THE ROLE OF HER4 IN RELATION TO TRASTUZUMAB RESISTANCE AND PROGNOSIS IN HER2 POSITIVE BREAST CANCER

SITI NORASIKIN BINTI MOHD NAFI

WOLFSON COLLEGE

MOLECULAR ONCOLOGY DEPARTMENT
WEATHERALL INSTITUTE OF MOLECULAR MEDICINE
UNIVERSITY OF OXFORD



SUPERVISOR: DR ANTHONY KONG
CO-SUPERVISOR: DR ROXANIS IOANNIS

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ABSTRACT

Background

Trastuzumab resistance imposes a major limitation to the successful treatment of HER2 positive breast cancer. The expression of HER4 and its prognostic value is controversial in breast cancer. Furthermore, its role in trastuzumab treatment and resistance in HER2 positive breast cancer has not been reported.

Methods

The effects of trastuzumab on HER4 cleavage and its localisation were studied in both parental and trastuzumab-resistant SKBR3 and BT474 cells using western blot, RT-PCR, nuclear fractionation and confocal microscopy. Tissue microarrays consisting of a cohort of HER2 positive breast cancer patients were stained for HER4 by immunohistochemistry and the results were correlated with patients' outcome. This study also assessed HER4 expression in the tumor samples from a window study of trastuzumab alone or in combination with neoadjuvant chemotherapy in HER2 positive breast cancer patients.

Results

Trastuzumab treatment upregulated HER4 mRNA, and increased expression of both 80 and 180 kDa HER4 protein isoforms, and induced nuclear translocation of 80kDa HER4 protein isoforms, which the results similar to heregulin stimulation. This was also seen in trastuzumab resistant cells although $HER4_{80kDa}$ and nuclear HER4 decreased upon overnight withdrawal of trastuzumab in resistant cell lines. In addition, knockdown of HER4 protein expression by specific siRNAs increased trastuzumab sensitivity and reversed trastuzumab resistance in SKBR3 and BT474 cells, confirming the importance of HER4 in trastuzumab response. This study also showed that trastuzumab-induced HER4 nuclear translocation is due to HER4 activation and cleavage since γ-secretase inhibitor (GSi) and neratinib prevented the process when combined with trastuzumab treatment, correlating with an increased apoptosis and decreased cell viability. There was also increased nuclear HER4 expression in tumors from both BT474 xenografts and from patients with breast cancer treated with trastuzumab monotherapy. Furthermore, nuclear HER4 predicted poor clinical response to trastuzumab monotherapy in patients

undergoing a window study and was a poor prognostic factor in HER2 positive breast cancer.

Conclusions

This study suggests HER4 activation, cleavage and nuclear translocation play a key role in trastuzumab resistance in HER2 positive breast cancer. Nuclear HER4 could be a novel predictive and prognostic biomarker in HER2 positive breast cancer patients.

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ABBREVIATIONS

ADAM	A disintegrin and metalloprotenaise
ADCC	antibody dependent cellular cytotoxicity
AR	Amphiregulin
BAD	Bcl-2 related protein
Bp	Base pair
BSA	Bovine serum albumin
BTC	Betacellulin
cDNA	complementary deoxyribonucleic acid (DNA)
Cm	Centimeter
CO_2	Carbon dioxide
CT value	threshold cycle value
Chk	Checkpoint kinase
Crk	CT10 regulator of kinase
DAB	3,3'-Diaminobenzidine
DAPI	4' 6-diamidino-2-phenylindole
DCIS	Ductal carcinoma *in situ*
DEPC	Diethylpyrocarbonate
DFS	Disease-free survival
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
ECL	Enhanced chemiluminesence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EPR	Epiregulin
Erk	Extracellular signal-regulated kinases
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum

FISH	Fluorescence *in situ* hybridization
FITC	Fluorescein isothiocyanate
Fw	forward
Gab1	Grb2-associated adaptor protein 1
GSi	γ -secretase inhibitor
Grb2	Growth factor receptor-bound protein 2
HER1	Human EGF receptor-1 (ERBB1/EGFR)
HER2	Human EGF receptor-2 (ERBB2/neu)
HER3	Human EGF receptor-3 (ERBB3)
HER4	Human EGF receptor-4 (ERBB4)
HERA trial	Herceptin adjuvant trial
HRG	Heregulin
IHC	immunohistochemistry
IRS	immunoreactive score
IP	immunopreciptation
Kg	Kilograms
L	Litre
MAPK	Mitogen-activated protein (MAP) kinases
Mg	Milligram
Min	Minute
Mmol	Milimole
mRNA	messenger ribonucleic acid (RNA)
Mw	molecular weight
NK	Natural killer
Ng	Nanogram
Opti-MEM	Opti-Minimal Essential Medium
OS	Overall-free survival
PI3K	Phosphatidyllinositol 3-kinase
PBS	Phosphate buffered saline
PBST	PBS Tween
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PI	Propidium Iodide

PKB	Protein Kinase B
PKC	Protein Kinase C
PLC	Phospholipase C
PS	Penicillin-Streptomycin
PVDF	Polyvinylidene Fluoride
QRT-PCR	Quantitative Real Time PCR
Ras	A G protein (a small GtPase)
Rb gene	Retinoblastoma gene
RTK	Receptor tyrosine kinase
rpm	revolutions per minute
RPMI medium	Roswell Park Memorial Institute medium
Rv	reverse
SDS-PAGE	SDS polyacrylamide gel electrophoresis
STAT	Signal transducer and activator of transcription
siRNA	small interfering RNA
Src	Oncogene of the chicken Rous sarcoma
SH2	Src homology 2
SH3	Src homology 3
SHP	Src homology 2 domain-bearing protein tyrosine phosphatase
SEM	Standard Error Mean
TACE	Tumor necrosis factor-α converting enzyme
TBE	Tris Boric EDTA
TE	Tris-EDTA
TKI	Tyrosine kinase inhibitors
TMA	tumor microarray
Δ	Delta
ºC	Celcius

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CHAPTER ONE

Introduction

CHAPTER 1: INTRODUCTION

1.1 HER protein kinase family and their ligands

The HER (EGFR/ErbB) family includes four receptors namely HER1 (ERBB1/EGFR), HER2 (ErbB2/neu), HER3 (ErbB3) and HER4 (ErbB4) (Baselga and Swain, 2009; Linggi and Carpenter, 2006a). The receptors have been identified as crucial regulators involved in cell proliferation, survival, migration and differentiation (Yarden and Sliwkowski, 2001; Zhang et al., 2009). The activation of the HER receptors is a ligand dependent and involves various ligands, which is discussed in Section 1.1.2 (Lemmon, 2009). Figure 1.1 depicts the formation of several homo- or heterodimeric receptor combinations after activation upon ligand-binding (Cooper et al., 2011). Although HER family members share a similarity in the way they are activated, there are still some differences that distinguish them. The four members of the HER family will now be discussed in more detail below.

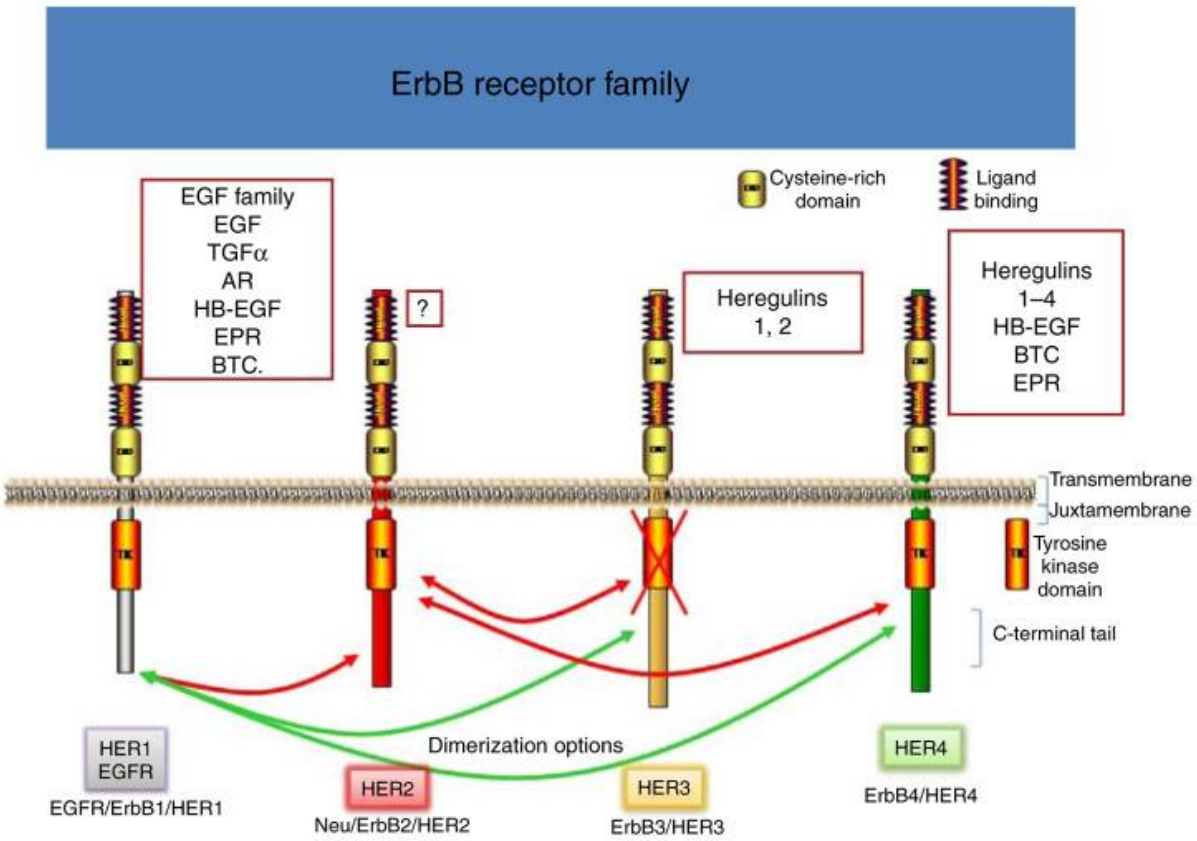


Figure 1.1 Schematic diagram of HER (ErbB) receptor family. Figure taken from (Cooper et al., 2011).

1.1.1 HER protein kinases

1.1.1.1 EGFR

The first HER family receptor identified was the HER1, which is primarily known as EGFR or ErbB1 (Citri and Yarden, 2006; Masuda et al., 2012). The interaction of HER1 with other HER receptors occurs after it changes from the closed conformation to the active form after a ligand binds to its extracellular domain, leading to its dimerisation with one of the other HER receptor partners (Barros et al., 2010; Garrett et al., 2002). Several EGFR tyrosine residues that are phosphorylated upon ligand binding have been identified (Citri and Yarden, 2006; Olayioye et al., 2000). As stated in Section 1.1.3 (Figure 1.2), some of the HER1 phosphorylation sites are found to bind the adaptor proteins Grb2 and SHC, which allow the activation of protein effectors i.e. RAS and MAPK (Fan et al., 2005). These effectors are known to induce increased survival, proliferation, motility and invasiveness of the tumor cells. However, other HER1 phosphorylation sites could activate downstream proteins that negatively regulate the receptor. For example, HER1 phosphorylated tyrosine Y974 triggers receptor endocytosis and Y1045 triggers Cbl-dependent HER1 ubiquitination and proteosomal degradation (de Melker et al., 2004). It was also noted that the binding of SHP protein is responsible in dephosphorylating HER1 (Tran et al., 2003).

1.1.1.2 HER2

Among the HER family members, HER2 does not require any ligand binding for its activation due to the closed position of the ligand-binding site that is caused by a strong interaction between domain III and I (Citri and Yarden, 2006; Franklin et al., 2004; Garrett et al., 2003). The interaction between domain I and III also causes domain II and IV to be always exposed. The constant exposure of domain II allows association of

HER2 with other receptors, explaining the fact that HER2 is the preferred dimerisation partner. HER2 over expression has been reported to activate several downstream signalling pathways such as RAS-MAPK, PI3K/Akt and VEGF to induce proliferation activity, block apoptosis and to promote angiogenic activity respectively (Delord et al., 2005; Hermanto et al., 2001; Hoar et al., 2003; Yang et al., 2002; Zheng et al., 2004). The PI3K/Akt activity that is induced by HER2 over expression has been extensively studied. It promotes tumor aggressiveness due to an increase of cell cycle regulator cyclin D1 that leads to down regulation of $p27^{Kip}$ activity and thus increased cell cycle progression and tumor cell survival (Le et al., 2003; Neve et al., 2000; Serra et al., 2011). In addition, the activation of the PI3K/Akt pathway due to HER2 over expression also prevents cell death by blocking activation of apoptotic proteins (Zhou et al., 2001).

1.1.1.3 HER3

In contrast to the other receptors, HER3 carries mutations on four amino acids in its kinase domain, which causes this receptor to have inactivated tyrosine kinase (Koutras et al., 2010; Linggi and Carpenter, 2006a). Therefore, HER3 requires interaction with another HER partner in order to be activated and to initiate downstream pathways. It has been observed that the intracellular region of HER3 retains PI3K-linked domains, namely SH2 domains, which are the major factors to promote heterodimerisation with other HER family members (Prigent and Gullick, 1994). HER2 is commonlye co-expressed with HER3 and mediates PI3K signals in tumor progression (Alimandi et al., 1995; Stern, 2008). Over expression of HER3 has been associated with poor prognosis in different types of cancer such as breast, lung and ovary (Tanner et al., 2006; Way and Lin, 2005). Approximately 17.5% of all breast cancer patients overexpress HER3 which is strongly associated with poor prognosis

(Witton et al., 2003).

1.1.1.4 HER4

HER4 is the fourth member of HER family receptors, and the binding of its ligand to the extracellular domain of this receptor causes dimerisation with other HER receptors as well as tyrosine kinase activation in the carboxyl-terminal end of intracellular domain (Koutras et al., 2010). The alternative splicing of HER4 mRNA produces four different isoforms of this receptor, which namely as JM-a, JM-b, CYT-a and CYT-b (Hollmen and Elenius, 2010). The characteristics of each HER4 isoforms will be further discussed in Section 1.3.1.4. The JM-a region consists of Adam-17 (TACE) cleavage site that allows the first step of HER4 proteolytic cleavage (Elenius et al., 1997a; Muraoka-Cook et al., 2008). As a result, two different fragments are produced, the extracellular domain (ECD) and the intracellular domain (ICD/m80) (Rio et al., 2000). The ECD is released to the media while the m80 is subsequently cleaved at the transmembrane domain by γ (gamma)-secretase (Ni et al., 2001; Williams et al., 2004). The m80 is released to the cytosol, and where it is present as a soluble protein of 80kDa (s80). Later, the s80 is translocated into the nucleus, where it functions as a co-activator or co-repressor due to interaction with several transcription factors (Linggi and Carpenter, 2006b; Sundvall et al., 2008a), which will be discussed in Section 1.3.1.5.

1.1.2 HER ligands

A list of epidermal growth factor (EGF) family of peptide growth factors have been identified, which include EGF, transforming growth factor-alpha (TGFα), amphiregulin (AR), betacellulin (BTC), heparin-binding EGF-like growth factor (HB-EGF), epiregulin (EPR), epigen (EPG), and the neuregulins (NRGs) or heregulins (Figure 1.1 & Table 1.1). These growth factors initiate biological response when binding the HER family receptors. Ligands such as EGF, TGF-α and AR have been shown to solely activate HER1 (Barros et al., 2010; Yotsumoto et al., 2009). However, there are also ligands that co-activate HER1 and HER4 such as BTC, HB-EGF and EPR (Elenius et al., 1997b; Riese and Stern, 1998). Both HER3 and HER4 can be activated by neuregulin-1 & -2 (Carraway et al., 1997). However, neuregulin -3 and -4 are ligands that exclusively bind to HER4 (Carpenter, 2003; Sundvall et al., 2008a).

Most of the HER receptor ligands exist as a single pass integral membrane proteins (Gullick, 2001). The precursors of the EGF ligands consist of three parts: an extracellular part, a transmembrane part and an intracellular part. The ectodomain shedding is mediated by ADAM (a disintegrin and metalloproteases) family members, resulting in the release of the extracellular part of the ligand precursors to initiate the ligand's signal (Blobel, 2005; Lee et al., 2003; Sunnarborg et al., 2002). As summarized in Table 1.1, the two most common proteolytic enzymes (sheddase) are Adam-10 and Adam-17, which are involved in the processing of most of the EGF ligand family members (Roskoski, 2014; Sahin et al., 2004). In addition, both Adam-10 and Adam-17 also process other growth factor receptors, cytokines, cytokine receptors, and cell adhesion molecules (Janes et al., 2005; Liu et al., 2006; Maretzky et al., 2005; Reiss et al.,

2005). It has been reported that ADAMs are involved in several processes including adipogenesis, fertilization, myogenesis, and neurogenesis (Rocks et al., 2008).

Table 1.1 The characteristic of HER receptor ligands and their proteolytic sheddases. Table taken from (Roskoski, 2014).

Ligand	Uniprot KB ID	Amino acids in precursor	Final size	Sheddase[a]
Amphiregulin	P15514	252	84	ADAM17
Betacellulin	P35070	178	80	ADAM10
EGF	P01133	1207	53	ADAM10
Epigen	Q6UW88	154	61	ADAM17
Epiregulin	O14944	169	49	ADAM17
HB-EGF	Q99075	208	86	ADAM17
TGFα	P01135	160	84	ADAM17
Nrg-1	Q02297	640	222	ADAM17
Nrg-2	O14511	850	293	?
Nrg-3	P56975	720	359	?
Nrg-4	Q8WWG1	115	61	?

Data is a summary from (Scheller et al., 2011) and (Blobel, 2005).

1.1.3 HER signaling pathways

As stated above, ligand binding and receptor dimerisation promotes the activation and tyrosine phoshorylation in the C-terminus of HER receptors. Each HER receptor exhibits a different pattern of C-terminal autophosphorylation sites (Figure 1.3) (Wilson et al., 2009). These sites interact with several adaptor proteins such as Shc, Crk, Grb2, Grb7 and Gab1; kinases such as Src, Chk and phosphatidylinositol 3-kinase (PI3K; via the p85 regulatory subunit); and the protein tyrosine phosphatases SHP1 and SHP2. These interactions mediate several HER signalling pathways and three major pathways are generally discussed; the phosphatidylinositol 3-kinase (PI3K)/Akt (PKB) pathway, the Ras/Raf/MEK/ERK1/2 pathway and the phospholipase C (PLCγ) pathway (Yarden and Pines, 2012).

1.1.3.1 The phosphatidylinositol 3-kinase (PI3K)/Akt (PKB) pathway

This PI3K/Akt pathway is involved in mediating cell survival and HER3 is the main receptor that activates this pathway (Dey-Guha et al., 2011). Figure 1.3 illustrates that, as compared to other HER receptors, tyrosine phosphorylation residues of HER3 have 6 binding sites for subregulatory PI3K subunit (p85) (Wilson et al., 2009). The PI3K is essential for phosphatidylinositol 4,5-bisphosphate (PIP_2) catalysis to form phosphatidylinositol 3,4,5-trisphosphate (PIP_3) which binds to the pleckstrin homology domain of Akt or protein kinase B (PKB) (Vanhaesebroeck et al., 2012). Since PIP_3 is confined on the plasma membrane, this directly translocates Akt into similar cellular compartment (Engelman, 2009). The activation of Akt is responsible for mTOR activation that has an important role in many cellular processes including that of cell survival (Engelman, 2009). Akt also has direct effects on apoptosis activity, by interacting with Bcl-2 related protein (BAD) (Downward, 2004). The Akt pathway is

inhibited by the activation of tumor suppressor, PTEN (Ramaswamy et al., 1999).

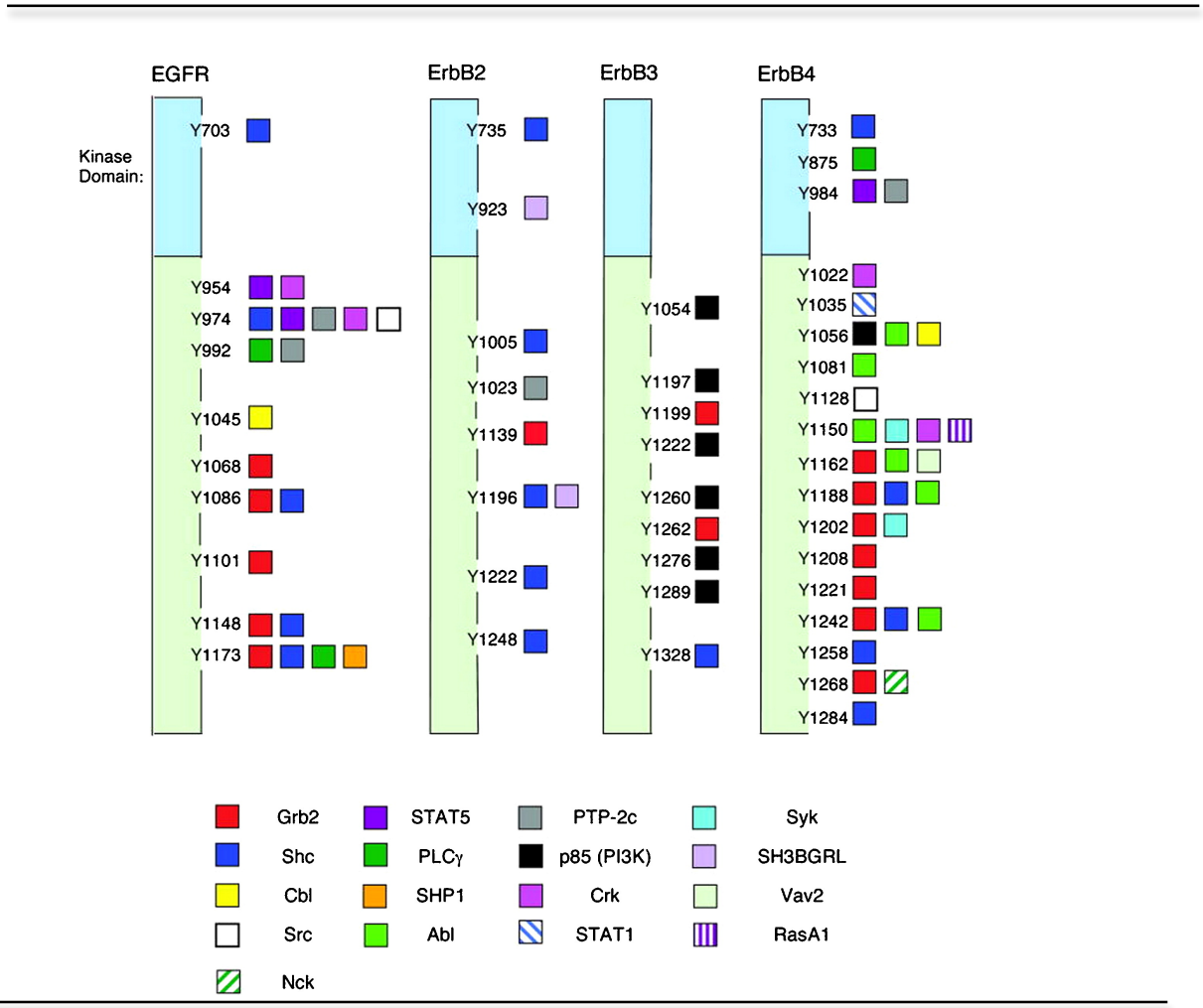


Figure 1.2 Signalling by the ErbB/HER protein tyrosine kinases.

Schematic diagram indicates various tyrosine residues of HER1, HER2, HER3 and HER4, which link to numerous signalling effectors. Schematic diagram taken from (Wilson et al., 2009).

1.1.3.2 The Ras/Raf/MEK/ERK1/2 pathway

This pathway participates in cell proliferation and is regulated by complex receptor protein-tyrosine kinase interactions. The activation of the Ras/Raf/MEK/ERK1/2 signalling is due to the binding of Grb2 or Shc to phosphotyrosines in the HER family (Roskoski, 2011, 2012). Several other protein adaptors those are responsible for the activation Ras/MAP kinase pathway including Nck 1 and Vav2 (Figure 1.3). These proteins have been characterized to contain the SH2 and SH3 domains that bind numerous protein regulators within diverse multiprotein signalling channels. The Crk splicing isoforms have different combinations of SH2 and SH3 domains; CrkI (SH2–SH3) and CrkII (SH2–SH3–SH3) and both isoforms regulate cell growth and motility (Sriram and Birge, 2010). The signal transducers and activators of transcription, namely STAT1 and STAT5 contain a single SH2 domain and play a role in tumor angiogenesis and cell survival (Li et al., 2003; Xia et al., 2002a). HER1 and HER4 have been reported to link with Src, a non-receptor protein-tyrosine kinase that is implicated in cell proliferation and survival (Roskoski, 2004; Wilson et al., 2009). The Abl1/Abl2 proto-oncogene contains SH2-SH3 domain responsible to suppress kinase activation (Panjarian et al., 2013). As shown in Figure 1.3, the Syk, a non-receptor protein links to HER4 receptor. However, the role of this adaptor receptor remains controversial (Stewart and Pietenpol, 2001).

1.1.3.3 The phospholipase C (PLCγ) pathway

It is seen that the tyrosine phosphorylation residues of HER1, HER2 and HER4 have several PLCγ phosphotyrosine binding sites (Wilson et al., 2009). The activation of PLCγ induces the release of Ca^{2+} from the endoplasmic reticulum and activates the protein-serine/threonine kinase C (PKC) (Martiny-Baron and Fabbro, 2007). PKC

expression affects various physiological responses that include angiogenesis, cell proliferation, cell death, cell migration, and cell adhesion. One of the downstream effectors of PKC is the Raf/MEK/ERK1/2 pathway, which induces cell proliferation (Roskoski, 2012). In addition, two SHP proteins, SHP 1 and SHP2 are negative regulators of signalling pathways that control proliferation, migration, and angiogenesis (Lopez-Ruiz et al., 2011). As shown in Figure 1.3, SHP1 binds to HER1 and deactivates HER1 phosphorylation.

1.2 HER family in breast cancer

1.2.1 Breast cancer: general overview

At present, breast cancer is the most common cancer among women. The United States is estimated to have approximately 232,340 of new breast cancer cases with 39,620 deaths among women in 2013 (www.cancer.org). Data from Cancer Research UK (2013) shows an estimate of 49,936 new cases were diagnosed and 11,684 breast cancer deaths were reported among women in the UK in 2011 (www.cruk.org/cancerstats). It is evident that there has been an improvement on the survival rate of breast cancer patients in the recent years. Overall, the numbers of death from breast cancer among women in the UK have been declining since the early 1970s (www.cruk.org/cancerstats). Many efforts contribute to this including improvements in the early detection of breast cancer as well as treatment strategies directed towards this malignancy.

Understanding the breast cancer molecular subtypes plays an important role in predicting breast cancer prognosis and in determining the treatment. Based on a hierarchical clustering analysis, Sorlie et al. categorised breast cancer gene expression into five major groups; HER2 (ERBB2) amplicon group, novel unknown gene group, basal epithelial cell-enriched cluster, normal breast-like cluster and luminal epithelial/ER+ve gene cluster (Figure 1.4) (Sorlie et al., 2001). This is useful for the identification of breast tumor subtypes, which are defined as follows:

1.2.1.1 Luminal A and luminal B.

Approximately 40% of breast cancer cases are diagnosed as luminal A (Blows et al., 2010; Perou and Borresen-Dale, 2010). This tumor subtype is ER+ and/or PR+ and

HER2- and is normally described as less aggressive than the other breast tumor subtypes. Some of the luminal B subtype has been reported to have over-expression of HER2 expression and high proliferation activity (Cheang et al., 2009). Luminal B accounts for approximately 10-20% of breast cancer cases (Perou and Borresen-Dale, 2010). These hormone-receptor positive breast cancers have improved survival with various hormone treatments including tamoxifen and aromatase inhibitors (Voduc and Nielsen, 2008).

1.2.1.2 Basal-like

The basal-like subtype was identified by high expression of keratins 5 and 17, laminin, and fatty acid binding protein 7 (Sorlie et al., 2001). The basal-like subtype is negative for expression of ER, PR and HER2 (Perou, 2011). These tumors are more common in African American women, premenopausal women, and those with a BRCA1 gene mutation (Voduc et al., 2010). It has been noted that these patients tend to have a worse prognosis since there are no FDA approved targeted therapies for this subtype yet.

1.2.1.3 HER2+ subtype

This group was identified by high expression of genes located in the HER2 amplicon at 17q22.24, including HER2 and GRB7 (Sorlie et al., 2001). HER2 over expression is normally determined by IHC and HER2 overexpressing is determined by FISH analysis (Figure. 1.5) (Tagliabue et al., 2010). HER2 over expressed breast cancers are usually described as aggressive tumors and are associated with poor prognosis compared to luminal breast cancers if not treated with anti-HER2 therapies. Details on HER2 over-expressing breast cancer and treatment directed to this subtype are described in the next section.

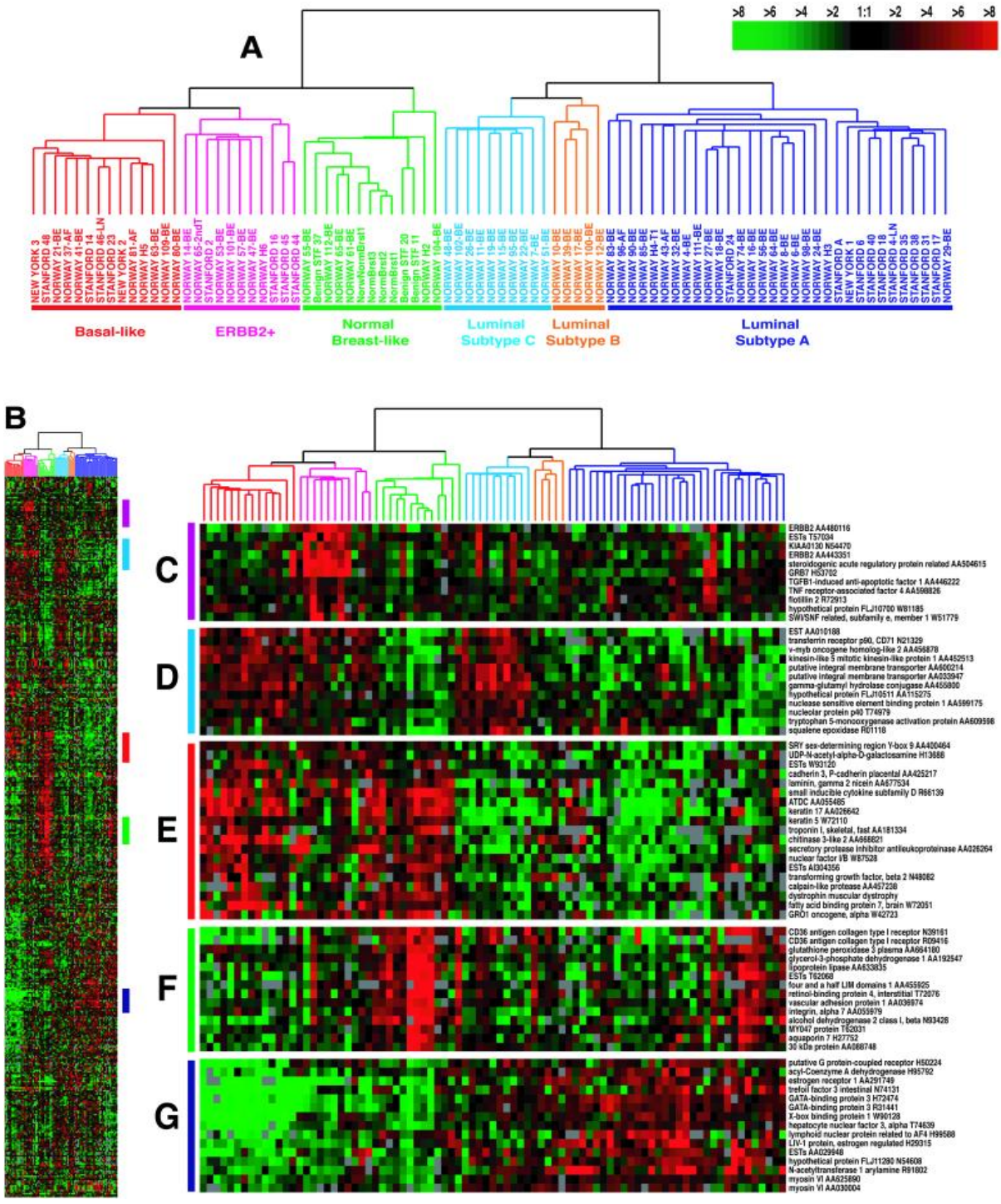


Figure 1.3 Classification of breast tumor subtypes according to different clusters gene.

The diagram is taken from (Sorlie et al., 2001).

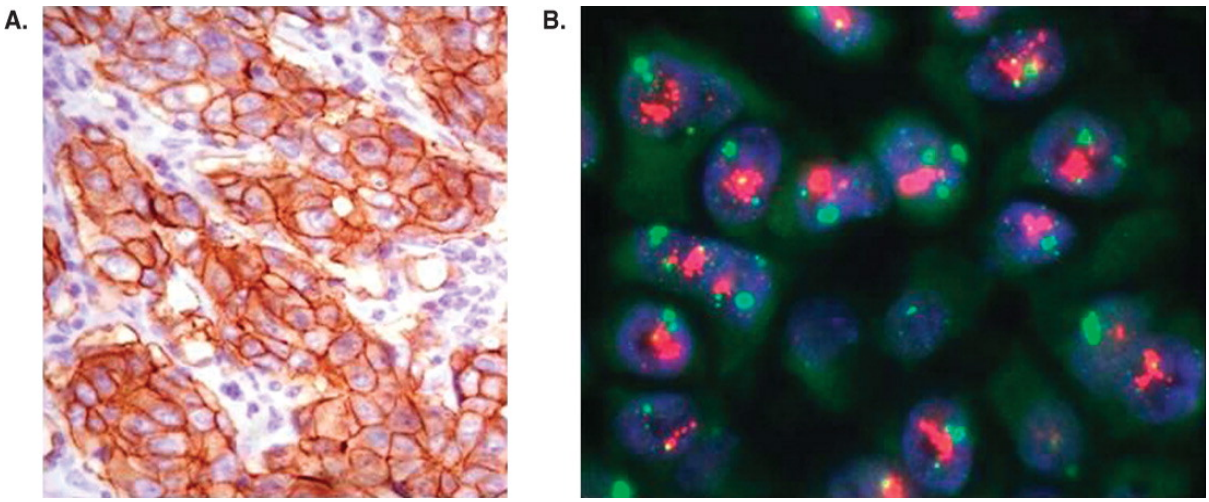


Figure 1.4 Evaluation of HER2 status as assessed by immunohistochemistry (IHC) and fluorescence *in situ* hybridisation (FISH).

Formalin-fixed paraffin-embedded section of HER2-positive breast cancer was probed with specific HER2 antibodies specific to IHC (HercepTest) (A) and FISH (B). (A) Representative IHC picture indicates positive HER2 staining indicated by brown chromogen uptake. (B) A representative FISH image showed HER2-specific probe (red), centromeric 17 probe (green) and DAPI (blue). Pictures taken from (Tagliabue et al., 2010).

1.2.2 HER2 over-expressing breast cancer

HER2 (ERBB2/neu) has been one of the most widely investigated tyrosine kinase receptors because of its well-characterised role in breast cancer (Baselga and Swain, 2009; Hynes et al., 2001; Ignatiadis et al., 2009). Generally, HER2 is over expressed in 15–25% of breast cancers (Barros et al., 2010). Higher tumor grade, larger size and lymph node positivity are features that are associated with HER2-overexpressing tumors (Al-azawi et al., 2011; Barnes, 1993; Glynn et al., 2010). Moreover, a significant correlation between HER2 over expression and reduced survival of breast cancer patients has been observed (Pegram and Slamon, 2000; Ryan et al., 2006). Even though HER2 over expression corresponds with aggressive tumor behavior, it has also been observed in pre-neoplastic breast lesions, i.e. atypical duct proliferations and ductal carcinoma *in situ*, indicating the presence of HER2 amplification at early stage of tumor genesis (Baselga and Swain, 2009). In addition, HER2 over expression has also been found to be a factor that mediates endocrine resistance, as evidenced by its crosstalk with ER signalling pathway (Yang et al., 2004). Therefore, the above findings have highlighted HER2 as an important target for therapeutic approach in breast cancer treatment.

1.2.3 HER2 targeted breast cancer therapy

1.2.3.1 Trastuzumab

Trastuzumab (Herceptin), a humanized monoclonal anti-HER2 antibody, was developed in order to combat HER2 activity (Slamon et al., 2001). Trastuzumab has been shown to benefit breast cancer patients with gene amplification of HER2. HER2

over expression is present in approximately 15-25% of all patients (Nahta and Esteva, 2006).

The initial studies of trastuzumab demonstrated a higher percentage reduction in recurrence and mortality among patients who diagnosed with late-stage breast cancer and received combination treatment of trastuzumab and chemotherapy (Baselga et al., 1999; Pusztai and Esteva, 2006; Slamon et al., 2001; Smith, 2001). In addition, treatment with trastuzumab also improves treatment for breast cancer patients diagnosed at an early stage of cancer. A study showed that trastuzumab significantly improves OS and DFS in HER2-positive women with early and locally advanced breast cancer (Moja et al., 2012). However, the study also observed increases heart problems among the patients. This causes an ongoing question as to whether trastuzumab in combination with hormonal therapy gives benefit to breast cancer patients with early stage disease (Neugut et al., 2014).

Trastuzumab has been found to target the HER2 extracellular domain IV (Cho et al., 2003). Several mechanisms of action of trastuzumab have been reported previously (Spector and Blackwell, 2009). Trastuzumab can enhance cytotoxic activity by inducing ADCC (antibody-dependent cellular cytotoxity) (Arnould et al., 2006). It has been reported that trastuzumab induces the ADCC signals when its Fc portion recognizes the Fcγ receptor of the NK cells, which then further recruits NK cells to attack the tumor cells (Lazar et al., 2006). Targeting the HER2 domain IV also causes trastuzumab to prevent HER2 cleavage mediated by ADAM10 (Molina et al., 2001). In addition, several HER2-mediated transduction signals are inhibited in tumors treated with trastuzumab such as the MAPK, PI3K-Akt, and cyclin D, indicating that trastuzumab could inhibit tumor proliferation and progression (Junttila et al., 2009; Yakes et al., 2002). The down regulation of PI3K activity induced by trastuzumab has been reported to be due to an

increased activity of PTEN (Nagata et al., 2004), which could induce upregulation of apoptotic activity and reduction of tumors (Dogan et al., 2011). Trastuzumab has also been observed to increase cell cycle arrest in G1 phase by inducing $p27^{Kip}$ (Le et al., 2003). Moreover, trastuzumab also blocks angiogenesis-promoted tumor growth via down regulation of several angiogenic markers including VEGF, transforming growth factor α, plasminogen activator inhibitor 1, and angiopoietin 1 and enhancing of anti-angiogenic factors as thrombospondin-1 (Wen et al., 2006). It has also been noted that trastuzumab increases the sensitivity to chemotherapy and radiotherapy treatments by inhibition of DNA repair that leads to increased DNA damage (Pietras et al., 1999).

As trastuzumab was shown to increase survival of breast cancer patients with HER2–over expression or gene amplification, the drug is now used as a standard approach in the management of this type of breast cancer. In 1998, the US Food and Drug Administration (FDA) approved trastuzumab in combination with chemotherapy for the treatment of breast cancer patients (Slamon et al., 1987; Slamon et al., 2001). However, a pitfall is that all of the patients seem to progress eventually when trastuzumab is given as a monotherapy for advanced or metastatic breast cancer (Slamon et al., 2001; Vogel et al., 2002)

1.2.3.2 Lapatinib

Lapatinib is a dual tyrosine kinase inhibitor that blocks both HER1 & HER2 (Alvarez and Hortobagyi, 2013; Liao et al., 2010). This was the second HER2 targeted agent approved by the FDA for metastatic HER2 positive breast cancer (Geyer et al., 2006). The large aniline quinazoline head group found in lapatinib allows access deep into the catalytic cleft of HER1, which causes delay in the dissociation rate of this inhibitor compared to the other quinazolines i.e. erlotinib (Wood et al., 2004). Lapatinib

has been shown to block the downstream MAPK/Erk1/2 and PI3K/Akt pathways (Xia et al., 2002b). Treatment with lapatinib has been found to prevent tumor growth in xenograft models (Konecny et al., 2006). The combination of lapatinib and trastuzumab enhanced anti-proliferative effects more than single drug treatment in HER2 over expressing cell lines (Konecny et al., 2006). Geyer et al. (2006) demonstrated that lapatinib plus capecitabine was superior to capecitabine alone in HER2 positive breast cancer patients that have progressed on trastuzumab and chemotherapy agents (Geyer et al., 2006).

