

DEC1: a potential biomarker of malignant transformation in oral leukoplakia

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Abstract: The purpose of this study was to analyze the differential expression of DEC1 in oral normal mucosa (NM), oral leukoplakia (OLK) and oral squamous cell carcinoma (OSCC). Surgically excised specimens from patients with OLK (n = 47), OSCC (n = 30) and oral normal mucosa (n=11) were immunostained for DEC1. The expression of DEC1 protein was evaluated, and its association with the clinicopathological features was analyzed. The expression of DEC1 in NM, OLK and OSCC tissues increased in turn, and significant differences were observed among the groups (P < 0.0001). In terms of the association between DEC1 expression and epithelial dysplasia, DEC1 expression was lower in hyperkeratosis without dysplasia (H-OLK) than in OLK with moderate to severe dysplasia (S-OLK), and these differences were significant (p < 0.05). The expression of DEC1 in OSCC with OLK was significantly higher than that in OSCC without OLK (p < 0.01). Therefore, DEC1 could be a potential biomarker of malignant transformation in the carcinogenesis of OSCC, which may provide a new research direction for the transformation of oral potentially malignant disorders (OPMDs) into OSCC.

Keywords: Leukoplakia, Oral; Mouth Neoplasms.

Declaration of Interests: The authors certify that they have no commercial or associative interest that represents a conflict of interest in connection with the manuscript.

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Introduction

Oral leukoplakia (OLK) is a white plaque in the oral cavity that cannot be wiped away and cannot be diagnosed as any other disease.¹ It is well known that OLK is the most common form of oral potentially malignant disorders (OPMDs).² According to its pathological features, OLK can be divided into epithelial hyperplasia or hyperkeratosis, with or without epithelial dysplasia.³ The probability of malignant transformation of OLK was 0.13–17.5% in one study, which was proportional to the degree of epithelial cell dysplasia.⁴ Oral epithelial cell dysplasia (OED) had malignant conversion rates of 6.6%–36.4%, which may increase the risk of OLK malignant transformation. A malignant conversion rate of less than 5% was associated with mild OED, while rates of 3–15% and 7–50% were associated with moderate and severe OED, respectively.⁵

Oral squamous cell carcinoma (OSCC), the most common head and neck malignancy, is the worst end point of OLK development.⁶ OSCC is a preventable disease, as approximately 62% of cases are transformed from

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OPMDs.⁷ Thus, more attention should be focused on the search for a reliable biomarker for the early detection of OSCC.

According to early research by our team, DEC1 expression was found to be correlated with the incidence and prognosis of OSCC.⁸ Human differentiated embryonic chondrocyte-expressed gene 1 (DEC1, also known as Bhlhe40/Bhlhb2/Stra13/Sharp2), a member of the basic helix-loop-helix (bHLH) transcription factor family, plays an important role in the development of chondrocytes and in the regulation of circadian rhythms.⁹ In previous studies, DEC1 was reported to be a useful marker in detecting the occurrence of tumors and evaluating tumor prognosis.^{10,11} In gastric cancer, DEC1 was shown to be highly expressed and positively correlated with the expression of KI67.¹⁰ DEC1 was further demonstrated to be essential for gastric cancer cell proliferation and for the promotion of tumor invasiveness in gastric cancer.¹⁰ Similarly, in studies on hepatocellular carcinoma and pancreatic ductal adenocarcinoma, DEC1 is often regarded as a potential biological marker for the occurrence and prognosis of cancer.¹¹ Therefore, in this study, we focused on the differential expression of DEC1 protein during the evolution of normal oral mucosa to OLK and then to OSCC.

Methodology

Patients and clinical specimens

Ethical approval for this study was obtained from the Medical Ethics Committee of Xiangya Hospital, Central South University. We reviewed all biopsies that were histologically diagnosed as OLK or OSCC at two institutions: the Department of Pathology, Xiangya Hospital (from May 2014 to May 2018), Central South University and the Department of Oral Pathology, Xiangya Stomatological Hospital, Central South University (from May 2014 to May 2018). We have read the Declaration of Helsinki and have followed the guidelines in this research. All clinicopathological information such as gender, age, site of lesion, size and pathological findings was obtained from medical records (Table 1).

