EVALUATION OF PALBOCICLIB IN ORAL SQUAMOUS CELL CARCINOMA AND THE ROLE OF PIK3CA IN CONFERRING RESISTANCE

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ABSTRACT

Lack of effective therapies remains a problem in the treatment of oral squamous cell carcinoma (OSCC), especially in patients with advanced tumors. OSCC development is driven by multiple aberrancies within the cell cycle pathway including amplification of cyclin D1 and loss of p16. Hence, cell cycle inhibitors of the CDK4/6-cyclin D axis are appealing targets for OSCC treatment. This study aimed to determine the potency of palbociclib and identify genetic features that are associated with palbociclib's response in OSCC. It was demonstrated that 80% of OSCC cell lines were sensitive to palbociclib at sub-micromolar concentrations. Palbociclib-treated cells were arrested at G_1 phase with concomitant reduction of phosphorylated-Rb. Consistently, it was found that palbociclib was effective in controlling tumor growth in mice. Importantly, this study identified palbociclib-resistant cells to harbour mutations in the *PIK3CA* gene. Using isogenic cell lines, *PIK3CA*-mutant cells were shown to be less responsive to palbociclib compared to wildtype cells. Despite the reduction of RB phosphorylation upon palbociclib treatment, *PIK3CA*-mutant cells upregulated CDK2 and cyclin E1 suggesting this to be the mechanism underlying resistance to palbociclib. Further, it was demonstrated that the combination of PF-04691502, a PI3K/mTOR inhibitor, with palbociclib completely controlled tumor growth in mice. This study provides a rationale for the inclusion of *PIK3CA* testing in clinical evaluation of CDK4/6 inhibitors and suggests combination approaches for further clinical studies.

ABSTRAK

Keberkesanan rawatan terhadap penyakit sel skuamus karsinoma mulut (OSCC) adalah terhad, terutamanya kepada tumor berperingkat lanjutan. Perkembangan OSCC didorong oleh perubahan molekular terhadap kitaran sel seperti penambahan kadar Cyclin D1 dan pengurangan p16. Oleh itu, perencat CDK4/6-cyclin D yang terdiri dalam kitaran sel adalah calon menarik sebagai rawatan OSCC. Kajian ini bertujuan untuk mengenal pasti keberkesan palbociclib dan ciri-ciri genetik OSCC yang berkaitan dengan respon terhadap palbociclib. Kajian ini menunjukkan bahawa 80% sel OSCC adalah sensitif terhadap palbociclib pada kepekatan sub-mikromolar. Sel yang dirawat palbociclib terbantut pada fasa G_1 dengan pengurangan fosforilasi Rb. Palbociclib juga didapati berkesan dalam mengawal pertumbuhan tumor pada model mencit. Selain itu, kajian ini mengenalpasti bahawa sel-sel yang kalis kepada palbociclib mempunyai mutasi dalam gen *PIK3CA*. Dengan menggunakan sel isogen, kajian ini membuktikan bahawa sel mutan *PIK3CA* kurang responsif kepada palbociclib berbanding dengan sel di mana *PIK3CA* adalah normal. Terdapat kenaikan pada kadar CDK2 dan cyclin E1 apabila palbociclib didedahkan pada sel-sel mutan *PIK3CA* walaupun fosforilasi RB berkurangan. Seterusnya, kajian ini membuktikan bahawa gabungan perencat PI3K / mTOR (PF-04691502) bersama palbociclib berjaya mengawal sepenuhnya pertumbuhan tumor pada mencit. Hasil kajian ini memberikan rasional untuk memasukkan ujian mutasi *PIK3CA* dalam penilaian klinikal perencat CDK4/6 serta mencadangkan pendekatan kombinasi bagi kajian klinikal selanjutnya.

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LIST OF SYMBOLS AND ABBREVIATIONS

µg	:	Microgram
µL	:	Microlitre
µM	:	Micromolar
ANOVA	:	Analysis of variance
ATP	:	Adenosine triphosphate
BSA	:	Bovine serum albumin
CCND1	:	Gene encoding Cyclin D1
CDK4/6	:	Cyclin-dependent kinase 4/6
CDKN2A	:	Cyclin-dependent kinase inhibitor 2A gene encoding p16
CKI	:	Cyclin-dependent kinase inhibitor
CNA	:	Copy number amplification
CT	:	Chemotherapy
DMEM	:	Dulbecco's Modified Eagle Medium
DMEM/F12	:	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
DMSO	:	Dimethyl sulfoxide
DNA	:	Deoxyribonucleic acid
E2F	:	E2 factor
EGFR	:	Epidermal growth factor receptor
ER	:	Estrogen receptor
ESR1	:	Estrogen receptor 1
FBS	:	Fetal bovine serum
FZR1	:	Fizzy And Cell Division Cycle 20 Related gened Cell Division Cycle 20 Related 1
GDNA	:	Genomic deoxyribonucleic acid

GDSC	:	Genomics of Drug Sensitivity in Cancer
GI_{50}	:	Half-maximal growth inhibitory concentration
GSK-3β	:	Glycogen synthase kinase 3 beta
h	:	Hour
HER2	:	Human epidermal growth factor receptor 2
HNSCC	:	Head and neck squamous cell carcinoma
HPV	:	Human papillomavirus
HRP	:	Horseradish peroxidase
IC_{50}	:	Half-maximal inhibitory concentration
ICD-10	:	10th revision of the International Statistical Classification of Diseases and Related Health Problems
IMRT	:	Intensity-modulated radiotherapy
JAK/STAT	:	Janus kinase/signal transducers and activators of transcription
MAPK	:	Mitogen-activated protein kinases
mg	:	Milligram
mL	:	Millilitre
mM	:	Millimolar
mm^3	:	Cubic millimetre
mTOR	:	Mammalian target of rapamycin
nm	:	Nanometre
NOD/SCID	:	Nonobese diabetic/severe combined immunodeficiency
NSCLC		Non-small-cell lung cancer
OSCC	:	Oral squamous cell carcinoma
p.o	:	Per oral
PCR	:	Polymerase chain reaction

PD-1	:	Programmed cell death protein 1
PDL-1	:	Programmed death-ligand 1
PFS	:	Progression-free survival
PI3K	:	Phosphoinositide 3- kinase
PIK3CA	:	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
pS6	:	Phospho-ribosomal protein S6
R/M	:	Recurrence/metastatic
Rb	:	Retinoblastoma
RCT	:	Radio-chemotherapy
RPM	:	Revolutions per minute
RT	:	Radiotherapy
S6	:	Ribosomal protein S6
S780	:	Serine 780
S795	:	Serine 795
S807/811	:	Serine 807/811
SD	:	Standard deviation
SDS-PAGE	:	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	:	Standard error of mean
TBS	:	Tris-buffered saline
TCGA	:	The Cancer Genome Atlas
TNM	:	Tumor-node-metastases
TP53	:	Tumor protein p53
TSN	:	Tobacco-specific nitrosamine
v/v	:	Volume per volume
VC	:	Vehicle control

w/v	Weight per volume

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CHAPTER 1 : INTRODUCTION

1.1 Background

Cancers that begin in the squamous cells lining the mucosal surfaces of the head and neck region such as the oral cavity, pharynx, larynx, salivary glands and nasal cavity, are collectively termed as head and neck squamous cell carcinoma (HNSCC). In 2018, oral squamous cell carcinoma (OSCC), the most predominant site of HNSCC, accounted for an estimated 354,864 new cases worldwide and was the 3rd most common cancer among men in the developing countries (Bray et al., 2018). The standard treatments for OSCC used since the past decades include surgery, radiation therapy, and chemotherapy. Despite advances in these treatment modalities to improve survival and locoregional control, the 5-year survival rate remains below 60% (Warnakulasuriya, 2009). Moreover, about 50% of OSCC patients present late at stage 3 and 4 (Rogers, Vedpathak, & Lowe, 2011), resulting in a significant drop in their survival rates (Edwards & Jones, 1999). The use of targeted therapy in OSCC is limited to cetuximab, a humanized antibody inhibiting the epidermal growth factor receptor (EGFR). However, the clinical benefit of cetuximab with chemotherapy is moderate, with only 2.7 months improvement in overall survival as compared to chemotherapy alone (Vermorken et al., 2008). Moreover, cetuximab treatment is challenged by primary and acquired resistance in OSCC patients (Rampias et al., 2014). More recently, immune checkpoint inhibitors, pembrolizumab and nivolumab, were approved for the treatment of OSCC. However, the efficacy of immunotherapy is only seen in less than 20% of patients (Chow et al., 2016). It is apparent that the treatment options for OSCC patients are currently limited, especially for patients with advanced disease and who are refractory to chemotherapy. This underscores the unmet need to increase effective treatment options for OSCC patients.

Increased cell cycle activity has been molecularly characterized in a large proportion of OSCC where mutations in p53, amplification of cyclin D1 and loss of p16 are commonly observed (Helsten et al., 2016; Leemans, Snijders, & Brakenhoff, 2018; B. Wang, Wang, Cao, & Li, 2015). Hence, the use of an inhibitor that could induce cell cycle arrest is a rational treatment agent for OSCC. Specific inhibitors of CDK4 and CDK6 kinases (CDK4/6 inhibitors) that arrest the G_1 phase of the cell cycle have been developed recently. Palbociclib is the first of its class to be approved for the use in estrogen receptor (ER)-positive and human epidermal growth factor receptor 2 (HER2)-negative metastatic breast cancer in combination with letrozole or fulvestrant (Cristofanilli et al., 2016; Finn et al., 2015). Although there are mounting evidence that palbociclib is beneficial in inhibiting this breast cancer subset, the evaluation of this drug in OSCC remains limited. It is known from a phase I trial that the combination of palbociclib with cetuximab is safe for the use in OSCC patients (Michel et al., 2016). Apart from this, other clinical trials are on-going (NCT02499120, NCT03194373, NCT03498378, NCT03024489, NCT03389477) but thus far, no further reports have been published on the efficacy of palbociclib in OSCC patients. Preclinical data evaluating palbociclib is also currently limited, warranting further evidence of its efficacy in OSCC models. Understanding the molecular changes that could influence therapeutic response is vital for identifying subgroups that are most likely to benefit from a targeted therapy. An example would be BRAF mutation, which play an oncogenic role in malignant melanoma development. Metastatic melanoma patients with BRAF V600E mutation have shown improved survival when given BRAF kinase inhibitor vemurafenib (Chapman et al., 2011). Given the limited number of studies, there is currently insufficient data of well-established molecular biomarkers that could predict OSCC response to palbociclib.

1.2 Importance of research

Evaluating palbociclib on OSCC models will enhance the understanding of its ability to inhibit OSCC growth, where this knowledge is currently lacking. By conducting palbociclib screening towards a panel of OSCC cell line models, this will help to delineate the mechanism of sensitivity and resistance of OSCC to palbociclib. As we can leverage on the genomic information of well-characterized OSCC models, this study will help to identify molecular biomarkers that could modulate the response of OSCC to palbociclib. Through the identification of well-established biomarkers of response, this information can help in the tailoring of palbociclib to the right niche of OSCC patients to achieve the maximal benefit of palbociclib towards OSCC patients. Taken together, the knowledge gained from this study will further inform on clinical studies particularly in identifying patients who may or may not respond to palbociclib.

1.3 Research aim and hypotheses

This study aimed to evaluate palbociclib, a CDK4/6 inhibitor, as an anti-cancer agent for OSCC using a panel of well-characterized cell lines and tumor xenograft models.

1.3.1 Specific objectives

There are four specific objectives in this study, as stated below:

- To investigate the sensitivity of OSCC cell lines towards palbociclib.
- To elucidate the effects of palbociclib on the molecular signaling pathways in OSCC cell lines.
- To investigate the *in vivo* antitumor effect of palbociclib treatment using xenograft models of OSCC.
- To evaluate potential biomarkers of response to palbociclib.

1.3.2 Research hypotheses

The hypotheses of this study are that a subset of OSCC cell lines is sensitive to the treatment of a CDK4/6 inhibitor, palbociclib. OSCC cell lines are inhibited by palbociclib through cytostasis that can be measured by reduction of cell proliferation and cell cycle arrests. In the *in vivo* model, palbociclib reduces tumor volumes as compared to its vehicle control. Based on the mutational status compared between palbociclib-sensitive and resistant cell lines, it is hypothesized that mutation in *PIK3CA* is associated with resistance to palbociclib in OSCC.

1.4 Thesis outline

This thesis is divided into five chapters. Chapter 2 (Literature review) gives details on OSCC in general, reviews the current treatment for OSCC patients, and discusses how the cell cycle is implicated in this disease. The development of CDK4/6 inhibitors and the rationale of using these for OSCC treatment is discussed. Finally, the lack of biomarkers to predict palbociclib response is reviewed. Chapter 3 (Methodology) outlines the experimental work that were carried out based on the objectives set for the study. Chapter 4 (Results) describes the findings of the study where the effects of palbociclib towards OSCC models are reported. This chapter also describes the identification of *PIK3CA* mutation as a possible mechanism of resistance towards palbociclib response. Finally, chapter 5 (Discussion) states the interpretation and explanation of the results, including acknowledgment of the study limitations, suggestions for future studies and drawing of the conclusions for this study.

CHAPTER 2 : LITERATURE REVIEW

2.1 Oral cancer

Oral cancers are cancers that begin in the oral cavity region (ICD-10 codes: C00-08) i.e lip (C00), base of tongue (C01), unspecified parts of tongue (C02), gum (C03), floor of mouth (C04), palate (C05), unspecified parts of mouth (C06), parotid gland (C07), and unspecified major salivary glands (C08). More than 90% of oral cancer cases are oral squamous cell carcinomas (OSCC) which are cancers that arise from squamous cells that line the mucosal surfaces of the oral cavity. The remaining 10% of oral cancer cases are adenocarcinomas (cancers arising in glandular tissue of the minor salivary and parotid glands), lymphomas (cancers arising in the lymph tissue surrounding the tonsil and base of tongue) and melanomas (cancers arising from melanocytes within the oral cavity).

2.1.1 Incidence, mortality and survival of OSCC

In 2018, OSCC accounts for an estimated 354,864 new cases worldwide and became the 3rd most common cancer among men in developing countries (Bray et al., 2018). OSCC has the highest incidence rate in countries of Southern Asia and the Pacific Islands such as India, Sri Lanka and Papua New Guinea and is the leading cause of cancer mortality among men in India and Sri Lanka. In Malaysia, OSCC occurred in 2362 individuals from 2007-2011 and is one of the most common cancers amongst Indians (Azizah, Nor Saleha, Noor Hashimah, Asmah, & Mastulu, 2016). The 5-year survival rate of OSCC patients in most countries was reported to be around 50% with lip cancers having the best survival and pharyngeal cancers having the poorest survival (Warnakulasuriya, 2009). OSCC patients presented with tumor-node-metastasis (TNM)-stage 1 tumors showed a 5-year survival rate of 80% however the survival rate decreases to 15% for patients with stage 4 tumors (Edwards & Jones, 1999). Unfortunately, about 65% of OSCC patients in Malaysia are presented late at stage 3 and 4 (Azizah, Nor Saleha, Noor Hashimah, Asmah, & Mastulu, 2016).

2.1.2 Risk factors

Several risk factors have been well-characterized to be associated with OSCC. Tobacco consumption has been known to be the leading risk factor of OSCC from ample evidence gathered throughout the past decades (Kumar, Nanavati, Modi, & Dobariya, 2016). Tobacco is known to be smoked or chewed and existed in various forms such as cigarettes, pipe, bidi, hookah, and betel quid (Ram et al., 2011). Regardless of its form of use, tobacco contains carcinogens that are known to cause OSCC such as the hydrocarbon benzpyrene and tobacco-specific nitrosamines (TSN). Metabolites of TSN have been shown in an animal study to covalently bind to DNA of keratinocytes forming DNA adducts that are responsible for the introduction of critical mutations (Varshney, 2003). Smoking is estimated to account for about 71% of deaths from oral cavity cancer (including pharynx) in high-income countries and 37% of deaths in low-income and middle-income countries (Danaei et al., 2005). A 10-year follow-up study of tobacco cessation in more than 12,000 individuals showed a significant fall in the incidence of leukoplakia and other lesions indicating reduction in OSCC risks (Gupta, Murti, Bhonsle, Mehta, & Pindborg, 1995).

Alcohol consumption is another important risk factor that has been implicated in the development of OSCC. Alcohol is estimated to account for about 33% and 14% of oral-cancer related deaths in high-income countries and middle/low-income countries (Danaei et al., 2005). Alcohol is shown to increase permeability and alters the morphology of oral mucosa. This leads to easier penetration of carcinogens into the mucosa (Kumar et al., 2016). Both alcohol and smoking are known to be synergistically associated with increased risk of OSCC (Blot et al., 1988). About 80% and 48% of oral-cancer related deaths in the high-income and middle/low-income countries respectively are attributed by the joint risk factors of smoking and alcohol (Danaei et al., 2005).

Human papillomavirus (HPV) infection has also been aetiologically linked to a subset of OSCC. Approximately 4% of OSCC cases are related to HPV infection (Machado et al., 2010), where 90-95% of this subset is due to HPV-16 type. HPV encodes two major oncoproteins, E6 and E7. The E6 and E7 proteins have been shown to bind and degrade p53 and Rb tumor suppressor proteins respectively. Loss of these critical proteins leads to cell cycle dysregulation with loss of control on DNA replication, DNA repair, and apoptosis (Kumar et al., 2016).

2.1.3 A review on the current management and treatment of OSCC

Clinical management of OSCC is dependent on the TNM staging and anatomical site of the tumor. Surgery remains the mainstay treatment for resectable OSCC regardless of disease stage. However, surgical resections of tumor in the oral cavity generally results in the impairment of functional and aesthetic aspects of the head and neck region. Several long-term postoperative complications have been strongly recognized in OSCC patients including difficulties in articulation, chewing and swallowing that can lead to nutritional deficits (Kerawala, 2010). Cranial nerves are also known to be at risk of post-surgery dysfunction leading to neurological complications such as loss of sensation and motor innervation of highly specialized organs such as the cheek and tongue (Kolokythas, 2010). Most importantly, OSCC resection is challenged by the failure of determining negative surgical margin which could result in disease recurrences (Looser, Shah, & Strong, 1978).

Radiotherapy (RT) is another standard treatment for OSCC, used as definitive treatment for early-stage tumors or as multimodal therapy with surgery and/or chemotherapy for locally advanced tumors (Tolentino et al., 2011). Although RT (alone or combination) has shown to increase the cure rate of oral malignancies, radiation of the oral cavity commonly produces adverse effects that manifest during or after completion of treatment (Otmani, 2007). The most prevalent oral complications induced by RT is

mucositis (ulceration), xerostomia (dry mouth), radiation caries, and osteoradionecrosis (bone death due to radiation) (Dörr, Hamilton, Boyd, Reed, & Denham, 2002; Mitchell & Logan, 1998; Möller, Perrier, Ozsahin, & Monnier, 2004; Paulino, Simon, Zhen, & Wen, 2000). RT is also commonly given in combination with chemotherapies (CT) such as cisplatin, carboplatin and 5-fluorouracil in the primary, adjuvant or salvage settings (Huang, 2013). In a study to compare the efficacy of postoperative irradiation between RT and radio-chemotherapy (RCT), the latter was found to significantly increase progression-free survival (36% versus 47%) and overall survival (40% versus 53%) in late-stage HNSCC patients (Bernier et al., 2004). However, the response was accompanied by significantly higher incidence of severe mucosal adverse effects in the group receiving RCT (41%) as compared to the group receiving just RT (21%).

Intensity-modulated radiotherapy (IMRT) is an advanced mode of RT that uses dose-delivery technology allowing higher precision of radiation while sparing more of the surrounding healthy tissues. IMRT has proven better toxicity outcome as compared to conventional RT especially in reducing the incidence of xerostomia, mucositis and dysphagia (Feng et al., 2010; T. Gupta et al., 2012; Nutting et al., 2011). Nevertheless, increased efficacy of IMRT as opposed to the conventional RT/RCT is still debatable. No significant differences were found in loco-regional control or overall survival of HNSCC patients treated with IMRT versus conventional RT in two randomized controlled trials (Feng et al., 2010; Ghosh–Laskar et al., 2016). Additionally, IMRT has cost implications for OSCC patients where patients in the United States undergoing IMRT had a mean cost of $101,099 compared to $42,843 for traditional RT (Razfar et al., 2016). Availability of IMRT is also limited especially in developing countries due to its high cost.

As the understanding of molecular mechanisms underlying cancer development are unraveled, this provides opportunities to develop biological therapies that target cancer cells specifically. The first targeted therapy to be approved for head and neck cancers is

cetuximab, a humanized monoclonal antibody that binds to the epidermal growth factor receptor (EGFR) protein. EGFR is frequently amplified and has an established role as a driver gene in HNSCC according to The Cancer Genome Atlas (TCGA), with mutation and copy number alteration (CNA) frequency of 4% and 11% respectively (Leemans et al., 2018). Inhibition of EGFR by cetuximab inactivates growth signaling pathways mediated by EGFR that links to several important downstream signaling pathways such as the MAPK, PI3K and JAK-STAT. Clinically, cetuximab is generally given (commonly as an adjuvant to CT) to OSCC patients with recurrent or metastatic (R/M) tumors. The clinical outcome of cetuximab however has been modest. Combining cetuximab with platinum-based CT and 5-fluorouracil (EXTREME regimen) was associated with a 2.7-month increase in the median survival from the CT-5-fluorouracil only group (Vermorken et al., 2008). Recently, the EXTREME-regimen did not add as much to the median overall survival of former standard treatments with platinum-based CT (Vermorken et al., 2008) and unexpectedly caused higher toxicity when used in R/M HNSCC patients outside of clinical trial (Lynggaard, Therkildsen, Kristensen, & Specht, 2015). Based on these reports, cetuximab has shown limited efficacy and its combination with standard therapies for OSCC poses risks to adverse side effects underscoring the need for exploring other targeted drugs for OSCC.

