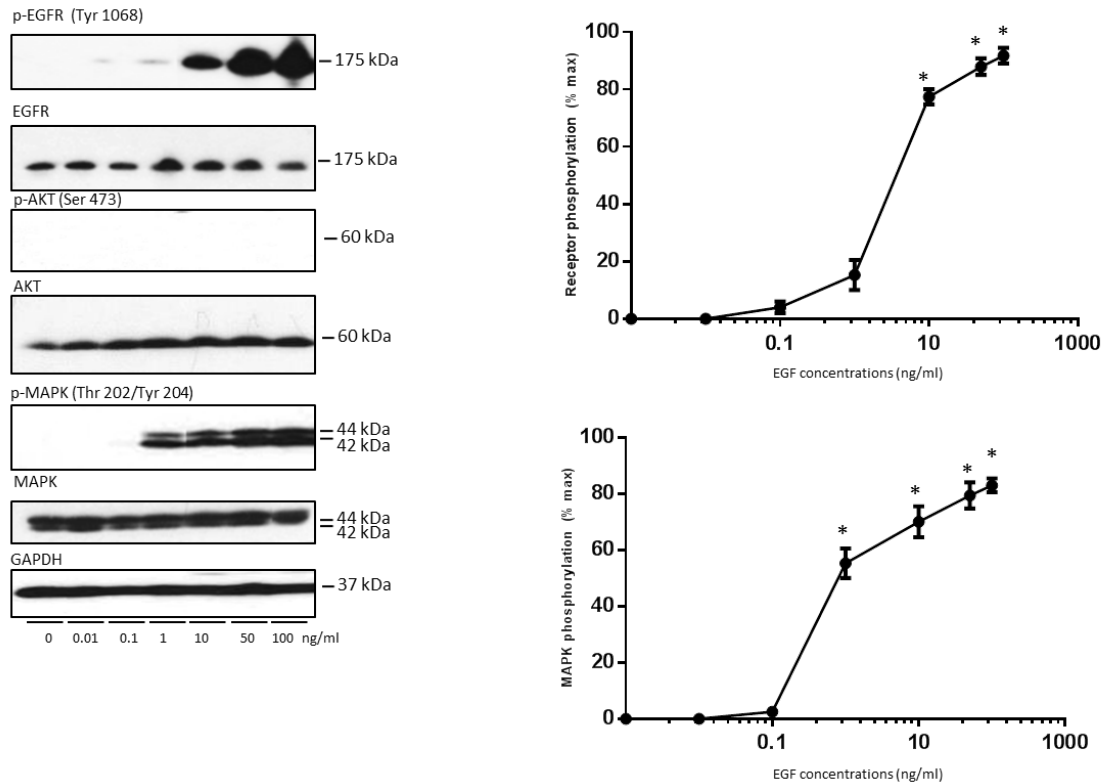


Figure 5.12: Activation of proteins involved in the EGF downstream signal transduction pathway in MDA-MB-231 cells. MDA-MB-231 cells were seeded both at 10×10^4 cells/ well in 1 ml of maintenance medium. Cells were allowed to attach by incubation for 24 hours. Cells were removed from the maintenance medium, washed with 0.5 ml of PBS and medium were replaced with 1 ml of DCCS medium every day for two days. Medium was replaced with 1 ml of DCCS supplemented with EGF at different concentrations of 0, 0.01, 1, 10, 50, 100 ng/ml for 15 minutes. After 15 minutes of incubation, cells were lysed with 50 μ l per well of RIPA buffer plus inhibitors. Aliquots of 10 μ g protein were electrophoresed on 12% denaturing polyacrylamide gel, followed by transfer to nitrocellulose membrane for 45 minutes at 100 mA. Membranes were incubated with EGFR (1:20,000 dilution), phosphorylated EGFR (1:20,000 dilution), AKT (1:5000 dilution), phosphorylated AKT (1:1000 dilution), MAPK (1:10,000 dilution), phosphorylated MAPK (1:10,000 dilution) and GAPDH (1:20,000 dilution). Representative results are shown are from one experiment in triplicate. Densitometric scanning of X-ray films determined the amount of each protein by analysis with LabWork 4.0 software. The mean \pm SEM after normalisation to GAPDH expression are shown. Asterisks denotes statistically significant increase in the phosphorylation of EGFR and MAPK with increasing EGF concentration (One way ANOVA, * $p < 0.05$).

Stimulation with EGF induced a concentration dependent increase in phosphorylation of EGFR, detected from 0.1 ng/ml in MDA-MB-231 cells. EGF did not affect the total level of EGFR after stimulation for 15 minutes. No phosphorylation of AKT was observed at any tested concentration of EGF in MDA-MB-231 cells. Phosphorylation of MAPK was not observed in the absence of EGF, MAPK phosphorylation was detected after stimulation with EGF at concentration of 1 ng/ml and higher.

Stimulation of triple-negative MDA-MB-231 breast cancer cells with EGF resulted in activation of EGFR mediated signalling pathways. This was demonstrated by phosphorylation of downstream proteins after treatment with certain concentrations of EGF. The results agree with the previous findings (section 5.2.10) that stimulation with EGF results in dramatic phosphorylation of EGFR and MAPK, whereas no detectable AKT phosphorylation was observed at any tested concentration and that MDA-MB-231 cells require high concentration of EGF to stimulate the maximum phosphorylation of EGFR and MAPK.

Taken these data together, stimulation of MDA-MB-231 cells with EGF resulted in activation of EGFR mediated signal transduction pathways, confirming that some EGF-mediated signalling is functional in this cell line. The inability of EGF to protect MDA-MB-231 against anoikis might be due to non-activation of PI3K and AKT pathway in this cell line by EGF. Despite the strong activation of the MAPK pathway, MDA-MB-231 cell survival may be dependent upon activation of PI3K and AKT pathway, which means that EGF is unable to protect cells against anoikis.

5.2.13 Effect of lapatinib on activation of both EGFR and HER-2 by EGF in SK-BR-3 cells

Lapatinib is a dual tyrosine kinase inhibitor which targets both EGFR and HER-2 pathways. It is used for treatment of HER-2 over-expressed breast tumours in both metastatic and adjuvant setting.

In this experiment to investigate the effectiveness of lapatinib and establish the concentration at which the maximum inhibition occurs in SK-BR-3 cells that EGF had protective effect, cells were cultured in attached conditions in full maintenance medium and incubated with different concentrations of lapatinib up to 20 μ M for 30 minutes. After 30 minutes of incubation, cells were stimulated with 50 ng/ml EGF for 15 minutes. Proteins were extracted after incubation for 15 minutes and analysed by western transfer for phosphorylation of EGFR, HER-2, AKT, and MAPK (Figure 5.13).

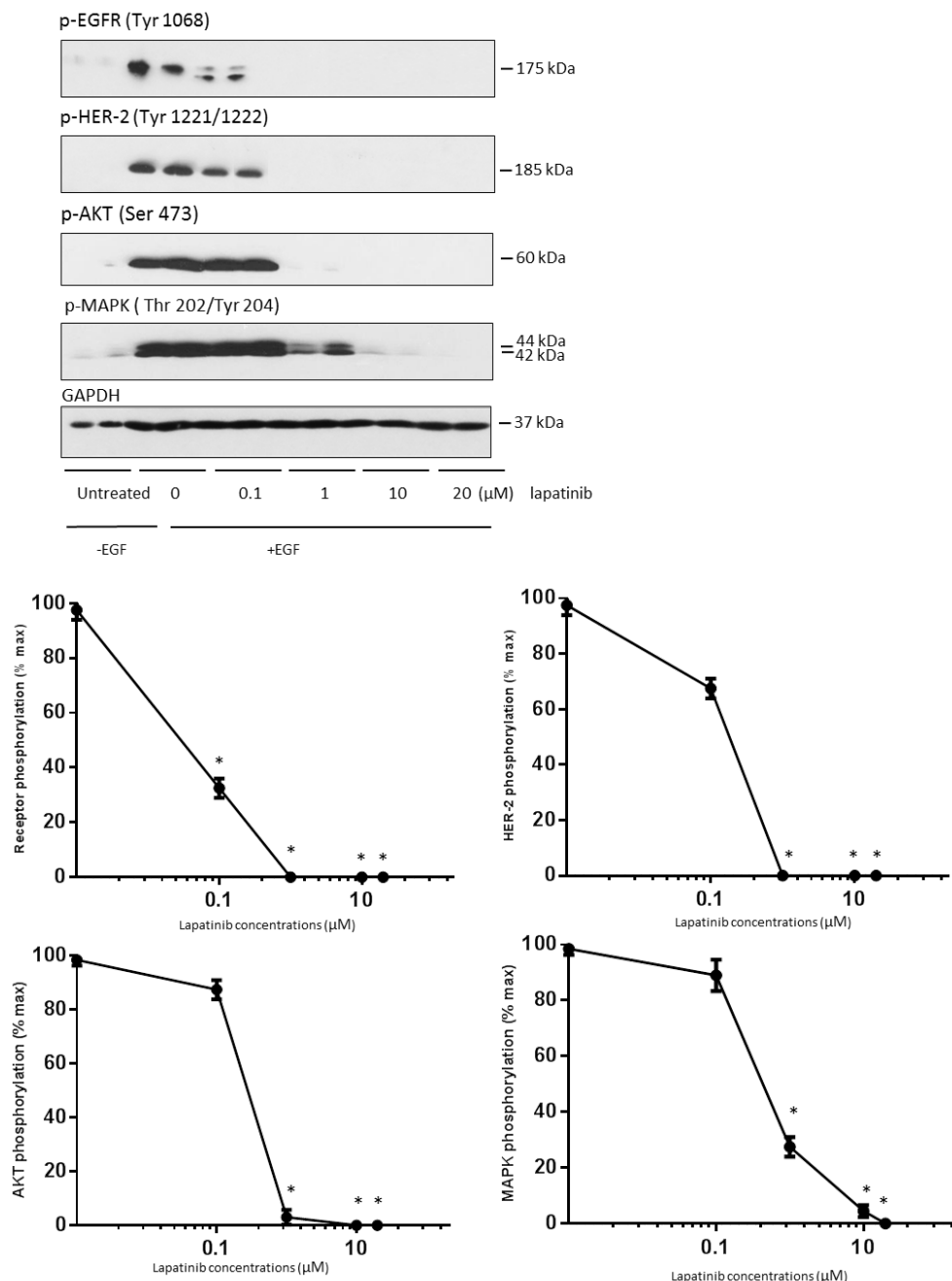


Figure 5.13: Effect of lapatinib on activation of both EGFR and HER-2 by EGF in SK-BR-3 cells. SK-BR-3 cells were seeded into 12 well tissue culture plates at a density of 10×10^4 cells/well in 1 ml of maintenance medium. Cells were incubated for 24 hours to allow attachment. Medium was aspirated and cells were washed with 0.5 ml of PBS. Medium was replaced daily with 1 ml/well of DCCS medium for two days. After two days of incubation, medium was replaced with DCCS supplemented with lapatinib at final concentrations of 0, 0.1, 1, 10, 20 μ M and cells were incubated with the lapatinib for 30 minutes. After 30 minutes treatment of cells with various concentrations of lapatinib, medium was replaced with the DCCS supplemented with 50 ng/ml of EGF and cells were stimulated for 15 minutes. Proteins were extracted with 50 μ l of RIPA buffer. Aliquots of 10 μ g protein were electrophoresed on a denaturing 12 % polyacrylamide gel and transferred to nitrocellulose membrane for 45 minutes at 100 mA. Membranes were incubated with phosphorylated EGFR (1:20,000 dilution), phosphorylated HER-2 (1:2000 dilution), phosphorylated AKT (1:15,000 dilution), phosphorylated MAPK (1:20,000 dilution) and GAPDH (1:20,000 dilution). Representative results are from duplicate experiment. Labwork 4.0 software used for densitometric analysis of the X-ray films and measurement were normalised with GAPDH. Asterisks represent the statistically significant reduction in phosphorylation of EGFR, HER-2, AKT and MAPK in the presence of lapatinib compared with in the absence of lapatinib (One way ANOVA, * $p < 0.05$).

Treatment of EGF stimulated cells with lapatinib resulted in a concentration dependent decrease in the phosphorylation of EGFR and HER-2, detected from a concentration of 0.1 μM lapatinib. There was an almost complete inhibition in phosphorylated AKT at concentrations of 1 μM and higher. Reduction in phosphorylation of MAPK was detected at concentrations of 1 μM , and complete inhibition was observed at higher concentrations.

Stimulation of SK-BR-3 cells with various concentrations of lapatinib resulted in inhibition of activation of both downstream components Akt and MAPK, which suggest that lapatinib is an effective inhibitor of signal transduction induced by EGF.

5.2.14 Inhibition of EGFR and HER-2 by lapatinib abrogates the protective effect of EGF against cell death in anoikis in SK-BR-3 cells

Based on the previous result shown in section 5.2.13, lapatinib completely inhibited the phosphorylation of EGFR but not the downstream proteins in SK-BR-3 cells at concentrations of 1 μM and complete inhibition of the downstream proteins was obtained at concentration of 10 μM . Hence the effect of lapatinib on the protective effect of EGF was investigated at two different concentrations of 1 μM (data not shown) and 10 μM .

Cells were cultured in non-attached conditions in serum-free medium for 30 minutes in the presence and absence of 1 μM and 10 μM lapatinib and in the presence and absence of 50 ng/ml EGF, proteins were extracted and analysed for cleaved PARP, phosphorylation of HER-2, EGFR, AKT and MAPK by using western transfer (Figure 5.14).

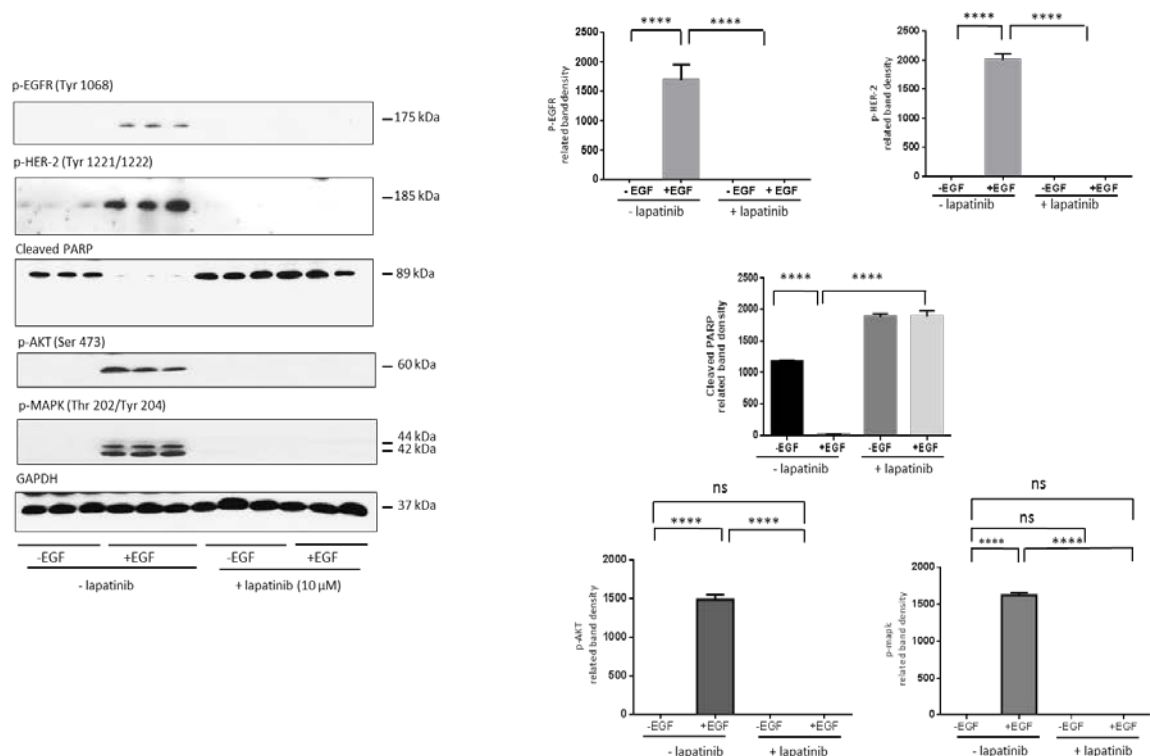


Figure 5.14: The effect of inhibition of EGFR and HER-2 by lapatinib on the protective effect of EGF in SK-BR-3 cells. SK-BR-3 cells were seeded into 12 well poly-HEMA coated plates at 20×10^4 cells/well in 2 ml of serum free medium, in the presence 10 μM of lapatinib or vehicle (DMSO) and in the presence and absence of 50 ng/ml of EGF. Proteins were extracted after incubation of 6 hours from SK-BR-3 cells with 50 μl per well of RIPA buffer. Aliquots of 10 μg protein were analysed by electrophoresis in 12% denatured gel and transferred for 45 minutes at 100 mA. Membranes were incubated with antibody against phosphorylated EGFR (1:20,000 dilution), phosphorylated HER-2 (1:2000 dilution), cleaved PARP (1:2000 dilution), phosphorylated AKT (1:10,000 dilution), phosphorylated MAPK (1:20,000 dilution) and GAPDH (1:20,000 dilution). Illustrated images are representative of cleaved PARP from a triplicate experiment that have been repeated at least two times. Labwork 4.0 software used for densitometric analysis of the X-ray films and measurement were normalised with GAPDH. Error bars are \pm SEM. Asterisks denotes there was a statistically significant increase in the amount of cleaved PARP, complete inhibition in phosphorylation of EGFR, HER-2, AKT and MAPK in the presence of lapatinib compared with in the absence of lapatinib (One way ANOVA, **** $p < 0.0001$).

As is shown in Figure 5.14, culture of SK-BR-3 cells in non-attached conditions in serum-free medium for 6 hours induced anoikis, which was inhibited in the presence of EGF at 50 ng/ml. In the presence of EGF, there was a statistically significant increase in the amount of cleaved PARP in the presence of 10 μM lapatinib. This was followed by complete inhibition of activation of downstream proteins AKT and MAPK in the presence of 10 μM lapatinib. Although in the absence of EGF, lapatinib at 1 μM concentration increased the cell death but was not enough to block the survival effect of EGF (data not shown). This observation agrees with the finding that higher concentration of lapatinib is

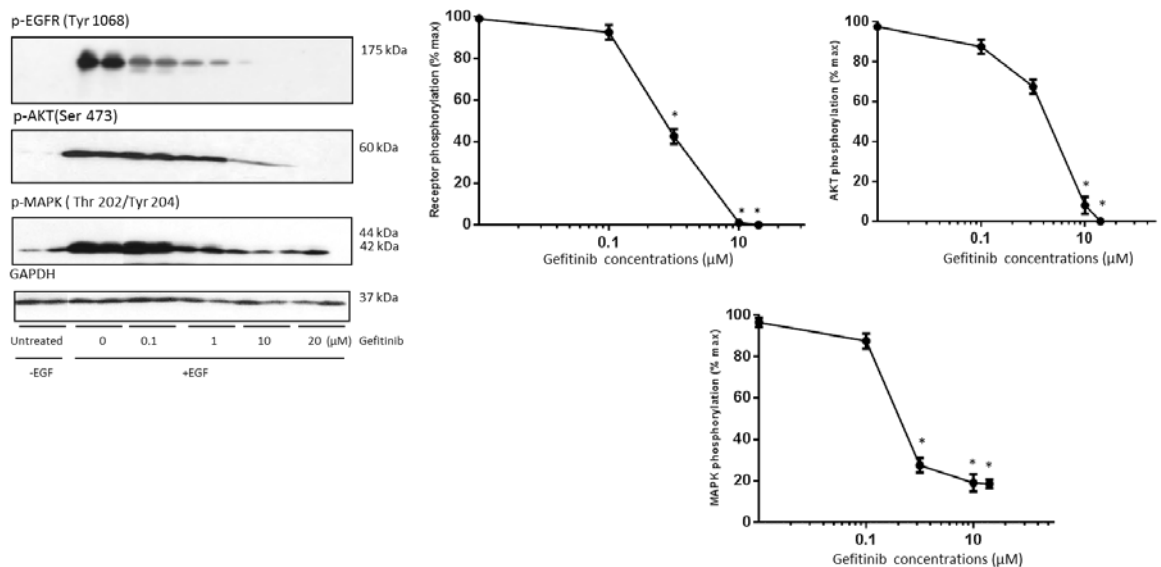
required as demonstrated to inhibit the downstream signal transduction PI3K/AKT and MAPK pathways in Figure 5.14.

5.2.15 Effect of inhibition of EGFR with gefitinib on anti-anoikis effect of EGF in oestrogen receptor-negative breast cancer cells

Gefitinib is a selective inhibitor of EGFR tyrosine kinase domain which inhibits EGFR autophosphorylation and downstream signal transduction; hence leading to inhibition in growth of EGFR overexpressed tumour cells (Thomas and Grandis 2004).

To investigate the effectiveness of gefitinib and establish the concentration at which the maximum inhibition occurs in the cells that EGF had protective effect, Hs578T and SK-BR-3 cells were cultured in attached condition in full maintenance medium and incubated with different concentrations of gefitinib up to 20 μM for 30 minutes. After 30 minutes incubation, cells were stimulated with 50 ng/ml of EGF for 15 minutes. Proteins were extracted and analysed by western transfer for phosphorylation of EGFR, AKT, and MAPK (Figure 5.15).

A Hs578T



B SK-BR-3

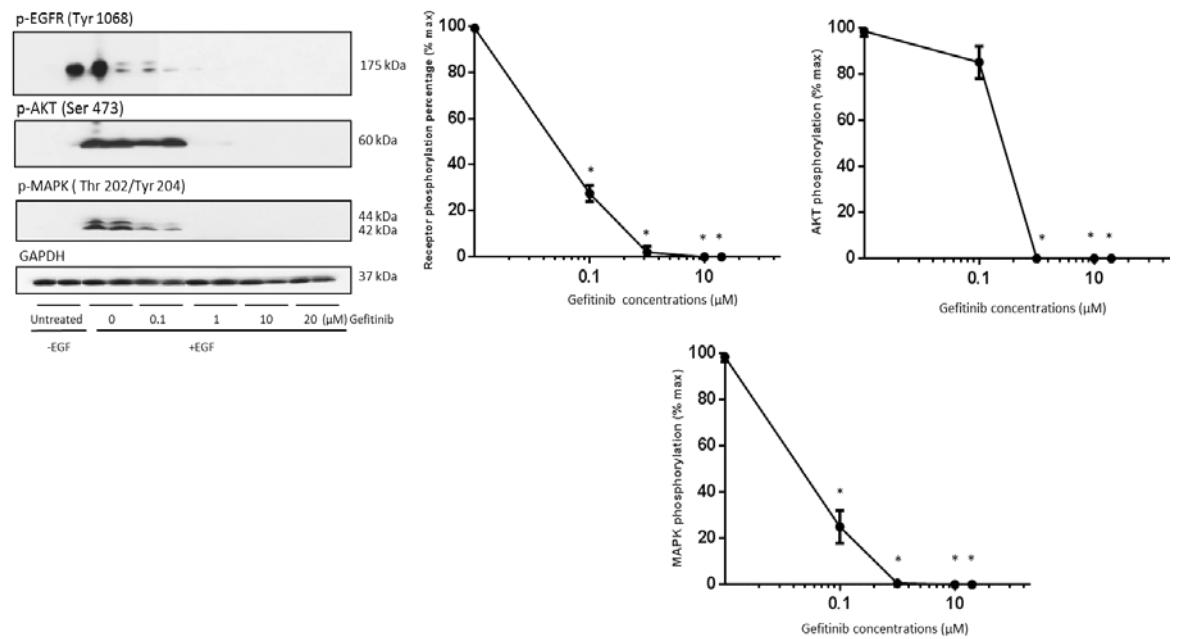


Figure 5.15: Effect of gefitinib on activation of EGFR by EGF in Hs578T and SK-BR-3 cells. Hs578T (A) and SK-BR-3 (B) cells were seeded into 12 well tissue culture plates at density of 10×10^4 cells/well and 20×10^4 cells/well in 1 ml of maintenance medium respectively. Cells were incubated for 24 hours to allow attachment. Medium was aspirated and cells were washed with 0.5 ml of PBS. Medium was replaced daily with 1 ml/well of DCCS medium for two days. After two days of incubation, medium was replaced with DCCS supplemented with gefitinib at final concentrations of 0, 0.1, 1, 10, 20 μ M and cells were incubated with the inhibitor for 30 minutes. After 30 minutes treatment of cells with various concentrations of inhibitor, medium was replaced with the DCCS supplemented with 50 ng/ml of EGF and cells were stimulated for 15 minutes. Proteins were extracted with 50 μ l of RIPA buffer. Aliquots of 10 μ g proteins were electrophoresed on a denaturing 12 % polyacrylamide gel and transferred into nitrocellulose membrane for 45 minutes at 100 mA. Membranes were incubated with phosphorylated EGFR (1:20,000 dilution), phosphorylated AKT (1:10,000 dilution), phosphorylated MAPK (1:20,000 dilution) and GAPDH (1:20,000 dilution). Representative bands are from duplicate experiment. Labwork 4.0 software used for densitometric analysis of the X-ray films and measurement were normalised with GAPDH. Asterisks represent the statistically significant reduction in phosphorylation of EGFR, AKT and MAPK in the presence of gefitinib compared with in the absence of gefitinib (One way ANOVA, * $p < 0.05$).

Following stimulation with 50 ng/ml of EGF in both Hs578T and SK-BR-3 cells, phosphorylation of EGFR was reduced in a concentration dependant fashion by increase in the concentration of gefitinib. In Hs578T cells, reduction in phosphorylation of EGFR was detected in the presence of gefitinib at concentration of 0.1 μ M, followed by complete inhibition at concentration of 10 μ M and higher. Reduction in AKT phosphorylation was detected in the presence of 10 μ M gefitinib and complete inhibition was observed at higher concentration in this cell line. Decreased phosphorylation of MAPK was detected at concentration of 1 μ M and higher, and decreased more with increasing gefitinib concentration.

In SK-BR-3 cells, reduction in phosphorylation of EGFR was observed in the presence of 0.1 μ M gefitinib and complete inhibition was detected at concentration of 1 μ M and higher. Complete inhibition in the phosphorylation of AKT was observed at concentration of 1 μ M and at higher concentrations in this cell line. Reduction in MAPK phosphorylation was detected at 0.1 μ M, followed by complete inhibition at 1 μ M and higher.

Based on these results, the complete inhibition of EGFR and downstream signal transduction components in both cell lines at specific concentrations suggest the inhibitory effect of gefitinib against activation of EGFR signalling pathway, and that SK-BR-3 cells are more sensitive to gefitinib than Hs578T cells as complete inhibition of phosphorylated EGFR and AKT was detected at concentration of 1 μ M, whereas complete inhibition in Hs578T cells observed at concentration of 20 μ M.

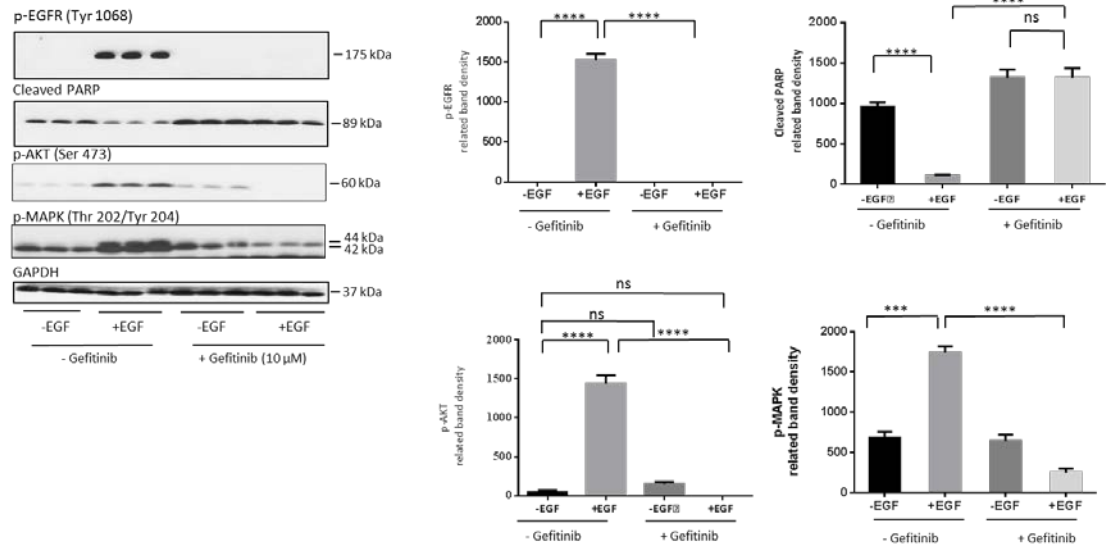
5.2.16 Inhibition of EGF receptor by gefitinib inhibitor on protective effect of EGF against anoikis in Hs578T and SK-BR-3 cells

To confirm that the EGFR mediates the anti-anoikis effect of EGF, the effect of EGF in anoikis was evaluated in the presence and absence of gefitinib which is a specific inhibitor of the EGFR.

Response to gefitinib has been linked to activating mutations in the *EGFR* gene in patients with lung adenocarcinoma. Hs578T and SK-BR-3 cells have been reported to have non-mutated EGFR gene based on the mutation analysis (Ikediobi, Davies et al. 2006).

Hs578T and SK-BR-3 cells which express high level of EGFR were cultured in non-attached condition in serum-free medium in the presence and absence of gefitinib, and in the presence and absence of EGF. Proteins were extracted after incubation for 6 hours for both Hs578T and SK-BR-3 cells and were analysed for cleaved PARP, phosphorylation of EGFR, AKT and MAPK by using western transfer (Figure 5.16).

A Hs578T



B SKBR-3

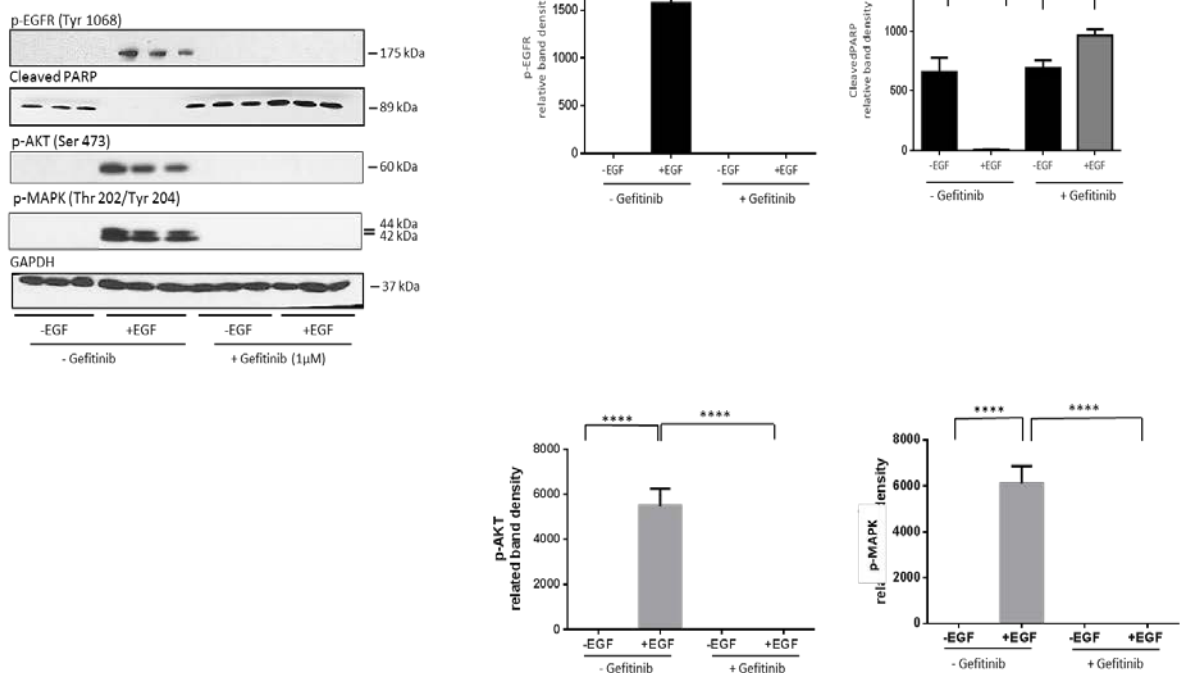


Figure 5.16: Effect of inhibition of EGFR with gefitinib on the anti-anoikis effect of EGF in Hs578T and SK-BR-3 cells. Hs578T and SK-BR-3 cells were seeded into 12 well poly-HEMA coated plates at 20×10^4 cells/well in 2ml of serum free medium, in the presence of 10 μ M of gefitinib or vehicle (DMSO) for Hs578T cells or in the presence of 1 μ M gefitinib or vehicle (DMSO) for SK-BR-3 cells, and in the presence and absence of 50 ng/ml EGF. After incubation of 6 hours, proteins were extracted from both Hs578T and SK-BR-3 cells with 50 μ l per well of RIPA buffer. Aliquots of 10 μ g protein were separated by electrophoresis in denaturing 12% polyacrylamide gels and transferred to nitrocellulose for 45 minutes at 100 mA. Membranes were incubated with antibody against phosphorylated EGFR (1:20,000 dilution), cleaved PARP (1:2000 dilution), phosphorylated AKT (1:10,000 dilution for Hs578T cells and 1:20,000 for SK-BR-3 cells), phosphorylated MAPK (1:10,000 dilution for Hs578T cells and 1:20,000 for SK-BR-3 cells) and GAPDH (1:20,000 dilution). Images shown are representative of cleaved PARP

from a triplicate experiment that have been repeated at least three times. Labwork 4.0 software used for densitometric analysis of the X-ray films and measurement were normalised with GAPDH. Error bars are \pm SEM. Asterisks denotes there was a statistically significant increase in the amount of cleaved PARP and complete inhibition in phosphorylation of EGFR, AKT in both cell lines along with full inhibition of MAPK phosphorylation in SK-BR-3 cells and significant reduction in phosphorylation of MAPK in Hs578T cells in the presence of gefitinib compared with in the absence of gefitinib (One way ANOVA, *** $p=0.0001$, **** $p<0.0001$).