Table 1. Clinicopathological informations.

Clinicopathological features	Number of samples	%
Age (years)		
>50	36	46.8
≤ 50	41	53.2
Gender		
Male	71	92.2
Famale	6	7.8
Site		
Tongue	56	72.7
Buccal	19	24.7
Gingiva	2	2.6
Histopathological grading		
Hyperkeratosis without dysplasia	12	15.6
Mild dysplasia	21	27.3
Moderate and severe dysplasia	14	18.2
OSCC risen from oral leukoplakia	12	15.6
OSCC	18	23.4
Habits		
Tobacco		
Alcohol		NA
Areca nut chewing		

NA: not available; OSCC: Oral squamous cell carcinoma.

Samples used in this study consisted of surgically excised specimens from 77 patients, among which 47 were diagnosed as OLK and 30 were diagnosed as OSCC. A previously reported oral epithelial dysplasia scoring system was used to grade the OED in this study.¹² Oral normal mucosa (NM) tissues were obtained from eleven healthy volunteers. All the normal mucosa specimens were derived from the extra normal oral mucosa, which was removed during surgery for maxillofacial trauma or cleft lip and palate. Histopathological diagnosis was confirmed by two experienced pathologists by reviewing the original histological sections stained with hematoxylin and eosin.

Immunohistochemistry

After the specimens were resected, they were fixed in 10% formalin, embedded in paraffin, sectioned

into 4 μm slices and placed on slides. The slides were deparaffinized for 60 minutes in a temperature chamber at 65°C. Following deparaffinization by immersion in xylene, the sections were immersed in alcohol then washed in distilled water. Antigen retrieval was performed as previously reported.¹³ Then, the slices were washed three times in phosphate buffer for 5 minutes each time.

Endogenous peroxidase activity was blocked by incubating the slices with hydrogen peroxide for 20 minutes at room temperature. The sections were then incubated with a rabbit monoclonal antibody against human DEC1 (1:1000; ab70723; Abcam, USA) at 4°C overnight. Histostain™ - SP Kits (Zsbio, Beijing, China) were used in this experiment. After a rewarming step at 37°C, the sections were washed with PBS, incubated with goat anti-rabbit secondary antibody (Zsbio, Beijing, China) and horseradish peroxidase (HRP, Dako, Denmark) for 20 minutes at 37°C and then stained with 3, 3'-diaminobenzidine (DAB) for 2 minutes and counterstained with hematoxylin. After that, the sections were dehydrated and sealed for observation under a microscope.

The expression of DEC1 was reflected by the mean of the integral optical density (MOD) value, as previously reported.¹³ MOD values greater than 5 were defined as high expression, while MOD values less than or equal to 5 were defined as low expression. The obtained sections were observed under by microscopy. The imaging system included a Leica DFC 420 CCD camera connected to a Leica DM IRE2 microscope (Leica Microsystems Imaging Solutions, Cambridge, UK). Using Leica Qwin Plus V3 software, five representative views were selected under 400x magnification and were saved. The MOD of each image was counted and measured by Image-Pro Plus V6.0 software (Media Cybernetics, Bethesda, MD, USA). Two researchers analyzed the expression and analyzed the clinical data in a blinded manner.

Statistical analysis

All statistical analyses were performed using Graphpad Prism v6.0 software (GraphPad Software, La Jolla, USA) and SPSS 24.0 (SPSS, Inc., Chicago,

USA). Group comparisons were analyzed using one-way analysis of variance (ANOVA). Nonparametric data comparisons between groups were made with the Mann-Whitney test, while unpaired t-tests were used for parametric data. The association between clinicopathological features and the expression of DEC1 was verified by Chi-square test. The expression in each group was reflected by the mean \pm SEM. The level of statistical significance was accepted at $p < 0.05$.

