

Cyclin D1 overexpression enhances chemosensitivity to TPF chemotherapeutic agents via the caspase-3 pathway in oral cancer

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Received January 9, 2020; Accepted July 15, 2020

DOI: 10.3892/ol.2020.12015

Abstract. Induction chemotherapy has been previously demonstrated to downgrade locally advanced or aggressive cancers and increase the likelihood of primary lesion eradication. Based on our previous phase 3 trial on TPF (docetaxel, cisplatin and fluorouracil) induction chemotherapy in patients with oral squamous cell carcinoma (OSCC), in which short-term prognostic and predictive values of cyclin D1 expression were reported, the present study aimed to determine the long-term predictive value of cyclin D1 expression in the same patients with OSCC who were eligible to receive TPF induction chemotherapy. In addition, the present study investigated the potential association between cyclin D1 expression and chemosensitivity to TPF agents during OSCC cell intervention, and the underlying apoptotic mechanism of action. In total, 232 patients with locally advanced OSCC from our previous trial with a median follow-up of 5 years were included for survival analysis using the Kaplan-Meier method and the log-rank test in the present study, where cyclin D1 expression in their tissues was detected by immunohistochemistry. Cyclin D1 knockdown, cytotoxicity assays assessing the efficacy of the TPF chemotherapeutic agents and measurements of caspase-3 and PARP activity in HB96, CAL27 and HN30 cell lines were performed. Patients with OSCC in the low cyclin D1 expression group exhibited significantly superior long-term

clinical outcomes compared with those in patients in the high cyclin D1 expression group [overall survival (OS), $P=0.001$; disease-free survival, $P=0.003$; local recurrence-free survival, $P=0.004$; distant metastasis-free survival (DMFS), $P=0.001$]. Furthermore, patients with stage clinical nodal stage 2 (cN2) OSCC in the high cyclin D1 expression group benefitted from TPF induction chemotherapy (OS, $P=0.024$; DMFS, $P=0.024$), whilst patients with cN2 OSCC in the low cyclin D1 expression group did not benefit from this chemotherapy. Overexpression of cyclin D1 expression was found to enhance chemosensitivity to TPF chemotherapeutic agents in OSCC by mediating caspase-3-dependent apoptosis. Based on these findings, TPF induction chemotherapy can benefit patients with cN2 OSCC and high cyclin D1 expression in terms of long-term survival from compared with standard treatment. In addition, OSCC cell lines overexpressing cyclin D1 are more sensitive to TPF chemotherapeutic agents in a caspase-3-dependent manner (clinical trial. no. NCT01542931; February 2012).

Introduction

Oral squamous cell carcinoma (OSCC) is the most common type of cancer in the oral and maxillofacial region and is estimated to account for ~80% of all oral and maxillofacial malignancies (1,2). The 5-year survival rate of patients with OSCC is only 50-60%, which is even lower in patients with locally advanced lesions (3,4). At present, the recommended treatment option for patients with locally-advanced resectable OSCC is radical surgery with postoperative radiation or chemoradiation, a decision that is dependent on the postoperative pathological findings (5). Therefore, there is a demand to improve the clinical outcomes of patients with OSCC. Induction chemotherapy has been documented to downgrade locally advanced or aggressive cancers and to increase the likelihood of primary lesion eradication (6). Docetaxel, cisplatin and 5-fluorouracil (5-FU; TPF) induction chemotherapy protocol has been shown to be superior compared that with only cisplatin and 5-fluorouracil in patients with head and neck squamous cell carcinoma (HNSCC) (7,8). Unfortunately, a previous clinical trial conducted, which investigated the effects of TPF induction chemotherapy in patients with

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Key words: oral squamous cell carcinoma, cyclin D1, docetaxel, cisplatin, fluorouracil, induction chemotherapy, chemosensitivity, apoptosis

clinical stages III and IVA OSCC, failed to observe significant improvements in clinical outcomes (9,10). However, subgroup analysis revealed that patients with cN2 OSCC and high cyclin D1 expression benefitted from TPF induction chemotherapy with respect to clinical outcomes (11).

Functionally, cyclin D1 combines with cyclin-dependent kinase 4/6 to form a complex to promote G₁-S phase cell cycle progression. This form of regulation participates in a number of cell processes, including promotion of cell proliferation, regulation of cell growth, modulation of mitochondrial activity, inhibition of DNA repair and acceleration of migration (12,13). Cyclin D1 has been found to be overexpressed in a large portion of malignant tumors, including 39-64% primary HNSCCs (14). In addition, previous studies have shown that cyclin D1 overexpression is a potential biomarker for predicting the prognosis of HNSCC, where it associates with occult lymph node metastasis (13,15,16). In a previous report, patients with OSCC and low cyclin D1 expression exhibited superior clinical outcomes compared with those in patients with high cyclin D1 expression (11). This previous study also revealed that only patients with stage N2 OSCC benefitted from TPF induction chemotherapy with respect to overall survival (OS) and distant metastasis-free survival (DMFS) (11). However, the mechanism underlying responses to TPF chemotherapeutic agents in patients with OSCC and its association with cyclin D1 overexpression remains poorly understood. Although it has been previously reported that cyclin D1 overexpression is associated with improved responses to cisplatin in HNSCC cell lines (17), cyclin D1 overexpression has also been reported to mediate cisplatin, platamin, neoplatin, cismaplat and cis-diamminedichloridoplatinum (II) therapy resistance (18-20).

