Comparative expression profiles of keratins and apoptosis regulating proteins in keratocystic odontogenic tumor, orthokeratinized odontogenic cyst, and dentigerous cyst

Teruyoshi Hayashi¹, Masahiro Wato², Tomio Iseki³, Akio Tanaka², Shosuke Morita³

¹First Department of Oral and Maxillofacial Surgery, Graduate School of Dentistry, Osaka Dental University, Hirakata, Japan,

²Department of Oral Pathology, Osaka Dental University, Hirakata, Japan

³First Department of Oral and Maxillofacial Surgery, Osaka Dental University, Hirakata, Japan

Abstract

Background: In addition to keratocystic odontogenic tumor (KCOT), orthokeratinized odontogenic cyst (OOC) has recently been recognized as a different jaw cyst entity whose lining epithelia are markedly keratinized. However, the odontogenic background of OOC remains still controversial. To clarify the difference in the lining epithelial natures of KCOT and OOC, we investigated differentiation modes for keratinization and neoplastic characteristics of these two cysts by immunohistochemistry for keratin subtypes and apoptosis-related proteins. **Methods:** Surgical specimens of KCOT (n = 20) and OOC (n = 15), as well as dentigerous cyst (DC, n = 15), which was used as a control, were examined by immunohistochemistry and western blotting for their expressions for cytokeratin (CK) 1, CK13, Bcl-2, and Bax. Immunohistochemical reactions were evaluated separately in two epithelial layers: upper spinous and basal. **Results:** Most of the spinous cells in the upper layer of KCOT and DC were CK13 positive (90–100%), while they were not as conspicuous in OOC (20%). In contrast, CK1 was positive in OOC (100%), while it was not positive in KCOT and DC. Basal layer cells in KCOT were positive for Bcl-2 (100%) but not for Bax (0%). Instead, they were not positive for Bcl-2 but positive Bax in OOC and DC. These immunohistochemical profiles in the three cystic lesions were confirmed by western blot analyses showing CK13 in KCOT and DC and CK1 in OOC. **Conclusions:** The CK expression modes were similar between KCOT and DC, indicating their odontogenic characteristics, which were not evident in OOC. The Bcl-2 positivity in KCOT suggested its neoplastic nature, though there was no neoplastic evidence in OOC and DC. [Oral Med Pathol 2012; 16: 67–73 doi: 10.3353/omp.16.67]

Key words: keratocystic odontogenic tumor, orthokeratinized odontogenic cyst, cytokeratin, Bcl-2, Bax

Correspondence: Masahiro Wato, Department of Oral Pathology, Osaka Dental University, 8-1, Kuzuhahanazono-cho, Hirakata 573-1121, Japan
Phone: +81-72-864-3057, Fax: +81-72-864-3157, E-mail: wato@cc.osaka-dent.ac.jp

Introduction

In 2005, the World Health Organization (WHO) recommended that the term keratocystic odontogenic tumor (KCOT) replace the term odontogenic keratocyst (OKC) (1), because KCOT was regarded as neoplastic according to its highly proliferative properties of cyst lining epithelial cells, high recurrence rates, and association with mutations of the PTC-1 gene or a loss of heterozygosity in the related genes (2–5). Besides KCOT, however, jaw cysts lined by orthokeratinized epithelia have been identified and termed as orthokeratinized odontogenic cyst (OOC) by several different research groups (6–8). To the best of our knowledge,

few studies have analyzed the histopathological features of KCOT and OOC to establish an accurate differential diagnosis (9–10).

