Original Article

# An immunohistochemical evaluation of podoplanin expression in oral leukoplakia and oral squamous cell carcinoma to explore its potential to be used as a predictor for malignant transformation

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## Abstract

**Background:** Oral leukoplakia (OL) is a potentially malignant disorder with increased risk for the development of oral squamous cell carcinoma (OSCC). Many cases of OSCC arise from the malignant transformation of preexisting OL. However, the risk of progression into OSCC and the possible prediction of malignant potential of OL remain inconclusive. Recent studies have shown that podoplanin, a mucin-like transmembrane glycoprotein specifically expressed in lymphatic endothelial cells, is expressed in various neoplasms including OSCC, indicating its possible biologic role in tumor cells. In this study, an evaluation of podoplanin expression in OL and OSCC has been carried out to assess its potential role as a biomarker to predict the possibility of malignant transformation in OL cases.

**Aims and Objectives:** To assess the usefulness of podoplanin as a potential biomarker for predicting the risk of malignant transformation in OL, by comparing its immunohistochemical expression in OL and OSCC.

**Materials and Methods:** Archival paraffin-embedded blocks of 25 OL cases with varying grades of dysplasia and 30 OSCC cases showing its varying grades were selected. Sections were subjected to immunohistochemical staining for podoplanin and compared with the control group for evaluation of results in the three groups.

**Results:** A statistically significant increase in podoplanin expression was observed from normal mucosa through OL to OSCC. In the OL cases, the podoplanin staining score progressively increased from mild dysplasia to carcinoma *in situ*, whereas in OSCC, well-differentiated group showed the maximum expression of podoplanin.

**Conclusion:** The progressive increase in podoplanin expression through the increasing grades of dysplasia in OL is suggestive of an increased risk for malignant transformation with increased expression of podoplanin in OL cases. A high podoplanin expression in the well-differentiated OSCC may indicate a vital role for podoplanin in the early stages of tumorigenesis.

**Keywords:** Biomarker, malignant transformation, oral leukoplakia, oral squamous cell carcinoma, podoplanin

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## INTRODUCTION

Head-and-neck cancer is one of the ten most common types of cancers worldwide, afflicting more than 500,000 individuals every year.[1] Oral squamous cell carcinoma (OSCC) represents 95% of all forms of head-and-neck cancer.

Oral SCC can arise either *de novo* or from preexisting potentially malignant disorders including oral leukoplakia (OL), erythroplakia, submucous fibrosis and lichenoid dysplastic lesions.[2,3] OL is the most prevalent premalignant lesion and has an overall increased risk for malignant transformation with a range of 17%–31%.[4-9]

Despite the currently available therapeutic strategies, the 5-year survival rate for OSCC is as low as 53%.[10] This was mainly due to the diagnosis of OSCC in the late stages. Early detection of oral cancer is the most efficient way to ensure patient survival.[11] Therefore, it is important to further understand the biology of OSCC development and to identify biologic markers that may be able to augment the staging system.[12,13]

Podoplanin is a mucin-like transmembrane glycoprotein that is highly and specifically expressed in lymphatic endothelial cells, but not in blood endothelium.[14] It has been shown that podoplanin deficiency disrupts normal lymphatic vasculature formation and causes lymphedema.[15] Recent studies have also shown that podoplanin may be expressed in certain tumor cells, including squamous cell carcinoma, raising a possibility that it may have some biologic functions in tumor cells as well.[16-19]

With this background, a study to explore the immunohistochemical expression of podoplanin in OL, OSCC and normal oral mucosa was undertaken. The expression of podoplanin was evaluated and compared through the varying grades of epithelial dysplasia cases in OL and also through the varying grades of OSCC to look for a correlation in its expression pattern from normal mucosa through OL to OSCC for a possibility to use it as a potential biomarker for malignant transformation of OL.

## MATERIALS AND METHODS

The archives in the department of oral pathology and microbiology were scanned to retrieve the paraffin-embedded blocks of histopathologically reported cases of 25 OL, 30 OSCC and 10 normal oral mucosa which were designated as Groups I, II and III, respectively. The OL group comprised five cases of mild dysplasia, ten cases of moderate dysplasia and five cases each of severe dysplasia and carcinoma *in situ* (Ca *in situ*). The OSCC group included ten cases each of well-differentiated OSCC and moderately and poorly differentiated OSCC. Diagnosis of all the 65 archival blocks was confirmed by viewing the H&E-stained sections prepared from these blocks.