1.2.3.3 Other HER2 targeted therapy

There are several other HER2 targeted therapies that have recently developed (Incorvati et al., 2013). In contrast to trastuzumab, pertuzumab is a monoclonal antibody that targets sub domain II of the HER2 extracellular domain and blocks heterodimerisation of HER2 with other HER receptors, especially HER2/HER3 dimer (Baselga et al., 2011; Franklin et al., 2004). The combination of trastuzumab and pertuzumab in HER2 positive breast cancer had a greater antitumor activity compared to trastuzumab alone in xenograft model (Scheuer et al., 2009). Trastuzumab emtansine (T-DM1) is a HER2-targeted immunoconjugate drug that links trastuzumab with an antimicrotubule cytotoxic chemotherapeutic agent in a covalent bond (Burris et al., 2011). This drug has been shown to improve progression-free survival compared to lapatinib for HER2-positive metastatic breast cancer patients who have progressed on a trastuzumab-containing regimen (Verma et al., 2012). Afatinib and neratinib are the two irreversible tyrosine kinase inhibitors targeting HER1, HER2, and HER4. Both panHER inhibitors have shown promising results in clinical trials (Burstein et al., 2010; Lin et al., 2012).

1.2.4 Trastuzumab resistance

Even though trastuzumab has been shown as a successful therapeutic approach for HER2 positive breast cancer, however, there is a group of patients that do not to respond to this treatment. It has been observed that only about 35% of patients diagnosed with HER2 over expression showed a response after single treatment with trastuzumab (primary or *de novo* therapeutic resistance) (Pohlmann et al., 2009). However, the response rate was enhanced after patients received combination treatment of trastuzumab and first-line chemotherapy (Kostler et al., 2005). In addition, patients treated with trastuzumab in a continuous manner are prone to develop drug resistance after a certain period of time, viewed as secondary trastuzumab resistance (Pohlmann et al., 2009).

Various mechanisms have been proposed to explain trastuzumab resistance of tumor cells (Nahta et al., 2006). It has been hypothesized that activation of other HER receptors (EGFR, HER3 & HER4) decrease trastuzumab efficiency to kill tumor cells (Dua et al., 2010; Spector and Blackwell, 2009; Wang et al., 2008). It has been previously observed that EGF, betacellulin, and heregulin stimulation alter the growth inhibitory effect of trastuzumab (Motoyama et al., 2002). Moreover, increased levels of ADAM17 have been shown to increase HER ligand release resulting in continuously maintained HER2 phosphorylation in tumors treated with trastuzumab via a PKB negative feedback loop (Gijsen et al., 2010). Cross talk with IGF-receptor also influences trastuzumab resistance due to down regulation of $p27^{Kip}$ (Kute et al., 2004; Nahta et al., 2004; Nahta et al., 2005). Additionally, it was shown that resistance to trastuzumab might be due to the loss of PTEN that controls PI3K/Akt signalling (Nagata et al., 2004). Trastuzumab resistance was also related to the presence of membrane-associated glycoprotein

(MUC4) that masks the binding epitope of this monoclonal antibody, thereby blocking the interaction between HER2 and trastuzumab (Nagy et al., 2005). Another resistance mechanism was described to be due to an HER2 cleavage mechanism that produces the p95 form of HER2, which contains no extracellular binding region for trastuzumab (Saez et al., 2006). This fragment is found to be more oncogenic than the full-length of HER2.

1.3 The controversial role of HER4 in breast cancer

Previous studies have reported that HER4 has proliferation activity, which supports the fact that HER4 has oncogenic properties (Cohen et al., 1996; Junttila et al., 2005; Tang et al., 1999). The HER4 CYT-2 isoform that lacks a PI3K binding site has been shown to promote proliferation activity (Kainulainen et al., 2000). It was observed that NIH 3T3 cells transfected with HER4 form colonies in soft agar, indicating that HER4 may play a role in proliferation (Cohen et al., 1996). Juntilla *et al.* (2005) further supported this by demonstrating that over expression of exogenous HER4 promotes the growth of their breast cancer model (Junttila et al., 2005). In addition, downregulation of HER4 prevents tumor growth *in vitro* and *in vivo* (Tang et al., 1999). Furthermore, Bieche et al. (2003) reported that breast cancer patients with tumors overexpressing HER4 had a shorter survival rate, suggesting HER4 to be an important marker for poor prognosis (Bieche et al., 2003).

In contrast, there were also observations indicating HER4 to be a tumor suppressor. Several studies reported that HER4 activity was closely associated with growth control and differentiation signalling (Kainulainen et al., 2000; Naresh et al., 2006; Sartor et al., 2001), which is contrary to the activity of the other HER receptors that promote cell growth. In contrast to HER2 mutant, HER4 mutant was found to prevent cell proliferation in a rodent fibroblast cell line (Penington et al., 2002). A reduction of proliferation caused by HER4 has been found to correlate with increased apoptosis activity (Naresh et al., 2006; Sartor et al., 2001). Moreover, Sartor *et al.* provided evidence that HER4 interrupts HER2 signalling to control tumor growth (Sartor et al., 2001).

In contrast to HER2, it was reported that HER4 expression correlates with anti-proliferative and pro apoptotic activity (Jones et al., 1999; Maatta et al., 2006; Muraoka-Cook et al., 2006b; Sundvall et al., 2008a), as well as low tumor grade, low proliferation rate and positive ER and PR scores (Barnes et al., 2005; Koutras et al., 2008; Witton et al., 2003). In addition, HER4 protein expression was also associated with an increased survival rate of breast cancer patients (Suo et al., 2002). Koutras et al. (2008) supported this by demonstrating a positive correlation of HER4 mRNA with prognostic indicators, i.e. DFS and OS (Koutras et al., 2008). However, Bieche et al. reported that breast cancer patients with tumors overexpressing HER4 had a shorter survival rate, suggesting HER4 to be an important marker for poor prognosis (Bieche et al., 2003). Taken together, it seems that the prognostic value of HER4 expression in relation to breast cancer survival has been controversial.

1.3.1 Possible reasons for controversial role of HER4

The controversial role of HER4 could be attributed to multiple reasons including:

1.3.1.1 Cell types

A mutation of the HER4 tyrosine kinase domain has been found to act as an oncogenic marker in several cancers such as lung, melanoma, osteosarcoma and oral squamous cancer (Bei et al., 2001; Kang et al., 2007; Prickett et al., 2009; Starr et al., 2006). However, HER4 also may act as a tumor suppressor, supported by the loss of HER4 observed in renal, prostate and pancreatic cancers (Mill et al., 2011a; Thomasson et al., 2004; Wheeler et al., 2008).

1.3.1.2 HER4 ligands

The HER ligands that are involved in the activation of HER4 include betacellulin, heparin binding (HB)-EGF, epiregulin, neuregulin (heregulin) type-1, -2, -3 and -4 (Riese and Stern, 1998; Sartor et al., 2001; Sundvall et al., 2008a). Some of these ligands are found to bind with HER1 and HER4 i.e. BTC, HB-EGF and EPR and some bind to HER3 and HER4 i.e. neuregulin-1 and neuregulin-2. However, neuregulin-3 and neuregulin-4 are the ligands specific for HER4. The different roles of HER4 ligands suggest that HER4 may have a different impact on breast cancer depending on which ligand is activating HER4 or other HER receptors. For example, Kawaguchi et al. suggested that betacellulin functions as an autocrine growth factor responsible for the cells growth in pancreatic cancer (Kawaguchi et al., 2000). However, it was further proven that betacellulin regulates the proliferation activity by HER1 homodimers and HER1/HER2 heterodimers signalling (Oh et al., 2011). The high level of HB–EGF has also been found in several types of cancers (Kramer et al., 2007; Ongusaha et al., 2004; Sloss et al., 2010) but it is also hypothesized that the ligand promotes tumor proliferation activity through HER1 signalling and not HER4. However, Sartor's study demonstrated that the expression of heregulin and HB-EGF prevents the growth of breast cancer cells that negatively express HER1 (Sartor et al., 2001), suggesting that HER4 ligands play a role in cell differentiation.

The presence of heregulin (neuregulin) has been noted in around 30% breast carcinoma and causes cancer growth and metastatic spread (Hayes and Gullick, 2008; Khurana et al., 2013; Marshall et al., 2006). Tsai et al. noted that the inhibition of heregulin prevents tumor growth in breast cancer (Tsai et al., 2003). Previous studies showed that heregulin mediates its cellular response through HER2/HER3 signalling (Ruan et al., 2012; Tsai et al., 2003). However, when heregulin activates HER4

signalling, the growth inhibitory effect has been observed in breast cancer cells (Muraoka-Cook et al., 2006a), suggesting that heregulin can also trigger cell differentiation signals.

A specific HER4 ligand, neuregulin-3 has been reviewed to play an important role during early stages of mammary development (Howard, 2008). In addition, co expression of HER4 and neuregulin-4, another ligand specific to HER4, was shown to be better indicator for survival of bladder cancer patients (Memon et al., 2004). However, the ligand is also highly expressed in gastrointestinal malignant lymphoma, suggesting the role of this ligand as a tumor oncogene (Ebi et al., 2011).

1.3.1.3 Dependency on other HER receptors

Figure 1.6 illustrates the changes of cancer phenotypes caused by different co-expression of HER4 with other HER receptors (Karamouzis et al., 2007). From the figure, it has been noted that HER4 plays an important role during the early development of breast carcinogenesis. Co-expression of HER2 and HER4 was significantly correlated with better outcomes for breast carcinoma *in situ* (Barnes et al., 2005). In addition, HER2 and HER4 dimerisation also appeared to be oncogenic in breast cancer (Mill et al., 2011b). However, during tumor progression, HER4 expression is downregulated and HER3 over expression was shown to have a significant impact on supporting cancer growth by interacting with HER1 and HER2 (Figure 1.6). This is not surprising since a number of previous studies have found a positive association of HER3 and an inverse association of HER4 with the other HER receptors in invasive breast cancers (Abd El-Rehim et al., 2004; Witton et al., 2003). While a few previous studies have shown that HER3 is a negative prognostic marker (Bieche et al., 2003; Sassen et al.,

2008), others have suggested that HER4 expression is associated with a favourable clinical outcome (Koutras et al., 2008; Pawlowski et al., 2000; Witton et al., 2003).

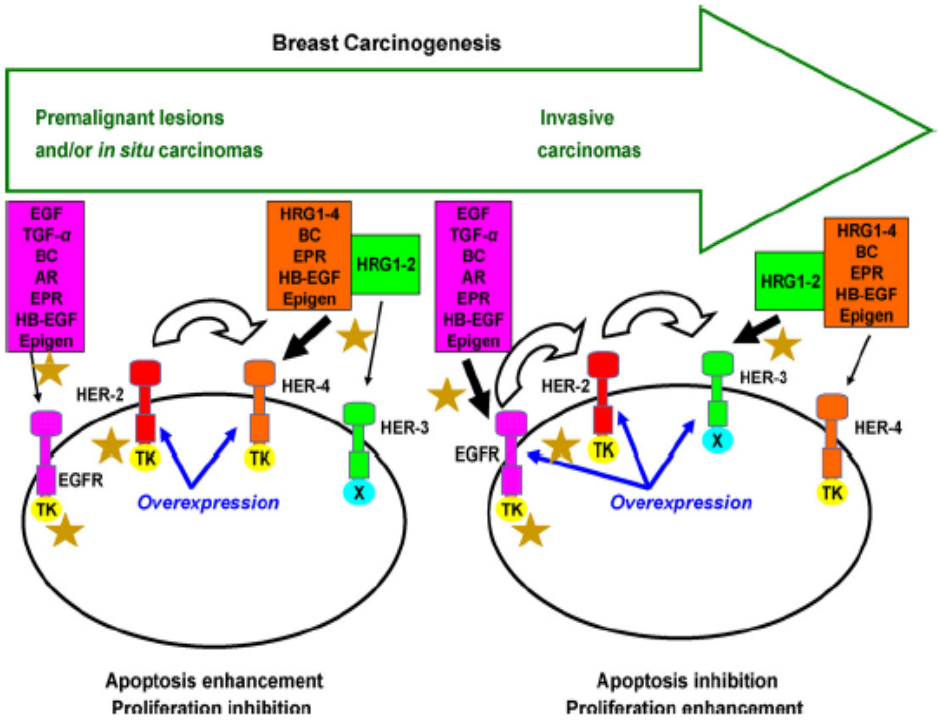


Figure 1.5 The role of HER3 and HER4 in breast carcinogenesis (Karamouzis et al., 2007).

In his review, Karamouzis et al noted the importance of HER4 over expression during the early stage of breast carcinogenesis that increases apoptosis and controls atypical proliferation caused by HER2. However, in the invasive phenotype, heregulin stimulates HER3 to be highly expressed compared to HER4. It is postulated that heregulin mediated HER3 and HER1 signalling inhibits apoptosis and causes more cancer cells to proliferate.

1.3.1.4 HER4 isoforms

As stated in section 1.1.1.4, the alternative mRNA spicing generates four different HER4 isoforms; the juxtamembrane JM-A and JM-B isoforms and cytoplasmic CYT-1 and CYT-2 isoforms (Hollmen and Elenius, 2010). These variants have been demonstrated to have different functional characteristics and mediate dissimilar cellular responses. As shown in Figure 1.1, the JM-A has longer sequences (23 amino acid long, at exon 16) compared to JM-B (13 amino acid long, at exon 16). In addition, in contrast to JM-B isoform, the JM-A is a fragment that could be processed and cleaved by the proteolytic enzyme, Adam-17 (Figure 1.1) (Rio et al., 2000). Whilst the HER4 JM-A isoform is highly expressed in kidney, salivary gland, trachea, thyroid gland, prostate and mammary gland, the expression of HER4-JM-B isoform is commonly observed in the brain and heart tissues (Hollmen and Elenius, 2010). HER4 JM-A CYT-2 isoform influences the growth of breast cancer cells, while over-expression of the non-cleavable HER4 JM-B CYT-2 promotes differentiation (Maatta et al., 2006; Sundvall et al., 2007).

The roles of both cytoplasmic HER4 isoforms are also different. In contrast to HER4 CYT-2, HER4 CYT-1 isoform has been shown to directly link to the PI3-K/Akt pathway (Elenius et al., 1999; Kainulainen et al., 2000), which supports cells differentiation. In contrast, HER4 CYT-2 is responsible for cell proliferation, as studied in both *in vitro* and *in vivo* experiments (Kainulainen et al., 2000; Muraoka-Cook et al., 2009). These studies prove that the functional diversity of HER4 isoforms that may account for the different and uncertain roles of HER4 receptor.

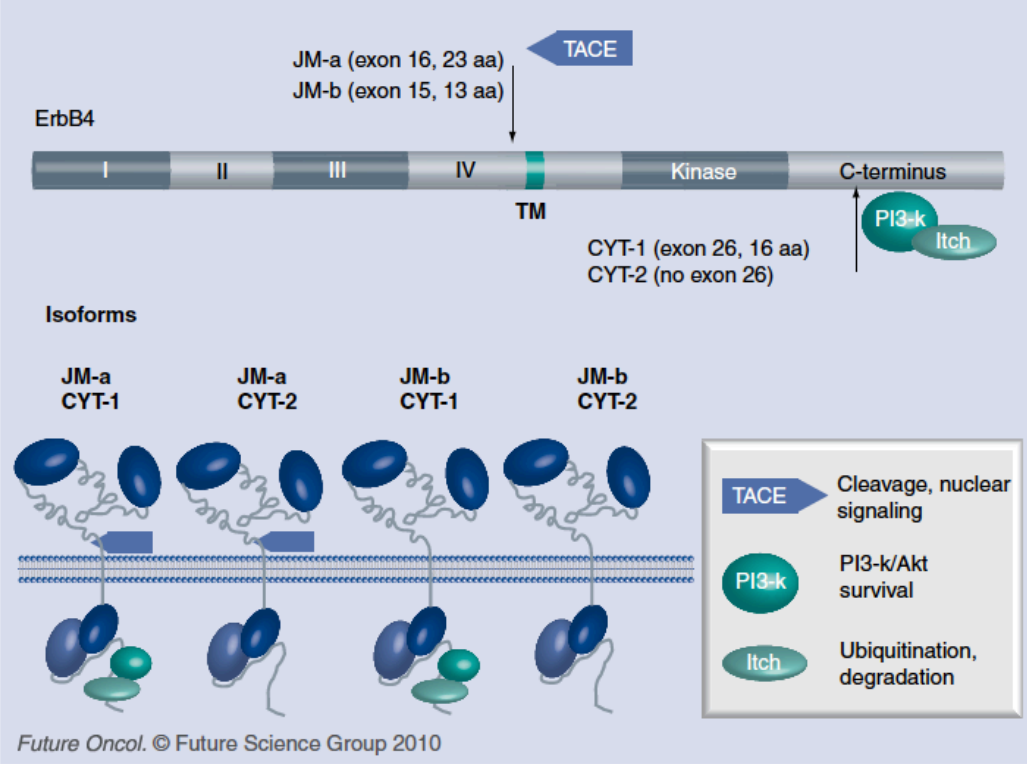


Figure 1.6 The structure of HER4 and its isoforms. Schematic diagram taken from (Hollmen and Elenius, 2010).

1.3.1.5 HER4 localisation

As stated in section 1.1.1.4, the proteolytic cleavage that is mediated by Adam-17 and γ-secretase, separates full length HER4 into two parts, the extracellular domain (ECD) and the intracellular domain (ICD). The ICD is normally recognized as an 80kDa fragment, which retains several tyrosine phosphorylation sites on its C-terminus (Figure 1.3) (Linggi et al., 2006).

The ICD has been reported to localize to different cellular compartments and has been shown to have a different role in mediating cellular response (Sundvall et al., 2008a; Tovey et al., 2006). Figure 1.6 (A) indicates HER4 interacts with the PI3K/Akt pathways and Ras/MAPK when it translocates into the cytoplasm, and supports cell proliferation and differentiation. These mechanisms are regulated by different HER4 cytoplasmic isoforms, CYT1- and CYT-2 (See Section 1.3.1.4) (Kainulainen et al., 2000). Thor et al. have found that cytosolic HER4 has a positive prognostic significance in predicting survival for node-negative breast cancer patients (Thor et al., 2009). In addition, there was also a significant correlation of cytosolic HER ICD with apoptotic cells in primary breast tumors, compared to membranous HER4 ICD (Naresh et al., 2006). However, it was also shown that apoptosis induction is mediated by mitochondrial HER4 ICD through BAK signalling (Vidal et al., 2005). It is also seen that HER4 signalling in the cytosol interacts with Itch and WWox ligase proteins that function for HER4 receptor stability (Figure 1.3) (Aqeilan et al., 2007; Sundvall et al., 2008b).

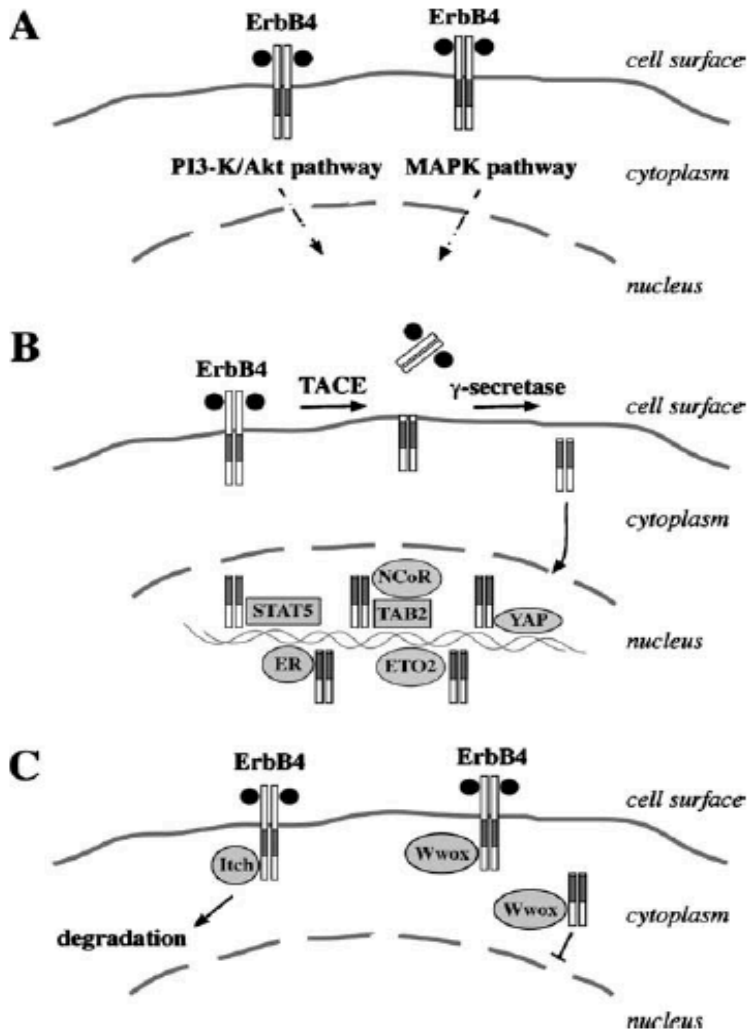


Figure 1.7 Signals mediated by HER4 activation (Sundvall et al., 2008a).

Depending on different cellular compartments, HER4 activation mediates diverse several cellular responses through several signals; (A) PI3-K/Akt and MAPK pathways. (B) Multiple transcription factors such as STAT5, YAP, ETO2, ER & TAB2-NCoR complex. (C) Itch and WWox ligase proteins that induce HER4 monoubiquitation, endocytosis and degradation, and stabilize the structure of HER4 full length. This blocks HER4 ICD formation and HER4 nuclear translocation.

In contrast to cytosolic and mitochondrial HER4 ICD, nuclear HER4 ICD is found in differentiated cells (Clark et al., 2005; Muraoka-Cook et al., 2006b). As shown in Figure 1.6, several transcription factors such as STAT5, YAP, ETO2, ER and the TAB2-NCoR have been reported to interact with nuclear HER4 ICD (Komuro et al., 2003; Linggi and Carpenter, 2006b; Sundvall et al., 2008a; Williams et al., 2004; Zhu et al., 2006). However, it was also observed that JM-a CYT-2, a cleaved form of HER4, has more oncogenic potential when it is located in the nucleus (Maatta et al., 2006). In a clinical study, Juntilla et al. observed that breast cancer patients with nuclear localisation of an HER4 ICD have a worse prognosis compared to patients with HER4 expression at cell surface (Junttila et al., 2005).

1.3.1.6 Dependency on blockage of HER receptors

It was hypothesized that the blockage of other HER receptors can modulate HER4 in different ways. For example, HER4 activation and its cleavage were induced after EGFR inhibition via treatment with AG1478 and Iressa, tyrosine kinase inhibitors specific to HER1 (Kong et al., 2008). HER4 is also thought to become important during trastuzumab treatment, since trastuzumab increases the release of HER4 ligand, heregulin and upregulates HER2 and HER4 dimerisation (Gijsen et al., 2010; Kong et al., 2008). Taken together, it is suggested that HER4 may be important during inhibition of other HER receptors. Although a recent study showed that HER4 could enhance trastuzumab response in metastatic breast carcinoma (Portier et al., 2013; Sassen et al., 2009), the exact mechanisms as well as how HER4 activation and its cleavage related to trastuzumab response were not reported.

1.4 Aims of study

The role of HER4 in relation to trastuzumab treatment and resistance remains unclear. Although HER4 expression has been shown to improve trastuzumab response (Portier et al., 2013; Sassen et al., 2009), there has been no detailed report on how trastuzumab affects HER4 expression, activation and cleavage and how these processes mediate sensitivity and resistance to trastuzumab in HER2 positive breast cancer cells. Therefore, the aim of this study is to elucidate the role and prognostic significance of HER4 expression and localisation in relation to trastuzumab treatment and resistance in HER2 positive breast cancer.

This study is carried out with four main objectives:

1. To investigate the role of HER4 expression in relation to trastuzumab treatment and resistance in HER2 positive breast cancer
2. To investigate the activation and cleavage of HER4 in relation to trastuzumab treatment and resistance in breast cancer cells
3. To investigate the role of HER4 localisation in relation to trastuzumab and neratinib treatment in HER2 positive breast cancer xenograft models.
4. To investigate the role of HER4 in relation to prognosis and trastuzumab response in HER2 positive breast cancer patients.

CHAPTER TWO

Materials and Methods

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

2.1.1 Cells

In this study, SKBR3 and BT474 cells were used as cell lines that represent over expression of HER2. MCF-7, which is a HER2 negative cell line, was used as the control. All cell lines were provided from Cancer Research UK (Lincolns' Inn Fields laboratory). The trastuzumab-resistant SKBR3 and BT474 were generated by my previous lab colleague, Merel Gijsen. The cells were treated with trastuzumab at 40μg/ml for more than 8 months (Gijsen et al., 2010).

2.1.2 Drugs/inhibitors

Table 2.1 List of drugs and inhibitors

Drug/inhibitor	Concentration used	Supplier
1. Trastuzumab	40μg/ml	Roche
2. Neratinib	100nM	Sellect
3. γ -secretase inhibitor IX (GSi)	10μM	Merck
4. Heregulin	100ng/ml	Roche

2.1.3 Solutions

1. PBS-Tween

800μl of Tween 20 was added to 400ml of PBS.

2. 5% BSA

1.25g of BSA were dissolved in 25ml PBS-Tween.

3. 1% BSA

0.25g of BSA were dissolved in 25ml PBS-Tween.

4. 4% PFA

The solution was prepared by adding 5ml of 16% PFA in 25ml PBS.

5. Cell lysis buffer

The solution was made up of 20 mM Tris-HCl (pH 7.5), 10mM EDTA, 150 mM NaCl, 0.1% Triton-X and one tablet of Complete Mini Protease Inhibitor Cocktail Tablet.

6. Running buffer

The solution was prepared freshly prior to gel electrophoresis. 25ml of 20x NuPAGE® MOPS SDS Running buffer was added to distilled water to make up 500ml of running buffer.

7. Transfer buffer

The solution was prepared by dissolving 192 mM glycine, 100ml of methanol, and 25 mM Tris in a total of 500ml of distilled water.

8. 1x Citrate buffer

The solution was prepared by diluting 50ml 10x Citrate buffer (pH6.0) (Sigma Aldrich) in 450ml of distilled water.

2.1.4 List of reagents and kits

Table 2.2.List of reagents and kits

Applications	**Reagents/kits**	**Supplier**
1. Cell culture	DMEM	Invitrogen
	RPMI	
	PBSA	
	FCS	PAA
	Trypsin-EDTA in PBSA	
2. siRNA transfection	Oligofectamin	Invitrogen
	Optimem serum-free media	
	HER4 siRNA	Qiagen
	All Star Negative siRNA	
3. Clonogenic assay	Methylene blue	Sigma
4. FACS analysis	Camptothecin	Sigma Aldrich
	Alexa Fluor® 647-Annexin V conjugate	Life Technologies
	FITC Annexin V Apoptosis Detection Kit I	BD Pharmingen
5. Cell viability	ISOTON ® II diluent	Beckman Coulter
6. Protein Assay	BIO-RAD Protein Assay	BioRad
7. Western blot	Tween® 20	Triton-X-100
	BSA	Sigma-Aldrich
	Beta-mercaptoethanol	Sigma

	Complete Mini Protease Inhibitor Cocktail Tablets	Roche
	Bradford dye, NuPAGE® 4-12% Bis-Tris Gel	Invitrogen
	Immobilon®-P Transfer Membrane	
	NuPAGE® LDS Sample Buffer	
	20x NuPAGE® MOPS SDS Running buffer	
	2.5 mm Blotting Filter Paper	
	1% non-fat dry, ECL	GE Healthcare
	Restore™ PLUS Western Blot Stripping Buffer	Thermo-Scientific
8. Cells fractionation	NE-PER Nuclear and Cytoplasmic Extraction kit	Thermo-Scientific
9. RT PCR	Total RNeasy Mini Kit	Qiagen
	iScript DNA synthesis kit	Applied Biosystems
	Sensimix™ SYBR Mastermix	Bioline
10. Confocal	4% PFA	Pierce/Thermo-Scientific
	0.2% Triton-X -100	Sigma Aldrich
	Natrium Borohydrate.	Sigma Aldrich
	1% BSA	Sigma Aldrich
	Fluoromount-G with DAPI	Southern

		Biotech
11. IHC	Histoclean	Merck
	Haematoxylin	
	10x Citrate buffer (pH6.0)	Sigma Aldrich
	ImmPRESS™ Reagent Anti-Rabbit Ig	Vector Laboratories
	DAB kit	

2.1.5 List of antibodies

Table 2.3 List of primary antibodies

Antibody	**Application**	**Concentration**	**Incubation time**	**Supplier**
1. HER4 (Rabbit polyclonal)	Western blot, IHC, confocal	1:500 (western blot, confocal), 1:200 (IHC)	Overnight	Neomarkers/ Thermo Scientific
2. HER4 C-18; SC-283 (Rabbit polyclonal)	Western blot	1:500	Overnight	Santa Cruz
3. HER4 HFR-1 (mouse monoclonal)	Western blot	1:500	Overnight	Abcam
4. Phospho-HER4 (Tyr1056)	Western blot	1:1000	Overnight	Santa Cruz
5. HER2	Western blot	1:1000	3 hours	Cell Signalling
6. Phospho-HER2 (Tyr1221/122	Western blot	1:1000	3 hours	Cell Signalling

2)				
7. GAPDH	Western blot	1:1000	3 hours	Santa Cruz
8. Histone-3	Western blot	1:1000	Overnight	Cell Signalling
9. PEN-2	Western blot	1:1000	3hours	Cell Signalling
10. β–actin	Western blot	1:1000	3 hours	Cell Signalling

Table 2.4 List of secondary antibodies

Applications	Antibody	Concentration
Western blot	HRP-Goat anti Mouse & HRP-Goat Anti Rabbit (Invitrogen)	1:10,000
Confocal	Alexa Fluor- 546-Goat Anti Rabbit & Alexa Fluor 488-Goat Anti Mouse (Invitrogen)	1:400
IHC	ImmPRESS™ Reagent Anti-Rabbit Ig (Vector laboratories)	1:500

2.1.6 Lists of primers

Table 2.5 List of primers

Primer	**Oligonucleotide sequence**	**Supplier**
HER2	Fw: 5′-AGC CTT GCC CCA TCA ACT G-3′	Invitrogen
	Rv: 5′-AAT GCC AAC CAC CGC AGA-3′	
HER4 (JM-a isoform)	Fw: 5′-CCA CCC ATG CCA TCC AAA-3′	
	Rv: 5′-CCA ATT ACT CCA GCT GCA ATC A-3′	
β–actin	Fw: 5′-ATC TGG CAC CAC ACC TTC TAC AAT-3′	
	Rv:5′-CCG TCA CCG GAG TCC ATC A-3′	

2.1.7 Lists of siRNAs

Table 2.6 List of siRNAs

siRNA name	**Sense strand/antisense strand**	**Supplier**
HER4 $siRNA_{10}$	5'-ACG UGU UAG UGG CUC UUA ATT-3' / 5'-UUA AGA GCC ACU AAC ACG UAG-3'	Qiagen
HER4 $siRNA_{11}$	5'-GGG AUU UGG CAG CCC GUA ATT-3' / 5'-UUA CGG GCU GCC AAA UCC CGA-3'	
All Star Negative	Sequences not provided by manufacturer	

2.2 Methods

2.2.1 Cell culture

2.2.1.1 Cell lines and growth condition

MCF-7, BT474 and SKBR3 cell lines were provided by cell services lab at Cancer Research UK (Lincoln's Inn Fields laboratory), which has a stringent quality control in cell authenticity and has incorporated short-tandem repeat (STR) profiling for cell line validation. Trastuzumab resistant cell lines were generated as previously reported (Canonici et al., 2013; Gijsen et al., 2010; Kong et al., 2008). The MCF-7, SKBR3, and trastuzumab-resistant SKBR3 cell lines were grown in DMEM containing 10% FCS and 1% penicillin-streptomycin. In contrast, BT474 and trastuzumab-resistant BT474 cell lines were maintained in RPMI 1640 containing 10% FCS and 1% penicillin-streptomycin. Trastuzumab-resistant cell lines (see Section 2.1.1) were grown in media with continuous treatment with 40μg/ml trastuzumab. All cells were grown in a humidified incubator at 37°C with 5% CO_2. Routinely, the cells were sub-cultured for up to 25 times and the medium was weekly changed for three times.

2.2.1.2 Drug treatment and ligand stimulation

For the experiments involving trastuzumab, the concentration used was 40μg/ml unless otherwise indicated. Exogenous heregulin was used as positive control to assess the effect of ligand activation on HER4. In addition, the cells were also treated with a 10μM GSi and 100nM neratinib for investigating HER4 activation and cleavage in relation to trastuzumab treatment.

2.2.1.3 siRNA transient transfection

The cells were transfected with siRNA against HER4 (Qiagen). To knockdown

HER4, two HER4 siRNA sequences were used: 5' -TCG GGA TTT GGC AGC CCG TAA-3' (HER4 siRNA 10) and 5'-CTA CGT GTT AGT GGC TCT TAA-3' (HER4 siRNA 11) (Table 2.6). Initially, cells were plated in a 6-well plate the day before the transfection. The transfection reagent in a final volume of 2ml/well was prepared an hour before being added to the cells and comprised 20nM HER4 siRNA, Oligofectamine, and Optimem serum-free medium. Oligofectamine was diluted in Optimem and after 5 mins at room temperature, the solution was added to 20nM siRNA in Optimem. The mixture was incubated for 20 mins at room temperature, before being added to antibiotic –free media in cell culture plate. The media was discarded from the cells and replaced with fresh growth media. The HER4 expression was determined at protein and mRNA levels at 72 hours post transfection. To determine the siRNA HER4 efficiency, scrambled siRNA with non-specific binding siRNA (All Star Negative siRNA) was used as a control (Table 2.6).

2.2.1.4 Cell counting

Approximately 10, 000 cells were plated in a 24-well plate, one day before treatment with different drugs at different concentrations. The cells were consequently counted after 3 days and 6 days of treatment. Cells were initially washed with PBS, before being detached by incubation with 500µl trypsin/EDTA for up to 5 mins. The trypsin solution was mixed thoroughly using a pipette to ensure cell detachment. 400µl of cell suspension from each well was diluted into isotone for counting. The cells were then counted using a Coultier counter (Beckman Coulter).

2.2.1.5 Colony formation assay

Following completion of treatment, approximately 2,000 cells were re-plated in a 6-well plate and were left to grow for two weeks to allow colony formation. The media

was replaced twice a week. After 14 days, the media was removed and cells were washed with PBS. Cells were fixed and stained with 0.2% methylene blue (Sigma) in 50 % methanol for at least 1 hour. The mixture was removed and stained cells were rinsed with water. The plates were left to dry at room temperature overnight and images of plates were obtained with a scanner. The number of colonies was quantified using Image J software.

2.2.1.6 FACS analysis

Following completion of treatment, cells were left for overnight incubation before proceeding with the assessment of apoptosis. 6μM Camptothecin (Sigma Aldrich) was added to untreated cells as a positive control. Untreated cells without Camptothecin were used as negative control. On the day of the experiment, the 6-well plate was placed on ice. The media was removed from the well and transferred into a new 5ml tube that had been placed on ice earlier. Cells were washed twice with ice-cold PBS and harvested by incubation with 500μl trypsin/EDTA for 10 mins at room temperature. PBS was added to stop trypsinisation and the cell suspension was centrifuged at 1000 rpm for 3 mins to remove the supernatant. The cell pellet was washed twice with ice-cold PBS, followed by a centrifugation step at 1,000 rpm for 3 mins. The rinsed cell pellet was resuspended in 350μl Annexin V Binding Buffer (BD Pharmingen). In addition, 0.4μl of Alexa Fluor® 647-Annexin V conjugate (Life Technologies) and 5μl of Propidium Iodide (PI) (BD Pharmingen) were added to the cell suspension. Cells resuspended in Annexin V buffer only were used for cell gating, whereas cells resuspended in Annexin V buffer with either Alexa Fluor® 647-Annexin V or PI were used for gating of each fluorescent. The analysis was later run using a flow cytometry detector, FACS Calibur (Becton Dickinson). The apoptotic cells were analysed

at the early (Annexin-V-positive, PI-negative) and the late apoptotic stage (Annexin-V-positive, PI-positive) within a total 20,000 of counted cells. The experiments were performed three times in duplicate. Analysis of results was performed using Summit 4.3 software (Dako Colorado).

2.2.2 Western blotting analysis

2.2.2.1 Protein extraction

Initially, confluent dishes were placed on ice and rinsed with PBS. RIPA-100 buffer, protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail 2 and 3 (Sigma) were added and cells scraped off. Subsequently, the lysates were centrifuged at 4°C for 10 mins at 13, 500 rpm to split the unnecessary cell pellet from the supernatant. The cell lysates were normalized according to the protein amount as determined using the Bradford assay. The lysates were then prepared for loading using 4X SDS with 10% BME.

2.2.2.2 Nuclear and cytoplasmic fractionation

This step was carried out using the NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Scientific). Initially, the media of the confluent cells was taken off and the cells were rinsed with PBS. The cells were then harvested using 200µl of ice-cold CER I. The cell suspension was immediately transferred into a centrifugation tube. 11µl of ice-cold CER II was added to the cell suspension and was incubated for 10 min. The tube was then centrifuged at 13, 500 rpm for 5 mins. The supernatant (cytoplasmic extract) was immediately transferred into a new tube and stored at -20°C. Meanwhile, the pellet in the tube was re-suspended with 100µl of ice-cold NER and incubated on ice for 40 min. Again, the suspension- containing tube was centrifuged at 13, 500 rpm for 10 min.

The supernatant containing the nuclear extract was immediately transferred into a new tube. Both, the cytoplasmic and nuclear extracts were then prepared for Western blot analysis. GAPDH and Histone-3 were used as positive control for cytoplasmic and nuclear fraction respectively.

2.2.2.3 Western blot

The western blotting technique was performed to demonstrate the protein expression of several markers. Prior to gel electrophoresis, the samples were boiled for 10 min in the hot-block with the temperature set at 95°C. Later, the samples were loaded on a NuPage gel and electrophoresis was run at 130 V for 2 hours. The proteins were then transferred to a PVDF membrane using semi-dry immunoblot at 12 V for 2 hours. The membrane was blocked with 3% BSA in PBS-Tween for 30 min prior to primary antibody incubation, which was prepared in the same solution. The antibody concentration and time of incubation were varied among the primary antibodies (rabbit plolyclonal or mouse monoclonal) used (Section 2.14). After the incubation, the membrane was washed four times for 10 min each with 1% non-fat dry milk in PBS-Tween. The membrane was incubated with HRP-Goat anti Mouse or HRP-Goat Anti Rabbit (Invitrogen) at a 1:10,000 dilution (Section 2.14) for 1 hour at room temperature and rinsed again for four times with 1% non-fat dry milk in PBS-Tween. To develop a film for visualization, the membrane was finally incubated with a chemiluminescent enhancer reagent (ECL).

2.2.2.4 Semi-quantitative assay and relative quantification

The visible protein bands observed on the Western blot were cropped and the intensity from the cropped area was quantified using Image J software. The intensity measured was then normalized to the intensity of β-actin. Relative quantification was

performed over the control sample.

2.2.3 Real Time (RT)-PCR assay

2.2.3.1 Decontamination

In order to remove the RNase (ribonucleases) contamination, the RNase*ZAP*® solution was applied to clean work surfaces, laboratory equipments, i.e. pipettors and other non-disposable plasticware and glassware. The equipment was then thoroughly rinsed with distilled water and dried. The sterile microcentrifuge tubes and pipette tips used were RNase-free, i.e. RNase-free elution tubes and RNase-free pipetting tips. All of them do not require pretreatment to inactivate the RNases. To ensure that all working solutions used in the experiment were RNase-free, 0.1% DEPC-treated water was used in preparation of the solutions i.e. 70% ethanol and TBE buffers.

2.2.3.2 Sample preparation

2.2.3.2.1 RNA extraction

Total RNA was isolated from the treated cell lines using Total RNeasy Mini Kit. All steps of RNA extraction were performed on ice and RNAse/DNAse free bench work. The cell lysate was centrifuged for 3 mins at maximum speed, 13,000 rpm in a microcentrifuge. The produced supernatant was transferred in a new collection tube. One volume of 70% ethanol (350µl) was added to the respective tube and mixed well by pipetting. The 700µl of the mixture was subsequently applied to RNeasy spin column placed in 2ml collection tube. The tube was then centrifuged for 15 seconds at 10,000 rpm to wash. The flow through was subsequently discarded.

700 µl of RW1 buffer was added into the same spin column and centrifuged for 15 seconds at 12,000 rpm. The flow through and collection tube were discarded.

