Underestimated \textit{PTCH1} mutation rate in sporadic keratocystic odontogenic tumors

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\textbf{SUMMARY}

Objectives: Keratocystic odontogenic tumors (KCOTs) are benign cystic lesions of the jaws that occur sporadically in isolation or in association with nevoid basal cell carcinoma syndrome (NBCCS). The protein patched homolog 1 gene (\textit{PTCH1}) is associated with NBCCS development and tumor genesis associated with this syndrome. However, previous studies have revealed that more than 85% of syndromic KCOTs and less than 30% of sporadic KCOTs harbor \textit{PTCH1} mutations. The significantly lower \textit{PTCH1} mutation rates observed in sporadic KCOTs suggest that they serve a minor role in pathogenesis. We aimed to discern the importance of \textit{PTCH1} mutations in sporadic KCOTs.

Materials and methods: \textit{PTCH1} mutational analysis was performed with 19 new sporadic KCOT cases by direct sequencing of epithelial lining samples separated from fibrous capsules. Using this approach, we further reexamined 9 sporadic KCOTs that were previously reported to lack \textit{PTCH1} mutations by our group.

Results: Nineteen \textit{PTCH1} mutations were detected in patient samples from 16/19 new cases (84%) all these mutations were absent in fibrous tissues and peripheral blood specimens from the same patients. We also identified four \textit{PTCH1} mutations in 3/9 patients (33%) that were previously undetected.

Discussion: These data indicated that \textit{PTCH1} mutations occur in sporadic KCOTs at a higher rate than previously suspected, owing to the masking effects of the attached stromal tissues in the test samples. These results suggest that the \textit{PTCH1} gene plays a significant role in the pathogenesis of sporadic KCOTs, which is comparable to that observed in NBCCS patients.

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\textbf{Introduction}

Keratocystic odontogenic tumors (KCOTs), previously known as odontogenic keratocysts, are benign, intraosseous, cystic lesions of odontogenic origin with high growth potential and propensity for recurrence [1]. In 2005, KCOTs were classified by the World Health Organization under a new classification scheme for head and neck tumors as benign neoplasms, based on evidence from various clinicopathological studies [2–7]. Although a great majority of KCOTs occur in isolation as single, non-syndromic cysts, they may also present as multiple cysts as a feature of the nevoid basal cell carcinoma syndrome (NBCCS). NBCCS, also known as Gorlin syndrome, is a rare autosomal-dominant disorder with complete penetrance and variable expressivity that can manifest as basal cell carcinomas (BCCs), medulloblastomas, KCOTs, or bifid ribs [8,9]. KCOT is one of the most consistent and representative signs of NBCCS, occurring in 65–100% of NBCCS patients [8].

The protein patched homolog 1 gene (\textit{PTCH1}) is the human homolog of the \textit{Drosophila} segment polarity gene and is responsible for NBCCS and some related sporadic tumors [10,11]. \textit{PTCH1} has been mapped to 9q22.3–31 (Genbank accession numbers: U43148 and U59464) and consists of 23 exons that encode a
1447-amino acid protein with 12 transmembrane regions, two extracellular loops, and a putative sterol-sensing domain (SSD) [10,11]. PTCH1 is the receptor for the Drosophila Hedgehog (HH) protein and thereby regulates the HH signaling pathway, which is implicated in the formation of embryonic structures and tumorigenesis. In the absence of HH, PTCH1 signals via the smoothened protein a seven transmembrane spanning G-protein-coupled receptor encoded by the SMO gene [12]. Consequently, PTCH1 mutations cause SMO activation, and may cause constitutive ligand-independent signal transduction, leading to neoplastic growth [13].

