Enhanced expression of podoplanin in ameloblastomas

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OBJECTIVE: Podoplanin, a mucin-type transmembrane glycoprotein, is specifically expressed by lymphatic but not blood vascular endothelial cells, and is also widely expressed in various specialized cell types throughout the body. Recent studies have demonstrated that it mediates a pathway leading to collective cell migration and invasion in vivo and in vitro. In the present study, we carried out an immunohistochemical investigation of podoplanin to clarify whether it is expressed in human ameloblastomas (AMs), which are characterized by locally aggressive behavior with a high rate of recurrence. In addition, we examined the localization of the epithelial marker E-cadherin and the mesenchymal marker vimentin to clarify whether AMs show epithelial–mesenchymal transition (EMT).

METHODS: Paraffin-embedded tissue specimens of 38 AMs were examined immunohistochemically using antibodies against podoplanin, E-cadherin, and vimentin.

RESULTS: Immunohistochemical reactivity for podoplanin was detected in the cell membrane and cytoplasm of most odontogenic tumor epithelial cells in AMs. Podoplanin was expressed strongly in peripheral columnar cells and slightly in central stellate reticulum-like cells. E-cadherin was expressed in central stellate reticulum-like cells and showed decreased expression in peripheral columnar cells. Immunoreactivity for E-cadherin was weak or negative in keratinizing cells of acanthomatous AMs, suggesting terminal differentiation of the tumor cells. Immunohistochemical reactivity for vimentin was found in stromal cells, but partial or no reaction was observed in neoplastic cells.

CONCLUSION: Expression of podoplanin in AMs is considered to be associated with neoplastic odontogenic tissues; this molecule might play a role in the collective cell migration of tumor nests in AMs. The pattern of expression of E-cadherin and vimentin suggests that invasion in AMs occurs in the absence of EMT. The migration and invasion mediated by podoplanin in AMs could be related to cytoskeletal reorganization.

Keywords: ameloblastoma; E-cadherin; immunohistochemistry; podoplanin; vimentin

Introduction

Tooth morphogenesis is a well-orchestrated process regulated by sequential and reciprocal interactions between epithelial cells lining the oral cavity and cranial neural crest-derived ectomesenchymal cells (1). Odontogenic tumors are derived from these tissues or from their derivatives, exhibiting considerable histologic variation, and are classified into several benign and malignant entities (2, 3). 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 (2, 3). Histologically, AMs occur in two main patterns, follicular and plexiform, and include acanthomatous, granular cell, basal cell, and desmoplastic variants (2, 3). Recent studies have identified genetic and molecular alterations in epithelial odontogenic tumors (3, 4); however, details of the mechanism of oncogenesis, cytodifferentiation, and tumor progression remain unknown (3).

Invasion, the movement of cells through cellular and extracellular matrix (ECM) barriers into neighboring tissue, is a complex process entailing alterations in cell–cell and cell–ECM interactions, remodeling of the ECM, reorganization of the cytoskeleton, and increased cell motility (5). The process of invasion requires expression and/or activation of multiple genes and their products (5–9). Epithelial–mesenchymal transition (EMT) is a complete switch of epithelial cell properties characterized by phenotypic conversion in which epithelial cells lose their polarity and cohesiveness and acquire migratory features and the characteristics of fibroblasts (6, 7). Such gain of migratory capability and autonomous cell survival underlies the development of invasive and metastatic tumors (10).

The exact role of EMT in tumor progression is still debatable, but is thought to be particularly important in cancers showing single-cell migration and early dissemination of tumor cells. In contrast, the invasion of large cell sheets into neighboring tissue, often called collective cell migration, is less well understood. These cell sheets

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maintain their expression of epithelial adhesion structures, but, nonetheless, invade into the surrounding tissue and thereby destroy the host organ (6).

