# Mutation in exon 7 of PTCH deregulates SHH/PTCH/SMO signaling: Possible linkage to WNT

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**Abstract.** The novel PTCH mutation and clinical manifestations within Gorlin syndrome family links PTCH haploinsufficiency and aberrant activation of the Wnt pathway. We report a family case with Gorlin syndrome, characterized by the usual phenotype features such as widespread basocellular tumors and craniofacial and bone malformations, but also including a less common appearance of craniopharyngioma. These clinical manifestations might be associated with a novel constitutional mutation of the PTCH gene, 1047insAGAA, which we found in exon 7. It changes the normal amino acid sequence leading to termination of the PTCH protein at exon 9. The analyzed tumors of the family show extensive loss of heterozygosity in the PTCH region, both basocellular and in particular craniopharyngioma, and in the latter a high expression of ß-catenin was detected. Our findings suggest involvement of the SHH/PTCH/SMO pathway in pathogenesis of the analyzed disorders, including its possible contribution to aberrant activation of the Wnt pathway in craniopharyngioma.

## Introduction

The Nevoid basal cell carcinoma syndrome (NBCCS) or Gorlin syndrome is a rare autosomal dominant disorder characterized by diverse tumors and malformations. Most frequent are multiple basal cell carcinomas (BCC), medulloblastomas, meningiomas, fibromas of the ovaries and heart; cysts of the jaws, pits of the palms and soles, and diverse developmental abnormalities, often including rib and craniofacial alterations and sometimes polydactyly, syndactyly, and spina bifida (1).

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This heritable disease is associated with mutations in PTCH, a human homologue of the *Drosophila* segment polarity gene patched, which maps to chromosome 9q22.3, and loss of heterozygosity (LOH) at this site in both sporadic and hereditary basal cell carcinomas, medulloblastomas and ovarian fibromas suggests that it functions as a tumor suppressor (2).

PTCH is a member of the Hedgehog/Patched (or SHH/SMO/PTCH) signaling pathway, which plays a fundamental role during development and appears to underlie many disease states when misregulated (3). PTCH gene plays a crucial role in the pathway auto regulation; in many cases of BCC, pathway misregulation appears to be caused by mutations of this gene, although other pathway genes may be altered instead, such as SHH. Increased PTCH expression in tumor tissues (especially in case of at least partly non-functional protein) is thought to be an indicator of the pathway misregulation (4).

Craniopharyngiomas account for 1-5% of all intracranial tumors. They are the most common intracranial neoplasm of non-glial origin in the pediatric population, accounting for ~9% of all pediatric tumors (5). Two clinicopathological forms are distinguished, the adamantinomatous and the papillary craniopharyngioma. There is no established genetic susceptibility, and generally genetic alterations in craniopharyngioma are poorly understood. A most consistent genetic feature appears to be that ß-catenin gene mutations are found in all cases of the adamantinomatous and in none of the papillary craniopharyngiomas (6).

The ß-catenin or CTNNB1 gene is a member of the Wnt pathway, another important pathway in the developmental process. Misregulation of the Wnt pathway during organogenesis results in severe abnormalities, but the pathway is also implicated in several neoplasms such as colon cancer and pilomatricoma (7). Mutations of the ß-catenin gene (associated with its high expression) in adamantinomatous craniopharyngioma are hypothesized to induce an aberrant reactivation of the Wnt pathway, which is responsible for specific histological structuring of the tumor (8).

However, potential activation of the Wnt pathway has also been associated with upregulation of the downstream genes in the Hedgehog/Patched pathway (4), so that both pathways could be implicated in craniopharyngioma. Among the scarce recently published data relevant to such hypothesis, however,

