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ORIGINAL ARTICLE

Genomic profiling of late-onset basal cell carcinomas from two brothers with nevoid basal cell carcinoma syndrome

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Abstract

Background Nevoid basal cell carcinoma syndrome (NBCCS) is an autosomal dominant genetic disorder. It is commonly caused by mutations in *PTCH1* and chiefly characterized by multiple basal cell carcinomas (BCCs) developing prior to the age of 30 years. In rare cases, NBCCS presents with a late onset of BCC development.

Objective To investigate BCC tumorigenesis in two brothers, who showed characteristic features of NBCCS but developed their first BCCs only after the age of 40 years. Two other siblings did not show signs of NBCCS.

Results We obtained blood samples from four siblings and nine BCCs from the two brothers with NBCCS. Whole exome sequencing and RNA sequencing revealed loss of heterozygosity (LOH) of *PTCH1* in eight out of nine tumours that consistently involved the same haplotype on chromosome 9. This haplotype contained a germinal splice site mutation in *PTCH1* (NM_001083605:exon9:c.763-6C>A). Analysis of germline DNA confirmed segregation of this mutation with the disease. All BCCs harboured additional somatic loss-of-function (LoF) mutations in the remaining *PTCH1* allele which are not typically seen in other cases of NBCCS. This suggests a hypomorphic nature of the germinal *PTCH1* mutation in this family. Furthermore, all BCCs had a similar tumour mutational burden compared to BCCs of unrelated NBCCS patients while harbouring a higher number of damaging *PTCH1* mutations.

Conclusions Our data suggest that a sequence of three genetic hits leads to the late development of BCCs in two brothers with NBCCS: a hypomorphic germline mutation, followed by somatic LOH and additional mutations that complete *PTCH1* inactivation. These genetic events are in line with the late occurrence of the first BCC and with the higher number of damaging *PTCH1* mutations compared to usual cases of NBCCS.

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Conflicts of interest

None declared by all authors.

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Introduction

Basal cell carcinomas (BCCs) are the most common skin cancers of the Caucasian population.[1] They grow slowly and rarely metastasize, with estimates as low as 0.05–0.88%.[2] Tumorigenesis is usually induced through hyperactivation of the Sonic Hedgehog (Hh) signalling pathway that is mostly caused by cumulative UV radiation (UVR) damage in basal cells along the sheathes of hair follicles.[3] Risk factors for developing BCCs include higher age (>60 years), fair skin types, immunodeficiency and long-term use of hydrochlorothiazide.[4,5] Nevoid BCC syndrome (NBCCS) is a rare genetic autosomal dominant disorder that is associated with the development of multiple BCCs.[6] 60% of cases are inherited and the rest result from *de novo* mutations.[7] The mean age of first BCC development in Western countries is in the early 20s, and most patients develop their first BCC before the age of 40.[6,8] In Japan, however, the mean age of first BCC appearance is 37.4 years for which the reason remains unknown.[9]

The commonest cause of NBCCS is pathogenic heterozygous germline variants in *PTCH1*, which encodes Patched-1, the key regulator of the G-protein coupled receptor Smoothened (SMO). Loss of Patched-1 leads to SMO activation with continuous signal transduction to GLI zinc finger transcription factors, resulting in hyperactivation of the Hedgehog (Hh) signalling pathway.[3] Damage to other Hh pathway genes has been found to cause NBCCS as well, including *PTCH2* and the Suppressor of Fused.[10,11] Recent studies suggest that BCCs harbour somatic driver mutations in other cancer genes, including *TP53*, the Hippo-YAP pathway genes, MYCN/FBXW7 signalling and the TERT-promoter region.[12,13] The average tumour mutational burden (TMB) in sporadic BCCs (spBCCs) is 65 mut/Mb, while the TMB of BCCs from NBCCS patients (NBCCS-BCCs) averages 21 mut/Mb.[12]

