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PTCH1 alterations are frequent but other genetic alterations are rare in sporadic odontogenic keratocysts

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Abstract

Objective: Odontogenic keratocysts (OKCs) are benign jaw lesions with high growth potential and propensity for recurrence. Our previous study revealed that *PTCH1* mutations, which were frequently detected in sporadic OKCs, might be underestimated due to the masking effect of the stromal components within the tested tissues. We aimed to confirm these results in larger scale and further present the unbiased view of the genomic basis of sporadic OKCs except *PTCH1*.

Materials and methods: We analyzed *PTCH1* mutations in additional 19 samples. Using whole-exome sequencing (WES), we further characterized the mutational landscape of five sporadic OKC samples lacking *PTCH1* mutation and loss of heterozygosity (LOH).

Results: Combined with our previously reported 19 cases, thirty of 38 (79%) cases harbored *PTCH1* mutations. Through whole-exome sequencing and integrative analysis, 22 novel mutations were confirmed among five *PTCH1*-negative samples. No recurrent mutations were identified in the WES samples and validation cohort of 10 OKCs.

Conclusions: Our data further confirmed the frequent *PTCH1* mutation and other rare genetic alterations in sporadic OKCs, highlighting the central role of SHH signaling pathway. In *PTCH1*-negative cases, other rare mutations scattered in a subset of OKCs were independent of the SHH pathway. These results suggested that an SHH inhibitor may be effective to treat the majority of OKCs.

KEYWORDS
PTCH1 mutation, odontogenic keratocysts, whole-exome sequencing

1 | INTRODUCTION

Odontogenic keratocysts (OKCs, also known as keratocystic odontogenic tumors) are locally aggressive cystic lesions of the jaw that are through to arise from the odontogenic epithelium (Partridge & Towers, 1987). Gorlin syndrome is an autosomal dominant disorder predisposing affected individuals to the development of multiple OKCs, and studies on patients with Gorlin syndrome have initially shown a pivotal role for the sonic hedgehog (SHH) pathway in OKC development. Further research has confirmed the central role of the SHH/patched1 (PTCH1)/smoothened (SMO) pathway in sporadic OKCs. PTCH1 normally acts to block the activity of SMO (Stone et al., 1996). In particular, the binding of SHH to the extracellular

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domain of PTCH1 relieves SMO inhibition, leading to activation of the GLI transcription factor that regulates the transcription of SHH target genes (Toftgard, 2000).

Surgical intervention remains the primary treatment of choice for OKCs. However, a high recurrence rate of up to 30% has been found following conservative treatments, such as enucleation and curettage, making it difficult to determine the optimal extent of surgical resection. Aggressive tumor resection can lead to the need for extensive reconstructive surgery and rehabilitation for patients with OKCs, causing significant morbidity and negatively impacting their quality of life (Li, 2011; Madras & Lapointe, 2008; Miller, Campbell, & Deas, 2011; Morgan, Burton, & Qian, 2005). Recently, the SHH pathway was identified as a potential therapeutic target with the introduction of a clinically effective small-molecule antagonist of the SMO receptor, vismodegib (Rudin, 2012). Indeed, a clinical trial in patients with Gorlin syndrome showed that vismodegib could shrink OKCs in some patients, highlighting the possibility of precision medicine for managing OKCs (Ally et al., 2014).

Recently, we reported frequent *PTCH1* (OMIM#601309) mutations detected in 16 of 19 (84%) sporadic OKCs (Qu et al., 2015). Approximately 20% of patients with sporadic OKCs do not appear to carry a *PTCH1* mutation; thus, the molecular alterations of this subgroup of lesions remain to be further studied. In this study, we further expanded the sample size to confirm *PTCH1* mutation frequency, providing the basis for targeted therapeutic strategy. Moreover, we applied whole-exome sequencing (WES) for evaluation of *PTCH1*-negative sporadic OKC samples in an attempt to detect novel genes involved and better understand the overall mutation landscape.

