ORIGINAL ARTICLE

Evaluating the efficacy of osteopontin expression as a prognostic marker in oral squamous cell carcinoma in the Indian subpopulation

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Received: 13-06-2011
Accepted: 16-06-2014

ABSTRACT

Aim: This study aimed to correlate the prognostic value of osteopontin (OPN) expression using both tissue and plasma samples from patients with clinically and histologically confirmed oral squamous cell carcinoma (OSCC). **Methods and Materials:** The study group comprised of sixty patients (*n* = 60), which were clinically and histologically diagnosed for oral squamous cell carcinoma (OSCC)**.** The Control group comprised of ten (*n* = 10) healthy volunteers. Plasma OPN levels were assayed using a quantitative enzyme-linked immunosorbent assay (OPN ELISA). Expression of OPN was also identified and evaluated by immunohistochemistry in tissue sections. These OPN expressions were then correlated with different parameters like age, sex, site, clinical presentation, tumor node metastasis (TNM) staging, histopathological grading and lymph node metastasis. **Statistical Analysis:** One-way analysis of variance (ANOVA) was used to evaluate the difference in tissue intensity and plasma OPN levels between the OSCC and the normal control groups. **Results:** The distribution of the plasma OPN levels and tissue OPN intensity in OSCC cohorts were compared to histopathological grades and analyzed. When evaluated OPN expression in tissue had higher intensity observed in OSCC (95% +ve) cases. And the mean plasma OPN concentration in OSCC cohort was more in comparison to the normal cohort. The results clearly showed that the plasma OPN levels and intensity grading in tissue correlated with tumor grades. **Conclusion:** The study highlights OPN as a biomarker for prognosis in OSCC in both plasma and tissue samples. We would like to emphasize on the evaluation of plasma OPN as a protocol of blood examination for all cancer patient, as it may serve as an indicator for tumor progression and potential risk of metastasis.

Key words: Biopsy, enzyme linked immunosorbent assay, immunohistochemistry, osteopontin, oral squamous cell carcinoma, plasma

INTRODUCTION

In the Indian subcontinent oral squamous cell carcinoma (OSCC), frequently shows metastasis to cervical lymph nodes and despite the availability of modern treatment modalities, the 5-year survival rate has not changed significantly.[1] Treatment options for head and neck cancers based on multiple factors such as age, sex, tumor site, TNM staging and histopathological grade help as an adjunct to guide therapy, but are not reliable predictors of the outcome. The most accurate clinical predictors for recurrence and metastasis at present are nodal staging (N-staging).[2] Unfortunately, many patients come with advanced stage at presentation and N-stage is not very discriminating in this group of patients. Thus, it becomes essential that other avenues be explored like immunohistochemistry of lesional tissue or plasma levels of the patient to check for prognostic tumor markers. One such marker is osteopontin (OPN), a secreted phospho-glycoprotein that binds $\alpha_v\beta_3$ integrin and some CD44 isoforms.[3] Although, molecular mechanisms are not very clear, OPN actions in carcinogenesis and metastasis

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Website: www.jomfp.in

DOI: 10.4103/0973-029X.141327

are linked to the changes in cell migrations, angiogenesis and apoptosis. In tumors of breast, prostate and stomach, OPN expression was evaluated in both tumor tissue and body fluids. Studies have concluded that high expression levels are usually associated with poor prognosis.[4] The clinical significance of osteopontin expression in T1 and T2 tongue cancers have also been correlated, making it a reliable candidate for assessing prognosis.[5]

In OSCC cases, OPN expressions assessed by using immunohistochemistry (IHC) were found to be progressively higher from normal tissues to epithelial hyperplasia, dysplasia and carcinoma *in situ*, suggesting the role of OPN in oral carcinogenesis.[6] Although, plasma OPN level has been considered as a prognostic marker for OSCC,[7,8] it has been also noted that plasma level could also be influenced by various inflammatory and fibrotic processes such as atherosclerosis, bone remodelling, angiogenesis, wound healing and tissue injuries.[9] Literature also confirms that tissue expression of OPN is a more reliable way to reflect the fidelity of OPN expression by the tumor.

Hence, we examined the preoperative plasma level of OPN and the OPN expression by immunohistochemistry (IHC) in the paraffin-embedded tumor tissues by incisional biopsy of the same patients and evaluated the prognostic significance of OPN expression in OSCC patients.

