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PTCH Mutations in Sporadic and Gorlin-syndrome-related Odontogenic Keratocysts

ABSTRACT

Odontogenic keratocysts are relatively common lesions that may occur in isolation or in association with nevoid basal cell carcinoma syndrome (or Gorlin syndrome). The *PTCH* gene has been reported to be associated with Gorlin syndrome. We investigated 10 cases of non-syndromic keratocysts and two other cases associated with Gorlin syndrome, looking for *PTCH* mutations. Four novel and 1 known *PTCH* mutations were identified in five individual patients. Of the 5 mutations identified, 2 were germ-line mutations (2619C>A; 1338_1339insGCG) in 2 cysts associated with Gorlin syndrome, and 3 were somatic mutations (3124_3129dupGTGTGC; 1361_1364delGTCT; 3913G>T) in 3 non-syndromic cysts. This report is the first to describe *PTCH* mutations in both non-syndromic and Gorlin-syndrome-related odontogenic keratocysts in Chinese patients, and suggests that defects of *PTCH* are associated with the pathogenesis of syndromic as well as a subset of non-syndromic keratocysts.

KEY WORDS: *PTCH*, mutation, odontogenic keratocyst, Gorlin syndrome.

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A supplemental appendix to this article is published electronically only at http://www.dentalresearch.org.

INTRODUCTION

The odontogenic keratocyst is an aggressive cystic lesion that has a putative significant growth potential and a propensity for recurrence (Browne, 1971; Li *et al.*, 1994). Although the great majority of keratocysts occur in isolation as single, non-syndromic cysts, they may also present as multiple cysts as a feature of the nevoid basal cell carcinoma syndrome (Gorlin snydrome, OMIM#109400). Gorlin syndrome is a rare autosomal-dominant disorder with variable clinical manifestations, such as basal cell carcinoma of the skin, keratocysts of the jaws, palmar or plantar pits, ectopic calcification of the falx cerebri, etc. (Gorlin, 1987; Kimonis *et al.*, 1997). Multiple jaw keratocysts are the most consistent and common manifestation of the syndrome, occurring in 65-100% of patients (Gorlin, 1987). The syndrome-associated keratocysts are found in both jaws with equal frequencies, in contrast to non-syndromic cysts, which are most frequently associated with the lower jaw (Lo Muzio *et al.*, 1999a). Keratocysts often represent the first manifestations of Gorlin syndrome, frequently preceding syndromic basal cell carcinomas, thus facilitating early diagnosis (Lo Muzio *et al.*, 1999b).

It is now believed that Gorlin syndrome is caused by germ-line mutations of the *PTCH* gene (Hahn *et al.*, 1996; Johnson *et al.*, 1996). In about 60-85% of individuals fulfilling the diagnostic criteria of the syndrome, it is possible to identify the underlying *PTCH* defect (Evans and Farndon, GeneReview at www.genetests.org, 2004).**(AQ)** The *PTCH* gene is the human homologue of the *Drosophila* segment polarity gene *patched* and has been localized to chromosome 9q22.3-q31 (GenBank accession numbers: U43148 and U59464; Hahn *et al.*, 1996; Johnson *et al.*, 1996). It encodes a 12-transmembranous-domain protein that physically binds at least 1 of the 3 known vertebrate Hedgehog molecules—Sonic hedgehog (*SHH*)—with high affinity, controlling cell fate and embryonic patterning in numerous tissues (Stone *et al.*, 1996; Hardcastle *et al.*, 1998). Apart from the high frequency of germ-line mutations of the *PTCH* gene detected in patients with the syndrome, somatic mutations of *PTCH* have also been identified in a range of sporadically occurring tumors, including those observed in the syndrome, *i.e.*, basal cell carcinomas (Gailani *et al.*, 1996; Hahn *et al.*, 1996; Johnson *et al.*, 1996), medulloblastoma (Xie *et al.*, 1997), and trichoepithelioma (Vorechovsky *et al.*, 1997). Similarly, several studies demonstrated the presence of *PTCH* mutations in syndromic and non-syndromic keratocysts (Lench *et al.*, 1997; Barreto *et al.*, 2000; Ohki *et al.*, 2004). However, the prevalence and range of *PTCH* mutations in odontogenic keratocysts remain to be established, in view of the limited number of cysts examined to date. The present study aimed to analyze *PTCH* mutations in a group of Chinese patients presenting with non-syndromic and Gorlin-syndrome-related keratocysts.

