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Original Article

Double-staining of E-cadherin and podoplanin offer help in the pathological diagnosis of indecisive early-invasive oral squamous cell carcinoma

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Abstract: Diagnosis of the early-invasive oral squamous cell carcinoma (OSCC) can be challenged in biopsies, and immunohistochemistry is commonly used in such settings. A double immunohistochemical staining (DIHC) containing both E-cadherin (E-cad) and podoplanin antibodies were developed and its use in the diagnosis of limited cancer in the early-invasive was evaluated. In this study, the expressions of E-cadherin and podoplanin were checked by the way of DIHC in 214 oral biopsy tissues including normal oral epithelial (NOE), oral epithelial dysplasia (OED), squamous carcinoma in situ (SCIS), and OSCC. Meanwhile, 17 indecisive cases whose original diagnoses were SCIS incidentally suspicious infiltration had been checked. Tumor specimens presented a significant loss of expression of E-cad when compared with normal epithelium. In all NOE and 62.5% of OED tissues, the expression of E-cad showed positive clearly and strongly in cell membrane, while podoplanin was showed negative.The expression of E-cad was showed positive in 35.6% of SCIS as the expressions of podoplanin became stronger. The expression of E-cad declined obviously and the expression of podoplanin became stronger in the 54.8% of OSCC. The expression of podoplanin was easier to be observed in the same slice due to the decreased expression of E-cad in malignant cell. By the same way, early-invasions were showed clearly in 5 cases of 17 indecisive cases. The decrease of E-cad and the increase of podoplanin had closely relationship with OSCC ($P<0.05$). The cocktail double staining of E-cad and podoplanin may offer an objective index for the decision of the early-invasive oral squamous cell carcinoma.

Keywords: Oral squamous cell carcinoma, E-cadherin, podoplanin, immunohistochemistry, antibody cocktail

Introduction

Oral and maxillofacial malignancies, as the fifth most common cancer of the world, whose incidence was increasing year by year. Especially, the surrounding tissues are more easily invaded by the oral squamous cell carcinoma, which accounts for approximately 80% of oral malignancies. Despite of improving therapy, the 5-year survival rate remains low at approximately 50%. So the early detection and diagnosis of OSCC is quite important to patients for treatment and prognosis, which would improve survival and quality of life [1-4]. It is an important process for tumor development from OED to OSCC, but there is no significant difference in OED and early OSCC in the clinical symptoms. The methods of treatment to OED and early OSCC were quite different, which means it's important to determine whether the organization is cancer or not in the biopsy tissues.

Malignant transformation in many carcinomas is associated with the loss of epithelial differentiation and the gain of a mesenchymal phenotype. Epithelial to esenchymal transition (EMT), which had well explain the occurrence, development, invasion and metastasis of tumors. Many studies suggest that the presence of EMT may be a predictor of OSCC progression [1, 5-7], so the occurrence of EMT can be monitored to detect the early stage of OSCC in a way, which can improve the survival rate of patients. E-cad and podoplanin are both important indexes in EMT process. E-cad was a calcium-dependent transmembrane glycoprotein which was expressed in most epithelial cells, and its function was establishing cell polarity and maintain-

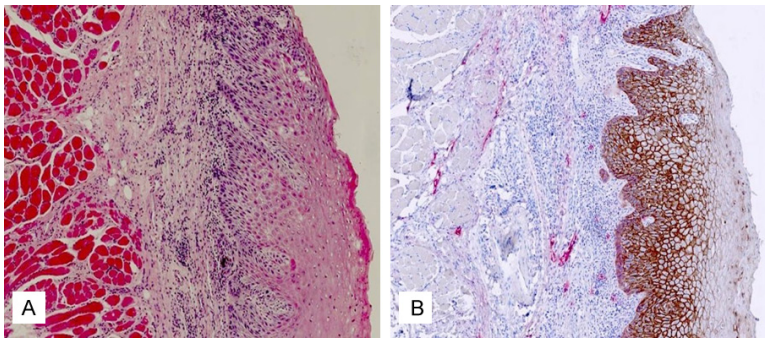

Figure 1. The expression of E-cad and podoplanin in the NOE (A, B, ×40). (A and B) Came from the same tissue (A. HE; B. MaxVision double staining). The signal of E-cad was brown, membrane coloring. The signal of podoplanin was red, membrane coloring.

