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Molecular mechanism of extracutaneous tumours in patients with basal cell nevus syndrome

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SUMMARY
Basal cell nevus syndrome (BCNS) is a rare genetic disorder accompanied by a broad variety of tumours, of which basal cell carcinomas and odontogenic keratocysts are the most common. BCNS is caused by a germline or postzygotic mutation in either PTCH1 or SUFU. As BCNS is a rare disease, it is difficult to establish whether less frequently occurring tumours are actually part of the syndrome. In this study, the molecular mechanism behind four extracutaneous tumours in patients with BCNS was elucidated. A leiomyoma of the testis and meningioma were confirmed to be associated with BCNS in two patients by presence of a second mutation or loss of heterozygosity in PTCH1. In a meningioma of a patient with a mosaic postzygotic PTCH1 mutation an association could not be conclusively confirmed. SUFU was probably not involved in the development of a thyroid carcinoma in a patient with a germline SUFU mutation. Hence, we have proven that meningioma and leiomyoma of the testis are rare extracutaneous tumours that are part of BCNS.

INTRODUCTION
Basal cell nevus syndrome (BCNS, OMIM #109400), also known as Gorlin syndrome, is a rare autosomal dominant disorder characterised by multiple basal cell carcinomas (BCCs), odontogenic keratocysts and calcification of the falx cerebri. Besides these symptoms, multiple developmental defects and a variety of other tumours have been described. BCNS is usually caused by a germline mutation in the patched-1 (PTCH1) gene. The PTCH1 gene encodes for the patched-1 protein, which acts as a tumour suppressor gene (TSG) in the sonic hedgehog (SHH) signalling pathway. Patched-1 inhibits the protein smoothened (SMO). If this inhibition is released, SMO can translocate into the cell and eliminate the binding and anchoring of the glial-derived oncogene (GLI) transcription factors by the suppressor of fused (SUFU). This, in turn, causes GLI transcription factors to become active (GLIA), which leads to proliferation, suppression of apoptosis and angiogenesis. Other causative mutations for the BCNS phenotype are germline mutations in SUFU or postzygotic mutations in PTCH1 or SMO. According to the two-hit hypothesis, a mutation needs to be accompanied by a second hit in the wild-type allele of a TSG leading to its inactivation, for example, a second mutation, gene loss or a promoter hypermethylation event, in order to result in loss of functionality and subsequent induction of tumorigenesis. In several BCNS-related tumours PTCH1 mutation with loss of heterozygosity (LOH) has been described. Alternatively, haploinsufficiency might occur. In many less frequently reported BCNS tumours, the molecular mechanism behind tumour formation has not been examined yet. In this study, we investigated the extracutaneous tumours of four individuals with a BCNS phenotype caused by a germline/postzygotic mutation in PTCH1 or SUFU using targeted next-generation sequencing (NGS).

Case 1
A male patient had numerous histopathological proven BCCs and a family history with a PTCH1 germline mutation, c.747–2A>G, located in the splice acceptor site of intron 5 (previously published). Besides BCCs, the patient also had segmentally distributed neurofibromas without other features of neurofibromatosis. DNA analysis on blood revealed no germline mutations in NF1 or SPRED1. However, on DNA extraction of three independent neurofibromas, a shared NF1 mutation was found (c.6522_6523dup p.(Thr2175Argfs*5), located in exon 43, NM_000267.3) and the patient was diagnosed with type I segmental mosaicism for neurofibromatosis.

In his late 40s, he presented with a meningothelial meningioma, which was surgically removed. SmMIP genetic analysis of the meningioma was performed to determine whether the meningioma developed as a result of a second hit in either PTCH1, NF1 or occurred sporadically through mutations in for example NF2, frequently involved in meningioma tumour formation. The variant allele frequency (VAF) of the PTCH1 germline mutation was 91% in the sample (90% tumour cells), indicating LOH. TSO500 confirmed this finding (table 1) and did not reveal mutations in NF1, NF2 or other high VAF of possible tumourigenesis initiating driver mutations. We, therefore, conclude LOH of PTCH1 was the oncogenic initiating event in the meningioma.

