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Orthokeratinized odontogenic cysts: A clinicopathologic study of 159 cases and molecular evidence for the absence of *PTCH1* mutations

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Abstract

Background: Orthokeratinized odontogenic cyst (OOC), a newly designated entity of odontogenic cysts, is an intraosseous jaw cyst that is entirely or predominantly lined by orthokeratinized squamous epithelium. The aim of this study was to report a large series of OOC to substantiate its clinicopathologic profiles and to investigate *PTCH1* mutations in OOCs.

Method: The clinicopathologic features of 167 OOCs from 159 patients were analyzed and the immunohistochemical expression of markers related to cell differentiation and proliferation was evaluated. Furthermore, *PTCH1* mutations were analyzed in 14 fresh samples of OOC.

Results: OOCs occurred mostly in the third and fourth decades (60.4%) with a male predilection (66.7%). The lesions developed more often in the mandible than maxilla, primarily in the posterior mandible and ramus. Eight patients (5.0%) showed multiple locations of either bilateral posterior mandible ($n = 6$) or both the maxilla and mandible. Radiographically, the majority of OOCs (91.2%) showed a well-demarcated, unilocular radiolucency with 14 multilocular cases (8.8%). A follow-up of 131 patients (123 treated by enucleation with or without marsupialization and eight by peripheral ostectomy) revealed no recurrence during an average period of 4.56 years after surgery. Immunohistochemistry indicated lower proliferative activity and a varying epithelial differentiation pattern in OOC compared with odontogenic keratocysts (OKC). No *PTCH1* mutation was detected, except for three known single nucleotide polymorphisms.

Conclusion: The clinicopathological and molecular differences between OOC and OKC justified their separation, and unlike OKCs, OOCs did not harbor *PTCH1* mutations, suggesting different pathogenesis underlying these two jaw cysts.

KEYWORDS
clinicopathology, odontogenic keratocyst, orthokeratinized odontogenic cyst, *PTCH1*

1 | INTRODUCTION

Orthokeratinized odontogenic cyst (OOC), first recognized as a variant of odontogenic keratocyst (OKC) in 1981,[1] presents as a jaw cyst

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showing the entire or prevalent orthokeratinization of its lining epithelium. Several studies have discussed the clinical and pathologic differences between OKC and OOC.[2–4] The new edition of the WHO classification of head and neck tumors published in 2017 has described OOC as a separate entity.[5] OOC represents approximately 10% of the cases previously coded as OKC and rarely recurs following conservative surgery.[6] Due to its relatively low prevalence, however, the clinicopathological features of OOC, especially data related to its recurrence in larger series with long term follow-up, require further clarification and confirmation.

The protein patched homolog 1 (*PTCH1*), the receptor of sonic hedgehog (SHH) pathway, is the human homolog of the *Drosophila* segment polarity gene which is believed to be the causative gene for nevoid basal cell carcinoma syndrome. The most consistent feature of this syndrome is multiple occurrences of OKCs involving the jaws, which accounts for 65%–100% of the syndrome patients.[7] SHH pathway is implicated in the formation of various embryonic structures and tumorigenesis.[8] *PTCH1* mutations may cause constitutive activation of SHH signaling, leading to aberrant and/or neoplastic growth. The ratio of *PTCH1* mutation in syndromic OKC was over 80%.[9] However, this ratio in sporadic OKC was underestimated over the years.[10] The traditional protocol using the full thickness of the cyst wall (both lining epithelium and fibrous capsule) only detected 30% of sporadic OKCs harboring *PTCH1* mutations.[11,12] Sequencing lining epithelial samples separated from the fibrous cystic wall, 16 out of 19 sporadic OKCs (~84%) were detected to harbor *PTCH1* mutations.[10] This result was later confirmed in additional 19 samples of sporadic OKC, also by our group,[10] indicating that *PTCH1* mutations occurred as frequently as over 80% in both syndromic and sporadic OKCs. So far there has been no report on mutational analysis of *PTCH1* in OOCs except for one study involving LOH analysis.[13] Here, we report a large series of 167 OOCs in 159 patients with a comprehensive clinicopathological analysis and present new molecular evidence for the absence of *PTCH1* mutations in OOCs.

