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Expression of SHH, PTCH and SMO to the Hedgehog signal in keratocystic odontogenic tumor

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The keratocystic odontogenic tumor (KCOT) is a benign odontogenic tumor of the jaw that occurs in association with nevoid basal cell carcinoma syndrome (NBCCS). In 2005, WHO recommended that the term KCOT replace the term odontogenic keratocyst (OKC), because of the aggressive biological behavior of KCOT, and its expression of the Patched 1 (PTCH) gene that is related to KCOT tumorigenesis. We investigated the co-expression of PTCH with that of sonic hedgehog (SHH), for which PTCH is a receptor, and of smoothened (SMO), a molecule downstream of PTCH signaling to determine if this signaling pathway might be intact in KCOT. We used immunohistochemistry, western blotting and RT-PCR analyses to examine the expression of SHH, PTCH and SMO in surgical specimens of 30 cases of KCOT, 8 cases of the orthokeratinized odontogenic cyst (OOC) and 10 cases of the dentigerous cyst (DC). We also did immunohistochemical analyses of two different epithelial layers, the upper spinous layer and the lower basal layer.

Immunohistochemical analysis revealed that the spinous layer of 28 cases and the basal layer of 15 cases were positive for SHH, the spinous layer of all cases and the basal layer of 23 cases were positive for PTCH, and the spinous layer of 28 cases and the basal layer of 15 cases were positive for SMO. Western blotting showed that SHH protein expression, with a molecular weight of 17 kDa, was detected in KCOT, OOC and DC ; a PTCH protein, with a molecular weight of 161 kDa, was observed in a specific pattern in KCOT, and an SMO protein, with a molecular weight of 46 kDa, was observed in KCOT. PCR analysis indicated a specific expression pattern of PTCH in KCOT, OOC and DC. Since PTCH, SHH and SMO were expressed in some cases of KCOT, we think that non-mutated molecules in the SHH pathway may be responsible for the development of KCOT, OOC and DC. (J Osaka Dent Univ 2015 ; 49(1) : 85–93)

Key words : Keratocystic odontogenic tumor ; Sonic hedgehog ; Patched 1 protein ; Smoothened protein

INTRODUCTION

In 1960, Gorlin and Goltz established that the keratocystic odontogenic tumor (KCOT)[1] is one of the main lesions of nevoid basal cell carcinoma syndrome (NBCCS).[2] In 1996, Hahn[3] and Johnson[4] reported that 9q22.3 is a causative gene of NBCCS, and Patched 1 (PTCH) was identified as the responsible gene. PTCH is a receptor for the signaling protein Hedgehog (HH).[5–7] The HH signaling pathway regulates proliferation, differentiation and morphogenesis of cells. Sonic hedgehog (SHH) has an important role in the process of morphogenesis during development. Loss of function mutations of PTCH and gain of function mutations in smoothened (SMO) result in constitutive activation of the pathway and are thought to facilitate oncogenesis.[8, 9]

Although the association between the pathogenesis of KCOT and PTCH has been investigated, there have been few studies that have simultaneously ex-

amined PTCH and HH signaling in KCOT. We therefore used immunohistochemical, western blotting (WB) and RT-PCR analyses to clarify if SHH, PTCH and SMO were simultaneously expressed in sporadic KCOT cases, and to compare their expression in KCOT with that in the control, the orthokeratinized odontogenic cyst (OOC), and the dentigerous cyst (DC).

MATERIALS AND METHODS

Materials

Specimens were obtained from patients in Osaka Dental University Hospital with KCOT (n = 30), OOC (n = 8) or DC (n = 10), which were classified on the basis of the 2005 WHO histological typing of odontogenic tumors. The KCOT specimens were obtained from 15 male and 15 female patients between 15 and 77 years of age, with an average age of 41 years. The OOC specimens were obtained from 6 male and 2 female patients between 18 and 71 years of age, with an average age of 36 years. The DC specimens were obtained from 6 male and 4 female patients between 9 and 78 years of age, with an average age of 46 years. Six of the KCOT cases were in the maxilla (2 in anterior teeth, and 4 in molars) and 24 in the mandible (4 in anterior teeth, 2 in premolars, and 18 in molars). One case of OOC was in a maxillary molar region and 7 were in the mandible (1 in a premolar and 6 in molar region). One case of DC was in a maxillary anterior and 9 were in mandibular molar region (Tables 1 and 2). This research was approved by the Ethics Committee of Osaka Dental University (Approval number 90332).

