Expression of hard α -keratins in adenomatoid odontogenic tumor: a case study with immunohistochemical analysis

Kentaro Kikuchi^{1, 2}, Toshiyuki Ishige^{1, 2}, Fumi Fuchinoue^{1, 2}, Yukari Obana^{1, 2}, Toshinori Oinuma^{1, 2}, Tsutomu Yamada^{1, 2}, Minori Yoshida³, Terukazu Ueki³, Norimichi Nemoto^{1, 2}

¹Department of Pathology, Nihon University School of Medicine, Tokyo, Japan ²Pathology Laboratory, Nihon University Itabashi Hospital, Tokyo, Japan ³Department of Oral Surgery, Nihon University School of Medicine, Itabashi Hospital, Tokyo, Japan.

> *Abstract*: Adenomatoid odontogenic tumor (AOT) is an uncommon entity of odontogenic origin, characterized by the formation of duct-like structures in the tumor. Although its histogenesis is still uncertain, it is also considered to be a rather hamartoma-like lesion. Here we report a case of AOT in a 22-year-old Japanese woman, the first documented case to have been analyzed for the presence of hard α-keratin (human hair protein). A positive reaction for human hair proteins was noted in the marginal area of calcified material, or at the interface or between areas of dystrophic calcification and columnar epithelial cells. Furthermore, melanin pigment was demonstrated histologically in this case, similar to several previously reported cases of AOT with melanin. This is the first reported case of AOT with hard α-keratin. These findings suggest that AOT may have the potential to differentiate into hair components.

[Oral Med Pathol 2008; 13: 7-14 doi: 10.3353/omp.13.7]

- *Key words*: hard α-keratin, PA-HP1, PA-HP2, immunohistochemistry, adenomatoid odontogenic tumor
- *Correspondence*: Kentaro Kikuchi, Department of Pathology, Nihon University School of Medicine, 30-1 Oyaguchi Kamimachi, Itabashi-ku, Tokyo 173-8610, Japan Phone: +81-03-3972-8111, Fax: +81-03-3972-8163, E-mail: kentak@med.nihon-u.ac.jp

Introduction

Adenomatoid odontogenic tumor (AOT) is a rare entity of odontogenic origin, accounting for only 2-7% of all odontogenic tumors (1-2). The earliest account of this lesion was by Ghosh in 1934, who described it as an adamantinoma of the upper jaw (3). The term AOT was adopted by the World Health Organization (WHO) in 1971. In the 2005 revision of the WHO classification, AOT was transferred from the category "arising from odontogenic epithelium with odontogenic ectomesenchyme" to another category, "without odontogenic ectomesenchyme", reflecting the fact that it should not be interpreted as an induction phenomenon, i.e. so-called dental hard tissue formation, although in very rare cases dentin-like material containing dentinal tubules may be present. Furthermore, occurrence of hyaline, dysplastic material or calcified osteodentin is likely the result of a metaplastic process. However, there have been few reports of calcified material including enamel or dentinlike hard tissue in AOT. Interestingly, Ide et al. (4) have reported that adenomatoid odontoma has overlapping histologic features of both AOT and complex odontoma. A recent study of odontoma cases by Tanaka et al. (5) revealed that frequent expression of human hair proteins in centers of mineralization forms calcified materials resembling Liesegang rings. Kusama *et al.* (6) demonstrated the expression of human hair proteins (hard α -keratins) in transitional and shadow cells or ghost cells in calcifying epithelioma, adamantinomatous craniopharyngioma (ACP) and calcifying cystic odontogenic tumor (CCOT or calcifying odontogenic cyst). No previous article has described the expression of hard α -keratins in AOT, although melanin pigmentation like that in cortex cells of the normal hair shaft is occasionally observed in some AOT cases (7-10). Here we report an unusual and rare case of AOT showing expression of hard α -keratins (PA-HP1 and PA-HP2) and melanin pigmentation.

Case report

A 22-year-old Japanese woman presented at the Department of Dental and Oral Surgery, Nihon University Itabashi Hospital, with swelling in the lower left canine region. Intra-oral examination showed that the left mandibular deciduous canine was retained but the permanent canine was missing. Radiographic examination demonstrated a well circumscribed radiolucency extending throughout the anterior mandible from the right second incisor to the left first premolar region (Fig. 1a), and also an impacted third molar in an area of expansile osteolysis with stippled calcifications (Fig. 1b). The clinical diagnosis was a mandibular tumor. The tumor was enucleated by intra-oral surgery with the patient under general anesthesia. After tumorectomy, the patient recovered and showed a good clinical course. The surgical specimen was a $30 \times 30 \times 15$ mm mass (Fig. 1c). Macroscopically, cut sections revealed the tumor to be a cystic mass with an unerupted tooth, and intraluminal proliferation and nodular growth were also evident (Fig. 1d).

