Molecular characterization of novel germline deletions affecting *SDHD* and *SDHC* in pheochromocytoma and paraganglioma patients

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Abstract

A major cause of paraganglioma and pheochromocytoma is germline mutation of the tumor suppressor genes *SDHB*, *SDHC*, and *SDHD*, encoding subunits of succinate dehydrogenase (SDH). While many SDH missense/nonsense mutations have been identified, few large deletions have been described. We performed multiplex ligation-dependent probe amplification deletion analysis in 126 point mutation-negative patients, and here we describe four novel deletions of *SDHD* and *SDHC*. Long-range PCR was used for the fine mapping of deletions. One patient had a 10 kb AluSg–AluX-mediated deletion including *SDHD* exons 1 and 2, the entire *TIMM8B* gene, and deletion of exons of *C11orf57*. A second patient had a deletion of *SDHD* exons 1 and 2 and exon 1 of the *TIMM8B* gene. A third patient showed a deletion of exon 2 of *SDHD*, together with a 235 bp MIRb–*Tensin* gene insertion. In a fourth patient, a deletion of exons 5 and 6 of the *SDHC* gene was found, only the second *SDHC* deletion currently known. The deletions of the *TIMM8B* and *C11orf57* genes are the first to be described, but do not appear to result in an additional phenotype in these patients. Four of the eight breakpoints occurred in Alu sequences and all three *SDHD* deletions showed an intron 2 breakpoint. This study underlines the fact that clinically relevant deletions may encompass neighboring genes, with the potential to modify phenotype. Gene deletions of *SDHD* and *SDHC* represent a substantial proportion of all mutations, and must be considered in paraganglioma patients shown to be negative for mutations by sequencing.

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Introduction

Paragangliomas of the head and neck occur as tumors of both the parasympathetically innervated head and neck paraganglia, most commonly in the carotid body, and of the sympathetic paraganglia and adrenal gland, frequently described as pheochromocytomas when occurring in the adrenal medulla, and as extra-adrenal paragangliomas. Head and neck paragangliomas may lead to significant morbidity due to compromised function of cranial nerves, but generally follow a benign

course. Sympathetic paragangliomas often present with hypertension, sweating, and palpitations due to catecholamine excess, and especially in cases with extra-adrenal localization, they may be metastatic and aggressive.

Although many head and neck paragangliomas and pheochromocytomas are apparently sporadic (i.e. no known family history), many patients will carry a germline mutation and worldwide up to 30% of all cases can be shown to have familial antecedents (Neumann *et al.* 2002).

The first gene shown to be involved in hereditary head and neck paragangliomas was *SDHD* (Baysal *et al.* 2000). *SDHD* encodes a subunit of the mitochondrial tricarboxylic acid cycle enzyme, succinate dehydrogenase (SDH), which also acts as the complex II component of the electron transport chain, locating SDH at the center of cellular metabolism. Further subunits of SDH were subsequently shown to be involved in both head and neck paragangliomas and pheochromocytoma (Niemann & Muller 2000, Astuti *et al.* 2001).

Although the Dutch *SDHD* founder mutations p.Asp92Tyr and p.Leu139Pro (Taschner *et al.* 2001) explain the majority of mutation-positive patients with paraganglioma in The Netherlands, a considerable number of patients are mutation negative by sequencing. While over 200 missense and nonsense mutations are listed in the SDH mutation database (Bayley *et al.* 2005; http://chromium.liacs.nl/lovd_sdh/), only 10 distinct large deletions of the *SDH* genes have been described (Baysal *et al.* 2004, McWhinney *et al.* 2004, Cascon *et al.* 2006, 2008, Amar *et al.* 2007, Fish *et al.* 2007, Pasini *et al.* 2008, Pigny *et al.* 2009). We wished to assess the nature and frequency of deletions of the SDH genes in the Dutch paraganglioma population.

