Somatic and Occult Germ-line Mutations in SDHD, a Mitochondrial Complex II Gene, in Nonfamilial Pheochromocytoma

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Abstract

Most pheochromocytomas are sporadic but about 10% are thought to be hereditary. Although the etiology of most inherited pheochromocytoma is well known, little is known about the etiology of the more common sporadic tumor. Recently, germ-line mutations of SDHD, a mitochondrial complex II gene, were found in patients with hereditary paraganglioma. We sought to determine whether SDHD plays a role in the development of sporadic pheochromocytomas and performed a mutation and deletion analysis of SDHD. Among 18 samples, we identified 4 heterozygous sequence variants (3 germ-line, 1 somatic). One germ-line SDHD mutation IVS1+2T>G (absent among 78 control alleles) is predicted to cause aberrant splicing. On reinvestigation, this patient was found to have a tumor of the carotid body, which was likely a paraganglioma. Another patient with malignant, extra-adrenal pheochromocytoma was found to have germ-line c.34G>A (G12S). However, this sequence variant was also found in 1 of 78 control alleles. The third, germ-line nonsense mutation R38X was found in a patient with extra-adrenal pheochromocytoma. The only somatic heterozygous mutation, c.242C>T (P81L), has been found in the germ line of two families with hereditary paraganglioma and is conserved among four eukaryotic multicellular organisms. Hence, this mutation is most likely of functional significance too. Overall, loss of heterozygosity in at least one of the two markers flanking SDHD was found in 13 tumors (72%). All of the tumors that already harbored intragenic SDHD mutations, whether germ-line or somatic, also had loss of heterozygosity. Our results indicate that SDHD plays a role in the pathogenesis of pheochromocytoma. Given the minimum estimated germ-line SDHD mutation frequency of 11% (maximum estimate up to 17%) in this set of apparently sporadic pheochromocytoma cases and if these data can be replicated in other populations, our observations might suggest that all such patients be considered for SDHD mutation analysis.

Introduction

Pheochromocytomas produce and secrete catecholamines and, hence, can cause hypertension. They derive from large plexomorphic chromaffin cells that most commonly arise from the adrenal medulla. In rare instances, pheochromocytomas are extra-adrenal. Most pheochromocytomas are sporadic but ~10% are hereditary (1, 2) and may be found in association with the MEN 2 (3) syndromes or VHL (3–5). Isolated familial forms of pheochromocytoma without other associated clinical features occur even less commonly (6). The etiology of most familial forms of pheochromocytoma is well known. Germ-line mutations in the RET proto-oncogene are found in >98% of MEN 2 patients with pheochromocytoma (5, 7), and germ-line mutations in the tumor suppressor gene VHL are found in >98% of VHL patients with pheochromocytoma (8, 9). In contrast, little is known about the etiology of the more common sporadic form of pheochromocytoma. Somatic RET mutations have been found in up to 10% of tumors and somatic VHL mutations in no more than 2% (10–13).

Pheochromocytomas are also referred to as adrenal paragangliomas. Paragangliomas originate from the neural crest-derived paraganglia of the autonomic nervous system. They are identified throughout the body in both adrenal and extra-adrenal sites. Hereditary paragangliomas are highly vascularized tumors located in the head and neck. They are most commonly found at the bifurcation of the carotid artery in the neck, also referred to as the carotid body. Recently, germ-line mutations of SDHD were found in patients with hereditary paragangliomas (14). SDHD, located on 11q23 (15), encodes the small subunit (cybS) of cytochrome b in the succinate-ubiquinone oxidoreductase complex (mitochondrial complex II). This enzyme complex plays an important role in both the tricarboxylic acid cycle and the aerobic respiratory chain of eukaryotic cell mitochondria. SDHD comprises four exons and three introns spanning over 19 kb (16).

We sought to determine whether SDHD plays a role in the development of sporadic cases of what has been defined as pheochromocytoma in the German-Polish Registry by analyzing the SDHD gene for sequence variants and microsatellite markers flanking the gene for LOH. Our results indicate that SDHD plays a role in the pathogenesis of pheochromocytoma.