### Immunohistochemistry

Tissue sections, 5-μm thickness, were obtained from each of the 65 selected paraffin blocks and were transferred onto 3-Amino Propyltriethoxy saline (APES)-coated slide. The slides were deparaffinized and rehydrated with graded concentrations of alcohol. Antigen retrieval was performed using a microwave oven by steaming the slides with 10 mmol/L citrate buffer (pH: 6.0) for 20 min. The slides were then immersed in methanol containing 3% hydrogen peroxide for 10 min to block the endogenous peroxidase activity followed by incubation in 10% horse serum (HRP) for 30 min at room temperature. The slides were then incubated with monoclonal antibody (D2-40 antipodoplanin – DAKO, USA) at 1:100 dilution for 1 h followed by the secondary antibody for ½ hour. The sections were then incubated with DAB chromogen for 5–10 min. Finally, the slides were washed and counterstained with Mayer's hematoxylin. After drying and mounting with dibutyl phthalate in xylene, the slides were subject to histomorphometric analysis for podoplanin expression.

### Evaluation of immunohistochemical staining

After immunostaining, the evaluation for podoplanin staining and expression was performed using a "labomed" binocular microscope with 10x eyepiece and 40x objective. The podoplanin expression was evaluated by two separate observers. Cell membrane immunoreactivity in the cells was considered as a positive expression of podoplanin. Podoplanin-positive lymphatic vessels were taken as positive controls for staining [Figure 1].

In OL group, mean quantitative scoring (MQS) for positive podoplanin expression in the epithelium was done using a scoring system described by Kawaguchi *et al*.[4] as follows:

- 0: No expression observed in any part of the epithelium
- 1: Expression restricted to the basal layer of the epithelium
- 2: Expression observed in the basal and suprabasal layers at one area
- 3: Suprabasal layer expression observed at two or three areas
- 4: Suprabasal layer expression observed at more than three focal areas.

Scoring was done for five high-power fields in each slide, and mean was calculated per slide. The staining intensity

of podoplanin expression was also rated for each of the ten sections separately on a scale of 0–3 as follows:

0 = negative – no staining; 1 = weak – faint staining; 2: moderate – staining between 2 extremes (dark brown and weak staining); 3: strong – dark brown staining of cells.

The Mean Staining Intensity Score (MSIS) was then calculated for each slide. The German Immunoreactive Score (IRS) for podoplanin expression was calculated by multiplying MQS and MSIS for each slide. An IRS score of 7 or higher was considered as high reactivity and 0–6 as low reactivity.[20] The above scorings were done for the mild, moderate and severe dysplasia and Ca *in situ* subgroups of OL, and the MQS, MSIS and mean IRS (MIRS) were calculated for each subgroup separately. The mean IRS for the whole group (OL) was also calculated and noted based on this. In all the four subgroups, the lymphatic microvessel density (LVD) was also noted by counting the number of D2-40-positive lymphatic vessels immediately below the basement membrane. The mean LVD (MLVD) for each of the subgroups and for the whole group (OL) was calculated.

In OSCC, MQS scoring was done based on a modification of the scoring criteria given by Rodrigo *et al*. as follows:[20]

- 0: Negative
- 1: Basal cells of the tumor islands alone or central portion of the island alone
- 2: Basal and parabasal cells of the island show positivity (two or more than two layers of cells)
- 3: Half of the island shows positivity
- 4: More than half, up to 3/4$^{th}$ of the island, shows positivity
- 5: The entire tumor islands show positivity.

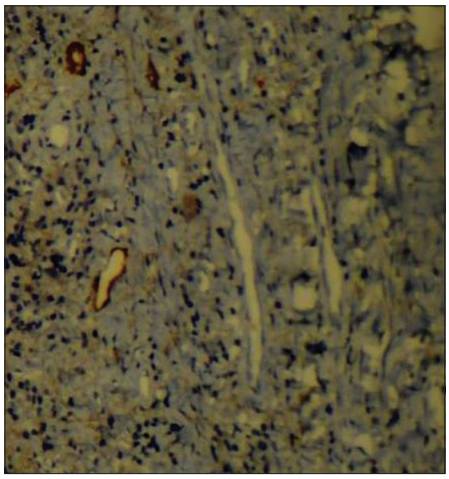


**Figure 1:** Podoplanin-positive lymphatic vessels taken as a positive control for the staining (immunohistochemistry podoplanin ×10). Note that the blood vessels in the field have not taken up the stain

The MSIS and the MIRS were also calculated in a manner similar to that of OL group. The MQS, MSIS, MIRS and MVLD were calculated separately for each of the three subgroups – well-differentiated OSCC and moderately and poorly differentiated OSCC and also for the whole group (OSCC). For each of the podoplanin-positive cases, the pattern of staining of the tumor islands (focal/diffuse) was also noted for comparison.

