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Immunohistochemical detection of phosphorylated JNK, p38 MAPK, and ERK5 in ameloblastic tumors

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BACKGROUND: To evaluate roles of mitogen-activated protein kinases (MAPKs) in oncogenesis and cytodifferentiation of odontogenic tumors, expression of phosphorylated JNK (p-JNK), p38 MAPK (p-p38 MAPK), and ERK5 (p-ERK5) was analyzed in ameloblastic tumors as well as in tooth germs.

METHODS: Ten tooth germs, 47 ameloblastomas, and 5 malignant ameloblastic tumors were examined immunohistochemically with the antibodies against p-JNK, p-p38 MAPK, and p-ERK5.

RESULTS: Immunoreactivity for p-JNK was detected in epithelial or neoplastic cells detached from the basement membrane in 7 tooth germs and 7 ameloblastomas, and the expression levels of p-JNK in ameloblastic tumors were significantly lower than that in tooth germs. Expression of p-p38 MAPK was found in epithelial or neoplastic cells in tooth germs and ameloblastic tumors except for two ameloblastomas, and increased expression was found in keratinizing cells of acanthomatous ameloblastomas. The expression level of p-p38 MAPK in ameloblastomas was significantly higher than the levels in tooth germs and malignant ameloblastic tumors. Immunoreactivity for p-ERK5 was found predominantly in epithelial or neoplastic cells near the basement membrane in tooth germs and ameloblastic tumors. The expression levels of p-ERK5 in ameloblastic tumors were slightly higher than that in tooth germs, and plexiform ameloblastomas showed significantly higher p-ERK5 expression than follicular ameloblastomas.

CONCLUSION: Expression of p-JNK, p-p38 MAPK, and p-ERK5 in tooth germs and ameloblastic tumors suggests that these MAPK signaling pathways contribute to cell proliferation, differentiation, or apoptosis in both normal and neoplastic odontogenic tissues. Altered expression of these phosphorylated MAPKs in ameloblastic tumors may be involved in oncogenesis and tumor cell differentiation.

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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, 4); however, the detailed mechanisms of oncogenesis, cytodifferentiation, and tumor progression remain unknown.

Mitogen-activated protein kinases (MAPKs) are components of signal transduction pathways activated by diverse extracellular stimuli, and four MAPK family molecules have been identified in mammals: ERK1/2 (extracellular signal-regulated kinase 1/2), JNK1–3 (c-Jun NH_2-terminal kinase 1–3), p38 MAPK (p38 MAPKα, β, γ, and δ), and ERK5 (5). These molecules are serine/threonine kinases modulated by the MAPK cascades, sequential activation pathways comprising MAPK kinase kinases (MAPKKKs), MAPK kinases (MAPKKs), and MAPKs, and sequential phosphorylation and activation of these components induce cell proliferation, differentiation, or apoptosis (5, 6). JNK, also known as stress-activated protein kinase (SAPK), was identified as a kinase that was activated by cell stresses and phosphorylated c-Jun at NH_2-terminal activating sites (7). p38 MAPK was originally described as a peptide that underwent phosphorylation in response to endotoxin treatment and osmotic shock (8). JNK and p38 MAPK are activated by environmental

stresses and inflammatory cytokines, and phosphorylated JNK (p-JNK) and p38 MAPK (p-p38 MAPK) are localized in the nucleus, controlling apoptotic cell death (5, 9). ERK5, also known as BMK1 (big MAPK 1), was cloned as a component of new MAPK pathways by a two-hybrid screen with an upstream activator (10). ERK5 is activated by various mitogenic stimuli, and activated and phosphorylated ERK5 (p-ERK5) induces its translocation from the cytoplasm to nucleus, leading to cell proliferation and differentiation (5, 11). These MAPKs are tightly controlled under physiologic conditions, and alterations of these molecules are proven to be involved in human diseases, including neurodegenerative disorders, autoimmune diseases, and neoplasms (6, 12–15).

