Germline Mutation in \textit{KIF1Bβ} Gene Associated with Loss of Heterozygosity: Usefulness of Next-Generation Sequencing in the Genetic Screening of Patients with Pheochromocytoma

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The genetic approach of pheochromocytomas and paragangliomas has changed in the last two decades. Nowadays, we know that more than 40% of patients have a germline mutation in one of the susceptibility genes identified to date. Our aim is to underline how genetic diagnosis by next-generation sequencing (NGS) can improve the management of patients affected by pheochromocytomas and paragangliomas in our routine diagnostic screening. We reported a case presentation and next-generation sequencing analysis supported by in silico studies and evaluation of mitochondrial status in \textit{KIF1Bβ} tissue. A 46-year-old male affected by a left secreting pheochromocytoma underwent surgery in 2017. After surgery, the normetanephrine levels decreased very slowly and a suspected abdominal lymph node was detected. We found a novel germline \textit{KIF1Bβ} gene mutation, c.4052C>T, p. Pro1351Leu associated with tumor loss of heterozygosity, and resulted likely-pathogenetic by in silico studies. This mutation was also associated with an increased number of mitochondria through the electron microscopy compared with wild-type tissues as suggestive for mitochondria neof ormation compensatory to the mitochondrial autophagic figures observed. Our results underline the usefulness of next-generation sequencing in the presence of multiple tumor predisposition genes and how, at the same time, its use may result challenging for the clinicians. To date, performing the genetic analysis according to the latest Consensus Statement is mandatory in patients affected by PHEO/PGL.

1. Introduction

Pheochromocytomas (PHEO) and paragangliomas (PGL) are rare tumors with an incidence around 2–5 patients per million per year. In 2017, the WHO Classification of Tumors of Endocrine Organs acknowledged this unpredictable behavior classifying PHEO and PGL among malignant category (ICD-O/3) [1]. Up to 40% of PHEO/PGL are caused by germline mutations in one of the current 14 main known susceptibility genes [2]. Moreover, mutations of these and other genes have been found at the somatic level, in tumor tissue. In the past, the search for germline mutations in patients with PHEO/PGL was clinically driven [3] but, with the advent of the next-generation sequencing (NGS), targeted panels have been created allowing the simultaneous analysis of the susceptibility genes, according to the Consensus Statement on the genetic diagnosis of PHEO/PGL [4].
In particular, we test simultaneously many susceptibility genes: EGLN1, EPAS1, FH, KIF1Bβ, MAX, NF1, RET, SDHA, SDHAF2, SDHB, SDHC, SDHD, TMEM127, and VHL. During the last two decades, knowledge of the genetic basis of PHEO and PGL has undergone important advances. Transcripome studies divided PHEO/PGL into two main clusters: cluster 1 included PHD2-, VHL-, SDHx-, IDH-, HIF2A-, MDH2-, and FH-mutated tumors while cluster 2 included RET-, MAX-, NF1-, TMEM127-, and KIF1Bβ-mutated tumors plus sporadic PHEO/PGL [5].

KIF1Bβ is a member of the kinesin 3 family genes with a specific cellular role in energy transport. The gene is located at chromosome 1p36.22 and encodes two isoforms, KIF1Ba and KIF1Bβ, the latter acting as a tumor suppressor gene necessary for neuronal apoptosis [6]. A KIF1Bβ germline mutation was described in a family affected by Charcot-Marie-Tooth (CMT) type 2A [7].

Li et al. [8] demonstrated that KIF1Bβ controls mitochondrial fission through the activation of calcineurin (CN) implicated in the dephosphorylation of dynamin-related protein (DRP1) at Ser636 position and that the translocation of DRP1 from the cytoplasm to the mitochondria results in mitochondrial fission and apoptosis. Also Ando et al. [9] reported the central role of KIF1Bβ in the mitochondrion-mediated apoptosis; in particular, they demonstrated that KIF1Bβ overexpression induces mitochondrial fragmentation and apoptosis interacting with a mitochondrial metallocysteine, YME1L1.