2 | MATERIALS AND METHODS

2.1 | Subjects and samples

In total, 1250 cases coded as keratinized (both orthokeratinized and parakeratinized) odontogenic cysts were reviewed from the files of the Department of Oral Pathology, Peking University Hospital and School of Stomatology, during the period of 2000–2020. One hundred fifty-nine cases were diagnosed as OOC by two pathologists reviewing the clinical and pathological records according to the criteria of the 4th edition of the WHO classification of head and neck tumors,[5] while 1091 cases were diagnosed as OKC. The epithelial lining of all these cases was entirely or predominantly orthokeratinized stratified squamous epithelium. Cases with focal areas of typical OKC lining were excluded. Clinical data including age, sex, location, radiographic features, treatment, and available follow-up data were recorded. The experimental protocols used in this study were reviewed and approved by the Ethics Committee of the Peking University Health Science Center. The data of OKCs for comparison were analyzed by same researcher using same method and published already.[14]

2.2 | Immunohistochemistry

Immunohistochemical expression of CKpan, CK5/6, CK10/13, Ki-67, and Bcl-2 was studied in 20 cases of OOC together with 20 cases of OKC using the avidin-biotin-peroxidase complex method. Tissue specimens were routinely fixed in 10% neutral formalin, processed, and embedded in paraffin. The antibodies anti-CKpan (ZM-0069, clone AE1/AE3), anti-CK5/6 (ZM-0313, clone OTI1C7), anti-CK10/13 (ZM-0314, clone DE-K13), anti-Ki-67 (ZM-0166, clone UMAB107), and anti-Bcl-2 (ZM-0010, clone D5) were purchased from ZSGB-BIO.

2.3 | Tissue sample preparation prior to sequencing

Fresh tissue samples from 14 OOCs were collected and immediately stored at −80°C until analysis. Ten freshly frozen samples of OKC (previously identified to harbor *PTCH1* mutations[10]) were also used as positive controls. The cyst walls of OOCs of approximately 0.8 cm × 0.8 cm × 0.3 cm in size were washed with phosphate-buffered saline solution and then incubated overnight at 4°C with Dispase II (1 U/ml; Roche), while the OKCs samples were incubated 1–2 h at room temperature. Under a dissecting microscope, the cyst linings were carefully separated from the fibrous capsules.

2.4 | Polymerase chain reaction and Sanger sequencing

Genomic DNA was isolated from the epithelial lining of 24 samples using a QIAamp DNA Mini Kit (Qiagen). Each of the 23 exons comprising the *PTCH1* gene (NM_000264.3) was amplified by polymerase chain reaction (PCR) with specific primers described in our previous study.[15] PCR assays were performed in a 25 μl reaction mixture containing 20 ng of template DNA, 200 μM dNTPs, 10 pM of each primer, and 1 U of Ex Taq DNA polymerase (Takara) in buffer (10 mM tris-HCl, 50 mM KCl, and 1.5 mM $MgCl_2$). Thermocycling conditions were optimized for each primer pair, and the following conditions were used: initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 30 s, 60–65°C for 30 s, 72°C for 30 s; and final extension at 72°C for 10 min. The amplified products were sequenced with an automatic sequencer (ABI 3730, Applied Biosystems) and analyzed by Chromas software. The Basic Local Alignment Search Tool website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to align the sequence. All experiments were performed in duplicate and repeated at least twice.

2.5 | Statistical analysis

All quantitative data were analyzed using the Statistical Package for Social Sciences software version 20.0 software (IBM Corp.). The student's *t*-test and chi-square test were used to compare the mean values of two groups and categorical data, respectively. Data are presented as the mean ± SEM. $p < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | Clinical findings

