Genetic predisposition to neural crest-derived tumors: revisiting the role of KIF1B

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Abstract

Objective: We previously described a family in which predisposition to pheochromocytoma (PCC) segregates with a germline heterozygous KIF1B nucleotide variant (c.4442G>A, p.Ser1481Asn) in three generations. During the clinical follow-up, one proband’s brother, negative for the KIF1B nucleotide variant, developed a bilateral PCC at 31 yrs. This prompted us to reconsider the genetic analysis.

Design and Methods: Germline DNA was analyzed by Next Generation Sequencing (NGS) using a multi-gene panel plus MLPA or by Whole Exome Sequencing (WES). Tumor-derived DNA was analyzed by SnapShot, Sanger sequencing or NGS to identify loss-of-heterozygosity (LOH) or additional somatic mutations.

Results: A germline heterozygous variant of unknown significance in MAX (c.145T>C, p.Ser49Pro) was identified in the proband’s brother. Loss of the wild type MAX allele occurred in his PCCs thus demonstrating that this variant was responsible for the bilateral PCC in this patient. The proband and her affected grandfather also carried the MAX variant but no second hit could be found at the somatic level. No other pathogenic mutations were detected in 36 genes predisposing to familial PCC/PGL or familial cancers by WES of the proband germline. Germline variants detected in other genes, TFAP2E and TMEM214, may contribute to the multiple tumors of the proband.

Conclusion: in this family the heritability of PCC is linked to the MAX germline variant and not to the KIF1B germline variant which, however, may have contributed to the occurrence of NB in the proband.
INTRODUCTION

We previously described a family in which predisposition to pheochromocytoma (PCC), segregates on 3 generations with a germline heterozygous nucleotide variant of KIF1B (c.4442G>A, p.Ser1481Asn) which encodes the kinesin-like protein KIF1B (1). KIF1B isoform β (KIF1Bβ) is a molecular motor protein which participates in the transport of synaptic vesicle precursors and is essential for neuronal survival and differentiation (2). In vitro, the p.Ser1481Asn variant decreases the ability of KIF1Bβ to promote the apoptosis of primary rat sympathetic neurons (3) and thus may facilitate tumorigenesis later on. Conversely enforced expression of KIF1B resulted in an induction of apoptosis of NB cells (4). Thus, the KIF1Bβ neuronal pro-apoptotic effect combined with the mapping of KIF1B on chromosome 1p36, a region frequently deleted in PCC and NB (5), suggested that KIF1B might function as a tumor suppressor gene (TSG) in these diseases (3). However, in our kindred, we did not identify a loss of the wild type (WT) allele of KIF1B at the somatic level in the PCC or NB of the proband, deviating from the Knudson two hits theory (1). At that time, we thus hypothesized that the p.S1481N variant of KIF1Bβ functions in haploinsufficiency in these tumors (1) but its exact mechanisms of action remain unclear (6).

Since our initial report, the large size KIF1B gene has been rarely incorporated in the PCC/PGL gene panels which are analyzed in patients with PCC or PGL by Next Generation Sequencing to identify familial tumours. Welander et al. (7) described one PCC patient with a germline variant of KIF1B classified as disease causing in their cohort thus representing a prevalence of 1.1%. The PCC had a sporadic presentation in this women who later presented with an endometrial carcinoma. Curras-Freixes et al (8) reported 3 germline variants of unknown significance (VUS) of KIF1B in their cohort of PCC patients, representing a prevalence of 0.66% (3/453) in line with our own estimation of the prevalence of KIF1B VUS at 1.3% (1/74 patients with PCC/PGL analyzed by NGS between 2017-19, unpublished data).
In conclusion, only 1-2% of the patients with PCC or PGL had a germline pathogenic variant of $KIF1B$ thus leading to frequent questioning on its involvement in the heritability of PCC/PGL (9-10).

At the time of our report in 2008, the proband’s two brothers, who did not carry the $KIF1B$ germline variant, were clinically asymptomatic (1). However, during the clinical follow-up, one proband’s brother developed a bilateral PCC at 31 yrs. This prompts us to extend the genetic analysis in this family and to reconsider the molecular pathogenesis of the PCC.
Materials and Methods

**Clinical Data.** The pedigree of the family has been updated in figure 1. In brief, the proband developed a NB of the broad ligament at 17 months which was treated by surgery, radiation therapy and chemotherapy. At age 22 y, she developed a right PCC plus a ganglioneuroma at the site of the original NB and also an ileal schwannoma. At that time, she had hypertension and high normetanephrines levels (exact data not available). Six years later, the patient underwent adrenal surgery for a left PCC associated with a mature ganglioneuroma. At the same time, a well-differentiated leiomyosarcoma arising from the mesosigmoid was detected and surgically removed. At 39 y, several cutaneous metastases of the leiomyosarcoma were surgically removed. At 42 y, a 9-cm moderately differentiated hepatic carcinoma was diagnosed and surgically removed. Finally, at 43 y an uterine leiomyoma, and two metastases (parietal and peritoneal) from the leiomyosarcoma were removed. Her paternal grand-father (I-1) had bilateral PCC at 70 y and her father (II-2) had a lung adenocarcinoma at 47 y and prostatic cancer at 54 y. The proband’s youngest brother (III-3) presented at 31 y with a cardiomyopathy complicated by a Takotsubo’s syndrome which led to the diagnosis of bilateral PCC. The proband’s paternal uncle (II-3) was diagnosed at 56 y with an oligosymptomatic adrenal nodule exhibiting a high $^{18}$F-DOPA uptake at PET (positron emission tomography).