Previous studies from our group and other investigators have revealed that greater than 85% of syndromic KCOTs and fewer than 30% of sporadic KCOTs harbor PTCH1 mutations, suggesting that PTCH1 plays a critical role in the pathogenesis of these jaw tumors [14–20]. However, the fact that PTCH1 mutations were apparently absent in over 70% of sporadic cases raises questions regarding its significance. We hypothesized that the presence of contaminating stromal tissues may conceal somatic PTCH1 mutations in the epithelium in the non-syndromic cysts. Thus, we analyzed PTCH1 mutations in sporadic KCOTs by using samples of epithelial linings separated from the connective tissue capsules.

Materials and methods

Subjects and samples

KCOT samples from 28 unrelated Chinese individuals were obtained from the Peking University Hospital and School of Stomatology. All cases in this series were diagnosed as sporadic KCOTs in histopathology from patients with no signs or histories of NBCCS. Samples were obtained from 19 new cases and 9 cases that were previously studied and reported by our group to lack PTCH1 mutations [16,18]. Besides, all samples were newly onset of KCOTs and not recurrent cases. Fresh tissue specimens and corresponding peripheral blood samples were collected and stored at -80 °C for subsequent analysis. Experimental protocols used in this study were reviewed and approved by the Ethics Committee of the Peking University Hospital. Informed consent was obtained from all subjects.

Separation of the epithelial linings from fibrous capsule tissues

Cyst walls of approximately 0.8 cm × 0.8 cm × 0.2 cm in size were prepared from specimens from each case. Tissue samples were washed three times with PBS. Tissue masses were incubated for 1 h at 4 °C in Dispase II (1 U/ml) (Roche, Basel, Switzerland). Epithelial linings of cyst walls were carefully separated from the associated fibrous capsule under a dissecting microscope. DNA was isolated from both the epithelial and stromal fragments.

DNA extraction, polymerase chain reaction (PCR), and direct sequencing

Genomic DNA from epithelial linings and fibrous capsules were isolated with the QiAamp DNA Mini Kit (Qiagen, Hilden, Germany). Each of the 23 exons comprising the PTCH1 gene was amplified by PCR with specific primers, as described in our previous study [16]. Briefly, PCR was performed using 50-μl reaction mixtures containing 200 μM dNTPs, 10 pmol of each primer, 1.25 U of Ex Tag DNA polymerase (Takara, Kyoto, Japan), 50 mM KCl, 10 mM Tris–HCl, 1.5 mM MgCl2, and approximately 100 ng of template DNA. 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, and elongation at 60–65 °C for 30 s, and a final extension at 72 °C for 10 min. The amplified products were sequenced directly with the primers used for the original PCR. Sequencing was performed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). All mutations detected were confirmed by reverse sequencing and at least two additional independent PCR experiments.

Results

Detachment of the epithelial linings from the connective tissue capsule

White and semitransparent epithelial flakes were detached from underlying connective tissue capsules (Fig. 1A).

PTCH1 mutations in fresh sporadic KCOTs

We detected 19 PTCH1 mutations in fresh patient samples from 16/19 cases (Patients 6, 7, and 16 carried two simultaneous mutations; Table 1), corresponding to a mutation frequency of approximately 84%. None of the 19 mutations detected in the DNA from the epithelial layers were present in the DNA from connective tissue or peripheral blood samples from the corresponding patients. Only 1 out of the 19 PTCH1 mutations observed has been previously reported in the Human Gene Mutation Database. Fig. 2C shows a frameshift PTCH1 mutation (c.1381_1414del) from Patient 3 (a 32-year-old woman) that was detected in the epithelial tissue, but not in the corresponding peripheral blood and connective tissues (Fig. 2A and B). Patient 1 carried a nonsense mutation (c.1581_1582del), which introduced a stop codon at amino acid residue 528. A duplication of 24 nucleotides followed position 3103 in exon 18 of Patient 2, resulting in an in-frame insertion of 8 amino acids between codons 1034 and 1041. We also identified a novel homoygous frameshift mutation in Patient 8 involving deletion of T at position 2362 in Exon 15, leading to a stop codon at amino acid residue 805. Three cases (Patients 6, 7, and 16) showed two coincident frameshift mutations, respectively (c.[2477del(+)]1611dup, c.[927dup(+)]1457dup, and c.[612del(+)]1396del). Additional information regarding mutations found in patient tissues is given in Table 1.

