CASE REPORT

A novel *TMEM127* mutation in a patient with familial bilateral pheochromocytoma

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Abstract

Objective: In this report, we describe a new patient with unexplained familial bilateral pheochromocytoma. Following the recent description of *TMEM127* as a new pheochromocytoma susceptibility gene, the aim of this study was to test the hypothesis of a causative *TMEM127* gene mutation in this patient.

Design: Pheochromocytoma susceptibility genes were analyzed in germline DNA and losses of heterozygosity (LOH) assessed by BAC array comparative genomic hybridization in tumor DNA. *SDHB* expression and S6 kinase (S6K) phosphorylation were analyzed by immunohistochemistry. Genome-wide expression microarray studies were performed, and vascular density was quantified after CD34 immunohistochemistry.

Results: A first germline variant was identified in the *SDHB* gene (c.158G>A; p.Gly53Glu). However, a positive *SDHB* immunostaining in the tumor indicated that this *SDHB* variant was a non-functional polymorphism. A novel *TMEM127* germline mutation (c.140C>A, p.Ala47Asp) associated with a 2q11 LOH was found. Transcriptome and immunohistochemical analyses showed that *TMEM127*-related pheochromocytoma clusterized with *NF1*-related and *RET*-related tumors in a large series of pheochromocytomas and paragangliomas, exhibited a reduced *TMEM127* mRNA expression and displayed a low vascularization. The phosphorylation of S6K observed in this tumor was suggestive of an activation of the MTOR pathway.

Conclusions: Pathological and genomic data demonstrated that a *TMEM127* gene mutation not previously described was causative of a new case of familial bilateral pheochromocytoma. This report highlights the importance of supplementary analyses on tumor tissue to provide an accurate pheochromocytoma/paraganglioma genetic testing result to affected patients.

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Introduction

Pheochromocytomas and paragangliomas are rare catecholamine-secreting tumors arising from the adrenal medulla (pheochromocytoma proper) or from extra-adrenal chromaffin tissues. Although usually sporadic, pheochromocytomas and paragangliomas can occur in the context of inherited cancer syndromes in ~30% of cases (1). These hereditary diseases include multiple endocrine neoplasia type 2, von Hippel–Lindau disease, neurofibromatosis type 1, and hereditary paragangliomas caused respectively by germline mutations in the

RET, *VHL*, *NF1*, *SDHB*, *SDHC*, *SDHD*, *SDHA* (*SDHx*), and *SDHAF2* genes (2–4). Some familial clusters of yet unexplained pheochromocytomas and/or paragangliomas remained, suggesting the existence of new genes predisposing to these tumors (5). Recently, Qin *et al.* (6) reported heterozygous germline mutations in *TMEM127* gene in seven patients affected by pheochromocytoma. They demonstrated that *TMEM127* is a new tumor suppressor gene involved in hereditary pheochromocytoma/paraganglioma syndrome, according to a two-hit model of inactivation (germline mutation associated with loss of wild-type allele).

Moreover, microarray-based expression profiling analyses showed that *TMEM127*-mutated tumors present a transcription signature comparable to that of *RET*- and *NF1*-mutated pheochromocytomas (characterized by enrichment for kinase receptor signaling pathways), differentiating them from *SDHx*- and *VHL*-related tumors, which are characterized by activation of the hypoxic pathway.

Here, we report a patient with familial bilateral pheochromocytoma, harboring a non-functional missense variant in the *SDHB* gene and a causative germline mutation in the *TMEM127* gene. This report illustrates the importance of clinical, pathological, and genomics data to interpret pheochromocytoma/paraganglioma genetic testing, and confirms that the *TMEM127* gene is a new tumor suppressor gene involved in pheochromocytoma predisposition.

Subjects and methods

Case history

The patient is a woman who reported hyperadrenergic symptoms (sweating, headache, and tachycardia) since she was 20 years old and who suffered from hypertension and diabetes during both her pregnancies. At the age of 44 years, she became permanently hypertensive. She had asthenia and presented unexplained weight loss, symptoms that led to consult a specialized physician. Her family history was remarkable. Her mother had bilateral adrenal surgery for pheochromocytoma at the age of 67 years and died from pancreatic cancer. Her brother died during childhood after general anesthesia.

