SYNERGISTIC GROWTH INHIBITION BY AFATINIB AND TRAMETINIB IN PRECLINICAL ORAL SQUAMOUS CELL CARCINOMA MODELS

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FACULTY OF DENTISTRY
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SYNERGISTIC GROWTH INHIBITION BY AFATINIB AND TRAMETINIB IN PRECLINICAL ORAL SQUAMOUS CELL CARCINOMA MODELS

ABSTRACT

Oral squamous cell carcinoma (OSCC) remains a challenging disease to manage due to limited efficacious treatments, hence finding more effective treatment approaches remains a priority. Given that the aberrant activation of epidermal growth factor receptor family receptors (ERBB) is a common event in OSCC and high expression of these receptor proteins are often associated with poor prognosis, this rationalizes the approach of targeting ERBB signaling pathways to improve the survival of OSCC patients. However, monotherapy with the pan-ERBB blocker afatinib has shown limited survival benefits. This study was carried out to identify mechanisms of afatinib resistance and to explore potential afatinib-based combination treatment with other targeted inhibitors in OSCC. Anti-proliferative effects of afatinib on a panel of OSCC cell lines were determined via crystal violet cytostatic assay, click-iT 5-ethynyl-2′-deoxyuridine staining and cell cycle analysis. Western blottings were performed to study the underlying mechanism of drug treatment as a single agent or in combination with the MEK inhibitor trametinib. Anti-tumor effects of single agent and combined treatment were evaluated by using OSCC xenograft models. In this study, afatinib inhibited OSCC cell proliferation via cell cycle arrest at the G_0/G_1 phase, and inhibited tumor growth in xenograft mouse models. Interestingly, the mitogen-activated protein kinase (ERK1/2) was reactivated *in vitro*, which possibly reduced the effects of ERBB inhibition. Concomitant treatment of OSCC cells with afatinib and trametinib synergized the anti-tumor effects in OSCC-bearing mouse models. These findings provide insight into the molecular mechanism of resistance to afatinib and support further clinical evaluation into the combination of afatinib and MEK inhibition in the treatment of OSCC.

Keywords: Oral squamous cell carcinoma (OSCC), ERBB family kinases, afatinib, trametinib, synergistic growth inhibition

PERENCATAN PERTUMBUHAN SECARA SINERGI OLEH AFATINIB DAN TRAMETINIB DALAM MODEL PRAKLINIKAL KARSINOMA SEL SKUAMUS MULUT

ABSTRAK

Karsinoma sel skuamus mulut (OSCC) merupakan penyakit yang mencabar kerana kekurangan rawatan yang efektif. Oleh itu, pencarian rawatan yang lebih berkesan untuk OSCC adalah amat penting dan perlu diberi keutamaan. Memandangkan pengaktifan *epidermal growth factor receptor family* (ERBB) sering berlaku kepada pesakit OSCC dan ekspresi yang tinggi bagi receptor protein ini sering dikaitkan dengan prognosis yang teruk, adalah wajar bagi kajian ini untuk menumpukan sasaran pada laluan isyarat ERBB bagi meningkatkan kemandirian pesakit OSCC. Walaupun begitu, monoterapi perencat ERBB (afatinib) menunjukkan kesan rawatan yang terhad. Oleh itu, kajian ini bertujuan untuk mengenal pasti mekanisme rintangan afatinib dan mencari rawatan kombinasi afatinib dengan perencat sasaran lain bagi OSCC. Kesan perencatan pertumbuhan oleh afatinib atas panel sel OSCC telah dikajikan dengan menggunakan *crystal violet cytostatic assay, click-iT 5-ethynyl-2′ -deoxyuridine staining* and analisis kitaran sel. Ujikaji biokimia juga dilaksanakan untuk menyelidik mekanisma rawatan afatinib secara tunggal atau kombinasi dengan perencat MEK (trametinib). Selain itu, kesan anti-tumor dalam rawatan tunggal dan kombinasi dikaji dengan menggunakan model mencit OSCC. Dalam kajian ini, afatinib bukan sahaja berupaya menghalang pertumbuhan sel OSCC melalui sekatan kitaran sel pada fasa G_0/G_1, tetapi juga memperlahankan pertumbuhan tumor dalam model mencit OSCC. Yang menariknya, rawatan afatinib mengaktifkan semula *mitogen-activated protein kinase* (ERK1/2) secara *in vitro*, yang mungkin berupaya mengurangkan kesan daripada rencatan ERBB. Rawatan sel OSCC dengan menggunakan afatinib bersama trametinib dapat meningkatkan kesan anti-tumor dalam model mencit dengan OSCC. Penemuan ini memberi gambaran mengenai mekanisme

molekul di mana keberkesanan monoterapi afatinib adalah terhad. Kajian ini juga mendorong penilaian klinikal atas rawatan kombinasi afatinib and perencat MEK pada OSCC.

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LIST OF SYMBOLS AND ABBREVIATIONS

AJCC	:	American Joint Committee on Cancer
AR	:	Amphiregulin
BIBW2992	:	(N-[4-]93-chloro-4-fluorophenyl)amino]-7-[[93S]-tetrahydro-3-furanyl]oxy]-6-quinaz-olinyl]-4-(dimethylamino)-2-butanamide)
BSA	:	Bovine serum albumin
c-MET	:	tyrosine-protein kinase Met
CASP8	:	Caspase 8
CCND1	:	Cyclin D1
CD8+	:	Cytotoxic T cells
CI	:	Combination index
CNA	:	Copy number alteration
CO_2	:	Carbon dioxide
$CuSO_4$	:	Copper sulfate
DMEM	:	Dulbecco's modified eagle's medium/Nutrient mixture F-12 HAM's medium
DMSO	:	Dimethyl sulfoxide
DNA	:	Deoxyribonucleiac acid
EdU	:	5-ethynyl-2'deoxyuridine
EGF	:	Epidermal growth factor
EGFR	:	Epidermal growth factor receptor
EMT	:	Epithelial-to-mesenchymal transition
EPHA2	:	Ephrin Type-A Receptor 2
EPG	:	Epigen
EPR	:	Epiregulin

ERBB	:	Epidermal growth factor receptor family receptors
ERK1/2	:	Extracellular signal-regulated kinase 1/2
Fa	:	Fraction-affected
FAT1	:	FAT atypical cadherin 1
FBS	:	Fetal bovine serum
FDA	:	Food and Drug Administration
FGFR1	:	Fibroblast growth factor receptor 1
GI_{50}	:	50% of maximal inhibition of cell growth
H&E	:	Hematoxylin and Eosin
HER2	:	Human epidermal growth factor receptor 2
HER3	:	Human epidermal growth factor receptor 3
HNSCC	:	Head and neck squamous cell carcinoma
HPV	:	Human papillomavirus
HRAS	:	HRas Proto-Oncogene, GTPase
IACR	:	International Agency for Research on Cancer
IACUC	:	International Animal Care and Use Committee
ICD	:	International Classification of Disease
IGFBP3	:	Insulin-like growth factor-binding protein 3
IGF-1R	:	Insulin-like growth factor 1 receptor
IGFR	:	Insulin-like growth factor receptor
IgG1	:	immunoglobulin G subclass 1
IU	:	International unit
IUCC	:	International Union Against Cancer
Jak/Stat	:	Janus kinase/Signal transducers and activators of transcription
KRAS	:	Kirsten rat sarcoma viral oncogene homolog
MAPK	:	Ras/Raf-mitogen-activated protein kinase

MEK	:	MAPK/ERK Kinase
MHC-1	:	Major histocompatibility complex class-1
MTD	:	maximum-tolerated dose
mTOR	:	Mammalian target of rapamycin
MTT	:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
R/M	:	Recurrent and/or Metastatic
NaCl	:	Sodium chloride
NCCN	:	National Comprehensive Cancer Network guidelines
NRGs	:	Neuregulins
NIH	:	National Institute of Health
NNK	:	carcinogen 4-(nitrosomethylamino)-1-(3-pyridyl)-1-butanone
NNN	:	N'nitrosonornicotine (NNN)
NOTCH1	:	Notch homolog 1
NP-40	:	Nonyl phenoxypolyethoxylethanol
NSCLC	:	Non-small cell lung carcinoma
OS	:	Overall survival
OSCC	:	Oral squamous cell carcinoma
p16	:	Cyclin-dependent kinase inhibitor 2A protein
PBS	:	Phosphate-buffered saline
PD-1	:	Programmed cell death protein-1
PEG	:	Polyethlene glycol
PFS	:	Progression-free survival
PI3K/AKT	:	Phosphatidylinositol-3-kinase/v-Akt Murine Thymoma Viral Oncogene
pRb	:	retinoblastoma proteins
PTEN	:	Phosphatase and tensin homolog

PVDF	:	Polyvinylidene difluoride
RTK	:	Receptor Tyrosine Kinase
SDS	:	Sodium dodecyl sulfate
SEM	:	Standard error of mean
SH2	:	Src Homology 2
SRC	:	Proto-oncogene tyrosine-protein kinase Src
STR	:	Short tandem repeat
TCGA	:	The Cancer Genome Atlas
TCL	:	Total cell lysates
TGF-α	:	Transforming growth factor
TKI	:	Tyrosine kinase inhibitor
TNM	:	Classification of malignant tumor
Tris	:	Trisaminomethane
VEGF	:	Vascular endothelial growth factor receptor
v/v	:	Volume/volume
w/v	:	Weight/volume

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CHAPTER 1: INTRODUCTION

1.1 Background

Oral squamous cell carcinoma (OSCC) is among the most prevalent cancers worldwide. Furthermore, the recurrent disease remains challenging mainly due to the limited therapeutic options (Specenier & Vermorken, 2010). While chemotherapy remains the most feasible standard-of-care for advanced OSCC, lack of response with these cytotoxic therapies remains a concern. Additionally, adverse effects resulting from the treatment severely impact the quality of life and patient outcome.

The epidermal growth factor receptor (EGFR) plays crucial roles in the tumorigenesis of OSCC by signaling through the PI3K/AKT kinase and Ras/Raf/MEK/ERK pathways, resulting in the increase in cell growth and migration, as well as inhibition of apoptosis (Marmor, Skaria, & Yarden, 2004). Of note, EGFR overexpression is found in ~90% of OSCC patients and this has been associated with poor prognosis and radioresistance (Rubin et al., 1998; Saki, Toulany, & Rodemann, 2013; Wheeler et al., 2012). As a result, many strategies for targeting EGFR have been developed for therapeutic purposes. Cetuximab, an EGFR-directed monoclonal antibody was approved by the US Food and Drug Administration (FDA) as monotherapy or for use in combination with radiation or chemotherapy for head and neck cancer (Bonner et al., 2010; Burtness et al., 2005). Despite the approval for clinical use, cetuximab in combination with platinum-based therapies only extends the median overall survival by ~3 months (Vermorken et al., 2008). Notably, the activation of other receptor tyrosine kinases (RTK) such as HER2 (ERBB2) and HER3 (ERBB3), as well as tyrosine-protein kinase Met (c-MET) and insulin-like growth factor receptor (IGFR) were found to confer resistance to cetuximab (Madoz-Gurpide et al., 2015; Wheeler et al., 2008; Zuo, Shi, Li, Chen, & Luo, 2010).

Therefore, there is a great need to identify effective therapeutic strategies that can improve the current approaches in inhibiting EGFR.

Recently, a novel web-based drug repurposing bioinformatics tool named DeSigN 1.0, was developed and used to identify drugs that could be potent in OSCC. In brief, the differentially expressed genes between OSCC cell lines and normal primary cultures from the oral mucosa were uploaded into the DeSigN 1.0 bioinformatics tool. Based on this gene signature and using a pattern-matching algorithm, DeSigN 1.0 identifies drugs that could be efficacious for OSCC. The efficacy of the identified drugs was ranked by a connectivity score, in which the closer the connectivity score is to 1, the more likely the drug is predicted to work. From this analysis, BIBW2992 (afatinib) was ranked at the fourth position with a connectivity score of 0.91 (Lee et al., 2017).

Afatinib has demonstrated more pronounced efficacy than gefitinib and erlotinib in non-small-cell lung carcinoma, owing to its irreversible binding not only to EGFR, but also to other ERBB family members (HER2, HER3, and HER4) (Solca et al., 2012; Sos et al., 2010). Of note, tumor growth control by afatinib was comparable to those treated with cetuximab in phase 2 clinical trial of head and neck squamous cell carcinoma (HNSCC) (Seiwert et al., 2014). Importantly, patients with recurrent and/or metastatic (R/M) HNSCC who received afatinib treatment demonstrated statistically improved progression-free survival (PFS), as compared to those who were treated with methotrexate (2.6 months versus 1.7 months). However, the difference in overall survival was not significant (Machiels et al., 2015). Further studies found that the use of afatinib in combination with standard chemotherapies such as 5-fluorouracil and paclitaxel resulted in promising anti-tumor activity in patients with R/M HNSCC, despite increased toxicities observed (Chung et al., 2016; Vermorken et al., 2013). While afatinib-based combination with inhibitors targeting the intracellular signaling pathways, such as AKT

(rapamycin), ERK (trametinib) and JAK/STAT (Pyridone 6) have been shown to improve tumor regressions in various preclinical cancer models, thus far the combinational effects of afatinib with other targeted drugs have not been reported for OSCC (Kim et al., 2012; Perera et al., 2009; Sun et al., 2014).

In this study, afatinib was demonstrated to be efficacious in inhibiting the proliferation of OSCC cells. The prolonged afatinib treatment reactivated ERK1/2 signaling, indicating a possible compensatory pathway that might confer resistance to afatinib in OSCC. Furthermore, in the study of evaluating the combinational potency of afatinib and a MEK inhibitor, trametinib in OSCC models, a remarkable synergistic effect of anti-tumor activity was shown by significant tumor growth inhibition. These findings demonstrate that a combination of afatinib and trametinib could improve tumor control in OSCC.

1.2 Aims

Given that afatinib monotherapy is not sufficient to control tumors in HNSCC patients, this study aims to identify the possible mechanisms of resistance and explore afatinib-based combination treatments with other targeted inhibitor to improve the survival benefit in OSCC.

1.3 Objectives

1. To elucidate the effects of afatinib on cellular responses and signaling pathways in OSCC.
2. To determine the co-targeting effects of key mechanisms by afatinib treatment in combination with trametinib in OSCC.

3. To compare the antitumor effects of afatinib treatment as a single agent and in combination with trametinib in xenograft models of OSCC.

CHAPTER 2: LITERATURE REVIEW

2.1 Oral Cancers

2.1.1 World incidence and incidence in Malaysia

Oral cancer is one of the most debilitating malignancies worldwide, which accounts for incidence of 354,864 and mortality of 117,384 in year 2018 (GLOBOCAN 2018) (Bray et al., 2018). This disease is most frequently diagnosed in countries in Melanesia and South Central Asia. Among of these countries, Papua New Guinea has the highest incidence rate worldwide in both male and female. Notably, among males, oral cancer is the leading caused of cancer death in India and Sri Lanka (Figure 2.1). Based on the epidermiology data obtained from GLOBOCAN 2018, a total of 667 of incidence and 327 of mortality was reported for Malaysia in year 2018 (Bray et al., 2018). According to the Malaysian National Cancer Registry Report 2007-2011 (https://nci.moh.gov.my/index.php/ms/pengumuman/340-malaysian-national-cancer-registry-report-2007-2011), oral cancer is the eighth and fourth most common cancer among Indian men and women respectively (Figure 2.2), likely related to the betel quid chewing and other risk habits practiced by this community that results in higher vulnerability to this cancer type (Ghani et al., 2011; Manan, Tamin, Abdullah, Abidin, & Wahab, 2016).