In Hs578T cells, stimulation with EGF in the absence of gefitinib induced strongly EGFR phosphorylation. In the presence of 10 μ M gefitinib, no EGFR phosphorylation was detected in response to stimulation with EGF, compared with in the absence of gefitinib. Hs578T cells underwent anoikis following incubation in non-attached conditions in serum-free medium for 6 hours. In the presence of 50 ng/ml EGF anoikis was inhibited almost completely. The protective effect of EGF against anoikis was significantly reduced in the presence of gefitinib which was demonstrated by significant increase in the amount of cleaved PARP in the presence of gefitinib compared to the absence of gefitinib. The increase in phosphorylation of AKT in response to stimulation with EGF was completely inhibited in the presence of gefitinib. Increase in the amount of MAPK phosphorylation in response to stimulation with EGF was reduced markedly in the presence of gefitinib.

In SK-BR-3 cells, stimulation with EGF in the absence of gefitinib induced phosphorylation of EGFR, whereas in the presence of 1 μ M gefitinib no EGFR phosphorylation was detected even after long exposure (Data not shown). In the presence of 50 ng/ml EGF anoikis was inhibited completely. The anti-anoikis effect of EGF was significantly reduced in the presence of gefitinib ($p<0.001$). This was shown by significant increase in the amount of cleaved PARP. The increase in phosphorylation of both AKT and MAPK in response to stimulation with EGF was completely inhibited in the presence of gefitinib.

The survival effect of EGF was completely inhibited with gefitinib, which can support the contention that the survival effect of EGF is mediated through the EGFR receptor.

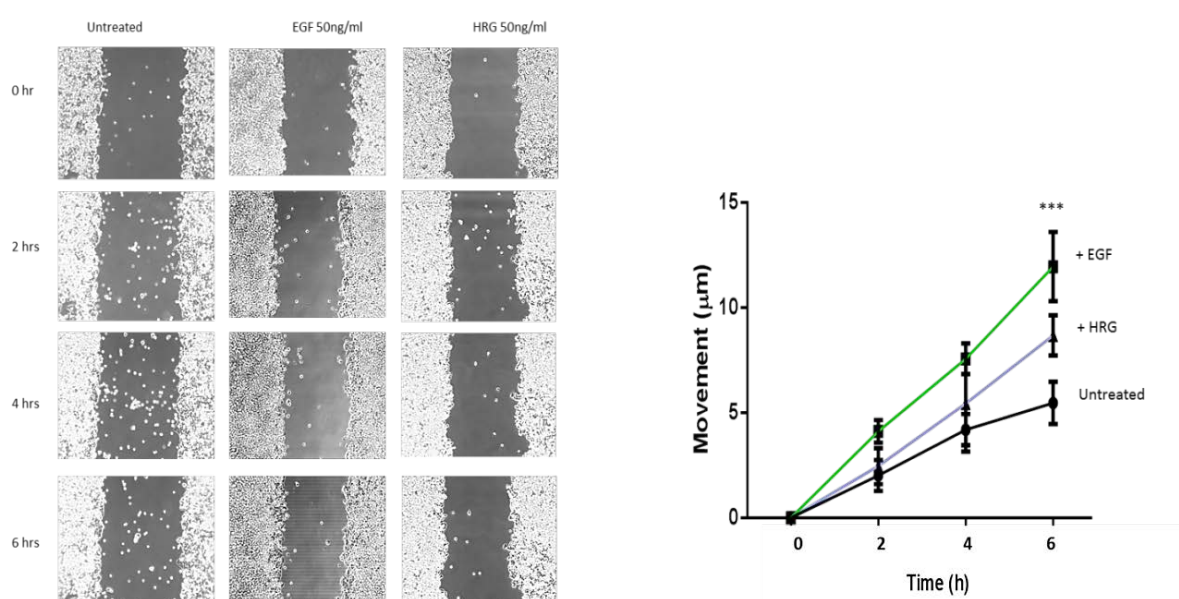
5.2.17 Effect of EGF and heregulin-1 on migration of oestrogen receptor-negative breast cancer cells

5.2.17.1 Effect of EGF and heregulin-1 on migration of triple-negative MDA-MB-231 and Hs578T breast cancer cells

One of the characteristics of malignancy and predisposition of metastasis is an increase in the motility of the cancerous cells. Hence, the effects of EGF and heregulin-1 on migration of two triple-negative MDA-MB-231 and Hs578T breast cancer cells were evaluated in an *in vitro* wound healing assay.

Cells were plated at high density in 24 well plates, under which two parallel lines had been scored underneath each wells and left for 24 hours in order to form a confluent monolayer. Cells were washed and withdrawn from the effect of growth factors for 16 hours. After 16 hours, cells were wounded and incubated for 2 hours in 0.01% BSA and phenol red-free DMEM. After 2 hours, the first images of the wounded cells were captured in the absence or presence of EGF and heregulin-1 and represent as 0 hour. Images of the cells were captured every two hours up to 6 hours of incubation. The width of the each wound was measured 20 times with Image J software and the average was calculated (Figure 5.17). Cell migration was evaluated by measuring the distance of the edge of the wound towards the centre of the wound.

A MDA-MB-231



B Hs 578T

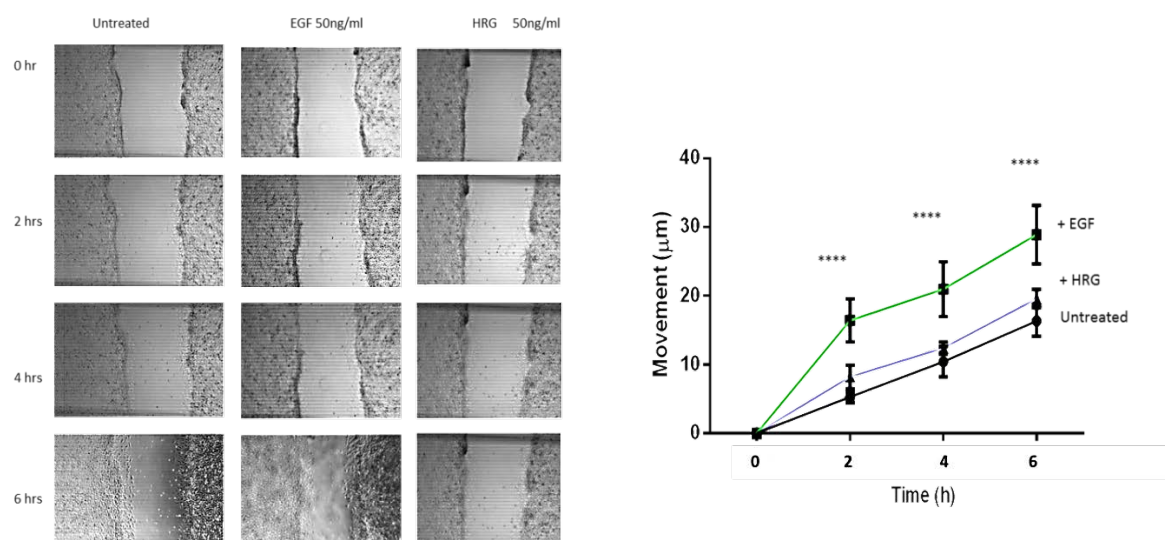


Figure 5.17: Effect of EGF and heregulin-1 on migration of triple-negative breast cancer cells. MDA-MB-231 (A) and Hs578T (B) cells were seeded at density of 40×10^4 cells/well into 24 well plates that had scored on the under surface with two parallel lines across the middle of each well. Cells were withdrawn in phenol red-free DMEM medium supplemented with 10% charcoal-treated newborn calf serum as described in Materials and Methods section. Monolayer cells were wounded with a sterile 2 μ l Gilson pipette tip through the centre of the well, perpendicular to the scored lines. Wounded cells were washed twice with 0.5 ml of phenol red-free DMEM and incubated in phenol red-free DMEM supplemented with 0.01 % of BSA for two hours. After two hours of incubation, cells were washed again and incubated with phenol red-free DMEM medium to remove any extra cell debris or detached cells and 0.01% BSA alone () or supplemented with 50 ng/ml of EGF (■) or in the presence of 50 ng/ml heregulin-1 (HRG) (▲) for various time points up to 6 hours. Image were captured at the beginning of the experiment (time 0) and after each two hours of treatment up to 6 hours. Three separate measurements in three different wells were made for each treatment condition. The distance between the wounds was measured using Image J software programme. The demonstrated values are the mean \pm SEM. Asterisks denotes there was a statistical significant increase in the cell migration in the presence of EGF compare with in the absence of EGF in both MDA-MB-231 and Hs578T cells (One way ANOVA, *** $p=0.0001$, **** $p<0.0001$).

As illustrated in the Figure 5.17, addition of 50 ng/ml EGF stimulated the movement of both MDA-MB-231 and Hs578T cells 2.4 fold and 3 fold respectively. In MDA-MB-231 cells, there was a statistically significant increase in the migration of cells after 6 hours of incubation, whereas, significant increase in migration of Hs578T cells was observed after 2, 4 and 6 hours of incubation with 50 ng/ml EGF. Heregulin-1 did not stimulate the movement of the MDA-MB-231 or Hs578T cells at any time analysed, as no significant stimulation of cell movement was detected at any of the incubation time. Results suggest that EGF has potential migratory effect on both triple-negative breast cancer cell lines.

5.2.17.2 Effect of EGF and heregulin-1 on migration of HER-2 positive SKBR-3 breast cancer cells

To examine the effectiveness of EGF and heregulin-1 on migration of HER-2 positive SKBR-3 breast cancer cells, *in vitro* wound healing assay was initially conducted as described in section 5.2.17.1.

Wounded SK-BR-3 cells were incubated in the presence and absence of 50 ng/ml EGF and in the presence and absence of 50 ng/ml heregulin-1 at 0, 2, 4 and 6 hours of incubation. Images of the cells were captured at each particular time. The width of the each wound was measured 20 times and the average of them was calculated. The cell migration was evaluated by measuring the distance of the edge of the wound towards the centre of the wound by using Image J software (Figure 5.18).

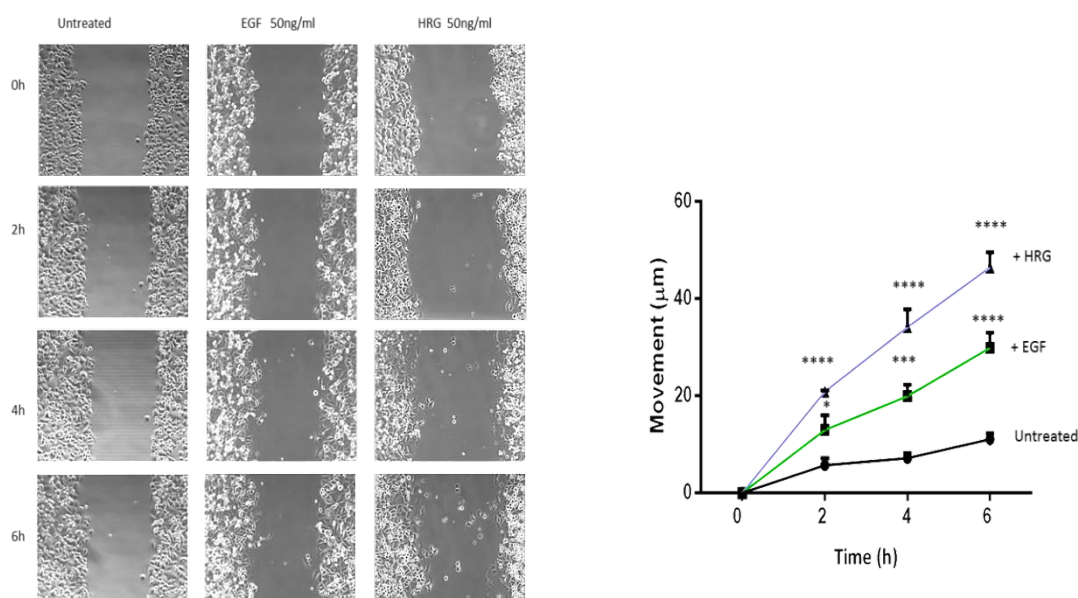
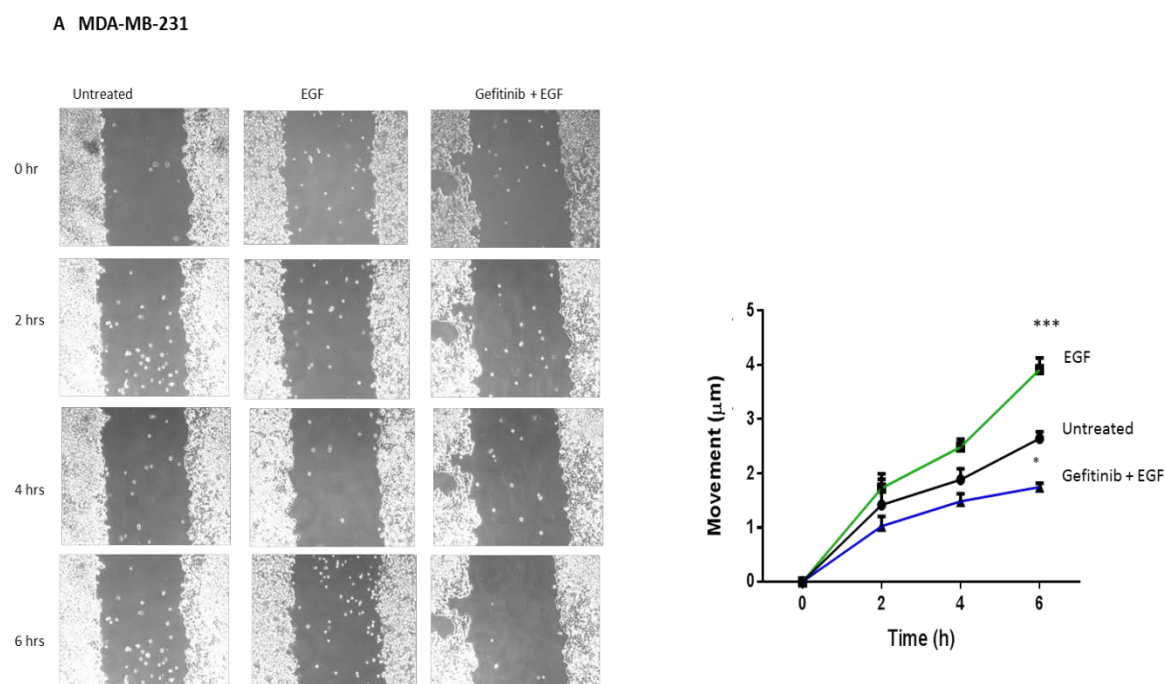


Figure 5.18: Effect of EGF and heregulin-1 on migration of SK-BR-3 cells. SK-BR-3 cells were seeded at density of 40×10^4 cells/well into 24 well plates that had scored on the under surface with two parallel lines across the middle of each wells. Cells were withdrawn in phenol red-free DMEM medium supplemented with 10% charcoal-treated newborn calf serum as described in Materials and Methods section. Monolayer cells were wounded with a sterile 2 μ l Gilson pipette tip through the centre of the well, perpendicular to the scored lines. Wounded cells were washed twice with 0.5 ml of phenol red-free DMEM and incubated in phenol red-free DMEM supplemented with 0.01 % of BSA for two hours. After two hours of incubation, cells were washed again and incubated with phenol red-free DMEM medium and 0.01% BSA alone () or supplemented with 50 ng/ ml of EGF (■) or in the presence of 50 ng/ ml heregulin-1(HRG) (▲) for various time points up to 6 hours. Image were captured at the beginning of the experiment (time 0) and after each two hours of treatment up to 6 hours. Three separate measurements in three different wells were made for each treatment condition. The distance between the wounds was measured using Image J software programme. The demonstrated values are the mean \pm SEM. Asterisks denotes there was a statistically significant increase in the cell migration in the presence of EGF and heregulin-1 compare with in the absence of EGF or heregulin-1 in SK-BR-3 cells (One way ANOVA, ** $p < 0.001$, **** $p < 0.0001$).

Addition of 50 ng/ml of EGF significantly enhanced migration of SK-BR-3 cells two fold after 6 and 8 hours of incubation. Addition of 50 ng/ml heregulin-1 stimulated migration of cells more than three fold after 4 and 6 hours of incubation, which indicates that heregulin-1 has more migratory effect than EGF on SK-BR-3 cells.

5.2.18 Effect of EGFR inhibitor on migration of triple-negative MDA-MB-231 and Hs578T breast cancer cells

In previous sections the potential migratory effect of EGF was evaluated on motility of oestrogen receptor-negative breast cancer cells. The migration stimulated was less than with IGF-1 but is comparable to that reported previously (Mezi, Todi et al. 2012). To investigate whether the migratory effect of EGF is inhibited by EGFR tyrosine kinase inhibitor, gefitinib in triple-negative breast cancer cells, wounded MDA-MB-231 and Hs578T cells were incubated in the presence and absence of 10 μ M gefitinib and in the presence and absence of 50 ng/ml EGF at 0, 2, 4 and 6 hours of incubation. Images of the cells were captured at each particular time. The width of the each wound was measured 20 times and the average of them was calculated. The cell migration was evaluated by measuring the distance of the edge of the wound towards the centre of the wound by using Image J software (Figure 5.19).



B Hs578T

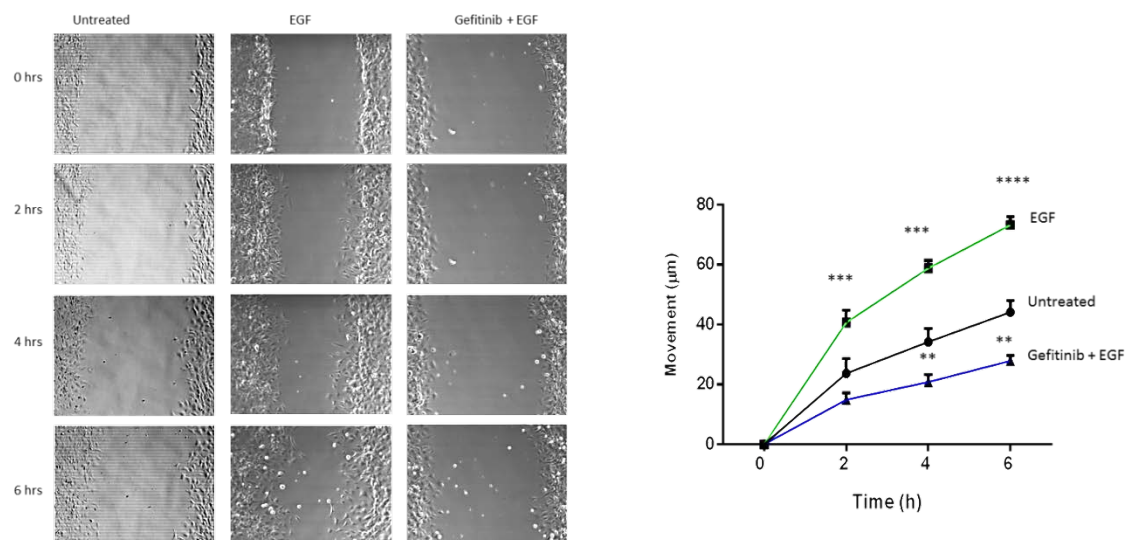


Figure 5.19: Effect of inhibition of EGFR with gefitinib on migration of triple-negative breast cancer cells. MDA-MB-231 (A) and Hs578T (B) cells were seeded at density of 40×10^4 cells/well into 24 well plates that had scored on the under surface with two parallel lines across the middle of each wells. Cells were withdrawn in phenol red-free DMEM medium supplemented with 10% charcoal-treated newborn calf serum as described in Materials and Methods section. Monolayer cells were wounded with a sterile 20-200 µl Gilson pipette tip through the centre of the well, perpendicular to the scored lines. Wounded cells were washed twice with 0.5 ml of phenol red-free DMEM and incubated in phenol red-free DMEM supplemented with 0.01 % of BSA for two hours. After two hours of incubation, cells were washed again and incubated with phenol red-free DMEM medium and 0.01% BSA alone () or supplemented with 50 ng/ ml of EGF (■), or in the presence of 10 µM gefitinib and 50 ng/ml EGF (▲) for various time points up to 6 hours. Image were captured at the beginning of the experiment (time 0) and after each two hours of treatment up to 6 hours. Three separate measurements in three different wells were made for each treatment condition. The distance between the wounds was measured using Image J software programme. The demonstrated values are the mean \pm SEM. Asterisks denotes there was a statistically significant increase in the cell migration in the presence of EGF compare with in the absence of EGF and there was significant reduction in the migration of cells in the presence of the gefitinib cells in both MDA-MB-231 and Hs578T cells (One way ANOVA, * $p < 0.05$, ** $P < 0.001$, *** $p = 0.0001$, **** $P < 0.0001$).

As it was demonstrated previously, there was a statistically significant increase in migration of both MDA-MB-231 and Hs578T cells in the presence of 50 ng/ml EGF. Treatment of cells with 10 µM gefitinib significantly reduced migration of MDA-MB-231 cells after 6 hours and migration of Hs578T cells after 4 and 6 hours of incubation. These results suggest that gefitinib has an inhibitory effect against migration of cells stimulated by EGF.

5.2.19 Effect of EGFR inhibitor on migration of SK-BR-3 cells

The ability of gefitinib to inhibit migration of SK-BR-3 cells stimulated by EGF was tested by wounding healing assay.

Wounded SK-BR-3 cells were incubated in the presence and absence of 10 μ M gefitinib and in the presence and absence of 50 ng/ml EGF at 0, 2, 4 and 6 hours of incubation. Images of the cells were captured at each particular time. The width of the each wound was measured 20 times and the average of them was calculated. The cell migration was evaluated by measuring the distance of the edge of the wound towards the centre by using Image J software (Figure 5.20).

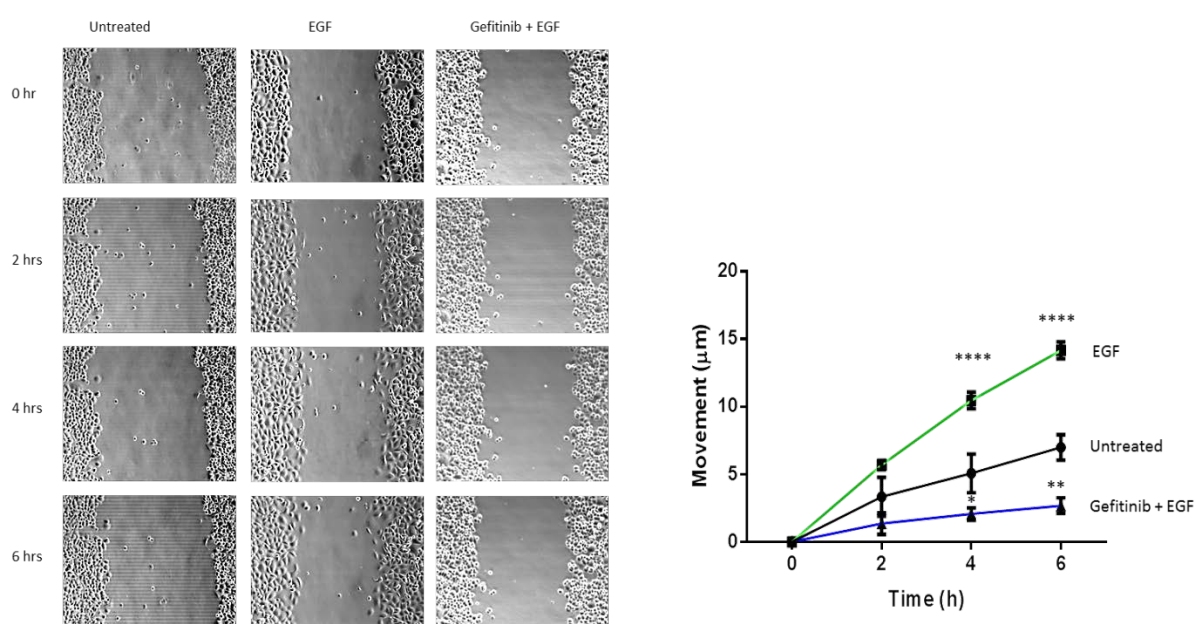


Figure 5.20: Effect of inhibition of EGFR with gefitinib on migration of SK-BR-3 cells. SK-BR-3 cells were seeded at density of 40×10^4 cells/well into 24 well plates that had scored on the under surface with two parallel lines across the middle of each wells. Cells were withdrawn in phenol red-free DMEM medium supplemented with 10% charcoal-treated newborn calf serum as described in Materials and Methods section. Monolayer cells were wounded with a sterile 2 μ l Gilson pipette tip through the centre of the well, perpendicular to the scored lines. Wounded cells were washed twice with 0.5 ml of phenol red-free DMEM and incubated in phenol red-free DMEM supplemented with 0.01 % of BSA for two hours. After two hours of incubation, cells were washed again and incubated with phenol red-free DMEM medium and 0.01% BSA alone () or supplemented with 50 ng/ ml of EGF (■), or in the presence of 10 μ M gefitinib and 50 ng/ ml EGF () for various time points up to 6 hours. Image were captured at the beginning of the experiment (time 0) and after each two hours of treatment up to 6 hours. Three separate measurements in three different wells were made for each treatment condition. The distance between the wounds was measured using Image J software programme. The demonstrated values are the mean \pm SEM. Asterisks denotes there was a statistically significant increase in the cell migration in the presence of EGF compared with in the absence of EGF and there was significant reduction in the migration of cells in the presence of the gefitinib in SK-BR-3 cells compared with in the absence of gefitinib (One way ANOVA, ** $P < 0.001$, **** $P < 0.0001$).

Figure 5.20 shows the inhibitory effect of gefitinib on the migration response of SK-BR-3 cells to EGF. Treatment of SK-BR-3 cells with gefitinib reduced significantly EGF stimulated movement two folds at 4 and 6 hours of incubation, which can suggest that blocking the EGFR by gefitinib, inhibits the migratory effect of EGF.

5.2.20 Effect of dual tyrosine kinase inhibitor of EGFR and HER-2 on migration of HER-2 positive SK-BR-3 cells

The ability of dual tyrosine kinase inhibitor lapatinib on inhibition of SK-BR-3 cell stimulated with EGF was tested by using wound healing assay.

Wounded SK-BR-3 cells were incubated in the presence and absence of 10 μ M lapatinib and in the presence and absence of 50 ng/ml EGF for 0, 2, 4 and 6 hours of incubation. Images of the cells were captured at each particular time. The width of the each wound was measured 10 times and the average of them was calculated. The cell migration was evaluated by measuring the distance of the edge of the wound towards the centre by using Image J software (Figure 5.21).

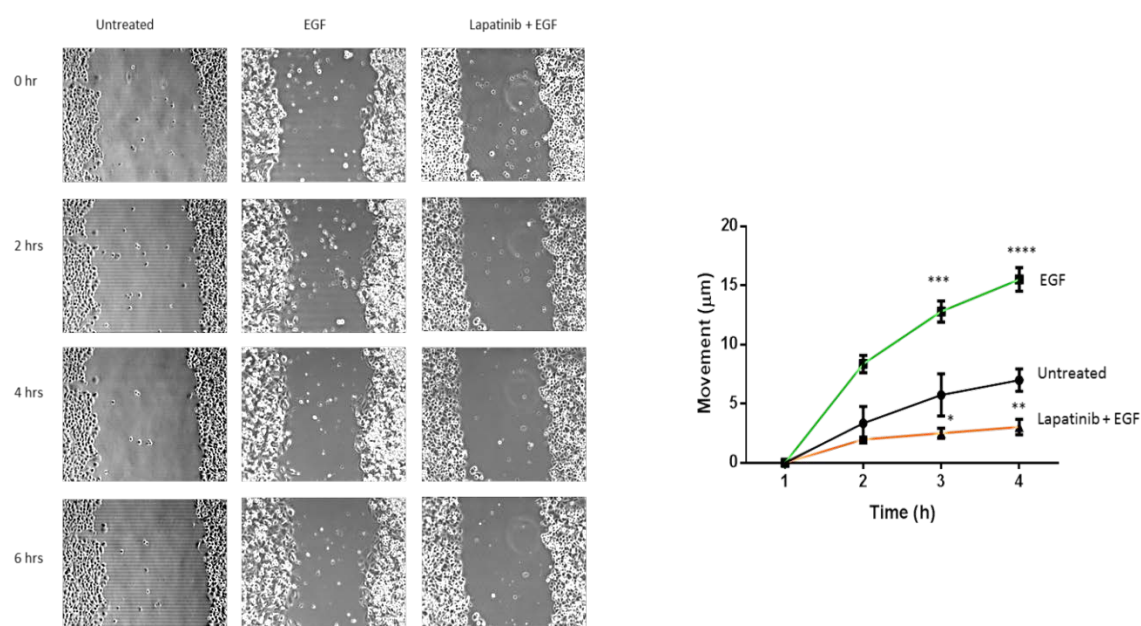


Figure 5.21: Effect of Lapatinib on migration of SK-BR-3 cells. SK-BR-3 cells were seeded at density of 40×10^4 cells/ well into 24 well plates that had scored on the under surface with two parallel lines across the middle of each wells. Cells were withdrawn in phenol red-free DMEM medium supplemented with 10% charcoal-treated newborn calf serum as described in Materials and Methods section. Monolayer cells were wounded with a sterile 2 μ l Gilson pipette tip through the centre of the well, perpendicular to the scored lines. Wounded cells were washed twice with 0.5 ml of phenol red-free DMEM and incubated in phenol red-free DMEM supplemented with 0.01 % of BSA for two hours. After two hours of incubation, cells were washed again and incubated with phenol red-free DMEM medium and 0.01% BSA alone () or supplemented with 50 ng/ ml of EGF (■), or in the presence of 10 μ M lapatinib and 50 ng/ml

EGF () for various time points up to 6 hours. Image were captured at the beginning of the experiment (time 0) and after each two hours of treatment up to 6 hours. Three separate measurements in three different wells were made for each treatment condition. The distance between the wounds was measured using Image J software programme. The demonstrated values are the mean \pm SEM. Asterisks denotes there was a statistically significant increase in the cell migration in the presence of EGF compared with in the absence of EGF, and significant reduction in migration of EGF stimulated cells in the presence of lapatinib compared with in the absence of lapatinib (One way ANOVA, * $p < 0.05$, ** $p < 0.001$, *** $p = 0.0001$, **** $P < 0.0001$).

EGF significantly stimulated the migration of SK-BR-3 cells which is supporting the previous findings. There was a statistically significant two fold reduction in the presence of 10 μ M lapatinib on stimulated cells with 50 ng/ml EGF at 4 and 6 hours of incubation. Result suggests that blocking both EGFR and HER-2 inhibit the migratory effect of EGF in SK-BR-3 cells.

5.3 Discussion

As mentioned in the discussion of chapter 3, avoidance of anoikis and increase in cell motility are significant characteristics of malignant epithelial tumour cells. Deregulation of growth factor receptors expression is known as one of the anoikis-resistant mechanisms, which triggers activation of cell survival pathways and induces migration and invasion of cells (Wells 2000). Overexpression of EGFR and HER-2 has been reported in contribution of lung tumours metastasis through activation of MAPK signalling which leads to degradation of Bim and subsequently block the anoikis pathway (Sharma, Bell et al. 2007, Paoli, Giannoni et al. 2013). In 2003, Reginato *et al* illustrated in human mammary epithelial cells MCF-10A that when EGFR is overexpressed, it mediates ERK pathway activation which in turns blocks the anoikis process through suppression of Bim (Reginato, Mills et al. 2003). In another study, overexpression of HER-2 in human breast cancer was showed to suppress Bim expression through activation of MAPK pathway which subsequently conferring anoikis insensitivity (Haenssen, Caldwell et al. 2010).