Based on results from a previous study, which documented survival benefits from TPF induction chemotherapy in patients with cN2 OSCC and high cyclin D1 expression (9), the present study aimed to determine the relationship between cyclin D1 expression and responses to TPF chemotherapy in OSCC cell lines.

Materials and methods

Cell culture. The present study used three OSCC cell lines: HB96 cells, which were previously established in our lab from an *in vitro* cellular carcinogenesis model of OSCC (21), CAL27 and HN30 cells. The CAL27 cell line was purchased from ATCC and the HN30 cell line was a gift from Professor Li Mao from the University of Maryland Dental School (Baltimore, MD, USA). All cell lines were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.). All cells were maintained in a 5% CO₂ humidified atmosphere at 37°C.

Patients and samples. From March 2008 to December 2010, 232 patients with clinical stage III and IVA OSCC (sex, 160 males and 72 females; age range, 26-75 years; mean age, 55.4±10.0 years) were enrolled into the present study. The patients participated in a previous phase 3 trial (clinical trial registration no. NCT01542931), which investigated the potential benefit of TPF induction chemotherapy prior to standard treatment for locally advanced OSCC. The present study is a follow-up study of our previous study (11). All

the patients were enrolled into the Department of Oral and Maxillofacial-Head and Neck Oncology at the Ninth Peoples' Hospital, Shanghai Jiao Tong University School of Medicine (Shanghai, China). The detailed protocol of the clinical trial has been previously described (9). Briefly, patients who met the criteria were randomly assigned to into the experimental group (n=105), who underwent TPF induction chemotherapy, radical surgery (tumor resection and neck dissection) and post-operative radiotherapy, or the control group (n=127), who underwent surgery and post-operative radiotherapy.

The pretreatment levels of cyclin D1 expression in the tumor tissues (taken before induction chemotherapy) were assessed using immunohistochemical staining as previously described, as well as the representative immunohistochemical images of cyclin D1 staining (11). Rabbit monoclonal antibody to cyclin D1 (1:150 dilution; cat. no. ab134175; Abcam) was used with the Dako Real™ EnVision™ Detection System, Peroxidase/DAB+, Rabbit/Mouse (cat. no. K5007; Agilent Technologies, Inc.). Staining for cyclin D1 expression was observed in the cellular nucleus using light microscopy. Cyclin D1 expression index was calculated on the basis of the proportion of stained cells using a semi-quantitative scale, described as follows: i) Negative, ≤10% stained cells; ii) Weakly positive, <50% of stained cells; and iii) Strong positive, ≥50% of stained cells. In accordance with previous studies (11,22,23), low cyclin D1 expression was defined as negative and weakly positive cyclin D1 expression whereas high cyclin D1 expression was defined as strong positive cyclin D1 expression. The present study was approved by the Ethics Committee of the Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine and written informed consent was obtained from each patient.

Cyclin D1 RNA interference. In total, two sets of small interfering (si)RNA oligonucleotides for cyclin D1 and a negative control oligonucleotide were designed and synthesized by Sangon Biotech Co., Ltd. Their sequences are as follows: siRNA1 sense, 5'-CCC GCAGAUUUC AUUGAA dtdt-3' and antisense, 5'-UUCAAUGAAAUCGUGCGGGdt dt-3'; siRNA2 sense, 5'-GUAUACUGCUCUAUUC CAAdt dt-3' and antisense, 5'-UUGGAAUAGAGCAGUAAUC dtdt-3' and siRNA-NC sense, 5'-UUCUCCG AACGUGUCACGU dt dt-3' and antisense, 5'-ACGUGACACGUUCGGAGAA dtdt-3'. The siRNAs (100 nM) were transiently transfected into HB96 and CAL27 cells using the Lipofectamine® 3000 transfection reagent, according to the manufacturer's protocol (Invitrogen; Thermo Fisher Scientific, Inc.). Western blotting was applied to measure the expression levels of cyclin D1 (Fig. S1). The time interval between transfection and subsequent experiments was 24 h.

Cyclin D1 gene transfection. The lentiviral overexpression vector pLVX-puro-hcyclin D1 (cat. no. V109020035) and the empty pLVX puro (cat. no. V109050901) plasmids were obtained from Shanghai Qihe Biotechnology Co., Ltd. The plasmids (0.28 μg/ml) were transfected into 293T cells (cultured in DMEM supplemented with 10% FBS in a 5% CO₂ humidified atmosphere at 37°C), and after ~7 days the supernatant containing the lentiviral particles was collected and filtered through a 4-μm filter. CAL27 and HN30 cells

