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Analysis of the neoplastic nature and biological potential of sporadic and nevoid basal cell carcinoma syndrome-associated keratocystic odontogenic tumor

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BACKGROUND: Keratocystic odontogenic tumor (KCOT), also known as odontogenic keratocyst, is a benign cystic neoplasm, which may be associated with nevoid basal cell carcinoma syndrome (NBCCS) and if it does, will occur as multiple cystic lesions. KCOT is locally destructive despite its bland histological features. However, the neoplastic nature of KCOT is not well established.Heparanase is an endo-D-glucuronidase enzyme that specifically cleaves heparan sulfate (HS) and the increase of its level in tumors promotes invasion, angiogenesis, and metastasis.

METHODS: To investigate the neoplastic character of KCOT, we studied the localization patterns of heparanase in KCOT, focusing on the differences between sporadic and NBCCS-associated KCOTs, by immunohistochemistry and *in situ* hybridization. To compare the expression pattern of these cysts with non-tumorous odontogenic developmental cyst, dentigerous cyst was included.

RESULTS: All the odontogenic cysts showed positive immunoreaction for heparanase protein in various intensities. The expression pattern of heparanase gene corresponded to that of protein expression. Interestingly, intense gene and protein expressions were observed in KCOT associated with NBCCS compared with sporadic ones and dentigerous cyst.

CONCLUSIONS: The results implied that heparanase expression may be correlated with the neoplastic properties of KCOT, particularly in NBCCS-associated cases.
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Introduction

Although the bulk of odontogenic tumors is benign, some of them will show locally destructive behavior. Keratocystic odontogenic tumor (KCOT), previously called odontogenic keratocyst in former times, is known as a benign but aggressive odontogenic neoplasm and it often associated with nevoid basal cell carcinoma syndrome (NBCCS). In the new WHO classification revised in 2005, KCOT is defined as benign intraosseous neoplasm of odontogenic origin with characteristic lining of parakeratinized squamous epithelium (1). It is generally agreed that the origin of KCOT is odontogenic epithelium, particularly the dental lamina and its remnants, and extensions of basal cells from overlying oral epithelium (2–4).

Heparanase is a mammalian endo-D-glucuronidase enzyme that specifically cleaves heparan sulfate (HS) chains. HS chains include repeating disaccharides composed of *N*-acetylglucosamine and uronic acid, and exist in extracellular matrix and cell surface attached to a heparan sulfate proteoglycan (HSPG) core protein.

It is reported that heparanase is involved in the remodeling of extracellular matrix in normal condition, and increases in tumor condition associated with invasion and metastasis, and poor prognosis in many kinds of cancers (5, 6).

In a previous study, we investigated the localization of heparanase gene and protein expression in ameloblastoma, suggesting the possible contribution of heparanase in the local invasiveness and secondary morphological changes (7). In the present study, we investigated the localization of heparanase gene and protein expression in KCOT to determine its neoplastic nature and local aggressiveness.

Materials and methods

Surgical materials

A total of 30 cases of paraffin-embedded blocks were selected for this study from the surgical pathology unit of the Department of Oral Pathology and Medicine, Graduate School of Medicine, Dentistry and Pharmaceutical Sciences of Okayama University (Okayama, Japan).

In the 20 cases of KCOT, 10 were associated with NBCCS. To compare the expression pattern of heparanase gene and protein of these cysts with non-tumorous odontogenic developmental cyst, 10 cases of dentigerous cyst were included. The cases of KCOT associated with NBCCS were taken from patients who were clinically diagnosed as NBCCS according to the diagnostic criteria (8). The clinical details are summarized in Table 1.

These cases were fixed in 10% neutral-buffered formalin, decalcified with citrate-buffered 10% formic acid if needed, and routinely processed and embedded in paraffin. Histopathological diagnosis was done based on the WHO histological typing (9). Four-micrometer-thick serial sections were made and used for hematoxylin–eosin staining, immunohistochemistry and *in situ* hybridization.

