Recurrence of Keratocystic Odontogenic Tumor: Clinicopathological Features and Immunohistochemical Study of the Hedgehog Signaling Pathway

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Key Words

Keratocystic odontogenic tumor • Recurrence • Immunohistochemistry • Sonic hedgehog • Patched • Smoothed

Abstract

Aims: Critical factors responsible for the recurrence of keratocystic odontogenic tumor (KCOT) were examined. **Methods:** The clinicopathological features were retrospectively studied in 74 patients with 75 sporadic KCOTs. From the 75 KCOTs, 23 were examined for the expression of Sonic Hedgehog (SHH), Patched and Smoothed (SMO) by immunohistochemistry. **Results:** Recurrence in multilocular lesions was more frequent than in unilocular lesions. Nine (64%) of 14 multilocular lesions recurred, in contrast to 2 (7%) of 27 unilocular lesions ($p = 0.0350$). The average length of recurrent lesions (62.8 ± 6.5 mm) was larger than that of nonrecurrent lesions (43.0 ± 4.0 mm; $p = 0.0363$). The immunoreactivity of proliferation-related SMO in KCOTs with recurrence was higher than that of those without recurrence ($p = 0.0475$), whereas the expressions of a ligand, SHH, and an inhibitory receptor, Patched, were not associated with KCOT recurrence. The expressions of SHH and SMO showed inverse cor-

relation in whole KCOT ($p = 0.0318$). **Conclusion:** These findings suggest that recurrence of KCOT is associated with multilocular large lesions and high SMO expression.

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Introduction

Keratocystic odontogenic tumor (KCOT) is a benign intraosseous tumor of odontogenic origin with a characteristic lining of parakeratinized stratified squamous epithelium that sometimes occurs in association with nevusoid basal cell carcinoma syndrome (NBCCS). KCOT is well known to have aggressive potential and infiltrative behavior. Recurrence rates were reported to be 5–62.5% [1, 2], but the clinical and pathological factors responsible for recurrence remain controversial.

Recent studies have demonstrated that the Patched (PTCH) gene is responsible for NBCCS. Mutations and loss of heterozygosity within the PTCH gene have been identified in sporadic KCOT as well as NBCCS-related neoplasm [3–6]. PTCH plays a key role in the regulation of the hedgehog (HH) signaling pathway, and HH signaling is emerging as one of the most important regulators

of oncogenic transformation. Although the roles of the HH signaling pathway in the development of KCOT are not well known, activation of this pathway may be related to the clinical behavior and outcomes of KCOT.

In the present study, therefore, we investigated the immunohistochemical expression of molecules related to the HH signaling pathway as well as the clinical and pathological features of 74 cases of KCOT in order to clarify critical factors responsible for KCOT recurrence.

Materials and Methods

Subjected Cases

The records of 74 patients with 75 sporadic KCOTs treated in the Department of Oral and Maxillofacial Surgery at the Hospital of Nara Medical University between October 1981 and December 2002 were retrospectively studied. One patient was diagnosed with 2 lesions. Patients associated with the signs of NBCCS were excluded from this study. All lesions fulfilled the criteria for KCOT as defined by the 2005 WHO classification of tumors [6]. Analysis included age, gender, location and size of lesion, surgical procedures, the presence of impacted teeth and daughter cysts (including epithelial islands). The site of involvement was divided into the following 9 regions: mandibular anterior, mandibular anterior-molar, mandibular molar, ramus, molar-ramus, maxillary anterior, maxillary anterior-molar, maxillary molar and maxillary sinus. In order to analyze the relationship between recurrence and clinicopathological findings, we excluded patients who were lost to follow-up within 12 months.

Tissue Samples for Immunohistochemistry

To analyze members of the HH signaling pathway in KCOTs, we examined the immunohistochemical expression of Sonic Hedgehog (SHH), PTCH and Smoothed (SMO). We selected 23 specimens of KCOTs from 75 banked KCOT cases at the Nara Medical University Hospital. These are specimens obtained at primary treatment and consisted of 17 lesions without recurrence and 6 lesions with recurrence. We also selected 5 archival specimens of orthokeratinized odontogenic cyst (OOC), which was described as a variant of KCOT in the 1995 WHO classification of tumors. The criterion for OOC is an intraosseous jaw cyst with an epithelial lining, all or a large portion of which exhibits orthokeratinization and in which basal cells show little tendency to palisade or polarize. Lesions containing focal areas of typical KCOT or various skin appendages were not included.

