Targeting EGFR signalling pathway in triple negative breast cancer

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Abstract

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Epidermal growth factor receptor (EGFR) is frequently overexpressed in the majority of triple negative breast cancer patients (TNBC). However, the molecular determinants behind their limited response to EGFR-targeted therapies are poorly understood. Here, both the acute and chronic responses of TNBC to the EGFR-targeted therapy, cetuximab (CTX), have been investigated.

The expression of EGFR has been analyzed in a cohort of 2000 breast cancer tumours from the public dataset as well as in a panel of breast cancer cell lines. Furthermore, the response of TNBC cell lines to CTX has been investigated using conventional biochemical methods. Finally, a comprehensive transcriptomic profiling of an acquired CTX-resistant TNBC model by RNA sequencing has been performed to understand the molecular determinants of acquired CTX resistance.

The results confirmed that EGFR is highly expressed in TNBC in comparison to non-TNBC breast cancer tumours and cell lines, which was associated with adverse clinical outcomes. Targeting EGFR in TNBC cell lines using CTX failed to completely inhibit the EGFR signalling pathway and was associated with an increase in ADAMs-mediated release of endogenous EGFR ligands, EGF and TGFα. Inhibition of ADAMs (ADAM10 and ADAM17) significantly enhanced the anti tumour efficacy of CTX both *in vitro* and *in vivo*. Furthermore, transcriptomic profiling of the acquired CTX-resistant TNBC cell line (MDA-MB-468CR) revealed an activation of several key oncogenic pathways and genes,

including the TGFβ/BMP pathway. Blocking BMP receptors (BMPRs) restored the sensitivity of resistant cells to CTX treatment.

Collectively, current findings offer alternative strategies that could enhance the CTX response in TNBC. We further reported that simultaneous targeting of both EGFR and BMPR pathways could overcome CTX resistance, which might have important implications for the treatment of TNBC.

Declaration

Unless otherwise indicated, the author at the Weatherall Institute of Molecular Medicine, University of Oxford, performed all the work described in this thesis. The work reported in this thesis has not been submitted for any other degree in this or any other university or institute of learning.

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Ashwag..

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Abbreviations

ADAM	A disintegrin and metalloproteinase
ADCC	Antibody-dependent cell-mediated cytotoxicity
AR	Androgen receptor
AREG	Amphiregulin
ATCC	American Type Culture Collection
BCSS	Breast cancer specific survival
BL1	Basal-like 1
BL2	Basal-like 2
BMP6	Bone morphogenetic protein 6
BSA	Bovine serum albumin
BTC	Betacellulin
CAF	Cancer-associated fibroblast
CK5/6	Cytokeratin 5/6
CNAs	Copy number aberrations
CTX	Cetuximab
DFS	Disease free survival
DMAC	N-N-Dimethylacetamide
DMEM	Dulbecco's modified Eagle medium
DMFS	Distant-metastasis-free survival
DMSO	Dimethyl sulfoxide
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGFRIII	EGFR variant III
EMT	Epithelial-to-mesenchymal transition
EPG	Epigen
EPR	Epiregulin
ER	Estrogen receptor
FBS	Foetal bovine serum
HB-EGF	Heparin-binding EGF
Her2	Human epidermal growth factor receptor 2

HGF	Hepatocyte growth factor
HGFR	Hepatocyte growth factor receptor
HNSCC	Head and neck squamous cell carcinoma
HR	Hazard ratio
IDC-NOS	Invasive ductal carcinomas not otherwise specified
IHC	Immunohistochemistry
IM	Immunomodulatory
IP	Intraperitoneal
Lambda PP	Lambda Protein Phosphatase
LAR	Luminal androgen receptor
M	Mesenchymal
mAbs	Monoclonal antibodies
mCRC	Metastatic colorectal cancer
MDGI	Mammary derived growth factor inhibitor
MSL	Mesenchymal stem-like
NRG	Neuregulin
NSCLC	Non-small cell lung cancer
OD	Optical density
ORR	Objective response rate
OS	Overall survival
PARP-1	Poly(ADP-ribose) polymerase-1
PARP-2	Poly(ADP-ribose) polymerase-2
PBS	Phosphate buffered saline
pCR	Pathological complete response
PDGFRα	Alpha-type platelet-derived growth factor receptor
PFS	Progression-free survival
PG	Propylene Glycol
PI	Propidium iodide
PR	Progesterone receptor
PTB	Phospho-tyrosine binding
RFS	Relapse-free survival
RNAi	RNA interference
RPMI-1640	Roswell Park Memorial Institute medium 1640

RR	Response rate
RTK	Receptor tyrosine kinase
SFKs	Src-family tyrosine kinases
SH2	Src homology 2
Src	Avian sarcoma viral oncogene homolog
TCGA	The Cancer Genome Atlas
TGF-α	Transforming growth factor-α
TKIs	Tyrosine kinase inhibitors
TM	Transmembrane domain
TNBC	Triple negative breast cancer
TrkC	Neurotrophic tyrosine kinase receptor type 3
VEGF	Vascular endothelial growth factor
VEGFR1	Vascular endothelial growth factor receptor 1

1 Introduction

1.1 Breast cancer

1.1.1 Breast cancer statistics

Breast cancer is the most common malignancy affecting women in the United States and Europe and is the second leading cause of cancer deaths in women after lung cancer (Siegel *et al.* 2014). Incidence rates of breast cancer have increased in the UK during the last decade. In 2011, 49,900 women were diagnosed with breast cancer and around 11,700 died from breast cancer in the UK. However, death rates for breast cancer have decreased by a fifth in the UK during the last ten years (Cancer Research UK, 2014).

1.1.2 Breast cancer classification

Breast cancer is a heterogeneous disease that is traditionally classified according to the histopathological criteria and immunohistochemical (IHC) markers such as estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (Her2) (Elston *et al.* 1999; Harris *et al.* 2007).

1.1.1.1 Histopathological and tumour grade classification

Breast cancer can also be classified based on the histological features of tumours. About 70-80% of breast cancers are classified as invasive ductal carcinomas not otherwise specified (IDC-NOS). On the other hand, around 25% are classified into different special subtypes such as lobular, tubular, medullary and inflammatory carcinomas (Ellis *et al*. 2003; Tavassoli *et al*. 2003). Gene expression profiling of different histological subtypes revealed that the majority of special subtypes are homogenous while the IDC-NOS contains all the molecular breast cancer subtypes (Weigelt *et al.* 2008).

Tumour grade is another histological feature of tumours that provides useful prognostic information. It involves an assessment of the degree of differentiation and proliferative activity of the tumours (Rakha *et al.* 2010).

1.1.1.2 Immunohistochemistry characterization

The standard characterization of breast cancers involves the IHC assessment of ER, PR and Her2 status. The determination of the status of these markers guides the selection of patients for treatments (Dawson *et al.* 2013). Endocrine therapy, including tamoxifen and aromatase inhibitors, is commonly used for the treatment of ER-positive breast cancers, which accounts for about 70% of breast cancer patients (Mohamed *et al.* 2013). Approximately 20% of breast cancer patients are Her2-positive (overexpression and/or amplification) and they receive different Her2 targeted therapies such as trastuzumab, pertuzumab, TDM1 and lapatinib (Arteaga *et al.* 2012). The remaining patients are defined as triple negative breast cancer (TNBC); patients whose tumours lack the expression of ER and PR as well as Her2 overexpression or gene amplification. There is still a lack of standard targeted therapy for this group (Dawson *et al.* 2009).

1.1.2.1 Molecular classification

The evolution of the new era of molecular breast cancer classification has been started with the expression profiling of 65 breast tumours from 42 individuals using cDNA microarray, which resulted in the identification of four distinct subtypes; luminal, basal-like, Her2-postive and normal breast (Perou *et al.* 2000). The luminal subtype was further divided into two subgroups: luminal A and luminal B. These five subtypes have been identified, based on their gene expression patterns that represent biologically distinct disease and are associated with different clinical outcomes **(Figure 1.1)** (Sorlie

et al. 2001; Sorlie *et al.* 2003). The breast cancer molecular subtyping based on the intrinsic gene sets was further refined based on a 50-gene subtype predictor (PAM50) (Parker *et al.* 2009; Nielsen *et al.* 2010). Further studies have identified a novel intrinsic subtype called claudin-low or mesenchymal-like breast cancers. Claudin-low tumours are ER, PR and Her2 negative and are enriched for markers associated with stem cell function and epithelial-to-mesenchymal transition (EMT) (Herschkowitz *et al.* 2007; Hennessy *et al.* 2009). The emergence of next generation sequencing technologies has allowed the incorporation of both genomic and transcriptomic analyses. Using this approach, Curtis C *et al*. (2012) have recently characterized the architecture of 2000 breast tumours as part of the Molecular Taxonomy of Breast Cancer International Consortium (METABRIC) (Curtis *et al.* 2012). Clustering analysis of these tumours revealed 10 novel molecular subgroups that are associated with distinct copy number aberrations (CNAs) and gene expression profiling as well as clinical outcomes **(Figure 1.2)** (Curtis *et al.* 2012). This clearly demonstrates the high level of heterogeneity and complexity in breast cancers. The focus of the current project is TNBC/basal-like breast cancer.

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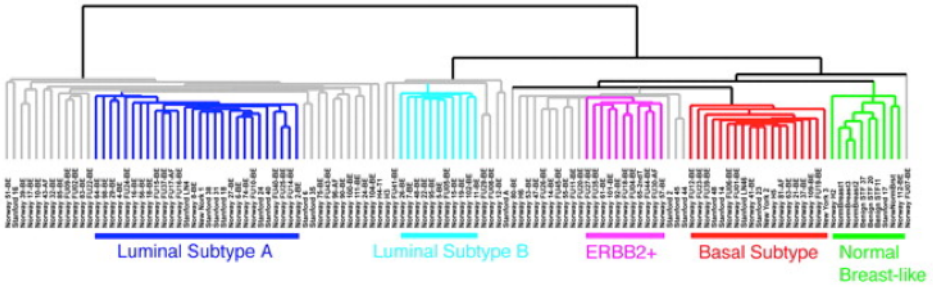

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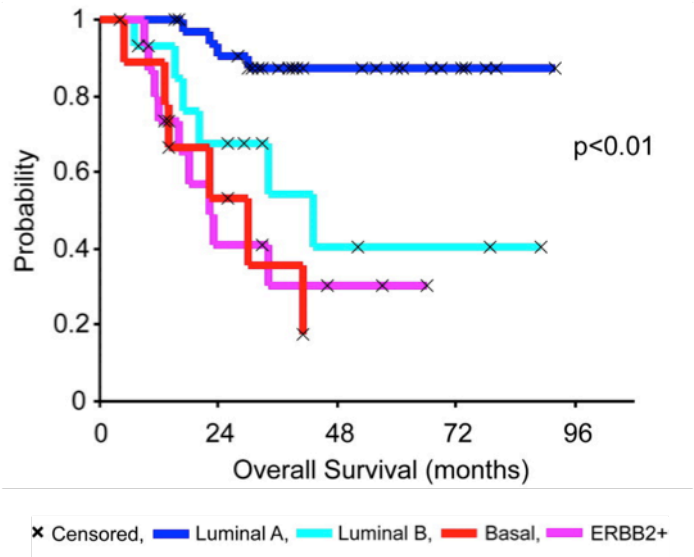

Figure 1.1 Molecular subtypes of breast cancer.
(A) Dendogram showing the five breast cancer clusters. **(B)** OS for patients from the distinct subtypes (Reproduced with permission from (Sorlie *et al.* 2003), Copyright (2003) National Academy of Sciences, U.S.A).

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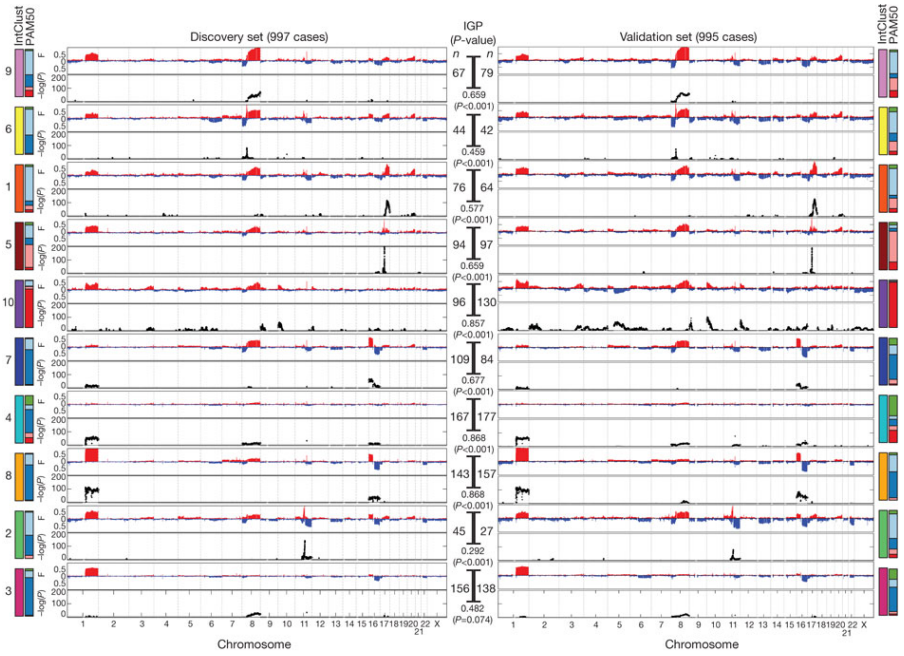

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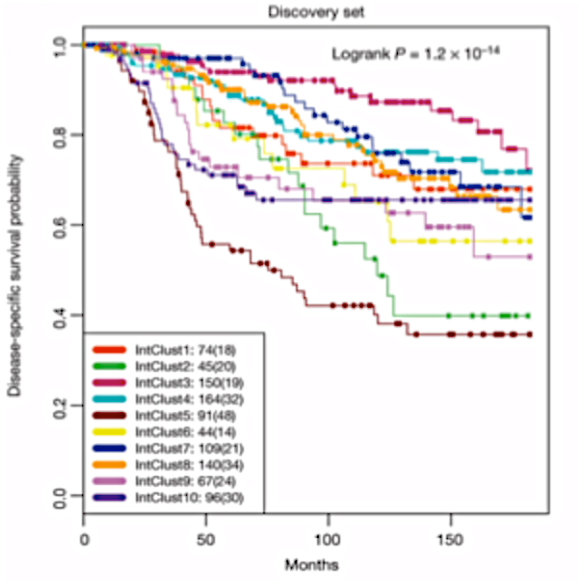

Figure 1.2 Architecture of 2000 breast cancers.
(A) Genomic and transcriptomic landscape of the 10 integrative subgroups from METABRIC data set **(B)** Kaplan–Meier plot of disease-specific survival for the integrative subgroups (Reprinted with permission from Macmillan Publishers Ltd: Nature (Curtis *et al.* 2012), copyright (2012).

1.2 Triple negative breast cancer (TNBC)

1.2.1 Characteristics of TNBC tumours

TNBC accounts for about 15-20% of newly diagnosed breast cancers (Bauer *et al.* 2007; Reis-Filho *et al.* 2008). They are usually high-grade IDC-NOS tumours, which are lymph node positive and are associated with African American women and younger age (<40 year) (Bauer *et al.* 2007; Dent *et al.* 2007; Morris *et al.* 2007; O'Brien *et al.* 2010; Anders *et al.* 2011). TNBCs are more likely to arise in *BRCA1* mutation carriers and have similar gene expression profiles to *BRCA1*-deficient tumours (Haffty *et al.* 2006). Additionally, TNBC patients are associated with poor prognosis and have a high risk of distant recurrence within the first 3 years of diagnosis, although the risk decreases after this time (Dent *et al.* 2007).

1.2.2 TNBC versus basal-like tumours

As stated above, Perou C. *et al*. (2000) identified the five intrinsic breast cancer subtypes including basal-like subtype (Perou *et al.* 2000). Basal-like tumours comprise about 10-25% of breast cancers and are not synonymous with TNBC. According to the recent data from three clinical trials, approximately 70-75% of TNBCs are basal-like tumours while the rest are not (Prat *et al.* 2011; Cheang *et al.* 2012). Recently, Prat, A. *et al*. (2013) have molecularly characterized TNBC and basal-like tumours from 12 publicly available breast cancer microarray data sets. Among the 412 TNBC tumours, 78.6% were basal-like and 68.5% of the basal-like tumours were TNBCs, using both the IHC-based and PAM50 subtyping systems (Prat *et al.* 2013).

Due to the limitations of using gene expression profiling in clinical practice, different efforts have been made to identify IHC-based surrogates for gnomically defined basal-like tumours. The term "core basal" has been used to define tumours that are triple negative and express cytokeratin 5/6 (CK5/6), epidermal growth factor receptor (EGFR), or both (Tischkowitz *et al.* 2007; Cheang *et al.* 2008). Evaluation of *CK5/6*, *EGFR* and *cKIT* expression in 21 basal-like breast tumours revealed that 62%, 57% and 29% of these tumours were positive for these biomarkers, respectively (Nielsen *et al.* 2004). Gazinska, P. *et al*. (2013) have also analyzed a cohort of 142 TNBCs and demonstrated that 56% were CK5/6 positive and 54% were EGFR positive (Gazinska *et al.* 2013). TNBC patients with core basal phenotype were associated with a significantly worse outcome in comparison to those that were negative to these 5 markers (Tischkowitz *et al.* 2007; Cheang *et al.* 2008). However, a recent study did not show a significant outcome between core basal and other TNBCs (Gazinska *et al.* 2013).

1.2.3 TNBC sub-classification

TNBC is a heterogeneous disease that consists of a highly diverse group of cancers. Recently, Lehmann, B. *et al*. (2011) have analyzed the gene expression profiles from 21 breast cancer data sets. Their analyses revealed that TNBCs is composed of 6 subtypes that display unique gene expression profiles. The TNBC subtypes include 2 basal-like (BL1 and BL2), an immunomodulatory (IM), a mesenchymal (M), a mesenchymal stem–like (MSL) and a luminal androgen receptor (LAR) subtype. The BL1 tumours were enriched in cell cycle and cell division components and DNA damage response signature while BL2 tumours were enriched in growth factor signalling such as epidermal growth factor (EGF) and hepatocyte growth factor (HGF) pathways as well as myoepithelial markers. The IM subtype was enriched for genes involved in immune cell processes. The

M and MSL subtypes expressed genes involved in cell differentiation, cell motility and growth factor pathways. Tumours within the LAR subtype expressed higher levels of androgen receptor (AR), numerous downstream AR targets and coactivators. It has been found that the relapse-free survival (RFS) varied significantly between TNBC subtypes but not distant-metastasis-free survival (DMFS) (Lehmann *et al.* 2011). Using differential gene expression, Lehmann, B. *et al*., (2011) has also designated representative TNBC cell lines to each TNBC subtype **(Table 1.1)** (Lehmann *et al.* 2011).

Table 1.1 Assignment of TNBC cell lines to subtypes.
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TNBC subtype	Cell line	Subtype correlation (*P* value)	Histology	Mutations[A]	Intrinsic subtype[B]	Basal subtype[C]
Basal-like						
BL1	HCC2157	0.66 (0.00)	DC	*BRCA1; STAT4; UTX*	Basal	Basal A
	HCC1599	0.62 (0.00)	DC	*BRCA2; TP53; CTNND1; TOP2B; CAMK1G*	Basal	Basal A
	HCC1937	0.28 (0.00)	DC	*BRCA1; TP53; MAPK13; MDC1*	HER2	Basal A
	HCC1143	0.26 (0.00)	IDC	*TP53*	Basal	Basal A
	HCC3153	0.24 (0.00)		*BRCA1*	Basal	Basal A
	MDA-MB-468	0.19 (0.06)	DC	*PTEN; RB1; SMAD4; TP53*	Basal	Basal A
	HCC38	0.19 (0.01)	DC	*CDKN2A; TP53*	Unclassified	Basal B
BL2	SUM149PT	0.30 (0.00)	INF	*BRCA1*	Unclassified	Basal B
	CAL-851	0.25 (0.00)	IGA	*RB1; TP53*	Basal	
	HCC70	0.24 (0.04)	DC	*PTEN; TP53*	Basal	Basal A
	HCC1806	0.22 (0.00)	ASCC	*CDKN2A; TP53; UTX*	Unclassified	Basal A
	HDQ-P1	0.18 (0.17)	IDC	*TP53*	Unclassified	
IM	HCC1187	0.22 (0.00)	DC	*TP53; CTNNA1; DDX18; HUWE1; NFKBIA*	Basal	Basal A
	DU4475	0.17 (0.00)	DC	*APC; BRAF; MAP2K4; RB1*	Unclassified	
Mesenchymal-like						
M	BT-549	0.21 (0.00)	IDC	*PTEN; RB1; TP53*	Unclassified	Basal B
	CAL-51	0.17 (0.00)	AC	*PIK3CA*	Unclassified	
	CAL-120	0.09 (0.00)	AC	*TP53*	Luminal B	
MSL	HS578T	0.29 (0.00)	CS	*CDKN2A; HRAS; TP53*	Unclassified	Basal B
	MDA-MB-157	0.25 (0.00)	MBC	*NF1; TP53*	Unclassified	Basal B
	SUM159PT	0.14 (0.00)	ANC	*PIK3CA; TP53 HRAS*	Unclassified	Basal B
	MDA-MB-436	0.13 (0.00)	IDC	*BRCA1; TP53*	Unclassified	Basal B
	MDA-MB-231	0.12 (0.00)	IDC	*BRAF; CDKN2A; KRAS; NF2; TP53; PDGFRA*	Unclassified	Basal B
LAR						
LAR	MDA-MB-453	0.53 (0.00)	AC	*PIK3CA; CDH1; PTEN*	Luminal A	Luminal
	SUM185PE	0.39 (0.00)	DC	*PIK3CA*	Luminal A	Luminal
	HCC2185	0.34 (0.00)		*PIK3CA*	Luminal A	Luminal
	CAL-148	0.32 (0.00)	AC	*PIK3CA; RB1; TP53; PTEN*	Luminal A	
	MFM-223	0.31 (0.00)	AC	*PIK3CA; TP53*	Luminal A/B	
Unclassified	HCC1395		DC	*ATR; BRCA2; CDKN2A; PTEN; FGFR1; PDGFRB; TP53*	Basal	
	BT20		IDC	*CDKN2A; PIK3CA; TP53*	HER2	Basal A
	SW527				Luminal B	

[A]Source: mutations taken from COSMIC database (www.sanger.ac.uk/genetics/CGP/cosmic/). [B]Molecular subtype determined by correlation with UNC/ intrinsic breast centroids (29). [C]Basal subtype obtained from Neve RM et al. (32). AC, adenocarcinoma; ANC, anaplastic carcinoma; ASCC, acantholytic squamous cell carcinoma; C, carcinoma; CS, carcinosarcoma; DC, ductal carcinoma; IDC, invasive ductal carcinoma; IGA, invasive galactophoric adeno-carcinoma; INF, inflammatory ductal carcinoma; MC, metaplastic carcinoma and MBC, medullary breast cancer.

1.2.4 Treatment strategies for TNBC

Due to the lack of molecular targets in the majority of TNBCs, chemotherapy is currently the mainstay therapeutic option for TNBC patients. Anthracycline with taxane chemotherapies are the most commonly used regimens in the neoadjuvant setting for TNBC (von Minckwitz *et al.* 2012), which have shown higher rates of pathological complete response (pCR) in TNBC, basal-like and Her2-positive tumours compared to luminal tumours (Rouzier *et al.* 2005; Liedtke *et al.* 2008). A prospective study has shown that TNBC patients who received neoadjuvant chemotherapy had a significantly higher pCR compared to non-TNBC patients (22% versus 11%, respectively; $P= 0.03$) but significantly lower 3-year progression-free survival (PFS) ($P<0.0001$) and overall survival (OS) rates ($P<0.0001$). On the other hand, TNBC patients with residual disease had an increased risk of visceral metastases and worse OS compared to non-TNBC patients (Liedtke *et al.* 2008). Profiling residual TNBCs after neoadjuvant chemotherapy has identified genetic alterations and targetable molecular lesions. This could be useful for setting combinatory adjuvant therapies to improve the outcome of those patients (Balko *et al.* 2014). In the adjuvant setting, anthracycline-based chemotherapy resulted in a significantly shorter metastasis-free survival (MFS) and breast cancer specific survival (BCSS) in TNBC and basal-like patients compared to non-TNBC patients (Tan *et al.* 2008).

The strong association of TNBC with *BRCA1* mutations supported the use of platinum-based regimens such as cisplatin and carboplatin as potential therapies in TNBC (Byrski *et al.* 2009; Byrski *et al.* 2010; Silver *et al.* 2010). However, a recent report from GEICAM 2006-03 study revealed that the addition of carboplatin to conventional chemotherapy did not improve the pCR in basal-like breast cancer patients (Alba *et al.* 2011).

Overall, chemotherapy regimens might be effective only in a subset of TNBC patients with early disease. However, TNBC patients with advanced disease may respond poorly to the current chemotherapeutic options. Therefore, there is an imperative need to investigate novel targeted therapies for TNBCs (Crown *et al.* 2012).

1.2.5 Targeted treatment for TNBC

During the last few years, investigators have been investigating multiple potential targets for the treatment of TNBC. The emerging targeted therapies, which are being currently investigated in TNBCs, are presented in **(Figure 1.3)**. Below is a summary of some of the targeted therapies that are currently being investigated in TNBC.

1.2.5.1 Src tyrosine kinase inhibitor

Avian sarcoma viral oncogene homolog (Src) tyrosine kinase, a member of the Src-family non-receptor tyrosine kinases (SFKs), plays a key role in many cellular signalling pathways. It is overexpressed in TNBC and is associated with metastatic progression (Biscardi *et al.* 1998; Fornier *et al.* 2011). TNBC cell lines were found to be sensitive to Src inhibition using an SFK kinase inhibitor, dasatinib (Finn *et al.* 2007). However, results obtained from a phase II clinical trial revealed a limited efficacy of dasatinib as a single-agent in unselected TNBC patients (Finn *et al.* 2011).

1.2.5.2 PI3K and mTOR inhibitors

Aberrant PI3KCA pathways including activating *PI3KCA* mutations, loss of *PTEN* and *AKT* mutation and/or amplification have been reported in TNBC patients (Creighton *et al.* 2010; Cancer Genome Atlas 2012; Shah *et al.* 2012). Multiple potential therapeutic inhibitors including PI3KCA inhibitors, AKT inhibitors and mTOR inhibitors are being

investigated in early phase trials for TNBC treatment, either as single agents or in combination with chemotherapy (reviewed in (Crown *et al.* 2012; Hirshfield *et al.* 2014).

1.2.5.3 VEGF/VEGFR inhibitors

Higher vascular endothelial growth factor (VEGF) expression level has been found in TNBC compared to non-TNBC tumours (Linderholm *et al.* 2009). In the neoadjuvant setting, the addition of bevacizumab, a VEGF monoclonal antibody (mAb), significantly increased the pCR from 27.9% to 39.3% for TNBC patients treated with epirubicin, cyclophosphamide and docetaxel (von Minckwitz *et al.* 2012). A meta-analysis of three randomized Phase III trials (E2100, AVADO, and RIBBON-1) revealed that the addition of bevacizumab to chemotherapy in the first-line setting increased the median PFS by 2.7 months compared with chemotherapy alone (P<0.0001) in TNBC patients. However, there was no improvement in the OS rate in these patients (P= 0.67) (O'Shaughnessy, J. *et al*. 2011). Nevertheless, the primary results of a randomized phase III trial (BEATRICE) did not recommend the addition of bevacizumab to chemotherapy in the adjuvant setting in TNBC patients (Cameron *et al.* 2013).

1.2.5.4 PARP inhibitors

Poly(ADP-ribose) polymerase-1 and -2 (PARP-1 and PARP-2) are members of PARPs family, which involve 18 multifunctional enzymes and are involved in single-strand DNA repair mechanisms (Ame *et al.* 2004). *BRCA-1* mutated and *BRCA-1* deficient tumours are highly sensitive to PARP inhibitors compared to *BRCA*-proficient tumours (Bryant *et al.* 2005; Farmer *et al.* 2005). Several PARP inhibitors are being evaluated in phase I, II and III clinical trials for the treatment of TNBC patients. Olaparib, an oral PARP

inhibitor, has been assessed as a single-agent in *BRCA1*-mutant and *BRCA2*-mutant advanced breast cancer patients. An objective response rate (ORR) of 41% and 22% has been obtained at doses of 400 mg and 100 mg twice daily, respectively (Tutt *et al.* 2010). However, in another trial, no confirmed ORR was observed in 26 TNBC patients treated with olaparib (Gelmon *et al.* 2011). Other PARP inhibitors such as iniparib and veliparib have also been evaluated with chemotherapy in TNBC patients (Reviewed in (Abramson *et al.* 2015). Despite the initial promising results in *BRCA*-associated TNBCs, clinical trials and biomarker studies are needed to identify TNBC patients without *BRCA* mutations who could response to PARP inhibitors (Metzger-Filho *et al.* 2012).

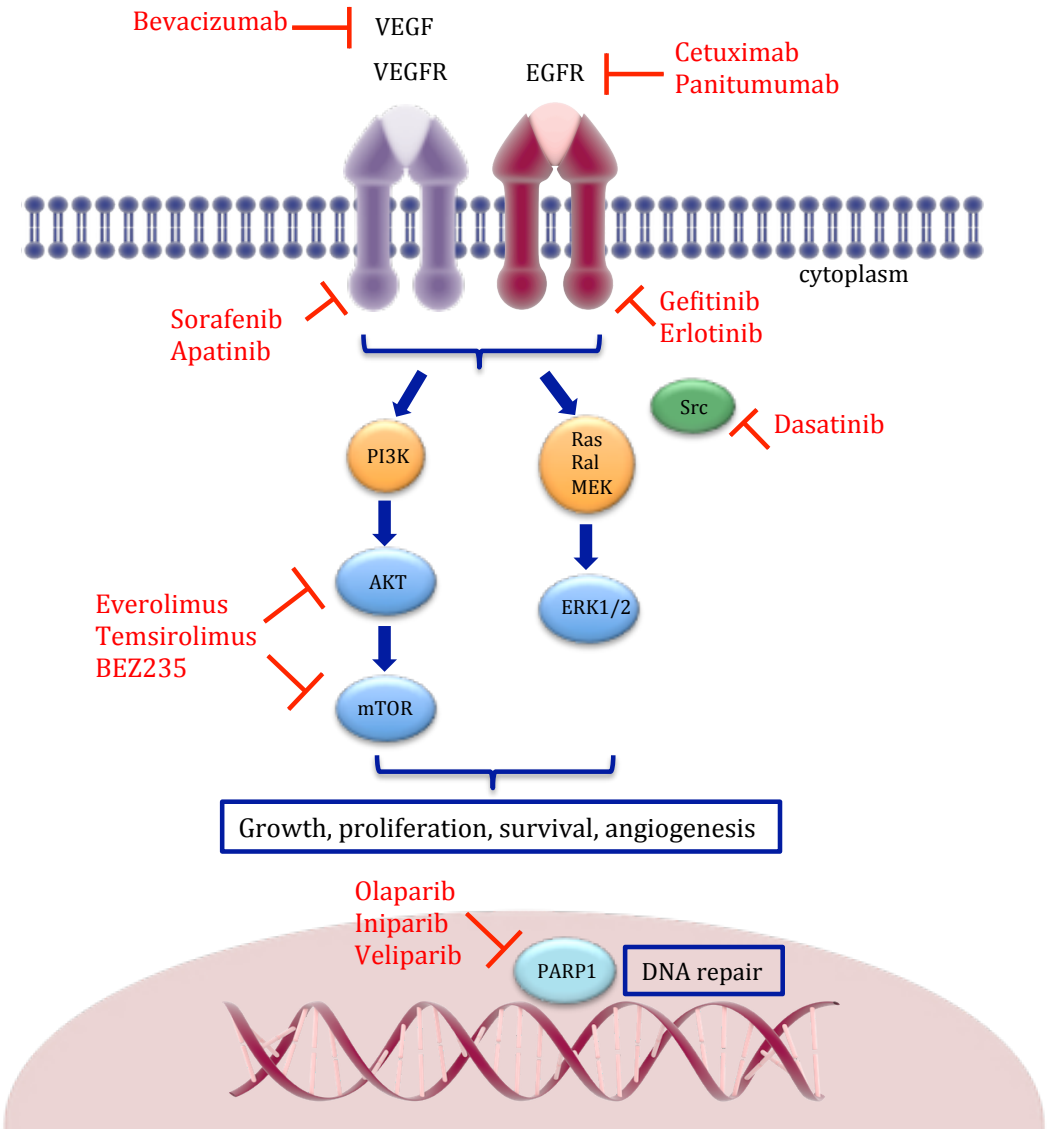


Figure 1.3 Targeted therapies being investigated in TNBC.
Several targeted therapies are being investigated in TNBC patients including; EGFR, VEGFR, Src, AKT, mTOR and PARP inhibitors.

1.3 ErbB signalling pathway

1.3.1 ErbB receptors

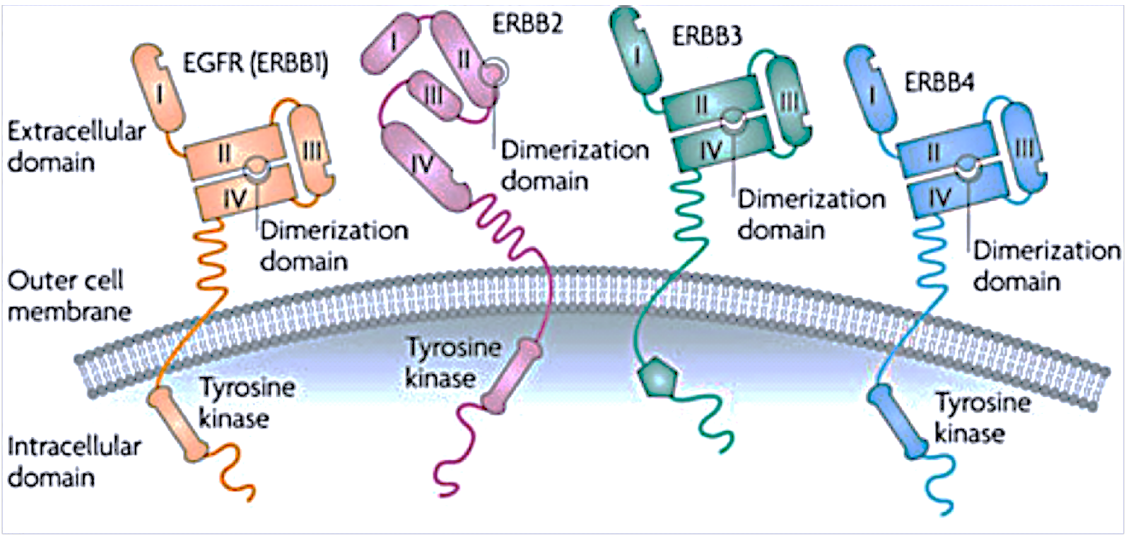
The human epidermal growth factor receptors family (Her; ErbB) consists of four transmembrane type I receptor tyrosine kinases (RTKs); EGFR (ErbB1), Her2 (ErbB2), Her3 (ErbB3) and Her4 (ErbB4). The four receptors share a common structure consisting of an extracellular domain (ectodoamin) that contains 2 cysteine-rich sub-regions (domains II and IV) and ligand-binding domains (domain I and III), an α-helical transmembrane domain (TM) and a c-terminal domain that contains the kinase and phosphorylation sites as illustrated in **(Figure 1.4A)** (Wells 1999; Olayioye *et al.* 2000; Linggi *et al.* 2006; Baselga *et al.* 2009). Her2 is ligand-less (Klapper *et al.* 1999), whereas Her3 has defective intrinsic kinase domain activity (Guy *et al.* 1994). However, they form heterodimers with each other as well as with other ErbB receptors to activate several downstream signalling pathways (Baselga *et al.* 2009).

1.3.2 ErbB ligands

The activation of ErbB receptors is mediated by the specific binding of 11 different growth factors, ErbB ligands. These ligands include EGF, transforming growth factor-α (TGF-α), heparin-binding EGF (HB-EGF), amphiregulin (AREG), betacellulin (BTC), epigen (EPG), epiregulin (EPR) and neuregulin 1–4 (NRG1–4). These are small soluble proteins that share an EGF-like motif of 3 disulfide bonds between 6 conserved cysteine residues. As shown in **(Figure 4.1B)**, each ligand binds to specific ErbB receptor(s) (Olayioye *et al.* 2000; Linggi *et al.* 2006).

All ErbB ligands exist as type I membrane-bound proteins that contain an EGF motif. In order for their activation, these pro-ligands are cleaved into soluble forms with the shedding of the ectodoamin by members of a disintegrin and metalloproteinase (ADAM) family (Harris *et al.* 2003; Blobel 2005). In human, 13 out of the 21 ADAMs have proteolytic activity in their metalloproteinase domains (Bode *et al.* 1993). Among the different ADAMs, ADAM17 is the major sheaddase for ErbB ligands such as TGFα, HB-EGF, AREG and EPR. Additionally, ADAM10 has been reported to induce the release of EGFR ligands such as BTC and EGF (Reviewed in (Blobel 2005).

A



B

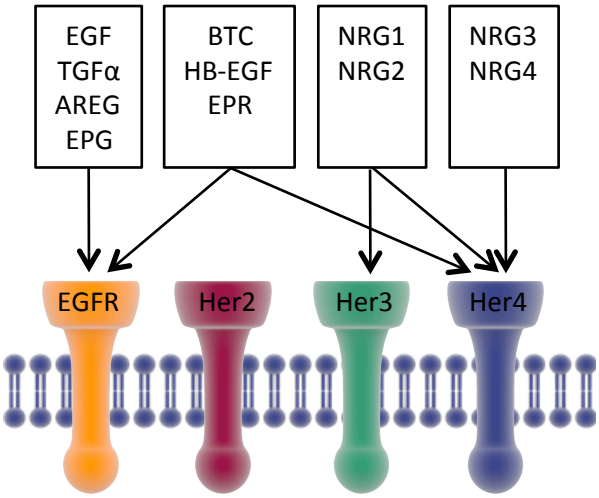


Figure 1.4 ErbB receptors and their ligands.
(A) ErbB family consists of four receptors: EGFR, Her2, Her3 and Her4 (ErbB1-4). Each receptor composed of extracellular, transmembrane and c-terminal domains. (Reprinted with permission from Macmillan Publishers Ltd: Nature Reviews Cancer (Baselga *et al.* 2009), copyright (2009). **(B)** Specificity of ErbB ligands. ErbB ligands bind to and activate specific receptors as indicated.

1.3.3 EGFR signalling pathway

Without ligand binding, EGFR exists in a tethered intramolecular conformation in which the dimerization subdomain (domain II) is covered by intramolecular interaction with subdomain IV (Linggi *et al.* 2006). Ligand binding to subdomains I and III induces a conformational rearrangement that exposes the dimerization subdomain with another receptor and allows homo- or hetero-dimerization **(Figure 1.5A)** (Ogiso *et al.* 2002; Linggi *et al.* 2006; Baselga *et al.* 2009). This brings the two cytoplasmic tyrosine kinase domains of the receptors together, resulting in an asymmetric tyrosine kinase activation and auto-phosphorylation of specific tyrosine residues **(Figure 1.5B)** (Linggi *et al.* 2006; Baselga *et al.* 2009). These phosphorylated tyrosine residues provide docking sites for Src homology 2 (SH2) or phospho-tyrosine binding (PTB) domains of adaptor proteins, kinases and phosphatases that activate several downstream pathways **(Figure 1.5C)** (Olayioye *et al.* 2000). The main EGFR signalling pathways are Ras/Raf/MAPK and PI3K/AKT. Additionally, EGFR also activates PLC-γ-CaMK/PKC and STATs pathways **(Figure 1.6)**.

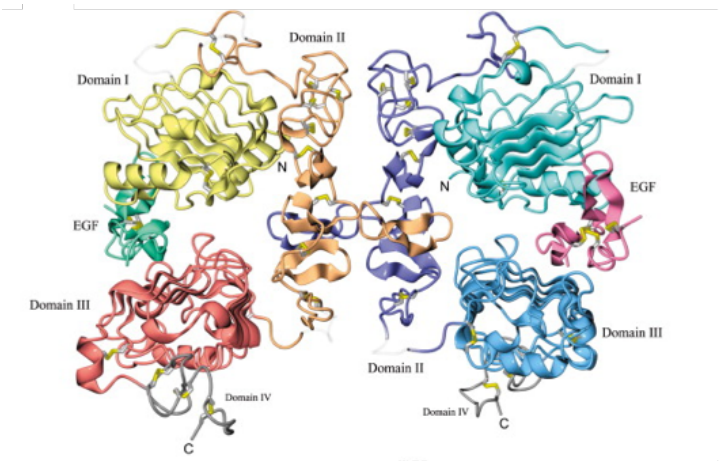
1.3.3.1 Ras/Raf/MAPK pathway

Following EGFR activation, GRB2/Sos complex adaptor proteins bind directly or indirectly to EGFR through SH2 adaptor proteins (Lowenstein *et al.* 1992; Batzer *et al.* 1994). This leads to a structural change in Sos, recruitment and activation of Ras with subsequent activation of Raf1, MAPK1 and MAPK2 (Hallberg *et al.* 1994; Liebmann 2001). This results in the regulation of specific transcription factors that induce cell migration and proliferation (Hill *et al.* 1995).