**Custom endocrine tumors NGS panel.** In compliance with the french regulation, each patient gave his/her written informed consent before performing genetic testing which is an integral part of the patient’s care. Moreover this protocol was reviewed and validated by the Ethical Committee (Comité de Protection des Personnes Nord Ouest IV) under the number HP 20/04. A custom panel based on the hybridization and capture technology (Haloplex, Agilent Technologies, Les Ulis, France) was designed to be compatible with the Illumina MiSeq platform (San Diego, CA). Probes covered the coding regions and intronic flanking sites of 21 susceptibility genes for endocrine tumors, either PCC/PGL (**VHL, RET, SDHA, SDHAF2, SDHB, SDHC, SDHD, TMEM127, KIF1B, MAX, PHD2**) or pituitary
tumors/hyperparathyroidism (MEN1, HRPT2, AIP, CASR, CYP24A1, GCM2, PTH, GNA11, AP2S1, CDKN1B). Genomic DNA extracted from blood cells was fragmented with restriction enzymes; then digested DNA was hybridized to Haloplex probes which resulted in circularization of DNA fragments and sample indexing. Target DNA was captured with streptavidin coated magnetic beads, ligated and eluted before bridge PCR amplification of the libraries. After quantification of enriched target DNA, samples were pooled for multiplexed sequencing. NGS sequencing data were aligned to hg19 human reference and annotated using two independent bioinformatic pipelines [alignment using bwa v0.7.15-r1140 followed by best practices for germline variant detection using GATK v3.7 (11) and SeqNext V4.4 (JSI) (12)]. Data were filtered using an in-house database (DVD) to remove recurrent sequencing errors. Regions with coverage <30X were re-analyzed using Sanger sequencing.

Somatic Cancer Panel analysis. DNA extracted from three separate regions of the frozen left PCC from proband (III-1) was analyzed for somatic nucleotide variants in hot spot regions of 48 commonly mutated cancer genes using the Illumina TruSeq Amplicon Cancer Panel (ABL1, AKT1, ALK, APC, ATM, BRAF, CDH1, CDKN2A, CSF1R, CTNNB1, EGFR, ERBB2, ERBB4, FBXW7, FGFR1, FGFR2, FGFR3, FLT3, GNA11, GNAQ, GNAS, HNF1A, HRAS, IDH1, JAK2, JAK3, KDR, KIT, KRAS, MET, MLH1, MPL, NOTCH1, NPM1, NRAS, PDGFR, PIK3CA, PTEN, PTPN11, RB1, RET, SMAD4, SMARCB1, SMO, SRC, STK11, TP53, VHL), run on Illumina MiSeq following standard protocols, and analyzed using the filter settings described above.

WES (Whole Exome Sequencing) analysis. Genomic DNA extracted from fresh blood cells from the proband and her father with QiAmp DNA mini kit (Qiagen) was captured using Agilent in-solution enrichment methodology with their biotinylated oligonucleotides probes library (SureSelect Clinical Research Exome V2, Agilent Technologies), followed by paired-end 75 bases massively parallel sequencing on Illumina HiSeq4000 (IntegraGen SA, Evry, France), as reported (13). Sequence capture, enrichment and elution were performed according to manufacturer’s instruction and protocols (SureSelect, Agilent) without modification, except for library preparation performed with NEBNext®
Ultra kit (New England Biolabs®). For library preparation, 600 ng of each genomic DNA were fragmented by sonication and purified to yield fragments of 150-200 bp. Paired-end adaptor oligonucleotides were ligated on repaired fragments then purified and enriched by 8 PCR cycles. A total of 1200ng of the purified Libraries were then hybridized to the SureSelect oligo probe capture library for 72 hr. After hybridization and washing, the eluted fraction was PCR-amplified, purified and quantified by qPCR. Each eluted-enriched DNA library was then sequenced on an Illumina HiSeq4000 as paired end 75bp reads. Image analysis and base calling was performed using Illumina Real Time Analysis (2.7.7) with default parameters.

**Bioinformatic analysis.** After demultiplexing and FASTQ generation, the paired end reads were trimmed using TrimGalore v0.4.4. The paired end reads were then aligned to hg19 human reference genome with BWA v0.7.15-r1140 (11). We applied the GATK v3.7 pipeline (12) for indel realignment, duplicate removal, and performed SNP and INDEL discovery as well as share genotype across both the proband and her father’s samples simultaneously according to GATK Best Practices recommendations (14,15). Variants were annotated and filtered with Agilent Technologies Bench Lab NGS v5.0.2. To select putative pathogenic variants, filters were applied as follows (FigS1) : (i) variants common to proband and her father were filtered out; (ii) low quality reads were filtered out; (iii) only heterozygous variants were retained; (iv) variants predicted as pathogenic (see FigS1) were retained; (v) variants in exonic regions or near splicing sites were retained; (vi) variants with a frequency ≥0.01 in the general population (using Exac database, release 0.3, 1000 Genomes Phase1 release v3.20101123, ESP6500SI-V2, 1000 Genomes Phase 3 release v5.20130502) were filtered out. Relevant nucleotide variants were validated by Sanger sequencing on Applied Biosystems 3730 platform.