Human podoplanin (T1alpha-2, agrus and gp36) is a type-1 transmembrane sialomucin-like glycoprotein consisting of 162 amino acids. Podoplanin was originally detected in puromycin-induced nephrosis on the surface of rat podocytes as a 38-kDa mucoprotein linked to the flattening of foot processes, and is now utilized as a specific marker for recognizing lymphatic vessels (6, 11–16). Enhanced expression of podoplanin has been demonstrated at the invasive front of a number of human carcinomas (12–16).

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 (15). Podoplanin also contributes to tumor invasion by binding ezrin, radixin, moesin proteins to activate RhoA, and remodeling the actin cytoskeleton of tumor cells, contributing to their increased motility (15–17). In addition, studies of transgenic mouse models have demonstrated that podoplanin shifts the pattern of invasion from that of single cells involving EMT to that involving large cell sheets in the absence of EMT (6, 17). We have also demonstrated enhanced expression of podoplanin in limited neoplastic myoepithelial elements of human salivary gland pleomorphic adenomas, characterized by benign but also invasive behavior (18).

Strong expression of podoplanin has also been detected in enamel epithelia of the cervical loop in areas of cell contact, and weakly in the center of the mouse tooth bud and neighboring stratum intermedium, suggesting a possible role of podoplanin in tooth development (11). On the other hand, a study of basal cell extension in the gingival epithelium has suggested that the former possesses characteristics of odontogenic epithelium (19). A previous study conducted in our laboratory showed positivity for podoplanin in basal cell extensions, which are frequently observed in gingival epithelium, but not in other types of oral epithelium (20). AM is believed to arise from epithelial cells of the developing tooth, including cells of the dental lamina and enamel organ and the differentiation level of AM cells remains at the cap/bell stage of tooth development (4).

The present study was designed to investigate the expression and distribution of cells expressing podoplanin in human AM tissues in an attempt to evaluate its role in tumor progression. We also examined the presence of the epithelial marker E-cadherin and the mesenchymal marker vimentin to investigate whether EMT is present in AMs, and how it might be associated with podoplanin expression.

Materials and methods
Paraffin-embedded tissue specimens of 38 AMs were examined immunohistochemically using antibodies against podoplanin, E-cadherin, and vimentin. The study protocol was reviewed and approved by the Research Ethics Committee of Meikai University Graduate School of Dentistry (A0832).

Tissue preparation
Specimens were collected from the archives of the Department of Diagnostic and Therapeutic Sciences, Division of Pathology. The paraffin-embedded tissue blocks were sliced into 4-μm thick sections for subsequent histologic examinations. The tissue sections were stained with hematoxylin and eosin for histologic diagnosis according to the WHO histologic classification of odontogenic tumors (2). The tumors comprised 38 AMs, which included nine follicular, 19 plexiform, and three unicystic types, and seven cases of the acanthomatous subtype. To compare the expression pattern of podoplanin protein with non-tumorous odontogenic cysts, 15 cases of dentigerous cyst were included. Data on patient age, gender, and lesion site were obtained from information submitted with the surgery request forms.

Immunohistochemistry
The serial sections were deparaffinized, and immersed in methanol with 0.3% (v/v) hydrogen peroxide for 15 min at room temperature to block endogenous peroxidase activity. After washing with running water and phosphate-buffered saline (PBS, pH 7.4), the sections were heated in a microwave oven while immersed in 0.01 M citrate buffer (pH 6.0) for 5 min at high power and then for 15 min at low power (for D2-40 and E-cadherin antigen retrieval). All sections were then incubated in 2% (w/v) bovine serum albumin (BSA) to block non-specific reactions. Appropriately diluted monoclonal antibody against podoplanin (D2-40; Dako North America, Inc., Carpinteria, CA, USA; supernatant, 1:50), polyclonal anti-E-cadherin (H-108; Santa Cruz Biotechnology; Santa Cruz, CA, USA; supernatant, 1:50), and monoclonal anti-vimentin (SP20; Nichirei Bio, Inc., Tokyo Japan; pre-diluted) were applied to each section for 1 h at room temperature. The sections were then incubated with peroxidase-labeled dextran polymer, Simple Stain MAX-PO (Nichirei Bio, Inc.) for 30 min, and the reaction products were visualized by immersing the sections in freshly prepared 0.03% diaminobenzidine solution containing 2 mM hydrogen peroxide for 6–8 min. Nuclei were lightly stained with Mayer’s hematoxylin. For control studies of the antibodies, the serial sections were treated with 2% BSA–PBS instead of the primary antibodies, and were confirmed to be unstained. Immunohistochemical reactivity for podoplanin was evaluated and classified into two groups: (+) positive, (+) positive in epithelial or neoplastic cells. The statistical significance of differences in the percentage of cases with different reactivity levels was analyzed by the Mann–Whitney U-test. Values of P < 0.01 were considered to indicate statistical significance.