MATERIALS & METHODS

Subjects and Samples

Samples of 12 keratocysts from 12 unrelated patients were obtained from the Department of Oral Pathology, Peking University School of Stomatology. The fresh tissue specimens were divided into two parts. The first part was fixed in 10% formalin and routinely processed for histological diagnosis. Ten lesions were classified as non-syndromic keratocysts, and 2 were from patients with Gorlin syndrome diagnosed according to established criteria (Kimonis *et al.*, 1997; see APPENDIX). The second part of the tissue specimen was immediately frozen and stored at -80°C for subsequent PCR and sequencing analysis. Peripheral blood was also collected from all patients. 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 Polymerase Chain-reaction (PCR)

Genomic DNA from frozen samples (25 mg) of cyst tissue was extracted with a DNeasy Tissue Kit (Qiagen).**(AQ)** DNA from peripheral blood was isolated with a Whole Blood Genomic DNA Mini Kit (V-gene Biotechnology Limited, Hangzhou, P.R. China). Each of the 23 exons of the *PTCH* gene was amplified separately with 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 2 pieces, 5′-AAAATGGCAGAATGAAAGCACC-3′, 5′-CTGAGGGTGTCC TGTGTCAC-3′ and 5′-CACACGCACGTGTACTACAC-3′, 5′-CTGATGAACTCCAAAGGTTCTG-3′. Exon 23 was also amplified in 2 pieces, 5′-AACCCAAGGAGGGAAGTGTG-3′, 5′-AAGCCGTCACAGTGGTGATG-3′ and 5′-TCTACTGAAGGG CATTCTGGC-3′, 5′-GAACCTTGTCCTCCTCTTTGC-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 (Dalian) Co., Ltd, P.R. 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 sec, 57°C for 30 sec, and 72°C for 30 sec in a thermal cycler (PTC-100; MJ Research, Watertown, MA, USA).

Direct Sequencing

PCR products were gel-purified with a Gel Extraction kit (Omega Bio-Tek, Doraville, USA) **(AQ)**, according to the manufacturer's protocol, and directly sequenced with 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 the cloning of purified PCR product into the plasmid vector pGEM-T (Promega, Madison, WI, USA). After transformation into the competent *E. coli* strain TOP10, colonies carrying recombinant plasmid were selected, and the plasmid DNA was isolated with the use of a Plasmid Miniprep Kit (Sigma, St. Louis, MO, USA). Plasmid DNA was sequenced with M13 universal forward and reverse primers. Sequencing analysis was performed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).**(AQ)** Any mutation detected was confirmed by reverse sequencing and by analysis of samples from at least 2 independent PCRs.