Table 1 Age and gender of the patients with cystic lesions

	Gender		Age	
	M	F	Average	Range
KCOT (n = 30)	15	15	41	15–77
OOC (n = 8)	6	2	36	18–71
DC (n = 10)	6	4	46	9–78

KCOT : Keratocystic odontogenic tumor, OOC : Orthokeratinized odontogenic cyst, DC : Dentigerous cyst.

Table 2 Location of the cystic lesions

	Maxilla		Mandible	
	Anterior	Premolar and molar	Anterior	Premolar and molar
KCOT (n = 30)	2	4	4	20
OOC (n = 8)	0	1	0	7
DC (n = 10)	1	0	0	9

KCOT : Keratocystic odontogenic tumor, OOC : Orthokeratinized odontogenic cyst, DC : Dentigerous cyst.

Immunohistochemical staining

All specimens were fixed in 10% formalin solution, dehydrated in a graded ethanol series and embedded in paraffin. Sections with a thickness of 2 μm were cut and mounted on silane-coated glass slides (MAS-GP ; Matsunami, Kishiwada, Japan). Samples were deparaffinized in Hemo-De® (Falma, Tokyo, Japan) and rehydrated through a graded ethanol series. For immunohistochemical staining of PTCH, antigen was retrieved by activation with 1% trypsin at room temperature for 30 minutes. Endogenous peroxidase activity was blocked with Blocking One (Nacalai Tesque, Tokyo, Japan) for 30 min at room temperature. The sections were then incubated with 1 : 2000 rabbit anti-human PTCH (Abcam, Cambridge, UK) overnight at 4°C.

For immunohistochemical staining of SHH and SMO, antigen retrieval was carried out by autoclaving at 121°C for 15 min in 0.01 M citrate buffer at pH 7.0 (Mitsubishi Kagaku Yatoron, Tokyo, Japan). After autoclaving, the slides were allowed to cool to room temperature and were then briefly washed with phosphate-buffered saline. Antigen was activated with 0.01% of trypsin at room temperature for 5 minutes. Endogenous peroxidase activity was blocked with Blocking One (Nacalai Tesque) for 30 min at room temperature. The sections were then incubated with 1 : 100 rabbit anti-human SHH (Santa Cruz Biotechnology, Texas, USA) or 1 : 100 anti-human SMO (Abcam) for 60 min at room temperature. Following antibody incubation, all prepared sections were incubated

with Envision$^+$ peroxidase dextran polymer (DAKO, Glostrup, Denmark) for 30 min at room temperature. The signals were then visualized using the DAKO Envision$^+$ Kit/HRP (DAB) (DAKO), and counterstained with hematoxylin.

Evaluation of immunohistochemical staining

The epithelial lining of tissue specimens was divided into spinous and basal layers to evaluate the results of immunohistochemical staining. The cells of the two layers were considered positive (+) or negative (–) for staining when a particular protein was, or was not expressed, respectively, in these cells (Fig. 1).

Western blotting

The specimens were fixed in 10% formalin solution, dehydrated in a graded ethanol series and embedded in paraffin. Proteins were extracted from the formalin-fixed, paraffin-embedded tissues for western blot analysis using the Qproteome FFPE Tissue Kit® (Qiagen, Hilden, Germany) according to Hayashi *et al.*[12] In brief, tissue sections from the KCOT, OOC and DC specimens were cut to ten tissue sections of 10 μm thickness and immersed in the Qproteome extraction buffer. The samples were incubated for 20 min at 100°C followed by 120 min at 80°C at an agitation speed of 750 revolutions per minute. After incubation, the samples were centrifuged at 14,000 × g for 15 min at 4°C, and the supernatants (protein lysates) were transferred to a new safe-lock tube and stored at –20°C.