Cytokeratins (CKs) are epithelial-specific intermediate filament proteins with subtypes 1–20. CK1, a basic form keratin, has a molecular weight of 68 kDa and is expressed in all suprabasal layers of the keratinized squamous epithelium, but not in the basal layer (11–12). CK13 is acidic with a molecular weight of 54 kDa and is expressed in the spinous layer of the non-keratinized squamous epithelium (11, 12). Specific CK subtype expression is associated with the development of odontogenic tumors and cysts and also aids in distinguishing these two cell types at the time of

Table 1. Age and gender of patients with cystic lesions

lesion	case number	age (years)		gender	
		median	range	male	female
keratocystic odontogenic tumor	20	41.2	15-77	10	10
orthokeratinized odontogenic cyst	15	31.5	24-71	8	7
dentigerous cyst	15	45.4	7-62	11	4

Table 2. Location of cystic lesions

lesion	case number	maxilla			mandible		
		anterior	premolar	molar	anterior	premolar	molar
keratocystic odontogenic tumor	20	1	1	1	1	2	14
orthokeratinized odontogenic cyst	15	1	1	2	0	1	10
dentigerous cyst	15	1	1	0	0	0	13

diagnosis (8, 13-18).

Bcl-2 and Bax are proteins that regulate apoptosis (19-21). Bcl-2 reported suppression of apoptosis is primarily expressed in lymphocytes and neurons (22-25). Bcl-2 overexpression has been reported in many types of tumors, including thyroid (26) and skin tumors (27).

In contrast, Bax, a 21 kDa protein that shares 21% homology with Bcl-2, is believed to be a major effector of apoptosis (28). In addition to homodimers, Bax forms heterodimers with Bcl-2. The function of Bax homodimer in activating cell death is antagonized by Bax-Bcl-2 heterodimers. Thus, the ratio of Bcl-2 to Bax regulates the susceptibility of cells to apoptosis-inducing stimuli (29). Bcl-2 and Bax expression is associated with the development of odontogenic tumors and cysts (30-37).

Since there have been no detailed investigations for keratinization and proliferation properties in the epithelial lining of KCOT and OOC, we analyzed their expression profiles for CK1, CK13, Bcl-2, and Bax by immunohistochemical and western blotting methods.

Materials and methods

Materials

Formalin-fixed, paraffin-embedded tissues from specimens of resected KCOT (n = 20) were collected from the

archives of the Osaka Dental University Hospital. The OOC and dentigerous cyst (DC) specimens were used as controls for comparison with the KCOT specimens.

The KCOT specimens were obtained from 10 male and 10 female patients (median age, 41.2 years; range, 15-77 years), the OOC specimens were obtained from eight male and seven female patients (median age, 31.5 years; range, 24-71 years), and the DC specimens were obtained from 11 male and four female patients (median age, 45.4 years; range, 7-62 years) (Table 1). The number of maxillary KCOT, OOC and DC specimens was three, four and two, respectively; the number of mandibular KCOT, OOC and DC specimens was 17, 11 and 13, respectively (Table 2). None of the patients with KCOT showed symptoms of nevroid basal cell carcinoma syndrome. This research was approved by the Ethics Committee of Osaka Dental University (Approval Number 090332).

Immunohistochemical staining

Tissue specimens from biopsied or excised lesions were fixed in 10% formalin solution and embedded in paraffin. Sections with a thickness of 4 µm were cut and mounted on silane-coated glass slides.

Antigen retrieval was performed by autoclaving at 121 °C for 15 min in retrieval buffer (pH 6.0) (Mitsubishi Kagaku Iatron, Tokyo, Japan). After autoclaving, the

Table 3. Comparative immunohistochemical profiles of cystic lesions

antigens	layers of lining epithelia	numbers of immunopositive cases (%)		
		keratocystic odontogenic tumor (n=20)	orthokeratinized odontogenic cyst (n=15)	dentigerous cyst (n=15)
cytokeratin 1	upper	1 (5)	15 (100)*	0 (0)
	basal	0 (0)	0 (0)	0 (0)
cytokeratin 13	upper	18 (90)	3 (20)*	15 (100)
	basal	0 (0)	0 (0)	0 (0)
Bcl-2	upper	0 (0)	0 (0)	0 (0)
	basal	20 (100)	0 (0)*	0 (0)*
Bax	upper	20 (100)	14 (93)	15 (100)
	basal	0 (0)	14 (93)*	15 (100)*