Table. Summary of *PTCH* Mutations and Polymorphisms in Odontogenic Keratocysts

Patient No.	Age/Sex	Exon/Intron	Nucleotide Change[a]	Consequence[a]
		Mutation[b]		
Non-syndromic-cyst #2	20/F	18	3124_3129dupGTGTGC	V1042_C1043dupVC
Non-syndromic-cyst #7	21/M	10	1361_1364delGTCT	X454fs
Non-syndromic-cyst #9	48/F	23	3913G>T	D1305Y
Syndrome-cyst #1	21/F	16	2619C>A	Y873X
Syndrome-cyst #2	37/M	9	1338_1339insGCG	Y446_L447insA
		Polymorphism[c]		
Non-syndromic-cyst #1	21/F	intron 10	IVS10-51G>C, IVS10-8T>C	--
		22	3583A>T	T1195S
		23	3944T>C	L1315P
Non-syndromic-cyst #2	20/F	14	2199A>G	S733S
Non-syndromic-cyst #3	18/M	Intron 5	IVS5-55C>T	--
		23	3944T>C	L1315P
Non-syndromic-cyst #4	17/M	23	3944T>C	L1315P
Non-syndromic-cyst #6	50/M	14	2199A>G	S733S
		23	3944T>C	L1315P
Non-syndromic-cyst #7	21/M	intron 10	IVS10-51G>C, IVS10-8T>C	--
		14	2199A>G	S733S
Non-syndromic-cyst #8	25/F	intron 5	IVS5-55C>T	--
		12	1686C>T	A562A
		23	3944T>C	L1315P
Non-syndromic-cyst #9	48/F	12	1686C>T	A562A
		intron 15	IVS15+9G>C	--
		23	3944T>C	L1315P
Non-syndromic-cyst #10	30/M	12	1686C>T	A562A
		14	2199A>G	S733S
Syndrome-cyst #1	21/F	intron 10	IVS10-51G>C, IVS10-8T>C	--
Syndrome-cyst #2	37/M	intron 10	IVS10-51G>C	--

[a] Nucleotide and amino-acid residue numbering is based on GenBank entry U59464.
[b] Gene mutation nomenclature recommended by den Dunnen and Antonarakis (2000) is applied.
[c] All the polymorphisms listed here have been previously reported by Xie *et al.* (1997), Richards *et al.* (1997), Bodak *et al.* (1999), Boutet *et al.* (2003), and Fujii *et al.* (2003).

RESULTS

PTCH gene mutations were examined in 10 non-syndromic and 2 syndromic keratocysts by direct sequencing. Four novel and 1 known *PTCH* mutations were identified in 5 cysts, 3 of which were non-syndromic cases, and 2 of which were associated with Gorlin syndrome (Table). These mutations were distributed throughout the gene. In addition, 8

previously reported polymorphisms were also found in 11 of the 12 cases (Table).

Somatic Mutations in Non-syndromic Keratocysts

Somatic mutations were identified in 3 out of 10 non-syndromic keratocysts. These 3 novel mutations were evident only in DNA isolated from the cyst tissues, and were not detected in samples from the corresponding peripheral blood. A duplication of 6 nucleotides following position 3129 in exon 18 was detected in a non-syndromic case (Fig. 1A). This mutation causes a duplicated insertion of valine and cysteine residues between codons 1043 and 1044. The second mutation involved deletion of 4 nucleotides at position 1361_1364 in exon 10 (Fig. 1B). This frameshift mutation introduces a stop codon at amino acid residue 454. Direct sequencing of exon 23 from another non-syndromic cyst revealed a G>T transversion at nucleotide 3913 (Fig. 1C). This caused a change from aspartic acid to tyrosine at codon 1305.

Germ-line Mutations in Syndromic Patients

A known nonsense mutation was detected in a 21-year-old female syndromic patient. Direct sequencing of both the cyst and peripheral blood samples revealed a C>A substitution at nucleotide 2619 in exon 16. This caused a tyrosine to a stop codon substitution at amino acid residue 873 (Fig. 2A). This patient had a family history of Gorlin syndrome and presented with multiple keratocysts, cleft lips and palate, bifid ribs, and multiple facial naevi. In addition, multiple recurrences of keratocysts in the upper and lower jaws were recorded in this patient over a period of 7 yrs. A triplet nucleotide insertion at position 1338 in exon 9 (Fig. 2B) was detected in both the jaw cyst and peripheral blood from the other syndromic patient. This mutation introduced an alanine between codon 446_447. In addition, it also created a novel restriction enzyme cleavage site for *MluI* (5′-ACGCGT-3′), thus allowing its presence to be confirmed by restriction-enzyme analysis (Fig. 2C). This 37-year-old male patient also had a family history of the syndrome and presented manifestations of recurrent multiple keratocysts, calcification of the falx cerebri, and multiple skin naevi.

