

REDD1 overexpression in oral squamous cell carcinoma may predict poor prognosis and correlates with high microvessel density

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Abstract. The association between the hypoxia-inducible gene termed regulated in development and DNA damage responses 1 (REDD1) and microvessel density (MVD) in human oral cancer has rarely been reported. The present study aimed to explore REDD1 expression in oral squamous cell carcinoma (OSCC), its clinical prognostic significance and its correlation with angiogenesis. REDD1 expression in 23 pairs of fresh-frozen OSCC and matched peritumoral mucosal tissues was quantified by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting. Furthermore, 74 formalin-fixed paraffin-embedded OSCC tissues were collected to detect REDD1 expression and CD34-positive MVD by immunohistochemistry (IHC). The association between REDD1 expression and MVD, patients' clinicopathological characteristics and cancer-associated survival rate was also evaluated using the log-rank (Mantel-Cox) test. The results from RT-qPCR and western blotting demonstrated that REDD1 expression was significantly higher in OSCC tissues compared with peritumoral mucosal tissues ($P<0.05$). In addition, the results from IHC revealed that REDD1 expression was higher in OSCC tissues compared with peritumoral tissues. Furthermore, REDD1 expression was associated with advanced clinical

stage, poorer tumor differentiation, lymphatic metastasis and tumor recurrence ($P=0.000$, $P=0.003$, $P=0.006$ and $P<0.001$, respectively). Additionally, REDD1 overexpression was positively correlated with MVD ($r=0.7316$; $P<0.001$). The results from Kaplan-Meier survival analysis demonstrated a significantly reduced disease-free survival and overall survival in patients with OSCC and high REDD1 expression ($P<0.001$). REDD1 may therefore serve as a novel prognostic biomarker, a key regulatory checkpoint that could coordinate angiogenesis and a new therapeutic target for patients with OSCC.

Introduction

Tumor development is frequently associated with its micro-environment and activation of abnormal signaling pathways in the tumor, such as Hedgehog, Wnt, Notch, transforming growth factor- β and AKT (1). For example, poor oxygenation or hypoxia is commonly found in solid tumors and affects tumor angiogenesis, heterogeneity, tumor progression and sensitivity to radiotherapy or chemotherapy (1,2). This phenomenon is frequent in head and neck squamous cell carcinomas, in particular in oral squamous cell carcinomas (OSCCs) (3,4), including that of the mouth floor, buccal mucosa, alveolar ridge, posterior molar triangle and hard palate, where the degree of tumor hypoxia has a crucial influence on chemoradiotherapy resistance, prognosis and patients' overall survival (OS) (5-8). Although early diagnosis and treatment methods have been developed in the past few years, local recurrence and lymph node metastasis remain the major factors influencing the prognosis of patients with OSCC (9), and the 5-year survival rate has not significantly improved over the past several decades (60%) (10). Furthermore, advanced clinical manifestations, subtle symptomatology and rapid disease progression also contribute to OSCC poor prognosis (11,12). It is therefore crucial to identify novel biomarkers that could provide a deeper understanding of the molecular mechanisms involved in oral carcinogenesis and OSCC progression, in order to identify better diagnostic methods and more effective prognostic indicators.

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Under hypoxic conditions, cancer cells exhibit reduced oxidative metabolism and initiate the protection of ATP by limiting certain energy-consuming processes, including protein synthesis of hypoxia-inducible factor (HIF) and mTOR (13). The effect of hypoxia on protein synthesis is partly mediated by the inhibition of mammalian target of rapamycin complex 1 (mTORC1) kinase (14), which is a central regulator of cell proliferation and protein translation (15,16). The hypoxia-inducible gene named regulated in development and DNA damage response 1 (REDD1, also known as DDIT4/RTP801/Dig1), which was first discovered in 2002, is induced by hypoxia and other cellular stress as an upstream inhibitor of mTORC1 signaling and as an essential regulatory factor contributing to multiple DNA damage, which are widely expressed in many human tissues (17,18). REDD1, which encodes a serine-rich 232-amino acid cytoplasmic protein with an unknown functional domain, is localized to the human chromosome 10q24.33 and has one open reading frame and acid-coded prediction (18). REDD1-mediated mTOR inhibition occurs in cells exposed to hypoxia and cells responding to energy stress, and depends on the presence of the functional tuberous sclerosis (TSC)1/TSC2 inhibitory complex (19,20).

To the best of our knowledge, the role of REDD1 in human oral cancer remains unclear. The present study aimed therefore to determine REDD1 expression in OSCC tissues, to evaluate whether REDD1 expression could be considered as a novel therapeutic target and a key regulatory checkpoint in OSCC and to explore its association with patients' clinicopathological characteristics and survival rate. In addition, since hypoxia is common in OSCC (7), the present study evaluated the association between REDD1 expression and microvessel density (MVD) in order to further understand the underlying mechanism of REDD1 in OSCC.

Materials and methods

Patients and tissue specimens. A total of 23 pairs of fresh-frozen (stored in liquid nitrogen) OSCC tissues and matched peritumoral mucosal tissues (distance from tumor edge, >2 cm) obtained from 23 patients with OSCC between January 2017 and December 2018, and 93 formalin-fixed paraffin-embedded tissue samples (74 primary OSCC and 19 peritumoral mucosa) obtained from 74 patients with OSCC between January 2007 and December 2012, were collected from the Affiliated Hospital of Qingdao University. Histopathological evaluation was performed independently by two pathologists. All diagnoses were made according to the pathology criteria of head and neck tumors of the 4th edition of the World Health Organization classification of tumors in 2017 (21). Clinical, demographic and pathological data from all patients are listed in Tables I and II. The study was approved and supervised by the Institutional Medical Ethics Committee of the Affiliated Hospital of Qingdao University, and written informed consent was obtained from each patient. Tumors were staged and graded according to clinical TNM stage and histologic grade of the National Comprehensive Cancer Network (NCCN) guidelines (22). There were 34 stage I/II tumors and 40 stage III/IV tumors. The 74 patients were followed up by interview at the clinic or by telephone for 5 years. The last follow-up time-point was in December 2017, where 26 patients

had died and 48 patients were alive. Among the 74 patients, 18 had tumor recurrence. The median age of the patients was 57.8 years (age range, 36-79 years).