Among the 159 OOC cases evaluated, the male ($n = 106$) to female ($n = 53$) ratio was 2:1. The patients age ranged from 13 to 78 years (average, 39.1 ± 1.1 years), with a peak in the third and fourth decades (60.4%). Teenage patients were rare (Figure 1A). The sex and age distribution of OOCs was somewhat different from that of OKCs. Compared to OKCs,[14] OOCs appeared to occur more often in male patients with a slightly older age of onset ($p < 0.05$). The posterior mandible, in particular the molar and ramus region, was the most affected site for OOCs ($n = 128$; 76.6%). Of 17 maxilla lesions, seven (41.2%) involved the sinus and two (11.8%) affected the nasal cavity. Eight patients (5.0%) had multiple lesions either affecting the bilateral posterior mandible ($n = 6$) or both the maxilla and mandible ($n = 2$) (Figure 1B). OOC patients with multiple lesions were proportionally more abundant than those with sporadic OKC[14] ($p < 0.05$).

Radiologically, typical OOCs showed well-demarcated, unilocular radiolucent lesions with or without impacted teeth (Figure 1C). However, the size of OOCs varied from approximately 1.0 to 9.8 cm in diameter, which might be related to the duration of the lesions. Small lesions could mimic lateral periodontal cysts or radicular cysts, whereas large lesions were difficult to distinguish from those of OKCs (Figure 1D–F). Fourteen (8.8%) OOCs were multilocular, a significantly lower incidence compared with that of OKC[14] ($p < 0.05$). In total, 86 OOC lesions (51.5%) were associated with impacted teeth.

Pain with swelling was the most common presenting symptom ($n = 69$, 43.4%). Nine patients (5.7%) displayed limited mouth opening and four (2.5%) had numbness of the lower lip. None of the patients were associated with nevoid basal cell carcinoma syndrome.

Simple enucleation was the primary treatment for OOCs ($n = 141$, 88.7%). Moreover, nine patients were treated with marsupialization followed by enucleation, and nine were treated with peripheral ostectomy. Post-operative complications were rare. Only one patient experienced pathological fracture at the inferior border of

