Original Article

Immunolocalization of osteopontin in dysplasias and squamous cell carcinomas arising from oral epithelium

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Abstract

Background: Early detection of oral squamous cell carcinoma (OSCC) remains one of the most efficient ways to ensure patient survival and improved quality of life. Although specific biomarkers related to OSCC have been investigated, a useful biomarker that assesses the transition potential of potentially malignant lesion to OSCC remains to be found. Osteopontin (OPN) has been recognized as an important factor in tumorigenesis and their expression in OSCC have been investigated earlier. In the present study, evaluation of OPN expression in premalignant and malignant lesions has been carried out to assess their possible role as a biomarker in the early diagnosis and prognosis of OSCC.

Objectives: The objective of this study is to evaluate the role of OPN as a biomarker in the diagnosis and prognosis of OSCC.

Materials and Methods: The study group consisted of archival paraffin-embedded blocks of ten cases each of varying grades of OSCC, oral epithelial dysplasias and epithelial hyperplasias. Sections were subjected to immunohistochemical staining for the biomarker OPN.

Results: A positive OPN expression was noticed in epithelial dysplasias and SCC arising from the oral epithelium. A progressive increase in the intensity of staining was seen with increasing grades of dysplasias and a decrease in OPN expression with an increase in grades was observed in OSCC.

Conclusion: The expression of OPN in full thickness of epithelium in severe dysplasias, carcinoma *in situ*, and in the superficial epithelium of OSCC suggest the possibility of considering OPN expression in full epithelial thickness in dysplasias as an indicator for malignant transformation.

Keywords: Biomarker, malignant transformation, oral dysplasia, oral squamous cell carcinoma, osteopontin

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INTRODUCTION

Oral cancer is one of the most deadly diseases in the world. The overall 5-year survival rates for oral cancer have remained low at approximately 50% for the past decades.[1] This was mainly due to the diagnosis of oral squamous cell carcinoma (OSCC) at late stages. Hence, early detection of oral cancer and its curable precursors remain one of the most efficient ways to ensure patient survival and improved quality of life.

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Osteopontin (OPN), a calcium-binding glycol-phosphoprotein, involved in a variety of physiological cellular functions has been shown to play an important role in tumorigenesis, tumor invasion and metastasis in various cancers.[2] Although the expression of OPN has already been studied in OSCC, only a few studies have been done in collaborating premalignancy with malignancy.[3] Hence, the OPN expression in premalignant and malignant lesions arising from the oral epithelium have been studied to assess their possible role as a biomarker in the early diagnosis and prognosis of OSCC.

MATERIALS AND METHODS

Study population

The study group consisted of paraffin-embedded blocks of thirty cases of OSCC (Group I), ten cases of oral dysplasias (Group II), and ten cases of epithelial hyperplasias (Group III) taken from the archives of the Department of Oral Pathology. Only epithelial hyperplasias associated with traumatic keratosis were included in the study and epithelial hyperplasias associated with potentially malignant lesions were excluded from the study. The normal oral mucosa from ten subjects (Group IV) obtained from patients whose oral mucosa were removed from the site of extraction of impacted third molars, were taken as control group. Thirty cases of OSCC included ten cases each from well-differentiated, moderately- and poorly-differentiated carcinomas. Grading was done based on Broders' criteria.[4] The ten cases of oral dysplasias were selected according to varying grades of dysplasias which included mild, moderate and severe based on the WHO classification.[5]

Immunohistochemistry

After clearing and dehydration, the tissue sections of 5-micron thickness were transferred to citrate buffer and autoclaved for antigen retrieval at 15 lbs pressure for 15 min. They were allowed to cool and washed in phosphate-buffered saline (PBS) solution for 5 min. Endogenous peroxidase blocking was done by dipping sections in freshly prepared 3% H_2O_2 for 10 min. After blotting the excess peroxide, the slides were treated with a protein block reagent. Sections were then incubated with mouse monoclonal primary antibody (anti-human-OPN-Leica) at room temperature for 1 h. The sections taken out were washed in three changes of PBS for 5 min each to remove the excess antibody. A drop of enhancer from the secondary antibody kit (Biogenex Pvt. Ltd.) was added, and the slides were incubated for 30 min followed by the addition of a drop of Streptavidin from the secondary antibody kit on the sections and incubated for 30 min. The sections were washed in 3 changes of PBS for 5 min each, and a drop of freshly prepared DAB (3'diaminobenzidine tetrahydrochloride a substrate chromogen) was added on both sections. Slides were washed in PBS to remove excess DAB and then counter-stained with hematoxylin. The tissue sections were mounted with DPX.

