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Development and Validation of a comprehensive mutation and deletion detection assay for *SDHB*, *SDHC*, and *SDHD*

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Abstract

Background—Lack of sequencing-validation and complexity of deletion testing hinder genetic diagnosis of *SDH*-associated paraganglioma/pheochromocytoma.

Methods—We developed sequencing assays and multiplex ligation-dependent probe amplification (MLPA) deletion-detection for *SDHB*, *SDHC* and *SDHD*. Clinical performance was validated on 141 blinded samples, previously tested at NIH.

Results—Sequencing and deletion-detection were highly reproducible and agreed with previous NIH results in 99.3% and 100%, respectively.

Conclusions—DNA sequencing combined with MLPA allows reliable and simplified genotyping of *SDHB*, *SDHC* and *SDHD*.

Keywords

SDHB; SDHC; SDHD; paraganglioma; pheochromocytoma; genotyping; DNA-sequencing; deletion detection; MLPA

Introduction

Succinate dehydrogenase (SDH) is a mitochondrial oxireductase complex consisting of four subunits: SDHA, SDHB, SDHC and SDHD (1, 2). Germline heterozygous mutations or deletions of *SDHB*, *SDHC* or *SDHD* cause an autosomal dominant tumor syndrome, manifesting when the second copy is somatically lost or mutated in sympathetic or parasympathetic ganglia (3). *SDHB* mutations are observed most commonly and lead (usually) to catecholamine-secreting paragangliomas (PGL) or pheochromocytoma (PC), while the rarer *SDHC* or *SDHD* tumors cause chiefly nonfunctioning head and neck PGL (4).

Between 30–50% of apparently sporadic and >90% of clinically hereditary PGL are caused by *SDH* germline mutations or deletions; the corresponding figures for PC are 1–10% and 20–30% (1, 3). Consequently, mutation and deletion testing of *SDHB*, *SDHC* or *SDHD* has become indispensable in patients with PGL, or, seemingly, hereditary PC (3, 4, 5, 6)

When both full gene sequencing and deletion detection are performed, testing is highly accurate in identifying affected individuals. However, deletion detection by traditional methods, e.g. Southern blotting, is labor intensive, costly and slow. In addition, clinical validation of sequencing assays, in particular for *SDHC* and *SDHD*, has proven challenging, because of the low numbers of affected patients. Testing is sometimes only available through research laboratories, resulting in difficult access, unpredictable turn-around times, possible need for independent confirmation for patient care, and potential rejection of charges by insurance carriers.

To address these problems, we developed an easier deletion detection method, using multiplex ligation-dependent probe amplification (MLPA), performed an extensive analytical validation, including quantitative measurements of repeatability of sequencing quality and MLPA allelic ratios, and performed clinical validation of full gene sequencing and MLPA deletion detection of *SDHB*, *SDHC* and *SDHD*, using a large cohort of well characterized samples obtained from collaborators at the National Institute of Health (NIH; Bethesda, MD).

Methods

Samples

This study was approved by the Mayo Clinic Institutional Review Board. We purified DNA from 15 de-identified normal volunteer blood samples. Fifty archival, de-identified normal DNA samples and two samples with known *SDHB* deletions were used for deletion detection validation.

We obtained, and tested blindly, 141 de-identified, coded DNA samples, which had previously been mutation/deletion tested at NIH.

Sequencing of *SDHB*, *SDHC*, and *SDHD*

We sourced primers for PCR-amplification and cycle sequencing of all 18 exons of *SDHB*, *SDHC* and *SDHD* from IDT (Integrated DNA Technologies, Coralville, IO) (Supplemental table 1). Amplification reactions of 25 µl contained: 100 ng of genomic DNA, 200 µmol/L dNTPs, 0.5U Platinum Taq Polymerase (both Invitrogen, Carlsbad, CA), 0.2 µmol/L primer mix and 1.5 mmol/L MgCl₂. The cycling conditions were: 1 × 95 C (10 min), 12 × 95 C (30 s), 64 C (30s) and 72 C (1 min), 12 × 95 C (30 s), 62 C (30 s) and 72 C (1 min), 12x 95 C (30 s), 58 C (30 s) and 72 C (1 min), 1x 72 C (5 min).

Cycle-sequencing was performed using nested primers and BigDyeTerminators™ v1.1 (Applied Biosystems, ABI, Foster City, CA). Electropherograms were generated on ABI-3730xl automated DNA sequencers (ABI). The traces were analyzed with Mutation Surveyor® (Softgenetics, LLC State College, PA).