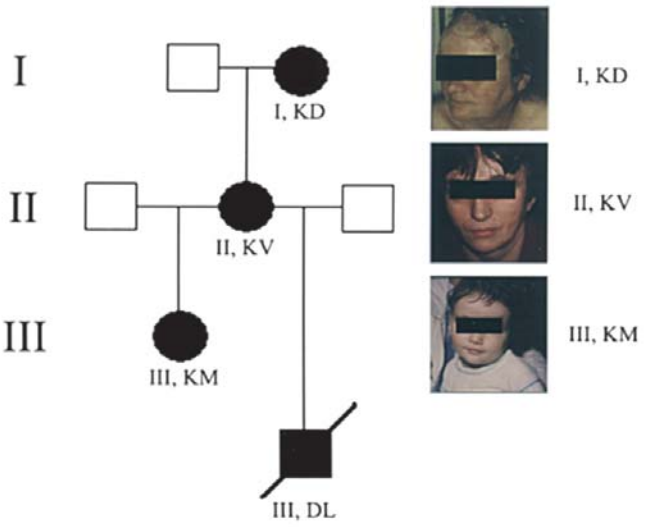


Figure 1. The family tree showing the affected members: grandmother (I-KD), mother (II-KV), daughter (III-KM), and son (III-LD) who died at the age of 9 months.

Table I. The family clinical data.

| Patient | Clinical features |
|---|---|
| I-KD | BCC, calcified falx cerebri, palm pits, calcified falx cerebri, strabismus (6) |
| II-KV | BCC, odontogenic keratocysts, facial dismorphology, ovarian cysts, strabismus (7) |
| III-KM | Craniopharyngioma at the age of 4 years (8), frontal bossing |
| III-LD | Medulloblastoma at the age of 8 months |

the most decided statement is made in a study on the clonal composition of adamantinomatous type, which claims 'little if any contribution of PTCH' in these tumors (9).

## Materials and methods

*Clinical evaluation of patients*. We present a family case, for which some data were published earlier (10-12). The family tree (Fig. 1) shows 4 affected members through three generations. Clinical data are summarized in Table I. Grandmother (I-KD): the patient was described previously (10). No genetic analyses were performed. Mother (II-KV): the patient was described previously (11).

Daughter (III-KM): at the age of 6 ophthalmologic disturbances and retarded development were indications for surgical excision of craniopharyngioma. Microneurosurgery successfully removed the tumor, which was a suprasellar cystic mass about 2 cm in size with calcifications (Fig. 2). After surgery ophthalmologic examination showed recovery, and neurological status has been normal for several years. Pathohistological analysis confirmed adamantinomatous

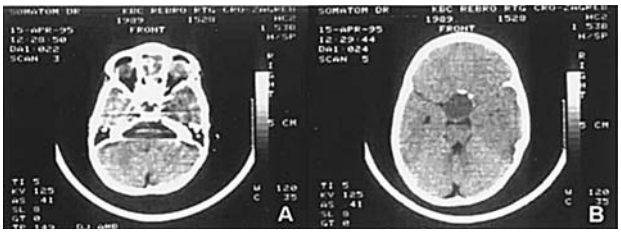


Figure 2. CT images before the surgical removal of craniopharyngioma in 1995.

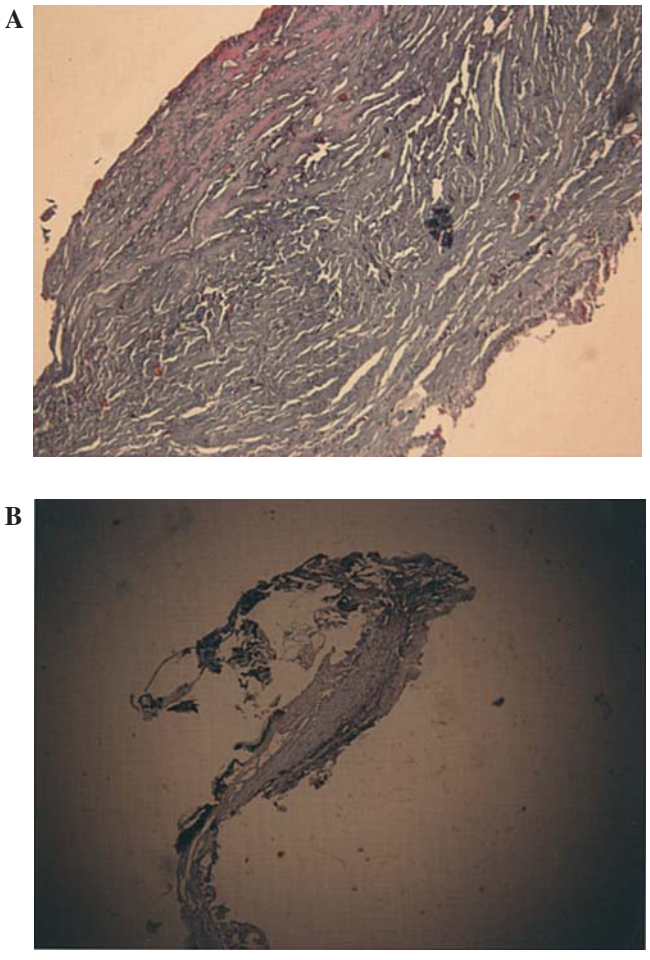


Figure 3. H&E staining (A) and EMA staining (B) of the daughter's craniopharyngioma slice.

