

Expression pattern of DKK3, dickkopf WNT signaling pathway inhibitor 3, in the malignant progression of oral submucous fibrosis

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Abstract. Oral squamous cell carcinoma (OSCC) is a common malignant neoplasm of the head and neck. Oral submucous fibrosis (OSF) is one of its pre-cancerous lesions; however, the key molecular events in the tumorigenesis of OSF remain elusive. Dickkopf WNT signaling pathway inhibitor 3 (DKK3) is one of the Wnt antagonists, and its downregulation and methylation have been reported in multiple malignancies, while no report of its expression in the carcinogenesis of OSF exists. In the present study, we investigated DKK3 expression at the protein and mRNA levels by immunochemical staining and semi-quantitative RT-PCR in normal oral, OSF and OSCC tissues. We found that DKK3 was readily expressed in normal oral mucous tissues, but was gradually increased in early, moderately advanced and advanced OSF tissues, and strongly expressed in OSCC tissues. DKK3 was localized in the cytoplasm during OSF progression. A rare mutation of DKK3 was observed in OSCC, along with increased copy numbers. Furthermore, through analysis of its co-expressed genes, DKK3 may deregulate Wnt signaling, p53 signaling, apoptosis, Ca²⁺ signaling and mitochondrial signaling pathways in OSCC pathogenesis. Thus, our results demonstrated that DKK3 is upregulated in the carcinogenesis of OSF, due to gain of copy number, which could be a potential tumor marker for the early detection of OSCC.

Introduction

Oral squamous cell carcinoma (OSCC) constitutes more than 90% of all malignant neoplasms in the oral cavity, with an increased number of new OSCC cases worldwide and varied geographical distribution (1,2). OSCC is prevalent in Southern Asia and Latin America, and is the most common cancer among the male population (3,4). In the high-risk areas, betel quid (BQ) chewing, tobacco and alcohol consumption have been well documented as etiologic factors for OSCC (4,5). A causal relationship between BQ chewing and the high incidence of oral cancer has been established, particularly its correlation with early oral premalignancies such as oral submucous fibrosis (OSF) (6,7). Despite the recent rapid improvement in multimodality treatment, the overall 5-year survival rate of OSCC remains poor. Thus, elucidation of molecular events during OSCC development and progression is needed for the early detection and therapy of OSCC.

Overactivation of Wnt/ β -catenin signaling has been identified in the tumorigenesis of multiple cancers including OSCC, and plays critical roles in cell proliferation, differentiation, adhesion and stemness (7,8). Wnt ligands, antagonists and mutations of APC and β -catenin have been well-defined for its activation. The dickkopf (DKK) family as Wnt antagonists, contains five members, DKK1, DKK2, DKK3, DKK4 and DKKL1, with different impacts on Wnt/ β -catenin signaling (9,10). However, the underlying mechanisms remain elusive. Notably, the secreted Wnt antagonist dickkopf-related protein 3 (DKK3) is emerging as a crucial regulator of human cancers (9). DKK3 is frequently reduced by promoter methylation in multiple solid and hematological cancers, acting as a functional tumor suppressor by affecting apoptosis and proliferation. Re-introduction of DKK3 expression in cancer cells may be a potential biomarker and effective therapeutic approach.

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There are several contradictory studies of *DKK3* in head and neck squamous cell carcinomas (HNSCC) and OSCC. Its tumor suppressive and oncogenic functions have been reported in OSCC (11-15). However, the expression pattern of *DKK3* in the malignancy progression of OSF has not been addressed. In the present study, we examined *DKK3* expression at the protein and mRNA levels in normal oral mucosa, OSF and OSCC tissues, as well as the correlation with clinicopathological features. We also analyzed its potential function in the pathogenesis of OSCC.

