**Oral Diseases (2008) 14**, 174–179. doi:10.1111/j.1601-0825.2007.01369.x

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## ORIGINAL ARTICLE

# *PTCH* germline mutations in Chinese nevoid basal cell carcinoma syndrome patients

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**OBJECTIVES: *PTCH*, the human homologue of the *Drosophila* segment polarity gene, patched, has been identified as the gene responsible for nevoid basal cell carcinoma syndrome. The aim of this study was to investigate *PTCH* gene mutation in Chinese patients with nevoid basal cell carcinoma syndrome.**

**MATERIALS AND METHODS: DNA was isolated from both odontogenic keratocyst tissue and peripheral blood of five patients with syndrome and one patient with only multiple odontogenic keratocysts, and mutational analysis of the *PTCH* gene performed by direct sequencing after amplification of all 23 exons by polymerase chain reaction (PCR).**

**RESULTS: A previously reported germline mutation (c.2619C>A) was identified in two familial cases involving the mother and the daughter, with the mother also carrying a novel somatic mutation (c.361_362insGAGC). Three novel germline *PTCH* mutations (c.1338_1339insGCG, c.331delG and c.1939A>T) were detected in three unrelated patients with syndrome. The patient with multiple odontogenic keratocysts who failed to fulfill the diagnostic criteria of the syndrome also carried a novel germline mutation (c.317T>G).**

**CONCLUSION: The frequent germline *PTCH* mutations detected in our series provide further evidence for the crucial role of *PTCH* in the pathogenesis of nevoid basal cell carcinoma syndrome in Chinese.**

*Oral Diseases* (2008) **14**, 174–179

**Keywords:** PTCH; mutation; nevoid basal cell carcinoma syndrome; odontogenic keratocyst

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Received 19 July 2006; accepted 29 December 2006

## Introduction

Nevoid basal cell carcinoma syndrome (NBCCS; Gorlin syndrome; MIM no. 109400) is a rare autosomal dominant disorder characterized primarily by multiple basal cell carcinomas (BCCs), odontogenic keratocysts (OKCs) of the jaws, and developmental defects, such as bifid ribs, intracranial calcification, and polydactyly (Gorlin, 1995). NBCCS also predisposes to a variety of low-frequency tumors such as ovarian fibroma, medulloblastoma, rhabdomyosarcomas, and cardiac fibromas (Gorlin, 1995; Kimonis *et al*, 1997). The gene responsible for this disorder is *PTCH*, the human homolog of the *Drosophila* segment polarity gene, patched (MIM no. 601309; Hahn *et al*, 1996; Johnson *et al*, 1996). *PTCH* has been mapped to 9q22.3-q31 and consists of 23 exons spanning approximately 50 kb and encoding a 1447-amino acid transmembrane glycoprotein (Hahn *et al*, 1996; Johnson *et al*, 1996; Stone *et al*, 1996). *PTCH* is involved in Sonic hedgehog (*Shh*) signaling, where it is thought to act as a receptor for *Shh* ligands (Stone *et al*, 1996). An important clue to the understanding of *PTCH* function comes from the study of its interactions with another membrane protein, smoothened (*Smo*). In the absence of *Shh* signal, *Ptch* represses the constitutive signaling activity of *Smo*, by forming a *Ptch–Smo* complex (Stone *et al*, 1996). Mutational inactivation of *Ptch* results in the failure of *Ptch* to inhibit *Smo*, leading to the constitutive activity of the *Shh* signaling pathway (Bale and Yu, 2001). *Shh* signaling pathway has been implicated in the formation of embryonic structures and tumorigenesis (Hardcastle *et al*, 1998). Therefore, a disorder of this pathway could result in an abnormal body conformation and tumorigenesis as seen in NBCCS patients.

