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ORIGINAL ARTICLE

Immunohistochemical detection of BH3-only proteins in ameloblastic tumors

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OBJECTIVE: To evaluate expression of BH3-only proteins in odontogenic tumors, expression of Bid, Bim, Bad, Noxa, and Puma was analyzed in ameloblastic tumors as well as in tooth germs.
METHODS: Nine tooth germs, 37 ameloblastomas, and five malignant ameloblastic tumors were examined immunohistochemically with antibodies against Bid, Bim, Bad, Noxa, and Puma.
RESULTS: Immunohistochemical reactivity for Bid, Bim, Bad, Noxa, and Puma was detected in the cytoplasm of cellular components in normal and neoplastic odontogenic tissues. Expression of these BH3-only proteins was evident in odontogenic epithelial cells near the basement membrane in tooth germs and ameloblastic tumors. Acanthomatous ameloblastomas showed no reactivity for Bid, Bim, Bad, Noxa, or Puma in keratinizing cells, whereas granular cells in granular cell ameloblastomas reacted with these BH3-only proteins. Basal and desmoplastic ameloblastomas and ameloblastic carcinomas showed immunoreactivity for the BH3-only proteins in most neoplastic cells.
CONCLUSION: Expression of Bid, Bim, Bad, Noxa, and Puma in tooth germs and ameloblastic tumors suggests that the BH3-only proteins have a role in apoptotic cell death of normal and neoplastic odontogenic epithelium. Distinctive expression patterns of these BH3-only proteins in ameloblastoma variants suggest that the BH3-only proteins might be involved in tumor cell differentiation of ameloblastomas.
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Keywords: ameloblastoma; apoptosis; BH3-only protein; immunohistochemistry

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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 (Sciubba *et al*, 2001; Philipsen *et al*, 2005). 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. Histologically, ameloblastoma shows considerable variation, including follicular, plexiform, acanthomatous, granular cell, basal cell, and desmoplastic variants (Sciubba *et al*, 2001; Philipsen *et al*, 2005). Malignant counterparts of ameloblastoma are classified into metastasizing ameloblastoma and ameloblastic carcinoma on the basis of metastatic spread and cytological malignant features (Philipsen *et al*, 2005). Recent studies have identified genetic and molecular alterations in these epithelial odontogenic tumors (Heikinheimo *et al*, 2002; Kumamoto, 2006); however, the detailed mechanisms of oncogenesis, cytodifferentiation, and tumor progression remain unknown.

Apoptosis, also known as programmed cell death or physiologic cell death, has diverse roles in development and tissue homeostasis, as well as in a variety of pathologic conditions (Kerr *et al*, 1972; Vaux and Korsmeyer, 1999; Johnstone *et al*, 2002). There are two alternative pathways that initiate apoptosis: one is mediated by death receptors on the cell surface, and the other is mediated by mitochondria (Hengartner, 2000). Bcl-2 family proteins that possess at least one of four conserved motifs known as Bcl-2 homology domains (BH1 to BH4) determine life or death of a cell by controlling the mitochondria-mediated apoptotic pathway. A large number of Bcl-2 family proteins can be divided into three subfamilies: anti-apoptotic members that exert anti-cell death activity and have all four BH domains, such as Bcl-2, Bcl-x, and Mcl-1; pro-apoptotic members that promote apoptosis and share sequence homology in BH1, BH2, and BH3 but not in BH4, such as Bax and Bak; and BH3-only proteins that initiate apoptosis and share sequence homology only in BH3,

such as Bid, Bim, Bad, Noxa, and Puma (Adams and Cory, 1998; Hengartner, 2000). BH3-only proteins activated by an apoptotic stimulus induce their translocation from the cytosol to mitochondria, where they interact with anti-apoptotic or pro-apoptotic members, facilitating apoptosis, and the pro-apoptotic activity of BH3-only proteins is stringently controlled by a variety of mechanisms (Adams and Cory, 1998; Vaux and Korsmeyer, 1999; Hengartner, 2000). Recent studies have revealed that aberrations of BH3-only proteins are linked to the pathophysiology of degenerative disorders, autoimmunity, and neoplasia (Bouillet *et al*, 1999; Yin *et al*, 1999; Bouillet *et al*, 2001; Ranger *et al*, 2003; Zinkel *et al*, 2003).