Utility of detecting PTCH1 mutations in detached epithelial layers

By sequencing exonic PTCH1 DNA sequences in separated epithelial layers, we successfully identified 4 mutations in 3/9 patients...
(Patients 17, 18 and 19) that we previously found to lack PTCH1 mutations. As observed with the new cases, all PTCH1 mutations identified with the reexamined cases were present only in the epithelial linings (Table 1). A c.2776T > A missense mutation in exon 17 previously described in a NBCCS case [21] was observed in the

### Table 1

Summary of PTCH1 mutations in sporadic keratocystic odontogenic tumors.

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age/sex</th>
<th>Exon No.</th>
<th>Nucleotide definition&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Amino acid definition&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Functional effect</th>
<th>Type</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>49/M</td>
<td>Exon11</td>
<td>c.1581_1582del</td>
<td>p.(Asn528&lt;sup&gt;⁄&lt;/sup&gt;)</td>
<td>Nonsense</td>
<td>Truncation</td>
<td>ICL2</td>
</tr>
<tr>
<td>2</td>
<td>23/M</td>
<td>Exon18</td>
<td>c.3103_3126dup</td>
<td>p.Val1034_Leu1041dup</td>
<td>In-frame duplication</td>
<td>Non-truncation</td>
<td>TM8</td>
</tr>
<tr>
<td>3</td>
<td>32/F</td>
<td>Exon10</td>
<td>c.1381_1414del</td>
<td>p.Asp461Leufs&lt;sup&gt;19&lt;/sup&gt;</td>
<td>Frameshift</td>
<td>Truncation</td>
<td>ICL1</td>
</tr>
<tr>
<td>4</td>
<td>10/M</td>
<td>Exon8</td>
<td>c.1086dup</td>
<td>p.Arg1029Hisfs&lt;sup&gt;123&lt;/sup&gt;</td>
<td>Frameshift</td>
<td>Truncation</td>
<td>ECL1</td>
</tr>
<tr>
<td>5</td>
<td>28/M</td>
<td>Exon11</td>
<td>c.3064_3073dup</td>
<td>p.Phe826Serfs&lt;sup&gt;4&lt;/sup&gt;</td>
<td>Frameshift</td>
<td>Truncation</td>
<td>ECL4</td>
</tr>
<tr>
<td>6</td>
<td>27/M</td>
<td>Exon12</td>
<td>c.2477del</td>
<td>p.Gly538Argfs&lt;sup&gt;89&lt;/sup&gt;</td>
<td>Frameshift</td>
<td>Truncation</td>
<td>ICL2</td>
</tr>
<tr>
<td>7</td>
<td>58/M</td>
<td>Exon6</td>
<td>c.927dup</td>
<td>p.Asn510Glnfs&lt;sup&gt;9&lt;/sup&gt;</td>
<td>Frameshift</td>
<td>Truncation</td>
<td>ECL1</td>
</tr>
<tr>
<td>8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>55/F</td>
<td>Exon10</td>
<td>c.1457dup</td>
<td>p.Leu487Profs&lt;sup&gt;10&lt;/sup&gt;</td>
<td>Frameshift</td>
<td>Truncation</td>
<td>TM3</td>
</tr>
<tr>
<td>9</td>
<td>62/M</td>
<td>Exon6</td>
<td>c.848dupA</td>
<td>p.Asn283Lysfs&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Frameshift</td>
<td>Truncation</td>
<td>ECL1</td>
</tr>