Nevoid BCC syndrome-BCCs are thought to develop according to the classic Knudson two-hit suppressor gene model: a heterozygous germline *PTCH1* mutation represents the first hit, followed by a somatic loss of heterozygosity (LOH) or UV-induced loss-of-function (LoF) mutation as a second hit.[14–16] The frequency of LOH in *PTCH1* among BCCs varies considerably among studies, ranging between 24% and 93% of studied tumours.[12,13,17] The majority of NBCCS cases are caused by non-sense mutations, frameshift indels, splice site mutations or exon losses. Coding and splice site mutations were identified in 61% of NBCCS.[16] Splice site mutations in *PTCH1* can cause NBCCS through formation of cryptic splice sites that lead to translation of a dysfunctional Patched-1 protein.[18,19]

We present a genetic analysis of four siblings from a family where two brothers have NBCCS with the first BCC developing after the age of 40 years. The aim of this study is to explore underlying somatic and germline mutations that may cause the unusually late development of BCCs in these two siblings with NBCCS and to explore common genetic patterns in tumorigenesis that will lead to a better understanding of the syndrome.

Materials and methods

Patients, blood and tissue sampling

The study was approved by the local Swiss Ethics committee (Ethikkommission Ostschweiz, EKOS 2017-00096) and conducted in accordance with the Declaration of Helsinki. Clinical assessment of the family members was performed at the Department of Dermatology of the Kantonsspital St. Gallen, Switzerland. Informed consent was retrieved from all patients prior to their inclusion into the study. Brother 1 and brother 2 fulfilled the clinical criteria of NBCCS. Brother 3 and the sister did not show any NBCCS symptoms. One family member rejected participation and was not included. Whole blood (EDTA) was acquired from all four siblings for DNA extraction from peripheral blood mononuclear cells (PBMCs). BCC tumour tissue was available from both brothers with NBCCS. From brother 1, we received one formalin-fixed paraffin-embedded (FFPE) BCC for DNA analysis (brother 1—BCC 1), one fresh BCC for DNA and RNA analysis (brother 1—BCC 2) and one healthy skin sample for RNA analysis. From brother 2, we received seven FFPE BCCs for DNA analysis (brother 2—BCC 1–7). All tumours were examined by an independent board-certified dermatopathologist. The BCCs were laser capture microdissected from their paraffin block (10-µm sections) using Arcturus PicoPure DNA Extraction Kit (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions. DNA extractions from PBMCs and tumour tissue were performed as previously described.[12] Punch biopsies were used for fresh BCC and healthy skin tissue. Sample details are provided in Table S1 (Supporting Information).

Sequencing and variant identification

We performed whole genome sequencing (WGS) on DNA from PBMCs of brother 1 and whole exome sequencing (WES) on DNA from PBMCs of the three other siblings in order to identify the germline variant predisposing to NBCCS. The sequencing libraries were prepared with Agilent SureSelect v5 exome enrichment kit (Agilent Technologies, Santa Clara, CA, USA) and

