Aberrant DKK3 expression in the oral leukoplakia and oral submucous fibrosis: a comparative immunohistochemical study

T. Al-dhohrah,[1,2] M. Mashrah,[3] Z. Yao,[1] J. Huang[1]

[1]Department of Oral Pathology, Xiangya Stomatology Hospital, Central South University, Changsha, Hunan, China
[2]Department of Oral and Maxillofacial Pathology, Faculty of Dentistry, Ibb University, Yemen
[3]Clinical fellow, Department of Oral and Maxillofacial Surgery, Xiangya Hospital, College of Stomatology, Central South University, Changsha, Hunan, China

Abstract

We aimed to assess and compare the expression of Dickkopf homolog 3 (DKK3), a possible tumor suppressor gene (TSG), in oral leukoplakia (OLK) and oral submucous fibrosis (OSF) using immunohistochemistry. Seventy-five cases of normal oral mucosa (NOM), OLK, OSF, and squamous cell carcinoma (OSCC) were studied. DKK3 was expressed in all cases of NOM, OLK and OSCC. There was steady increases in the percentage of the positive cells progressing toward OSCC. The expression was localized in the cytoplasm and cell membrane of cell affected by OLK with mild dysplasia and OLK with severe dysplasia. No significant association was observed between DKK3 expression and dysplastic status of OLK. Loss of DKK3 expression was observed in 15 of 30 cases in the OSF group, which was significantly associated with histological grade of OSF (P<0.0001). The percentage of positive cells gradually declined with the increasing severity of epithelial atrophy. A significant difference (P<0.01) was observed when comparing DKK3 expression among different groups of OLK and OSF cases. DKK3 may have diverse expressions in oral premalignant lesions. Loss of DKK3 expression in dysplastic/advanced stage of OSF may imply a high risk of progression to oral cancer.

Introduction

Despite the improvement in the diagnostic and therapeutic strategies, oral squamous cell carcinoma (OSCC) remains a major public health problem; it is the eighth most common cancer worldwide. The five-year survival rate of patients with OSCC remains almost unchanged.[1] Oral leukoplakia (OLK) and oral submucous fibrosis (OSF) are potentially malignant disorders with a high risk of transitioning to OSCC. Dysplasia occurs in 25% of OLK cases and 26% of OSF cases.[2-4] The potential for malignant transformation of OLK and OSF lesions with evidence of epithelial dysplasia ranges from 5%-15% and 3%-19%, respectively.[3,5] Therefore, attempts are being made to identify specific molecular biomarkers to predict which lesions have the potential to be malignant. The Wnt pathway plays a role in cell adhesion, proliferation, and differentiation.[6] Activation of the canonical Wnt pathway occurs by binding extracellular ligands to the frizzled receptor and the low-density lipoprotein (LRP5/6) co-receptors, which ultimately leads to stabilization of β-catenin. Consequently, the cytoplasmic level of β-catenin increases. β-catenin translocates to the nucleus, interacts with Tcf/LEF family, and activates transcription by replacing repressor complexes like the Groucho repressor complex.[6] The human Dickkopf family comprises four members (DKK1, 2, 3, and 4),[7] which function as inhibitors of the canonical Wnt pathway. DKK3 is a potential tumor suppressor gene (TSG) that is localized on 11p15, a locus often deleted in cancerous cells.[8] DKK3 interacts with the Krm receptors intracellularly,[9,10] which exerts its inhibitory effect on Wnt signaling either by binding to bTrCP, thus blocking β-catenin from entering the nucleus,[11] or by increasing the localization of β-catenin in the cell membrane.[12] DKK3 has been shown to be downregulated in various cancer tissues,[13-16] suggesting that this gene may be a TSG, while hypermethylation of its promoter correlates with the occurrence of cancers.[17,18] However, its overexpression has been reported to inhibit cell proliferation.[12,19]

Since the expression of DKK3 in potentially premalignant disorders has not been examined, in this study we aim to assess and compare immunohistochemical expression of DKK3 in OLK and OSF as well as correlation with clinicopathological features.