Our previous study has confirmed the expression of ERK1⁄2 in odontogenic tumors, suggesting that the classical MAPKs contribute to cell proliferation and differentiation of odontogenic tissues (16). In the present study, immunohistochemical expression of p-JNK, p-p38 MAPK, and p-ERK5 was examined in benign and malignant ameloblastic tumors as well as in tooth germs to evaluate the roles of these MAPKs in oncogenesis and cytodifferentiation of epithelial odontogenic tumors.

Materials and methods

Tissue preparation

Specimens were surgically removed from 52 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 one 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 WHO histological classification of odontogenic tumors (2). The tumors comprised 47 ameloblastomas and five malignant ameloblastic tumors. Ameloblastomas were divided into 24 follicular and 23 plexiform types, including 11 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 10 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. A part of these specimens was used in our previous study (16).

Immunohistochemistry

The serial sections were deparaffinized, immersed in methanol with 0.3% hydrogen peroxide, and heated in 0.01 M citrate buffer (pH 6.0) 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 rabbit anti-p-JNK polyclonal antibody (Cell Signaling Technology, Beverly, MA, USA; diluted at 1:50), rabbit anti-p-p38 MAPK monoclonal antibody (Cell Signaling Technology; Isotype IgG; diluted at 1:50), and rabbit anti-p-ERK5 polyclonal antibody (Biosource, Camarillo, CA, USA; diluted at 1:75) (15, 17, 18). The sections were allowed to react with peroxidase-conjugated anti-rabbit IgG 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 1–5 min. Nuclei were lightly stained with Mayer's hematoxylin. For control studies of the antibodies, the serial sections were treated with phosphate-buffered saline and normal rabbit IgG instead of the primary antibodies and were confirmed to be unstained.

Immunohistochemical reactivity for p-JNK, p-p38 MAPK, and p-ERK5 was evaluated and classified into four groups: (−) negative in epithelial or neoplastic cells, (±) weakly (less than 5% of epithelial or neoplastic cells) positive, (+) moderately (5–25% of epithelial or neoplastic cells) positive, and (++) strongly (more than 25% of epithelial or neoplastic cells) 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. Values of $P < 0.05$ were considered to indicate statistical significance.

Results

Immunohistochemical reactivity for p-JNK, p-p38 MAPK, and p-ERK5 in tooth germs and ameloblastic tumors is summarized in Table 1. Expression of p-JNK, p-p38 MAPK, and p-ERK5 was detected in the nuclei of cellular components in normal and neoplastic odontogenic tissues (Fig. 1–3). In tooth germs, p-JNK reactivity was found in some fibroblastic cells in dental papillae and dental follicles. Scattered reactivity in stellate reticulum was also detected in seven of 10 tooth germs (Fig. 1A). Ameloblastomas and malignant ameloblastic tumors showed p-JNK in some stromal fibroblasts (Fig. 1B–D). Scattered reactivity in central polyhedral cells was detected in seven of 47 ameloblastomas (Fig. 1C). The levels of immunohistochemical reactivity for p-JNK in ameloblastic tumors were significantly lower than that in tooth germs (ameloblastomas: $P < 0.001$, malignant ameloblastic tumors: $P < 0.01$, Table 1).

Immunoreactivity for p-p38 MAPK in tooth germs was found in inner enamel epithelium (Fig. 2A). Some fibroblastic cells and many endothelial cells in dental papillae and dental follicles were also reactive. All but two ameloblastomas and all metastasizing ameloblastomas showed scattered reactivity for p-p38 MAPK in neoplastic cells (Fig. 2B and C). Increased expression of p-p38 MAPK was found in keratinizing cells in acanthomatous ameloblastomas (Fig. 2C). Ameloblastic carcinomas showed reactivity for p-p38 MAPK in some neoplastic cells (Fig. 2D). The level of immunohisto-