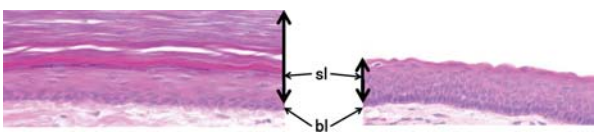

Fig. 1 Schematic diagram of the epithelial lining of tissue specimens that were divided into spinous and basal layers for evaluation of the results of immunohistochemical staining. This shows the epithelial lining of a normal tissue specimen stained with hematoxylin and eosin stain, indicating the spinous (sl) and basal layers (bl) that were used for evaluation of the results of immunohistochemical staining.

The protein lysates were boiled in sample buffer solution with 2-ME (2 ×) for 5 min at 90°C for SDS-PAGE (Nacalai Tesque) analysis. These protein lysates (containing 10 μg protein) were electrophoresed through Mini-PROTEAN® TGX™ Precast Gels (Bio-Rad, Tokyo, Japan) (SHH at 12.5%, PTCH and SMO at 7.5%) for 30 min at a constant 200 V and 0.01 A. Proteins were then transferred onto polyvinylidene difluoride membranes (Immun-Blot™ PVDF membrane ; Bio-Rad) using a wet system at 200 V and 0.1 A for 90 min. The membranes were blocked with Blocking One (Nacalai Tesque) for 30 min at room temperature. The membranes were then incubated overnight at 4°C with primary antibodies diluted in Can Get Signal® (Toyobo, Osaka, Japan) and washed three times with TBST. These membranes were then incubated in alkaline phosphatase (ALP)- conjugated goat anti-rabbit IgG polyclonal antibody (Nichirei, Tokyo, Japan) for 30 min at room temperature. The membranes were again washed three times with TBST and bound antibodies were visualized using the BCIP-NBT Solution Kit (Nacalai Tesque).

RT-PCR

The specimens were fixed in Optimal Cutting Temperature Compound (Sakura Finetek, Tokyo, Japan) at –20°C. Ten tissue sections of 10 μm thickness were cut from the KCOT, OOC and DC specimens and immersed in NucleoSpin Tissue Solution (Macherey-Nagel, Duren, Germany) as described by Tsuji *et al.*[13] DNA was purified by precipitation. PTCH primers described by Diniz *et al.*[14] were mixed with the samples in AccuPower® Optimal Cutting Temperature Compound (Sakura Finetek) (Table 3). To amplify PTCH DNA, 40 cycles of denaturation at 94°C for 1 min, an-

Table 3 Primer pairs used to study polymorphic microsatellite markers for multiple genetic loci encompassing the 9q22–31 chromosome region

PTCH	GCTATGACTTGTTCTGTGACAA	CACTACATTGTTCAAGGGTCA
PTCH	GGTTTTAGTTTGCATTTCCC	CAAACTGGAATTACAGCACTG
PTCH	CCCTCAAATTGCTGTCTAT	AGATTGATTGATACAAGGATTTG

nealing at 55°C for 2 min, and extension at 72°C for 1 min were performed using a DNA thermal cycler (Takara, Otsu, Japan). After amplification, the sample was analyzed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

RESULTS

Immunohistochemical evaluation of SHH, PTCH and SMO expression in KCOT cases

The expression of SHH, PTCH and SMO in epithelial layers of tissues from KCOT patients and control OOC and DC patients was analyzed using immunohistochemistry. For this analysis, the epithelial lining of the tissue specimens was divided into the upper spinous layer and the lower basal layer (Fig. 1). The results for the KCOT patient tissues (n = 30) were as follows. The upper spinous layer of 28 cases, and the basal layer of 15 cases were positive for SHH. The upper spinous layer of all cases, and the basal layer of 23 cases were positive for PTCH. The upper spinous layer of 28 cases, and the basal layer of 15 cases were positive for SMO (Fig. 2).

These results from KCOT patients were compared with data from control OOC and DC patients. In the OOC specimens (n = 8), the upper spinous layer of 7 cases, and the basal layer of 5 cases were positive for SHH. The upper spinous layer of all cases, and the basal layer of 5 cases were positive for PTCH. The upper spinous layer of 7 cases, and the basal layer of 5 cases were positive for SMO (Fig. 3). In the DC specimens (n = 10), the upper spinous layer of all cases and the basal layer of 9 cases were positive for SHH. The upper spinous layer of all cases and the basal layer of 7 cases were positive for PTCH. The upper spinous layer of 9 cases and the basal layer of 8 cases were positive for SMO (Fig. 4 and Table 4).

