Original Article

Role of podoplanin expression in deciding the invasive potential of ameloblastoma – A retrospective IHC study

Sindhu M. Ganvir, Pratima G. Khobragade*, Swati A. Bamane, Ramnivas Kumavat, Amit Dalmia

Department of Oral Pathology and Microbiology, Government Dental College and Hospital, Nagpur, India

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ABSTRACT

Background: The aim of this study was to investigate the podoplanin expression in epithelial odontogenic tumors both non-aggressive and aggressive, tumors with and without ectomesenchyme and remnants of the odontogenic epithelium from dental follicles (DF) of unerupted teeth and to examine its role in progression and invasion of tumors.

Method: Thirty paraffin embedded specimens AM (15 were non-aggressive ameloblastomas and 15 ameloblastoma showing aggressive behavior), 15 paraffin embedded specimens of AOT, 15 paraffin embedded specimens of CEOT, 15 paraffin embedded specimens of CCOT were obtained and were analyzed by immunohistochemistry using anti-human podoplanin. Podoplanin expression in odontogenic epithelial cells was evaluated using a scoring method, along with calculation of the percentage of positive odontogenic cells.

Results: Podoplanin was expressed strongly at the invasive front (in the peripheral odontogenic epithelial cells) of most tumors and dental follicles. Membranous expression of podoplanin in ameloblastomas was stronger in cases of ameloblastomas showing aggressive behavior than (NA) non-aggressive ameloblastomas.

Conclusion: Expression of podoplanin at the invasive front (in peripheral cells) of odontogenic tumors considered to be associated with neoplastic odontogenic tissues. This molecule might play a role in progression and local invasion of odontogenic tumors. The migration and invasion mediated by podoplanin in odontogenic tumors could be related to cytoskeletal reorganization.

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1. Introduction

Ameloblastoma (AM) is one of the most frequent odontogenic tumors and is characterized by benign but locally aggressive behavior with a high rate of recurrence.^{1,2} Histologically, AMs occur in many patterns, follicular, plexiform, and include acanthomatous, granular cell and basal cell variants.^{1,2} Recent studies have identified genetic and molecular alterations in epithelial odontogenic tumors^{2,3} however, details of the mechanism of oncogenesis, cytodifferentiation, and tumor progression remain unknown.²

Podoplanin is a transmembrane glycoprotein that has been largely used as an identifying marker of the lymphatic endothelium.⁴ Published findings indicate that this protein has a

relatively broad spectrum of reactivity including its expression in a wide variety of benign and malignant oral tumors. In oral squamous cell carcinoma, the strong expression of podoplanin by malignant cells was associated with lymph node metastasis and poor clinical outcome.^{5–15}

A previous study has demonstrated that Chinese hamster ovary cells, which overexpress podoplanin, are retained in the lungs, suggesting that podoplanin plays a role in tumor metastasis because of its platelet aggregation-inducing activity.¹⁶ The expression of podoplanin in normal and tumorous odontogenic cells is a recent topic of study.

In view of the above considerations, the aim of this study was to investigate the expression of podoplanin in two groups of odontogenic tumors: those exclusively composed by epithelial neoplastic components and those composed by epithelial and ectomesenchymal tumoral cells, to examine role of podoplanin in aggressive behavior of ameloblastoma compared to non-aggressive one and also to determine the potential role of podoplanin in benign tumor progression by attempting to identify an association

* Corresponding author at: Department of Oral Pathology and Microbiology, Government Dental College and Hospital, Near New Bus Stand, Behind Talathi Office, Vidyanagar, Bramhapuri, Chandrapur, Maharashtra 441206, India. Tel.: +91 09503833379.

E-mail address: pratimak237@gmail.com (P.G. Khobragade).

between podoplanin expression in odontogenic epithelial cells of ameloblastomas and dental follicles (DF).

