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PTCH1 and *SMO* Gene Alterations in Keratocystic Odontogenic Tumors

ABSTRACT

Keratocystic odontogenic tumors (KCOTs, previously known as odontogenic keratocysts) are aggressive jaw lesions that may occur in isolation or in association with nevoid basal cell carcinoma syndrome (NBCCS). Mutations in the *PTCH1* (*PTCH*) gene are responsible for NBCCS and are related in tumors associated with this syndrome. Mutations in the *SMO* gene have been identified in basal cell carcinoma and in medulloblastoma, both of which are features of NBCCS. To clarify the role of *PTCH1* and *SMO* in KCOTs, we undertook mutational analysis of *PTCH1* and *SMO* in 20 sporadic and 10 NBCCS-associated KCOTs, and for *SMO*, 20 additional cases of KCOTs with known *PTCH1* status were also included. Eleven novel (1 of which occurred twice) and 5 known *PTCH1* mutations were identified. However, no pathogenic mutation was detected in *SMO*. Our findings suggest that mutations are rare in *SMO*, but frequent in *PTCH1* in sporadic and NBCCS-associated KCOTs. Abbreviations: NBCCS, nevoid basal cell carcinoma syndrome; KCOTs, keratocystic odontogenic tumors; BCCs, basal cell carcinomas.

KEY WORDS: *PTCH1*, *SMO*, nevoid basal cell carcinoma syndrome, keratocystic odontogenic tumors, *PTCH*.

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INTRODUCTION

Keratocystic odontogenic tumors (KCOTs, previously known as odontogenic keratocysts) are aggressive, non-inflammatory jaw cysts with a putative high growth potential and a propensity for recurrence (Browne, 1971; Li *et al.*, 1994). Considerable insight into their genesis has come from the discovery that mutations of the *PTCH1* gene (*PTCH*; MIM# 601309) are associated with nevoid basal cell carcinoma syndrome (NBCCS; MIM# 109400), an autosomal-dominant disorder presenting a spectrum of developmental abnormalities such as palmar/plantar pits, calcified falx cerebri, bridged sella, bifid ribs, and an increased susceptibility to different neoplasms, including multiple basal cell carcinomas (BCCs), KCOTs, medulloblastoma, and ovarian fibroma (Hahn *et al.*, 1996; Johnson *et al.*, 1996).

Mutations in *PTCH1* are associated with the majority of NBCCS and are found in tumors associated with this syndrome, as has been most convincingly demonstrated for BCCs and medulloblastomas (Raffel *et al.*, 1997; Wolter *et al.*, 1997). In addition to *PTCH1*, somatic mutations in the *SMO* gene (MIM# 601500) have also been identified in BCCs and in medulloblastomas (Reifenberger *et al.*, 1998; Xie *et al.*, 1998). These findings provide additional insight into the role of the sonic hedgehog pathway in NBCCS and associated tumors. Hedgehog signaling is a key regulator of embryonic development controlling cellular proliferation and fate. Binding of sonic hedgehog (SHH) to its receptor, patched (PTCH1), is thought to relieve normal inhibition by PTCH1 of smoothened (SMO), a seven-span transmembrane protein with homology to a G-protein-coupled receptor (Stone *et al.*, 1996). Thus, loss of PTCH1 function by the inactivation of *PTCH1* mutations, as well as aberrant activation of SMO by the activation of *SMO* mutations, could cause constitutive, ligand-independent signal transduction that may lead to neoplastic growth (Toftgard, 2000).

KCOTs are among the most prominent features of NBCCS, which are found in 65-100% of affected individuals (Gorlin, 1987). This fact leads to the obvious hypothesis that KCOTs are caused by genetic alterations, both in syndromic and sporadic cases (Lench *et al.*, 1997; Barreto *et al.*, 2000; Pavelic *et al.*, 2001; Ohki *et al.*, 2004). To assess further the role of *PTCH1* and *SMO* in the pathogenesis of KCOTs, we describe here the screen of *PTCH1* and *SMO* gene mutations in a large series of Chinese persons with sporadic and NBCCS-associated KCOTs.