Table 1 Immunohistochemical reactivity for phosphorylated JNK, p38 MAPK, and ERK5 in tooth germs and ameloblastic tumors

	p-JNK						*p-p38 MAPK*					*p-ERK5*				
	n	−	±	+	++		−	±	+	++		−	±	+	++	
Tooth germ	10	3(30)	7 (70)	0 (0)	0 (0)	***	0 (0)	4 (40)	6 (60)	0 (0)	*	0 (0)	1 (10)	9 (90)	0 (0)	
Ameloblastoma	47	40(85)	7 (15)	0 (0)	0 (0)		2 (4)	4 (9)	41 (87)	0 (0)		0 (0)	4 (9)	31 (65)	12 (26)	*
Follicular type	24	20 (83)	4 (17)	0 (0)	0 (0)		2 (8)	2 (8)	20 (84)	0 (0)		0 (0)	4 (17)	16 (66)	4 (17)	
Plexiform type	23	20 (87)	3 (13)	0 (0)	0 (0)		0 (0)	2(9)	21 (91)	0 (0)		0 (0)	0 (0)	15 (65)	8 (35)	
Acanthomatous subtype	11	8 (73)	3 (27)	0 (0)	0 (0)	**	1 (9)	0 (0)	10 (91)	0 (0)	**	0 (0)	3 (27)	7 (64)	1 (9)	*
Granular subtype	6	6 (100)	0 (0)	0 (0)	0 (0)		1 (17)	2 (33)	3 (50)	0 (0)		0 (0)	1 (17)	4 (66)	1 (17)	
Basal cell subtype	3	3 (100)	0 (0)	0 (0)	0 (0)		0 (0)	0 (0)	3 (100)	0 (0)		0 (0)	0 (0)	1 (33)	2 (67)	
Desmoplastic subtype	4	4 (100)	0 (0)	0 (0)	0 (0)		0 (0)	0 (0)	4 (100)	0 (0)		0 (0)	0 (0)	4 (100)	0 (0)	
Non-cellular variation	23	19 (83)	4 (17)	0 (0)	0 (0)		0 (0)	2 (9)	21 (91)	0 (0)		0 (0)	0 (0)	15 (65)	8 (35)	
Malignant ameloblastic tumor	5	5 (100)	0 (0)	0 (0)	0 (0)		0 (0)	3 (60)	2 (40)	0 (0)		0 (0)	0 (0)	2 (40)	3 (60)	
Metastasizing ameloblastoma	2	2 (100)	0 (0)	0 (0)	0 (0)		0 (0)	1 (50)	1 (50)	0 (0)		0 (0)	o (0)	2 (100)	0 (0)	
Ameloblastic carcinoma	3	3 (100)	0 (0)	0 (0)	0 (0)		0 (0)	2 (67)	1 (33)	0 (0)		0 (0)	0 (0)	0 (0)	3 (100)	

Immunohistochemical reactivity: (−) negative in epithelia l or neoplastic cells, (±) weakly (less than 5% of epithelial or neoplastic cells) positive, (+) moderately (5–25% of epithelial or neoplastic cells) positive, and (++) strongly (more than 25% of epithelial or neoplastic cells) positive. Values in parentheses denote percentage values. Statistical significance: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

