

Investigating the role of ADAM10 and ADAM17 in cetuximab resistance in head and neck squamous cell carcinoma

Sedigeh Kareemaghay

University of Oxford, Lincoln College

This thesis is submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Trinity term
August 2014

Supervisors:
Professor Len Seymour
Dr. Anthony Kong
Dr. Ioannis Roxanis

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ABSTRACT

Epithermal Growth Factor Receptor (EGFR) is overexpressed in up to 90% of head and neck squamous cell carcinoma (HNSCC). Cetuximab is the first and the only anti-EGFR monoclonal antibody which received approval from FDA for the treatment of HNSCC. However, most patients either do not respond to cetuximab or develop acquired resistance. The aim of my D.Phil. study was to investigate the role of ADAM10 and 17 in resistance mechanisms of cetuximab in HNSCC. Chronic exposure to cetuximab led to an activation of HER receptors and downstream signalling pathways in HNSCC cell lines. Higher levels of ADAM10 and 17 and their substrates, BTC and NRG-1 were found in cetuximab resistant cells compared to their parental cells, suggesting the involvement of ADAM-mediated ligands' release in reactivation of HER receptors. Inhibition or knockdown of ADAM10 and 17 enhanced cetuximab response and reversed cetuximab resistance in HNSCC cells. In addition, results from this study showed that the combination of cetuximab with EGFR-TKIs had greater effect in parental cells and reversed cetuximab resistance in HNSCC cells. Upregulation of ADAM10 and 17 also was observed in HNSCC TMAs compared to normal head and neck TMAs. High nuclear ADAM10 and cytoplasmic ADAM17 expression levels were associated with shorter DFS. By evaluating tumour excision samples from HNSCC patients who underwent a cetuximab window study high ADAM10 and 17 expression levels were found to be associated with poor response to cetuximab. In conclusion cetuximab-induced ADAM-mediated ligands' release is a potential mechanism of resistance to cetuximab in HNSCC. Thus, targeting ADAM10/17 or subsequent HER activations may represent an important strategy in overcoming resistance to cetuximab in HNSCC. Results from this study also suggest the implication of ADAM10 and 17 as potential prognostic and predictive biomarkers in HNSCC although further validation is required.

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LIST OF ABBREVIATION

- ADAM: A Disintigrin And Metalloprotease
- ADH: Alcohol Dehydrogenase
- AEs: Adverse Events
- ALDH2: Aldehyde Dehydrogenase 2
- AR: Amphiregulin
- ARF: Alternate Reading Frame
- ATCC: American Type Culture Collection
- BAG3: BCL2-associated athanogene 3
- Bax: Bcl-2–associated X protein
- Bcl-2: B-cell lymphoma 2 protein
- bFGF: Basic Fibroblast Growth Factor
- BID: Twice a day
- BIS-TRIS: Bis (2-hydroxyethyl) amino-tris (hydroxymethyl) methane
- BSA: Bovine Serum Albumin
- BSC: Best Supportive Care
- BTC: β-cellulin
- c-Cbl: Cbl proto-oncogene, E3 ubiquitin protein ligase
- CCND1: Cyclin D1
- CCR: Complete Clinical Response
- CDK: Cyclin Dependent Kinase
- CDKN: Cyclin Dependent Kinase Inhibitor
- cDNA: Complementary DNA
- c-erbB: Erythroblastic Leukemia Viral Oncogene Homolog
- CO_2: Carbon Dioxide

- CRC: Colorectal Cancer
- CRT: Chemo-Radiation Therapy
- CYP2E1: Cytochrome P450 2E1
- DAB: 3,3'-Diaminobenzidine
- DCR: Disease Control Rate
- dH_2O: de-ionised H_2O
- DLT: Dose-Limiting Toxicity
- DMEM／F-12: DMEM F-12 Nutrient Mixture (Ham)
- DMEM: Dulbecco’s Modified Eagle Medium
- DMSO: Dimethyl Sulfoxide
- DNA: Deoxyribonucleic Acid
- DNA-PK: DNA-dependent protein kinase
- DNase: Deoxyribonuclease
- dNTP: Deoxyribonucleoside Triphosphates
- DR5: Death Receptor-5
- ECL: Enhanced Chemiluminescence
- ECL: Enhanced Chemi-Luminescence
- EDTA: Ethylenediaminetetraacetic Acid
- EGF: Epidermal Growth Factor
- EGFR: Epithermal Growth Factor Receptor
- ELISA: Enzyme-Linked Immunosorbant Assay
- EPR: Epiregulin
- ERK: Extracellular-Signal-Regulated Kinase
- ESCC: Esophageal Squamous Cell Carcinoma
- FBS: Fetal Bovine Serum Gold

- FBXW7: F-Box and WD Repeat Domain Containing 7
- FDA Food and Drug Administration
- FDG: Fluorodeoxyglucose
- FDG-PET: Fluorodeoxyglucose-Positron Emission Tomography
- FFPE: Formalin Fixed Paraffin Embedded
- FISH: Fluorescence In Situ Hybridization
- HB-EGF: Heparin-Binding EGF-like growth factor
- HCC: Hepatocellular Carcinomas
- HER: Human Epidermal Growth Factor Receptor
- HGFR: Hepatocyte Growth Factor Receptor
- HNSCC: Head and Neck Squamous Cell Carcinoma
- HOXA11: Homeobox Protein
- HPV: Human Papillomavirus
- HR: Hazard ratio
- IgG: Immunoglobulin G
- IHC: Immunohistochemistry
- IL-8: Interleukin-8
- IMRT: Intensity-Modulated RT
- ING1: Inhibitor of Growth Family, member 1
- IR: Ionizing radiation
- IRS: Immunoreactive score of Remmele and Stegner
- KCL: Potassium chloride
- KH_2PO_4: Potassium phosphate monobasic
- LCIS: Lobular Carcinoma In Situ
- LDS: Lithium Dodecyl Sulfate

- mAb: Monoclonal Antibody
- MAPK: Mitogen-Activated Protein Kinase
- MDGI: Mammary Derived Growth Inhibitor
- MDM2: Murine Double Minute 2
- MEK: Mitogen-Activated ERK Kinase
- MET: Mesenchymal Epithelial Transition Factor
- miRNA: Micro Ribonucleic Acid
- MMPs: Matrix Metalloproteinases
- mRNA: Messenger Ribonucleic Acid
- MTT: 3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrzolium bromide
- $Na_2H_2P_2O_7$: Sodium pyrophosphate dibasic
- Na_2HPO_4: Sodium phosphate dibasic
- NaCl: Sodium chloride
- NaF: Sodium fluoride
- NNK: N-nitrosamines 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
- NNN: N'-nitrosonornicotine
- NRGs: Neuregulins
- NSCLC: Non-Small Cell Lung Cancer
- ORR: Objective Response Rate
- OS: Overall Survival
- OSCC: Oral Squamous Cell Carcinoma
- p21(WAF1/CIP1): cyclin-dependent kinase inhibitor 1/CDK interacting protein1
- p27(KIP1): cyclin-dependent kinase inhibitor 1B
- PAH: Polycyclic Aromatic Hydrocarbons

- pAKT: Phosphorylated AKT
- PBS: Phosphate Buffered Saline
- PBST: PBS-Tween-20
- PDCD4: Programmed Cell Death 4
- PEGFR: Phosphorylated EGFR
- pERK: Phosphorylated ERK
- PFA: Paraformaldehyde
- PFS: Progression Free Survival
- PHER: Phosphorylated HER
- PI3K-PKB-AKT: Phosphatidylinositol 3′ Kinase-Protein Kinase B
- PIK3CA: Phosphatidylinositol-4,5-Bisphosphate 3-Kinase, Catalytic Subunit Alpha
- PIK3R1: Phosphatidylinositol 3-Kinase Regulatory Subunit Alpha
- PLC-PKC: Phospholipase C-Protein Kinase C
- PR: Partial Responses
- pRb: Retinoblastoma Protein
- PSA: Prostate-Specific Antigen
- PTEN: Phosphatase and Tensin Homolog
- PVDF: Polyvinyl Difluoride
- qRT-PCR: Quantitative Reverse Transcriptase Polymerase Chain Reaction
- Ras: Rat Sarcoma
- Ras-MAPK: Ras–Mitogen-Activated Protein Kinase
- RIPA Buffer: Radio-Immunoprecipitation Assay Buffer
- RNA: Ribonucleic Acid

- ROS: Reactive Oxygen Species
- RPM: Revolutions Per Minute
- RT: Radiotherapy
- RT: Reverse Transcription
- RTK: Receptor Tyrosine Kinase
- RTKs: Receptor Tyrosine Kinases
- RT-PCR: Reverse Transcriptase Polymerase Chain Reaction
- SCC: Squamous Cell Carcinoma
- SD: Stable Disease
- SDS: Sodium Dodecyl Sulphate
- SFKs: Src Family Kinases
- SIB-IMRT: Simultaneous Integrated Boost Intensity-Modulated RT
- siRNA: Small interfering RNA
- STAT: Signal Transducer and Activator of Transcription
- SUV: Standardized Uptake Value
- TGF-α: Transforming Growth Factor -α
- TIMPs: Tissue Inhibitors of Metalloproteinases
- TKIs: Tyrosine Kinase Inhibitors
- TMAs: Tissue Microarrays
- TNFRSF10B: Tumour Necrosis Factor Receptor Superfamily, Member 10B
- Tp53: p53 Tumour Suppressor Protein
- Tp63: Tumour Protein 63
- TRIS-HCL: Tris (hydroxymethyl) aminomethane hydrochloride
- TTP: Time To Progression

- VEGF: Vascular Endothelial Growth Factor
- VEGFR1: Vascular endothelial growth factor receptor-1
- 5-FU: 5-florouracil
- ^{18}F-FDG: ^{18}F-Fluorodeoxyglucose
- ΔSUVmax: Delta Maximal Standardized Uptake Values

ACKNOWLEDGEMENTS

My special thanks to Professor Len Seymour and Dr. Anthony Kong for providing me with the opportunity to be a member of their lab. I am very grateful to them for all their valuable advice and support during my doctoral research in Oxford. I owe my deepest gratitude to Dr. Kong for his kind and constant supervision, guidance and patience throughout my project. Without his insightful comments and suggestions the accomplishment of this thesis would not have been possible. I am deeply indebted to Professor Jean-Pascal Machiels and Dr. Sandra Schmitz for providing me with precious tumour excision samples from HNSCC patients. I would also like to thank Dr. Ioannis Roxanis, Dr. Syed Haider and Mrs Helen Turley for their help with IHC experiments and data analysis. I also wish to thank all other members of group for their help during the time of my project. Finally, I would like to express my sincere thanks to all members of the department of oncology and Lincoln College who supported me to fulfil my dream.

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strategy in overcoming resistance to cetuximab in HNSCC. Results from this study also suggest the implication of ADAM10 and 17 as potential prognostic and predictive biomarkers in HNSCC although further validation is required.

CHAPTER 1

GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

1.1. Head and Neck Cancer

Head and neck cancers are a group of tumours which arise from craniofacial bones, soft tissues, salivary glands, skin and mucosal membranes. However, nearly 90% of these tumours arise from the squamous epithelium which lines the surfaces of paranasal sinuses, nasal cavity, oral cavity, larynx and pharynx which includes nasopharynx, oropharynx and hypopharynx (figure 1.1). These types of tumours are generally known as Head and Neck Squamous Cell Carcinomas (HNSCCs) (Pai *et al.* 2009). HNSCC is the sixth most common malignancy worldwide (Leemans *et al.* 2011), accounting for about 5% of all cancers (Tejani *et al.* 2010). The current treatments for HNSCC patients include surgery, chemotherapy, radiotherapy, cetuximab or their combinations. This is based on the patient status, primary tumour site, stage of the disease and prior therapies. However, the 5 years overall survival rate in these patients is only 40–50% (Leemans *et al.* 2011).

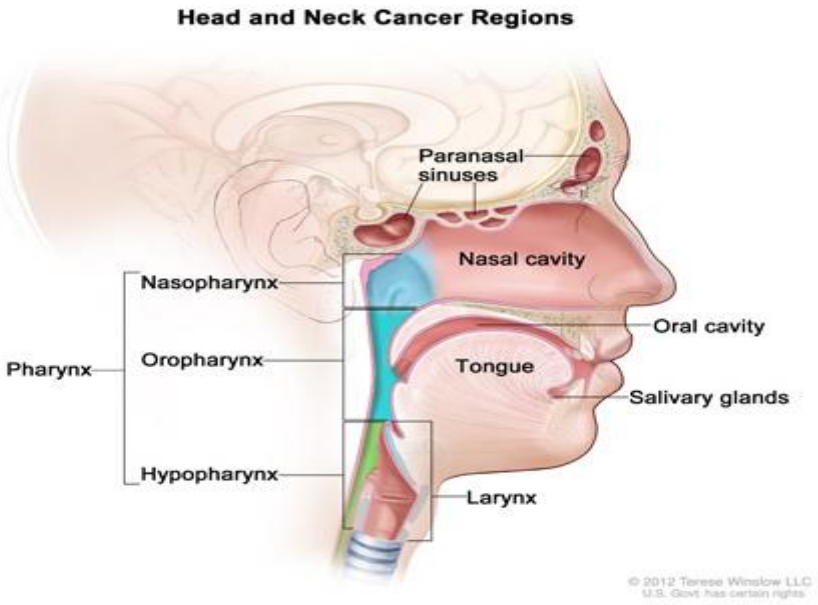


Figure 1.1: Head and neck cancer regions.

(http://www.cancer.gov/cancertopics/factsheet/Sites-Types/head-and-neck)

1.2. Risk factors for HNSCC

Tobacco and alcohol use are known to be the most important risk factors for HNSCC (Leemans *et al.* 2011). Studies have also shown the involvement of Human Papillomavirus (HPV) infection in about 25% of all HNSCC cases (Tejani *et al.* 2010). As not all the individuals exposed to the above mentioned carcinogens develop the disease, therefore it is believed that genetic predisposition also plays an important role in HNSCC carcinogenesis (Cloos *et al.* 1996, Lacko *et al.* 2014).

1.2.1. Tobacco

Smoked tobacco contains 60 carcinogens, including Polycyclic Aromatic Hydrocarbons (PAH), N-nitrosamines, aromatic amines, 1,3-butadiene, benzene, aldehydes and ethylene oxide being the most important ones (Hecht, 2003, Anantharaman *et al.* 2007). Smokeless forms of tobacco are also known to contain 28 carcinogens, with N-nitrosamines 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N'-nitrosonornicotine (NNN) being the two most important ones (Hecht, 2003, Xue *et al.* 2014). The mechanism by which unburned tobacco could lead to cancer formation is less known, however, various studies have explained the mechanisms of tobacco smoke carcinogenesis. Hecht (2003) has suggested that exposure to carcinogenic compounds which are present in tobacco smoke, leads to the formation of covalent bonds between these carcinogens and DNA. This reaction leads to the formation of products known as DNA adducts which then results in miscoding during the process of DNA replication, formation of permanent mutations and eventually causing cancer (figure1.2) (Hecht, 2003).

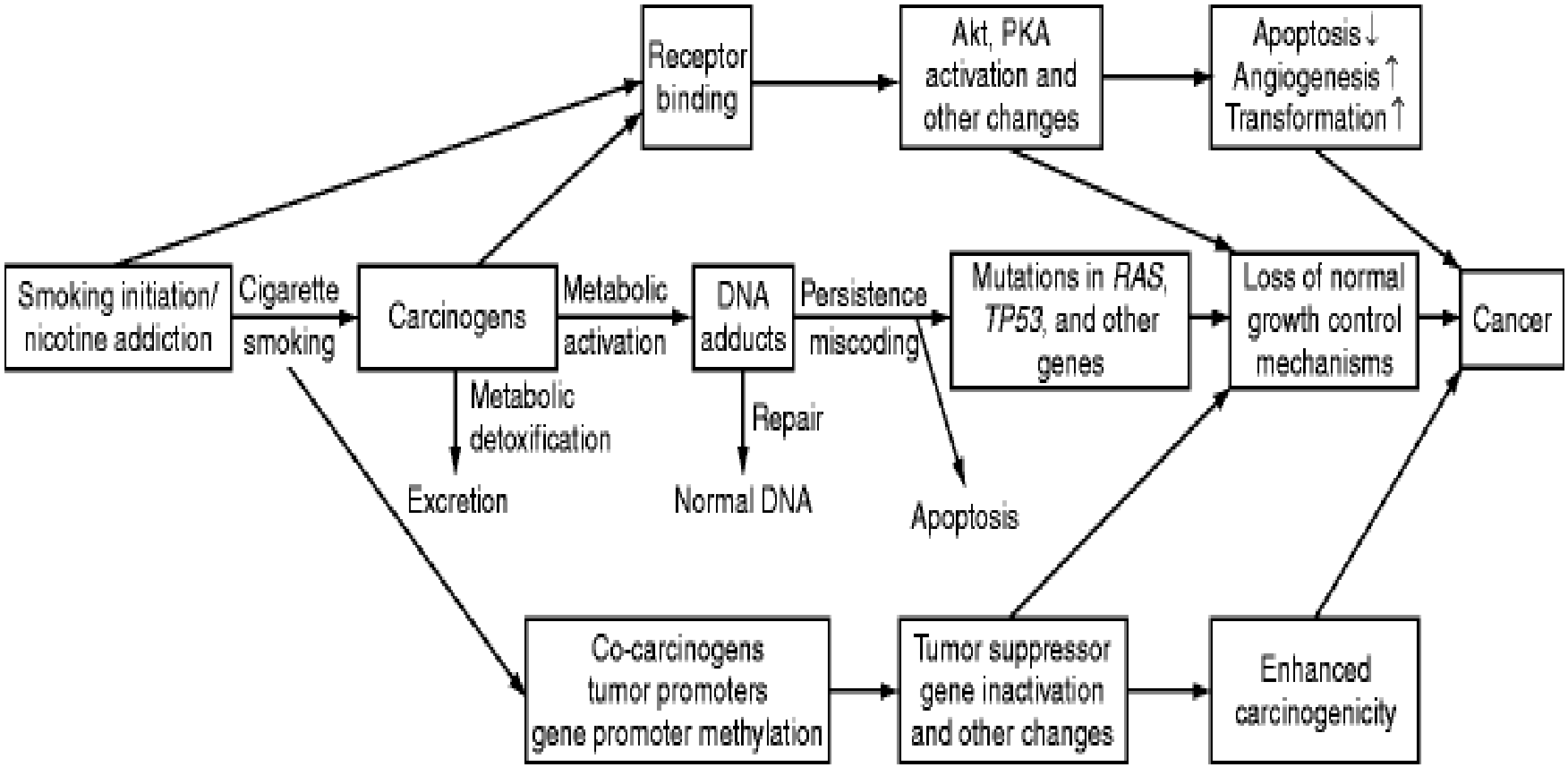


Figure 1.2: Mechanisms of tobacco carcinogenesis (Hecht,S.S 2003).

1.2.2. Alcohol

Chronic alcohol consumption is known as a significant risk factor for different types of cancers particularly cancers of upper aerodigestive tract which includes the oral cavity, pharynx, larynx and esophagus. Alcohol metabolism is shown to be the main factor in the development of alcohol-related tumours. During the process of metabolism, alcohol is oxidized to acetaldehyde by Alcohol Dehydrogenase (ADH) enzymes, microsomal enzyme Cytochrome P450 2E1 (CYP2E1) or by certain microbes which live in the human gastrointestinal tract. Acetaldehyde is then oxidized to acetate by the enzyme Aldehyde Dehydrogenase 2 (ALDH2). In addition to acetaldehyde other carcinogens such as Reactive Oxygen Species (ROS) which is produced by CYP2E1 are also generated during alcohol metabolism pathway. These carcinogens could potentially interact with DNA leading to the formation of DNA adducts and subsequently interfering with DNA replication (figure 1.3) (Seitz and Becker, 2007).

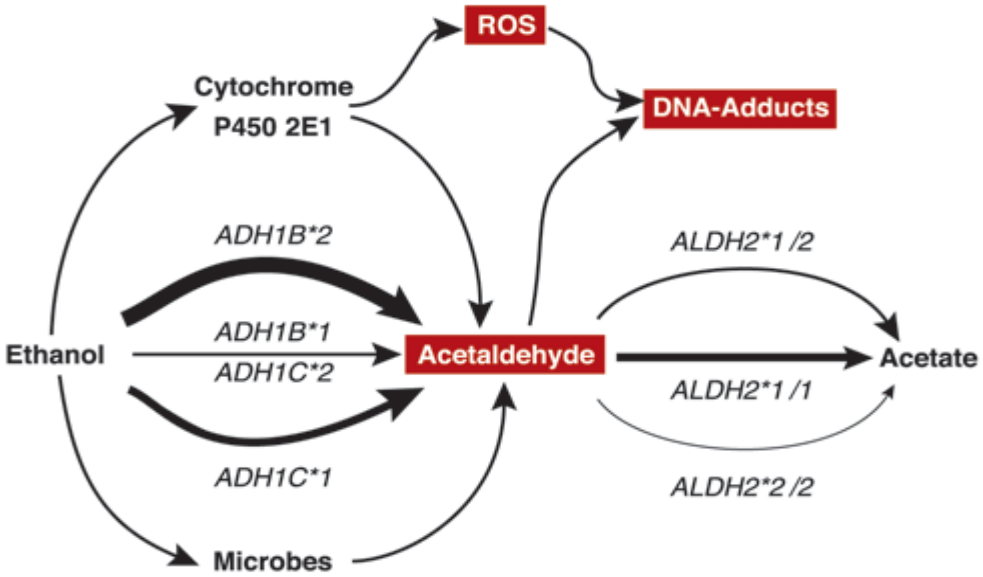


Figure 1.3: Pathways of ethanol metabolism and their roles in carcinogenesis (Seitz and Becker, 2007).

1.2.3. Human papillomavirus infection

HPV is a small, non-enveloped, double-stranded DNA tumour virus which belongs to papovaviridae family of viruses. The early region of the viral genome encodes for six non-structural viral regulatory proteins (E1, E2, E4, E5, E6 and E7) and the late region of the viral genome encodes for two structural viral capsid proteins known as L1 and L2. The early proteins play important roles in viral genome replication, transcription, cell cycle, cell signalling and apoptosis. They are also involved in structural modification and immune modulation of the infected cell. L1 and L2 are essential for virus transmission, spread and survival (Graham 2006, Zheng and Baker, 2006). Among the different types of HPVs, specific types such as HPV-16, HPV-18, and HPV-31 are known as high-risk oncogenic types, being responsible for the development of cervical and anal cancers (Zur Hausen 2002, Zheng and Baker, 2006). Studies by different groups have also shown the involvement of HPV in the development of HNSCC (Ha *et al.* 2002, Snijders *et al.* 1992, Klussmann *et al.* 2001, Gillison *et al.* 2000, Herrero *et al.* 2003). HPV infection is known to be responsible for about 25% of HNSCC cases (Sedaghat, *et al.* 2009). In the majority of HPV-positive HNSCC cases, tumours found to be localized in the oropharynx and tonsils (D'Souza and Dempsey, 2011). Among the high-risk oncogenic types of HPV, HPV-16 is the most important one in HNSCC cases. HPV16-related tumourigenesis is mediated by the expression of the viral oncoproteins E6 and E7. The E6 viral protein binds to p53 leading to its inactivation and degradation. The E7 oncoprotein binds to pRb and inactivates this tumour suppressor protein. Inactivation of pRb consecutively leads to the overexpression of p16 tumour suppressor protein

p16INK4A. HPV E6/E7 oncoprotein expression eventually leads to the deregulation of the cell cycle and causes uncontrolled cellular growth (Braakhuis, *et al.* 2004). HPV-positive HNSCC patients have a different pattern of disease compared to HPV-negative patients. HPV-positive HNSCC patients tend to be younger with less consumption of tobacco and alcohol (Suh *et al.* 2014). In relation to prognostic significance of HPV, HPV-positive tumours appear to have a better prognosis in comparison with HPV-negative cases (Lajer and Buchwald 2010). Vermorken *et al.* (2014) also showed that HNSCC patients with HPV-positive tumours had longer overall survival compared to HPV- negative tumours. This suggests that the HPV status could act as a prognostic factor in recurrent/metastatic HNSCC (Vermorken *et al.* 2014). HPV-positive patients also have been shown to respond better to chemotherapy and radiotherapy (RT) compared to HPV-negative patients (Fakhry *et al.* 2008). Pogorzelski *et al.* (2014) investigated the significance of HPV status on determining response to cetuximab in HNSCC. Results from their *in vivo* and *in vitro* experiments did not show any correlation between HPV status and response to cetuximab (Pogorzelski *et al.* 2014). Currently treatments are the same regardless of HPV status in these patients, but as previously described HPV associated HNSCC patients have a higher response rate to standard treatment. However, these therapies are associated with considerable toxicities. Therefore, there are two ongoing phase III clinical trials (De-ESCaLATE HPV and RTOG 1016) where clinical researchers are investigating the possibility of decreasing chemotherapy use in HPV-positive patients. In these studies, HPV-positive oropharynx cancer

patients are treated either with RT plus cetuximab or Chemo-Radiation Therapy (CRT).

1.2.4. Genetic variations

Genetic variations have a major impact in HNSCC tumourigenesis and are responsible for the transformation of normal epithelium cells to hyperplasia, dysplasia, carcinoma *in situ* and eventually invasive tumour (Nagai, 1999).

1.2.4.1. Inherited disorders

High incidence of HNSCC has been reported in certain inherited diseases. Trizna and Schantz (1992) have claimed that patients with Lynch-II syndrome, Bloom syndrome, Fanconi's anemia, xeroderma pigmentosum, ataxia telangiectasia, and Li-Fraumeni syndrome have a higher risk of developing head and neck cancer (Trizna and Schantz 1992). Kutler and colleagues (2003) also believe that patients with Fanconi anemia have a higher chance of developing an aggressive form of HNSCC at a younger age (Kutler *et al.* 2003). This is believed to be due to chromosomal instability and immune-deficiencies in these patients (Kaplan *et al.* 1985, Minhas *et al.* 2003).

1.2.4.2. MicroRNAs

MicroRNAs (miRNAs) are small (18-25 nucleotides long) non-coding RNA molecules which have a role in transcriptional and post-transcriptional regulation of gene expression (Lu *et al*. 2008, Bentwich *et al*. 2005). These

non-coding RNA molecules have been shown to play a role in cancer formation and progression (Calin *et al*. 2004, Lu *et al*. 2005, Tsuchiya *et al*. 2006). Studies have suggested the involvement of tumour suppressing miRNA downregulation and oncogenic miRNA upregulation in miRNA associated carcinogenesis (Lu *et al*. 2008). Overexpression of miR-21 has been reported in lung, breast, stomach, prostate, colon, brain, esophagus, pancreas and head and neck cancer (Lu *et al*. 2008, Cervigne *et al*. 2009, Avissar *et al*. 2009). miR-21 has shown to regulate the Phosphatase and Tensin homolog (PTEN) gene and consequently the PI3K/AKT pathway as well as Programmed Cell Death 4 (PDCD4) gene, thus playing a role in cell transformation. In addition, miR-21 overexpression has been reported to be involved in chemo-resistance in human cholangiocarcinoma cell lines (Meng *et al.* 2006). Cervigne *et al*. (2009) have reported that overexpression of miR-345 and miR-181b have significant roles in malignant transformation and oral cancer progression (Cervigne *et al*. 2009). miR-345 is known to inhibit the expression of anti-apoptosis protein BCL2-associated Athanogene 3 (BAG3) (Tang *et al*. 2011). miR-181 downregulates the Homeobox protein (HOXA11) which is a repressor of cell differentiation (Naguibneva *et al*. 2006). Yang *et al*. (2011) have also shown the correlation between miR-181 overexpression with lymph node metastasis, vascular invasion, and a poor survival in Oral Squamous Cell Carcinoma (OSCC) (Yang *et al*. 2011). miR-211 overexpression has also shown to be associated with poor prognosis in OSCC (Chang *et al*. 2008). Overexpression of miR-106b family has been observed in different types of tumours. miR-106b family contributes to cancer formation by regulating p21/CDKN1A , thus promoting cell cycle progression

and tumour growth (Ivanovska *et al*. 2008). miR-205 is known to target PTEN tumour suppressor gene and thus PI3K/AKT pathway. Low expression level of miR-205 has been shown to correlate with poor survival in HNSCC patient (Childs *et al*. 2009). The expression level of miR-205 has been suggested as a potential prognostic marker in HNSCC (Childs *et al*. 2009) and predictive biomarker for radio-sensitivity of patients with nasopharyngeal carcinoma (Qu *et al*. 2012). miRNA expression ratio (miR-221:miR-375) is found to be able to distinguish between normal and tumour tissue in HNSCC (Avissar *et al*. 2009). This group claimed that this ratio maybe considered as a diagnostic biomarker in HNSCC (Avissar *et al*. 2009).

1.2.4.3. Genetic mutations

Tumour Protein p53 (TP53) gene is located on chromosome 17p13.1 and encodes for tumour suppressor protein p53 which regulates cell division by inducing growth arrest or apoptosis in various tumours. Boyle *et al.* (1993) reported the presence of mutation of the p53 gene in 40-45% of primary HNSCCs (Boyle *et al*. 1993). It is believed that these patients have poorer responses to chemotherapy and have a higher risk of loco-regional recurrence when treated with radiation therapy (Koch *et al*. 1996, Cabelguenne *et al*. 2000). In many of the patients with wild type p53, indirect inactivation of p53 has been reported. This is shown to be through various mechanisms such as expression of HPV-E6. The E6 protein binds to p53 and induces its degradation (Rothenberg and Ellisen 2012, Münger *et al*. 1992). Overexpression of the human Murine Double Minute 2 (MDM2), an important regulator of p53, due to the amplification of the MDM2 gene is another

mechanism which leads to the degradation of p53 (Shangary and Wang 2008, Rothenberg and Ellisen 2012).

Cyclin-Dependent Kinase Inhibitor 2A (CDKN2A) gene encodes two tumour suppressor proteins, $p14^{ARF}$ and $p16^{INK4a}$ which differ in their first exon (Rutter *et al.* 2003). The $p14^{ARF}$, an Alternate Reading Frame (ARF) product of the CDKN2A, is an upstream regulator of p53. It inhibits MDM2 and thus blocks degradation of p53 and results in p53 stabilization and activation. Therefore deletion of CDKN2A could also be responsible for the disruption of the p53 pathway (Rothenberg and Ellisen 2012). The $p16^{INK4a}$ regulates the Rb pathway by inhibiting the cell cycle progression in the G1-S transition (Ohtani *et al.* 2004). *CDKN2A* inactivation by mutation, deletion or epigenetic inactivation has been reported in 75%-85% of HNSCC (Perez-Sayans *et al.* 2009, Reed *et al.* 1996, Smeets *et al.* 2009, Rothenberg and Ellisen 2012). Inactivation of *CDKN2A* and consequent loss of p16/INK4A expression leads to an increased cell proliferation and tumour formation. Studies have shown that loss of p16/INK4A expression is associated with worse prognosis. These patients have been reported to have reduced disease-free and overall survival rates (Bova *et al.* 1999, Rothenberg and Ellisen 2012).

Cyclin D1 is encoded by CCND1 gene on chromosome 11q13 and it is involved in cell cycle regulation. Cyclin D1 is believed to play an important role in HNSCC and has been shown to be associated with poor prognosis in HNSCC (Namazie *et al.* 2002). Amplification of CCND1 gene has been observed in about 1/3 of HNSCC patients which is known to be more frequent

in HPV-negative tumours (Smeets *et al.* 2006, Sheu *et al.* 2009, Rothenberg and Ellisen 2012). Overexpression of cyclin D1 has also been reported in about 80% of HNSCC cases (Leemans *et al.* 2011, Rothenberg and Ellisen 2012). Overexpression of cyclin D1 has been found to be associated with increased lymph node metastasis, higher tumour grade and reduced disease-free and overall survivals (Bova *et al.* 1999). This group also reported that patients with both cyclin D1 overexpression and loss of p16INK4A expression have a higher chance of early recurrence and reduced survival (Bova *et al.* 1999).

Using high-throughput next-generation sequencing techniques, researchers have reported novel truncating and missense mutations in NOTCH1 gene in 10% to 15% of the HNSCC tumours (Stransky *et al.* 2011, Agrawal *et al.* 2011). *NOTCH1* is located on chromosome 9q34.3 and encodes neurogenic locus Notch homolog protein 1 which is a member of the Notch family of transmembrane receptors (NOTCH1– 4). These receptors play important roles in determining cell fates during development and any dysregulation of NOTCH receptors' activities can lead to tumourigenesis.

Tumour Protein 63 (TP63) is a member of p53 gene family. This gene is located on chromosome 3q28 and encodes for p63 transcription factor which is a crucial factor for normal development. Studies have shown that the overexpression of p63 occurs in about 80% of HNSCC (Rocco *et al.* 2006).

*FBXW7 (*F-box and WD repeat domain containing 7) is a tumour suppressor gene and is located on chromosome 4q31.23. It encodes for FBXW7 which is a member of the F-box family of proteins and mediates the ubiquitin-dependent degradation of different oncoproteins such as cyclin E1, c-Myc, c-Jun, and NOTCH1 (Akhoondi *et al.* 2007, Agrawal *et al.* 2011). Two indels and four missense mutations in FBXW7 gene have been reported in HNSCC (Agrawal *et al.* 2011).

Tumour Necrosis Factor Receptor Super Family, Member 10B (TNFRSF10B) gene which is also known as KILLER/DR5 is located on chromosome 8p21.3. This region contains a number of putative tumour suppressor genes and it is known as a translocations site in head and neck cancer. A loss of function mutation in KILLER/DR5 gene leads to a loss of its apoptotic function (Pai *et al.* 1998).

Inhibitor of Growth family, member 1 (ING1) gene is located on chromosome 13q34 and encodes a tumour suppressor protein which is involved in p53 signalling pathway. This nuclear protein interacts with p53 and as a result it induces cell growth arrest and apoptosis. Gunduz *et al*. (2000) have reported three missense mutations in the ING1 gene in tumour tissues of HNSCC (Gunduz *et al*. 2000).

PTEN gene is located on chromosome 10q23.31 and acts as a tumour suppressor gene. PTEN is a key regulator of the PI3K signalling pathway and thus plays an important role in the regulation of cell cycle (Rothenberg and

Ellisen 2012). *PTEN* is mutated in various advanced cancers at high frequency. Mutations in *PTEN* have also been reported in 7% of HNSCC tumour samples (Poetsch *et al*. 2002, Suh *et al*. 2014). Another important regulator of the PI3K signalling pathway is PIK3CA (Phosphatidylinositol-4,5-Bisphosphate 3-Kinase, Catalytic Subunit Alpha) gene which is mapped to chromosome 3q26.3. Activating mutations in the *PIK3CA* gene have been reported in 6%–11% of HNSCC cases (Qiu *et al*. 2006). These mutations seem to have a higher incidence in HPV-positive HNSCC cases (Rothenberg and Ellisen 2012).

Mutations in the Ras proto-oncogenes (HRAS, KRAS and NRAS) have been reported in 20–30% of all cancers (Bos 1989). Whole-exome sequencing studies in recent years have led to the discovery of activating missense mutations in the HRAS gene in 3%–5% of HNSCC samples (Stransky *et al.* 2011, Agrawal *et al.* 2011). Studies have shown that mutations in HRAS gene are associated with tobacco chewing (Saranath *et al.* 1991). The association between *HRAS* mutations and HPV infection has also been reported (Anderson *et al.* 1994).

Mesenchymal Epithelial Transition Factor (MET) which is also known as Hepatocyte Growth Factor Receptor (HGFR) or more commonly c-MET, is a transmembrane receptor tyrosine kinase which is encoded by c-*MET* proto-oncogene on chromosome 7q21-31 (Organ and Tsao 2011). Upon binding to its ligand, Hepatocyte Growth Factor (HGF), c-MET activates its downstream signalling pathways thus plays important roles in cell proliferation, survival

migration and invasion (Organ and Tsao 2011, Suh *et al*. 2014). Alterations in *c-MET* due to gene mutation or amplification have been reported in various types of malignancies. *c-MET* mutations have been found in renal (Schmidt *et al.* 1997, Jeffers *et al.* 1997), lung (Ma *et al.* 2005) and head and neck cancers (Di Renzo *et al.* 2000, Ghadjar *et al.* 2009) and amplification of *c-MET* has been observed in gastric and esophageal carcinomas (Hara *et al.* 1998, Kuniyasu *et al.* 1992, Miller *et al.* 2006).

Overexpression of c-MET due to gene amplification or transcriptional upregulation has also been reported in different types of tumours (Organ and Tsao 2011) such as HNSCC (Marshall and Kornberg 1998, Kim *et al.* 2006). Overexpression of c-MET has been observed in more than 80% of HNSCC and increased copy numbers has been reported in about 13% of tumour samples (Knowles *et al*. 2009, Seiwert *et al*. 2009). c-MET overexpression was shown to be associated with reduced disease-free and overall survival (Zhao *et al*. 2011, Lo *et al*. 2006). *c-MET* amplification has been shown to promote resistance to EGFR TKIs (Engelman *et al*. 2007, Bean *et al.* 2007). In recent years different groups have also claimed that c-MET overexpression is responsible for resistance to radiation, cisplatin and cetuximab (De *et al*. 2011, Sun *et al*. 2011, Krumbach *et al*. 2011, Suh *et al*. 2014).

Signal Transducers and Activators of Transcription (STAT) family proteins are cytoplasmic transcription factors which have major roles in embryonic development, cell differentiation, immune response and organogenesis (Levy, 1999, Kamran *et al*. 2013). STAT3 gene is a member of STAT gene family

and is located on chromosome 17q21.31. STAT3 regulates cellular proliferation, invasion, migration, and angiogenesis and is found to be activated in different tumour types, resulting in an increased tumour growth and metastasis (Kamran *et al*. 2013). Overexpression of STAT3 has also been observed in HNSCC (Grandis *et al*. 2000). Increased STAT3 expression in HNSCC tumours promotes cell cycle and inhibits apoptosis and thus leads to the proliferation of cancer cells.

Overexpression of Epidermal Growth Factor Receptor (EGFR) has been reported in various cancer types, compared to normal tissues. This has been shown to be associated with an induced tumour growth (Nicholson *et al*. 2001). EGFR is known as a prognostic indicator for ovarian, cervical, bladder, esophageal as well as head and neck cancers. Overexpression of EGFR correlates with reduced recurrence-free and overall survival rates in these tumours (Nicholson *et al*. 2001). Unlike Non-Small Cell Lung Carcinoma (NSCLC), *EGFR* activating mutations are rare in HNSCC whereas the EGFR gene amplification has been observed in 10-30% of HNSCC cases. In addition, 1-7% of HNSCC patients have been reported to have EGFR point mutations (Grandis *et al*. 1993, Loeffler-Ragg *et al*. 2006, Temam *et al*. 2007). EGFRvIII is a mutant form of EGFR which results from an in-frame deletion of exons 2–7 in the extracellular domain. Expression of EGFRvIII has been observed in 42% of HNSCC patients and it is believed to be responsible for increased tumour growth (Sok *et al*. 2006).

1.3. Epithermal Growth Factor Receptor

EGFR is a member of erbB Receptor Tyrosine Kinases (RTKs) family, which is also known as human epidermal growth factor receptor family (HER). c-erbB-2/Her-2, c-erbB-3/Her-3 and c-erbB-4/Her-4 are the other members of erbB receptor family (Bazley & Gullick 2005). HER family receptors play an important role in cell proliferation, differentiation, survival and migration.

EGFR (c-erbB1/Her1) gene is located on the short arm of chromosome 7 at position 12 and encodes for a 170 kDa EGFR glycoprotein. EGFR glycoprotein consists of a single polypeptide chain which is 1186 amino acids long (Voldborg *et al*. 1997). EGFR is composed of three domains; the cysteine-rich extracellular ligand binding domain, the hydrophobic transmembrane domain and the intracellular domain (Voldborg *et al*. 1997). The extracellular domain itself consists of four subdomains: I, II, III and IV, which are also known as L1, CR1, L2 and CR2 respectively. CR1 and CR2 are homologous cysteine-rich regions whereas L1 and L2 form the binding sites for EGFR ligands (Garrett *et al*. 2000, Ogiso et al. 2002). The intracellular domain consists of juxtamembrane domain, tyrosine kinase domain and regulatory region domain (figure 1.4) (Flynn *et al.* 2009).

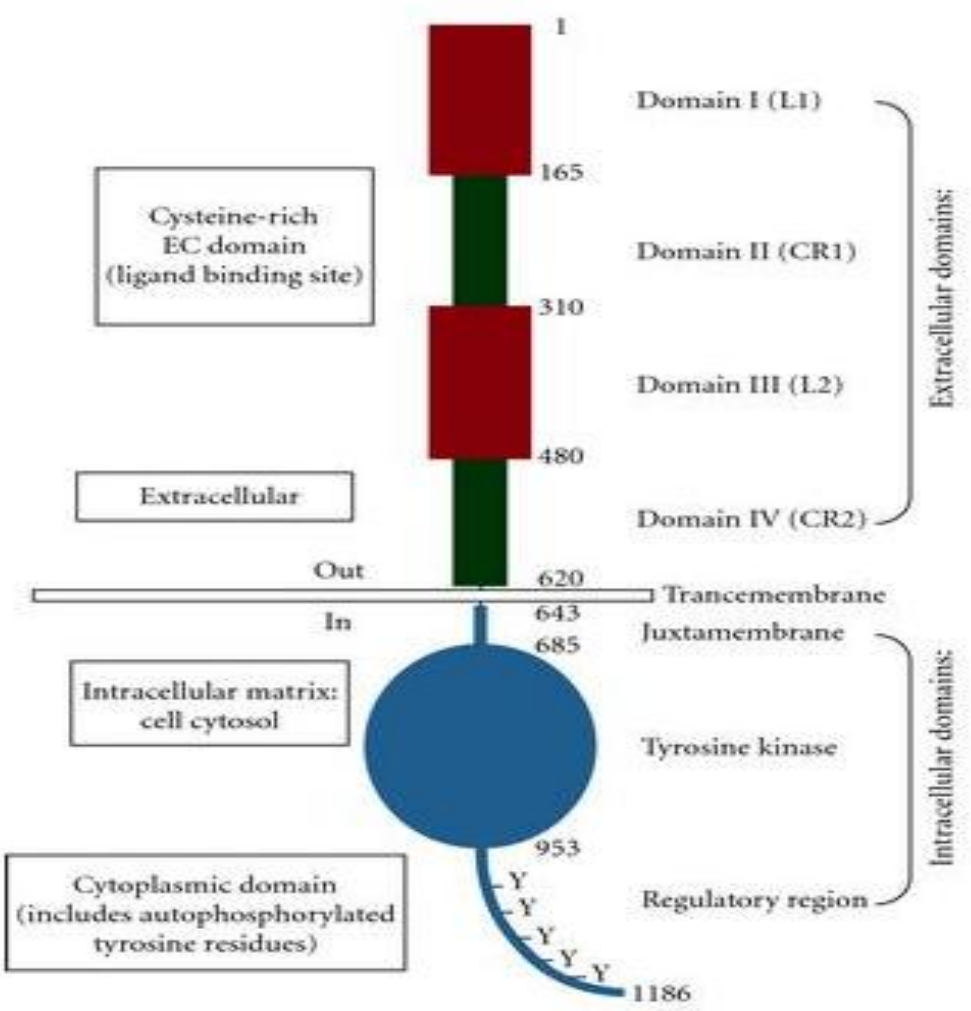


Figure 1.4. Structure of EGF receptor representing extracellular domain, transmembrane domain, intracellular domain and their sub-domains (Flynn *et al.* 2009).

1.4. HER family ligands

Up to date 11 ligands have been identified to bind to HER family receptors (Linggi & Carpenter 2006). Epidermal Growth Factor (EGF), Transforming Growth Factor-α (TGF-α), Amphiregulin (AR) and Epigen only bind to EGFR. Heparin-Binding EGF-like Growth Factor (HB-EGF), Epiregulin (EPR) and Betacellulin (BTC) bind to EGFR and erbB4 receptors. Neuregulins (NRGs) 1

and 2 bind to both erbB3 and erbB4 receptors, whereas NRG-3 and 4 only bind to erbB4 receptor (table 1.1). No specific ligand has been identified to bind to erbB2 receptor (Linggi & Carpenter 2006).

	ErbB-1	ErbB-2	ErbB-3	ErbB-4
EGF	+	−	−	−
TGF-α	+	−	−	−
HB-EGF	+	−	−	+
Amphiregulin	+	−	−	−
Betacellulin	+	−	−	+
Epigen	+	−	−	−
Epiregulin	+	−	−	+
Neuregulin-1	−	−	+	+
Neuregulin-2	−	−	+	+
Neuregulin-3	−	−	−	+
Neuregulin-4	−	−	−	+

Table 1.1. HER family receptors and their specific ligands.

Each HER family ligand is synthesized as an inactive membrane-bound precursor which would be processed, cleaved and released as an active growth factor by metalloprotease(s) in particular the ADAM (A Disintigrin And Metalloprotease) family of proteases (Ohtsu *et al.* 2006).

1.5. A Disintigrin And Metalloprotease

1.5.1. Structure of ADAM proteins

ADAMs are zinc-dependant membrane-bound metalloproteases which are 750 amino acids long (Arribas & Esselens 2009). Their multi-domains structure consists of N-terminal signal sequence, pro-domain, metalloprotease domain, disintegrin domain, cysteine-rich domain, EGF-like domain, transmembrane domain and cytoplasmic tail (figure 1.5) (Arribas & Esselens 2009).

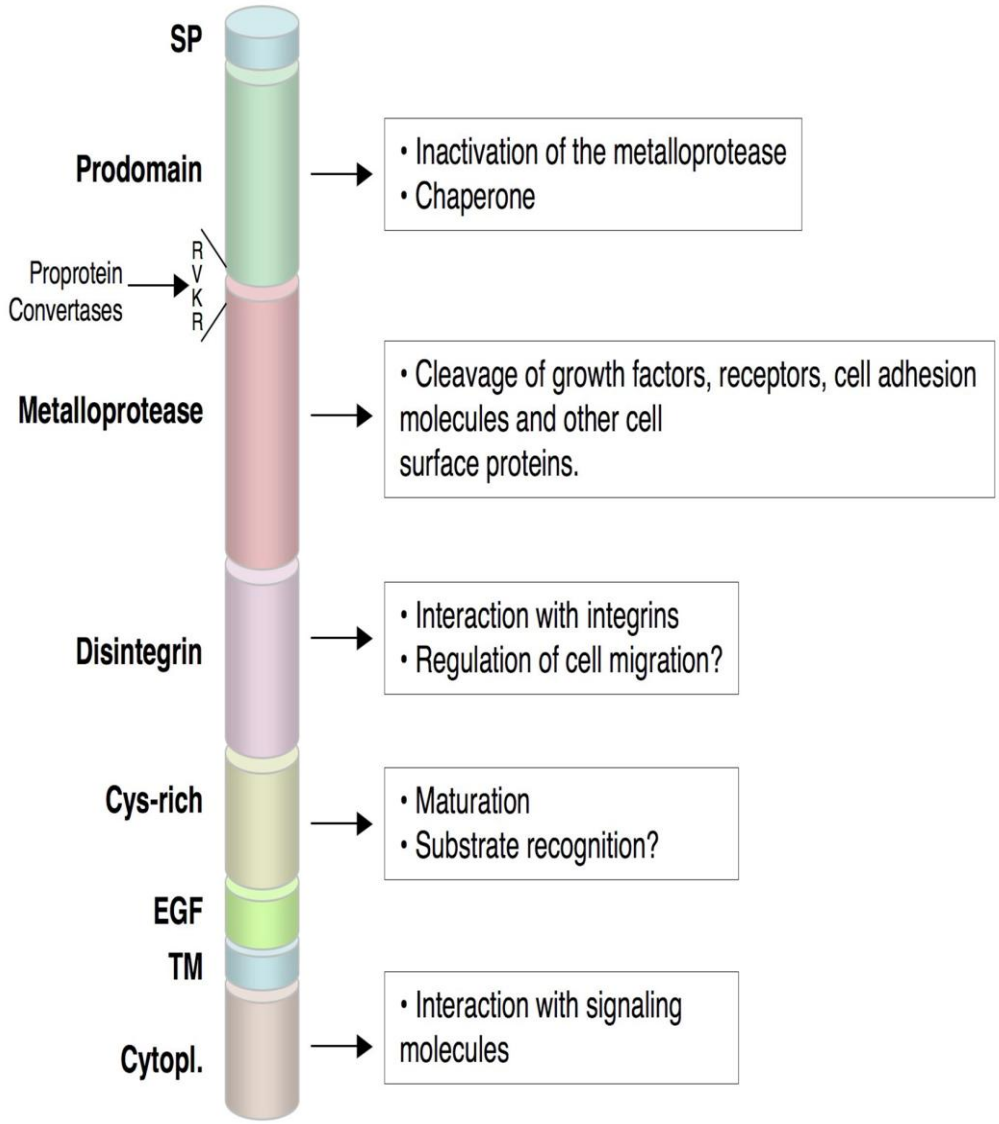


Figure 1.5. Schematic diagram representing ADAM's multi-domains structure and their proposed functions (Arribas & Esselens, 2009).

1.5.2. Function of ADAM proteins

Up to date 25 human ADAMs have been identified; however, only 13 members of family have been shown to be proteolytically active (Arribas & Esselens 2009). The proteolytically active ADAMs are involved in the cleavage of the ectodomains of membrane-bound growth factors, receptors, cytokines, chemokines and adhesion molecules and thus play important roles in cell proliferation, differentiation, adhesion and migration (Alfandari *et al.* 2009).

1.5.3. ADAMs inhibition

As described previously ADAM family members play a vital role in the regulation of various cell surface proteins and the deregulation of these metalloproteases could lead to autoimmune disorders, cardiovascular diseases, infection, inflammation, neurodegeneration as well as cancer formation and progression. Therefore, in recent years ADAM family has been investigated by many researchers for their therapeutical potential and they are considered as interesting targets for new treatments. A number of ADAM inhibitors have been developed in recent years which are listed in table 1.2.

Selective ADAM inhibitors

Inhibitor	Target ADAM	Company
INCB3619	10, 17	Incyte, Wilmington, DE
INCB7839	10,17	Incyte
WAY-022	17	Wyeth-Aherst, Pearl River, NY
GI254023X	10	Glaxo Smith Kline
GW280264X	10,17	Glaxo Smith Kline
TMI-2	17	Pfizer
KB-R7785	12	Organon, Osaka, Japan

Table 1.2. Selective ADAM inhibitors (Duffy *et al.* 2011).

1.5.4. ADAMs and their roles in EGFR signalling

ADAM10 and ADAM17 are the key members of the ADAM family and have been shown to play an important role in EGFR signalling. Sahin *et al.* (2004) studied the role of ADAMs in EGFR ligands shedding in knockout mice and showed that ADAM17 is the key regulator in cleavage of TGFα, HB-EGF, amphiregulin, and epiregulin where as ADAM10 is crucial in EGF and BTC shedding (Sahin *et al.* 2004). ADAM17 has also been shown to be a key factor in regulating the cleavage of neuregulin, which is a specific ligand for erbB3 and erbB4 receptors (Murphy 2008). Thus, each specific ADAM cleaves its own substrate and the release of the ligands would in turn stimulate erbB dimerization with subsequent activation of downstream signalling pathways (figure 1.6).

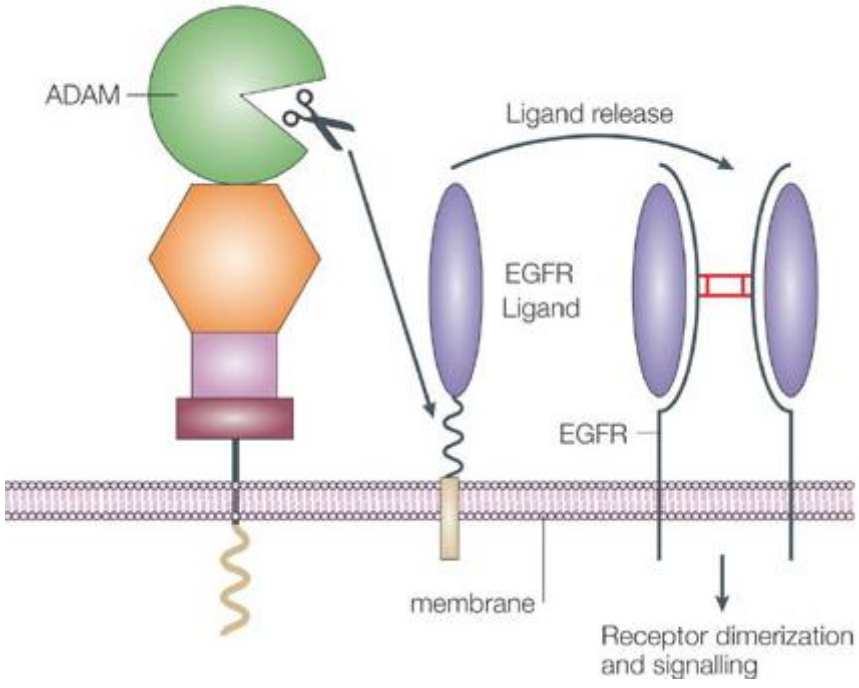


Figure 1.6. Schematic diagram showing ADAM induced EGFR ligand release and EGFR receptor dimerization (adapted from Blobel, Nature Reviews, Molecular Cell Biology, 2005).

1.6. EGFR signalling

ADAM-mediated release of EGFR ligands is the first step in activation of EGFR signalling. The binding of a specific ligand to the receptor stimulates the homodimerization or heterodimerization of EGFR with other members of HER family receptors (Voldborg *et al*. 1997). Ligand-induced dimerization of receptors then leads to the phosphorylation of specific tyrosine residues in the carboxyl terminal tail of tyrosine kinase domain (figure 1.7) (Zhang *et al.* 2006).

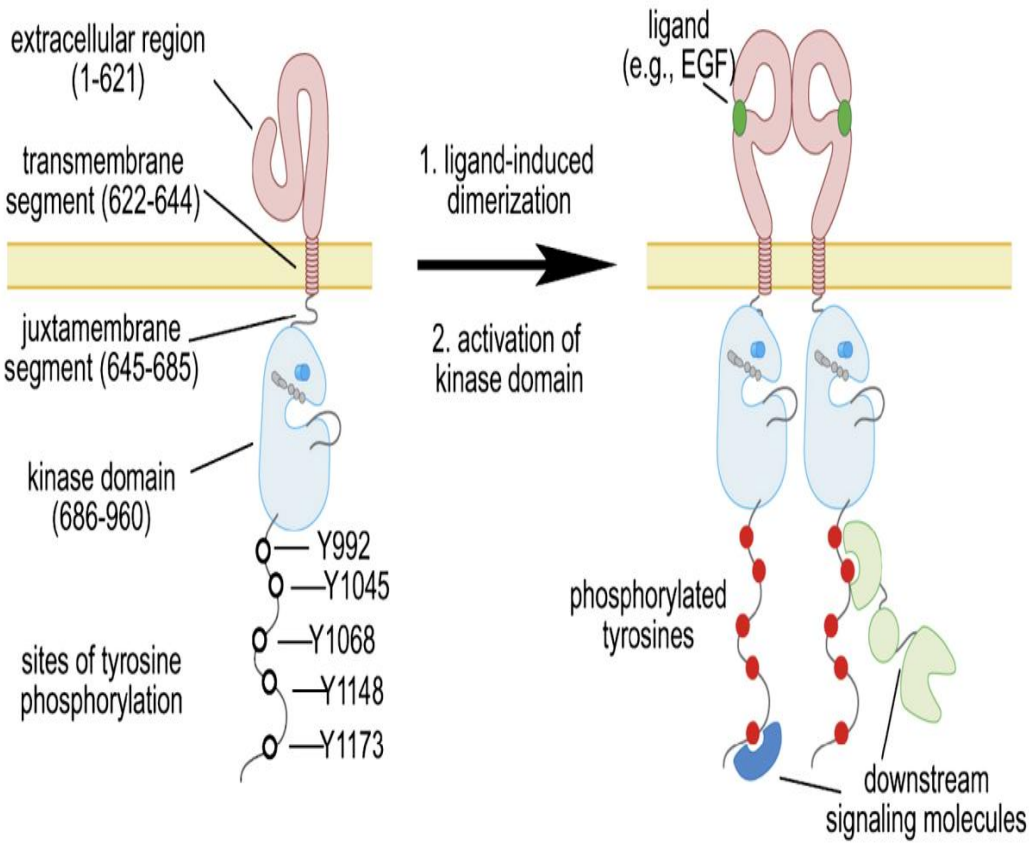


Figure 1.7. Ligand-induced dimerization and activation of EGFR (Zhang *et al.* 2006).

Tyrosine phosphorylation stabilizes the receptor construction and generates a docking site for the recruitment of a range of signalling proteins to the phosphorylation site (Zhang *et al.* 2006). This leads to the activation of various downstream signalling pathways mainly Ras–Mitogen-Activated Protein Kinase (Ras-MAPK), Phosphatidylinositol 3′ Kinase-Protein Kinase B (PI3K-PKB/AKT), Phospholipase C-Protein Kinase C (PLC-PKC) and Signal Transducer and Activator of Transcription (STAT) pathways (figure 1.8). This eventually triggers the activation of nuclear transcription factors leading to the production of proteins which are involved in cellular proliferation, differentiation, migration and apoptosis (Marmor *et al.* 2004).

Therefore EGFR is believed to be important in the mammalian embryonic and postnatal development and any dysregulation of the EGFR signalling pathways due to EGFR overexpression, gene amplification or gene mutation could result in oncogenesis.

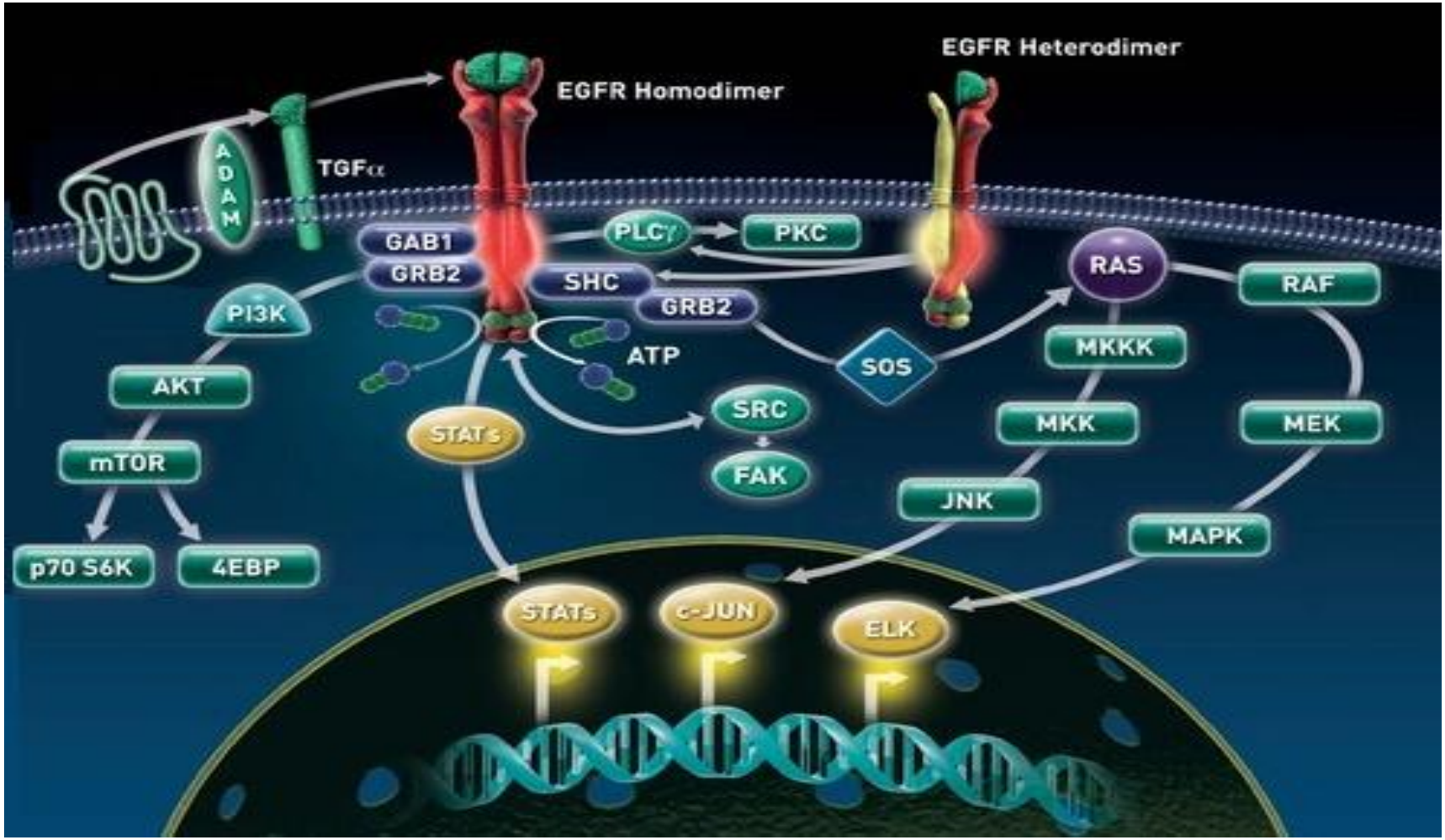


Figure 1.8. EGFR signal transduction (Adapted from Lilleby *et al.* 2011).

