OVEREXPRESSION OF CYCLIN D1 IN ORAL CANCER INCREASES TPF CHEMOTHERAPEUTIC DRUGS VIA ACTIVATING THE CASPASE-3 PATHWAY

GEORGE A[*1], SIDRAH[2], ALI A[3], ZAMAN M[4], AKHTAR R[4], MEMON Y[5], AAMIR K[4], RAMZAN A[4]

[1]Department of Pathology, Dow Ojha Campus, Karachi, Pakistan
[2]Department of Pathology, Bilawal Medical College For Boys, Liaquat University of Medical and Health Sciences Jamshoro, Hyderabad, Pakistan
[3]Department of Pathology, Jhalwan Medical College Khuzdar, Balochistan, Pakistan
[4]Department of Pathology, Liaquat University of Medical and Health Sciences Jamshoro, Hyderabad, Pakistan
[5]Department of Dental Surgery, Liaquat University of Medical and Health Sciences Jamshoro, Hyderabad/ Dental Surgeon Bhittai Hospital Latifabad, Hyderabad. Pakistan
Corresponding author email address: dranitageoarge842@gmail.com

Abstract: *Previous studies have indicated that induction chemotherapy can limit the possibility of early lesion eradication and lessen the severity of globally advanced tumors. This study aimed to ascertain if cyclin D1 expression in OSCC patients who were qualified for TPF induction chemotherapy might be regarded as a valid prognostic indicator for long-term outcomes. This study aimed to determine whether or not there was a connection between OSCC cell intervention and the production of cyclin D1 and chemosensitivity to TPF medicines through the apoptotic mechanism. The current work used immunohistochemistry to determine the expression of cyclin D1 in various tissues. To ascertain the survival rate of 232 people diagnosed with locally advanced oral squamous cell carcinoma (OSCC), the study utilized the log-rank test and Kaplan-Meier analysis. The participants in this historical clinical trial were observed for a mean duration of five years. Cytotoxicity assays were employed to assess the therapeutic effectiveness of TPF chemotherapy drugs. The activity of caspase-3 and PARP was evaluated in the HB96, CAL27, and HN30 cell lines following cyclin D1 inhibition, respectively. Long-term clinical outcomes were significantly better for patients with OSCC whose cancer cells expressed low amounts of cyclin D1 than those whose cells expressed high protein levels. Time to initial illness occurrence, time to initial local recurrence, time to mortality, and overall survival were among the metrics evaluated. The groups with low and high expression levels of cyclin D1 showed significantly different survival rates for disease-free survival, overall survival (OS), DMFS, local recurrence-free survival, and local recurrence-free survival (p0.001, 0.003, 0.004, and 0.001, respectively). Patients with cN2 oral squamous cell carcinoma (OSCC) with higher cyclin D1 expression had better distant metastasis-free survival (OS, P=0.024; DMFS, P=0.024) and overall survival (OS, P=0.024; DMFS, P=0.024) with TPF induction chemotherapy. However, patients with cN2 OSCC and low cyclin D1 expression did not show this survival advantage (P=0.024). Research has shown that oral squamous cell carcinoma (OSCC) is more sensitive to TPF-based chemotherapeutic drugs using caspase-3-mediated apoptosis that occurs when cyclin D1 expression rises. Based on these results, TPF induction chemotherapy may provide longer-term benefits than traditional treatment approaches for patients with cN2 OSCC and high cyclin D1 expression. Through a caspase-3-dependent mechanism, oral squamous cell carcinoma (OSCC) cell lines become more susceptible to TPF chemotherapeutic drugs when cyclin D1 is overexpressed.*

Keywords: Oral Cancer, Caspase3 Pathway, Cyclin D1 Overexpression, Chemosensitivity, TPF Chemotherapeutic Agents

Introduction

More than 80% of cancers in the oral and maxillofacial region are squamous cell carcinomas (OSCC) (Kademani, 2007; Petersen, 2003). It is estimated that between 50% and 60% of people with oral squamous cell carcinoma (OSCC) will survive for five years. However, it should be noted that patients with locally advanced malignancies have a significantly worse survival rate (Chinn and Myers, 2015; Siegel et al., 2019). Individuals at an advanced stage of locally acquired oral squamous cell carcinoma (OSCC), a condition that can be cured, usually get radiation therapy or chemotherapy after undergoing surgery (Wood, 2015). The selection of the therapy regimen will be based on the pathological findings observed after the surgical procedure. Hence, it is imperative to enhance the prognosis for individuals diagnosed with oral squamous cell carcinoma (OSCC). The research has demonstrated that induction

chemotherapy can effectively treat symptoms related to locally progressed or metastatic malignancies and can delay the need for surgical intervention in cases with worrisome tumors (Devisetty and Wong, 2013). Research from the past has indicated that patients with head and neck squamous cell carcinoma (HNSCC) benefit more from induction chemotherapy consisting of docetaxel, cisplatin, and 5 fluorouracil (TPF) than from cisplatin and 5 fluorouracil administered alone (Posner et al., 2007; Vermorken et al., 2007). The effects of TPF induction chemotherapy on patients with IVA OSCC and clinical stages III were investigated in recent clinical research (Zhong et al., 2015; Zhong et al., 2013). Nevertheless, this investigation did not reveal any clinical outcomes that were statistically significant.
Patients with cN2 OSCC who had TPF induction therapy showed better clinical outcomes and higher expression

levels of cyclin D1 (Sherr et al., 2016). The cyclin D1 protein interacts with cyclin-dependent kinase 4/6 to promote the cell cycle's progression from the G1 phase to the S phase. Numerous cellular processes, such as those that promote cell growth and proliferation, improve migration, alter mitochondrial function, prevent DNA repair, and other related activities, are linked to this specific control style (Ramos-Garcia et al., 2017; Sherr et al., 2016). A considerable percentage of early head and neck squamous cell carcinomas (HNSCCs), ranging from 39% to 64%, have been found to overexpress cyclin D1 (Hardisson, 2003). A possible biomarker for determining the prognosis of patients with head and neck squamous cell carcinoma that has spread to undiagnosed lymph nodes is elevated expression of cyclin D1 (Capaccio et al., 2000; Huang et al., 2012; Ramos-Garcia et al., 2017). According to earlier research, patients with oral squamous cell carcinoma (OSCC) who had lower tumor levels of cyclin D1 expression had better survival rates than patients with greater tumor levels of cyclin D1 expression (Sherr et al., 2016).