Total RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA isolation and RT-qPCR were performed as described previously (23,24). Total RNA was extracted from 23 fresh-frozen OSCC and matched peritumoral mucosal tissues using an RNAPrep pure tissue kit (Tiangen Biotech Co., Ltd.) according to the manufacturer's instructions. Each sample was performed in triplicate. The concentration and integrity of RNA preparations were assessed with an EON analyzer (BioTek Instruments, Inc.) using Gene5 software (version 5; BioTek Instruments, Inc.). The sequences of the primers used were as follows: REDD1 forward, 5'-GAGCCTGGAGAGCTCGGACT-3' and reverse, 5'-CTGCATCAGGTTGGCACACA-3'; and β -actin forward, 5'-CCCTGGAGAAGAGCTACGAG-3' and reverse, 5'-GGAAGGAAGGCTGGAAGAGT-3' (Genomics). RT-qPCR amplification reactions were performed using a PCR Real-Time system (Bio-Rad CFX96; Bio-Rad Laboratories, Inc.) as follows: Denaturation at 95°C for 15 min, followed by 39 cycles of 95°C for 10 sec, 53°C for 30 sec and 72°C for 30 sec, with a final extension at 72°C for 10 min. The relative expression levels of REDD1 were normalized to the endogenous control β -actin and were expressed as $2^{-\Delta\Delta C_q}$ (25). Each run included a standard curve and a buffer blank control without template to test for contamination of analytical reagent. PCR products were also visualized using an AlpaImagerHP System (ProteinSimple) following electrophoresis on 1% agarose gels with ethidium bromide dye.

Western blotting. Tissue proteins from 23 fresh-frozen OSCC and matched peritumoral mucosal tissues were extracted as previously described (26). The tissue of each sample (20 mg) was rapidly lysed in an ice-cold RIPA lysis containing protease and phosphatase inhibitor (Beyotime Institute of Biotechnology) for 30 min. The lysates were centrifuged at 12,000 \times g for 25 min at 4°C. The protein concentration was measured using the Micro BCA Protein Assay kit (Beyotime Institute of Biotechnology). Protein was denatured by boiling for 10 min before electrophoresis. The protein sample (20 μ g) was separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were blocked with TBS containing 0.1% Tween-20 and 5% skimmed milk powder on a shaker for 2 h at room temperature, and incubated overnight at 4°C with the following primary antibodies: Polyclonal rabbit anti-REDD1 (1:1,000; cat. no. 10638-1-AP; ProteinTech Group, Inc.) and monoclonal mouse anti- β -actin (1:5,000; cat. no. 66009-1; ProteinTech Group, Inc.). Membranes were then incubated with horseradish peroxidase-conjugated secondary antibody (1:5,000; cat. no. SA00001-1; ProteinTech Group, Inc.). Clarity™ Western enhanced chemiluminescence substrate and imaging system (Bio-Rad Laboratories, Inc.) were used to detect the signal on the membrane. The data were analyzed via densitometry using ImageJ software V1.6.0 (National Institutes of Health) and normalized to expression of the internal control β -actin. Each experiment was repeated three times and the data represent the means of the three experiments.

Table I. Clinicopathological characteristics of the 23 patients with oral squamous cell carcinoma.

Clinicopathological characteristics	n (%)
Age, years	
≤57.8	11 (47.83)
>57.8	12 (52.17)
Sex	
Male	16 (69.57)
Female	7 (30.43)
Tumor size, cm	
≤4	13 (56.52)
>4	10 (43.48)
TNM stage	
I, II	10 (43.48)
III, IV	13 (56.52)
Histological grade	
G1	12 (52.17)
G2	9 (39.13)
G3	2 (8.70)
Lymphatic metastasis	
Negative	9 (39.13)
Positive	14 (60.87)

Tissue microarray construction and immunohistochemistry (IHC). After interpreting the known pathological results of each paraffin-embedded tissue block, tissue microarrays (TMAs) were constructed by selecting one representative block from each case specimen and taking two core tissue regions from morphologically representative areas of the block (26). Several different TMA blocks were constructed with 1-mm-diameter cylinders in new wax (97.5% paraffin and 2.5% beeswax mixed at 55°C for 10 min), each containing 42 cylinders. The blocks then were sectioned at a thickness of 4 µm and placed on slides coated with 3-aminopropyltriethoxysilane for immunohistochemistry (IHC).

IHC was performed on TMA sections as previously described (24). The primary antibodies used for IHC were as follows: Monoclonal rabbit anti-CD34 (1:200; cat. no. ab81289; Abcam) and polyclonal rabbit anti-REDD1 (1:100; cat. no. 10638-1-AP; ProteinTech Group, Inc.). Following deparaffination and rehydration, slides were heated for 5 min at 95°C for antigen retrieval in a microwave oven in EDTA buffer. The sections were then washed with phosphate buffer saline (PBS), and the endogenous peroxidase activity was blocked with 3% H₂O₂ for 10 min at room temperature. Following PBS washes, slides were blocked with 0.5% BSA at room temperature for 20 min, respective primary antibodies were incubated with anti-REDD1 or anti-CD34 antibodies overnight at 4°C in a humidity chamber, followed by the addition of the secondary antibody (1:1,000; cat. no. SA00001-1; ProteinTech Group, Inc.) at 37°C for 30 min. Following PBS washes for 3 min, the reaction product was visualized using 3'-diaminobenzidine substrate kit (Dako; Agilent Technologies, Inc.) for 5 min at room temperature. Subsequently, the sections

were counterstained with hematoxylin for 2 min at room temperature. Negative control sections were incubated with PBS instead of primary antibody. The slides were covered, sealed and examined under a light microscope (Olympus Corporation). Five fields of view (magnification, x100) were randomly selected and photographed. IHC staining for REDD1 and CD34 was independently analyzed by two pathologists, without knowledge of the patients' clinical information. The IHC scoring criteria for REDD1 used in the present study was the same as previously described (26). The scaled scores were based on the estimated proportion of positive tumor cells and analyzed by Image Pro Plus 6.0 (Media Cybernetics, Inc.). The scores were classified as 0, 1, 2, 3 or 4 for 0, <10, 10-33, 33-66 or >66% of positively stained cells proportion, respectively. The score intensity indicated the average intensity of the positive tumor cells and was defined as negative, weak, intermediate or strong, for scores of 0, 1, 2 or 3, respectively. The proportion of positively stained cells and intensity scores were then added to obtain a total score ranging between 0 and 7. All specimens were divided into three groups for further statistical analyses according to the following criteria: i) Negative/weak expression, 0-3 points; ii) moderate expression, 4-5 points; and iii) strong expression, 6-7 points.