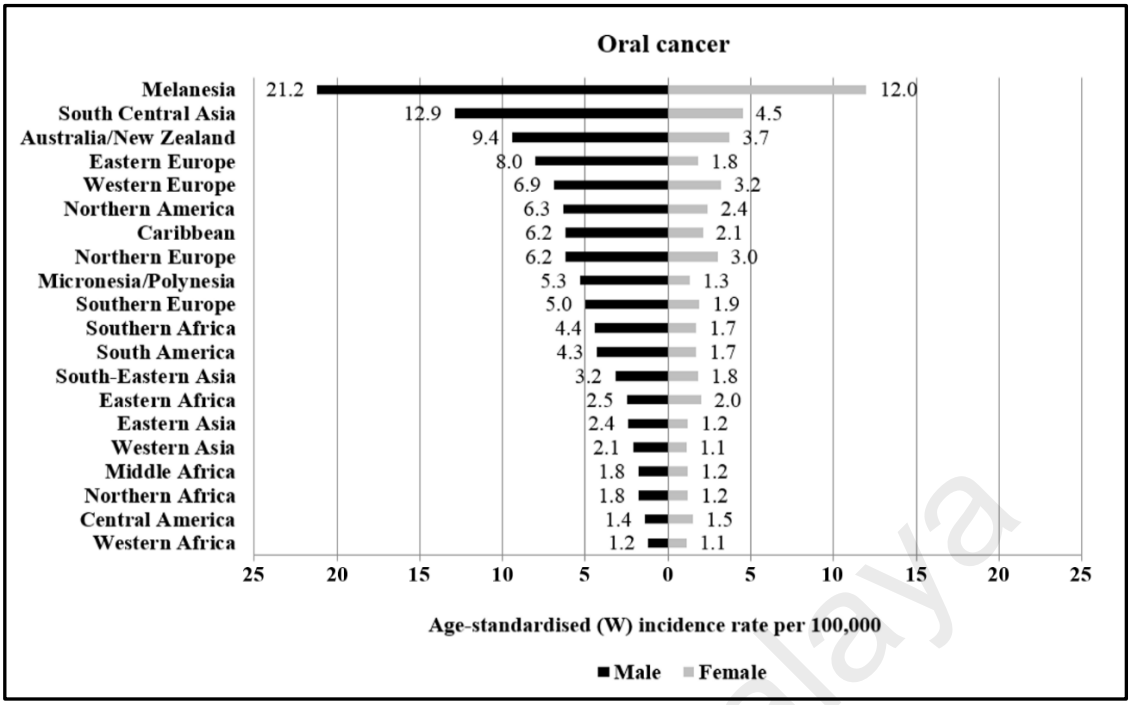

Figure 2.1: Region-specific Age-standardized incidence rates by sex for oral cancer in 2018 (GLOBOCAN 2018). Figure is modified from (Bray et al., 2018)

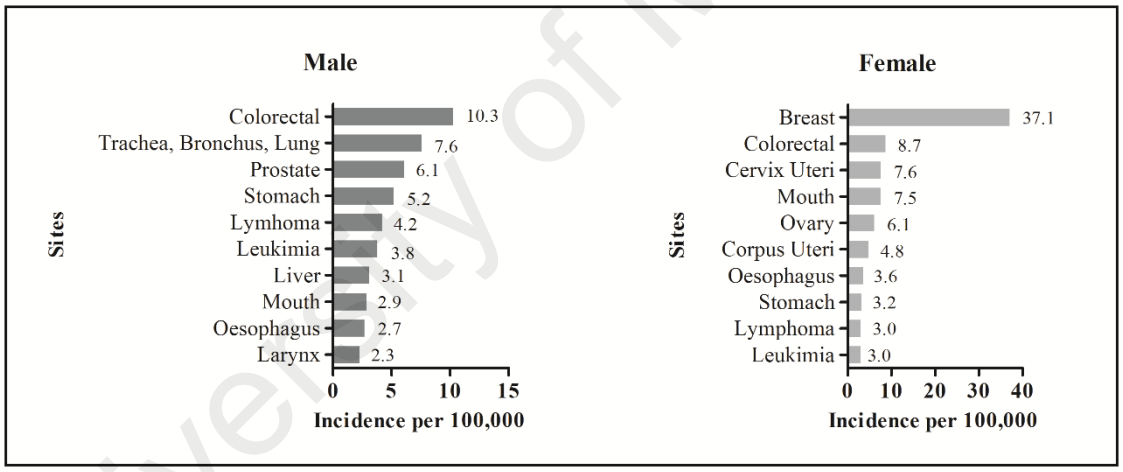

Figure 2.2: Age-standardized incidence rate for the ten most common cancers amongst Indians by sex In Malaysia (2007-2011). Figure is modified from Malaysian National Cancer Registry Report 2007-2011).

2.1.2 Associated risk factors

There are numerous risk factors for oral cancer, namely tobacco, excessive alcohol drinking, betel quid chewing and human papillomavirus (HPV) infection. Of these, tobacco smoking contributes to about 25% of oral cancer (Hashibe et al., 2007; Petti, 2009). Carcinogenesis mechanism of tobacco have been extensively studied and

numerous carcinogens contained in tobacco were found. Particularly, tobacco-specific nitrosamines such as carcinogen N'nitrosonornicotine (NNN) and 4-(nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK) produce DNA adducts which cause deleterious mutations to oncogenes and tumor suppressor that result in the onset of tumorigenesis. Additionally, the binding of these nitrosamines to nicotinic acetylcholine receptors mediate cell proliferation and anti-apoptosis by inducing numerous key oncogenic pathways, resulting in the development of cancer (Xue, Yang, & Seng, 2014).

Besides tobacco smoking, alcohol drinking has been determined as risk habit for cancer of oral cavity by the International Agency for Research on Cancer (IACR), in which the amount and frequency of alcohol drinking is associated with a higher risk of developing oral cancer (LoConte, Brewster, Kaur, Merrill, & Alberg, 2018). The mechanism underlying the carcinogenic effects of alcohol has been linked to the acetaldehyde, the primary metabolite of alcohol that forms DNA adducts (Boffetta & Hashibe, 2006; LoConte et al., 2018). Acetaldehyde can also bind to enzymes that are essential for DNA repair and methylation. Notably, excessive consumption of alcohol has been associated with aberrant DNA methylation that modulates the expression of oncogenes and tumor suppressor (Boffetta & Hashibe, 2006; Xie, Feng, & Mao, 2019). Additionally, alcohol acts as a solvent to carcinogenic agents from tobacco, which in turn increases the risk of oral malignancies in tobacco users by ~3-fold (Hashibe et al., 2009). Notably, more than 80% of oral malignancies are attributable to both of these habits (Blot et al., 1988; Hashibe et al., 2009).

Betel quid chewing is another major risk habit which is particularly common in some countries in South-Central Asia (India, Sri Lanka, Bangladesh and Pakistan) and East Asia (Taiwan and China), and this lifestyle habit has a significant association with an increased risk to oral cancer (Silverman, 2003; Warnakulasuriya, 2009). Betel quid which

is also known as paan, usually comprises of betel leaf from the *Piper betel* plant wrapped around the areca nut, lime, calcium hydroxide and tobacco (Goldenberg et al., 2004). Some compounds in the betel quid namely alkaloids, polyphenols, tannins, metal ions, and safrole are genotoxic, cytotoxic and may also stimulate cell proliferation (Lin, Chen, Lai, Huang, & Chen, 1997; Lin et al., 2000). In populations such as Taiwan, China, and Papua New Guinea, the risk of oral cancer can be attributed to areca nut alone (Lu et al., 1996; Merchant et al., 2000). However, the mixing of tobacco to betel quid can further increase the likelihood of developing oral malignancy, whereby the users have ~5 to 6 times increase in risk (Silverman, 2001).

Besides, oral cancers can also be attributable to human papillomavirus (HPV) infections (Shah, Candela, & Poddar, 1990), although the number of affected patients are relatively small globally (2.2%) (Serrano, Brotons, Bosch, & Bruni, 2018). Amongst the many genotypes of HPV, oral cancer is associated with the high-risk HPV-16 and 18 that accounts for 66–82% and 26–34% of the HPV-positive oral cancers respectively (Kreimer, Clifford, Boyle, & Franceschi, 2005; Syrjanen et al., 2011). The oncoproteins E6 and E7 encoded by HPV-16 and -18 are responsible for tumorigenesis, mainly by inactivating the tumor suppressor genes that are crucial in controlling the cell cycle. Particularly, E6 enhances ubiquitin-degradation of p53, consequently abrogating its tumor suppressor functions (Maruyama et al., 2014; Termine et al., 2008). E6 also interacts with several pro-apoptotic proteins to prevent apoptosis, whereas, E7 blocks the interaction between retinoblastoma proteins (pRb) and E2F transcription factor leading to enhanced cell proliferation. (Klingelhutz, Foster, & McDougall, 1996; Narisawa-Saito & Kiyono, 2007). It is crucial to distinguish HPV-infected OSCC patients because they usually respond better to chemotherapy and radiotherapy (Kimple et al., 2013; Ziemann et al., 2015).

2.1.3 Classification of oral cancer

Oral cancer is a subset of malignancies of head and neck cancers. According to International Classification of Disease (ICD-10) by World Health Organization (WHO), it is classified by codes and affects any part of the oral cavity, which includes the lips (C00), tongue (C01-02), gum (C03), floor of mouth (C04), palate (C05), cheek (C06), and other parts of the oral cavity (C06-C10) (https://icd.who.int/browse10/2016/en). Notably, more than 90% of the malignancies of the oral cavity arising from the squamous cells of the lining mucosa (Silverman, 2001). The primary tumor cells can spread to local lymph nodes and worsen the prognosis. Generally, patients at early stages have better prognosis with 5-year survival rate of ~80% at stage I, as compared to 40% survival for stage IV patients (Siegel, Miller, & Jemal, 2016). Unfortunately, most OSCC is diagnosed at late stages that frequently involve aggressive metastasis leading to a high mortality rate.

Examination of histopathological features and clinical staging assessment are the gold standard methods to determine the clinical extent of oral cancer. Histopathological grading system measures degree of differentiation, pattern of invasion, lymphovascular and perineural invasion that are associated with disease prognosis. Whereas, the TNM Classification of Malignant Tumors (TNM) that includes the tumor size, depth of invasion and nodal/distant spread (International Union Against Cancer (UICC)/ American Joint Committee on Cancer (AJCC)) (Amin et al., 2017), is widely used in clinic not only to determine the stages of the oral malignancies, but also for the selection of therapeutic approaches and prediction of the treatment outcome.

2.1.4 Treatment

Treatment for oral cancer involve multidisciplinary modalities and is mainly dependent on disease stage. According to the National Comprehensive Cancer Network guidelines (NCCN), Version 1 (2018) (Colevas et al., 2018), patients at all stages are generally treated by resection of the primary tumors. In advanced-stage disease, alongside surgery on the primary tumor, removal of lymph nodes will be conducted to check the presense of a positive nodal spread (Tankere et al., 2000). Often, external radiation beam can be administered as a postoperative treatment to patients for several reasons, namely large primary tumors, close resection margin or signs of invasion to the lymph nodes (Huang & O'Sullivan, 2013; Mendenhall, Dagan, Bryant, & Fernandes, 2019). Chemotherapeutic drugs such as cisplatin, carboplatin, 5-fluorouracil (5-FU), paclitaxel, docetaxel or hydroxyurea (Cohen & Lippard, 2001; Herbst & Khuri, 2003; Tong, Poot, Hu, & Oda, 2000), are used as pre-operative induction treatment, or combined with radiation before or after surgery. Chemo-radiotherapy has been used as a standard adjuvant treatment for advanced oral cancers to prevent disease recurrence (Huang & O'Sullivan, 2013). However, partial or lack of response to the cytotoxic therapies still remain a concern. Additionally, the adverse effects of the treatment severely impact the quality of life of cancer patients.

As a majority of oral cancers have an aberrantly high level of the epidermal growth factor receptor (EGFR), a drug targeting this receptor called cetuximab has been approved by the United States of America Food and Drug Administration (US-FDA) to treat locoregional advanced (LA) OSCC in combination with radiotherapy, as well as metastatic or recurrent patients, either as single treatment or in combination with chemotherapy. Cetuximab is an immunoglobulin G subclass 1 (IgG1) monoclonal antibody against EGFR, which is the central regulator of cell proliferation. However, the efficacy of cetuximab was limited with an objective response rate of 13% in a

monotherapy setting and 36% in combination with chemotherapeutic drugs (Vermorken et al., 2008; Vermorken et al., 2007). Notably, some patients acquire resistance to cetuximab with only five months of time-to-treatment failure (Vermorken et al., 2008).

A newer treatment option is the use of immunotherapies, in the form of checkpoint inhibitors targeting programmed cell death protein 1 (PD-1) receptor called pembrolizumab and nivolumab. These two drugs are particularly effective in treating tumors with the high endogenous levels of PD-1 and its ligand (PD-L1), by blocking the protective mechanism of tumor evasion from the immune system. Thus far, pembrolizumab and nivolumab are the only two drugs that had been approved as the second line treatment to R/M oral cancers (Bauml et al., 2017; Ferris et al., 2016).

2.1.5 Molecular alterations

Oral carcinogenesis is complex and the development involves the accumulation of genetic alterations in both oncogenes and tumor suppressors. While genetic aberrations found in multiple tumor types were broadly categorized to multiple recurrent chromosomal changes (C class) and somatic mutations (M class), majority of OSCC are featured by copy number alterations (CNA) that include recurrent focal gains (3q, 7p, 8q, 9q, 11q, 20q) and losses (3p, 5q, 8p, 18q) (Chong et al., 2017; Ciriello et al., 2013; Pickering et al., 2013; Su et al., 2017). These regions of CNA contain many candidates and known oncogenes and tumor suppressors. Notably, high-level amplification of some genes is strongly associated with high gene expression such as cyclin D1 (*CCND1*) and *EGFR*, which are the established oncogenic drivers of OSCC (Pickering et al., 2013). Besides, co-amplification of these 2 genes is associated with poor survival in OSCC (Chong et al., 2017).

Apart from chromosomal instabilities, previous whole exome sequencing studies in The Cancer Genome Atlas (TCGA) also revealed numerous somatic mutations (Cancer Genome Atlas Network, 2015). Such gene alterations are dominated by tumor suppressor genes where *TP53* and *CDKN2A* are the most frequently mutated genes, whereas *PIK3CA* mutation is the most common oncogenic mutation. Other mutated genes such as *NOTCH1*, *HRAS*, *EPHA2*, *CASP8*, and *FAT1* are frequently altered in OSCC, which could be essential in modulating the cancer hallmarks such as cell proliferation, apoptosis and cell cycle control (Cancer Genome Atlas Network, 2015; Pickering et al., 2013; Su et al., 2017).

While gene mutation and protein expression are significant factors that can modulate the therapeutic responses, knowing the druggable targets or pathways is helpful to provide insight into developing new targeted therapy. Of note, 80% of tumors harbor at least 1 aberrant event that can be potentially targeted by FDA approved drugs. High frequency of co-occurrence of targetable genes in tumor suggested combination of targeted treatment to improve the clinical outcome of single-agent treatment (Pickering et al., 2013). While EGFR overexpression is commonly seen in OSCC, numerous genetic alterations of downstream pathways were identified and these aberrations could potentially contribute to limited clinical responsiveness to cetuximab (EGFR inhibitor) in OSCC. Hence, targeting this pathway through combination targeted treatment should be pursued.

2.2 ERBB family receptors and dysregulation in oral cancer

2.2.1 ERBB family receptors signaling pathway

Epidermal growth factor receptor family receptor (ERBB family receptors) consists of EGFR, ERBB2 (HER2), ERBB3 (HER3) and ERBB4 (HER4). These transmembrane

receptors possess an N-terminal extracellular ligand binding region, which is joint to a single transmembrane segment that contains a cytoplasmic tyrosine kinase domain flanked by an intracellular juxtamembrane region and a C-terminal tail (Jorissen et al., 2003; Lemmon, 2009). Activation of the ERBB signaling is initiated when the ligand binds to the N-terminal domain of monomeric receptor. The ligands of the ERBB family receptors are broadly divided into three groups based on their receptor specificity. The first group consists of a wide range of ligands, including epidermal growth factor (EGF), transforming growth factor-alpha (TGF-α), epigen (EPG) and amphiregulin (AR) that bind only to EGFR. The second group recognizes both EGFR and HER4, in which the ligands include heparin-binding EGFR (HB-EGF), beta-cellulin (BTC) and epiregulin (EPR). Members of neuregulins (NRGs) form the third group, in which NRG-1 and NRG-2 bind to HER3 and HER4, whereas NRG-3 and NRG-4 are specific ligand to HER4 only (Hynes & Lane, 2005). The ligand/receptor binding subsequently induces the formation of kinase-active dimers. While EGFR and HER4 can form homodimer or heterodimer with other ERBB receptors, HER2 has no known ligand and always acts as a dimeric partner for other ERBB receptors (Wilson, Gilmore, Foley, Lemmon, & Riese, 2009). Whereas, HER3 is the only member that lacks kinase activity and therefore, it must dimerize with other kinase-active ERBB receptors (Sierke, Cheng, Kim, & Koland, 1997). Upon receptor dimerization, the tyrosine residues at C-terminal of ERBB proteins are phosphorylated. This allows the docking of numerous signaling molecules that contain Src homology 2 (SH2) or phosphotyrosine-binding domains to the receptors, subsequently stimulates a cascade activation of downstream effectors. There are three main downstream signaling pathways in ERBB dependent pathway: phosphatidylinositol-3-kinase/v-Akt Murine Thymoma Viral Oncogene (PI3K/AKT), RAS/RAF/ mitogen-activated protein kinase (RAS/RAF/MEK/ERK) and signal

transducers and activators of transcription (STAT) pathways which are important to promote cell proliferation and survival (Figure 2.3) (Yarden & Pines, 2012).