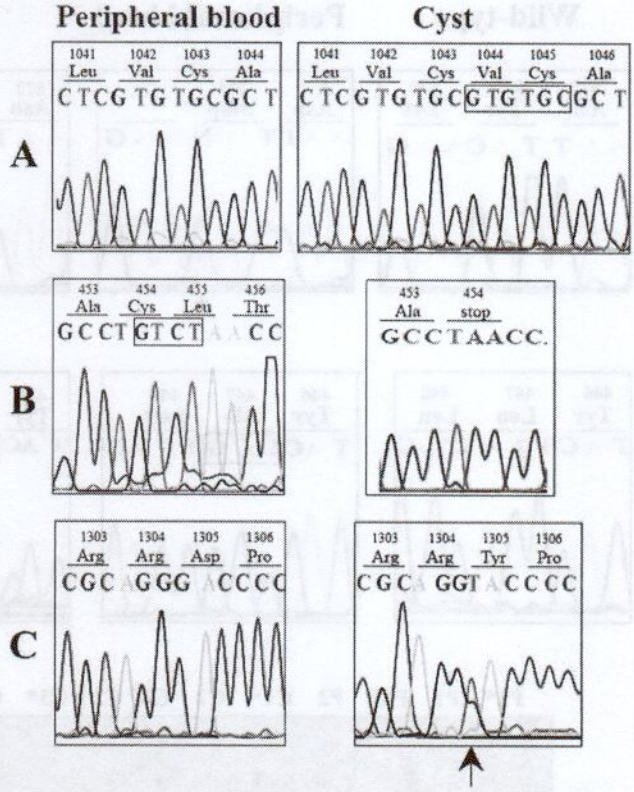

Figure 1. Three somatic mutations of *PTCH* identified in 3 non-syndromic keratocysts. Sequences of both peripheral blood (left) and cyst (right) are shown. **(A)** In non-syndromic cyst #2, a dupliction of 6 nucleotides (highlighted in small box) caused a duplicating insertion of valine and cysteine after codon 1043 in exon 18, but the patient's peripheral blood showed the wild-type sequence. **(B)** Sequencing of exon 10 of non-syndromic cyst #7 revealed a deletion of 4 nucleotides (highlighted in small box), causing a frameshift and introducing a stop condon at amino acid residue 454. This mutation was absent in the patient's peripheral blood. **(C)** Non-syndromic cyst #9 showed a missense mutation (G>T, arrow) in codon 1305, causing a change from aspartic acid to tyrosine, but the patient's peripheral blood showed the wild-type sequence.

DISCUSSION

The *PTCH* protein serves as a receptor for the secreted Sonic hedgehog (*SHH*) protein, and inhibits the signaling pathway by repressing the activity of Smoothened (*SMO*), another transmembranous protein (Stone *et al.*, 1996). According to this model, *PTCH* inhibition of *SMO* is lifted upon binding of *SHH* to *PTCH* or following mutational inactivation of *PTCH*. The *SHH* signaling pathway plays an important role in mammalian embryonic development of structures such as the neural tube, axial skeleton, limbs, lungs, skin, hair follicles, and teeth (Hardcastle *et al.*, 1998). *SHH* signaling also regulates growth and determines the shape of teeth (Dassule *et al.*, 2000). Addition of exogenous *SHH* protein directly to tooth germs during early development may result in abnormal epithelial invagination. Recent experiments showed that at the stage of initiation of odontogenesis in the murine mandibular arch, the *SHH* target genes such as *PTCH* and *GLI-1* are up-regulated in the diastema mesenchyme (Cobourne *et al.*, 2004). Odontogenic keratocysts almost certainly arise from derivatives of the dental lamina, an embryonic structure that normally differentiates into tooth buds and enamel-producing cells during odontogenesis (Browne, 1971; Gorlin, 1987). The remnants of dental lamina usually regress at later stages of development. The precursor cells of jaw cysts may fail to involute, due to a genetic alteration. Alternatively, dental lamina remnants may remain quiescent in childhood and adult life, unless a genetic event creates a hyperproliferative clone of cells. The epithelium lining keratocysts does show higher proliferative activity than the linings of other types of jaw cysts (Li *et al.*, 1994). The demonstration that *PTCH* is a tumor suppressor gene, probably responsible for Gorlin syndrome and its related sporadic neoplasms, prompted researchers to investigate the role of this gene in the pathogenesis of keratocysts. Loss of heterozygosity in the 9q22.3 region has been demonstrated with high frequency in both syndromic and non-syndromic keratocysts (Levanat *et al.*, 1996). There have been three reports in the literature involving *PTCH* mutational analysis of DNA derived from keratocyst tissues (Lench *et al.*, 1997; Barreto *et al.*, 2000; Ohki *et al.*, 2004). Lench *et al.* (1997) identified *PTCH* mutations in 5 out of 16 syndrome-related keratocysts. These 5 mutations were all germ-line

