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LYMPHANGIOGENESIS IN ORAL SQUAMOUS CELL CARCINOMA AND POTENTIALLY MALIGNANT ORAL LESIONS

SAUMYA CHANDRA[*1], MADHU MATI GOEL[2], ANNU MAKKER[3], SEEMA NAYAK[4], PRIYANKA SHILPI[5], VIKRAM BHATIA[6] AND M.L.B. BHATT[7]

[1,2,3,4,5,6] *Department of Pathology, King George's Medical University, Lucknow*
[7] *Department of Radiation Oncology Dr. Ram Manohar Lohia Institute of Medical Sciences, Lucknow*

ABSTRACT

Podoplanin is a mucin-like glycoprotein that is important in lymphangiogenesis but not in blood vessel formation. The aim of this study is to determine the role of podoplanin in malignant transformation of PMOLs to oral cancer and in progression of oral cancer. In this report, LVD defined as of podoplanin detected positive lymphatic vessels and MVD as the density of CD-31 positive microvessels. The objectives of this study were to clarify the clinical and prognostic significance of both LVD and MVD in PMOLs and oral cancer. The current results suggested that LVD is a more useful tool than MVD. We can conclude that podoplanin is involved in malignant transformation to oral cancer in PMOLs patients and can be a predictor for lymph node metastasis in oral cancer patients.

KEYWORDS: **Potentially malignant oral lesions (PMOLs), Epithelial Dysplasia, Malignant Transformation, Oral cancer, Lymph Node**

SAUMYA CHANDRA
Department of Pathology, King George's Medical University, Lucknow

INTRODUCTION

Development of oral cancer is a multistep process involving accumulation of genetic, epigenetic, and metabolic alterations resulting from exposure to carcinogens[1,2]. Oral leukoplakia and oral submucous fibrosis are the most common premalignant oral disorders [3,4]. It comprises several histopathologically diverse lesions with a variable risk of development of oral squamous cell carcinoma 5. Studies with follow-up periods of up to 7 years reported malignant transformation rates from 3.0% to 17.5% [6,7]. The identification of reliable markers that indicate a high risk of malignant transformation in oral leukoplakia and oral submucous fibrosis is particularly important because of the poor prognosis of oral squamous cell carcinoma [8]. Generally, the dysplasia status is recognised as a useful parameter for malignant transformation to oral cancer [9,10,11]. However, histopathological grading has limited value in predicting the risk of developing cancer. Recent studies have identified podoplanin as a potential marker for malignant progression from oral leukoplakia to invasive carcinoma [12]. A prominent feature of malignant behavior is the capability of tumor cells to metastasize to other organs. Metastases of various human carcinomas, including oral squamous cell carcinoma, occur primarily through the lymphatic system. Cross-talk involved in tumor cell-cell adhesion and tumor cell-stromal contact is recognized as an important condition for invasion and metastases [9]. Most of oral cavity cancers are squamous cell carcinomas and, although they are accessible to biopsy and early identification, at the time of diagnosis, most of them have already metastasized. Metastatic spread to regional lymph nodes through the lymphatic system is one of the major pathways by which head and neck squamous cell carcinoma (OSCC) disseminates. The mechanisms that tumors use to metastasize are well documented concerning the hematogenous spread, but lymphatic spread is not so well understood. However, recent findings show its importance in several human malignancies, including HNSCC[13].