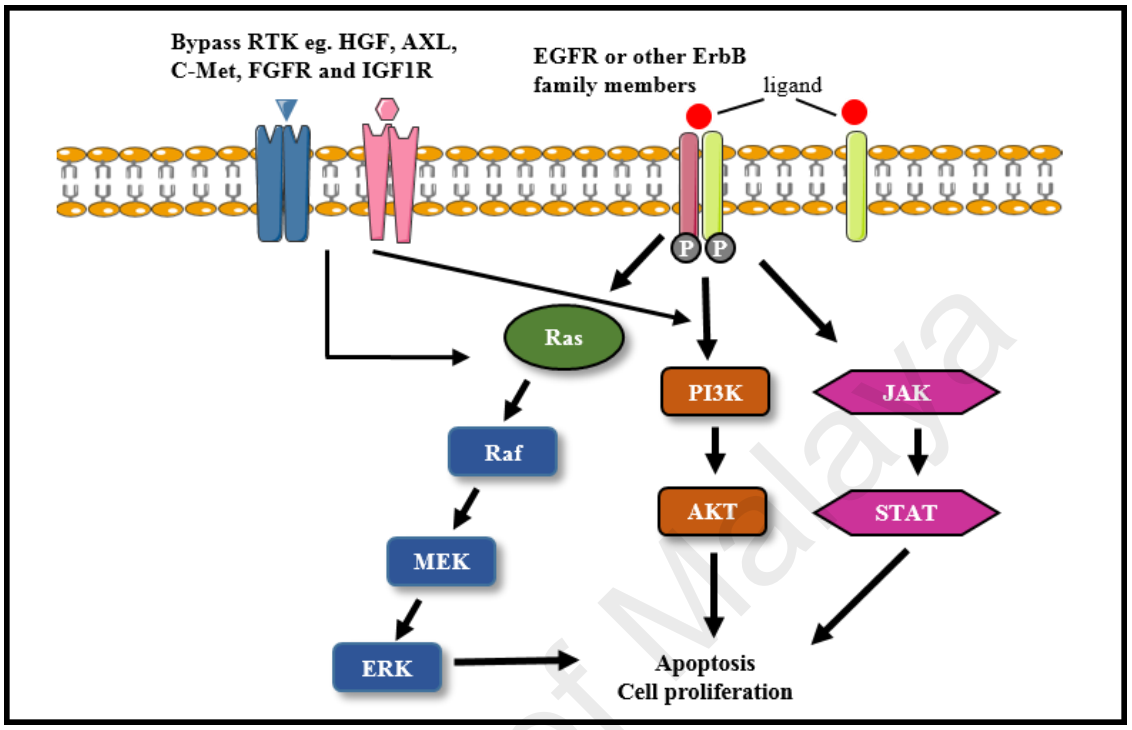

Figure 2.3: Schematic representation of ERBB-dependent signaling pathways and bypass RTK signaling activations. Homodimerization or heterodimerization of ERBB receptors upon the ligand binding induces the activation of RAS/RAF/MEK/ERK, PI3K/AKT and JAK/STAT pathways, which eventually promote cell proliferation and survival. Other RTKs can induce the downstream signalings via ErbB-independent way. Figure is modified from (Liu et al., 2018).

2.2.2 Dysregulation of ErbB signaling in cancers

Many types of solid malignancies such as non-small cell lung carcinoma (NSCLC), head and neck, colorectal, bladder and pancreatic cancers, are linked with aberrant ERBB signal transduction that could be due to gene amplification, somatic mutations and/or transcriptional upregulation (Arteaga & Engelman, 2014). While mutations of *EGFR* and other family members are relatively rare in OSCC (Leemans, Braakhuis, & Brakenhoff, 2011), ERBB family receptors are found to be highly expressed (Yewale, Baradia, Vhora, Patil, & Misra, 2013). About 90% of patients have a high level of EGFR, while ~50%

and 23% overexpress ERBB2 and ERBB3 respectively (Fong, Chou, Hung, Wu, & Kao, 2008; Takikita et al., 2011). Notably, increased ERBB expressions are strongly associated with shorter overall survival and loco-regional relapse in OSCC (Xia et al., 1999). Besides, ionizing radiation triggers the bindings of nuclear EGFR to DNA-dependent protein kinase and prevents the repair of DNA damage (Dittmann et al., 2005). Overexpression of ERBB-specific ligand is another mechanism by which cancer aberrantly activates ERBB. For instance, TGF-α is co-overexpressed with EGFR in various tumor types, including oral cancer (Rubin et al., 1998). Besides, oncogenic addiction to ERBB has been linked to the aberrantly high NRG-1 as seen in head and neck cancer (Wilson, Lee, Berry, Shames, & Settleman, 2011). The activation of ERBB due to either genetic aberrations of ERBB receptor or the overexpression of ligand intimately links to the enhanced downstream signaling of PI3K/AKT and MEK/ERK that resulting in tumorigenesis. Taken together, ERBB is considered as a promising target of anti-cancer therapy.

2.2.3 Inhibition of ERBB pathways

A number of inhibitors that target EGFR in different mode of action have been developed to improve the control of tumor growth. Monoclonal humanized antibodies are directed against the extracellular domains of EGFR receptors and thereby preventing the interaction of receptor-ligand to transmit signals. Another group of agents is small molecule inhibitors that target the cytoplasmic tyrosine kinase domain, leading to the inhibition of auto-phosphorylation of receptor and the subsequent blockade of activation of the intracellular signaling cascade.

Thus far, cetuximab is the only FDA approved EGFR-targeted drug for oral cancer. However, the clinical outcome was modest in which it only improved about 3 months of

median overall survival when used in combination with chemotherapy (Vermorken et al., 2008). While cetuximab monotherapy is also approved to treat the locally advanced or metastatic stage of the disease, the response rate was 10–13% despite the overexpression of EGFR in OSCC (Baselga et al., 2005; Vermorken et al., 2007). The limited clinical efficacies were likely linked to the intrinsic and acquired resistance to cetuximab which remains the main challenge in oral cancer treatment (Misale, Di Nicolantonio, Sartore-Bianchi, Siena, & Bardelli, 2014; Wheeler et al., 2008).

Despite many other EGFR-targeting monoclonal antibodies (panitumumab) and tyrosine kinase inhibitors (erlotinib and gefitinib) were approved for other cancer types, none of these drugs significantly improved the overall survival of OSCC patients in clinical trials (Cohen et al., 2003; Siu et al., 2007; Vermorken et al., 2007). Several findings demonstrated that activation of other EGFR members was induced as the result of EGFR inhibition, leading to drug resistance (Landi & Cappuzzo, 2013; Siu et al., 2007). Hence, targeting multiple EGFR family members by lapatinib and afatinib could be a promising strategy to overcome resistance. Of note, afatinib effectively killed intrinsic or acquired cetuximab resistance cell lines of OSCC (De Pauw et al., 2018), suggesting a promising therapeutic drug to improve survival of OSCC patients.

2.3 Afatinib

2.3.1 Effects in the molecular and cellular level

Afatinib which is known as BIBW2992 (N-[4-]93-chloro-4-fluorophenyl)amino]-7-[[93S]-tetrahydro-3-furanyl]oxy]-6-quinaz-olinyl]-4-(dimethylamino)-2-butanamide), is a pan-inhibitor of ERBB family receptors (Solca et al., 2012). It acts as an ATP-competitive inhibitor that covalently binds to the ErbB receptor, leading to the inhibition of tyrosine kinase activity. The irreversible bindings result in the blockade of

autophosphorylation of EGFR, ERBB2, ERBB4, and transphosphorylation of ERBB3, subsequently inactivating downstream signaling pathways to prevent tumor cell growth and enhance apoptosis. In cell-free based assays, afatinib demonstrated remarkable potency to inhibit the enzymatic activity of wild type ERBB receptors, as well as EGFR mutant L858R and T790M (0.2 to 25 nM) (Kumar, Petri, Halmos, & Boggon, 2008; Solca et al., 2012). Notably, the potency of afatinib was either comparable or superior to gefitinib, erlotinib or lapatinib that only targets the EGFR and HER2.

Afatinib has been approved by the Food and Drug Administration (FDA) as the first line therapy for NSCLC patients whose tumors have non-resistant EGFR-mutations (Exon 19 deletions, L858R, L861Q, G719X, and S768I). Although *EGFR* mutations are rare in OSCC, the ERBB family receptor may still represent a rationale for therapeutic targets due to the aberrant copy number amplification and over-expression. Therefore, several research work have been conducted to delineate the anti-tumor roles played by afatinib in OSCC. Of note, OSCC cell lines are highly responsive to afatinib with nanomolar potency, in which the sensitivity was associated with amplified EGFR genes (Young et al., 2015). Consistent with that, a web-based tool named DeSigN that correlates the gene expression profile with the drug response from 140 drugs from the database of Genomic of Drug Sensitivity in Cancer, has predicted that afatinib is likely efficacious in a series of Asian-derived OSCC cancer cell lines (ORL series) due to the high expression of ERBB receptors (Fadlullah et al., 2016; Lee et al., 2017) (Table 2.2). Moreover, a previous study found that afatinib was capable to establish cytotoxicity on gefitinib (EGFR-TKI) resistant cell lines (Young et al., 2015).

Importantly, the efficacy of afatinib has been demonstrated in a number of OSCC preclinical models. While afatinib was capable of controlling tumor growth effectively that was comparable with cetuximab, the *in vivo* anti-tumor effect was more potent than

other EGFR-targeting drugs such as lapatinib, erlotinib, and neratinib (Young et al., 2015). Hence, several preclinical studies explored if afatinib could improve the limited outcome of the standard OSCC treatments. While the addition of afatinib to cetuximab had no indication of synergism, an additive effect was seen when combined with radiotherapy *in vitro* and in *vivo* (Macha et al., 2017; Schutze et al., 2007; Young et al., 2015). Moreover, significant enhancement of cisplatin treatment was found when combined with afatinib in OSCC (Brands et al., 2016; Longton, Schmit, Fransolet, Clement, & Michiels, 2018).

Table 2.1: Drug response prediction by DeSigN based on the gene expression pattern of OSCC. Table is modified from (Lee et al., 2017)

Rank	Drug	Known Drug Targets	Connecitivity Score	*p* value
1	GSK-650394	SGK3	1.00	0.000
2	pyrimethamine	Dihydrofolate reductase (DHFR)	0.98	0.090
3	RDEA119	MEK1/2	0.91	0.011
4	**BIBW2992 (afatinib)**	**EGFR, ERBB2**	**0.91**	**0.024**
5	CGP-082996	CDK4	0.90	0.015
6	lapatinib	EGFR, ERBB2	0.85	0.025
7	PF-562271	FAK	0.84	0.030
8	bosutinib	SRC, ABL, TEC	0.81	0.045
9	PD-0325901	MEK1/2	0.80	0.039

2.3.2 Clinical trials

2.3.2.1 Monotherapy

Afatinib was first studied in OSCC patients in phase I clinical trial and the maximum-tolerated dose (MTD) was determined to be 40 or 50 mg/day with daily administration (Gordon et al., 2013). Subsequently, numerous phase II and III trials have been carried out to determine the anti-tumor activity, progression-free survival and disease-free survival in R/M and LA oral cancers (Table 2.3). Notably, in a phase II clinical trial (NCT00514943), afatinib (50 mg/day) showed comparable tumor shrinkage to cetuximab

in HNSCC patients who had progressed disease after platinum-containing therapy (afatinib 16.6% versus cetuximab 10.1%, $p = 0.03$) (Seiwert et al., 2014).

In a phase III clinical trial named LUX-Head and Neck 1 (NCT01345682), afatinib monotherapy improved progression-free survival (PFS) of R/M HNSCC patients who had progressed on or after first-line platinum-based therapy, as compared to methotrexate treatment (afatinib: 2.6 months versus methotrexate: 1.7 months). However, the median overall survival (OS) was not significantly improved (Machiels et al., 2015). A similar study that focuses on Asian population is currently ongoing (LUX-Head and Neck 3). While afatinib showed comparable potency to approved therapeutic drugs in OSCC, in the evaluation of afatinib as adjuvant therapy on locally advanced patients, the LUX-Head & Neck 2 (NCT01345669) and 4 (NCT02131155) trials were halted due to the unlikeliness of efficacy benefit compared to the placebo group (http://clinicaltrials.gov/).

Notably, subgroups of patients from the aforementioned LUX-Head and Neck 1 trial have shown increased benefit from afatinib monotherapy (Machiels et al., 2015). The treatment benefit of afatinib was more prominent in p16-negative and EGFR-amplified or EGFR-therapy naïve patients, denoting predictive biomarkers for afatinib treatment. Interestingly, better progression-free survival with afatinib treatment was seen in patients with low HER3 and high phosphatase and tensin homolog (PTEN), suggesting constitutive PI3K-activation might antagonize the afatinib activity in which further investigation is warranted (Cohen et al., 2017). In the effort to achieve more precise treatment, a biomarker-driven trial is currently being conducted (NCT03088059). Patients whose tumors are p16 negative, EGFR/HER2 amplification or mutation and high PTEN, are selected to compare the objective response rate between the treatment afatinib and chemotherapeutic drugs. To date, the clinical outcome from these trials are not reported yet.

2.3.2.2 Afatinib-based combination treatment

To date, clinical assessments of afatinib-based combination treatments in OSCC patients remain limited. In a phase 1 clinical trial (NCT01732640), although combination of afatinib with standard cytotoxic chemotherapeutic drugs such as paclitaxel and 5-FU demonstrated promising preliminary antitumor activity, the toxicity issues are concerning (Chung et al., 2016; Vermorken et al., 2013). Of note, numerous preclinical studies have highlighted that the combination of afatinib with other targeted inhibitors is a promising approach in various cancer types. Therefore, clinical evaluations have been carried out to assess such combinations in patients. For example, a combination of afatinib with sirolimus (mammalian target of rapamycin (mTOR) inhibitor) has demonstrated potent activity in NSCLC patients with EGFR mutation-positive (NCT00993499). The combination of dasatinib (proto-oncogene tyrosine-protein kinase Src (SRC) kinase inhibitor) and afatinib has also been assessed in Phase I trial in patients with lung carcinoma (NCT01999985). Following the synergistic anti-tumor effects observed in preclinical models (Sun et al., 2014), a Phase I/II trial is currently being conducted to assess the activity of afatinib plus selumetinib (MEK inhibitor) in patients with NSCLC, pancreatic, gastrointestinal or colorectal cancers (NCT02450656). Apart from targeting the intracellular signaling pathways, other clinical studies also explore the combination of afatinib with agents that target the receptor tyrosine kinases such as vascular endothelial growth factor receptor (VEGFR) and insulin-like growth factor 1 receptor) (IGF-IR) in advanced solid tumors (NCT00998296; NCT02198291).

While afatinib-based combination treatments are clinically evaluated in various human malignancies, such studies in oral cancer are very limited (Table 2.3). Several clinical trials are currently at early phases, one of these is to elucidate the antitumor activity of afatinib in combination with cetuximab in patients with R/M OSCC. Notably, in a

previous phase I clinical trial, this combination regimen demonstrated potent preliminary antitumor activity in a number of solid tumors including HNSCC (Gazzah et al., 2018). Following the recent findings that revealed the immunomodulatory role played by afatinib, a clinical trial is currently underway to determine the objective response resulting from the combination of afatinib and anti-PD-1 inhibitor (pembrolizumab). Notably, these trials are driven by the preclinical evidence by which afatinib potentiates the sensitivity of cetuximab or pembrolizumab (De Pauw et al., 2018; Watanabe et al., 2019). Given that afatinib monotherapy has limited clinical benefit, perhaps a better understanding of the mechanism underlying the resistance of afatinib is useful to provide insight into the development of effective combination regimen.

2.3.3 Mechanism of resistance

Study of mechanisms underlying the resistance to afatinib is emerging, in which many of these were focused on lung malignancies. Of note, *EGFR* secondary mutation T790M is the major acquired resistance mechanism, in which about 50% of the patients with lung cancers have been detected with this particular mutation (Tanaka et al., 2017; Wu et al., 2016). Similarly, another two *EGFR* mutations L792F and C797S were also found in lung tumors which acquired resistance to afatinib (Kobayashi et al., 2017), suggesting the significance of these mutations in developing resistance to afatinib is this cancer type.