Results
Specimens from 38 patients diagnosed as having AM at our department were selected. The patients ranged in
age from 6 to 76 years, with a mean of 34.9 years. The most common location was the mandibular molar-ramus in 17 cases, followed by the mandibular molar in eight cases, mandibular incisor-molar in eight cases, maxillary molar in three cases and mandibular incisor and maxillary incisor-molar in one case each.

Immunohistochemical reactivity for podoplanin, E-cadherin, and vimentin in the AMs is summarized in Table 1. Expression of podoplanin in follicular and plexiform AMs was variable; overall, positivity for podoplanin was often observed on the cell membrane, at cell–cell boundaries, and in the cytoplasm of peripheral columnar cells, and was slight or negative in central stellate reticulum-like cells (Figs 1A and 2A). Three of the AMs, two follicular and one plexiform, including one of the acanthomatous subtype, were negative for podoplanin. No difference in reaction was seen between the two patterns (Figs 1A and 2A). The unicystic AMs showed a reaction in the basal layer of the cystic lining, and no reaction in the upper layers (Fig. 3A). In the acanthomatous variant, expression of podoplanin was lost in keratinized areas (Fig. 3D).

In the case of dentigerous cyst, podoplanin protein was negative in 60% of the cases (nine cysts), but some areas with inflammatory reaction were weakly positive (six cysts, 40%) in the basal layer of the lining epithelium in all cases (Fig. 3F). The level of immunohistochemical reactivity for podoplanin in AMs was significantly higher than that in dentigerous cyst (P < 0.01).

Although positivity for E-cadherin was usually found on the membrane at cell–cell boundaries of central angular or stellate reticulum-like cells, slight or decreased reactivity was evident at cell–cell boundaries and in the cytoplasm of peripheral columnar cells (Figs 1B and 2B). A strong reaction in the upper layer of the cystic lining, and decreased expression in the basal layer were observed in the unicystic variant (Fig. 3B). Expression of the molecule was weak or lost in the keratinized areas of acanthomatous AMs (Fig. 3E). In addition, tumor nests were evident within large samples that did not show expression of E-cadherin.

In the case of dentigerous cyst, E-cadherin protein was usually found on the membrane at cell–cell boundaries of central angular or stellate reticulum-like cells, slight or decreased reactivity was evident at cell–cell boundaries and in the cytoplasm of peripheral columnar cells through the invasive front of the tumor nests. Positivity for vimentin was not evident within the tumor cell, or in stromal cell in all cases of dentigerous cyst.

### Discussion

Invasion and metastasis are not unique to cancer, as they also occur during embryonic development, in healthy organs, and in many non-cancerous diseases. The term invasion indicates penetration into neighboring territories and their occupation; consequently invasion and metastasis are major prognosis markers (21). The glycoprotein podoplanin is well established as a lymphatic-specific marker that is widely used in histopathology, and has been reported to be associated with tumor-induced platelet aggregation and tumor metastasis (14). In addition, enhanced podoplanin expression has been demonstrated in squamous cell carcinoma of the lung, uterine cervix, and head and neck, oral leukoplaikia, and pleomorphic adenoma (12, 17, 18, 22).