Previous research (Sherr et al., 2016) has shown that in patients with stage N2 oral squamous cell carcinoma (OSCC), TPF induction chemotherapy improves disease-free survival (DMFS) and overall survival (OS) results. The precise regulatory mechanism underlying the response to TPF chemotherapeutic agents in individuals diagnosed with oral squamous cell carcinoma (OSCC) remains elusive; nonetheless, existing evidence suggests a potential association with the upregulation of cyclin D1. Head and neck squamous cell carcinoma (HNSCC) cell lines have been shown to respond more favorably to the chemotherapy drug cisplatin when their levels of cyclin D1 are increased (Åkervall et al., 2004). Nevertheless, this category encompasses the utilization of platinum-based pharmaceutical agents, namely cisplatin, platin, neoplatin, cismaplat, and cis-diamminedichloroplatinum (II). This study's primary goal was to investigate the connection between oral squamous cell carcinoma (OSCC) patient lines' susceptibility to TPF treatment and the expression of cyclin D1 (Kothari and Mulherkar, 2012; Nakashima and Clayman, 2000; Warenius et al., 1996). Building upon the discoveries elucidated in a recent investigation (Zhong et al., 2013), our objective was to investigate the correlation between augmented cyclin D1 expression and enhanced longevity in individuals afflicted with cN2 OSCC who underwent TPF induction chemotherapy.

Methodology

Cell Culture

Three distinct oral squamous cell carcinoma (OSCC) cell lines were used in this investigation: CAL27, HN30, and HB96. Using a cellular carcinogenesis model, oral squamous cell carcinoma (OSCC)--specific cell lines were created in the lab (reference 21). On the other hand, the ATCC provided the CAL27 cell line for use in commercial applications. The cell lines were grown in 10% fetal bovine serum (FBS)-supplemented Dulbecco's Modified Eagle Medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.). The experiment utilized a 5% carbon dioxide concentration to maintain each cell's humidity and temperature at 37 degrees Celsius.

Patients and Samples

A cohort of 233 people with invasive verrucous carcinoma (IVA) or stage III oral squamous cell carcinoma (OSCC) were included in this investigation. The participants ranged in age from 26 to 75, with a mean age of 55. A total of 320 participants—160 men and 72 women—were included in the study. Before beginning standard treatment for locally advanced OSCC, the patients were included in a phase 3 clinical trial (NCT01542931) to assess the possible advantages of TPF induction chemotherapy. The patients were formally admitted to the Pakistani Oral and Maxillofacial-Head and Neck Oncology Unit. As Reference (9) mentioned, a comprehensive summary of the clinical trial methodology is provided below. The patients who satisfied the criteria for inclusion were allocated randomly to one of two groups: the radical surgery group (n = 105), which underwent post-operative radiation after neck dissection and tumor excision, or the control group (n = 127), which received surgery exclusively.

Immunohistochemical labeling was used to investigate cyclin D1 expression levels in tumor tissues before induction chemotherapeutic treatment. Images of cyclin D1 staining by immunohistochemistry were also included in the evaluation (11). Using a rabbit monoclonal antibody, we used the Dako RealTM EnVisionTM Detection System, Rabbit/Mouse Peroxidase/DAB+ (catalog number K5007; Agilent Technologies, Inc.) to detect cyclin D1. For detection, we used the Dako RealTM EnVisionTM Detection System with a dilution of the antibody of 1:150. Cells expressing cyclin D1 were identified using light microscopy and a cytokeratin-staining reagent. Cyclin D1 expression was roughly quantified using the proportion of stained cells. A value of 0% on this scale denoted entirely unstained cells; 10% represented a moderate quantity of unstained cells, and 50% represented a substantial quantity of stained cells. Cyclon D1, low expression levels, have been characterized as moderately positive or negative. In contrast, those high expression levels have been considered significantly optimistic or optimistic after Pakistan's hospital's ethics committee approved the research, and each subject provided written informed consent.

Cyclin D1 RNA Interference

The company Sangon Biotech Co., Ltd. developed and produced oligonucleotides known as small interfering (si) RNA, which were specifically engineered to target cyclin D1. Furthermore, a control oligonucleotide was devised for this study. They are made up of the events that come next. A 5'-CCCGCAGAUUUCAUUGAA dtdt3' sense sequence and a 5'-UUCAAUGCGUGCGGGdt 3' antisense sequence make up the first small interfering RNA (siRNA). The sense sequence of the second siRNA, siRNA2, is 5'-GUAUACUGCUAUUCCAAdt 3', while the antisense sequence is 5'-UUGGAAUAGCAGUAAUCdt3'. Finally, the antisense sequence 5'UUCUCCGAACGUGUGUCACGUdt 3' is present in the siRNA identified as siRNA. Thermo Fisher Scientific, Inc.'s Lipofectamine® 3000 transfection reagent (Invitrogen) was used to transiently transfect the HB96 and CAL27 cell lines with 100 nM siRNAs. Cyclin D1 expression levels were assessed using Western blotting, as depicted in Figure S1. There was a 24-hour gap between using the transfection method and carrying out the experiments.

Cyclin D1 Gene Transfection

Pakistan Biotechnology Co., Ltd. kindly provided the empty

pLVX puro (catalog number V109050901) and the lentiviral overexpression vector pLVX-puro-cyclin D1 (catalog number V109020035) plasmids. Following plasmid transfection at a 0.28 g/ml concentration, 293T cells were cultured in DMEM media supplemented with 10% fetal bovine serum for seven days. The culture was kept in a controlled environment at 37 degrees Celsius with a relative humidity of 5% CO_2. Following the isolation of lentiviral particles from the supernatant, a 4m filter was utilized. After exposure to the vector supernatant with a 5×10^7 TU/ml concentration, the CAL27 and HN30 cell media were supplemented with 1 g/l of puromycin obtained from Life Technologies (Thermo Fisher Scientific, Inc.).