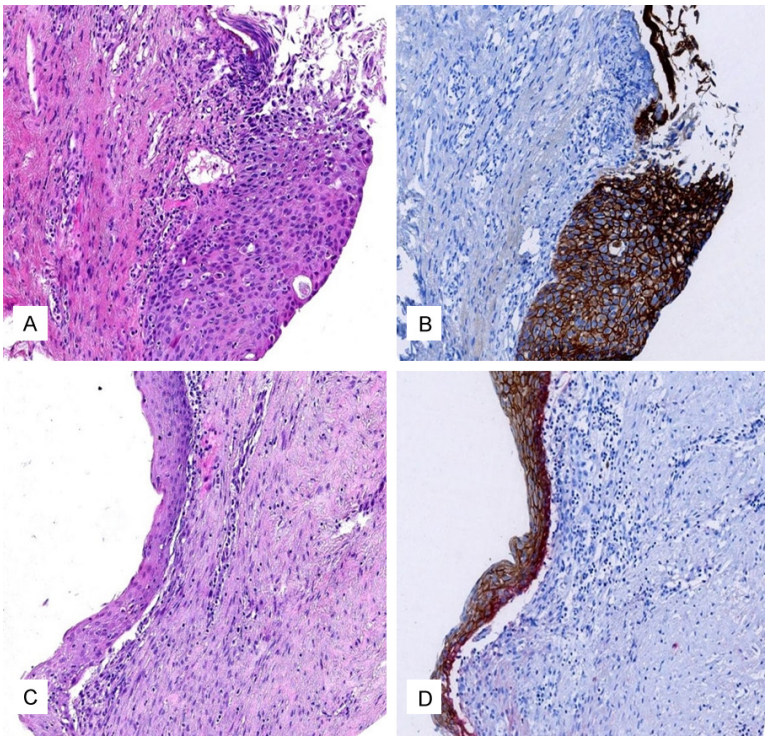

Figure 2. The expression of E-cad and podoplanin in the OED. (A-D) Came from the same tissue (A, C. HE, ×100, A and C were from one slice; B, D. MaxVision double staining, ×100, B and D were from one slice). The signal of E-cad was brown, membrane coloring; the signal of podoplanin was red, membrane coloring. (A) HE staining of squamous epithelium with the OED. (B) The expression of E-cad in the OED didn't decrease and the expression of podoplanin was negative compared with D. (C) HE staining of squamous epithelium with the relative normal tissue in the same tissue slice. (D) The expression of E-cad and podoplanin in the relative normal tissue (internal control) was observed in the same tissue slice.

ing normal tissue structure [8]. E-cad expression was thought to affect, or be affected by Wnt pathway signalling [9]. The loss of E-cad expression was known to be a marker of the changes of EMT in epithelial cells [10]. Diniz Freitas found that the transfection of E-cad protein can inhibit the invasion of tumor cells, and the reduced expression of E-cad was a malignant symbol [11]. Lots of research also showed that the level of the expression of E-cad in squamous cell carcinoma (SCC) or at the invasive frontier of the SCC is decreased [12-14].

Podoplanin is a 38-KDa type-I transmembrane glycoprotein consisting of 162 amino acids, nine of which form the intracellular domain. The extracellular domain is highly O glycosylated with sialic acid, α-2,3 linked to galactose, forming the main part of the protein carbohydrate moieties [15]. Podoplanin was used as a marker for lymphatic endothelial cells [16], but its expression also has been found in squamous cell carcinomas of several tumor cells, including the uterine cervix, skin, oral cavity, esophagus, and germ cell tumors [17-19]. Wicki et al. have shown that podoplanin can change the actin cytoskeleton of tumor cells or induce collective cell migration by filopodia formation via the down regulation of the activities of small Rho family GTPases to invade and migrate [20]. The loss of E-cad has already been recognized as an important step in the process of tumor progression, while the podoplanin can induce tumorigenesis in the absence of cadherin and EMT. In this study, DIHC was used to detect the expression of E-cad and podoplanin in oral squamous epithelium in order to assist the diagnosis of oral squamous cell lesions.

