Immunohistochemical Examination of Patched and Sonic Hedgehog in Odontogenic Keratocysts Associated with Basal Cell Nevus Syndrome

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About 5% of odontogenic keratocysts (OKC) arise in association with basal cell nevus syndrome (BCNS), a genetical disorder caused by aberrant function of patched1 (ptch1) gene. Ptch1 is a receptor of Sonic hedgehog (Shh), whose signaling plays a role in proliferation of developing odontogenic epithelium.

OKC specimens from five BCNS patients were examined immunohistochemically for expression of Ptc1, Ptc2, Shh and CyclinD1. Ptc1 and Shh staining was distinct in the lining epithelium, and 20-50% of basal/suprabasal cells were positive for CyclinD1. Ptc2 staining was as weak as none. Loss of heterozygosity (LOH) in ptch1 and ptch2 loci was probed; in which one case showed no LOH for ptch1 while three cases showed no LOH for ptch2. Co-expression of Shh and its receptor in OKC epithelium implicates that autocrine or paracrine activation of hedgehog signaling within the lining cells is involved in genesis of OKC associated with BCNS.

Key words: odontogenic keratocyst, basal cell nevus syndrome, Patched, Sonic hedgehog

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Introduction

Odontogenic keratocyst (OKC) is a relatively common developmental cyst that exhibits histologically distinctive appearances. The cyst wall is lined with a uniform, parakeratinized epithelium that consists of 5-10 layers of cells with flat rete ridges. The basal cells have a vertically elongated shape that is reminiscent of ameloblasts. The cyst is thought to arise from remnants of dental lamina, but the mechanism underlying its development is largely unknown.

OKC sometimes arises as a symptom of basal cell nevus syndrome (BCNS), an autosomal dominant disorder characterized by multiple developmental abnormalities and susceptibility to a variety of tumors including basal cell carcinoma of skin (1-3). About 5 percent of OKC were from BCNS patients and the other 5 percent were from patients with multiple OKC but with no other features of the syndrome (1, 2).

BCNS is caused by aberrant function of the product of patched1 (ptch1) gene that is mapped to chromosome 9q22.3 (4, 5). Ptc1 is a homologue of the Drosophila segment polarity gene patched, which encodes a receptor of a secretory signaling factor hedgehog (hh) and plays a key role in embryogenesis and organogenesis (6-8). Ptc1 appears to act as a tumor suppressor gene since the loss of heterozygosity (LOH) of 9q22 region is frequently observed in several malignant tumors, including basal cell carcinoma (9), medulloblastoma (10) and bladder carcinoma (11).

In the course of tooth development, ptc1 and its homologue ptc2 are both expressed in the odontogenic epithelium that is destined to differentiate into ameloblasts along with strong expression of sonic hedgehog (shh) and their downstream effector gene, gli transcription factors (12-14). The hedgehog signaling is suggested to mediate the proliferation, cell polarity of preameloblasts and their differentiation into mature ameloblasts (14).

As well as its essential role in normal odontogenesis, aberrant hedgehog signaling is proposed to be the major cause of OKC, as LOH in the ptc1 locus is
frequently observed in the lining epithelial cells of OKC and even in some sporadic OKC cases (15). This is a convincing indication that OKC may develop as a result of abnormal activation or inactivation of Ptch1 due to its gene alteration in the residual dental epithelial cells, but this hypothesis needs to be reinforced by accumulation of data on a wide variety of cases.

In the present study, archival OKC specimens from 5 patients, who had been clinically diagnosed as BCNS, were collected and analyzed for the expression of Shh, Ptch1 and Ptch2 by immunohistochemical examination. The expression of CyclinD1, which is a cell-proliferation regulator thought to be under the control of hedgehog signaling (16), was also examined. In an attempt to confirm the diagnosis of BCNS, LOH analyses on the *ptch1* and *ptch2* loci were conducted.