Tumor angiogenesis (new blood vessel growth) is well established in the literature partly owing to empiric logic that a neoplasm, being an autonomous new growth, has to have new and more supporting and feeding vessels and partly to the wide availability of a multitude of specific blood vascular markers. On the other hand, tumor lymphangiogenesis (the formation of tumor-associated lymphatic vessels) has been a controversial issue. This has been partly attributed to the lack of a specific lymphatic endothelial marker. To understand the mechanisms underlying lymphangiogenesis it is essential to understand how lymph vessels develop and find specific markers for them. During embryonic development endothelial cells express lymphatic vascular endothelial receptor (LYVE-1) and vascular endothelial growth factor receptor (VEGFR-3) and, afterwards, the expression of the homeobox gene *Prox1* commits these cells to the lymphatic lineage [14]. Members of VEGF family are closely related to the lymphatic vessels spread. Lymphangiogenesis largely depends on VEGFC signalling and the activity of the receptor VEGFR-3 [15,16]. Another molecule involved in this process is Podoplanin, is a mucin-type transmembrane glycoprotein that is specifically expressed in lymphatic endothelial cells but not in blood endothelial cells [17,18]. Enhanced over expression of podoplanin has been correlated with a poor clinical outcome[25]. The aim of this study was to investigate lymphatic and micro vessel densities and their correlation with clinicopathological parameters in PMOLs and & OSCC including overall survival and malignant transformation respectively. For that purpose, Podoplanin was used to detect LVD and CD-31 to detect MVD.

MATERIALS AND METHODS

Patients and sample collection

The study included a total of 215 prospective cases of oral cancer (n=86), potentially malignant oral lesions (n=79) [including 54 cases of leukoplakia (LKP) and 25 cases of oral submucous fibrosis (OSMF)] and 50

cases of normal healthy controls collected from Department of Oral and Maxillofacial Surgery, Surgery, Radiotherapy and Otolaryngology after obtaining the Institutional Ethical approval and informed written consent from the patients. Healthy oral tissues were obtained from patients undergoing cosmetic surgery, who otherwise did not have any infective or inflammatory oral lesion. The relevant clinical and demographical data was recorded.

Follow up

The patients were diagnosed histologically and followed-up every 2 months in the 1st year, every 3 months in the 2nd year and every 4-6 months thereafter. Overall survival was measured from the date of histological diagnosis to death or last follow-up to a maximum of 5 years.

Histopathological examination

All oral tissues were fixed in 10% neutral buffered formalin and processed for histopathological examination as described [19]. 5um thick sections were cut and stained with haematoxylin and eosin (H&E). Sections were reviewed by two independent pathologists and histological diagnosis was made as per WHO criteria [11].

Immunohistochemistry

Immunohistochemistry was performed using primary antibodies to Podoplanin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and CD-31(Biogenex CA, USA) Sections were deparaffinised in xylene followed by hydration in graded ethanol. Secondary antibody kit used was Novo link min polymer detection kit (Novacastra, Leica Biosystem Newcastle Ltd, UK). Formalin-fixed, paraffin-embedded tissues sections (3-4µm thick) were taken on 3-aminopropyl triethoxysilane (APTES) coated glass slides. Sections were immersed in antigen-retrieval solution (Citrate buffer pH 6.0) and antigen retrieval was done in the antigen-retrieval system (Biogenex Laboratories, Inc, CA, US) at 98°C for 15 min. The sections were then brought to room temperature. Endogenous peroxidase activity was blocked in 3% hydrogen peroxide for 5 min and nonspecific binding sites were blocked with protein block for 5 min. Sections were covered with 50 µl of individual primary antibody, kept in moist chamber and slides were incubated over night at 4°C. Slides were then washed with tris buffer saline (TBS), followed by a 30 minutes incubation with post primary block at room temperature (RT). Sections were then washed again in TBS and incubated with secondary antibody for 30 min at RT. 3, 3-diamino-benzidine was used as chromogen for visualization of antigen antibody complex. Sections were counterstained with haematoxylin, dehydrated through graded ethanol, cleared in xylene and mounted with DPX. Negative control slides omitting the primary antibody were included in all batches. Section from tonsillar tissue served as positive control for podoplanin and pyogenic granuloma for CD-31.