Evaluation of immunohistochemical staining

For the evaluation of OPN immunostaining, the superficial epithelium was divided into three zones and the underlying connective tissue was divided into two zones.

Zones in the epithelium

- Zone I - Basal and parabasal layers
- Zone II - Middle layer
- Zone III - Superficial layer.

Zones in the connective tissue

- Zone IV - Superficial connective tissue
- Zone V - Deep connective tissue.

The immunopositive reaction was semiquantitatively evaluated with reference to the method of Grizzle *et al*.[6] Evaluation was carried out at three different areas in four groups. First, the cells were classified individually from level 0 to level 3 based on the intensity of immunostaining for OPN determined from several views selected at random in each area (level 0 - negative, level 1 - weak staining of cytoplasm, level 2 - distinct staining of cytoplasm and level 3 - remarkable staining of cytoplasm). The cell number was counted for each value of intensity. The total product of intensity and proportion of all levels in one view was taken as the score in each case. The results obtained were statistically analyzed. The mean values of scoring of OPN in varying grades of oral dysplasias and OSCC were compared using Chi-square test. Two sample *t*-test was carried out to compare the mean values of scoring of OPN in oral dysplasias with varying grades of OSCC.

RESULTS

Our observation based on the immunohistochemical expression of OPN in the four groups studied showed that the expression of OPN was negative in normal and hyperplastic epithelium while a positive OPN expression was noticed in oral dysplasias and OSCC. Normal muscle cells and the salivary duct cells were taken as internal positive controls.

OPN expression was noticed in all the ten cases of oral dysplasias included in the study as brownish granules in the cytoplasm of epithelial cells [Figure 1]. There was a progressive increase in the expression of OPN from mild

dysplasia to severe dysplasia [Figure 2]. This increased expression was noticed in the intensity of staining as well as in the distribution of positive cells. In mild dysplasia, the expression of OPN was restricted to basal and parabasal cell layers (Zone I) with majority of the cells showing an intensity level of 1. In moderate dysplasia, the expression of OPN was seen in Zone I as well as in Zone II, and majority of cells showed an intensity level of 2. In severe dysplasia, the expression of OPN was appreciated along the entire thickness of epithelium (Zone I, Zone II and Zone III) and their staining intensity varied between level 2 and 3. The number of positive cells and intensity of staining was considered for scoring the expression of OPN in these cases. Based on the scoring, the cases of oral dysplasias were divided into low- and high-score groups using the median of the total score (1.3317 in oral dysplasias) as the cutoff value. In the case of mild dysplasias, all the cases were of low score whereas, in moderate dysplasias, two cases showed low score and one showed high score. All cases of severe dysplasia showed high score. In carcinoma *in situ*, there was a positive OPN expression in all the three zones of epithelium with the majority of the cells showing a varying intensity of level 2 and level 3. An increase in the expression of OPN with increasing grades of dysplasia was observed, and the statistical analysis using Chi-square test showed that the result was statistically significant with a $P = 0.026$ [Table 1].

