Immunohistochemical Analysis of Benign Mixed and Mesenchymal Odontogenic Tumors

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Benign mixed and mesenchymal odontogenic tumors (ameloblastic fibroma, ameloblastic fibrodentinoma, ameloblastic fibro-odontomas, odontogenic fibroma and odontogenic myxoma) were immunohistochemically examined using antibodies against amelogenin, cytokeratin 19, bcl-2, hepatocyte growth factor (HGF), c-Met, transforming growth factor- β (TGF- β), TGF- β receptors and Ki-67 to know cytodifferentiation, epithelial-mesenchymal interaction and proliferative activity. Expression of amelogenin was detected in odontogenic epithelial cells and enamel matrices in ameloblastic fibro-odontomas, and cytokeratin 19 was expressed in odontogenic epithelial cells, indicating odontogenic epithelial properties. Expressions of bcl-2, HGF, TGF- β , T β R I and T β R II were detected in odontogenic epithelial and mesenchymal cells in mixed and mesenchymal odontogenic tumors, while expression of c-Met was detected in odontogenic epithelial cells. Odontogenic epithelial and mesenchymal cells sporadically showed positive reactions for Ki-67 in mixed and mesenchymal odontogenic tumors, and no apparent differences were obtained among these tumors or between the components. Conclusion: Immunohistochemical localization of HGF, TGF- β and their receptors suggests that these growth factor signaling are effective in the epithelial component in paracrine and autocrine manner in mixed and mesenchymal odontogenic tumors. These results of growth factor signaling suggest that certain epithelial-mesenchymal interactions are related to neoplastic cell growth in these tumors.

Key words: cytodifferentiation, epithelial-mesenchymal interaction, immunohistochemistry, mesenchymal odontogenic tumors, mixed odontogenic tumors, proliferative activity

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Introduction

Mixed odontogenic tumors are a group of lesions composed of odontogenic epithelial and mesenchymal components with inductive interactions leading to the formation of dentin and then enamel, and include ameloblastic fibroma, ameloblastic fibrodentinoma, ameloblastic fibro-odontoma, ontoameloblastoma, and odontoma (1-3). A sequence of ameloblastic fibroma, ameloblastic fibrodentinoma and ameloblastic fibro-odontoma is the representative mixed odontogenic tumor and occurs in children and young adults without gender difference (2, 4). These tumors most frequently involve the premolar-molar region of the mandible (1, 2, 4).

Radiographs of these tumors show well-defined

radiolucency, and ameloblastic fibrodentinoma and ameloblastic fibro-odontoma exhibit various degrees of radiopaque materials (3). Histopathologically, ameloblastic fibroma consists of proliferating odontogenic epithelium and cellular mesenchymal tissue resembling dental papilla, and ameloblastic fibrodentinoma and ameloblastic fibro-odontoma also exhibit formation of dentin and enamel (1, 3, 4).

Mesenchymal odontogenic tumors are originated from odontogenic mesenchymal tissues with or without odontogenic epithelial cells, and comprise odontogenic fibroma, odontogenic myxoma and benign cementoblastoma (3). Odontogenic fibroma and odontogenic myxoma are the representative mesenchymal odontogenic tumors, and

possess considerably different characteristics. Odontogenic fibroma occurs in a wide age range with a female predilection, whereas odontogenic myxoma most frequently occurs in the second or third decade without gender difference (5, 6). A frequent site of odontogenic fibroma is the maxilla, while odontogenic myxoma is common in the maxilla and mandible (6, 7, 8). Radiographically, odontogenic fibroma usually shows a unilocular radiolucency, while odontogenic myxoma reveals multiple radiolucent areas, called a "soap-bubble appearance" (5, 7, 9). Histologically, odontogenic fibroma consists of a more cellular fibrous tissue containing varying amounts of inactive odontogenic epithelium, and odontogenic myxoma consists of rounded and angular mesenchymal cells in abundant mucoid stroma with or without inactive-looking odontogenic epithelium (3, 10).