Western Blotting Assay

According to the previously published procedure (24), total protein was isolated and lysed. The cells were lysed in a 2X lysis solution that contained 80% confluent HB96, CAL27, or HN30 cells, 125 mM Tris-HCl (pH 6.8), ice-cold 5% w/v SDS, and 24.75% glycerol. Every operation was performed in a cold chamber. The Bradford assay (Pierce; Thermo Fisher Scientific, Inc.) determined the total protein concentration in compliance with the manufacturer's procedure. SDS-PAGE separated proteins at 10–12% (15 g per lane). Proteins were analyzed after being transferred electrophoretically from a gel to PVDF membranes (EMD Millipore) with a pore size of 0.22m (provided by Bio-Rad Laboratories, Inc.). The blocking procedure was applied to the membranes for an hour at room temperature. This was accomplished by employing a blocking buffer of 0.02% Tween-20 in TBS mixed with 5% dry skim milk. The primary antibodies were incubated in a buffer solution at 4 degrees Celsius for an entire day. After incubation with blocking serum overnight, anti-mouse antibodies (1:5,000; Cat. no. 7076) from Cell Signaling Technology, Inc. were added.

Chemiluminescent detection was performed by employing anti-rabbit antibodies (diluted at a ratio of 1:5,000; catalog number 7704; Cell Signaling Technology, Inc.) in combination with peroxidase-linked IgG secondary antibodies (incubated at room temperature for 1 hour) and a lumpiest ECL substrate solution kit (sb-wb011; Share-Bio, Inc.). In summary, the PVDF membranes underwent scanning and evaluation utilizing a sophisticated chemiluminescence detection apparatus, specifically the AmershamTM Imager 600 manufactured by GE Healthcare, as an integral component of the experimental procedure. Cell Signaling Technology, Inc provided the loading control solution of β-actin (1:10,000). The primary antibodies employed in this study were obtained from Cell Signaling Technology, Inc. The antibodies used in this study consisted of a monoclonal antibody against cyclin D1 derived from rabbits and a rabbit antibody specific to the cleaved fragment of human PARP at Asp214 (diluted at 1:500; catalog number 5625P).

Cytotoxicity Assay and Chemotherapeutic Agents

The cells that completed transfection were distributed in 96-well plates, each containing a density of 2×10^3 cells. The plates were incubated at 37 degrees Celsius for eight to twelve hours. The concentrations of glutamine, streptomycin, and penicillin in the incubation media were significantly low. Subsequently, the cells were subjected to increasing doses of 5-fluorouracil (5FU), cisplatin, or docetaxel (10-fold, 3-fold, and 2-fold, respectively). The temperature during the experiment was maintained at a constant value of 37 degrees Celsius for 72 hours. The IC50 value was employed for each cell line to determine the optimal dosage. The outcome above was achieved by assessing cellular viability in samples subjected to different medication dosages. The Cell Counting Kit-8 (CCK-8) solution from Dojindo Molecular Technologies, Inc. was added to each well by the manufacturer's instructions after the liquid portion above the sediment was discarded. After being incubated for two hours at 37 degrees Celsius, the 96-well plates were removed. A wavelength of 450 nm was used for absorbance measurements to determine the cells' vitality. Three separate times were used to experiment.

Statistical Analysis

The data analysis was conducted using SPSS 27.0 for Windows, a software developed by SPSS, Inc. After the initial two-year period of therapy, participants were subjected to assessments at three-month intervals. These assessments were conducted every six months from the third to the fifth year. Subsequently, individuals were evaluated annually until either their death or data removal. The time interval from random assignment to the occurrence of death was applied in the calculation of overall survival (OS). To determine disease-free survival (DFS), locoregional recurrence-free survival (LRFS), and distant metastasis-free survival (DMFS) by evaluating the incidence of recurrence and distant metastasis, the researchers used random assignment dates. The log-rank test was used for comparative analysis, and the Kaplan-Meier method was used for survival analysis. The present study aims to compare two groups characterized by high and low cyclin D1 expression. This analysis will be performed using two statistical tests, with each group having its distinct set of baseline values. The non-parametric data were subjected to the Bonferroni test after implementing the Kruskal-Wallis test. A statistically significant difference was discovered at the significant level of 0.05.

Results

Cyclin D1 Expression and Treatment Outcomes

There were no statistically significant variations found in terms of alcohol intake, pathologic grade, smoking history, original tumor site, T stage, N stage, or primary tumor site between the groups exhibiting high and low levels of cyclin D1 expression (Table SI). Approximately 50% of the stained cells exhibited either negative or weakly positive staining, suggesting a diminished presence of cyclin D1 in the cellular composition of these subjects. Conversely, cells exhibiting high levels of cyclin D1 expression were identified as strongly positive, constituting around 50% of the stained cell population. In contrast to patients with low cyclin D1 expression, individuals with high levels of expression had noticeably lower disease-free survival, local recurrence-free survival, and distant metastasis-free survival times. The follow-up period's median length was 67 months, with a deviation of 55 months falling between the first and third quartiles and 75 months above the latter. The potential benefits of TPF induction therapy were investigated in a later subgroup study of patients whose long-term prognosis was anticipated based on their cyclin D1 expression levels. Based on the findings, it can be