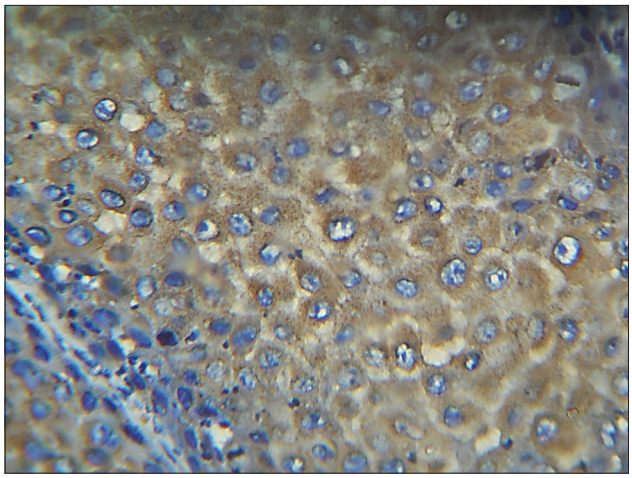


Figure 1: Intracellular cytoplasmic expression of osteopontin in epithelial cells, Immunohistochemistry ×40. Note the granular appearance of positive expression

Analysis of OPN expression in OSCC revealed that there was a positive immunoreaction in all the three grades of OSCC. In early invasive squamous cell carcinoma (SCC), there was

Table 1: Scoring of osteopontin expression in oral dysplasias with its *P* value

Dysplasia	Scores		*P*
	Low score, *n* (%)	High score, *n* (%)	
Mild (3)	3 (100.0)	0	0.026
Moderate (3)	2 (66.7)	1 (33.3)	
Severe (4)	0	4 (100.0)	

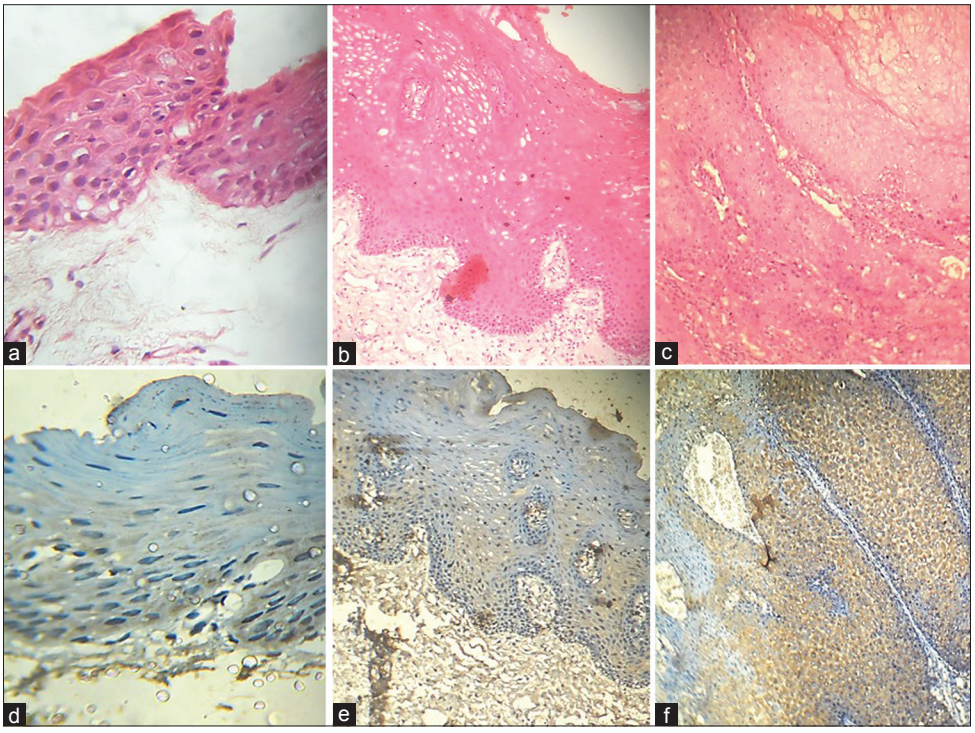


Figure 2: Progressive increase in osteopontin expression in varying grades of oral epithelial dysplasias. (a) Mild dysplasia, (b) moderate dysplasia, (c) severe dysplasia ([a-c], H&E), (d) expression restricted to Zone I in mild dysplasia, (e) moderate dysplasia showing expression extending till Zone II and (f) severe dysplasia showing expression extending till Zone III ([d-f], immunohistochemistry-osteopontin)

a positive expression of OPN in the superficial epithelium, with the invasive front showing OPN expression along the entire thickness. In the invasive front, the intensity of staining decreased from Zone I to Zone III with maximum intensity in Zone I. The epithelium away from the invasive front showed a decreased intensity of staining (level 1), and the staining was restricted to Zone I and Zone II.