MATERIALS & METHODS

Participants and Tumors

In total, 50 KCOT samples from 50 unrelated Chinese persons were obtained from Peking University Hospital and School of Stomatology, 20 of which have been previously described for *PTCH1* mutations (11 *PTCH1* mutations were

identified in 5 of 14 sporadic and 6 of 6 NBCCS-associated KCOTs) (Gu *et al.*, 2006; Yuan *et al.*, 2006). The remaining samples included 20 sporadic and 10 NBCCS-associated KCOTs. Additionally, a total of ten affected relatives and 12 unaffected family members belonging to the kindreds of five NBCCS probands (NB9, NB10, NB12, NB13, NB16) were also investigated for the familial segregation of the mutations identified. Diagnosis of NBCCS was established according to previously described clinical criteria (Kimonis *et al.*, 1997). Fresh tissue specimens were collected and frozen at –80°C. Peripheral blood was collected from all participants and 100 unaffected donors after they provided informed consent. The study protocol was approved by the Ethical Committee of Peking University Health Science Center.

DNA Isolation and Mutation Analysis

Genomic DNA from tumors and peripheral blood was isolated by means of a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). PCR was performed with 24 pairs of intronic primers covering 22 coding exons (exons 2-23) of *PTCH1* (U59464.1) and 13 pairs of intronic primers covering all exons of *SMO* (U84401.1; see APPENDIX). PCR products were analyzed by denaturing high-performance liquid chromatography (DHPLC) to screen for *PTCH1* and *SMO* mutations (APPENDIX). Samples showing an abnormal elution profile were subjected to direct sequencing (ABI Prism 3100 Genetic Analyzer, Applied Biosystems, Foster City, CA, USA). Any mutation detected was confirmed by reverse-sequencing and by analysis of samples from at least 2 independent PCRs. We evaluated the pathogenic role of novel missense and intronic changes by testing all available family members' DNA to determine whether the variant segregated with disease, or by PCR-RFLP or DHPLC to screen 200 control chromosomes to rule out polymorphic variants.

Total RNA Isolation and RT-PCR

Where the variants involved the exon-intron junction, we used RT-PCR to determine whether the variants were likely to affect splicing. Total RNA was extracted from 4 tumors (NB7, NB8, NB9, NB10) by means of an RNeasy Protect Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized with the SuperScript™ III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). A 2-μL quantity of cDNA was used to amplify products designed to cover the regions carrying the predicted splicing changes (exons 8-13 and exons 13-17; see APPENDIX). PCR and sequence conditions were standardized as described above. RT-PCR products were visualized on a 1.5% agarose gel.

Table. Summary of *PTCH1* Mutations in 20 Sporadic and 10 NBCCS-associated KCOTs (GenBank U59464.1); Numbering of Nucleotides, +1 = A of ATG Codon

Sample ID	Age/Sex	Exon/Intron	Nucleotide Definition	Amino Acid Definition	Functional Effect	PCR-RFLP	References	Characterization	Phenotype[a]
NB7	36/F	Intron 9	c.1347+6G>A	p.Val406_Gln501del	Exons 9 & 10 skipping; loss of 96 amino acids	—	This study	Germ line	mKCOT,PP
NB9	9/M	Exon 16	c.2619C>A	p.Tyr873X	Nonsense	—	Boutet *et al.*, 2003; Gu *et al.*, 2006	Germ line	FMH,mKCOT, BCCs, PP, TC
		Intron 10	c.1504-1G>A	p.Leu450_Glu534del	Exons 10 & 11 skipping; loss of 85 amino acids	Sfc I (loss)	This study	Germ line	
NB10	44/F	Intron 15	c.2560+1G>T	p. Val 751AspfsX49	Exon 15 skipping; premature stop	Mse I (gain)	This study	Germ line	FMH, mKCOT, PP, FC, TC
NB11	14/F	Exon 6	c.863G>A	p.Gly288Asp	Missense	Bcl I (gain)	D'Errico *et al.*, 2000	Germ line	mKCOT, PP, FB, TC
		Exon 14	c.2196_2197del	p.Ser733IlefsX4	Frameshift		Wicking *et al.*, 1997	Germ line	
NB12	43/M	Exon 9	c.1247C>G	p.Thr416Ser	Missense	Bsr I (gain)	This study	Somatic	mKCOT, PP, TC
NB13	22/M	Exon 20	c.3440T>G	p.Phe1147Cys	Missense	HpyCH4V (gain)	This study	Germ line	FMH, mKCOT, PP, FC, FB, TC
NB14	36/F	Exon 19	c.3244_3246dup	p.Pro1082dup	In-frame duplication	—	This study	Germ line	FMH, mKCOT, PP, FC, BS, BR, TC
NB16	42/M	Exon 21	c.3499G>A	p.Gly1167Arg	Missense	—	Barreto *et al.*, 2000; Pastorino *et al.*, 2005; Teh *et al.*, 2005	Germ line	FMH, mKCOT, BCCs, PP
KC19	56/M	Exon 7	c.983delA	p.His328LeufsX14	Frameshift	—	This study	Somatic	sKCOT
		Exon 9	c.1325dupT	p.Ala443GlyfsX54	Frameshift	—	This study	Somatic	
KC21	29/M	Exon 11	c.1558_1574del	p.His520ArgfsX4	Frameshift	—	This study	Somatic	sKCOT
		Exon 16	c.2635delG	p.Asp879MetfsX24	Frameshift	—	This study	Somatic	
KC22	73/F	Exon 9	c.1247C>G	p.Thr416Ser	Missense	Bsr I (gain)	This study	Somatic	sKCOT
KC30	10/F	Exon 3	c.403C>T[b]	p.Arg135X	Nonsense	—	Wicking *et al.*, 1997; Pruvost-Balland *et al.*, 2006	Somatic	mKCOT
KC33	33/F	Exon 18	c.3162dupG	p.Ile1055AspfsX90	Frameshift	—	This study	Somatic	sKCOT