Another common mechanism of resistance to afatinib is the activation of alternative pathways that bypass EGFR-dependent signaling. In particular, Kirsten rat sarcoma viral oncogene homolog (KRAS) amplification and overexpression were found upon acquisition of afatinib resistance (Eberlein et al., 2015; Yamaoka et al., 2017). Other studies have also revealed that upregulated insulin-like growth factor-binding protein 3(IGFBP3), signal transducer and activator of transcription (STAT) and fibroblast growth

Table 2.2: Clinical trials (phase II and III) of afatinib monotherapy and combination in oral cancer (http://clinicaltrials.gov/)

Clinical trial.gov identifier	Study	Study regimen	Study population	Primary Endpoint	Status of clinical trials
Monotherapy					
NCT00514943	Randomised, phase II	Afatinib versus cetuximab	Recurrent and/or metastatic	Tumor Shrinkage	Completed[a]
NCT01345682	Randomised, phase III (LUX-Head and Neck 1)	Afatinib versus methotrexate	Recurrent and/or metastatic	Progression-free Survival	Completed[b]
NCT01856478	Randomised, phase III (LUX-Head and Neck 3)	Afatinib versus methotrexate	Recurrent and/or metastatic (in Asian patients)	Progression-free Survival	Active, not recruiting
NCT01345669	Randomised, phase III (LUX-Head and Neck 2)	Afatinib versus placebo (as adjuvant after chemo-radiotherapy)	Locally Advanced	Disease-free survival	Terminated
NCT02131155	Randomised, phase III (LUX-Head and Neck 4)	Afatinib versus placebo (as adjuvant after chemo-radiotherapy)	Locally Advanced (in Asian patients)	Disease-free survival	Terminated
NCT01427478	Randomised, phase II	Afatinib maintenance versus placebo maintenace	Locally Advanced	Disease-free survival	Active, not recruiting
NCT01824823	Randomised, phase II	Afatinib versus placebo (as adjuvant after chemo-	Locally Advanced	Disease-free survival	Active, not recruiting
NCT03088059	Non randomised, phase II	Biomarker-based Study	Recurrent and/or metastatic	Progression-free Survival, Objective response rate	Recruiting
NCT02465060	Non randomised, phase II	Targeted Therapy Directed by Genetic Testing	Advanced refractory	Objective response rate	Recruiting
Combination					
NCT02979977	Single arm, phase II	Afatininb plus cetuximab	Recurrent and/or metastatic	Tumor shrinkage	Recruiting
NCT03695510	Single arm, phase II	Afatinib plus Pembrolizumab	Recurrent and/or metastatic	Objective response rate	Not yet recruiting

[a] Seiwert et al., 2014; [b] Machiels et al., 2015

factor receptor 1 (FGFR1) mediated acquired resistance to afatinib (Azuma et al., 2014; Kim et al., 2012; Yamaoka et al., 2017). Furthermore, PI3K/AKT and ERK pathways activation together with epithelial-mesenchymal transition (EMT) features were noticed in afatinib-resistant cells (Coco et al., 2015). In gastric cancer, overexpression of ephrin type-A receptor 2 (EPHA2) and reactivation of MAPK contributed to sustain cell survival in the presence of afatinib, suggesting a crucial role of resistance development (Gao, Shen, Chen, & Liu, 2018).

Despite various mechanisms underlying afatinib resistance have been uncovered, thus far such studies have not been done on OSCC. It is crucial to understand the underlying mechanism that compensates the ERBB inhibition and discover new treatment strategy such as dual targeting to overcome the therapeutic limitation of afatinib for optimal future application in OSCC treatment.

CHAPTER 3: MATERIALS AND METHODS

3.1 Study design

This study determined the effects of afatinib and its mechanism of action on OSCC cell lines and xenograft models. The synergistic effect of afatinib with another drug, trametinib was also investigated to determine if this could improve the efficacy of afatinib.

3.2 Cell Lines

3.2.1 Oral cancer cell lines

Human oral cancer cell lines 'ORL series' were established from Malaysian oral cancer patients that possess unique molecular-genetics profiles of OSCC and provided by Cancer Research Malaysia. These ORL cell lines (ORL-48, -115, -136, -150, -153, -156, -174, -188, 196, -204, -207 and -215) were derived from tumors and spontaneously immortalized (Fadlullah et al., 2016; Hamid et al., 2007). Another human-derived oral cancer cell line from a Caucasian patient, CAL27 was a kind gift from Dr. J. Silvio Gutkind (University of California, San Diego, USA). All the cell lines were cultured for *in vitro* studies and *in vivo* xenograft inoculations. The authenticity of all cell lines was confirmed by short tandem repeat (STR) profiling. Cell line information is summarized in Appendix A. Genetic aberrations such as copy number alteration and mutation status of the ERBB family receptors of these OSCC cell lines are listed in Appendix B.

3.2.2 Maintenance of cell lines

ORL cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM)/Nutrient mixture F-12 HAM's medium (Hyclone, Logan, Utah, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Gibco, Auckland, NZ), 100 IU Penicillin/Streptomycin (Gibco) and 0.5 µg/ml hydrocortisone (Sigma-Aldrich, St Louis, MO, USA). CAL27 was cultured in DMEM medium (Gibco)

supplemented with 10% (v/v) heat-inactivated FBS and 100 IU Penicillin/Streptomycin. All cell lines were cultured at 37ºC in a humidified atmosphere of 5% CO_2.

3.3 Determination of drug effects in OSCC cell lines by *in vitro* assays

3.3.1 Crystal violet-cytostatic assay

OSCC cells were seeded with a density of 9000 to 40,000 cells per well in triplicates in a 24-well plate (Appendix C). After overnight incubation (day 0), cells were treated with afatinib in a serial 4-fold dilution of the working stock concentration (640 nM) for 72 hours, and 0.01% (v/v) dimethyl sulfoxide (DMSO; Sigma-Aldrich) was used as the control. Following 72 hours of incubation, 2% (w/v) crystal violet (Sigma Aldrich) was added to each well to stain the viable cells. The resulting crystals were then dissolved in 500 µl of 100% DMSO and measured at a wavelength of 590 nm by using the Synergy™ HI Microplate Reader (BioTek Instruments, Winooski, VT, USA). The untreated cells after 24 hours of cell seeding (day 0) were stained and measured as mentioned above. Drug concentrations for 50% of maximal inhibition of cell growth (GI_{50}) were calculated using the formula: $((X_i-X_0)/(X_{DMSO}-X_0))*100$, whereby X_0 is the absorbance value at day 0, X_i is the absorbance value after 72 hours of drug treatment, and X_{DMSO} represnts the absorbance value of vehicle control after 72 hours (Holbeck, Collins, & Doroshow, 2010). Three independent experiments were conducted and the result is represented as mean ± SEM.

3.3.2 Click-iT proliferation assay

The impact of afatinib on cell proliferation was determined by the Click-iT assay (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, ORL-48, ORL-115, ORL-156, ORL-207 and CAL27 that were seeded on glass cover-slips with a density of 100,000 cells in 12-well plate. Cells were treated with 0.025% (v/v) DMSO and indicated drugs concentrations (1, 10, 100 nM) for 24 hours. Then, cells were

processed to allow incorporation of 5-ethynyl-2′-deoxyuridine (EdU) for 2 hours. Following that, cells were fixed with 3.7% (v/v) formaldehyde (in phosphate-buffered saline (PBS)) for 15 minutes. After washing with 3% (w/v) bovine serum albumin (BSA), cells were incubated with 0.5% (v/v) Triton-X solution (Sigma Aldrich) for 20 minutes at room temperature. Cells were next stained with the Click-iT reaction cocktail (1X Click-iT reaction buffer, $CuSO_4$, Alexa Fluor azide 488, reaction buffer additive) for 30 minutes in the dark. Subsequently, the Click-iT reaction cocktail was washed off and cells were incubated with 5 µg/ml of 1X Hoechst 33342 solution in the dark for 30 minutes. After rinsing with PBS,the cover-slips were mounted with Vectashield mounting medium (Vector Laboratories Inc, Burlingame, CA, USA) and kept protected from light. For analysis, images of EdU positive and Hoechst 33342 stained cells were captured with a fluorescent microscope (Olympus, Shinjuku-ku, Tokyo, Japan) with double bandpass filter. Images were captured from at least 5 random fields of each experiment and quantified using QuickCount® software (Cancer Research Malaysia, Subang Jaya, Selangor, Malaysia; (Tiong et al., 2018)). The number of Edu positive cells and Hoechst 33342 stained cells were counted for each image and the percentage of EdU positive cells was calculated using the following formula: number of EdU positive cells/number of Hoechst 33342 stained cells x 100. EdU positive cells represent cells that are undergoing DNA synthesis, whereas Hoechst 33342 stained cells represent total cells. This assay was performed at least twice for each cell line.

3.3.3 Cell cycle analysis

ORL-48 and CAL27 were seeded with a density of 200,000 cells in 60 mm^3 culture dish. Given that afatinib exhibited most pronounced anti-proliferative effects at the concentration of 100 nM in Click-iT assay, ORL-48 and CAL27 were treated with the same concentration for the indicated time-points (0, 12, 24, 48 and 72 hours) to analyse cell cycle progression. After cells were harvested and centrifugated at 2,500 rpm for 5

minutes, pellets were resuspended and fixed in 70% ethanol for 16 hours in -20°C. Prior to analysis, fixed cells were pelleted, washed in 1X PBS and stained with 10 µg/mL propidium iodide and 20 µg/ml RNase for 15 minutes on ice and in the dark. As propidium iodide stains both DNA and RNA, RNase was added to degrade RNA for accurate analysis. DNA content of at least 10,000 cells was measured with FACSCalibur^{TM} flow cytometer (BD Biosciences, San Jose, CA, USA). After gating the live cells, single cell population was gated using area and width parameters. To determine the percentage of cells in each cell cycle phase, the area parameter histogram was analysed using ModFit software (Verity Software House, Topsham, Maine, USA).

3.4 Determination of drug efficacy in preclinical OSCC xenograft models

3.4.1 Development of OSCC xenograft models

All animal studies were performed in accordance with National Institute of Health (NIH) guide for the care and use of laboratories animals. The protocols were approved by the Animal Ethics Committees of the National University of Malaysia (UKM) (Ethical approval code: CRM/2016/CHEONG/18-MAY/762-JUNE-2016-JUNE-2019). Female NOD/SCID mice (BioLASCO, Nangang Dist., Taipei, Taiwan) at 4–6 weeks of age were were housed in appropriate sterile filter-capped cages, and fed and watered *ad libitum*. For subcutaneous OSCC model establishment, 2 X 10^6 cells were injected to both flanks of the mice. The mice were evaluated every other day for general behavioral abnormalities, signs of illness or discomfort. Tumor volume was determined using the formula: $LW^2/2$ whereby L and W represent the length and the width of the tumor respectively. Body weight was measured and the tumor volume was recorded by the same operator using digital calipers throughout the study.

3.4.2 *In vivo* mouse experiments and analysis

When tumor volume reached ~150-200 mm^3, mice were randomly split into treatment (n=5) and control (n=5) groups. Prior to treatment, afatinib and trametinib were prepared by dissolving in DMSO to obtain 50 mg/ml and 10 mg/ml respectively. Afatinib was further diluted in 0.5% (w/v) of methylcellulose (Sigma Aldrich), and administered at 10 mg/kg/day via oral gavage (Li et al., 2008; Quesnelle & Grandis, 2011; Regales et al., 2009; Schutze et al., 2007; Young et al., 2015). Meanwhile, trametinib was diluted in PBS containing 5.2% (v/v) of polyethylene glycol (PEG; Sigma Aldrich) and 5.2% (v/v) of Tween 80 (Sigma Aldrich), and was given via intraperitoneal injection at 1 mg/kg (Yamaguchi K et al., 2016). For combination treatment, the same dose of respective drugs was given via the administration routes described above. The control groups were treated with equivalent concentrations of DMSO (1-2%) dissolved in the same diluent as mentioned above. All animals were treated once daily for 22 days and tumors were measured 2 times weekly. At the end of the study, all mice were euthanized and all tumors were harvested. Non-necrotic tumor tissues were snap-frozen and stored in liquid nitrogen. A portion of the tumor tissue was fixed in formalin and embedded in paraffin for hematoxylin and eosin (H&E) histopathological evaluation using the Anneroth multifactorial grading system (Anneroth G et al., 1987) by a board-certified pathologist.

3.5 Evaluation of the mechanism of action of afatinib on OSCC cells

3.5.1 Western blotting

Following treatment with 100 nM afatinib or DMSO control (0.025 % (v/v)) at different time (0, 5, 10, 30 minutes and 1, 3, 6, 12, 24 hours), cells were stimulated by 100 ng/ml of epidermal growth factor (EGF) for 30 minutes. Then, total cell lysates (TCL) were extracted with RIPA buffer (50mM Tris pH8, 1% (v/v) NP-40, 0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate and 150 mM NaCl) supplemented with Halt Protease and Phosphatase Inhibitor Cocktail (Pierce Biotechnology, Rockford, IL, USA) on ice.

TCL was collected by centrifugation and protein concentration was quantified using the BSA method (Thermo Scientific, Waltham, MA, USA). To conduct western blot analysis, 25 µg of the TCL was used to resolve total proteins by SDS-PAGE (refer to Appendix D) at 100 V for 90 minutes. Subsequently, proteins were transferred onto PVDF membranes (Millipore, Burlington, MA, USA) at 400 mA for 1 hour on ice. Membranes were blocked with 5% (w/v) skim milk in Tris-buffered saline with 0.1% (v/v) Tween 20 (TBST) for one hour. After three times washing in TBS supplemented with 0.1% Tween 20 (Sigma Aldrich; TBST) the membranes were probed with the indicated primary antibodies (1:1000 dilution in 1% (w/v) BSA) (refer to Appendix E) overnight at 4°C. After three times washing in TBST, membranes were incubated with the corresponding secondary antibody (1:10,000 dilution in 5% milk) (refer to Appendix E) for 1 hour at room temperature. This was followed by washing three times in TBST before detection by WesternBright Quantum HRP substrate (Advansta Inc, San Jose, CA, USA) and visualization using the FluorChem^{TM} HD2 imaging systems (Alpha Innotech, San Jose, CA, USA). Spectra multicolor broad range protein ladder (Thermo Fisher Scientific, Waltham, MA, USA) was used as marker to identify the molecular weight of the protein signals. TCL of ORL-136 that has constitutively active EGFR was used as a positive control for the detection of phosphorylated EGFR. To normalize for loading, the blots were stripped and re-probed with anti-tubulin antibody (1:1000 dilution in 1% BSA) and processed as described above. To determine the drug combination effect on ERBB mechanisms, cells were treated with afatinib (100 nM) and varying concentrations of trametinib (10, 20 and 40 nM) for 24 hours. Following the drug treatments, cells were stimulated by 100 ng/ml of epidermal growth factor (EGF) for 30 minutes. TCL was collected and processed as mentioned above.

3.6 Evaluation of the synergistic potential of afatinib and trametinib in combination

3.6.1 Crystal violet-cytostatic assay

ORL-48 and CAL27 were seeded with a density of 13,000 and 10,500 respectively in each well of 24-well plate and treated with a concentration range of trametinib in a serial 4-fold dilution of the working concentration (40 nM) for 72 hours. The crystal violet-cytostatic assay was performed as mentioned in section 3.3.1 to determine the GI_{50} of trametinib. Then, the same cell number of respective cell lines was seeded as described above. Cells were treated with a range of afatinib in a serial 4-fold dilution of working concentrations (ORL-48: 40 nM; CAL27: 320 nM) for 72 hours, with or without the addition of respective GI_{50} of trametinib (ORL-48: ~3 nM; CAL27: ~11 nM) that have been determined and shown in Appendix F. Following that, the crystal violet-cytostatic assay was performed and the percentage of cell viability across the tested concentration was calculated by using the formula: $((X_i-X_0)/(X_{DMSO}-X_0))*100$, whereby X_0 is the absorbance value at day 0, X_i is the absorbance value after 72 hours of drug treatment, and X_{DMSO} represents the absorbance value of vehicle control after 72 hours (Holbeck et al., 2010). Next, five parameter fit curves were plotted and the GI_{50} of afatinib in cells treated by afatinib alone or in combination with trametinib was determined. Three independent experiments were conducted and the result is represented as mean ± SEM.

3.6.2 *3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide* (MTT)

ORL-48 and CAL27 were seeded at a density of 3000 and 5000 respectively per well in 96 well plates in triplicate. Varying concentrations of afatinib (a serial 4-fold dilution of 400 nM) and trametinib (a serial 4-fold dilution of 200 nM), either as single or in combination were added to the wells. After 72 hours of incubation, cells were added with 5 mg/ml of *3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide* (MTT) (Sigma Aldrich) and incubated for 4 hours. Subsequently, the resulting purple formazan was

dissolved in 100 µl of 100% DMSO. Absorbance at 570 nm was measured by using the Synergy^{TM} HI Microplate Reader (BioTek Instruments).