To date, over 100 *PTCH* germline mutations associated with NBCCS have been reported, most (73%) identifying nonsense or frameshift mutations leading to the synthesis of a truncated *Ptch* protein (Lindstrom *et al*, 2006). These mutations appear to be mainly clustered into the large extracellular loops and the large intracellular loop of the Ptch protein, but no apparent genotype–phenotype correlations have been established

(Wicking *et al*, 1997a; Lindstrom *et al*, 2006). Most of the accumulated data, so far, have been generated mainly from Caucasian and African–American populations. Among Asian populations, studies have mainly been performed in Japanese individuals (Minami *et al*, 2001; Fujii *et al*, 2003; Tanioka *et al*, 2005) with only few reports documenting *PTCH* mutations in Chinese NBCCS patients (Lam *et al*, 2002; Chung *et al*, 2003). Herein we present data on five novel and one known *PTCH* mutations while screening five Chinese NBCCS patients and one patient with multiple OKCs who does not have clinical features of NBCCS.

## Materials and methods

*Patients and samples*

Three unrelated and two familial patients (involving the mother and the daughter) were diagnosed as having NBCCS according to the established criteria (Kimonis *et al*, 1997), which included the presence of at least two major features of the syndrome such as multiple OKCs of the jaws (five cases), multiple BCCs (one case), calcification of falx (two cases), bifid rib (two cases) and having a first-degree relative with NBCCS (four cases, Table 1). Of the five NBCCS patients, one familial (P2) and one unrelated (P3) case have been reported previously by our group (Gu *et al*, 2006). One patient with multiple OKCs who does not have other features of NBCCS was also included in this study. The removed OKC tissue specimens as well as their corresponding peripheral blood samples were collected from all patients and stored at −80°C for subsequent polymerase chain reaction (PCR) and sequencing analysis. One hundred unrelated control DNA samples were obtained from 100 normal volunteers accessioned from the Blood Transfusion Center, Peking University First Hospital. Informed consent was obtained from all study participants and the study protocol was approved by the Ethical Committee of Peking University Health Science Center.

*DNA extraction and PCR*

Genomic DNA from frozen samples (25 mg) of cyst tissue was extracted with a DNeasy Tissue Kit (Qiagen, Valencia, CA, USA). DNA from peripheral blood was isolated with a Whole Blood Genomic DNA Mini Kit (V-gene Biotechnology Limited, Hangzhou, China). Each of the 23 exons of the *PTCH* gene was amplified separately using specific primers as previously described (Chidambaram *et al*, 1996; Hahn *et al*, 1996; Xie *et al*, 1997), except for exon 14 and exon 23. Exon 14 was amplified in two pieces, 5′-AAAATGGCAGAATGAAAGCACC-3′, 5′-CTGAGGGTGTCCTGTGTCAC-3′ and 5′-CACACGCACGTGTACTACAC-3′, 5′-CTGATGAACTCCAAAGGTTCTG-3′. Exon 23 was also amplified in two pieces, 5′-AACCCAAGGAGGGAAGTGTG-3′, 5′-AAGCCGTCACAGTGGTGATG-3′ and 5′-TCTACTGAAGGGCATTCTGGC-3′, 5′-GAACCTTGTCCTCCTCTTTGC-3′. PCRs were performed in a final volume of 50 $\mu$l containing approximately 100 ng of template DNA, 200 $\mu$M dNTPs, 10 pmol of each primer, 1.25 U of *Taq* polymerase [TaKaRa Biotechnology Co., Ltd, Dalian, China], 50 mM KCl, 10 mM Tris–HCl, and 1.5 mM $MgCl_2$. Amplification was performed for 35 cycles at 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s in a thermal cycler (PTC-100; MJ Research, Watertown, MA, USA).

*Direct sequencing*

Polymerase chain reaction products were gel-purified with a Gel Extraction kit (Omega Bio-Tek, Doraville, GA, USA) according to the manufacturer's protocol and directly sequenced using the same primers as for the original PCR amplification. When insertion or deletion of multiple nucleotides occurred and direct sequencing from the PCR products became difficult, further mutation detection was pursued in a subset of samples by cloning purified PCR product into the plasmid vector pGEM-T (Promega, Madison, WI, USA). After transformation into competent *Escherichia coli* strain TOP10, colonies carrying recombinant plasmid were selected, and the plasmid DNA isolated using a Plasmid Miniprep Kit (Sigma, St Louis, MO, USA). Plasmid DNA was sequenced using M13 universal forward and reverse primers. Sequencing analysis was performed on an 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 two independent PCRs.