Materials and Methods

Patients and DNA Samples. Genomic DNA from tumor and peripheral blood leukocytes was obtained from 18 unrelated patients (14 female, 4 male) who underwent surgery for sporadic pheochromocytoma at the University of Freiburg, Germany. All of the samples were obtained with informed consent. The patients were selected from a population-based register that currently holds 170 sporadic pheochromocytomas.4 These 18 patients were chosen because they were the only ones who had paired germ-line DNA and frozen pheochromocytoma in the sample bank. All of the 18 pheochromocytomas were classified as being sporadic because of the absence of germ-line mutations specific for MEN 2 (RET, localized to 10q11.2) and VHL syndrome (VHL, 3p25–26; Ref. 5, 8). Furthermore, the patients’ personal and family histories, as well as meticulous physical examinations, biochemical tests, and imaging studies, were not suggestive of MEN 2, VHL, or NF 1 (3, 13, 17). Of the 18 tumors, 4 pheochromocytomas were extra-adrenal, 3 of which were malignant. Another intra-adrenal pheochromocytoma also was classified as malignant. Malignancy was operationally defined as local infiltration of adjacent tissues.

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3 The abbreviations used are: MEN 2, multiple endocrine neoplasia type 2; VHL, von Hippel-Lindau syndrome; LOH, loss of heterozygosity.


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cent tissues and/or metastatic disease. Relevant clinical information is summarized in Table 1.

Genomic DNA from peripheral blood leukocytes from a region-matched, race-matched control group was available. This group consisted of 39 individuals who were admitted to the Department of General Surgery, Halle, Germany, for nonpheochromocytoma-related diseases. Informed consent was given in all of the cases.

DNA extraction was performed using the QIAamp tissue kit (Qiagen, Santa Clarita, CA) according to the manufacturer’s instruction.

**Mutation Analysis.** PCR amplification was carried out in 1 × PCR buffer (Qiagen) that contained 200 μmol/liter deoxynucleotide triphosphate, 0.6 μmol/liter of each primer (14), 2.5 units of Taq polymerase (Qiagen), and 100–200 ng of tumor DNA template in a 50 μl volume. PCR conditions were: one cycle of 15 min at 95°C; 35 cycles of 1 min at 95°C, 1 min at 58°C, and 1 min at 72°C; followed by one cycle of 10 min at 72°C.

PCR amplicons were gel (Bio-Rad Laboratories, Hercules, CA) and column (Wizard PCR Prep; Promega, Madison, WI) purified and subjected to semi-automated sequencing using the above primers, dye terminator technology, and the ABI377xl or PE3700. If sequencing revealed a variant, the corresponding leukocyte DNA was examined in the same manner to determine whether the sequence variant was somatic or germ-line. Each sample with a sequence variant was subjected to repeat mutation analysis that used a separate PCR reaction.

**LOH Analysis.** All of the PCR reactions were carried out using 0.6 μM each of forward and reverse primer in 1 × PCR buffer (Qiagen), 4.5 mM MgCl2 (Qiagen), 1 × Q-buffer (Qiagen), 2.5 units of HotStarTaq polymerase (Qiagen), and 200 μM deoxynucleotide triphosphate (Life Technologies, Inc., Gaithersburg, MD) in a final volume of 50 μl. Reactions were subjected to 35 cycles of 94°C for 1 min, 55–60°C for 1 min, and 72°C for 1 min followed by one cycle of 10 min at 72°C.

PCR amplicons were separated by electrophoresis through 6% denaturing polyacrylamide gels using an Applied Biosystems model 377 automated DNA sequencer (Applied Biosystems and Perkin-Elmer Corp., Norwalk, CT). Scoring of LOH was performed by inspection of the GeneScan collection and analysis software (GeneScan; Applied Biosystems, Norwalk, CT). LOH was defined as an absence or presence of a specific DNA peak relative to a control peak. The wild-type allele was defined by the presence of a peak at the same position as the control, the mutant chromosome represented a reduction of the control peak, and the mutant allele was defined by the absence of a control peak.