[a] FMH = family medical history; mKCOT = multiple keratocystic odontogenic tumors; BCCs = multiple BCCs; PP = palmar/plantar pits; FC = calcification of falx cerebri; BS = bridged sella; BR = bifid rib; VA = vertebral anomalies; FB = frontal bossing; TC = telecanthus; sKCOT = sporadic keratocystic odontogenic tumor.

[b] This mutation demonstrated homozygosity.

RESULTS

We detected 16 *PTCH1* mutations (1 of which was identified twice) in 5 of 20 sporadic and 8 of 10 NBCCS-associated KCOTs, 11 of which were novel (Table). The 16 mutations consisted of 6 frameshift, 2 nonsense, 3 aberrant splicing, 4 missense, and 1 in-frame duplication mutations. In addition, 13 previously described polymorphism sites of the *PTCH1* gene were identified (APPENDIX). In contrast, no pathogenic mutation was found in the splicing and coding regions of the *SMO* gene in the total cohort of 50 KCOT samples tested. But 8 *SMO* polymorphisms were identified in 18 out of 50 participants, among which 3 (c.536C>T, c.IVS537+18C>T, c.582A>G) were newly identified (APPENDIX).

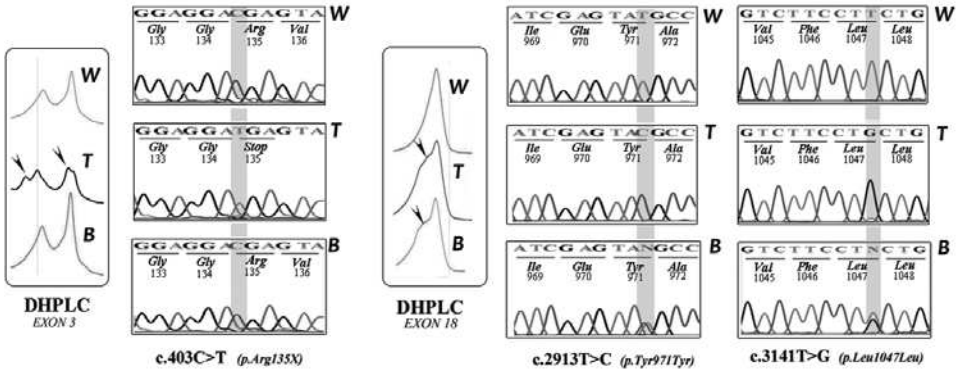

Figure 1. Identification of *PTCH1* mutations in KC30. Nonsense mutation (c.403C>T) showed a homozygous pattern in DNA samples of KCOT, but was absent in peripheral blood DNA **(left)**. Two concomitant polymorphisms (c.2913T>C; c.3141T>G) were homozygous in the tumor, but heterozygous in blood DNA **(right)**, suggesting allelic loss (W, wild-type; T, KCOT tissue; B, peripheral blood).