Similarly, the above parameters were calculated for each of the ten slides of the normal oral mucosa group.

Based on the results obtained above, the statistical comparison of podoplanin expression between the three broad study groups, namely, OL, OSCC and normal oral mucosa was analyzed using one-way ANOVA test, with the help of Statistical Package for the Social Sciences (SPSS) (IBM-SPSS version 20.0, Chicago, IL, USA). Statistical significance was at $P \leq 0.05$. Similarly, intragroup podoplanin expression status in the OL and OSCC was analyzed using Kruskal–Wallis test. The mean positive podoplanin staining values were also compared and analyzed among the four subgroups of Group I and the three subgroups of Group II separately using Chi-square test.

## RESULTS

Based on the results obtained, the percentage positivity of podoplanin expression in the three groups was compared. OL showed a positivity of 76% (19 out of 25), OSCC showed 100% positivity ($n$ = 30), while the normal mucosa showed 60% positivity (six out of ten cases). The differences in these values were found to be statistically significant ($P$ = 0.003) [Table 1].

Our observation based on the MIRS for podoplanin expression in the three main study groups – OL, OSCC and normal mucosa – showed that OSCC showed the

**Table 1: Podoplanin positivity in the three study groups with P value**

| Podoplanin expression | Groups | | | | | | *P* |
|---|---|---|---|---|---|---|---|
| | OL (*n*=25), *n* (%) | | OSCC (*n*=30), *n* (%) | | Normal oral mucosa (*n*=10), *n* (%) | | |
| Positive | 19 | 76 | 30 | 100 | 6 | 60 | 0.003 |
| Negative | 6 | 24 | 0 | 0 | 4 | 40 | |

OSCC: Oral squamous cell carcinoma, OL: Oral leukoplakia

highest MIRS for podoplanin expression (9.15 ± 3.54). The OL group showed an MIRS of 3.76 ± 3.19, whereas normal mucosa showed the least expression of podoplanin (0.83 ± 0.80) [Figure 2]. These differences in the MIRS among the three groups were found to be highly statistically significant ($P < 0.001$) [Table 2].

The MIRS for podoplanin expression was evaluated and compared among the three subgroups of OL – mild, moderate and severe dysplasia and Ca *in situ*. There was a definite increase in the MIRS from mild dysplasia to Ca *in situ*; however, the differences were found to be statistically insignificant ($P = 0.211$). Mild dysplasia showed the least score (2.00 ± 2.00), whereas Ca *in situ* showed the highest (6.26 ± 3.78) [Graph 1 and Figure 3].

**Table 2: Scoring of podoplanin expression in the three study groups with *P* value**

| Groups | *n* | Mean IRS±SD | *P* |
|---|---|---|---|
| OL | 25 | 3.76±3.19 | <0.001 |
| OSCC | 30 | 9.15±3.54 | |
| Normal Oral mucosa | 10 | 0.83±0.80 | |

IRS: Immunoreactive Score, SD: Standard deviation, OSCC: Oral squamous cell carcinoma, OL: Oral leukoplakia

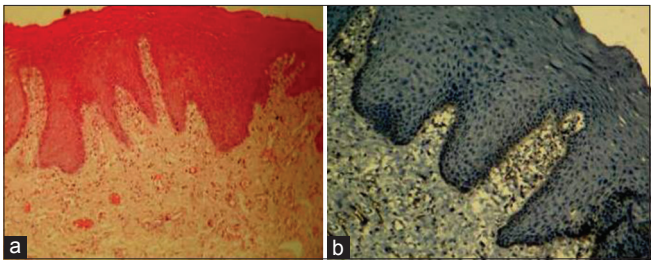



**Figure 2:** Weak podoplanin expression in normal oral mucosa. (a) Normal oral mucosa (H and E, ×10), (b) Very faint expression of podoplanin confined to the basal cells of the oral epithelium (immunohistochemistry podoplanin, ×10)

The MIRS for podoplanin expression was also calculated and compared for the three subgroups in OSCC – well, moderately and poorly differentiated. It was observed that the MIRS decreased from well differentiated (11.03 ± 3.41) to poorly differentiated (7.60 ± 2.37). This difference, however, was found to be statistically insignificant ($P = 0.119$) [Graph 2 and Figure 4].

