Immunohistochemical expression of osteonectin, matrix metalloproteinases-9 and Ki-67 in ameloblastoma

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Abstract

Background: Ameloblastoma (AM) is a benign odontogenic neoplasm characterized by local invasiveness and recurrence. We have evaluated the immunohistochemical expressions of osteonectin (ON), matrix metalloproteinase-9 (MMP-9) and Ki-67 in AM to understand the biologic behavior of this neoplasm.

Aim: The aim of this study is to evaluate the expression of ON, MMP-9 and Ki-67 in AM.

Materials and Methods: The study sample included archival tissues embedded in paraffin blocks histopathologically diagnosed as AM (20 cases). Three serial sections of each tissue specimen were stained separately with ON, MMP-9 and Ki-67 (immunohistochemistry stain) and evaluated. The data were analyzed using Spearman rank correlation test.

Results: All AM cases 20/20 (100%) exhibited positive immunostaining for ON, MMP-9 and Ki-67.

Conclusion: Increased expression of ON, MMP-9 and Ki-67 in AM indicates that these molecules might play a role in the regulation and aggressive nature of this neoplasm.

Keywords: Ameloblastoma, Ki-67, matrix metalloproteinase-9, osteonectin

INTRODUCTION

Ameloblastomas (AMs) are benign, slow-growing, aggressive neoplasms of epithelial origin mainly affecting the posterior mandibular region, with a poorly understood potential for rare metastasis.[1,2] The relative frequency of AM ranged from 11% to 92%, and it was noted to be 60% among odontogenic tumors, in the Indian scenario.[3,4] The clinical, radiological and histological features of this neoplasm have been well characterized.[5] They are capable of reaching large sizes with extensive bone erosion and destruction or conversely infiltrating into the medullary spaces causing local invasion and leading to high-recurrence rates.[6-8]

Many markers have been used to study the biological nature of this neoplasm. Osteonectin (ON)/secreted protein acidic and rich in cysteine (SPARC) is involved in the regulation of important physiological processes

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such as cell proliferation and cell migration that require the modulation of cellular-extracellular matrix (ECM) and cell-growth factor interactions. It also participates in pathological responses to tumorigenesis, tumor invasion and progression.[9]

Matrix metalloproteinases (MMPs) are proteinases that participate in ECM degradation that release mitogenic factors accounting for increased tumor cell proliferation, thus contributing to the local invasiveness of these tumors.[10,11]

Ki-67 is a well-known invasion and cell proliferation marker that helps in predicting aggressiveness. It is a nuclear nonhistone protein which is required for maintaining the cell cycle.[12,13]

This study aims to evaluate the expression of ON along with MMP-9 and Ki-67 to confirm its contribution to the aggressive and infiltrative behavior of these challenging, commonly occurring neoplasm.

MATERIALS AND METHODS

This laboratory-based immunohistochemistry (IHC) study was conducted for 6 months, the study sample involved the use of buffered formalin fixed, paraffin-embedded tissues of histologically diagnosed cases of AM retrieved from the archives of the Department of Oral and Maxillofacial Pathology and Microbiology, Narayana Dental College and Hospital, Nellore, Andhra Pradesh, India. Since paraffin-embedded tissue specimens were used in the study, ethical clearance was not sought. Cases with complete patient record were included in the study. Necrotic tumor areas or areas with the deterioration of tissue morphology due to processing were discarded in the analysis.

A total of 20 cases of AM were evaluated immunohistochemically for ON, MMP-9 and Ki-67 expression. Six cases of breast cancer were used as positive controls for ON (2 cases), MMP-9 (2 cases) and Ki-67 (2 cases). For negative control, TBS solution was used instead of primary antibody.

Two to three serial sections of 4–5 μm thickness were made and taken onto Poly-L-Lysine coated slides. The sections were deparaffinized, rehydrated and immersed in deionized water for 5 min. The slides were placed in the slide tank containing citrate buffer solution for immunostaining. The sections were covered with an appropriate volume of peroxidase block solution (containing 3% hydrogen peroxide) for 15 min.

Following this, the slides were gently washed with Tris-buffered saline and kept for 5 min in the same solution. Universal protein blocking was done with appropriate volume of Power Block™ solution for 15 min. The sections were covered with appropriate volume of respective primary antibody solution. For negative control, TBS solution was used instead of primary antibody. Application of Super Enhancer™ Reagent was done followed by application of Poly-HRP Reagent. Application of appropriate volume of freshly prepared substrate chromogen solution was carried out until acceptable color intensity has been reached. The slides were then counterstained with Harris Hematoxylin stain.