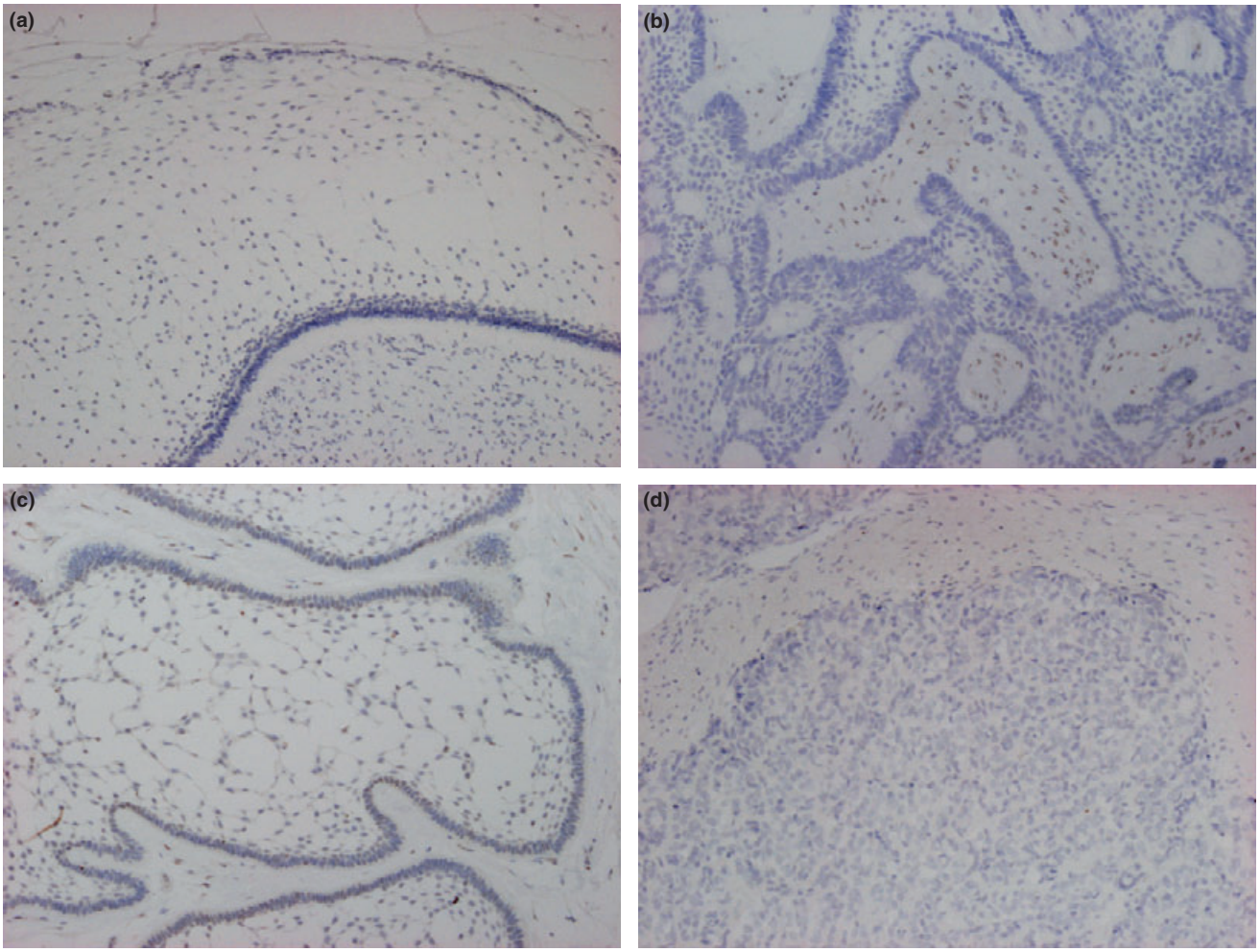

Figure 1 Immunohistochemicalreactivity for phosphorylated JNK. (a) Tooth germ showing scattered reactivity in stellate reticulum. Some mesenchymal cells in dental papilla and dental follicle are also positive (×70). (b) Plexiform ameloblastoma showing reactivity in many stromal cells (×90). (c) Follicular ameloblastoma showing scattered reactivity in central polyhedral cells. Some stromal cells are also positive (×90). (d) Ameloblastic carcinoma showing reactivity in some stromal cells (×90).