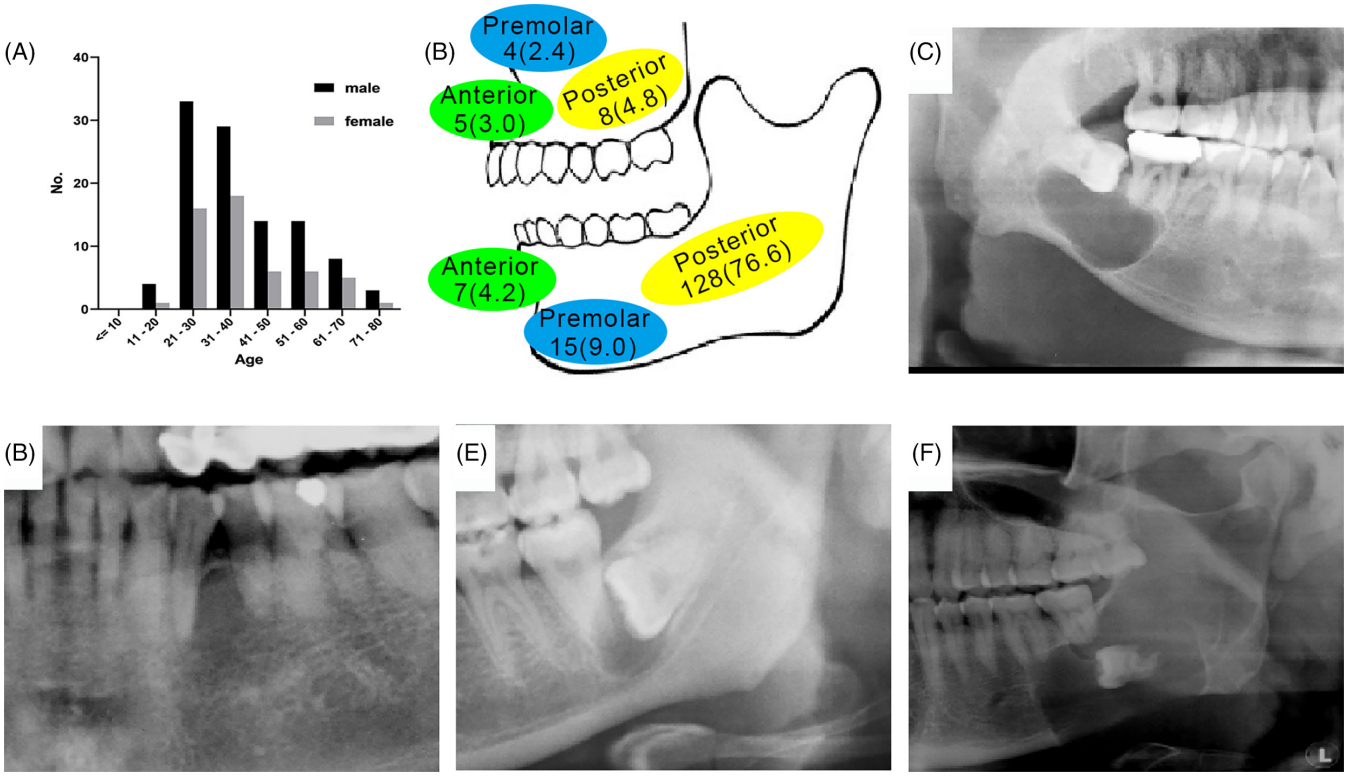

FIGURE 1 The clinical and radiographic features of orthokeratinized odontogenic cyst (OOC). The peak incidence age of OOC was third and fourth decades and hardly occurred in teenagers. And male attacked OOC more than female (A). Cases that crossed the midline were not included in the analysis in the location distribution analysis (B) and percentage is shown in parentheses. OOC showed various radiographic appearance: typical one with well-demarcated, unilocular radiolucent lesion (C), untypical lesions may in the lateral periodontal regions (D) or around the crown (E). It can be multilocular in panorama (F)

TABLE 1 Results of follow-up

Follow-up time	*n*		
	Cases	No recurrence	Recurrence
<1 year	30	30	0
1–3 years	38	38	0
4–6 years	22	22	0
7–9 years	19	19	0
≥10 years	22	22	0
Total	131	131	0

the mandible, and one had an oral and nasal fistula. Related follow-up data were available for 131 patients, among whom, 123 were treated by enucleation with or without marsupialization and eight were treated by peripheral ostectomy. None of the patients recurred during an average follow-up period of 4.56 ± 0.38 years (range, 0.5–19 years; Table 1).

3.2 | Pathological findings

Although OOCs had similar clinical and radiographical features as those of other odontogenic cysts, morphological features of OOCs were distinctive and diagnostic. The lining of the OOC consisted of stratified squamous epithelium that was mostly thin and uniform. The surface was entirely or at least prevalently orthokeratinized, often described as "onion-skin-like" keratin layers (Figure 2A). A prominent, well-developed granular cell layer was observed immediately beneath the surface keratin layer. The basal layer cells were flattened or cuboidal and palisading basal cells and reverse nuclear polarity were not observed (Figure 2B). Focal areas became thickened and atypical owing to persistent chronic inflammation. The detachment of the lining epithelium from the fibrous capsule, one of the histopathological traits of OKC, was rarely seen in OOCs. No epithelial islands or daughter cysts were observed in the dense fibrous capsules.

Immunohistochemical expression of pan-keratin (AE1/AE3), CK5/6, CK10/13, Ki67, and Bcl-2 were examined in both OOCs and OKCs. Although pan-keratin and CK5/6 expressed in all layers in both cyst types, CK10/13 expression observed in all layers of OOCs was absent in the basal cell layers of OKCs (Figure 2C,D). Bcl-2 expression was negative or weakly positive in OOCs but was apparent in the basal cell layer of OKCs (Figure 2E,F). The proliferative activity, represented by Ki67 expression, was higher in OKC than in OOC, respectively.

