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Original

Localization of Oxytalan fiber, Type III Collagen and BMP Family in Conventional and Desmoplastic Ameloblastoma

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Abstract:The histologic hallmark distinguishing desmoplastic ameloblastoma (DA) from conventional ameloblastoma (CA) is its pronounced stromal desmoplasia, and this formed the basis of this investigation. To elucidate the stromal characteristics, localization patterns of oxytalan fibers, type III collagen and BMP family in DA (*n*=8) was compared with CA (*n*=24), and periodontal ligament (PL) (*n*= 8). Oxytalan fibers formed apico-occlusal bundles in PL, thick radial bundles around tumor nests in DA, and as scanty fibers in CA. Type III collagen was identified in PL, strongly expressed in DA stroma, but weakly in CA. BMP-2, -3, -4 and -7 expression patterns in tumor epithelium and stroma were more pronounced in DA (including sites of bone formation), than CA. No immunoreactivity for BMP-5 and -6 were detected. Current findings suggest that the stroma in DA is neoplastic and derived from odontogenic ectomesenchyme, and recommends its reclassification as an odontogenic epithelial-ectomesenchymal neoplasm.

Key words: Odontogenic neoplasm, Ameloblastoma, Matrix proteins, BMP family

Introduction

Odontogenic tumors are a rare but unique group of neoplasms thought to be related to the odontogenic apparatus because they contain odontogenic epithelial and/or ectomesenchymal tissues that morphologically resemble those occurring during tooth organogenesis. Hence, the histologic appearances of these tumors often recapitulate the various stages of the developing tooth germ [1-3)]. For this reason, in the revised WHO *Histological Typing of Odontogenic Tumors*, odontogenic tumors are classified according to whether there is odontogenic epithelial, ectomesenchymal or epithelial-ectomesenchymal tissue participation[2)]. The ameloblastoma is classified as an odontogenic epithelial neoplasm because of its putative origin from the enamel organ[2-4)]. Hence the dominating histological feature in the conventional or classical ameloblastoma is an odontogenic tumor epithelium comprising a peripheral layer of polarized pre-ameloblast-like cells enclosing centrally, stellate reticulum-like cells, and these are disposed as islands and plexiform strands in a mature connective tissue stroma. However, in some histological subtypes of ameloblastoma, notably the desmoplastic variant, pronounced stromal desmoplasia and frequent osteoplasia also constitute important histological characteristics[5, 6)].

There are various types of matrix proteins expressed during tooth morphogenesis, and their functional roles and mechanisms of action have been detailed elsewhere[7-12)]. Among these matrix proteins, type III collagen and oxytalan connective tissue fibers purportedly formed the most significant components in the periodontal ligament and odontogenic ectomesenchyme[11-16)]. Hence, these proteins are often used as markers to establish the functional characteristics of these tissues.

Bone morphogenetic protein (BMP) was originally described by Urist, who demonstrated the ability of a bone inductive extract to induce bone formation in ectopic sites in rodents[17)]. Since then at least six related members of this family have been identified and

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Table 1. Grade of staining intensity for matrix proteins in conventional and desmoplastic ameloblastoma

MP	Conventional ameloblastoma		Desmoplastic ameloblastoma	
	Epithelium	Stroma	Epithelium	Stroma
OF	-	±	-	++
Col III	-	±	-	++
BMP-2	+	±	++	++
BMP-3	±	±	++	++
BMP-4	±	±	++	++
BMP-5	-	-	-	-
BMP-6	-	-	-	-
BMP-7	±	±	++	++

MP: Matrix protein; OF: Oxytalan fibers; Col III: Collagen type III; BMP: Bone morphogenetic protein;
Grade of staining intensity (-: negative; ±: weak and focal; +: moderate; ++: strong)

are named BMP-2 through BMP-7[18]. These molecules formed part of the TGF-β superfamily. The BMP family plays multiple roles in the differentiation of many tissues, and in the formation, development and patterning of several different organ systems including tooth germs. In the latter, BMP family acts as regulators in the sequential and reciprocal epithelial-mesenchymal interactions during tooth morphogenesis[19-24]. These interactions in turn determine cell growth and differentiation in the developing tooth germ[19-24].

