

Genetic Analysis of Gorlin Syndrome Using a Multiplex PCR Gene Panel, and Its Potential Clinical Utility for Liquid Biopsy

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Research

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Abstract

Background: Gorlin syndrome is an autosomal dominant, rare disease caused by mutations in *PTCH1*, *PTCH2*, and *SUFU* with various symptoms in multiple organs making early diagnosis challenging. In this study, we generated a Gorlin syndrome gene panel that could help to overcome the difficulties in diagnosing Gorlin syndrome using a single test.

Results: This gene panel is time- and cost-efficient and highly reliable with a high-quality score of 30, on-target ratio, and coverage depth, and could detect more mutations than whole-exome sequencing of the same patient. Although the current *in silico* prediction tools have a limited genetic database of gene mutations in rare hereditary diseases, five prediction tools were used to identify pathological mutations. Pathogenic gene mutations were detected not only in *PTCH1* but also in *PTCH2* in five out of 12 patients with Gorlin syndrome diagnosed based on clinical symptoms.

Conclusions: Using this gene panel, we showed the same gene mutation in the patients and their asymptomatic relatives; hence, it has enabled a highly reliable genetic diagnosis of Gorlin syndrome at a low cost requiring only blood sample.

Background

Gorlin syndrome, nevoid basal cell carcinoma syndrome (NBCCS; OMIM109400), is a rare genetic disease with an estimated prevalence of 1 in 30,000 to 1 in 250,000 worldwide [1]. This disease shows an autosomal dominant inheritance with various clinical symptoms such as odontogenic keratocysts (OKCs) of the maxilla and mandible, developmental abnormalities, and palmar pits. It is occasionally accompanied by malignant tumors such as medulloblastoma and basal cell carcinoma (BCC) [2]. Several clinical criteria have been used for the diagnosis of Gorlin syndrome, including the diagnostic criteria proposed by Kimonis et al [3]. However, it is often difficult to diagnose patients in childhood because some of the symptoms that are important for diagnosing Gorlin syndrome are not yet clear and do not meet the diagnostic criteria. Furthermore, cases have been reported in which children develop Gorlin syndrome, even though their parents are asymptomatic [4]. In this case, mutations in the causative genes of germ cells have been reported. Therefore, genetic diagnosis is very important for early and accurate detection of Gorlin syndrome [5, 6].

The genes responsible for Gorlin syndrome are patched 1 (*PTCH1*) [7], patched 2 (*PTCH2*) [4], and suppressor of fused (*SUFU*) [8]. *PTCH1* and *PTCH2* encode the receptor for Sonic hedgehog (SHH) protein [9]. Based on an analysis of mutations in these three genes, Gorlin syndrome is caused by upregulation of the hedgehog (Hh) pathway. *PTCH1*, *PTCH2*, and *SUFU* are negative regulators of the Hh pathway. The membrane receptors PTCH1 and PTCH2 inhibit Smoothed (SMO) activity in the absence of ligand binding. After ligand binding, SMO is activated and induces the dissociation of SUFU/ glioma-associated oncogene (GLI) complexes, allowing the transcriptional activation of GLI target genes. SUFU sequesters

the full-length GLI proteins in the cytoplasm to restrict their activity and promotes the formation of truncated GLI proteins to repress the target gene expression.

Aberrant Hh signaling is associated with the development and progression of multiple types of cancer and is involved in the maintenance of cancer stem cells (CSCs) [10–12], while *PTCH1*, *PTCH2*, and *SUFU* function as tumor suppressors, thereby negatively regulating the Hh signaling pathway [13]. Homozygous inactivation of the tumor suppressor Patched (*PTCH*) induces tumorigenesis and the formation of multiple BCCs and other neoplasms [14, 15]. The association of CSCs with several solid tumors has been previously investigated [16, 17]. Leprieur et al. [18] has reported that the paracrine mechanism of CSCs produces full-length SHH protein and induces tumorigenesis. Gain-of-function mutations in the *SMO* gene are associated with BCC carcinogenesis, whereas loss-of-function mutations in the *PTCH1*, *PTCH2*, and *SUFU* genes, which negatively regulate the Hh pathway, are associated with carcinogenesis [19]. Indeed, *SMO* heterozygous mutations (c.1234C > T [p.Leu412Phe]) have been reported only in cases of BCC with Gorlin syndrome [20]. However, the possibility of a causative gene mutation in Gorlin syndrome cannot be ruled out. The current study suggests that there may be an undiscovered mutation in the *PTCH1* gene in Gorlin syndrome. Double mutations in *PTCH1* and *PTCH2* were previously shown in two out of four patients with Gorlin syndrome, while multi-layered mutations in multiple genes in the Hh signaling pathway may alter Hh signal activation levels causing Gorlin syndrome [21].

Although genetic mutation identification requires skilled personnel and is a time-consuming process, molecular genetic testing for Gorlin syndrome is of paramount importance for clinical diagnosis, disease prognosis, treatment, and family genetic counseling. When performing genetic testing, the conventional Sanger-sequencing method is expensive and time-consuming. Next-generation sequencing (NGS) overcomes these challenges and enables parallel sequencing of multiple genes in multiple samples in a single assay. Application of NGS to a custom panel of target genes known to be associated with the disease provides relatively rapid results with cost-efficacy and improves diagnostic accuracy [22]. Multigene panel testing can be a very useful screening tool for patients, especially those with a family history of cancer or hereditary disease [23].

No previous development or demonstration of the usefulness of a custom genetic panel test for the diagnosis of Gorlin syndrome has been reported to date. Herein, the development of a custom gene panel test for Gorlin syndrome is demonstrated and the results compared with existing total exome sequence data of patients with Gorlin syndrome. Furthermore, the frequency of multi-layer mutations in the Hh pathway genes was clarified in patients with Gorlin syndrome. The results of this study provide undiagnosed individuals with Gorlin syndrome with the opportunity to undergo genetic testing, enabling early diagnosis and treatment.

