

Expression of podoplanin in predicting the biological behavior of oral squamous cell carcinoma – A clinicopathological correlation

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Abstract

Background: Podoplanin is a mucin-like transmembrane glycoprotein that been highly and specifically expressed in the lymphatic endothelial cells, but not in the endothelium of blood vessels. Studies have shown that podoplanin expression in tumor cells including those of squamous cell carcinomas and a relationship with the clinicopathological features. This raises a possibility that podoplanin might have a biological function in oral squamous cell carcinoma and its expression could be used as a biomarker for diagnosis and prognosis.

Methods: Formalin-fixed paraffin-embedded tissue blocks and histopathology reports of 50 cases of OSCC were used in this study. These were evaluated immunohistochemically for the expression of podoplanin using D2-40, a monoclonal antibody. The association between podoplanin expression and tumor site, size and degree of differentiation, lymph node metastasis, and prognosis was analyzed.

Results: In this study, we observed that podoplanin was highly expressed in OSCC, but we found no significant association between podoplanin expression and clinicopathological characteristics and survival.

Conclusion: The role of podoplanin as a marker for lymph node metastasis is questionable. To determine the role of podoplanin as a prognostic marker, further prospective studies are required with a longer follow-up period. Further studies using a combination of markers are required to predict tumor invasiveness and occult metastasis in OSCC.

Introduction

Oral squamous cell carcinoma is one of the most common malignancies involving the head-and-neck region. The development of OSCC is a multistep process which involves various genetic, epigenetic, and metabolic alterations resulting from exposure to various carcinogens.^[1,2] Consumption of tobacco, alcohol, and betel constitutes the main risk factors. A significant proportion of OSCC develops from premalignant lesions.^[1,3] In spite of various advances in diagnosis and treatment, the survival rates have not improved significantly. After diagnosis, the 5-year survival rate remains around 15–50%. The prognosis of OSCC remains poor due to diagnosis at a late stage, high rates of primary site recurrence, and metastasis to regional lymph nodes. Early diagnosis can improve the prognosis and reduce the mortality significantly.^[1,3]

OSCC spreads locoregionally in its advanced stages, where the tumor cells travel to the cervical lymph nodes through the lymphatic system. The presence of metastasis to lymph node is the most important indicator for the prognosis of OSCC. Histopathological evaluation has been the gold standard in the diagnosis of OSCC.^[1,3] However, this may be inadequate due to the high frequency of recurrence. Therefore, it is important to further understand the characteristics and pathogenesis of OSCC and to improve the diagnostic capabilities.^[1,4]

Studies have shown that changes occur at molecular level before the occurrence of any clinical signs and symptom. Therefore, it is important to identify biological markers which may augment the clinical staging system.^[1,5]

Podoplanin is a mucin-like transmembrane glycoprotein which has been highly and specifically expressed in the

lymphatic endothelial cells, but not in the endothelium of blood vessels.^[5-7]

It has been seen that deficiency of podoplanin disrupts the normal lymphatic vasculature formation. Studies have revealed the expression of podoplanin in certain tumor cells, including those of squamous cell carcinomas and a relationship with tumor invasion.^[5-7] This raises a possibility that podoplanin may have a biological function in tumor cells and its expression could be used as a biomarker for diagnosis and prognosis.

The purpose of this study was to determine the expression of podoplanin in oral squamous cell carcinoma and the potential associations between its expression and clinicopathological characteristics.

Methods and Methodology

Study design and setting

This study included of 50 cases (prospective and retrospective) of histologically proven OSCC who had undergone surgical resection and neck dissection by the Department of OMFS, FDS, Ramaiah University of Applied Sciences, and Department of Surgical Oncology, M.S Ramaiah Teaching and Memorial Hospitals, Bengaluru, from July 2012 to February 2017. Formalin-fixed paraffin-embedded (FFPE) tissue blocks and histopathology reports of OSCC cases were retrieved from the Department of Pathology, M.S Ramaiah Teaching and Memorial Hospitals. Immunohistochemical evaluation of these cases was then done for the expression of podoplanin using monoclonal antibody (D2-40).

Patients who had undergone prior chemotherapy and/or radiotherapy, patients with incomplete case records, and cases whose primary tumor blocks were unavailable were excluded from the study. Ethics approval was taken from the Ethics Committee and Institutional Review Board. Informed consent was obtained from all patients.