Several studies addressed the involvement of EGF in induction of cell motility and invasion in various cancers (Price, Wilson et al. 1996, Shibata, Kawano et al. 1996, Wells 2000). Overexpression of EGFR and HER-2 reported to be related to the ability of the mammary carcinoma cells to invade and metastasis (Yamaguchi, Wyckoff et al. 2005, Palmieri, Bronder et al. 2007). In 2006, Garcia *et al* reported that treatment of MCF-7 breast cancer cells with EGF induced cell motility through activation of both AKT and ERK pathways (Garcia, Franklin et al. 2006). In breast cancer, the correlation between overexpression of EGF and its receptors with higher incidence of distant metastasis has been reported in several studies (Ikediobi, Davies et al. 2006, De Luca, Carotenuto et al. 2008, Giltane, Moeder et al. 2009). Several *in vitro* mouse breast cancer model studies have been reported significant role of EGFR signalling inhibitors in reduction of bone and brain metastasis (Gril, Palmieri et al. 2008, Molli, Adam et al. 2008).

In this chapter, the role of EGF-like ligands, namely: EGF and heregulin-1 in the survival and migration of oestrogen receptor-negative breast cancer cells was investigated. The first part of this chapter discussed the effect of EGF and heregulin-1 on survival of oestrogen receptor-negative breast cancer cells in

non-attached conditions. Amongst oestrogen non-responsive breast cancer cells studied in this chapter, SK-BR-3 cells, Hs578T and MDA-MB-231 expressed high level of EGFR and downstream key components of EGFR signalling pathway was activated in response to stimulation with EGF in a concentration-dependent manner. Stimulation with EGF powerfully suppressed anoikis in Hs578T and SK-BR-3 breast cancer cells cultured in non-attached conditions, as evidenced by inhibition of PARP cleavage in these cell lines. In contrast, EGF did not show a protective effect against anoikis on MDA-MB-231 cells, which may be explained by the inability of EGF to induce autophosphorylation of EGFR and activation of the PI3K and AKT pathway in this cell line despite the activation of the MAPK pathway. These results suggest that survival signals need to be transmitted through PI3K and AKT but not the MAPK pathway.

It has been reported that HER-2-HER-3 heterodimers are potent activators of PI3K pathways (Holbro *et al*, 2003; Chan *et al*, 2005). Frequent co-expression of HER-3 with HER-2 in breast cancer has been reported and contribution of HER-3 in HER-2-mediated breast carcinogenesis has speculated (Bieche, Onody *et al*. 2003, Witton, Reeves *et al*. 2003, Sassen, Rochon *et al*. 2008, Park, Jung *et al*. 2014). Treatment of cells with heregulin-1 showed powerful effect on suppression of anoikis in HER-2 positive SK-BR-3 cells, which supports the supposition that HER-2 and HER-3 formed heterodimers in response to stimulation with heregulin-1 and activated the downstream key component AKT and MAPK pathways. Following treatment of triple-negative MDA-MB-231 and Hs578T cells with heregulin-1 did not show a protective effect, which suggests that the survival signals must be through EGFR. The survival effect of EGF was found to be concentration-dependent in Hs578T and SK-BR-3 cells. In addition, interference with the function of both EGFR and HER-2 and also EGFR with tyrosine kinase inhibitors reduced the survival effect of EGF accompanied by complete inhibition of activation of two main downstream effector proteins, AKT and MAPK.

The sensitivity of gefitinib in inhibition of growth in non-small lung cancer cell lines has been reported and that this sensitivity reported to be more associated with EGFR signal transduction pathway for mediation of cell proliferation and survival (Ono, Hirata *et al*. 2004). To our knowledge, effect of EGFR inhibitor

gefitinib and also dual tyrosine kinase inhibitor lapatinib on survival of anchorage-independent oestrogen receptor-negative breast cancer cells was not studied before. Hence, the effect of gefitinib and lapatinib was investigated on survival of oestrogen receptor-negative breast cancer cells in non-attached condition. In this chapter, gefitinib and lapatinib have been shown to play important role in abrogating the survival effect of EGF in non-attached oestrogen receptor-negative breast cancer cells. In SK-BR-3 cells, lapatinib demonstrated to play important role in the anti-anoikis effect of EGF, as observed by significant induction of cell death followed by block the two key downstream effector AKT and MAPK proteins. This result is agreed with other findings that lapatinib induces apoptosis and blocks activation of both downstream effectors AKT and MAPK in HER-2 positive BT-474 breast cancer cells (Xia, Mullin et al. 2002).

Role of gefitinib in induction of apoptosis in SK-BR-3 has been reported (Normanno, Campiglio et al. 2002). Here, we have tested the effect of gefitinib on induction of anoikis in SK-BR-3 cells, and to see whether the survival effect is transmitted via EGFR and HER-2 hetero-dimerisation or through EGFR. Gefitinib significantly induced reduction in survival effect of EGF, as significant increase in the amount of cleaved PARP was observed. This was consistent with the previous finding that gefitinib induce apoptosis (Normanno, Campiglio et al. 2002). The increase in phosphorylation of both AKT and MAPK in response to stimulation with EGF was completely inhibited in the presence of gefitinib in SK-BR-3 cells. Induction of cell death by gefitinib can in part be explained due to complete inhibition of PI3K and AKT pathway. Interestingly, in SK-BR-3 cells, both lapatinib and gefitinib abrogated the EGF survival effect which was accompanied by complete inhibition of the AKT and MAPK pathways. The result confirms that the survival effect of EGF is transmitted through EGFR. In Hs578T cells in the presence of gefitinib, the survival effect of EGF was significantly reduced, which was shown by significant increase in the amount of cleaved PARP. In this cell line, phosphorylation of AKT was completely inhibited in the presence of gefitinib but MAPK phosphorylation was inhibited partially.

The second part of this chapter demonstrated the migratory effect of EGF and heregulin-1 on oestrogen receptor-negative breast cancer cells with the

monolayer wound-healing assay. The migratory effect of EGF was observed in all three cell lines studied and was found to be higher in Hs578T and SK-BR-3 than in MDA-MB-231 cells, as evidenced by significant increase in migration of all three cell lines. Stimulation with heregulin-1 resulted in an increase migration of SK-BR-3 cells, which can explained be due to hetero-dimerization of HER-2 and HER-3. Heregulin-1 had no effect on motility of MDA-MB-231 or Hs578T cells, which is consistent with results of this chapter that heregulin-1 is not able to activate MAPK pathway in these cell lines, as activation of MAPK pathway reported to plays important role in migration of breast cancer cells (Garcia, Franklin et al. 2006).

Treatment of MDA-MB-231, Hs578T and SK-BR-3 cells with gefitinib inhibited significantly the migratory effect of EGF. Gefitinib has shown more inhibitory effect in SK-BR-3 and Hs578T cells than in MDA-MB-231 cells. Treatment of SK-BR-3 cells with lapatinib resulted in significant inhibition of EGF stimulated migration. Interestingly, gefitinib and lapatinib has shown the similar significant inhibitory effect in SK-BR-3 ($p < 0.001$).

Further experiments could investigate the effect of lapatinib in the presence of heregulin-1 in survival and migration of HER-2 positive SK-BR-3 cells, to evaluate the inhibitory effect of this inhibitor on the effect of heregulin-1.

In conclusion, these findings emphasise the activity of EGF signal transduction pathway in survival and migration of oestrogen receptor-negative breast cancer cells and that EGFs may contribute to the metastatic potential of breast cancer cells by activation of survival and migratory pathways. The inhibitory effect of both EGFR inhibitor and dual tyrosine kinase inhibitor against cell survival and migration might be useful as therapeutic targeted therapies.

Chapter 6. Expression of human epidermal growth factor receptors 1 and 2 in oestrogen receptor-negative breast tumours

6.1 Introduction

It is well known that aberration in tyrosine kinase activity of epidermal growth factor receptor family particularly, EGFR and HER-2 are considered as one of the significant reasons for development and progression of human cancer (Dhomen, Mariadason et al. 2012). Overexpression of EGFR and HER-2 has been reported frequently in breast tumours (Garcia, Vizoso et al. 2003, Memon, Sorensen et al. 2006). Overexpression of EGFR has been reported to be associated with aggressive phenotypes and poor clinical outcome in breast tumours and its over expression is correlated with increased proliferation of malignant cells and resistance to apoptosis (Rimawi, Shetty et al. 2010). Nickerson *et al* in 2010 reported the overexpression of EGFR in triple-negative breast cancer patients and its association with increase in metastasis (Wiseman, Makretsov et al. 2005, Foley, Nickerson et al. 2010).

In 2005, Wiseman *et al* reported the high expression of EGFR, HER-2 and HER-3 in oestrogen receptor-positive breast tumours and their correlation with decreased disease survival of patients whose tumours express high levels of EGFR, HER-2 and HER-3 (Wiseman, Makretsov et al. 2005). Amplification of HER-2 has been reported in 15-30% of breast cancers and is associated with poor prognosis. Patients with lymph node metastasis are more likely to have HER-2 positive tumours than those that are node-negative. HER-2 amplification is observed more frequently in oestrogen receptor-negative breast cancers than in oestrogen receptor-positive breast cancers (Hynes and Stern 1994, Howe and Brown 2011).

This chapter is focused on analyzing the expression of EGFR and its association with HER-2, expression of proliferation marker Ki-67, apoptotic marker, cleaved caspase-3 along with various clinico-pathological criteria on oestrogen receptor-negative breast tumours by immunohistochemistry.

6.2 Results

6.2.1 Expression of EGFR in normal breast tissue

EGFR expression was detected in all 7 normal breast tissues investigated by immunohistochemistry. In general, EGFR expression was weak to moderate with a mean histoscore of 123.43 and a median histoscore of 55. Immuno-reaction was membranous in epithelial cells that line the acini (Figure 6.1).

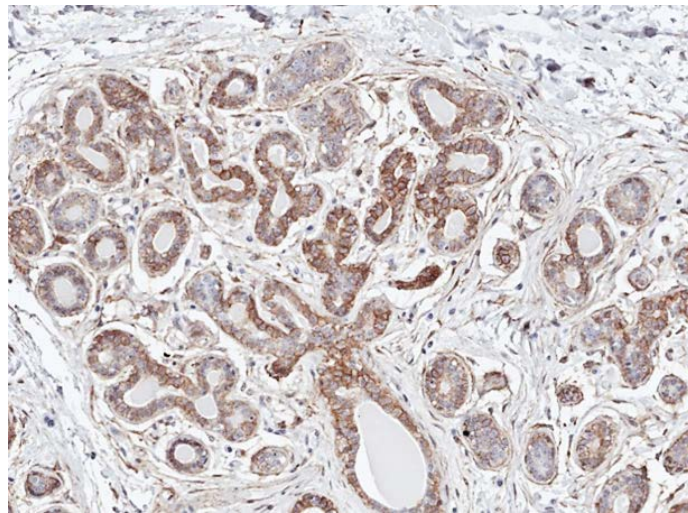


Figure 6.1: Expression of EGFR in normal breast tissue. Section from normal breast tissue with histoscore of 383 was analysed for expression of EGFR by immunohistochemistry. Membranous immuno-reaction of EGFR was detected in epithelial line of the acini. Photograph magnification is x200.

6.2.2 Expression of EGFR in *in situ* carcinoma of the breast

EGFR immuno-reaction was detected in 32 out of 37 carcinomas *in situ* (86.48%). The immuno-reaction of EGFR was membranous (Figure 6.2). In general, most of EGFR positive tumours showed weak to moderate expression of EGFR with a mean histoscore of 159 and a median histoscore of 72. However, 3 tumours were strongly immuno-reactive for EGFR with a maximum histoscore of 735.

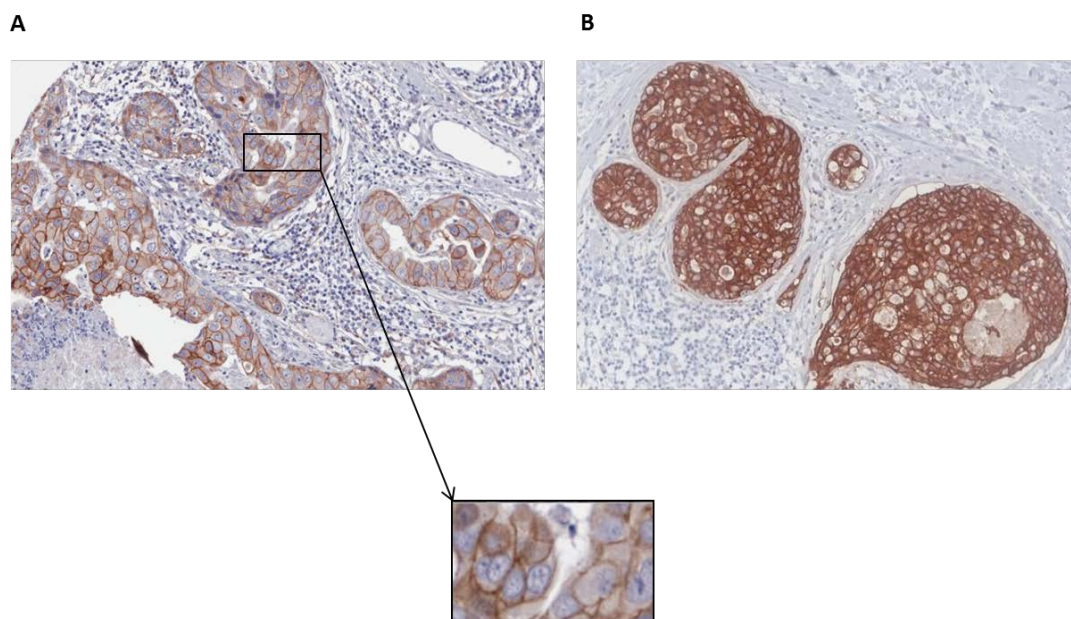


Figure 6.2: Expression of EGFR in *in situ* breast carcinoma. Section of (A) comedo and (B) solid type of carcinoma *in situ* with histoscores of 255 and 735 respectively were analysed by immunohistochemistry for expression of EGFR. The inset shows the membranous immuno-reaction of EGFR in *in situ* neoplastic cells. Magnification is x200 for inset and x20 for the image.

6.2.3 Expression of EGFR in invasive carcinoma of the breast

All invasive breast tumours included in the study were analysed for EGFR expression in duplicate cores and the mean values were used for the statistical analyses. The consistency of expression between the duplicate cores was tested; there was a strong correlation between EGFR expression measured in the duplicate cores (Pearson correlation, $p=0.001$, Figure 6.3).

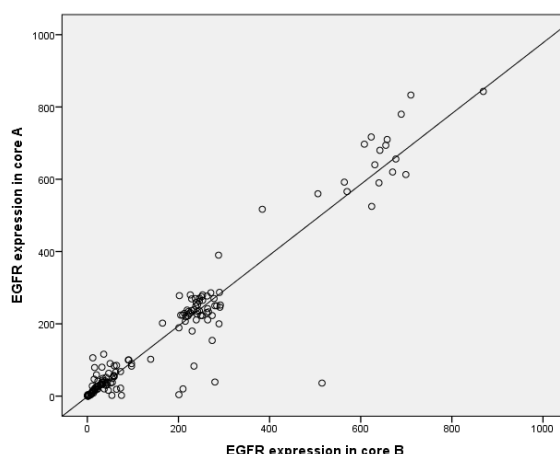


Figure 6.3: Expression of EGFR in the duplicate cores of invasive tumours. Two cores (A and B) of one millimetre in diameter were taken from each tumour for construction of TMA blocks. EGFR immuno-reactive sections were analysed for EGFR independently. There was a strong correlation of EGFR expression between the two cores tested in each tumours (Pearson's rho correlation $p=0.001$).

EGFR immuno-reaction was demonstrated in 77 tumours of the 79 invasive lesions investigated (97%). In general, the majority of EGFR positive tumour cells were moderately immuno-reactive with a mean histoscore of 202 and median histoscore of 115. However, 6 tumours were strongly positive for expression of EGFR with a maximum histoscore of 856. The immuno-reaction for EGFR was membranous (Figure 6.4).

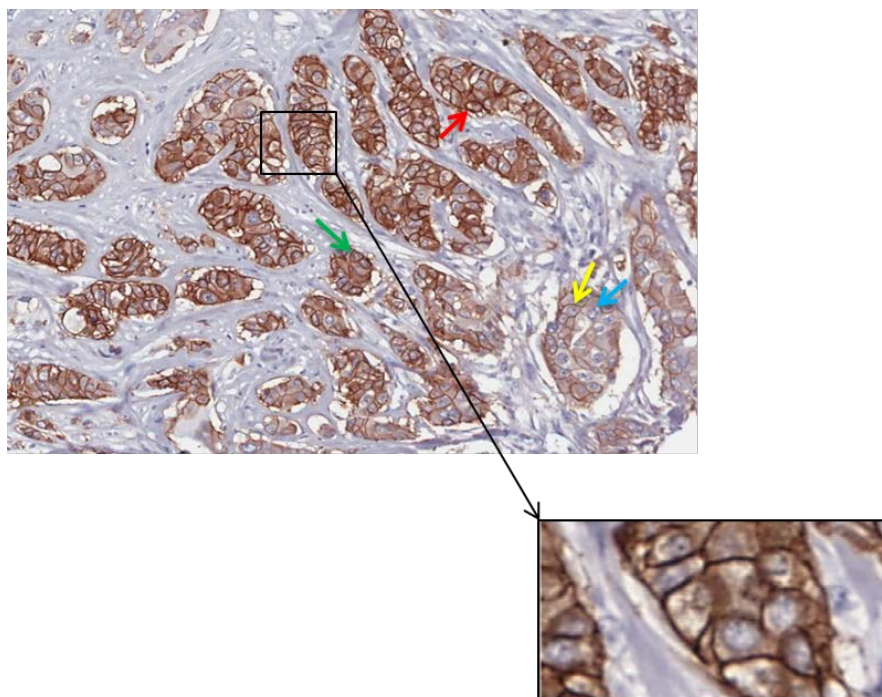


Figure 6.4: Expression of EGFR in invasive breast cancer. Sections of invasive breast carcinomas with histoscore of 578 were tested for EGFR expression by immunohistochemistry. Specific membranous immuno-reaction was demonstrated in the neoplastic cells. Representative arrows indicate the malignant epithelial cells with EGFR expression absent (blue), weakly positive (yellow), moderately positive (green) and strongly positive (red). Specific membranous immuno-reaction was demonstrated. Magnification is x20 and x200 for insets.

6.2.4 EGFR expression and presence of lymph node metastasis

To explore the possible role of EGFR in breast cancer cell metastasis, the expression of EGFR was tested in the primary tumours and their corresponding tumour cells with axillary lymph node metastases in 26 patients. There was a significant correlation between the expression of EGFR in the primary tumour cells and tumour cells with metastatic lymph node deposits (Pearson's rho correlation, $p=0.030$, Figure 6.5). There is a significant difference in the expression levels of EGFR in primary tumours and metastatic counterparts. EGFR is expressed at higher levels in primary tumours than the corresponding metastatic tumour cells (Wilcoxon signed ranking test, $p=0.02$).

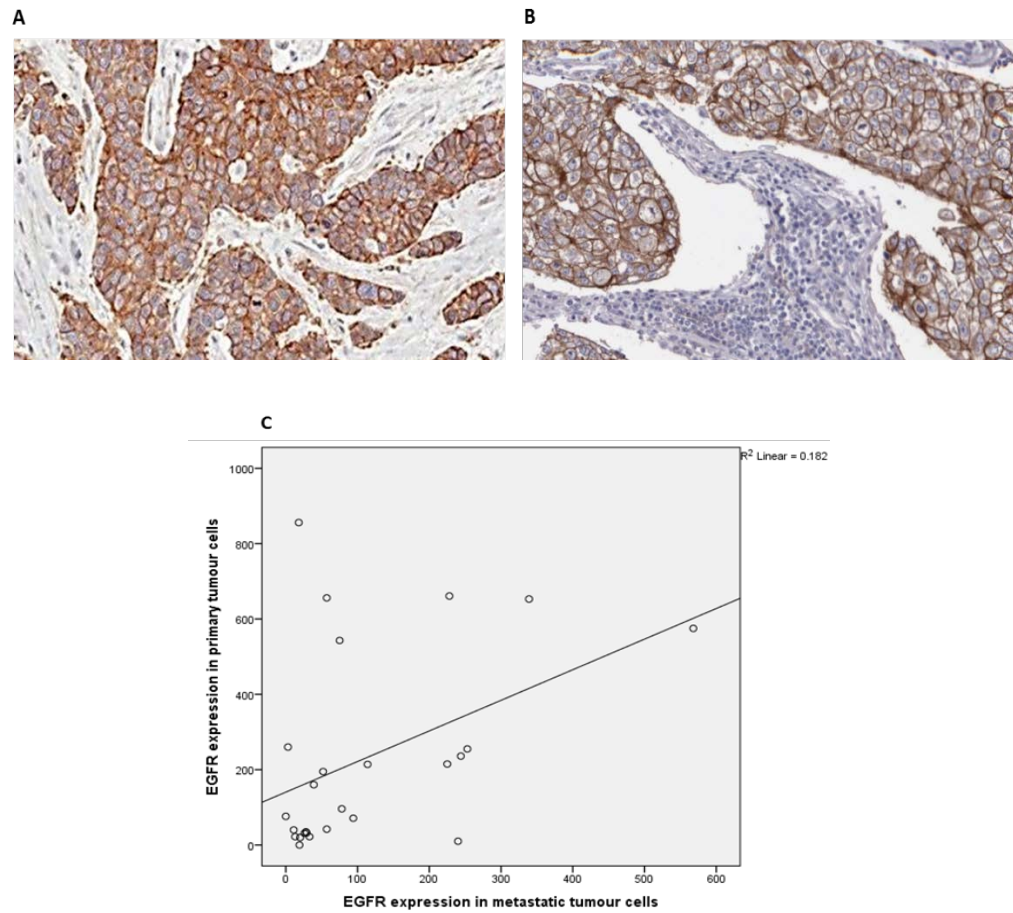


Figure 6.5: EGFR expression in primary breast tumour cells and metastatic tumour cells in lymph nodes. EGFR expression was analysed by immunohistochemistry in both primary (A) and metastatic (B) tumour cells of the same patients in 26 tumours. There was a significant association between EGFR expression in primary and metastatic tumour cells ($p=0.030$) (C). Magnification is x200.

The association of EGFR expression with the number of axillary lymph node metastasis were investigated. There was no significant correlation between EGFR expression and the number of axillary lymph node metastasis (Spearman's rho correlation, $p=0.236$).

6.2.5 Expression of EGFR in various breast lesions

Expression of EGFR was investigated in normal breast tissues and various types of breast carcinomas (Figure 6.6). Amongst different types of breast lesions, EGFR was mostly expressed in invasive breast tumours.

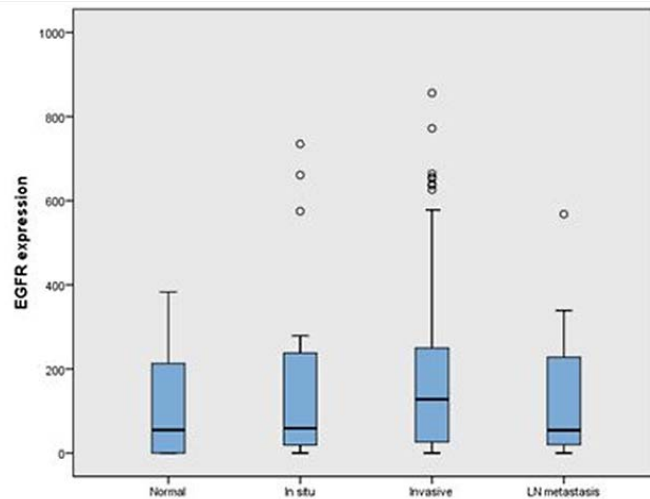


Figure 6.6: Expression level of EGFR in different breast lesions. Expression of EGFR in normal breast tissues, *in situ* carcinomas, invasive carcinomas and metastatic breast tumours (LN metastasis). The horizontal bars represent the median values, the boxes represent the 50th percentiles, whiskers represent the range of data, the dots refer to outlier values.

6.2.6 EGFR expression and clinico-pathological criteria

The relationship between EGFR expression and clinical and pathological features of breast tumours was investigated. There was no significant correlation between EGFR expression and age of patients (Pearson's rho correlation, $p=0.840$, Figure 6.7).

The data illustrated in figure 6.7 suggests that there may be a trimodal distribution of EGFR expression. The association between age and EGFR expression in each of the identified EGFR subgroups (0-200, 200-400 and >400) was evaluated. There was no significant association between EGFR expression and patient's age in any of the EGFR subgroups (Pearson *rho* correlation $p=0.118$, $p=0.477$ and $p=0.344$).

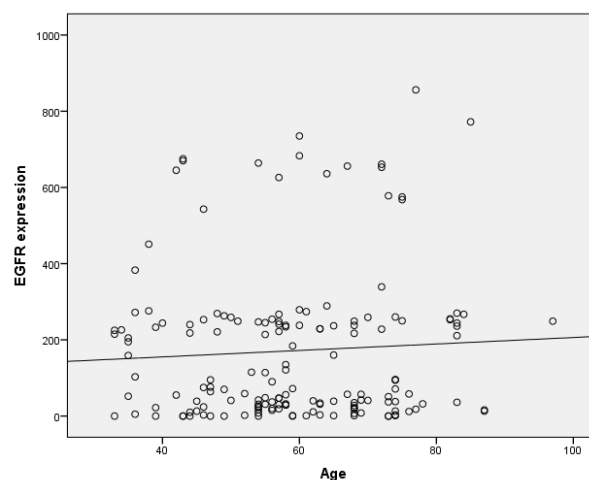


Figure 6.7: Association of EGFR expression with patient age. EGFR expression was not associated significantly with age of patients, ($p=0.840$).

There was no correlation between EGFR expression and size of the tumour (Pearson's rho correlation, $p=0.997$) or grade of tumour (Spearman's rho correlation, $p=0.350$). There was no significant difference in expression level of EGFR in lower and high grade tumours (Mann-Whitney test, $p=0.585$, Figure 6.8). This implies that EGFR expression is independent of the size of the tumours and tumour grade.

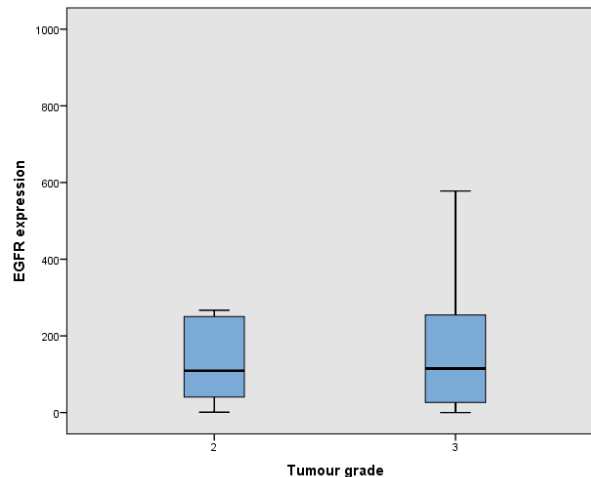


Figure 6.8: Association of EGFR expression with tumour grade. EGFR expression showed no significant association with the histological grades of invasive tumours ($p>0.05$). The horizontal bars represent the median values, the boxes represent the 50th percentiles and the whiskers represent the range of data.

6.2.7 Association between expression of EGFR and expression of Ki-67 protein in malignant breast tumours

To investigate the role of EGFR in proliferation of breast tumour cells, association between EGFR expression and expression of the proliferation markers Ki-67 was investigated. The statistical analysis was tested on 142 cases which include *in situ* carcinoma, invasive carcinoma and lymph node metastasis tumour cells. The results show that there was no significant correlation between the expression of EGFR and Ki-67 in all the breast lesions analysed (Pearson correlation, $p=0.058$).

6.2.7.1 Association between expression of EGFR and expression of Ki-67 in *in situ* breast carcinoma

Expression of EGFR and Ki-67 was investigated in 37 *in situ* breast carcinomas, of which 6 tumours positive for EGFR were negative for Ki-67 and 5 tumours that expressed Ki-67 were negative for expression of EGFR (Figure 6.9).

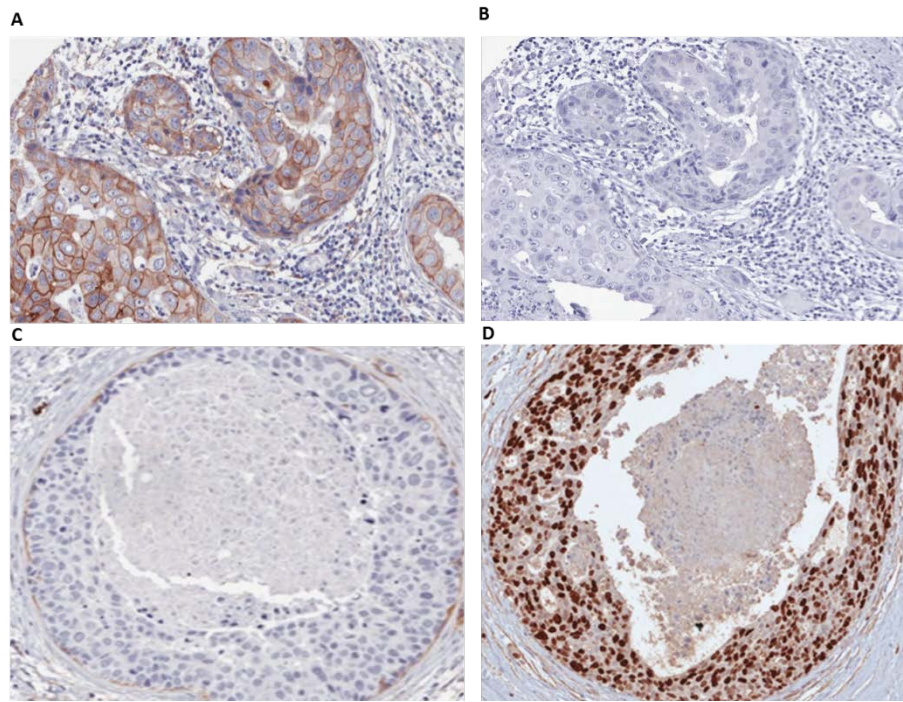


Figure 6.9: EGFR expression and expression of Ki-67 in *in situ* breast cancer. Sections from comedo type of *in situ* breast carcinomas were tested for EGFR (A, C) and Ki-67 (B, D) expression by immunohistochemistry. Magnification is x200.

The association between expression of EGFR and Ki-67 were investigated in *in situ* breast carcinomas. There was no correlation between expression of EGFR and Ki-67 in *in situ* tumours (Pearson correlation, p -value=0.752, Figure 6.10).

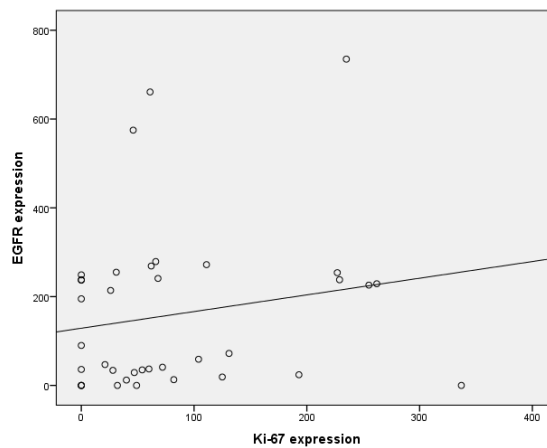


Figure 6.10: Association of EGFR expression and expression of Ki-67 in *in situ* breast carcinoma. EGFR and Ki-67 expression were compared in 37 *in situ* breast carcinomas. There was no association between EGFR expression and expression of Ki-67 in *in situ* breast carcinomas (p =0.752).

6.2.7.2 Association between expression of EGFR and expression of Ki-67 in invasive breast tumours

To investigate whether EGFR is associated with proliferation of invasive breast tumours, the expression of Ki-67 and EGFR was measured in invasive breast tumours. Out of 79 invasive tumours, 77 were positive for both expression of EGFR and Ki-67. There was a significant correlation between EGFR expression and Ki-67 expression in invasive breast carcinoma investigated (Pearson's rho correlation, $p=0.024$, Figure 6.11).