Materials And Methods

Research subjects

Information regarding relevant medical, dental, and family histories, such as removal of jawbone cysts and skin lumps, were obtained from patients who visited the Tokyo Dental College Suidobashi Hospital, Ichikawa General Hospital, and Chiba Dental Center between October 2014 and April 2020. Clinical examinations, including oral inspection and examination of the skin for basal cell nevi or BCCs, as well as for cysts and pits on the palms and soles, were performed. Radiographs of the chest and skull, as well as an orthopantomogram, were taken. All 12 patients met the diagnostic criteria for NBCCS reported by Kimonis et al. [3] (Fig. 1). In addition, three natural blood relatives with no clinical symptoms were included in the analysis.

Design of the custom gene panel

The AmpliSeq Custom DNA Panel (Illumina Inc., San Diego, CA, USA) was designed to target four genes (*PTCH1*, *PTCH2*, *SMO*, and *SUFU*) using the Design Studio (<http://designstudio.illumina.com/>) (Illumina) software. The targets comprised the coding exons, exon/intron junctions, and the untranslated regions (UTRs) of the four target genes covering 99.83% (15,620 bp) of the desired target area (15,648 bp) aligned to the reference human genome (UCSC, hg 19/GRCh37). The missing regions included a 12 bp region in the intron of *PTCH1* (position 98278743–98278754 on chromosome 9) and a 16 bp region in the intron of *SMO* (position 128828968–128828983 on chromosome 7). All exons in *PTCH2* and *SUFU* were covered.

Quality control (QC) of the extracted genomic DNA samples

Genomic DNA was extracted from gingiva (normal tissue), odontogenic keratocysts (OKCs), and peripheral blood samples. Gingival-derived fibroblasts were collected from the gingiva of seven patients with Gorlin syndrome (G1, G2, G3, G4, G5, G6, and G7). OKC-derived fibroblasts were collected from the OKCs of four patients with Gorlin syndrome (G1, G2, G3, and G7).

Those fibroblasts were cultured, and genomic DNA was extracted using Biotechnology Explorer Kit PV92 PCR/Informatics (Bio-Rad Laboratories, Hercules, CA, USA). Genomic DNA was extracted from gingiva (normal tissue) and OKC of five patients with Gorlin syndrome (G8, G9, G10, G11, and G12) using NucleoSpin® Tissue kit (MACHEREY-NAGEL, Düren, Germany).

Genomic DNA of eight subjects (five patients and three clinically asymptomatic natural relatives) was isolated from peripheral blood using the NucleoSpin® Blood kit. The DNA of each sample was processed according to the manufacturer's protocol. The QC was performed using a TapeStation analysis software A.02.01 (Agilent Technologies, Palo Alto, CA, USA). The extracted genomic DNA was subjected to quantification and qualification using Genomic DNA Screen Tape and reagent kits (Agilent Technologies, Palo Alto, CA, USA) (Supplementary Fig. 1).

Library preparation and amplicon sequencing

The library was prepared according to the manufacturer's instructions (Illumina). Each three pools contained 32, 33, and 35 different primers corresponding to four target gene regions. PCR reactions (20 cycles) were performed using the three pools at 99°C for 15 s and 80°C for 8 min. After each PCR product

(amplicon) was mixed into one, phosphorylation and adaptor ligation were performed according to the manufacturer's protocol (Illumina). The final mixtures were purified using AMPure XP beads (per 1× volume; Beckman Coulter, Inc., Brea, CA, USA). The final purified reaction mixture (library) was quantified and qualified on a 4200 Tape Station system using High Sensitivity D1000 Screen Tape and reagent kit (Supplementary Fig. 2). All library samples were sequenced on a MiSeq (Illumina) analyzer using the dual index sequencing method (MiSeq® Reagent Nano Kit v2, Illumina). Subsequently, MiSeq Reporter, Burrows-Wheeler Aligner v0.7.17, and Genome Analysis Toolkit v4.1.6.0 were used for alignment and variant calling.

***In silico* prediction**

Data analysis, including alignment to the hg19 human reference genome and variant calling, was performed using the MiSeq Reporter (Illumina). These variants were then annotated using Illumina Variant Studio v3.0 data analysis software and BaseSpace Sequence Hub® App, DNA Amplicon App (Illumina). Integrative Genomic Viewer v2.8.10 (Broad Institute and the Regents of the University of California) (<https://software.broadinstitute.org/software/igv/>) [24–26] was used to visualize the sequences and check for the presence of variants. Bioinformatics analysis was performed using five prediction tools: Mutation Taster (<http://neurocore.charite.de/MutationTaster/>) [27], PolyPhen-2 (<http://genetics.bwh.harvard.edu/pph/>) [28], SIFT (<http://sift.jcvi.org/>), PROVEAN (<http://provean.jcvi.org/seqsubmit.php>) [29], and PANTHER (<http://www.pantherdb.org/tools/csnpscoreForm.jsp>) [30, 31].

Results

Pathological findings and diagnosis

Twelve patients with cystic lesions were enrolled who visited the Tokyo Dental College Suidobashi Hospital, Chiba Dental Center, and Ichikawa General Hospital and were diagnosed histologically with OKCs. The resected OKCs were histopathologically examined by oral pathologists.

The cyst epithelium was relatively flat at the base and consisted of several layers of thin, parakeratinized stratified squamous epithelium (Fig. 2A–G). The basal layer of the cyst was palisaded with prominent, polarized, and intensely stained nuclei of uniform diameter (Fig. 2A–G).