Materials and methods

Tissue specimens. OSCC (n=55), OSF (n=45), and normal oral mucosa (n=15) tissue specimens were obtained at the time of surgical resection at Xiangya Second Hospital and Xiangya Hospital, Central South University (Changsha, China) and Shanghai Ninth People's Hospital, Shanghai Jiaotong University School of Medicine (Shanghai, China) from January 2013 to June 2014. The patient informed consents were obtained under a protocol reviewed and approved by the Institutional Review Boards of the Xiangya School of Medicine or Shanghai Jiaotong University School of Medicine. The clinical diagnosis and pathologic stage of the OSF were determined in terms of the Pingborg criteria (16). OSCC was diagnosed according to the World Health Organization criteria of 2005. Fifteen normal specimens were obtained from healthy oral mucosa. Forty-five cases of OSF were incident, newly diagnosed without OSCC or neoplastic disease. OSF was classified into 3 grades: early stage (E, n=15), moderately advanced stage (M, n=15) and advanced stage (A, n=15). All collected tissues were divided into two parts, one of which was frozen immediately at -80°C after careful removal of the tumor mass, OSF tissue in the epithelium layer and grossly normal-looking epithelium, and the other part was fixed in 4% buffered formalin solution for pathologic diagnosis and immunohistochemical staining. Clinicopathologic staging of OSCC was determined by the TNM classification of the International Union Against Cancer in 2009. The results of the immunostained formalin-fixed, paraffin-embedded (FFPE) sections were evaluated separately by two pathologists (C.Z. and Z.Y. at the Department of Oral Pathology of the respective affiliate hospital).

Immunohistochemistry. Immunohistochemical staining was performed on 4- μ m serial sections from FFPE specimens. After deparaffinization and hydration, the slides were treated with endogenous peroxidase in 3% H₂O₂ for 20 min. The sections were then blocked for 30 min at 37°C with 1.5% blocking serum in phosphate-buffered saline (PBS) before reacting with *DKK3* (1:100 dilution; #10365-1-AP; ProteinTech) at 4°C in a moist chamber overnight. Negative control slides were duplicate sections in the absence of primary antibodies. For evaluating *DKK3* expression, a scoring method was used. A mean percentage of positive cells was determined by the examination of 500 cells in at least 5 areas at a magnification of $\times 400$. Cells were assigned to 1 of the 5 following categories according to the percentage of positive cells (PP): 0, <5%; 1, 5-24%; 2, 25-49%; 3, 50-75%; and 4, >75%. The intensity of the *DKK3* staining (SI) was then scored as follows: 0, negative (-); 1, weak (+); 2, moderate (++); and 3, intense (+++). The final immunoreactive score (IRS = SI + PP) was as follows: -, 0

or 1; +, 2 or 3; ++, 4 or 5; and +++, 6 or 7. The stained tissues were scored blindly regarding clinical patient data. Statistical analyses were performed with SPSS 19.0 software. Statistical significance was evaluated by the Chi-square test (χ^2). The significance level was set at 0.05.

Reverse-transcription polymerase chain reaction (RT-PCR). TRIzol reagent (Invitrogen Life Technologies, Karlsruhe, Germany) was used to extract total RNAs. Reverse transcription polymerase chain reaction (RT-PCR) was performed using a kit from Promega (Madison, WI, USA). Real-time PCR was performed to detect *DKK3* expression, according to the manufacturer's protocol (HT7500 system; Applied Biosystems). Primers for amplifying *DKK3* mRNA sequences were synthesized as previously described. The 161-bp mRNA of *DKK3* was amplified by PCR with forward primer, 5'-CAC CCTCAATGAGATGTTCC and reverse primer, 5'-TGGTCTC ATTGTGATAGCTG. *GAPDH* as a control for all the samples was shown in our previous studies (17,18). PCR amplification was performed with denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec in 32 cycles. The PCR products were visualized on 2% agarose gels under ultraviolet transillumination.