Multiplex ligation-dependent probe amplification (MLPA) gene deletion analysis of *SDHC* and *SDHD* was carried out in 126 paraganglioma–pheochromocytoma patients who had previously tested negative for point mutations of *SDHB*, *C*, and *D* by sequencing. Three deletions of *SDHD* and one of *SDHC* were identified. We proceeded to map the deletions in each patient. Two patients with apparently identical deletions of *SDHD* by MLPA analysis proved to have distinct deletions on further characterization. The clinical phenotype of these patients is described.

Materials and methods

Patients

The Molecular Genetics Laboratory at the Leiden University Medical Center received referrals of 251 index paraganglioma or pheochromocytoma patients for molecular testing of the *SDHD/B* and/or *C* genes between 2000 and 2008. Informed consent was obtained for DNA testing according to protocols approved by LUMC Ethics Review Board. A total of 126 patients were found to be negative for pathogenic mutations by sequencing. These patients were subsequently analyzed for deletions of the SDH genes by MLPA.

MLPA

The P226 MLPA kit includes probes for the all exons and the promoter of the *SDHB*, *SDHC*, and *SDHD* genes, in addition to control probes located on diverse chromosomes, a total of 27 probes (<http://www.mrc-holland.com>). MLPA analysis was performed according to the MRC-Holland protocol (Schouten *et al.* 2002), except that all reagents in the kit were used at half of the recommended volume and hybridization time was reduced from 16 to 2.5 h.

Mapping of deletions

Long-range PCR was used to analyse the final exons and 3' region of the *SDHC* gene, and the first exons and 5' region of the *SDHD* gene. Primers were designed using the Primer 3 program (<http://frodo.wi.mit.edu/>) on repeat masked sequences (<http://www.girinst.org/censor/index.php>), (primer sequences available on request). Alu sequences were analyzed using Repbase (Jurka 2000). Long-range PCR was carried out using Takara LA Taq (Takara Bio Inc., Lucron Bioproducts B.V., Gennep, The Netherlands) following the manufacturer's recommendations except that the final volume was reduced to 20 µl. Resulting PCRs with anomalous fragments were characterized in detail by a further round of PCR mapping and/or restriction mapping using the enzymes, StuI, ScaI, NdeI, and BamHI. After analysis of the resulting PCR fragments/restriction patterns, primers were designed for precise breakpoint characterization and sequencing. The sequenced PCR product was analyzed using the Multalin program (Corpet 1988). Sequence analysis was performed according to standard procedures (details available upon request). Deletion nomenclature follows HGVS guidelines.

Results

MLPA analysis

While the majority of patients undergoing SDH mutation analysis will carry missense and nonsense mutations, some point mutation-negative patients may carry whole-gene or exon deletions. In this study, we describe the molecular characterization of four deletions of the *SDHC* and *SDHD* genes identified by MLPA analysis. These deletions (4/130) represented 3% of all mutations identified in index patients. Two patients appeared to have a deletion of the *SDHD* promoter, together with exons 1 and 2 (patients 1 and 2). One patient showed deletion of *SDHD* exons 1 and 2 (patient 3). Another patient (patient 4) showed

deletion of *SDHC* exons 5 and 6. A founder deletion of exon 3 of the *SDHB* gene was identified in nine patients and has been described elsewhere (Bayley *et al.* 2009). When all 13 patients with *SDHB*, *SDHC*, or *SDHD* deletions are taken in to account, deletions (13/138) represented ~10% of all mutations identified in index patients, indicating that deletions can represent a significant proportion of all mutations in paraganglioma (PGL) patient groups (Table 1).

Molecular characterization of deletions

We used a long-range PCR strategy to map deletions. Forward primers were designed in undeleted regions of the genes. Reverse primers were located at increasing distances from the forward primers, with steps of ~5 or 8 kb (Fig. 1a and b). This approach yields an allele-specific PCR product that can be directly further characterized by restriction mapping or sequencing.