More recently, cancer treatment has shifted to the employment of our own immune system to attack cancer cells. Pembrolizumab and nivolumab are the first two immunotherapies to be approved for the treatment of R/M HNSCC. Both humanized monoclonal antibodies target the immune checkpoint protein called program death receptor 1 (PD1) that functions to inhibit the activation of T cells by binding to ligand PDL1 commonly expressed by cancer cells. PD1 inhibitors function by preventing PD1/PDL1 bindings and release the ‘immune brake’ for regulation of anti-tumor T cell response. Although clinical activity of PD1 inhibitors are promising, most of R/M

HNSCC patients remain unresponsive to this therapy with objective response rate of less than 20% (Bauml et al., 2017; Ferris et al., 2016; Seiwert et al., 2016). Additionally, emerging data has demonstrated that a significant subset of patients who initially responded to immune checkpoint inhibitors eventually relapsed after a period of response (Schachter et al., 2016; Zaretsky et al., 2016). Given these limitations, further work is still needed to better understand the mechanism of effective anti-tumor response and factors that can facilitate primary and acquired resistance to immune checkpoint therapies.

Taken together, the current treatment options for OSCC patients remain limited and are commonly linked to poor quality of life. The efficacy of available targeted therapy is restricted to only a subset of patients. Therefore, it is necessary to increase the treatment options for OSCC patients by testing and developing new effective therapies.

2.2 Excessive cell proliferation is a hallmark of cancer cells

For a cell to divide, it needs to undergo the cell cycle which consists of four primary phases (G_1, S, G_2 and M phase). In the G_1 phase, cells grow physically larger and undergo metabolic changes to prepare for cell division. At the late G_1 phase, cells are assessed by the first cell cycle checkpoint for errors in conditions necessary for cell division such as cell size, nutrient reserves, growth factors and DNA integrity (Barnum & O'Connell, 2014). Cells lacking these factors will leave the cell cycle and enter the quiescent phase (G_0). Those which passes the G_1 checkpoint will be committed to cell division by transiting to the S phase where DNA synthesis takes place to produce two sister chromatids from each chromosome. Cells will then enter the second gap phase (G_2) for further cell growth and assemble cytoplasmic organelles and proteins necessary for mitosis. Prior to the mitotic (M) phase, there is an additional checkpoint to assess if DNA is completely and correctly copied during the S phase. If errors are detected, cells will either attempt to repair the damaged DNA, resume DNA replication or undergo

programmed cell death. During the M phase, cells divide its copied DNA and cytoplasm into two new cells.

Apart from cell cycle checkpoints described earlier, the process of cell division is intricately controlled by highly specialized cell cycle regulators such as cyclin-dependent kinases (CDK) and cyclins. CDK4 and its functional homolog CDK6 are the two cyclin-dependent-kinases (CDKs) that are known to play critical roles during the G_1/S transition. CDK4/CDK6 activation requires binding to its regulatory subunits, D-type cyclins. The active CDK-cyclin D complex will then phosphorylate retinoblastoma (Rb) protein, a tumor suppressor that functions to inhibit G_1/S progression. Hyperphosphorylated Rb undergo changes in its conformation and releases transcription factor E2F, resulting in active transcription of genes necessary for DNA replication in S phase such as cyclin E and A. Progression of G_1/S phase is controlled by CDK inhibitor (CKI) called p16 which is encoded by the *CDKN2A* gene. Its inhibitory mechanism involves binding and inactivating CDK4/6-cyclin D complex, leading to inactivity of Rb and cell cycle arrests (Liggett Jr & Sidransky, 1998).

In OSCC, the key regulators of the cell cycle mentioned above are frequently altered. These genetic alterations include amplification of cyclin D1 and loss of p16 which are the direct key regulators of the G_1/S progression of the cell cycle. Cyclin D1 is encoded by the oncogenic gene *CCND1*. In OSCC, cyclin D1 is frequently altered through copy number amplifications located at chromosome 11q13. From TCGA data, about 28% of the analyzed HNSCC tumors were found to have *CCND1* amplification (Figure 2.1) (Leemans et al., 2018). The alteration frequency of *CCND1* in HNSCC is the second highest after breast cancer amongst the 38 different cancer types that were analyzed from clinical sequencing of 10,000 patients (Figure 2.2) (Zehir et al., 2017). Given the function of cyclin D in the cell cycle as described earlier, its overexpression is associated with enhanced G_1/S progression through CDK-dependent pathway (Baldin, Lukas, Marcote,

Pagano, & Draetta, 1993; Quelle et al., 1993). Moreover, HNSCC tumors demonstrated significant association between high cyclin D1 expression with advanced tumor stage and lymph node metastasis (Dhingra, Verma, Misra, Srivastav, & Hasan, 2017).

Loss of p16 is also frequently observed in OSCC or HNSCC patients. Encoded by the *CDKN2A* gene on the human chromosome 9p21, p16 functions to inhibit the cell cycle activity by binding to CDK4 or CDK6 and disrupting the association with cyclins D1, D2 or D3 (Witkiewicz, Knudsen, Dicker, & Knudsen, 2011). According to TCGA, *CDKN2A* is altered in 49% of the 279 sequenced HNSCC cases (Figure 2.1). The most common alteration is deep deletion (26.88%), followed by missense/ truncating mutation (21.51%) and multiple alterations (1.08%) (Cancer-Genome-Atlas-Network, 2015). These alterations contribute to p16 loss-of-function to inhibit G_1/S cell cycle progression. The functional status of p16 has been identified as an important prognostic factor in both HPV-negative and positive HNSCC. Patients with p16-negative tumors from different sites of the oral cavity demonstrated significantly poorer survival when compared to the p16-positive group (Stephen et al., 2013).

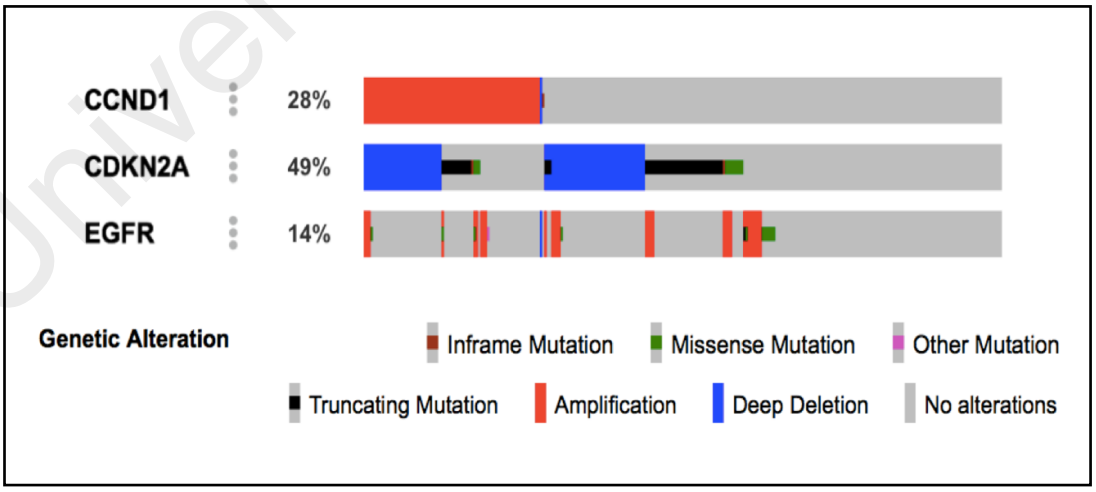

Figure 2.1 Alteration frequencies of *CCND1*, *CDKN2A* and *EGFR* in 279 sequenced HNSCC patient from TCGA cohort. Graph was generated from CBioPortal (https://www.cbioportal.org/).

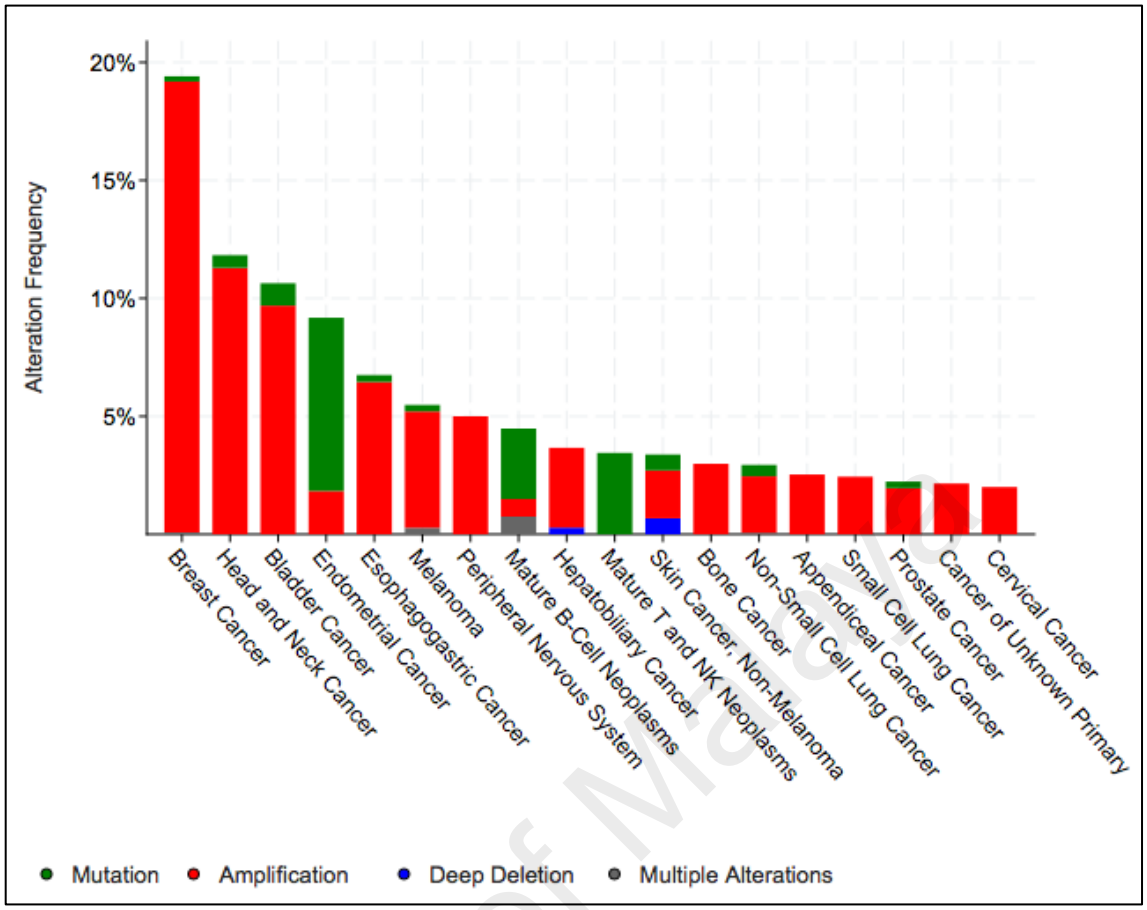

Figure 2.2: Alteration frequency of *CCND1* in different cancer types from the MSK-IMPACT clinical sequencing cohort of 10,000 patients (Zehir et al., 2017). Graph was generated from CBioPortal (https://www.cbioportal.org/).

Given the frequent alterations in the cell cycle pathway in OSCC and their association with poor clinical outcome in patients, these serve as biological rationales of using a CDK4/6 inhibitor for the treatment of OSCC.

2.3 Development of CDK inhibitors

Inhibitors targeting CDKs have been developed around 30 years ago when the fundamentals of CDK biology was discovered (Asghar, Witkiewicz, Turner, & Knudsen, 2015). The initial development was focused on non-specific CDK inhibitors targeting multiple CDKs at a time. Two prominent examples are flavopiridol (also known as alvocidib) targeting CDK1/2/4/6/7/9 and roscovitine (also known as seliciclib) targeting CDK1/2/5/7/9. These two inhibitors were extensively investigated in early phase clinical

trials however were found to be highly toxic to patients (Byrd et al., 2005; Le Tourneau et al., 2010). Moreover, flavoporidol demonstrated low levels of clinical activity as seen in a phase II studies in several tumor types (Bible et al., 2012; Carvajal et al., 2009; Lin et al., 2009), and roscovitine treatment had to be discontinued in a phase II study in advanced non-small cell lung cancer after it failed to improve progression-free survival (PFS) of patients (Asghar et al., 2015). Following the first generation of CDK inhibitors, improvement was carried out to increase the selectivity for CDK1 and 2 or increase the overall potency of the multiple CDK inhibitors. Dinaciclib, a highly potent inhibitor of CDK1/2/5/9 with less selectivity towards CDK4/6/7 is one of the most studied second generation CDK inhibitor. It demonstrated promising results in a phase I study (Nemunaitis et al., 2013) and managed to progress to phase II studies of solid cancers (Mita et al., 2014; Stephenson et al., 2014). Unfortunately, results were disappointing and the treatment of dinaciclib was terminated due to poor clinical activity.

Failure of the many non-specific CDK inhibitors can be attributed to several reasons. First, given that these inhibitors target multiple CDKs with poor selectivity, the mechanism of action underlying these inhibitors were not clearly understood (Kalra, Joshi, Munshi, & Kumar, 2017). This lack of clarity impeded the ability to develop them in the clinic and to design effective combination strategies. Second, due to the non-specific nature of these inhibitors, identification of biomarkers that can predict response to these agents were challenging (Asghar et al., 2015). This has led to the lack of appropriate patient selection and contributed to the failure of these agents in the clinic. Third, there was a lack of therapeutic window as many of these non-specific CDK inhibitors target several proteins that are known to be critical in the development of normal cells. Examples are CDK1 and CDK9 that are known to play critical roles in cell proliferation and survival (Asghar et al., 2015).

More recently, investigation has focused on CDK inhibitors that are highly selective towards CDK4/6 which are direct regulators of the G_1/S progression. Palbociclib is the first CDK4/6 specific inhibitor to demonstrate clinical efficacy for the treatment of ER-positive and HER2-negative metastatic breast cancers followed by abemaciclib and ribociclib. All three inhibitors share the same mechanism of action by specifically targeting CDK4 and 6 at low nanomolar concentrations with very minimal binding to other CDKs. Differences between the three inhibitors include chemical structure (Appendix A), pharmacokinetics and clinical toxicity (Klein, Kovatcheva, Davis, Tap, & Koff, 2018). This study focused on palbociclib, as it currently has the most clinical evidence of efficacy.

2.3.1 Mechanism of target

Palbociclib selectively binds to the specialized adenosine triphosphate (ATP)−binding pocket of CDK4 and CDK6 with specific residue reactions in the ATP-binding cleft (Asghar et al., 2015). This prevents CDK4/6 from binding to their regulatory subunits (cyclin D) making them inactivated kinases. Without CDK4/6 being activated, phosphorylation of downstream Rb is reduced and E2F transcription factor stays inactivated. This prevents the production of proteins necessary for S phase and causes cancer cells to be arrested at the G_1 phase (Figure 2.3).

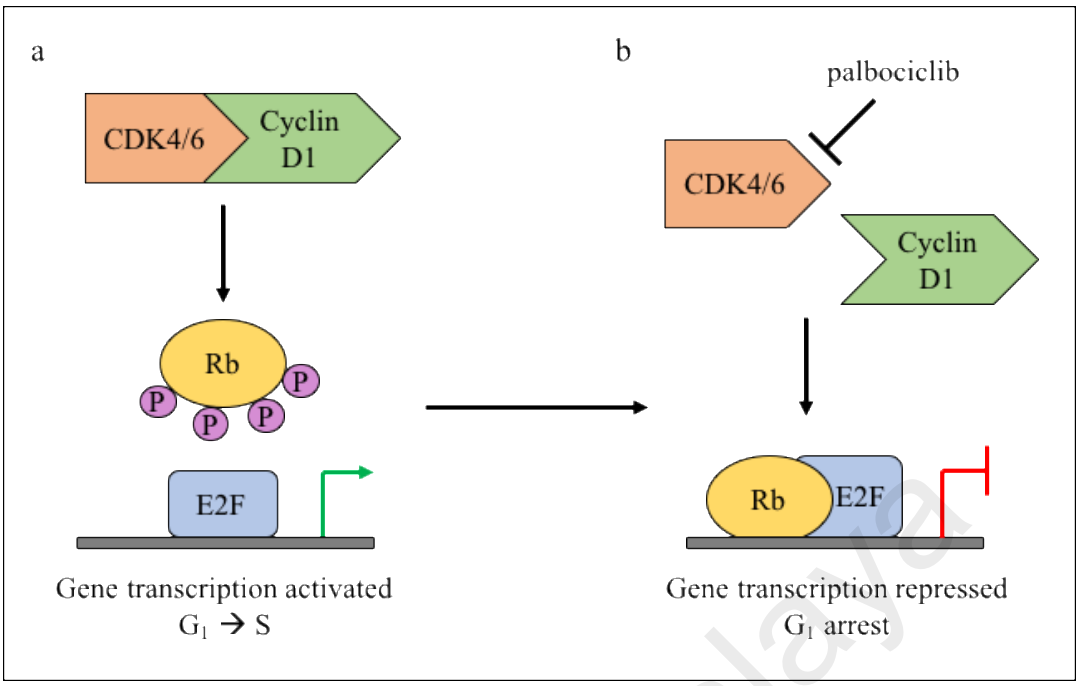

Figure 2.3 Schematic illustrating the mechanism of palbociclib in arresting the cell cycle. In *a*, CDK4/6 binds to cyclin D1 to form an active CDK4/6-cyclin D1 complex. The active complex then phosphorylates Rb and causes hyperphosphorylated Rb to release the E2F transcription factor. This allows E2F to synthesize S-phase initiating proteins. In *b*, palbociclib competitively binds to the ATP-binding pockets of CDK4/6. This prevents the formation of active CDK4/6-cyclin D1 complex and reduces phosphorylation of Rb protein. E2F transcription factor remains intact with Rb and cell cycle is arrested at G_1.

2.3.2 Clinical efficacy of palbociclib

In 2016, palbociclib gained accelerated approval by FDA based on findings from the phase 2 PALOMA-1/TRIO-18 study involving 165 post-menopausal ER-positive, HER2-negative, metastatic breast cancer patients (Finn et al., 2016). Palbociclib in combination with letrozole was evaluated against letrozole alone as a first-line treatment. Median PFS was 10.2 months (95% CI 5.7–12.6) for the letrozole group and 20.2 months (13.8–27.5) for the palbociclib plus letrozole group (hazard ratio 0.48, 95% CI 0.319–0.748; p=0.0004), which correlates to a doubled PFS in favour of the combination group. The most common adverse event was grade 3-4 neutropenia in the combination group but was not associated with any overlapping infections. Additionally, the safety profile of the combination group was similar to the overall safety profile of the study.

In addition to the benefit of palbociclib as first-line treatment in combination with letrozole, PALOMA-3 trial evaluated the role of palbociclib in patients who progressed during initial endocrine therapy (Cristofanilli et al., 2016). The study recruited 521 patients and they are randomized to either receive palbociclib plus fulvestrant or fulvestrant alone. Median PFS was 9.5 months (95% CI 9.2–11.0) in the fulvestrant plus palbociclib group and 4.6 months (3.5–5.6) in the fulvestrant plus placebo group (hazard ratio 0.46, 95% CI 0.36–0.59; p<0·0001). The study concluded that fulvestrant plus palbociclib was associated with significant and consistent improvement in PFS compared with fulvestrant alone in endocrine-resistant patients. The toxicity profile was similar to the PALOMA-1/TRIO-18 study, where most adverse events were grade 3-4 neutropenia.

Although approval of palbociclib is currently limited to ER-positive and HER2-negative breast cancer patients, it is being actively investigated in clinical studies of other types of cancers such as non-small cell lung cancer, mantle cell lymphoma, leukemia and HNSCC (Gopalan et al., 2014; Kadia et al., 2018; Michel et al., 2016; Niesvizky et al., 2015). In a phase I study for HNSCC, palbociclib was investigated for its safety in combination with cetuximab in patients with R/M HNSCC. The study determined that 125 mg/day of palbociclib taken daily for 21 days of each 28-day cycle with cetuximab was safe in patients. Palbociclib-cetuximab is now being studied in a randomized, multi-center, phase II trial to see whether the combination approach is superior to cetuximab in prolonging the overall survival of HPV-negative R/M HNSCC patients [NCT02499120]. Other approaches that are currently being evaluated in early phase clinical trials are combination of palbociclib with carboplatin [NCT03194373], triple combination of palbociclib, cetuximab and avelumab (anti PD-L1 inhibitor) for R/M HNSCC [NCT03498378] and triple combination of palbociclib, cetuximab and IMRT for locally advanced unresectable HNSCC [NCT03024489]. The ‘Los Tres Paso’ or ‘three steps’ phase II study is another approach to evaluate the results of treating HPV-unrelated

HNSCC patients with neoadjuvant single-agent palbociclib, followed by chemoradiation (either cisplatin + IMRT or cetuximab + IMRT), followed by adjuvant single-agent palbociclib [NCT03389477].

