Identification of Known and Novel *PTCH* Mutations in Both Syndromic and Non-syndromic Keratocystic Odontogenic Tumors

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Abstract

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Aim To clarify the role of *PTCH* in patients with NBCCS-related and non-sydromic keratocystic odontogenic tumors.
Methodology Mutation analysis was undertaken in 8 sporadic and 4 NBCCS-associated KCOTs.
Results Four novel and two known mutations were identi-fied in 2 sporadic and 3 syndromic cases, two of which being germline mutations (c.2179delT, c.2824delC) and 4 somatic mutations (c.3162dupG, c.1362-1374dup, c.1012 C>T, c.403C>T).

Conclusion Our findings suggest that defects of *PTCH* are associated with the pathogenesis of syndromic as well as a subset of non-syndromic KCOTs.

Keywords keratocystic odontogenic tumor, mutation, nevoid basal cell carcinoma syndrome, *PTCH*

Introduction

Nevoid basal cell carcinoma syndrome (NBC-CS, also known as Gorlin syndrome, OMIM NO. 109400) is a rare autosomal dominant disorder characterized by multiple basal cell carcinomas, keratocystic odontogenic tumors (KCOTs), medulloblastoma, ovarian fibroma, and developmental defects such as palmar/plantar pits, calcified falx cerebri, bridged sella, bifid ribs, scoliosis, and frontal bossing, etc (Gorlin, 2004). *PTCH* gene (OMIM NO.601309), the human homologue of the *Drosophila* segment polarity gene, has been iden-tified as the gene responsible for NBCCS. *PTCH* gene consists of 23 exons spanning approximately 34 kbp. It encodes a 1447 amino acid protein containing 12 transmembrane-spanning domains and 2 large extracellular loops as well as a large intracellular loop (Calzada-Wack *et al*., 2002). It is involved in the Sonic hedgehog (SHH) signaling pathway which has been implicated in the for-mation of embryonic structures and tumorigenesis. Mutation of *PTCH* resulting in aberrant activity of SHH pathway may lead to NBCCS as well as some related sporadic tumors, such as basal cell carcinoma, KCOTs, medulloblastoma, and tricho-epithelioma *et al* (Bale *et al*., 2001; Pietsch *et al*., 1997; Wolter *et al*., 1997).

KCOTs are common aggressive cystic jaw tu-mors with a high growth potential and a propensity for recurrence. The majority of KCOTs arise spo-radically in single form while multiple lesions often occur when associated with NBCCS. It has been reported that nearly 75% NBCCS-associated KCOTs harbored *PTCH* mutations, while only a few reports documented *PTCH* mutations in Chi-nese patients with sporadic KCOTs (Gu *et al*., 2006; Sun *et al*., 2008). The aim of this study was to search for *PTCH* mutations in both non-syndromic and syndrome-associated KCOTs. Mu-tations in 2 out of 8 sporadic KCOTs and 3 out of 4 NBCCS-associated KCOTs suggested that defects of *PTCH* may play a crucial role in the genesis of both sporadic and NBCCS-associated KCOTs.

Methods and materials

Patients and samples

Fragments from KCOT samples were obtained from Peking University Hospital and School of Stomatology. Eight lesions were histological classified as sporadic KCOT (KC1–KC8), and 4 were from patients with NBCCS (NB1–NB4) diagnosed according to the established criteria (Kimonis *et al*., 1997). Fresh tissue specimens as well as their corresponding peripheral blood samples were collected for subsequent analysis. The study protocol was approved by the Ethical Committee of Peking University Health Science Center. Informed consents were obtained from all patients.

DNA extraction and polymerase chain reaction

Genomic DNA from tumors and peripheral blood was extracted with a QIAamp DNA Mini Kit (Qiagen, Germany). All 23 exons and the exon-intron boundaries of the *PTCH* gene were amplified by polymerase chain reaction (PCR) with specific primers described previously (Gu *et al*., 2006; Sun *et al*., 2008). PCR was performed in a volume of 50 μL containing 100 ng of DNA, 10 pmol/L of each primer, 2.5 mmol/L of dNTPs, 1 unit of Taq DNA polymerase in PCR buffer. Amplification was performed for 35 cycles at 95℃ for 30 seconds, 61℃ for 30 seconds and 72℃ for 45 seconds to 3 minutes.