Although the expression patterns of some matrix proteins including amelogenin[7], collagen[10-12], fibronectin[12] and BMP family[19-24] have been studied in relation to odontogenesis, much less is known of their distribution in odontogenic tumors. Previous studies have investigated the patterns of expression of specific collagen (IV) chains in the basement membrane of various types of odontogenic tumors including the desmoplastic variant of ameloblastoma, and found that these proteins may serve as diagnostic markers for distinguishing benign odontogenic tumors from their malignant counterparts, and as indicators of the infiltrative and progressive growth potential of these neoplasms [25, 26]. The distribution patterns of other matrix proteins including tenascin[27, 28], fibronectin[27, 28], laminin [28], type VII collagen[28], amelogenin[29, 30] have also been studied in the odontogenic tumors including ameloblastomas. However, reports on the localization of type III collagen[31], oxytalan fibers[32, 33] and BMP family[24, 34] in odontogenic tumors are limited. In this study, we investigated the localization of all these three proteins in two types of odontogenic tumors, namely conventional ameloblastoma and desmoplastic ameloblastoma in an attempt to gain some insights into the stromal characteristics of the latter.

Materials and Methods

Tissue samples

The source of the sample used in this study was from the surgical pathology files of the Department of Oral Pathology and Medicine, Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences Okayama University. Archival formalin-fixed, paraffin embedded tissue blocks of 24 ameloblastomas (12 follicular, and 12 plexiform) and 8 desmoplastic ameloblastomas were used as experimental samples. 8 nonlesional periodontal tissues of jaw bones served as normal controls. Both experimental and control samples were retrieved and new 4μm sections prepared for routine staining with hematoxylin-eosin, special staining with Gomori's aldehyde fuchsin following oxidation with potassium monopersulfate to demonstrate oxytalan fibers[35], and for immunohistochemistry. All tumors were histologically diagnosed and classified according to the WHO *Histological Typing of Odontogenic Tumours* and other established criteria in current use[2-5].

Monoclonal antibodies

Rat monoclonal antibodies for human BMP-2 (N-14), BMP-3 (N-19), BMP-4 (N-16), BMP-5 (N-19), BMP-6 (N-19), BMP-7 (N-19) (Santa Cruz, CA, USA), and type III collagen were used in this study.

Immunohistochemistry

For immunohistochemical staining, deparaffinized sections were immersed in 0.3% methanol containing 1% hydrogen peroxide for 30 min to block endogenous peroxidase and rinsed in 0.05 M TBS (5 min, 3 times) before immersing in blocking solution (Funakoshi, Japan) for 10 min at room temperature. Thereafter the sections were covered with the primary antibody and incubated overnight at 4°C. The immunoreaction was performed using a Vectastain peroxidase ABC kit (Vector Laboratories, Burlingame, CA, USA). The antigenic sites were

demonstrated by reacting sections with a mixture of 0.05% 3,3'-diaminobenzidine tetrahydrochloride (Vector Laboratories, Burlingame, CA, USA) in 0.05M Tris-HCl buffer, pH 7.6, containing 0.01% H_2O_2 for 7 min. The nuclei were counterstained with Mayer's hematoxylin. For negative control, sections were reacted with normal rat serum or with the secondary antibody alone. All the control sections were negative. Positive staining controls were included for each antibody and, where present in the specimens, internal staining controls were also checked for appropriate reactions with each antibody.

Results

Table 1 compares the intensity of staining for oxytalan fibers, type III collagen and BMP family between the conventional and desmoplastic ameloblastoma. The localization patterns of these proteins in the periodontal ligament, conventional and desmoplastic ameloblastoma are detailed below.

Localization of type III collagen and oxytalan fibers

Periodontal ligament (n = 8)

Type III collagen was identified as fine fibrils in the periodontal ligament and cementum of the control samples (Fig 1-A). Oxytalan fibers formed slender bundles running in an apico-occlusal direction in the periodontal ligament, and inserting into the cementum in these same samples (Fig. 1-B).