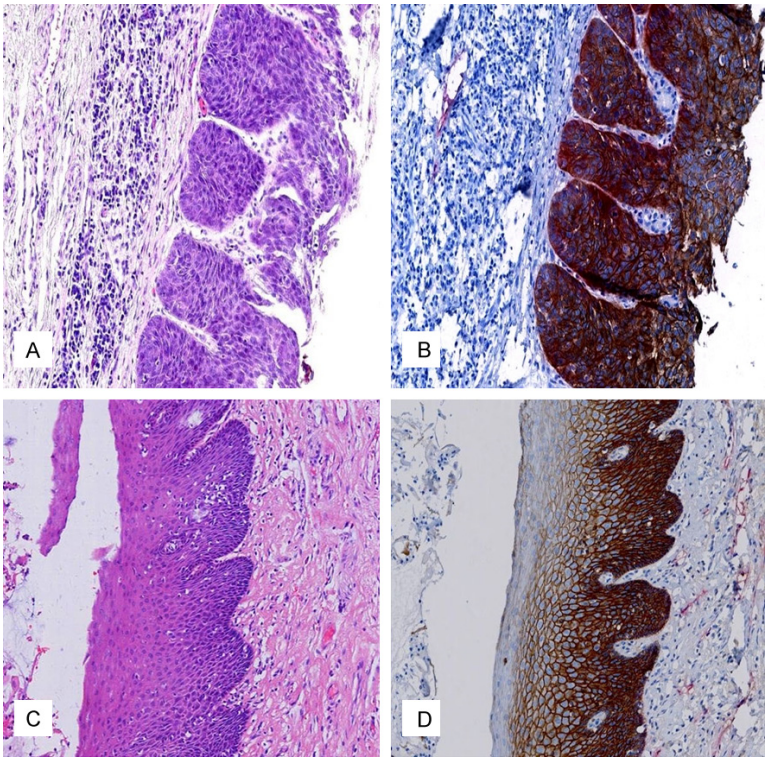

Figure 3. The expression of E-cad and podoplanin in the SCIS. (A-D) Came from the same tissue (A, C. HE, ×100, A and C were from one slice; B, D. MaxVision double staining, ×100, B and D were from one slice). The signal of E-cad was brown, membrane coloring; the signal of podoplanin was red, membrane coloring. (A) HE staining of squamous epithelium with the SCIS. (B) The expression of E-cad in the SCIS did not decrease and the expression of podoplanin was positive compered with D. (C) HE staining of the squamous epithelium. (D) The expression of E-cad and podoplanin in the squamous epithelium (internal control) was observed in the same tissue slice.

Materials and methods

Cases

231 cases of Paraffin-embedded tissue specimens were selected in this study, including 30 cases of inflammation, 66 cases of OED, 45 cases of SCIS, 73 cases of OSCC and 17 cases tissue specimens whose original diagnoses were SCIS with suspicious infiltration that were examined histologically by hematoxylin and eosin (H&E) staining. All the cases were chosen from the biological sample library of the Department of Pathology, the First Affiliated Hospital of Zhengzhou University from 2011 to 2015. All tissues were fixed in formalin, embedded in paraffin, 3 μm thick cut, and stained with hematoxylin-eosin (H&E) and immunohistochemical. All the HE slices were chosen and detected by two senior pathologists.

Immunohistochemistry and double staining

Immunohistochemical staining: in this study, the MaxVision method was used as the immunohistochemical double staining. The anti-E-cad rabbit monoclonal antibody (concentrated type) and podoplanin mouse monoclonal antibody (concentrated type) were purchased from Zsbio Demo Store, Co. Ltd. China and Fuzhou Mai Xin Biotech. Co. Ltd. China, respectively. Each batch was stained with phosphate buffered saline (PBS) instead of primary antibody as the negative control and known positive sections as the positive control.

The cocktail method was used as the immunohistochemical double staining, and the process was as followed. Briefly, the dewaxed and hydrated sections were placed in a water bath that contained about 1.5 L of citrate buffer (pH about 6.40) at a high temperature (1000 W) heating for the antigen repair. 3% H_2O_2 was dropwised into the sections, incubated at the room temperature for 10 min, to inactivate endogenous peroxidase activity. The sections were dropped the goat serum, incubated at the room temperature for 20 minutes, to close the nonspecific binding sites. These sections were discarded serum and added primary antibodies (E-cad:podoplanin=5:1), incubated at 4°C overnight. Next day, these sections were at room temperature for one hour, thoroughly washed in PBS, then incubated with biotinylated secondary antibody (mouse:rabbit=1:1) at room temperature for 30 minutes. Then these sections were added AP-red developer (1:20), colored 30 minutes. After that, the sections were added diaminobenzidine (DAB) reagent (1:20), colored 5 minutes. The sections were dropped into hematoxylin for half of minute and rinsed with tap water for 5 minutes. At last, the excess water was removed, and the sections was

