Original Article

Assessment of podoplanin lymphatic vessel density in oral epithelial dysplasia

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Abstract

Background: Oral squamous cell carcinoma (OSCC) can be preceded by the appearance of lesions which have the potential to develop into cancer. Recently, it was suggested that the tumor-associated lymphatic vessels formation plays an active role in tumor progression and metastasis of several human malignancies including OSCC. There is the view that, in an individual lesion, the more severe the dysplasia, the greater the likelihood is of progression to malignancy.

Aim: This study is aimed to investigate podoplanin (PDPN) immunoexpression in lymphatic vessels of oral epithelial dysplasia (OED) and to assess the lymphatic vessel density (LVD) in histologic grades of OED.

Materials and Methods: The study group comprised thirty histopathologically diagnosed cases of OED with various grades of differentiation and thirty cases of clinically normal oral mucosa. After immunohistochemical staining, cases of OED were immunohistochemically analyzed quantitatively for PDPN (D2-40) LVD.

Statistical Analysis: The statistical analysis was done using Kruskal Wallis analysis of variance; pair-wise Tukey's *post hoc* test was applied to evaluate the significant differences among the mean values in different groups. Results with "$P < 0.05$" were considered to be statistically significant at 95% of confidence level.

Results: PDPN LVD scores increased with increasing grades of dysplasia. Pair-wise comparisons of the PDPN LVD scores and the histopathologic grades of OED were found to be statistically significant ($P < 0.05$).

Conclusions: Increase in PDPN LVD in OED represents a promising tool for more wide spread studies of tumor lymphangiogenesis and its role in progression of dysplastic lesion to human cancer.

Keywords: Carcinoma, cell differentiation, epithelial dysplasia, lymphangiogenesis, lymphatic vessels

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INTRODUCTION

Squamous cell carcinoma of the oral cavity was defined by Rajendran *et al.* (2012) as "a malignant epithelial neoplasm exhibiting squamous differentiation as characterized by the formation of keratin and/or the presence of intercellular bridges."[1] Despite advances in diagnostic and therapeutic modalities, the prognosis of oral squamous cell carcinoma (OSCC) still remains poor with 5-year survival rates of <50%.[2] This is mainly due to the lack of early detection of OSCC, the high incidence of metastasis, recurrence after surgery, and chemoresistance of OSCC.[2] OSCC is frequently preceded by the precursor lesions.[3]

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Prevention and early detection of such oral potentially malignant disorders (OPMDs) have the potential of not only decreasing the incidence but also improving the survival of those who develop oral cancer.[4]

Podoplanin (PDPN) is a 38 transmembrane sialoglycoprotein consisting of 162 amino acids belonging to type 1 transmembrane sialomucin-like glycoproteins.[5] PDPN consists of a small transmembrane domain and an intracellular domain. The latter interacts with protein kinase C (PKC) and proteins of the ezrin, radixin, and meosin family, which were shown to influence cancer cell motility and invasive potential.[6] PDPN is also involved in lymphatic vessel formation and does not influence formation of blood vessels.[7] D2-40 can also been used for immunohistochemical (IHC) studies of tumor lymphangiogenesis. An increase in the number of lymphatic vessels in the tumor stroma has been shown to correlate with lymph node metastasis.[8] Enhanced PDPN expression in OSCC which was significantly associated with a poor pathologic grade of differentiation.[9] Furthermore, OSCC may arise from oral epithelial dysplasia (OED). However, the stage of tumor growth and invasion at which lymphangiogenesis may begin is still not certain. Therefore, the present study was performed to study the expression of PDPN in OED and study the presence of lymphangiogenesis in OED.

MATERIALS AND METHODS

The study group comprised thirty histopathologically diagnosed cases of OED with various grades of differentiation and thirty cases of normal oral mucosa. The normal oral mucosa samples were obtained during crown lengthening and disimpaction procedures.

Relevant information (viz. age, sex, and site of the lesion) was recorded on the case history pro forma. Informed consent was obtained from all the patients.

An ethical clearance was obtained from the institutional ethical committee, and an informed consent from patients was obtained for the present study. Grading of OED was done according to the system suggested by the WHO (2005).[10]

Immunostaining with PDPN[11] (DAKO cytomation; monoclonal mouse; Anti human D2-40; Clone: D2-40; Isotype: IgG1, kappa; Ready-to-use) was performed using Sensitive novalink Polymer Diaminobenzidine (DAB) detection kit.[12]

Procedure

Two sections of OED of 3–4 μ thickness were obtained from formalin fixed paraffin embedded tissues using a semi-automated microtome (Thermoscientifics, Microm HM 340E). One section was stained with hematoxylin and eosin, and another was immunostained with primary antibody against PDPN (D2-40). For routine hematoxylin and eosin staining, sections were placed on slides coated with egg albumin. For IHC staining, sections were placed on 3-aminopropyl triethoxy silane coated slides.