Conventional ameloblastoma (n=24)

Twelve cases each of follicular and plexiform ameloblastoma were examined. In both these histological variants, type III collagen showed weakly positive immunoreactivity in the stromal tissues. (Oxytalan fibers were few and scantily distributed in the stromal tissues of these tumors.

Desmoplastic ameloblastoma (n=8)

Type III collagen was strongly expressed in the stromal tissues of all cases of desmoplastic ameloblastomas (Fig. 2-A). Oxytalan fibers formed dense bundles with a radial pattern around the odontogenic epithelial tumor nests of these neoplasms (Fig. 3-b).

Localization of BMP family

Conventional ameloblastoma (n=24)

In both the follicular and plexiform ameloblasoma cases studied, some peripheral cells of small odontogenic epithelial tumor nests and bud-like structures showed positive immunoreactivity for

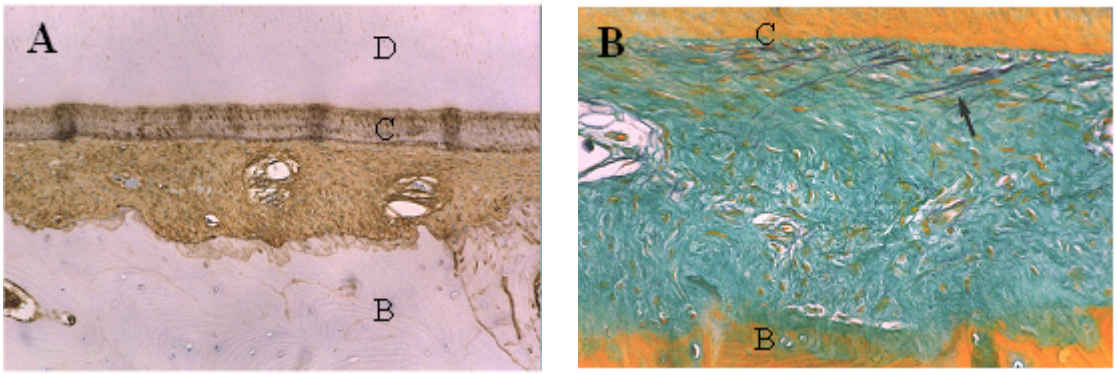

Figure. 1. Histological sections of normal periodontal ligament (A, B). A: Type III collagen (COL III) was identified as fine fibrils in the periodontal ligament and cementum (C). B: Oxytalan fibers (OF) (arrow) formed slender bundles running in an apico-occlusal direction in the periodontal ligament, and inserting into the cementum (C) in these same samples. (A: anti type III collagen, x100; B: aldehyde fuchsin stain, x200). [B: Bone; D: Dentine].