2. Subjects and methods

All paraffin-embedded tissue specimens from 30 ameloblastomas (15 ameloblastoma showing aggressive behavior some of them shows lymph node involvement and 15 were non-aggressive), 15 adenomatoid odontogenic tumor, 15 CEOT, 15 CCOT and 15 DF were retrieved from the Department of Oral Pathology and Microbiology, Government dental college and hospital, Nagpur. The inclusion criteria were as follows: (i) patients with microscopically confirmed diagnoses of solid/multicystic ameloblastoma or unicystic ameloblastoma, AOT, CEOT, CCOT determined by the sum of the clinical, radiographic, and microscopic data; (ii) DF of patients with asymptomatic, unrequited and non-inflamed teeth confirmed both clinically and microscopically; (iii) the availability of the paraffin block with a sufficient amount of tissue for microscopic analysis. These samples were analyzed by immunohistochemistry for podoplanin. The diagnosis of ameloblastoma, AOT, CEOT, CCOT was reviewed according to the World Health Organization histological classification of odontogenic tumors.¹ This study was approved by the Ethics Committee of the Government Dental College and Hospital, Nagpur.

2.1. Immunohistochemistry

Formalin-fixed 4 μ m sections of Ameloblastomas, AOT, CEOT, CCOT and DF of unerupted teeth were obtained from the pathology archive and subjected to immunohistochemistry analysis using anti-podoplanin antibodies to determine their expression in odontogenic cells. After antigen retrieval using 10 mM citrate buffer, pH 6.0, in EZ antigen retriever for 10 min of 2 cycles, endogenous peroxidase activity was blocked by incubating in 3% H₂O₂ for 20 min. Each ameloblastoma or dental follicle section was incubated for 1 h in a 1:200 dilution of the primary monoclonal anti-podoplanin antibody (D2-40 clone, code#3619-1; Dako North America, Inc., Carpinteria, CA, USA). Primary antibody dilutions were made in a bovine serum albumin (code#A2153; Sigma, Co., St Louis, MO, USA) solution to block non-specific reactions. After the primary antibody incubation, each section was incubated using the Advance HRP Link System (code #K4067; Dako North America, Inc.) for 30 min at 37 °C. The antibodies podoplanin was detected using 3,3 diaminobenzidine tetrahydrochloride (cod# D-5637; Sigma). Sections were counterstained with Mayer's hematoxylin before being dehydrated and cover slipped. Lymph node was used as the positive control. The primary antibody was omitted during immunohistochemical staining for the negative control.

2.2. Immunostaining evaluation

Cytoplasmic and/or membranous podoplanin expression by epithelial odontogenic cells in each ameloblastoma, AOT, CCOT, CEOT and dental follicle was assessed by the evaluation of the staining intensity and percentage of podoplanin positive cells, according to the method used by Etamad-Moghadam et al.¹⁷

All the immunohistochemical-stained sections were evaluated by two observers to eliminate interobserver bias and any disagreement were resolved with a pentahead microscope.

2.3. Statistical analysis

Statistical analysis was done using statistical software SPSS (Statistical Package of Social Software) version 16.0, to make the study statistically significant. After clinical observations, the data

Table 1

Comparison of mean age of the participants between test groups.

	Mean	SD	P* value
Aggressive ameloblastoma	42.2667	12.76976	<0.001
Non-aggressive ameloblastoma	44.0000	13.36306	
AOT	18.7333	4.83243	
CCOT	56.6667	7.79805	
CEOT	41.9333	16.49877	
Dental follicle	31.5333	8.56794	

* One-way ANOVA test.

Table 2

Gender distribution of test groups.

	Sex		P* value
	Female	Male	
Groups			
Aggressive ameloblastoma	5	10	0.16
	12.5%	20.0%	
Non-aggressive Ameloblastoma	4	11	
	10.0%	22.0%	
AOT	11	4	
	27.5%	8.0%	
CCOT	6	9	0.16
	15.0%	18.0%	
CEOT	7	8	
	17.5%	16.0%	
Dental follicle	7	8	
	17.5%	16.0%	
Total	40	50	
	100.0%	100.0%	

* Chi-square test.

collected was tabulated and all observed results were then subjected to various statistical analyses as per requirement.