**Search for large rearrangements.** The analysis of large rearrangements was performed with the Multiplex Ligation-dependent Probe Amplification (MLPA) technology for FH and SDHx genes and by multiplex PCR (QMPSF) for VHL. MLPA probes (ref. SALSA MLPA Probemix P198, P226 and P429) and
reagents were manufactured and supplied by MRC-Holland (Amsterdam, The Netherlands). The MLPA procedure was conducted according to manufacturer’s specifications. The amplified probes were analyzed on a 3130XL DNA Analyzer (Life Tech, ThermoFisher). Regarding FH analysis, the proband DNA sample was tested twice in the same run along with 10 negative samples and one positive control with a total deletion of the FH gene. Data were interpreted with Coffalyser software (MRC Holland).

**LOH analysis.** DNA from formalin-fixed paraffin-embedded (FFPE) tumor samples was extracted using the QIAAmp DNA FFPE kit (Qiagen, France). Tissue samples of the proband available for LOH analysis are detailed in Table S1. LOH of selected nucleotide variant was searched by SNaPshot analysis as previously described (16). PCR and extension primers details are available upon request. Extension products were analyzed on Applied Biosystems 3730 along with GeneScan 120LIZ molecular marker using the Genemapper software. In addition, visualization of the variant peaks on Sanger sequence traces was done using the Mutation Quantifier tool (Surveyor program, Softgenetics).

**Immunohistochemical analysis.** Fumarate Hydratase expression was assessed on paraffin-embedded tumors using an anti-FH antibody 1:1000 dilution as previously described (17).

**In silico analysis.** Prediction of the missense variant of MAX protein was carried out with the Phyre2 server (Protein Homology/analogY Recognition Engine V2.0). We compared the deduced human amino-acid 3D structure with the 3D resolved structure of the *Homo sapiens* MAX protein (99% sequence identity) using The PyMOL Molecular Graphics System (v2.0, Schrödinger, LLC). The SuperPose server v.1.0 (18) was used to estimate the structural homology, measuring the average distance between the backbones of superimposed proteins.
RESULTS

During the clinical follow-up of the family, patient III-3, who did not harbor the *KIF1B* c.4442G>A nucleotide variant in his germline DNA, developed bilateral PCC at 31 yrs. His germline DNA was analyzed by NGS using a custom endocrine tumor panel which includes 11 major susceptibility genes for familial PCC/PGL (*PHD2, KIF1B*, *SDHA, SDHB, SDHC, SDHD, SDHAF2, VHL, MAX, TMEM127* and *RET*). The only heterozygous variant identified was in *MAX* (NM_002382.5): c.145T>C, p.Ser49Pro. A large rearrangement of *SDHA, SDHB, SDHC, SDHD, SDHAF2, FH* and *VHL* was excluded by MLPA or QMPSF analysis, respectively. Using the ACMG guidelines and Varsome tool (19), the *MAX* nucleotide variant was classified as a VUS. The secretory profile of his tumors consisted of increased urinary normetanephrines at 1979 nmol/mmol of creatinine (8 x the upper limit range) with normal total metanephrines at 182 nmol/mmol of creatinine (<190), reminiscent of the pattern observed in patients with a *MAX* pathogenic variant (20). The p.Ser49Pro MAX variant is predicted to show the Proline 49 with an opened cycle which is impossible, leading probably to the precipitation of the protein (Fig. 2). Proline is a constrained amino acid due to its pyrodylin cycle hindering the rotation of the φ angle with the previous amino-acid and thus altering the amino acid connection. Therefore, this variant is likely deleterious.

We thus reanalyzed the germline DNAs of the proband (III-1), her father (II-1) and grandfather (I-1), who had been previously found to be carriers of the *KIF1B* c.4442G>A nucleotide variant (1), using the NGS panel. All also carried the *MAX* c.145T>C variant at the heterozygous level. No other pathogenic variant in the nine other susceptibility genes for PCC/PGL was identified (Fig. 1). We also excluded a large rearrangement of *SDHA, SDHB, SDHC, SDHD, SDHAF2, FH* and *VHL* in the proband by MLPA or QMPSF analysis, respectively. The *MAX* c.145T>C variant was also detected by Sanger sequencing at the heterozygous level in the germline DNA of the proband’s paternal uncle (II-3), who was *KIF1B* negative (Fig.1). Interestingly, II-3 recently developed, at age 56, a 11-mm hypervascular nodule in his right adrenal which remains clinically silent but strongly uptakes [18F]-DOPA during PET,
suggestive of PCC or adrenomedullary nodule. Plasma free normetanephrines were at the upper limit of the normal values. Pre-operatively, patient I-1 had high urinary normetanephrines (5x the upper limit range) and chromogranin-A levels (x3 the upper limit range). By contrast, patient II-2, who had a regular follow-up by PET imaging due to his lung and prostatic cancers, never demonstrated an adrenal uptake of $^{18}$F-Deoxy-Glucose. Moreover, his levels of metanepherines/normetanephrines were in the normal range at each follow-up, both elements being in disfavor of a PCC.