1.7. EGFR and its role in HNSCC

Overexpression of EGFR has been observed in about 90% of HNSCC (Cassell & Grandis 2010) as well as other epithelial malignancies such as lung, breast, brain, colon, ovarian, cervical and bladder (Harari 2004). EGFR overexpression is known to be associated with advanced clinical stage, poor patient prognosis and resistance to hormonal therapy, chemotherapy, and radiation (Arteaga 2002). Therefore EGFR is considered to be a major therapeutic target in HNSCC.

1.8. EGFR targeted therapy

Many groups of researchers have been studying HNSCC biology in order to improve current therapies or develop more effective strategies. These studies have led to the introduction of targeted therapies against EGFR such as use of anti-sense oligonucleotides, tyrosine kinase inhibitors (TKIs) and monoclonal antibodies (mAbs).

1.8.1. EGFR antisense DNA

EGFR antisense DNA is a synthetic sequence of DNA which is constructed in the antisense direction to a sequence of DNA in EGFR. EGFR antisense DNA directly blocks the expression of EGFR in cancerous cells leading to the inhibition of tumour cells proliferation and tumour growth. Therefore several pre-clinical studies and clinical trials have been going on in recent years to assess the efficacy of antisense DNA in HNSCC patients. Lai *et al.* (2009)

conducted a phase I clinical trial in order to investigate the safety and therapeutic effects of an EGFR antisense gene sequence under U6 promoter control. 17 patients with advanced HNSCC who did not respond to standard therapies were entered into the trial. These patients were given four weekly intratumoral EGFR antisense injections with escalated doses of 60-1,920 microg/injection. None of these patients developed grades 3-4 or dose-limiting toxicities. Two patients achieved Complete Clinical Response (CCR), three patients had Partial Responses (PR) and two patients had Stable Disease (SD). Compared with patients who had progressive disease, these 7 patients with disease control had tumours with higher EGFR and lower STAT3 expression levels at baseline (Lai *et al.* 2009). An ongoing phase I/II clinical trial is currently investigating the safety and efficacy of combining EGFR antisense DNA with radiation and concurrent cetuximab for treatment of patients with locally advanced HNSCC who are not the right candidates to receive chemotherapy (clinicaltrials.gov-NCT00903461).

1.8.2. EGFR tyrosine kinase inhibitors

1.8.2.1. Erlotinib (Tarceva)

Erlotinib is a reversible EGFR TKI which received FDA approval in 2013 as a first-line treatment for patients with metastatic NSCLC who have EGFR exon 19 deletions or exon 21 (L858R) substitution mutations. The anti-tumour activity of erlotinib in HNSCC was first reported by Pollack *et al.* in 1999 (Pollack *et al.* 1999). Results from their pre-clinical study demonstrated *in vivo* inhibition of EGFR phosphorylation in murine xenografts of a human

HNSCC cell line after 1 hour treatment with 100 mg/kg of erlotinib. They claimed that EGFR phosphotyrosine reduction was associated with tumour growth inhibition. This group also investigated the toxicity and efficacy of combining erlotinib with cisplatin *in vivo* and claimed that the combination treatment produced additive anti-tumour effects without increased toxicity (Pollack *et al.* in 1999). In a phase II clinical trial which was carried out by Soulieres *et al.* (2004), the safety and efficacy of erlotinib was investigated in patients with advanced recurrent or metastatic HNSCC. 115 patients were entered into the trial and treated with an initial dose of 150 mg/d of erlotinib. Treatment with erlotinib led to PR in 5 patients (4.3%) and stabilized the disease in 44 patients (38.3%) for a median duration of 16.1 weeks. In this study the median Progression Free Survival (PFS) was 9.6 weeks and the median Overall Survival (OS) was 6.0 months. Drug-related skin toxicity and diarrhoea was observed in 79% and 37% of the patients respectively. This group reported the correlation between the severity of skin toxicity and OS. The median survival of patients who developed grade 2-4 rash was 7.4 months whereas the survival of patients with grade 0 to 1 skin toxicity was 4 to 5 months respectively (Soulieres *et al.* 2004).

Anti-tumour activity of erlotinib in combination with existing RT and chemotherapy regimens has also been investigated in HNSCC. In a phase II trial, 45 recurrent or metastatic HNSCC patients were treated with a combination of erlotinib and cisplatin (Siu *et al.* 2007). The response rate was reported to be 21%, with one CCR and eight PR and disease stabilization was observed in 21 patients. Median OS was 7.9 months and median PFS was

3.3 months. The combination treatment had a favourable toxicity profile and patients with higher grade skin toxicities had better survival outcomes (Siu *et al.* 2007).

Martins *et al.* (2013) conducted a randomized phase II trial to evaluate the efficacy of combining cisplatin-RT with erlotinib in patients with locally advanced HNSCC. 204 patients were entered into the trial and the CCR rate was 40% in patients receiving cisplatin 100 mg/m^2 combined with 70 Gy of RT and 52% in patients with the addition of erlotinib 150 mg/d to the same doses of CRT. Although the addition of erlotinib did not result in an increased toxicity of cisplatin and RT, it failed to increase the CCR rate and PFS significantly (Martins *et al.* 2013). There is currently an ongoing randomised, placebo-controlled phase II trial to investigate the efficacy of adding erlotinib to the combination of docetaxel and either cisplatin or carboplatin in controlling HNSCC (clinicaltrials.gov-NCT01927744).

1.8.2.2. Gefitinib (Iressa)

Gefitinib, is a selective reversible EGFR TKI, similar to erlotinib. It is the first EGFR-targeted TKI which received approval from FDA for the treatment of NSCLC patients with EGFR mutations. Interestingly, its FDA approval was subsequently withdrawn although it remains approved and used in Europe and other countries. Gefitinib has also been investigated in HNSCC and its ability in inhibition of cell proliferation in a dose-dependent manner has been reported by different groups (Di Gennaro *et al.* 2003, Shintani *et al.* 2003a

and 2003b). Mandal *et al.* (2002) have reported the anti-metastatic activity of gefitinib in HNSCC and breast cancer cells (Mandal *et al.* 2002). In addition, various phase I clinical trials were conducted in order to evaluate the safety and tolerability of gefitinib in different solid tumours including HNSCC and showed that it was well tolerated (Herbst *et al.* 2002, Baselga *et al.* 2002, Ranson *et al.* 2002, Nakagawa *et al.* 2003). Cohen *et al.* (2003) conducted a pashe II clinical trial using gefitinib (ZD1839) in 52 patients with recurrent or metastatic HNSCC. Of 47 assessable HNSCC patients with incurable disease, the response rate was only 10.6% and the Disease Control Rate (DCR) was 53%. In about half of the patients the median Time To Progression (TTP) was 3.4 months and median survival was 8.1 months. In this study 24 patients developed skin rash which was found to be associated with statistically significant improvements in TTP and OS (Cohen *et al.* 2003). This group also conducted another phase II trial where they treated 70 patients with recurrent or metastatic HNSCC with a lower dose of gefitinib 250 mg/d (Cohen *et al.* 2005) instead of 500mg/d which was used previously (Cohen *et al.* 2003). In this study the response rate was even lower. Of these 70 patients only 1 PR was observed and the median PFS and OS were reported to be 1.8 and 5.5 months, respectively (Cohen *et al.* 2005). Although gefitinib showed some clinical activities, it failed to produce a significant survival benefit. Therefore a number of studies assessed the effect of combining gefitinib with standard chemotherapy and RT in HNSCC. In a phase II study by Hainsworth *et al.* (2009) patients with locally advanced HNSCC were treated with combination of gefitinib (250 mg/d) and CRT. The combined treatment was associated with a moderate increase in toxicity and

produced the survival results similar to that observed with CRT alone (Hainsworth *et al.* 2009). Gregoire *et al.* (2011) has also conducted a phase II study to evaluate the efficacy and safety of gefitinib in combination with cisplatin and RT or as a maintenance therapy to standard cisplatin/RT in stage III/IV non-metastatic HNSCC. Although gefitinib (250 or 500 mg/d) was well tolerated, it did not show a very promising efficay in combination with CRT compared to placebo or as a maintenance therapy (Gregoire *et al.* 2011). Argiris *et al.* (2013) conducted a phase III randomised, placebo-controlled trial of docetaxel with gefitinib in recurrent or metastatic HNSCC. 270 patients entered into this trial. These patients received weekly docetaxel 35 mg/m^2 IV every 28 days plus placebo (Arm A, n=136) or gefitinib 250 mg/d (Arm B, n=134) until disease progression. 6 and 7.3 months median OS was observed in arm A and arm B respectively. In this study combination treatment produced a higher rate of grade 3/4 diarrhoea. Overall, although the addition of gefitinib to docetaxel was well tolerated, it did not improve the clinical benefits in these patients (Argiris *et al.* 2013).

1.8.2.3. Lapatinib (Tykerb)

Lapatinib is a reversible dual TKI against EGFR and HER2 which has received approval from FDA for the treatment of patients with breast cancer.
Del Campo *et al.* (2011) conducted a randomised phase II study where they assessed the clinical effects of treatment with lapatinib in patients with locally advanced HNSCC (Del Campo *et al.* 2011). 107 therapy-naive patients entered into the trial and received lapatinib or placebo for 2–6 weeks prior to

CRT. Treatment with lapatinib did not have any significant effect on apoptosis, however, it decreased Ki67 proliferative index. In this study 40 patients received lapatinib (n=24) or placebo (n=16) for more than 4 weeks. The Objective Response Rate (ORR) was 17% (n=4/24) and 0% (n=0/16) respectively. No significant difference in ORR was observed between lapatinib (70%) and placebo (53%) after CRT (Del Campo *et al.* 2011). In another phase II study, the safety and efficacy of lapatinib was investigated in patients with recurrent or metastatic HNSCC (de Souza *et al.* 2011). Out of 45 patients who entered into the trial, 27 patients were EGFR inhibitor naive (arm A) and 18 patients had prior exposure to an EGFR inhibitor (arm B). Both groups were treated with lapatinib (1500 mg/d) but none of the EGFR inhibitor naive or refractory patients had complete or partial responses. 41% of arm A and 17% of arm B patients had SD. No difference was observed in median PFS (52 days for both groups) but median OS was 288 days for arms A and 155 days for arms B (de Souza *et al.* 2011). The safety and efficacy of concurrent CRT in combination with lapatinib was evaluated in patients with locally advanced HNSCC by Harrington *et al.* (2013). In this randomised phase II study, 67 patients took part. Group 1 received concurrent CRT and placebo followed by placebo and group 2 received concurrent CRT and lapatinib followed by lapatinib. The combination of lapatinib with CRT was well-tolerated. At 6 months follow-up, the CRR was 36% in group 1 and 53% in group 2. At 18 months follow-up the PFS rates were 41% versus 55% and OS rates were 57% versus 68% in group 1 and 2 respectively. The difference between the two groups was greatest in patients with p16 negative tumours.

The median PFS was reported to be greater than 20.4 months in group 2 versus 10.9 months in group 1 (Harrington *et al.* 2013).

1.8.2.4. Dacomitinib (PF-00299804)

Dacomitinib is an irreversible next generation small molecule TKI that targets EGFR, HER2 and HER4 receptors. In a phase II trial which was conducted by Abdul Razak *et al.* (2013) the efficacy of dacomitinib was evaluated in patients with recurrent or metastatic HNSCC. 69 patients entered into the trial and received dacomitinib at 45 mg/d in 21-day cycles. 8 patients had PR and 36 patients had SD which lasted for at least 24 weeks in 9 patients. The median PFS was 12.1 weeks and the median OS was 34.6 weeks. Diarrhoea, acneiform dermatitis and fatigue were the most common grade 3-4 treatment-related AEs (Abdul Razak *et al.* 2013). There is currently an ongoing phase 1 clinical trial where investigators assessing the effects of dacomitinib with RT and with or without chemotherapy in HNSCC patients (clinicaltrials.gov-NCT01737008).

1.8.2.5. Afatinib (Gilotrif, BIBW2992)

Afatinib is a second generation irreversible inhibitor that targets the entire HER family receptors. Afatinib received approval from FDA in 2013. It is approved as a first-line treatment for patients with metastatic NSCLC whose tumours harbor exon 19 deletions or exon 21 (L858R) substitution mutations

in EGFR gene (Keating 2014). Seiwert *et al.* (2014) conducted a randomized phase II clinical trial of afatinib versus cetuximab in patients with recurrent or metastatic HNSCC. 121 patients who failed platinum-containing therapy received either afatinib (50 mg/d) (n=61) or cetuximab (400 mg/ m^2 initial loading dose followed by 250 mg/ m^2 /w) (n=60) in stage I of this trial. The mean tumour shrinkage was 10.4% with afatinib and 5.4% with cetuximab. ORR was 16.1% with afatinib and 6.5% with cetuximab. The median PFS was 13 versus 15 weeks in afatinib versus cetuximab groups. DCR were 50% with afatinib and 56.5% with cetuximab. Upon disease progression or drug-related AEs at stage I, 68 patients (32 from afatinib arm and 36 from cetuximab arm) were allowed to cross over to other treatment (stage II). At this stage DCR was 38.9% with afatinib and 18.8% with cetuximab and median PFS was 9.3 versus 5.7 weeks in afatinib versus cetuximab arms. Results from this study demonstrated that afatinib has clinical activity similar to cetuximab in HNSCC and patients found to respond to sequential therapy with afatinib/cetuximab or vice versa after afatinib or cetuximab failure (Seiwert *et al.* 2014). Based on these results, Machiels *et al.* (2014) has conducted a phase III, LUX-Head & Neck 1 study where they are currently investigating afatinib versus methotrexate in patients with recurrent or metastatic HNSCC who failed platinum-based chemotherapy (Machiels *et al.* 2014). In another phase III trial, LUX-Head & Neck 2, which is currently recruiting patients, afatinib is investigated as an adjuvant therapy after CRT in patients with locally advanced HNSCC (clinicaltrials.gov-NCT01345669).

1.8.3. EGFR monoclonal antibodies

1.8.3.1. Zalutumumab

Zalutumumab is a novel high affinity fully human IgG1 mAb against EGFR. Zalutumumab has shown promise for treatment of HNSCC. Bastholt *et al.* (2007) conducted a phase I/II study to evaluate the safety and tolerability of zalutumumab (HuMax-EGFr) in 28 patients with advanced HNSCC. This group suggested that zalutumumab was well tolerated up to the highest tested dose of 8 mg/kg. Skin rash was the most frequent AE (n=16) and no DLTs were observed (Bastholt *et al.* 2007). Machiels *et al.* (2011) conducted a randomised phase III trial where HNSCC patients either received zalutumumab (8mg/kg initial dose followed by increasing titrated doses of 4mg/kg every 14 days until the development of grade 2 skin rash) plus Best Supportive Care (BSC) (group 1: 191 patients, 67%) or BSC with optional methotrexate (group 2: 95 patients, 33%). All 286 patients who entered into the trial had recurrent or metastatic disease which progressed within 6 months of platinum-based therapy. Median OS was 6.7 months in group 1 and 5.2 months in group 2. Although zalutumumab did not improve the OS, however, group 1 had longer PFS compared to group 2. Skin rash, anaemia and pneumonia were the most common grade 3/4 AEs and tumour haemorrhage, pneumonia and dysphagia were the most common serious AEs (Machiels *et al.* 2011). Saloura *et al.* (2014) conducted a phase II trial of zalutumumab for patients with platinum-refractory recurrent or metastatic HNSCC. Median OS was 5.3 months and median PFS was 2.1 months. In this study two deaths (2/90) occurred which was beleived to be treatment-related. skin rash,

hypomagnesaemia and pneumonitis were grade 3/4 drug-related AEs (Saloura *et al.* 2014). Currently there is an ongoing trial where investigators assessing whether addition of zalutumumab to primary curative RT could enhance the loco-regional control in HNSCC (clinicaltrials.gov-NCT00496652).

1.8.3.2. Nimotuzumab (h-R3)

Nimotuzumab is a humanized IgG1 anti-EGFR mAb which has shown to have anti-proliferative, anti-angiogenic and pro-apoptotic activity in EGFR overexpressing tumours (Crombet-Ramos *et al.* 2002). Results from a phase I study showed no severe toxicity in 12 patients with advanced epithelial cancers who received nimotuzumab (Crombet-Ramos *et al.* 2003). In a phase I/II trial, 24 patients with advanced HNSCC received nimotuzumab (50,100 ,200,400 mg) in combination with RT. The combination treatment was well tolerated and significantly increased the OS where high doses of nimotuzumab (200 or 400 mg) were used (Crombet-Ramos *et al.* 2004). Rodriguez *et al.* (2010) conducted a phase II trial where they treated 106 patients with advanced HNSCC with nimotuzumab plus RT (group 1) or RT plus placebo (group 2). The median OS rates were 12.5 and 9.47 and CCR rates were 59.5 and 34.2%, for group 1 and 2 respectively. Combination treatment was well tolerated and most common AEs were asthenia, fever, headache and chills with no rash (Rodriguez *et al.* 2010). In another phase II trial, 92 patients with advanced HNSCC were treated either with RT versus RT + nimotuzumab (group A) or CRT versus CRT + nimotuzumab (group B).

Loco-regional response was 37% versus 76% in group A and 70% versus 100% in group B. The OS rates at 48 months follow-up were 13% versus 34% and 21% versus 47% in group A and B respectively. 65% reduction in risk of death was achieved with CRT plus nimotuzumab (Babu *et al.* 2010).

1.8.3.3. ABT-806

Studies have shown the association between mutant EGFR, EGFR variant III, and induced cell proliferation and tumour volume as well as cetuximab resistance in HNSCC (Sok *et al.* 2006). ABT-806 is a humanized IgG1 mAb which was raised against a truncated version of EGFR (EGFR vIII). However, ABT-806 has also been shown to bind to wild type EGFR on the cell surface of the cells which have EGFR gene amplification (Johns *et al.* 2002, Mishima *et al.* 2001, Luwor *et al.* 2006). Studies have reported that ABT-806 inhibits the growth of human xenografts which express EGFR vIII or amplified EGFR (Mishima *et al.* 2001, Luwor *et al.* 2006). A phase I clinical trial of ABT-806 was conducted by Cleary *et al.* (2012) where 26 patients with advanced solid tumours including HNSCC were treated with 2-24 mg/kg of ABT-806 every other week. Dyspnea (15%) and hypotension (12%) were ABT-806−related grade 3/4 AEs and nausea (23%), vomiting (19%), fatigue (15%), and rash (12%) were reported to be likely due to the administration of ABT-806. No typical EGFR-inhibitor related rash was reported in these patients. 2 head and neck cancer, 2 colorectal cancer and 1 lung cancer patients achieved SD (> 8 weeks) and 1 HNSCC patient with EGFR amplification had stable disease for 23 weeks (Cleary *et al.* 2012).

1.8.3.4. MEHD7945A

MEHD7945A is a novel dual target human IgG1 antibody against EGFR and HER3 (Schaefer *et al.* 2011). In a pre-clinical study by Huang *et al.* (2013), MEHD7945A was shown to have the ability to overcome acquired resistance to cetuximab and erlotinib in lung and head and neck cancer tumour cells. Cervantes-Ruiperez *et al.* (2012) conducted a phase I trial of MEHD7945A in patients with refractory/recurrent epithelial tumours. In this study 36 patients (CRC=12, NSCLC=9, HNSCC=10, pancreatic=5) received intravenous MEHD7945A (1-30 mg/kg) every 2 weeks. In patients who received 14 mg/kg of MEHD7945A no drug-related G≥3 AEs have been reported. Rash/dermatitis (53%), diarrhoea (36%), fatigue (22%), paronychia (19%), dry skin, nausea, and decreased appetite (all 17%), asthenia and stomatitis/oral pain (all 14%) were the most common drug-related G1-2 AEs. Anti-tumour activity of MEHD7945A was observed in 2 HNSCC, 2 NSCLC and 4 CRC patients. 7 of these patients had previous treatment with EGFR inhibitors. 2 HNSCC patients had PR and other 6 patients had SD for at least 8 weeks (Cervantes-Ruiperez *et al.* 2012). There are two other MEHD7945A trials which are currently recruiting patients. In one study investigators are assessing MEHD7945A versus cetuximab in patients with recurrent or metastatic HNSCC (clinicaltrials.gov-NCT01577173). In another trial, the effect of combining MEHD7945A with cisplatin and 5-FU or paclitaxel and carboplatin is being evaluated in patients with recurrent or metastatic HNSCC (clinicaltrials.gov-NCT01911598).

1.8.3.5. Symoo4

Sym004 is a mixture of two anti-EGFR mAbs which target distinct non-overlapping epitopes in the extracellular domain III of EGF receptor (Pedersen *et al.* 2010). In pre-clinical study Sym004 has shown to overcome acquired resistance to cetuximab in NSCLC cells *in vitro* and *in vivo* (Iida *et al.* 2013). Sym004 has also been investigated in clinical setting. In a phase II trial by Machiels *et al.* (2013) the efficacy and safety of Sym004 was evaluated in HNSCC patients who failed anti-EGFR based therapies. In this study 26 patients received Sym004 (12 mg/kg/w IV) until disease progression. 10/26 patients had hypomagnesaemia ≥ G 3 and 11/26 patients developed skin rash ≥ G 3 during treatment. Median PFS was 82 and OS was 156 days. Tumour shrinkage was observed in 8/20 evaluable patients and SD was achieved in 14 patients, but no objective response was reported (Machiels *et al.* 2013).

1.8.3.6. Panitumumab (Vectibix)

Panitumumab is a fully human IgG2 mAb against EGFR which received approval from FDA for the treatment of metastatic colorectal cancer (Ruthann *et al.* 2007). Following promising results from pre-clinical studies in HNSCC (Kruser *et al.* 2007), the effects of combining panitumumab with paclitaxel, carboplatin and Intensity-Modulated RT (IMRT) was investigated in the clinical setting (Wirth *et al.* 2010). 19 patients with advanced HNSCC entered into this phase I study and received fixed doses of panitumumab (2.5 mg/kg) and carboplatin (AUC 1.5) plus paclitaxel (15 mg/m ^{2}, 3 patients and 30 mg/m ^{2},

16 patients) with IMRT on a weekly basis. DLT occurred in only one patient treated with a high dose of paclitaxel. Most common toxicities were oral pain, xerostomia, dysphagia, weight loss, dermatitis, nausea and acneiform rash and grade 3 or more mucositis were observed in all patients. All patients had PR, overall CCR rate was 95% and 95% of patients remained disease free at median follow-up time of 21 months (Wirth *et al.* 2010). In another study Wirth *et al.* (2013), assessed the effects of adding panitumumab to docetaxel/cisplatin as a first-line treatment for recurrent or metastatic HNSCC. 103 patients entered into this randomised phase II study. Arm 1 (n=52) received docetaxel/cisplatin (both at 75 mg/m^2) plus panitumumab (9 mg/kg) and arm 2 (N=51) received docetaxel/cisplatin (both at 75 mg/m^2) alone. Median PFS, OS and ORR were 6.9, 12.9 and 44 % in arm 1 and 5.5, 13.8 and 37% in arm 2 respectively. Grade 3/4 AEs were 73% in Arm 1 versus 56% in Arm 2 (Wirth *et al.* 2013). In a phase II randomised trial (CONCERT-1) Giralt *et al.* (2012) assessed the efficacy and safety of treatment with CRT (cisplatin 100 mg/m^2, 3 cycles + standard fractionation RT) alone (arm 1, n=63) or CRT plus panitumumab (9 mg/kg) (arm2, n=87) as a first-line treatment in locally advanced HNSCC patients. 36% of the arm 2 patients and 24% of the arm 1 patients died. In summary, this treatment regimen was associated with increased toxicity and did not produce any survival benefit (Giralt *et al.* 2012). In CONCERT-2 trial treatment with panitumumab (9 mg/kg) plus RT (70-72 Gy) was compared with CRT (cisplatin 100 mg/m^2 + XRT; 70-72 Gy) in 151 patients with locally advanced HNSCC. The 2-year loco-regional control rate was 51% for panitumumab + RT and 61% for CRT. PFS and OS results also favoured the CRT arm. Grade 3+ AEs were 85%

and 81% in panitumumab + RT and CRT respectively (Giralt *et al.* 2012). In a randomised phase III trial (SPECTRUM trial) Vermorken *et al.* (2013) studied the efficacy and safety of adding panitumumab to cisplatin and fluorouracil regimen as a first-line treatment for patients with recurrent or metastatic HNSCC. 657 patients received up to six, 3-week cycles of cisplatin (100 mg/m^2, IV, day 1) and fluorouracil (1000 mg/m^2, IV, days 1-4) ± panitumumab (9 mg/kg). Although addition of panitumumab did not significantly improved median OS (11.1 versus 9 months), it improved median PFS (5.8 versus 4.6 months). Grade 3/4 AEs; skin/eye toxicity, diarrhoea, hypomagnesaemia, hypokalaemia and dehydration were more common in patients who received panitumumab. 14 and 2 deaths were reported in panitumumab and control group respectively. These investigators also assessed the p16 status in 443 patients and found that panitumumab improved the median OS in patients with p16-negative patients (n=344) (11·7 versus 8·6 months). This was not observed in p16 positive patients (n=99). Results from this study suggested the potential of p16 status as a prognostic and predictive marker in panitumumab and chemotherapy treatments (Vermorken *et al.* 2013).

1.8.3.7. Cetuximab (Erbitux)

Cetuximab is a recombinant human/mouse chimeric monoclonal IgG1 antibody which has a higher affinity for EGFR relative to EGF and TGF-α (Kim *et al*. 2001). Therefore it binds to the receptor and induces its internalization and degradation which leads to the downregulation of the receptor (Martinelli *et al*. 2009). By blocking the ligand binding site, cetuximab also inhibits the

dimerization and activation of the receptor which consecutively results in a lack of activation of downstream signalling pathways (Martinelli *et al*. 2009). Studies have shown that treatment with cetuximab reduces the expression of cyclin D1 and induces the expression of cyclin dependent kinase inhibitors p21 (WAF1/CIP1) and p27 (KIP1). This leads to the cell cycle arrest at the G1 phase and subsequent inhibition of cancerous cell proliferation (Wu *et al*. 1996, Huether *et al.* 2005, Martinelli *et al*. 2009). Cetuximab has also been shown to provoke apoptosis by inducing the expression level of pro-apoptotic Bcl-2–associated X (Bax) protein, pro-apoptotic caspase-3, caspase-8 and caspase-9 or by inhibiting the expression level of anti-apoptotic B-cell lymphoma 2 (Bcl-2) protein (Mandal *et al*. 1998, Martinelli *et al*. 2009). Cetuximab has also been shown to inhibit angiogenesis by down-regulating pro-angiogenic proteins, mainly interleukin-8 (IL-8), Vascular Endothelial Growth Factor (VEGF) and basic Fibroblast Growth Factor (bFGF) (Perrotte *et al*. 1999, Martinelli *et al*. 2009). Exposure to cetuximab has been shown to inhibit cancerous cell invasion and metastasis. This is believed to be due to the down-regulation of Matrix Metalloproteinase-9 (MMP-9) which is a metastasis associated protein (Inoue *et al*. 2000).

In 2006, FDA approved cetuximab for treatment of non-metastatic HNSCC in combination with RT as a first-line therapy or as a single drug following standard therapy. In 2011, FDA approved cetuximab for treatment of metastatic head and neck cancer in combination with platinum-based therapy plus 5-florouracil (5-FU) as a first-line therapy (http://www.fda.gov/).

1.8.3.7.1. Cetuximab and radiotherapy

Bonner *et al.* (2006) conducted a phase III trial to compare RT alone with RT plus cetuximab on 424 patients with loco-regionally advanced head and neck cancer (Bonner *et al.* 2006). These patients were randomly assigned to receive treatment with either high dose of RT alone (213 patients) or high dose of RT combined with weekly administration of cetuximab (211 patients). Patients were treated with 400 mg/m^2 dose of cetuximab prior to initiation of RT followed by weekly treatment with 250 mg/m^2 during RT. The median duration of loco-regional control was prolonged by cetuximab from 14.9 months in RT alone arm to 24.4 months among patients treated with cetuximab plus RT. Combination treatment also improved the median duration of OS from 29.3 to 49.0 months and prolonged PFS significantly (Bonner *et al.* 2006). However, up to date RT plus cetuximab hasn't been compared to platinum-based CRT in locally advanced head and neck cancer. Therefore the well established platinum-based CRT regimen is still considered as the standard treatment for these patients where surgery is not an option (Specenier and Vermorken 2013). Nevertheless, at present there are two ongoing clinical trials where clinical researchers are comparing cetuximab plus RT versus CRT. In a phase II trial investigators are comparing cetuximab and RT versus cisplatin and RT as a first line treatment for locally advanced HNSCC patients (clinicaltrials.gov-NCT01216020). Other group has conducted a phase III trial to compare the combination of cetuximab plus RT versus CRT in HPV associated oropharynx cancer (clinicaltrials.gov-NCT01302834).

1.8.3.7.2. Cetuximab and chemotherapy

In a randomised phase III trial by Burtness *et al.* (2005), 117 patients with recurrent or metastatic HNSCC were assigned to receive cisplatin (100 mg/m^2 every 4 weeks) with cetuximab (200 mL/m^2 on day 1 followed by 125 mL/m^2/w) in arm A or placebo (200 mL/m^2 on day 1 followed by 125 mL/m^2/w) in arm B. Median PFS was prolonged from 2.7 months in arm B to 4.2 months in arm A. Median OS was improved very slightly from 8.0 months in arm B to 9.2 months in arm A. Although no significant improvement was observed in PFS and OS, the addition of cetuximab improved the response rate (26% in arm A versus 10% in arm B) in this trial (Burtness *et al.* 2005).

The efficacy and safety of cetuximab in combination with cisplatin for the treatment of recurrent or metastatic platinum refractory HNSCC patients was evaluated in two phase II trials conducted by Herbst *et al.* (2005) and Baselga *et al.* (2005). 132 patients entered into the trial by Herbst *et al.* (2005). These patients were treated with cisplatin/paclitaxel or cisplatin/fluorouracil. 30 patients who had PR or CCR continued to receive standard therapy. 51 patients had SD (group1) and 25 had progressive disease (group2). These patients in addition to 54 patients who developed progressive disease within 90 days from the end of platinum-based therapy (group3) were treated with cetuximab and cisplatin. Patients received cetuximab with initial dose of 400 mg/m^2 on day 1 followed by 250 mg/m^2/w IV and cisplatin 75 or 100 mg/m^2 IV on day 1 every 21 days. The response rates were 18%, 20%, and 6% in groups 1, 2 and 3 respectively. The median PFS was 7.4, 4.2 and 4.1months and the median OS was 11.7, 6.1 and 4.3 months in these groups

respectively. Anemia, skin rash, leukopenia, fatigue and nausea were the most common drug-related toxicities. In this study, 5% of the patients were reported to have a grade 3/4 hypersensitivity reaction to cetuximab (Herbst *et al.* 2005). In a study by Baselga *et al.* (2005) 96 patients were treated with cetuximab (initial dose of 400 mg/m^2 IV on day 1 followed by 250 mg/m^2/w IV) and cisplatin or carboplatin (based on their treatment schedule before entering into the trial). The response rate was 10% and DCR was 53%. The median TTP was 85 days and OS was 183 days in these patients. The most common reported AE was cetuximab-related skin toxicities (Baselga *et al.* 2005). Results from these two studies indicate the potential of cetuximab in reversing resistance to chemotherapy.

In the Erbitux in First-Line Treatment of Recurrent or Metastatic Head and Neck Cancer (EXTREME) trial which was conducted by Vermorken *et al.* (2008) 442 patients with recurrent or metastatic HNSCC were randomised. 220 patients received cisplatin (100 mg/m^2 on day 1) or carboplatin (5mg/mL/min on day 1) plus fluorouracil (1000 mg/m^2 per day for 4 days) every 3 weeks for a maximum of 6 cycles. The second group (222 patients) received identical chemotherapy regimen plus cetuximab (initial dose of 400 mg/m^2 followed by 250 mg/m^2/w) for a maximum of 6 cycles. Improved OS, PFS and response were observed in patients who had cetuximab plus platinum-fluorouracil chemotherapy compared to platinum-based chemotherapy plus fluorouracil alone (Vermorken *et al.* 2008). Tumour HPV/p16 status is an important prognostic factor in loco-regionally advanced HNSCC. Therefore this group investigated the outcome of the EXTREME trial according to tumour HPV/p16 status in order to assess its prognostic value in

recurrent/metastatic HNSCC. Their results showed that patients with p16 or HPV-positive tumours had longer OS compared to p16 or HPV-negative tumours (Vermorken *et al.* 2014).

1.8.3.7.3. Cetuximab and chemo-radiotherapy

Pfister *et al.* (2006) conducted the first phase II clinical trial of a combination of cetuximab with CRT in loco-regionally advanced HNSCC. 21 patients received concomitant boost RT (1.8 Gy/d weeks 1-6; boost: 1.6 Gy 4-6 hours later weeks 5-6; 70 Gy total to gross disease), cisplatin (100 mg/m^2 IV weeks 1 and 4), and cetuximab (400 mg/m^2 IV week 1, followed by 250 mg/m^2 weeks 2-10). The 3-year OS, PFS, and loco-regional control rates were 76%, 56%, and 71% respectively. Grade 3/4 cetuximab-related skin rash and hypersensitivity were observed in 10% and 5% of patients respectively. However, due to significant AEs such as myocardial infarction, bacteremia, atrial fibrillation and two early deaths (one due to pneumonia and one for an unknown cause) this study was prematurely closed (Pfister *et al.* 2006). In 2011, Merlano *et al.* published the results from a phase II trial which was conducted to assess the efficacy of adding cetuximab to CRT in stage III–IV M0 HNSCC. 45 patients received RT as a single daily fraction (up to 70 Gy), three cycles of cisplatin (20 mg/m^2/day), fluorouracil (200 mg/m^2/day) and cetuximab (initial dose of 400 mg/m^2 followed by 250 mg/m^2/w). 33 patients (73%) developed grade 3 radiodermatitis. 32 patients (71%) had CCR and PFS and OS was 21+ months and 32.6+ respectively (Merlano *et al.* 2011). Tong *et al.* (2012) studied the tolerability and efficacy of combining cetuximab with simultaneous integrated boost Intensity-Modulated RT (SIB-IMRT) and 5-

fluorouracil plus hydroxyurea CRT regimen. In this phase II trial 65 patients with loco-regionally advanced HNSCC received SIB-IMRT (for a total dose of 60-73.5 Gy) and concurrent treatment with 5-fluorouracil (600 mg/m^2/d), hydroxyurea (500 mg, twice a day) and cetuximab (250 mg/m^2 every two weeks). Mucositis (46%), leukopenia (18%), anemia (18%) and dermatitis (17%) were the most common grade 3/4 toxicities in this study. The 2-year loco-regional control, distant control, PFS, EFS and OS were 79, 83, 72, 63 and 80%, respectively. Results from this study also demonstrated the correlation between p16 overexpression with significantly increased distant control, PFS, EFS and OS in 48 patients (Tong *et al.* 2012). Ang *et al.* (2014) conducted a randomised phase III trial (RTOG-0522) where they studied the effect of combined treatment with cetuximab and cisplatin-based CRT in stage III/IV non-metastatic HNSCC patients. 940 patients were received either CRT (72 Gy plus cisplatin 100 mg/m^2) with or without weekly cetuximab. The 2-year PFS was 64.3% in patients who received CRT (group 1) and 63.4% in group which received cetuximab in addition to CRT (group 2). The 2-year OS rates were 79.7% and 82.6% in groups 1 and 2 respectively. The 2-year loco-regional relapse rate was 19.8% and 24.5% and the 2-year distant metastasis rate was 12.0% and 7.6% respectively (Ang et al. 2014). Addition of cetuximab to cisplatin-based chemo-radiotherapy failed to improve PFS, OS or LRC in HNSCC patients whereas studies have shown that the addition of cetuximab to radiation (Bonner *et al.* 2006) or platinum-based chemotherapy (Vermorken *et al.* 2008) could improve survival in these patients (Bonner *et al.* 2006, Vermorken *et al.* 2008). Ionizing radiation (IR) is believed to provoke the translocation of EGFR into nucleus where it interacts with DNA-dependent

protein kinase (DNA-PK). This has shown to induce the activity of DNA-PK and thus triggering the repair of DNA double-strand breaks. Studies have shown that cetuximab impairs the nuclear translocation of EGFR therefore it inhibits IR-induced DNA-PK activation. This leads to the inhibition of DNA repair and as a subsequence improves cellular radio-sensitivity (Bandyopadhyay *et al.* 1998, Dittmann *et al.* 2005). As cetuximab and platinum derivatives have comparable radio-sensitization activities the addition of cetuximab to CRT cannot make resistant tumours sensitive to the treatment or improve the response of sensitive tumours (Ang *et al.* 2014). In addition, in RTOG-0522 trial patients who were treated with CTX+CRT had higher rates of mucositis and local dermatitis compared to the patients who were treated with CRT. This led to an incomplete drug administration in 26.4% of patients and radiation interruptions in 26.9% of patients, which could explain the trend toward a higher local regional failure rate in the experimental arm (Ang *et al.* 2014).

1.8.3.7.4. Proposed mechanisms of resistance to cetuximab

As described, cetuximab demonstrated synergism with RT and chemotherapy and showed some efficacy with CRT in HNSCC patients. However, only a subset of patients respond to cetuximab and a large number of patients either do not respond to the drug or develop resistance at some point throughout the treatment. Therefore in recent years there has been growing interest in understanding the mechanisms of resistance to cetuximab and several mechanisms have been proposed by different groups.

Wheeler *et al*. (2008) studied the involvement of HER receptors in the development of resistance to cetuximab in NSCLC and HNSCC. They have shown that the dysregulation of c-Cbl-mediated internalization, ubiquitinylation and degradation of the EGFR is responsible for an induced EGFR expression in cetuximab-resistant clones (Wheeler *et al*. 2008). This group has also reported the hyperactivation of HER2, HER3 and c-MET in resistant cells (Wheeler *et al*. 2008). An increased phosphorylation of HER2 has also been shown to be responsible for the development of resistance to cetuximab in bladder cancer (Quesnelle and Grandis 2011). In another study by Yonesaka *et al*. (2011) HER2 activation was also found to be involved in cetuximab resistance in colorectal cancer. This group showed that activation of HER2 signalling through *HER2* amplification or heregulin overexpression blocks the downregulation of ERK1/2 signalling in the presence of cetuximab. This results in the suppression of growth inhibition and eventually leads to cetuximab resistance (Yonesaka *et al*. 2011). Nuclear EGFR has also been shown to play a role in acquired resistance to cetuximab (Li *et al.* 2009). Results from this study have shown that an upregulation of HER ligands initiates EGFR translocation to the nucleus in cetuximab-resistant clones of NSCLC. Induced nuclear EGFR expression as well as overexpression of cyclin D1 and B-Myb in cetuximab resistant NSCLC cells was proposed by this group as a mechanism of resistance to cetuximab. This group has also suggested the involvement of Src Family Kinases (SFKs) in mediating the nuclear localization of EGFR (Li *et al.* 2009). It has been reported that an induced activation of SFKs in cetuximab-resistant NSCLC cells is responsible for the hyperactivation of EGFR and thus resulting in the activation of HER3

and PI3K/AKT pathway (Wheeler *et al*. 2009). Nevo *et al.* (2009) have shown that Mammary-Derived Growth Inhibitor (MDGI) plays an important role in cetuximab resistance by inducing the intracellular accumulation of EGFR in breast and lung cancers (Nevo *et al.* 2009). Induced expression of Vascular Endothelial Growth Factor (VEGF), a key contributor to angiogenesis, has been reported in cetuximab-resistant A431 cell line (Viloria-Petit *et al.* 2001). Overexpression of VEGFR1, Vascular Endothelial Growth Factor Receptor-1, has also been shown by Bianco *et al.* (2008) in human colon carcinoma, hormone refractory prostate adenocarcinoma and mammary gland carcinoma (Bianco *et al.* 2008). Studies have also shown the involvement of mutations in the oncogenes KRAS, BRAF and PIK3CA as well as the tumour suppressor gene PTEN in response to cetuximab in metastatic colorectal cancer (Bardelli & Siena 2009). Sok, *et al.* (2006) have reported that HNSCC cells which express mutant EGFR, EGFR variant III, showed induced cell proliferation and tumour volume, suggesting the involvement of EGFRvIII in resistance to cetuximab (Sok, *et al.* 2006). Montagut *et al.* (2012) have reported a point mutation (S492R) in the EGFR extracellular domain which was shown to play a role in the development of resistance to cetuximab in HNSCC (Montagut *et al.* 2012).

The aim of this study was to further investigate the mechanisms of resistance to cetuximab in HNSCC, in order to propose suitable approaches to overcome its limitation in clinic.

1.9. AIM AND OBJECTIVES

Our group has previously investigated the mechanisms of resistance to EGFR TKI, gefitinib, and anti-HER2 monoclonal antibody, trastuzumab, in breast cancer cell lines (Kong *et al.* 2008, Gijsen *et al*. 2010, Feldinger *et al.* 2014). Results from these studies demonstrated the involvement of ADAM-mediated ligand release in the development of acquired resistance to these targeted therapies. Therefore similar methodologies were used to study the mechanisms of resistance to anti-EGFR monoclonal antibody, cetuximab, in HNSCC.

1.9.1. Overall D.Phil. aim

Investigating the role of ADAM10 and ADAM17 in relation to cetuximab treatment and resistance in HNSCC.

1.9.2. Objectives

1.9.2.1. Comparing the effects of cetuximab treatment on HER receptors' signalling in parental and resistant HNSCC cell lines.

- Assessing the effects of cetuximab on several parental HNSCC cell lines.
- Generation of cetuximab resistant HNSCC cell lines.
- Comparing the effects of cetuximab on HER receptor expression and activation in relation to downstream signalling in parental and resistant HNSCC cell lines.

1.9.2.2. Investigating the role of ADAM10 and ADAM17 in relation to cetuximab treatment and resistance.

- Investigating the effect of cetuximab on ADAM10 and ADAM17 proteins expression levels.
- Investigating the effect of cetuximab on release of HER ligands; NRG-1 and BTC.
- Investigating the effect of ADAM10 and ADAM17 knockdown and inhibition in parental and cetuximab resistant cells.

1.9.2.3. Investigating the effect of reversible EGFR TKI, gefitinib, with or without cetuximab in parental and cetuximab resistant cells.

1.9.2.4. Investigating the effect of treatment with an irreversible panHER inhibitor, afatinib, and its combination with cetuximab in parental and cetuximab resistant cells.

1.9.2.5. Assessing the role of ADAM10 and ADAM17 as prognostic and predictive biomarkers in human patients.

CHAPTER 2

MATERIALS & METHODS

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Cell Lines

SCC4, SCC9, SCC15 and SCC25 head and neck squamous cell carcinoma cell lines were used in this study. SCC4 and SCC25 cell lines were purchased from ATCC (American Type Culture Collection). SCC9 and SCC15 cell lines were kindly provided by Dr. Stephan Feller (University of Oxford). The epidermoid carcinoma cell line A431 which is known to express high level of EGFR was used as a positive control cell line. A431 cell line was provided by Cancer Research UK. These cell lines were not tested for mycoplasma contamination and were not subject to short tandem repeat (STR) analysis. Cetuximab-resistant SCC9 and SCC15 cell lines were generated by exposing the cells to increasing concentrations of cetuximab until 20 µg/mL over 24 months and they are currently maintained at this dose.

2.1.2. Primary Antibodies

Antibody	Manufacturer	Dilution
EGFR	Cell Signaling	1:1000
PEGFR	Santa Cruz	1:500
HER2	Cell Signaling	1:500
PHER2	Cell Signaling	1:500
HER3	Abcam	1:2000
PHER3	Cell Signaling	1:500
AKT	Cell Signaling	1:1000
PAKT	Cell Signaling	1:1000
ERK	Cell Signaling	1:1000
PERK	Cell Signaling	1:1000
ADAM 10	Abcam	1:500
ADAM 17	Abcam	1:500
BTC	Abcam	1:500
NRG-1	Abcam	1:500
ß-ACTIN	Cell Signaling	1:1000

Table 2.1. List of primary antibodies used in this study.

2.1.3. Secondary Antibodies

Antibody	Manufacturer	Dilution
ECL Anti-Mouse	GE Health Care	1:2500
ECL Anti-Rabbit	GE Health Care	1:2500

Table 2.2: List of secondary antibodies used in this study.

2.1.4. Drugs & Inhibitors

Drugs & Inhibitors	Manufacturer
Cetuximab (Erbitux)	Merck Serono
Gefitinib (Iressa)	Tocris
Afatinib (BIBW2992)	Selleckchem
TAPI-1 (ADAM17 and other metalloproteases inhibitor) Primary Targets: MMPs and TACE Primary Targets IC_{50}: 8.09 µM and 3.61 µM blocking constitutive and muscarinic receptor-stimulated sAAPα release in HEK 293 cells expressing M3 muscarinic receptors, respectively Ref: http://www.emdmillipore.com/INTL/en	Calbiochem
INCB3619 (ADAM10/17 Inhibitor) Targets (IC_{50}, nM): ADAM10 (22), ADAM17 (14), ADAM33 (1026), ADAM9 (>5000), MMP1 (>5000), MMP2 (35), MMP3 (3560), MMP7 (>5000), MMP9 (304), MMP12 (17), MMP14 (772) Ref: Zhou et al. 2006.	Incyte

Table 2.3. List of drugs and inhibitors used in this study.

2.1.5. Primer Sequences

The following primers were designed by Ms Merel Gijsen (University of Oxford) and they were purchased from Invitrogen.

Gene	Primer Sequence (5'-3')	Direction
ADAM10	ATATTACGGAACACGAGAAGCTG	Forward
ADAM10	TCAATCGCTTTAACATGACTGG	Reverse
ADAM17	CCTTTCTGCGAGAGGGAAC	Forward
ADAM17	CACCTTGCAGGAGTTGTCAG	Reverse
Actin	AATGGCAATGAGCGGTTC	Forward
Actin	GGATGCCACAGGACTCCAT	Reverse

Table 2.4. Gene specific sense and antisense primer sequences.

2.1.6. Solutions and Buffers

Solutions/Buffers	Composition
1X PBS	140 mM NaCl 2.7 mM KCL 8.0 mM Na_2HPO_4 1.5 mM KH_2PO_4 Made up in dH_2O & sterilised by autoclaving
1X PBST (PBS-Tween-20)	0.2% Tween-20 in 1 X PBS
Blocking Buffer	5% BSA in 1X PBST
Cell Lysis Buffer	150 mM NaCl 10 mM EDTA (pH 8) 20 mM Tris-HCL (pH 7.5) 10 mM $Na_2H_2P_2O_7$ 100 mM NaF 1% Triton-X-100 Complete Mini Protease Inhibitor Cocktail Tablet Made up in dH_2O
Transfer buffer	192 mM glycine 25 mM Tris-HCL 20 % methanol Made up in dH_2O
Loading Buffer	90% LDS sample buffer (4X) 10% Beta-mercaptoethanol
1X Running buffer	5% NuPAGE® MOPS SDS Running buffer (20X) 95% dH_2O

Table 2.5. List of solutions and buffers used in this study.

2.1.7. Reagents

Reagent	Manufacturer
Albumin, from bovine serum	Sigma-Aldrich
All Stars Negative Control siRNA	Qiagen
Beta-mercaptoethanol	Sigma-Aldrich
BIO-RAD Protein Assay	Bio-rad
Bovine serum albumin	Invitrogen
Citroclear	National Diagnostics
Complete Mini Protease Inhibitor Cocktail Tablet	Roche
Dimethyl sulfoxide, 99.9%, A.C.S. spectrophotometric grade (DMSO)	Sigma-Aldrich
DMEM (1X) Dulbecco's Modified Eagle Medium (DMEM) + 4.5 g/L D-Glucose, +L-Glutamine,+Pyruvate	Gibco
DMEM/F-12 (1:1) (1X) Dulbecco's Modified Eagle Medium F-12 Nutrient Mixture (Ham)	Gibco
DNase and DNase dilution solution	Bio-Rad
ECL detection reagents	GE Healthcare
Fetal Bovine Serum Gold	PAA
Glycine	Sigma-Aldrich
Hematoxylin QS	Vector Laboratories
High Capacity cDNA Reverse Transcription kit	Applied Biosystems
Human Betacellulin DuoSet ELISA Development Kit	R & D Systems
Human NRG1-beta 1/HRG1-beta 1 DuoSet ELISA Development Kit	R & D Systems
Hydrocortisone	Sigma-Aldrich
ImmPACT DAB Peroxidase Substrate	Vector Laboratories
ImmPRESS Anti-Rabbit Ig (peroxidase)	Vector Laboratories
ISOTON® II diluents	Beckmann Coulter
LDS sample buffer (4X)	Invitrogen
Methylene blue (0.2%)	Sigma-Aldrich
Normal horse serum blocking solution (2.5%)	Vector Laboratories
Novex® sharp protein standard	Invitrogen
NuPAGE® 4-12% Bis-Tris Gels	Invitrogen
NuPAGE® MOPS SDS Running buffer (20X)	Invitrogen
Oligofectamine™ Reagent	Invitrogen
OPTI-MEM® I + GlutaMAX™-I	Gibco

Reagent	Manufacturer
Penicillin-Streptomycin (100X)	PAA
Purified human IgG	R & D Systems
RIPA Buffer	Sigma-Aldrich
RNeasy Mini kit	Qiagen
SensiMix™ SYPR Hi-ROX Mastermix kit	Bioline
Thiazolyl Blue Tetrazolium Bromid	USB corporation
Triton X-100	Sigma-Aldrich
Trypsin-EDTA (10x) 0.5% / 0.2% in PBS	PAA
Tween-20	Sigma-Aldrich

Table 2.6. List of reagents used in this study.

2.1.8. siRNAs

FlexiTube siRNA	Target Sequence	Manufacturer
ADAM17-7	CAGGATGTAATTGAACGATTT	Qiagen
ADAM17-8	AAGAAACAGAGTGCTAATTTA	Qiagen
ADAM10-5	TTGGTGGGCAGTATTACTTAT	Qiagen
ADAM10-6	GAGAGATATATTAAAGACCGA	Qiagen

Table 2.7. List of siRNAs used in this study.

2.2. Methods

2.2.1. Cell culture

2.2.1.1. Maintenance of cell lines

A431 cell line was cultured in DMEM supplemented with 10% fetal bovine serum and 1% antibiotic penicillin-streptomycin. SCC4, SCC9, SCC15 and SCC25 cell lines were cultured in DMEM/F-12 supplemented with 10% fetal bovine serum, 1% antibiotic penicillin-streptomycin and 400 ng/mL hydrocortisone. All cells were grown at 37ºC and 5% CO_2.

2.2.1.2. Passaging of cell lines

The cells were subcultured every 3 days. To passage the cells in a 150 cm^2 tissue culture flask, they were washed with 20 mL of PBS and incubated with 2 mL of Trypsin-EDTA at 37ºC for 10-15 minutes. Once the cells had become detached, they were re-suspended in 8 mL of medium to stop trypsinisation. The cells were then diluted and plated in new tissue culture flasks.

2.2.2. Western blotting

2.2.2.1. Protein extraction

Confluent 10cm tissue culture dishes of cells were placed on ice, medium was removed from the dishes and cells were washed with cold PBS. Cells were lysed by scraping them in 350 µL of cold lysis buffer (composition described in table 2.5) or 1X Sigma's RIPA Buffer with 1% Triton-X-100 (for expression

levels of ADAM10 and ADAM17) with a sterile disposable cell scraper. Lysates were centrifuged at 13,500 rpm for 10 minutes at 4ºC. The supernatants obtained by centrifugation were transferred into eppendorf tubes.

2.2.2.2. Protein concentration quantification

In order to measure the total protein concentration of a sample, the Bradford protein assay was performed. The Bradford protein assay is based on the binding of Coomassie Brilliant Blue G-250 dye to proteins. Formation of protein-dye complex converts the dye which is in the doubly protonated red cationic form (Amax = 470 nm) to a stable unprotonated blue form (Amax = 595 nm) which could be detected at 595 nm using microplate reader. (http://www.bio-rad.com/webroot/ web/pdf/lsr/literature/4110065A.pdf).

In this experiment Bovine Serum Albumin (BSA) was used as a protein standard. In order to generate a standard curve, various concentrations of BSA (0-10 µg/mL) were prepared by diluting the reagent in dH_20. Bio-Rad reagent was diluted with dH_20 in 1:5 ratios. 1µl of standard proteins or samples were mixed with 200 µl of diluted Bio-Rad reagent and the mixtures were then added in a 96-well plate (the experiment was done in duplicate). The plate was incubated at room temperature for 5 minutes and the absorbance was measured at the wavelength of 595 nm using a microplate reader. After quantification of protein concentrations, equal amounts of protein samples were prepared in loading buffer in 1:4 ratios and boiled for 10 minutes at 95ºC.

2.2.2.3. SDS-Polyacrylamide gel electrophoresis

For electrophoresis, a gel-tank was filled with running buffer and equal amounts of proteins were loaded into each well of a precast gel. 5 µL of the Novex® sharp protein standard was also loaded into a well to run simultaneously with the samples to determine the molecular weight of proteins. The gel electrophoresis was performed for 2 hours at 130 volts.

2.2.2.4. Semi dry Transfer

Proteins were transferred onto a 0.45 µm pore Immobilon PVDF transfer membrane (Millipore) as follows. Two filter papers (Bio-Rad) and one membrane were cut to the same size as the gel. The filter papers were soaked in transfer buffer and the membrane was soaked in methanol. One piece of the filter papers was put onto the platinum anode of a Bio-Rad Trans-Blot Semi-Dry Transfer Cell, followed by the membrane and then the gel before the other piece of filter paper was put on top of all the others. The cathode plate and the safety cover were placed on the apparatus and transfer was performed at 15 volts for 2 hours.

2.2.2.5. Probing of Western blot and detection

The membrane was blocked for 1 hour in 5% blocking buffer followed by incubation for 3 hours in the primary antibody diluted in 5% blocking buffer. The membrane was washed 4 × 10 minutes in 1% milk in PBST and was incubated for 1 hour in the secondary antibody diluted in 5% milk in PBST. Finally the membrane was washed 4 × 15 minutes in 1% milk in PBST. The

ECL (Enhanced Chemi-Luminescence) method was used to detect the proteins. For this purpose the membrane was incubated with the ECL solution for 2 minutes, drained and wrapped in cling film and exposed to RX autoradiograph film (FUJIFILM) for various time points depending on the strength of the signal.

2.2.3. Cell Count Experiment

The cells were seeded in a 24-well plate at a density of 5000 cells per well and allowed to attach overnight at 37ºC and 5% CO_2. The cells were then treated with indicated drugs for relevant time points. At the end of the treatment, the cells were washed with PBS and incubated with 500 µL of Trypsin-EDTA at 37ºC for 10-15 minutes. 400 µL of cells were then added into Coulter cup containing 19.6 mL of isotone and the number of viable cells were counted using Coulter Cell Counter.

2.2.4. MTT cell proliferation assay

The cells were seeded in a 96-well plate at a density of 2000 cells per well for incubation with relevant drugs. The total volume per well did not exceed 200 µL. At the end of treatment, 50 µL of 5 mg/mL MTT in PBS was added to each well and cells were incubated with the MTT compound for 2-4 hour at 37ºC and 5% CO_2. The media was then discarded carefully and 200 µL of DMSO was added to each well. The plate was placed on the shaker for 15 minutes and the optical density was measured at 560 nm and 670 nm using a microplate reader.

2.2.5. Transient Knockdown

ADAM10 and ADAM17 genes knockdown was performed by reverse transfection method using oligofectamine transfection reagent. For this purpose SCC15 cells were exposed to various concentrations of ADAM10 and ADAM17 oligonucleotides, scrambled negative control oligonucleotide or transfection reagent alone (Mock). The oligonucleotides were diluted to the desired concentrations using Opti-MEM to a final volume of 185 µL. The reagents were mixed and incubated at room temperature for 5 minutes. 3 µL of oligofectamine reagent was then diluted in Opti-MEM to a final volume of 15 µL. The mixture was incubated for 5 minutes at room temperature. The diluted oligonucleotide was mixed with the diluted oligofectamine reagent and was incubated at room temperature for 20 minutes. For mock control, 15 µL of diluted oligofectamine reagent was mixed with 185 µL of Opti-MEM only. At this stage, medium was removed from tissue culture flask and cells were washed with 20 mL of PBS and incubated with 2 mL of Trypsin-EDTA at 37ºC for 10-15 minutes. Once cells had become detached they were resuspended in antibiotic free medium in order to stop trypsinisation. Cells were then seeded in 6-well plate at a density of 150,000 cells per well. The total volume per well did not exceed 800 µL. After the formation of oligonucleotide-transfection reagent complex, 200 µL of the mixture was added to the cells. Cells were incubated for 24 hours at 37ºC and 5% CO_2. Transfection solution was then removed and cells were cultured in antibiotic free medium until analysis.

2.2.6. mRNA analysis

In order to validate the efficiency of ADAM10 and ADAM17 genes knockdown, their mRNA levels were analysed by qRT- PCR technique.

2.2.6.1. RNA extraction

In this experiment RNeasy Mini kit was used to extract RNA. After 48 hours of transfection, the medium was removed from each well and cells were washed with PBS. 350 µL of RNeasy Lysis Buffer was then added to each well and the cells were lysed by pipetting the mixture up and down several times. 350 µL of 70% ethanol was then added to the cells and the cell lysates were transferred into the RNeasy spin columns. The columns were centrifuged for 15 seconds at 8000 x g and the flow-through was discarded. 700 µL of RW1 wash buffer was then added to each column and was centrifuged for 15 seconds at 8000 x g and the flow-through was discarded. The columns were then washed with 500 µL of RPE wash buffer and were centrifuged for 15 seconds at 8000 x g and the flow-through was then discarded. This washing step was performed twice but this time the columns were centrifuged for 2 minutes. At this stage RNeasy spin columns were placed in new collection tubes and 50 µL of RNase-free water was then added to each column. The columns were centrifuged for 1 minute at 8000 x g and the eluted RNAs were stored at -80ºC till further analysis.

2.2.6.2. Reverse Transcription

Reverse transcription was performed using a High Capacity cDNA Reverse Transcription Kit. 2 µg of RNA was diluted in 50 µL of Nuclease-free H_2O and RT master mix was prepared according to table 2.8.

Reagent	**Volume (µL)**
10× RT Buffer	10
25× dNTP Mix (100 mM)	4
10× Random Primers	10
MultiScribe™ Reverse Transcriptase	5
Nuclease-free H_2O	21
Total per reaction	50

Table 2.8. Reverse transcription master mix components.

50 µL of master mix was then added to the tubes containing 50 µL of total RNA. The mixture was centrifuged for 2 minutes and was kept on ice. The reverse transcription reaction was then performed in the thermal cycler (Applied Biosystems) with the program presented in table 2.9. The cDNA tubes were then stored at 2 to 6ºC.

	Temperature (°C)	**Time (Minutes)**
Step 1	25	10
Step 2	37	120
Step 3	85	5
Step 4	4	-

Table 2.9. The thermal cycler program for reverse transcription reaction.

2.2.6.3. qRT-PCR

The quantitative RT-PCR reaction was performed using SensiMix™ SYBR Hi-ROX Kit. The PCR master mix was prepared according to table 2.10.

Reagent	**Volume (µL)**
2x SensiMix™ SYBR	10
100 µL Forward Primer	1
100 µL Reverse Primer	1
Nuclease-free H_2O	3
cDNA Template	5
Total per reaction	20

Table 2.10. PCR master mix components.

The qRT-PCR reaction was then performed in Rotor Gene RG 3000 thermal cycler (Corbett Research) with the program listed in table 2.11.

In this experiment actin gene was used as an internal control.

Cycle	**Temperature (ºC)**	**Time**
1	95	10 Min
40	95 55-60 72	15 Sec 15 Sec 15 Sec

Table 2.11. The thermal cycler program for qRT-PCR reaction.

2.2.7. Clonogenic assay

The cells were seeded in a 6-well plate at a density of 200,000 cells per well and were allowed to attach overnight at 37ºC and 5% CO_2. The cells were then treated with drugs/inhibitors for 3 days. For transient knockdown experiments, the cells were transfected for 3 days as previously described. At the end of the treatment/transfection, the cells were harvested and 800-1000 cells were re-plated in duplicate. The cells were then incubated at 37ºC for 10-14 days to form colonies. In order to fix and stain the cells, the medium was removed and the cells were washed with PBS. The cells were then incubated with 0.2% methylene blue in 50% ethanol for 30 minutes and were then rinsed with tap water. The plates were left to dry at room temperature overnight and the number of colonies was determined using digital colony counter pen.