RESULTS

In this study, all the study samples were observed by two observers. The counting of positively stained cells was done independently under ×10, ×20 and ×40 objectives. Reliability analysis was done to test the consistency between the two observers. Since no interobserver bias was observed between the values of the two observers, the values of the first observer were taken for analysis.

Interpretation of immunostaining

The anti-ON, MMP-9 and Ki-67 antibody expression by the tumor cells showed deposition of brown pigmentation in the cytoplasm (ON and MMP-9) and nucleus (Ki-67) that were indicative of positive immunoreactivity [Figures 1-3].

Evaluation of osteonectin and matrix metalloproteinase-9 immunostaining

The entire tumor section was assessed for the immunohistochemical evaluation. A semi-quantitative assessment as proposed by Krajewska *et al.* (1996) was employed to evaluate the immunostaining of ON and MMP-9 by considering both the intensity (I) of staining and the percentage of positive cells (P). The percentage (P) of positively stained tumor cells was determined by screening the entire tumor section, and each section was assigned to one of the following immunoscore categories.

Scoring system for percentage of positive cells

0. 0%–4%
1. 5%–24%
2. 25%–49%
3, 50%–74%
4. 75%–100%.

Scoring system for intensity of staining

0. Negative
1. Light yellow
2. Yellow-brown
3. Dark brown.

Staining intensity was measured based on the scoring system. For each section, a final score was obtained by multiplying the percentage of positive cells (P) by the staining intensity scores (I) as proposed by Shen *et al.* (2010).

Thus, the final immunoscores for ON and MMP-9 were then assigned to one of the following groups of staining expression.

- Weak (+)-0–2
- Moderate (+ +)-3–5
- Strong (+ + +)-6–8
- Very strong (+ + + +)-9–12.

Evaluation of Ki-67 immunostaining

Ki-67 immunostaining was quantitatively evaluated by the method proposed by Mitrou *et al.* The average numbers of positively stained nuclei were counted in each stained section in 10 high-power microscopic fields (×400). The final immunoscores were calculated by adding up all the positively stained nuclei in 10 high-power fields and dividing it by 10.

Scoring system for Ki-67 immunostaining

- Weak (+)–<5 positive epithelial cells
- Moderate (++)–6–10 positive cells
- Strong (+++)–11–20 positive cells
- Very strong (++++)–>21 positive cells.

Statistical analysis

Interobserver agreement was evaluated using kappa statistics as follows: it was considered as poor agreement when the kappa value was <0.40, values between 0.40 and 0.59 was considered as fair agreement, between 0.60 and 0.74 as good agreement, and between 0.75 and 1.00 as excellent agreement.

The differences in statistical values in the immunoscores between ON, MMP-9 and Ki-67 were compared and analyzed using Spearman rank correlation test for the tumor components of AM. $P < 0.005$ is considered to be statistically significant.

All AM cases 20/20 (100%) exhibited positive immunostaining for ON, MMP-9 and Ki-67 [Table 1 and Graph 1]. The total number and percentage distribution of cases based on the IHC expression of ON,

Table 1: Percentage of positive and negative cases of osteonectin, matrix metalloproteinases-9 and Ki-67 immunostaining in 20 cases of ameloblastoma

| Cases	Total cases	Positive	Percentage	Negative
Osteonectin	20	20	100	Nil
MMP-9	20	20	100	Nil
Ki-67	20	20	100	Nil

MMP: Matrix metalloproteinase

MMP-9 and Ki-67 staining in the tumor component of AM is given in Table 2 and Graph 2. The statistical analysis yielded a $P = 0.004$, 0.09 and 0.004, all of which were significant when the expression was compared between (a) ON and MMP-9, (b) ON and Ki-67 and (c) MMP-9 and Ki-67 immunostaining, respectively, in the tumor component of 20 AM cases [Tables 3-5].

DISCUSSION

AM is the most frequently occurring odontogenic tumor, and although classified as a benign neoplasm, it is a locally invasive and destructive tumor of the jaw bone with a relatively high rate of recurrence and even metastasizes in rare conditions.[14] Immunohistochemical markers such as ON, MMP-9 and Ki-67 were used in the present study to understand the biologic behavior and mechanisms underlying the local invasiveness associated with this neoplasm.