Some immunohistochemical studies have been carried out in mixed or mesenchymal odontogenic tumors (11-18). Since these mixed and mesenchymal odontogenic tumors are rare, detailed examination on these tumors has not been carried out until recently. Mixed odontogenic tumor, ameloblastic fibroma, ameloblastic fibrodentinoma, and ameloblastic fibro-odontoma, are odontogenic epithelium with odontogenic ectomesenchyme, with or without dental hard tissue formation by epithelial-mesenchymal interaction, but mesenchymal odontogenic tumors, odontogenic fibroma and odontogenic myxoma are odontogenic ectomesenchyme with or without included inactive-looking odontogenic epithelium. It is thought that the epithelial islands of odontogenic fibroma or odontogenic myxoma sometimes appears to be included by chance rather than playing any essential role in the pathogenesis of the lesion (3). In the present study, we immunohistochemically examined the representative mixed and mesenchymal odontogenic tumors using antibodies against amelogenin, cytokeratin 19, bcl-2, hepatocyte growth factor (HGF), c-Met, transforming growth factor- β (TGF- β), TGF- β receptors and Ki-67, to characterize the cytodifferentiation, epithelial-mesenchymal interaction and proliferative activity of

these tumors.

Material and methods

Tissue preparation

Specimens were surgically removed from seven patients with mixed or mesenchymal odontogenic tumor at Tohoku University Dental Hospital. The clinical characteristics of the seven patients are shown in Table 1. Tumor tissues were fixed in 10% buffered formalin for a few days and embedded in paraffin wax. Some specimens with calcified tissues were decalcified in Plank-Rychlo solution or 10% formic acid solution for 2 or 3 days. Serial sections, 3mm thick, were taken from the tissue blocks and processed for routine histopathological and subsequent immunohistochemical studies. Tissue sections were stained with hematoxylin and eosin, and tumors were diagnosed according to the World Health Organization (WHO) histological typing of odontogenic tumors (3). Seven surgical specimens consisted of 1 ameloblastic fibroma, 1 ameloblastic fibrodentinoma, 2 ameloblastic fibro-odontomas, 1 odontogenic fibroma and 2 odontogenic myxomas.

Immunohistochemical studies

The serial sections were deparaffined and immersed in methanol with 0.3% hydrogen peroxide to eliminate endogenous peroxidase activity. For antigen retrieval, the sections for bcl-2, c-Met, TGF- β and Ki-67 immunostaining were boiled by autoclave (121°C, 2 atm) in 0.01 M citrate buffer (pH 6.0) for 10 min, and the sections for cytokeratin 19 immunostaining and HGF immunostaining were warmed by water bath (37°C) in 0.1% trypsin (pH 7.6) for 30 min. After treatment with normal goat or rabbit serum for 15 min to block non-specific binding, the sections were incubated with primary antibodies at 4°C overnight. The applied antibodies are listed in Table 2. The standard streptavidin-biotin-peroxidase complex method was performed to bind the primary antibodies with the use of Histofine SAB-PO kits (Nichirei, Tokyo, Japan). Reaction products were visual-

Table 1: Clinicopathological data of mixed and mesenchymal odontogenic tumors

Case No.	Age	Sex	Location (tooth formula)	Clinical finding	Radiological finding	Treatment	Histopathological diagnosis
1	3	M	Maxilla (52)	Unerupted 52	Radiolucency	Extirpation	Ameloblastic fibroma
2	8	M	Maxilla (16)	Unerupted 16	Radiolucency with radiopaque material	Extirpation	Ameloblastic fibrodentinoma
3	10	F	Mandible (37)	No symptom	Radiolucency with radiopaque material	Extirpation	Ameloblastic fibro-odontoma
4	15	M	Mandible (37, 38)	Swelling	Radiolucency with radiopaque material	Extirpation	Ameloblastic fibro-odontoma
5	19	F	Mandible (34, 35)	Swelling	Radiolucency	Extirpation	Odontogenic fibroma
6	17	F	Maxilla (25, 26)	No symptom	Radiolucency	Extirpation	Odontogenic myxoma
7	21	M	Mandible (37)	No symptom	Radiolucency	Extirpation	Odontogenic myxoma

ized by immersing the sections for 1 to 10 min in 0.03% diaminobenzidine (DAB) solution containing 2 mM hydrogen peroxide. Nuclei were lightly counterstained with 1% methyl green. Control sections were treated with phosphate-buffered saline (PBS), normal rabbit IgG and mouse IgG instead of the primary antibodies and were confirmed to be unstained.