Results

Patient information

Samples were roughly divided into three main groups according to their clinical and pathological diagnosis: the NM group ($n = 11$), the OLK group ($n = 47$) (after OLK excision, patients in the OLK group were observed for approximately 1–2 years during which no malignant changes were observed) and the OSCC group ($n = 30$, no lymph node metastasis). According to the different histological manifestations and the OED classification system, the OLK group could be further subdivided into the hyperkeratosis without dysplasia subgroup (H-OLK, $n = 12$), the mild dysplasia subgroup (M-OLK, $n = 22$) and the moderate to severe dysplasia subgroup (S-OLK, $n = 13$). The OSCC group included OSCC arising from OLK (OLK-OSCC; OLK with carcinogenesis at focal sites was included in the OLK-OSCC subgroup, $n = 12$) and the other OSCC (OSCC not arising from OLK) subgroup ($n = 18$) (Table 2).

Expression level of DEC1 protein in the three main groups

Differences in the expression of DEC1 were observed among the three main groups ($p < 0.0001$). DEC1 immunohistochemical staining was observed in both the cytoplasm and nucleus but was predominantly seen in the basal and stratum spinosum layers.

In the OLK group ($\text{MOD} = 15.14 \pm 1.930$), the expression of DEC1 in the epithelial layer was significantly higher than that in the NM ($\text{MOD} = 0.1648 \pm 0.09857$) but was lower than that in the OSCC group ($\text{MOD} = 23.52 \pm 3.051$) ($p < 0.0001$, Figure 1).

Table 2. The samples were grouped according to histopathological grading and the specific clinical details after grouping.

Clinicopathological features	Hyperkeratosis without dysplasia	Mild dysplasia	Moderate and severe dysplasia	OSCC risen from oral leukoplakia	OSCC	Total	Control
Gender							
Male	12	19	13	11	16	71	
Female	0	2	1	1	2	6	
Age(years)							
Age range	35–67	29–64	27–85	36–69	40–67	27–85	
Mean age	49.00	46.48	51.64	50	54.11	50.14	NA
Anatomic site							
Buccal	2	8	3	0	6	19	
Tongue	10	13	11	12	10	56	
Gingiva	0	0	0	0	2	2	
Numbers	12	21	14	12	18	77	11

NA: not available; OSCC: oral squamous carcinoma; N: no.