2 | MATERIALS AND METHODS

2.1 | Patients and samples

Thirty-eight patients with sporadic OKCs were obtained from Peking University School and Hospital of Stomatology, nineteen of which were previously described in a study of *PTCH1* mutations (Qu et al., 2015). Informed consent was obtained, and all methods were performed in accordance with the relevant guidelines of the Ethics Committee of the Peking University Health Science Center (No. 2016-09, Date of Approval: February 26, 2016).

2.2 | Epithelium separation, DNA extraction, and *PTCH1* sequencing

The epithelial linings were separated from the associated fibrous capsule, as described previously (Qu et al., 2015). Genomic DNA was isolated, and each of the 22 exons comprising the *PTCH1* gene was amplified by polymerase chain reaction (PCR) with specific primers and conditions, as described in our previous report (Qu et al., 2015). Sanger sequencing was performed on an ABI PRISM 3730 Genetic Analyzer (Applied Biosystems).

2.3 | LOH assay

Eight cysts without *PTCH1* mutation were screened for LOH. Five microsatellite markers (D9S253, D9S197, D9S196, D9S287, and *PTCH1* intra, exon 1a) were examined. PCR was performed using a fluorescent forward primer. Primer sequences were obtained from the National Center for Biotechnology Information UniSTS database. Genomic instability was detected with an ABI PRISM 3730 Genetic Analyzer. A given informative marker was considered to display LOH when a ≥ 2-fold difference was observed in the relative allele height ratio between the lesion and matched stroma. Cysts with loss of at least two loci were considered to exhibit LOH.

2.4 | Whole-exome sequencing (WES) and bioinformatics analysis

Five cysts without *PTCH1* mutations or LOH satisfied WES requirements. The sample was prepared using Agilent SureSelect Human All Exon (50M) Kit (Agilent Technologies) by following the manufacturer guide. DNA sequencing was performed on an Illumina HiSeq2500 instrument (Illumina Inc) using standard protocols.

Sequencing data were mapped to the human reference genome using the Burrows–Wheeler Aligner (BWA) (Li & Durbin, 2009). Variants were called with Genome Analysis Toolkit (GATK) (McKenna et al., 2010). For detection of somatic single-nucleotide variants (SNVs) and indels, paired sequencing data for each lesion and the matched normal sample were analyzed using MuTect2 (Cibulskis et al., 2013) and were annotated with ANNOVAR.

We filtered out all known polymorphisms from 1,000 Genomes and dbSNP. Highly confident variants require the following conditions: (a) non-silent somatic variants; (b) genes mutated in at least two OKCs; (c) minimum depth of 10× and minimum variant support depth of 4×; and (d) somatic SNVs present at a frequency of ≥10% or indels at a frequency of ≥20% in the lesions (Yap et al., 2014).

Functional impact predictions were performed using PolyPhen-2 (genetics.bwh.harvard.edu/pph2/) and SIFT (sift.jcvi.org/www/SIFT_enst_submit.html). In addition, we used phenolyzer (http://phenolyzer.usc.edu) to predict the disease-causing genes of OKCs (Yang, Robinson, & Wang, 2015).

2.5 | Sequence validation

Cyst-specific variants were confirmed by Sanger sequencing, and primer sequences are shown in Tables S1–S2. Through a literature review and phenolyzer prediction, we focused on two potential disease-causing genes (*CDON* and *MAPK1*). The confirmed DNA variants and full length of *CDON* and *MAPK1* were further assessed in a validation set of other 10 OKCs, including three *PTCH1*-negative cysts and seven *PTCH1*-positive samples.

