SDHA Immunohistochemistry Detects Germline *SDHA* Gene Mutations in Apparently Sporadic Paragangliomas and Pheochromocytomas

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Context: Pheochromocytoma-paraganglioma syndrome is caused by mutations in *SDHB*, *SDHC*, and *SDHD*, encoding subunits of succinate dehydrogenase (SDH), and in *SDHAF2*, required for flavination of SDHA. A recent report described a patient with an abdominal paraganglioma, immunohistochemically negative for SDHA, and identified a causal germline mutation in *SDHA*.

Objective: In this study, we evaluated the significance of SDHA immunohistochemistry in the identification of new patients with *SDHA* mutations.

Setting: This study was performed in the Erasmus Medical Center in Rotterdam (The Netherlands) and the Université Paris Descartes in Paris (France).

Methods: We investigated 316 pheochromocytomas and paragangliomas for SDHA expression. Sequence analysis of *SDHA* was performed on all tumors that were immunohistochemically negative for SDHA and on a subset of tumors immunohistochemically positive for SDHA.

Results: Six tumors were immunohistochemically negative for SDHA. Four tumors from Dutch patients showed a germline c.91C→T *SDHA* gene mutation (p.Arg31X). Another tumor (from France) carried a germline *SDHA* missense mutation c.1753C→T (p.Arg585Trp). Loss of the wild-type *SDHA* allele was confirmed by loss of heterozygosity analysis. Sequence analysis of 35 SDHA immunohistochemically positive tumors did not reveal additional *SDHA* mutations.

Conclusions: Our results demonstrate that SDHA immunohistochemistry on paraffin-embedded tumors can reveal the presence of *SDHA* germline mutations and allowed the identification of *SDHA*-related tumors in at least 3% of patients affected by apparently sporadic (para)sympathetic paragangliomas and pheochromocytomas. **(*J Clin Endocrinol Metab* 96: E1472–E1476, 2011)**

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Abbreviations: LOH, Loss of heterozygosity; SDH, succinate dehydrogenase.

Pheochromocytomas and paragangliomas are rare tumors that originate from neural crest-derived cells (1). Intraadrenal tumors are called pheochromocytomas, whereas similar extraadrenal tumors are called paragangliomas. Based on location, paragangliomas are subdivided into parasympathetic and sympathetic paragangliomas and are classified as functional or nonfunctional, depending on their catecholamine production.

Succinate dehydrogenase (SDH), also known as mitochondrial complex II, is involved in the citric acid cycle and electron transport chain and is composed of four subunits: SDHA, SDHB, SDHC, and SDHD (2). Previously, *SDHAF2*, *SDHB*, *SDHC*, and *SDHD* mutations have been associated with paragangliomas and pheochromocytomas (3–7). Initially, no genetic link between SDHA and paragangliomas could be established, and *SDHA* mutations were only known to be involved in Leigh syndrome (8–11). However, we recently identified the first heterozygous germline *SDHA* mutation (p.Arg589Trp), associated with an abdominal paraganglioma (12).

Patients with *SDHB*, *SDHC*, and *SDHD* mutations can be identified using SDHB immunohistochemistry, because their tumors are immunohistochemically negative for SDHB (13). The *SDHA*-related tumor we described was also immunohistochemically negative for SDHB and, in addition, lacked expression of SDHA. In contrast, *RET*-, *NF1*-, *SDHB*-, and *SDHD*-related tumors were uniformly immunohistochemically positive for SDHA (12). These results suggested that SDHA immunohistochemistry might be an appropriate and efficient technique to diagnose new *SDHA*-mutated pheochromocytomas and paragangliomas. The aim of this study was to validate the usefulness of SDHA immunohistochemistry in the identification of patients with *SDHA* mutations.