Immunohistochemical Evaluation of Podoplanin and CD-31

The immunohistochemical positive reaction of Podoplanin and CD31 antibodies was evaluated considering its expression in the cytoplasm of lymphatic and blood endothelial cells, respectively. Lymphatic vessels could generally appear as distorted and overlapped structures in cancer setting, the packed vessels were assumed as one lymphatic unit. Fields showing intense vascularisation (*i.e.* the 'Hot Spots') were identified on low power, provided they were not in areas of hemorrhage or necrosis.The number of vessels were counted at 40X magnification. A mean of 5 hot spot fields were taken both for lymphatic vessel density (LVD) and microvessel density (MVD)(figure 3).

Statistical Analysis

Statistical analysis was performed using SPSS 16 software for windows (SPSS, Inc, Chicago, IL, USA). LVD and MVD data was described in term of mean ± standard deviation. Data was examined for statistical significance using the T-Student test, the One-Way ANOVA. For the all test a 'p' value <0.05 was considered significant. Survival between two groups was compared by Kaplan-Meier method and the difference in survival was done by Log rank test. A two-sided ($\alpha=2$) $p<0.05$ was considered statistically significant.

RESULTS

1. Patient's characteristics, LVD and MVD

In PMOL group there were 77.2% (n=61) men and 22.8% (n=18) women and the average age was 35 years with a range from 16-55 year. 68.4% (n=54) were leukoplakia and 38.89% (n=21) were OSMF. Primary oral sites were as follows: 17.7% (n=14) in tongue, 64.6% (n=51) in buccal mucosa, 5.1% (n=4) in gingiva, 5.1% (n=4)) in the lip, 6.3% (n=5) in palate and 1.3% (n=1) in retromolar region. In leukoplakia 61.11% (n=33) were leukoplakia with dysplasia, 38.89% (n=21) were leukoplakia without dysplasia, in OSMF group 48% (n=12) with dysplasia and 52% (n=13) without dysplasia (table2). Patients with oral squamous cell carcinoma there were 70.9% (n=61) men and 29.1% (n=25) women, and the average age was 50 years, with a range from 25–65 years. Primary tumor sites were as follows: 36.0% (n=31) in tongue, 27.9% (n=24) in the buccal mucosa, 7.0% (n=6) in the floor of mouth, 6.0% (n=5) in gingiva, 7.0% (n=6) in the lip, 7.0% (n=6) in the palate, 2.3% (n=2) in retromolar region and 7.0% (n=6) in tonsil. 65.12% (n=56) tumors were histologically well differentiated, 29.06% (n=25) were moderately differentiated, and 5.81% (n=5) were poorly differentiated. Patients were grouped by Tumor and Node stages as follows: 17.44% (n=15) in T1, 11.62% (n=10) in T2, 47.67% (n=41) in T3 and 9.3% (n=20) in T4 and 55.82% (n=48) in N0, 44.18% (n=38) in N1 (table 3). Table 1 shows mean LVD and MVD in normal, PMOLs and oral squamous cell carcinoma, The mean LVD in OSCC (22.34 ± 6.32) was significantly higher than that in PMOLs (10.14 ± 3.54) and normal (6.20 ± 5.19; $p<0.001$).The mean MVD in OSCC 21.79 ± 2.61 was significantly higher than that in PMOLs (8.48 ± 3.68) and normal (8.28 ± 4.89; $p<0.001$).

2. Correlation of clinicopathological parameters with LVD and MVD in patients with PMOLs

Table 2 summarizes the correlation of LVD and MVD with clinicopathological parameters in patients with PMOLs and in those showing malignant transformation. Buccal mucosa was the most common site. LVD shows significant increase in density with dysplasia status of leukoplakia and oral submucous fibrosis with changing from no dysplasia (9.43±3.23) to dysplasia (12.58±3.02, $p<0.0001$). No significant association of MVD was found with dysplasia status ($p>0.18$). No significant correlation of LVD and MVD was found with age, sex, tobacco, alcohol and smoking habit. 16.5% (13/79) patients with oral leukoplakia were converted into oral cancer. The mean LVD of oral leukoplakia converted (13.53±3.64) to oral cancer patients was significantly higher as compared to patients those not converted (9.46±3.13, $p<0.001$) (Fig. 2). None of the patients with OSMF show malignant transformation. However, the mean MVD did not show significant difference between two groups though it was comparatively higher in converted malignant than not converted.