The MLVD – the mean number of lymphatic vessels at the invasive front – was also evaluated in the three groups and tabulated [Table 3]. It was observed that the MLVD was highest in OSCC (42.00 ± 21.22). OL showed the highest value (27.32 ± 18.91), whereas normal mucosa showed the least MLVD (5.50 ± 4.35). These differences were found to be statistically highly significant ($P < 0.001$) [Figure 5].

## DISCUSSION

Head-and-neck squamous cell carcinoma (HNSCC) is one of the most common types of cancer, with an incidence of nearly 550,000 cases worldwide annually. OSCC is the most common type of HNSCC. Oral cancer development

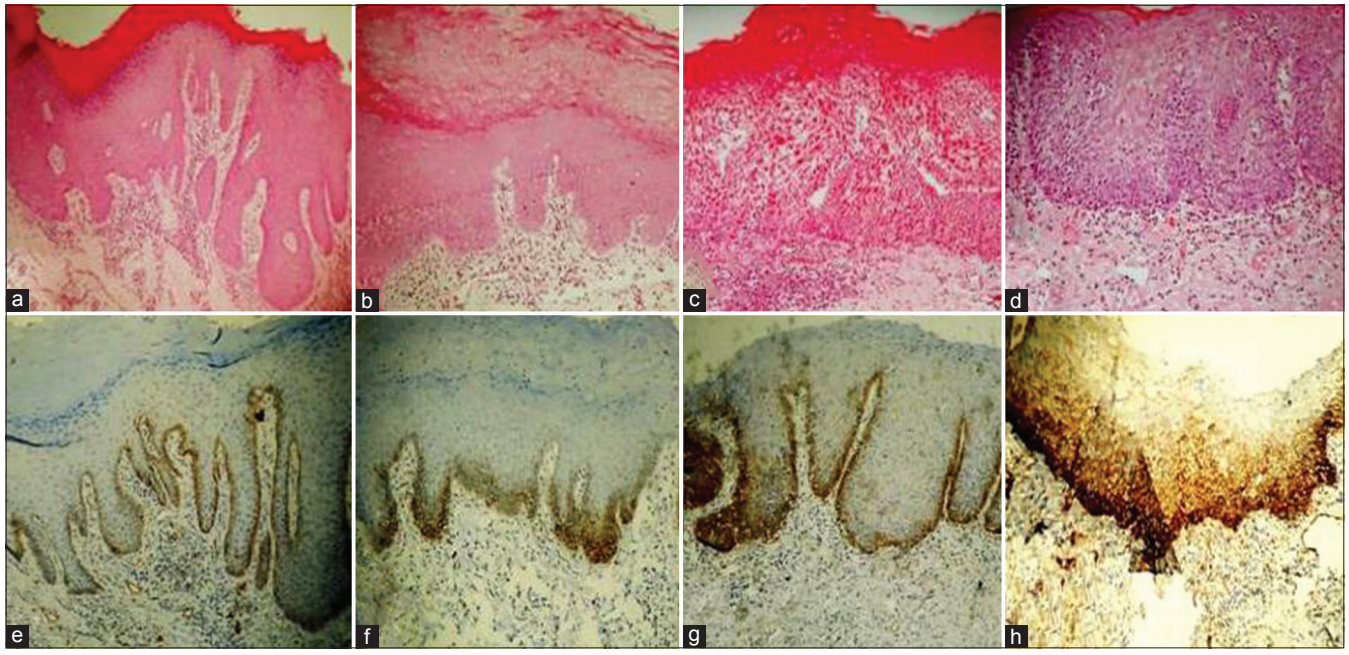
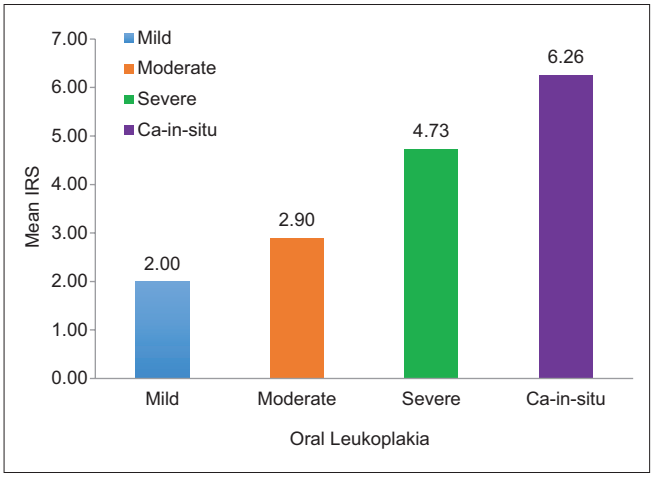
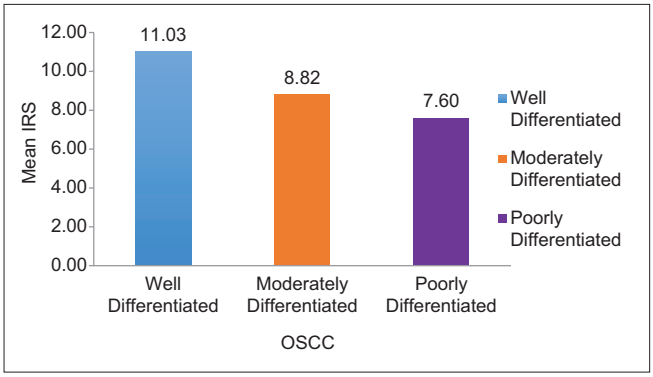