TABLE 1 Summary of *PTCH1* mutations in a subset of 14 cases from 19 sporadic OKCs

Patient No.	Age/sex	Exon No.	Nucleotide change[a] (NM_000264.4)	Amino acid definition[b]	Functional effect	Structure
20	43/M	Exon 5	c.742delC	p.Leu248Serfs*2	Frameshift	ECL1
21	46/M	Exon 3	c.465_466delTC	p.Gln156Thrfs*7	Frameshift	ECL1
22	35/M	Exon 17	c.2884dupA	p.Arg962Lysfs*31	Frameshift	ECL4
23	35/F	Exon 10	c.1396_1397insTGGGACT GCTCCAACTCC	p.Ser465_ Gln466insLeuGlyLeuLeuGlnLeu	Inframe insertion	ICL1
24	68/M	Exon 10	c.1410delG	p.Leu471Trpfs*20	Frameshift	ICL1
25	67/M	Exon 16	c.2566delC	p.Gln856Argfs*47	Frameshift	ECL4
26	33/F	Exon 15	c.2436_2451del	p.Pro813Tyrfs*12	Frameshift	ECL4
26	33/F	Exon 20	c.3344delC	p.Ala1115Valfs*24	Frameshift	ICL5
27	22/F	Exon 21	c.3501delG	p.Leu1168Trpfs*23	Frameshift	TM12
28	39/F	Exon 18	c.3158_3161dupCCGG	p.Ile1055Argfs*91	Frameshift	ICL4
29	32/M	Exon 18	c.3127T>C	p.Cys1043Arg	Missense	ECL6
30	51/F	Exon 15	c.2419dupA	p.Thr807Asnfs*22	Frameshift	ECL4
31	28/M	Exon 21	c.3451_3468delinsCA	p.Tyr1151Glnfs*35	Frameshift	ECL6
32	33/M	Exon 18	c.2893_3124dup	p.Val1042Glyfs*28	Frameshift	TM8
33[c]	61/M	Exon 10	c.1441_1447del	p.Val481Glnfs*8	Frameshift	TM3

[a]Gene mutation nomenclature is applied according to the guidelines of the Human Genome Variation Society.
[b]Nucleotide and amino acid residue numbering are based on Genbank entry U59464.1;+1 = A of ATG Codon.
[c]The mutation demonstrated homozygosity.