Evaluation of immunostaining

Immunostainings for amelogenin, cytokeratin 19, bcl-2, HGF, c-Met, TGF- β , TGF- β type I receptor (TbR I)

and TGF- β type II receptor (TbR II) were evaluated by detection of immunoreactivities. Labeling indices for Ki-67 immunostaining (Ki-67-LI) were calculated by counting the positive cells among more than 200 odontogenic epithelial cells and mesenchymal cells in randomly selected fields.

Results

Immunohistochemical reactivity for amelogenin was detected in the cytoplasm of odontogenic epithelial cells and enamel matrices in ameloblastic fibro-odonto-

Table 2: Antibodies used

Antibody	Clonality	Source	Dilution	Pretreatment
Amelogenin	Polyclonal	Sangi, Otaru, Japan	1: 200	—
Cytokeratin19	Monoclonal	Dako, Glostrup, Denmark	1: 50	Trypsin
bcl-2	Monoclonal	Dako	1: 20	Autoclave
Hepatocyte growth factor (HGF)	Polyclonal	IBL, Fujioka, Japan	1: 20	Trypsin
c-Met	Monoclonal	Novocastra, Newcastle, UK	1: 20	Autoclave
Transforming growth factor (TGF)- β	Monoclonal	Genzyme/Techne, Cambridge, MA, USA	1: 10	Autoclave
TGF- β type I receptor (TbR I)	Polyclonal	Santa Cruz, Santa Cruz , CA, USA	1: 50	—
TGF- β type II receptor (TbR II)	Polyclonal	Santa Cruz	1: 50	—
Ki-67	Monoclonal	Immunotech, Marseille, France	pre-diluted	Autoclave

Fig. 1: Immunohistochemical reactivity for amelogenin, cytokeratin (CK) 19 and bcl-2 in mixed and mesenchymal odontogenic tumors. A: Ameloblastic fibro-odontoma showing amelogenin expression in odontogenic epithelial cells and enamel matrix (¥ 150). B: Odontogenic fibroma showing no expression of amelogenin (¥ 150). C: Ameloblastic fibro-odontoma showing CK 19 expression in odontogenic epithelial cells (¥ 150). D: Odontogenic myxoma showing bcl-2 expression in odontogenic epithelial cells and mesenchymal cells (¥ 150).

Fig. 2: Immunohistochemical reactivity for hepatocyte growth factor (HGF), transforming growth factor- β (TGF- β) and their receptors in mixed and mesenchymal odontogenic tumors. A: Ameloblastic fibroma showing HGF expression in odontogenic epithelial cells and mesenchymal cells (¥ 150). B: Odontogenic myxoma showing c-Met expression in odontogenic epithelial cells (¥ 150). C: Ameloblastic fibro-odontoma showing TGF- β expression in odontogenic epithelial cells and mesenchymal cells (¥ 150). D: Odontogenic fibroma showing transforming growth factor- β type I receptor expression in odontogenic epithelial cells and mesenchymal cells (¥ 150).

Fig. 3: Immunohistochemical reactivity for Ki-67 in mixed and mesenchymal odontogenic tumors. Ameloblastic fibro-odontoma (A) and odontogenic myxoma (B) showing Ki-67 expression in sporadic odontogenic epithelial cells and mesenchymal cells (¥ 150).

mas, and mesenchymal components were devoid of amelogenin staining (Fig. 1A). No expression of amelogenin was confirmed in the ameloblastic fibroma, ameloblastic fibrodentinoma, odontogenic fibroma and odontogenic myxomas (Fig. 1B). Expression of cytokeratin 19 was found in the cytoplasm of odontogenic epithelial cells in all mixed and mesenchymal odontogenic tumors,

and mesenchymal cells were devoid of cytokeratin 19 staining (Fig. 1C). Immunohistochemical reactivity for bcl-2 was detected in the cytoplasm of odontogenic epithelial and mesenchymal cells in mixed and mesenchymal odontogenic tumors (Fig. 1D).

