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Source / Izvornik: **Croatian medical journal, 1999, 40, 533 - 538**

Journal article, Published version

Rad u časopisu, Objavljena verzija rada (izdavačev PDF)

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

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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 (NBCCS) or Gorlin syndrome is a rare autosomal dominant disorder characterized by predisposition to basocellular carcinomas and several other tumors, including ovarian fibroma and medulloblastoma (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 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-

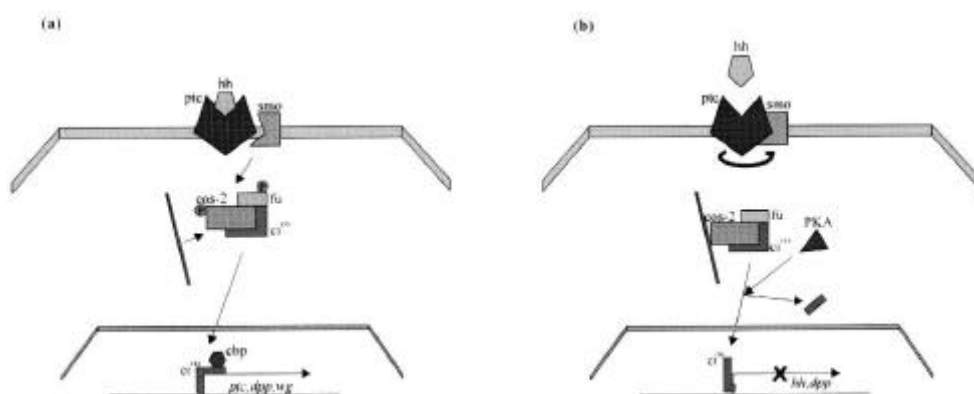


Figure 1. Signalling through the *Shh*/PTCH pathway. (a) Hh binds to *ptc* transmembrane protein relieving *smo*, another transmembrane protein. *Smo* transmits the signal to the cytosol, downregulating cAMP dependent PKA (protein kinase A) and causing *Cos-2* (Costal 2) and *fu* (fused) phosphorylation and dissociation from microtubules. Activated, non-proteolysed form of *Ci* (*Cubitus interruptus*), translocates to the nucleus induces gene expression with involvement of *cbp* (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^{155} is proteolysed into a repressor form Ci^{75} through PKA inhibiting *hh* and *dpp* (decapentaplegic protein) transcription in the nucleus.

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 autoregulation 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.

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 hematoxylin/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 μ L microcentrifuge tubes with 36 μ L 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 μ L 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- μ L reaction volumes containing 0.3-0.5 μ mol/L of forward and reverse primers, 50 μ mol/L dNTPs, 0.5 units of Taq polymerase (Perkin Elmer, Norwalk, USA), 0.5 μ Ci 32 P-dCTP (Amersham, Buckinghamshire, UK) and 1.25 mmol/L $MgCl_2$ 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 D9S287; denaturation at 96°C for 5 min + 30 cycles (denaturation at 94°C for 45 s; annealing at 55°C for 45 s; extension at 72°C for 1 min) + 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 for D9S127; 35 cycles (denaturation at 94°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 D9S180 (14) and denaturation at 95°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;

D8S180 – locus 9q22.3, 220-230 bp, sense 5'-AGC TAT TTT TGG GGG CTG AG, antisense 3'-CAG TGG TTT GGA ATC GAA CC;

D9S287 – locus 9q22.3, 168-180 bp, sense 5'-AGG ATG CTC CTC ACG C, antisense 3'-ACC ACT ACA TTG TTC AAG GG;

D9S196 – locus 9q22.3, 254-260 bp, sense 5'-GGG ATT ACA CCT CAA AAC CA, antisense 3'-ACC ACA CTG CGG GAC TT;

smo, 140 bp fragment size, sense 5'-CTG GTA CGA GGA CGT GGA GG, antisense 5'-AGG GTG AAG AGC GTG CAG AG.

For LOH analysis, 5 μ L of each PCR reaction was added to 15 μ L of loading buffer (95% deionized formamide/20 mmol/L EDTA/0.05% bromophenol 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 500 V. Electrophoreses were run for 8 to 12 hours at 10-15 V/cm in 1xTBE buffer (89 mmol/L Tris, 89 mmol/L boric acid, 20 mmol/L EDTA). Gels were autoradiographed for 24 to 36 h at -20°C or silver stained (16).