Denaturing highperformance liquid chromatogramphy analysis and sequencing

PCR products were separated through 2% agarose gels and purified using the Takara gel extraction kit. PCR products were analyzed by denaturing highperformance liquid chromatography (DHPLC) to screen for *PTCH* mutations. Samples showing an abnormal elution profile were subjected to direct sequencing. When insertion or deletion of multiple nucleotides were detected by direct sequencing, the purified products were ligated into pGEM-T vector (Promega, USA), and the resulting clones were sequenced on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, USA).

Total RNA extraction and reverse transcriptase polymerase chain reaction

Where two different heterozygous mutations were detected in one case, reverse transcriptase polymerase chain reaction (RT-PCR) was used to determine whether two variants were located in one or two alleles. Total RNA was extraction from tumor of NB3 patient by means of Trizol^TM reagent (Invitrogen, USA). cDNA were synthesized with Superscript^TM Ⅲ First-Strand synthesis system (Invitrogen, USA). A 2 μL quantity cDNA was used to amplify segment (E3-17) designed to cover the region carrying the two mutational changes by forward primer 5'-TGGGATTAAAAGCAGCGAA C-3' and reverse primer 5'-AGCCCCAGGCTCGT ATAGTT-3'. PCR and cloning sequencing conditions were standardized as described above. RT-PCR product was visualized and purified on a 2% agarose gels.

Results

Six *PTCH* mutations in 3 NBCCS-associated and 2 sporadic KCOTs were identified, four of which were novel. The 6 mutations consisted of 2 nonsense, 3 frameshift and 1 inframe duplication mutations. A summary of the results obtained is shown in Table 1.

Mutations in sporadic KCOTs

We identified 2 somatic mutations in 8 sporadic KCOTs. One known frameshift mutation (c.3162 dupG) in exon 18 was detected in KC1 (Figure 1), which was predicted to introduce a stop codon at amino acid residue 1144, located at the sixth extracellular loop of PTCH protein. One novel inframeshift mutation was identified (c.1362-1374 dup) in exon 10 of patient KC2. Cloning sequencing revealed a duplication of 12 bp nucleotides. It led to a four amino acid duplication in sterol-sensing domain of PTCH protein and was highly conserved across species through alignment of the amino acid sequences of protein orthologs of human (Hs), chicken (Gg), mouse (Mm), Xenopus (Xl), and zebra fish (Dr) (GenBank Accession No: U59464.1, U40074, U46155, AF302765, and AJ007742).

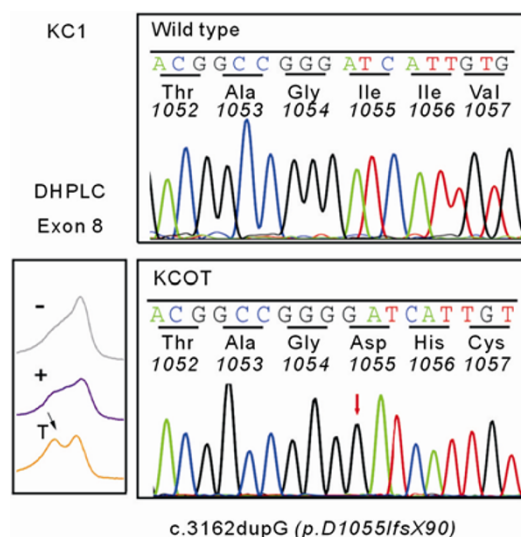**Table 1**   Summary of *PTCH* mutations in 8 sporadic and 4 NBCCS-associated KCOTs

Patient	Age/sex	Exon	Mutation[a]	Effect on coding[a]	Characteration	Patient's phenotype and history
KC1	46/M	18	c.3162dupG	p.D1055IfsX90	Somatic, known	Multiple KCOTs, recurrent history
KC2	16/M	10	c.1362-1374dup	p.L455-L458dup	Somatic, novel	Single KCOT
NB1	53/M	7	c.1012C>T	p.Q338X	Somatic, novel	Single KCOT, basal cell carcinomas of skin, calcification of falx cerebri, bifid rib, recurrent history
NB2	15/F	14	c.2179delT	p.C727VfsX19	Germline, novel	Multiple KCOTs, bifid rib, multiple skin naevi, recurrent history
NB3	26/F	3	c.403C>T	p.R135X	Somatic, known	Multiple KCOTs, calcification of falx cerebri, bridged sella, frontal bossing, palmar/planter pits, multiple skin naevi, recurrent history
		17	c.2824delC	p.R942GfsX20	Germline, novel	

[a] Nucleotide and amino-acid residue numbering is based on Genbank entry U59464.