1.3.3.2 PI3K/AKT pathway

EGFR lacks PI3K docking sites and EGFR-dependent activation of PI3K occurs through EGFR-Her3 dimerization (Carpenter *et al.* 1993; Yarden *et al.* 2001). The PI3K consists of two subunits; the regulatory p85 subunit that interacts directly with specific ErbB docking sites through its SH2 domain (Yu *et al.* 1998; Yu *et al.* 1998) and the catalytic p110 subunit that catalyzes the phosphorylation of phosphatidylinositol 4,5-diphosphate to phosphatidylinositol 3,4,5-triphosphate, which in turn phosphorylates and activates AKT. Activation of AKT induces cell survival, proliferation, growth and invasion (Vivanco *et al.* 2002).

A



B

EGFR kinase domain	
Y845	STAT5B/ SRC
Y891	SRC
Y920	SRC
Y974	AP-2
Y992	PLCγ/ PTP1B/ PTP2C/ SHP2
Y1045	CBL
Y1046	
Y1047	
Y1057	
Y1068	GRB2/ SHC
Y1086	GRB2/ SHC/ DOK-R/ ABL
Y1101	
S1142	
Y1148	GRB2/ SHC/ PTP1B/ DOK-R
Y1173	GRB2/ SHP1/ SHC/ PLCγ

C

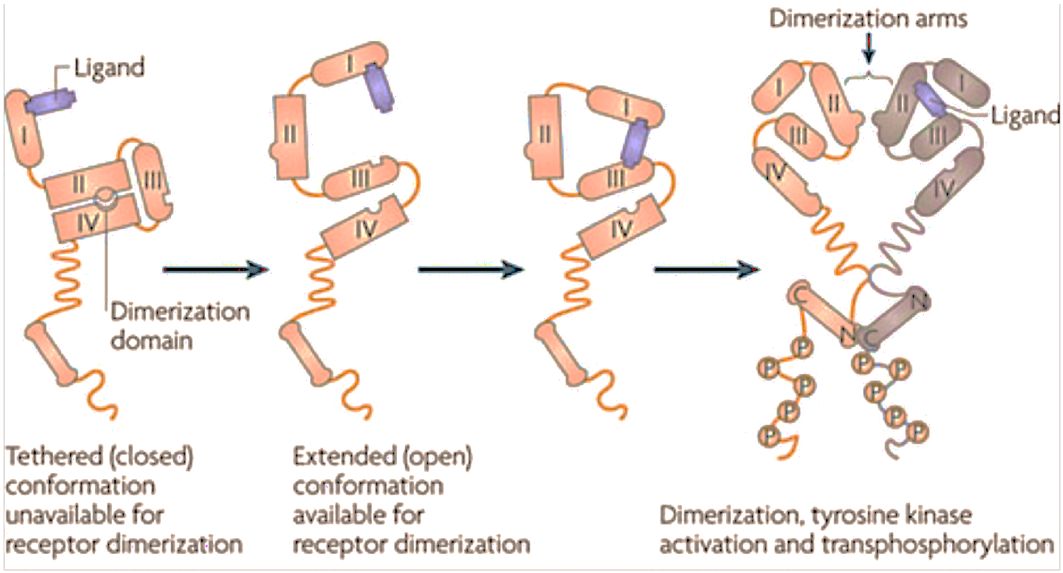


Figure 1.5 EGFR: structure, phosphorylation sites, dimerization and activation.
(A) Ribbon diagram of 2:2 EGF-EGFR complex ((Reprinted with permission from Elsevier: Cell (Ogiso *et al.* 2002), copyright (2002). **(B)** Schematic presentation of the phosphotyrosine residues in EGFR c-terminal domain. **(C)** Ligand-binding to ErbB receptors and subsequent conformational changes and dimerization (Reprinted with permission from Macmillan Publishers Ltd: Nature Reviews Cancer (Baselga *et al.* 2009), copyright (2009).

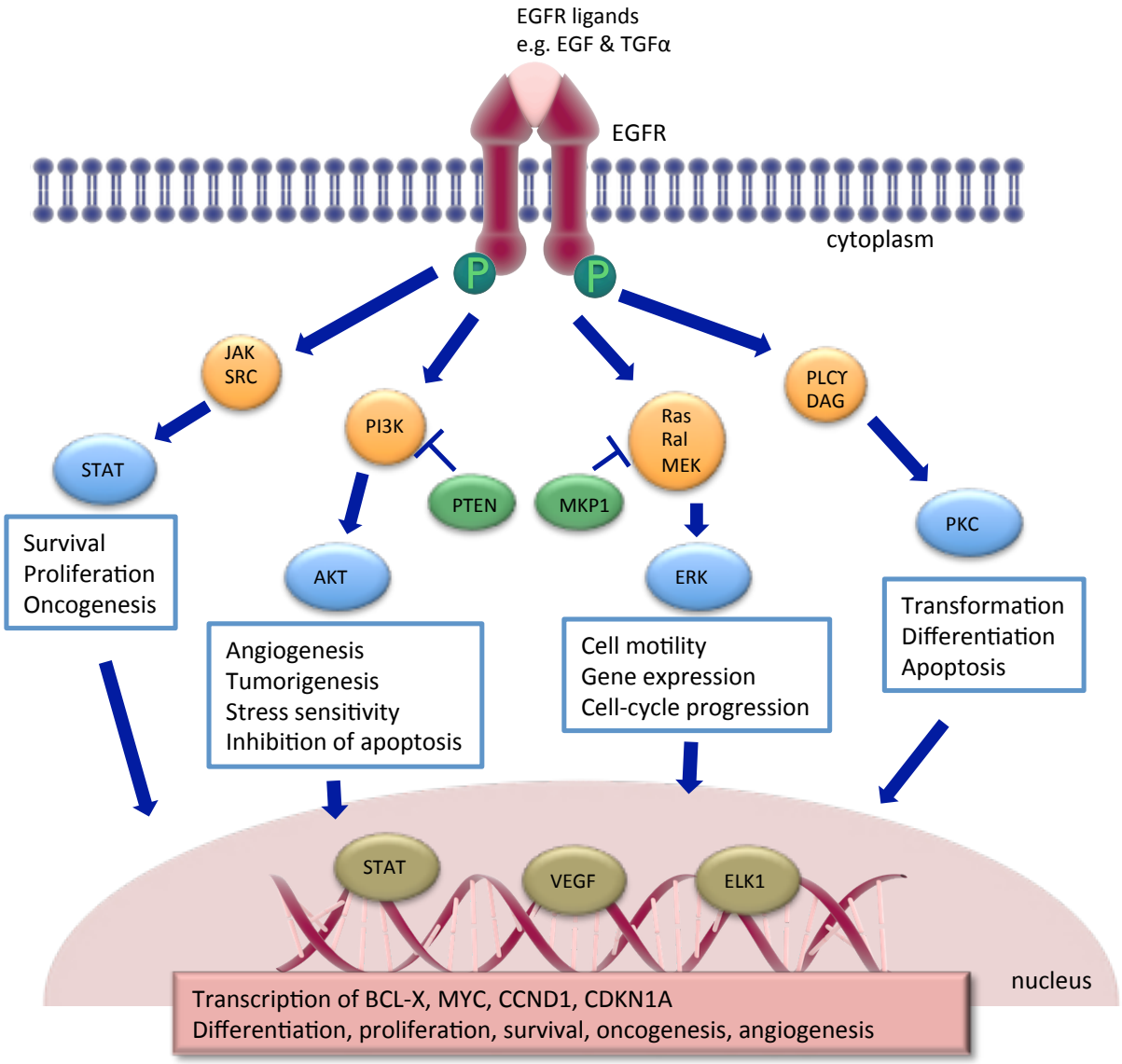


Figure 1.6 The EGFR signalling pathway.
(Adapted with permission from Macmillan Publishers Ltd: Nature Reviews Cancer (Nyati *et al.* 2006), copyright (2006).

1.3.4 EGFR in triple negative breast cancer

EGFR is highly expressed in different epithelial tumours such as prostate, gastric, colorectal, lung, ovarian and breast cancers (Baselga 2002; Mendelsohn *et al.* 2006) and is correlated with poor response to therapy, disease progression and poor survival (Brabender *et al.* 2001). The Cancer Genome Atlas (TCGA) data revealed a high prevalence of genetic aberrations in *EGFR*, including mutation, deletion or amplification in a variety of tumours, especially in glioblastoma, lung cancers and head and neck cancers **(Figure 1.7)** (source: cBioPortal.org; (Cerami *et al.* 2012). In breast cancer, a meta-analysis of 5232 patients from 40 different studies revealed that an average of 45% breast cancer patients were positive for EGFR (range 14-91%) (Klijn *et al.* 1992) and another study indicated that 47% of 370 breast cancer patients were EGFR positive (Fox *et al.* 1994). In metaplastic breast carcinomas, analysis of 47 patients showed that 32/47 (68%) of patients had overexpression of *EGFR* and 11 of them (23%) harboured *EGFR* amplification (Reis *et al.* 2006). However, recent data from the TCGA indicated that only 18 and 5 out of 463 breast cancer patients harbor *EGFR* up-regulation and amplification respectively (source: cBioPortal.org).

Several studies have shown high EGFR expression (up to 72%) in TNBC/basal-like tumours (Nielsen *et al.* 2004; Livasy *et al.* 2006; Meche *et al.* 2009; Viale *et al.* 2009). However, an analysis of a series of 930 breast cancers by IHC has shown only 13% of the cases (82/614 interpretable cores) were EGFR positive. In basal cytokeratin positive cases, 44% (41/93) were EGFR positive. Additionally, EGFR expression is significantly associated with poor survival ($P<0.01$) and is an independent negative prognostic factor (relative risk= 1.54, $P=0.01$) from other co-variables including nodal status and tumour size (Nielsen *et al.* 2004). Kreike, B. *et al*. (2007) analyzed 97 TNBC tumours by gene

expression profiling and IHC staining of selective markers including EGFR. All tumours were classified as basal-like based on their gene expression profiling and 27% (26/97) of the cases were EGFR positive (Kreike *et al.* 2007). Further analysis of basal-like tumours revealed 5 clusters based on their gene profiling. EGFR expression varied between these clusters (I: 3% (1/29), II: 26% (5/18), III: 50% (11/22), IV: 50% (6/12) and V: 21% (3/14). Another study by Meche, A. *et al*. (2009) found a positive EGFR expression in 41.66% (5/12) of basal-like tumours (Meche *et al.* 2009). More recently, M. *et al*. (2013) evaluated 136 TNBC patients and reported that EGFR was expressed in 60.3% (82/136) cases and EGFR-positive patients had significantly worse disease free survival (DFS) ($P = 0.005$) and OS ($P = 0.009$) (Zhang *et al.* 2013) **(Figure 1.8)**. These findings implicated that EGFR could be a molecular target for TNBC/basal-like breast cancer treatment. Therefore, several clinical trials are evaluating EGFR-targeted therapies in these patients as will be discussed below.

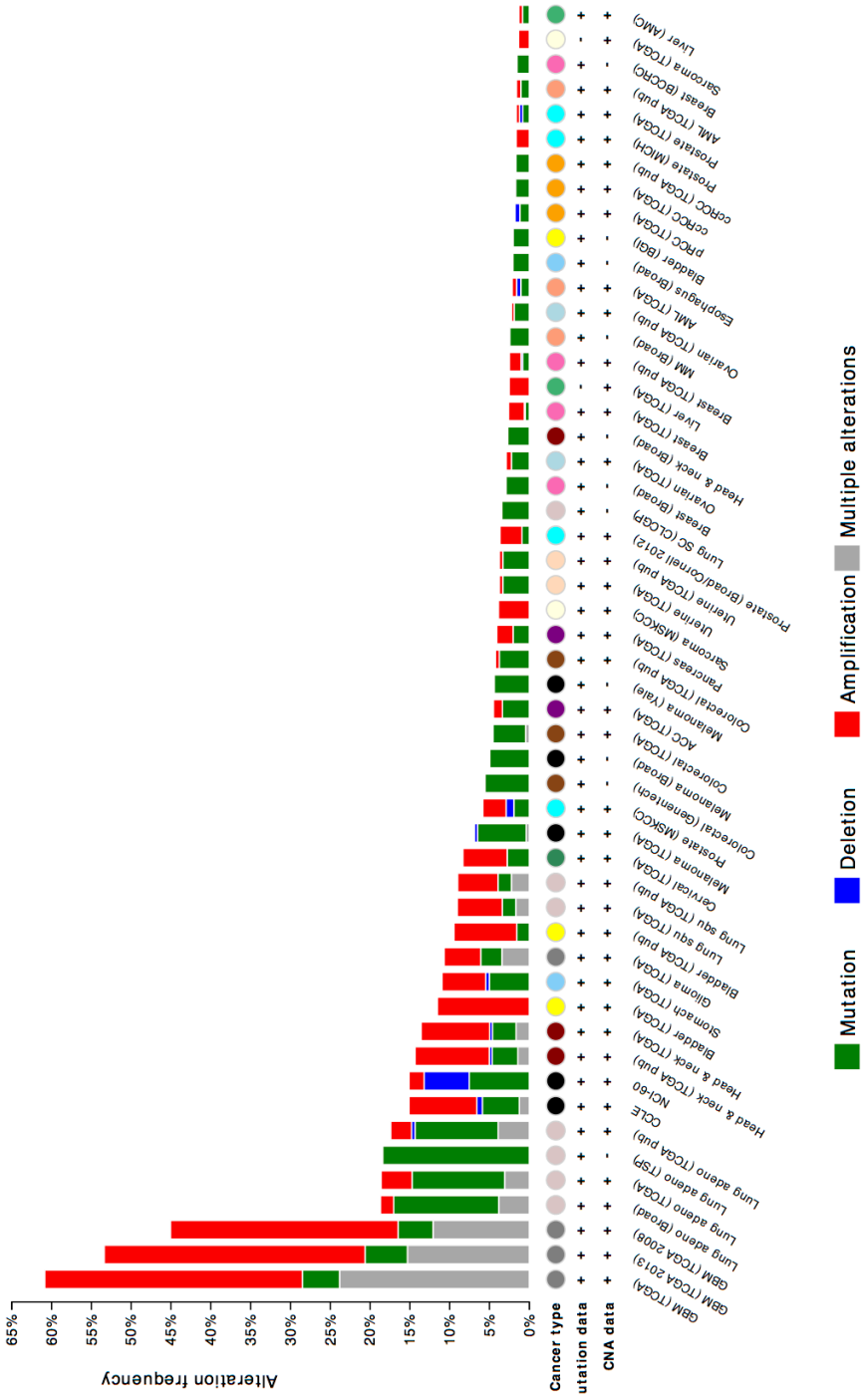


Figure 1.7 Somatic alteration of *EGFR* in cancer.

Percentage of each listed tumour type harboring amplification, somatic mutation, deletion and multiple alterations in *EGFR* gene. Plot was generated from the cBioPortal for Cancer Genomic (http://www.cbioportal.org/public-portal/).

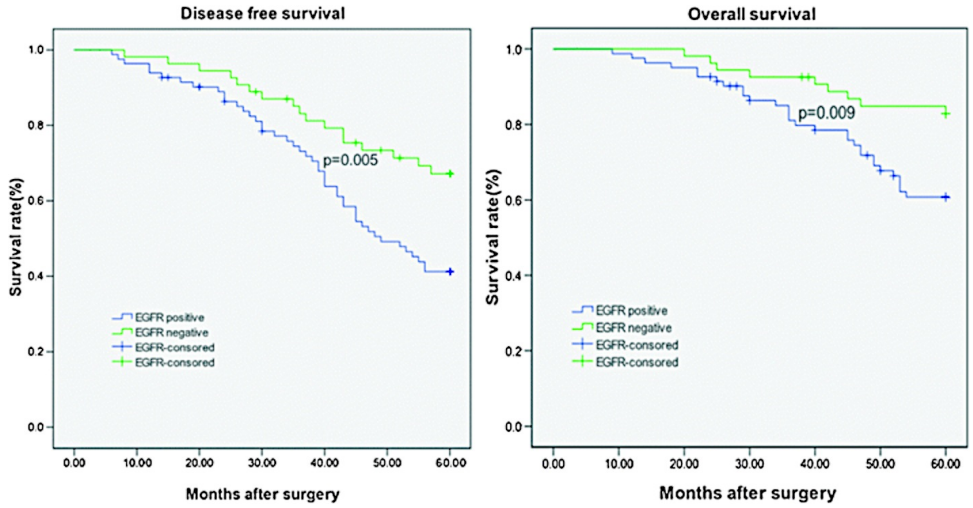


Figure 1.8 The DFS and OS of TNBC patients based on EGFR expression status. (Reprinted with permission from Springer: Targeted Oncology (Zhang *et al.* 2013), copyright (2013).

1.3.5 EGFR targeted therapies in TNBC

There are two main classes of EGFR-targeted therapies: the anti-EGFR monoclonal antibodies (mAbs) such as cetuximab (CTX) and the tyrosine kinase inhibitors (TKIs) such as gefitinib (Reviewed in (Ciardiello *et al.* 2008) **(Figure 1.9)**.

1.3.5.1 TKIs

TKIs such as gefitinib and erlotinib are selective small molecule inhibitors that compete with ATP binding by reversible binding to the ATP binding site in the intracellular tyrosine kinase domain of EGFR and thus inhibit EGFR phosphorylation (Baselga *et al.* 2000; Ciardiello *et al.* 2008).

Gefitinib (Iressa™, AstraZeneca) is approved for the treatment of advanced NSCLC patients. In advanced breast cancer patients, evaluation of gefitinib monotherapy in phase II clinical trial revealed a stable disease in 38.7% (12/31) patients but no complete or partial responses were reported. The IHC evaluation of tumour samples indicated an inhibition of both EGFR and MAPK phosphorylation but no effect on AKT phosphorylation. Authors concluded that the lack of significant clinical activity is due to the lack of EGFR dependency in the investigated patients rather than lack of receptor inhibition (Baselga *et al.* 2005). Different phase II clinical trials have evaluated gefitinib in combination with docetaxel in advanced metastatic breast cancer patients, which revealed a response rate ranged between 39-54% (Ciardiello *et al.* 2006; Dennison *et al.* 2007). Recent data from another phase II clinical trial (NCT00319618) comparing gefitinib plus docetaxel versus placebo plus docetaxel indicated a partial response in 1/9 versus 4/9 patients respectively. However, the study was closed early because of drug-related toxicity (Engebraaten *et al.* 2012).

Erlotinib (Tarceva™, OSI pharmaceuticals) is approved for the treatment of NSCLC and pancreatic patients. In a phase II clinical trial, Dickler, M.N. *et al*. (2009) have shown a minimal activity of erlotinib in unselected metastatic breast cancer patients (Dickler *et al.* 2009). Combining erlotinib with bendamustine in metastatic TNBCs was highly toxic and resulted in severe prolonged lymphopenia (Layman *et al.* 2013). There are currently ongoing clinical trials evaluating gefitinib and erlotinib in TNBC patients, including the safety and effects of gefitinib in triple-negative, EGFR positive metastatic breast cancer (NCT01732276) and a study of erlotinib and metformin in triple negative breast cancer (NCT01650506).

1.3.5.2 Monoclonal antibodies

The mAb CTX has been approved by the FDA for the treatment of metastatic colorectal cancer (mCRC) and head and neck cancers (Wheeler *et al.* 2010). In TNBC, CTX efficacy has been investigated as a single agent as well as in combination with different chemotherapeutic agents in phase I and II clinical trials. In a phase II randomized clinical trial (TBCR001), 102 metastatic TNBC patients were divided into 2 arms: CTX alone (arm 1A), CTX with carboplatin after progression with CTX alone (arm 1B) and combined CTX with carboplatin from the beginning (arm 2). The response rates of 6%, 16% and 17% were observed in patients in arms 1A, 1B and 2, respectively. The OS was 7.5 and 10.4 months in arms 1 and 2, respectively. Gene expression analysis of pre- and post-treatment revealed an activation of the EGFR pathway in the majority of TNBC patients and only a minority showed pathway inhibition. Authors concluded that patients might have alternative mechanisms of EGFR activation (Carey *et al.* 2012). Another phase II trial (BALI-1) evaluated cisplatin versus cisplatin with CTX in 173 metastatic TNBC patients. The addition of CTX to cisplatin doubled the ORR from 10%

to 20% and prolonged the PFS by 2.2 months and OS by 3.5 months compared to cisplatin alone (Baselga *et al.* 2013). Panitumumab, an EGFR mAb, is currently being investigated in a phase II clinical trial with gemcitabine and carboplatin in metastatic TNBC patients (ClinicalTrials.gov NCT00894504).

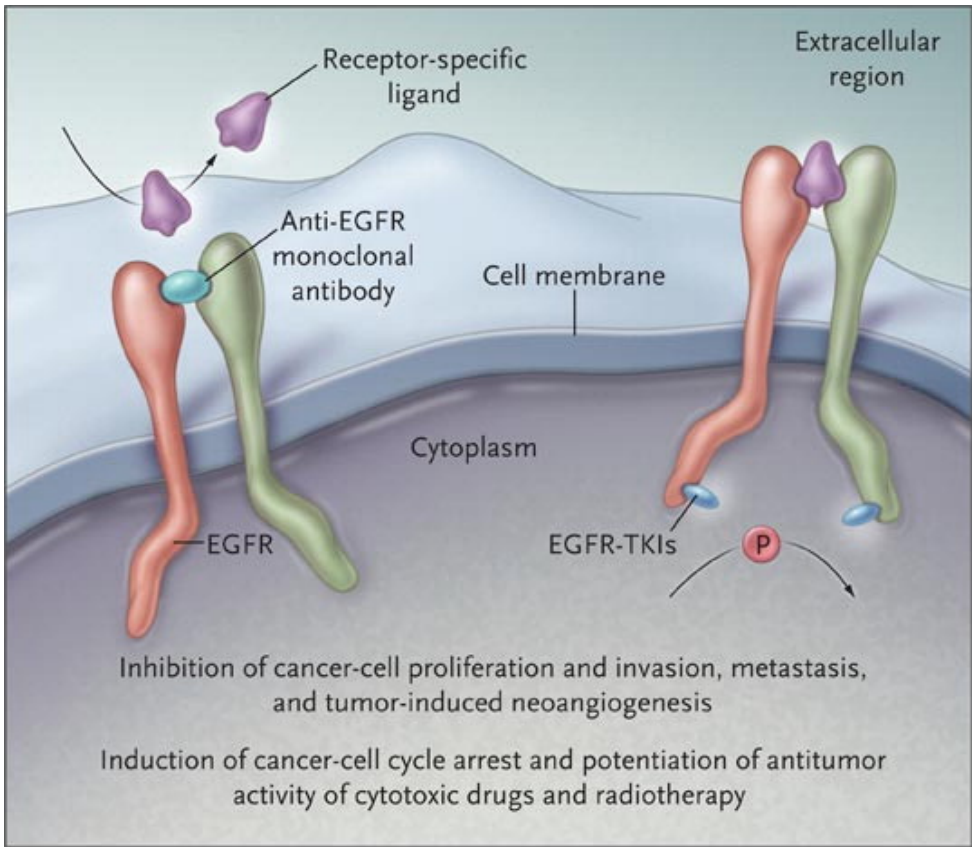


Figure 1.9 Mechanisms of action of EGFR targeted therapies in cancer.
Anti-EGFR mAbs bind to the extracellular domain and prevent ligand binding while TKIs compete with ATP for binding the intracellular tyrosine kinase domain (Reprinted from (Ciardiello *et al.* 2008), Copyright Massachusetts Medical Society).

1.4 Cetuximab (CTX)

Cetuximab (C225, Erbitux®) is a human/murine chimeric anti-EGFR mAb that specifically binds to subdomain III of the extracellular domain of EGFR. Therefore, it blocks EGF binding and prevents the formation of the required EGFR extended conformation for dimerization and subsequent EGFR phosphorylation **(Figure 1.10).** It also enhances EGFR internalization and degradation, induces cell cycle arrest in G1 phase by increasing the protein level of $p27^{kip1}$ inhibitor of cyclin-dependent kinases and the inhibition of PCNA, induces apoptosis by altering the Bax (proapoptotic) to Bcl-2 (antiapoptotic) ratio and increasing caspases expression, decreases the expression of pro-angiogenic factors and by inducing antibody-dependent cell-mediated cytotoxicity (ADCC) (Sato *et al.* 1983; Li *et al.* 2005; Wheeler *et al.* 2010; Brand *et al.* 2011). In 2004, FDA has approved CTX+/- irinotecan for the treatment of mCRC. In 2006, FDA also approved it as a treatment for head and neck squamous cell carcinomas (HNSCC) patients (Wheeler *et al.* 2010).

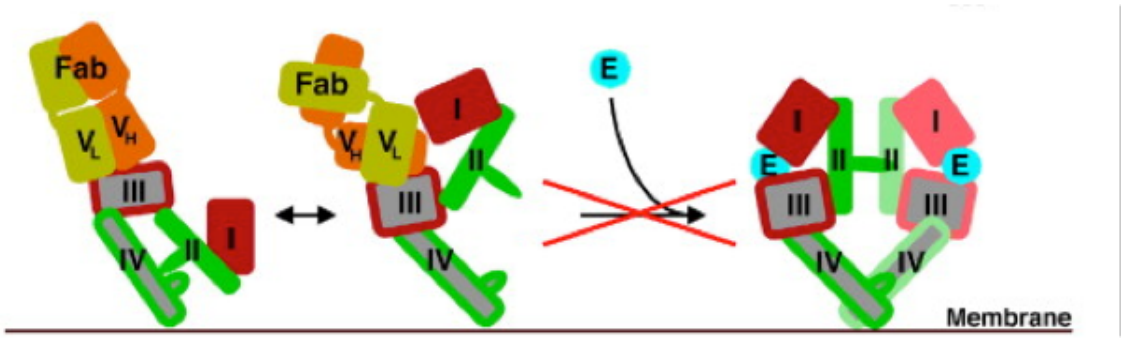


Figure 1.10 Mechanism of CTX action.
CTX binds to and blocks domain III of the extracellular region of EGFR and prevents the formation of the extended EGFR conformation that is required for ligand binding (Reprinted with permission from Elsevier: Cancer Cell (Li *et al.* 2005), copyright (2005).

1.4.1 Predictive biomarkers of response to CTX treatment

Efforts have been made in order to find biomarkers that would predict response to CTX. In Her2 overexpressing breast cancer patients, Her2 expression predicted response to trastuzumab, anti-Her2 therapy (De *et al.* 2013). In contrast, EGFR expression did not correlate with response to CTX in colorectal cancer patients (Chung *et al.* 2005; Lenz *et al.* 2006). In addition, *EGFR* mutations were not shown to predict response to CTX (Tsuchihashi *et al.* 2005). However, increased *EGFR* copy number is associated with CTX response in mCRC tumours. Moroni, M. *et al*. (2005) has found an increase in *EGFR* copy number in 88.8% (8/9) of colorectal cancer patients who responded to CTX but only 5% (1/20) of the non-responders had increased *EGFR* copy number (Moroni *et al.* 2005). Other studies have also confirmed the association between the increase in *EGFR* copy number and response to CTX (Frattini *et al.* 2007; Cappuzzo *et al.* 2008; Personeni *et al.* 2008). Furthermore, increased *EGFR* copy number also correlated with the response of advanced non-small cell lung cancer (NSCLC) patients to CTX (Hirsch *et al.* 2008). However, a phase III clinical trial (BMS-099) did not reveal an association between response to CTX in advanced NSCLC patients and *EGFR* copy number (Khambata-Ford *et al.* 2010). Increased expression of EGFR ligands in the tumours (AREG and EPR) is also a predictive marker of response to CTX in mCRC (Khambata-Ford *et al.* 2007).

1.4.2 Mechanisms of acquired CTX-resistance

The efficacy of cancer therapeutics is often limited by the rapid emergence of acquired resistance despite the initial good response. Several studies have highlighted the molecular mechanisms underlying acquired CTX resistance in different tumour models.

Ciardiello, F. *et al*. (2004) have developed acquired CTX-resistant GEO colon cancer cells *in vivo* by chronic exposure to CTX. Resistant cells exhibited an increase in VEGF protein expression compared to the parental cells. Blocking the vascular endothelial growth factor receptor 1 (VEGFR1) signalling in these resistant cells using a TKI offered a potential strategy to overcome CTX resistance (Ciardiello *et al.* 2004). Further investigations by Bianco, R. *et al*. (2008) have shown an increase in *VEGFR1* expression in CTX-resistant cells and silencing *VEGFR1* restored their sensitivity to CTX (Bianco *et al.* 2008).

The expression of the EGFR variant III (EGFRIII), which lacks the ligand-binding domain, has been shown to contribute to CTX resistance in HNSCC (Sok *et al.* 2006). *EGFR* promoter methylation has also been investigated in mCRC patients who received CTX and chemotherapy. Compared with unmethylated tumours, the methylation in the *EGFR* promoter was associated with a lower RR and a higher rate of disease progression as well as shorter PFS and OS in patients (Scartozzi *et al.* 2011). Another study has identified a mutation in *EGFR* extracellular domain (S492R), which was responsible for acquired CTX resistance in colorectal cancer cells by preventing the binding of CTX to the extracellular domain of EGFR (Montagut *et al.* 2012).

EGFR ubiquitination has also been reported as a mechanism of acquired resistance to CTX. Lu, Y. *et al* (2007) have found an increase in EGFR association with the ubiquitin ligase Cbl, resulting in an increase in EGFR ubiquitination and degradation in the acquired CTX-resistant colorectal cancer cells compared to their parental cells (Lu *et al.* 2007). Using a similar approach, Moreover, these resistant cells exhibited an activation of the other ErbB receptors (Her2 and Her3) as well as cMET (Wheeler *et al.* 2008). Yoneska, K. *et al*. (2011) have developed an acquired CTX-resistant colorectal cancer cells, which also exhibited an activation of Her2 through *ERBB2* amplification and NRG1 up-regulation (Yonesaka *et al.* 2011).

Activation of SFKs has been found in acquired CTX-resistant NSCLC cells, which enhanced the EGFR activation of Her3 and PI3k/AKT pathway. However, using the SFKs inhibitor, dasatinib, restored CTX sensitivity in the resistant cells (Wheeler *et al.* 2009). The same group has found that acquired CTX-resistant NSCLC cells expressed higher levels of nuclear EGFR compared to their parental cells. This nuclear translocation was mediated by SFKs activity and its inhibition led to the loss of nuclear EGFR and restored their sensitivity to CTX (Li *et al.* 2009). Brand, T.M. *et al*. (2014) have recently found that inhibiting nuclear translocation of EGFR resulted in an accumulation of EGFR on the plasma membrane of TNBC cell lines and enhanced their sensitivity to CTX (Brand *et al.* 2014).

The mammary derived growth factor inhibitor (MDGI) is a small cytosolic protein involved in fatty acid binding. Nevo, J. *et al*. (2009) have shown that MDGI overexpression resulted in intracellular accumulation of EGFR and thus induced CTX resistance (Nevo *et al.* 2009).

KRAS mutation has also been associated with CTX resistance. The analysis by Lièvre, A. *et al*. (2006) of 30 CTX-treated mCRC patients revealed a significant association between *KRAS* mutation and resistance to CTX therapy (Lievre *et al.* 2006). This finding has been further confirmed by the analysis of another 59 mCRC patients treated with CTX, which also indicated an association of *KRAS* mutation with resistance to CTX (Di Fiore *et al.* 2007). Moreover, xenografts derived from HNSCC cells that retro-engineered to express *PIK3CA* and *RAS* were also resistant to CTX. However, combining CTX with mTOR inhibitors, downstream of EGFR, PIK3CA and RAS, increased the anti-tumour activity of CTX (Wang *et al.* 2014). The proteosomal degradation of PTEN with subsequent constitutive activation of AKT was involved in acquired CTX resistance in NSCLC cells (Kim *et al.* 2010).

Brand, T.M. *et al*. (2014) have shown that acquired CTX-resistant NSCLC clones exhibited an increase in the expression and phosphorylation of AXL, a member of the TAM family of RTKs, as well as its association with EGFR in comparison to the parental cells. Furthermore, resistant clones were sensitive to AXL inhibition using both anti-AXL mAb and TKI (Brand *et al.* 2014).

Increased expression of EGFR ligands has been shown to be involved in acquired resistance to CTX treatment in HNSCC and colon cancer but not in TNBC models. Hatakeyama, H. *et al*. (2010) have found a significant increase of HB-EGF expression in the acquired CTX-resistant HNSCC cells compared to their parental cells. Additionally, stimulation of parental cells with HB-EGF induced resistance to CTX, while knocking down HB-EGF in the resistant cells restored the sensitivity to CTX in the resistant cells (Hatakeyama *et al.* 2010). Similarly, Troiani, T. *et al*. (2013) have found that TGFα and HB-EGF were significantly increased in the acquired CTX-resistant colorectal cancer

cells. However, knockdown TGFα but not HB-EGF, restored their sensitivity to CTX. In addition, overexpression of TGFα induced EGFR-MET interaction that contributed to CTX resistance (Troiani *et al.* 2013).

Collectively, CTX has shown a promising success as a therapeutic option. However, identification of the mechanism(s) and the alternative pathway(s) that are involved in CTX resistance is still not fully understood.

1.5 Aims and objectives

As discussed in **(1.4.1 and 1.4.2)**, predictive biomarkers of response to CTX as well as the mechanisms of acquired CTX resistance have been investigated in different cancers, including colorectal cancer, NSCLC and HNSCC. However, the underlying mechanism(s) behind the poor response of TNBC patients have not yet been identified. The overall aim of this project is to investigate the acute and chronic responses of TNBC cell lines to CTX treatment in order to identify the underlying resistance mechanism(s) and hence, to develop further strategies to enhance the initial response and to overcome the acquired resistance in TNBC patients.

The specific objectives of this project are as follows:

1. Investigate EGFR expression in TNBC patients and cell lines

2. Investigate the acute response of TNBC cell lines to CTX treatment

2.1 Correlation of *EGFR* expression in TNBC cell lines and their sensitivity to CTX

2.2 The acute effect of CTX treatment on TNBC cell lines on the EGFR signalling pathway and its ligands to identify the possible mechanisms of escaping the drug effect

3. Investigate the underlying mechanism of acquired CTX resistance in TNBC

3.1 Development of acquired CTX-resistant TNBC cell line

3.2 Characterization of the resistant cell line

3.3 Comparison of the cell signalling profile between the parental and resistant cells using the high-throughput phospho-RTK array.

3.4 Transcriptome profiling of parental and resistant cells using high-throughput next generation RNA-Seq to identify patterns of differential expression between CTX-sensitive and CTX-resistant cells genome-wide.

3.5 Identification of the underlying mechanism(s) of acquired CTX resistance and the possible combination(s) of different drugs that could overcome CTX resistance.

2 Materials and Methods

2.1 Materials

2.1.1 Human cell lines

MDA-MB-361, ZR-75-1, MDA-MB-468, HCC1806, HCC70, BT-549, MDA-MB-231, MDA-MB-453 breast cancer cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). MCF7, SKBR3, BT474 breast cancer cell lines and A431 epidermoid carcinoma cell line were obtained from cell services at Cancer Research UK (Lincoln's Inn Fields laboratory). MCF10a and T47D cell lines were kindly obtained from Professor Adrian Harris (University of Oxford). HCC1937, HCC1143 and Hs578T cell lines were kindly provided by Dr. Patricia McGowan and Professor Michael Duffy (St. Vincent's University Hospital, Ireland).

2.1.2 Drugs

CTX (Erbitux, Merck KGaA) was purchased from Oxford University Hospitals NHS Trust pharmacy and stored in aliquots at 4°C as 5 mg/ml stock. ADAM10/17 dual inhibitor (INCB3619) was kindly provided by Incyte. It was dissolved in dimethyl sulfoxide (DMSO) to 5 mM stock and stored at 4°C for *in vitro* experiments. For *in vivo* experiment, INCB3619 was dissolved in (10% DMAC, 30% PG). K02288 was purchased from Biofocus, dissolved in DMSO to 1 mM and stored at -20°C. cMET TKI (INCB28060) was kindly obtained from Dr Valentine Macaulay (University of Oxford) as 10 mM stock in DMSO.

2.1.3 Growth factors

Human EGF and TGFα (Sigma-Aldrich) were dissolved to 100 µg/ml stocks in phosphate buffered saline (PBS) and stored at -20°C. Recombinant human bone morphogenetic

protein 6 (BMP6) (Peprotech) was dissolved in MilliQ containing 0.1% BSA and stored at -20°C.