ON/SPARC is a multifaceted collagen and hydroxyapatite binding glycoprotein. Its main function is to modulate cell-matrix interactions, cell functions and regulation of matrix remodeling through metalloproteinases, etc.[9] It has antiadhesion property; hence, it induces transcription of matrix remodeling metalloproteinases involved in the degradation of the basement membrane, breaks down interstitial connective tissue matrices causing tumor invasion and metastasis.[15-17]

A study conducted by Jeen *et al.*, Shen *et al.* in odontogenic tumors, noted the strong expression of ON in some stages of tooth development, AM s, CEOT and AOT cases. They concluded that ON/SPARC regulate calcification process and subsequent hard-tissue formation, hence strongly express in developing tooth germ and odontogenic tumors.[18] ON expression is modulated by
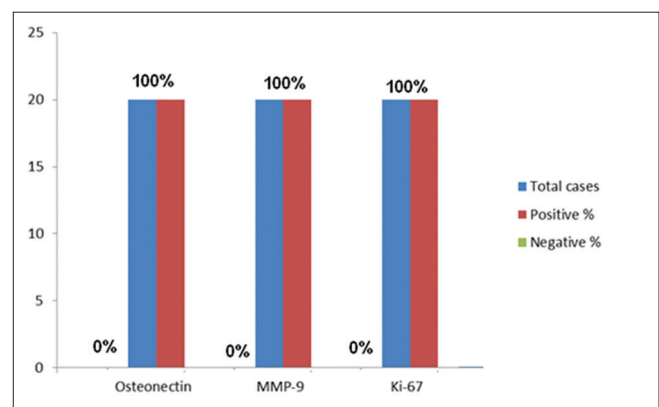
Graph 1: Percentage of positive and negative immunostaining of osteonectin, matrix metalloproteinase-9 and Ki-67 in 20 cases of ameloblastoma

Table 2: Total number and percentage distribution of cases based on immunohistochemical expression of osteonectin, matrix metalloproteinases-9 and Ki-67 in the tumor component of ameloblastoma

Markers	Total number of cases	Weak (+) (%)	Moderate (++) (%)	Strong (+++) (%)	Very strong (++++) (%)
Osteonectin	20	2 (10)	2 (10)	14 (70)	2 (10)
MMP-9	20	1 (5)	1 (5)	15 (75)	3 (15)
Ki-67	20	-	5 (25)	15 (75)	-

+: Weak staining, ++: Moderate staining, +++: Strong staining, ++++: Very strong staining, MMP: Matrix metalloproteinases

Table 3: Spearman rank correlation between immunohistochemical expression of osteonectin and matrix metalloproteinases-9 staining in the tumor components of 20 ameloblastoma cases

Number of cases	Marker I	Marker II	ρ	P	Significance
20	Osteonectin	MMP-9	0.617	0.004	Statistically significant

MMP: Matrix metalloproteinase

Table 4: Spearman rank correlation between immunohistochemical expression of osteonectin and Ki-67 staining in tumor components of 20 ameloblastoma cases

Number of cases	Marker I	Marker II	ρ	P	Significance
20	Osteonectin	Ki-67	0.569	0.009	Statistically significant

Table 5: Spearman rank correlation between immunohistochemical expression of matrix metalloproteinases-9 and Ki -67 staining in tumor components of 20 ameloblastoma cases
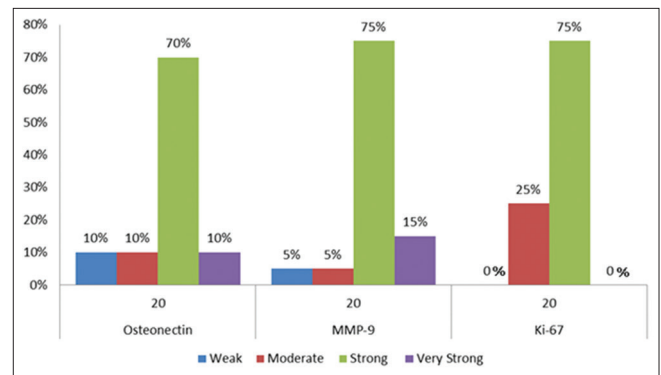
Number of cases	Marker I	Marker II	ρ	P	Significance
20	MMP-9	Ki-67	0.067	0.020	Statistically significant