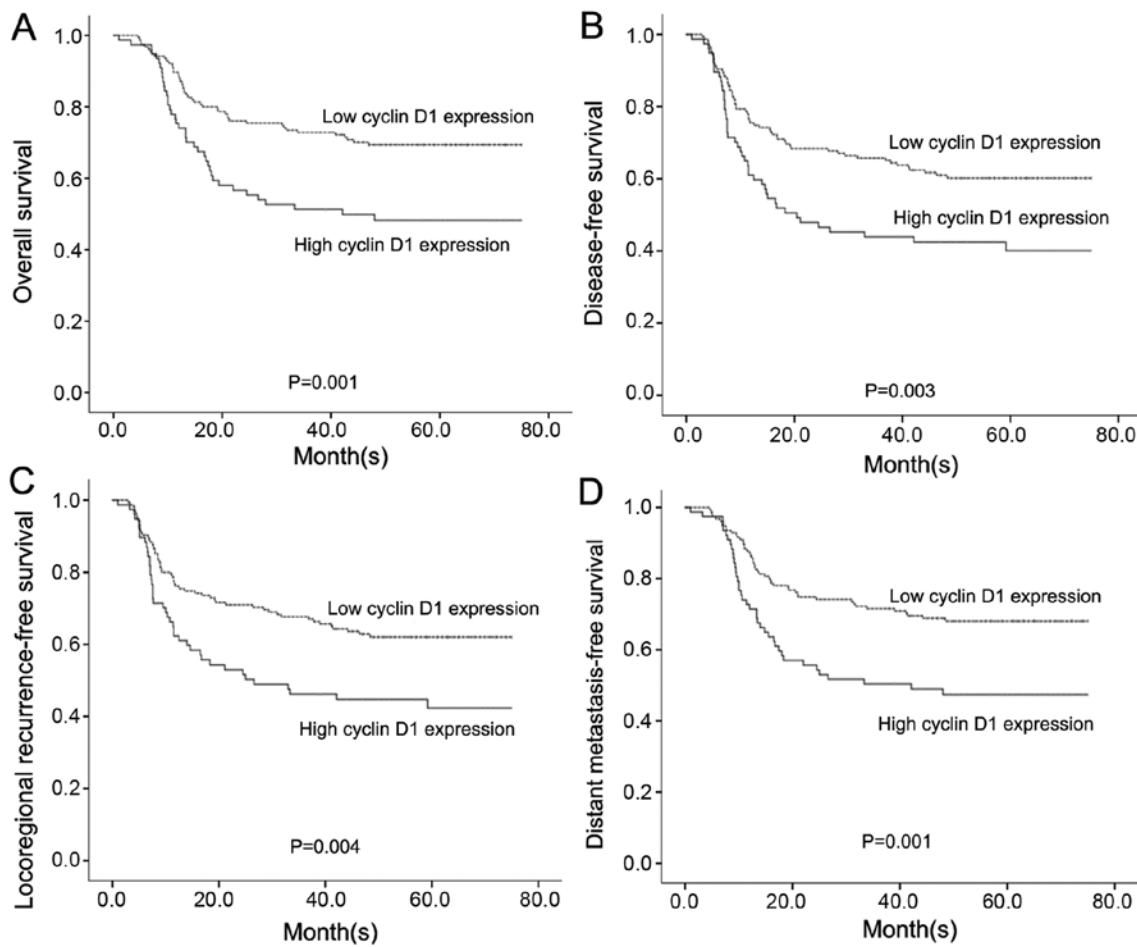


Figure 1. Survival analysis of patients with oral squamous cell carcinoma as a function of tumor cyclin D1 expression. Patients with low cyclin D1 expression exhibited superior (A) overall, (B) disease-free, (C) locoregional recurrence-free and (D) distant metastasis-free survival rates compared with those of patients with high cyclin D1 expression.

were then treated with the vector supernatant (5×10^7 TU/ml) and screened with puromycin (Life Technologies; Thermo Fisher Scientific, Inc.), which was added to the medium at a final concentration of $1 \mu\text{g/ml}$. Western blotting was applied to measure the expression levels of cyclin D1 (Fig. S1).

Western blotting assay. Total protein was extracted from collected cells (HB96, CAL27 or HN30) at 80% confluency and lysed in ice cold 2X lysis buffer containing 125 mM Tris-HCl (pH 6.8), 5% w/v SDS and 24.75% glycerol, as previously described (24). All procedures were performed on ice. Total protein concentration was determined using the Bradford assay according to the manufacturer's protocol (Pierce; Thermo Fisher Scientific, Inc.) Extracted proteins ($15 \mu\text{g/lane}$) were separated using 10-12% SDS-PAGE and then transferred electrophoretically onto $0.22\text{-}\mu\text{m}$ PVDF membranes (EMD Millipore) using a wet transfer system (Bio-Rad Laboratories, Inc.). The membranes were blocked with blocking buffer containing 5% dry skimmed milk in TBS with 0.02% Tween-20 at room temperature for 1 h and incubated overnight with primary antibodies at 4°C before being incubated with anti-mouse (1:5,000; cat. no. 7076; Cell Signaling Technology, Inc.) or anti-rabbit (1:5,000; cat. no. 7704; Cell Signaling Technology, Inc.) IgG secondary antibodies conjugated to horseradish peroxidase

at room temperature for 1 h for chemiluminescent detection (LumiBest ECL substrate solution kit; cat. no. sb-wb011; Share-Bio, Inc.). Finally, the PVDF membranes were scanned and analyzed using an enhanced chemiluminescence detection system (AmershamTM Imager 600; GE Healthcare). β -actin (1:10,000; cat. no. 1226; Cell Signaling Technology, Inc.) was used as a loading control. The primary antibodies used were as follows: Rabbit anti-cyclin D1 monoclonal antibody (1:500; cat. no. ab16663; Abcam); rabbit anti-cleaved fragment of human PARP (Asp214; 1:500; cat. no. 5625P; Cell Signaling Technology, Inc.) and cleaved fragment of caspase-3 (Asp175, 1:500; cat. no. 9664P; Cell Signaling Technology, Inc.).