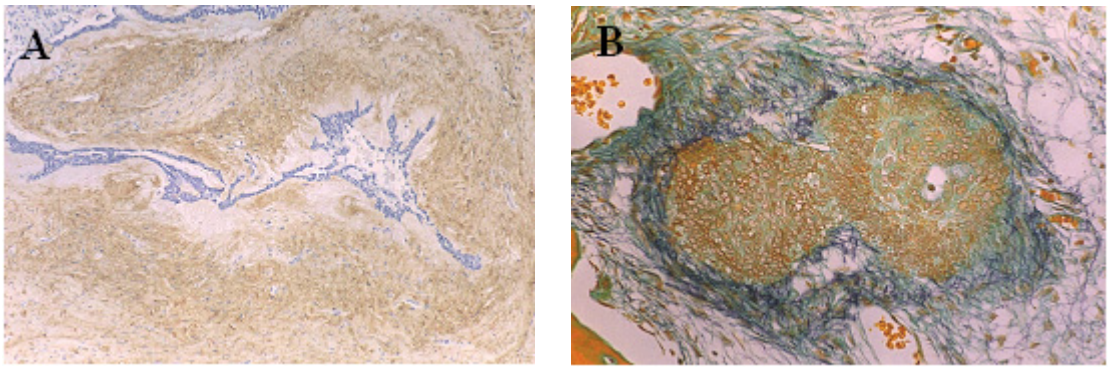

Figure. 2. Histological sections of desmoplastic ameloblastoma (A, B). A: Type III collagen (COL III) showed strong expression in the stromal tissues. B: Oxytalan fibers (OP) formed thick radial bundles around the odontogenic epithelial tumor nests. (A: anti type III collagen, x100; B: aldehyde fuchsin stain, x200).

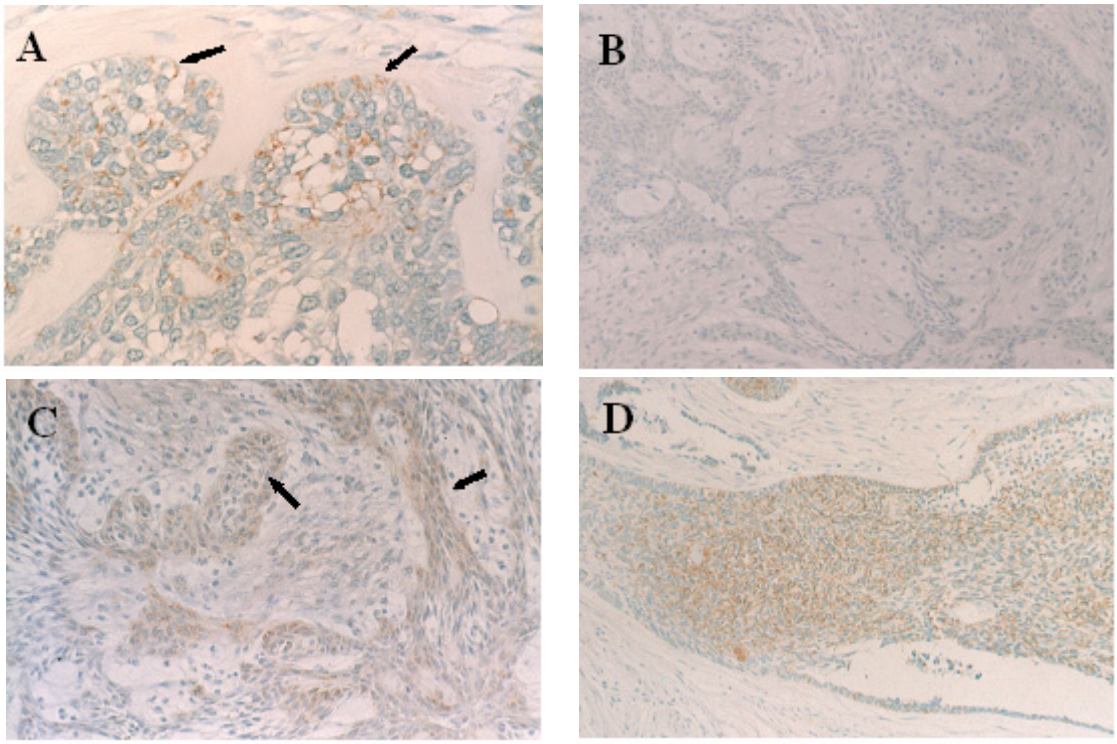

Figure. 3. Histological sections of conventional ameloblastoma and BMP localization (A-D). A, C: Some peripheral cells of small odontogenic epithelial tumor nests and bud-like structures (arrows) showed positive immunoreactivity for BMP-2 and -4. D: BMP-7 was usually localized in the peripheral epithelial cells (arrows) of the tumor nests, and occasionally as a strong intracellular staining within their central cells. (A: anti BMP-2, x400; B: anti BMP-3, x100; C: anti BMP-4, x200; D: anti BMP-7, x100).