Results

Increased expression levels of *DKK3* in OSCC and HNSCC. We firstly investigated *DKK3* expression in OSCC and HNSCC using the open access cancer microarray database Oncomine (<https://www.oncomine.org/resource/login.html>). Through analysis of studies by Peng *et al*, Ye *et al* and Ginos *et al*, the results revealed that *DKK3* expression at the mRNA level was 1.377-fold higher in OSCC than that in normal oral mucosa tissues (P=0.002) (19), 1.433-fold higher in tongue squamous cell carcinoma (TSCC) tissues than that in normal tongue mucosa tissues (P=2.63E-4) (20), 1.831-fold higher in HNSCC tissues than that in normal buccal mucosa (P=1.93E-5) (21) (Fig. 1A-C). By analyzing microarray data from the GENT dataset (<http://medical-genome.kribb.re.kr/GENT/search/search.php>), *DKK3* was found to be overexpressed in HNSCC tissues, compared with the level in the corresponding normal oral mucosa tissues (Fig. 1D). These results suggest that *DKK3* upregulation may play a crucial role in OSCC development.

Genetic alteration of *DKK3* in OSCC. We next assessed genetic alterations of *DKK3* in HNSCC including OSCC using The Cancer Genome Atlas (TCGA) database. *DKK3* was overexpressed in two TCGA HNSCC cohorts, with a rare mutation (Fig. 2A). Copy number analysis using Oncomine database showed an increased *DKK3* gene copy number in OSCC tissues (P=1.75E-4), compared with that noted in normal oral mucosa tissues (Fig. 2B). Although deletion of *DKK3* was observed in the HNSCC TCGA cohort, there was no statistically significant difference (Fig. 2C). These results suggest that gain of *DKK3* gene copy number may lead to increased *DKK3* expression level in OSCC.

***DKK3* upregulation at the protein level in the carcinogenesis of OSF.** To evaluate *DKK3* protein expression in malignant progression of OSF, we firstly performed immunohistochemical