3.6.3 Chou-Talalay's synergistic analysis

Synergistic effect of the drug combination was determined by calculating the combination index (CI) value. The fraction-affected (Fa) value of each combination in MTT assay (section 3.5.2) was calculated and analyzed according to the Chou–Talalay method (Chou, 2010) using CompuSyn software (ComboSyn, Inc., Paramus, NJ, USA). A CI value below 1 represents synergism, above 1 represents antagonism, while CI equals to 1 means additive effect between these 2 drugs.

3.7 Comparison of efficacy of single and combination treatments in preclinical OSCC xenograft models

3.7.1 Drug efficacy on OSCC xenografts

Efficacy of the single agent and combination treatment was indicated by tumor growth inhibition (TGI). Tumor inhibition was determined by the formula: %TGI = 100 - (T/C X 100), where T = mean tumor volume of treatment group at the end of treatment/ mean volume of treatment group at the start of treatment, while C = mean tumor volume of control group at the end of treatment/mean volume of control group at the start of treatment (Sanceau J et al., 2002).

3.8 Statistical analysis

All statistical analysis was performed using IBM SPSS Statistics for Window, version 21.0 (IBM Corp., NY City, NY, USA). Independent t-test was used to determine the significant difference between the control and afatinib-treated groups in afatinib efficacy *in vivo* study. One-Way ANOVA was conducted to perform comparisons between the control and different treatment groups in Click-iT proliferation assay, cell cycle analysis as well as single/combination treatments in the mouse xenograft study. Nonlinear

regression analysis was carried out to demonstrate the dose-response of the drug combination. Significant *p* values were indicated as $p < 0.05$*, $p < 0.01$** or $p < 0.001$***.

CHAPTER 4: RESULTS

4.1 Effects of afatinib in OSCC *in vitro* and *in vivo*

4.1.1 Inhibition of cell growth by afatinib

In this study, the response of OSCC cells to afatinib was evaluated by measuring the cell growth after 72 hours of afatinib treatment. The result showed that all OSCC cell lines were markedly sensitive to afatinib, with mean GI_{50} ranging from ~3 to 45 nM (Figure 4.1, Table 4.1). Majority of the cell lines tested had amplified EGFR, except for CAL27 that had the highest GI_{50} among the tested cell lines (Appendix B). While majority of these cell lines have wild type *ERBB*, no link was observed between the drug response and mutation status of the ERBB family members.

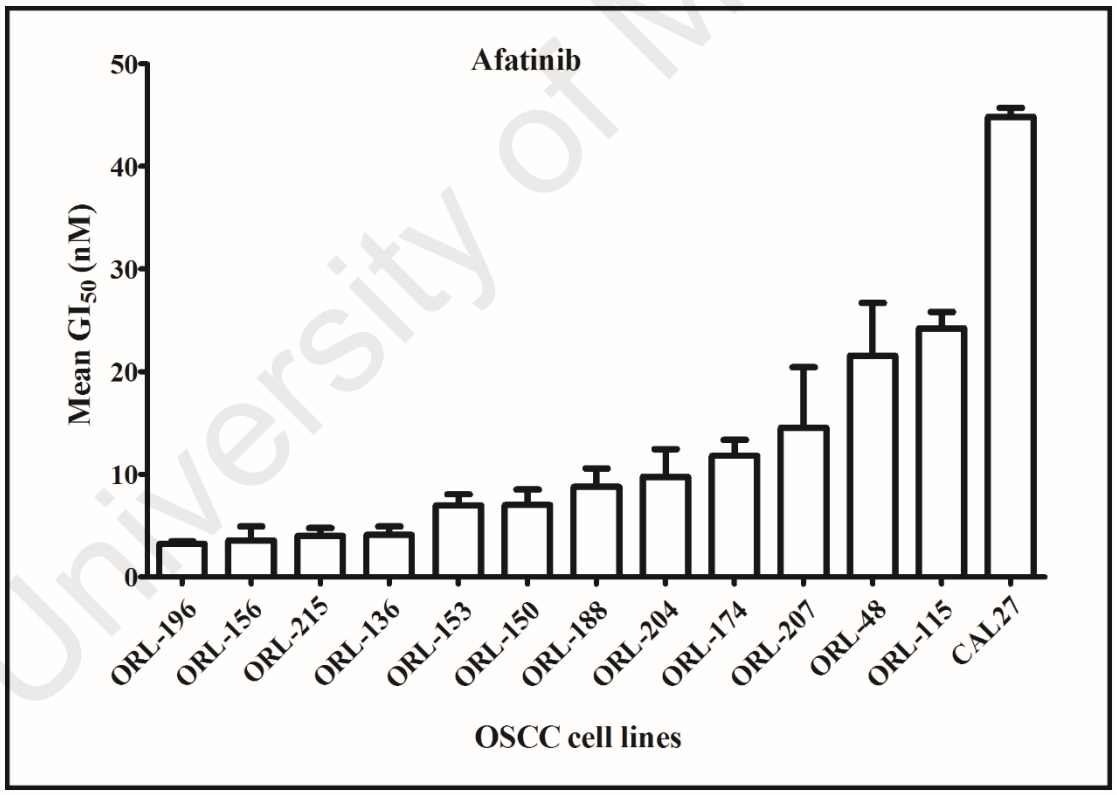

Figure 4.1: OSCC cell lines are sensitive to afatinib. The crystal violet-cytostatic assay showed that all the 13 OSCC cell lines treated with afatinib had GI_{50} less than 1 µM.

Table 4.1: Mean GI_{50} of afatinib and population doubling time of OSCC cell lines

Cell Lines	Mean G_{I50} (nM) (n=3)	Population doubling time (hour)
ORL-196	3.2 ± 0.2	17.4 ± 0.4[a]
ORL-156	3.6 ± 1.1	12.3 ± 0.1[a]
ORL-215	4.0 ± 0.6	18.9 ± 0.0[a]
ORL-136	4.1 ± 0.7	37.7 ± 2.1[a]
ORL-153	7.0 ± 0.8	18.5 ± 3.7[a]
ORL-150	7.0 ± 1.2	25 ± 0.6[a]
ORL-188	8.8 ± 1.3	27.5 ± 0.2[a]
ORL-204	9.8 ± 2.2	16.3 ± 0.1[a]
ORL-174	11.8 ± 1.3	27.9 ± 0.3[a]
ORL-207	14.5 ± 4.2	17.9 ± 1.3[a]
ORL-48	21.6 ± 4.2	15 ± 0.7[a]
ORL-115	24.5 ± 1.0	18.2 ± 3.1[a]
CAL27	43.2 ± 0.6	35[b]

[a] Data was obtained from *Fadullah et al., 2016*

[b] Data was obtained from *Gioanni et al., 1988*

Of these 13 tested cell lines, ORL-136 and CAL27 had the longest population doubling time (Table 4.1). Although the proliferation rate of both cell lines are similarly slow, ORL-136 had ~13.5 times lower GI_{50} than CAL27. Besides, ORL-115, ORL-153 and ORL-215 which have similar population doubling times showed different GI_{50} concentrations when tested with afatinib, Taken together, these observations indicates no clear correlation between drug response (GI_{50}) and population doubling time.

4.1.2 Afatinib blocks DNA synthesis of OSCC cells

To evaluate the impact of cell proliferation, a subset of these cell lines (ORL-48, CAL27, ORL-207, ORL-115, and ORL-156) was tested by measuring active DNA

synthesis in drug-treated (1-100nM) cells compared to control (0.05% DMSO) cells for 24 hours, using the Click-iT assay. Results showed that the percentage of EdU positive cells (in red) was significantly reduced upon afatinib treatment, indicating inhibition of cell proliferation in all OSCC cell lines tested (Figure 4.2). Notably, 100 nM of afatinib over 24 hours was able to reduce proliferating cells by ~73-89% in ORL cell lines (p <0.001) and ~15% in CAL27 compared to DMSO-treated cells (p = 0.009).

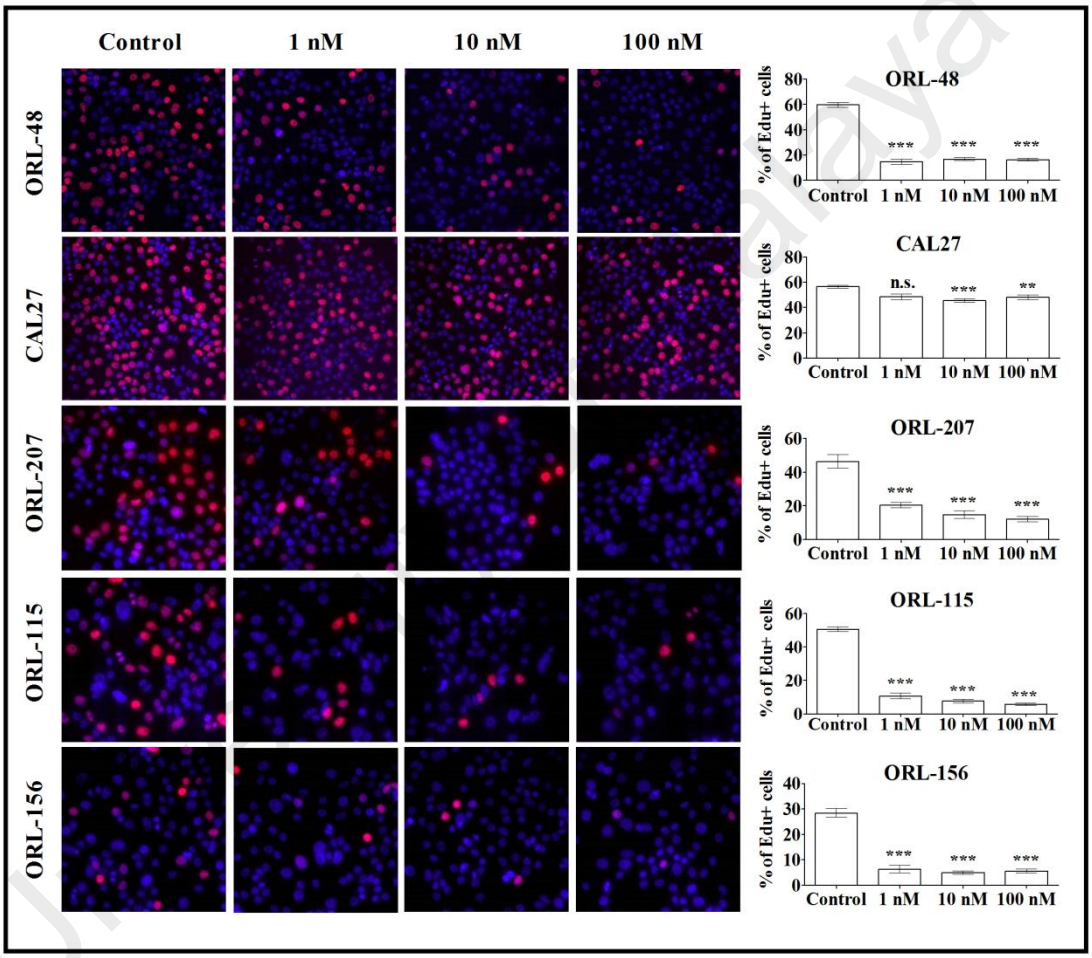

Figure 4.2: Afatinib blocks DNA synthesis. OSCC cells were treated with afatinib for 24 hours and subsequently stained with AlexaFluor 488-labeled EdU. Hoechst 33342 staining (blue color) shows total cells. Click-iT proliferation assays demonstrated a reduction of actively proliferating cells (red color) (left). Percentage of EdU positive cells which represents actively proliferating cells were significantly reduced in afatinib-treated cells compared to DMSO control (right). Significant p values were indicated as p <0.01** or p <0.001***, whereas n.s. means not significant.

4.1.3 Afatinib induces cell cycle arrest at G_0/G_1 phase

To determine if the anti-proliferative effects by afatinib were due to the arrest in the cell cycle, afatinib-treated ORL-48 and CAL27 cells (100 nM) were stained with propidium iodide. Based on the flow cytometry analysis, afatinib induced a G_0/G_1 cell cycle arrest as early as 12 hours post-treatment (p <0.001), in which the cell population at this phase significantly increased compared to 0 hour. Consistently, corresponding reduction of the cell population in S and G_2/M phases were observed in both cell lines tested (Figure 4.3).

4.1.4 Afatinib efficaciously suppressed OSCC tumor growth

As both ORL-48 and CAL27 are tumorigenic in NOD/SCID mice, hence they were used to further investigate the efficacy of afatinib in mouse xenograft models. The results showed that daily treatment of afatinib at 10 mg/kg significantly inhibited tumor growth relative to controls in both ORL-48 and CAL27 xenografts (p <0.001) (Figure 4.4).

4.2 Mechanism of action of afatinib on OSCC cells

ERBB family receptors activate numerous pathways, including PI3K/AKT and Ras/ERK pathways which are crucial for cell growth. To confirm if these pathways are targeted upon afatinib treatment, both ORL-48 and CAL27 cells were treated with 100 nM afatinib with or without stimulation of EGF. As shown in Figure 4.5, afatinib was able to completely inhibit the phosphorylation of EGFR, HER2 and HER3 irrespective of EGF stimulation as early as 5 minutes post-treatment. Notably, a time-dependent increased of total HER3 was observed in both ORL-48 and CAL27. The results also

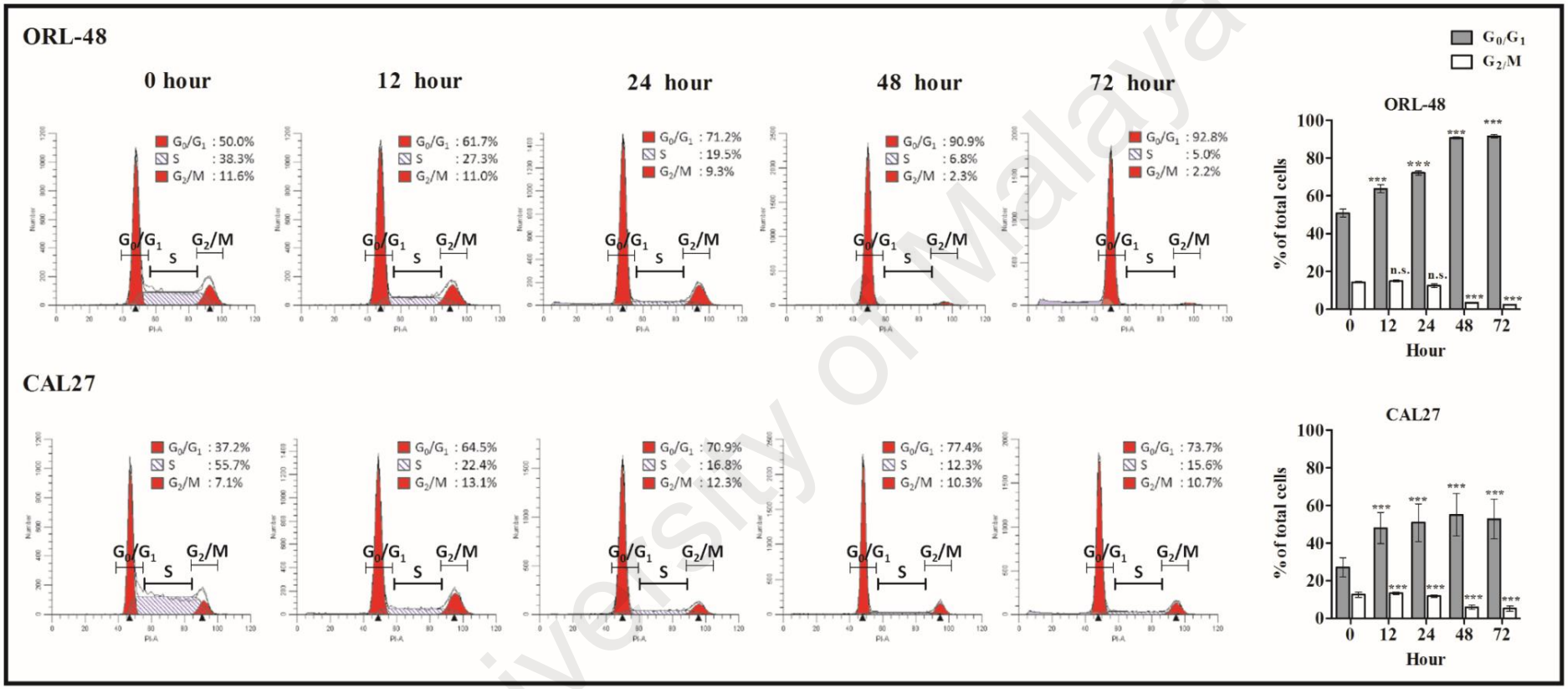

Figure 4.3: Afatinib inhibited cell cycle progression. ORL-48 and CAL27 were treated with 100 nM afatinib in time dependent manner. Cell cycle analysis showed a marked increase of cell population in G_0/G_1 phase, with the corresponding decrease in S and G_2/M phases in both ORL-48 and CAL27. Significant *p* values were indicated as $p < 0.001$***.