Mutations in NBCCS-associated KCOTs

A total of 4 mutations (3 novel and 1 known) were detected in 3 patients with NBCCS. DNA sequencing from KCOT tissues and peripheral blood revealed two were germline mutations and two were somatic mutations. Patient NB1, a 53-year-old male patient with typical NBCCS phenotypes, carried a nonsense mutation in exon 7 (c.1012C>T). The mutation introduces a stop codon and leads to a premature termination at codon 338. This mutation was somatic due to the absence from peripheral blood. Cloning sequencing of samples from NB2 revealed a threonine deletion at nucleotide 2179 in exon 14. This frameshift mutation introduces a stop codon at amino acid residue 745, located in the third intracellular loop. Two mutations were identified in patient NB3, one is a germline frameshift mutation in exon 17 (c.2824 delC) and the other is a somatic nonsense mutation in exon 3 (c.403C>T), resulting in premature stops at codon 961 and 135 respectively (Figure 2A). RT-PCR and cloning sequencing revealed these two mutations occurred in the same allele of the *PTCH* gene (Figure 2B).

Discussion

PTCH, which has been described as a tumor suppressor gene, is predicted to participate in tumorigenesis in NBCCS and related sporadic neoplasms. So far, several studies have provided evidence that *PTCH* might take part in the pathogenesis of NBCCS-associated and sporadic KCOTs (Barreto *et al*., 2000; Gu *et al*., 2006; Sun *et al*., 2008). In this study, we identified 6 additional *PTCH* mutations in 2 out of 8 sporadic and 3 out of 4 NBCCS-associated KCOTs, consisted of 2 nonsense, 3 frameshift and 1 inframe duplication mutations. The 2 somatic nonsense mutations, happened in exon 3 and 7 (c.403C>T and c.1012C>T), both resulted in PTCH protein premature stop at the first large extracellular loop, which has been summarized as one of the "hot" spots associated with hedgehog ligand binding

Figure 1   *PTCH* mutations in KC1

A frameshift mutation (c.3162dupG, arrow) was detected in patient KC1, which introducing a stop codon at amino acid residue 1144.

(Lindstrom *et al*. 2006). Of the 3 frameshift mutations, 2 novel germline mutations in patient NB2 and NB3 (c.2179delT and c.2824delC) were predicted to lead to premature termination of PTCH at the large intracellular loop and the second large extracellular loop respectively. Patient KC1 had multiple KCOTs but did not have other feathers of NBCCS. A somatic frameshift mutation (c.3162 dupG) in KC1 was predicted to introduce a stop codon at amino acid residue 1144, located at the sixth extracellular loop of PTCH protein. The inframe duplication mutation (c.1362-1374dup) in a 16-year-old patient (KC2) with a single KCOT caused a four amino acid duplication at the junction of transmembrane 2 and intracellular loop 1 of the PTCH protein. Transmembrane domains 2–6 were described as sterol-sensing domain and highly conserved across species. It is also a "hot" region analyzed by *PTCH* mutation database (Lindstrom *et al*., 2006). Of the six mutations identified, five

(c.403C>T, c.1012C>T, c.2179delT, c.2824delC, c.3162dupG) resulted in truncation of the PTCH protein. These results were in consistent with the previous reports that most *PTCH* mutations lead to a premature termination of the protein (Li *et al*., 2008; Wicking *et al*., 1997) .

It is interesting that a NBCCS patient (NB1) only harbored a somatic mutation (c.1012C>T) in the KCOT sample while the mutation was absent from corresponding peripheral blood. Instead of postulating that there would be another germline mutation which failed to be detected due to methodology, we could speculate that this somatic mutation (p.Q338X) might occur early in embryonic development and represent germline mosaicism in consideration of his typical clinical manifestations, such as basal cell carcinomas, calcification of falx cerebri, bifid rib, and recurrent history.