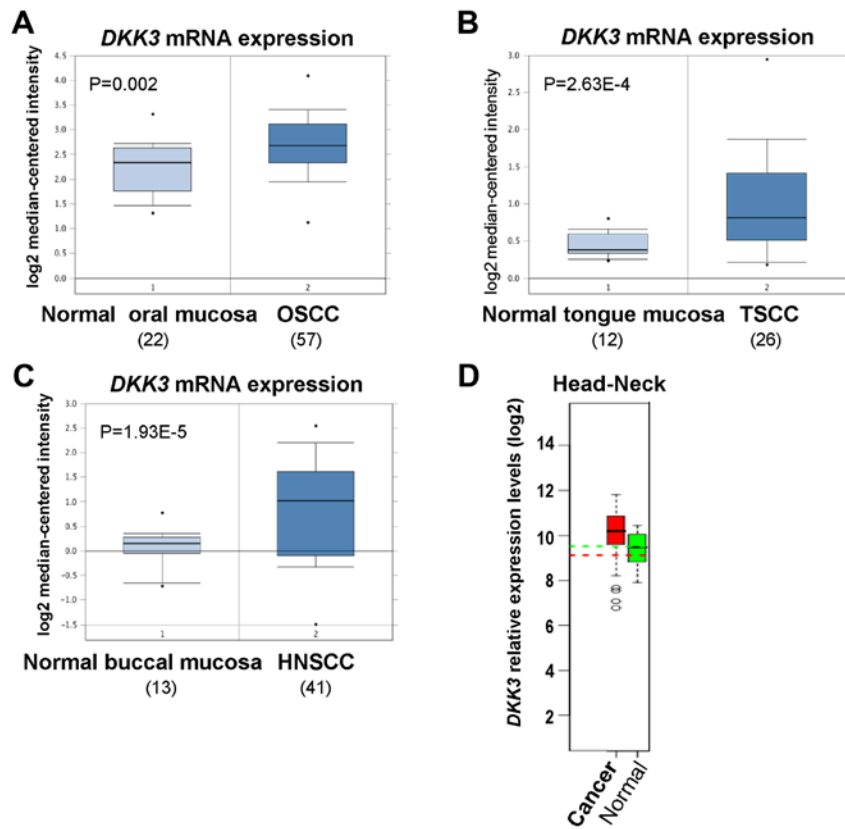


Figure 1. Analysis of *DKK3* mRNA expression in OSCC and HNSCC using Oncomine and GENT database. Box-plot diagrams were analyzed to compare the *DKK3* mRNA levels in normal oral, tongue and buccal mucosa tissue with that in (A) OSCC, (B) TSCC and (C) HNSCC using the Oncomine dataset. The vertical axis represents the log2 median value. The upper (75%) and lower (25%) quartiles are represented by the upper and lower borders of the boxes, respectively. (D) *DKK3* mRNA expression levels in HNSCC and normal head-neck tissues in the GENT database.

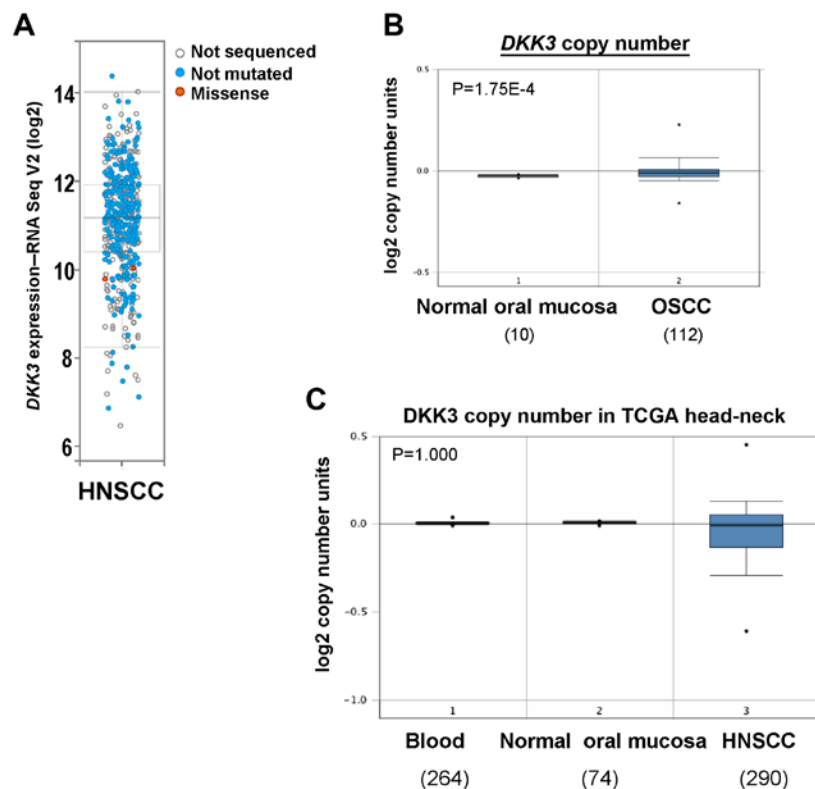


Figure 2. *DKK3* gene mutation and copy number analysis in OSCC and HNSCC. (A) *DKK3* expression and mutation status from the TCGA HNSCC cohorts. (B) *DKK3* copy number analysis in OSCC using the Oncomine dataset. (C) *DKK3* copy number analysis in the TCGA Head-Neck cohort using the Oncomine database.

Table I. DKK3 expression in the carcinogenesis of OSF.

Group	n	DKK3				DKK3 expression (%)	Mean DKK3 score
		-	+	++	+++		
Normal	15	9	3	2	1	33.33	1.73
OSF	45	10	12	12	11	77.78	3.73
E	15	4	3	4	4	73.33	3.53
M	15	3	4	4	4	80.00	3.67
A	15	3	2	5	5	80.00	4.00
OSCC	55	2	17	17	19	96.36	4.45

DKK3, dickkopf WNT signaling pathway inhibitor 3. OSF, oral submucous fibrosis: E, early stage; M, moderately advanced stage; A, advanced stage. OSCC, oral squamous cell carcinoma.