extrapolated that TPF induction chemotherapy provided the most significant advantages for persons who satisfied the following conditions: cN2 OSCC, heightened cyclin D1 expression, substantial mortality vulnerability, and progressed distant metastases. Patients who received TPF induction chemotherapy and had cN2 OSCC and low cyclin D1 expression did not experience an improvement in survival rates. Statistically significant is the two-year difference between the two groups regarding OS and DMFS (Figure 1) (P=0.024).
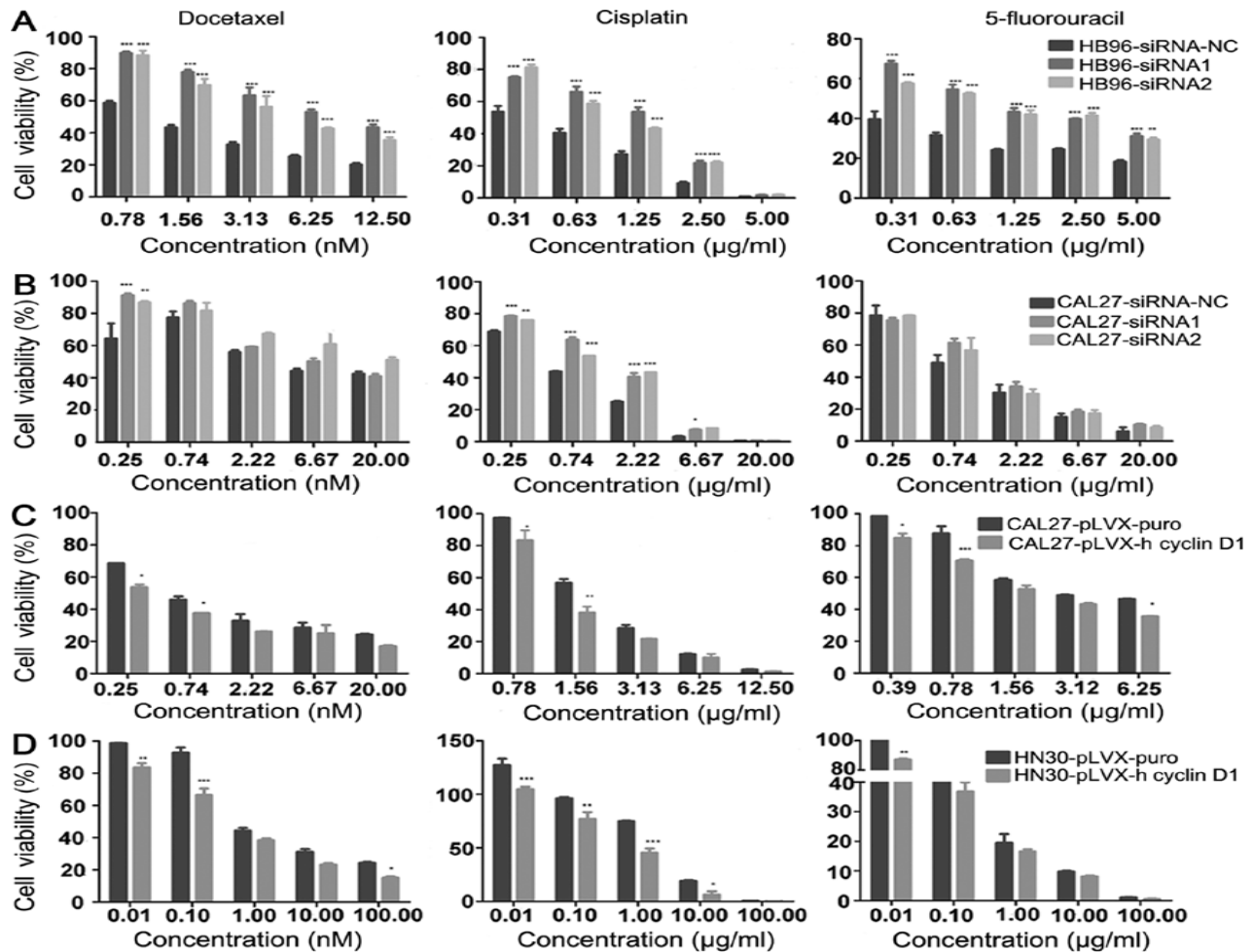
Figure 1. *A correlation has been discovered between the expression of cyclin D1 and the ability to respond to docetaxel, cisplatin, and 5-fluorouracil (5-FU). The assessment of cell viability was conducted on HB96 and CAL27 cells at the 72-hour time point after the transfection process using siRNA1 and siRNA2, both of which possess a specific affinity for cyclin D1. The treatment approach incorporated the utilization of docetaxel, cisplatin, and 5-fluorouracil (5FU) at different concentrations. The assessment of cell viability was conducted 72 hours after the transfection of cyclin D1 overexpression vectors into HN30 and CAL27 cells. Subsequently, these cells were treated with different dosages of docetaxel, cisplatin, and 5-FU. Statistical significance was assessed by employing the significance levels of P0.05, P0.01, and P0.001 to compare the experimental and control groups. Small interfering RNA (siRNA), also referred to as negative control RNA (NC RNA), is an alternative terminology for the same biological entity. The widely used abbreviation for five fluorouracil ions is 5FU.*

Cyclin D1 Expression Upregulation Increases OSCC Cell Sensitivity to Docetaxel, Cisplatin, and 5-FU.

The study aimed to assess the association between the expression of cyclin D1 and the sensitivity of oral squamous cell carcinoma (OSCC) cell lines toward TPF chemotherapeutic agents. This study aimed to offer empirical data supporting the clinical observations about patients diagnosed with cN2 oral tongue squamous cell carcinoma who exhibited good in vitro outcomes following TPF induction chemotherapy. Therefore, the CAL27 cell line was employed. The subject of the inquiry has a direct lineage to an individual who was diagnosed with cN2 oral tongue squamous cell carcinoma (Gioanni et al., 1988).