Cytotoxicity assay and chemotherapeutic agents. Transfected cells (2×10^3 per well) were seeded into 96-well plates and cultured in $100 \mu\text{l}$ medium without glutamine and penicillin-streptomycin at 37°C for 8-12 h before being exposed to 2X, 3X or 10X drug gradient concentrations (depending on the respective IC_{50} for each cell line, which was calculated according to the cell viability after treatment with different drug gradient concentrations) of docetaxel, cisplatin or 5-FU at 37°C for 72 h. The supernatant of each well was then removed before $100 \mu\text{l}$ Cell Counting Kit-8 (CCK-8) solution was added according to the manufacturer's

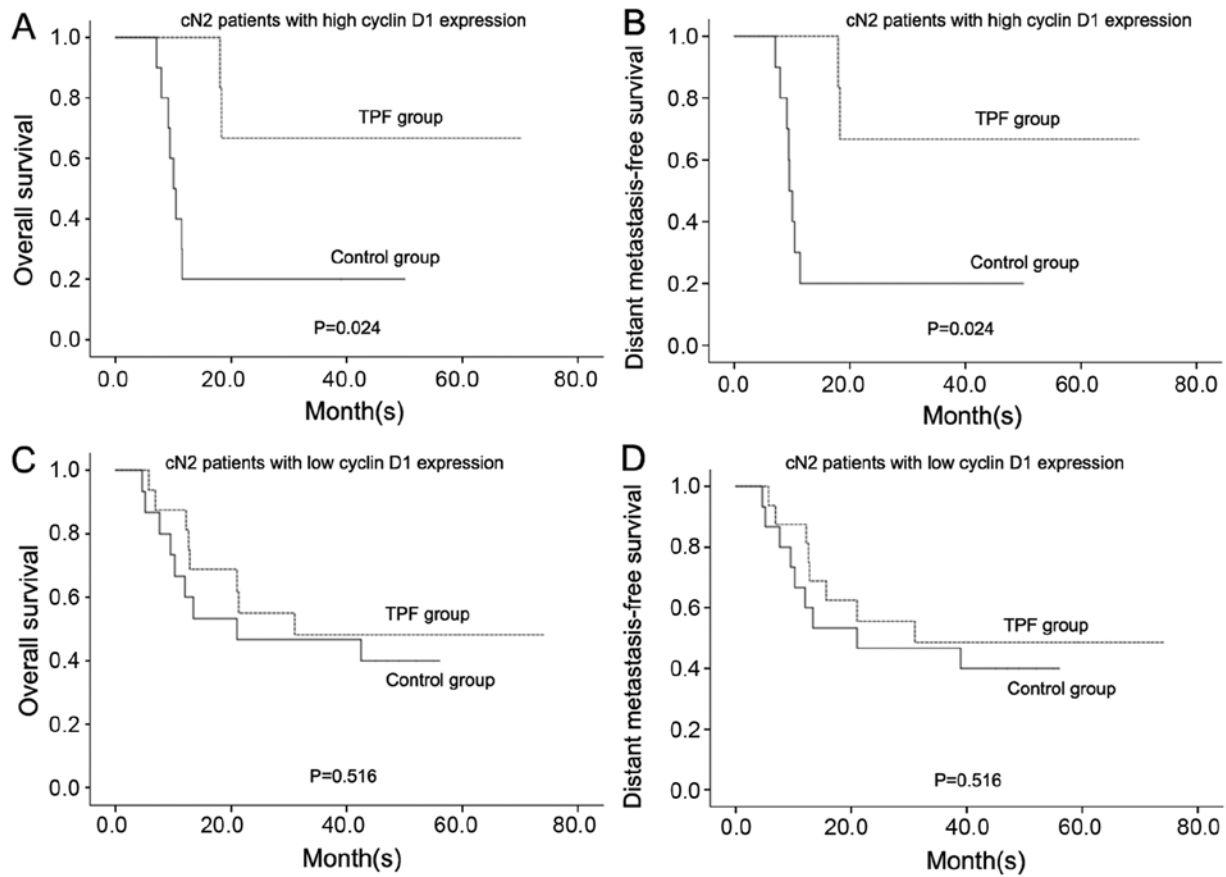


Figure 2. Survival comparison between patients with cN2 OSCC with low and high cyclin D1 expression in the experimental and control groups. Patients with cN2 OSCC and high cyclin D1 expression in the experimental group exhibited superior survival rates compared with those in patients in the control group with respect to (A) overall and (B) distant metastasis-free survival. Patients with cN2 OSCC and low cyclin D1 expression did not benefit from TPF induction chemotherapy with respect to (C) overall and (D) distant metastasis-free survival. OSCC, oral squamous cell carcinoma.

protocol (Dojindo Molecular Technologies, Inc.), consisting of fresh medium with 10% CCK-8 solution. Subsequently, the 96-well plates were incubated at 37°C for an additional 2 h. Absorbance values were then read at 450 nm, which was used to calculate cell viability. This experiment was performed in triplicate.