Table II. Correlation of DKK3 expression and the clinicopathological features of the OSCC cases.

Clinicopathological features	Total (n)	DKK3		P-value
		+	-	
Age (years)				>0.05
<50	43	42	1	
≥50	12	11	1	
Gender				<0.01
Male	52	52	0	
Female	3	1	2	
Tumor site				>0.05
Tongue	40	39	1	
Others	15	13	2	
Primary tumor				>0.05
T1+T2	12	11	1	
T3+T4	43	42	1	
TNM stage				>0.05
I+II	12	11	1	
III+IV	43	42	1	
Differentiation grade				>0.05
Well	20	19	1	
Moderate-poor	35	34	1	

DKK3, dickkopf WNT signaling pathway inhibitor 3; OSCC, oral squamous cell carcinoma.

staining using a DKK3-specific antibody in normal oral mucous, OSF and OSCC tissues. Six of 15 (33.3%) normal oral mucous cases showed DKK3 positivity in the cytoplasm, and 35 of 45 (77.8%) OSF tissues showed cytoplasmic DKK3 expression, including tissues from 11 of 15 (73.3%) early stage, 12 of 15 (80.0%) moderately advanced stage and 12 of 15 (80.0%) advanced stage, as well as 53 of 55 (96.4%) OSCC (Fig. 3A). The average values of DKK3 expression varied in the different tissue samples, including the mean score of 1.73 in normal oral mucosa

tissues, 3.73 in OSF tissues and 4.45 in OSCC tissues (Fig. 3B; Table I). DKK3 expression level was gradually increased in normal oral, OSF and OSCC tissues ($P<0.05$).

The correlation between DKK3 expression and the clinicopathological features of the OSCC cases was analyzed, including age, gender, tumor site, primary tumor, TNM stage and differentiation grade. DKK3 expression between male patients and female patients was statistically significant (Table II). These data suggest that DKK3 is upregulated at the protein level in the carcinogenesis of OSF.

DKK3 mRNA expression is elevated in the carcinogenesis of OSF. DKK3 expression at the mRNA level was examined in normal oral mucosa and OSF tissues, as well as OSCC and their paired adjacent tissues by semi-quantitative RT-PCR. We found that *DKK3* was expressed in normal oral mucosa (Fig. 4A) and OSF tissues in different stages (Fig. 4B) with varied levels. *DKK3* expression was detected in OSCC and their adjacent OSF tissues. Results showed that *DKK3* expression levels were higher in OSCC tissues, than their adjacent OSF tissues (Fig. 4C). Moreover, the *DKK3* expression level was upregulated in OSCC tissues, compared with the paired adjacent normal tissues (Fig. 4D). Increased mRNA expression level of *DKK3* in OSCC tissues was further confirmed by real-time RT-PCR, compared with their adjacent normal or OSF tissues (Fig. 4E). Therefore, the *DKK3* mRNA expression level is increased in the carcinogenesis of OSF.

Analysis of co-expressed genes with DKK3 in OSCC. To further investigate the potential function of DKK3 in OSCC, average linkage hierarchical clustering was applied to identify the co-expressed gene set with DKK3 in the Oncomine database. One study comparing DKK3 expression data for normal oral mucosa and OSCC tissues was selected for analysis, by using human genome U133A array with 12,624 genes measured. Nineteen genes were co-expressed with DKK3 in OSCC with correlation rate from 0.803 to 0.954 (Fig. 5): TSPYL5, MND4, ZNF415, NEFH, PCDH7, TMEM38B, HSPB11, BAG2, LGR5, col10orf137, PMPCB, GUF1, RPAP3, IGKV3-20, IGL, IGHM, CARS2, RYR3 and ZNF107. Several of these genes are related to Wnt and p53 signaling, apoptosis, Ca^{2+} and mitochondrial signaling pathways.