MVD was calculated according to the positive staining of CD34 marker on vascular endothelial cells (VECs) according to the method described by Weidner *et al* (27). The number of highest density area of CD34-positive expressing cells was designated as hot spots at low magnification, and VEC cluster that was clearly distinguished from the surrounding tumor cells and connective tissue was counted as a microvessel in high power fields. The microvessel numbers in five high power fields of each hot spot and in five hot spots of each section were recorded as the MVD value. In the present study, the median MVD value of all specimens was used to distinguish between tissues with high and low levels of vascular expression.

Statistical analysis. All data were statistically analyzed using SPSS statistical software version 17.0 (SPSS, Inc.). The results are expressed as the mean ± standard deviation. Unpaired Student's t-tests were used for comparisons of two groups of data. Multiple comparisons were evaluated by one-way ANOVA followed by Student-Newman-Keuls test. Pearson's χ^2 test was used to examine the association between REDD1 expression and the patients' clinicopathological characteristics, and to examine the association between MVD and the patients' clinicopathological characteristics, and was also used to compare REDD1 expression between OSCC and peritumoral mucosal tissues. Spearman's correlation analysis and linear regression were used to examine the correlation between MVD counts and REDD1 expression. Survival analysis according to the different REDD1 expression groups was performed using the Kaplan-Meier method and compared by the log-rank (Mantel-Cox) test. P<0.05 was considered to indicate a statistically significant difference.

Results

REDD1 mRNA expression in 23 fresh-frozen OSCC and matched peritumoral mucosal tissues. The mRNA expression level of REDD1 in OSCC tissues and matched peritumoral

Table II. Association between REDD1, MVD expression and clinicopathological characteristics of 74 patients with OSCC.

Clinicopathological characteristics	Cases (%)	REDD1 expression		P-value	MVD		P-value
		Negative/weak (0-3)	Moderate/strong (4-7)		≤35	>35	
Age, years							
≤57.8	36 (48.65)	22	14	0.610	20	16	0.481
>57.8	38 (51.35)	21	17		18	20	
Sex							
Male	50 (67.57)	29	21	0.978	27	23	0.511
Female	24 (32.43)	14	10		11	13	
Tumor size, cm							
≤4	42 (56.76)	25	17	0.777	22	20	0.839
>4	32 (43.24)	18	14		16	16	
TNM stage							
I, II	34 (45.95)	28	6	<0.001	22	12	0.034
III, IV	40 (54.05)	15	25		16	24	
Histological grade							
G1	39 (52.70)	29	10	0.003	25	14	0.011
G2	28 (37.84)	13	15		12	17	
G3	7 (9.46)	1	6		1	5	
Lymphatic metastasis							
Negative	40 (54.05)	29	11	0.006	27	13	0.003
Positive	34 (45.95)	14	20		11	23	
Tumor recurrence							
Yes	18 (24.32)	0	18	<0.001	3	15	0.002
No	56 (75.68)	43	13		35	21	
OSCC tissues	74	43 (58.1%)	31 (41.9%)	<0.001	38	36	
Peritumoral mucosa	19	19 (100%)	0 (0%)				

MVD, microvessel density; OSCC, oral squamous cell carcinoma; REDD1, regulated in development and DNA damage responses 1; TNM, Tumor-Node-Metastasis.

mucosa was detected by RT-qPCR. The results demonstrated that REDD1 mRNA level was significantly higher in the 23 OSCC tissues compared with matched peritumoral mucosa (Figs. 1A and E and S1; $P<0.05$). Furthermore, the mRNA expression level of REDD1 was associated with histopathological grade. In particular, the difference in REDD1 expression in highly, moderately and poorly differentiated (G1, G2 and G3) OSCCs was statistically significant (Fig. 1B and F; $P<0.01$). The melting curves for REDD1 expression are presented in Fig. 1C and D to show that it had no non-specific amplification.

Western blotting analysis of REDD1 expression in primary OSCC and matched mucosal tissues. The protein expression of REDD1 varied greatly among the 23 fresh-frozen OSCC tissues. Compared with matched peritumoral mucosa tissues, the protein expression of REDD1 was significantly increased in the 23 fresh-frozen OSCC tissues (Figs. 2A and 2C and S2; $P<0.001$). In addition, there were statistically significant differences of REDD1 expression in highly, moderately and poorly differentiated (G1, G2 and G3) OSCCs (Fig. 2B and D; $P<0.01$).

REDD1 and CD34 IHC in OSCC and peritumoral mucosal tissues. REDD1 and CD34 IHC staining was performed on 74 primary OSCC and 19 peritumoral mucosal specimens. The results demonstrated that REDD1 was mainly expressed in tumor cells from OSCC tissues, whereas CD34 was mainly expressed in VECs of blood vessels (Figs. 3A-D and 4A-F). The expression of REDD1 protein was different in tumors and peritumoral tissues, and exhibited heterogeneity in tumor tissues. The percentage of OSCC tissues with negative/weak and moderate/strong REDD1 expression was 58.1% (43/74; Fig. 3B; Table II) and 41.9% (31/74; Fig. 3C and D; Table II), respectively; however, REDD1 expression was weak or negative in all peritumoral mucosal tissues (0/19; Fig. 3A; $P<0.001$; Table II). In addition, REDD1 expression was significantly increased in G2 and G3 OSCC tissues compared with G1 tissues in a grade-dependent manner ($P<0.01$ and $P<0.001$, respectively; Fig. 3E and F). Furthermore, the median MVD count was 35 among all OSCC tissues, and the numbers of samples with low and high MVD were 38 (of 74; $MVD \leq 35$) and 36 (of 74; $MVD > 35$), respectively.

