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Histopathological and immunohistochemical study in keratocystic odontogenic tumors: Predictive factors of recurrence

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Abstract. The aim of the present study was to identify the most useful markers for predicting recurrence of keratocystic odontogenic tumors (KCOTs). A total of 65 tumor samples from 63 patients diagnosed with typical parakeratinized cysts and KCOTs between 1992 and 2014 were retrospectively studied. Clinical and histopathological data and treatment modality were reviewed. In addition, the expression profiles of Ki-67, cluster of differentiation (CD)34 and podoplanin were assessed using immunohistochemistry. The association between these factors and the rate of KCOT recurrence was evaluated. The presence of daughter cysts, epithelial islands and high Ki-67, CD34 and podoplanin expression levels were revealed to be associated with tumor recurrence. In particular, univariate analysis revealed that high CD34 expression levels were significantly associated with tumor recurrence (P=0.034), as was conservative surgical treatment (P=0.003). Multivariate analysis revealed that conservative treatment was the greatest independent risk factor for tumor recurrence (odds ratio=13.337; P=0.018). These results suggest that overexpression of CD34 may be a potent predictor of tumor recurrence and radical treatment of the teeth that are in contact with the tumors is recommended in order to prevent tumor recurrence.

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Abbreviations: KCOT, keratocystic odontogenic tumor; CD, cluster of differentiation; MVD, microvesssel density; NBCCS, nevoid basal cell carcinoma syndrome; WHO, World Health Organization; LI, labeling index

Key words: keratocystic odontogenic tumor, recurrence, marker of proliferation Ki-67, cluster of differentiation 34, podoplanin

Introduction

Keratocystic odontogenic tumors (KCOTs) are classified as benign odontogenic tumors by the World Health Organization (WHO) (1). A KCOT is defined as a benign unicystic or multicystic intraosseous tumor of odontogenic origin, with a characteristic lining of parakeratinized stratified squamous epithelium that is noted for its locally aggressive nature and capacity for recurrence (1,2). The high rate of recurrence is due to the neoplastic nature of these tumors, including a high rate of proliferative activity and angiogenesis, and the presence of daughter cysts and epithelial islands (3-5). The incomplete surgical removal of the epithelial components of KCOTs due to their fragility is also a factor contributing to recurrence (3,6).

Various markers of proliferation as well as microvessel density (MVD) have previously been examined with regard to their capacity to predict tumor recurrence in immunohistochemical studies (3-8). One such marker of proliferation is Ki-67, a prototypical cell cycle-associated nuclear protein that is expressed by proliferating cells in all phases of the cell cycle, with the exception of G_0, and is rapidly degraded following mitosis, with a half-life of ≤1 h (7,8). The immunohistochemical detection of Ki-67 has been used to evaluate the proliferative potential of tumor cells, and to predict the recurrence of KCOTs (7,8).

MVD is determined by detecting the expression levels of cluster of differentiation (CD)34, and is frequently used to quantify angiogenesis in benign and malignant oral tumors, including KCOTs (4,9,10). In our previous study, it was reported that CD34 expression was associated with tumor growth in oral squamous cell carcinoma (10).

Podoplanin is a transmembrane glycoprotein that is specifically expressed by lymphatic endothelial cells, and its expression is associated with lymph node metastasis in head and neck squamous cell carcinoma (11). Podoplanin has also been detected in a variety of neoplastic tissues, and its expression may be associated with the extracellular matrix signaling pathways, neoplastic nature and proliferative capacity of KCOTs (5,12,13).

Although these markers are useful for predicting tumor recurrence, to the best of our knowledge, no previous studies have examined their utility in the context of surgical treatment

procedures for KCOTs. In the present study, clinicopathological and immunohistochemical analyses were combined in order to investigate how these factors may be associated with KCOT recurrence.

Materials and methods

Patients. The present study was approved by the independent ethics committee of Nagasaki University Hospital (Nagasaki, Japan; approval no. 15061127). The medical records of patients who were diagnosed with a typical parakeratinized cyst and KCOT between 1992 and 2014 at Nagasaki University Hospital according to the WHO classification, and who underwent a complete surgical tumor excision, were retrospectively reviewed in the current study. Paraffin embedded samples from these patients were obtained from the pathology department of Nagasaki University Hospital. Information regarding patient gender and age, the site and duration of the lesion prior to treatment, surgical modality and the time to follow-up and tumor recurrence was obtained from the patient records. Patients for whom the follow-up period was <6 months, and those who were diagnosed with nevoid basal cell carcinoma syndrome (NBCCS), were omitted from the current study. One patient with multiple lesions was selected for the present study as they had not satisfied the diagnostic criteria for NBCCS (14).