In well-differentiated SCC, the superficial epithelium showed variation in expression of OPN. At the invading front, OPN expression was seen in all the three zones (Zone I, Zone II and Zone III). A decrease in the expression was noticed with increase in the distance from the invading front. The intensity of OPN expression also varied according to the zones with Zone I showing maximum intensity and Zone III showing minimum intensity. All superficial islands and deeper islands with keratin showed positive OPN expression though variation in intensity was noticed between the cases. Deeper islands with no keratin formation showed negative OPN expression. In larger islands, the keratin and surrounding cells showed positive OPN expression, but the peripheral cells lining the island showed negative expression [Figure 3]. In moderately-differentiated OSCC, superficial epithelium was positive for OPN expression. At the invasive front, a reduced intensity in staining was noticed when compared to well-differentiated OSCC. A heterogeneous pattern of staining was noticed in moderately-differentiated OSCC with mixture of OPN positive and negative cells. Positive expression was noticed in island showing attempted keratin pearl formation. In poorly-differentiated OSCC, a heterogeneous pattern of staining was noticed with majority of cells showing negative OPN staining. Positive cells showed only faint cytoplasmic staining [Figure 4].

The scoring of the OPN expression was calculated in the same way as that of dysplasia. All the thirty cases of OSCC were divided into low- and high-score groups using the median of the total score (1.2667 in OSCC) as the cutoff value. In well-differentiated SCC, two cases showed low score and eight cases scored high. In the case of moderately-differentiated SCC, six cases showed low score and four cases showed high score. In the case of poorly-differentiated SCC, eight cases showed low score and two cases showed high score [Graph 1]. This suggests that the expression of OPN decreases with increasing grades of OSCC [Graph 2]. Statistical analysis using Chi-square test showed that the difference in expression of OPN between grades of OSCC was statistically significant with a $P = 0.024$ [Table 2].

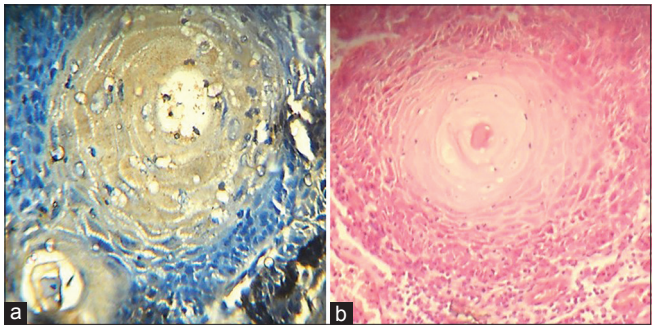


Figure 3: Well-differentiated squamous cell carcinoma (a) positive osteopontin expression seen in keratin and surrounding epithelial cells. Note the negative osteopontin expression in the peripheral cells, ×40, (b) H&E, ×40

DISCUSSION

The OSCC, the most common malignant neoplasm arising from the oral epithelium, is a major health problem in developing countries. OPN, a secreted phosphor-glycoprotein was found to be overexpressed in a variety of human malignancies and was also detected in a high percentage of premalignant and malignant oral lesions. Our study was aimed to assess the OPN expression in premalignant and malignant lesions arising from the oral epithelium and to explore the role of OPN in the early detection of oral cancer.