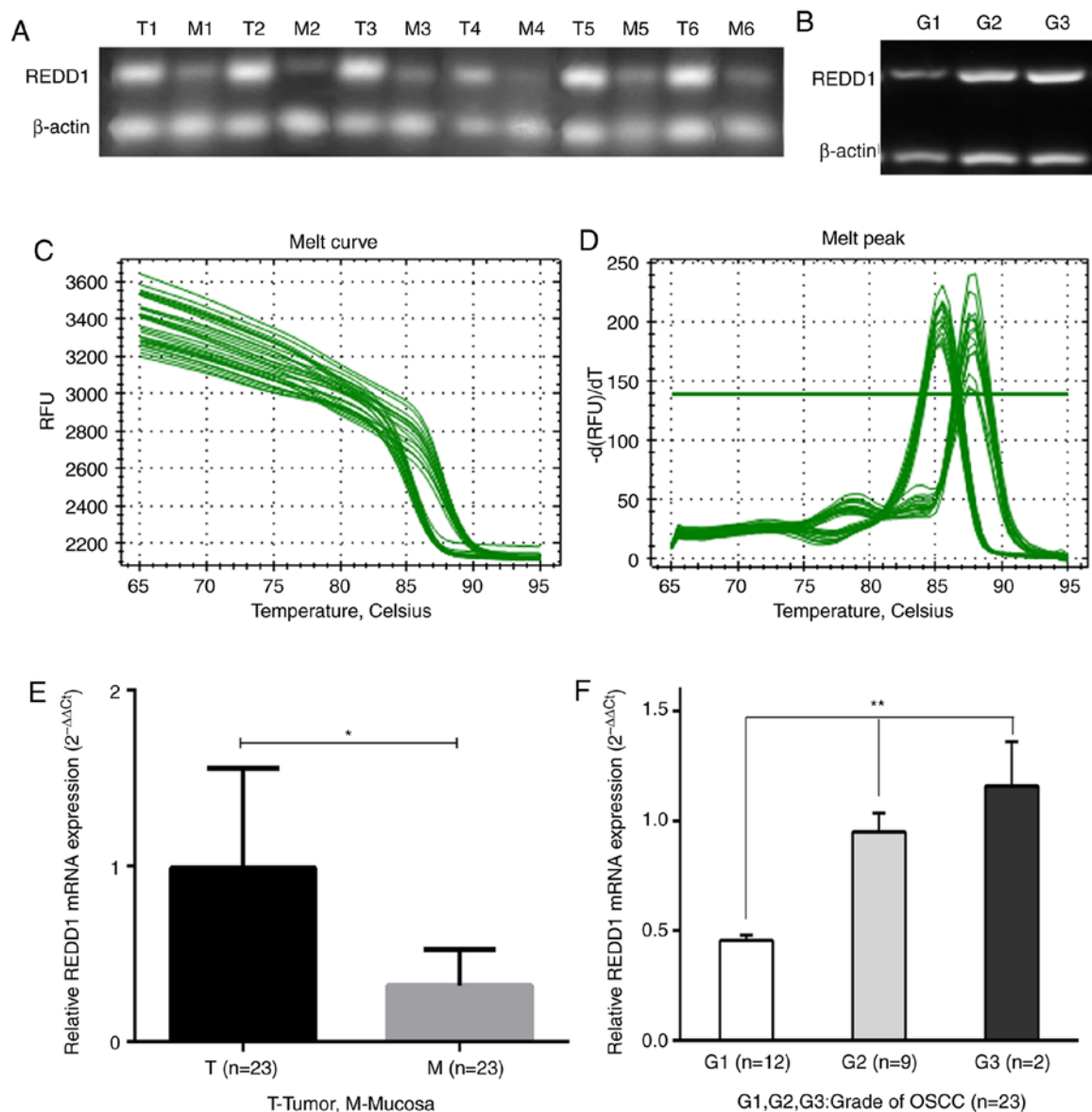


Figure 1. High mRNA expression of REDD1 in OSCC tissues. (A) Representative bands of REDD1 and β -actin in OSCC and matched peritumoral mucosa tissue following agarose gel electrophoresis and RT-qPCR. REDD1 expression level was higher in OSCC tissue (T1-T6) compared with matched peritumoral mucosa (M1-M6). (B) Representative bands of REDD1 mRNA relative to β -actin levels in OSCC tissues of different histological grades. REDD1 mRNA expression was higher in poorly-differentiated OSCC tissue (G3) compared with highly-differentiated OSCC (G1). (C) Melt curves for the expression of REDD1 and β -actin were assessed in OSCC tissue and matched peritumoral mucosa by RT-qPCR. (D) Melt peak showed a single peak indicating the specific amplification of mRNA. (E) Evaluation of Ct values for RT-qPCR expressed as $2^{-\Delta\Delta C_t}$. The relative expression of REDD1 was significantly higher in 23 OSCC tissues compared with the matched peritumoral mucosa. * $P < 0.05$. (F) Relative expression ($2^{-\Delta\Delta C_t}$) of REDD1 was significantly higher in poorly-differentiated OSCC tissue (G3, G2) compared with highly-differentiated OSCC (G1) in 23 fresh-frozen OSCC tissues. ** $P < 0.01$. M, mucosa; OSCC, oral squamous cell carcinoma; REDD1, regulated in development and DNA damage responses 1; RFU, relative fluorescence unit; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; T, tumor.

Associations between REDD1 expression or MVD and patients' clinicopathological characteristics. VECs staining for CD34 in areas of neovascularization was used to detect the MVD in OSCC specimens. The association between REDD1 expression or MVD count and patients' clinicopathological characteristics was analyzed in 74 OSCC tissues. The results demonstrated that both REDD1 expression and MVD counts were significantly associated with clinical TNM stage ($P < 0.001$ and $P = 0.034$, respectively), histological grade ($P = 0.003$ and $P = 0.011$, respectively), lymphatic metastasis ($P = 0.006$ and $P = 0.003$, respectively) and tumor recurrence ($P < 0.001$ and $P = 0.002$, respectively; Table II). However, REDD1 expression

and MVD counts were not associated with age, sex and tumor size ($P > 0.05$; Table II).