2.2.8. Preparation of conditioned media samples

In order to investigate the levels of the ligands BTC and NRG-1 secreted into the media, the cells were seeded in 10 cm tissue culture dishes at a density of ~1,000,000 cells in full media and were allowed to attach overnight at 37ºC and 5% CO_2. The cells were then treated with relevant drugs/inhibitors for different time points in serum-free media. In order to investigate the effect of transient knockdown on the release of ligands, the cells were transfected for 24 hour as previously described. At the end of the treatment/transfection, the media were collected into 15 mL tube and were centrifuged at 2300 rpm at 4ºC for 5 minutes. The supernatant were then transferred into Amicon Ultra centrifugal filter units (Millipore) and were centrifuged at 3200 rpm at 4ºC for

30 minutes. The proteins concentrations were determined by Bradford protein assay and the samples were then investigated by either western blot as described previously or by ELISA kit according to manufacturer's instructions.

2.2.9. Immunohistochemistry (IHC)

2.2.9.1. Preparation of the paraffin-embedded cell pellet

SCC15 cells were transfected with either ADAM10 or ADAM17 siRNAs as described previously. At day 3, the cells were trypsinised for 10-15 minutes. Trypsinization was stopped by adding the media into the cell culture dishes and the cells were then transferred into a 15 mL tube and were centrifuged at 2300 rpm at 4ºC for 10 minutes. The media was removed and 1 mL of 4% PFA was then added to the cells and were mixed by pipetting up and down gently. The cells were incubated with PFA at 4ºC overnight. The next day, the cells were spun down and the supernatant was removed and 1mL of PBS was added to the cells. The samples were kept at 4ºC till performing the next step. In order to prepare the cells to be embedded in the paraffin blocks, samples were transferred into microcentrifuge tubes and they were incubated in 60ºC water bath for 5 minutes. Samples were then centrifuged at 1000 rpm at room temperature for 5 minutes. The supernatant was then removed by gently tapping the tubes on tissue paper. The tubes were placed back in 60ºC water bath and 600 µL of 4% PFA + Agarose was added to the cells. The samples were centrifuged at 1000 rpm for 1 minute at room temperature and were left to cool down at room temperature or in the cold room. The cell pellets were then processed and embedded in paraffin. The samples were

then cut into 4 µm sections, mounted on X-tra Adhesive Micro Slides (Leica) and were incubated at 37ºC overnight.

2.2.9.2. Deparaffinization

For this purpose the slides were incubated at 60ºC for 10 minutes. The slides were then incubated in two washes of citroclean, two washes of 100% ethanol, one wash of 50% ethanol and subsequently one wash of dH_2O for 5 min each.

2.2.9.3. Antigen retrieval

At this stage the slides were placed in 1 mM EDTA, pH 8.0 and were boiled in pressure cooker for 30 minutes and were maintained at a sub-boiling temperature for 15 minutes. The slides were then incubated at room temperature for 15 minutes.

2.2.9.4. Staining

In order to stain the samples, the slides were washed with PBS two times for 5 minutes each. The slides were dried carefully with tissue paper, placed in a humidified chamber and were blocked with 2.5% normal horse serum blocking solution for 30 minutes at room temperature. The blocking solution was then removed and the slides were incubated with primary antibody for 1 hour at room temperature. The slides were then washed with PBS two times for 5 minutes each and were incubated with ImmPRESS Anti-Rabbit Ig (peroxidase) for 30 minutes at room temperature. The slides were washed again with PBS two times for 5 minutes each and were incubated with ImmPACT DAB

Peroxidase Substrate (50 µL of DAB Chromogen in 1 mL of DAB Diluent) for 2-5 minutes. The slides were then washed with dH_2O and counterstained with Hematoxylin for about 2 minutes. Subsequently the slides were dried carefully with tissue paper and were covered with Aquatex mounting medium (VWR) and cover slips (VWR).

2.2.9.5. IHC scoring

ADAM10 and ADAM17 expression levels were assessed by IHC in two cohorts of HNSCC patients; TMAs consisting of normal and tumour samples from a series of head and neck patients undergoing routine treatments in Oxford and tumour samples from HNSCC patients undergoing cetuximab window study. Their immunostaining was scored by Dr. Ioannis Roxanis and me. The percentage of positive cells (PP) (1 point: 0-10%, 2 points: 11-50%, 3 points: 51-80%, 4 points: 81-100%) and the staining intensity (SI) (1 point: weak, 2 points: moderate, 3 points: strong) were determined. The results were then evaluated according to immunoreactive score of Remmele and Stegner (IRS) system. SI was multiplied with PP to achieve a score of 1 - 12.

2.2.10. Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 software. The means or medians between two groups were analysed by unpaired T-test or Mann-Witney Test respectively. One-way Anova with Bonferroni's Multiple Comparison Test was used for comparing the means of more than two groups. Statistically significant changes were represented by asterisks; $P \leq 0.05$ *****, $P \leq 0.01$ ******, $P \leq 0.001$ *******, $P \leq 0.0001$ ********

CHAPTER 3

Comparing the effects of cetuximab treatment on HER receptors signalling in sensitive and resistant HNSCC cell lines

3.1. INTRODUCTION

As discussed in chapter 1, a vast number of head and neck cancer tumours overexpress EGFR. Therefore in recent years cetuximab, anti-EGFR mAb, has been introduced into clinical practice as a treatment for this type of malignancy. Cetuximab is now established as the only approved targeted agent either alone or in combination with radiation or chemotherapy for the treatment of HNSCC.

Although some clinical responses have been observed in a number of patients treated with cetuximab, intrinsic and acquired resistance to this drug remains as a common problem. Therefore it would be interesting to further understand the resistance mechanisms to cetuximab in order to propose suitable strategies to overcome drug resistance.

In this study the mechanisms of acquired resistance to cetuximab in relation to HER signalling was investigated in HNSCC. For this purpose cetuximab resistant cell lines were established using primary cetuximab sensitive cell lines. Following chronic exposure to cetuximab western blot analysis was performed to assess and compare the activity of HER receptors and their downstream signalling pathways versus parental cell lines.

3.2. RESULTS

3.2.1. Basal expression of HER family receptors and downstream signalling molecules in HNSCC cell lines

In this study the basal expression levels of HER receptors and downstream signalling proteins were analysed in 4 SCC cell lines (SCC4, 9, 15 and 25). The characteristics of these cell lines are summarized in table 3.1. As it can be seen from figure 3.1, HNSCC cell lines express various levels of HER family receptors and signalling proteins (figure 3.1).

HNSCC Cell Line	Primary Site	Coding Mutations	Examples Of Tested genes With No Mutations	HPV Status
SCC-4	Tongue	CDKN2A TP53	BRAF EGFR ERBB2 KRAS PIK3CA PIK3R1	-
SCC-9	Tongue	CDKN2A TP53		-
SCC-15	Tongue	TP53		-
SCC-25	Tongue	CDKN2A TP53		-

Table 3.1. Characteristics of HNSCC cell lines.

(www.sanger.ac.uk/genetics/CGP/CellLines/)

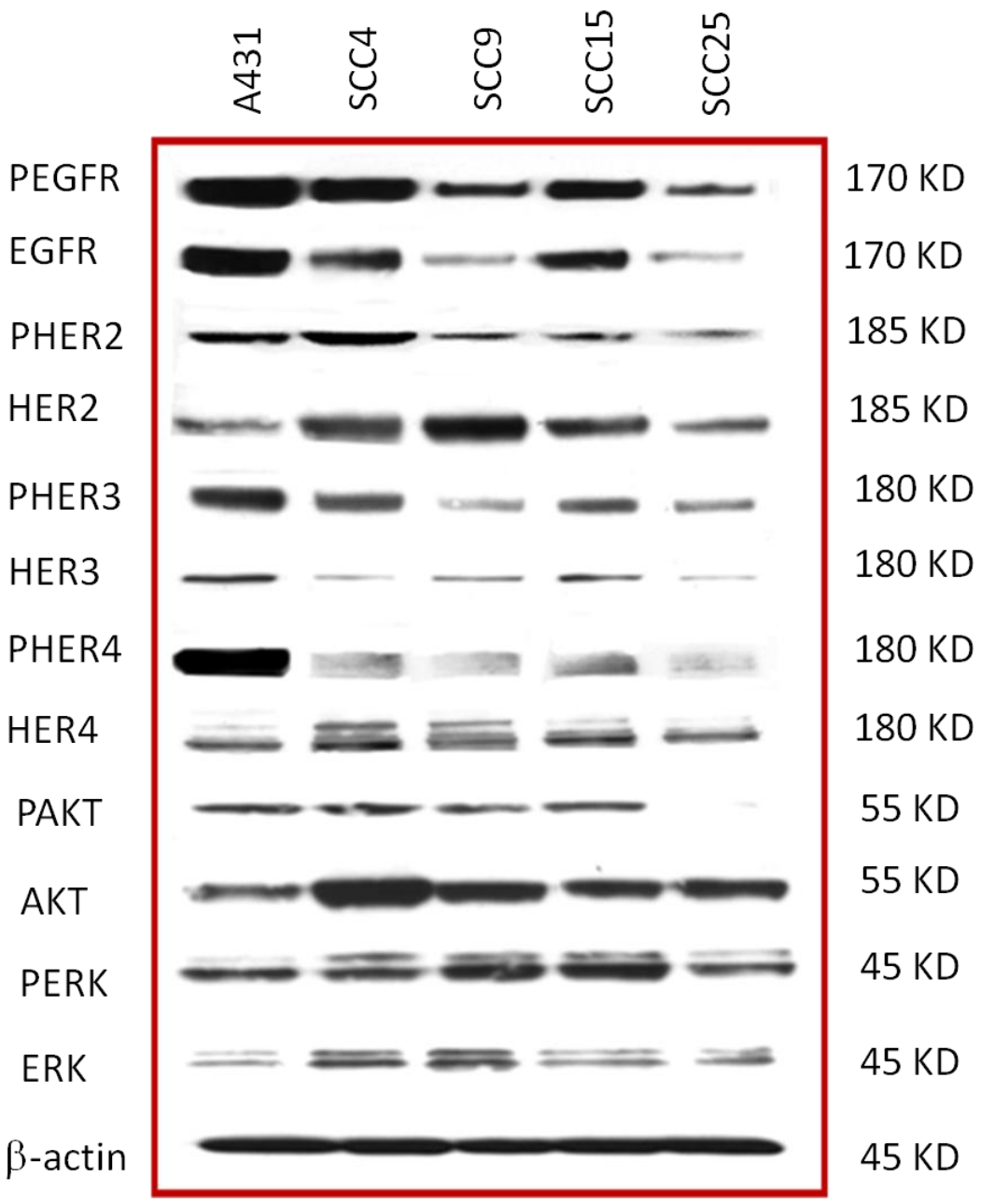


Figure 3.1. The basal expression of HER family receptors and downstream signalling proteins in HNSCC cell lines.

Cells were lysed for western blot analysis assessing the basal levels of the indicated proteins. A431 cell line which is known to express high level of EGFR was used as a positive control. β-actin was used as a loading control for each gel but only one representative band is shown.

3.2.2. HNSCC cell lines sensitivity to cetuximab

To assess the sensitivity of HNSCC cell lines to cetuximab, these cells were treated with different concentrations of cetuximab. All HNSCC cell lines were sensitive to cetuximab despite expressing various levels of HER receptors (figure 3.2).

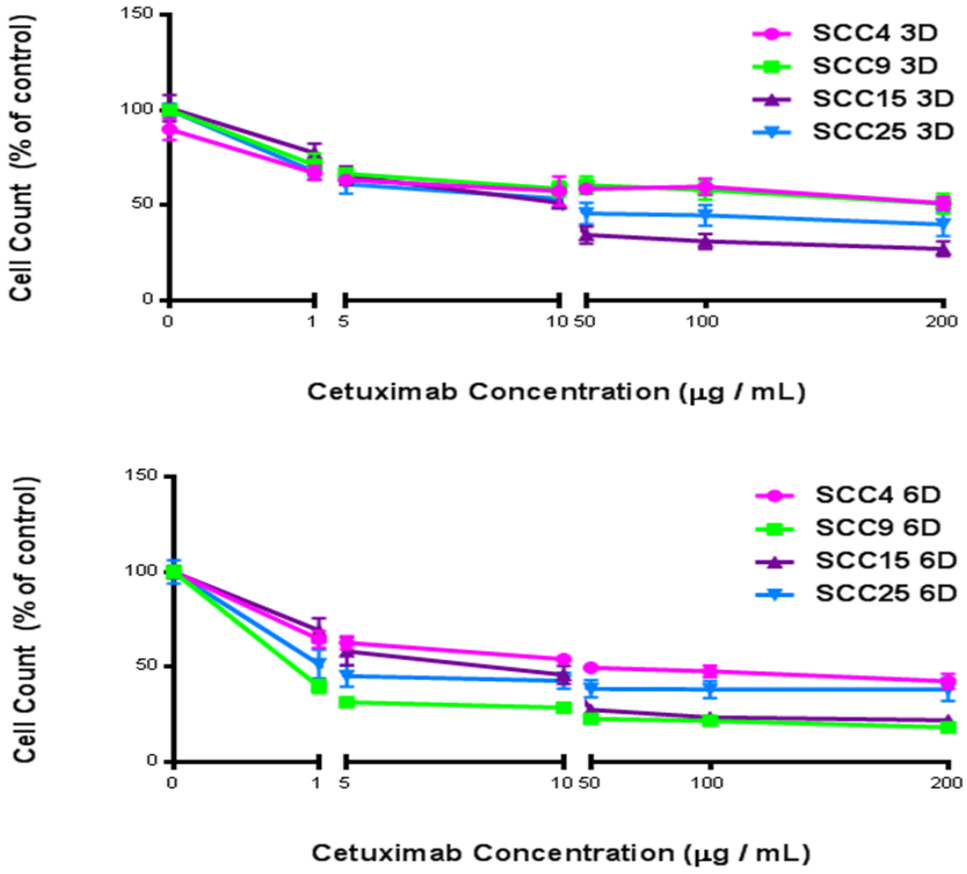


Figure 3.2. Relative sensitivities of HNSCC cell lines to cetuximab.

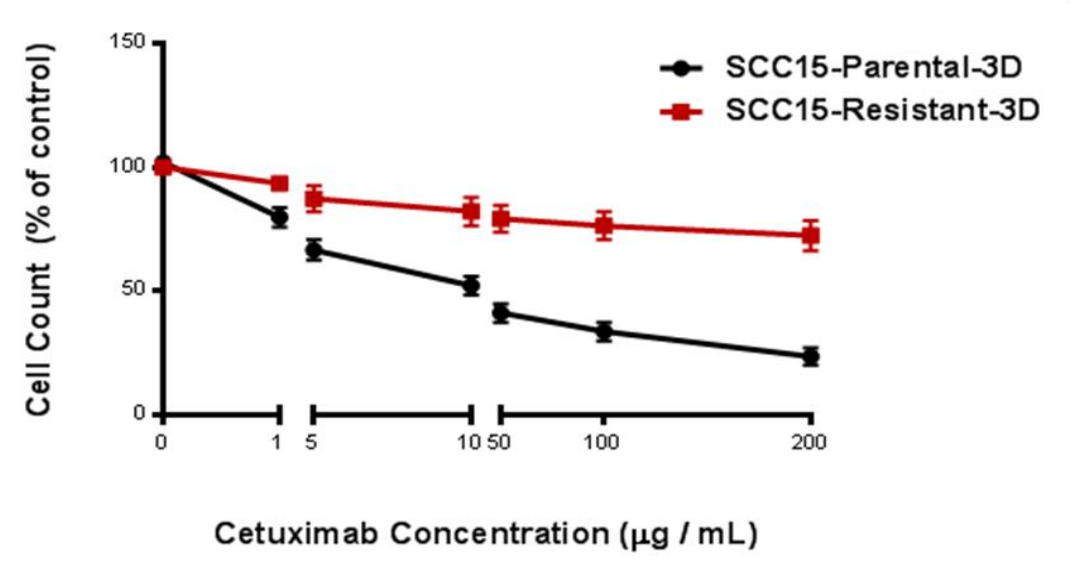
HNSCC cells were treated with 0, 1, 5, 10, 50, 100 and 200 µg/mL of cetuximab for 3 and 6 days and the number of cells was counted using cell counter. Three independent experiments were performed in triplicate and the percentage of viable cells were calculated relative to the untreated cells and presented as the mean ± S.D.

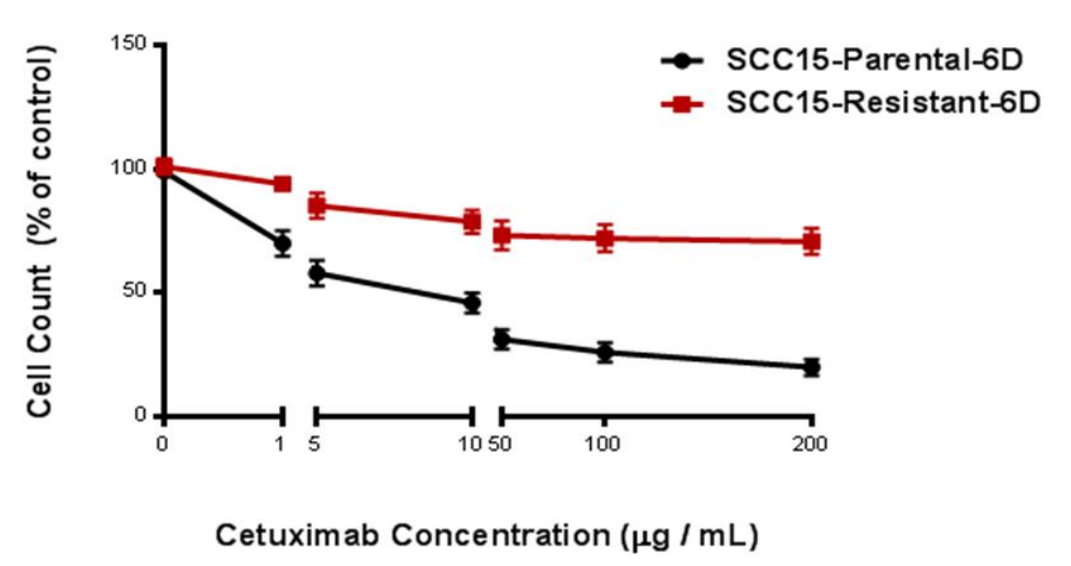
Among the tested HNSCC cell lines with various levels of HER receptors expression and different sensitivities to cetuximab, SCC9 cell line which express low level of EGFR and SCC15 cell line with high level of EGFR were chosen for further investigation in this project.

3.2.3. Establishment of cetuximab resistant cell lines

Cetuximab resistant cell lines were generated by continuously exposing SCC15 and SCC9 cells to the increasing concentrations of cetuximab up to 20 μg/mL over 24 months. The sensitivities of established cell lines to cetuximab were compared between parental and resistant cells. 10 μg/mL of cetuximab reduced the cell number by 48% at 3 days and 55% at 6 day in parental SCC15 cell line. However, in resistant cells 10 μg/mL of the drug reduced the cell number only by 18% at 3 days and 22% at 6 days (figure 3.3A). In parental SCC9 cell line, the cell number was reduced by 47% and 67% at 3 and 6 days respectively. However, in resistant cells only about 10% reduction was observed (figure 3.3B).

A


SCC15-Parental-3D
SCC15-Resistant-3D
Cell Count (% of control)
150
100
50
0
0
1
5
10
50
100
200
Cetuximab Concentration (µg / mL)


SCC15-Parental-6D
SCC15-Resistant-6D
Cell Count (% of control)
150
100
50
0
0
1
5
10
50
100
200
Cetuximab Concentration (µg / mL)

B

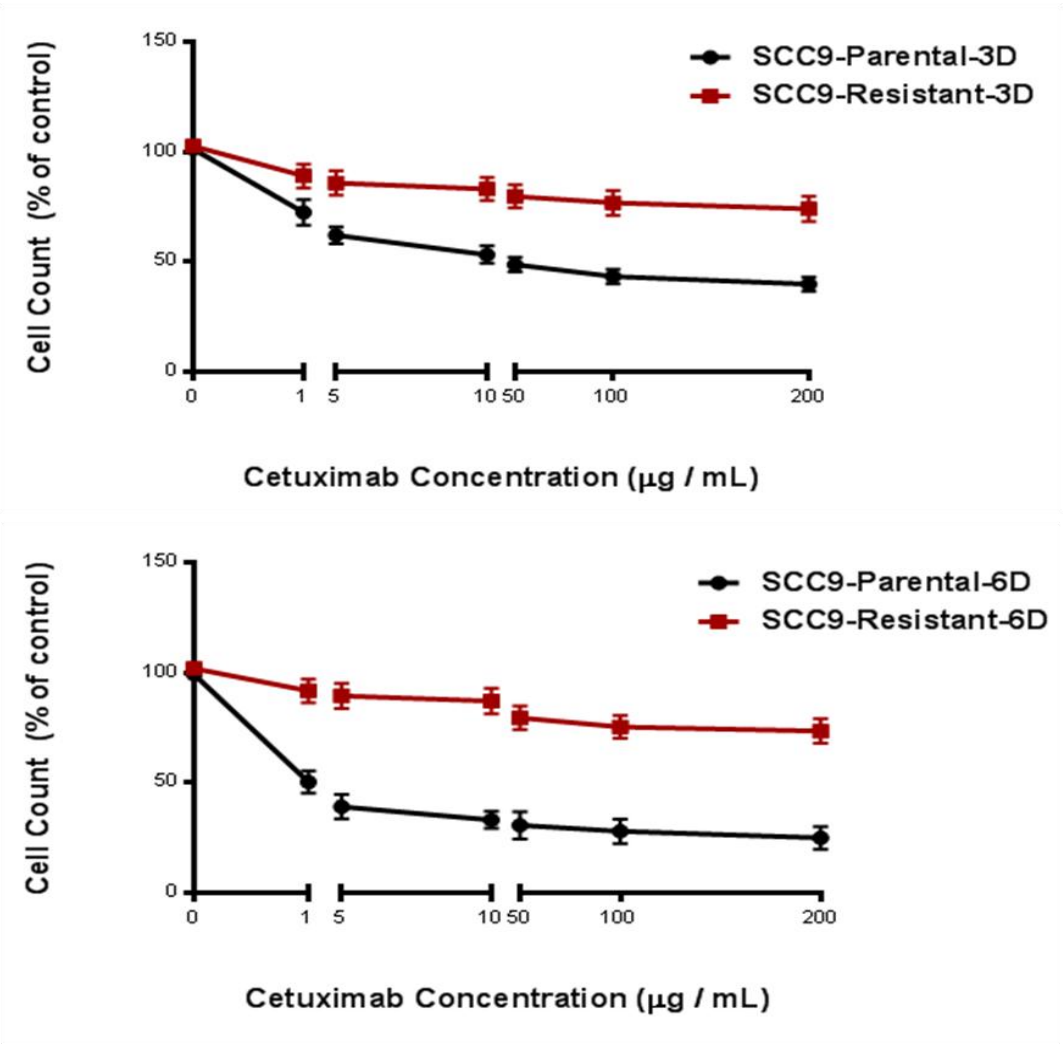


Figure 3.3. Comparison of the sensitivities to cetuximab between parental and acquired resistant cell lines.

SCC9 and SCC15 parental and cetuximab resistant cells were treated with 0, 1, 5, 10, 50, 100 and 200 µg/mL of cetuximab for 3 and 6 days and the number of cells was counted using the cell counter. Three independent experiments were performed in triplicate and the percentage of viable cells were calculated relative to the untreated cells and presented as the mean ± S.D.

3.2.4. Chronic exposure to cetuximab leads to the reactivation of HER receptors and their downstream signalling pathways

In order to investigate the expression and activation of HER receptors upon cetuximab treatment, SCC9 and SCC15 cell lines were treated with 10 µg/mL of cetuximab for different time points. In SCC15 cell line cetuximab downregulated EGFR, HER2 and HER3 at 6 days (figure 3.4). The inhibition of pHER3 and pAKT occurred within 24 hours before being reactivated at 3 days. Cetuximab also decreased pERK after 1h treatment, however, the reactivation was observed at 24 hours (figure 3.4, top panel). In SCC9 cell line cetuximab downregulated EGFR at 6 days and HER2 at 3 days. However, downregulation of HER3 and AKT occurred at shorter time points. Compared to IgG control, a pronounced effect was not observed in pHER3 level, however, a decrease in the level of its downstream protein, pAKT, was observed at 6 days which was consistent with downregulation of total AKT. Cetuximab was also very effective in inhibition of pERK in SCC9 cell line which occurred after only 1 hour treatment and unlike SCC15 there was minimal reactivation for 6 days (figure 3.4, bottom panel). The effect of chronic exposure to cetuximab was also analysed in this study. The reactivation of EGFR, HER2, HER3 and their downstream signalling pathways was observed in both cetuximab resistant cell lines (figure 3.4).

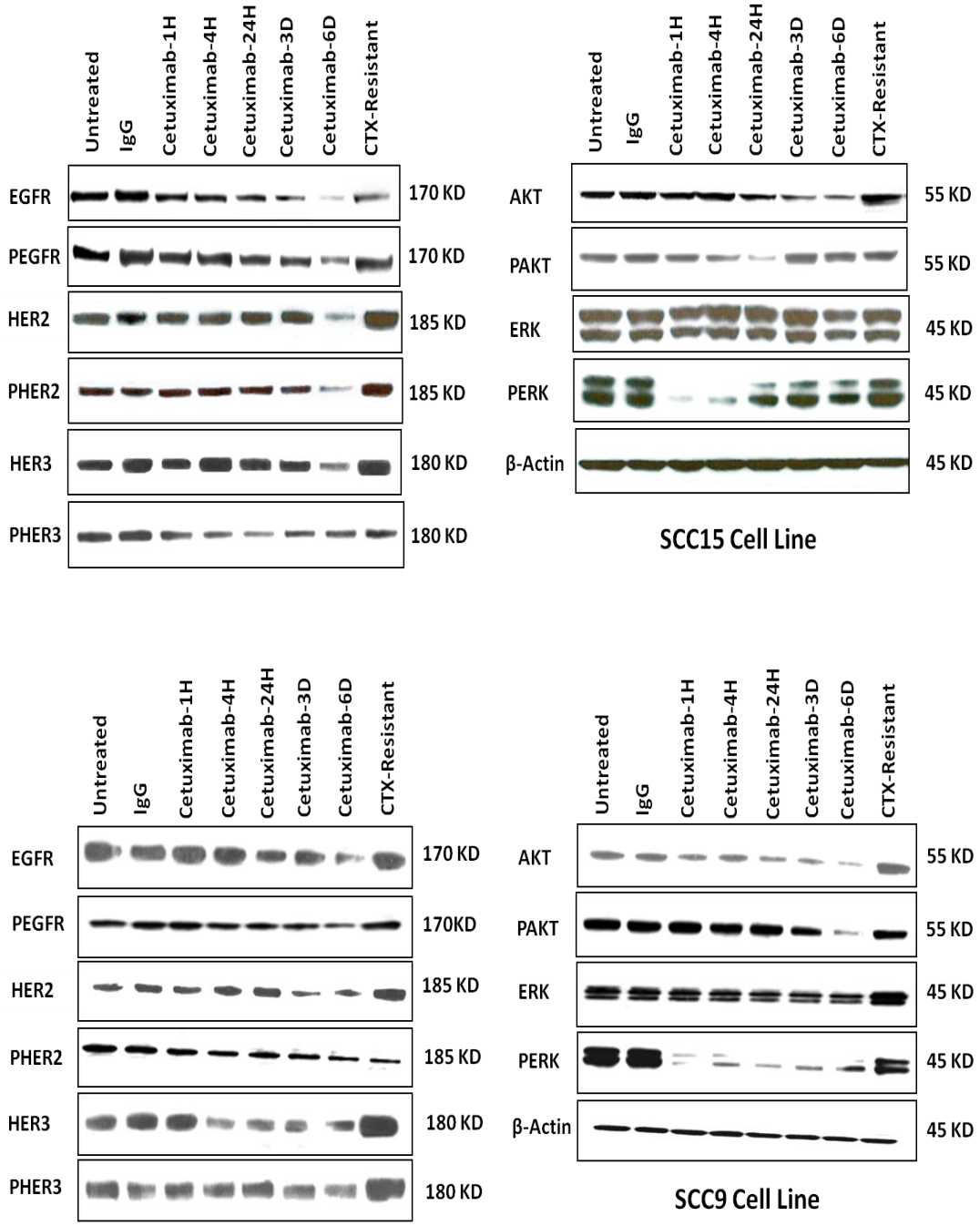


Figure 3.4: Chronic exposure to cetuximab leads to the reactivation of EGFR, HER2, HER3 and downstream signalling pathways

SCC15 (top) and SCC9 (bottom) cells were treated with 10 µg/mL of cetuximab for indicated time points before being lysed for western blot analysis. Resistant cells were continuously exposed to 20 µg/mL of cetuximab. 10 µg/mL of human IgG was used as a control. β-actin was used as a loading control for each gel but only one representative band is shown. Western blots are representative of three independent experiments.

3.3. DISCUSSION

In this study the effects of cetuximab on HER receptors' expression and activation in relation to downstream signalling pathways were compared in sensitive and cetuximab resistant HNSCC cell lines. Although SCC9 and SCC15 cell lines express different basal levels of HER receptors, both cell lines had similar sensitivities to cetuximab regardless of EGFR expression. Compared to SCC15 cell line, SCC9 cell line with a higher level of HER2 and a similar level of pHER2 (figure 3.1) was less sensitive to cetuximab. Downregulation of HER2 and subsequent inhibition of pHER2 were more pronounced in SCC15 cell line. However, direct comparison has not been done. Downregulation of HER3 occurred earlier in SCC9 cell line which express lower level of HER3 and pHER3 compared to SCC15 cell line. However, this was not the case for pHER3 as its inhibition was observed at 24h in SCC15 cell line. Both cell lines expressed similar levels of total AKT and pAKT and although similar sensitivies to cetuximab was observed for AKT, inhibition of pAKT occurred earlier in SCC15 cell line, consistent with its upstream protein, pHER3. Both cell lines expressed a similar level of pERK which was inhibited by cetuximab. However, its reactivation occurred at 24h in SCC15 cell line, whereas this was observed at 6 days in SCC9 cell line.

Although the two cell lines behaved differently with short-term cetuximab treatment, in both cell lines chronic exposure to cetuximab led to the activation of EGFR, HER2 and HER3 as well as their downstream signalling proteins, AKT and ERK. These results are consistent with the findings by Wheeler *et al.* (2008) who studied the involvement of HER family receptors in

acquired resistance to cetuximab in human NSCLC and HNSCC. By means of high-throughput comparative analysis this group assessed the activity of 42 RTKs in cetuximab resistant clones compared to their parental cell lines. Their results showed the upregulation of EGFR, HER2, HER3 as well as hepatocyte growth factor receptor (HGFR, c-MET) in both cetuximab resistant NSCLC and HNSCC cell lines. By performing immunoprecipitation analysis, this group also studied the EGFR binding partners. An induced dimerization of EGFR with HER2, HER3 and cMET was observed in cetuximab resistant cell lines compared to the parental cells. This group has suggested that chronic exposure to cetuximab was responsible for the upregulation of EGFR which in turn led to an induced heterodimerization of EGFR with HER2, HER3 and cMET and activation of these receptors (Wheeler *et al.* 2008).

In summary, results from this study showed increased activity of EGFR, HER2 and HER3 in cetuximab resistant cell lines. In theory, in order for a HER receptor to get activated its ligand must bind to it to initiate its homo or heterodimerization with other receptors. But ligand itself needs to be cleaved and released to be able to bind to the receptor. As described in chapter 1, the majority of HER ligands are processed by ADAM family members. Therefore it would be interesting to investigate the role of two important members of this family, ADAM10 and ADAM17 in this process. In the following chapter the role of ADAM10 and ADAM17 in release of HER ligands and subsequent activation of HER receptors will be discussed.

CHAPTER 4

The role of ADAM10 and ADAM17 in relation to cetuximab treatment and resistance

4.1. INTRODUCTION

As described previously, prolonged cetuximab treatment led to the activation of EGFR, HER2, HER3 and downstream signalling pathways. Our group has previously shown that drug-induced ligand release is responsible for the activation of alternative HER receptors and thus mediating resistance to EGFR TKIs in breast cancer cell lines (Kong *et al*. 2008). Our group has also investigated the mechanisms of acquired resistance to anti-HER2 monoclonal antibody trastuzumab in HER2-positive breast cancer cell lines. The results showed that treatment with trastuzumab induces ADAM-mediated ligand release, leading to the activation of alternative HER receptors and downstream signalling pathways (Gijsen *et al.* 2010). The role of ADAM10 in relation to trastuzumab treatment and resistance in HER2-positive breast cancer has also been investigated in our lab. Trastuzumab treatment increased the expression level of ADAM10 *in vitro* and *in vivo*. Results from this study showed that inhibition of ADAM10 enhanced response to trastuzumab in parental and trastuzumab resistant breast cancer cell lines (Feldinger *et al.* 2014).

Based on these studies, it was hypothesized that the reactivation of EGFR, HER2 and HER3 upon prolonged cetuximab exposure might be due to ADAM-mediated HER ligand release. Thus, this study was done to investigate the role of ADAM10 and ADAM17, two important members of ADAM family proteases, in relation to cetuximab treatment and resistance in HNSCC.

4.2. Results

4.2.1. Cetuximab induces the level of ADAM10 and ADAM17 in HNSCC

In this study the acute and chronic effect of cetuximab treatment on ADAM10 and ADAM17 proteins level was investigated. A marked increase in the level of mature (93 kDa) and precursor (125 kDa) ADAM17 and ADAM10 was observed in SCC15 and SCC9 cells when they were treated with 10 µg/mL of cetuximab for 1 and 24h (figure 4.1, left panels). By comparing the levels of ADAM10 and ADAM17 between parental and cetuximab resistant cells, a consideralbe increase in the levels of both proteins was also observed in the resistant cells (figure 4.1, right panels).

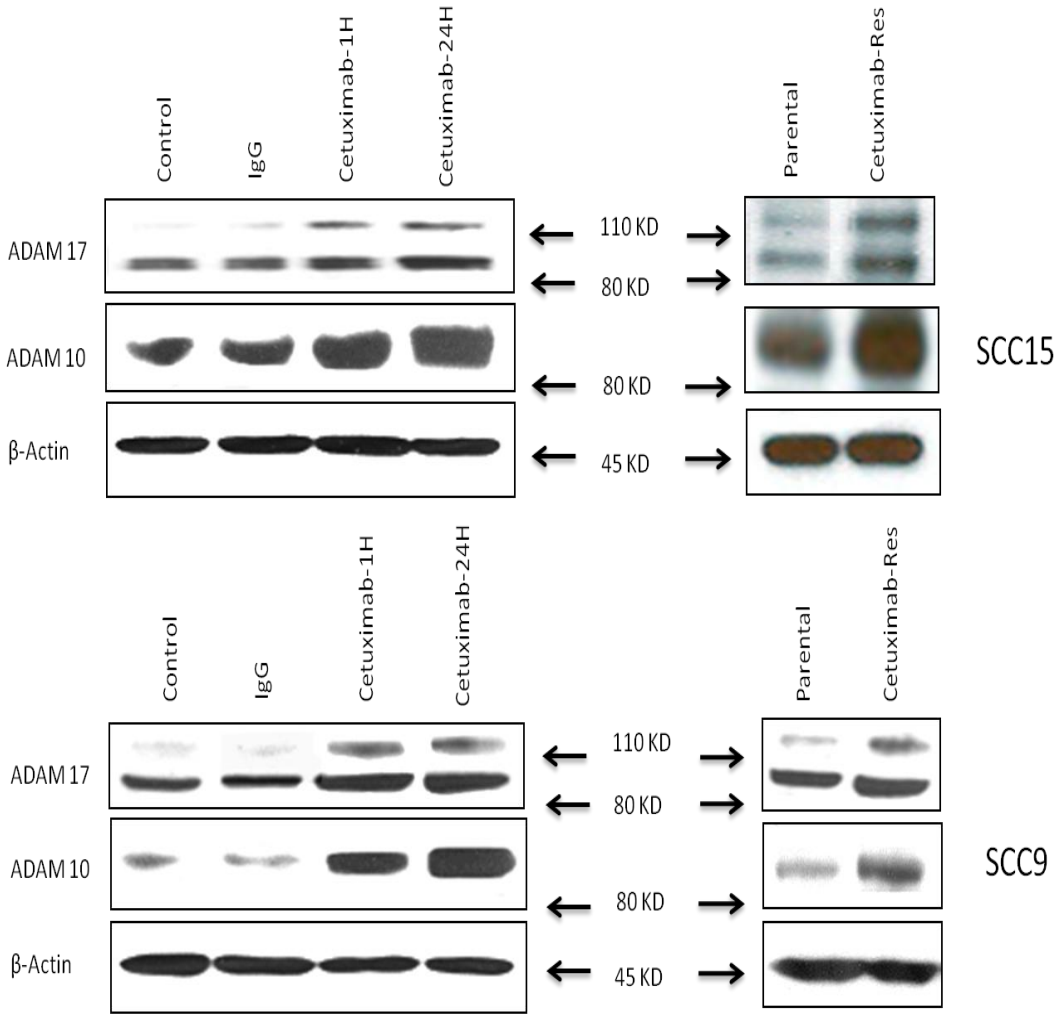
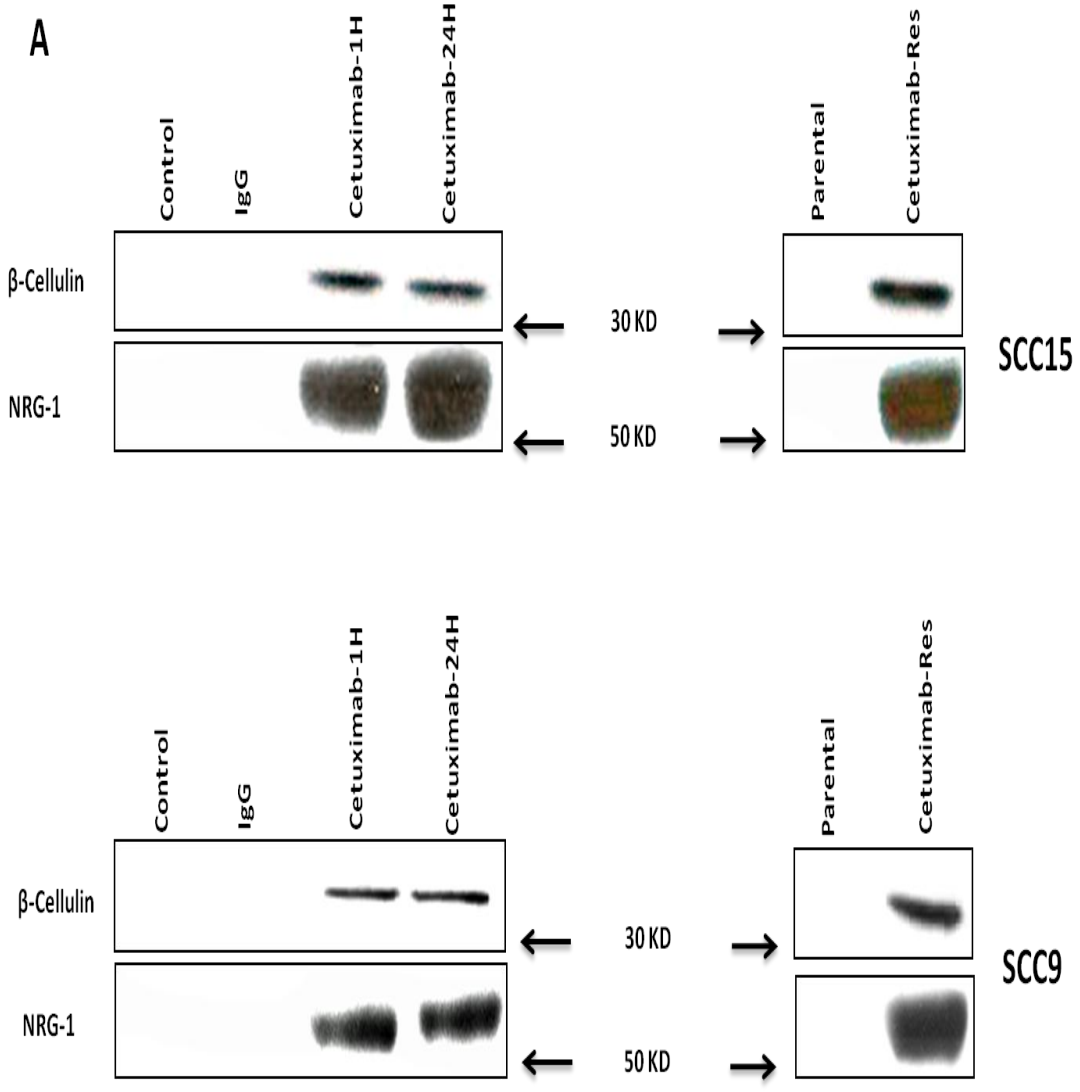


Figure 4.1: Exposure to cetuximab induces the level of ADAM10 and ADAM17 expression in sensitive and cetuximab resistant cell lines

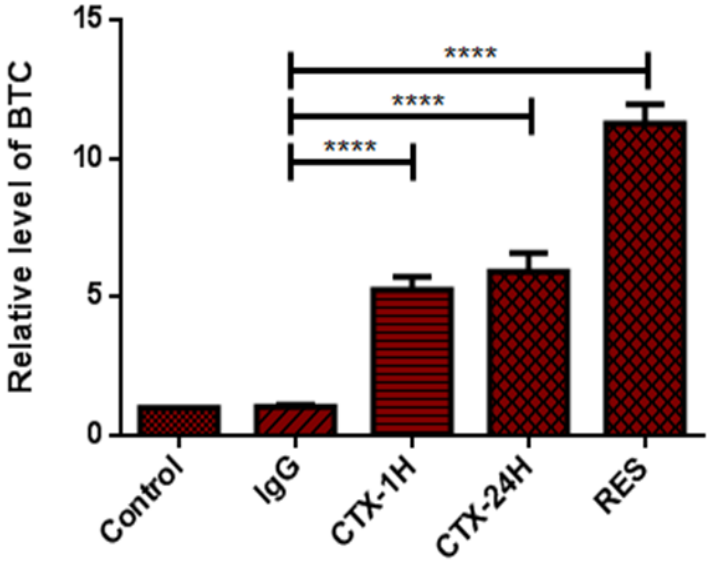
The parental SCC9 and SCC15 cells were treated with 10 µg/mL of cetuximab for 1h and 24h and the levels of ADAM10 and ADAM17 proteins expression were analysed by western blot (left panels). The expression level of ADAM proteins was compared between the parental and cetuximab resistant cell lines which were continuously exposed to 20 µg/mL of cetuximab (right panels). β-actin was used as a loading control for each gel but only one representative band is shown. Western blots are representative of three independent experiments.

4.2.2. Exposure to cetuximab induces the release of HER ligands into the media

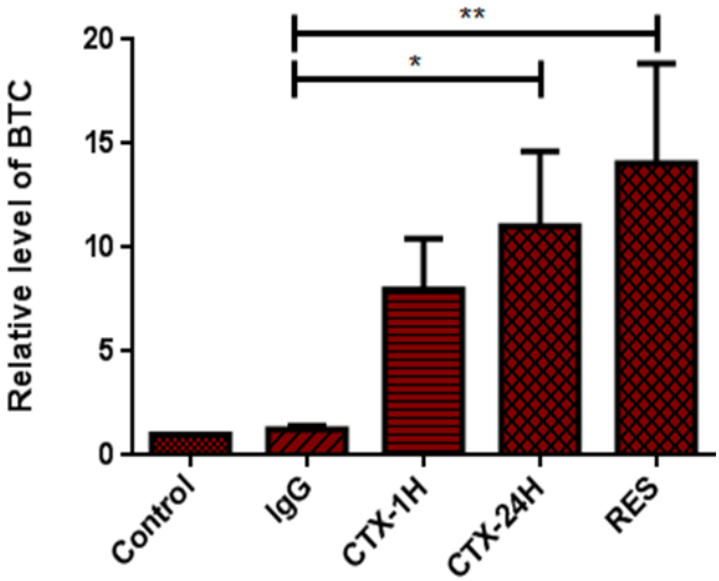
The levels of BTC (an ADAM10 substrate) and NRG-1 (an ADAM17 substrate) were investigated by western blot analysis (figure 4.2A) and ELISA (figure 4.2B&C). Cetuximab treatment after 1 and 24 hours significantly induced the release of these ligands into the media in both HNSCC cell lines. The levels of these ligands were also significantly higher in the media of the resistant cells compared to the parental cells (figures 4.2).



B

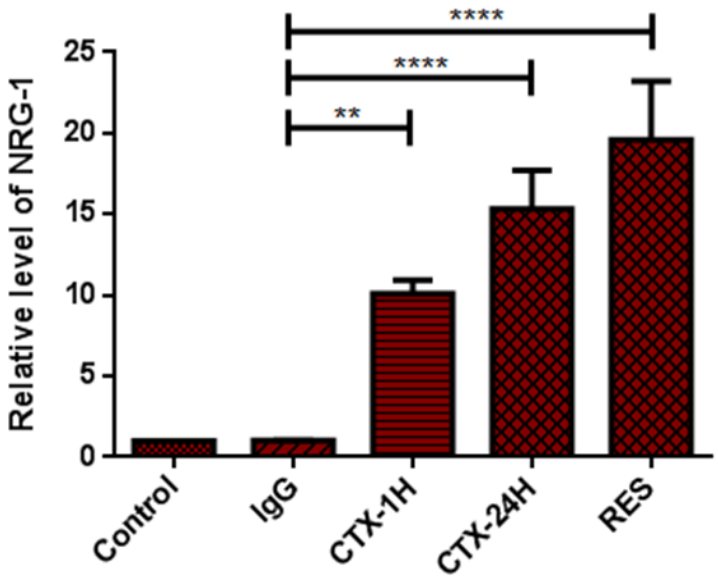

Relative level of BTC
15
10
5
0
****
****
****
Control
IgG
CTX-1H
CTX-24H
RES

SCC15

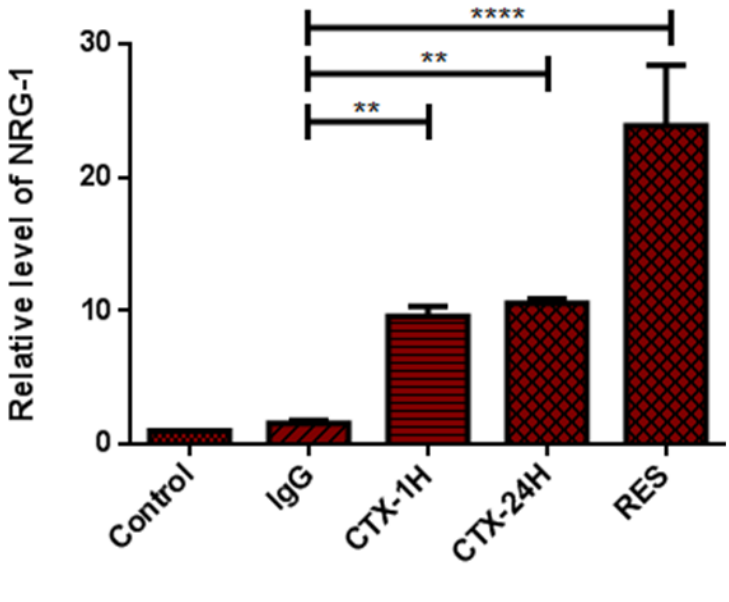

Relative level of BTC
20
15
10
5
0
**
*
Control
IgG
CTX-1H
CTX-24H
RES

SCC9

C


****
****
**
Relative level of NRG-1
25
20
15
10
5
0
Control
IgG
CTX-1H
CTX-24H
RES

SCC15


****
**
**
Relative level of NRG-1
30
20
10
0
Control
IgG
CTX-1H
CTX-24H
RES

SCC9

Figure 4.2: Expression level of HER ligands upon treatment with cetuximab

The parental SCC15 and SCC9 cells were treated with 10 µg/mL of cetuximab for 1h and 24h before the level of BTC and NRG-1 was analysed in concentrated medium by western blot (A, left). The levels of these ligands were compared between parental and cetuximab resistant cell lines which were continuously exposed to 20 µg/mL of cetuximab (A, right).

The levels of these ligands upon cetuximab treatment were also analysed by ELISA. In this experiment parental cells were treated with 10 µg/mL of cetuximab for 1h and 24h and the level of BTC (B) and NRG-1 (C) was compared between the parental and cetuximab resistant cell lines. The resistant cells were exposed to 20 µg/mL of cetuximab.

10 µg/mL of human IgG was used as a control. Western blots are representative of three independent experiments. For ELISA experiment, three independent experiments were performed in triplicate. The means between the groups were analysed by Anova with Bonferroni's Multiple Comparison Test and statistically significant changes were represented by asterisks ($P \leq 0.05$ *, $P \leq 0.01$ **, $P \leq 0.0001$ ****).

4.2.3. Stimulation with exogenous HER ligands render sensitive cell lines resistant to cetuximab

In order to show the involvement of HER ligands in acquired resistance to cetuximab, SCC9 and SCC15 cells were stimulated with 100 ng/mL of NRG-1 or BTC while treated with 10 µg/mL of cetuximab for 3 days. Cetuximab reduced the cell proliferation of both cell lines significantly. However, cetuximab treatment with exogenous stimulation of cells with NRG-1 or BTC rendered both cell lines resistant to the drug (figures 4.3).

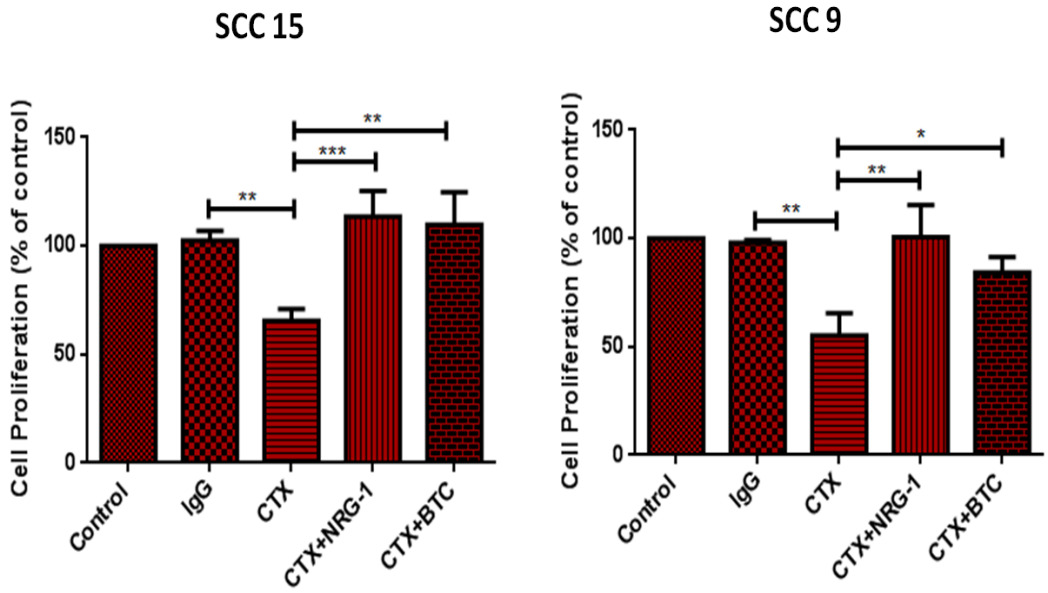


Figure 4.3: Stimulation with exogenous HER ligands, BTC and NRG-1, renders sensitive cell lines resistant to cetuximab

SCC9 and SCC15 cells were treated with either 10 µg/mL of cetuximab alone or in combination with 100 ng/mL of NRG-1 or BTC for 3 days. The cell proliferation was assessed by MTT assay. 10 µg/mL of human IgG was used as a control. Three independent experiments were performed in triplicate. The means between the groups were analysed by Anova with Bonferroni's Multiple Comparison Test and statistically significant changes were represented by asterisks ($P \leq 0.05$ *, $P \leq 0.01$ **, $P \leq 0.001$ ***).

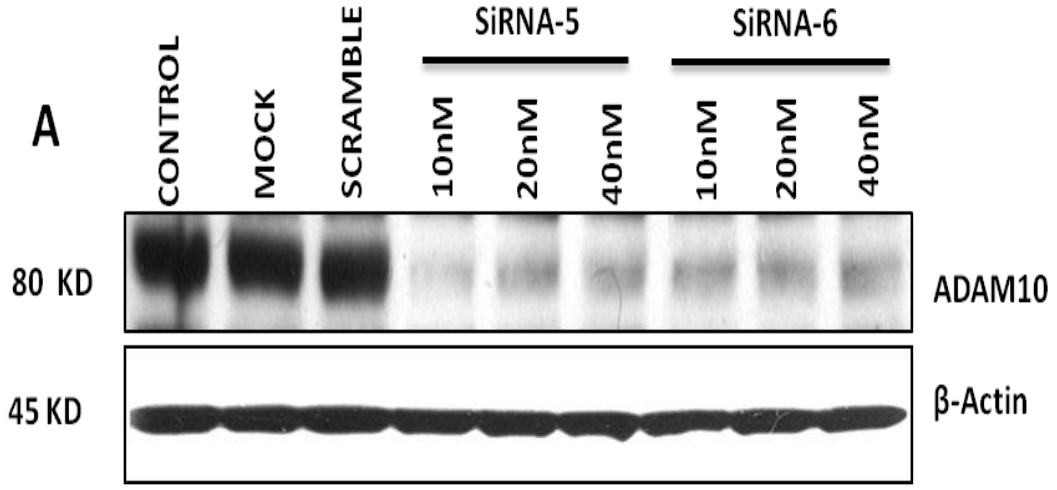
4.2.4. The effects of ADAMs knockdown and inhibition on HER ligands Shedding in parental and cetuximab resistant cell lines

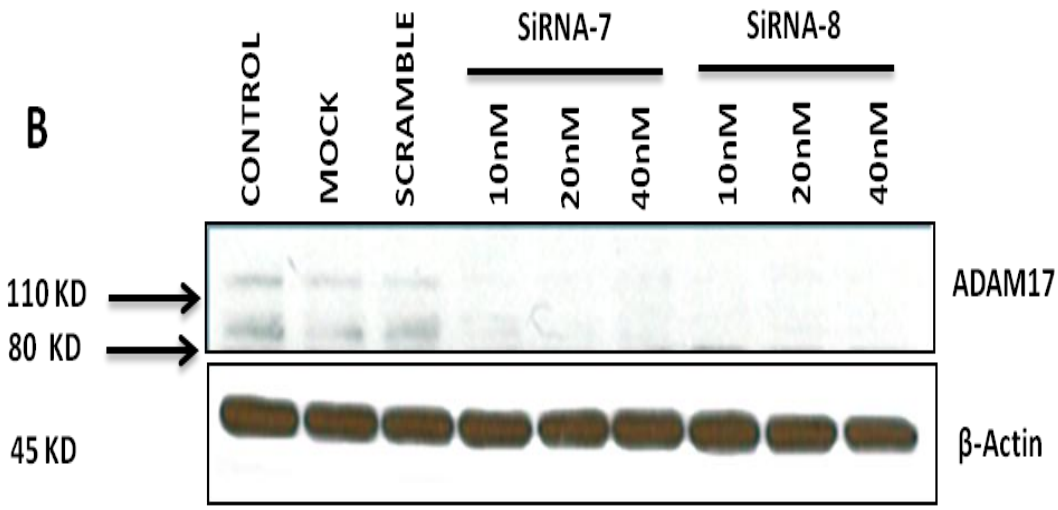
The above results showed that treatment with cetuximab induced an upregulation of ADAM10 and ADAM17 as well as the shedding of their substrates, BTC and NRG-1, respectively into the media. Thus I proceeded to investigate the effects of the knockdown or inhibition of ADAMs on ligand release in relation to cetuximab treatment. For this purpose the optimization of ADAM10 and ADAM17 knockdown was first carried out.

4.2.4.1. Optimization of ADAM10 and ADAM17 knockdown

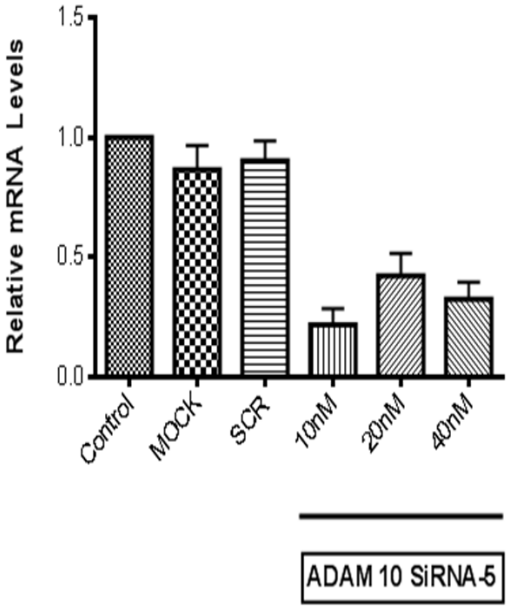
In this study two specific siRNAs against ADAM10, Hs-ADAM10-5 and Hs-ADAM10-6, and two specific siRNAs against ADAM17, Hs-ADAM17-7 and Hs-ADAM17-8, were used. In order to determine the lowest effective concentration of the siRNAs, 3 concentrations of each siRNA were used for optimization and ADAM10 and ADAM17 proteins knockdown was analysed by western blot analysis after 72h of transfection. 10 nM of siRNA-5 and siRNA-6 (figure 4.4A) as well as 10 nM of siRNA-7 and siRNA-8 (figure 4.4B) led to an efficient knockdown of ADAM10 and ADAM17 proteins respectively. The efficiency of the chosen concentrations of siRNAs was also analysed at mRNA level. For this purpose the mRNA knockdown of ADAMs was measured by qRT-PCR after 48h of transfection. The results suggested that 10 nM of each siRNA was the most effective concentration to achieve the maximal mRNA knockdown (figure 4.4C&D). To optimize the time of exposure to transfection reagent, the cells were transfected with 10 nM of relevant siRNAs for 24, 48 or 72 hours. In these experiments 72h seems to

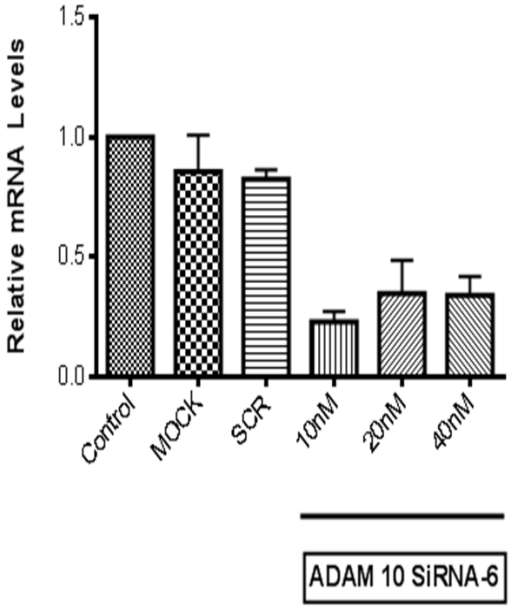
be the optimal time to achieve the maximal knockdown of ADAM10 and ADAM17 proteins (figure 4.4E&F). Furthermore, to assess the specificity of each siRNA, the cells were transfected with ADAM10 siRNAs and were blot for ADAM17 (figure 4.4G) or transfected with siRNAs against ADAM17 and were blot for ADAM10 (figure 4.4H). ADAM10 knockdown did not have any effect on ADAM17 protein level and ADAM17 knockdown also did not affect ADAM10 protein level (figure 4.4G&H).