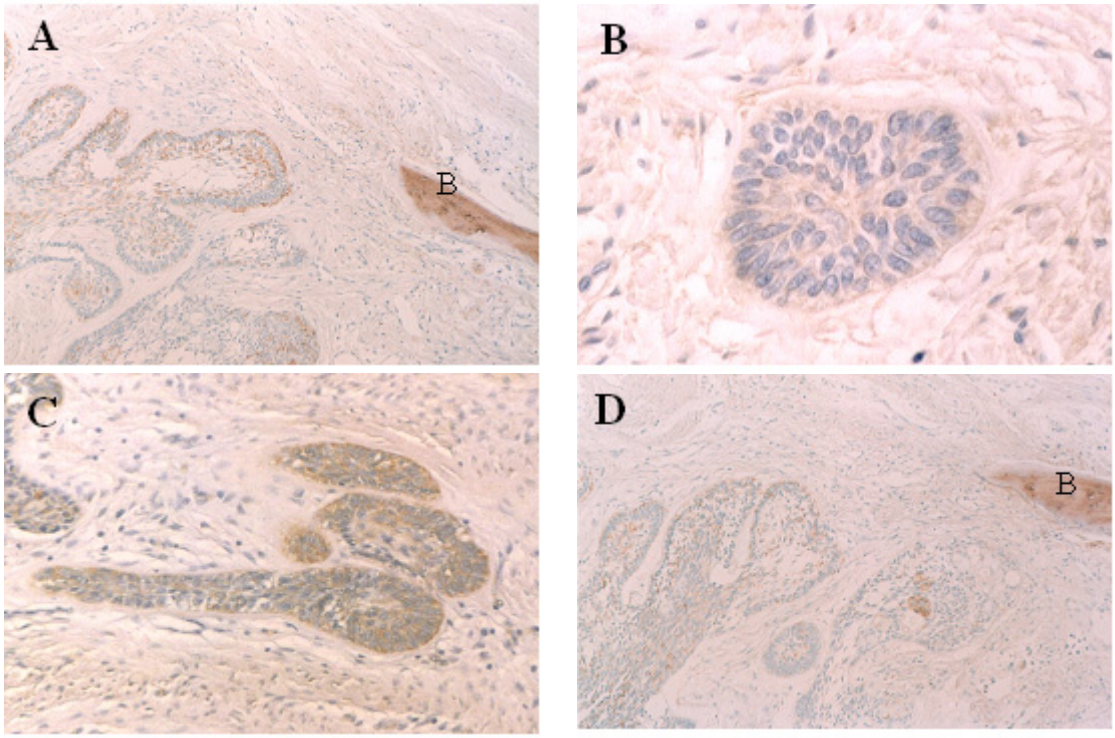

Fig. 4. Histological sections of desmoplastic ameloblastoma and BMP localization. (A-D). A-D: Some peripheral cells of small odontogenic epithelial tumor nests and bud-like structures (arrows) show strong positive immunoreactivity for BMP-2, -3, -4, and -7. BMP-7 is usually localized in the peripheral epithelial cells (arrows) of the tumor nests, and occasionally as a strong intracellular staining within their central cells. A diffuse immunopositivity for BMP-2, -3, -4 and -7 was detected in the stroma, especially around areas of new bone formation (A: anti BMP-2, x100; B: anti BMP-3, x400; C: anti BMP-4, x200; D: anti BMP-7, x100).

BMP-2 and -4 (Figs.3-A, C). For BMP-7, the expression was usually localized in the peripheral epithelial cells of these tumor nests. However, in some tumors, a strong intracellular staining was detected within the central cells of these tumor nests (Fig. 3-D). No immunoreactivity for BMP-5 and -6 were seen in all cases of conventional ameloblastoma studied.

Desmoplastic ameloblastoma *(n=8)*

In the desmoplastic ameloblastoma, the same pattern of localization for BMP-2, -4 and -7 was observed in the peripheral cells of small odontogenic epithelial tumor nests and bud-like structures (Figs. 4-A, C and D), but the intensity of staining was comparatively stronger than in the conventional ameloblastoma. Similarly, BMP-7 was usually localized in the peripheral epithelial cells of these tumor nests, but occasionally showed strong intracellular staining in their central cells (Fig. 4-D). No immunoreactivity for BMP-5 and 6 were seen in all cases of desmoplastic ameloblastoma studied.