craniopharyngioma, visualized as structures with strong connective tissue, squamous epithelia with gaps of cholesterol crystals, rigid cytoplasm and hyperchromatic oval nuclei (Fig. 3A). Craniopharyngioma samples were tested for EMA (Fig. 3B) and chromogranine antibodies to determine the position of tumor tissue in the sample. EMA positive and chromogranine negative areas were selected for analysis. Son (III-LD): at the age of 8 months medulloblastoma was found and removed, patient died a month later. No genetic analyses were performed.

For this study we have analyzed PTCH exons (dHPLC and sequencing) in blood and tumors of the mother (BCC) and daughter (craniopharyngioma), performed

Table II. Summary of LOH analysis of the tumor samples.

| Patient sample | D9S127 | D9S180 | PTCH intra | D9S287 | D9S196 |
|---|---|---|---|---|---|
| II-KV BCC | ho | het | het | LOH (8) | LOH |
| III-KM craniopharyngioma | ho | LOH | ho | LOH (8) | ho |

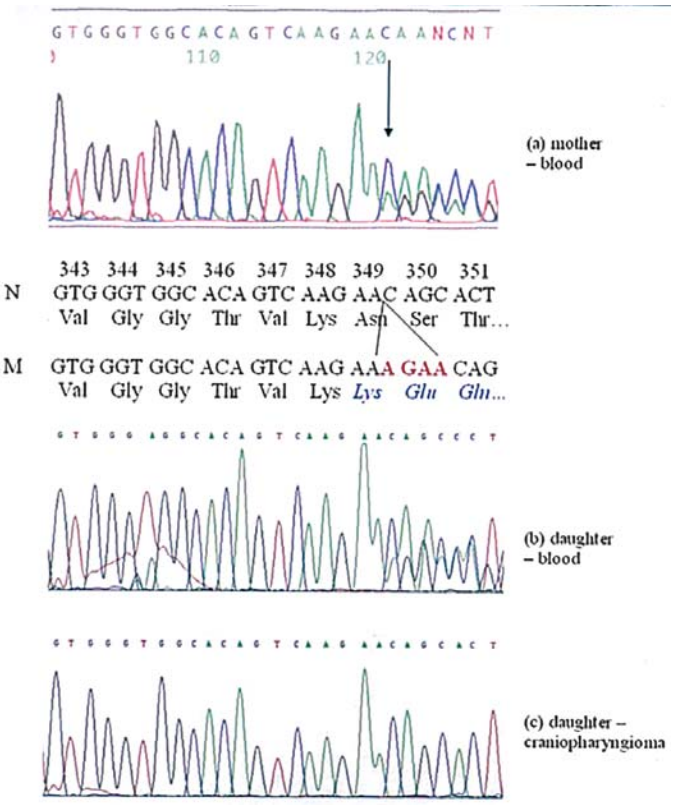


Figure 4. Sequence analysis of PTCH exon 7. Constitutional mutation 1047insAGAA, causing frameshift at codon 349 and stop at 436, is seen in the mother's (a) and daughter's (b) blood samples. The codon and aminoacid sequence (N) (upper row) corresponds to the wild-type databank (www.ncbi.nlm.nih.gov), whereas the lower row describes the mutated allele. The same locus in the daughter's craniopharyngioma (c) reveals deletion of the mutated allele.