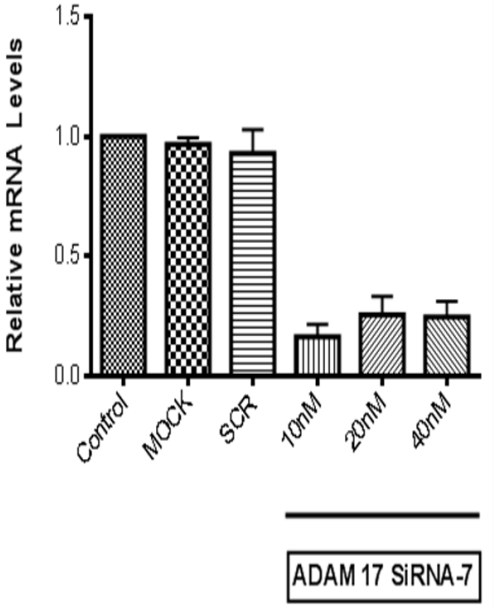


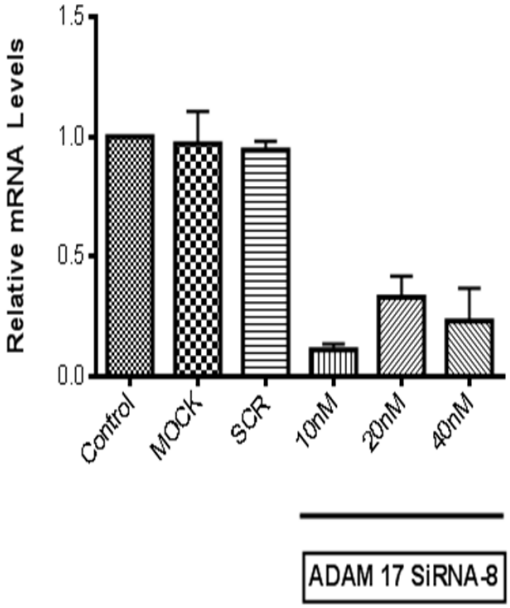
C

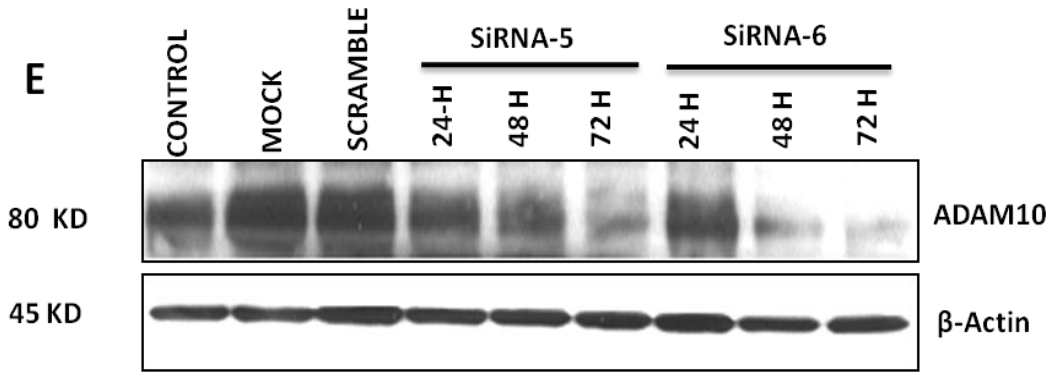

Relative mRNA Levels
1.5
1.0
0.5
0.0
Control
MOCK
SCR
10nM
20nM
40nM
ADAM 10 SiRNA-5

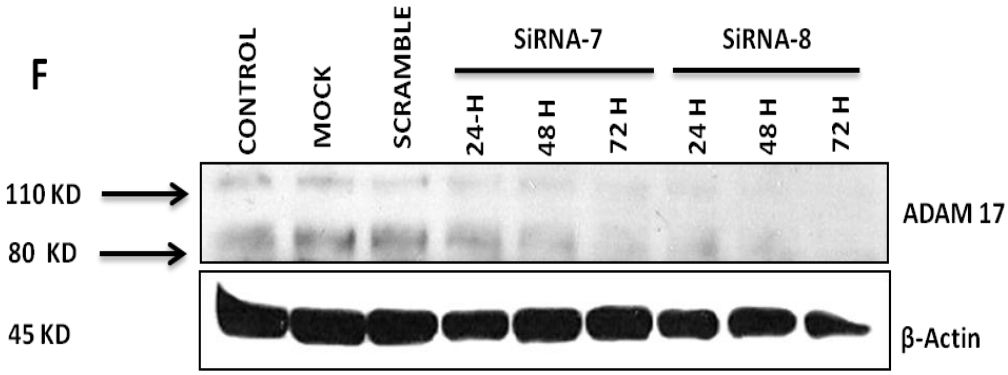

Relative mRNA Levels
1.5
1.0
0.5
0.0
Control
MOCK
SCR
10nM
20nM
40nM
ADAM 10 SiRNA-6

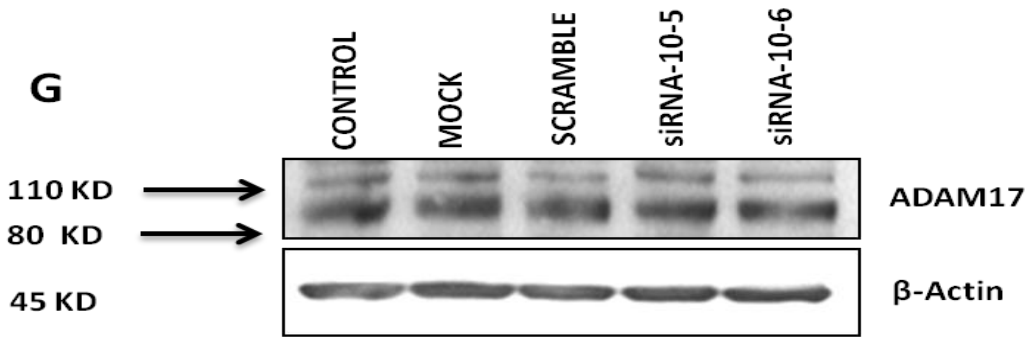
D


Relative mRNA Levels
1.5
1.0
0.5
0.0
Control
MOCK
SCR
10nM
20nM
40nM
ADAM 17 SiRNA-7


Relative mRNA Levels
1.5
1.0
0.5
0.0
Control
MOCK
SCR
10nM
20nM
40nM
ADAM 17 SiRNA-8


E
CONTROL
MOCK
SCRAMBLE
SiRNA-5
SiRNA-6
24-H
48 H
72 H
24 H
48 H
72 H
80 KD
45 KD
ADAM10
β-Actin


F
CONTROL
MOCK
SCRAMBLE
SiRNA-7
SiRNA-8
24-H
48 H
72 H
24 H
48 H
72 H
110 KD
80 KD
45 KD
ADAM 17
β-Actin


G
CONTROL
MOCK
SCRAMBLE
siRNA-10-5
siRNA-10-6
110 KD
80 KD
45 KD
ADAM17
β-Actin

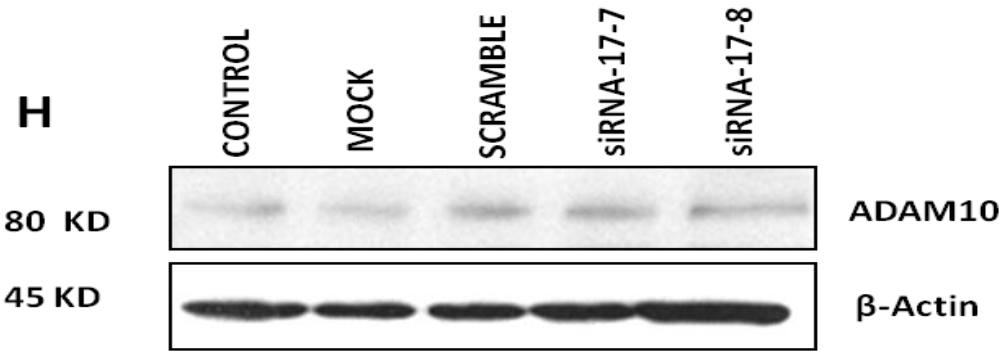

H
CONTROL
MOCK
SCRAMBLE
siRNA-17-7
siRNA-17-8
80 KD
45 KD
ADAM10
β-Actin

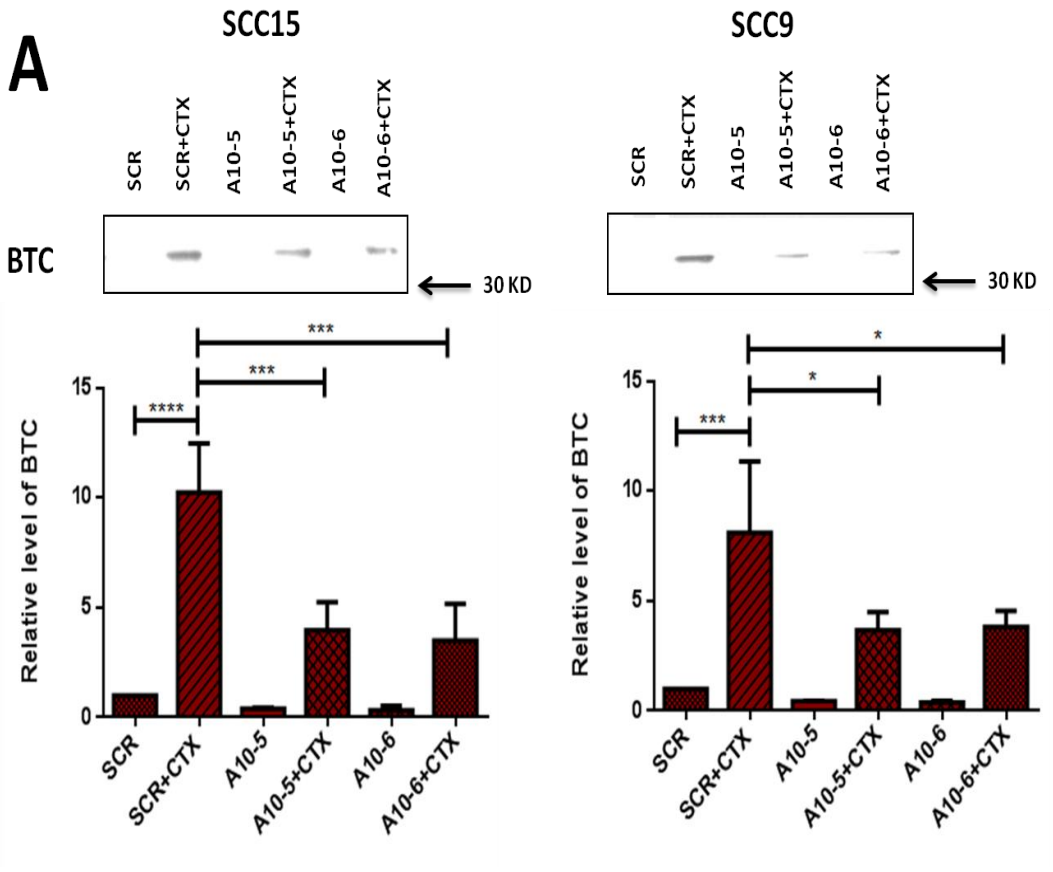
Figure 4.4. Optimization of ADAM10 and ADAM17 knockdown in SCC15 cell line

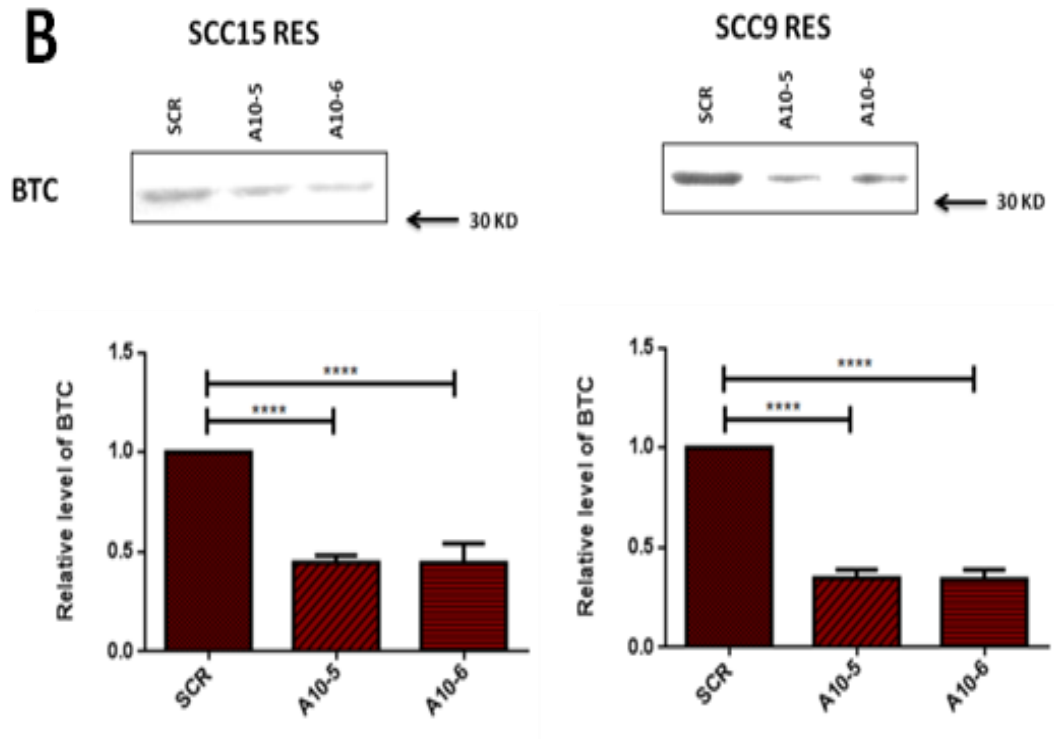
SCC15 cells were transfected with the indicated concentrations of ADAM10 siRNAs 5 and 6 or ADAM17 siRNAs 7 and 8. The protein levels of ADAMs were analysed by western blot 72h post transfection (A&B) and the mRNA levels were measured by qRT-PCR 48h post transfection (n=3, mean ± S.D) (C&D). The optimization of transfection durations was performed using 10 nM of each siRNA for 24h, 48h and 72h (E&F). SCC15 cells were transfected for 72h with 10 nM of ADAM10 siRNAs or ADAM17 siRNAs and were blotted for ADAM17 (G) and ADAM10 (H) respectively. Scrambled siRNA was used as a negative control. Mock-transfected control contained transfection reagent only without any siRNA. β-actin was used as a loading control. Western blots are representative of three independent experiments.

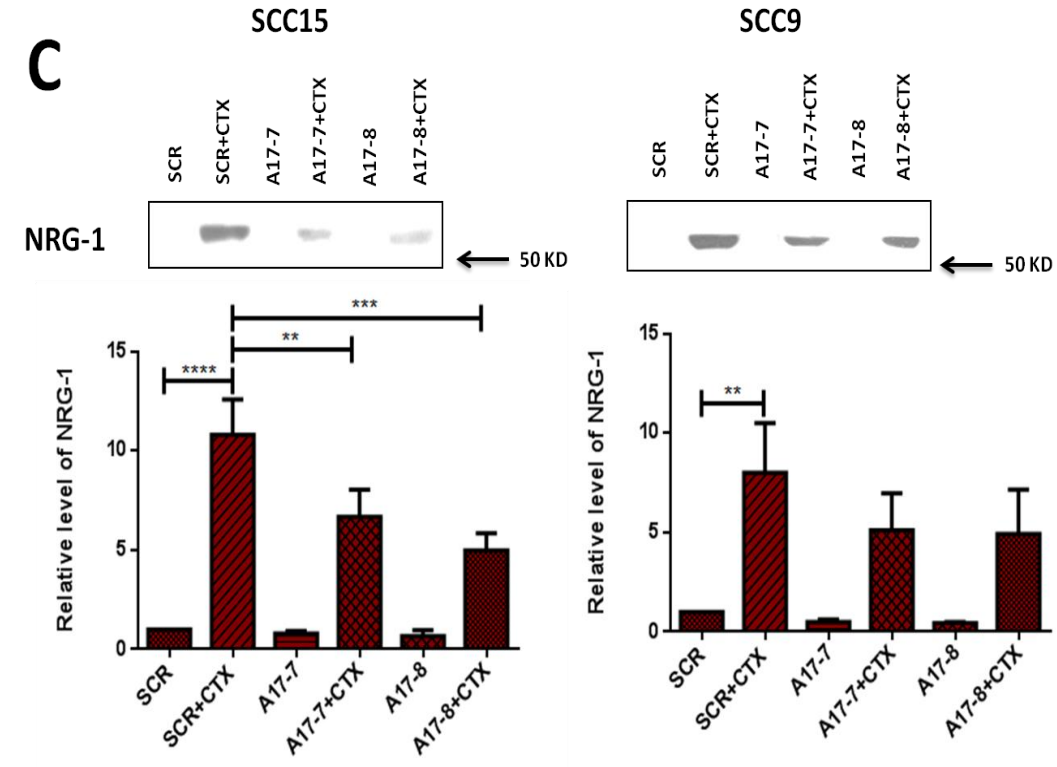
4.2.4.2. ADAMs knockdown and inhibition decreased the shedding of HER ligands in response to cetuximab treatment

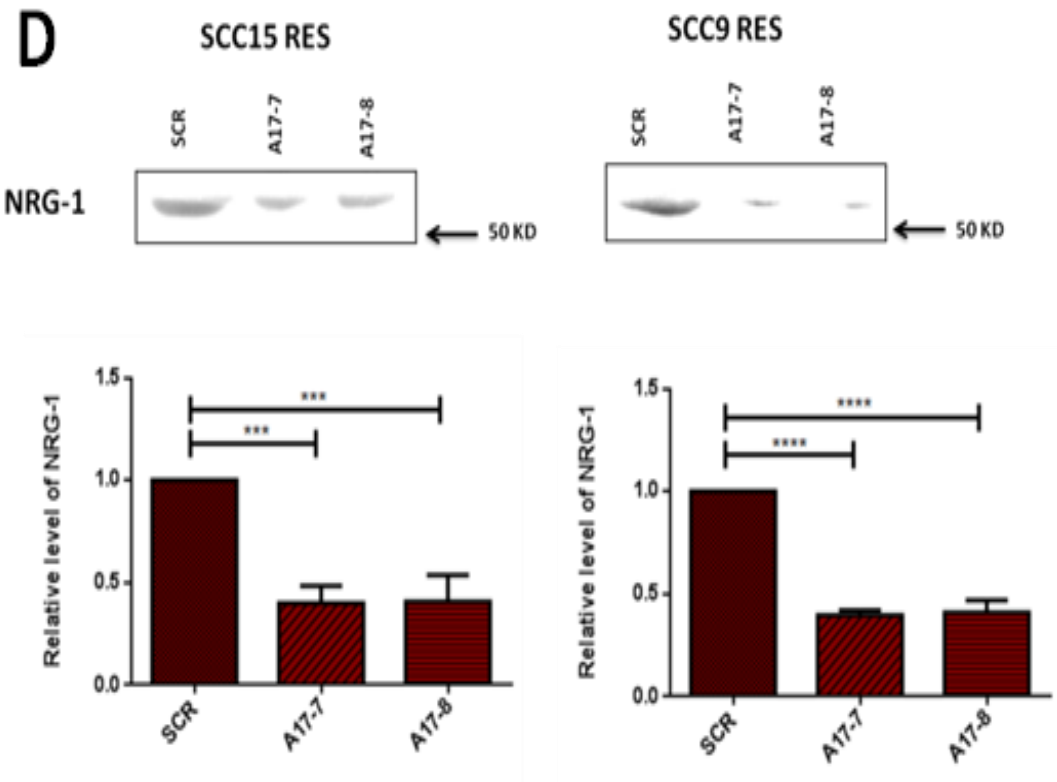
SCC9 and SCC15 cells were transfected with two specific siRNAs against ADAM10 (figure 4.5A) and ADAM17 (figure 4.5C) and the levels of their substrates in the media were assessed by western blot analysis (top panel) and ELISA (bottom panel). The cetuximab resistant cell lines were also transfected with two specific siRNAs against ADAM10 (figure 4.5B) and ADAM17 (figure 4.5D). Treatment with cetuximab significantly increased the release of BTC into the media of the SCC9 and SCC15 cell lines. When the cells were transfected with the indicated ADAM10 siRNAs while treated with cetuximab, a significant decrease in the level of BTC (a substrate of ADAM10) in the media was observed compared to the cells which were transfected with scrambled siRNA. In the cetuximab resistant cell lines, there was a significant decrease in the level of BTC in the media of the cells transfected with

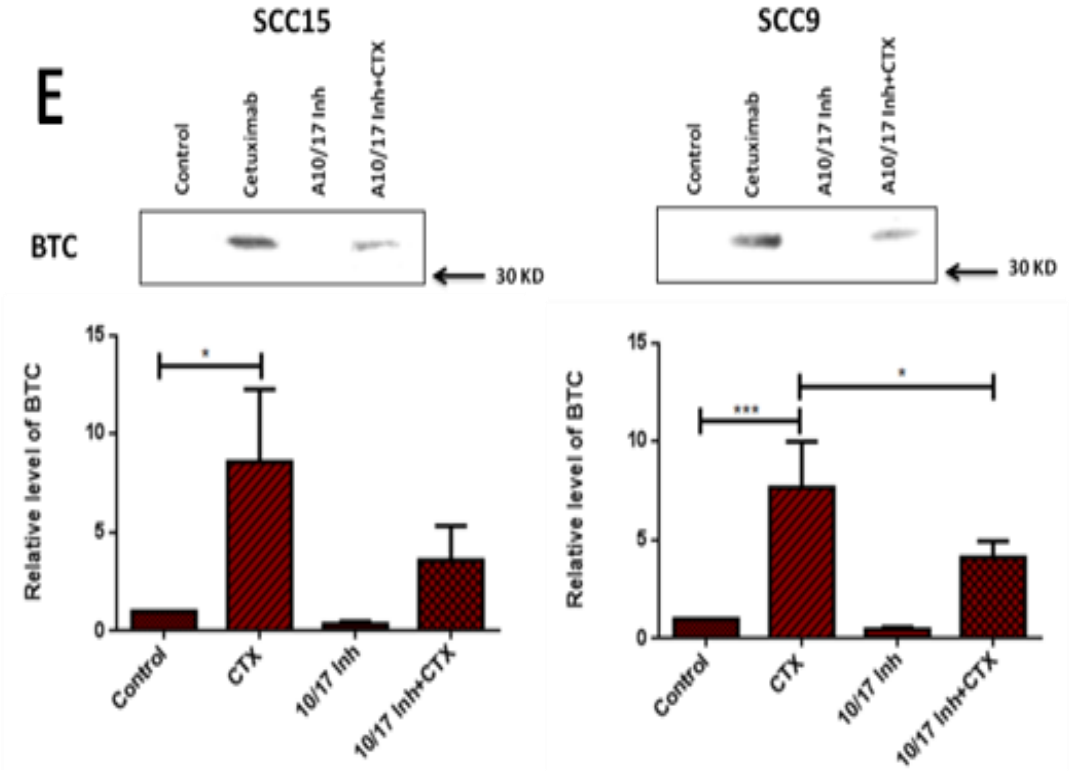
ADAM10 siRNAs compared to the negative siRNA control. Cetuximab also significantly induced the shedding of NRG-1, a substrate of ADAM17 into the media of the parental cell lines. Transfection of cells with two specific ADAM17 siRNAs in the presence of cetuximab reduced the level of NRG-1 in the media of both cell lines, however, this was only significant in SCC15 cell line. Compared to the negative siRNA control, ADAM17 knockdown significantly inhibited the release of NRG-1 in the cetuximab resistant cell lines. The inhibition of ADAM10 and ADAM17 by ADAM10/17 dual inhibitor (INCB3619) also reduced the level of BTC in the media of the parental (figure 4.5E) and cetuximab resistant (figure 4.5F) SCC9 and SCC15 cell lines, shown by western blot (top panel) and ELISA (bottom panel). However, the reduction was only significant in parental SCC9 cell line using ELISA method. ADAM10/17 inhibitor also significantly inhibited the release of NRG-1 into the media of the parental cell lines (figure 4.5G). Although reduced level of NRG-1 was observed in the media of resistant cell lines upon treatment with ADAM10/17 inhibitor, this was not statistically significant using ELISA method (figure 4.5H).

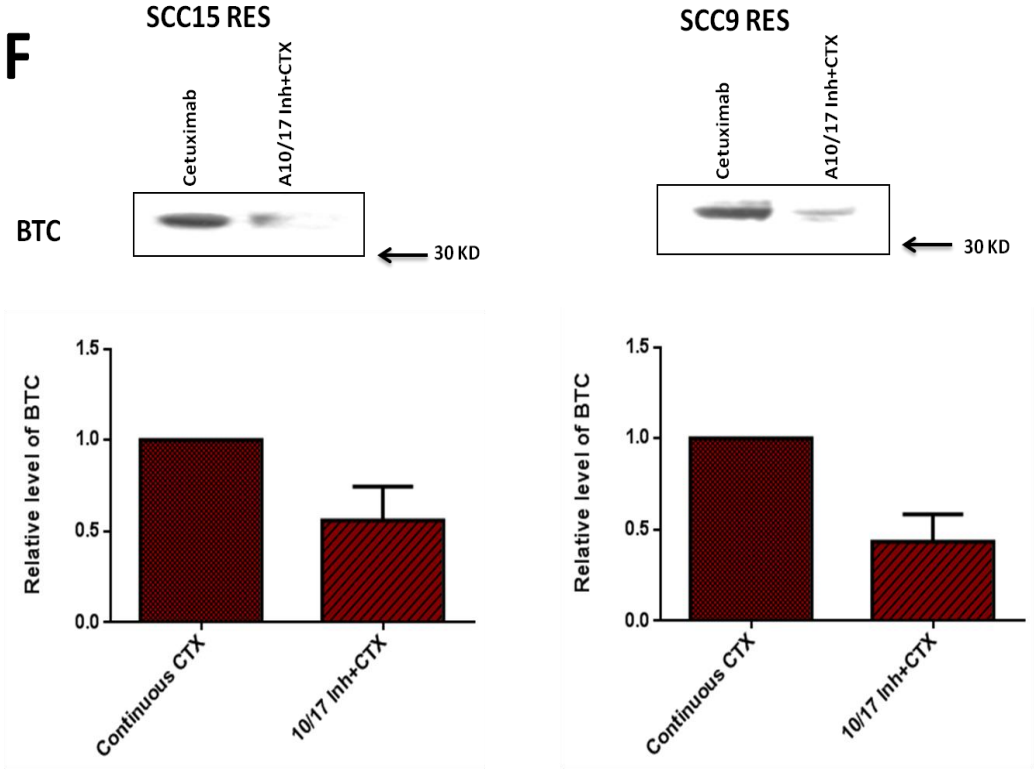

A
SCC15
SCC9
SCR
SCR+CTX
A10-5
A10-5+CTX
A10-6
A10-6+CTX
BTC
30 KD
Relative level of BTC
***
****
*

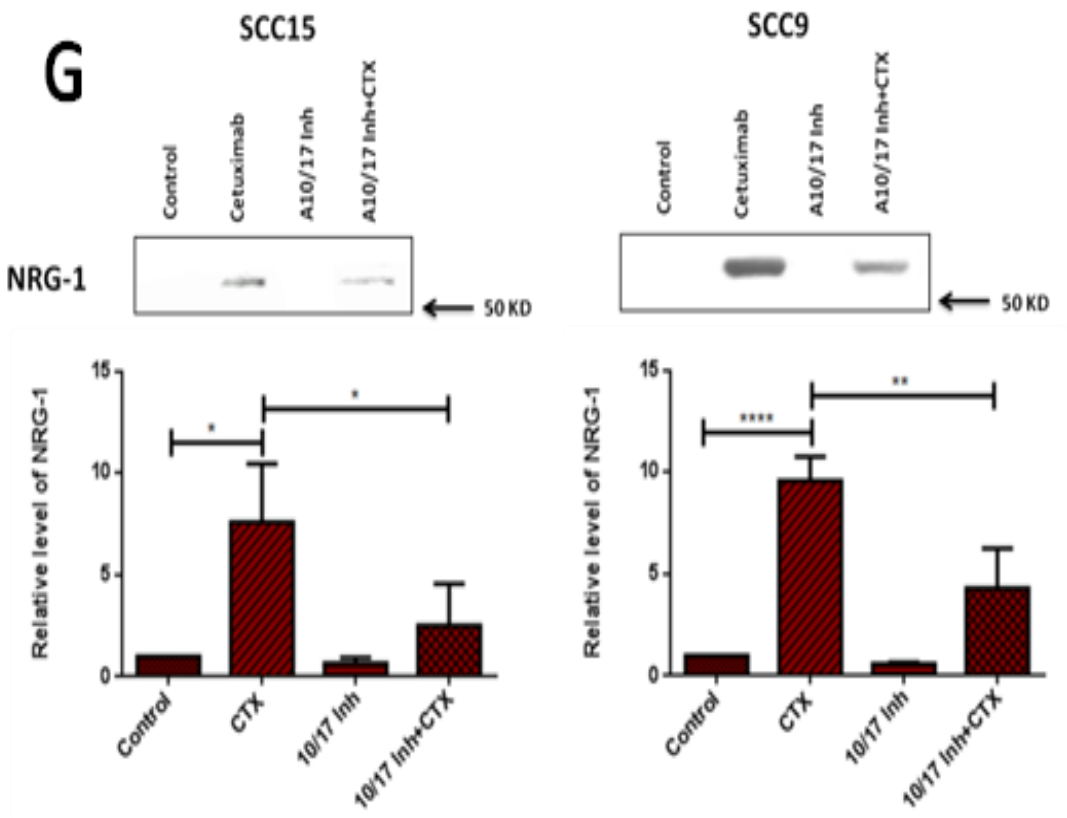

B
SCC15 RES
SCC9 RES
SCR
A10-5
A10-6
BTC
30 KD
Relative level of BTC
****


C
SCC15
SCC9
SCR
SCR+CTX
A17-7
A17-7+CTX
A17-8
A17-8+CTX
NRG-1
50 KD
Relative level of NRG-1
****
**
***


D
SCC15 RES
SCC9 RES
SCR
A17-7
A17-8
NRG-1
50 KD
Relative level of NRG-1
***
****


E
SCC15
SCC9
Control
Cetuximab
A10/17 Inh
A10/17 Inh+CTX
BTC
30 KD
Relative level of BTC
Control
CTX
10/17 Inh
10/17 Inh+CTX


F
SCC15 RES
SCC9 RES
Cetuximab
A10/17 Inh+CTX
BTC
30 KD
Relative level of BTC
Continuous CTX
10/17 Inh+CTX


G
SCC15
SCC9
Control
Cetuximab
A10/17 Inh
A10/17 Inh+CTX
NRG-1
50 KD
Relative level of NRG-1
Control
CTX
10/17 Inh
10/17 Inh+CTX

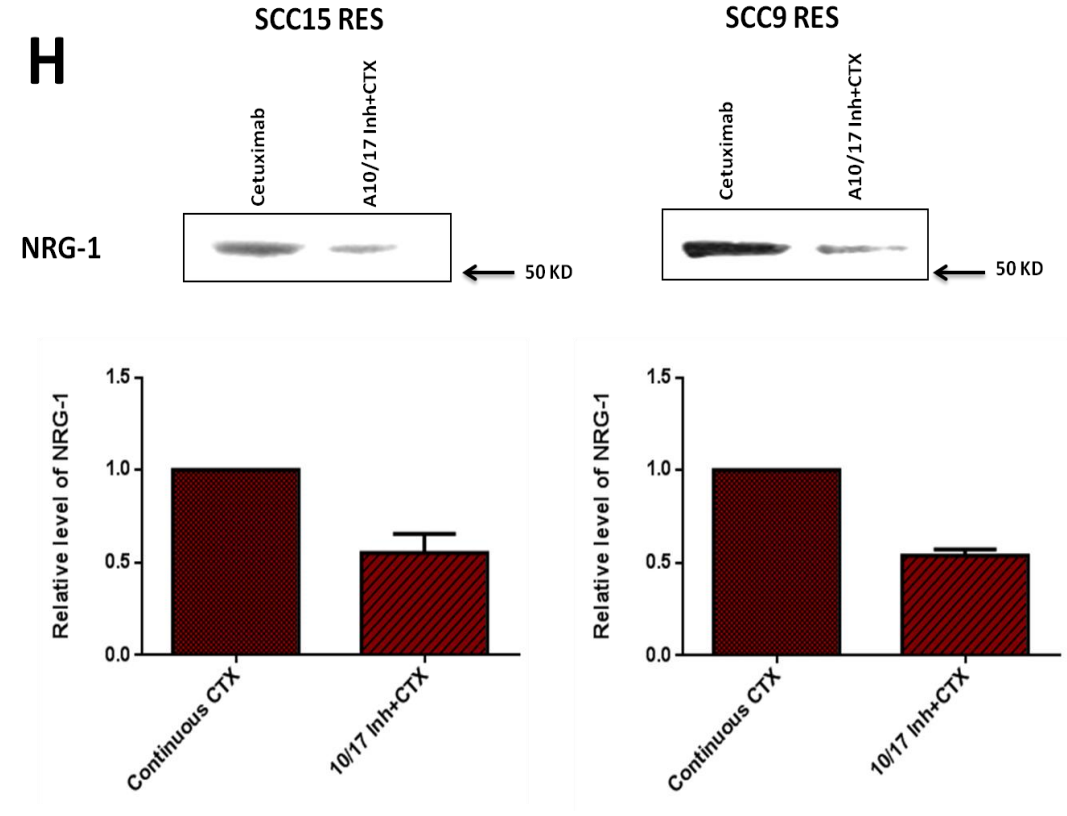

H
SCC15 RES
SCC9 RES
Cetuximab
A10/17 Inh+CTX
NRG-1
50 KD
Relative level of NRG-1
Continuous CTX
10/17 Inh+CTX

Figure 4.5: ADAMs knockdown or inhibition decreases the shedding of ligands in response to cetuximab in parental and cetuximab resistant cell lines

SCC9 and SCC15 cells were transfected with either 10 nM of two specific siRNAs against ADAM10 (A10-5 & A10-6) (A) or ADAM17 (A17-7 & A17-8) (C) or treated with 10 μM of ADAM10/17 inhibitor (E& G) alone or in combination with 10 μg/mL of cetuximab for 24h. For cetuximab resistant cell lines, the cells were transfected with 10 nM of two specific siRNAs against ADAM10 (B), ADAM17 (D) or treated with 10 μM of ADAM10/17 inhibitor (F&H) for 24h while continuously treated with 20 μg/mL of cetuximab. The conditioned media were then collected as described in the materials and methods section and the levels of BTC and NRG-1 were assessed by western blot (top panels) and ELISA (bottom panels). Western blots are representative of three independent experiments. For ELISA, three independent experiments were performed in triplicate. The means/medians between the groups were analysed by Anova with Bonferroni's Multiple Comparison Test (more than two groups) or Mann-Witney Test (two groups) and statistically significant changes were represented by asterisks ($P \leq 0.05$ *, $P \leq 0.01$ **, $P \leq 0.001$ ***, $P \leq 0.0001$ ****).

4.2.5. The effect of ADAM10 and ADAM17 inhibition in HNSCC cell lines

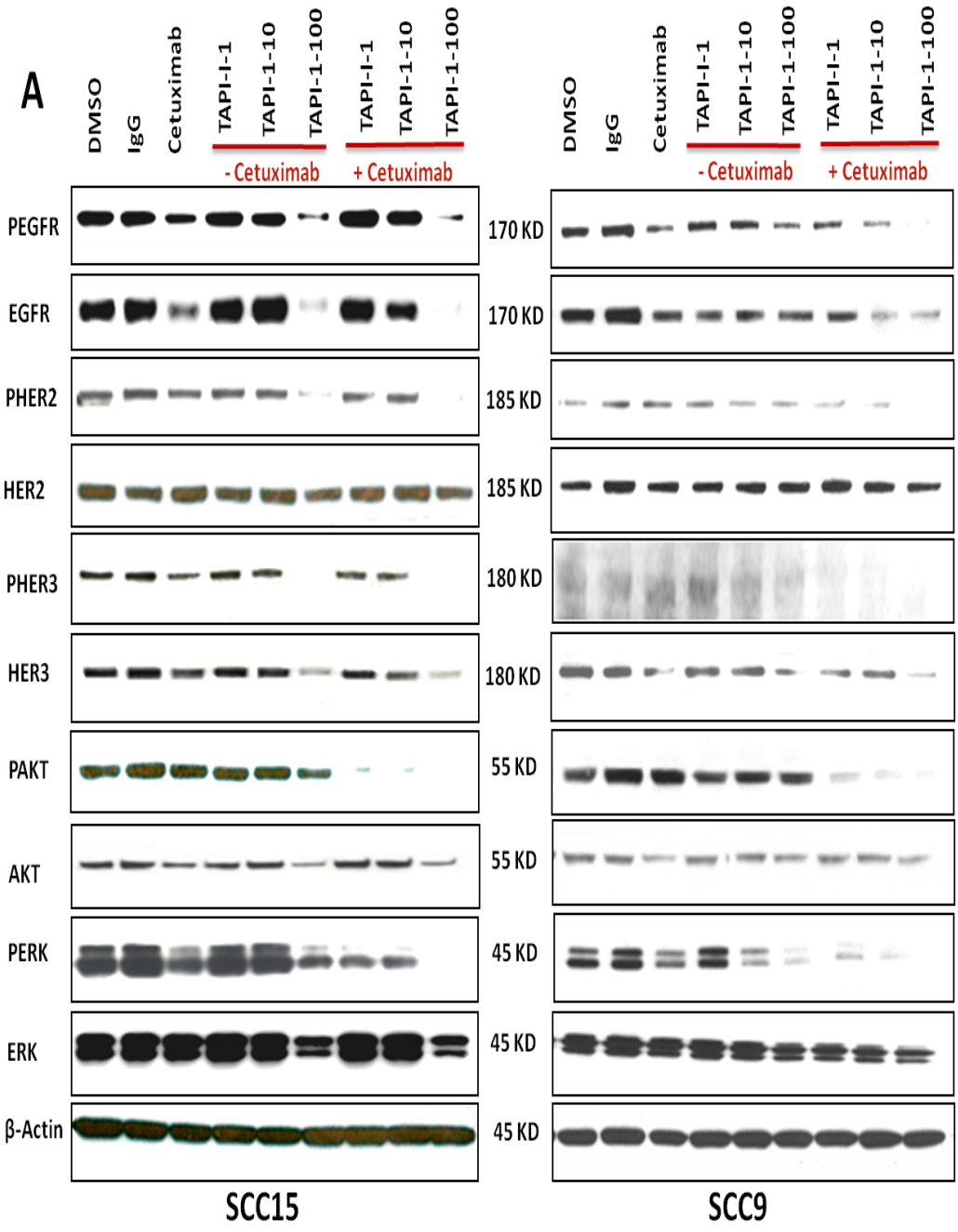
In order to investigate the role of ADAM10 and ADAM17 in relation to HER receptors' signalling in the parental and cetuximab resistant cell lines, their inhibition was carried out using TAPI-1 (ADAM17 and other metalloproteases inhibitor) and ADAM10/17 dual inhibitor (INCB3619).

4.2.5.1. TAPI-1

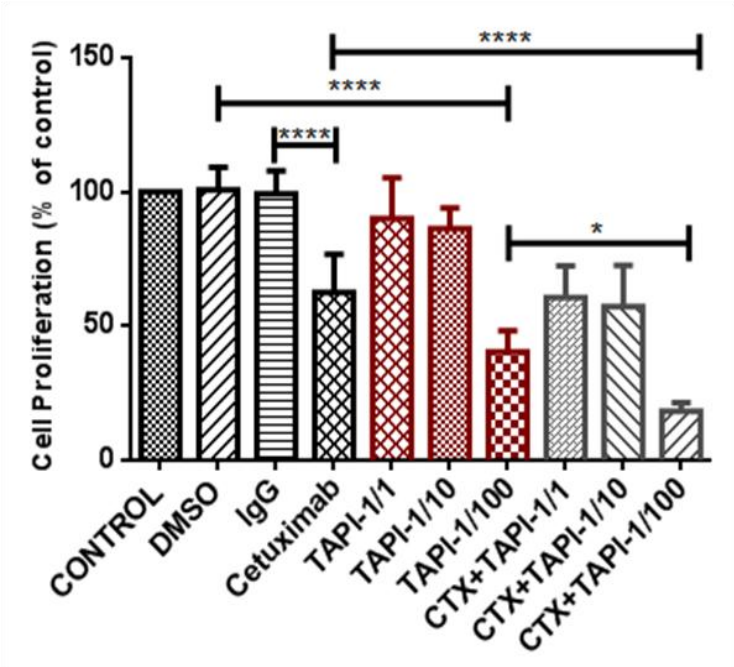
4.2.5.1.1. ADAMs inhibition had a significant effect on targeting HER receptors' signalling in parental cell lines

The effect of TAPI-1 with or without cetuximab on HER receptors' signalling was assessed in SCC15 and SCC9 cell lines. In SCC15 cells cetuximab and TAPI-1 (100 μM) downregulated EGFR but the effect was greater when the cells were exposed to the combination of both drugs, correlated with a decrease in pEGFR level. In SCC9 cells cetuximab or TAPI-1 alone did not have any effect on EGFR. However, a noticeable downregulation of EGFR was observed when cetuximab was combined with TAPI-1 (10 & 100 μM). Combination of cetuximab with TAPI-1(100 μM) also had a pronounced effect on inhibition of pEGFR in SCC9 cells. In SCC15 cells cetuximab did not have a perceptible effect on pHER2 but TAPI-1 (100 μM) alone or its combination with cetuximab inhibited pHER2 considerably. Whereas in SCC9 cell line the inhibition of pHER2 was observed only if cetuximab was combined with the highest concentration of TAPI-1. Noticeable inhibition of pHER3 was observed when SCC15 cells were treated with TAPI-1 (100 μM) alone or its combination with cetuximab whereas in SCC9 cell line only combination treatment had a great effect on pHER3. Cetuximab did not have any effect on HER3, AKT or ERK in SCC15 cells but TAPI-1 (100 μM) alone or its combination with cetuximab downregulated these proteins although not very considerable. Combination of cetuximab with TAPI-1 (100 μM) downregulated HER3 in SCC9 cells, however, did not affect AKT or ERK proteins level. In both cell lines the combination treatment was found to be more effective in the inhibition of pAKT and pERK compared to single drug

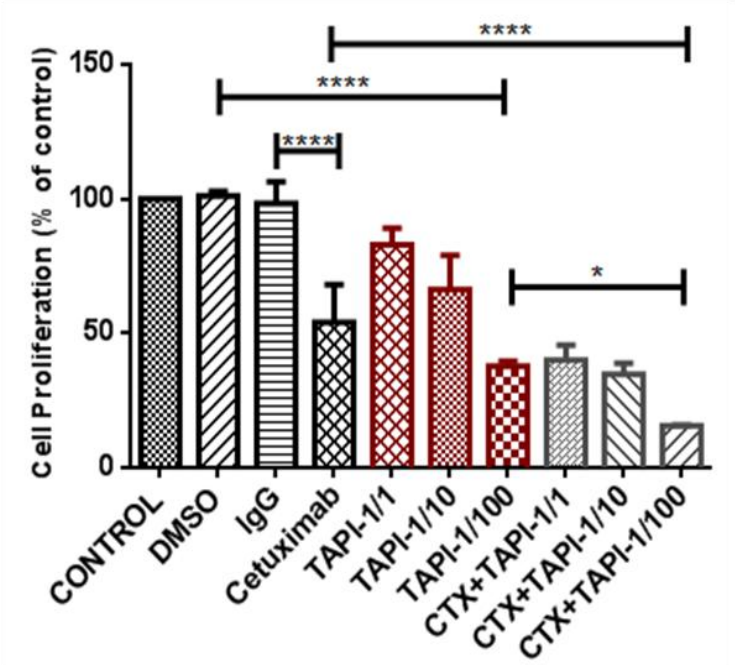
(figure 4.6A). The effect of TAPI-1 on the cell proliferation was also investigated. Cetuximab and TAPI-1(100 μM) decreased the cell proliferation of both parental cell lines significantly but a greater inhibition was observed when cells were treated with the combination of both drugs (figure 4.6B).



B


Cell Proliferation (% of control)
150
100
50
0
****
****
****
*
CONTROL
DMSO
IgG
Cetuximab
TAPI-1/1
TAPI-1/10
TAPI-1/100
CTX+TAPI-1/1
CTX+TAPI-1/10
CTX+TAPI-1/100

SCC15


Cell Proliferation (% of control)
150
100
50
0
****
****
****
*
CONTROL
DMSO
IgG
Cetuximab
TAPI-1/1
TAPI-1/10
TAPI-1/100
CTX+TAPI-1/1
CTX+TAPI-1/10
CTX+TAPI-1/100

SCC9

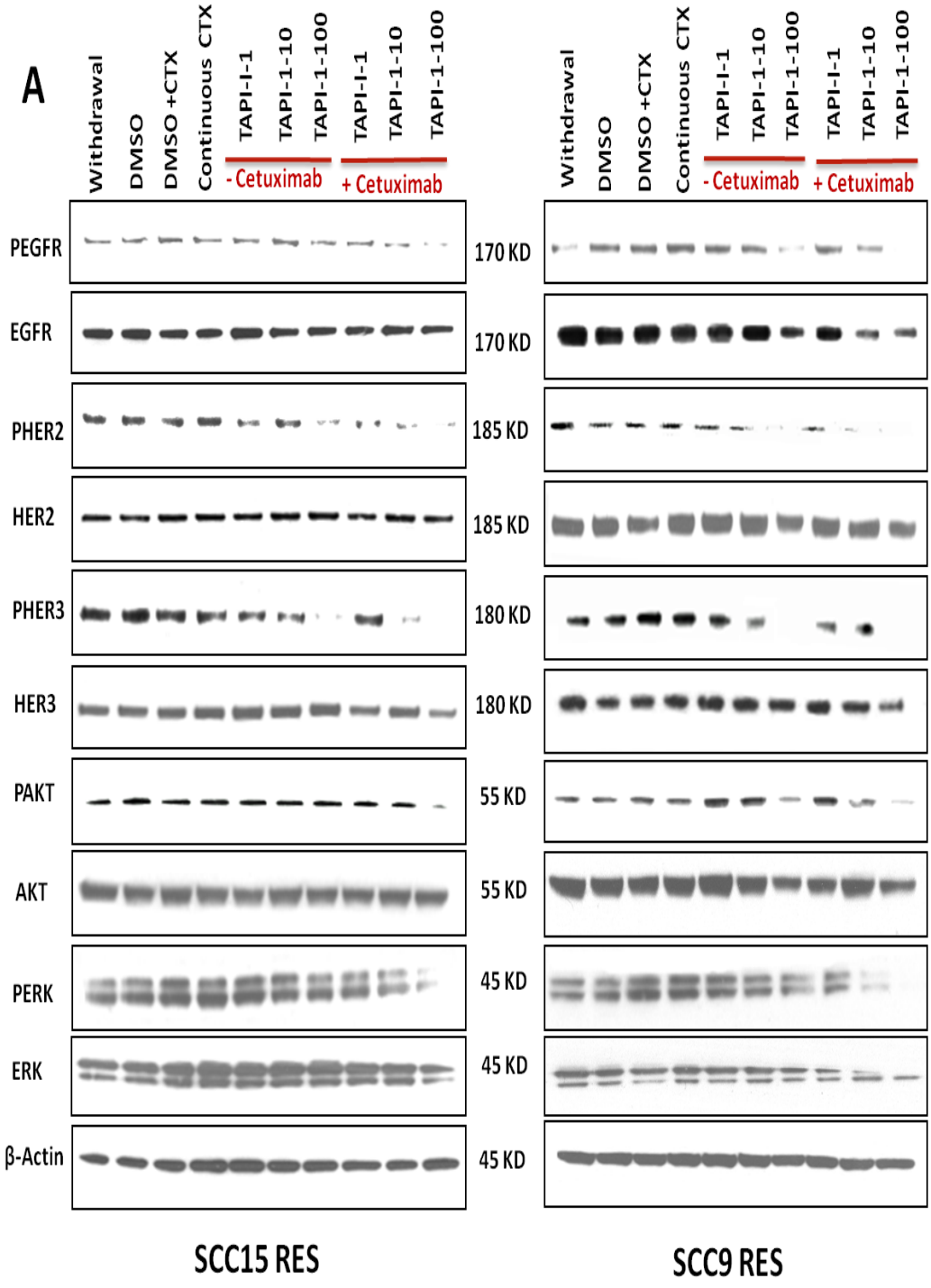
Figure 4.6: The effect of TAPI-1 in parental cell lines

SCC15 and SCC9 cells were treated with 10 µg/mL of cetuximab, TAPI-1 (1, 10 or 100 µM) or their combinations for 72h. The expression and phosphorylation of the indicated proteins were analysed by western blot analysis (A) and the cell proliferation was assessed by MTT assay (B). Three independent experiments were performed in triplicate and the cell proliferation was calculated relative to the untreated cells and presented as the means ± S.D. The means between the groups were analysed by Anova with Bonferroni's Multiple Comparison Test and the statistically significant changes were represented by asterisks ($P \leq 0.05$ *, $P \leq 0.0001$ ****). 10 µg/mL of human IgG was used as a control. DMSO was used as a solvent control for TAPI-1. β-actin was used as a loading control for each gel but only one representative band is shown. Western blots are representative of three independent experiments.

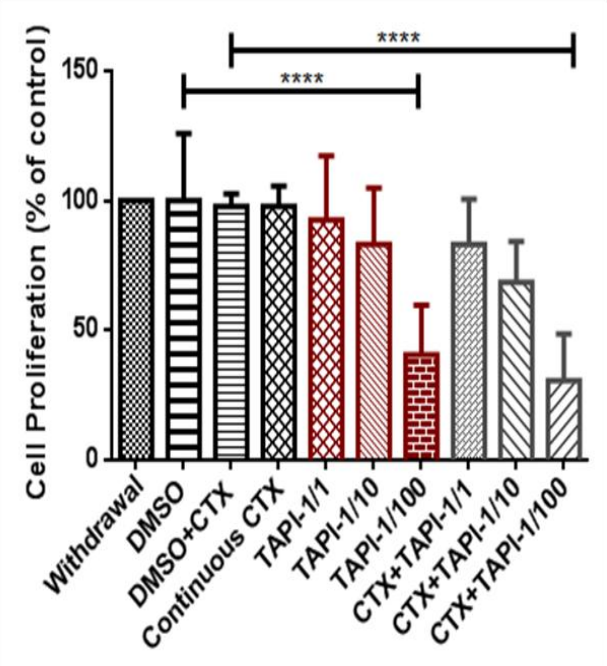
4.2.5.1.2. Inhibition of ADAMs was very effective in targeting HER receptors' signalling in cetuximab resistant cell lines

In SCC15 and SCC9 cetuximab resistant cell lines inhibition of pEGFR, pHER2 and pERK was observed when the cells were treated with higher concentration of TAPI-1 while continuously being exposed to cetuximab. In SCC15 resistant cells, the combination of cetuximab with TAPI-1 (10 & 100 µM) seemed to be more effective in inhibition of pHER3 compared to either drug alone. However, in SCC9 resistant cells the combination treatment did not seem to have a greater effect compared to TAPI-1 alone. The combination of cetuximab with higher concentration of TAPI-1 also had greater effect on inhibition of pAKT in SCC9 resistant cell lines but a pronounced inhibition of pAKT was only observed when SCC15 resistant cells were treated with TAPI-1 (100 µM) in combination with cetuximab (figure

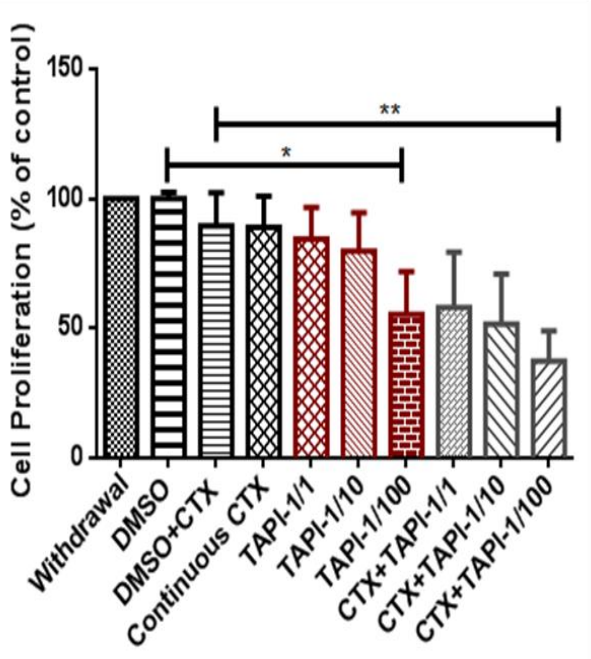
4.7A). In resistant cell lines TAPI-1 (100 μM) significantly decreased the cell proliferation in the presence or absence of cetuximab (figure 4.7B).



B



SCC15 RESISTANT



SCC9 RESISTANT

Figure 4.7. The effect of TAPI-1 in cetuximab resistant cell lines

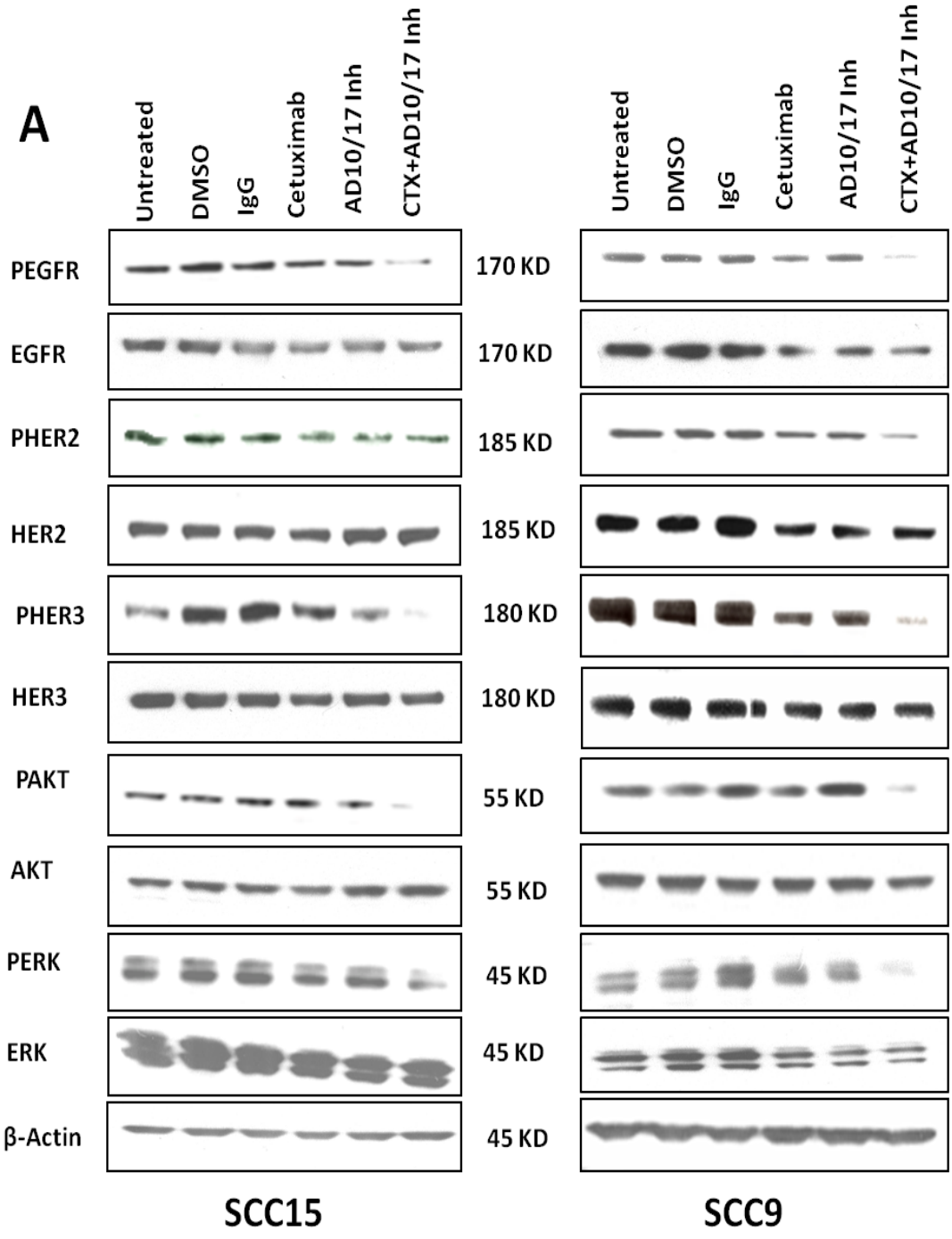
The resistant SCC15 and SCC9 cells were treated with TAPI-1 (1, 10 or 100 μM) for 72h in the presence or absence of cetuximab (20 μg/mL). The expression and activation of proteins were analysed by western blot analysis (A) and the cell proliferation was assessed by MTT assay (B). Three independent experiments were performed in triplicate and cell proliferation was calculated relative to the untreated cells and presented as the means ± S.D. The means between the groups were analysed by Anova with Bonferroni's Multiple Comparison Test and statistically significant changes were represented by asterisks ($P \leq 0.05$ *, $P \leq 0.01$ **, $P \leq 0.0001$ ****). 10 μg/mL of human IgG was used as a control. DMSO was used as a solvent control for TAPI-1. β-actin was used as a loading control for each gel but only one representative band is shown. Western blots are representative of three independent experiments.

4.2.5.2. ADAM10/17 dual inhibitor

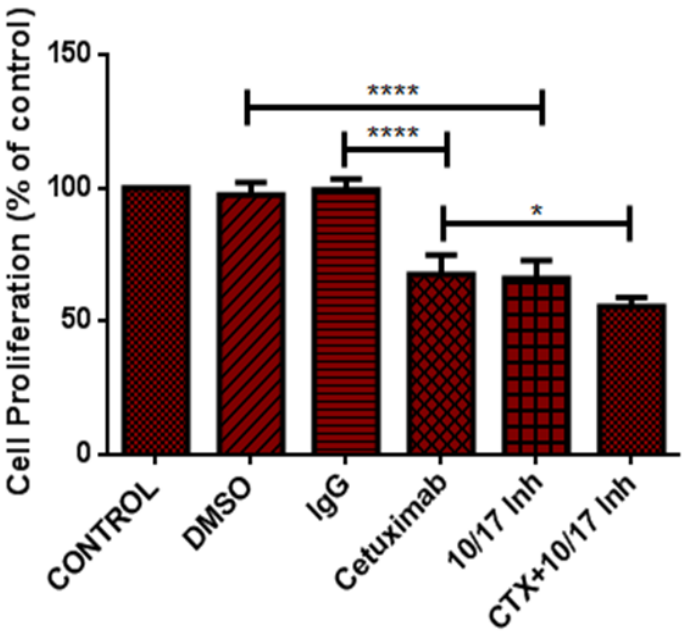
4.2.5.2.1. The combination of cetuximab with ADAM10/17 dual inhibitor has a greater effect on inhibition of HER receptors compared to either drug alone

The effect of ADAM10/17 dual inhibitor on HER receptors' signalling was investigated in SCC15 and SCC9 cell lines. The greatest inhibition of pEGFR, pHER3 and their downstream pathways, pERK and pAKT was observed upon the combination treatment of cetuximab with ADAM10/17 inhibitor. The combination treatment also led to the inhibition of pHER2 in SCC9 cell line but there was no difference in the combination treatment compared to cetuximab alone or ADAM10/17 inhibitor alone in SCC15 cells (figure 4.8A). The results from MTT assay showed that the combination treatment was more effective in reducing the cell proliferation compared to cetuximab alone in both cell lines (figure 4.8B). The effect of combining cetuximab with ADAM10/17 inhibitor on

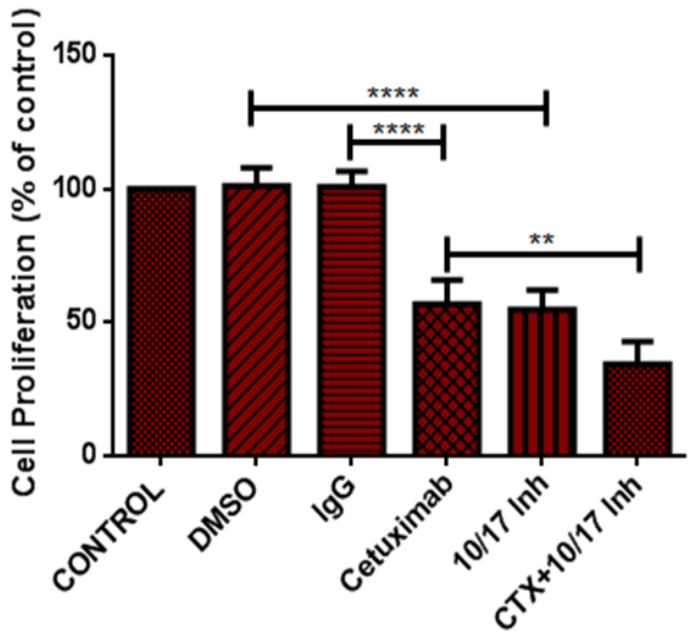
colony formation was also assessed in this study. In both parental cell lines the combination treatment led to a greater decrease in the number of colonies compared to cetuximab alone. Although the combination treatment seemed to be more effective compared to ADAM10/17 inhibitor alone, this was not statistically significant (figure 4.8C&D).