RNeasy spin column was transferred into a new 2 ml collection tube. 500µl RPE buffer was pipetted into the column and centrifuged for 15 seconds at 12,000 rpm. This was followed by discarding the flow through. Another 500µl of RPE buffer was then added into the same RNeasy column and centrifuged for 2 mins at 10 000 rpm in order to dry the silica gel membrane of the RNeasy column. The flow through and tube were discarded. The column was then transferred to a new 2ml collection tube and centrifuged at 13,000 rpm for 1 min. The flow through and tube were discarded. The RNeasy column was moved to a new 1.5ml tube. In order to elute RNA, 50µl RNAse-free water was pipetted directly onto the silica gel membrane of the column. The column was then centrifuged for 1 min at 13,000 rpm. The RNA was then stored at -80ºC until it was used.

2.2.3.2.2 Quantification RNA

The purity of the extracted RNA was determined by measuring the absorbance ratio 260 nm (A260) to 280 nm (A280) using Nanodrop system. RNA samples with a purity ratio of 1.8 – 2.0 were selected for further analysis by RT-PCR and the RNA concentration was measured in ng/µl.

2.2.3.2.3 cDNA synthesis

The preparation of the cDNA synthesis reaction was performed using the iScript DNA synthesis kit and was done on ice. An example condition of the reaction is described in Table 2.7. The iScript cDNA synthesis reaction was incubated using the thermal cycle programmed as follow: 5 mins at 45ºC, 30 mins at 42ºC, 5 mins at 85ºC and hold at 4ºC. The cDNA products were then stored at -80ºC until use.

Table 2.7 iScript cDNA synthesis condition.

Component	Volume
5X iScript Reaction Mix	4µl
iScript Reverse Transcriptase	1µl
Total RNA (100ng)	4µl*
nuclease free water	11µl**
Total	20µl

*The volume of total RNA used for reverse-transcriptase condition was varied according to the different concentration value of Total RNA.

**The nuclease free water was added to make up the reaction volume of 20µl.

2.2.3.3 RT-PCR

Quantitative RT-PCR assays were performed using standard SYBR-Green methodology. The primer sequences were obtained from a previous study (Junttila et al., 2003) and purchased from Invitrogen. Using the Sensi-Mix SYBR kit, the reaction was prepared in a total volume of 50µl. The preparation was done on ice. The Sensi-Mix SYBR condition for each sample was varied according to different concentration used of primers and cDNA template. An example condition of Sensi-Mix SYBR reaction is described in Table 2.8. The RT-PCR assay was carried out using the Mx3005P QPCR System**.** Thermal cycling was initiated with 5 mins at 95ºC, followed by 40 cycles of 10 seconds at 95ºC and 1 min at 60°C.

Table 2.8 Sybr-Green RT-PCR condition

Component	Concentration	Volume/reaction (μl)
2X Sensi-MixTM SYBR Green QPCR Master Mix	2X	25.0
Primer (forward), see Section 2.1.5	100nM	0.5
Primer (reverse), see Section 2.1.5	100nM	0.5
cDNA	10ng	5.0
PCR-grade water (was added to make up 50μl of mixture)		19.0
	Total	50.0

2.2.3.4 C_T value and relative quantification

The exponential route of C_T value was converted into a linear form using the $2^{-\Delta\Delta CT}$-relative calculation methods. β-actin mRNA was used for normalization for gene expression. HER2 mRNA and HER4 mRNA levels from treatment groups were quantified relative to the respective untreated cells.

2.2.4 Confocal staining

2.2.4.1 Slide preparation

Approximately 10, 000 cells were seeded onto coverslips that had been put onto a 24-well plate. On the following day, the cells were treated with trastuzumab alone or in combination with GSi or neratinib for 1h. Subsequently, the medium containing drugs was withdrawn from the cells and the cells were immediately rinsed with PBS twice.

The cells were then ready for fixation by incubation with 4% PFA for 10 min, followed by incubation with 0.2% Triton-X-100 for 5 min and incubation with Natrium Borohydrate. During the process, the cells were rinsed with PBS twice between the steps. Following this, the cells were incubated with 1% BSA to block unspecific binding of antibodies for an hour. Subsequently, HER4 and HER2 antibodies (dilution at 1:500) were added and incubated for 3 hours. Then the fluorescent 488- and 546-secondary antibodies were applied and incubated for 1 hour (Material 2.1.6). The cells were then rinsed with PBS and distilled water, twice each. The coverslip holding the treated cells was than mounted onto a microscope slide using the Fluoromount-G with DAPI.

2.2.4.2 Confocal microscopy

The immunofluorescence slides were assessed using confocal microscopy (Zeiss LSM 780) to determine the localisation of HER4. DAPI staining was used to indicate nuclear localisation. Analysis for HER4 localisation was done using ZEN 2011 LSM software.

2.2.5 Immunohistochemistry staining

2.2.5.1 Cell pellet preparation

MCF-7, SKBR3, and BT474 cells were seeded in 10cm dishes. HER4 siRNA knockdown was performed in BT474 cells for a period of 72 hours to later show the specificity of the HER4 antibody in immunohistochemistry. Cells were trypsinized, resuspended in media and centrifuged at 1,000 rpm for 3 mins. After removal of the medium, cell pellets were resuspended and fixed in 1 ml 4% PFA overnight at 4 °C. Cells were re-pelleted by centrifugation at 1,000 rpm for 3 mins and resuspended in 500 µl

PBS. 2% agarose/formalin was liquefied in the microwave. The melted agarose was placed in a 60°C water bath for equilibration while preparing the cell pellet. After pre-heating at 60°C for five mins, the cell suspension was again re-pelleted by centrifugation at 1,000 rpm for 3 mins to remove the PBS. 500 µl of melted agarose/formalin was added and immediately vortexed to re-suspend the cell pellet, followed by a centrifugation at 1,000 rpm for 1 mins. The tube was placed on ice to solidify the suspension for 1 min. Later, the gel with the cell pellet was removed from the tube. The pellet was then subjected to processing procedures in the Histopathology Laboratory of the John Radcliffe Hospital, Oxford. The processed samples were embedded in a paraffin block, which was then trimmed and sectioned with a microtome to obtain 4µm sections. The ribbons of sectioned tissue were floated in a 40°C water bath and placed on X-tra™ Adhesive slides (Leica), and air-dried.

2.2.5.2 Xenograft models

HER4 immunostaining was carried out in two sets of *in vivo* experiments:

1. Xenograft experiments comparing trastuzumab treatment with vehicle control was described earlier (Kramer-Marek et al., 2012). For treatment group (n=5), mice received trastuzumab intraperitoneally with an initial dose of 50 mg/kg and maintenance doses at 25 mg/kg for a period of 2 weeks. Control mice were given saline vehicle.
2. Details of the animal experiments involving neratinib and trastuzumab have been described in previous report (Canonici et al., 2013; Kramer-Marek et al., 2012). BT474 tumors were implanted into BALC/nu/nu mice and the mice were divided into four different groups according to different drug treatments; control group (n=4) and treatment groups (n=5 for each condition) were treated with

neratinib (10mg/kg), trastuzumab (10mg/kg), or a combination of neratinib (10mg/kg) and trastuzumab (10mg/kg). Tumor samples were collected after 16 days of treatment (Canonici et al., 2013; Kramer-Marek et al., 2012).

2.2.5.3 Experiments involving human tissues

a. Tissue microarrays (TMA) of HER2 positive breast cancer patients were obtained from Oxford Radcliffe Biobank, a cohort study that was approved by the UK Human Tissue Act 2004. Patients' histological information including age, sex, tumor grade, menopausal, nodal and ER status and patients' data on relapse free as well as overall survival was obtained from the medical records.

b. A set of formalin-fixed paraffin embedded tissues was obtained from a clinical trial conducted at UOM Patologia Mammaria-Az. Instituti Ospitalieri di Cremona. This study was approved by local ethical approval (Protocol CE-21392/2012). This retrospective study included consecutive cases of HER2 positive breast cancer patients that underwent chemotherapy and trastuzumab treatment and was performed from April 2010 to December 2012. Patients' clinical information was obtained from accessible medical records.

2.2.5.4 Immunohistochemistry (IHC) techniques

The immunohistochemistry technique was performed to assess the expression of HER4. The procedure was used on formalin-fixed paraffin embedded tissue sections. To assess the specificity of the reaction, negative controls (incubation without primary antibody) were included. 4μm thick tissue sections mounted on poly-L-lysine slides were deparaffinised in a 60°C oven for 10 mins, followed by immersing the sections in

two changes of Histoclean (6 mins each). The sections were then gradually hydrated in graded alcohol solutions: two changes of absolute alcohol (5 mins each) and 5 mins in 50% alcohol. The tissue sections were then rinsed in PBS for 5 mins each.

To recover tissue antigenicity, the sections were incubated in 10mM citrate buffer, pH 6.0, in a pressure cooker for 30 mins. Sections were allowed to cool at room temperature for 30 mins. Subsequently, 3 drops of blocking reagent (normal horse serum blocking solution from ImmPRESS™ Anti-Rabbit Ig reagent kit) were applied onto each section and incubated for 15 mins. The sections were then rinsed with PBS twice. HER4 primary antibody (rabbit polyclonal; Neomarkers) was diluted in RMPI at 1:200. The dilution was applied to each section and incubated at 4°C overnight. After the primary antibody incubation, the sections were rinsed with PBS twice. The sections were then incubated with 2 drops of ImmPRESS™ Anti-Rabbit Ig reagent (150µl) and incubated for 30 min. The sections were rinsed with PBS twice. For visualization, the slides were covered with DAPI for 5 mins and then by washed in distilled water. The slides were counterstained with haematoxylin, dehydrated and mounted.

2.2.5.5 IRS scoring

The IHC slides were then reviewed by light microscopy using 100x, 200x and 400x magnification. All IHC slides were reviewed together with a consultant breast pathologist, Dr Roxanis Ionannis, to determine a HER4 scoring value. HER4 expression level was scored semi-quantitatively using the immunoreactive score (IRS), which considers staining intensity as well as distribution. The final IRS was determined by multiplying the intensity scores (Table 2.9) with the percentage of positive cells (Table 2.9), giving the outcomes between 0 (no staining) and 12 (maximum score) (Han et al., 2008).

Table 2.9 The score for staining intensity and percentage of positive cells.

Score for staining intensity	Score for distribution of positive cells
0 = negative 1 = weak 2 = moderate 3 = strong	0=<1% 1=1%–10% 2=11%–50% 3=51%–80% 4= >80% positive cells.

2.2.6 Statistical Analysis

For *in vitro* experiments, statistical analyses were performed using t-Test for a comparison between two groups and one-way ANOVA with Bonferonni's correction test for comparison among multiple groups. Data are shown as means ± SEM. In *in vivo* models, the Mann-Whitney (Wilcoxon rank-sum test) was used to compare HER4 protein level between two groups due to small number of mice in each group; while the Kruskal Wallis rank-sum test with Dunn's correction was applied on the analysis involving more than two groups in xenograft experiments involving neratinib and trastuzumab (Canonici et al., 2013; Kramer-Marek et al., 2012). The above data analysis was done using the GraphPad Prism 6 for MAC OS X, and p values < 0.05, <0.01 and <0.001 were considered as statistically significant and were marked as *, ** and ***, respectively.

The correlation of HER4 scoring with the other clinical parameters was assessed using Fisher's Exact test. The Kaplan-Meier survival curves analysis and Cox proportional hazard models were carried out to correlate HER4 expression with disease-free and overall survivals. The univariate and multivariate Cox proportional analysis followed a protocol from Machleidt et al. (2013), which using the clinical univariable that has a $p < 0.1$ for the multivariate analysis (Machleidt et al., 2013). Data from the analysis were presented in hazard ratios (HR) with 95% confidence intervals (CI) and $p<0.05$ indicated statistical significance. These Fisher's Exact, Kaplan-Meier survival curves and Cox proportional hazard models analyses were performed using IBM SPSS Statistics 20.0. The results were discussed and validated with Dr. Syed Haider (a postdoctoral research fellow in the bioinformatics department), using the R statistical environment (v.2.14.1) (R package: survival v2.36-14).

CHAPTER THREE

The effect of trastuzumab treatment on HER4 in HER2 positive breast cancer cells and xenograft models

CHAPTER 3: THE EFFECT OF TRASTUZUMAB TREATMENT ON HER4 IN HER2 POSITIVE BREAST CANCER CELLS AND XENOGRAFT MODELS

3.1 Introduction

Trastuzumab is a monoclonal antibody that was developed to combat HER2 activity (Slamon et al., 2001). However, activation of other HER receptors i.e. EGFR and HER3 have been reported to reduce trastuzumab efficiency in decreasing cell survival (Dua et al., 2010; Spector and Blackwell, 2009; Wang et al., 2008). Unlike other HER receptors, the role of HER4 in relation to trastuzumab treatment and resistance remains unclear. Previous studies have demonstrated that the dimerisation between HER4 and HER2 appeared to be oncogenic (Mill et al., 2012; Wang et al., 1998). However, HER4 over-expression was shown to decrease cell proliferation and induce apoptosis in breast cancer cells that lack HER2 expression (Sartor et al., 2001), suggesting that HER4 activity may not necessarily be dependent on HER2. Another finding indicated that higher expression of HER4 increases trastuzumab sensitivity in patients with metastatic HER2 positive breast cancer (Sassen et al., 2009). In addition, a recent study showed that patients with HER2/HER4 co-over-expression had a better outcome after neo-adjuvant trastuzumab therapy and adjuvant trastuzumab therapy (Portier et al., 2013). These studies suggest that HER4 expression may play a key role in relation to trastuzumab treatment and resistance in HER2 positive breast cancer. However, there have been no detailed reports on how trastuzumab affects HER4 expression, activation and cleavage or how these processes mediate sensitivity and resistance to trastuzumab. Therefore, the aim of this study is to elucidate the role and prognostic significance of

HER4 cleavage and localisation in relation to trastuzumab treatment and resistance in HER2 positive breast cancer.

This chapter provides findings of the effect of trastuzumab on HER4 expression and signalling in HER2 over-expressed SKBR3 and BT474 cells, using the MCF-7 cell line as the control cell line since it has low levels HER2. The effect of trastuzumab treatment on HER4 was also compared with the exogenous heregulin stimulation because our lab has previously shown that heregulin (HER4 ligand) is released during trastuzumab treatment (Gijsen et al., 2010; Kong et al., 2008). The levels of HER4 protein and mRNA were analysed using western blot and RT-PCR in breast cancer cells treated with different doses of trastuzumab for various durations. HER4 localisation before and after trastuzumab treatment was determined by confocal microscopy and nuclear fractionation. In addition, HER4 expression and its localisation were compared in trastuzumab parental and acquired resistant SKBR3 and BT474 cells. HER4 expression and localisation was also assessed using immunohistochemistry in BT474 xenograft samples from mice treated with trastuzumab in comparison to the control.

3.2 Results

3.2.1 HER4 basal expression in HER2 positive breast cancer cells in comparison with the control cell line

SKBR3 and BT474 breast cancer cell lines were chosen as models of HER2 over-expressed breast tumors. MCF-7 cells were used as a negative control since they have a low level of HER2. Figure 3.1A shows the basal levels of HER4 in all cell lines. From the western blot, the basal expression of HER4 in SKBR3 cells was comparable to the expression in MCF-7 cells (Figure 3.1A). However, it seems that HER4 was lower in the BT474 cells. Figure 3.1B shows HER4 localisation in MCF-7, SKBR3 and BT474, as

assessed using confocal microscopy. In all cell lines, HER4 was seen to be localised at the cytoplasm.

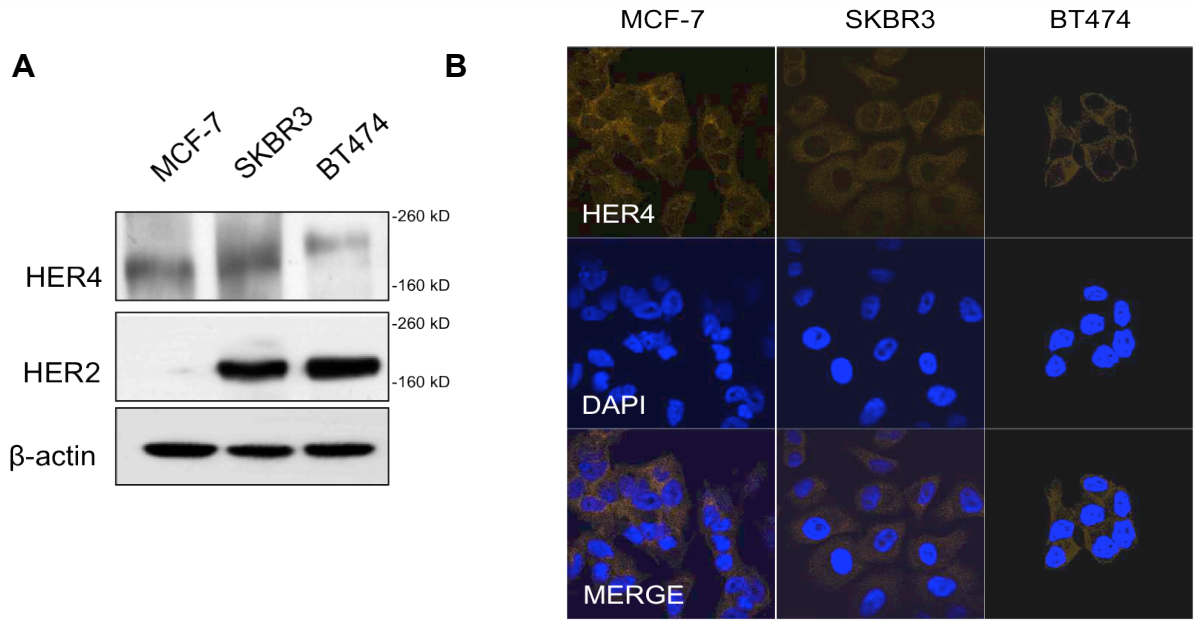


Figure 3.1 HER4 basal expression in MCF-7, SKBR3 and BT474 cells.

(A) Cell lysates from MCF-7, SKBR3 and BT474 cells were used to perform western blot analysis, probing for HER4 and HER2 with β-actin used as the loading control. (B) Confocal images of HER4 and HER2 localisation in MCF-7, SKBR3 and BT474 cells.

3.2.2 Effects of heregulin stimulation on HER4 expression and its localisation

To assess the effect of heregulin on HER4, the SKBR3 and BT474 cell lines were stimulated with heregulin at different time points. Exogenous heregulin stimulation after 30 and 60 mins in SKBR3 cells increased HER4 proteins at 180kDa and 80kDa, as assessed by the western blot (Figure 3.2A). In BT474 cells, the increase in 180kDa and faint 80 kDa bands was seen from 15 mins. There were also additional bands seen at 160kDa, 110kDa and 120kDa (Figure 3.2B). From RT PCR analysis, heregulin stimulation from 30 to 60 mins induced HER4 mRNA upregulation in SKBR3 and BT474 cells, which was statistically significant compared to the control.

Confocal microscopy experiments were performed to assess the effect of exogenous heregulin on HER4 localisation. From the confocal images (Figure 3.3A), heregulin stimulation increased HER4 intensity in the nucleus in SKBR3 cells from 15 mins. Nuclear HER4 intensity in SKBR3 after 60 mins stimulation with heregulin was significantly increased compared to the control ($n=3$, $p<0.05$, Figure 3.3A, right panel). In the BT474 cell, the intensity of cytoplasmic HER4 was seen to be higher than nuclear HER4 intensity, but no obvious change on HER4 intensity in the cytoplasmic could be detected with different time points of heregulin stimulation in BT474 cells (Figure 3.3B). A relative quantification of nuclear HER4 intensity shows a slightly increase of nuclear HER4 after 60 mins heregulin stimulation as compared to the control, but this was not significant (Figure 3.3 C, right panel).

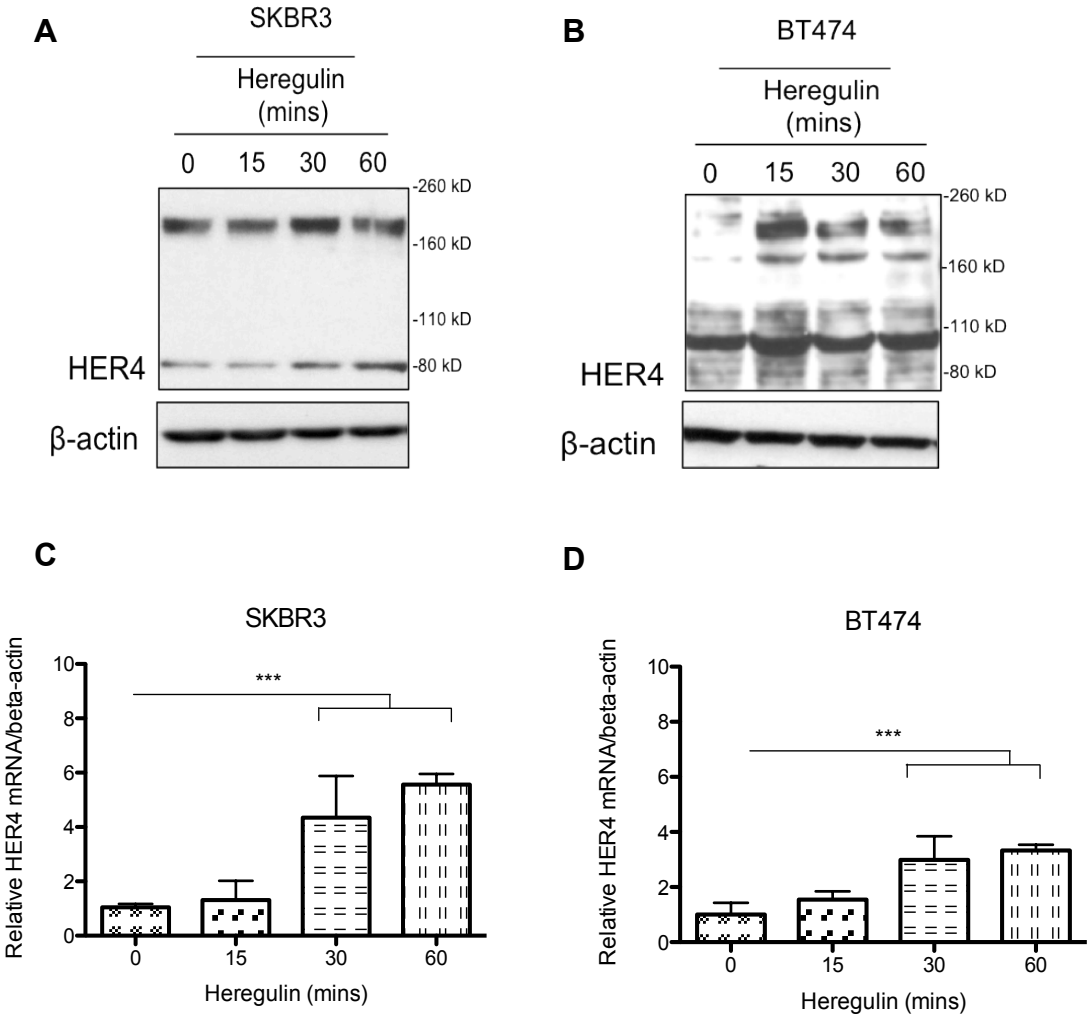


Figure 3.2 HER4 expression is upregulated following exogenous heregulin stimulation at different time points in SKBR3 and BT474 cells.

(A, B) SKBR3 and BT474 cells were lysed for western blot analysis after 100 ng/ml heregulin stimulation for 0, 15, 30 or 60 mins. Equal amounts of protein were loaded on each lane of the SDS gels and the membranes were probed with a cytoplasmic anti-HER4 (Neomarkers) antibody and β-actin was used as the loading control. (C, D) HER4 mRNA levels of SKBR3 and BT474 cells were measured by RT-PCR following heregulin stimulation. β-actin mRNA was used for normalisation and the quantification was relative to the untreated cells. Graphs represent mean ± SEM of relative HER4 mRNA. *P* value was calculated using a Bonferroni correction for multiple comparisons (*** denotes $p<0.001$).

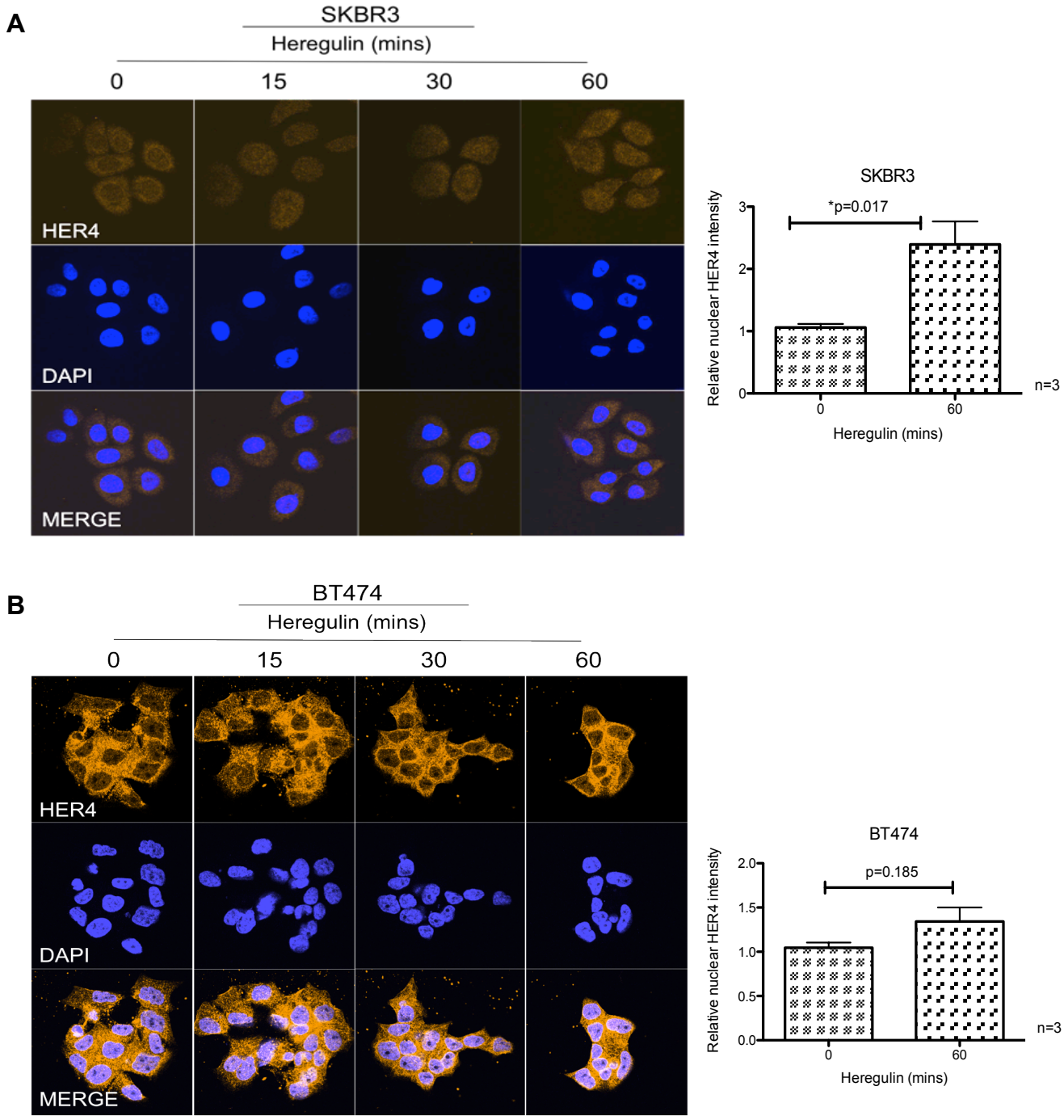


Figure 3.3 The effect of exogenous heregulin on HER4 localisation in SKBR3 and BT474 cells.

SKBR3 cells (A) and BT474 cells (B) were stimulated with exogenous heregulin at 100ng/ml for 0, 15, 30 or 60 mins before being fixed for confocal microscopy experiments. Representative confocal images (left panel) show HER4 localisation as

indicated by HER4-Alexa Fluor 546 staining; DAPI staining was used as a control for nuclear localisation. The right panel of each figure indicates a relative quantification graph of HER4 intensity at nuclear compartment (mean ± SEM) in cells stimulated with heregulin at 0 and 60 mins, as quantified from three representative confocal images using Image J software. A t-test was applied to determine the significant difference of nuclear HER4 intensity between the two groups ($p<0.05$).

3.2.3 Effects of trastuzumab treatment and heregulin stimulation on HER4 expression and localisation in MCF-7 cells

In primary resistant HER2 negative MCF-7 cells, one-hour treatment of trastuzumab and heregulin induced an upregulation of HER4 expression at 180kDa (Figure 3.5A, upper panel). There was a faint band of HER4 m80 appearing at a longer time of exposure but no obvious increase in the level after trastuzumab treatment or heregulin stimulation. However, treatment with trastuzumab and heregulin significantly upregulated HER4 mRNA level compared to the untreated MCF-7 cells ($n=3$, $p<0.001$, Figure 3.5B, bottom panel). An hour-long treatment with trastuzumab and stimulation with heregulin increased the intensity of HER4 in the cytoplasmic compared to the untreated MCF-7, $p< 0.05$ (Figure 3.5C).

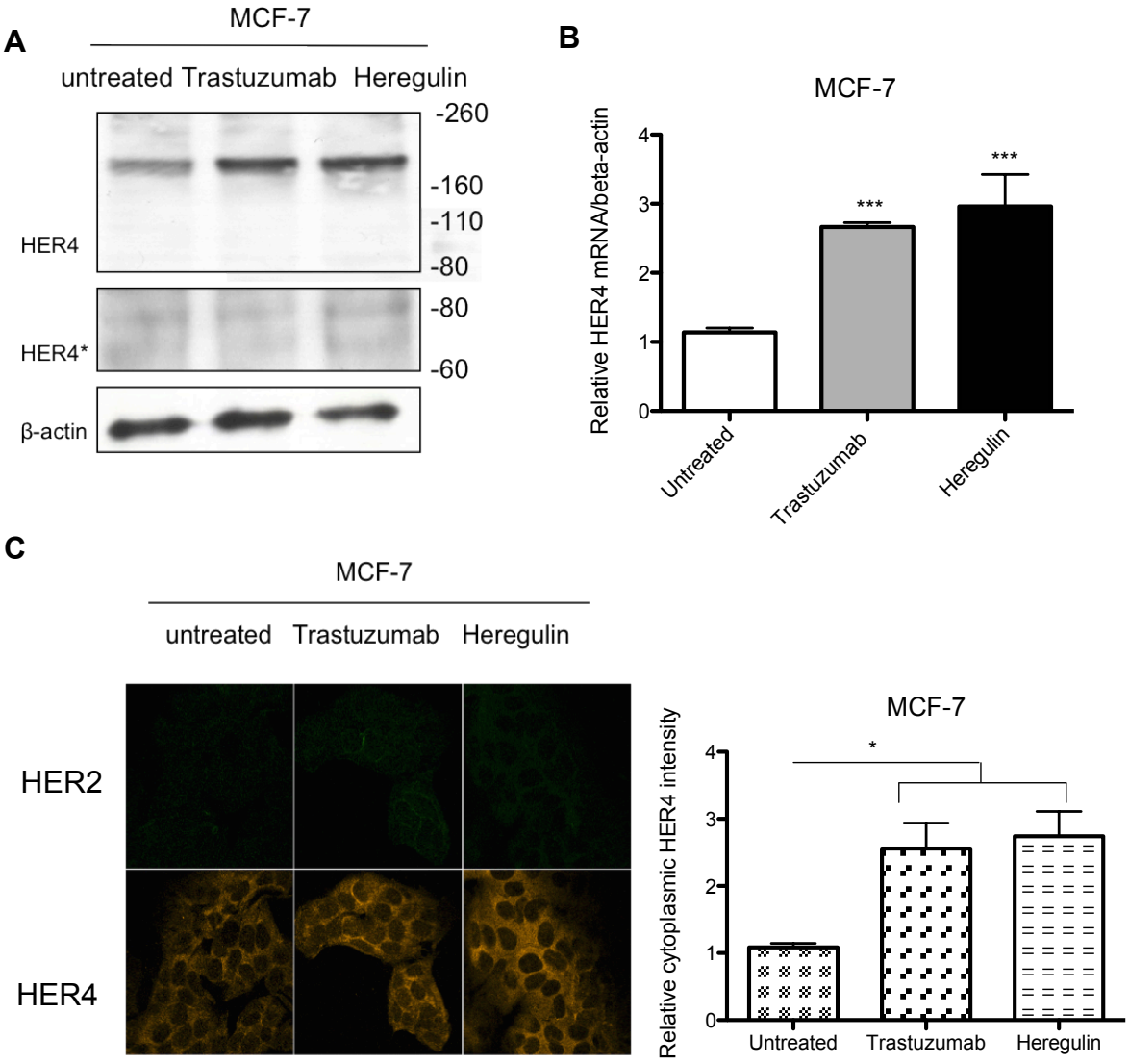


Figure 3.4 Trastuzumab treatment and heregulin stimulation increase HER4 expression in MCF-7 cells.

MCF-7 was treated with 40μg/ml trastuzumab and 100ng/ml heregulin for a duration of one hour. (A) Cells were lysed for western blot analysis with equal amounts of protein loaded on each lane of the SDS gels and membranes were probed with cytoplasmic anti-HER4 (Neomarkers) and β-actin. HER4* indicates that the membrane was exposed for a longer time to obtain a band at 80 kDa. (B) RNA was extracted from cells after trastuzumab treatment and heregulin stimulation to assess HER4 mRNA

expression via RT-PCR experiment. The HER4 mRNA of each group was quantified relative to the untreated cells and normalised to β-actin. The graph represents mean ± SEM of HER4 mRNA. The significant differences of HER4 mRNA among the group were noted at *p<0.05 and ***p<0.001, as *p* value was calculated using a Bonferroni correction for multiple comparisons.(C) Cells were fixed for a confocal microscopy experiment. Cells were stained with HER4-Alexa Fluor 546 to determine HER4 localisation, with HER2-Alexa Fluor 488 staining used as a control. A quantification of cytoplasmic intensity of HER4 was done using Image J software; the graph of relative cytoplasmic intensity (mean ± SEM) among the treatment group is shown in the right panel (*p<0.05). *P* value was determined using a Bonferroni correction for multiple comparisons (*** denotes p<0.001).

3.2.4 Effects of trastuzumab treatment on HER4 expression in SKBR3 and BT474

Similar to exogenous heregulin stimulation, one-hour trastuzumab treatment resulted in an upregulation of HER4 protein level (Figure 3.4A, upper panel), as shown by two different cytoplasmic anti-HER4 antibodies. The semi-quantitative blot of Neomarkers' anti-HER4 cytoplasmic antibody showed that the levels of $HER4_{180kDa}$ and $HER4_{80kDa}$ were statistically significantly increased in SKBR3 cells treated with one hour of trastuzumab compared to the untreated cells ($n=3$, $p<0.01$, Figure 3.4A, bottom panel). In BT474 cell lines, one hour of trastuzumab resulted in an upregulation of HER4 at 180kDa, 120kDa and 110kDa and 80kDa, as was similarly seen in heregulin stimulation (Figure 3.4B, upper panel). These bands were not detected in SKBR3 cells but the additional bands were also detected using another anti-HER4 antibody from Santa Cruz (Figure 3.4A & B). In addition, siRNA experiments in Chapter 4 (Figure 4.4) demonstrated that these bands are specific to HER4. There was also an increase in HER4 phosphorylation in SKBR3 and BT474 cells following one hour of heregulin stimulation and trastuzumab treatment (Figure 3.4A & B, upper panel).

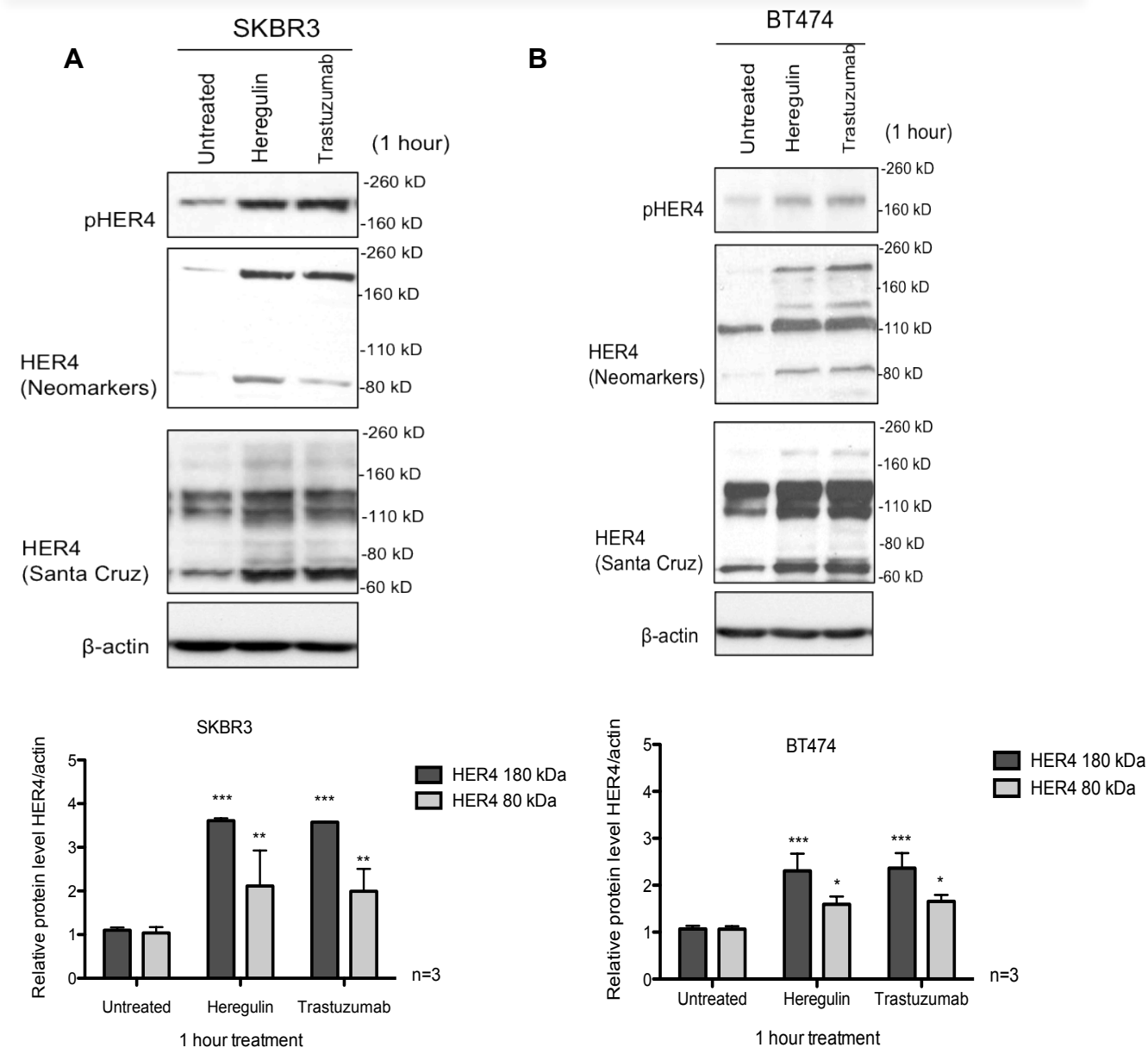


Figure 3.5 HER4 expression is upregulated following heregulin stimulation and trastuzumab treatment.

(A & B) SKBR3 and BT474 cells were treated with 40μg/ml trastuzumab or stimulated with heregulin (100ng/ml) for one hour, before cells being lysed for western blot analysis. Membranes were blotted with two different anti-HER4 antibodies; HER4 (Neomarkers) and HER4 C18 (Santa Cruz: SC283) as well as β-actin. The quantification of HER4 protein level was done for an anti-HER4 antibody from Neomarkers after it

was normalised to β-actin (bottom panels). Mean ± SEM from three independent experiments are shown in the graph. To determine the significant differences in HER4 protein between groups, *p* value was calculated using a Bonferroni correction for multiple comparisons (* $p<0.05$, ** $p<0.01$ and *** $p<0.001$).

3.2.5 Dose-response effects of trastuzumab on HER4 expression

An increase in the 80kDa and 180kDa HER4 bands was seen in both SKBR3 and BT474 treated with increasing concentrations of trastuzumab for one hour (Figure 3.6A). In addition, the additional 110 and 120kDa of HER4 bands were also detected in BT474 cells (Figure 3.6A, right panel). As HER2 is a target for trastuzumab treatment, the expression of HER2 was used as treatment control. However, no obvious changes of HER2 protein could be seen with increasing doses of trastuzumab for one hour in both SKBR3 and BT474 cells (Figure 3.6A & B, upper panel). Moreover, in both SKBR3 and BT474 cells, the significant differences in HER4 mRNA upregulation were noted at higher trastuzumab doses (40μg/ml and 80μg/ml) compared to the control ($n=3$, $p<0.001$, Figure 3.6B). Higher trastuzumab doses also resulted in HER2 mRNA downregulation, which was statistically significant in BT474 cells ($n=3$, $p<0.001$, Figure 3.6B).