The molecular analysis of NBCCS associated tumors showed that a "two-hit" hypothesis is appli-
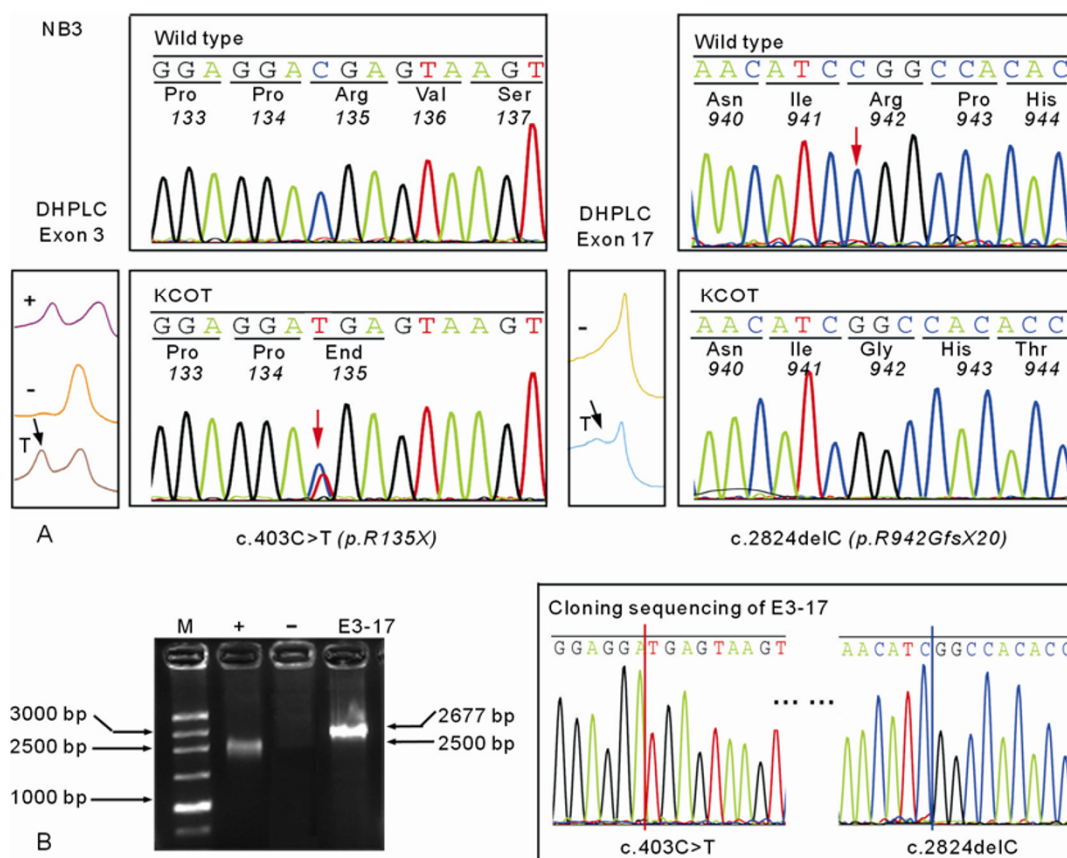
Figure 2 *PTCH* mutations in NB3

(A): Two coincident mutations were identified in NB3. One is a somatic nonsense mutation in exon 3 (c.403C>T, arrow) which caused a stop at codon 135, the other is a germline frameshift mutation (c.2824delC) in exon 17. (B): RT-PCR amplified fragment (2677bp) spanning the region from exon 3 to exon 17 (left) and cloning sequencing of E3-17 indicated these two variants occurring in the same allele of *PTCH* (right).

cable to their pathogenesis (Levanat *et al.*, 1996; Shear, 2002). These syndrome associated tumors presumably arise from precursor cells that contain a hereditary "first hit". Additional somatic mutation, loss of heterozygosity, or epigenetic silence of the other allele, acting as the "second hit", would introduce functional inactivity of PTCH protein. In addition to this, haploinsufficiency of *PTCH* is documented in several kinds of tumors, revealing that inactivation only one allele of *PTCH* gene might result in tumor genesis (Soufir *et al.*, 2006). In the present study, we demonstrated a patient with syndrome (NB3) carried two different mutations (c.403C>T, somatic and c.2824delC, germline). Further analysis by RT-PCR revealed that these two variants located in the same allele of *PTCH*. This result indicates the possibility that inactivity of *PTCH via* haploinsufficiency and two-hit mechanism might both be involved in the pathogenesis of KCOTs.

Conclusion

In conclusion, *PTCH* mutations could be identified in both sporadic and NBCCS-associated KCOTs. The result provides evidence for its crucial role in tumorigenesis of KCOTs even when they are not syndrome-related. Pathogenesis of KCOTs which did not carry *PTCH* mutations remains to be elucidated.

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