Immunohistochemical staining

Sections were placed on hot plate at 60°C for 10 min. Dewaxing was done using xylene followed by rehydration in ethyl alcohol. Then, the slides were rinsed with distilled water for 5 min.

Antigen retrieval

Tri sodium citrate buffer solution (1000 ml distilled water with 2.94 g of Tri sodium citrate buffer) at pH 6 was used for antigen retrieval immunostaining.[13]

Blocking

The slides were covered with 3% hydrogen peroxide block (RE7165) reagent. The sections were incubated for 10 min at room temperature in humid chamber and rinsed well with buffer wash. The tissues were then covered with protein block reagent (RE 7166) and incubated for 5–10 min at room temperature in humid chamber. The tissues were then gently blotted.

Labeling

Tissues were covered with primary antibody (Anti human D2-40, Mouse monoclonal IgG1 kappa Dako USA, Ready-to-use). The slides were incubated for 1 h at room temperature in humid chamber and rinsed well with wash buffer. Tissues were covered with post primary (RE7167) reagent and incubated for 30 min at room temperature in the humid chamber. The slides were then rinsed well with wash buffer. Sections were covered with Novalink polymer horse radish peroxidase (RE7168) reagent and incubated for 30 min, at room temperature in the humid chamber. The slides were then rinsed well with wash buffer.

Tissues were covered with Substrate Solution (1 μl DAB chromogen) DAB-3-3 (RE7169) reagent mixed with 6 μl of substrate buffer DAB (RE 7171) reagent and incubated for 10 min, at room temperature in the humid chamber. The slides were rinsed well with wash buffer. The sections were counterstained with hematoxylin (RE7172), dehydrated, cleared and mounted.

The immunopositivity of PDPN was determined by the presence of brown-stained lymphatic vessels. Any lymphatic vessel or lymphatic vessel cluster positive for

PDPN antigen and clearly separate from an adjacent cluster was considered to be a single, countable microvessel. Vessel lumen was not necessary for a structure to be defined as a microvessel.

Lymphatic vessel density quantification

Quantitative analysis of the tumoral microvessel density was performed according to modified protocol given by Weidner *et al*. Lymphatic vessel density (LVD) was defined as the number of PDPN-positive vessels per optical field (an optical field corresponds to an examination area of 0.15 mm^2).

First, all slides were screened using a low-magnification objective lens to identify the areas that contained the highest number of positively stained vessels (3 hot spots). Then, the number of vessels was counted by 2 observers in three hot spots using (×400) magnification lens. The total number of lymphatic vessels counted at ×400 magnification were divided by the area of the objective (0.15 mm^2 for 40 × objective-Olympus CX21*i*).

Statistics

The statistical analysis was done using statistical software SPSS version 21.0 Spss software 2017 (IBM Inc, Chicago, Illinois, USA). Percentage distribution was done for demographic data. Descriptive statistics was used for discrete variables and summarized as mean with standard deviation and as number with percentage. Kruskal–Wallis test, analysis of variance and pair-wise Tukey's *post hoc* test were applied to evaluate the significant differences among the mean values in different groups. Results with "$P < 0.05$" were considered to be statistically significant at 95% of confidence level.

RESULTS

On computing the demographic details pertaining to the age group in OED, majority of the patients with OED 16 (53.33%) were in the age group of 20–40 years with a mean age of 42.66 years. On analyzing the demographic data pertaining to the gender in OED, a definite male predominance was noted accounting for 26 (86.66%) cases. On studying the site distribution of the lesions, buccal mucosa 18 (60.00%) was the predominant site for OED, followed by gingivobuccal sulcus 5 (16.66%), labial mucosa 5 (16.66%) and alveolus 2 (6.66%) [Table 1].

PDPN LVD scores were found to increase from mild (13.55) [Figures 1 and 2], moderate (22.81) [Figures 3 and 4] to severe (44.15) [Figures 5 and 6] dysplasia. The differences in PDPN LVD scores were found to be statistically significant among histopathologic grades of OED ($P < 0.05$). Pair-wise comparisons of the PDPN LVD scores and the histopathologic grades of OED were found to be statistically significant ($P < 0.05$) among mild versus severe and moderate versus severe [Table 2].

IHC positivity was absent in the normal oral mucosa tissue.