2.1.4 Antibodies

Table 2.1 List of primary antibodies

Name	Species	Application	Source
EGFR (C74B9) mAb	Rabbit	WB: 1:1000	Cell Signalling Technology (2646)
EGFR (D38B1) mAb	Rabbit	IHC: 1:100	Cell Signalling Technology (4267)
Phospho-EGFR (Tyr 1173) pAb	Rabbit	WB: 1:500 IHC: 1:200	Santa Cruz Biotechnology (sc-101668)
Phospho-EGFR (Tyr 1045) (11C2) mAb	Mouse	WB: 1:1000	Abcam (ab24928)
AKT pAb	Rabbit	WB: 1:1000	Cell Signalling Technology (9272)
Akt (pan) (C67E7) mAb	Rabbit	IHC 1:300	Cell Signalling Technology (4691)
Phospho-Akt (Ser473) pAb	Rabbit	WB: 1:1000	Cell Signalling Technology (9271)
Phospho-Akt (Ser473) (D9E) mAb	Rabbit	IHC: 1:50	Cell Signalling Technology (4060)
p44/42 MAPK (ERK1/2) pAb	Rabbit	WB: 1:1000 IHC: 1:200	Cell Signalling Technology (9102)
Phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) pAb	Rabbit	WB: 1:1000	Cell Signalling Technology (9101)

Her3 (1B2E) mAb	Rabbit	WB: 1:500	Cell Signalling Technology (4754)
Phospho-Her3 (Tyr1289) (21D3)	Rabbit	WB: 1:500	Cell Signalling Technology (2842)
Her2 (29D8) mAb	Rabbit	WB: 1:500	Cell Signalling Technology (2165)
Phospho-Her2 (Tyr1221/22) pAb	Rabbit	WB: 1:500	Cell Signalling Technology (2249)
β-actin (13E5) mAb	Rabbit	WB: 1: 3000	Cell Signalling Technology (4970)
SMAD5 (D4G2) mAb	Rabbit	WB: 1:1000	Cell Signalling Technology (12534)
Phospho-SMAD1 (Ser463/465)/ SMAD5 (Ser463/465)/ SMAD8 (Ser426/428) pAb	Rabbit	WB: 1:1000	Cell Signalling Technology (9511)
Ki67 mAb (SP6)	Rabbit	IHC: 1:400	Vector Laboratories (VP-RM04)
Cleaved caspase-3 mAb (ASP175) (5A1E)	Rabbit	IHC: 1:300	Cell Signalling Technology (9664)
MET (L41G3) mAb	Mouse	WB: 1:1000	Cell Signalling Technology (3148)
Phospho-MET (Tyr1349) pAb	Rabbit	WB: 1:1000	Cell Signalling Technology (3121)

2.1.5 Primers

Table 2.2 List of Primers used for RT-qPCR

Primer		Sequence
CCND1	Forward	5'-GAGGAAGAGGAGGAGGAGGA-3'
CCND1	Reverse	5'-GAGATGGAAGGGGGAAAGAG-3'
ACTIN	Forward	5'-ATTGGCAATGAGCGGTTC-3'
ACTIN	Reverse	5'-GGATGCCACAGGACTCCAT-3'
BMPR1A	Forward	5'-TTTATGGCACCCAAGGAAAG-3'
BMPR1A	Reverse	5'-TGGTATTCAAGGGCACATCA-3'
BMPR2	Forward	5'-GAGTGAAGGAAGCACCGAAG-3'
BMPR2	Reverse	5'-CCCCAAATTATTTCCCTCGT-3'
TGFBR1	Forward	5'-GATGGGCTCTGCTTTGTCT-3'
TGFBR1	Reverse	5'-CAAGGCCAGGTGATGACTTT-3'
SMAD5	Forward	5'-AACCTGAGCCACAATGAACC-3'
SMAD5	Reverse	5'-GTGGCATATAGGCAGGAGGA-3'
ZFYVE16	Forward	5'- CCGAGCATGTGGGAAAGTAT-3'
ZFYVE16	Reverse	5'- TGAGACTGGCAAAGTTGCTG-3'
ZFYVE9	Forward	5'-GTGTGTTGGATTGGCAGATG-3'
ZFYVE9	Reverse	5'-AACACCTGGGTCTTGCATTC-3'

2.1.6 Commercial reagents

Table 2.3 List of commercial reagents and their usage

Application	Reagent	Source
Cell culture	Dulbecco's modified Eagle medium (DMEM)	Life Technologies
	Roswell Park Memorial Institute medium 1640 (RPMI-1640)	Life Technologies
	Penicillin/Streptomycin	Life Technologies
	Foetal bovine serum (FBS)	PAA Laboratories
	Trypsin-EDTA (10×)	PAA Laboratories
	DMSO	Sigma-Aldrich
Cellular protein extraction	cOmplete protease inhibitor cocktail tablets	Roche
	Phosphatase inhibitor cocktail 2	Sigma-Aldrich
	Phosphatase inhibitor cocktail 3	Sigma-Aldrich
	Triton X-100 (t-octylphenoxy-polyethoxyethanol)	Sigma-Aldrich
Cell viability assay	CellTiter-Blue® cell viability assay kit	Promega
Clonogenic assay	Methylene blue	Sigma-Aldrich
	Ethanol	Sigma-Aldrich
Caspase 3/7 activity	Caspase-Glo® 3/7 Assay kit	Promega
ELISA	Human EGF DuoSet	R&D Systems
	Human TGF-alpha	R&D Systems
	Substrate reagent pack: hydrogen peroxide and tetramethylbenzidine	R&D Systems
	Stop solution 2N sulfuric acid	R&D Systems
	Probumin bovine serum albumin (BSA) diagnostic grade powder	Millipore

Flow cytometry	Propidium iodide solution (PI) (1 mg/ml)	Sigma-Aldrich
	Ethanol	Sigma-Aldrich
	Ribonuclease A, from bovine pancreas	Sigma-Aldrich
Immunohistochemistry	100x Citrate buffer pH 6.0	Abcam
	Aquatex mounting agent	Merck
	Histo-clear	National Diagnostics
	Hydrogen peroxide (30%)	Sigma-Aldrich
	Haematoxylin QS	Vector Laboratories
	ImmPACT DAB peroxidase substrate	Vector Laboratories
	ImmPRESS Anti-Mouse Ig (peroxidase) polymer detection kit	Vector Laboratories
	ImmPRESS Anti-Rabbit Ig (peroxidase) polymer detection kit	Vector Laboratories
In vivo experiment	IsoFlo (isoflurane, USP)	Abbott Laboratories
	Baytril 10% oral solution	Bayer Animal Health
	N-N-Dimethylacetamide (DMAC)	Sigma-Aldrich
	Propylene Glycol (PG)	Sigma-Aldrich
	Matrigel basement membrane matrix	BD Bioscience
	ALZET osmotic pump	Charles River
Medium concentrating	Amicon Ultra-15, Ultracel 10 kDa centrifugal filter	Millipore
	Amicon Ultra-15, Ultracel 3 kDa centrifugal filter	Millipore
Protein concentration assay	Bio-Rad protein assay	Bio-Rad

	BSA solution (50 mg/ml)	Sigma-Aldrich
Real-time PCR	SensiMix SYBR Hi-ROX kit	Bioline
Reverse transcription	High capacity cDNA reverse transcription kit	Life Technologies
RNA extraction	DNase I	Bio-Rad
	Ethanol, absolute, molecular biology grade	Fisher Scientific
	RNeasy Mini kit	Qiagen
Phospho-RTK array	Proteome Profiler human phospho-RTK array kit	R&D Systems
SDS-PAGE	Novex Sharp pre-stained protein standard	Life Technologies
	NuPAGE 4-12% bis-tris gel	Life Technologies
	NuPAGE LDS sample buffer (4×)	Life Technologies
	NuPAGE MOPS SDS running buffer (20×)	Life Technologies
Western blot	Amersham ECL western blotting detection reagents	GE Healthcare
	Immobilon-P 0.45 µm PVDF membrane	Millipore
	BSA	Sigma-Aldrich
	Glycine	Sigma-Aldrich
	TWEEN20 (Polyoxyethylene (20) sorbitan monolaurate)	Sigma-Aldrich
	Methanol, absolute	VWR International
Protein phosphatase depletion	**Lambda Protein Phosphatase (Lambda PP)**	NEWENGLAND BioLabs

2.1.7 Solutions

- Cell lysis buffer: 20 mM Tris (pH 7.4), 10 mM EDTA (pH 8.0), 150 mM NaCl, 10 mM $Na_2H_2P_2O_7$, 100 mM NaF, 1% (v/v) Triton X-100, supplemented with 1% (v/v) phosphatase inhibitor cocktail 2, 1% (v/v) phosphatase inhibitor cocktail 3 and 1 cOmplete protease inhibitor cocktail tablet per 10 ml of cell lysis buffer
- LDS loading buffer: 10% (v/v) β-mercaptoethanol in 4× NuPAGE LDS sample buffer
- PBS: 136.8925 mM NaCl, 3.3534 mM KCl, 12.0199 mM $NaHPO_4$, 1.8371 mM KH_2PO_4, pH 7.2
- PBSA: Phosphate buffered saline tablet dissolved in 100 ml of Milli-Q water per tablet
- Propidium iodide (PI) staining solution: 40 µg/ml propidium iodide, 100 µg/ml DNAse free RNAse A in PBSA
- Semi-dry blotting transfer buffer: 25 mM Tris (pH7.5), 192 mM glycine, 20% (v/v) methanol
- TBS: 136.8925 mM NaCl, 2.6827 mM KCl, 24.7647 mM Tris, pH 7.5
- TBST: 0.1% (v/v) Tween 20 in TBS
- Trypsin-EDTA (0.25%): 5 ml of 10× Trypsin-EDTA in 45 ml of PBSA

2.2 Methods

2.2.1 Cell culture

2.2.1.1 Maintenance

MCF10a, MCF7, SKBr3, MDA-MB-361, MDA-MB-468, Hs578T, MDA-MB-231, MDA-MB-453, A431 cell lines were maintained in DMEM medium supplemented with 10% (v/v) FBS, penicillin (100 units/ml) and streptomycin (100 µg/ml). T47D, BT474, ZR-75-1, HCC1937, HCC1143, HCC1806, HCC70, BT-549 cell lines were maintained in RPMI-1640 medium supplemented with 10% (v/v) FBS, penicillin (100 units/ml) and streptomycin (100 µg/ml). All cell lines were maintained in a humidified incubator containing 5% CO_2 at 37°C.

2.2.1.2 Routine passaging

After washing cells twice with PBSA, cells were detached by incubating with 0.25% trypsin-EDTA solution at 37°C for few minutes (3-5 minutes) until they detached. Detached cells were diluted in complete fresh medium and reseeded at a density of 1:10 in 10 ml complete medium.

2.2.1.3 Cryopreservation

For long-term storage, cells in the growing phase were trypsinizated, resuspended in complete medium, spun down at 0.2 g for 5 minutes and resuspended in freezing medium. Aliquots were frozen at -80°C in propanol filled Cryo 1°C freezing container (Nalgene) overnight before being stored in liquid nitrogen.

2.2.1.4 Frozen cell recovery

Cells stored in liquid nitrogen were recovered by rapid thawing in a 37°C water bath and transferred immediately into a flask containing pre-warmed complete medium. The medium was replaced after 24 hours with fresh complete medium.

2.2.2 Development of CTX-resistant cell line

Acquired CTX-resistant MDA-MB-468 cell line, MDA-MB-468CR, was generated by chronic exposure of parental MDA-MB-468 cells to increasing doses of CTX (2.5, 5, 7.5 and up to 10 μg/ml for more than 6 months. Fresh CTX-supplemented media were replaced every 4-5 days.

2.2.3 Cell viability assay

Cell viability was assessed using CellTiter-Blue® cell viability assay kit (Promega) according to the manufacturer's protocol. Approximately 500 - 2000 cells per well were seeded in a 96-well black, clear bottom tissue culture plate (Corning Incorporated) in triplicate and incubated overnight to allow cells to attach. The next day, media were replaced with drug-containing media and incubated for 6 days. At the end of treatment, culture media were discarded and replaced with 100 μl medium and 20 μl CellTiter-Blue® Reagent, shacked for 10 seconds, incubated for 1-2 hours at 37°C, shacked for 10 seconds and fluorescence intensity was measured at 560 nm excitation and 590 nm emission using Spectramax m2e microplate reader (Molecular Devices). Fluorescent readings were subtracted from the background reading (empty well containing medium and CellTiter-Blue® Reagent) and calculated as the mean percentage of viable cells relative to the untreated control cells (mean± S.E.).

2.2.4 Clonogenic assay

Clonogenic assay was performed as described previously (Franken *et al.* 2006). Approximately 1000 cells per well were seeded in a 12-well tissue culture plate (Corning) and allowed to adhere overnight. The following day, cells were treated with the indicated drugs for 12 days. After washing twice with PBSA, cells were stained with 0.2% methylene blue in 60% (v/v) ethanol for 1 hour. Wells were then washed twice with PBSA and twice with tap water before they were allowed to dry. Colonies were photographed using the AlphaImager HP system and quantified using ImageJ software. Results of three independent experiments were calculated as an average of the percentage of covered area in treated cells relative to the untreated control and were reported as means±S.E.

2.2.5 Cellular protein extraction

Approximately 400,000 cells were seeded in 10 cm tissue culture plates and were allowed to adhere overnight. The next day, the media were replaced with fresh media containing drugs indicated in the experiments and incubated for the specified times. At the end of treatment, the media were removed; the cells were washed twice with ice-cold PBSA and were scraped into ice-cold lysis buffer **(Section 2.1.7)**. Lysates were incubated for 30 minutes at 4°C with rotation and were then centrifuged at 17000 g for 10 minutes at 4°C. Supernatants were collected into new eppendorf tubes and were used immediately for western blotting or stored at -80°C for later use.

2.2.6 Protein concentration assay

Serial standard protein solutions were prepared by diluting 50 mg/ml standard BSA solution into 2, 4, 6, 8 and 10 mg/ml using deionized water. Protein assay stock solution

was diluted 1:5 in deionized water. Diluted protein assay solution (500 µl) was mixed with 1 µl of standard BSA solution or cell lysate. A 200-µl duplicate of each sample mixture was transferred to 96-well microplate and the optical density (OD) at 595 nm was measured using µQuant microplate reader (Bio-tek Instruments). Diluted protein assay solution without sample protein was used as a blank. The concentration of protein samples was calculated using the standard curve; an example is shown in **(Figure 2.1)**.

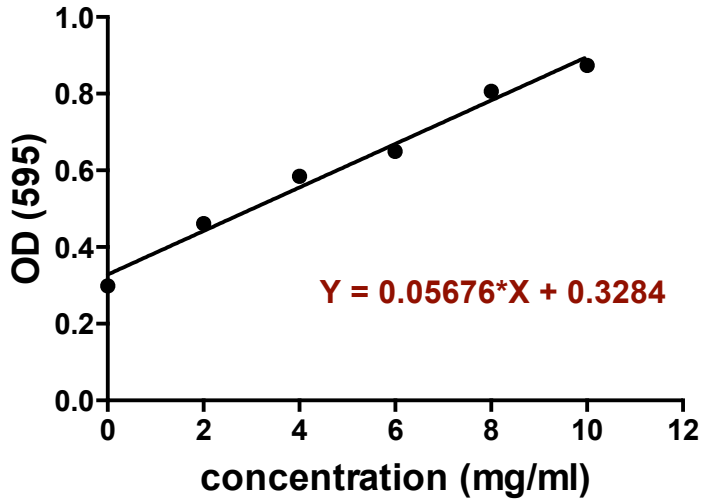


Figure 2.1 Representative standard curve of protein concentration assay

2.2.7 Western blotting

After protein quantification of cell lysates, protein contents were diluted into an equal concentration using cell lysis buffer **(Section 2.1.7)**. A mixture of one part LDS loading buffer **(Section 2.1.7)** and three parts of cell lysate was prepared and heated onto a thermal plate for 10 minutes at 90°C. Equal amounts of proteins (ranging from 15 to 80 µg) were loaded in 4-12% Bis-Tris gels, resolved by SDS-PAGE, transferred to 0.45 µm PVDF membrane using Trans-blot SD semi-dry transfer cell (Bio-Rad). The membranes

were blocked with 5% BSA in TBST for at least 1 hour followed by incubation with primary antibodies **(Section 2.1.4)** in 5% BSA in TBST overnight at 4°C with rotation. The membranes were then washed four times (10 minutes each) with 5% non-fat dry milk in TBST and were probed with secondary goat horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (Section 2.1.4) diluted in 5% non-fat dry milk in TBST for one hour at room temperature. After rinsing four times with 1% non-fat dry milk in TBST, protein-antibody complexes were detected by chemiluminescence with the Amersham ECL Detection Reagents using Fusion system (Vilber Lourmat). Experiments were independently repeated at least three times.

2.2.8 Protein phosphatase depletion

Lambda protein phosphatase kit (NEW ENGLAND BioLabs) was used to release the phosphate groups from tyrosine residues in EGFR and ERK1/2. In brief, cell lysates (30 μg) were mixed with 1X MnCl2, 1X NEBuufer and lambda protein phosphatase in a total volume of 20 μl. The mixtures were incubated for 1 hour at 30°C, boiled for 10 minutes at 95°C, mixed with the LDS loading buffer and separated by SDSPAGE as in **(Section 2.2.7)**.

2.2.9 Western blot quantification

The intensity of western blot bands was quantified using ImageJ software; values for each protein were normalized to β-actin values and presented as mean±S.E. relative to untreated control.

2.2.10 RTK array

MDA-MB-468 and MDA-MB-468CR cells were grown on 10-cm culture dishes to approximately 70% confluency and were treated with CTX for the indicated times. After treatment, the cells were washed twice with ice-cold PBSA and were lysed with ice-cold Lysis Buffer 17 provided in the RTK array kit (R&D Systems). The samples were prepared according to the manufacturer instructions and were incubated with the nitro-cellulose membranes overnight at 4°C on a rocking platform shaker. Next day, the membranes were washed three times with 1X Wash Buffer and were incubated with the Anti-Phospho-Tyrosine-HRP Detection Antibody for 2 hours at room temperature. After three times washing, arrays were incubated with chemi reagent mix for one minute and exposed to X-ray films. Scanned images were quantified using ImageQuant software and expressed as fold change relative to the untreated cells.

2.2.11 Preparation of culture media for ELISA

The cells were seeded on 10-cm culture dishes to approximately 70% confluence. The plates were then washed twice with 0.05% serum-containing medium prior to treatment and were treated with indicated drugs in 0.05% serum-containing medium for the indicated times. The culture media were collected and spun at 135 g for 3 minutes to remove dead cells. The supernatants were then collected and concentrated by centrifugation using Amicon Ultra-15 10 kDa or 3 kDa centrifugal filters at 1280 g for 35 minutes. The residues were collected and stored at −80°C for next step.

2.2.12 ELISA

Protein concentration was quantified as described previously **(Section 2.2.6)**. EGF and TGFα levels in the media were measured using Human EGF DuoSet and Human TGF-

alpha Kits (R&D Systems) according to the manufacturer's protocols. In brief, the ELISA plate was coated overnight with capture antibodies. After blocking the plate with the Reagent Diluent for 1 hour, 100 µl of concentrated medium or standard protein was loaded on each well. The plate was then incubated at room temperature for 2 hours before being washed 3 times and incubated with detection antibody for another 2 hours. Following this, the plate was washed and incubated with streptavidin labelled with horseradish peroxidase for 20 minutes. A substrate solution was added and incubated for 20 minutes and the reaction was stopped using a stop solution. The absorption was measured using Spectramax m2e microplate reader (Molecular Devices). A seven-point standard curve was produced using 2-fold serial dilution of standard EGF (range 3.9 – 250 pg/ml) or TGFα (range 7.8 – 500 pg/ml) and was plotted using fourth order polynomial function in GraphPad Prism 6 (Example of standard curve; **Figure 2.2**). Each experiment was done in duplicate for 3 times independently. The data were normalized and plotted as means±S.E. relative to untreated controls.

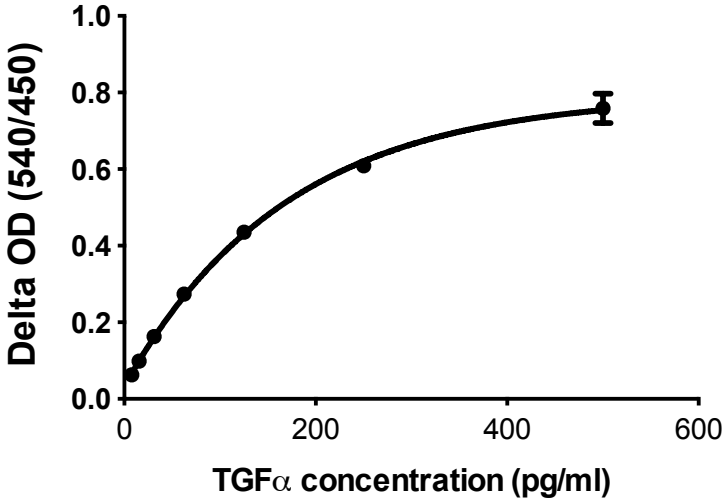


Figure 2.2 Representative standard curve of TGFα ELISA

2.2.13 Cell cycle analysis by flow cytometry

Approximately 100,000 cells per well were seeded in a 6-well tissue culture plate and were allowed to adhere overnight. The next day, the cells were treated with the indicated drugs for 72 hours. At the end of the treatment, the cells were washed twice with PBSA before being trypsinzed and collected with ice-cold PBS. The cells were washed twice in PBSA and re-suspended in 1 ml of PBSA before being fixed by the drop-wise addition of 3 ml of pre-chilled absolute ethanol with gentle vortex and incubated for 24 hours at 4°C. The fixed cells were pelleted by centrifugation at 135 g for 5 minutes and were washed twice with ice-cold PBSA. They were then re-suspended in a PI staining solution (Section 2.1.7) and were incubated in the dark for 30 minutes before being analyzed by CyAn ADP analyzer. PI was measured by excitation at 405 nm and emission detection at 675 nm. Data were analyzed using FlowJo software and the results of three independent experiments were plotted as means±S.E.

2.2.14 Measuring caspase 3 and 7 activity

Caspase 3/7 activity was measured using Caspase-Glo® 3/7 Assay (Promega) according to the manufacturer's protocol. Approximately 1000 cells per well were seeded in white wall 96-well tissue culture plate (Corning) and were allowed to adhere overnight. After 24 hours, the media were replaced with drug-containing media and were incubated for 24 hours at 37°C. At the end, Caspase-Glo® 3/7 reagent (100 µl) was added to each well and was gently mixed for 30 seconds before being incubated for 60 minutes at room temperature. The luminescence was measured using a Spectramax m2e microplate reader (Molecular Devices). Three independent experiments were done with three technical replicates and the data were plotted as means±S.E.

2.2.15 Spheroids' experiments

MDA-MB-468CR cells (5×10^3/well) were seeded in a low attachment, round-bottom 96-well plate (Costar) with the addition of 2.5% matrigel (BD Biosciences). The plates were then centrifuged for 10 minutes at 375 g and were incubated at 37°C overnight. The next day, cells were treated with the indicated drugs for 16 days. The pictures of the spheroids were taken regularly and the volumes of the spheroids were assessed using ImageJ software (http://imagej.nih.gov/ij/). The data were analyzed using GraphPad Prism® v6.0 software (GraphPad).

2.2.16 *In vivo* experiment

The xenograft experiment was carried out under Home Office regulations and was permitted by the Home Office license. Female BALB/c nu/nu mice were purchased from Harlan. At the age of 6-8 weeks old, MDA-MB-468 cells (2.5×10^6 cells suspended in 25 µl of serum-free medium and 25 µl matrigel) were injected subcutaneously at the mammary fat pad of the mice under general anaesthesia induced by isoflurane. Xenograft tumour growth was monitored three times per week using digital calipers before the treatment. Tumour volume was calculated using the formula $V = L\times W\times H\times 0.52$ (L= length, W= width, and H= height). When half of the tumours reached an average volume of 150 - 200 mm^3, mice were randomized according to their tumour volume (n=5 per group) into 4 groups and were treated as described in **(Table 2.4)** for a total of three weeks. On day 21, the mice were euthanised by cardiac puncture under isoflurane general anaesthesia and death was confirmed by neck dislocation. The blood was collected in EDTA tubes (Sarstedt) and was placed on ice. The blood plasma was collected by centrifugation at 845 g at 4°C for 15 minutes before being snap-frozen in liquid nitrogen and stored at -80°C. Residual tumours were removed and weighed. Half

of the tumours were snap-frozen in liquid nitrogen and were stored at -80°C. The other half was fixed in 4% paraformaldehyde at 4°C for 48 hours and was stored in 70% ethanol at 4°C until being processed at the Oxford Centre for Histopathology Research.

Table 2.4 Treatment groups for *in vivo* experiment

Group	Treatment	Route of treatment	Frequency of treatment
a) Control	1. 10% DMAC + 30% PG in sterile water	Subcutaneous using ALZET osmotic pump, model 2004	Constant delivery rate of 0.25 µl/hour
	2. PBS	Intraperitoneal (IP)	3 times per week
b) CTX	1. CTX: 50 mg/kg body weight	IP	3 times per week
	2. 10% DMAC + 30% PG in sterile water	Subcutaneous using ALZET osmotic pump, model 2004	Constant delivery rate of 0.25 µl/hour
c) INCB3619	1. INCB3.16: 30 mg/kg body weight/day	Subcutaneous using ALZET osmotic pump, model 2004	Constant delivery rate of 0.25 µl/hour
	2. PBS	IP	3 times per week
d) CTX+ INCB3619	1. CTX: 50 mg/kg body weight	IP	3 times per week
	2. INCB3619: 30 mg/kg body weight/day	Subcutaneous using ALZET osmotic pump, model 2004	Constant delivery rate of 0.25 µl/hour

2.2.17 Immunohistochemistry

2.2.17.1 Deparaffinising and rehydration

The slides were incubated for 10 minutes at 60°C and were then rinsed twice in histo-clear solution (National Diagnostics) for 5 minutes, twice with 100% ethanol for 5 minutes followed by washing in 70% and 50% ethanol for 5 minutes and finally tap water for 5 minutes.

2.2.17.2 Antigen retrieval

The rehydrated slides were left in a vessel containing 1x Citrate buffer solution (pH 6.0) (Abcam). The vessel was heated to 125°C for 2 minutes in a pressure cooker. After cooling, the slides were rinsed in water and were kept in PBS until blocking.

2.2.17.3 Staining

The slides were blocked with normal horse serum (ImmPRESS kit, Vector Laboratories) for 30 minutes at room temperature and then they were washed twice in PBS for 5 minutes. The primary antibodies were diluted in 3% BSA in PBS and were applied to the slides, which were incubated at 4°C overnight. The slides were washed twice in PBS with gentle agitation for 5 minutes and were incubated in 0.3% hydrogen peroxide in PBS for 15 minutes to quench background HRP. After two 5-minute wash in PBS, the mouse or rabbit secondary antibodies (ImmPRESS kit, Vector Laboratories) were applied and were incubated for 1 hour at room temperature. Two washings in PBS followed this with gentle agitation for 5 minutes. The ImmPACT DAB peroxidase substrate (Vector Laboratories) was diluted according to manufacturer's protocol and was applied to the slides for 30-60 seconds before being washed with tap water. The

Haematoxylin QS counterstain was applied to the slides for 30 seconds and was then washed with tap water. The cover glass was mounted on the stained samples slides using Aquatex mounting agent (Merck). The slides were allowed to dry and scored by a consultant breast pathologist (Dr. Ioannis Roxanis, Oxford University Hospitals).

2.2.18 Total RNA isolation

Total RNA isolation was extracted using RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. The cells in 6-well tissue culture plate were washed twice with ice-cold PBS, harvested by RLT buffer, mixed with 1 volume of 70% ethanol and transferred to RNeasy Mini spin column. The samples were centrifuged for 15 seconds at 8000×g, followed by DNAase I (Bio-0Rad) treatment for 15 minutes to eliminate genomic DNA. The columns were washed twice with RW1 buffer, twice with RPE buffer and the concentrated RNA was eluted in 40 µl nuclease-free water and stored at −80°C.

2.2.19 Reverse transcription

Isolated RNA was converted to cDNA using High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer's instructions. A 2X RT master mix was mixed with an equal volume of isolated RNA (maximum of 2 µg) in 200 µl PCR tube. The mixture was incubated in a thermal cycler (GeneAmp, PCR System 9700, Applied Biosystems) sat up at 25^0C for 10 minutes, 37^0C for 120 minutes, and 85^0C for 5 minutes. After incubation, the cDNA was stored at -20^0C.

2.2.20 Quantitative real time qPCR (RT-qPCR)

The RT-qPCR was performed using SensiMix™ SYBER Hi-ROX qPCR master mix (Bioline) and specific primers for each of the target transcript **(Section 2.1.5).** The

primers were designed using Primer3 Input (http://primer3.ut.ee/) and were synthesized by Invitrogen. The cDNA (100 ng) was mixed with SensiMix™ SYBER Hi-ROX qPCR master mix (Bioline) and the specific primers (1 μM) for each of the target transcripts. The RT-qPCR was performed using 7900HT Fast Real-Time PCR system (Applied Biosystems) as follows: 2 minutes at 50°C, 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 minutes at 60°C. A dissociation stage was performed after a complete amplification: 15 seconds at 95°C, 20 seconds at 60°C and 15 seconds at 95°C. Each experiment was repeated three times, in triplicate each time. The *ACTIN* expression was used for normalization as an internal control. The relative expression of the transcript was determined by quantifying target transcript compared to control gene *actin*, using the ΔΔCt method:

$$\text{Relative expression}_{\text{transcript}} = 2^{-\Delta\Delta Ct}$$

Where $\Delta\Delta Ct_i = \text{Transcript}_{\text{normalized (i)}} = (Ct_{(i)} - Ct_{actin\,(i)}) - (Ct_{(cont)} - Ct_{actin\,(cont)})$ and, for sample i, "$Ct_{(i)}$" is the mean of the instrument Ct triplicate results for sample i; and "$Ct_{actin\,(i)}$" is the mean of *ACTIN* Ct triplicate results.

2.2.21 High throughput sequencing

The total RNA samples from MDA-MB-468 cells treated with different conditions (the control, treated with CTX for 1 hour and 24 hours) and MDA-MB-468CR cells were prepared as described previously **(Section 2.2.17)** and the RNA concentration was measured using nanodrop (Thermo Scientific). The samples were sent to the Core Genomics Facilities as part of the service at the Wellcome Trust Centre for Human Genetics, Oxford for library preparation and sequencing using HiSeq-2000 platforms (Illumina).

2.2.22 Bioinformatics analyses

2.2.22.1 Mapping pipeline

The RNA-seq data were mapped using in-house analysis pipelines, which allowed sequential mapping of the raw data to specific RNA databases and for calculation of the expression level of the mapped transcripts. The analysis pipeline was initiated by trimming the adapter sequences from the raw sequence data using the FASTX-tool kit (http://hannonlab.cshl.edu/fastx_toolkit/). The sequences were mapped sequentially to 2009 human genome release, hg19 RNA database using TOPHAT (http://ccb.jhu.edu/software/tophat/index.shtml)

2.2.22.2 The quantification of gene expression

This analysis was done by Dr. Francesca Buffa (Applied Computational Genomics Group, Department of Oncology, University of Oxford). Using EdgeR package, contrasts were defined to derive differentially expressed transcripts between the different conditions. Then, pairwise comparisons between each condition (1 hour and 24 hours CTX-treatment and MDA-MB-468CR) was performed against the control untreated MDA-MB-468 and reported as the average log2 FC and their *P* values.

2.2.23 The analyses of the METABRIC data set

The analysis of the METABRIC data set was done by Dr. Syed Haider (Applied Computational Genomics Group, Department of Oncology, University of Oxford). The METABRIC breast cancer dataset was preprocessed, summarized and quantile-normalized from the raw expression files generated by Illumina BeadStudio (R packages: beadarray v2.4.2 and illuminaHuman v3.db_1.12.2). The raw METABRIC files

were downloaded from the European genome-phenome archive (EGA) (Study ID: EGAS00000000083). The data files of one METABRIC sample were not available at the time of analysis, and were therefore excluded. Multiple probes to gene mapping were performed by keeping the most variable probe for the corresponding gene. Log2 scaled data was used for mRNA abundance plots and the subsequent correlation analysis (Spearman's rank correlation). The METABRIC analysis and visualisation were prepared in R statistical environment (version 3.0.1). The Cox proportional hazards model was fitted to estimate the hazard ratio (HR), The Wald test was used to test the significance of the outcome difference between the low- and high-expression groups. The risk groups were established by median dichotomisation.

This study makes use of data generated by the Molecular Taxonomy of Breast Cancer International Consortium, which was funded by Cancer Research UK and the British Columbia Cancer Agency Branch (Curtis *et al.* 2012).

2.2.24 Statistical analyses

The statistical analysis was performed and the graphs were generated using GraphPad Prism® version 6.0 software (GraphPad). The differences in the means between different groups were tested using one-way ANOVA test and Bonferroni's multiple comparisons tests for experiments involving more than 2 groups and Student t-test (parametric) or Mann-Whitney (non-parametric) if only two groups. The statistical significance is represented by: $P<0.05$ *, $P<0.01$ **, and $P<0.001$ ***.

3 The inhibitory effect of CTX in TNBC is counteracted by the release of active EGFR ligands

3.1 Introduction

EGFR is frequently overexpressed in TNBC patients and its overexpression is associated with worse clinical outcomes (Nielsen *et al.* 2004; Dogu *et al.* 2010). Targeting EGFR using either mAbs (e.g CTX) or TKIs (e.g. erlotinib and lapatinib) has been investigated in TNBC. Both *in vitro* and *in vivo* data indicated that TNBC and basal-like cell lines are sensitive to CTX-treatment (Hoadley *et al.* 2007; Oliveras-Ferraros *et al.* 2008; Jung *et al.* 2011). On the other hand, clinical trials showed a limited efficacy of CTX in the management of TNBC and basal-like patients as a single agent (Carey *et al.* 2012). However, combining CTX with carboplatin (Carey *et al.* 2012) or cisplatin (Baselga *et al.* 2013) was associated with 17% and 20% ORR, respectively, compared to chemotherapy alone.

In this chapter, I have analyzed the expression of ErbB receptors from publicly available data as well as in a panel of breast cancer cell lines. I also investigated the sensitivity of TNBC cell lines to CTX-treatment and correlated it with basal *EGFR* expression. In addition, I investigated the CTX effect on EGFR signalling in TNBC cell lines.

3.2 Expression of ErbB receptors in breast cancer patients

The expression level of ErbB receptors was analyzed using published expression data derived from a large cohort of breast cancer tumours (n=1988, METABRIC dataset) for whom clinical outcome data were also available (Curtis *et al.* 2012). The median expression levels of *EGFR*, *ERBB2*, *ERBB3* and *ERBB4* were calculated across the cohort. Tumours in this cohort were further sub-classified, based on their triple negative status (ER, *PR* and *ErbB2* negative), ER status (ER-positive versus ER-negative), *ErbB2* status (Her2-positive versus Her2-negative) and also using the PAM50 gene expression profiler (Parker *et al.* 2009). The highest *EGFR* expression was found in TNBC and basal-like tumours (median log2 mRNA abundance 7.18 and 7.1, respectively) compared to the other subtypes **(Figure 3.A).** Further analysis of the *EGFR* expression within the PAM50 subtypes revealed a significant difference in *EGFR* expression between Her2-positive and basal-like (log2 fold change (FC)= -0.66, 95% CI= -0.81 to -0.49, adjusted *P* value (P_{adj})<0.0001), luminal A and basal-like (log2 FC= -1.21, 95% CI= -1.33 to -1.08, P_{adj}<0.0001) and luminal B and basal-like tumours (log2 FC= -1.42, 95% CI= -1.55 to -1.28, P_{adj}<0.0001). On the other hand, *ERBB2* mRNA expression was the highest in Her2-positive breast cancer tumours (median log2 mRNA abundance= 13.15) and the lowest in TNBC and basal-like tumours (median log2 mRNA abundance= 10.01 and 9.9, respectively) **(Figure 3.1B).** In addition, *ERBB3* mRNA expression was the highest in both luminal A and luminal B subtypes but the lowest in basal-like and TNBC patients **(Figure 3.1C).** On the other hand, *ERBB4* expression did not vary among the different breast cancer subtypes **(Figure 3.1D).**

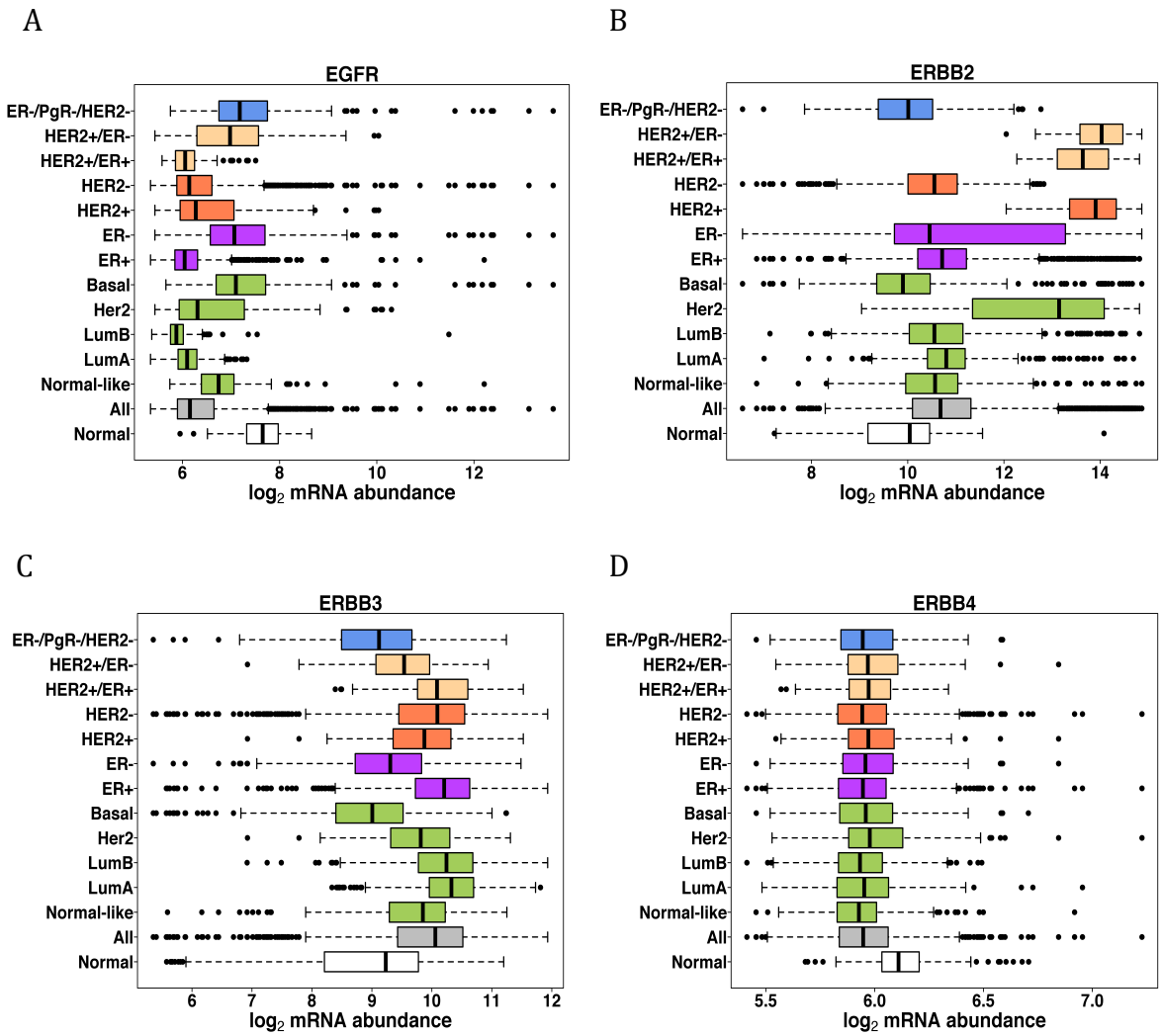


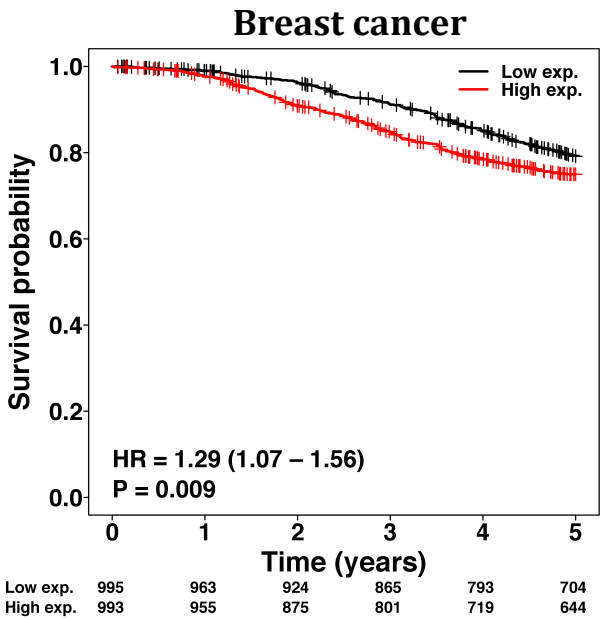
Figure 3.1 Expression of ErbB receptors in a cohort of 1988 breast cancer tumours.

Published expression data (Curtis *et al.* 2012) were used to analyze the expression of **(A)** *EGFR*, **(B)** *ERBB2*, **(C)** *ERBB3* and **(D)** *ERBB4* in a cohort of breast cancer tumours (n=1988).

3.3 High *EGFR* expression is associated with poor survival in breast cancer patients

As shown in **(Figure 3.1)**, *EGFR* expression is higher in TNBC compared to non-TNBC breast cancer patients. The prognostic value of *EGFR* expression was further assessed in TNBC patients as well as the cohort of breast cancer patients. The patients were divided into two categories: high *EGFR* expression (above median) and low *EGFR* expression (below median). The Kaplan-Meier analysis revealed that high *EGFR* expression was significantly associated with poor OS in all breast cancer patients (HR= 1.29, 95% CI= 1.07-1.56, *P*=0.009) **(Figure 3.2A)**. The multivariate analyses of *EGFR* expression, adjusted for clinicopathological features known to correlate with outcome (age, tumour size, grade, stage and node status), showed that *EGFR* expression remained significantly associated with poor outcome after adjustment for age (*P*=0.004), tumour size (*P*=0.002), stage (*P*=0.025), node status (*P*=0.001) and grade (*P*=0.039) **(Table 3.1)**. This indicates that tumour *EGFR* expression is an independent prognostic marker in breast cancer patients at least with respect to these clinical variables. A similar but not significant association was also observed in TNBC patients (HR=1.46, 95% CI=0.97-2.21, *P*=0.071) **(Figure 3.2B and Table 3.1)**. On the other hand, there was no significant correlation between the expressions of other ErbB receptors (*ERBB2*, *ERBB3* and *ERBB4*) and OS in TNBC patients **(Table 3.2)**.

A



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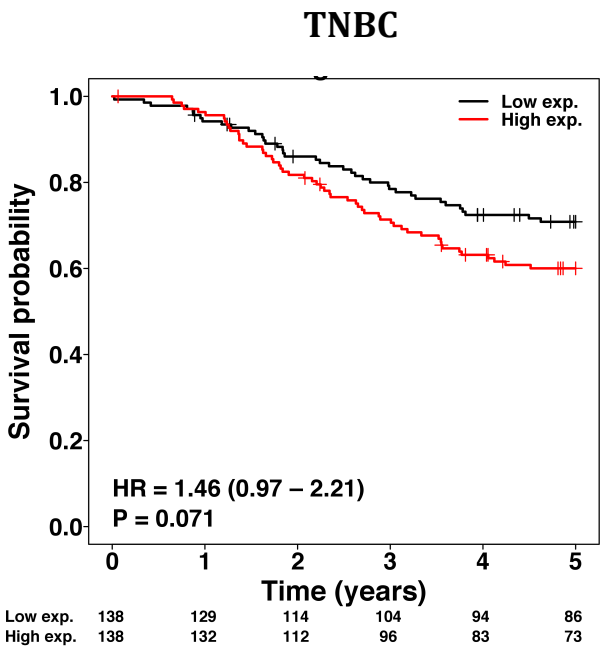


Figure 3.2 Tumour EGFR expression level is associated with adverse clinical outcome in breast cancer.

Kaplan-Meier analysis of OS in **(A)** breast cancer and **(B)** TNBC patients showing a significant association between tumour expression level of *EGFR* and poor OS in a cohort of 1988 breast cancer patients.