*Restriction enzyme analysis*

To characterize any unreported *PTCH* gene alterations (missense mutations in particular) identified in the present study as being novel mutations rather than polymorphisms, 100 unrelated control DNAs were tested together with each of the identified mutant samples by restriction-enzyme analysis using 8% polyacrylamide gel electrophoresis.

## Results

*PTCH* gene mutations were identified in all the six patients examined (Table 1). Direct sequencing of DNA from OKC tissues obtained from the patients and from peripheral blood revealed five germline mutations and one somatic mutation. Of the six mutations identified, five (four germline and one somatic) were novel and one germline mutation has been reported in a French NBCCS family (Boutet *et al*, 2003). In addition, five types of known *PTCH* polymorphisms were detected in five of the six patients (Table 1).

An identical germline mutation was identified in the two familial patients [P1 (mother) and P2 (daughter)]. A C > A substitution was detected at nucleotide 2619 (Figure 1a). This causes a tyrosine to stop codon substitution at amino acid residue 873. The mother carried an additional mutation (361_362insGAGC), which was only present in the OKC tissue but not in the peripheral blood. This somatic mutation was a 4-base insertion, resulting in a frameshift and a premature stop at codon 140 (Figure 1b). The later alteration was absent from any of the daughter's DNA samples.

**Table 1** Summary of *PTCH* mutations and polymorphisms in NBCCS patients

*Mutations*

| *Case no.* | *Age/sex* | *Exon* | *Mutation*[a] | *Effect on coding*[a] | *Patient's phenotype and family history* |
|---|---|---|---|---|---|
| P1 | 47/F | 16 | c.2619C > A | p.Y873X | Multiple OKCs, multiple BCCs, calcification of falx, ovarian fibromas, first member in the family affected, her daughter (P2) also affected |
| | | 2 | c.361_362insGAGC | p.L121fsX140 | |
| P2[b] | 21/F | 16 | c.2619C > A | p.Y873X | Multiple OKCs, bifid rib, cleft of lip and palate, multiple facial naevi, her mother (P1) affected with NBCCS |
| P3[b] | 37/M | 9 | c.1338_1339insGCG | p.Y446_L447insA | Multiple OKCs, calcification of falx, multiple skin naevi, positive family history (father, brother and sister affected with NBCCS) |
| P4 | 15/M | 2 | c.331delG | p.A111fsX116 | Multiple OKCs, bifid rib, first member in the family affected |
| P5 | 60/F | 14 | c.1939A > T | p.S647C | Multiple OKCs, multiple epidermal cysts, first member in the family affected, her two sons also affected with NBCCS |
| P6 | 13/M | 2 | c.317T > G | p.L106R | Multiple OKCs, no other symptoms of NBCCS, no family history |

*Polymorphisms*[c]

| Case no. | Exon/intron | Nucleotide change | Effect on coding |
|---|---|---|---|
| P1 | 23 | c.3944T > C | p.L1315P |
| P2 | Intron10 | c.IVS10-51G > C c.IVS10-8T > C | – |
| P3 | Intron10 | c.IVS10-51G > C | – |
| P5 | 23 | c.3944T > C | p.L1315P |
| | 12 | c.1665T > C | p.N555N |
| | Intron10 | c.IVS10-8T > C | – |
| | Intron15 | c.IVS15 + 9G > C | – |
| P6 | 23 | c.3944T > C | p.L1315P |
| | 12 | c.1665T > C | p.N555N |

[a]Nucleotide and amino acid residue numbering is based on GenBank entry U59464. Gene mutation nomenclature recommended by den Dunnen and Antonarakis (2000) is applied.
[b]P2 and P3 are previously reported by Gu *et al* (2006).
[c]The PTCH polymorphisms listed here have been previously reported by Xie *et al* (1997), Boutet *et al* (2003) and Fujii *et al* (2003).