In our study, we found that the OPN protein though negative in normal and hyperplastic epithelium showed

Table 2: Scoring of osteopontin expression in oral squamous cell carcinomas with its *P* value

Grades	Scores		*P*
	Low score, *n* (%)	High score, *n* (%)	
Grade I SCC (10)	2 (20.0)	8 (80.0)	0.024
Grade II SCC (10)	6 (60.0)	4 (40.0)	
Grade III SCC (10)	8 (80.0)	2 (20.0)	

SCC: Squamous cell carcinoma

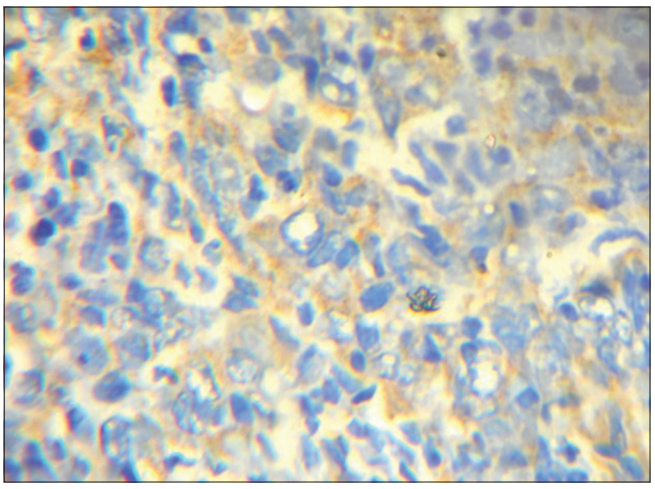
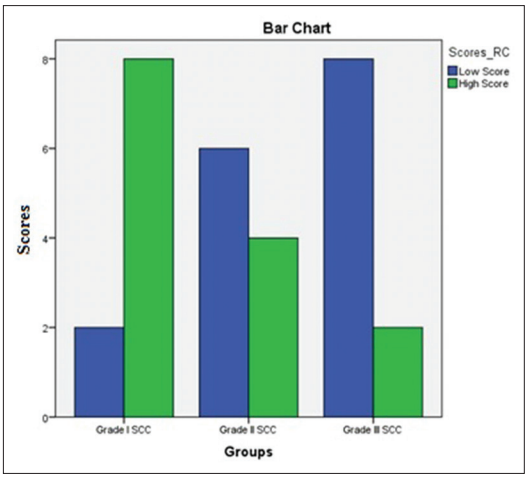
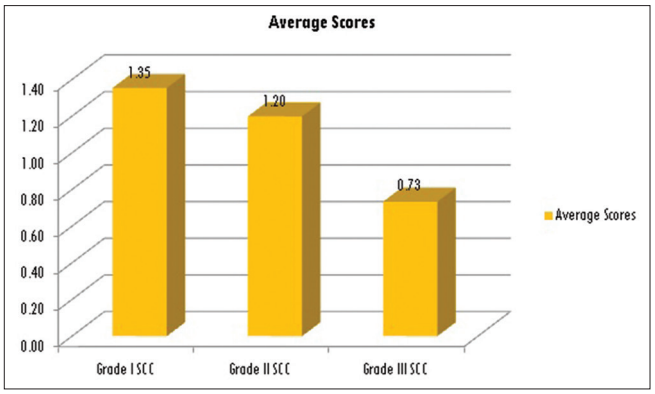


Figure 4: Poorly-differentiated oral squamous cell carcinoma positive cells showing faint cytoplasmic and heterogeneous pattern of staining with majority of cells showing negative osteopontin staining, ×40



Graph 1: Comparison of low- and high-score group in varying grades of oral squamous cell carcinoma



Graph 2: Decreased expression of osteopontin with increasing grades of oral squamous cell carcinoma

a positive expression in oral dysplasias and OSCC. The difference in expression of OPN between neoplastic and nonneoplastic epithelial proliferation is in accordance with the view that transformed epithelial cells secrete OPN. The lack of expression in normal oral mucosa had been previously reported.[3,7] Staibano *et al.* noted that OPN expression was virtually undetectable in normal laryngeal mucosa and laryngeal hyperplasia.[8] However, Chiu *et al.* reported OPN expression confined to the basal and prickle cell layers in normal mucosa adjacent to SCC.[9] As normal appearing mucosa adjacent to a malignant lesion would have already undergone cellular transformation, the increased expression noted can be considered as expression of OPN by transformed epithelial cells. Devoll *et al.* demonstrated intracellular and intercellular immunoreactivity in 75% of oral epithelial hyperplasias.[3] However, our findings were similar to many of the previous studies which showed negative OPN expression in oral hyperplasias.[9,10]

As with the previous studies, all the ten cases of oral dysplasias showed a positive expression of OPN. We noted a progressive increase in expression of OPN from mild dysplasia to severe dysplasia. In cases of oral dysplasias studied, increased expression was noticed in the intensity as well as in the distribution of OPN. A similar increase in expression with increasing grades of dysplasias had been reported in oral dysplasias by Devoll *et al.* and in laryngeal dysplasias by Staibano *et al.* The expression of OPN in high percentage of dysplasias suggests that OPN expression may present an early event in tumor progression.