3. Results

3.1. Clinical features

The age of patients with ameloblastoma ranged from 19 to 65 years (mean of 44 years) (Table 1). A predominance of men (22%) was observed in relation to women (10%) (Table 2). The distribution of tumors according to the histopathological type and according to aggressive behavior is listed in Table 3. Eight male and seven female patients comprised the samples of DF from the unerupted teeth selected for this study. Their ages ranged from 9 to 32 years. The vast majority of DF, 80%, were from posterior teeth, 12.5% were from anterior teeth, and 7.5% did not have this information available (Table 4).

3.2. Podoplanin expression in odontogenic epithelial cells from ameloblastomas, AOT, CEOT, CCOT and dental follicles

The expression of podoplanin in follicular ameloblastomas was predominantly observed in the peripheral columnar cells of

Table 3

Distribution of ameloblastoma according to histopathologic type.

Type	Number (N)	Percentage (%)
Aggressive ameloblastoma	15	50
Unicystic ameloblastoma	3	10
Follicular ameloblastoma	6	20
Plexiform ameloblastoma	3	10
Acanthomatous ameloblastoma	3	10
Total	30	100

Fig. 1. (a, b) Follicular ameloblastoma (podoplanin expression was observed in peripheral cells while central cells were negative for podoplanin).

tumoral islands, while the reticulum stellate-like cells exhibited virtually no immunostaining (Fig. 1a, b). The outer cells of the plexiform AMs expressed podoplanin based on immunohistochemistry staining. In a few specimens, its expression reached the central cells (Fig. 2a, b). The expression of podoplanin was quite variable in the unicystic AMs. Its positivity was restricted to the basal and supra-basal layers of the cystic lining in some tumors with no reaction in the upper layer (Fig. 3a, b). The acanthomatous areas did not express podoplanin (Fig. 4).

The majority of epithelial cells composing the strands and islands of adenomatoid odontogenic tumors strongly expressed podoplanin in the transmembrane. In some cells, this expression was observed in the cytoplasm either. Duct-like and rosette shaped structures were also positive for podoplanin (Fig. 5a) while foci of calcification were negative (Fig. 5b).

In the calcifying epithelial odontogenic tumor, peripheral cells were positive for podoplanin. However the epithelial odontogenic cells in a more central location were mildly positive for the protein while eosinophilic material and calcification areas were negative (Fig. 6a, b).

Calcifying cystic odontogenic tumors presented positivity for podoplanin in the epithelial odontogenic cells of the cystic lining. Ghost cells within the tumor and the neoplastic fibrous wall did not express the protein (Fig. 7a, b).

Fig. 2. (a, b) Plexiform ameloblastoma.

Table 4
Distribution of groups with respect to staining intensity.

Groups	Staining intensity			P* value
	Low	Medium	High	
Aggressive ameloblastoma	0	5	10	0.36
	0%	12.2%	28.6%	
Non-aggressive ameloblastoma	1	8	6	
	7.1%	19.5%	17.1%	
AOT	3	8	4	
	21.4%	19.5%	11.4%	
CCOT	4	6	5	
	28.6%	14.6%	14.3%	
CEOT	4	7	4	
	28.6%	17.1%	11.4%	
Dental follicle	2	7	6	
	14.3%	17.1%	17.1%	
Total	14	41	35	
	100.0%	100.0%	100.0%	

* Chi-square test.

Fig. 3. (a, b) Unicystic ameloblastoma only basal and suprabasal layer express podoplanin.

Fig. 4. Acanthomatous ameloblastoma (acanthomatous area did not express podoplanin).

Fig. 5. (a, b) Adenomatoid odontogenic tumor (rosette and ductal pattern and calcification).