The epithelium-connective tissue interface was relatively flattened and often thin and loose. The epithelial lining was weakly attached or separated from the underlying connective tissue (Fig. 2D–F). The epithelial surface was corrugated (Fig. 2A, B, and G). Some of the cyst lumens contained large amounts of keratin debris (Fig. 2C, E, and F). Inflammation was absent or meager (Fig. 2A–G).

From these findings, the cysts were diagnosed as parakeratotic-type OKCs, and each patient had met at least one other major diagnostic criterion proposed by Kimonis et al. [3] and showed symptoms of palmar

pit (Fig. 1).

Sequencing quality assessment

First, the Gorlin syndrome gene panel was assessed for reliability, and the accuracy of analysis was confirmed by comparing the whole-exome sequencing (WES) results of seven samples with the results of the gene panel prepared in this study. The quality score 30 (Q30), on-target ratio, and coverage depth were investigated.

Phred's base call and score, a program to verify the certainty of the read base sequence, was used (Table 1) [32]. The Q30 and coverage depth were evaluated; all samples tested had a Q30 of 90% or higher and an average coverage depth of $\geq 1,000$. The calculated QC parameters and average coverage depth for all exon targets are shown in Supplementary Tables S1 and S2. These parameters could be decoded with 99.83% certainty in all exon regions of the four target genes (Supplementary Table S3). Only four bases in the exon1 region of *PTCH1* could not be decoded.

Gene panel amplicon sequencing data analysis

The gene panel sequencing detected 83 variants. All 69 variants identified using WES in a previous study were detected in this gene panel sequence (Fig. 3) [21]. Five prediction tools were used for analysis to predict whether the 83 variants would result in protein dysfunction. Pathogenic mutations were found in the *PTCH1* gene in all 12 patients with Gorlin syndrome, and *PTCH2* pathogenic variants were found in five patients with Gorlin syndrome. Among the pathogenic variants, 11 in *PTCH1* and 4 in *PTCH2* were detected using both WES and the custom gene panel developed in this study. The variant in the 5'-UTR (c.297_298insGGC) in the *PTCH1* region could only be detected in this gene panel. The *PTCH1* and *PTCH2* mutations are summarized in Tables 2 and 3, respectively. We did not observe any pathogenic germline variants in *SUFU* or *SMO*.

PTCH1 pathogenic mutations in patients with Gorlin syndrome

We identified *PTCH1* pathogenic germline mutations in all 12 patients with Gorlin syndrome (Table 2). A schematic diagram of the structure of the protein patched homolog 1 (Q13635), which encodes 1,447 amino acids, is shown in Fig. 4A. Amino acid substitution sites representing pathogenic germline mutants are also shown to identify mutant types (Fig. 4A). Most pathogenic germline mutations were located in the transmembrane or near the transmembrane region. The amino acid substitution sites differed in each case.

PTCH2 pathogenic mutations in patients with Gorlin syndrome

Of the 12 Gorlin syndrome cases we examined, *PTCH2* pathogenic germline mutations were identified in 5 cases of Gorlin syndrome, and as a result, *PTCH1* and *PTCH2* double mutations were detected in 5 of 12 cases (Table 3). A schematic diagram of the structure of the protein patched homolog 2 (Q9Y6C5), which encodes 1,203 amino acids, is shown in Fig. 4B. Amino acid substitution sites representing

pathogenic germline mutants are also shown to identify mutant types (Fig. 4B). No specific amino acid substitution site was observed in the *PTCH2* pathogenic germline mutants.

Evaluation of variants using the American College of Medical Genetics and Genomics (ACMG)-Association for Molecular Pathology (AMP) variant-classification guidelines

The ACMG and the AMP have previously developed guidance on the interpretation of sequence variants [33, 34]. A total of 18 variants in *PTCH1* and *PTCH2* showed pathogenicity at least once using five prediction tools (Mutation Taster, PolyPhen-2, SIFT, PROVEAN, and PANTHER). Supplementary Fig. 3 shows the results of the ACMG-AMP classification assessment for each variant. As shown in Table 2, all patients observed in *PTCH1* were predicted to be pathogenic using the Mutation Taster analysis. PolyPhen-2 analysis revealed that mutations in G3, G4, G6, G8, and G10 were predicted as probably or possibly damaging. SIFT analysis showed that mutations in G4 and G6 were deleterious. Mutations in G3, G4, G6, G8, and G10 were deleterious using PROVEAN and PANTHER analysis. As shown in Table 3, for mutations in *PTCH2*, Mutation Taster predicted G1 and G3 mutations as disease causing. PolyPhen-2 analysis showed that mutations observed in G1, G3, G5, G7, and G9 were possibly damaging. SIFT analysis revealed that mutations in G3, G5, G7, and G9 were predicted to be deleterious. PROVEAN analysis predicted mutations in G1, G3, G5, G7, and G9 to be deleterious. PANTHER analysis predicted that mutations in G3, G5, G7, and G9 were deleterious.

Identification of Gorlin syndrome mutations in each family

The gene panel sequencing results were compared for each of the three families of natural blood relatives (Fig. 5). The p.Pro1211Pro (c.3633C > T) synonymous variant in the *PTCH1* region was detected in the parent-child case of Gorlin syndrome in the son (G2) and the mother (G12) in case 1 (Fig. 5A). The *PTCH1* p.Pro1211Pro (c.3633C > T) variant was predicted to be disease-causing because it contained a change in the splice site as shown by Mutation Taster (Table 2). Mutations were also examined in asymptomatic blood relatives, and pathogenic germline variants were detected (Fig. 5B and C).

The p.Gln361Profs*77 (c.1079_1080insCCCT) change in *PTCH1* in case 2 was functionally pathogenic *in silico*. This variant was detected in the father (G10), the older brother (G8), and the younger brother (G8 brother), but not the mother (G8 mother) (Fig. 5B). The younger brother (G8 brother) was a 5-year-old child who did not develop clinical symptoms.