Table 3.1 Multivariate cox proportional hazards analyses of *EGFR* with clinical parameters in the METABRIC breast cancer cohort

Variable	HR	95% CI	*P* value	n
All patients				
Age	1.33	1.09 – 1.61	0.004	1988
Size	1.34	1.10 – 1.62	0.002	1968
Node	1.36	1.12 – 1.65	0.001	1982
Stage	1.29	1.03 – 1.62	0.025	1474
Grade	1.22	1.00 – 1.48	0.039	1899
TNBC patients				
Age	1.46	0.96 – 2.20	0.07	276
Size	1.48	0.97 – 2.26	0.06	272
Node	1.36	0.89 – 2.07	0.15	274
Stage	1.42	0.86 – 2.35	0.16	203
Grade	1.43	0.94 – 2.16	0.08	274

Table 3.2 Expression of other ErbB receptors is not associated with poor patient survival in TNBC patients.

Receptor	HR	95% CI	*P* value
ERBB2	1.12	0.74 – 1.68	0.60
ERBB3	0.99	0.66 – 1.49	0.96
ERBB4	1.00	0.66 – 1.49	0.98

3.4 Expression of EGFR in breast cancer cell lines

As shown above, *EGFR* expression is higher in TNBC tumours than non-TNBC tumours. Next, I assessed the basal EGFR expression in a panel of TNBC and non-TNBC breast cancer cell lines. TNBC cell lines were selected based on Lehmann, B. *et al*. (2011) classification of TNBC tumours and cell lines in to include at least one cell line of each TNBC subtype except the IM **(Section 1.2.3 and Table 1.1)** (Lehmann *et al.* 2011) as follows; BL1 (MDA-MB-468, HCC1937 and HCC1143), BL2 (HCC1806 and HCC70), M (BT549), MSL (Hs578T and MDA-MB-231) and LAR (MDA-MB-453)**.** The non-TNBC cell lines included luminal breast cancer cell lines (MCF7 and T47D), Her2 overexpressed breast cancer cell lines (Her2 3+) (SKBr3 and BT474), Her2 moderately expressed breast cancer cell lines (Her2 2+) (MDA-MB-361 and ZR751) and the normal mammary epithelial cells (MCF10a). The human epidermoid A431 cell line was used as an EGFR positive control.

EGFR mRNA expression was plotted relative to the basal expression in MCF10a cell line. A431 and MDA-MB-468 cell lines expressed the highest EGFR level at both mRNA and protein levels. TNBC cell lines, HCC1143, HCC1937 and HCC1806 and the ErbB2 positive BT474 cell line expressed similar levels of EGFR. MDA-MB-453 and ZR751 cells were EGFR negative **(Figure 3.3A and B)**. These data were in line with previous publications (deFazio *et al.* 2000; Hochgrafe *et al.* 2010; Ferraro *et al.* 2013). In accordance with the *EGFR* expression in TNBC tumours, the basal *EGFR* mRNA expression varied within the investigated TNBC cell lines **(Figure 3.3A and B)**. Moreover, TNBC cell lines expressed significantly higher *EGFR* compared to non-TNBC cell lines, at least within the investigated breast cancer cell lines (P=0.02, Mann-Whitney t-test) **(Figure 3.3C)**. Furthermore, TNBC cell lines of basal-like subtypes (BL1 and BL2) had a significantly

higher *EGFR* mRNA level compared to the mesenchymal-like (M, MSL) and LAR subtypes (*P*=0.01, Mann-Whitney t-test) **(Figure 3.3D)**.

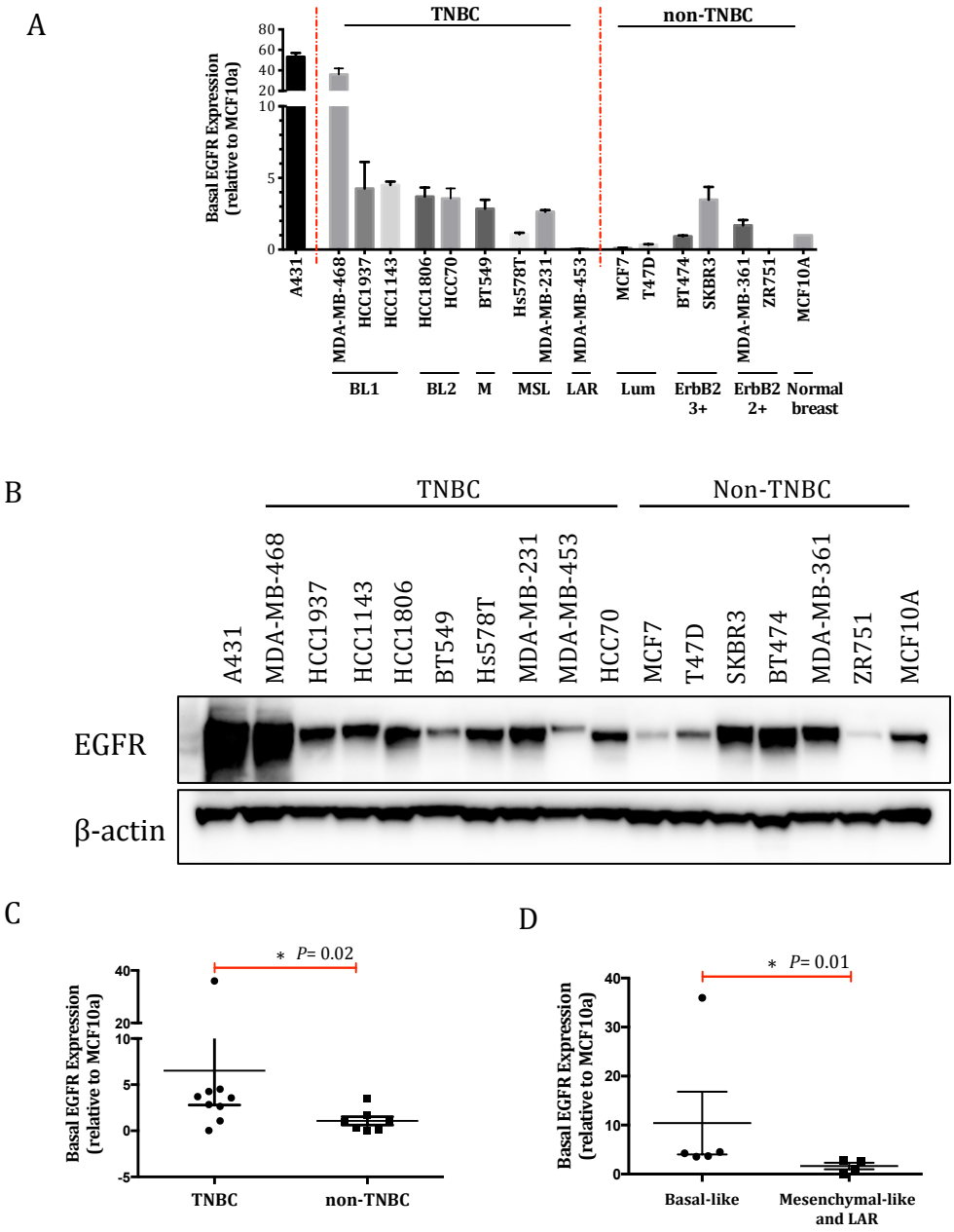


Figure 3.3 Basal EGFR expression in breast cancer cell lines.
(A) Basal *EGFR* mRNA expression as assessed by RT-qPCR in a panel of cell lines, including TNBC and non-TNBC cell lines with A431 cells as EGFR positive control. The graph shows the basal mRNA level as mean±S.E relative to MCF10a cell line (n=3). **(B)** EGFR protein expression as assessed by western blot in the same panel of cell lines as in (A). **(C)** Comparison of basal *EGFR* mRNA in TNBC (n=9) versus non-TNBC cell lines (n=7) and **(D)** basal-like versus mesenchymal-like and LAR TNBC cell lines.

3.5 Sensitivity of TNBC cell lines to CTX treatment

Basal-like breast cancer cell lines are more sensitive to EGFR inhibitors than luminal breast cancer cell lines (Hoadley *et al.* 2007). To investigate the sensitivity of TNBC cell lines to CTX treatment, the same panel of TNBC cell lines discussed in section 3.4 was treated with increasing doses of CTX (range 5 – 200 µg/ml) for 6 days. As shown in **(Figure 3.4 and Table 3.3)**, MDA-MB-468 cell line was the most sensitive cell line to CTX followed by the HCC70 cell line. In contrast, the other investigated TNBC cell lines showed minimal or no response to CTX-treatment **(Figure 3.4 and Table 3.3).** The EGFR over-expressed A431 cell line was used as a positive control, which is sensitive to CTX treatment (Matar *et al.* 2004).

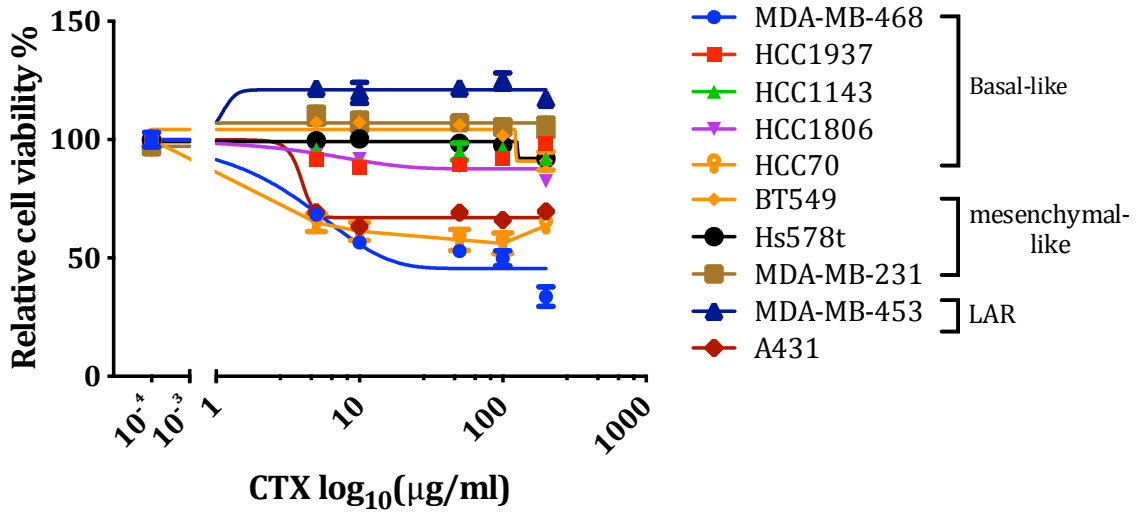


Figure 3.4 Sensitivity of TNBC cell lines to CTX-treatment.
A panel of TNBC cell lines as well as A431 cell line were treated with CTX (range 5 – 200 µg/ml) for 6 days and cell viability was assessed using CellTiter-Blue® kit. Percentage of viable cells were calculated relative to untreated control cells and expressed as mean±S.E (n=3).

Table 3.3 The percentage of viable cells relative to untreated control cells after CTX-treatment.

Data presented as means±S.E., n=3.

Cell line	CTX (µg/ml)				
	5	**10**	**50**	**100**	**200**
MDA-MB-468	68.4±3.0	56.6±2.1	52.9±0.9	49.8±3.2	33.6±4.1
HCC1937	91.8±1.5	88.2±1.8	89.3±2.4	91.9±1.7	98.4±2.0
HCC1143	95.9±2.4	90.2±2.8	94.9±3.6	96.4±2.7	91.9±2.6
HCC1806	93.5±2.3	91.8±1.7	88.8±2.0	91.6±1.2	82.4±1.5
HCC70	64.9±3.8	61.3±3.8	57.6±4.4	56.2±4.4	63.7±3.3
BT549	107.2±1.8	107.4±1.8	106.1±1.5	101.7±1.1	91.0±3.8
Hs578T	99.6±1.3	100.3±0.8	98.4±0.9	97.7±1.1	92.1±2.1
MDA-MB-231	110.2±3.5	107.5±3.6	107.1±2.7	105.3±3.0	105.2±3.8
MDA-MB-453	121.7±2.0	119.8±4.4	121.8±2.1	124.8±3.4	117.1±2.9
A431	69.3±1.1	63.3±1.0	69.1±2.6	66.1±1.7	69.9±0.9

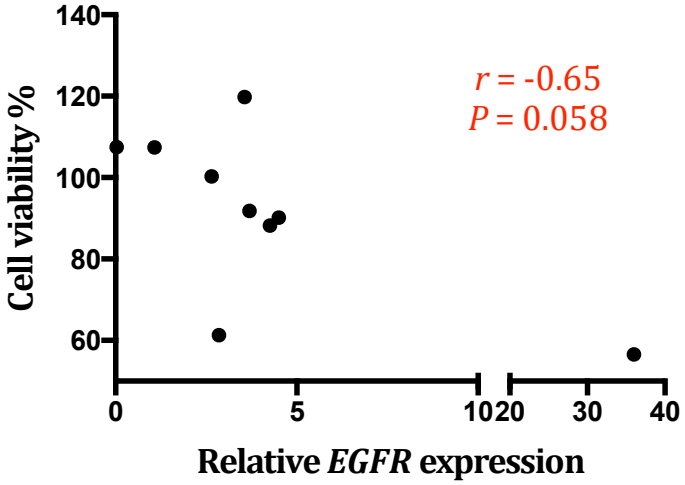
3.6 The correlation between *EGFR* expression and CTX-sensitivity in TNBC cell lines

As shown in **(3.5)**, TNBC cell lines varied in their basal EGFR expression and also their sensitivity to CTX treatment. A negative correlation has been observed between basal *EGFR* expression and percentage of viable cells after CTX-treatment (10 μg/ml) (***r***=-0.65) **(Figure 3.5A).** There was no correlation between CTX-sensitivity at different doses and *EGFR* expression (data not shown). However, more cell lines should be included to confirm whether there is any significant correlation between *EGFR* expression and sensitivity to CTX.

3.7 Correlation between TNBC subtypes and CTX-sensitivity in TNBC cell lines

Basal-like TNBC cell lines (BL1 and BL2) expressed a significantly higher *EGFR* level compared to other subtypes (mesenchymal-like and LAR) (P=0.01, Mann-Whitney t-test) **(3.4).** A comparison of the percentage of viable cells treated with CTX between basal-like and other subtypes (mesenchymal-like and LAR) revealed that the basal-like TNBC cells were significantly more sensitive to CTX than the mesenchymal and LAR subtypes at both 10 and 50 μg/ml (P<0.05, One-way ANOVA with Bonferroni's multiple comparison post tests) **(Figure 3.5B)**. However, there was no significant difference at 5, 100 and 200 μg/ml. This could be explained by the very low dose of CTX (5 μg/ml) that was below the optimal dose and at 100 and 200 μg/ml CTX, all the cell lines reached a plateau of sensitivity and there was no further decrease in cell viability **(Figure 3.5B).**

A



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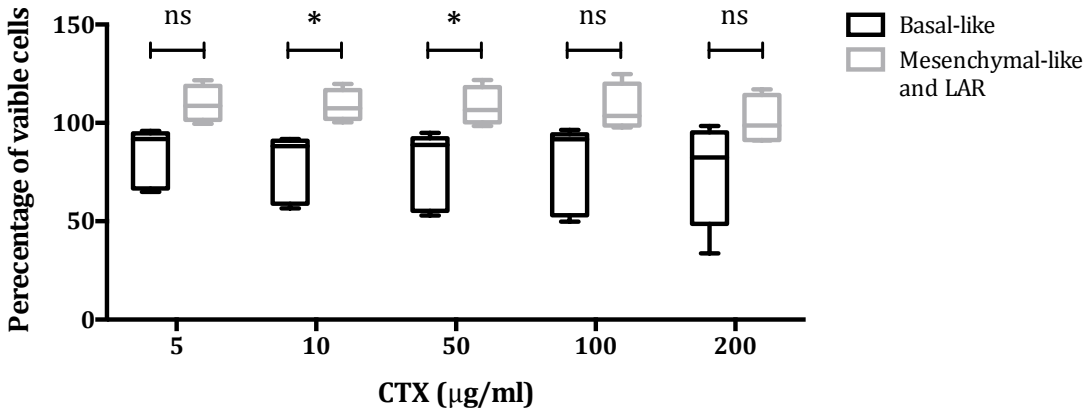


Figure 3.5 Correlation of CTX-sensitivity and EGFR expression and TNBC subtype. **(A)** Correlation between basal *EGFR* mRNA expression and percentage of viable TNBC cells treated with CTX (10 μg/ml) for 6 days. **(B)** Comparison between the sensitivity of basal-like versus mesenchymal-like and LAR TNBC subtypes to CTX treatment at different doses (range 5 – 200 μg/ml). The percentages of viable cells were plotted as mean±S.E.

3.8 Effect of CTX on EGFR signalling pathway in TNBC cells

To investigate the effect of CTX on EGFR signalling pathway in TNBC cell lines, the CTX-sensitive MDA-MB-468 cells were treated with CTX (10 and 100 μg/ml) for 1, 24 and 72 hours. As shown in **(Figure 3.6)**, CTX treatment did not affect the total EGFR level but induced a minor decrease of $pEGFR^{1173}$ and $pEGFR^{1045}$ after 72 hours of treatment **(Figure 3.6A)**. Following 1 hour of treatment, although CTX did not decrease $pEGFR^{1173}$ and $pEGFR^{1045}$ levels, it decreased pAKT by 30% at 10 μg/ml and by 40% at 100 μg/ml CTX. In addition, there was a significant decrease in pERK1/2 to 0.1 (P<0.0001) at 10 μg/ml and to 0.06 (P<0.0001) at 100 μg/ml CTX after 1 hour CTX treatment **(Figure 3.6A).** Nevertheless, there was a reactivation of pERK1/2 (0.6 and 0.5 at 10 and 100 μg/ml respectively) after 24 hours of CTX treatment **(Figure 3.6A)**. For pAKT, there was an increase in pAKT at a CTX concentration of 100 μg/ml after 24 hours, which was not evident for a CTX concentration of 10 μg/ml compared to 1 hour CTX treatment **(Figure 3.6A)**.

As there was a decrease in the downstream pathways, pAKT and pERK1/2 after 1 hour CTX treatment but not in the $pEGFR^{1173}$ and $pEGFR^{1045}$, further control experiments were done to assess the specificity of $pEGFR^{1173}$ antibody. First, lambda protein phosphatase treatment was utilized to treat the MDA-MB-468 lysates followed by western blot analysis. This treatment resulted in the depletion of all $pEGFR^{1173}$ in the treated lysates but not in the untreated lysates **(Figure 3.6B).** This indicated that the $pEGFR^{1173}$ antibody is specific for the phosphorylated EGFR level but not the total EGFR. In addition, as shown in **(Figure 3.6C)**, EGF stimulation induced an increase in $pEGFR^{1173}$ level, which was correlated with an increase in AKT and ERK1/2 phosphorylation in MDA-MB-468 cells. In contrast, pretreatment of cells with CTX for

30 minutes abolished EGF-induced phosphorylation of EGFR. This confirmed that CTX targeted EGFR in MDA-MB-468 cells.

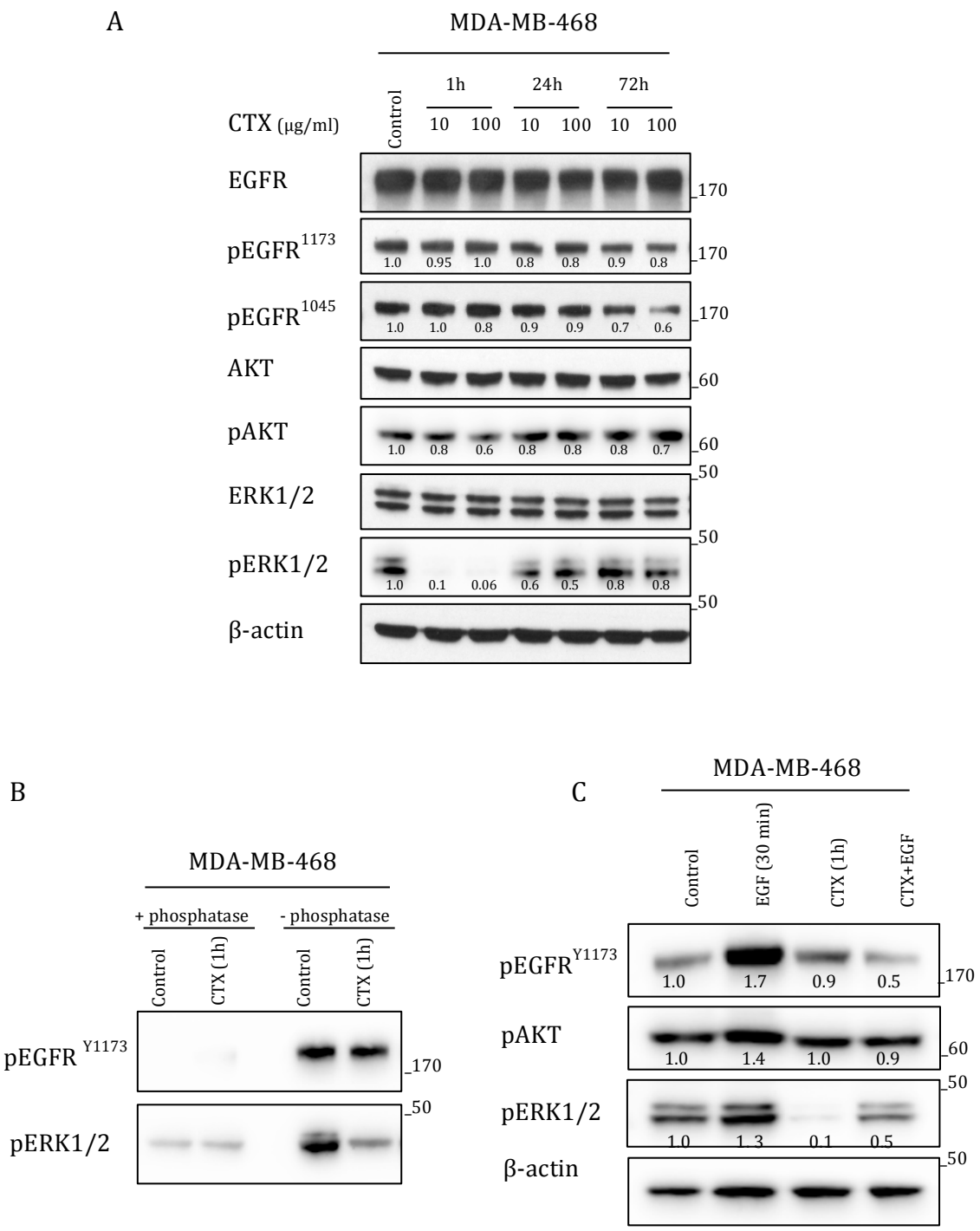


Figure 3.6 Effect of CTX on EGFR signalling pathway.

(A) MDA-MB-468 cells were treated with CTX (10 and 100 μg/ml) for the indicated times and cell lysates were subjected to western blot analysis and were immunoblotted with the indicated antibodies. **(B)** Lysates of MDA-MB-468 cells (control and CTX-treated) were subjected to phosphatase treatment followed by western blot analysis and blotting for pEGFR^{Y1173} and pERK1/2. **(C)** MDA-MB-468 cells were stimulated with EGF (100 ng/ml, 30 minutes), treated with CTX (10 μg/ml, 1 hour) or treated with CTX + EGF stimulation and subjected to western blot analysis as in (A).

3.9 Effect of CTX on other ErbB receptors in TNBC cells

To further investigate the effect of CTX on other ErbB receptors, Her2 and Her3, MDA-MB-468 cells were treated with CTX as in **(Section 3.9)**. Due to the very low expression of Her2 in these cells, it was difficult to detect pHer2. As shown in **(Figure 3.7)**, CTX induced an initial decrease of pHer3 with subsequent reactivation after 24 hours. This was more evident for a CTX concentration of 100 µg/ml, and this correlated with pAKT level shown above **(Figure 3.6A)**.

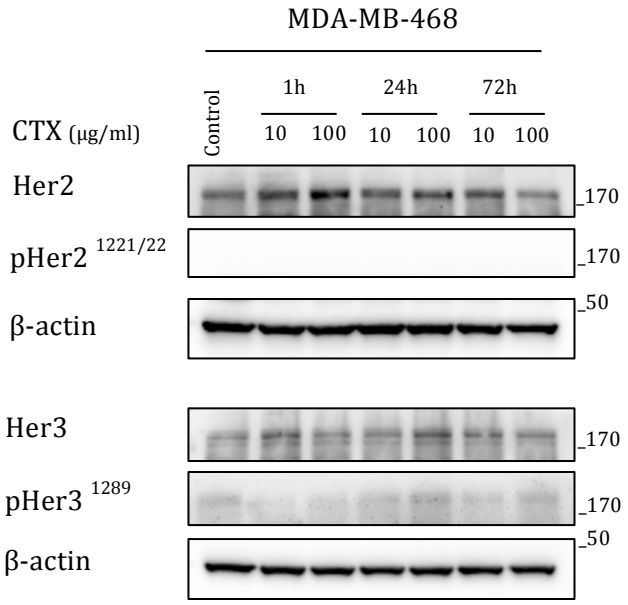


Figure 3.7 Effect of CTX treatment on Her2 and Her3 phosphorylation.
MDA-MB-468 cells were treated with CTX (10 and 100 µg/ml) for the indicated times and cell lysates were subjected to western blot analysis and were immunoblotted with the indicated antibodies. β-actin was used as a loading control.

3.10 CTX induces the release of EGFR ligands

As shown in **(Figure 3.6)**, CTX failed to decrease EGFR phosphorylation in CTX-sensitive MDA-MB-468 cells within 24 hours. Next, I assessed the effect of CTX treatment of different TNBC cell lines (MDA-MB-468, MDA-MB-231, BT-20, BT-549 and HCC1806) on the level of soluble EGFR ligands, EGF and TGFα, in the media. In the MDA-MB-468 cell line, CTX-treatment significantly increased the level of EGF by 4.4±0.7-fold (P<0.0001, Paired t-test, n=6) and TGFα by 15.2±1.7-fold (P<0.0001, Paired t-test, n=6) in the media after 24 hours treatment **(Figure 3.7A and B)**. The other cell lines, MDA-MB-231 and HCC1806 also significantly released EGF and TGFα in the media after 24 hours of CTX-treatment **(Figure 3.7A and B)**. On the other hand, CTX-treatment did not induce a significant increase of EGFR ligands in the BT-549 cell line **(Figure 3.7A and B)**.

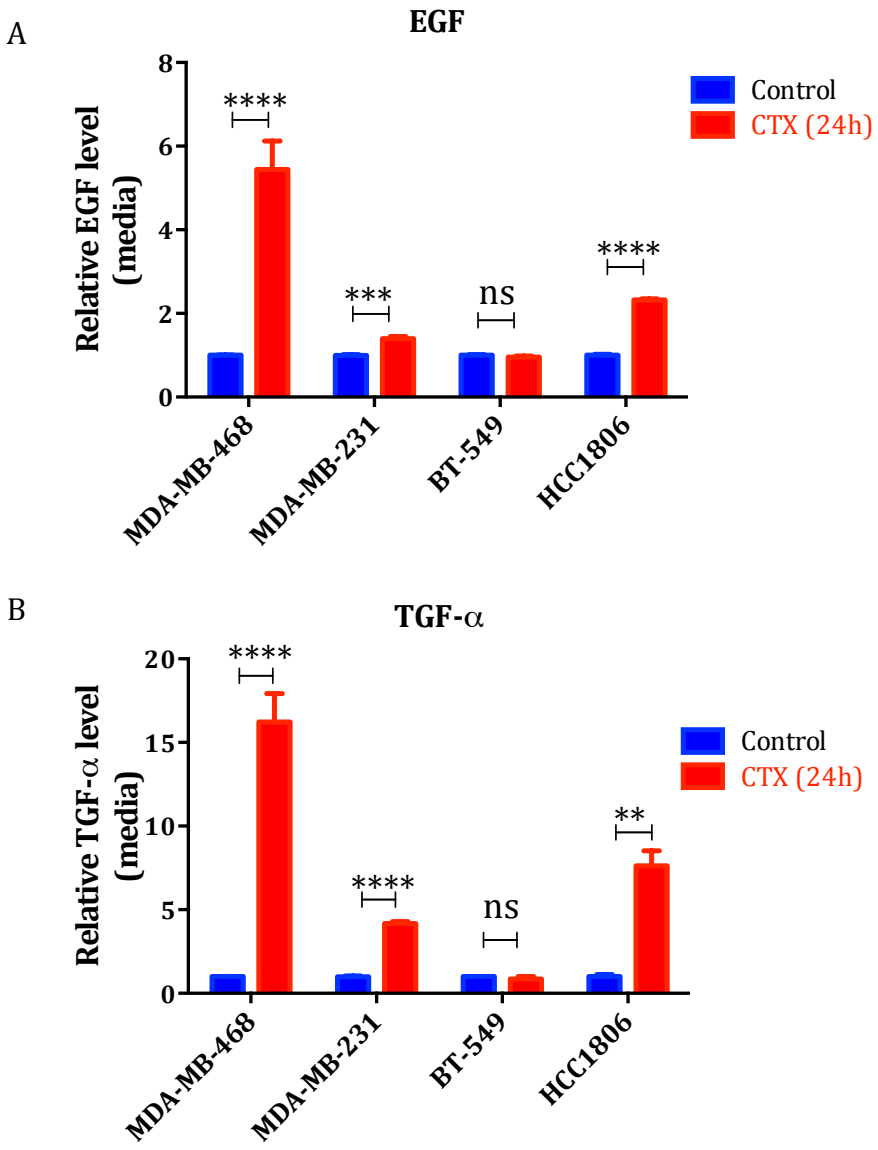


Figure 3.8 Elevation of EGFR ligands in the media induced by CTX-treatment.
The levels of (A) EGF and (B) TGFα in the media in different TNBC cell lines treated with CTX (10 μg/ml) for 24 hours measured by ELISA. Relative values are expressed as mean±S.E., Paired t-test (n=3).

3.11 Effect of EGFR ligands on response to CTX-treatment in TNBC cell lines

As CTX-treatment triggered the release of EGFR ligands, EGF and TGFα, in the media **(Shown in 3.9)**, it was asked whether this increase of endogenous ligands in the media would affect the response of TNBC cells to CTX. Therefore, MDA-MB-468 cells were treated with CTX for 24 hours and stimulated with increasing doses of exogenous EGF (10, 50, 100 and 200 ng/ml) for 30 minutes. As shown in **(Figure 3.8A)**, treatment with CTX (10 μg/ml) for 24 hours induced a decrease in both pAKT and pERK1/2 to 0.7 and 0.6, respectively **(Figure 3.8A)**. However, stimulation of CTX-treated MDA-MB-468 cells with increasing doses of exogenous EGF inhibited the effect of CTX and resulted in a dose-dependent increase of EGFR phosphorylation in both tyrosine 1173 and tyrosine 1045 residues **(Figure 3.8A)** as well as an increase in both pAKT and pEEK1/2 in a dose-dependent manner **(Figure 3.8A)**. Moreover, although CTX significantly inhibited the cell viability of MDA-MB-468 to 56.3±2.0 % (P<0.0 001), exogenous stimulation with both EGF and TGFα significantly reduced the effect of CTX on cell viability and increased the percentage of viable cells in a dose-dependent manner (P<0.05) **(Figure 3.8B)**. Collectively, these results indicated that the CTX effect might be inhibited by the release of its ligands, which counteracted its effect on the EGFR signalling pathway.

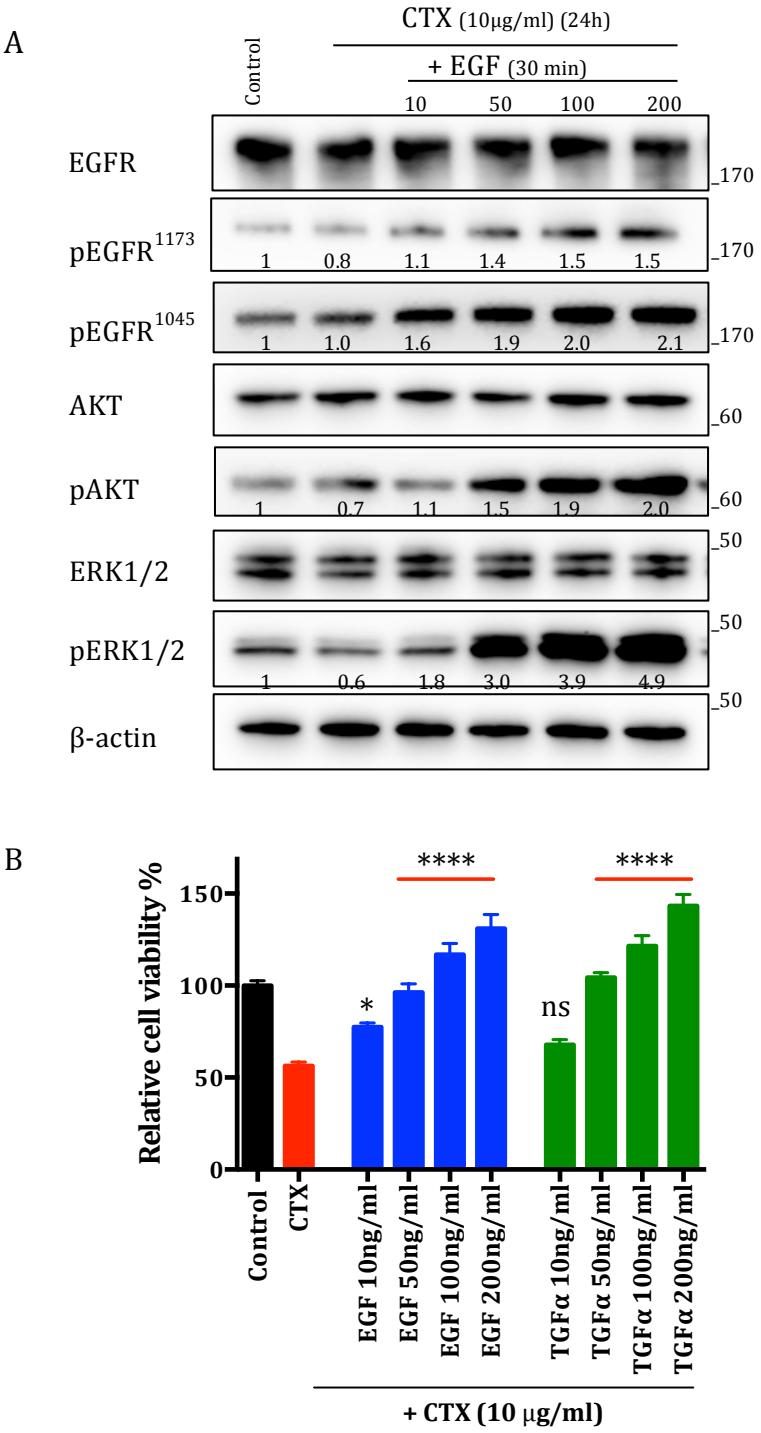


Figure 3.9 Effect of EGF on response to CTX-treatment.
(A) MDA-MB-468 cells were treated with CTX (10 µg/ml) for 24 hours and stimulated with the indicated concentrations of EGF for 30 minutes. Lysates were immunoblotted with the indicated antibodies. Quantifications are the average of three biological replicates. **(B)** Relative cell viability of MDA-MB-468 cells that were treated with CTX ± increasing concentrations of EGF or TGFα (10, 50, 100, 200 ng/ml) for 6 days was measured using CellTiter-Blue® (Promega) and calculated as relative to the control untreated cells and plotted as the mean±S.E. (n=3).

3.12 Discussion

Several studies support the fact that EGFR is highly expressed in some breast cancer tumours, particularly in basal-like and TNBC tumours and that its expression is associated with poor prognosis (Tsutsui *et al.* 2002; Nielsen *et al.* 2004; Meche *et al.* 2009; Zhang *et al.* 2013). Alterations in *EGFR* were identified in 39/825 (4.7%) breast cancer patients and in 17/81 (21%) basal-like breast cancer patients in the TCGA dataset (Cerami *et al.* 2012; Shah *et al.* 2012). In this chapter, the expression of *EGFR* in a publicly available cohort of 1988 breast cancer patients (METABRIC dataset) was analyzed (Curtis *et al.* 2012). Furthermore, this cohort was sub-classified into different categories based on ER, *PR* and *ErbB2* status as well as the PAM50 gene expression profiling as indicated in **(Section 3.2).** This analysis confirms the previous observation that *EGFR* is highly expressed in a subset of breast cancer patients and is significantly associated with adverse clinical outcome. Moreover, tumour *EGFR* expression is a potential independent prognostic marker from other clinicopathological features of breast cancer including age, size, node, stage and grade. However, these analyses were not statistically significant in TNBC and basal-like patients. This could be attributed to the number of TNBC and basal-like tumours being considerably fewer (n=276 and 330, respectively) than the total tumours in the cohort (n=1988).

Targeting EGFR using CTX has shown a poor response rate in TNBC patients. In TBCRC001 phase II clinical trial, response rate was 6% (2/31) and 17% (21/71) to CTX and CTX plus carboplatin, respectively (Carey *et al.* 2012). A parallel randomized phase II study revealed an increase in the overall response rate from 10% to 20% by adding CTX to cisplatin and prolonging the PFS from 1.5 to 3.7 months (Baselga *et al.* 2013). Collectively, their results indicated that CTX failed to completely inactivate the EGFR

signalling pathway despite the high *EGFR* expression in those patients. This could be attributed to the patient selection criteria and the lack of biomarkers that predicts response to CTX in TNBC. TNBC is a highly heterogeneous disease and understanding the molecular differences will help to identify the appropriate treatments. Recently, Lehmann *et al*. (2011) have identified 6 subtypes within TNBC tumours displaying distinct gene expression profiles. Furthermore, they aligned different TNBC cell lines as models of each subtype that have different sensitivities to targeted therapies (Lehmann *et al.* 2011). In this chapter, a panel of TNBC cell lines that contains at least one cell line from each subtype were used and their sensitivity to CTX treatment were investigated. These cell lines varied in their basal EGFR expression as well as their sensitivity to CTX treatment. In colorectal cancer, EGFR expression does not correlate with response to CTX (Cunningham *et al.* 2004; Saltz *et al.* 2004; Chung *et al.* 2005). Nevertheless, the amplification of *EGFR* gene predicts the response to CTX in colorectal cancer (Moroni *et al.* 2005; Personeni *et al.* 2008) as well as NSCLC (Hirsch *et al.* 2008). This study found that, although there is a positive correlation between basal EGFR expression and sensitivity to CTX in TNBC cell lines, this was not statistically significant. Moreover, both A431 and MDA-MB-468 cell lines that harbour *EGFR* amplification were the most sensitive cell lines to CTX within the investigated panel. However, further investigations with a larger panel of TNBC cell lines and/or patient-derived TNBC xenografts with variable CTX sensitivity is required to confirm the association between *EGFR* amplification and CTX response. This might be helpful to select a small subset of TNBC patients who could benefit from CTX in the clinical settings. In line with previous findings (Ferraro *et al.* 2013), current results also found that the HCC70 cell line is sensitive to CTX. This cell line expresses moderate level of EGFR without amplification. However, its sensitivity could be explained by the findings by Lehmann *et al*. (2011)

that the BL2 TNBC subtype, including HCC70, is enriched in EGF pathway. Brand *et al*. (2014) showed that although TNBC cell lines, which they have investigated, are resistant to CTX (treated with 100 nmol/L CTX for 72 hours) but are dependent on EGFR for their proliferation (Brand *et al.* 2014). Collectively, the current data indicated that multiple factors might contribute to CTX sensitivity and further investigations are required to find the molecular biomarkers that predict sensitivity to CTX treatment. A biomarker study of CTX with pre- and post-CTX treatment biopsies in TNBC will be particularly useful to identify further the factors that predict sensitivity and resistance to CTX-treatment.