Previous studies have confirmed the presence of apoptotic cells and apoptosis-related factors, such as Bcl-2 family proteins (IAP), inhibitor of apoptosis protein family proteins, p53, Fas ligand, tumor necrosis factor α (TNFα), and TNF-related apoptosis-inducing ligand (TRAIL), in tooth germs and ameloblastic tumors, suggesting that apoptotic cell death has an important role in oncogenesis or cytodifferentiation of odontogenic epithelium (Slootweg, 1995; Kumamoto, 1997; Kumamoto and Ooya, 1999; Kumamoto *et al*, 2001b; Sandra *et al*, 2001; Kumamoto and Ooya, 2004, 2005a). We showed that the expression of caspase-9, an apoptosis initiator in the mitochondria-mediated apoptotic pathway, in ameloblastic tumors was higher than that of caspase-8, an apoptosis initiator in the death receptor-mediated apoptotic pathway, and these findings suggested that the mitochondrial pathway has a more important role in apoptotic cell death than the death receptor-mediated pathway in odontogenic tumors (Kumamoto and Ooya, 2005a,b). In the present study, the immunohistochemical expression of Bid, Bim, Bad, Noxa, and Puma was examined in benign and malignant ameloblastic tumors as well as in tooth germs to evaluate the roles of these BH3-only proteins in oncogenesis and cytodifferentiation of epithelial odontogenic tumors.

Materials and methods

The study protocol was reviewed and approved by the Research Ethics Committee of Tohoku University Graduate School of Dentistry.

Tissue preparation

Specimens were surgically removed from 42 patients with epithelial odontogenic tumors at the Department of Oral and Maxillofacial Surgery, Tohoku University 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 WHO histological classification of odontogenic tumors (Philipsen *et al*, 2005). The tumors comprised 37 ameloblastomas and five malignant ameloblastic tumors. Ameloblastomas were divided into 17 follicular and 20 plexiform types, including six acanthomatous, five granular cell, three basal cell, and three desmoplastic subtypes. Malignant ameloblastic tumors were classified into two metastasizing ameloblastomas and three ameloblastic carcinomas. Specimens of nine 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, immersed in methanol with 0.3% hydrogen peroxide, and heated in 1 mM ethylenediamine tetraacetic acid buffer (pH 9.0; for Bid, Bad, and Puma) or 0.01 M citrate buffer (pH 6.0; for Bim and Noxa) 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-Bid polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; diluted at 1:50), rabbit anti-Bim polyclonal antibody (Lab Vision, Fremont, CA, USA; diluted at 1:20), rabbit anti-Bad polyclonal antibody (Cell Signaling Technology, Beverly, MA, USA; diluted at 1:20), goat anti-Noxa polyclonal antibody (Santa Cruz Biotechnology; diluted at 1:50), and rabbit anti-Puma polyclonal antibody (Cell Signaling Technology; diluted at 1:50). The sections were allowed to react with peroxidase-conjugated anti-rabbit IgG (for Bid, Bim, Bad, and Puma) or anti-goat IgG (for Noxa) 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 1–3 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 and goat IgG instead of the primary antibodies and were confirmed to be unstained. Immunohistochemical reactivity for BH3-only proteins was evaluated and classified into three groups: (–) negative, (+) scatteredly positive, and (++) diffusely positive.

Results

Immunohistochemical reactivity for Bid, Bim, Bad, Noxa, and Puma in tooth germs and ameloblastic tumors is summarized in Table 1. Expression of these BH3-only proteins was detected in the cytoplasm of cellular components in normal and neoplastic odontogenic tissues (Figures 1–3). In tooth germs, reactivity for Bid, Bim, Bad, Noxa, and Puma was found in inner enamel epithelium (Figure 1). Bid and Puma reactivity was scatteredly recognized in other epithelial components (Figure 1a). Many endothelial cells and some fibroblastic cells in dental papillae and dental follicles were also reactive with Bid and Puma. Bad reactivity was found in some endothelial cells, and Noxa reactivity was found in many fibroblastic cells.