Since MAX behaves as a TSG (20), we analyzed the tumor DNAs of patient III-3 and identified loss of the MAX WT allele in both tumors (Fig. 3) suggesting that this variant is indeed responsible for the bilateral PCC in this patient. In contrast, no LOH of the WT allele of MAX was found in the 2 PCCs of the proband (III-1), using DNA obtained from three independent samples, FFPE samples from left and right PCCs (Fig. 3), and one fresh frozen sample from the left PCC (Fig. S2). The oldest of the samples (the right PCC) in fact showed complete absence of the variant allele (Fig. 3), which could be due to allelic dropout in the PCR, a well-recognized cause of errors in DNA from suboptimal samples (21). Although the estimated proportion of tumor cells of the left PCC FFPE sample was high (Fig. 3), this estimate was not available from the bulk frozen specimen from this tumor nor the right PCC, so it is unclear to what extent normal cell admixture may have contributed to the allelic count. We also excluded, by Sanger sequencing, the presence of an additional somatic MAX mutation, which might have functioned as the second hit in the absence of LOH in the DNA extracted from the left PCC of the proband. We further examined the DNA from the three separate fragments from the left PCC to search for additional somatic mutations in 48 cancer genes using NGS. The three fragments displayed similar variant allele frequencies across these genes, suggesting that the left PCC was homogeneous with respect to both genetic and cell composition. Moreover, these fragments lacked areas of allelic imbalance, in favor of high level of normal cells in these fragments. Only a few VUS were detected (Table S2); however, no variant frequency suggestive of LOH was observed in these three samples. Finally, FH expression evaluated by immunohistochemistry was conserved on the uterine leiomyoma
and pelvic leiomyosarcoma of the proband (Fig S3), which strongly suggests the absence of somatic pathogenic variants of \textit{FH} responsible for these 2 tumors in the proband.

Since the proband (III-1) and her father (II-2) shared the same genotype for \textit{MAX} and \textit{KIF1B} despite very different phenotypes (Fig.1), we considered that the proband may carry additional variants in known/new susceptibility genes that modified cancer predisposition, which might have been inherited from her mother or have occurred \textit{de novo}. WES was performed on the germline DNA of III-1 and II-2. First, the proband had no pathogenic variant in \textit{NF1}, \textit{IDH1}, \textit{IDH2}, \textit{FH}, \textit{MDH2} or \textit{SLC25A11} which predispose to familial PCC/PGL nor in a group of 30 cancer-predisposing genes (22) i.e. \textit{APC}, \textit{ATM}, \textit{BAP1}, \textit{BARD1}, \textit{BMPR1A}, \textit{BRCA1}, \textit{BRCA2}, \textit{BRIP1}, \textit{CDH1}, \textit{CDKN2A}, \textit{CDK4}, \textit{CHEK2}, \textit{EPCAM}, \textit{GREM1}, \textit{MITF}, \textit{MLH1}, \textit{MSH2}, \textit{MSH6}, \textit{MUTYH}, \textit{NBN}, \textit{PALB2}, \textit{PMS2}, \textit{POLD1}, \textit{POLE}, \textit{PTEN}, \textit{RAD51C}, \textit{RAD51B}, \textit{SMAD4}, \textit{STK11} and \textit{TP53}. Thus, we decided to focus on the nucleotide variants which were present in the germline of III-1 but absent in II-2 in agreement with our working hypothesis. One hundred twenty-five nucleotide variants were identified as unique to III-1 (Fig S1). Class 1 and 2 variants (benign or probably benign) were filtered out using Varsome, leading to a list of 24 variants all classified as VUS (Class 3) (Table 1). After interrogation of several resources such as PUBMED (for a link between the gene of interest and cancer), UniProt (for information on encoded protein function), HGMD (for information on germline mutations currently identified), TCGA (for somatic mutations catalogue), Protein Atlas and CTEX databases (for detailed information on tissue expression), the list was narrowed down to 5 variants occurring in 5 genes (Table 1 in red). \textit{KLH7} and \textit{PKM} were good candidate genes for hereditary PCC since they encode proteins expressed in adrenals (23) and are mutated (though rarely) at the somatic level in PCC (TCGA PCPG). \textit{RIPK3}, \textit{TFAP2E} and \textit{TMEM214} were good candidates for the non-neural crest tumors since they are mutated in sarcomas (TCGA SARC), myomatous neoplasms and in hepatocarcinomas (TCGA LIHC) at the somatic level. No protein expression data was available for \textit{TMEM214}, \textit{TFAP2E} and \textit{RIPK3} in the Protein Atlas (23). Sanger sequencing confirmed the presence of the five nucleotide variants in the germline of the
proband and her mother but absence in her father as expected (Table 2), none were de novo. Patient III-3 was heterozygous only for the KLHL7 variant.