Immunohistochemistry

Immunohistochemistry was performed as previously described [7]. Consecutive 4- μ m sections were cut from each block, and immunostaining was performed using the immunoperoxidase technique following antigen retrieval with pepsin (Dako, Carpinteria, Calif., USA) treatment for 20 min. After endogenous peroxidase block by 3% H₂O₂-methanol for 15 min, specimens were rinsed with phosphate-buffered saline (PBS), and incubated at room temperature with anti-SHH antibody (Santa Cruz Biotechnology, Santa Cruz, Calif., USA), anti-PTCH antibody (Santa Cruz) and anti-SMO antibody (Santa Cruz) for 2 h. The applied

Table 1. Antibodies used for immunohistochemistry

	Clonality	Source	Dilution
SHH	polyclonal (rabbit)	Santa Cruz Biotechnology	1:100
PTCH	polyclonal (goat)	Santa Cruz Biotechnology	1:100
SMO	polyclonal (goat)	Santa Cruz Biotechnology	1:100

antibodies are listed in table 1. The specimens were rinsed with PBS and incubated at room temperature for 1 h with secondary antibody conjugated to peroxidase diluted at 0.5 μ g/ml (anti-goat IgG; Medical & Biotechnological Laboratories Co. Ltd., Nagoya, Japan). All specimens were then rinsed with PBS and color developed using diaminobenzidine solution (Dako). After washing with water, specimens were stained with Meyer's hematoxylin (Sigma, St. Louis, Mo., USA). Immunostaining of all specimens was performed to ensure the same condition of antibody reaction and diaminobenzidine exposure.

Evaluation of Immunoreactivity

Immunoreactivity was evaluated according to the proportion of positive cells and average intensity of positive cells in the intermediate layer [8]. A proportion score was assigned as the estimated proportion of positive cells in the intermediate layers, ranging from 0 to 3 as follows: 0 = no positive cells; 1 = 1–25% positive cells; 2 = 25–50% positive cells; 3 = >50% positive cells. An intensity score was assigned as the average staining intensity of positive cells, ranging from 0 to 3 as follows: 0 = negative; 1 = weak; 2 = moderate; 3 = strong. Proportion score and intensity score are added to obtain a total score (TS) (0, 2–6). We classified TS into 3 grades as follows: grade 0 = TS 0; grade 1 = TS 2–4; grade 2 = TS 5–6.

Statistical Analysis

The clinicopathological parameters were analyzed by Student's t test, Pearson's χ^2 test and Fisher's exact test. The statistical significance of differences in the percentages of cases with different reactivity levels was analyzed by the Mann-Whitney U test for differences among 2 groups or the Kruskal-Wallis test for differences among 3 groups. $p < 0.05$ were considered significant. Statistical analyses were performed using Microsoft Excel with the Statcel2 statistical add-on package (OMS Co., Tokyo, Japan).

Results

Clinicopathological Findings

The 74 patients included 49 men and 25 women (ratio 1.96:1), aged from 8 to 83 years (average 41.1 years). One patient was diagnosed with 2 lesions. Fifty-three patients (71.6%) presented with symptoms (swelling, pain, nasal discharge and fistula formation), whereas the remaining

Table 2. Anatomic locations of KCOTs

Location in maxilla	KCOTs ¹	Location in mandible	KCOTs ¹
Anterior	5	Anterior	9
Anterior-molar	4	Anterior-molar	2
Molar	9	Molar	26
Sinus	2	Ramus	8
		Molar-ramus ²	10
Total	20		55

¹ 74 patients with 75 lesions, excluding NBCCS patients.
² Combined lesion between the molar and the ramus.

Table 3. Clinicopathological findings of KCOTs

	KCOTs ¹
Figure	
Unilocular	43
Multilocular	19
Unknown	13
Association with impacted teeth	
With	16
Without	43
Unknown	16
Size	
<20 mm	9
20–40 mm	17
40–60 mm	16
60–80 mm	8
>80 mm	4
Mean ± SE	41.8 ± 2.9
Unknown	21
Daughter cysts ²	
With	10
Without	65
Total	75

¹ 74 patients with 75 lesions, excluding NBCCS patients.
² Including epithelial islands.