sequenced using Illumina 2500 (Illumina Inc., San Diego, CA, USA) with 2 × 100 bp reads. The whole genome of one of the brothers was sequenced using the BGISEQ-500 (BGI) platform with 2 × 100 bp reads. The raw FASTQ reads were aligned to the GRCh37-hg19 genome using BWA-MEM with default parameters.[20] The next steps of the file processing were performed using GATK software according to GATK Best Practices from the Broad Institute.[21] Calling of somatic SNVs was carried out with the GATK Mutect2 caller which used germline variant database gnomAD (http://gnomad.broadinstitute.org) with default parameters.[22] The resulting VCF files were filtered using GATK FilterMutectCalls and GATK VariantFiltration tools, resulting in high-quality somatic 'PASS' variants with at least one read from each strand and a minimum variant allele frequency of VAF >0.05. Finally, somatic variants were annotated with Oncotator software.[23] Mutational signatures of the studied samples were assessed using the MutationalPatterns R package (https://bioconductor.org/packages/release/bioc/html/MutationalPatterns.html; The R Foundation, Vienna, Austria).[24] The germline variant calls produced with GATK HaplotypeCaller were filtered with the BCFtools utility (http://samtools.github.io/bcftools)[25] for a minimum coverage (i.e. calls with fewer than 4× reads) and hard-filtered for quality (variants with quality <20). The resulting high-quality call set was then annotated for impact on protein sequence and/or splicing using the ANNOVAR tool (http://www.openbioinformatics.org/annovar).[26] Additional annotation of specific variants and estimated population frequencies was achieved through annotation with ANNOVAR against dbSNP135 (http://www.ncbi.nlm.nih.gov/snp), population frequency estimates from the 1000 Genomes Project (http://www.1000genomes.org), the National Institutes of Health Heart, Lung and Blood Institute Grand Opportunity Exome Sequencing Project (https://esp.gs.washington.edu/drupal/) and ~2500 control exomes that have been processed through the same bioinformatics analysis pipeline.[27]

VariantMaster tool was used to identify the germline variants most likely predisposing the two brothers to NBCCS.[28] Analysis was run in the mode of autosomal dominant inheritance with full penetrance. The variants were subjected to the following filtering parameters: The Genome Aggregation Database all population allele frequency (gnomAD_ALL): <0.001; SIFT version 2 score (ljb2_sift): <0.05; PolyPhen version 2 score (ljb2_pp2hvar): >0.95; Clinical GENetics software application variant (CGEN_-variants): 0; Variant Function (ExonicFunc): synonymous, intronic; 5UTR, 3UTR were excluded; Quality Score (QS): >100. Additionally, we annotated with ANNOVAR and manually checked all the non-coding variants from WGS sample in *PTCH1* gene and 2kbp upstream. Copy number analysis for the tumours was performed from WES and WGS data using NEXUS software.[29] The assessment of the tumour cell fraction with somatic driver events is described in detail in Appendix S1 (Supporting Information).

Sanger sequencing

Primers were designed for exon 9 of the *PTCH1* gene using the open-source software Primer3 2.3.4 (http://primer3.sourceforge.net; primer generation via Microsynth AG, Switzerland) and exon templates from the human genome assembly (GRCh37/hg19, *PTCH1* gene accession no. NCBI 5272). Exons were amplified via PCR with Phusion DNA Polymerase (New England Biolabs, Ipswitch, MA, USA) according to the manufacturer's instructions. The resulting fragments were run through agarose gel 2%. Sequence analysis was performed with Geneious r9 software, V9.1.8 (Biomatters Ltd., Auckland, New Zealand).

Assessment of TMB

To calculate the TMB, we divided the total number of mutations in our tumour samples by the length of the target region in Mb. NBCCS-BCC and spBCC TMB and patient age were retrieved from previously published BCC sequence analyses performed by Bonilla *et al.*, 2016.[12] All WES samples had a mean of VAF >0.2.

RNA-seq analysis

RNA extraction from one fresh BCC sample and adjacent healthy skin was performed using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Hilden, Germany) according to the manufacturer instructions. For sequencing, BGI-SEQ500 (BGI) with 2 × 100 bp was used. 53.5 millions paired-end reads were aligned using STAR software to the human genome Hg19.[30] Allele-specific RNA expression was estimated via ASEQ software.[31]

Results

Clinical presentation

Two brothers (brother 1 and brother 2) presented with multiple BCCs, which had started as small initial lesions that had first appeared after the age of 40 years (listed in Table S2, Supporting Information). Both had odontogenic keratocysts, palmar pitting and more than five BCCs, fulfilling the clinical criteria for NBCCS.[8] The other two siblings (brother 3 and sister 2) showed no NBCCS-related symptoms. The youngest sister (sister 1) had died of melanoma before the age of 30 years. Family records and patient statements presented no indication of NBCCS-related symptoms in other first-grade family members (Fig. 1).

