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Immunolocalization of cell signaling molecules in the granular cell ameloblastoma

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BACKGROUND: Bone morphogenic protein (BMP) and Wnt signaling pathway molecules play important roles in cytodifferentiation and cell proliferation. We attempted to localize these signaling molecules in the granular cell ameloblastoma.

MATERIALS AND METHODS: Four samples of paraffin-embedded ameloblastoma with granular cells were studied. Immunohistochemistry was performed to detect basement membrane type heparan sulfate (HS) (JM403), cell surface type HS (10E4), heparanase, Wnt-5a, Wnt-2, β-catenin, and BMP-4.

RESULTS: In all four samples, strong expression of β-catenin and Wnt-5a was detected within the granular cells, while BMP-4 expression was weak and Wnt-2 was negative. Immunoreactivities of basement membrane type HS, cell surface type HS, and heparanase were variable within granular cells in ameloblastoma.

CONCLUSION: Granular cells in ameloblastoma exhibit abnormal biological behaviors, particularly synthesis and secretion of protein. Synthesis of signaling molecules is upregulated, but secretion is arrested in some cases, while both are lost in other cases.

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Keywords: signaling molecules; granular cell; ameloblastoma

Introduction

The granular cell ameloblastoma is an unusual variant showing marked transformation in the cytoplasm of tumor cells, which are usually stellate reticulum-like cells. The transformed cells possess very coarse, granular, eosinophilic cytoplasm (1, 2). Previous ultrastructural and immunohistochemical studies of cytoplasmic granules of common ameloblastoma have revealed that they are lysosomal aggregates (1, 3). Lysosomal aggregation within the cytoplasm is caused by dysfunction of either a lysosomal enzyme or lysosome-associated protein involved in enzyme activation, enzyme targeting, or lysosomal biogenesis. These defects lead to the accumulation of substrate that would normally be degraded in the endosome–lysosome system (4). Many authors have already reported the immunohistochemical findings of granular cell ameloblastoma (3–8). However, the contents of the lysosomal aggregates within the granular cells in ameloblastoma are largely undefined.

Wnts are a family of secreted glycoproteins distributed throughout the tissue by binding heparan sulfate proteoglycans (HSPG) (9), and they constitute one of the most important families of signaling molecules during development. These proteins also have vital roles in adult tissues, including the regulation of cell proliferation and motility, generation of cell polarity, and specification of cell fate (10). The 19 Wnt family proteins have been identified (10). Wnt-2 is an important member of the Wnt family and is upregulated in various carcinogenesis (11–13). In non-small-cell-lung cancer cells, selective apoptosis were induced through inhibition of Wnt-2-mediated signaling pathway (13). In the previous study of granular cell ameloblastoma, the granularity of these cells resulted from apoptotic cell death of neoplastic cells (8). It may be assumed that Wnt-2 has an important role in granular cells. β-catenin was originally identified based on its association with cadherin adhesion molecules. It is now widely recognized as an essential element of the Wnt signaling cascade (14). On the contrary, Wnt-5a is a β-catenin antagonist that inhibits the canonical Wnt signaling pathway by degrading β-catenin (15). In ameloblastoma, β-catenin and Wnt signaling pathways play important roles in cell–cell adhesion and signal transduction, which causes cytodifferentiation of tumor cells (16).

Bone morphogenic proteins (BMPs) belong to the transforming growth factor-β (TGF-β) superfamily. Recent studies have revealed that BMPs play critical roles in cellular proliferation and differentiation (17, 18).

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In several neoplasms including ameloblastoma, expression of BMP is an indicator of tumor progression and development (19–21). Studies on normal development and tumor progression suggested that BMP family protein and Wnt signaling protein may play a role in oncogenesis, epithelial–mesenchymal interaction, and cellular proliferation (16, 18).

HSPG is the main protein that ubiquitously present in the extracellular matrix. HSPG bound various growth factors, signaling molecules, and cytokines through their sugar chain which protected these molecules from enzymatic degradation (22–26). There are different types of heparan sulfate (HS) chains, which are present within the cell surface as well as in the extracellular matrix. JM403 and 10E4 are the basement membrane type HS and cell surface type HS, respectively. These epitopes have a specific chemical structure with different localization pattern (24). Wnt family protein and BMP-4 are molecules bound to HS chains (9, 22). Nevertheless, specific epitopes binding of these molecules have not been so far reported in the literatures. Although 10E4 has been reported in odontogenic tumor including ameloblastoma (26), the expressions of these two most common HS epitopes and their relations to the granular cells in ameloblastoma is still unknown.