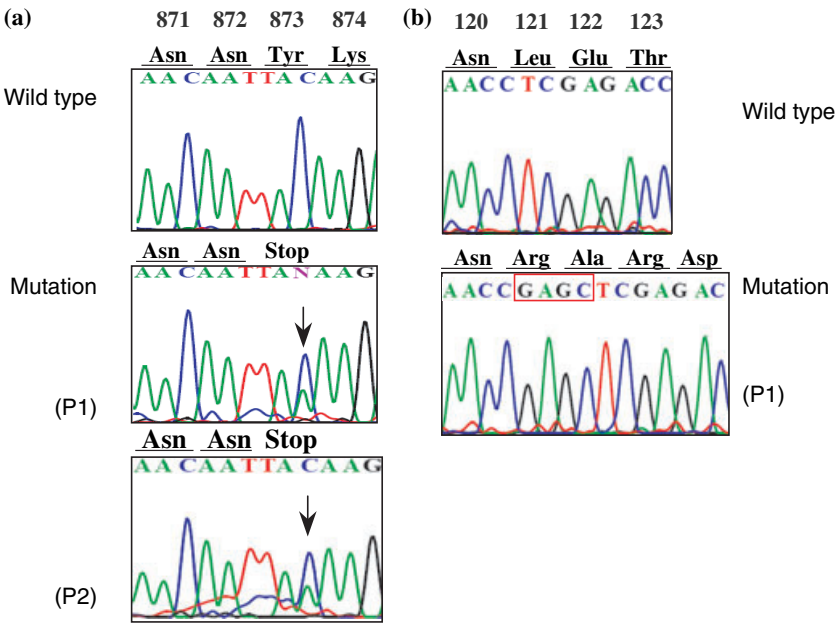


**Figure 1** (**a**) A common *PTCH* germline mutation is present in both the mother (P1) and the daughter (P2) from a NBCCS family. Both patients showed a germline C to A substitution (arrows) introducing a stop condon at amio acid residue 873 in exon 16. (**b**) Cloned DNA sequencing of exon 2 from the cyst of the mother (P1) disclosed a 4-base insertion (red box) resulting in a frameshift and a premature stop codon at 140 amino acids downstream. This insertion was absent in the mother's peripheral blood and in both the peripheral blood and cyst of the daughter

The three unrelated NBCCS patients each carried a unique germline mutation. A triplet nucleotide insertion at position 1338 in exon 9 was detected in both the jaw cyst and peripheral blood from a 37-year-old female NBCCS patient (P3). This mutation introduces an alanine between codon 446_447. Direct DNA sequencing of samples from a 15-year-old male NBCCS patient (P4) revealed a G (guanosine) base deletion at residue 331 in exon 2. This frameshift mutation introduces a stop codon at amino acid residue 116. An A > T substitution at nucleotide 1939 in exon 14 was detected in DNA samples from a 60-year-old female NBCCS patient (P5).