Somatic tumour mutational burden

The mean coverage of the nine BCCs from the siblings was 59×, and the estimated average mutational burden was 32 muts/Mb. We compared the number of tumorigenic events, including LOH and damaging mutations, on the *PTCH1* gene with those of NBCCS-BCCs and spBCCs from previously published data.[12] Here, we found a statistically significant difference between NBCCS patients with late-onset BCCs and spBCCs ($P = 0.004$), regular NBCCS and spBCC ($P = 0.002$), but not between

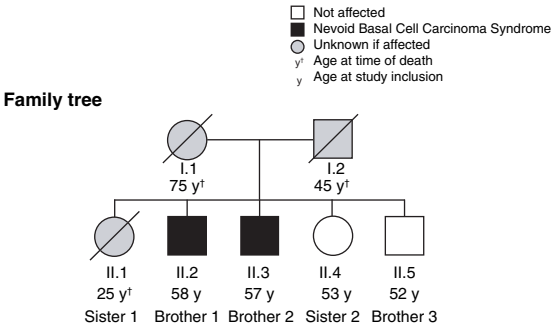

Figure 1 Family pedigree. Brother 1 (II-2) and brother 2 (II-3) have nevoid basal cell carcinoma syndrome (NBCCS) with multiple late-onset basal cell carcinomas, jaw cysts and pitting of palms and soles. Sister 2 (II-4) and brother 3 (II-5) have no NBCCS-related symptoms. Sister 1(II-1) died of melanoma. It is unclear if the parents (I-1 and I-2) or sister 1 had any evidence of NBCCS. Roman numerals I-II indicate first and second generation, respectively. Arabic numbers show the order of birth.

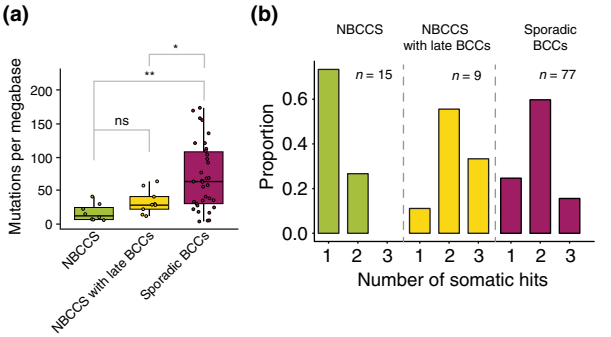

Figure 2 Tumour mutational burden and somatic hits of patients with basal cell carcinoma. (a) Box plots illustrating the age and tumour mutational burden (TMB, on *y*-axis) of basal cell carcinomas (BCCs) found in nevoid BCC syndrome (NBCCS) patients (n = 15, left), in BCCs from our two NBCCs patients (n = 9, centre) and in sporadic BCCs (n = 77, right). The Mann–Whitney *U*-test was used for comparison (*P*: ns—not significant, * <0.05, ** <0.01) (b) Proportion of number of somatic events per tumour (hits) including loss of heterozygosity (LOH) and damaging mutations found in *PTCH1*. BCCs of regular NBCCS patients often have one somatic event (mostly LOH), while late-onset BCCs of NBCCS patients and usual sporadic BCCs similarly require 2–3 somatic hits.

NBCCS patients with late-onset BCCs and regular NBCCs (P = 0.38; Fig. 2a). However, the numbers of somatic damaging events on *PTCH1* of NBCCS patients with late-onset BCCs are more similar to those of spBCCs than of regular NBCCS patients (Fig. 2b). Regression analyses have also suggested that the TMB positively correlates with age in spBCCs (R = 0.4, P = 0.018, Spearman's rank correlation) but not in NBCCS (R = −0.49, P = 0.22), indicating different mechanisms of tumorigenesis (Fig. S1, Supporting Information).