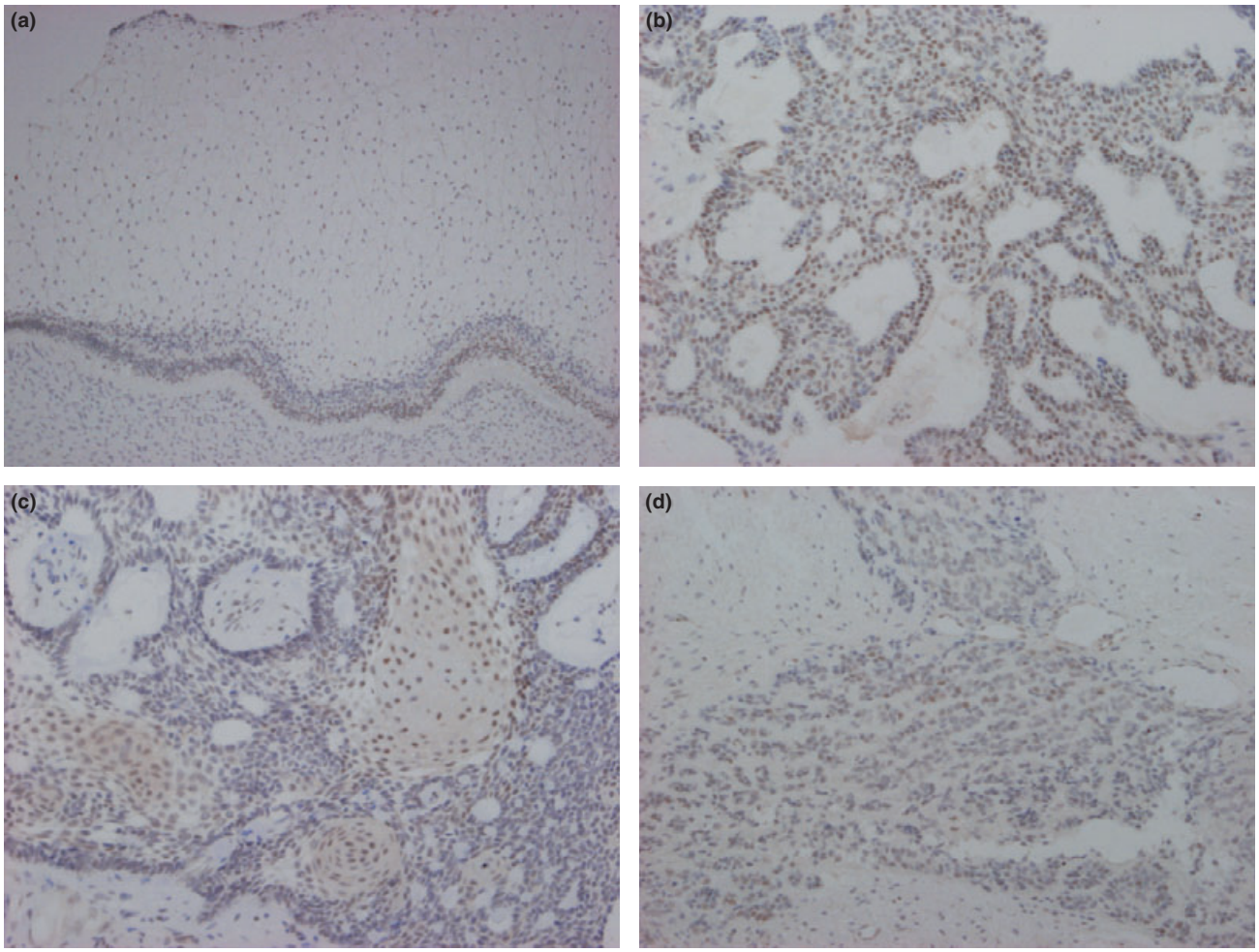

Figure 2 Immunohistochemical reactivity for phosphorylated p38 MAPK. (a) Tooth germ showing reactivity in inner enamel epithelium. Some mesenchymal cells in dental papilla and dental follicle are also positive (×60). (b) Plexiform ameloblastoma showing scattered reactivity in neoplastic cells. Some stromal cells are also positive (×100). (c) Acanthomatous ameloblastoma showing increased reactivity in keratinizing cells (×100). (d) Ameloblastic carcinoma showing scattered reactivity in neoplastic cells. Some stromal cells are also positive (×100).

chemical reactivity for p-p38 MAPK in ameloblastomas was significantly higher than the levels in tooth germs and malignant ameloblastic tumors (tooth germs: $P < 0.05$, malignant ameloblastic tumors: $P < 0.01$, Table 1). In the stroma of these ameloblastic tumors, some fibroblasts and many endothelial cells were also reactive with p-p38 MAPK.