Tissue processing and immunohistochemical analysis

The diagnosed cases of OSCC with and without lymph node metastasis were taken and confirmed with routine hematoxylin and eosin stain. IHC staining was done using standard operating protocol using a mouse monoclonal antibody (D2-40) (from Pathnsitu) as the primary antibody for podoplanin and subjected to the various steps involved in the immunohistochemistry staining.

Two pathologists evaluated the IHC-stained slides and samples were blinded to avoid any bias. The cytoplasm and/or membrane immunoreactivity of podoplanin was considered positive and interpretation of the same was done by selecting five HPF for each of the stained section under $\times 400$.

The interpretation of the immunoreactivity of podoplanin was evaluated by employing the scoring criteria given by Yuan *et al.* 2006 (a) staining intensity was graded as negative (0), weak (1), moderate (2), and strong (3) and (b) quantity of positive tumor cells was scored as 0 (0%), 1 (1–10%), 2 (11–30%), 3 (31–50%), 4 (51–80%), and 5 (81–100%).^[5]

The data were converted to an immunoreactive score (IRS) by multiplying the staining intensity and quantity of positive tumor cells. The final scores were put on the scale ranging from 0 to 15 as 0 (no expression), ≤ 4 (weak expression), and > 4 (strong expression).^[5]

Statistical analysis

Statistical analysis of the data was performed using version 22 of SPSS software. Chi-square test was employed to check the correlation between podoplanin expression and site, size and degree of differentiation, lymph node metastasis, and prognosis. $P \leq 0.05$ was considered as statistically significant.

Results

Patient characteristics

FFPE blocks of 50 cases of histologically proven OSCC were included in this study. Out of the 50 cases of OSCC, one was excluded as it did not meet the inclusion criteria. The 49 remaining cases analyzed in this study included 28 female (57.1%) and 21 male (42.9%) patients. Age of the patients ranged from 33 years to 80 years with a mean age of 52.63 ± 11.95 years. OSCC involving the buccal mucosa and mandibular GBS was the most frequently recorded site with 16 cases (32.7%) each, followed by tongue (15 cases, 30.6%), and last, maxillary and mandibular GBS (2 cases; 4.1%) [Table 1]. All the cases included in this study had undergone surgical resection and neck dissection with or without reconstruction. The patients were followed up for a period of 6 months–4 years.

Expression of podoplanin in OSCC

Expression of podoplanin was seen both in the membrane and cytoplasm, with the membrane showing stronger staining than the cytoplasm. In the normal squamous epithelium adjacent to the tumors cells, podoplanin expression was not detectable or it was extremely low in basal cells. However, some of the hyperplastic and dysplastic areas adjacent to the tumors cells showed high expression of podoplanin in the basal cell layers.

In 9 cases (18.4%), podoplanin expression was not detected. A weak expression of podoplanin was seen in 22 cases (44.9%), while 18 cases (36.7%) of OSCC exhibited a strong expression. An IRS of less than or equal to 4 was considered as weak expression, while a score > 4 was considered as strong expression [Figure 1].

Podoplanin expression and its association with clinicopathologic characteristics

The expression of podoplanin was not significantly associated with the age ($P = 0.56$), gender ($P = 0.68$), and site ($P = 0.89$) of the tumor [Table 2].

Our study showed that of the 9 cases with no podoplanin expression, 4 cases were of T2 stage followed by 2 cases each of T1 and T4 stages. In 11 cases of stage T2, the tumor cells

Table 1: Patient and tumor characteristics

Characteristic	No. (%)
Patients	
Male	21 (42.9%)
Female	28 (57.1%)
Age	
Mean	52.63±11.95
Tumor site	
Buccal mucosa	16 (32.7%)
Mandibular GBS	16 (32.7%)
Maxillary and mandibular GBS	2 (4.1%)
Tongue	15 (30.6%)
cT	
T1	9 (18.4%)
T2	27 (55.1%)
T3	4 (8.2%)
T4	9 (18.4%)
cN	
N0	24 (49%)
N1	25 (51%)
pT	
T1	8 (16.3%)
T2	19 (38.8%)
T3	6 (12.2%)
T4a	16 (32.6%)
Pn	
N0	23 (46.9%)
N1	6 (12.2%)
N2a	2 (4.1%)
N2b	13 (26.5%)
N2c	5 (10.2%)
Stage	
I	2 (4.1%)
II	12 (24.5%)
III	7 (14.3%)
IV A	28 (57.1%)
Degree of differentiation	
MDSCC	35 (71.4%)
WDSCC	14 (28.6%)

showed a strong expression of podoplanin, which was diagnosed in only three cases with stage T4 tumors. Twelve cases with tumors of stage T2 had a weak podoplanin expression, while only four cases with an advanced tumor of stage T4 exhibited weak podoplanin expression. In this study, no significant association