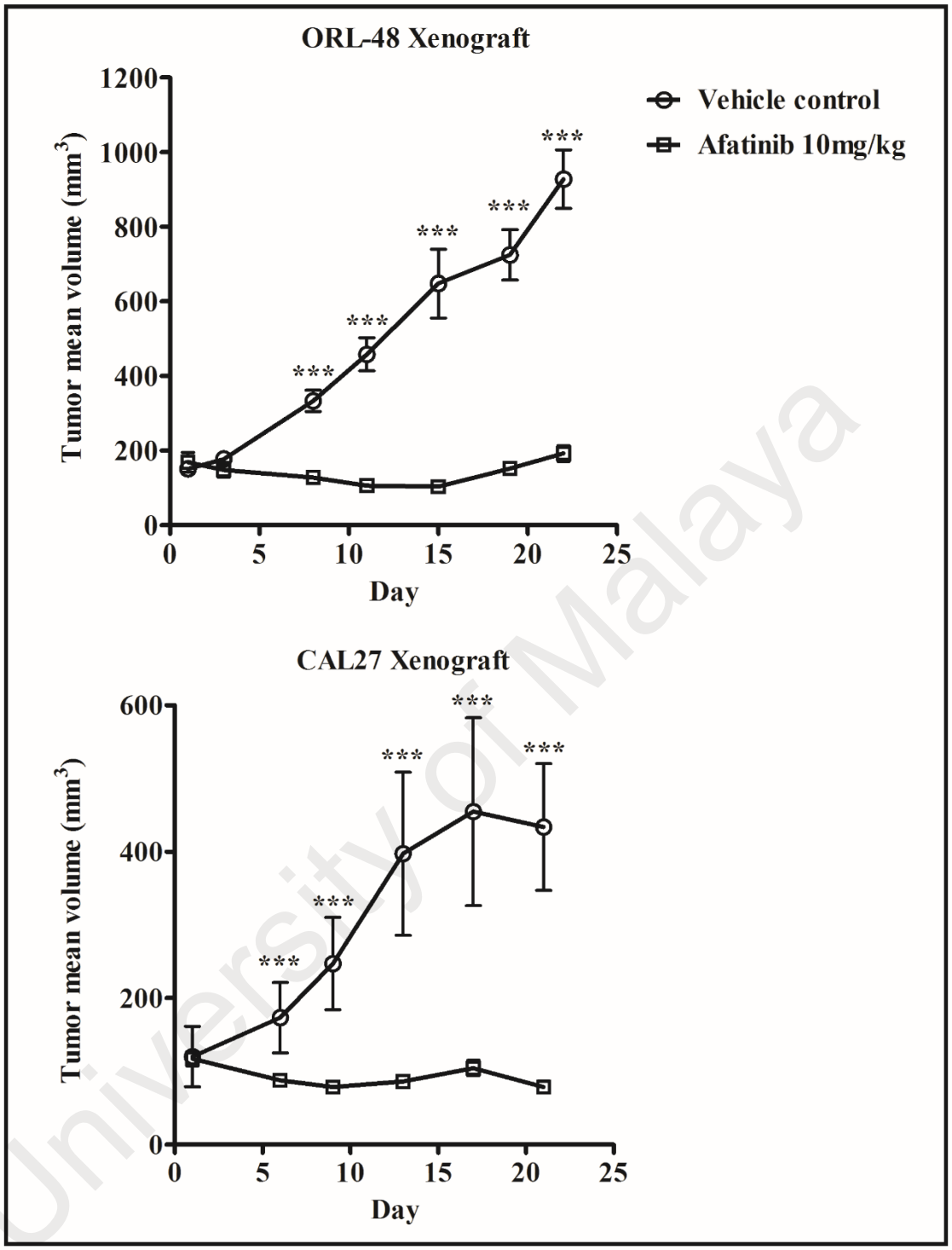

Figure 4.4: Afatinib inhibits OSCC tumor growth. Subcutaneous models of ORL-48 and CAL27 were treated with either vehicle control (2% of DMSO) (n=5) or afatinib (10 mg/kg) (n=5) daily via oral gavage for 22 days. Treatment of afatinib markedly regressed tumor growth in mice bearing ORL-48 and CAL27. Significant difference between the control and treatment groups at each time-point of tumor measurement is represented by *** (p <0.001).

showed blockage of AKT activity at Ser473 phosphorylation site in a time-dependent manner, but not at the phosphorylation site of Thr308. Following the rapid suppressed of ERBB phosphorylation by afatinib, inactivation of the downstream ERK1/2 was observed. However, the prolonged afatinib exposure was not able to result in sustained ERK1/2 inhibition, whereby there was a rebound in phosphorylation of ERK1/2 as early as 12 hours post treatment in both cell lines.

4.3 Combinational effect of afatinib and trametinib in OSCC *in vitro* and *in vivo*

4.3.1 Trametinib potentiated effects of afatinib in OSCC cells

Given that reactivation of ERK1/2 was observed *in vitro*, anti-tumor effects could possibly be improved by co-targeting the ERBB family receptors and ERK1/2 was. To test this hypothesis, the FDA approved drug trametinib that targets MEK which could potently inhibit downstream ERK1/2 was selected. Notably, trametinib has also been shown to significantly reduce the ERK1/2 activation in OSCC patients (Uppaluri et al., 2017). Both ORL-48 and CAL27 were treated with increasing concentration of afatinib, either with or without the addition of a GI_{50} dose of trametinib of the respective cell lines (Appendix F). In the presence of trametinib, a significant reduction of viable cells (in blue) treated with afatinib was observed (Figure 4.6). The sensitization of cells to afatinib when combined with trametinib was also reflected by the decrease of GI_{50} of afatinib by ~10-40-fold in the presence of trametinib compared to cells being treated with afatinib alone.

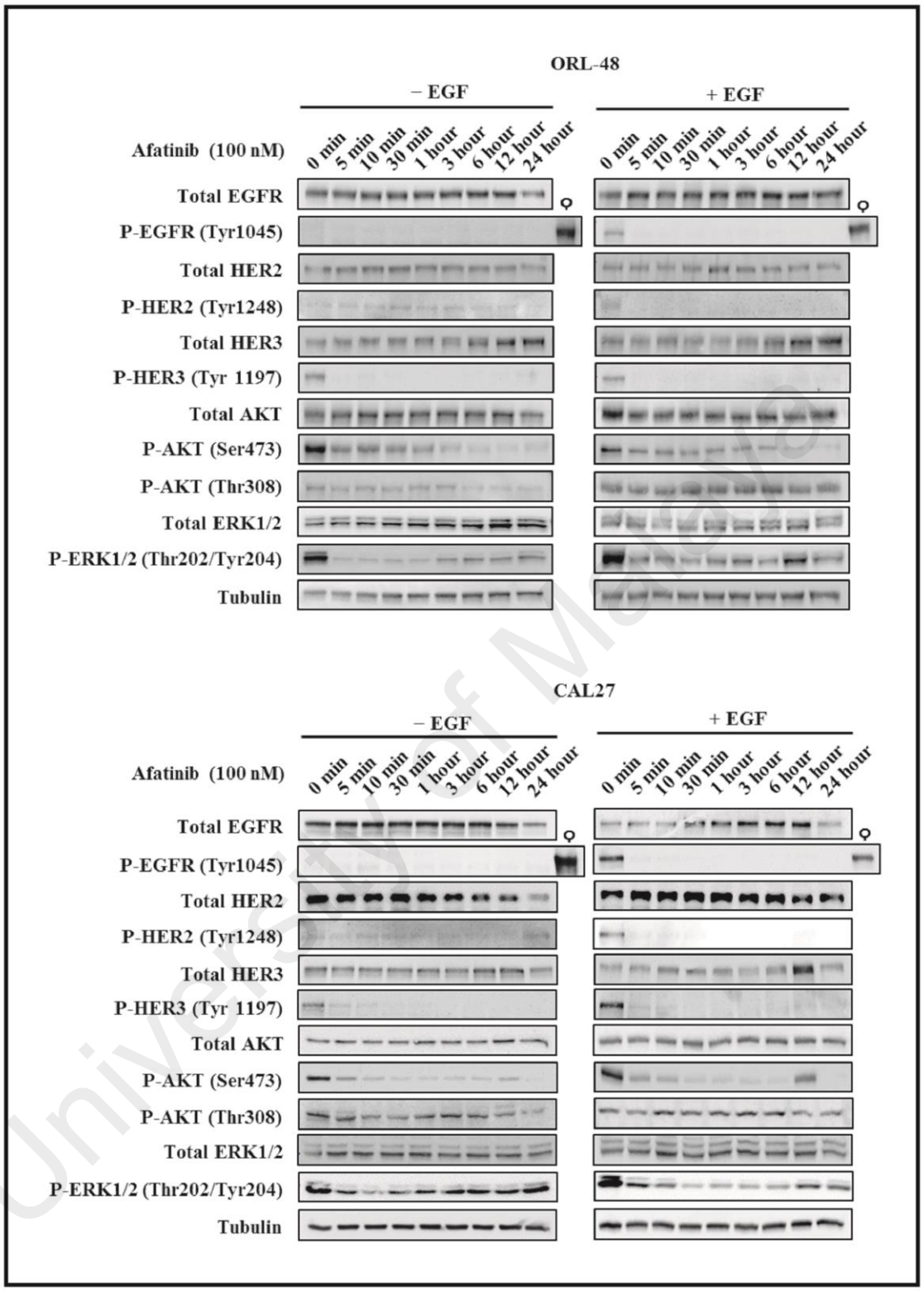

Figure 4.5: Afatinib inactivates ERBB family receptor and downstream signaling. Western analysis were performed on the total protein lysates of ORL-48 and CAL27 treated with afatinib (100 nM) in a time-dependent manner, with or without EGF stimulation. Total protein lysate of ORL-136 (♀) was used as a positive control of P-EGFR detection. The phosphorylation of ERBB family receptors (EGFR, HER2, and HER3) and downstream AKT were inhibited by afatinib in a time-dependent manner, whereas ERK1/2 activity was transiently inactivated in both ORL-48 and CAL27.

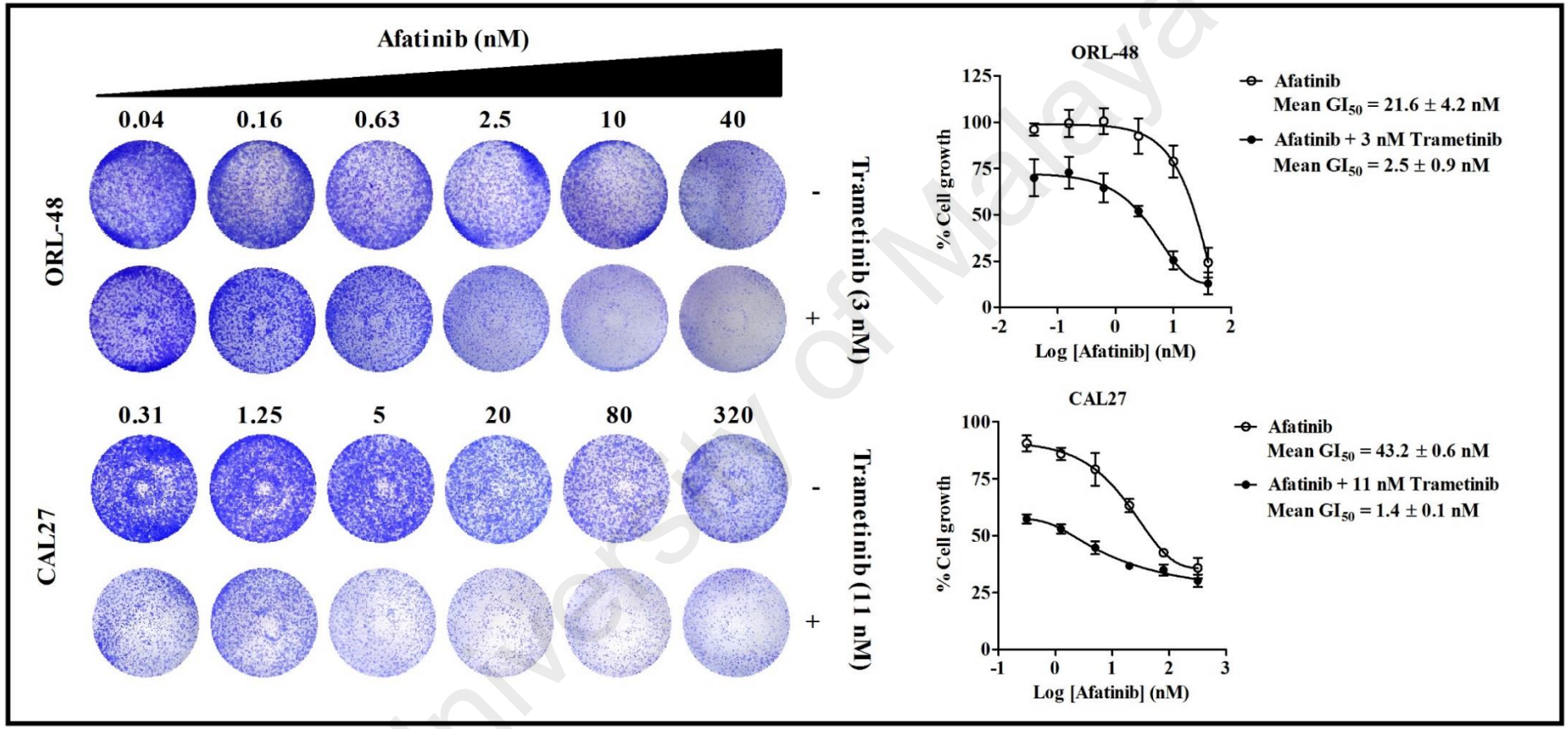

Figure 4.6: Trametinib potentiates afatinib effects in OSCC cells. ORL-48 and CAL27 were treated with various concentration of afatinib, with or without trametinib (GI_{50} of respective cell lines) for 72 hours. Crystal violet staining (purple formazan) shows viable cells (left). The five parameter fit curve indicated the inhibitory effects on cell growth with the treatment of afatinib alone or in combination with trametinib. The GI_{50} of afatinib in both ORL-48 and CAL27 were markedly decreased when combined with trametinib, as compared to cells treated with afatinib alone (right).

4.3.2 Combination treatment of afatinib and trametinib showed synergism

Next, validation of the combinational potential of the two inhibitors was carried out by performing a matrix combination dose response screen in ORL-48 and CAL27 to simultaneously assess single-agent activity and the synergistic interaction across a range of drug concentrations. Results demonstrated synergistic effects at the drug concentrations tested, with CI values that were less than 1 (Figure 4.7A). To further confirm if this dual-inhibition could successfully diminish the reactivation of ERK1/2 resulting from afatinib treatment, the protein lysates of ORL-48 and CAL27 treated either with afatinib, trametinib or combination of both drugs were assessed. As observed in Figure 4.7B, inhibition of ERBB receptors by afatinib single treatment at 24 hours was consistent with observation earlier. As expected, trametinib alone had no effect on the activity of ERBB family receptors and AKT even at 40 nM. Since trametinib directly targets the MEK pathway, phosphorylation of ERK1/2 was markedly reduced as expected on both cell lines. A complete blockade was seen as low as 10 nM in ORL-48, whereas the inhibition in CAL27 was dose-dependent and complete inhibition of ERK1/2 phosphorylation was only seen with the use of drug combination. The result also showed that a combination of afatinib and trametinib inhibited phosphorylation of the ERBB family receptors and AKT. While afatinib alone did not block the phosphorylation of ERK1/2 efficiently, sustained inhibition of ERK1/2 phosphorylation was observed when a combination of afatinib and trametinib was used.