KIF1Bβ was identified as a susceptibility gene for neuroendocrine diseases about ten years ago, but, to date, only a few variants have been associated with cluster 2 PHEO/PGL [10, 11].

2. Materials and Methods

2.1. Clinical Report. A 46-year-old male affected by a left incidentally detected PHEO was referred to our unit in 2017. A computed tomography (CT) scan and a magnetic resonance imaging (MRI) confirmed the presence of a solid lesion in the left adrenal gland, 8.5 cm in size, with absolute and relative washout of 20% and 10%, respectively, at enhanced CT scan.

The urine test showed high levels of urinary metanephrines (MNu: 4033 mcg/24h, normal value (nv) < 320) and urinary normetanephrine (NMNu: 5234 mcg/24h, nv < 390), confirming the presence of a PHEO. At the patient’s history, there was no evidence of tumor risk factors.

The patient underwent surgery in November 2017; the surgical course was uneventful, and the histological examination confirmed the presence of a left PHEO. At pathology report, Ki67% was <1% and necrosis and vascular invasion were absent. Pheochromocytoma of Adrenal gland Scaled Score (PASS) [12] was not reported.

The patient gave his written informed consent to genetic testing, and all the major susceptibility genes were assayed (EGLN1, EPAS1, FH, KIF1Bβ, MAX, NF1, RET, SDHA, SDHAF2, SDHB, SDHC, SDHD, TMEM127, and VHL) by NGS. Only a variant of uncertain significance (VUS) in exon 38 of KIF1Bβ was found.

One month after surgery, the levels of urinary metanephrines were still elevated (MNu 118 mcg/24h; NMNu 946 mcg/24h). Subsequent controls confirmed the increase in NMNu up to 1026 mcg/24h in March 2018. The patient performed an abdominal MRI which revealed the presence of an enlarged lymph-node, close to the area of surgery, that showed a weak positive uptake at 123I-metaiodobenzylguanidine (123I-MIBG) scintigraphy and at 18F-fluoro-dihydroxyphenylalanine (18F-DOPA) positron emission tomography (PET).

At the latest control, after two years from surgery, we observed a normal level of NMNu (389 mcg/day). A control abdominal MRI has been planned to evaluate the state of the abdominal lymph-node.

2.2. Molecular Analysis. After obtaining informed consent, genomic DNA of the patient was extracted by the QIA synthome CDX kit (Qiagen). DNA quality and quantity were measured by the Qubit ds assay on the Qubit 2.0 fluorometer (Thermo Fisher Scientific).

A panel of 14 genes was designed using the online Sure Design software (Agilent Technologies). A predesigned Haloplex Pheochromocytoma panel containing the genes EGLN1, EPAS1, FH, KIF1Bβ, MAX, NF1, RET, SDHA, SDHAF2, SDHB, SDHC, SDHD, TMEM127, and VHL was used. The size of the final target region was 38,813 kb with 2170 amplicons, and the mean sequence coverage was 98.53 at 20X priori 99.41% coverage of the target region.

Libraries were generated using the Agilent HaloPlex Target Enrichment protocol, according to the manufacturer’s instructions and sequenced as 150 bp paired-end reads on the Illumina Miseq platform.

Reads quality was checked with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc), and reads were aligned to the reference human genome hg19 with BWA-MEM alignment [13]. Genome Analysis Toolkit (GATK) [14] was used to recalibrate base qualities and realign aligned reads around indelions (InDels).

Finally single-nucleotide variants (SNVs) and InDels were identified by the Unified Genotyper module of GATK and functional annotation by ANNOVAR [15].

BASH and R custom scripts based on BedTools CoverageBed analysis [16] were used to obtain the list of low coverage (≤20X) regions per sample.

Clinically relevant variants have been classified according to the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG) guidelines [17].