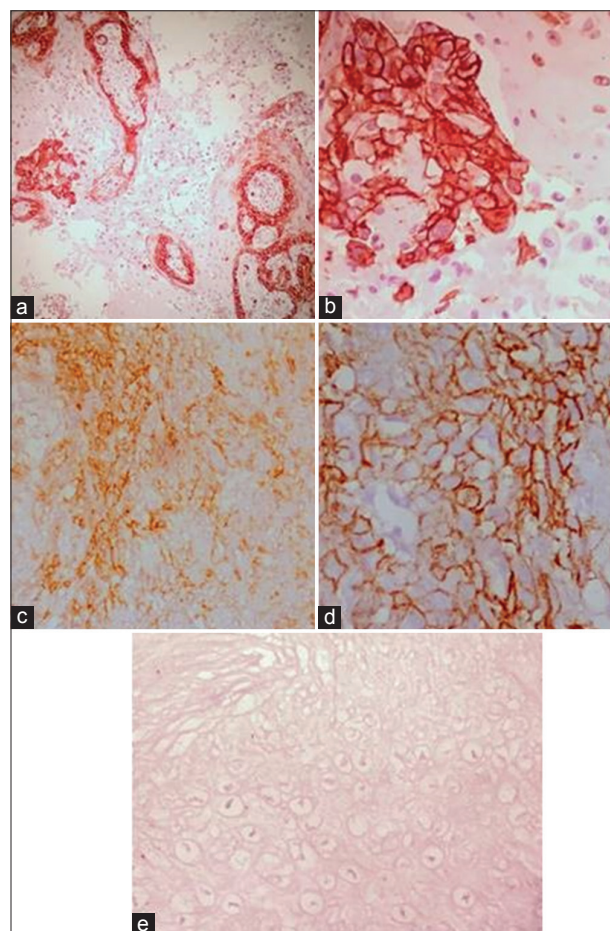


Figure 1: Photomicrographs showing immunohistochemical staining of podoplanin in OSCC. (a and b) Strong expression of podoplanin under $\times 10$ and $\times 40$; (c and d) weak expression of podoplanin under $\times 10$ and $\times 40$. (e) Negative expression of podoplanin under $\times 40$

was found between podoplanin expression and clinical tumor size ($P = 0.99$) [Table 2].

Podoplanin expression did not have a significant association with the pathological tumor size ($P = 0.56$) [Table 2]. Our study showed weak expression of podoplanin in eight cases of tumor size T4a and three cases of T3 compared to strong expression in only four cases of T4a and two of T3 tumor with comparable number of cases of both weak and strong podoplanin expression in smaller tumor sizes (T1 and T2).

The Chi-square test revealed an association between cN stage and podoplanin expression ($P = 0.09$) [Table 2 and Graph 2]. Among the 25 cases with lymph node metastasis, 12 showed a strong expression of podoplanin followed by 11 cases of weak expression, while only 2 showed no expression. However, six cases with no detectable cervical lymph node metastasis showed strong podoplanin expression while 11 showed weak expression. Seven cases with no lymph node metastasis had no expression of podoplanin.

Table 2: Correlation of podoplanin expression and clinicopathological characteristics

Clinicopathological characteristics	Podoplanin expression			Total	Chi-square value	P-value
	No expression	Strong expression	Weak expression			
Age (in years)					4.84	0.564
35-44	2	8	6	16		
45-54	3	5	7	15		
55-64	2	3	8	13		
65 and above	2	2	1	5		
Sex					0.76	0.68
Females	5	9	14	28		
Males	4	9	8	21		
Site					2.21	0.89
Buccal mucosa	2	7	7	16		
Mandibular GBS	4	4	8	16		
Maxillary and mandibular GBS	0	1	1	2		
Tongue	3	6	6	15		
cT					0.77	0.99
T1	2	3	4	9		
T2	4	11	12	27		
T3	1	1	2	4		
T4	2	3	4	9		
pT					6.72	0.56
T1	2	3	3	8		
T2	2	9	8	19		
T3	1	2	3	6		
T4	4	4	8	16		
cN					4.75	0.09
N0	7	6	11	24		
N1	2	12	11	25		
pN					7.38	0.49
N0	6	8	9	23		
N1	0	1	5	6		
N2a	0	1	1	2		
N2b	3	6	4	13		
N2c	0	2	3	5		
Tumor staging					3.69	0.718
I	1	0	1	2		
II	1	6	5	12		
III	1	2	4	7		
IV	6	10	12	28		
Tumor grade					2.203	0.332
MDSCC	6	11	18	35		
WDSCC	3	7	4	14		
Prognosis					0.236	0.889
Alive	8	15	18	41		
Deceased	1	3	4	8		

