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Immunohistochemical detection of MT1-MMP, RECK, and EMMPRIN in ameloblastic tumors

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BACKGROUND: To evaluate the roles of matrix-degrading proteinase regulators in progression of odontogenic tumors, expression of membrane-bound matrix metalloproteinase (MMP) MT1-MMP, MMP inhibitor RECK and MMP inducer EMMPRIN was analyzed in ameloblastic tumors as well as in tooth germs.

METHODS: Tissue specimens of 11 tooth germs, 40 ameloblastomas, and five malignant ameloblastic tumors were examined immunohistochemically with the use of antibodies against MT1-MMP, RECK, and EMMPRIN.

RESULTS: Immunohistochemical reactivity for MT1-MMP, RECK and EMMPRIN was detected predominantly in odontogenic epithelial cells near the basement membrane in tooth germs and benign and malignant ameloblastic tumors. The level of immunoreactivity for MT1-MMP was slightly higher in benign and malignant ameloblastic tumors than in tooth germs. RECK expression was lower in ameloblastomas than in tooth germs. Follicular ameloblastomas showed significantly lower expression of RECK than plexiform ameloblastomas, and immunoreactivity for RECK in acanthomatous ameloblastomas was slightly lower than that in other cellular variants.

CONCLUSION: Expression of MT1-MMP, RECK and EMMPRIN in tooth germs and ameloblastic tumors suggests that these normal and neoplastic epithelial components control MMP-dependent extracellular matrix (ECM) degradation during tooth development and tumor progression via epithelial-mesenchymal interactions.

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Introduction

Tumors arising from epithelium of the odontogenic apparatus or from its derivatives or remnants exhibit considerable histological variation and are classified into several benign and malignant entities (1, 2). Ameloblastoma is the most frequently encountered tumor arising from odontogenic epithelium and is characterized by a benign but locally invasive behavior with a high risk of recurrence (1, 2). Histologically, ameloblastoma shows considerable variation, including follicular, plexiform, acanthomatous, granular cell, basal cell and desmoplastic variants (2). Malignant counterparts of ameloblastoma are classified into metastasizing ameloblastoma and ameloblastic carcinoma on the basis of metastatic spread and cytological malignant features (2). Recent studies have identified genetic and molecular alterations in these epithelial odontogenic tumors (3–5); however, the detailed mechanisms of oncogenesis, cytodifferentiation and tumor progression remain unknown.

Matrix metalloproteinases (MMPs) are a family of zinc- and calcium-dependent proteolytic enzymes that degrade extracellular matrix (ECM) macromolecules, such as collagens, gelatins, fibronectin, tenascin and laminin, at physiological pH (6). More than 20 different members are currently known and are classified according to their substrate specificity and sequence homology into five subfamilies: collagenases (MMP-1, -8 and -13), gelatinases/type IV collagenases (MMP-2 and -9), stromelysins (MMP-3 and -10), membrane-type MMPs (MMP-14, -15, -16, -17, -24 and -25), and other MMPs (MMP-7, -11, -12, -19, -20 and others) (6, 7). Most MMPs are secreted as inactive proenzymes with a prodomain and are then activated by proteolytic cleavage of the prodomain (7, 8). Under physiologic conditions, these enzymes play central roles in ECM regulation during embryonic development and tissue remodeling (9, 10). Additionally, they participate in the destruction of ECM associated with inflammatory processes, such as rheumatoid arthritis and periodontal disease (11, 12), as well as ECM destruction associated with tumor invasion and metastasis (6, 13, 14). MT1-MMP (membrane type 1-matrix metalloproteinase; MMP-14) is a

membrane-bound MMP that can degrade several components of the ECM, including collagens, laminin and fibronectin (15, 16). This molecule also participates in the activation of MMP-2 through formation of a trimolecular complex with tissue inhibitor of metalloproteinases (TIMP)-2 and pro-MMP-2 at the cell surface (15, 17).