Materials and Methods

Patients and tissues

Seventy-five formalin-fixed paraffin-embedded blocks with histologically proven NOM, OSF, OLK, and OSCC were collected retrospectively. The samples consisted of 5 NOM, 14 OSF without dysplasia, 16 OSF with dysplasia, 18 OLK with mild dysplasia, 12 OLK with moderate/severe dysplasia, and 10 OSCC. All patients

in this study provided informed consent, and the study was approved by the School of Medicine, Central South University. Thorough clinicopathological characteristics were recorded. OSCC was diagnosed according to WHO criteria of 2005.[20] According to Pindborg's criteria,[21] OSF cases were further categorized as early stage (n=5), moderately advanced (n=16), or advanced (n=9). NOM samples were obtained from the buccal mucosa during the surgical removal of third molars.

Immunohistochemical staining

The biopsy samples were fixed in 10% neutral buffered formalin, left 24 h at room temperature, routinely processed for histology and embedded in paraffin wax. Four-micrometer-thick sections were cut, dewaxed in xylene, and dehydrated in a graded alcohol. After the slides were rinsed, endogenous peroxidase activity was blocked by treatment with 3% H_2O_2 for 15 min at room temperature. The slides were pretreated with citrate buffer (10% citrate buffer stock in distilled water, pH 6.0) and microwaved for 20 min to retrieve the antigen. Non-reactive staining was blocked by 1.0% goat serum in Tris-buffered saline (pH 6.0) applied for 3 min. Primary rabbit polyclonal antibodies against human DKK3 (AP1523a, Abgent, San Diego, CA, USA; 1:50) were diluted in phosphate-buffered saline (PBS, pH 7.4)

and then incubated for 1 h at 37°C in a humidified chamber. After the slides were rinsed with PBS, they were incubated with a secondary antibody (biotinylated polylink; Dako, Hamburg, Germany) for 30 min at 37°C in a humidified chamber and then rinsed with PBS. DKK3 expression was detected by a diaminobenzidine chromogen system (Dako). The sections were counterstained with hematoxylin, dehydrated, and then mounted.

Image analysis

DKK3 expression and epithelial thickness was measured in all tissue samples using ImageJ, Java-based image processing software (National Institute of Mental Health, Bethesda, MD, USA). DKK3 expression was scored as previously described by Carmona *et al.*[22] The mean values for each group of measurements were compared using the Student's *t*-test.

Statistical analysis

Statistical analysis was performed with SPSS 17.0 (SPSS Inc., Chicago, IL, USA). The chi-square test was used to determine the relationship between DKK3 expression and clinicopathological parameters. DKK3 expression was compared between different OLK and OSF groups using the Mann-Whitney U test. Differences with a probability value of less than 0.05 were considered to be statistically significant.

Results

DKK3 staining was observed in 60 of the samples and was lost in 15. In NOM, DKK3 was localized only in the cell membrane of suprabasal cell layers, whereas the basal cell layer was negatively stained except for some scattered spots of weak DKK3 expression. (Figure 1 A,B). DKK3 expression was observed in all OLK cases (Table 1). It was localized in both the cell membrane and cytoplasm. However, the percentage of cytoplasmic expression in cases of OLK with severe dysplasia was higher compared to OLK with mild dysplasia. In contrast, the membranous expression of the suprabasal cells was higher in OLK with mild dysplasia compared to OLK with severe dysplasia (Figure 1 C-F). No statistical difference was observed (P=0.323) (Table 1).

DKK3 expression was detected in 15 of 30 tissue samples of patients with OSF (Table 2). DKK3 expression was detected in 13 cases of OSF without dysplasia and was lost in 1, whereas DKK3 was detected in 2 cases of OSF with dysplasia and was lost in 14. A significant difference was observed when comparing DKK3 staining among cases of OSF with and without dysplasia (Figure 2 A-F). DKK3 expression was observed in 4 out of 5 patients in the early stage, 9 out of 16 in the moderately advanced stage, and 2 out of 9 in the advanced stage. However, it was lost in 1, 7, and 7 cases, respectively. There was a gradual decline in DKK3 expression while epithelial atrophy progressed, but this was not statistically significant. No DKK3 expression was detected in the cell membrane of any OSF case. In the early stage of OSF, DKK3 expression was localized in the cytoplasm of the basal and suprabasal layers, but it was only localized in the cytoplasm of the basal layer in the moderately advanced

Table 1. Clinicopathological characteristics and DKK3 expression in patients with oral leukoplakia.