Mutational signatures

Mutational signatures are important to understand the aetiology of cancers.[32] A framework for signature correlation is provided in the COSMIC database (https://cancer.sanger.ac.uk/cosmic/signatures). As expected, most SNVs in BCCs presented C:G>T:A mutations in UVR-specific trinucleotide contexts (Fig. S2, Supporting Information). Concurringly, seven BCCs predominantly showed the mutational signature 7, which is known to be UVR-associated.[32–34] Two of the studied tumours showed a lower proportion of UVR-associated signature mutations, which could be explained by endogenous DNA damage.

Somatic tumour driver events

Eight out of nine BCCs presented with LOH of *PTCH1*, and we found additional somatic damaging *PTCH1* mutations in all BCCs (Table S3, Supporting Information). The average number of hits in *PTCH1* was 3.2 including one germline mutation, one LOH and one to three somatic LoF mutations (Fig. 3 and Fig. S3, Supporting information). Other driving events likely contributing to tumorigenesis in late-onset BCCs included mutations in *TP53* tumour suppressor gene (5/9 tumours), trisomy of chromosome 6 (3/9) and oncogenic mutations in *MYCN* gene (3/9). Additionally, mutations in *RAC1*, *PTPN14* and *PREX2* were identified in single tumours.

Germline mutations

Whole exome sequencing of DNA from PBMCs from four family members revealed four deleterious variants that segregated with the disease phenotype, i.e. were only found in brother 1 and brother 2: *NME2* p.E152A, *MTCL1* p.P1178R, *ZFX2* p.R1277W and *PTCH1* exon 9: splice site. Of those, only the *PTCH1* mutation was reported in the ClinVar database (dbSNP ID: rs186008764), however, with conflicting interpretations of pathogenicity. The variant is reported as a splice site mutation, located at the −6 bp position from acceptor site at 3′ of exon 9 (NM_001083605:exon9:c.763-6C>A, Fig. 4). Sanger sequencing confirmed the heterozygous splice site mutation (Fig. S4a,b, Supporting Information). In order to estimate its damage, we used the SPANR and MaxEntScan splice site effect prediction tools.[35,36] Both programs predicted that the mutation carries a significant likelihood of aberrant splicing. The GnomAD database (http://gnomad.broadinstitute.org) reports that the mutation has very low overall allele frequency of 0.0003715 (0.0007 in the European population). Furthermore, there are no known cases of homozygosity, which further suggests that it is a pathogenic variant.[37] Importantly, LOH consistently resulted in retention of the splice site mutation in all of our BCCs with LOH. This further supports a causal role of the observed *PTCH1* splice site mutation. Exploring non-coding rare variations in *PTCH1* and 2kbp upstream through WGS, we identified one additional

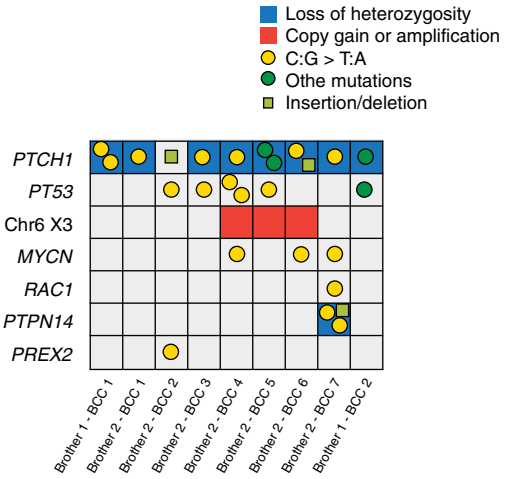

Figure 3 Putative driver mutations and number of somatic events on *PTCH1*. Grid diagram displaying putative driver events in the nine studied BCCs. Only damaging mutations and relevant somatic copy number alterations are shown. The left axis lists names of affected genes, the bottom axis individual tumours. Mutation types are defined in the figure legend (right).

