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Immunohistochemical demonstration of an enamel sheath protein, sheathlin, in odontogenic tumors

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Abstract Enamel proteins can be useful markers for assessment of the functional differentiation of neoplastic epithelium and the nature of extracellular matrices in odontogenic tumors. In the present study, we examined immunohistochemical localization of sheathlin, a recently cloned enamel sheath protein, in various odontogenic tumors to evaluate functional differentiation of tumor cells and the nature of hyalinous or calcified matrices in odontogenic neoplasms. Distinct immunolocalization of sheathlin was observed in the immature enamel of the tooth germ at the late bell stage. Secretory ameloblasts facing the enamel matrix also showed positive staining in their cytoplasm. Definite localization of sheathlin was demonstrated in the enamel matrix in odontogenic tumors with inductive dental hard tissue formation such as ameloblastic fibroodontomas and odontomas. Immunoexpression of sheathlin was, furthermore, demonstrated in eosinophilic droplets in solid nests of adenomatoid odontogenic tumor (AOT) and ghost cells in the epithelial lining of calcifying odontogenic cyst (COC). In AOT, cells facing the eosinophilic droplets also expressed the protein in their cytoplasm. There was neither intracellular staining for sheathlin in the tumor cells nor extracellular staining in the matrix of ameloblastomas and calcifying epithelial odontogenic tumors. Dentin, dysplastic dentin-like hyaline material and cementum in the tumors examined were negative for sheathlin. These results show that immunodetection of sheathlin is a useful marker for functional differentiation of secretory ameloblasts and enamel matrix, which is often hard to differentiate from other hard tissues in odontogenic tumors. Our findings from the view point of sheathlin expression support that the tumor cells of ameloblastomas do not attain full differentiation into functional ameloblasts. It is very interesting that epithelial cells in odontogenic tumors can differentiate into functional ameloblasts without induction by odontogenic mesenchyme, as shown by immunoexpression of sheathlin in eosinophilic droplets within solid epithelial sheets in AOT and ghost cells in the epithelial lining of COC where inductive participation of mesenchymal cells was most unlikely.

Key words Enamel sheath · Sheathlin · Odontogenic tumors

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Introduction

The list of matrix proteins that are reported to be present during enamel development is now extensive, including amelogenin, enamelin, ameloblastin, tuftelin, and others [17]. These proteins are produced by ameloblasts in different stages and considered to play various roles in the modulation of mineral deposition and crystal growth during tooth morphogenesis. For example, amelogenin forming the bulk of the organic matrix of immature enamel is biosynthesized by secretory ameloblasts and involved in the control of crystal growth and the support of growing crystals during amelogenesis. Thus, enamel proteins can be useful markers for evaluating the functional differentiation of neoplastic epithelium and the nature of extracellular matrices in odontogenic tumors. Immunohistochemical studies using antibodies to the enamel proteins may provide us with indispensable information to discuss histogenesis and classification of the odontogenic tumors.

However, there have been only a few studies on immunohistochemical localization of enamel proteins in various odontogenic tumors and cysts [14, 18]. Saku et al. [18] showed amelogenin and enamelin in small mineralized foci and in tumor cells surrounding them in adenomatoid odontogenic tumor (AOT), calcifying epithelial odontogenic tumor (CEOT) and calcifying odonto-

genic cyst (COC), but not in tumor cells in ameloblastoma. They suggested that tumor cells of AOT, CEOT, and COC showed ameloblastic differentiation, but ameloblastoma cells did not attain functional maturation as secretory phase ameloblasts. However, Mori et al. [14] described extensive immunoexpression of amelogenin in tumor cells of various tumors even including ameloblastoma.

Sheathlin is a recently cloned sheath protein in porcine enamel [10]. It is an analogue of ameloblastin in rat incisors and amelin in rat molars [5, 11]. Immunohistochemical study using antibodies that recognize different regions of the protein showed that (1) sheathlin is synthesized in secretory ameloblasts and secreted into immature enamel matrix with post-translational and post-secretory modifications, and (2) cleaved N-terminal polypeptides concentrate in the prism sheath [26]. It is supposed that sheathlin and/or its cleavage products play important roles in amelogenesis, such as mediation of the contact of ameloblasts to the mineralized matrix and modulation of crystal growth [26].

In the present study, we examined immunohistochemical localization of sheathlin in various odontogenic tumors to assess the usefulness of the protein as a new marker and to discuss the functional differentiation of tumor cells and the nature of hyalinous or calcified matrices in odontogenic tumors.