3. Correlation between density of lymphatics and micro vessels and clinicopathological parameters with OSCC patients

Table3 summarizes the correlation of LVD and MVD with clinicopathological parameters in patients with OSCC. The LVD of poorly differentiated tumors (31.0±7.21) was significantly higher than that in the moderately differentiated tumors (25.28±6.54), and the LVD in the moderately differentiated tumors was significantly higher than that in well-differentiated tumors (20.25±4.91). The LVD in tumor stage I (19.06±3.39) was significantly lower than that in the stage II group (20.90±5.76), and the LVD in stage III (22.14±6.24) were lower than that in stage in IV (25.90±7.05). The lymph node metastasis group (24.41±6.91) was significantly higher than non-lymph node metastasis group (19.71±4.28,$P < 0.0001$). There was no significant association between LVD and age, sex, and primary site. No significant association was found between MVD and age, sex, primary site, tumor stage, lymph node status. No significant association of LVD and MVD was found with tobacco, alcohol and smoking habit. Patients were followed-up for a minimum of 48 months. During this period 41.86% (36/86) patients died due to oral cancer. Significant difference in overall survival was observed when comparing

patients with high v/s low LVD (Log rank, test, p<0.001). Similarly, patients with higher MVD also had significantly lower survival (p<0.05) as compared to those with lower MVD (Fig.1). Further, survival analysis revealed significantly lower survival (Hazard ratio=0.28, 95% CI=0.12-0.50) (Hazard ratio=0.43, 95% CI=0.21-0.82) those with higher LVD (Log rank: χ^2=15.33, p<0.001) and higher MVD (Log rank: χ^2=6.52, p=0.011) respectively as compared to lower LVD and lower MVD,

Table 1
Lymphatic and microvessel density levels (Mean ± SD) of three groups

Density	Normal (n=50)	PMOLs (n=79)	OSCC (n=86)	F value	p value
LVD	6.20 ± 5.19 (0-22)	10.14 ± 3.54 (0-19)	22.34 ± 6.32 (9-38)	189.64	p<0.001
MVD	8.28 ± 4.89 (2-25)	8.48 ± 3.68 (2-16)	21.79 ± 2.61 (13-27)	348.91	p<0.001

Table 2
Correlation of LVD and MVD with clinicopathological parameters in patients with PMOLs

Clinical characteristics	N (%)	LVD (Mean ±SD)	MVD (Mean±SD)
Age			
<35	31(39.24%)	9.74±3.80	8.03±4.23
>35	48(60.76%)	10.39±3.38	8.72±3.71
p-value		0.42	0.96
Sex			
Male	61(77.2)	10.34±3.45	8.52±4.08
Female	18(22.8)	9.44±3.83	8.2±3.38
p-value		0.34	0.53
Site			
Buccal mucosa	51(64.6%)	9.56±3.44	8.13±3.83
Gingiva	4(5.1%)	10.25±0.95	8.5±2.38
Lip	4(5.1%)	10.75±2.06	9.00±6.37
Palate	5(6.3%)	11.4±4.50	9.6±3.78
Retromolar region	1(1.3%)	6	6
Tongue	14(17.7%)	11.85±3.95	9.21±4.28
ANOVA p-value		0.24	0.82
Tobacco			
Absent	26(32.9%)	10.18±3.86	8.35±3.90
Present	53(67.1%)	10.03±2.83	8.65±4.00
p-value		0.86	0.67
Alcohol			
Absent	29(36.7%)	10.89±2.73	9.24±3.84
Present	50(63.3%)	9.7±3.89	8.00±3.92
p-value		0.14	0.20
Smoking			
Absent	21(26.6%)	11.33±3.35	9.47±4.00
Present	58(73.4%)	9.7±3.86	8.08±3.55
p-value		0.07	0.24
PMOLs_dysplasia status			
Leukoplakia	54(68.4%)		
Leukoplakia with dysplasia	33(61.11%)	12.58±3.02	9.24±4.81
Leukoplakia without dysplasia	21(38.89%)	9.43±3.23	8.61±3.21
Oral submucous fibrosis	25(31.6%)	7.52±1.91	7.28±2.82
Oral submucous fibrosis with Dysplasia	12(48%)	8.00±1.79	7.25±2.2
Oral submucous fibrosis without Dysplasia	13(52%)	7.08±2.01	7.3±3.3
p-value		<0.0001	0.18
Status _PMOLs			
Converted to malignancy	13(16.5%)	13.53±3.64	10.00±4.33
No conversion	66(83.5%)	9.46±3.13	8.15±3.7
p-value		<0.0001	0.10