Heparanase, the mammalian endoglucoronidase enzyme that selectively cleaves the HS at specific site is well known to play an important role in invasion of cells associated with inflammation and cancer metastasis (23, 27–29). In ameloblastomas, heparanase contributes to local invasiveness and secondary changes (28).

In this study, we attempted to analyze the expression pattern of the BMP and Wnt family molecules in the granular cells that present in ameloblastoma. We observed that all these signaling molecules are localized in the granular cells in ameloblastoma.

Material and methods

Histopathological samples

Archival formalin-fixed, paraffin-embedded blocks from four cases of ameloblastoma with characteristics granular cells were selected from the Surgical Pathology Unit of Department of Oral Pathology and Medicine, Graduate school of Medicine, Dentistry and Pharmaceutical Science of Okayama University, Japan. Granular cell ameloblastoma was diagnosed histologically by routine hematoxylin and eosin (HE) stained slides, according to WHO histological typing of odontogenic tumors. Serial 4-μm sections were cut and used for immunohistochemistry procedures.

Immunohistochemistry

Four-micrometer sections mounted on salinized slides were used for immunohistochemical staining. Briefly, sections were deparaffinized in a series of xylene for 15 min and rehydrated in graded ethanol solutions. Endogenous peroxidase activity was blocked by incubating the sections in 0.3% H_2O_2 in methanol for 30 min. Antigen retrieval of paraffin section was achieved by microwaving the section immersed in 10 mM citrate buffer solution pH 6.0 (for JM403, Wnt-5a, Heparanase, and BMP-4 immunostaining), or by autoclaving the section in Tris-ethylene diamine tetra acetic acid (EDTA) solution pH 9.0 (β-catenin immunostaining) or in 10 mM citrate buffer solution pH 6.0 (for Wnt-2 immunostaining) at 121°C for 5 min, or by treating the section with 0.1% trypsin for 5 min (for 10E4 immunostaining). After treatment with normal serum, the sections were incubated with primary antibodies at 4°C overnight. The tagging of primary antibody was achieved by subsequent application of anti-goat/mouse IgG and avidin–biotin complexes [mouse/goat ABC kit (Vector Laboratories, Inc., Burlingame, CA, USA)] or Envision peroxidase detecting reagent (Dako, Carpinteria, CA, USA). Visualization of immunohistochemical reaction was performed by developing the enzyme complex with DAB/H_2O_2 solution (Histofine DAB substrate; Nichirei, Japan) and counterstained with Mayer's hematoxylin.

The antibodies used are listed in Table 1.

Table 1 Characteristics of antibodies used in immunohistochemical studies

Antibody	*Clonality*	*Supplier*	*Dilution*
β-catenin	Rabbit polyclonal	Dako (Carpenteria, CA, USA)	1:150
Wnt-5a	Goat polyclonal	R&D Systems (Minneapolls, MN, USA)	1:25
BMP-4	Goat polyclonal	Santacruz Biotechnology, Inc. (Santacruz, CA, USA)	1:100
Wnt-2	Goat polyclonal	Santacruz Biotechnology, Inc. (Santacruz, CA, USA)	1:500
JM403[a]	Mouse monoclonal	Seikagaku Corp. (Tokyo, Japan)	1:500
10E4[b]	Mouse monoclonal	Seikagaku Corp. (Tokyo, Japan)	1:1000
Heparanase	Mouse monoclonal	Kindly provided by Dr M. Nakajima	1:1500

[a]Basement membrane type heparan sulfate.
[b]Cell surface type heparan sulfate.

Results

Immunohistochemical staining of granular cells

Strong immunoreactivity for β-catenin with a granular appearance was observed in granular cells, but weak immunoreactivity was found in peripheral cuboidal cells (Fig. 1a). Wnt-5a showed strong positive reaction in granular cells of ameloblastoma, and reactivity was also observed in peripheral cuboidal cells (Fig. 1b). Weak BMP-4 immunoreactivity with a granular appearance was observed within the cytoplasm of granular cells, whereas no reactivity was seen in peripheral cuboidal cells (Fig. 2a). Wnt-2 expression was completely absent in granular cells but was positive in peripheral cells (Fig. 2b). JM403, a specific antibody against the basement membrane type HS chain, strongly stained the granular cells and peripheral cells (Fig. 3a). 10E4, another cell surface type HS chain specific antibody, gave negative immunostaining in the granular cells but positive in cuboidal cells (Fig. 3b). HS chain specific