Materials and methods

Materials

For immunohistochemical assessment of the expression of sheathlin, the following odontogenic tumors with or without ectomesenchymal components were selected from the pathology file from the Department of Oral Pathology, Hiroshima University School of Dentistry: ameloblastomas (*n*=10), CEOTs (*n*=5), ameloblastic fibro-odontomas (*n*=4), AOTs (*n*=10), COCs (*n*=10) and odontomas (*n*=10). Five human fetal tooth germs of various developmental stages were examined for control staining. The materials were fixed in 10% buffered formalin and routinely processed to be embedded in paraffin.

Antibodies

Polyclonal antibodies (antibody M and N) raised against synthetic peptides of sheathlin (N against N-terminal residues 27–42 and M against residues 93–102 of sheathlin) were used. Detailed procedures for production of the antibody have been described previously [15].

Immunostaining of sheathlin

Serial 4.5-µm sections mounted on silicon-coated glass slides were used for immunohistochemical staining of sheathlin with a three-step SAB method using a commercially available streptavidin–biotin kit (Nichirei, Tokyo, Japan). The rabbit anti-pig sheathlin polyclonal antibody was applied (0.5 µg/ml) overnight at 4°C [26]. Peroxidase activity was developed with 0.02% 3-3' diaminobenzidine hydrochloride (DAB) in Tris-HCl containing 0.006% H_2O_2. Mayer's hematoxylin was used for counterstaining. As negative control, the primary antibody was substituted with normal rabbit serum at the same dilution.

Results

Two antibodies, M and N, showed similar immunostainability in the odontogenic tumors examined.

Tooth germ

Distinct immunolocalization of sheathlin was observed in immature enamel of tooth germs (Fig. 1). Secretory ameloblasts facing the enamel matrix also showed slight positive staining in their cytoplasm (Fig. 1). There was no immunoreactivity in the remaining components of tooth germs such as odontoblasts, predentin, dental papilla, and dental follicle.

Ameloblastoma

Regardless of growth pattern of the tumor, i.e., follicular or plexiform, there was neither intracellular staining for sheathlin in the tumor cells nor extracellular staining in the matrix of ameloblastomas examined (Fig. 2).

Calcifying epithelial odontogenic tumor

Tumor cells were negative for sheathlin (Fig. 3). Globular eosinophilic amyloid-like material characteristic of CEOT and calcified foci often seen in the eosinophilic material were also negative for the immunostaining of sheathlin (Fig. 3).

Adenomatoid odontogenic tumor

Distinct immunostaining of sheathlin was seen in a homogeneous eosinophilic substance in tumor cell nests (Fig. 4a). Positive reaction was most intensive at the periphery of the eosinophilic material. Mineralized foci, which were often seen in the eosinophilic material, were negative for sheathlin (Fig. 4b). Tumor cells facing the eosinophilic substance usually expressed the protein in their cytoplasm. Columnar tumor cells forming duct-like microcysts and convoluted structures and small polygonal cells between the duct-like structures were generally negative for sheathlin (Fig. 4b). Dysplastic dentin-like hyaline material in the stroma was negative for sheathlin. No obvious enamel formation was seen in the cases examined.

Ameloblastic fibro-odontoma

Neither epithelial component forming slender strands and islands nor cellular mesenchymal component resembling the dental papilla showed immunoexpression of sheathlin (Fig. 5a). In the areas where inductive hard tissue formation occurred, immature enamel and neighbor-