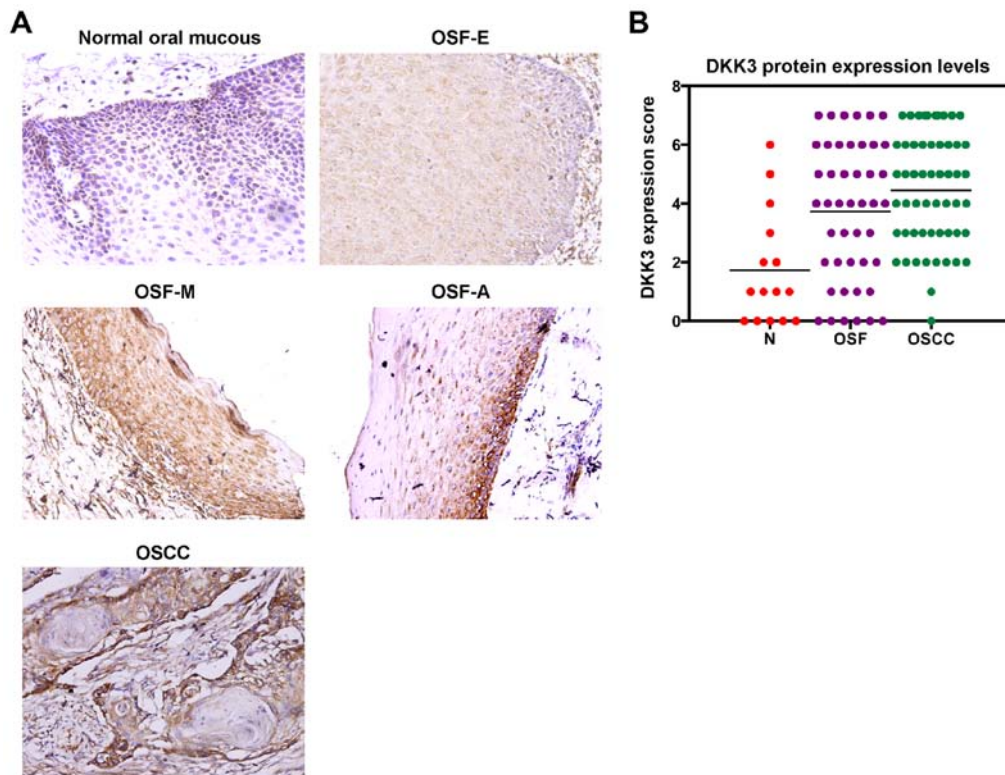


Figure 3. Immunohistochemical staining of DKK3 in normal oral mucosa, OSF and OSCC tissues. (A) Normal oral tissues exhibited weak DKK3 protein expression in the cytoplasm. OSF early (E), moderately advanced (M), and advanced (A) stage tissues showed DKK3-positive expression in cytoplasmic parts. Primary OSCC showed very strongly cytoplasmic immunoreaction for DKK3. Original magnification, x400. (B) The average values of DKK3 expression in the different tissue samples. N, normal oral tissues; OSF, oral submucous fibrosis; OSCC, oral squamous cell carcinoma; DKK3, dickkopf WNT signaling pathway inhibitor 3.

Discussion

Cumulative genetic and epigenetic aberrations in cancer genes contribute to OSCC tumorigenesis. Thus, identification of key genes involved in OSCC initiation and progression, particularly malignant progression of precancerous OSF lesions, is vital. As one of the Wnt antagonists, DKK family members antagonize the Wnt signaling pathway by interacting with the Wnt co-receptors LRP5 and LRP6, further preventing their interaction with Wnt ligands (10,22,23). Unlike other family members, only DKK3 and DKKL1 contain sgy-domain thus are divergent members of the DKK family (10), suggesting possible Wnt-independent function of DKK3 other than as a Wnt antagonist in tumorigenesis.