The splice donor variant (c.1503 + 1G > C) of *PTCH1* in case 3 was present only in the daughter (G9) (Fig. 5C). The p. Thr988Met (c.2963C > T) in *PTCH2*, present in both the mother (G9 mother) and her daughter (G9) (Fig. 5C), was predicted to be functionally pathogenic *in silico*.

Discussion

In this study, we generated a genetic panel for the diagnosis of Gorlin syndrome to establish a new, cost-effective, easy, and reliable method for genetic diagnosis. This gene panel has the following advantages:

1. **High reliability.** The entire exons of the three Gorlin syndrome causative genes *PTCH1*, *PTCH2*, and *SUFU* and exon regions of the BCC driver gene, *SMO*, can be sequenced with high accuracy. The reliability of the sequence obtained from this gene panel will be very high if the QC of the extracted patient DNA is good and the library purification is of high quality. The average Q30 of this panel was $\geq 90\%$, the average on-target ratio was $\geq 90\%$, and the average coverage depth was $\geq 1,000$ (Supplementary Table S1).
2. **Easy to handle, time-saving, and cost-effective.** All the analysis pipelines can be executed with a graphical user interface using the program BaseSpace Sequence Hub, and hence, no previous experience is needed to analyze the sequences. It only requires approximately 2 days to obtain results from patients' blood samples.
3. **Tailorable to many diseases.** This gene panel can be used to diagnose a wide range of diseases.

Regarding the high reliability of the gene panel, the standard coverage depth of WES is usually $\geq 30\times$, but that of the gene panel is $\geq 1000\times$, suggesting that the gene panel is much more reliable. Should a high-quality library is produced with a Q30 score of ≥ 90 , the reliability of the gene sequence obtained by panel analysis is then high. NGS analysis using a custom panel limited the region of target gene analysis and provided deeper coverage than WES analysis. In this way, the gene panel is more accurate than the typical WES test; hence, it is possible to identify new mutations that cannot be identified using WES. In addition, the panel-based gene analysis method established in this study can be applied for mutation analysis of the OKC tissue; it is possible to diagnose the cyst itself and evaluate the possibility of a future recurrence.

When the causative gene mutations in patients with Gorlin syndrome were comprehensively investigated using NGS exome analysis, it was found that the proportion of patients with both *PTCH1* and *PTCH2* mutations was higher than expected. In many cases, the *PTCH2* mutation has not been examined after patients were found to have the *PTCH1* mutation; thus, the double mutation might have been possibly overlooked. The gene panel developed in this study can accurately detect mutations in *PTCH1* and *PTCH2* at once, eliminating the possibility of not being able to detect duplicate mutations, as described above. In addition, *SMO* mutations, which are relatively common in BCC and frequently occur in Gorlin syndrome, have never been identified as the causative gene mutation of the Gorlin syndrome. This gene panel can be used to discover syndromes caused by *SMO* gene mutations.

The gene panel is composed of multiple PCR-based gene amplifications of the entire target gene exons in one tube. Thus, each step of the examination procedure is relatively simple. As the reliability of the panel is assured, it is easy to use owing to the user-friendly analysis program BaseSpace Sequence Hub. Furthermore, NGS analysis using a custom panel can accurately obtain 15,000 bp of base information at once, which is a great advantage in terms of time, human resources, and cost. Therefore, this panel is easy to use and time- and cost-effective.

As Gorlin syndrome is caused by hyperactivation of the Hh pathway due to loss-of-function mutations in Hh suppressor genes (*PTCH1*, *PTCH2*, and *SUFU*), these mutations are often observed in tumors.

Activation mutations in these Hh pathway genes are involved in BCC and several types of jawbone cysts [2]. However, the relationship between mutations in these genes and pathogenesis has not been well elucidated, probably owing to the relatively difficult analysis of these causative gene mutations. In the future, a highly accurate application of this gene panel for the comprehensive diagnosis of BCC and jawbone cysts may become common [35]. If more patients are examined using this gene panel technique, the relationship between SMO gene mutations, or even SUFU gene mutations, and pathological conditions may also be identified, along with association between SUFU and SMO gene mutations and pathological conditions.

However, clinical outcomes should never be based solely on *in silico* predictions. It has been reported that *in silico* pathogenicity predictions tend to have false positive results and low specificity [36, 37]. The accuracy of *in silico* tools remains low while the use of computational information in clinical practice is limited by strict guidelines [33]. This necessitates the use of multiple *in silico* tools. In this study, we used the five *in silico* tools described in the ACMG-AMP 2015 guidelines. Variants shown as pathogenic variants using the five pathogenicity prediction tools were evaluated according to the ACMG-AMP guidelines for variant classification. As a result of this classification, some limitations were observed. First, owing to the lack of case reports, variant data for rare diseases are lacking compared to those for cancer. Variant databases for patients with Gorlin syndrome have not been prepared, and it was difficult to evaluate the data with the endpoints of the ACMG-AMP guidelines. Second, data collected was limited because the gene region to be analyzed differs depending on the research center and the analysis method. There are ethnic differences in each disease, and disease mutation databases for each ethnic group are needed. The results of the custom gene panel analysis of blood from three clinically asymptomatic natural relatives who were related to the patients with Gorlin syndrome suggest that custom gene panel testing could be clinically applied as a tool for early diagnosis and then treatment of patients with Gorlin syndrome. In particular, the patient with asymptomatic Gorlin syndrome can be diagnosed using prophylactic tests and informed about the future possibility of developing the disease.