Immunohistochemistry. Paraffin-embedded sections of resected KCOT tissue specimens cut at 4 μm were deparaffinized in xylene and were soaked in 10 mmol/l citrate buffer (pH 6.0) and placed in an autoclave at 121˚C for 5 min for antigen retrieval. Endogenous peroxidase was blocked by incubation with 0.3% H_2O_2 in methanol for 30 min. Immunohistochemical staining was performed using the Envision system (ENVISION+; Dako; Agilent Technologies, Inc., Glostrup, Denmark). The following antibodies were used as primary antibodies: Monoclonal antibody for Ki-67 (dilution, 1:50; cat. no., M7240; Dako, Agilent Technologies, Inc.), CD34 (undiluted; cat. no., M716529; Dako, Agilent Technologies, Inc.) and podoplanin (dilution, 1:50; cat. no., M3619; Dako, Agilent Technologies, Inc.) derived from mouse. The sections were then washed in Dulbecco's PBS, followed by incubation with the primary antibodies at 4˚C overnight. Following the incubation of sections with secondary antibodies (undiluted; cat. no., K4001; Dako, Agilent Technologies, Inc.) for 30 min at room temperature, the reaction products were visualized by immersing the sections in diaminobenzidine solution, and the samples were counterstained with Meyer's hematoxylin and mounted. Negative controls were created by the replacement of the primary antibody with phosphate-buffered saline (PBS). Ki-67 expressions were evaluated by light microscopy in the basal to suprabasal cell layers by calculating the mean number of positive cells in 5 randomly selected visual fields of each tissue section. The MVD was determined from CD34 expression levels in the stromal cells bordering the tumor parenchyma, by evaluating the number of CD34-positive capillaries in the 200-μm region immediately below the epithelium, as previously described (4). The neoplastic character of the tumor tissues was assessed using podoplanin labeling in the basal to suprabasal cell layers by calculating the total immunoreactivity score as the product of proportional scores, which were based on the estimated fraction of positively labeled tumor cells for all tumor cells (0, none; 1, <25%; 2, 25-50%; 3, >50%) (5). All immunohistochemical assessments were performed blind by two examiners, and based on the results of these assessments, the present study compared the responses and characterized the tumors.

Statistical analysis. The associations between marker expression levels and the patient clinicopathological features were analyzed using Fisher's exact test. Continuous data are presented as the median with interquartile range (IQR). Multiple logistic regression analysis and univariate and multivariate logistic regression analyses were performed in order to identify independent factors for predicting tumor recurrence. Predictors that were not determined to be associated with recurrence by the univariate analysis were not included in the multivariate analysis. $P<0.05$ was considered to indicate a statistically significant result.

Results

Clinical characteristics of patients with KCOTs. Medical records were reviewed for a total of 65 tumors in 63 patients who were treated during the aforementioned 22-year period, and the clinicopathological features are summarized in Table I. Males and females accounted for 58.7 and 41.3% of patients, respectively. The median age of the patients was 41 years (range, 10-87 years). Regarding the site of tumor involvement, 44 patients (69.8%) had mandibular tumors, 18 (28.6%) had maxillary tumors, and 1 (1.6%) had tumors involving the maxilla and the mandible. Radiographic examination using panoramic and computed tomography revealed unilocular radiolucency in 53 tumors (81.5%), with the remainder (18.5%) being multilocular. The median tumor size was 35 mm (range, 5-120 mm). Single tumors were detected in 62 patients (98.4%), whereas 1 patient (1.6%) had three tumors. Recurrence was observed for 13/65 tumors (20.0%) and the median time to recurrence was 36 months (range, 10-137 months). Of the 65 tumors, 55 (84.6%) were treated with surgical enucleation, and 10 (15.4%) with marsupialization and subsequent enucleation. The median follow-up period for marsupialization was 5.5 months (range, 5-14 months). In 24 tumors (36.9%), enucleation was used in combination with peripheral ostectomy with a bone bur (Table II). The most frequently used treatment modality for cases in which the roots of teeth were in contact with the margins of the primary tumor was conservative (no extraction; 37 cases; 56.9%), which included 29 cases of no treatment and 8 cases of apicoectomy; radical treatment (extraction) was administered for 28 tumors (43.1%), including 22 extractions and 6 cases in which there was no contact with the root (solitary tumor; Table III). No patients underwent a partial mandibulectomy or maxillectomy.