2.4 Lack of biomarker of response to palbociclib

Palbociclib was first found to preferentially inhibit the proliferation of luminal estrogen receptor-positive subtype of human breast cancer cell lines (Finn et al., 2009). This subset was associated with high cyclin D1 levels, low p16 expression and has intact Rb pathway making it susceptible for CDK4/6 inhibition. This observation has guided the patient selection for palbociclib evaluation in which only patients of the ER-positive and HER2-negative subset were enrolled in the PALOMA studies (Finn et al., 2016) (Cristofanilli et al., 2016). Indeed, these studies confirmed that palbociclib have significant activity in ER-positive breast cancers. However, the genetic alterations that are implicated in this subtype group such as p16 loss and cyclin D1 amplification did not show to correlate with higher activity of palbociclib in the PALOMA-1 study (Finn et al., 2016). To date, ER-positivity and HER2-negativity are currently the only markers used to select breast cancer patients for palbociclib treatment in the clinic. Evaluation on potential novel biomarkers has failed to predict patients' response to palbociclib (Garrido-Castro & Goel, 2017; Guarducci et al., 2017). Notably, there were patients from the ER-positive subtype that remained unresponsive to palbociclib therapy with objective response rate of 42.1% (Finn et al., 2016) and a growing number of patients have shown acquired resistance to palbociclib (Cristofanilli et al., 2016). For these reasons, identification of novel biomarkers is crucial to accurately identify subgroup of patients that are more likely to respond to the drug.

In OSCC, there are no biomarker-driven eligibility specifications for the clinical trials that are currently underway. Patient selection criteria was based on HPV status of patients due to the ability of the E7 viral oncogene of HPV to bind and degrade retinoblastoma

pocket proteins including Rb, p130 and p107 leading to the continuous induction of S-phase (Kumar et al., 2016). Given the limited number of preclinical studies of palbociclib on OSCC, there remains to be lack of well-characterized biomarkers that could support the clinical development of palbociclib for OSCC patients.

2.5 Gap of knowledge and the importance of study: a summary

OSCC is the third most common cancer among men in the developing countries. The current treatment for OSCC is associated with poor quality of life and offer limited survival benefit. Due to the perturbation of the cell cycle activity that are commonly found in OSCC, inhibitors targeting the CDK4/6 could be a rational approach. Palbociclib has demonstrated encouraging results in phase II clinical trials for ER-positive and HER2-negative breast cancer patients in combination with hormonal therapies. In HNSCC, palbociclib was reported as safe to be used with cetuximab while different combinatorial approaches are also under evaluation. While palbociclib seems to be promising in the treatment of OSCC, biomarkers that can delineate sensitivity or resistance of OSCC to palbociclib is currently not characterized and established at the preclinical or clinical stages. Using a panel of well-characterized OSCC cell line models, this study enables the characterization of OSCC response to palbociclib and to further characterize genetic alterations that could confer sensitivity or resistance to palbociclib. Findings from this study will help to support the clinical development of palbociclib for OSCC, guide patient selection, and suggest strategies for combinatorial approaches.

CHAPTER 3 METHODOLOGY

3.1 Overview of study design

Figure 3.1 depicts an overview of the study design based on four specific objectives.

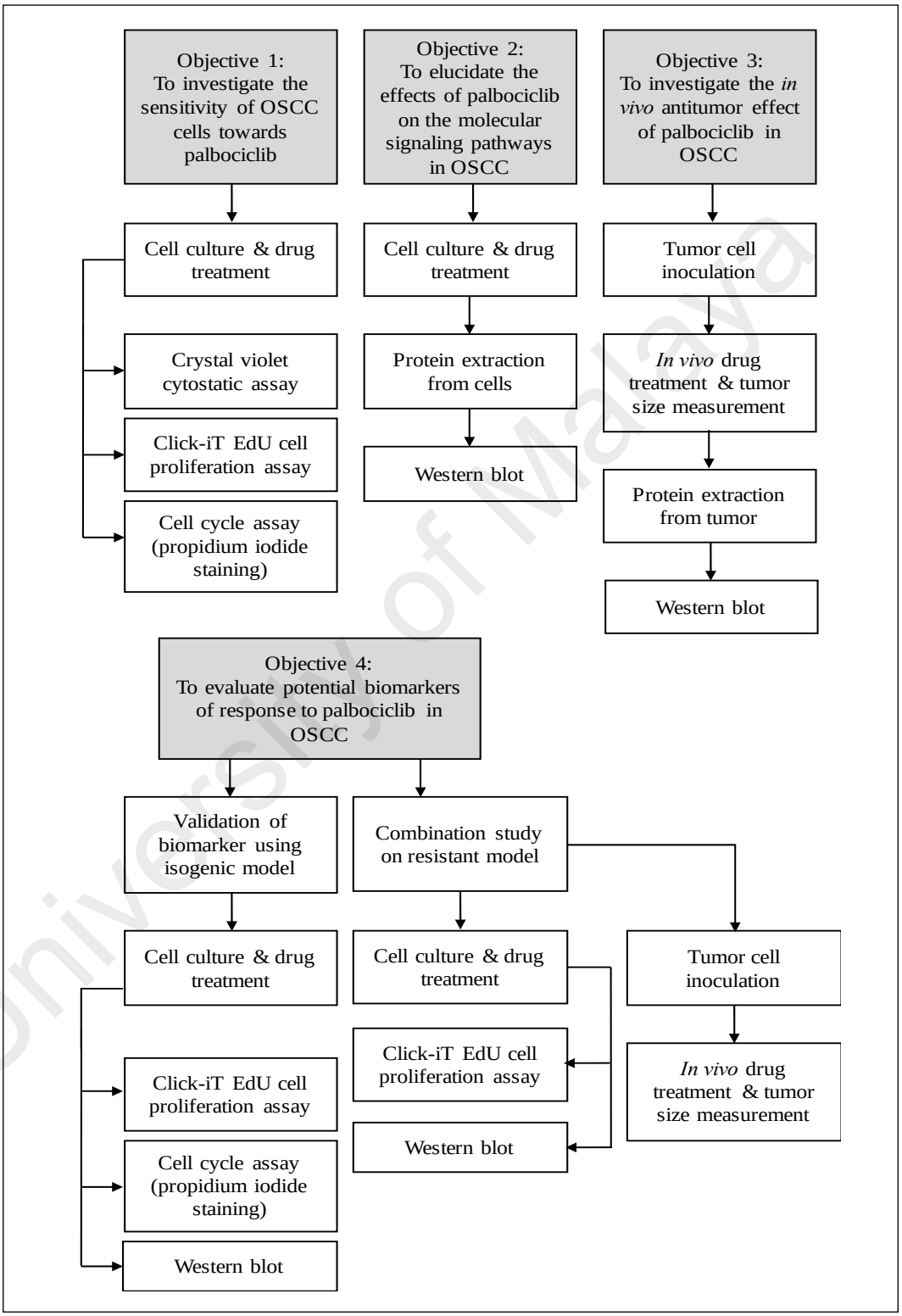

Figure 3.1: Overview of study design and methodologies

3.2 *In vitro* validation of palbociclib's efficacy in OSCC

3.2.1 Chemical reagents

Palbociclib was purchased from LC Laboratories, MA, USA. Ribociclib, abemaciclib and PF-04691502 were purchased from Selleckchem, TX, USA. For *in vitro* use, all reagents were dissolved into 10 mM stocks (palbociclib and PF-04691502 in DMSO; ribociclib and abemaciclib in water) and kept as small aliquots in -20°C until further use.

3.2.2 Cell culture and maintenance

The ORL series used in this study were established from Malaysian oral cancer patients as previously reported (Fadlullah et al., 2016; Hamid et al., 2007). The demographic information of the patients from whom the ORL lines were derived is provided in Appendix B. All ORL cell lines were cultured in Dulbecco's Modified Eagle's Medium/Nutrient mixture F12-Ham's medium (DMEM/F12; Hyclone, Utah, USA) supplemented with 10% (v/v) of heat-inactivated fetal bovine serum (FBS; Gibco, Auckland, NZ) and 500 ng/ml of hydrocortisone (Sigma-Aldrich, MO, USA). Generation of CAL27-mutant was described previously (Z. Wang et al., 2014). CAL27, CAL27-mutant and MCF-7 cells were grown in DMEM (Gibco, Auckland, NZ) supplemented with 10% (v/v) of heat-inactivated FBS. All cell lines have been authenticated as described previously (Appendix C) (Fadlullah et al., 2016). Cells were cultured at 37°C in a 5% CO_2 humidified atmosphere.

3.2.3 Crystal violet cytostatic assay

The cytostatic effect of palbociclib on a panel of OSCC lines was determined by crystal violet cytostatic assay. Briefly, 8 x 10^3 cells were seeded in triplicates in 12-well plates and following overnight incubation, cells were exposed to palbociclib at concentrations ranging from 0.008-2 µM or vehicle control (VC) of 0.5% (v/v) DMSO. After 72 h of incubation, crystal violet solution consisting of 2% (w/v) crystal violet (Sigma Aldrich,

MO, USA), 10% (v/v) formaldehyde (Merck Millipore, MA, USA) and distilled water was added into each well and the plates were further incubated for an additional 10 minutes with agitation. After, crystal violet solution was removed from each well and cells were washed 2 times with water and dried overnight. The following day, the resulting crystals were dissolved in 500 µl of DMSO and absorbance was measured at 590 nm using a Synergy H1 Multi-Mode reader (BioTek Instruments, VT, USA). The concentration of drug to cause 50% reduction in cell growth were calculated as GI_{50} = $((Abs_p - Abs_0)/(Abs_{DMSO} - Abs_0))*100\%$, where Abs_0 is the absorbance value of untreated cells at day 0, and Abs_p and Abs_{DMSO} are the absorbance values of palbociclib-treated and VC-treated cells after 72 h, respectively.

3.2.4 Click-iT cell proliferation assay

Cell proliferation was determined by the Click-iT EdU assay (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Briefly, 7 x 10^4 cells per well were grown overnight on glass coverslips and treated with 0.06-0.5 µM palbociclib or 0.5% (v/v) DMSO for 24 h. Cells were incubated with 10 µM 5-ethynyl-2′-deoxyuridine (EdU) for 2-6 h prior to fixation with 3.7% (v/v) formaldehyde. The cells were permeabilized with 0.1% (v/v) Triton X-100 in phosphate buffer, followed by EdU detection *via* a copper-catalyzed reaction and nuclei staining by Hoechst 33342. The coverslips were then mounted on glass slides using VECTASHIELD® Mounting Medium (Vector Laboratories, Burlingame, CA, USA). Slides were examined on an upright Olympus IX71 microscope (Olympus, Japan) with double bandpass filters to detect fluorescent-stained nuclei (Hoechst 33342: excitation 360 -370 nm and emission 420 nm) and Alexa labelled EdU (Alexa 647: excitation 650 nm and emission 667 nm). Images were captured from 10 randomly chosen fields of each experiment and analysed with the QuickCount® software (Tiong et al., 2018). The number of EdU-positive cells and Hoechst 33342-stained cells were counted and the percentage of EdU-positive cells was calculated (from

three independent experiments) using the following formula: number of EdU positive cells/number of Hoechst 33342 stained cells × 100. EdU-positive cells broadly represent cells that are undergoing DNA synthesis, whereas Hoechst 33342 stained cells represent all cells in the same field.

3.2.5 Cell cycle assay (propidium iodide staining)

Briefly, 7 x 10^4 cells were seeded per well in 12-well plates and treated with 0.06-0.5 µM palbociclib or 0.5% (v/v) DMSO on the following day for 24 h. All floating and attached cells were harvested and fixed in 70% (v/v) ethanol for 16 h at -20°C. Prior to analysis, fixed cells were pelleted and washed in cold PBS followed by staining with 10 µg/ml propidium iodide solution containing 20 µg/ml RNase for 30 minutes at room temperature in the dark. Stained cells were analysed by BD FACSCanto II™ flow cytometer (BD Biosciences, MA, USA) with 10,000 events of gated single cell populations collected for each reading. The distribution of DNA in different phases was determined using the ModFit software (Verity Software House, USA). The percentage of cells in each phase was obtained from three independent experiments.

3.3 Evaluation of molecular signaling pathways

3.3.1 Western blotting

For dose-dependent western blot analysis, cells were treated with palbociclib at concentrations of 0.06, 0.25 and 0.5 µM or 0.5% (v/v) DMSO for 24 h. For time-dependent analysis, cells were treated with 0.5 µM palbociclib for 0.2, 0.5, 1, 6, 12, and 24 h or treated with 0.5% (v/v) DMSO for 24 h. Treated cells were lysed on ice in lysis buffer (5 M NaCl, 10% (v/v) NP-40, 1 M Tris pH 8.0, 0.5 mM DTT) supplemented with HALT Protease and Phosphatase Inhibitor Cocktail (Pierce Biotechnology, IL, USA). Cell lysates were then centrifuged at 14,000 rpm for 10 minutes at 4°C prior to estimation of protein content using the BCA method (Thermo Scientific, MA, USA). For western

blot analysis, 30 µg of total cellular proteins were resolved on a 12% (w/v) SDS-PAGE gel and electrotransferred onto Immobilon-P membrane (PVDF; Millipore, MA, USA) at 100 V for 1.5 h on ice. Membranes were blocked with 5% (v/v) skimmed milk in Tris-buffered saline with 0.1% (v/v) Tween 20 (TBST; Sigma Aldrich, MO, USA) for 1 h and then probed overnight at 4°C with the indicated primary antibodies at 1:1000 dilution in 1% (v/v) BSA in TBST (pRb (S780), pRb (S795), total Rb1, Cyclin D1, Cyclin E1, CDK2, CDK4, CDK6, pAKT (S473), total AKT1, pS6, total S6 (Cell Signaling Technology, MA, USA) and α-tubulin (Sigma-Aldrich, MO, USA). Membranes were next washed 3 times in TBST for 5 minutes each. Membranes were then incubated with the corresponding secondary HRP-conjugated antibodies (Southern Biotech, AL, USA) at 1:10,000 dilution in 5% (v/v) skimmed milk in TBST for 1 h at room temperature. This was followed by 3 washes in TBST prior to detection by WesternBright Quantum HRP substrate (Advansta Inc, CA, USA) and visualization using the FluorChem^{TM} HD2 imaging systems (ProteinSimple, CA, USA).

3.4 *In vivo* mouse efficacy study

3.4.1 Subcutaneous xenograft in NOD/SCID mice

Six weeks-old female NOD/SCID mice were implanted with 2 x 10^6 cells, subcutaneously in both flanks. When the tumors reached the size of approximately 100-200 mm^3, the mice were randomized into experimental groups; vehicle control, palbociclib (150 mg/kg body weight, suspended in DMSO 5% and Cremophor-EL 10%, 500 µl p.o) or PF-04691502 (10 mg/kg body weight, suspended in 4% DMSO and 0.5% methylcellulose, 200 µl p.o). All procedures involved in the animal study have been reviewed and approved by the Animal Ethics Committee of Universiti Kebangsaan Malaysia (Ethics approval number: CARIF/2016/CHEONG/18-MAY/762-JUNE-2016-JUNE-2019). Detailed procedure for mouse studies is available in Appendix D.

3.4.2 Drug treatment and tumor measurement

For *in vivo* experiments, palbociclib and PF-04691502 were dissolved into 133 mg/ml and 25 mg/ml stocks respectively in DMSO and stored in -20 °C until further use. Treatment was administered daily for 21 days and tumor measurements were taken using a digital calliper every 3-4 days throughout the treatment duration. Tumor volumes (V) were calculated as V = ½ length x $(\text{width})^2$. Growth curves were plotted as an average tumor volume of each experimental group against the set time points. At the end of therapy, mice were sacrificed and tumors were harvested for tissue processing and histopathological analysis.

3.4.3 Protein extraction from tumor

At endpoint, mice were terminated and tumors were quickly resected and snap frozen in liquid nitrogen. Frozen tumors were dissected into smaller pieces on dry ice and weighed. The piece of tumor was then cut into smaller fragments. While frozen, the tumor fragments were homogenized in 2.5 µl/mg complete lysis buffer (as described in 3.3.1) using a glass 1 ml Dounce tissue grinder on ice. Lysates were then transferred into a 1.5 ml Eppendorf tube and sonicated three times (5 seconds each) at low setting on ice. Lysates were next centrifuged at 13,000 rpm for 10 minutes at 4°C to remove insoluble materials.

3.5 Investigation of potential biomarker of response

3.5.1 Identification of *PIK3CA* mutation as a resistant biomarker

To identify candidate molecular biomarkers, gene mutational analysis from RNA sequencing data were compared between palbociclib-sensitive and palbociclib-resistant OSCC cell lines. The sensitivity of OSCC cell lines was determined from palbociclib screening using the crystal violet cytostatic assay as described in 3.2.3. Mutations that were present in the resistant group were first identified. Several filters were then applied

to generate a shortlisted mutation list that is implicated in the resistant group. Any mutations that were present in the normal oral keratinocytes (N-231, N232 and N-235) (Fadlullah et al., 2016) and those found in the sensitive group were removed. Synonymous mutations (gene mutations that do not change the amino acid sequence) were also removed from the mutation list. Shortlisted mutations are then ranked based on the significance of differences of average GI50 between sensitive and resistant groups. This process is depicted as a flow diagram in Appendix E. Having identified *PIK3CA* mutations to be enriched in palbociclib-resistant cell lines, this was validated in a larger population of cancer cells, the dataset from Genomics of Drug Sensitivity in Cancer (GDSC). The analysis was performed using 789 palbociclib-treated cancer cell lines of all available cancer types in the database. These cell lines were grouped based on their *PIK3CA* mutational status (wild-type or having any mutations in *PIK3CA* gene) and their geometric means of IC_{50} were compared. Significant differences were determined using a 95% confidence interval.

3.5.2 Validation of *PIK3CA*-mutated cell line

DNA Sanger sequencing was performed to validate the presence of *PIK3CA* mutation in CAL27, a cell line that was previously engineered to stably express *PIK3CA* H1047R mutation (CAL27-mutant cells) (Z. Wang et al., 2014). This was generated by infection with pLESIP HA-PIK3CA H1047A lentivirus. Genomic DNA of CAL27-mutant cells was first extracted using DNeasy Blood & Tissue Kit (Qiagen, USA) and quantified by Nanodrop spectrophotometer (Thermofisher Scientific, MA, USA). Extracted gDNA was then purified using QIAquick PCR Purification Kit (Qiagen, USA) prior to gene amplification by polymerase chain reaction (PCR) assay and sequencing of DNA (external service). The presence of the mutation was also confirmed by western blotting (described in 3.3.1), by evaluating the expression of phosphorylated Akt between CAL27 and CAL27-mutant.

3.5.3 Validation of *PIK3CA* mutation as a resistance biomarker by *in vitro* cell proliferation assays

To determine the differential response between CAL27 and CAL27-mutant cells towards palbociclib, cell proliferation assays such as Click-iT EdU assay (described in 3.2.4) and cell cycle assay (described in 3.2.5) was performed. Western blotting assay (described in 3.3.1) was performed to elucidate the mechanism of resistance instigated by *PIK3CA* mutation.

3.5.4 Investigation of combined inhibition of CDK4/6 and PI3K/mTOR

To investigate whether concurrent inhibition of the PI3K/mTOR pathway would re-sensitize palbociclib-resistant cell lines, cells were treated with either vehicle control, palbociclib alone, PF-04691502 alone, or combination of the two inhibitors. The effect of the treatments was evaluated by the Click-iT EdU assay (described in 3.2.4), western blotting (described in 3.3.1) and mouse efficacy study (described in 3.4).

3.6 Statistical analysis

All data are expressed as mean ± SD from three independent experiments unless stated otherwise. Statistical analysis was performed using IBM SPSS Statistics for Windows, version 21.0 (IBM Corp., NY, USA). Significant differences between groups were analyzed using 2-tailed independent *t*-test or one-way ANOVA. *p* values of less than 0.05 were considered statistically significant.

CHAPTER 4 RESULTS

4.1 Validation of palbociclib's efficacy in OSCC

4.1.1 Palbociclib inhibits growth of OSCC cell lines in culture

To examine the effect of palbociclib towards OSCC, a panel of OSCC cell lines was treated with 0-2 μM of palbociclib for 72 h. Among the sixteen OSCC cell lines tested, thirteen of them showed sensitivity to palbociclib with GI_{50} of less than 1 μM, while three lines with GI_{50} of more than 1 μM indicated resistance to palbociclib based on the cytostatic assay (Figure 4.1). The sensitive and resistant groups were significantly different from each other with ~5-fold differences in average GI_{50} values (p<0.01).

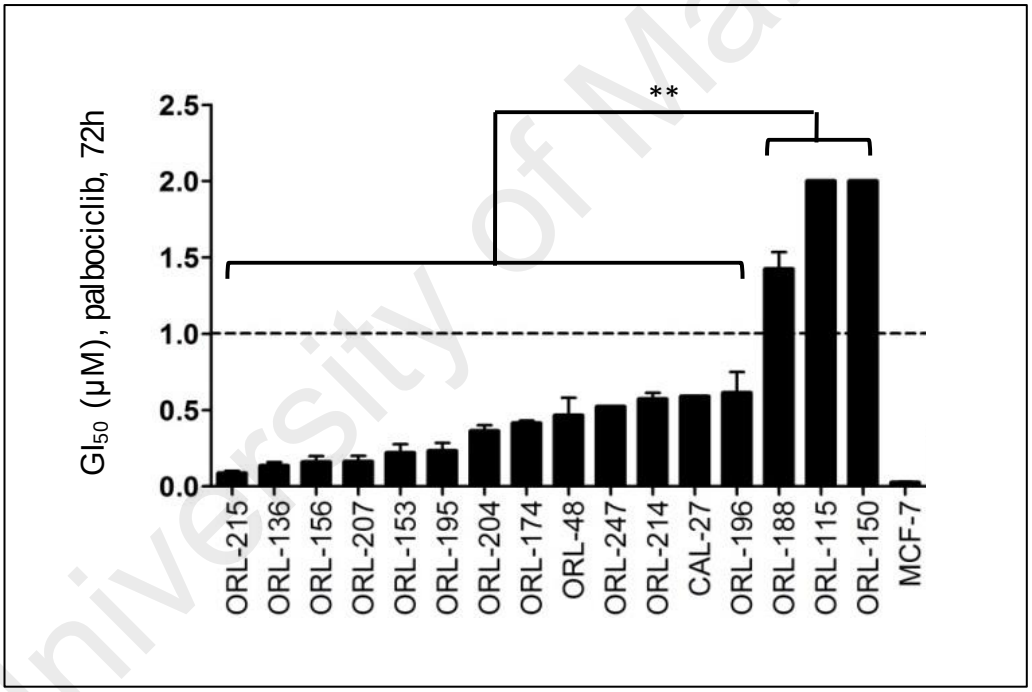

Figure 4.1: The GI_{50} of palbociclib (μM) on a panel of OSCC cell lines based on crystal violet cytostatic assay. Thirteen of the cell lines tested showed GI_{50} of less than 1 μM, while three lines (ORL-188, ORL-150 and ORL-115) indicated resistance (GI_{50}>1 μM). Cell lines were treated with 72 h of 0.008 to 2 μM of palbociclib. The MCF-7 cell line was used as a positive (sensitive) control to palbociclib treatment. Data are shown as mean ± SD from at least two independent experiments; ** p<0.01 denotes significant difference between the sensitive and resistant groups.