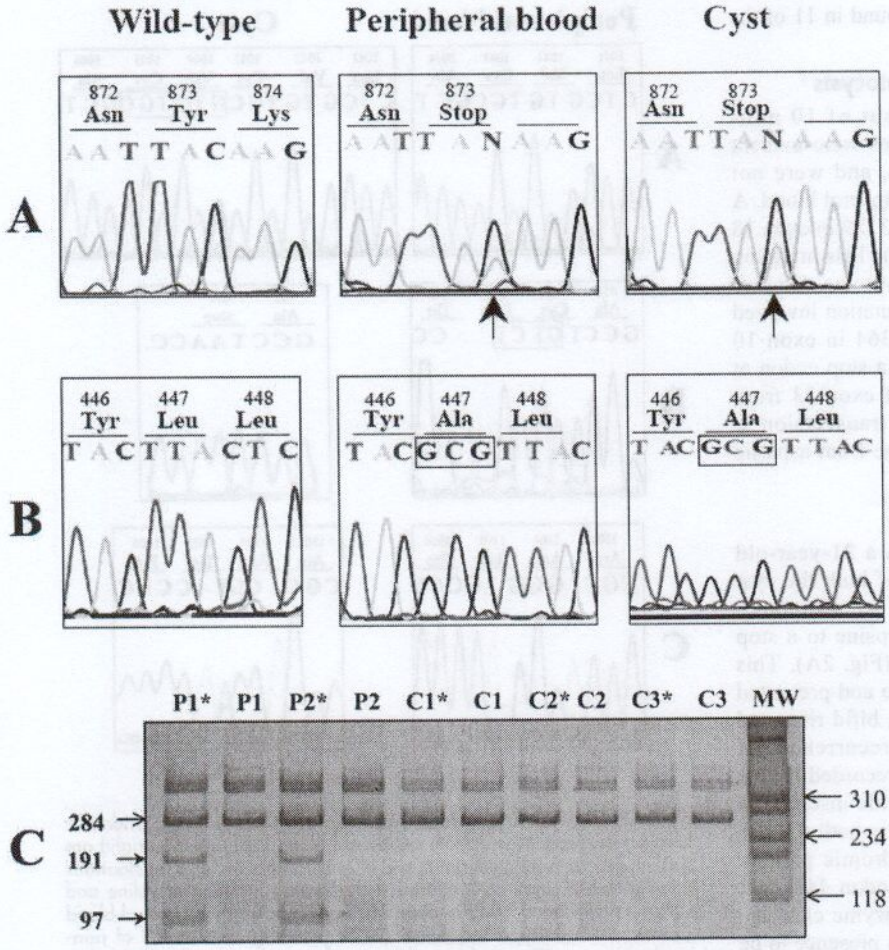

Figure 2. Two germ-line mutations of the *PTCH* gene identified in two patients with Gorlin syndrome (**A,B**). Sequences of the wild-type control (left), the patient's peripheral blood (middle), and cyst (right) are shown. (A) The peripheral blood and cyst of syndrome patient #1 showed a C to A substitution (arrows), introducing a stop condon at amino acid residue 873 in exon 16. (B) Sequencing of exon 9 revealed an insertion of 3 nucleotides (highlighted in small box) between codons 446 and 447, causing an insertion of alanine in the peripheral blood and cyst of syndrome patient #2. (**C**) *MluI* digestion of exon 9 PCR products from the cyst (P1) and peripheral blood (P2) of syndrome patient #2 and the unrelated control DNAs (C1-C3). The insertion created a restriction site with fragments of 97 and 191 bp, as well as the undigested fragment of 284 bp. In the unrelated controls, only the 284-bp fragment was seen. MW, molecular-weight ladder; *enzyme digest.

mutations, 4 of which caused frameshifts and premature protein truncation. In a subsequent study of 3 non-syndromic and 3 syndrome-related keratocysts, *PTCH* mutations were found in 3 cysts, with 2 germ-line mutations associated with Gorlin syndrome and 1 somatic mutation in a non-syndromic cyst (Barreto *et al.*, 2000). A more recent study by Ohki *et al.* (2004) failed to identify any *PTCH* mutations in 4 syndromic keratocysts, but 13 mutations were detected in 7 out of the 18 non-syndromic keratocysts. It is not clear, however, whether these identified mutations were somatic or germ-line, since the constitutional DNA of these cases was not available for analysis.

In the present study, we have identified 4 novel and 1 known *PTCH* mutations in 5 cysts, 2 of which were associated with Gorlin syndrome. A known germ-line mutation found in a syndrome patient was a nonsense mutation (2619C>A) resulting in *PTCH* protein truncation in the second extracellular loop. An identical germ-line mutation has been previously reported in a French Gorlin syndrome patient (Boutet *et al.*, 2003). 