Histopathological and immunohistochemical analysis. The presence of one or more daughter cyst (Fig. 1A) or epithelial island (Fig. 1B) in the cyst wall was observed in 32/65 tumors (49.3%), of which 7 recurred during the follow-up period. No daughter cysts or epithelial islands were observed in the cyst wall of 33 tumors (50.7%), and 6 of these cases demonstrated recurrence during the follow-up period.

Hematoxylin-eosin staining (Fig. 2A) and immunohistochemical analysis (Fig. 2B-D) revealed the presence of

Table I. Clinicopathological characteristics of 65 keratocystic odontogenic tumors in 63 patients.

Characteristic	Value
Gender, n (%)	
Male	37 (58.7)
Female	26 (41.3)
Age, years	
Range	10-87
Median	41
Site of involvement, n (%)	
Maxilla	18 (28.6)
Mandible	44 (69.8)
Mixed	1 (1.6)
Number of tumors, n (%)	
Single	62 (98.4)
Multiple	1 (1.6)
X-ray results, n (%)	
Unilocular	53 (81.5)
Mutilocular	12 (15.5)
Tumor size, mm	
Range	5-120
Median	35
Follow-up period, months	
Range	6-252
Median	16
Daughter cysts/epithelial islands, n (%)	
Absent	33 (50.7)
Present	32 (49.3)
Recurrence, n (%)	
No	52 (80.0)
Yes	13 (20.0)
Recurrence period, months	
Range	10-137
Median	36

Ki-67-positive cells in the basal and suprabasal cell layers of the tissue samples. The median Ki-67 labeling indexes (LI) was 7.5% (IQR=1.8-17.26) in all tumors, and 5.0% (IQR=0-17.3) and 12.5% (IQR=7.5-17.1) in non-recurrent and recurrent tumors, respectively. There were 4 tumors (13.8%) with an LI ≤7.5% and 9 (39.1%) with an LI >7.5% that demonstrated recurrence (Fig. 2B). CD34-positive blood vessels were observed in the connective tissues and the MVD was 6.5% (IQR=3-10) in all tumors, and 5.0% (IQR=3-9.25) and 8.5% (IQR=6.25-14.5) in non-recurrent and recurrent tumors, respectively. There were 3 tumors with an MVD <6.5 and 10 with an MVD >6.5 that demonstrated recurrence (Fig. 2C; Table IV). Podoplanin was expressed in the cell membrane and cytoplasm of the majority of cells in the basal and suprabasal cell layers. Recurrence was observed in 3 tumors with scores of 0 or 1, and in 10 tumors with scores of 2 or 3 (Fig. 2D; Table V).

Table II. Surgical modality used in the treatment of keratocystic odontogenic tumors (n=65).

Factor	Value
Marsupialization and subsequent enucleation, n (%)	10 (15.4)
Follow-up period of marsupialization, months	
Range	5-14
Median	5.5
Enucleation alone, n (%)	55 (84.6)
Peripheral ostectomy, n (%)	
Absent	41 (63.1)
Present	24 (36.9)

Table III. Surgical modality used when the root of the tooth was in contact with the margin of the primary keratocystic odontogenic tumor (n=65).

Surgical modality	No. of cases (%)
Conservative treatment (non-extracted)	37 (56.9)
No treatment	29 (44.6)
Apicoectomy	8 (12.3)
Radical treatment (extracted)	28 (43.1)
No contact with primary tumors	6 (9.3)
Extraction	22 (33.8)

Table IV. Ki-67 and CD34 immunoreactivity (MVD) in keratocystic odontogenic tumors.

Factor	No. of cases	Ki-67 LI, % (IQR)	MVD, % (IQR)
Non-recurrence	52	5.0 (0-17.3)	5.0 (3-9.25)
Recurrence	13	12.5 (7.5-17.1)	8.5 (6.25-14.5)
Total	65	7.5 (1.8-17.3)	6.5 (3-10)

Data are presented as the median with interquartile range. CD34, cluster of differentiation 34; MVD, microvessel density; LI, labeling index.

Table V. Podoplanin immunoreactivity in keratocystic odontogenic tumors.