p-ERK5 was expressed in many cells of inner enamel epithelium and some cells of other epithelial components in tooth germs (Fig. 3A). Some fibroblastic cells in dental papillae and dental follicles were also reactive. Ameloblastomas and metastasizing ameloblastomas showed p-ERK5 reactivity in many peripheral columnar or cuboidal cells and some central polyhedral cells (Fig. 3B and C). Decreased p-ERK5 reactivity was found in keratinizing cells in acanthomatous ameloblastomas and granular cells in granular cell ameloblastomas (Fig. 3C). Basal cell ameloblastomas showed p-ERK5 reactivity in many neoplastic cells, and desmoplastic ameloblastomas were reactive in some neoplastic cells. The level of immunohistochemical reactivity for p-ERK5 in plexiform ameloblastomas was significantly higher than that in follicular ameloblastomas ($P < 0.05$, Table 1). Ameloblastic carcinomas showed p-ERK5 reactivity in most neoplastic cells (Fig. 3D). The level of immunohistochemical reactivity for p-ERK5 in malignant ameloblastic tumors was significantly higher than that in tooth germs ($P < 0.05$, Table 1). In these ameloblastic tumors, some stromal fibroblasts were also reactive with p-ERK5.

Discussion

Mitogen-activated protein kinases are conserved during evolution and play an essential role in diverse intracellular signaling processes in organisms ranging from yeast to vertebrates (5). Components of the JNK signaling pathway are required for dorsal closure in *Drosophila* and eyelid closure in mouse (19). *Drosophila p38 MAPK* gene has a role in wing morphogenesis, and p38 MAPK in vertebrates participates in various cell differentiation processes, including neuronal, myogenic,