The proteolytic activity of MMPs is suppressed by various endogenous inhibitors, α-macroglobulin, TIMPs and RECK (reversion-inducing cysteine-rich protein with Kazal motifs) (7, 18). RECK is a membrane-anchored MMP inhibitor containing serine-protease inhibitor-like domains and negatively regulates at least three members of the MMP family, MMP-2, -9 and MT1-MMP (18, 19). In addition to regulation by the proteolytic activation of proenzymes, such as MT-1-MMP and by the inhibitory effect of endogenous inhibitors, such as TIMPs and RECK, MMPs are also regulated transcriptionally as a result of the action of various cytokines, growth factors, hormones, and other mediators (6, 7). EMMPRIN (ECM metalloproteinase inducer) is a cell surface MMP inducer belonging to the immunoglobulin superfamily and stimulates nearby fibroblasts to produce MMP-1, -2, -3 and -9 (7, 20). These cell membrane-associated MMP mediators, MT1-MMP, RECK and EMMPRIN, contribute to the extent of ECM degradation in physiological and pathological conditions (7, 18).

Previous studies have confirmed the presence of MMP-1, -2 and -9, and TIMP-1 and -2 in ameloblastomas, suggesting that these matrix-degrading proteinases contribute to the local invasiveness of odontogenic tumors (21, 22). In the present study, immunohistochemical expression of MT1-MMP, RECK and EMMPRIN was examined in benign and malignant ameloblastic tumors as well as in tooth germs to evaluate the role of these cell membrane-associated MMP regulators in the progression of epithelial odontogenic tumors.

Materials and methods

Tissue preparation

Specimens were surgically removed from 45 patients with epithelial odontogenic tumors at the Department of Oral and Maxillofacial Surgery, Tohoku University Dental Hospital and affiliated hospitals. The specimens were fixed in 10% buffered formalin for 1 to several days and were embedded in paraffin. The tissue blocks were sliced into 3-μm-thick sections for routine histological and subsequent immunohistochemical examinations. Tissue sections were stained with hematoxylin and eosin for histological diagnosis according to the World Health Organization histological classification of odontogenic tumors (2). The tumors comprised 40 ameloblastomas and five malignant ameloblastic tumors. Ameloblastomas were divided into 23 follicular and 17 plexiform types, including 10 acanthomatous, six granular cell, three basal cell, and four desmoplastic subtypes. Malignant ameloblastic tumors were classified into two metastasizing ameloblastomas and three ameloblastic carcinomas. Specimens of 11 tooth germs of the mandibular third molars, enucleated for orthodontic reasons at the initial stage of crown mineralization, were similarly prepared and compared with the epithelial odontogenic tumors.

Immunohistochemistry

The serial sections were deparaffinized and immersed in methanol with 0.3% hydrogen peroxide. Sections for MT1-MMP and EMMPRIN immunostaining were heated in 1 mM ethylenediamine tetraacetic acid (EDTA) buffer (pH 8.0) for 10 min by autoclave (121°C, 2 atm). Then, the sections were incubated with primary antibodies at 4°C overnight. The applied antibodies were mouse anti-MT1-MMP monoclonal antibody (Fuji, Takaoka, Japan; subclass IgG1; diluted at 1:15), goat anti-RECK polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; diluted at 1:40), and rabbit anti-EMMPRIN polyclonal antibody (Zymed, San Francisco, CA, USA; prediluted). The sections were allowed to react with peroxidase-conjugated anti-mouse IgG (for MT1-MMP), anti-goat IgG (for RECK), or anti-rabbit IgG (for EMMPRIN) polyclonal antibody (Histofine Simple Stain MAX-PO; Nichirei, Tokyo, Japan) for 45 min and reaction products were visualized by immersing the sections in 0.03% diaminobenzidine solution containing 2 mM hydrogen peroxide for 2–3 min. Nuclei were lightly stained with methylgreen. For control studies of the antibodies, the serial sections were treated with phosphate-buffered saline, mouse anti-OPD4 (CD45RO) monoclonal antibody (subclass IgG1; Dako, Glostrup, Denmark), and normal goat and rabbit IgG instead of the primary antibodies and were confirmed to be unstained.