Tumor DNA from 8 different FFPE tumors from the proband (Table S1) were analyzed by Snap Shot to screen for LOH of the candidate genes. No LOH of KLH7 WT allele was found in the PCC of the proband nor in those of her brother III.3 (Fig. 3). Unfortunately, the data were not informative for PKM (not shown). Regarding TFAP2E, LOH of the WT allele was found in the DNA from the uterine leiomyoma whereas no LOH occurred in the parietal and peritoneal metastasis of the leiomyosarcoma (Fig. 4). By contrast, a LOH of the WT allele of TMEM214 was found in the parietal and peritoneal metastasis of the leiomyosarcoma (Fig. 4) whereas the data were not informative in the hepatocarcinoma (not shown).

**DISCUSSION**

The occurrence of a bilateral PCC at a young age in a relative (patient III.3) who did not carry the germline KIF1B c.4442G>A variant suggested that other susceptibility gene(s) might be implicated in the PCC/PGL predisposition of this family. Indeed, analysis of a panel of 11 classic susceptibility genes for PCC/PGL (17,18) by NGS led to the identification of a heterozygous variant of MAX: c.145T>C, p.Ser49Pro. This variant was also found in the germline of the two other individuals with PCC in this family, III-1 and I-1. This variant has not been previously found in the germline of patients with PCC/PGL or at the somatic level based on the LOVD Leiden and TCGA database, respectively. Using the pathogenicity criteria proposed by the ACMG, this variant was classified as a VUS rendering the interpretation more complex. However, several elements are in favor of its pathogenic role. First, the secretory pattern of the tumors of patient III-3 and I-1 is compatible with those classically observed in patients with MAX pathogenic variants (20). Secondly, loss of the WT allele of MAX in both PCCs of III-3 suggests the role of MAX gene and causality of the p.Ser49Pro variant in the pathogenesis of the PCCs. Thirdly, introduction of a proline at position 49 seems incompatible with a native 3D conformation for the MAX protein. In addition, although histology of the adrenal nodule identified in
patient II.3 is presently not available, its imaging pattern is compatible with a PCC. If confirmed in the follow-up, this diagnosis provides support to the pathogenic role of the MAX variant since he is WT for KIF1B.

The interpretation of the pathogenesis of the PCCs occurring in the proband and her grandfather is more intricate. They both have the MAX p.Ser49Pro plus the KIF1B p.Ser1481Asn germline variants. We were unable to identify LOH of KIF1B or MAX WT alleles in the proband’s PCC despite the analyses of multiple separate fragments from the most recently removed PCC which was not fixed in Bouin’s reagent known to be deleterious for DNA (21). However, as discussed above, our additional data on this tumor does not allow us to rule out the contribution of high levels of nontumoral tissue to the lack of detectable LOH. Although we cannot exclude that both KIF1B and MAX may contribute to the PCC phenotype or to the clinical variability in this family, KIF1B pathogenic variants have rarely been described in patients with PCC/PGL since our initial report in 2008 (1, 7, 8). Only one, a p.Tyr835Cys variant, was reported by Welander et al. in a 54-yr old woman with a unilateral PCC and an endometrial carcinoma (7). This patient had no germline variants in any of the 11 other major susceptibility genes for PCC/PGL but no somatic LOH of the wild-type allele was found, so preventing any definitive conclusion on the pathogenic relevance of this novel variant.

Given the phenotypic variability of this family, with multiple non-PCC/PGL cancers in patient III.1, and to evaluate the possibility that other susceptibility events were at play, we performed WES on the germline DNAs from patient III.1 and II.2. Our working model was that patient III.1 had de novo or maternally-inherited mutations in new/known susceptibility genes for hereditary cancers or, alternatively, in “modifier genes” which could have an impact on cancer-promoting pathways (24). These additional nucleotide variants, combined with the KIF1B and MAX germline variants, might explain the very severe phenotype of this patient. WES excluded a pathogenic mutation in other genes predisposing to familial PCC/PGL and also in 30 other hereditary cancer susceptibility genes (22). Among the 125 variants which were private to the proband most were classified as benign or
probably benign and were filtered out; the 24 remaining variants were all classified as VUS. Based on bibliographic data, tissue pattern of expression, biological function of the encoded protein, and a catalogue of somatic mutations, we narrowed down the list to 5 variants in 5 different genes which may be implicated either in the pathogenesis of PCC (KLHL7 and PKM) or sarcomas (RIPK3, TMEM214 and TFAP2E). We did not detect a LOH of the WT allele of KLHL7 or PKM in the proband’s PCC. Thus, we cannot assign any causality in the pathogenesis of the proband’s PCCs to one of these variants.