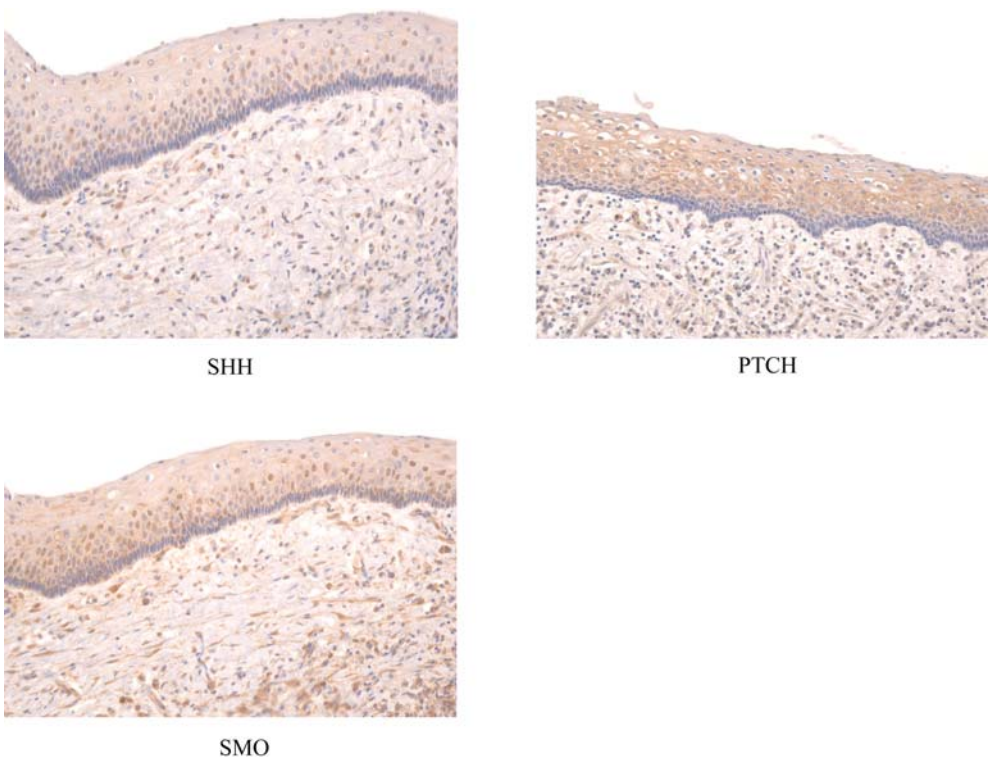

Fig. 2 Comparison of the immunohistochemical expression of SHH, PTCH and SMO in a keratocystic odontogenic tumor (KCOT). Representative immunoperoxidase staining is seen of SHH, PTCH and SMO in the KCOT specimens. KCOT cystic spaces are lined by flat and uniform epithelia composed of stratified squamous cells with six to eight cell layers. The basal cells are cuboidal or columnar-shaped and arranged in a palisading fashion. The interface of the lining epithelium and the underlining connective tissue is usually flat without rete ridge formation. Although the luminal surface cells are basically flat and show parakeratosis, occasionally they exhibit a wavy or corrugated appearance. Spinous cells and cells in the basal layer were positive for SHH, PTCH and SMO. (×200)