Correlation between REDD1 expression and MVD in OSCC tissues. To investigate the correlation between REDD1 expression and MVD in OSCC tissues, Spearman's correlation analysis and linear regression were used. Among the 43 cases of OSCC with negative/weak REDD1 expression, 33 presented with low MVD and the other 10 cases exhibited high MVD (Table III, Fig. 4A and B). Conversely, in the 31 samples with moderate/strong expression, 26 had low MVD, whereas the other five cases presented with a low MVD count (Table III,

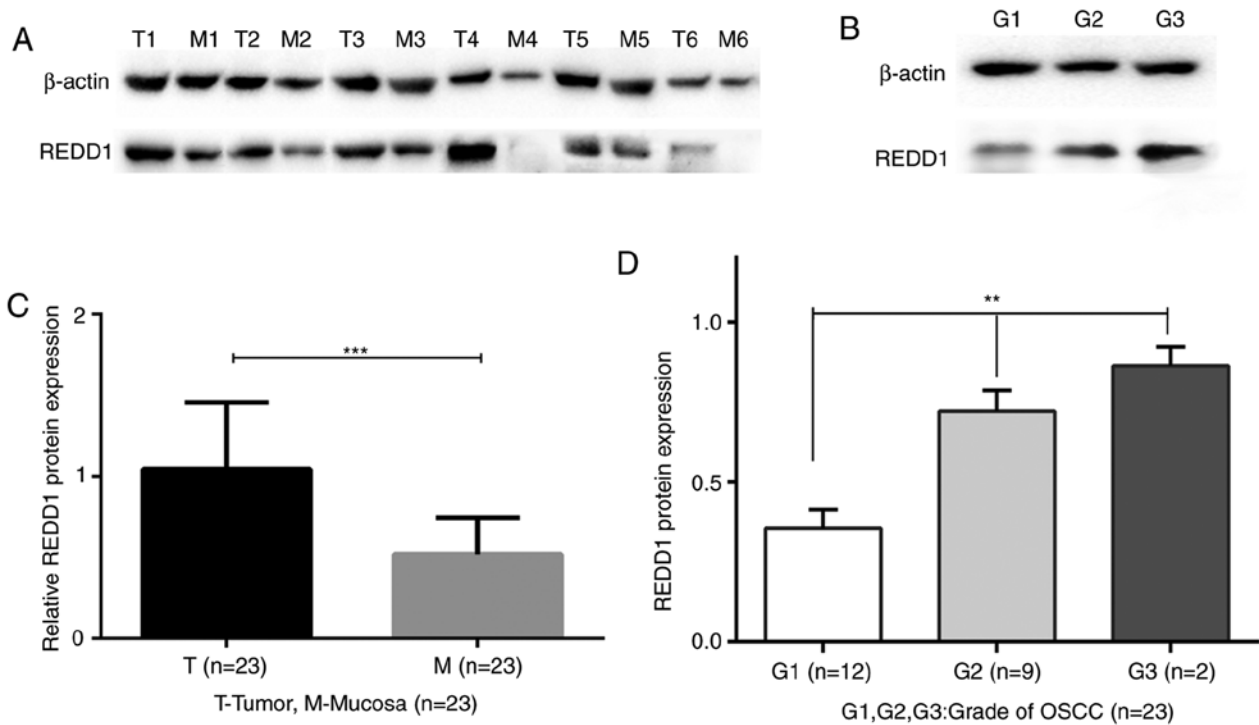


Figure 2. REDD1 is overexpressed in OSCC tissues. (A) REDD1 was highly expressed in OSCC tissues (T1-T6), but not in matched peritumoral mucosa (M1-M6). (B) Representative REDD1 expression was different in highly, moderately and poorly differentiated (G1, G2 and G3) OSCCs in 23 fresh-frozen OSCC tissues. (C) Relative REDD1 expression was significantly higher in the 23 OSCC tissues compared with the 23 matched peritumoral mucosa. (D) REDD1 expression was significantly higher in poorly-differentiated OSCC tissue (G3 and G2) compared with highly differentiated OSCC (G1) in 23 fresh-frozen OSCC tissues. ** $P < 0.01$ and *** $P < 0.001$. M, mucosa; OSCC, oral squamous cell carcinoma; REDD1, regulated in development and DNA damage responses 1; T, tumor.

Fig. 4C-F). The results demonstrated that REDD1 expression and MVD count were positively correlated in OSCC samples ($P < 0.0001$; $r = 0.7316$; Fig. 4G). Furthermore, moderate or strong REDD1 expression in tumors was significantly correlated with higher MVD, compared with weak or negative REDD1 expression in tumors ($P < 0.0001$; Table III). These results suggested that REDD1, as an upstream gene of MVD, may be considered as a key regulatory checkpoint that could coordinate vascular growth signaling inputs.

Kaplan-Meier analysis of disease-free survival (DFS) and OS in patients with OSCC according to REDD1 expression. The results demonstrated that patients with negative/weak REDD1 expression presented significantly increased DFS and OS rates compared with patients with moderate/strong REDD1 expression ($P < 0.0001$, respectively; Fig. 5A and B). REDD1 overexpression may therefore serve as a biomarker for prognosis of patients with OSCC.

Discussion

REDD1 protein is present at low level in most mature tissues (18). Previous studies reported that REDD1 could be a transforming oncogene in solid cancer types, such as pancreatic, ovarian and breast cancer (28-30). The results from the present study demonstrated that REDD1 expression was significantly higher in OSCC tissues compared with peritumoral mucosa. In addition, poorly differentiated OSCC was associated with higher REDD1 expression, whereas it was not the case for highly differentiated OSCC. Furthermore, patients

with high REDD1 expression had a more advanced clinical stage, higher rate of lymphatic metastasis and recurrence, and shorter DFS and OS. These results suggested that REDD1 may function as an oncogene in OSCC, and that changes in REDD1 expression may serve a role in oral carcinogenesis and OSCC progression, which is consistent with findings from previous studies in prostate cancer (31), breast cancer (32) and ovarian carcinoma (33). In addition, the present study demonstrated that REDD1 expression in low grade OSCC (G1) was lower than that in high grades (G2 and G3) OSCC, which was consistent with previous studies reporting (32,33) that REDD1 expression is reduced in certain slow-growing tumors, including certain low-grade ovarian cancers (34).