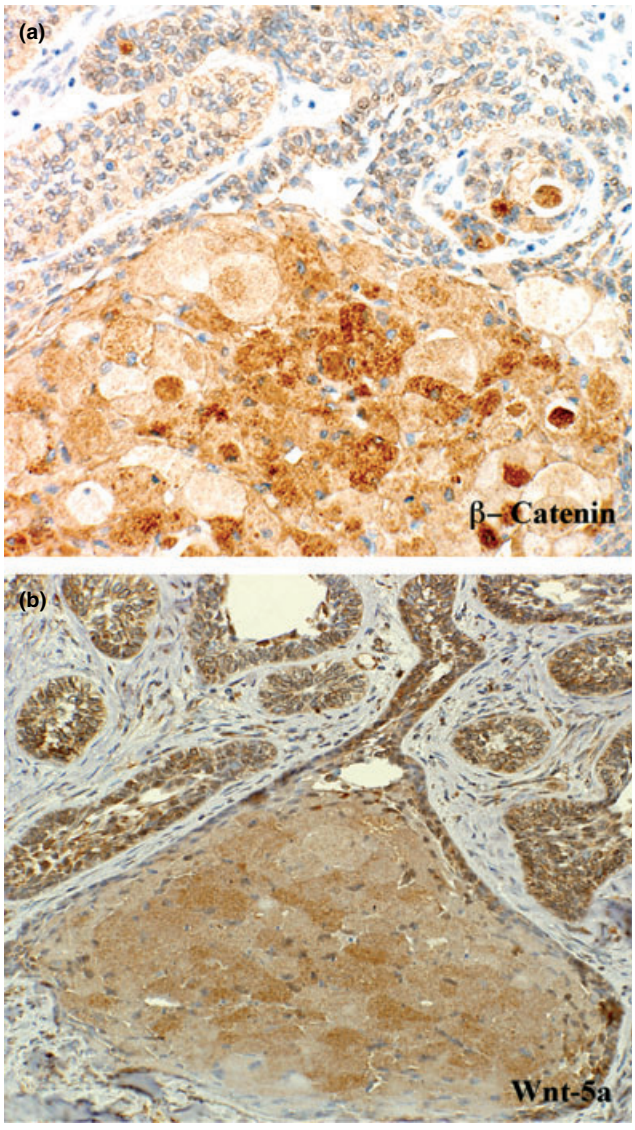

Figure 1 (a) β-catenin is strongly positive and shows granular appearance in granular cells but is weak in peripheral cuboidal cells. (b) Wnt-5a is positive in both granular cells and cuboidal cells. a: 100× and b: 130×.

endoglucoronidase heparanase showed weak positive reactivity in granular cells in ameloblastoma, and also in peripheral cells (Fig. 3c).

The immunoreactivity for all of these markers is summarized in Table 2.

Discussion

The Wnt/β-catenin signaling pathway is integrally associated with tumor development and progression (11). Wnt family proteins are secreted ligands that control various cellular and tissue proliferation patterns during normal development. The activation of this signal transduction pathway is a known and major contributing factor to oncogenesis. These proteins are covalently linked to lipids and cell surface proteoglycans (9). A recent study has suggested that HSPGs influence morphogens trafficking and stabilize Wnt proteins, and