B

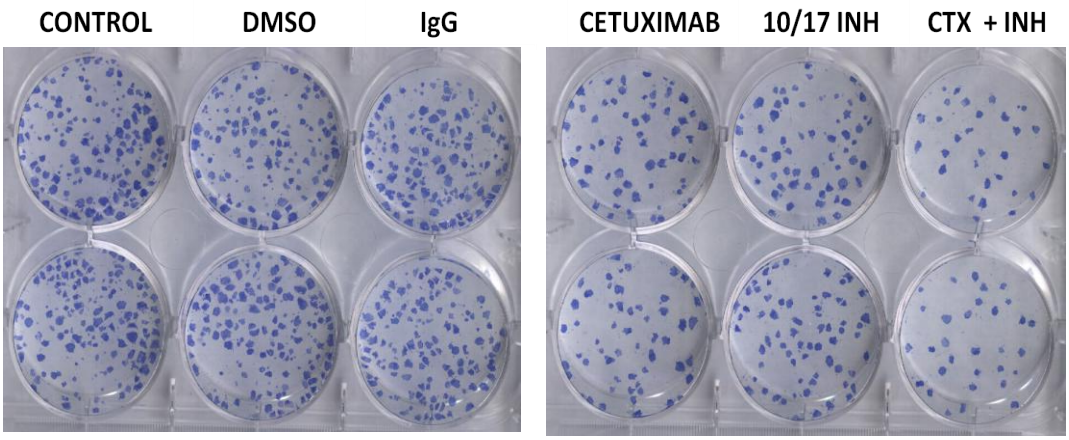

Cell Proliferation (% of control)
150
100
50
0
****
****
*
CONTROL
DMSO
IgG
Cetuximab
10/17 Inh
CTX+10/17 Inh

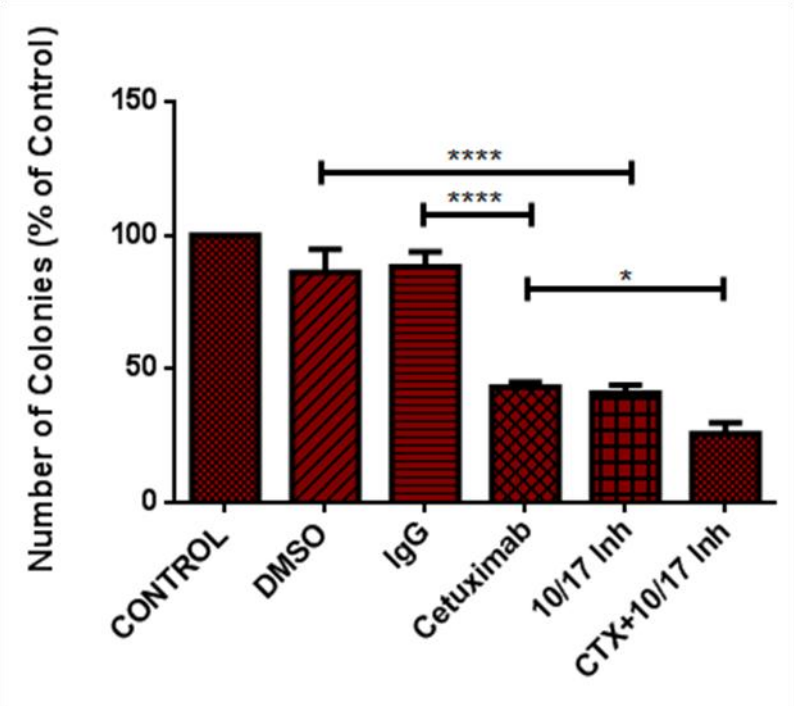
SCC15


Cell Proliferation (% of control)
150
100
50
0
****
****
**
CONTROL
DMSO
IgG
Cetuximab
10/17 Inh
CTX+10/17 Inh

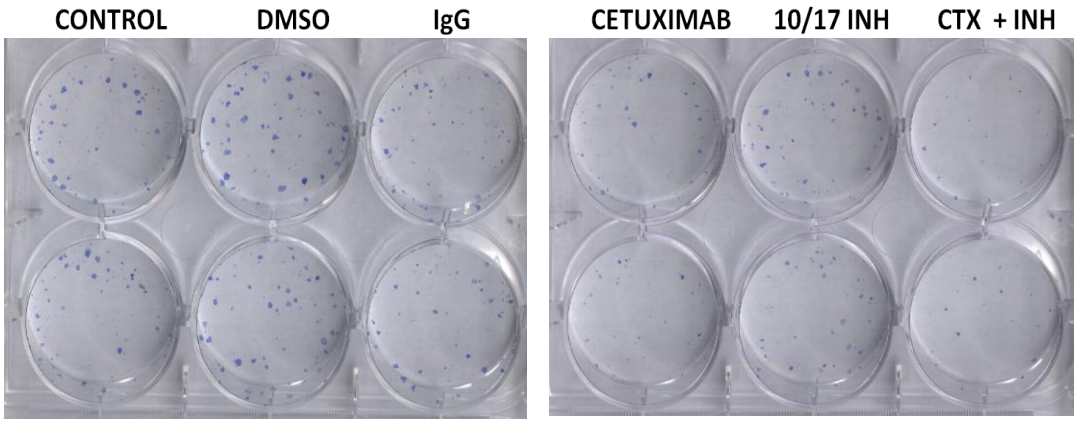
SCC9

C SCC 15


CONTROL
DMSO
IgG
CETUXIMAB
10/17 INH
CTX + INH


Number of Colonies (% of Control)
150
100
50
0
****
****
*
CONTROL
DMSO
IgG
Cetuximab
10/17 Inh
CTX+10/17 Inh

D SCC9


CONTROL
DMSO
IgG
CETUXIMAB
10/17 INH
CTX + INH

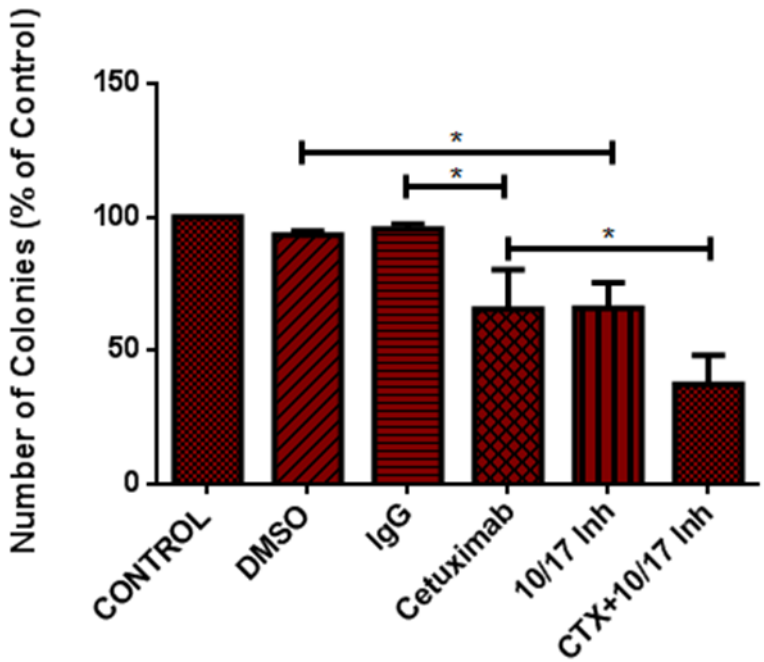

Number of Colonies (% of Control)
150
100
50
0
*
*
*
CONTROL
DMSO
IgG
Cetuximab
10/17 Inh
CTX+10/17 Inh

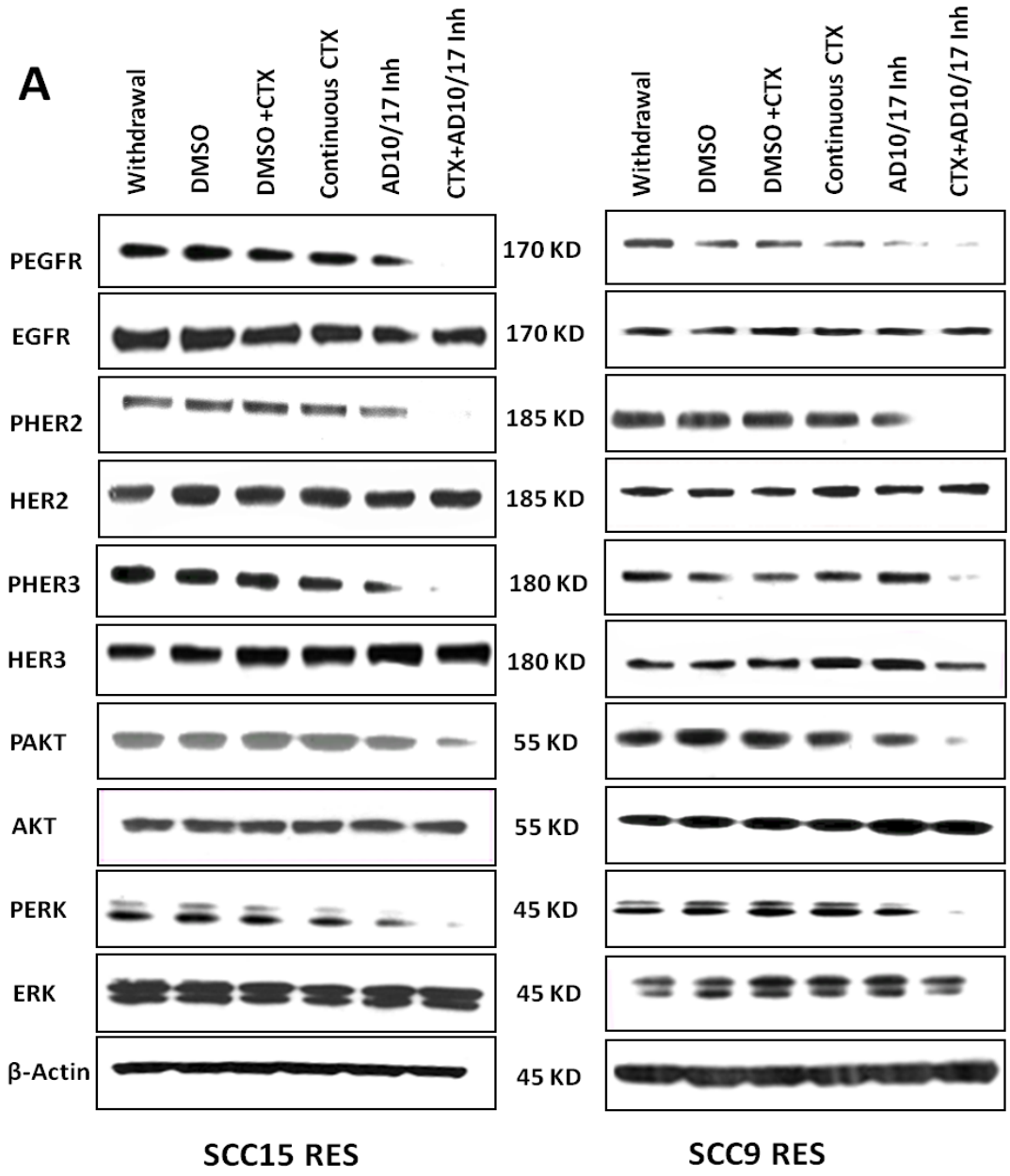
Figure 4.8: The effect of ADAM10/17 Inhibition in the parental SCC15 and SCC9 cell lines

SCC15 and SCC9 cells were treated with 10 µg/mL of cetuximab, 10 µM of ADAM10/17 inhibitor or their combination for 72h. The expression and phosphorylation of the indicated proteins were analysed by western blot analysis (A) and the cell proliferation was assessed by MTT assay (B). For clonogenic assay, the cells were harvested at the end of the treatment and were re-plated in duplicate. The cells were then incubated at 37ºC for 10-14 days to form colonies before being stained with 0.2% methylene blue (C&D). Three independent experiments were performed in duplicate and the cell proliferation and colony numbers were calculated relative to the untreated cells and presented as the mean ± S.D. The means between the groups were analysed by Anova with Bonferroni's Multiple Comparison Test and statistically significant changes were represented by asterisks ($P \leq 0.05$ *, $P \leq 0.01$ **, $P \leq 0.0001$ ****). 10 µg/mL of human IgG was used as a control. DMSO was used as a solvent control for ADAM10/17 inhibitor. β-actin was used as a loading control for each gel but only one representative band is shown. Western blots are representative of three independent experiments.

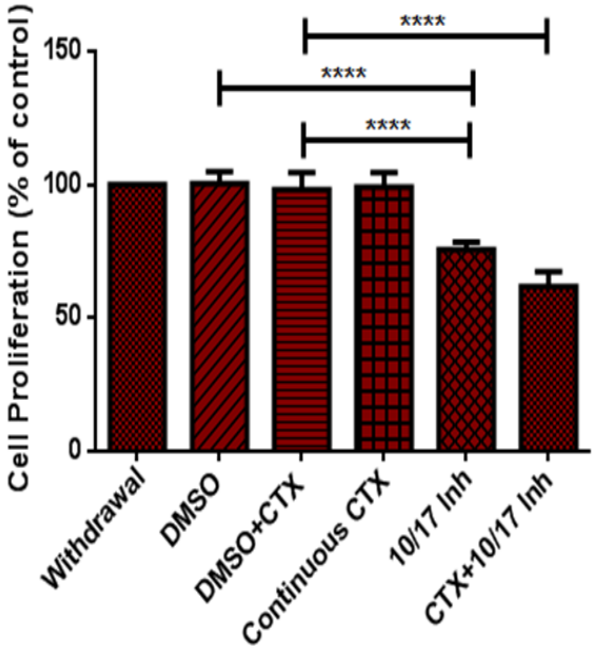
4.2.5.2.2. ADAM10/17 inhibition led to a significant inhibition of HER receptors in cetuximab resistant cell lines

In SCC15 and SCC9 cetuximab resistant cell lines a marked inhibition of all HER receptors as well as their downstream signalling pathways, pAKT and pERK, was observed when cells were treated with a combination of cetuximab and ADAM10/17 inhibitor (figure 4.9A). ADAM 10/17 Inhibitor was also very effective in decreasing the cell proliferation in both resistant cell lines, in the presence or absence of cetuximab (figure 4.9B). A significant decrease in the number of colonies was observed when SCC15 resistant cells were treated with ADAM 10/17 Inhibitor ± continuous cetuximab (figure 4.9C).

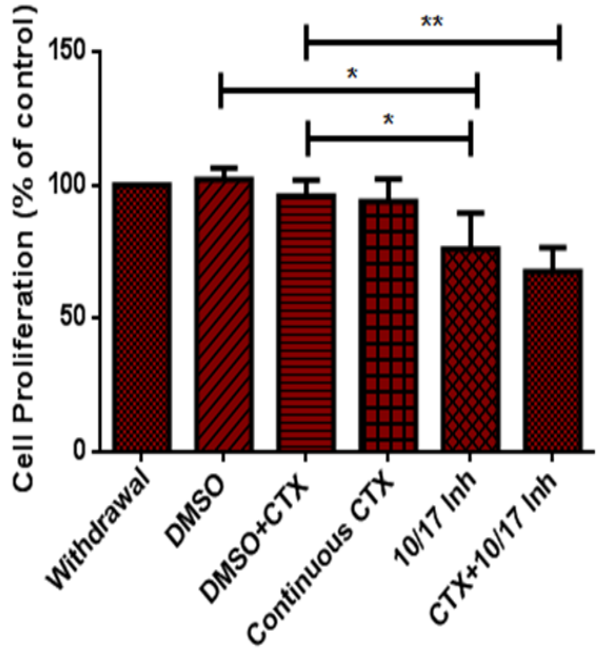
In SCC9 resistant cell line the combination treatment was found to be better than continuous cetuximab in decreasing colony numbers (figure 4.9D).



B

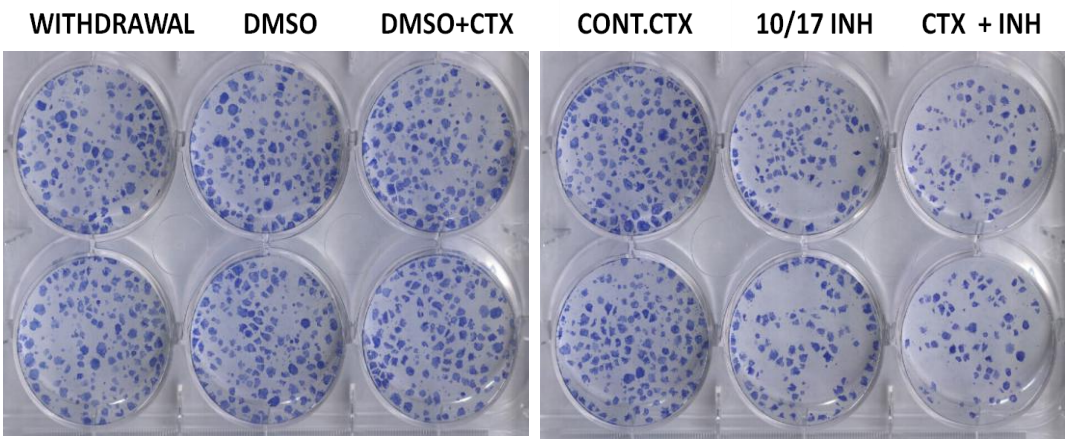


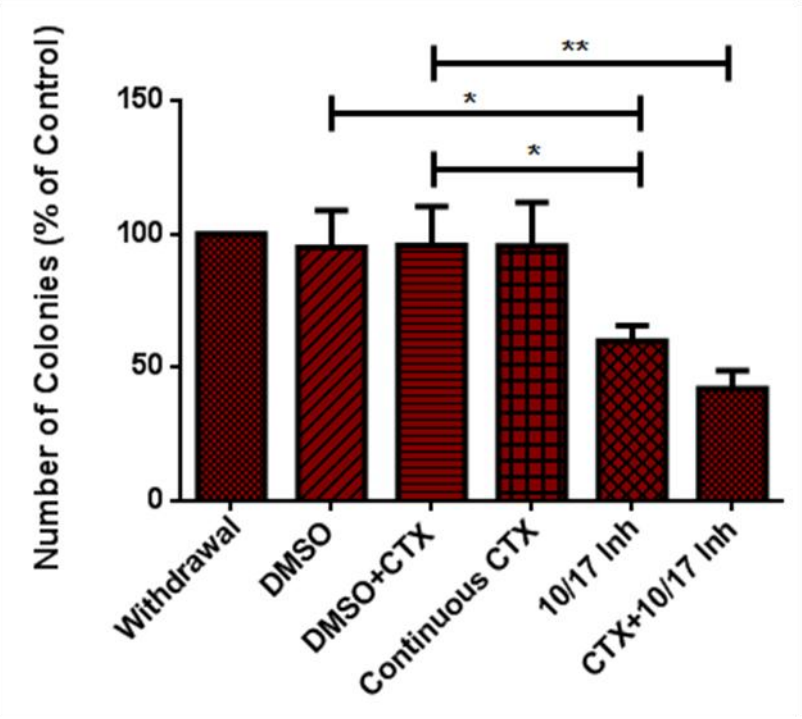
SCC15 RES



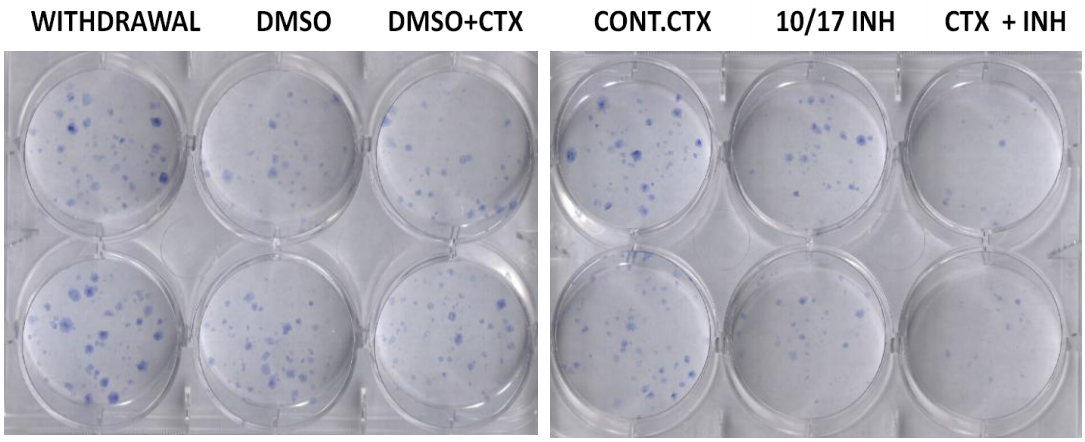
SCC9RES

C SCC 15 RES


WITHDRAWAL
DMSO
DMSO+CTX
CONT.CTX
10/17 INH
CTX + INH


Number of Colonies (% of Control)
150
100
50
0
**
*
*
Withdrawal
DMSO
DMSO+CTX
Continuous CTX
10/17 Inh
CTX+10/17 Inh

D SCC 9 RES


WITHDRAWAL
DMSO
DMSO+CTX
CONT.CTX
10/17 INH
CTX + INH

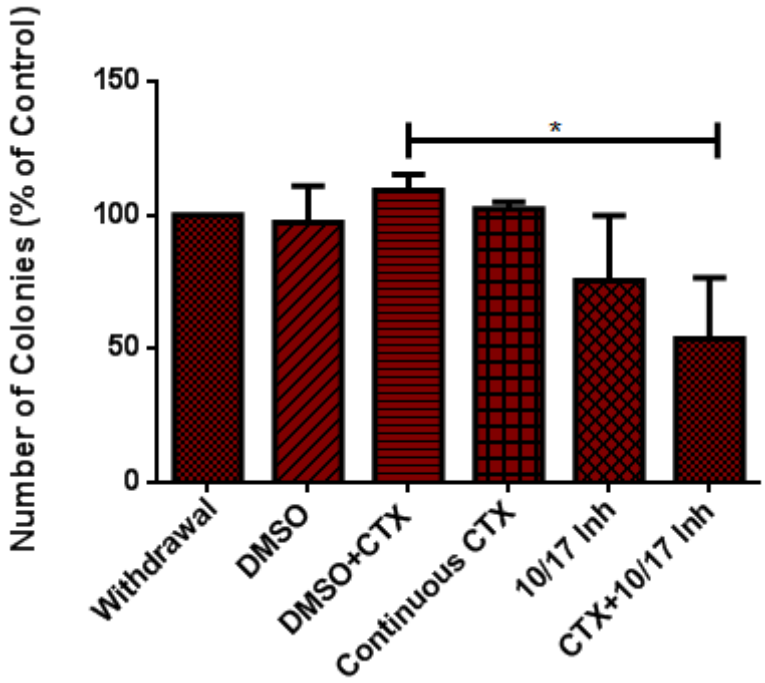

Number of Colonies (% of Control)
150
100
50
0
*
Withdrawal
DMSO
DMSO+CTX
Continuous CTX
10/17 Inh
CTX+10/17 Inh

Figure 4.9: The effect of ADAMs inhibition in cetuximab resistant cell lines

SCC15 and SCC9 resistant cells were treated with 10 μM of ADAM10/17 inhibitor for 72h in the presence or absence of cetuximab. The expression and phosphorylation of the indicated proteins were analysed by western blot analysis (A) and the cell proliferation was assessed by MTT assay (B). For clonogenic assay, the cells were harvested at the end of the treatment and were re-plated in duplicate. The cells were then incubated at 37ºC for 10-14 days to form colonies before being stained with 0.2% methylene blue (C&D). Three independent experiments were performed in duplicate and the cell proliferation and colony numbers were calculated relative to the untreated cells and presented as the mean ± S.D. The means between the groups were analysed by Anova with Bonferroni's Multiple Comparison Test and statistically significant changes were represented by asterisks ($P \leq 0.05$ *, $P \leq 0.01$ **, $P \leq 0.0001$ ****). 10 μg/mL of human IgG was used as a control. DMSO was used as a solvent control for ADAM10/17 inhibitor. β-actin was used as a loading control for each gel but only one representative band is shown. Western blots are representative of three independent experiments.

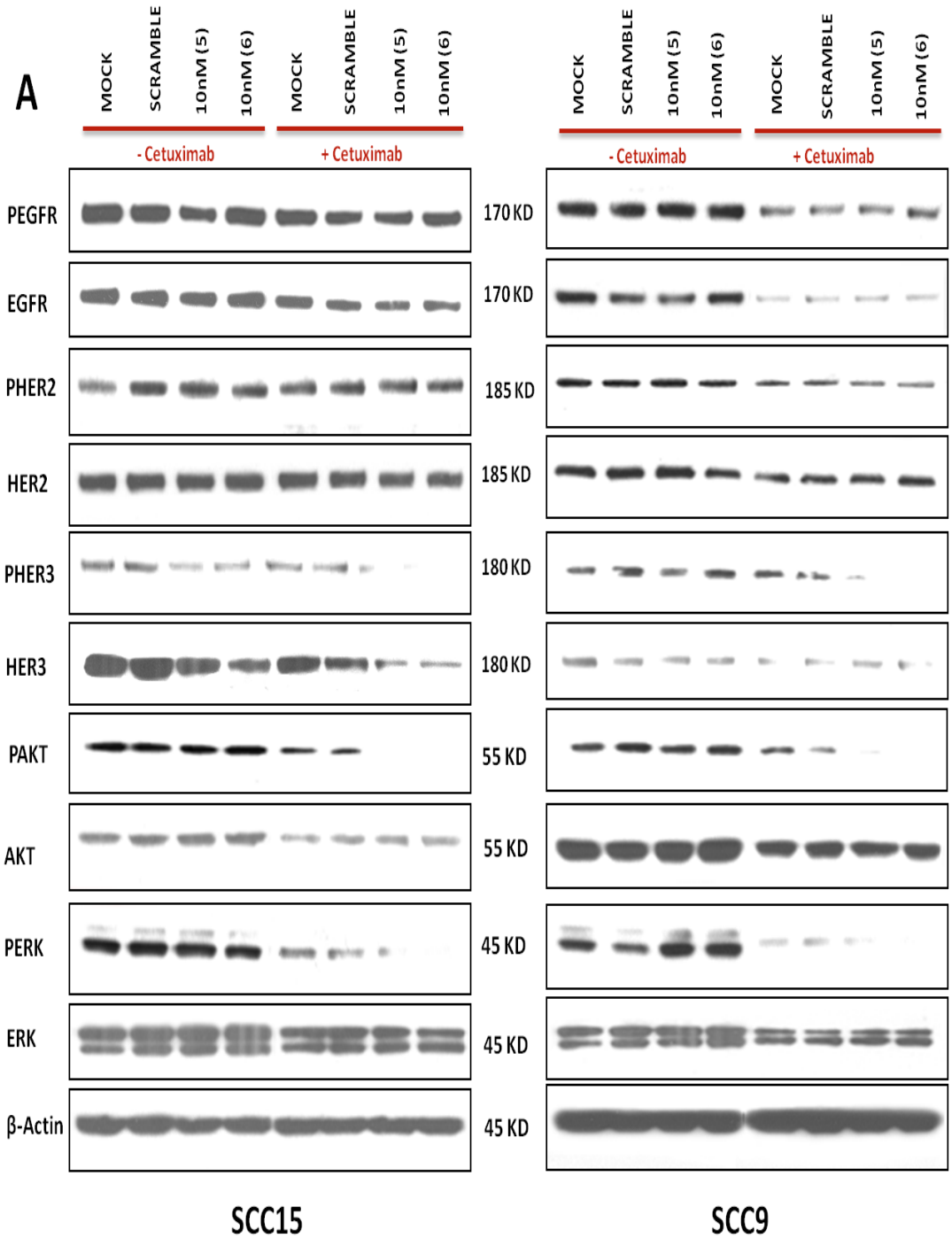
4.2.6. The effect of ADAMs knockdown on HER signalling in HNSCC cell lines

As the inhibitors are not very specific, I also used siRNAs against ADAM10 and 17 in order to investigate their role in relation to HER receptors signalling.

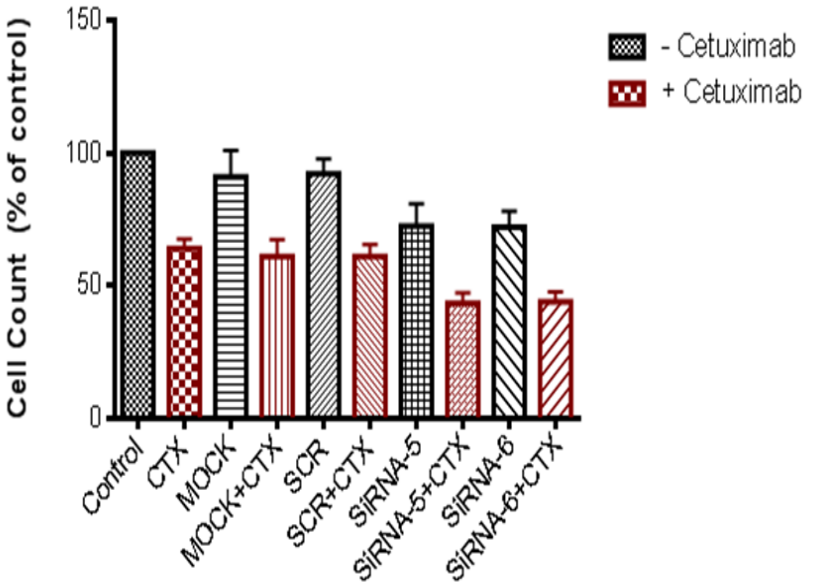
4.2.6.1. The effects of ADAM10 knockdown on HER receptors signalling

4.2.6.1.1. Combination of cetuximab with ADAM10 Knockdown had significant effects in parental cell lines

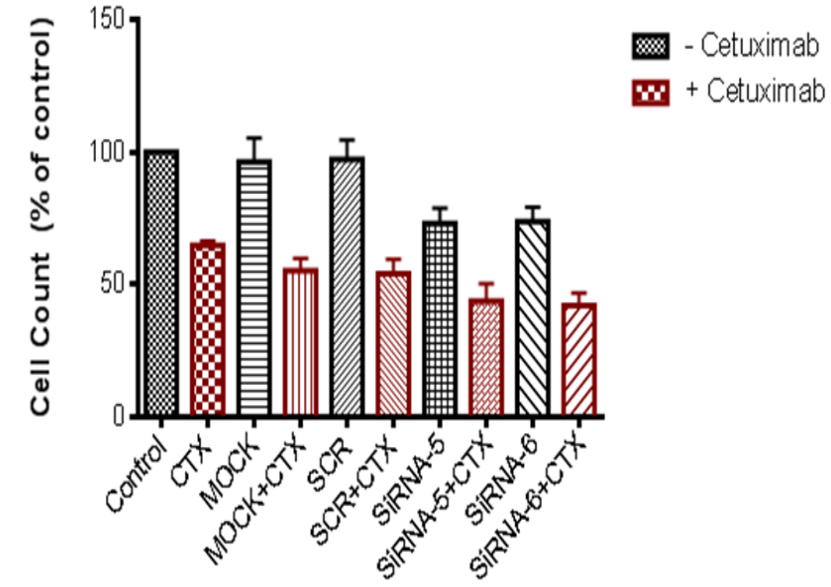
HER receptors' signalling status was analysed after transfection of SCC15 and SCC9 cells by siRNAs against ADAM10 (siRNAs 5&6). The effect of combining siRNAs with cetuximab was also assessed in this study. The results showed that siRNA alone did not have a considerable effect on HER receptor signalling in these cell lines. However, when combined with cetuximab a great inhibition of pHER3, pAKT and pERK was observed (figure 4.10A). The effect of knockdown on the cell number was also tested in the parental cell lines. ADAM10 knockdown reduced the cell number up to 30% compared to the scramble control. Up to 60% reduction in the cell number was observed when the cells were treated with the combination of cetuximab and ADAM10 siRNAs knockdown. However, the combination treatment did not seem to be statistically significant compared to the cetuximab or siRNA alone (figure 4.10B). Both cetuximab and ADAM10 siRNAs resulted in a significant reduction in the number of colonies in both cell lines. However, the combination treatment had a significantly greater effect compared to cetuximab alone or ADAM10 knockdown alone (figure 4.10C&D).


A
MOCK
SCRAMBLE
10nM(5)
10nM(6)
MOCK
SCRAMBLE
10nM(5)
10nM(6)
- Cetuximab
+ Cetuximab
MOCK
SCRAMBLE
10nM(5)
10nM(6)
MOCK
SCRAMBLE
10nM(5)
10nM(6)
- Cetuximab
+ Cetuximab
PEGFR
EGFR
PHER2
HER2
PHER3
HER3
PAKT
AKT
PERK
ERK
β-Actin
170 KD
170 KD
185 KD
185 KD
180 KD
180 KD
55 KD
55 KD
45 KD
45 KD
45 KD
SCC15
SCC9

B

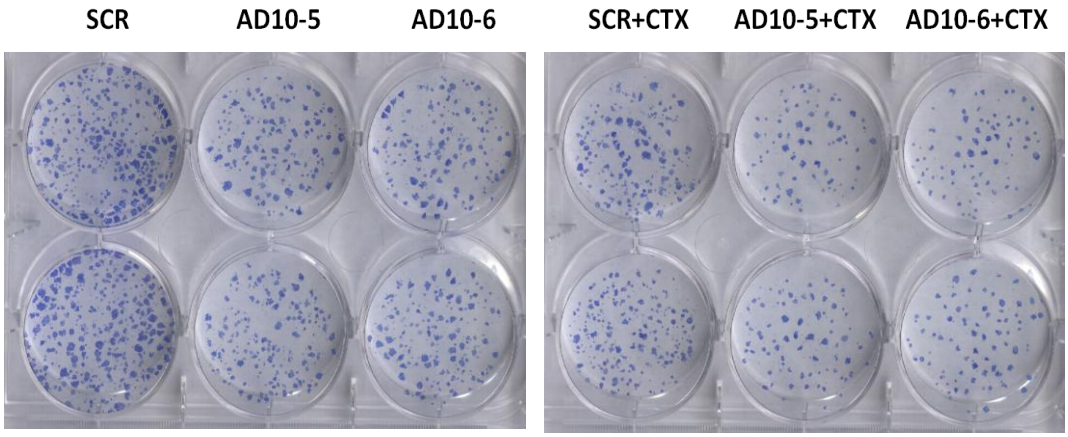

Cell Count (% of control)
150
100
50
0
- Cetuximab
+ Cetuximab
Control
CTX
MOCK
MOCK+CTX
SCR
SCR+CTX
SiRNA-5
SiRNA-5+CTX
SiRNA-6
SiRNA-6+CTX

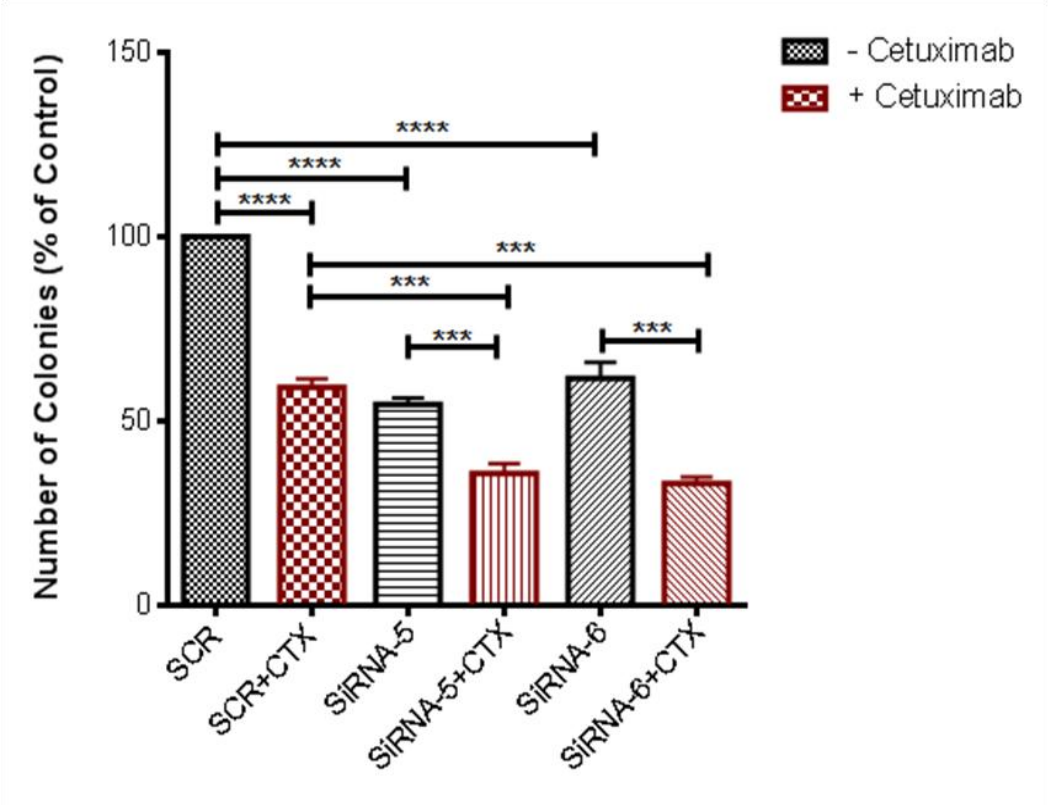
SCC15


Cell Count (% of control)
150
100
50
0
- Cetuximab
+ Cetuximab
Control
CTX
MOCK
MOCK+CTX
SCR
SCR+CTX
SiRNA-5
SiRNA-5+CTX
SiRNA-6
SiRNA-6+CTX

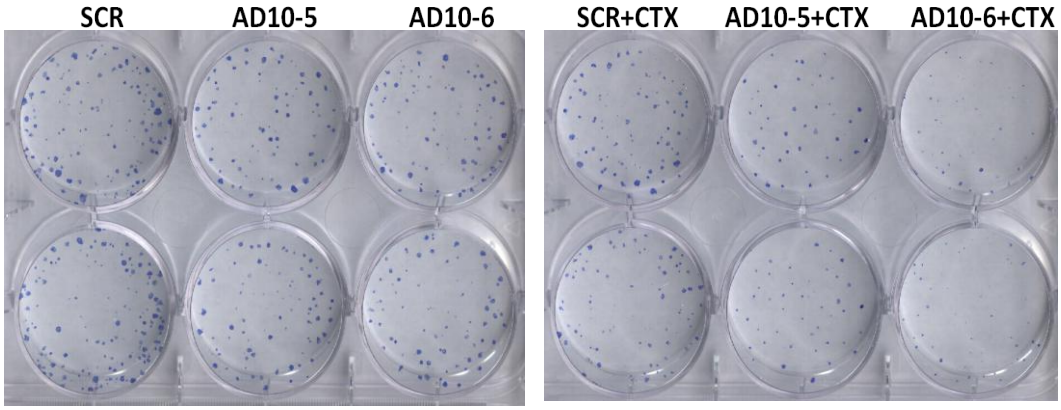
SCC9

C SCC 15


SCR
AD10-5
AD10-6
SCR+CTX
AD10-5+CTX
AD10-6+CTX


- Cetuximab
+ Cetuximab
Number of Colonies (% of Control)
150
100
50
0
****
****
****
***
***
***
***
SCR
SCR+CTX
SiRNA-5
SiRNA-5+CTX
SiRNA-6
SiRNA-6+CTX

D SCC 9


SCR
AD10-5
AD10-6
SCR+CTX
AD10-5+CTX
AD10-6+CTX

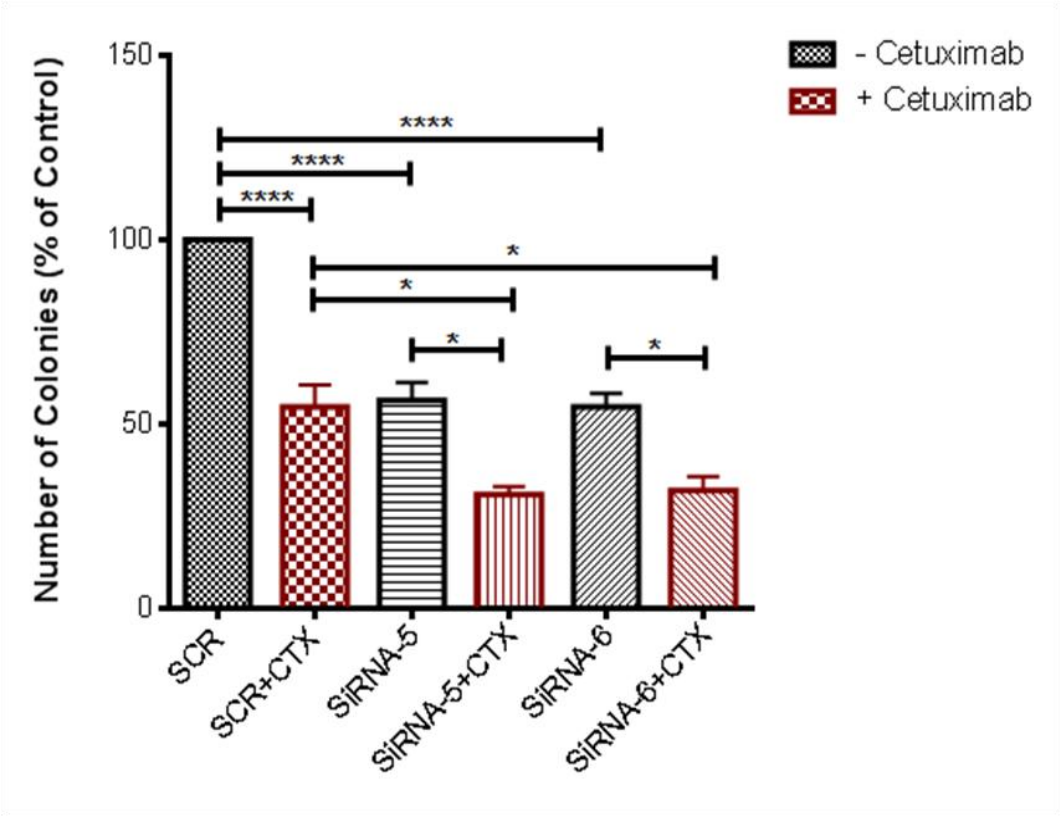
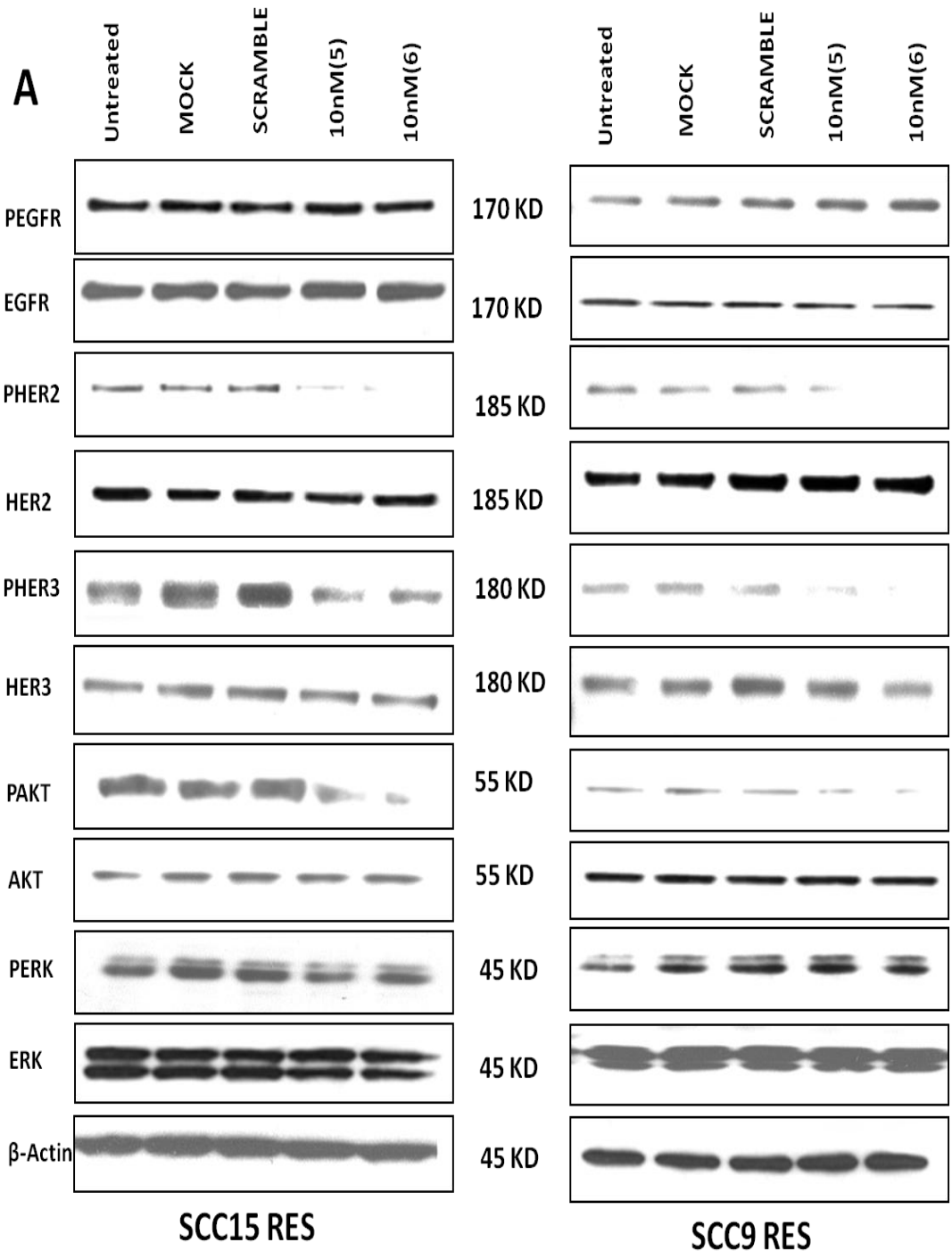

- Cetuximab
+ Cetuximab
Number of Colonies (% of Control)
150
100
50
0
****
****
****
*
*
*
*
SCR
SCR+CTX
SiRNA-5
SiRNA-5+CTX
SiRNA-6
SiRNA-6+CTX

Figure 4.10. The effect of ADAM10 knockdown in parental cell lines

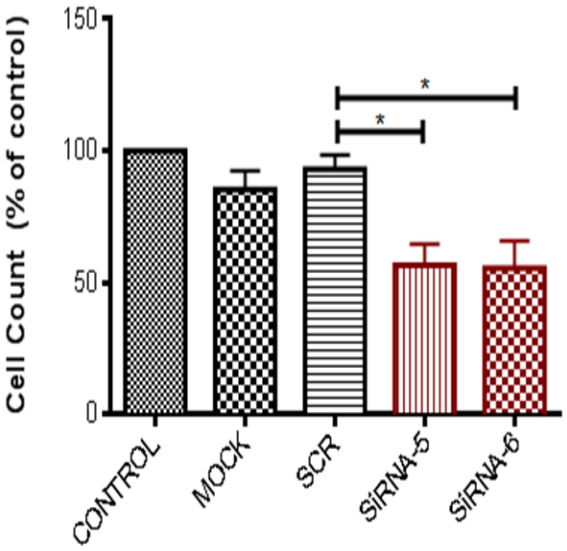
SCC15 and SCC9 cells were treated with either 10 μg/mL of cetuximab, 10 nM of ADAM10 siRNAs (siRNAs 5&6) or their combinations for 72h. At the end of transfection western blot (A) or cell count (B) experiments were performed. For clonogenic assay, the cells were harvested at the end of the transfection and were re-plated in duplicate. The cells were then incubated at 37ºC for 10-14 days to form colonies before being stained with 0.2% methylene blue (C&D). Three independent experiments were performed in duplicate and the percentage of viable cells and colony numbers were calculated relative to the untreated cells and presented as the means ± S.D. The means between the groups were analysed by Anova with Bonferroni's Multiple Comparison Test and statistically significant changes were represented by asterisks ($P \leq 0.05$ *, $P \leq 0.001$ ***, $P \leq 0.0001$ ****). Scrambled siRNA was used as a negative control. Mock-transfected control contained transfection reagent alone. β-actin was used as a loading control for each gel but only one representative band is shown. Western blots are representative of three independent experiments.

4.2.6.1.2. ADAM10 knockdown can overcome resistance to cetuximab

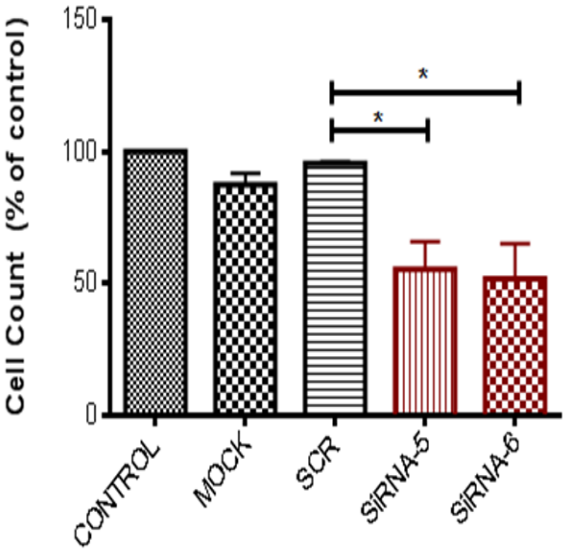
The effect of ADAM10 knockdown on HER receptors signalling status was also investigated in cetuximab resistant cells. Transfection with ADAM10 siRNAs led to a pronounced inhibition of pHER2, pHER3 and pAKT in both resistant cell lines (figure 4.11A). The siRNAs against ADAM10 also had a significant effect in reducing the number of cells (figure 4.11B) and colonies (figure 4.11C&D) in both resistant cell lines (figure 4.11).


A
Untreated
MOCK
SCRAMBLE
10nM(5)
10nM(6)
PEGFR
EGFR
PHER2
HER2
PHER3
HER3
PAKT
AKT
PERK
ERK
β-Actin
170 KD
170 KD
185 KD
185 KD
180 KD
180 KD
55 KD
55 KD
45 KD
45 KD
45 KD
SCC15 RES
SCC9 RES

B

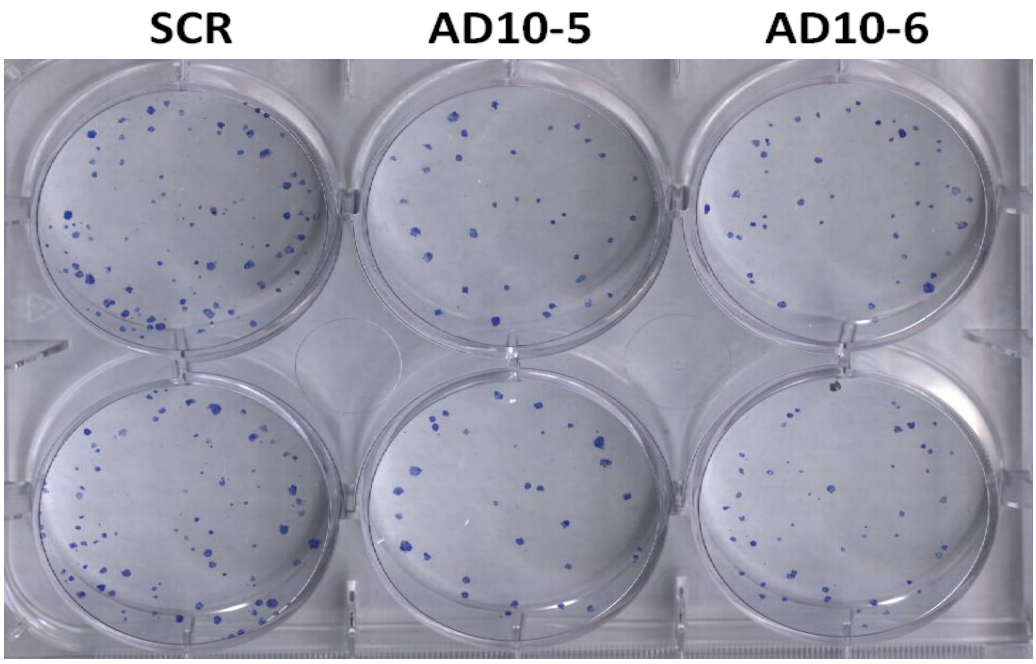


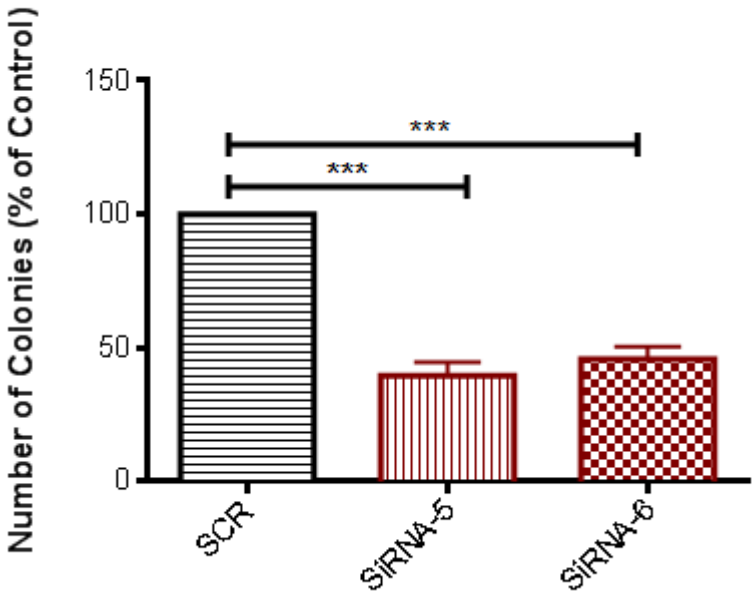
SCC15 Resistant



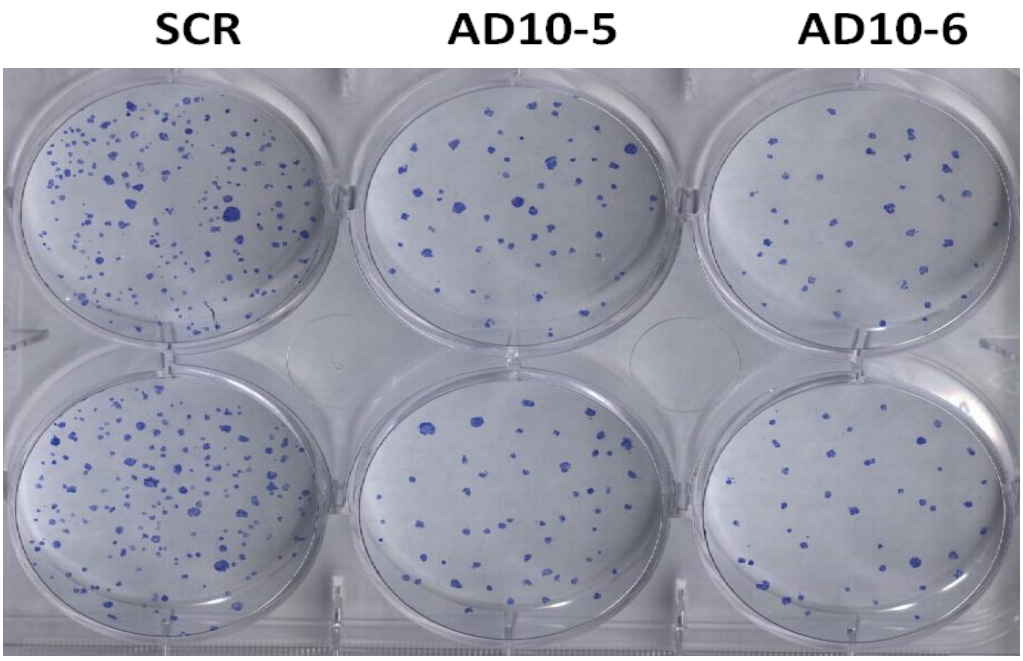
SCC9 Resistant

C SCC 15 RES


SCR
AD10-5
AD10-6


Number of Colonies (% of Control)
150
100
50
0
***
***
SCR
SiRNA-5
SiRNA-6

D SCC 9 RES


SCR
AD10-5
AD10-6

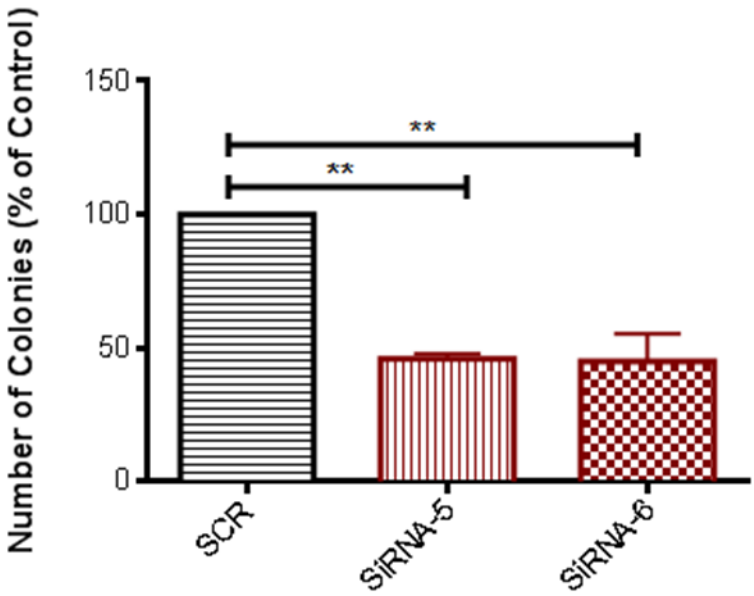

Number of Colonies (% of Control)
150
100
50
0
**
**
SCR
SiRNA-5
SiRNA-6

Figure 4.11. The effect of ADAM10 knockdown in cetuximab resistant cell lines

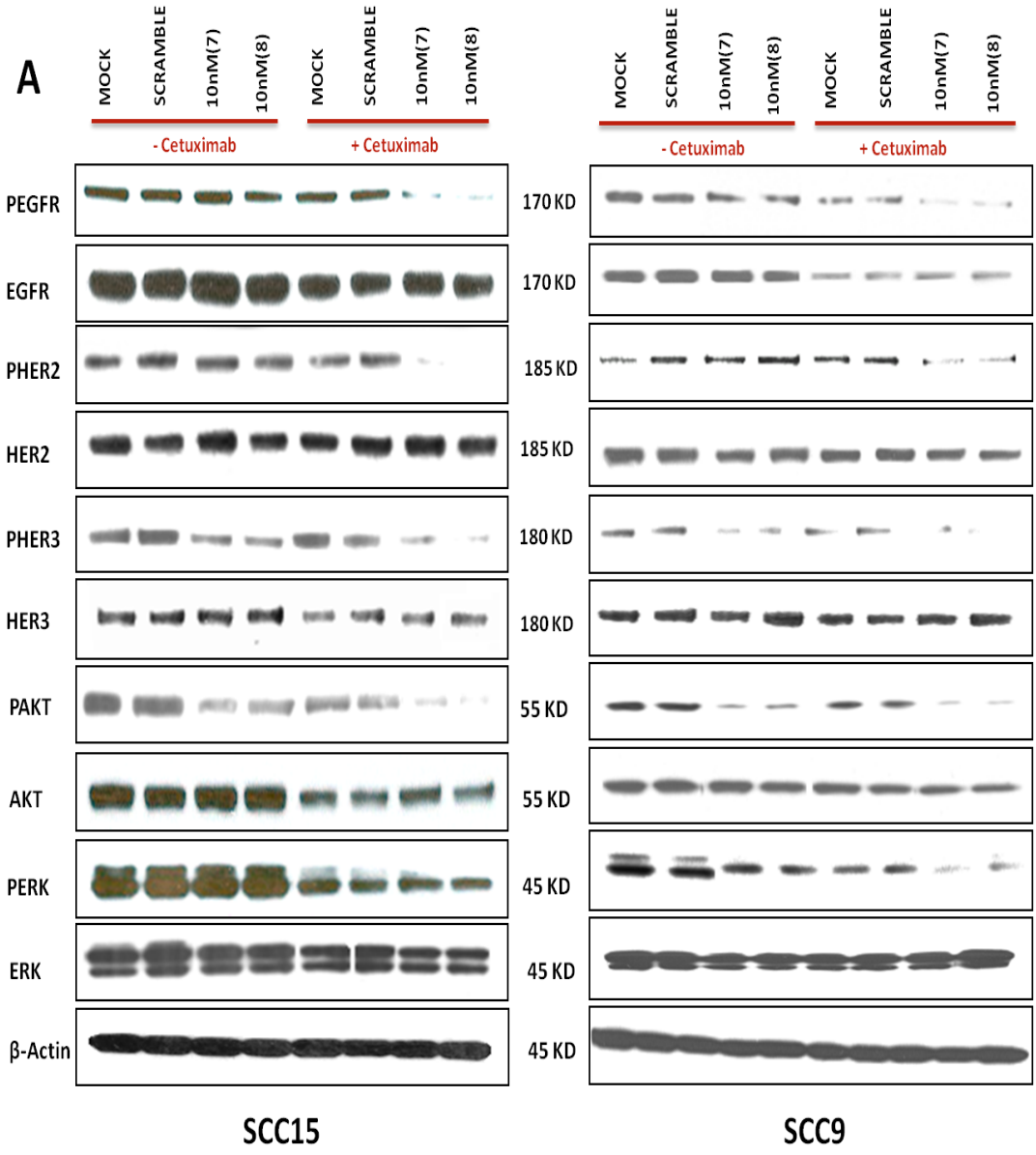
SCC15 and SCC9 cetuximab resistant cells were transfected with 10 nM of siRNAs while continuously being treated with 20 µg/mL of cetuximab. After 72h, western blot (A) or cell count (B) experiments were performed. For clonogenic assay, the cells were harvested at the end of the transfection and were re-plated in duplicate in the absence of cetuximab. The cells were then incubated at 37ºC for 10-14 days to form colonies before being stained with 0.2% methylene blue (C&D). Three independent experiments were performed in duplicate and the percentage of viable cells and colony numbers were calculated relative to the untreated cells and presented as the means ± S.D. The means between the groups were analysed by Anova with Bonferroni's Multiple Comparison Test and statistically significant changes were represented by asterisks ($P \leq 0.05$ *, $P \leq 0.01$ **, $P \leq 0.001$ ***). Scrambled siRNA was used as a negative control. Mock-transfected control contained transfection reagent alone. β-actin was used as a loading control for each gel but only one representative band is shown. Western blots are representative of three independent experiments.