MMP: Matrix metalloproteinase

Graph 2: Total number and percentage distribution of cases based on the immunohistochemistry expression of osteonectin, matrix metalloproteinase-9 and Ki-67 staining in the tumor component of ameloblastoma

growth factors such as transforming growth factor beta 1 (TGF-β1) (TGF-β superfamily), fibroblast growth factor-2, interleukin-1, platelet-contained growth factor, insulin-like growth factor-1, indicating a potential role in modulating invasion and metastasis.[15]

In the present study, ON expression was noted in all the 20 cases (100%) of AMs with varying staining intensities and percentages of positive cells. The staining was localized to the cytoplasm of the basal columnar cells and also to the stellate reticulum-like cells in tumor component. Positive immunostaining was noted in the surrounding stromal cells, such as fibroblasts, endothelial cells, inflammatory cells, osteoblasts and osteocytes, similar to a study conducted by Shen *et al.*[17]

Further, the presence of ON/SPARC in normal tooth germs and its role in odontogenic tumors still remains controversial, with some researchers supporting its participation in hard-tissue formation/resorption and some vouching for its role in neoplastic progression. Further

studies in this regard need to clarify the role of ON/SPARC in odontogenic tumors.

MMPs, also called matrixins, are a family of zinc and calcium-dependent proteolytic enzymes that degrade ECM macromolecules, such as collagens, gelatins, fibronectin, tenascin and laminin, at physiological pH.[19,20] Under physiological conditions, these enzymes play central roles in ECM regulation during embryonic development and tissue remodeling.[21] They can bring about a breach in the basement membrane by cleaving of Type IV collagen as a preface to invasion. In many aggressive tumors, it has been noted that there is abnormal expression of MMP-1, 2 and 9.[22] The strong expression of MMPs and loss or weak expression of TIMPs in AMs reflect its aggressive nature and high potential to recur.[23]

In the present study, MMP-9 expression was noted in all the 20 (100%) cases of AMs with different staining intensities which were similar to study conducted by Ribeiro *et al.* but in contrast to the study conducted by Kumamoto *et al.*[11,21] Strong immunostaining was observed in most of the cases similar to studies conducted by Shen *et al.*, Pinheiro *et al.*, Zhong and Tue, Qian and Huang and Teronen *et al.* but contrasted with those of Kumamoto *et al.* who observed weak immunostaining of MMP-9 in AMs.[17,21,23-29]

The immunostaining was localized to the cytoplasm of the basal columnar cells/peripheral cells and stellate reticulum-like cells/central tumoral cells of nests, follicles and cords of neoplastic odontogenic epithelium similar to
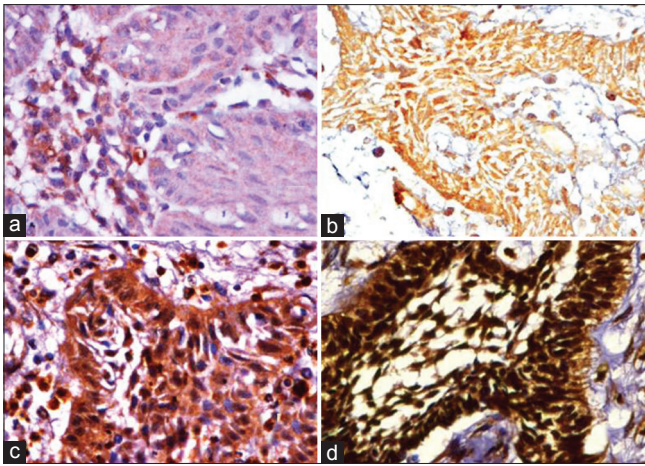
Figure 1: Osteonectin – Photomicrograph showing (a) weak staining, (b) moderate staining, (c) strong staining, (d) very strong staining of the tumor cells and the subjacent stromal component (×40)