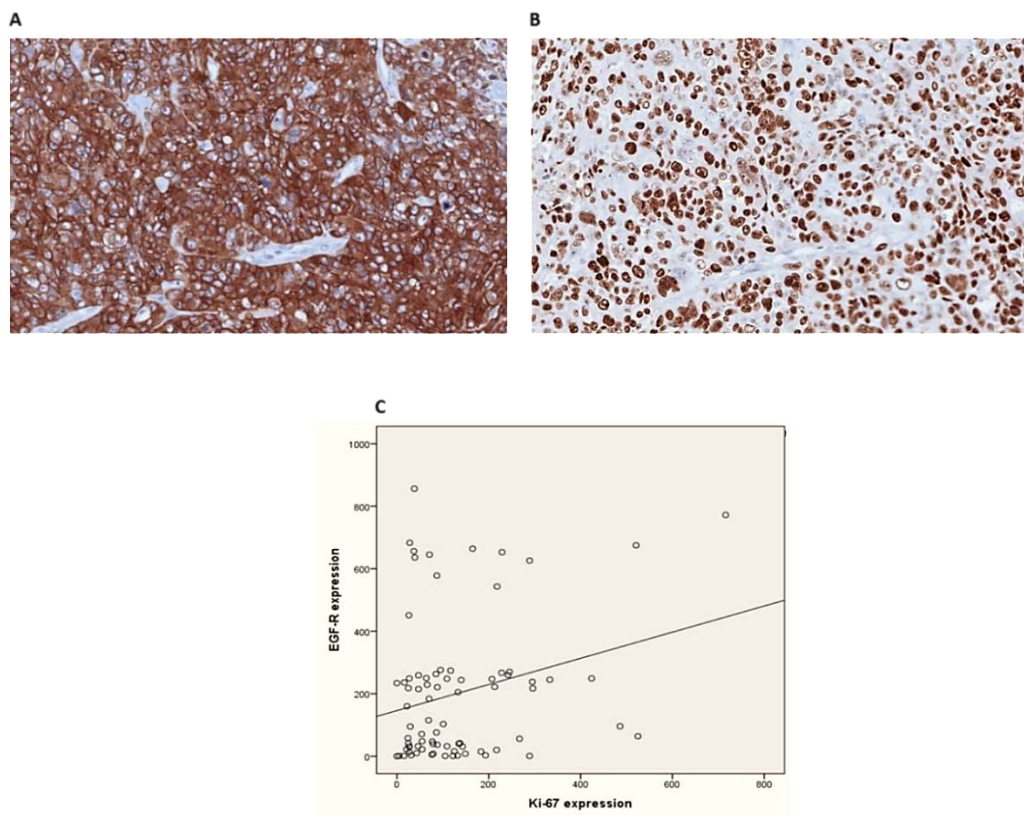


Figure 6.11: Association of EGFR expression and expression of Ki-67 in invasive breast cancers. EGFR (A) expression and Ki-67 (B) expression were analysed by immunohistochemistry. Significant association was detected between EGFR and Ki-67 expression(C) ($p=0.024$).

6.2.7.3 Association between expression of EGFR and Ki-67 in breast tumour cells with involved lymph nodes metastasis

The association between expression of EGFR and Ki-67 in 26 breast tumour cells with axillary lymph nodes was investigated (Figure 6.12).

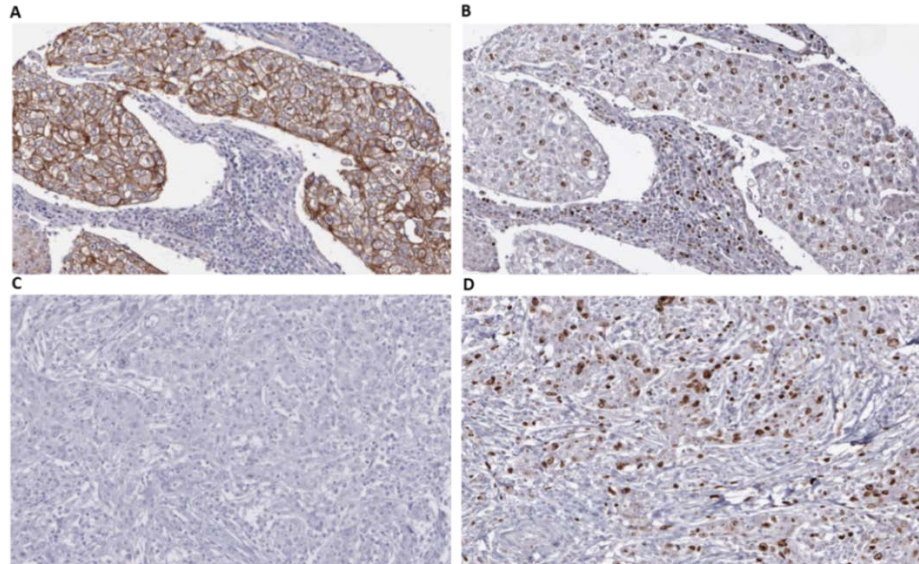


Figure 6.12: EGFR expression and expression of Ki-67 in breast tumour cells with involved lymph nodes. Expression of EGFR (A, C) and Ki-67 (B, D) was analysed in tumours involved with axillary lymph nodes metastatic tumour cells by immunohistochemistry. Magnification is x40.

There was no association between EGFR expression and expression of Ki-67 in tumour cells with involved axillary lymph nodes metastatic deposits (Pearson's *rho* correlation, $p=0.654$, Figure 6.13).

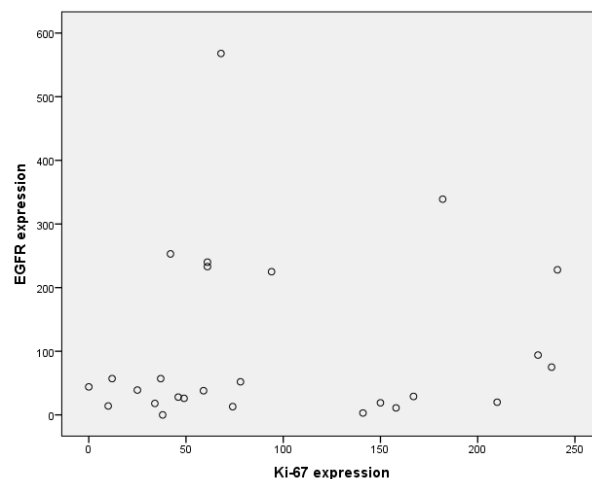


Figure 6.13: Association of EGFR expression and expression of Ki-67 in breast tumour cells with involved lymph nodes. EGFR histoscores were shown against Ki-67 histoscores in 26 breast tumour cells with involved lymph nodes. There was no association between EGFR expression and Ki-67 ($p=0.654$).

6.2.8 EGFR expression and HER-2 protein expression

The association between the expression of EGFR and HER-2 was investigated. Expression of HER-2 has been evaluated on 142 cases which include *in situ* carcinomas, invasive carcinomas and lymph node metastasis tumours. EGFR was expressed in 110 out of 142 tumours and there was no significant correlation between the expression of EGFR and HER-2 expression in all the breast lesions analysed (Pearson's rho correlation, $p= 0.988$).

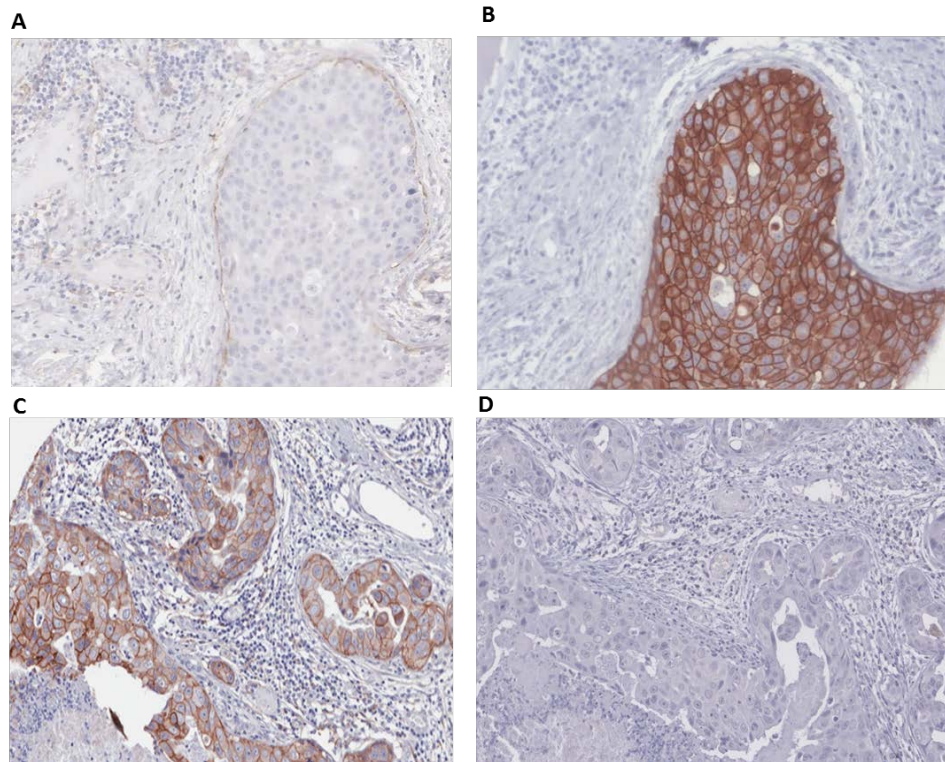


Figure 6.14: Expression of EGFR and HER-2 in *in situ* breast carcinomas. Parallel sections of solid ductal carcinoma *in situ* (A and B) comedo carcinoma *in situ* (C and D) were analysed by immunohistochemistry for expression of EGFR (A, C) and HER-2 (B, D). Magnification is x20.

Out of 37 *in situ* breast carcinoma, 28 tumours were positive for HER-2. There was no correlation between expression of EGFR and HER-2 in *in situ* breast carcinoma (Pearson' rho correlation, $P=0.136$, Figure 6.15).

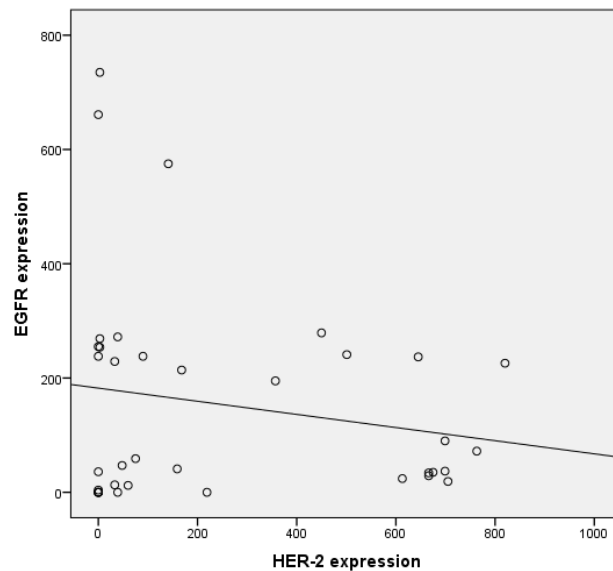


Figure 6.15: Association of EGFR expression with HER-2 expression in *in situ* breast carcinomas. EGFR histoscores were shown against HER-2 histoscores in 37 *in situ* breast carcinomas and no significant correlation was detected ($p=0.136$).

Expression of HER-2 and EGFR was investigated in invasive breast tumours. Out of 79 invasive tumours, 43 tumours were positive for expression of HER-2 with mean histoscore of 143.65 (figure 6.16).

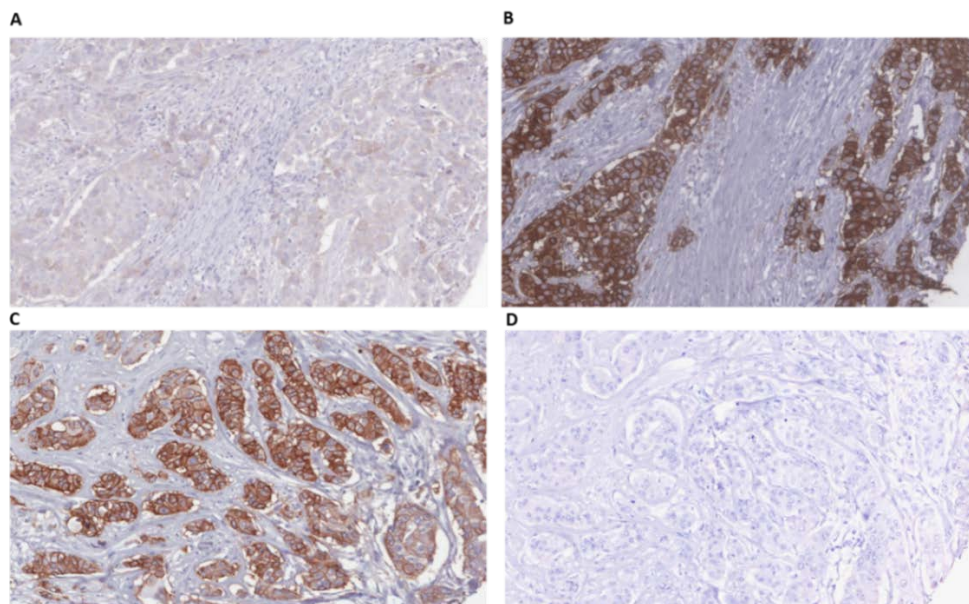


Figure 6.16: Expression of EGFR and HER-2 in invasive breast tumours. Parallel sections of invasive breast tumour were analysed by immunohistochemistry for expression of EGFR (A, C) and HER-2 (B, D) Magnification is x200.

The association between expression of EGFR and HER-2 was investigated in 79 invasive breast tumours. There was a significant inverse association between expression of EGFR and HER-2 expression in invasive breast tumours (Pearson's rho correlation, $p=0.044$, Figure 6.17).

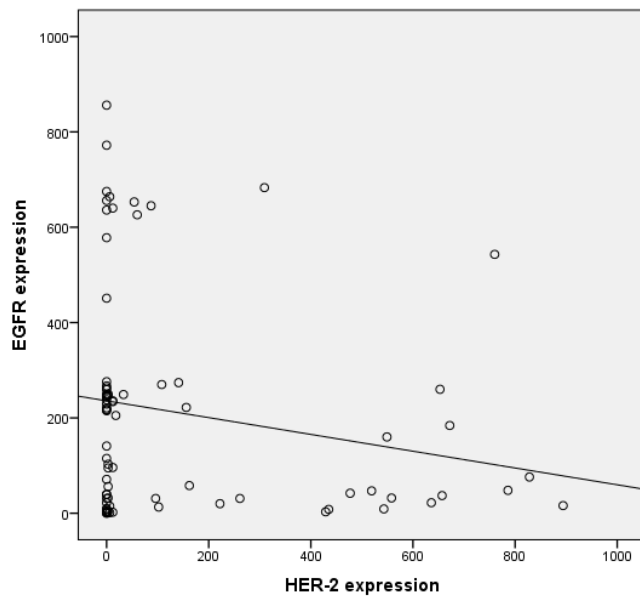


Figure 6.17: Association between expression of EGFR and HER-2 in invasive breast tumours. Expression of EGFR compared with the expression of HER-2 in 79 invasive breast tumours ($p=0.044$).

Out of 26 tumour cells with involved lymph node metastatic deposits, 15 tumour cells were positive for expression of HER-2 with mean histoscore of 199.46 (Figure 6.18).

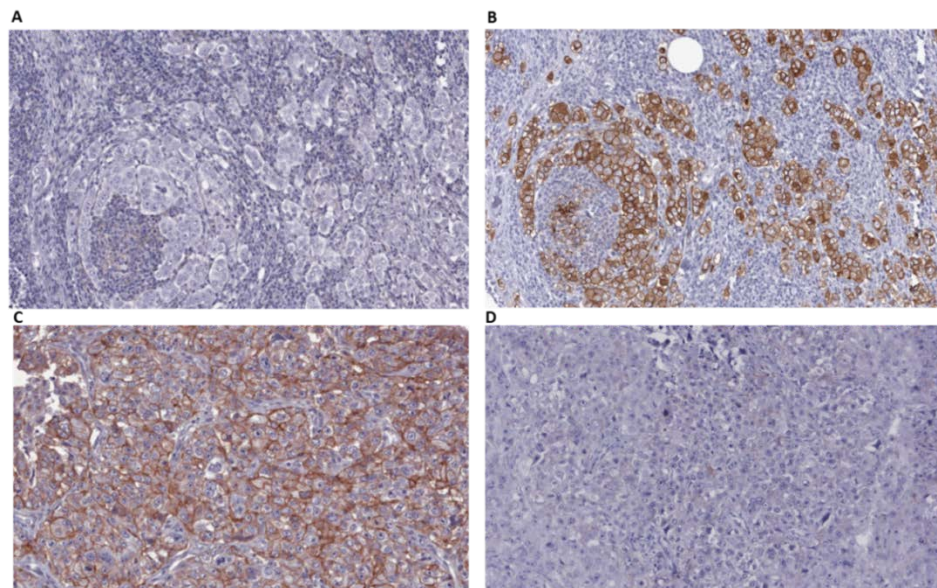


Figure 6.18: Expression of EGFR and HER-2 in breast tumour cells with involved lymph nodes. Parallel sections breast tumour cells with axillary lymph node deposits were analysed by immunohistochemistry for expression of EGFR (A, C) and HER-2 (B, D). Magnification is x20.

Association between expression of EGFR and HER-2 expression was investigated in breast tumour cells with involved lymph nodes and significant inverse correlation was detected (Pearson's rho correlation, $p=0.045$, Figure 6.19).

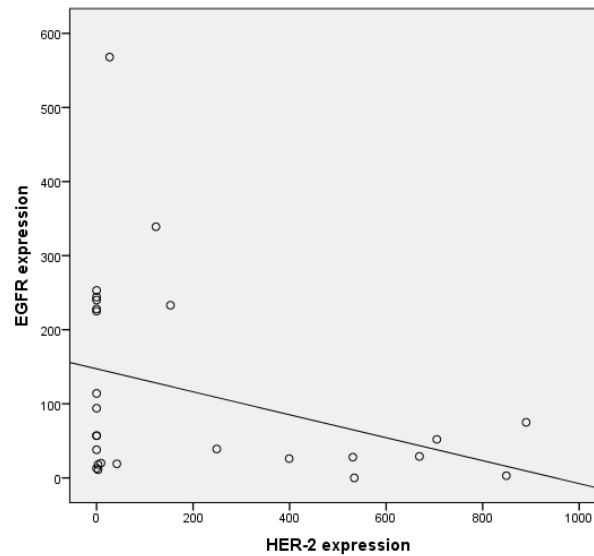


Figure 6.19: Association of EGFR expression with HER-2 expression in breast tumour cells with involved lymph nodes. The expression between EGFR and HER-2 was compared in 26 tumour cells that metastasized to axillary lymph nodes. Expression of EGFR was inversely associated with expression of HER-2 in breast tumour cells with involved lymph nodes ($p=0.045$).

6.2.9 EGFR and expression of cleaved caspase-3 protein

Based on the previous findings of this study, mediation of the survival effect of EGF through EGFR in oestrogen receptor-negative breast cancer cells was demonstrated. The association between EGFR expression and expression of cleaved caspase-3 was tested. The expression of cleaved caspase-3 in 79 invasive breast tumours was evaluated. Amongst the 78 EGFR positive tumours, 71 were negative for cleaved caspase-3 and only 7 were positive for cleaved caspase-3. One EGFR negative tumour was negative for cleaved caspase-3 (cross tabulation). There were no significant difference between expression levels of EGFR and cleaved caspase-3 (Mann-Whitney test, $p=0.966$, Figure 6.20).

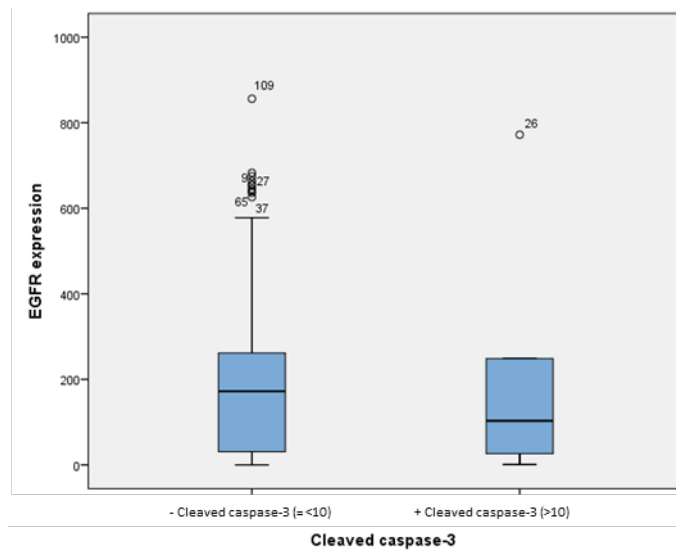


Figure 6.20: EGFR and expression of cleaved caspase-3 in invasive breast tumours.

Seventy one tumours out of 78 EGFR positive tumours were negative for cleaved caspase-3 and 7 tumours were positive for cleaved caspase-3. The cut off point for positive cleaved caspase-3 histoscore was >10 . The whiskers represent the range of data, the boxes represent the 50th percentiles, the horizontal bars represent the median values and the dots refer to outlier values.

6.3 Discussion

Immunohistochemical expression of EGFR was demonstrated in all 7 cases of normal breast tissue, in a high proportion of *in situ* breast carcinoma (86.48%) with mean histoscore of 160 and in 78.5% of invasive breast cancers investigated. The mean expression level of EGFR in invasive breast tumours was 202.

In 2005, Wiseman *et al* demonstrated that expression of EGFR was independent from patient's lymph node status (Wiseman, Makretsov et al. 2005). In the current study, the possibility of correlation between EGFR expression and number of lymph node metastasis was investigated and no significant association was found (Pearson's rho correlation, $p=0.236$). This result would agree with the previous findings that EGFR expression is not related to the lymph node status of the patient. There was a significant association between EGFR expression in primary breast tumours with their metastatic counterparts (Pearson's rho correlation, $p=0.03$). However, primary tumour cells expressed EGFR at higher levels than corresponding metastatic tumour cells of the same patients (Wilcoxon signed ranking test, $p=0.02$).

Riwambi *et al* (2010) reported the high proliferation of EGFR expressing breast tumour cells in a large cohort of malignant breast tumours including oestrogen receptor-positive and negative breast tumours (Rimawi, Shetty et al. 2010). In current study, the association between EGFR expression and expression of proliferation marker Ki-67 was tested. There was a significant correlation between EGFR expression and Ki-67 expression in invasive breast tumours ($p=0.046$). This result agrees with the contention that EGFR-positive tumours have higher proliferative rate than other breast tumours (Rimawi, Shetty et al. 2010).

The association between EGFR expression and the other clinico-pathological parameters was considered. Witton *et al* in 2003 reported a significant correlation between EGFR expression and tumour grade (Moulder, Yakes et al. 2001). Similarly, Wiseman *et al* also reported the significant correlation between expression of EGFR and HER-2 with grade of tumours in cohort of breast tumours but no association with size of the tumour was found (Wiseman, Makretsov et al. 2005). In the present study, EGFR expression did not exhibit

significant association with size of tumours ($p=0.997$), which would agree with the conclusion of the previous study. Unlike the previous study of EGFR on breast carcinoma, expression of EGFR was not associated with tumour grade ($p=0.350$) or age of the patient ($p=0.840$). There was no significant differences in the expression level of EGFR in low and high grade tumours ($p=0.940$).

The present study demonstrated a significant inverse correlation between EGFR and HER-2 expression in invasive and metastatic HER-2 positive breast tumour cells ($p=0.044$ and $p=0.045$ respectively). This is the first time that an inverse correlation has been observed. In 2001, Esteva *et al* reported a positive correlation between co expression of EGFR and HER-2 in breast tumours (Esteva, Hortobagyi *et al.* 2001). Conversely, another study reported no significant association between EGFR and HER-2 in HER-2 positive breast tumours (Wiseman, Makretsov *et al.* 2005).

Based on findings described in this thesis, EGFR mediated the survival effect of EGF in oestrogen receptor-negative breast cancer cells. It has been demonstrated that overexpression of EGFR in invasive breast tumours is associated with their resistance to apoptosis (Rimawi, Shetty *et al.* 2010). Hence, the correlation between expression of EGFR and expression of cleaved caspase-3 was tested on oestrogen receptor-negative breast tumours. Most of the EGFR positive tumour cells were negative for cleaved caspase-3. However, only 7 tumours were positive for both EGFR expression and cleaved caspase-3. This supports the contention that EGFR plays an important role in mediation of cell survival in oestrogen receptor-negative breast tumour cells (Rimawi, Shetty *et al.* 2010).

In conclusion, EGFR and HER-2 are expressed at moderate levels in oestrogen receptor-negative neoplastic breast tissues. The results suggest that EGFR plays a significant role in mediation of survival and proliferation but not metastasis of oestrogen receptor-negative breast tumour cells.

Chapter 7. The effect of combined IGF-1 and EGF in oestrogen receptor-negative breast cancer

7.1 Introduction

Insulin-like growth factor 1 (IGF-1) and epidermal growth factor (EGF) stimulate migration of oestrogen receptor-negative breast cancer cells through interaction via type I IGF receptor and EGFR respectively as discussed in chapters 3 and 5.

Activation of IGF-IR and EGFR play important role in initiation and progression of breast cancer. Several studies addressed the possibility that interaction between IGF-IR and EGFR is important in EGFR-targeted therapy resistance in breast cancer (Camirand, Zakikhani et al. 2005, Jones, Gee et al. 2006, Jin and Esteva 2008, van der Veeken, Oliveira et al. 2009). It has been reported that increased IGF-IR signalling plays a significant role in breast cancer resistance to anti-EGFR therapy and that one of the mechanisms of resistance to anti-EGFR therapy is the direct interaction between IGF-IR and EGFR (Camirand, Zakikhani et al. 2005, Jones, Gee et al. 2006). Studies reported that in breast cancer association between IGF-IR and EGFR activity synergistically induce cellular proliferation (Hijazi, Thompson et al. 2000, Tsai, Shamon-Taylor et al. 2003, Ahmad, Farnie et al. 2004, Hirsch, Shen et al. 2006).

In this chapter, the effect of both IGF-1 and EGF in combination on migration of oestrogen receptor-negative breast cancer cells was investigated and the effects of the combined treatment with inhibitors against IGF-IR and EGFR tested on cell migration of these cells.

7.2 Results

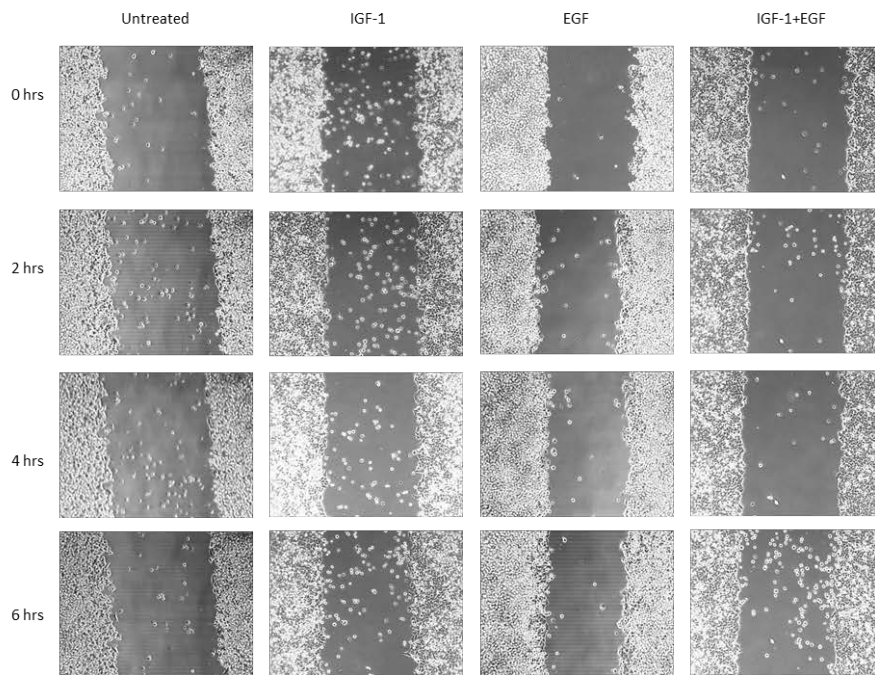
7.2.1 *Effect of both combinations of IGF-1 and EGF on migration of oestrogen receptor-negative breast cancer cells*

7.2.1.1 *Effect of combination of IGF-1 and EGF on migration of triple-negative MDA-MB-231 and Hs578T breast cancer cells*

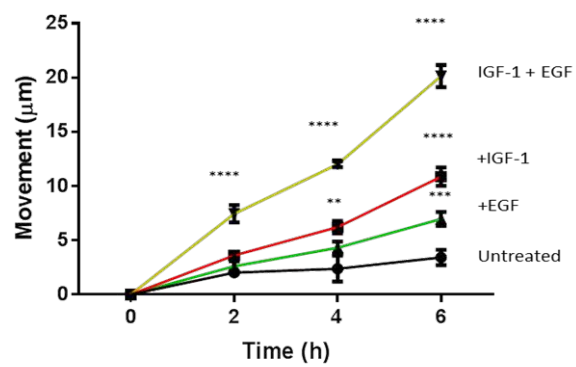
The results of the previous chapters indicated the significant migratory effect of IGF-1 and EGF on their own in each MDA-MB-231, Hs578T cell lines. The ability of IGF-1 and EGF in combination to stimulate cell migration was compared to the effect of either ligand on its own.

Scratched monolayer MDA-MB-231, Hs578T cells were incubated in the presence and absence of 50 ng/ml IGF-1 or 50 ng/ml EGF or both IGF-1 and EGF for 0, 2, 4 and 6 hours. Images of the cells were captured at each particular time. The width of the each wound was measured 20 times and the average of them was calculated. The cell migration was evaluated by measuring the distance of the edge of the wound towards the centre of the wound with Image J software (Figure 7.1).

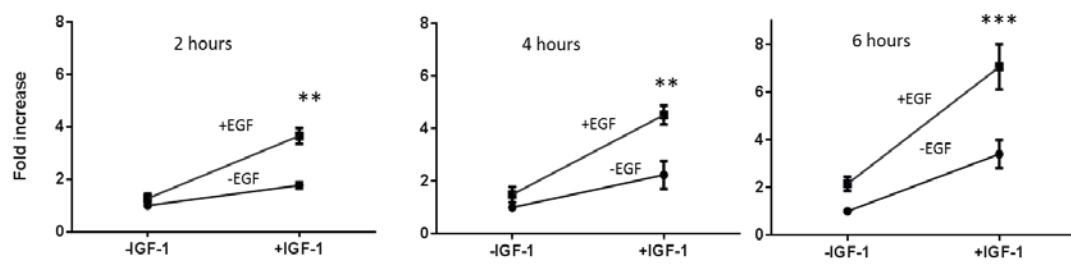
A MDA-MB-231



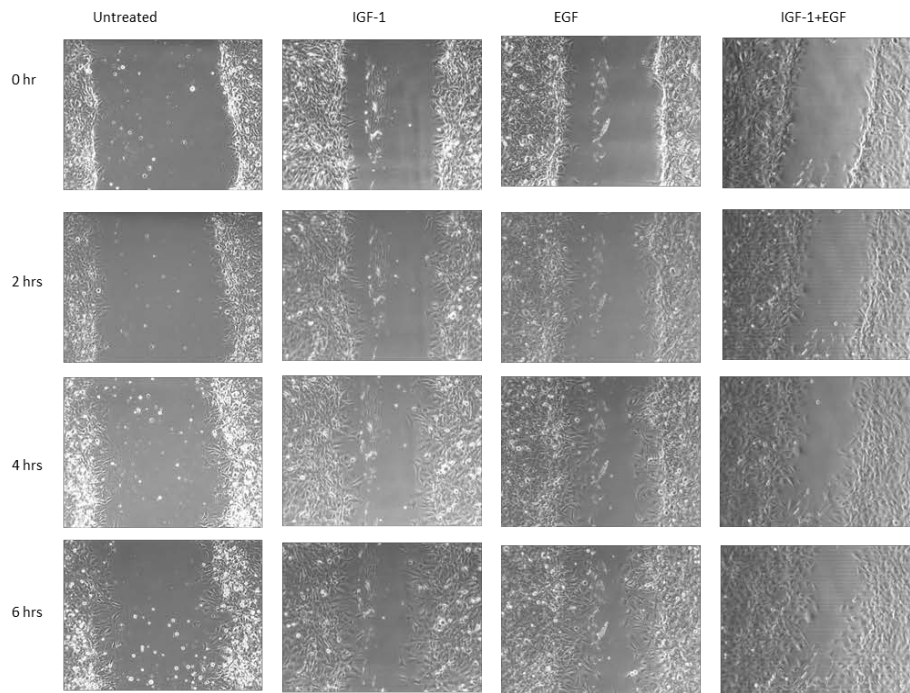
B



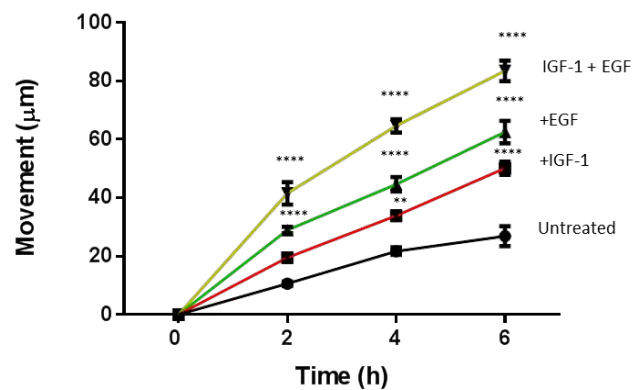
C



D Hs578T



E



F

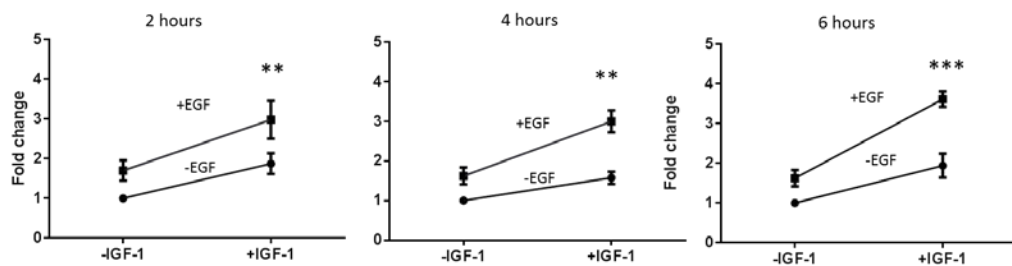


Figure 7.1: Effect of IGF-1 and EGF in combination on migration of MDA-MB-231 and Hs578T cells. MDA-MB-231 (A) and Hs578T (D) cells were seeded at density of 40×10^4 cells/well into 24 well plates that had scored on the under surface with two parallel lines across the middle of each wells. Cells were withdrawn in phenol-red free DMEM medium supplemented with 10% charcoal-treated new born calf serum as described in Materials and Methods section. Monolayer cells were wounded with a sterile 20-200 μ l Gilson pipette tip through the centre of the well, perpendicular to the scored lines. Wounded cells were washed twice with 0.5 ml of

phenol red-free DMEM and incubated in phenol red-free DMEM supplemented with 0.01 % of BSA for two hours. After two hours of incubation, cells were washed again and incubated with 0.01% BSA alone () or with 50 ng/ml IGF-1 (■), 50ng/ml EGF (■) or in the presence of 50 ng/ml IGF-1 and 50 ng/ml EGF in combination () for various time points up to 6 hours. Image were captured at the beginning of the experiment (time 0) and after each two hours of treatment up to 6 hours. Three separate measurements in three different wells were made for each treatment condition. The distance between the wounds was measured using Image J software programme. The demonstrated values are the mean \pm SEM. Asterisks denotes statistically significant increase in migration of cells in the presence of IGF-1 or EGF compared with in the absence of IGF or EGF and that IGF-1 and EGF in combination markedly increase more cell movement compared to each ligand alone in both MDA-MB-231 and Hs578T cells (**B, E**) (One way ANOVA, ** $p < 0.001$, *** $p = 0.0001$, **** $p < 0.0001$). Similar results were obtained in three independent experiments. Results are shown as the fold increase in migration stimulated by IGF-1 in the absence and presence of EGF (**C, F**). The mean of \pm SEM, are shown. The interactions were statistically significant (** $p < 0.001$, *** $p = 0.0001$ by two way ANOVA with log transformation).