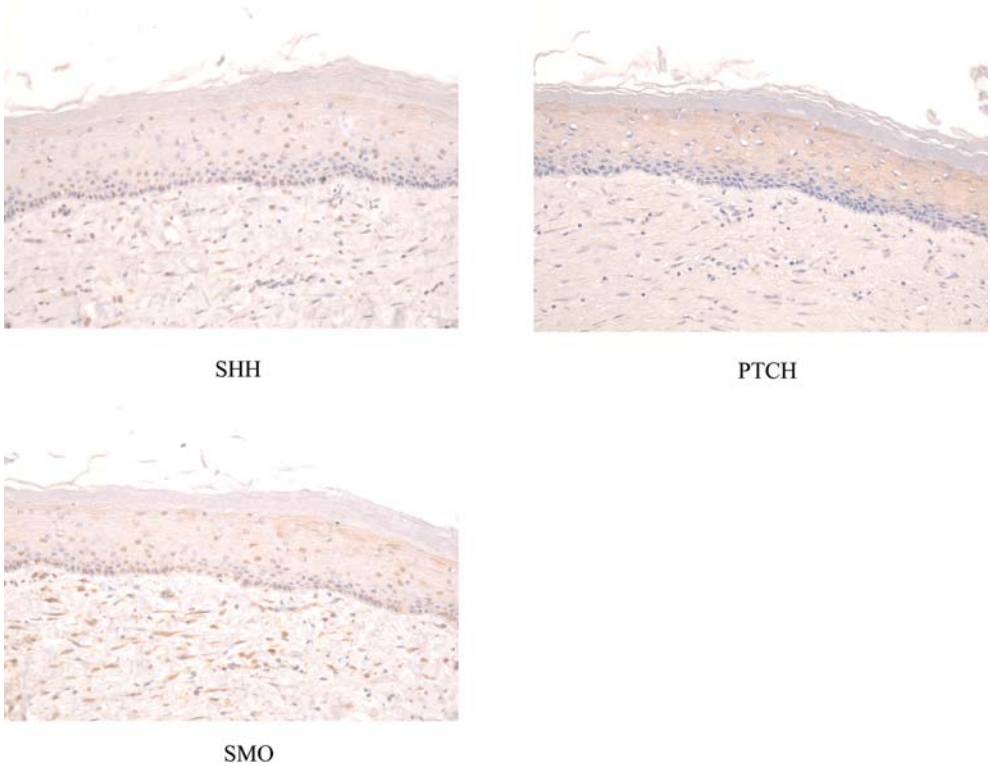

Fig. 3 Comparison of the immunohistochemical expression of SHH, PTCH and SMO in an orthokeratinized odontogenic cyst (OOC). These show representative immunoperoxidase staining of SHH, PTCH and SMO in OOC specimens. OOC cystic spaces are lined by a regular orthokeratinized stratified squamous epithelium. The basal cells are not cuboidal or columnar-shaped, but rather flat and not arranged in a palisading fashion. The basal line of the epithelia was usually flat. Spinous cells and cells in the basal layer were positive for SHH, PTCH and SMO. (× 200)

Table 4 Comparative immunohistochemical profiles of the cystic lesions

		KCOT (n = 30)	OOC (n = 8)	DC (n = 10)
SHH	Spinous layer	28 (93%)	7 (88%)	10 (100%)
	Basal layer	15 (50%)	5 (63%)	9 (90%)
PTCH	Spinous layer	30 (100%)	8 (100%)	10 (100%)
	Basal layer	23 (77%)	5 (63%)	7 (70%)
SMO	Spinous layer	28 (93%)	7 (88%)	9 (90%)
	Basal layer	15 (50%)	5 (63%)	8 (80%)

KCOT : Keratocystic odontogenic tumor, OOC : Orthokeratinized odontogenic cyst, DC : Dentigerous cyst.

Western blotting

The expression of SHH, PTCH and SMO proteins in these samples was next analyzed by western blotting. SHH, PTCH and SMO immunoreactive bands with molecular weights of 17 kDa, 161 kDa and 46 kDa, respectively, were detected in KCOT samples. An SHH immunoreactive band of 17 kDa was also confirmed in both the OOC and DC samples (Fig. 5). However, no PTCH or SMO immunoreactive bands of 161 kDa or 46 kDa, respectively, were detected in either sample (Figs. 6 and 7).