Immunohistochemical reactivity for MT1-MMP, RECK and EMMPRIN was evaluated and classified into three groups: (−) negative, (+) positive and (++) strongly positive. The statistical significance of differences in the percentages of cases with different reactivity levels was analyzed by the Mann–Whitney *U*-test for differences between two groups or the Kruskal–Wallis test for differences among three or more groups. *P*-values < 0.05 were considered to indicate statistical significance.

Results

Immunohistochemical reactivity for MT1-MMP, RECK and EMMPRIN in tooth germs and ameloblastomas is summarized in Table 1. Expression of MT1-MMP was detected in the cell membrane and cytoplasm of cellular components in normal and neoplastic odontogenic tissues (Fig. 1). In tooth germs, MT1-MMP expression was strong in inner and outer enamel epithelium and dental lamina and weak in stratum intermedium and stellate reticulum (Fig. 1a). Some endothelial cells in dental papillae and dental follicles were also weakly reactive. Benign and metastasizing ameloblastomas showed strong MT1-MMP reactivity in peripheral columnar or cuboidal cells and weak reactivity in central polyhedral cells

Table 1 Immunohistochemical reactivity for MT1-MMP, RECK and EMMPRIN in tooth germs and ameloblastic tumors

		MT1-MMP		*RECK*			*EMMPRIN*	
	n	+	++	−	+	++	+	++
Tooth germ	11	6 (55)	5 (45)	0 (0)	3 (27)	8 (73)	3 (27)	8 (73)
Ameloblastoma	40	10 (25)	30 (75)	6 (15)	22 (55)	12 (30) *	7 (18)	33 (82)
Follicular type	23	6 (26)	17 (74)	5 (22)	13 (56)	5 (22)	5 (22)	18 (78)
Plexiform type	17	4 (24)	13 (76)	1 (6)	9 (53)	7 (41) *	2 (12)	15 (88)
Acanthomatous subtype	10	4 (40)	6 (60)	5 (50)	4 (40)	1 (10)	3 (30)	7 (70)
Granular subtype	6	1 (17)	5 (83)	0 (0)	3 (50)	3 (50) *	1 (17)	5 (83)
Basal cell subtype	3	2 (67)	1 (33)	0 (0)	1 (33)	2 (67)	1 (33)	2 (67)
Desmoplastic subtype	4	1 (25)	3 (75)	0 (0)	4 (100)	0 (0)	1 (25)	3 (75)
Non-cellular variation	17	2 (12)	15 (88)	1 (6)	10 (59)	6 (35)	1 (6)	16 (94)
Odontogenic carcinomas	5	0 (0)	5 (100)	0 (0)	2 (40)	3 (60)	1 (20)	4 (80)
Metastasizing ameloblastoma	2	0 (0)	2 (100)	0 (0)	1 (50)	1 (50)	0 (0)	2 (100)
Ameloblastic carcinoma	3	0 (0)	3 (100)	0 (0)	1 (33)	2 (67)	1 (33)	2 (67)

Immunohistochemical reactivity: −, negative; +, positive; ++, strongly positive.
Values given in parentheses are percentage values.
Statistical significance: $*P < 0.05$.

