Involvement of Patched (PTCH) Gene in Gorlin Syndrome and Related Disorders: Three Family Cases

Mima Šitum, Sonja Levanat, Ivana Crniga, Božidar Pavelić, Darko Macan, Jakša Grgurević, Mirjana Mubrin-Končar, Jasna Lipozenić

Department of Dermatovenerology, Zagreb University School of Medicine; 1Division of Molecular Medicine, Ruđer Bošković Institute, Zagreb; 2School of Dental Medicine, University of Zagreb, Zagreb; 3Department of Oral and Maxillofacial Surgery, University Hospital Dubrava, Zagreb; and 4Department of Nuclear Medicine, Sestre Milosrdnice University Hospital, Zagreb, Croatia

Aim. To find genetic alterations in PTC or other genes of the Shh/PTCH pathway in tumorous and non-tumorous samples from three families and to correlate them with the varying expression of disorders in presented nevoid basal cell carcinoma syndrome (NBCCS) phenotypes.

Method. DNA was extracted from archival paraffin-embedded tissues, tumor tissue or peripheral blood leukocytes, and the loss of heterozygosity (LOH) and single strand conformational polymorphism analysis was performed using PCR with primers for polymorphic 9q22.3 markers (D9S196, D9S287, D9S180, D9S127); PTCH exons 3, 6, 8, 13, 15, 16; and smo (smoothened) exon 1. G-banding technique was used for cytogenetic analysis of the peripheral blood lymphocytes.

Results. We found a LOH for PTCH in several cases and variability in smo in one case. In one case NBCCS could reasonably be ascribed to hemizygous PTCH inactivation, while in other two families this typical correlation between the syndrome phenotype and the observed genetic alterations could not be established.

Conclusions. Further analysis of relatively sparse cases of NBCCS is needed before the symptoms of the syndrome could be convincingly explained by genetic alterations in the Shh/PTCH signalling pathway.

Key words: allelic loss; basal cell nevus syndrome; Gorlin syndrome; LOH, loss of heterozygosity; signal pathways

Among the increasing number of medical conditions for which inherited predispositions are suspected, underlying genetic alterations are most readily demonstrated for those disorders that have an increased incidence in heritable syndromes.

Nevoid basal cell carcinoma syndrome (NBCS) or Gorlin syndrome is a rare autosomal dominant disorder characterized by predisposition to basalcellular carcinomas and several other tumors, including ovarian fibroma and medullo-blama (1). Developmental defects are another prominent feature of the syndrome and include pits of the palms and soles, jaw keratocysts and other dental malformations, midline brain malformations, strabismus, spine and rib abnormalities, ectopic calcifications, and macrocephaly with a characteristic coarse facies and generalized overgrowth (1). However, the syndrome is particularly noted for its extensive interfamilial variability as well as intrafamilial variability with respect to the manifestation and severity of the phenotype (2). The criteria for the diagnosis of NBCCS include presence of at least two major features of the syndrome, such as multiple basal cell carcinomas, or onset of basocellular carcinomas before the age of 20, or pits of palms and soles, jaw cysts, calcification of falx cerebri, and having a first degree relative with NBCCS (1).

This heritable condition has been associated with constitutional hemizygous inactivation of the recently cloned patched (PTCH) gene, which is a human homologue of ptc, Drosophila segment polarity gene (patched) (3). The gene appears to have a tumor suppressor role, at least in some malignancies for which the syndrome predisposes, as has been most convincingly demonstrated for basocellular carcinomas (4). Its protein product, located in the plasma membrane, is a part of the newly discovered Shh/PTCH signalling pathway (Sonic hedgehog, Shh – human homologue of hedgehog, corresponding to the hedgehog/patched pathway in Drosophila) (5). It has a receptor role for the Shh ligand through close connection to smoothened (smo), another membrane protein component (5). PTCH suppresses smo's continuous signalling into cyto-
plasm by binding to it. That prevents smo from inducing expression of several genes, which are called the pathway downstream genes. While mechanisms and functions of this downstream section largely remain to be elucidated, little doubt remains that the genes involved are capable of inducing cell division. Their expression is triggered when Shh arrives at the membrane and relieves smo by binding to PTCH (Fig. 1) (6). However, the Shh signal is normally of transient nature due to an auto regulation process effected by PTCH, which is itself among the upregulated downstream genes (7).