With respect to stromal expression, a diffuse immunopositivity for BMP-2, -3, -4 and -7 was detected in the stroma, especially around areas of new bone formation (Fig. 4-A, B, C, and D). No immunoreactivity for BMP-5 and -6 were seen.

Discussion

In the revised WHO *Histological Typing of Odontogenic Tumours*, odontogenic tumors are grouped according to their putative tissue(s) of origin into odontogenic epithelial, epithelial-ectomesenchymal, and ectomesenchymal neoplasms[2)]. The ameloblastoma is grouped under the first category i.e. benign neoplasm of odontogenic epithelium without odontogenic ectomesenchyme because of the widely accepted view that it develops from the enamel organ and its derivatives[2-6)]. The conventional or classical form of ameloblastoma is composed of islands and nests of odontogenic epithelium in a mature connective tissue stroma. Since its original description, the histological spectrum of the ameloblastoma has expanded to include granular, basal, clear cell, acanthomatous, and more recently the desmoplastic subtype[1-6)]. The latter, first described as a variant of ameloblastoma by Eversole in 1984[5)], differs from the conventional ameloblastoma in many respects. Clinically and radiographically, this tumor variant mimics benign fibro-osseous lesions of the jaws in presenting as a slowly expansive, painless bony swelling, and exhibiting a mixed radiolucent-radiopaque appearance[6)]. Microscopically, it is characterized by pronounced stromal desmoplasia.[2, 5, 6)]. Some authors considered this desmoplastic change as a stromal fibroblastic reaction in ameloblastoma[5, 6)]. Others demonstrated the presence of oxytalan fibers, and alluded to an origin from the periodontal ligament[32, 33)] (to be discussed further later). We believed that the mesenchymal component of the desmoplastic ameloblastoma does not represent ordinary supporting connective tissues, but is *pari pasu* of its entire neoplastic process. A probable odontogenic ectomesenchymal origin was also speculated here, and we sought to validate this by examining the various mesenchymal elements for expression of oxytalan fibers, type III collagen and BMP family. The reasons for selecting these protein markers are discussed below.

Oxytalan fiber is the name first coined by Fullmer to describe the occurrence of 10 -12 nm wide microfibrils that formed apico-occlusal bundles in the connective tissues of the periodontal ligament[13)]. These fibers perform several functions including anchoring, maintenance of elasticity, and as a guideline for cell migration. Subsequent to Fullmer's report, oxytalan fibers were also identified in neoplasms of putative periodontal ligament origin, notably benign fibro-osseous jaw lesions[36)]. Recently, oxytalan fibers were also detected in the stroma of desmoplastic ameloblastoma, and some of these fibers extended from the periodontal ligament to penetrate the tumor stroma [33, 34)]. These observations led the investigators to suggest that the desmoplastic ameloblastoma tumor epithelium probably arose from the epithelial cell rests of Malassez, while its stroma developed from the periodontal ligament mesenchymal tissues[33, 34)]. In the present study, oxytalan fibers were also identified as fine fibrils scattered throughout the intervening stroma in all 8 cases of desmoplastic ameloblastoma, but were scanty in the conventional ameloblastomas studied. Another added observation in the desmoplastic ameloblastoma was the tendency of these fibers to concentrate around tumor epithelial nests to form thick radial bundles. This distinctive feature led us to speculate that the peritumoral oxytalan fibrous condensation may be the result of an inductive interaction between two neoplastic components i.e odontogenic tumor epithelium and ectomesenchymal tissues.