Primary tumors of head and neck squamous cell carcinoma generally show heterogeneous expression of podoplanin with two patterns of staining: diffuse expression in most living tumor cells, and focal expression at the proliferating periphery of tumor cell nests, with no expression in the central areas (13). Podoplanin is also highly expressed in some hyperplastic and dysplastic lesions adjacent to primary tumors, which could imply its early overexpression in head and neck tumorigenesis (13). In the present study, a similar pattern was observed in AMs, with predominantly heterogeneous expression on the surfaces of peripheral columnar cells, and decreased or absent expression on...
stellate reticulum-like central cells. There was weak or no expression in keratinized areas of the acanthomatous subtype of AM. Interestingly, large tumors showed decreased expression of podoplanin in some peripheral

Figure 1 Immunohistochemical reactivity for podoplanin (A), E-cadherin (B), and vimentin (C), in a plexiform ameloblastoma. (A) Expression of podoplanin is evident in peripheral cuboidal neoplastic cells. Some stellate reticulum-like cells are also positive (10×). (B) E-cadherin is expressed strongly on the surfaces of central stellate reticulum-like cells, and slightly at the cell-cell boundaries of peripheral columnar cells (10×). (C) Vimentin is not expressed in tumor cells, but some stromal cells show strong reactivity for the molecule (10×).

Figure 2 Immunohistochemical reactivity for podoplanin (A), E-cadherin (B), and vimentin (C), in a follicular ameloblastoma. (A) Expression of podoplanin is exclusively evident in peripheral cuboidal neoplastic cells of tumor nests (4×). (B) E-cadherin is expressed on the surfaces of central stellate reticulum-like cells, and slightly at the cell-cell boundaries of peripheral columnar cells (4×). (C) Vimentin is not expressed in tumor cells, but some stromal cells show a strong immunoreaction (4×).
cells. The present study is the first to have demonstrated the expression of podoplanin in AMs.

In the case of dentigerous cysts, immunoreaction for podoplanin was monotonous and weak in some areas in the basal layer of the lining epithelium; while lymphatic vessels were positive for the antibody in the same lesions. Interestingly, areas with inflammatory reaction showed increased expression of podoplanin in the basal layer compared with areas where inflammatory changes were absent. In this study, immunoreactivity for

![Figure 3](image-url)
podoplanin in dentigerous cyst was significantly lower than that in AMs.

The particular localization of podoplanin expression raises the question of whether factors secreted from stromal cells might induce podoplanin expression. A previous study has demonstrated that epidermal growth factor (EGF), fibroblast growth factor 2, and transforming growth factor-beta (TGF-β) can upregulate the expression of podoplanin in MCF7 cells (17), raising the possibility that stroma-derived growth factors may induce specific expression of podoplanin.

In the invading tumor front, EGF receptor (EGFR) expression in AMs is higher than that in epithelial elements of radicular cysts and granulomas (3), and TGF-β and its receptors (types I and II) are also expressed in AM, suggesting that they play an important role in cell differentiation and matrix formation via regulation or dysregulation of epithelial–mesenchymal interactions (23, 24). Histologically, podoplanin-positive cells were located specifically in the basal region of AM tumor nests, close to the surrounding stromal cells; considering their localization, it is reasonable to speculate that the stromal microenvironment contributes to regulation of the invasion of AM tumor cells.

We found that AMs retained their expression of E-cadherin in the central areas of tumor nests, although there was decreased expression in areas of cell–cell contact at the periphery; these findings are in line with previous studies (25). We observed a decrease or loss of E-cadherin in keratinized areas of acanthomatous AM, which might have been caused by terminal differentiation of the tumor cells, rather than tumor progression or malignant potential (25). Moreover, we were able to identify tumor nests within large tumors that had lost E-cadherin expression. Given the remarkable complexity of E-cadherin regulation, it would be difficult to think of a single model that could account for the loss of E-cadherin function, whereas it seems more likely that a combination of genetic, epigenetic, transcriptional, and post-transcriptional mechanisms all cooperate to weaken E-cadherin-dependent cell–cell adhesion, and thus promote invasion (8).