As shown in the Figure 7.1, movement of MDA-MB-231 cells in response to stimulation with 50 ng/ml IGF-1 increased significantly at 4 and 6 hours of incubation by 2 and 3 fold respectively. EGF stimulated significantly the movement of the MDA-MB-231 cells at 6 hours of incubation by 2 fold, in which the cell movement was lower than the respond of the cells to stimulation with 50 ng/ml IGF-1. The combination of 50 ng/ml IGF-1 and 50 ng/ml EGF enhanced synergistically more movement of MDA-MB-231 cells at 2, 4 and 6 hours of incubation by 3, 4 and 5 fold respectively. This result indicates that IGF-1 and EGF in combination stimulate significantly more migration in MDA-MB-231 cells compared to the migratory effect of either ligand on its own ($p < 0.0001$ by one way ANOVA).

In Hs578T cells, 50 ng/ml IGF-1 stimulated significantly migration of the cells at 4 and 6 hours of incubation by 1.4 and 2 fold respectively. In response to 50 ng/ml EGF, the movement of Hs578T cells was stimulated after 2, 4 and 6 hours by 1, 2 and 3 fold respectively, which was more than in response to 50 ng/ml IGF-1. The combination of 50 ng/ml IGF-1 and 50 ng/ml EGF increased more migration of the cells than either ligand on its own ($p < 0.0001$ by one way ANOVA).

The results shown that the combination of IGF-1 and EGF triggers more migration of triple-negative breast cancer cells than the combination of the two stimuli alone. These two ligands have a synergistic effect on migration of MDA-MB-231 cells ($p = 0.0001$ by two way ANOVA). Combination of IGF-1 and EGF resulted in higher migration in Hs578T cells compared to MDA-MB-231 cells.

7.2.1.2 Combination effect of IGF-1 and EGF on migration of HER-2 positive SK-BR-3 breast cancer cells

Previously in this study, migratory effect of either IGF-1 or EGF in SK-BR-3 cells was demonstrated. Here, the effect of IGF-1 and EGF in combination was investigated on migration of SK-BR-3 cells (Figure 7.2).

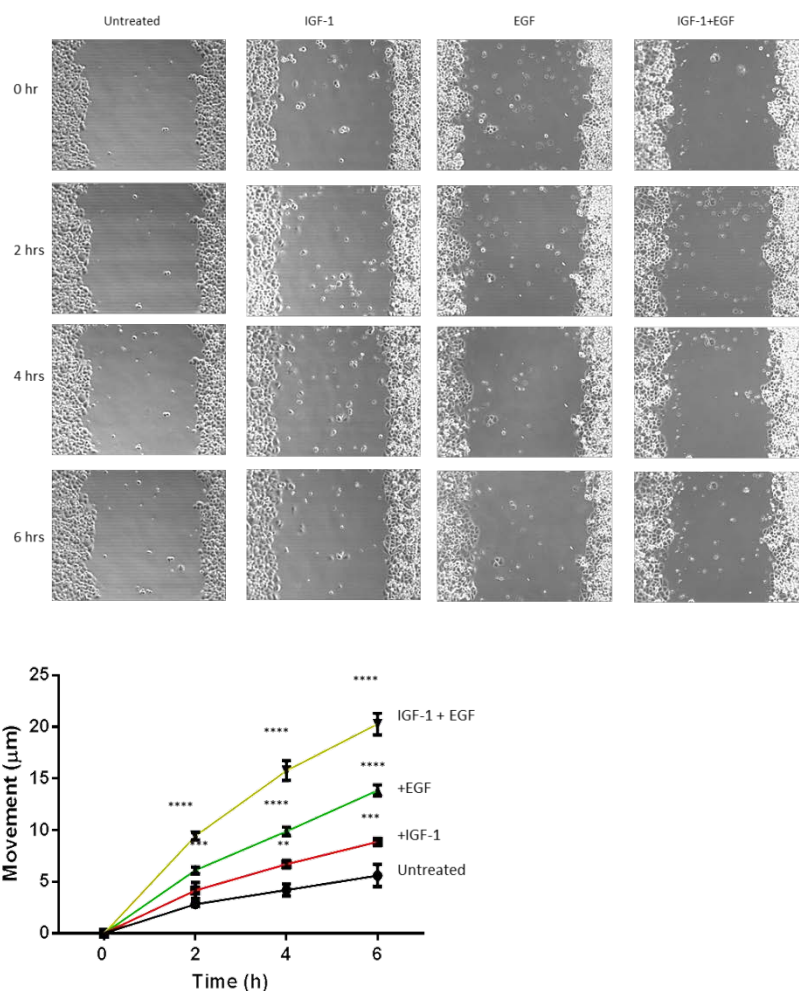


Figure 7.2: Effect of IGF-1 and EGF in combination on migration of SK-BR-3 cells. SK-BR-3 cells were seeded at density of 40×10^4 cells/well into 24 well plates that had scored on the under surface with two parallel lines across the middle of each wells. Cells were withdrawn in phenol free DMEM medium supplemented with 10% charcoal-treated new born calf serum as described in Materials and Methods section. Monolayer cells were wounded with a sterile 20-200 μ l Gilson pipette tip through the centre of the well, perpendicular to the scored lines. Wounded cells were washed twice with 0.5 ml of phenol red-free DMEM and incubated in phenol red-free DMEM supplemented with 0.01 % of BSA for two hours. After two hours of incubation, cells were washed again and incubated with 0.01% BSA alone () or with 50 ng/ml IGF-1 (■), 50ng/ml EGF (■) or in the presence of 50 ng/ml IGF-1 and 50ng/ml EGF in combination (■) for various time points up to 6 hours. Image were captured at the beginning of the experiment (time 0) and after each two hours of treatment up to 6 hours. Three separate measurements in three different wells were made for each treatment condition. The distance between the wounds was measured using Image J software programme. The demonstrated values are the mean \pm SEM. Asterisks denotes statistically significant increase in migration of cells in the presence of IGF-1 or EGF compared with in the absence of IGF or EGF. IGF-1 and EGF in combination markedly increased higher migration of the cells compared to each ligand alone in SK-BR-3 cells (One way ANOVA, ** $p < 0.01$, *** $p = 0.0001$, **** $p < 0.0001$). Similar results were obtained in three independent experiments.

Stimulation of SK-BR-3 cells with 50 ng/ml EGF increased the cell migration significantly 1.5, 2 and 3 fold at 2, 4 and 6 hours of incubation, which was higher than migration of the cells stimulated with 50 ng/ml IGF-1. The combination of 50 ng/ml IGF-1 and 50 ng/ml EGF induced higher cell movement at 2, 4 and 6 hours of incubation by 3, 3.5 and 4 fold respectively.

Results indicate that the combination of IGF-1 and EGF induced a migratory response higher than two stimuli used alone in SK-BR-3 cells.

7.2.2 Effect of combination of IGF-IR and EGFR inhibitors on migration of triple-negative breast cancer cells

As demonstrated previously, inhibitory effect of CP-751, 871, an antibody against type I IGF receptor and gefitinib, an EGFR tyrosine kinase inhibitor on migration of MDA-MB-231 and Hs578T cells were demonstrated individually. In this chapter, ability of IGF-IR and EGFR inhibitor in combination on inhibition of migration of MDA-MB-231, Hs578T cells investigated.

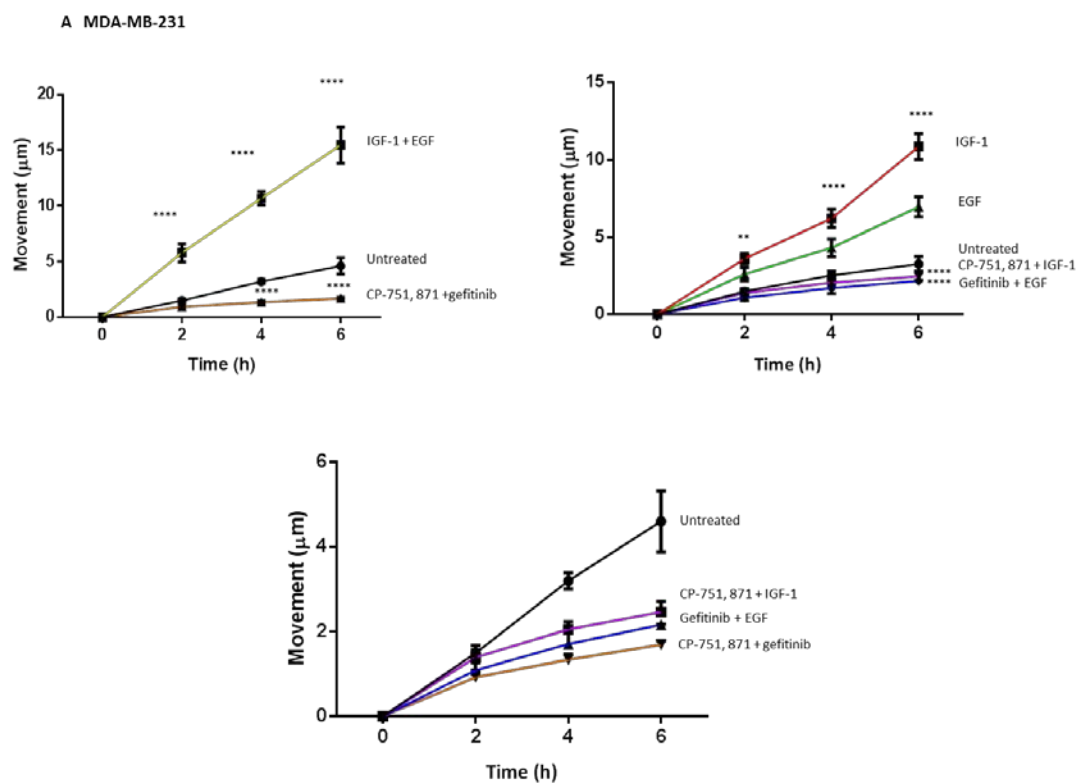
Wounded MDA-MB-231 and Hs578T cells were incubated in the presence and absence of 50 ng/ml IGF-1 or 50 ng/ml EGF or in combination. In addition, cells were treated with 10 μ M gefitinib or 10 μ g/ml CP-751, 871 or in combination, in the presence of 50 ng/ml IGF-1 or 50 ng/ml EGF or in combination at 0, 2, 4 and 6 hours of incubation. Images of the cells were captured at each particular time. The width of each wound was measured 20 times and the average of them was calculated. The cell migration was evaluated by measuring the distance of the edge of the wound towards the centre by using Image J software (Figure 7.3).

As shown in the Figure 7.3, treatment of MDA-MB-231 cells with CP-751, 871 or gefitinib induced significant reduction of migration of cells in response to IGF-1 or EGF respectively. The combination of inhibitors resulted in reduction of MDA-MB-231 cell movement in response to both IGF-1 and EGF, but the reduction was not significantly higher compared to treatment of cells with either inhibitor alone.

Treatment of Hs578T cells with CP-751, 871 or gefitinib significantly reduced the migration of cells in response to IGF-1 or EGF respectively after 6 hours of incubation. Treatment of cells with combination of both CP-751, 871 and

gefitinib resulted in reduction of cell movement in response to IGF-1 and EGF by three fold after 2 hours of incubation and more than six fold after 4 and 6 hours of incubation. The combination of both inhibitors significantly induced higher reduction in cell movement compared to treatment of cells with either inhibitor alone as illustrated in Figure 7.3.

These results suggest that combination of CP-751, 871 and gefitinib triggered higher inhibitory effect on migration of cells compared with the either inhibitor used alone in Hs578T but not in MDA-MB-231 cells.



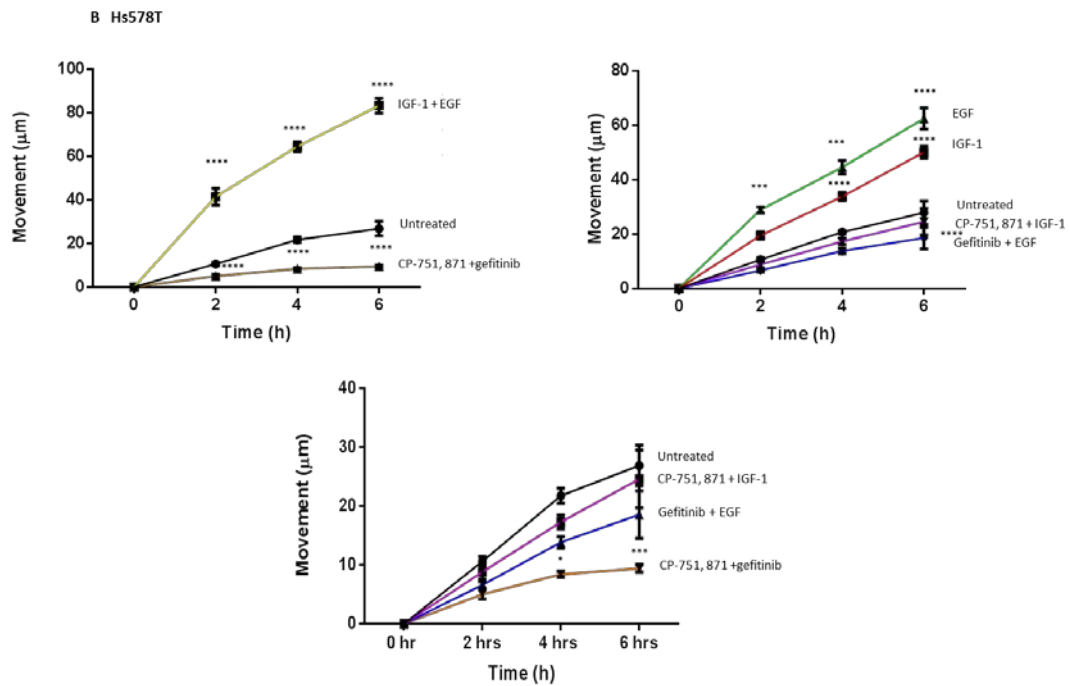


Figure 7.3: Effect of IGF-IR and EGFR inhibitors in combination on migration of MDA-MB-231 and Hs578T cells. MDA-MB-231 (A) and Hs578T (B) cells were seeded at density of 40×10^4 cells/well into 24 well plates that had scored on the under surface with two parallel lines across the middle of each wells. Cells were withdrawn in phenol red-free DMEM medium supplemented with 10% charcoal-treated new born calf serum as described in Materials and Methods section. Monolayer cells were wounded with a sterile 20-200 μ l Gilson pipette tip through the centre of the well, perpendicular to the scored lines. Wounded cells were washed twice with 0.5 ml of phenol red-free DMEM and incubated in phenol red-free DMEM supplemented with 0.01 % of BSA for two hours. After two hours of incubation, cells were washed again and incubated with 0.01% BSA alone () or 50 ng/ml IGF-1 (-■-) or 50 ng/ml EGF (-■-) or IGF-1 and EGF in combination () in the presence of 10 μ g/ml CP-751, 871 and 50 ng/ml IGF-1 (), or in the presence of 10 μ g/ml CP-751, 871 and 50 ng/ml IGF-1 in combination with 10 μ M of gefitinib and 50 ng/ml EGF () for various time points up to 6 hours. Image were captured at the beginning of the experiment (time 0) and after each two hours of treatment up to 6 hours. Three separate measurements in three different wells were made for each treatment condition. The distance between the wounds was measured using Image J software programme. The demonstrated values are the mean + SEM. Asterisks denotes the significant decrease in motility of cells in the presence of CP-751, 871 or gefitinib compared with in the absence of either CP-751, 871 or gefitinib. Combination of both inhibitors markedly decreased the migration of the cells more than each inhibitor alone in Hs578T cells but not MDA-MB-231 cells. Similar results were obtained in three independent experiments (One way ANOVA, * $p < 0.05$, *** $P = 0.0001$, **** $P < 0.0001$).

7.2.3 Effect of combination of IGF-IR and EGFR inhibitors on migration of HER-2 positive SK-BR-3 breast cancer cells

Previously in this study, the inhibitory effect of CP-751, 871 or gefitinib was investigated on migration of SK-BR-3 cells. Here, inhibitory effect of both inhibitors in combination was studied on migration of SK-BR-3 cells stimulated with both IGF-1 and EGF (Figure 7.4).

Treatment of SK-BR-3 cells with CP-751, 871 or gefitinib resulted in significant decrease in the migration of cells, which was demonstrated earlier in this study. The effect of combination of inhibitors in reduction of cell movement stimulated with both IGF-1 and EGF was almost three fold after 2 hours and more than five fold after 4 and 6 hours of incubation. The effect of combined inhibitors did not induced significantly higher reduction in SK-BR-3 cell movement compared to the treatment of cells with each inhibitor alone.

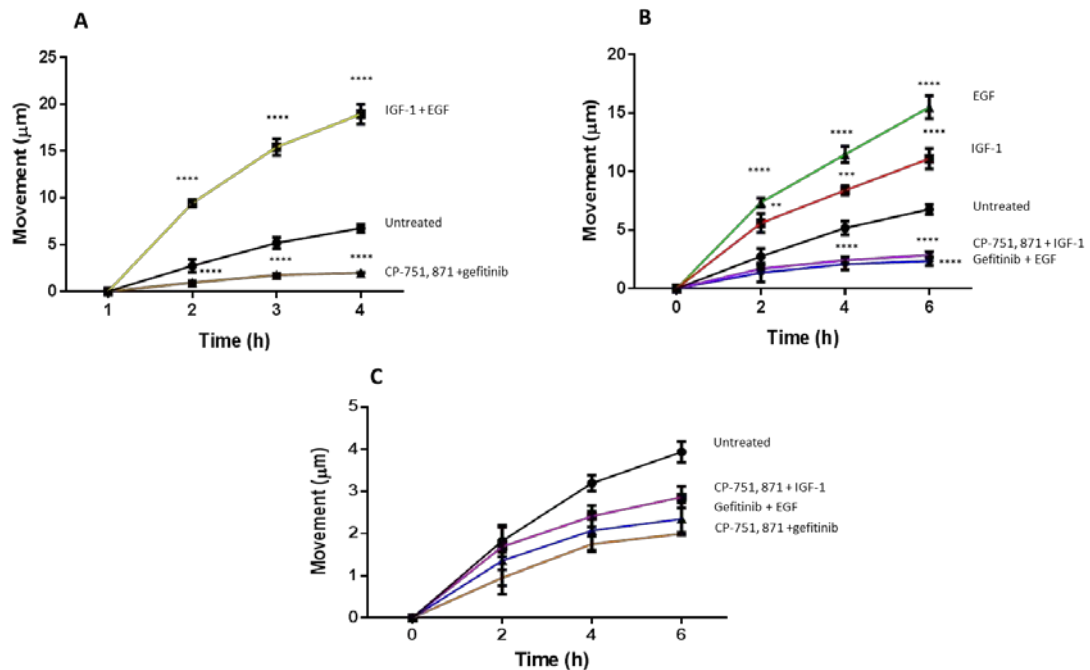


Figure 7.4: Effect of IGF-IR and EGFR inhibitors in combination on migration of SK-BR-3 cells. SK-BR-3 cells were seeded at density of 40×10^4 cells/well into 24 well plates that had scored on the under surface with two parallel lines across the middle of each wells. Cells were withdrawn in phenol red-free DMEM medium supplemented with 10% charcoal-treated new born calf serum as described in Materials and Methods section. Monolayer cells were wounded with a sterile 20-200 µl Gilson pipette tip through the centre of the well, perpendicular to the scored lines. Wounded cells were washed twice with 0.5 ml of phenol red-free DMEM and incubated in phenol red-free DMEM supplemented with 0.01 % of BSA for two hours. After two hours of incubation, cells were washed again and incubated with 0.01% BSA alone () or 50 ng/ml IGF-1 (-■-) or 50 ng/ml of EGF (-■-) or IGF-1 and EGF in combination () in the presence of 10 µg/ml CP-751, 871 and 50 ng/ml IGF-1 (), or in the presence of 10 µg/ml CP-751,871 and 50 ng/ml IGF-1 in combination with 10 µM of gefitinib and 50 ng/ml EGF () for various time points up to 6 hours. Image were captured at the beginning of the experiment (time 0) and after each two hours of treatment up to 6 hours. Three separate measurements in three different wells were made for each treatment condition. The distance between the wounds was measured using Image J software programme. The demonstrated values are the mean \pm SEM. Asterisks denotes the significant decrease in motility of cells in the presence of CP-751, 871 or gefitinib compared with in the absence of either CP-751, 871 or gefitinib (B). Combination of both inhibitors decreased the migration of the cells (A) but did not induce significantly higher reduction in cell motility compared to either inhibitor alone (C). Similar results were obtained in three independent experiments (One way ANOVA, **p<0.001, *** P=0.0001, ****P<0.0001).

7.2.4 Association of IGF-IR and EGFR in malignant breast lesions

The association between expression of IGF-IR and EGFR in both *in situ* and invasive breast carcinoma was investigated. The expression of IGF-IR and EGFR was compared in 37 *in situ* breast carcinomas (Figure 7.5).

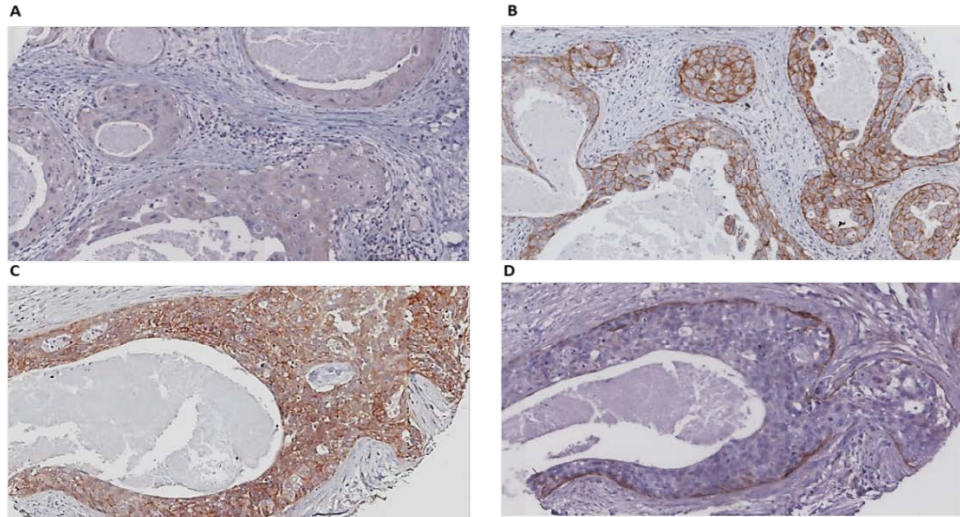


Figure 7.5: Expression of IGF-IR and EGFR in *in situ* carcinoma of the breast. Sections of comedo types of ductal carcinoma *in situ* were analysed for IGF-IR (A, C) and EGFR (B, D) expression by immunohistochemistry. Magnification x200.

Both IGF-IR and EGFR expressed in 29 *in situ* breast carcinomas. The association between expression of IGF-IR and EGFR expression was investigated in *in situ* carcinoma of the breast. The expression of IGF-IR was not significantly associated with the expression of EGFR in *in situ* breast carcinoma (Pearson's rho correlation, $p=0.501$, Figure 7.6).

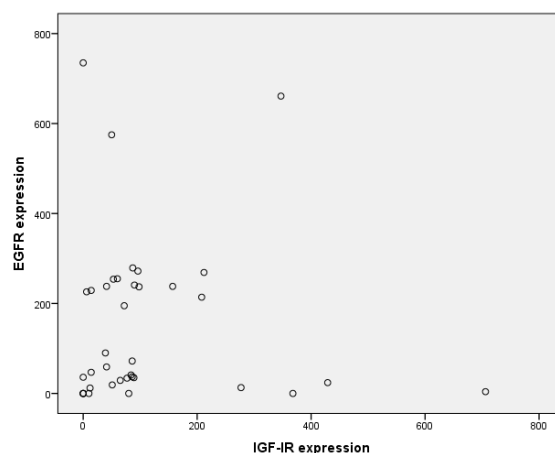


Figure 7.6: Association of IGF-IR and EGFR expression in *in situ* carcinoma of the breast. IGF-IR histoscores are shown against EGFR histoscores in 37 *in situ* lesions. No significant correlation was detected (Pearson correlation, $p=0.501$).

The expression of IGF-IR and EGFR was compared in 79 invasive breast tumours (Figure 7.7). Both IGF-IR and EGFR were detectable in 73 tumours (92%).

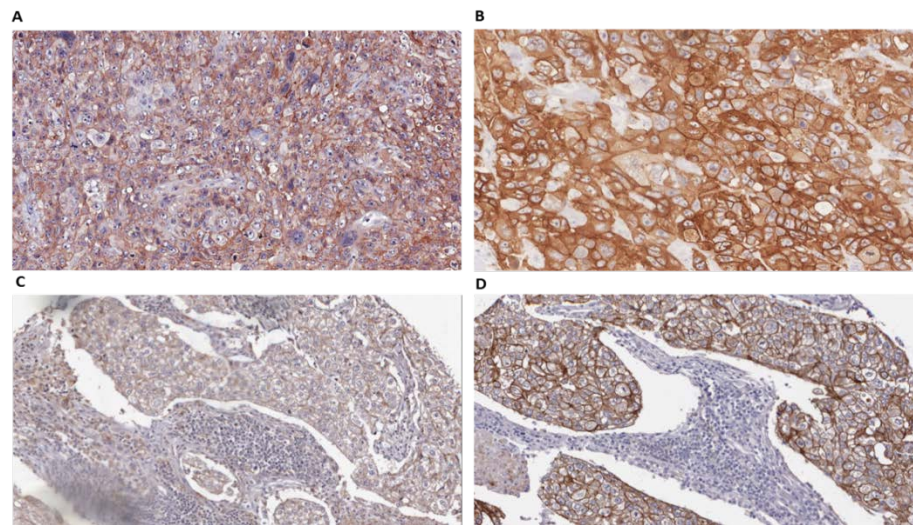


Figure 7.7: Expression of IGF-IR and EGFR expression in invasive breast cancer. Parallel section of invasive breast tumour (A, B) and metastasized breast tumour cells to axillary lymph nodes (C, D) were analysed for IGF-IR (A, C) and EGFR (B, D) expression by immunohistochemistry. Immuno-reaction is membranous. Magnification x20.

The relationship between IGF-IR and EGFR expression was investigated in 79 invasive breast tumours. There was a significant association between IGF-IR expression and expression of EGFR in invasive breast cancer (Pearson correlation rho, $p=0.002$, Figure 7.8 A). There was no significant association between the expression of IGF-IR and EGFR in breast cancer cells that had metastasized to axillary lymph node (Pearson correlation, $p=0.879$, Figure 7.8 B).

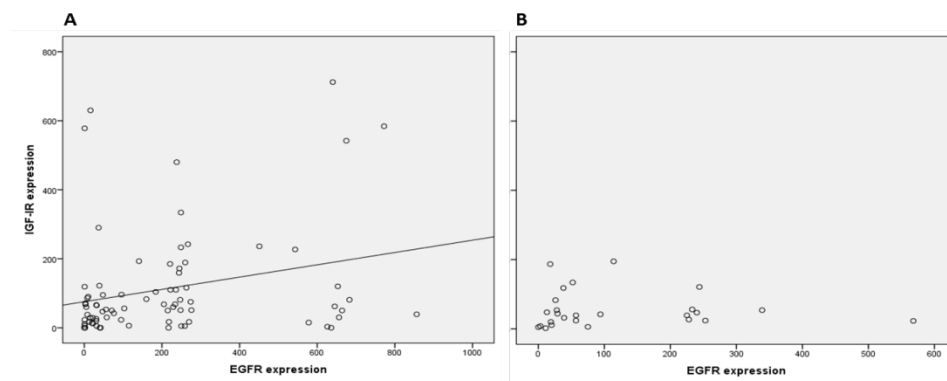


Figure 7.8: Association of IGF-IR and EGFR expression in malignant breast lesions. IGF-IR histoscores are shown against EGFR histoscores for 79 invasive breast cancers (A) and 26 lesions of axillary lymph node metastatic deposits (B). Expression of IGF-IR is significantly correlated with expression of EGFR in invasive breast cancers (Pearson correlation, $p=0.002$).

7.2.5 IGF-IR expression and HER-2 protein expression

The association between expression of IGF-IR and expression of HER-2 in *in situ*, invasive and lesions of axillary lymph node deposits was investigated.

There was no association between expression of IGF-IR and HER-2 expression in overall tumours (Pearson's rho correlation, $p=0.531$).

Association between IGF-IR expression and expression of HER-2 was investigated in 37 *in situ* breast carcinomas. There was no association between IGF-IR expression and expression of HER-2 in *in situ* breast carcinomas (Pearson's rho correlation, $p= 0.590$, Figure 7.9).