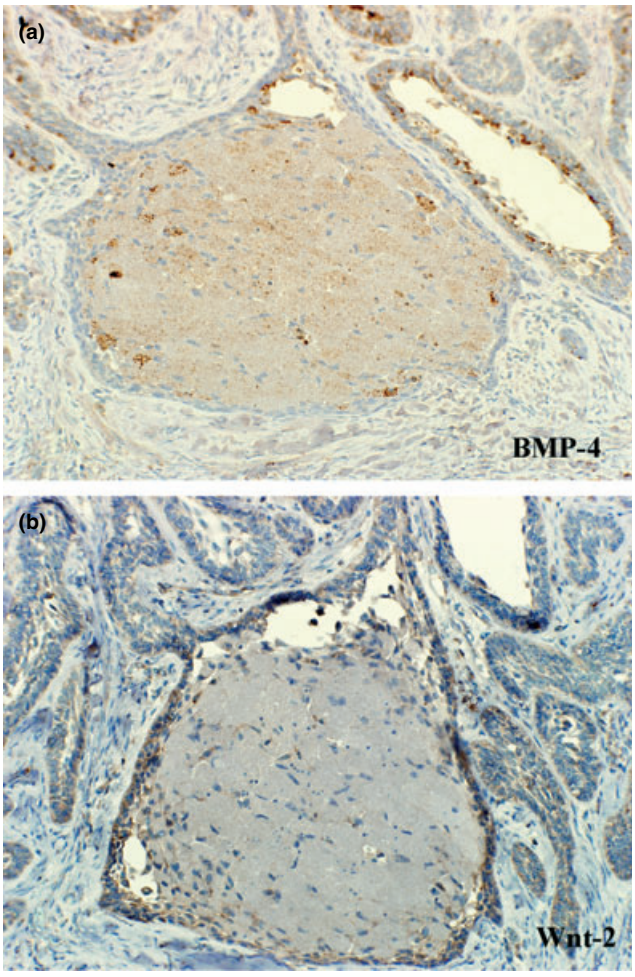

Figure 2 (a) BMP-4 is weakly positive with granular appearance in granular cells but negative in peripheral cuboidal cells. (b) Wnt-2 is completely negative in granular cells, but is positive in peripheral cuboidal cells. a and b: 130×.

disperse them throughout the tissue (9). Wnt-5a is a representative ligand that activates a β-catenin-independent pathway in the Wnt signaling (30). Wnt-5a is also a β-catenin antagonist that inhibits the canonical Wnt pathway by degrading β-catenin (15). Our results show that Wnt-5a expression is positive in both granular and neoplastic cells, and also suggest its co-localization with HS molecules within the granular cells. From this data, we speculate that may be, the secretion process of Wnt-5a stopped after being synthesized, resulting in accumulation of these molecules within the cytoplasm by autophagosome.

β-catenin is a cadherin-binding protein involved in cell–cell adhesion and also functions as transcriptional activator complexes in the nucleus with TCF/LEF family protein (12). It is now widely recognized that β-catenin is an essential element of the Wnt signaling pathway (14). Our data show that β-catenin was strongly expressed by granular cells. This finding suggests that the newly synthesized β-catenin in granular cells could not reach the cell surface and accumulated in the cytoplasm. Finally, the β-catenin was autophagocytosed in cytoplasm and stored within the cell.

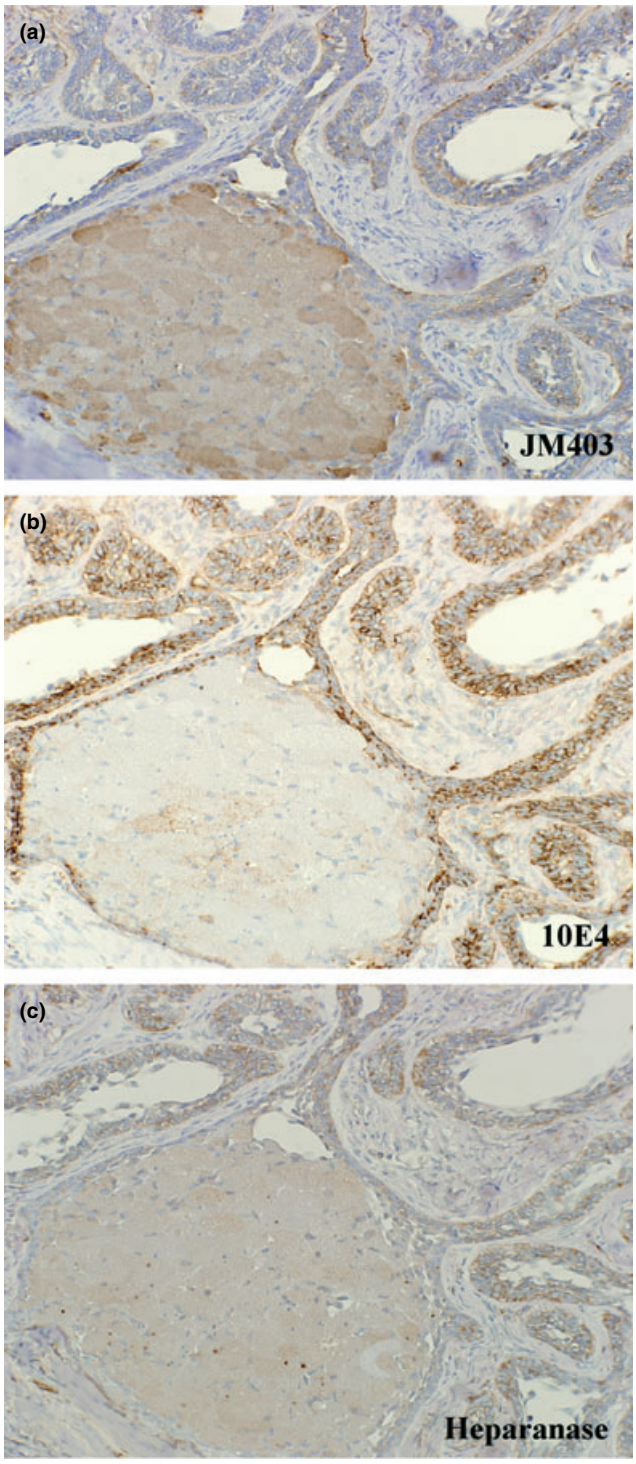

Figure 3 (a) Strong immunoreactivity of basement membrane type HS (JM403) in granular cells. (b) Cell surface type HS (10E4) is negative in granular cells. (c) Heparanase is weakly positive in granular cells. Peripheral cuboidal cells in all cases show positive reaction. a, b, and c: 130×.