Table3
Correlation of LVD and MVD with clinicopathological parameters in oral cancer

Clinical characteristics	N (%)	LVD (mean ±SD)	MVD(mean ±SD)
Age			
<50	42 (48.83%)	20.85±5.48	21.88±2.63
>50	44 (51.17%)	23.75±6.79	21.70±2.61
Sex			
Male	61 (70.9%)	22.11±6.59	21.85±2.35
Female	25 (29.1%)	22.88±5.68	21.64±3.20
p-value		0.61	0.73
Site			
Tongue	31 (36.0%)	23.33±1.09	22.12±0.42
Buccal mucosa	24 (27.9%)	20.50±6.35	21.50±2.39
Floor of mouth	6 (7.0%)	22.33±1.63	19.16±1.83
Gingiva	5 (5.8%)	17.60±5.54	22.20±3.89
Lip	6 (7.0%)	22.66±6.65	21.80±2.63
Palate	6 (7.0%)	22.83±7.19	21.30±4.27
Tonsil	6 (7.0%)	21.41±2.34	23.00±0.45
Retromolar region	2 (2.3%)	22.73±4.24	24.50±.071
p-value		0.47	0.15
Tumor stage			
T1	15 (17.44%)	19.06±3.39	21.93±2.55
T2	10 (11.62%)	20.90±5.76	22.00±3.12
T3	41 (47.67%)	22.14±6.24	21.70±2.75
T4	20 (23.32%)	25.90±7.05	21.75±2.24
ANOVA p-value		0.01	0.98
Node stage			
N1	38 (44.18%)	24.41±6.91	21.55±3.01
N2	48 (55.82%)	19.71±4.28	21.97±2.25
p-value		<0.0001	0.45
Histological grade			
WD	56 (65.12%)	20.25±4.91	21.28±2.67
MD	25 (29.06%)	25.28±6.54	22.60±2.36
PD	5 (5.81%)	31.0±7.21	23.40±1.51
p-value		<0.0001	0.04
Tobacco			
Absent	21 (24.4%)	22.55±6.35	22.14±2.30
Present	65 (75.6%)	21.66±6.32	21.67±2.71
p-value		0.57	0.48
Alcohol			
Absent	31 (36.0%)	22.90±6.33	21.89±2.84
Present	55 (64.0%)	21.32±6.26	21.61±2.17
p-value		0.26	0.28
Smoking			
Absent	22 (25.6%)	22.46±6.83	22.46±6.83
Present	64 (74.4%)	21.95±4.64	21.95±4.64
p-value		0.74	0.74
Status _oral cancer			
Death	36(41.86%)	23.75±5.50	22.25±2.27
Alive	50(58.13%)	21.32±7.14	21.46±2.28
p-value		0.07	0.16