3.2.6 Treatment duration effects of trastuzumab on HER4 expression

A time-course experiment was performed to assess the effect of trastuzumab treatment on HER4 expression in SKBR3 and BT474 cells in comparison with the acquired resistant cells generated in the lab as previously described (Gijsen et al., 2010). The acquired drug resistance of SKBR3 and BT474 cells was first confirmed (Figure 3.7A). Compared to untreated SKBR3 cells, trastuzumab treatment statistically significantly increased $HER4_{180kDa}$ and $HER4_{80kDa}$ levels from one hour and a further increase was seen with longer treatment and in acquired resistant SKBR3 cells (Figure 3.7B). The increase in $HER4_{180kDa}$ and $HER4_{80kDa}$ levels was also seen in BT474 cells but a statistical significance for both bands was also seen in resistant cells. In contrast to trastuzumab-resistant SKBR3 cells that were continuously treated with trastuzumab, an

overnight trastuzumab withdrawal reduced $HER4_{180kDa}$ and $HER4_{80kDa}$ protein levels ($p<0.001$) (Figure 3.7B, bottom panel). Trastuzumab withdrawal was also seen to reduce HER4 m80 protein levels in the resistant BT474 cells but the difference was not statistically significant (Figure 3.7C). There was also a statistically significant increase in HER4 mRNA levels after trastuzumab treatment and upon acquired resistance in both cell lines (Figure 3.7D). Withdrawal of trastuzumab from the resistant SKBR3 cells decreased HER4 mRNA level ($p<0.001$) (Figure 3.7D, left panel), which was not seen in BT474 cells (Figure 3.7 D, right panel).

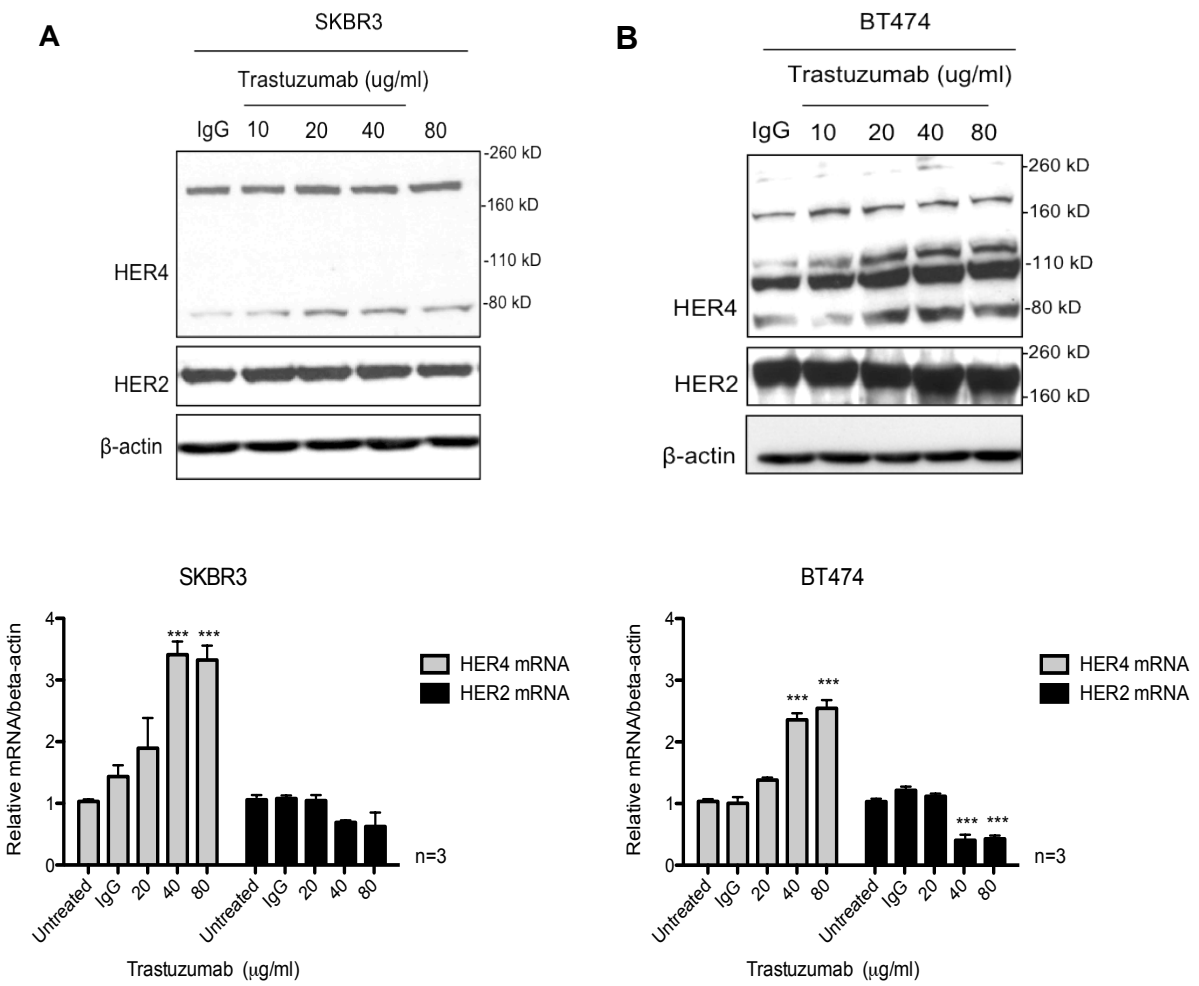
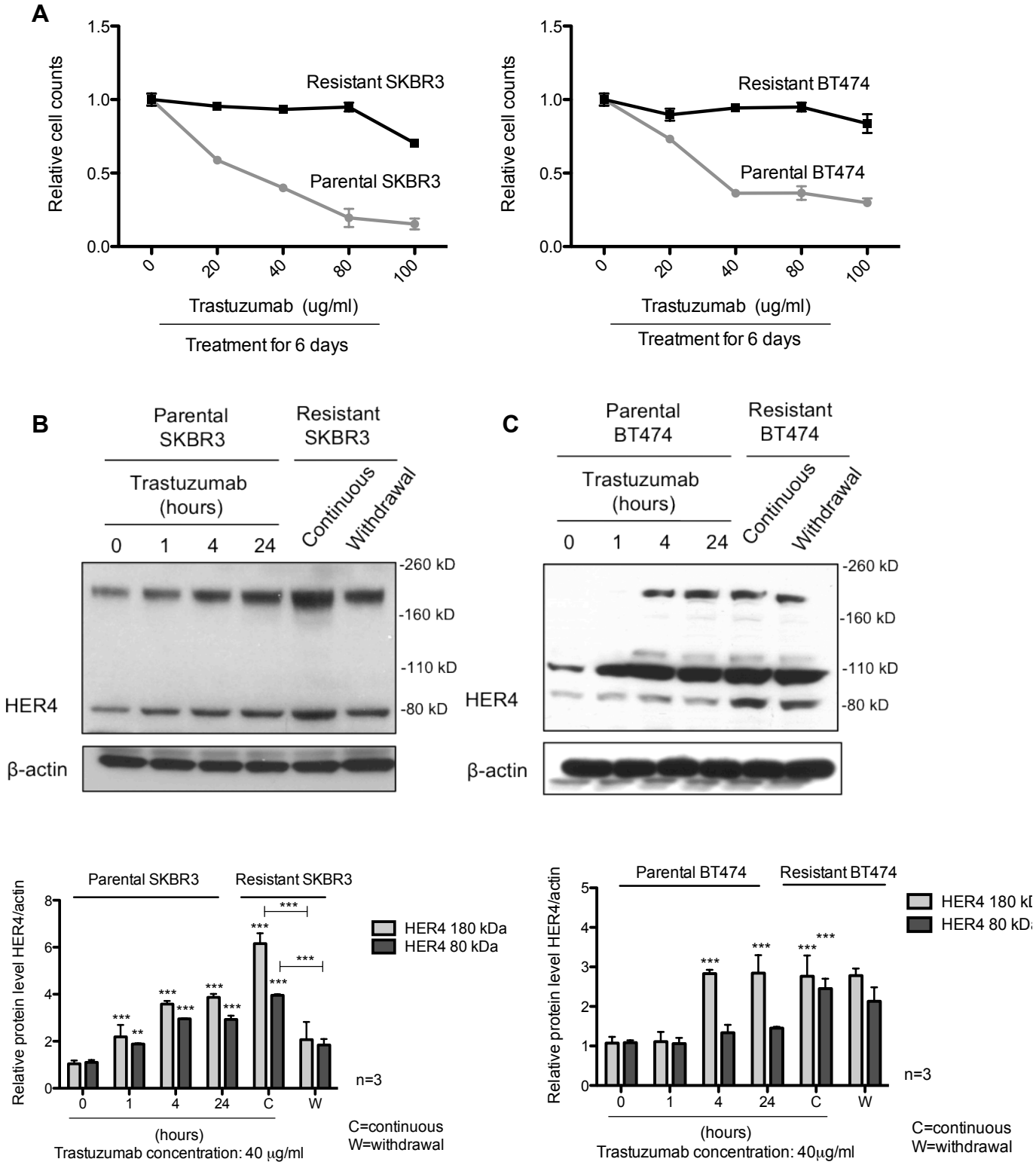


Figure 3.6 HER4 expression is upregulated after trastuzumab treatment in a dose dependent-manner.

(A & B) SKBR3 and BT474 cells were treated with increasing concentrations of trastuzumab for one hour and the lysates were prepared for western blot analysis by probing the membranes with anti-HER4 antibody (Neomarkers). HER2 was used as a treatment control. HER4 expression in relation to trastuzumab dose-response was also determined at mRNA level via RT-PCR (the bottom panel of each figure). The significant differences of HER4 mRNA among the group were noted at $p<0.001$***, as determined by a Bonferroni correction for multiple comparisons. The graph represents mean ± SEM.


A
Relative cell counts
Resistant SKBR3
Parental SKBR3
Trastuzumab (ug/ml)
Treatment for 6 days
Resistant BT474
Parental BT474
B
Parental SKBR3
Resistant SKBR3
Trastuzumab (hours)
Continuous
Withdrawal
HER4
β-actin
-260 kD
-160 kD
-110 kD
-80 kD
C
Parental BT474
Resistant BT474
Relative protein level HER4/actin
HER4 180 kDa
HER4 80 kDa
n=3
(hours)
Trastuzumab concentration: 40 μg/ml
C=continuous
W=withdrawal

D

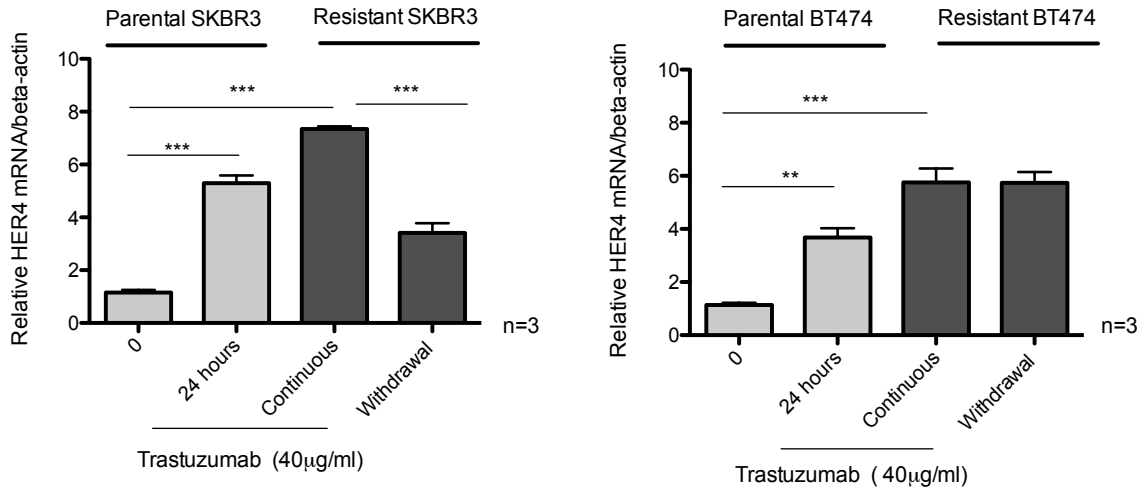


Figure 3.7 Trastuzumab treatment upregulates HER4 protein and mRNA levels at different time points.

HER4 expression was analysed in HER2 positive breast cancer cell lines after treatment with trastuzumab at different time points and in comparison with acquired resistant cells. (A) The cell count experiments were done in parental and resistant SKBR3 and BT474 cells after 6 days of treatment with increasing doses of trastuzumab as indicated. The description of the resistant cells is stated in Section 2.1.1 (B, C) Parental SKBR3 and BT474 cells were treated with trastuzumab at 0, 1, 4 and 24 hours in comparison with acquired trastuzumab resistant cells (with or without trastuzumab 24 hr withdrawal). Cells were lysed for western blot and membranes were probed with anti-HER4 (Neomarkers) and β-actin. The bottom panel of each figure indicates a relative quantification graph of HER4 intensity at 180 kDa and 80 kDa, as quantified from 3 representative blots using Image J software. β-actin was used for normalisation and the quantification was relative to the untreated cells. (D) HER4 mRNA levels of parental and trastuzumab resistant SKBR3 and BT474 cells were measured by RT-PCR.HER4 mRNA was quantified relative to the untreated cells, and normalised to β-actin. Mean ± SEM from three independent experiments are shown in the graph. *P* value was calculated using a Bonferroni correction for multiple comparisons (*p<0.05, **p<0.01 and ***p<0.001).

3.2.7 Effects of trastuzumab treatment on HER4 localisation in HER2 positive cells

The effect of trastuzumab treatment on HER4 localisation was assessed in HER2 positive breast cancer cell lines (Figure 3.8 & Figure 3.9). Compared to the untreated SKBR3 cells (30% ± 1.92), the percentage of the cells that had nuclear HER4 staining was increased to 83.33% ± 5.09 ($p<0.01$), 96.22% ± 2.32 ($p<0.01$) and 99.33% ± 0.66 ($p<0.01$) after trastuzumab treatment at 1, 4 and 24 hours respectively (Figure 3.8 A & B). There was also increased HER4 nuclear translocation in acquired resistant SKBR3 cells (Figure 3.8A), which was statistically significant ($p<0.01$) compared to untreated parental SKBR3. However, an overnight trastuzumab withdrawal caused a significant decrease in the percentage of nuclear HER4 positive cells compared to the continuously treated cells ($p<0.001$) (Figure 3.8A & C). Fractionation experiments showed that trastuzumab treatment upregulated mainly $HER4_{180kDa}$ but not $HER4_{80kDa}$ in the cytoplasmic fraction (Figure 3.8B). However, there was an increased $HER4_{80kDa}$ level in the nuclear fraction, correlated with HER4 localisation seen in the confocal experiments (Figure 3.8B).

To ensure that trastuzumab induced nuclear translocation in BT474, as it does in SKBR3, nuclear HER4 intensity was assessed by from a confocal microscopy in trastuzumab parental and resistant BT474 cells (Figure 3.9). Nuclear HER4 in trastuzumab-resistant BT474 was slightly increased, as compared to untreated parental BT474; this was shown to be significant, $p<0.05$ (Figure 3.9 A). In addition, cells that have positive nuclear HER4 staining were higher after trastuzumab treatment at 24 hours and trastuzumab resistant compared to untreated cells (Figure 3.9 A & B). However, only the number of cells with positive nuclear HER4 staining in acquired

resistant BT474 was statistically significantly higher compared to untreated parental BT474 ($n=3$, $p<0.05$, Figure 3.9 C). An overnight trastuzumab withdrawal in fact caused a decrease in the number of cells with positive nuclear HER4 intensity, but this was not significant (Figure 3.9 B & C).

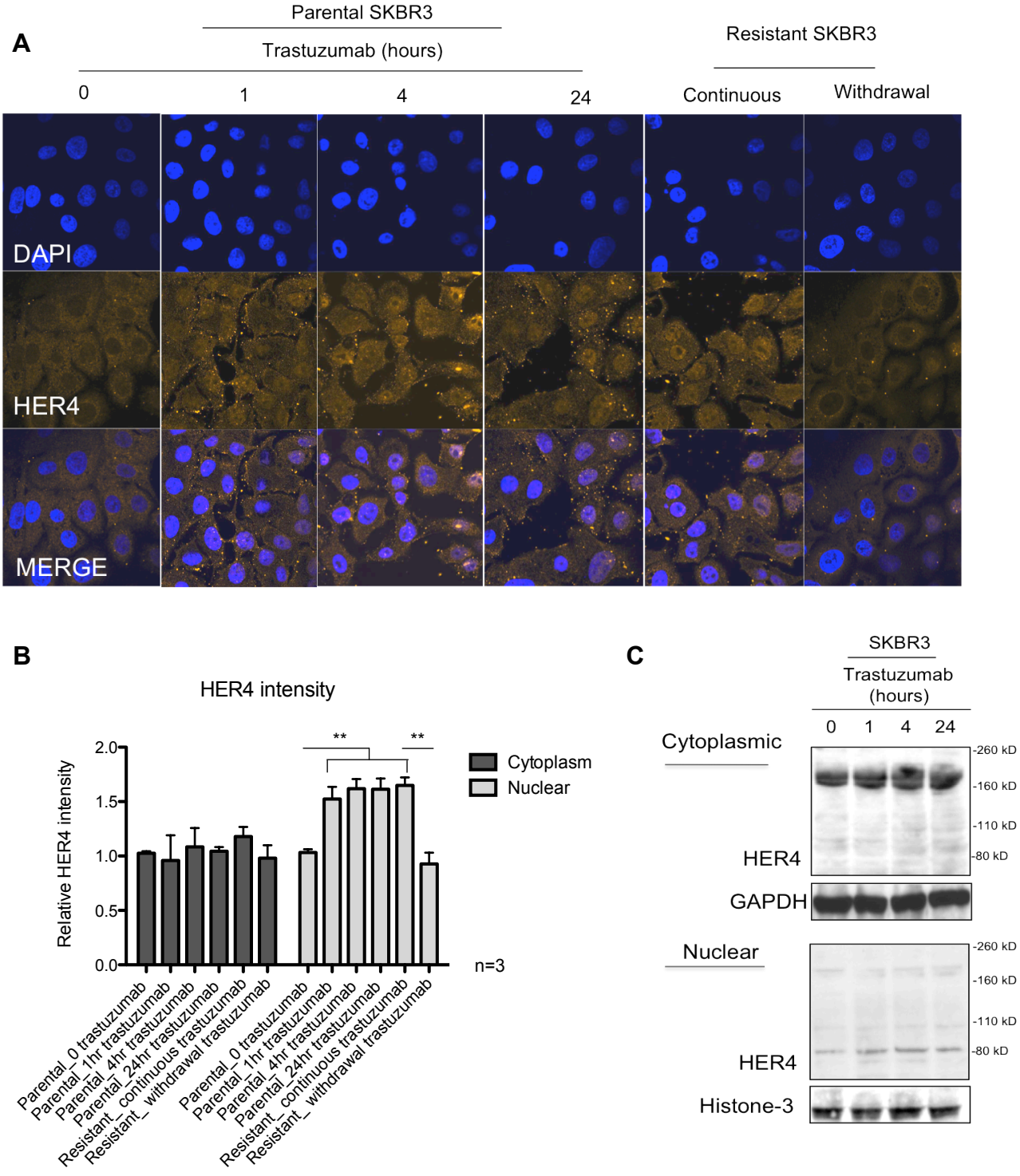
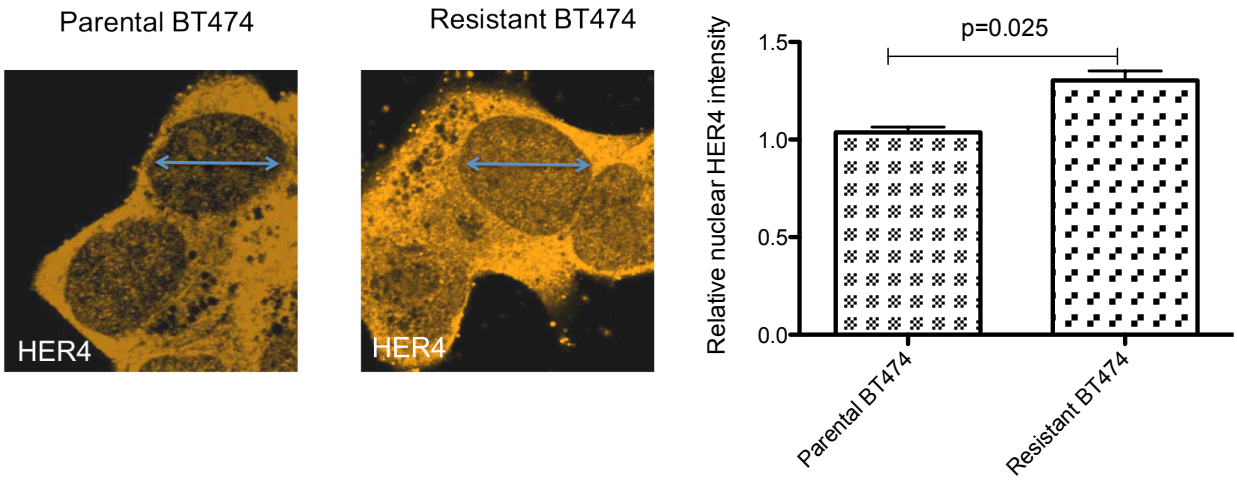


Figure 3.8 Trastuzumab treatment and resistance induces nuclear HER4 localisation in SKBR3 cells.

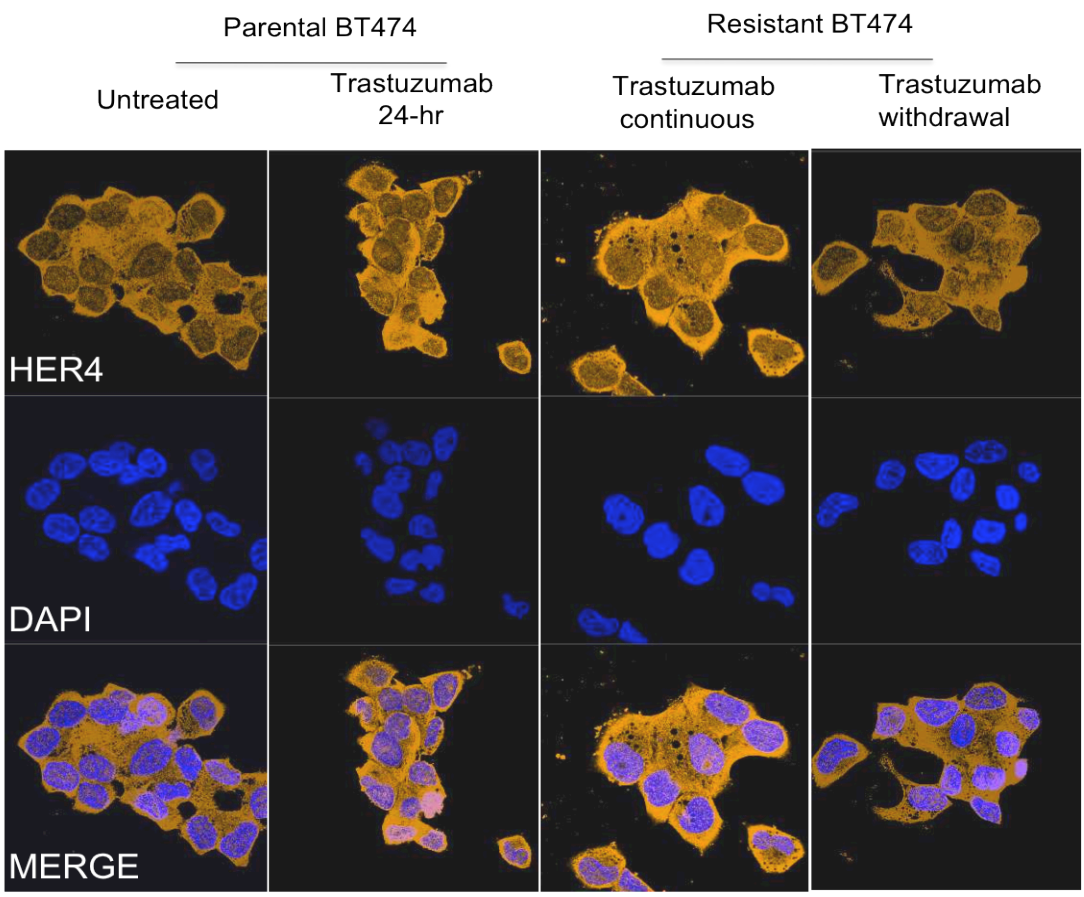
(A) Parental and acquired trastuzumab resistant SKBR3 cells were plated and left overnight to adhere. Parental cell lines were treated with 40µg/ml trastuzumab for a

period of 0, 1, 4 and 24 hours. Resistant cells were grown in media with and without trastuzumab for an overnight incubation. After treatment was completed, cells were fixed for a confocal microscopy experiment. Cells were stained with HER4-Alexa Fluor 546 to determine HER4 localisation. DAPI staining was used to determine nuclear localisation. (B) The graph showing relative HER4 intensity at cytoplasmic and nuclear compartments. Mean ± SEM from three independent experiments is shown in the graph and *p* value was calculated using a Bonferroni correction for multiple comparisons (**p<0.01). (B) Western blot analysis was performed on the cytoplasmic and nuclear fractions of SKBR3 cells isolated using a NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Scientific). After cell fractionation, GAPDH and Histone-3 levels were used as the loading controls for cytoplasm fraction and nuclear fraction respectively.

A


Parental BT474
Resistant BT474
HER4
HER4
p=0.025
Relative nuclear HER4 intensity
1.5
1.0
0.5
0.0
Parental BT474
Resistant BT474

B


Parental BT474
Resistant BT474
Untreated
Trastuzumab 24-hr
Trastuzumab continuous
Trastuzumab withdrawal
HER4
DAPI
MERGE

C

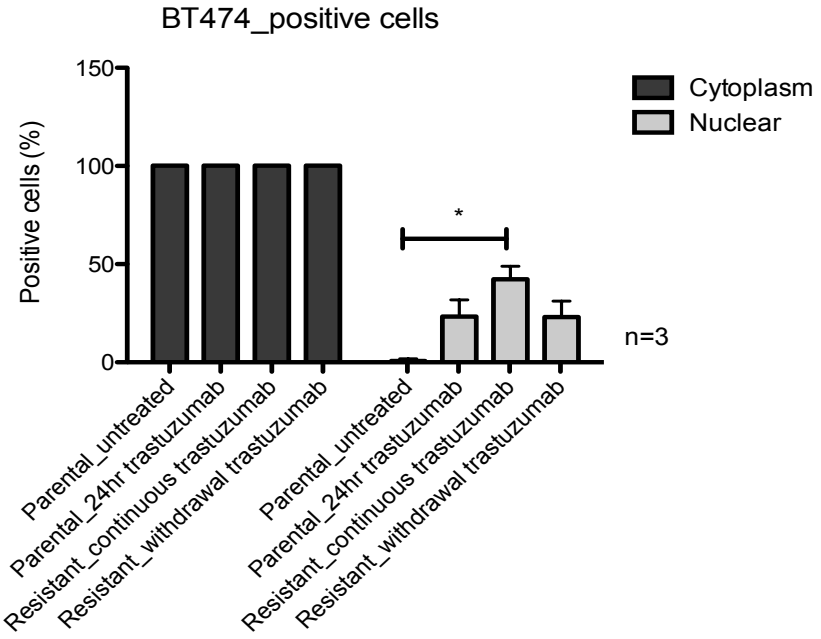


Figure 3.9 The effect of trastuzumab on HER4 localisation in BT474 parental and resistant cells.

Parental and acquired trastuzumab resistant BT474 cells were plated and left overnight to adhere. Parental cell lines were treated with 40µg/ml trastuzumab for 24 hours. Resistant cells were grown in media with and without trastuzumab for an overnight incubation. After treatment was completed, cells were fixed for confocal microscopy experiment. Cells were stained with HER4-Alexa Fluor 546 to determine HER4 localisation. DAPI staining was used to determine nuclear localisation. (A) The fluorescence intensity profile for HER4 was along the indicated line at higher magnification 400x. Relative nuclear HER4 intensity (mean ± SEM) between two groups are shown in the graph and a paired t-test was applied to determine significant differences of HER4 protein between groups (* $p<0.05$). (B) Representative of confocal images detecting HER4 expression is shown under magnification 100x. (C) Quantification of the proportion of BT474 parental and resistant cells positive for HER4, cytoplasmic and nuclear. Values are presented as the mean ± SEM, from 3 independent experiments, *$p<0.05$.

3.2.8 HER4 immunostaining on cell pellets and xenograft models

Trastuzumab treatment and resistance was shown to induce HER4 upregulation and nuclear localisation in the in vitro experiments, suggesting the important role of nuclear HER4 in mediating trastuzumab resistance. To validate this, the effect of trastuzumab on HER4 expression and localisation was examined in BT474 xenograft models treated with either control or trastuzumab (Kramer-Marek et al., 2012). Prior to HER4 immunostaining on xenograft tumor tissue, HER4 IHC staining was first optimised in cell pellets from HER2 positive and negative breast cancer cells. It is shown in Figure 3.10A that SKBR3, MCF-7 and BT474 cell pellets had positive HER4 immunostaining. In addition, the staining intensity of HER4 was seen mostly in the cytoplasm in all cell pellets (Figure 3.10A). To determine the specificity of HER4 immunostaining, IHC HER4 staining was done in paraffin-embedded cell pellets from BT474 cells treated with either the negative control siRNA (Scr siRNA) or specific HER4 siRNA. Figure 3.10B demonstrates that HER4 staining was absent in BT474 transfected with specific HER4 siRNA compared to the negative control. HER4 immunostaining on cell pellets was also optimised at different serial dilutions of the primary antibody (Figure 3.10C). The optimum concentration of the primary antibody was determined to be 1:200, which was used for HER4 staining in xenograft tumors.

Figure 3.11 shows positive HER4 immunoreactivity at cytoplasmic and nuclear localisation of untreated BT474 xenograft tumors. From Figure 3.11A, cytoplasmic HER4 intensity scored up to 3+ (strong intensity) in tumors derived from BT474 xenograft mice. Under 400x magnification, the intensity for nuclear HER4 was observed between 0 (negative staining) to 2+ (moderate staining) (Figure 3.11B). Using IRS scoring (Section 2.6.6.3), HER4 expression was shown to increase in the cytoplasm in

the trastuzumab-treated group compared to the control group, but the difference was not statistically significant between the groups (Figure 3.12A). Trastuzumab-treated mice had statistically higher nuclear HER4 intensity compared to the control ($p<0.05$) (Figure 3.11B).

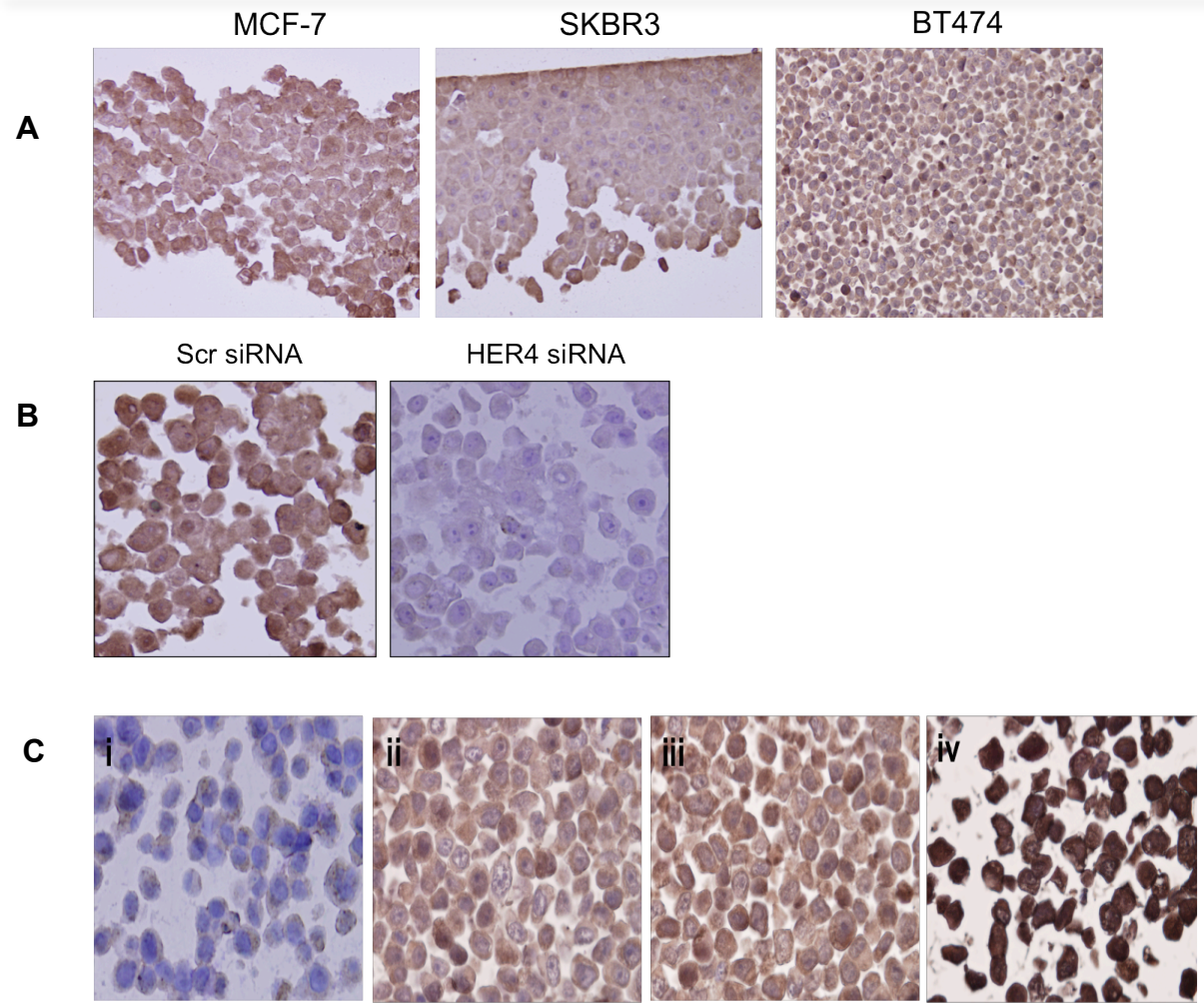


Figure 3.10 HER4 immunostaining on cell pellet.

MCF-7, SKBR3 and BT474 cells were grown to 80% confluency and the cells were trypsinised for cell pellet preparation. (A) Representative of HER4 immunostaining on MCF-7, SKBR3 and BT474 cell pellets. (B) To assess the specific staining of HER4, HER4 immunostaining was compared between BT474 cells treated with negative control siRNA (Scr siRNA) and HER4 siRNA. (C) The optimisation of HER4 staining was done at different serial dilutions of the primary antibody (anti-HER4 Neomarker): 0 (i), 1:500 (ii), 1:200 (iii) and 1:100 (iv). An overnight incubation was done at 4°C. Magnification: 200x.

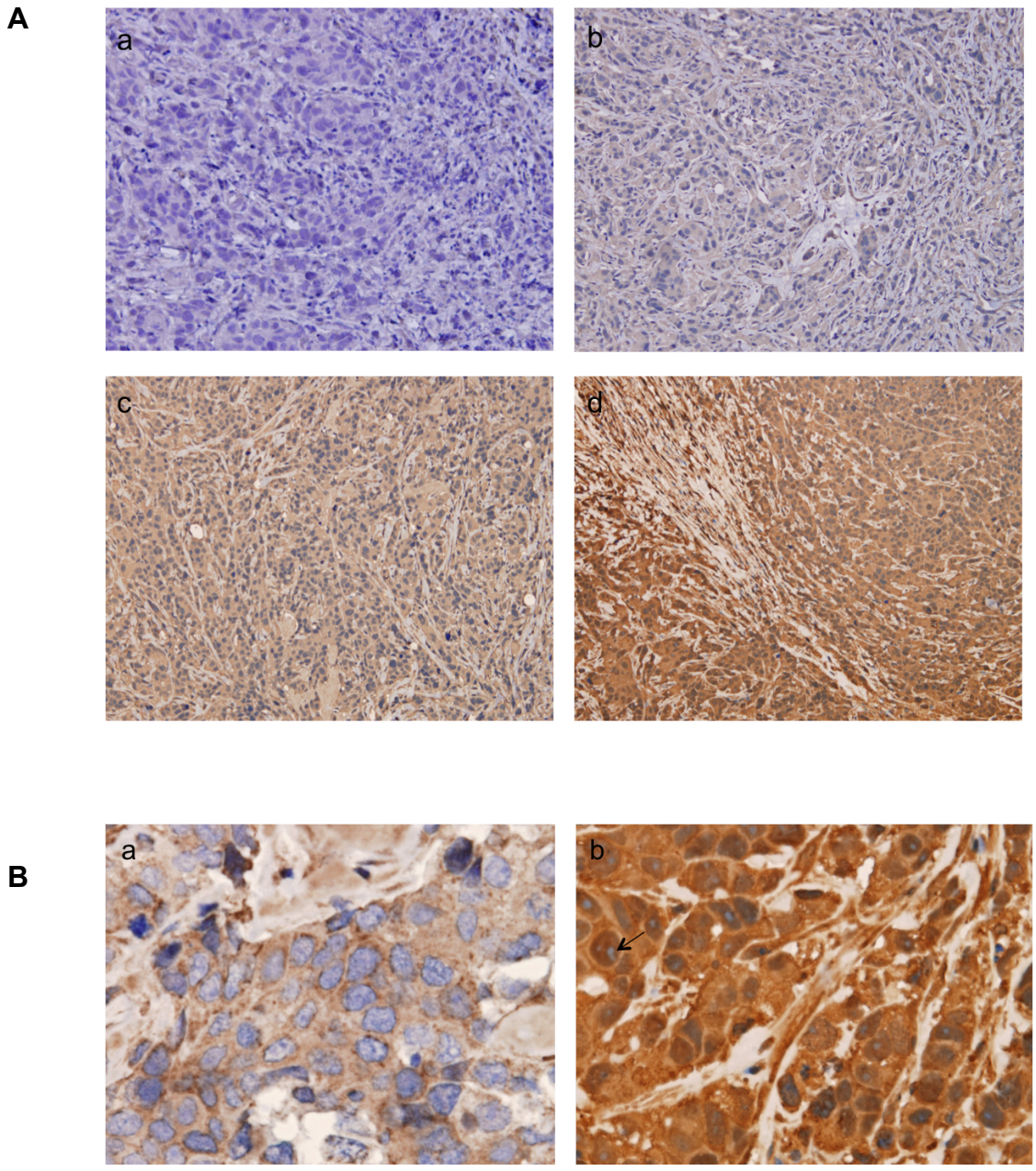


Figure 3.11 HER4 IHC staining in paraffin-embedded tumors from xenograft mice.

Tumors were extracted from mice treated with 5 doses of trastuzumab (n=5) and saline-control (n=3) over 2.5 weeks (Kramer-Marek et al., 2012). These tumors were paraffin-embedded and analysed by IHC for detection of HER4 expression. (A) HER4 IHC staining shows negative to strong cytoplasmic reactivity in tumor cells (magnification, 100x). The scoring for HER4 cytoplasmic was given as 0 for negative staining (a), 1+ for weak staining (b), 2+ for moderate staining (c) and 3+ for strong

staining (d). (B) Representative HER4 IHC staining of nuclear reactivity (magnification, 400x). Nuclear HER4 was scored as 0 for negative staining (a), and 2+ for moderate staining (b). The arrow indicates an example of negative staining on nucleoli.