Conclusion

We successfully generated a genetic panel for Gorlin syndrome with cost-effectiveness, ease of performance, and reliable genetic diagnostic methods for Gorlin syndrome. This gene panel can be used not only for patient diagnosis, but also for screening asymptomatic relatives to provide a genetic diagnosis in the *PTCH1*, *PTCH2*, *SMO*, and *SUFU*. Although further research is needed, this genetic panel may prove to be an excellent genetic diagnostic tool not only for Gorlin syndrome but also for patients with other tumors such as BCCs and OKCs.

Abbreviations

ACMG: American College of Medical Genetics and Genomics; BCC: basal cell carcinoma; BCNS: basal cell nevus syndrome; CSC: cancer stem cells; NBCC: nevoid basal cell carcinoma; NBCCS: nevoid basal cell

carcinoma syndrome; NGS: next-generation sequencing; SIFT: sorting intolerant from tolerant; SUFU: suppressor of fused; WES: whole-exome sequencing

Declarations

Data and code availability

The DNA data obtained in this study are available at the National Bioscience Database Center (NBDC) hum JGA000099 and JGAS000308.

Ethics approval and consent to participate

All procedures were reviewed and approved by the ethics committees of the Tokyo Dental College (approval no. 527,974-1,974-2) and Tokyo Dental College Ichikawa General Hospital (approval no. I 15-78, I19-85, I19-86). Written informed consent was obtained from 12 patients with Gorlin syndrome and three of their blood relatives between October 2014 and April 2020. The data were analyzed anonymously in accordance with the Declaration of Helsinki.

Consent for publication

Written consent was obtained from the legal guardian and the adult participants themselves.

Availability of data and materials

The data that supports the findings of this study are available in the supplementary material of this article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Y.N. contributed to data acquisition, analysis, and interpretation drafted and critically revised the manuscript. S.O., T.A. contributed to conception, design, interpretation, drafted and critically revised the manuscript. K.H, M.T., A.K., and T.N. contributed to acquisition, interpretation data and critically revised the manuscript. All authors gave their final approval and agree to be accountable for all aspects of the work.

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Tables

Due to technical limitations, table 1 to 3 PDFs are only available as a download in the Supplemental Files section.

Figures

Fig. 1

Case (n=12)	Birth year	Sex	Major criteria						Gene panel samples			
			BCC	OKC	Palmar/plantar pit	Skeletal anomaly	Calcification of the falx cerebri	First-degree relative with Gorlin syn.	Normal tissue (n=12)	OKC (n=7)	OKC site (n=7)	Blood (n=5)
G1	1953	M	+	+	+	+	+	+	+	+	R mandible	—
G2	1987	M	+	+	+	+	+	+	+	+	L maxilla	+
G3	1983	M	—	+	+	+	+	+	+	+	R mandible	—
G4	1995	M	—	+	+	—	—	+	+	—	—	—
G5	2002	M	—	+	+	+	+	+	+	—	—	—
G6	2002	M	—	+	+	—	—	+	+	—	—	—
G7	2000	M	—	+	+	—	+	—	+	+	R maxilla	—
G8	2009	M	—	+	+	+	+	+	+	+	R maxilla	+
G9	2003	F	—	+	+	+	+	—	+	+	L maxilla	+
G10	1981	M	—	+	+	+	+	+	+	+	R maxilla	+
G11	1978	F	—	+	+	—	+	—	+	—	—	—
G12	1954	F	+	+	+	+	+	+	+	—	—	+

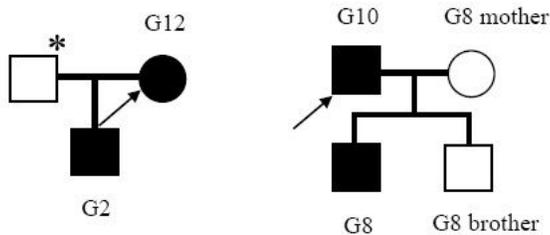


Figure 1

Six major criteria proposed by Kimonis et al. (1997) and samples of the panel sequencing of 12 patients. Six major criteria: BCC) At least two basal cell carcinomas or one under the age of 20 years; OKC) Odontogenic keratocysts of the jaw diagnosed using histology; Palmar/plantar pit) At least three palmar or plantar pits; Skeletal anomaly) Bifid, fused, or markedly splayed ribs; Calcification of the falx cerebri) Bilamellar calcification of the falx cerebri; and First-degree relative with Gorlin syndrome) Self-

explanatory. Right panel: G2 and G12, and G8 and G10 are natural relatives of patients with Gorlin syndrome as shown in the family trees. *: not available for gene panel testing.