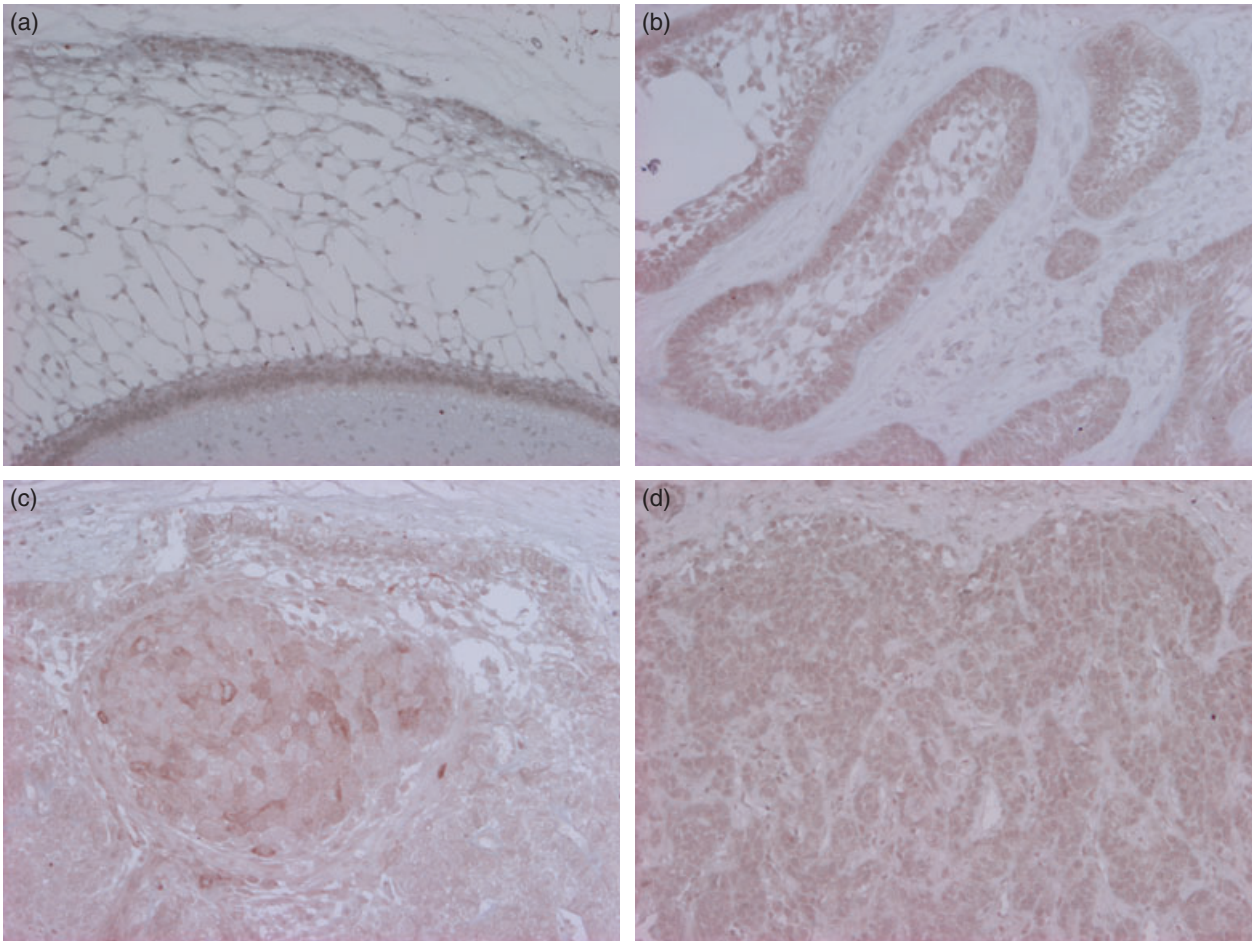

Figure 1 Immunohistochemical reactivity for MT1-MMP. (a) Tooth germ showing strong reactivity in inner and outer enamel epithelium and weak reactivity in stratum intermedium and stellate reticulum (×100). (b) Follicular ameloblastoma showing strong reactivity in peripheral columnar cells and weak reactivity in central polyhedral cells (×115). (c) Granular cell ameloblastoma showing reactivity in some granular cells as well as in other neoplastic cells (×110). (d) Ameloblastic carcinoma showing reactivity in most neoplastic cells (×115).