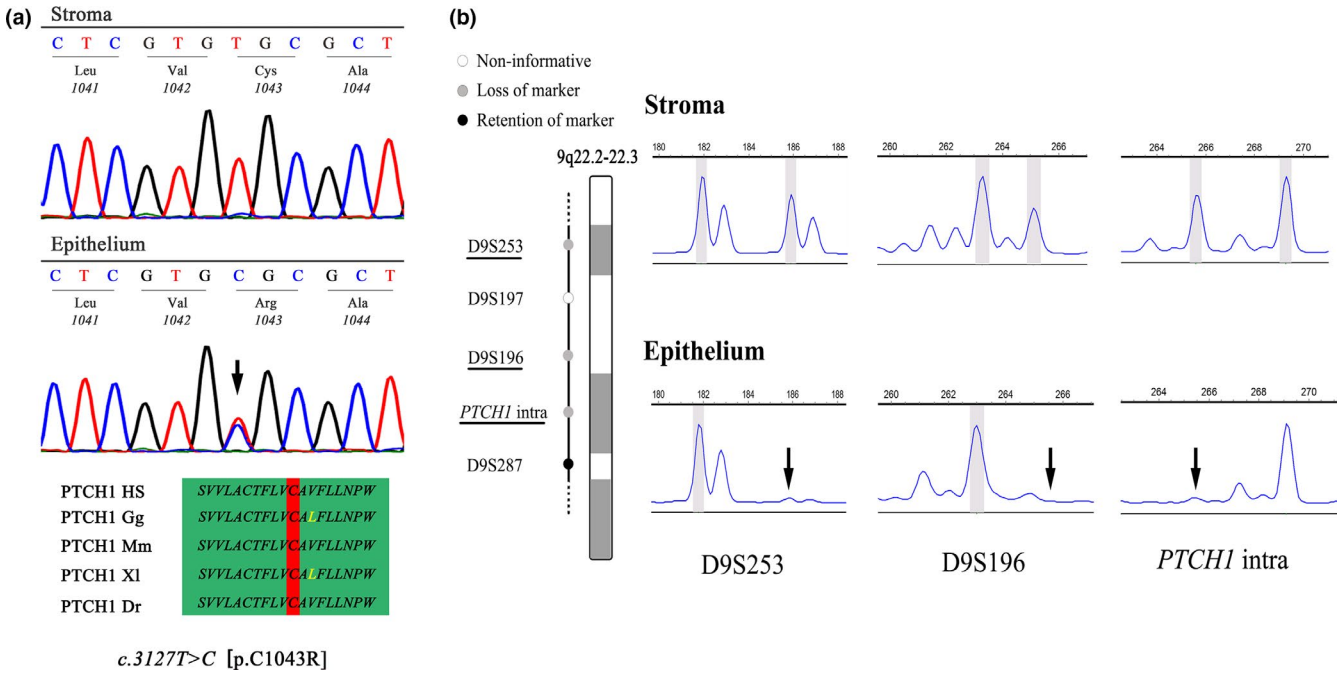

FIGURE 1 *PTCH1* alterations identified in the detached epithelial layers of fresh OKCs. (a) A missense *PTCH1* mutation (c.3127T>C) was detected in the epithelial layer of patient 29, resulting in amino acid substitutions in a highly conserved domain, as demonstrated by sequence alignment. The variant was absent from the stromal tissues. (b) LOH assay for patient 34, showing loss of three DNA markers (D9S253, D9S196, and PTCH1 intra) in the *PTCH1* locus when a ≥2-fold difference was observed in the relative allele height ratio between the matched stroma and epithelial lining (arrows) [Colour figure can be viewed at wileyonlinelibrary.com]

2.6 | Pathway enrichment analysis

We performed pathway enrichment analysis with KOBAS (v3.0) by examining the distribution of the non-synonymously mutated genes identified within Kyoto Encyclopedia of Genes and Genomes (KEGG). Significantly altered pathways were determined by measuring *p* values calculated on the basis of hypergeometric distribution with the Benjamini correction (corrected *p* value < 0.05).

TABLE 2 Overview of somatic variants found in the discovery set of 5 sporadic OKCs

		Filter steps									
			In CDS/Splicing site								
Sample ID	SNV/Indel	Not in dbSNP or 1,000 Genomes	Missense	Frameshift	Splice site	Nonsense	Synonymous	Total	Highly confident variants[a]	Amino acid change[b]	Variants confirmed
18	109	68	20	0	0	4	6	30	14	10	9
19	41	20	7	0	0	0	4	11	1	1	0
36	50	26	9	0	2	1	2	14	1	1	0
37	104	72	27	1	0	0	6	34	8	7	7
38	128	77	21	2	1	5	7	36	7	6	6
Total	**432**	**263**	84	3	3	10	25	**125**	**31**	**25**	**22**

Abbreviation: CDS, coding sequence.

[a](i) depth ≥ 10×, variant support depth ≥ 4×; (ii) SNV rate ≥ 10% or Indel rate ≥ 20%; (iii) non-silent variants; (iv) recurrent variants.

[b]Variants with "Possibly damaging" to "Damaging" predicted functional impacts on the encoded protein, as predicted by effect prediction tool SIFT and PolyPhen-2, have been referred to as variants with "Amino acid change".

3 | RESULTS

3.1 | Mutation and LOH of *PTCH1* in sporadic OKCs

We detected 15 *PTCH1* mutations in the fresh epithelium from 14 of 19 cases (one OKC sample carried two simultaneous mutations; Table 1). Combined with our previous research (in which *PTCH1* mutations were observed in 16 of 19 sporadic cases) (Qu et al., 2015), a total of 30 out of 38 patients carried *PTCH1* mutations, corresponding to a mutation frequency of 79%. Overall, all the mutations were determined to be somatic because of their absence in the matching stromal samples. Patient 29 carried a missense mutation (c.3127T>C), resulting in a change from cysteine to arginine at codon 1043 (Figure 1a). Most of the remaining variants identified were frameshift mutations that were predicted to cause the premature termination of the PTCH1 protein.

The remaining eight cysts (patients 17–19 and 34–38) lacked *PTCH1* alterations. Overall, two cysts (patients 17 and 34) showed LOH for at least two loci, which suggested that they may have been caused by LOH of *PTCH1* instead of mutations (Figure 1b).