Somatic *PTCH1* Mutations in Sporadic KCOTs

We identified 6 novel (c.983delA, c.1325dupT, c.1558_1574del, c.2635delG, c.1247C>G, c.3162dupG) and 1 known (c.403C>T) *PTCH1* mutations in 5 out of 20 sporadic KCOTs investigated. All mutations were somatic because of their absence in the matching blood samples. Two cases (KC19, KC21) were found to be compound heterozygous for 2 different frameshift mutations (c.[983delA(+)1325dupT] and c.[1558_1574del (+)2635delG]). Another novel frameshift mutation (c.3162dupG) was detected in a sporadic case (KC33). All these frameshift mutations were predicted to cause premature termination of PTCH1. One known nonsense mutation (c.403C>T) demonstrated a homozygous pattern in a tumor (KC30) and was absent in peripheral blood. The 2 concomitant polymorphisms (c.2913T>C, c.3141T>G) detected in this case were homozygous in a tumor but heterozygous in blood (Fig. 1). Analysis of these data suggests loss of 1 allele of *PTCH1* and mutation of the other, since it is unlikely that both alleles harbor the same point mutations. One novel missense mutation (c.1247C>G) identified in a sporadic individual (KC22) also occurred in one of the 3 KCOT lesions of a NBCCS individual (NB12, Table). This mutation was absent in the matching constitutional DNA of NB12. It affected a moderately conserved amino acid and was not detected in a total of 200 control chromosomes; therefore, it is unlikely to be a rare polymorphism.

Germ-line *PTCH1* Mutations in Persons with NBCCS

In total, 9 germ-line *PTCH1* mutations (5 novel and 4 known) were identified in seven out of ten persons with NBCCS. One known nonsense mutation (c.2619C>A) was detected in a person with NBCCS (NB9) and was found to segregate with the disease in his family. Another known frameshift mutation (c.2196_2197delCT) was identified in NB11. This person also had a known missense mutation (c.863G>A), which hits a highly conserved residue (APPENDIX). One known missense mutation (c.3499G>A) was detected in NB16 and was found to segregate with the disease in the family. Two novel mutations, one missense (c.3440T>G) and one in-frame duplication (c.3244_3246dup), were detected in two persons (NB13, NB14), respectively. All these missense/in-frame duplication mutations were located in highly conserved regions across species (APPENDIX) and were not detected in a total of 200 control chromosomes. We have therefore classified these variants as pathogenic.

Four variants identified in four persons with NBCCS (c.1347+6G>A, NB7; c.1504-1G>A, NB9; c.2251-3C>G, NB8; c.2560+1G>T, NB10) occurred at the exon-intron junction. Splicing events in NB7 and NB9 were analyzed by RT-PCR with primers designed to amplify exons 8-13. An RNA sample from KC15 (no *PTCH1* mutation detected) was used as control. The expected wild-type RT-PCR product of 744 bp and a shorter fragment (588 bp) that derives from the skipping of exon 10 were detected in all cases investigated (KC15, NB7, NB9) (Fig. 2A). NB7 also exhibited an additional shorter fragment (456 bp), which is the result of the skipping of exons 9 and 10 (Fig. 2A). It resulted in the in-frame deletion of 96 amino acids (406 to 501) and the subsequent loss of part of the first extracellular loop and sterol-sensing domain. The RT-PCR products of NB9 showed a different shorter fragment (489 bp), and sequence analysis revealed that both exons 10 and 11 had been skipped (Fig. 2A), resulting in an 85-amino-acid deletion and the removal of part of the sterol-sensing domain. Possible splicing alterations in NB8 and NB10 were analyzed by RT-PCR with primers to amplify exons 13-17. While KC15 (control) and NB8 showed only the wild-type RT-PCR product of 977 bp, NB10 gave an additional shorter fragment (667 bp). This smaller product was the result of the skipping of the entire exon 15 (310 bp) (Fig. 2B). This resultant transcript contained a stop codon leading to the subsequent termination of PTCH1 49 amino acids downstream (Fig. 2B).

DISCUSSION

We previously reported 11 *PTCH1* mutations in 5 of 14 sporadic and 6 of 6 NBCCS-associated KCOTs (Gu *et al.*, 2006; Yuan *et al.*, 2006). Here we present 16 additional *PTCH1* mutations in 5 of 20 sporadic and 8 of 10 NBCCS-associated KCOTs. In total, we have detected 26 *PTCH1* mutations (2 mutations, c.2619C>A and c.1247C>G, occurred twice) in 10 out of 34 (29.4%) sporadic and 14 out of 16 (87.5%) NBCCS-associated KCOTs. The 26