Materials and Methods

The resected mass was fixed in 10% neutral buffered formalin, embedded in paraffin wax, and stained routinely with hematoxylin-eosin. Tissue sections were then prepared for immunohistochemical analysis using antibodies (MA-HP1, PA-HP1 and 2) supplied by Professor Kaoru Kusama, Division of Pathology, Department of Diagnostic and Therapeutic Science, Meikai University School of Dentistry, Sakado, Saitama, Japan. Kusama et al. (6) previously demonstrated the immunohistochemical specificity of these antibodies, and all three react only with the hair shaft in sections of normal skin and dermoid cyst. Deparaffinized sections were immersed in absolute methanol containing 0.3% H₂O₂ for 20 min at room temperature to block endogenous peroxidase activity, then treated with 2% bovine serum albumin for 20 min to block non-specific reactions. The sections were subsequently incubated with rabbit polyclonal antibodies against human hair proteins (6), PA-HP1 and 2 (10 g/ml), a monoclonal antibody against type II neutral/basic hard α -keratin, MA-HP1 (1:50 dilution), and monoclonal antibodies against soft keratin (as an epithelial series marker, including squamous epithelium) and highand low-molecular-weight cytokeratins (clone 34 ßE12, 1:50, Dako, Glostrup, Denmark, and clone AE1, 1:2, Shandon/Lipshaw Co., Pittsburgh, PA, USA) for 60 min at room temperature. After washing with phosphate-buffered saline (PBS, pH 7.4), biotinylated goat anti-rabbit IgG (H+L) (1:200, Vector Laboratories, Burlingame, CA, USA) or biotinylated horse anti-mouse IgG (H+L) (1:200, Vector) was applied to each section for 30 min. An appropriate dilution of streptavidin-peroxidase (Gibco BRL Life Technologies, Invitrogen Co., Carlsbad, CA) was applied to the tissue sections for 30 min, after which they were immersed in 0.05% 3,3'-diaminobenzidine tetrahydrochloride in 0.05 M Tris-HCl buffer (pH 7.6) containing 0.01% H₂O₂ (DAB solution) for 10 min and counterstained with Mayer's hematoxylin.

Results

Microscopically, the tumor had a well-defined fibrous capsule and consisted of whorls and strands of epithelium (Fig. 2a), with tubule-like microcysts (Fig. 2b). Eosinophilic materials were found in some gland-like spaces bordered by



Fig. 1. Clinical findings of adenomatoid odontogenic tumor. (a) Panoramic radiograph before therapy; (b) CT image; (c) surgical specimen; (d) cut surface of (c). A well circumscribed radiolucent lesion in the mandible, extending from the left first premolar to the right second incisor (a). CT showed an impacted tooth in an area of expansile osteolysis with stippled calcifications (b). A mass was easily removed from the surrounding mandibular bone, and the surgical specimen measured 30 × 30 × 15 mm (c). On cut section, the tumor appears as a cystic mass with a tooth. Note the intraluminal proliferation and nodular growth features (d).

ameloblast-like cells (Fig. 2c). Calcified materials were evident in the epithelial sheets (Fig. 2d), and several eosinophilic tumor droplets were noted in solid nodules of cuboidal epithelial cells (Fig. 2e). Dystrophic calcification was juxtaposed with tall columnar epithelial cells (Fig. 2f) and gland-like spaces (Fig. 2g). The tall columnar epithelial cells were positively stained with periodic acid-Schiff (PAS) and diastase resistant (data not shown). There was no evidence of dentin, enamel matrix or enamel formation, or of ghost cells in the tumor. An unusual histological feature was the presence of prominent calcified materials of various sizes in the tumor nests. Immunohistochemistry with the three antibodies, PA-HP1, PA-HP2, and MA-HP1, gave similar staining patterns. However, the staining reaction with MA-HP1 was very weakly positive (data not shown). PA-HP1 and PA-HP2 (human hard keratins/human hair protein) were reactive only with hair shafts (or hair roots) in normal hair follicles of head skin as a control specimen (Figs. 3a-3b & 3d-3e). Antibodies against human soft keratin, and high- (Figs. 3c & 3f) and low-molecular-weight cytokeratins (data not shown) were not reactive with hair shafts.