3.3 | *PTCH1* mutation detection

PTCH1 mutations were evaluated in 14 frozen tissue samples of OOCs and 10 OKCs (Table 2). The examined epithelial lining samples were separated from the fibrous walls (Figure 3A,B). While the 10 OKC samples, previously tested for *PTCH1* mutations in one of our

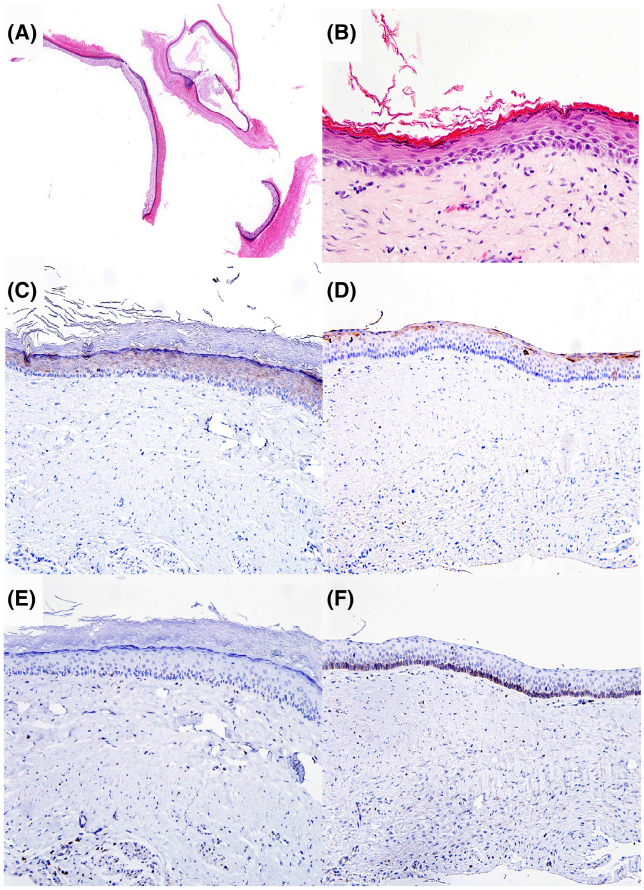

FIGURE 2 The histopathological findings in orthokeratinized odontogenic cysts (OOC). The surface of epithelium in OOC was entire or prevalent thick orthokeratinized without parakeratinized area (A, magnification: 12.5×) with the flattened basal cells layer and clear prominent granular cell layer (B, magnification: 400×). CK10/13 expressed in major of OOC epithelium with basal layer patchy positive (C, magnification: 200×) but the upper part of epithelium positive in Odontogenic keratocyst (OKC), that is surface parakeratinized cells and some granular cells (D, magnification: 200×). The Bcl-2 expression was negative in OOC epithelium (E, magnification: 200×). OKC showed consistent positive for Bcl-2 in basal cell layer (F, magnification: 200×).

previous studies,[10] showed identical results in the present study, none of the 14 OOC samples harbored *PTCH1* mutations. However, three variants found in our cohort fit the single nucleotide polymorphism (SNP) category (Figure 3C–E). The minor allele frequencies of three SNPs were greater than 0.01. A missense variant of exon 23, rs357564 (NM_000264.4:c.3944C>T, NP_000255.2:p.Pro1315Leu), was widely detected in 13 of the 14 samples. Two other SNPs (rs1805155 and rs2227970) were synonymous variants located in exons 12 and 14, respectively.

4 | DISCUSSION

OKC has a typical histological feature of parakeratinized epithelial linings and tends to recur following conservative surgery.[5] In contrast,

TABLE 2 Clinical information and *PTCH1* sequencing analysis

				PTCH1 sequencing analysis		
				Single nucleotide polymorphism		
No.	Age	Sex	Location	rs1805155	rs2227970	rs357564
OOC						
1	29	Female	Bilateral	–	–	Heterozygous
2	26	Male	Left	Heterozygous	–	–
3	44	Female	Left	Heterozygous	–	Heterozygous
4	25	Female	Right	–	–	Heterozygous
5	38	Male	Left	Heterozygous	–	Heterozygous
6	34	Male	Left	–	–	Homozygous
7	33	Female	Left	–	–	Homozygous
8	20	Male	Left	–	–	Heterozygous
9	35	Female	Right	–	–	Homozygous
10	48	Male	Right	Heterozygous	–	Heterozygous
11	76	Male	Left	–	–	Homozygous
12	55	Male	Right	–	–	Homozygous
13	31	Female	Right	–	–	Homozygous
14	31	Female	Right	Heterozygous	Heterozygous	Homozygous