3.2 | Identification of cyst-specific somatic variants in a discovery cohort

On average, 5.13 Gb of high-quality sequence data were generated per sample. Overall, 98.3% of the sequence reads were aligned to the human reference genome (hg19/GRCh37). In both the lesion and normal samples, a 57-fold mean coverage was achieved with 95% of the bases on average covered by at least 10-fold. The summary statistics of WES are presented in Table S3.

As shown in Table 2, we detected 125 somatic mutations in CDSs or splicing sites (Table S4), corresponding to an average 0.48 mutations per megabase (range, 0.22–0.72; Figure 2a), a considerably smaller number compared with other types of malignant tumors (Bonilla et al., 2016; Stransky et al., 2011). All 125 mutations were confirmed in only a single individual, such that no recurrent alteration was identified in the discovery cohort. According to the screening of candidate genes, we shortlisted 31 highly confident DNA variants, among which 25 variants were predicted to be functionally damaging (Table S5). Twenty-two of 25 variants were verified to be heterozygous SNVs by Sanger sequencing (Table 3). Moreover, phenolyzer identified 10 genes that were predicted as possible disease-causing genes based on the 22 confirmed variants (Figure 2b).

3.3 | Identification of candidate driver mutations in the validation cohort

The 22 novel variants and two full-length predicted disease-causing genes (*MAPK1* and *CDON*) were subjected to a validation screen in a set of other 10 OKCs, including three *PTCH1*-negative and seven *PTCH1*-positive samples. We failed to find recurrent mutations in the validation set, thereby indicating that the 22 novel variants were not likely to be hotspot mutations. Moreover, *MAPK1* and *CDON*