*P<0.05 compared with keratocystic odontogenic tumor

slides were allowed to cool to room temperature and were then briefly washed with phosphate-buffered saline. Endogenous peroxidase activity was blocked with 3% hydrogen peroxidase. The sections were incubated with mouse anti-human CK1 monoclonal antibody (clone 34 β B4, diluted at 1:20, Novocastra, Newcastle, UK), mouse anti-human CK 13 monoclonal antibody (DE-K13, 1:25, Dako, Glostrup, Denmark), mouse anti-human Bcl-2 oncoprotein monoclonal antibody (124, 1:100, Dako) and rabbit anti-human Bax oncoprotein polyclonal antibody (1:25, Dako). These antibodies were then incubated for 60 min at room temperature. Subsequently, the prepared sections were incubated with Envision+ peroxidase dextran polymer (Dako) for 30 min at room temperature. The sections were then visualized with 3, 3'-diaminobenzidine tetrahydrochloride and hydrogen peroxide, and then counterstained with hematoxylin.

Evaluation of immunohistochemical staining

The epithelial lining of tissue specimens was divided into an upper spinous layer and basal layer to evaluate the results of immunohistochemical staining. The spinous layer and basal layer cells were considered positive (+) or negative (–) when a particular protein was or was not expressed in these cells.

Results were expressed as ratio of positive cases. Statistical difference was determined by a two-sided Student's *t*-test. Differences with $P < 0.05$ were considered significant.

Gel electrophoresis and immunoblotting analysis

Proteins were extracted from the formalin-fixed, paraffin-embedded tissues for the western blot analysis using the Qproteome FFPE Tissue Kit® (Qiagen Inc., Hilden, Germany). Tissue sections cut at 100 μ m thickness from the KCOT, OOC and DC specimens were immersed in the Qproteome extraction buffer. The samples were incubated for 20 min at 100 °C followed by 2 hrs at 80 °C at an agitation speed of 750 revolutions per minute. After incubation, the samples were centrifuged at 12,000 $\times g$ for 15 min, and the supernatants were transferred to a new safe-lock tube and stored at -20 °C. Protein lysates were boiled with a sample buffer for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (2% SDS, 25% glycerol, 0.5% 2-mercaptoethanol, 0.01% bromophenol blue, and 62.5 mM Tris-hydrochloric acid, pH 6.8) (Bio-Rad Laboratories, Inc, Hercules, CA, USA) for 5 min. Whole protein lysates (containing 10 μ g protein) were electrophoresed in 12.5% SDS gel for 30 min at a constant voltage of 200V, and then transferred onto polyvinylidene difluoride membranes (Immun-Blot® PVDF membrane, Bio-Rad) using a wet system at 200V for 45 min. The membranes were blocked with 5% skimmed milk (Wako Pure Chemical Industries, Osaka, Japan) in a mixture of Tris-buffered saline and 0.01% Tween 20 (TBST) (Dako) for 1 hr at room temperature to avoid non-specific antibody binding. The membranes were then incubated for 16 hrs at 4 °C with primary antibodies diluted in Can Get Signal® (Toyobo Co.

Fig. 1. Comparative immunohistochemical profiles between CK1, CK13, Bcl-2, and Bax in keratocystic odontogenic tumor (KCOT). (a) Hematoxylin and eosin (HE) stain and immunoperoxidase stain for cytokeratin (CK) 1 (b), CK13 (c), Bcl-2 (d), and Bax (e), hematoxylin counterstain, $\times 200$. KCOT cystic spaces were lined by flat and uniform epithelia composed of stratified squamous cells with six- to eight- cell layers. The basal cells were cuboidal or columnar-shaped and arranged in a palisading fashion. The interface of the lining epithelium and the underlining connective tissue was usually flat without rete ridge formation. The luminal surface cells were basically flat and showed parakeratosis but occasionally exhibited a wavy or corrugated appearance. CK1 was not positive in spinous cells of the upper layer (b), while those cells were positive for CK13 (c). Bcl-2 was positive in columnar cells of the basal layer but not positive in the upper layer (d). Bax was not positive in the basal layer but was positive in the upper layer (e).