For SSCP analysis, 4-9 μ L 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 dismorphology (Fig. 2a) and dental deformations (with several jaw cysts), anomalies of ribs and bones, and



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

palmoplantall 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 anomalies and spina bifida occulta, 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, *smo* was analyzed in all family members. A single SSCP variability in *smo* 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

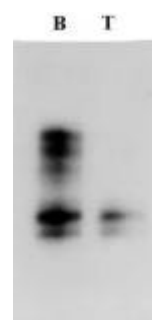


Figure 2b. LOH analysis for polymorphic marker D9S287 in blood leukocytes (B) and basocellular caecinomas (T) of the same patient shows loss of upper allele in the tumor sample.

developmental defects is the NBCC syndrome, in which one allele of the PTCH gene is constitutionally inactivated, either by inheritance or as *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 dismorphology, 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

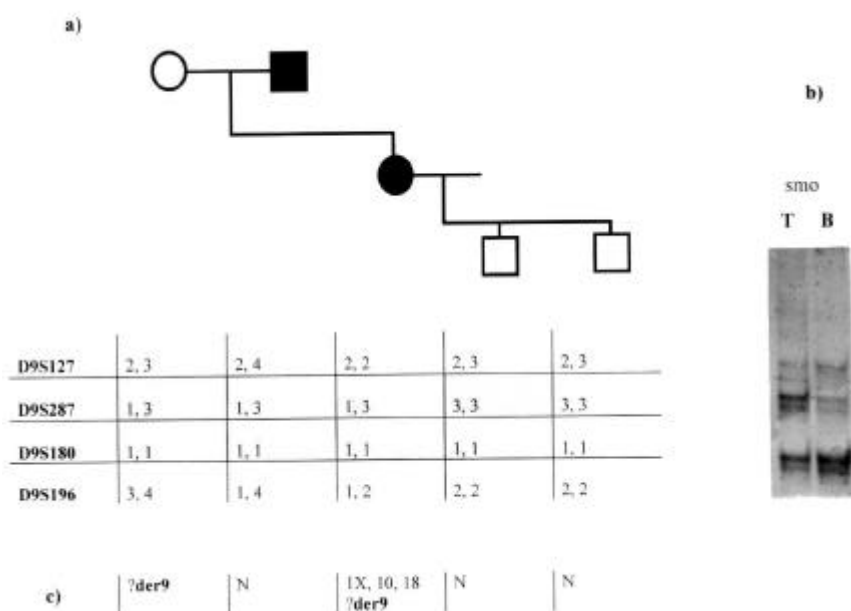


Figure 3. Analysis of the second family with Gorlin syndrome. (a) Pedigree of a Gorlin syndrome family with chromosome 9q22 haplotypes. Affected members are shown as black symbols and non-affected as opened symbols. Under the symbols corresponding to each family member are the results of typing for polymorphic markers as follows: D9S127 shows transmission of allele 2 to all family members, transmission of allele 3 of D9S287 to all family members, D9S180 was uninformative – all members were homozygous, father and daughter shared the allele 1 of D9S196, but the loss of suspicious allele in daughter's tumor was not detected. (b) SSCP variability of *smo* in daughter's BCC tumor (T) in comparison with the DNA from blood leukocytes (B). (c) Cytogenetic analysis detected chromosomal instability in only two family members with possible chromosome 9 derivative (?der9). Other members had normal karyotype (N).

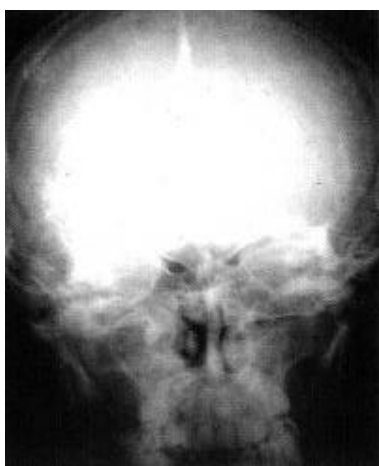


Figure 4a. Calcified falx cerebri with lesion in mandible corresponding to odontogenic keratocyst seen in X-ray film of a boy from the third family.

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.

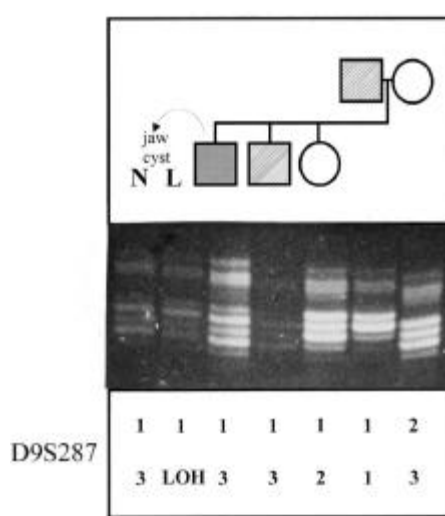


Figure 4b. Typing for D9S287 on the third family where under the symbols for each family member are the results of typing for D9S287 in blood leukocytes. Female family members were not affected. The father had several jaw keratocysts, which were removed during lifetime but not analyzed for LOH. Members of the family (father and a son) who had same features of Gorlin syndrome (many keratocysts) are marked in same way by shaded symbols. LOH analysis was performed only on twins' keratocysts. The Gorlin syndrome diagnosis was accepted only for the son (left dark symbol) with calcification in the falx cerebri (figure 4a) and LOH was detected only in his jaw keratocyst. Allele 3 (column L) was missing from the keratocyst lining in comparison to the surrounding tissue (column N) or constitutional DNA (column under dark symbol). LOH was not detected in keratocysts of other son (data not shown). According to this analysis, allele 1 was affected allele, alleles 2 and 3 were wild-type alleles, and father was homozygous and noninformative for this marker.

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Received: September 7, 1999

Accepted: October 24, 1999

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