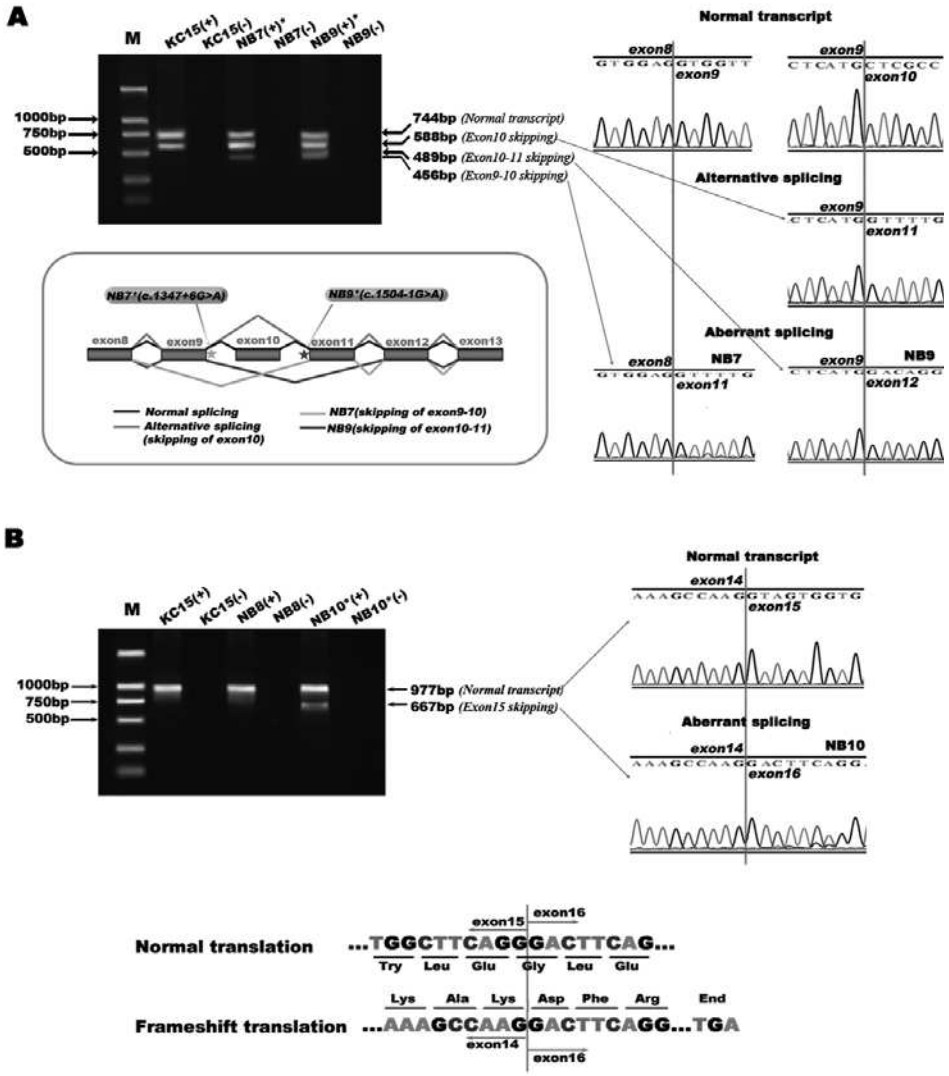

Figure 2. RT–PCR and sequencing analysis of aberrant splicing of *PTCH1*. **(A)** (upper left) Agarose electrophoresis of RT-PCR products (exons 8-13) with total RNA extracted from KCOT samples of KC15 (no *PTCH1* mutation identified), NB7 (c.1347+6G>A), and NB9 (c.1504-1G>A). The lanes are as follows: M (Marker DL2000); KC15, NB7, NB9 in the presence (+) and absence (-) of reverse transcriptase. (right) DNA sequencing of RT-PCR products. The consistent alternative transcript (588 bp) is the result of the skipping of exon 10; the extra bands of NB7 (456 bp) and NB9 (489 bp) are the result of the skipping of exons 9-10 and exons 10-11, respectively. (lower left) Schematic representation of abnormal splicing identified in NB7 and NB9. The positions of point mutations are indicated by asterisks. **(B)** Exons 13-17 amplified by RT–PCR with total RNA extracted from KC15, NB8 (c.2251-3C>G), and NB10 (c.2560+1G>T). Sequence analysis of the additional fragment of NB10 (667 bp) showed skipping of the entire exon 15, resulting in a frameshift translation and a stop codon (below in the Fig.).