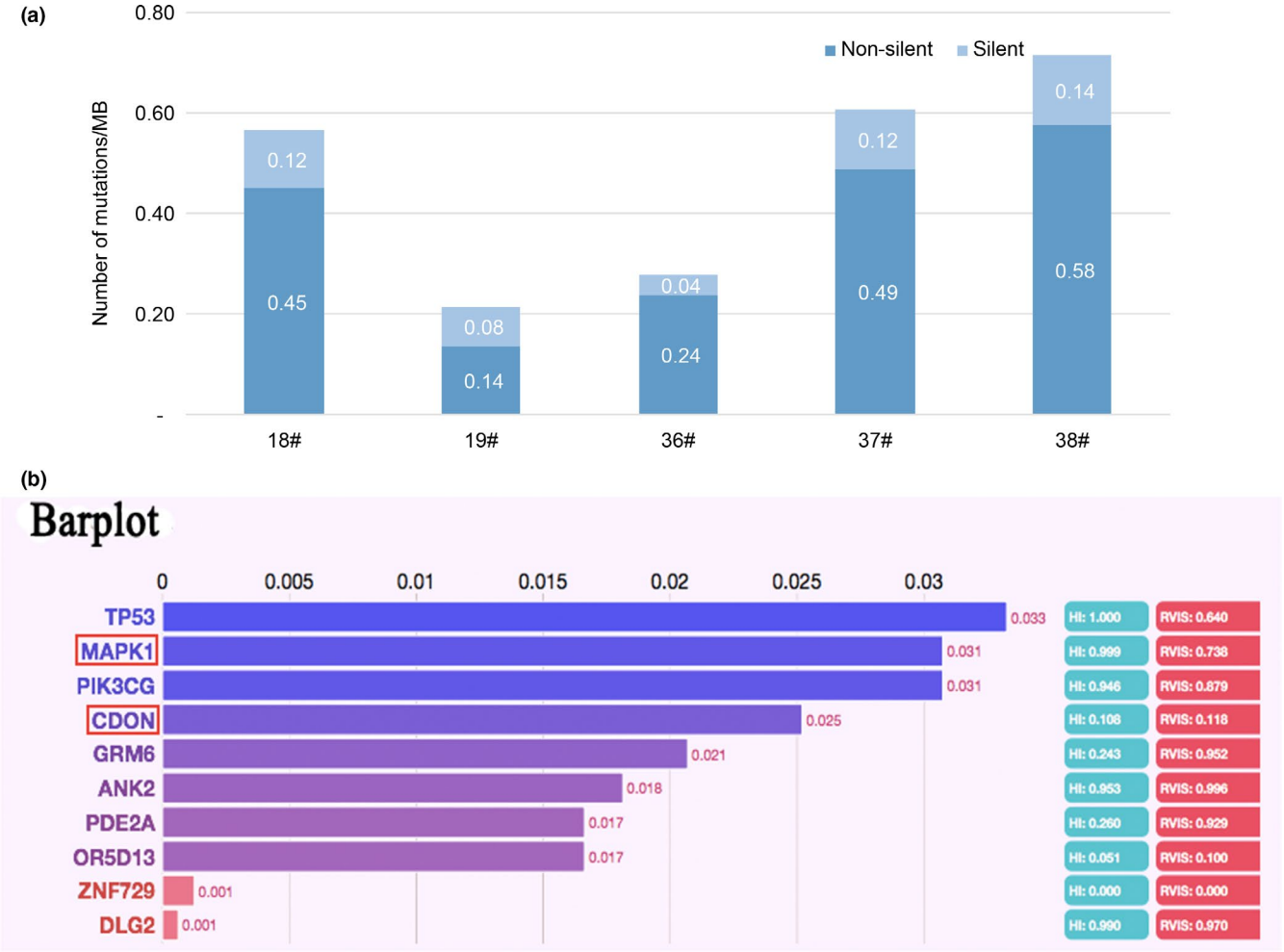

FIGURE 2 Number of mutations in cases by WES and prediction of disease-causing genes. (a) Somatic mutation frequencies for five *PTCH1*-negative OKCs. (b) Ten predicted genes in *PTCH1*-negative OKCs listed by phenolyzer and ranked according to the score, with a higher score indicating a greater chance to be a disease-causing gene. Two full-length genes validated in 10 tumors (*CDON* and *MAPK1*) are marked [Colour figure can be viewed at wileyonlinelibrary.com]

mutations represent either rare drivers for pathogenesis or passenger mutations and are thus unlikely to represent common driver mutations in OKCs.

3.4 | Altered pathway in sporadic OKCs

Pathway analyses revealed several significant altered pathways, including "Basal cell carcinoma" pathway (Table S6). Hedgehog signaling was a vital component in "Basal cell carcinoma" pathway, which supported the essential role of SHH signaling in pathogenesis of OKCs.

4 | DISCUSSION

The present study, together with our previous data (Qu et al., 2015), confirmed the high *PTCH1* mutation rate (approximately 80%) in sporadic OKCs. Given the key role of PTCH1 in regulating the SHH signaling pathway, these results provide evidence that SHH signaling plays a critical role in OKC development. Importantly, the SHH pathway inhibitor vismodegib has been used as an adjunctive therapy in patients with Gorlin syndrome to reduce OKC size and define the margins needed for surgical resection (Ally et al., 2014). Thus, since approximately 80% of the sporadic OKCs analyzed in these studies harbor *PTCH1* mutations, our results indicated that this inhibitor could also be effective for treating a significant number of sporadic OKCs.

We compiled a list of 56 *PTCH1* mutations observed in 44 out of 90 patients with sporadic OKCs by our group (Table S7) and have included an additional 15 mutations that were identified in this study from 14 of 19 cases. Consistent with previous studies, most of the mutations identified (58/71; 82%) are predicted to result in the expression of truncated PTCH1 protein (Figure 3). A significantly higher frequency (32/71; 45%) clustered in the two large extracellular loops, where hedgehog ligand binding occurs (Lindstrom, Shimokawa, Toftgard, & Zaphiropoulos, 2006). Another "hot"