To further validate the inhibitory effect of palbociclib towards cell proliferation, Click-iT EdU proliferative assay was performed on representative palbociclib-sensitive cells (CAL27 and ORL-48) and palbociclib-resistant cells (ORL-150 and ORL-115). These cell lines were selected based on their tumor-forming ability in mice as characterized previously (Fadlullah et al., 2016). Relative to the vehicle control (VC) group, palbociclib treatment (0.06 to 0.5 μM) for 24 h reduced the number of EdU-positive cells (represented as red stained-cells) in CAL27 and ORL-48 (Figure 4.2 (a)), suggesting a decrease in the DNA synthesis activity and blockade of cell cycle progression from G_1 to S phase. As expected, the reduction of EdU-positive cells was not as pronounced in ORL-150 and ORL-115 cells where the reduction of dividing cells were less than 50% even at the highest drug concentration (Figure 4.2 (b)).

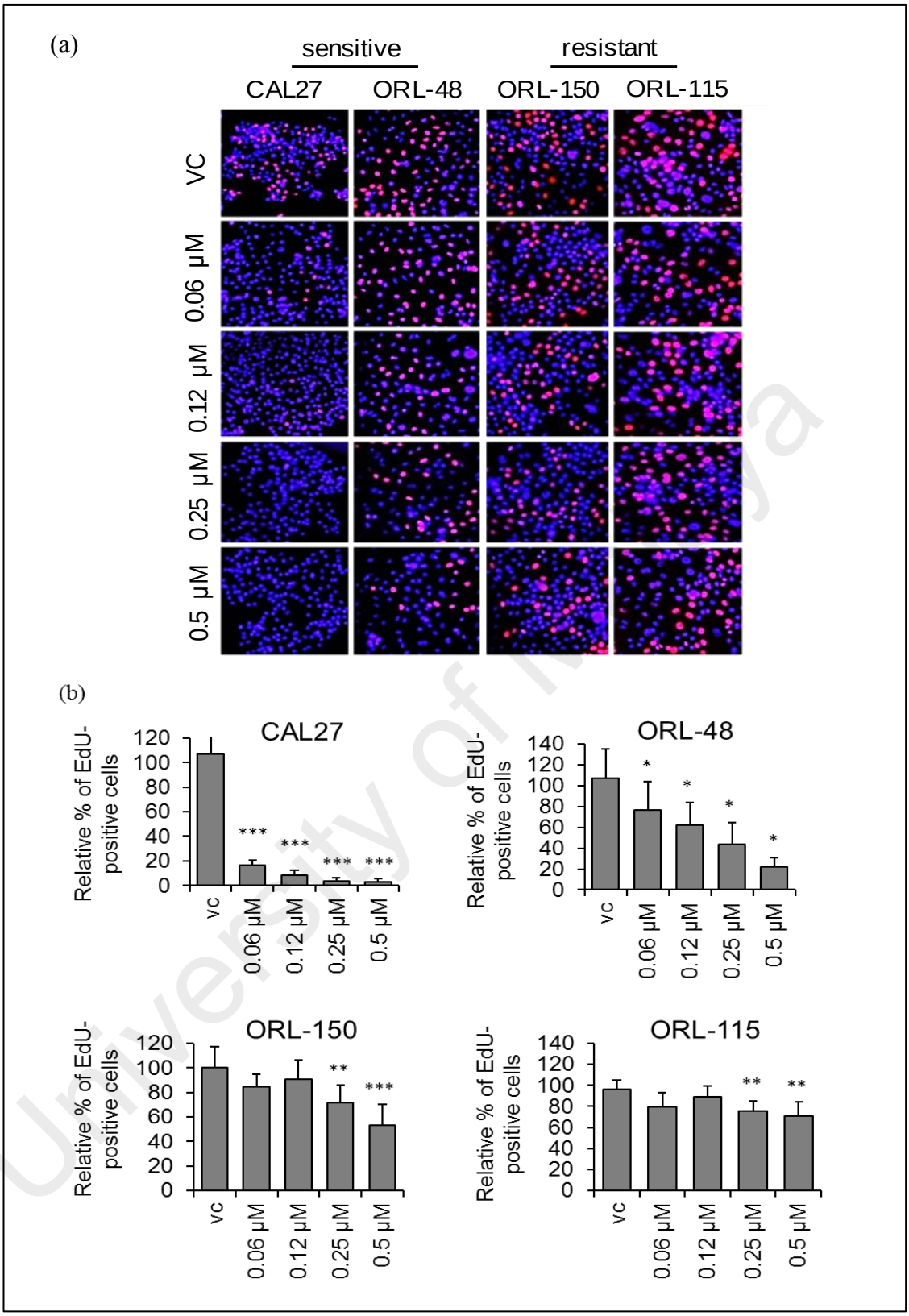

Figure 4.2: Validation of the cytostatic effect of palbociclib in representative sensitive (CAL27 and ORL-48) and resistant (ORL-150 and ORL-115) cell lines using Click-iT EdU assay. (a) Cells were treated with 0.06 to 0.5 μM palbociclib for 24 h, fixed and stained with Alexa Fluor azide (red) and Hoecsht 33342 (blue). Blue represents the total number of cells in any field and red represents proliferating cells that have incorporated the EdU label as shown in the representative image. (b) The number of EdU-positive cells (which represents actively dividing cells) were quantified as relative percentage. Palbociclib exhibited anti-proliferative effects on CAL27 and ORL-48 as demonstrated by the reduction in the relative percentage of EdU-positive cells, but not observed in ORL-115. Data are shown as mean ± SD of ten fields from duplicate wells and are representative of two independent experiments; * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ denote significant differences from VC.

4.1.2 Anti-proliferative activity of palbociclib is associated with G_1 cell cycle arrest

As a selective inhibitor of CDK4/6, palbociclib is known to prevent the initiation of S-phase of the cell cycle. To confirm this, the anti-proliferative effect of palbociclib was investigated through cell cycle analysis following propidium iodide staining in palbociclib-sensitive cells, CAL27 and ORL-48. The cells were treated with palbociclib (0.06 to 0.5 μM) for 24 h. Palbociclib treatment increased the percentage of cells in the G_0/G_1 phase, and concurrently reduced the percentage of S-phase cells in a dose-dependent manner for both cell lines (Figure 4.3). This indicates the inhibition of S-phase initiation by palbociclib, which resulted in the arrest of cell cycle progression at the G_0/G_1 phase in OSCC cells.

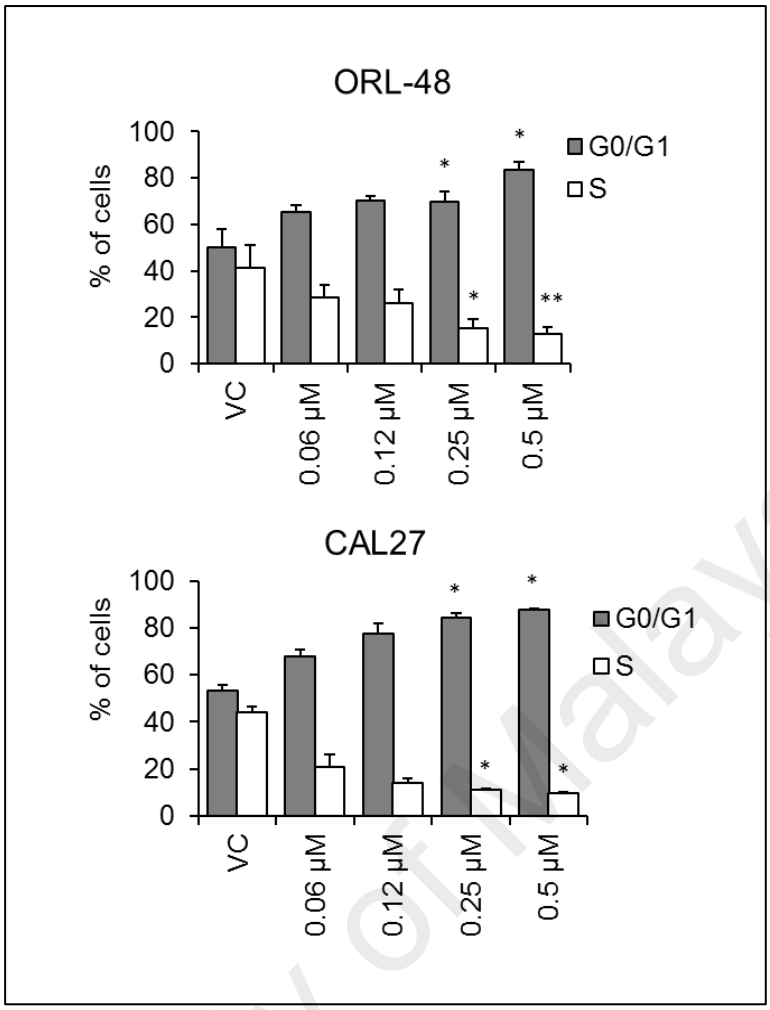

Figure 4.3: Percentage of cell population in the G_0/G_1 and S phases of the cell cycle upon palbociclib treatment. Palbociclib increased cell population in G_0/G_1 and decreased cell population in S phase in ORL-48 and CAL27 cells. Cells were treated with 0.06 to 0.5 µM palbociclib for 24 h, fixed and stained with propidium iodide. Data are shown as mean ± SD of triplicate wells and are representative of two independent experiments; * $p<0.05$ and ** $p<0.01$ denote significant differences from VC.

4.1.3 Palbociclib reduces Rb protein levels and Rb phosphorylation

The Rb pathway is critical in regulating the initiation of DNA replication. The biochemical effect of palbociclib towards proteins involved in this pathway was further studied. A dose-dependent protein expression analysis was first conducted on palbociclib-sensitive (CAL27 and ORL-48) and palbociclib-resistant cells (ORL-150 and ORL-115) on Rb phosphorylation at S780, S795 and S807/811 as these sites are known to be phosphorylated by CDK4/6 (Zarkowska & Mittnacht, 1997). It was observed that the

treatment of palbociclib, especially at 0.5 μM, reduced Rb-phosphorylation at all three phosphorylation sites in CAL27, ORL-48 and ORL-150 (Figure 4.4), indicating the restoration of Rb activity in blocking cell cycle progression upon palbociclib treatment. By contrast, ORL-115 retained the expression and phosphorylation of Rb at all doses tested (0.06 – 0.5 μM), validating the resistance of this cell line towards palbociclib as noted in the proliferative assays described above.

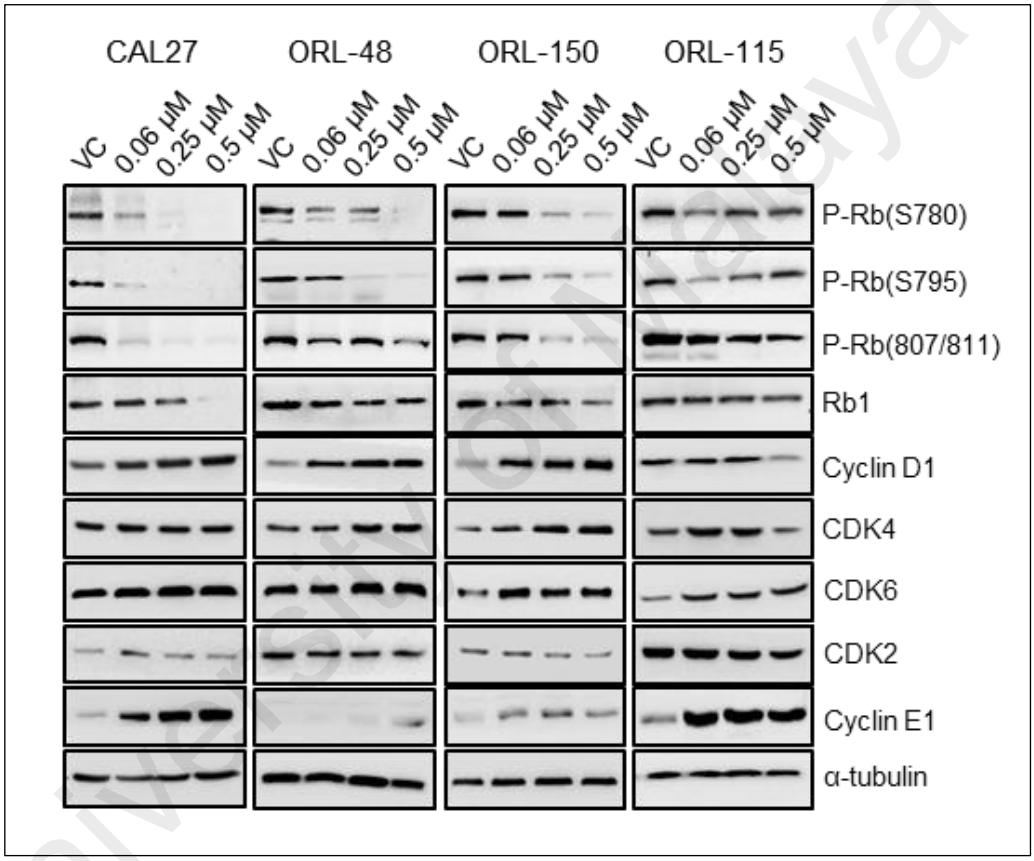

Figure 4.4: Dose-dependent western blot analysis of palbociclib treatment towards sensitive (CAL27 and ORL-48) and resistant cells (ORL-150 and ORL-115). Cells were treated with 0.06 to 0.5 μM palbociclib for 24 h prior to protein extraction and western blotting. 30 μg proteins were used from each sample. Results shown are representative of at least two independent experiments.

As the greatest effect of Rb-dephosphorylation was observed at 0.5 μM dose, this concentration was used to conduct a time-dependent protein expression analysis on palbociclib-sensitive cells (CAL27 and ORL-48). Complete dephosphorylation of Rb was observed by 24 h treatment, particularly on S780 and S795 (Figure 4.5). Additionally, palbociclib steadily downregulated the expression levels of Rb in a time-dependent

manner culminating in low Rb expression at 24 h treatment in both cell lines. It was also noted that protein levels of cyclin D1, CDK4 and 6 were slightly increased with time as a result of palbociclib treatment, consistent with observations in other studies on breast and colorectal cancers (Herrera-Abreu et al., 2016; Li, Qi, Bellail, Hao, & Liu, 2014).

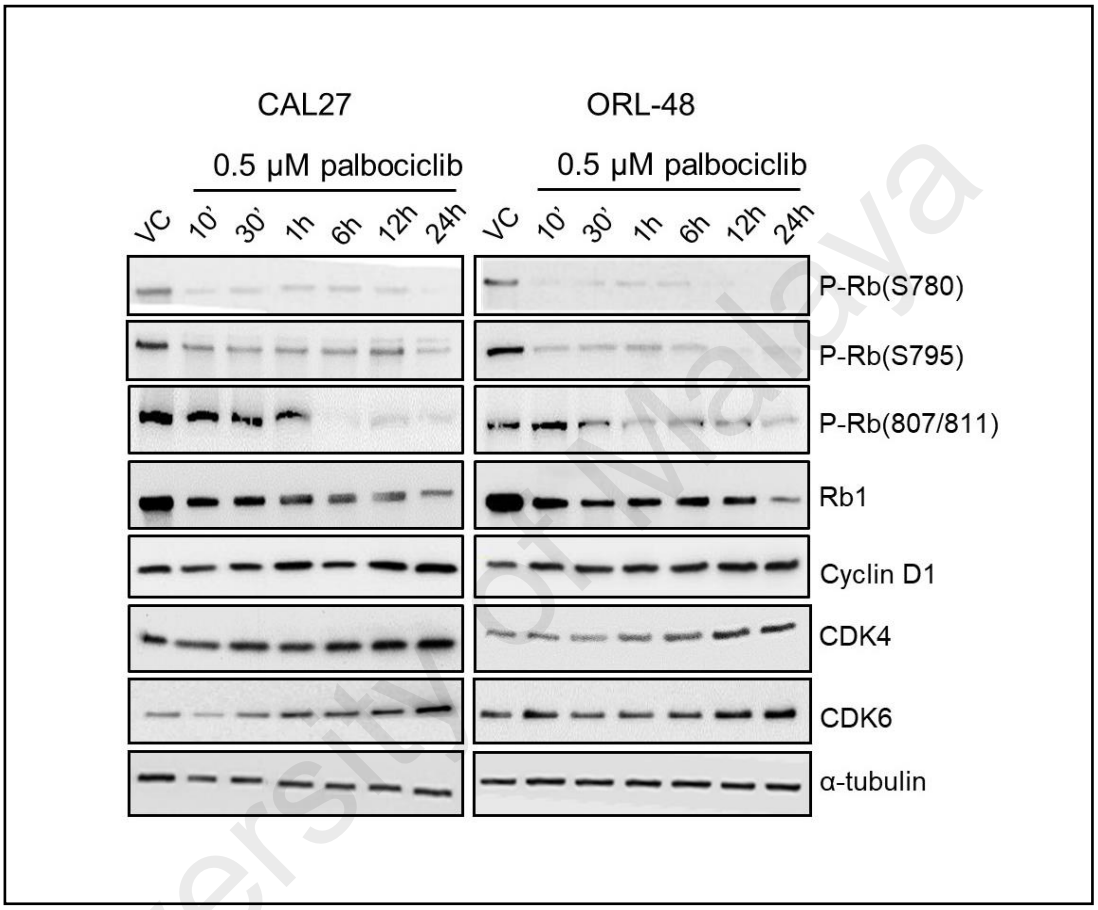

Figure 4.5: Time-dependent western blot analysis on palbociclib-sensitive cells (CAL27 and ORL-48). Cells were treated with 0.5 μM palbociclib for 10 min to 24 h prior to protein extraction and western blotting. 30 μg proteins were used from each sample. Results shown are representative of at least two independent experiments.

4.1.4 Palbociclib inhibits tumor growth in subcutaneous xenograft model

Due to palbociclib's potent inhibition towards OSCC cells as seen *in vitro*, its *in vivo* efficacy was next investigated in subcutaneous xenograft models using CAL27 and ORL-48 cells. Mice were randomized to receive either 150 mg/kg of palbociclib or vehicle control (VC) when tumors reached an average volume of 100-150 mm^3. It was observed that palbociclib treatment for 21 days significantly inhibited the growth of CAL27 (VC:

477.5 ± 101.2 mm^3 vs. palbociclib: 159.76 ± 22.6 mm^3; p = 0.007) and ORL-48 (VC: 755.06 ± 51.07 mm^3 vs. palbociclib: 68.47 ± 15.07 mm^3, $p = 1x10^{-7}$) xenograft tumors (Figure 4.6 (a & b)). Palbociclib (150 mg/kg) treatment did not result in the significant loss of body weight (Appendix F). Next, the expression and phosphorylation of Rb protein in xenograft tumors that were harvested at the end of the study were determined. As shown in Figure 4.6 (c), palbociclib treatment resulted in the reduction of phosphorylated Rb at S795 phosphorylation site in CAL27 xenograft tumors. The effect was more apparent in ORL-48 xenograft tumors and reduction of Rb phosphorylation can be seen at both S780 and S795 phosphorylation sites, consistent with the observation that tumor control was more significant in this cell line compared to CAL27 (Figure 4.6 (a)). Although marked reduction in Rb phosphorylation at S780 and S795 were observed, Rb phosphorylation was not affected in S807/811 in the tumor (Figure 4.6 (c)) that was consistent with observations *in vitro* for ORL-48 (figure 4.4). In addition, palbociclib caused reduction in the Rb levels of both CAL27 and ORL-48 tumors, consistent with what was observed *in vitro*. These observations confirmed that palbociclib could inhibit the CDK4/6-Rb pathway, which eventually led to tumor control in mice.