2.3. Loss of Heterozygosity (LOH) Analysis. To determine whether there was LOH at the mutation position (c.4052C>T), we undertook sequence analysis of KIF1Bβ exon 38 and flanking intronic regions (NM_003002.2) in tumor DNA from the tissue removed at surgery, with standard PCR amplification. PCR products were sequenced by standard direct sequencing with a BigDye version 1.1 kit (Applied Biosystems, Foster City, California). Sequencing reactions were analyzed using a model 310 ABI PRISMA
genetic analyzer, and the data were processed by sequencing analysis (Applied Biosystems, Foster City, California).

2.4. Three-Dimensional (3D) Mutation Prediction. KIF1Bβ
3D structures were modeled using I-TASSER (Iterative Threading ASSEMBly Refinement https://zhanglab.ccmr.med.umich.edu/I-TASSER/), a hierarchical approach to protein structure and function prediction. The analysis provides structural templates from the Protein Data Bank (PDB). Function insights of the target are then defined by COACH (https://zhanglab.ccmr.med.umich.edu/COACH/) to predict protein-ligand binding site [18–20].

The * pdb files generated from I-TASSER were loaded and visualized with ChemDraw software (version 8; Cambridge Software; PerkinElmer, Inc., Waltham, MA, USA).

Electron Microscopy. Tissue specimens (2 × 2 mm) were washed with PBS and were directly fixed in Karnovsky (cold 2.5% glutaraldehyde and 2% formaldehyde) in 0.1 M sodium cacodylate buffer (pH 7.4) overnight at 4°C and postfixed in cold 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) for 1 h at room temperature. The samples were dehydrated in graded acetone, passed through propylene oxide, and embedded in epoxy resin. Ultrathin sections were stained with gadolinium acetate and alkaline bismuth subnitrate and examined under a JEM 1010 electron microscope (Jeol, Tokyo, Japan) at 80 kV. Photomicrographs were taken with a MegaView III (Soft Imaging System, Muenster, Germany) digital camera connected with a personal computer with dedicated software (AnalySIS, Soft Imaging Software, Muenster, Germany) [21]. Results of electron microscopy studies were compared with those obtained by analyzing 3 randomly selected wild-type (wt) PHEO (Table 1, [22]). To analyse the number of mitochondria, five cytoplasmic fields for each tumor were chosen at random and a count by point was performed [23].

Parental Genetic Analysis. Genetic analysis was later extended to patient’s mother and father who consented to be studied. After informed consent, genomic DNA was screened for direct research of the described KIF1Bβ mutations, using PCR and sequencing.

3. Results

3.1. Mutation Analyses. NGS analysis of the 14 genes included in the panel revealed a novel germline, heterozygous missense mutation in the exon 38 of KIF1Bβ gene, causing the substitution of proline with leucine at position 1351 (c.4052C > T). The alteration was confirmed by Sanger sequencing and loss of heterozygosity (LOH) was detected in the tumor tissue (Figure 1, [24]).

The analysis of variance by querying different databases, i.e., Clinvar and Intervar, allowed us to classify the variant as uncertain (or unknown) significance. We subsequently assessed the pathogenic potential of novel transversion using VarSome, a powerful suite which simultaneously queries the main tools of in silico analysis (Table 2, [25, 26]). Finally, the effect of the mutation P1351L on the structure of the protein was also examined. 3D modeling was generated from the aa 480 up to the C-terminal of the protein (aa 1770) using I-TASSER, and 91% of the residues were modeled at an accuracy of >90% (Figure 2, [27]). A comparison between wt KIF1Bβ protein and mutant protein P1351L revealed an overlap of two kinase domains 1B (aa 799–846; aa 899–928) with a reduction of surface of about 34% (light blue). In addition, a rotation of the predicted DUF3694 domain (aa 1220–1366) probably related to the Kinase domain stabilization (blue) was observed. Moreover, a delocalization of the Forkhead-associated (FHA) domain (aa 512–581) (green), essential for the nuclear localization of the protein, was also observed. The results have been fully confirmed by gene ontology analysis derived from COACH. In particular, a possible cytosolic accumulation of the protein, a loss of microtubule function with consequent displacement of cytoplasmic organelles mainly of mitochondria, and a loss of cell motility during the formation of the central nervous system (CNS) were predicted.