Even before PTCH was cloned, loss of heterozygosity (LOH) in 9q22.3 region to which the gene maps had been reported for basocellular carcinomas (8), as well as for odontogenic keratocysts (9), the jaw lesions most commonly found in NBCCS. The LOH finding in typical NBCCS-related and sporadic malignancies indicates PTCH tumor suppressor role, whereas in cysts it suggests a similar two-hit mechanism through which the suspected gene might be involved in a number of developmental malformations found in the syndrome (10). As an obvious common finding in all these disorders, PTCH inactivation was perceived to be the necessary and perhaps initiating event for their onset (6).

Reexamination of such hypotheses in the context of Shh signalling not only supported the idea in general, but also provided a few plausible details regarding processes which may trigger the aberrant cell proliferation. But it has also indicated that other mechanisms might be alternatively or concurrently involved in the genesis of disorders under consideration. As a confirmation for this anticipated possibility of different initiating events that could deregulate Shh/PTCH signalling pathway, basocellular carcinomas were reported with smo alterations instead of PTCH inactivation (11).

In this study we describe three families with varying expression of disorders that are commonly associated with NBCCS, and analyze alterations observed in their genetic material. Better understanding of the Shh signalling functions, in particular of its role in the notoriously variable NBCCS phenotype, largely depends on persistent examination of the relatively sparse syndrome cases.

Figure 1. Signalling through the Shh/PTCH pathway. (a) Hh binds to ptc transmembrane protein relieving smo, another transmembrane protein. Smo transmits the signal to the cytosol, downregulating cAMP dependent PKA (protein kinase A) and causing Cos-2 (Costal 2) and fu (fused) phosphorylation and dissociation from microtubules. Activated, non- proteolyzed form of Ci (Cubitus interruptus), translocates to the nucleus induces gene expression with involvement of cdp (CREB-binding protein). (b) In the absence of Hh, smo is repressed by ptc and no signal is transmitted to the cytosol. Consequently, Cos-2/fu/Ci physical complex remains bound to the microtubules via Cos-2 in the cytosol. Ci\textsuperscript{155} is proteolyzed into a repressor form Ci\textsuperscript{155} through PKA inhibiting hh and dpp (decapentaplegic protein) transcription in the nucleus.

### Material and Methods

**Tissue Samples and DNA Extraction**

Samples were collected from patients’ peripheral blood leukocytes, tumor tissue after surgery, or paraffin-embedded tissue slices. DNA was extracted by standard phenol-chloroform method (12).

**Microdissection**

Archival paraffin-embedded tissue was stained in hematoxilin/eosin and used as a control of microdissection of unstained paraffin embedded tissues slices.

Microdissected paraffin-embedded cyst linings were separated from the surrounding tissue (1x1x2 mm) into 500 µL microcentrifuge tubes with 36 µL of digestion buffer (0.9% Tween-20, 0.9% Triton X-100, 5 mmol/L EDTA, 2 mmol/L DTT, 10 mmol/L TrisHCl, pH 7.5) and 4 µL of proteinase K 20 mg/mL (Boehringer Mannheim, Wien, Austria). Digestion solution was overlaid with mineral oil, centrifuged briefly to ensure submersion of the tissue flakes in the digestion buffer and placed at 65°C overnight. The tubes were then boiled for 10 min and used in further procedures (13).

**Loss of Heterozygosity and Single Strand Conformational Polymorphism Analysis**

In order to perform loss of heterozygosity (LOH) and single strand conformational polymorphism (SSCP) analysis we amplified polymorphic 9q22.3 markers (D9S196, D9S287, D9S180, D9S127). PTCH exons 3, 6, 8, 13, 15, 16 (3,14), and smo exon 1 by polymerase chain reaction (PCR) (15).