Statistical analysis. Data analysis was performed using SPSS18.0 for Windows (SPSS, Inc.). Following initial treatment, patients were monitored every 3 months for the first 2 years, every 6 months in the subsequent 3-5 years and once a year thereafter until death or censoring of data. OS was calculated from the date of random assignment to the date of death. Disease-free survival (DFS), locoregional recurrence-free survival (LRFS) and DMFS were calculated from the date of random assignment to recurrence, locoregional recurrence and distant metastasis, respectively, or death from any causes. Survival analysis was conducted using the Kaplan-Meier method and compared using log-rank test.

χ^2 test was performed to compare the differences between the low and high cyclin D1 expression groups based on the different baseline factors. Bonferroni test was performed following Kruskal-Wallis test for the comparison of non-parametric data. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Cyclin D1 expression and treatment outcomes. Among the 232 patients with OSCC, no significant differences could be found between the high and low cyclin D1 expression groups in terms of gender, age, primary tumor site, T stage, N stage, pathologic grade, tobacco use or alcohol consumption (Table SI). In accordance with IHC staining, patients with low cyclin D1 expression were defined as negative and weak positive cyclin D1 expression (<50% of stained cells), whilst high cyclin D1 expression was defined as strong positive cyclin D1 expression ($\geq 50\%$ of stained cells). During the follow-up period (first quartile, 55 months; median, 67 months; third quartile, 75 months), patients with low cyclin D1 expression exhibited significantly superior long-term clinical outcomes compared with those in patients with high cyclin D1 expression (Fig. 1; Table SII) with respect to OS ($P=0.001$), DFS ($P=0.003$), LRFS ($P=0.004$) and DMFS ($P=0.001$).

Subgroup analysis was subsequently performed to identify patients with different levels of cyclin D1 expression who may benefit from TPF induction chemotherapy with respect to long-term prognosis. Only patients with cN2 OSCC and high cyclin D1 expression, who were at high risk for distant metastasis and death, were found to be able to benefit from TPF induction chemotherapy. These patients benefited from TPF induction chemotherapy with respect to OS ($P=0.024$)