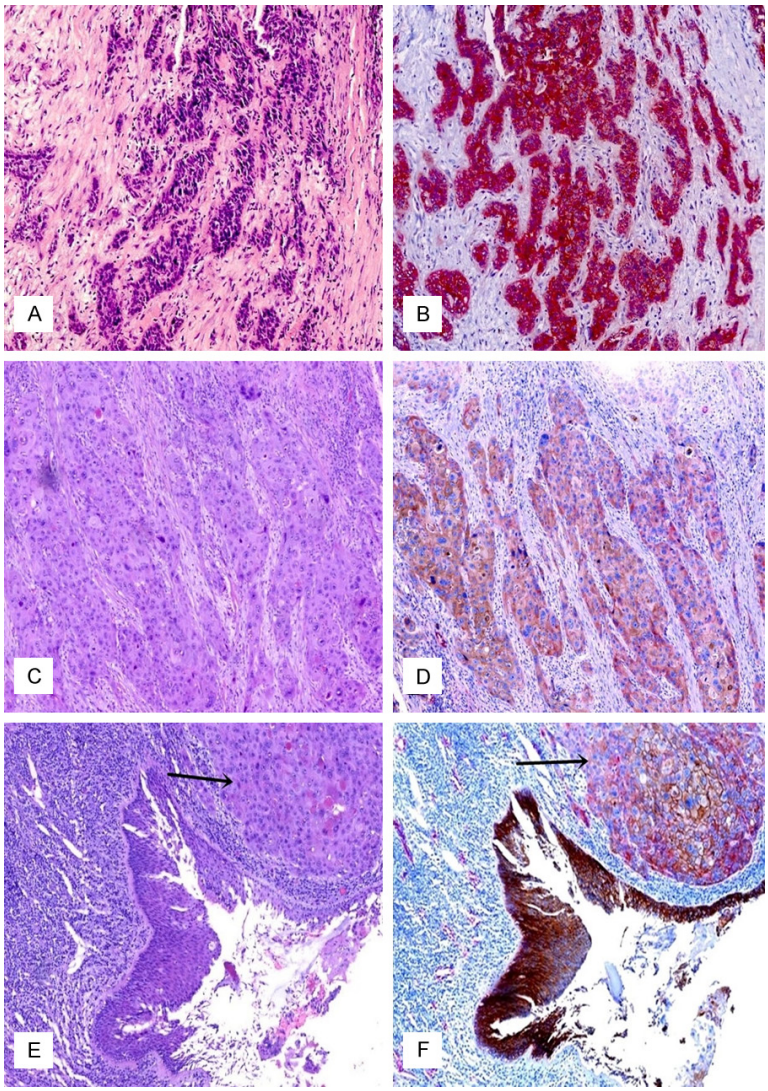

Figure 4. The expression of E-cad and podoplanin in the OSCC. (A and B) Came from the same tissue; (C-F) Came from the same tissue (A, C, E: HE, ×100, ×400, ×400; B, D, F: MaxVision double staining, ×100, ×400, ×400). The signal of E-cad was brown, membrane coloring; the signal of podoplanin was red, membrane and cytoplasm coloring. (A, C) HE staining of squamous epithelium with the OSCC. (B, D) The expression of E-cad in the OSCC decreased and the expression of podoplanin was positive. (E) HE staining of squamous epithelium with the relative normal tissue in the OSCC. (F) The expression of E-cad and podoplanin in the relative normal tissue (internal control) was observed in the OSCC, and the area of the OSCC could also been seen (indicated by the arrow '↑'), clearly.

sealed (the sections washed with PBS and distilled water has been omitted in every step).

Evaluation of immunoreactivity

Immunoreactivity was judged independently by two examiners. Immunoreactivity of E-cad was assessed as followed. We made the reduced staining of E-cad in the target tissue as positive in cell membrane or cytoplasm, compared with the surrounding normal squamous epithelium. The expression of podoplanin in the cell membrane or cytoplasm was observed, which was regarded as positive. In this study, the positive signal of podoplanin was red and E-cad was brown. In NOE, OED and SCIS, the decreased expression of E-cad was divided into three grades. Grade 0, no depressed signal was found. Grade 1, the depressed signal was found only in the basal layer or the spinous layer. Grade 2, the depressed signal was found not only in the base layer or the spinous layer but also somewhere else. Similarly, the expression of podoplanin was also divided into three grades. Grade 0, no positive signal was found. Grade 1, the positive signal appeared in the basal or the spinous layer. Grade 2, the positive signal appeared not only in the basal layer or the spinous layer but also somewhere else. In the OSCC, the decreased expression of E-cad was divided into three grades. Grade 0, no depressed signal was found. Grade 1, the depressed signal was found only around the invasion of the tumor. Grade 2, the depressed signal was found not only around the invasion of the tumor, but also somewhere else. In the OSCC, the expression of podoplanin was also divided into three grades. Grade 0, no expression was found. Grade 1, the expression was found only around the invasion of the tumor. Grade 2, the expression was found not only around the invasion of the tumor, but also somewhere else. In the analysis of the double-stained sections of E-cad and podoplanin, grade 0 and grade 1 were regarded as negative while grade 2 was regarded as positive (E-cad, decreased or absent expression. Podoplanin, expression) in

Table 1. The expression of E-cad and podoplanin in NOE, OED, SCIS and OSCC (case)