**Material and methods**

**Specimens**

Archival surgical specimens in the file of Dental Hospital of Tokyo Medical and Dental University between 1999 and 2002 were collected from 5 patients who had been clinically diagnosed as BCNS. The diagnoses of BCNS had been given at the other institutes. All the specimens were processed for pathological inspection according to the routine laboratory protocol. The excised tissues were fixed in 10% formalin for one to two days, dehydrated in series of alcohol and embedded in paraffin. Decalcified specimens were inappropriate for PCR and immunohistochemistry, and thus they were laid aside from the examination.

**Immunohistochemical staining and its evaluation**

Sections were cut at 4 μm thickness after hydration by passage through xylene and graded alcohols. Immunohistochemical staining was performed using a Histofine Kit (Nichirei). Tissue specimens were autoclaved at 121°C for 15 minutes in sodium citrate buffer. Endogenous peroxidase was inactivated by incubating the sections in 0.3% hydroxide in methanol for 15 min. Blocking was performed in 10% bovine serum in phosphate buffered saline (PBS) for 1 hour. The specimens were incubated in PBS with primary antibody at 4°C overnight. After washing, incubation with biotin-coupled 2nd antibody was done at room temperature for 1 hour, and peroxidase-coupled streptavidin was then applied and allowed to stand for 15 min. The coloration was performed with diaminobenzidine tetrahydrochloride substrate. Counterstaining was omitted.

Antibodies used in this study were anti-patched1 (H-267, Santa Cruz, 1:100 dilution), anti-patched2 (N-19, Santa Cruz, 1:100 dilution), anti-sonic hedgehog (5E1, Hybridoma Bank, University of Iowa, 1:50 dilution) and anti-cyclin D1 (DCS-6, DAKO, 1:200 dilution).

Staining intensities in the epithelial cells were evaluated in comparison with the mesenchymal staining and graded into weak or none (±), fair (+) and strong (++). Regions that accompanied secondary changes, including inflammatory cell infiltration, epithelial thickening or atrophic changes, were excluded from the evaluation and only the part of cyst epithelia which exhibited the typical features of OKC were selected for examination.

The labeling index of CyclinD1 was calculated by evaluating the positive cells lined on the basal or suprabasal cell layers. When either basal or suprabasal cells were positively stained, the vertical line was considered as positive. When both basal and suprabasal cells were negative for staining, the vertical line was considered negative. At least 50 basal cells with the corresponding suprabasal cells were counted, and the number of positive vertical lines was divided by total vertical lines.

**Microdissection, PCR and LOH analysis**

Six μm thick sections were prepared, deparaffinized and slightly stained with methyl green. Cyst lining epithelium and the underlying connective tissue were separately collected by manual microdissection in a protein digestion solution (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 20 mg Proteinase K) under light microscopy. Protein digestion was performed at 55°C overnight and then Proteinase K was inactivated by heating at 95°C for 10 min. These crude extracts were used as templates for PCR.

Primers for PCR were;

- D1S463F: AGCTACAATAACTCAGTACATGGAA,
- ROX-D1S463R (labeled with ROX): TTCCTTGACTCTTCTAGCAGC,
- D9S287R: CACGCCACTGATCTAGGCT,
- D9S280F: TCTTTTTCGCTTCCCACCCA,
- ROX-D9S287R: CACGCCACTTCTTCTAGCAGC,
- D9S280F: CAGTGTTTGGTGATGGCACC,
- ROX-D9S180R: AGCTATTTTTGAGGCTAGAG.

PCR was carried out in 20 μl volumes containing 1 μl of sample solution with advantage polymerase mixture (Clontech). PCR parameters were 35 cycles of 94°C 30 sec, 55°C 30 sec and 68°C for 30 sec. Expected sizes of the PCR products were approximately 220 bp for D1S463, 170 bp for D9S287, 150 bp for D9S280 and 230 bp for D9S180. The PCR products were verified by 1.5% agarose gel electrophoresis and correctly amplified samples were electrophoresed on the fluorescent DNA sequencer (ABI prism 3100) to analyze for LOH.