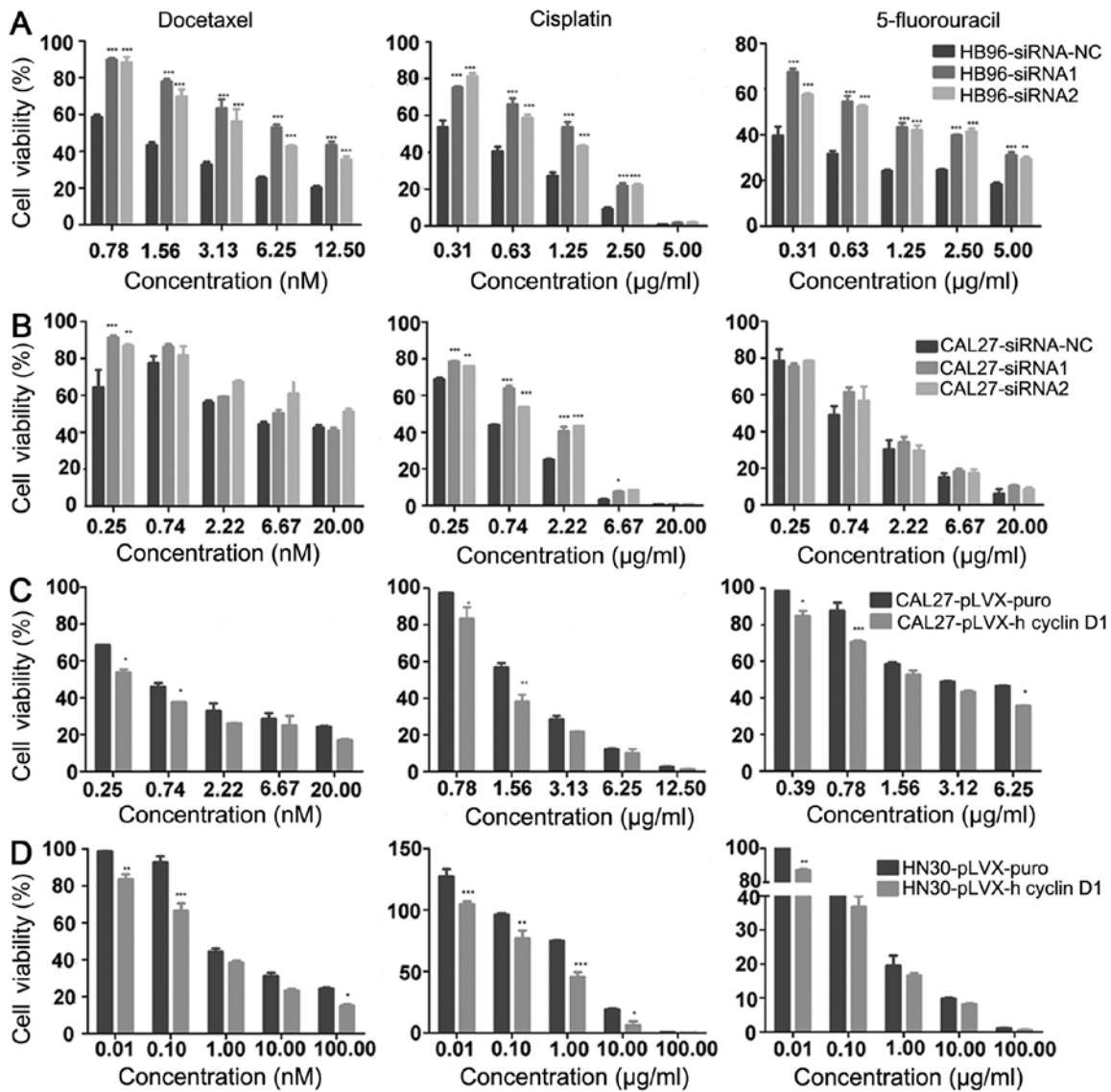


Figure 3. Association between cyclin D1 expression and sensitivity to chemotherapeutic agents docetaxel, cisplatin and 5-FU. After (A) HB96 and (B) CAL27 cells were transfected with siRNA1 and siRNA2 against cyclin D1, cell viability was measured following treatment with a series of concentrations of docetaxel, cisplatin and 5-FU for 72 h. After (C) CAL27 and (D) HN30 cells were transfected with cyclin D1 overexpression vectors, cell viability was measured following treatment with a series of concentrations of docetaxel, cisplatin and 5-FU for 72 h. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. NC. 5-FU, 5-fluorouracil; siRNA, small interfering RNA; NC, negative control.

and DMFS ($P=0.024$) whereas the patients with cN2 OSCC with low cyclin D1 expression did not benefit from TPF induction chemotherapy (Fig. 2).

Upregulation of cyclin D1 expression enhances sensitivity to docetaxel, cisplatin and 5-FU in OSCC cells. To support the clinical findings of patients with cN2 OSCC benefiting from TPF induction chemotherapy *in vitro*, the association between cyclin D1 expression and sensitivity to the TPF chemotherapeutic agents was analyzed in OSCC cell lines, especially the CAL27 cell line, which was originally established from a patient with cN2 oral tongue squamous cell carcinoma (25). CCK-8 assay was used to determine cell viability of OSCC cell lines after the down- or upregulation of cyclin D1 expression following treatment with chemotherapeutic agents docetaxel, cisplatin or 5-FU for 72 h at different concentrations, which was dependent on their respective IC_{50} values for each cell

line (Table SIII). Following the downregulation of cyclin D1 expression in HB96 cells, sensitivity to docetaxel, cisplatin or 5-FU was found to be significantly reduced compared with cells transfected with the siRNA-NC, resulting in increased cell viability at all doses in the siRNA groups (Fig. 3A). Following the downregulation of cyclin D1 expression in CAL27 cells, a significant reduction in the sensitivity to docetaxel and cisplatin was observed at low doses (0.25 nM for docetaxel and $< 2.5 \mu\text{g/ml}$ for cisplatin), but not at high doses, and no reduction in the sensitivity to 5-FU was observed at all doses (Fig. 3B). After the CAL27 and HB96 cells with cyclin D1 expression knocked down were treated with docetaxel, cisplatin and 5-FU altogether, increased cell viability was observed but the difference was not significant (Fig. S2). By contrast, when cyclin D1 was overexpressed in CAL27 and HN30 cells (this was not performed in HB96 cells since they already had high cyclin D1 expression), significantly increased sensitivity