	No. of patients (n=30) (%)	DKK3 expression		χ^2	P value
		+(%)	++(%)		
Sex				0.139	0.709
Female	3 (10)	1 (33.3)	2 (66.7)		
Male	27 (90)	17 (63.0)	10 (37.0)		
Age (years)				0.362	0.547
<46	17 (60)	11 (64.7)	6 (35.3)		
>46	13 (43.3)	7 (53.8)	6 (46.2)		
Dysplastic status				0.978	0.323
Mild dysplasia	18 (60)	9 (50.0)	9 (50.0)		
Severe dysplasia	12 (40)	9 (75.0)	3 (25.0)		
Habits				0.536	0.464
Alcohol	21 (70)	14 (66.7)	7 (33.3)		
Alcohol and smoking	9 (30)	4 (44.4)	5 (55.6)		

Table 2. Clinicopathological characteristics and DKK3 expression in patients with oral submucous fibrosis.

	No. of patients	DKK3 expression		χ^2	P value
		+(%)	-(%)		
Sex				0.000	1.000
Male	26	13 (50)	13 (50)		
Female	4	1 (25)	3 (75)		
Age (years)				2.143	0.143
<31	14	5 (35.7)	9 (64.3)		
>31	16	10 (62.5)	6 (37.5)		
Grade				4.563	0.130
Early	5	4 (80)	1 (20)		
Moderate	16	9 (56.3)	7 (43.7)		
Advanced	9	2 (22.2)	7 (77.8)		
Dysplasia status				19.286	0.0001*
Dysplasia	16	2 (12.5)	14 (87.5)		
No dysplasia	14	13 (92.9)	1 (7.1)		
Habit				0.556	0.456
Betel nut	18	10 (55.6)	8 (44.4)		
Betel nut and smoking	12	5 (41.7)	7 (58.3)		
Duration				0.133	0.715
<8 years	15	8 (53.3)	7 (46.7)		
>8 years	15	7 (46.7)	8 (53.3)		
Mouth opening				0.635	0.426
<28	9	3 (33.3)	6 (77.7)		
>28	21	12 (57.1)	9 (42.9)		
Stage/atrophy°				0.362	0.547
Early stage/atrophic	2	2	0		
Moderate stage/atrophic	7	4	3		
Sever stage/atrophic	9	2	7		

*Statistically significant at P<0.05; °relation between epithelial atrophy and DKK3 expression according to Pindborg's criteria.

and advanced stages (Figure 2 A-F). Cytoplasmic DKK3 expression was detected in all OSCC cases (Figure 3 A,B). The relationship between DKK3 expression and the various clinicopathological characteristics of OLK and OSF was studied. Only the dysplastic status of OSF, which shows significant correlation with DKK3 expression, other shows no significance (Tables 1 and 2). When DKK3 expression was compared between OLK groups and OSF groups, significant differences were observed (P<0.05) (Table 3).

Discussion

An important aim of this study was to assess and compare DKK3 expression in OLK and OSF patients. The current study is the first to investigate DKK3 expression in both OLK and OSF. In NOM, DKK3 was expressed on the cell membrane at suprabasal layers and was absent at the basal layer. In OLK, the percentage of DKK3-positive cytoplasmic expression in mild dysplasia was less compared with severe dysplasia. However, this was not statistically significant (P=0.323). Further, the staining was localized in the cytoplasm of the basal layer in both mild and severe dysplasia. Our finding was in line with the discovery[23] that DKK3 expression gradually increases from NOM to OLK and from OLK to OSCC. Additionally, DKK3 was only expressed in the cytoplasm in OSF cases. This was in agreement with Fujii *et al.*[23] findings regarding the location of the expression either in the cell membrane of the NOM or in the cytoplasm of the OLK and OSF. The precise mechanism by which DKK3 expression shifts from cell membrane to the cytoplasm is not fully clear. However, this does not appear to be through inhibition of the canonical Wnt/β-catenin signaling pathway.[23] The most striking finding in the present study was the loss of DKK3 expression in 50% of OSF cases. In contrast to OLK cases, the percentage of positive cytoplasmic expression was higher in cases of OSF without dysplasia than in cases of OSF with dysplasia (P=0.0001).