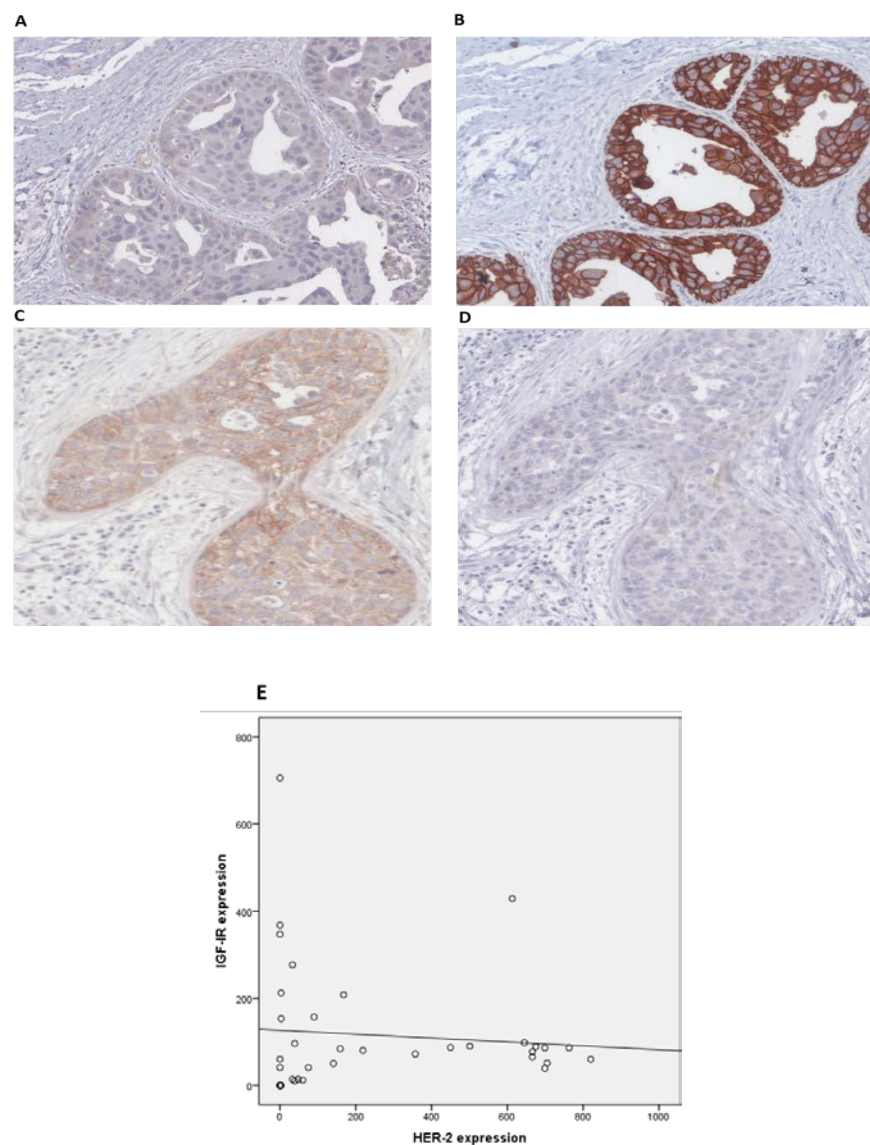


Figure 7.9: Association of IGF-IR expression with HER-2 expression in *in situ* breast carcinomas. IGF-IR expression (A, C) and HER-2 expression (B, D) were analysed in *in situ*

breast carcinoma by immunohistochemistry. IGF-IR expression showed no significant association with expression of HER-2 in *in situ* breast carcinomas (**E**), ($p=0.590$).

Expression of IGF-IR and HER-2 was investigated in 79 invasive breast tumours. There was no association between expression of IGF-IR and HER-2 expression in invasive breast tumours (Pearson's rho correlation, $p=0.498$, Figure 7.10).

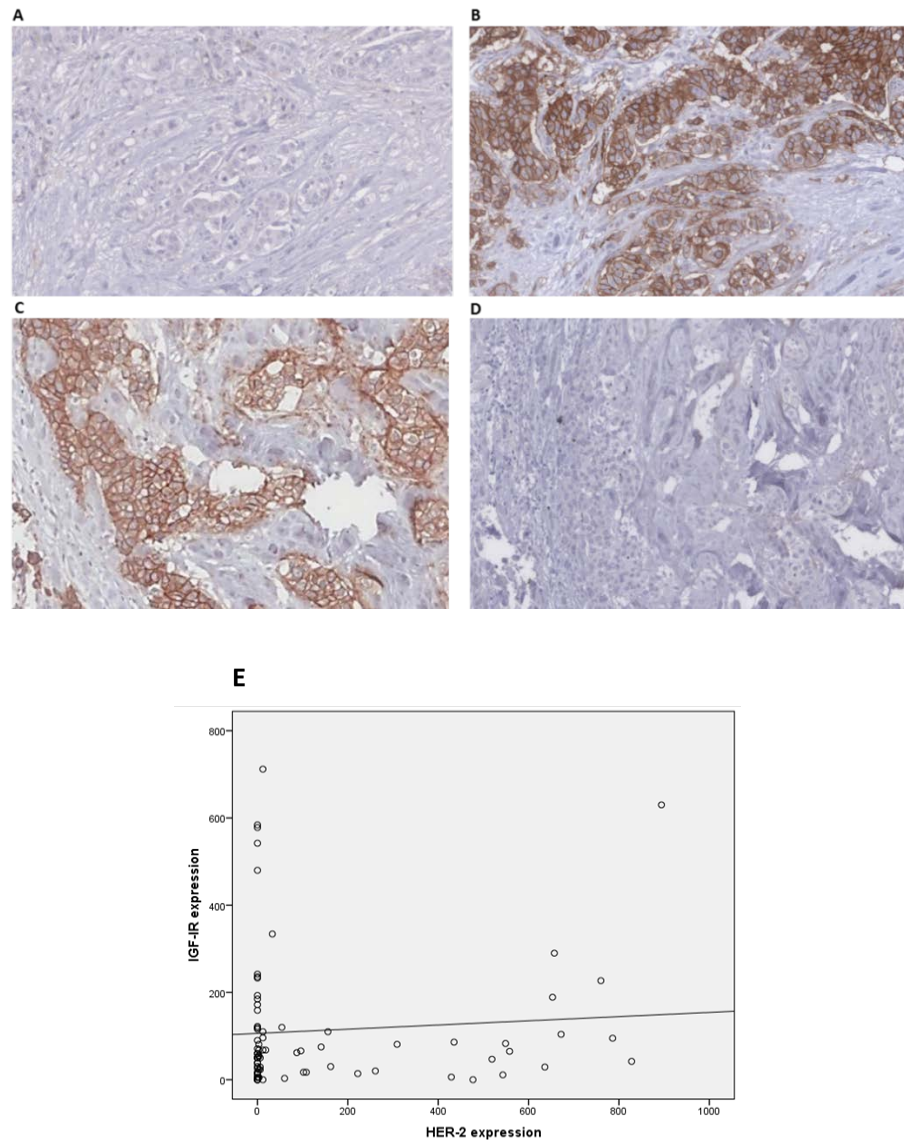


Figure 7.10: Expression of IGF-IR and HER-2 expression in invasive breast cancer. Sections of invasive breast tumours were analysed for IGF-IR (**A, C**) and HER-2 (**B, D**) expression in by immunohistochemistry. Magnification x20. No correlation was found between expression of IGF-IR and HER-2 in 79 cases of invasive breast tumours (**E**) (Pearson's rho correlation $p=0.498$).

Association between expression of IGF-IR and HER-2 expression was investigated in 26 breast tumour cells with the involved lymph node and no

significant correlation was detected between expression of IGF-IR and HER-2 expression (Pearson's rho correlation, $p=0.174$, Figure 7.11).

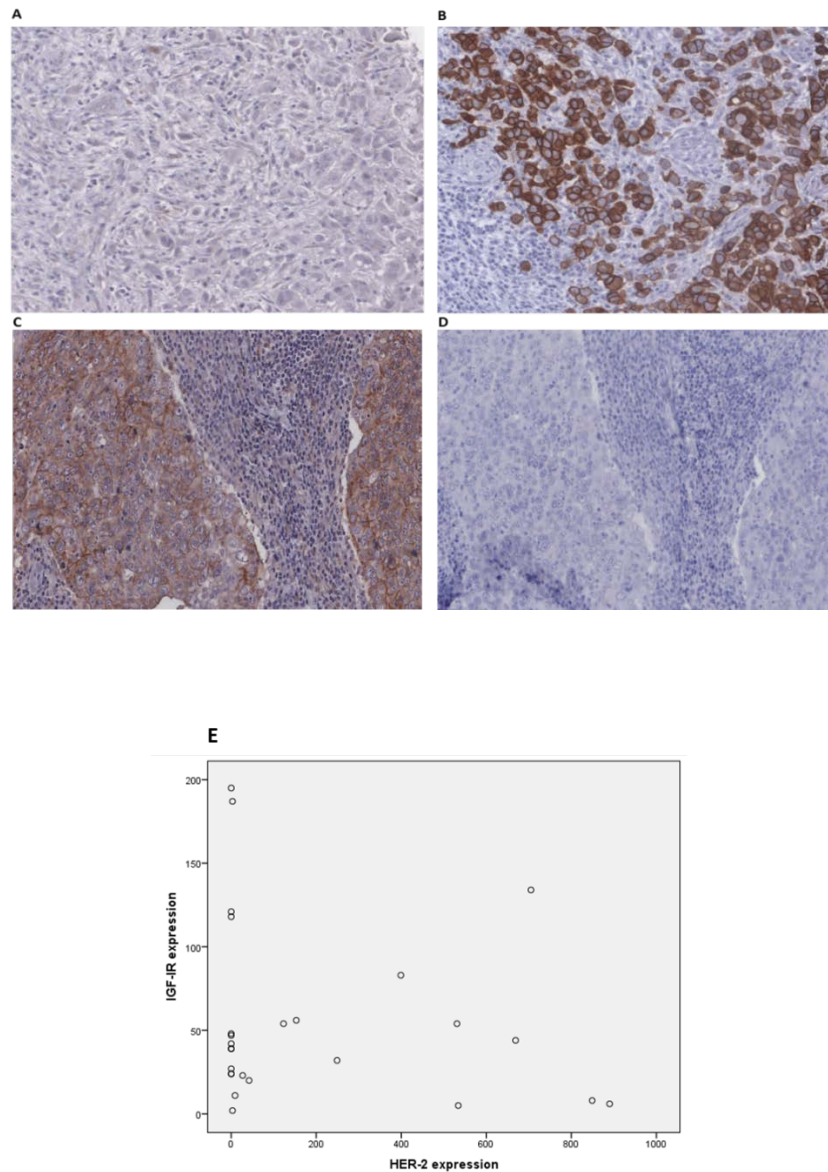


Figure 7.11: Association of IGF-IR expression with HER-2 expression in breast tumour cells in the involved lymph node. IGF-IR expression (A, C) and HER-2 expression (B, D) were analysed in breast carcinoma with metastatic deposit by immunohistochemistry. IGF-IR expression showed no significant association with expression of HER-2 in metastatic breast tumour cells (E) ($p=0.174$).

7.3 Discussion

The effect of the combination of IGF-1 and EGF on migration of oestrogen receptor-negative breast cancer cells were studied in this chapter.

One study compared the effect of IGF-1 and EGF on migration of MDA-MB-231 breast cancer cells and reported that migratory effect of IGF-1 was more than the migratory effect of EGF (Mezi, Todi et al. 2012). Results of the current study regarding the effect of each IGF-1 and EGF ligand on migration of MDA-MB-231 cell line, as discussed in previous chapters are in agreement with the findings of previous group, who observed that IGF-1 had more migratory effect than EGF in MDA-MB-231 cells (Mezi, Todi et al. 2012). However, this group did not test the effect of combination of IGF-1 and EGF on migration of MDA-MB-231 cells. No previous study has tested the effect of the combination of IGF-1 and EGF on the migration of oestrogen receptor-negative breast cancer cells.

In the current study, the effect of the combination of IGF-1 and EGF on migration of MDA-MB-231, Hs578T and SK-BR-3 cells was investigated. Although IGF-1 and EGF each alone stimulated significantly the migration of oestrogen receptor-negative breast cancer cells, the combination of both ligands stimulated higher migration of breast cancer cells than either motogen alone. Additionally, the inhibitors against IGF-IR and EGFR abolished IGF-1 and EGF-mediated migration. The migratory effect of the IGF-1 and EGF in combination was inhibited more significantly using combination of both anti-IGF-IR and anti-EGFR inhibitors compared to inhibitory effect of either inhibitor alone only in Hs578T cells. But, the combination of inhibitors did not show significantly higher inhibitory effect on movement of MDA-MB-231 or SK-BR-3 cells. This result is agreed with the findings of previous study that IGF-IR and EGFR inhibitors in combination are more effective on inhibition growth of colorectal cancer cells (Thomas and Grandis 2004).

The correlation between expression of IGF-IR and EGFR in both *in situ* and invasive breast carcinoma was investigated. There was no significant correlation detected in *in situ* breast carcinomas, whereas, a strong significant association was found between expression of IGF-IR and EGFR in invasive

breast carcinomas ($p=0.002$). In addition, correlation between expression of IGF-IR and HER-2 was studied. There was no significant association between expression of IGF-IR and HER-2 expression in *in situ*, invasive or metastatic HER-2 positive breast tumours ($p>0.05$). This result is not in agreement with the conclusion of previous report that IGF-IR expression was associated with HER-2 negativity (Yerushalmi, Gelmon et al. 2012).

Taken these data together, combination of IGF-1 and EGF demonstrated more effect on migration of oestrogen receptor-negative breast cancer cells than either ligand alone. Hence, targeting both IGF-IR and EGFR signal transduction pathway might be a valuable therapeutic approach, especially as both receptors are expressed in the same tumours.

Chapter 8. Expression of trefoil proteins (TFF1 and TFF3) in oestrogen receptor-negative breast cancer

8.1 Introduction

Trefoil proteins are small secretory proteins that are expressed in epithelial cells of gastrointestinal mucosa and other mucus secreting mucosae. TFF1 is synthesised mainly in the stomach and colon, while TFF3 is expressed in the goblet cells of small and large intestines (May, Semple et al. 2004, Westley, Griffin et al. 2005). The main functions of trefoil proteins are mucosal protection and repair through interaction with mucins and induction of cellular migration for the process of restitution. Overexpression of trefoil proteins is reported in several human solid tumours including breast cancer. Studies show that amongst the three trefoil proteins, TFF1 and TFF3 are expressed at high level in breast tumours whereas TFF2 is generally not expressed (Poulsom, Hanby et al. 1997, May, Semple et al. 2004). In addition, both TFF1 and TFF3, in particular TFF1 is identified as oestrogen-responsive genes which are most frequently expressed in breast tumours. It has been reported that overexpression of TFF3 induce the oestrogen-independent growth in breast cancer cells (Kannan, Kang et al. 2010). TFF3 expression at mRNA level has been reported in HER-2 positive BT-474 and MDA-MB 361 breast cancer cell lines, but not in HER-2 positive SK-BR-3 cells (Wilson, Roberts et al. 2002). In a recent study, expression of TFF3 reported to be higher in HER-2 positive breast tumours (Yue, Xiang et al. 2013).

Expression of TFF1 and TFF3 in oestrogen-responsive breast tumour has been studied and the involvement of TFF3 in progression and dissemination of oestrogen responsive breast tumours is reported (Ahmed, Griffiths et al. 2012). However, immunohistochemical expression of TFF3 protein and its association with IGF-IR, EGFR, HER-2 and proliferation marker, Ki-67 in oestrogen receptor-negative breast cancer tissues has not been reported. There are no reports that describe expression of TFF1 and TFF3 by immunofluorescence in oestrogen receptor-positive and negative breast cancer cells.

In this chapter, the expression of TFF1 and TFF3 proteins in a series of oestrogen non-responsive breast tumours were investigated by immunohistochemistry, also expression of TFF1 and TFF3 proteins in different breast cancer cell lines were analysed by immunofluorescence and western transfer analysis.

8.2 Result

8.2.1 Expression of TFF1 and TFF3 in *in situ* carcinoma of the breast

Expression of TFF1 and TFF3 was studied in all 37 *in situ* breast carcinomas. Out of 37 cases, TFF1 expression was not detected in any of the *in situ* cases, but TFF3 expression was detected in 7 tumours (8.8%). The expression was cytoplasmic and the majority of the positive tumour cells expressed TFF3 at weak or moderate levels with mean histoscore of 106 and median histoscore of 90, respectively (Figure 8.1). However, out of 7 tumours, one tumour was strongly immuno-reactive for TFF3. There is a significant correlation in the expression levels in between the duplicate cores (Pearson's rho correlation, $p < 0.01$).

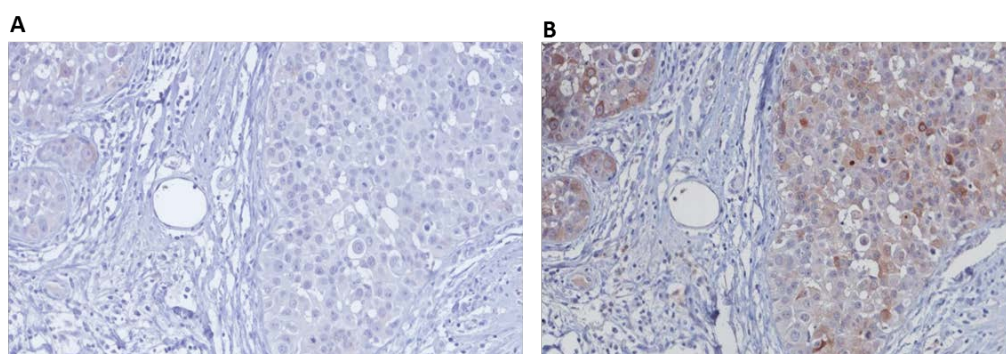


Figure 8.1: Expression of TFF1 and TFF3 in *in situ* breast carcinoma. Parallel sections of solid *in situ* carcinoma having histoscore of 4 (left side) and 213 (right side) were analysed in oestrogen receptor-negative breast tumours by immunohistochemistry for expression of TFF1 (A) and TFF3 (B) antibody. Magnification is x20.

8.2.2 Expression of trefoil proteins in invasive carcinoma of the breast

Consistency of TFF1 and TFF3 expression in duplicate cores was tested. It has been observed that there is a significant correlation in expression levels of duplicate cores (Pearson correlation, $p < 0.01$). This implies that further analysis can be performed and therefore mean values are taken into consideration.

Out of the 79 investigated invasive lesions, TFF3 immuno-reaction was detected in 6 tumours (7.5%). TFF1 was expressed at very low levels, therefore evaluation of TFF1 for further analysis was not been considered. Most of the TFF3 positive tumours were either weak or moderately immuno-reactive with mean histoscore of 232 and median histoscore of 245, respectively (Figure 8.2). However, 3 tumours were strongly immuno-reactive for TFF3 with maximum histoscore of 583. The immuno-reaction was cytoplasmic.

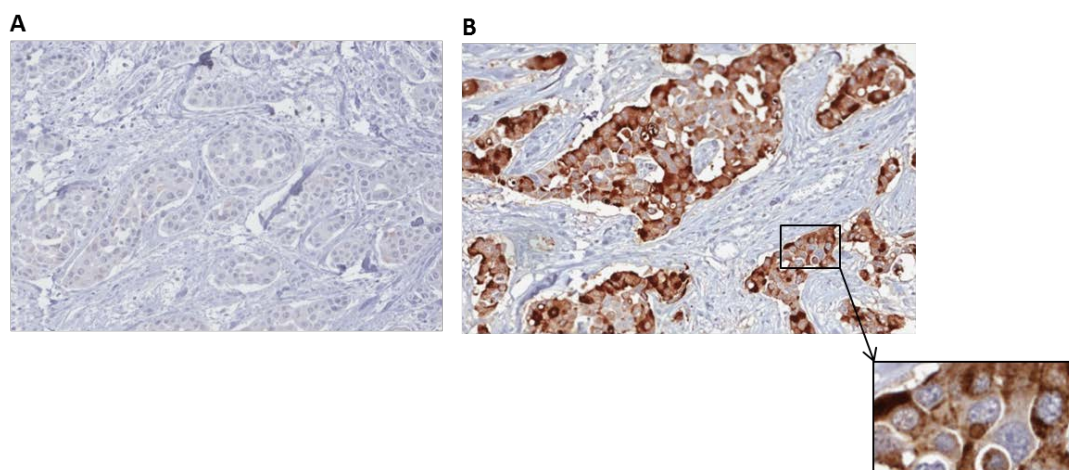
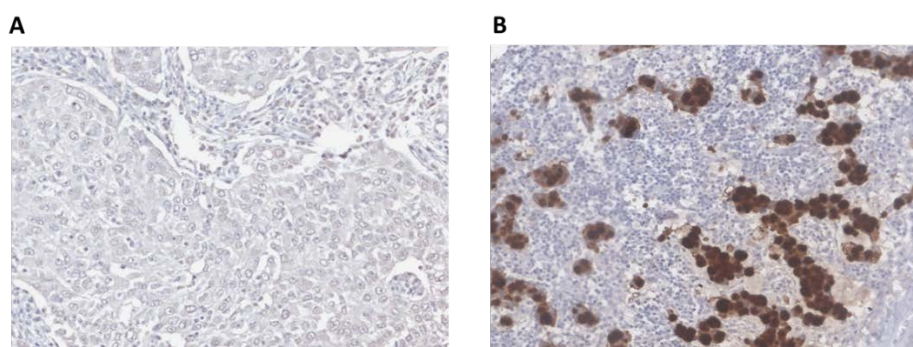


Figure 8.2: Expression of TFF1 and TFF3 in invasive breast carcinoma. Sections of an invasive breast carcinoma were analysed in oestrogen receptor-negative breast tumours by immunohistochemistry for expression of TFF1 (A) and TFF3 (B) with histoscore of 15 and 583, respectively. Magnification is x20 and for inset is x200.

8.2.3 *TFF3 expression and presence of lymph node metastasis*

To investigate whether TFF3 has role in further progression of breast cancer, expression of TFF3 was tested in the primary tumours and their corresponding axillary lymph node metastases in 26 patients. There was no correlation between expression of TFF3 in primary tumour cells and tumour cells with involved lymph node (Pearson's rho correlation, $p=0.134$, Figure 8.3).



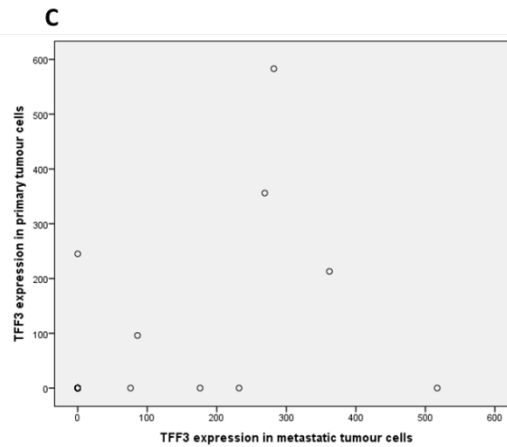


Figure 8.3: TFF3 expression in primary breast tumours and tumour cells in involved lymph node. TFF3 expression was analysed by immunohistochemistry in both primary (A) and metastatic (B) tumour cells from the same patients in 26 tumours having histoscore of 0 and 517, respectively. TFF3 histoscores in the primary tumour cells of 26 patients are shown against its histoscore in the same tumour cells that metastasized to axillary lymph nodes (C).

The association between expression of TFF3 and number of axillary lymph node metastasis was tested. There was no association between expression of TFF3 and number of axillary lymph node metastasis (Spearman's rho correlation, $p=0.640$, Figure 8.3). Out of 26 tumour cells for which involved lymph node was identified; TFF3 was detected only in 5 tumours. There was no significant difference between the expression level of TFF3 in primary tumours with detected axillary lymph nodes and tumours without detectable axillary lymph nodes (Mann-Whitney test, $p=0.932$).

8.2.4 Association between TFF3 expression and expression of IGF-IR and EGFR

To evaluate the relationship between the expression of TFF3, IGF-IR and EGFR, their association has been tested in 79 invasive breast tumours (Figure 8.4). There was no association between expression of TFF3 and IGF-IR expression (Pearson's rho correlation, $p=0.921$). There was no association between expression of TFF3 and EGFR expression (Pearson's rho correlation, $p=0.938$).

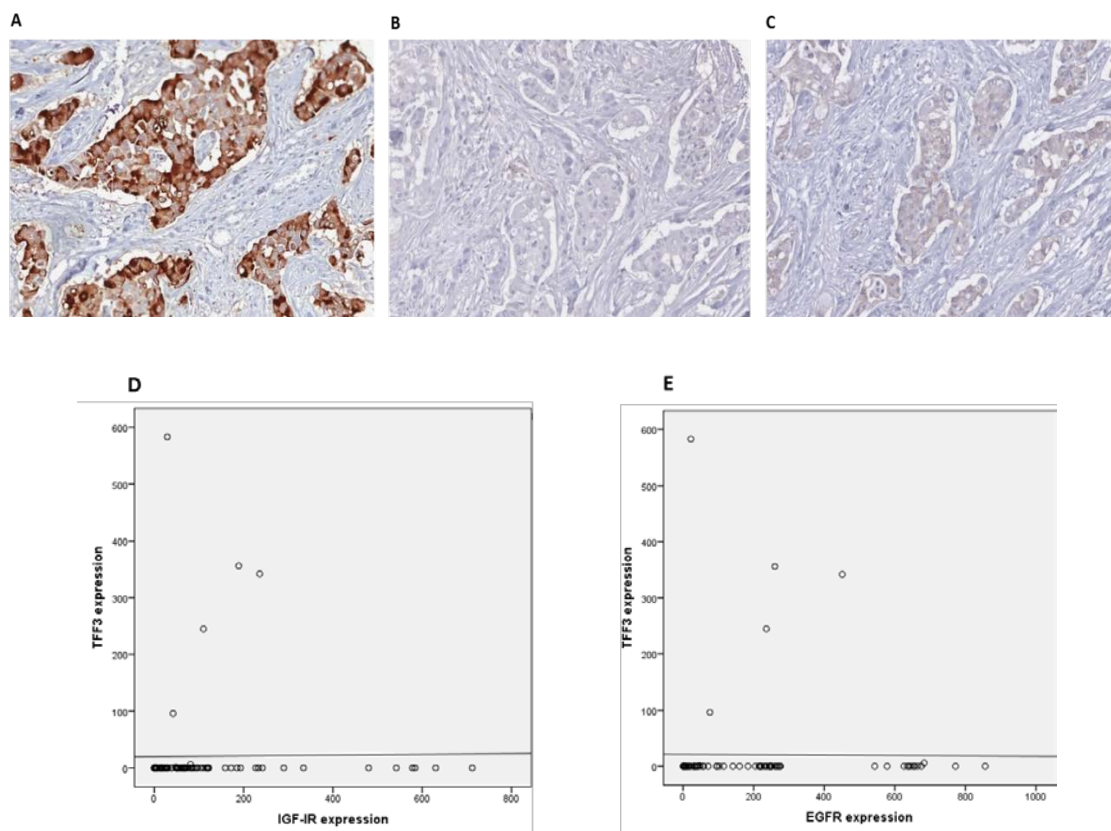


Figure 8.4: Association between expression of TFF3 and IGF-IR and EGFR expression in invasive breast tumours. TFF3 expression (A) and expression of IGF-IR (B) and EGFR (C) was analysed in invasive breast tumours by immunohistochemistry. No correlation between TFF3 expression and expression of IGF-IR ($p=0.921$) or EGFR ($p=0.938$) was detected (D, E).

8.2.5 Association between HER-2 expression and expression of TFF3

Few literatures have reported the relationship between expression of TFF3 and HER-2. In one immunohistochemical study, it has been reported that HER-2 positive breast tumours have expressed high level of TFF3. In the *in vitro* part of the same study, inhibition of HER-2 shown to significantly down regulate TFF3 expression in HER-2 overexpressed SK-BR-3 breast cancer cells (Yue, Xiang et al. 2013). In another study, expression of TFF3 reported to be up regulated in HER-2 positive breast cancer cell lines (Wilson, Roberts et al. 2002). Here, the association between expression of TFF3 and HER-2 in *in situ* breast carcinomas, invasive tumours and breast tumour cells with involved axillary lymph nodes was investigated.

Expression levels of TFF3 and HER-2 was been compared in 37 *in situ* breast carcinomas. There was no association between TFF3 expression and expression of HER-2 in *in situ* breast carcinomas (Pearson correlation, $p=0.46$, Figure 8.5).

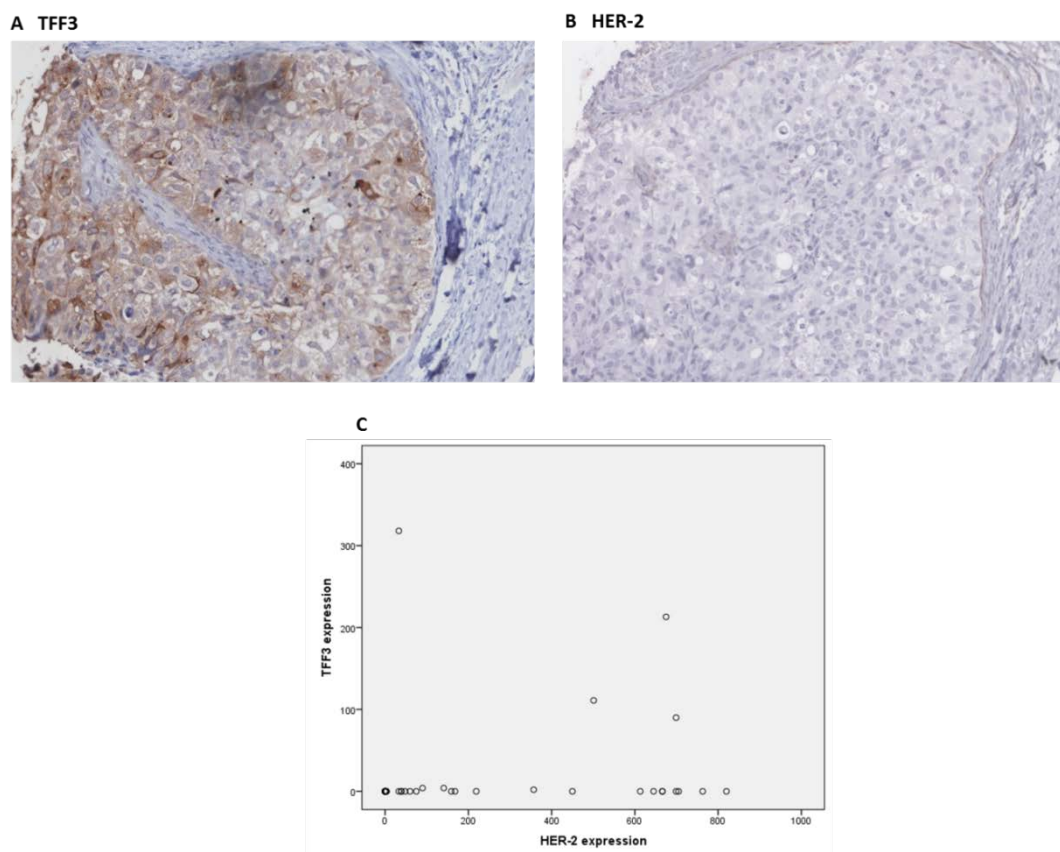


Figure 8.5: Association of TFF3 expression with HER-2 expression in *in situ* breast carcinomas. TFF3 expression (A) and HER-2 expression (B) were analysed in *in situ* breast carcinoma by immunohistochemistry. TFF3 expression showed no significant association with expression of HER-2 ($p=0.46$).

Expression of TFF3 and HER-2 was investigated in 79 invasive tumours. Out of 6 TFF3 positive tumours, 5 tumours were positive for expression of HER-2.

Association between expression of TFF3 and HER-2 was investigated in invasive tumours. There was a significant association between expression of TFF3 and HER-2 expression (Pearson's rho correlation, $p=0.02$, Figure 8.6 C).

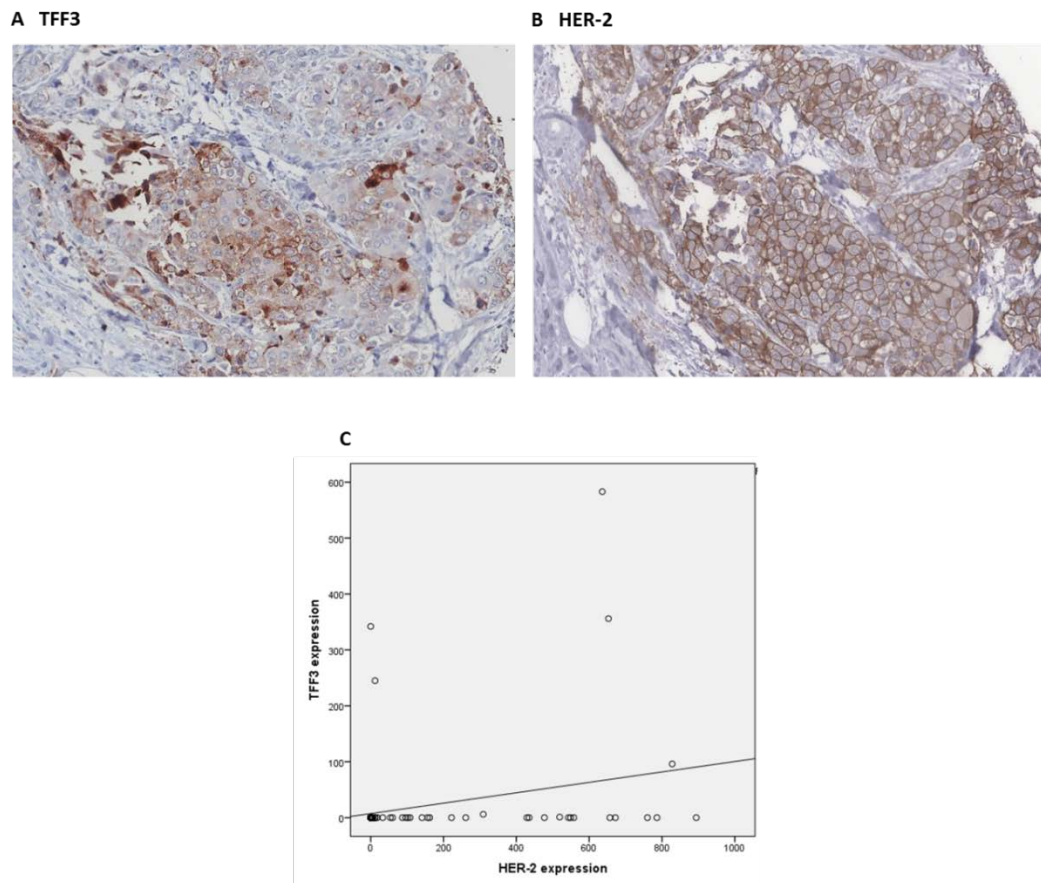


Figure 8.6: Association between expression of TFF3 and HER-2 expression in invasive breast tumours. TFF3 expression (A) and HER-2 expression (B) were analysed in invasive breast tumours by immunohistochemistry. TFF3 histoscores are shown against HER-2 histoscores in the 79 invasive tumours. The correlation between TFF3 expression and HER-2 expression was significant ($p=0.02$).