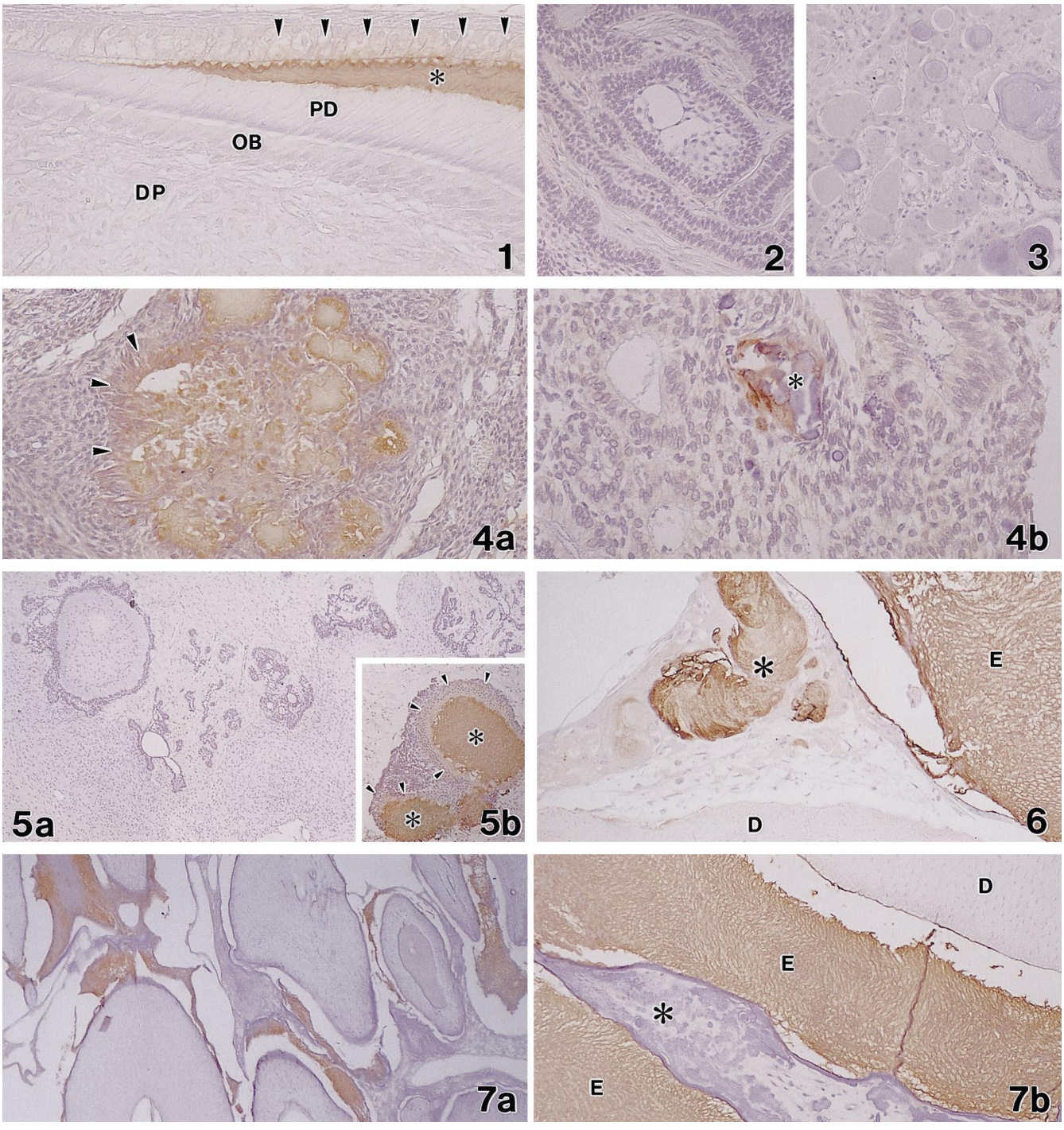

Fig. 1 Immunostaining of sheathlin in a tooth germ. Distinct positive reaction is observed in immature enamel (*). Secretory ameloblasts facing the enamel matrix also show slight positive staining in their cytoplasm (*arrowheads*). There is no positive staining in the remaining components of the tooth germ such as odontoblasts (*OB*), predentin (*PD*), and dental papilla (*DP*). ×280

Fig. 2 Immunostaining of sheathlin in ameloblastoma. There is neither intracellular staining for sheathlin in the tumor cells nor extracellular staining in the matrix. ×140

Fig. 3 Immunostaining of sheathlin in calcifying epithelial odontogenic tumor. Tumor cells are negative for sheathlin. Globular eosinophilic amyloid-like material and calcified foci are also negative. ×140

Fig. 4. Immunostaining of sheathlin in adenomatoid odontogenic tumor. **a** Distinct immunostaining of sheathlin is seen in eosinophilic droplets in tumor cell nests. Positive reaction is most intensive at the periphery of the eosinophilic material. Tumor cells facing the eosinophilic substance often express the protein in their cytoplasm (*arrowheads*). **b** Columnar tumor cells forming duct-like microcysts and convoluted structures and small polygonal cells between the duct-like structures are generally negative for sheathlin. Mineralized foci (*), which are often seen in the eosinophilic material, are usually negative for sheathlin. **a** ×140, **b** ×280

ing ameloblastic cells were stained positively for the protein (Fig. 5b).