DKK3 downregulation by CpG methylation has been reported in various cancer types.[17,24-26] It is suggested that DKK3 is involved in SCC carcinogenesis in the head, neck, and oral regions.[23,27,28] However, its specific function has yet to be investigated. Pannone *et al.*[27] reported that DKK3 is epigenetically inactivated in oral cancer. Katase *et al.*[28] showed that by performing a genome-wide loss of heterozygosity (LOH) analysis. An allele at the DKK3 locus is frequently deleted in head and neck SCC suggesting that DKK3 functions as a TSG. DKK3 knockdown in OSCC-derived cells resulted in reduced cell migration and invasion and

may have an oncogenic function that is independent of Wnt signaling.[29] An intriguing question was raised: Why was DKK3 expressed in all OLK cases, but loss of protein expression was reported in 50% of OSF cases? To answer this question, we should first understand the cause of OSF development, which is still not clear. Many studies show a close association between chewing areca nuts and OSF development.[30] Therefore, it is tempting to hypothesize that there may be underlying factors responsible for loss of DKK3 expression in OSF with dysplasia. The possible explanation for aberrant DKK3 expression may be the chemi-

Table 3. A comparative account of DKK3 expression in different stages of oral leukoplakia and oral submucous fibrosis.

Groups	Rate (%)	χ^2 (Mann-Whitney test)	Z	P value
OSFWD	16 (26.7)	OSFWD *vs* OLKWSD	4.970	0.0001*
OSFWTD	14 (23.3)	OSFWD *vs* OLKWMD	4.476	0.0001*
OLKWSD	18 (30.0)	OSFWTD *vs* OLKWSD	1.899	0.058*
OLKWMD	12 (20.0)	OSFWTD *vs* OLKWMD	2.512	0.012*
		OSFWD *vs* OSFWTD	4.543	0.0001*

OSFWTD, oral submucous fibrosis without dysplasia; OSFWD, oral submucous fibrosis with dysplasia; OLKWMD, oral leukoplakia with mild dysplasia; OLKWD, oral leukoplakia with moderate/sever dysplasia. *Statistically significant at P<0.05.
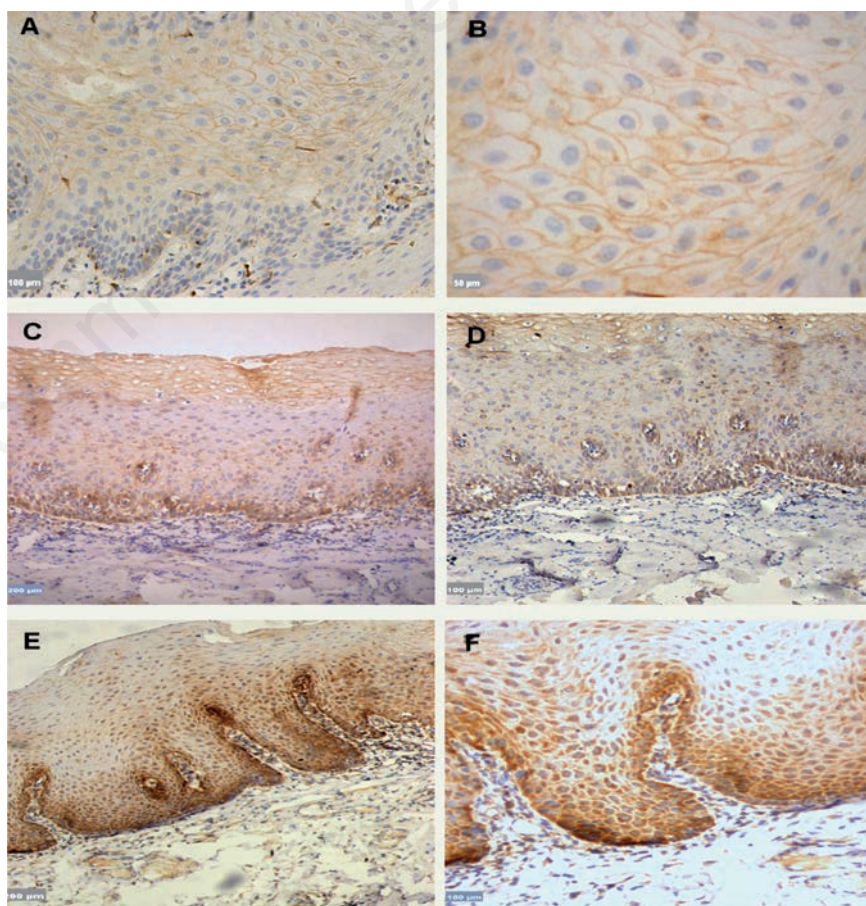