Group / Result	NOE (n=30)	OED (n=66)	SCIS (n=45)	OSCC (n=73)	Total (n=214)
A	28 (93.3%)	43 (65.2%)	10 (22.2%)	7 (9.6%)	88
B	2 (6.7%)	6 (9.1%)	11 (24.4%)	10 (13.7%)	29
C	0 (0%)	8 (12.1%)	8 (17.8%)	40 (54.8%)	56
D	0 (0%)	9 (13.6%)	16 (35.6%)	16 (21.9%)	41

NOE, normal oral epithelial; OED, oral epithelial dysplasia; SCIS, squamous carcinoma in situ; OSCC, oral squamous cell carcinoma. A. The expression of E-cad didn't decrease and podoplanin was negative. B. The expression of E-cad decreased and podoplanin was negative. C. The expression of E-cad decreased while podoplanin was positive. D. The expression of E-cad didn't decrease and podoplanin was positive.

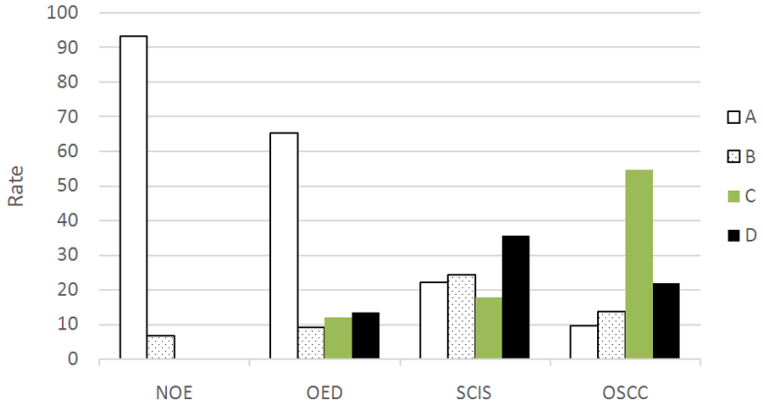

Figure 5. The rate of the expression of E-cad and podoplanin in NOE, OED, SCIS and OSCC (%). NOE, normal oral epithelial; OED, oral epithelial dysplasia; SCIS, squamous carcinoma in situ; OSCC, oral squamous cell carcinoma. A. The expression of E-cad did not decrease and podoplanin was negative. B. The expression of E-cad decreased and podoplanin was negative. C. The expression of E-cad decreased and podoplanin was positive. D. The expression of E-cad did not decrease and podoplanin was positive.

Table 2. Comparison of the probabilities of the four experimental groups

Contrast experiment group	χ^2	p
NOE and OED	10.088	0.018
NOE and SCIS	37.247	0.000
NOE and OSCC	67.798	0.000
OED and SCIS	20.747	0.000
OED and OSCC	49.988	0.000
SCIS and OSCC	16.177	0.001

NOE, normal oral epithelial; OED, oral epithelial dysplasia; SCIS, squamous carcinoma in situ; OSCC, oral squamous cell carcinoma.

the NOE, OED and SCIS. Grade 0 was regarded as negative while grade 1 and grade 2 were regarded as positive (E-cad, decreased or absent expression. Podoplanin, expression) in the OSCC. Finally, the probability of results were recorded as the following four letters. A, the expression of E-cad did not decrease and podoplanin was negative. B, the expression of E-cad decreased and podoplanin was negative. C, the expression of E-cad decreased and podoplanin was positive. D, the expression of E-cad did not decrease and podoplanin was positive.

Statistical analysis

All statistical analyses were performed using SPSS 21.0 software. Correlations between biomarker co-expression patterns and histopathological featureswere analysed using the chi-square test. A value of $P<0.05$ was considered statistically significant. The Bonferroni method was used to correct the level of the inspection When 3 or more groups were analyzed by chi-square test.

Results

The expression of E-cad did not reduce and the podoplanin was negative in almost all NOE and the most OED (**Figures 1A**, **1B** and **2A-D**). The expression of E-cad didn't reduce and the podoplanin was positive (**Figure 3A-D**) in the most SICS, while E-cad decreased and podoplanin was positive in the most OSCC (**Figure 4A-D**). The probability of classification A, B, C and D in NOE, OED, SCIS, and OSCC were shown in **Table 1** and **Figure 5**. There was statistically significant between the four groups ($\chi^2=106.976$, $P<0.05$). The Bonferroni method was used to correct the level of the inspection when 3 or more groups were analyzed by chi-square test. Then significant differences were found between the NOE group and the SCIS group or the NOE group and the OSCC group, respectively ($P<0.008$). Significant differences were also