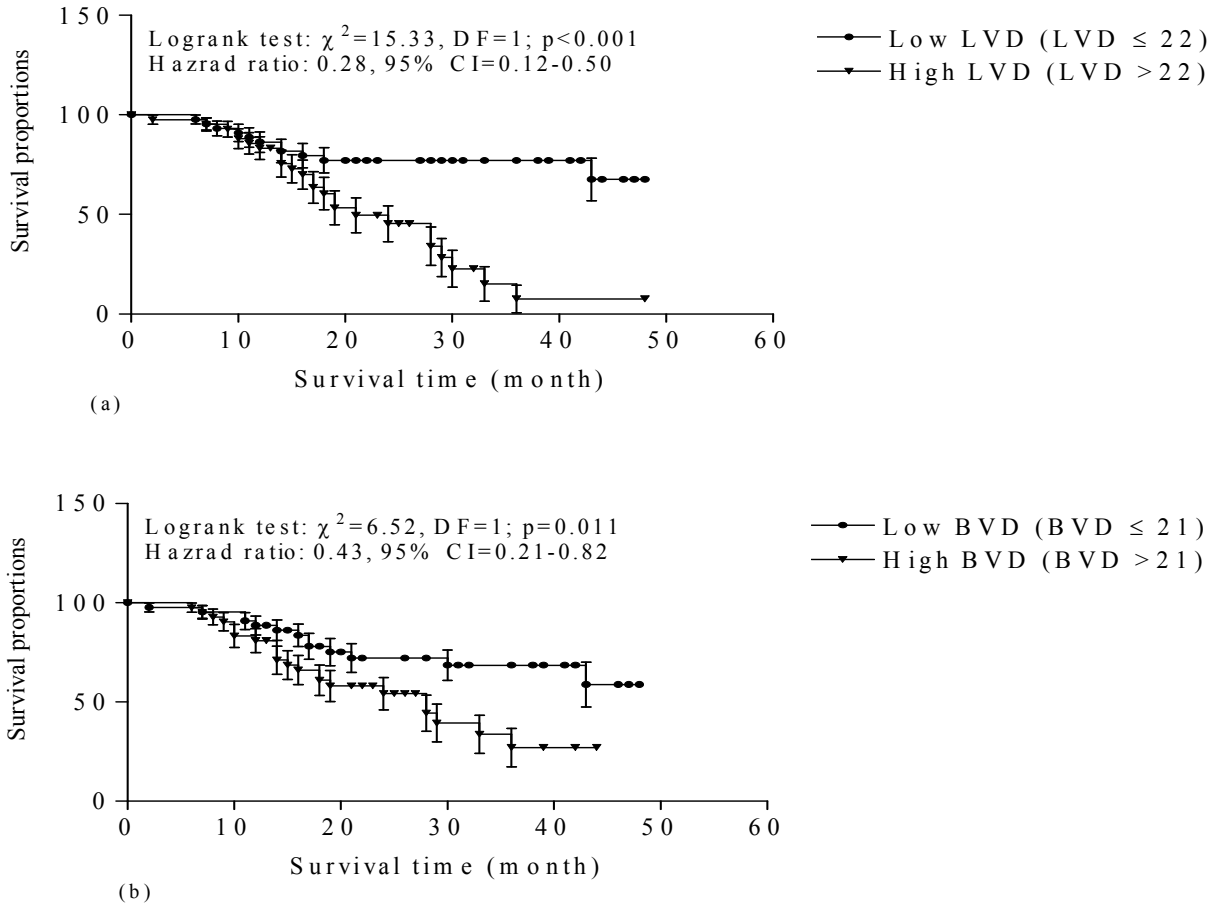
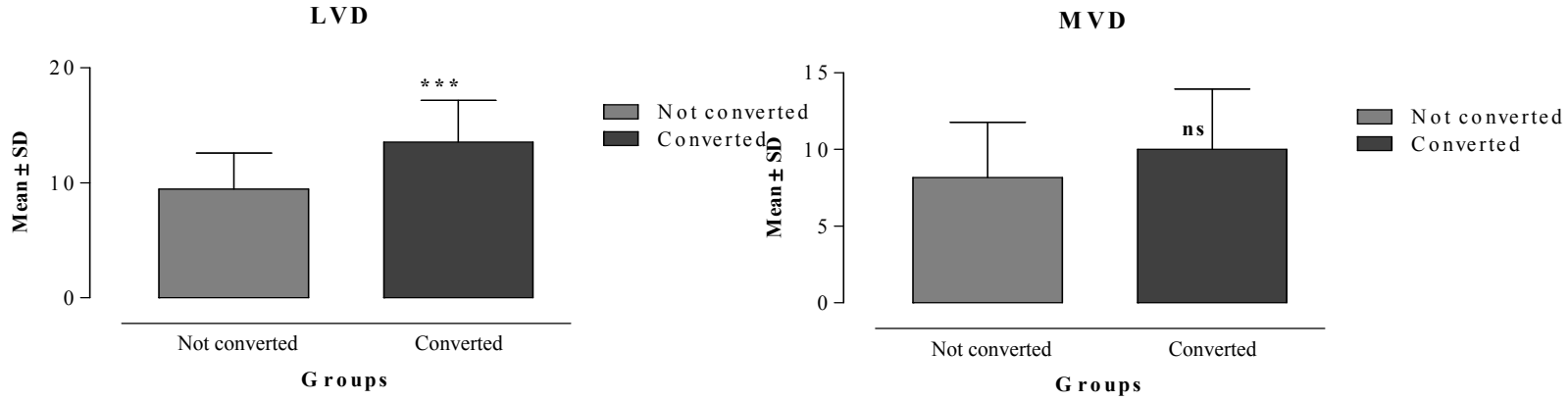