DISCUSSION

OSCC can be preceded by the appearance of lesions which have the potential either to develop into cancer or portend the development of cancer in the oral cavity.[14] OPMDs include a variety of lesions and conditions characterized by an increased risk for malignant transformation to OSCC.[15] The concept of a step-wise transition from OPMD to OSCC is well established, but it can be difficult to predict if and when an OPML will undergo full transformation and result in a tumor. The presence of epithelial dysplasia in OPMD is generally accepted as one of the most reliable predictors of malignant development. However, histopathologic diagnosis is subjective and lacks sensitivity.[16]

Thus, the challenge in the field of oral precancer is to predict which lesions will eventually develop into carcinoma.[17]

Table 1: Distribution of oral epithelial dysplasia according to age group, gender, and site

	Total number of cases (%)	Total (%)
Age (years)		
20-40	16 (53.33)	30 (100)
41-60	12 (40.00)	
>60	2 (6.66)	
Gender		
Male	26 (86.66)	30 (100)
Female	4 (13.34)	
Site		
Buccal mucosa	18 (60.00)	30 (100)
Gingival sulcus	5 (16.66)	
Labial mucosa	5 (16.66)	
Alveolus	2 (6.66)	
Tongue	0	

Table 2: Comparison of podoplanin lymphatic vessel density with histologic grades of oral epithelial dysplasia using one-way ANOVA and pair-wise comparison by Tukey's *post hoc* test

	Grades of oral epithelial dysplasia	*n*	Mean	SD	ANOVA *f*	*P*
Podoplanin LVD	Mild	15	13.558	3.103	12.350	0.000
	Moderate	9	22.815	3.236		
	Severe	6	44.150	28.870		
Pair wise comparisons by Tukey's *post hoc* test						
Mild versus moderate					0.215	
Mild versus severe					0.000	
Moderate versus severe					0.010	

LVD: Lymphatic vessel density, SD: Standard deviation

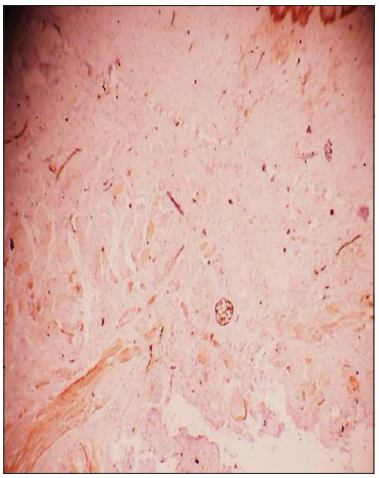

Figure 1: Immunohistochemical image showing hotspot in mild dysplasia (×40 magnification)

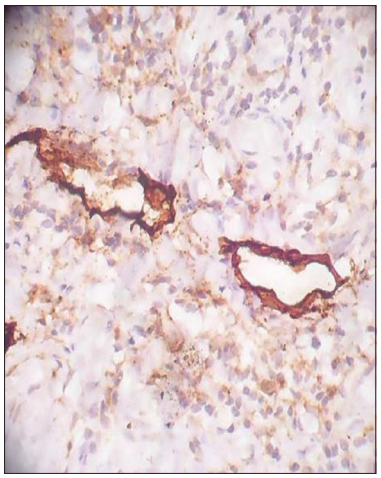

Figure 2: Podoplanin-positive lymphatic vessels in mild dysplasia (×400 magnification)

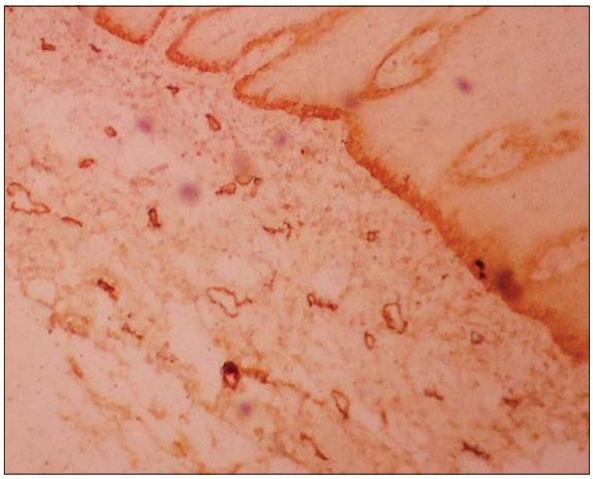

Figure 3: Immunohistochemical image showing hotspot in moderate dysplasia (×40 magnification)

Figure 4: Podoplanin-positive lymphatic vessels in moderate dysplasia (×400 magnification)

It is well established that tumor growth is associated with angiogenesis, and vascular density is correlated with a higher risk of hematogenous metastasis. This relationship is based on the idea that the more the number of vessels present, the greater is the probability of vascular invasion. It is unclear whether tumor induces lymphangiogenesis or permeates preexisting lymphatic vessels. Apart from migratory activity of tumor cells, the composition of extracellular matrix and the expression of adhesion molecules; growth factors secreted by tumor cells or by an inflammatory infiltrate influence angiogenesis. In commonly used hematoxylin staining, lymphatic vessels are often not clearly distinguishable from capillaries and post capillary venules. Thus, in order to assess the LVD in OED, we performed this IHC study.[18]

The commercially available antibody D2-40, specifically detecting PDPN in archival paraffin-embedded human tissues, represents a promising tool for more widespread studies of tumor lymphangiogenesis and its role in human cancer progression.