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 *SHH* ligand. The novel germ-line mutation (1338_1339insGCG) identified from the other syndrome patient resulted in an insertion of alanine in the second transmembrane domain of the *PTCH* protein, a region known as the sterol-sensing domain (SSD). Analysis of recent data from *Drosophila* suggests that the SSD may play a role in mediating intracellular *PTCH* trafficking as a means of regulating Smoothened (Strutt *et al.*, 2001). Thus, insertion of an amino acid residue in the SSD may disturb signaling transduction in the *SHH* signaling pathway. We also identified 3 novel somatic mutations in 3 non-syndromic keratocysts. One deletion mutation (1361_1364delGTCT) resulted in a frameshift and premature protein truncation in the SSD. The other was a duplication mutation (3124_3129dupGTGTGC), which introduced 2 extra amino acid residues in the 8th transmembrane domain. The third one was a missense mutation (3913G>T), which caused exchange of an acidic amino acid (Asp) for a neutral polar amino acid (Tyr) near the C terminus of the *PTCH* protein. To characterize further the 4 novel mutations identified in the present study, we tested 100 unrelated control DNA samples by PCR-SSCP analysis and found that the abnormal SSCP migration bands seen in the 4 novel mutant samples were absent from the control DNAs (see APPENDIX). Therefore, these newly identified mutations are unlikely to be rare polymorphisms. Thus, analysis of our data provides further evidence that defects of *PTCH* are involved in the formation of syndromic as well as non-syndromic keratocysts, although further studies are now required to identify how these mutations may impair *PTCH* function.

Eight polymorphisms, previously reported in North American (Xie *et al.*, 1997), European (Richards *et al.*, 1997; Bodak *et al.*, 1999; Boutet *et al.*, 2003), and Japanese (Fujii *et al.*, 2003) populations, were also identified in 11 of the 12 patients in the present series. Some of these polymorphic variants (*i.e.*, 1686C>T, 2199A>G, 3944T>C, IVS10-51G>C, IVS10-8T>C) occurred with a high frequency, and eight

patients had 2 or more polymorphisms. A recent study has reported that certain haplotypes of *PTCH* polymorphisms could mediate susceptibility to basal cell carcinomas (Strange *et al.*, 2004). Further studies are needed to define such an association between susceptibility to odontogenic keratocyst formation and *PTCH* polymorphisms.