Patient 1 appeared to have a deletion including exons 1 and 2 of the *SDHD* gene and extending into the promoter. An anomalous PCR fragment of around 5.5 kb was shown by 5' mapping, indicating a deletion of ~10 kb (Fig. 2a). Restriction mapping refined the deleted region to an interval of ~1 kb. Primers proximal to the breakpoint were used to amplify the fragment and sequencing revealed the breakpoint. Analysis of the breakpoint regions using Repbase indicated that the breakpoints were located in AluSg and AluSx repeats. Alignment of the Alu sequences indicated 77% identity and revealed a 21 bp continuous mismatch, with the breakpoint precisely at the 3' end (Fig. 2b). The deletion of 10 kb not only included *SDHD* exons 1 and 2, but also resulted in the deletion of the entire *TIMM8B* gene (translocase of inner mitochondrial membrane 8 homolog B), which lies proximal to *SDHD* in the opposite orientation. The deletion also encompassed exons 2–5 of *C11orf57* gene.

Patient 2 also appeared to have a deletion of the promoter and exons 1 and 2 of *SDHD* by MLPA analysis. Mapping showed that the deletion in this case was considerably smaller with an anomalous fragment, indicating a deletion size of ~2.5 kb. A further round of PCR mapping and sequencing identified the breakpoint (Fig. 3). Analysis of the breakpoints showed that both occurred in unique sequence with no clear sequence homology. The deletion not only included exons 1 and 2 of *SDHD*, but also resulted in the deletion of exon 1 of the *TIMM8B* gene.

A further patient, patient 3, showed an anomalous PCR fragment, which indicated a deletion of ~1 kb. This patient appeared to have a deletion spanning exons 1 and 2. Primers were designed around the deletion and

Table 1 Summary of deletions in four human paraganglioma patients

Pat	Deletion length (bp)	HGVS description	Deleted exon(s) SDHD	Deleted exon(s) SDHC	Deleted exon(s) TIMM8B	Deleted exon(s) C11orf57	Upstream BP	Downstream BP
1	10 336	c.1-8828_169 + 442del	1 + 2	NA	1 + 2	2–5	AluSg	AluSx
2	2522	c.1-1427_169 + 29del	1 + 2	NA	1	None	Unique	Unique
3	1100	c.52 + 5_169 + 325delins235	2	NA	None	None	Unique	AluSx
4	7930	c.242-241_510 + 1978del	NA	5 + 6	NA	NA	AluSx	Unique

Deletion length is indicated in bp; HGVS, human genome variation society nomenclature; BP, breakpoint sequence types; Unique, nonrepetitive sequence; NA, not applicable.

Figure 1 Long-range PCR-mapping strategy of the (a) *SDHD* and (b) *SDHC* genes. F and R indicate approximate positions of the forward and reverse primers used.

the PCR fragment was directly sequenced. Analysis of the sequence showed only deletion of exon 2 (Fig. 4a). The 5' breakpoint was located in intron 1, 4 bp from exon 1, in unique sequence. MLPA incorrectly suggested an exon 1 deletion due to the partial location of the probe in intron 1 (Fig. 4b). The 3' breakpoint was located in the same AluSx repeat as patient 1, but at a different location. In addition to the deletion, an insertion of 235 bp was found. This fragment mapped to intron 24 of the *Tensin* gene on chromosome 2, and included a 3' portion of a mammalian-wide interspersed repeat (MIRb). The 5' and 3' arms of this fragment suggest the mechanism of insertion (Fig. 4c).

A final patient was identified with a deletion spanning the last exons of *SDHC*, exons 5 and 6. Mapping this deletion required several forward primers due to the large size of intron 5 of *SDHC* (Fig. 1b). A deletion of approximately 7.5 kb was found, spanning exons 5 and 6 and including the bulk of the 3' untranslated regions (3' UTR). The 5' breakpoint was located in an AluSx repeat, and the 3' breakpoint was located in unique sequence in the distal portion of the 3' UTR (Fig. 5).