The expression of podoplanin in the odontogenic epithelial cells of DF was observed predominantly in the remnants of the odontogenic epithelium island, the peripheral epithelial cells were predominantly immunostained for podoplanin (Fig. 8a, b).

3.3. Podoplanin expression in aggressive and non-aggressive ameloblastoma

Membranous immunostaining of podoplanin was analyzed separately between aggressive and non-aggressive ameloblastoma.

The vast majority of central cells from the islands of non-aggressive ameloblastomas displayed absence or weak immunoreactivity for podoplanin (Figs. 1–3), while tumors showing aggressive behavior and lymph node positivity showed strong immunostaining of central cells for podoplanin. The membranous expression of podoplanin was higher and more frequent in the odontogenic epithelium (both in central and peripheral cells) of ameloblastoma showing aggressive behavior than non-aggressive (Fig. 9a, b).

4. Discussion

The distribution of podoplanin immunoreaction in the odontogenic tumors found in this study is corroborated by previous

Fig. 6. (a, b) CEOT with eosinophilic material and calcification.

Fig. 7. (a, b) CCOT with ghost cells.

investigations.^{7,8,10,13,14} Its expression had been studied only in few exclusively epithelial odontogenic tumors.^{7,10,13,18,19} The unique exception of this study is comparison between ameloblastomas showing aggressive behavior and lymph node involvement with non-aggressive one and podoplanin expression in epithelial odontogenic tumors, mixed tumors and dental follicle. Thus, to contribute to this line of investigation the immunostaining pattern of podoplanin in 60 epithelial odontogenic tumors, 15 mixed odontogenic benign tumors and 15 dental follicles were analyzed.

In this study, all ameloblastomas displayed membranous and/or cytoplasmic expression of podoplanin, and its expression was consistent with the cell type and tumor location. The epithelial tumoral cells in intimate contact with the stroma were stained strongly positive for podoplanin (Figs. 1–3), while the central cells of the islands were negative (Fig. 1a, b). These results corroborated the previous reports of González-Alva et al.⁷ and Zustin et al.¹³

The expression of podoplanin was basically restricted to the peripheral epithelial cells of the odontogenic neoplasias, indicating that this protein probably has a role in the process of tumoral invasion. It is reinforced by the fact that the podoplanin-positive structures, such as peripheral cells of ameloblastic islands, duct like structures of AOT and epithelial remnants of dental lamina.

The plexiform ameloblastoma resembles the tooth germ in the dental lamina stage when the differentiation process of the odontogenic epithelium has not initiated.²⁰ This lack of cellular differentiation may reflect the homogeneity of podoplanin expression found in these benign epithelial tumors, confirming previous results obtained by other authors.^{7,18} Interestingly, a similar pattern of podoplanin expression in the odontogenic epithelium was found in murine developing teeth where pre-ameloblasts strongly express podoplanin until they differentiate into mature ameloblasts, at which time the podoplanin immunoreactivity decreases.^{21,22}

All adenomatoid odontogenic tumors showed membranous and cytoplasmic podoplanin expression in the central epithelial odontogenic cells, including the duct-like structures (Fig. 5). On the other hand, the podoplanin-negative structures (acanthomatous areas of ameloblastomas, calcification foci of Pindborg tumor, and ghost cells of CCOT) are quiescent cells (Figs. 4, 6b, 7b).

These findings appear to provide the evidence of a relationship between podoplanin and odontogenic cellular activity (i.e. the protein is expressed during intense proliferative activity in odontogenic cells and when these cells reach maturity or a stable state, there is a reduction or lack of podoplanin immunoreactivity).

Our results reinforce this hypothesis because the odontogenic tumoral cells with low mitotic activity, such as the central stellate

Fig. 8. (a, b) Epithelial remnants of dental lamina and reduced enamel epithelium of dental follicle.

reticulum-like cells, keratinized acanthomatous cells did not express podoplanin.