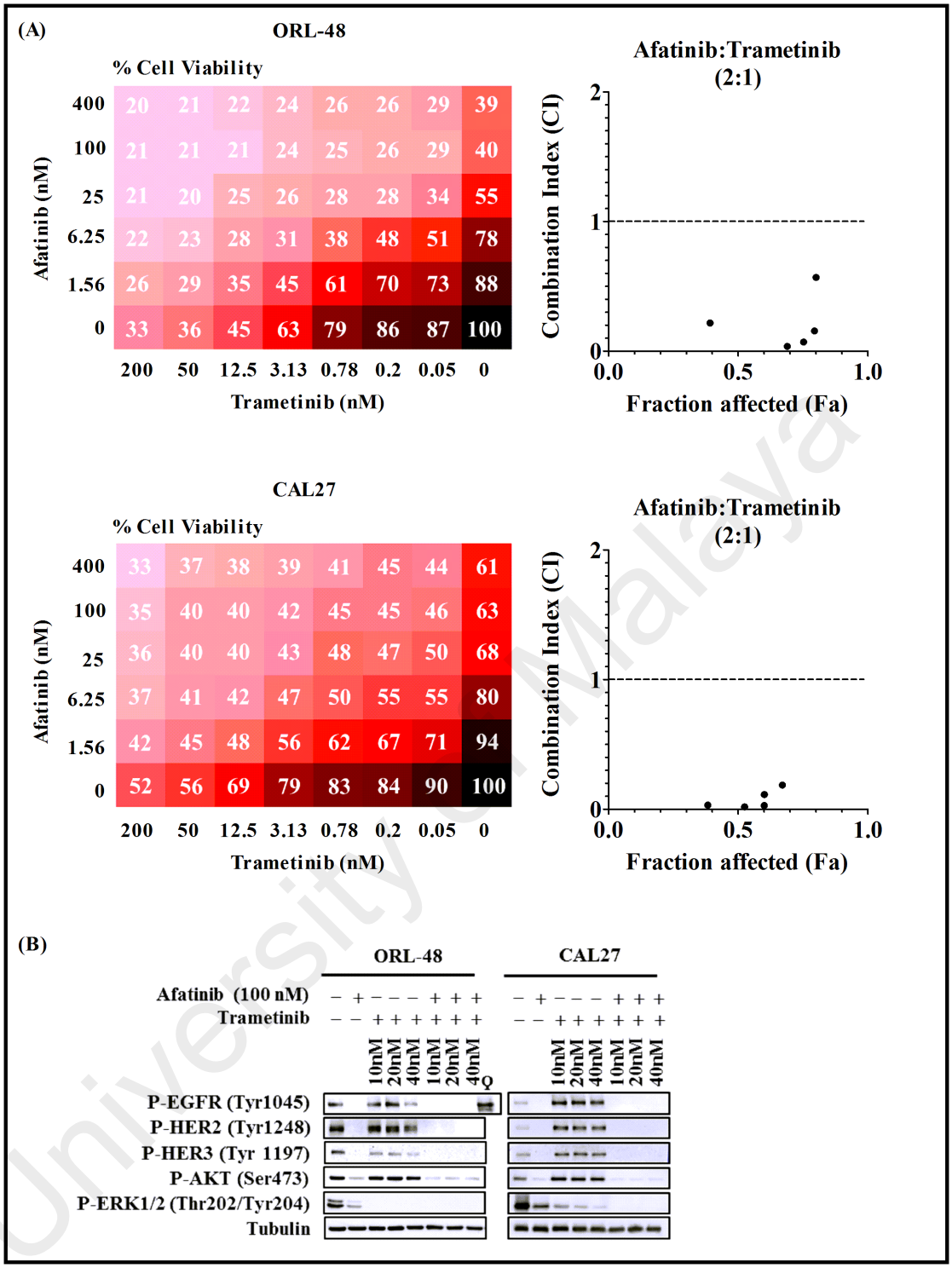

Figure 4.7: Combination of afatinib and trametinib shows synergism. (A) The number in the matrix represents the % of cell viability post-treated by various concentrations of afatinib and trametinib for 72 hours in the MTT colorimetric assay (left). The Fa-CI curve was generated based on the matrix data according to Chou-Talalay's method. Synergistic growth inhibition (CI <1) was induced by a combination of afatinib and trametinib (right). (B) Western analysis was performed using total protein lysates of ORL-48 and CAL27 cells treated with single or combination treatment of afatinib/trametinib at indicated concentration for 24 hours, with EGF stimulation. Total protein lysate of ORL-136 (♀) was used as a positive control for P-EGFR. Combination treatment had more complete inhibition of phosphorylated ERK1/2 compared to afatinib single treatment.

4.3.3 Co-targeting by afatinib and trametinib improved tumor regression in OSCC preclinical models

To further validate the combined effects of afatinib and trametinib observed in cell line models, drug efficacies were assessed in the OSCC mouse model. As seen in Figure 4.8A & B, although ORL-48 and CAL27 xenografts were sensitive to single treatment of afatinib and trametinib, the anti-tumor effect of combination treatment was more potent than either single agent used alone in the mouse models, ORL-48: A vs A+Tr (p =0.003); Tr vs A+Tr (p <0.001) and CAL27: A vs A+Tr (p =0.047); Tr Vs A+Tr (p =0.001). Consistently, this was also reflected by tumor growth inhibition (TGI) values of 76.4% and 89.5% for afatinib, 88.9% and 90.3% for trametinib, 96.9% and 97.8% for combination in ORL-48 and CAL27 xenografts respectively (Table 4.2). From the histopathological examination, no significant differences were observed in the histological grade of malignancy between the untreated and drug-treated tumors in ORL-48 and CAL27 mouse models. Despite that, predominant keratinous material was noticed in the representative tumor of ORL-48 xenograft model after 22-day combination treatment. More importantly, tumor cells were hardly found in this tumor sample. Due to the small size of CAL27 tumor in the combination group, histopathological examination was not able to be done (Appendix G).

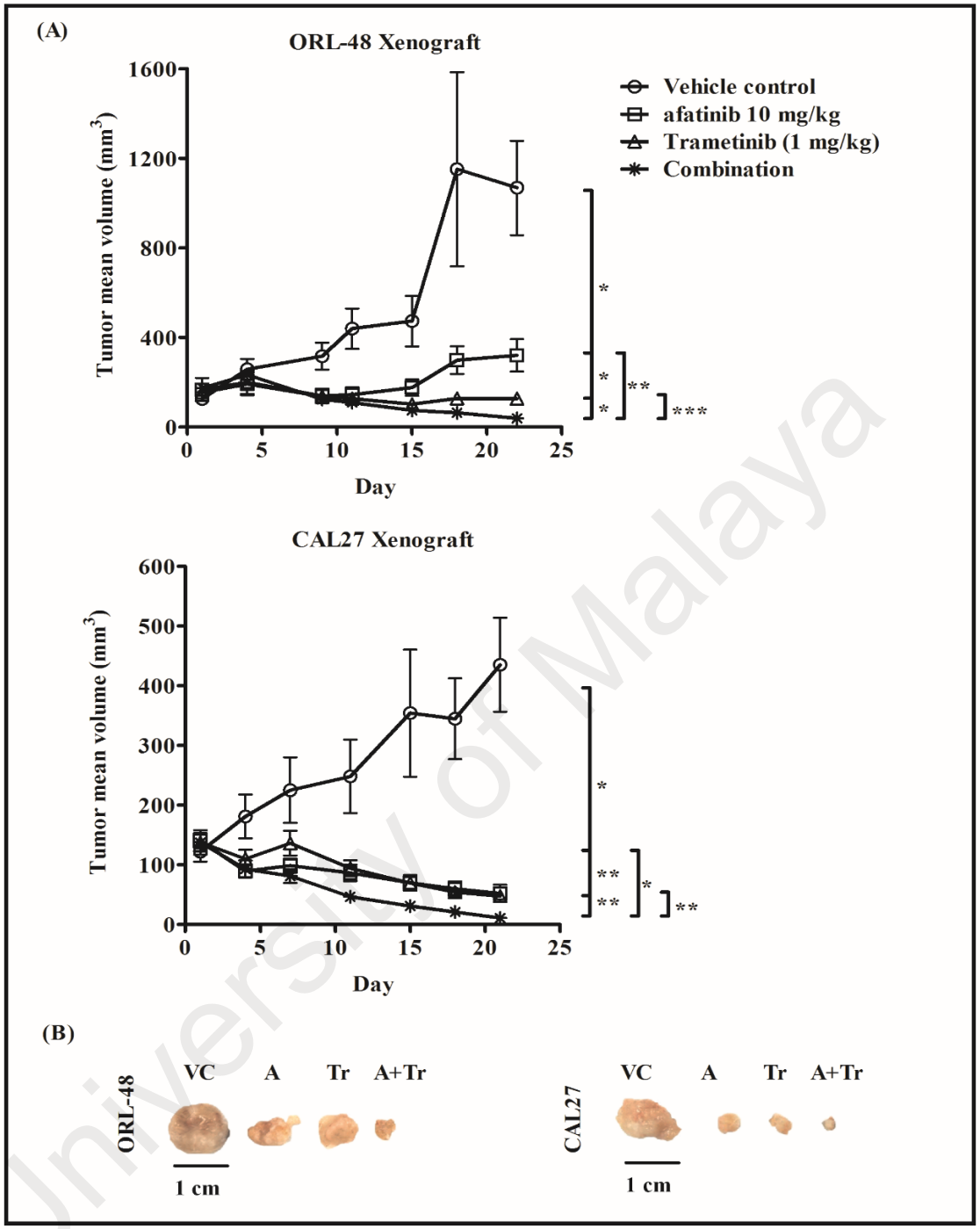

Figure 4.8: Combination treatment of afatinib and trametinib improved the anti-tumor effect. Subcutaneous models of ORL-48 and CAL27 were treated with either vehicle control (1 to 2% of DMSO), afatinib (10 mg/kg), trametinib (1 mg/kg), or their combination (afatinib and trametinib) daily. (A) Data points indicate mean tumor volume (mm^3) of OSCC xenografts over 22 days of treatment. (B) Representative tumors of OSCC xenografts harvested at the endpoint treatment of control (VC), afatinib (A), trametinib (Tr), or combination (A + Tr). The efficacy study demonstrated that combination treatment of afatinib and trametinib had a more potent anti-tumor effect than either single agent alone. Significant *p* values were indicated as $p < 0.05$*, $p < 0.01$** or $p < 0.001$***.

Table 4.2: Tumor growth inhibition of single agent and combination treatment at day 22 in OSCC mouse xenografts

OSCC Bearing Mice Model	Treatment group	Tumor growth inhibition (%)
ORL-48	Afatinib	76.4
	Trametinib	88.9
	Combination	96.9
CAL27	Afatinib	89.5
	Trametinib	90.3
	Combination	97.8

4.3.4 Toxicity evaluation of afatinib and trametinib treatment

To evaluate the toxicity of afatinib and trametinib as a single agent or in combination, the body weight of mice in each treatment arm was measured twice a week. Body weight of mice during the treatment period was compared to the starting body weight prior to drug treatments. As seen in Figure 4.9, mice body weight throughout the treatment period was minimal, whereby none of the measurements reduced below 20% of the initial body weight, which is the maximum threshold set by the Institutional Animal Care and Use Committee (IACUC) guidelines (National Research Council, 2011) as being acceptable. From our observation, all mice remained active and did not show sign of sickness due to drug treatments, however, mice co-treated with afatinib and trametinib had a watery discharge of feces.

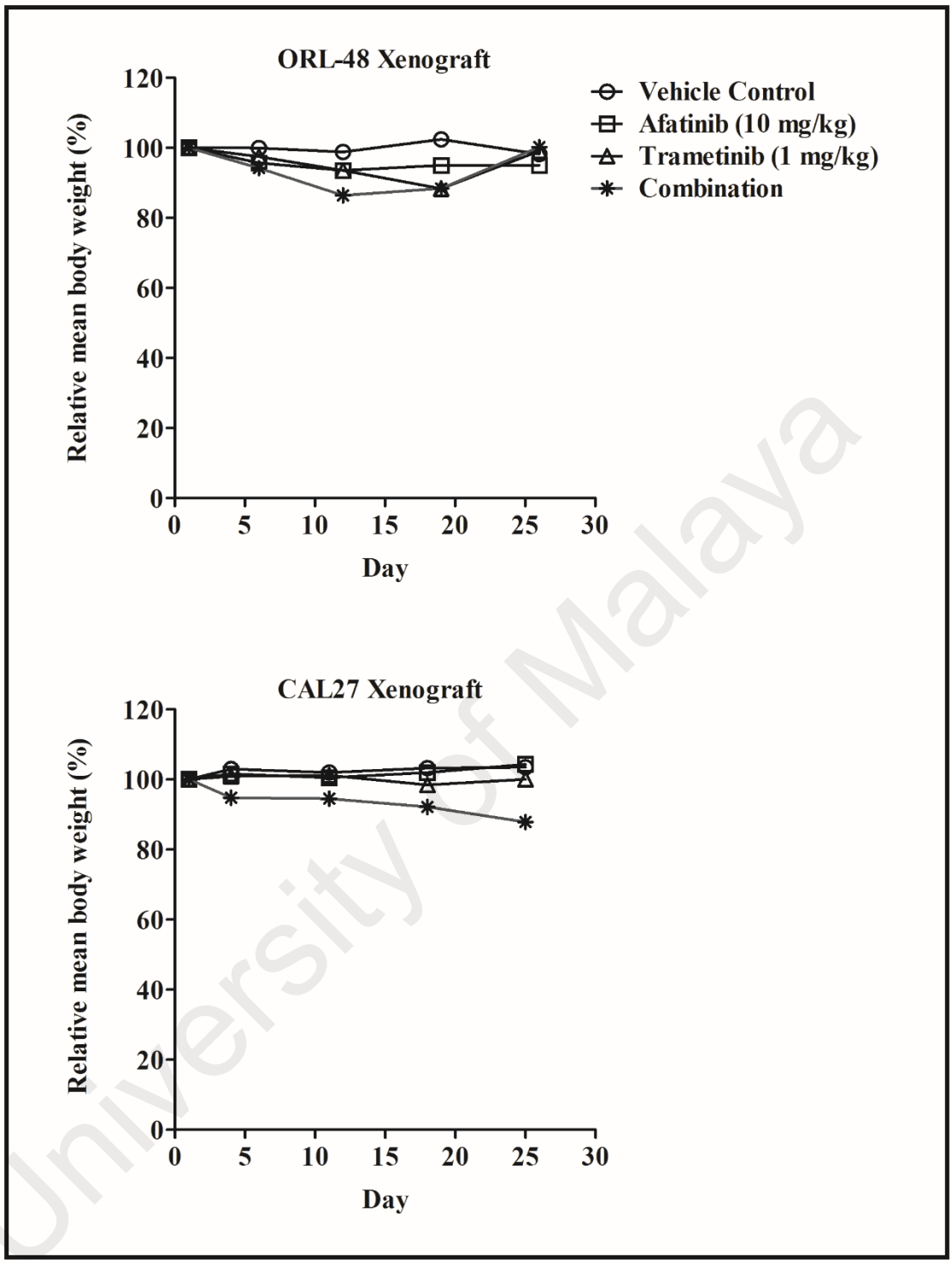

Figure 4.9: Body weight of mice during the treatment. Treatment of afatinib and trametinib did not distinctly change the body weight of the mice throughout the treatment period.

CHAPTER 5: DISCUSSION

5.1 Afatinib effects in OSCC *in vitro and in vivo*

5.1.1 Anti-proliferative effects of afatinib

ERBB dysregulation is a frequent event in OSCC, by which elevated protein expression and amplified copy number of EGFR, HER2, and HER3 have been associated with poor prognosis (Ang et al., 2002; Ganly et al., 2007; Takikita et al., 2011). Therefore, targeting the ERBB receptors with a pan-inhibitor such as afatinib could provide maximum blockade of the ERBB activated pathways for more pronounced anti-tumor effect compared to inhibitors that target only a single receptor within the family.

Afatinib binds irreversibly to the ERBB receptors and is known to affect cancer cell proliferation. Results here showed that afatinib induced cell cycle arrest in OSCC via the blockade of G_0/G_1 population, and this observation is consistent to those reported in cancers of colorectal and pharynx (Khelwatty, Essapen, Seddon, & Modjtahedi, 2011; Schutze et al., 2007). Although majority of the tested cell lines in this study had *EGFR* amplification, it is noteworthy that CAL27 which did not have amplified *EGFR* was the cell line that had the highest GI_{50} towards afatinib. This could suggest that afatinib might be a better therapeutic option for cancer with amplified *EGFR* (Young et al., 2015). In fact, OSCC patients who have a high level of *EGFR* gained more pronounced survival benefits from afatinib monotherapy in a phase III clinical trial (Machiels et al., 2015).

Notably, afatinib consistently suppressed the proliferation of OSCC cell lines with wild type and mutated *ERBB*. This finding was in line with previous finding in NSCLC preclinical studies (Li et al., 2008), suggesting that OSCC patients can be benefited from afatinib treatment regardless of the mutation status of *ERBB*. However, NSCLC patients with wild type *EGFR* demonstrated marked toxicity that resulting in dose limitation in clinic. While such observation is not known in OSCC, this subset of patient needs to be

monitored closely when afatinib is evaluated in clinic. Although a previous study demonstrated that afatinib-resistance cells had slower proliferation rate (Yamaoka et al., 2017), no link between the sensitivity to afatinib and population doubling time of OSCC cell lines was observed in this study.