Detection of *SDHB*, *SDHC*, and *SDHD* deletions

A combination of MLPA and Luminex® xTAG (Luminex Molecular Diagnostics, Inc., Toronto, Ontario, Canada) technologies was used for deletion detection. DNA (400 ng) was denatured at 95 C (5 min), followed by addition of M13-tailed probes for each exon of *SDHB*, *SDHC*, *SDHD* and control genes at 25 C (see below). Samples were denatured at 95 C (5 min) before probe-hybridization at 60 C (>=16 h). Ligase-65 (MRC-Holland, Amsterdam, Netherlands) was then added for probe-ligation at 54 C for 15 min. The ligase was inactivated at 95 C (5 min) and the ligation-mixes were added to pre-made PCR-mixes containing universal M13-primers (forward primer 5' biotin-labeled). PCR consisted of 23 cycles of 95 C (30 s), 60 C (30 s) and 72 C (60 s); final elongation at 72 C (20 min). PCR-

products were mixed with xTAG beads, 2000 of each bead per sample, denatured at 96 C (2 min) and hybridized at 37 C (1 h). The hybridization buffer was 1x TMAC (300 ml 5M tetramethyl ammonium chloride, 2.5 ml of 20% Sarcosyl, 25 ml of 1M TRIS pH 8.0, 4 ml of 0.5M EDTA pH 8.0 and 168.5 ml of water). Hybridized products were incubated with 25 µl of a 1:125 mixture of Streptavidin R-phycoerythrin and 1x TMAC (15 min) at room temperature in the dark, and then applied to a Luminex 200 instrument (Luminex Molecular Diagnostics, Inc.). Two lasers with different wavelengths detect the bead identity and the reporter molecule signal. For each probe, deviations of >20% from a 1:1 ratio between signals from normal control samples and patient samples indicate possible deletions or insertions/duplications. Less than 4% of normal samples fall outside this ratio. Data was analyzed semi-automatically with Gene Marker® (Softgenetics), using *CNNTB* (3p22.1), *DMD* (Xp21.1), *HIRA* (22q11.2), *NPC-E17* (18q11.2), and *TRNSFR7* (12p13.31) as reference/control genes.

Analytical and Clinical Validation

Intra- and inter-assay precision for sequencing and deletion detection were assessed by running one DNA sample 3 times in the same run, and 10 times in different runs. We reviewed the traces for mutations and tracked the Mutation Surveyor quality scores for these samples, for the fresh normal samples, and the samples used in the clinical validation (see below), to calculate the coefficient of variation (CV) for the quality scores of each exon. The quality scores indicate the fraction of noise in a trace (e.g. a score of 20 corresponds to a noise of 1/20=5%).

The freshly collected 15 normal DNA and the 50 archival DNA samples were used to verify absence of false-positive sequencing and deletion testing, respectively, and to establish the signal cut-off ratios for deletion detection (see above). The two samples with known deletions were used for initial verification of these cut-offs. One of these patients had a complete *SDHB* deletion (Figure 1), while the other's deletion encompassed part of the promotor and exon 1 of *SDHB*. Reproducibility of signal ratios for MLPA was assessed by calculating the CVs for the signal ratios for each tested exon and control gene.

Clinical validation utilized the 141 samples provided by NIH; 99 had been characterized for *SDH* mutations by DNA-sequencing, while 42 had been tested for deletions by quantitative multiplex PCR. After completion of assay validation, our blinded genotyping results were send back to NIH, where they were compared with the original results.

Results

Within and between run agreement of DNA sequencing and deletion detection was 100%. We did not detect any mutations or deletions in the normal samples and correctly identified both known *SDHB* deletions in the two unblinded samples. The average sequencing quality scores for bidirectional sequencing of *SDHB*, *SDHC* and *SDHD* ranged from 17–71, with intraassay and interassays CVs of 0–40.8% and 1.2–75.4%, respectively. For MLPA, the average signal ratios for control genes in normal samples and in samples of patients with deletions ranged from 0.980–1.025, with total CVs of 2.85–5.22%. For *DMD* the average ratios were 0.913–0.992 for females and 0.591–0.599 for males, with respective CVs of 5.04–7.76% and 7.00–11.00%. For undeleted and deleted *SDH* exons the average ratios were 0.932–1.077 and 0.646–0.708, respectively, with corresponding CVs of 3.49–15.34% and 4.38–6.86% (Supplemental table 2).

The results of the blinded validation portion of the study are summarized in the Table. The 141 samples contained 60 disease-causing mutations or big deletions, which were all correctly identified in our study. We found one additional mutation (confirmed), not

previously found by NIH, corresponding to an agreement of 61/60 (99.2%) for disease-causing DNA changes between NIH and us. We also found an additional NSV (normal sequence variant) in one sample. Overall agreement between methods for deletions, mutations and NSVs was 75/76 (99.3 %), for a total genotyping agreement of 98.6%.

Discussion

We have developed and comprehensively validated a method for complete genotyping of *SDHB*, *SDHC* and *SDHD*. The deletion detection component is less labor intensive and faster than many alternative approaches. Moreover, due to the high number of beads counted during Luminex® detection, the results are semi-quantitative, with control limits, highly reproducible, and easily amenable to standard quality control measures, thereby reducing the potential for mis-interpretation and facilitating quality assurance. With regards to DNA-sequencing, our collaboration with NIH allowed us to validate the performance for all three disease-causing *SDH* genes in actual patient samples, despite the relative rarity of this disorder, in a rigorous blinded fashion.