HGF and TGF- β were expressed in the cytoplasm of odontogenic epithelial and mesenchymal cells in mixed

Table 3: Immunohistochemical findings of mixed and mesenchymal odontogenic tumors

Case No.	Diagnosis	Immunohistochemical reactivity								Labeling index (%) Ki-67
		Amelogenin	CK19	bcl-2	HGF	c-Met	TGF-b	TbR I	TbR II	
1	Ameloblastic fibroma									
	Epithelial cell	–	+	+	+	+	+	+	+	0.56
	Mesenchymal cell	–	–	+	+	–	+	+	+	4.00
2	Ameloblastic fibrodentinoma									
	Epithelial cell	–	+	+	+	+	+	+	+	—
	Mesenchymal cell	–	–	+	+	–	+	+	+	NA
	Dentinoid	–	–	–	–	–	–	–	–	NA
3	Ameloblastic fibro-odontoma									
	Epithelial cell	+	+	+	+	+	+	+	+	1.30
	Mesenchymal cell	–	–	+	+	–	+	+	+	1.60
	Enamel matrix	+	–	–	–	–	–	–	–	—
	Dentinoid	–	–	–	–	–	–	–	–	—
4	Ameloblastic fibro-odontoma									
	Epithelial cell	+	+	+	+	+	+	+	+	1.10
	Mesenchymal cell	–	–	+	+	–	+	+	+	1.50
	Enamel matrix	+	–	–	–	–	–	–	–	—
	Dentinoid	–	–	–	–	–	–	–	–	—
5	Odontogenic fibroma									
	Epithelial cell	–	+	+	+	+	+	+	+	1.00
	Mesenchymal cell	–	–	+	+	–	+	+	+	0.90
6	Odontogenic myxoma									
	Epithelial cell	–	+	+	+	+	+	+	+	1.20
	Mesenchymal cell	–	–	+	+	–	+	+	+	1.30
7	Odontogenic myxoma									
	Mesenchymal cell	–	–	+	+	–	+	+	+	1.53

–: negative, +: positive, NA: not available

and mesenchymal odontogenic tumors (Figs. 2A and 2C). Immunoreactivity for c-Met was detected in the cell membrane of odontogenic epithelial cells in mixed and mesenchymal odontogenic tumors (Fig. 2B). Expression of TbR I and TbR II was detected in the cell membrane and cytoplasm of odontogenic epithelial and mesenchymal cells in mixed and mesenchymal odontogenic tumors (Fig. 2D).

Odontogenic epithelial and mesenchymal cells sporadically showed positive reactions for Ki-67 in the nuclei in mixed and mesenchymal odontogenic tumors (Fig. 3). In mixed odontogenic tumors, the mean Ki-67-LI of odontogenic epithelial cells and mesenchymal cells were $0.99 \pm 0.38\%$ and $2.37 \pm 1.42\%$, respectively. In mesenchymal odontogenic tumors, the mean Ki-67-LI of odontogenic epithelial cells and mesenchymal cells were $1.10 \pm 0.14\%$ and $1.24 \pm 0.32\%$, respectively. These immunohistochemical results are summarized in Table 3.

Discussion

Amelogenin is a major enamel matrix protein produced by secretory ameloblasts and plays an important role in enamel formation (19, 20). A variety of odontogenic tumors have been proven to show amelogenin expression (11, 16, 18, 21–23). Some investigators have suggested that ameloblastic fibroma and ameloblastic fibrodentinoma will ultimately differentiate or mature further into ameloblastic fibro-odontoma and then continue maturation into a completely differentiated odontoma (1, 18). On the other hand, some investigators have suggested that ameloblastic fibroma, ameloblastic fibrodentinoma, ameloblastic fibro-odontoma and odontoma are all classified as so-called odontogenic mixed tumor, but basically different (2, 14). In the present study, expression of amelogenin was detected in ameloblastic fibro-odontomas, but not in ameloblastic fibroma or ameloblastic fibrodentinoma. These results suggest that odontogenic epithelial cells of ameloblastic fibro-odontoma show ameloblastic differentiation in association with