3.2. Genetic Family Analysis. We found the same KIF1Bβ mutation in the father. He performed a CT scan and measurement of urinary metanephrines that were both negative.

3.3. Mitochondrial Status. Since KIF1Bβ is involved in the regulation of mitochondrial fission and apoptosis [8, 9] we assessed in tumor specimens the number and ultrastructure of mitochondria by electron microscopy as well as the proliferative index.

Ultrastructural examination of the tumor specimens bearing the KIF1Bβ gene mutation revealed the presence of several swollen mitochondria with disrupted cristae and cleared matrix. Numerous autophagic vacuoles were also observed, some of which contained mitochondrial remnants (Figures 3(a)–3(b), [28]). By comparison, such abnormalities were not observed in a specimen of wt tumors (Figure 3(c), [28]). Moreover, performing the count by point of the mitochondria, we observed that the number of these organelles was almost two folds higher in KIF1Bβ-mutated tumor than in the nonmutated pheochromocytoma specimens (20.5 ± 2.1 and 10.4 ± 2.0 mitochondria/field, respectively).

4. Discussion

4.1. Role of KIF1Bβ. KIF1Bβ gene variants have been associated to the development of neural and nonneural tumors [10]. Germline KIF1Bβ variants have been rarely reported in patients with PHEO, while somatic variants have been more frequently found in tumor tissues.

The pathogenic mechanisms by which KIF1Bβ mutations cause the occurrence of neural and nonneural neoplasms are only partially known. Global transcription analysis of KIF1Bβ -mutant PHEO revealed that these tumors are transcriptionally related to RET- and NF1-mutated
PHEO (Cluster 2) but independent from SDH- and VHL-associated tumors (Cluster 1). Furthermore, KIF1Bβ-mutant tumors are uniquely enriched for pathways related to glutamate metabolism and the oxidative stress response [10]. Additionally, kinesin KIF1Bβ acts downstream to prolyl hydroxylase to induce apoptosis; therefore, germline

Table 1: Clinical characteristics of patients with nonmutated pheochromocytoma used for electron microscopy analysis.

<table>
<thead>
<tr>
<th></th>
<th>Wt tumors</th>
<th>Age at diagnosis (years)</th>
<th>Sex (F/M)</th>
<th>PHEO/PGL</th>
<th>Secretion (A/NA)</th>
<th>Metastatic (yes/no)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt1</td>
<td>Right PHEO</td>
<td>39</td>
<td>F</td>
<td>A</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Wt2</td>
<td>Left PHEO</td>
<td>69</td>
<td>F</td>
<td>NA</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Wt3</td>
<td>Right PHEO</td>
<td>52</td>
<td>F</td>
<td>NA</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

Wt: wild-type; F: female; M: male; A: adrenergic; NA: noradrenergic (according to Eisenhofer et al. 2011) [22, 23].

Table 2: DANN pathogenicity score resulting from the simultaneous query of different in silico prediction tools.