Figure 1. A,B) DKK3 expression in NOM, which was localized in the cell membrane of suprabasal cell layers, however absence of staining was detected in the basal layer; scale bars: A) 100 μm; B) 50 μm. C,D) OLK Mild dysplasia, DKK3 staining was localized in the cytoplasm of the basal layer and in the cell membranes of suprabasal layer; scale bars: C) 200 μm; D) 100 μm. E,F) OLK severe dysplasia, the staining was localized in the cytoplasm as in mild dysplasia; however, the percentage of cytoplasmic positive cells were higher in severe dysplasia compared with mild dysplasia; scale bars: E) 200 μm; F) 100 μm.
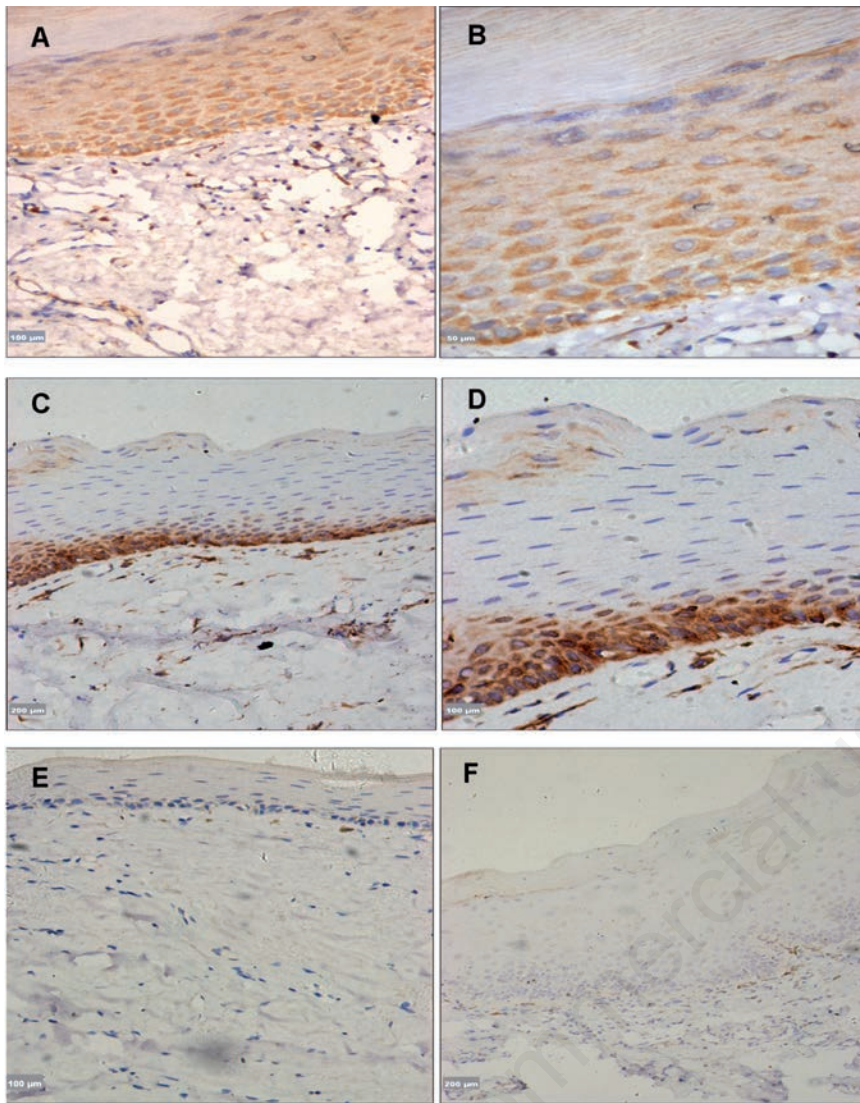
Figure 2. A,B) OSF without dysplasia/early stage, DKK3 expression was localized in the cytoplasm of basal and suprabasal layers; scale bars: A) 100 μm; B) 50 μm. **C,D)** OSF without dysplasia/moderately advanced, DKK3 was localized in the cytoplasm of basal layer; however, negative staining was observed in the suprabasal layers; scale bars: C) 200 μm; D) 100 μm. **E)** OSF advanced stage, complete loss of DKK3 expression in the basal and suprabasal layer; scale bar: 100 μm. **F)** OSF with dysplasia/ moderately advanced stage, showing absence of DKK3 staining; scale bar: 200 μm.
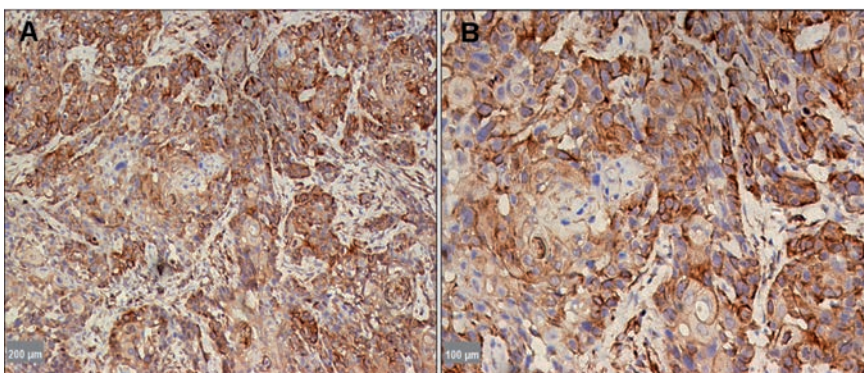