RT-PCR

RT-PCR analysis indicated that PTCH was expressed in the KCOT, OOC and DC samples. A specific pattern of PTCH mRNA expression was observed in

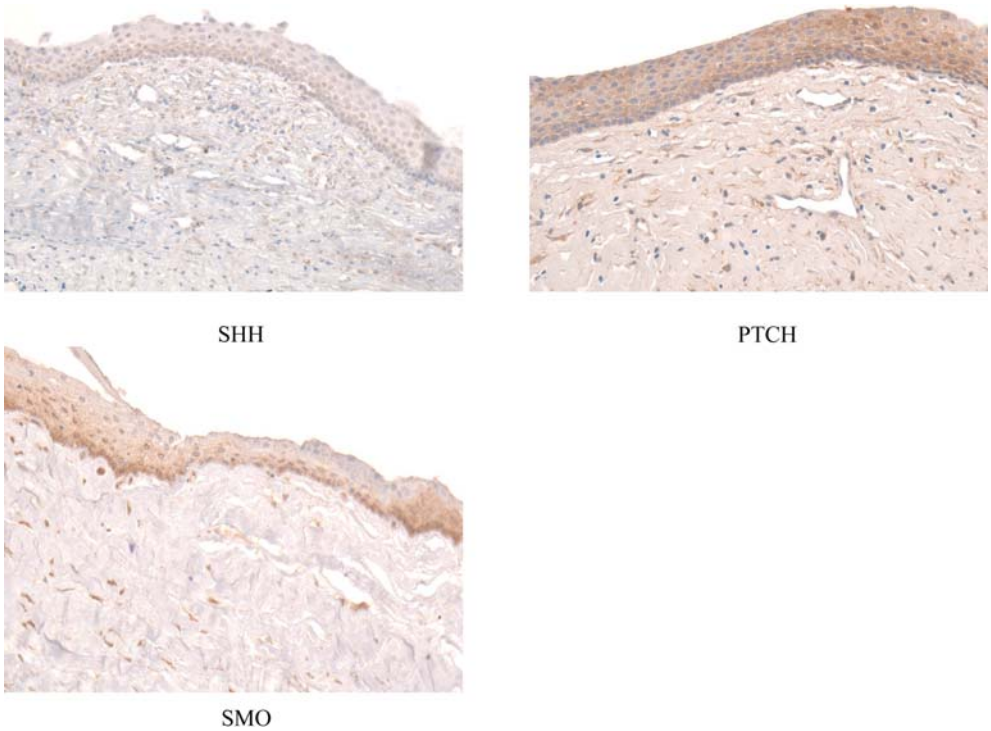

Fig. 4 Comparison of the immunohistochemical expression of SHH, PTCH and SMO in a dentigerous cyst (DC). These show representative immunoperoxidase staining of SHH, PTCH and SMO in DC specimens. The lining epithelium of dentigerous cysts typically consisted of flattened stratified squamous epithelium with a discernible alignment of basal cells. Spinous cells and cells in the basal layer were positive for SHH, PTCH and SMO. (×200)

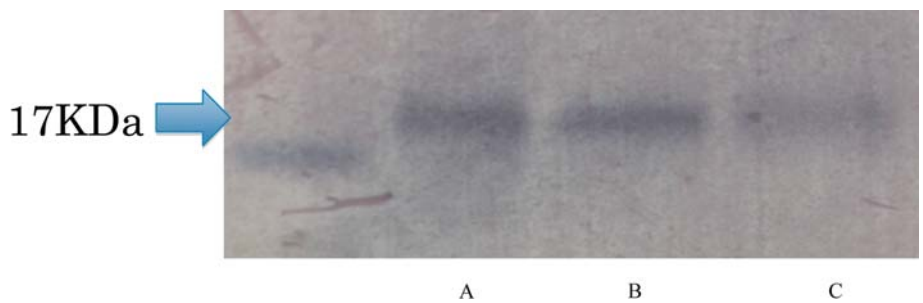

Fig. 5 Western blot analysis of SHH expression in a keratocystic odontogenic tumor (KCOT), orthokeratinized odontogenic cyst (OOC) and dentigerous cyst (DC). SHH protein expression in KCOT (Lane A), OOC (Lane B) and DC (Lane C) was analyzed by western blotting. An immunoreactive band of molecular weight 17 kDa was visible in all tissues.