**Results**

**Clinicopathological findings**

A summary of the clinicopathological information is given in Table 1. All five cases showed multiple jaw cysts. The excised cyst tissues showed distinct histological features that met the criteria for OKC: cysts lined
with 5-10 layers of ortho- or parakeratinized squamous epithelia with nuclear palisading. Secondary inflammatory changes were sometimes seen, but these regions were omitted from examination. The patients are currently leading a normal life, and occurrence of malignant tumor including basal cell carcinoma has not been noted.

Immunohistochemical findings

Immunoreactivity to anti-Shh antibody was observed in the lining epithelium, which exhibited a diffuse cytoplasmic staining at a slightly elevated level in the outer prickled cell layer. The staining in the basal cell layer was weak. Immunoreactivity to anti-Ptch1 antibody was observed mainly in the prickled cell layer, but some staining was observed also in the suprabasal layer. The staining showed membranous or cytoplasmic pattern, which was consistent with the features of Ptch protein as a cell surface receptor molecule. Ptch1 staining in the basal cell layer was weak. Ptch2 staining was barely seen, except as occasional dotted staining in a small population of epithelial cells. Several other antigen-retrieval methods did not improve the results with the anti-Ptch2 antibody. CyclinD1 exhibited distinct nuclear staining mainly in the suprabasal layer, with occasional staining in the basal cell layer. These patterns of expression showed little difference between the cases. Epithelial components of OKC showed strong staining of Ptch1 in four cases. Ptch2 staining was very weak in all the cases. Shh immunoreactivity was observed at a fair or high level in all the cases. CyclinD1 labeling index was 20-50%, implicating a raised proliferation activity in the epithelial component of OKC. No significant correlation was observed between the staining intensities of Ptch1, Ptch2, Shh and CyclinD1 counts.

LOH assay for ptch1 and ptch2 loci

Genomic DNA was extracted separately from the epithelial cells and the mesenchymal cells to use for PCR with fluorescent labeled primers, targeting the polymorphic genomic markers close to ptch1 or ptch2 loci. LOH analyses were performed using a DNA sequencer. For ptch1, only one sample yielded an informative amplification, in which no LOH was detected. For ptch2, three samples yielded successful amplification, in which one is homogenous and two were heterogeneous with no LOH.

Discussion

Interactions within the dental epithelial cells, as well as those between epithelium and mesenchyme, play a key role in tooth germ development (14). Groups of dental epithelial cells express shh and/or its receptor ptch1 and ptch2, and activation of hedgehog signaling cascade
occurs autonomously within the dental epithelium, which governs proliferation, differentiation and polarization of dental epithelial cells (13, 14). Removal of smoothened, the hedgehog signal transducer, causes reduced proliferation of dental epithelium, which leads to impaired morphogenesis of tooth germ (14).

LOH in the *ptch1* gene locus is frequently observed in the lining epithelium of OKC, both with and without BCNS (15). Some cases of dentigerous cysts also showed LOH in the *ptch1* locus (17). In *ptch1* heterozygous knock out mice, development of jaw cysts was observed at a high frequency compared to the wild type mice (18). These observations gave way to a definite suggestion that dysregulation of hedgehog signaling induces the remnants of dental epithelial cells to proliferate and develop into OKC.

We collected five cases of OKC associated with BCNS, the diagnoses of which were drawn from their family history and the general symptoms. Since the diagnoses had not been backed up by genomic examination, we tried to detect LOH in the *ptch1* locus. We succeeded in amplifying the short tandem repeat (STR) marker fragment only in one case, and no LOH was found in this case. Our cases are archival pathological specimens, and the failure of PCR appeared due to insufficient preservation of genomic DNA and contaminants in the DNA extracts.