CTX is a mAb that binds to EGFR domain III and prevents ligand-induced phosphorylation of EGFR with subsequent activation of the downstream pathways. It is also reported to hinder the exposure of the dimerization arm on domain II, thus preventing EGFR dimerization with other ErbB receptors (Sato *et al.* 1983; Li *et al.* 2005). Here, the effect of CTX on EGFR signalling pathway was evaluated in the CTX-sensitive cell line, MDA-MB-468, after treatment CTX-treatment for 1, 24 and 72 hours. Current results indicated that CTX induced only a minimal inhibition of EGFR phosphorylation at tyrosine residue 1173. However, there was a higher decrease in EGFR phosphorylation at tyrosine residue 1045 after 72 hours. An earlier time point (30 minutes) has also been evaluated and did not show an effect of CTX on EGFR phosphorylation (data not shown). However, earlier time points (less than 30 minutes) have not been investigated. On the other hand, there was an immediate de-phosphorylation of the downstream AKT and ERK1/2 within 1 hour. This is in line with the previously unexplained phenomenon associated with CTX-treatment (Mandic *et al.* 2006; Song *et al.* 2009). Mandic, R. *et al*. (2006) have found an increase in EGFR

phosphorylation (tyrosine 1173) induced by CTX-treatment in HNSCC cell lines for 13 hours in low-serum medium. However, there was a decrease in ERK1/2 phosphorylation (Mandic *et al.* 2006). Moreover, Song, J.Y. *et al*. (2009) have shown that CTX induced an initial decrease in ERK1/2 phosphorylation with subsequent reactivation after 4 hours in A431 cells (Song *et al.* 2009). One of the possible explanations is that CTX induces nuclear translocation of EGFR (Liao *et al.* 2009) and therefore, the decrease in EGFR phosphorylation on the cell membrane could not be detected in the total cell lysate. Another explanation is that EGFR phosphorylation is maintained by the robust recycling of EGFR, which maintains the total and phosphorylated EGFR unchanged. There is also a possibility that the minor change in EGFR phosphorylation cannot be detected by western blotting. Therefore, more sensitive assays such as ELISA or mass spectrometry might detect this minor change. Additionally, there might be other off targets of CTX that are affected by treatment. In comparison to untreated cells, CTX did not induce a de-phosphorylation of EGFR. However, pre-treatment of EGF-stimulated cells with CTX induced a decrease in pEGFR, pAKT and pERK1/2 compared to EGF-stimulated cells **(Figure 3.6B)**. This indicates that CTX is able to block EGFR signalling pathway. As previously mentioned, CTX prevents the formation of heterodimers between EGFR and other ErbB receptors (Li *et al.* 2005). As shown in **(Figure 3.7)**, CTX treatment reduced Her3 phosphorylation within the first hour with subsequent reactivation. As EGFR lacks PI3K docking sites and the activation of PI3k/AKT pathway occurs through EGFR-Her3 dimerization (Carpenter *et al.* 1993; Yarden *et al.* 2001), the decrease in AKT phosphorylation could be mediated by the decrease in Her3 phosphorylation. Recently, it has been shown that combining CTX with MM-121/SAR256212, an anti-HER3 mAb, enhanced the response of HNSCC cell lines to CTX and exerted a more potent inhibition of EGFR and the downstream AKT and ERK

phosphorylation (Jiang *et al.* 2014). Therefore, the role of Her3 in mediating response and resistance to CTX could be investigated in the future to elucidate this effect of CTX on the downstream AKT and ERK phosphorylation without affecting EGFR phosphorylation.

Interestingly, it was found in this study that CTX treatment triggers the release of endogenous EGFR ligands, EGF and TGFα, in both CTX-sensitive and –resistant cell lines. However, ligand release was higher in the sensitive cell line compared to resistant cells. This supports the previous findings in which the level of EGFR ligands EREG and AREG in tumours correlates with sensitivity to CTX in metastatic KRAS wild-type colorectal cancer patients (Jacobs *et al.* 2009). It has been reported that CTX has a higher affinity for EGFR than its ligands, EGF and TGFα (Kawamoto *et al.* 1983; Gill *et al.* 1984). Nevertheless, I found that stimulation of CTX-treated cells with exogenous ligands counteracted the effect of CTX on the sensitive cells and inhibited the de-phosphorylation of both AKT and ERK1/2 induced by CTX. The increase in plasma AREG and EGF was associated with worse clinical outcomes in mCRC (Loupakis *et al.* 2014), which could be explained by the increase in ADAM17 metalloprotease shedding activity that would decrease EGFR ligands in tissue and increase them in blood plasma (Van Schaeybroeck *et al.* 2011). Moreover, it has been found that both TGFα and HB-EGF are significantly secreted by the acquired CTX-resistant GEO cells compared o their parental GEO cells. However, silencing of TGFα but not HB-EGF in the acquired CTX-resistant GEO cells restored their sensitivity to CTX (Troiani *et al.* 2013). Collectively, these findings indicated that the effect of CTX on EGFR phosphorylation is complex due to compensatory feedback mechanisms and the induction of several parallel processes simultaneously. Furthermore, the current results highlighted the role of EGFR ligands

in predicting the response to CTX and their involvement in CTX resistance. Therefore, inhibiting their release might be an approach to increase CTX-sensitivity.

4 ADAM10 and 17 inhibition decreases the release of EGFR ligands and enhances the *in vitro* and *in vivo* response to cetuximab in TNBC

4.1 Introduction

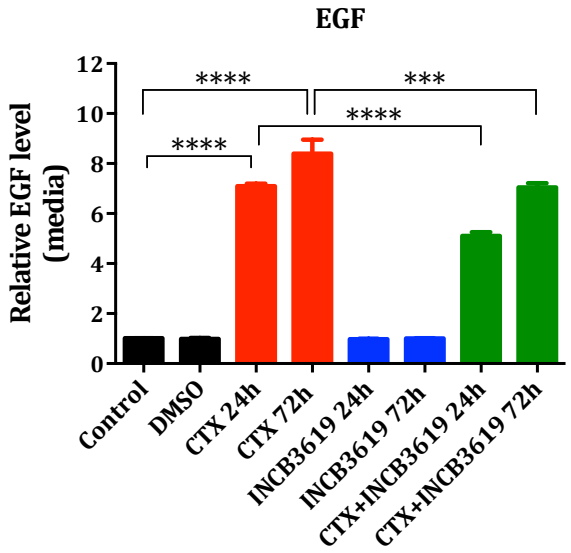
The release of active EGFR ligands is mediated by different metalloproteases. Both ADAM10 and ADAM17 are involved in the release of active EGFR ligands and cancer progression (Kataoka 2009). Emerging evidence suggested the involvement of ADAM17 in breast cancer progression. Analysis of ADAM17 protein level in 153 breast cancer patients revealed that high levels were associated with higher tumour grade (grade 3) and poor OS (McGowan *et al.* 2008). Moreover, ADAM17 expression was significantly higher in TNBC compared to non-TNBC tumours. Pre-treatment with an ADAM17 inhibitor enhanced the response to different therapies including the pan-Her inhibitor, neratinib (McGowan *et al.* 2013). Furthermore, Kenny, P.A. and Bissell, M.J. (2007) reported that inhibition of ADAM17 activity or knockdown of ADAM17 reverted breast cancer cell line malignancy mainly by preventing the release of EGFR ligands, EGF and AREG (Kenny *et al.* 2007).

In the previous chapter, I showed that CTX triggers the release of endogenous EGFR ligands such as EGF and TGFα in both CTX sensitive and resistant cell lines. Moreover, stimulating the sensitive cells with exogenous ligands inhibited the anti-proliferative effect of CTX. Therefore, this chapter aims to investigate the effect of combining ADAM10/17 inhibition using a dual ADAM10/17 inhibitor, INCB3619, and CTX in TNBC cell lines both *in vitro* and *in vivo*.

4.2 Inhibition of ADAM10 and ADAM17 activities reduces CTX-induced ligand release

As shown in the previous chapter **(Section 3.9 and Figure 3.7)**, CTX induces the release of EGFR ligands. CTX treatment significantly increased the level of both EGF and TGFα in the media by 6.0±0.3-fold (P<0.0001) and 15.48±2.8-fold (P<0.0001) after 24 hours and by 7.4±0.3-fold (P<0.0001) and 22.16±2.8-fold after 72 hours (P<0.0001) compared to untreated control cells **(Figure 4.1A and B)**. However, combining CTX and INCB3619 significantly reduced EGF level in the media by 1.98±0.3-fold (P<0.0001) and by 1.3±0.3-fold (P<0.001) after 24 and 72 hours, respectively, compared to CTX alone **(Figure 4.1A)**. Moreover, this combination also reduced the relative TGFα in the media by 8.1±2.8-fold (P=0.069) after 24 hours and by 9.5±2.8-fold (P=0.016) after 72 hours compared to CTX alone **(Figure 4.1B)**.

A



B

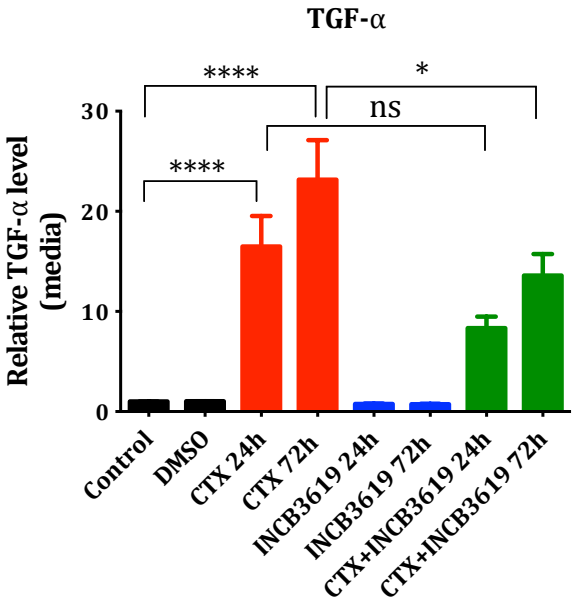


Figure 4.1 Inhibition of ADAM10 and ADAM17 reduce CTX-induced increase of EGFR ligands in the media.

The relative levels of **(A)** EGF and **(B)** TGFα in the media in MDA-MB-468 cells that were treated with CTX (10 μg/ml), INCB3619 (5 nM), or their combination for 24 and 72 hours. Relative values are expressed as means±S.E. (One-way ANOVA with Bonferroni's multiple comparison posttests, n=3).

4.3 Effect of ADAM10/17 inhibition on EGFR signalling pathway in CTX-treated cells

Treatment of MDA-MB-468 cell line with CTX failed to block EGFR signalling pathway as shown in **(Section 3.8).** This could be due to the significant release of EGFR ligands induced by CTX resulting in EGFR activation **(Section 3.9 and 4.2).** Therefore, I investigated the effect of combining CTX with INCB3619 on EGFR phosphorylation. As shown in **(Figure 4.2)**, co-treatment with INCB3619 induced a minor decrease in EGFR phosphorylation at tyrosine 1173 from 0.9 to 0.7 and at tyrosine 1045 from 0.8 to 0.5 after 24 hours treatment compared to CTX only. Additionally, the combined treatment enhanced the inhibition of pAKT after 1 hour (fraction relative to control = 0.2) and after 24 hours treatment (fraction relative to control = 0.3) compared to 0.7 and 0.8 for single CTX treatment, respectively **(Figure 4.2)**. Moreover, while CTX-treatment reduced pERK1/2 to arbitrary values of 0.2 and 0.6 after 1 and 24 hours treatment respectively, combined CTX and INCB3619 treatment further reduced pERK1/2 to 0.07 and 0.3 after 1 hour and 24 hours of treatment, respectively **(Figure 4.2)**. Since both ADAM10 and ADAM17 regulate different signalling pathways, therefore the additive effect of INCB3619 due to an inhibition of other signalling pathway(s) could not be excluded.

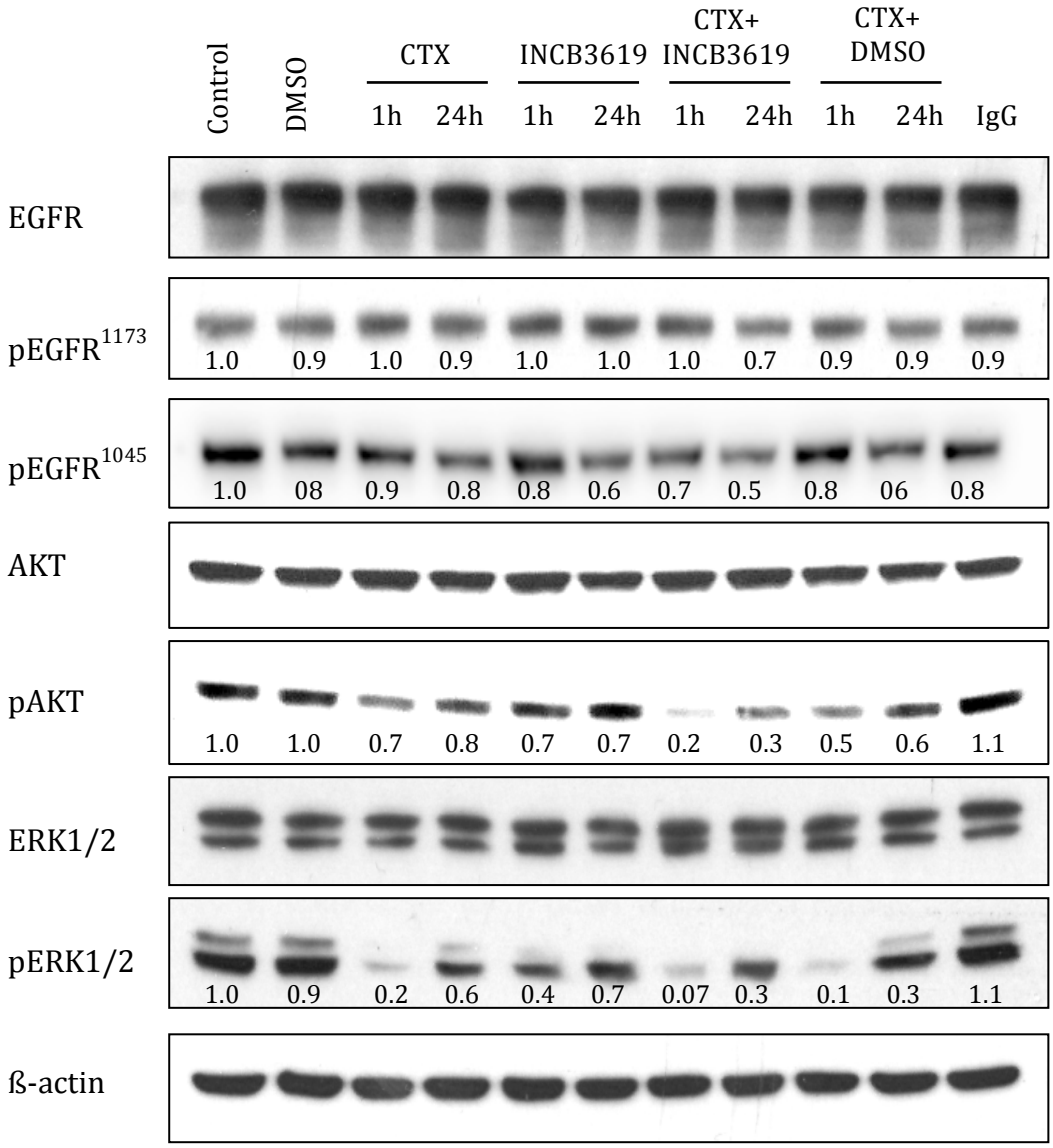


Figure 4.2 Effect of CTX and INCB3619 combination on EGFR signalling.
MDA-MB-468 cells were treated with CTX (10 µg/ml), INCB3619 (5 nM), or their combination for 1 and 24 hours. Lysates were immunoblotted with the indicated antibodies. Quantifications are the average of three independent experiments.

4.4 Anti-tumour effect of combining ADAM10/17 inhibition and CTX treatment in TNBC cell lines *in vitro*

4.4.1 CTX and INCB3619 combination reduced cell viability

The effect of CTX (red), INCB3619 (blue) and their combination (green) on cell viability was further investigated in CTX- primary sensitive (MDA-MB-468, HCC70 and A431), and primary resistant (MDA-MB-231) cell lines and also in cells with acquired CTX-resistance (MDA-MB-468CR). In MDA-MB-468 cell line, while CTX alone significantly decreased the percentage of viable cells to 67.5±3.5% (P<0.0001) and INCB3619 alone to 82.4±2.3% (P<0.001), their combination significantly reduced the percentage of viable cells to 52.4±0.9% (P<0.0001). The differences between CTX alone or INCB3619 alone with their combination were also significant (P<0.01 and P<0.0001, receptively) **(Figure 4.3A)**. A similar result has been found in A431 cell line in which the combination of CTX and INCB3619 induced a significant reduction in the percentage of viable cells compared to CTX or INCB3619 alone (P<0.01 and P<0.0001, receptively) **(Figure 4.3B)**. On the other hand, although HCC70 cell line was sensitive to both CTX (P<0.0001) and INCB3619 (P<0.0001), their combination did not induce further statistically significant reduction in cell viability despite a trend towards reduction **(Figure 4.3C)**. Moreover, neither CTX or INCB3619 monotherapy nor their combination affected the viability of primary CTX-resistant MDA-MB-231 cell line **(Figure 4.3D)**. Finally, I investigated the effect of combining CTX and INCB3619 on the acquired CTX-resistant cell line, MDA-MB-468CR, which has been derived from the primary sensitive MDA-MB-468 cell line by continuous exposure to CTX **(Detailed in Chapter 5)**. Interestingly, while CTX and INCB3619 reduced the percentage of viable cells by 2.8 and

3.8% compared to untreated cells, their combination significantly reduced the percentage of viable cells by 27.1±4.5% (P<0.0001) compared to untreated control cells **(Figure 4.3E).**

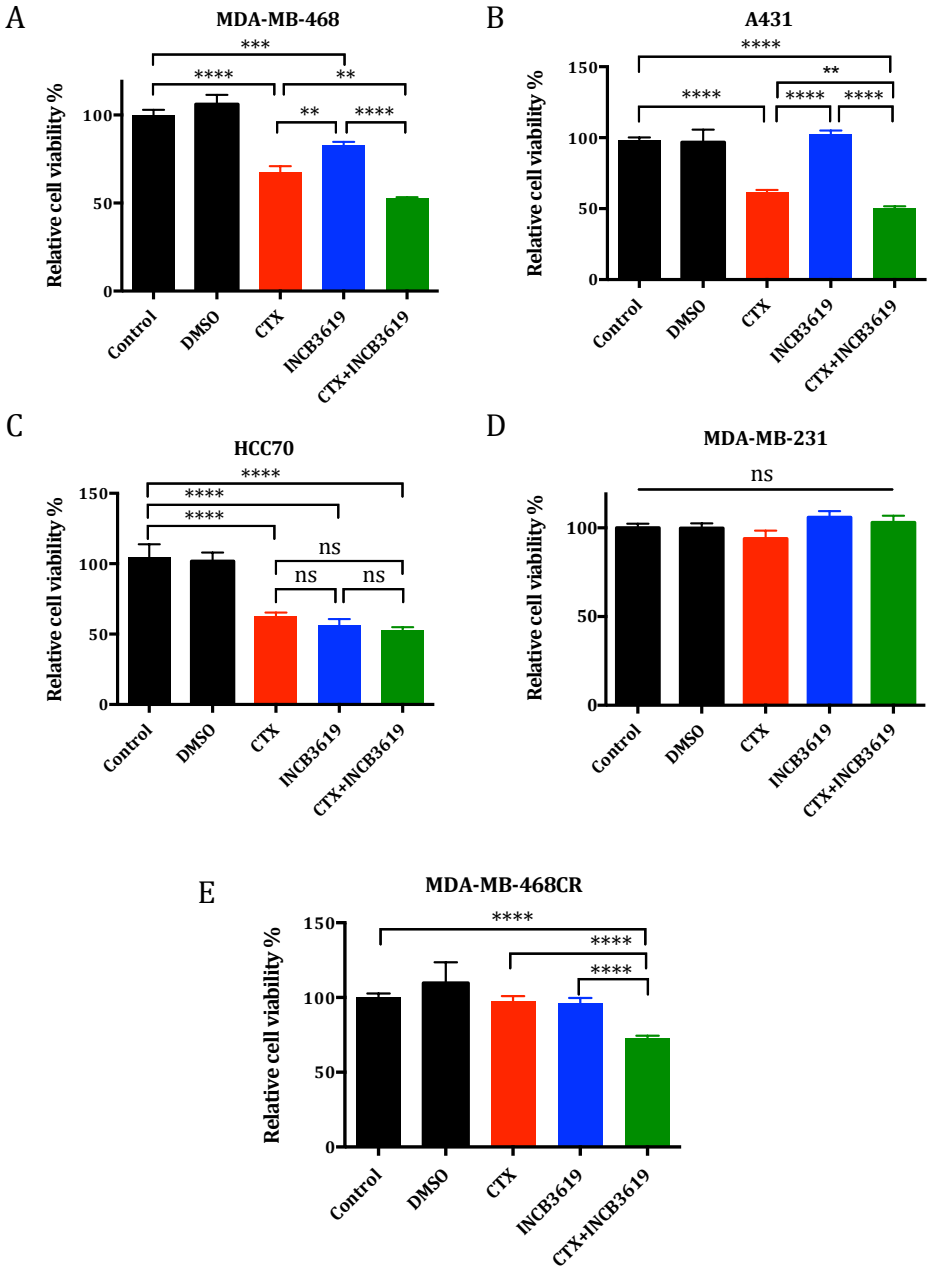


Figure 4.3 Effect of CTX and INCB3619 combination on cell viability of TNBC and A431 cell lines.

(A) MDA-MB-468, **(B)** A431, **(C)** HCC70, **(D)** MDA-MB-231 and **(E)** MDA-MB-468CR cell lines were treated with CTX (10 µg/ml), INCB3619 (5 nM), or their combination for 6 days. Relative cell viability was measured using CellTiter-Blue® (Promega) and calculated as relative to the control untreated cells and plotted as the mean±S.E (n=3).

4.4.2 CTX and INCB3619 combination inhibits cell proliferation

Clonogenic assays were used to determine the effect of CTX (red), INCB3619 (blue) or their combination (green) on cell proliferation of the three cell lines that showed a significant reduction of cell viability by the combination of both drugs: (a) MDA-MB-468, (b) A431 and (c) MDA-MB-468CR cell lines.

In accordance with cell viability, combined treatment with CTX and INCB3619 significantly reduced the colonogenicity of MDA-MB-468 cells by 22.1±5.7% (P<0.01) and by 28.4±5.7% (P<0.001) compared to CTX and INCB3619, respectively **(Figure 4.4A and D)**. Similarly, there was a significant inhibition in the proliferation of A431 cells with combined treatment compared to CTX alone (P<0.05) or INCB3619 alone (P<0.001) **(Figure 4.4B and E)** and the proliferation of MDA-MB-468CR cells compared to CTX alone (P<0.0001) or INCB3619 alone (P<0.001) **(Figure 4.4C and F).**

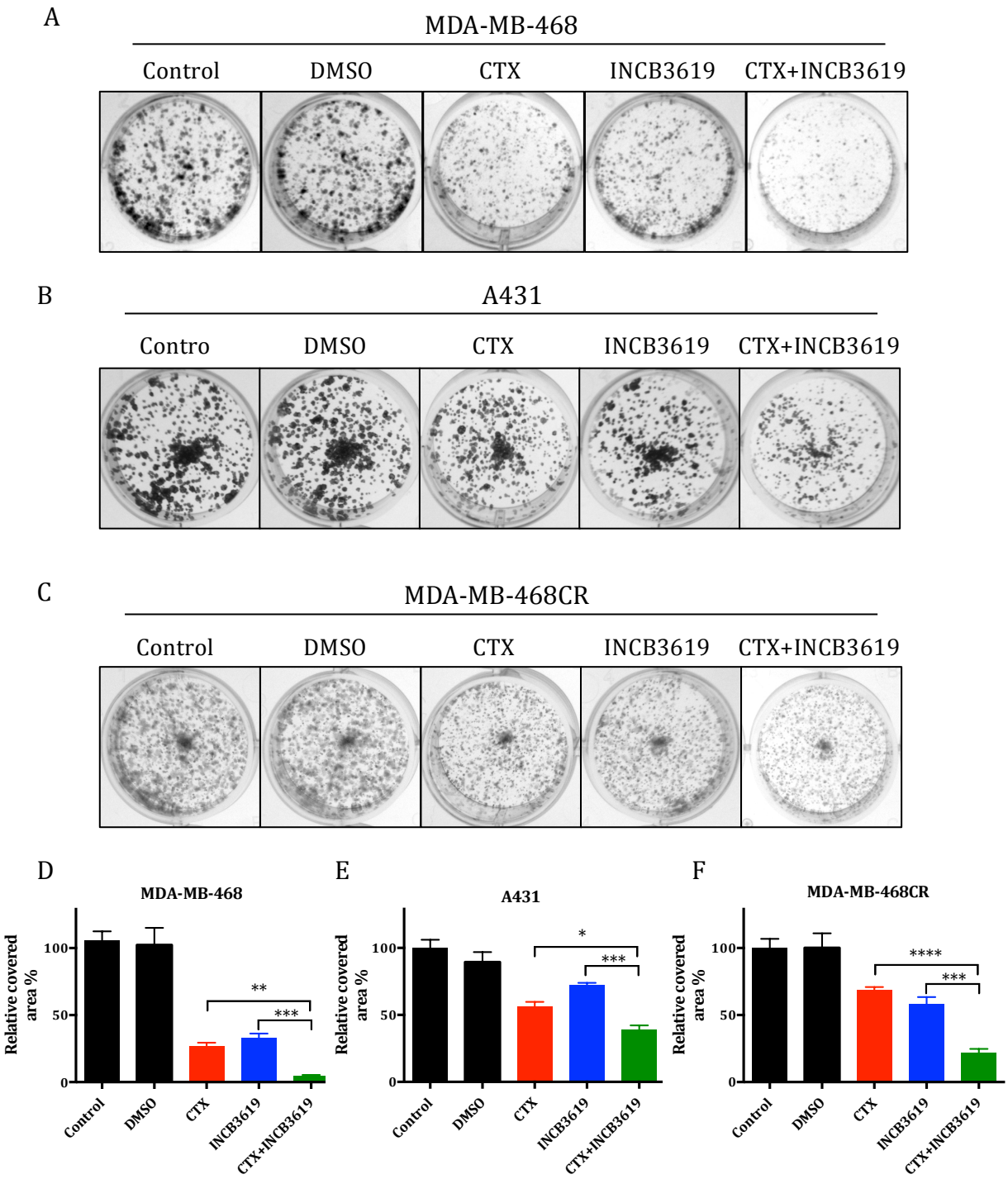


Figure 4.4 Effect of CTX and INCB3619 combination on cell proliferation.
Representative images of colonies of **(A)** MDA-MB-468, **(B)** A431 and **(C)** MDA-MB-468CR, which were treated with CTX (10 µg/ml), INCB3619 (5 nM), or their combination for 12 days. **(D, E, F)** Quantification of the covered area of (A, B, C, respectively) relative to their untreated control cells were plotted the means±S.E (n=3).

4.4.3 CTX and INCB3619 combination induces apoptosis

Activation of effector caspases such as caspase 3 and caspase 7 induced cell apoptosis (Kurokawa *et al.* 2009). In MDA-MB-468 cells, treatment with CTX (10 µg/ml) or INCB3619 (5 nM) did not induce a significant increase in caspase 3 and 7 activities. However, their combination significantly increased their activities by 60.7±13.5% (P<0.01) and by 45.5±13.5% (P<0.5) compared to untreated control and CTX-treated cells, respectively **(Figure 4.5)**.

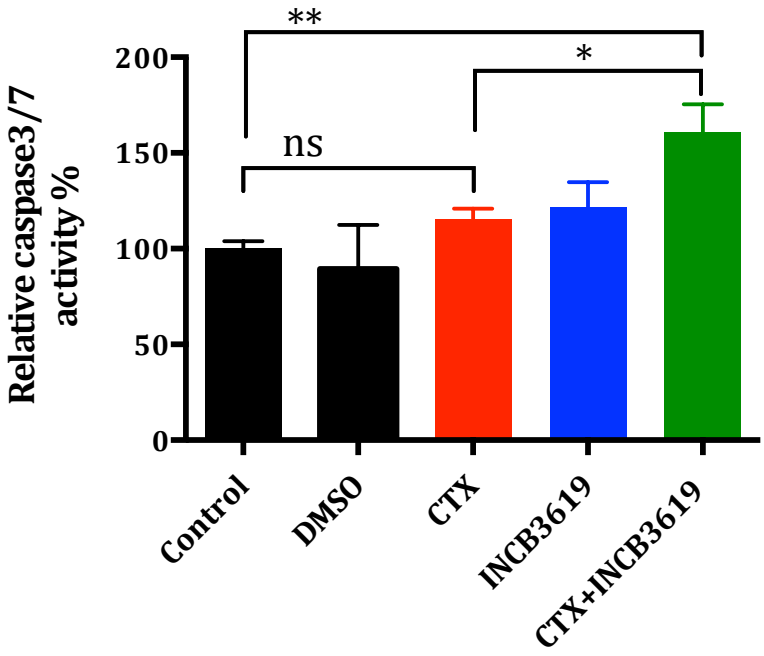


Figure 4.5 Effect of CTX and INCB3619 combination on caspase 3/7 activities.
MDA-MB-468 cells were treated with CTX (10 µg/ml), INCB3619 (5 nM), or their combination for 24 hours and caspase 3/7 activities were assessed using Caspase-Glo® 3/7 Assay (Promega) as described in **(Section 2.2.13)**. Caspase 3/7 activities were calculated as percentage mean±S.E relative to untreated control cells (n=3).

4.4.4 CTX and INCB3619 combination induces G1 arrest and reduced cyclin D1 expression

Propidium iodide (PI) staining was used to assess the stages of the cell cycle in MDA-MB-468 cells treated with CTX (10 μg/ml), INCB3619 (5 nM) or their combination for 72 hours. As shown in **(Figure 4.6)**, neither CTX nor INCB3619 induced G1 arrest compared to untreated control cells. Nevertheless, combining both CTX and INCB3619 induced a significant G1 arrest compared to untreated control cells ($P<0.01$) and CTX-treated cells ($P<0.05$). However, the G1 arrest induced by both drugs was not statistically significant compared to INCB3619-treated cells.

Cell cycle is regulated by cyclins and cyclin-dependent kinases (Nigg 1995). Cyclin D1 is required for the G1/S transition (Sherr 1993). Here, I found that the G1 arrest induced by combining CTX and INCB3619 in MDA-MB-468 cell line was associated with a significant decrease in cyclin D1 (*CCND1*) expression mRNA level compared to untreated control cells or CTX and INCB3619 single treatments ($P<0.05$) **(Figure 4.6B)**. On the other hand, neither CTX nor INCB3619 affected cyclin D1 mRNA expression **(Figure 4.6B)**.

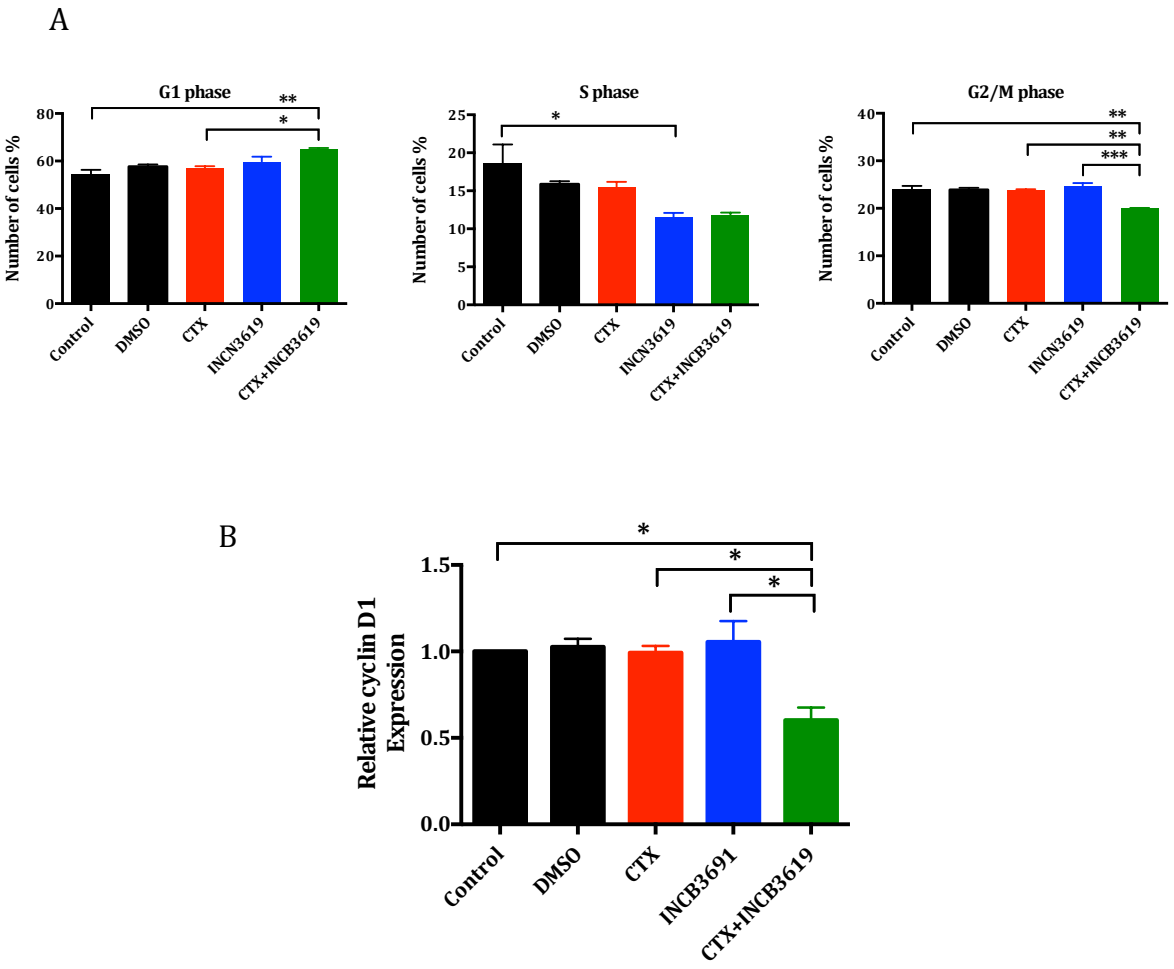


Figure 4.6 Effect of CTX and INCB3619 combination on cell cycle.
MDA-MB-468 cells were treated with the indicated drugs for 72 hours and were **(A)** stained with PI and analyzed using CyAn FACS analyzer (mean±S.E, n=3) or **(B)** RNA was extracted and cyclin D1 expression was analyzed by RT-qPCR (means±S.E, n=3).

4.5 The inhibition of ADAM10/17 enhances anti-tumour effect of CTX in TNBC xenografts

As shown above **(Section 4.4)**, the inhibition of ADAM10/17 using INCB3619 increased the response of MDA-MB-468, A431 and MDA-MB-468CR cells to CTX-treatment *in vitro*. Therefore, the anti-tumour effect of this combination was further investigated *in vivo.* MDA-MB-468 xenograft model was used to validate the *in vitro* data since it is frequently used as a TNBC xenograft model (MacDiarmid *et al.* 2007; Bartholomeusz *et al.* 2010; Jung *et al.* 2011).

MDA-MB-468 cells were injected subcutaneously at the mammary fat pad of 32 nude mice. Prior to treatment, two mice were euthanized because of animal welfare considerations. In addition, 10 of the mice had very small or no tumour. The remaining 20 mice were randomized into four groups; control (vehicle and PBS), CTX, INCB3619 or CTX plus INCB3619 as described in **(Table 2.4)**. CTX was injected at 50 mg/kg body weight (IP) as described previously (Jung *et al.* 2011) and INCB3619 at 30 mg/kg body weight/day using ALZET osmotic pumps (Zhou *et al.* 2006). The total treatment continued for 21 days only as the compatible osmotic pump with mice body weight was suitable for only 21 days of treatment.

4.5.1 Tumour growth

Tumour growth was monitored and tumour volume was measured 3 times weekly as described in **(Section 2.2.15)**. As indicated in **(Figure 4.7A and B)**, CTX alone induced tumour regression in MDA-MB-468 xenograft mice. However, this regression was not statistically significant compared to the tumours from control mice (P=0.26). Moreover,

INCB3619 alone did not induce a noticeable change in tumour growth relative to control tumours. However, the combination of both CTX and INCB3619 significantly induced tumour regression compared to the control group (P=0.008) and INCB3619-treated group (P=0.04) but not CTX-only group **(Figure 4.7A and B)**. The tumour regression in both CTX and combination groups were correlated with the final tumour volumes in which the tumour volume in the combination group was significantly smaller than that of control group (P=0.004) and INCB3619 group (P=0.01) but not CTX-treated group **(Figure 4.7C)**. These *in vivo* results support the *in vitro* data and indicated that combining CTX and INCB3619 is superior to CTX alone at least in CTX sensitive cells.

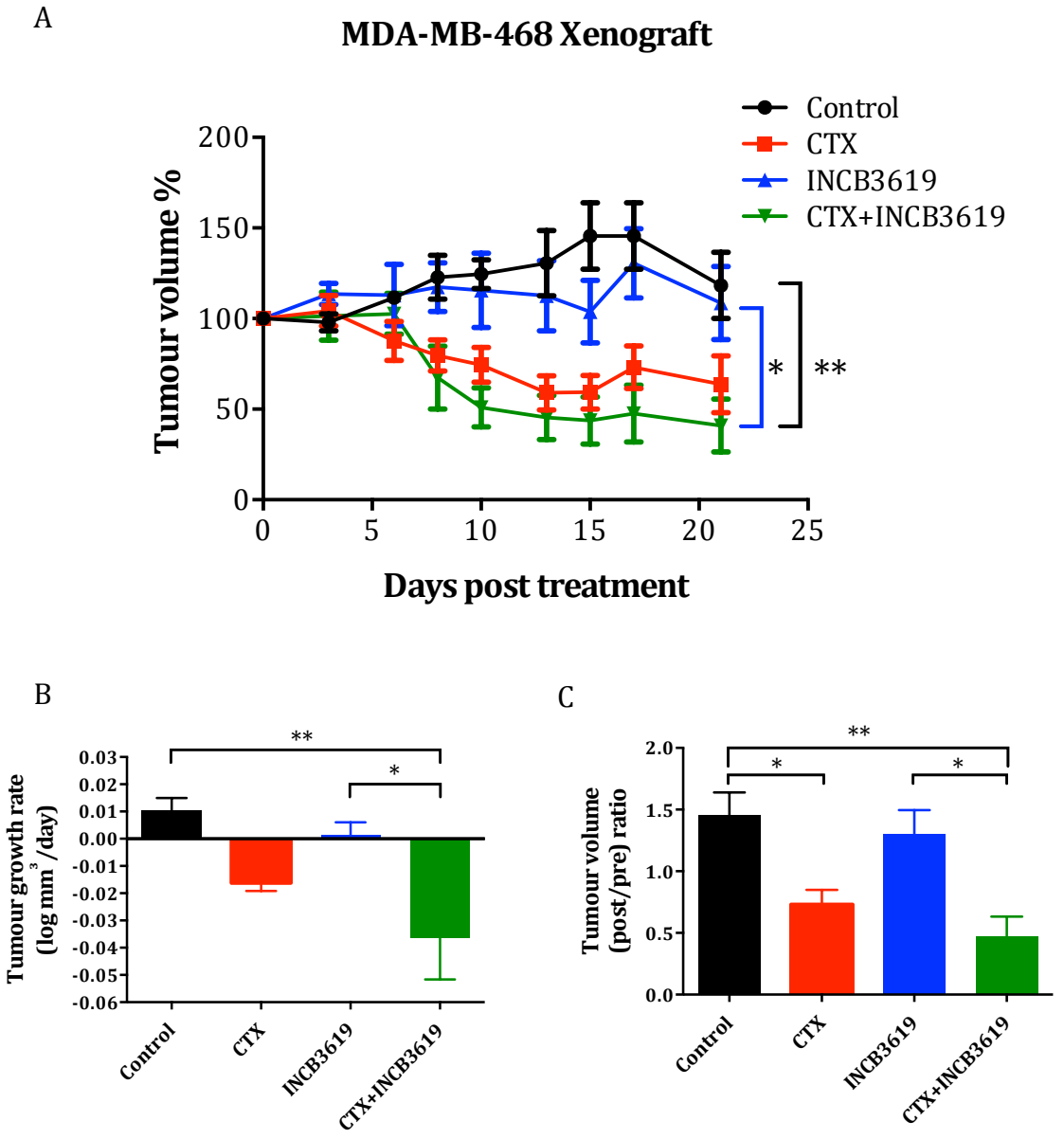


Figure 4.7 The *in vivo* effect of CTX and INCB3619 combination on MDA-MB-468 tumour growth.

MDA-MB-468 xenograft was established in BALB/c nude mice. After reaching the appropriate tumour volume, mice were randomized into 4 groups (n=5) and treated with vehicle, CTX (50 mg/kg, IP), INCB3619 (30 mg/kg, subcutaneous), or their combination for 21 days. **(A)** Tumour size was measured in each mouse and the tumour volume was normalized, calculated and plotted as relative to the initial tumour volume (Mean±S.E) **(B)** Statistical analysis of the tumour growth using non-linear fit **(C)** An illustration of the ratios of post-treatment to pre-treatment tumour volume of each group. Mean differences between the groups were analyzed by ANOVA with Bonferroni's multiple comparison test.