Clinical investigations revealed high concentrations of urinary fractionated metanephrines (193.9 $\mu\text{mol}/24\text{ h}$, normal range <3.7 , with a predominant elevation of normetanephrines). Computed tomography scan disclosed two heterogeneous adrenal masses (40 $\times$ 26 mm on the left side and 150 $\times$ 50 mm on the right side) suggestive of bilateral pheochromocytoma (Fig. 1A). Whole body metaiodobenzylguanidine scintigraphy was positive for both suspected pheochromocytomas, and no extra-adrenal tumor localization was detected. Following bilateral open adrenalectomy, histology confirmed the diagnosis of bilateral pheochromocytoma, with no indication of malignancy. Post-operative recovery was uneventful under hormonal supplementation. The patient is currently tumor free with normal metanephrine excretion 14 years following adrenalectomy.

DNA samples

Patient signed a written informed consent for germline and somatic DNA analyses as well as for the collection of tumor samples at the time of surgery. Tumor samples were immediately frozen in liquid nitrogen to be included

Figure 1 (A) Computed tomography (CT) of the abdomen after contrast enhancement revealed two highly vascularized heterogeneous adrenal masses (40 $\times$ 26 mm mass on the left side and a necrotic bilobed 150 $\times$ 50 mm mass on the right side) suggestive of bilateral pheochromocytoma. (B) Sequence chromatograms of *SDHB* gene showed the normal sequence in control DNA, the heterozygous variant c.158G>A identified in the patient leukocyte DNA, and the predominant allele in tumor DNA indicating LOH at the *SDHB* locus. (C) Positive SDHB immunostaining is observed in the patient's tumor as well as in a *RET*-mutated pheochromocytoma compared to a negative immunostaining in an *SDHB*-mutated tumor. Calibration bar: 100 μm .

in the COMETE network. Ethical approval for the study was provided by the institutional review board (CPP Paris-Cochin, January 2007). A Caucasian reference population was used to obtain 170 control DNAs. Germline DNAs were extracted from leukocytes according to standard protocols. Tumor DNAs and RNAs were extracted using AllPrep DNA/RNA Mini Kit (Qiagen).

Genetic testing

Mutation analysis for *RET*, *VHL*, *SDHB*, *SDHC*, and *SDHD* genes was performed by direct sequencing. *VHL*, *SDHB*, *SDHC*, and *SDHD* genes were also analyzed for the presence of large deletions by Multiplex Ligation-dependent Probe Amplification (MLPA) method as described previously (2, 7). The four exons and the intron–exon boundaries of *TMEM127* gene in the germline and tumor DNAs were directly sequenced (primers available on request).

Microarray and BAC array comparative genomic hybridization

Microarray and BAC array comparative genomic hybridization (CGH) analyses were performed as previously described (3, 8).

Immunohistochemistry

Paraffin blocks were cut and 6 μm -thick sections were mounted on Superfrost plus slides. Immunohistochemical analyses were performed using the following antibodies: anti-SDHB (HPA002868, Sigma–Aldrich),

anti-CD34 (Clone QBEND 10, Immunotech, Marseille, France 1/100) for the quantification of vascular density (8), anti-S6 kinase (S6K) (sc-8418, Santa Cruz Biotechnologies), and anti p-S6K (sc-7984-R, Santa Cruz Biotechnologies, Santa Cruz, CA, USA).

Results

Identification of a non-functional SDHB variant

In 2008, genetic testing was proposed to the patient, accordingly to international recommendations for patients with paraganglioma/pheochromocytoma (9, 10). The search for germline mutation in *VHL*, *RET*, *SDHC*, and *SDHD* genes was negative, but a heterozygous missense variant was identified in the *SDHB* gene (c.158G>A; p.Gly53Glu; Fig. 1B). This variant was previously reported as a possible polymorphism in the NCBI's Entrez system (dbSNP reference: rs34916635) and in the TCA cycle gene mutation database (11). However, it appeared homozygous after *SDHB* direct sequencing from tumor DNA (Fig. 1B) suggesting a loss of heterozygosity (LOH) at the *SDHB* locus (1p36), which was further confirmed by BAC array CGH (Fig. 2B). To assess the functionality of this variant, we therefore performed SDHB immunohistochemistry on the tumor tissue (Fig. 1C) (12). Such analysis of *SDHB* expression revealed a clear cytoplasmic SDHB staining in the patient's tumor, comparable to that observed in non-SDHx pheochromocytomas, clearly confirming that the c.158G>A *SDHB* variant is a non-functional polymorphism.