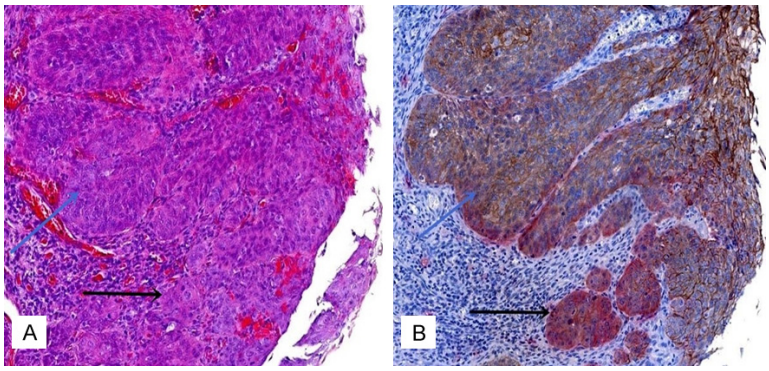

Figure 6. The expression of E-cad and podoplanin in the SCIS with suspicious infiltration (A, B, ×100). (A and B) Came from the same tissue (A. HE; B. MaxVision double staining). The signal of E-cad was brown, membrane coloring; the signal of podoplanin was red, membrane and cytoplasm coloring. (A) HE staining of squamous epithelium with SCIS with suspicious infiltration. (B) The expression of E-cad in SCIS with suspicious infiltration (indicated by the dark arrow '↑') decreased and the expression of podoplanin was strongly positive compared with the SCIS (indicated by the blue arrow '↑'), which made the area of the suspicious infiltration to be clearly distinguished.

found between the OED group and the SCIS group or the OED group and the OSCC group (P<0.008). There was also significant difference between the SCIS group and the OSCC group (P<0.008). But there was no statistically significant difference between the NOE group and the OED group (P>0.008). The specific χ^2 values were shown in **Table 2**.

Then 17 cases whose original diagnoses were SCIS incidentally indecisive infiltration were tested by this double immunohistochemistry staining method, and the indecisive infiltration epithelia in these 5 cases were found that they all had the same expression features as the OSCC. The expression of E-cad was decreased in SCIS incidentally suspicious infiltration and podoplanin was strongly positive compared with the SCIS, which made the area of the suspicious infiltration to be clearly distinguished (**Figure 6A**, **6B**). And these 5 cases were verified as OSCC later via re-biopsy or follow-up visits.

In this study, the expression of podoplanin in OED incidentally canceration has also been found observable difference from the OED whose expression of podoplanin was mainly in the basal layer or the spinous layer, while the expression of podoplanin in OED incidentally canceration was mainly in the area of the canceration. The following phenomenon had also been observed occuring in the canceration of the OED. The expression of podoplanin of the canceration was strongly po-sitive and the expression of E-cad of the canceration decreased (**Figure 7C**, **7D**) or not (**Figure 7A**, **7B**), comparing with the noncancerous area, which made the cancerous area clearly distinguished.

The coloring sites of E-cad were analyzed at the same time, and almost all the squamous epithelial cells stained in cell membrane in NOE, except some cells of the basal layer colored in cytoplasm. Statistical analysis was performed in the OED group, the SCIS group and the OSCC group. Then the difference of cytomembrane and cytoplasm coloring of E-cadherin among the three groups was found being statistically significant (χ^2=13.587, P<0.05). The specific cases were shown in **Table 3**, and the specific frequency was shown in **Figure 8**. Then the groups was analyzed by chi-square test and the Bonferroni method was used to correct the level of the inspection, and there was significant difference between the OED group and the OSCC group (χ^2=13.348, P<0.0167). But there was no significant difference between the OED group and the SCIS group (χ^2=5.100, P>0.0167) or between the SCIS group and the OSCC group (χ^2=1.226, P>0.0167). Therefore, the different location of E-cad in the OED and the OSCC had been considered as the statistical and the clinical significance. These results might suggest that the expression of E-cad decreased gradually in the process of OED to invasive cancer, especially in cell membrane. But there was no significant difference in cytoplasmic expression among the three groups.

Discussion

The diagnosis of the most diseases is based on the basic morphology of HE staining in pathology, including oral pathology. However, many pathologists agree with the viewpoint that the diagnosis largely depends on the results of the IHC for some difficult diagnostic cases, especially when the morphological characteristic is