Fig. 2

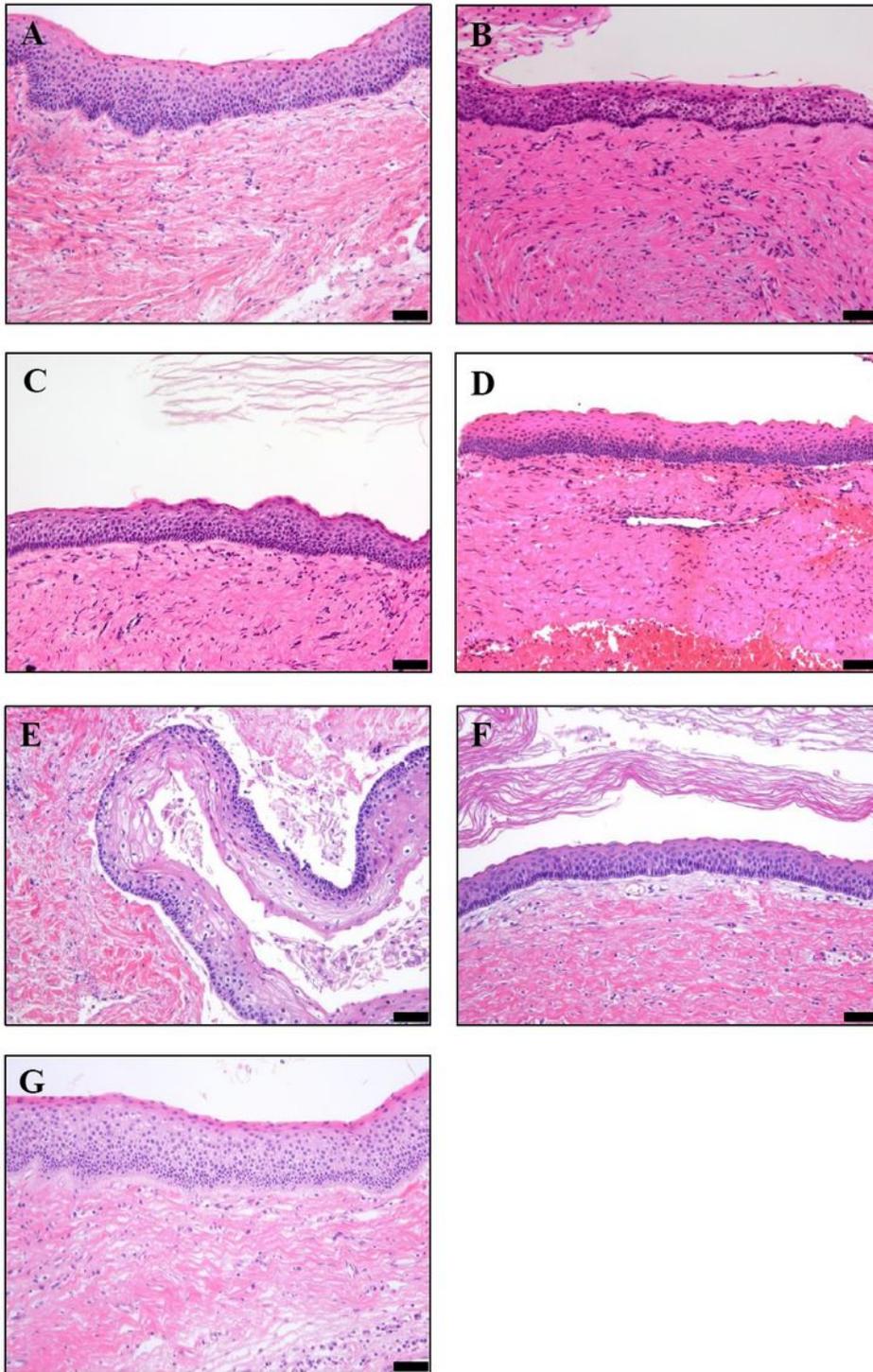


Figure 2

Histopathological findings of the odontogenic keratocyst samples in seven patients with Gorlin syndrome. The samples were stained using HE. The cystic epithelia of all odontogenic keratocysts are composed of several layers of thin parakeratinized and stratified squamous epithelium with a relatively

flat basal area. Individual cases (A) G1, (B) G2, (C) G3, (D) G7, (E) G8, (F) G9, and (G) G10. Scale bar: 50 μ m.