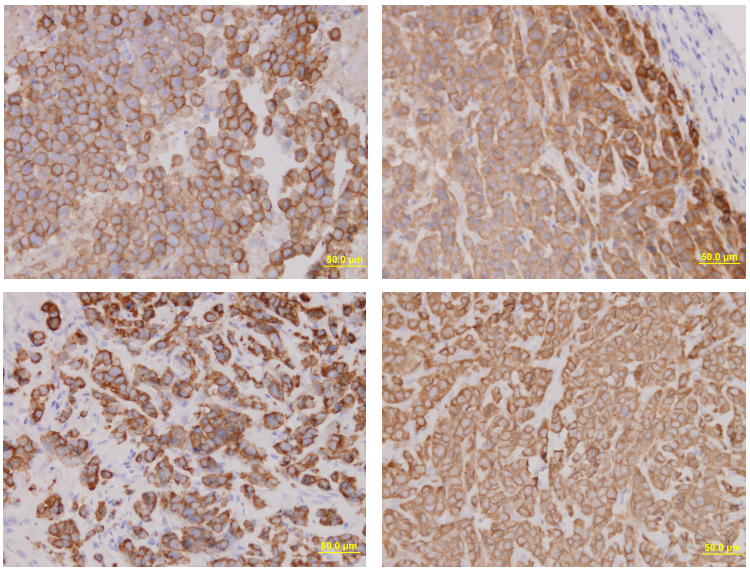
4.5.2 Molecular biomarkers

Tumours were collected from the euthanized mice and paraffin-embedded before using IHC to stain the following molecular markers: EGFR, pEGFR, pAKT, pERK, Ki-67 (proliferation marker), cleaved caspase3 (apoptotic marker). The percentage of necrotic areas in the tumours was also measured. Representative images of IHC staining of these biomarkers are shown in **(Figure 4.8)**.

The combination of both CTX and INCB3619 resulted in a significant decrease in total EGFR protein compared to the control ($P<0.05$) and CTX-treated ($P<0.01$) groups **(Figure 4.9A)**. However, there was no significant difference in pEGFR protein expression among the different treated groups **(Figure 4.9B)**. Moreover, none of the investigated biomarkers as well as the necrotic areas showed a significant difference between different groups **(Figure 4.9C-G)**. Interestingly, the cells expressed various levels of all these biomarkers within each tumour. Johansson, A *et al*. 2012 has reported a modulation of CTX sensitivity was mediated by CAF-derived soluble factors (Johansson *et al.* 2012). Here, it was shown that there was an increase in the cancer-associated fibroblast (CAF) to tumour ratio in CTX-treated tumours. However, this was not statistically significant **(Figure 4.9H)**.

A

EGFR

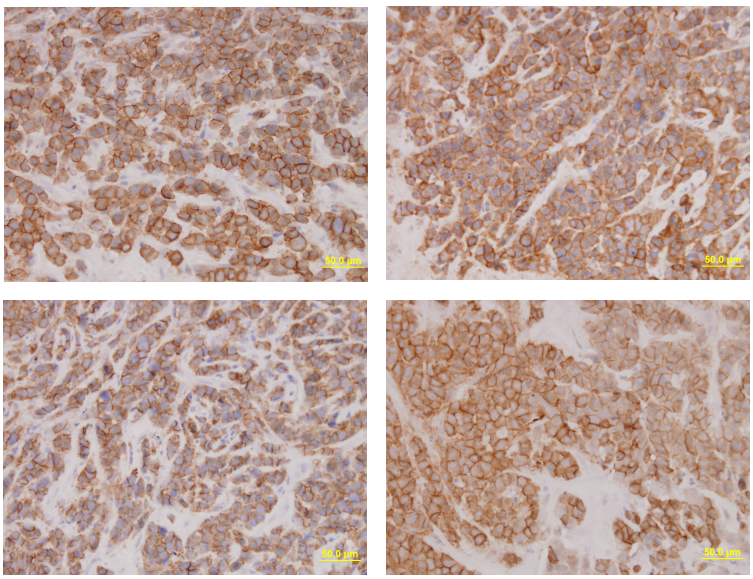


Control **INCB3619**

CTX **CTX+INCB3619**

B

pEGFR^{1173}



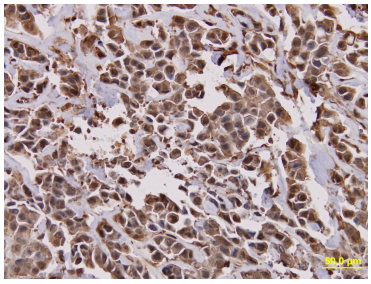
Control **INCB3619**

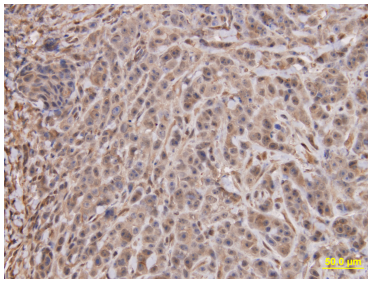
CTX **CTX+INCB3619**

C

AKT

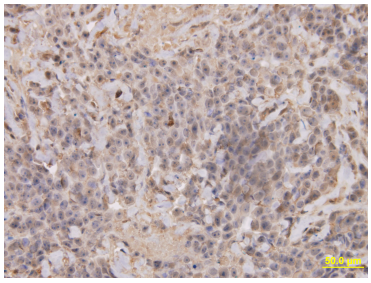
Control

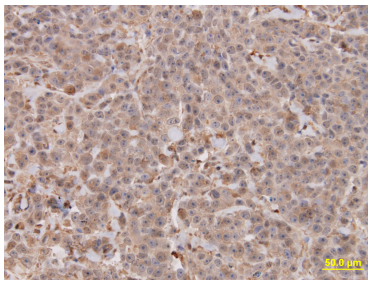




INCB3619

CTX



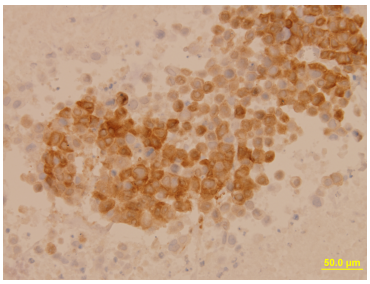


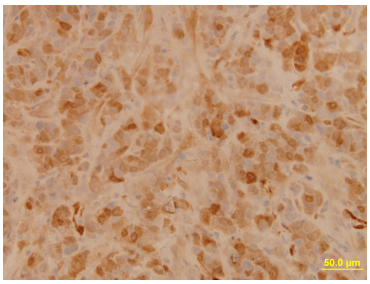
CTX+INCB3619

D

pAKT

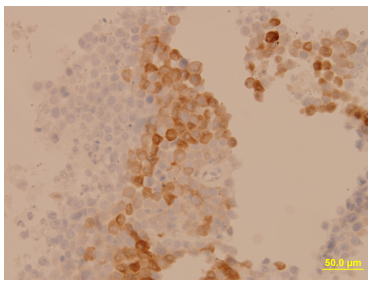
Control

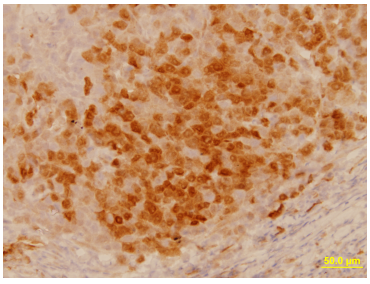




INCB3619

CTX

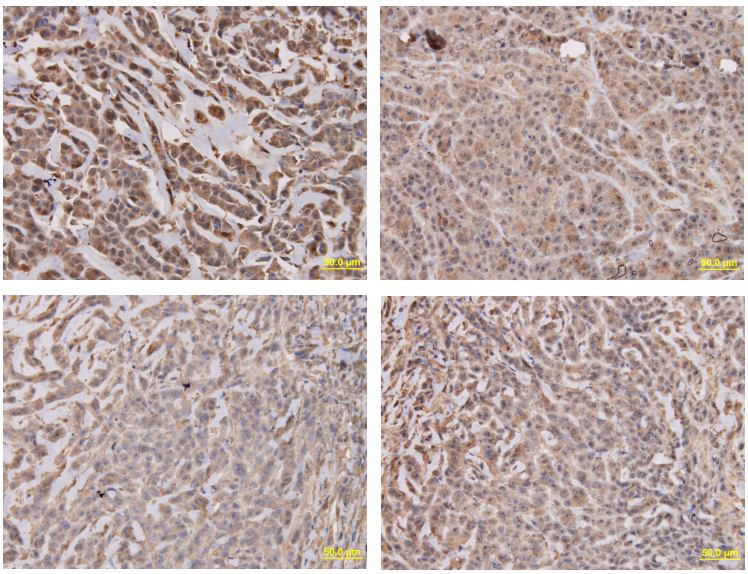




CTX+INCB3619

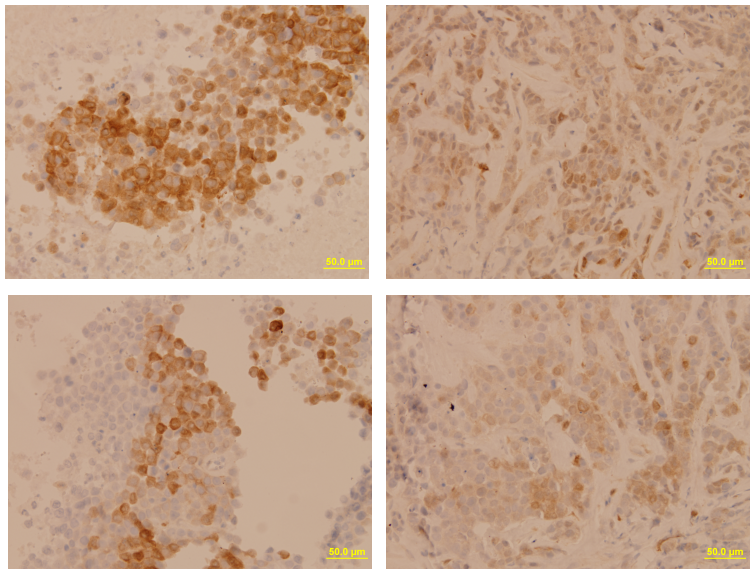
E

ERK1/2



F

pERK1/2



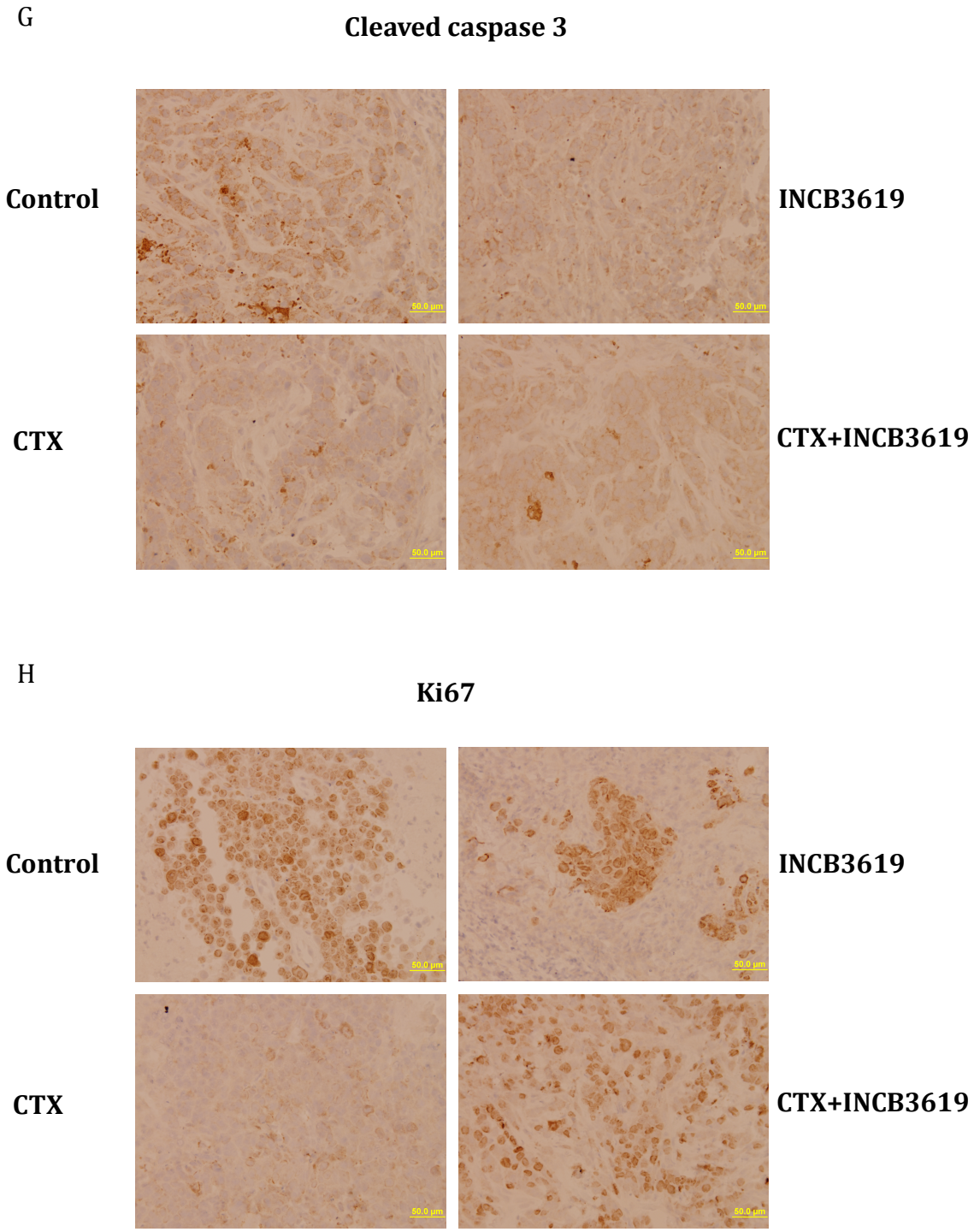
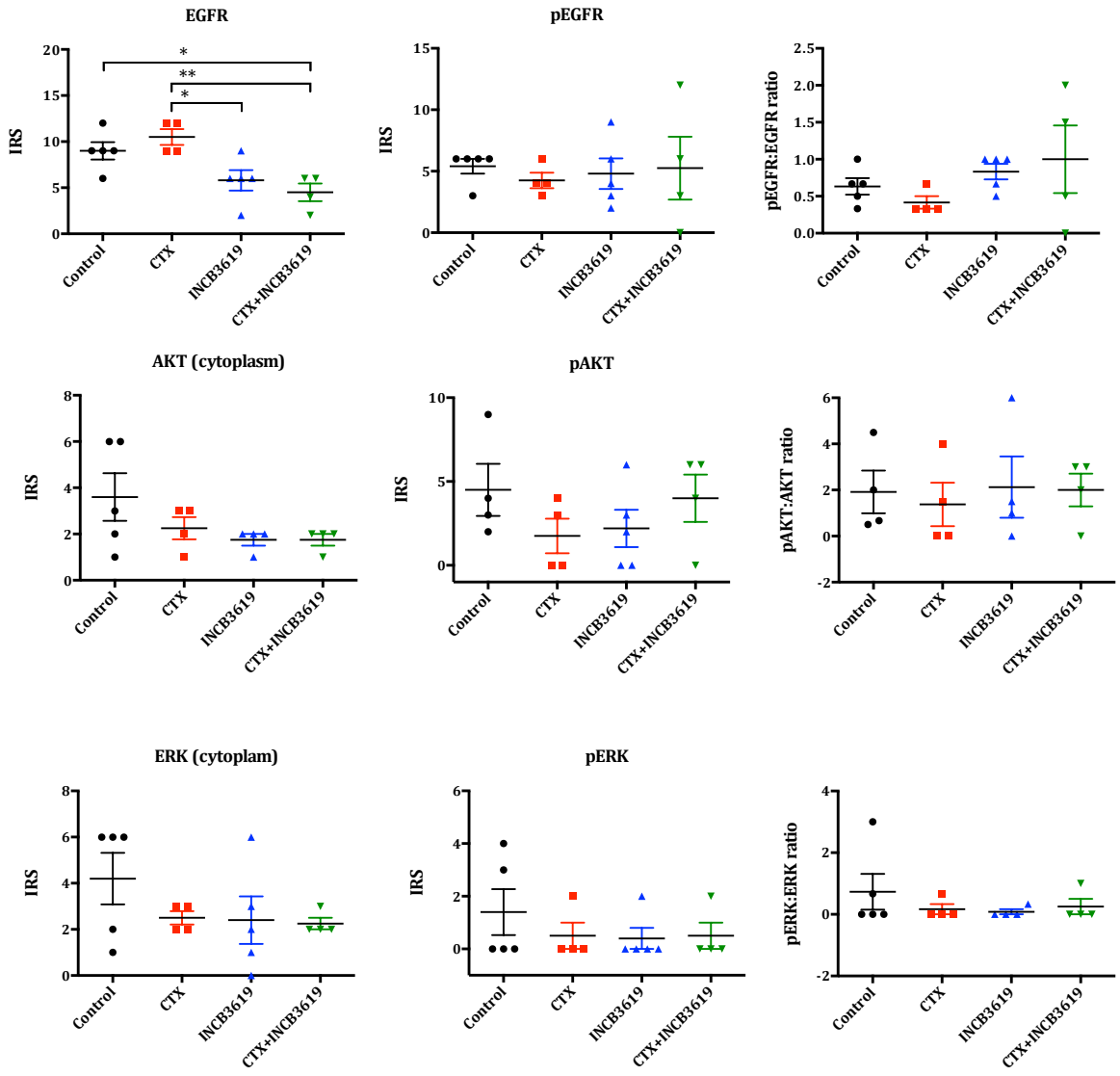


Figure 4.8 Representative images of IHC staining of the *in vivo* xenografts.
Mice were treated with the indicated drugs. Mouse xenograft tumours were sectioned into 4 μm slides. IHC staining was performed to detect **(A)** EGFR, **(B)** pEGFR^{Y1173}, **(C)** AKT, **(D)** pAKT, **(E)** ERK1/2, **(F)** pERK1/2, **(G)** cleaved caspase-3 and **(H)** Ki67. Representative images of each treatment group were shown.


EGFR
IRS
20
15
10
5
0
*
**
*
Control
CTX
INCB3619
CTX+INCB3619
pEGFR
IRS
15
10
5
0
Control
CTX
INCB3619
CTX+INCB3619
pEGFR:EGFR ratio
2.5
2.0
1.5
1.0
0.5
0.0
Control
CTX
INCB3619
CTX+INCB3619
AKT (cytoplasm)
IRS
8
6
4
2
0
Control
CTX
INCB3619
CTX+INCB3619
pAKT
IRS
10
5
0
Control
CTX
INCB3619
CTX+INCB3619
pAKT:AKT ratio
6
4
2
0
-2
Control
CTX
INCB3619
CTX+INCB3619
ERK (cytoplam)
IRS
8
6
4
2
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Control
CTX
INCB3619
CTX+INCB3619
pERK
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pERK:ERK ratio
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CTX+INCB3619

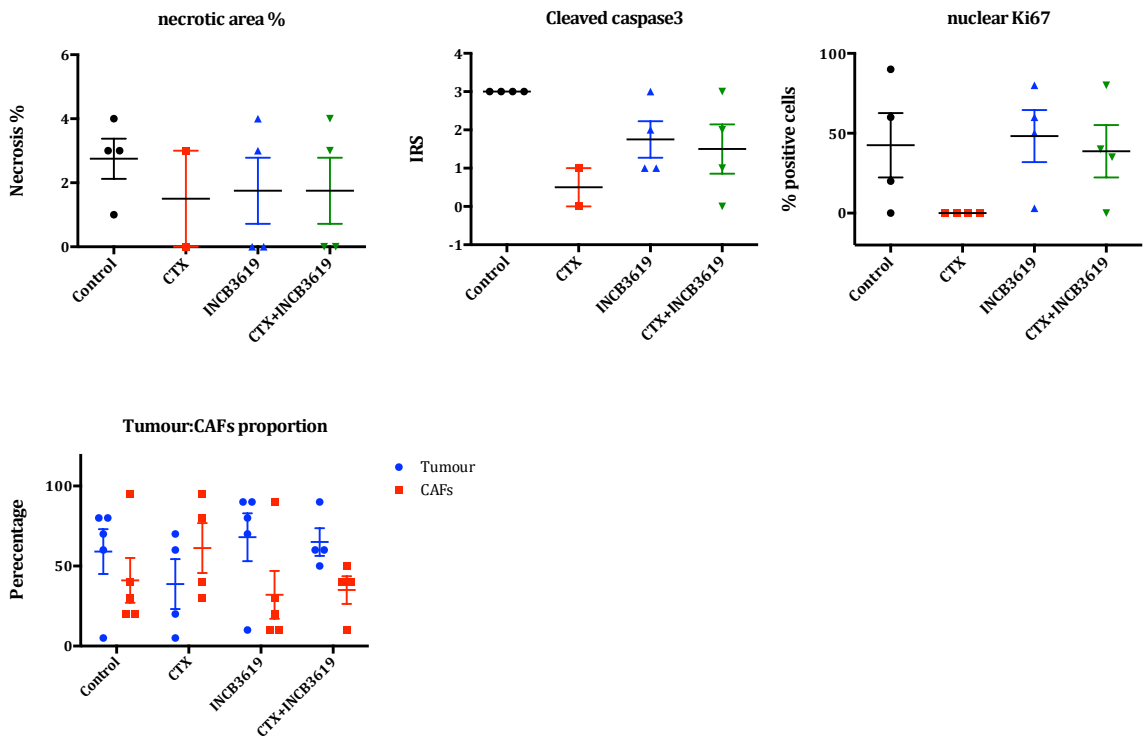


Figure 4.9 Effect of CTX, INCB3619 and their combination on EGFR signalling, proliferation, apoptosis and necrosis *in vivo*.

Total and phosphorylated EGFR, AKT and ERK1/2 as well as cleaved caspase3 were scored in terms of the staining intensity and the percentage of stained cells (IRS). Ki-67 was scored as the percentage of positive nuclear stained cells. Necrosis was scored as the percentage of necrotic region in a tumour. The scores were then grouped according to the treatments that the mice received and data are plotted as (means±S.E). The author and a consultant pathologist performed all scorings.

4.6 Discussion

Inhibition of ADAM17 activity using small molecule inhibitors or ADAM17 knockdown by siRNA has been reported to inhibit the shedding of TGFα (Kenny *et al.* 2007; McGowan *et al.* 2013), while the shedding of EGF is reduced in ADAM10−/− cells (Sahin *et al.* 2004). In the previous chapter, CTX was found to trigger the release of endogenous EGFR ligands **(Section 3.9)**, which could be mediated by ADAMs such as ADAM10 and 17. Kyula JN *et al*. (2010) has previously shown that chemotherapeutic treatment of colorectal cancer induced the release of EGFR ligands through ADAM17 activation, which is involved in drug-resistance (Kyula *et al.* 2010). In consistence, inhibition of ADAM17 using a selective ADAM17 inhibitor (McGowan *et al.* 2013) or a dual ADAM10/17 inhibitor (Kyula *et al.* 2010) enhanced the response to different cytotoxic drugs including the pan-HER TKI, neratinib. Furthermore, our lab has shown previously that ADAM17 also maintained Her2 phosphorylation in Her2 positive breast cancer cell lines, which were treated with Herceptin (Gijsen *et al.* 2010). Therefore, this chapter focused on the effect of inhibiting this ligand shedding by using a dual ADAM10/17 inhibitor, INCB3619, on the response of TNBC cell lines to CTX both *in vitro* and *in vivo*. As expected, INCB3619 inhibited the release of both EGF and TGFα induced by CTX-treatment **(Figure 4.1)** but this inhibition of EGFR ligands release only resulted in a minor decrease in EGFR phosphorylation, which was not statistically significant **(Figure 4.2)**. This could be explained by the presence of pre-existing EGFR ligands and growth factors in the medium, which would not be affected by concurrent treatment of INCB3619 with CTX. EGFR phosphorylation could be maintained by these pre-existing EGFR ligands or the presence of other ErbB ligands that are not regulated by ADAM10 or ADAM17. Nevertheless, combined treatment with CTX and INCB3619 enhanced the

response to CTX-treatment in at least two out of the three CTX sensitive cell lines **(Figure 4.3 and 4.4)**. This variation in response to the combined treatment could be due to the heterogeneity between cancer cell lines. Interestingly, co-treatment with INCB3619 also decreased the cell viability as well as colony formation in the acquired CTX-resistant cell line, MDA-MB-468CR **(Figure 4.3 and 4.4)**. This could be due to the activation of other ErbB receptors in the acquired CTX-resistant cells (Wheeler *et al.* 2008; Yonesaka *et al.* 2011), which might be also through ADAMs. However, further investigations are required to identify the role of ADAM10 and ADAM17 in acquired CTX resistance.

The combination of both CTX and INCB3619 induced apoptosis *in vitro* as indicated by the increase in caspase3/7 activities **(Figure 4.5)**. This is in agreement with previous finding in colorectal cells in which a dual ADAM10/17 inhibitor combined with chemotherapy synergistically activates apoptosis (Kyula *et al.* 2010). In addition to the release of EGFR ligands, both ADAM10 and ADAM17 are required for Notch signalling (Bozkulak *et al.* 2009). Hyper-activation of Notch signalling has been shown in TNBC (Lee *et al.* 2008) and contributes to their resistance to EGFR inhibition (Dong *et al.* 2010). Furthermore, the combination also induced a cell cycle arrest in G1 phase and a significant decrease in cyclin D1 expression. Several signalling pathways mediate the activation of cyclin D1 transcription including EGFR (Nyati *et al.* 2006) and Notch signalling pathways (Ronchini *et al.* 2001). Thus, the inhibition of both ADAM10 and ADAM17 has the potential to block ErbB and other signalling pathways that are important in cancer progression such as Notch pathway.

The inhibitory effect of CTX has been evaluated in different tumours *in vivo*. CTX significantly reduced tumour growth in the gastric NCI-N87 tumour-bearing mice (Hotz

et al. 2012) and the A431 tumour-bearing mice (Goldstein *et al.* 1995). However, Ferraro DA *et al*. (2013) has shown only a partial inhibitory effect of CTX alone *in vivo* using TNBC model (Ferraro *et al.* 2013). The current *in vivo* data revealed a statistically insignificant regression of tumour growth in MDA-MB-468 tumour-bearing mice that were treated with CTX compared to control mice **(Figure 4.7)**. CTX has been reported to exert ADCC response *in vivo* (Kurai *et al.* 2007; Hara *et al.* 2008) and thus this observed tumour regression could be partially due the CTX-induced ADCC response. In contrast, INCB3619 failed to inhibit the tumour growth, which is line with the previous findings in BT474 (Liu *et al.* 2006), MDA-MB-231 breast tumour-bearing mice (Fridman *et al.* 2007) and the A549 NSCLC (Fridman *et al.* 2007) and contradicting the findings by Zhou, B,S. *et al.* (2006) in the same A549 NSCLC (Zhou *et al.* 2006). In fact, Fridman J.S (2007) have evaluated the tumour growth for only 29 days while Zhou, B.S. *et al.* (2006) have evaluated tumour growth for up to 135 days post-inoculation. Therefore, these data implicated that INCB3619 has a weak anti-tumour effect *in vivo* and can be only observed after long-term treatment. One of the limitations of this *in vivo* study was the using of ALZET osmotic pumps for delivering the INCB3619. Although it has the advantage of delivering constant rate of the drug over time, it has a limited life span and cannot be used for longer than that. Therefore, this *in vivo* study could not be evaluated for longer than 21 days. Combining INCB3619 with different therapeutic agents such as gefitinib (Fridman *et al.* 2007), cisplatin (Fridman *et al.* 2007), paclitaxel (Fridman *et al.* 2007), trastuzumab (Liu *et al.* 2006) and lapatinib (Witters *et al.* 2008) has shown superior effect *in vivo* than single agents. Consistently, the current findings indicated that the combination of CTX and INCB3619 treatments resulted in a significant tumour regression compared to control untreated mice.

The IHC staining of the tumours collected from the mice revealed a decrease in total EGFR in both INCB3619 and the combination groups but not in CTX-treated group. However, there was hardly any difference in pEGFR, pAKT and pERK1/2 staining among different treatments. This could be attributed to the small number of mice per group or the instability of the phosphorylated proteins (Baker *et al.* 2005). Additionally, there was heterogeneity in pEGFR, pAKT and pERK1/2 staining within each tumour and only the predominant score was used for the analyses. Ki67 expression varied within each group and there was no difference between different treatments. In CTX-treated mice, there were 4 tumours only and two of them had only very few tumour cells (about 20 cells). Therefore, the results might not be reliable in this group. Although *in vitro* data indicated an increase in caspase3 and 7 activities in the combined CTX and INCB3619 treatment, *in vivo* data did not reveal a significant difference among the different groups. This could be due to the small number of mice or the short period of the *in vivo* experiment, which might not be enough to induce a significant difference between different groups. However, it should clarify here that the small number of tumour cells that has been observed in some slides might not represent the actual tumour as these initial IHC slides have been cut from the edge of these tumours. Further cutting and preparation of the IHC slides indicated the presence of more tumour cells. Therefore, repeating the IHC staining for these biomarkers might be required to confirm these findings. Lastly, the tumours analyzed here were the residual tumours after 21 days of treatment. Therefore, these tumours could represent the resistant clones and could have a different protein expression compared to those tumours if they were to be harvested after a very short exposure of the treatment (e.g. after few hours).

In summary, this chapter indicated that the inhibition of ADAM10 and 17 activities prevents CTX-induced release of EGFR ligands and enhanced the effect of CTX on EGFR signalling. However, this might not be the only mechanism of the additive effect that has been seen both *in vitro* and *in vivo*. ADAM10 and 17 are also involved in shedding and regulating other signalling pathways such as notch and Met signalling pathways. Therefore, the additive effect could be due to the inhibition of multiple pathways rather than a single pathway.

5 Investigating the transcriptome changes in acquired CTX-resistance in triple negative breast cancer using next generation RNA-sequencing technology

5.1 Introduction

Despite the initial response to different EGFR-targeted therapies, including CTX, patients develop acquired resistance over the time. Several studies highlighted the molecular mechanisms underlying CTX resistance in different cancers **(discussed in section 1.4.2)**.

In TNBC, Carey, R. *et al*. (2012) showed that CTX as a single agent induced limited efficacy in a phase II clinical trial, TBCRC001, and the majority of the patients exhibited an activation of the EGFR pathway (Carey *et al.* 2012). This could be explained by lack of effectiveness in targeting EGFR, rebound activation of EGFR through alternative mechanisms or activation of parallel signalling pathways (Carey *et al.* 2012). However, to the best of my knowledge, the mechanisms of acquired CTX resistance in TNBC remain poorly understood.

In this chapter, I show that I have developed a preclinical model of acquired CTX-resistant TNBC and used next generation RNA sequencing (RNA-seq) technology to identify the full spectrum of changes in the transcriptome of the model. These changes potentially included novel pathways associated with acquired CTX resistance in TNBC.

5.2 Development of acquired CTX-resistant TNBC cell line

In chapter 3, the sensitivity to CTX was investigated in several TNBC cell lines. MDA-MB-468 cell line was the most sensitive TNBC cell line to CTX treatment **(Figure 3.4)**. In order to investigate the underlying mechanism(s) of acquired CTX-resistance in TNBC, a model of acquired CTX-resistant, named MDA-MB-468CR, was derived from the parental CTX-sensitive MDA-MB-468 cell line. MDA-MB-468CR was developed by continuous exposure of MDA-MB-468 cells to increasing doses of CTX and maintained at 10 μg/ml CTX as described in **(Section 2.2.2) (Figure 5.1A)**. As shown previously **(Figure 3.4)**, CTX treatment of the parental MDA-MB-468 cells significantly reduced the percentage of viable cells in a dose-dependent manner (up to a maximum of 33.6±4.1 % of viable cells at 200 μg/ml CTX) **(Figure 5.1B)**. In contrast, inhibition of cell viability induced by CTX significantly decreased in MDA-MB-468CR cells compared to MDA-MB-468 cells (maximum of 76.3±2.8 % of viable cells at 200 μg/ml CTX) **(Figure 5.1B and Table 5.1)**. Interestingly both parental and CTX-resistant cells were morphologically similar **(Figure 5.1C)**.

Table 5.1 Percentage of viable cells in MDA-MB-468 and MDA-MB-468CR cells after CTX treatment with the indicated concentrations for 6 days.

CTX (μg/ml)	MDA-MB-468	MDA-MB-468CR	*P* value
5	68.3±2.9	95±3.4	< 0.0001
10	56.6±2.1	89.3±4.6	< 0.0001
50	52.5±0.8	83.2±4.9	< 0.0001
100	49.8±3.2	82.5±2.7	< 0.0001
200	33.65±4.1	76.3±2.8	< 0.0001

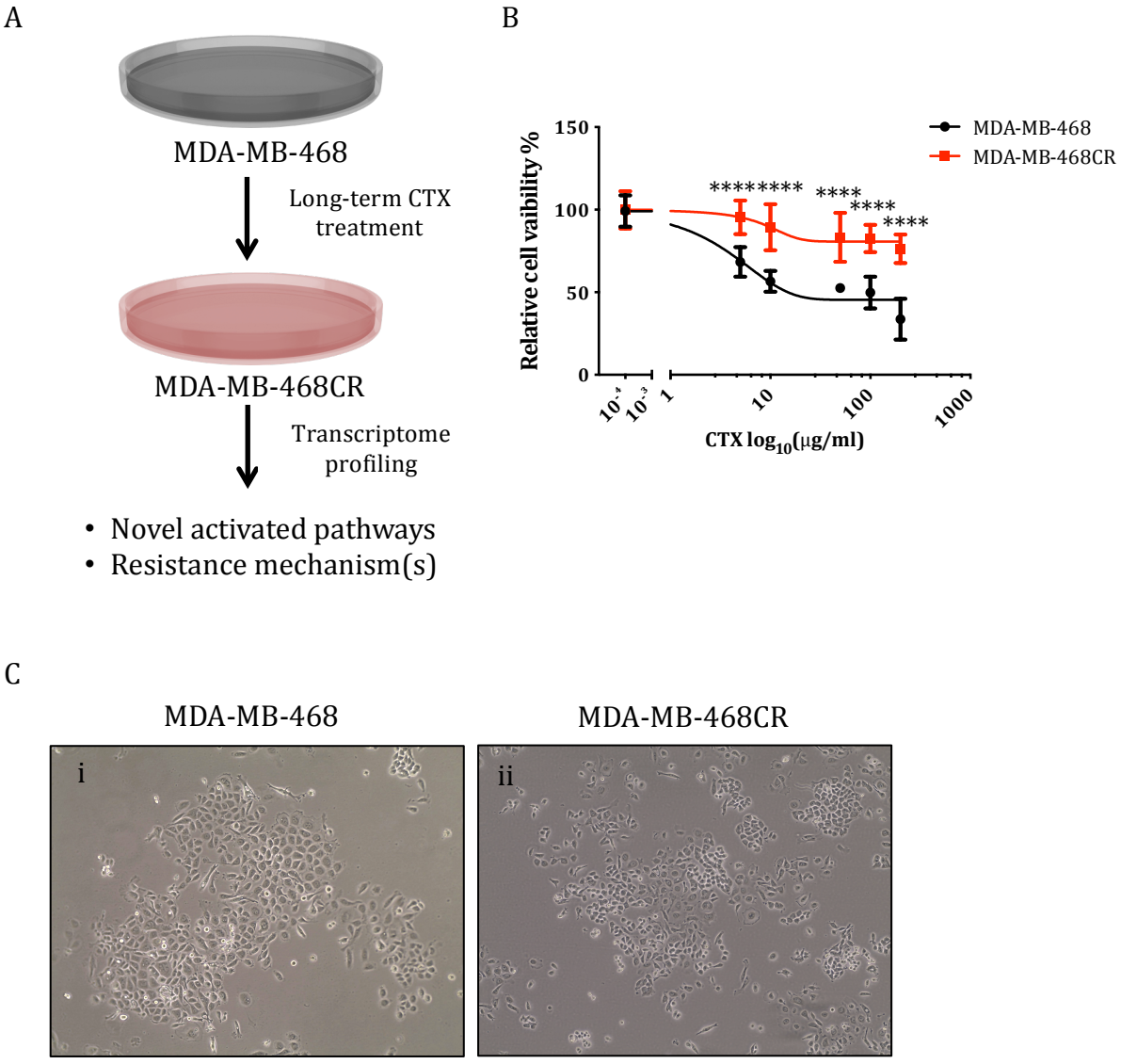
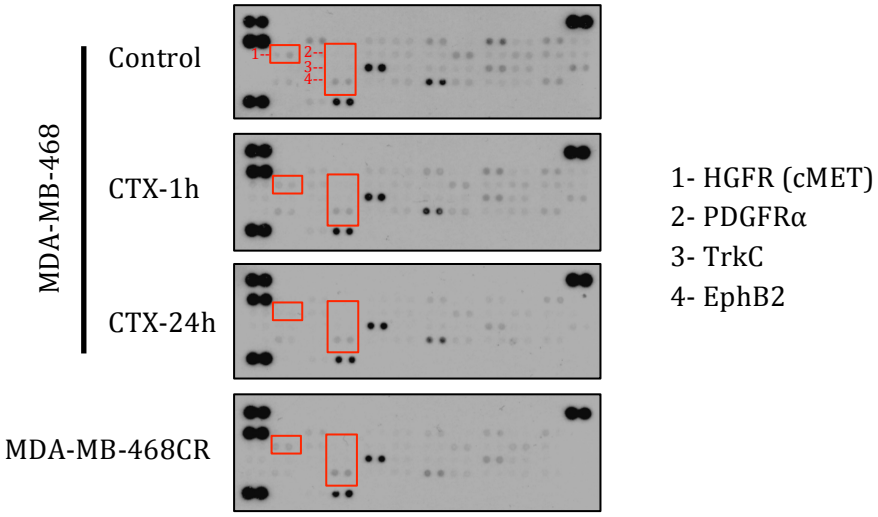


Figure 5.1 Development of acquired CTX-resistant MDA-MB-468CR cells.
(**A**) Acquired CTX-resistant MDA-MB-468CR cell line was developed from the parental MDA-MB-468 cells by continuous exposure to increasing doses of CTX for >6 months. (**B**) Both MDA-MB-468 and MDA-MB-468CR cells were treated with increasing doses of CTX (5-200 µg/ml) for 6 days and cell viability was assessed using CellTiter-Blue® cell viability assay kit. Results were shown as means±S.E relative to the untreated cells (Two-way ANOVA with Bonferroni's multiple comparisons tests, n=3). (**C**) Acquired CTX-resistant MDA-MB-468CR cells were morphologically indistinguishable from the parental MDA-MB-468 cells under light microscopy.

5.3 Profiling of receptor tyrosine kinases (RTKs) phosphorylation

Activation of alternative RTKs such as Her2, Her3 and cMET has been reported to be involved in the development of acquired resistance to CTX in different cancers (Wheeler *et al.* 2008; Yonesaka *et al.* 2011). Therefore, I tested whether the activation of other RTKs is also involved in acquired CTX-resistance in TNBC cells. Using a high-throughout phospho-RTK array system, the phosphorylation status of a panel of 42 RTKs in both MDA-MB-468 (control, 1 hour and 24 hours CTX treatment) and MDA-MB-468CR cells was investigated **(Figure 5.2A)**. Consistent with my previous observation **(Figure 3.7),** the quantification and comparative analysis revealed a significant reduction in the phosphorylation of Her3 after 1 hour of treatment **(Figure 5.2B and Table 5.2).** In addition, the analysis revealed an increase in the phosphorylation of hepatocyte growth factor receptor (HGFR, cMET), Ephrin type-B receptor 2 (EphB2), alpha-type platelet-derived growth factor receptor (PDGFRα), neurotrophic tyrosine kinase receptor type 3 (TrkC) by 1.69, 1.41, 1.38 and 1.24 –fold, respectively, in MDA-MB-468CR relative to the parental MDA-MB-468 cells **(Figure 5.2B and Table 5.2)**.

A



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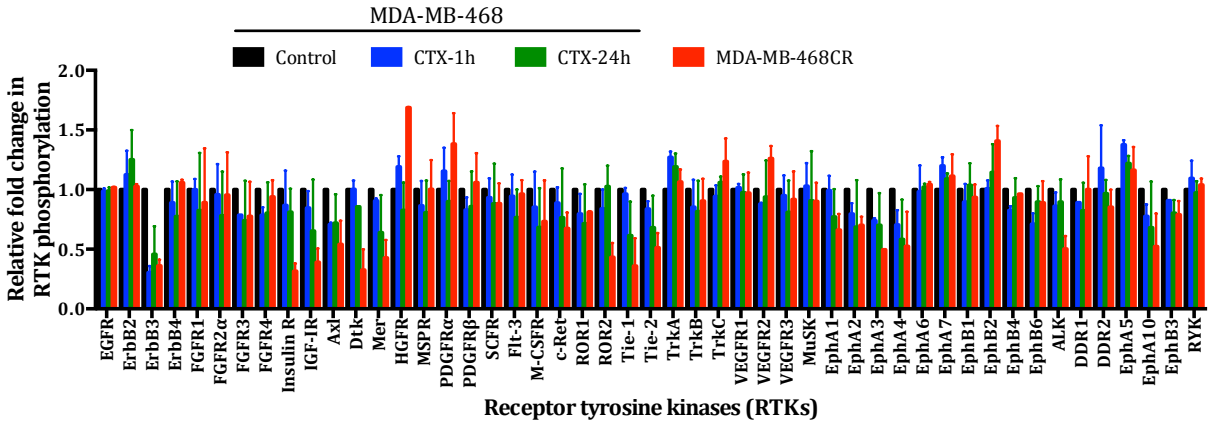


Figure 5.2 Phospho-receptor tyrosine kinase (RTK) array in parental MDA-MB-468 and acquired CTX-resistant MDA-MB-468CR cells.