DKK3 downregulation by promoter CpG methylation has been reported in multiple types of cancer, including thyroid (24), lung (25,26), gastric (27), colon (27), hepatocellular (27,28), breast (29,30), ovarian (31), cervical (32) and renal (33,34) cancers, as well as acute myeloid leukemia (35) and glioma (36). Various studies found that DKK3 is a putative Wnt signaling inhibitor. In OSCC, DKK3 possesses tumor-suppressive function or oncogenic function. DKK3 was found to be epigenetically inactivated in oral cancer, along with other Wnt antagonists WIF1 and SFRPs (12). DKK3 deletion was detected in HNSCC samples using Oncomine and TCGA databases, although no statistical significance and significantly prolonged overall survival were observed, suggesting that DKK3 may function as a tumor-suppressor gene (TSG) in OSCC. Recent studies provide strong evidence that DKK3

plays an oncogenic role in OSCC. DKK3 protein expression was found to be increased in dysplasia and further in OSCC, and was found to be correlated with OSCC metastasis and poor prognosis, independent of the Wnt signaling pathway (13-15).

In the present study, we found that DKK3 was statistically significantly upregulated in OSCC development by bioinformatic analysis. A rare genetic mutation of DKK3 was found in OSCC. *DKK3* gene copy number was increased in OSCC, compared to normal oral mucosa, thus further causing DKK3 upregulation in OSCC. We also found that DKK3 either at the protein level or at the mRNA level was expressed in normal oral mucosal tissues, and the levels were gradually increased in the different stages of OSF and OSCC tissues with BQ chewing, which is consistent with other reports. By analyzing co-expressed genes with DKK3, potential biological functions and molecular mechanisms of DKK3 in OSCC pathogenesis were shown. For example, DKK3 activates the Wnt signaling pathway by binding to and activating LGR5 (37,38); DKK3 was found to inhibit the p53 signaling pathway by modulating TSPYL5 (39) and ZNF415 (40); DKK3 also suppressed apoptosis through the mitochondrial signaling pathway.

In summary, we provide evidence that the DKK3 expression level is increased in the carcinogenesis of OSF. DKK3 copy number gains are responsible for its upregulation in OSCC. The present study revealed the oncogenic role of DKK3 in the malignant progression of OSF, and sheds light on the development of a valuable tumor biomarker for the early detection of OSCC.