Fig. 3

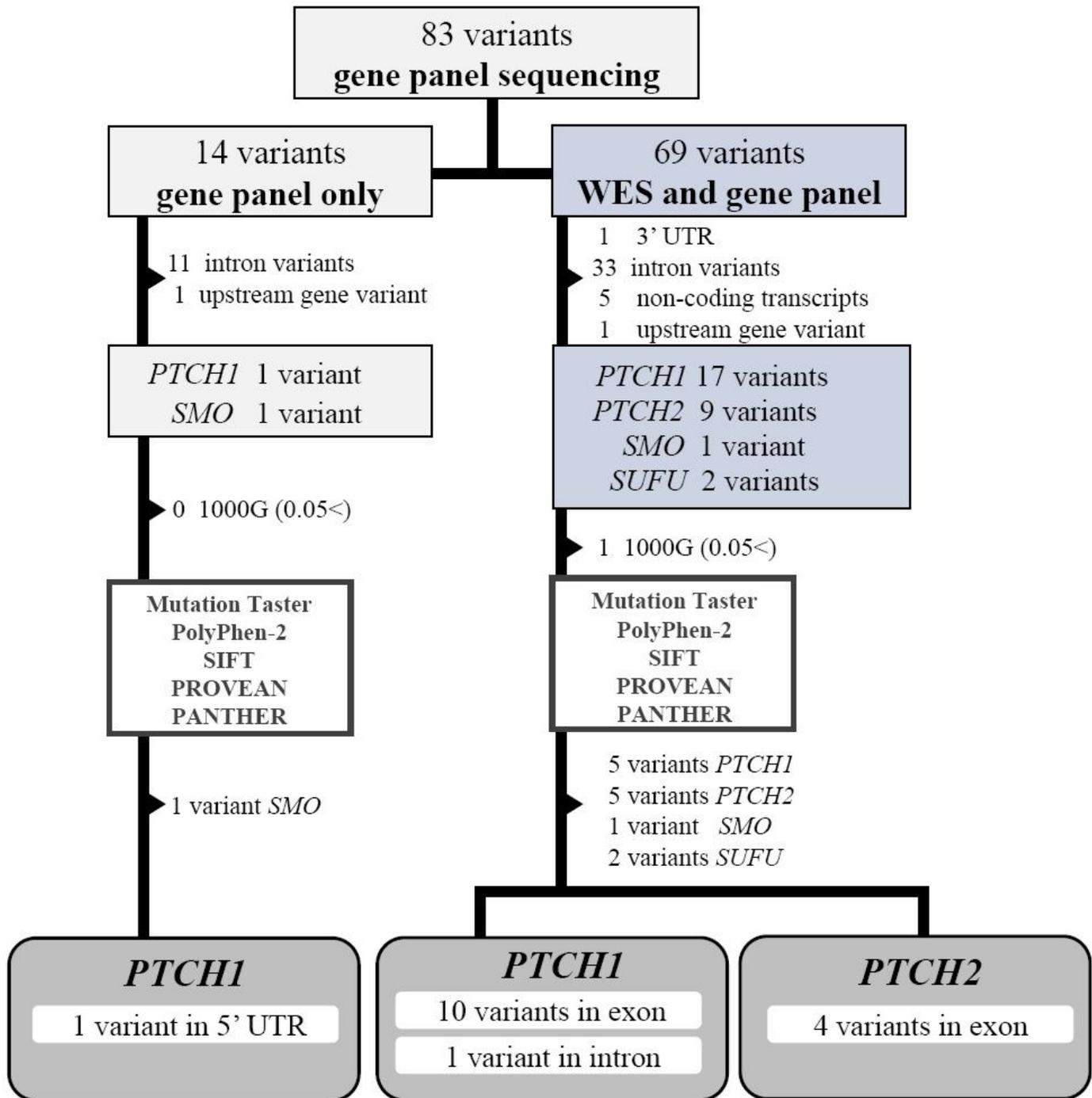


Figure 3

Comparison of gene panel and whole-exome sequencing (WES) analyses of seven patients. Custom gene panel sequencing of four genes detected 83 variants; 69 of the 83 variants were detected using both WES and gene panel sequencing (right column). The in silico analysis for pathogenicity prediction excluded

non-pathogenic 3'-UTRs, intronic variants, non-coding transcripts, and upstream gene variants. The variant (5'-UTR of PTCH1) previously reported in Gorlin syndrome was not excluded. In addition, common variants with a frequency higher than 0.05 in the 1000 Genomes Project (<http://www.1000genomes.org>) were excluded. Fourteen novel variants were detected using gene panel sequencing alone, as shown in the left column. In silico analysis for pathogenicity prediction excluded intron and upstream gene variants. The dark gray box shows the results of the in silico analysis of pathogenicity prediction. The results showed that only PTCH1 and PTCH2 had pathogenic variants.