Clinical profile

Patient 1, a 53-year-old male, presented with a left-sided mass in the neck at the age of 48 years, which on magnetic resonance imaging (MRI), appeared to be a carotid body paraganglioma. There was no known family history of PGL. A wait-and-scan strategy was followed. At the age of 49 years, 24 h urinary catecholamine screening showed an increased excretion of norepinephrine and vanillylmandelic acid (VMA). The patient showed no symptoms of catecholamine excess and no hypertension. An abdominal MRI showed an irregular enlarged left adrenal, suspected to be a pheochromocytoma. Subsequent resection of the left adrenal and histological examination showed hyperplasia of the adrenal medulla, but not sufficiently characteristic to allow a diagnosis of pheochromocytoma. Postoperatively, urinary catecholamine excretion was normalized, suggesting the left adrenal had been the source of catecholamine excess. Two years later, MRI and computed tomography scans showed a ^{123}I -MIBG positive lesion ($0.9 \times 1.5 \times 1.1$ cm) dorsally from the

Figure 2 (a) Patient 1. The structure of *SDHD* and proximal genes is shown. Genomic DNA is represented by horizontal lines with exons as boxes, the 3' untranslated regions (UTR) as narrow boxes, and the direction of transcription indicated by arrows. The dotted line shows the extent of the deletion with the sequence of the breakpoint below. Arrows represent the orientation of the Alu sequences. (b) Alignment of Alu sequences shows the continuous region of mismatch and the breakpoints (arrow).

Figure 3 The deletion of exons 1 and 2 of *SDHD* and exon 1 of *TIMM8B* in patient 2.

sternal manubrium and under the brachiocephalic vein, suspected to be a paraganglioma. There was no increased urinary catecholamine excretion. Since surgical resection of this nonfunctioning lesion would imply a sternotomy, patient asked for a wait-and-scan strategy with reconsideration of surgical resection in the case of growth. The lesion is currently indolent.

The second patient presented with a right-sided mass in the neck at the age of 42. An MRI of the head and neck revealed bilateral carotid body tumors. Five other family members are also affected, all with head and neck paragangliomas. In the following year, 24 h urinary screening for excretion of catecholamines showed increased excretion of norepinephrine, dopamine, and VMA. He had no symptoms of catecholamine

excess and had no hypertension. An MRI scan of the abdomen showed a 2.6×2.6 cm nodular lesion in the left adrenal and a 2.0×0.9 cm lesion in the right adrenal. Both lesions showed an increased uptake of ¹²³I-MIBG, suggesting a bilateral pheochromocytoma. Bilateral adrenalectomy was performed and histological examination confirmed the diagnosis of bilateral pheochromocytoma. A few months after this surgery, a resection of the left carotid body tumor was successfully performed. The postoperative course was uneventful.

Patient 3 presented with bilateral carotid body tumors at the age of 58. There was no catecholamine excess; imaging of the thorax–abdomen has not been carried out. The family history provided no clear indication of paraganglioma.

Figure 4 (a) The deletion/insertion affecting exon 2 of *SDHD* in patient 3. Sequences proximal to the breakpoint are shown, with the black box representing the insertion. (b) The sequence of *SDHD* with the 235 bp insertion of intron 24 of the *Tensin* gene. The sequence highlighted in grey is the insert, with short sequences (in bold and boxed) indicating regions of homology to *SDHD* are also found in the inserted sequence. These 'sticky ends' suggest the mechanism of insertion. The sequence in italic and underlined is the 3' portion of a mammalian-wide interspersed repeat (MIRb). (c) The 5' breakpoint in intron 1, proximal to exon 1. The boxes indicate the 5' and 3' portions of the exon 1 MLPA probe with the double backslash indicating the exact breakpoint.

Figure 5 The deletion of exons 5 and 6 of *SDHC* in patient 4.

Patient 4, carrying the *SDHC* deletion, had a right-sided carotid body paraganglioma at the age of 26. No other tumors were detected on MRI of the head and neck. The patient had no family history of PGL. Recently, 24 h urinary screening for excretion of catecholamines had shown repeatedly increased excretion of norepinephrine, dopamine, and VMA. She was asymptomatic and did not have hypertension. An MRI of the thorax, abdomen, and pelvis showed no additional paragangliomas/pheochromocytomas. Therefore, it was concluded that the catecholamines might be produced by the carotid body paraganglioma. Alpha-blockade was started. Patient was referred to the vascular surgeon for evaluation of resectability of the carotid body paraganglioma.