Case 2
A female patient presented with multiple BCCs and OKCs. Mid 40s, she presented with a mixed type meningioma, which was surgically removed. The clinical suspicion of BCNS could not be confirmed genetically, since no variant was detected after PTCH1 and SUFU analysis in DNA isolated from...
blood. To exclude BCNS on the basis of postzygotic mosaicism, formalin fixed, paraffin embedded (FFPE) samples from two BCCs and the meningioma were analysed. All samples demonstrated the same *PTCH1* mutation in exon 15 (c.2359G>T, p.Glu787*) and the patient was diagnosed with type I segmental mosaicism for BCNS. In both BCCs either a second hit or LOH of *PTCH1* was seen (table 1). To test the hypothesis that the postzygotic mutation contributed to the development of the meningioma, we sought for other variants or possible LOH of *PTCH1* in the meningioma sample. Only the known mutation was found with the smMIP-NGS approach and TSO500 confirmed the increased presence of this *PTCH1* variant (VAF 39%, 90% tumour cells compared with an undetectable variant in blood). Furthermore, TSO500 identified a loss of heterozygosity of the *PTCH1* mutation, that is, c.1022+1G>A, located in the splice donor site of intron 8, in peripheral blood. In the literature, only a few patients have been described with a *SUFU* germline mutation, including the mutation detected here, and in one of them a thyroid carcinoma was reported. To test the hypothesis that the *SUFU* germline mutation could be underlying to thyroid carcinoma development, smMIP analysis on resection material of the thyroid carcinoma (90% tumour cells) was performed and the *SUFU* germline mutation was detected. No additional variants were detected in *SUFU*. TSO500 analysis performed on the thyroid carcinoma sample confirmed the germline *SUFU* mutation c.1022+1G>A with a VAF of 43.9% without an additional *SUFU* mutation. Several additional relevant mutations were found table 1, of which *BRAF* c.1799T>A, p.V600E (33%) was assumed to be the most likely oncogenic driving event in the thyroid carcinoma.

**Case 4**

A male patient presented with multiple BCCs, numerous trichoepithelioma and milia on the face, and epidermoid cysts from his early 60s. A year later, he developed a papillary thyroid carcinoma. Because of the high number of BCCs and the typical coarse facial features BCNS was suspected, but *PTCH1* analysis of the blood DNA revealed no pathogenic mutation. Additional analysis of *SUFU* detected a heterozygous germline mutation, that is, c.1022+1G>A, located in the splice donor site of intron 8, in peripheral blood. In the literature, only a few patients have been described with a *SUFU* germline mutation, including the mutation detected here, and in one of them a thyroid carcinoma was reported.

### DISCUSSION

In this study, the molecular mechanism underlying the development of extracutaneous tumours in four individuals with a BCNS phenotype was elucidated. Two individuals, one with a *PTCH1* germline mutation (case 1) and one with a postzygotic *PTCH1* mutation (case 2), presented with a meningioma. The meningioma that developed in the patient with a heterozygous germline *PTCH1* mutation (case 1) was initiated by LOH of *PTCH1*. This is consistent with previous findings in one patient. In the other meningioma (case 2), no second hit (mutation) or LOH

### Table 1 Results of TSO 500 NGS analysis and smMIP analysis of four extracutaneous tumours in BCNS patients

<table>
<thead>
<tr>
<th>Case</th>
<th>Tumour, % tumour cells</th>
<th>Gene</th>
<th>cdna</th>
<th>Protein</th>
<th>Allele frequency</th>
<th>Depth</th>
<th>Genomic position (GRCh37)</th>
<th>Exon</th>
<th>Consequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Meningioma, 90% tumour cells</td>
<td>PTCH1</td>
<td>c.747–2A&gt;G</td>
<td>p.?</td>
<td>90%</td>
<td>82</td>
<td>98 242 872</td>
<td>i5</td>
<td>Splice Acceptor SNV</td>
</tr>
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<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Meningioma, 90% tumour cells</td>
<td>MSH6</td>
<td>c.1052_1053 dup</td>
<td>p.V352Tfs*20</td>
<td>9%</td>
<td>148</td>
<td>48 026 172</td>
<td>4</td>
<td>Frameshift</td>
</tr>
<tr>
<td>3</td>
<td>Leiomyoma, 80% tumour cells</td>
<td>PTCH1</td>
<td>c.2308C&gt;T</td>
<td>p.R770*</td>
<td>52%</td>
<td>168</td>
<td>98 229 650</td>
<td>15</td>
<td>Nonsense</td>
</tr>
<tr>
<td>4</td>
<td>Thyroid carcinoma, 90% tumour cells</td>
<td>BRAF</td>
<td>c.1799T&gt;A</td>
<td>p.V600E</td>
<td>33%</td>
<td>177</td>
<td>140 453 136</td>
<td>15</td>
<td>Missense</td>
</tr>
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</tr>
</tbody>
</table>

Only (likely) pathogenic (class 4 and 5) mutations are listed. *LOH confirmed with multiplex ligation-dependent probe amplification analysis. I would prefer to use soething else than a * to mark this footnotes as there are many * signs in the table because the sign is used in mutation nomenclature. BCNS, basal cell nevus syndrome; LOH, loss of heterozygosity; NGS, next-generation sequencing.
of PTCH1 could be detected. Still, involvement of PTCH1 in the tumourigenesis of this meningioma could be considered due to the fact that the postzygotic mutation is more prominently present in the meningioma. Haploinsufficiency of PTCH1, however, is less likely as a cause of tumourigenesis: according to the haploinsufficiency theory, mutations in TSG leading to haploinsufficiency usually occur in genes involved in DNA repair or chromosomal segregation, which is not the case for PTCH1.16

TSO500 NGS analysis also identified an additional pathogenic driver variant in NF2, which is a commonly mutated in meningiomas.12 Consequently, LOH of NF2 could also have been the tumour initiating event of the meningioma.