21 (28.4%) were unexpectedly discovered on routine radiophotographs of dental treatment. Follow-up periods ranged from 0 to 183 months (mean 40.3 months). Twenty-five patients (33.8%) were lost to follow-up within 12 months, and were excluded from analysis of recurrence. Of the 75 sporadic KCOTs, 55 lesions (73.3%) were found in the mandible and 20 (26.7%) in the maxilla (table 2). The mandibular molar regions (34.6%) were most fre-

Table 4. Clinicopathological findings of KCOTs with recurrence

	KCOTs ¹		
	without recurrence	with recurrence	total
Figure			
Unilocular ²	25	2	27
Multilocular ²	9	5	14
Unknown	8	0	8
Association with impacted teeth			
With	12	2	14
Without	19	5	24
Unknown	11	0	11
Size			
<20 mm	6	0	6
20–40 mm	7	1	8
40–60 mm	11	2	13
60–80 mm	5	4	9
>80 mm	3	0	3
Mean ± SE	43.0 ± 4.0 ³	62.8 ± 6.5 ³	46.6 ± 3.7
Unknown	10	0	10
Daughter cysts ⁴			
With	5	1	6
Without	37	6	43
Primary treatment			
Enucleation	34	6	40
Marsupialization	5	1	6
Simple fenestration	3	0	3
Total	42	7	49

¹ Totally 49 patients with follow-up longer than 12 months, excluding NBCCS patients
² Statistically significant: $p = 0.0350$.
³ Statistically significant: $p = 0.0363$.
⁴ Including epithelial islands.

quently involved. Radiographic findings of 62 lesions showed that 43 (69.4%) showed unilocular radiolucency and 19 (30.6%) were multilocular (table 3). Sixteen lesions (27.1%) were associated with an impacted tooth in 59 lesions. The maximal length of the cavity measured on the panoramic radiograph in 54 KCOTs was variable. It ranged from 6 to 100 mm (mean ± SE 41.8 ± 2.9 mm).

Enucleation and primary closure were performed in 65 lesions, and 7 lesions required a combination of marsupialization followed with enucleation. Simple fenestration was performed in 3 lesions associated with an impacted tooth for its eruption. Marginal or segmental resection was not performed in any lesions. Pathological review showed that 10 lesions had epithelial islands and/or daughter cysts (table 3) and none of the lesions showed evidence of malignant transformation.

Table 5. Immunohistochemical expression of SHH, PTCH and SMO in KCOT and OOC

	Total	SHH		PTCH		SMO ¹	
		0-1	2	0-1	2	0-1	2
KCOT							
Total	29	26	3	20	9	19	10
Without recurrence	23	21	2	16	7	18	5 ²
With recurrence	6	5	1	4	2	1	5 ^{2, 3}
OOC							
Total	4	2	2	1	3	4	0 ³

¹ SMO-positive cases were treated with enucleation (9 cases) and marsupialization (1 case); SMO-negative cases were treated with enucleation (18 cases) and marsupialization (1 case; $p = 1.0000$).

² Statistically significant: $p = 0.0105$ (Fisher's exact test).

³ Statistically significant: $p = 0.0476$ (Fisher's exact test).

Factors for Recurrence

In the 49 patients followed up for longer than 12 months, factors responsible for recurrence were examined. A summary of recurrence and clinicopathological findings is presented in table 4. Recurrence occurred in 7 patients (14.3%), of which 2 experienced recurrence twice. As the primary treatment, enucleation and primary closure were performed in the 6 patients with recurrence. In the remaining patient, marsupialization was performed the first time and we planned to perform enucleation after the lesion reduced; however, it grew larger while observing its progress and we treated it as recurrence. There was no correlation between treatment methods and recurrence. All recurrent lesions were treated by enucleation and primary closure. The mean period until first recurrence was 34 months (range 13–79 months). No significant difference was found between the recurrence rates of KCOTs in the mandible and maxilla (12.2 and 5.2%, respectively). Five recurrences were multilocular and 2 were unilocular. The recurrence rate of multilocular lesions was 35.7%, which was significantly higher than that of unilocular lesions (7.4%; $p = 0.0350$). The mean length of the cavity on the panoramic radiograph in the recurrent group was 62.8 ± 6.5 mm, significantly larger than in the nonrecurrent group (43.0 ± 4.0 mm; $p = 0.0363$). The presence of neither daughter cysts nor impacted teeth was statistically related to recurrence.

Expression of HH Signal Factors

The immunoreactivity of PTCH, SHH and SMO is summarized in table 5. In most KCOTs and OOCs, positive immunostaining of PTCH, SHH and SMO was detected in the intermediate layer, but rarely in the superfi-

cial and basal layers. Immunostaining was seen in the cytoplasm of epithelial cells (fig. 1). The immunoreactivity of SMO in KCOTs with recurrence was significantly higher ($p = 0.0105$) than in those without recurrence (table 5). In SMO-positive cases, surgical procedures were not correlated with recurrence. There was no distinct difference of SHH or PTCH reactivity between KCOTs with and without recurrence. The immunoreactivity of SMO of KCOTs was not significantly different from that of OOC. Immunoreactivity of SMO in OOC was significantly lower than in KCOTs with recurrence ($p = 0.0476$), but this was not the case when compared to KCOTs without recurrence.