Antibodies

Monoclonal antibody against human heparanase, which recognizes both proform and mature forms as previously reported (6), was used. For antibody detection, mouse IgG ABC kit (Vectastain Elite ABC kits; Vector Laboratories, Burlingame, CA, USA) was used. DAB (Histofine DAB substrate; Nichirei, Tokyo, Japan) was used to visualize the immunoreactions.

Probe

Digoxigenin 11-UTP-labeled single-stranded RNA probes were prepared using DIG labeling kit (Roche Diagnostic GmbH, Penzberg, Germany) according to the manufacturer's instruction. For the generation of single-stranded heparanase RNA probe, 571-bp fragment of human heparanase cDNA (bases 261–832 of the total cDNA) (GenBank accession no. AF144325) was produced by RT-PCR, subcloned into pCR21 (Invitrogen, Carlsbad, CA, USA) and amplified by PCR.

In situ *hybridization*

In situ hybridization was performed as previously described (6). Sections were deparaffinized in xylene, rehydrated in ethanol and incubated with 3 mg/ml of proteinase K (Roche Diagnostics) in 10 mM Tris–HCl (pH8.0) and 1 mM EDTA for 15 min at 37°C. Acetylation of the sections was performed by incubating in 0.1 M triethanolamine–HCl buffer (pH 8.0) at room temperature. Hybridization solution contains 50% deionized formamide, 10% dextran sulfate, 1× Dehardt's solution, 600 mM NaCl, 0.25% SDS, 250 mg/ml of *Escherichia coli* tRNA (proteinase treated), 10 mM dithiothreitol, and 0.1–0.2 mg/ml of digoxigenin-UTP-labeled RNA probe. The probe was placed on the sections, covered with parafilm, incubated at 50°C. After hybridization, the sections were washed in a series of Standard Saline Citrate (SSC) at 50°C, incubated with 1.5% blocking reagent in DIG1 buffer for 60 min. Anti-digoxigenin-AP Fab fragment (1:800) (Roche Diagnostics) in DIG1 buffer was applied to the sections and incubated for 60 min at room temperature. Coloring solution, containing 337.5 μg/ml of nitro blue tetrazolium and 165-μg/ml of 5-bromo-4-chloro-3-indolyl phosphate in DIG3 buffer, was mounted on the sections and incubated at 37°C. Counter staining was performed with methyl green.

Table 1 Surgical materials

Diagnosis	*No. of cases*	*Location*
Sporadic keratocystic odontogenic tumor	10	4 maxilla 6 mandible
Keratocystic odontogenic tumor associated with Nevoid basal cell carcinoma syndrome	10	6 maxilla and mandible 3 maxilla 1 mandible
Dentigerous cyst	10	3 maxilla 7 mandible

Total: 30 cases.

Results

Heparanase gene and protein expression were observed in all lesions. Both heparanase protein and gene were positive in the epithelium of the cyst wall in varying degrees.

The details of heparanase protein and mRNA expression in each case are summarized in Table 2 and discussed as follows.

Expression of heparanase protein in KCOT and dentigerous cyst

Both sporadic and NBCCS-associated KCOTs showed positive reactions to heparanase in the lining epithelium. Interestingly, there were some differences in the expression patterns between the two (Fig. 1).

In sporadic KCOT, heparanase expression was comparatively weak and uneven. However, basal and keratinized cells showed moderate expression.

On the other hand, NBCCS-associated KCOT showed very intense and even expression of heparanase

Table 2 Summary of immunohistochemistry and *in situ* hybridization

	Heparanase	
Diagnosis	*Immunohistochemistry*	*In situ* hybridization
Sporadic keratocystic odontogenic tumor		
E	(+)	(+)
C	(−)	(−)
Keratocystic odontogenic tumor associated with nevoid basal cell carcinoma syndrome		
E	(++)	(++)
C	(−)	(−)
Dentigerous Cyst		
E	(+)	(+)
C	(−)	(−)

E, epithelium; ++, strongly positive; C, connective tissue; +, positive; −, negative.