MATERIAL AND METHODS

Case selection

Controls

The control group comprised of ten (n = 10); age and sex matched volunteers from whom, in the form of biopsies, normal mucosae and blood samples for plasma were collected.

Study group

The study group comprised of 60 patients, of which 84% were males and 16% were females. The age of the patients ranged from 23-68 years with average age at 48.67 years, who were clinically and histologically diagnosed for oral squamous cell carcinoma. Clinical evaluation including TNM classification was done and fine needle aspiration cytology (FNAC) and histopathological examination confirmed the presence of oral squamous cell carcinoma. The grading was done according to malignancy grading system proposed by Anneroth's criteria.[10]

Inclusion criteria

- Those cases which were histopathologically diagnosed as OSCC
- All the patients who were ready to undergo the required surgical procedure.

Exclusion criteria

- Those cases which showed cervical lymph node enlargement due to acute or chronic infection without any clinical evidence of oral malignancy
- Those cases which undergoing treatment for recurrence including chemotherapy and radiotherapy.

Immunohistochemistry

Thick tissue sections of 4 μm were used for immunohistochemistry procedure. For negative control, phosphate-buffered saline (PBS) solution was used instead of primary antibody. Sections were washed briefly in running water and lightly stained with Mayer's hematoxylin. A section of gall bladder was used as the positive control in each run. Positive OPN immunostaining was defined as detectable immunoreactivity in the perinuclear and/or other cytoplasmic regions in atleast 10% of the cancer cells.

Plasma OPN assay

The enzyme linked immunosorbent assay (ELISA) technique was used to quantify OPN in K-EDTA plasma from OSCC patients and in respective controls. The plasma levels of OPN were determined with the human OPN ELISA kit according to the manufacturer's instructions. Samples were brought to a room temperature and rested till completely thawed. After short vortex and visual check, samples were centrifuged at 5000 rpm and 4°C. Assay buffer provided in ELISA kits was used to dilute plasma samples to a desired proportion. Each sample was tested in duplicate and the results were quantified using a standard curve.

RESULTS

The study consisted of a cohort of 60 OSCC patients and 10 normal patients with average age at 48.67 years. The tumor site, divided the subjects into four categories; buccal mucosa, gingiva, lips and tongue. The tumor sites at buccal mucosa and gingiva were contributing 90% of the total cases. The following chart displays the distribution of cases in OSCC cohort according to clinical sites [Figure 1]. The OSCC cohort was then divided into three categories according to histopathological grading (well-differentiated, moderately differentiated, poorly differentiated) [Figure 2] and clinical staging was also done (TNM Staging) [Table 1]. The habit parameter listed OSCC cohorts for tobacco chewing (T), smoking (S) and alcohol (A). The T category (tobacco chewing in various forms including gutkha, mawa, etc) was found to be pre-dominant [Table 2].

The comparative evaluation of distribution of OSCC and normal cohorts was done from the reading of plasma OPN samples. The OSCC cohort was distributed with mean OPN concentration at 0.11 and standard deviation of 0.049. The Control group was found to be distributed with mean OPN

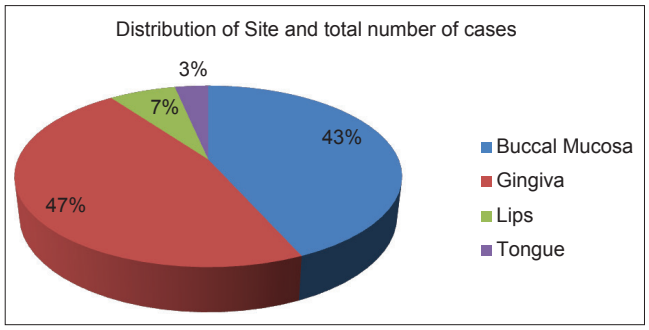


Figure 1: The clinical site distribution of the cases of OSCC, where buccal mucosa and gingiva contributed to 90% of the total cases

Table 1: Distribution of cases according to clinical staging using TNM classification

Clinical stage	Cases	%
T1N0M0	4	6.7
T1N1M0	10	16.7
T1N2M0	4	6.7
T2M0N0	2	3.3
T2N0M0	2	3.3
T2N1M0	26	43.3
T2N2M0	8	13.3
T3N2M0	4	6.7

