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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.[1] Besides these symptoms, multiple developmental defects and a variety of other tumours have been described.[2] 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 glioma-associated oncogene (GLI) transcription factors by the suppressor of fused (*SUFU*). This, in turn, causes GLI transcription factors to become active (GLIA),[3] 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*.[2 4 5] According to the two-hit hypothesis,[6] 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 tumourigenesis. In several BCNS-related tumours *PTCH1* mutation with loss of heterozygosity (LOH) has been described.[7–9] Alternatively, haploinsufficiency might occur.[10] 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).[11] 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.[12] 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

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

Case	Tumour, % tumour cells	Gene	cdna	Protein	Allele frequency	Depth	Genomic position (GRCh37)	Exon	Consequence
1	Meningioma, 90% tumour cells	PTCH1	c.747–2A>G	p.?	90%	82	98 242 872	i5	Splice Acceptor SNV
		MSH6	c.1052_1053 dup	p.V352Tfs*20	9%	148	48 026 172	4	Frameshift
2	Meningioma, 90% tumour cells	PTCH1	c.2359G>T	p.E787*	39%	273	98 229 599	15	Nonsense
		NF2	c.301del	p.Y101Ifs*22	69%	136	30 035 135	3	Frameshift
	Basal cell carcinoma number 1 (without the * please) *	PTCH1	c.2359G>T	p.E787*	38%	133	98 229 599	15	Nonsense
		PTCH1	c.2524_2548delins23	p.(Lys842Aspfs*5)	11%	114	98 229 434	15	Frameshift
		PTCH1	c.2588_2589delinsAA	p.(Trp863*)	24%	37	98 224 253	16	Nonsense
	Basal cell carcinoma number 2 (without the * please)*	PTCH1	c.2359G>T	p.E787*	67%* Change this footnote sign please	226	98 229 599	15	Nonsense
3	Leiomyoma, 80% tumour cells	PTCH1	c.2308C>T	p.R770*	52%	168	98 229 650	15	Nonsense
		PTCH1	c.2542_2544del	p.F848del	36%	122	98 229 413	15	In Frame Deletion
4	Thyroid carcinoma, 90% tumour cells	SUFU	c.1022+1G>A	p.?	44%	155	104 359 302	i8	Splice Donor SNV
		BRAF	c.1799T>A	p.V600E	33%	177	140 453 136	15	Missense
		ARID1A	c.1558C>T	p.Q520*	33%	300	27 057 850	3	Nonsense

Only (likely) pathogenic (class 4 and 5) mutations are listed.[19]
*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.

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 *PTCH1* mutation in blood). Furthermore, TSO500 identified a loss of function variant in *NF2*, c.301del p.(Y101Ifs*22), with a high VAF of 69%. These findings did not provide enough evidence to conclude which mutation, in *PTCH1* or *NF2*, initiated tumourigenesis of the meningioma.

Case 3

A male patient presented with multiple BCCs and OKCs since his early teenage years. Mutation analysis of the peripheral blood detected a single heterozygous mutation in *PTCH1*, ie, c.2308C>T p.(Arg770*), located in exon 15. In his late teenage years, he presented with a testicular leiomyoma that was surgically removed (figure 1). To confirm the contribution of the germline *PTCH1* mutation to the development of the leiomyoma, smMIP mutation analysis of the leiomyoma (80% tumour cells) was performed and revealed the germline mutation and a second variant in *PTCH1*, ie, c.2542_2544del p.(Phe848del). These variants were also detected using the TSO500 analysis (*PTCH1*, c.2308C>T, VAF 52.4% and *PTCH1*, c.2542_2544del, VAF 36.1%) (table 1). No other explanatory driver mutations were found. These data imply that the leiomyoma was initiated due to a somatic second hit (mutation) in the *PTCH1* gene.

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,[13] and in one of them a thyroid carcinoma was reported.[14] 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.

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.[15] In the other meningioma (case 2), no second hit (mutation) or LOH

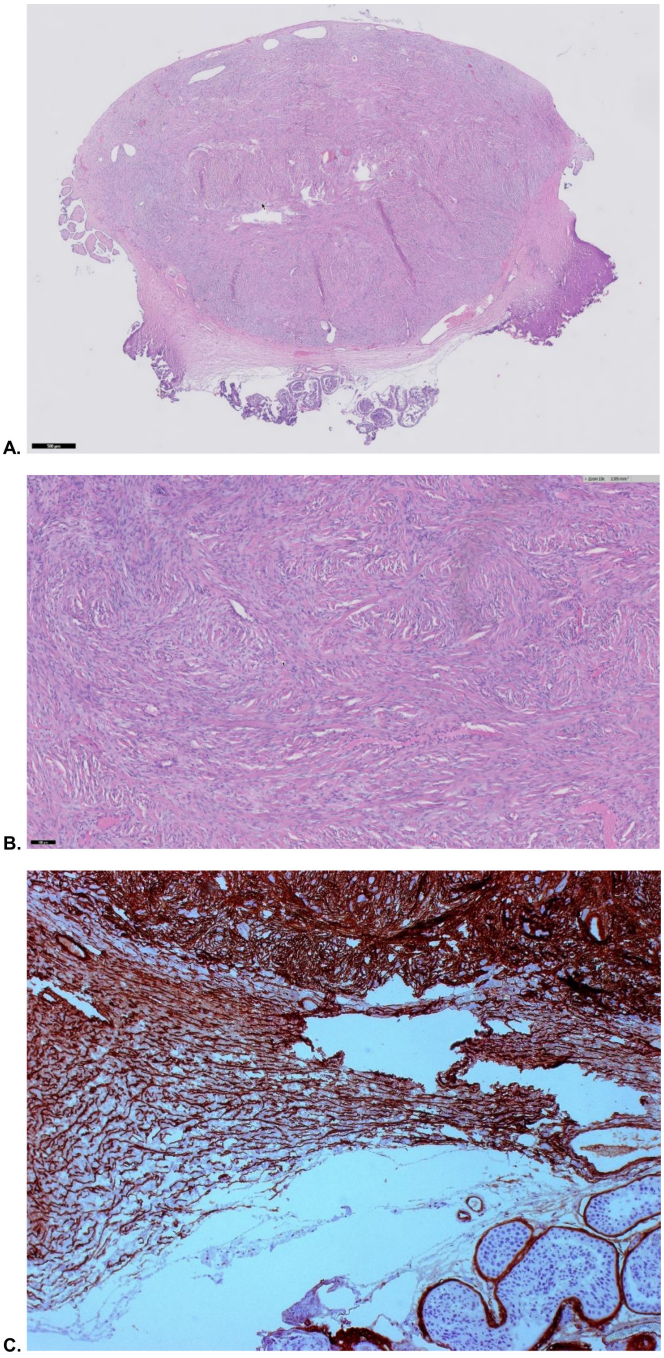

Figure 1 (A) Excision sample of the testicular tumour shows a well-defined, non-encapsulated lesion originated from the tunica albuginea. H&E stain, original magnification. (B) The lesion consists of intersecting bundles of non-atypical spindle-shaped cells in a background of collagenous fibres. There are scattered blood vessels of which the walls show continuity with the lesion. There is no mitotic activity or necrosis. H&E stain, ×100. (C) Positive alpha-smooth muscle actin staining led to the diagnosis of testicular leiomyoma.

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-DNA-FFPE-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 *NF1* 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.[23] Variant filtering and interpretation was achieved with Alamut V.2.11 (Interactive Biosoftware) and included public databases like the Genome Aggregation Database (gnomAD) 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.[22] 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 immuneoncology 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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