Patients and Methods

Patients and tumor samples

This study included a series of 316 tumors (202 pheochromocytomas, 43 sympathetic paragangliomas, 65 parasympathetic paragangliomas, and six metastases) diagnosed between 1978 and 2009. Of these tumors, 167 came from the archives of the Erasmus Medical Center (Rotterdam, The Netherlands), 92 were collected by the COMETE Network (Paris, France), and the remaining tumors came from various Dutch and foreign centers. Of the 202 pheochromocytomas, 129 were apparently sporadic and 73 were syndrome-related tumors. Of the 65 parasympathetic paragangliomas, 40 were apparently sporadic and 24 were syndrome-related tumors due to germline mutations in different susceptibility genes, and one tumor had a somatic *IDH1* mutation (14). In addition, of the 43 sympathetic paragangliomas, 24 occurred sporadically and 19 were syndrome related. Four metastases were seen in patients with a sporadic presentation and two in patients with an *SDHB* mutation. Clinical data of all patients is shown in Supplemental Tables 1 and 2 (published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). The tumors were used anonymously, in accordance with the code of conduct *Proper Secondary Use of Human Tissue* established by the Dutch Federation of Medical Scientific Societies (http://www.federa.org). The French study was formally approved by the institutional review board (Comité de Protection des Personnes Paris-Cochin, January 2007). DNA was isolated from anonymous healthy subjects, consisting of Dutch blood donors (227 for exon 2 and 116 for exon 13) and normal French volunteers (119 for exon 2 and 370 for exon 13).

Immunohistochemistry

Immunohistochemistry was performed for SDHA and SDHB, using a 1/1000 dilution of the SDHA monoclonal antibody ab14715 (Abcam, Cambridge, UK) and a 1/500 dilution of the SDHB polyclonal antibody HPA002868 (Sigma-Aldrich, St. Louis, MO). The antibodies were applied to routine formalin-fixed and paraffin-embedded archival tissues, processed as described previously (13). Tumors received a negative score if the nontumorous cells from the fibrovascular network surrounding the tumor cells stained positive (internal positive control), and the tumor cells were negative as previously described (13). Tumors were scored as positive if the tumor cells had the same intensity as internal positive-control cells. Following these guidelines, no equivocal cases were seen, and there were no discrepancies between observers. The immunohistochemistry results were evaluated by two independent observers: R.d.K. and E.K. in Rotterdam or J.F. and N.G. in Paris.

Sequence analysis

Sequence analysis of *SDHA* (NM_004168) was performed on all tumors immunohistochemically negative for SDHA (primers available on request) and on 35 SDHA immunohistochemically positive tumors (21 Dutch and 14 French). DNA was isolated according to the manufacturer's instructions (Gentra Systems, Minneapolis, MN, or AllPrep DNA/RNA Mini Kit from QIAGEN, Venlo, The Natherlands). The entire *SDHA* coding sequence, including intron-exon boundaries, was analyzed for mutations, taking into account the *SDHA* pseudogenes (NCBI: NR_003263, NR_003264, NR_003265). When a mutation was demonstrated in the tumor DNA, germline DNA of the same patient was also tested, isolated from paraffin-embedded, histologically normal tissue surrounding the tumor or from leukocytes.

Loss of heterozygosity (LOH)

A microsatellite marker, located at position 1,004,307–1,004,351 bp on chromosome 5 (University of California Santa Cruz Genome Browser; February 2009 GRCh37/hg19 Assembly), was selected for LOH analysis of the *SDHA* gene (primers are available on request). LOH was performed on tumor and normal DNA from patients presenting with SDHA-negative tumors, as described previously, using fluorescence-labeled primers (Invitrogen, Paisley, UK) and ABI 3130-XL genetic analyzer (Applied Biosystems, Foster City, CA) for analysis (15).

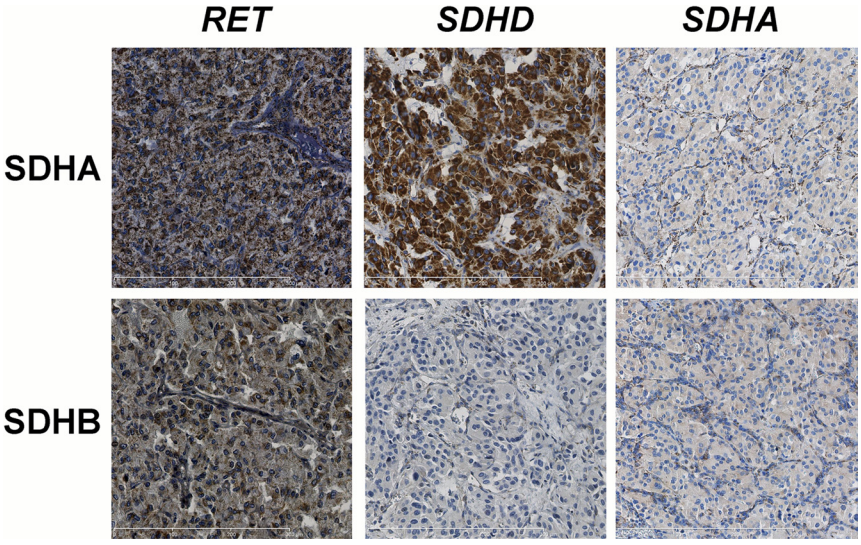

FIG. 1. *Upper panel*, SDHA immunohistochemistry showing positive staining in a *RET*- and *SDHD*-mutated tumor. The *SDHA*-mutated tumor displays negative staining of the tumor cells, whereas the fibrovascular stromal cell staining is positive. *Lower panel*, SDHB immunohistochemistry shows positive immunohistochemical staining in the *RET*-related tumor, whereas the *SDHD*- and *SDHA*-mutated tumors are negative.