Calcifying odontogenic cyst

Although epithelial cells were generally negative for sheathlin, ghost cells in the epithelial lining showed distinct immunoreactivity (Fig. 6). While dysplastic dentin laid down in the wall was negative for sheathlin, immature enamel in three cases with odontomas showed intensive immunostainability of sheathlin (Fig. 6).

Odontoma

Immature enamel was strongly positive for sheathlin (Fig. 7a, b). Other components such as dentin, cementum, and pulpal tissue were devoid of immunoreactivity (Fig. 7a, b). Cementum deposition on sheathlin-positive enamel matrix was the common feature in odontomas (Fig. 7b).

Control experiments

No immunostaining for sheathlin was seen in negative controls.

Discussion

Immunoexpression of sheathlin was first described in various odontogenic tumors in the present study. It has been reported that sheathlin is synthesized as a 55-kDa core protein and modified into a 70-kDa protein post-translationally in secretory ameloblasts, and secreted into the enamel matrix, especially in the prism sheath, with immediate post-secretory degradation of C-terminal polypeptides [26]. The antibodies used in the present study recognize N-terminal side residues (N against residues 27–42 and M1 against residues 93–102). Immunohistochemical and immunocytochemical studies of sheath proteins in the rat and pig using these antibodies showed positive staining in the secretory machinery of the secretory ameloblast and the entire thickness of the enamel matrix, especially the peripheral region of the enamel rod [15, 25, 26]. Well corresponding with those reported findings, the present study demonstrated that sheathlin was also expressed in the immature enamel and secretory ameloblasts facing the enamel matrix in the human tooth germ. In odontogenic tumors, distinct localization of sheathlin was demonstrated in enamel matrix formed in tumors with inductive hard tissue formation such as odontomas and ameloblastic fibroodontomas. In contrast to the distinct staining of sheathlin in enamel matrix, no positive reaction was seen in other hard tissues such as dentin, cementum and bone. Sheathlin was also expressed in neoplastic epithelial cells most of which were related to enamel matrix. Immunodetection of sheathlin is, therefore, considered to be a useful marker for functional differentiation of secretory ameloblasts and enamel matrix, which is often hard to differentiate from other hard tissues in odontogenic tumors.

Although the term “ameloblastoma” is a widely accepted name, it has been disputed that tumor cells of ameloblastoma differentiate into functional ameloblasts, worthy to be named “ameloblastoma”, because ultrastructural, histochemical and in vitro studies failed to provide definite proof that the tumor cells are actually neoplastic ameloblasts [1]. Analyses of the expression of enamel proteins give additional information for cellular differentiation of ameloblastomas. Saku et al. [18] demonstrated that neither amelogenin nor enamelin was expressed in ameloblastoma and concluded that ameloblastoma cells do not attain functional maturation as secretory phase ameloblasts. However, Mori et al. [14] reported positive expression of amelogenin in follicular ameloblastomas, especially in peripheral columnar cells. They also described immunoexpression of amelogenin in cystic areas of ameloblastoma, centrally located cells of nests and even granular cells. Furthermore, Snead et al. [23] demonstrated amelogenin mRNA expression in ameloblastomas by means of Northern and in situ hybridization analyses. Our findings from the view point of sheathlin expression support that the tumor cells of ameloblastomas do not attain full differentiation into functional ameloblasts.

One of the characteristic features of CEOT is the presence of homogeneous hyaline materials in which calcification is often found. Although the amyloid-like nature of the materials has been accepted based on their positive staining with Congo red, green birefringence under polarized light, and fluorescence with Congo red or Thioflavine T, there is considerable controversy as to the true nature of the hyaline material, including keratin [2], basal lamina [4], and enamel matrix [2, 12, 13]. Our previous electron microscopic and immunohistochemical study of CEOT demonstrated that the materials consisted of a dense accumulation of randomly oriented fibrils, and there was no positive staining with anti-laminin, type-IV collagen and keratin antibodies [24]. Mori et al. [14] studied the histochemical and immunohistochemical

◀ **Fig. 5.** Immunostaining of sheathlin in ameloblastic fibro-odontoma. **a** Neither epithelial component nor cellular mesenchymal component shows immunoexpression of sheathlin. **b** In the areas where inductive changes are present, immature enamel (*) and neighboring ameloblastic cells (*arrowheads*) are stained positively for sheathlin. ×40