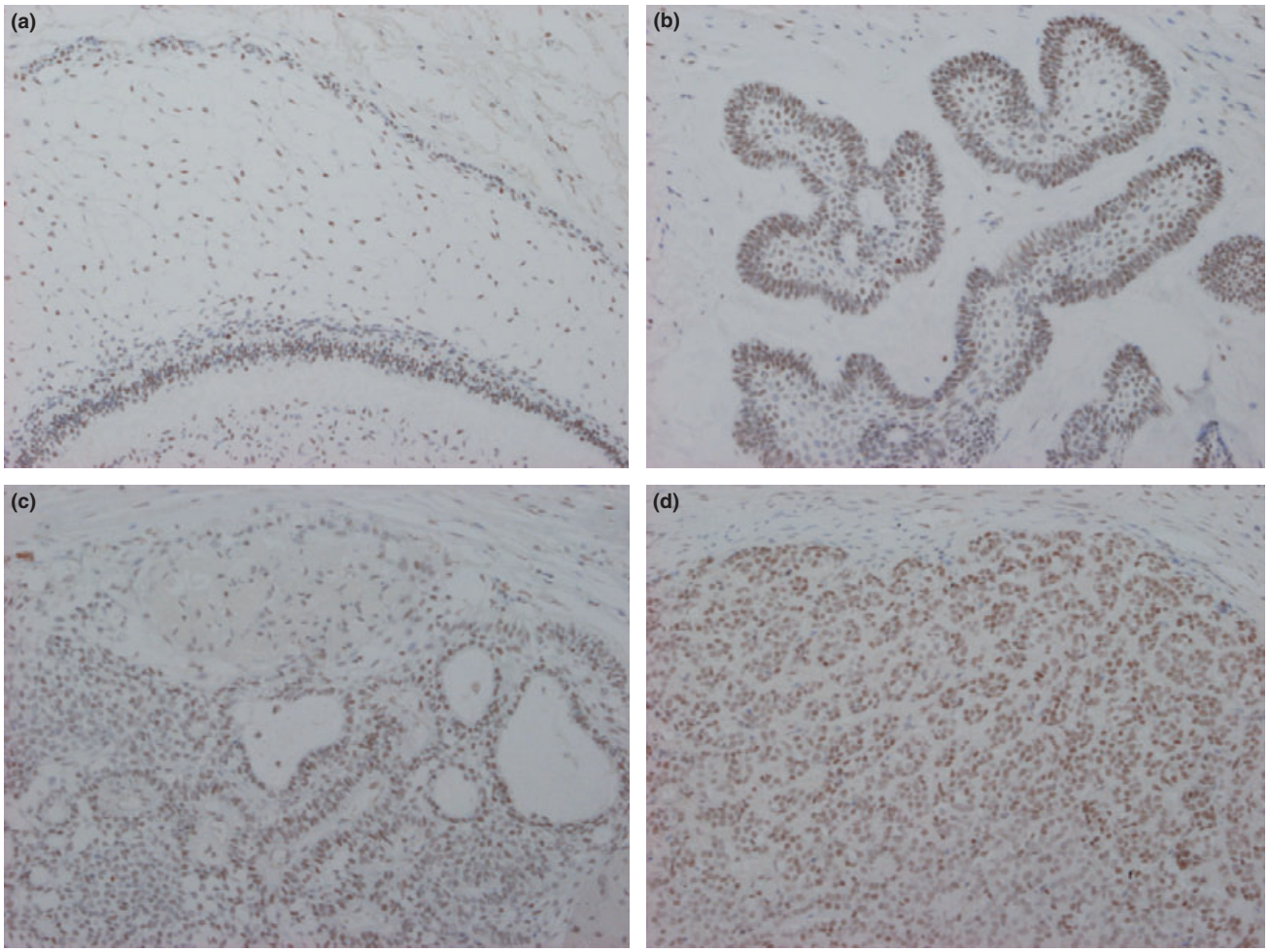

Figure 3 Immunohistochemical reactivity for phosphorylated ERK5. (a) Tooth germ showing reactivity in many cells of inner enamel epithelium and some cells of other epithelial components. Some mesenchymal cells in dental papilla and dental follicle are also positive (×85). (b) Follicular ameloblastoma showing reactivity in many peripheral columnar cells and some central polyhedral cells. Some stromal cells are also positive (×100). (b) Granular cell ameloblastoma showing decreased reactivity in granular cells (×100). (d) Ameloblastic carcinoma showing reactivity in most neoplastic cells. Some stromal cells are also positive (×100).

and adipocytic differentiation (20). *ERK5* gene regulates body size through control of cell growth in *Caenorhabditis elegans*, and the ERK5 pathway has an essential role in neuronal differentiation in *Xenopus* embryonic development (11). *JNK1/JNK2* double-mutant mice exhibit defective neuronal tube morphogenesis and reduced apoptosis in hind brain regions (21). *p38 MAPK*α-deficient mice show placental defects and insufficient erythropoiesis (22). *ERK5* null mice display defective cardiovascular development (23). Gene disruption in these experimental mice results in embryonic lethality (21–23). Thus, functions of these MAPKs are needed for developmental processes. In the present study, distinct patterns of immunohistochemical reactivity for p-JNK, p-p38 MAPK, and p-ERK5 in human tooth germs were found in epithelial components, suggesting that these MAPKs contribute to cell proliferation, differentiation, or apoptosis of odontogenic epithelium during tooth development.

JNK activation occurs in response to cellular stresses, such as ultraviolet irradiation, heat shock, reactive oxygen species, inflammatory cytokines, hyperosmolarity, and direct DNA damage, and participates in the apoptotic signaling pathway by mediating many proapoptotic and antiapoptotic molecules, including p53 and Bcl-2 family members (5, 11). Enhanced activation of the JNK pathway has been linked to neurodegenerative disorders, such as polyglutamine disease, Alzheimer's disease, and Parkinson's disease (13, 24, 25). Activation of JNK is consistently observed in oncogene-expressing cells, and constitutively activated JNK has been detected in human malignancies, such as glioblastoma and leukemia (14, 26). A recent study has revealed that cultured ameloblastoma cells show no expression of p-JNK in the absence of a stimulus, and treatment with concentrated conditioned media induces phosphorylation of JNK (27). Expression of p53 and Bcl-2 family members has been confirmed in ameloblastic tumors