<tr>
<td>10</td>
<td>25/F</td>
<td>Exon7</td>
<td>c.955dup</td>
<td>p.Met319Asafs&lt;sup&gt;118&lt;/sup&gt;</td>
<td>Frameshift</td>
<td>Truncation</td>
<td>ECL1</td>
</tr>
<tr>
<td>11</td>
<td>83/M</td>
<td>Exon2</td>
<td>c.239_262del</td>
<td>p.Phe88Asafs&lt;sup&gt;28&lt;/sup&gt;</td>
<td>Frameshift</td>
<td>Truncation</td>
<td>N-terminus</td>
</tr>
<tr>
<td>12</td>
<td>25/F</td>
<td>Exon15</td>
<td>c.2332dup</td>
<td>p.Thr778Asafs&lt;sup&gt;12&lt;/sup&gt;</td>
<td>Frameshift</td>
<td>Truncation</td>
<td>ECL4</td>
</tr>
<tr>
<td>13</td>
<td>31/M</td>
<td>Exon11</td>
<td>c.1540_1555del</td>
<td>p.Asp514Profs&lt;sup&gt;23&lt;/sup&gt;</td>
<td>Frameshift</td>
<td>Truncation</td>
<td>TM4</td>
</tr>
<tr>
<td>14</td>
<td>26/M</td>
<td>Exon9</td>
<td>c.1341dup</td>
<td>p.Leu448Thrfs&lt;sup&gt;49&lt;/sup&gt;</td>
<td>Frameshift</td>
<td>Truncation</td>
<td>TM2</td>
</tr>
<tr>
<td>15</td>
<td>49/M</td>
<td>Exon23</td>
<td>c.4264del</td>
<td>p.Arg1422Glufs&lt;sup&gt;9&lt;/sup&gt;</td>
<td>Frameshift</td>
<td>Truncation</td>
<td>C-terminus</td>
</tr>
<tr>
<td>16</td>
<td>72/M</td>
<td>Exon4</td>
<td>c.612del</td>
<td>p.(Tyr204&lt;sup&gt;⁄&lt;/sup&gt;)</td>
<td>Nonsense</td>
<td>Truncation</td>
<td>ECL1</td>
</tr>
<tr>
<td>17&lt;sup&gt;d&lt;/sup&gt;</td>
<td>46/M</td>
<td>Exon10</td>
<td>c.1396del</td>
<td>p.Gln464Argfs&lt;sup&gt;25&lt;/sup&gt;</td>
<td>Frameshift</td>
<td>Truncation</td>
<td>ICL1</td>
</tr>
<tr>
<td>18&lt;sup&gt;d&lt;/sup&gt;</td>
<td>32/F</td>
<td>Exon6</td>
<td>c.750_751insGGAATTCCCTGGAAGAAGAAGACAGAAGTAACAAATTTTGAACTACCC</td>
<td>p.Lys251Glyfs&lt;sup&gt;30&lt;/sup&gt;</td>
<td>Frameshift</td>
<td>Truncation</td>
<td>ECL1</td>
</tr>
<tr>
<td>19&lt;sup&gt;d&lt;/sup&gt;</td>
<td>38/M</td>
<td>Exon17</td>
<td>c.2776T &gt; A</td>
<td>p.Trp926Arg</td>
<td>Missense</td>
<td>Non-truncation</td>
<td>ECL4</td>
</tr>
</tbody>
</table>

<sup>a</sup> Gene mutation nomenclature is applied according to the guidelines of the Human Genome Variation Society.

<sup>b</sup> Nucleotide and amino acid residue numbering is based on Genbank entry U59464.1; +1 = A of ATG Codon.

<sup>c</sup> The mutation demonstrated homozygosity.

<sup>d</sup> Patients were previously reported as none PTCH1 mutations by our group.

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**Fig. 2.** PTCH1 mutation identified in detached epithelial layers of fresh KCOTs. Patient 3 revealed a frameshift mutation (c.1381_1414del) in the epithelial layer (C), while it is absent in both peripheral blood (A) and stromal tissues (B).