Results

Immunohistochemistry

SDHA immunohistochemistry of the 316 tumors revealed seven tumors with SDHA-negative tumor cells (Fig. 1), including the previously described *SDHA*-mutated paraganglioma (patient 297) (12). The other six SDHA immunohistochemically negative tumors included one pheochromocytoma (patient 132), one abdominal paraganglioma (patient 291), one malignant bladder sympathetic paraganglioma (patient 146), one thoracic sympathetic paraganglioma (patient 161), one vagal parasympathetic paraganglioma (patient 162), and one carotid body parasympathetic paraganglioma (patient 187). As expected, SDHB immunohistochemistry was negative in all seven tumors. None of these tumors harbored a mutation in the *SDHB*, *SDHC*, or *SDHD* genes.

Mutation analysis

Mutation analysis of *SDHA* was performed on five of the six new SDHA immunohistochemically negative tumors. In one case, mutation analysis could not be performed due to an insufficient DNA quality. Four tumors from Dutch patients showed a novel c.91C→T *SDHA* gene mutation (NCBI: NM_004168), leading to a truncated protein (p.Arg31X). One French patient harbored a novel c.1753C→T mutation leading to a missense change (p.Arg585Trp). Pseudogenes differ by at least two nucleotides from the *SDHA* gene within each of the amplicons, confirming that these two new mutations did occur in the *SDHA* gene. Corresponding germline DNA, isolated from formalin-fixed paraffin-embedded normal tissue (four Dutch patients) or from leukocytes (one French patient) confirmed the presence of the germline mutation in all five patients (Fig. 2). The sequence chromatogram of the tumor DNA displayed the mutation almost exclusively, indicating loss of the wild-type allele (Fig. 2C). Among the healthy control population, both novel mutations were identified in respectively two of 692 (c.91C→T; 0.3%, only present in Dutch controls) and one of 972 (c.1753C→T; 0.1%) alleles of healthy subjects.

LOH analysis

Four of the six patients were heterozygous for the marker alleles, and all corresponding tumor DNA showed LOH (Fig. 2). The Dutch p.Arg31X patients did not share the same alleles, providing no evidence of relatedness. In addition, expression of the mutant allele was confirmed by RT-PCR (Supplemental Fig. 1).

Discussion

In the present study, we investigated a series of 316 apparently sporadic and syndrome-related pheochromocytomas and paragangliomas for SDHA protein expression and found an additional six negative tumors (2.2% overall). These were exclusively present in apparently sporadic cases, yielding a percentage of 3% in this group (six additional SDHA-negative tumors of 198 apparently sporadic cases).

Sequence analysis of the SDHA-negative tumors revealed two novel *SDHA* mutations [c.91C→T (n = 4) and c.1753C→T (n = 1)], which were found in the tumor DNA as well as in corresponding germline DNA of the affected patients. In accordance with Knudson's two-hit hypothesis, all SDHA immunohistochemically negative *SHDA*-mutated tumors showed loss of the wild-type allele. This confirms that *SDHA*, as we described previously, acts as a *bona fide* tumor suppressor gene (12).

Interestingly, both *SDHA* mutations found in this study were also identified in a healthy control group. The p.Arg31X occurred in 0.3% of the control cases, which is 10-fold lower than that observed in the apparently sporadic tumor group (3%). However, the p.Arg31X mutation appears to be unequivocally involved in the patho-