4.2.6.2. The effect of ADAM17 knockdown on HER receptor signalling

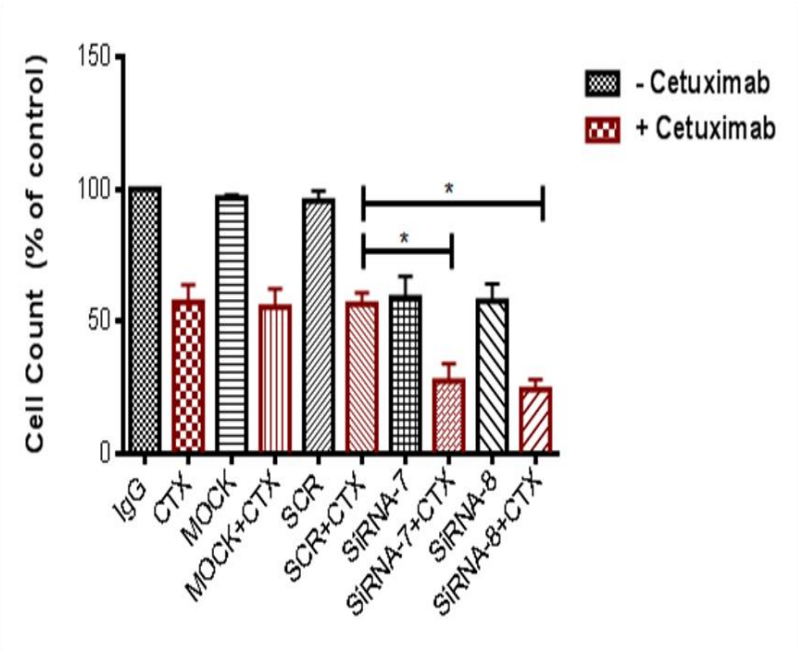
4.2.6.2.1. ADAM17 inhibition seems to have a greater effect on HER receptor signalling compared to its close homolog ADAM10

The effect of ADAM17 knockdown was also assessed by transfecting SCC15 and SCC9 cells with two specific siRNAs against ADAM17. The siRNAs alone led to an inhibition of pHER3 and pAK in both cell lines when compared to the scramble control but the inhibition was more pronounced with the combination of ADAM17 knockdown and cetuximab treatment. In addition, a considerable inhibition of pEGFR, pHER2 and pERK was observed when cetuximab was combined with ADAM17 siRNAs compared to cetuximab or knockdown alone (figure 4.12A). The effect of ADAM17 knockdown on the

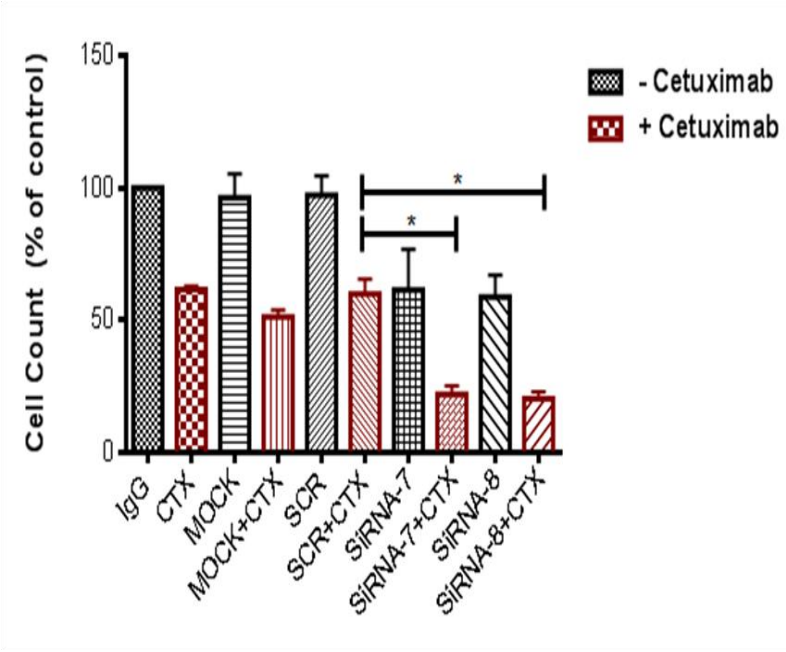
cell number was also assessed in this study. When the cells were transfected with ADAM17 siRNAs, up to 40% reduction in the cell count was achieved. However, the combination treatment had a significant effect in both cell lines by reducing the cell numbers up to 80% (figure 4.12B). In addition, the combination of cetuximab with ADAM17 siRNAs had greater effect in reducing the number of colonies compared to either condition alone (figure 4.12C&D).



B

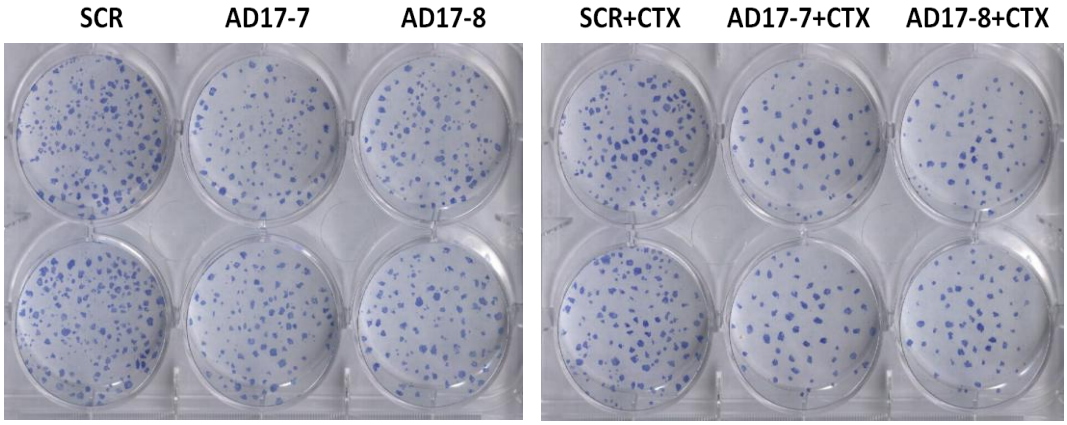

Cell Count (% of control)
150
100
50
0
- Cetuximab
+ Cetuximab
*
*
IgG
CTX
MOCK
MOCK+CTX
SCR
SCR+CTX
SiRNA-7
SiRNA-7+CTX
SiRNA-8
SiRNA-8+CTX

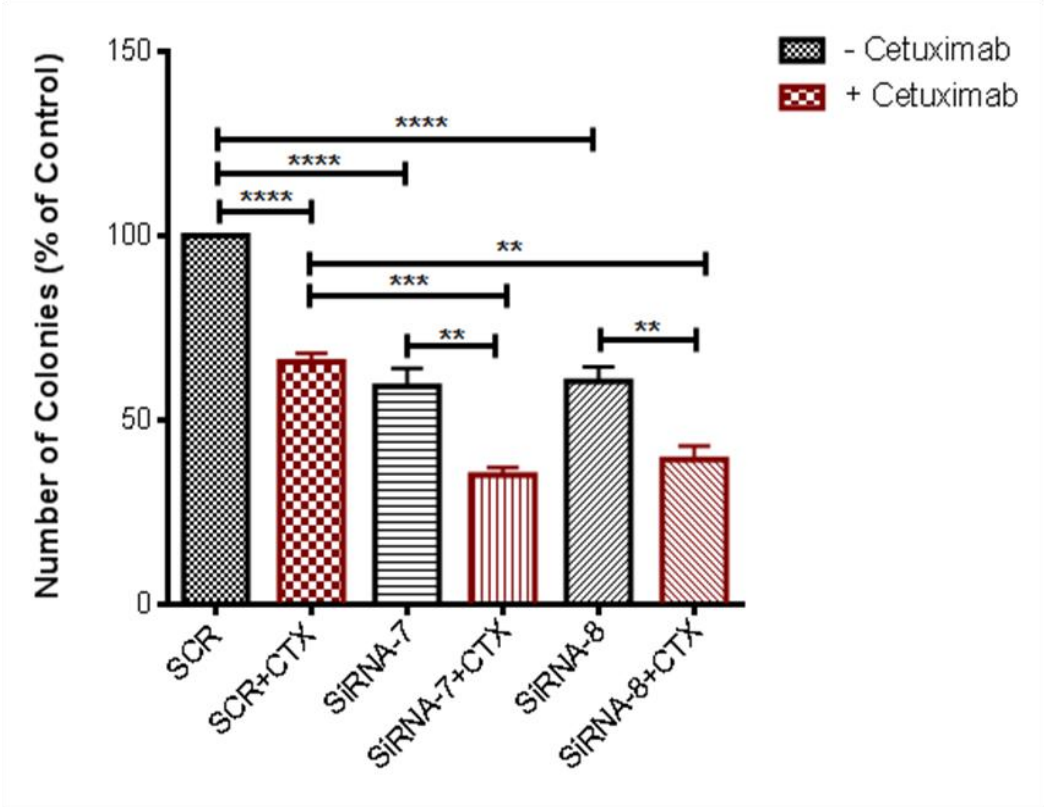
SCC15


Cell Count (% of control)
150
100
50
0
- Cetuximab
+ Cetuximab
*
*
IgG
CTX
MOCK
MOCK+CTX
SCR
SCR+CTX
SiRNA-7
SiRNA-7+CTX
SiRNA-8
SiRNA-8+CTX

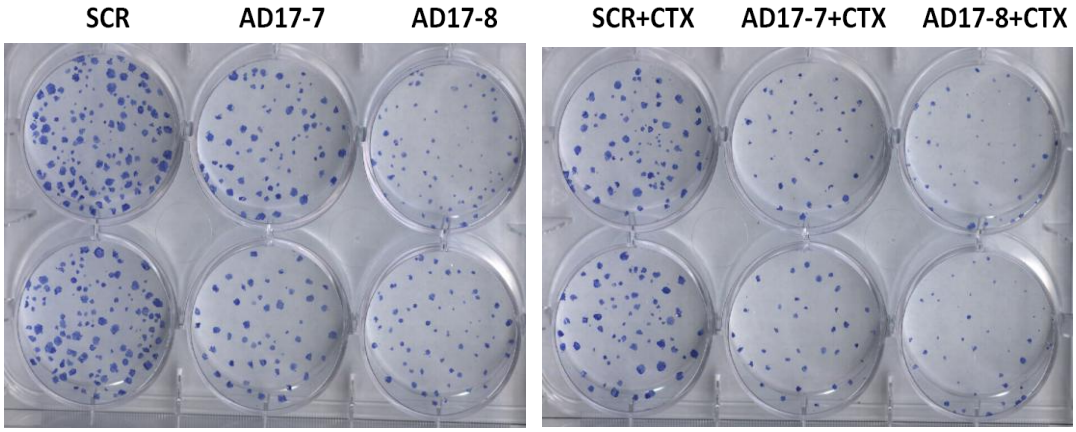
SCC9

C SCC 15


SCR
AD17-7
AD17-8
SCR+CTX
AD17-7+CTX
AD17-8+CTX


- Cetuximab
+ Cetuximab
Number of Colonies (% of Control)
150
100
50
0
****
****
****
**
***
**
**
SCR
SCR+CTX
SiRNA-7
SiRNA-7+CTX
SiRNA-8
SiRNA-8+CTX

D SCC 9


SCR
AD17-7
AD17-8
SCR+CTX
AD17-7+CTX
AD17-8+CTX

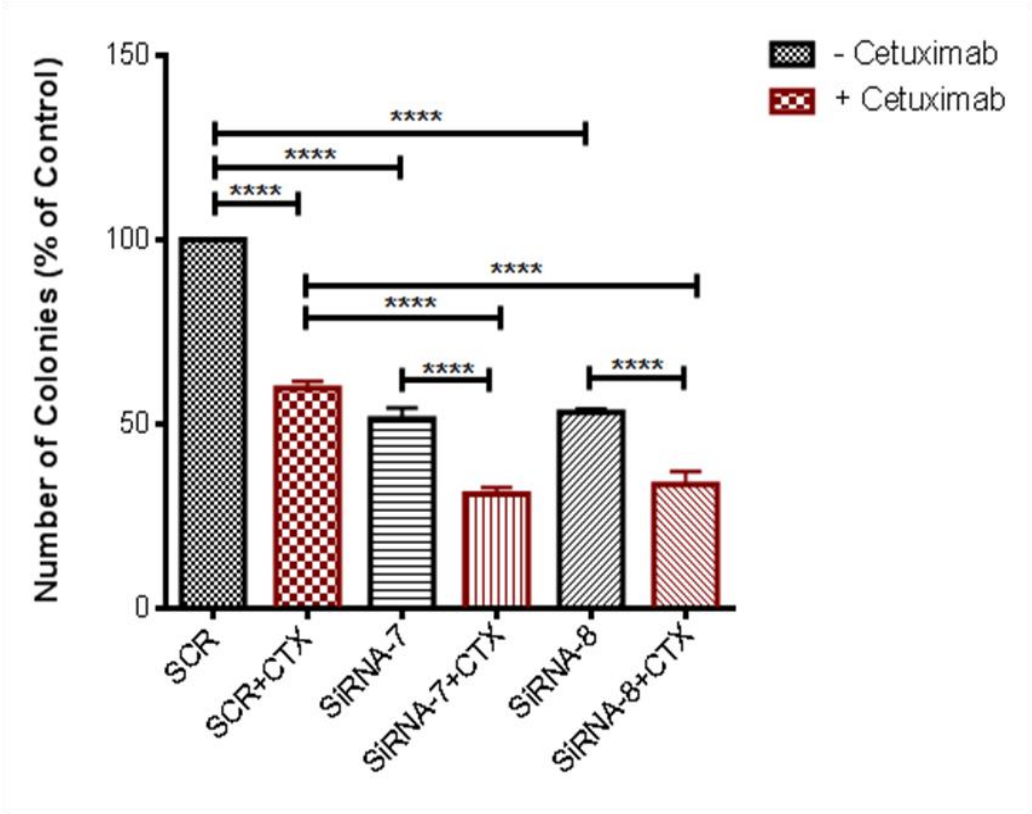
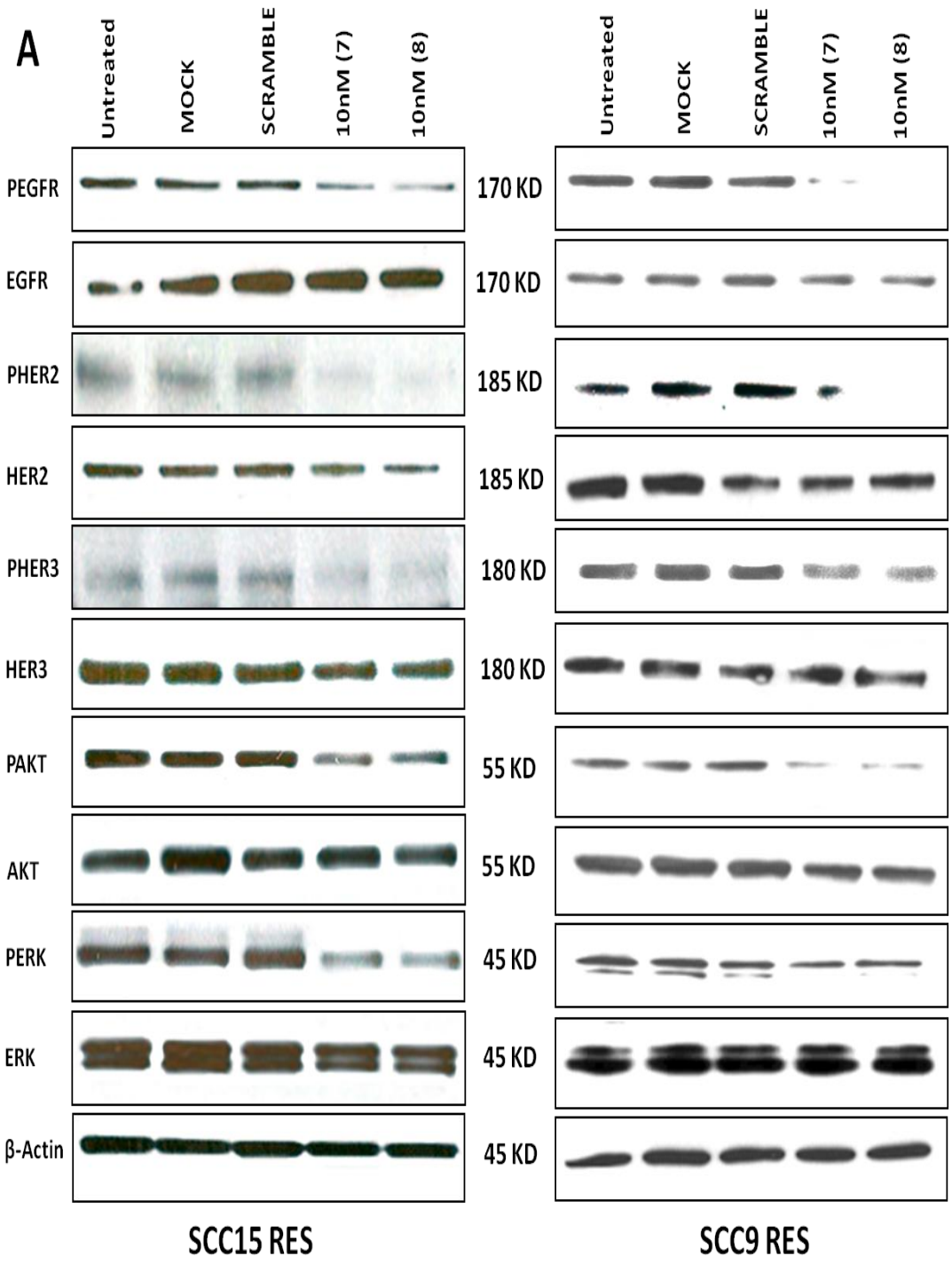

- Cetuximab
+ Cetuximab
Number of Colonies (% of Control)
150
100
50
0
****
****
****
****
****
****
****
SCR
SCR+CTX
SiRNA-7
SiRNA-7+CTX
SiRNA-8
SiRNA-8+CTX

Figure 4.12: The effect of ADAM17 knockdown in parental cell lines

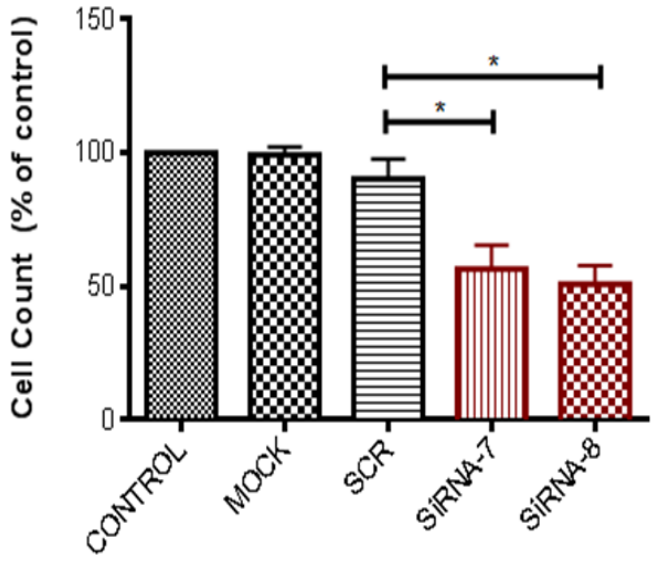
SCC15 and SCC9 cells were treated with either 10 µg/mL of cetuximab, 10 nM of ADAM17 siRNAs (siRNAs 7&8) or their combinations for 72h. At the end of transfection, western blot (A) or cell count (B) experiments were performed. For clonogenic assay, the cells were harvested at the end of the transfection and were re-plated in duplicate. The cells were then incubated at 37ºC for 10-14 days to form colonies before being stained with 0.2% methylene blue (C&D). Three independent experiments were performed in duplicate and the percentage of viable cells and colony numbers were calculated relative to the untreated cells and presented as the means ± S.D. The means between the groups were analysed by Anova with Bonferroni's Multiple Comparison Test and statistically significant changes were represented by asterisks ($P \leq 0.05$ *, $P \leq 0.01$ **, $P \leq 0.001$ ***, $P \leq 0.0001$ ****). Scrambled siRNA was used as a negative control. Mock-transfected control contained transfection reagent alone. β-actin was used as a loading control for each gel but only one representative band is shown. Western blots are representative of three independent experiments.

4.2.6.2.2. ADAM17 knockdown was very effective in overcoming resistance to cetuximab in HNSCC

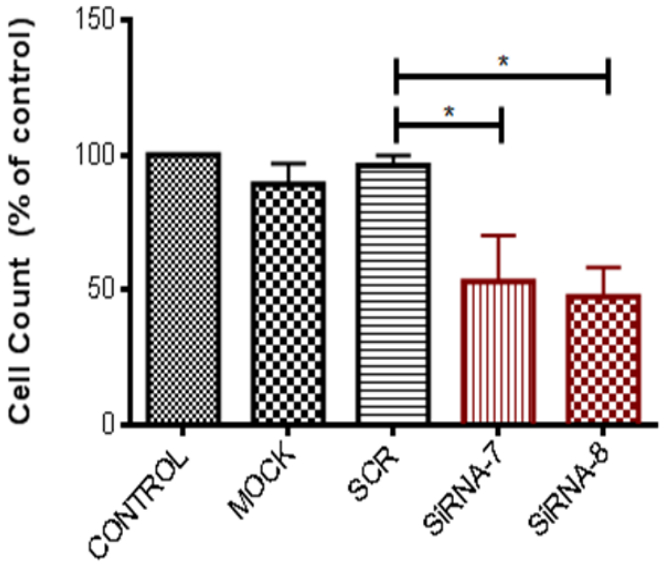
The effect of ADAM17 knockdown on HER receptors' signalling status was further tested in cetuximab resistant cells. Transfection with ADAM17 siRNAs led to the inhibition of all receptors and their downstream signalling pathways in both resistant cell lines (figure 4.13A). Consistent with these results, ADAM17 knockdown resulted in a significant reduction in the number of cells (figure 4.13B) and the number of colonies in resistant cell lines (figure 4.13C&D).


A
Untreated
MOCK
SCRAMBLE
10nM (7)
10nM (8)
PEGFR
EGFR
PHER2
HER2
PHER3
HER3
PAKT
AKT
PERK
ERK
β-Actin
170 KD
170 KD
185 KD
185 KD
180 KD
180 KD
55 KD
55 KD
45 KD
45 KD
45 KD
SCC15 RES
SCC9 RES

B

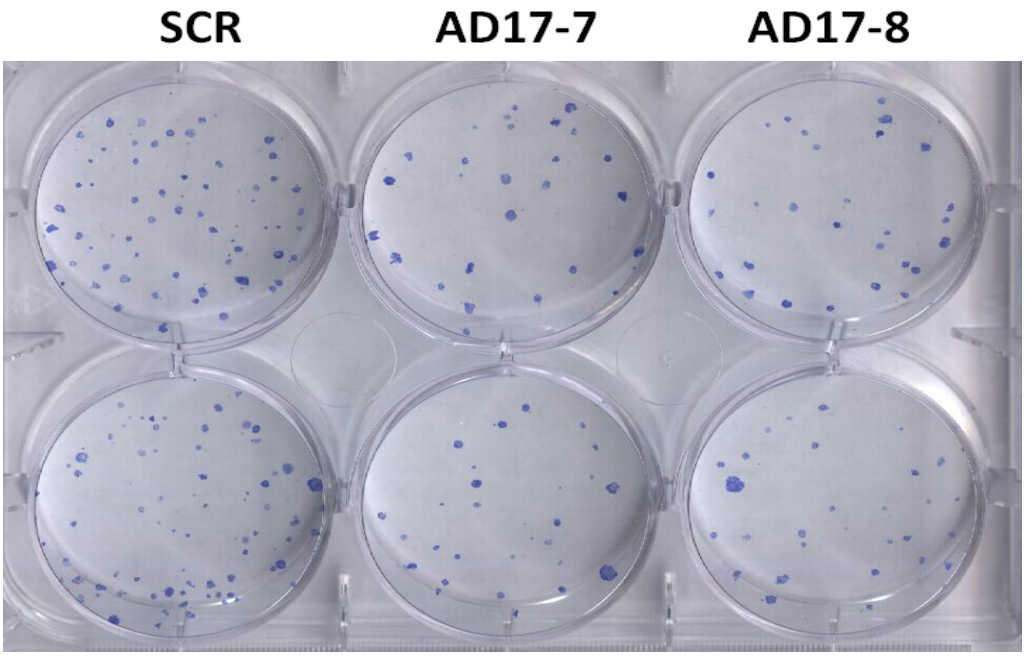


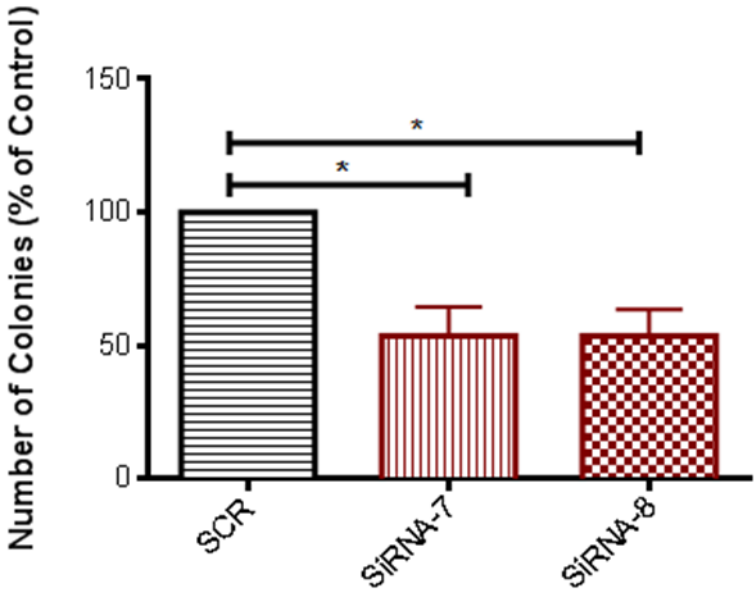
SCC15 Resistant



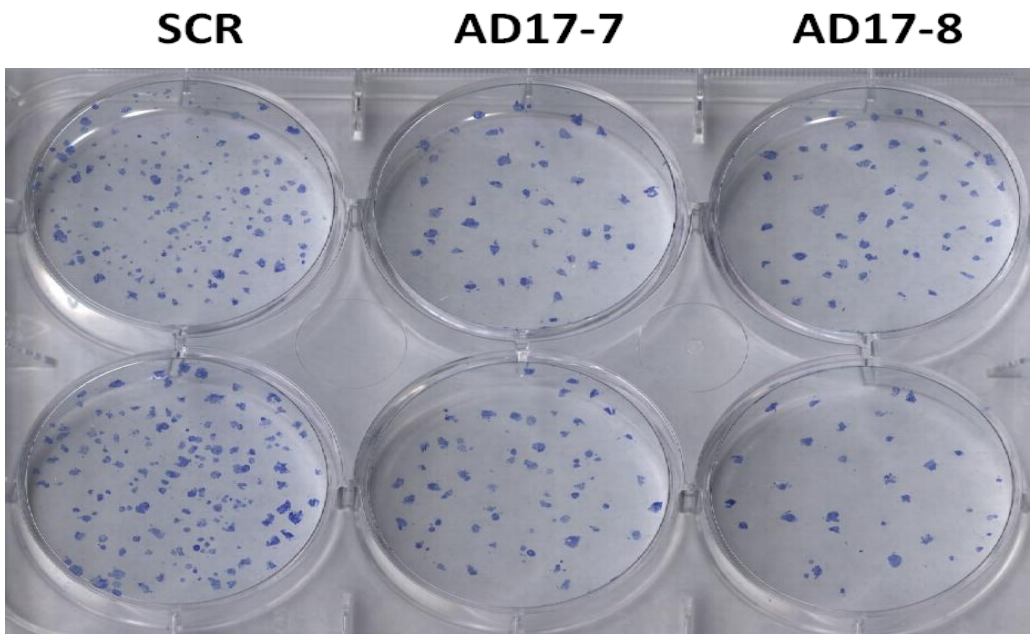
SCC9 Resistant

C SCC 15 RES


SCR
AD17-7
AD17-8


Number of Colonies (% of Control)
150
100
50
0
*
*
SCR
SiRNA-7
SiRNA-8

D SCC 9 RES


SCR
AD17-7
AD17-8

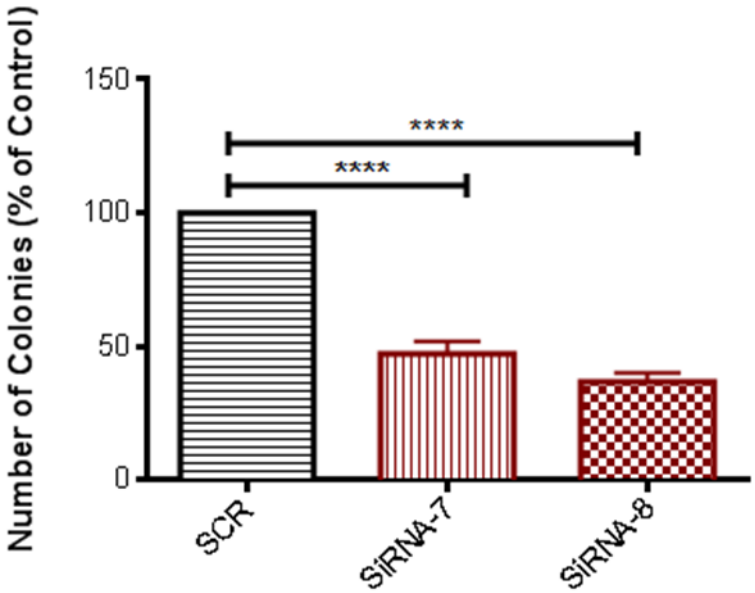

Number of Colonies (% of Control)
150
100
50
0
****
****
SCR
SiRNA-7
SiRNA-8

Figure 4.13: The effect of ADAM17 knockdown in cetuximab resistant cell lines

SCC15 and SCC9 cetuximab resistant cells were transfected with 10 nM of siRNAs while continuously being treated with 20 µg/mL of cetuximab. After 72h, western blot (A) or cell count (B) experiments were performed. For clonogenic assay, the cells were harvested at the end of the transfection and were re-plated in duplicate in the absence of cetuximab. The cells were then incubated at 37ºC for 10-14 days to form colonies before being stained with 0.2% methylene blue (C&D). Three independent experiments were performed in duplicate and the percentage of viable cells and colony numbers were calculated relative to the untreated cells and presented as the mean ± S.D. The means between the groups were analysed by Anova with Bonferroni's Multiple Comparison Test and statistically significant changes were represented by asterisks ($P \leq 0.05$ *, $P \leq 0.0001$ ****). Scrambled siRNA was used as a negative control. Mock-transfected control contained transfection reagent alone. β-actin was used as a loading control for each gel but only one representative band is shown. Western blots are representative of three independent experiments.

4.2. DISCUSSION

The results from this study showed that acute and chronic exposure to cetuximab induces the release of BTC and NRG-1 into the conditioned media in HNSCC cell lines. The upregulation of HER ligands upon treatment with HER-targeted mAbs also have been reported in other types of tumours. Our group has previously shown an increased shedding of NRG-1 and BTC into the media after treatment with trastuzumab in HER2+ breast cancer cell lines (Gijsen *et al.* 2010, Feldinger *et al.* 2014). An induction of TGF-α expression upon treatment with trastuzumab also has been observed in tissue samples of patients with progressive breast cancer (Valabrega *et al.* 2005). The overexpression of TGF-α and subsequent activation of EGFR also has been shown to play a crucial role in the development of resistance to cetuximab in colon cancer cells (Rajput *et al.* 2007). Li *et al.* (2009) evaluated the expression levels of HER family ligands and reported an upregulation of EGF, HB-EGF, BTC and amphiregulin in cetuximab resistant clones of NSCLC (Li *et al.* 2009).

Involvement of HER family ligands in acquired resistance to EGFR targeted TKIs has also been evaluated in a number of studies. Our group has previously shown that acute treatment with gefitinib leads to the inhibition of HER3 and AKT phosphorylation in breast cancer cell lines. However, prolonged gefitinib treatment led to the ligand-dependent reactivation of HER3 and AKT (Kong *et al.* 2008). In another study by Zhou *et al.* (2006), heregulin-induced, HER2-mediated HER3 activation was also shown to be

responsible for gefitinib resistance in NSCLC cells (Zhou *et al.* 2006). By comparing non-malignant lung tissues with normal tissues, this group has also reported an increased ADAM17 protein expression and ADAM10 activation (Zhou *et al.* 2006).

Our group has previously shown that increased expression of ADAM17 (Gijsen *et al.* 2010) and ADAM10 (Feldinger *et al.* 2014) upon treatment with trastuzumab was associated with HER ligands' release (Gijsen *et al.* 2010, Feldinger *et al.* 2014). In my D.Phil. study, cetuximab was shown to be responsible for increased expression levels of ADAM10 and ADAM17 in HNSCC cells. Therefore, the role of ADAM10 and ADAM17 in the release of their substrates was further investigated by using ADAM10 and ADAM17 inhibitors and specific siRNAs. The results showed that ADAM10 and ADAM17 inhibition prevented cetuximab-mediated HER ligands' shedding in the parental and cetuximab resistant cell lines. Furthermore, the effect of ADAM10 and ADAM17 inhibition on HER receptors' signalling was also evaluated. In order to assess the effect of ADAM17 inhibition in the parental cell lines, TAPI-1 which is an inhibitor against ADAM17 and other metalloproteases was used. The effect of increased concentrations of TAPI-1 with fixed concentration of cetuximab was assessed. In SCC15 cell line no difference was detected between the different concentrations of TAPI-1 compared with its combination with cetuximab concerning the effect on HER2, pHER3, HER3, AKT and ERK levels. This was also the case in parental SCC9 cells concerning HER2, AKT and ERK. This might be due to the fact that the concentrations used in these experiments were too high to show the

difference. In SCC15 resistant cells treatment with TAPI-1 in the presence of cetuximab was more effective compared with TAPI-1 alone. This was also observed in SCC9 resistant cells with the exception of pHER3, where highest concentration of TAPI-1 produced the same results in the presence or absence of cetuximab. These results suggest that continuing cetuximab upon resistance in combination with ADAM10/17 inhibition was more effective than withdrawing cetuximab. In breast cancer continued trastuzumab treatment after resistance in combination with lapatinib or capecitabine significantly improved the overall response compared with lapatinib or capecitabine monotherapy (Blackwell *et al.* 2012, von Minckwitz *et al.* 2012).

Due to the lack of specific inhibitor against ADAM10, dual ADAM10/17 inhibitor was used to assess the effects of ADAM10 inhibition on HER receptors signalling. The results from the western blot as well as the MTT and clonogenic assays all demonstrated the additive effects of ADAM10/17 inhibitor in combination with cetuximab on targeting HER signalling in parental cell lines. ADAM10/17 inhibition also reversed cetuximab resistance in HNSCC cell lines. As ADAM inhibitors are not very specific, their inhibition using specific siRNAs were also assessed in this study. In the parental cell lines, the combination of cetuximab with ADAMs siRNAs had additive effects compared to single agent. ADAM10 and ADAM17 knockdown also could overcome cetuximab resistance in HNSCC.

The significance of ADAM10 and 17 inhibitions in enhancing response to HER-targeted therapies has been shown in other malignancies. Our group showed that the combination of trastuzumab with TAPI-1, ADAM17 inhibitor INCB4298 or ADAM10/17 inhibitor INCB3619 decreased HER2 phosphorylation and had additive effect on inhibition of cell viability in breast cancer cells (Gijsen *et al.* 2010). ADAM10 inhibitor INCB8765 also was shown to enhance response to trastuzumab in HER2-positive breast cancer cell lines (Feldinger *et al.* 2014). Zhou *et al.* (2006) showed that targeting ADAMs by INCB3619 inhibitor and thus inhibiting ligand release restored gefitinib sensitivity in NSCLC cell lines. In addition, INCB3619 sensitized A549 cells to paclitaxel and had anti-tumour activity in the A549 xenograft model which was more significant in combination with paclitaxel (Zhou *et al.* 2006). The therapeutic effect of ADAM17 inhibition has also been shown in triple negative breast cancer (McGowan *et al.* 2013). This group showed that inhibition of ADAM17 reduced EGFR ligand release, decreased the level of pEGFR and inhibited cell proliferation. In addition, pre-treatment with ADAM17 inhibitor increased response to afatinib, gefitinib, neratinib, carboplatin and doxorubicin in triple negative breast cancer (McGowan *et al.* 2013).

In summary, Short-term cetuximab treatment induces an upregulation of ADAM10 and ADAM17 and HER ligands' release in HNSCC cells. Furthermore, higher levels of ADAM10 and ADAM17 as well as their substrates, BTC and NRG-1, are found in the resistant cells compared to their parental cells, suggesting that cetuximab-induced ADAM-mediated ligands'

release is a potential mechanism of resistance to cetuximab in HNSCC. Inhibition or knockdown of ADAM10 and ADAM17 enhance cetuximab response and reverse cetuximab resistance in HNSCC cells. Therefore, the significance of ADAM10 and ADAM17 as prognostics and predictive biomarkers in HNSCC patients was further investigated and will be presented in chapter 6.

CHAPTER 5

The effect of EGFR tyrosine kinase inhibitors in HNSCC cell lines

5.1. INTRODUCTION

As previously described, cetuximab-induced autocrine ligand release led to the activation of EGFR, HER2 and HER3 in HNSCC cell lines tested. This could explain the limited efficacy of cetuximab as an EGFR targeted mAb in inhibition of its target. One approach to overcome this limitation is to block ADAM-mediated ligand release by specific inhibitors or use siRNAs against ADAMs as shown in chapter 4. One approch which might improve responce to cetuximab and overcome its limitation is to combine cetuximab with an EGFR inhibitor which targets its tyrosine kinase domain. Another approach which might have synergistic/additive effect in parental and cetuximab resistant cell lines is to combine cetuximab with a panHER inhibitor which targets all HER receptors.

Among the various number of small molecule TKIs against EGFR, gefitinib (Iressa), a reversible TKI, is the first EGFR-targeted agent which received approval from FDA for the treatment of NSCLC patients. The activity and tolerability of gefitinib monotherapy has been tested in HNSCC, however, the results from phase II trials demonstrated the limited efficacy of gefitinib in HNSCC. Therefore there has been a great interest in assessing the efficacy of combining TKIs with mAbs. In this project the effect of combining cetuximab with gefitinib was investigated in HNSCC cell lines.

In recent years panHER inhibitors such as neratinib, dacomitinib and afatinib has attracted a lot of attention in cancer treatment. These are second

generation irreversible inhibitors that target the entire HER family receptors. Among these inhibitors afatinib received approval from FDA as a first-line treatment for metastatic NSCLC patients with EGFR mutations. Results from phase II clinical trials in HNSCC demonstrated the clinical activity of afatinib as a single agent in a group of patients with refractory/metastatic HNSCC (Ferrarotto & Gold 2014). Based on the promising results from these studies, afatinib is currently undergoing phase III clinical trials for the treatment of locally advanced, recurrent or metastatic HNSCC (NCT01345682 & NCT01345669, clinicaltrials.gov). Therefore, in this project the efficacy of afatinib in overcoming acquired resistance to cetuximab was assessed in HNSCC.

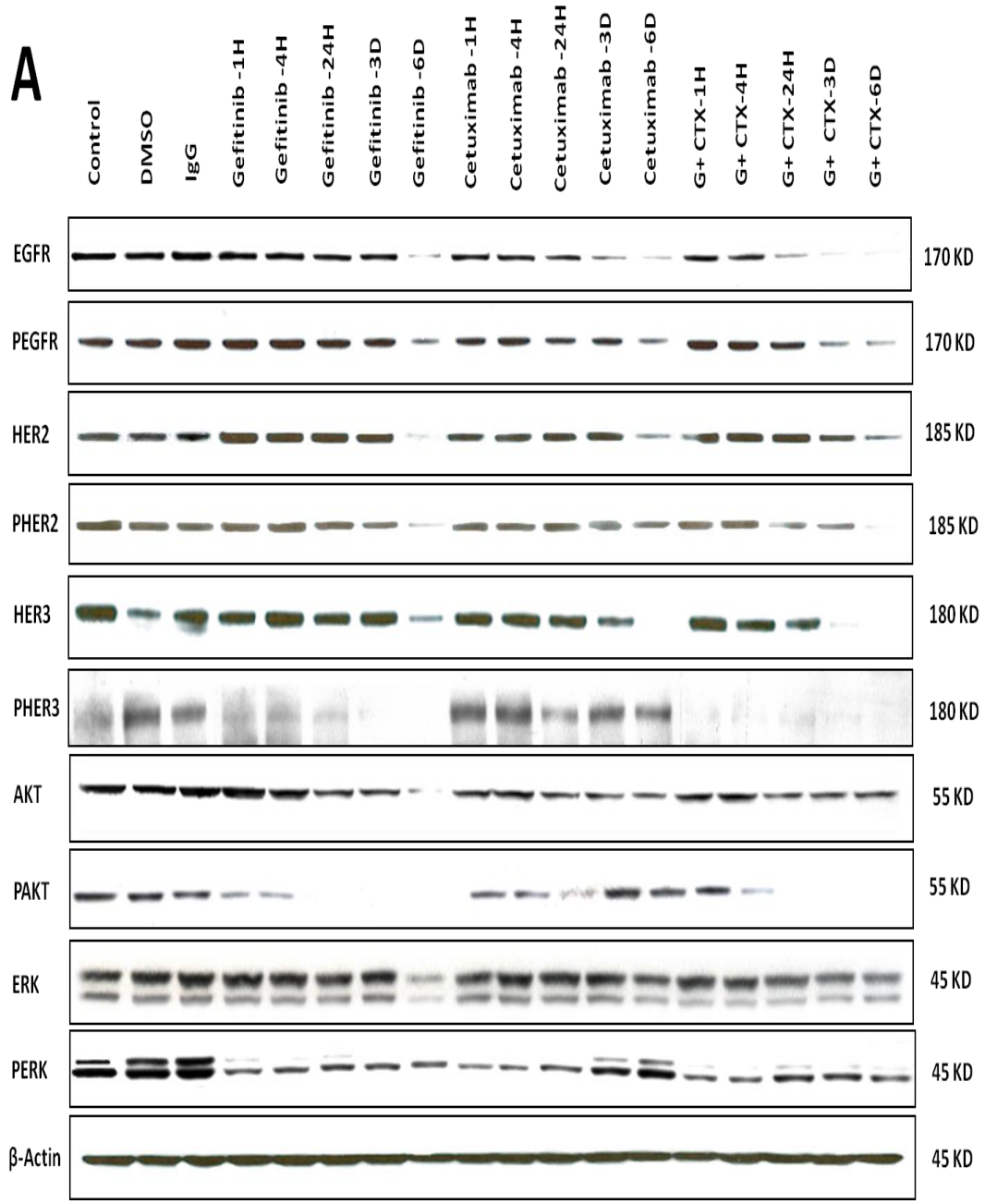
5.2. RESULTS

5.2.1. Gefitinib

5.2.1.1. Gefitinib was very effective in targeting HER receptors signalling in sensitive HNSCC cell line

HER receptors' signalling was investigated in SCC15 cells upon treatment with gefitinib and its combination with cetuximab. As a monotherapy cetuximab and gefitinib downregulated EGFR after 3 and 6 days of treatment respectively. However, downregulation of the EGFR occurred earlier at 24h in combination treatment. This was also the case for pEGFR since a decrease was observed at 3 days for combination treatment instead of 6 days for either single agent alone. Gefitinib, cetuximab or their combination downregulated HER2 at 6 days but the greatest decrease in pHER2 was seen in the combination treatment. Compared to DMSO control, gefitinib decreased pHER3 and pAKT after 1h treatment which was sustained after 6 days. For cetuximab, the inhibition of pHER3 and pAKT was observed at 24h but their reactivation occurred at 3 days. When the cells were treated with the combination of both drugs, this was not the case and a greater inhibition was achieved than either drug alone. Gefitinib monotherapy was more effective in downregulation of AKT and ERK at 6 day and was effective in inhibiting pERK after 1h treatment. A decrease of pERK after 1h treatment with cetuximab was also observed although its reactivation occurred after 3 days of treatment, which was to a lesser degree in the combination treatment (figure 5.1A). The effect of gefitinib, cetuximab and their combination on the cell number was also assessed in the parental HNSCC cells. Treatment with

gefitinib led to a greater inhibition of the cell number compared to cetuximab, although the effect of combination treatment was not more than gefitinib as a single agent (figure 5.1B).



B

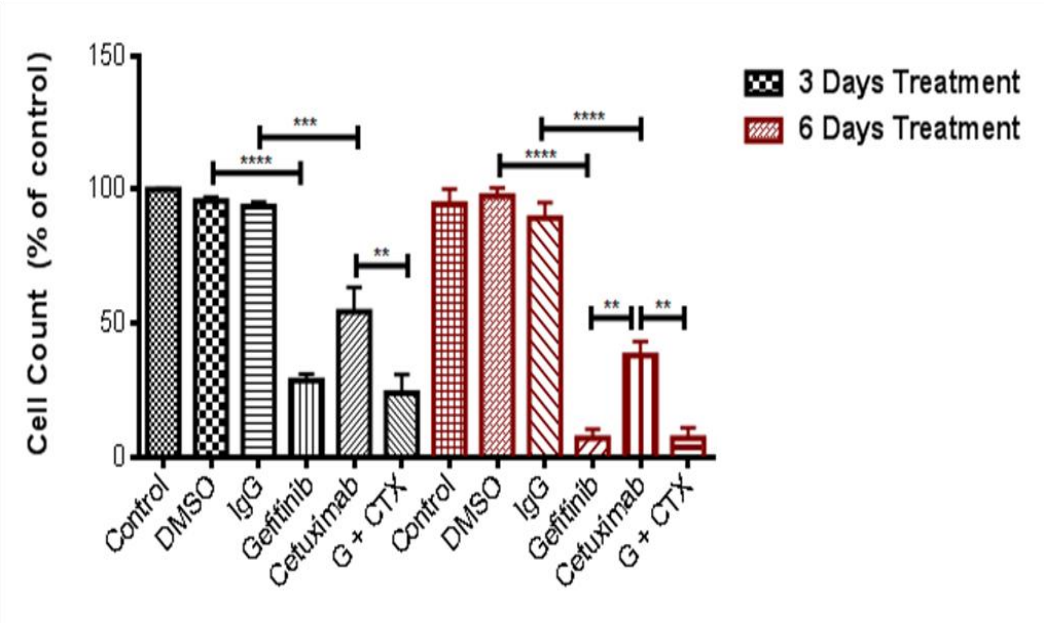
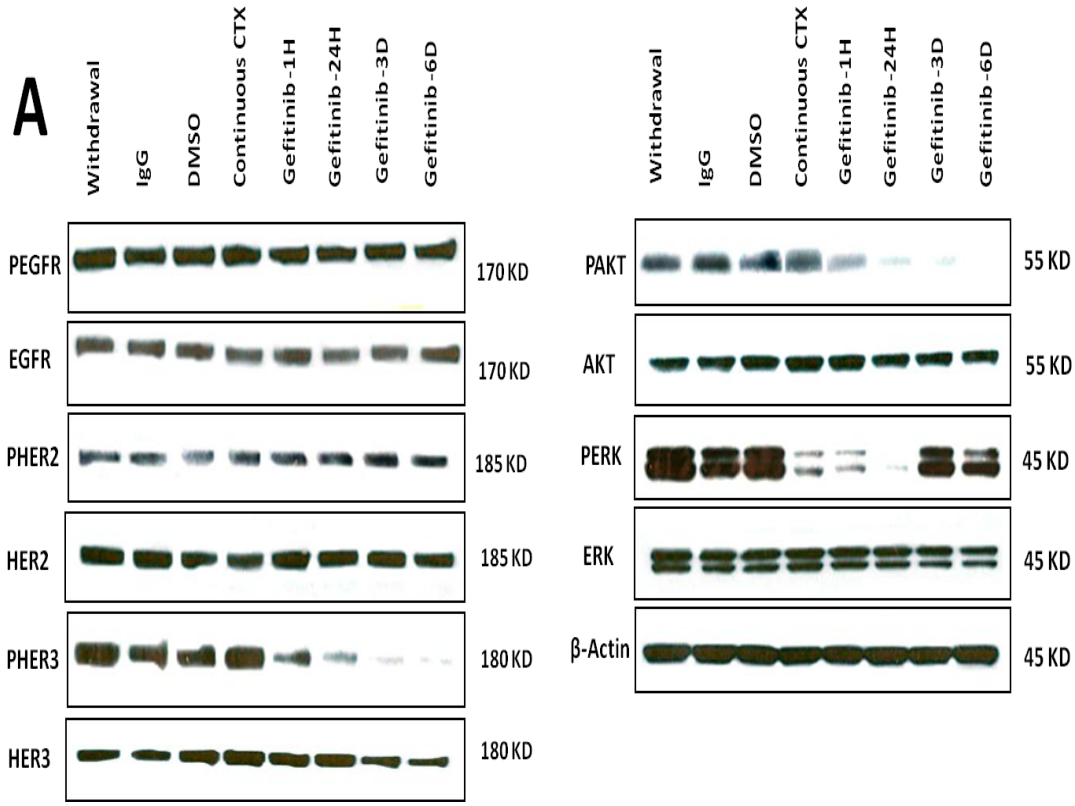


Figure 5.1. The effect of gefitinib with or without cetuximab on HER receptors signalling and cell count in sensitive SCC15 cell line

SCC15 cells were treated either with 1 μM of gefitinib, 10 μg/mL of cetuximab or their combinations for 1h, 4h, 24h, 3D and 6D for western blot analysis (A) and 3 and 6 days for cell counting experiment (B). Three independent cell counting experiments were performed in triplicate and the percentage of viable cells were calculated relative to the untreated cells and presented as the mean ± S.D. The means between the groups were analysed by Anova with Bonferroni's Multiple Comparison Test and statistically significant changes were represented by asterisks ($P \leq 0.01$ **, $P \leq 0.001$ ***, $P \leq 0.0001$ ****). 10 μg/mL of human IgG was used as a control. DMSO was used as a vehicle control for gefitinib. β-actin was used as a loading control for each gel but only one representative band is shown. Western blots are representative of three independent experiments.

5.2.1.2. Gefitinib significantly inhibited the activation of HER receptors signalling in cetuximab resistant HNSCC cell line

Gefitinib decreased pHER3 and pAKT in cetuximab resistant SCC15 cells but not pEGFR and pHER2. Inhibition of pERK was also observed at 24h, however, its reactivation occurred after 3 days (figure 5.2A). The results from the cell count experiments showed a statistically significant reduction in the cell number, which is consistent with effects on HER receptor and downstream signalling pathways (figure 5.2B).



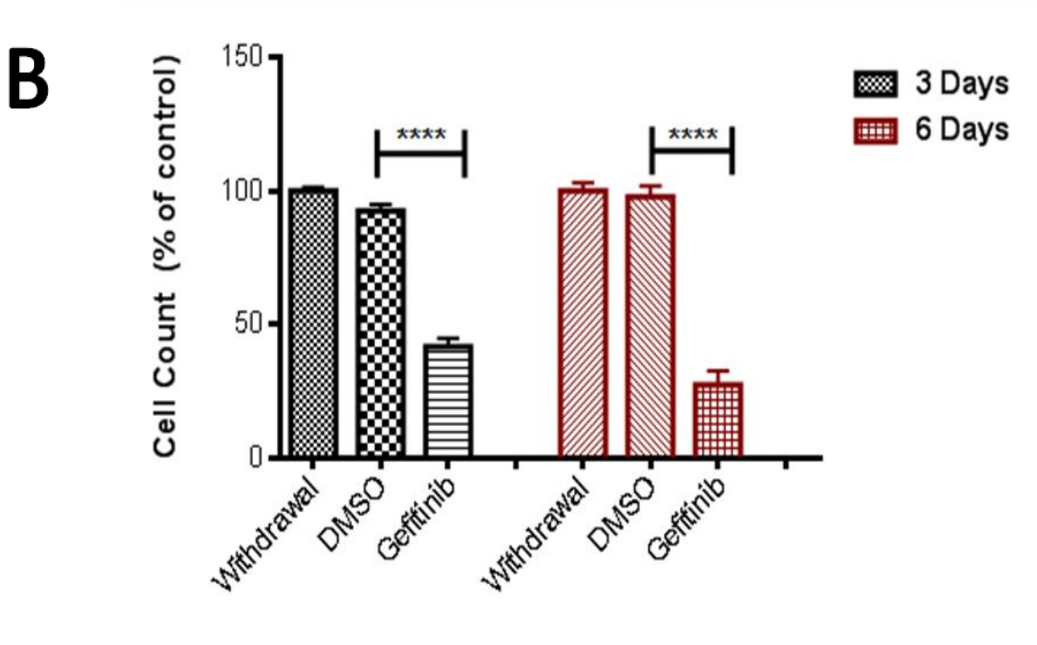


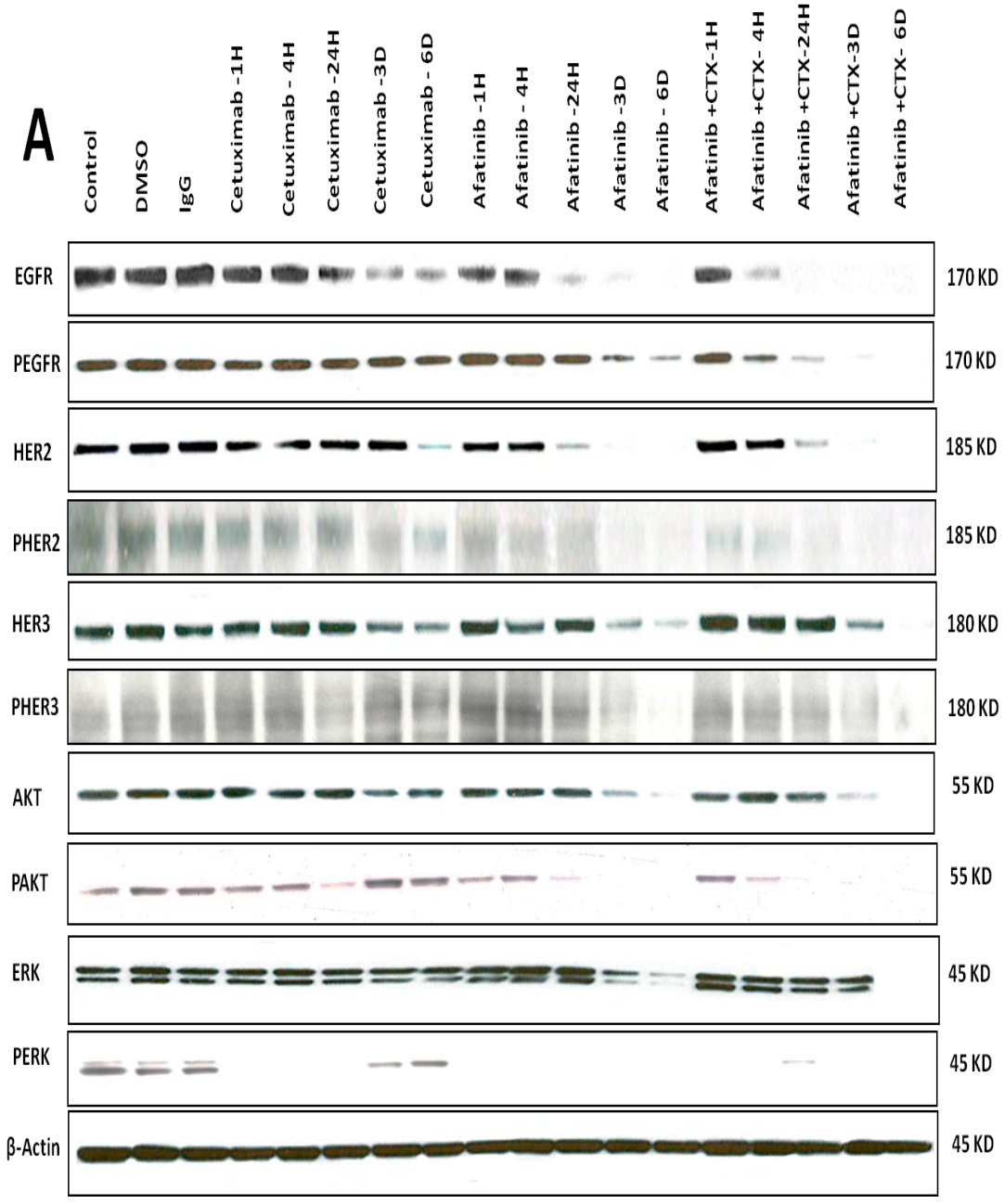
Figure 5.2. The effect of gefitinib on HER signalling and cell count in cetuximab resistant SCC15 cell line.

Resistant SCC15 cells were treated either with continuous cetuximab (20 µg/mL) or 1 µM of gefitinib after withdrawal of cetuximab. Treatments were carried out for 1h, 24h, 3d and 6d and the expression and activation of the indicated proteins were analysed by western blot analysis (A). Resistant cells were treated with 1 µM of gefitinib after withdrawal of cetuximab. The number of cells was counted after 3 and 6 days using cell counter (B). Three independent cell count experiments were performed in triplicate and the percentage of viable cells were calculated relative to the untreated cells and presented as the mean ± S.D. The means between the groups were analysed by Anova with Bonferroni's Multiple Comparison Test and statistically significant changes were represented by asterisks ($P \leq 0.0001$ ****). 10 µg/mL of human IgG was used as a control. DMSO was used as a vehicle control for gefitinib. β-actin was used as a loading control for each gel but only two representative bands are shown. Western blots are representative of three independent experiments.

5.2.2. Afatinib (BIBW2992)

5.2.2.1. Afatinib seems to have a greater effect on HER receptors signalling compared to cetuximab

Downregulation of EGFR was observed at 3 days with cetuximab treatment and 24h with afatinib, this occurred earlier at 4h with the combination of both drugs. Afatinib was more effective in downregulation of total HER2 and its combination with cetuximab did not seem to have any additive effect. Both afatinib and cetuximab downregulated HER3 and AKT at 3 and 6 days, but the combination treatment had greater effect on HER3 at 6 days. Afatinib and cetuximab also decreased pHER3 and pAKT after 24h treatment although there was reactivation after 3 days of cetuximab treatment but this was not observed with combination treatment. Treatment with afatinib alone or its combination with cetuximab also led to the downregulation of ERK. There was reactivation of pERK with cetuximab treatment at 3 and 6 days, which was not seen in afatinib or its combination with cetuximab (figure 5.3A). The results of the cell count experiments showed that combining cetuximab with afatinib had a significantly greater inhibitory effect on the number of sensitive cells at 3 days than either drug alone. However, the effect of combination treatment did not appear to be more effective compared to afatinib alone at 6 days although it remained to be better than cetuximab alone (figure 5.3B).