Among the 26 cases with lymph node metastasis, 10 had strong podoplanin expression in their primary tumors while 13 had weak expression of podoplanin. Of the 23 cases without lymph node metastasis, eight showed a strong expression of the marker, whereas nine showed a weak expression. However, there was no statistically significant association between greater podoplanin expression and lymph node metastasis ($P = 0.49$) [Table 2 and Graph 1].

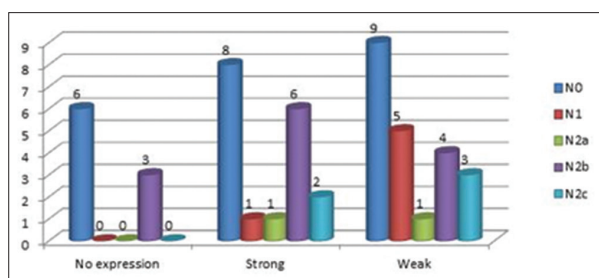
The results of the Chi-square test showed that an increased expression of podoplanin was seen in tumors with a higher stage. Of the 18 cases with strong podoplanin expression, 10 cases were of advanced Stage IVA, followed by six cases of Stage II and two belonging to Stage III. Twelve cases of Stage IVA, followed by five of Stage II, four of Stage III, and one of Stage I, had weak expression of podoplanin. However, in our study, there was no significant association between a stronger expression and higher stage of the tumor in comparison to a weak expression ($P = 0.718$) [Table 2].

Immunostaining of podoplanin was assessed in 35 cases of MDSCC and 14 cases of WDSCC. MDSCC showed positivity in 29 cases and WDSCC in 11 cases. A higher expression of podoplanin was observed in MDSCC compared to WDSCC, however, it did not reach a level of significance ($P = 0.332$) [Table 2] as the number of cases of MDSCC was more compared to those of WDSCC.

One of the aims of our study was to determine whether expression of podoplanin in the primary tumors of OSCC is a reliable parameter to predict the prognosis of the patient with respect to survival. In our study, expression of podoplanin did not have a significant correlation with the survival of the patient ($P = 0.889$) [Table 2]. Our analysis showed an increased expression of podoplanin in patients who were alive. Among the deceased, three cases showed a strong expression while four showed weak expression of podoplanin. One case which did not show podoplanin expression did not survive.

Discussion

Podoplanin is a mucin-like transmembrane glycoprotein which has been highly and specifically expressed in the lymphatic endothelial cells, but not in the endothelium of blood vessels. It has been seen that the deficiency of podoplanin disrupts normal lymphatic vasculature formation.^[5-7]



Graph 1: Graph illustrating the association of podoplanin expression and pathological nodal status (pN)

A significant proportion of oral squamous cell carcinoma develops from premalignant lesions.^[3] In oral leukoplakia, high expression of podoplanin was associated with an increased risk of progression to an invasive tumor, suggesting that podoplanin could serve as a biomarker for predicting the risk for the development of OSCC in patients with oral leukoplakia.^[8-10] This evidence supports that podoplanin has an important role in oral tumorigenesis and malignant transformation.^[11] A study by Funayama *et al.* showed that podoplanin was expressed less in normal tissues compared to tissues of OSCC. They also reported that the closer the pre-malignant lesion is to the malignant lesion, a higher expression of podoplanin was seen.^[12] In this study, high podoplanin expression was seen in areas of dysplasia and hyperplasia near the tumor tissue, this probably indicates their propensity for malignant transformation.