Using the CCK-8 test, the current study evaluated the relative survival of many oral squamous cell carcinoma (OSCC) cell lines. Following a 72-hour therapeutic intervention involving varying dosages of docetaxel, cisplatin, and 5FU, the present work analyzed the modulation of cyclin D1 expression, specifically examining up- and down-regulation. Based on the IC50 values obtained for each cell type, our findings indicate that transfection with an empty vector resulted in a notable increase in cell viability compared to treatment with the administered medications. This observation was particularly evident when examining drug concentrations at lower levels, as depicted in Figure 2C and D.
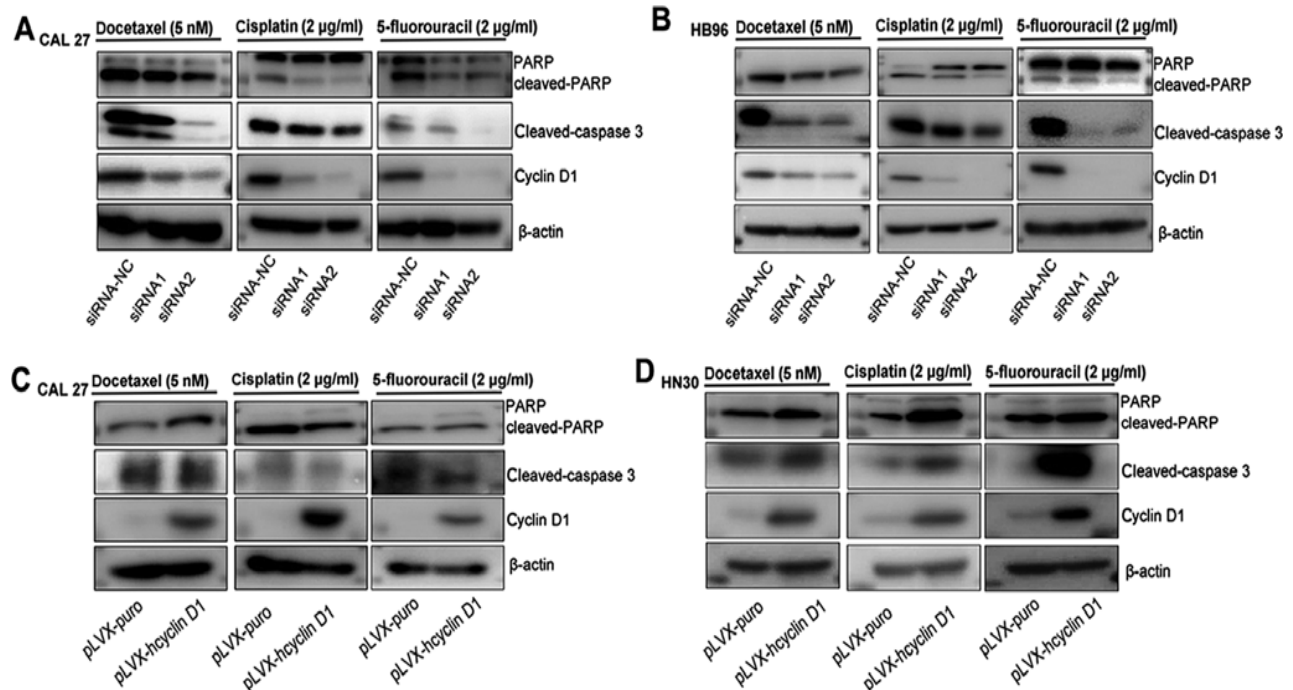
Figure 2. This work explores any possible relationship between the expression of cyclin D1 and cleaved caspase-3 and PARP levels after treatment with docetaxel, cisplatin, and 5FU. After receiving 5 nM docetaxel, 2 g/ml cisplatin, or 2 g/ml 5FU for 72 hours, cleaved caspase-3 and PARP protein levels were assessed. Two small interfering RNAs (siRNAs) were introduced into the CAL27 and HB96 cell lines to target cyclin D1 specifically. These siRNAs were referred to as siRNA1 and siRNA2. Seventy-two hours after transfecting CAL27 and HN30 cells with a cyclin D1 overexpression vector and treating them with 5 nM docetaxel, 2 g/ml cisplatin, or 2 g/ml 5FU, the amounts of cleaved caspase-3 and PARP proteins were measured. Poly (ADP-ribose) polymerase (PARP), short interfering RNA (siRNA), and fluorouracil (5-fluorouracil) are denoted by their corresponding acronyms as follows.

Increased Cyclin D1 Expression in OSCC Cells Through Caspase-3-Dependent Apoptosis Enhances Sensitivity to Docetaxel, Cisplatin, and 5FU.

Following treatment with docetaxel, cisplatin, and 5FU, the enzymatic activity of PARP and caspase-3 was assessed in CAL27, HN30, and HB96 cells. Following the transfection of siRNA-NC to lower cyclin D1 expression, CAL27, and HB96 cells showed significant decreases in cleaved caspase-3 and PARP levels. As shown in Figures 4A and B, this effect was seen after a 72-hour treatment regimen that included the administration of docetaxel, cisplatin, and 5-FU. Following a treatment duration of 72 hours involving the administration of docetaxel, cisplatin, and 5FU, it was shown that the levels of cleaved caspase-3 and PARP were increased in HN30 cells that exhibited overexpression of cyclin D1. In contrast, the administration of cisplatin did not increase cleaved PARP levels. However, the treatment with docetaxel and 5FU did lead to an enhancement of cleaved PARP levels in CAL27 cells that overexpress cyclin D1. No significant differences were seen in the amounts of cleaved caspase-3 among the three treatments, as depicted in Figures 2C and 2D

Discussion

Patients with cN2 OSCC who had high levels of cyclin D1 expression had better long-term survival rates after receiving TPF induction chemotherapy as opposed to standard treatment. When patients with cN2 OSCC with low cyclin D1 expression were treated with TPF induction chemotherapy, there was no discernible improvement in response compared to standard care. The following in vitro investigations provide evidence that oral squamous cell carcinoma (OSCC) cells expressing increased levels of cyclin D1 are more prone to the effects of the chemotherapeutic drugs 5-fluorouracil (FUR), cisplatin, and docetaxel. The activation of caspase-3 mediates this susceptibility. Cyclin D1 is present in most malignancies, and its carcinogenic properties have been well-established. Previous research has provided evidence indicating that it can impede the mechanisms responsible for DNA repair (Dozier et al., 2017; Zhong et al., 2010) while concurrently promoting cellular proliferation and migration. The prognosis for patients diagnosed with pancreatic adenocarcinoma, colorectal carcinoma, ovarian cancer, and breast cancer is significantly poorer when they exhibit elevated levels of cyclin D1 (Qie and Diehl, 2016; Sales et al., 2014). The study conducted by a previous researcher (reference 9) employed a randomized design to investigate the level of cyclin D1 expression in patients before undergoing treatment. In this study, treating oral squamous cell carcinoma (OSCC) involves the administration of TPF induction chemotherapy. The primary objective of this study was to identify the optimal therapeutic approach for individuals diagnosed with oral squamous cell carcinoma (OSCC) and exhibiting elevated levels of cyclin D1 expression.