While afatinib was demonstrated to have remarkable anti-tumor effects in preclinical models, clinical studies showed that the overall survival of patients appears to be limited, as demonstrated in recent clinical trials. For example, the LUX-Head & Neck-1 phase 3 clinical trial on afatinib showed that the overall survival (OS) of patients treated with afatinib monotherapy was not significantly improved compared to methotrexate-treated patients (Machiels et al., 2015). Similarly, other phase 3 clinical trials (LUX-Head & Neck 2 (NCT01345669) and 4 (NCT02131155)) trials were terminated due to the unlikeliness of treatment benefit from afatinib monotherapy when compared to the placebo group (https://clinicaltrials.gov/). Hence, an understanding of the molecular mechanisms underlying afatinib resistance affords an opportunity to improve its efficacy in cancer patients.

5.1.2 Mechanism underlying reduced effects of afatinib

From the *in vitro* study, whilst afatinib was able to inhibit ERBB signaling resulting in the inhibition of downstream molecules, ERK1/2 was re-phosphorylated at later time points suggesting that this could be a compensatory pathway that can overcome the inhibition effect of afatinib. Indeed, these findings are in agreement with recent studies in NSCLC which have demonstrated that ERK1/2 activation mediated resistance to afatinib (Coco et al., 2015; Ma et al., 2016). Development of resistance via the upregulated activation of ERK has also been demonstrated in other EGFR inhibitors, such as erlotinib and WZ4002 in lung carcinomas (Ercan et al., 2012; Ma et al., 2016). Besides,

EMT which was likely linked to activation of ERK, was also seen in afatinib-resistant lung cancer cells (Coco et al., 2015). Apart from ERBB receptors, ERK1/2 is also a common downstream effector of many other receptor pathways including insulin growth factor type 1 receptor, MET and fibroblast growth factor receptor (Gotoh, 2008; Knowles et al., 2009; Pollak, 2008). Perhaps, these parallel signaling could also activate ERK1/2 in OSCC cells.

Concurrent with the inhibition of ERBB and AKT phosphorylations, a time-dependent increase in the levels of HER3 was observed in this study. This could be due to a negative feedback loop where the prolonged blockade of ERBB receptors and inhibition of AKT may transcriptionally upregulate HER3 as demonstrated by others previously (Gala & Chandarlapaty, 2014). Previous study showed that the feedback upregulated HER3 can be induced via FOXO-dependent mechanism (Chakrabarty, Sanchez, Kuba, Rinehart, & Arteaga, 2012; Chandarlapaty et al., 2011; Garrett et al., 2011), Moreover, FOXO transcription factors are negatively regulated by AKT only at the phosphorylation Ser473 site but not at Thr308 (Vadlakonda, Dash, Pasupuleti, Anil Kumar, & Reddanna, 2013). Perhaps, this explain the observation in which the inhibition of AKT occurred only at Ser473 while HER3 is upregulated. Notably, several studies have demonstrated HER3 signaling as one of the major mechanism of resistance to EGFR inhibitors. For example, compensatory feedback expression of HER3 and activation contributed to the incomplete response to cetuximab in HNSCC and colon cancers (Bosch-Vilaro et al., 2017; Chakrabarty et al., 2012; Garrett et al., 2011). Similarly, transcriptionally upregulated HER3 has also been involved in resistance to MEK/RAF inhibitor in melanoma and thyroid cancer (Abel et al., 2013; Montero-Conde et al., 2013). Given that the survival benefit of afatinib treatment was more pronounced in OSCC patients with low HER3 expression, upregulation of HER3 might be associated with afatinib resistance. However, the role of HER3 in conferring resistance to afatinib in this study is not clear. Due to the

inherently impaired kinase activity of HER3 (Jura, Shan, Cao, Shaw, & Kuriyan, 2009), it must bind with other kinase-active dimeric partners such as EGFR, HER2 and c-MET to activate downstream signals (Tanizaki, Okamoto, Sakai, & Nakagawa, 2011). Interestingly, HER2 and HER3 are phosphorylated in the HER2/HER3 heterodimer, although the mechanism is not fully understood. Whereas, phosphorylation of EGFR and c-MET by HER3 has not been reported thus far. Nevertheless, as the phosphorylation of EGFR, HER2 and HER3 remained inhibited by afatinib, the reactivation of ERK1/2 mediated by the activation of these signaling receptors is unlikely. Further investigation is needed to gain a better understanding of resistant mechanism observed here.

5.2 Combination effects of afatinib and trametinib

Targeted therapies remain a considerable challenge in OSCC treatment when used as a single agent, due to the modest activity that ultimately resulting in relapse while on these treatments (Ferris et al., 2016; Wheeler et al., 2008). The limited therapeutic benefits can be improved by co-targeting key oncogenic pathways. Indeed, a successful example of the combination of targeted therapies in other cancers is the dual inhibition of BRAF and MEK that resulted in improved overall survival of patients with BRAF-mutant melanoma (Long et al., 2015; Robert et al., 2015). Recent studies demonstrated strong synergistic effect of anti-tumor activity by afatinib when combined with other cancer targeted drugs, such as rapamycin (mTOR inhibitor), selumetinib (MEK inhibitor), dasatinib (SRC inhibitor), and pyridone 6 (JAK/Stat inhibitor) on preclinical cancer models from diverse tumor types for instance, lung, colorectal and melanoma (Kim et al., 2012; Perera et al., 2009; Sun et al., 2014; Yoshida et al., 2014). However, thus far, such studies have not been done on OSCC.

Here, reactivation of ERK1/2 signaling was demonstrated as a possible mechanism of resistant to afatinib treatment and provide the data showed that trametinib potentiates the afatinib treatment *in vitro* (Figure 5.1). Additionally, the combination of afatinib and trametinib resulted in improved regression of OSCC tumor xenografts, likely due to the amplified inhibition of the ERBB and MEK pathway as hypothesized earlier in section 4.3.1. This also implies that inhibition of the compensatory reactivated ERK1/2 could achieve better tumor control. In fact, synergistic anti-tumor effects were also seen previously in NSCLC KRAS mutant xenograft model, in which the tumor control was more potent in this drug combination than single-agent treatment (Sun et al., 2014). Besides, other studies had demonstrated that trametinib alone was not sufficient to control tumor progression of lung and pancreatic cancers but when in combination with other drugs, synergistic drug effects were observed (Manchado et al., 2016; Witkiewicz et al., 2016).

Despite having a remarkable anti-tumor response from the dual inhibition, it is also important to consider the side effects of this combination treatment. Based on the observations in the drug efficacy study, mice of all treatment group remained active with no marked weight loss throughout the treatment period. However, mice treated with combination drugs encountered diarrhea problems. Similarly, diarrhea has been reported to be a common adverse effect seen in patients treated with afatinib or trametinib, hence combining both drugs might intensify this effect suggesting that this is one potential adverse event that needs to be monitored closely when testing this drug combination in the clinic (Infante et al., 2012; Yang et al., 2013).

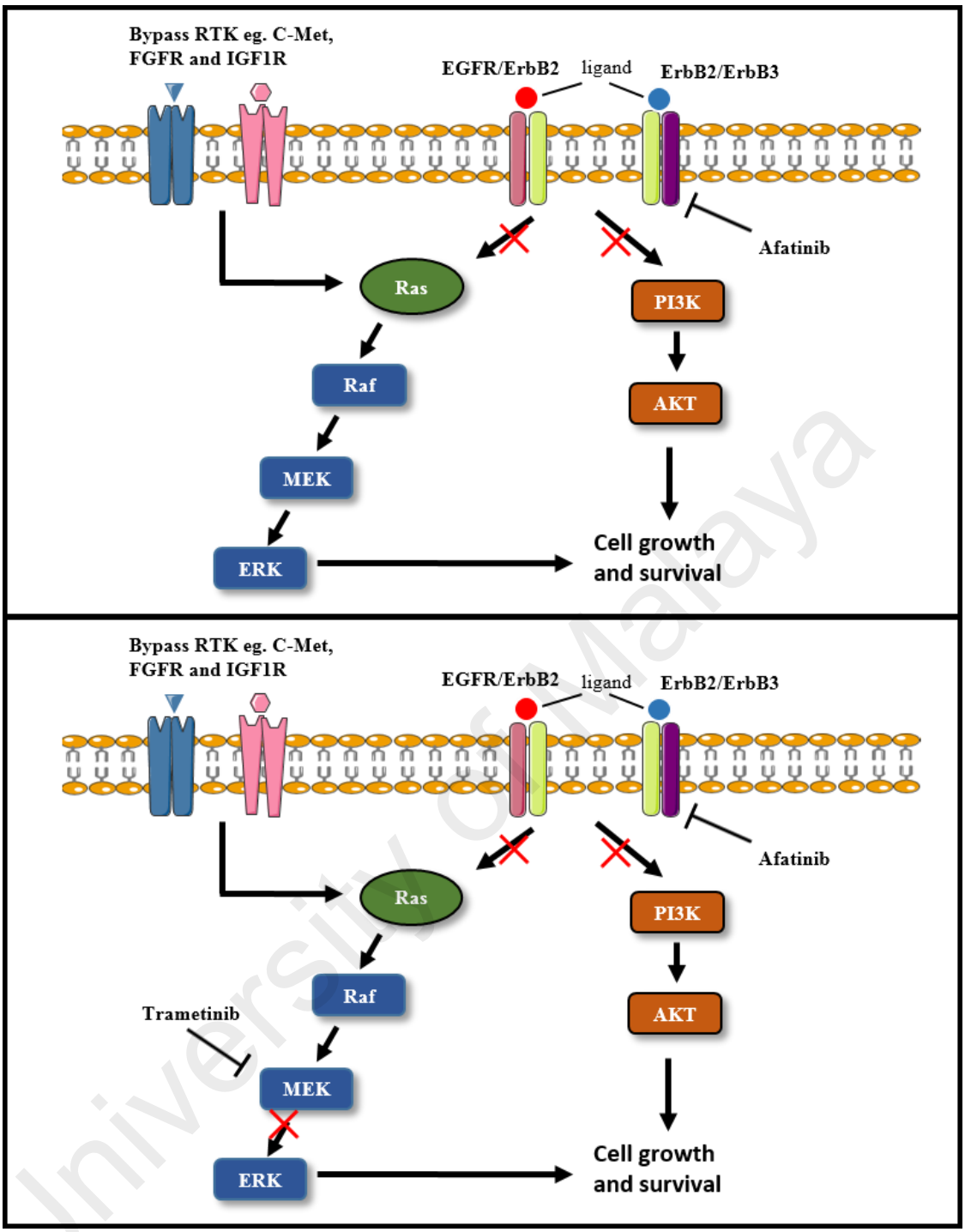

Figure 5.1: Reactivation of Erk1/2 via the induction of bypass RTK. Inhibition of kinase activity of ERBB receptors by afatinib prevents signal transduction to RAS/ERK and PI3K/AKT pathways. Resistance occurs when other bypass RTK reactivates the RAS/ERK pathway, while ERBB kinase activity is inhibited (above). Addition of MEK inhibitor, trametinib blocks the ERK1/2 pathway, subsequently suppresses the tumor cell growth and survival (bottom).

CHAPTER 6: SUMMARY

6.1 Summary

This study elucidated the inhibitory activity and the possible resistance mechanism of afatinib in preclinical OSCC models. This is probably the largest study that describes the afatinib sensitivity using the unique cell line models derived from Malaysia OSCC patients. In this investigation, inhibition of ERBB receptors activities by afatinib significantly prevented the cell cycle progression and thereby halted cancer cell growth of OSCC. Comparing to the control group, afatinib exhibited significant anti-tumor effects on OSCC xenograft models. Furthermore, this study provide evidence that resistance to afatinib could be mediated by reactivation of ERK1/2. This is the first time that the combination of afatinib and trametinib was shown to be able to markedly improve the anti-tumor effects in OSCC mouse models.

Despite new generations of ERBB pan-inhibitors such as dacomitinib and neratinib are emerging, the efficacy of these drugs on OSCC are still in early phases of evaluation. (Elicin & Ozsahin, 2016; Schneider et al., 2016). Hence, improving afatinib through combination treatment to overcome drug resistance remains a rational strategy, and could also inform on the possible resistant mechanisms of these new generation inhibitors. Currently, several MEK inhibitors are under development or in clinical trial, aiming to reduce the adverse effects while retaining the drug efficacy (Cheng & Tian, 2017). Notably, a combination of afatinib with another MEK inhibitor, selumetinib is currently being evaluated in clinical trial for lung cancer (NCT02450656), hence our findings provide the basis for considering the combination of afatinib and trametinib in OSCC for clinical investigation.

6.2 Study limitations

This study had a few limitations. Primarily, mechanistic study to elucidate the activities of targeted proteins upon single or combination treatment was only conducted *in vitro*. Such assessments were difficult to perform on drug treated-tumors from preclinical efficacy studies, mainly due to the significant shrinkage of tumors after 22-days treatment and thereby resulting in insufficient protein lysates to examine the phosphorylated levels of ERBB receptors, AKT and ERK1/2 via western analysis. Unfortunately, this is an inherent limitation of such *in vivo* study and therefore optimizing the time window to harvest tumors would be necessary to determine the status of targeted signaling pathways. Although afatinib also targets HER4, the signals of HER4 expression and its phosphorylation level were extremely weak in western blot, likely due to the inherently low abundancy of this protein in the cell lines. Hence, assessment of HER4 was challenging in this research work.

6.3 Recommendations for future study

This study has demonstrated afatinib-based combination treatment improved anti-tumor effects by co-targeting ERBB and MEK signaling pathways. With this data as a basis, future studies should be focused on developing strategies to maximize the clinical use of the combination treatment of afatinib and trametinib.

One of the possible strategies is developing a useful biomarker for afatinib-based therapies. Firstly, as the current study identified reactivation of ERK1/2 mediated resistance to afatinib, therefore further verification of using phosphorylated ERK1/2 as a biomarker is warranted. Secondly, the LUX-Head and Neck 1 phase 3 trials demonstrated that patients with low HER3 expression had more pronounced benefits from afatinib

monotherapy (Machiels et al., 2015). Subsequently, the current study demonstrated that afatinib up-regulated HER3 which possibly induced afatinib resistance via ERK1/2 reactivation. Taken together, these findings suggest that HER3 expression level could be a potential predictive biomarker. While low HER3 is predictive for the benefit to afatinib monotherapy, high HER3 would be useful to predict the likelihood to respond well to the combination of afatinib and trametinib. Therefore, validation of the predictive values of HER3 expression in afatinib-based therapies regardless of monotherapy or combination should be pursued.

Despite that targeted therapy commonly yields rapid anti-tumor response, however in most patients the drug efficacy is not durable. To address this challenge, immunotherapy such as immune checkpoint blockade that is capable to produce a more stable response has been combined with molecular therapy. This strategy is rationale because such combinations have demonstrated synergistic anti-tumor response in preclinical cancer models, mainly due to the immunomodulatory effects by the molecular targeted inhibitors. In previous findings, poor response to PD-1/PD-L1 inhibitor has been linked with down-regulated major histocompatibility complex class-1 (MHC-1) expression on NSCLC which could be possibly modulated by activated EGFR/MEK/ERK pathway. In concordance with that, erlotinib and trametinib which efficiently decreased the phosphorylated EGFR and ERK, markedly increased MHC-1 expression as well as infiltrating CD8+ T cells in HNSCC and NSCLC models (Watanabe et al., 2019). Besides, several EGRF-TKIs were capable to enhance T cell-mediated killing and improve anti-PD-1 therapy, in which afatinib treatment is more prominent as compared to erlotinib and gefitinib (Lizotte et al., 2018). Collectively, these studies have suggested that afatinib and trametinib are potent immunomodulatory agents.

Indeed, clinical assessments of afatinib combine with anti–PD-1 therapy (pembrolizumab) are currently on-going for NSCLC and HNSCC (NCT02364609, NCT03157089 and NCT03695510). Of note, such combination in HNSCC from a retrospective study was encouraging, in which the overall response rate (ORR) was significantly improved in patients treated in combination (58.5%) (Lizotte et al., 2018), as compared to previously reported ORR of pembrolizumab (16%) and afatinib (10%). Furthermore, this combination resulted in a significant reduction of average tumor size (82.9%, n=41) without associated increased toxicity. Besides, a number of clinical trials are also actively recruiting patients to assess the efficacy of combining trametinib and pembrolizumab for NSCLC and melanoma (http://www.clinicaltrial.gov/). However, thus far no investigation has been done on the combination of immune checkpoint inhibitor together with afatinib and trametinib. Hence, future investigations of the concurrent inhibition of ERBB/MEK/ERK and immune checkpoints for OSCC treatment are warranted.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

This research study has been published in *Targeted Oncology* in February 2019 (doi: 10.1007/s11523-019-00626-8) (Appendix H). This work was also presented in the Annual Scientific Congress of Malaysian Oncology Society (ASCOMOS) in 2016.