Podoplanin expression has been explored previously in various other squamous cell carcinomas, such as hypopharyngeal, esophageal, and laryngeal carcinomas. In esophageal SCC, podoplanin positivity was associated with lymph node metastasis, recurrence, and overall survival rate.^[13] Huber *et al.* found that expression of podoplanin had an association with a higher pathological grade and a positive lymph node status in tumors involving the oral cavity and oropharynx, but they did not find any association with the tumor stage and survival.^[14] In contrast to these studies, laryngeal SCC showed that higher podoplanin expression was inversely correlated with the T classification, stage, and pathological grade of the tumor.^[11] Similarly, Dumoff *et al.* reported a strong correlation between low podoplanin expression and both lymph node metastasis and lymphatic invasion in uterine cervical cancer. These findings suggest that there may be a variation in the biological function of podoplanin in different types of cancer.^[7]

In this study, we focussed on the expression of podoplanin in tumor cells of OSCC to investigate its relationship with clinicopathological parameters and prognosis. We found that podoplanin was highly expressed in oral squamous cell carcinoma with 40 out of 49 cases showing expression of the marker. Only nine cases showed no expression of podoplanin. This could be probably related to processing errors of the tissues. The specific staining of both the tumor cells and lymphatic endothelial cells in our study is consistent with findings in the previous studies.^[5,7]

Our study included 21 male and 28 female patients with age ranging from 33 to 80 years [Table 1]. There was no significant association of podoplanin expression with the age and gender of the patients [Table 2]. This finding is consistent with that of the previous studies.^[7,13,15] Only one study of SCCs involving the tongue reported that podoplanin expression is predominately seen in patients ≤ 40 years of age.^[16]

In the present study, we had 16 cases of OSCC of buccal mucosa and mandibular GBS each followed by 15 cases involving the tongue. Two cases showed involvement of maxillary as well as mandibular GBS [Table 1]. The number of cases showing podoplanin expression was almost equal in all the three sites. Therefore, our study showed no significant correlation between the podoplanin expression and the tumor site [Table 2]. Ciurea

et al. reported similar findings in their study.^[15] In contrast to our findings, de Vicente *et al.* found that SCC involving the floor of the mouth and tongue developed neck lymph node metastasis more frequently, especially when there was high a podoplanin expression.^[17]

Our study revealed a stronger expression of podoplanin in clinically smaller tumor size cT2 (23 cases) compared to cT4 (7 cases). However, the number of cases of cT2 tumors (27 cases) was more in our study than those of cT4 tumors (9 cases). This association between the tumor size and podoplanin expression was not found to be of statistical significance ($P < 0.05$) [Table 2]. Lee and Park had reported that podoplanin expression was higher in the early clinical stages (cT1, cT2) compared to the advanced stages (cT3, cT4). However, it was not of statistical significance ($P \leq 0.05$).^[18] High expression of podoplanin in early stages may indicate that overexpression of this marker occurs early in tumorigenesis, suggesting an important role of podoplanin in the early stages of oral carcinogenesis.^[1,5]

Some studies have shown that high levels of podoplanin expression in primary oral squamous cell carcinoma are associated with an advanced pathological stage (pT3, pT4) compared to early stages (pT1, pT2).^[5,7,15] Larger size tumors have a higher invasive and metastatic potential. The presence of this marker in the tumor cells is, therefore, helpful for pathological diagnosis and further treatment planning.^[19] Our study showed weak expression of podoplanin in advanced pathological stages (pT3, pT4) with equal distribution of cases of both weak and strong expression in smaller tumors (pT1, pT2) [Table 2]. These findings did not correlate with the previous studies.

Metastasis in OSCC primarily occurs through the lymphatic system, and the extent of lymph node involvement is an important factor for deciding the treatment and for prognosis of the patients.^[13] One of the greatest challenges is the presence of occult metastases in clinical N0 necks of patients with OSCC. Occult regional lymph node metastases can also be found in cases with small primary tumors. Therefore, a reliable marker, which can aid in predicting the chance of regional lymph node metastases, could be a great asset in making a decision for an elective treatment of a clinical N0 neck.^[7,16] The role of podoplanin, a lymphatic biomarker, has, therefore, been evaluated.

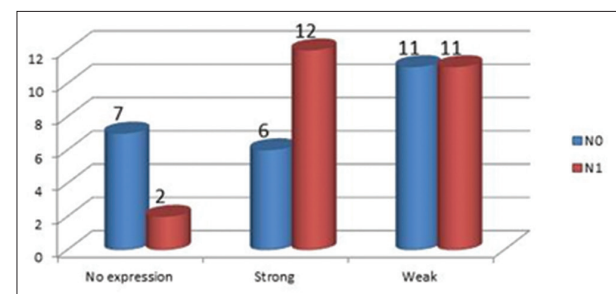
In this study, we found the presence of podoplanin expression in clinical N0 tumors with six cases showing a strong expression, 11 showing weak expression, and seven showing no expression [Table 2]. Pathologically, eight cases without lymph node metastasis showed strong podoplanin expression, nine showed weak expression, while six showed no expression of the marker [Table 2]. The tendency to express podoplanin when there is no evidence of nodal metastasis may probably be because of greater tumor invasiveness and presence of occult metastasis. In this study, all patients with and without lymph node metastasis had undergone neck dissection. Therefore, it still remains a question whether these tumors had any evidence of occult metastasis.