Ameloblastomas showed reactivity for Bid, Bim, Bad, Noxa, and Puma in peripheral columnar or cuboidal

Table 1 Immunohistochemical reactivity for BH3-only proteins in tooth germs and ameloblastic tumors

	Bid	*Bim*	*Bad*	*Noxa*	*Puma*
Tooth germ (n = 9)					
Epithelial cells					
Inner enamel epithelium	++	++	++	+/++	++
Outer enamel epithelium	+	–	–	–	+
Stellate reticulum	–/+	–	–	–	+
Stratum intermedium	–/+	–	–	–	+
Dental lamina	+	–	–	–	+
Mesenchymal cells					
Dental papilla	+/++	–	+	+/++	+/++
Dental follicle	+/++	–	+	+/++	+/++
Ameloblastoma (n = 37)					
Neoplastic cells					
Peripheral cells	++	++	++	+/++	++
Central cells	–/+	–/+	–	+	–
Keratinizing cells	–	–	–	–	–
Granular cells	++	++	++	++	++
Basal cell variant	++	++	++	++	++
Desmoplastic variant	++	++	++	++	++
Stromal cells	+/++	–	+	+/++	+/++
Metastasizing ameloblastoma (n = 2)					
Neoplastic cells					
Peripheral cells	++	++	++	+/++	++
Central cells	–	–	–	–	+
Stromal cells	+/++	–	+	+/++	+/++
Ameloblastic carcinoma (n = 3)					
Neoplastic cells	++	++	++	++	++
Stromal cells	+/++	–	+	+/++	+/++

–, negative; +, scatteredly positive; ++, diffusely positive.

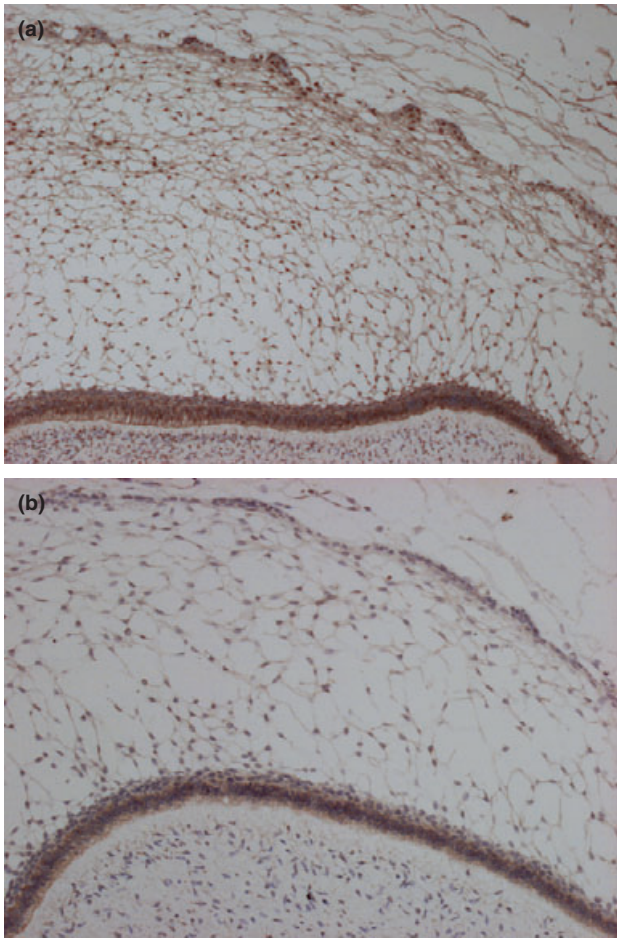

Figure 1 Immunohistochemical reactivity for BH3-only proteins in tooth germs. (**a**) Tooth germ showing strong Bid reactivity in inner enamel epithelium and weak reactivity in other epithelial components (×70). (**b**) Tooth germ showing Bad reactivity in inner enamel epithelium (×85)

neoplastic cells (Figure 2a–d). Some central polyhedral neoplastic cells were reactive with Bid, Bad, and Puma. Acanthomatous ameloblastomas showed no reactivity for Bid, Bim, Bad, Noxa, or Puma in keratinizing cells (Figure 2c), while granular cells in granular cell ameloblastomas were reactive with these BH3-only proteins (Figure 2d). Basal cell and desmoplastic ameloblastomas showed reactivity for Bid, Bim, Bad, Noxa, and Puma in most neoplastic cells (Figure 2e,f), and staining intensity of Bim and Noxa was weak in desmoplastic ameloblastomas. Metastasizing ameloblastomas showed reactivity for Bid, Bim, Bad, Noxa, and Puma in peripheral columnar or cuboidal neoplastic cells (Figure 3a). Some central polyhedral neoplastic cells were reactive with Puma. Ameloblastic carcinomas showed reactivity for Bid, Bim, Bad, Noxa, and Puma in most neoplastic cells (Figure 3b). In the stroma of these ameloblastic tumors, many endothelial cells and some fibroblasts were also reactive with Bid and Puma. Bad reactivity was found in some endothelial cells, and Noxa reactivity was found in many fibroblasts.