In AOT, the staining reactions of PA-HP1 and PA-HP2 were noted mainly in the marginal area of the calcified









Fig. 4. Comparative immunohistochemistry for hard a-keratin between normal skin and adenomatoid odontogenic tumor. (a, d) normal hair follicles of head skin, \times 40; (**b**, c, e, f) adenomatoid odontogenic tumor, (b, e) \times 200, (c, f) \times 400. Immunoperoxidase stains with PA-HP1 (b, c) and PA-HP2 (e, f), hematoxylin counterstain. In adenomatoid odontogenic tumor, stain-ing with PA-HP1 (b) and PA-HP2 (e) was mainly in the marginal area of the calcified material. Higher magnification showed positive reactions with PA-HP1 (c) and PA-HP2 (f) at the interface between the annular calcification and spindleshaped epithelial cells (c) or between the dystrophic calcification and spindle-shaped epithelial cells (f).



Fig. 5. Immunohistochemistry for hard α -keratin with PA-HP1 in adenomatoid odontogenic tumor. (a, b) Immunoperoxidase stain with PA-HP1, hematoxylin counterstain. PA-HP1 gave weakly-positive stainings in the cytoplasm of spindle and columnar epithelial cells around the calcified material (a: arrows), strongly positive in the interface between round calcifications and epithelial cells (a: arrowhead), and weakly positive in the cytoplasm of polyhedral cells around the dystrophic calcification in part (b).

material (Figs. 4b-4c & 4e-4f), being weakly positive in areas of dystrophic calcification adjacent to odontogenic epithelial cells (Fig. 5a) and strongly positive at the interface between dystrophic calcification and odontogenic epithelial cells (Fig. 5a) in tumor nests. There was also weak positivity in the cytoplasm of polyhedral cells around the prominent areas of dystrophic calcification (Fig. 5b).

Histochemistry also indicated marked melanin pigmen-

tation in and around the dystrophic calcification (Fig. 6a), as evidenced by positive Masson-Fontana staining (Fig. 6b) that was bleached by hydrogen peroxide (Fig. 6c) and a negative Berlin blue reaction for ferric irons (data not shown). Melanin pigmentation was found in peripheral or central parts of the calcified material (Figs. 6d-6e), odontogenic epithelial nests (Fig. 6f) and in some droplets (Fig. 6g). Moreover, positive Masson-Fontana staining was



Fig. 6. Melanin pigmentation in adenomatoid odontogenic tumor. (a, **c-g**) Adenomatoid odontogenic tumor; (b) normal abdominal skin. Masson-Fontana stain before (a, b, d-g) and after (c) bleaching by hydrogen peroxide. $(a, c) \times 40$, (b) \times 100, (d) \times 200, (e-g) \times 400. Reaction for Masson-Fontana stain was seen in the markedly calcified area(a), and in melanin pigmentation in normal abdominal skin (b). Masson-Fontana staining in AOT was bleached by hydrogen peroxide (c). Therefore, the black staining was considered to be melanin. Melanin pigmentation was noted in irregularly calcified materials in part (d), near the center of the slightly annular dystrophic calcification (e), nodular aggregates of cells (f), and some areas of tumor droplets (g).



Fig. 7. Melanophages and melanocytes in adenomatoid odontogenic tumor. Masson-Fontana stain before (a, c, d) and after (b) bleaching by hydrogen peroxide. $(a, b) \times 200$, $(c, b) \times 200$ d) × 400. Reaction for Masson-Fontana staining was seen in the tumor nests and stromal connective tissue (a), and was bleached by hydrogen peroxide (b). Melanocytelike cells with a dendritic form were present in para-vessels of stromal connective tissue (c, arrow) and minimal stroma of tumor nests (d, arrow), and melanophages were noted in the same area (d, arrowhead).

found in calcified materials within convoluted ductal spaces and minimal stromal connective tissue (Fig.7a), and was bleached by hydrogen peroxide (Fig. 7b). Melanocyte-like cells with a dendritic form were noted in perivascular connective tissue (Fig. 7c) and in the stroma of tumor nests (Fig. 7d), along with melanophage-like round cells (Fig. 7d). Congo red staining for amyloid was negative in calcified material within the tumor nests (data not shown).

The postoperative histopathological diagnosis was adenomatoid odontogenic tumor with prominent hard tissue formation. Immunohistochemical analysis of the tumor revealed melanin pigmentation and expression of hard α -keratins (human hair proteins) at the boundary between dystrophic calcification and odontogenic epithelial cells.