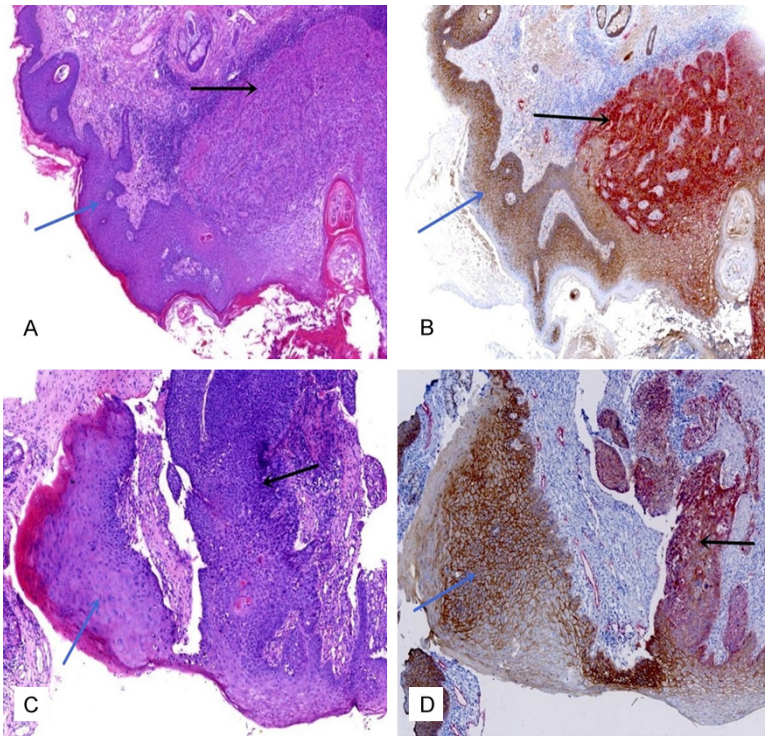

Figure 7. The expression of E-cad and podoplanin in the OED with canceration (A-D, ×200). (A and B) Came from the same tissue while (C and D) came from the same tissue, too (A, C, HE; B, D, MaxVision double staining). The signal of E-cad was brown, membrane or cytoplasmcoloring; the signal of podoplanin was red, membrane and cytoplasm coloring. (A, C) HE staining of squamous epithelium in OED with canceration. (B) The expression of E-cad in canceration of the OED (indicated by the dark arrow '↑') was colored in cytoplasm and didn't decrease, while the expression of podoplanin was positive when compared with the noncancerous area (indicated by the blue arrow '↑'), which made the area of the cancerousto be clearly distinguished. (D) The expression of E-cad in canceration of the OED (indicated by the dark arrow '↑') was colored in membrane and decreased, while the expression of podoplanin was positive compared with the noncancerous area (indicated by the blue arrow '↑'), which also made the area of the cancerousto be clearly distinguished.

Table 3. The expression of E-cad in cytoplasm or cytomembrane of the OED, SCIS and OSCC (case)

Group result	OED (n=66)	SCIS (n=45)	OSCC (n=73)	Total (n=184)
Cytomembrane	26	11	13	50
Cytoplasm	35	25	40	100
Negative	5	9	20	34

OED, oral epithelial dysplasia; SCIS, squamous carcinoma in situ; OSCC, oral squamous cell carcinoma.

not clear or the pathological diagnoses need to base on a variety of clinical manifestations and clinical inspection results. With the development and progress of the immunohistochemical, the DIHC, especially the cocktail double staining, also becomes an important technical to solve these problems. In this study, the DIHC was used to analyze the expression of E-cad and podoplanin in NOE, OED, SCIS and OSCC, and the predictive ability of these molecules in the development of OSCC was also studied. The descension of E-cad expression and the increase of podoplanin expression have a close relationship with squamous epithelial lesions. E-cad is a calcium-dependent transmembrane glycoprotein which plays an important role in maintaining epithelial cell morphology, normal tissue structural integrity and polarity [8, 21]. It can inhibit the produce of matrix metalloproteinases (MMPs) in the host and tumor cells, preventing the degradation of various protein components that compose the basement membrane (BM) or the matrix around tumor cells, and preventing tumor cells breakthrough the matrix or the basement membrane barrier [22]. The cancerous epithelial cells reduce the expression of E-cad, leading MMPs produced by the host and the tumor cells. MMPs degrade the protein of the BM or the matrix around the tumor cells, destruct the adhesion of cells, and promote the tumor cells infiltration or metastasis. Recent studies find that podoplanin can be expressed in a variety of cancer epithelium, and the expression is often associated with increased invasion of cancer tissue. Wicki found that podoplanin induced collective cell migration, invasion, and spreading by filopodia formation via the downregulation of the activities of small Rho family GTPases [20, 23]. They believed that E-cad was present in podoplanin-expressing tumor cells in the invading front of carcinomas, but podoplanin did not necessarily prevent the loss of E-cad expression in the invasive tumor cells. These results raise the intrigu-