BMPs belonging to the TGF-β superfamily have been found to play a critical role in cellular proliferation and differentiation (17, 18). BMPs and Wnt signaling molecules are important differentiation factors associated with epithelial–mesenchymal interaction (18). Some

Table 2 Summary of immunohistochemical study

Antibody	*Immunoreactivity of granular cells in ameloblastoma*
β-catenin	+ +
Wnt-5a	+ +
BMP-4	+/–
Wnt-2	–
JM403[a]	+ +
10E4[b]	–
Heparanase	+/–

+ +, strong reaction; +, positive reaction; –, negative reaction; +/–, weak reaction.
[a]Basement membrane type heparan sulfate.
[b]Cell surface type heparan sulfate.

authors reported that these two signaling molecules interact in the nucleus. Some data of interaction placed BMPs upstream of Wnts, whereas other experimental results placed BMPs downstream of or parallel to Wnts (31). In various types of neoplasm including osteosarcoma, oral carcinoma, salivary gland tumors, and ameloblastoma, BMP and Wnt-2 expression in tumors reflects that these molecules are responsible not only for osteogenesis but also for oncogenesis (12, 19, 21, 32, 33). Inhibition of Wnt-2 resulted to selective apoptosis of the cancer cells in non-small-cell-lung cancer (13). In this experiment, BMP-4 showed weak positive reactivity whereas Wnt-2 was completely negative in granular cells. But peripheral cuboidal cells were positive to Wnt-2. These results suggest that these granular cells lost their functional capacity and Wnt-2 is responsible for the apoptotic cell death. The synthesis and secretion processes of BMP and Wnt signaling molecules may be impaired and inactivated within the granular cells in ameloblastoma. These findings also support the hypothesis that BMP and Wnt signaling pathways have some interactions with each other (31).

In this study, strong immunoreactivity of basement membrane type HS (JM403) in granular cells and irregularly strong immunoreactivity in some ameloblastoma cells were observed. However, the cell surface type HS (10E4) was completely negative in granular cells, but positive on the cell surface and basement membrane of neoplastic cells. These results suggest that the synthesis and secretion patterns of these two HS molecules are completely different. It is possible that because of the loss of function of the granular cells, overproduced basement membrane type HS, JM403, could not be secreted, causing an overload of the HS that was ultimately phagocytosed in the cytoplasm. For the cell surface type HS, 10E4, there was a possibility that both synthesis and secretion processes were arrested, which may explain why the HS was not accumulated within the granular cells.

Heparanase is an endo-β-D-glucoronidase that specifically cleaves the HSPGs chain (23, 27, 28). In ameloblastoma, heparanase is overexpressed both at mRNA level and protein levels, resulting in invasion and secondary morphological changes of ameloblastoma (28). The activation process of the latent heparanase

takes place within the lysosome (29). The present study showed weak positive reactivity of heparanase in granular cells, suggesting that because of inactivation of lysosomal enzymes, the activation process of the latent heparanase may be stopped resulting in storage of these molecules within the lysosomes.

Conclusion

This study provides some insights on the biological behaviors of granular cells in ameloblastoma. We speculate that the synthesis and secretion processes for the cell signaling molecules within the granular cells may be altered two ways. First, these cells may be able to synthesize signaling molecules such as β-catenin and Wnt-5a, but their transportation or secretion process is impaired. As a result, the molecules accumulate within the cytoplasm as autophagosomes. Second, both synthesis and secretion processes are arrested for BMP-4 and Wnt-2, and these molecules are thus not accumulated within the granular cells in ameloblastoma. In case of HS, the synthesis of basement membrane type HS is increased but the synthesized HS cannot be secreted extracellularly possibly because of cellular inactivation, and the molecules accumulate within the cell cytoplasm. In the case of cell surface type HS, both synthesis and secretion process may be arrested. The experimental results obtained in this study indicate that signaling pathways related to cell proliferation and differentiation are loss or inactive in the granular cells, suggesting that these cells are functionally inactive and their synthesis and secretion activities have become irregular.

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