A
Control
DMSO
IgG
Cetuximab -1H
Cetuximab - 4H
Cetuximab -24H
Cetuximab -3D
Cetuximab - 6D
Afatinib -1H
Afatinib - 4H
Afatinib -24H
Afatinib -3D
Afatinib - 6D
Afatinib +CTX-1H
Afatinib +CTX- 4H
Afatinib +CTX-24H
Afatinib +CTX-3D
Afatinib +CTX- 6D
EGFR
170 KD
PEGFR
170 KD
HER2
185 KD
PHER2
185 KD
HER3
180 KD
PHER3
180 KD
AKT
55 KD
PAKT
55 KD
ERK
45 KD
PERK
45 KD
β-Actin
45 KD

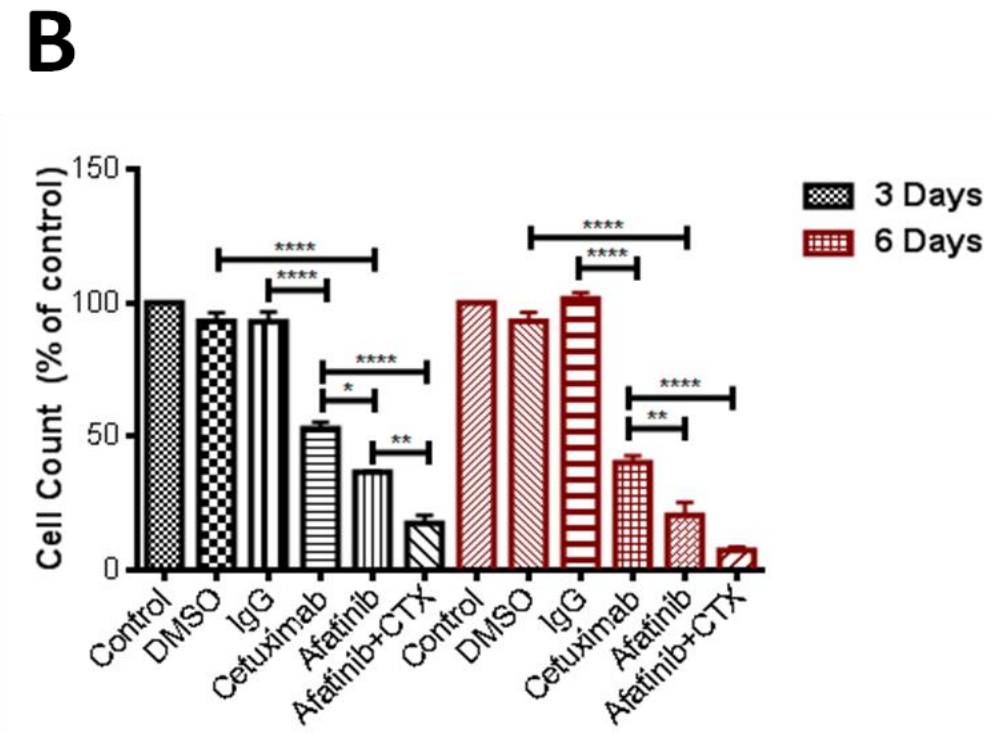
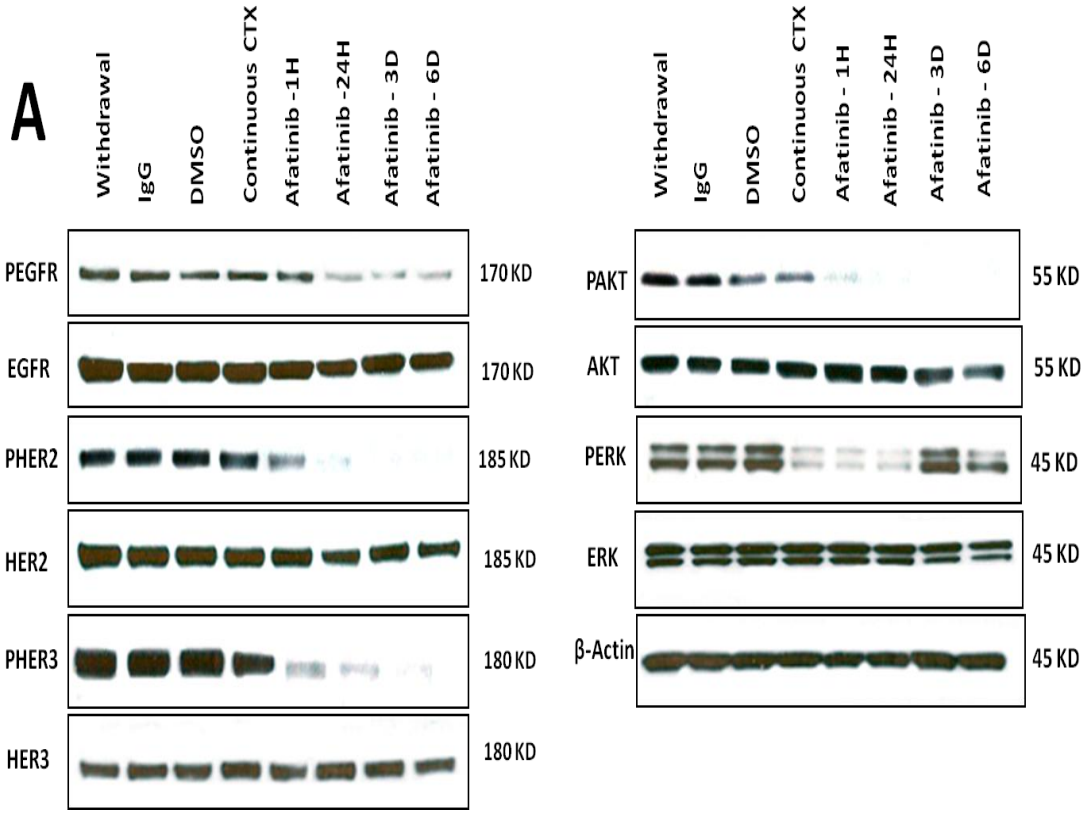


Figure 5.3. The effect of afatinib with or without cetuximab on HER receptors signalling and cell count in sensitive cell line.

SCC15 cells were treated either with 1 μM of afatinib, 10 μg/mL of cetuximab or their combinations. The expression and activation of the indicated proteins were then analysed by western blot after 1h, 4h, 24h, 3D and 6D treatments (A). Cell count experiment was performed after 3 and 6 days treatments (B). Three independent cell count experiments were performed in triplicate and the percentage of viable cells were calculated relative to the untreated cells and presented as the mean ± S.D. The means between the groups were analysed by Anova with Bonferroni's Multiple Comparison Test and statistically significant changes were represented by asterisks ($P \leq 0.05$ *, $P \leq 0.01$ **, $P \leq 0.0001$ ****). 10 μg/mL of human IgG was used as a control. DMSO was used as a vehicle control for afatinib. β-actin was used as a loading control for each gel but only one representative band is shown. Western blots are representative of three independent experiments.

5.2.2.2. Treatment with afatinib led to the inhibition of HER receptors signalling in cetuximab resistant cell line

As shown in Fig 3.4, chronic exposure to cetuximab leads to the reactivation of EGFR, HER2, HER3 and downstream signalling pathways. Therefore, it was hypothesized that afatinib would inhibit HER receptors signalling in cetuximab resistant cells. The results showed that afatinib inhibited pEGFR, pHER2, pHER3 and pAKT within 24 hours in cetuximab resistant SCC15 cell line (figure 5.4A). The pERK was increased after withdrawal of cetuximab but remained suppressed if afatinib was added. However, there was reactivation of pERK at 3 days in the absence of other the activation of EGFR, HER2 or HER3. Afatinib also significantly decreased the number of cetuximab resistant cells compared to DMSO control, consistent with the effects on HER receptors and downstream signalling pathways (figure 5.4B).


A
Withdrawal
IgG
DMSO
Continuous CTX
Afatinib -1H
Afatinib -24H
Afatinib - 3D
Afatinib - 6D
PEGFR
170 KD
EGFR
170 KD
PHER2
185 KD
HER2
185 KD
PHER3
180 KD
HER3
180 KD
Withdrawal
IgG
DMSO
Continuous CTX
Afatinib - 1H
Afatinib - 24H
Afatinib - 3D
Afatinib - 6D
PAKT
55 KD
AKT
55 KD
PERK
45 KD
ERK
45 KD
β-Actin
45 KD

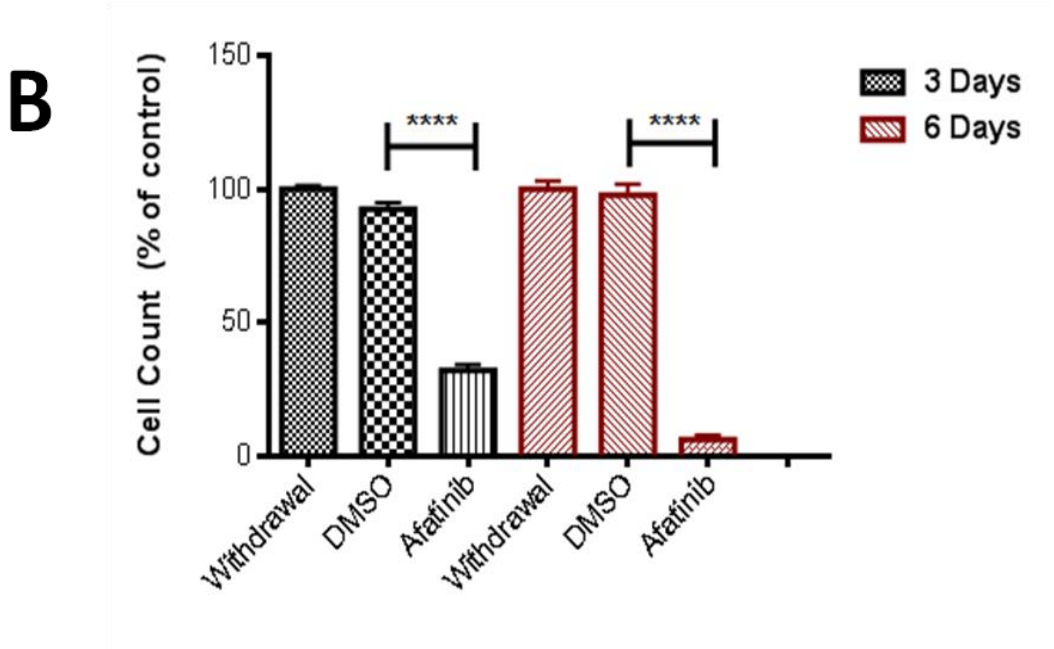


Figure 5.4. The effect of afatinib on HER signalling in resistant cell line

Resistant cells were treated with continuous cetuximab (20 μg/mL) or 1μM of afatinib after withdrawal of cetuximab. Cells were treated for 1h, 24h, 3D and 6D before being lysed for western blot analysis (A). Resistant cells were treated with 1μM of afatinib after withdrawal of cetuximab and the number of cells was counted after 3 and 6 days using cell counter (B). Three independent cell count experiments were performed in triplicate and the percentage of viable cells were calculated relative to the untreated cells and presented as the mean ± S.D. The means between the groups were analysed by Anova with Bonferroni's Multiple Comparison Test and statistically significant changes were represented by asterisks ($P \leq 0.0001$ ****). 10 μg/mL of human IgG was used as a control. DMSO was used as a vehicle control for afatinib. β-actin was used as a loading control for each gel but only two representative bands are shown. Western blots are representative of three independent experiments.

5.3. DISCUSSION

As described in previous chapters, acute cetuximab monotherapy failed to produce a considerable effect on inhibition of EGFR phosphorylation and prolonged cetuximab exposure led to the activation of other HER receptors. It was shown in this study that treatment with cetuximab induced the release of HER ligands in an autocrine manner. This process was shown to be ADAM mediated. Therefore it was hypothesized that inhibitions of ADAMs, dual targeting of EGFR or inhibition of other members of HER receptors family could improve the effect of cetuximab in HNSCC. Results from chapter 4 demonstrated the beneficial effect of combing cetuximab with ADAM10 and ADAM17 inhibitors. In this part of the project the effects of combining cetuximab with gefitinib and afatinib on HER receptors signalling as well as cell viability were assessed. Experiments were performed to see whether combining two distinct classes of inhibitors, TKIs versus mAb, against the same target could have additive or synergistic effects in targeting EGFR compared to cetuximab monotherapy. The effects of reversible EGFR TKI, gefitinib, and irreversible pan-HER TKI, afatinib, in overcoming resistance to cetuximab in HNSCC cell lines also were evaluated.

Treatment with gefitinib alone seemed to be more effective than cetuximab in inhibition of the cell viability of parental cells. However, this is not the case in clinic. Although gefitinib showed some clinical activities, it failed to produce significant survival benefits. HNSCC patients seem to gain survival benefit from cetuximab and not gefitinib as indicated by FDA approval of cetuximab in HNSCC and not gefitinib. The anti-tumour activity of cetuximab has been

suggested to be due to antibody-dependent cellular cytotoxicity (ADCC) (Kimura *et al.* 2007).

Even though HER3 is not the direct target of gefitinib, however, the inhibition of pHER3 and pAKT was observed after 1h treatment with gefitinib which was sustained even after 6 days. This is maybe because the HER family receptors perform their signalling activities through heterodimerization and activation of other members of the family. This finding is noteworthy as previous studies have reported the failure of TKIs to effectively target HER3/PI3K/AKT signalling *in vitro* and *in vivo* (Sergina *et al.* 2007). Results from a study by Sergina *et al.* (2007) which was performed in HER2-driven breast cancer cells demonstrated that gefitinib does not produce sustained inhibition of HER3 signalling (Sergina *et al.* 2007). Different groups have suggested that the reactivation of HER3/AKT is responsible for the development of resistance to TKIs (She *et al.* 2003, Bianco *et al.* 2003, Haas-Kogan *et al.* 2005, Sergina *et al.* 2007).

Although the combination of cetuximab with gefitinib seems to be more effective in targeting HER receptor signalling compared to either single agent, the combination treatment was not more effective than gefitinib alone at 6 day. This could be due to the high dose of gefitinib (1µM) which was tested in this study. By using lower concentration of the drug and thus avoiding non-specific kinase inhibition likely differences between monotherapy and combination treatment might be more pronounced. As described previously, the effect of combination treatment on the cell number was also not more than

gefitinib as a single agent. This could be due to the limitation of the detection technique which was used in these experiments.

The effectiveness of various combination treatments has also been investigated by other groups in different human cancer cell lines. Matar *et al.* (2004) investigated the effect of combining cetuximab with gefitinib in colon, prostate and breast carcinoma cells. For their growth assay they used increasing concentrations of gefitinib (0.1, 1, 10 μM) and/or cetuximab (0.5, 5, 50 nM) for 72 hours. For western blot analysis and TUNEL assay they treated cells with gefitinib (0.1 & 1 μM) and/or cetuximab (0.5 & 5 nM) for 2 hours and 3 days respectively. This group has reported that the combination treatment had a synergistic effect on the cell proliferation and the induction of apoptosis but this was not observed in all cell lines tested. They claimed that the synergism effect of combination therapy was dependent to EGFR expression levels as well as the sensitivity of cell lines to each individual drug. Reduced EGFR, MAPK, and AKT activation upon combination treatment was also reported. Administration of gefitinib along with cetuximab also led to a complete and permanent regression of tumours in the A431 xenograft model, but only if suboptimal doses of drugs were used. They claimed that tumour regrowth upon discontinuation of treatment was due to the highest doses of cetuximab (Matar *et al.* 2004).

In another study, Huang *et al.* (2004) studied the combination of cetuximab with either gefitinib or erlotinib in HNSCC, NSCLC and prostate cancer cells. In their study different cell lines were treated with cetuximab (5 nM) in

combination with gefitinib (0.5 μM) or erlotinib (0.5 μM) for 48-72h before performing western blot analysis. In order to investigate the number of viable cells this group performed MMT assay. In their study an enhanced growth inhibition was observed with the combination of cetuximab plus gefitinib or erlotinib. This group also reported that the phosphorylation of EGFR, ERK and AKT was inhibited more rigorously with combined treatment, leading to an induced apoptosis in HNSCC cell lines tested. In their *in vivo* experiments they showed that the combination treatment had significant effects on growth inhibition of human lung cancer xenografts (Huang *et al.* 2004).

Although studies by Matar *et al.* (2004) and Huang *et al.* (2004) have shown promising results, Fischel *et al.* (2005) has reported that the combination of cetuximab with gefitinib induces an antagonistic effect. This group assessed the effect of combination treatment in two EGFR+ tumour cell lines; head and neck (CAL33) and vulval (CAL39). For cell proliferation assay they treated their cell lines with cetuximab (0.01-100 nM), gefitinib (0.01-100 μM) or their combinations for 5 days. For western blot analysis cells were treated with cetuximab (1 nM) and/or gefitinib (0.01-100 μM) for 48h. Their results showed that the combination treatment was antagonistic regardless of the types of cell lines. Combination treatment also increased the expression level of EGFR in the tested cell line (CAL33) (Fischel *et al.* 2005). Results from these studies indicate the importance of factors such as detection techniques, cell types, optimal drug dose and treatment schedule in determining the true and accurate effect of combination therapies.

In this project the effects of afatinib in HNSCC was also investigated. Afatinib was found to be more effective in targeting HER receptor and reducing the cell number of parental cells compared to cetuximab alone. This is because afatinib is an irreversible pan-HER inhibitor which targets the tyrosine kinase domains of all HER family receptors and thus it is expected to have a greater inhibitory effect. However, the combination treatment seems not to be more effective compared to afatinib monotherapy in our cell line. This also could be due to the single dose of afatinib (1μM) used in this study. As these experiments and also the experiments concerning gefitinib have been done in only one HNSCC cell line, further experiments using more cell lines with optimal dose of drugs will be required to validate the efficacy of combination treatment in HNSCC cell lines.

In this study although combination treatments did not produce considerable inhibitory effects compared to single agent, nevertheless both TKIs showed interesting results in targeting cells which have developed resistance to cetuximab mAb. Gefitinib was very effective in cetuximab resistant cell line and led to the inhibition of pHER3 and its downstream pathway pAKT. This was consistent with the significant reduction in the number of resistant cells. As described above, reactivation of HER3 and AKT has shown to be responsible for gefitinib resistance in breast cancer cell lines. This was found to be due to inability of gefitinib to cause sustained inhibition of the receptor and its downstream signalling. However, in this study gefitinib was able to constantly inhibit HER3 signalling up to 6 days and thus overcoming resistance to cetuximab in HNSCC.

Consistent with our results, Huang *et al.* (2004) showed that both gefitinib and erlotinib retain their capacity to inhibit the growth of NSCLC and HNSCC cells that have developed resistant to cetuximab. Although treatment with gefitinib or erlotinib did not have any effect on pEGFR, a pronounced inhibition of pMAPK and pAKT was reported in cetuximab resistant cell lines (Huang et al. 2004).

As expected, afatinib seemed to be more effective in inhibition of HER receptor signalling in cetuximab resistance cells compared to gefitinib although the direct comparison on their ability (± cetuximab) on HER signalling as well as the inhibition of cell viability and proliferation have not been done. This study shows promising results on the potential of TKIs in overcoming resistance to cetuximab. However, these results need to be validated in more HNSCC cell lines.

Studies have shown that the irreversible TKIs are highly effective in overcoming T790M (acquired secondary EGFR mutation that confers resistance to erlotinib and gefitinib) mediated resistance in NSCLC (Kwak et al. 2005). In recent years a number of less frequent EGFR mutations have been identified which are known to be associated with acquired resistance to TKIs. Bean *et al.* (2008) have reported a novel mutation in the exon 21 of EGFR gene (T854A) in *EGFR* mutant lung adenocarcinoma patients who had developed acquired resistance to EGFR TKIs. This group showed the potency of afatinib in overcoming such resistance (Bean *et al.* 2008). Ercan

et al. (2012) also reported two novel secondary EGFR mutations, L718Q and L844V, in TKI resistant cells. These EGFR mutant cells reported to be sensitive to neratinib (EGFR/HER2/HER4 TKI) and afatinib (Ercan *et al.* 2012). Concomitant EGFR inhibition by irreversible TKIs and mAbs also has shown promising pre-clinical results. Regales *et al.* (2009) investigated the effect of afatinib-cetuximab combination in overcoming EGFR TKI resistance mutation, T790M, in mouse models of lung cancer. Treatment of EGFRL858R+T790M mice with combination of afatinib and cetuximab led to an induced tumour regression. Results from experiments on xenografts-derived lysates showed greater inhibitory effects on total and phospho EGFR with combination treatment (Regales *et al.* 2009). Takezawa *et al.* (2012) studied the effects of combining afatinib and cetuximab in *in vitro* and *in vivo* models of acquired resistance to erlotinib in EGFR mutant NSCLC lacking T790M mutation. The combination treatment led to a greater inhibition of the cell growth and decreased the phosphorylated levels of EGFR, HER2, HER3, AKT and ERK, compared with single drug. By investigating lysates derived from their xenografts model, this group has also reported the depletion of total and phospho-EGFR with combination treatment. Decrease in HER2 and HER3 phosphorylation has also been reported, however, this was found to be reactivated after 48h (Takezawa *et al.* 2012). In a study by Quesnelle and Grandis (2011) the effects of afatinib in overcoming resistance to cetuximab was investigated in bladder cancer. Results from *in vitro* experiments showed that afatinib retained its ability to inhibit the growth of the cetuximab resistant cells. Afatinib also had an additive growth inhibitory effect in cetuximab resistant xenografts (Quesnelle and Grandis 2011).

In summary, gefitinib was very effective in targeting HER signalling and enhanced response to cetuximab in parental HNSCC cell line. Gefitinib also significantly inhibited the activation of HER signalling in cetuximab resistant cell line. As expected, afatinib found to have a greater effect on HER signalling compared to cetuximab and could reverse resistance to cetuximab. Results from this study demonstrated the potential of TKIs in overcoming resistance to cetuximab in HNSCC.

CHAPTER 6

Assessing the role of ADAM10 and ADAM17 as prognostic and predictive biomarkers in HNSCC patients

6.1. INTRODUCTION

Although cetuximab is now considered as an established treatment for HNSCC and colorectal cancer patients, not all patients benefit from this drug and the response rate is low. Therefore it would be very important to identify biomarkers that can predict response to cetuximab in these patients. The significance of KRAS gene mutation (exon 2, codon 12 and 13) as a predictive marker of resistance to EGFR-targeted mAbs, cetuximab and panitumumab, has been reported in colorectal cancer (Siena *et al.* 2009). However, mutation in KRAS gene is very rare in HNSCC (Boeckx *et al.* 2014) and there is a lack of reliable predictive biomarkers of response/resistance to cetuximab in HNSCC.

As described in chapter 4, upregulation of ADAM10 and ADAM17 occurred during acute and chronic exposure to cetuximab. It was hypothesized that the expression levels of ADAMs might correlate with tumour progression and may predict response to cetuximab in HNSCC. In order to test this hypothesis ADAM10 and ADAM17 expression levels were assessed in tissue microarrays (TMAs) from a cohort of HNSCC patients who underwent routine treatments as well as cetuximab treated excision samples from HNSCC patients who underwent a cetuximab window study.

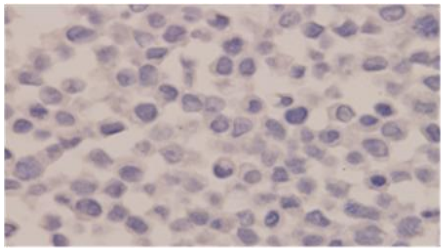
6.2. RESULTS

6.2.1. ADAM10 and ADAM17 antibodies verification

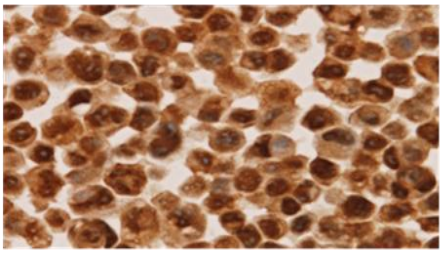
In order to verify ADAMs antibodies' specificity and also to determine the optimal concentrations of antibodies, knockdown was performed in SCC15 cell line using specific siRNAs against ADAM10 and ADAM17. Two concentrations of each antibody, 1:100 and 1:200 dilution, were tested in this experiment. No staining was observed in the 2nd antibody controls (figure 6.1A & 6.1B, top panels), indicating the specificity of primary antibodies. Compared to the scrambled control (figure 6.1A & 6.1B, middle panels), siRNAs knockdown reduced the staining intensity of ADAM10 (figure 6.1A, bottom panel) and ADAM17 (figure 6.1B, bottom panel) in SCC15 cell pellet (figure 6.1). 1:200 dilution of ADAM10 antibody and 1:100 dilution of ADAM17 antibody were chosen for staining of TMAs and cetuximab treated patients' samples.

A

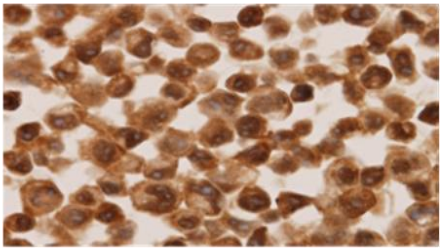
ADAM10



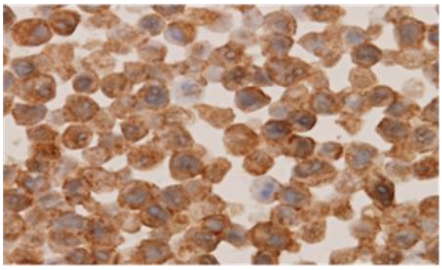
2nd Antibody Control



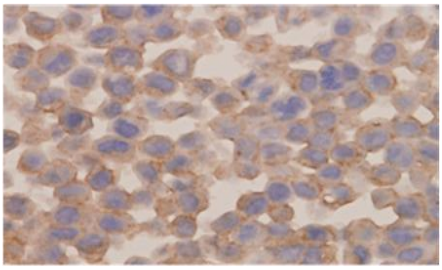
Scramble / AD10 Ab (1:100)



Scramble / AD10 Ab (1:200)



AD10 KD / AD10 Ab (1:100)



AD10 KD / AD10 Ab (1:200)

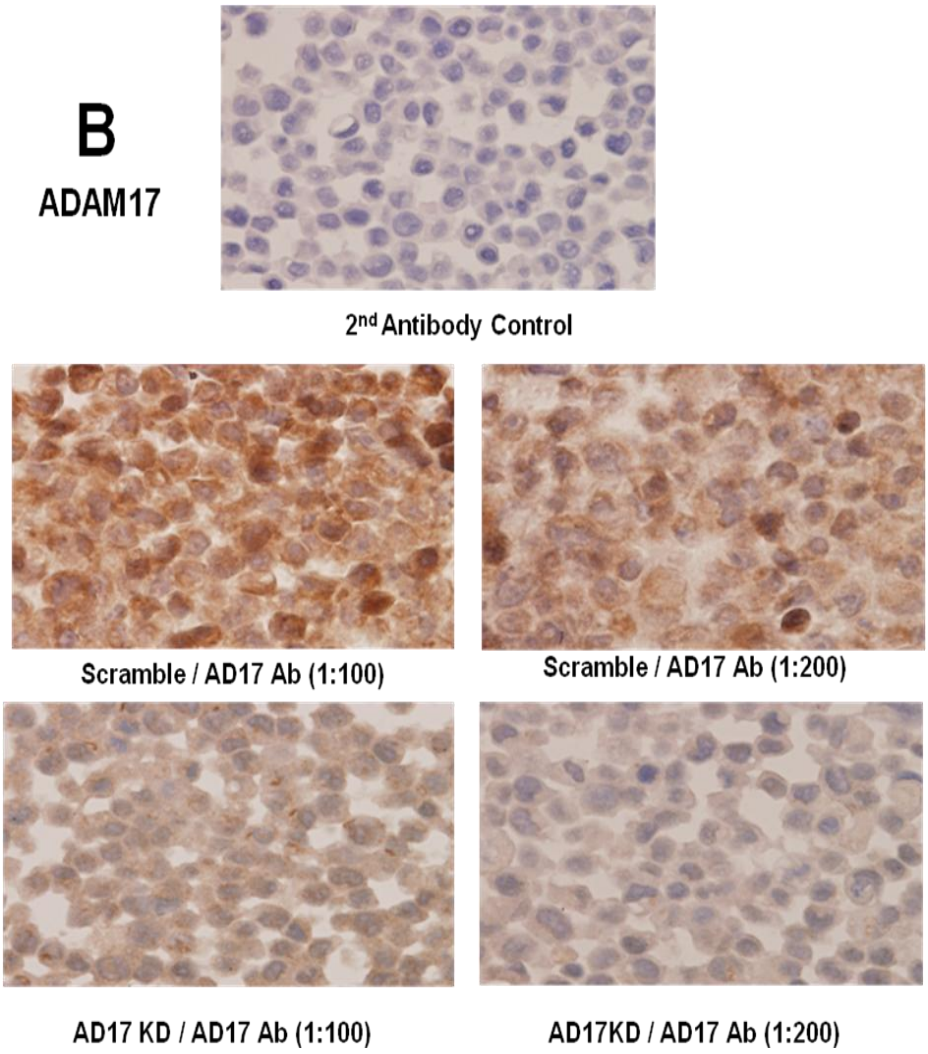


Figure 6.1. ADAM10 and ADAM17 antibodies verification in SCC15 cell pellet

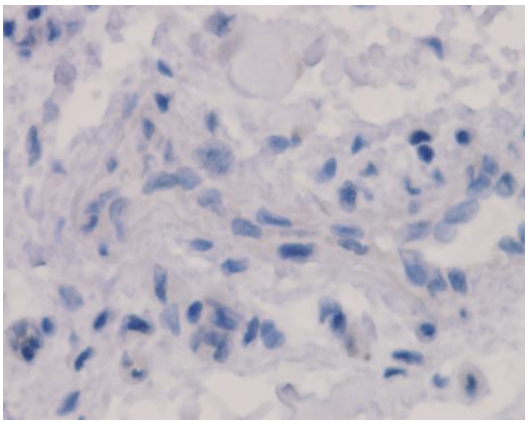
SCC15 cells were transfected for 72 h either with 10 nM of scrambled siRNA, 10 nM of ADAM10 siRNA or 10 nM of ADAM17 siRNA. At the end of transfection SCC15 cell pellets were generated as described in chapter 2. The control (not transfected) cell pellets were also generated from the parental SCC15 cells and were stained with 2nd antibody alone (A&B, Top panels) to assess for non-specific staining. Cells transfected with negative siRNAs (scramble) were stained with indicated concentrations of ADAM10 (A, middle panel) or ADAM17 (B, middle panel) antibody. Cells transfected with ADAM10 siRNA were stained with ADAM10 antibody (A, bottom panel) and cells which were transfected with ADAM17 siRNA were stained with ADAM17 antibody (B, bottom panel). Two concentrations for each primary antibody were tested as indicated in the figures.

6.2.2. ADAMs expression pattern in head and neck tissue samples

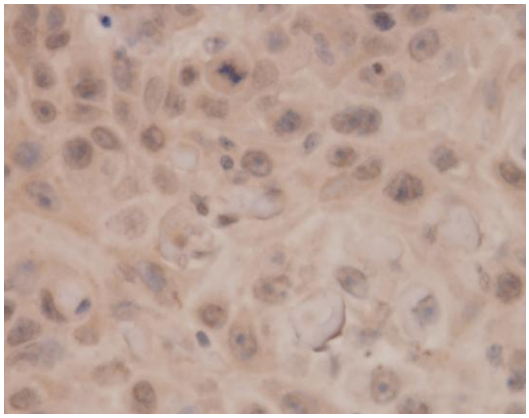
Following the optimization of the antibodies' concentration in the cell pellets, the expression levels of ADAM10 and ADAM17 were assessed in tissue samples from several different cohorts of HNSCC patients. One cohort is the TMAs consisting of normal and tumour samples from a series of head and neck patients undergoing routine treatments in Oxford. These samples were obtained from Oxford Radcliffe Biobank. The second cohort is the tumour samples from HNSCC patients undergoing a cetuximab window study provided by our collaborators; Professor Jean-Pascal Machiels and Dr. Sandra Schmitz at Cliniques Universitaires Saint-Luc (UCL) in Brussels.

Samples were stained with ADAM10 and ADAM17 antibodies and the expression of these metalloproteases was heterogeneous and was semi-quantitatively scored according to IRS scoring system as described in chapter 2. IRS scores of 0 (non-detectable staining) to 12 (maximum staining) based on the intensity and percentage of staining were assigned to each tumour. Representative expression patterns of ADAM10 (figure 6.2A) and ADAM17 (figure 6.2B) are shown.

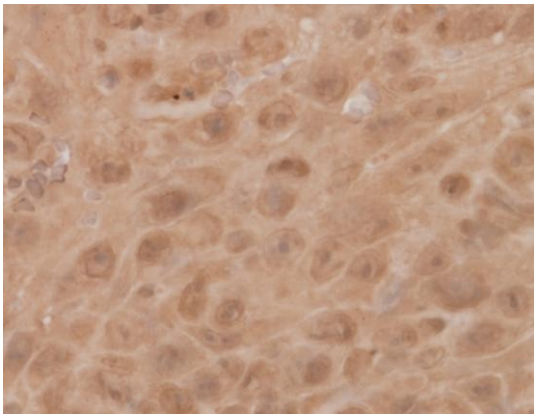
A ADAM10



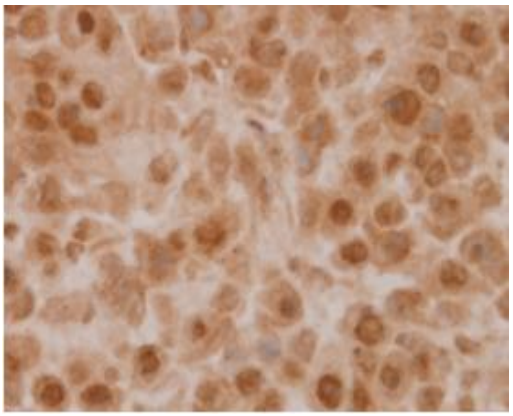
Negative



Weak

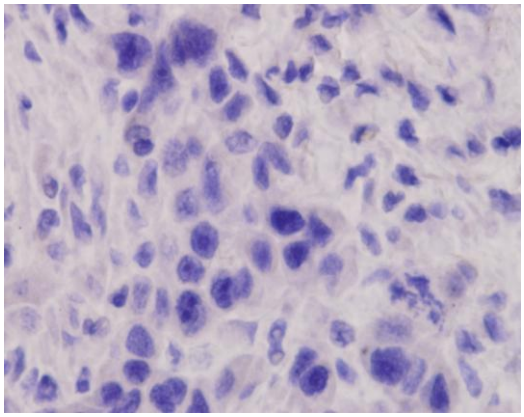


Moderate

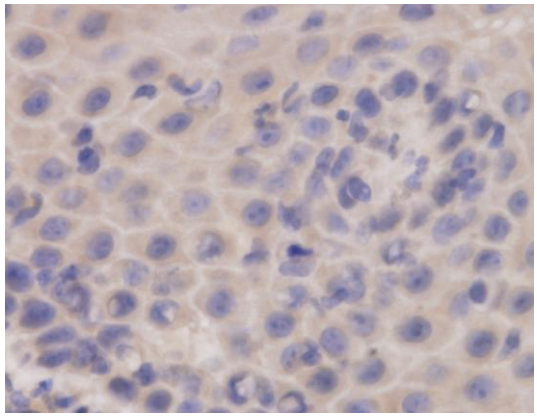


Strong

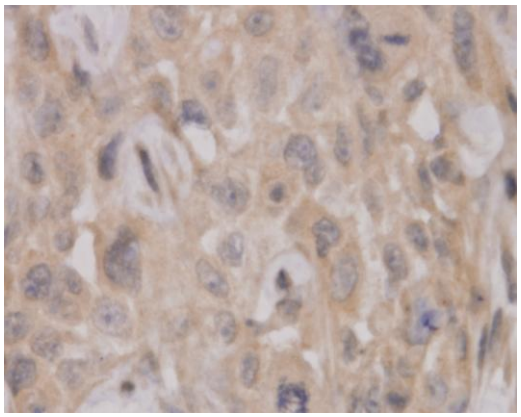
B **ADAM17**



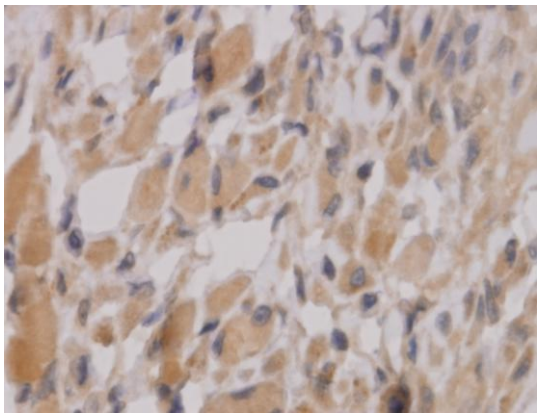
Negative



Weak



Moderate



strong

Figure 6.2. Representative expression patterns of ADAM10 and 17 in HNSCC patients' samples

Tumour samples were stained with 1:200 dilution of ADAM10 antibody or 1:100 dilution of ADAM17 antibody. Representative staining of ADAM10 and ADAM17 are shown in section A and B respectively.

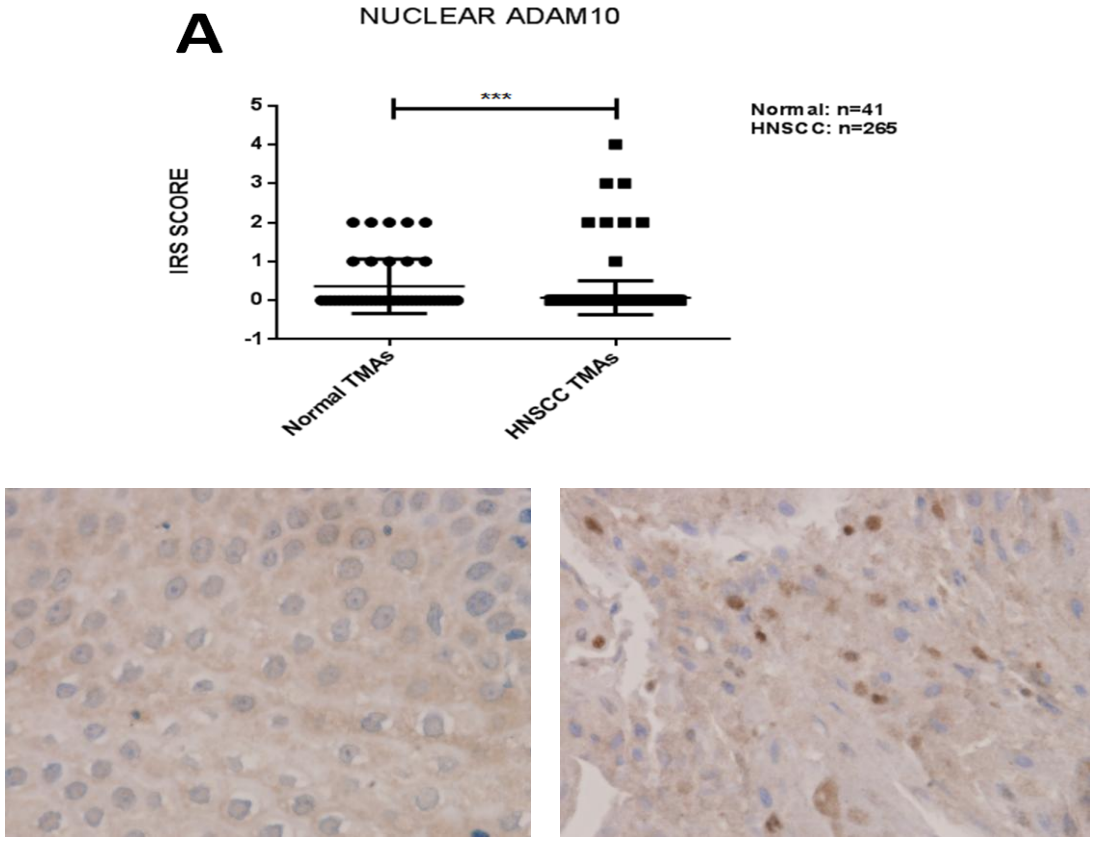
6.2.3. TMAs from a cohort of HNSCC patients in Oxford

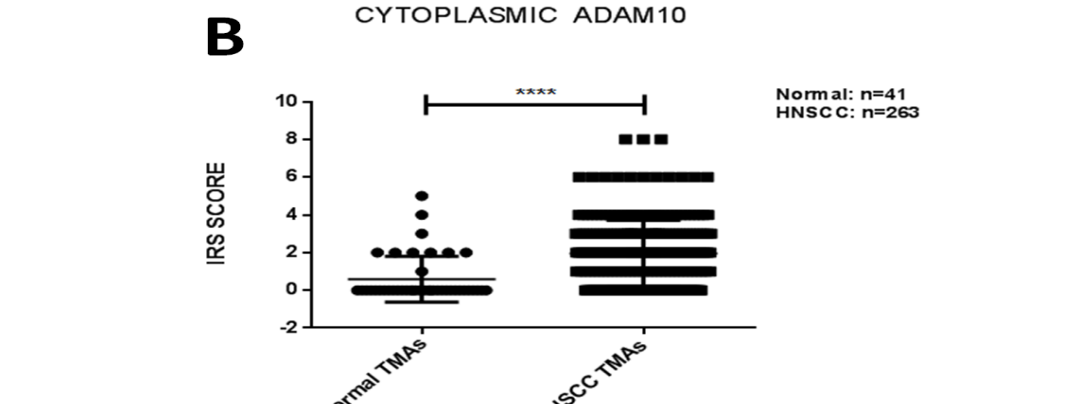
6.2.3.1. Basal expressions of ADAM10 and ADAM17 in head and neck TMAs

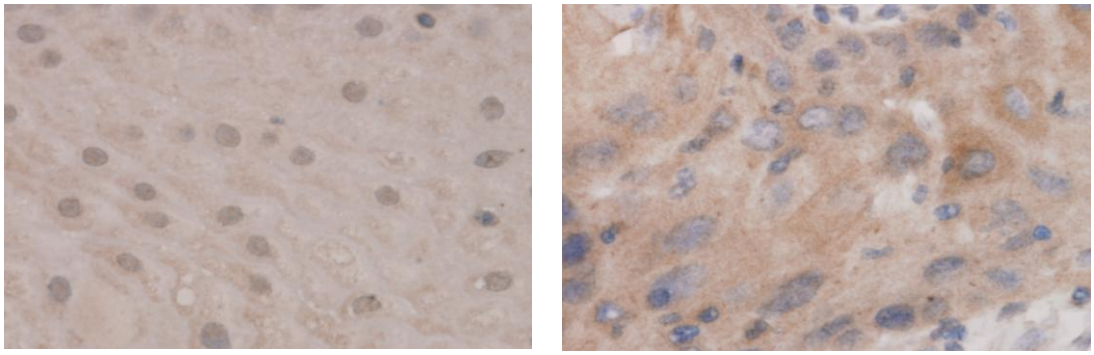
The basal expression levels of ADAMs were compared between normal and tumour samples from the TMAs obtained from Oxford. The characteristic of the patients and tumours are shown in table 6.1. Significantly higher expression levels of nuclear (figure 6.3A) and cytoplasmic (figure 6.3B) ADAM10 were observed in tumour samples compared to the normal tissues. Higher expression level of cytoplasmic ADAM17 was also observed in tumour tissues compared to normal samples, however, this was not statistically significant (figure 6.3C). No nuclear staining was detected for ADAM17. One representative staining is shown for each condition (figure 6.3).

Characteristics	Total Number of Patients
Gender	
Male	198
Female	67
Disease Recurrence	
Positive	110
Negative	155
Overall Outcome	
Alive	142
Dead	123
Size Of The Primary Tumour	
1-2 cm	109
3-4 cm	156
Regional Lymph Node Metastasis	
Positive	202
Negative	58
Unknown	5
Clinical Staging	
I & II	36
III & IV	229
Tumour Grade	
Poor	86
Moderate	167
Well	12
Tumour Site	
Oral Cavity	75
Larynx	44
Pharynx	146

Table 6.1. The patients and tumour characteristics of the cohort of HNSCC patients in Oxford.


A
NUCLEAR ADAM10
***
Normal: n=41
HNSCC: n=265
IRS SCORE
5
4
3
2
1
0
-1
Normal TMAs
HNSCC TMAs


B
CYTOPLASMIC ADAM10
****
Normal: n=41
HNSCC: n=263
IRS SCORE
10
8
6
4
2
0
-2
Normal TMAs
HNSCC TMAs



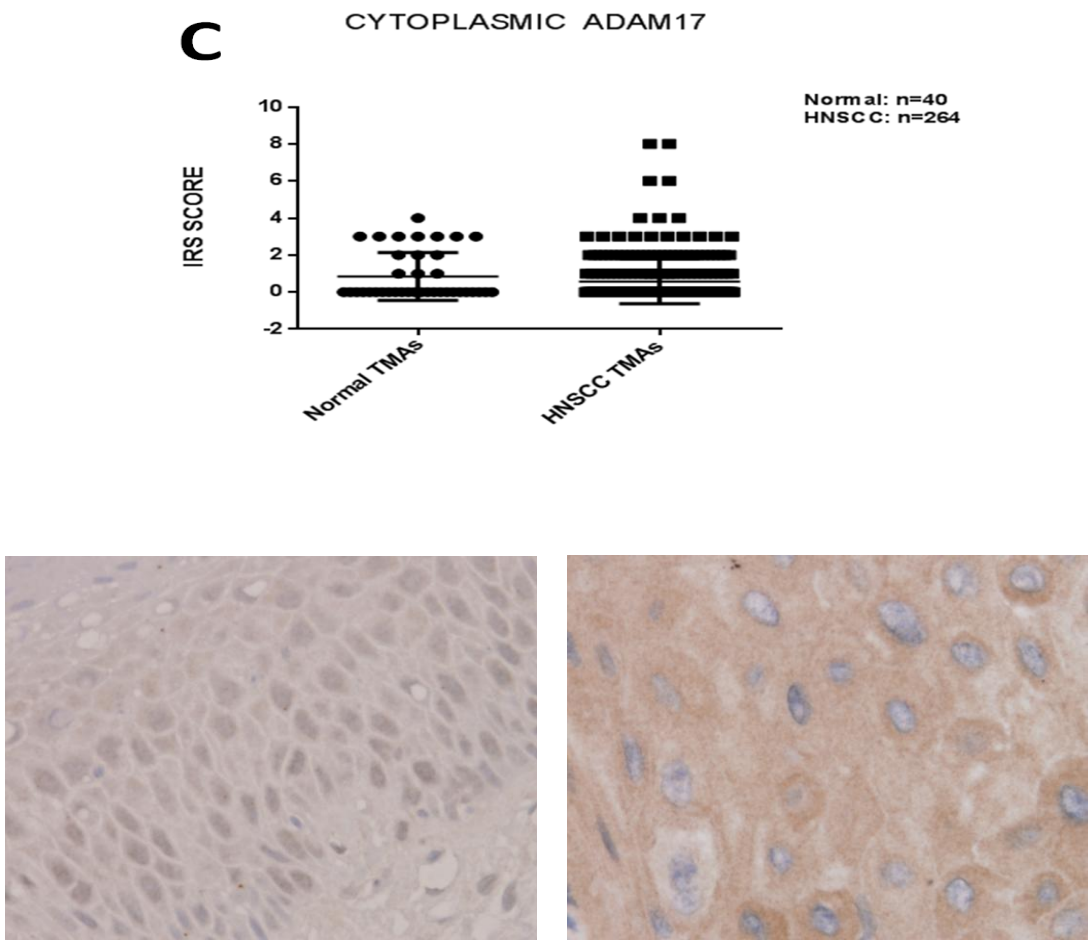


Figure 6.3. ADAM10 and ADAM17 basal expression levels in head and neck TMAs

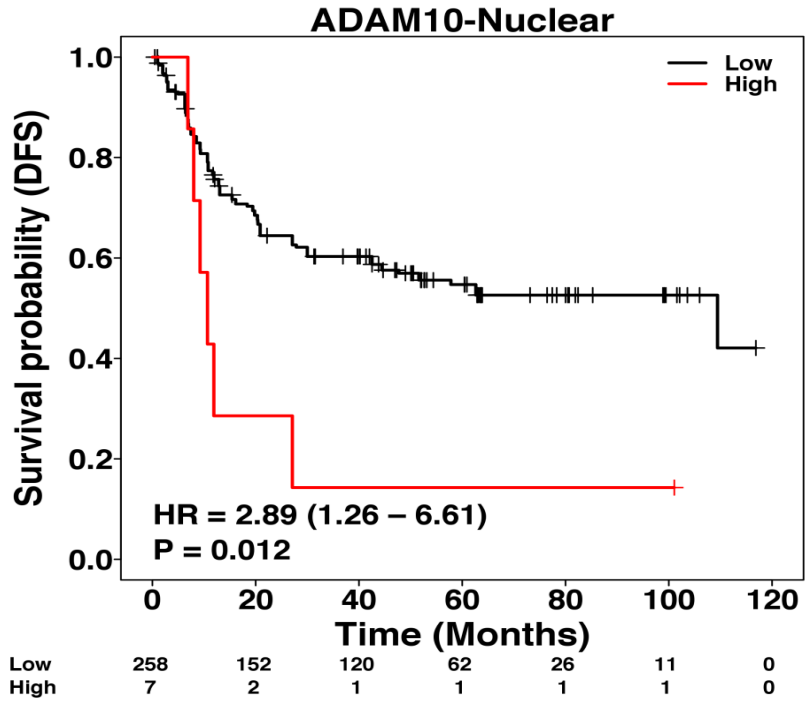
Normal and tumour tissues were stained for ADAM10 (A&B) and ADAM17 (C) and the immunostaining was scored according to IRS system. 1:200 dilution of ADAM10 and 1:100 dilution of ADAM17 antibodies were used. The means between the groups were analysed by unpaired T-test and statistically significant changes were represented by asterisks ($P \leq 0.001$ ***, $P \leq 0.0001$ ****). Representative staining of nuclear ADAM10 (A), cytoplasmic ADAM10 (B) and cytoplasmic ADAM17 (C) are shown.

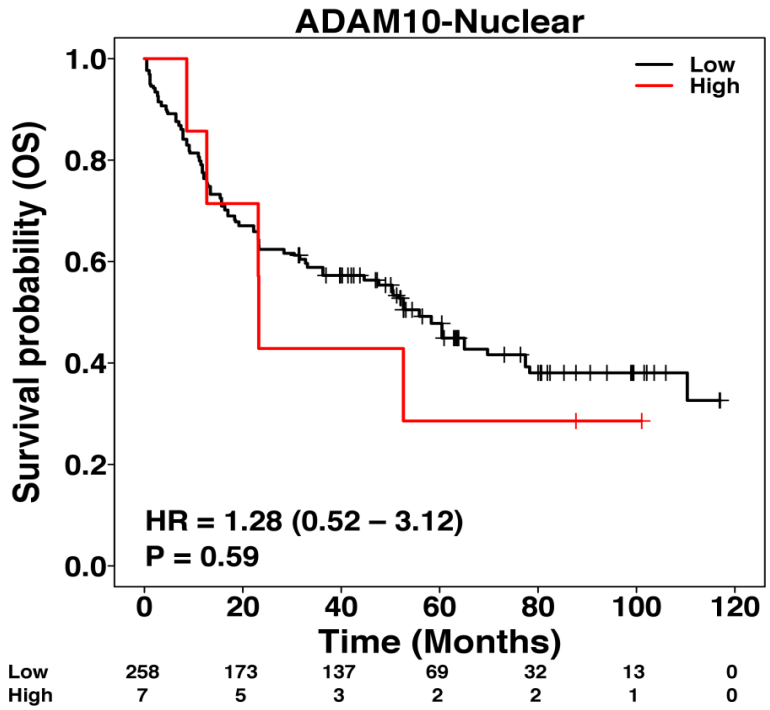
6.2.3.2. HNSCC patients with high nuclear ADAM10 or high cytoplasmic ADAM17 expressions have shorter disease-free survival

In order to assess the prognostic value of ADAM10 and ADAM17 in HNSCC TMAs survival analysis was performed by Dr. Syed Haider using Kaplan-Meier method and the Cox proportional hazards model. Low ADAM10 expression was defined as either no or weak staining in less than 10% of tumour cells (IRS of ≤ 1) as per previous work in breast cancer done in our lab (Feldinger *et al.* 2014). The cohort was dichotomised into low (IRS score ≤ 1) and high (IRS score > 1) risk groups and the nuclear and cytoplasmic expression was expressed separately. The same cut-off point was used for ADAM17 expression. Higher nuclear ADAM10 expression was correlated with poorer disease-free survival (DFS) compared to low nuclear ADAM10 expression (HR = 2.89, 95% CI = 1.26-6.61, p = 0.012). A trend towards worse overall survival (OS) for higher nuclear ADAM10 expression was also observed, however, this was not statistically significant (HR = 1.28, 95% CI = 0.52-3.12, p = 0.59) (figure 6.4A). The differences between high and low ADAM10 cytoplasmic expression were not significant for DFS (HR = 1.04, 95% CI = 0.71-1.52, p = 0.828) and OS (HR = 1.05, 95% CI = 0.75-1.46, p = 0.784) (figure 6.4B). Although there was no association between ADAM17 cytoplasmic expression with OS (HR = 0.92, 95% CI = 0.59 -1.42, p = 0.696), a significantly worse DFS (HR = 1.69, 95% CI = 1.09 -2.63, p = 0.019) was observed for the patients in the ADAM17 high expression group compared to the patients in the low ADAM17 expression group (figure 6.4C). The results from multivariate analysis adjusted for sex, stage and grade suggested that nuclear ADAM10 was an independent prognostic factor for DFS (p= 0.048)

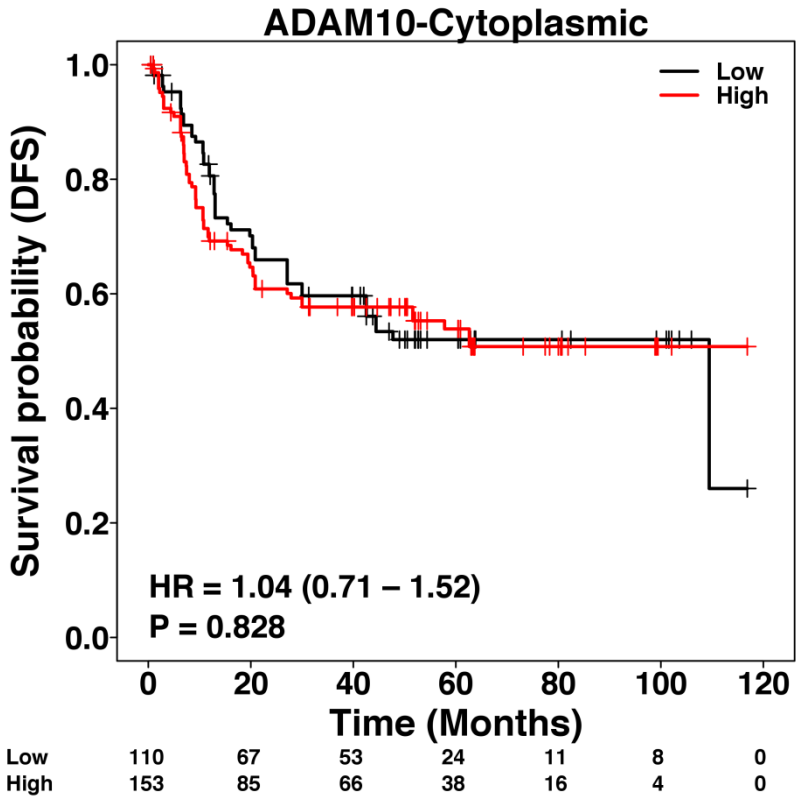
(table 6.2). The cytoplasmic ADAM17 also remained as an independent prognostic indicator for DFS (p= 0.013) (table 6.3).

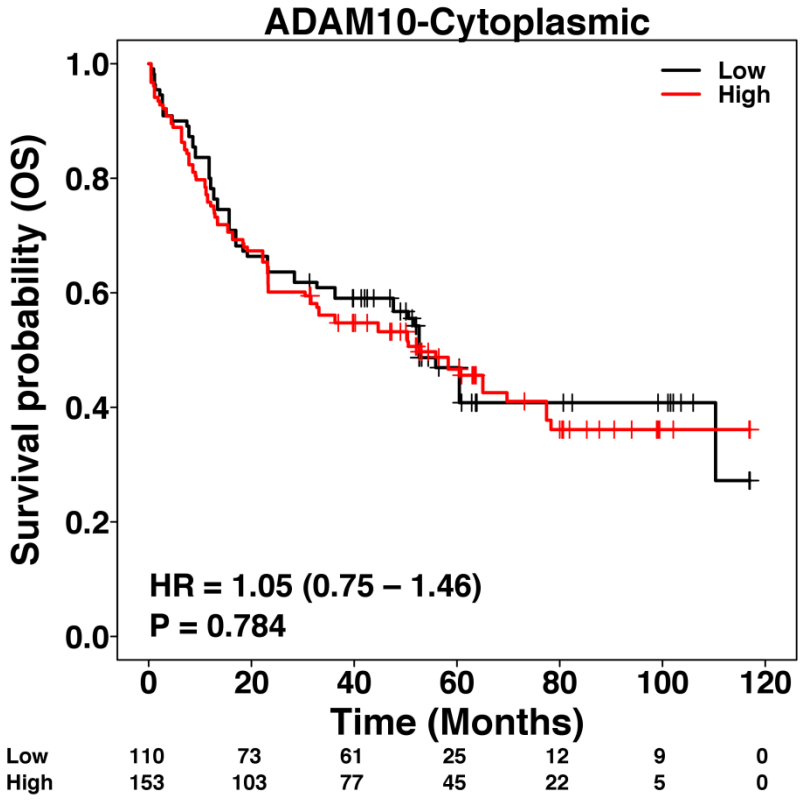
A



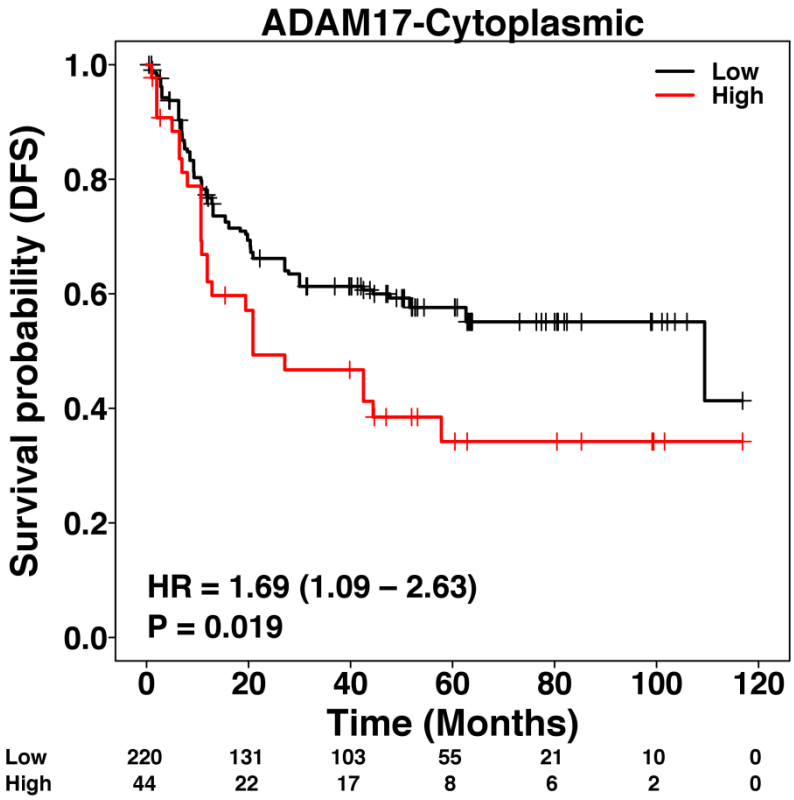


B


ADAM10-Cytoplasmic
Low
High
Survival probability (DFS)
1.0
0.8
0.6
0.4
0.2
0.0
HR = 1.04 (0.71 – 1.52)
P = 0.828
0
20
40
60
80
100
120
Time (Months)
Low 110 67 53 24 11 8 0
High 153 85 66 38 16 4 0


ADAM10-Cytoplasmic
Low
High
Survival probability (OS)
1.0
0.8
0.6
0.4
0.2
0.0
HR = 1.05 (0.75 – 1.46)
P = 0.784
0
20
40
60
80
100
120
Time (Months)
Low 110 73 61 25 12 9 0
High 153 103 77 45 22 5 0

C


ADAM17-Cytoplasmic
Low
High
Survival probability (DFS)
1.0
0.8
0.6
0.4
0.2
0.0
HR = 1.69 (1.09 – 2.63)
P = 0.019
0
20
40
60
80
100
120
Time (Months)
Low 220 131 103 55 21 10 0
High 44 22 17 8 6 2 0

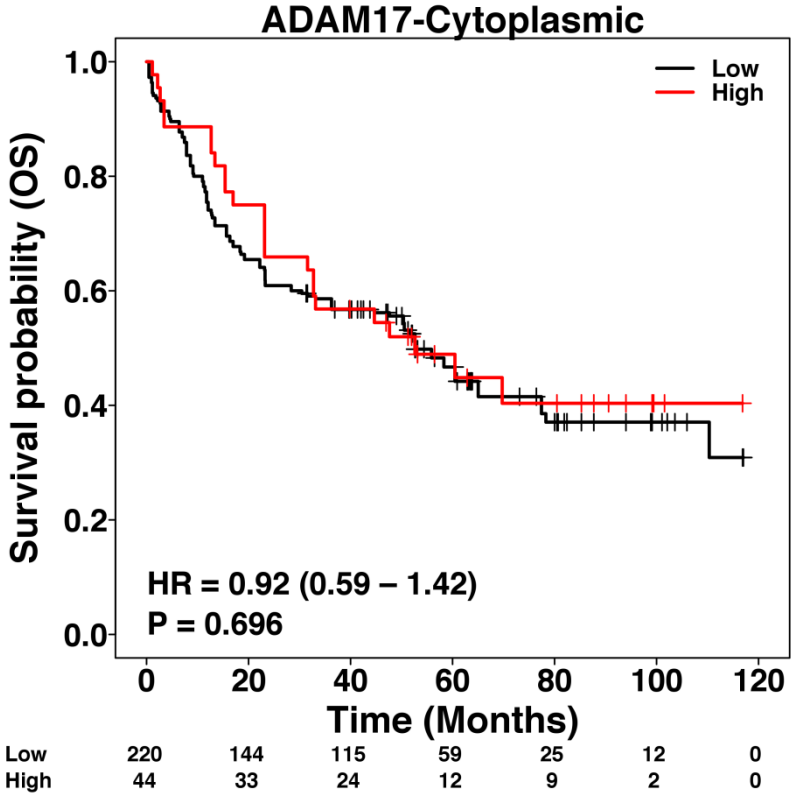

ADAM17-Cytoplasmic
Low
High
Survival probability (OS)
1.0
0.8
0.6
0.4
0.2
0.0
HR = 0.92 (0.59 – 1.42)
P = 0.696
0
20
40
60
80
100
120
Time (Months)
Low 220 144 115 59 25 12 0
High 44 33 24 12 9 2 0

Figure 6.4. ADAM10 and ADAM17 are prognostic biomarkers in HNSCC patients

Survival analysis using Kaplan-Meier method and the Cox proportional hazards model was performed for nuclear ADAM10 (n=265) (A), cytoplasmic ADAM10 (n=263) (B) and cytoplasmic ADAM17 (n=264) (C). Based on the IRS scores for ADAM10 and ADAM17, DFS and OS of patients were plotted and the differences between the groups were evaluated by Log-rank test.