Ltd., Osaka, Japan) and washed three times with TBST. These membranes were then incubated in alkaline phosphatase (ALP)-conjugated goat anti-mouse IgG polyclonal antibody or ALP-conjugated goat anti-rabbit IgG polyclonal antibody (Nichirei Corporation, Tokyo, Japan) for 30 min at 25 °C. Then they were again washed three times with TBST,

Fig. 2. Comparative immunohistochemical profiles between CK1, CK13, Bcl-2 and Bax in orthokeratinized odontogenic cyst (OOC). (a) HE stain and immunoperoxidase stain for CK1 (b), CK13 (c), Bcl-2 (d) and Bax (e), hematoxylin counterstain, $\times 200$. OOC cystic spaces were lined by a regular orthokeratinized stratified squamous epithelium. The basal cells were not cuboidal or columnar-shaped but rather flat and not arranged in a palisading fashion. The basal line of the epithelia was usually flat. The luminal surface of the lining epithelium was flattened with orthokeratosis, and spinous cells in the upper layer were positive for CK1 (b) but not for CK13 (c). Bcl-2 was neither positive in the basal layer nor in the upper layer (d), while Bax was positive in the whole layer (e).

and bound antibodies were visualized with 5-bromo-4-chloro-3-indoxyl phosphate and nitro blue tetrazolium chloride (Dako).

Results

Immunohistochemical evaluations

Immunohistochemical positive reactions in the cystic linings were evaluated separately for the basal layers with cuboidal, columnar, or flat epithelial cells and for the upper

Fig. 3. Comparative immunohistochemical profiles between CK1, CK13, Bcl-2 and Bax in dentigerous cyst (DC). (a) HE stain and immunoperoxidase stain for CK1 (b), CK13 (c), Bcl-2 (d) and Bax (e), hematoxylin counterstain, $\times 200$. The lining epithelium of dentigerous cysts typically consisted of flattened stratified squamous epithelium with a discernible alignment of basal cells (a). CK13 (c) was positive in the spinous layer but not positive in the basal layer. CK1 (b) and Bcl-2 (d) were not positive in the whole layer, while Bax was positive in the whole layer (e).

layers with spinous epithelial cells. Numbers of cases with positive stainings for the four antigens were summarized in all three kinds of cystic jaw lesions in Table 3.

In KCOT specimens ($n = 20$), CK1 was positive in spinous cells in the upper layer of only one case (5%). The basal cells were not positive for CK1 in all of the cases (Fig. 1b). CK13 was positive in the upper layer of 18 cases (90%) (Fig. 1c). Bcl-2 was not positive in the upper layer but positive in the basal layer in all of the cases (Fig. 1d). Bax was positive in the upper layer (100%) but not at all in the basal layer (Fig. 1e), while basal layer cells were Bcl-2 positive (Fig. 1d).

In the OOC specimens ($n = 15$), spinous cells in the

upper layer were CK1 positive, while the basal layer was not positive for CK1 in all cases (Fig. 2b). CK13 was positive in the upper layer of 3 cases (20%) but not positive at all in the basal layer (0%) (Fig. 2c). Bcl-2 was not positive in the whole layer in all of the fifteen cases examined (Fig. 2d). Bax was positive both in the upper and basal layer in 14 cases (93%) (Fig. 2e).