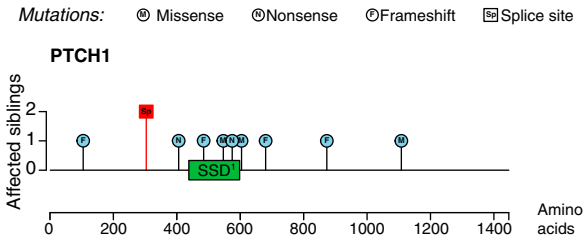

Figure 4 Mapping of the splice site mutation. Protein-level lollipop of damaging *PTCH1* mutations. The left scale indicates the number of siblings affected by each mutation. Somatic mutations were found in basal cell carcinomas (BCCs) from the two brothers with Nevoid BCC syndrome (blue circles). The red box signifies the location of the germline splice site mutation found in both brothers. Most somatic mutations are located close to or within the sterol-sensing domain (SSD, green box), which is required for maintaining *PTCH1* receptor function. The germline splice site mutation is located before the SSD.

candidate variant (NC_000009.11:g.98206399T>A) which ClinVar annotates with uncertain significance (two reports). The variant was excluded from further consideration because while it is rare in the general population (AF = 0.006187) it is common in the Ashkenazi Jewish population (AF = 0.01896).

To investigate the relevance of the splice site mutation for NBCCS RNA sequencing (RNA-seq) was performed on one fresh BCC sample (brother 1—BCC 2) and matched healthy skin. We measured the expression of four allele-specific heterozygous single-nucleotide variants (SNVs) that were located in expressed regions of the gene. The most likely phase of each SNV was inferred using expression data from tumour tissue with LOH on Chromosome 9. In healthy skin of brother 1, the expression analysis of the mutated haplotype yielded only 21 reads, while the wild-type (wt) haplotype yielded 38 reads (ASEQ tool).[31] Next, we compared the splicing patterns of healthy skin from our NBCCS patient with three healthy reference skin samples. Here, we could not detect any splicing aberrations around exon 9 (not shown). Our results may have been limited by low coverage due to weak expression of *PTCH1* in differentiated keratinocytes. Nevertheless, the wt haplotype had a markedly higher expression of *PTCH1* than the allele with the splice site mutation ($P = 0.018$, one-sided exact binomial test), suggesting that transcription of mutated *PTCH1* may be at least partially impaired.

Discussion

We studied the genetic mechanisms that lead to an unusually late onset of BCC development in two members of the family with NBCCS. One major NBCCS criterion is the appearance of the first BCC before the age of 30 years.[6] Our NBCCS siblings developed no tumours before the age of 40 years.

The average mutational load in our BCCs was 32 mut/Mb. This is significantly lower than that in spBCCs (65 muts/Mb) and tends to be higher than that in BCCs of common NBCCS patients without statistical significance (21 muts/Mb).[12] Interestingly, the numbers of somatic driver events in *PTCH1* of late-onset BCCs were similar to those found in spBCCs and higher compared to typical NBCCS patients. Since the two brothers had started to develop their BCCs later than typical NBCCS patients, more UVR damage may have accumulated, as the overall TMB has been shown to increase with age.[38] Mutational signature analysis of our tumours revealed a dominance of the UVR-associated COSMIC signature 7 with C:G>T:A mutations, as typically found in BCCs.[12]

Analysis of somatic SNVs showed that eight out of nine BCCs underwent LOH of *PTCH1* and carried various tumour-specific damaging *PTCH1* mutations on the remaining allele with germline splice site mutation. We hypothesize that after LOH of *PTCH1* the remaining allele was initially sufficient to preserve the SMO-suppressing function of Patched 1. In this state, the affected basal cells of the skin may have acquired a mildly increased proliferative potential, favouring neoplastic transformation. Accumulation of cells in a precancerous state (i.e. with a single *PTCH1* allele) increases the risk that one LoF mutation in one of these cells results in complete LoF of *PTCH1* (Fig. 5). Importantly, we found additional secondary somatic driver events (such as *TP53* and *MYCN* mutations) that are known to be involved in BCC tumorigenesis in NBCCS patients.[12]