(Fig. 1b). No or little MT1-MMP reactivity was found in keratinizing cells of acanthomatous ameloblastomas and granular cell ameloblastomas exhibited MT1-MMP expression in some granular cells (Fig. 1c). Basal and desmoplastic ameloblastomas and ameloblastic carcinomas showed MT1-MMP expression in

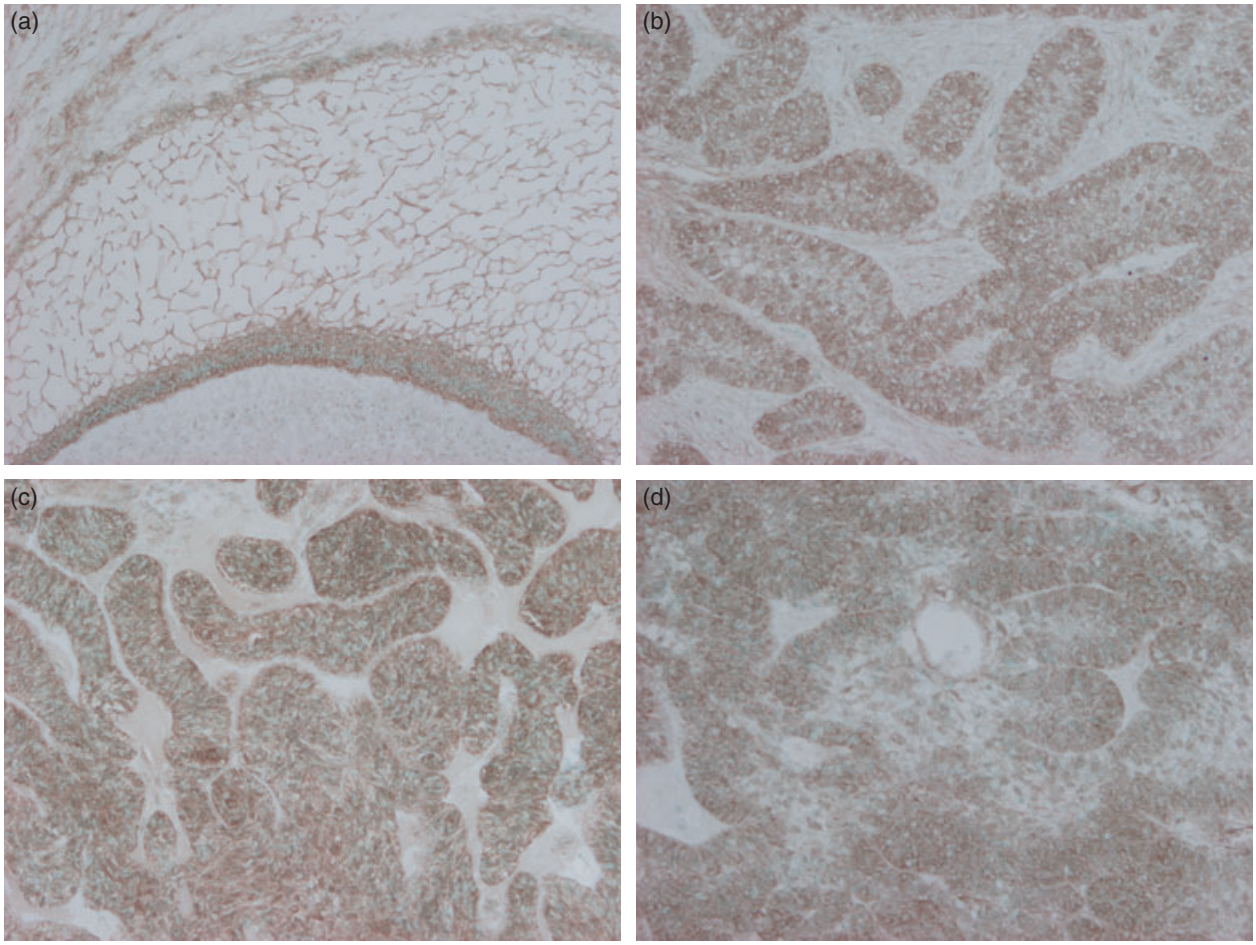

Figure 2 Immunohistochemical reactivity for RECK. (a) Tooth germ showing strong reactivity in inner enamel epithelium and stratum intermedium and weak reactivity in outer enamel epithelium and stellate reticulum (×75). (b) Follicular ameloblastoma showing reactivity in many peripheral columnar cells and some central polyhedral cells (×125). (c) Basal cell ameloblastoma showing reactivity in most neoplastic cells (×110). (d) Metastasizing ameloblastoma showing reactivity in many peripheral columnar cells and some central polyhedral cells (×125).

most neoplastic cells (Fig. 1d). In these epithelial odontogenic tumors, some stromal endothelial cells had weak MT1-MMP reactivity.