Hypoxia is known to be present during tumor growth, such as ovarian, colorectal, brain cancer and small cell lung cancer (13,35), which is also the case in OSCC. Since hypoxia and increased oxidative stress can induce REDD1 overexpression (18), REDD1 gene was initially identified as a stress response gene. However, REDD1 was subsequently confirmed to also be induced in response to glucocorticoid treatment (36), nutrient deprivation (37) and other stress conditions (19,38,39). Because one reason is that tumor growth occurs at a faster rate than angiogenesis, hypoxia and nutrient deficiencies are known to persist in the cancer microenvironment (35). Under continuous hypoxia, cancer cells exhibit reduced oxidative metabolism and self-limiting energy expenditure (13), which stimulates REDD1 protein synthesis. In the present study, the results from IHC demonstrated that high REDD1 expression was significantly associated with increased MVD. This may be due to the fact that angiogenesis occurs at a much slower

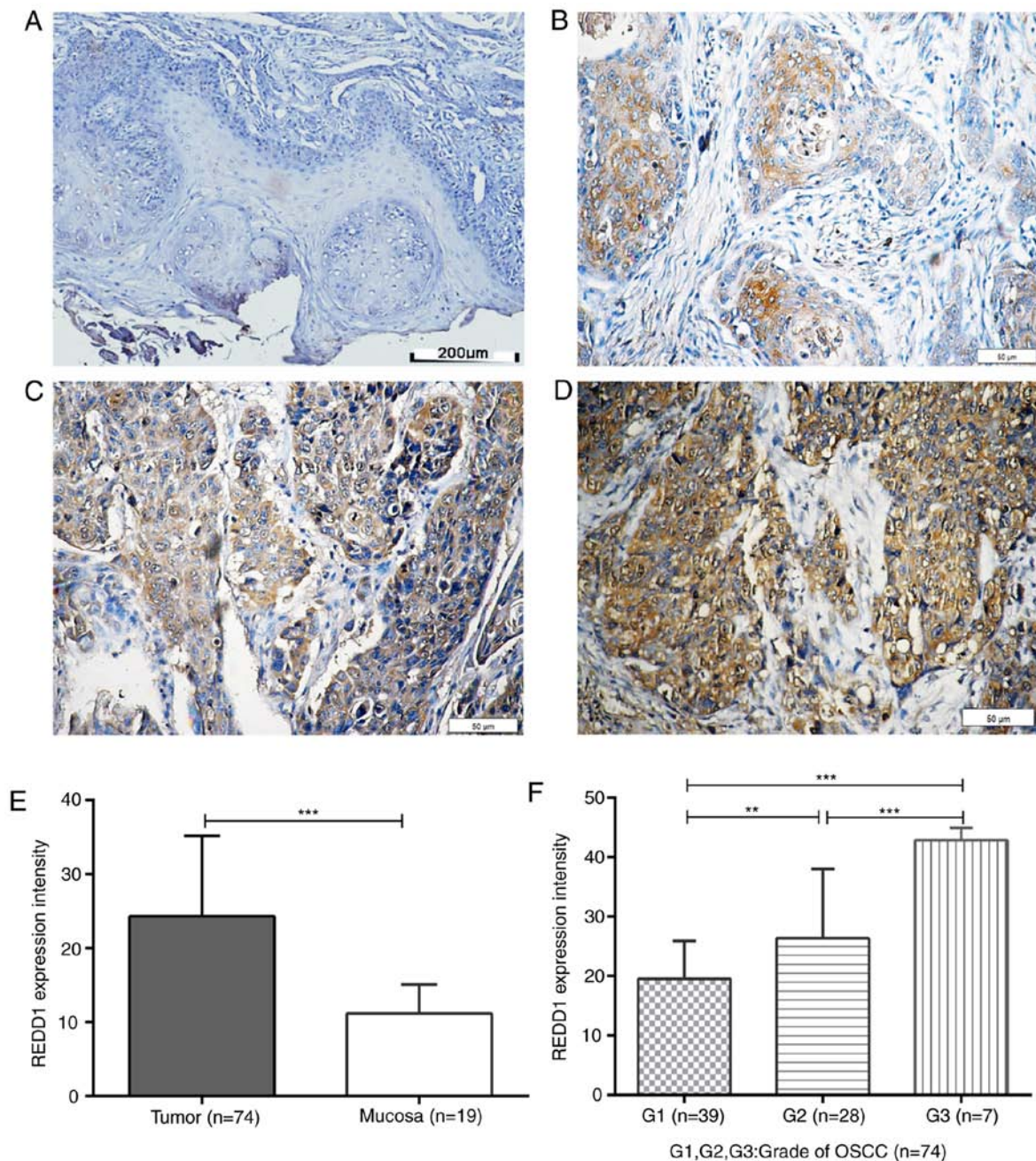


Figure 3. IHC staining for REDD1 in OSCC and peritumoral mucosal tissues. Paraffin-embedded sections of OSCC and adjacent tissues were stained using an anti-REDD1 polyclonal antibody. (A) Representative REDD1 IHC staining of negative/weak expression in peritumoral mucosal specimen. Scale bar, 200 μ m. (B) Representative REDD1 IHC staining of weak expression in highly-differentiated OSCC tissue (G1). Scale bar, 50 μ m. (C) Representative REDD1 IHC staining of moderate expression in moderately-differentiated OSCC tissue (G2). Scale bar, 50 μ m. (D) Representative REDD1 IHC staining of moderate/strong-expression in poorly differentiated OSCC tissue. Scale bar, 50 μ m. (E) REDD1 expression intensity in 74 primary OSCC and 19 peritumoral mucosal specimens. (F) REDD1 expression intensity in differently-differentiated OSCC tissue (G1, n=39; G2, n=28; G3, n=7). **P<0.01, ***P<0.001. IHC, immunohistochemistry; OSCC, oral squamous cell carcinoma; REDD1, regulated in development and DNA damage responses 1.

rate than tumor growth, which inevitably diminishes the blood supply to the tumor, resulting in hypoxia and poor tumor nutrition, and increased REDD1 expression. Conversely, hypoxia also leads to angiogenesis in tumors (35). Despite active angiogenesis, tumor novel vessels are highly irregular and leaky and function poorly (35). Even in the case of vascular-rich tumors, these defective vascular properties induce tumor ischemia, resulting in continued hypoxia that leads to stable REDD1 expression (35), which is another reason that hypoxia leads to REDD1 overexpression. This phenomenon was also

observed in the study, although MVD expression was higher in the moderately and poorly differentiated OSCC tissues and REDD1 expression was increased accordingly.