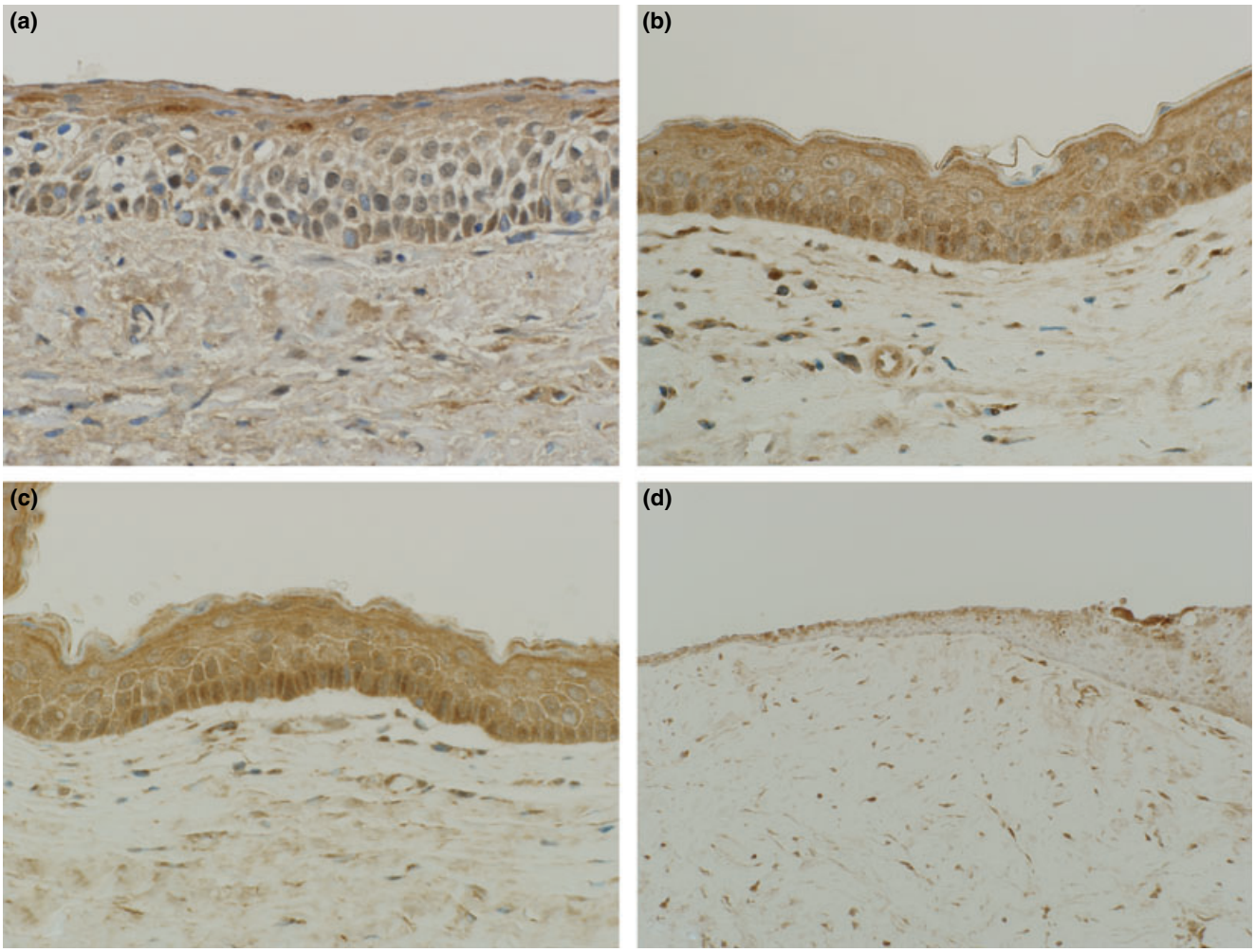

Figure 1 Heparanase protein expression in KCOT and dentigerous cyst. In sporadic KCOT, heparanase expression pattern was weak and uneven, localized within keratinized cells (a). In NBCCS-associated KCOT, intense and uneven expression of heparanase was observed not only in cytoplasm but also in nucleus of basal cells (b and c). In dentigerous cyst, heparanase was positive in the lining epithelium (d).

protein. Moreover, the protein expression was observed not only in cytoplasm but also in the nucleus of the basal cells. No significant difference in the heparanase protein expression pattern between primary and recurrent cases.

In the case of dentigerous cyst, heparanase protein was weakly positive in lining epithelium. Connective tissue beneath the epithelium was almost negative or weak.

Expression of heparanase mRNA in KCOT and dentigerous cyst

Comparing the results of immunohistochemistry, difference in intensity was the most distinct feature (Fig. 2).

In sporadic KCOT, heparanase mRNA expression was limited within the basal cell layer, whereas in NBCCS-associated KCOT, intense expression was observed in the entire lining epithelium. Significant difference in intensity was observed also in mRNA expression pattern between sporadic and NBCCS-associated KCOT.

In dentigerous cyst, heparanase mRNA expression was detected in the whole layer of the lining epithelium uniformly. But it was not detected in the connective tissue.

Discussion