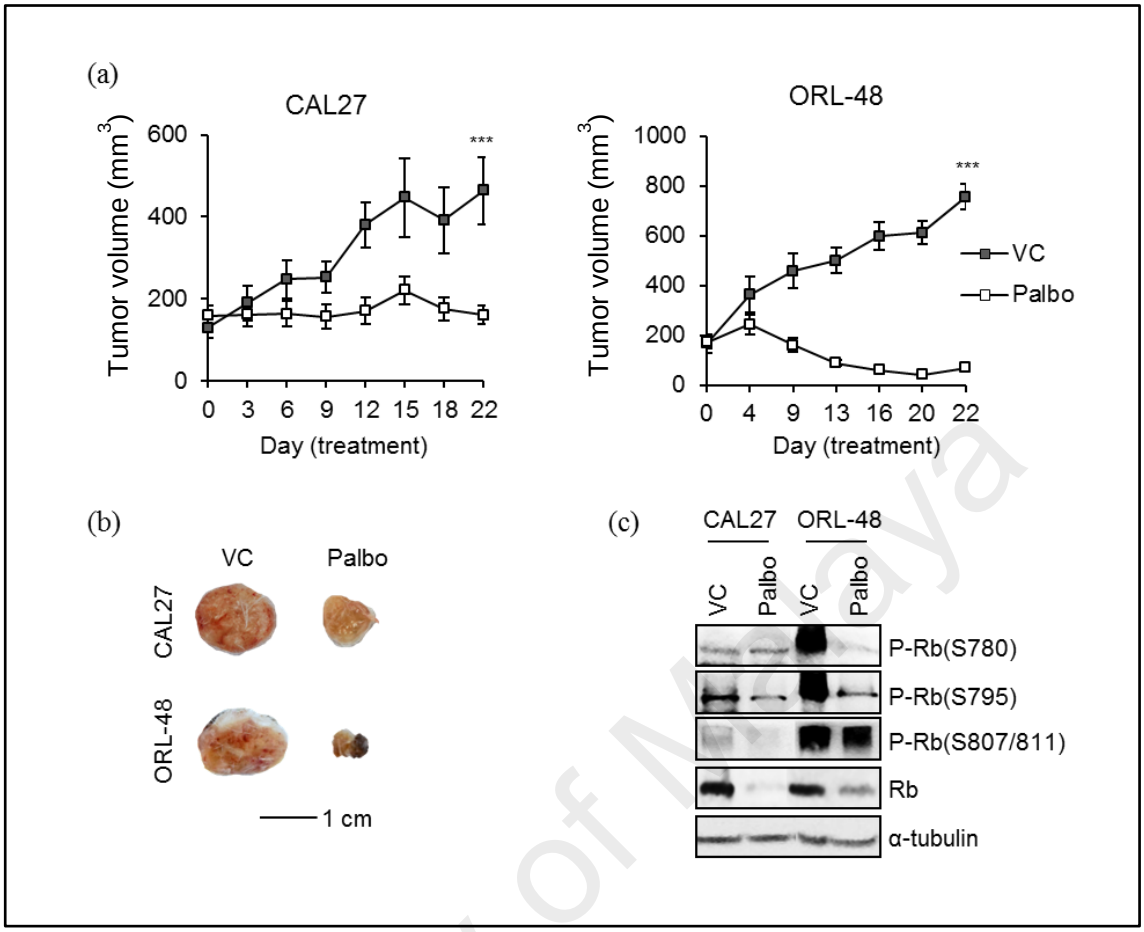

Figure 4.6: *In vivo* efficacy of palbociclib in CAL27 and ORL-48 xenograft model. (a) Mice bearing CAL27 or ORL-48 xenograft tumors were treated with either vehicle control (VC) or 150 mg/kg palbociclib (Palbo) for 21 d. Palbociclib significantly inhibited the growth of OSCC subcutaneous xenograft ORL-48 and CAL27 tumors based on the average tumor volume. Results shown are mean ± SEM (n=10 tumors per group). Symbol *** denotes $p<0.001$. (b) Representative tumors harvested at the end of treatment showed decreased tumor volume in the palbociclib treatment group as compared to VC. (c) Western blot analysis on proteins extracted from CAL27 and ORL-48 xenograft tumors at end-point showed 150 mg/kg palbociclib treatment (daily for 21 d) reduced Rb phosphorylation in mouse tumors particularly at S780 and S795 phosphorylation sites. 30 µg proteins were used from each sample. Results shown are representative of at least two independent experiments.

4.2 Investigation of *PIK3CA* mutation as a biomarker of response

4.2.1 Identification of *PIK3CA* as biomarker of resistance

Based on the cytostatic assay in Figure 4.1, a subset of OSCC cell lines demonstrated resistant to palbociclib. Hence it was investigated whether certain genetic features could lead to palbociclib resistance. To achieve this, the mutational profile between palbociclib-sensitive and resistant cells was compared (as depicted in Appendix E) and *PIK3CA* mutation was found to be highly implicated in the resistant group (mutation list available in Appendix G). Two out of three cells in the resistant group carry mutations in the *PIK3CA* gene (H1047L and Q546R in ORL-115 and ORL-150 respectively) (Fadlullah et al., 2016; B. Lee et al., 2018), while none of the palbociclib-sensitive cell lines had any mutations in this gene. Cell lines carrying mutant *PIK3CA* had significantly higher GI_{50} as compared to cell lines with wildtype *PIK3CA*, suggesting an association between *PIK3CA* mutations with palbociclib's resistance (p<0.001; Figure 4.7 (a)). This observation was validated with a larger data set from Genomics of Drug Sensitivity in Cancer (GDSC) comprising of 852 palbociclib-treated cancer cell lines of 30 cancer types in the database. These cell lines were grouped based on their *PIK3CA* mutational status (WT or having any mutations in *PIK3CA* gene; Appendix H) and their geometric mean of IC_{50} were compared. Cancer cells having any mutation in the *PIK3CA* gene have a higher geometric mean IC_{50} as compared to WT (8.23 μM and 5.81 μM respectively), suggesting that they are more resistant to palbociclib as compared to WT cell lines. The difference however was not statistically significant (p=0.101; Figure 4.7 (b)). This led to further investigation on the role of *PIK3CA* mutations in conferring resistance to palbociclib in OSCC.

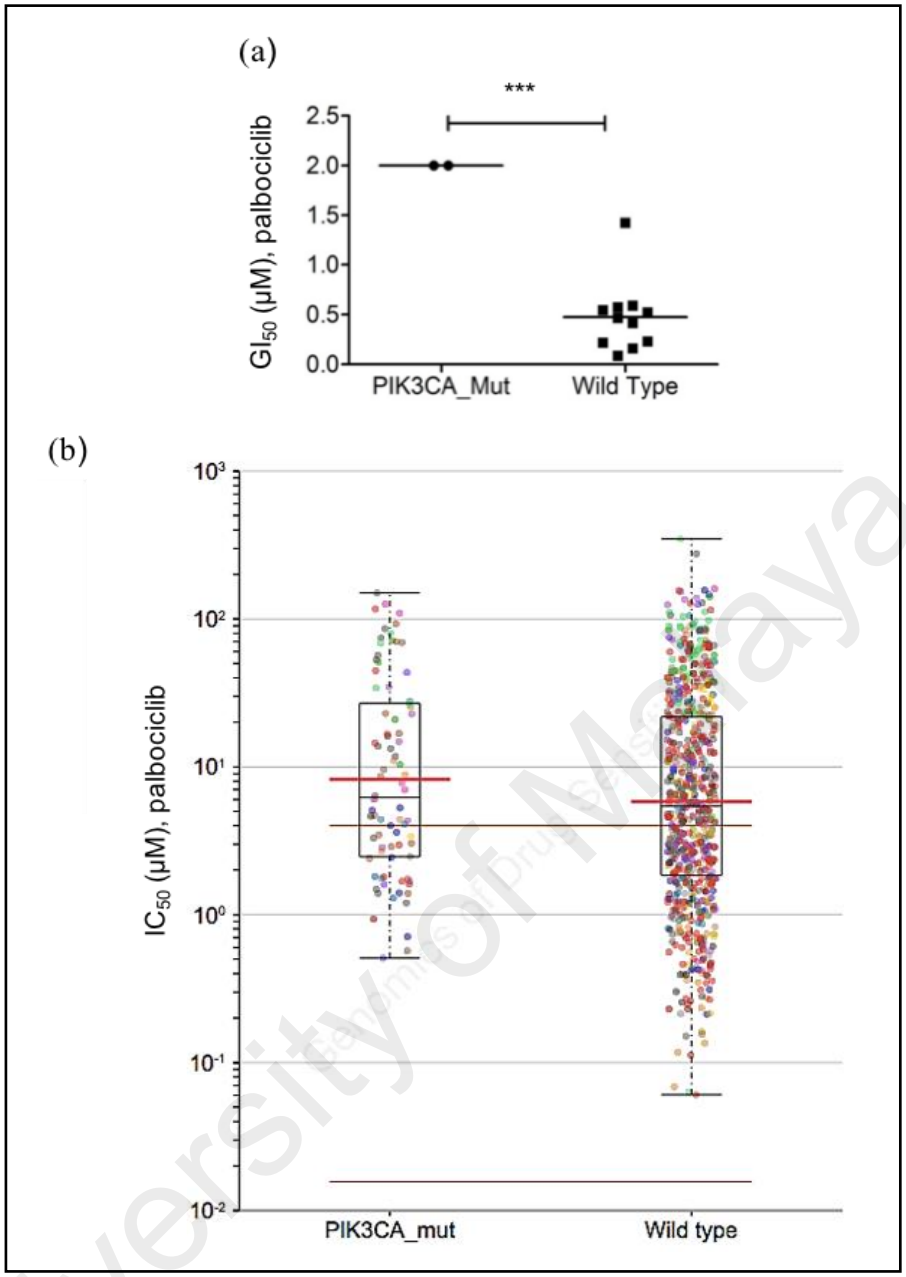

Figure 4.7: GI_{50} and IC_{50} of palbociclib-treated cell lines according to their *PIK3CA* mutational status (a) Comparison of the GI_{50} of *PIK3CA*-mutant and wild type cell lines treated with palbociclib by cytostatic assay shown in Figure 4.1. This analysis revealed a significant association of *PIK3CA* mutation with palbociclib resistance. Symbol *** denotes $p<0.001$. (b) Comparison of the geometric mean IC_{50} of palbociclib between *PIK3CA*-mutant and wild type cell lines in the Genomics of Drug Sensitivity in Cancer (GDSC) database. Cancer cells having any mutation in the *PIK3CA* gene have higher geometric mean IC_{50}, implying them to be more resistant to palbociclib compared to wild-type cancer cells. A total number of 852 palbociclib-treated cancer cell lines from 30 different cancer types (represented by the different colored circles) are included in the analysis. The bottom and upper brown line represent the minimum and maximum palbociclib concentration used in the screening (Adapted from (Yang et al., 2013)).

4.2.2 Validation of isogenic CAL27 cell line

To test whether *PIK3CA* mutation confers resistance to palbociclib in OSCC, CAL27 cells that were previously engineered to stably express *PIK3CA* (H1047R) alteration (Z. Wang et al., 2014) were used for subsequent studies. The mutational status of *PIK3CA* in isogenic CAL27-mutant and CAL27 cell lines were verified by Sanger sequencing, where the CAL27-mutant cells have base substitution from A (wild-type; WT) to G resulting in the replacement of histidine (H) to arginine (R) at position 1047 (Figure 4.8 (a)). The presence of the *PIK3CA*-H1047R mutation was also confirmed by western blotting, whereby increase Akt phosphorylation was observed in CAL27-mutant cells (Figure 4.8 (b)).

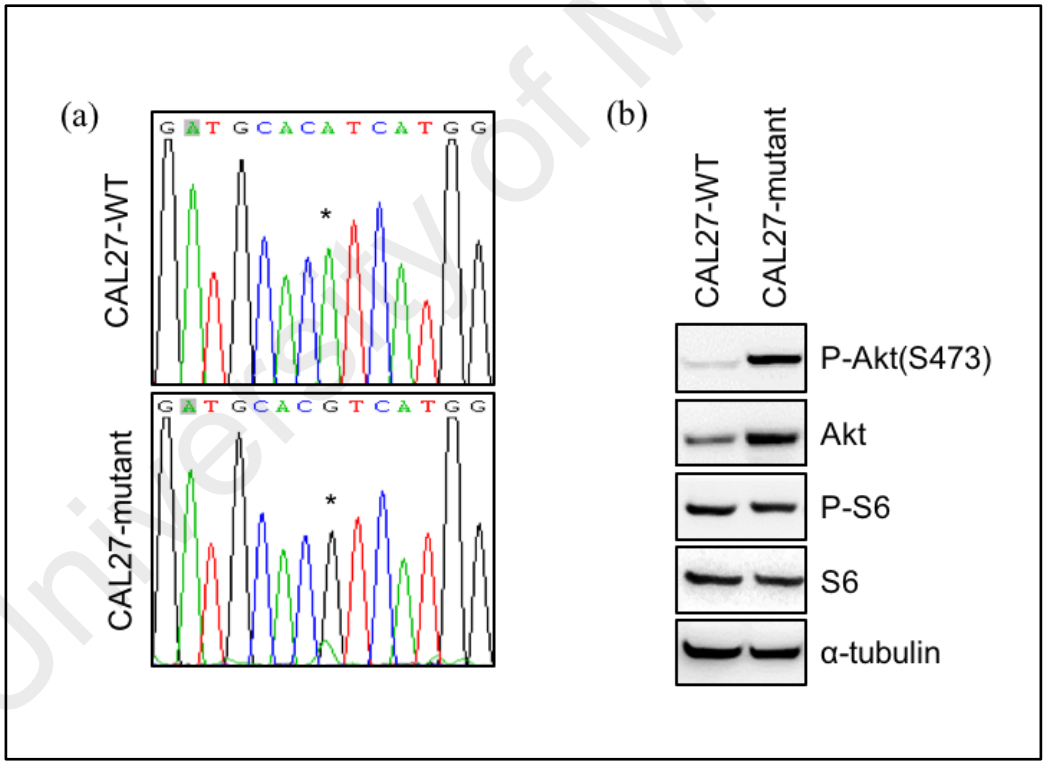

Figure 4.8: Confirmation of *PIK3CA* (H1047R) alteration in previously engineered CAL27 cells (a) DNA chromatogram of CAL27 and CAL27-mutant cells confirmed base substitution from A (WT) to G (mutant) resulting in the change of amino acid R at position 1047 indicated by the asterisks. (b) Proteins extracted from CAL27-mutant cells showed over-activation of Akt(S473) but not in CAL27 cells by western blotting.

4.2.3 PIK3CA mutation decreases response to palbociclib

The differential sensitivity to palbociclib in these isogenic cells was first evaluated by EdU proliferation assay. The inhibition of cell proliferation was more pronounce in CAL27 cells as the percentage of EdU-positive cells were observed to be lower by ~4-fold compared to CAL27-mutant cells at a dose of 0.5 μM (p<0.001; Figure 4.9 (a&b)).

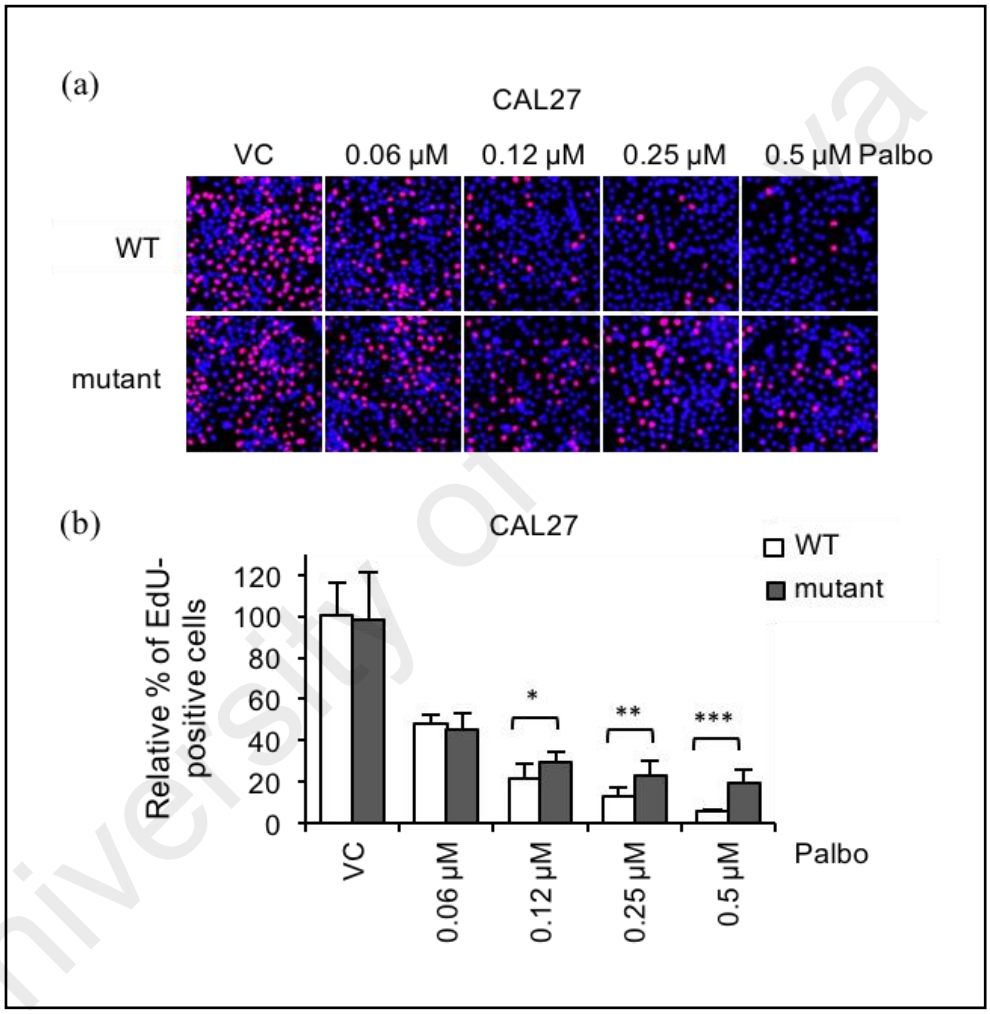

Figure 4.9: Click-iT EdU assay performed on CAL27 (WT) and CAL27-mutant cells (a) Cells were treated with 0.06 to 0.5 μM palbociclib for 24 h, fixed and stained with Alexa Fluor azide and Hoecsht 33342. Blue represents the total number of cells in any field and red represents proliferating cells that have incorporated the EdU label (EdU-positive cells) as shown in the representative image. Less inhibition by palbociclib (24 h) was observed in CAL27-mutant cells as indicated by the increased percentage of dividing cells as compared to CAL27. (b) Data of relative percentage of EdU-positive cells are shown as mean ± SD of ten fields from duplicate wells and are representative of two independent experiments; * p<0.05, ** p<0.01 and *** p<0.001 denote significant differences between two groups.

In addition, the effect of *PIK3CA* mutation towards two other clinically approved CDK4/6 inhibitors was examined (O'Shaughnessy et al., 2018; Sledge Jr et al., 2017). Isogenic CAL27 cells were treated with ribociclib and abemaciclib (0.25 – 0.5μM) and it was found that EdU-positive cells were also significantly lower in CAL27 cells as compared to CAL27-mutant cells (Figure 4.10). The results indicated that the presence of *PIK3CA* mutation reduced the sensitivity of CAL27 to CDK4/6 inhibitors and this observation was not restricted to palbociclib.

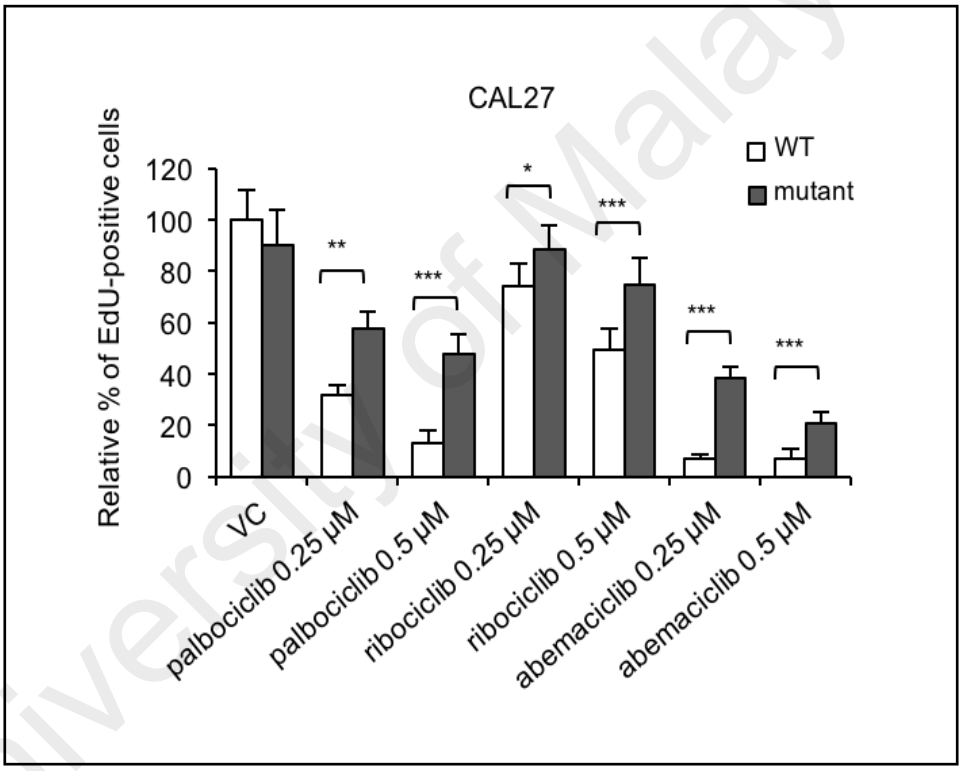

Figure 4.10: CAL27-mutant cells were less sensitive to all available specific CDK4/6 inhibitors (palbociclib, ribociclib and abemaciclib) at 0.25 and 0.5 μM by Click-iT EdU assay. Data are shown as mean ± SD of ten fields from duplicate wells and are representative of two independent experiments; * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ denote significant differences between two groups.

Flow cytometry was next performed to evaluate the differences in G_1 cell cycle arrest on the respective WT and mutant cells. As demonstrated previously, palbociclib treatment arrests cells at the G_0/G_1 phase. Based on Figure 4.11, the percentages of cells in the G_0/G_1 phase for CAL27-mutant cell line were lower than that of the CAL27 at all doses tested,

with statistical significance at 0.5 μM palbociclib (p=0.02). Concurrently, CAL27-mutant cell line showed a greater percentage of cells in the S phase than that of CAL27 cells at 0.5 μM treatment (p=0.03), indicating higher cell cycle activity consistent with observations of resistance to palbociclib.