**Figure 3:** Progressive increase in podoplanin expression in varying grades of oral epithelial dysplasias (four subgroups of oral leukoplakia). (a) Mild dysplasia, (b) Moderate dysplasia, (c) Severe dysplasia, (d) Carcinoma *in situ* ([a-d], H&E, ×10), (e) Expression restricted to basal layer in mild dysplasia, (f and g) Expression in more than one focus of basal and parabasal layers in moderate and severe dysplasias, (h) Maximum expression and staining in carcinoma *in situ*, ([e-h], immunohistochemistry podoplanin ×10)

is a multistep process with an accumulation of genetic, epigenetic and metabolic alterations due to exposure to carcinogens.[21] Oral potentially malignant diseases carry an increased risk of malignant transformation. The most prominent among them is OL. Although the grade of epithelial dysplasia is thought to be an indicator for the risk of development of OSCC in OL, there are reports of oral cancers developed from OL that lacked dysplastic changes in the epithelium.[9] This has forced researchers to search for additional objective markers to identify the high-risk lesions of OL that require timely intervention and management.




**Graph 1:** Comparison of mean immunoreactive scores for podoplanin expression among the four subgroups in oral leukoplakia




**Graph 2:** Comparison of mean immunoreactive scores for podoplanin expression among the three subgroups in oral squamous cell carcinoma

Our quest for a potential biomarker for predicting the risk of malignant transformation in OL and for predicting the chances of lymph node metastases as well as tumor progression in OSCC led to a glycoprotein named podoplanin – a 43-kDa type I transmembrane sialomucin-like glycoprotein. Originally detected on the surface of podocytes, it has been shown to be expressed in lymphatic endothelium, but not in blood-vessel endothelium.[14,22] It has, therefore, been utilized as a good marker for recognizing lymphatic vessels and therefore lymphangiogenesis.[23,24] Podoplanin expression in normal oral mucosa has been reported to be either absent or restricted to the basal epithelial layers. It has been suggested that podoplanin may be associated with tissue remodeling and repair in inflammatory lesions. Studies have also reported podoplanin expression in various neoplasms including OSCC.

We undertook the present study to evaluate the MIRS for podoplanin expression in three main groups. Among the three main study groups, OSCC showed the maximum