Figure 3. DKK3 expression in OSCC, which was localized in the cytoplasm; however, the staining was completely absent in the nucleus. Scale bars: A) 200 μm; B) 100 μm.

cals in areca nuts: arecoline, arecaidine, guvacine, and guvacoline. These compounds have been proposed to be highly cytotoxic and genotoxic to cultured human buccal epithelial cells.[31] Teh *et al*.[32] demonstrated that genomic instability is due to LOH in several chromosomal loci containing known oncogenes and TSGs associated with head and neck carcinogenesis in OSF tissues. Whether this reflects that DKK3 is instable or hypermethylation occurs in the promoter remains to be confirmed by molecular biology techniques. Surprisingly, a gradual loss/reduced expression of DKK3 in OSF cases, from overexpression in the early stage to partial loss in the moderately advanced stage, and then to severe loss in the advanced stage was observed. In other words, DKK3 expression progressively decreased with increased severity of epithelial atrophy from the early to advanced stages of OSF. However, the results were not significant (P=0.547). In their investigation in colorectal cancer, Zitt *et al*. reported that DKK3 positive samples showed a higher mean microvessel count than did DKK3 negative samples. Suggesting that DKK3 can be considered a putative pro-angiogenic protein in colorectal cancer.[33] Since OSF occurs because of the stromal changes, which undergo progressive hyalinization, decrease in vascularity and cellularity.[34] We hypothesized that DKK3 expression in OSF samples may decrease with the decrease in vascularity of the stromal layer. Recently, DKK3 is the clinical target of gene therapy in prostate,[19] gastric scirrhous[35] and renal fibrosis.[36] Federico *et al*. demonstrated that DKK3 might serve as a diagnostic marker to identify the degree of atrophy and fibrosis in human patients with different types of chronic kidney diseases.[36] Based on the Federico *et al*. findings as well as our study findings, DKK3 may serve as a diagnostic marker to identify different stages of OSF based on the severity of epithelial atrophy. Further, it may also help to assess the OSF carcinogenesis based on the dysplastic status of OSF, as most predictions about the onset of malignancy are currently based on the severity of dysplasia.[37,38]

We note several limitations to this study. The number of patients was relatively small; only single antibody was investigated. Therefore, further well-designed study with large sample size and multiple markers are needed.

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