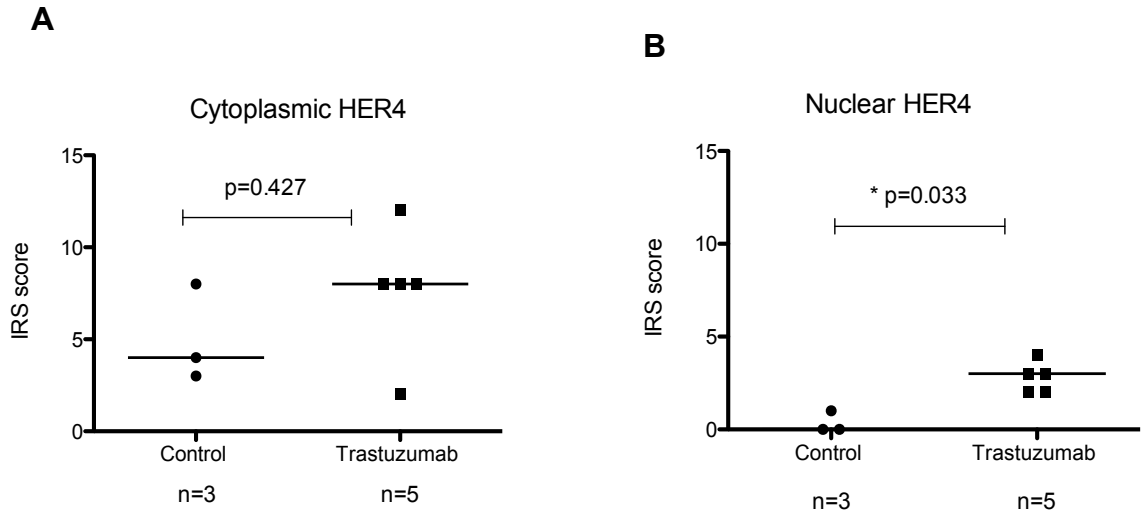


Figure 3.12 Nuclear and cytoplasmic HER4 expression is upregulated after trastuzumab treatment in BT474 xenograft models.

HER4-IHC staining was done on the xenograft tissue slides that were obtained after the mice were sacrificed (Kramer-Marek et al., 2012). HER4 expression was scored semiquantitatively using the immunoreactive score (IRS) as described in Section 2.2.6.3. (A & B) The cytoplasmic and nuclear HER4 scorings of the trastuzumab-treated and control groups are presented in a graph. The Mann-Whitney (Wilcoxon rank-sum test) was used to determine the difference in IRS scores between the untreated and trastuzumab-treated group. The statistical significance was denoted as *$p<0.05$.

3.3 Discussion

The data presented in this chapter provides evidence that trastuzumab treatment and resistance induces increased HER4 expression and nuclear translocation in HER2 positive breast cancer cells. To better understand the mechanism, this study initially characterised the basal levels of HER4 in HER2 positive and negative breast cancer cell lines. HER4 basal expression was noted in both types of cell lines. This finding is consistent with previous works that found HER4 present at moderate to low levels, in contrast to high expression of EGFR and HER2 in human breast carcinoma (Carpenter, 2003).

A number of previous reports have confirmed that heregulin; a HER4 ligand induces HER4 activation and cleavage, as evident by the presence of an 80kDa HER4 fragment and its translocation into the nuclear compartment (Linggi et al., 2006; Muraoka-Cook et al., 2006b; Naresh et al., 2006; Sundvall et al., 2007; Zhou and Carpenter, 2000). In this chapter, experiments utilising exogenous heregulin stimulation were carried out to investigate HER4 expression and nuclear translocation in HER2 negative and positive breast cancer cells. One hour of heregulin stimulation in MCF-7 cells appeared to increase HER4 mRNA and protein levels, which supports previous reports (Junttila et al., 2005; Kong et al., 2008). Heregulin stimulation also seemed to upregulate HER4 mRNA and protein levels in SKBR3 and BT474 cells. Plus, $HER4_{80kDa}$ upregulation upon heregulin stimulation was also shown in these HER2-positive cells.

It is observed that HER4 mRNA and protein level were upregulated in HER2 positive cells stimulated with heregulin for 30 mins. The increase in HER4 protein is seen so rapidly and we believed that this was due to the limitation of the exogenous

ligand stimulation. It is too far to conclude that exogenous heregulin stimulation affects the *de novo* synthesis of HER4 protein. However, we suggest that a further investigation should be carried out to investigate any possible mechanistic that can explain the above finding.

Data from confocal images indicates that heregulin stimulation seemed to induce increased nuclear HER4 localisation, which is obviously seen in SKBR3 cells compared to BT474. This observation could be due to the level expression of HER4 that is lower in BT474 cells than SKBR3 cells. It is known that it may be difficult to detect subtle changes in fluorescence intensity in confocal images. It is possible that the changes in nuclear intensity were too small to be detected by the confocal images. In addition, the cytoplasmic HER4 expression was much higher than the nuclear compartment in BT474 cells. The increase of $HER4_{80kDa}$ and nuclear localisation in response to heregulin stimulation is most likely due to proteolytic cleavage as previously reported (Ni et al., 2001). However, this needs to be confirmed by the reversal of the process by a γ-secretase inhibitor (see Chapter 5).

This study further investigated the effect of trastuzumab treatment on HER4 expression and nuclear translocation. Similar to exogenous heregulin stimulation, one hour of trastuzumab treatment resulted in an upregulation of HER4 protein levels at 180 kDa and 80 kDa as well as increased HER4 phosphorylation in SKBR3 and BT474 cells. It seems that trastuzumab induces HER4 activation and its cleavage, similar to heregulin stimulation. This is not surprising since it has previously been shown that trastuzumab induces an endogenous release of heregulin (Gijsen et al., 2010).

Prolonged treatment with trastuzumab increased the levels of $HER4_{180kDa}$ and $HER4_{80kDa}$, as evident in trastuzumab-resistant SKBR3 and BT474 cells. However, an overnight trastuzumab withdrawal significantly reduced HER4 protein (180 kDa and

80kDa) and mRNA levels in trastuzumab-resistant SKBR3. Trastuzumab-induced HER4 nuclear translocation in parental and resistant SKBR3 cell lines was further supported by observations of increased nuclear intensity and nuclear fragments, as assessed in confocal microscopy and cell fractionation experiments respectively. This finding suggests the important role of nuclear HER4 in mediating trastuzumab resistance in HER2 positive breast cancer. Despite the increase in m80 in acute and prolonged trastuzumab treatment in BT474 cells, confocal images failed to detect changes in nuclear intensity with short trastuzumab treatment, although this was seen in resistant cells. However, trastuzumab treatment for 2.5 weeks (Kramer-Marek et al., 2012) significantly increased nuclear HER4 expression compared to the untreated group in xenograft mice bearing HER2 positive BT474 breast tumors.

Taken together, data from this chapter provides evidence that trastuzumab treatment and resistance induces an upregulation of HER4 expression and nuclear translocation in HER2 positive breast cancer cells and xenograft models. To further confirm whether m80 induction and nuclear translocation is due to HER4 activation and cleavage, further study was designed to assess the effect of inhibiting HER4 activity by neratinib and preventing its cleavage by a secretase inhibitor (Chapter 4 and 5).

CHAPTER FOUR

The effect of HER4 silencing with and without trastuzumab in HER2 positive cells

CHAPTER 4: THE EFFECT OF HER4 SILENCING WITH AND WITHOUT TRASTUZUMAB IN HER2 POSITIVE CELLS

4.1 Introduction

Whilst numerous studies have shown HER4 to have an anti-proliferative activity in breast cancer cells (Kainulainen et al., 2000; Naresh et al., 2006; Sartor et al., 2001), HER4 downregulation has also been demonstrated to induce a tumor growth inhibitory effect, suggesting that targeting this marker may be important in improving cancer treatment. In lung cancers, inhibiting HER4 expression using a monoclonal antibody has been demonstrated to prevent tumor growth and increase apoptosis (Starr et al., 2006). The growth of melanoma cells with HER4 mutation is delayed after treatment with HER4 shRNA and lapatinib (HER inhibitor) (Prickett et al., 2009). In addition, the growth inhibitory effect due to endogenous HER4 silencing was also demonstrated in breast tumor cell lines (Tang et al., 1999).

As discussed in Chapter 3, HER4 nuclear translocation occurs during trastuzumab treatment and resistance and HER4 inhibition prevents tumor growth. Therefore, it will be interesting to assess the effect of HER4 silencing on cellular response when combined with trastuzumab treatment. This chapter further investigates the effect after HER4 downregulation via transient knockdown with specific siRNAs, in relation to trastuzumab treatment in HER2 positive breast cancer. The effect of these treatments on cell counts, colony formation and apoptosis was assessed by cell viability, FACS and clonogenic assay.

4.2 Results

4.2.1 Optimisation of HER4 knockdown by transient siRNA transfection in MCF-7, SKBR3 and BT474 cells

Two independent HER4 siRNAs, HER4 $siRNA_{10}$ and HER4 $siRNA_{11}$ were chosen in this study based on their efficient knockdown (more than 80%, an information provided by the manufacturer (Qiagen)). To ensure valid RNAi data, dose-dependent and time-dependent experiments were performed. Figure 4.1A shows that HER4 could be successfully knocked-down at a concentration of 20nM compared to a concentration of 10nM in MCF-7, SKBR3 and BT474 cells. Analysis by western blot indicated that greater efficiency for HER4 knockdown could be achieved after 48 hours compared to 24 hours (Figure 4.1 B). Therefore, following optimisation, HER4 siRNA transfection experiments in the rest of this chapter were performed at the optimum concentration of 20nM for 48 hours.

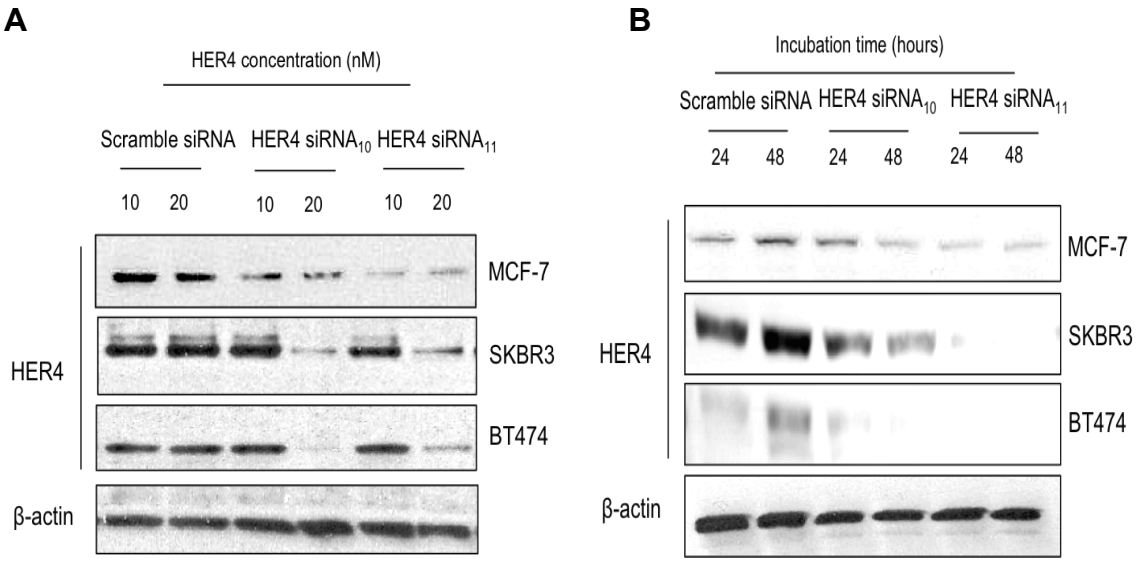


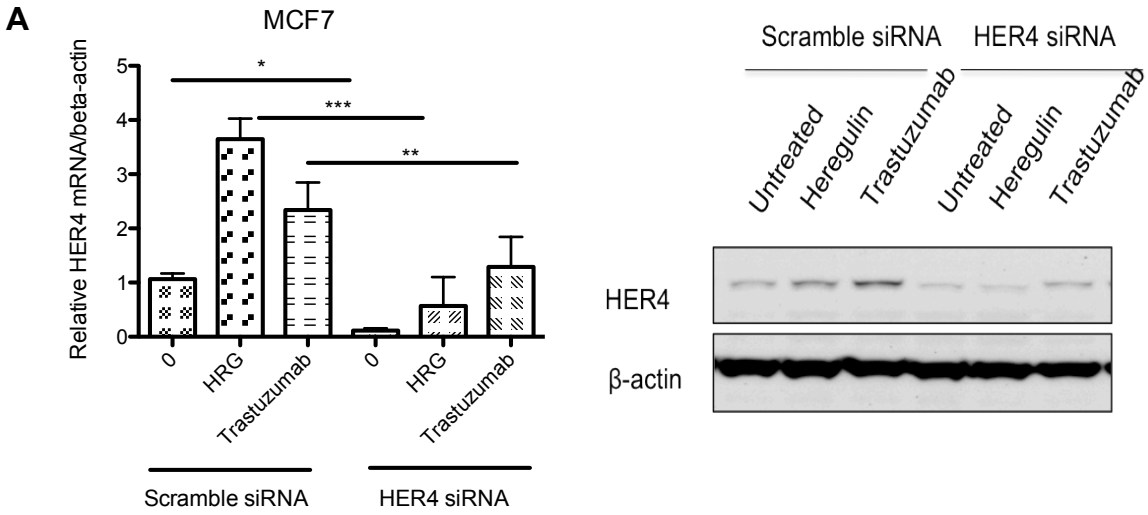
Figure 4.1 The optimisation of dose and time for HER4 siRNA transfection.

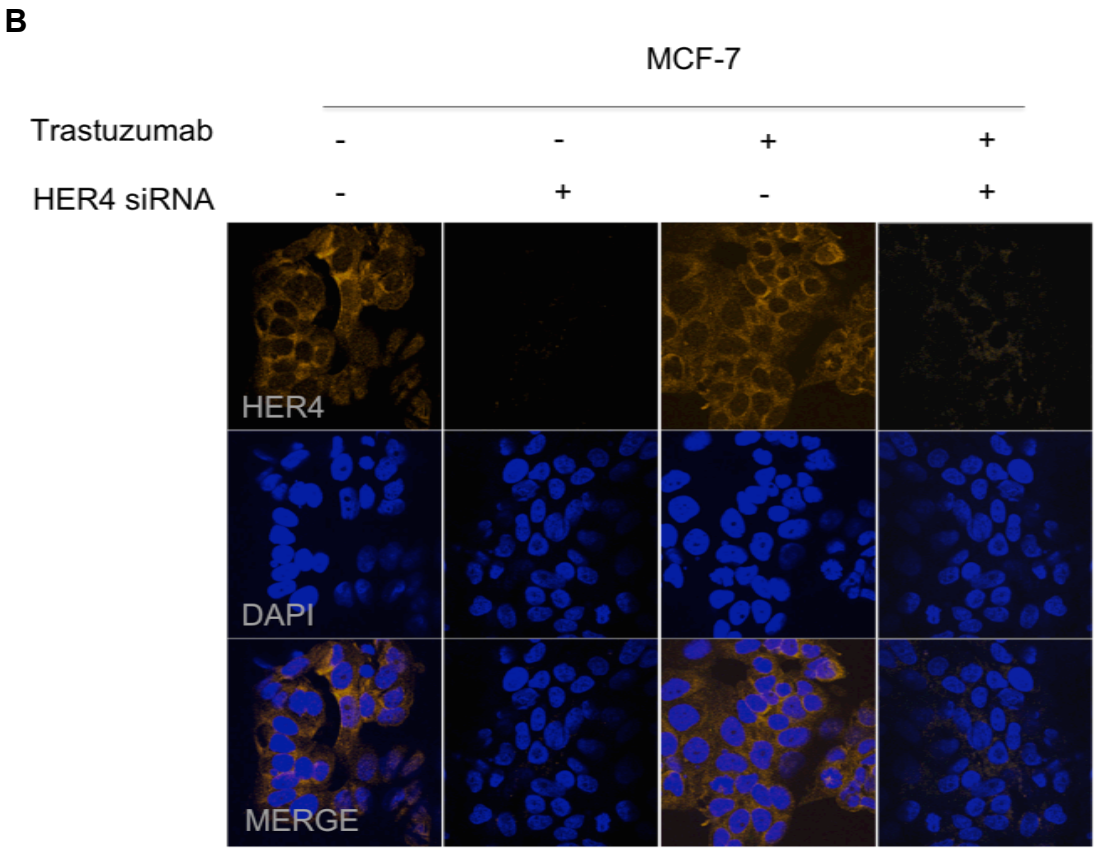
Transient transfection of HER4 siRNA was performed in MCF-7, SKBR3 and BT474 cell lines, using two different subtypes of HER4 siRNA11 and HER4 siRNA$_{10}$. Transfection with scramble (All Star Negative siRNA) was included as a negative control. Efficiency of HER4 knockdown was optimised at different concentrations and incubation times, as cells were transfected with HER4 siRNAs at 10 and 20 nM (A) for a period of 24 and 48 hours (B). Cell lysate was prepared and loaded onto an SDS gel for western blot analysis. The membrane of each cell line was probed for HER4 and β-actin. Representative blots are shown (A & B).

4.2.2 The effect of HER4 knockdown in MCF-7 cells

The effect of HER4 knockdown in relation to trastuzumab treatment was investigated in HER2 negative breast cancer cells, MCF-7. Transfection of MCF-7 cells with HER4 siRNA significantly reduced HER4 mRNA levels, as compared to the scramble ($p<0.05$, Figure 4.2A, left panel), and a similar effect was observed for HER4 protein level from western blot analysis (Figure 4.2A, right panel). In addition, transfection with HER4 siRNA also decreased HER4 protein and mRNA level that was induced by trastuzumab and heregulin treatment (Figure 4.2A). However, combination treatement of HER4 siRNA and trastuzumab upregulated HER4 mRNA and protein level compared to the other treatment groups (Figure 4,2A). From the confocal images, it was a marked decrease in HER4 intensity following depletion of HER4 in MCF-7 cells (Figure 4.2B). In addition, knockdown of HER4 also reduced HER4 cytoplasmic intensity induced by trastuzumab.

To determine if HER4 downregulation in MCF-7 cells contributed to trastuzumab-sensitivity, cell counts were evaluated 3 days post transfection. As shown in Figure 4.2C, no obvious changes were seen in cell counts after transfection with HER4 siRNAs in MCF-7 compared to the scramble. The combination treatment of trastuzumab and HER4 siRNAs reduced the number of cell counts compared to trastuzumab with scramble although this was not statistically significant (Figure 4.2C).


A
MCF7
Relative HER4 mRNA/beta-actin
0
1
2
3
4
5
*
***
**
0
HRG
Trastuzumab
0
HRG
Trastuzumab
Scramble siRNA
HER4 siRNA
Scramble siRNA
HER4 siRNA
Untreated
Heregulin
Trastuzumab
Untreated
Heregulin
Trastuzumab
HER4
β-actin


B
MCF-7
Trastuzumab
HER4 siRNA
- - + +
- + - +
HER4
DAPI
MERGE

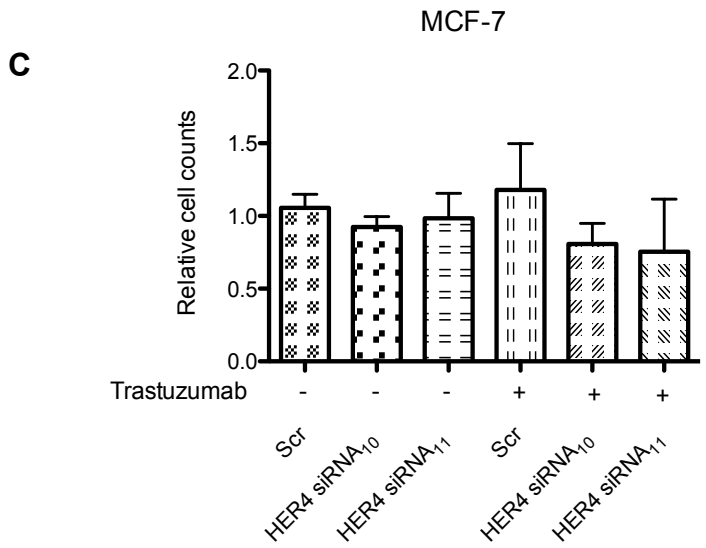


Figure 4.2 HER4 mRNA and protein expression are upregulated after transient HER4 siRNA in HER2 negative breast cancer cells.

(A) MCF-7 cells were transfected with HER4 siRNA 20nM with 100ng/ml heregulin and 40μg/ml trastuzumab for 72-hour incubation. Cell lysates and RNA extracts were obtained to proceed with western blot analysis and RT-PCR. The graph (left panel) indicates mean ± SEM of HER4 mRNA, whose treatment group was quantified relative to the untreated cells and normalised to β-actin. The significant differences of HER4 mRNA among the group were noted at *$p<0.05$ and ***$p<0.001$, as determined by 1-way ANOVA test with Bonferroni correction. The right panel indicates representative western blot of HER4 and β-actin. (B) Representative confocal images show HER4 localisation as indicated by HER4-Alexa Fluor 546 staining. DAPI staining was used as a control for nuclear localisation. (C) MCF-7 cells were plated on 24-well plates and transfected with HER4 siRNA 20nM with and without 40μg/ml trastuzumab for 72 hours. Cells were counted for cell viability assessment, following the completion of treatment. Data was obtained from three independent experiments.

4.2.3 Assessing HER4 mRNA level after transient HER4 knockdown with or without trastuzumab treatment in HER2 positive breast cancer cells

To further understand the role of HER4 in trastuzumab-treated HER2 positive breast cancer cell lines, we performed knockdown of HER4 by transient transfection with specific HER4 siRNAs, with or without trastuzumab treatment. HER4 $siRNA_{10}$ and HER4 $siRNA_{11}$ significantly diminished endogenous HER4 mRNA expression in SKBR3 cells compared to the scramble-untreated cells ($n=3$, $p<0.001$, Figure 4.3A), confirming the efficiency of knockdown using these siRNAs. HER4 siRNAs also significantly reduced expression of the total HER4 mRNA in BT474 cells ($p<0.001$, Figure 4.3B). Although the addition of trastuzumab in breast cancer cell lines transfected with HER4 siRNAs significantly increased the levels of HER4 mRNA compared to HER4 knocked-down cells without trastuzumab ($n=3$, $p<0.001$), the levels were much lower than cells without HER4 knockdown (Figure 4.3A & B).

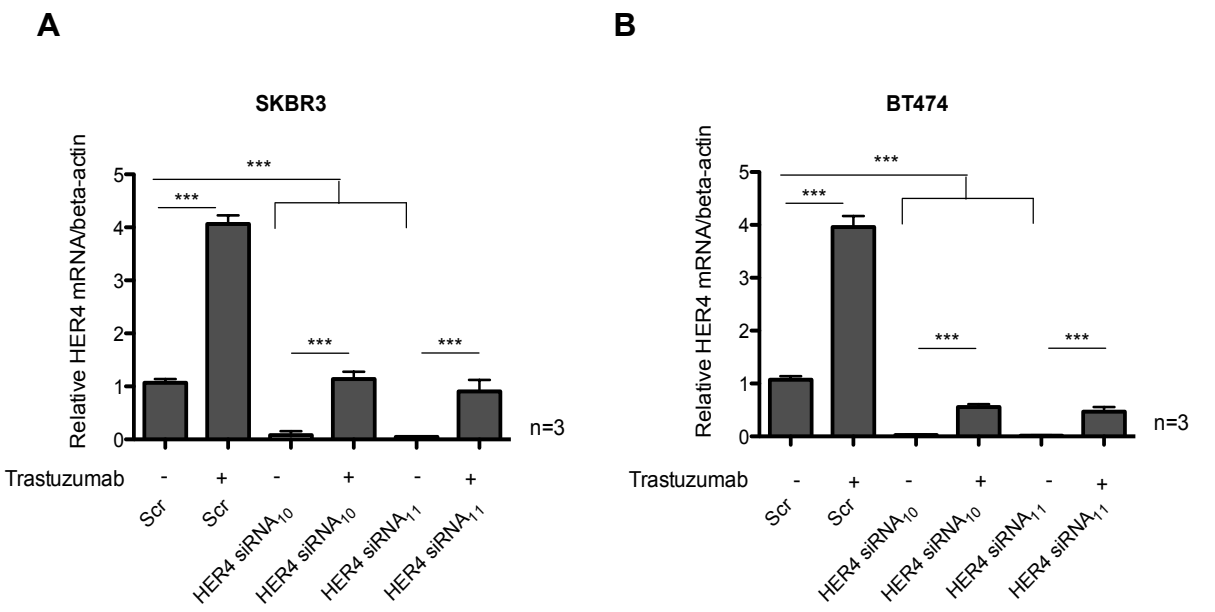


Figure 4.3 Transient knockdown of HER4 in HER2 positive breast cancer cell lines.

(A & B) Transient knockdown of HER4 was performed in SKBR3 and BT474 by transfection of two independent subtypes of HER4 siRNA, HER4 siRNA10 and HER4 siRNA11. Scramble (scr) was used as the transfection control. The optimum siRNA concentration at 20nM with an incubation time of 48 hours was applied, as determined earlier. After transfection was completed, 40µg/ml trastuzumab was added to the media and incubated for the next 24 hours. The mRNA levels were measured after 72 hours post-transfection, as assessed by RT-PCR. The scramble (scr) siRNA was used to normalise for transfection efficiency. Expression of HER4 is relative to the untreated scramble control and is normalised to β-actin. In all cases, each data point represents the mean ± SEM of HER4 mRNA from three independent experiments.

4.2.4 Assessing HER4 protein level after transient HER4 knockdown with or without trastuzumab treatment in HER2 positive breast cancer cells

Efficient knockdown of HER4 also led to the expected decrease in the total HER4 protein compared to the control (Figure 4.4A & B). The immunoblot using two different HER4 antibodies showed that the bands around 180 kDa, 110 kDa and 80kDa (also known as m80) could be knocked-down by siRNAs, indicating that these are specific HER4 bands. However, there was only a small decrease in the total HER4 when trastuzumab was added to HER4 siRNA transfected cells (no decrease of $HER4_{180kDa}$ level could be detected with Neomarkers' antibody in SKBR3 cells). This indicates that the combination treatment with trastuzumab seems to decrease the effect of siRNAs since trastuzumab upregulated HER4 expression in HER2 positive cell lines as shown previously. HER4 knockdown lead to a decrease in HER4 phosphorylation in trastuzumab treated and untreated cells (Figure 4.4A & B), as seen in both SKBR3 and BT474 cells. Although HER4 knockdown had no effect on HER2 level, there was a decrease in pHER2 level, indicating the dependency of HER2 activation on HER4 in these cells.

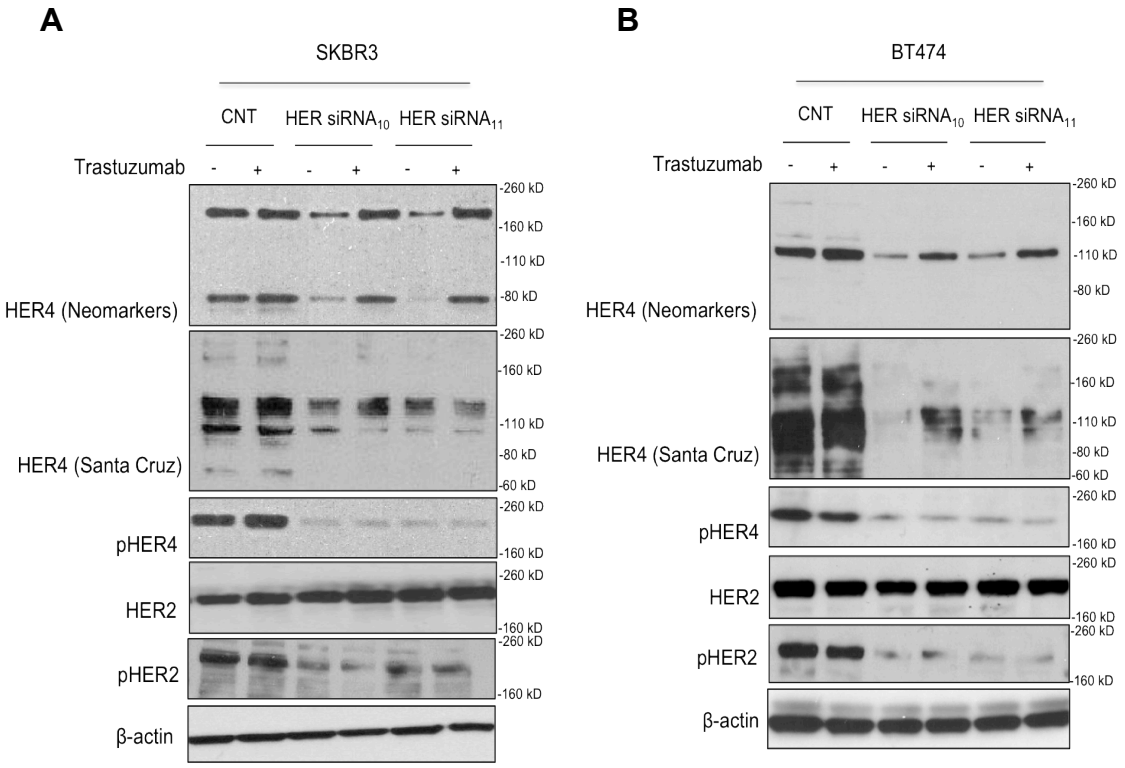


Figure 4.4 Transient transfection of HER4 siRNA decreases HER4 protein in trastuzumab parental HER2 positive cell lines.

(A & B) SKBR3 and BT474 cells were transfected with HER4 siRNA$_{10}$ and siRNA$_{11}$, with and without 40µg/ml trastuzumab treatment for 72 hours before performing western blot analysis. Membranes were probed with two different anti-HER4 antibodies, HER4 (Neomarkers) and HER4 C18 (Santa Cruz: SC283), pHER4 (Tyr 1056, Santa Cruz), HER2 (Cell Signalling) and pHER2 (Tyr1221/1222, Cell Signaling).

4.2.5 HER4 expression by confocal microscopy after transient HER4 knockdown with or without trastuzumab treatment

Although HER4 silencing could not completely prevent trastuzumab-induced increase in the mRNA and protein levels of HER4, they were less than the control siRNA treated cells (Figure 4.3 and 4.4). To investigate this observation further, the effect of HER4 knockdown with or without trastuzumab treatment was also examined on HER4 localisation via confocal microscopy. As shown in Figure 4.5A, HER4 siRNAs reduced HER4 intensity in SKBR3 parental cells, with and without trastuzumab treatment. Similar results were observed in another HER2-overexpressing cell line, BT474 (Figure 4.5 B). HER4 siRNAs were also able to decrease HER4 fluorescence intensity in the cells that became resistant to trastuzumab (see Section 2.1.1)

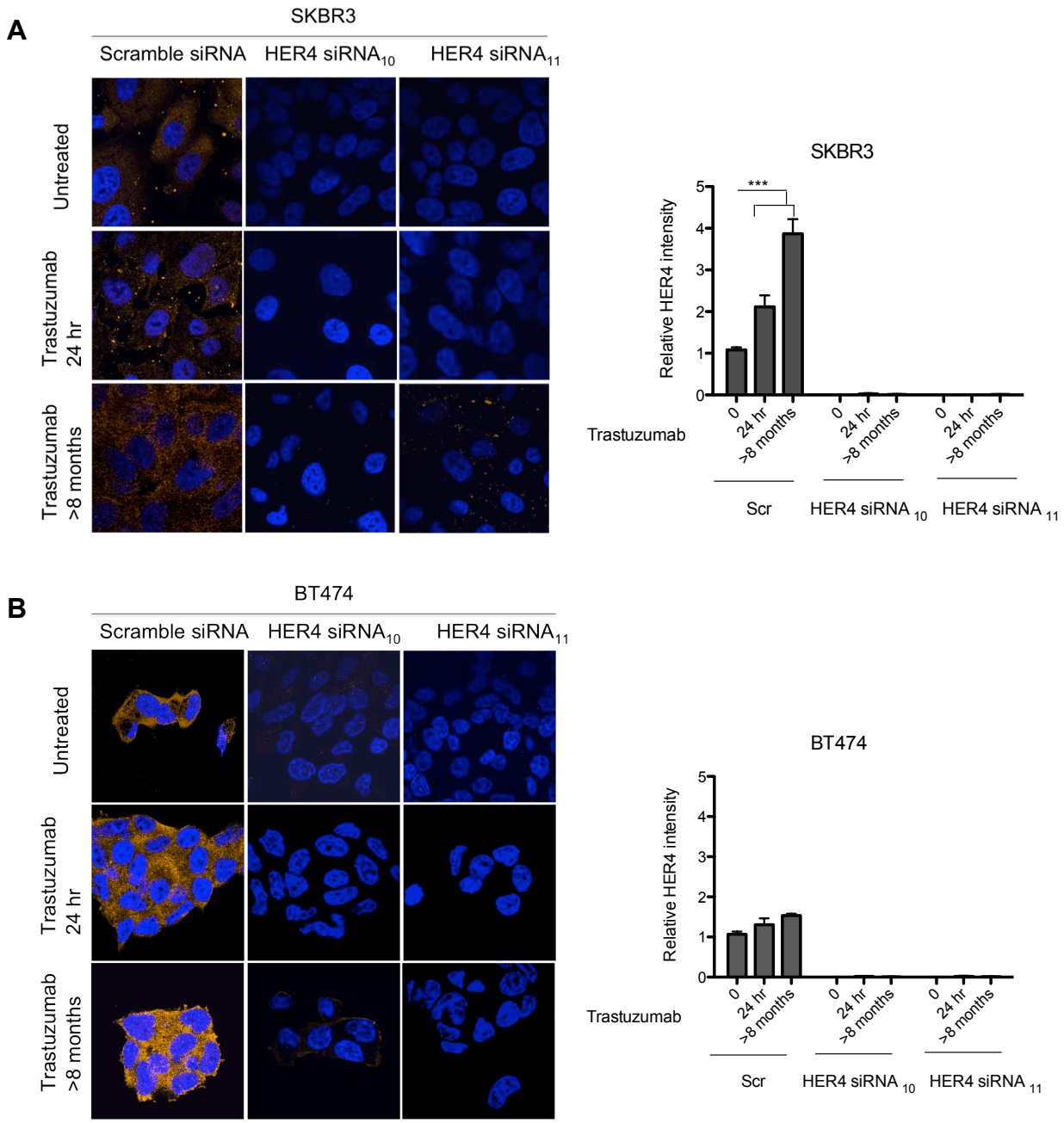


Figure 4.5 HER4 cytoplasmic and nuclear intensity is decreased after transient transfection with specific HER4 siRNAs with and without trastuzumab.

(A, B) Trastuzumab parental and resistant cells were transfected with 20nM HER4 siRNA$_{10}$ and HER4 siRNA$_{11}$ with an addition of 40μg/ml trastuzumab for a 72-hour incubation. Cells were fixed for confocal microscopy experiments to stain for HER4-Alexa Fluor 546 and DAPI. Representative confocal images are shown in the left panel.

The right panel of each figure indicates a relative quantification graph of HER4 intensity (mean ± SEM), as quantified from three representative confocal images using Image J software. *P* value was calculated using a Bonferroni correction for multiple comparisons ($P<0.05$).

4.2.6 The effect of HER4 knockdown on cell viability, with or without trastuzumab treatment

The effect of HER4 knockdown with or without trastuzumab treatment was further assessed using cell viability experiments in SKBR3 and BT474. In both cell lines, knockdown of HER4 alone had a minimal effect in decreasing the relative number of cell counts, which is not significant compared to the scramble control (Figure 4.6). There was a statistically significant reduction of the cell counts after trastuzumab treatment, compared to the untreated-scramble cells ($p<0.01$ in SKBR3 and $p<0.001$ in BT474). However, HER4 knockdown (HER4 $siRNA_{10}$ and siRNA11) with trastuzumab seemed to further enhance the reduction of the cell counts compared to trastuzumab alone, which was statistically significant (Figure 4.6A). As shown in Figure 4.6B, combination treatment of trastuzumab and HER4 siRNAs in BT474 cells also statistically significantly reduced the cell counts compared to trastuzumab alone.

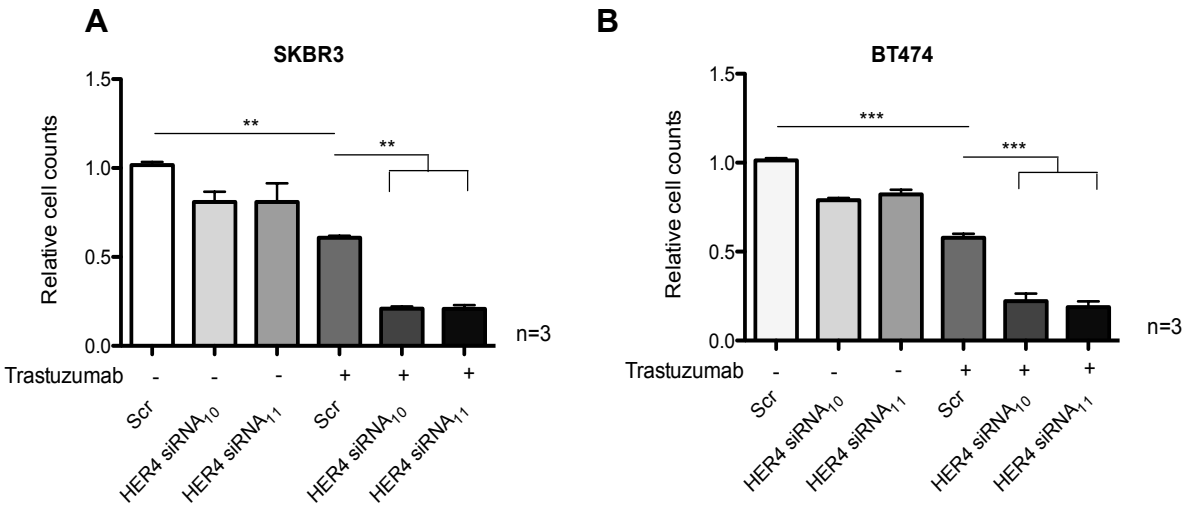


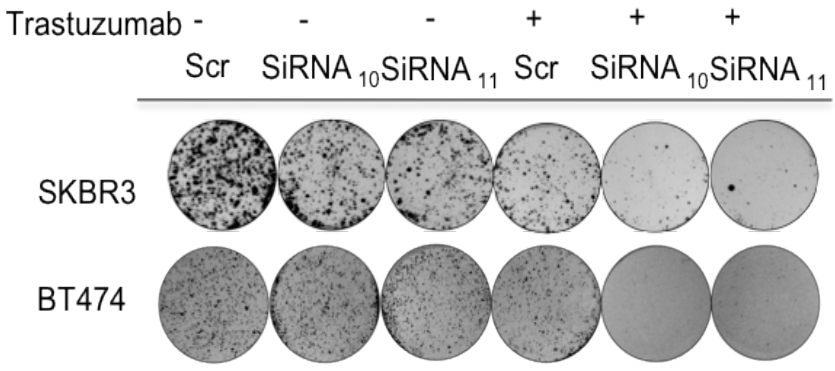
Figure 4.6 HER4 knockdown enhances trastuzumab effect on cell viability in HER2 positive breast cancer cells.

Cell count experiments were performed in SKBR3 (A) and BT474 (B) cells after 72 hours of HER4 siRNA transfection in combination with/without 40μg/ml trastuzumab treatment. The number of cell counts were normalised relative to the untreated control. The graphs indicate the mean ± SEM from the three independent experiments with the differences between groups assessed using a Bonferroni correction for multiple comparisons . *P* value is shown as **p<0.01, ***p < 0.001.

4.2.7 The effect of HER4 knockdown on colony formation, with or without trastuzumab treatment

Figure 4.7 demonstrates the effect of transient HER4 siRNAs, with and without trastuzumab in SKBR3 and BT474 cells on colony formation. SKBR3 cells transfected with HER4 siRNA alone showed a significant reduction of tumor colony formation compared to those transfected with the scramble control ($p<0.001$, Figure 4.7A). However, HER4 knockdown alone in BT474 cells had a minimal effect on decreasing colony number. The decreased colonies after trastuzumab treatment ($p<0.001$) is comparable to cells transfected with single HER4 siRNAs. However, in both cell lines, the combination of HER4 siRNA transfection with trastuzumab significantly reduced the formation of tumor colonies compared to the scramble siRNA (with or without trastuzumab), $p<0.001$ (Figure 4.7A & B).