Association between expression of TFF3 and HER-2 was investigated in 26 breast tumour cells in involved axillary lymph nodes. There was no significant correlation detected between expression of TFF3 and HER-2 expression (Pearson's rho correlation, p -value=0.662, Figure 8.7).

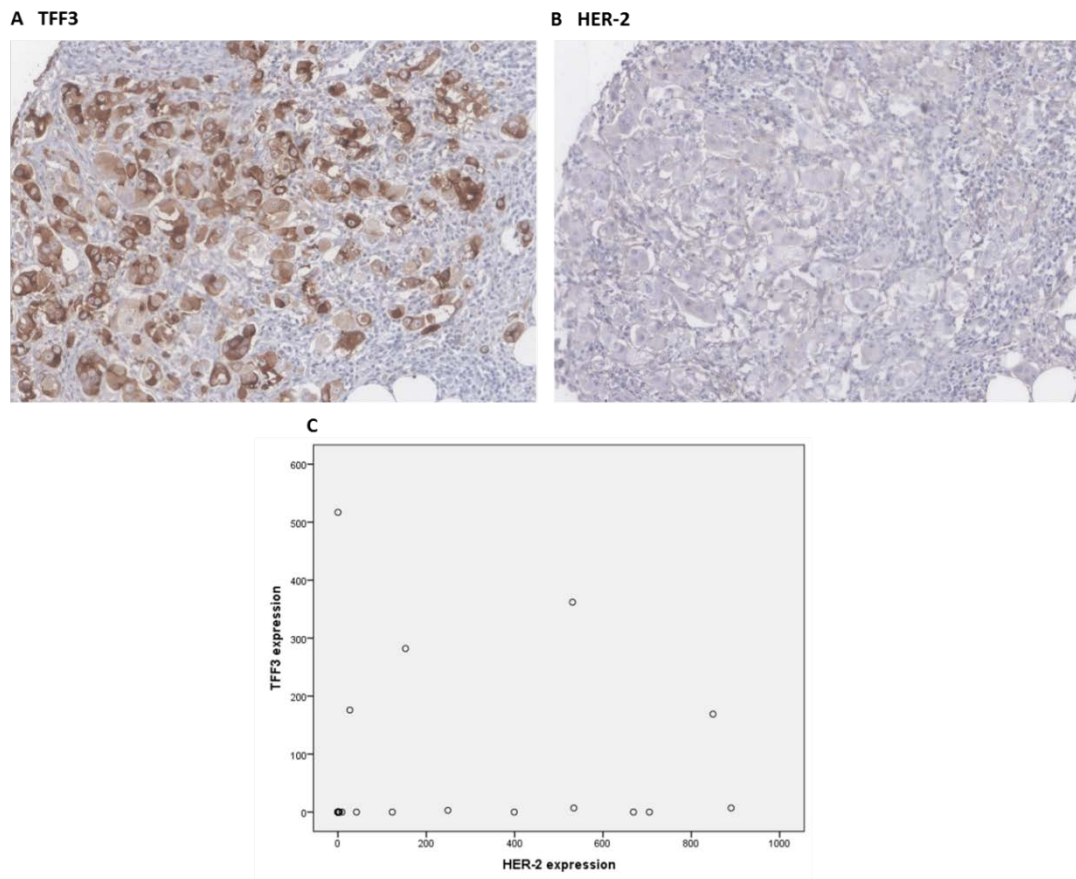


Figure 8.7: Association of TFF3 expression with HER-2 expression in breast tumour cells in involved lymph node. Sections of breast tumours with metastatic lymph node deposits were analysed for expression of TFF3 (A) and HER-2 (B) by immunohistochemistry. TFF3 expression showed no significant association with expression of HER-2 in metastatic breast tumour cells (C) ($p=0.662$).

8.2.6 Association between expression of TFF3 and Ki-67 protein

Association between expression of TFF3 and expression of proliferation marker, Ki-67 was investigated in breast tumours including *in situ* carcinomas, invasive tumours and tumour cells with involved lymph nodes.

Expression of TFF3 and Ki-67 was analysed in 37 *in situ* carcinoma of the breast. There was no significant association between expression of TFF3 and Ki-67 expression (Pearson's rho correlation, $p=0.789$, Figure 8.8 C). However, TFF3 positive tumours were expressed Ki-67 at low level (Figure 8.8 C).

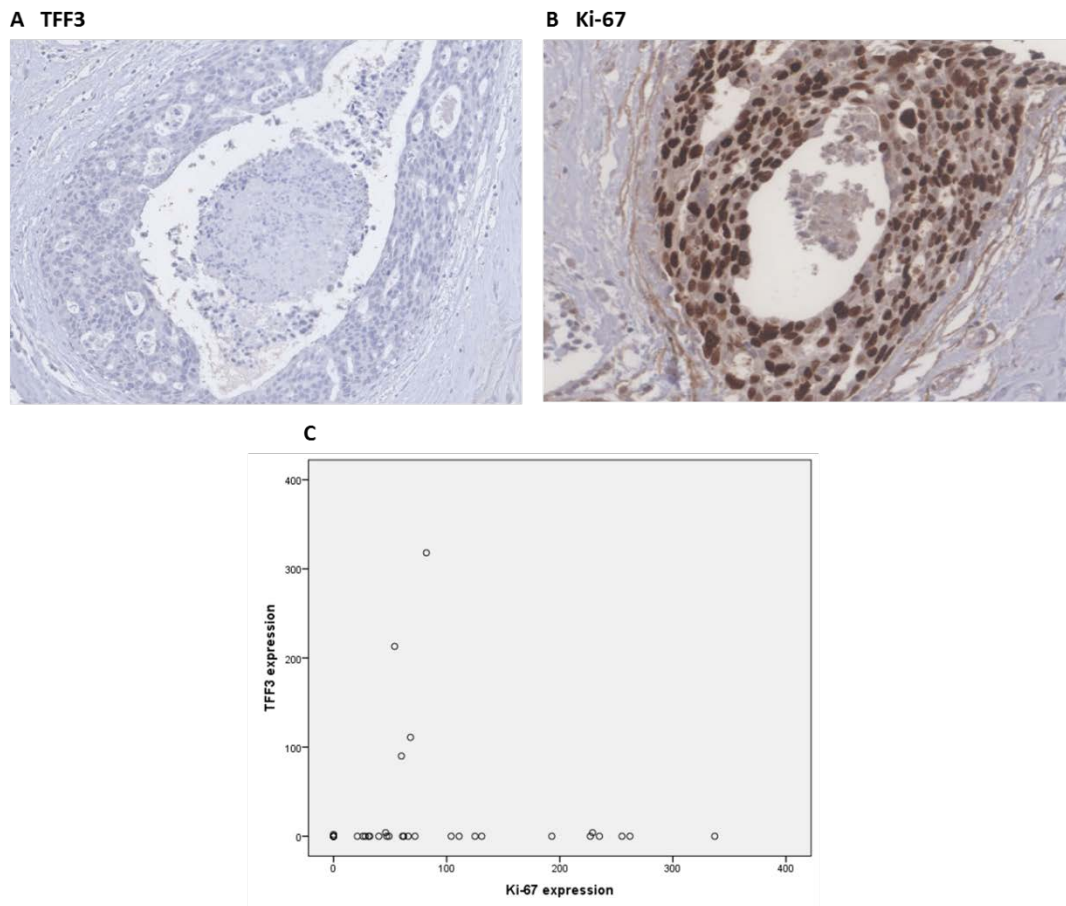


Figure 8.8: Association of TFF3 expression with Ki-67 expression in *in situ* breast carcinomas. TFF3 expression (A) and Ki-67 expression (B) were analysed in *in situ* breast carcinoma by immunohistochemistry. TFF3 expression showed no significant association with expression of Ki-67 in *in situ* breast carcinomas ($p=0.789$).

Expression of TFF3 and Ki-67 was compared in 79 invasive breast tumours. Majority of Ki-67 positive tumours were negative for expression of TFF3. However, 5 tumours positive for expression of TFF3 also expressed Ki-67 at weak and moderate level. There was no significant association between expression of TFF3 and Ki-67 expression in invasive breast tumours (Pearson's rho correlation, $p=0.839$, Figure 8.9).

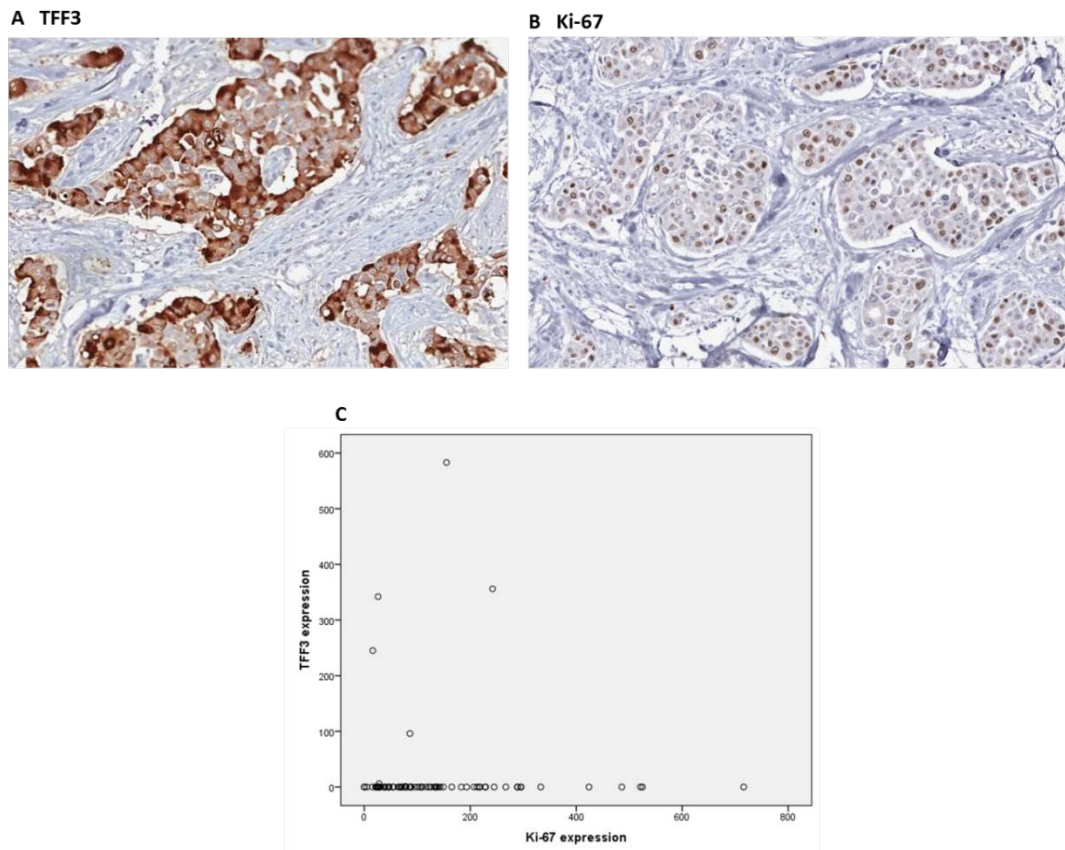


Figure 8.9: Association of TFF3 expression and expression of Ki-67 in invasive breast tumours. Parallel sections of invasive breast tumours were analysed for expression of TFF3 **(A)** and Ki-67 **(B)** by immunohistochemistry. There was no association between expression of TFF3 and expression of Ki-67 ($p=0.839$).

Expression of TFF3 and Ki-67 was analysed in 26 breast tumour cells with metastatic lymph node deposits. There was no significant correlation between expression of TFF3 and Ki-67 expression in tumour cells with involved lymph node (Pearson's rho correlation, $p=0.183$, Figure 8.10 C).

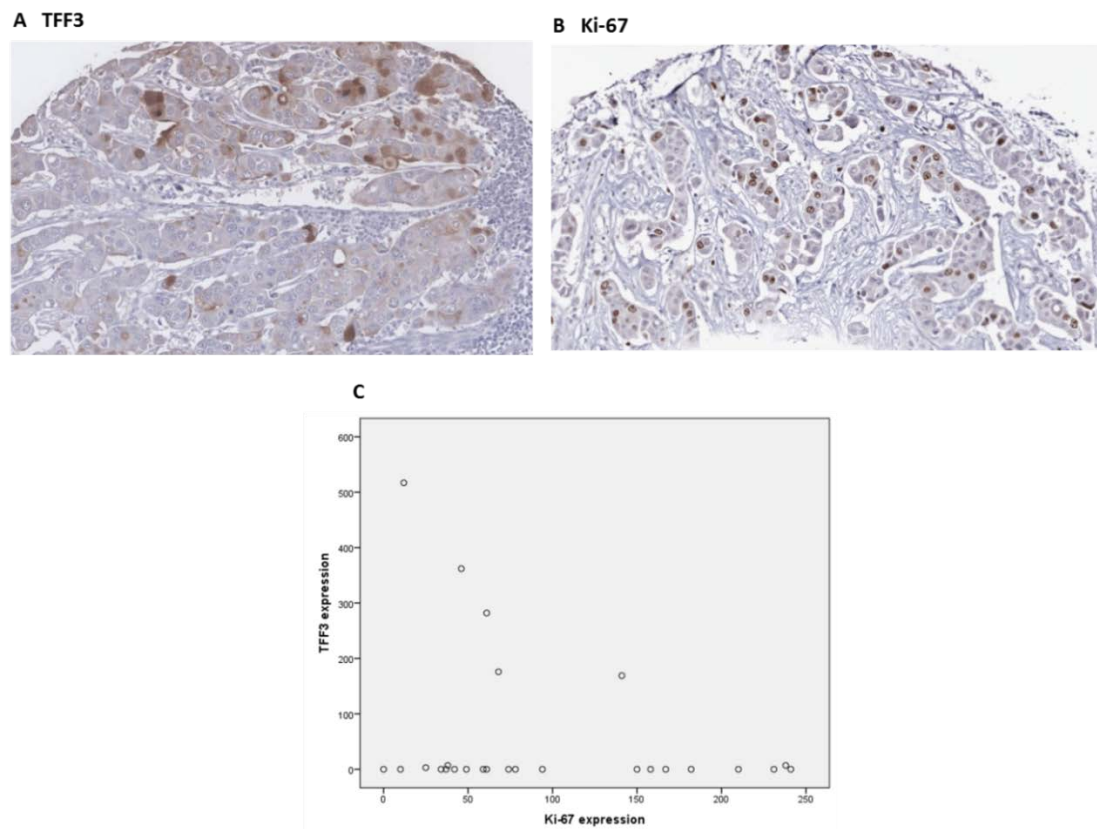


Figure 8.10: Association of TFF3 expression with Ki-67 expression in breast tumour cells in involved axillary lymph nodes. Expression of TFF3 (**A**) compared with expression of Ki-67 (**B**) in 26 breast tumours with involved lymph nodes by immunohistochemistry. TFF3 expression showed no significant association with expression of Ki-67 in tumour cells with metastatic deposits ($p=0.183$).

8.2.7 Expression of TFF1 and TFF3 in oestrogen receptor-positive and negative breast cancer cell lines

Expression of TFF1 and TFF3 was evaluated by western transfer analysis in different breast cancer cell lines (Figure 8.11). In MCF-7 cells, TFF1 protein was detected but not TFF3 protein. BT-474 cells expressed both TFF1 and TFF3 at reasonable amount. EFM-19 cells did not show expression of TFF1 or TFF3. Expression of TFF1 and TFF3 was not detected in any of the oestrogen receptor-negative SK-BR-3, Hs578T and MDA-MB-231 breast cancer cells. Expression of TFF1 and TFF3 was observed in HCC-1419 cells, which is agreed with the previous findings. TFF1 expressed at higher level than TFF3 in HCC-2218 cell. Different results in the expression level of TFF1 and TFF3 in EFM-19 cells might be due to different sensitivities of the antibodies that have been used in western and immunofluorescence experiments.

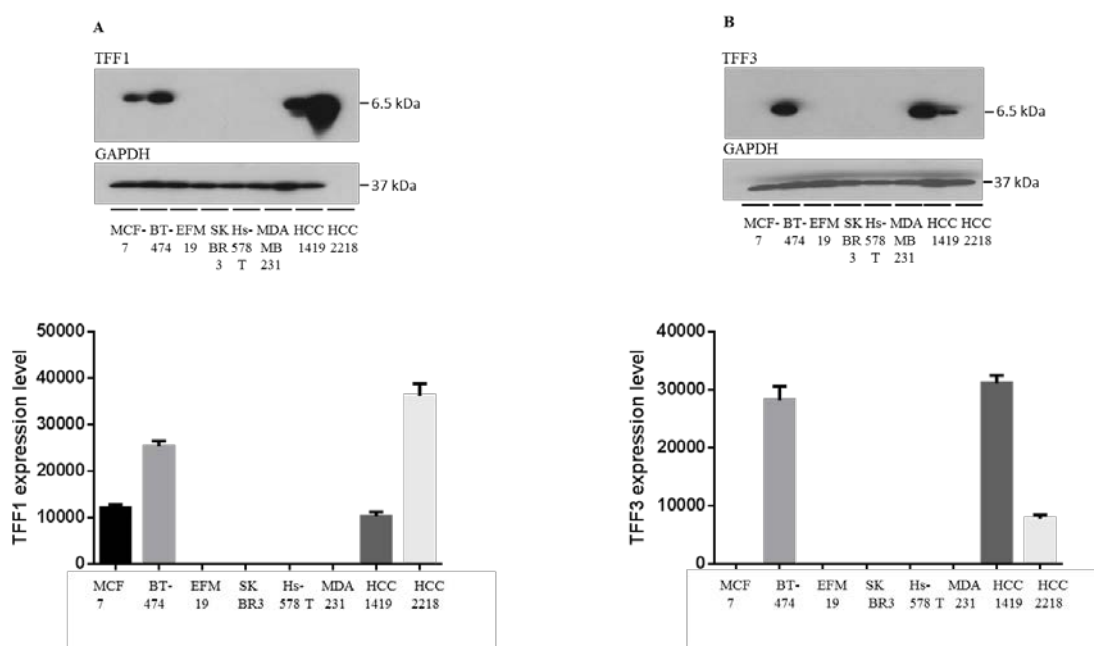


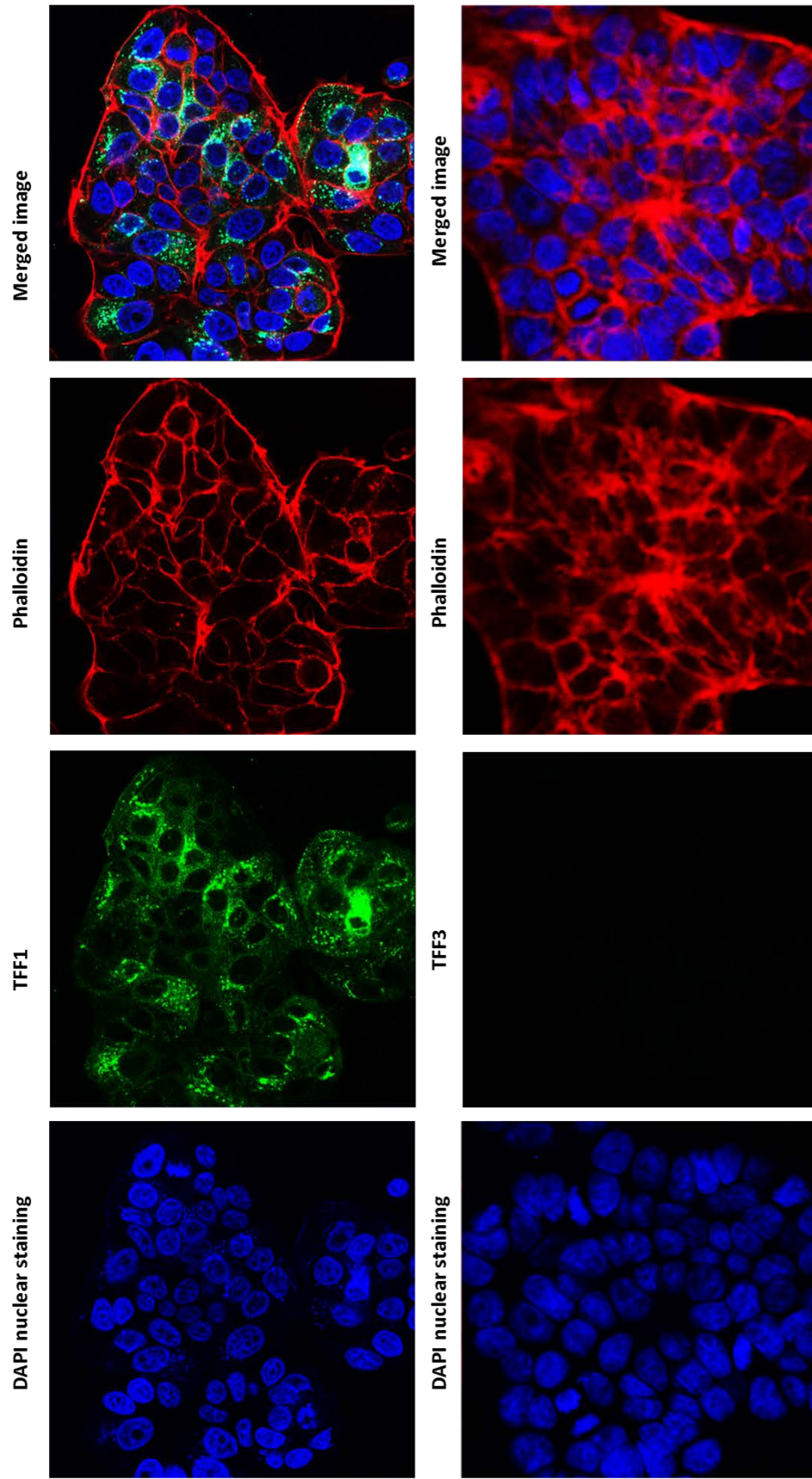
Figure 8.11: Expression of TFF1 and TFF3 in different breast cancer cell lines by western transfer analysis. MCF-7, BT-474, EFM-19, SK-BR-3, Hs578T, MDA-MB-231, HCC-1419 and HCC-1418 cells were cultured to approximately 80% confluence and their protein extracted with 1 ml of RIPA buffer per 75 cm² tissue culture flask as described in Material and Methods Section. Aliquots containing 20 µg of extracted protein were electrophoresed on a 12% denaturing polyacrylamide gel and transferred in to PVDF membrane at 100 mA for 10 minutes. Membrane was incubated with (A) TFF1 (1 in 10 dilution), (B) TFF3 (1 in 20 dilution) and GAPDH (1 in 20,000 dilution). Densitometric scanning of X-ray films determined the amount of each protein by analysis with LabWork 4.0 software. The mean \pm SEM after normalisation to GAPDH expression are shown.

8.2.8 Cellular localization of TFF1 and TFF3 expression in oestrogen receptor-positive and negative breast cancer cell lines

Expression of TFF1 and TFF3 was analysed by immunofluorescence in oestrogen receptor-positive, HER-2 negative MCF-7 cells, and HER-2 positive EFM-19 and BT-474 cells. The expression of TFF1 and TFF3 were also analysed in oestrogen receptor-negative but HER-2 positive HCC-1419 and SK-BR-3 cell lines.

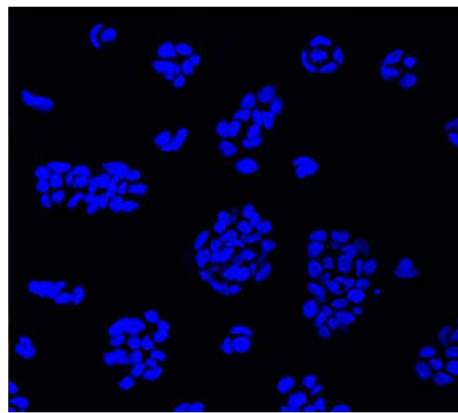
In MCF-7 cells, expression of TFF1 was detected, but there were no detectable expression of TFF3 in this cell line. In EFM-19 cells, TFF1 expression was not detected, but expression of TFF3 was detected. In HCC-1419 and BT-474 cells, expression of both TFF1 and TFF3 was detected. Neither TFF1 nor TFF3 expression was detected in SK-BR-3 cells. Expression of both TFF1 and TFF3 was cytoplasmic. As illustrated in Figure 8.12 and Figure 8.13, the vesicular expression of both TFF1 and TFF3 makes them distinct from nuclear expression.

A MCF-7



B EFM-19

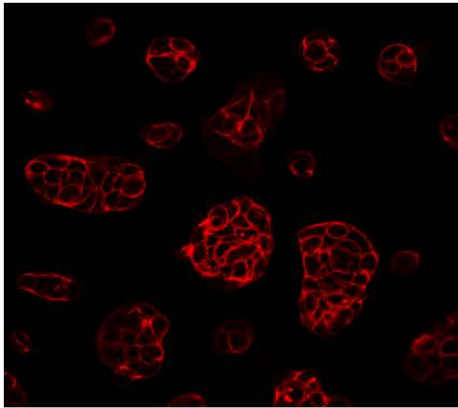
DAPI nuclear staining



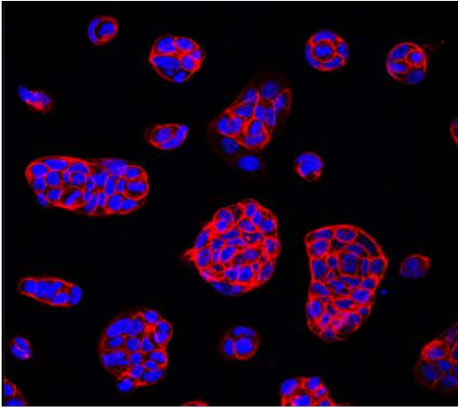
TFF1



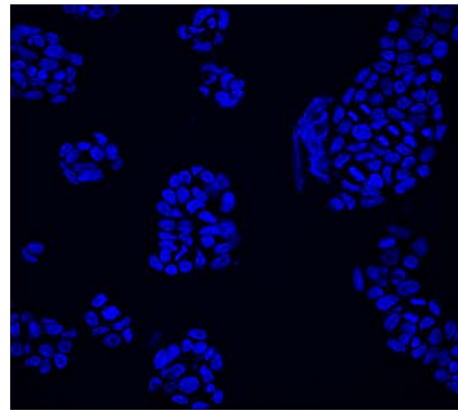
Phalloidin



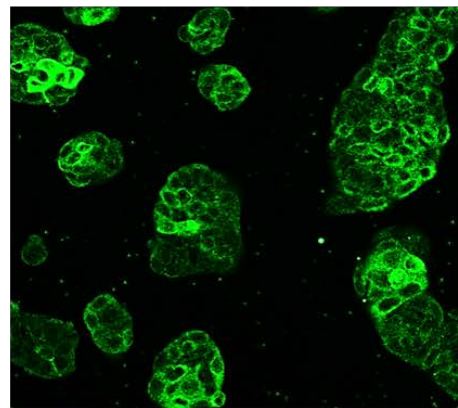
Merged image



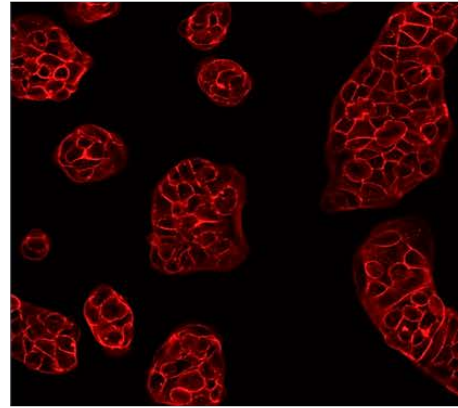
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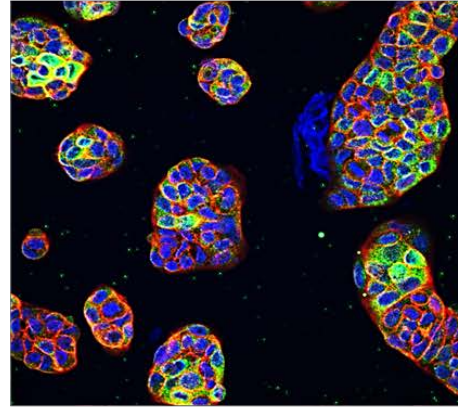
TFF3