PCR was performed in 25-µL reaction volumes containing 0.3-0.5 µmol/L of forward and reverse primers, 50 µmol/L dNTPs, 0.5 units of Taq polymerase (Perkin Elmer, Norwalk, USA), 0.5 µCi 3P-dCTP (Amersham, Buckinghamshire, UK) and 1.25 mmol/L MgCl\textsubscript{2} in 1xPCR buffer (Perkin Elmer, Norwalk, USA) under the following conditions: denaturation at 98°C for 5 min + 35 cycles (denaturation at 95°C for 30 s; annealing at 55°C for 30 s; extension at 72°C for 40 s) + extension at 72°C for 10 min for D9S196; 35 cycles (denaturation at 94°C for 1 min; annealing at 56°C for 1 min; extension at 72°C for 2 min) + extension at 72°C for 5 min + 35 cycles (denaturation at 95°C for 30 s; annealing at 58°C for 30 s; extension at 72°C for 45 s) + extension at 72°C for 10 min for smo (15).

The size and sequence of PCR primers were as follows:

D9S127 – locus 9q31, 149-159 bp, sense 5’-AGA TTG ATT GAT ACA AGG ATT TG, and antisense 3’-CCC TCA AAT TTG CTG CTG TCT AT;
D9S180 – locus 9q22.3, 220-230 bp, sense 5’-AGC TAT TTT TGG GAG CGT AG, antisense 3’-CAG TCG TTT GGA ATC GAA CC;

D9S287 – locus 9q22.3, 168-180 bp, sense 5’-AGG ATG GTA TCT TTC ACT ACA, antisense 3’-ACC ACT ACA CGG GCT GTG AG CTG GAG;

D9S196 – locus 9q22.3, 254-260 bp, sense 5’-GGG ATT ACA CCTCAA AAC CA, antisense 3’-ACC ACA CTG CGG GAC TT; smn, 140 bp fragment size, sense 5’-CTG GTA CGA GGA CGT GGA GC, antisense 5’-AGG GTG AAG AGC GTG CAG AG.

For LOH analysis, 5 µL of each PCR reaction was added to 15 µL of loading buffer (95% deionized formamide/20 mM EDTA, 0.05% bromphenol blue/0.05% xylene cyanol), heated to 95°C for 2 min and electrophoresized in 6-8% denatured polyacrylamide gel (1 mm/32 cm/40 cm) at 1,750 V or 8-12% native polyacrylamide gel (1 mm/32 cm/40 cm) at 1,750 V. Electrophoreses were run for 2 min and electrophoresed in 6-8% denatured polyacrylamide gel (1 mm/32 cm/40 cm) at 1,750 V or 8-12% native polyacrylamide gel (1 mm/32 cm/40 cm) at 1,750 V. Electrophoreses were run for 5 hours at 10-15 V/cm in 1xTBE buffer (89 mmol/L Tris, 89 mmol/L boric acid, 20 mM/L EDTA). Gels were autoradiographed for 24 to 36 h at -20°C or silver stained (16).

For SSCP analysis, 4-9 µL of PCR products were denatured for 10 min at 42°C in 1x alkali denaturing buffer (50 mM NaOH, 1 mM EDTA) and loaded on (1 mm/16 cm/18 cm) native 6% or 9% polyacrylamide gels (3% cross-linking). Electrophoreses were run for 5 hours at 15-20 V/cm.

Data Analysis

DNA was visualized by silver staining method as previously described (16) or by autoradiography using 32P-dCTP or SYBR Green I Nucleic Acid Gel Stain on Spreadex gels (Elchrom Scientific, Cham, Switzerland).

Allelic loss was scored when DNA in the surrounding tissue or DNA from leukocytes was heterozygous and one of the expected bands in the DNA from the cyst or tumor was completely absent or markedly reduced in intensity (9). SSCP variability was identified from the single stranded region; samples showing variation in sizes were compared with other family members to assess segregation of the alleles, or with normal DNA from the same patient in the case of tumors (3).

Cytogenetic Analysis

G-banding technique (17) was used for cytogenetic analysis of the peripheral blood lymphocytes (PBL’s) from patients and untreated family members.

Results

We report here on diverse findings in three family cases related to the Gorlin syndrome. In the first case we have data for the Gorlin syndrome phenotype of a female patient with widespread basocellular carcinomas on her face, neck and arms, which she began removing at the age of 14. She also has characteristic facial dysmorphology (Fig. 2a) and dental deformations (with several jaw cysts), anomalies of ribs and bones, and palmoplantus pits. During childhood, she behaved as a mentally retarded person, she could not see with one eye, and had kidney problems. Her father died of sarcoma, her grandfather had lung carcinoma and mother suffered of gastric carcinoma. In the patient’s tumors we found LOH for D9S287 (Fig. 2b) which implies mutation in the region where Gorlin syndrome gene maps (18).