Out of the thirty cases of OED included in the study, the occurrence of OED in the younger age is suggestive of heavy indulgence in the risk of tobacco chewing habits at a very younger age.

On analyzing the site of the lesion, buccal mucosa 18 (60.00%) was the predominant site for OED. This site-specific occurrence could be attributed to the prevalent habit of tobacco and quid chewing which is placed in the sulcus. This causes a constant irritation of mucosa by the quid ingredients along with the release of detrimental carcinogens.

A significant increase was found in LVD from the normal mucosa to OED. The difference in PDPN LVD scores was

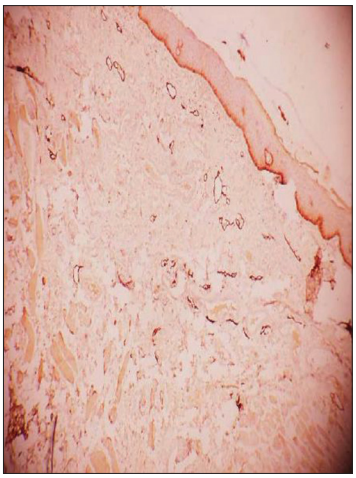

Figure 5: Immunohistochemical image showing hotspot in severe dysplasia (×40 magnification)

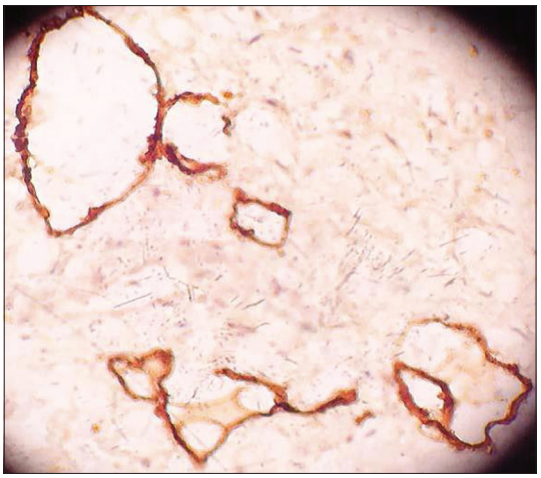

Figure 6: Podoplanin positive lymphatic vessels in severe dysplasia (×400 magnification)

found to be statistically significant among histopathologic grades of OED ($P < 0.05$). Pair-wise comparison of the PDPN LVD scores and the histopathologic grades of OED was found to be statistically significant ($P < 0.05$) among the various groups.

Chandra *et al*.[19] showed significant increase in density with dysplasia status of leukoplakia and oral submucous fibrosis with changing from no dysplasia.

Similar results were reported by Ali in his study in OSCC.

Our results herein reported strongly suggest that lymphatic vessel proliferation occurs early in precancerous lesions. This suggests that the molecular armamentarium of proliferating lymphatic neovascularization begins early in the intraepithelial lesions. Increased density of lymphatic vessels associated with severe dysplasia suggested an essential role of lymphangiogenesis in the progression noninvasive to invasive lesions.

Such a detailed analysis of correlation between PDPN LVD and histologic grades of OED is not documented to the best of our knowledge. One unique feature of our study is the experimental design, which assessed the PDPN LVD in Normal Oral mucosa and OED and compared them. Normal mucosal staining of PDPN showed a lack of expression. Thus, the expression of PDPN increases from normal oral mucosa to OED.

Some variances exist among various studies which could result from the differences in the sample size, lack of uniformity in selection of grades of OED, differences in IHC protocols, differences in evaluation of PDPN positivity, and LVD among observers.

However, our study had a relatively small sample size was unicentric. For clinical application, prospective studies involving larger number of patients are needed to further evaluate the clinical utility of PDPN as a biomarker for oral cancer risk assessment providing additional value beyond the clinical and histological marker.

On establishment of the role of PDPN on invasion and tumorigenesis, PDPN can serve as an effective therapeutic target.

CONCLUSIONS

Early lymphatic vessel proliferation occurring in precancerous lesions suggests that the molecular armamentarium of proliferating lymphatic neovascularization begins early in the intraepithelial lesions.

Assessment of PDPN expression and LVD in OED may aid in identifying the patients with higher risk of tumor initiation, progression, and developing regional metastasis. Thus, this study suggests the utility of PDPN as a biomarker for cancer risk assessment, as it detects the early changes and thus providing additional value beyond current clinical and histopathological evaluations.

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Conflicts of interest

There are no conflicts of interest.

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