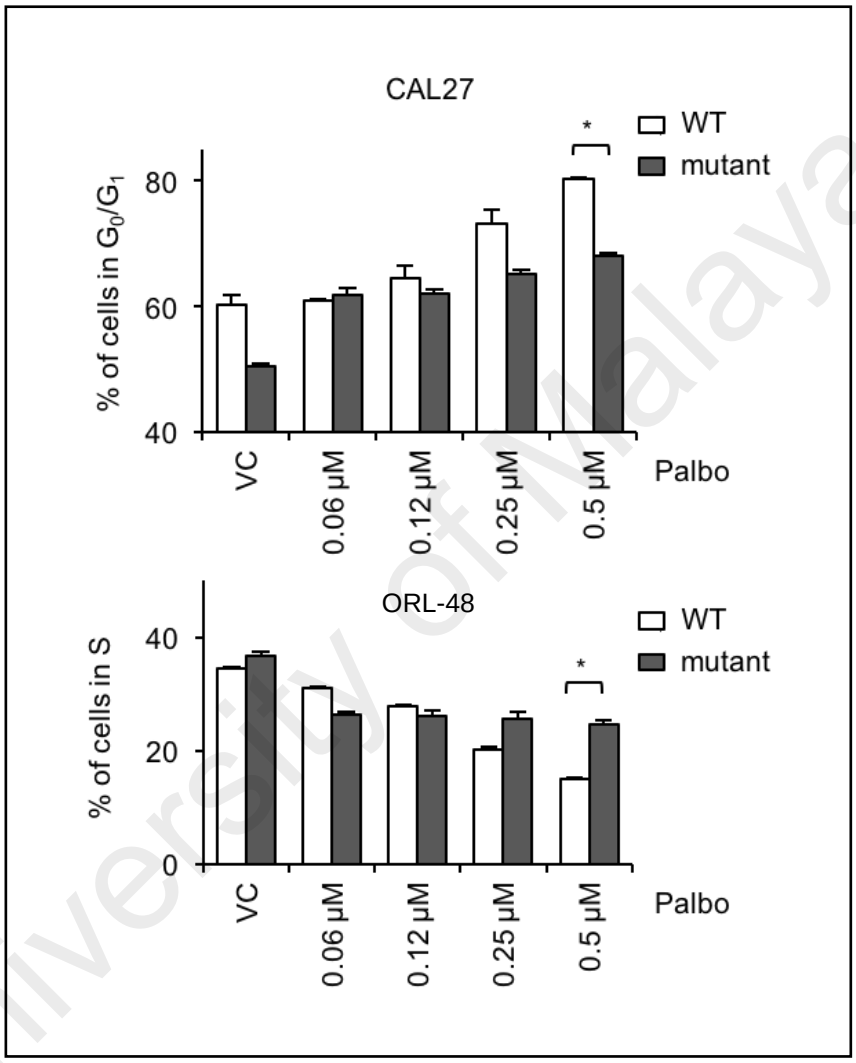

Figure 4.11: Cell cycle analysis (propidium iodide staining) performed on CAL27 and CAL-27-mutant cells. Reduced G_0/G_1 and increased S phase cell population were seen in CAL27-mutant cells as compared to CAL27 following 24 h of 0.06-0.5 μM palbociclib treatment. Data are shown as mean ± SD of triplicate wells and are representative of two independent experiments; * p<0.05, ** p<0.01 and *** p<0.001 denote significant differences between two groups.

4.2.4 *PIK3CA*-mutated cells escape CDK4/6 inhibition by CDK2 pathway

To investigate the biochemical mechanism that caused CAL27-mutant cells to exhibit higher resistance to palbociclib, proteins were extracted from the isogenic cells after a 24 h treatment of escalating doses of palbociclib (0.06 to 0.5 μM) and proteins within the Akt and Rb pathway were examined by western blotting (Figure 4.12). Due to the presence of the *PIK3CA*-H1047R mutation, CAL27-mutant cells had significantly higher phosphorylation of Akt as compared to CAL27 cells across all palbociclib doses tested. The reduction of Rb phosphorylation particularly at the S795 and S807/811 phosphorylation sites were not as marked in CAL27-mutant cells treated with palbociclib when compared to CAL27 cells. Less reduction of Rb phosphorylation at the S780 site was also detected, however it was only apparent at the 0.06 μM treatment. The lack of reduction in Rb phosphorylation that occurred evidently in S807/811 and S795 but less in S780 in the CAL27-mutant cells suggested an S-phase entry through phosphorylation of Rb by CDK2/cyclin E1 complex (Kitagawa et al., 1996; Schmitz, Hirt, Aebi, & Leibundgut, 2006; Zarkowska & Mittnacht, 1997). Palbociclib treatment (0.06 to 0.5 μM) caused reduction of CDK2 expression in CAL27 cells, however CDK2 expression sustained throughout the range of palbociclib dose tested in the CAL27-mutant cells. Cyclin E1, which partners with CDK2 to phosphorylate Rb, showed increased levels of expression in the CAL27-mutant cells as compared to CAL27 when palbociclib treatment was given. On the contrary, there were no changes of CDK4, CDK6 and cyclin D1 protein levels between CAL27 and mutant cells across all treatment doses, suggesting that the CDK2/cyclin E1 is potentially a compensatory pathway that led to Rb phosphorylation in CAL27-mutant cells resulting in an escape from palbociclib treatment.

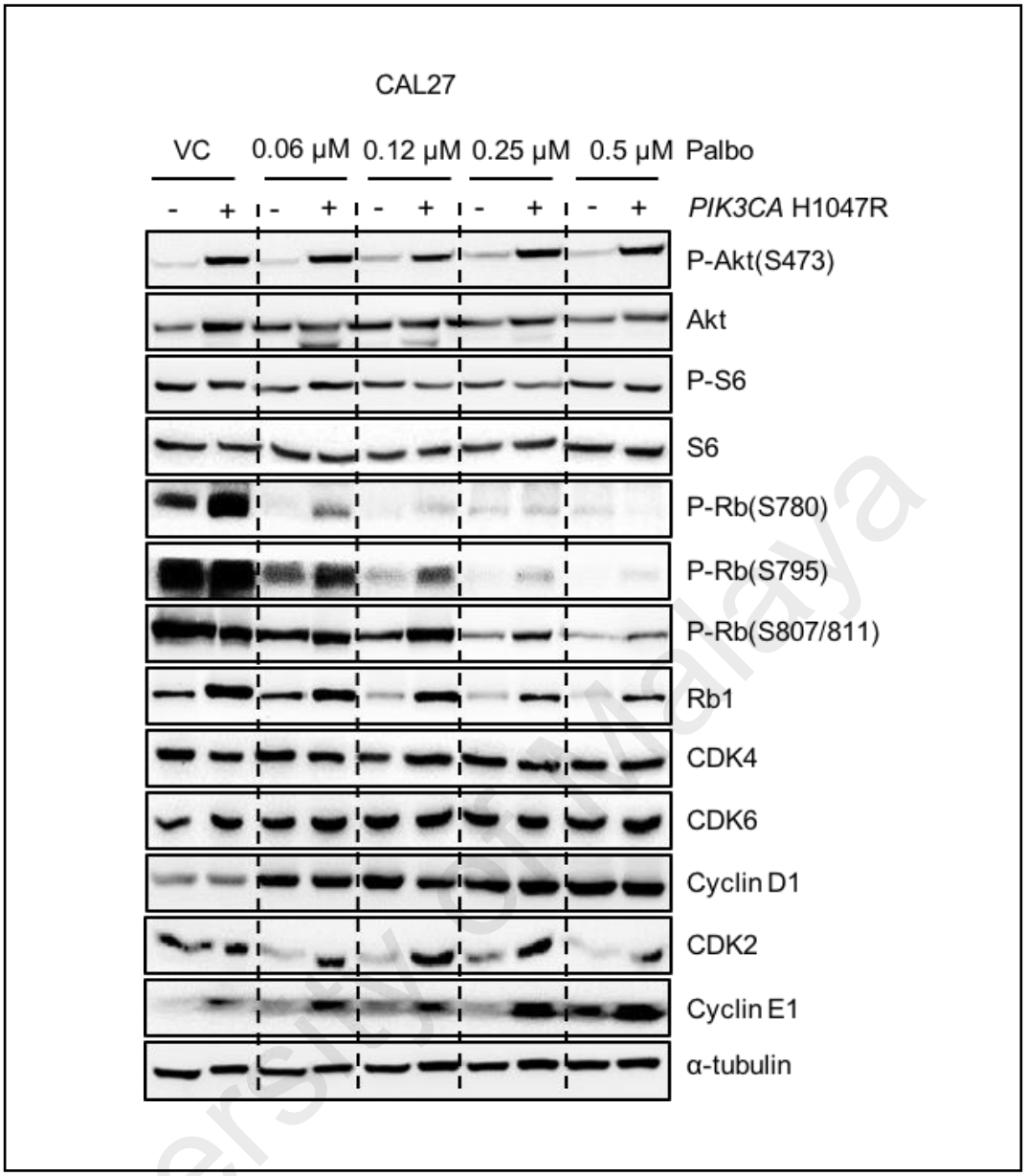

Figure 4.12: Western blot analysis performed on proteins extracted from CAL27 and CAL27-mutant cells upon 24 h of 0.06-0.5 μM palbociclib treatment. 30 μg proteins were used from each sample. CAL27 and CAL27-mutant cells were indicated by '-' and '+'. Results shown are representative of at least two independent experiments.

4.2.5 Combination of CDK4/6 and Pi3K inhibitors sensitizes *PIK3CA*-mutant ORL-115

Since *PIK3CA* mutation could confer resistance to palbociclib, the next aim was to determine whether the *PIK3CA*-mutant cells could be sensitized to palbociclib with a combination treatment targeting the PI3K pathway. Using ORL-115 which harbors the *PIK3CA* H1047L mutation, combining palbociclib with a PI3K/mTOR inhibitor (PF-

04691502) substantially inhibited cell growth where the lowest percentage of EdU-positive cells was observed in the combination group (Figure 4.13 (a&b)). Western blot analysis indicated that a single treatment of palbociclib in ORL-115 resulted in the upregulation of cyclin E1, sustained levels of CDK2, and phosphorylation of Rb especially at S795 and S807/811 sites (Figure 4.13 (c)). Inhibiting both CDK4/6 and PI3K pathways prevented the upregulation of cyclin E1 and reduced CDK2, with the concomitant accumulation of non-phosphorylated Rb at all three sites suggesting an improved growth inhibition in ORL-115. Additionally, co-treatment with PF-04691502 resulted in the reduction of other cell cycle kinases such as CDK4, CDK6 and cyclin D1 as compared to palbociclib treatment alone.

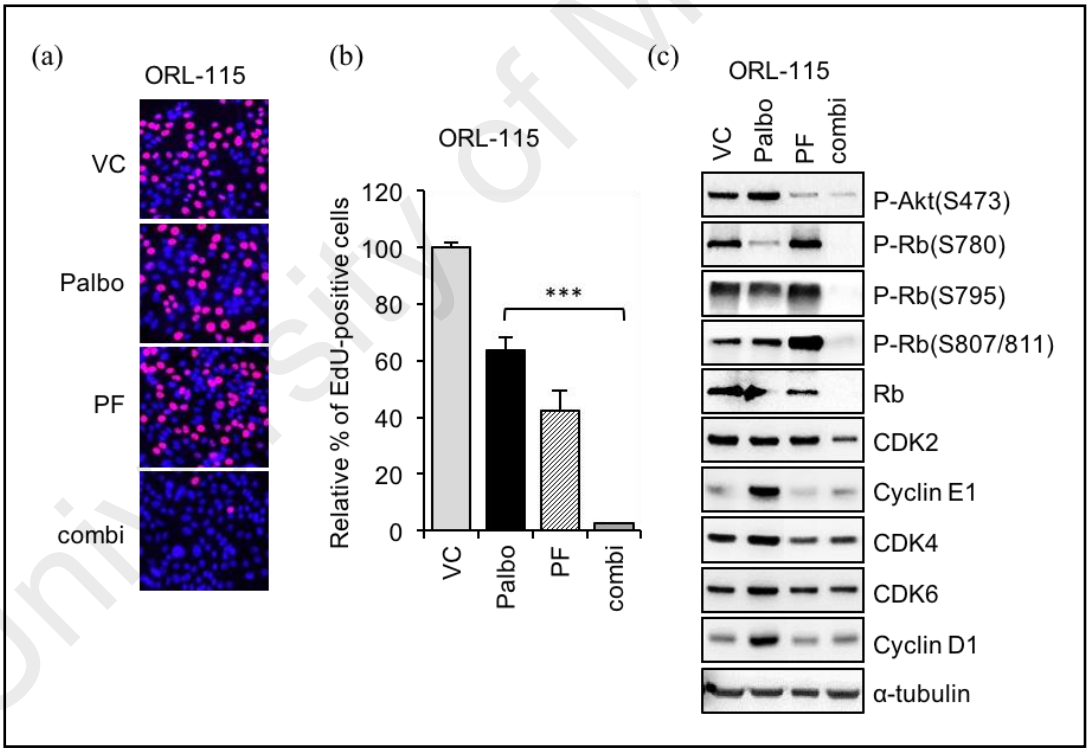

Figure 4.13: Combined activity of palbociclib and PF-04691502 (PI3K/mTOR inhibitor) in ORL-115 cells. (a) ORL-115 (naturally carrying a H1047L *PIK3CA* mutation) were treated with either VC, 0.5 µM palbociclib, 0.25 µM PF-04691502, or combination of 0.5 µM palbociclib and 0.25 µM PF-04691502 (combi) for 24 h prior to Click-iT EdU assay. Blue represents the total number of cells in any field and red represents proliferating cells that have incorporated the EdU label as shown in the representative image. (b) Data shown as mean ± SD of ten fields from duplicate wells and are representative of two independent experiments; *** $p<0.001$ denote significant differences of two groups. (c) Western blot analysis of proteins extracted from ORL-115 cells treated with 0.5 µM palbociclib and 0.25 µM PF-04691502 (24h) showed reduction of Rb phosphorylation and prevention of cell cycle kinases upregulations. 30 µg proteins were used from each sample. Results shown are representative of at least two independent experiments.

Consistently *in vivo*, ORL-115 was less responsive to palbociclib, as sustained tumor growth was still observed in animals treated with palbociclib, with tumor volume steadily increasing from 112 mm^3 (day 1) to 296 mm^3 (day 22) throughout the treatment period (Figure 4.14 (a)). Only when the combined treatment was given, a sustained tumor reduction was achieved from 124 mm^3 (day 1) to 76 mm^3 (day 22). ORL-115 tumors that were treated with PF-04691502 also demonstrated slower tumor growth as compared to palbociclib however, this was not as marked as compared to the combination treatment of palbociclib and PF-04691502 (Figure 4.14 (a&b)). The combination treatment of palbociclib (150 mg/kg) with PF-04691502 (10 mg/kg) did not result in the significant loss of body weight (Appendix I).

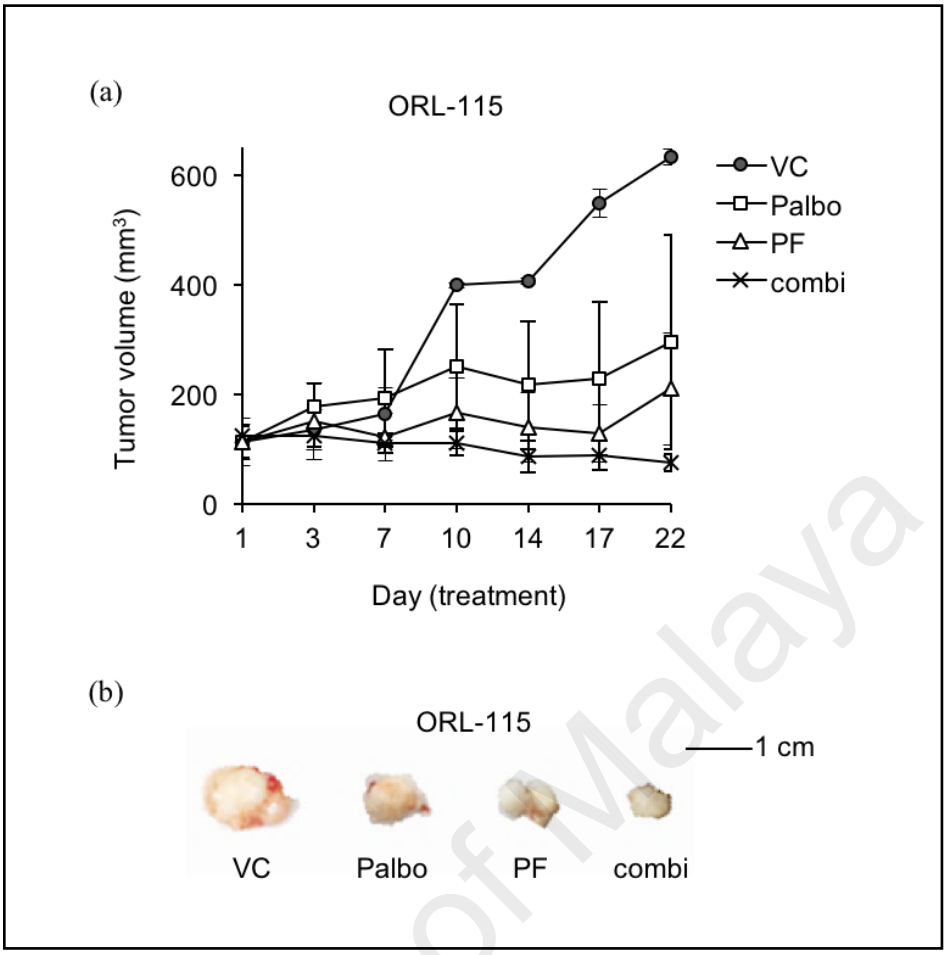

Figure 4.14: Combined activity of palbociclib and PF-04691502 in ORL-115 tumor xenografts. (a) Mice bearing ORL-115 tumor xenografts were treated with either VC, palbociclib (150 mg/kg), PF-04691502 (10 mg/kg), or combination of both for 21d. Only the combination treatment resulted in the gradual decrease of tumor volumes. Results shown are mean ± SEM (n=10 tumors per group). (b) Representative ORL-115 xenograft tumor harvested at the end of the experiment showed the greatest tumor inhibition from the combination group.

CHAPTER 5 DISCUSSION

5.1 Efficacy of palbociclib in OSCC

The standard therapy for OSCC patients such as surgery and chemoradiotherapy are commonly linked to poor quality of life and risks of adverse events. Furthermore, most recurrent or metastatic OSCC patients do not respond to the currently available targeted therapies such as cetuximab and pembrolizumab where response rates from different trials were reported to be less than 20% for both agents. Further, in patients who respond initially, many develop resistance during their treatment. To extend the treatment options for OSCC patients, drugs that can effectively inhibit progression of this disease should be developed. In recent years, cell cycle inhibitors with targeted activity towards specific components of the cell cycle have been approved in the clinical setting for breast cancers (Finn et al., 2016; O'Shaughnessy et al., 2018). Here, we describe the effects of the CDK4/6 inhibitor palbociclib on OSCC cell lines and tumor models. We found that ~80% of OSCC cell lines responded to palbociclib with concomitant reduction of RB phosphorylation, which resulted in inhibition of cell proliferation and G_1 cell cycle arrest. The *in vitro* observations were confirmed by significant tumor control in subcutaneous mouse models. The efficacy of palbociclib shown in this study is similar to those observed in preclinical models of other cancers. A study from Fry et al., demonstrated the anti-proliferative activity of palbociclib in human breast carcinoma cell line *in vitro* through induction of G_1 cell cycle arrests and reduction in Rb phosphorylation. This anti-tumor activity was further validated in various human tumor xenografts including breast carcinoma and colon carcinoma (Fry et al., 2004). More recently, Bollard et al., reported the efficacy of palbociclib in cell line models of liver cancer where growth inhibition and reversible cell cycle arrests were observed. Similarly, anti-tumor activity were also seen in both genetically engineered mouse model and human xenograft model of liver cancer (Bollard et al., 2017).

Based on the data from this and other studies, palbociclib has shown great potential in suppressing cell growth of various cancer models. In addition to its cytostatic properties mainly affecting the CDK4/6-Rb pathway, recent findings have also provided novel mechanistic basis for the effects of palbociclib. Qin et al., has demonstrated the ability of palbociclib in inhibiting the migration and invasion of two molecular subtypes of breast cancer cells, using the cell line models T47D (ER-positive) and MDA-MB-231 (ER-negative) (Qin et al., 2015). This observation was accompanied by the suppression of vimentin and Snail expression levels which are two crucial molecules involved in EMT progression via the c-Jun/COX-2 pathway. The study further demonstrated palbociclib to reduce lung metastasis of MDA-MB-231-Luc cells in animal models and decreased COX-2 and c-Jun expression in tumor tissues. However, the crosstalk between the c-Jun/COX-2 and the CDK4/6-Rb pathway was not studied or discussed in the study. Separately, palbociclib has also been shown to exert cytotoxic activity by inducing apoptosis and autophagy in hepatocellular carcinoma Hep3B cells (Hsieh et al., 2017). Palbociclib induced phosphorylation of apoptosis signal-regulating kinase 1 (ASK1) and c-Jun N-terminal kinase (JNK) within the 5′ AMP-activated protein kinase (AMPK) axis. Using CDK4/6 knockdown Hep3B cells, palbociclib treatment still resulted in the activation of the AMPK pathway, suggesting that the cytotoxic activity elicited by palbociclib was independent of CDK4/6.