In the DC specimens ($n = 15$), spinous layer and basal layer cells were not positive for CK1 in all cases (Fig. 3b). However, spinous cells in the upper layer were positive for CK13 (100%), while basal layer cells were not positive for CK13 (0%) (Fig. 3c). There was no positive staining for Bcl-2 in any layers (0%) (Fig. 3d). In contrast, Bax was positive in the whole layer of all cases (100%) (Fig. 3e).

Immunoblotting analysis

In KCOT samples, expressions of CK13, Bcl-2, and Bax were identified as bands of 54 kDa, 26 kDa, and 21 kDa, respectively. However, there was no band for CK1 around 68 kDa (Fig. 4). In OOC samples, bands corresponding to CK1 and Bax were obtained at 68 kDa and 21 kDa, respectively. However, there were no bands for CK13 at 54 kDa and Bcl-2 at 26 kDa (Fig. 5). In DC samples, the expressions of CK13 and Bax were confirmed by the presence of bands of 54 kDa and 21 kDa, respectively. However, those of CK1 (68 kDa) and Bcl-2 (26 kDa) were not obtained (Fig. 6).

Discussion

This study aimed to investigate comparatively the characteristics of the epithelial lining of KCOT and OOC by immunohistochemical and western blot analyses for CK1, CK13, Bcl-2 and Bax. Since the CK1 and CK13 expression profiles of KCOT resembled those of DC, it is considered that KCOT and DC share their odontogenic characteristics. KCOT was also characterized by the consistent Bcl-2 expression in the basal layer, which was neither obtained in OOC nor in DC, and thus OOC and DC are not neoplastic. The present study revealed the different disease properties between neoplastic and non-neoplastic, and between odontogenic and non-odontogenic lesions.

CK1 was expressed in all suprabasal layer cells of the keratinized squamous epithelium but not in the basal layer (11). Aragaki *et al.* reported that all suprabasal layer cells were CK1 positive in all cases (100%) of the OOC specimens and no CK1 positive in 75% cases of the KCOT specimens (8), which was consistent with the present results.

CK13 was not expressed in the basal cells but was expressed in the upper non-keratinized squamous epithelium (7). In addition, the expression of CK13 has been confirmed in the lining epithelia of odontogenic cysts (9–12). According to Stoll *et al.*, the expression rates for CK13 in the lining epithelia were 100% in radicular cysts, 87% in DC, and 80% KCOT (11). Hayakawa *et al.* reported that almost the entire spinous layer in KCOT was CK13 positive (13). The CK13 positivity may indicate their odontogenic origins as demonstrated also in ameloblastoma by Wato *et al.* (18). On

Fig. 4. Western blot analysis of CK1, CK13, Bcl-2, and Bax in KCOT. Lane a, CK1; lane b, CK13; lane c, Bcl-2; lane d, Bax. Immunoalkaline phosphatase stain. There was no positive reaction for CK1 around its expected molecular weight of 68 kDa (lane a): A band for CK13 was obtained at 54 kDa (lane b). An immunopositive band for Bcl-2 was clearly visible at 26 kDa (lane c). There was a band with molecular mass of 21 kDa indicating Bax (lane d).

Fig. 5. Western blot analysis of CK1, CK13, Bcl-2 and Bax in OOC. Lane a, CK1; lane b, CK13; lane c, Bcl-2; lane d, Bax. Immunoalkaline phosphatase stain. There was a positive band for CK1 at 68 kDa (lane a), while no band for CK13 was found around 54 kDa (lane b). Although there was no immunopositive band for Bcl-2 around 26 kDa (lane c), a positive signal for Bax was found at 21 kDa (lane d).