<table>
<thead>
<tr>
<th>Prediction tool</th>
<th>Score</th>
<th>Converted rank score</th>
</tr>
</thead>
<tbody>
<tr>
<td>MutationTaster dbNSFP version 4.0</td>
<td>Prediction disease causing</td>
<td>Accuracy 1</td>
</tr>
<tr>
<td>Mutation assessor dbNSFP version 4.0</td>
<td>Prediction medium</td>
<td>Score 2.635</td>
</tr>
<tr>
<td>FATHMM-MKL dbNSFP version 4.0</td>
<td>Coding prediction damaging</td>
<td>Coding score 0.9891</td>
</tr>
<tr>
<td>FATHMM-XF dbNSFP version 4.0</td>
<td>Coding prediction damaging</td>
<td>Coding score 0.8661</td>
</tr>
<tr>
<td>LRT dbNSFP version 4.0</td>
<td>Prediction deleterious</td>
<td>Score 0</td>
</tr>
<tr>
<td>DEOGEN2 dbNSFP version 4.0</td>
<td>Prediction damaging</td>
<td>Score 0.8068</td>
</tr>
<tr>
<td>EIGEN dbNSFP version 4.0</td>
<td>Prediction pathogenic</td>
<td>Raw coding score 0.8034</td>
</tr>
<tr>
<td>EIGEN PC dbNSFP version 4.0</td>
<td>Prediction pathogenic</td>
<td>PC raw coding score 0.798</td>
</tr>
<tr>
<td>SIFT dbNSFP version 4.0</td>
<td>Prediction damaging</td>
<td>Score 0.032, 0.03</td>
</tr>
<tr>
<td>SIFT4G dbNSFP version 4.0</td>
<td>Prediction damaging</td>
<td>Score 0.039, 0.04</td>
</tr>
<tr>
<td>PROVEAN dbNSFP version 4.0</td>
<td>Prediction damaging</td>
<td>Score -8.16, -8.3</td>
</tr>
<tr>
<td>REVEL dbNSFP version 4.0</td>
<td>Prediction pathogenic</td>
<td>Score 0.699</td>
</tr>
<tr>
<td>PrimateAI dbNSFP version 4.0</td>
<td>Prediction damaging</td>
<td>Score 0.8779</td>
</tr>
<tr>
<td>MetaSVM dbNSFP version 4.0</td>
<td>Prediction damaging</td>
<td>Score 0.186</td>
</tr>
<tr>
<td>MetaLR dbNSFP version 4.0</td>
<td>Prediction damaging</td>
<td>Score 0.5961</td>
</tr>
<tr>
<td>FATHMM dbNSFP version 4.0</td>
<td>Prediction tolerated</td>
<td>Score -0.9, -0.81, -0.89</td>
</tr>
<tr>
<td>MVP dbNSFP version 4.0</td>
<td>Prediction uncertain</td>
<td>Score 0.7537</td>
</tr>
<tr>
<td>MutPred dbNSFP version 4.0</td>
<td>Prediction uncertain</td>
<td>Score 0.66</td>
</tr>
</tbody>
</table>

The value range is 0 to 1, with 1 given to the variants predicted to be the most damaging (http://varsome.com/variant/hg19/kif1b%3Ac.4052C>T) (according to Quang et al. 2015, [26]).

Figure 1: Wild-type (a) and patient electropherogram showing the germline mutation p.Pro1351Leu in exon 38 of the KIF1Bβ gene (b) and the somatic mutation showing the LOH (c).

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alterations compromising KIF1Bβ functions allow certain neuronal progenitor cells to escape the developmental culling [8]. Not all the KIF1Bβ-related tumors are associated with LOH, thus suggesting haploinsufficiency or epigenetic silencing of the wt allele in some tumors [10].

4.2. New Likely Pathogenetic KIF1Bβ Mutation. The transversion (A > T) identified is a novel mutation and, as in such cases, the question arises whether it has to be considered pathogenic or not. The patient's family history was negative for the presence of neural or nonneural neoplasms, so we performed several in silico studies to answer this question. The bioinformatic tools classified the variant as pathogenic. The finding of LOH in the tumor tissue strengthened this assumption, and the adrenergic biochemical characteristic [23] of our patient's tumor is in agreement with the cluster 2 genetic profile.