We propose that in this family the genetic susceptibility to PCC/PGL is linked to the MAX nucleotide variant which is however associated with an incomplete penetrance since patient II-2 did not develop any symptomatic adrenal lesion. The KIF1B isoform β p.Ser1481Asn variant, which is partly defective in the apoptotic culling of neural crest progenitors, may also contribute to the occurrence of NB in the childhood of patient III-1, similar to other rare observations in NB (3). Finally, the leiomyosarcoma and hepatic carcinoma of the proband, and her father’s lung adenocarcinoma may be MAX and KIF1B independent. The involvement of TFAP2E and TMEM214 variants is an attractive hypothesis but the pathways acting in disease development remain to be determined.
Conflict of interest statement:

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Legends for Figures and Tables

Figure 1. Phenotype and genotype of the family. The proband is patient III.1

Figure 2. (a) 3D structure of the human native MAX protein (pdb code: 1AN2) (amino-acids 22-104); (b) 3D structural prediction of the human Ser49Pro mutated MAX protein (amino-acids 22–104)

Figure 3. Search for a LOH of MAX and KLHL7 in the DNA extracted from the pheochromocytomas of patients III.1 and III.3 by SnapShot analysis.

Figure 4. Search for a LOH of TFAP2E and TMEM214 in the DNA extracted from the different tumors from patient III.1 by SnapShot analysis.
figure 1

180x119mm (300 x 300 DPI)
**MAX, NM_002382: c.145t>c**

**Patient III-3**

| Blood DNA | Tumor DNA | Tumor DNA |
|-----------|-----------|-----------|
| Blood DNA | Tumor DNA | Right phe removed in 2017 | Right phe removed in 2017 |
| Left phe removed in 2017 | Tumor cells: 63% | Tumor cells: 63% |

**Patient III-1**

| Blood DNA | Tumor DNA |
|-----------|-----------|
| Blood DNA | Tumor DNA |
| Left phe removed in 2003 | Tumor cells: 90% |
| Right phe removed in 1997 | Tumor cells: ? Bouin fixative |

**KLHL7, NM_018846: c.1319g>a**

**Patient III-3**

| Blood DNA | Tumor DNA | Tumor DNA |
|-----------|-----------|-----------|
| Blood DNA | Tumor DNA | Right phe removed in 2017 |
| Left phe removed in 2017 | Tumor cells: 63% |
| Right phe removed in 2017 | Tumor cells: 63% |

**Patient III-1**

| Blood DNA | Tumor DNA | Tumor DNA |
|-----------|-----------|-----------|
| Blood DNA | Tumor DNA | Right phe removed in 1997 |
| Left phe removed in 2903 | Tumor cells: 90% |
| Right phe removed in 1997 | Tumor cells: ? Bouin fixative |

**Figure 3**
TFAP2E, NM_178548: c.1087c>t

Patient III-1

**Blood DNA**

**Tumor DNA**

Pelvic Leiomyosarcoma

Tumor cells: 7%

Uterine Leiomyoma

Tumor cells: 90%

Tumor DNA

Hepatocarcinoma

Tumor cells: 80%

---

TMEM214, NM_017727: c.787g>a

**Blood DNA**

**Tumor DNA**

Leiomyosarcoma

Peritoneal metastasis

Tumor cells: 95%

Leiomyosarcoma

Peritoneal metastasis

Tumor cells: 95%

**Tumor DNA**

Uterine Leiomyoma

Tumor cells: 90%

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Figure 4
Table 1. List of the 24 nucleotide variants identified by WES in patient III.1 germline DNA and classified as VUS