Fig. 6 Immunostaining of sheathlin in calcifying odontogenic cyst. Ghost cells (*) show distinct immunoreactivity. While dysplastic dentin (*D*) laid down in the wall is negative for sheathlin, immature enamel (*E*) in cases with odontomas shows intensive immunostainability. ×140

Fig. 7. Immunostaining of sheathlin in odontoma. **a** Immature enamel is strongly positive for sheathlin. Other components such as dentin, cementum, and pulpal tissue are devoid of immunoreactivity. **b** Cementum deposition (*) is seen on sheathlin positive enamel matrix (*E*). *D* dentin. **a** ×40, **b** ×140

expression of intermediate filament proteins and enamel proteins in CEOT and concluded that the hyaline materials included enamel proteins. The positive staining for enamel proteins was also described as small immunoreactive patches at the periphery of the hyaline masses by Saku et al. [18]. Thus, in combination with the present study, it is considered that the hyaline material includes, most likely, enamel proteins such as amelogenin and enamelin, but not sheathlin. The absence of sheathlin may be explained by the independent synthetic and secretory pathways between amelogenin/enamelin and sheathlin or the different synthetic and secretory phases of cellular differentiation.

AOT is classified as a hamartomatous odontogenic tumor made up of odontogenic epithelium with odontogenic ectomesenchyme accompanied by dental hard tissue formation. Although the formation of the enamel matrix has been described in the tumor, it is very rare. In the present study, there was no case with enamel matrix that could be expected to be positive for sheathlin. Deposition of eosinophilic materials is frequently found between epithelial cells arranged in solid nests, in duct-like structures, and sometimes in the midst of opposing rows of cells arranged in a rosette pattern. It has been suggested that the material may be a basement-membrane-like material [3], abortive enamel [14, 16, 18, 22], or dentin matrix [19]. The present study shows sheathlin in the eosinophilic droplets in the solid nests and in the cytoplasm of the tumor cells surrounding the droplets. Comparing this immunohistochemical localization of sheathlin with those in tooth germs, the tumor cells are considered to differentiate to the degree of functional ameloblasts and secrete sheath proteins in the hyaline material. The enamel nature of the material is supported by the immunohistochemical studies on amelogenin and enamelin [18]. In the present study, mineralized foci in the hyaline materials were negative for sheathlin, while the peripheral area of eosinophilic material and the surrounding cells were positive for the protein. This disappearance with calcification may be explained by the fate of sheathlin during the normal amelogenesis that sheathlin is quickly degraded and eliminated from enamel with its maturation [25]. Histological features of the high columnar cells forming a duct-like structure give an impression that the cells differentiate into functional ameloblasts. However, no obvious expressions of enamel proteins in these cells were seen in either the present study or previous studies [14, 18]. The columnar tumor cells forming duct-like structures without sheathlin-positive hyalinous materials seem not to fully differentiate into secretory ameloblasts, as seen in ameloblastomas.

The most remarkable feature of COC is the presence of ghost cells that often undergo calcification. Although ghost cells have been considered to represent a form of keratinization, as indicated by its synonym; keratinizing and calcifying epithelial cyst, it has also been suggested that the cells were involved only in a degenerative procedure [9] or in the formation of enamel matrix [7, 14]. The present study showed distinct immunopositivity of sheathlin in ghost cells, as seen in the enamel matrix of odontoma in the wall of COC (Fig. 6). Mori et al. [14] described positive staining of amelogenin in ghost cells. These findings suggest that abnormal accumulation of enamel proteins may account for the presence of ghost cells in COC.

It is very interesting that epithelial cells in odontogenic tumors can differentiate into functional ameloblasts without induction by odontogenic mesenchyme. Namely immunoexpression of sheathlin was observed in eosinophilic droplets within solid epithelial sheets in AOT and ghost cells in the epithelial lining of COC where inductive participation of mesenchymal cells was most unlikely. In the neoplastic transformation of odontogenic epithelium, cellular differentiation to secretory ameloblasts may occur without the mesenchymal participation.

Cementum deposition on sheathlin-positive enamel matrix was the common feature in odontomas. Since Slavkin and his coworkers [20, 21] proposed the epithelial origin of cementum, many studies have been carried out to provide evidence for roles of enamel-related proteins on root cementum. Recently, Hammerström and his colleagues [6, 8] postulated that amelin, a rat molar analogue of sheathlin, might be one of the key proteins coupled to the process of cementogenesis. The present finding observed in odontomas may support these hypotheses.

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