4.3. Family Screening. On the basis of these results, we asked the patient’s parents to undergo genetic testing which we performed after their informed consent. The same KIF1Bβ mutation was detected in the father which resulted negative in the biochemical and imaging screening.
In view of the KIF1Bβ mutation rarity, the penetrance is unknown and a low one, explaining the father’s negative clinical picture can only be hypothesized. A further explanation might reside in a gene maternal imprinting similar to that found for SDHD and SDHAF2 [29, 30]. Indeed, in the manuscript by Yeh et al., the family pedigree may be consistent with a maternal imprinting [10]. Nonetheless, the father’s clinical follow-up remains mandatory.

4.4. Patient’s Clinical Course. At diagnosis the biochemical phenotype was in line with the cluster 2 secreting pattern. However, at proband’s follow up, MNu was found normal while NMNu, although decreased, was still elevated, prompting us to perform additional studies to explain this unexpected result. The biochemical phenotype detected after surgery may reflect the extra-adrenal localization of the disease. MRI, 123I-MIBG scintigraphy, and 18F-DOPA-PET suggested the presence of a metastatic lymph-node responsible for the increase in NMNu. No additional lesions were detected. During the follow up, the lymph-node was found slightly shrunk while NMNu normalized very slowly. The patient, who at present is normotensive and without any symptom, will undergo a life-long follow up in view of his genetic status, looking for PHEO recurrence, metastatic disease development, or the occurrence of other KIF1Bβ-linked tumors. The reason for the NMNu normalization two years after surgery is difficult to explain; it might be due to a tumor dedifferentiation or to a tissue necrosis.

4.5. First Evaluation of Mitochondrial Features in KIF1Bβ-Mutated Pheochromocytoma. To investigate the consequences of this mutation at the cellular level, we performed a deep analysis of the mitochondrial status in the tumor, finding a significantly higher aberrant morphology and number compared with wt tissues. This result is in line with the finding that KIF1Bβ altered activity is involved not only in neurodegenerative diseases but also in mitochondrial morphological aberration-associated with some tumors [20]. Previous data have shown that KIF1Bβ plays a role in the regulation of mitochondrial apoptosis. In particular, low expression of KIF1Bβ could lead to a reduction of mitochondrial fission and of subsequent apoptosis [8, 9]. We here observed an increased number of mitochondria (almost two fold higher) and the presence of autophagic vacuoles in the

Figure 3: Representative ultrastructural micrographs of a pheochromocytoma bearing the KIF1Bβ mutation (a, b) in comparison with a wild-type tumor (c). Gene mutation is associated with the occurrence of numerous swollen mitochondria (dashed arrows) with reduction of cristae and matrix clearing. Several autophagic vacuoles (black arrows) containing remnants of organelles, including mitochondria (a) can also be seen. Magnification: x12,000.
mutated tumor as compared with wt tissues. Considering our results, it is reasonable to hypothesize that the mutation found in KIF1Bβ is associated with a decrease in the physiological mitochondrial fragmentation and with the permanence of aberrant nonfunctional mitochondria in autophagic vacuoles, finally resulting in a decrease in the mitochondrial-induced apoptosis which may support tumor progression. It is possible to speculate that, to restore the mitochondrial homeostasis, tumor cells might activate a compensatory mitochondrial neoformation leading to the increased number of these organelles. Further studies are needed to corroborate this hypothesis. Nonetheless, the present results are in line with the previous studies [8, 9] and represent the first observation performed in mitochondria of KIF1Bβ-mutated tissue. The evaluation of CN, DRP1, and YME1L1 expression in future studies on mutated tissues could improve our understanding of the role of KIF1Bβ alterations in human diseases.

5. Conclusions

In conclusion, we report a novel KIF1Bβ variant in a patient with PHEO. Our study confirms the importance of using target NGS in the genetic analysis of cancer patients in order to screen all the main susceptibility genes. At the same time, it recalls the necessity of a strict collaboration between geneticists and clinicians for a correct interpretation of the results and application of personalized medicine in view of an appropriate management of these patients. For the first time, we analyzed the mitochondrial features in a KIF1Bβ-mutated tissue.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors do not have any conflicts of interest to disclose.

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