The current study has demonstrated sensitivity of OSCC cell line and xenograft models towards palbociclib. The potential of palbociclib in inhibiting tumorigenesis is further supported by many other studies that has looked into different properties of palbociclib. Furthermore, a recent Phase I clinical trial on OSCC has reported tumor response (assessed by RECIST1.1 imaging) in OSCC patients treated with palbociclib. This includes tumors that were previously resistant to cetuximab or cisplatin (Michel et al.,

2016), strongly suggesting that palbociclib could be a promising therapy for OSCC patients.

5.2 *PIK3CA* mutation as biomarker of response

Understanding the molecular changes that could influence therapeutic response is vital for identifying subgroups that are most likely to benefit from palbociclib treatment. Amongst the sixteen cell lines used in the screening, three cell lines showed resistance to palbociclib. To evaluate if resistance was associated with mutations, the genomic information of the well-characterized cell lines were utilized (Fadlullah et al., 2016; B. Lee et al., 2018; Martin et al., 2014). By comparing different mutational status between sensitive and resistant groups, this study found *PIK3CA* mutations to be significantly associated with resistance of OSCC cell lines to palbociclib. This association was also consistent in the panel of palbociclib-treated pan cancer cell lines that are available in GDSC database albeit not statistically significant (Yang et al., 2013). Using CAL27 isogenic cell lines, we confirmed that mutations in *PIK3CA* indeed confer resistance to palbociclib, where *PIK3CA*-mutated CAL27 showed higher cell proliferation, reduced G_1 cell cycle arrest and higher Rb phosphorylation compared with WT cells upon palbociclib treatment. In the *PIK3CA* mutant line, this resistance was accompanied by an increase in cyclin E and CDK2 expressions.

PIK3CA mutations have been shown to increase expression of cyclins and their dependent kinases. For example in a non-tumorigenic human breast epithelial cell line, *PIK3CA* mutations resulted in the upregulation of cyclin D1, resulting in an increase in cell proliferation (Gustin et al., 2009). Further, upregulation of cyclin D1 and CDK2-dependent Rb phosphorylation by PI3K activation was also reported in MCF7 in response to CDK4/6 inhibition (Herrera-Abreu et al., 2016). The effect of *PIK3CA* mutations was also observed to increase cyclin E expression, where PI3K-dependent upregulation of cyclin E1 levels was associated with acquired resistance to CDK4/6 inhibition in

pancreatic ductal adenocarcinoma (Franco, Witkiewicz, & Knudsen, 2014). PI3K activation can drive the cell cycle through regulating the cell cycle inhibitors including p21 and p27 as reported by others previously (Medema, Kops, Bos, & Burgering, 2000) (Graff et al., 2000) (Zhou et al., 2001). Further, mutant *PIK3CA* could also directly regulate cyclin D levels by inhibiting glycogen synthase kinase-3β (GSK-3β) and resulting in the stabilization and localization of cyclin D1 to the nucleus (Diehl, Cheng, Roussel, & Sherr, 1998; Gustin et al., 2009). In breast cancer patients, the effect of *PIK3CA* mutation in predicting palbociclib's response was studied in the PALOMA 3 trial where *PIK3CA* mutations were detected in the circulating DNA. Results however did not show *PIK3CA* mutations to be associated with palbociclib treatment effect in breast cancer and the hunt for a biomarker for palbociclib response is still on-going.

Based on our observation that activation of PI3K confers resistance to palbociclib, we demonstrated that a combination of a PI3K/mTOR inhibitor with palbociclib resulted in complete tumor growth control in animal model. Whilst PI3K/mTOR inhibition alone was also able to delay tumor growth, it was obvious that blocking PI3K signaling restored sensitivity to palbociclib in OSCC models. Concurring with our *in vitro* observations, complete tumor growth control was observed concomitantly with reduction of cyclin E1 and CDK2. This is consistent with data from other studies where PI3K/mTOR inhibitor synergistically cooperated with CDK4/6 inhibitor in suppressing cell proliferation by reducing critical cell cycle regulatory proteins such as cyclin E1, cyclin D1, CDK2 and cyclin A (Bonelli et al., 2017; Franco, Witkiewicz, & Knudsen, 2014; O'Brien et al., 2014).

5.3 Other biomarker of response to CDK4/6 inhibitors

Previous studies have reported several other biomarkers that may determine the response to CDK4/6 inhibition. The most studied biomarkers are within the CDK4/6-Rb pathway such as p16, cyclin D1 and Rb. One of the earliest preclinical study of palbociclib

has reported sensitivity in the ER-positive subset of breast cancer cell lines which associates with higher levels of cyclin D1 and lower levels of p16 (Finn et al., 2009). Loss of p16 and amplification of cyclin D1 have been characterized as two of the most common alterations in HNSCC with frequencies of 49% and 28% respectively (Cancer-Genome-Atlas-Network, 2015). Notably, all of the cell lines used in this study is known to have inactivation of p16 (Fadlullah et al., 2016; Martin et al., 2014), and 75% of the cell lines have amplification of *CCND1* (B. Lee et al., 2018). Although around 80% of our OSCC cell lines showed sensitivity to palbociclib, neither loss of p16 nor amplification of *CCND1* were found to be associated with resistance. In a phase II study of single-agent palbociclib in 37 breast cancer patients with Rb-positive tumors, both p16 nuclear expression and *CCND1* amplification failed to predict clinical benefit or prolonged progression-free survival (DeMichele et al., 2015). In a larger cohort, this hypothesis was assessed through immunohistochemical staining of p16 and cyclin D1 and resulted in similar findings (Finn et al., 2015).

Loss of Rb function is another biomarker of resistance that has been reported by others. Studies have shown that cancer cell lines with deficient Rb protein eliminated the G_1 cell cycle checkpoint, rendering resistance to CDK4/6 inhibition (Lukas, Aagaard, Strauss, & Bartek, 1995; Lukas, Parry, et al., 1995). No antitumor activity of palbociclib was seen in Rb-deficient cells (Fry et al., 2004) and the same was true in explants derived from human breast tumors (Dean et al., 2012). Similarly, patients with Rb loss did not show to benefit from CDK4/6 inhibition (Bosco & Knudsen, 2007). In HNSCC, loss of the Rb protein is not characterized as a common event, with *RB1* mutational frequency of 4% (Cancer-Genome-Atlas-Network, 2015). In the current study, none of the cell lines used were reported to have any alterations in the *RB1* gene including those in the resistant group. However, it is interesting to note that ORL-115 (palbociclib-resistant cell line) is known to be related with HPV infection which may interfere with Rb function. Further

validation is needed to confirm the role of HPV in conferring palbociclib resistance in this cell line.

Alterations in other genes such as *ESR1, TP53* and *FZR1* and their link with resistance to CDK4/6 inhibitors were previously evaluated. Mutations in the *ESR1* gene was commonly observed to predict breast cancer resistance towards aromatase inhibitors (P. Wang et al., 2016). Hence it was evaluated whether *ESR1* mutations in breast cancer patients would predict resistance to palbociclib. Result from the PALOMA-3 trial has shown that *ESR1* mutation was not associated with palbociclib treatment effect (Fribbens et al., 2016). In a phase I study, *TP53* mutations were more commonly found in non-responding tumors to abemaciclib. Given the role of p53 in cellular growth and senescence, there is a strong biological rationale behind this observation. However, validation in a larger patient cohort is required. Finally, downregulation of FZR1 which plays a role in regulating late mitosis and G1/S phase were found to have synergistic effect with the loss of Rb in mediating resistance to CDK4/6 inhibitors. The study suggested that not only Rb status but also FZR1 levels and functional activity were important biomarkers that might determine how cancer cells will respond to CDK4/6 inhibitors. This however awaits further confirmation in randomized patient datasets

5.4 Study limitations and recommendation of future studies

Several limitations that may influence the results of the study have been identified. Based on these limitations, recommendation of improvement and further studies are discussed below:

5.4.1 Caveats of preclinical cancer cell line model

Cancer cell lines and xenografts are vital preclinical models that are vastly used in cancer research prior to human studies. The limitation of using preclinical cancer models have been widely reviewed and recognized. This includes the inadequacy of tumor cell

characterization to fully recapitulate human disease, incapacity to mimic the actual human tumor microenvironment, poor appreciation of pharmacokinetics and pharmacodynamics, genetic drifts after many passages, and mouse-specific tumor evolution (Begley & Ellis, 2012; Ben-David et al., 2017). Although associated with limitations, preclinical cancer models are indispensable as tools to discover and investigate cancer drugs and their biology. As an improvement to cell line cultures, a newer strategy in culturing patient tissues such as the three dimensional 'cancer organoids' has shown potential to more accurately model the human tumor. Cancer organoids contain multiple cell types that organize similarly to the primary tissue and function specifically to the parent organ (Xu et al., 2018). The tumoroid establishment and culture system of organoids depends on the type of cancer. To date, cancer organoids have been established in diverse cancer types such as gastrointestinal cancer, liver cancer, pancreatic cancer, breast cancer, bladder cancer and prostate cancer (Boj et al., 2015; Broutier et al., 2017; S. H. Lee et al., 2018; Puca et al., 2018; Sachs et al., 2018; Vlachogiannis et al., 2018). This technology can be applied in the future as a way to improve the current preclinical model of OSCC.

5.4.2 Sample size and representation

The number of OSCC cell lines used in this study is limited and may not recapitulate the heterogeneity of OSCC patients. By increasing the number of cell lines in the screening, this may result in the identification of additional biomarkers of response. Results from this study have shown that majority of OSCC cell lines responded sensitively to palbociclib and this may be due to the activation of the cell cycle pathway in OSCC. This study can be expanded to other types of cancers that are also characterized to have increased cell cycle activity. One such example is bladder cancer where amplification of cyclin D1 and loss of p16 are common molecular events with alteration frequency of 10.6% and 22.5% respectively (Zehir et al., 2017). Screening of palbociclib

towards different types of cancers that are reliant on CDK4/6 activation would also enable the identification of more biomarkers of response.

5.4.3 Validation of biomarker of response

In this study, the effect of *PIK3CA* H1047R mutation towards palbociclib's response was only demonstrated in a single model of isogenic cell line. Validation on more models of isogenic *PIK3CA* H1047R cell line could further strengthen the study. Furthermore, the effect of this biomarker towards palbociclib response using the isogenic *PIK3CA* H1047R model was not validated in the in vivo system, where a more accurate drug response should be achieved in view that the animal model would incorporate biological factors such as pharmacokinetics, pharmacodynamics and immune systems. The *in vivo* validation involves inoculating CAL27 and CAL27-mutant cell lines subcutaneously in mice, and observe tumor responses between the two groups of mice treated with palbociclib.

While this study only validates the differential response of palbociclib using an isogenic *PIK3CA* H1047R model, different mutational hotspot sites of *PIK3CA* such as E545K and E542K should also be further investigated. This will increase the understanding of whether different mutations of the *PIK3CA* gene will confer different response to palbociclib.

5.5 Conclusion

In summary, we demonstrate that palbociclib is effective in controlling tumor growth in ~80% of OSCC cell lines tested. Our data also showed that *PIK3CA* mutational status may represent a biomarker for response to palbociclib and across other clinically approved CDK4/6 inhibitors. Finally, we demonstrated that simultaneous inhibition of PI3K/mTOR and CDK4/6 is effective in controlling tumor growth in resistant OSCC. Given that clinical studies on CDK4/6 inhibitors in OSCC have been initiated, our study provides a rationale for testing biomarkers of response and suggests possible combinatorial approaches for patients who may not respond to CDK4/6 inhibitors as single agents.

REFERENCES

Asghar, U., Witkiewicz, A. K., Turner, N. C., & Knudsen, E. S. (2015). The history and future of targeting cyclin-dependent kinases in cancer therapy. *Nature reviews Drug discovery, 14*, 130-146.

Azizah, A. M., Nor Saleha, I. T., Noor Hashimah, A., Asmah, Z. A., & Mastulu, W. (2016). Malaysian National Cancer Registry Report 2007–2011. *Malaysia cancer statistics, data and figure. Putrajaya: National Cancer Institute, Ministry of Health*.

Baldin, V., Lukas, J., Marcote, M. J., Pagano, M., & Draetta, G. (1993). Cyclin D1 is a nuclear protein required for cell cycle progression in G1. *Genes & development, 7*, 812-821.

Barnum, K. J., & O'Connell, M. J. (2014). Cell cycle regulation by checkpoints. In *Cell Cycle Control* (pp. 29-40). Humana Press, New York, NY.

Bauml, J., Seiwert, T. Y., Pfister, D. G., Worden, F., Liu, S. V., Gilbert, J., ... & Kang, H. (2017). Pembrolizumab for platinum-and cetuximab-refractory head and neck cancer: results from a single-arm, phase II study. *Journal of clinical oncology: official journal of the American Society of Clinical Oncology, 35*(14), 1542-1549.

Begley, C. G., & Ellis, L. M., (2012). Drug development: Raise standards for preclinical cancer research. *Nature, 483*(7391), 531.

Ben-David, U., Ha, G., Tseng, Y. Y., Greenwald, N. F., Oh, C., Shih, J., ... & Golub, T. R. (2017). Patient-derived xenografts undergo mouse-specific tumor evolution. *Nature genetics, 49*(11), 1567.

Bernier, J., Domenge, C., Ozsahin, M., Matuszewska, K., Lefèbvre, J. L., Greiner, R. H., ... & Cognetti, F. (2004). Postoperative irradiation with or without concomitant chemotherapy for locally advanced head and neck cancer. New England Journal of Medicine, 350(19), 1945-1952.

Bible, K. C., Peethambaram, P. P., Oberg, A. L., Maples, W., Groteluschen, D. L., Boente, M., ... & Maguire, J. L. (2012). A phase 2 trial of flavopiridol (Alvocidib) and cisplatin in platin-resistant ovarian and primary peritoneal carcinoma: MC0261. *Gynecologic oncology, 127*(1), 55-62.

Blot, W. J., McLaughlin, J. K., Winn, D. M., Austin, D. F., Greenberg, R. S., Preston-Martin, S., ... & Fraumeni, J. F. (1988). Smoking and drinking in relation to oral and pharyngeal cancer. *Cancer research, 48*(11), 3282-3287.

Boj, S. F., Hwang, C. I., Baker, L. A., Chio, I. I. C., Engle, D. D., Corbo, V., ... & Gracanin, A. (2015). Organoid models of human and mouse ductal pancreatic cancer. *Cell, 160*(1-2), 324-338.

Bollard, J., Miguela, V., de Galarreta, M. R., Venkatesh, A., Bian, C. B., Roberto, M. P., ... & Nakagawa, S. (2017). Palbociclib (PD-0332991), a selective CDK4/6

inhibitor, restricts tumour growth in preclinical models of hepatocellular carcinoma. *Gut*, 66(7), 1286-1296.

Bonelli, M. A., Digiacomo, G., Fumarola, C., Alfieri, R., Quaini, F., Falco, A., ... & Ulivi, P. (2017). Combined inhibition of CDK4/6 and PI3K/AKT/mTOR pathways induces a synergistic anti-tumor effect in malignant pleural mesothelioma cells. *Neoplasia, 19*(8), 637-648.

Bosco, E. E., & Knudsen, E. S., (2007). RB in breast cancer: the crossroads of tumorigenesis and treatment. *Cell cycle, 6*(6), 667-671.

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R. L., Torre, L. A., & Jemal, A. (2018). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: a cancer journal for clinicians, 68*(6), 394-424.

Broutier, L., Mastrogiovanni, G., Verstegen, M. M., Francies, H. E., Gavarró, L. M., Bradshaw, C. R., ... & Georgakopoulos, N. (2017). Human primary liver cancer–derived organoid cultures for disease modeling and drug screening. *Nature medicine, 23*(12), 1424.

Byrd, J. C., Peterson, B. L., Gabrilove, J., Odenike, O. M., Grever, M. R., Rai, K., ... & Cancer and Leukemia Group B. (2005). Treatment of relapsed chronic lymphocytic leukemia by 72-hour continuous infusion or 1-hour bolus infusion of flavopiridol: results from Cancer and Leukemia Group B study 19805. *Clinical Cancer Research, 11*(11), 4176-4181.

Cancer-Genome-Atlas-Network. (2015). Comprehensive genomic characterization of head and neck squamous cell carcinomas. *Nature, 517*(7536), 576.

Carvajal, R. D., Tse, A., Shah, M. A., Lefkowitz, R. A., Gonen, M., Gilman-Rosen, L., ... & O'Reilly, E. M. (2009). A phase II study of flavopiridol (Alvocidib) in combination with docetaxel in refractory, metastatic pancreatic cancer. *Pancreatology, 9*(4), 404-409.

Chapman, P. B., Hauschild, A., Robert, C., Haanen, J. B., Ascierto, P., Larkin, J., ... & Hogg, D. (2011). Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *New England Journal of Medicine*, 364(26), 2507-2516.

Chow, L. Q., Haddad, R., Gupta, S., Mahipal, A., Mehra, R., Tahara, M., ... & Keam, B. (2016). Antitumor activity of pembrolizumab in biomarker-unselected patients with recurrent and/or metastatic head and neck squamous cell carcinoma: results from the phase Ib KEYNOTE-012 expansion cohort. *Journal of Clinical Oncology, 34*(32), 3838-3845.

Cristofanilli, M., Turner, N. C., Bondarenko, I., Ro, J., Im, S. A., Masuda, N., ... & Iwata, H. (2016). Fulvestrant plus palbociclib versus fulvestrant plus placebo for treatment of hormone-receptor-positive, HER2-negative metastatic breast cancer that progressed on previous endocrine therapy (PALOMA-3): final analysis of the multicentre, double-blind, phase 3 randomised controlled trial. *The Lancet Oncology, 17*(4), 425-439.

Danaei, G., Vander Hoorn, S., Lopez, A. D., Murray, C. J., Ezzati, M., & Comparative Risk Assessment collaborating group (Cancers. (2005). Causes of cancer in the world: comparative risk assessment of nine behavioural and environmental risk factors. *The Lancet, 366*(9499), 1784-1793.

Dean, J. L., McClendon, A. K., Hickey, T. E., Butler, L. M., Tilley, W. D., Witkiewicz, A. K., & Knudsen, E. S. (2012). Therapeutic response to CDK4/6 inhibition in breast cancer defined by ex vivo analyses of human tumors. *Cell Cycle, 11*(14), 2756-2761.

DeMichele, A., Clark, A. S., Tan, K. S., Heitjan, D. F., Gramlich, K., Gallagher, M., ... O'Dwyer, P. (2015). CDK 4/6 inhibitor palbociclib (PD0332991) in Rb+ advanced breast cancer: phase II activity, safety, and predictive biomarker assessment. *Clin Cancer Res, 21*(5), 995-1001.

Dhingra, V., Verma, J., Misra, V., Srivastav, S., & Hasan, F. (2017). Evaluation of cyclin D1 expression in head and neck squamous cell carcinoma. *Journal of Clinical and Diagnostic Research: JCDR, 11*(2), EC01.

Diehl, J. A., Cheng, M., Roussel, M. F., & Sherr, C. J. (1998). Glycogen synthase kinase-3β regulates cyclin D1 proteolysis and subcellular localization. *Genes & development, 12*(22), 3499-3511.

Dörr, W., Hamilton, C. S., Boyd, T., Reed, B., & Denham, J. W. (2002). Radiation-induced changes in cellularity and proliferation in human oral mucosa. *International Journal of Radiation Oncology* Biology* Physics, 52*(4), 911-917.

Edwards, D. M., & Jones, J. (1999). Incidence of and survival from upper aerodigestive tract cancers in the UK: the influence of deprivation. *European Journal of Cancer, 35*(6), 968-972.

Fadlullah, M. Z. H., Chiang, I. K. N., Dionne, K. R., Yee, P. S., Gan, C. P., Sam, K. K., ... Cheong, S. C., (2016). Genetically-defined novel oral squamous cell carcinoma cell lines for the development of molecular therapies. *Oncotarget, 7*(19), 27802.

Feng, F. Y., Kim, H. M., Lyden, T. H., Haxer, M. J., Worden, F. P., Feng, M., ... & Bradford, C. R. (2010). Intensity-modulated chemoradiotherapy aiming to reduce dysphagia in patients with oropharyngeal cancer: clinical and functional results. *Journal of Clinical Oncology, 28*(16), 2732.

Ferris, R. L., Blumenschein Jr, G., Fayette, J., Guigay, J., Colevas, A. D., Licitra, L., ... & Worden, F. (2016). Nivolumab for recurrent squamous-cell carcinoma of the head and neck. *New England Journal of Medicine, 375*(19), 1856-1867.

Finn, R. S., Dering, J., Conklin, D., Kalous, O., Cohen, D. J., Desai, A. J., ... & Los, G. (2009). PD 0332991, a selective cyclin D kinase 4/6 inhibitor, preferentially inhibits proliferation of luminal estrogen receptor-positive human breast cancer cell lines in vitro. *Breast Cancer Research, 11*(5), R77.

Finn, R. S., Crown, J. P., Lang, I., Boer, K., Bondarenko, I. M., Kulyk, S. O., ... & Shparyk, Y. (2015). The cyclin-dependent kinase 4/6 inhibitor palbociclib in combination with letrozole versus letrozole alone as first-line treatment of

oestrogen receptor-positive, HER2-negative, advanced breast cancer (PALOMA-1/TRIO-18): a randomised phase 2 study. *The lancet oncology, 16*(1), 25-35.

Finn, R. S., Martin, M., Rugo, H. S., Jones, S. E., Im, S. A., Gelmon, K. A., ... & Gauthier, E. R. (2016). PALOMA-2: Primary results from a phase III trial of palbociclib (P) with letrozole (L) compared with letrozole alone in postmenopausal women with ER+/HER2–advanced breast cancer (ABC). *Journal of Clinical Oncology.*

Franco, J., Witkiewicz, A. K., & Knudsen, E. S. (2014). CDK4/6 inhibitors have potent activity in combination with pathway selective therapeutic agents in models of pancreatic cancer. *Oncotarget, 5*(15), 6512.