REDD1 is expressed in response to numerous stress conditions and is also an important regulator of the response to a number of transcription factors, including p53, p63, activating transcription factor 4, Sp1 and HIF-1 (18,40,41). Previous studies reported that REDD1 expression is upregulated in numerous types of cancer, including ovarian cancer (33,34), breast cancer (32), pancreatic ductal adenocarcinoma (42) and

Table III. Correlation between REDD1 expression and MVD in 74 oral squamous cell carcinoma tissues.

REDD1 expression	Groups (%)	MVD		r	P-value
		≤35	>35		
Negative/weak (0-3)	43 (58.11)	33	10	0.7316	<0.0001
Moderate/strong (4-7)	31 (41.89)	5	26		

MVD, microvessel density; REDD1, regulated in development and DNA damage responses 1.

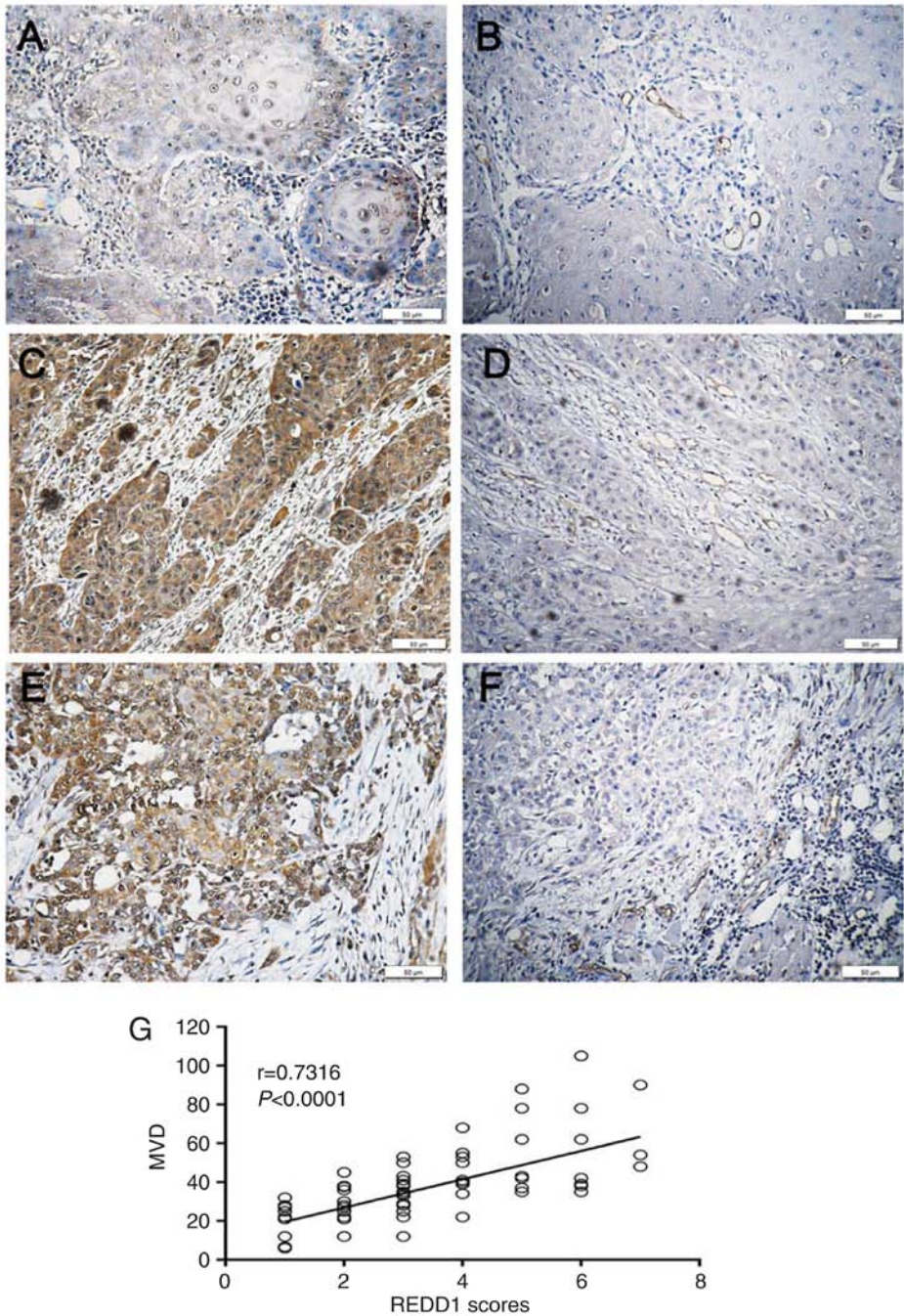


Figure 4. Representative image showing the positive association between REDD1 expression and MVD in OSCC tissues. CD34 was mainly expressed in vascular endothelial cells, which was assessed to determine MVD values. The median MVD count was 35. (A) REDD1 negative/weak expression in highly-differentiated tumors. (B) MVD ≤35 in highly-differentiated tumors. (C) REDD1 moderate expression in moderately differentiated tumors. (D) MVD >35 in moderately differentiated tumors. (E) REDD1 strong-expression in poorly-differentiated tumors. (F) MVD >35 in poorly-differentiated tumors. Scale bar, 50 μm. (G) Results demonstrated that REDD1 expression and MVD were positively correlated ($r=0.7316$; $P<0.001$). MVD, microvessel density; OSCC, oral squamous cell carcinoma; REDD1, regulated in development and DNA damage responses 1.