Discussion

Human hair proteins comprise two groups: hard α -keratins forming microfibrous intermediate filaments, and non-filamentous matrix proteins (11). The hard α -keratins are further subdivided into two families: type I acidic (40-50 kDa) and type II neutral/basic (55-65 kDa) (11). Although epithelial soft keratins have been well studied, little is known about hard α -keratins and their associated proteins, probably because of the lack of commercially available antibodies against human hair proteins.

AOT is a relatively rare slow-growing lesion thought to arise from odontogenic epithelium because of its predilection for tooth-bearing bone, with varying degrees of inductive change in connective tissue. Ide *et al.* (4) have reported that adenomatoid odontoma has overlapping histologic features of both AOT and complex odontoma, and that the tumor cells of AOT usually differentiate toward an apparent ameloblastic phenotype, but fail to attain further functional maturation. Immunohistochemical studies of AOT have revealed enamel proteins (amelogenin, enamelin, sheath line) in the mineralized particles, hyaline droplets, and eosinophilic material of the tumor cells (12-14). Enamel proteins have also been detected in the ghost cells of calcifying cystic odontogenic tumor (CCOT or calcifying odontogenic cyst) (13-14). Meanwhile, the presence of enamel proteins and lymphoid enhancer factor 1 (LEF-1) in ACP indicates that it has not only morphological, but also functional differentiation into odontogenic epithelium (15). An elevated level of LEF-1 in the lip furrow epithelium of developing transgenic animals triggers these cells to invaginate, sometimes leading to inappropriate development of hair follicle and tooth cell fates (16). Kusama et al. (6) reported that antibodies against human hair proteins (hard α -keratins) were reactive with ghost, shadow or transitional cells in CCOT, ACP and calcifying epithelioma. The present case of AOT was positive for PA-HP1 and 2 at the boundary between the calcified material and odontogenic epithelial cells, indicating differentiation into hair in some of the tumor cells. Human hair proteins are composed of hard keratins and matrix proteins, and it is thought that the Wnt- β -catenin-Tcf (T cell factor)/LEF pathway is involved in the expression of hard keratins (17-18). Interestingly, the Wnt signaling pathway is involved in the development of teeth (19-20), and activation of the pathway plays an important role in terminal hair shaft differentiation (16, 21-23). Tanaka et al. (5) recently reported the expression of human hair proteins in ghost cells and calcified materials in odontoma, and also the presence of β -catenin and LEF-1 in the tumors. The present case of AOT was not a pure neoplasm in part, and it appeared to have hamartoma-like components with hair differentiation. Reported cases of AOT showing a positive reaction for enamel proteins (14) had a hair protein

expression pattern similar to that in the present case. Although there is currently no supporting evidence, it has been suggested that AOT might be an ameloblastoma-like, rather than a hamartoma-like lesion.

In addition to microscopically-evident melanin pigmentation, the calcified material, minimal connective tissue and odontogenic epithelial cells showed positive Masson-Fontana staining. In a comprehensive review of the literature, we found 4 cases of AOT with concomitant melanin pigmentation (7-10). However, the reason for melanin pigmentation in AOT, including other odontogenic tumors, is unclear and of considerable interest. Melanin pigmentation, like that seen in cortex cells of the normal hair shaft, is occasionally observed concomitantly in CCOT (24-25). In fact, we have frequently observed that mature cystic teratoma of the ovary with teeth and hair shows melanin pigmentation in ossified or bony structures. Furthermore, calcifying epithelioma arising from skin also shows melanin pigmentation in the tumor. The significance of melanin in odontogenic tumors and its role with their development still remain unclear.

In the present case, part of the tumor component of AOT might have had the potential to differentiate into hair. To our knowledge, this is the first report to demonstrate the presence of hair protein in AOT immunohistochemically. Further studies using a large number of AOT cases are needed to elucidate the mechanisms involved.

Acknowledgments

We thank Prof. Kaoru Kusama, Division of Pathology, Department of Diagnostic and Therapeutic Sciences, Meikai University School of Dentistry, Sakado, Saitama, Japan, for his advice regarding use of the antibodies MA-HP1, PA-HP1, and PA-HP 2.