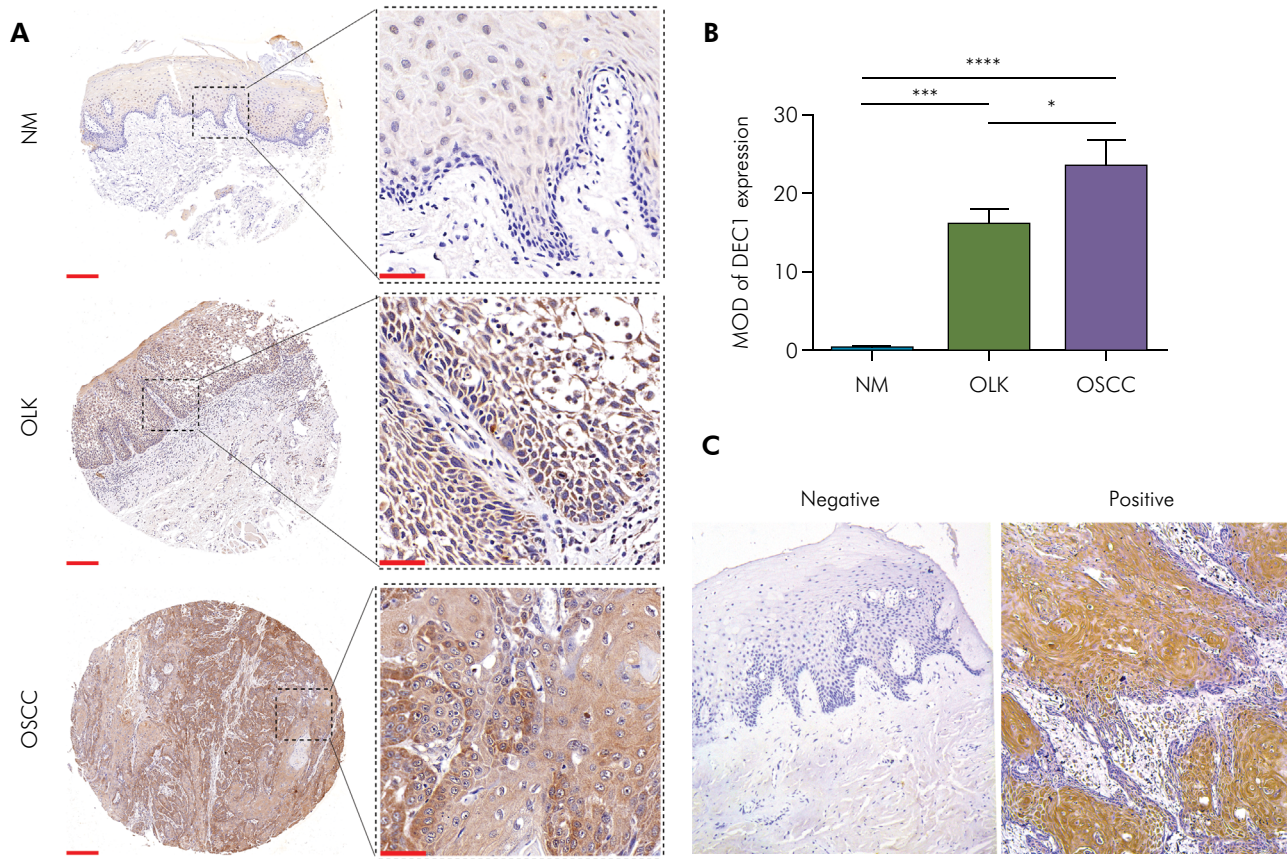


Figure 1. Immunohistochemical expression of DEC1 in oral normal mucosa (NM), oral leukoplakia (OLK) and oral squamous cell carcinoma (OSCC): (A) DEC1 immunohistochemical staining was observed in both the cytoplasm and nucleus and was successively increased in NM, OLK and OSCC. Scale bar (left side) = 200 μ m, scale bar (right side) = 50 μ m. (B) The MOD of DEC1 expression increased in turn, and the difference was statistically significant. The difference between NM and OSCC was the most obvious, followed by differences between NM and OLK, and OLK and OSCC. *indicates $p < 0.05$, *** indicates $p < 0.001$, ****indicates $p < 0.0001$, the bar on the column refers to SEM: Structural Equation Modeling.