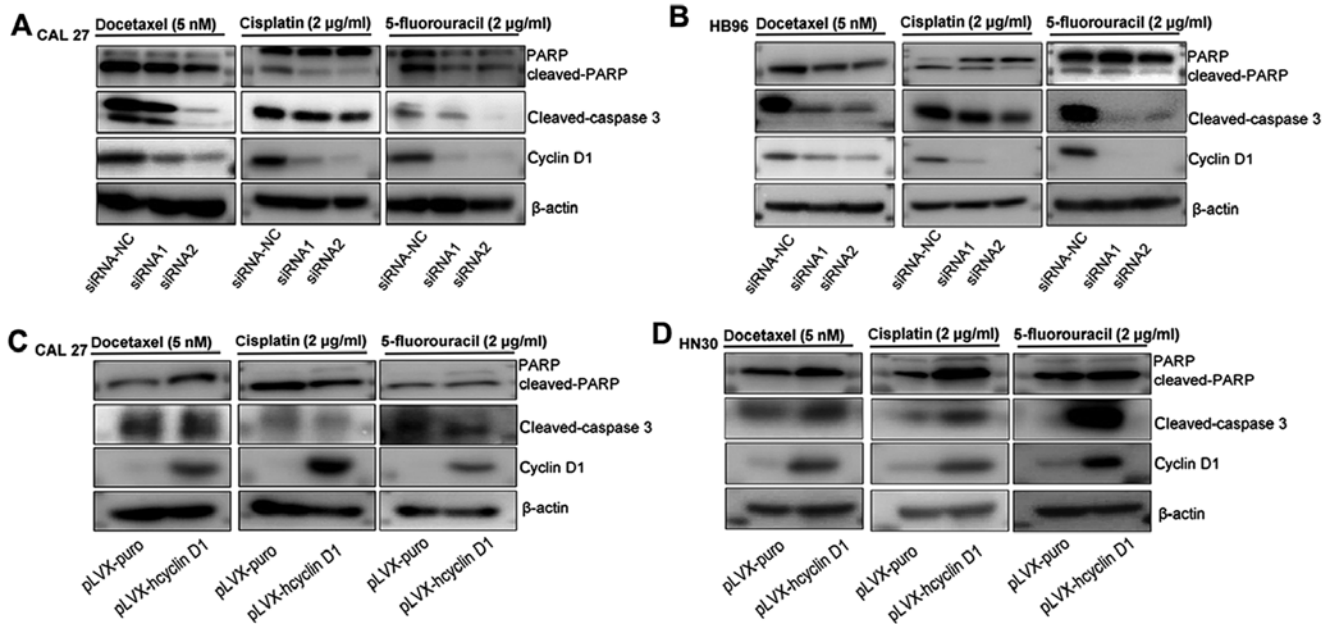


Figure 4. Association between cyclin D1 expression and cleaved caspase-3 and PARP levels following treatment with chemotherapeutic agents docetaxel, cisplatin and 5-FU. After (A) CAL27 and (B) HB96 cells were transfected with siRNA1 and siRNA2 against cyclin D1, cleaved caspase-3 and PARP protein levels were measured after treatment with 5 nM docetaxel, 2 μg/ml cisplatin or 2 μg/ml 5-FU for 72 h. After (C) CAL27 and (D) HN30 cells were transfected with the cyclin D1 overexpression vector, cleaved caspase-3 and PARP protein levels were measured following treatment with 5 nM docetaxel, 2 μg/ml cisplatin or 2 μg/ml 5-FU for 72 h. 5-FU, 5-fluorouracil; siRNA, small interfering RNA; NC, negative control; PARP, poly (ADP-ribose) polymerase.

to these agents was found compared with cells transfected with the empty vector, with decreased cell viability, especially at low doses (Fig. 3C and D).

Upregulation of cyclin D1 expression enhances sensitivity to docetaxel, cisplatin and 5-FU via caspase-3-dependent apoptosis in OSCC cells. Apoptotic protein levels (caspase-3 and PARP) were next measured in CAL27, HN30 and HB96 cells that were treated docetaxel, cisplatin and 5-FU. After CAL27 and HB96 cells transfected with cyclin D1 expression knocked down were treated with docetaxel, cisplatin and 5-FU for 72 h, the levels of cleaved caspase-3 and PARP levels were found to be reduced compared with cells transfected with siRNA-NC (Fig. 4A and B). By contrast, after the HN30 cells overexpressing cyclin D1 were treated with docetaxel, cisplatin and 5-FU for 72 h, cleaved caspase-3 and PARP levels were increased compared with cells transfected with the empty plasmid; however, in CAL27 cells overexpressing cyclin D1 treated with the three agents, an increase in cleaved PARP was observed in cells treated with docetaxel and 5-FU, but not in those treated with cisplatin, and no differences in cleaved caspase-3 with the three agents were observed (Fig. 4C and D).

Discussion

In the present study it was found that patients with cN2 OSCC and high cyclin D1 expression conferred long-term survival benefits from TPF induction chemotherapy compared with those who received standard treatment. By contrast, patients with cN2 OSCC and low cyclin D1 expression did not benefit from TPF induction chemotherapy compared with those who received standard treatment. *In vitro* studies subsequently confirmed that OSCC cells overexpressing cyclin D1 were

more sensitive to chemotherapeutic agents docetaxel, cisplatin and 5-FU via the caspase-3-dependent pathway.

Cyclin D1 serves an oncogenic role in the majority of malignant tumors, with previously documented roles including the inhibition of DNA repair, enhancements in cell proliferation and migration (26,27). Patients with cancer harboring high cyclin D1 expression have been reported to exhibit inferior clinical outcomes compared with those with low cyclin D1 expression, including breast cancer, pancreatic adenocarcinoma, colorectal carcinoma and OSCC (28,29). To determine the optimal treatment protocol with which to improve the clinical outcomes of patients with OSCC and high cyclin D1 expression, patients from a previous randomized trial (9) involving TPF induction chemotherapy in OSCC were chosen for the measurement of cyclin D1 expression in the pretreatment samples. Only patients with cN2 OSCC and high cyclin D1 expression benefitted from TPF induction chemotherapy compared with those who received standard treatment, whilst patients in other subgroups did not benefit from TPF induction chemotherapy. Of note, patients with cN2 OSCC have a relatively higher risk of distant metastasis compared with patients with cN0 and cN1 OSCC (9). OSCC cells with high cyclin D1 expression tend to be more aggressive compared with those with low cyclin D1 expression, which was demonstrated in previous studies, where cyclin D1 overexpression increased oral cancer cell migration and cell motility (13,30). Therefore, patients with cN2 OSCC and high cyclin D1 expression may have a high risk for distant metastasis. Induction chemotherapy has been previously shown to benefit patients with HNSCC with respect to DMFS (31,32). As demonstrated by results in the present study, patients with cN2 OSCC and high cyclin D1 expression exhibited higher DMFS after being treated with TPF induction chemotherapy compared with standard treatment, which translated into improvements in OS.