A



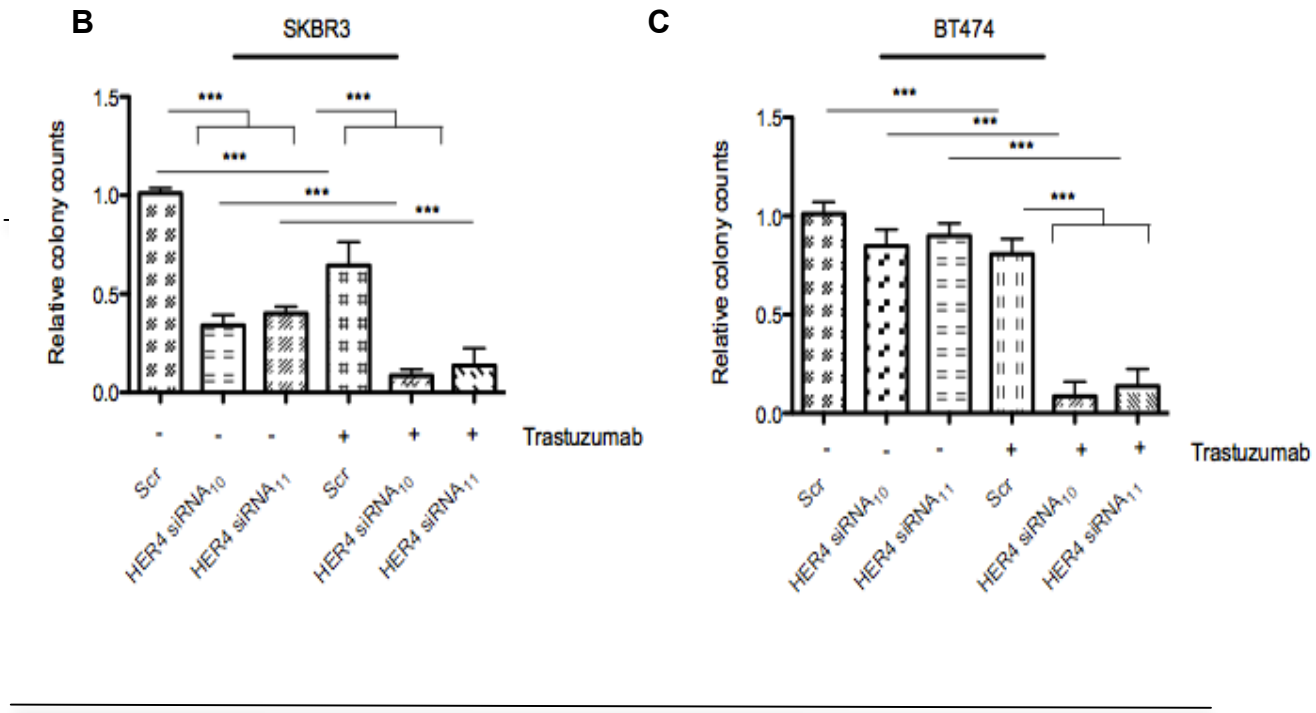


Figure 4.7 Transient HER4 knockdown increases trastuzumab response to prevent colony formation in HER2 positive breast cancer cells.

The effect of 20nM HER4 siRNA and/or 40µg/ml trastuzumab in HER2 positive breast cancer was studied using colony formation assay. Cells were counted 48h after transfection, and 2000 cells were grown in duplicates of 6-well plates. 7 days later, colonies were counted using Image J software. Colony numbers of cells transfected with scramble siRNA are set as 1. The graph indicates mean ± SEM of ratio colony numbers, the differences between groups were calculated using a Bonferroni correction for multiple comparisons and a p value is shown as ***$p < 0.001$.

4.2.8 The effect of HER4 knockdown on apoptosis with or without trastuzumab treatment

To correlate with the observations from cell counts and colony formation assay, an apoptosis assay via FACS analysis was performed. As shown in Figure 4.8, HER4 silencing alone did not induce a significant increase in the percentage of cells that were positively stained with Annexin V, as compared to the scramble control in SKBR3 and BT474 cells. However, trastuzumab treatment in SKBR3 and BT474 induced apoptosis since the percentage of cells that were positively stained with Annexin V significantly increased in trastuzumab-treated SKBR3 ($p<0.001$) and BT474 cells ($p<0.05$), as compared to untreated cells. When HER4 silencing was combined with trastuzumab, there was a statistically significant increase in cell death in SKBR3 ($p<0.001$) and BT474 ($p<0.05$) cells, as compared to trastuzumab alone.

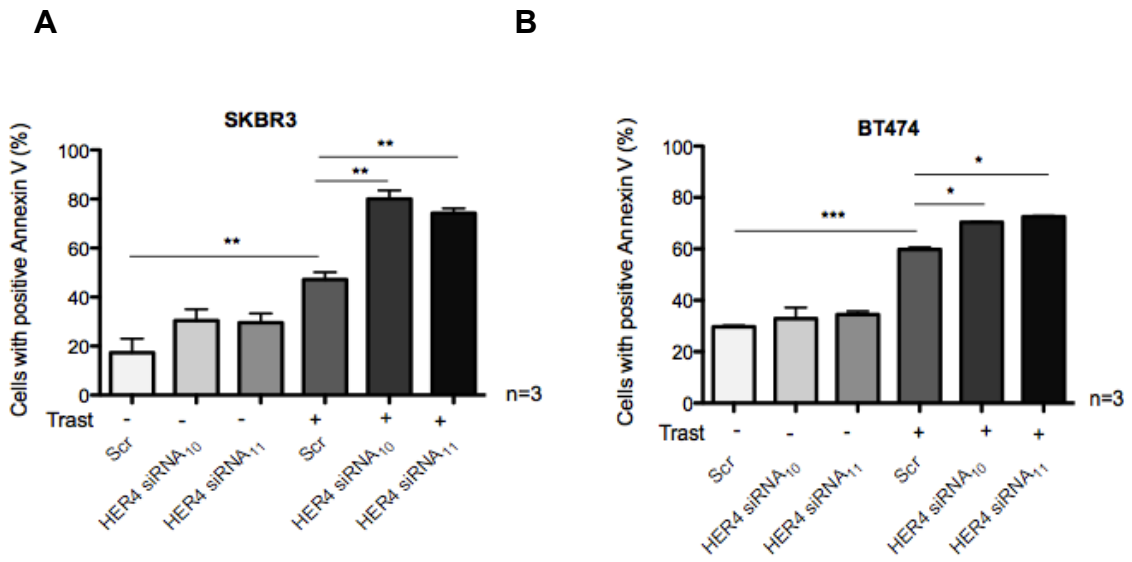


Figure 4.8 The combination of HER4 siRNAs transfection and trastuzumab enhances apoptosis compared to trastuzumab alone in HER2 positive cell lines.

SKBR3 and BT474 cells transfected with transient HER4 siRNA (with and without trastuzumab) for 72 hours were stained with Annexin V and the percentage of positive cells was assessed using FACS analysis. Mean ± SEM from three independent experiments are shown in the graph (*p<0.05, ***p<0.001).

4.2.9 HER4 protein level after transient HER4 knockdown in trastuzumab resistant cells

HER4 was also knocked-down in trastuzumab resistant SKBR3 and BT474 cells in the presence or absence of continuous trastuzumab treatment to assess its effect. HER4 and its phosphorylation decreased after both of the trastuzumab-resistant cell lines were transfected with HER4 siRNAs, with and without combination treatment with trastuzumab (Figure 4.9A and 4.9B). Although the total HER2 remained unchanged for each treatment, pHER2 was decreased in trastuzumab-resistant SKBR3 and BT474 cells transfected with HER4 siRNAs (Figure 4.9A and 4.9B). In contrast to pHER4, pHER2 increased when trastuzumab was withdrawn from both trastuzumab-resistant cells transfected with HER4 siRNAs (Figure 4.9A and 4.9B). Furthermore, HER4 knockdown did not decrease pHER2 when trastuzumab was withdrawn from both resistant cells (Figure 4.9A and 4.9B).

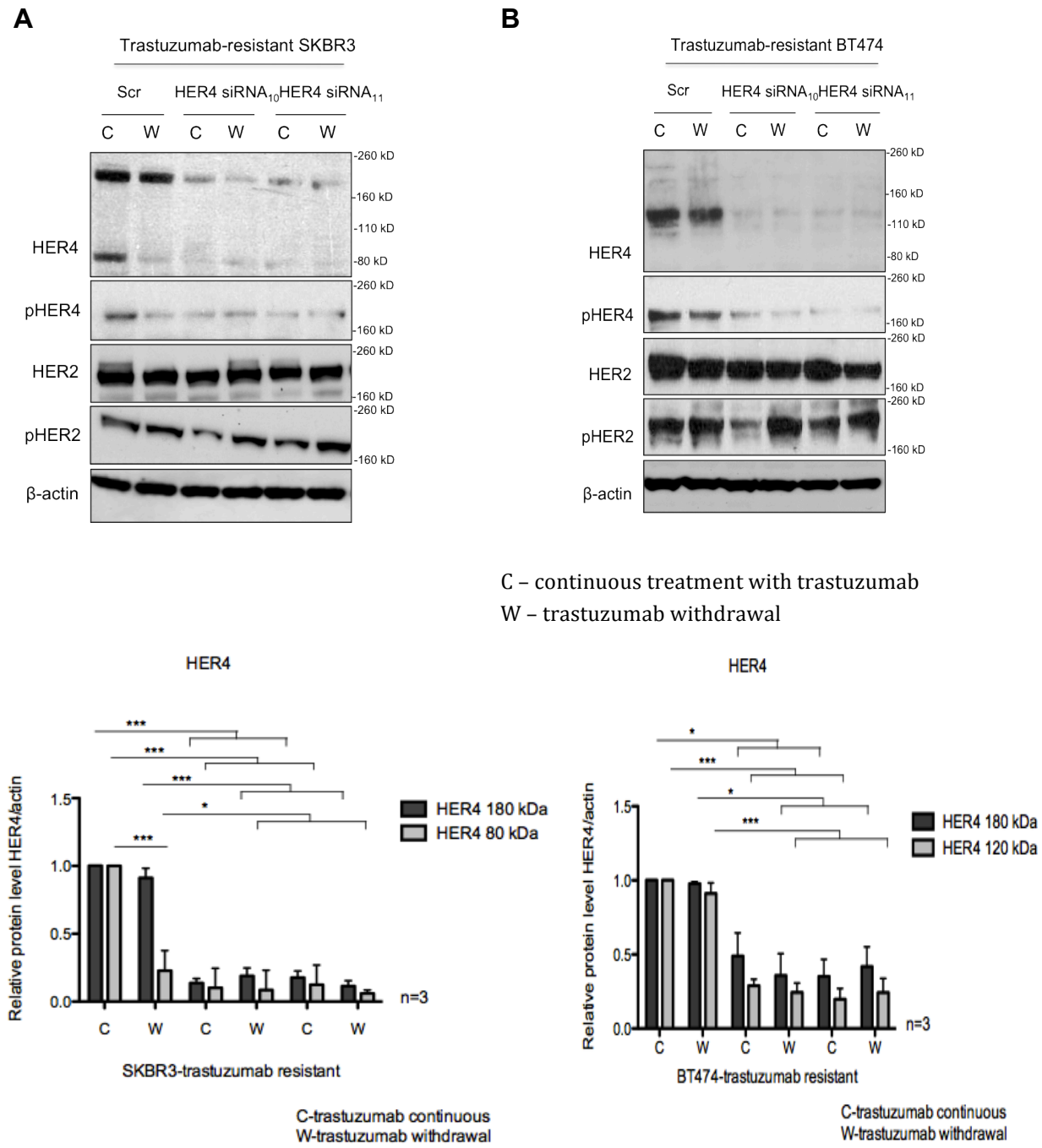


Figure 4.9 Transient knockdown of HER4 in trastuzumab resistant cell lines.

Trastuzumab-resistant cell lines were lysed for western blot analysis after transfection with 20nM HER4 siRNAs with/without 40μg/ml trastuzumab for 48 hours. Equal amounts of protein were loaded on an SDS gel and membranes were probed with HER4

(Neomarkers) and HER4 C18 (Santa Cruz: SC283), pHER4 (Tyr 1056, Santa Cruz), HER2 (Cell Signalling) and pHER2 (Tyr1221/1222, Cell Signaling) and β-actin.

4.2.10 The effect of HER4 knockdown on cell counts in trastuzumab resistant cells

The assessment of cell counts revealed a decrease in the number of trastuzumab-resistant cells transfected with HER4siRNA$_{10}$ ($p<0.001$) and HER4siRNA$_{11}$ ($p<0.001$), compared to trastuzumab-resistant SKBR3 and BT474 cells transfected with the scramble control. However, trastuzumab withdrawal in the transfected cells significantly enhanced the number of cells in trastuzumab-resistant SKBR3 and BT474 cells treated with HER4 siRNAs (Figure 4.10A and 4.10B).

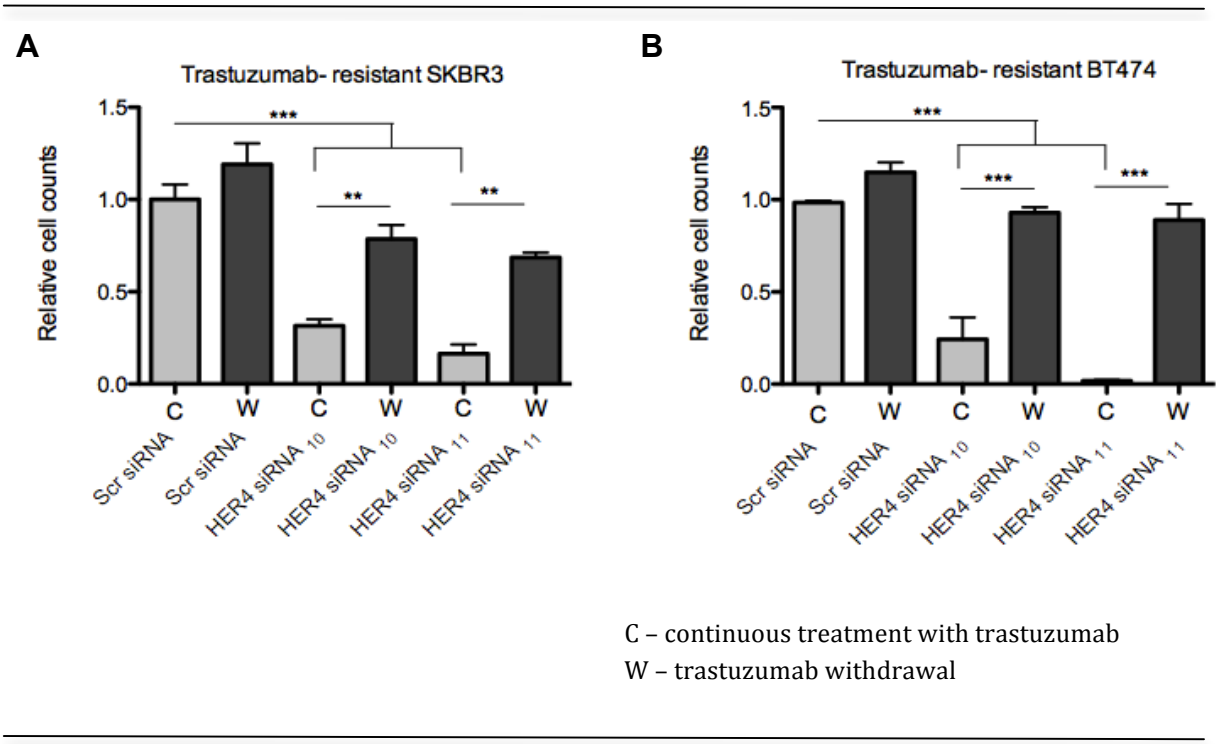


Figure 4.10 HER4 siRNA transfection in trastuzumab resistant cell lines reversed trastuzumab resistance.

Cell count experiments were performed in resistant SKBR3 and BT474 cells after HER4 siRNA transfection. The relative cell counts (normalised to the scr control) are shown in the graph. Mean ± SEM of the three independent experiments are shown (**$p<0.01$, ***$p<0.001$

4.3 Discussion

In my study, the transient transfection with specific HER4 siRNAs was successful in knocking-down HER4 at mRNA and protein levels in HER2 positive cells as well as MCF-7 cells. Although HER4 silencing could not completely prevent a trastuzumab-induced increase in the mRNA and protein levels of HER4, they were less than the control siRNA treated cells. Since we also observed trastuzumab causes an upregulation of HER4 in the negative control MCF-7 cells following HER4 inhibition, it is suggested that further investigation is required to see any potential impact of the transfection process in response to trastuzumab. In order to see this, it might be crucial to include a mock control, which to perform transfections without siRNA. In addition, a reduction of HER4 intensity evident from confocal microscopy also confirmed the knockdown efficiency of HER4 in all cell lines before and after treatment with trastuzumab.

The results of this chapter further demonstrate the importance of HER4 in HER2 positive breast cancer in relation to trastuzumab treatment. HER4 downregulation through transient siRNA transfection in SKBR3 and BT474 cells significantly decreased cell viability in trastuzumab-treated cancer cells compared to untreated cells. However, no obvious change in cell viability was seen after HER4 knockdown in the MCF-7. The above findings suggest that HER4 knockdown could increase trastuzumab sensitivity in HER2-positive breast cancer cells. The growth inhibitory effect due to HER4 downregulation has been previously reported in melanoma cells with mutated HER4 (Prickett et al., 2009) and in estrogen receptor-positive breast cancer cells (Tang et al., 1999) but no study has been performed in HER2 positive breast cancer cells or in relation to trastuzumab treatment.

The growth inhibition effect of HER4 silencing in trastuzumab-treated cells was further confirmed by decreased proliferation shown by a reduction in colony formation and increased apoptosis shown by increased number of cells positive for Annexin V. It has been reported that inhibition of HER2 by trastuzumab induces apoptosis (Milella et al., 2004) and this is is in agreement with our study. However, as been suggested earlier, transfection with mock control is recommended in the future study as to prove whether the increase in apoptosis is due to trastuzumab alone or the trasfection process itself. This is important as to confirm the combination of HER4 depletion followed by trastuzumab resulted in a significantly stronger induction of apoptosis.

HER4 silencing could also reverse trastuzumab resistance in HER2 positive breast cancer cells. However, HER4 knockdown had a minimal effect when trastuzumab was withdrawn from the resistant cells. The results indicate that the cells only became more dependent on HER4 signalling during trastuzumab treatment and HER4 knockdown may be more effective with continuing trastuzumab treatment after resistance is acquired.

Trastuzumab could not decrease HER2 phosphorylation as reported by our lab and other authors (Junttila et al., 2009; Scaltriti et al., 2009). Data from this chapter demonstrates that HER4 silencing (with and without trastuzumab treatment) decreased the pHER2 level in parental cell lines with or without trastuzumab. This suggests that HER2 signalling is partly dependent on HER4 and HER4 knockdown could deactivate HER2. An interaction between HER2 and HER4 was reported to be oncogenic (Mill et al., 2012). Therefore, HER4 is an important target in HER2 positive breast cancer and this study has proven that inhibition of HER4 increases trastuzumab sensitivity and reverses its resistance in these cells.

In Chapter 3, it was shown that trastuzumab-treated and resistant HER2 positive cells induced an upregulation of $HER4_{180kDa}$ as well as its cleaved fragment, $HER4_{80kDa}$ level (Chapter 3). Since trastuzumab induces the release of endogenous heregulin (Gijsen et al., 2010) and this could induce γ-secretase-mediated HER4 cleavage and nuclear localisation (Ni et al., 2001), it was hypothesised that inhibition of γ-secretase in trastuzumab-treated HER2 positive cells could inhibit cell proliferation through prevention of HER4 cleavage. In the next chapter, the combination effect of γ-secretase inhibitor with trastuzumab treatment on HER4 signalling and cleavage in HER2 positive breast cancer cells will be reported.

CHAPTER FIVE

The effect of γ-secretase and neratinib on HER4 cleavage and localisation

CHAPTER 5: THE EFFECT OF γ-SECRETASE AND NERATINIB ON HER4 CLEAVAGE AND LOCALISATION

5.1 Introduction

HER4 cleavage in response to γ-secretase activity has been previously reported, as demonstrated by the presence of HER4 cytoplasmic domain at nuclear localisation (Lee et al., 2002; Ni et al., 2001; Vidal et al., 2005; Williams et al., 2004). As trastuzumab has been shown to induce HER4 expression, cleavage and nuclear translocation in HER2 positive breast cancer cells (Chapter 3), experiments utilising an inhibitor against γ-secretase (GSi) were carried out to study the role of this protease in mediating HER4 cleavage in response to trastuzumab. We used PEN-2, a subunit protein for gamma-secretase as a marker to determine the efficiency of GSi treatment. Furthermore, the effect of GSi on HER4 nuclear localisation and cell survival in HER2 positive breast cancer cells was also investigated, since data from Chapter 4 indicate that downregulation of HER4 by specific siRNAs decreased cell viability and improved trastuzumab response as well as reversed its resistance in HER2 positive breast cancer cells.

Neratinib is an irreversible TKI targeting tyrosine kinase activities of EGFR/HER1, HER2 and HER4 (Wissner and Mansour 2008). We have previously reported that neratinib is effective in overcoming acquired resistance to trastuzumab (Canonici et al., 2013), but its effect on HER4 cleavage and localisation was not investigated. This chapter also investigated the role of this panHER inhibitor on HER4 cleavage and its nuclear translocation in HER2 positive-breast cancer, *in vivo* and *in vitro*.

5.2 Results

5.2.1 Effects of GSi on HER4 expression in HER2 positive breast cancer cells

To investigate the effect of GSi on HER4 expression and cleavage, protein levels of HER4 were assessed by Western blot. First, GSi treatment reduced PEN-2 protein level in SKBR3 and BT474 (Figure 5.1A &B). As shown in Figure 5.1, HER4 protein levels at 180 kDa and 80kDa were upregulated in SKBR3 and BT474 cells treated with trastuzumab compared to the controls, in accordance with the previous data on HER4 protein expression. $HER4_{80kDa}$ expression was reduced when GSi was added to SKBR3 and BT474 cells. Interestingly, GSi also prevented trastuzumab–induced $HER4_{80kDa}$ in HER2 positive cell lines, compared to trastuzumab alone or trastuzumab with DMSO (Figure 5.1B).

As shown in Figure 5.1C & D, treatment with GSi alone did not reduce HER4 mRNA expression compared to the control (IgG+DMSO). However, when GSi was combined with trastuzumab, the combination treatment after 24 hours prevented the increase of HER4 mRNA level induced by trastuzumab ($p<0.001$ in SKBR3, $p<0.05$ in BT474). (Figure 5.1C &D).

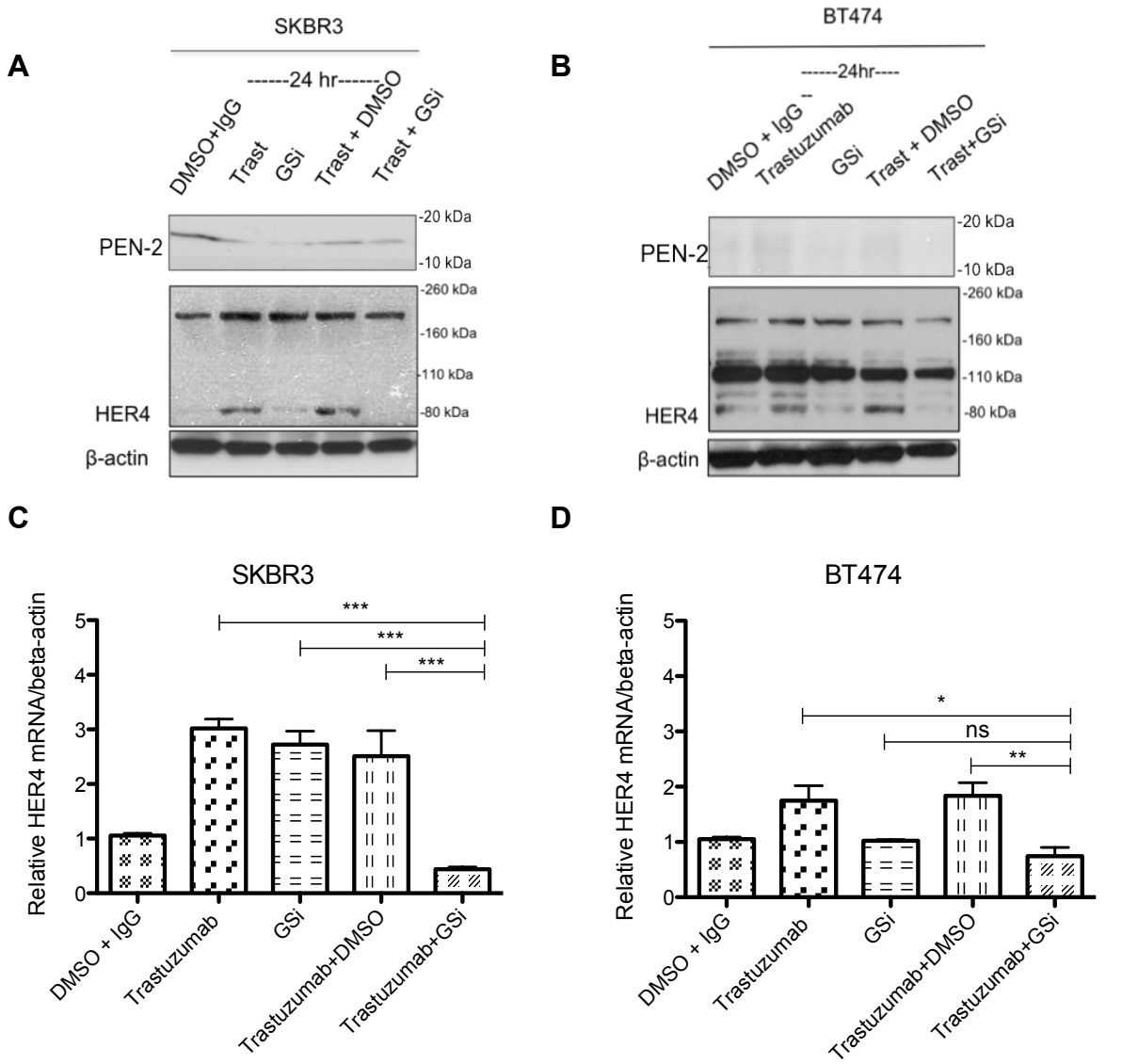


Figure 5.1 GSi decreased trastuzumab-induced $HER4_{80kDa}$ protein level and HER4 mRNA level in HER2 positive breast cancer cells.

SKBR3 and BT474 cells were treated with 10μM GSi with or without 40μg/ml trastuzumab for 24 hours, before performing Western blot analysis and RT PCR to investigate HER4 expression. (A, B) Representative Western blots from membranes that were blotted with HER4 (Neomarkers) and β-actin. (C, D) HER4 mRNA levels of cells were measured by RT-PCR following 24 hours treatment with GSi and/or trastuzumab treatment. Graphs represent means with ± SEM of relative HER4 mRNA. *P* value was

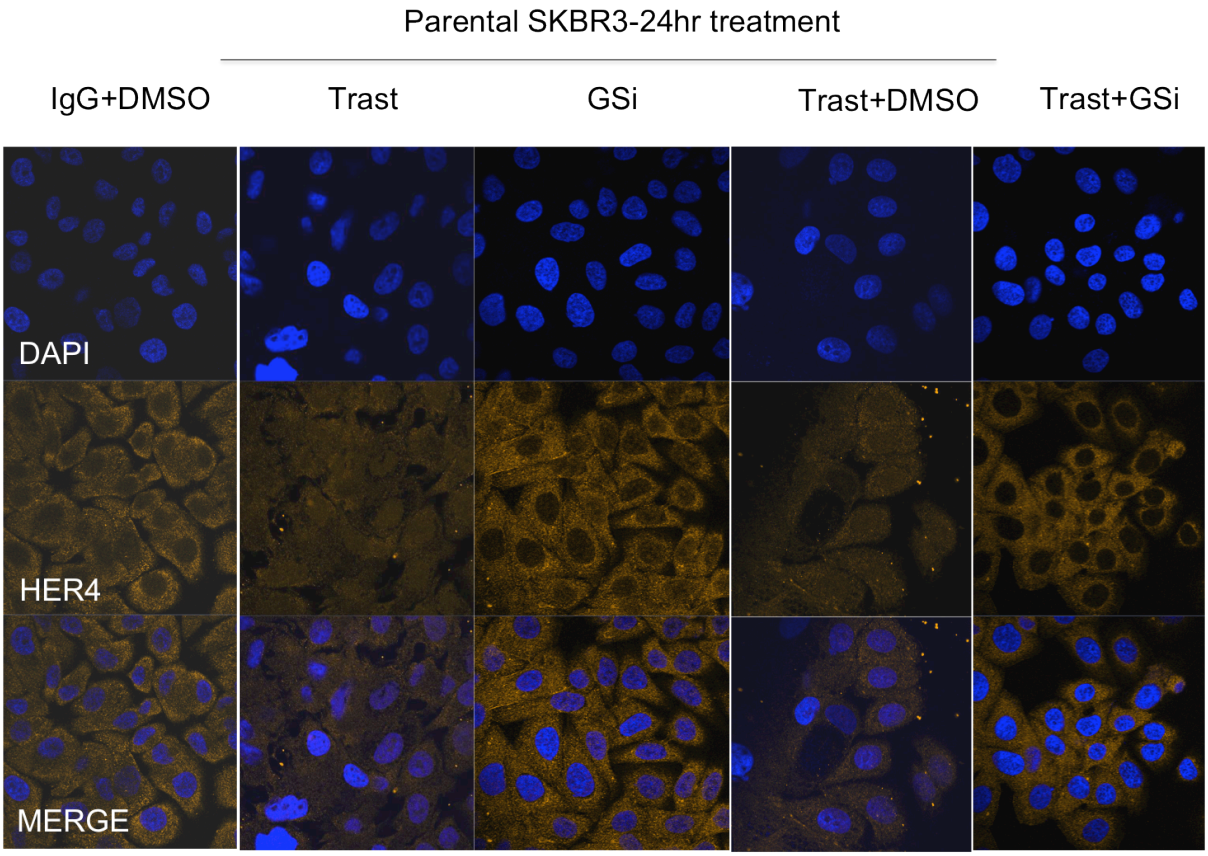
calculated using a Bonferroni correction for multiple comparisons to determine significant difference of HER4 mRNA levels between groups (*** denotes $p<0.001$).

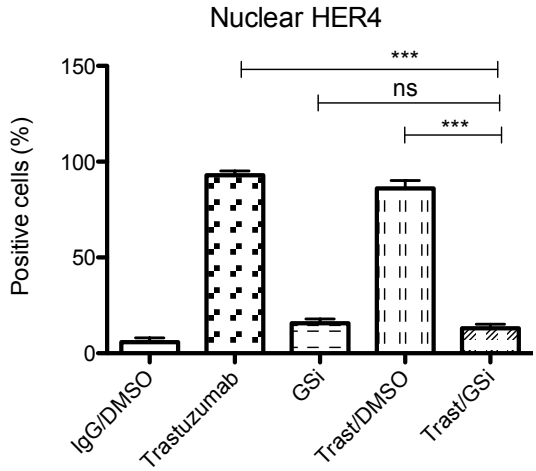
5.2.2 Effects of GSi on HER4 localisation in trastuzumab parental cell lines

Previous data have shown that trastuzumab treatment enhanced HER4 nuclear staining in parental SKBR3 cells, as assessed by confocal microscopy (Chapter 3, Figure 3.2). Therefore, SKBR3 cells were chosen as a model to examine the effect of GSi on nuclear HER4 localisation induced by trastuzumab. To look at the localisation of HER4 after GSi treatment, confocal images were taken. Figure 5.2A illustrates the confocal images from SKBR3 cells treated with GSi, with or without 24-hour treatment with trastuzumab. GSi alone decreased HER4 intensity slightly in the nucleus compared to the control. In addition, when GSi was added to trastuzumab, it decreased nuclear HER4 intensity induced by trastuzumab. As shown in Figure 5.2A (bottom panel), the number of cells that has positive nuclear HER4 staining was significantly decreased as compared to trastuzumab alone or trastuzumab+DMSO ($p<0.001$). In BT474 cells, no obvious changes on cytoplasmic intensity were seen after treatment with GSi, in addition with or without trastuzumab (Figure 5.2B). The effect of GSi on nuclear localisation in the BT474 was unable to be detected since nuclear intensity in BT474 was too faint compared to cytoplasmic intensity.

.

A


Parental SKBR3-24hr treatment
IgG+DMSO
Trast
GSi
Trast+DMSO
Trast+GSi
DAPI
HER4
MERGE


Nuclear HER4
Positive cells (%)
150
100
50
0
***
ns
***
IgG/DMSO
Trastuzumab
GSi
Trast/DMSO
Trast/GSi

B

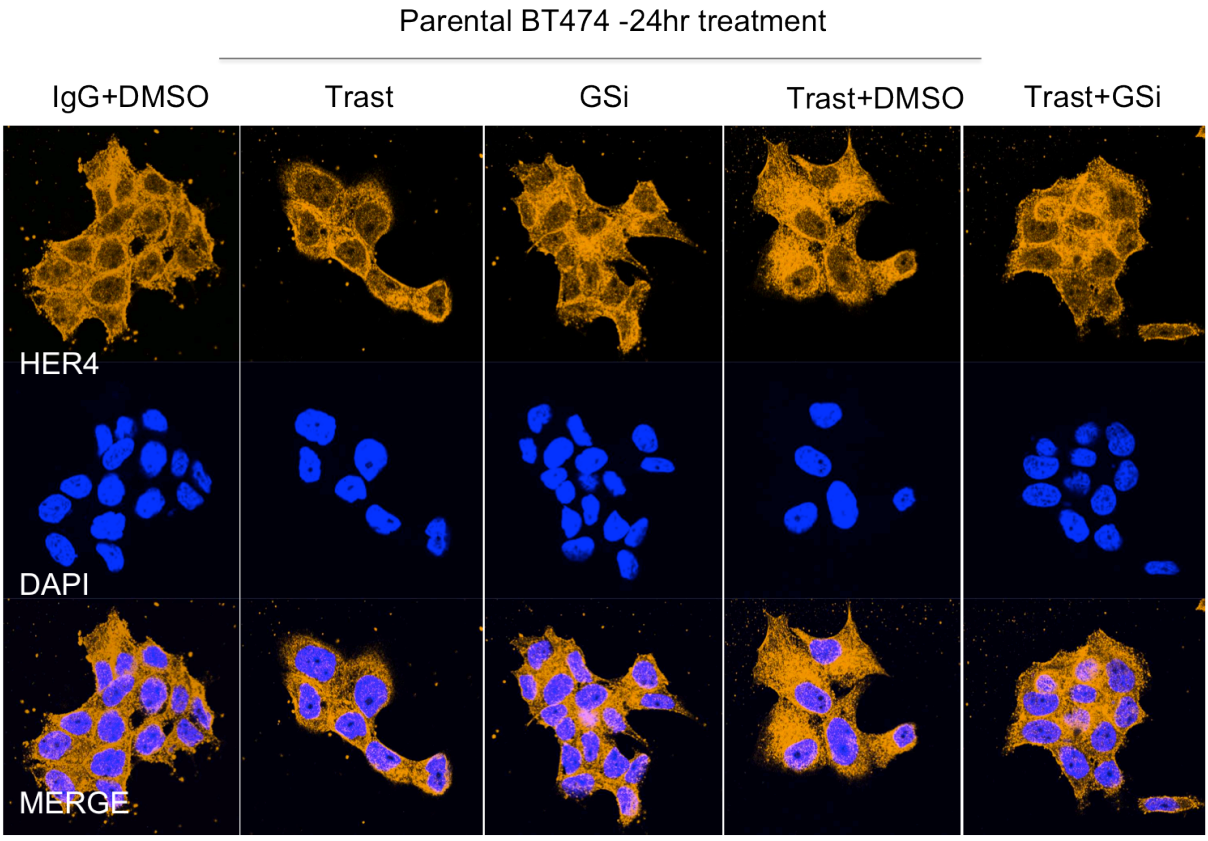


Figure 5.2 GSi prevents nuclear localisation induced by trastuzumab.

Confocal immunofluorescence microscopy was performed in SKBR3 (A) and BT474 (B) cells after 24hr treatment with 10μM GSi in combination with/without 40μg/ml trastuzumab. A representative confocal image indicates HER4 localisation as determined using HER4-Alexa Fluor 546 staining. DAPI staining was used as a control for nuclear localisation. (A, bottom panel) A graph shows the percentage of positive cells stained with nuclear HER4, as assessed from three independent experiments. 1-way ANOVA test with Bonferroni's multiple comparison was applied to determine significant differences of HER4 protein between groups (***$p<0.001$).

5.2.3 Effects of GSi on cell viability in parental SKBR3 and BT474

Next, cell viability was assessed via cell count evaluation in SKBR3 cells treated with trastuzumab and inhibitors against γ-secretase (GSi). The cell viability of trastuzumab-treated SKBR3 cells decreased compared to the untreated cells after 3 days of treatment (Figure 5.3A, left panel). SKBR3 cells treated with trastuzumab in combination with GSi had a further reduction in cell viability more than the cells treated with trastuzumab alone or trastuzumab with DMSO, which was statistically significant compared to the other groups (Figure 5.3A). To determine whether the decrease in cell viability was attributable to apoptosis, SKBR3 cells were stained with FITC conjugated Annexin V and PI and evaluated by FACS analysis. As shown in Figure 5.3B, there was also a significantly increased number of apoptotic SKBR3 cells treated with GSi and trastuzumab, as compared to trastuzumab monotherapy or trastuzumab with DMSO ($p< 0.001$).

Since GSi decreased $HER4_{80kDA}$ in BT474 cells, as seen in Figure 5.1D, the effect of this inhibitor in relation to trastuzumab was also determined on this cell. It was observed that treatment with trastuzumab after 3 days resulted in a significant decrease of cell viability ($n=3$, $p<0.01$, Figure 5.3C). Trastuzumab with GSi treatment decreased cell viability compared to control; however, this was not statistically significant compared to trastuzumab alone, GSi alone or trastuzumab+DMSO. This result was further supported by the percentage of cells with positive Annexin V staining (Figure 5.3D)

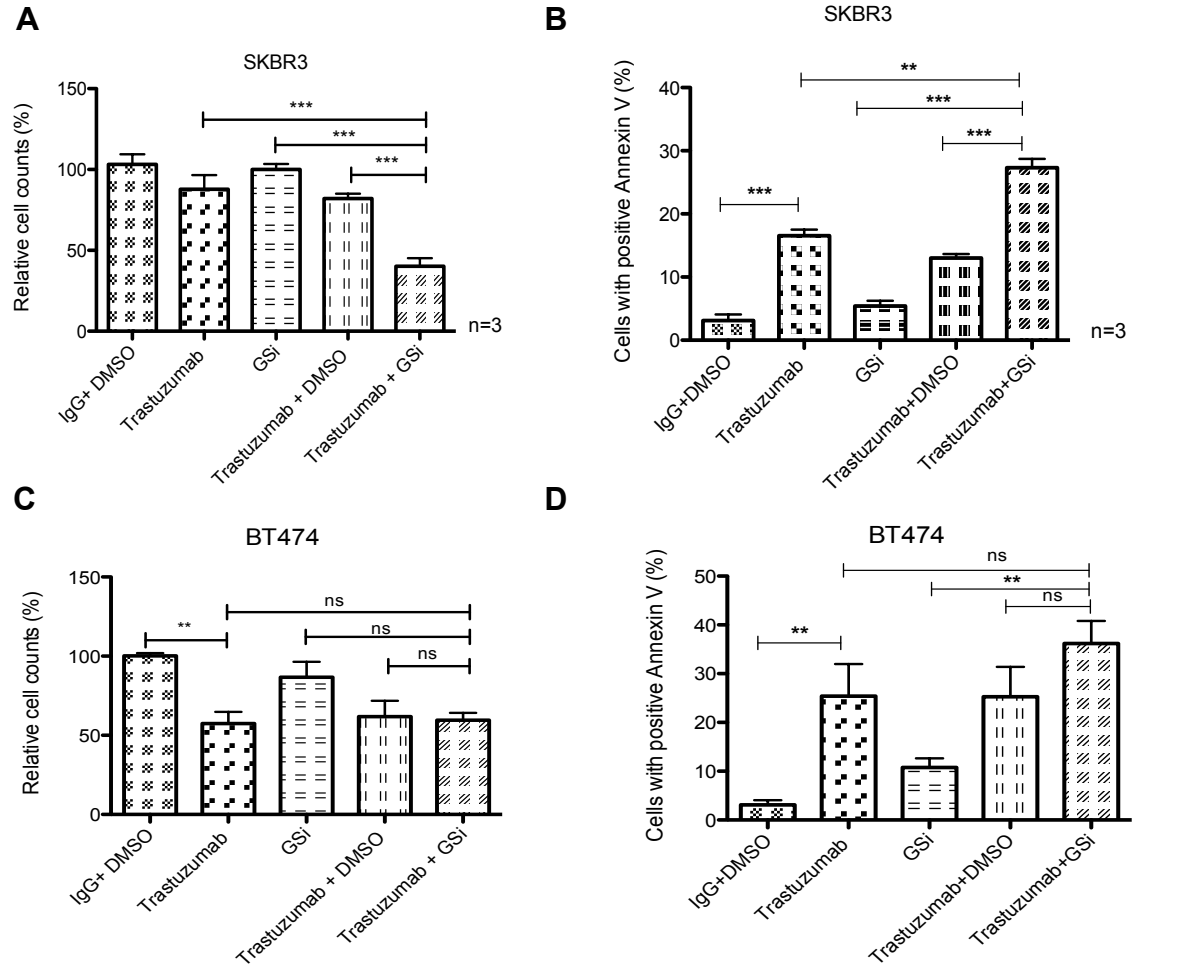


Figure 5.3 Combination treatment of GSi and trastuzumab decreased cell counts with apoptosis induction in trastuzumab-parental cells.