Discussion

The apoptotic program is required for normal development of almost all multicellular organisms to control cell number and tissue size (Vaux and Korsmeyer, 1999; Hengartner, 2000). Genetic studies in *Caenorhabditis elegans* have shown that deficiency of EGL-1, the only BH3-only protein in this species, impedes all developmentally programmed cell death of somatic cells (Adams and Cory, 1998; Vaux and Korsmeyer, 1999). Targeted elimination of *Bim* results in embryonic lethality in more than half of deficient mice, and survivors show abnormal hematopoietic cell differentiation and subsequent glomerulonephritis (Bouillet *et al*, 1999). Thus, BH3-only proteins have roles in developmental processes. Apoptotic cell death is known to play an important role in tooth development (Kindaichi, 1980; Vaahtokari *et al*, 1996). Anti-apoptotic and pro-apoptotic members of Bcl-2 family proteins and mitochondria-mediated apoptosis signaling molecules have been identified in tooth germ tissues (Slootweg and de Weger, 1994; Kumamoto and Ooya, 1999, 2005b). In the present study, immunohistochemical reactivity for Bid, Bim, Bad, Noxa, and Puma was found in human tooth germs, suggesting that these BH3-only proteins have a role in apoptotic cell death during tooth development.

Apoptosis normally eliminates cells with damaged DNA or aberrant cell cycles, and evasion of apoptosis by aberrations of cell death regulators underpins tumor