Compared to patients who received conventional treatment, patients who did not meet the criteria for cN2 OSCC and had high levels of cyclin D1 expression did not see statistically significant benefits from the administration of

TPF induction chemotherapy. When cN2 oral squamous cell carcinoma (OSCC) is diagnosed instead of cN0 or cN1 OSCC, there is a positive correlation with a higher chance of distant metastases (Zhong et al., 2013). Research has demonstrated that oral cancer cells with higher expression levels of cyclin D1 typically behave more aggressively than cells with lower expression levels (Fusté et al., 2016; Ramos-Garcia et al., 2017). This finding provides evidence that the heightened expression of cyclin D1 promotes the movement and mobility of cells associated with oral cancer. Patients who have been diagnosed with cN2 oral squamous cell carcinoma (OSCC) and exhibit increased levels of cyclin D1 expression may be at a higher risk of developing distant metastases. Previous studies have shown that the survival rate without dental caries, as measured by When head and neck squamous cell carcinoma (HNSCC) patients get induction therapy, their Dental Caries-Free Survival (DMFS) index improves (Ma et al., 2012; Pignon et al., 2009). The study revealed that individuals diagnosed with cN2 OSCC and exhibiting high levels of cyclin D1 expression experienced considerably prolonged life durations when subjected to TPF induction chemotherapy, as compared to those who underwent conventional treatment.

Table SIII demonstrates the enhancements implemented universally for the OS product line. The findings indicated that the downregulation of cyclin D1 expression resulted in a notable decrease in the susceptibility of HB96 cells to docetaxel, cisplatin, and 5FU. The observed outcome presents a significant disparity compared to the effects observed following siRNA-NC transfection into the same cell populations. The groups treated with siRNA exhibited a significant enhancement in cell viability, as depicted in Figure 3A. The sensitivity of CAL27 cells to 2.5 g/ml cisplatin and 0.25 nM docetaxel was significantly reduced due to the suppression of cyclin D1 expression. Nevertheless, there was no observed improvement at higher dosages. Furthermore, Figure 3B demonstrates no alteration in the sensitivity to 5FU across the various administered doses. The study observed that the administration of docetaxel, cisplatin, and 5FU enhanced cell viability in CAL27 and HB96 cells. This effect was attributed to the inhibition of cyclin D1. The phenomenon above is observed in Figure S2; nonetheless, it is crucial to acknowledge that it did not attain statistical significance. On the other hand, it was found that cyclin D1 overexpression significantly raised sensitivity in CAL27 and HN30 cells. HB96 cells were not included in the study because of their history of high cyclin D1 expression. By evaluating the sensitivity of OSCC cell lines in a lab setting, it was possible to anticipate how well TPF chemotherapeutic medications would work in treating patients with node-positive oral squamous cell carcinoma (OSCC). The patient with stage 2 oral tongue squamous cell carcinoma, whose tumor accounted for 25% of the total tissue, provided the CAL27 cell line.

The present investigation revealed that cells exhibiting overexpression of cyclin D1 had a heightened vulnerability towards the chemotherapeutic drugs docetaxel, cisplatin, and 5FU, as opposed to cells transfected with inert plasmids. The results of the experiment indicated that oral squamous cell carcinoma (OSCC) cells that were transfected with non-targeting siRNA (siRNA-NC) exhibited a higher level of resistance to various chemotherapeutic treatments as compared to cells in which cyclin D1 had been knocked down. Increased cyclin D1 expression was linked to increased sensitivity of oral squamous cell carcinoma (OSCC) cells to docetaxel, cisplatin, and 5-fluorouracil (5-FU) chemotherapy treatments. DNA replication and transcription process is impeded by the cross-linking effect of ciprofloxacin, resulting in a deceleration of cell cycle progression, specifically during the transition into and out of the S/G2 phase (Jamieson and Lippard, 1999). The decrease in intracellular deoxythymidine triphosphate (dTTP) reserves can be attributed to the impact of 5-fluorouracil (5′FU), which inhibits the activity of thymidylate synthase and subsequently reduces the synthesis of pyrimidines (Longley et al., 2003). The pharmacological mechanism of docetaxel involves the inhibition of microtubule depolymerization, resulting in apoptosis and the termination of the G2/M phase of the cell cycle (Garcia et al., 1994). The biological rationale for using TPF induction therapy with cyclin D1 overexpression is highly persuasive. The individuals diagnosed with cN2 oral squamous cell carcinoma (OSCC) who exhibited elevated levels of cyclin D1 demonstrated a more favorable response to the induction treatment consisting of docetaxel, cisplatin, and fluorouracil (TPF).

The significance of this discovery lies in its relevance to the continuing debates about the impact of cyclin D1 expression on the effectiveness of induction chemotherapy. In a recent study by Akervall et al. (2017), a total of twenty-three squamous cell carcinoma (SCC) cell lines were examined. The phenomenon of heightened responsiveness to cisplatin was associated with the overexpression of cyclin D1. An investigation was started by Perisanidis et al. (Perisanidis et al., 2012) to learn more about how cyclin D1 overexpression affects the effectiveness of induction chemoradiotherapy using mitomycin and 5-FU. Considering different levels of cyclin D1 expression, no discernible variations in patient responses were seen. However, it is essential to note that out of the patient group, seven individuals (constituting 36% of the total) were found to have progressed to the pathogenic N2 stage. The comparison of the clinical effectiveness of induction chemotherapy-radiotherapy and standard care for patients diagnosed with cN2 OSCC has posed difficulties in terms of prognostication. It was determined how well docetaxel, cisplatin, and 5-fluorouracil made oral squamous cell carcinoma (OSCC) cells more sensitive to cyclin D1 overexpression. Consequently, the amounts of PARP and cleaved caspase-3 proteins in the blood were determined using a quantification analysis. A higher apoptosis rate was seen in oral squamous cell carcinoma (OSCC) cells that expressed cyclin D1 in response to docetaxel, cisplatin, and 5-fluorouracil (5FU).

Increased amounts of poly (ADP-ribose) polymerase and cleaved caspase-3 served as indicators of this (PARP). When cyclin D1 expression was inhibited, oral squamous cell carcinoma (OSCC) cells responded to different chemotherapies by showing decreased cleaved caspase-3 and PARP levels. The potential upregulation of cyclin D1 in oral squamous cell carcinoma (OSCC) cells may have led to the activation of the caspase-3 pathway, potentially elucidating the heightened susceptibility of these cells to docetaxel, cisplatin, and 5FU. Prior research has indicated that upregulation of cyclin D1 (Cowan et al., 2015; Pirkmaier et al., 2003; Smith et al., 2011) renders rhabdoid

tumors, lymphomas, and breast cancer more susceptible to the chemotherapeutic agents fenretinide and bortezomib. Cancer cells exhibiting an atypically elevated expression of cyclin D1 may display increased susceptibility to the initiation of apoptosis during chemotherapy. Individuals diagnosed with oral squamous cell carcinoma (OSCC) and exhibiting elevated levels of cyclin D1 expression have been the primary subjects of scientific investigations exploring the potential efficacy of chemotherapeutic agents or compounds that selectively target cyclin D1 (Ramos-Garcia et al., 2017). Further research is required to investigate the exact methodologies employed in targeting cyclin D1. The present study is constrained by the fact that sensitivity testing for the three chemotherapeutic medicines was conducted on distinct sets of cells from oral squamous cell carcinoma (OSCC), and different researchers executed the intervention targeting cyclin D1.