To explain the clinical benefit from TPF induction chemotherapy in Patients with cN2 OSCC, *in vitro* experiments on the sensitivity to TPF chemotherapeutic agents was performed in OSCC cell lines. The CAL27 cell line was originally derived from a patient with cN2 oral tongue squamous cell carcinoma (25). Sensitivity to the chemotherapeutic agents docetaxel, cisplatin and 5-FU in OSCC cells following cyclin D1 overexpression was found to be increased compared with cells transfected with empty plasmids. By contrast, sensitivity to these chemotherapeutic agents was decreased in OSCC cells following cyclin D1 knockdown compared with cells transfected with siRNA-NC. These findings suggest that the OSCC cells overexpressing cyclin D1 are more sensitive to docetaxel, cisplatin and 5-FU. The anticancer activity of cisplatin is to combine with DNA to form adducts by cross-linking, in turn inhibiting DNA replication and transcription, blocking G₂ phase entry or S/G₂ phase progression (33). By contrast, 5-FU inhibits the synthesis of pyrimidine by inhibiting thymidylate synthase, leading to the depletion of intracellular dTTP library (34). Docetaxel inhibits microtubule depolymerization to arrest the cell cycle at G₂/M phase and induce apoptosis (35). These molecular basis promotes the clinical application combining cyclin D1 overexpression with TPF induction chemotherapy. Although controversies remain regarding the association between cyclin D1 expression and responses to induction chemotherapy, the present study demonstrated a positive association between cyclin D1 overexpression and sensitivity to TPF induction chemotherapy in patients with cN2 OSCC. Akervall *et al* (17) previously studied 23 SCC cell lines and demonstrated that cyclin D1 overexpression is associated with favorable responses to cisplatin, which is in agreement with results from the present study. In addition, Perisanidis *et al* (36) analyzed the influence of cyclin D1 overexpression on the effectiveness of induction chemoradiotherapy with mitomycin and 5-FU, which found no differences in responses among patients with different cyclin D1 expression levels. However, in their cohort of patients, only seven patients were reported to be at the pathological N2 stage (36). Therefore, it was difficult to predict the clinical value of induction chemoradiotherapy compared with standard treatment in patients with cN2 OSCC.

To elucidate the potential mechanism underlying the increased sensitivity to the chemotherapeutic agents docetaxel, cisplatin and 5-FU in OSCC cells following cyclin D1 overexpression, cleaved caspase-3 and PARP protein levels were measured. In OSCC cells overexpressing cyclin D1 overexpression, increased cleaved caspase-3 and PARP levels were detected after the cells were treated with docetaxel, cisplatin and 5-FU, suggesting increased apoptosis. In OSCC cells following cyclin D1 knockdown, decreased cleaved caspase-3 and PARP levels were detected after treatment with these chemotherapeutic agents. Therefore, the increased sensitivity to docetaxel, cisplatin and 5-FU in OSCC cells following cyclin D1 overexpression may be due to activation of the caspase-3 pathway. Cyclin D1 overexpression has been reported to correlate with increased sensitivity to the chemotherapeutic agents fenretinide and bortezomib by activating apoptosis in breast cancer, rhabdoid tumors and lymphomas (37-39). Therefore, in some types of cancers overexpressing cyclin

D1, chemotherapeutic agents may exert their effects by activating apoptosis. In addition, the possibility of using chemotherapeutic agents or molecules targeting cyclin D1 have also been studied to treat patients with OSCC and cyclin D1 overexpression (13). However, the detailed mechanism of targeting cyclin D1 remains poorly understood and warrants further investigation.

The limitation of the present study is that the sensitivity experiments in OSCC cells and cyclin D1 intervention were performed using each of the three chemotherapeutic drugs alone, instead of combined treatment. Although many combinations with different concentrations have been attempted, differences in cell viability among the control, single agent and three agents altogether were not satisfactory. The possible reason is that these three agents all operate via different molecular mechanisms in OSCC cells. When added together into the OSCC cells following cyclin D1 manipulation, the mechanism became too complex to be elucidated fully, which requires further investigation. Another limitation of the present study is that data obtained using the OSCC cell lines for the *in vitro* experiments could not be translated into the patients with OSCC treated with the chemotherapeutic agents. Concentrations of chemotherapeutic agents used for the OSCC cells did not correspond to the concentrations obtained in the serum samples of patients with OSCC.

In conclusion, the present study demonstrated that patients with cN2 OSCC and high cyclin D1 expression exhibited long-term survival benefits from TPF induction chemotherapy compared with those who received standard treatment. In addition, OSCC cells overexpressing cyclin D1 were found to be more sensitive to TPF chemotherapeutic agents via the caspase-3-dependent pathway.

Acknowledgements

The authors would like to thank Professor Li Mao (University of Maryland Dental School, Baltimore, MD, USA) for providing the HN30 cell line as a gift.

Funding

The present study was supported by The National Natural Science Foundation of China (grant nos. 81972525 and 81672660), The Shuguang Program of the Shanghai Municipal Education Commission (grant no. 17SG18), The Shanghai Municipal Commission of Health and Family Planning (grant no. 2018BR41) and The Program of Shanghai Academic/Technology Research Leader (grant no. 19XD1422300).

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

LZ and ZZ were responsible for the study design, interpretation of the data and revision of the manuscript. YH and WS were

responsible for data acquisition, analysis of the work presented and the preparation of the manuscript. TZ, YL, DZ, LW, JL and CZ participated in the clinical management of patients and laboratory experiments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Review Board of the Ninth People's Hospital of Shanghai Jiao Tong University School of Medicine (Shanghai, China; approval nos. 2008-12 and 2014-41) and written informed consent was obtained from all participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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