In the second family, father and daughter are affected (Fig. 3a). Father has rib abnormalities and spina bifida oculta, whereas his daughter has numerous basocellular carcinomas over back, neck and arms. She also has polycystic ovaries and distorted blood clotting. In her two children, the syndrome is not expressed yet. Our tests did not show any loss or variation of affected NBCCS alleles in the daughter’s children, which could suggest that disease does not have genetic consequences. Until now we have not detected any LOH in daughter’s tumors or constitutional DNA (Fig. 3a and b), nor any SSCP aberrant band of PTCH exons. Exons 3, 6, 8, 13, 15 and 16 did not show any variability (data not shown). Therefore, smn was analyzed in all family members. A single SSCP variability in smn was found in daughter’s basocellular carcinomas (Fig. 3b). Cytogenetic analysis revealed chromosomal instability in the mother’s and daughter’s karyotype with suspected clonally changed chromosome 9 homologue in both of them (Fig. 3c). Other family members have numerically and structurally normal karyotype. Observed chromosomal instability could be one of the features of the syndrome, but in this case the connection is difficult to explain.

The third case is a family with insufficient evidence for Gorlin syndrome diagnosis, but with numerous dental problems and bone malformations. The twin boys have multiple keratocysts in the jaws, which their dentist started to excise when they were between 10 and 12 years old. One of them has calcified falx cerebri (Fig. 4a) which is a characteristic feature of NBCCS patients (1). Their mother has cysts in liver, and in kidney. One of their grandfathers removed jaw cysts at late age (data not available). In their family history there is no evidence of Gorlin syndrome diagnosis. Interestingly, in the keratocysts of the boy with falx cerebri LOH for D9S287 was detected as a typical genetic defect for NBCCS syndrome.

Discussion

A prominent candidate for genetic studies of hereditary syndromes that involve both cancer predisposition and

Figure 2a. Characteristic facial dysmorphology of a Gorlin syndrome patient with skin basocellular carcinomas on the neck and face.

Figure 2b. LOH analysis for polymorphic marker D9S287 in blood leukocytes (B) and basocellular carcinomas (T) of the same patient shows loss of upper allele in the tumor sample.
developmental defects is the NBCCS syndrome, in which one allele of the PTCH gene is constitutionally inactivated, either by inheritance or as a de novo event occurring very early in development (19). While this haploinsufficiency may by itself have some minor effects on organogenesis and growth, most NBCCS-related disorders have been associated with the increased likelihood of full PTCH inactivation in individual cells by local damage to the remaining normal allele (20).

To correlate possible extent and modes of PTCH inactivation with the phenotype expression, all the syndrome disorders are grouped in three sets. Oncogenesis is most easily explained. Isolated somatic loss of the remaining normal PTCH allele appears quite likely during adult life (or even childhood), and it locally disables PTCH tumor suppressor role (the second hit of the Knudson’s hypothesis) (21), leading to syndrome-associated tumors, in particular to multiple loci of basocellular carcinomas (22). Disorders of the second set include malformed spine and ribs and keratocysts of the jaws, and appear in multiple, random clusters within the same patient. This mosaic manifestation may be a consequence of an early loss or inactivation (e.g. fetal or embryonic) of the normal PTCH allele (23) in a progenitor cells of the relevant tissue (second hit of the Crosby’s hypothesis) (10). Disorders of this set, as well as the syndrome associated malignancies, appear sporadically with a much lower incidence and severity because in the general population they require somatic inactivation of both PTCH alleles.

Finally, symmetrical defects occurring to some extent in most of the patients, such as overgrowth, macrocephaly and facial dysmorphology, belong to the third set and have been hypothesized to result from per-}

turbation of dosage-sensitive pathways during development (24).

The described patient from the first family is an example of the thoroughly expressed syndrome, in which the LOH observed for basocellular carcinomas convincingly indicates PTCH aberrations as the underlying genetic cause. However, PTCH inactivation can no longer be analyzed outside the context of Shh signalling, which helps explain some related phenomena, but complicates understanding of others (25). PTCH is a part of physical complex located on a membrane, which sends signals into the cytoplasm through a still not well understood cascade of steps.