TABLE 3 Details of 22 somatic variants confirmed in *PTCH1*-negative KCOTs

Gene	Patients	Description	Transcript	Nucleotide change	Amino acid definition	Function effect
ANK2	18	Ankyrin 2, neuronal	NM_001148	c.9271G>T	p.E3091*	Nonsense
STAMBPL1	18	STAM binding protein-like 1	NM_020799	c.503G>T	p.R168L	Missense
PIP5K1A	18	Phosphatidylinositol-4-phosphate 5-kinase, type I, alpha	NM_001135637	c.1146G>T	p.R382S	Missense
GRM6	18	Glutamate metabotropic receptor 6	NM_000843	c.2424C>G	p.Q808H	Missense
DDX27	18	DEAD (Asp-Glu-Ala-Asp) box polypeptide 27	NM_017895	c.1865A>T	p.E622V	Missense
SRCAP	18	Snf2-related CREBBP activator protein	NM_006662	c.7727C>A	p.S2576*	Nonsense
ZNF729	18	Zinc finger protein 729	NM_001242680	c.3560C>A	p.P1187H	Missense
CDON	18	Cell adhesion associated, oncogene regulated	NM_001243597	c.3406C>A	p.V1136F	Missense
TP53	18	Tumor protein p53	NM_000546	c.772G>T	p.E258*	Nonsense
OR5D13	37	Olfactory receptor family 5 subfamily D member 13	NM_001001967	c.746C>T	p.A249V	Missense
GABRG2	37	Protein tyrosine phosphatase type IVA, member 3	NM_198904	c.1363A>T	p.I455F	Missense
DLG2	37	Disks, large homolog 2 (Drosophila)	NM_001142700	c.1598G>A	p.S533F	Missense
MAPK1	37	Mitogen-activated protein kinase 1	NM_002745	c.461T>C	p.N154S	Missense
PAX7	37	Paired box 7	NM_001135254	c.947C>A	p.S316Y	Missense
ITSN2	37	Intersectin 2	NM_019595	c.4489C>A	p.L1497I	Missense
RYR1	37	ryanodine receptor 1	NM_000540	c.2689G>C	p.D897H	Missense
DPYSL5	38	Dihydropyrimidinase-like 5	NM_001253723	c.349C>T	p.R117*	Nonsense
PDE2A	38	Phosphodiesterase 2A, cGMP-stimulated	NM_002599	c.9G>T	p.Q3H	Missense
PIK3CG	38	Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit gamma	NM_001282426	c.1666G>T	p.E556*	Nonsense
SLC16A12	38	Solute carrier family 16, member 12	NM_213606	c.1183C>G	p.P395A	Missense
EP400	38	E1A-binding protein p400	NM_015409	c.8272delC	p.P2758Hfs*19	Frameshift
TET1	38	Tet methylcytosine dioxygenase 1	NM_030625	c.1069G>T	p.E357*	Nonsense

mutation region was the highly conserved sterol-sensing domain (SSD) (20/71; 28%), which harbors transmembrane domains 2–6 (Lindstrom et al., 2006). However, no mutations were found in the large intracellular loop, which mediates the non-canonical hedgehog pathway (Yu, Hong, Qu, Chen, & Li, 2014). After a careful and comprehensive analysis, neither *PTCH1* mutation hotspots nor apparent genotype–phenotype correlations could be established.

We performed WES in five cysts and obtained 125 somatic variants. Although we failed to find the same gene mutated in more than one cyst, 22 novel SNVs were confirmed. CDON, a membrane-associated SHH-binding protein (Tenzen et al., 2006; Yao, Lum, & Beachy, 2006), serves as a co-receptor with PTCH1 to promote SHH pathway activity (Izzi et al., 2011). MAPK1 (extracellular signal-regulated kinase [ERK]) is downstream of MEK1 in the ERK pathway and is associated with cell proliferation. Activation of the ERK pathway, as a consequence of SHH-induced gene expression, occurs frequently in human BCC. Thus, mutations in *CDON* and *MAPK1* may directly activate SHH and ERK signaling pathway, respectively, and contribute to OKC formation.