enamel formation. Ameloblastic fibroma and ameloblastic fibrodentinoma are capable of enamel formation, but our results might reflect the periods of amelogenin synthesis or secretion. In this study, no expression of amelogenin was confirmed in odontogenic fibroma and odontogenic myxomas. These findings supported no ability of enamel formation in these mesenchymal odontogenic tumors. Cytokeratins are intermediate filaments that function as the skeletons of epithelial cells (24). Cytokeratin 19 has been detected characteristically in odontogenic epithelial cells of tooth germs and epithelial odontogenic tumors (22, 23, 25, 26). In the present study, expression of cytokeratin 19 was detected in odontogenic epithelial cells in all mixed and mesenchymal odontogenic tumors, indicating that cytokeratin 19 revealed odontogenic epithelial properties also in mixed and mesenchymal odontogenic tumors. The *Bcl-2* gene is a proto-oncogene, and its product protein functions as a suppressor of apoptosis (27). *Bcl-2* is known as one of the regulators of apoptosis associated with odontogenesis and growth in odontogenic tumors (23, 28, 29). In the present study, *bcl-2* expression was recognized in both odontogenic epithelial and mesenchymal cells in mixed and mesenchymal odontogenic tumors. These features suggest that these odontogenic cells in mixed and mesenchymal odontogenic tumors are avoided by apoptotic cell death and that *bcl-2* would be associated with the proliferation or survival of these odontogenic cells in mixed and mesenchymal odontogenic tumors.

Growth factors are widely associated with the growth and differentiation of normal cells, and derangement of these functions can result in various pathologic conditions, including neoplasia (30). HGF is a broad-spectrum and multifunctional cytokine, which has mitogenic, motogenic and morphogenic functions in various cells via its receptor tyrosine kinase, c-Met, which is encoded by the *c-Met* oncogene (31-34). HGF and/or c-Met overexpression has been reported in many tumors and is therefore considered to play a role in oncogenesis, differentiation, tumor invasion and metastasis (35-37). In the present study, HGF expression was detected in odontogenic epithelial and mesenchymal cells in mixed and mesenchymal odontogenic tumors, while c-Met expression was detected in odontogenic epithelial cells. These features suggest the HGF signaling is effective in the epithelial component in paracrine and autocrine manner in mixed and mesenchymal odontogenic tumors. TGF- β plays important roles in regulating cell growth, differentiation and function by working as a potent growth inhibitor (38, 39). TGF- β signal is transduced by binding to two kinds of receptors, T β R I and T β R II, with serine/tyrosine kinase activity (40, 41). Mutations in TGF- β -related genes and altered expression of these product proteins have been identified in several tumors (42, 43). In the present study, expression of TGF- β , T β R I and T β R II

was detected in odontogenic epithelial and mesenchymal cells in mixed and mesenchymal odontogenic tumors, suggesting that the TGF- β signaling was transduced in these tumors. During embryonic development, HGF and TGF- β function as mediators of epithelial-mesenchymal interactions to induce morphogenesis of various organs (34, 38, 44). Expression of HGF, TGF- β and their receptors has been found along tooth development (34, 38). Our immunohistochemical features of HGF, TGF- β and their receptors suggest that these growth factor signaling are effective in the epithelial component in paracrine and autocrine manner in mixed and mesenchymal odontogenic tumors. These results of growth factor signaling suggest that certain epithelial-mesenchymal interactions might be related to neoplastic cell growth not only in mixed odontogenic tumors but also in mesenchymal odontogenic tumors.

Analysis of cell proliferation in situ provides important indices of cell turnover in various tissues or tumors (29, 45, 46). Ki-67 antigen is expressed in proliferative cells through G₁, S, G₂, and M phases and provides a reliable index of cellular proliferation (47). In mixed odontogenic tumors, the malignant tumor has shown much higher proliferative activity than benign tumors (15). In the present study, odontogenic epithelial and mesenchymal cells sporadically showed positive reactions for Ki-67 in mixed and mesenchymal odontogenic tumors. Proliferative activities of these benign mixed and mesenchymal odontogenic tumors are thought to be low, and there were no apparent differences among these tumors or between the components.

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