A role of podoplanin in invasion and metastasis has been suggested. This hypothesis is based mainly on the observation that high expression of podoplanin is consistently correlated with the presence of metastasis (16). In addition, podoplanin increases the migration of MCF7 cells and HaCaT keratinocytes associated with downregulation of E-cadherin expression; these findings suggest that podoplanin does not suppress the cadherin switch and can mediate tumor invasion by an alternative pathway (6, 17). Podoplanin might favor tumor invasion through its ability to remodel actin in the cytoskeleton of tumor cells, contributing to their increased motility; this association, the podoplanin–cytoskeleton, seems to be mediated by ezrin, which is markedly phosphorylated in the presence of podoplanin overexpression (7, 26). In addition, human podoplanin expressed in immortal HaCaT keratinocytes fails to trigger a complete EMT but induces scattering associated with increased plasma membrane motility and reduced cell–cell cohesion (17).

These findings indicate that podoplanin-induced RhoA activation in epithelial cells precedes EMT rather than being a consequence of it (7).

Ameloblastomas are characterized by benign but locally invasive behavior with a high rate of recurrence (2, 25, 27, 28). One important class of molecules within the invasive tumor front is the matrix metalloproteinases (MMPs). MMPs can be secreted by neighboring cells and can become localized and activated on the surface of migrating endothelial cells (29). AMs express MMPs -1, -2, and -9, which degrade the ECM and basement membrane components, and their inhibitors – tissue inhibitors of metalloproteinases (TIMPs) -1 and -2 – have also been recognized in AMs (3, 27, 28). Gene profiling studies of AMs have demonstrated overexpression of MMPs, suggesting that gap-junction communications may be sparse, and that cell adhesion is lost in AMs, as has been described for many types of neoplasia. Such alterations in the cell membrane environment could also increase the locally aggressive growth of AMs (4, 28).

Wicki and Christofori (6) showed that invasion of podoplanin-expressing tumor cells was correlated with overexpression of MMPs, and that this could be repressed by specific inhibitors of MMPs. Podoplanin has also been found to promote tumor cell invasion by inducing collective cell migration via downregulation of the activities of small Rho family GTPases (30); however, its particular pattern of staining restricted to the basal layers suggests that expression of podoplanin alone may not be sufficient to promote tumorigenesis (12).

Our results have revealed podoplanin expression in the benign odontogenic tumor, AM, showing that podoplanin expression is not restricted to specific types of malignancy, such as testicular germ-cell tumor and peritoneal mesothelioma, nor to specific histologic types of tumor such as squamous cell carcinoma. Moreover, it is evident that podoplanin would be applicable as a marker for classification of odontogenic tumors. Of interest was that podoplanin-positive cells were observed in the peripheral layer of tumor nests, but were absent in keratinized areas of the acanthomatous AM subtype, probably because of terminal differentiation of the tumor cells resulting from maturation and/or degenerative changes. Based on these concepts, podoplanin may be a useful marker of cells with the capacity for further maturation (22).

The large amount of accumulated data on the expression of podoplanin indicates that it may not only function as a specific diagnostic marker for some malignancies, but may also be associated with tumor progression in diverse types of human cancer (6, 16).

In conclusion, the pattern of staining for podoplanin observed in AMs, the expression of the epithelial marker E-cadherin, and the slight or lack of expression of the mesenchymal marker vimentin in tumor cells suggest a role of podoplanin in tumor invasiveness through collective cell migration in which the cadherin switch or EMT may not be involved. In addition, the specific staining of podoplanin in AM could be a useful tool for
classification of odontogenic tumors. Further investigation of more cases should be carried out in an attempt to determine the role of podoplanin in AMs, as well as in other odontogenic tumors.

Conflicts of interest

The author(s) indicate no potential conflicts of interest.

References