References

- Philipsen HP, Birn H. The adenomatoid odontogenic tumor. Ameloblastic adenomatoid tumor or adeno-ameloblastoma. *Acta Pathol Microbiol Scand* 1969; **75**: 375-98.
- Philipsen HP, Reichart PA, Nikai H. The adenomatoid odontogenic tumor (AOT): An update. *Oral Med Pathol* 1998; 2: 55-60.
- Ghosh LS. Adamantinoma of the upper jaw. *Am J Pathol* 1934; **10**: 773-89.
- Ide F, Kusama K. Adenomatoid odontoma. Oral Surg Oral Med Oral Pathol 2002; 94: 149-50.
- Tanaka A, Okamoto M, Yoshizawa D, et al. Presence of ghost cells and the Wnt signaling pathway in odontomas. J Oral Pathol Med 2007; 36: 400-4.
- Kusama K, Katayama Y, Oba K, *et al.* Expression of hard α-keratins in pilomatrixoma, craniopharyngioma, and calcifying odontogenic cyst. *Am J Clin Pathol* 2005; **123**: 376-81.
- Aldred MJ, Gray AR. Apigmented adenomatoid odontogenic tumor. Oral Surg Oral Med Oral Pathol 1990; 70: 86-9.
- 8. Effiom OA, Odukoya O. Adenomatoid odontogenic tumor:

a clinico-pathological analysis and melanin pigmentation study of 31 Nigerian cases. *Niger Postgrad Med J* 2005; **12**: 131-5.

- Takeda Y. Pigmented adenomatoid odontogenic tumor. Report of an undescribed case and review of the literature of pigmented intraosseous odontogenic lesions. *Virchows Arch A Pathol Anat Histopathol* 1989; **415**: 571-5.
- Water A, George-Diolombi G, Chazal M, Ango A. Melanin in a dentigerous cyst and associated adenomatoid odontogenic tumor. *Cancer* 1990; 66: 786-8.
- Nakamura A, Arimoto M, Takeuchi K, Fujii T. A rapid extraction procedure of human hair proteins and identification procedure of human hair proteins and identification of phosphorylated species. *Biol Pharm Bull* 2002; 25: 569-72.
- Murata M, Cheng J, Horino K, Hara K, Shimokawa H, Saku T. Enamel proteins and extracellular matrix molecules are co-localized in the pseudocystic stromal space of adenomatoid odontogenic tumor. *J Oral Pathol Med* 1999; 29: 483-90.
- Abico Y, Murata M, Ito Y, *et al.* Immunohistochemical localization of amelogenin in human odontogenic tumors, using a polyclonal antibody against bovine amelogenin. *Med Electron Microsc* 2001; 34: 185-9.
- Takata T, Zhao M, Uchida T, Kudo Y, Sato S, Nikai H. Immunohistochemical demonstration of an enamel sheath protein, sheathlin, in odontogenic tumors. *Virchows Arch* 2000; 436: 324-9.
- Sekine S, Takata T, Shibata T, *et al.* Expression of enamel proteins and LEF1 in adamantinomatous craniopharyngioma: evidence for its odontogenic epithelial differentiation. *Histopathology* 2004; 45: 573-9.
- Zhou P, Byrne C, Jacobs J, Fuchs E. Lymphoid enhancer factor 1 directs hair follicle patterning and epithelial cell fate. *Genes Dev* 1995; **9**: 570-83.
- DasGupta R, Fuchs E. Multiple roles for activated LEF/ TCF transcription complexes during hair follicle development and differentiation. *Development* 1999; 126: 4557-68.
- Merrill BJ, Gat U, DasGupta R, Fuchs E. Tcf3 and Lef1 regulate lineage differentiation of multipotent stem cells in skin. *Genes Dev* 2001; 15: 1688-705.
- Sarkar L, Sharpe PT. Expression of Wnt signaling pathway genes during tooth development. *Mech Dev* 1999; 85: 197-200.
- Jarvinen E, Salazar-Ciudad I, Birchmeier W, Taketo MM, Jernvall J, Thesleff I. Continuous tooth generation in mouse is induced by activated epithelial Wnt/β-catenin signaling. *Proc Natl Acad Sci USA* 2006; **103**: 18627-32.
- Ridanpaa M, Fodde R, Kielman M. Dynamic expression and nuclear accumulation of β-catenin during the development of hair follicle-derived structures. *Mech Dev* 2001; **109**: 173-81.
- Tsuji H, Ishida-Yamamoto A, Takahashi H, Iizuka H. Nuclear localization of β-catenin in the hair matrix cells and differentiated keratinocytes. *J Dermatol Sci* 2001; 27: 170-7.
- Dunn SM, Keough RA, Rogers GE, Powell BC. Regulation of hair follicle keratin intermediate filament gene promoter. *J Cell Sci* 1998; 111: 3487-96.

- 24. Buchner A, David R, Carpenter W, Leider A. Pigmented lateral periodontal cyst and other pigmented odontogenic lesions. *Oral Dis* 1996; **2**: 299-302.
- Somes JV. A pigmented calcifying odontogenic cyst. Oral Surg Oral Med Oral Pathol 1982; 53: 395-400.

Received July 9, 2008 Accepted September 7, 2008