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APPENDICES

Details of Patient Description and the Diagnostic Criteria for Gorlin Syndrome

Twelve odontogenic keratocyst samples from 12 unrelated patients were obtained from the Department of Oral Pathology, Peking University School of Stomatology. The diagnosis of keratocyst was established histologically in all cases (Kramer *et al.*, 1992). Ten lesions were classified as non-syndromic keratocysts, since the patients presented with only solitary jaw cysts and did not show any other manifestations of Gorlin syndrome. Two cysts were from patients with Gorlin syndrome diagnosed according to the established criteria (Kimonis *et al.*, 1997; Appendix Table). These two patients fulfilled the diagnostic criteria by displaying at least 2 of the major features shown in the Appendix Table.

SSCP Analysis and Restriction Enzyme Analysis of Unrelated Normal Controls for Certifying Novel Mutations Identified in the Present Study

In the present study, 4 novel and 1 known *PTCH* mutations were identified in five individual patients. To characterize any unreported *PTCH* gene alterations identified in the present study as being novel mutations rather than polymorphisms, we tested 100 unrelated control DNA samples (obtained from 100 normal volunteers with their informed written consent, accessed from the Blood Transfusion Center, Peking University First Hospital), together with each of the identified mutant samples by, respectively, PCR-SSCP analysis and, when a restriction site was available, by restriction-enzyme analysis.

SSCP Analysis

Briefly, an aliquot of 5-8 μL of PCR product, mixed with SSCP loading dye at a ratio of 1:3, was denatured at 95°C for 10 min and snap-cooled on ice until loaded onto a neutral 6-10% polyacrylamide gel (acrylamide:bis ratio by weight, 49:1) with 10% glycerol. The electrophoresis was carried out on a D-Code Mutation Detection System (Bio-Rad, Hercules, CA, USA) at 30 W for 6 hrs at 4°C. The gels were silver-stained and visually inspected for potentially abnormal variants.

We found that the abnormal SSCP migration bands seen in the 4 novel mutant (3124_3129dupGTGTGC, 1361_1364delGTCT, 3913G>T, and 1338_1339insGCG) samples were absent in the control DNAs (Appendix Figs. 1A, 1B). These results suggest that the 4 newly identified alterations of *PTCH* gene are mutations rather than polymorphisms. Furthermore, 3 (3124_3129dupGTGTGC, 1361_1364delGTCT, and 3913G>T) of the 4 novel mutations were somatic, that is, the *PTCH* gene alterations detected in the cyst tissue samples were absent from their matched constitutional DNA. Thus, these mutations are unlikely to be rare polymorphisms.