Reliable *SDH* genotyping, including deletion detection is, alongside the more widely available *VHL* and *MEN2* testing, another step towards comprehensive genetic characterization of PGL and PC patients, conditions that are now recognized to have a much larger genetic component than previously thought (3,7). Identifying affected patients is important, as their tumors have an increased propensity for recurrence. Moreover, it allows screening of pre-symptomatic family members, enabling affected individuals to be entered in surveillance programs. Since the chances of metastatic spread and potentially fatal adrenergic storm increase in parallel with tumor size, this might reduce morbidity and mortality (1).

While we can offer clinicians a comprehensive *SDH* genotyping service, clinical judgment can be helpful in selecting the *SDH* gene that should be tested first. We suggest a tiered approach, based on the location of tumor, presence of malignancy, family history and tumor hormonal status (Figure 2). *SDHB* mutations/deletions are far more common than those of *SDHC* or *SDHD* and should be sought first in patients with hormonally active tumors, i.e. chest/abdominal/pelvic. *SDHD* mutations are mostly found in patients with head and neck PGL; PGL in chest (10), pelvis and abdomen, or seemingly sporadic PC are less prevalent. Finally, *SDHC* mutations are very rare and usually result in head and neck PC (1, 6, 8).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. MLPA deletion detection

The top half of the figure depicts the signals of a patient sample (blue peaks) compared to multi-run averaged female control signals (red peaks).

The bottom half of the figure shows the peak ratios of averaged control signal to the signal generated in a run by control samples (male, left upper and female, right upper) and the response ratios observed in the sample of a male patient with a complete deletion of the SDHB gene (left lower) and a male patient with a deletion of exon 1 of *SDHB*. The horizontal green lines and the green shaded area on each graph indicate the expected normal range for the signal ratios.

Of note is the difference between both normal control and patient sample with regards to the signal ratio of the DMD control gene. The DMD gene is located on the X-chromosome and is therefore only present in a single copy in male patients, thus serving as a convenient control gene for the expected deviation of a heterozygous deletion from the established normal signal ratio range.

Figure 2. Decision tree and workflow for *SDH* genotyping

The flow chart depicts a suggested decision tree and workflow for *SDH* genotyping in patients with suspected or known familial paraganglioma (PGL) or pheochromocytoma (PC). This includes most patients with PGL. With regards to PC, activating mutations in *RET*, and inactivating mutations or deletions in *VHL* and *NF1* also have to be considered in suspected familial cases, and this is indicated at the top of the decision tree. However, an in-depth discussion and depiction of workflow for all familial variants of chromaffin tumors is beyond the scope of this article and the figure concentrates on the *SDH* genotyping.

Table
Comparison of original genotyping with blinded re-testing in 141 patients with suspected *SDH* mutations/deletions

Sequence variants	Original genotyping result (N of patients)				Blinded retesting result (N of patients)				Total	Method agreement (%) *
	<i>SDHB</i>	<i>SDHC</i>	<i>SDHD</i>	Combination#	Total	<i>SDHB</i>	<i>SDHC</i>	<i>SDHD</i>	Combination#	
Total N of samples sequenced	-	-	-	-	99	-	-	-	-	99
Matches Genebank ref-sequence	-	-	-	-	44	-	-	-	-	43
Normal sequence variants (NSV)	3	3	4	5	15	3	3	3	6	15
Disease-causing mutations, no NSV	27	0	2	0	29	27	0	3	0	30
Missense	20	0	1	0	21	20	0	2	0	22
Nonsense	7	0	1	0	8	7	0	1	0	8
Disease-Causing mutations + NSV	2	0	0	9	11	2	0	0	9	11
Missense + one NSV	1	0	0	5	6	1	0	0	5	6
Nonsense + one NSV	1	0	0	4	5	1	0	0	4	5
Total N of samples tested for deletions					42					42
No deletions					22					22
Deletions	20	0	0	0	20	20	0	0	0	20
Total N of disease-causing mutations and deletions detected	49	0	2	9	60	49	0	3	9	61
Total N of all sequence variants and deletions detected	52	3	6	14	75	52	3	6	15	76
Total genotyping **										98.6 **

* Calculated as follows for each row: $100 \times \text{total original} / (0.5 \times \text{total original} + 0.5 \times \text{total retesting})$.

** Calculated as follows: $100 \times \text{N of sequence variants and deletions in agreement between both methods} / (\text{total N of sequenced samples} + \text{total N of deletion tested samples})$. I.e. in this case: $100 \times 139 / (99+42)$.

Combination category applies to NSV in >1 *SDH* gene. No combinations of mutations in more than one *SDH* gene were observed.

No additional patients with NSVs were found, but one additional NSV (in *SDHD*) was detected during blinded retesting in one patient with a *SDHD* NSV