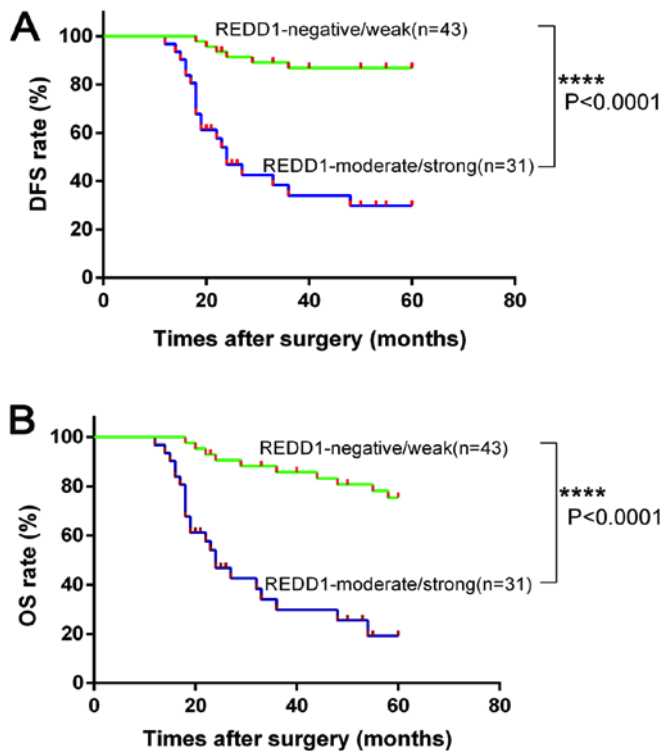


Figure 5. Kaplan-Meier analysis of DFS and OS in patients with oral squamous cell carcinoma. Patients with negative/weak expression of REDD1 had higher (A) DFS and (B) OS rates compared with patients with moderate/strong expression. **** $P<0.0001$. DFS, disease free survival; OS, overall survival; REDD1, regulated in development and DNA damage responses 1.

bladder urothelial carcinoma (43). REDD1-mediated signaling abnormalities may also disrupt energy homeostasis and regulation of tumorigenesis through multiple pathways (31). Hypoxia-induced mTOR regulation has been reported to be essential for the regulation of HIF activity and the regulation of HIF-induced REDD1 (20). The HIF pathway is therefore considered a master regulator of angiogenesis (35). However, to the best of our knowledge, correlation between high REDD1 expression and angiogenesis has not yet been investigated in OSCC. REDD1 activation can inhibit mTORC1 via TSC1/2, which acts as a negative regulator of mTORC1 activity (31,43). Simultaneously, REDD1 can also inhibit mTORC1 activity via the TSC1/2 complex in the H1299 lung cancer cell line (44). Similarly, the present study demonstrated that REDD1 expression was significantly higher in OSCC samples compared with normal tissue and that MVD was increased, indicating that HIF-inducible REDD1 may have a regulatory role in hypoxic signaling in OSCC. These findings suggested that mTORC1 inhibition may occur via the stable expression of REDD1, which may promote angiogenesis by modulating HIF transcription. Hypoxia and HIF activation have profound effects on tumor biology, and HIF-1 α and HIF-2 α are associated with numerous cancers, including cancers of the brain, breast, colon, head and neck, liver, lung, skin and pancreas (5,45,46), for which poor prognosis is associated with metastasis. The present study highlighted the importance of REDD1 in the hypoxia-dependent regulation of MVD. Further investigation on the association between REDD1, mTOR and HIF-1 α is therefore required. The results from this study also indicated

that, like hypoxia, REDD1 induction may stimulate angiogenesis.

Defective angiogenesis and decrease in blood supply cause increased tumor glycolysis (22,47). Subsequently, cancer cells have a reduced oxidative metabolism and initiate ATP protection by limiting protein synthesis, which is an energy-consuming process (47,48). These cells can therefore exhibit chemoresistance and radioresistance, affecting the chemoradiosensitivity of the entire tumor and further reducing the 5-year survival rate of patients with cancer (49,50). Most patients with advanced OSCC need chemoradiotherapy following surgery; however, REDD1 overexpression may affect radiotherapy and chemotherapy efficacy. The findings from the present study suggested that patients with OSCC and high REDD1 expression may have a poor prognosis, be prone to recurrence, would exhibit reduced sensitivity to postoperative radiochemotherapy, as previously described (43,51,52). This hypothesis will be further investigated in a future study. Although the present study revealed that REDD1 overexpression increases MVD in OSCC tissues, highly irregular, leaky and dysfunctional blood vessels might not increase sensitivity to chemotherapy and radiotherapy (35). The present study demonstrated that patients with OSCC and high REDD1 expression had poorer prognosis and shorter DFS and OS compared with patients with low REDD1 expression, suggesting that REDD1 expression may affect the 5-year survival of patients with OSCC. Thus, it can be concluded that the upregulation of this marker might predict poor survival in OSCC.

In conclusion, the present study demonstrated that the expression of the mTORC1 inhibitor REDD1 was positively correlated with tumor MVD. REDD1 may therefore be considered as a key regulatory checkpoint that could coordinate vascular growth signaling inputs. In addition, REDD1 overexpression may serve as a biomarker of adverse prognosis in OSCC progression. REDD1, which is a part of a network of signaling molecules comprising the mTOR pathway, may also represent a novel therapeutic target that could be used alone or in combination with other therapies targeting MVD, in order to stop the development and progression of OSCC.

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Availability of data and material

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

Authors' contributions

YF, KS and NW performed the experiments and wrote the manuscript. WS, LC and BP participated in the design of the study and collected data. CW and NW performed the analysis of the patients' clinicopathological characteristics. All authors contributed to the writing of the manuscript and approved the final version.

Ethics approval and consent to participate

All patients provided their full consent to participate in the present study. This study was approved by the Institutional Medical Ethics Committee of the Affiliated Hospital of Qingdao University, and all procedures were performed in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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