(A) MDA-MB-468 cells were untreated or treated with CTX (10 µg/ml) for 1 hour and 24 hours and MDA-MB-468CR cells were continuously treated with CTX. The cell lysates were analyzed with the phospho-RTK array (R&D systems) according to manufacturer's protocol. **(B)** Quantification of the phospho-RTKs relative to the control untreated MDA-MB-468 cells (n=2).

Table 5.2 Quantification of changes in phospho-RTKs after CTX treatment (n=2)

RTK	Control	CTX-1h	CTX-24h	MDA-MB-468CR
EGFR	1	0.99	0.99	1.02
Her2	1	1.12	1.25	1.03
Her3	1	0.30	0.66	0.36
Her4	1	0.89	0.77	1.06
FGFR1	1	1.00	0.82	0.89
FGFR2α	1	0.96	0.78	0.95
FGFR3	1	0.79	0.74	0.78
FGFR4	1	0.79	0.80	0.94
Insulin R	1	0.87	0.81	0.32
IGF-IR	1	0.85	0.65	0.39
Axl	1	0.71	0.72	0.54
Dtk	1	1.00	0.86	0.33
Mer	1	0.91	0.64	0.43
HGFR	1	1.19	0.83	**1.69**
MSPR	1	0.86	0.81	1.00
PDGFRα	1	1.15	0.90	**1.38**
PDGFRβ	1	0.83	0.86	1.06
SCFR	1	0.93	0.88	0.88
Flt-3	1	0.94	0.77	0.96
M-CSFR	1	0.85	0.68	0.73
c-Ret	1	0.89	0.76	0.67
ROR1	1	0.80	0.71	0.81
ROR2	1	0.84	1.03	0.43

Tie-1	1	0.96	0.61	0.36
Tie-2	1	0.84	0.68	0.51
TrkA	1	1.27	1.19	1.06
TrkB	1	0.85	0.82	0.90
TrkC	1	0.94	1.06	**1.24**
VEGFR1	1	1.02	0.97	0.97
VEGFR2	1	0.88	0.94	1.26
VEGFR3	1	0.95	0.81	0.92
MuSK	1	1.03	0.91	0.90
EphA1	1	0.99	0.77	0.66
EphA2	1	0.80	0.69	0.70
EphA3	1	0.74	0.70	0.50
EphA4	1	0.70	0.58	0.52
EphA6	1	0.98	1.02	1.04
EphA7	1	1.20	1.09	1.11
EphB1	1	0.90	1.04	0.93
EphB2	1	1.01	1.14	**1.41**
EphB4	1	0.83	0.93	0.96
EphB6	1	0.71	0.90	0.89
ALK	1	0.86	0.90	0.50
DDR1	1	0.89	0.82	1.00

5.4 Transcriptome analysis of CTX-sensitive and acquired CTX-resistant TNBC cells using next generation RNA sequencing (RNA-seq)

5.4.1 Comprehensive analysis of acquired CTX-resistant transcriptome

In order to gain a global overview of the aberrant changes in acquired CTX-resistant TNBC MDA-MB-468CR cells, the transcriptome landscapes of both MDA-MB-468 (control, 1 hour and 24 hours CTX treatment) and MDA-MB-468CR cells were profiled. Total RNAs of three biological replicates were processed by the Genomics Core service facility at the Wellcome Trust Centre for Human Genetics in Oxford for high throughput next generation RNA-seq using Illumina Hiseq2000 platform (paired end, 100 bp length reads for the first replicate and 51 bp length reads for the two other replicates). The raw data were mapped to the reference human genome 2009 release (hg19) using TOPHAT as described in **(Section 2.2.21.1)**. The total count reads and the total mapped reads in each sample are summarized in **(Table 5.3)**.

Table 5.3 Summary of total and mapped reads from the RNA-seq data

Sample	Total reads	Mapped reads	(%)
MDA-MB-468 Control 1	51346069	45086245	87.8
MDA-MB-468 CTX-1H 1	44154931	36803002	83.3
MDA-MB-468 CTX-24H 1	48840000	38839515	79.5
MDA-MB-468CR 1	49644107	45134989	90.9
MDA-MB-468 Control 2	47289008	46678423	98.7
MDA-MB-468 CTX-1H 2	48241030	47739893	98.9
MDA-MB-468 CTX-24H 2	46235014	45484375	98.4
MDA-MB-468CR 2	44091943	43711861	99.1
MDA-MB-468 Control 3	49136474	48549951	98.8
MDA-MB-468 CTX-1H 3	51087975	50583067	99.0
MDA-MB-468 CTX-24H 3	48248859	47547977	98.5
MDA-MB-468CR 3	47476681	46988863	98.9

5.4.2 Differential expression analyses

Mapped reads from the three replicates were used to calculate the average differential gene expression using the edgeR (Bioconductor, Seattle, WA, USA) package as described in **(Section 2.2.21.2)**. The numbers of deregulated transcripts at 1 hour and 24 hours CTX-treated MDA-MB-468 and in MDA-MB-468CR relative to untreated control MDA-MB-468 cells (Log2 FC≥ 1.2 and FDR< 0.01) are summarized in **(Table 5.4)**.

Table 5.4 Differentially expressed genes (Log2 FC≥ 1.2 and FDR < 0.01)

	Genes			
	Total	**Deregulated**	**Up-regulated**	**Down-regulated**
MDA-MB-468				
CTX 1 hour	21653	2 (0.01%)	0 (0%)	2 (0.01%)
CTX 24 hours	21653	28 (0.13%)	17 (0.08%)	11 (0.05)
MDA-MB-468CR	21653	3906 (18%)	3113 (14.3%)	793 (3.6%)

5.4.2.1 One-hour CTX treatment

At 1 hour treatment, CTX induced a significant down-regulation of two genes: FBJ murine osteosarcoma viral oncogene homolog (*FOS*) (Log2 FC= -2.0, FDR=2.14^{-22}) and early growth response 1 (*EGR1*) (Log2 FC= -1.8, FDR=2.9^{-32}) and there was no significant up-regulation of any gene in comparison to untreated MDA-MB-468 cells.

5.4.2.2 Twenty-four hours CTX treatment

As shown in **(Table 5.4)**, 24 hours of CTX treatment induced a significant deregulation of 28 genes (0.13%) of which 17 genes (0.08%) were up-regulated and 11 genes (0.05%) were down-regulated (log2 FC ≥1.2, FDR <0.01). The deregulated genes in 24 hours CTX-treated MDA-MB-468 are listed in **(Supplementary Table S.1)**.

5.4.2.3 MDA-MB-468CR

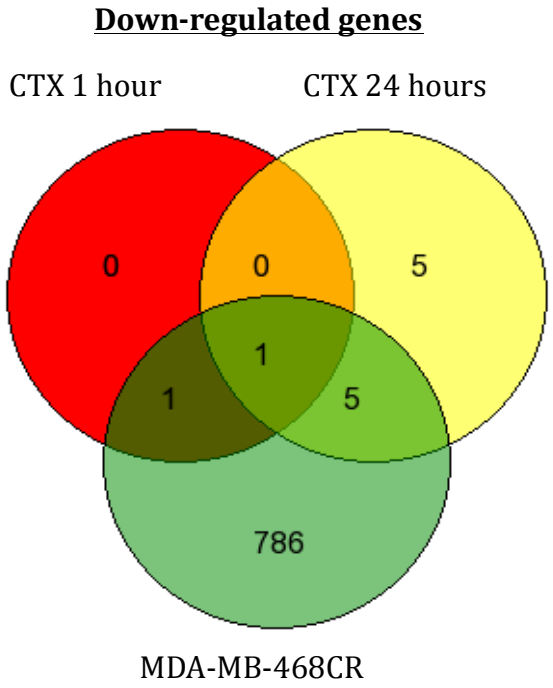
In comparison to MDA-MB-468 cells, MDA-MB-468CR cells exhibited a significant deregulation of 3906 genes (18.0%) including up-regulation of 3113 genes (14.3%) and down-regulation of 793 genes (3.6%) (Log2 FC ≥1.2, FDR <0.01) **(Table 5.4)**. A list of

the top 100 up-regulated and down-regulated genes in MDA-MB-468CR is listed in **(Supplementary Table S.2)**.

5.4.2.4 The overlap between significantly deregulated genes

Here, the overlap between the significantly up-regulated and down-regulated genes between the 1 hour, 24 hours CTX-treated MDA-MB-468 cells and MDA-MB-468CR was compared. *EGR1* was the common down-regulated gene in 1 hour, 24 hours CTX-treated cells and also in MDA-MB-468CR cells (Log2 FC= -1.8, -2.87 and -3.19, respectively) **(Figure 5.3A and Supplementary Figure S1)**. On the other hand, there was no any significant up-regulated gene in 1 hour CTX treatment and the comparison between 24 hours CTX treatment and resistant cells revealed an overlapping of 5 genes (*DDIT4L*, *FAM107A*, *IL32*, *KLHL38* and *NCALD*) **(Figure 5.3B)**.

A



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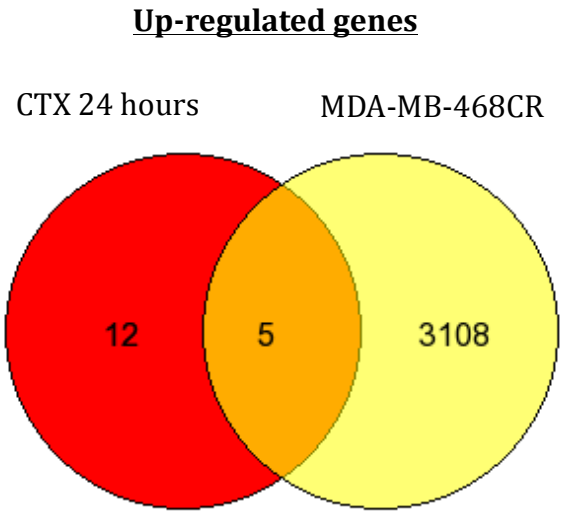


Figure 5.3 The overlapping of significant deregulated genes.
Venn diagram of **(A)** down-regulated and **(B)** up-regulated genes in the indicated conditions.

5.4.3 Pathway analysis and gene ontology

As hypothesized, acquired CTX-resistance might involve an up-regulation of alternative pathway(s). Therefore, further analyses were focused on the significantly up-regulated genes in MDA-MB-468CR. Given that a large number of genes were induced in MDA-MB-468CR cells **(Section 5.4.2.3)**, pathway analysis was performed on the up-regulated genes in MDA-MB-468CR cells (Log2 fold-change ≥1.2, FDR<0.01) to determine gain and possible activation of pathways that might be involved in acquired CTX-resistance. Pathway analyses revealed a significant activation of a range of key pathways including the ErbB signalling pathway, the p53 signalling pathway, the phosphatidylinositol signalling system and the transforming growth factor beta (TGFβ)/bone morphogenetic protein (BMP) signalling pathway **(Table 5.5).** These data indicated that CTX-resistance is potentially due to alteration of several pathways and mechanisms rather than a single mechanism. However, due to the time limitation, the rest of this project focused on one of the up-regulated pathways (TGFβ/BMP signalling pathway).

Table 5.5 Pathway analysis for the top up-regulated genes in MDA-MB-468CR compared to MDA-MB-468

The top 20 most significantly enriched pathways (P<0.05) are shown using Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang da *et al.* 2009; Huang da *et al.* 2009).

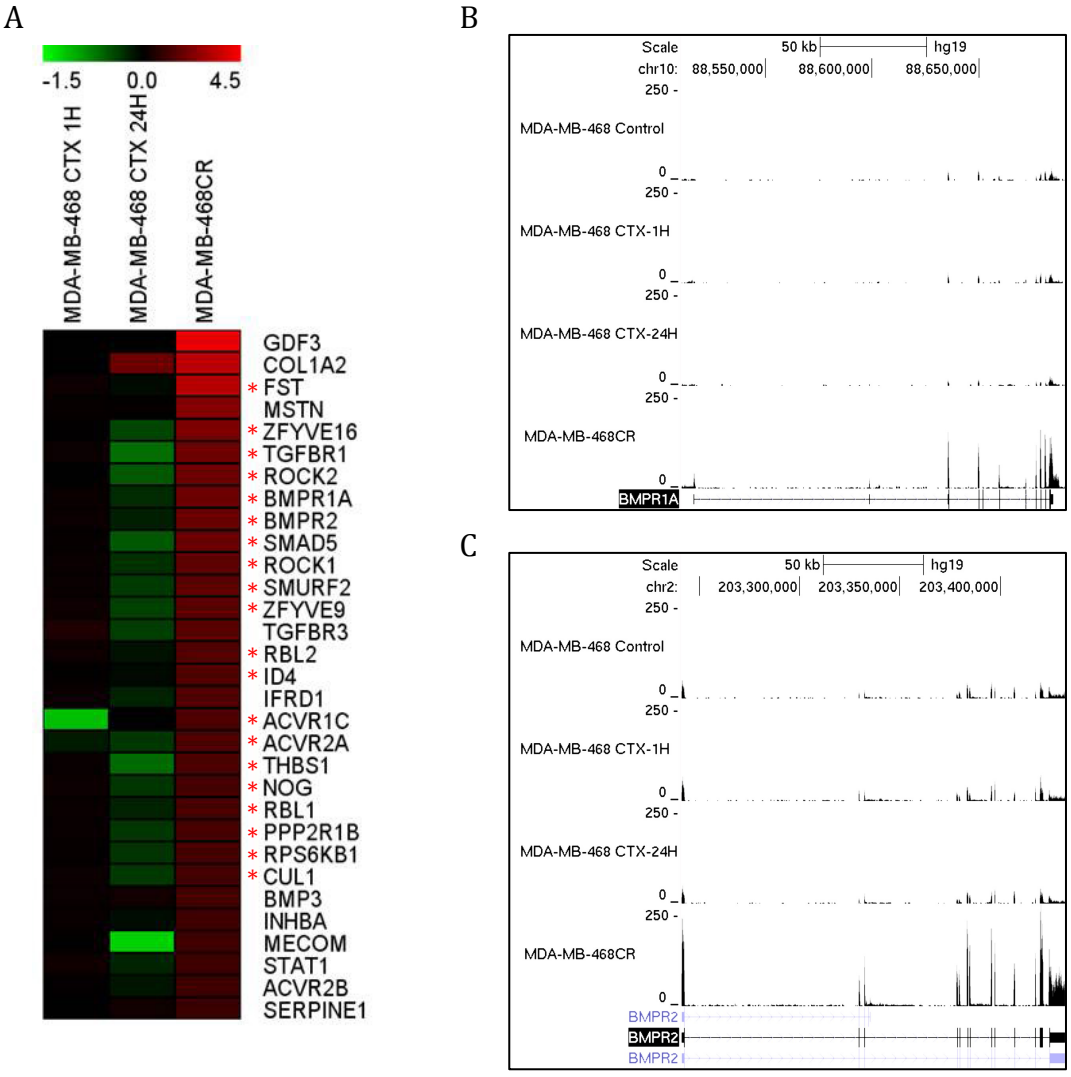
Pathway	No. of up-regulated genes	%	P value
Regulation of actin cytoskeleton	44	1.7	0.00012
Ubiquitin mediated proteolysis	31	1.2	0.00026
ErbB signalling pathway	22	0.8	0.00056
Gap junction	22	0.8	0.00078
P53 signalling pathway	18	0.7	0.00120
Colorectal cancer	20	0.8	0.00230
Phosphatidylinositol signalling system	18	0.7	0.00320
TGF-beta/BMP signalling pathway	20	0.8	0.00350
Glioma	16	0.6	0.00390
Toll-like receptor signalling pathway	22	0.8	0.00410
Melanoma	17	0.6	0.00520
Focal adhesion	36	1.4	0.00620
Progesterone-mediated oocyte maturation	19	0.7	0.00710
Chronic myeloid leukemia	17	0.6	0.00900
Prostate cancer	19	0.7	0.01000
MAPK signalling pathway	44	1.7	0.01100
Renal cell carcinoma	16	0.6	0.01100
Cell cycle	23	0.9	0.02400
RNA degradation	13	0.5	0.02400
Adherens junction	16	0.6	0.02500

5.5 Up-regulation of TGFβ/BMP pathway in the acquired CTX-resistant MDA-MB-468CR cells

As shown in **(Table 5.5)**, pathway analysis indicated a significant up-regulation of the TGFβ/BMP pathway in MDA-MB-468CR cells compared to the parental MDA-MB-468 cells whereby the expression of 20 genes within this pathway was significantly increased (*P*=0.003). These up-regulated genes included genes encoding ligands, receptors and downstream genes involved in TGFβ/BMP signalling pathway **(Figure 5.4A).** Among the significantly up-regulated genes, there was an up-regulation in the expression of two bone morphogenetic protein receptors (BMPRs), *BMPR1A* and *BMPR2* (Log2 FC= 1.89 and 1.88, respectively) **(Figure 5.4B and C)** and *TGFBR1* (Log2 FC= 1.96) **(Supplementary Figure S2).** Additionally, there was a significant increase in *SMAD5* mRNA expression (Log2 FC= 1.84) **(Supplementary Figure S3).** The mRNA expression level of particular genes including the two BMPRs (*BMPR1A* and *BMPR2*), *TGFBR1*, *SMDA5*, *ZFYVE9* and *ZFYVE16* in both MDA-MB-468 and MDA-MB-468CR cells was also confirmed using RT-qPCR **(Figure 5.4D-I).**

Ligand binding of TGFβ superfamily induces the phosphorylation of type I TGFβ and BMPRs through type II receptors, resulting in the recruitment and phosphorylation of SMADs and subsequent activation of SMAD and non-SMAD pathways (Wrana *et al.* 1994) (Schmierer *et al.* 2007). As illustrated in **(Figure 5.5)**, although there was an initial decrease in SMAD1/5/8 phosphorylation after 1 hour of CTX treatment, they were reactivated at 24 hours as well as in MDA-MB-468CR compared to untreated MDA-MB-468 cells. Both TGFβ and BMP mediate AKT phosphorylation (Bakin *et al.* 2000; Gangopahyay *et al.* 2011). In parallel, pAKT increased in MDA-MB-468CR cells relative

to MDA-MB-468 cells **(Figure 5.5)**. Collectively, these data confirmed the activation of BMPRs in CTX-resistant MDA-MB-468CR cells, which may be involved in their resistance.



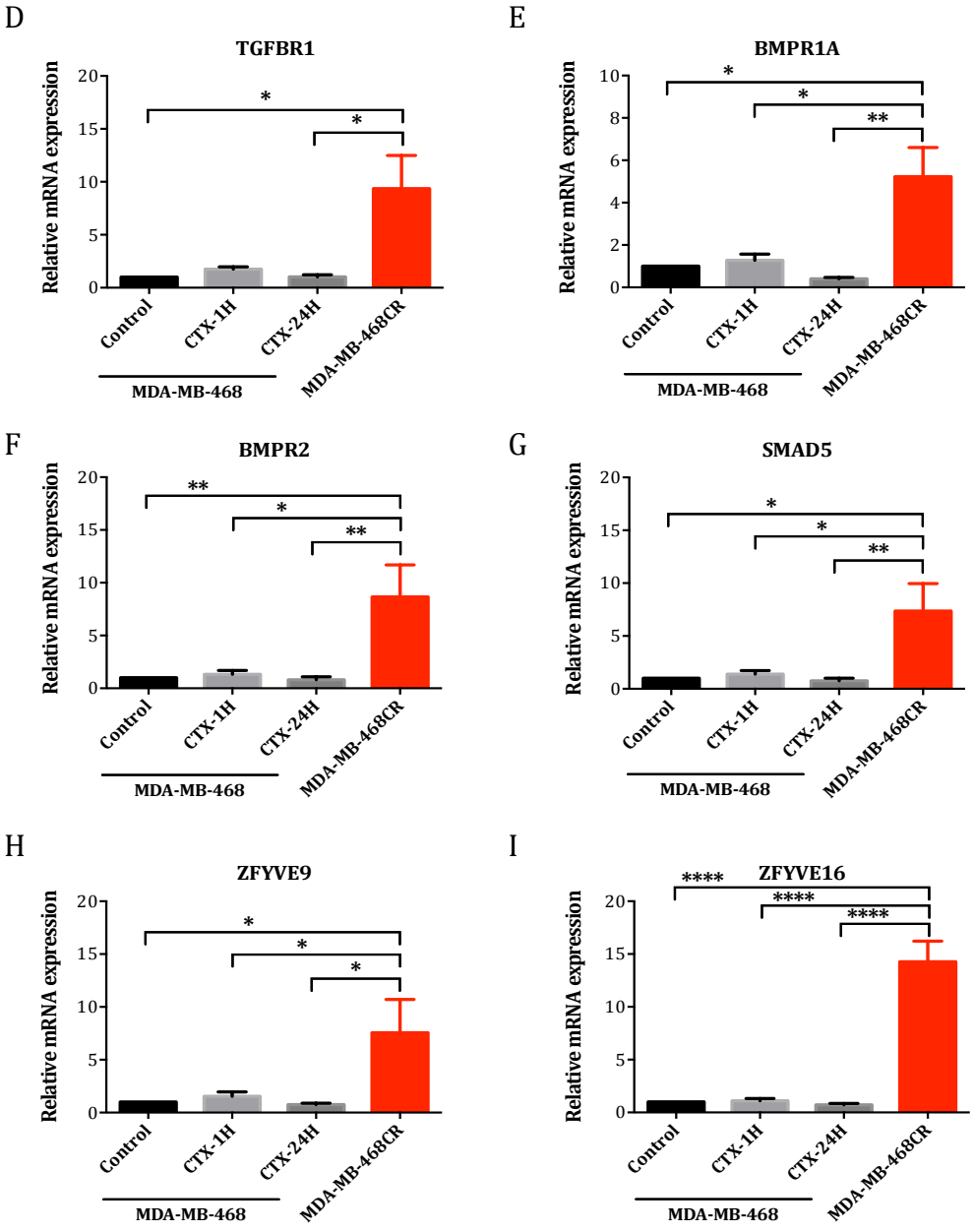


Figure 5.4 TGFβ/BMP signalling pathway in MDA-MB-468CR.
(A) Heat map of the expression of up-regulated genes in TGFβ/BMP signalling pathway in MDA-MB-468CR cells (* indicates genes from DAVID analysis). A snapshot of the UCSC genome browser at **(B)** *BMPR1A* and **(C)** *BMPR2* gene loci. RT-qPCR validation of **(D)** *TGFBR1*, **(E)** *BMPR1A*, **(F)** *BMPR2*, **(G)** *SMAD5*, **(H)** *ZFYVE9* and **(I)** *ZFYVE16* mRNA expression in MDA-MB-468 (control, 1 hour and 24 hours CTX treatment) and MDA-MB-468CR cells (means±S.E, one-way ANOVA with Bonferroni's multiple comparisons tests, n=3).

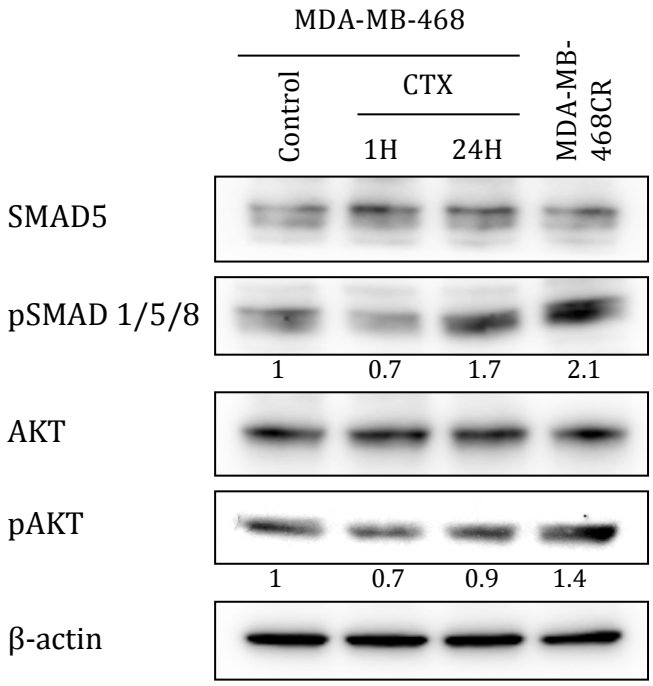


Figure 5.5 Activation of SMADs in MDA-MB-468CR.
Western blot analysis of total SMAD5, phosphorylated SMAD1/5/8, total AKT and pAKT in MDA-MB-468 (Control, 1 hour, 24 hour CTX treatment) and MDA-MB-468CR cells.

5.6 Inhibition of BMPRs overcomes acquired CTX-resistance in MDA-MB-468CR cells

As shown above, there was a significant increase in the expression of BMPRs that was correlated with SMAD5 phosphorylation in MDA-MB-468CR cells. Therefore, I tested whether inhibiting BMPRs could overcome CTX resistance in MDA-MB-468CR cells. Recently, a selective BMPR inhibitor (K02288) has been developed which specifically inhibits BMP-induced SMAD pathway (Sanvitale *et al.* 2013). Treatment of MDA-MB-468CR cells with K02288 inhibited SMAD1/5/8 phosphorylation **(Figure 5.6A)**. In MDA-MB-468CR cells, combining CTX treatment with increasing doses of K02288 resulted in a significant decrease in the cellular viability to 35.3±12.3% and 11.4±4.4% (both $P<0.0001$) at 5 and 10 μM respectively compared to the control untreated cells. Similarly, there was a significant decrease in the percentage of viable MDA-MB-468 cells to 12.8±9.2% and 0.3±6.8% (both $P<0.0001$) at 5 and 10 μM respectively compared to control **(Figure 5.6B)**.

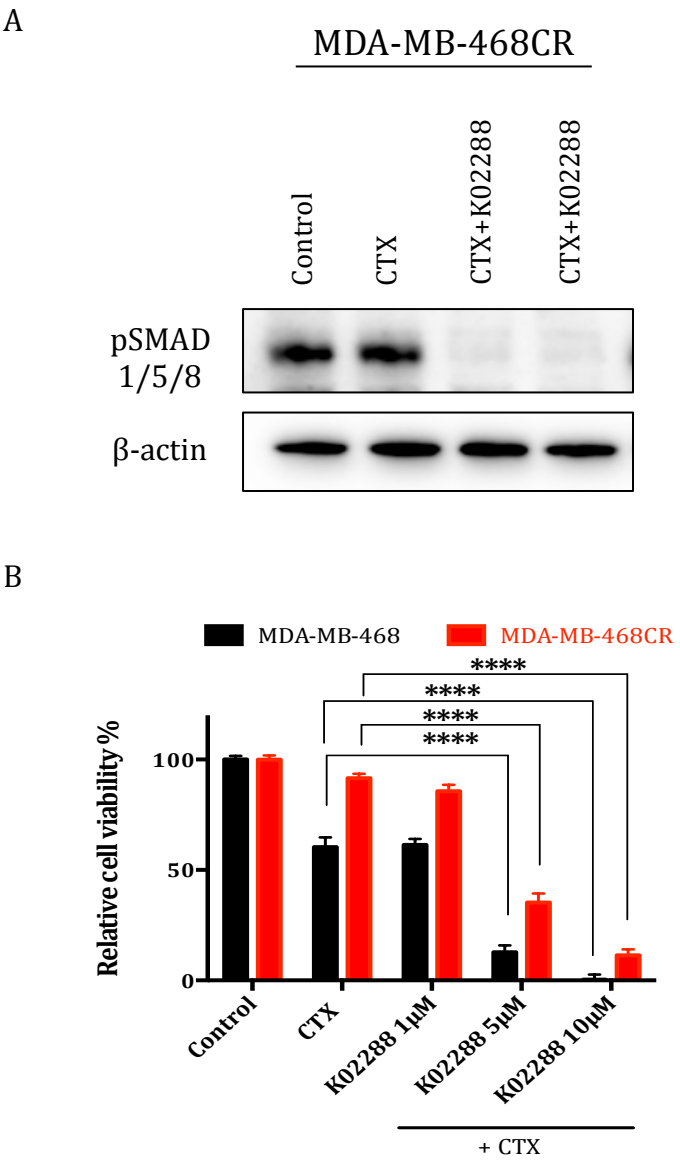


Figure 5.6 BMPR inhibitor (K02288) enhances CTX response in MDA-MB-468 cells and reverses CTX resistance in MDA-MB-468CR cells.

(**A**) SMAD1/5/8 phosphorylation in MDA-MB-468CR cells that were treated with CTX for 24 hours +/- K02288 (1 and 5 μM) for 30 minutes (**B**) MDA-MB-468 and MDA-MB-468CR cells were treated with CTX (10 μg/ml) and increasing doses of K02288 (1, 5 and 10 μM) for 6 days. Cell viability was then assessed using CellTiter-Blue® cell viability assay kit and results were calculated and plotted as means±S.E relative to untreated cells (P<0.01 **, P<0.0001 ****, one-way ANOVA with Bonferroni's multiple comparisons tests, n=3).

5.7 BMPR ligand reduces CTX efficacy in parental sensitive cells

As shown in **(Section 5.6)**, inhibition of BMPRs using K02288 restored the sensitivity of MDA-MB-468CR to CTX as well as increased the response of MDA-MB-468 cells to CTX **(Figure 5.6B)**. On the other hand, stimulation of MDA-MB-468 with the BMPR ligand, BMP6 (10 and 100 ng/ml), significantly increased the cell viability (136.6±4.5% and 147.5±3.4%, respectively) compared to the control untreated cells (P<0.0001) **(Figure 5.7)**. Furthermore, BMP6 stimulation of CTX-treated cells reversed the inhibitory effect of CTX on MDA-MB-468 cells and significantly increased the percentage of the viable cells to 87.9±3.9% (P<0.01) and to 94.7±2.1% (P<0.0001) (CTX + BMP6 10 ng/ml and CTX + BMP6 100 ng/ml, respectively) compared with CTX alone (70.8±1.8%) **(Figure 5.7)**.

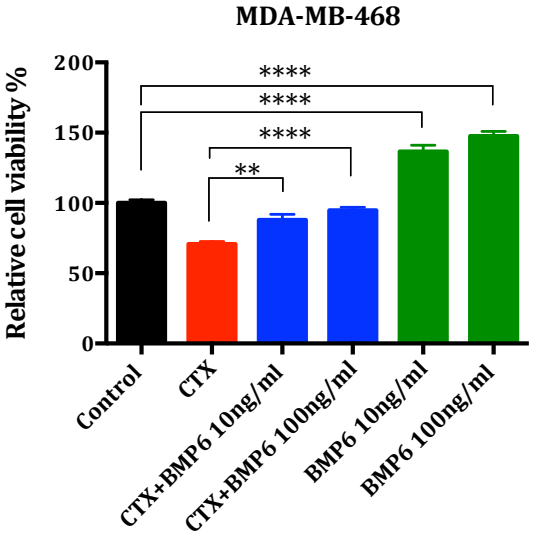
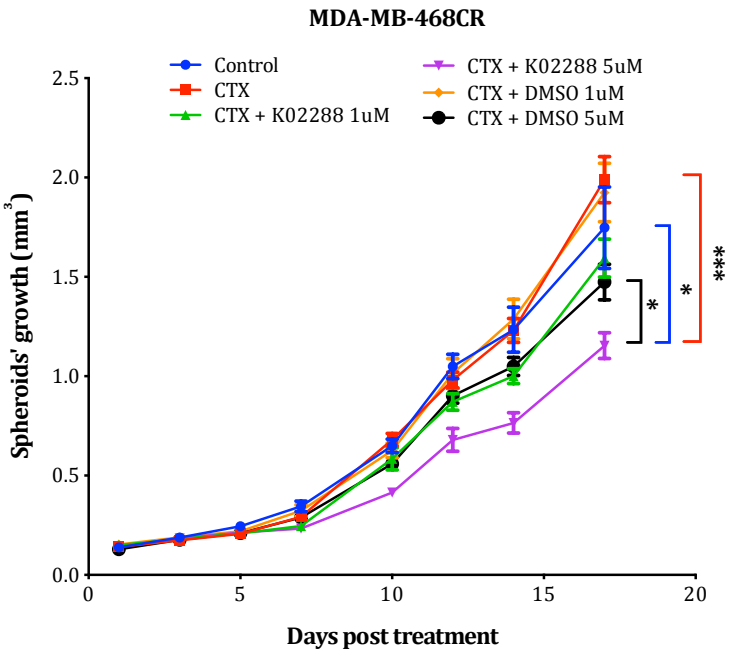


Figure 5.7 Effect of BMP6 stimulation on CTX sensitivity.
Parental MDA-MB-468 cells were treated with CTX (10 µg/ml) and/or stimulated with BMP6 (10 and 100 ng/ml) for 6 days and cell viability was assessed using CellTiter-Blue® cell viability assay kit (P<0.01 **, P<0.0001 ****, one-way ANOVA with Bonferroni's multiple comparisons tests, n=3).

5.8 Overcoming acquired CTX-resistance using BMPR inhibitor in a 3D model

Combining the BMPR inhibitor (K02288) with CTX significantly reduced the cell viability of acquired CTX-resistance in MDA-MB-468CR **(Figure 5.6)**. Furthermore, MDA-MB-468CR spheroids were treated with CTX and/or K02288 and the spheroids' growth was assessed regularly. As displayed in **(Figure 5.8A and B)**, continuous exposure to CTX did not affect the spheroids' growth compared to the control. On the other hand, combining CTX and K02288 (5 μM) significantly reduced the spheroids' volume in MDA-MB-468CR cells compared to the control (P<0.05) and to CTX treatment (P<0.001) **(Figure 5.8A and B).**

A



B

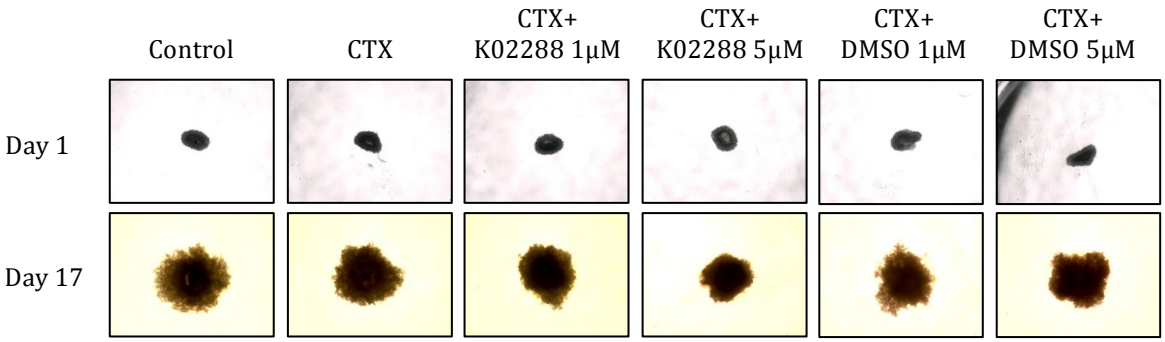


Figure 5.8 Effect of CTX and K02288 combination on the growth of MDA-MB-468CR spheroids' growth.

(A) MDA-MB-468CR spheroids were treated with CTX (10 µg/ml) +/- K02288 (1 or 5 µM) and spheroid volume of 20 individual spheroids per treatment was monitored for 17 days. Data were analysed using ImageJ software (http://imagej.nih.gov/ij/) (P<0.05 *, P<0.001 ***, two-way ANOVA with Bonferroni's multiple comparisons tests). **(B)** Representative MDA-MB-468CR spheroids before and after treatment with CTX (10 µg/ml) +/- K02288 (1 or 5 µM) for 17 days.

5.9 The MDA-MB-468CR cells are sensitive to cMET inhibition

The analysis of phosphorylation of different RTKs in both MDA-MB-468 and MDA-MB-468CR using phospho-RTK array revealed an increase in cMET phosphorylation in the resistant cells compared to the parental MDA-MB-468 cells **(Section 5.3)**. Since only pMET was increased by > 1.5 fold compared to the control, further analysis was done on cMET. At protein level, both the pro-cMET and cMET were increased in MDA-MB-468CR cells. However, I could not detect pMET by western blot, which could be due to the sensitivity of the antibody. In fact, other researchers had similar issues with detecting pMET by western blot in our lab **(Figure 5.9A).** Additionally, RNA-seq data revealed a significant increase in *cMET* mRNA level in the resistant MDA-MB-468CR cells compared to the parental MDA-MB-468 cells (Log2 FC= 2.33) **(Figure 5.9B).**

Moreover, co-treatment with CTX and the cMET TKI, INCB28060, significantly reduced the cell viability of both MDA-BM-468 and MDA-MB-468CR relative to CTX alone in a dose-dependent manner (P<0.0001) **(Figure 5.9C and D).** These preliminary data suggested that inhibition of cMET could potentiate the effect of CTX and overcome the acquired CTX resistance.

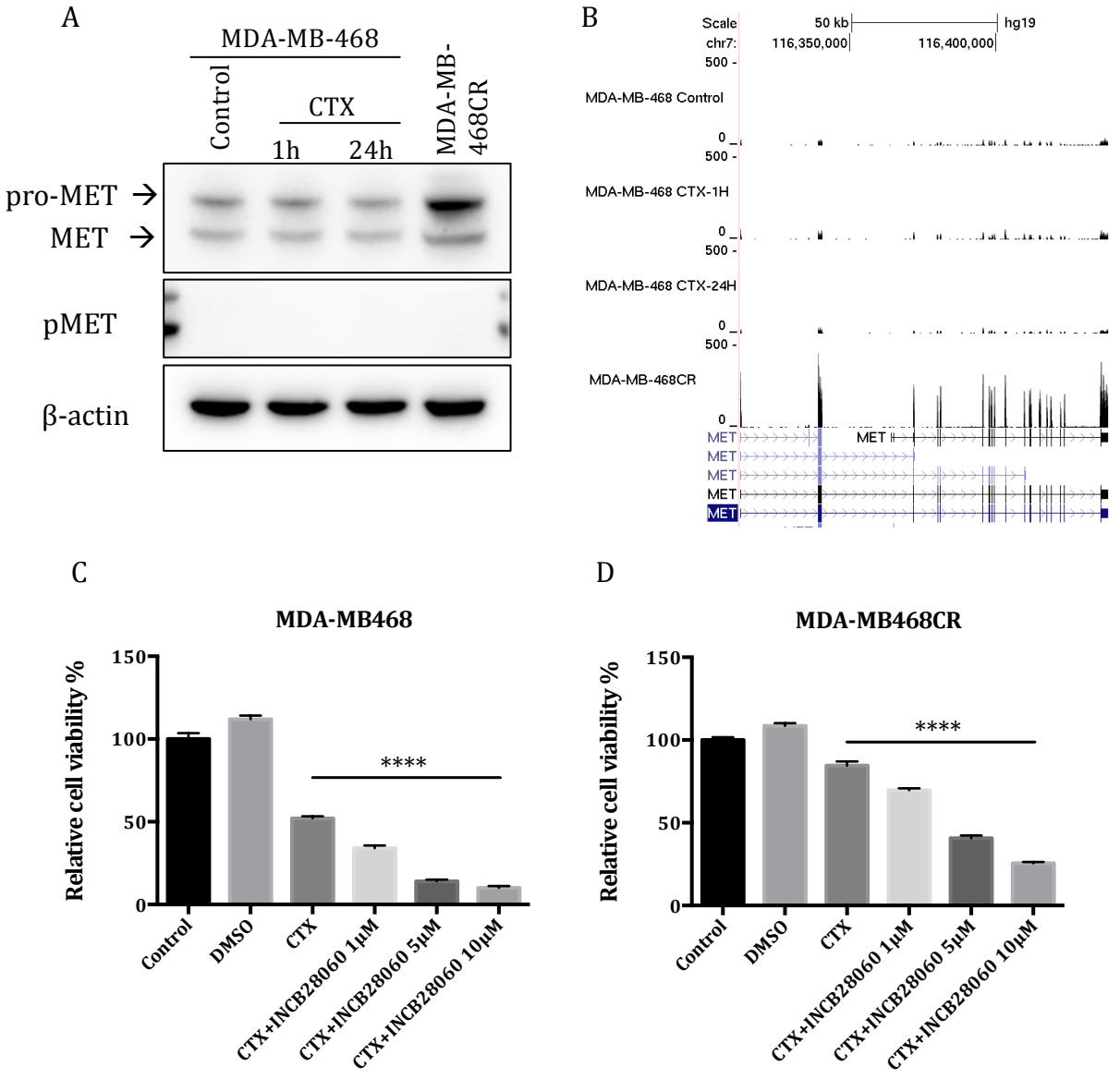


Figure 5.9 cMET expression in the acquired CTX resistant MDA-MB-468CR cells. **(A)** Western blot analysis of total and phospho MET in MDA-MB-468 and MDA-MB-468CR that were untreated or treated with CTX (10 μg/ml) for 1 hour and 24 hours and the MDA-MB-468CR cells. **(B)** A snapshot of the UCSC genome browser at *cMET* gene loci. Assessment of cell viability using CellTiter-Blue® cell viability assay kit of **(C)** MDA-MB-468 and **(D)** MDA-MB-468CR that were treated with CTX+/- INCB28060 for 6 days. The percentage of viable cells was shown as mean±S.E relative to the untreated cells (One-way ANOVA with Bonferroni's multiple comparisons tests, n=3).