We also evaluated the expression of podoplanin in the odontogenic epithelial remnants of DF from unerupted teeth. The epithelial component of a dental follicle consists of the epithelial rests of the dental lamina and the reduced enamel epithelium.²³ These both represent quiescent and mature odontogenic epithelium that has performed its function and often remains dormant in the ectomesenchymal tissue indefinitely. Therefore, the epithelium of the dental follicle was used as a control group to compare to AM islands and cords. Similar to the odontogenic epithelium of ameloblastomas, membranous and/or cytoplasmic podoplanin immunoreactivity was observed in the peripheral cells of dental lamina remnants (i.e., those adjacent to the surrounding connective tissue) (Fig. 8a). In the reduced enamel epithelium, the basal cells were positive for podoplanin while the reduced ameloblasts exhibited no staining (Fig. 8b). After the conclusion of the secretory activity, the ameloblasts reduce their size and the number of cellular organelles as a consequence of ending their secreting and proliferative activities, assuming a more quiescent state. Our results showed that at this moment, the so-called reduced ameloblasts do not express podoplanin. These data support the concept that podoplanin may be necessary for the proliferative activity of the epithelial odontogenic cells. Some

Fig. 9. (a, b) Aggressive ameloblastoma.

authors suggested that podoplanin may be associated with cellular proliferative activity based on its expression during odontogenesis.^{21,23} During this process, the protein expression is restricted to areas with high mitotic rates, such as the dental lamina, the terminal portion of Hertwig's sheath and pre-ameloblasts. In ameloblastomas also proliferative activity is virtually restricted to the outer layer of epithelial islands. This pattern of staining was observed in our study either.

While comparing ameloblastomas showing aggressive behavior with non-aggressive one we found that membranous expression of podoplanin was significantly stronger and intense in ameloblastomas showing aggressive behavior compared to non-aggressive one. This protein was intensely expressed in areas of high cellular proliferative activity and also in the central cells. Nevertheless, the current study is unique in separately analyzing podoplanin expression between aggressive and non-aggressive ameloblastoma.

In this study results showed an enhanced podoplanin expression in areas of local invasion of ameloblastomas, suggesting a role of this protein in such process. The tumoral invasion relies on intense active remodeling of the cellular actin cytoskeleton. In both human keratinocytes and in MCF7 breast cancer cells, the forced expression of podoplanin led to a dramatic change of cellular morphology with a significant decrease of cellular stress fibers and a concomitant formation of filopodia-like membrane protrusions.¹⁵

Adding to these findings with increased membranous podoplanin expression in the peripheral cells of ameloblastomas, it is

possible to speculate that this protein may be associated with the remodeling of the cytoskeleton of neoplastic cells.

5. Conclusion

Based on previous studies, as well as the evidence provided here, it may be hypothesized that the membranous expression of podoplanin may be the evidence of local invasion of epithelial odontogenic cells in ameloblastomas. Indeed, the conflicting results about the role of podoplanin in odontogenic tumors reinforce the importance of elucidating how this molecule participates in the growth of these tumors.

The data from this current study suggest that odontogenic tumors expressing podoplanin have a tendency to be somewhat less histologically differentiated and that they have a more infiltrative pattern of invasion and more prone to show lymph node metastasis. However, in this study, podoplanin expression failed to correlate with poor patient survival.

These findings suggest that overexpression of podoplanin is likely to be related to the biological aggressiveness of various odontogenic tumors, so blocking of podoplanin activity or its associated signaling could be a novel therapeutic approach for the treatment of aggressive odontogenic tumors.

Summary describing a key finding of the study

This study assesses the role of podoplanin (a transmembrane glycoprotein) in deciding invasive potential and aggressive behavior of various odontogenic tumors which could be useful for therapeutic approach of various aggressive odontogenic tumors.

Conflicts of interest

The authors have none to declare.

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