TNM: Tumor node metastasis

Table 2: Distribution of cases according to habit (tobacco, smoke, alcohol)

Habit	Cases	%
T	38	63.3
S	2	3.3
T+S	4	6.7
T+A	2	3.3
S+A	4	6.7
T+S+A	10	16.7

T: Tobacco, S: Smoke, A: Alcohol

expression at 0.10 and standard deviation of 0.035. The mean plasma OPN concentration in OSCC cohort was more in comparison to the normal cohort [Graph 1]. The distribution of the plasma OPN levels in OSCC cohorts was then compared with respect to histopathological grades viz. well, moderate and poor differentiated. The comparison clearly showed that the plasma OPN levels in poorly differentiated grade were higher compared to those from moderately and well differentiated grades [Graph 2]. The OSCC cohort afterwards, was divided into two categories according to their age viz. ≤50 years and 51 years and above. The mean OPN expression in ≤50 years was 0.10 compared to 0.13 in the 51+ year category. The Mann-Whitney U test showed a significant difference in OPN expression between the two groups with *P* value of 0.048 [Figure 3].

For OPN expression quantification using immunohistochemistry (IHC) on tissue samples, the number of cytoplasmic positive squamous neoplastic cells were

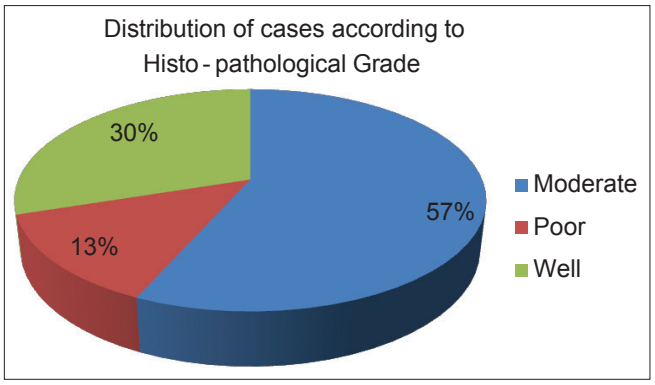


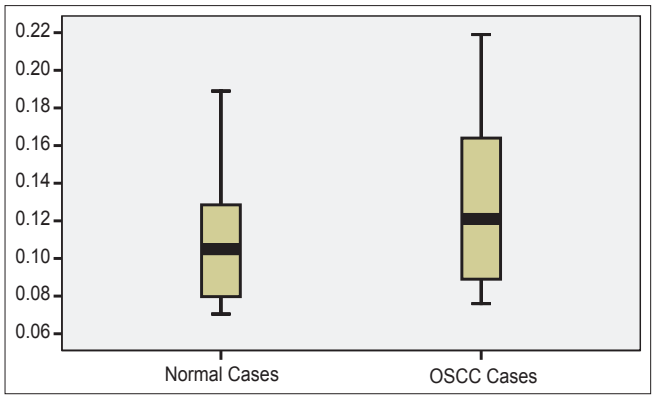
Figure 2: The OSCC cohort was divided according to histo-pathological grading

counted under light microscopy using Grizzle *et al.* protocol[11] [Figure 4]. Cases were assessed along with its histopathological grading and accordingly its positivity was enumerated relating to intensity [Table 3]. When evaluated OPN expression had higher intensity observed in OSCC (95% +ve) cases than in normal epithelium (NE - 60% +ve) cases [Figure 5]. The average total score for OPN expression in the OSCC cases was 1.65 ± 1.05, whereas in NE it was 0.72 ± 0.51 [Graph 3].