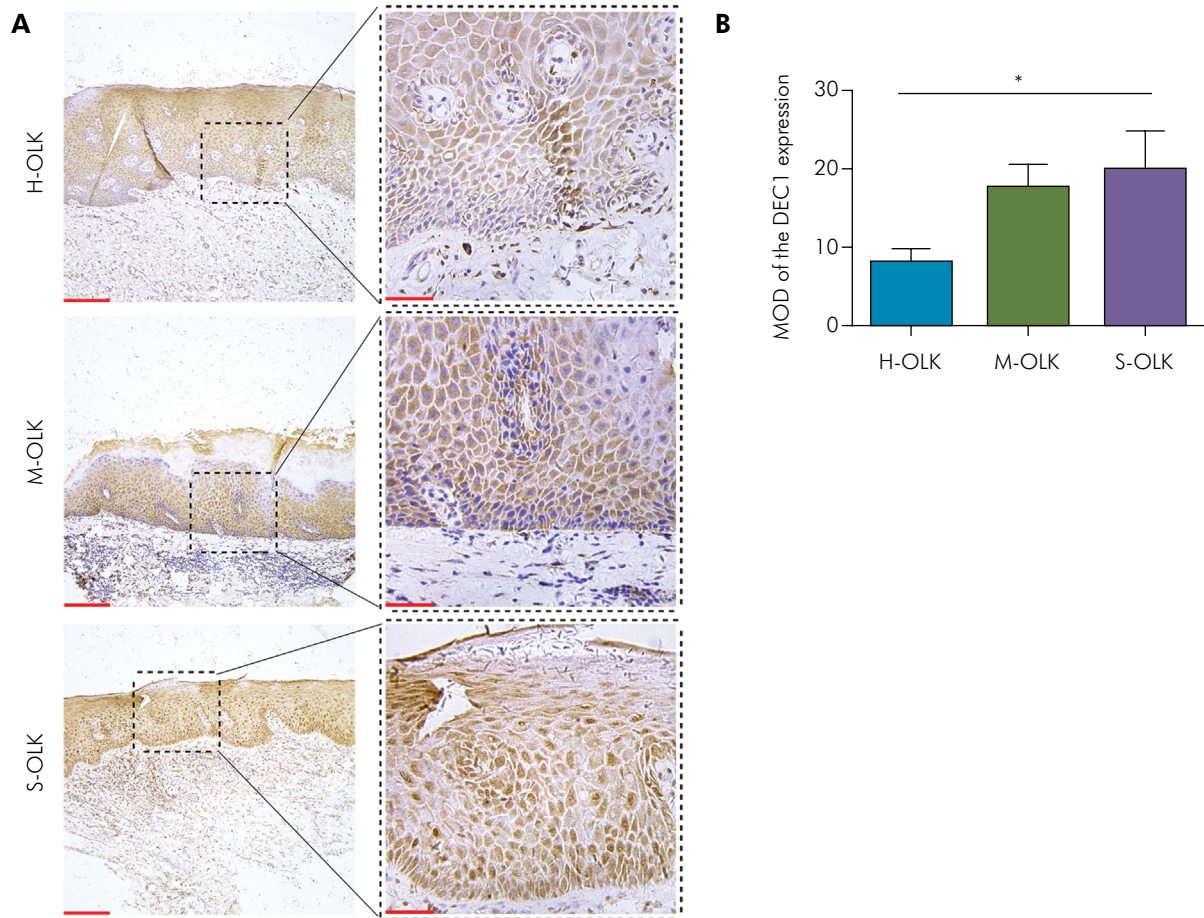


Figure 2. The connection between the expression of DEC1 and the degree of epithelial dysplasia with oral leukoplakia (OLK): (A) Immunohistochemical staining for DEC1 in the OLK group. Scale bar (left side) = 100 μ m, scale bar (right side) = 25 μ m. (B) In the OLK group, the expression of DEC1 in the mild dysplasia subgroup (M-OLK) was higher than that in the hyperkeratosis without dysplasia subgroup (H-OLK) and lower than that in the moderate to severe dysplasia subgroup (S-OLK). The difference between H-OLK and S-OLK was statistically significant. *indicates $p < 0.05$, the bar on the column refers to SEM: Structural Equation Modeling.

Expression level of DEC1 protein in OLK tissues

In the OLK group, the expression of DEC1 was lowest in H-OLK (MOD = 8.561 ± 1.935), slightly higher in M-OLK (MOD = 16.97 ± 2.906) and was highest in S-OLK (MOD = 17.43 ± 4.140), but the differences were not statistically significant ($p = 0.0628$). The difference in DEC1 expression between the hyperkeratosis without dysplasia subgroup and the moderate to severe dysplasia subgroup was significant ($p < 0.05$, Figure 2).

Expression level of DEC1 protein in OSCC tissues

In the OSCC group, the expression of DEC1 in the OLK-OSCC subgroup (MOD = 33.52 ± 5.406) was slightly higher than that in the OSCC subgroup (MOD = 16.99 ± 3.045). The difference was statistically significant ($p = 0.0077$, Figure 3).

Association between the expression level of DEC1 protein and clinicopathologic features

Differences in DEC1 expression between groups according to age, sex and anatomical location were not observed ($p > 0.05$, Table 3).