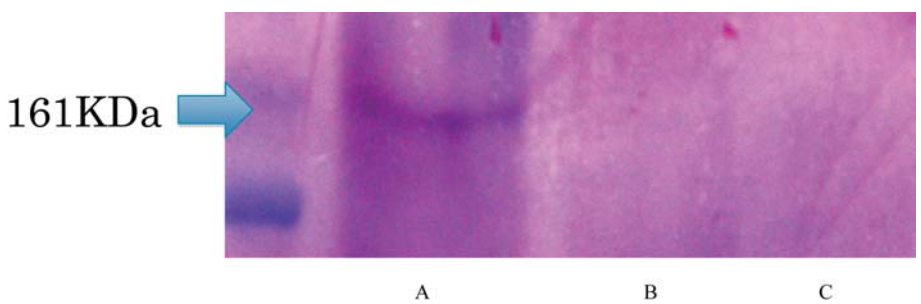

Fig. 6 Western blot analysis of PTCH in a keratocystic odontogenic tumor (KCOT), orthokeratinized odontogenic cyst (OOC) and dentigerous cyst (DC). The PTCH protein expression in KCOT (Lane A), OOC (Lane B) and DC (Lane C) was analyzed by western blotting. An immunoreactive band of molecular weight 161 kDa was detected in KCOT, but not in OOC or in DC tissue.

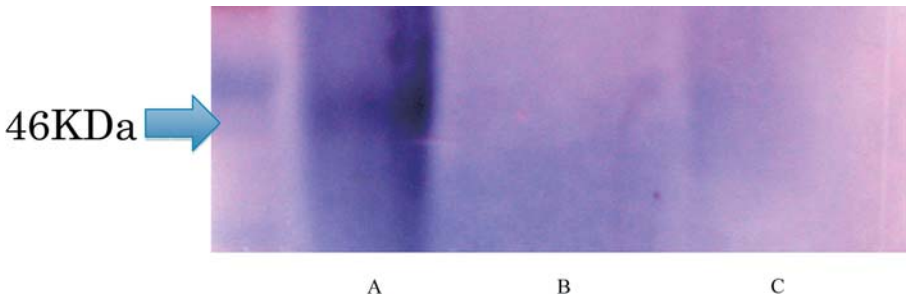

Fig. 7 Western blot analysis of SMO in a keratocystic odontogenic tumor (KCOT), orthokeratinized odontogenic cyst (OOC) and dentigerous cyst (DC).
SMO protein expression in KCOT (Lane A), OOC (Lane B) and DC (Lane C) was analyzed by western blotting. An immunoreactive band of molecular weight 46 kDa was detected in Lane A but was not in Lanes B or C.

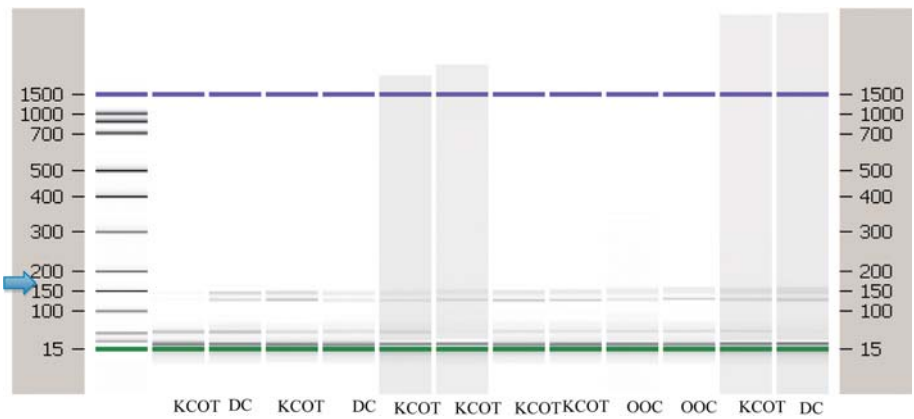

Fig. 8 RT-PCR analysis of PTCH mRNA expression in a keratocystic odontogenic tumor (KCOT), orthokeratinized odontogenic cyst (OOC) and dentigerous cyst (DC). PTCH mRNA expression was analyzed using fluorescent-labeled primers, yielding PCR products that varied in size from 20 to 205 base pairs. KCOT : A band of 1, 3, 5–8, 11 ; OOC : A band of 9, 10 ; DC : A band of 2, 4, 12.