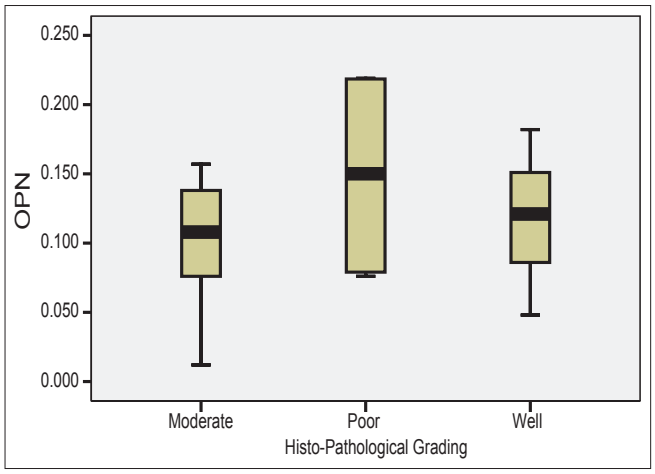
DISCUSSION

In the present study, an effort was made to evaluate the efficacy of plasma OPN and tissue OPN expressions, as a prognostic marker in 60 cases of OSCC with different histological grading and 10 cases of NE. Semi-quantitative evaluation of immunopositive reaction of OPN and scoring for each case revealed that OPN expression was significantly higher in carcinoma cells than in normal. Intercellular and cytoplasmic staining was observed, while nuclei and cytoplasmic vacuole remained unstained. As there was significant difference between OSCC and normal cohorts ($P < 0.05$ Sig), the value suggests that OPN expression increases with malignant transformation. These results were consistent with the study by Muramatsu *et al.*[12], showing that proliferation and invasion were reduced by inhibition of OPN in BSC-OF cells. According to our study that reported by us earlier, the authors have concluded and highlighted OPN's role as a biomarker for malignancy in the form of invasion using IHC.[13]

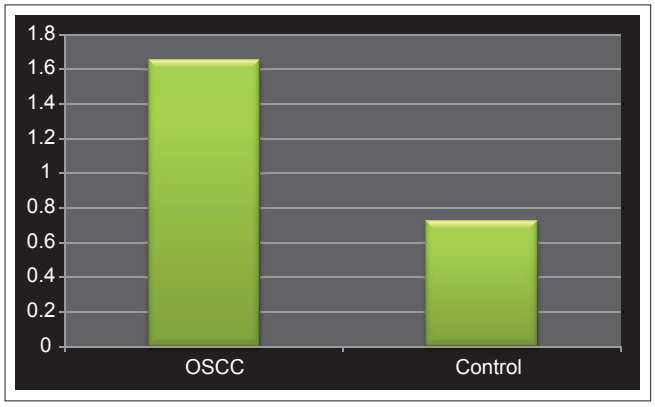
Plasma levels of OPN in patients with head and neck cancer have been found to be elevated and correlate with progression.[7,8] In another study, Devoll *et al.* reported that OPN was not detected in normal oral mucosa but was detectable in a significant percentage of the tissues with dysplasia, carcinoma *in situ* and squamous cell carcinomas of oral epithelium by IHC study.[6] In our study, there was a distinct increase of plasma OPN in OSCC patients clearly indicating its association with its aggressive behavior, which has already been established by Chien *et al.*[14] Moreover, OPN levels in patients with OSCC were higher than in controls. In the present study, OPN concentrations correlated strongly



Graph 1: The box-plot showing comparative distribution of OSCC and normal cohorts



Graph 2: The distribution of the plasma OPN levels in OSCC cohorts were compared with respect to histo-pathological grades viz. well, moderate and poorly differentiated



Graph 3: Comparision of OPN intensity between OSCC and Control cohorts

with tumor stage. Levels of OPN were significantly lower in OSCC patients with tumor stage T1 compared to those with stage T2 or stages T3-4 with the medians 31, 82 and 116 μg/L respectively, which simulated with the clinical staging study by Chien *et al.*[5] OSCC patients with tumor grades I-II had lower concentrations of OPN in plasma compared to those with

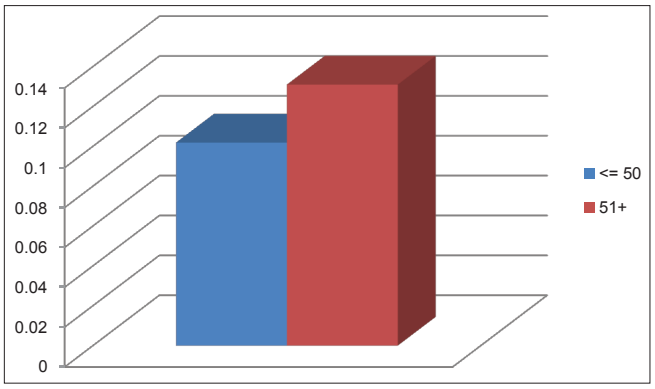


Figure 3: OSCC cohort was divided into two categories according to their age viz. <=50 years and 51 years & above and correlated with OPN expression