Based mostly on evidence with the Drosophila homologue, it has been suggested that PTCH protein represses expression of several genes – such as BMPs (bone morphogenetic proteins) and Wnt (human homologue of Drosophila wingless), including itself – by antagonizing smo, which would otherwise initiate an intracellular cascade leading to their transcription (24). However, the repression occurs only in the absence of the Shh protein, which binds to PTCH and relieves smo from repression, thereby initiating transcription of the target genes (25). Such Shh signalling normally operates only in limited tissue regions, over which this secreted protein spreads by slow diffusion. Since PTCH is itself among the target genes, its upregulated expression eventually results in complete binding of Shh, terminating the signal. PTCH mutations can obviously cause deregulation of the process, so that target genes become expressed independently of the Shh signalling activity. In addition, any deficiency of PTCH transcription might allow Shh to spread into an inappropriate domain (6).

Although in the downstream part of the pathway many details remain unclear, it is known that some target
genes can increase cell proliferation (25), which is the first step towards understanding the pathway role in NBCCS-related disorders. The observed defects are generally consistent with the known expression sites and proposed functions of the Shh/PTCH pathway in development (6). This new insight in mechanism of PTCH involvement in Gorlin syndrome-related disorders suggests the possibility that most if not all of them can be induced by mutations of other genes in the Shh pathway as well (25). But unresolved complexities of the pathway still impede proper understanding of all aspects necessary for a comparative evaluation of various modes of its deregulation and for an estimate of relative importance of PTCH in ensuing disorders. Still, even such a rudimentary concept of pathway deregulation may help understand initially puzzling reports that a number of different PTCH mutations seem to be in no correlation to the variable phenotype expression of NBCCS. However, occasional evidence of chromosomal instability should also be analyzed for possible contribution to this variability (26).

The first case described here appears to be a typical NBCCS with constitutional hemizygous PTCH inactivation, but the other two families may require more complex explanation in order to link observed genetic alterations with the resulting disorder manifestations.

In the second case, the two children of an affected mother showed no evidence of the syndrome. The unusual feature is the finding of smo alterations instead of PTCH inactivation in her basocellular carcinomas. While this is not the first report of smo induced basocellular carcinomas, it would be worthwhile exploring whether her other syndrome manifestations can be attributed to smo. She might have inherited the syndrome from her father, but the data we have are insufficient for genetic linkage confirmation. Cytogenetic analysis revealed chromosomal instability, which was also found in her mother who had no symptoms of the syndrome.

Finally, the twins from the third family, with dental and skeleton malformations — that were not sufficient for a positive diagnosis of the syndrome — should be followed up to see whether constitutional inactivation of a PTCH allele can have so limited phenotype expression. Although the LOH found in cyst lining could be attributed to local PTCH alterations, the presence of cysts in the other twin precludes the simple mosaic-type explanation for inactivation of both alleles and indicates an inherited predisposition.

Our findings contribute to the previous observations of involvement of the Shh/PTCH signaling pathway in tumorous and developmental malformations, confirming the importance of this pathway in various stages during development and possible overlapping of different pathways during lifetime. It also demonstrates that in Gorlin syndrome and related disorders, where one of the most prominent features is variability in phenotype expression, possible alterations in more than one pathway gene have to be considered.

Due to the very low incidence of the syndrome, there is no alternative to thorough and persistent observation of the few available NBCCS patients, in order to collect further data on the syndrome phenotypes and the associated genetic damage. Their condition may be utilized for further exploration of the Shh pathway and PTCH role in various disorders, and understanding of the complex genetic mechanisms of growth and development. In two of the three examined families the typical correlation between hemizygous PTCH inactivation and the syndrome was not found, so further analysis and observation is needed before the symptoms can be explained by genetic alterations in Shh/PTCH pathway.
References


Received: September 7, 1999
Accepted: October 24, 1999

Correspondence to:
Sonja Levanat
Division of Molecular Medicine
Ruđer Bošković Institute
Bijenikova 54
HR-10000 Zagreb
Croatia
levanat@rudjer.irb.hr