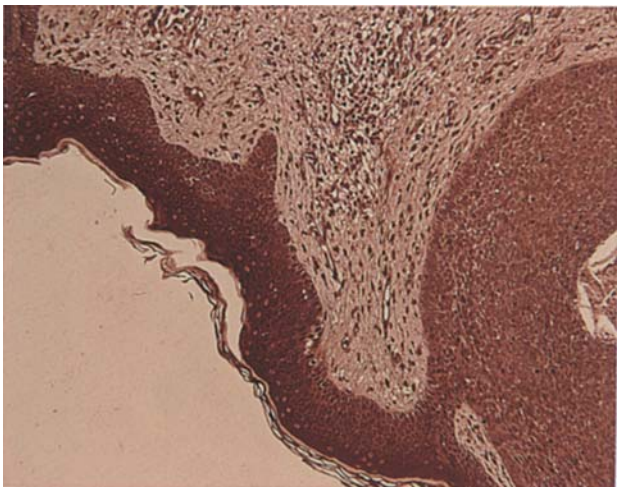

Figure 5. Immunohistochemical staining of the mother's BCC with PTCH antibody.

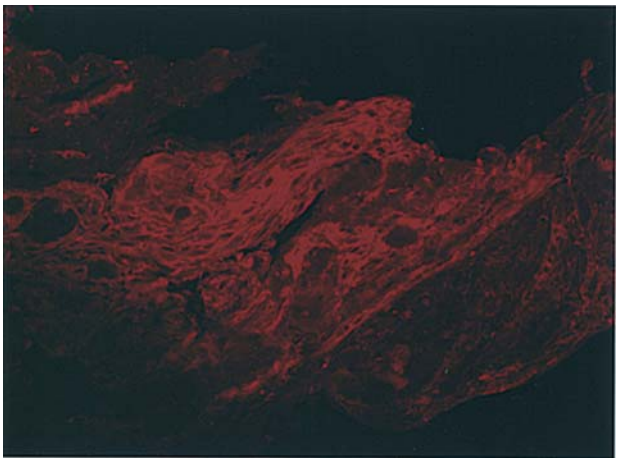

Figure 6. Immunohistochemical staining of the daughter's craniopharyngioma with ß-catenin antibody.

immunohistochemical staining of the tumors, and extended previous LOH analysis (12) of the tumors.

*LOH and sequencing*. DNA samples from blood leukocytes and fresh or paraffin tumor tissues were extracted by standard methods. DNA samples were typed for five short tandem repeat polymorphisms, D9S127, D9S180, PTCHintra, D9S287, D9S196, spanning chromosome region 9q22.3-31. PCR reaction was performed in a 25 $\mu$l reaction mixture containing 100 ng of template DNA or 1-5 $\mu$l of crude extract from paraffin-embedded tissue prepared after microdissection, 200 $\mu$mol/l deoxynucleoside triphosphate (dNTP), 1.5 mmol/l $MgCl_2$, 10 pmol/l each primer, 1.25 U Taq polymerase (AmpliTag Gold® DNA polymerase, Roche Molecular Systems, Inc., Mannheim, Germany) in 10 mmol/l Tris-HCl buffer. The PCR system (Gene Amp PCR system 2400, Applied Biosystems, Foster City, CA, USA) was set for 25-35 cycles, with the parameters described previously (11).

For LOH analysis, fluorescent forward primers were used. Five PCR products (1 $\mu$l each) were mixed with HiDi formamide (Applied Biosystems) and loaded on ABI PRISM-310 automatic sequencer (Applied Biosystems), and the analysis was made using Gene Mapper, version 3.0.

PCR products for the 23 exons were analyzed with dHPLC (denaturing high-performance liquid chromatography) WAVE DNA fragment analysis system (Transgenomic, Omaha, USA). Briefly, PCR products were subjected to an

additional denaturation for 10 min at 95˚C, followed by 30 min reannealing, and were loaded on the dHPLC.

Only the exons with heteroduplex were additionally sequenced. Before sequencing, amplified PCR products were purified with the QIAquick purification kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. PCR products were then sequenced in both directions by using the same primers and the Big Dye terminator cycle sequencing kit (Applied Biosystems, Weiterstadt, Germany). Sequencing analysis was performed on an automatic sequencer (ABI PRISM-377 DNA, version 2.1.1, PE, Applied Biosystem Division).

*Immunohistochemistry*. Immunohistochemical analysis was performed on 5 $\mu$m paraffin slides. The slides were deparaffinized, placed into prewarmed Epitope retrieval solution (DakoCytomation, code no. OA315) and kept at 95-99˚C for 40 min. The slides were cooled down to room temperature and then washed in PBS 3x5 min.