Figure 1
4 years overall survival in OSCC patients according to (a) low and high LVD (b) and low and high MVD

Figure 2
Mean LVD and MVD of PMOLs with conversion status



*ns p>0.05 or *** p<0.001- Not converted vs. Converted*

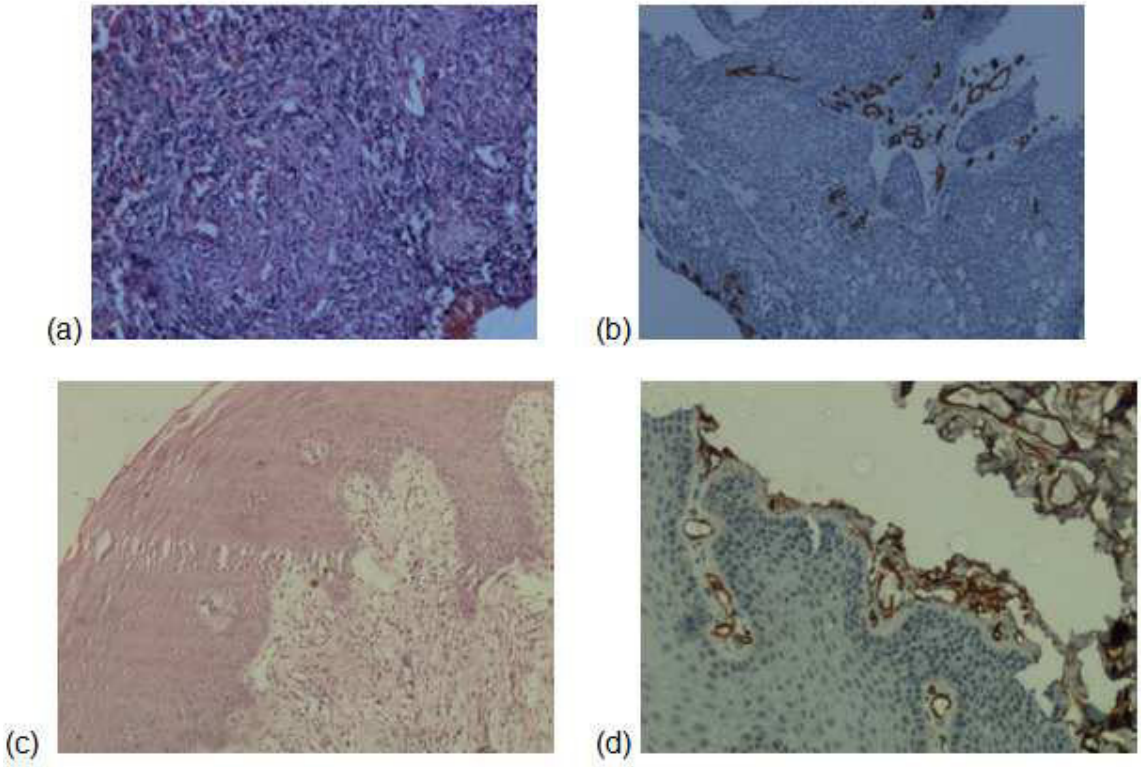


Figure3

Immunohistochemical staining of Podoplanin. (a) Histological section of oral squamous cell carcinoma (H and E, ×100), (b) Podoplanin positivity in lymphatic vessels in tissue section of oral squamous cell carcinoma (H and E, ×100) (c) Histological section of leukoplakia (LKP) with dysplasia (H and E, ×100); (d) Podoplanin detected lymphatic vessels positivity in LKP with dysplasia Immunohistochemistry (IHC ×100)

DISCUSSION

The lymphatic system is the primary pathway of metastasis for oral cancers, and the extent of lymph node involvement is a crucial prognostic factor for the patient's outcome. The study on lymphangiogenesis in PMOLs is still in preliminary stage. Current work provides an approach whether podoplanin expression in lymphatic vessels in pretreatment biopsies could serve as a biomarker to predict the risk of malignant transformation in patients with oral leukoplakia and OSMF. Funayama et al, 2011 describe similar findings that LVD in oral leukoplakia is strongly associated with dysplasia ($p<0.001$) [20]. Similar to our findings, Kawaguchi et al, 2008 have also shown that dysplasia status had a strong impact in terms of malignant transformation in oral leukoplakia. But we do not found any significant association with either clinicopathological parameters or malignant transformation in case oral submucous fibrosis [12]. In case of OSCC, LVD was found to be significantly associated with tumor stage node status and histological grading but did not influence other clinicopathological parameters (age, sex, tobacco, alcohol and smoking). In contrast to the study by Adhemar Longatto Filho et al., 2007, we do not found any significant association between clinicpathological parameters and MVD in oral cancer and PMOLs. Our findings suggest that patients who have an elevated LVD value