**Fig. 3.** Utility of detecting PTCH1 mutations in detached epithelial layers. Patient 19 showed a missense mutation (T > A, arrow) in the epithelium (B), which is absent in the corresponding fibrous capsule (C). When the epithelial lining attach to the capsule, we failed to identify the mutation (A).
epithelial lining of Patient 19 (Fig. 3B) but not in the corresponding fibrous capsule (Fig. 3C). When epithelial linings and fibrous tissues were mixed as done in our previous studies, we failed to identify the c.2776T > A missense mutation (Fig. 3A). Patient 17 carried 2 concomitant frameshift mutations (c.808_812del; c.2924del). Another novel mutation (c.750_751insGGAATTCCTGGAAGAGTTAAATAAAC) was detected in Patient 18.

Discussion

Odontogenic keratocysts arise from remnants of dental lamina, an embryonic structure that normally differentiates into tooth buds and enamel-producing cells during odontogenesis [8,22]. The remnants of dental lamina usually regress at later stages of development. The precursor cells of jaw cysts may fail to involute, because of a genetic alteration. The PTCH1 gene is predicted to participate in tumorigenesis in NBCCS and related sporadic neoplasms, including somatic keratocysts. Previous studies have revealed that the frequency of PTCH1 inactivation in sporadic KCOTs (<30%) is much lower than that of syndromic KCOTs (>85%). The potential causes of observed lower PTCH1 mutation rates in sporadic KCOTs are as follows:

(1) A likely possibility is that PTCH1 mutations in patient samples may not be detectable by standard sequencing methods if epithelial tissues are contaminated with associated stromal tissues. The presence of fibrous tissues may cause the selective amplification of the normal alleles of the PTCH1 gene from DNA isolated from keratocyst tissue, leading to the failure of detecting PTCH1 mutations.

(2) Some authors have also argued that sporadic KCOTs may represent the NBCCS syndrome in its least expressed form [23,24]. We consider that with increasing age and disease progression, the cellular capacity for DNA mismatch repair gradually declines, thereby reducing genome stability and furthering the risks for acquiring additional genetic mutations. Somatic cases in which PTCH1 mutations are currently not detected perhaps represent the early stage of NBCCS, although such mutations may eventually occur as the disease develops and other abnormalities associated with the syndrome may gradually appear.

(3) KCOT, as a complex disease, may be caused by mutations in multiple genes, including PTCH1. Reports have shown that other genes, such as PTCH2 [25] and SUFU [26,27] might be involved in NBCCS pathogenesis.

To avoid potential sample contamination with stromal tissues, we detached the epithelial layers from their underlying connective capsules in the samples. In 16/19 cases (84%), we were able to detect PTCH1 mutations. All the alterations occurred in epithelial samples, but not in the corresponding stromal tissues, demonstrating that they are somatic. Previous studies (Table 2) have revealed that the PTCH1 mutation frequency in sporadic KCOTs (25–50%) is nearly equivalent to that observed in syndromic KCOTs [25,26,27]. This result suggests that lower PTCH1 mutation rates detected in KCOTs may be caused by the presence of confounding fibrous tissues and that epithelial separation can assist in accurately identifying driver mutations in sporadic KCOTs.

We compiled a list of 33 PTCH1 mutations observed in 25/71 patients with sporadic KCOTs by our group