Figure 2: Matrix metalloproteinase-9 – Photomicrograph showing (a) weak staining, (b) moderate staining, (c) strong staining, (d) very strong staining of the tumor cells and the subjacent stromal component (×40)
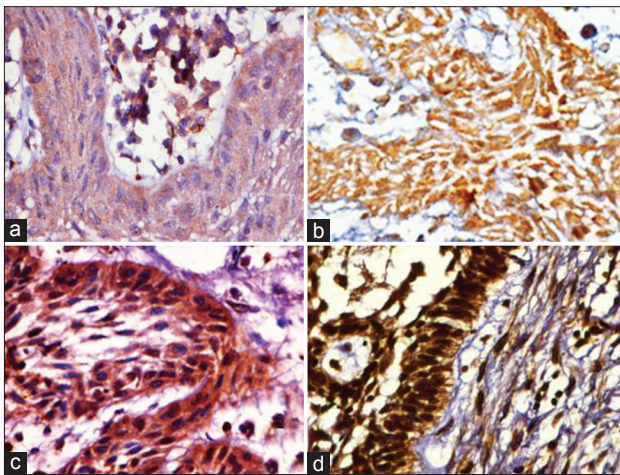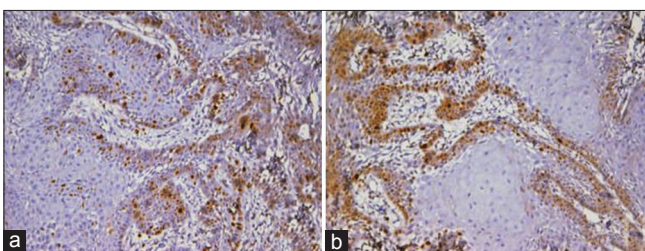

Figure 3: Ki-67 – Photomicrograph showing strong staining in peripheral columnar as well as parabasal cells (a) ×20 (b) ×40

observation of Shen *et al*.[17] The results were in contrast to studies conducted by Pinheiro *et al*. and Kumamoto *et al*. where the expression of MMP-9 was restricted to the peripheral tumoral cells.[21,26]

Although MMPs, particularly MMP-9, promote tumor angiogenesis and regulate cell breakdown to facilitate

invasiveness and metastasis of carcinoma cells, its role in neoplastic progression in odontogenic tumors *per se* in AMs needs to be defined precisely.

It is suggested that ON/SPARC could interact with MMP-9 and induce a series of invasion of the pericellular microenvironment by the neoplastic cells and participate in the proteolysis of ECM of AM. MMP-9 and ON/SPARC both being matricellular proteins with lot of interdependency between them either directly or through other ECM molecules, is known to act synergistically in degradation of ECM and angiogenesis.[17,30]

Ki-67 protein (also known as MKI67) is a cellular marker for proliferation. The proliferative activity of a tumor or a tissue is determined by the number of cells in the cycle and the time taken to complete the cell cycle.[31,32] There is a strong correlation between the proliferation rate of tumor cells and the clinical, behavioral outcome, i.e., its aggressiveness.[31,33,34] Proliferation rate can be assessed to determine the likelihood of recurrences or aggressive behavior and could serve as an added factor in determining the management and outcome of this neoplasm.[35]

Proliferative markers such as Ki-67 and PCNA have been used to study the proliferation rate of AM in studies conducted by Ong'uti *et al*., Sandra *et al*., Abdel-Aziz and Amin, Carreón-Burciaga *et al*., Jaafari-Ashkavandi *et al*. and Meer *et al*.[32-37] The superiority of Ki-67 in comparison with PCNA was demonstrated by McCormick and Hall and Gerdes *et al*.[38,39]

In the present study, Ki-67 expression was noted in all the 20 (100%) cases. Ki-67 positivity was localized to nuclei, predominantly in basal cells with occasional suprabasal positive nuclei seen in the stellate reticulum-like cells. The quantitative method of evaluation of Ki-67 staining as proposed by Mitrou *et al*. was followed in the present study.[40] Increased Ki-67 expression, as noted in our and other studies does prove its role in aggressive behavior of AMs. IHC procedures are technique sensitive, and standardization was a tedious procedure. Once this limitation was overcome, the study gave accurate results.

CONCLUSION

The present study using immunohistochemical markers ON, MMP-9 and Ki-67 helped us understand the biologic behavior of AM. Further studies are required to address the role of ON in tumorigenesis and the interactions of both ON/SPARC and MMP-9 with factors which could bring an increase in Ki-67 expression. This may provide

a better understanding of this neoplasm, leading to the development of effective preventive, diagnostic and treatment approaches.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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