Fribbens, C., O'Leary, B., Kilburn, L., Hrebien, S., Garcia-Murillas, I., Beaney, M., ... & Jiang, J. (2016). Plasma ESR1 mutations and the treatment of estrogen receptor-positive advanced breast cancer.

Fry, D. W., Harvey, P. J., Keller, P. R., Elliott, W. L., Meade, M., Trachet, E., ... & Toogood, P. L. (2004). Specific inhibition of cyclin-dependent kinase 4/6 by PD 0332991 and associated antitumor activity in human tumor xenografts. *Molecular cancer therapeutics, 3*(11), 1427-1438.

Garrido-Castro, A. C., & Goel, S. (2017). CDK4/6 inhibition in breast cancer: mechanisms of response and treatment failure. *Current breast cancer reports, 9*(1), 26-33.

Ghosh–Laskar, S., Yathiraj, P. H., Dutta, D., Rangarajan, V., Purandare, N., Gupta, T., ... & Agarwal, J. P. (2016). Prospective randomized controlled trial to compare 3-dimensional conformal radiotherapy to intensity-modulated radiotherapy in head and neck squamous cell carcinoma: Long-term results. *Head & neck, 38*(S1), E1481-E1487.

Gopalan, P. K., Pinder, M. C., Chiappori, A., Ivey, A. M., Gordillo Villegas, A., & Kaye, F. J. (2014). A phase II clinical trial of the CDK 4/6 inhibitor palbociclib (PD 0332991) in previously treated, advanced non-small cell lung cancer (NSCLC) patients with inactivated CDKN2A. *Journal of Clinical Oncology.*

Graff, J. R., Konicek, B. W., McNulty, A. M., Wang, Z., Houck, K., Allen, S., ... & Vessella, R. L. (2000). Increased AKT activity contributes to prostate cancer progression by dramatically accelerating prostate tumor growth and diminishing p27Kip1 expression. *Journal of Biological Chemistry, 275*(32), 24500-24505.

Guarducci, C., Bonechi, M., Boccalini, G., Benelli, M., Risi, E., Di Leo, A., ... Migliaccio, I. (2017). Mechanisms of Resistance to CDK4/6 Inhibitors in Breast Cancer and Potential Biomarkers of Response. *Breast Care (Basel), 12*(5), 304-308.

Gupta, P. C., Murti, P. R., Bhonsle, R. B., Mehta, F. S., & Pindborg, J. J. (1995). Effect of cessation of tobacco use on the incidence of oral mucosal lesions in a 10–yr follow-up study of 12 212 users. *Oral diseases, 1*(1), 54-58.

Gupta, T., Agarwal, J., Jain, S., Phurailatpam, R., Kannan, S., Ghosh-Laskar, S., ... & Chaturvedi, P. (2012). Three-dimensional conformal radiotherapy (3D-CRT) versus intensity modulated radiation therapy (IMRT) in squamous cell carcinoma

of the head and neck: a randomized controlled trial. *Radiotherapy and Oncology, 104*(3), 343-348.

Gustin, J. P., Karakas, B., Weiss, M. B., Abukhdeir, A. M., Lauring, J., Garay, J. P., ... & Mohseni, M. (2009). Knockin of mutant PIK3CA activates multiple oncogenic pathways. *Proceedings of the National Academy of Sciences, 106*(8), 2835-2840.

Hamid, S., Lim, K. P., Zain, R. B., Ismail, S. M., Lau, S. H., Mustafa, W. M. W., ... & Cheong, S. C. (2007). Establishment and characterization of Asian oral cancer cell lines as in vitro models to study a disease prevalent in Asia. *International journal of molecular medicine, 19*(3), 453-460.

Helsten, T., Kato, S., Schwaederle, M., Tomson, B. N., Buys, T. P., Elkin, S. K., ... & Kurzrock, R. (2016). Cell-cycle gene alterations in 4,864 tumors analyzed by next-generation sequencing: Implications for targeted therapeutics. *Molecular cancer therapeutics, 15*(7), 1682-1690.

Herrera-Abreu, M. T., Palafox, M., Asghar, U., Rivas, M. A., Cutts, R. J., Garcia-Murillas, I., ... & Bellet, M. (2016). Early adaptation and acquired resistance to CDK4/6 inhibition in estrogen receptor–positive breast cancer. *Cancer research, 76*(8), 2301-2313.

Hsieh, F. S., Chen, Y. L., Hung, M. H., Chu, P. Y., Tsai, M. H., Chen, L. J., ... & Shiau, C. W. (2017). Palbociclib induces activation of AMPK and inhibits hepatocellular carcinoma in a CDK4/6-independent manner. *Molecular oncology, 11*(8), 1035-1049.

Huang, S. H. (2013). Oral cancer: Current role of radiotherapy and chemotherapy. *Medicina oral, patologia oral y cirugia bucal, 18*(2), e233.

Kadia, T. M., Konopleva, M. Y., Garcia-Manero, G., Benton, C. B., Wierda, W. G., Bose, P., ... & Pemmaraju, N. (2018). Phase I Study of Palbociclib Alone and in Combination in Patients with Relapsed and Refractory (R/R) Leukemias.

Kalra, S., Joshi, G., Munshi, A., & Kumar, R. (2017). Structural insights of cyclin dependent kinases: Implications in design of selective inhibitors. *European journal of medicinal chemistry, 142*, 424-458.

Kerawala, C. J. (2010). Complications of head and neck cancer surgery–Prevention and management. *Oral oncology, 46*(6), 433-435.

Kitagawa, M., Higashi, H., Jung, H. K., Suzuki-Takahashi, I., Ikeda, M., Tamai, K., ... & Taya, Y. (1996). The consensus motif for phosphorylation by cyclin D1-Cdk4 is different from that for phosphorylation by cyclin A/E-Cdk2. *The EMBO journal, 15*(24), 7060-7069.

Klein, M. E., Kovatcheva, M., Davis, L. E., Tap, W. D., & Koff, A. (2018). CDK4/6 inhibitors: the mechanism of action may not be as simple as once thought. *Cancer Cell.*

Kolokythas, A. (2010). Long-term surgical complications in the oral cancer patient: a comprehensive review. Part I. *Journal of oral & maxillofacial research, 1*(3).

Kumar, M., Nanavati, R., Modi, T. G., & Dobariya, C. (2016). Oral cancer: Etiology and risk factors: A review. *Journal of cancer research and therapeutics, 12*(2), 458.

Le Tourneau, C., Faivre, S., Laurence, V., Delbaldo, C., Vera, K., Girre, V., ... & Gianella-Borradori, A. (2010). Phase I evaluation of seliciclib (R-roscovitine), a novel oral cyclin-dependent kinase inhibitor, in patients with advanced malignancies. *European journal of cancer, 46*(18), 3243-3250.

Lee, B. K. B., Gan, C. P., Chang, J. K., Tan, J. L., Fadlullah, M. Z., Abdul Rahman, Z. A., ... & Tan, A. C. (2018). GENIPAC: A Genomic Information Portal for Head and Neck Cancer Cell Systems. *Journal of dental research, 97*(8), 909-916.

Lee, S. H., Hu, W., Matulay, J. T., Silva, M. V., Owczarek, T. B., Kim, K., ... Shen, M. M. (2018). Tumor Evolution and Drug Response in Patient-Derived Organoid Models of Bladder Cancer. *Cell, 173*(2), 515-528.

Leemans, C. R., Snijders, P. J., & Brakenhoff, R. H. (2018). The molecular landscape of head and neck cancer. *Nature Reviews Cancer.*

Li, C., Qi, L., Bellail, A. C., Hao, C., & Liu, T. (2014). PD‐0332991 induces G1 arrest of colorectal carcinoma cells through inhibition of the cyclin‐dependent kinase‐6 and retinoblastoma protein axis. *Oncology letters, 7*(5), 1673-1678.

Liggett Jr, W. H., & Sidransky, D. (1998). Role of the p16 tumor suppressor gene in cancer. *Journal of clinical oncology, 16*(3), 1197-1206.

Lin, T. S., Ruppert, A. S., Johnson, A. J., Fischer, B., Heerema, N. A., Andritsos, L. A., ... & Moran, M. E. (2009). Phase II study of flavopiridol in relapsed chronic lymphocytic leukemia demonstrating high response rates in genetically high-risk disease. *Journal of Clinical Oncology, 27*(35), 6012.

Looser, K. G., Shah, J. P., & Strong, E. W. (1978). The significance of "positive" margins in surgically resected epidermoid carcinomas. *Head & neck surgery, 1*(2), 107-111.

Lukas, J., Aagaard, L., Strauss, M., & Bartek, J. (1995). Oncogenic aberrations of p16INK4/CDKN2 and cyclin D1 cooperate to deregulate G1 control. *Cancer research, 55*(21), 4818-4823.

Lukas, J., Parry, D., Aagaard, L., Mann, D. J., Bartkova, J., Strauss, M., ... & Bartek, J. (1995). Retinoblastoma-protein-dependent cell-cycle inhibition by the tumour suppressor p16. *Nature, 375*(6531), 503.

Lynggaard, C. D., Therkildsen, M. H., Kristensen, C. A., & Specht, L. (2015). The EXTREME regimen for recurrent/metastatic head and neck squamous cell carcinoma (R/M HNSCC): treatment outcome in a single institution cohort. *Acta Oncologica, 54(7), 1071-1075.*

Machado, J., Reis, P. P., Zhang, T., Simpson, C., Xu, W., Perez-Ordonez, B., ... & Irish, J. C. (2010). Low prevalence of human papillomavirus in oral cavity carcinomas. *Head & neck oncology, 2*(1), 6.

Martin, D., Abba, M. C., Molinolo, A. A., Vitale-Cross, L., Wang, Z., Zaida, M., ... & Gutkind, J. S. (2014). The head and neck cancer cell oncogenome: a platform for the development of precision molecular therapies. *Oncotarget*, *5*(19), 8906.

Medema, R. H., Kops, G. J., Bos, J. L., & Burgering, B. M. (2000). AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27 kip1. *Nature*, *404*(6779), 782.

Michel, L., Ley, J., Wildes, T. M., Schaffer, A., Robinson, A., Chun, S. E., ... & Adkins, D. (2016). Phase I trial of palbociclib, a selective cyclin dependent kinase 4/6 inhibitor, in combination with cetuximab in patients with recurrent/metastatic head and neck squamous cell carcinoma. *Oral oncology*, *58*, 41-48.

Mita, M. M., Joy, A. A., Mita, A., Sankhala, K., Jou, Y. M., Zhang, D., ... Shapiro, C. L. (2014). Randomized phase II trial of the cyclin-dependent kinase inhibitor dinaciclib (MK-7965) versus capecitabine in patients with advanced breast cancer. *Clin Breast Cancer*, *14*(3), 169-176.

Mitchell, M. J., & Logan, P. M. (1998). Radiation-induced changes in bone. *Radiographics*, *18*(5), 1125-1136.

Möller, P., Perrier, M., Ozsahin, M., & Monnier, P. (2004). A prospective study of salivary gland function in patients undergoing radiotherapy for squamous cell carcinoma of the oropharynx. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology*, *97*(2), 173-189.

Nemunaitis, J. J., Small, K. A., Kirschmeier, P., Zhang, D., Zhu, Y., Jou, Y. M., ... & Bannerji, R. (2013). A first-in-human, phase 1, dose-escalation study of dinaciclib, a novel cyclin-dependent kinase inhibitor, administered weekly in subjects with advanced malignancies. *Journal of translational medicine*, *11*(1), 259.

Niesvizky, R., Badros, A. Z., Costa, L. J., Ely, S. A., Singhal, S. B., Stadtmauer, E. A., ... & Spicka, I. (2015). Phase 1/2 study of cyclin-dependent kinase (CDK) 4/6 inhibitor palbociclib (PD-0332991) with bortezomib and dexamethasone in relapsed/refractory multiple myeloma. *Leukemia & lymphoma*, *56*(12), 3320-3328.

Nutting, C. M., Morden, J. P., Harrington, K. J., Urbano, T. G., Bhide, S. A., Clark, C., ... & Adab, F. (2011). Parotid-sparing intensity modulated versus conventional radiotherapy in head and neck cancer (PARSPORT): a phase 3 multicentre randomised controlled trial. *The lancet oncology*, *12*(2), 127-136.

O'Brien, N. A., Tomaso, E. D., Ayala, R., Tong, L., Issakhanian, S., Linnartz, R., ... & Slamon, D. J. (2014). In vivo efficacy of combined targeting of CDK4/6, ER and PI3K signaling in ER+ breast cancer.

O'Shaughnessy, J., Petrakova, K., Sonke, G. S., Conte, P., Arteaga, C. L., Cameron, D. A., ... & Lindquist, D. (2018). Ribociclib plus letrozole versus letrozole alone in patients with de novo HR+, HER2− advanced breast cancer in the randomized MONALEESA-2 trial. *Breast cancer research and treatment*, *168*(1), 127-134.

Otmani, N. (2007). Oral and maxillofacial side effects of radiation therapy on children. *Journal of the Canadian Dental Association*, *73*(3).

Paulino, A. C., Simon, J. H., Zhen, W., & Wen, B. C. (2000). Long-term effects in children treated with radiotherapy for head and neck rhabdomyosarcoma. *International Journal of Radiation Oncology* Biology* Physics*, *48*(5), 1489-1495.

Puca, L., Bareja, R., Prandi, D., Shaw, R., Benelli, M., Karthaus, W. R., ... Beltran, H. (2018). Patient derived organoids to model rare prostate cancer phenotypes. *Nature Communication*, *9*(1), 2404.

Qin, G., Xu, F., Qin, T., Zheng, Q., Shi, D., Xia, W., ... & Deng, W. (2015). Palbociclib inhibits epithelial-mesenchymal transition and metastasis in breast cancer via c-Jun/COX-2 signaling pathway. *Oncotarget*, *6*(39), 41794.

Quelle, D. E., Ashmun, R. A., Shurtleff, S. A., Kato, J. Y., Bar-Sagi, D. M. F. R., Roussel, M. F., & Sherr, C. J. (1993). Overexpression of mouse D-type cyclins accelerates G1 phase in rodent fibroblasts. *Genes & development*, *7*(8), 1559-1571.

Ram, H., Sarkar, J., Kumar, H., Konwar, R., Bhatt, M. L. B., & Mohammad, S. (2011). Oral cancer: risk factors and molecular pathogenesis. *Journal of maxillofacial and oral surgery*, *10*(2), 132.

Rampias, T., Giagini, A., Siolos, S., Matsuzaki, H., Sasaki, C., Scorilas, A., & Psyrri, A. (2014). RAS/PI3K crosstalk and cetuximab resistance in head and neck squamous cell carcinoma. *Clinical Cancer Research*, *20*(11), 2933-2946.

Razfar, A., Mundi, J., Grogan, T., Lee, S., Elashoff, D., Abemayor, E., & John, M. S. (2016). IMRT for head and neck cancer: Cost implications. *American journal of otolaryngology*, *37*(6), 479-483.

Rogers, S. N., Vedpathak, S. V., & Lowe, D. (2011). Reasons for delayed presentation in oral and oropharyngeal cancer: the patients perspective. *British Journal of Oral and Maxillofacial Surgery*, *49*(5), 349-353.

Sachs, N., de Ligt, J., Kopper, O., Gogola, E., Bounova, G., Weeber, F., ... & Korving, J. (2018). A living biobank of breast cancer organoids captures disease heterogeneity. *Cell*, *172*(1-2), 373-386.

Schachter, J., Ribas, A., Long, G. V., Arance, A., Grob, J. J., Mortier, L., ... & Larkin, J. (2017). Pembrolizumab versus ipilimumab for advanced melanoma: final overall survival results of a multicentre, randomised, open-label phase 3 study (KEYNOTE-006). *The Lancet*, *390*(10105), 1853-1862.

Schmitz, N. M. R., Hirt, A., Aebi, M., & Leibundgut, K. (2006). Limited redundancy in phosphorylation of retinoblastoma tumor suppressor protein by cyclin-dependent kinases in acute lymphoblastic leukemia. *The American journal of pathology*, *169*(3), 1074-1079.

Seiwert, T. Y., Burtness, B., Mehra, R., Weiss, J., Berger, R., Eder, J. P., ... & Cheng, J. D. (2016). Safety and clinical activity of pembrolizumab for treatment of recurrent

or metastatic squamous cell carcinoma of the head and neck (KEYNOTE-012): an open-label, multicentre, phase 1b trial. *The lancet oncology, 17*(7), 956-965.

Sledge Jr, G. W., Toi, M., Neven, P., Sohn, J., Inoue, K., Pivot, X., ... & Koh, H. (2017). MONARCH 2: abemaciclib in combination with fulvestrant in women with HR+/HER2− advanced breast cancer who had progressed while receiving endocrine therapy. *Journal of clinical oncology*, 35(25), 2875-2884.

Stephen, J. K., Divine, G., Chen, K. M., Chitale, D., Havard, S., & Worsham, M. J. (2013). Significance of p16 in site-specific HPV positive and HPV negative head and neck squamous cell carcinoma. *Cancer and clinical oncology*, 2(1), 51.

Stephenson, J. J., Nemunaitis, J., Joy, A. A., Martin, J. C., Jou, Y. M., Zhang, D., ... Edelman, M. J. (2014). Randomized phase 2 study of the cyclin-dependent kinase inhibitor dinaciclib (MK-7965) versus erlotinib in patients with non-small cell lung cancer. *Lung Cancer, 83*(2), 219-223.

Tiong, K. H., Chang, J. K., Pathmanathan, D., Hidayatullah F. M. Z., Yee, P. S., Liew, C. S., ... Cheong, S. C. (2018). QuickCount®: a novel automated software for rapid cell detection and quantification. *BioTechniques, 65*(6), 322-330.

Tolentino, E. D. S., Centurion, B. S., Ferreira, L. H. C., Souza, A. P. D., Damante, J. H., & Rubira-Bullen, I. R. F. (2011). Oral adverse effects of head and neck radiotherapy: literature review and suggestion of a clinical oral care guideline for irradiated patients. *Journal of Applied Oral Science, 19*(5), 448-454.

Varshney, H. (2003). Biochemical and immunological studies on DNA modified with nicotine (Doctoral dissertation, Aligarh Muslim University).

Vermorken, J. B., Mesia, R., Rivera, F., Remenar, E., Kawecki, A., Rottey, S., ... & Peyrade, F. (2008). Platinum-based chemotherapy plus cetuximab in head and neck cancer. *New England Journal of Medicine*, *359*(11), 1116-1127.

Vlachogiannis, G., Hedayat, S., Vatsiou, A., Jamin, Y., Fernández-Mateos, J., Khan, K., ... & Rata, M. (2018). Patient-derived organoids model treatment response of metastatic gastrointestinal cancers. *Science*, *359*(6378), 920-926.

Wang, B., Wang, T., Cao, X. L., & Li, Y. (2015). Critical genes in head and neck squamous cell carcinoma revealed by bioinformatic analysis of gene expression data. *Genet Mol Res*, *14*(4), 17406-17415.

Wang, P., Bahreini, A., Gyanchandani, R., Lucas, P. C., Hartmaier, R. J., Watters, R. J., ... & Kurland, B. F. (2016). Sensitive detection of mono-and polyclonal ESR1 mutations in primary tumors, metastatic lesions, and cell-free DNA of breast cancer patients. *Clinical cancer research*, *22*(5), 1130-1137.

Wang, Z., Martin, D., Molinolo, A. A., Patel, V., Iglesias-Bartolome, R., Sol Degese, M., ... & Gutkind, J. S. (2014). mTOR co-targeting in cetuximab resistance in head and neck cancers harboring PIK3CA and RAS mutations. *JNCI: Journal of the National Cancer Institute*, *106*(9).

Warnakulasuriya, S. (2009). Global epidemiology of oral and oropharyngeal cancer. *Oral oncology*, *45*(4), 309-316.

Witkiewicz, A. K., Knudsen, K. E., Dicker, A. P., & Knudsen, E. S. (2011). The meaning of p16ink4a expression in tumors: functional significance, clinical associations and future developments. *Cell cycle*, *10*(15), 2497-2503.

Xu, H., Lyu, X., Yi, M., Zhao, W., Song, Y., & Wu, K. (2018). Organoid technology and applications in cancer research. *Journal of hematology & oncology*, *11*(1), 116.

Yang, W., Soares, J., Greninger, P., Edelman, E. J., Lightfoot, H., Forbes, S., ... & Ramaswamy, S. (2012). Genomics of Drug Sensitivity in Cancer (GDSC): a resource for therapeutic biomarker discovery in cancer cells. *Nucleic acids research*, *41*(D1), D955-D961.

Zaretsky, J. M., Garcia-Diaz, A., Shin, D. S., Escuin-Ordinas, H., Hugo, W., Hu-Lieskovan, S., ... & Saco, J. (2016). Mutations associated with acquired resistance to PD-1 blockade in melanoma. *New England Journal of Medicine*, *375*(9), 819-829.

Zarkowska, T., & Mittnacht, S. (1997). Differential phosphorylation of the retinoblastoma protein by G1/S cyclin-dependent kinases. *Journal of Biological Chemistry*, *272*(19), 12738-12746.

Zehir, A., Benayed, R., Shah, R. H., Syed, A., Middha, S., Kim, H. R., ... & Hellmann, M. D. (2017). Mutational landscape of metastatic cancer revealed from prospective clinical sequencing of 10,000 patients. *Nature medicine*, *23*(6), 703.

Zhou, B. P., Liao, Y., Xia, W., Spohn, B., Lee, M. H., & Hung, M. C. (2001). Cytoplasmic localization of p21 Cip1/WAF1 by Akt-induced phosphorylation in HER-2/neu-overexpressing cells. *Nature cell biology*, *3*(3), 245.