				PTCH1 sequencing analysis		
				Mutations		
No.	Age	Sex	Location	Nucleotide definition	Functional effect	Type
OKC						
1	49	Male	Right	c.1581_1582del	Nonsense	Truncation
2	23	Male	Right	c.3103_3126dup	In-frame duplication	Non-truncation
3	32	Female	Right	c.1381_1414del	Frameshift	Truncation
4	10	Male	Bilateral	c.1086dup	Frameshift	Truncation
5	55	Female	Right	c.2362del	Frameshift	Truncation
6	83	Male	Right	c.259_262del	Frameshift	Truncation
7	31	Male	Right	c.1540_1555del	Frameshift	Truncation
8	46	Male	Right	c.808_812del c.2924del	Frameshift Frameshift	Truncation Truncation
9	62	Female	Left	c.848dupA	Frameshift	Truncation
10	38	Male	Left	c.2776 T>A	Missense	Non-truncation

jaw cysts with entirely or predominantly orthokeratinized epithelial linings show less aggressive behavior with little tendency to recur.[1–4] The new edition of the WHO classification of head and neck tumors designated the latter group as OOC, a new entity of odontogenic cysts.

After reviewing the reported OOCs in English literature, we found that more than 300 cases have been reported in different populations since 1981 (Table S1).[1–4,6,16–21] Thirty-eight cases in present study overlapped with our previous reports.[6,22] After removing duplicated data, a total of 280 reported cases were analyzed as follows. Age of onset ranged from 5 to 73 years (average, 25.0–38.9 years), and the male-to-female ratio was 2.1 to 1. More than half of these cases were associated with impacted teeth and only 24 cases showed a multilocular radiolucency. Only five cases (1.7%) were reported to recur following conservative surgery. These data are in general agreement with the present series.

Compared with OKCs,[14] OOC appeared to be more common in older and male patients. Multiple lesions in OOC patients ($n = 8$, 5.0%) were more common than those in patients with sporadic OKC ($n = 14$, 1.7%).[14] Meanwhile, multilocular radiolucencies could be seen in OOCs, but they were less common. In the present study, a total of 131 patients were followed up after surgical treatment (123 were treated by enucleation with or without marsupialization) during an average period of over 4.5 years. No recurrence was recorded in our series. The overall outcome of patients suggested that enucleation and/or marsupialization should be the recommended treatment for OOCs.

The morphology of OOC was unique and easy to diagnosis. Atypical cases mainly need to be differentiated from OKC. The difference of