Despite extensive efforts to modify concentrations, the control group, the single-agent treatment group, and the three-agent combination treatment group exhibited similar levels of cell viability. There are several potential explanations for this phenomenon, one of which is the differential biochemical mechanisms by which the three medications specifically target cells from oral squamous cell carcinoma (OSCC). The comprehensive understanding of the consequences resulting from the alteration of cyclin D1 in OSCC cells is challenging due to the intricate nature of the underlying mechanism. Hence, additional research is needed to achieve a comprehensive comprehension. It is advised that patients diagnosed with oral squamous cell carcinoma (OSCC) who are taking chemotherapy should exercise caution when interpreting the results of in vitro studies conducted using OSCC cell lines. The concentrations of chemotherapeutic medicines in OSCC cells did not exhibit consistency with the corresponding values seen in patient serum. When compared to traditional treatment methods, it was seen that TPF induction chemotherapy resulted in higher long-term survival rates among patients diagnosed with cN2 OSCC and exhibiting elevated cyclin D1 expression. Through a caspase-3-dependent mechanism, there is a correlation between the overexpression of cyclin D1 and increased sensitivity of oral squamous cell carcinoma (OSCC) cells to TPF chemotherapeutic agents.

Conclusion

Based on these results, TPF induction chemotherapy may provide longer-term benefits than traditional treatment approaches for patients with cN2 OSCC and high cyclin D1 expression. Through a caspase-3-dependent mechanism, oral squamous cell carcinoma (OSCC) cell lines become more susceptible to TPF chemotherapeutic drugs when cyclin D1 is overexpressed.

Declarations

Data Availability statement
All data generated or analyzed during the study are included in the manuscript.
Ethics approval and consent to participate
Approved by the department Concerned.

Author Contribution

ANITA GEORGE
Conception of Study, Development of Research Methodology Design, Study Design,, Review of manuscript, final approval of manuscript
SIDRAH
Data entry and Data analysis, drafting article
AZHAR ALI
Study Design, Review of Literature
MUHAMMAD ZAMAN
Data acquisition, analysis.
REHAN AKHTAR
Coordination of collaborative efforts.
YASMEEN MEMON
Supervision, funding acquisition.
KIRAN AAMIR
Manuscript revisions, critical input.
AAMIR RAMZAN
Data entry and Data analysis, drafting article.

References

Åkervall, J., Kurnit, D. M., Adams, M., Zhu, S., Fisher, S. G., Bradford, C. R., and Carey, T. E. (2004). Overexpression of cyclin D1 correlates with sensitivity to cisplatin in squamous cell carcinoma cell lines of the head and neck. *Acta oto-laryngologica* **124**, 851-857.

Capaccio, P., Pruneri, G., Carboni, N., Pagliari, A. V., Quatela, M., Cesana, B. M., and Pignataro, L. (2000). Cyclin D1 expression is predictive of occult metastases in head and neck cancer patients with clinically negative cervical lymph nodes. *Head & Neck: Journal for the Sciences and Specialties of the Head and Neck* **22**, 234-240.

Chinn, S. B., and Myers, J. N. (2015). Oral cavity carcinoma: current management, controversies, and future directions. *Journal of clinical oncology* **33**, 3269.

Cowan, A. J., Frayo, S. L., Press, O. W., Palanca-Wessels, M. C., Pagel, J. M., Green, D. J., and Gopal, A. K. (2015). Bortezomib and fenretinide induce synergistic cytotoxicity in mantle cell lymphoma via apoptosis, cell cycle dysregulation, and IκBα kinase down-regulation. *Anti-cancer drugs* **26**, 974.

Devisetty, K., and Wong, S. J. (2013). Neoadjuvant versus induction chemotherapy: more than semantics. *Journal of clinical oncology: official journal of the American Society of Clinical Oncology* **31**, 2971-2972.

Dozier, C., Mazzolini, L., Cénac, C., Froment, C., Burlet-Schiltz, O., Besson, A., and Manenti, S. (2017). CyclinD-CDK4/6 complexes phosphorylate CDC25A and regulate its stability. *Oncogene* **36**, 3781-3788.

Fusté, N. P., Fernández-Hernández, R., Cemeli, T., Mirantes, C., Pedraza, N., Rafel, M., Torres-Rosell, J., Colomina, N., Ferrezuelo, F., and Dolcet, X. (2016). Cytoplasmic cyclin D1 regulates cell invasion and metastasis through the phosphorylation of paxillin. *Nature communications* **7**, 11581.

Garcia, P., Braguer, D., Carles, G., Khyari, S. E., Barra, Y., de Ines, C., Barasoain, I., and Briand, C. (1994). Comparative effects of taxol and Taxotere on two different human carcinoma cell lines. *Cancer chemotherapy and pharmacology* **34**, 335-343.

Gioanni, J., Fischel, J.-L., Lambert, J.-C., Demard, F., Mazeau, C., Zanghellini, E., Ettore, F., Formento, P., Chauvel, P., and Lalanne, C.-M. (1988). Two new human tumor cell lines derived from squamous cell carcinomas of the tongue: establishment, characterization and response to cytotoxic treatment. *European Journal of Cancer and Clinical Oncology* **24**, 1445-1455.

Hardisson, D. (2003). Molecular pathogenesis of head and neck squamous cell carcinoma. *European Archives of Oto-Rhino-Laryngology* **260**, 502-508.

Huang, S.-F., Cheng, S.-D., Chuang, W.-Y., Chen, I.-H., Liao, C.-T., Wang, H.-M., and Hsieh, L.-L. (2012). Cyclin D1 overexpression and poor clinical outcomes in Taiwanese oral cavity squamous cell carcinoma. *World journal of surgical oncology* **10**, 1-7.