Immunohistochemical analysis on BCC and craniopharyngioma was performed for PTCH. Primary anti-PTCH-CTP rabbit antibody (a kind gift from Dr Allen Bale) diluted 1:200 was applied. Secondary goat anti-rabbit antibody (Dako, code no. Z0421) was used with PAP, rabbit (Dako, Code No. Z0133), and DAB (Dako).

ß-catenin immunofluorescence was performed for craniopharyngioma with ß-catenin mouse antibody (BD Transduction Laboratories, category no. 610153) diluted 1:100, and slides were covered with fluorescence mounting medium (Dako, code no. S3023) immediately after treatment with the secondary Cy3-conjugated donkey anti-mouse antibody (Johnson Immunoresearch Laboratories, code no. 715-165-150).

## Results

*Mutation analyses of PTCH*. After dHPLC screening of blood samples, which showed alterations only in exon 7 both for the mother (II-KV) and the daughter (III-KM), direct sequencing revealed the same constitutional mutation in both subjects. It is a novel mutation 1047insAGAA in exon 7, which leads to frameshift stop at codon 436, resulting in truncated protein (Fig. 4a and b).

Sequencing exon 7 in tumor tissue, we found loss of the mutated allele in the daughter's craniopharyngioma (Fig. 4c), whereas in the mother's BCC both mutated and wild-type alleles were present (sequence not shown, similar to Fig. 4a).

Complete results of the previous and present LOH analyses of these tumors are shown in Table II. In the mother's BCC, LOH was found for two markers (D9S287 and D9S196) from the set of five markers, as well as in the daughter's craniopharyngioma (D9S180 and D9S287). In addition, it can be seen that craniopharyngioma is not heterozygous for any of the markers used (due to constitutional homozygosity for three markers).

*Expression analyses*. Immunohistochemically we showed high PTCH expression in BCC (Fig. 5), while no PTCH reactivity could be detected in craniopharyngioma. However, in craniopharyngioma fluorescent staining showed highly positive reaction for ß-catenin (Fig. 6).

## Discussion

We presented a family case with a novel germline PTCH mutation of exon 7 in two generations. This mutation is the only constitutive alteration of the PTCH gene exons found in the mother and her daughter. Whereas the daughter has obviously inherited the mutation, it cannot be established whether the same is true for the mother. A different PTCH mutation has recently been reported at the same codon in sporadic BCC (13).

The role of the PTCH mutation in clinical manifestations within our Gorlin syndrome family was not easy to assess. Its immediate consequence, PTCH haploinsufficiency, has generally been hypothesized as the most likely cause of developmental malformations in Gorlin syndrome (14). However, the possibility that cancerogenesis might be associated with such PTCH haploinsufficiency is a more recent and debatable hypothesis (15).

In this case, neither the mother's BCC nor the daughter's craniopharyngioma are caused by the classical second hit, which would disable the wild-type homologue of the mutated allele and result in full PTCH inactivation. In BCC the mutated locus is the same as in the patient's blood, and in craniopharyngioma only the mutated allele is deleted. However, LOH analysis shows that in both cases PTCH locus in tumor tissues has been additionally altered. Although less specific, this is strong evidence that the gene is involved in development of these tumors.

The decisive role of the SHH/SMO/PTCH pathway in BCC genesis is generally recognized, and it is certainly not always attributable to PTCH inactivation. In our case, high PTCH immunostaining of BCC epithelial tissue indicates an increased pathway activity (16), which is consistent with the PTCH haploinsufficiency, although it might have actually been induced by some other and undetected alteration of another pathway gene.

However, for the craniopharyngioma, the role of the SHH/SMO/PTCH pathway is unclear. The tumor has been reported as one of less pronounced features of Gorlin syndrome (1), but no PTCH alterations were found in a recent study of a sizable set of adamantinomatous craniopharyngioma in general population (9).

Even the constitutional PTCH mutation of our patient is not found in her craniopharyngioma, which is due to loss of heterozygosity at the mutated site. On the other hand, this LOH, together with two other markers showing LOH in the region (and the high homozygosity of the region), strongly suggest malfunctioning of the SHH/SMO/PTCH pathway in this tumor. Furthermore, such malfunctioning may cause aberrant activation of the Wnt pathway through downstream targets from Gli family, which could perhaps account for the increased ß-catenin immunostaining.

Finally, the loss of mutated exon 7 allele, which we confirmed in several samples from the tumor, indicates monoclonal origin of this craniopharyngioma.

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