| Chromosome | dbSNP   | Zygosity   | Gene       | Protein       | HGVS cDNA-level nomenclature | Read depth (infoDP) | Alleles 1 reads | Alleles 2 reads | VARSOME Analysis on 11/15/2019 | GnomAD Exomes NON CANCER allele Frequency | GnomAD Exomes NON CANCER allele Frequency PopMax | Variant | Classification |
|------------|---------|------------|------------|---------------|------------------------------|---------------------|-----------------|-----------------|------------------------------|------------------------------------------|------------------------------------------|---------|----------------|
| 1          | rs145119239 | Heterozygous | TAFAP2E    | p.Arg363Cys    | NM_178548.4:c.1077C>T       | 98                  | 19              | 19              | PP3                          | 3.018e-03                                | 4.542e-03                                | varso.me/4RN  | VUS             |
| 1          | rs41264882  | Heterozygous | EDEM3      | p.Pro392Leu    | NM_003119960.2:c.575C>T     | 96                  | 19              | 19              | PP3                          | 9.475e-03                                | 1.463e-02                                | varso.me/4tg    | VUS             |
| 1          | rs757692903 | Heterozygous | TMCC2      | p.Ala344Val    | NM_014858.4:c.1031C>T       | 94                  | 57              | 57              | PM2 + PP3                   | 8.445e-06                                | 1.947e-05                                | varso.me/4th    | VUS             |
| 2          | rs199779856 | Heterozygous | TMMEM214    | p.Gly265Ser    | NM_017727.5:c.767G>A        | 85                  | 45              | 45              | PP3                          | 9.511e-04                                | 2.014e-03                                | varso.me/lh    | VUS             |
| 2          | rs148295709 | Heterozygous | ABHD1      | p.Tyr372Cys    | NM_033604.4:c.1131A>G       | 165                 | 67              | 67              | PP3                          | 1.896e-03                                | 3.542e-03                                | varso.me/10t   | VUS             |
| 3          | rs759213536 | Heterozygous | BSN        | p.Met3497Thr   | NM_003458.4:c.1040T>C       | 144                 | 73              | 73              | BP4                          | 2.534e-04                                | 5.780e-05                                | varso.me/lum   | VUS             |
| 4          | rs150771247 | Heterozygous | WFS1       | p.Arg226His    | NM_006005.3:c.683G>A        | 93                   | 53              | 53              | PP2 + PP3                   | 7.305e-07                                | 1.517e-04                                | varso.me/lkx   | VUS             |
| 5          | rs200904107 | Heterozygous | TMCO6      | p.Tyr153His    | NM_001300980.1:c.459C>A     | 75                   | 41              | 41              | PP3                          | 2.570e-03                                | 7.801e-05                                | varso.me/luu    | VUS             |
| 7          | rs75959437  | Heterozygous | KHL17      | p.Gly404Asp    | NM_018846.5:c.1319G>A       | 67                   | 32              | 32              | PM2 + PP2 + PP3             | NA                                        | NA                                      | varso.me/khaj  | VUS             |
| 7          | rs144546424 | Heterozygous | PSIP4      | p.Gly905Ser    | NM_004577.4:c.268G>A        | 110                  | 17              | 17              | PP3 + BP1                   | 1.993e-04                                | 1.207e-02                                | varso.me/DFdeg | VUS             |
| 7          | rs144454424 | Heterozygous | CPA1       | p.Gly466Asp    | NM_001868.4:c.497G>A        | 179                  | 99              | 99              | PP3                          | 1.644e-03                                | 3.099e-03                                | varso.me/kmgu  | VUS             |
| 10         | rs750208278 | Heterozygous | SEC24C     | p.Arg1033Gln   | NM_004922.4:c.3096G>A       | 34                   | 20              | 20              | PM2 + PP3 + PP3             | 1.688e-05                                | 3.892e-05                                | varso.me/lf5a  | VUS             |
| 11         | rs148772626 | Heterozygous | RSF1       | p.Val1120Leu   | NM_016578.3:c.3585G>A       | 50                   | 32              | 32              | PP3                          | 3.400e-05                                | 4.894e-05                                | varso.me/lsg   | VUS             |
| 12         | rs16412499  | Heterozygous | POXH4      | p.Arg236His    | NM_211596.3:c.947G>A        | 44                   | 25              | 25              | PP3 + BS1                   | 7.428e-03                                | 1.021e-02                                | varso.me/lslj  | VUS             |
| 14         | rs148658719 | Heterozygous | RIPK3      | p.Arg422*      | NM_006871.4:c.1264C>T       | 205                  | 88              | 88              | PP3                          | 1.579e-06                                | 8.399e-06                                | varso.me/lf4f  | VUS             |
| 14         | rs757205606 | Heterozygous | FOXA1      | p.Ala230Thr    | NM_004496.5:c.649G>A        | 100                  | 52              | 52              | PM2 + PP3 + BP1             | 4.703e-05                                | 1.055e-04                                | varso.me/lstq  | VUS             |
| 15         | rs201174904 | Heterozygous | TMEM251     | p.Met1147Thr   | NM_001098821.3:c.341T>C     | 89                   | 40              | 40              | PM2 + PP3 + BP1             | NA                                        | NA                                      | varso.me/lstq  | VUS             |
| 15         | rs637984298 | Heterozygous | TRPM1      | p.Pro798Ser    | NM_012552020.1:c.2392C>T    | 58                   | 18              | 18              | PM2 + PP3                   | 3.906e-05                                | 1.364e-05                                | varso.me/lw    | VUS             |
| 15         | rs201048858 | Heterozygous | C2CD4B     | p.Pro27Arg     | NM_000107595.3:c.80C>G      | 70                   | 30              | 30              | PP3 + BS1                   | 5.001e-03                                | 4.330e-03                                | varso.me/lf2   | VUS             |
| 15         | rs180714047 | Heterozygous | PKNM       | p.Glu455*      | NM_001313618.2:c.1419G>C    | 92                   | 37              | 37              | PP3 + BS4                   | 7.042e-04                                | 1.229e-02                                | varso.me/lf5s  | VUS             |
| 16         | rs151159316 | Heterozygous | ABCC11      | p.Glu83*       | NM_0033226.2:c.2491C>T      | 44                   | 22              | 22              | PP3                          | 2.415e-03                                | 4.652e-03                                | varso.me/kvt   | VUS             |
| 19         | rs772237409 | Heterozygous | PKNP       | p.Asn461Ser    | NM_007554.4:c.1382A>G       | 67                   | 31              | 31              | PM2 + PP3 + BP1             | 6.000e+00                                | NA                                      | varso.me/lf0p  | VUS             |
| 22         | rs201399907 | Heterozygous | SBFL1      | p.Arg130817p   | NM_002972.4:c.3922C>T       | 92                   | 47              | 47              | PP3                          | 6.464e-04                                | 1.916e-03                                | varso.me/ftdo  | VUS             |
| X          | rs140505250 | Heterozygous | ARR3       | p.Pro351Arg    | NM_004312.3:c.1052C>G       | 102                  | 52              | 52              | PP3                          | 1.591e-04                                | 2.696e-03                                | varso.me/lfsf  | VUS             |
Table 2. Distribution of the 5 VUS in the germline DNA of the different family members