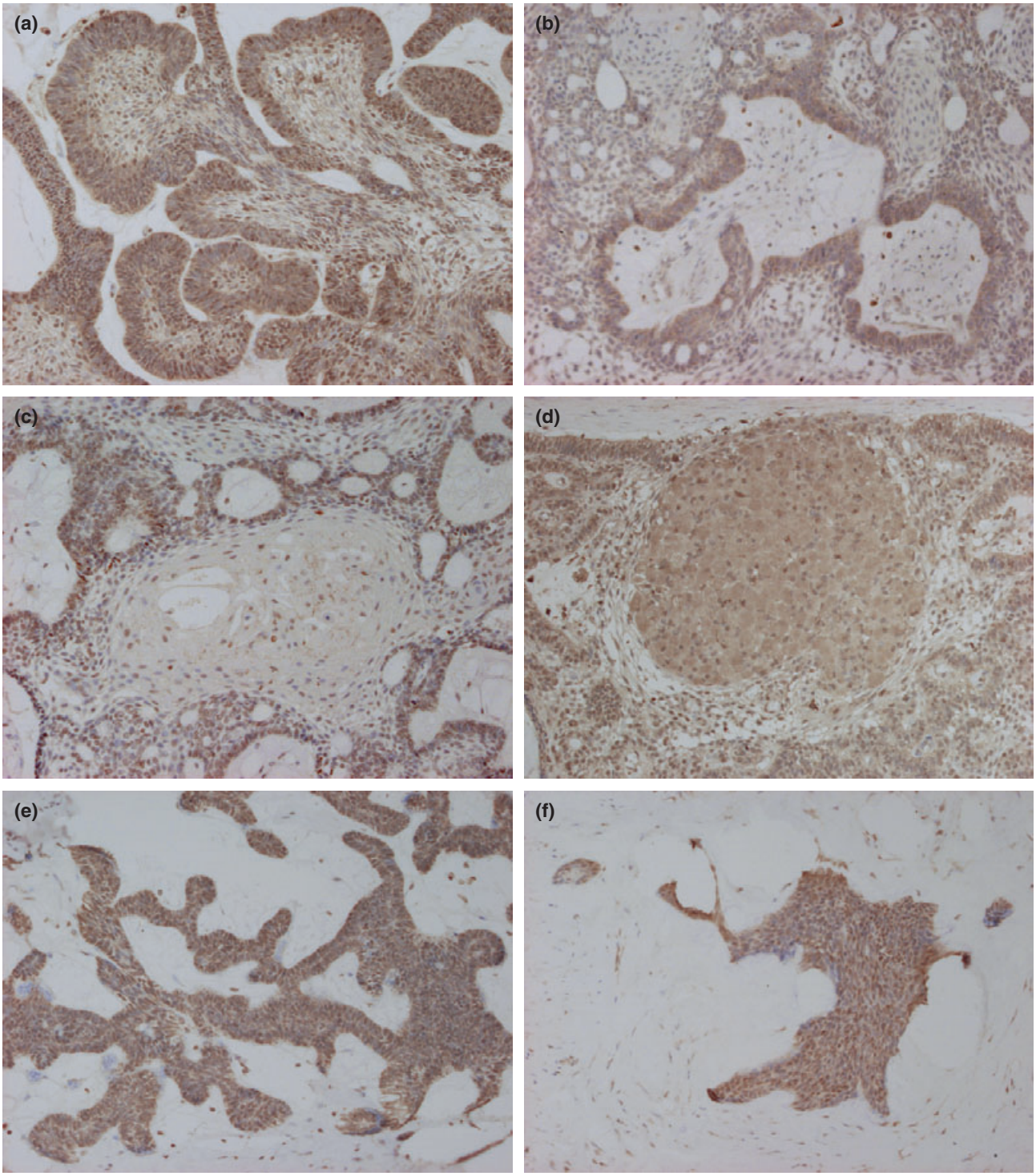

Figure 2 Immunohistochemical reactivity for BH3-only proteins in ameloblastomas. (**a**) Follicular ameloblastoma showing Bid reactivity in peripheral columnar neoplastic cells (×95). (**b**) Plexiform ameloblastoma showing Bad reactivity in peripheral cuboidal neoplastic cells (×95). (**c**) Acanthomatous ameloblastoma showing no Bim reactivity in keratinizing cells (×95). (**d**) Granular cell ameloblastoma showing Bid reactivity in granular cells as well as peripheral columnar neoplastic cells (×95). (**e**) Basal cell ameloblastoma showing Bim reactivity in most neoplastic cells (×85). (**f**) Desmoplastic ameloblastoma showing Puma reactivity in most neoplastic cells (×80)

development (Adams and Cory, 1998; Johnstone *et al*, 2002). BH3-only proteins could act as tumor suppressors, and disruption of their functions has been shown to contribute to tumorigenesis in various lineages (Hoque *et al*, 2003; Jansson *et al*, 2003; Ranger *et al*, 2003; Zinkel *et al*, 2003; Karst *et al*, 2005; Tagawa *et al*, 2005). Our previous studies have revealed that the mitochondrial apoptotic pathway has a more important role than the death receptor-mediated pathway in apoptotic reactions of ameloblastic tumors (Kumamoto

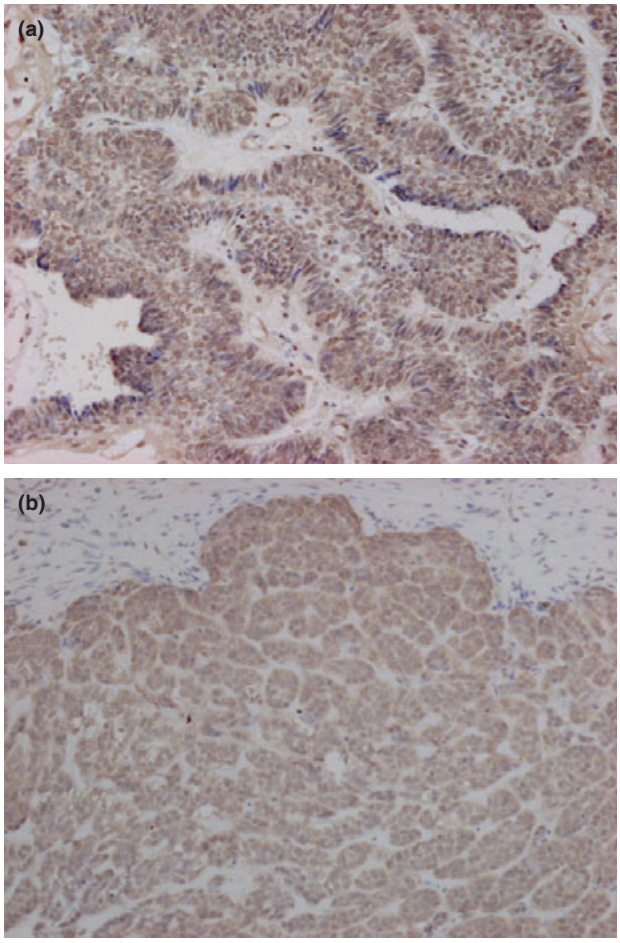

Figure 3 Immunohistochemical reactivity for BH3-only proteins in malignant ameloblastic tumors. (**a**) Metastasizing ameloblastoma showing Noxa reactivity in peripheral columnar neoplastic cells (×95) (**b**). Ameloblastic carcinoma showing Puma reactivity in most neoplastic cells (×95)