	Number of patients				
Factor	Total	Score 0	Score 1	Score 2	Score 3
Non-recurrence	52	6	8	16	22
Recurrence	13	0	3	2	8

Uni- and multivariate analyses of tumor recurrence. A univariate analysis revealed that high CD34 expression levels

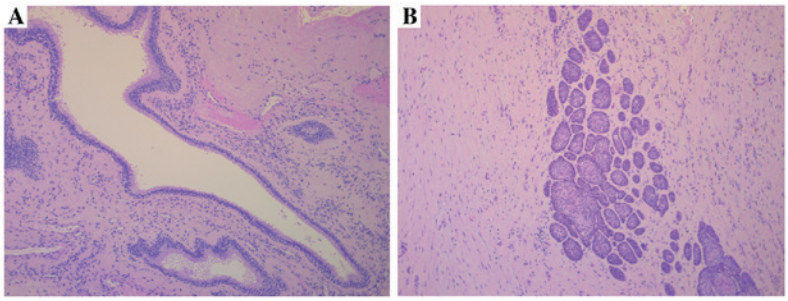

Figure 1. Representative images of (A) daughter cysts and (B) epithelial islands in keratocystic odontogenic tumor tissues. Original magnification, x100.

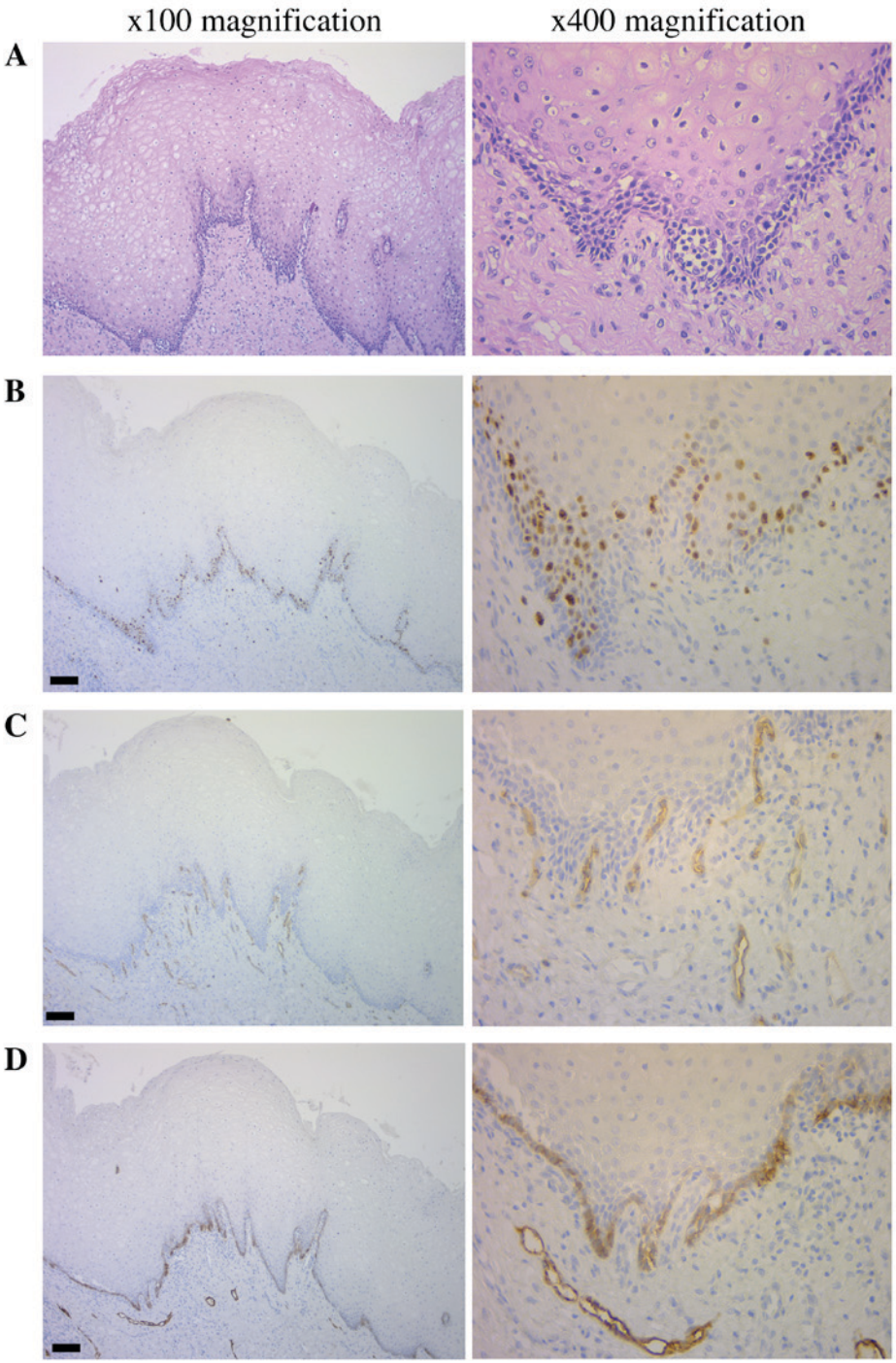

Figure 2. Representative images of Ki-67, CD34 and podoplanin expression in KCOT tissues, as determined by immunohistochemical staining. (A) Recurrent KCOT with hematoxylin-eosin staining. (B) Nuclear Ki-67 expression (labeling index, 28.5%). (C) CD34 expression in blood vessels (microvessel density, 19.5). (D) Cytoplasmic podoplanin expression (score, 1). KCOT, keratocystic odontogenic tumor; CD34, cluster of differentiation 34.

Table VI. Univariate analysis of keratocystic odontogenic tumor recurrence.

Variable	Recurrence, n		Recurrence rate (%)	P-value
	-	+		
Gender				0.127
Male	27	10	27.0	
Female	25	3	10.7	
Age, years				0.764
≤41	25	7	21.9	
>41	27	6	18.2	
Tumor size, mm				0.356
≤35	29	5	17.2	
>35	23	8	25.8	
Tumor site				0.485
Maxilla	15	3	16.7	
Mandible	37	10	21.3	
Radiographic findings				0.447
Unilocular	43	10	18.9	
Multilocular	9	3	25.0	
Daughter cyst + epithelial islands				0.764
Absent	27	6	18.1	
Present	25	7	21.9	
Ki-67, %				0.096
≤7.5	29	4	12.1	
>7.5	23	9	28.1	
CD34, MVD				0.034
≤6.5	29	3	9.4	
>6.5	23	10	30.3	
Podoplanin score				0.542
0-1	14	3	17.6	
2-3	38	10	20.8	
Surgical modality				0.317
Marsupialization + enucleation	7	3	30.0	
Enucleation alone	45	10	18.1	
Surgical modality				0.003
Conservative	25	12	32.4	
Radical	27	1	3.6	
Peripheral osteotomy				0.431
Absent	32	9	21.9	
Present	20	4	16.7	

Ki-67, marker of proliferation Ki67; CD34, cluster of differentiation 34; MVD, microvessel density.

