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_1339ins-GCG, 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.

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

Introduction

Neviod 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 (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 (Hahn et al., 1996; Johnson et al., 1996; 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, Ptc represses the constitutive signaling activity of Smo, by forming a Ptc–Smo complex (Stone et al., 1996). Mutational inactivation of Ptc results in the failure of Smo to inhibit, 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 Ptc protein (Lindstrom et al., 2006). These mutations appear to be mainly clustered into the large extracellular loops and the large intracellular loop of the Ptc protein, but no apparent genotype–phenotype correlations have been established.
separately using specific primers as previously described. PTCH (V-gene Biotechnology Limited, Hangzhou, China). Isolated with a Whole Blood Genomic DNA Mini Kit (Qiagen, Valencia, CA, USA). DNA from peripheral blood was extracted with a DNeasy Tissue Kit (Qiagen, Hilden, Germany). DNA extraction and PCR amplification in two pieces, 5'-TGAAGCACC-3', 5'-CTGAGGGTGTCCTGTGTCAC-3', 5'-AAAATGGCAGAATGAA-3', 5'-AAAATGGCAGAATGAA-3', 5'-TCTACTGAAGGGACATTCTGGC-3', 5'-GAACCTTGTCCTCTCTTTTCG-3'. PCRs were performed in a final volume of 50 μl containing approximately 100 ng of template DNA, 200 μM 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 MgCl2. 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 Mini prep 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_362insGAG), 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.
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) PvuII digestion of exon 14 PCR products from the peripheral blood (Pb) and cyst (Pc) of this patient (P5) and the unrelated control DNAs (C1–C6). The missense mutation shown in (a) creates an extra restriction site for PvuII 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 Fnu4HI and PvuII 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 Ptc 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

<table>
<thead>
<tr>
<th>Exon/intron</th>
<th>Mutation(^a)</th>
<th>Effect on coding(^a)</th>
<th>References</th>
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<tbody>
<tr>
<td>Intron 5</td>
<td>c.IVS5+1del8</td>
<td>aberrant splicing</td>
<td>Lam et al (2002)</td>
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\(^a\)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 Ptc1 protein truncation in the second extracellular loop. The second extracellular loop of Ptc1 is known to be an important domain that interacts with Shh (Gailani et al., 1996). Thus, Ptc1 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 Ptc1 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 in-frame insertion mutation (c.1338_1339 ins GCC; 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 syndrome 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 germine mutations in Chinese NBCCS patients as demonstrated here provide further evidence for its crucial role in the pathogenesis of this syndrome.

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References