and Ooya, 2005a,b). In the present study, immunohistochemical reactivity for Bid, Bim, Bad, Noxa, and Puma was detected in all benign and malignant ameloblastic tumors, suggesting that these BH3-only proteins are associated with apoptotic cell death in epithelial odontogenic tumors. These BH3-only proteins were expressed predominantly in neoplastic cells near the basement membrane in ameloblastic tumors, similarly to anti-apoptotic members in the Bcl-2 family, Bcl-2 and Bcl-x, and proliferation markers, such as Ki-67 and DNA topoisomerase IIα (Slootweg, 1995; Kumamoto and Ooya, 1999; Kumamoto *et al*, 2001a; Sandra *et al*, 2001). These features suggest that apoptosis initiated by the BH3-only proteins might be suppressed by interactions with other Bcl-2 family proteins. 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 (Kumamoto, 1997; Kumamoto *et al*, 2001b). In the present study, immunoreactivity for Bid, Bim, Bad, Noxa, and Puma did not apparently differ among tooth germs, ameloblastomas, and malignant ameloblastic tumors. Our previous studies have detected increased apoptotic cell death and decreased expression of apoptosis suppressors in keratinizing cells in acanthomatous ameloblastomas and granular cells in granular cell ameloblastomas (Kumamoto, 1997; Kumamoto and Ooya, 1999; Kumamoto *et al*, 2001b; Kumamoto and Ooya, 2004). In the present study, Bid, Bim, Bad, Noxa, and Puma were expressed in granular cells in granular cell ameloblastomas but not in keratinizing cells in acanthomatous ameloblastomas. These features suggest that cell death mechanisms differ among these ameloblastoma variants during neoplastic cell differentiation.

Bid is cleaved by caspase-8 on activation of cell surface death receptors, and the truncated Bid translocates from the cytosol to mitochondria and induces cytochrome c release, activating the mitochondrial apoptotic pathway (Hengartner, 2000; Shibue and Taniguchi, 2006). Ameloblastic tumors have shown expression of death ligands (Fas ligand, TNFα, and TRAIL) and their receptors with death domains (Fas, TNF receptor I, and TRAIL receptor 1 and 2), but caspase-8 expression is extremely limited in these tumors (Kumamoto *et al*, 2001b; Kumamoto and Ooya, 2005a). In the present study, Bid reactivity was detected in neoplastic cells of ameloblastic tumors. These features suggest that death signals mediated by death receptors might be transmitted through the mitochondria-mediated apoptotic pathway in epithelial odontogenic tumors. Bad is sequestered by 14-3-3 scaffold proteins after phosphorylation by kinases, such as Akt and protein kinase A, and its activation by dephosphorylation due to the lack of growth factors allows it to translocate to the mitochondria and to exert pro-apoptotic effects (Shibue and Taniguchi, 2006). Our previous study showed that activated Akt is expressed in ameloblastic tumors (Kumamoto and Ooya, 2007), and the present study detected Bad expression in ameloblastic tumors. These features suggest that pro-apoptotic effects of Bad might be suppressed by Akt in epithelial odontogenic tumors. Transcription of *Noxa* and *Puma* is directly induced by p53, pointing to their involvement in the p53-induced apoptotic pathway (Vousden and Lu, 2002; Shibue and Taniguchi, 2006). *Noxa*, *Puma*, and *Bim* are also directly upregulated by the transcription factor E2F-1, which is normally constrained by RB (Hershko and Ginsberg, 2004). Expression of p53, E2F-1, and RB has been found predominantly in neoplastic cells neighboring the basement membrane in ameloblastic tumors (Slootweg, 1995; Kumamoto and Ooya, 2006), similarly to the expression of Noxa, Puma, and Bim shown in the present study, suggesting that functions of these BH3-only proteins are possibly affected by the tumor suppressors p53 and RB.

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