Identification of a TMEM127 mutation associated with LOH

In 2010, *TMEM127* germline mutations were reported in seven patients affected by pheochromocytoma (6).

We thus analyzed the *TMEM127* gene by direct sequencing in our patient and identified a germline heterozygous missense mutation (c.140C>A, p.Ala47Asp) in leukocyte DNA that appeared homozygous in tumor DNA (Fig. 2A). This variant was not found in 340 control chromosomes. In the patient's tumor, LOH at the *TMEM127* locus (2q11) was confirmed by BAC array CGH experiments (Fig. 2B) performed in a series of 202 pheochromocytomas/paragangliomas (comprising 75 inherited tumors and 127 sporadic tumors) collected by the COMETE network. It is worth noting that in this tumor, no LOH was found at the *SDHA* (5p15) or *SDHAF2* (11q13) loci, thus excluding the involvement of these recently identified extra-adrenal paraganglioma susceptibility genes for our patient (Fig. 2B). In the whole series of 202 tumors, a 2q11 LOH was observed in nine additional tumors, including one *SDHB*-related paraganglioma (c.200+1G>A) and eight tumors with an apparently sporadic presentation. These nine additional candidates were thus submitted to *TMEM127* sequencing. Only one synonymous variant (c.621G>A; p.Ala207Ala (dbSNP reference: rs 3852673)) was identified in three other patients (heterozygosity frequency = 31%). Among the healthy control population, a new missense variant (c.121A>G; p.Ile41Val) was identified. This variant has not yet been annotated and should be considered as a rare polymorphism (frequency <1%).

Microarray and immunohistochemical analyses of the patient's tumor

The transcriptome of the patient's tumor was analyzed by genome-wide expression microarray in the context of a large study that included 188 tumors of the COMETE cohort (3, 8). The unsupervised clustering performed with genes' expression involved in energy metabolism (combination of oxidative phosphorylation and glycolytic pathways) or in hypoxia pathway classified the

Figure 2 (A) Sequence chromatograms of *TMEM127* gene obtained in the patient's leukocyte and tumor DNAs compared to a control DNA. Direct sequencing of the patient's leukocyte DNA shows the *TMEM127* heterozygous mutation c.140C>A and the predominance of the mutant allele in the tumor tissue indicating LOH at the *TMEM127* locus. (B) BAC array CGH analysis reveals 1p, 2q, 11p, and 21q losses and gain of 1q and 20pq in *TMEM127* tumor. Green and red lines indicate chromosomal losses and gains respectively. The arrow points to the *TMEM127* locus at 2q11.

TMEM127-related tumor in the subgroup of *RET*- and *NF1*-related pheochromocytomas (data not shown), further confirming the absence of any functional *SDHx* gene mutations in this tumor. The evaluation of angiogenesis by CD34 immunohistochemistry showed that the vascular density in the *TMEM127*-related tumor (13 ± 2 blood vessels/ 0.65 mm^2) was comparable to that previously reported in 4 *RET/NF1* tumors (mean = 9 ± 2 blood vessels/ 0.65 mm^2) and significantly lower than in 15 *SDHx/VHL*-related tumors (mean = 28 ± 3 blood vessels/ 0.65 mm^2) (Fig. 3A) (8). Analysis of *TMEM127* mRNA levels based on microarray expression data was performed in the patient's pheochromocytoma and compared to tumors with LOH at 2q11 locus ($n=8$) or without LOH at 2q11 ($n=179$). We observed an overall 35% reduction in *TMEM127* expression levels in tumors harboring a 2q11 LOH as compared to tumors with no LOH at the *TMEM127* locus. There was a significant association between *TMEM127* level and the presence of the 2q11 LOH ($P=4.10^{-5}$, *T*-test). In the patient's tumor, a 61% reduction in *TMEM127* mRNA was observed (Fig. 3B).