Using WES, we identified a heterozygous germline mutation at the acceptor splice site of *PTCH1* which we confirmed via

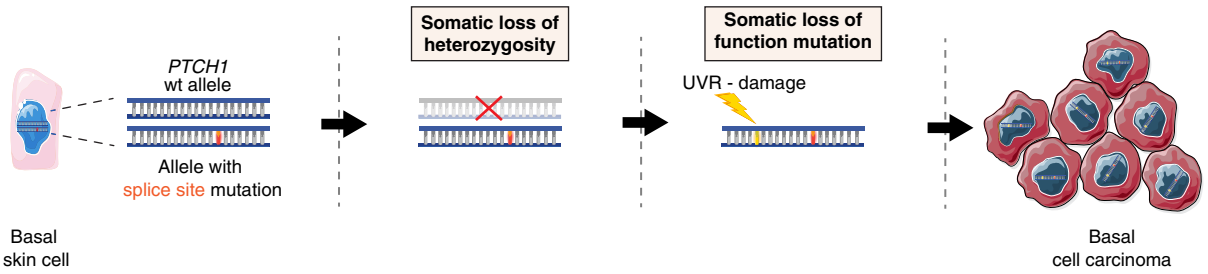

Figure 5 Three-hit hypothesis of late-onset basal cell carcinoma (BCC) development in nevoid BCC syndrome (NBCCS) patients. Diagram of a proposed mechanism for late-onset BCC tumour growth in NBCCS patients. From left to right: A keratinocyte carries a heterozygous germline splice site mutation (first hit). Two additional genetic events, i.e. loss of heterozygosity (second hit) and UVR damage (third hit), cause malignant transformation into BCC.

Sanger sequencing. The mutation segregated with the autosomal dominant disease phenotype. Interestingly, the splice site mutation was retained on the mutated allele in all cases of LOH (8/8) on chromosome 9. Furthermore, the mutation is located in a splice site of exon 9 of *PTCH1*, which encodes the sterol-sensing domain (SSD) of Patched-1 and is required for binding the sonic hedgehog ligand.[39] The importance of the SSD is supported by its presence in the majority of isoforms (20/27) listed in the GTex Portal (https://gtexportal.org/home/gene/PTCH1). Analysis of RNA-seq data from healthy skin of brother 1 revealed reduced *PTCH1* expression of the mutated allele. The generally low level of *PTCH1* expression in healthy skin was a limiting factor for further investigating a possible causative role of the splice site mutation. Therefore, we cannot fully rule out that the observed mutation is a haplotype marker associated with reduced expression of *PTCH1*, which could be driven by a not-yet-identified genetic event.

Conclusion

We propose a three-hit hypothesis of genetic events on *PTCH1* to explain the late development of BCCs in two brothers with NBCCS. Our analyses suggest a germline heterozygous hypomorphic splice site mutation predisposed brother 1 and brother 2 to NBCCS. LOH resulted in the loss of the wild-type haplotype, followed by LoF mutations on the remaining hypomorphic allele that completed the inactivation of Patched-1 protein. Furthermore, we found a significantly lower TMB compared to spBCCs, higher numbers of driver mutations in *PTCH1* than in regular NBCCS, and markedly reduced *PTCH1* expression of the mutated allele in healthy skin. Our findings encourage evaluation of genetic testing for NBCCS patients with multiple BCCs even if they appear after the age of 40 years.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Methods.
Figure S1. Tumor mutational burden and age of patients with basal cell carcinoma.
Figure S2. Mutational signature heatmap.
Figure S3. Tumor purity and driver events.
Figure S4. Sanger sequencing of splice site mutation.
Table S1. Sample details.
Table S2. List of basal cell carcinomas of two brothers with Nevoid Basal Cell Carcinoma Syndrome.
Table S3. Somatic point and small indel mutations identified in the nine studied basal cell carcinomas as possible driver events.