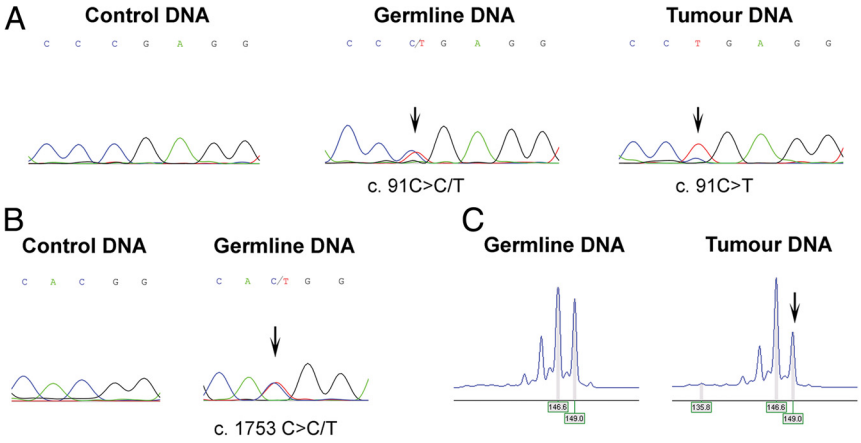

FIG. 2. A, *SDHA* sequence electropherogram showing a c.91C→T (p.Arg31X) mutation present in germline and tumor DNA of patient 161 but not in control DNA. The *SDHA* sequence electropherogram of the tumor DNA revealed predominantly the mutated allele (T), indicating relative loss of the wild-type SDHA allele. B, Sequence electropherogram displaying the c.1753C→T (p.Arg585Trp) mutation in the germline DNA of patient 291 compared with a control DNA. C, Microsatellite electropherogram demonstrating LOH, with loss of the larger allele (149, indicated by the *arrow*) in tumor DNA compared with germline DNA.

genesis of these tumors because the mutation leads to a truncated protein, and all *SDHA*-mutated tumors show loss of the wild-type allele, causing loss of SDHA expression. In addition, the p.Arg585Trp mutation was also found in one in 972 alleles in a healthy control group. Hence, the occurrence of these mutations in healthy controls suggests a low penetrance of paragangliomas in patients with *SDHA* mutations, which could be putatively explained by the rarity of loss of the 5p15 (*SDHA*) locus (12). Furthermore, none of the affected *SDHA* mutation carriers that we identified had a family history of the disease, comparable to most of the newly diagnosed *SDHB* mutation carriers presenting with a paraganglioma (16). Therefore, the majority of germline *SDHA* mutation carriers in the normal healthy population will most likely not develop the disease.

Negative SDHA staining was expected in the tumors with the c.91C→T, because this mutation leads to a truncated SDHA protein. In contrast, the c.1753C→T missense mutation does not lead to a truncated protein, but the SDHA staining was also negative. This could be due to a conformational change of the mutated SDHA protein destroying the antigenic epitope for the antibody. The SDHA antibody used was developed against cow complex II so was not directed against a specific peptide. To determine whether SDHA is present, but is not recognized by the used antibody, additional SDHA antibodies directed against other SDHA epitopes should be used.

All other tumors (n = 309) were immunohistochemically positive for SDHA, in accordance with our previous results, which showed SDHA expression in *RET*-, *VHL*-, *SDHB*-, and *SDHD*-mutated tumors (12). In addition, 35 apparently sporadic tumors with positive immunohistochemical SDHA staining were analyzed for *SDHA* gene mutations, but none were found. Because the SDHA antibody does not cross-react with other proteins, it is unlikely that false-positive staining occurred.

We recently demonstrated that *SDHB*-, *SDHC*-, and *SDHD*-related tumors all show loss of SDHB immunohistochemical expression, whereas *RET*-, *VHL*-, *NF1*-, and *TMEM127*-related tumors were immunohistochemically SDHB positive (13, 17). It was suggested that absence of functional SDHC or SDHD leads to impairment of complex II formation and degradation of SDHB. The current results, showing absence of SDHB expression in *SDHA*-mutated tumors, are in accordance with this explanation. In contrast, whereas *SDHB*-, *SDHC*- and *SDHD*-related tumors were immunohistochemically negative for SDHB, these tumors showed positive staining for SDHA. These findings suggest that the SDHB protein is degraded when the complex is disrupted, whereas the SDHA protein remains intact.

In conclusion, this study provides additional evidence that *SDHA* is a *bona fide* tumor suppressor gene responsible for a significant number of genetically determined paragangliomas and pheochromocytomas (3% of apparently sporadic tumors). Although the number of identified mutation carriers is still low, current observations suggest that *SDHA* mutations are not associated with a particular paraganglia location or with a familial presentation. The results of this study show that, in the absence of familial or clinical indications for a specific form of inherited pheochromocytoma or paraganglioma, SDHA immunohistochemistry on tumor tissue can detect patients carrying germline *SDHA* mutations.

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