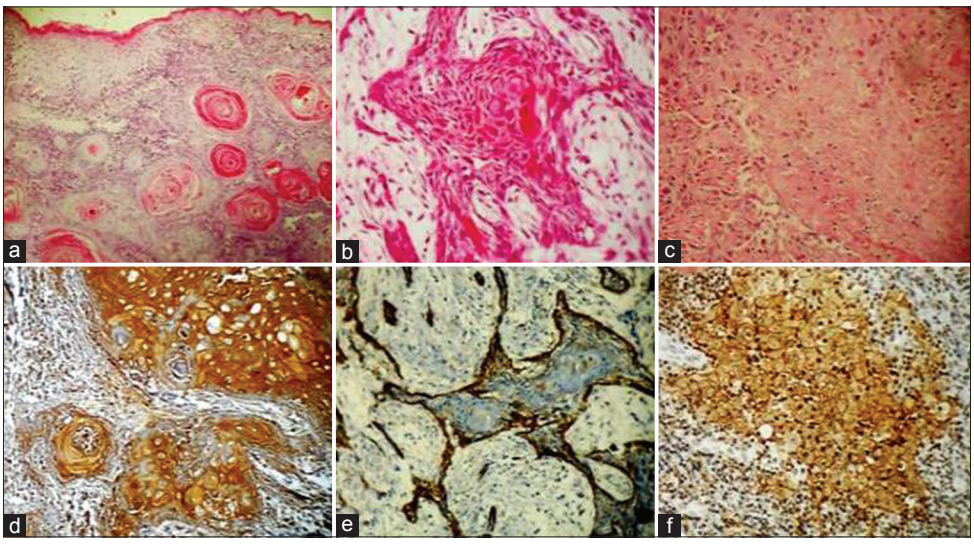



**Figure 4:** Progressive decrease in podoplanin expression in varying grades of oral squamous cell carcinoma. (a) Well differentiated, (b) moderately differentiated, (c) poorly differentiated ([a-c], H&E, ×40), (d) Intense expression and staining in tumor islands in well-differentiated group where both central and peripheral cells of the islands show strong expression, (e) Less intense expression and restricted to peripheral cells in moderately differentiated group, (f) Least expression and weak staining of tumor cells in poorly differentiated group, ([d-f], immunohistochemistry podoplanin, ×40)

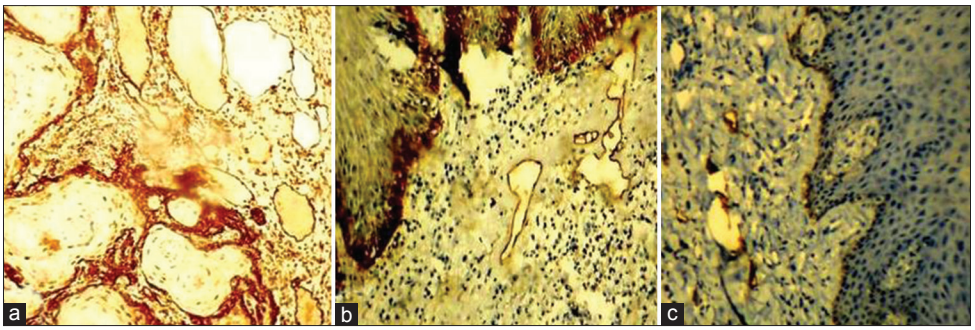



**Figure 5:** Mean lymphatic microvessel density in the three study groups. (a) Maximum mean lymphatic microvessel density was observed in well-differentiated squamous cell carcinoma, (b) Mean lymphatic microvessel density in oral leukoplakia group was found to be lesser than in (a), (c) Least mean lymphatic microvessel density in normal oral mucosa, ([a-c], immunohistochemistry podoplanin, ×10)

**Table 3: Scoring of mean lymphatic microvessel density in the three study groups**

| Groups | *n* | MLVD±SD | *P* |
|---|---|---|---|
| OL | 25 | 27.32±18.91 | <0.001 |
| OSCC | 30 | 42.00±21.22 | |
| Normal oral mucosa | 10 | 5.50±4.35 | |

MLVD: Mean lymphatic microvessel density, SD: Standard deviation, OSCC: Oral squamous cell carcinoma, OL: Oral leukoplakia

expression of podoplanin (9.5 ± 3.54), whereas the normal mucosa showed the least score for podoplanin expression (0.83 ± 0.80). Thus, it was observed that there was a progressive increase in podoplanin expression from normal mucosa to OL to OSCC. This difference was found to be statistically highly significant ($P < 0.001$). A comparison of the percentage positivity of podoplanin expression among the three groups also revealed a progressive positivity from normal oral mucosa (60%) to OL (76%) to OSCC (100%). This difference, too, was found to be statistically significant ($P = 0.003$). The above results were more or less in agreement with those obtained in an earlier study carried out by Parhar *et al*.[25]