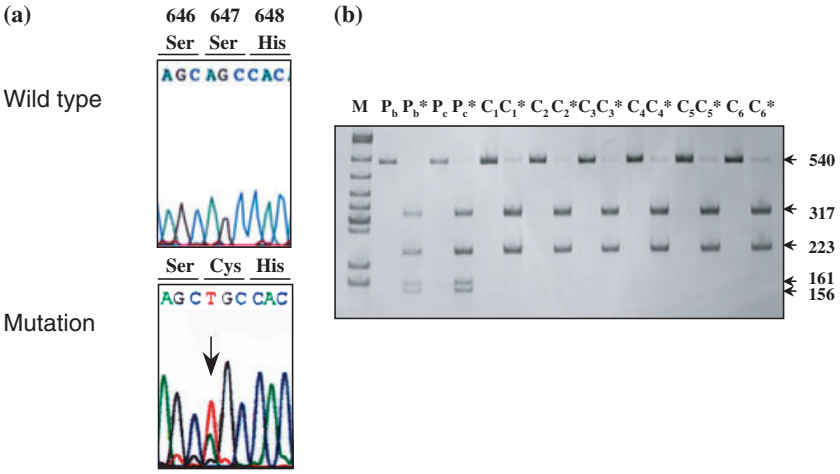


**Figure 2** (**a**) Sequencing of exon 14 of a patient with syndrome (P5) reveals a germline missense mutation (A > T, arrow) in codon 647 causing a change from serine to cysteine. (**b**) *Pvu*II digestion of exon 14 PCR products from the peripheral blood ($P_b$) and cyst ($P_c$) of this patient (P5) and the unrelated control DNAs ($C_1$–$C_6$). The missense mutation shown in (**a**) creats an extra restriction site for *Pvu*II with two additional fragments of 161 and 156 bp (arrows). In the unrelated controls, only one restriction site with fragments of 317 and 223 bp is seen (arrows). The undigested fragment is 540 bp in size. M, molecular-weight ladder; *enzyme digest

This missense mutation causes a serine to cysteine substitution at codon 647 (Figure 2a).

A T > G substitution at nucleotide 317 in exon 2 was also detected in a patient (P6) with multiple OKCs who did not have clinical features of NBCCS. This missense mutation causes a leucine to arginine substitution at codon 106. To further characterize the two novel missense mutations (c.317T > G/p.L106R and c.1939A > T/p.S647C) identified in this study, we tested 100 unrelated control DNA samples by restriction enzyme analysis using *Fnu*4HI and *Pvu*II respectively. As predicted, the abnormal restriction sites present in the PCR products from samples of the patients were absent in the PCR product from the control DNAs (Figure 2b).

## Discussion

In this study, we identified *PTCH* mutations in all the 5 Chinese patients with NBCCS. In a recent review by Evans and Farndon (GeneReview at http://www.genetests.org, 2004), mutations in the *PTCH* gene have been found in 60–85% of NBCCS patients. Our detection rate of *PTCH* mutations seems higher than previously reported. This may be due to the difference in methodology. In most of the previous reports, patients were initially screened by single-strand conformation polymorphism (SSCP), and only samples showing SSCP variants were sequenced. However, several factors such as gel composition, temperature, running time, and size of the DNA fragments could influence the sensitivity (Jordanova *et al*, 1997; Salazar *et al*, 2002). We therefore performed direct sequencing without prior screening by SSCP. It is also possible that the difference in ethnicity influences the frequency of *PTCH* mutation in NBCCS patients, as most of the accumulated data on *PTCH* mutations have been reported mainly from Caucasian and African–American patients. Interestingly, the two reports in literature describing four Chinese NBCCS families (Lam *et al*, 2002; Chung *et al*, 2003) have identified four different germline *PTCH* mutations in each individual family (Table 2). These data, together with ours, appear to raise the possibility of a high frequency of *PTCH* mutations in Chinese NBCCS patients.

Of the six mutations identified, three (c.331delG, c.361_362insGAGC and c.2619C > A) resulted in truncation of the *Ptch* protein. This is in keeping with the previous reports that most *PTCH* mutations lead to a premature termination of the protein (Wicking *et al*, 1997a; Lindstrom *et al*, 2006). Although no apparent hot-spot mutations have been reported (Chidambaram *et al*, 1996; Hahn *et al*, 1996; Johnson *et al*, 1996; Xie *et al*, 1997; Boutet *et al*, 2003), a recent review of all published *PTCH* mutations has demonstrated that the *PTCH* gene harbors mutational hot spot residues and regions, such as the large extracellular and intracellular loops of the Ptch protein (Lindstrom *et al*, 2006). In this study, one nonsense mutation (c.2619C > A/p.Y873X) was identified in a Chinese NBCCS family involving both the mother (P1) and the daughter (P2). The identical germline mutation has also been previously reported in a French NBCCS patient (with clinical manifestations of multiple OKCs and BCCs; Boutet *et al*, 2003). We believe that this is one of the rare recurrent mutations identified in the *PTCH* gene. This