**Results**

**SDHD Mutation Analysis in Pheochromocytomas.** Among 18 samples, we identified four heterozygous sequence variants in four distinct samples (Fig. 1). Three of these were germ-line, and the remaining one was somatic. A germ-line *SDHD* mutation IVS1+2T>G (Fig. 1B) was detected in a 39-year-old male. Although RNA was not available, this variant is predicted to cause aberrant splicing. We then contacted this patient’s general practitioner, who reported that this patient, at 43 years of age, had recently been diagnosed with a tumor of the carotid body, which was likely a paraganglioma, and was scheduled for surgery. The IVS1+2T>G variant was not found among 78 control alleles. Another patient, a 17-year-old female, was found to have germ-line missense mutation G12S (c.34G>A, exon 1; Fig. 1A). Clinically, she had an extra-adrenal, intra-abdominal pheochromocytoma. After surgery, catecholamine levels were normal but increased again. At 21 years of age, she underwent reoperation because of recurrent extra-adrenal, intra-abdominal pheochromocytoma. Because there was nodal involvement within the jugular fossa, this pheochromocytoma was classified as malignant. We then analyzed 78 control alleles and found this sequence variant in 1 allele from one patient with intestinal lipoma. This patient’s general practitioner was contacted, and the clinical absence of pheochromocytoma and paraganglioma was confirmed. A third patient, a 33-year-old female, was found to have germ-line nonsense mutation R38X (c.112C>T, exon 2; Fig. 1C). Clinically, she had two extra-adrenal pheochromocytomas, one intra-abdominal tumor, and one thoracic tumor.

The only somatic mutation c.242C>T, P81L (exon 3; Fig. 1D) was
found to occur in a heterozygous state in a pheochromocytoma from a 42-year-old female. This missense mutation was not found in corresponding germ-line DNA.

**LOH Analysis.** Overall, LOH (Fig. 2) in at least one of the two 11q23 markers was found in 13 tumors (72%). LOH of both markers was found in 3 tumors (17%). The remaining 10 tumors showed LOH of one or the other 11q23 marker. All of the tumors already harboring intragenic SDHD mutations also showed LOH.

**Discussion**

In our series of 18 apparently sporadic pheochromocytoma samples, we identified one (6%) somatic SDHD mutation and three (17%) germ-line SDHD variants. The only somatic mutation, P81L, is almost certainly pathogenic. It has also been found in the germ-line of two families with hereditary paraganglioma (14). This missense mutation is highly conserved in four eukaryotic multicellular organisms, and the proline-leucine substitution in that position would be predicted to alter cyBS conformation (14). This tumor with somatic P81L also has LOH of the remaining wild-type allele, which lends additional support that the missense mutation is pathogenic and that SDHD is a tumor suppressor gene that plays some role in the pathogenesis of sporadic pheochromocytoma.

The nonsense germ-line R38X mutation is almost certainly pathogenic because it would likely result in a very short nonfunctional protein. This mutation was also found by Baysal et al. (14) in the germ line of one family with hereditary paraganglioma. The germ-line IVS1+2T>G mutation occurs at one of the highly conserved consensus splice sequences and is almost certainly functionally significant inasmuch as it would be predicted to result in aberrant splicing. Unfortunately, despite great efforts, no RNA is available. This germ-line variant has not been found in 78 regional control alleles, and Baysal et al. (14) did not note the presence of this variant in 200 of their control chromosomes either. That this patient developed a carotid body tumor, almost certainly a paraganglioma, 4 years subsequent to his diagnosis of isolated pheochromocytoma and that the tumor had LOH of the remaining allele lend strong support that this germ-line variant is actually an occult germ-line SDHD mutation that predisposed to both the pheochromocytoma and the carotid body tumor. Finally, the G12S germ-line variant that occurred in a teenager with adrenal and extra-adrenal pheochromocytoma is somewhat of a dilemma because it has been found in one regional control who did not have any evidence of neuroendocrine tumors at the age of 72. Baysal et al. (14), on the other hand, did not note G12S in their 200 normal control chromosomes. Nonetheless, we can postulate either that this is a pathogenic but low-penetration germ-line missense mutation that predisposes to pheochromocytoma or that this variant could be a very rare polymorphism. Codon 12 is conserved between *Homo sapiens* and the domestic cattle (14). In that position, an alteration from a very small to a larger neutral and polar amino acid might have some consequence; although without direct functional assay, it is open to speculation. Given that the corresponding tumor had LOH of the wild-type allele also lends some support that G12S may be somehow pathogenic. Nonetheless, the conservative estimate of occult germ-line SDHD mutations that are likely pathogenic would be 11% if we discount the G12S variant (see below).