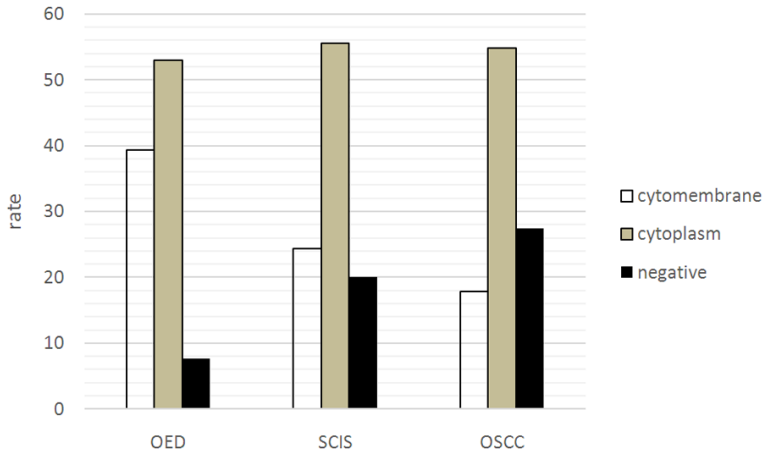

Figure 8. The expression rate of E-cad in cytoplasm or cytomembrane in OED, SCIS and OSCC (%). OED, oral epithelial dysplasia; SCIS, squamous carcinoma in situ; OSCC, oral squamous cell carcinoma.

noses were SCIS with suspicious infiltration were examined, and the expression of E-cad was found in the SCIS incidentally suspicious infiltration decreased and the expression of podoplanin was strongly positive compared with the SCIS, then the area of the suspicious infiltration was clearly distinguished. The expression of podoplanin was strongly positive and the expression of E-cad decreased or not in the cancerous area of the OED had also been found. The phenomenon above might have a relationship with the invasion of the cancer.

ing possibility that podoplanin is able to induce tumor cell invasion without dissolving epithelial adherens and tight junctions but downregulates RhoA activity, thereby ablating stress fibers, inducing filopodia formation, and promoting the tumor cells migration and invasion. Wicki concluded that the enhanced invasion properties of podoplanin-expressing cells appeared to depend on the activity of MMPs. So the expression trend of E-cad and podoplanin suggests their involvement in oral carcinogenesis via Wnt pathway dysregulation and RhoA pathway, respectively.

Our study showed that the expression of E-cad didn't reduce while the podoplanin was negative in almost all the NOE and the most OED. The expression of E-cad was reduced while podoplanin was negative or positive in the most SICS.In the most OSCC, E-cad decreased and the expression of podoplanin was positive. The experimental results were almost consistent with the previous findings [1, 13, 24, 25]. The phenomenon had been found that E-cad didn't reduce while podoplanin was positive in the most OED and SCIS, but E-cad reduced while podoplanin was positive in the OSCC. It indicated that the following decreased adhesive cellular bond, which might have helpful in the invasion of the tumor cells. SCIS was developed from OED, whose result of DICH was that E-cad was reduced while podoplanin was negative in our study. So we speculated that many of the SCIS had no or little invasion at the early time while the OSCC had invasion that made E-cad reduced. Then the cases whose original diagDIHC, especially cocktail double staining, was a mixture that made two antibodies into cocktail antibody and colored the two antibodies on one slice at the same time. DIHC not only made the tissue stained, but also clearly located the different parts of the expression in the biopsies when a variety of antibodies were mixed. In this study, we examined whether the expression of E-cad reduced via observing the degree of E-cad staining and the surrounding normal squamous epithelium which was as internal control, to avoid the inherent difference of the tissue itself in E-cad coloring. Podoplanin was a specific marker of LEC, which was used as an internal control to facilitate observation of the difference in staining results due to the cause of the tissue itself, operational errors or reagent causes. In this study, these two antibodies, E-cad and podoplanin, were selected as a couple. Although the same coloring part was showing but in different colors, the observation of the colors was relatively easy. There were also some new requirements for technical aspects to raise the diagnostic rate at the same time.

The coloring sites of E-cad were analyzed and we found that almost all the squamous epithelial cells were stained at cell membrane in the NOE, except that some cells of the basal layer were stained at cytoplasm. The expression of E-cad was decreased gradually in the progression from OED to OSCC, especially decreased in cell membrane. Previous studies had already found that the expression of E-cad was significantly reduced in the OEDs and OSCC [1, 13, 14]. Our study was consistent with the previous

studies. But we found that there was no significant difference in the expression of cytoplasmic among the three groups.

In conclusion, the cocktail double staining with the combination of E-cad and podoplanin may improve the specificity and sensitivity of detection to the early oral squamous cell carcinoma and make the early oral squamous cell carcinoma easier to observe. This method may also improve the diagnostic accuracy and efficiency of SCIS with micro or inconspicuous invasion in oral, which displays an important clinical value.

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Disclosure of conflict of interest

None.

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