Finally, Qin *et al.* (6) reported that *TMEM127*-mutated tumors displayed increased phosphorylation of MTOR target S6K, as compared with normal adrenal or tumors without *TMEM127* mutation. We thus performed immunohistochemistry to evaluate the expression of S6K and the presence of its phosphorylated form in the patient's tumor and confirmed a putative activation of the MTOR pathway in this tissue (Fig. 3C).

Figure 3 (A) Quantification of vascular density revealed a decreased number of blood vessels in *TMEM127*-related and *RET/NF1*-related pheochromocytomas compared with *SDH/VHL*-related tumor tissues. Data are means $\pm$ S.E.M. (B) Microarray evaluation of relative *TMEM127* expression in *TMEM127*-related pheochromocytoma compared to tumors with or without LOH in the 2q11 region (*TMEM127* locus). Data are means $\pm$ S.E.M. (C) Immunohistochemical analysis of S6K total expression (left) and phosphorylation shows that this protein is both present and phosphorylated in the nucleus of *TMEM127* tumor cells.

Discussion

In this report, we describe a patient affected by familial bilateral pheochromocytoma, harboring a non-functional variant in the *SDHB* gene (p.Gly53Glu) and a new *TMEM127* gene mutation (p.Ala47Asp) associated with 2q11 LOH. The presence of these variants could not be confirmed in the patient's tumor mother who was also affected by bilateral pheochromocytoma, as she died several years ago. However, genetic and expression data indicate that the *TMEM127* germline mutation identified in the patient is causative of her familial predisposition to pheochromocytoma and could be used with confidence for the genetic counseling of her first relatives.

This report underlines the importance of complementary tests to provide validated genetic results. Herein, we demonstrate that *SDHB* immunohistochemistry was positive in this case and confirms that such a pathological analysis is a powerful means to orientate the genetic testing among the nine genes that are now to be considered (13) and to validate the functionality of *SDHx* unknown variants (12).

Most of our data are in accordance with the recent publication on *TMEM127* gene by Qin *et al.* (6). The family history and the bilateral and benign nature of the pheochromocytoma are in keeping with the clinical characteristics of the first seven patients carrying a *TMEM127* germline mutation. This report confirms that *TMEM127* gene is a tumor suppressor gene following the two-hit Knudson model with LOH in the tumor DNA. Using the transcriptome analysis of the large series of pheochromocytomas and paragangliomas collected by the COMETE network, we were also able to confirm the cluster association between the *TMEM127*-related pheochromocytoma and *NF1*- and *RET*-related tumors. By the same token, the evaluation of angiogenesis assessed by CD34 immunohistochemistry revealed, in the *TMEM127*-related pheochromocytoma, a low vascular density as observed in the group of *RET/NF1*-related tumors. Analysis of *TMEM127* transcript levels indicated a 61% reduction for the tumor associating germline *TMEM127* mutation and LOH at *TMEM127* locus compared with wild-type *TMEM127* tumors. This down-regulation was slightly lower than that previously described (78%), but is also in favor of the instability of the mutant transcript. Interestingly, in patients with no *TMEM127* mutation but with 2q11 LOH, *TMEM127* mRNA levels were also decreased by 35%, thus showing that 2q11 LOH itself had a dosage effect. Such a correlation has already been reported for many other genes, such as 19p13 LOH and *GNG7* expression in esophageal cancer (14) or 3p14 LOH and *FHIT* expression in breast cancer (15).

Finally, as previously reported, we observed a putative activation of the mTOR pathway characterized by the phosphorylation of S6K in tumor cells. Whether analysis of these immunohistochemical criteria could

be used as a predictive tool for the presence of a *TMEM127* mutation will have to be evaluated in large cohorts.

Qin *et al.* (6) reported that 3% of apparently sporadic pheochromocytomas were caused by *TMEM127* gene mutations. In the COMETE series of 127 sporadic pheochromocytomas and paragangliomas, we limited the *TMEM127* gene sequencing to the eight patients harboring a 2q11 LOH (6%) and found no mutation. The exact prevalence of *TMEM127*-related pheochromocytomas will need to be defined by the *TMEM127* genotyping of further large series. Anyhow, the present knowledge suggests that *TMEM127* genotyping should now be added to *VHL* and *RET* analyses for patients with bilateral pheochromocytoma and/or a family history and with a positive tumor SDHB immunostaining.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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