| Gene            | TMEM214 | KLHL7 | PKM  | RIPK3 | TFAP2E |
|-----------------|---------|-------|------|-------|--------|
| nucleotide variant | c.787G>A | c.1319G>A | c.143>G | c.1284C>T | c.1087C>T |
| protein change  | p.Gly263Ser | p.Gly440Asp | p.Ser55* | p.Arg422* | p.Arg363Cys |
| Patients        |         |       |      |       |        |
| III.1           | +       | +     | +    | +     | +      |
| III.2           | +       | wild type | +    | +     | wild type |
| III.3           | wild type | +     | wild type | wild type | wild type |
| II.1            | +       | +     | +    | +     | +      |
| II.2            | wild type | wild type | wild type | wild type | wild type |
| II.3            | wild type | wild type | wild type | wild type | wild type |
| I.1             | wild type | wild type | wild type | wild type | wild type |
Table S1 - List of the tissue samples from the proband III-1 analyzed by SnapShot to identify a LOH

| Tissue samples of patient III-1 | TMEM214          | RIPK3           | TFAP2E          | KLHL7           | PKM             | MAX             |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Left PCC (2003)                 | c.787G>A        | c.1264C>T       | c.1087C>T       | c.1319G>A       | c.14C>G         | c.145T>C        |
| Right PCC (1997)                | NI              | NI              | no LOH          | NI              | no LOH          | NI              |
| Leiomyosarcoma (LMS) of the mesosigmoide (2003) | NI              | no LOH          |                 |                 |                 |                 |
| cutaneous metastasis of the LMS (2014) | NI              | no LOH          |                 |                 |                 |                 |
| parietal metastasis of the LMS (2018) | LOH             | no LOH          |                 |                 |                 |                 |
| peritoneal metastasis of the LMS (2018) | LOH             | no LOH          |                 |                 |                 |                 |
| Uterine leiomyoma (2018)        | no LOH          | NI              | LOH             |                 |                 |                 |
| Hepato-carcinoma (2017)         | NI              | no LOH          |                 |                 |                 |                 |

**Footnotes:** LOH, Loss of the Wild Type allele, NI: not interpretable,
Table S2- List of the VUS identified by NGS in 3 separate fragments from the left PCC of the proband

| Gene Name | Mutation type   | Nucleotide | Protein    | Fragment A |
|-----------|-----------------|------------|------------|------------|
|           |                 |            |            | Depth of coverage | VAF (%) |
| ATM       | missense_variant| c.1810C>T  | p.Pro604Ser| 250        | 57.6     |
| GNA11     | missense_variant| c.508T>A   | p.Leu170Met| 210        | 1.43     |
| HNF1A     | frameshift_variant| c.872delC| p.Pro291fs | 247        | 50.61    |
| JAK3      | missense_variant| c.2164G>A  | p.Val722Ile| 257        | 48.64    |
| KIT       | missense_variant| c.1621A>C  | p.Met541Leu| 279        | 54.12    |
| PIK3CA    | frameshift_variant| c.3095delA| p.Glu1032fs| 272        | 5.88     |
### Table S2- List of the VUS identified by NGS in 3 separate fragments from the left PCC of the proband III-1

| Depth of coverage | VAF (%) | Depth of coverage | VAF (%) |
|-------------------|---------|-------------------|---------|
| 257               | 54.09   | 249               | 63.05   |
| 207               | 0.97    | 179               | 5.03    |
| 250               | 44.62   | 254               | 45.28   |
| 257               | 51.36   | 257               | 47.86   |
| 275               | 50.18   | 275               | 48.36   |
| 273               | 4.4     | 263               | 3.8     |
68257 detected variants from proband WES data

Not present in Father

DP ≥ 20 & QUAL ≥ 100 *

Zygosity: Heterozygous

Pathogenicity in any:
PolyPhen2 prediction, HumDlv, HumVar: probably damaging, possibly damaging,
MutationTaster prediction: disease causing automatic, disease causing,
MutationAssessor prediction: High, Medium,
PROVEAN prediction: damaging

Coding effect: all except synonymous

Population Frequency as rare:
ESP6500 < 1%
ExAC < 1%
1000G Phase 1 < 1%
1000G Phase 3 < 1%

125 selected variants for manual curation

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| Sequence | Max genotype | Individual ID | DNA source |
|----------|--------------|---------------|------------|
| S40S (W7) | Proband’s mother | germline |
| S40P | Proband’s father (II-4) | germline |
| S40P | Proband’s brother (II-2) | germline |
| S40P | Proband (III-1) | germline |
| S40P | Proband’s left phoe (III-1) | tumor |

150x80mm (300 x 300 DPI)