**Table 2** Germline *PTCH* mutations detected in Chinese NBCCS patients in literature

| *Exon/intron* | *Mutation*[a] | *Effect on coding*[a] | *References* |
|---|---|---|---|
| 10 | c.1468_1469insA | p.L490fsX496 | Lam *et al* (2002) |
| 15 | c.2392_2393insC | p.F798fsX828 | Lam *et al* (2002) |
| Inron 5 | c.IVS5 + 1del8 | aberrant splicing | Lam *et al* (2002) |
| Inron 18 | c.IVS18-2A > G | aberrant splicing | Chung *et al* (2003) |

[a]Nucleotide and amino acid residue numbering is based on GenBank entry U59464. Gene mutation nomenclature recommended by den Dunnen and Antonarakis (2000) is applied.

mutation is predicted to result in *Ptch* protein truncation in the second extracellular loop. The second extracellular loop of *Ptch* is known to be an important domain that interacts with *Shh* (Gailani *et al*, 1996). Thus, *Ptch* protein truncation in this region may inactivate its ability to bind the *Shh* ligand. So far, however, no founder effect of *PTCH* gene mutations has been described in the literature (Wicking *et al*, 1997a; Boutet *et al*, 2003). The identification of this mutation in patients displaying different clinical features (P1, P2 and the reported French patient) does pinpoint the possible involvement of modifier genes and/or environmental factors. Interestingly, an additional mutation (c.361_362insGAGC) was also detected in the OKC tissues of the mother (P1) from this family, but the mutation was not identified in her peripheral blood sample. This somatic mutation causes *Ptch* protein truncation in the first extracellular loop, which again may influence its ability of *Shh* ligand binding. It has been proposed that *PTCH* may function as a tumor suppressor gene and its inactivation may undergo a two-hit mechanism (Levanat *et al*, 1996). Thus the two mutations detected in this syndrome patient (P1) could represent a hereditary 'first hit' and a somatic 'second hit', although the possibility of somatic mosaicism could not be ruled out.

The significance of the two missense mutations (c.317T > G/p.L106R and c.1939A > T/p.S647C) and the one inframe insertion mutation (c.1338_1339 ins GCG; Gu *et al*, 2006) will not become completely clear until a functional analysis of these mutations are performed. However, these sequence variations have not been found in 100 unrelated normal Chinese individuals and therefore they are unlikely to be rare polymorphisms. It is interesting to note that one of the missense mutations (c.317T > G/p.L106R) was detected in a patient with multiple OKCs who failed to fulfill diagnostic criteria of NBCCS after careful clinical and radiological examinations. Multiple OKCs are the most consistent and common manifestation in NBCCS, occurring in 65–100% of patients (Gorlin, 1995; Kimonis *et al*, 1997). They often represent the first sign of NBCCS, thus facilitating early diagnosis (Lo Muzio *et al*, 1999). It has been suggested that patients with multiple OKCs alone might represent the syndrome in its least expressed form (Browne, 1971; Li *et al*, 1995). Therefore, clinical surveillance of this teenage patient (P6) is important, because the identified germline missense mutation could represent a *de novo* mutation responsible for the later development of other syndromic symptoms.

The demonstration that *PTCH* gene is mutated in individuals with NBCCS has provided the potential to determine, at the molecular level, if such mutations are inherited or have arisen *de novo*. In this study we have shown that both familial and *de novo* mutations could occur in Chinese NBCCS patients. When neither parent of a proband with an autosomal dominant condition has the clinical evidence of the disorder or disease-causing mutation, it is likely that the proband has a *de novo* mutation (Wicking *et al*, 1997b). In the present series, three probands were the first members in the family to be affected by the syndrome, therefore, the mutations identified in these individuals are probably *de novo* mutations. However, confirmation of the diagnostic status of relatives of these three patients was mainly based on clinical and radiological examinations and parental DNA was not available for analysis. Thus the precise estimate of the new mutation rate of the *PTCH* gene in NBCCS patients awaits further investigation. In conclusion, the frequent *PTCH* germline mutations in Chinese NBCCS patients as demonstrated here provide further evidence for its crucial role in the pathogenesis of this syndrome.

## Acknowledgements

This work was supported by the Research Grants from National Nature Science Foundation of China (30625044 and 30572048) and Municipal Nature Science Foundation of Beijing (7032031). The authors would like to thank the patients who contributed to this study.

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