Discussion

In this study, we describe the molecular characterization of four deletions of the *SDHC* and *SDHD* genes identified by MLPA analysis. Deletion analysis of *SDHD* and *SDHC* by MLPA detected four deletions in a point mutation-negative group of 126 patients, so deletions of these genes represent 3% of all mutations identified. We have described a founder deletion of exon 3 of the *SDHB* gene in nine patients of this same group of point mutation-negative patients (Bayley et al. 2009). While founder mutations are common in the Dutch population (Taschner et al. 2001, Zeegers et al. 2004), as once again shown by the identification of the *SDHB* exon 3 deletion, other populations also show founder effects (Cascon et al. 2008, Peczkowska et al. 2008b, Opocher et al. 2009), indicating that while the general frequency of SDH deletions across all paraganglioma patient populations cannot yet be established, the proportion of deletions found in the Dutch population may have wider relevance.

When all deletions found during MLPA screening are considered, 13 out of 139 mutation-positive patients carried a deletion, representing 9.3% of all

mutations identified and indicating that deletions can represent a substantial proportion of all mutations in PGL patient groups.

While MLPA is a fast and efficient deletion scanning method, the case of patient 3 illustrates that not all MLPA probes are entirely reliable. In this case, the probe for exon 1 of *SDHD*, partially located in intron 1, was affected by the deletion of intron 1, incorrectly indicating the deletion of exon 1 (Fig. 4b). In the case of a patient with a single exon deletion, a similar effect might lead to an incorrect diagnosis, indicating that deletions of single exons should be confirmed by independent techniques.

MLPA can reliably detect large deletions, but only further characterization can definitively identify unique mutations. We used a long-range PCR strategy to map deletions. While a number of techniques have been applied to map breakpoints and each has advantages and disadvantages, the long-range PCR mapping strategy used here allows the rapid extension of the genomic region covered by the MLPA set. While no deletions larger than 10 kb were available to test a more extensive primer-mapping set, our experience suggests that mapping of deletions over a region of 100–200 kb can be rapid and cost effective using this approach. In several cases, the initial characterization and confirmation of the deletions could be completed in a single experiment. In other cases, a second round of fine mapping produced a PCR product suitable for sequence analysis and breakpoint identification.

The long-range PCR mapping strategy used here allowed, in several cases, the initial characterization and confirmation of the deletions to be completed in a single experiment. In other cases, a second round of fine mapping produced a PCR product suitable for sequence analysis and breakpoint identification.

The Alu repeat is the most abundant repetitive element in the human genome, accounting for over 10% of the mass of the human genome, and is often found in gene-rich regions. A full-length Alu is about 300 bp with two similar regions of 120–150 bp

separated by an A-rich sequence, and most have a poly(A) tail flanked by direct repeats (Batzer & Deininger 2002). The most common form of Alu-mediated deletion is by homologous unequal recombination of Alu sequence oriented in the same direction. This is clearly the case in patient 1, in which two highly homologous Alu sequences align closely in the same orientation except at the location of the breakpoint (Fig. 2b), suggesting torsion and breakage of DNA at the mismatch. Breakpoints occur more often in the left arm of Alu repeats (Lehrman *et al.* 1987), and this is confirmed here with all breakpoints. Alu sequences are involved in three out of the four deletions described, although when one breakpoint occurred in unique sequence, no obvious homology to the Alu sequence could be found.

The insertion seen in patient 3 included a portion of a MIR. MIR elements originally amplified 130 million years ago and around 370 000 copies are present in the human genome (Jurka *et al.* 1995). They are often truncated and are rarely involved in homologous unequal recombination. The involvement in this case appears to be coincidental, as the inserted repeat is truncated and the inserted fragment has short regions of homology at the insertion sites.