The 22 variants and two full-length genes *CDON* and *MAPK1* were sequenced in a validation cohort consisting of three *PTCH1*-negative and seven *PTCH1*-positive samples. We failed to find any mutations among these samples. Recently, Franca et al reported some other mutations except *PTCH1* in 18 OKCs by next-generation sequencing 2,856 cancer hotspot mutations in 50 oncogenes and tumor suppressor genes (Franca et al., 2018). Similar to our finding, these mutations

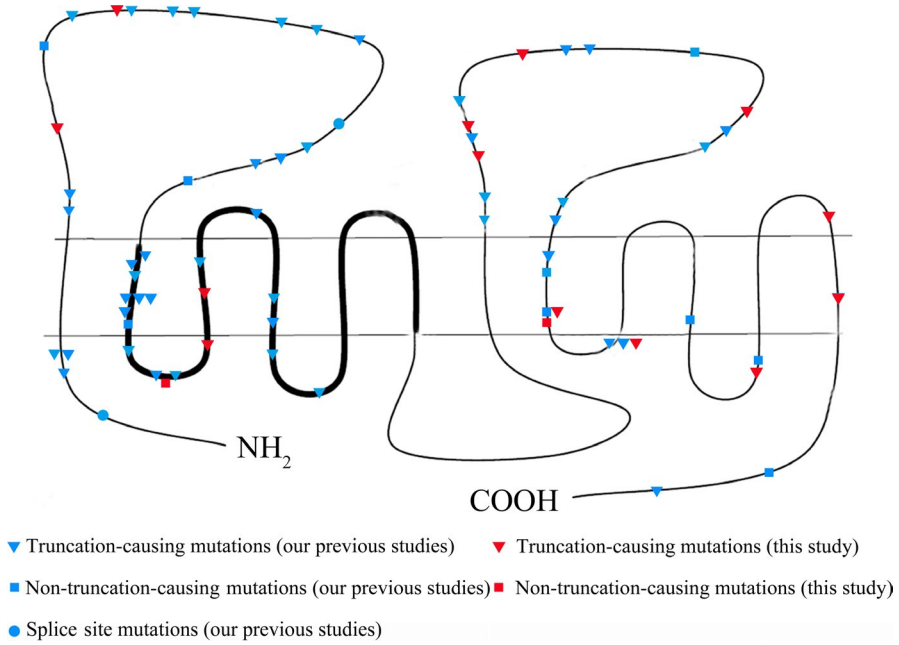

FIGURE 3 Distribution pattern of 71 *PTCH1* mutations related to the different domains of the patched protein. The thick line indicates the SSD [Colour figure can be viewed at wileyonlinelibrary.com]

occurred in one sample each and they failed to detect recurrent hotspot mutations. The mutations detected by Franca were different from that found in our study suggesting that these cancer hotspot mutations might occur rarely in OKCs. Thus, we inferred that mutations occurring in the SHH pathway regulator *PTCH1* (~80%) likely play a central role in OKCs, whereas other rare mutations (~20%) scattered in remaining OKC cases were independent of the SHH pathway. Our findings confirmed the functional involvement of the SHH pathway in the majority of OKCs and highlighted the usefulness of treatment with molecularly targeted drugs that inhibit this pathway, such as vismodegib.

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CONFLICT OF INTEREST

Declared none.

AUTHOR CONTRIBUTIONS

Li T and Chen F designed the study. Qu J and Zhang J collected samples, performed high-throughput sequencing data analysis and drafted the manuscript. Zhang H and Li X conducted quality control of sequencing data. Hong Y, Zhai J and Wang Y performed sample and high-throughput sequencing library preparation.

DATA AVAILABILITY STATEMENT

All data could be available in this article and its additional files. The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive (Genomics, Proteomics & Bioinformatics 2017) (Wang et al., 2017) in BIG Data Center (Nucleic Acids Res 2018) (BIG Data Center Members, 2019), Beijing Institute of Genomics (BIG), Chinese Academy of Sciences, under accession numbers CRA001584, CRA001584, that are publicly accessible at http://bigd.big.ac.cn/gsa.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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