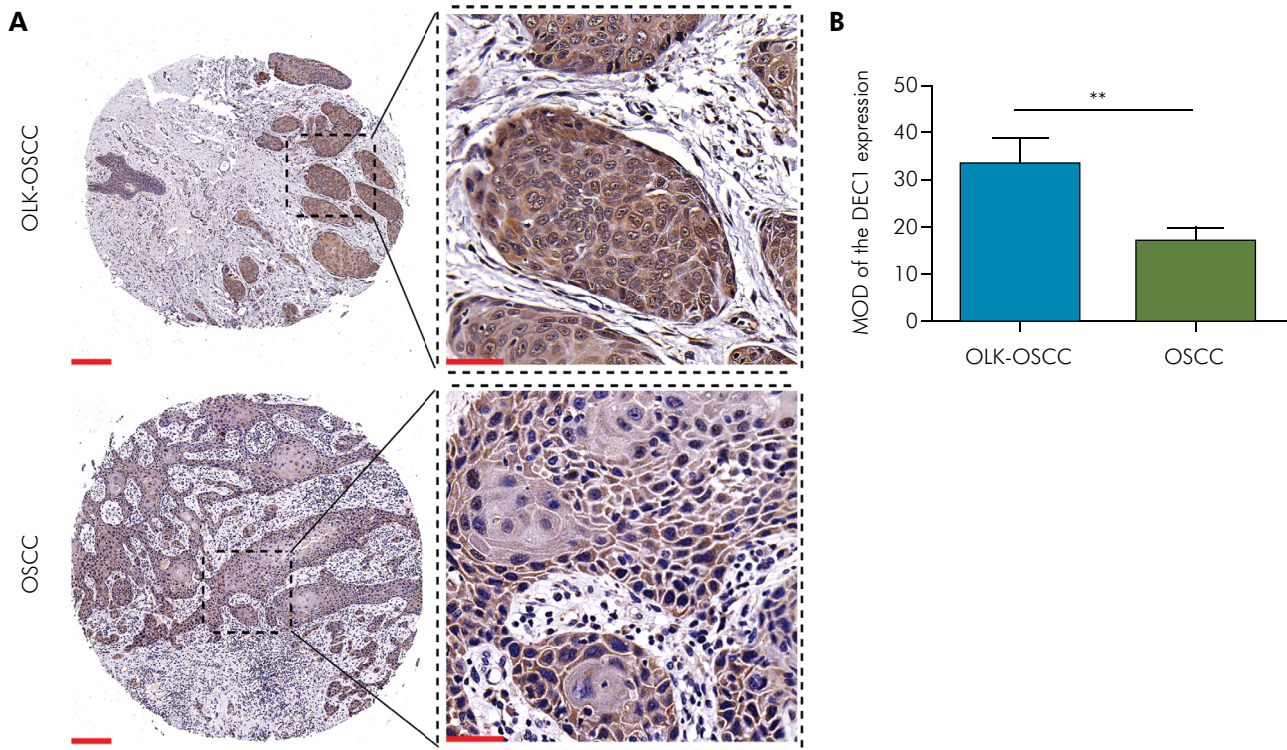


Figure 3. Differential expression of DEC1 between oral squamous cell carcinoma (OSCC) that originated from oral leukoplakia (OLK) and other OSCC: (A) Immunohistochemical staining for DEC1 in OSCC and OLK-OSCC. Scale bar (left side) = 200 μ m, scale bar (right side) = 50 μ m. (B) The expression of DEC1 in the OSCC group in OSCC that originated from OLK was higher than that in OSCC that did not, and the difference was statistically significant. **indicates $p < 0.01$, the bar on the column refers to SEM: Structural Equation Modeling.

Table 3. Analysis of association between clinicopathological information and DEC1 expression.

Clinicopathological features	High expression of DEC1	%	Low expression of DEC1	%	Total	Control	p-value
Age (years)							> 0.05
>50	28	22	8	78	36		
≤ 50	32	22	9	78	41		
Gender							> 0.05
Male	54	76.1	17	23.9	71		
Female	6	100	0	0	6	NA	
Site							> 0.05
Tongue	42	75	14	25	56		
Buccal	16	84.2	3	15.8	19		
Gingiva	2	100	0	0	2		
Histopathological grading							> 0.05
Hyperkeratosis without dysplasia	8	66.7	4	33.3	12		
Mild dysplasia	17	81	4	19	21		
Moderate and severe dysplasia	10	71.4	4	28.6	14	—	
OSCC risen from oral leukoplakia	12	100	0	0	12		
OSCC	13	72.2	5	27.8	18		

NA: not available; N: no; OSCC: Oral squamous cell carcinoma; High expression means $MOD > 5$; Low expression means $MOD \leq 5$.