Immunoreactivity for RECK was detected in the cell membrane and cytoplasm of cellular components in normal and neoplastic odontogenic tissues (Fig. 2). In tooth germs, RECK was expressed strongly in inner enamel epithelium and stratum intermedium and weakly in outer enamel epithelium, stellate reticulum and dental lamina (Fig. 2a). Some endothelial cells in dental papillae and dental follicles were also weakly reactive. In 34 ameloblastomas and two metastasizing ameloblastomas, RECK reactivity was detected in many peripheral columnar or cuboidal cells and some central polyhedral cells (Fig. 2b, d). The level of immunohistochemical reactivity for RECK was significantly lower in ameloblastomas than in tooth germs ($P < 0.05$; Table 1). Five follicular ameloblastomas and one plexiform ameloblastoma showed no RECK expression and the level of RECK immunoreactivity was significantly lower in follicular ameloblastomas than in plexiform ameloblastomas ($P < 0.05$; Table 1). Basal and desmoplastic ameloblastomas and ameloblastic carcinomas showed RECK expression in most neoplastic cells (Fig. 2c). In these epithelial odontogenic tumors, some stromal endothelial cells showed weak RECK reactivity.

The EMMPRIN immunoreactivity was detected on the cell membrane of normal and neoplastic odontogenic epithelial cells (Fig. 3). In tooth germs, EMMPRIN was expressed in inner enamel epithelium and stratum intermedium (Fig. 3a). Benign and metastasizing ameloblastomas showed EMMPRIN reactivity in peripheral columnar or cuboidal cells (Fig. 3b). Keratinizing cells in acanthomatous ameloblastomas showed no EMMPRIN expression, whereas granular cells in granular cell ameloblastomas were reactive with EMMPRIN (Fig. 3c). Basal and desmoplastic and ameloblastic carcinomas showed EMMPRIN expression in most neoplastic cells (Fig. 3d).

Discussion

Extracellular matrix provides an essential framework upon which cells grow, migrate and differentiate, and it's essential that ECM undergoes remodeling during