A positive OPN expression was noticed in all the three grades of OSCC, and the expression was found to be decreasing with increasing grades of OSCC. Literature review revealed the presence of OPN expression in various human carcinomas such as ovarian, gastric, breast, liver, lung, renal and head and neck cancers.[11-20] A positive expression of OPN in all the three grades of OSCC has also been reported earlier. Matsuzaki *et al.* found a negative expression of OPN in keratinized cells suggesting a loss of OPN expression with differentiation.[21] This finding was supported by Routray *et al.*, who noted that the intensity of OPN expression increased significantly with loss of differentiation of tumor cells.[7] Contrary to this finding, our study showed a decrease in expression of OPN with increasing grades of OSCC suggesting a positive correlation between the expression of OPN protein and degree of differentiation. Further, the keratin pearls and surrounding oral epithelial cells in well- and moderately-differentiated SCCs showed positive OPN expression. This was consistent with the previous studies which showed positive OPN expression of keratin pearls and neoplastic cells surrounding it.[21]

In SCCs, an increase in OPN expression was noticed in superficial epithelium at the invading front when compared to the rest of the epithelium. Matsuzaki *et al.* and Routray *et al.* also noted a definite, increase in expression at the invasive front than the neighboring epithelial dysplasia. A reduction in the proliferation and invasion brought about by the inhibition of OPN in oral cancer cell lines had also been demonstrated by Muramatsu *et al.*[22] The significant increase in the expression at the invading front correlates OPN secretion with invasive potential of transformed cells and can be considered as evidence for the direct role of OPN in transforming dysplastic lesions into frank malignancies. A zonal variation in the intensity of staining was also noticed in the superficial epithelium. The maximum intensity of OPN staining was noticed in zone 1 with a progressive decrease in staining from Zone 1 to Zone 3 which only suggest an increased secretion of OPN by basal cells which are directly involved in the early invasion of the malignant epithelial cells to the

underlying connective tissue. A heterogeneous pattern of OPN immunostaining showing a mixture of OPN positive and negative cells was noticed in moderately- and poorly-differentiated OSCC. Devoll *et al*. also observed a heterogeneous pattern of OPN staining with areas of OPN positive cells separated by areas of nonreactive cells.

Based on our study, it can be inferred that the presence of OPN expression in oral epithelial cells is indicative of cellular transformation. In addition, the malignant potential of a precancerous lesion can be assessed based on the intensity of staining as well as the distribution of positive cells within the layers of epithelium by immunostaining using the biomarker, OPN. A dysplastic epithelium showing a positive OPN expression in the entire thickness of epithelium seems to have an increased chance of transformation into more differentiated OSCCs. The role of biomarker, OPN in the assessment of malignant transformation of severe dysplasias to poorly-differentiated OSCC, is not certain. The superficial epithelium could not be assessed in the poorly-differentiated SCCs considered in our study, as majority of the cases were ulcerative lesions which lacked a superficial epithelium. Since the number of cases included in the present study was limited, our findings need to be verified with more number of cases and more studies done in this regard, might in future add more authenticity to our findings.

CONCLUSION

OPN seems to be a promising biomarker in predicting the malignant potential of a premalignant lesion. OPN expression in full epithelial thickness in severe dysplasias and their increased expression at the invading front in OSCC suggest their role in early phase of oral carcinogenesis. Hence, identification of OPN expression and distribution in transformed epithelial cells in dysplastic lesions might be of great value in the early detection of oral cancer.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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