### Table 2

<table>
<thead>
<tr>
<th>First author, Year (Ref. No.)</th>
<th>No. cases</th>
<th>PTCH1 mutations no.</th>
<th>Mutation frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Levanat*, 1996 [31]</td>
<td>14</td>
<td>4</td>
<td>29</td>
</tr>
<tr>
<td>Lench*, 1996 [32]</td>
<td>6</td>
<td>3</td>
<td>50</td>
</tr>
<tr>
<td>Barreto*, 2000 [19]</td>
<td>3</td>
<td>1</td>
<td>33</td>
</tr>
<tr>
<td>Zedana, 2001 [33]</td>
<td>8</td>
<td>3</td>
<td>38</td>
</tr>
<tr>
<td>Gu*, 2006 [14]</td>
<td>10</td>
<td>3</td>
<td>30</td>
</tr>
<tr>
<td>Sun*, 2008 [16]</td>
<td>20</td>
<td>5</td>
<td>25</td>
</tr>
<tr>
<td>Pan*, 2009 [17]</td>
<td>8</td>
<td>2</td>
<td>25</td>
</tr>
<tr>
<td>Pan*, 2009 [34]</td>
<td>8</td>
<td>2</td>
<td>25</td>
</tr>
<tr>
<td>Pan*, 2010 [35]</td>
<td>29</td>
<td>9</td>
<td>31</td>
</tr>
<tr>
<td>Guo*, 2013 [18]</td>
<td>29</td>
<td>13</td>
<td>45</td>
</tr>
<tr>
<td>This study</td>
<td>19</td>
<td>16</td>
<td>84</td>
</tr>
</tbody>
</table>

* PTCH1 mutations were previously reported by other groups.

** PTCH1 mutations were previously reported by our group.

![Fig. 4. Distribution pattern of 56 PTCH1 mutations in relation to the different domains of the Patched protein. The thick line indicates the SSD.](image-url)
(Supplemental Table 1) [17,18] and have added an additional 23 mutations identified in this study from 19/28 cases. A total of 56 mutations were identified, consisting of 40 frameshift, 5 nonsense, 5 missense, 2 splice-site mutations, and 4 in-frame insertion/deletion/duplication mutations. Consistent with previous studies [21], most mutations identified (45/56; 80.36%) are predicted to result in the expression of truncated PTCH1 proteins (Fig. 4). A significantly higher frequency (26/56; 46.43%) was clustered into the two large extracellular loops where hedgehog ligand binding occurs [21]. Another ‘hot’ region was the highly conserved sterol-sensing domain (17/56; 30.36%), which harbors transmembrane domains 2–6 [21], while no mutations in the large intracellular domain were found [18]. Careful analysis revealed no PTCH1 hot spots, and no apparent genotype–phenoype correlations could be established.

A disorder in the Sonic hedgehog (SHH) pathway involving PTCH1 could result in an aberrant cell cycle progression and neoplastic growth [28]. Pan et al. [17] found that PTCH1 mutations associated with a KCOT subgroup was linked to increased proliferation, suggesting the possibility that such mutations could lead to a phenotype of higher recurrence. These data indicates that the prevalence of PTCH1 alteration (84%) may explain the behavior of jaw cysts, which may be similar to low-grade neoplasms.

Medulloblastoma also occurs in NBCCS and is typically of the nodular/desmoplastic type, hemispheric in location, and arises as a direct consequence of PTCH1 mutations and activation of the SHH pathway in all cases [29]. In contrast, SHH pathway aberrations (PTCH1, SUFU, SMO) are found in only about 15% of patients with sporadic medulloblastoma [30]. Histologically, the nodular/desmoplastic medulloblastoma is composed of a heterogeneous architecture with regions of dense intercellular reticulin and nodules containing tumor cells with a neuronal phenotype and decreased growth fraction [29]. In light of the current study, these observations raise the possibility that higher PTCH1 mutation rates may also be observed in somatic nodular/desmoplastic medulloblastoma if tumor cells are carefully separated from contaminating reticulin, although further studies are needed to investigate this possibility. In conclusion, this study provides evidence that PTCH1 plays a critical role in KCOT tumorigenesis, even when they are not syndrome-related and that prior studies have likely underestimated the PTCH1 mutation rate in KCOT. Nevertheless, nearly 20% of somatic KCOTs did not harbor PTCH1 mutations and the underlying genetic alteration(s) driving pathogenesis remain to be elucidated.

**Conflict of interest statement**

None declared.

**Acknowledgements**

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**Appendix A. Supplementary material**

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.oraloncology.2014.09.016.

**References**