Jamieson, E. R., and Lippard, S. J. (1999). Structure, recognition, and processing of cisplatin− DNA adducts. *Chemical reviews* **99**, 2467-2498.

Kademani, D. (2007). Oral cancer. *In* "Mayo Clinic Proceedings", Vol. 82, pp. 878-887. Elsevier.

Kothari, V., and Mulherkar, R. (2012). Inhibition of cyclin D1 by shRNA is associated with enhanced sensitivity to conventional therapies for head and neck squamous cell carcinoma. *Anticancer research* **32**, 121-128.

Longley, D. B., Harkin, D. P., and Johnston, P. G. (2003). 5-fluorouracil: mechanisms of action and clinical strategies. *Nature reviews cancer* **3**, 330-338.

Ma, J., Liu, Y., Huang, X.-L., Zhang, Z.-Y., Myers, J. N., Neskey, D. M., and Zhong, L.-P. (2012). Induction chemotherapy decreases the rate of distant metastasis in patients with head and neck squamous cell carcinoma but does not improve survival or locoregional control: a meta-analysis. *Oral oncology* **48**, 1076-1084.

Nakashima, T., and Clayman, G. L. (2000). Antisense inhibition of cyclin D1 in human head and neck squamous cell carcinoma. *Archives of Otolaryngology–Head & Neck Surgery* **126**, 957-961.

Perisanidis, C., Perisanidis, B., Wrba, F., Brandstetter, A., El Gazzar, S., Papadogeorgakis, N., Seemann, R., Ewers, R., Kyzas, P. A., and Filipits, M. (2012). Evaluation of immunohistochemical expression of p53, p21, p27, cyclin D1, and Ki67 in oral and oropharyngeal squamous cell carcinoma. *Journal of oral pathology & medicine* **41**, 40-46.

Petersen, P. E. (2003). The World Oral Health Report 2003: continuous improvement of oral health in the 21st century–the approach of the WHO Global Oral Health Programme. *Community Dentistry and oral epidemiology* **31**, 3-24.

Pignon, J.-P., Le Maitre, A., Maillard, E., Bourhis, J., and Group, M.-N. C. (2009). Meta-analysis of chemotherapy in head and neck cancer (MACH-NC): an update on 93 randomised trials and 17,346 patients. *Radiotherapy and oncology* **92**, 4-14.

Pirkmaier, A., Yuen, K., Hendley, J., O'Connell, M. J., and Germain, D. (2003). Cyclin d1 overexpression sensitizes breast cancer cells to fenretinide. *Clinical cancer research* **9**, 1877-1884.

Posner, M. R., Hershock, D. M., Blajman, C. R., Mickiewicz, E., Winquist, E., Gorbounova, V., Tjulandin, S., Shin, D. M., Cullen, K., and Ervin, T. J. (2007). Cisplatin and fluorouracil alone or with docetaxel in head and neck cancer. *New England Journal of Medicine* **357**, 1705-1715.

Qie, S., and Diehl, J. A. (2016). Cyclin D1, cancer progression, and opportunities in cancer treatment. *Journal of molecular medicine* **94**, 1313-1326.

Ramos-Garcia, P., Gil-Montoya, J., Scully, C., Ayén, A., González-Ruiz, L., Navarro-Triviño, F., and González-Moles, M. (2017). An update on the implications of cyclin D1 in oral carcinogenesis. *Oral Diseases* **23**, 897-912.

Sales, K., Giudice, F., Castilho, R., Salles, F., Squarize, C., Abrahao, A., and Pinto Jr, D. (2014). Cyclin D 1-induced proliferation is independent of beta-catenin in H ead and N eck C ancer. *Oral Diseases* **20**, e42-e48.

Sherr, C. J., Beach, D., and Shapiro, G. I. (2016). Targeting CDK4 and CDK6: from discovery to therapy. *Cancer discovery* **6**, 353-367.

Siegel, R. L., Miller, K. D., and Jemal, A. (2019). Cancer statistics, 2019. *CA: a cancer journal for clinicians* **69**, 7-34.

Smith, M. E., Das, B. C., and Kalpana, G. V. (2011). In vitro activities of novel 4-HPR derivatives on a panel of rhabdoid and other tumor cell lines. *Cancer Cell International* **11**, 1-10.

Vermorken, J. B., Remenar, E., Van Herpen, C., Gorlia, T., Mesia, R., Degardin, M., Stewart, J. S., Jelic, S., Betka, J., and Preiss, J. H. (2007). Cisplatin, fluorouracil, and docetaxel in unresectable head and neck cancer. *New England Journal of Medicine* **357**, 1695-1704.

Warenius, H. M., Seabra, L. A., and Maw, P. (1996). Sensitivity to cis-diamminedichloroplatinum in human cancer cells is related to expression of cyclin D1 but not c-raf-1 protein. *International journal of cancer* **67**, 224-231.

Wood, D. E. (2015). National Comprehensive Cancer Network (NCCN) clinical practice guidelines for lung cancer screening. *Thoracic surgery clinics* **25**, 185-197.

Zhong, L.-p., Zhang, C.-p., Ren, G.-x., Guo, W., William Jr, W. N., Hong, C. S., Sun, J., Zhu, H.-g., Tu, W.-y., and Li, J. (2015). Long-term results of a randomized phase III trial of TPF induction chemotherapy followed by surgery and radiation in locally advanced oral squamous cell carcinoma. *Oncotarget* **6**, 18707.

Zhong, L.-p., Zhang, C.-p., Ren, G.-x., Guo, W., William Jr, W. N., Sun, J., Zhu, H.-g., Tu, W.-y., Li, J., and Cai, Y.-l. (2013). Randomized phase III trial of induction chemotherapy with docetaxel, cisplatin, and fluorouracil followed by surgery versus up-front surgery in locally advanced resectable oral squamous cell carcinoma. *Journal of clinical oncology* **31**, 744.

Zhong, Z., Yeow, W.-S., Zou, C., Wassell, R., Wang, C., Pestell, R. G., Quong, J. N., and Quong, A. A. (2010). Cyclin D1/cyclin-dependent kinase 4 interacts with filamin A and affects the migration and invasion potential of breast cancer cells. *Cancer research* **70**, 2105-2114.