Distinct immunostaining of Patch1 in the epithelium was observed in most (4/5) of the cases. Furthermore, Shh was detected in the epithelium of all the cases, indicating that autonomous hedgehog signaling is retained within the epithelial cells of OKC. It is not defined whether OKC develops as elevated or reduced activity of hedgehog signaling. Heterogeneous knock-out mice that presumably express less *ptch1* develop more jaw cysts (18), suggesting that OKC arises as the result of reduced reception of hedgehog protein. Contrary to this, co-expression of Shh and Patch1 in the OKC epithelium observed in our study indicates that an autocrine or paracrine hedgehog signaling can be maintained within the OKC epithelium. We assume that mitosis-inducing activity of hedgehog signaling observed during the course of tooth germ development may be erroneously committed to proliferation of dormant dental epithelial remnants. Since Patch1 expression is observed in most of the odontogenic tumors and cysts (19), its aberrant function may be a key element for pathogenesis of many odontogenic lesions. As seen in normal oral epithelium, expression of CyclinD1 in OKC was mainly observed in the suprabasal layer (20). The CyclinD1 labeling indices of OKC were as high as comparable to that of normal oral epithelium, which is approximately 40% (data not shown and (20)), suggesting high proliferation activity of OKC epithelial cells. Al-

![Fig. 1: Immunohistochemical staining of OKC in BCNS patients. (A) Shh showed diffuse staining in the lining epithelium. (B) Ptch1 showed a membranous staining pattern in the lining epithelium. (C) Ptch2 staining was very weak. (D) CyclinD1 is positive in the nuclei of population of basal and suprabasal cells. Original magnification: ×100](image-url)
though CyclinD1 is under the control of many other signaling cascades, abundant CyclinD1 expression in OKC epithelial cells may be attributed to abnormal increase of hedgehog signaling. As the expression of Ptch1 in the suprabasal layer is relatively low compared to that in the prickle cell layer, hedgehog signaling in OKC appears to have the other roles besides proliferation.

Ptch2 is assumed to compensate for Ptch1 function, but their expression only partially overlaps, providing a subtle mechanism to coordinate the readout of hedgehog signaling (21). In tooth germ, both ptch1 and ptch2 are expressed in the dental epithelium, but the expression pattern is different, suggesting their different role in odontogenesis (14, 21). We could not detect Ptch2 expression in OKC lining epithelium, nor LOH of ptch2 locus. Ptch2 did not appear to associate with pathogenesis of our OKC cases.

LOH of ptch1 locus is frequently observed even in sporadic OKC, but whether its development is caused by aberrant Ptch1 function is yet to be clarified. The two-hit model insists that BCNS-associated OKC arises as a result of loss of wild ptch1 allele, whereas sporadic OKC requires two hits on the pair of wild alleles (15). But the mutation of ptch1 in sporadic OKC has not been proved so far. BCNS-associated OKC shows a significantly high labeling index of CyclinD1, whereas sporadic OKC almost always shows a low labeling index of CyclinD1 (22), suggesting different mechanisms of their pathogenesis. Contrary to that report, our preliminary data showed a relatively high labeling index of CyclinD1 in sporadic OKCs, and the epithelia also expressed Shh and Ptch1 at the comparable level to syndromic OKCs, suggesting the involvement of similar mechanism among sporadic and syndromic OKCs. An interesting issue is whether patients of non-syndromic OKC carry minor genetic mutations in ptch1 or the other genes in the hedgehog signaling cascade.

In conclusion, we demonstrated that both Ptch1 and Shh are fairly expressed in the epithelial cells of OKC associated with BCNS. Ptch1 LOH was not detected in one case of clinically diagnosed BCNS, emphasizing the importance of genomic examination and data collection of BCNS-suspected cases.

Acknowledgments
We thank Shoko Shiraki, Miwako Hamagaki and Haruka Terasaki for their technical assistance.

References


(Accepted for publication February 17, 2004)