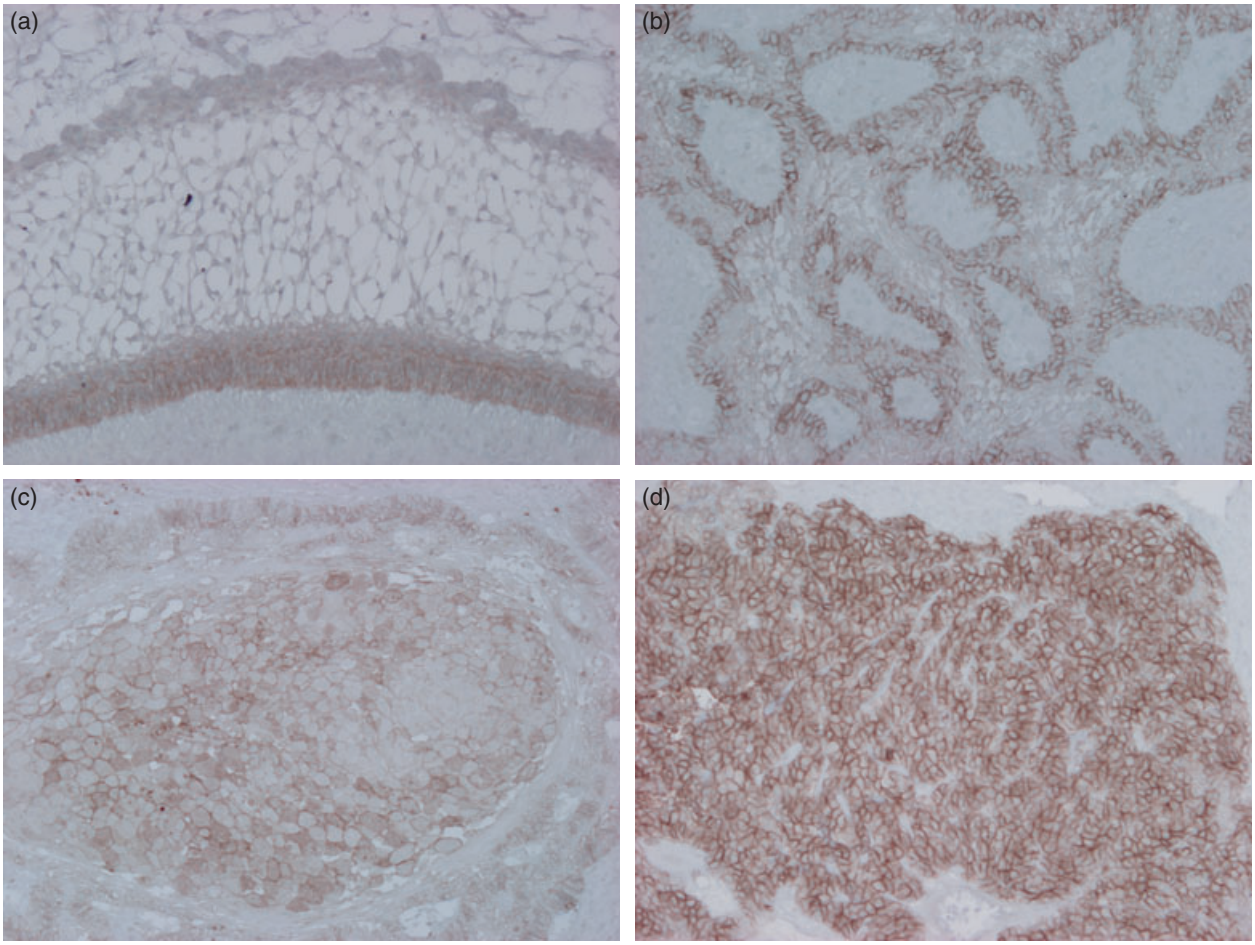

Figure 3 Immunohistochemical reactivity for EMMPRIN. (a) Tooth germ showing reactivity in inner enamel epithelium and stratum intermedium and weak reactivity in outer enamel epithelium and stellate reticulum (×105). (b) Plexiform ameloblastoma showing reactivity in peripheral neoplastic cells (×115). (c) Granular cell ameloblastoma showing reactivity in granular cells as well as in peripheral columnar cells (×95). (d) Ameloblastic carcinoma showing reactivity in most neoplastic cells (×115).

developmental processes (9, 10). MMPs function in cell-ECM interactions during the development of various organs and tissues (10, 23). MT1-MMP is strongly expressed in embryonic skeletal and periskeletal tissue (24, 25), and *MT1-MMP*-deficient mice cause severe defects in skeletal development and angiogenesis because of inadequate collagen turnover, leading to death by 3 weeks of age (26, 27). Mice lacking RECK gene die in utero with disorganized mesenchymal tissue architecture and defective vascular development (28). Expression of MMP-1, -2, -3, -9 and MT1-MMP have been detected temporally and spatially during tooth development, indicating that these MMPs have roles in tooth morphogenesis and eruption (23, 29–33). In the present study, immunoreactivity for MT1-MMP, RECK and EMMPRIN was recognized chiefly in epithelial components of tooth germs, suggesting that these membrane-associated molecules are involved in ECM degradation and physiological epithelial cell migration during tooth development by controlling MMP activities.