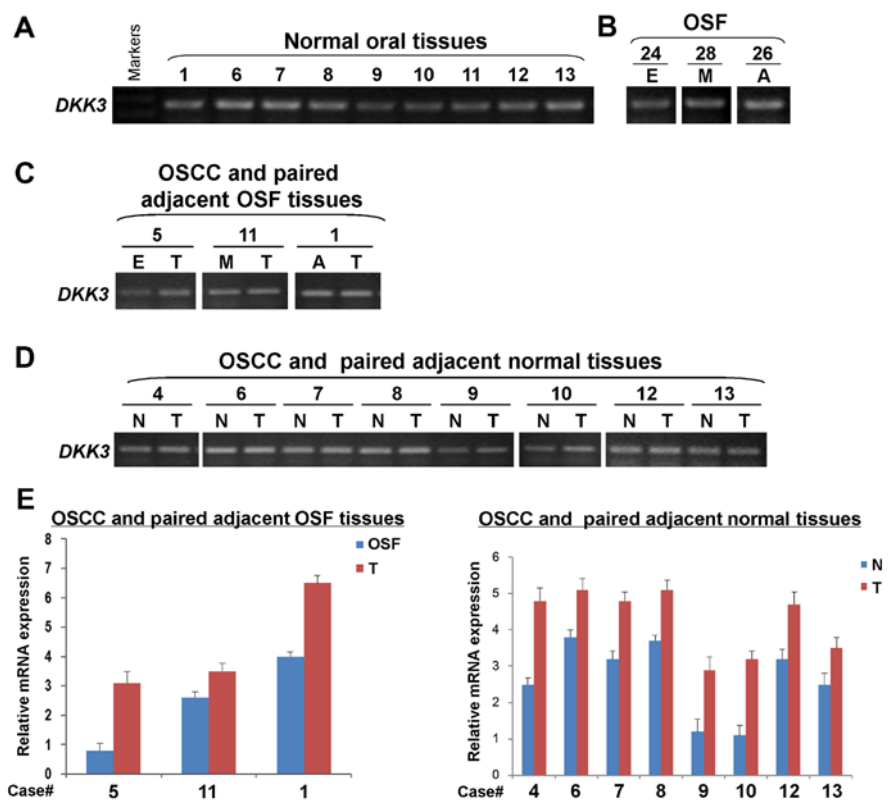


Figure 4. Detection of *DKK3* mRNA expression in normal oral mucosa, OSF and OSCC tissues. Semi-quantitative RT-PCR assessed *DKK3* expression in (A) normal oral mucosa tissues, (B) OSF tissues, (C) OSCC and paired adjacent OSF tissues, as well as (D) OSCC and paired adjacent normal tissues. GAPDH was used as an internal control. (E) Quantitative real-time RT-PCR was used to confirm *DKK3* expression in samples from OSCC and paired adjacent OSF or normal tissues. *DKK3*, dickkopf WNT signaling pathway inhibitor 3. OSF, oral submucous fibrosis: E, early stage; M, moderately advanced stage; A, advanced stage. OSCC, oral squamous cell carcinoma (T); N, normal mucosa tissue.

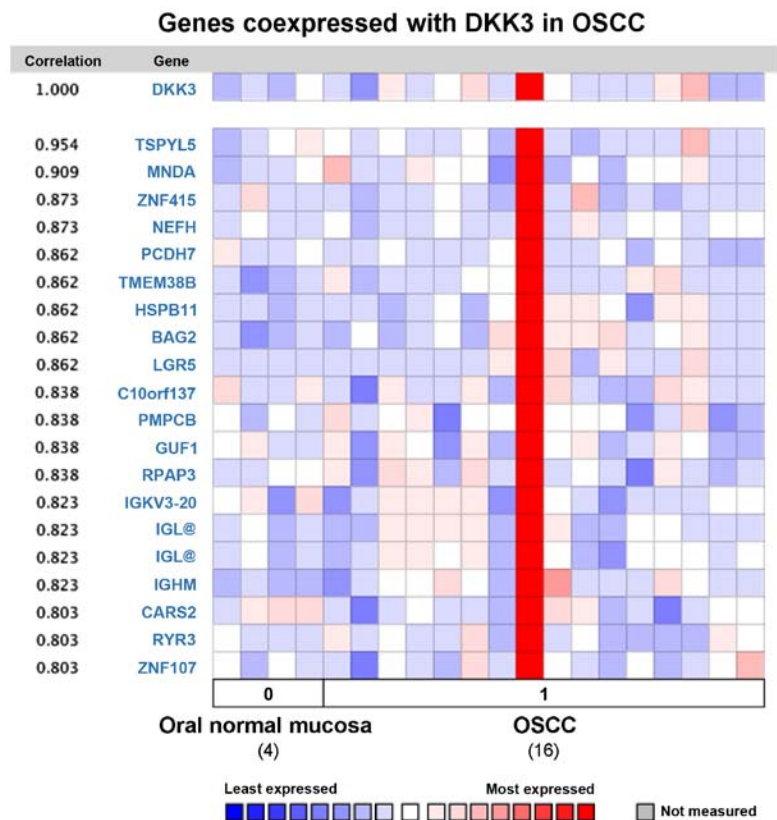


Figure 5. Co-expression gene analysis of *DKK3* in OSCC using the Oncomine database. Heat map of the co-expression profile of *DKK3* in OSCC tissues, compared with oral normal mucosa tissues. *DKK3*, dickkopf WNT signaling pathway inhibitor 3; OSCC, oral squamous cell carcinoma.

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