One individual (case 3) presented with a testicular leiomyoma, a rare benign smooth muscle tumour that has not been previously reported in patients with BCNS. Leiomyomas that have been described in BCNS were located in the stomach, in the kidney and in an ovary.17 18 Only in the ovarian leiomyoma mutation analysis was performed and it revealed a second hit in PTCH1, resulting in LOH.17 We found a second variant in PTCH1 in the testicular leiomyoma, but could not demonstrate whether both variants were located on the different alleles. The pathogenicity of this somatic second hit has not yet been proven and is based on theoretical variant classification (ACMG guidelines, variant of unknown clinical significance (class 3)).19 However, no other driver gene mutations were found and it is very likely that this second variant in PTCH1 is the second hit promoting tumourigenesis.

Thyroid carcinoma is rarely detected in patients with BCNS. After an extensive literature search, we found four reports of patients with a BCNS phenotype who developed a thyroid carcinoma. One patient with a SUFU germline mutation received chemotherapy for a medulloblastoma and developed a papillary thyroid carcinoma, of which no further genetic analysis was performed.14 In one patient with a medullary thyroid carcinoma at 32 years old,20 no germline mutation in PTCH1 was detected and SUFU was not analysed.20 The patient had no odontogenic keratocysts but did develop a medulloblastoma in childhood, features that are more linked to heterozygous SUFU patients.21 In two other patients with BCNS features and a thyroid carcinoma, no additional information was given regarding genetic analyses.22 In our patient, no second mutation or LOH in SUFU in the thyroid carcinoma was found, and therefore, we cannot conclude that the thyroid carcinoma is induced by SUFU loss of function. NGS analysis, however, did reveal an activating mutation in BRAF, which is a common driver of papillary thyroid carcinoma. This mutation thus is most likely the initiating oncogenic event in this case.

In conclusion, elucidating the molecular mechanisms underlying less common tumours in rare syndromes can provide evidence for associations between specific tumours and a syndrome. This is the first report proving that PTCH1 can be responsible for the development of a leiomyoma of the testis. This information is important to completely understand the pathogenesis of BCNS and also to raise awareness for physicians treating patients with BCNS that also leiomyoma of the testis can be associated with BCNS. We confirmed previous findings that meningiomas are associated with BCNS caused by a PTCH1 germline mutation, but did not find any evidence that thyroid carcinomas are associated with a germline SUFU mutation.

METHODS

DNA extraction

DNA was extracted from peripheral blood and FFPE tumour samples using the DNeasy Blood&Tissue Kit (Qiagen) and The Maxwell RSC-DNAFFPE-Kit (Promega), respectively. DNA from peripheral blood was analysed with the BigDye V.1.1 sequencing kit, ABI3730 DNA analyzer (Applied Biosystems, primer sequences on request) and multiplex ligation-dependent probe amplification analysis for the PTCH1 gene (kit-P067-B3, MRC Holland). DNA from tumour samples was analysed using single molecule molecular inversion probes (smMIPs) limited to
genes of the SHH signalling pathway followed by NGS and NGS with TruSight Oncology500 panel (TSO500, Illumina).

SmMIPs (826 probes, available on request) were limited to PTCH1, PTCH2, SMO and SUFU (respectively, NCBI RefSeq: NM_000264.3, NM_003738.4, NM_005631.4 and NM_01619.3). Also NFI and SPRED1 were analysed using smMIPs (respectively, NCBI RefSeq: NM_000267.3 and NM_152594.3). Mutation detection was performed using the NextSeq-500 (Illumina) with manufacturer’s materials and protocols. Library preparations (ThermoFisherScientific) and sequencing was performed as described earlier. Variant filtering and interpretation was achieved with Alamut V.2.11 and the Catalogue Of Somatic Mutations in Cancer (COSMIC). Variant classification was performed according to the American College of Medical Genetics and Genomics (ACMG) guidelines. SmMIPs were used to identify germline/postzygotic mutations and identify a shared mutation in the different tumour samples from an individual patient.

**TSO500**

The TSO500 panel (20028216; Illumina) was used to confirm mutations found and identify other relative pan-cancer genes in the tumour samples. The TSO500 panel included full coding of 523 pan-cancer genes and detected single nucleotide variants, indels, copy number variations, fusions and immunoenoclogy biomarkers as well as tumour mutational burden and microsatellite instability. Library preparations were performed using genomic DNA according to the manufacturers’ instructions. Data analysis was performed using the TSO500 local app, and variants were classified subsequently using the inline Varsome application (https://varsome.com).

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**Contributors** BJA and GMJMR contributed equally to this work. BJA, JHMS, EV and GMJMR processed and sequenced the samples. GMJMR and MvG contributed equally to this work. BJA, JHMS, EV and GMJMR contributed into interpreting the data, and critically reviewing the manuscript. E-MAH performed histopathological assessment and provided images. BJA, JHMS, EV and GMJMR contributed into interpreting the data, writing the manuscript and critically reviewing the manuscript. E-MAH and EV contributed equally to this work.

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**Patient consent for publication** Not applicable.

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**REFERENCES**