mutations consisted of 10 frameshift, 2 nonsense, 3 aberrant splicing, 4 in-frame insertion/deletion/ duplication, and 7 missense mutations (APPENDIX). In line with a recent review on *PTCH1* mutation (Lindstrom *et al.*, 2006), 13 of the detected mutations were predicted to result in premature termination of PTCH1 protein, and a significantly higher frequency of mutations (9/26) was clustered into the two large extracellular loops where hedgehog ligand binding occurs. Another 'hot' region was the highly conserved sterol-sensing domain (5/26), which harbors the transmembrane domains 2–6. Through careful analysis, however, no apparent genotype–phenotype correlations could be established. Although no *PTCH1* mutation hot-spots have been reported in the literature, we identified here 2 recurrent *PTCH1* mutations, one of which (c.2619C>A), a germ-line nonsense mutation seen in two unrelated Chinese families, has also been previously reported in a French person with NBCCS (Boutet *et al.*, 2003). Thus, analysis of our data, together with reports from other groups (Lench *et al.*, 1997; Barreto *et al.*, 2000; Ohki *et al.*, 2004), indicates that defects of *PTCH1* are involved in the pathogenesis of syndromic as well as sporadic KCOTs.

The *PTCH1* gene is thought to function as a tumor suppressor gene, at least in some of the malignancies associated with NBCCS, as has been most convincingly demonstrated for BCCs (Gailani *et al.*, 1996). The molecular analyses of KCOTs and BCCs in NBCCS showed that a two-hit hypothesis is applicable to their pathogenesis (Levanat *et al.*, 1996; Barreto *et al.*, 2000). In the present study, we demonstrated that two persons with the syndrome carried 2 different mutations (NB9, nonsense plus aberrant splicing; NB11, frameshift and missense mutations), respectively. In one sporadic case (KC30), we found that the tumor may lose the normal copy of *PTCH1* while retaining a mutant copy (c.403C>T). Two other sporadic cases (KC19, KC21) showed 2 coincident frameshift mutations, respectively. These results indicate the possibility that inactivation of *PTCH1 via* a two-hit mechanism may occur in a subset of KCOTs.

Alternative splicing in *PTCH1* is thought to be a complex event, since multiple isoforms of *PTCH1* mRNA by alternative first exons have been identified (Kogerman *et al.*, 2002; Nagao *et al.*, 2005a). Additional tissue-specific or disease-related splicing variants have also been described involving exons 1–5, exon 10, and exon 12b (Nagao *et al.*, 2005b). We describe here an alternate splicing product of exon-10-skipping in sporadic and NBCCS-associated KCOTs with/without *PTCH1* mutations. A previous study observed identical consistent alternate splicing of exon 10 in all cultured normal and NBCCS patient lymphocyte and normal keratinocyte cell lines (Smyth *et al.*, 1998). Its effect on *PTCH1* function, however, is currently unknown. Whether it is a common phenomenon or is functional in another context remains to be elucidated. This study also identified 3 instances of disease-associated aberrant splicing in *PTCH1*. The mutation

in NB7 at the donor splice site of intron 9 (c.1347+6G>A) resulted in the skipping of exons 9 and 10, which leads to destruction of extracellular loop 1 and the removal of transmembrane domains 2-3. Another instance of aberrant splicing identified in NB9 (c.1504-1G>A) was the skipping of exons 10 and 11, which ablated transmembrane domains 2-4. Although the functional consequences of these 2 aberrant splicing changes are yet to be investigated, the importance of extracellular loop 1 in binding of the *SHH* ligand and the role of the sterol-sensing domain, which is made up of transmembrane domains 2-6, in mediating the potent modulating effect of cholesterol on SHH/PTCH signaling suggest that the receptor form lacking exons 9-10 or exons 10-11 may show altered signaling properties. The mutation in NB10 at the 5′ end of intron 15 (c.2560+1G>T) resulted in the entire skipping of exon 15 and premature truncation of PTCH1.

The non-detection of *PTCH1* mutations in many sporadic KCOTs and even in some typical familial cases also underlines the non-exploration of other genetic events. The idea can be examined in more detail in the context of SHH signaling, whereby PTCH1 acts to restrain the activity of the G-protein-coupled receptor, SMO. Inactivation of PTCH1 allows for hedgehog ligand-independent activation of SMO, with the subsequent activation of transcription factors of the GLI family. Given that activation of SMO, like inactivation of PTCH1, up-regulates transcription of hedgehog target genes, it is not surprising that activating mutations in the *SMO* gene have been identified in BCCs and in medulloblastomas. However, the failure to detect *SMO* mutation in a total of 50 KCOTs (including 16 NBCCS-associated cases), as demonstrated here in this study, suggests that it is an extremely rare event in this tumor. The role of other components of SHH signaling remains to be elucidated.

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