and conservative treatment were significantly associated with tumor recurrence (P=0.034 and P=0.003, respectively). The presence of daughter cysts or epithelial islands and the expression of Ki-67 and podoplanin, were not associated with recurrence; however the rate of each was observed to increase in tumor tissue that were positive for these factors, as compared with tissues that were negative (Table VI). A multivariate analysis revealed that conservative treatment was the only independent predictor of tumor recurrence (odds ratio=13.337; P=0.018; Table VII).

Discussion

Investigation of the pathological and neoplastic characteristics and the proliferative and angiogenic activities of KCOTs may provide a means of predicting tumor recurrence and reveal

Table VII. Multivariate analysis of regional keratocystic odontogenic tumor recurrence.

Parameter	Odds ratio	95% CI	P-value
CD34 (≤6.5 vs. >6.5)	4.366	0.992-19.206	0.051
Surgical modality (conservative vs. radical)	13.337	1.565-113.647	0.018

CD34, cluster of differentiation 34; CI, confidence interval.

novel treatment approaches. Therefore, the present study aimed to identify the most useful markers associated with KCOT recurrence.

The association between the tumor histopathological features and KCOT recurrence was examined, and the presence of daughter cysts or epithelial islands showed a high recurrence rate compared with the absence of them, but the result was not significant. Previous studies have reported that the presence of daughter cysts is significantly associated with a high rate of tumor recurrence (6), as well as a high frequency of allelic loss in tumor suppressor genes, suggesting a neoplastic nature (15). However, another study refuted these results (8). The disparity may be due to the number of cases examined in each of these studies.