Discussion

A systematic review and meta-analysis performed by Tan N et al. revealed that the prevalence of OLK was 4.11% and that the malignant transformation rate was 0.13–17.5%.¹³ However, because the risk of malignant transformation of OLK is difficult to assess, it is impossible to completely prevent malignant transformation by early ablative surgery. Molecular diagnosis has been widely used in the study of various diseases to assess disease risk, presence and therapeutic efficacy. Accordingly, research on potential biomarkers that can detect the risk of malignant transformation for OLK is meaningful. In previous studies, EZH2 was reported to be a predictor of oral cancer development from OLK; E-cadherin and tenascin are also potential biomarkers of malignant transformation in oral leukoplakia, and the expression of Podoplanin in oral leukoplakia was reported to be significantly associated with the degree of epithelial atypical hyperplasia.^{14,15,16,7}

A few studies have been performed to determine the role of DEC1 in the malignant transformation of potentially malignant disorders in addition to OPMDs. In the present study, we observed that the expression of DEC1 increased gradually in normal mucosa, OLK and OSCC, while no statistically significant difference was observed regarding age, gender, and the site of occurrence.

Carcinogenesis of oral leukoplakia is often accompanied by some clinical manifestations, histopathological changes, gene mutations and changes in the expression levels of certain genes, which can predict the risk of malignant transformation to some extent (currently, the grade of oral epithelial dysplasia is the standard by which malignant transformation is predicted). Previous studies have shown that high-risk clinical factors for oral leukoplakia canceration include: female sex, long-standing cases, lack of smoking habit, high-risk sites (body of the tongue, floor of the mouth, corner of the mouth), nonhomogenous variant, lesions greater than >200 mm² in size, and the presence of dysplasia.¹⁸ Additionally, P53 mutations, cell motility, and hypoxia in the oral environment are important risk factors for malignant transformation and can be detected early in the development of

OSCC.^{15,16,19} DEC1, as a transcription factor, can regulate epithelial-to-mesenchymal transition and the response to hypoxia in addition to its ability to regulate chondrocytes and circadian rhythms.^{20,21} Furthermore, preliminary research by our team discovered that expression levels of DEC1 were associated with different prognoses of OSCC.⁸ Taken together, DEC1 is a promising molecular biomarker of carcinogenesis, which may explain the differences in its expression among oral normal mucosa, OLK and OSCC.

In our study, the expression of DEC1 was not significantly associated with the degree of epithelial dysplasia, although it was slightly increased with the degree of epithelial dysplasia in OLK. However, the difference between H-OLK and S-OLK was statistically significant. Two possible reasons may explain this phenomenon. One is that DEC1 may be involved in the regulation of cell senescence, which leads to canceration, while the degree of epithelial dysplasia is a pathological classification of leukoplakia from the perspective of cell morphology.²² Therefore, we propose the hypothesis that DEC1 promotes canceration by regulating the aging process of cells rather than by changing the degree of atypical hyperplasia, which may require more evidence from further studies. The other is that the small sample size in each subgroup may have led to bias, and thus more reliable results should be anticipated in large-scale investigations.

Additionally, we observed that the expression of DEC1 in OSCC with OLK was significantly higher than that in OSCC without OLK. Moreover, the pathological diagnosis of specimens from the OLK-OSCC subgroup was incipient carcinoma. Therefore, this difference may be due to the finding that DEC1 may show an upward trend during the initiation of a carcinoma and a downward trend during the development of carcinoma. Previous studies showed that the expression of DEC1 in poorly differentiated OSCC was lower than that in highly differentiated OSCC.²³ Paradoxically, in a study on the association between the prognosis of OSCC and DEC1 expression, the expression level of DEC1 in OSCC was significantly higher than that in the normal mucosa.⁸ Thus, the elucidation of specific signaling pathways related

to DEC1 expression and cancer prognosis requires further study.

CONCLUSION

In summary, the DEC1 protein expression was sequentially increased in NM, OLK and OSCC, and thus this protein may be used as a novel biomarker to detect the risk of malignant transformation from OLK to OSCC.

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