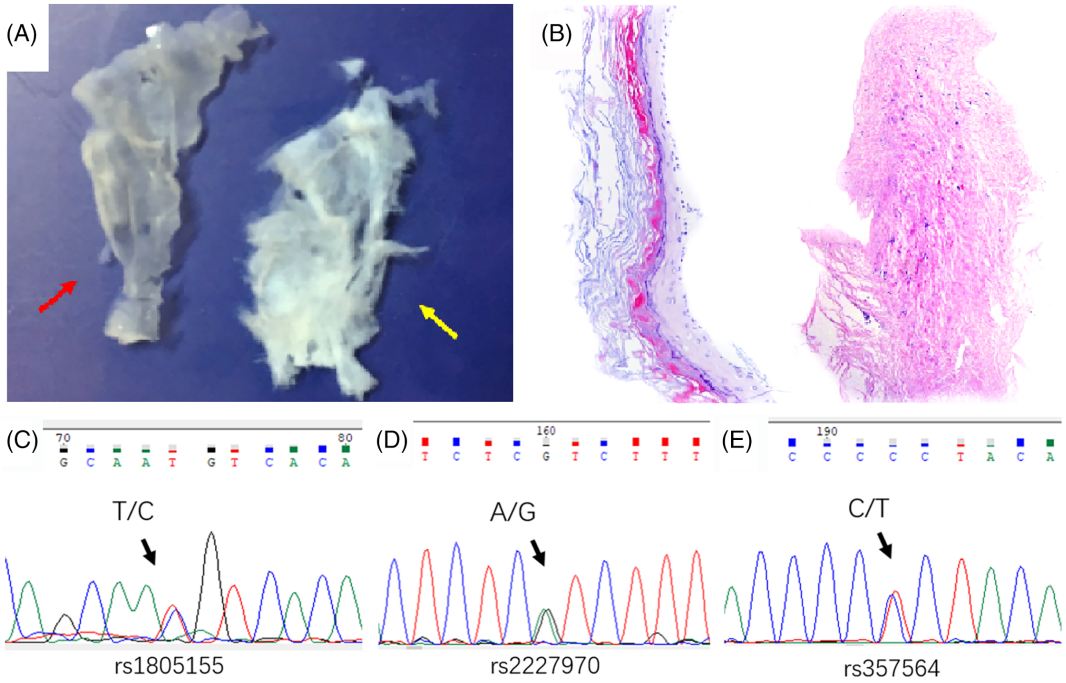

FIGURE 3 Division of epithelial lining and fibrous capsule and sequencing results. The macroscopy of separated epithelium (A, red arrow) and fibrous capsule (A, yellow arrow) and frozen slides showed separated epithelium was complete and pure (B). Three high frequency single nucleotide polymorphisms, rs1805155 (C), rs2227970 (D), and rs357564 (E), were detected.

attachment of the lining epithelium and the fibrous capsule in two diseases may result to the different treating time while separating the epithelium and stroma. Previous studies showed that immunohistochemical expression of various markers related to epithelial cell proliferation and differentiation varies between OOCs and OKCs.[2,6,23,24] The present study was in general agreement with previous immunohistochemical findings, and a lower proliferative activity in OOC linings was again confirmed. Interestingly, uniformly higher Bcl-2 expression in basal cells of the OKC lining epithelium was noted but not in OOCs. Bcl-2, regulated by the SHH downstream protein Gli1,[25] encodes a protein that prevents apoptosis by enabling cell survival independent of cell division.[26] And it was consistent with previous literature that lower expression of Bcl-2 in OOCs may result in mild biological behavior and a lower tendency to recur.[27] The differential expression between OKCs and OOCs might again suggest the possible different pathogeneses underlying the two types of odontogenic cysts.

Based on our previous studies, as well as reports from other groups, more than 80% of OKCs, both syndromic and sporadic, harbor *PTCH1* mutations.[9] The results of 10 samples of OKCs with *PTCH1* mutations as positive control was identical to previous result by our group.[28] In contrast, *PTCH1* mutations were not detected in 14 OOCs by sequencing analysis using frozen specimens of the lining epithelium separated from the fibrous capsules. Although one missense variant (rs357564, c.3944C>T, p.Pro1315Leu) identified in the present series, known as a SNP at exon 23, might modulate the association between the use of oral contraceptives and breast cancer risk,[29] abnormalities related to *PTCH1* frequently detected in OKCs were not seen in OOCs. However, in one previous study,[13] loss of heterozygosity of the *PTCH1* gene was detected in four out of seven OOC samples. Although no sequencing data was provided, this could raise the possibility of other forms of *PTCH1* alterations in OOCs.

In conclusion, the present study reported a large series of OOCs and confirmed their consistent clinicopathological features as a new entity, most notably exhibiting little if any tendency to recur following enucleation. Compared with OKCs, OOCs in the present series appeared to occur more often in older and male patients. Multiple involvement of the jaws in OOCs was more common than that in sporadic OKCs. The absence of *PTCH1* mutations was confirmed by sequencing epithelial lining samples from 14 OOCs, which was in sharp contrast to the results for OKCs, suggesting different molecular pathogeneses underlying these two seemingly entirely keratinized jaw cysts.

AUTHOR CONTRIBUTIONS

Yan-Jin Wang: Conceptualization; data curation; formal analysis; investigation; validation; visualization; writing – original draft. **Jian-Yun Zhang:** Investigation; validation; visualization; writing – review and editing. **Qing Dong:** Data curation; methodology; supervision. **Tie-Jun Li:** Conceptualization; funding acquisition; project administration; resources; supervision; writing – review and editing.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest, real or perceived, financial or non-financial in this article.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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