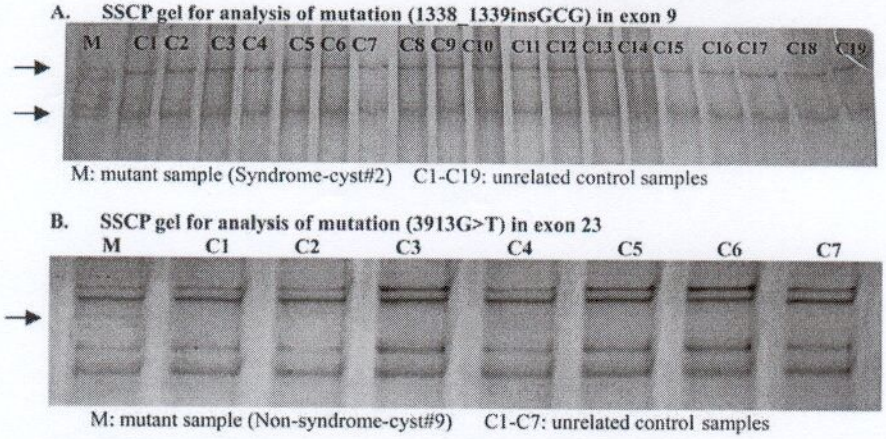
Restriction-enzyme Analysis

In addition, 1 novel germ-line mutation identified in a syndrome case (Syndrome cyst #2) was a triplet insertion of 3 nucleotides, which created a *MluI* restriction site (5′-ACGCGT-3′). *MluI* digestion of the PCR products (exon 9) from the patient and 100 unrelated control DNAs was performed, and the digestion products were analyzed by 8% polyacrylamide gel electrophoresis. Appendix Fig. 2 shows a polyacrylamide gel confirming the presence of this mutation in the patient and its absence from normal controls (a similar gel is presented in the manuscript as Fig. 2C).

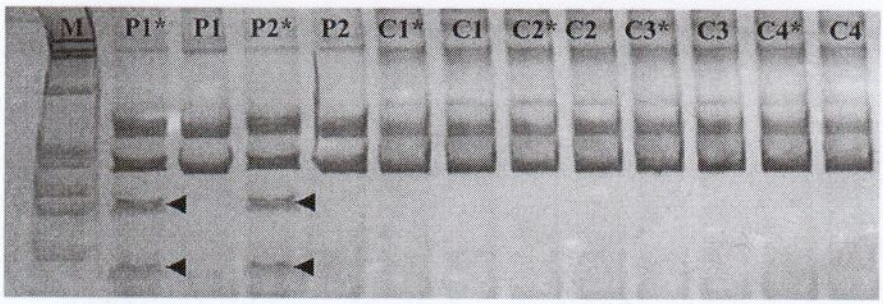
APPENDIX REFERENCES

Kimonis VE, Goldstein AM, Pastakia B, Yang ML, Kase R, DiGiovanna JJ, *et al.* (1997). Clinical manifestations in 105 persons with nevoid basal cell carcinoma syndrome. *Am J Med Genet* 69:299-308.

Kramer I, Pindborg JJ, Shear M (1992). WHO histological typing of odontogenic tumours. Berlin: Springer-Verlag, pp. 35-36.



Appendix Figure 1. (AQ) (A) SSCP gel for analysis of mutation (1338_1339insGCG) in exon 9. M, mutant sample (Syndrome cyst #2). C1-C19, unrelated control samples. **(B)** SSCP gel for analysis of mutation (3913G>T) in exon 23. M, mutant sample (Non-syndrome cyst #9). C1-C7, unrelated control samples.



Appendix Figure 2. Polyacrylamide gel electrophoresis of *MluI* digestion of the PCR products (exon 9). M, molecular-weight marker. P1, cyst. P2, peripheral blood. C1-C4, unrelated normal controls. *Enzyme digest. Arrows = extra fragments of 97 and 191 bp detected in the cyst and peripheral blood samples of the patient (Syndrome cyst #2).

Appendix Table. Diagnostic Criteria for Gorlin Syndrome (from Kimonis *et al.*, 1997)

Diagnosis of Gorlin syndrome made in the presence of two major or one major and two minor criteria:
Major criteria
1. More than 2 basal cell carcinomas or one under the age of 20 years
2. Odontogenic keratocysts of the jaw proven by histology
3. Three or more palmar or plantar pits
4. Bilamellar calcification of the falx cerebri
5. Bifid, fused, or markedly splayed ribs
6. First-degree relative with Gorlin syndrome
Minor criteria
Any one of the following features:
1. Macrocephaly determined after adjustment for height
2. Congenital malformations: cleft lip or palate, frontal bossing, "coarse face", moderate or severe hypertelorism
3. Other skeletal abnormalities: Sprengel deformity, marked pectus deformity, marked syndactyly of the digits
4. Radiological abnormalities: bridging of the sella turcica, vertebral anomalies such as hemivertebrae, fusion or elongation of the vertebral bodies, modeling defects of the hands and feet, or flame-shaped lucencies of the hands or feet
5. Ovarian fibroma
6. Medulloblastoma