(28–30). In the present study, immunohistochemical reactivity for p-JNK was detected scatteredly in central polyhedral neoplastic cells in some ameloblastomas, and malignant ameloblastic tumors were not reactive with p-JNK in neoplastic cells. The p-JNK expression levels in ameloblastic tumors were significantly lower than the level in tooth germs. These features suggest that decreased activation of JNK might be involved in oncogenesis of odontogenic epithelium.

p38 MAPK is activated by environmental stresses and inflammatory cytokines, similarly to JNK, and p-p38 MAPK can control many different substances, such as p53, p73, cyclin D, and $p21^{WAF1/Cip1}$, promoting apoptosis or cell cycle arrest (5, 20). p38 MAPK has an important role in activation of the immune response and appears to be involved in human immunological diseases, such as asthma (6). The role of p38 MAPK in neoplasms is complex, and the p38 MAPK pathway promotes or suppresses cell transformation and neoplastic cell proliferation (15, 31, 32). In cultured ameloblastoma cells, phosphorylation of p38 MAPK has reported to be upregulated on treatment with conditioned medium, similarly to JNK (27). In the present study, immunohistochemical reactivity for p-p38 MAPK in ameloblastomas was found in scattered neoplastic cells in most cases, and the p-p38 MAPK expression level in ameloblastomas was significantly higher than that in tooth germs. Malignant ameloblastic tumors showed limited p-p38 MAPK reactivity in neoplastic cells, and the p-p38 MAPK expression level in malignant ameloblastic tumors was statistically lower than that in ameloblastomas. Our previous studies have revealed that the number of apoptotic cells in ameloblastomas is greater than that in tooth germs, and apoptotic reactions are less frequent in malignant ameloblastic tumors than in ameloblastomas (29, 33). These features suggest that phosphorylation of p38 MAPK might contribute to apoptosis of neoplastic cells in these ameloblastic tumors. Previous studies have confirmed expression of apoptosis-related factors, p53 and p73, and cell cycle regulators, cyclin D1 and $p21^{WAF1/Cip1}$, in ameloblastic tumors (28, 34, 35), and p-p38 MAPK is thought to modulate these molecules associated with cell death and proliferation in these ameloblastic tumors. In the present study, immunohistochemical reactivity for p-p38 MAPK was obvious in keratinizing cells of acanthomatous ameloblastomas. Our previous studies have revealed that keratinizing cells and granular cells in ameloblastoma variants show increased apoptotic reactions as compared with other types of neoplastic cells (29, 33). These features suggest that p38 MAPK activation might be associated with specific differentiation of ameloblastoma cells.

ERK5 is activated not only by stress stimuli, such as oxidative stress and hyperosmolarity, similarly to JNK and p38 MAPK, but also by proliferative stimuli, such as serum and growth factors, and activated ERK5 leads to activation of transcription factors involved in cell proliferation, including MEF2C, c-Fos, and Fra-1 (5, 11). ERK5 and ERK2 synergize to activate the transcription factor NF-κB, promoting cell transformation, and the ERK5 pathway is essential for tumor-associated angiogenesis (12, 18). In the present study, immunohistochemical reactivity for p-ERK5 in ameloblastic tumors was found predominantly in neoplastic cells near the basement membrane, similarly to the distribution of proliferative cells (28, 29, 34, 36), and the expression levels of p-ERK5 in ameloblastic tumors were slightly higher than that in tooth germs. These features suggest that ERK5 activation might participate in oncogenesis of odontogenic epithelium via regulation of cell proliferation. A previous study has shown overexpression of *c-Fos* oncogene, a target of ERK5, in ameloblastomas on cDNA microarray and RT-PCR (3), and *c-Fos* overexpression is possibly affected by p-ERK5 in ameloblastomas. In the present study, p-ERK5 immunoreactivity was significantly higher in plexiform ameloblastomas than in follicular ameloblastomas. Our previous studies have revealed that Ki-67-labeled proliferating cells are slightly greater in plexiform ameloblastomas than in follicular ameloblastomas, similar to p-ERK5 reactivity (29, 36). These features suggest that activated ERK5 might influence tissue structuring of ameloblastomas.

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