Detection of palpable cervical nodes during clinical examination is important for assessing the extent of the disease and helps in planning the surgical treatment. Our study revealed an association between podoplanin expression in the primary tumor and clinical nodal status. An increased expression of podoplanin was seen in cN1 (23 cases) compared to cN0 (17 cases) [Table 2 and Graph 2].

The presence of lymph node metastasis is an important factor to provide adjuvant chemo or radiotherapy following surgical treatment. Podoplanin expression was seen in 23 out of 26 cases with lymph node metastasis and 17 out of the 23 cases without lymph node metastasis. This was not of statistical significance ($P \leq 0.05$) [Table 2 and Graph 1]. There was no significant association seen between stronger podoplanin expressions with nodal status compared to weaker expression. Our findings are in accordance with the studies by Sgaramella *et al.* and Lee and Park.^[16,18] In contrast to our findings, Yuan *et al.* and Kreppel *et al.* found a significant correlation between high levels of podoplanin expression and lymph node metastasis.^[5,7]

The results of our study showed an increased expression of podoplanin in advanced Stages (III, IV A: 28 cases) of the tumor than with early Stages (I, II: 12 cases). However, the number of cases of advanced Stages (III, IV A: 35 cases) was more than those of early tumor Stages (I, II: 14 cases). Therefore, it did not reach a level of statistical significance ($P \leq 0.05$) [Table 2]. Furthermore, there was no association between stronger podoplanin expressions in higher stages of the tumor in comparison to weak expression. In 2010, Kreppel *et al.* reported that expression of podoplanin was associated with a higher tumor stage.^[7]

We studied the expression of podoplanin in 35 cases of MDSCC and 14 cases of WDSCC and found that a higher expression of podoplanin was observed in MDSCC compared to WDSCC. This finding was not of a statistical significant level ($P \leq 0.05$) [Table 2]. This was mainly because of the greater number of cases of MDSCC in our study. Similar to our findings, Ciurea *et al.* recorded a higher intensity of podoplanin expression in MDSCC and PDSCC compared to WDSCC.^[15] Prasad *et al.* found that high levels of podoplanin expression are suggestive of an immature status in the differentiation process of OSCC.^[19] In contrast to these findings, de Vicente *et al.*



Graph 2: Graph illustrating the association of podoplanin expression and clinical nodal status (cN)

found high expression of podoplanin in highly differentiated tumors.^[17]

An important parameter in our study was the association of podoplanin expression with prognosis. We followed up patients for a period of 6 months–4 years. However, in our study, there was an increased expression of this marker in patients who were alive and podoplanin expression did not have a significant correlation with the survival of the patient. This could be because the number of patients with podoplanin expression who were disease free (33 cases) at the end of our follow-up period was significantly more than the number of deceased (7 cases). A follow-up period of at least 5 years for all the patients could probably show a correlation.

Almeida *et al.* and de Vicente *et al.* had similar findings in their studies, where expression of podoplanin was not found to be a significant prognostic factor for patients with OSCC.^[17,20] On the contrary, Yuan *et al.* found that cases with lymph node metastasis and high levels of podoplanin had a shorter disease-specific survival.^[5,9,10] Kreppel *et al.* in their study recorded that the 5-year overall survival was significantly lower for patients with high levels of podoplanin expression compared to patients with low and moderate expression.^[7,9,10]

Conclusion

In this study, we observed that podoplanin was highly expressed in OSCC, but we found no significant association between podoplanin expression and clinicopathological characteristics and survival. However, high podoplanin expression in dysplastic tissues adjacent to the tumor and in the early clinical stages indicates its role in carcinogenesis.

In our study, we had an almost equal distribution of cases with and without lymph node metastasis showing podoplanin expression. Podoplanin expression in N0 cases probably indicates tumor invasion and occult metastasis. This, however, requires further investigation. Podoplanin expression was marginally more in cases with lymph node metastasis in clinical as well as pathological staging. However, there was no statistical significance. Therefore, the role of podoplanin as a marker for lymph node metastasis needs to be further explored. To determine the role of podoplanin as a prognostic marker, further prospective studies are required with a longer follow-up period.

Ethical approval

All procedures performed in this study were in accordance with the ethical standards of the Institutional Research Committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

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