Fig. 4

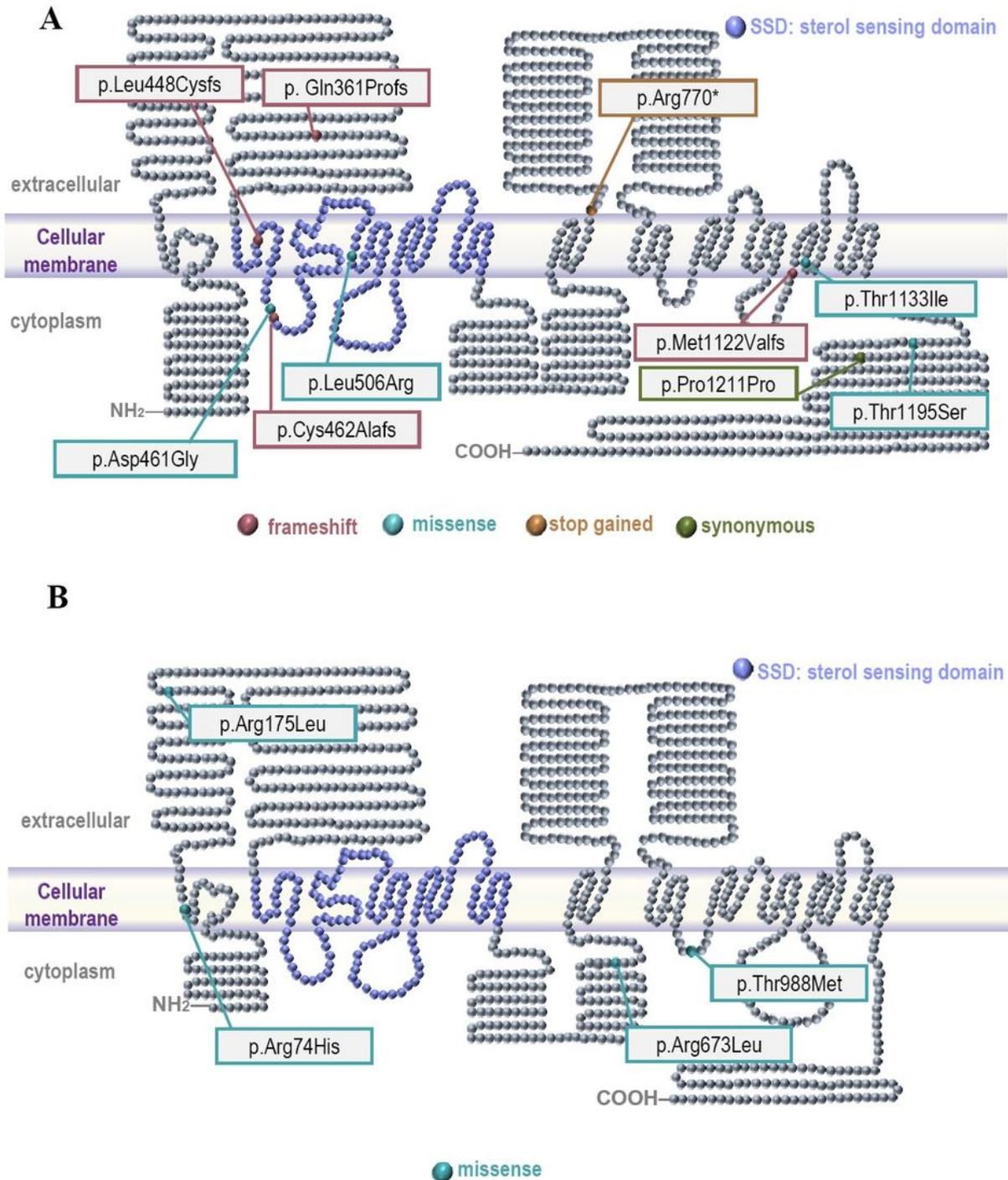


Figure 4

PTCH1 and PTCH2 pathogenic variants in 12 patients with Gorlin syndrome. (A) Amino acid change sites of PTCH1 pathogenic variants in 12 patients with Gorlin syndrome. (B) Amino acid change sites of PTCH2 pathogenic variants present in 5 of 12 patients with Gorlin syndrome. Circles indicate a single amino acid. Purple circles show sterol-sensing domain (SSD). Each variant is represented by colored circles as follows: red, frameshift; blue, missense; brown, stop gained; green, synonymous.

Fig. 5

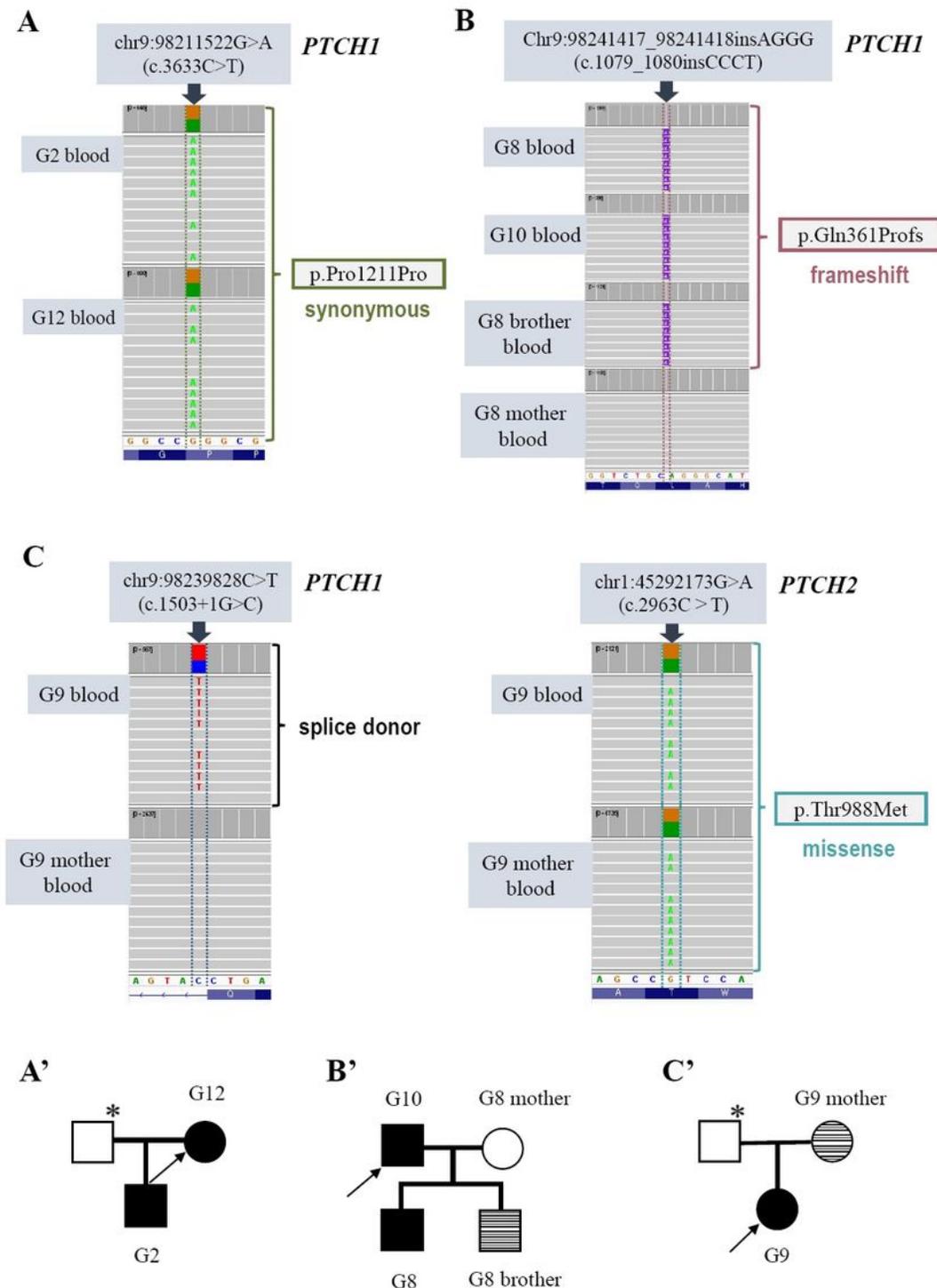


Figure 5

Common pathogenic variants among natural blood relatives (A) Family case 1: Inheritance pattern of the p.Pro1211Pro mutation in PTCH1. The same PTCH1 missense mutation was present in the mother (G12) and even in the son (G2). The family tree is shown in Fig. 5A'. (B) Family case 2: Inheritance pattern of the p.Gln361Profs mutation in PTCH1. The G8 younger brother (G8 brother) with no clinical symptoms showed the same mutation as his father (G10) and older brother (G8) who had Gorlin syndrome. The family tree is shown in Fig. 5B'. (C) Family case 3: Splice donor variant in PTCH1 was only present in the daughter (G9). The missense variant p.Thr988Met mutation in PTCH2 was inherited by the daughter (G9) from her mother (G9 mother). The family tree is shown in Fig. 5C'. *This sample in the family trees was not available for gene panel testing. A shaded symbol in the family trees indicates the person had no clinical symptoms.

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