KCOT, OOC and DC samples (Fig. 8).

DISCUSSION

KCOT was initially classified as a developmental cyst under the name odontogenic keratocyst (OKC) in the WHO Histological Typing of Odontogenic Tumours of 1992.[15] However, OKC with a parakeratinized lining epithelium was reclassified as an odontogenic tumor abbreviated KCOT in the WHO classification of 2005, because KCOT shows high proliferative activity and relapse rate.[16]

The HH signaling pathway was identified by analysis of gene clusters responsible for segmentation of Drosophila. HH was isolated as the gene cluster that participated in segmental polar decisions and such genes are called segment polarity genes.[17] Three mammalian HH signaling proteins are known ; Sonic hedgehog (SHH), Indian hedgehog (IHH) and Desert hedgehog (DHH).[17] SHH has an important biological role in many organs and tissues including the central nervous system, limbs, lungs, stomach, teeth and hair follicles. PTCH is a 12-pass transmembrane protein with two large extracellular loops, and SMO is a 7-pass transmembrane protein.[18,19] In the basal state, HH is not bound to PTCH, and PTCH inhibits the signaling of SMO, which is adjacent to PTCH in the membrane. However, binding of HH to PTCH removes its inhibitory effect on SMO, and GLI, which is a downstream transcription factor, becomes activated, shifts from the cell membrane to the nucleus, and causes transcriptional activity.[20] HH signaling is subject to negative feedback by PTCH, which was shown by

transfection of active PTCH into cells.[21, 22]

Misaki *et al.*[23] reported an immunohistochemical analysis of the expression of SHH in KCOT, in which they found that the cytoplasm of cells in the spinous layer (sl) were positive for SHH expression, and that the cells of the basal layer (bl) were diffusely positive for SHH. Cells of the bl were also positive for PTCH. In our study, 93% (28/30) of the KOT cases were SHH positive in the sl, and 50% (15/30) in the bl. 100% (30 /30) of the KCOT cases were positive for PTCH in the sl, and 77% (23/30) in the bl, while 93% (28/30) of the KCOT cases were positive for SMO in the sl, and 50% (15/30) in the bl. The expression of SHH, PTCH and SMO in the control OOC and DC tissues differed from that in KCOT. SHH is known to be an essential factor in the early development of the tooth and has a specific function in all processes up to the formative period.[24, 25] The concurrent expression of SHH, PTCH and SMO in these tissues prior to tooth eruption is similar to that observed in KCOT.

The expression of SHH, PTCH and SMO in KCOT was further confirmed by western blotting. Although SHH expression was detected by western blotting in OOC and DC, PTCH and SMO expression was not. The reason why PTCH and SMO expression was not detected may have been due to a problem with tissue collection, in that a sufficient amount of the thin epithelia may not have been collected from these tissues. Another possibility is that SMO was cleaved during the protein extraction. The expression of SHH, PTCH and SMO in KCOT as assessed by western blotting was similar to that as assessed by immunohistochemistry. Our data also suggest that SHH was expressed in OOC and DC.

RT-PCR analysis of the present study indicated the expression of PTCH mRNA in KCOT, OOC and DC. In cells, when HH is not bound to PTCH, PTCH suppresses downstream SMO signaling. However, when HH is bound to PTCH, then a downstream transcription factor is activated by removal of the inhibition of SMO. This factor then moves from the cell membrane to the nucleus and activates transcription.[26] HH signaling can be regulated by negative feedback via active PTCH.[27, 28] Equivalent expression of these proteins was detected with both immunohistochemical staining and western blotting.

Barreto *et al.*[29] and Sun *et al.*[30] reported a mutation of PTCH in sporadic cases of KCOT. Nakano[31] reported that KCOT was mutated in about 30% of the cases examined. Endo *et al.*[32] reported that no PTCH mutation could be detected in three of the 25 NBCCS patients. These results suggest that mutation of PTCH is involved in sporadic KCOT. Future analysis of PTCH mutation will contribute to understanding of the role of PTCH in KCOT.

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