Multivariate Analysis For DFS	Hazard Ratio	P-value
Nuclear ADAM10	2.39	0.048
Sex	2.42	0.002
Stage (baseline = Stage 1)		
Stage=2	0.24	0.034
Stage=3	0.71	0.544
Stage=4a	0.84	0.745
Stage=4b	7.38	0.003
Grade (baseline = well differentiated)		
Grade=moderate	0.83	0.617
Grade=moderate-poor	0.28	0.017
Grade=poor	0.56	0.137

Table 6.2. Multivariate analysis of nuclear ADAM10 expression adjusted for sex, stage and grade in HNSCC patients.

Multivariate Analysis For DFS	Hazard Ratio	P-value
Cytoplasmic ADAM17	1.77	0.013
Sex	2.27	0.004
Stage (baseline = Stage 1)		
Stage=2	0.26	0.046
Stage=3	0.76	0.626
Stage=4a	0.92	0.882
Stage=4b	10.21	0.000
Grade (baseline = well differentiated)		
Grade=moderate	0.81	0.567
Grade=moderate-poor	0.25	0.010
Grade=poor	0.55	0.123

Table 6.3. Multivariate analysis of cytoplasmic ADAM17 expression adjusted for sex, stage and grade in HNSCC patients.

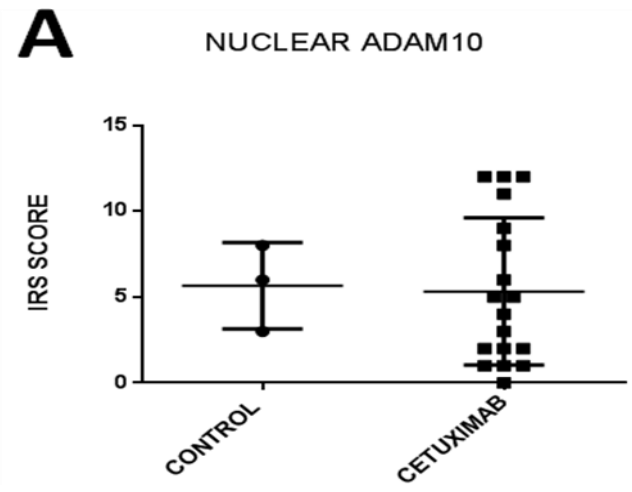
6.2.4. Tumour samples from HNSCC patients who underwent a window study of cetuximab treatment

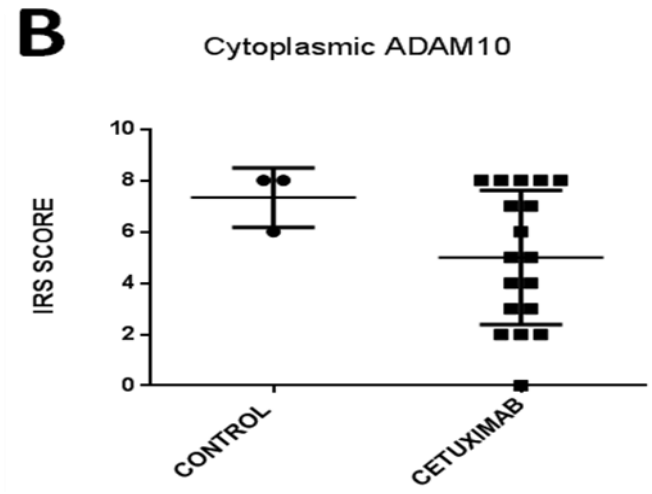
6.2.4.1. The expression levels of ADAM10 and ADAM17 in tumour samples from HNSCC patients treated with cetuximab monotherapy

From our collaborators in Brussels, we obtained formalin-fixed paraffin-embedded tumour excision samples from 21 HNSCC patients who underwent a cetuximab window study. The characteristics of these patients have been previously described (Schmitz *et al.* 2013). Briefly, 18 patients received 3

doses of cetuximab before primary surgery. These patients were treated with 400 mg/m^2 of cetuximab on day -15 and 250 mg/m^2 on day -8 and -1 prior to surgery. The tumour biopsies were performed before and after cetuximab treatment (just before excision surgery). Unfortunately, the biopsy samples before and after cetuximab window study were all used for previous research work and only the post-cetuximab treated excision samples were provided by the collaborators. However, three patients did not receive cetuximab and their tumour samples were used as the controls.

The expression levels of ADAMs were compared between post-cetuximab treated (n=18) and the control untreated patients (n=3). The samples were stained with either ADAM10 or ADAM17 antibodies and were scored as described previously. No statistically significant differences were observed in the expression level of ADAM10 (nuclear and cytoplasmic) (figure 6.5A&B) or ADAM17 (cytoplasmic) (figure 6.5C) between the control and post-cetuximab treated samples (figure 6.5).





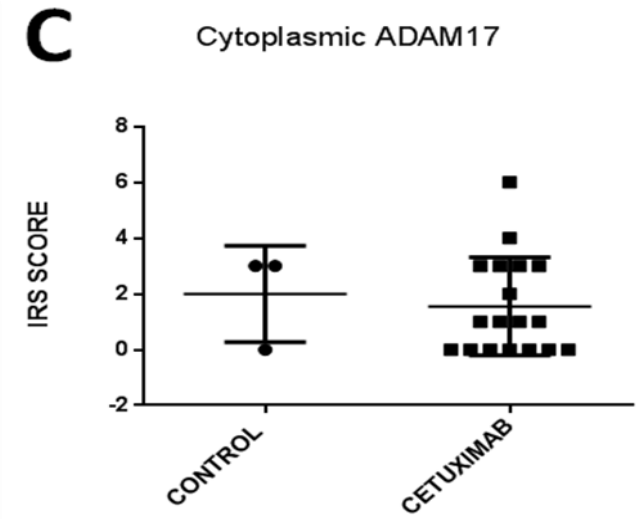


Figure 6.5. The expression levels of ADAM10 and ADAM17 in tumour samples from patients who underwent a cetuximab window study

Untreated and post-cetuximab treated samples from a cetuximab window study were stained for ADAM10 (A&B) or ADAM17 (C) by IHC procedure and the immunostainings were scored according to IRS system. 1:200 dilution of ADAM10 and 1:100 dilutions of ADAM17 antibodies were used.

6.2.4.2. Correlation of ADAMs expression with patients' characteristics

The associations between ADAM10 and ADAM17 expression levels with a number of co-variables were tested by Fisher's exact test. To choose a suitable cut-off point, we initially thought about using the same cut-off point as the retrospective Oxford series, i.e. IRS 0-1 versus IRS ≥ 2. However, most of the ADAM10 staining from cetuximab-treated samples was higher than those in the Oxford retrospective series. For example, only one patient had a cytoplasmic ADAM10 staining of IRS 0-1. This is not too surprising since previously our lab had shown that another ErbB inhibitor, trastuzumab induces an upregulation of ADAM10 (Feldinger *et al.* 2014). It is possible that cetuximab increased the ADAM10 expression but this could not be assessed in this study as we did not have pre and post-cetuximab treated samples as stated earlier. Therefore, we used alternative cut-off points. For ADAM10 staining, samples were grouped into high and low ADAM10 groups using two cut off points. The first of which was using median IRS score (median=6) and the groups were split into either IRS ≥ median (i.e. IRS 6-12) versus IRS < median (i.e. IRS 0-5). The second cut-off point was using the top tertile's IRS scores (i.e. IRS ≥ 8) versus the lower two tertiles' IRS scores (i.e. IRS < 8). For ADAM17, the staining was much weaker than ADAM10 staining. Attempt was also used to split the groups based on median IRS score. However, only 1 patient had an IRS ≥ median. Therefore, the cut-off point from the retrospective Oxford cohorts was used: IRS scores of 0-1 were placed in low ADAM17 expression group and samples with IRS score of ≥ 2 were classified as high ADAM17 expression group. No significant association was observed between the two classification factors except for tobacco use. With the top

tertile's cut-off point there was a significant relationship between smoking and nuclear ADAM10 expression level (P=0.0359) (table 6.4, 6.5, & 6.6).

Variables	Nuclear ADAM10		Total	P-value	Nuclear ADAM10		Total	P-value
	Low IRS 0-5	High IRS 6-12			Low IRS Lower Two Tertiles	High IRS Top Tertile		
Gender								
Male	9	6	15	1	11	4	15	1
Female	2	1	3		2	1	3	
Total	11	7	18		13	5	18	
HPV Status								
HPV -	10	6	16	1	12	4	16	0.4902
HPV +	1	1	2		1	1	2	
Total	11	7	18		13	5	18	
Tobacco								
Tobacco -	7	1	8	0.0656	8	0	8	0.0359*
Tobacco +	4	6	9		5	5	10	
Total	11	7	18		13	5	18	
Alcohol								
Alcohol -	8	4	12	0.6267	9	2	11	0.3260
Alcohol +	3	3	6		4	3	7	
Total	11	7	18		13	5	18	
Clinical Staging								
Stage I&II	7	6	13	0.5956	9	4	13	1
Stage III&IV	4	1	5		4	1	5	
Total	11	7	18		13	5	18	
Surgical Margins								
R 0	9	7	16	0.4967	11	5	16	1
R 1	2	0	2		2	0	2	
Total	11	7	18		13	5	18	
Extracapsular Spread								
ECS 0	9	4	13	0.3260	10	3	13	0.5827
ECS 1	2	3	5		3	2	5	
Total	11	7	18		13	5	18	
Tumour Site								
Oral Cavity	9	6	15	1	11	4	15	1
Larynx	2	1	3		2	1	3	
Total	11	7	18		13	5	18	
Disease Recurrence								
DR -	9	6	15	1	11	4	15	1
DR +	2	1	3		2	1	3	
Total	11	7	18		13	5	18	

Table 6.4. Correlation of nuclear ADAM10 expression with patients' characteristics and outcome.

Variables	Cytoplasmic ADAM10		Total	P-value	Cytoplasmic ADAM10		Total	P-value
	Low IRS 0-5	High IRS 6-12			Low IRS Lower Two Tertiles	High IRS Top Tertile		
Gender								
Male	8	7	15	1	11	4	15	1
Female	2	1	3		2	1	3	
Total	10	8	18		13	5	18	
HPV Status								
HPV -	9	7	16	1	11	5	16	1
HPV +	1	1	2		2	0	2	
Total	10	8	18		13	5	18	
Tobacco								
Tobacco -	6	2	8	0.1880	7	1	8	0.3137
Tobacco +	4	6	10		6	4	10	
Total	10	8	18		13	5	18	
Alcohol								
Alcohol -	7	4	11	0.6305	9	2	11	0.3260
Alcohol +	3	4	7		4	3	7	
Total	10	8	18		13	5	18	
Clinical Staging								
Stage I&II	7	6	13	1	9	4	13	1
Stage III&IV	3	2	5		4	1	5	
Total	10	8	18		13	5	18	
Surgical Margins								
R 0	9	7	16	1	12	4	16	0.4902
R 1	1	1	2		1	1	2	
Total	10	8	18		13	5	18	
Extracapsular Spread								
ECS 0	7	6	13	1	9	4	13	1
ECS 1	3	2	5		4	1	5	
Total	10	8	18		13	5	18	
Tumour Site								
Oral Cavity	8	7	15	1	11	4	15	1
Larynx	2	1	3		2	1	3	
Total	10	8	18		13	5	18	
Disease Recurrence								
DR -	8	7	15	1	10	5	15	0.5221
DR +	2	1	3		3	0	3	
Total	10	8	18		13	5	18	

Table 6.5. Correlation of cytoplasmic ADAM10 expression with patients' characteristics and outcome.

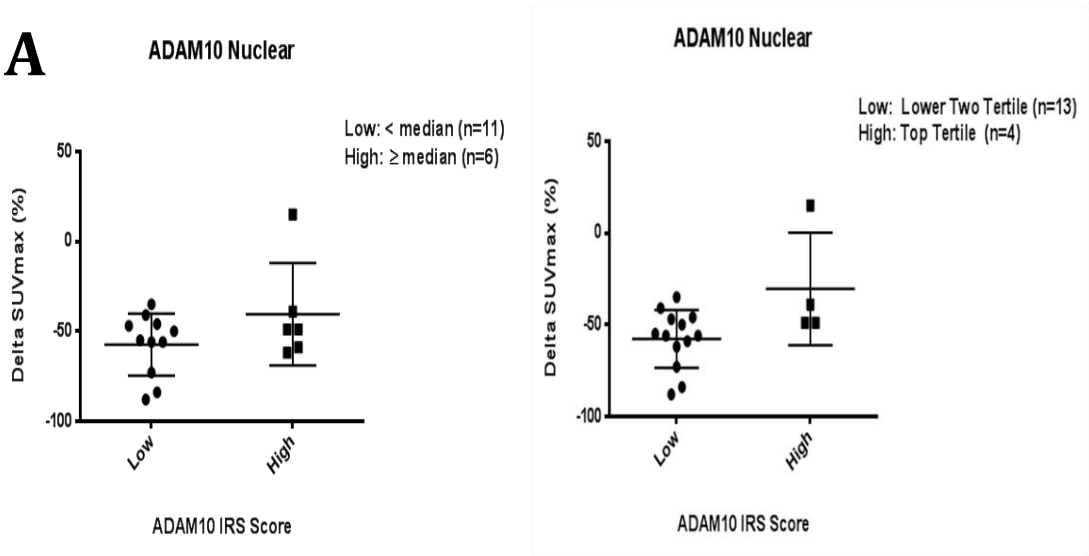
Variables	Cytoplasmic ADAM17		Total	P-value
	Low IRS 0-1	High IRS 2-12		
Gender				
Male	9	6	15	1
Female	2	1	3	
Total	11	7	18	
HPV Status				
HPV -	9	7	16	0.4967
HPV +	2	0	2	
Total	11	7	18	
Tobacco				
Tobacco -	6	2	8	0.3665
Tobacco +	5	5	10	
Total	11	7	18	
Alcohol				
Alcohol -	7	4	11	1
Alcohol +	4	3	7	
Total	11	7	18	
Clinical Staging				
Stage I&II	8	5	13	1
Stage III&IV	3	2	5	
Total	11	7	18	
Surgical Margins				
R 0	10	6	16	1
R 1	1	1	2	
Total	11	7	18	
Extracapsular Spread				
ECS 0	7	6	13	0.5956
ECS 1	4	1	5	
Total	11	7	18	
Tumour Site				
Oral Cavity	9	6	15	1
Larynx	2	1	3	
Total	11	7	18	
Disease Recurrence				
DR -	9	6	15	1
DR +	2	1	3	
Total	11	7	18	

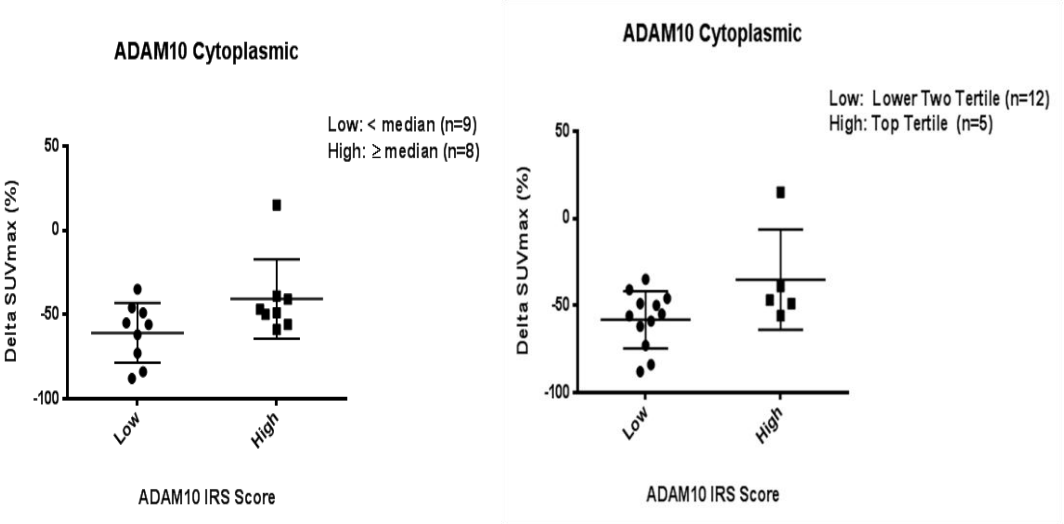
Table 6.6. Correlation of cytoplasmic ADAM17expression with patients' characteristics and outcome.

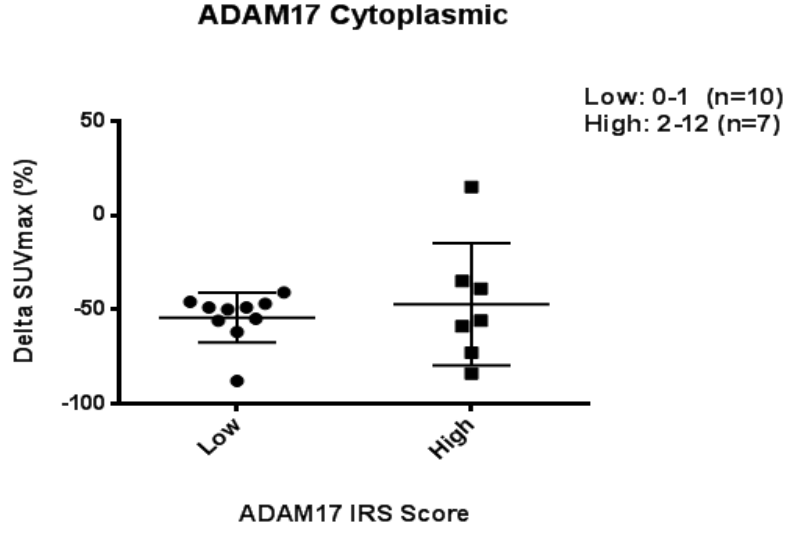
6.2.4.3. Predictive significance of ADAM10 and ADAM17 in HNSCC

The predictive value of ADAM10 and 17 expression in relation to cetuximab response was assessed by evaluating the correlation between ADAM10 and 17 staining with changes in ^{18}F-Fluorodeoxyglucose (^{18}F-FDG) uptake in the FDG-PET quantified by standardized uptake values (SUV). The percentage change in SUV, Delta maximal SUV (ΔSUVmax = [(SUVmax after – SUVmax before) / SUVmax before)] * 100)] was correlated with ADAM10 and ADAM17 expression levels. Low ADAM10 (nuclear and cytoplasmic) and ADAM17 (cytoplasmic) IRS scores were associated with ~ 60% decrease in ΔSUVmax compared to ~ 40% decrease in high IRS group, however, this was not statistically significant (figure 6.6A). In addition to SUV, the predictive value of the expression levels of ADAM10 and ADAM17 on the proliferative rates of cetuximab treated tumours was also assessed by analyzing the percentage change in the proliferation biomarker Ki-67. Lower ADAM10 and ADAM17 staining was associated with a greater decrease in the percentage change of Ki67, ΔKi67 value (ΔKi67= [(Ki67after – Ki67 before) / Ki67 before)] * 100). Although an overall trend was observed, statistically significant changes were only observed with the top tertile's cut-off points for both nuclear and cytoplasmic ADAM10 (figure 6.6 B).

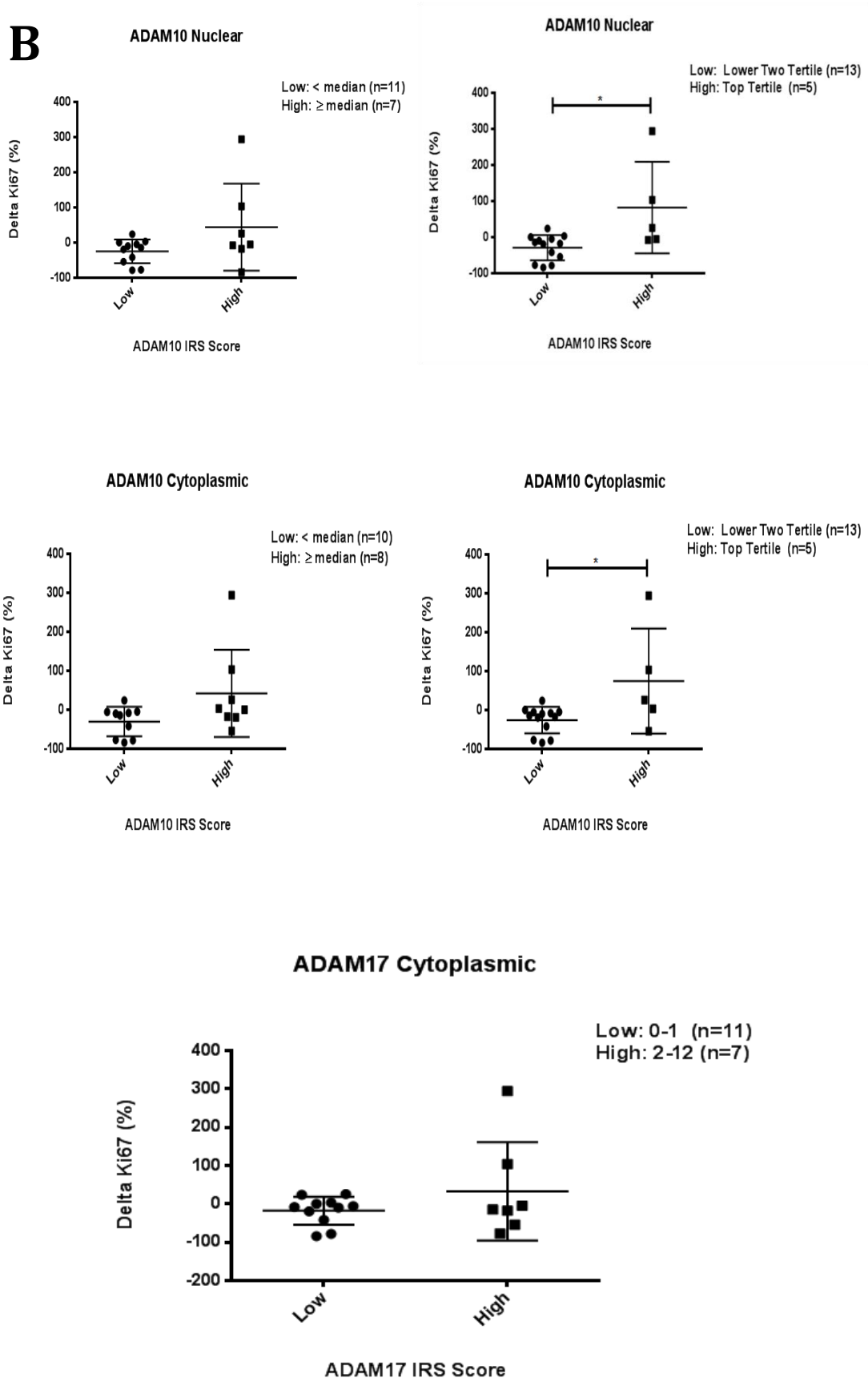
A


ADAM10 Nuclear
Low: < median (n=11)
High: ≥ median (n=6)
Delta SUVmax (%)
50
0
-50
-100
Low
High
ADAM10 IRS Score
ADAM10 Nuclear
Low: Lower Two Tertile (n=13)
High: Top Tertile (n=4)
Delta SUVmax (%)
50
0
-50
-100
Low
High
ADAM10 IRS Score


ADAM10 Cytoplasmic
Low: < median (n=9)
High: ≥ median (n=8)
Delta SUVmax (%)
50
0
-50
-100
Low
High
ADAM10 IRS Score
ADAM10 Cytoplasmic
Low: Lower Two Tertile (n=12)
High: Top Tertile (n=5)
Delta SUVmax (%)
50
0
-50
-100
Low
High
ADAM10 IRS Score


ADAM17 Cytoplasmic
Low: 0-1 (n=10)
High: 2-12 (n=7)
Delta SUVmax (%)
50
0
-50
-100
Low
High
ADAM17 IRS Score

B


ADAM10 Nuclear
Low: < median (n=11)
High: ≥ median (n=7)
Delta Ki67 (%)
400
300
200
100
0
-100
Low
High
ADAM10 IRS Score
ADAM10 Nuclear
Low: Lower Two Tertile (n=13)
High: Top Tertile (n=5)
*
Delta Ki67 (%)
400
300
200
100
0
-100
Low
High
ADAM10 IRS Score
ADAM10 Cytoplasmic
Low: < median (n=10)
High: ≥ median (n=8)
Delta Ki67 (%)
400
300
200
100
0
-100
Low
High
ADAM10 IRS Score
ADAM10 Cytoplasmic
Low: Lower Two Tertile (n=13)
High: Top Tertile (n=5)
*
Delta Ki67 (%)
400
300
200
100
0
-100
Low
High
ADAM10 IRS Score

ADAM17 Cytoplasmic
Low: 0-1 (n=11)
High: 2-12 (n=7)
Delta Ki67 (%)
400
300
200
100
0
-100
-200
Low
High
ADAM17 IRS Score

Figure 6.6. Predictive significance of ADAM10 and ADAM17 in HNSCC

Cetuximab treated samples were stratified into low and high IRS score groups according to the indicated cut off points. ADAM10 and ADAM17 expression levels and their correlation with ΔSUVmax and ΔKi67 are shown in part A and part B respectively.

6.3. DISCUSSION

Biomarkers are important in detecting cancer, evaluating prognosis and predicting response to cancer treatment. Studies in recent years have suggested the potential of ADAM family members as cancer biomarkers.

ADAMs as diagnostic markers

Overexpression of ADAM12 has been reported in human glioblastoma (Kodama *et al.* 2004), liver (Le Pabic *et al.* 2003), gastric, colon and breast cancers (Iba *et al.* 1999) as well as bladder (Fröhlich *et al.* 2006) and lung cancer (Shao *et al.* 2014). Roy *et al.* (2004) have shown the role of ADAM12 in diagnosis of breast cancer and prediction of disease status. In their study urine samples from breast cancer patients were analyzed and compared with control samples. Results showed that ADAM12 protein level were higher in patients samples compared to the control and that increased significantly with disease progression (Roy *et al.* 2004). This group also assessed the potential of urinary ADAM12 in identifying women at a higher risk of developing breast cancer (Pories *et al.* 2008). For this purpose urine samples from women with atypical hyperplasia and Lobular Carcinoma In Situ (LCIS) were compared with samples from healthy individuals. ADAM12 level was found to be significantly higher in women with atypical hyperplasia and LCIS compared to healthy controls (Pories *et al.* 2008). The significance of ADAM12 as a diagnostic marker for bladder cancer has been reported by Fröhlich *et al.* (2006). This group found a higher level of ADAM12 in the patients' urine samples compared with samples from healthy controls. Reduction in the level

of ADAM12 was observed in the majority of patients whose tumours were removed by surgery. Interestingly increased level of ADAM12 has been reported in patients with recurrence disease (Fröhlich *et al.* 2006). Compared to normal controls, a significantly higher expression of ADAM12 has also been reported in serum and urine samples of patients with SCLC patients (Shao *et al.* 2014). In this study patients with extensive-stage disease had a significantly higher level of ADAM12 compared to patients with limited disease (Shao *et al.* 2014). Overexpression of ADAM28 has been reported in renal carcinoma (Roemer *et al.* 2004) and NSCLC (Ohtsuka *et al.* 2006). The importance of ADAM28 in cancer diagnosis was reported by Kuroda *et al.* (2010). This group claimed that they have observed a higher serum level of ADAM28 in patients with NSCLC compared to healthy controls. A positive correlation was observed between ADAM28 serum level with tumour stage, size, recurrence and lymph node metastasis (Kuroda *et al.* 2010).

ADAMs as prognostic markers

ADAMs have been investigated by different groups for their potential as prognostic markers. Upregulation of ADAMs has been reported in different tumours and often correlates with tumour progression and aggressive disorder (Duffy *et al.* 2009). As described above ADAM28 is highly expressed in NSCLC (Ohtsuka *et al.* 2006). Among the 11 ADAM metalloproteinases which were screened for their mRNA expression levels, overexpression of ADAM28 was found to correlate with cell proliferation and lymph node metastasis in NSCLC (Ohtsuka *et al.* 2006). O'Shea *et al.* (2003) studied the

involvement of ADAM9 in breast cancer. They found that ADAM9 mRNA is expressed more often in breast carcinomas compared to normal breast tissues. Their results also showed that the induced expression level of the 84 kDa mature form of ADAM9 correlates with breast nodal status since a significantly higher expression level was observed in node-positive compared to node-negative primary cancers. This is also positively correlated with HER-2 protein levels (O'Shea *et al*. 2003). By investigating the expression levels of ADAM9 protein and mRNA, Fritzsche *et al.* (2008) have shown an upregulation of ADAM9 in prostate cancer. ADAM9 protein overexpression was found to be associated with shortened prostate-specific antigen RFS (Fritzsche *et al.* 2008). Association between ADAM9 overexpression and poor survival has also been reported in pancreatic ductal adenocarcinoma (Grützmann *et al.* 2004). ADAM15 is also implicated in human cancer. In a study by Kuefer *et al*. (2006), ADAM15 protein expression was assessed in TMAs and its overexpression was found in several types of adenocarcinoma, in particular in breast and prostate cancer samples. This group also found a strong correlation between ADAM15 protein expression level and breast and prostate cancer metastasis (Kuefer *et al.* 2006). ADAM8 was found to be significantly overexpressed in cancerous tissues compared with normal tissue in patients with renal cancer. The expression level of ADAM8 was associated with distant metastases and short survival in these patients (Roemer *et al.* 2004). Overexpression of ADAM8 has also been reported in lung cancer, particularly in advanced-stage IIIB/IV adenocarcinoma. Compared to healthy controls, higher serum levels of ADAM8 have been observed in patients' samples (Ishikawa *et al.* 2004). Overexpression of ADAM8 has been reported

in metastatic HNSCC tumours (Stokes *et al.* 2010). Zielinski *et al.* (2012) have also reported an increased expression of ADAM8 in HNSCC and have suggested its correlation with worse survival (Zielinski *et al.* 2012). High expression level and increased activity of ADAM8 and ADAM19 has been observed in human primary brain tumour and has been found to be associated with tumour invasiveness (Wildeboer *et al.* 2006). Upregulation of ADAM19 has also been reported in renal cancer patients (Roemer *et al.* 2004). Different studies have shown the importance of ADAM10 in various types of tumours. Yavari *et al.* (1998) have reported the overexpression of ADAM10 in pheochromocytomas and neuroblastomas (Yavari *et al.* 1998). Researchers have also shown the involvement of ADAM10 in prostate cancer (Karan *et al.* 2003, McCulloch *et al.* 2004, Arima *et al.* 2007). It is believed that the nuclear translocation of ADAM10 is responsible for tumour growth and progression in prostate cancer patients (Arima *et al.* 2007). Overexpression of ADAM10 has also been reported in Oral Squamous Cell Carcinoma (OSCC) (Ko *et al.* 2007, Jones *et al.* 2013). The reduced migration and invasion of OSCC upon silencing of ADAM10 indicates the importance of ADAM10 in OSCC invasion (Jones *et al.* 2013). The upregulation of ADAM10 and its correlation with tumour progression and metastasis have also been described in tongue squamous cell carcinoma (kai *et al.* 2014). The role of ADAM10 in breast cancer formation and progression has also been investigated in recent years. Increased expression level of ADAM10 in breast cancer has been reported by Liu *et al.* (2006). The expression level of ADAM10 has shown to be associated with aggressive disease and its inhibition was found to decrease cell growth, invasion and

migration in breast cancer tumours (Mullooly *et al.* 2012). Our group has also shown the correlation between high level of ADAM10 expression with poor relapse-free survival in patients with HER2+ breast cancer (Feldinger *et al.* 2014). Yue *et al.* (2013) have reported the overexpression of ADAM10 in hepatocellular carcinoma. The inhibition of proliferation, migration and invasion of HepG2 human hepatoma cells upon ADAM10 knockdown confirmed the significance of ADAM10 in tumour formation and progression in hepatocellular carcinoma (Yuan *et al.* 2013). Studies have also shown the overexpression of ADAM10 in colon cancer, which is believed to be associated with higher tumour stage (Knösel *et al.* 2005) and induced liver metastases (Gavert *et al.* 2013). The expression of ADAM10 has been found to be responsible for lymph node and distant metastasis as well as poor prognostic outcome in gastric cancer patients (Wang *et al.* 2011). The contribution of ADAM10 in the progression of pancreatic cancer (Gaida *et al.* 2010) osteosarcoma (Zhao *et al.* 2014), melanoma (Lee *et al.* 2010) and NSCLC (Guo *et al.* 2012) have also been reported. Overexpression of ADAM17 is known to have a major role in the development of Hepatocellular Carcinomas (HCC) (Ding *et al.* 2004) and is shown to decrease cisplatin-induced apoptosis in HCC through EGFR/PI3K/AKT pathway (Wang *et al.* 2013). Higher expression levels of ADAM17 mRNA and protein have been observed in Esophageal Squamous Cell Carcinoma (ESCC) compared to normal esophageal tissues (Liu *et al.* 2013). This is known to be an important factor in the formation, invasion and metastasis of ESCC (Liu *et al.* 2013). ADAM17 overexpression has been reported in colon cancer and was found to be associated with tumour growth and angiogenesis (Blanchot-Jossic *et al.*

2005). Involvement of ADAM17 in renal cancer has been reported by Roemer *et al.* (2004). Silencing of ADAM17 was shown to inhibit tumour cell growth, migration, invasion and inflammation in human renal carcinoma cell lines (Franovic *et al.* 2006). ADAM17 and its association with the disease progression have also been observed in ovarian cancer (Tanaka *et al.* 2005). ADAM17 was reported to be upregulated in breast cancer (Santiago-Josefat *et al.* 2007) and its upregulation was associated with poor prognosis (Kenny and Bissell 2007, McGowan *et al.* 2008). It is believed that ADAM17 plays a key role in breast cancer progression via the activation of the EGFR-PI3K-AKT signalling pathway (Zheng *et al.* 2009). McGowan *et al.* (2013) have also reported an upregulation of ADAM17 in triple negative breast cancer in comparison with non-triple negative cases (McGowan *et al.* 2013). ADAM17 is also known to induce brain tumour growth and invasion (Zheng *et al.* 2007) via EGFR-PI3K-AKT signalling pathway activation (Zheng *et al.* 2012). ADAM17 also plays a role in the growth and progression of gastrointestinal stromal tumour by shedding EGFR ligands and subsequent EGFR activation (Nakagawa *et al.* 2009). In comparison with non-cancerous gastric mucosa, higher levels of ADAM17 mRNA and protein have been detected in gastric cancer tissues. This was found to be associated with tumour invasion, lymph node metastases and distant metastases and was correlated with poor prognosis (Zhang *et al.* 2012). In NSCLC, it is reported that ADAM17 contributes to the tumourigenicity by regulating the expression of EGFR protein through the activation of Notch1 signalling (Zhou *et al.* 2006, Baumgart *et al.* 2010). Furthermore, ADAM17 downregulation resulted in a significant inhibition of cell proliferation and invasion *in vitro* as well as tumour

growth *in vivo* (Lv *et al.* 2014). Compared with normal epithelia, a higher expression level of ADAM17 mRNA has been observed in HNSCC. This was found to correlate with tumour stage and regional lymph node metastasis (Kornfeld *et al*. 2011). Lin *et al.* (2012) suggested that ADAM17 induces prostate cancer cell proliferation by targeting cyclin E, CDK2, p21 and p27, thus activating the EGFR/PI3K/AKT signalling pathway (Lin *et al*. 2012). In another study, ADAM17 was shown to play a role in prostate cancer cell invasion through cleavage of TGF-α (EGFR ligand) and consequent activation of EGFR-MEK-ERK signalling pathway (Xiao *et al*. 2012).

ADAMs as predictive markers

The predictive potentials of ADAM9, ADAM10, ADAM11 and ADAM12 in relation to tamoxifen treatment have been evaluated in breast cancer patients with recurrent disease. Patients with higher ADAM9 and ADAM11 mRNA levels were reported to respond better to tamoxifen therapy (Sieuwerts *et al*. 2005).

In this project the signifcance of ADAM10 and ADAM17 as prognostic and predictive biomarkers was investigated in HNSCC. IHC staining of Oxford head and neck tissue samples and tumour samples from HNSCC patients undergoing cetuximab window study demonstrated the nuclear and cytoplasmic localization of ADAM10 and cytoplasmic localization of ADAM17 in these samples. In gastric cancer cells, ADAM10 has been shown to localize in the cytoplasm or nucleus (Wang *et al.* 2011). Nuclear localization of

ADAM10 has also been reported in prostate cancer and has been shown to be associated with tumour growth and progression (Arima *et al.* 2007).

Compared to the normal tissues, higher expression levels of ADAM10 and ADAM17 were observed in tumour samples of TMAs from a cohort of HNSCC patients undergoing routine treatment. High nuclear ADAM10 and high cytoplasmic ADAM17 expression levels were associated with shorter DFS. Based on the results from multivariate analysis adjusted for sex, stage and grade, nuclear ADAM10 and cytoplasmic ADAM17 expression levels were found to be independent prognostic factors for DFS.

No differences were observed in the expression levels of ADAM10 and ADAM17 between the untreated and post-cetuximab treated tumour samples from HNSCC patients who underwent a window study of cetuximab before their surgery (Schmitz *et al.* 2013). However, pairs of pre- and post-cetuximab treated biopsy samples were not available from these patients to evaluate the effect of cetuximab on the expression levels of ADAM10 and ADAM17. Since only post-cetuximab excision samples are available with a few untreated samples not derived from the same patients, we could not accurately assess whether cetuximab could upregulate ADAM10 and ADAM17 in these patients.

In this study the predictive values of both ADAMs were also investigated. Results showed that patients with low ADAM10 and ADAM17 expression respond better to cetuximab in term of a greater reduction of Ki67 but no statistical difference was observed in the reduction of SUV despite a trend. This could be due to a small number of patients in this window study. The

associations between ADAM10 and ADAM17 expression levels with characteristics of cetuximab treated patients were also investigated in this study and a significant relationship was observed between smoking and nuclear ADAM10 expression level. The involvement of smoking in overexpression of ADAM10 or ADAM17 hasn't been reported, however, association between smoking and the activity of MMPs and ADAMs has been suggested (Heijink *et al.* 2011). It has been reported that cigarette smoke decreases the expression of TIMPs which play an important role in the regulation of MMPs and ADAMs' activity (Heijink *et al.* 2011). Lemjabbar-Alaoui *et al.* (2011) have reported that tobacco smoke is responsible for the activation of ADAM17 and subsequent amphiregulin release and EGFR phosphorylation in lung cells (Lemjabbar-Alaoui *et al.* 2011).

This study had a number of limitations. Since patients' tissue samples were limited, the secondary antibody staining alone (with no primary control) was performed in SCC15 cell pellet which is very different from human normal tissues with significantly more heterogeneity and surrounding tumour microenvironment (fibrous tissues, vessels, etc). In addition there was a lack of negative control (cell line or tissue sample which is known not to express the protein of interest) and positive control (cell line or tissue which is known to express the protein of interest). Background staining was detected in various types of cells such as fibroblast, lymphocytes, muscles, blood vessels and nerves. However, as all samples were stained with relevant antibodies on the same day and under the same experimental conditions, the staining of invasive tumours was evaluated by comparing the intensity and distribution of ADAMs expression levels between samples. Another limitation noted in the

evaluated samples was the presence of noticeable heterogeneity within and between samples. Heterogeneity in the expression of proteins in different parts of tumours is a common problem and has been observed in other types of tumours such as breast cancer. Use of TMAs has also been associated with some criticisms. Due to tumour heterogeneity it remains in debate whether these small sections of tissues can represent the entire tumour.

Determining clinically important cut-off points to validate the positivity of prognostic and predictive biomarkers has been associated with some challenges even for pre-specified biomarkers such as HER2 in breast cancer. According to FDA guideline breast tumours are defined as HER2+ if immunostaining of 3+ is observed in at least 10% of tumour cells. However, the criteria was changed to 3+ IHC staining in > 30% of invasive tumour cells by the American Society of Clinical Oncology and the College of American Pathologists (ASCO/CAP) in 2007 (Wolff *et al.* 2007). At that time, ASCO/CAP also changed the guideline for HER2 testing by FISH. The ratio of HER2/CEP17 was modified from ≥ 2.0 to >2.2 for HER2 gene amplification (Wolff *et al.* 2007). However, the criteria have now been changed back to the original FDA guideline (Wolff *et al.* 2013). This demonstrates the challenges involved in determining the optimal cut-off points for well studied and established biomarker such as HER2, which could be even more complicated for new biomarkers such as ADAM family members. Up to date, there is no published guideline for determining an optimal cut-off point for ADAM10 and ADAM17 proteins, which is a typical problem with novel tumour biomarkers. Therefore, in this exploratory study different cut-off points were tested as

described above. However, these cut-off points need to be refined and validated in larger independent cohorts.

In general, although IHC is a well-established and widely used technique, it is associated with a number of limitations and drawbacks. There are various factors which can affect the quality of the staining of tissue sections. Type of specimen, time from excision to sample preparation and fixation, method and length of storage as well as the quality of tissue sectioning and slide preparation are the factors which can affect the accuracy of the test results considerably. Other factors such as selection and validation of specific antibody as well as optimisation of antibody dilution and length of incubation also play important roles in achieving accurate results. In addition methodologies which are used in different steps of IHC experiments such as deparaffinization and antigen retrieval techniques and use of different reagents, buffers and detection system can all lead to variability of IHC results. In addition evaluation of immunostaining is very subjective and can influence the interpretation of the test results to a great extent (Bilous *et al.* 2003).

In summary, upregulation of ADAM10 and ADAM17 was observed in tumours compared to normal tissues in HNSCC. High nuclear ADAM10 and high cytoplasmic ADAM17 expression levels were associated with shorter DFS. Results from this study also showed that high ADAM10 and ADAM17 expression were associated with poorer response to cetuximab in HNSCC. However, more studies are required to further validate the prognostic and predictive significance of ADAM10 and ADAM17 in HNSCC patients.

CHAPTER 7

GENERAL DISCUSSION

7. GENERAL DISCUSSION

Overexpression of erbB family members including EGFR has been reported in several epithelial malignancies including HNSCC (Bei *et al*. 2004, Schartinger *et al.* 2004, Bei *et al.* 2001, Harari *et al.* 2007). Therefore, inhibition of EGFR signalling is an important strategy for cancer therapies. Up to date a various number of EGFR targeted therapies have been investigated in the pre-clinical and clinical settings in HNSCC. However, cetuximab remains as the only FDA approved EGFR inhibitor for HNSCC, and resistance to cetuximab is a common problem. Therefore many groups have been investigating the resistance mechanisms to cetuximab, in order to propose suitable approaches to overcome its limitation in clinic.

As described in chapter 1, several mechanisms of resistance have been proposed. In a study by Rajput *et al.* (2007) the overexpression of TGF-α has been shown to be involved in the development of resistance to cetuximab in colon cancer cells (Rajput *et al.* 2007). In another study an upregulation of EGF, HB-EGF, BTC and amphiregulin have been reported in cetuximab resistant NSCLC clones (Li *et al.* 2009). However, to the best of my knowledge the role of HER ligands' sheddases, ADAM10 and ADAM17 in cetuximab resistance has not been reported.

In my project the involvement of these ADAM proteases in the development of resistance to cetuximab was investigated in HNSCC. The results showed that exposure to cetuximab promotes the shedding and release of HER ligands, BTC and NRG-1 into the media. Cetuximab was also shown to be

responsible for increased expression levels of ADAM10 and ADAM17. The role of ADAM10 and ADAM17 in the release of their substrates was shown by using inhibitors and specific siRNAs against these metalloproteases. ADAMs inhibition reduced cetuximab-mediated HER ligands upregulation in the parental and cetuximab resistant HNSCC cell lines. Inhibition of ADAMs was found to be very effective in targeting HER receptors' signalling in the parental cell lines and could overcome resistance to cetuximab in HNSCC. Based on these findings ADAM-mediated ligands release is proposed as a novel mechanism of resistance to cetuximab in HNSCC. Therefore, inhibition of these metalloproteases may be considered as a promising approach to improve response or overcome resistance to cetuximab in HNSCC patients who do not respond to cetuximab or develop acquired resistance to the drug.

In recent years several other strategies have been proposed to enhance response to cetuximab in different cancers. One approach is the dual targeting of EGFR, using inhibitors from two distinct mechanisms of action such as mAbs and TKIs. It was hypothesized that such combination treatment could result in additive or synergistic inhibitory effects since cetuximab monotherapy which targets the EGFR ligand binding site failed to effectively inhibit the phosphorylation of EGFR and other HER receptors in my study. Therefore, the effect of combining cetuximab, EGFR mAb, with gefitinib, EGFR TKI, was investigated in HNSCC. Gefitinib was found to be very effective in overcoming resistance to cetuximab. Studies by other groups (discussed in chapter 5) have also shown the promising pre-clinical activity of

combination treatment, which have led to the evaluation of this strategy in the clinical setting.

Baselga *et al.* (2006) evaluated the safety and activity of combining cetuximab with gefitinib in patients with advanced CRC, HNSCC and NSCLC and the combined anti-EGFR therapy was found to be well tolerated and showed promising clinical activity (Baselga *et al.* 2006). In another study, the combination of cetuximab and gefitinib was investigated in patients with advanced or metastatic NSCLC and 31% of the patients achieved stable disease although none achieved a partial response (Ramalingam *et al.* 2008).

Another proposed strategy to improve cetuximab response is to target multiple HER family receptors using panHER inhibitors. As shown in my study, cetuximab induced escape pathways via ligand-dependent activation of multiple ErbB receptors. Therefore, the combination treatment of cetuximab with a panHER inhibitor was hypothesized to have a greater inhibitory effect compared to cetuximab alone.

As afatinib is currently in phase III clinical trial for the treatment of HNSCC, its combination with cetuximab was evaluated in my D.Phil. study. The results showed the effectiveness of afatinib as a single agent or in combination with cetuximab in targeting HER receptor signalling in HNSCC cells. Afatinib could also overcome acquired resistance to cetuximab in HNSCC cells.

The anti-tumour activity of afatinib in combination with cetuximab has been investigated in the clinical settings. In a study by Janjigian *et al.* (2011), the safety and activity of combined treatment with afatinib and cetuximab was investigated in NSCLS patients, including patients who had prior treatment with erlotinib or gefitinib. The combination treatment was well tolerated and 8/22 patients who received the higher dose of cetuximab (500 mg/m^2/w with afatinib 40 mg/d) had partial response (Janjigian *et al.* 2011). However, so far there has been no study done to assess the effect of combination of cetuximab with afatinib in HNSCC patients.

Although the combination therapies to target HER signalling seems to have promising efficacy in both the pre-clinical and clinical setting, this strategy is associated with a number of limitations and drawbacks. It is necessary to choose and optimize the most efficient doses of drugs and treatment schedules. This would be very crucial for achieving the maximum efficacy and minimizing potential treatment-related toxicities. Since multi-targeted therapies are expected to be associated with a higher incidence of treatment-related adverse effects, the selection of individuals who may benefit from the combination therapies is crucial. In addition, although using a combination of inhibitors with distinct mechanisms of actions to target HER signalling pathways might have additive or synergistic effects compared to cetuximab alone in the pre-clinical models, this strategy may not yield optimal outcomes in clinic. This is because there may only be a small subset of HNSCC patients who may benefit from the combination of cetuximab with TKIs. However, it will be a challenge to select and stratify patients who would

benefit from particular EGFR inhibitor monotherapy (cetuximab or reversible TKIs or irreversible TKIs) or their combination. In addition, HNSCC tumours are heterogeneous and there are various other pathways involved in the process of HNSCC tumour formation which are not affected by HER-specific inhibitors. Therefore, inhibition of other pathways such as c-MET and VEGFR may be able to overcome the limitations of HER-targeted therapies in HNSCC patients.

Alterations in c-MET gene have been reported in various types of cancers such as HNSCC (Marshall and Kornberg 1998, Di Renzo et al. 2000, Kim et al. 2006, Ghadjar et al. 2009, Seiwert et al. 2009, Knowles et al. 2009) and have been found to be responsible for aggressive phenotype and poor prognosis (Ma et al. 2003, Peruzzi and Bottaro 2006). Involvement of *c-MET* amplification in the development of acquired resistance to erlotinib and gefitinib has been shown in NSCLC (Bean *et al.* 2007, Engelman *et al.* 2007). In a recent study, Song *et al.* (2014) have reported that cetuximab-induced c-MET activation is responsible for the resistance to cetuximab in colon cancer cells (Song *et al.* 2014). As described previously hyperactivation of c-MET has also been reported in cetuximab resistance in NSCLC and HNSCC cell lines (Wheeler *et al*. 2008). The results from these studies suggest that combining c-MET inhibitors with cetuximab or other EGFR inhibitors might improve patients' response and may possibly overcome resistance to these targeted therapies in clinic.

VEGF and its receptors are involved in blood vessels' formation and growth in normal tissues. They are believed to be the key regulators in tumour angiogenesis which itself is associated with increased tumour invasion and metastases (Neufeld *et al*. 1999, Inai *et al*. 2004, Vincenzi *et al*. 2006). High level of VEGF is believed to correlate with poor prognosis in patients with various types of tumours (Poon *et al*. 2001, Vincenzi *et al*. 2006) including head and neck cancer (Eisma *et al.* 1997, Wakisaka *et al.* 1999, Smith *et al.* 2000). As mentioned previously overexpression of VEGF has shown to be involved in the development of resistance to cetuximab in A431 cell line (Viloria-Petit *et al.* 2001). Vallböhmer *et al.* (2005) have reported that high level of *VEGF* expression was associated with cetuximab resistance in patients with colorectal cancer (Vallböhmer *et al.* 2005). Therefore combination of cetuximab with anti-angiogenesis agents might enhance patient's outcome in clinic.

As described previously, inhibition of ADAM metalloproteinases could also enhance response to HER targeted therapies and was shown to have promising inhibitory effects in pre-clinical studies. Inhibition of metalloproteinase has also been widely studied in the clinical setting. Like ADAMs, Matrix Metalloproteinases (MMPs) belong to zinc-dependant membrane-bound metalloproteases. Overexpression of MMPs has been observed in different malignancies and is believed to be associated with aggressive disease and tumour metastasis (Chambers and Matrisian 1997, Kahari and Saarialho-Kere 1999, Ray and Stetler-Stevenson 1995, Kleiner and Stetler-Stevenson 1999). Due to the involvement of MMPs in

carcinogenesis, their therapeutic potential for treatment of cancer patients have been studied for many years (Hidalgo and Eckhardt 2001, Coussens *et al.* 2002).

Tissue inhibitors of metalloproteinases (TIMPs) are endogenous inhibitors of MMP. TIMP-1, TIMP-2, TIMP-3, and TIMP-4 are the four members of the family and are involved in the regulation of the enzymatic activity of all identified MMPs (Gomez *et al.* 1997). TIMPs have shown some therapeutic effects in targeting cancer cells therefore, they were considered for clinical development in the past (Bian *et al.* 1996, Ahonen *et al.* 1998). However, the development of these natural inhibitors was associated with technical complications leading to the production of synthetic MPIs (Coussens *et al.* 2002). First-generation MPIs showed poor bioavailability and second-generation MPIs also produced disappointing results in clinical trials (Coussens *et al.* 2002). In a phase I clinical trial, the safety of MPIs was investigated and prolonged treatment with MPIs was found to be responsible for the inflammation and musculoskeletal pain which were observed in participants (Coussens *et al.* 2002). In addition these broad-spectrum MPIs were also associated with lack of specificity. Therefore more selective MPIs have been developed in recent years. However, up to date all phase III clinical trials evaluating the efficacy of MPIs have failed due to the adverse side effects and limited efficacy of these inhibitors (Zucker *et al.* 2000, Overall and Lopez-Otin 2001, Coussens *et al.* 2002, Fingleton 2003, Overall and Kleifeld 2006). The results from these studies indicate that more specific

inhibitors are needed to effectively target tumour invasion and metastasis in cancer patients.

As mentioned above TIMPs are natural inhibitors of MMP but they also have an inhibitory effect against ADAM family. TIMP-1 inhibits ADAM10 where as TIMP-3 inhibits both ADAM10 and ADAM17 (Amour *et al.* 2000). As described before the development of TIMPs are associated with technical limitations (Coussens *et al.* 2002) therefore, in recent years low molecular weight synthetic inhibitors have been developed to target ADAM family members for the treatment of cancer and other diseases such as inflammatory diseases (Seals and Courtneidge 2003, Duffy *et al.* 2011). Among the various selective ADAMs inhibitors (chapter 1, table 1.2), INCB3619 (ADAM10 and 17 inhibitor, Incyte) and INCB7839 (second-generation inhibitor of ADAM10 and ADAM17 with identical specificity profile to INCB3619, Incyte) (Witters *et al.* 2008) are the most evaluated inhibitors for their anti-tumour potential.

In pre-clinical studies ADAMs selective inhibitors have shown interesting results in targeting cancer cells. As discussed in chapter 4, INCB3619 inhibitor restored gefitinib sensitivity in NSCLC cell lines and sensitized A549 cells to paclitaxel and had anti-tumour activity in the A549 xenograft model ± paclitaxel (Zhou *et al.* 2006). In my D.Phil. project, INCB3619 was shown to enhance cetuximab response and reversed cetuximab resistance in HNSCC cell lines. In a pre-clinical study by Fridman *et al.* (2007) INCB7839 was shown to inhibit the cleavage of HER2 in HER2+ breast cancer xenograft

tumour models and improved response to trastuzumab (Fridman *et al.* 2007). INCB7839 combination with lapatinib has been shown to have a synergistic growth inhibitory effect in HER2+ human breast cancer xenografts (Witters *et al.* 2008).

The anti-tumour activity of INCB7839 has also been investigated in clinic. In a phase Ib clinical trial, the safety and activity of INCB7839 was investigated in patients with NCSLC, HNSCC, breast cancer, colorectal cancer and hormone refractory prostate cancer (Infante *et al.* 2007). 30 patients who were refractory to standard treatment entered into the trial and received 100, 200 or 300 mg of INCB7839 (BID). The drug was well tolerated and none of the patients developed musculoskeletal pain or inflammation which has been reported in studies with MPIs. 6 out of 20 evaluable patients had SD (Infante *et al.* 2007). In another clinical trial the efficacy of combining INCB7839 with trastuzumab was investigated in breast cancer patients (Newton *et al.* 2010). 51 patients with metastatic HER2+ breast cancer who did not have prior treatment with chemotherapy received trastuzumab and INCB7839 (100-300 mg BID). HER2 cleavage was reduced upon treatment with INCB7839 and CRR was increased in 13/26 evaluable patients. p95+ patients (HER2+ patients with truncated HER2 receptor) who were expected to be resistant to trastuzumab also became sensitive to trastuzumab in the presence of INCB7839. This group also studied the effect of combination treatment with INCB7839 + trastuzumab + docetaxel in 13 patients which was found to be well tolerated (Newton *et al.* 2010).

The results from these studies suggested that more recent generation of metalloproteinases inhibitors are not associated with major AEs and thus the classical drawbacks of broad-spectrum inhibitors such as musculoskeletal pain can be prevented by developing more specific and selective drugs. The promising activity of ADAMs inhibitors also demonstrates the therapeutic potential of ADAMs inhibitors for the treatment of various types of tumours.

In my project ADAM10/17 dual inhibitor (INCB3619) decreased cetuximab-induced HER ligands release in the parental and cetuximab resistant cell lines. ADAM10/17 inhibitor monotherapy did not have any great effect on HER receptor signalling in any of the cell lines tested, although it significantly decreased cell proliferation and colony formation. This suggests that other signalling pathways such as Notch-signalling pathway might also be affected by ADAM10/17 inhibition. The results from my study demonstrated that ADAM10/17 inhibitor has greater effect in combination with cetuximab in the parental as well as cetuximab resistant cell lines. Based on these observations it is hypothesized that the combination treatment might have a greater inhibitory effect in the clinical setting.

Despite the promising results shown in this report, there are some limitations in my study. Most of the experiments in this project were performed in one or two HNSCC cell lines. Due to the heterogeneous nature of HNSCC tumours, it would be advantageous to validate the results in larger panel of cell lines. It would also be interesting to assess the combination of cetuximab with TKIs or ADAM10/17 inhibitor in the HNSCC xenograft models in the future.

The results from my study showed that cetuximab induced the release of BTC and NRG-1 in HNSCC. Since these are also ligands for HER4, the activation of this receptor upon prolonged treatment with cetuximab is expected. However, the role of HER4 in relation to cetuximab treatment and resistance was not investigated in my project. Our lab has recently shown the involvement of HER4 in trastuzumab resistance in HER2+ breast cancer (Nafi *et al.* 2014). Trastuzumab was shown to be responsible for an upregulation of HER4 and was found to induce the cleavage and translocation of HER4 into the nucleus. Blocking the cleavage of HER4 by γ-secretase inhibitor and targeting HER4 activity by pan-HER inhibitor, neratinib, improved the response to trastuzumab in breast cancer (Nafi *et al.* 2014). Therefore, it would be interesting to study the effect of cetuximab-induced HER ligands' release on HER4 activity and to evaluate the potential of using pan-HER inhibitors or ADAM10/17 inhibitors to target HER4 in HNSCC.

Investigating the role of ADAMs as prognostic and predictive biomarkers was also associated with some limitations. Only limited number of cetuximab treated excision samples from HNSCC patients who underwent a cetuximab window study was available. Therefore larger number of samples is needed to validate the results of this preliminary study. The effect of cetuximab in upregulation of ADAM10 and ADAM17 could not be evaluated precisely due to lack of pre- and post-cetuximab treated tumour samples. In addition as described in chapter 6, there is currently no published guideline for determining clinically important cut-off points for ADAM0 and ADAM17.

Therefore, the cut-off points used in this project are just exploratory and needs to be validated in larger independent cohorts.

In summary, results from this project showed increased activity of EGFR, HER2 and HER3 in cetuximab resistant HNSCC cell lines. Cetuximab treatment induced upregulation of ADAM10 and ADAM17 and HER ligands' release in HNSCC cells. Higher levels of ADAM10 and ADAM17 as well as their substrates, BTC and NRG-1 were found in the resistant cells compared to their parental cells. Thus ADAM-mediated ligands' release is believed to be responsible for the reactivation of HER receptors upon prolonged treatment with cetuximab. Inhibition or knockdown of ADAM10 and ADAM17 enhanced cetuximab response and reversed cetuximab resistance in HNSCC cell lines. In addition, results from this study showed that the combination of cetuximab with gefitinib or afatinib had greater effect in the parental cells and reversed cetuximab resistance in HNSCC cells. High nuclear ADAM10 and high cytoplasmic ADAM17 expression levels were associated with shorter DFS and poor response to cetuximab in HNSCC. It is proposed that further validation should be done to evaluate the prognostic and predictive significance of ADAM10 and ADAM17 in HNSCC patients.

CHAPTER 8

LITERATURE REFERENCES

8. LITERATURE REFERENCES

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