Phalloidin



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C BT-474

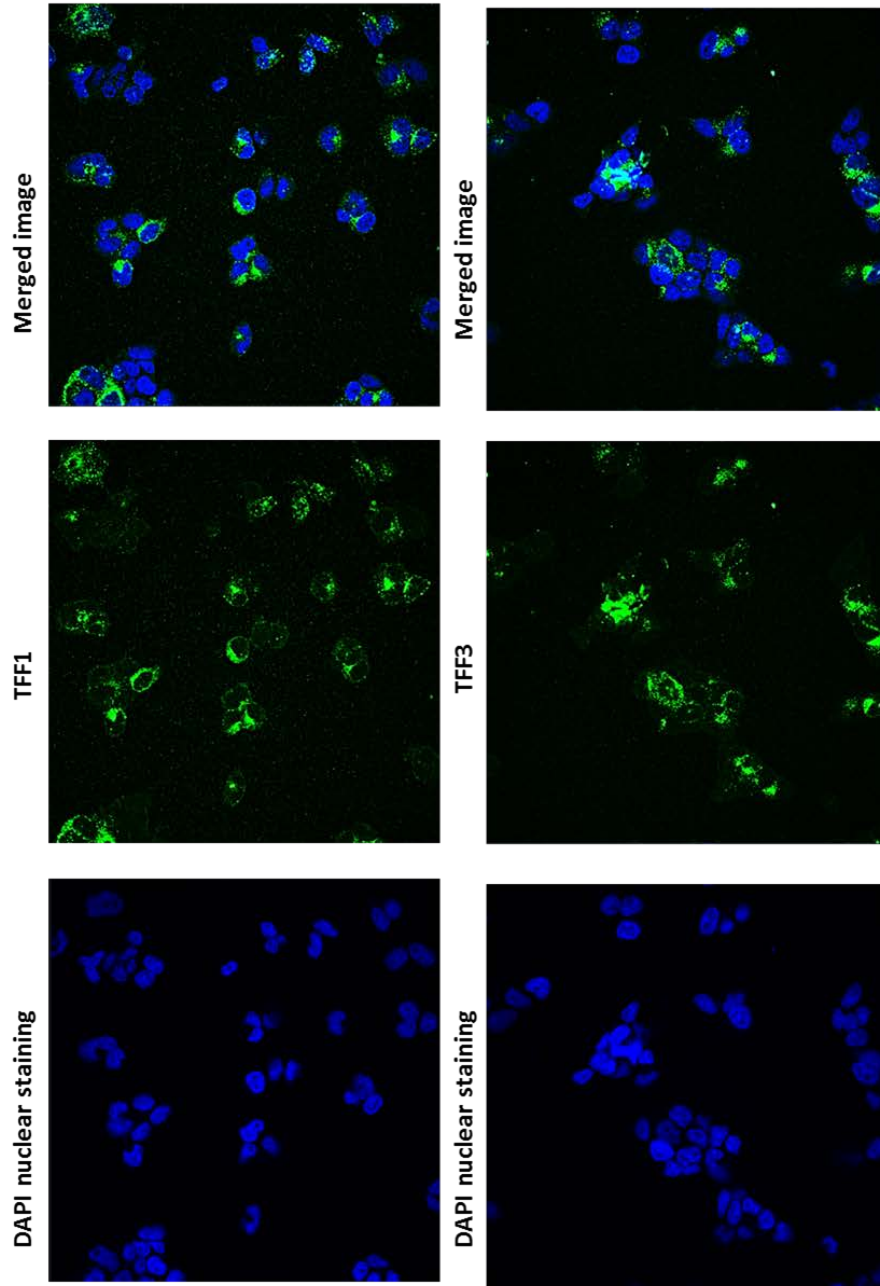
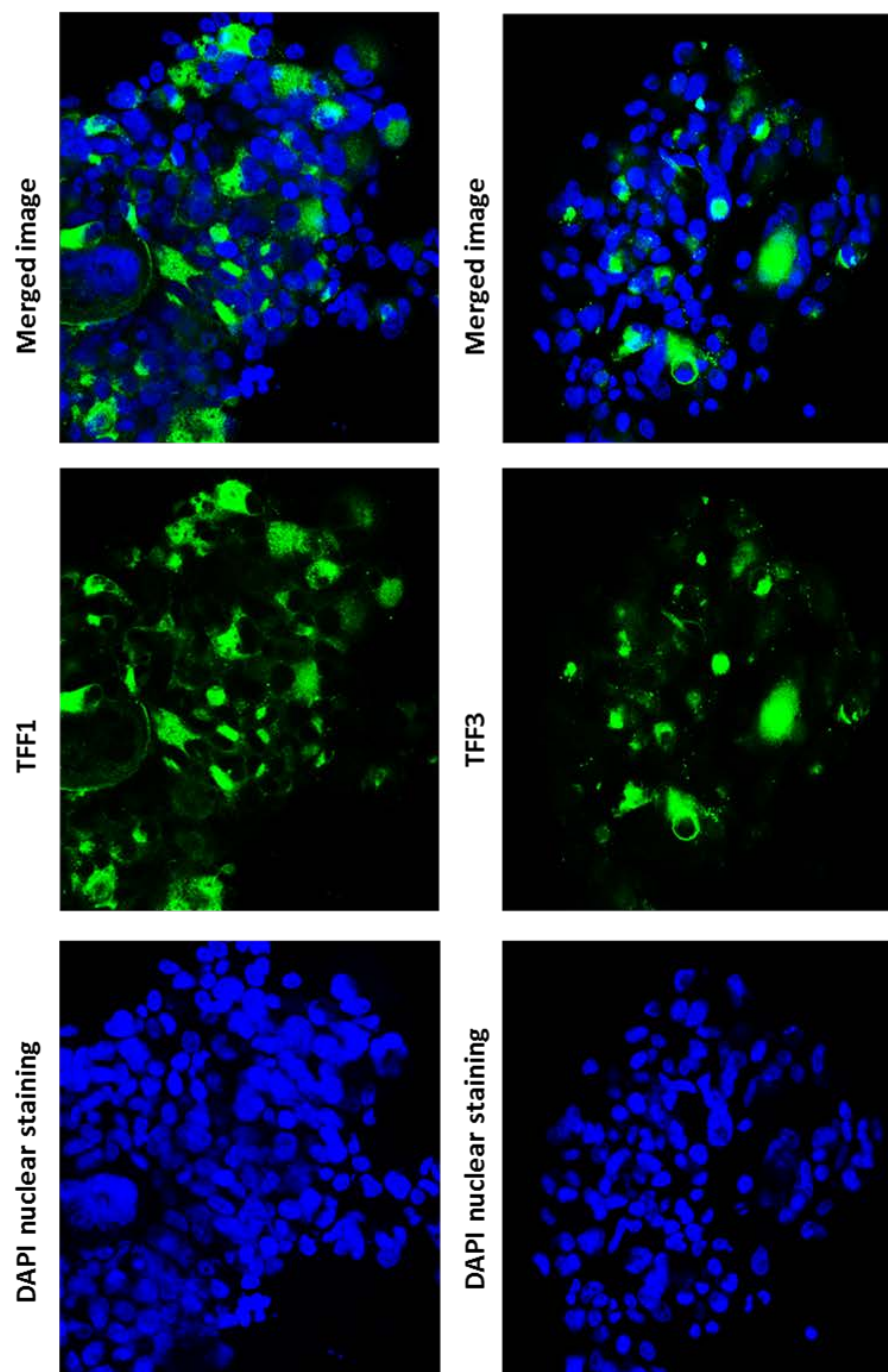


Figure 8.12 Immunofluorescence staining of TFF1 and TFF3 in MCF-7, EFM-19 and BT-474 cells. Expression of TFF1 and TFF3 were tested in three oestrogen receptor-positive breast cancer cell lines: MCF-7 (A), EFM-19 (B) and BT-474 (C) cells. Cells were plated into 6 well plates and incubated for 24 hours. After 24 hours of incubation cells were washed with PBS and fixed with cold methanol at room temperature for 20 minutes. Cells were then blocked and followed by incubation with the primary antibody TFF1 (1 in 10 dilution) and TFF3 (1 in 20 dilution). Cells were incubated for 15 minutes with 4 μ l of phalloidin 568. Results demonstrate the cytoplasmic expression of TFF1 in MCF-7 and BT-474 cells. TFF3 expression was cytoplasmic and detected in EFM19 and BT-474 cells. Vesicular expression of both TFF1 and TFF3 is illustrated in the Figure.

A HCC-1419



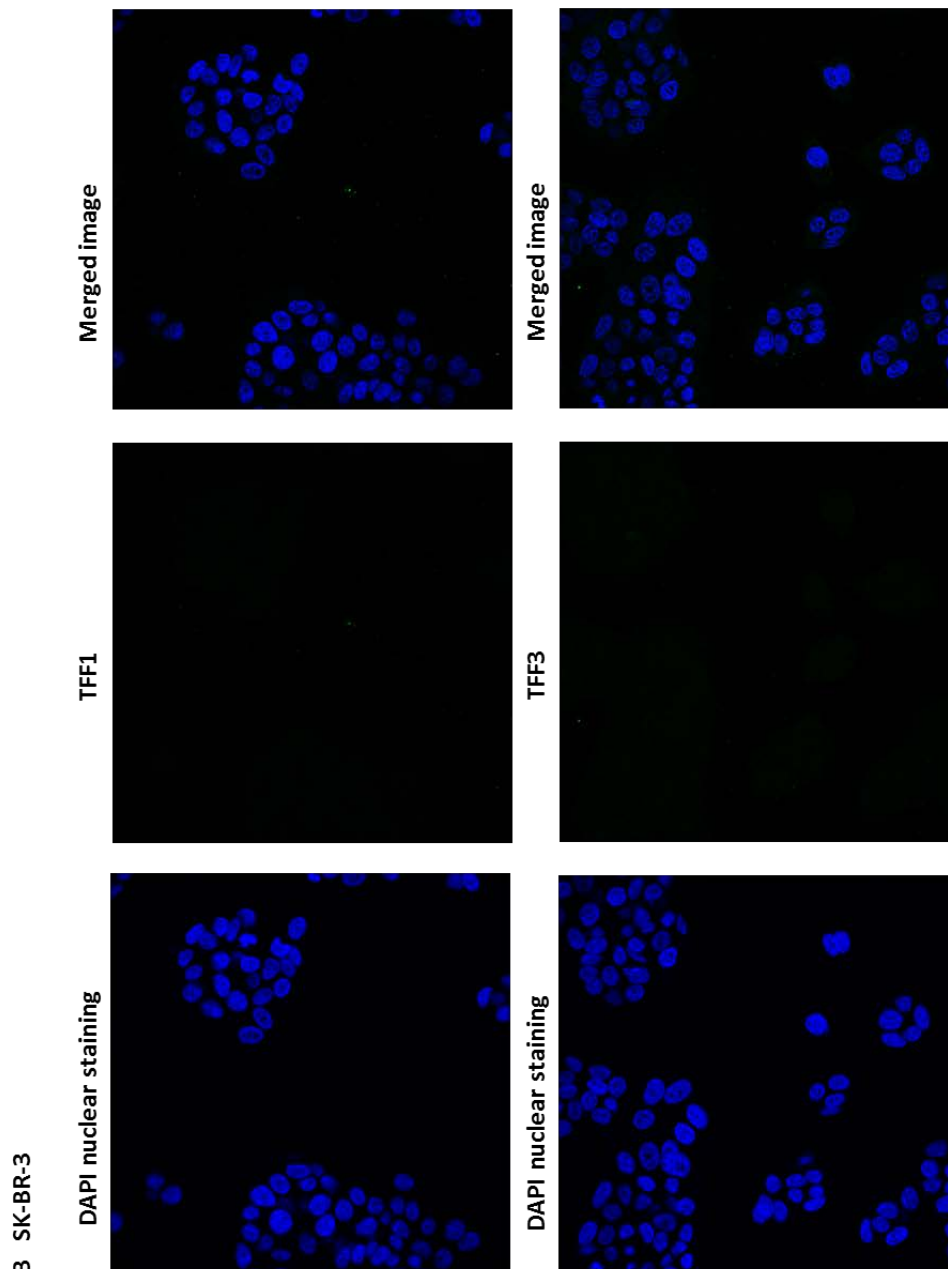


Figure 8.13: Immunofluorescence staining of TFF1 and TFF3 in HER-2 positive breast cancer cells. Expression of TFF1 and TFF3 were tested in two oestrogen receptor-negative but HER-2 positive breast cancer cell lines: HCC-1419 (A) and SK-BR-3 (B) cells. Cells were plated into 6 well plates and incubated for 24 hours. After 24 hours of incubation cells were washed with PBS and fixed with cold methanol at room temperature for 20 minutes. Cells were then blocked and followed by incubation with the primary antibody TFF1 (1 in 10 dilution) and TFF3 (1 in 20 dilution). Results demonstrate the cytoplasmic expression of TFF1 and TFF3 in HCC-1419 cells. Vesicular expression of TFF1 and TFF3 is illustrated in HCC-1419. Neither TFF1 nor TFF3 expression was detected in SK-BR-3 cells.

8.3 Discussion

Both TFF1 and TFF3, particularly TFF1 are oestrogen-responsive genes in breast cancer cells (Masiakowski, Breathnach et al. 1982, May and Westley 1997, May and Westley 1997). In contrast to the reduction in TFF1 expression in gastric tumours, TFF1 expression is not reduced in breast tumours and its expression in breast cancer cells is under the control of oestrogen. The exact role of trefoil proteins in tumourigenesis is controversial. Different biological roles have been assigned to trefoil proteins including tumour suppressive and tumour promoting depending partly upon the tissue. TFF1 and TFF3 can help tumour cell dissemination by stimulating the migration of breast cancer cells (May and Westley 1997). Over expression of TFF3 has been reported to enhance the cellular proliferation, survival and migration and anchorage-independent growth of the oestrogen receptor-positive breast cancer cells (Kannan, Kang et al. 2010). In a recent immunohistochemical study, the involvement of TFF3 in progression and dissemination of oestrogen receptor-positive breast tumours was reported (Ahmed, Griffiths et al. 2012).

Expression of TFF1 was investigated in a series of oestrogen receptor-negative breast tumours. TFF1 expression was detected at very low level in few cases where majority of breast tumours were negative for expression of TFF1, which is consistent with the conclusion that TFF1 is oestrogen-responsive element and its expression is under control of oestrogen (Masiakowski, Breathnach et al. 1982, May and Westley 1997, May and Westley 1997). Hence, further analysis has not considered.

TFF3 expression was detected in 7 *in situ* breast carcinomas with mean histoscore of 106 and 6 invasive breast tumours with mean histoscore of 232. The majority of TFF3 positive tumours were weakly to moderately cytoplasmic immuno-reactive. In the present study, the association between TFF3 expression in primary tumour cells and the related metastatic cells in the lymph nodes was investigated. There was no correlation between expression of TFF3 in primary tumour cells with their corresponding metastatic cells in the lymph nodes ($p=0.134$). The association between TFF3 and proliferation marker, Ki-67 was evaluated. There was no significant association between their expressions in any tested tumour type. TFF3 positive tumours were expressed Ki-67 at low level.

No correlation between expression of TFF3 and expression of EGFR or IGF-IR was found ($p>0.05$). There was a significant correlation between TFF3 expression and expression of HER-2 in invasive breast tumours ($p= 0.02$). This result would agree with the recent finding that TFF3 expression is higher in HER-2 positive breast tumours (Yue, Xiang et al. 2013). There was no significant correlation between TFF3 and HER-2 expression in *in situ* carcinomas ($p=0.466$) or breast tumours with involved axillary lymph nodes ($p=0.662$).

Cellular localization of TFF1 and TFF3 demonstrated by immunofluorescence on different oestrogen receptor-positive and oestrogen receptor-negative breast cancer cells. Cytoplasmic with vesicular expression of TFF1 was detected in oestrogen receptor-positive MCF-7 and BT-474 cells and oestrogen receptor-negative HCC-1419 cells, whereas, TFF3 expression was detected in EFM-19, BT-474 and HCC-1419 cells and also shown to be cytoplasmic and vesicular. TFF1 or TFF3 expression was not detected in HER-2 positive SK-BR-3 cells. Cytoplasmic expression of TFF3 would agree the immunohistochemical findings of this study that TFF3 expression is tend to be cytoplasmic in oestrogen receptor-negative breast tumours.

Expression of TFF1 and TFF3 was evaluated by western transfer analysis in different types of breast cancer cell lines. MCF-7 and HCC-1419 cells showed the similar expression results as observed in immunofluorescence. TFF1 expression was observed in MCF-7 cells but not TFF3, and in HCC-1419 expression of both TFF1 and TFF3 was detected. Expression of TFF1 or TFF3 was not observed in EFM-19 cells by western transfer, whereas, TFF3 expression was detected in EFM-19 by immunofluorescence. One possibility for this discordance might be different sensitivities of TFF3 antibodies that have used for detection of TFF3 expression by western transfer and immunofluorescence.

In 2002, Wilson *et al* investigated the association between TFF3 and HER-2 expression in series of HER-2 positive and negative breast cancer cell lines. TFF3 expression was not found in any of the HER-2 negative breast cancer cells but was found to express at high level in two of three HER-2 positive BT-474 and MDA-MB-361 cells. Expression of TFF3 was not detected in HER-2

positive SK-BR-3 cells. The study has concluded that TFF3 is up regulated in HER-2 positive breast cancer cells. However, one of the limitations in their study is that they did not consider the oestrogen receptor status of the tested cell lines and did not demonstrate an association between TFF3 and HER-2 expression in primary tumour cells (Wilson, Roberts et al. 2002). Conversely, recently Yue *et al* reported the expression of TFF3 in HER-2 positive SK-BR-3 cells and that silencing HER-2 induced downregulation of TFF3. In the same study, the high expression of TFF3 was reported in a cohort of HER-2 positive breast cancer patients (Yue, Xiang et al. 2013). In contrast to Yue *et al* study, but in agreement with Wilson *et al* findings, expression of TFF3 was not detected in SK-BR-3 cells by western transfer analysis or immunofluorescence, in the present study (Wilson, Roberts et al. 2002).

In conclusion, TFF3 but not TFF1 is expressed in oestrogen receptor-negative breast tumours and there is a significant association between TFF3 and HER-2 in oestrogen receptor-negative tumours which suggests that expression of TFF3 is not always under the control of oestrogen and might be regulated by other factors in oestrogen receptor-negative breast tumours.

Chapter 9. General discussion

The purpose of the current study was to investigate the role of insulin-like growth factor 1 and epidermal growth factors in the survival and migration of oestrogen receptor-negative breast cancer cells. An additional aim was to evaluate the expression level of the type I IGF receptor, EGFR, HER-2, TFF1, TFF3 and their association with biomarkers of proliferation and survival in oestrogen receptor-negative primary breast tumours and their associated metastases.

Studies have addressed the contribution of IGFs in the development, survival and invasion of different cancers, including breast cancer. In 2010, Key *et al* has reported that higher level of serum IGF-1 is associated with an increased risk of breast cancer (Key, Appleby *et al.* 2010). In 2011, Davison *et al* demonstrated the protective effect of IGF-1 on staurosporine-induced apoptosis in attached triple-negative breast cancer cell lines (Davison, de Blacquiere *et al.* 2011). Ravid *et al* (2005) reported that IGF-1 prevents reduction in a number of viable MCF-7 cells cultured in non-attached condition through maintaining mitochondrial activity, which was assessed with an MTT assay. However, the study did not investigate directly if the cells had died or not, the mode of cell death or the mechanism by which IGF-1 inhibits the cell death (Ravid, Maor *et al.* 2005). There have been no studies specifically on the protective effect of IGF-1 against anoikis in oestrogen receptor-negative breast cancer cells. The current *in vitro* data show that IGF-1 has a powerful effect on the prevention of anoikis in non-attached condition in triple-negative breast cancer cells. This was demonstrated by the induction of PARP cleavage and activation of casapase-3 as a result of loss of attachment in triple-negative breast cancer cells, which was consistent with the induction of anoikis.

In 2011, Davison *et al* demonstrated that IGF-IR and insulin receptor are expressed at relatively high levels in triple-negative breast cancer cell lines (Davison, de Blacquiere *et al.* 2011). Hence, it was interesting to compare the effect of an inhibitor that targets both IGF-IR and insulin receptor with an antibody that targets only IGF-IR in triple-negative breast cancer cells.

Targeting both IGF-IR and the insulin receptor was reported to be more effective in inhibition of growth in triple-negative breast cancer cells than

targeting type I IGF receptor alone (Law, Habibi et al. 2008). In 2011, Beate *et al* reported the sensitivity of triple-negative breast cancer cell lines to small dual tyrosine kinase inhibitor of type I IGF receptor and the insulin receptor BMS-754807. In the same study, Beate *et al* demonstrated that treatment of triple-negative tumour grafts with the anti-IGF-IR and the insulin receptor inhibits the growth of tumour grafts and when combined with cytotoxic agents result in complete tumour regression (Litzenburger, Creighton et al. 2011). In the current study, survival effects of IGF-1 were inhibited by BMS-754807 in triple-negative breast cancer cells. This was followed by complete inhibition of downstream PI3K and AKT pathway and noticeable reduction in activation of MAPK in these cell lines. This result supports the contention that dual tyrosine kinase inhibitor are effective in inhibition of anti-anoikis effect of IGF-1 in triple-negative breast cancer cells.

Sachdev *et al* (2009) reported that blocking the type I IGF receptor signal transduction pathway increases the anoikis, as measured the trypan blue exclusion in a metastatic variant of MDA-MB-435 breast cancer cells (Sachdev, Zhang et al. 2009). Interference of the IGF-IR activity with inhibitory antibody CP-751,871 reduced significantly the survival effect of IGF-1 in triple-negative breast cancer cells but showed partial inhibition in activation of PI3K and AKT in Hs578T cells. Taken together, these results indicate that dual tyrosine kinase inhibitor is more effective in inhibition of downstream PI3K and AKT pathway in triple-negative breast cancer cells than type I IGF receptor inhibitor. This conclusion is in agreement with previous report that targeting both the type I IGF receptor and the insulin receptor might be more efficient in inhibition of growth in triple-negative breast cancer cells (Law, Habibi et al. 2008).

In the present study, IGF-1 was required to prevent anoikis in triple-negative breast cancer cells and loss of IGF-1 survival signals could not compensated with other serum components in the culture medium. This result indicates that IGF-1 plays significant role in preventing anoikis in triple-negative breast cancer cells.

Studies have reported the role of IGF-1 as a potent inducer of migration in different types of human cancer cells. In 2010, Metalli *et al* demonstrated the migratory effect of IGF-1 in head and neck cancer cells in which the IGF-1-

induced motility effect could be inhibited through using anti-IGF-IR antibody (Metalli, Lovat et al. 2010). Similarly, it was reported that in pancreatic cancer, IGF-1 stimulated the migration of seven different pancreatic cancer cells (Tomizawa, Shinozaki et al. 2010). In the current *in vitro* study using monolayer wound-healing migration assay, IGF-1 induced migration of oestrogen receptor-negative breast cancer cells, which is in agreement with the published reports (Bartucci, Morelli et al. 2001, de Blaqui re, May et al. 2009). The migratory effect of IGF-1 was found to be higher in two triple-negative breast cancer cells compared to HER-2 positive breast cancer cells.

Sachdev *et al* (2009) reported that disruption of type I IGF receptor results in inhibition of invasion of lung cancer cells. Other study reported the growth inhibitory effect of dual tyrosine kinase inhibitor on triple-negative breast tumourgraft (Litzenburger, Creighton et al. 2011). Migratory effect of IGF-1 was significantly inhibited by treatment of the cells with anti-IGF-IR inhibitor, which is in agreement with the previous findings (Doerr and Jones 1996, Sachdev, Zhang et al. 2009), but greater inhibitory effect was observed in all oestrogen receptor-negative breast cancer cells by treating them with the dual tyrosine kinase inhibitor of IGF-IR and the insulin receptor.

In this study, the expression level of IGF-IR in series of oestrogen receptor-negative primary breast tumours and their associated metastases was investigated; also association between IGF-IR expression with biomarkers of proliferation and survival in breast tumours was evaluated. IGF-IR expression was detected at high percentage of *in situ* and invasive breast carcinomas. Expression of IGF-IR was detected also in normal breast tissues. There was no association between expression of IGF-IR and tumour size or grade. This finding is in agreement with previous studies which showed that expression of IGF-IR is not associated with tumour size or grade in oestrogen receptor-positive and negative breast tumours (Koda, Sulkowski et al. 2003).

In the current study, data demonstrated that there was a correlation between IGF-IR expression in primary tumours with the corresponding metastatic tumour cells of the same patients. The expression of IGF-IR was found to be higher in primary tumours than the corresponding metastatic tumours, which is consistent with the previous findings that expression of IGF-IR was more frequent in

primary breast tumours than lymph node metastasis (Koda, Sulkowski et al. 2003). Further evaluation could investigate the expression of IGF-IR in a greater number of oestrogen receptor-negative primary tumours and their associated tumour cells metastasized to the lymph nodes.

Data from this study indicates that IGF-IR expression is important in proliferation of invasive breast tumour cells as an association with Ki-67 proliferation marker was observed. These data are in agreement with a previous *in vitro* study that IGF-IR mediates the proliferation of triple-negative breast cancer cells (Davison, de Blacquiere et al. 2011).

The expression of IGF-IR was associated with biomarker of survival in oestrogen receptor-negative tumours. In the majority of the IGF-IR positive tumour cells, cell death was not observed as no cleaved caspase-3 was detected. This result is consistent with the previous *in vitro* finding of this study regarding mediation of cell survival through IGF-IR. Although another study reported that activated IGF-IR and insulin receptor has a higher correlation with survival of breast tumours (Law, Habibi et al. 2008). Further analysis could attempt to evaluate the expression of phosphorylated IGF-IR and insulin receptor in primary and their associated metastases tumour cells and compare the expression of both total and activated IGF-IR in these tumour cells and their relationship with survival outcome.

In the present study, the effects of EGF and heregulin-1 were examined on survival of oestrogen receptor-negative MDA-MB-231 and Hs578T breast cancer cells. EGF but not heregulin-1 demonstrated a powerful protective effect against anoikis in Hs578T cells. Although neither EGF nor heregulin-1 showed anti-anoikis effect in one of the triple-negative MDA-MB-231 breast cancer cells, which may be because EGF does not induce autophosphorylation of EGFR and activation of the PI3K and AKT pathway in this cell line as demonstrated in the result chapter 5.

The anti-anoikis effect of EGF is mediated through the EGFR based on the results discussed in chapter 5 that showed the same effect of dual tyrosine kinase inhibitor of EGFR and HER-2, lapatinib and an inhibitor of only EGFR, gefitinib on survival of HER-2 positive breast cancer cells. These observations confirm that the survival effect of EGF is transmitted through EGFR. Inhibition of

either EGFR or both EGFR and HER-2 hetero-dimers effectively reduced the survival effect of EGF which was followed by complete inhibition of downstream effectors PI3K/Akt and MAPK pathways in HER-2 positive cells.

EGF showed a powerful migratory effect on oestrogen receptor-negative breast cancer cells, which is in agreement with previous published reports that EGF induces migration in breast cancer cells (Price, Tiganis et al. 1999, Garcia, Franklin et al. 2006). It has been reported that heregulin-1 induces migration and invasion in HER-2 positive breast cancer cells by induction of epithelial-mesenchymal transition through activation of PI3K and AKT pathway (Cheng, Zha et al. 2009). In the current study, stimulation with heregulin-1 resulted in a significant increase in migration of SK-BR-3 cells, possibly due to formation of HER-2 and HER-3 heterodimers. This result is consistent with the previously mentioned findings that heregulin-1 induced migration and invasion of SK-BR-3 cells (Cheng, Zha et al. 2009). Heregulin-1 did not have a significant effect on the motility of triple-negative breast cancer cells, even though one of them, MDA-MB-231 cells expresses detectable levels of HER-3. One of the possibilities might be due to inability of heregulin-1 in activation of MAPK pathway as discussed in result chapter 5.

In vivo mouse mammary cancer model studies have been reported significant role of EGFR signalling inhibitors in reduction of bone and brain metastasis (Gril, Palmieri et al. 2008, Molli, Adam et al. 2008). In the current study, treatment of triple-negative breast cancer cells with anti-EGFR inhibitor resulted in inhibition of the migratory effect of EGF in these cell lines. Treatment of HER-2 positive breast cancer cells with either dual tyrosine kinase inhibitor or EGFR inhibitor showed similar inhibitory effects on migration of these cells.

A very recent study reported the involvement of new mechanism in lapatinib resistant HER-2 positive breast cancer cells, which is heregulin-mediated EGFR-HER-3 signalling axis and that HER-2 positive breast cancer cells are no longer depend on HER-2-HER-3 signalling axis (Xia, Petricoin et al. 2013). This group claimed that the reason for HER-2 positive breast cells to become resistant to lapatinib is due to incomplete inhibition of EGFR by lapatinib which leads to transactivation of HER-3 with EGFR, in which lapatinib is not able to inhibit, as HER-3 which is a cognate receptor of heregulin is up regulated in

lapatinib resistant HER-2 positive breast cancer cell models. Further experiments could attempt to investigate the effect of lapatinib in the presence of heregulin-1 on survival and migration of HER-2 positive SK-BR-3 cells, to evaluate the inhibitory effect of this inhibitor on the effect of heregulin-1.

EGFR expression was evaluated in a series of oestrogen receptor-negative breast primary tumours and their associated tumour cells with involved lymph nodes. EGFR expression was detected in normal breast tissues and in a high percentage in *in situ* and invasive breast carcinomas. Wiseman *et al* (2005) reported the association between EGFR with tumour grade in breast tumours but did not find a significant association between EGFR and size of the tumour (Wiseman, Makretsov *et al.* 2005). In the present study, no association was found between expression of EGFR and tumour size, which is in agreement with the previous study. Unlike the mentioned study from the other group, no association between EGFR expression and tumour grade or age of the patients was detected.

In 2005, Wiseman *et al* reported the expression of EGFR regardless of patient's lymph node status (Wiseman, Makretsov *et al.* 2005). Similarly, in the current study, no significant association was found between expression of EGFR and number of lymph node metastasis. There was a significant correlation between expression of EGFR in primary tumours with the corresponding metastatic tumour cells of the same patients. However, primary tumour cells expressed EGFR at higher levels than corresponding metastatic tumour cells of the same patients.

In 2010, Riwambi *et al* reported the high proliferative rate of EGFR expressing tumours compared to EGFR-negative tumours in a large cohort of oestrogen receptor-positive and negative malignant breast tumour cells (Rimawi, Shetty *et al.* 2010). The significant association between EGFR expression and expression of Ki-67 in invasive breast carcinomas that has been shown in this study could be explained by the ability of EGFR in promoting the proliferation of invasive oestrogen receptor-negative breast tumour cells. This finding agrees with the previous mentioned findings from other groups (Rimawi, Shetty *et al.* 2010). According to the discussed findings in the *in vitro* part of the current study, EGFR mediates survival of oestrogen receptor-negative breast cancer

cells. Immunohistochemical data showed that high expression of EGFR is not associated with expression of apoptotic protein cleaved caspase-3. In the majority of EGFR positive tumours, cleaved caspase-3 was not detected. This result is in agreement with the previous finding that EGFR may be responsible for mediation of survival in oestrogen receptor-negative breast tumours.

In one study a significant effect of both IGF-1 and EGF on migration of MDA-MB-231 breast cancer cells was compared and reported the stronger migratory effect of IGF-1 compared to the migratory effect of EGF (Mezi, Todi et al. 2012). Results of the current study regarding the effect of each IGF-1 and EGF ligand on migration of MDA-MB-231 cell line which has already been discussed separately are in agreement with the findings of previous group, who observed that IGF-1 had more migratory effect than EGF in MDA-MB-231 cells but this group did not test the effect of combination of IGF-1 and EGF on migration of MDA-MB-231 cells. No previous study tested the effect of the combination of IGF-1 and EGF on the migration of oestrogen receptor-negative breast cancer cells. Although IGF-1 and EGF each alone stimulated significantly the migration of oestrogen receptor-negative breast cancer cells, the combination of both ligands stimulated higher migration of breast cancer cells than either mitogen alone. Additionally, in one of the triple-negative breast cancer cells, Hs578T cells, the migratory effect of the combination of IGF-1 and EGF was inhibited more significantly using combination of both anti-IGF-IR and anti-EGFR inhibitors compared to inhibitory effect of either inhibitor alone.

Further experiment could investigate the effect of IGF-1 and EGF in combination on survival of oestrogen receptor-negative breast cancer cells and evaluate the combined effect of anti-IGF-IR and anti-EGFR inhibitors on anoikis to further support the importance of interaction between IGF-IR and EGFR in cell survival.

A significant association was found between expression of IGF-IR and EGFR in invasive breast tumours. There was no significant association between expression of IGF-IR and HER-2 in invasive HER-2 positive breast tumours. However, there was a significant inverse correlation between EGFR and HER-2 expression in both invasive and metastatic HER-2 positive breast tumours.

Expression of TFF1 and TFF3 has been reported in breast tumour cells and their expression is identified to be regulated by oestrogen (May and Westley 1997, Ahmed, Griffiths et al. 2012). Expression of both TFF1 and TFF3 was investigated in oestrogen receptor-positive and negative breast cancer cell lines and in series of oestrogen receptor-negative breast tumours.

TFF1 and TFF3 expression were evaluated at protein level by western transfer analysis. TFF3 expression was observed in two oestrogen receptor-negative cells which are positive for HER-2. Expression of TFF3 was not detected in HER-2 positive SK-BR-3 cells, which is agreed with the previous finding that TFF3 mRNA was not detected in SK-BR-3 cells (Wilson, Roberts et al. 2002).

In 2012, Ahmed *et al* reported the expression of TFF3 in normal oestrogen receptor-positive breast epithelial cells (Ahmed, Griffiths et al. 2012). In the current study expression of TFF1 and TFF3 was not detected in normal breast tissues. TFF1 expression was detected at low levels in a few breast tumours but the majority of oestrogen receptor-negative tumours were negative for expression of TFF1, which agrees with the previous published reports that TFF1 is an oestrogen-inducible gene and is expressed in oestrogen receptor-positive breast tumours (May and Westley 1997). Therefore, in this study investigation of TFF1 was not considered further.

Interestingly, TFF3 expression was observed in weak to moderate level in *in situ* breast carcinoma and in moderate to strong level in invasive breast tumours. TFF3 expression was not correlated with proliferation of oestrogen receptor-negative breast tumours, as no association was found between TFF3 expression and expression of proliferation marker Ki-67. TFF3 positive tumours expressed Ki-67 at low level, which agrees with the previous observation that TFF3 positive tumours express low mitotic index and act against cell division in malignant epithelial breast tumour (Ahmed, Griffiths et al. 2012). Expression of TFF3 was reported to be higher in HER-2 positive breast tumours than other tumour types (Yue, Xiang et al. 2013). Data from the current study demonstrated the significant association between expression of TFF3 and expression of HER-2 in invasive breast tumours, which is agreed with a recent report that TFF3 expression is high in HER-2 positive breast tumours (Yue, Xiang et al. 2013). There was no association of TFF3 expression with

expression of EGFR or IGF-IR in invasive breast tumours. Followed these investigations, cellular localization of TFF1 and TFF3 investigated in oestrogen receptor-positive and negative breast cancer cell lines by immunofluorescence to confirm the cytoplasmic expression of these proteins as it was observed by immunohistochemistry.

In conclusion, both IGF-1 and EGF have a powerful survival and migratory effect in oestrogen receptor-negative breast cancer cells and their receptors show significant association with biomarkers of survival and proliferation in oestrogen receptor-negative breast tumours. Combination of both IGF-1 and EGF mitogens stimulate migration of oestrogen receptor-negative breast cancer cells, which is more effective than the stimulation achieved by either mitogen alone. Thus the inhibition of both IGF-IR and EGFR signal transduction pathway would be predictive to increase the effectiveness of therapies in oestrogen receptor-negative particularly triple-negative breast cancers.

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