Discussion

Recurrence rates of KCOT are reported from 5 to 62.5% [1, 2]. Recurrence usually occurs within the first 5 years and is reported as long as 41 years after removal [1, 2, 9]. In the present study, recurrence occurred in 7 patients with a recurrence rate of 14.3%. The interval from treatment to recurrence ranged from 13 to 79 months (mean 34 months). Recurrence rates may be influenced by several factors, including the length of the follow-up period, treatment modalities, the site of involvement, the size of lesions, histopathological findings, number of cases investigated and others [10].

We showed that the recurrence rate of multilocular lesions is significantly higher than that of unilocular lesions, and that the average length of recurrent lesions is significantly larger than that of nonrecurrent lesions. These findings suggest that recurrence is partly related to inadequate treatment, because multilocular and large le-

Fig. 1. Immunohistochemical examination of PTCH, SHH and SMO in keratocystic odontogenic cysts. Positive immunostaining of SHH, PTCH and SMO was detected in the intermediate layer, but rarely in the superficial and basal layers. Immunostaining was seen in the cytoplasm of epithelial cells. Immunohistochemical grading (TS; proportion score + intensity score) of SHH, PTCH and SMO in the representative lesion was grade 1 (4; 1 + 3), grade 2 (6; 2 + 4) and grade 2 (6; 2 + 4), respectively. Scale bar = 100 μ m.

sions are difficult to access and the fragments are easily overlooked. A previous study also showed that recurrent odontogenic keratocysts (OKCs) can be attributed to incomplete resection of the lesion [11].

Although Myoung et al. [10] reported that OKCs located in the mandibular molar region had a significantly higher recurrence rate (75%) than those of other regions, no significant difference was found among the recurrence rates at various sites of involvements in the present study. No correlation between recurrence and the presence of

daughter cysts or impacted teeth was noted in the present study, which corresponded with previous reports [12].

The treatment of KCOT remains controversial. It is reported that treatment methods influence the outcomes of KCOT, and that enucleation showed a significantly higher recurrence rate than resection [12–14]. In the present study, however, enucleation with primary closure was chosen as the primary treatment and marsupialization followed by enucleation was also applied in a few cases. Marginal or segmental resection was not performed in

any lesions. Recurrence rates after enucleation and marsupialization were 15 and 17%, respectively. We consider that these results are acceptable and recurrent lesions were successfully treated.

In order to investigate the influence of the HH signaling pathway on the clinical behavior and outcomes of KCOT, we immunohistochemically examined the expression pattern of PTCH, SHH and SMO in sporadic KCOTs. Our study showed that the recurrence of KCOT is related to SMO expression. SMO is known to activate GLI1, which results in upregulation of the transcription of cellular proliferation genes [15]. In the present study, we confirmed that the case with strong SMO expression showed higher Ki67 labeling than SMO-negative cases. Moreover, SMO expression was often found at the solitary epithelial nest in the stroma (data not shown). Therefore, overexpression of SMO could increase cell proliferation and be related to recurrence. However, a previous study reported no statistical difference in SMO as well as SHH and PTCH reactivity between KCOTs with and without recurrence [5]. This discrepancy may be caused by the difference of our evaluation of immunoreactivity. We precisely evaluated both the proportions and average intensities of positive cells and classified them by our scoring method. Analysis of the immunohistochemical examination of SMO expression by our scoring method might have an impact on the prediction of clinical outcomes of KCOTs.

It is still obscure whether OOC should be categorized as a variant of KCOT. Although OOC was classified as a variant of OKC in the 1995 version of the WHO classification, the new WHO classification (2005) [6], in which the term KCOT is recommended rather than OKC, does not refer to the designation of OOC. Our results showed that immunoreactivity of SMO in OOC is significantly lower than in KCOTs with recurrence, but not in KCOTs without recurrence. This suggests that the characteristics of the HH signaling pathways of OOC and KCOTs without recurrence might be similar, whereas KCOTs with recurrence might possess aggressive behaviors substantially different from OOC and KCOTs without recurrence. Therefore, we think that the classification of OOC might still need further examination, including classification as odontogenic developmental cysts [16–18].

In conclusion, the present study showed that recurrence of KCOT occurred at a significantly higher rate in multilocular and large lesions, and was also related to SMO expression.

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