The LOH frequency at the SDHD locus on 11q23 is worthy of note, as well. LOH in pheochromocytomas has been reported on 1p (42–71%), 3p25 (16–56%), 17p (24%), and 22q (31–53%; Refs. 19–22). However, LOH on chromosome 11 in pheochromocytomas has not been reported to date. We found LOH in at least one of the two investigated markers on 11q23 in 72% of pheochromocytomas, a significant frequency. Compared with this high frequency of LOH, the frequency of functional mutations in SDHD (17%) is relatively low. The 6% somatic mutation frequency in SDHD, however, is comparable with that of RET and VHL mutations. We suspect that in the tumors with apparent hemizygous deletion, other epigenetic mechanisms, e.g., methylation of CpG islands in this region, may also play a role in silencing SDHD similar to the inactivation of 14–3-3ζ in breast cancer (23). Because the promoter of SDHD has not been identified yet (16), no further attempt to address this question has been undertaken. Another explanation of our LOH data is that the LOH actually reflects deletion of genes that neighbor SDHD and not SDHD itself.

The molecular basis for sporadic pheochromocytomas has been somewhat elusive. Allelotyping studies have revealed regions of LOH (above). Despite relatively high frequencies of LOH in the VHL gene region (3p25), <2% of sporadic pheochromocytomas carry somatic VHL mutations. Similarly, somatic RET mutations have been shown to occur in 0–10% of sporadic pheochromocytomas (10, 12, 24). Although our data need to be confirmed in a larger series, they strongly argue for a role of SDHD in the pathogenesis of sporadic pheochromocytoma. It was of particular interest that we found not only a somatic mutation but also occult germ-line variants, suggesting a familial predisposition. Among prior series of apparently sporadic pheochromocytoma presentations, occult germ-line mutations in VHL and RET were found only in <5% and <2%, respectively (10, 13). Thus, the minimum estimate of occult germ-line SDHD mutation frequency of 11% (17%) if the variant of unknown significance is considered) found in these patients is significant. This mutation frequency is clinically important in the context that a preliminary survey of the entire Registry, which comprises ~100 nonfamilial pheochromocytoma cases, also reveals an occult germ-line SDHD mutation frequency of 10%. From this current study alone, it is not known whether germ-line SDHD mutations in isolated pheochromocytoma cases would predict for the development of extra-adrenal disease or whether they could also predispose to a familial pheochromocytoma only syndrome. Of interest, among the Registry cases as a whole, 70% of those with germ-line SDHD mutations had intra-adrenal pheochromocytomas only, and 30% had extra-adrenal disease. Families with

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pheochromocytomas only (6) who do not have germ-line mutations in RET and VHL should be scanned for mutations in SDHD on a research basis in the first instance. Additionally, if further study proves that occult germ-line SDHD mutations occur with the frequency reported here or higher among apparently sporadic pheochromocytoma cases, then routine SDHD mutation analysis might be considered in all such presentations as well. Whether the presence of germ-line SDHD mutations can predict for extra-adrenal disease or for the future occurrence of extra-adrenal tumors in cases that present intra-adrenal pheochromocytomas should be the subject of further study.

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References


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