Cells were treated with 10μM GSi in combination with/without 40μg/ml trastuzumab for 3-day period, after which cells were collected and cell count and apoptosis assays were performed. The percentage of cell number was normalised relative to the untreated control. As described in Section 2.2.1.5, the apoptotic positive control was treated with 6 mM camptothecin and the analysis was carried out at a 24 hour time-point. Graphs indicate means with ± SEM from three independent experiments with the differences between groups were calculated using a Bonferroni correction for multiple comparisons (*** denotes $p<0.001$).

5.2.4 Effects of GSi on HER4 localisation in trastuzumab resistant cells

Since nuclear HER4 was seen in trastuzumab resistant SKBR3 and BT474 cells (Chapter 3), this study further investigated the effect of GSi on HER4 localisation in these cells. As shown in Figure 5.4A, the addition of GSi onto trastuzumab resistant SKBR3 cells that were continuously treated with trastuzumab decreased nuclear intensity, which is similar to that observed in resistant cells after trastuzumab withdrawal. It was also seen that GSi decreased HER4 intensity in both the cytoplasmic and nuclear compartments in resistant cells without trastuzumab. Compared to control, the number of cells that had positive nuclear HER4 staining was significantly decreased in trastuzumab resistant (with and without trastuzumab withdrawal) in addition with GSi.

The effect of GSi on HER4 localisation was also examined in trastuzumab resistant BT474 cells (Figure 5.5A). No change on cytoplasmic intensity could be detected after the addition of GSi in resistant BT474 cells, with or without trastuzumab withdrawal (Figure 5.5A). Even though there was a trend that GSi decreased the number of positive cells with nuclear staining in trastuzumab resistant BT474 cells, this was not statistically significant (Figure 5.5B).

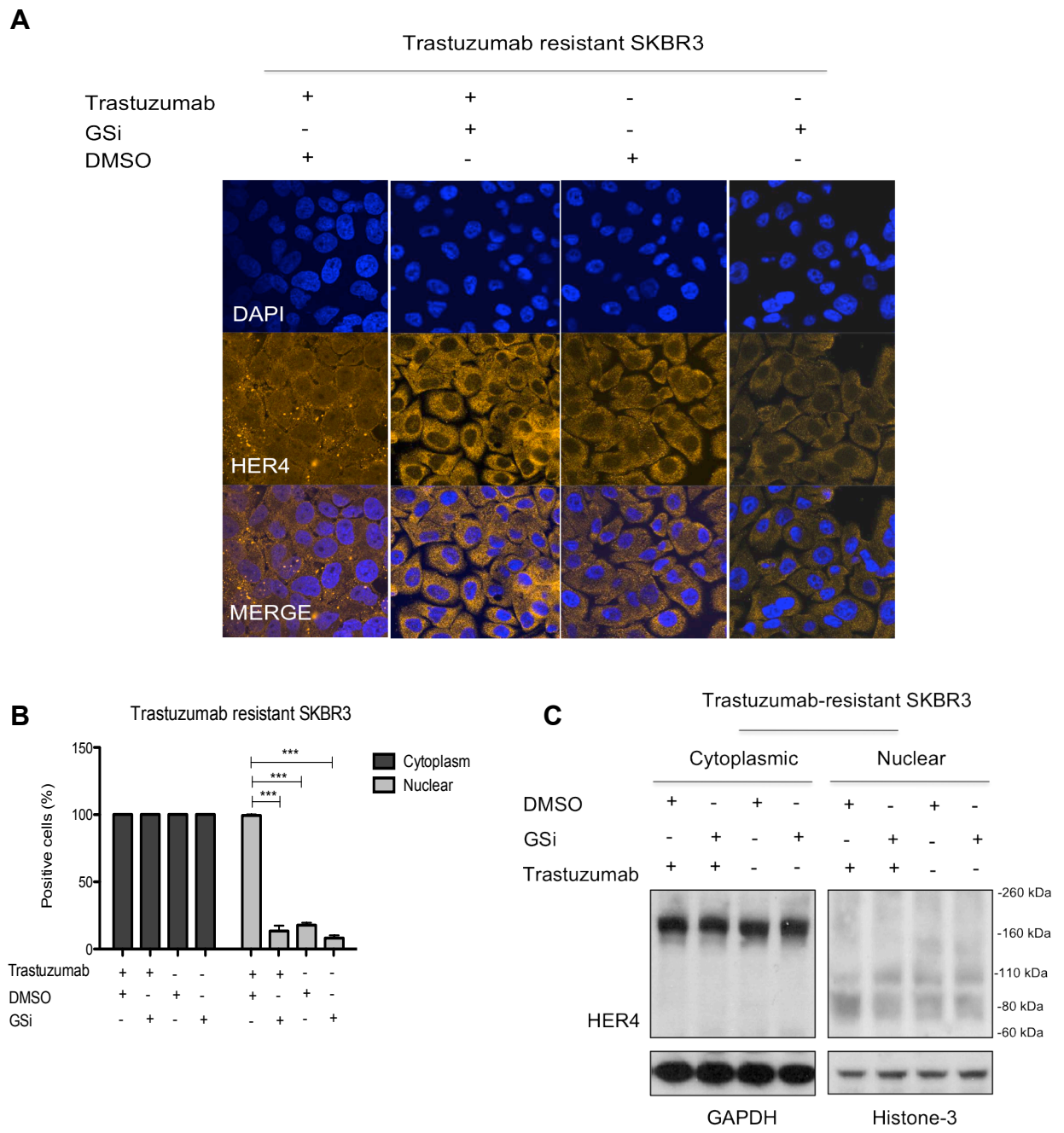


Figure 5.4 The effect of GSi in trastuzumab resistant SKBR3.

Trastuzumab resistant SKBR3 cells were grown in media with and without trastuzumab. Later, 10μM GSi was added into the media and cells were incubated for overnight. Cells were then fixed for confocal experiment to stain for HER4-Alexa Fluor 546 and DAPI. (B) A graph shows the percentage of positive cells stained with cytoplasmic and nuclear HER4, as assessed from three independent experiments. *P*

value was calculated using a Bonferroni correction for multiple comparisons (*** denotes $p<0.001$). (C) Western blot analysis was performed on the cytoplasmic and nuclear fractions of SKBR3 cells isolated using NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Scientific). After cell fractionation, GAPDH and Histone-3 levels were used as the loading controls for cytoplasm fraction and nuclear fraction respectively.

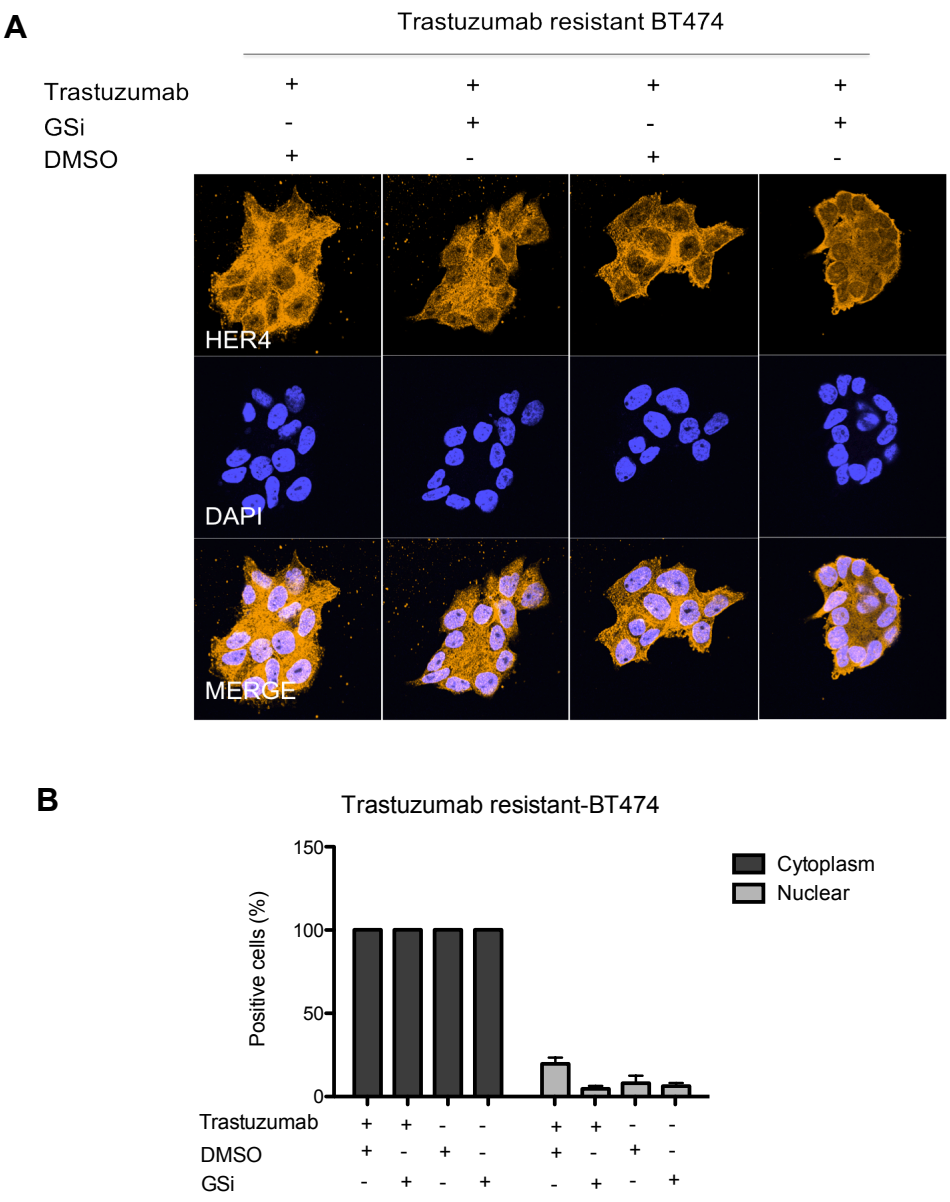


Figure 5.5 The effect of GSi in trastuzumab resistant BT474.

Resistant BT474 cells was continuously treated with trastuzumab and withdrawn for an overnight incubation. 10μM GSi was added and the cells were incubated for overnight. Cells were then fixed for confocal experiment to stain for HER4-Alexa Fluor 546 and DAPI. (B) A graph shows the percentage of positive cells stained with cytoplasmic and nuclear HER4, as assessed from three independent experiments. *P* value was calculated

using a Bonferroni correction for multiple comparisons to determine significant differences of HER4 protein between groups (***$p<0.001$). (C) Western blot analysis was performed on the cytoplasmic and nuclear fractions of SKBR3 cells isolated using NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Scientific). After cell fractionation, GAPDH and Histone-3 levels were used as the loading controls for cytoplasm fraction and nuclear fraction respectively.

5.2.5 Effects of GSi on cellular response in trastuzumab resistant cells

Cell viability was assessed after the addition of 24 hours GSi in trastuzumab resistant cells. It was seen that treatment with GSi decreased the cell counts in resistant SKBR3, with and without trastuuzmab (Figure 5.6A). However, the percentage of cell viability between treatment group and control-DMSO was not statistically significant. No changes on cell counts were seen after GSi treatment in resistant BT474, (Figure 5.6B).

Even though GSi did not reduced cell numbers significantly, the clonogenic assay showed a significant decreased in colonies after GSi treatment in resistant SKBR3 that is continuously treated with trastuzumab compared to control, $p<0.001$ (Figure 5.6C). Similarly, GSi also prevented colony formation in resistant SKBR3 cells with trastuzumab withdrawn. However, this had no effect on colony formation in trastuzumab resistant BT474 (data not shown)

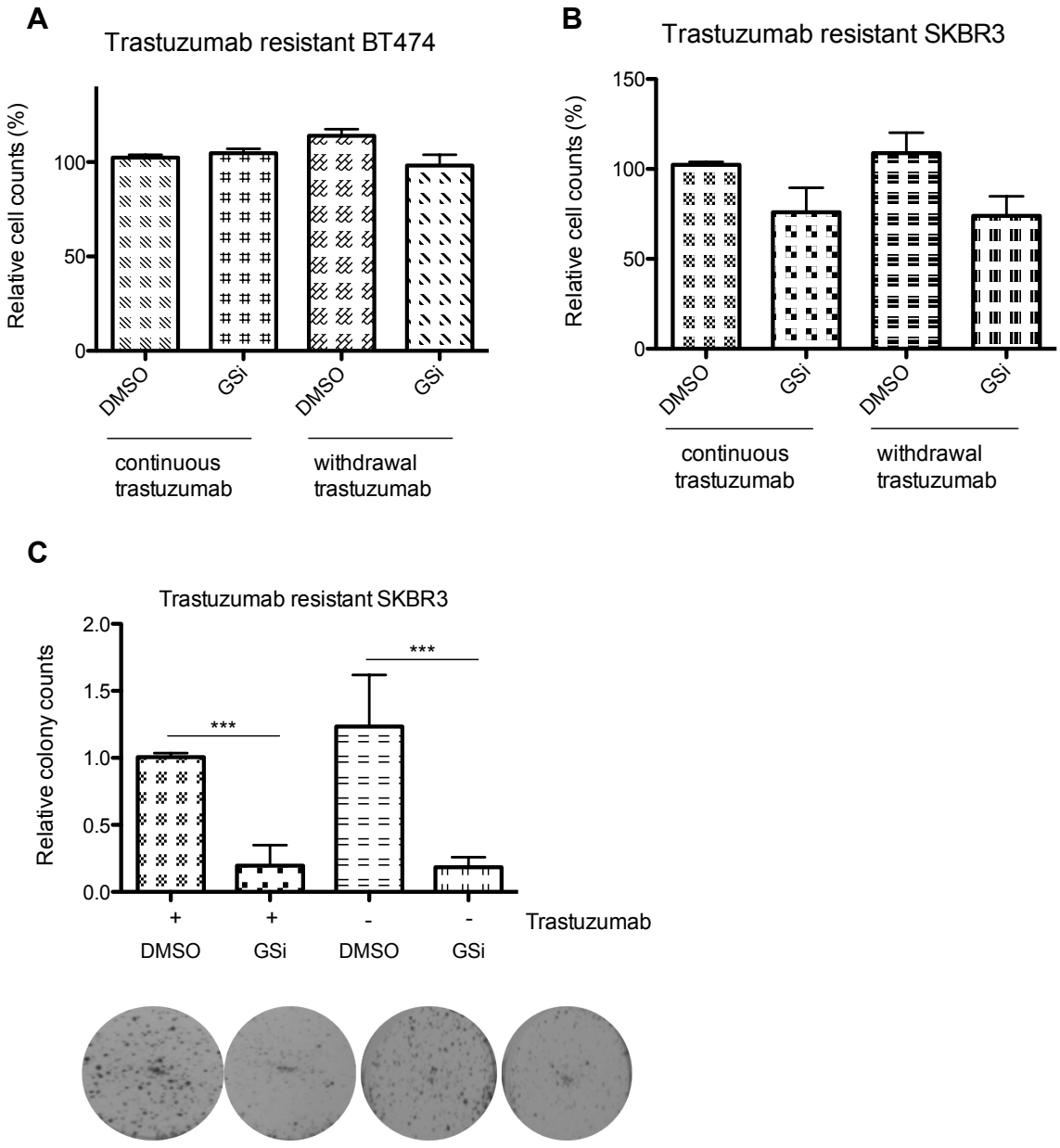


Figure 5.6 GSi decreases cell counts and prevents colony formation in resistant SKBR3 cells but not in BT474 cells.

The effect of 10μM GSi on trastuzumab-resistant SKBR3 and BT474 was studied using cell viability and colony formation assay. The cell number for the treatment group was normalised relative to the untreated control. (A&B) Graphs indicate means ± SEM of relative cell counts from three independent experiments with the differences between

groups were assessed by 1-way ANOVA test. (C) The relative ratio of colony counts in trastuzumab resistant SKBR3 is shown on the upper panel and representative colony pictures are shown on the bottom panel. Visible colony were counted using Image J software and normalised relative to the untreated trastuzumab-resistant SKBR3. Graph indicates means with ± SEM. *P* value was calculated using a Bonferroni correction for multiple comparisons (*** denotes $p<0.001$).

5.2.6 Effects on neratinib on HER4 expression and activation in HER2 positive breast cancer cells

As shown in Figure 5.7A, neratinib led to an expected decrease in pHER4 after 24h treatment of treatment with and without trastuzumab treatment in parental SKBR3 cells. The treatment also decreased the intensity of several bands around 180kDa, 110kDa and 80kDa (Figure 5.7A&B), which was observed in both SKBR3 and BT474 cells. Neratinib also blocked the upregulation of HER4 induced by trastuzumab resistance (Figure 5.7B).

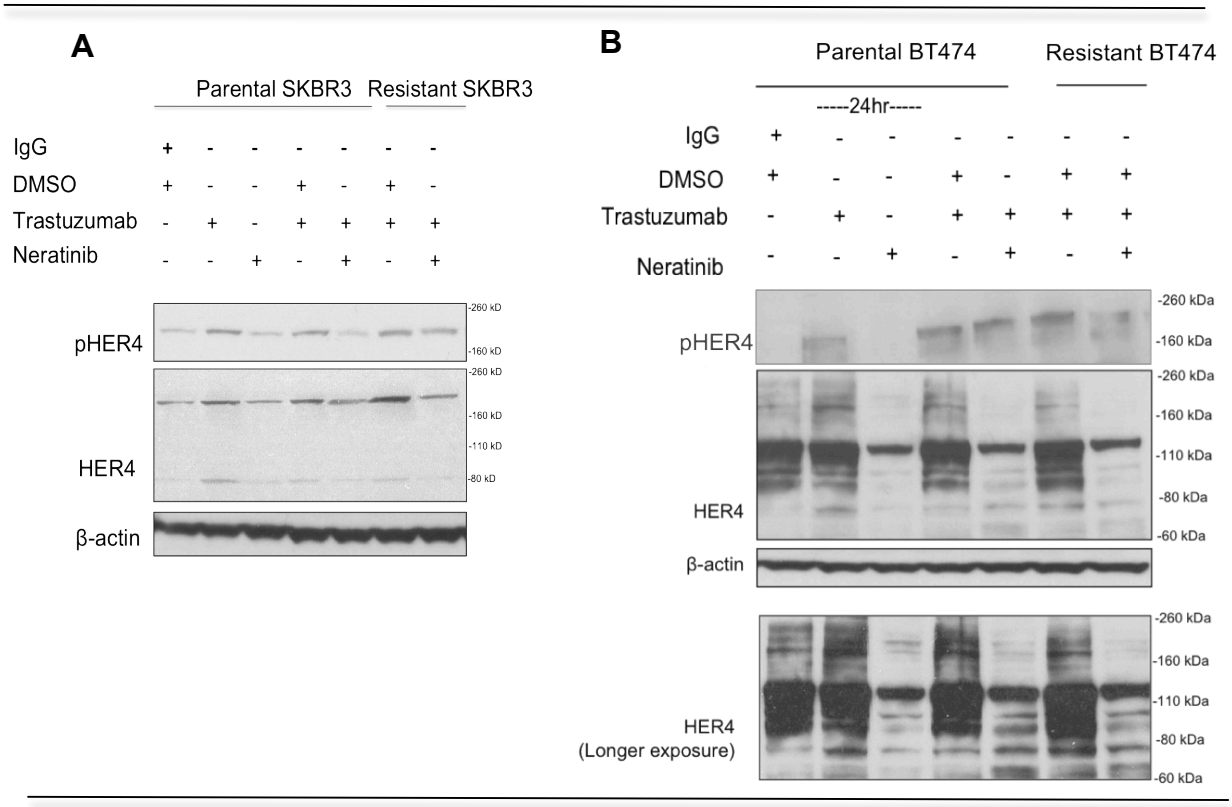


Figure 5.7 Total HER4 and its phosphorylation are decreased after neratinib treatment in HER2 positive breast cancer.

Trastuzumab parental and resistant cells were treated with 100 nM neratinib and/or 40μg/ml trastuzumab. Cells were lysed for Western blot analysis. Representative Western blots from membranes that were blotted with HER4 (Neomarkers), pHER4 (Santa Cruz) and β-actin.

5.2.7 Effects on neratinib on HER4 localisation in parental and resistant SKBR3 cells

This study also examined the effect of neratinib on HER4 localisation in SKBR3 cells. Figure 5.8 (left panel) demonstrates that 24-hr treatment with neratinib decreased nuclear HER4, compared to control. However, the decrease in the percentage of cells with positive nuclear HER4 after single neratinib treatment was not statistically significant compared to control SKBR3 cells (Figure 5.8, right panel). When neratinib was added with trastuzumab, the treatment decreased the % positive of cytoplasmic and nuclear HER4 compared to trastuzumab alone or trastuzumab with DMSO (Figure 5.8, left panel), which was statisitically significant ($n=3$, $p<0.001$, Figure 5.8 right panel). Neratinib also decreased nuclear HER4 % positivity in trastuzumab resistant SKBR3 ($n=3$, $p<0.001$, Figure 5.8 right panel) .

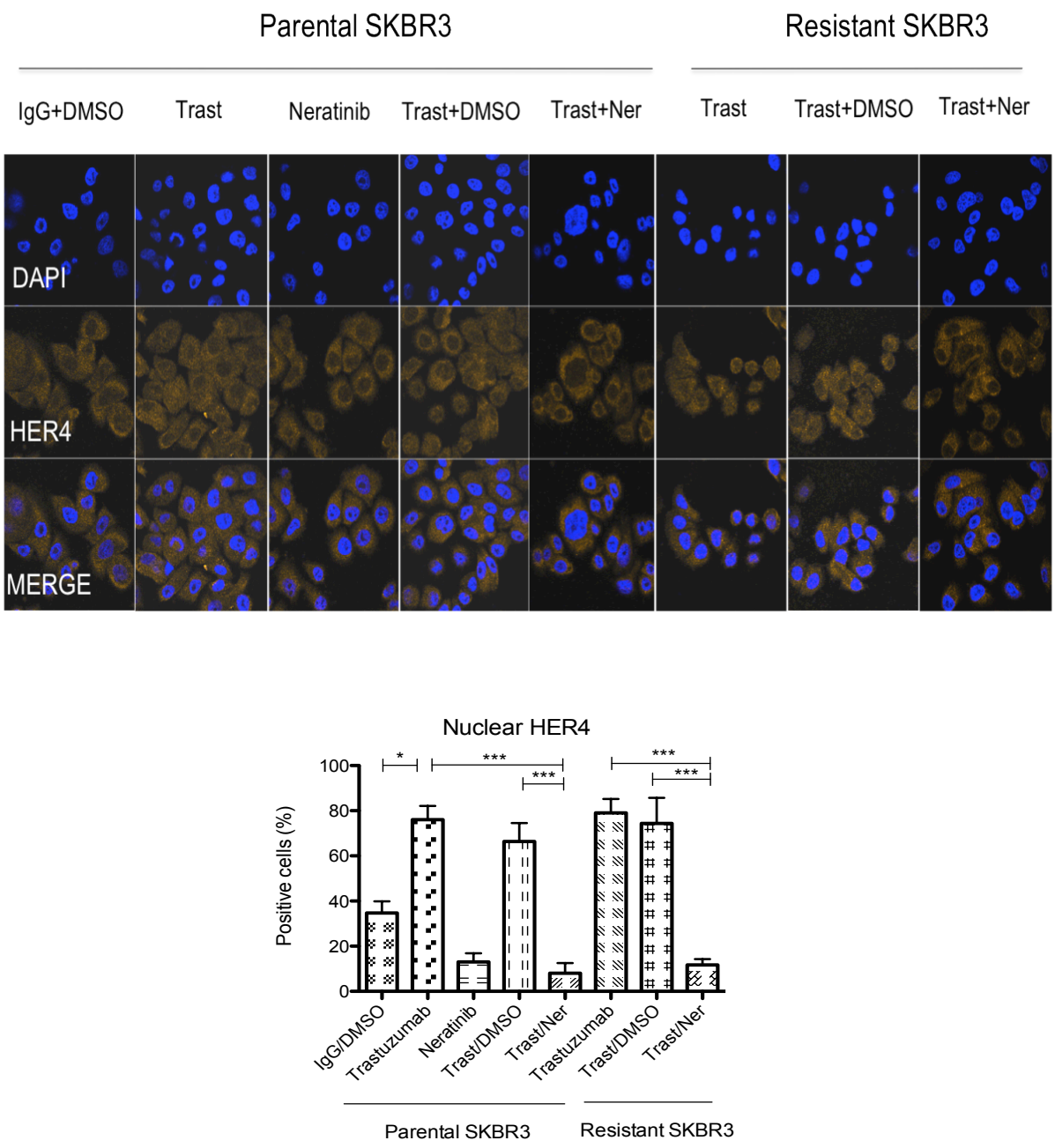


Figure 5.8 Neratinib prevents nuclear HER4 translocation induced by trastuzumab.

Parental and trastuzumab resistant SKBR3 cells were treated with 100 nM neratinib and/or 40μg/ml trastuzumab, before being fixed for confocal microscopy and lysed for cellular fractionation experiments. (A) Representative confocal images from different

treatment conditions are shown. DAPI staining was used to indicate nuclear staining. (B) Western blot analysis probing for HER4 (Neomarkers) was performed on the cytoplasmic and nuclear fractions. GAPDH and Histone-3 were used as the loading control for cytoplasm fraction and nuclear fraction, respectively.

5.2.8 Effects on neratinib on colony formation in parental and trasuzumab resistant SKBR3 cells

Previous work from the lab has shown that the combination treatment of neratinib and trastuzumab is more effective in decreasing cell viability compared to either treatment alone in HER2 amplified breast tumor cells, as assessed by the cell viability experiments (Canonici et al., 2013). As neratinib was observed to decrease nuclear HER4 localisation in parental and trastuzumab resistant SKBR3 cells, this study further investigated the effect of neratinib treatment on colony formation in these cells, which was not previously done by Canonici et al.

Figure 5.9 demonstrates that neratinib prevented SKBR3 colony formation compared to untreated or DMSO-treated cells ($n=3$, $p<0.001$, Figure 5.9 B). Neratinib, when combined with trastuzumab also showed similar effect in decreasing colony formation as compared to trastuzumab alone or trastuzumab +DMSO ($n=3$, $p<0.001$, Figure 5.9 B). Figure 5,9 also indicates trastuzumab withdrawal caused an increase of colony counts ($n=3$, $p<0.05$, Figure 5.9B), which is similar with that observed from cell viability experiments in Chapter 4 (Figure 4.10). However, neratinib decreased colony counts in trastuzumab resistant SKBR3 cells that had continuous treatment with trastuzumab or with trastuzumab withdrawal ($n=3$, $p<0.001$, Figure 5.9 B).

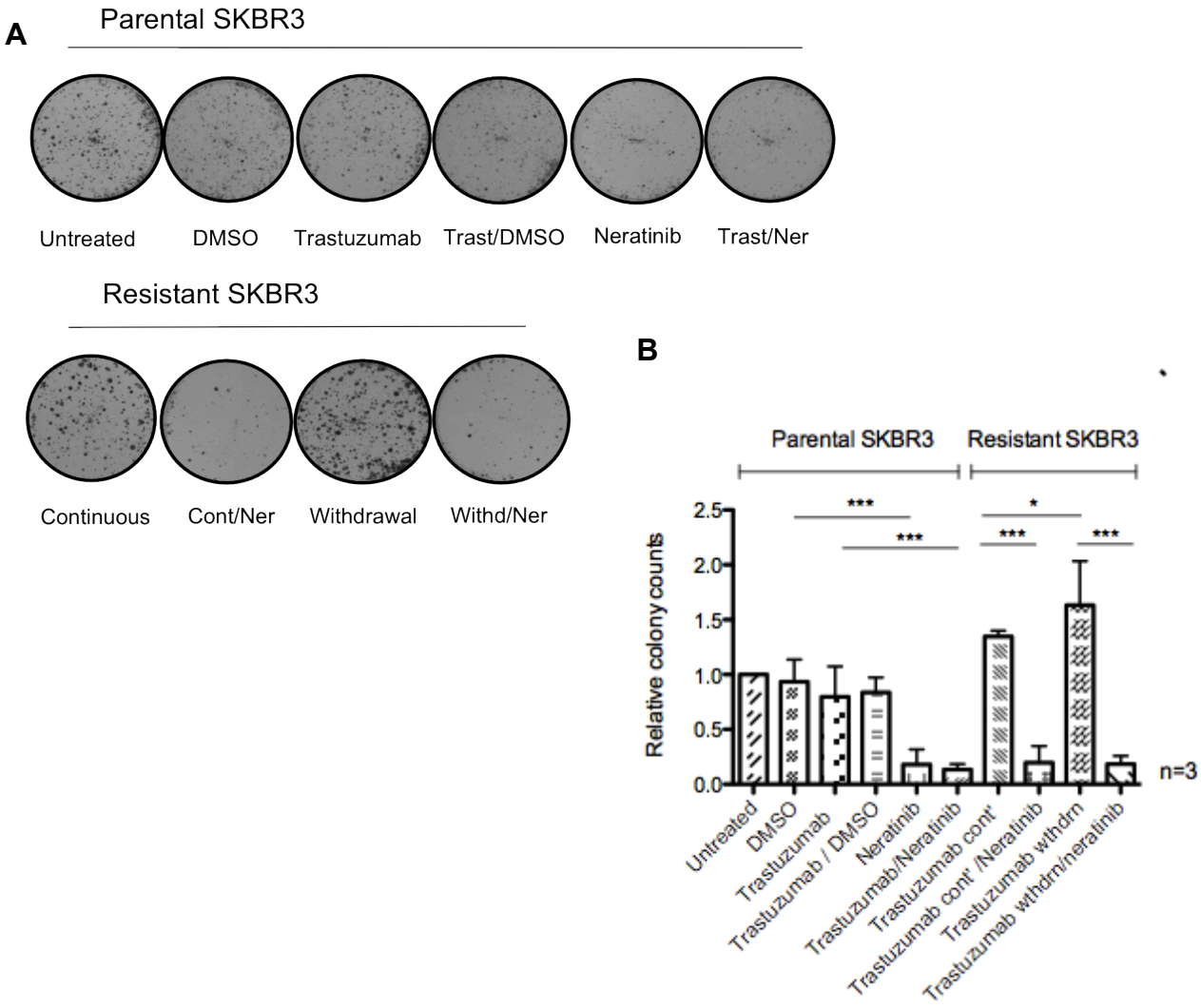


Figure 5.9 Neratinib treatment in SKBR3 prevents colony formation.

Parental SKBR3 and cells resistant to trastuzumab were treated with 100 nM neratinib and/or 40μg/ml trastuzumab for 72 hours and re-plated in duplicate for colony formation assay for 7 days. Representative colony pictures are shown on the upper panel and the relative ratio of colony counts are shown on the bottom panel. Visible colonies were quantified using Image J software and normalised relative to the untreated parental SKBR3. *P* value was calculated using a Bonferroni correction for multiple comparisons (*p < 0.05, ***p < 0.001).

5.2.9 HER4 expression in BT474 xenograft mice treated with neratinib and trastuzumab

Figure 5.11A illustrates HER4 immunostaining on BT474 xenograft tumors treated with either the control, trastuzumab alone, neratinib alone or trastuzumab with neratinib (Canonici et al., 2013). Neratinib alone was seen to decrease cytoplasmic and nuclear HER4 (Figure 5.11B). From this figure, there was also an increase in cytoplasmic and nuclear HER4 expression after trastuzumab treatment in xenograft tumors. However, this was prevented by the combination treatment of trastuzumab with neratinib (Figure 5.11A). The difference in the nuclear HER4 expression in the combination treatment of trastuzumab and neratinib was statistically significant compared to trastuzumab alone ($p<0.05$, Figure 5.11B).

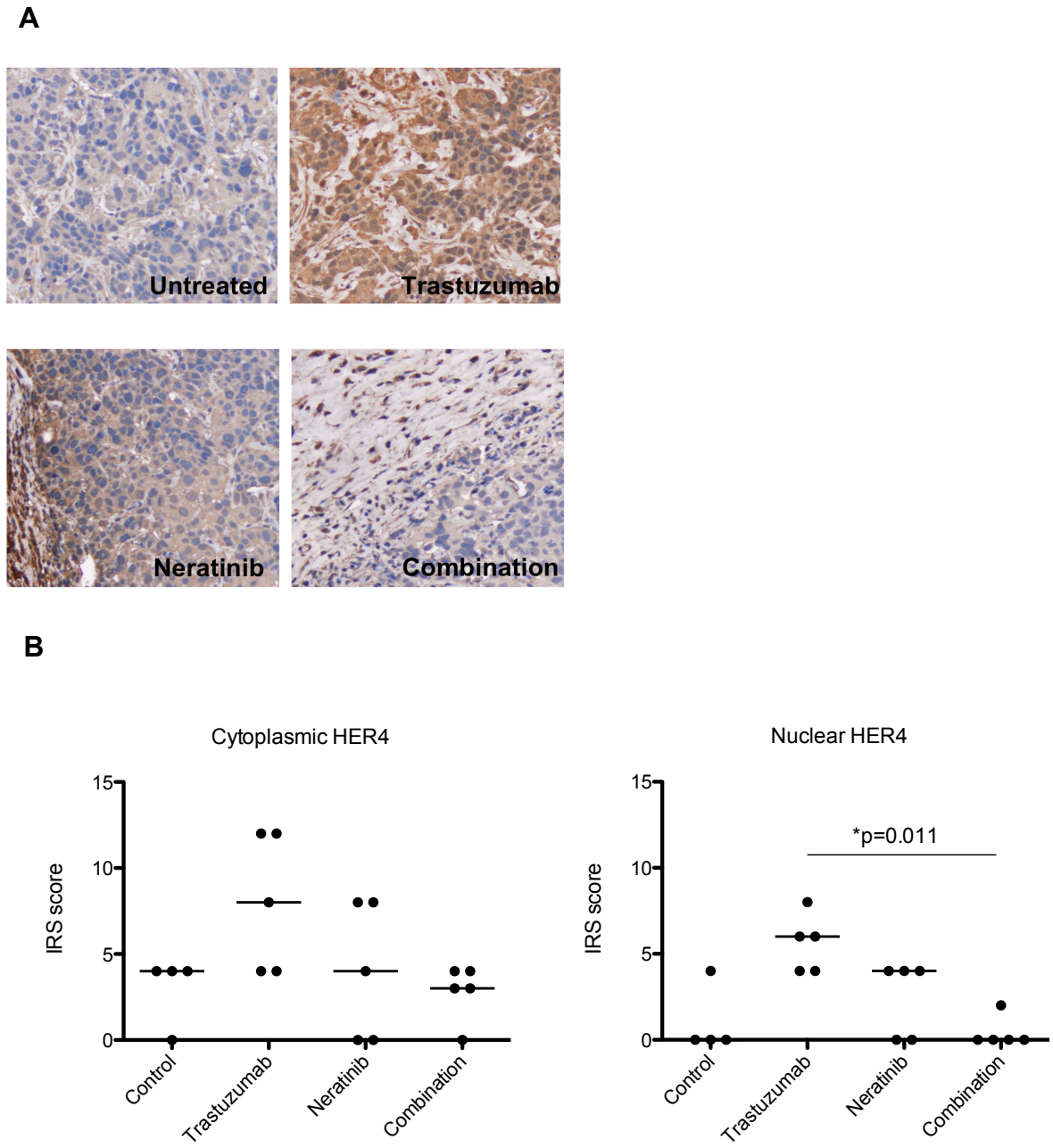


Figure 5.10 HER4 expression in BT474 xenograft models treated with trastuzumab and neratinib.

BT474 xenografts were treated with vehicle control, neratinib, trastuzumab or their combination (Canonici et al., 2013) and the tumor slides were stained for HER4

expression using IHC. (A) Representative tumor sections from formalin-fixed, paraffin embedded tumor tissues of animals receiving different treatments are shown. (B) The IRS scores of HER4 immunohistochemical staining of cytoplasmic, membranous and nuclear localisation of the tumors are shown in the bottom panels. Kruskal-Wallis rank-sum test was used to determine the difference in HER4 IRS scores among treatment groups (*$P<0.05$).

5.3 Discussion

It was seen in Chapter 3 that trastuzumab treatment leads to an increase of HER4 nuclear intensity. In addition, nuclear HER4 is also observed to be increased in trastuzumab resistant SKBR3 cells, suggesting that nuclear HER4 may mediate resistance to trastuzumab. The controversial effect of HER4 may be related to its cellular localisation since it has been shown that upon HER4 cleavage, it is translocated to the nucleus, interacting with different transcription factors (Aqeilan et al., 2007; Elenius et al., 1999; Linggi and Carpenter, 2006b; Sundvall et al., 2010; Williams et al., 2004; Zhu et al., 2006). To further validate the trastuzumab effect on HER4 activation and cleavage leading to its nuclear translocation, this study used GSi and neratinib to decrease HER4 cleavage and inhibits its activity in trastuzumab-treated cells. γ-secretase has been described to mediate HER4 cleavage and its nuclear localisation (Lee et al., 2002; Ni et al., 2001; Vidal et al., 2005; Williams et al., 2004) while neratinib has been reported to decrease HER4 tyrosine kinase activity (Burstein et al., 2010; Canonici et al., 2013; Ito et al., 2012; Rabindran et al., 2004)

This chapter demonstrates that there is a reduction of m80 and decreased nuclear HER4 translocation in SKBR3 when treated with GSi in combination with trastuzumab, correlated with an increase in apoptosis and decreased cell proliferation. Our study provides evidence that HER4 nuclear translocation during trastuzumab treatment is due to its proteolytic cleavage mediated by γ-secretase. It has been reported that GSi inhibited heregulin-dependent tumor growth (Feng et al., 2007; Ni et al., 2001). Our lab has previously shown that trastuzumab induces endogenous heregulin (Gijsen et al., 2010). Chapter 3 has shown that trastuzumab induces HER4 nuclear translocation similar to heregulin stimulation, which can be prevented by GSi (Chapter 5). However, it

cannot be excluded that the growth inhibition by GSi could also be due to its effect on other signalling pathways, e.g. Notch or E-cadherin (Olsauskas-Kuprys et al., 2013; Yoo et al., 2012).

Previously, it has been shown that neratinib increased trastuzumab sensitivity and reversed trastuzumab resistance in HER2 positive breast cancer cells (Canonici et al., 2013). However, the report did not show the neratinib effect on HER4 expression and nuclear translocation. Here, in this chapter, we showed that whereas trastuzumab increased HER4 expression (both $HER4_{180kDa}$ and $HER4_{80kDa}$) and its nuclear translocation, neratinib had an opposite effect to trastuzumab and prevented the induction of m80 and HER4 nuclear translocation. This highlights the importance in understanding the molecular mechanisms of actions and resistance between different anti-HER2 therapies. The opposing effects of neratinib and trastuzumab on HER4 could be one of the reasons why their combination was more effective than either drug alone in preclinical models (Canonici et al., 2013).

CHAPTER SIX

The role of HER4 as a prognostic factor in HER2 positive breast cancer patients

CHAPTER 6: THE ROLE OF HER4 AS A PROGNOSTIC AND PREDICTIVE FACTOR IN HER2 POSITIVE BREAST CANCER PATIENTS

6.1 Introduction

In the previous chapters, the role of HER4 in HER2 positive breast cancer in relation to trastuzumab treatment was investigated in cancer cell lines, and in xenograft model. The results showed that the upregulation of HER4 occurred during trastuzumab treatment and upon resistance. It is hypothesized that HER4 expression might be relevant as a biomarker which may predict prognosis and trastuzumab response in HER2 positive breast cancer patients. In this chapter, the role of HER4 in HER2 overexpressing breast cancer patients was further investigated.

The role of HER4 in relation to trastuzumab therapy has been recently studied (Portier et al., 2013; Sassen et al., 2009). Sassen et al. demonstrated that HER4 expression is associated with improved overall survival in trastuzumab-treated patients (Sassen et al., 2009). In addition, a recent study showed that patients with HER2/HER4 co-over-expression had a better outcome after neo-adjuvant trastuzumab therapy and adjuvant trastuzumab therapy (Portier et al., 2013). However, both studies do not relate their findings to HER4 localisation. To assess the prognostic value of HER4 expression in different cell compartments, this study stained TMAs from a well-annotated cohort of HER2 positive breast cancer patients for HER4 expression. This chapter also investigated the predictive role of HER4 expression in another set HER2 positive breast cancer patients who received trastuzumab monotherapy followed by its combination with neoadjuvant chemotherapy. The findings reported here suggest an important role

for HER4 localisation in HER2 positive breast cancer in relation to trastuzumab treatment.