may have an increased risk for lymph node metastasis, supporting the previous observations that LVD is responsible for the predominant lymphatic spread in oral cancer. A high LVD value may identify patients who are more susceptible to lymphogenous spread [21]. Increased MVD values reportedly are associated with lymph

node metastasis, advanced tumor stage, and a poor prognosis in many kinds of malignancies [22, 23, 24]. However, in head and neck tumors, its clinical significance is controversial. In this study, higher LVD and MVD values were correlated significantly with lower survival rates[9].

Thus, preoperative biopsies followed by additional staining for podoplanin may be important prognostic indicators for disease progression and may be crucial for deciding on therapeutic strategies for patients with PMOLs and oral cancer. Here we have demonstrated that LVD is associated with development of the PMOLs to cancer sequence in the oral cavity. From the biomarker standpoint, the potential utility of podoplanin expression depends on whether it can provide additional value beyond current clinical and pathologic assessments. The present study showed highly expressed podoplanin, in oral cancer and potentially malignant disorders. The early occurrence of podoplanin expression in oral tumorigenesis, the strong association between high podoplanin expression and cancer development, and the role of podoplanin in promoting cell invasion, suggest that podoplanin may be used as a biomarker for oral cancer risk assessment and a potential chemoprevention target. However, the results have to be validated in a larger cohort of patients. The use of the LVD and MVD as a marker of potential tumour invasion in pre-neoplastic disease is an attractive proposition. Prevention is always better than cure, and the identification of premalignant lesions with intervention to prevent malignant transformation may and could possibly be used as a follow-up tool.

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CONFLICT OF INTEREST: None

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