The median LI for Ki-67 for basal and suprabasal cell layers was previously demonstrated to be 4.5-13.8% in recurrent tumors, representing a significant association (4,7,8). Ki-67 is a marker that is often used to assess cell proliferation in aggressive tumors such as oral cancer or ameloblastoma (16,17). In the present study, the median LI for Ki-67 was ~12.5% in the recurrent group, as compared with ~5.0% in the non-recurrent group; however, univariate analysis determined that there was no significant difference between the two groups. The discrepancy between the previously reported values and the results of the present study may be due to variations in the evaluations. In the present study, the median positive cell rate was used as cut off value for univariate analysis, but different statistical analysis was used in other studies (4,7) or 10% for Ki-67 positive cells were used as cut off value for high expression (8).

Angiogenesis is essential for the proliferation of tumor cells (4) and is evaluated by MVD, of which has previously been implicated in oral squamous cell carcinoma (10). When nutrient consumption in the tumor parenchyma exceeds the local supply of nutrients, the tumor cells enter a hypoxic state and produce vascular endothelial growth factor to ensure the provision of the necessary nutrients and oxygen by promoting angiogenesis (10,18). Previous studies have reported that the typical diffusion range for nutrients and oxygen is 70-200 μm (4); therefore, the tumor cells that are located >100 μm from blood vessels often become hypoxic (19). From these studies, angiogenesis in the 200 μm region immediately below the epithelium was investigated in the present study, and it was identified that the number of blood vessels positive for CD34 expression was associated with the rate of tumor recurrence. Previous studies have reported that CD34 expression is a histopathological marker of tumor aggressiveness (4,9,20); however, to the best of our knowledge, the present study is the first to demonstrate that angiogenesis directly beneath the epithelium is a significant factor in predicting the recurrence of KCOTs.

The expression of podoplanin in KCOT tissues reflects the neoplastic activity of the tumor, including cell proliferation and local invasiveness (5,12,13,21). Podoplanin-positive cells are also involved in extracellular matrix remodeling, which is associated with cell growth (13,21). In the present study, ~91.8% of all tumors examined were positive for podoplanin expression; however, univariate analysis revealed that there was no significant difference in podoplanin expression levels between the recurrent and non-recurrent tumor tissues. Previous studies have indicated that podoplanin expression levels are also indicative of tumor aggressiveness (5,12,13,21), and that they are decreased following marsupialization or decompression (13). In the present study, no significant difference in the rate of recurrence was identified between those tumors treated with marsupialization and subsequent enucleation and those treated by enucleation alone, which may also suggest that podoplanin expression levels are not useful as a marker for KCOT recurrence.

Although there have been numerous retrospective studies that have investigated the association between the type of surgical procedure administered and the rate of tumor recurrence (6,22-24), to the best of our knowledge, no previous studies specifically examined the correlation between tumor recurrence and the surgical modality used when the tooth root was in contact with the margins of the primary tumor. The results of the current study demonstrated that conservative treatment was significantly associated with tumor recurrence; therefore, teeth that remain in contact with primary tumors may present a risk for recurrence, as previously suggested (23). The majority of primary KCOTs occur in the mandibular molar region; an apicoectomy against mandibular molars is often inaccurate because it is anatomically difficult to access compared with anterior region (23). Although peripheral osteotomy with a bone bur has previously been used to prevent recurrence (23,24), it was identified in the present study that the use of peripheral osteotomy was not associated with the rate of tumor recurrence.

Multivariate analysis identified conservative treatment to be the only independent factor for predicting KCOT recurrence. Therefore, it was hypothesized that the surgical modality may be more useful for predicting KCOT recurrence, compared with histopathological factors or molecular markers. Although NBCCS was omitted and solitary KCOT cases were selected to reduce biases associated with tumor recurrence, residual confounding effects may remain due to the retrospective study design. Additionally, a potential limitation of the current study was the relatively small number of patient cases that were

examined, due to the low incidence of KCOT amongst oral lesions. Therefore, further prospective and intergroup studies are required.

In conclusion, overexpression of CD34 may be a potent marker of tumor recurrence and the radical treatment (extraction) of teeth that are in contact with tumors is a promising approach for preventing the recurrence of KCOTs. However, as KCOT is more prevalent in younger patients, this may not be a widely acceptable treatment due to cosmetic and occlusional complications; therefore, a more elaborate peripheral osteotomy with a bone bur may be required when apicoectomy is selected.

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