In two of the patients described here, genes proximal to *SDHD* are also affected by the deletions. Deletions affecting neighboring genes may influence phenotypes, as seen in the case of the *VHL* gene, in which a reduced risk for renal cell carcinoma was noted when the actin regulator gene *HSPC300* was deleted together with the *VHL* gene (Maranchie *et al.* 2004, Cascon *et al.* 2007).

While little is known of the function of *TIMM8B*, homology to *TIMM8A* suggests that the gene may encode a mitochondrial intermembrane chaperone, with a role in the import and insertion of hydrophobic transmembrane proteins into the mitochondrial inner membrane. Mutations in *TIMM8A* cause Mohr–Tranebjærg syndrome (MTS; OMIM 304700), a rare X-linked condition characterized by dystonia, progressive sensorineural hearing loss, and several other manifestations including visual disabilities, mental deterioration, and behavioral abnormalities. Two families have been described with the coincidence of sensorineural hearing loss and paraganglioma (Badenhop *et al.* 2001), but no mutations of *TIMM8B* were found. No additional abnormalities are currently known in the patients described here, but these patients have one intact allele, in contrast to MTS. The deletion in patient 1 also encompassed *C11orf57*, a protein-encoding gene of unknown function. This gene has conserved orthologs in many mammalian species but all are uncharacterized, and no paralogs have been

identified in the human genome. This patient currently has no additional clinical features, providing no clues for this gene as to associated pathology or function. While we have not currently noted altered or additional phenotypes in these patients, vigilance is indicated due to the potential for clinical phenotypes that are not yet manifested or may display reduced penetrance.

Although in our study, only three patients are described with deletions of *SDHD* and only one patient with a deletion of *SDHC*, the phenotype of these patients does not seem to be essentially different from the phenotype described for point mutations of *SDHD* (Amar *et al.* 2005, Benn *et al.* 2006, Havekes *et al.* 2009) and *SDHC* (Schiavi *et al.* 2005, Mannelli *et al.* 2007). *SDHD* mutations are typically associated with multifocal head and neck paragangliomas and usually benign adrenal and extra-adrenal paragangliomas. *SDHC* mutations appear to be a rare cause of mainly head and neck paragangliomas (Schiavi *et al.* 2005). Only four cases among all the *SDHC* patients reported to date developed paragangliomas outside the head- and -neck regions (Mannelli *et al.* 2007, Pasini *et al.* 2008, Peczkowska *et al.* 2008a). Interestingly, in our study, all three patients carrying a *SDHD* deletion have unilateral or bilateral carotid body paragangliomas, and no other head and neck paragangliomas. In addition, one patient had a unilateral pheochromocytoma and likely a mediastinal paraganglioma, and the other patient had a bilateral pheochromocytoma at relatively young age. Since all three patients had (bilateral) carotid body tumors and two out of the three patients had intra- and extra-adrenal paragangliomas (~25% series reported by others at the age of 40–50 years, e.g. Benn), this might indicate a higher penetrance of paragangliomas, although our numbers are clearly too small to allow any firm conclusion. Remarkably, the patient with the *SDHC* deletion has a unilateral carotid body paraganglioma secreting catecholamines. In general, only about 5% of head and neck paragangliomas has been reported to secrete catecholamines (Erickson *et al.* 2001). Interestingly, Niemann *et al.* (2003) reported one case of a malignant catecholamine secreting carotid body paraganglioma. While the clinical correlates described above must be tentative, given the number of patients described in this study, as further patients with deletions are identified, a clearer picture of the clinical implications of SDH deletions should emerge.

In summary, we show that deletions of SDH genes account for a nontrivial proportion of all mutations in patients with head and neck paragangliomas and/or pheochromocytoma. This clearly indicates that deletion screening should be seriously considered as a follow-up

to sequencing in all point mutation-negative patients. We also show that deletions can include proximal genes. While in the case of the patients described these do not appear to result in additional phenotypes, unusual phenotypes in patients carrying gene deletions indicate further characterization.

Declaration of interest

The authors declare that there is no conflict of interest that could prejudice the impartiality of the research reported.

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