5.10 Discussion

The development of acquired resistance to different cancer therapies including CTX is a major challenge in cancer treatment. Therefore, understanding the underlying mechanisms behind resistance might aid in designing novel combinatorial strategies to improve the therapeutic outcomes. Extensive efforts have been made during the last decade to investigate the mechanisms of acquired CTX resistance in different cancers including colorectal cancer (Montagut *et al.* 2012; Troiani *et al.* 2013), NSCLC (Wheeler *et al.* 2008; Yonesaka *et al.* 2011) and HNSCC (Hatakeyama *et al.* 2010). In TNBC patients, CTX has shown a limited efficacy as a single treatment and patients tend to progress (Carey *et al.* 2012). However, little is known about the mechanisms of primary as well as acquired CTX resistance in TNBC.

In this chapter, a model of acquired CTX-resistant TNBC cell line (MDA-MB-468CR) has been developed from the parental CTX-sensitive cell line (MDA-MB-468) to investigate the mechanisms of acquired CTX resistance. The resistant cells showed a significant shift in their sensitivity to CTX **(Figure 5.1A).** A model of acquired CTX-resistant colorectal cancer cells was morphologically similar to the parental cells (Montagut *et al.* 2012). Similarly, MDA-MB-468CR cells were also reported to be morphologically similar to MDA-MB-468 cells **(Figure 5.1B).** Oncogenic shifting and activation of parallel pathway(s) are common mechanisms of resistance to different anti-cancer therapies including CTX (Wheeler *et al.* 2008; Yonesaka *et al.* 2011). Using the phospho-RTK array system revealed an activation of four receptors (cMET, EphB2, PDGFR and TrkC) in MDA-MB-468CR cells **(Figure 5.3B)**. Amplification of *cMET* has been implicated in acquired resistance of lung cancer to gefitinib (Engelman *et al.* 2007). Additionally, there was a significant association between high cMET expression and PFS but not with

the overall response rate in CTX-treated mCRC patients (Inno *et al.* 2011). Wheeler, DL. *et al.* (2008) have reported a strong cMET activation, which was associated with an increase in EGFR/cMET dimerization in acquired CTX-resistant NSCLC cell lines. Nevertheless, neither cMET inhibition nor *cMET* knockdown restored their sensitivity to CTX and therefore might not be a critical element in acquired CTX resistance in their model (Wheeler *et al.* 2008). In MDA-MB-468CR cells, the current data indicated a significant increase of *cMET* mRNA and protein levels **(Figure 5.9A and B)** as well as an increase in cMET phosphorylation **(Figure 5.3A and B)**. Moreover, the addition of cMET TKI increased the response of both MDA-MB-468 and MDA-MB-468CR to CTX treatment **(Figure 5.9C and D)**. These interested observations suggested a potential role of cMET in acquired CTX resistance in TNBC. However, due to time limitations, further investigations of the role of cMET are being carried our by Dr Norma O'Donovan as a joint project.

Furthermore, CTX resistant cells, MDA-MB-468CR, exhibited an increase in EphB2, PDGFRα and TrkC phosphorylation. EphB2 is a member of the Eph RTK family that is divided into two types, EphA and EphB (Frisen *et al.* 1999). To my knowledge, EphB2 has not been reported to be associated with EGFR signalling. However, up-regulation of EphA2 has been induced by ligand-activated EGFR (Pedersen *et al.* 2005). Additionally, ligand activation of EGFR increased EGFR-EphA2 co-localization in EGFR-expressing cells (Larsen *et al.* 2007). PDGFRα is a member of the platelet-derived growth factor receptors, which includes PDGFRα and PDGFRβ (Williams 1989). Interestingly, the expression of PDGFRβ has been found to be higher after CTX treatment in rectal cancer patients compared with the pre-treatment samples (Erben *et al.* 2008). Nevertheless, there was not any correlation between sensitivity to CTX treatment and PDGFRα

expression. A recent study has reported a transactivation of TrkC by EGFR signalling (Puehringer *et al.* 2013). However, to the best of my knowledge, the effect of CTX on neither PDGFRα nor TrkC phosphorylation has been reported before.

Recent advances in genomic technologies provided a powerful tool to identify novel components of alternative pathways and helped to identify mechanisms of drug resistance (Huang *et al.* 2012; Huber-Keener *et al.* 2012; Balko *et al.* 2014). Using next-generation RNA-seq, Huber-Keener, K. J., *et al.*, have compared the transcriptome of tamoxifen- sensitive and resistant MCF-7 cells and identified multiple genes and cellular pathways that were up-regulated in tamoxifen-resistant cells. In addition, a comprehensive study of the molecular profiling of TNBC residual disease after neo-adjuvant chemotherapy has identified molecular alterations that could be targeted using currently available therapies (Balko *et al.* 2014).

In this study, the transcriptome of MDA-MB-468 cells that were treated with CTX as well as the acquired CTX-resistant MDA-MB-468CR cells were revealed using the RNA-seq technology. The analyses showed a significant deregulation of the transcription factor, *EGR1*, in both CTX-treated MDA-MB-468 and MDA-MB-468CR cells. Interestingly, *EGR1* has been reported as a tumour suppressor as well as an oncogene (Baron *et al.* 2006). This is in accordance with previous findings (Jing *et al.* 2009). Therefore, further investigations are required to conclude its role in relation to CTX response and resistance.

Given that the resistant cells, MDA-MB-468CR, harboured a significant up-regulation of a large number of genes, pathway analysis approach was used to investigate the up-regulated pathways rather than single genes that might be involved in CTX-resistance. This approach indicated an up-regulation of several pathways **(Section 5.4.3 and Table**

5.4). Another approach would be the large-scale RNA interference (RNAi) genetic screen to identify the determinant novel components of the signalling pathways that are involved in acquired CTX resistance. However, due to the time constraint and financial limitation, I have only have been able to validate the up-regulation of TGFβ/BMP signalling pathway in relation to CTX treatment and resistance in this project.

The paradox of TGFβ/BMP signalling pathway in cancer is that it acts as a tumour suppressor as well as an oncogene (Derynck *et al.* 2001; Tang *et al.* 2003). Balko JM. *et al.* (2014) have recently shown an activation of TGFβ responsive genes in drug-resistant residual disease of TNBC patients who did not achieve complete pathological response after neoadjuvant chemotherapy (Balko *et al.* 2014). The activation of TGFβ pathway has been reported to be involved in resistance to different drugs including ALK inhibitor and EGFR inhibitors. Bedi, A., *et al.* (2012) showed that CTX-resistant head and neck patients and cell lines exhibited a significant increase in TGFβ level which exerts an extrinsic inhibition of the ADCC and provides EGFR-independent survival through AKT activation. In addition, using a TGFβ antibody improved the anti-tumour efficacy of CTX in the HNSCC xenograft (Bedi *et al.* 2012). On the other hand, loss of MED12 induced an up-regulation of TGFβ receptor 2 (*TGFBR2*), which mediated resistance to EGFR TKI, gefetinib, in NSCLC cells (Huang *et al.* 2012). Emerging evidence suggested the involvement of BMP signalling in invasiveness and metastasis of different cancers (Helms *et al.* 2005; Bailey *et al.* 2007; Katsuno *et al.* 2008). However, to my knowledge, involvement and activation of BMPRs has not yet been reported to be involved in resistance to EGFR targeted therapies. Therefore, the activation of BMPRs and its consequences have been further investigated in this project. The bioinformatics analysis of current RNA-seq data indicated an up-regulation of several genes within the

TGFβ/BMP pathway. This was confirmed using the standard RT-qPCR analysis of the expression of some of these genes. Also, these data correlated with the increase in SMAD1/5/8 and AKT phosphorylation in the resistant cells relative to the parental cells. This is in parallel with the previous studies that indicated that both TGFβ and BMP mediate AKT phosphorylation (Bakin *et al.* 2000; Gangopahyay *et al.* 2011), which provides an intrinsic EGFR-independent survival of tumour cells (Bedi *et al.* 2012). Collectively, these data confirmed the activation of BMPRs in CTX-resistant MDA-MB-468CR cells, which may be involved in their resistance.

Huang, S. *et al.* (2012) have shown that combining the inhibition of TGFBR with TKIs could be a new strategy to treat tumours with elevated TGFβ signalling and reverse resistance to targeted cancer therapies such as gefitinib (Huang *et al.* 2012). Moreover, combining CTX and TGFβ antibody enhanced the anti-tumour efficacy of CTX in both parental CTX-sensitive and derived acquired CTX-resistant HNSCC xenografts (Bedi *et al.* 2012). Consistently, the current data indicated that combining CTX and BMPR inhibitor significantly improved the anti-tumour efficacy of CTX in both parental CTX-sensitive and derived acquired CTX-resistant MDA-MB-468 cells and significantly reduced the growth of MDA-MB-468CR spheroids in 3D models. Furthermore, the anti-tumour efficacy of CTX in parental MDA-MB-468 cells was counteracted by stimulation with the BMPR ligand (BMP6). This is in contrast to the previous findings, which have shown that induction of BMP6 significantly inhibits cell proliferation of the MDA-MB-231 breast cancer cell line (Hu *et al.* 2013). This could be explained by the differences between these two cell lines, which differ in their gene expression and ontologies as a result of the heterogeneity between TNBC subtypes (Lehmann *et al.* 2011).

Overall, the current study has identified the activation of the TGFβ/BMP pathway as a key component in CTX-resistance and showed that inhibiting BMPRs using a selective BMPR inhibitor (K02288) could overcome CTX-resistance in our preclinical TNBC model. Given that activation of BMPR/SMAD pathway has been reported to be involved in the progression of estrogen-positive breast cancer (Helms *et al.* 2005) and chemo-resistant TNBC patients exhibited an activation of TGF/BMP pathway, these results provide a rationale for combinatorial targeting of both EGFR and the TGFβ/BMP receptors in CTX-resistant TNBC.

6 Discussion

6.1 Summary and conclusion

TNBC accounts for about 15-20% of breast cancers, which has worse outcomes in comparison to hormone receptor positive tumours (Bauer *et al.* 2007; Reis-Filho *et al.* 2008; Metzger-Filho *et al.* 2012). In this study, the analysis of the *EGFR* expression in breast cancer tumours from the METABRIC dataset, as well as the panel of breast cancer cell lines, confirmed the previous observation that EGFR is highly expressed in TNBC and basal-like compared to non-TNBCs tumours and cell lines. Due to the lack of defined molecular target in TNBCs, chemotherapy remains the main therapeutic option for those patients. Therefore, targeting EGFR could be an alternative therapeutic option for those patients. Preclinical data suggested that some TNBC cell lines are sensitive to CTX with or without chemotherapeutic agents (Hoadley *et al.* 2007; Oliveras-Ferraros *et al.* 2008), while other studies have shown contradictive results (Brand *et al.* 2014). Moreover, targeting EGFR using CTX has shown limited efficacy in TNBC patients (Carey *et al.* 2012; Baselga *et al.* 2013). Therefore, it is highly important both to understand the underlying mechanism(s) behind the poor response of TNBC patients to CTX and to find the biomarkers that predict the response to CTX in order to select the subset of TNBC patients who would benefit from CTX treatment.

In this project, the sensitivity of a panel of TNBC cell lines to CTX was investigated and correlated with EGFR expression. The data from this study revealed a variation in their EGFR expression as well as their sensitivity to CTX treatment. This suggests that EGFR expression may not predict CTX sensitivity in some of these cells or that some of the TNBC cells might not be dependent on EGFR signalling for their survival and growth. However, a recent study indicated that, although some of the TNBC cell lines were resistant to CTX, they were still dependent on EGFR for their proliferation (Brand *et al.*

2014). On the other hand, CTX has shown promising results in patients with different tumours such as HNSCC and mCRC, indicating its efficacy in the treatment of other cancers. However, even in these patients, only a small subset of patients responds to CTX. Collectively, these findings suggested that there is still a poor understanding of patient selection criteria for CTX treatment in different cancer types; and there may be other factors, which might contribute to the poor response of TNBC cells to CTX.

EGFR amplification, but not expression, has been shown to be predictive of CTX response in HNSCC and colon cancer (Cunningham *et al.* 2004; Chung *et al.* 2005; Moroni *et al.* 2005; Hirsch *et al.* 2008; Personeni *et al.* 2008). In this study, there was a positive, but not statistically significant, correlation between basal *EGFR* expression in TNBC cells and their sensitivity to CTX. This interesting finding could be investigated further in a larger panel of TNBS cell lines and correlated with the expression of EGFR and other ErbB receptors, as well as other tyrosine kinase receptors that have been reported to interact with EGFR, such as cMET.

The analysis of the EGFR signalling pathway in this study confirmed the previous findings, whereby CTX decreases the phosphorylation of the downstream biomarkers, AKT and ERK1/2, but can not significantly decrease EGFR phosphorylation in CTX sensitive cells. As discussed in **(Section 3.12)**, this could be attributed to different reasons and feedback loops that maintain EGFR phosphorylation. Importantly, the current study shows that CTX triggers the release of endogenous EGFR ligands, which could counteract the inhibitory effect of CTX on pEGFR. This is supported by the data showing that the efficacy of CTX is reduced by the presence of exogenous ligands in a dose-dependent manner. Several studies have shown that combining CTX with other EGFR mAbs that recognize different epitopes has a superior effect to using CTX alone,

both in inhibiting the EGFR signalling and in cell proliferation (Kamat *et al.* 2008; Hong *et al.* 2010; Ferraro *et al.* 2013). Together, these findings suggest that CTX, as a single agent, might not be able to completely block ligand binding to EGFR signalling due to the compensatory release of EGFR ligands.

Different ADAMs, such as ADAM10 and ADAM17, mediate the release of the active ligands from the endogenous EGFR pro-ligands. Recent studies showed that inhibiting ADAM17, or both ADAM10 and ADAM17, enhances the response of TNBC cells to different cytotoxic agents and to ErbB TKIs (McGowan *et al.* 2013). Therefore, in this study, it was hypothesized that inhibiting the release of endogenous EGFR ligands, through the inhibition of ADAMs' activities, might potentiate the effect of CTX treatment. As expected, using the dual ADAM10/17 inhibitor, INCB3619, significantly reduced the CTX-induced release of EGFR ligands. This was correlated with a minor but enhanced inhibitory effect on EGFR, AKT and ERK1/2 phosphorylation. In addition, combining INCB3619 and CTX increased the anti-tumour effect of CTX in the primary CTX-sensitive cell lines as well as in the acquired CTX-resistant cells *in vitro*. Further validations using a xenograft model also confirmed the *in vitro* data. Although INCB3619 significantly reduced the CTX-induced release of endogenous EGFR ligand and was additive in the anti-tumour effect with CTX, the combination could only reduce the pEGFR level slightly more than CTX alone. One of the possible explanations is that, although INCB3619 could reduce the CTX-induced release of the endogenous EGFR ligand, it would not have an effect on the existing EGFR ligands in the medium, since it was started together with CTX during experiments. In addition, the possibility could not be excluded that the enhanced anti-tumour effect could be attributed to the inhibition of other signalling

pathways that are regulated by ADAM10 and ADAM17, such as notch (Bozkulak *et al.* 2009), which has been shown to be hyper activated in TNBC (Lee *et al.* 2008).

Targeting EGFR using either mAbs or TKIs has shown promising responses in different cancers. However, the development of acquired resistance to these agents could limit their efficacy. Studies of preclinical models provide mechanistic insights that help designing combinatory treatment or new treatment strategies to overcome both intrinsic and acquired resistance. Comprehensive studies have been conducted in several preclinical acquired CTX-resistant models and patient tumour specimens. However, to my knowledge, little is known about the underlying mechanisms of acquired CTX resistance in TNBC.

In this project, a model of acquired CTX-resistant TNBC cell line was developed in order to identify the molecular alterations that might be involved in the development of CTX resistance. After prolonged treatment with CTX, the parental MDA-MB-468 cells acquired drug resistance with reduced sensitivity to CTX treatment. Removal of CTX for several passages did not affect their sensitivity (data not shown) but they were morphologically similar to their parental cells. The RTK array profiling of the phosphorylation status of 49 RTKs revealed an activation of 4 receptors including cMET, which has been previously shown to be involved in acquired CTX resistance in an NSCLC model. Further studies of the role of the activated tyrosine kinase receptors in the resistant cells might provide greater insight into the acquired resistance mechanism(s) to CTX.

Furthermore, transcriptome profiling of both parental and acquired CTX-resistant cells revealed a deregulation of several pathways, including an up-regulation of the TGFβ/BMP signalling pathway. The increase in *TGFβ* expression has been involved in

the acquired CTX resistance in head and neck patients and cell lines (Bedi *et al.* 2012). However, the up-regulation and activation of BMPRs have not been reported in acquired CTX resistance. In the MDA-MB-468CR cells, there was an up-regulation of TGFBRs and BMPRs. The increase in BMPRs expression was associated with the increase in SMADs phosphorylation. Moreover, inhibiting BMPRs using a selective BMPR small molecule inhibitor overcame CTX resistance and restored their sensitivity. Further validations, using siRNAs, could be performed in the future to determine the involvement of each receptor. Additional *in vivo* validation might also be important to confirm these findings. Collectively, these results provide a rationale for targeting both EGFR and TGFBR/BMPRs in acquired CTX-resistant TNBC. Given that the majority of TNBC cell lines were primary resistant to CTX treatment and combining CTX with ADAM10/17 inhibitor had an additive effect only in CTX-sensitive cells, targeting both TGBRs and/or BMPRs with CTX could be further investigated in TNBC cell lines. Lehmann, B.D *et al.* (2011) have shown that both M and MSL TNBC tumours were enriched in cell differentiation pathways including genes of the TGFβ/BMP signalling pathway (Lehmann *et al.* 2011). Therefore, targeting this pathway could be an effective approach in a subpopulation of TNBCs either alone or in combination with other chemotherapies and targeted therapies. Recently, Balko JM. *et al.* (2014) have shown an activation of TGFβ responsive genes in drug-resistant residual disease of TNBC tumours (Balko *et al.* 2014) and inhibiting TGBR1 in TNBC cell lines and xenografts prevented the development of paclitaxel-resistant cancer stem-like cells (Bhola *et al.* 2013). Collectively, results from the current study along with the previous studies suggest that TNBC is a heterogeneous disease that consists of several subtypes and this variation should be considered in the further investigation of the efficacy of different targeted therapies including the EGFR and TGFβ/BMP inhibitors.

6.2 Future work

The lack of an identified molecular target, and the discrepancies in the preclinical data and the clinical trials findings, might be attributed to the heterogeneity of TNBCs. Previously, EGFR has been reported to be overexpressed in up to 70% of TNBCs. However, recent data from the TCGA showed that EGFR is overexpressed in only 22% of the basal-like/TNBC tumours. Analysis of the METABRIC data s*et al*so revealed heterogeneity in the expression of EGFR within breast cancer tumours. Moreover, the expression of EGFR alone might not be a sufficient predictive biomarker for EGFR targeted therapies. It would have been very useful to have CTX-treated human TNBC samples to confirm the preclinical findings in this study. However, since CTX is not an approved treatment for TNBC patients, and there are only a few clinical trials on CTX (either as a single agent or in combination with chemotherapies) in TNBC and basal-like patients, it was not possible to obtain patient samples for further investigations.

One way to compensate for this limitation would be to use patient-derived tumour xenograft models, which have been used increasingly as a tool in cancer research (Hidalgo *et al.* 2014). These models, along with the advantages and the development in the next generation of sequencing and genome wide siRNA, could be a very useful approach to further assess the biomarkers that predict response to CTX treatment in TNBC, as well as the molecular determinant of primary and acquired resistance.

The interesting additive effect of inhibiting ADAM10/17 on the response to CTX in this project could be further investigated to determine the molecular mechanisms by which inhibiting ADAMs could increase the response to CTX, other than the effect on EGFR ligands. This could be due to an inhibition of the release of different cytokines and pro-

ligands that are ADAM-mediated, as well as the inhibition of the cleavage and subsequent activation of different receptors. This could be done *in vitro* or *ex vivo* using tumour cells cultured from xenograft tissues.

Finally, the development of acquired CTX-resistant models derived from different TNBC cell lines and/or patient derived xenografts could be used to validate our current data from the MDA-MB-468CR cells. Furthermore, combining data from genomic, transcriptomic and proteomic profiling of these cell lines, along with genome-wide siRNA screening, could help in identifying the genes and pathways that are involved in the development of acquired CTX resistance in TNBC and in designing new strategies to overcome CTX resistance.

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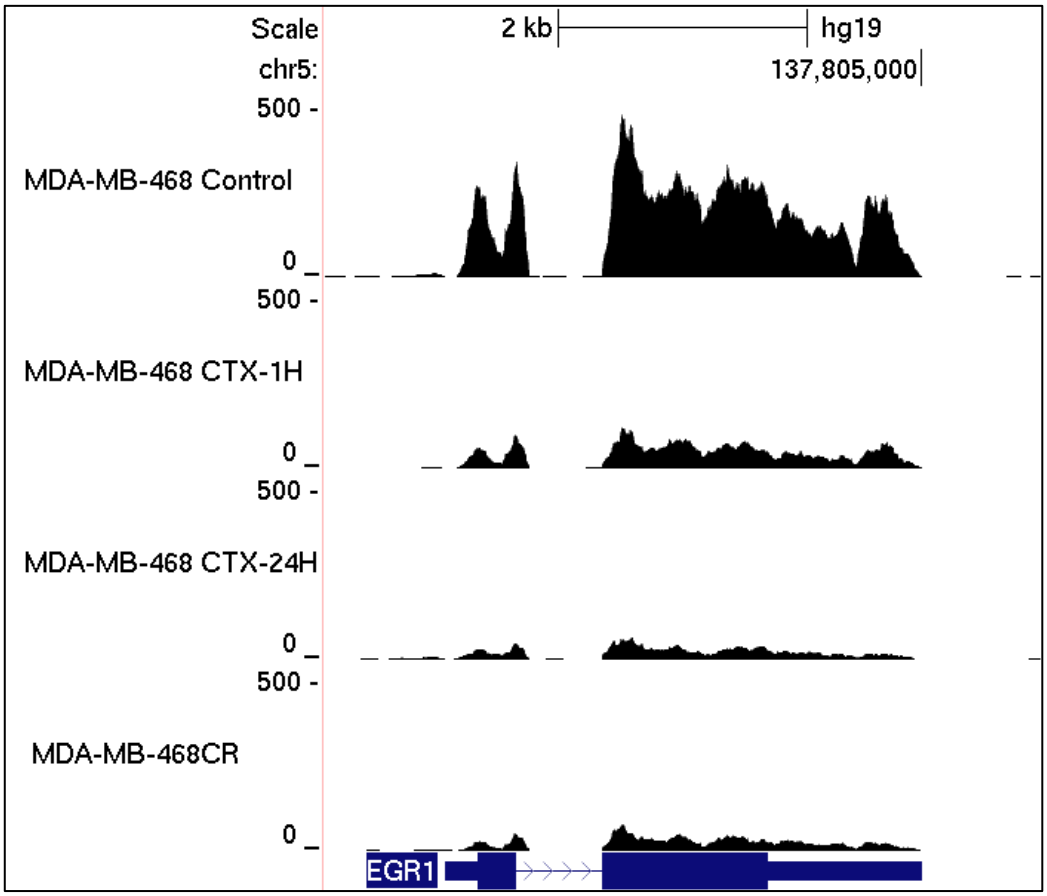
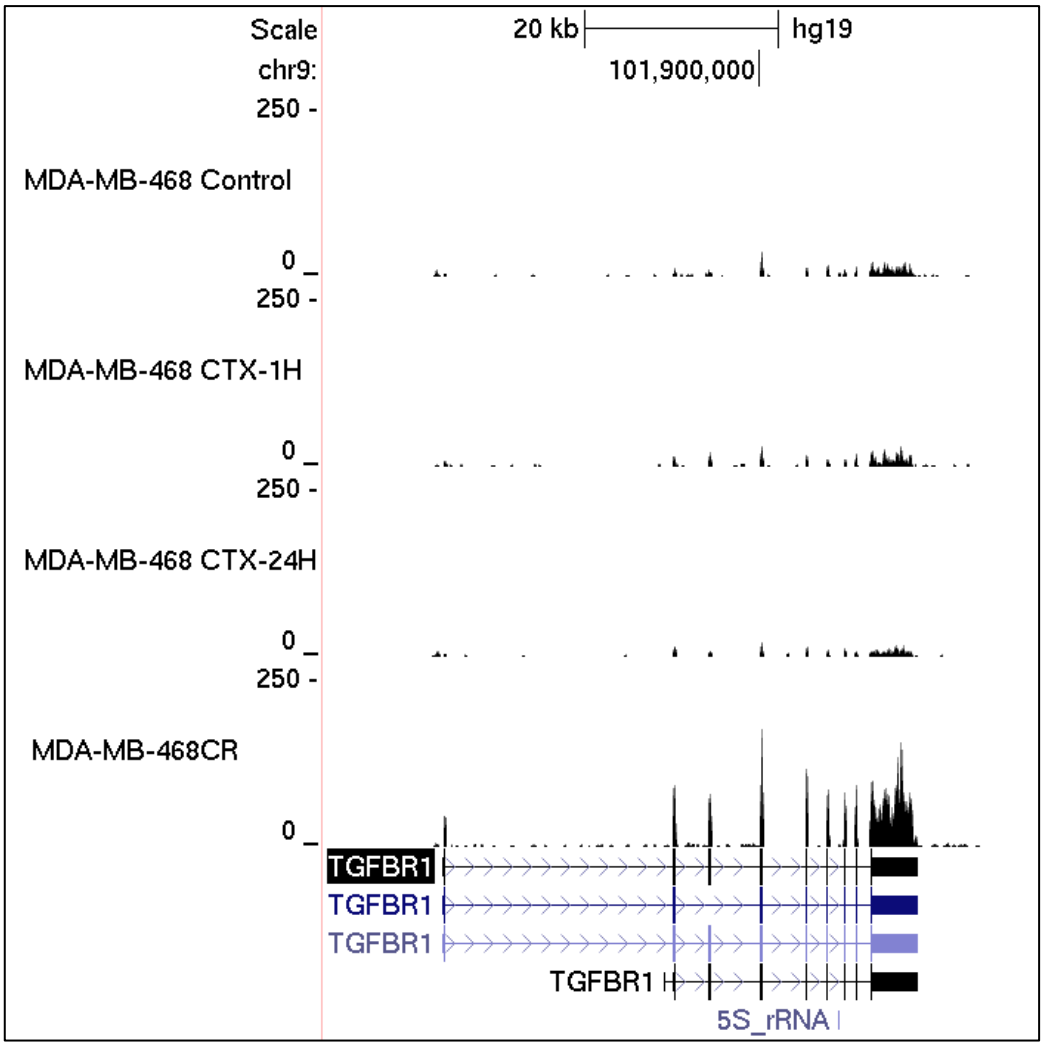
8 Appendix

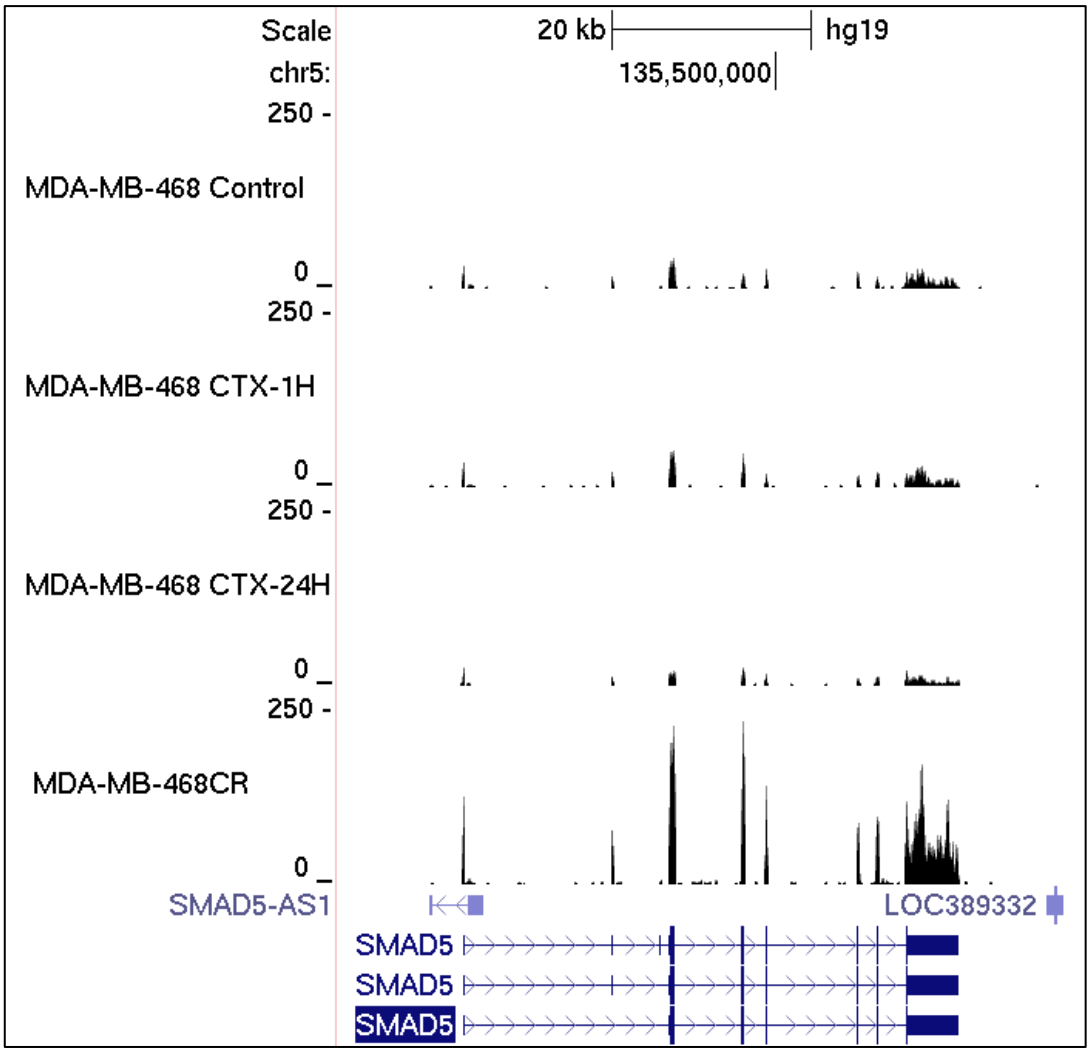
Supplementary Figure S1. A snapshot of the UCSC genome browser at FOS gene loci.



Supplementary Figure S2. A snapshot of the UCSC genome browser at TGFBR1 gene loci.



Supplementary Figure S3. A snapshot of the UCSC genome browser at SMAD5 gene loci.



Supplementary Table S.1 Differentially expressed genes induced by 24 hours CTX treatment in MDA-MB-468 cells

Gene ID	Full name	Log2 FC	FDR
Up-regulated genes (24 hours)			
HIST1H2BA	Histone cluster 1, H2ba	5.53	0.0006
LOC100288570	Glycosylphosphatidylinositol anchor attachment protein 1 homolog (yeast) pseudogene	5.29	0.0088
LOC440895	Two pore channel 3 pseudogen	5.29	0.0088
S100A7L2	S100 calcium binding protein A7-like 2	2.05	0.0097
KLHL38	kelch-like family member 38	1.47	1.87E-07
USH1G	Usher syndrome 1G (autosomal recessive)	1.45	7.65E-05
CXCL17	Chemokine (C-X-C motif) ligand 17	1.36	0.0028
DDIT4L	DNA-damage-inducible transcript 4-like	1.35	0.0002
IL32	Interleukin 32	1.34	5.76E-05
FAM107A	Family with sequence similarity 107, member A	1.32	8.74E-05
PLCH2	Phospholipase C, eta 2	1.28	0.0014
CYP4F12	Cytochrome P450, family 4, subfamily F, polypeptide 12	1.24	0.0010
ADHFE1	Alcohol dehydrogenase, iron containing, 1	1.23	0.0021
ALDH3A1	Aldehyde dehydrogenase 3 family, member A1	1.23	3.11E-11
LOC101927495	Uncharacterized LOC101927495	1.22	0.0058
CYP4Z1	Cytochrome P450, family 4, subfamily Z, polypeptide 1	1.22	1.07E-10
NCALD	Neurocalcin delta	1.21	0.0014
Down-regulated genes (24 hours)			
EGR1	Early growth response 1	-2.87	4.908E-74
IL1R2	Interleukin 1 receptor, type II	-1.95	0.0004
KIAA1199	KIAA1199	-1.51	2.72E-05
FOSL1	FOS-like antigen 1	-1.42	8.34E-13
ZBED2	Zinc finger, BED-type containing 2	-1.42	0.0006
IL7R	Interleukin 7 receptor	-1.38	0.0031
ACOX2	Acyl-CoA oxidase 2, branched chain	-1.35	5.94E-07
DOK7	Docking protein 7	-1.32	4.78E-08
AREG	Amphiregulin	-1.32	3.46E-07
ETV4	Ets variant 4	-1.31	6.24E-12
DUSP6	Dual specificity phosphatase 6	-1.29	7.58E-09

Supplementary Table S.2 The top differentially up-regulated genes induced MDA-MB-468CR relative to MDA-MB-468 cells

Gene ID	Full name	Log2 FC	FDR
LRP2	Low density lipoprotein receptor-related protein 2	8.51	2.52E-88
DLC1	Deleted in liver cancer 1	8.51	2.32E-23
SDC2	Syndecan 2	8.35	2.06E-21
CACNA2D1	Calcium channel, voltage-dependent, alpha 2/delta subunit 1	7.95	6.13E-16
LDOC1	Leucine zipper, down-regulated in cancer 1	7.53	9.82E-38
TMEM47	Transmembrane protein 47	7.23	5.72E-11
PLAG1	Pleiomorphic adenoma gene 1	6.82	4.36E-09
PLCB1	Phospholipase C, beta 1 (phosphoinositide-specific)	6.56	1.83E-11
PROX1	Prospero home box 1	6.54	6.87E-21
KCNT2	Potassium channel, subfamily T, member 2	6.48	1.26E-10
CPQ	Carboxypeptidase Q	6.47	1.59E-57
NELL2	NEL-like 2 (chicken)	5.91	1.12E-11
ABCB11	ATP-binding cassette, sub-family B (MDR/TAP), member 11	5.74	3.48E-06
TKTL1	Transketolase-like 1	5.69	8.04E-07
BCAT1	Branched chain amino-acid transaminase 1, cytosolic	5.54	6.95E-16
DCD	Dermcidin	5.47	7.92E-05
ZCWPW2	Zinc finger, CW type with PWWP domain 2	5.45	0.0011
GFRA1	GDNF family receptor alpha 1	5.37	4.57E-43
BTNL9	Butyrophilin-like 9	5.31	1.26E-11
ZC4H2	Zinc finger, C4H2 domain containing	5.31	3.49E-34
GPC6	Glypican 6	5.20	0.0005
LOC643733	Caspase 4, apoptosis-related cysteine peptidase pseudogene	5.16	6.70E-28
RNF150	Ring finger protein 150	5.15	1.80E-76
FMO1	Flavin containing monooxygenase 1	5.14	7.37E-05
MIR3125	MicroRNA 3125	5.11	0.0020
NPR3	Natriuretic peptide receptor C/guanylate cyclase C (atrionatriuretic peptide receptor C)	4.99	3.81E-92
PURG	Purine-rich element binding protein G	4.96	0.0031
MIR3651	MicroRNA 3651	4.94	0.0016
MIR4440	MicroRNA 4440	4.91	0.0004
LOC101927150	Uncharacterized LOC101927150	4.90	0.0004
ANKRD34C	ankyrin repeat domain 34C	4.89	0.0003

MSH4	mutS homolog 4	4.87	0.0005
MIR30C2	MicroRNA 30c-2	4.86	0.0003
LOC151171	Uncharacterized LOC151171	4.84	0.0014
ANGPT1	angiopoietin 1	4.81	6.48E-14
PIK3C2G	Phosphatidylinositol-4-phosphate 3-kinase, catalytic subunit type 2 gamma	4.81	0.0010
TMEM89	Transmembrane protein 89	4.80	0.0042
OR13J1	Olfactory receptor, family 13, subfamily J, member 1	4.79	0.0006
MIR630	MicroRNA 630	4.74	0.0007
PDCL2	phosducin-like 2	4.73	0.0023
TMSB15A	thymosin beta 15a	4.68	9.90E-54
ALPK2	Alpha-kinase 2	4.68	1.43E-66
MIR374B	MicroRNA 374b	4.66	0.0032
SLC26A10	Solute carrier family 26, member 10	4.63	0.0025
PGR	Progesterone receptor	4.62	0.0082
FRMPD4	FERM and PDZ domain containing 4	4.61	0.0016
LINC00968	Long intergenic non-protein coding RNA 968	4.60	1.76E-25
TAS2R31	Taste receptor, type 2, member 31	4.56	0.0052
COL19A1	Collagen, type XIX, alpha 1	4.50	0.0016
CNTN5	contactin 5	4.50	0.0016
LOC255130	Uncharacterized LOC255130 (LOC255130), non-coding RNA	4.49	1.37E-08
MIR181A1HG	MIR181A1 host gene (non-protein coding)	4.48	0.0032
TEX15	Testis expressed 15	4.48	0.0031
MIR3662	MicroRNA 3662	4.46	0.0037
ETNPPL	Ethanolamine-phosphate phospho-lyase	4.45	2.57E-33
HEPH	hephaestin	4.44	2.34E-08
MIR3939	MicroRNA 3939	4.42	0.0113
ART4	ADP-ribosyltransferase 4 (Dombrock blood group)	4.38	0.0100
TSPEAR-AS1	TSPEAR antisense RNA 1	4.37	1.30E-09
MIR374C	MicroRNA 374c	4.35	0.0060
CHRM5	Cholinergic receptor, muscarinic 5	4.35	0.0040
RTN1	reticulon 1	4.35	4.95E-16
CD200R1	CD200 receptor 1	4.34	0.0029
LOC101929633	Uncharacterized LOC101929633	4.34	0.0096
ADAMTS5	ADAM metallopeptidase with thrombospondin type 1 motif, 5	4.31	8.80E-37
MIR421	MicroRNA 421	4.28	0.0125
TRPM3	Transient receptor potential cation channel, subfamily M, member 3	4.23	0.0163

GIPC2	GIPC PDZ domain containing family, member 2	4.22	0.0171
GNGT1	Guanine nucleotide binding protein (G protein), gamma transducing activity polypeptide 1	4.20	0.0172
SNORD51	Small nucleolar RNA, C/D box 51	4.19	0.0296
SERPIND1	serpin peptidase inhibitor, clade D (heparin cofactor), member 1	4.18	0.0113
TSPEAR	thrombospondin-type laminin G domain and EAR repeats	4.18	4.10E-30
C1orf186	Chromosome 1 open reading frame 186	4.17	0.0211
CIB4	Calcium and integrin binding family member 4	4.17	0.0105
SLC5A9	Solute carrier family 5 (sodium/sugar cotransporter), member 9	4.16	0.0132
C21orf90	Chromosome 21 open reading frame 90	4.16	0.0001
DLEU7-AS1	DLEU7 antisense RNA 1	4.15	0.0069
LGR5	Leucine-rich repeat containing G protein-coupled receptor 5	4.14	0.0172
LDHAL6B	Lactate dehydrogenase A-like 6B	4.14	0.0080
MIR135B	MicroRNA 135b	4.14	0.0164
SCARNA8	Small Cajal body-specific RNA 8	4.14	0.0099
GDF3	Growth differentiation factor 3	4.12	0.0195
LOC442497	Uncharacterized LOC442497	4.11	1.02E-10
LINC00359	Long intergenic non-protein coding RNA 359	4.11	0.0170
WNT16	Wingless-type MMTV integration site family, member 16	4.10	0.0228

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