6.2 Results

6.2.1 Assessing the role of HER4 in HER2 positive breast cancer patients

The specificity of the HER4 staining by IHC was first validated using cell pellets (Chapter 3). This study assessed HER4 expression on well-annotated cohort HER2 positive breast cancer patients (see Section 2.2.5.3, Chapter 2). The characteristics of the patients and tumor parameters are listed in Table 6.1. Tumor staining was scored by IRS method (see Section 2.2.5.5, Chapter 2) and the median expression was used as the cut-off value; 6 for cytoplasmic HER4 and 1 for nuclear HER4. The results showed that high cytoplasmic HER4 (IRS score ≥ 6) was statistically significantly correlated with better overall survival (OS) and relapse-free survival (RFS) than that of patients with low cytoplasmic HER4 (IRS score <6) (OS: HR = 0.19, 95% CI = 0.04-0.80, p = 0.024; RFS: HR = 0.27, 95% CI = 0.09-0.85, p = 0.026) (Figure 6.1A). In contrast, Kaplan-Meier analysis of nuclear HER4 expression demonstrated an inverse correlation between nuclear HER4 and patient survival (IRS score ≥ 1 versus IRS score = 0, OS: HR = 13.63, 95%CI = 2.88-64.53, p < 0.001; RFS: HR = 6.08, 95%CI = 1.78-20.81, p = 0.004) (Figure 6.1B).

From the univariate analysis, nodes status is a variable that was selected for multivariate analyses with cytoplasmic and nuclear HER4 (Table 6.2) (see Section 2.2.6, Chapter 2). In a multivariate analysis with nuclear HER4-derived groups adjusted for nodal status, nuclear HER4 remained an independent prognostic factor for OS (p =

0.003) and RFS (p = 0.001); whereas cytoplasmic HER4 appeared to be a weak prognostic predictor of OS (p = 0.006) and RFS (p = 0.04) (Table 6.2).

Table 6.1 Correlations of cytoplasmic and nuclear HER4 expression with HER2 positive breast cancer patients' characteristics.

Characteristics	Cytoplasmic HER4			Nuclear HER4		
	NEG (<6)	POS (≥6)		NEG (0)	POS (≥1)	
Tumor grade	11	10				
1, 2	24	28		12	9	
3	35	38		35	17	
			p=0.796	47	26	p=0.430
Tumor size						
<2 cm	12	12		16	8	
≥2 cm	22	26		31	17	
	34	38	p=0.805	47	25	p=1.000
Age						
<55 years	10	16		19	6	
≥55 years	25	22		27	20	
	35	38	p=0.328	46	26	p=0.132
Menopausal						
Pre	6	9		12	3	
Post	29	29		35	23	
	35	38	p=0.569	47	26	p=0.228
Nodes						
Neg	15	21		23	13	
Pos	16	17		23	10	
	31	38	p=0.632	46	23	p=0.798
ER						
Neg	6	14		14	5	
Pos	27	24		31	20	
	33	38	p=0.113	45	25	p=0.405

P-value was calculated using independent T-test to correlate cytoplasmic and nuclear HER4 scoring with patient characteristics and tumor histology in a cohort of HER2 positive breast cancer patients.

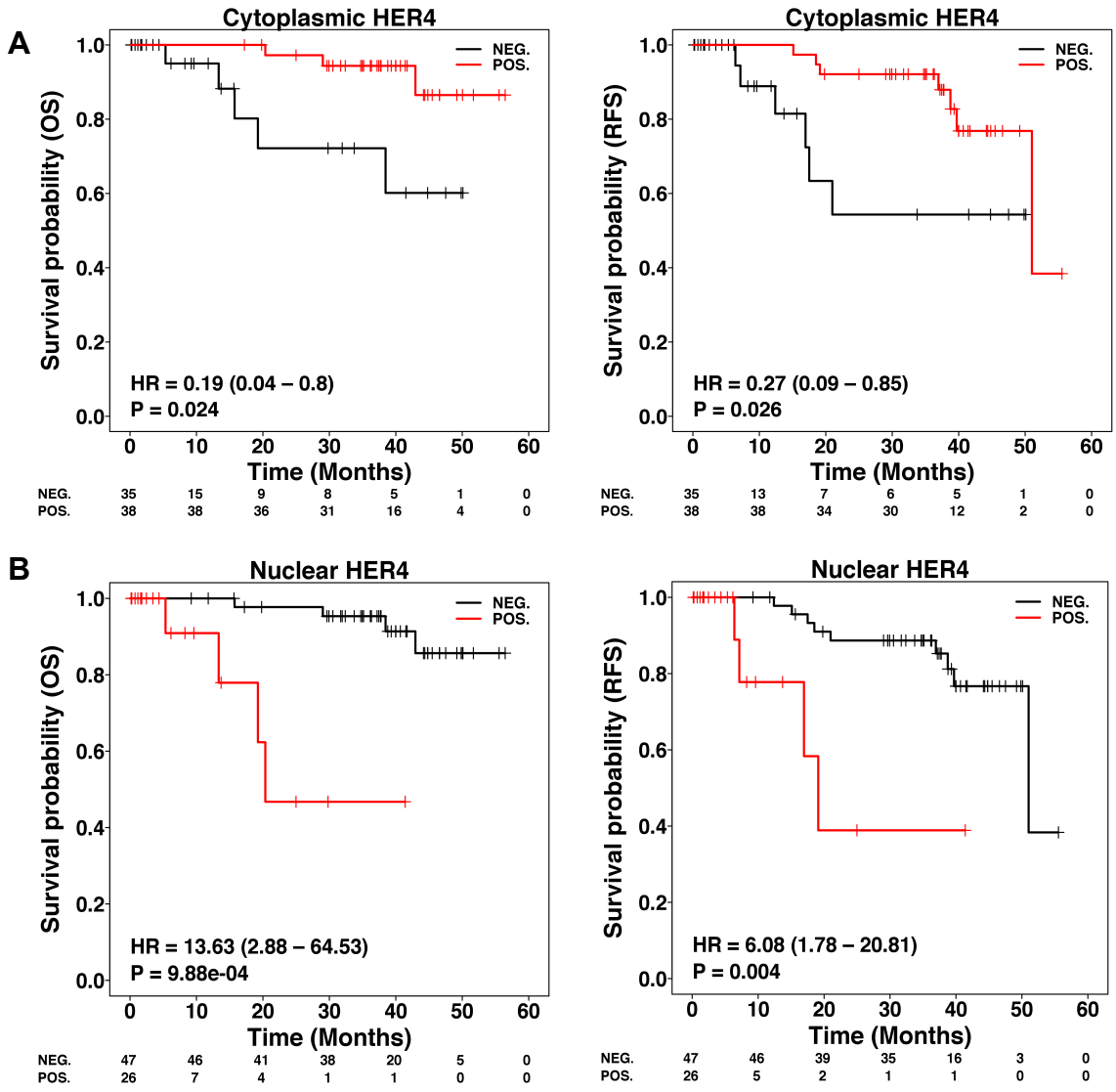


Figure 6.1 Nuclear HER4 predicts poor trastuzumab response and disease free survival in HER2 positive breast cancer patients.

TMAs containing paraffin embedded breast tumors were examined for HER4 expression by IHC. Patients were split into two groups according to the negative cytoplasmic HER4 (<6) and positive cytoplasmic HER4 (≥6) (A) and negative nuclear HER4 (0) and positive nuclear HER4 (≥1) (B) staining as defined in the method section. Survival curve analysis (Kaplan-Meier test) estimates the overall survival (OS) and relapse-free survival (RFS) of the two groups according to the cytoplasmic and nuclear HER4 staining in breast cancer patients (n= 73).

Table 6.2 Univariate and multivariate analysis for cytoplasmic and nuclear HER4 in a cohort of HER2 positive breast cancer patients.

	OS (n=73)		RFS (n=73)	
Variables	HR (95%CI)	p-value	HR (95%CI)	p-value
A. Univariate analysis				
Cytoplasmic HER4	0.19 (0.04-0.80)	0.002*	0.27 (0.09-0.85)	0.026*
Nuclear HER4	13.63 (2.88-64.53)	<0.001*	6.08 (1.78-20.81)	0.004*
Tumor grade	1.179 (0.32-4.22)	0.800	1.247 (0.44-3.47)	0.672
Age	1.073 (1.01-1.13)	0.21	1.033 (0.98-1.08)	0.186
Menopausal	1.81 (0.396-8.27)	0.320	0.552 (0.12-2.52)	0.444
Nodes	0.153 (0.01-1.24)	0.079*	0.092 (0.80-1.06)	0.062*
ER	0.963 (0.23-4.03)	0.959	0.392 (0.12-1.24)	0.112
B. Multivariate analysis				
Cytoplasmic HER4	0.245 (0.56-1.07)	**0.06**	0.313 (0.96-1.022)	**0.04**
Nodes	4.35 (0.50-37.53)	0.18	2.713 (0.71-10.29)	0.14
Nuclear HER4	2.269 (1.37-3.87)	**0.003**	2.978 (1.59-5.57)	**0.001**

Nodes	4.879 (0.57-41.31)	0.146	3.196 (0.87-11.74)	0.080

P-values and hazard ratio (HR) with 95% confidence interval (CI) were calculated from Cox proportional hazards regression analysis. The parameters with a p <0.1 (marked with *) were chosen for the multivariable analysis. The parameter's selection follows a statistical analysis protocol from (Machleidt et al., 2013). P<0.05 calculated from multivariable analysis was highlighted, as shown in the table.

6.2.2 Assessing the role of nuclear HER4 in trastuzumab-treated breast cancer patients

This study also assessed HER4 expression in HER2 positive breast cancer patients who underwent a window study as outlined in Figure 6.2. The samples were obtained from our collaborators in Italy and the trial was conducted with appropriate ethical approval (Protocol CE-21392/2012) (see Methods 2.2.5.3). The characteristics of the patients are listed in Table 6.3.

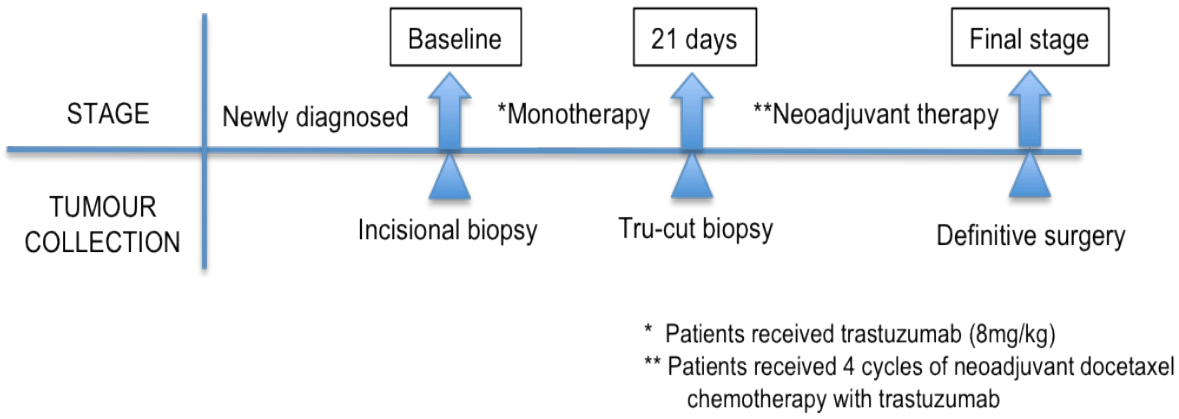


Figure 6.2 Schematic illustration of the window of opportunity study.

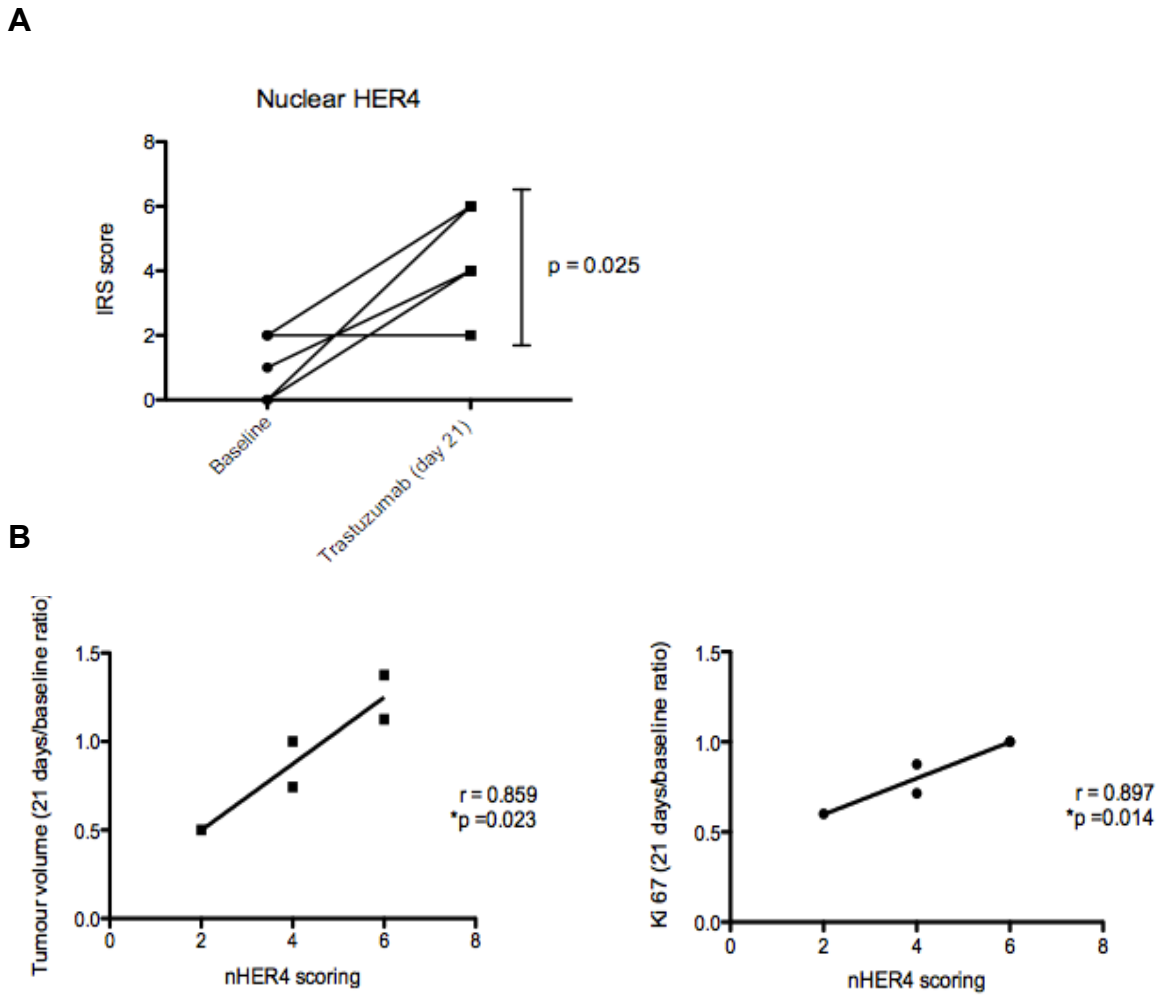
A schematic diagram shows a study conducted among HER2 positive breast cancer patients who received trastuzumab monotherapy followed by neoadjuvant chemotherapy with trastuzumab before surgery. Tumor specimens were collected at the baseline, 21 days after trastuzumab monotherapy and at the final stage after completed 4 cycles of neoadjuvant docetaxel chemotherapy 100 mg/m2 with 6mg/kg trastuzumab.

Table 6.3 Baseline characteristics and variables of HER2 positive patients underwent a window study of trastuzumab monotherapy and neoadjuvant therapy.

Characteristics (n=17)	Value
Median age (range)	59 (39-79)
Median clinical tumor size (range)	40 (15-80)
Tumor grade	
1, 2	5 (29%)
3	12 (71%)
Menopausal status	
Pre	5 (29%)
Post	12 (71%)
Oestrogen receptor	
Negative	8 (47%)
Positive	9 (53%)
Progesterone receptor	
Negative	5 (29%)
Positive	12 (71%)

There were 5 out of 17 patients underwent biopsy before and after trastuzumab monotherapy treatment. All samples had higher cytoplasmic staining (IRS score ≥6) (data not shown). After trastuzumab monotherapy, there was a statistically significant upregulation of nuclear HER4 expression (p=0.025) in the tumor biopsies collected at day 21 (mean IRS score=3.20, 95%CI 1.84 to 4.56) compared to the biopsies from the initial diagnosis (mean IRS score=0.80, 95%CI -0.56 to 2.16) (Figure 6.3A). In addition, higher nuclear HER4 expression at day 21 was correlated with a poorer clinical response to trastuzumab monotherapy, as defined by a bigger tumor volume (r=0.859, p=0.023) and higher Ki67 level (r=0.897, p=0.014) (Figure 6.3A).

However, there was no significant difference in the tumor nuclear HER4 expression between baseline and those after neoadjuvant chemo with trastuzumab (Figure 6.4A). In addition, nuclear HER4 after neoadjuvant chemotherapy did not correlate with tumor volume and Ki67 status obtained from definitive surgery (Figure 6.4B).



Patients	HER4 score	Tumor volume (21 days/baseline ratio)	Ki67 (21 days/baseline ratio)
1	2	0.5	0.6
2	4	1.0	0.7
3	4	0.7	0.9
4	6	1.1	1.0
5	6	1.4	1.0

Figure 6.3 Nuclear HER4 expression after neo-adjuvant treatment among HER2 positive breast cancer patients.

Nuclear HER4 expression was analysed in paired tissue samples (baseline and at 21 days) from 5 patients who received one dose of trastuzumab monotherapy. (A) The differences of HER4 IRS score between groups were assessed by paired t-test,

(*$p<0.05$). (B) Nuclear HER4 expression of tumors was correlated with Ki67 staining and tumor size, as assessed by linear regression. The graphs represent means ± SEM. Table of individual data are shown below the graphs.

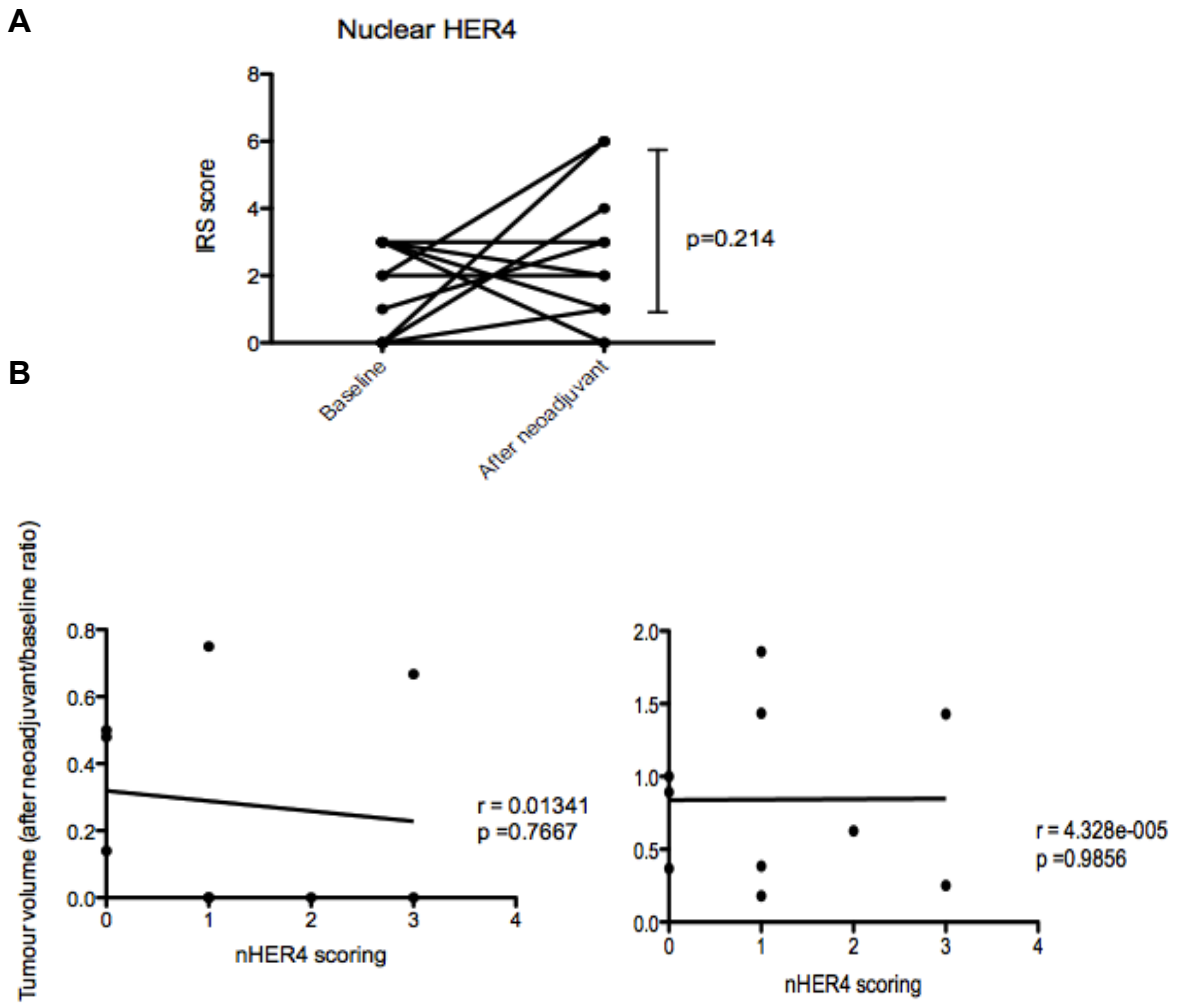


Figure 6.4 Nuclear HER4 expression after neo-adjuvant treatment among HER2 positive breast cancer patients.

(A) Nuclear HER4 expression in tumor samples from patients who received neoadjuvant chemotherapy with trastuzumab was compared to the nuclear HER4 expression at the baseline. Analysis on HER4 IRS score was done in paired tissue samples from 14 patients using paired t-test. (B) Nuclear HER4 expression of tumors from definitive surgical samples after patients underwent neoadjuvant chemotherapy with trastuzumab was correlated with Ki67 staining and tumor size, as assessed by linear regression. The graphs represent means ± SEM.

6.3 Discussion

From Hollmen and Eleinus's review, the prevalence of HER4 expression in breast cancer varied from 19% to 92%, depending on different breast cancer subtypes, and different experiment settings (Hollmen and Elenius, 2010). As stated in Chapter 1 (Section 1.3), the role of HER4 in predicting survival of breast cancer patients has been noted contradicted. Whilst a number of previous studies showed HER4 predicts better outcomes in breast carcinoma (Barnes et al., 2005; Koutras et al., 2008; Suo et al., 2002; Witton et al., 2003), however, it was also shown that this marker could be important for poor prognosis (Bieche et al., 2003). One of the reasons of the discrepancy between different studies could be related to the different prognostic significance of HER4 localisation. HER4 ICD cytosol and HER4 cytoplasmic variants have been shown to be associated with improved survival of breast cancer patients (Machleidt et al., 2013; Thor et al., 2009). In this study, I have shown a similar pattern that HER2 positive breast cancer patients with high level of cytoplasmic HER4 had a better prognosis. However, positive nuclear HER4 was correlated with poorer outcomes. This is in agreement with findings from Juntilla' study although the study did not specifically look at HER2 positive breast cancer patients (Junttila et al., 2005). This suggested that the different reports of HER4 on breast cancer survival might be due to failure to look at clinical significance of HER4 localisation in these patients. However, data from my study was not able to correlate HER4 expression with any other known prognostic biomarkers such as ER and lymph node status.

Even though HER4 expression has been demonstrated to predict drug sensitivity in trastuzumab treated patients (Portier et al., 2013; Sassen et al., 2009), however, both studies did not relate their findings in relation to HER4 localisation. As nuclear HER4

has been shown to be an adverse prognostic factor in HER2 positive breast cancer in this study, the effect of trastuzumab on HER4 expression and the predictive role of nuclear HER4 expression were further investigated in HER2 positive breast cancer patients who received trastuzumab monotherapy followed by its combination with neoadjuvant chemotherapy. The results demonstrated a significant upregulation of nuclear HER4 expression following trastuzumab treatment, and nuclear HER4 upregulation was correlated with poorer clinical response to trastuzumab, confirming a role of nuclear HER4 in mediating trastuzumab resistance in breast cancer patients.

In summary, cytoplasmic HER4 has been shown to correlate with improved survival of breast cancer patients whereas nuclear HER4 correlates with poorer relapse-free survival in a cohort of HER2 positive breast cancer patients. For patients who underwent a window study of trastuzumab monotherapy, trastuzumab treatment increased nuclear HER4 expression and pre-treatment high nuclear HER4 predicted poor response to trastuzumab at day 21. Therefore, results from this chapter indicate that targeting HER4 and prevents its nuclear translocation might enhance trastuzumab response and overcome acquired trastuzumab resistance in HER2 positive breast cancer patients.

CHAPTER SEVEN

Discussion

CHAPTER 7: Discussion

7.1 General discussion

HER2 (ERBB2/neu) has been widely investigated because of its well-defined role in breast cancer (Baselga and Swain, 2009; Hynes et al., 2001; Ignatiadis et al., 2009). Trastuzumab, a HER2-targeted therapy has been shown to increase survival for early and metastatic breast cancer patients with over-expression or amplification of HER2 (Piccart-Gebhart et al., 2005; Romond et al., 2005; Slamon et al., 2001). However, the response rate to trastuzumab monotherapy is less than 30% (Vogel et al., 2002) and primary or acquired resistance remains a challenge. The resistance mechanisms of trastuzumab have been extensively studied in order to improve the outcome after trastuzumab treatment. One of the mechanisms of resistance could be due to the role of other HER receptors (HER1, HER3 and HER4) (Gijsen et al., 2010). These receptors bind to HER2 by homo- and hetero-dimerisation and take part in cells' regulation.

In contrast to other HER family receptors (HER1-3), the role of HER4 in HER2 positive breast cancer remains controversial and unclear. Several reports have linked HER4 overexpression with both good and bad outcomes in breast cancer patients (Lodge et al., 2003; Machleidt et al., 2013; Suo et al., 2002; Witton et al., 2003). HER4 expression has been shown to correlate with trastuzumab sensitivity and better outcome after neo-adjuvant trastuzumab therapy and adjuvant trastuzumab (Portier et al., 2013; Sassen et al., 2009). However, to better understand the role of HER4 during trastuzumab resistance, this study is designed to investigate on how trastuzumab impacts on HER4 expression, activation and cleavage.

From Chapter 3, trastuzumab treatment has been shown to upregulate HER4 expression at 180 kDa and 80 kDa in SKBR3 and BT474 cells, the common HER4 variants reported from the previous studies (Maatta et al., 2006; Muraoka-Cook et al., 2009). Depending on different antibody and different cell lines (Chapter 3), we also observed the appearance of HER4 at multiple bands at 180kDa, 120kDa, 110 kDa and 80 kDa. No study indicated HER4 variants at 110 and 120 kDa. However, these bands were confirmed to be specific to HER4 due to the siRNA experiments (Chapter 4). From the recent publications, other HER receptors were also reported to have multiple variants. This including HER2 that was observed express at 185, 150, 110 and 95 kDa of molecular weight (Recupero et al., 2013; Ward et al., 2013). In addition, multiple HER3 variants were also demonstrated from a study using the Schwann cell model (Adilakshmi et al., 2011). We therefore suggest that a future study could be carried out to characterise HER4 variants at 110 and 120 kDa whether they could represents as different HER4 isoforms or different HER4 cleaved fragments.

In contrast to the full-length receptor, HER4 at 80 kDa is normally described as the cleaved fragment of HER4 that is known to translocate to the nucleus (Linggi and Carpenter, 2006b; Muraoka-Cook et al., 2006b; Naresh et al., 2006; Sundvall et al., 2007). Data from Chapter 3 also demonstrates that trastuzumab-induced $HER4_{80kDa}$ correlated with increased HER4 nuclear intensity from confocal images and increased expression of cleaved HER4 fragment (m80) in the nuclear fraction of trastuzumab treated cells. The xenograft experiments also showed that nuclear HER4 staining was significantly increased in the tumors of the mice treated with trastuzumab compared to the untreated group. Furthermore, trastuzumab resistant SKBR3 and BT474 cells showed an increased intensity of nuclear HER4 compared to the parental cells.

The above results suggests the hypothesis that HER4 activation and its cleavage could play a key role in mediating resistance to trastuzumab in HER2 positive breast cancer. To better understand this role, a study was design to investigate the effect of HER4 downregulation via transient knockdown with specific siRNAs, in relation to trastuzumab treatment and resistance in HER2 positive breast cancer. Although HER4 has been shown to have an anti-proliferative activity in breast cancer cells (Kainulainen et al., 2000; Naresh et al., 2006; Sartor et al., 2001), data from Chapter 4 showed that HER4 downregulation by siRNA decreased cell viability and enhanced trastuzumab sensitivity as well as reversing resistance in HER2 positive breast cancer cells. This suggested that during trastuzumab treatment, HER4 may mediate trastuzumab resistance and reduce its inhibitory effect.

Juntilla et al (2005) observed more HER4 ICD fragments in the nucleus compartment compared to the cell surface. The study has addressed a functional connection between HER4 ICD fragments and ER signalling pathways, which was further supported by several reports (Han and Jones, 2014; Junttila et al., 2005; Zhu et al., 2006). In addition, other studies also demonstrated that nuclear HER4 ICD acts as a nuclear chaperone for STAT5A, indicating HER4 promotes apoptotic cellular response (Clark et al., 2005; Williams et al., 2004). Although the above studies providing support for the general concept of an interaction between HER4 and the transcriptional factors, these previous studies have not specifically mentioned whether these molecular cross talks are involved in mediating trastuzumab resistance. Since our study hypothesized that HER4 involves in trastuzumab resistance, we therefore suggested future investigations are required to provide new insights into the mechanisms leading to this resistance that may lead to improved trastuzumab therapy.

To investigate the mechanism of HER4 nuclear translocation during trastuzumab treatment, I utilized an inhibitor against γ-secretase, since the enzyme has been shown to mediate HER4 cleavage and its nuclear localisation (Ni et al., 2001). Data from Chapter 5 provides evidence that HER4 nuclear translocation during trastuzumab treatment is due to its proteolytic cleavage mediated by γ-secretase since there was a reduction of m80 and decreased nuclear HER4 translocation in HER2 positive breast cancer cells when treated with γ-secretase inhibitor (GSi) in combination with trastuzumab. Kong's lab has previously shown that trastuzumab induces the release of endogenous heregulin, a HER4 ligand (Gijsen et al., 2010). This study has shown that trastuzumab induced HER4 nuclear translocation similar to exogenous heregulin stimulation, which could be prevented by GSi and correlated with an increase in apoptosis and decreased cell proliferation. This is consistent with reports that GSi could inhibit heregulin-dependent tumor growth (Feng et al., 2007; Ni et al., 2001). However, this may also be due to the non-specific effects of the inhibitor on other proteins since γ-secretase also helps to process several other proteins including Notch or E-cadherin (Olsauskas-Kuprys et al., 2013; Yoo et al., 2012). As far as we concerned, there is a recent study examined the effect of GSi in clinical work (Schott et al., 2013). This study investigated the combination effect of a GSi in combination with docetaxel chemotherapy in patients with advanced breast cancer. Treatment with GSi enhanced efficacy of docetaxel and reduced tumor growth.

To assess whether HER4 cleavage and nuclear translocation during trastuzumab is due to its tyrosine kinase activity, I also utilized neratinib, which is an irreversible panHER inhibitor that has an effect against HER4 tyrosine kinase activity (Burstein et al., 2010; Rabindran et al., 2004). It has previously been shown that neratinib increased trastuzumab sensitivity and reversed trastuzumab resistance in HER2 positive breast

cancer cells (Canonici et al., 2013), but the study did not investigate its effect on HER4 expression and its nuclear translocation. Here, it was shown that whereas trastuzumab increased HER4 levels ($HER4_{180kDa}$ and $HER4_{80kDa}$) and its nuclear translocation, neratinib had an opposite effect to trastuzumab and prevented the induction of m80 and HER4 nuclear translocation (Chapter 5). This highlights the importance in understanding the molecular mechanisms of actions and resistance between different anti-HER2 therapies. The opposing effects of neratinib and trastuzumab on HER4 could be one of the reasons why their combination was more effective than either drug alone in preclinical models (Canonici et al., 2013).

The prognostic value of HER4 expression in relation to breast cancer survival has been controversial and this could be related to HER4 isoforms and its localisation (Barnes et al., 2005; Junttila et al., 2005; Machleidt et al., 2013; Sassen et al., 2008; Suo et al., 2002; Thor et al., 2009; Tovey et al., 2004). Based on HER4 localisation, data from Chapter 6 indicates that high level of cytoplasmic HER4 had a better prognosis whereas positive nuclear HER4 was correlated with poorer outcomes in HER2 positive breast cancer patients. Since nuclear HER4 translocation occurs after its activation and cleavage, it is possible that HER4 nuclear localisation is a reporter of its tyrosine kinase activity, which could be more important than HER4 expression in relation to the prognosis and trastuzumab resistance in HER2 positive breast cancer patients. These suggested that the different reports of HER4 on breast cancer survival might be due to failure to look at clinical significance of HER4 localisation in these patients. As data from Chapter 6 demonstrated nuclear HER4 as an adverse prognostic factor in HER2 positive breast cancer, I further investigated the predictive role of nuclear HER4 expression in relation to trastuzumab treatment and showed that nuclear HER4 upregulation was correlated with poorer clinical response to trastuzumab in breast cancer patients.

Therefore, future studies should be done to further validate the predictive role of nuclear HER4 in trastuzumab sensitivity and resistance in breast cancer patients.

7.2 Limitation of study

Despite the promising results of the role of HER4 in relation to trastuzumab treatment and resistance shown in this thesis, there are a few limitations to my study.

1. Unlike HER2, the major problem for HER4 detection is due to the lack of specific antibodies to recognise its different epitopes. HER4 has been shown to be expressed at different levels; low, moderate or high according to different antibodies used (Hollmen and Elenius, 2010). The HER4 antibodies that are commercially used in previous studies are mAb-1479 (Hollmen et al., 2009), sc-283 (Hollmen et al., 2009), Cell Signaling (Gijsen et al., 2010) and HFR-1 (Tovey et al., 2006). HER4 E200 clone was claimed to be one of the best antibodies since it has high specificity and sensitivity to detect HER4 (Portier et al., 2013). However, based on the optimisation, I found that NeoMarkers and SC-283 are the best antibodies to assess HER4 expression in HER2 positive breast cancer. In contrast to SC-283, this study used HER4 Neomarkers in most experiments since it has variety applications to western blot, confocal and IHC protocols (See Section 2.1.5). However, both antibodies identified the C-terminus of HER4 protein. It is hoped that future studies should also use an antibody that recognize epitope specific to the N-terminal of HER4 protein. This could be done for the confocal microscopy experiments in conjunction with the experiments using C-terminal antibody (Chapter 3) to see the difference in HER4 localisation using this antibody. It is anticipated that there will not be increased nuclear intensity with N-terminal antibody following trastuzumab treatment, in contrast to the C-terminal antibody.

2. Although the aim of this study is to investigate the role of HER4 in relation to trastuzumab resistance, it did not involve an investigation in a panel of primary trastuzumab resistant breast cell lines. My study used the two most common cell lines used for HER2 positive breast cancer models (parental and acquired trastuzumab-resistant SKBR3 and BT474 cells). It would be essential to consider whether HER4 expression could predict response in relation to the primary trastuzumab resistance in a wider panel of breast cell lines with different HER2 expression.
3. The other limitation that exists for HER4 detection could be due to its own unique character compared to other HER receptors since it has four different isoforms produced from HER4 mRNA splicing; two extracellular juxtamembrane domains known as JM-A and JM-B isoforms and two intracellular cytoplasmic domains namely as CYT-1 and CYT-2 that can induce HER4 translocation into different cellular compartments (Junttila et al., 2000; Kainulainen et al., 2000; Thor et al., 2009; Veikkolainen et al., 2011). I did not focus my study on the role of HER4 isoforms in HER2 positive breast cancer since there is a lack of validated antibodies that could detect different HER4 isoforms and there are controversial reports on the role of these isoforms in human breast cancer.
4. Since the role of HER4 has been studied in HER2 positive breast cancer in relation to trastuzumab, it may be important to assess the role of HER2-HER4 dimerisation in addition to the results presented in this thesis. To investigate this, co-immunoprecipitation and FRET experiments were initially carried out. However, several experiments were not successful and a decision was made not to pursue these experiments further.

5. A number of ligands can activate HER4 receptor, which include heparin binding-EGF, beta-cellulin and epiregulin, the ligands that also bind to HER1 (Carpenter, 2003; Zhou and Carpenter, 2000) as well as heregulin -1 & -2 that can activate HER3 and HER4 (Carraway et al., 1997) and heregulin -3 and -4 which exclusively bind to HER4 (Carpenter, 2003; Sundvall et al., 2008a). In my study, exogenous heregulin-1 was shown to upregulate HER4 expression and induced nuclear HER4 induction. However, I did not assess the effects of all the HER4 ligands on HER4 expression and localisation, which may vary for different ligand. It will be interesting to assess whether a ligand that could activate both EGFR and HER4 (e.g. betacellulin) will have a different effect compared to specific HER4 ligand like heregulin-3. Furthermore, different HER4 ligands may have different roles in mediating trastuzumab response and its resistance.
6. As trastuzumab treatment induced nuclear HER4 upregulation in BT474 xenograft models, it would be interesting to assess the efficacy of HER4 knockdown via stable transfection of shRNA to see whether this could enhance trastuzumab response and overcome its resistance in HER2 positive breast cancer *in vivo.* However, due to time limitation, this was not done. The effect of HER4 inhibition using neratinib treatment +/- trastuzumab was studied for BT474 *in vivo* models (Chapter 6).
7. The prevention of nuclear HER4 translocation was seen after treating the HER2 positive cells with GSi. However, γ-secretase also cleaves other membrane proteins such as E-cadherin and Notch. Therefore, GSi could have non-specific effects on other targets, which may affect HER4 expression and localisation. It will be important to assess the effect of GSi on other subtrates to see their effects on HER4 but this is beyond the scope of this thesis. It will also be important to assess the effect of γ-secretase knockdown on HER4 to confirm the GSI studies.

8. The other limitations of my study are that only two sets of clinical breast cancer samples were available for HER4 immunostaining with the small number of patients involved. However, a study using these samples could still show the statistically significant results, as shown in data from Chapter 6, which leads to a hypothesis that HER4 expression is a potential prognostic and predictive biomarker. However, we do not have a large set of pre and post trastuzumab treatment samples or samples containing pairs of tumor tissues before and after trastuzumab resistance to further confirm the role of nuclear HER4. It was hoped that further studies could be done to further validate HER4 as a prognostic and predictive biomarker, including using samples from Breast Cancer Campaign Tissue Bank and randomized controlled adjuvant trastuzumab trial like FinHER study (Joensuu et al., 2009).

7.3 Conclusion and future direction

In summary, this study provides a first report on the effect of trastuzumab treatment in HER4 cleavage and its nuclear translocation in HER2 positive breast cancer. Downregulation of HER4 and strategies to inhibit HER4 cleavage and nuclear translocation (by GSi and neratinib) enhances trastuzumab response and reverses its resistance. Furthermore, it was shown that nuclear HER4 is an adverse prognostic factor and may predict poor trastuzumab response in HER2 positive breast cancer.

There are several aspects, which are still in need of further investigations: the role of HER4 ligands in relation to trastuzumab treatment and resistance in HER2 positive breast cancer. Moreover, it is important to assess how HER4 relates to HER2 function and its activity and response to anti HER2 targeted therapy. These could provide a more precise picture of the role of HER4 and may offer useful insights to cancer treatment in HER2 positive breast cancer and other cancers. In addition, further validation is

required in large independent randomized trials to further determine the prognostic and predictive role of HER4 in relation to anti-HER2 treatment in breast cancer patients.

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APPENDIX

LIST OF PUBLICATION AND PRESENTATIONS

1. **Siti Norasikin Mohd Nafi,** Daniele Generali, Gabriela Kramer-Marek, Merel Gijsen, Carla Strina, Mariarosa Cappelletti, Daniele Andreis, Syed Haider, Ji-Liang Li, Esther Bridges, Jacek Capala, Roxanis Ioannis, Adrian L Harris and Anthony Kong. *Nuclear HER4 mediates acquired resistance to trastuzumab and is associated with poor outcome in HER2 positive breast cancer.* The paper is under review by Oncotarget.

2. **Siti Norasikin Mohd Nafi,** Merel Gijsen, Gabriela Kramer-Marek, Jacek Capala, Roxanis Ioannis and Anthony Kong. *Investigating the role of HER4 in relation to Trastuzumab treatment and resistance in HER2 positive breast cancer.* Poster presentation at the 24th EORTC - NCI - AACR Symposium on Molecular Targets and Cancer Therapeutics, November 2012, Dublin.

3. **Siti Norasikin Mohd Nafi** , Merel Gijsen, Gabriela Kramer-Marek, Jacek Capala, Roxanis Ioannis and Anthony Kong. *HER4 cleavage and nuclear localisation mediates Trastuzumab resistance in HER2 positive breast cancer.* Poster presentation at the AACR Annual Meeting 2013, Washington.