Keratocystic odontogenic tumor, previously known as odontogenic keratocyst, is a benign cystic lesion, but it often shows locally destructive behavior and high recurrence rate, in spite of its bland histology (10). This may be attributed to the active proliferation of the lining epithelium (11, 12). Recent studies demonstrated that PTCH gene, which is mapped onto chromosome 9q22.3-q31 and thought to be a tumor suppressor gene, was involved in the etiology of KCOT (13–16).

Some articles support the tumorous character of KCOT, which shows a higher expression of bcl-2, p53, p63 observed in immunohistochemical studies (11, 12, 17).

Heparanase is an endo-D-glucuronidase that specifically cleaves HS. Heparanase is necessary for the natural

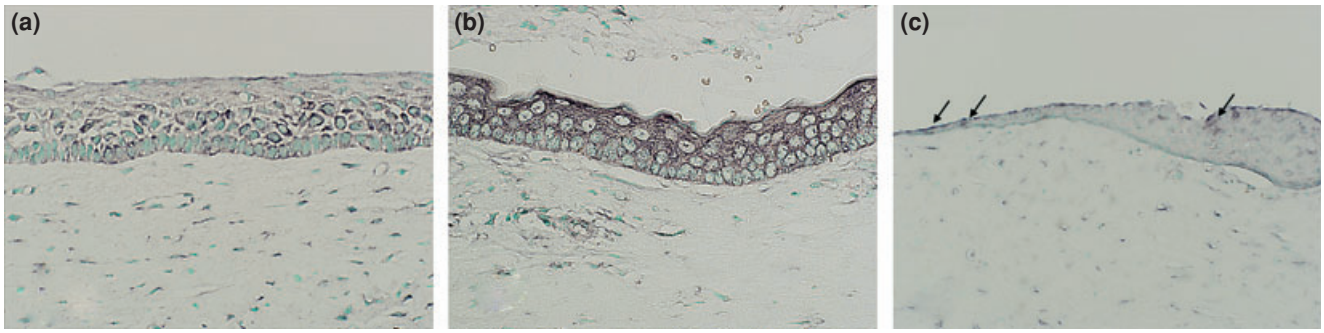

Figure 2 Heparanase mRNA expression in KCOT and dentigerous cyst. In sporadic KCOT, heparanase mRNA expression pattern was weak and uneven (a), whereas it was very intense and diffusely observed in NBCCS-associated cases (b). In dentigerous cyst, heparanase mRNA expression was weakly observed (arrows, c).

turn over of the HS (18); however, it may function not only as a digestive enzyme but may also be involved in cell signaling. The target of heparanase, HS exist in basement membrane and extracellular matrix attached to HSPGs. These HS can bind many kinds of biologically active molecules, growth factors, cytokines, and cell adhesion molecules; thus, they play important roles in cell adhesion and intercellular signaling (19), and as a reservoir of these molecules (20).