An evaluation and comparison of MIRS scores for podoplanin expression among the four subgroups of OL was undertaken. Although we observed that the podoplanin staining score progressively increased from mild dysplasia (2.00 ± 2.00) to Ca *in situ* (6.26 ± 3.78), this rise was not statistically significant. These results obtained were in accordance with those reported in a previous study, which also showed that the epithelial score of podoplanin increased as the severity of epithelial dysplasia increased. The pattern of podoplanin staining was also noted in each of the four subgroups of OL. While mild dysplasia displayed a staining pattern restricted to the basal layers, the moderate and severe dysplasias showed staining of the basal cells in 1–3 focal areas, with Ca *in situ* showing the maximum foci of epithelial podoplanin staining. The earlier study by Logeswari *et al*.[26] reported that, among the podoplanin-positive cases of OL, 42% of them showed podoplanin expression restricted to the basal epithelial layer, whereas 12.5% of the positive cases showed podoplanin expression extending up to the parabasal layer.

Based on the results obtained from our study and comparing them with the previous studies, with regard to podoplanin expression in OL in general as well as in its subgroups, it could be hypothesized that the expression of podoplanin in the upper layers (layers above the basal layer; a positive podoplanin expression restricted to the basal layer is the pattern observed in the positive cases of normal mucosa in our study) of the epithelium may carry a significantly higher risk of development of carcinoma when compared with cases where the expression of podoplanin was restricted to the basal epithelial layer alone. Yuan *et al*.[27] and Kawaguchi *et al*.[4] have expressed the opinion that, in epithelial dysplasia, high podoplanin expression could be associated with an increased risk of development of cancer. Thus, we can suggest that the podoplanin expression can indeed be used for serving as a marker to predict the risk for the development of a transformation into OSCC in patients with OL.

We also evaluated the MIRS separately for the three subgroups in OSCC. It was observed that well-differentiated group showed the maximum expression of podoplanin (11.03 ± 3.41), and there was a progressive decrease in this value to poorly differentiated group (7.60 ± 2.37). However, these results were statistically not significant. On observing the pattern of podoplanin staining in the three subgroups, it was found that the well-differentiated group showed both central and peripheral staining of the tumor islands in the stroma. These results are in agreement with those reported in similar previous studies by Kanh and Marks[14] and Rodrigo *et al*.[20] who concluded that podoplanin expression was higher in the early-stage tumors. Parhar *et al*.[25] have also reported similar findings. Thus, a gradual decrease in the epithelial podoplanin expression from well-differentiated

OSCC to poorly differentiated OSCC could indicate a higher proportion of tumor-initiating cells (TICs) in well-differentiated OSCCs. This points to a role for podoplanin in initiating tumorigenesis rather than in the invasive potential of the cancer.

Because podoplanin has been reported to be expressed in lymphatic endothelium (not the blood-vessel endothelium) and is often used as a marker for lymphangiogenesis,[14] we studied the MLVD in all the three groups as well as in the subgroups. We observed that there was a progressive increase in the MLVD from normal mucosa ($5.50 \pm 4.35$) to OSCC ($42.00 \pm 21.22$), and this was found to be statistically significant ($P < 0.001$). We had observed the MLVD in the advancing front of the lesion (immediately below the basement membrane in OL and the surrounding tumor islands in OSCC). Our results agree with the finding of Parhar *et al*.,[25] suggesting that lymphatic vessel proliferation occurs in early dysplasias, progressively increases through Ca *in situ* and points to an essential role of lymphangiogenesis in the progression of OL into invasive carcinoma.

## CONCLUSION

Based on our study, we conclude that a progressive and significant increase of podoplanin expression is evident from normal oral mucosa to OL and OSCC. Increased podoplanin expression correlates with a higher grade of epithelial dysplasia, and therefore indicates an increased risk for transformation of that lesion into OSCC. This makes podoplanin useful as a potential biomarker to predict the risk of malignant transformation in OL cases. Podoplanin expression is found to be highest in the well-differentiated OSCC, compared to the other two grades. This makes us infer that podoplanin may play a vital role in the early stages of tumorigenesis, tumor progression and metastasis. These findings led us to state that podoplanin is involved in the process of oral carcinogenesis, more in the early stages as was evident from its progressively increasing expression through the increasing grades of epithelial dysplasia and a high expression in OSCC, but showing a decrease in poorly differentiated SCC. It can be recommended on the basis of our study that podoplanin can be used as a predictive marker to assess the risk of malignant transformation of the OL cases and their progression to squamous cell carcinomas.

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Nil.

### Conflicts of interest

There are no conflicts of interest.

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