Type III collagen occurs in the periodontal ligament with a significant proportion compared to other connective tissue constituents, and therefore confers a functional characteristic to this tissue[16)]. It has also been localized in the dental follicle and ectomesenchymal tissues, and has been implicated to play some roles during tooth formation [8, 9)]. In the present study, type III collagen was strongly expressed by the mesenchymal cells of the desmoplastic ameloblastoma, but was weakly positive in the stroma of the conventional ameloblastoma. This observation indirectly supports an ectomesenchymal origin for the stromal cells of the desmoplastic ameloblastoma, and further confirms that these tissues do not represent ordinary supporting connective tissues.

The BMP family members are multifunctional proteins with diverse in vivo biological roles that range from regulating formation of bone and cartilage, to the development and patterning of several different organ systems including tooth germs, and all these have been extensively studied[17-24)]. In contrast, little is known of the roles of BMP family in diseased states, and most reports were related to bone neoplasms and soft tissue sarcomas [37-39)], and only one report describing its distribution in odontogenic tumors

has been documented to date[34]. In this previous study, antibody against BMP extracted from bovine bone was used to demonstrate the expression of this protein in odontogenic tumors[34]. All ameloblastomas and adenomatoid odontogenic tumors reportedly showed a negative reaction[34]. In the present study, immunolocalization patterns of BMP-2, -3, -4 and -7 in both the conventional and desmoplastic ameloblastomas were studied, and compared with those of published reports on the developing tooth germ in order to help elucidate their functional significance[19-23]. In all these instances, the desmoplastic ameloblastoma demonstrated a stronger immunoexpression pattern than the conventional ameloblastoma. In our analysis, we found that the expression patterns of BMP-2, -4 and -7 in the peripheral cells of tumor nests and bud-like structures of these neoplasms correlate closely with those in tooth germ at the initial and bud stage [21-23]. Furthermore, BMP-7 localized in the central cells of ameloblastoma tumor nests, also corresponds with its expression in the stelllate reticulum of cap stage tooth germ[21]. In the developing tooth germ, BMP-2, -4, and -7 formed parts of an intricate signaling network regulating tooth initiation and shape development[19, 22, 23]. From a functional viewpoint, there is a possibility that these same proteins also play regulatory roles in determining the shape development of ameloblastomas. Furthermore, it has been shown that BMP-2 induces functional differentiation of ameloblasts during tooth morphogenesis [20]. A similar expression pattern noted in the ameloblastoma implies that these proteins are also involved in the functional differentiation of the tumor cells. In the desmoplastic ameloblastoma, BMP-2, -3, -4 and -7 showed a comparatively stronger immunoreactivity in the stromal cells than those in the conventional ameloblastoma. This finding suggests that these proteins may be engaged in the functional characterization of the stromal tissues. Aberg et al reported that during tooth morphogenesis, BMP-3 expression is restricted to ectomesenchymal cells, in particular, those dental follicle cells which terminally differentiate into cementoblasts and secrete the cementum matrix[22]. Therefore, strong immunolocalization of BMP-2, -3, -4 and -7 in the mesenchymal tissues around the bone-like structures of the desmoplastic ameloblastoma also suggest the possibility of these BMP family members participating in the formation of hard tissues in this neoplasm.

In summary, the localization of oxytalan fibers, type III collagen and BMP family in the desmoplastic ameloblastoma was studied and compared with those of the conventional ameloblastoma in an effort to shed light on the stromal characteristics of the former. The expression patterns of these proteins were compared with those of the developing tooth germ in an attempt to understand better the functional significance of these proteins within the neoplastic environment. From the results of this study, we concluded that there is sufficient evidence to indicate that the mesenchymal cells of the desmoplastic ameloblastoma are active cells that might be from the neural crest-derived ectomesenchyme, and that they might function in a neoplastic manner alongside their odontogenic epithelial counterpart. Based on these findings, we recommend a relook into the revised WHO *Histological Typing of Odontogenic Tumours*, and reclassifying the desmoplastic ameloblastoma in the category of 'neoplasm with odontogenic epithelium and ectomesenchyme'. In line these recommendations, we would also like to suggest renaming this neoplasm as '*desmoplastic epithelial odontogenic tumor*' to emphasize its mixed odontogenic tissue origin.

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