Heparanase will cleave HS and make 10–20 sugar residue materials that are more active than the native HS (21). It is known that heparanase is rare in the normal tissue and often increases in tumors and promotes tumor invasion, angiogenesis and metastasis (6, 22–26).

In this study, both sporadic and NBCCS-associated KCOT showed heparanase protein and mRNA expression in varying degrees. In all cases, both heparanase protein and mRNA expression were limited to the lining epithelium and the expression of heparanase in the connective tissue of the cyst wall was minimal.

In the case of dentigerous cyst, immunopositive reaction for heparanase protein was observed in odontogenic epithelium. The expression pattern was monotonous, but in some areas with inflammatory reaction, the immunoreaction showed a weaker reaction. The mRNA of heparanase corresponds to that of protein expression.

Interestingly, some differences in the heparanase expression pattern and intensity were observed between sporadic and NBCCS-associated KCOT, not only in the protein level, but also in the mRNA level. Heparanase protein and mRNA were detected in the lining epithelium of sporadic and NBCCS-associated cases. In sporadic case, heparanase expression was uneven and limited, whereas very intense and even pattern in NBCCS-associated cases.

It is reported that heparanase expression will increase in many kinds of tumors, including odontogenic tumors (7, 22–26).The increased heparanase expression was thought to be somewhat concerned with the invasive property.

The mechanisms in the release of heparanase during tumor proliferation lies in the cleavage of HS causing severe anomaly in some important signaling pathways or growth factors (21). The possible mechanisms are as follows: (i) release of the bioactive molecules captured in HS, which results in profound effects on cellular functions (27, 28). (ii) the cleavage of HS by heparanase changes the signaling pathway and growth factor controls, via: (1) shedding of proteoglycans extracellular domain; (2) fragmentation of HS; and (3) removal of sulfate from 6-O position from HS (19). Kim et al. reported that the lining epithelium of KCOT showed intense BMP-4 expression, which was not expressed in dentigerous cyst (10). Supporting these theories, the intense expression of heparanase may be related to the aggressive growth attitude of KCOT.

The nuclear heparanase expression was observed only in NBCCS-associated KCOT. It is reported that nuclear heparanase expression is observed in human cell lines and breast and esophageal epithelial cells (24), and also observed in squamous metaplastic cells in ameloblastoma (7).

Very recently, Kobayashi et al. reported that heparanase was transferred to nucleus from cytoplasm during esophageal cell differentiation, and it correlates with keratinocyte differentiation. And it has been reported that nuclear translocation of heparanase is likely to correlate with cell differentiation and favorable prognosis in patients with gastric, esophageal and head and neck squamous cell carcinomas (29, 30). NBCCS-associated KCOT showed intense heparanase expression both in cytoplasm and nucleus. These facts will well explain the nature of KCOT, which shows locally destructive behavior regardless of its bland, and well differentiated histology. We used dentigerous cyst as a control, and weak heparanase protein and mRNA expression were observed, which may imply non-aggressive nature of this developmental cyst. These results altogether suggest that heparanase is involved in the aggressive behavior of NBCCS-associated KCOT, although it remains unknown whether heparanase overexpression leads first or secondary to aggressive alteration. This is the first report that showed the difference between heparanase expression pattern in KCOT and dentigerous cyst both in protein and

mRNA levels. The results may support the neoplastic nature of KCOT, particularly those that are NBCCS associated.

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