Fig. 6. Western blot analysis of CK1, CK13, Bcl-2 and Bax in DC. Lane a, CK1; lane b, CK13; lane c, Bcl-2; lane d, Bax. Immunoalkaline phosphatase stain. No immunopositive signals were obtained for CK1 (lane a), while a band for CK13 was definitely demonstrated at 54 kDa (lane b). There was no visible band for Bcl-2 (lane c). In contrast, a band for Bax at 21 kDa was clearly observed (lane d).

the other hand, Aragaki *et al.* reported that the upper layer cells in KCOT were CK13 positive, whereas those of OOC were not CK13 positive (8). Similarly, Koizumi *et al.* reported that OKC exhibited intense positive stainings for CK13 in the upper layer cells including parakeratinized cells, while OOC exhibited patchy stainings for CK13 limited to the superficial keratinized cells and granular cells (14). Meara *et al.* reported that the upper layer of linings of OKC was 100% positive for CK13 (16). Silva *et al.* reported that the occasional CK13 positivity in the intermediate epithelial layers of OOC distinguished OOC from OKCs in which intense stainings for CK13 were observed in the upper layer (17). In the present study, CK13 positive profiles in DC were noted because they were similar to those in KCOT but not to OOC. Our present results suggest that KCOT and DC resemble each other in the cellular differentiation but that the keratinization mode of OOC is different from that of KCOT and DC.

Bcl-2, which has a molecular weight of 26 kDa, is expressed in the mitochondrial and nuclear membranes and contributes to the inhibition of apoptosis (24). Piattelli *et al.* reported that this protein could possibly contribute to the development of odontogenic cyst because of its inhibitory effect on apoptosis (30). Bcl-2 expression has also been reported in the epithelial lining of OKC and calcifying odontogenic cyst. In previous studies, all spinous layer and basal layer cells in OOC specimens were not positive for Bcl-2 (8, 31-32), whereas basal layer cells in all KCOT specimens were not positive for Bcl-2 (8, 32-34). In addition, Kumamoto *et al.* (35) and Mitsuyasu *et al.* (36) reported that ameloblastoma cells exhibited intense positive staining for Bcl-2. In this study, the basal cells in KCOT were Bcl-2 positive but upper layer cells were not positive for Bcl-2. In contrast, basal layer and spinous layer cells in the OOC and DC specimens were Bcl-2 negative in all cases. It is believed that apoptosis is inhibited in bl of KCOT, but not in OOC (30-33). Taken together, our results and previous reports suggest that KCOT possesses neoplastic properties, whereas OOC does not.

Bax, a protein of the Bcl-2 family, has a molecular weight of 21 kDa and is an effector of apoptosis (38). Rangiani *et al.* reported that all spinous layer cells of KCOT specimens and all spinous layer and basal layer cells of the OOC specimens were Bax positive (31). Suzuki *et al.* reported that the cytoplasm of basal and parabasal cells in radicular cyst was Bax positive (37). Finally, Kolara *et al.* reported significant differences between OKC and DC in Bax expression (34). In this study, Bax-positive spinous layer cells were observed in 14 cases of the KCOT specimens; this finding was consistent with that of Rangiani *et al.* (31). It is thought that apoptosis is not inhibited in the basal cells of KCOT (31). The observed difference between Bax expression in basal cells of KCOT and OOC is also consistent with the finding of Rangiani *et al.* (31). In addition, KCOT exhibits different expression patterns of both epithelium- and apoptosis-related proteins compared with OOC. In addition, OOC could be distinguished from DC by their characteristic expression patterns of CK1 and

CK13, but the expression of apoptosis-related proteins in the OOC specimens was similar to that in the DC specimens.

In conclusion, the expression patterns of CK1, CK13, Bcl-2, and Bax were valuable for confirming that KCOT is an odontogenic tumor and DC is an odontogenic cyst, but that OOC is not neoplastic. However, whether OOC is odontogenic or not needs further examination. These immunohistochemical stainings are helpful in the differential diagnosis of KCOT and OOC.

Acknowledgments

We would like to thank the members of the First Department of Oral and Maxillofacial Surgery, Osaka Dental University, for their advice and assistance. We would also like to thank members of the Department of Oral Pathology, Osaka Dental University, for their advice concerning histopathology.

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