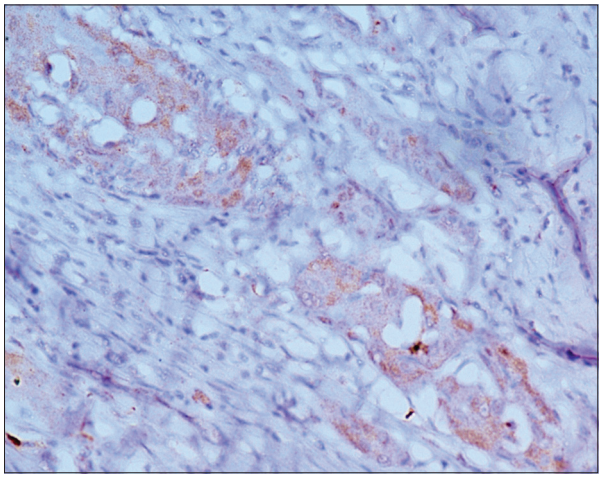


Figure 4: Immunostaining for osteopontin expression in oral squamous cell carcinoma with positive osteopontin immunoreactivity seen in the cytoplasm of the neoplastic squamous epithelial cells (IHC, ×200)

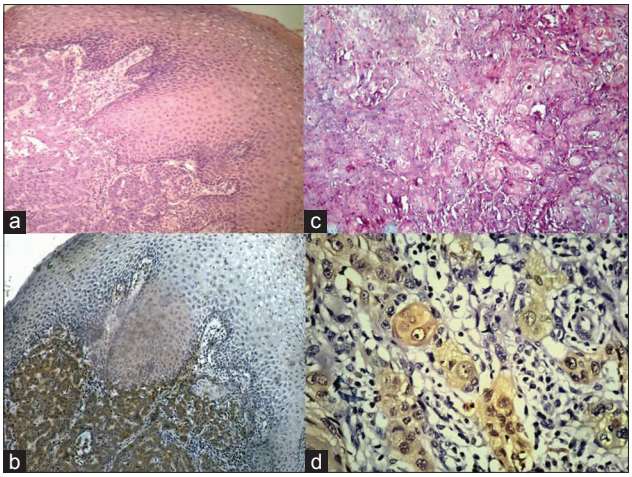


Figure 5: (a) Photomicrograph showing invasion and well differentiated squamous cell carcinoma (H&E, ×100) (b) Photomicrograph of same section showing positive immunostaining for OPN (H&E, ×100) (c) Photomicrograph showing nuclear and cellular pleomorphism with mitotic figures in the invading islands (H&E, ×40) (d) Photomicrograph of same section exhibiting characteristic cytoplasmic OPN immunostaining in neoplastic cells (IHC, ×400)

Table 3: Percentage positivity of various grades of intensity distribution observed in OSCC and normal cases

Group	Intensity			Total % positive cases
	Grade I	Grade II	Grade III	
OSCC	23	19	15	95 (57/60)
Normal	6	-	-	60 (6/10)

OSCC: Oral squamous cell carcinoma

grade III with the medians 116 μg/L. This close correlation with clinico-pathological data clearly indicates that plasma OPN is associated with tumor progression in OSCC patients.

CONCLUSION

Various malignancies, such as esophageal squamous cell carcinoma, hepatocellular carcinoma, metastatic breast cancer and gastric cancer, have already established significant correlations between plasma OPN levels and tumor aggressiveness.[15-17] This over expression of plasma OPN in our study is corroborative of the malignant phenotype, by increasing cell transformation, migration and invasion and is associated with oncogenesis and dissemination of various cancers.[4,18] In our IHC study, OPN expression also significantly correlated with more aggressive tumor behavior in the cohort of OSCC patients. In conclusion, high expression level of OPN in either the tumor or the plasma of the OSCC patients is associated with tumor progression. These results suggest that OPN expression is an important prognostic factor of OSCC.

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How to cite this article: Ingale Y, Routray S, Kheur SM, Kheur M, Mohanty N. Evaluating the efficacy of osteopontin expression as a prognostic marker in oral squamous cell carcinoma in the Indian subpopulation. J Oral Maxillofac Pathol 2014;18:11-5.

Source of Support: Nil. **Conflict of Interest:** None declared.