The MT1-MMP was originally identified as a specific MMP-2 activator on the surface of invasive neoplastic cells (15). Overexpression of MT1-MMP in neoplastic cells as well as in stromal cells has been demonstrated in a variety of malignancies, including lung, colorectal, head and neck, and breast carcinomas, and the expression of MT1-MMP has been correlated with the activity of MMP-2, suggesting that MT1-MMP is involved in carcinoma invasion (34, 35). Interestingly, the expression and activities of MMP-2, -9, and MT1-MMP in prostate carcinoma cells present in bone are apparently related to bone matrix turnover and metastatic tumor growth (36). Increased expression of MMP-2, -9 and MT1-MMP has been associated with tumor angiogenesis and malignant potential in malignant glioma and melanoma (37, 38). In the present study, immunoreactivity for MT1-MMP in ameloblastomas and malignant ameloblastic tumors was more obvious in neoplastic cells rather than in stromal cells. Our previous study revealed that ameloblastomas expressed MMP-2 and TIMP-2, which form complexes with MT1-MMP, predominantly in stromal cells (21). These features suggest that MT1-MMP contribute to neoplastic cell progression via epithelial-mesenchymal interactions. In this study, the expression levels of MT1-MMP in benign and malignant ameloblastic tumors were slightly higher

than that in tooth germs, suggesting that this MMP-2 activator might play a role in tumorigenesis or malignant transformation of odontogenic epithelium.

The gene of the only known membrane-bound MMP inhibitor, *RECK*, was isolated by cDNA expression cloning as a gene inducing morphological reversion in a mouse fibroblast cell line transformed by activated *Ras* oncogene (39). Subsequently, not only *Ras* but also several other oncogenes, including *Myc*, *Src* and *Fos*, have been shown to downregulate *RECK* gene (18). Reduced expression of RECK has been demonstrated in many types of human tumors, including hepatocellular, pancreatic, colorectal and breast carcinomas, and a positive correlation between RECK expression and survival of patients has been noted (40–42). Restoration of RECK expression in malignant cells suppresses their invasive, metastatic and angiogenic activities (18, 40, 41). Our previous study demonstrated that the expression of TIMP-1 and -2 in ameloblastomas is higher in stromal components than in neoplastic cells (21). In the present study, RECK expression in ameloblastomas and malignant ameloblastic tumors was predominantly found in neoplastic cells, suggesting that this molecule controls the activities of MMP-2, -9 and MT1-MMP in these epithelial odontogenic tumors (21, 22). However, ameloblastomas showed decreased RECK expression as compared with tooth germ tissues, suggesting that downregulation of this MMP inhibitor participates in tumor development and progression. In this study, follicular ameloblastomas showed significantly lower RECK expression than plexiform ameloblastomas and immunoreactivity in acanthomatous ameloblastomas was slightly lower than that in other cellular variants. These features suggest that RECK expression in ameloblastomas might contribute to tissue structuring and cell differentiation of the epithelial odontogenic tumors.

The EMMPRIN, also known as CD147, tumor collagenase stimulatory factor, M6 antigen, basigin and neurothelin, was initially characterized as a factor on the surface of neoplastic cells that induces MMP production in fibroblasts (7, 20, 43). Upregulation of EMMPRIN has been demonstrated in lung, bladder and breast carcinomas compared with their normal counterparts, suggesting that EMMPRIN may play a role in promoting MMP-dependent tumor aggression (44, 45). The expression and distribution of EMMPRIN in rheumatoid arthritis and giant cell tumor of bone indicate that this MMP mediator is associated with the development and progression of these bone lesions (46, 47). EMMPRIN expression has been detected in some ameloblastomas, indicating a collagenase-stimulating effect (48). In the present study, immunoreactivity for EMMPRIN in ameloblastomas and malignant ameloblastic tumors was recognized in neoplastic cells, suggesting that EMMPRIN might participate in tumor cell progression of these epithelial odontogenic tumors by inducing MMP in stromal cells. No distinctive differences in EMMPRIN immunoreactivity were found among tumor types or subtypes.

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