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# An immunohistochemical procedure to detect patients with paraganglioma and phaeochromocytoma with germline *SDHB*, *SDHC*, or *SDHD* gene mutations: a retrospective and prospective analysis

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JF, A-PG-R, RRK, and WNMD led the study design. PK, KP, PJP, MM, J-PB, AP, SN, AAV, APB, ERM, TM, CB, JB, LA, DA, EM, WWH, M-PFMVP, AL, JWML, and A-PG-R provided data and tumour samples. FHN, JG, JF, EK, RAO, JR, HD, B-JP, FT, FF, and JF did the mutation analyses. FHN, JG, EMCAB, HFBMS, PD did the analysis and quality control of immunohistochemistry. WCJH contributed to the statistical evaluation of the study. FHN, JG, JF, EK, A-PG-R, RRK, and WNMD drafted the report. All authors revised the report.

**Conflicts of interest**

The authors declared no conflicts of interest.

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## Summary

**Background—**Phaeochromocytomas and paragangliomas are neuro-endocrine tumours that occur sporadically and in several hereditary tumour syndromes, including the phaeochromocytoma–paraganglioma syndrome. This syndrome is caused by germline mutations in succinate dehydrogenase B (*SDHB*), C (*SDHC*), or D (*SDHD*) genes. Clinically, the phaeochromocytoma–paraganglioma syndrome is often unrecognised, although 10–30% of apparently sporadic phaeochromocytomas and paragangliomas harbour germline *SDH*-gene mutations. Despite these figures, the screening of phaeochromocytomas and paragangliomas for mutations in the *SDH* genes to detect phaeochromocytoma–paraganglioma syndrome is rarely done because of time and financial constraints. We investigated whether SDHB immunohistochemistry could effectively discriminate between *SDH*-related and non-*SDH*-related phaeochromocytomas and paragangliomas in large retrospective and prospective tumour series.

**Methods—**Immunohistochemistry for SDHB was done on 220 tumours. Two retrospective series of 175 phaeochromocytomas and paragangliomas with known germline mutation status for phaeochromocytoma-susceptibility or paraganglioma-susceptibility genes were investigated. Additionally, a prospective series of 45 phaeochromocytomas and paragangliomas was investigated for SDHB immunostaining followed by *SDHB*, *SDHC*, and *SDHD* mutation testing.

**Findings—**SDHB protein expression was absent in all 102 phaeochromocytomas and paragangliomas with an *SDHB*, *SDHC*, or *SDHD* mutation, but was present in all 65 paraganglionic tumours related to multiple endocrine neoplasia type 2, von Hippel–Lindau disease, and neurofibromatosis type 1. 47 (89%) of the 53 phaeochromocytomas and paragangliomas with no syndromic germline mutation showed SDHB expression. The sensitivity

and specificity of the SDHB immunohistochemistry to detect the presence of an *SDH* mutation in the prospective series were 100% (95% CI 87–100) and 84% (60–97), respectively.

**Interpretation—**Phaeochromocytoma–paraganglioma syndrome can be diagnosed reliably by an immunohistochemical procedure. *SDHB*, *SDHC*, and *SDHD* germline mutation testing is indicated only in patients with SDHB-negative tumours. *SDHB* immunohistochemistry on phaeochromocytomas and paragangliomas could improve the diagnosis of phaeochromocytoma–paraganglioma syndrome.

## Introduction

Phaeochromocytomas and paragangliomas are rare, usually benign, highly vascularised tumours that both originate from neural-crest-derived chromaffin cells. The term phaeochromocytoma is reserved for intra-adrenal tumours, whereas similar but extra-adrenal tumours are termed paragangliomas. Paragangliomas are subdivided into sympathetic and parasympathetic paragangliomas, depending on their location and catecholamine production. Parasympathetic paragangliomas are located in the head and neck region, and usually do not produce catecholamines, whereas sympathetic paragangliomas are situated along the sympathetic trunk in the abdomen, and usually produce catecholamines.[1]

Phaeochromocytomas and paragangliomas occur sporadically and in the context of several inherited tumour syndromes, including multiple endocrine neoplasia type 2 (MEN2, with *RET* gene germline mutations), von Hippel–Lindau (VHL) disease (caused by germline mutations in the *VHL* gene), neurofibromatosis type 1 (NF1, with *NF1* gene germline mutations), and the phaeochromocytoma–paraganglioma syndrome.[2,3] The latter syndrome is the most frequent hereditary condition with manifestation of paragangliomas, and is caused by germline mutations in the *SDHB*, *SDHC*, or *SDHD* genes. The syndrome is characterised by the familial occurrence of phaeochromocytomas or paragangliomas, usually at a young age, and often by multifocal disease with an increased risk of recurrence and an increased frequency of malignancy in the case of *SDHB* mutations.[4] *SDHB*, *SDHC*, and *SDHD* encode three of four subunits of mitochondrial complex II, the succinate-ubiquinone oxido reductase (succinate dehydrogenase) enzyme located at the crossroads between the mitochondrial aerobic electron transport chain and the tricarboxylic acid cycle.[5] Recent studies showed that SDH inactivation induces angiogenesis and tumorigenesis through the inhibition of hypoxia-inducible factors (HIF)-prolyl hydroxylase.[6] The *SDHB*, *SDHC*, and *SDHD* genes are bona fide tumour-suppressor genes, as biallelic inactivation is found in phaeochromocytoma–paragangliomasyndrome tumours (inherited inactivating germline mutation and acquired inactivating mutation of the corresponding wild-type allele in the tumour).[7]

With the exception of the NF1 syndrome, where the cutaneous café-au-lait spots are characteristic,[8] patients with inherited phaeochromocytomas and paragangliomas often go without clinical detection. In large published series of patients with phaeochromocytomas and paragangliomas, it has been shown that 25–30% of patients have an inherited form and 12% of patients with an apparently sporadic phaeochromocytoma and paraganglioma have unexpected germline mutations in *VHL*, *SDHB*, or *SDHD* genes.[3,7–9] The underdiagnosis of

patients with inherited phaeochromocytoma and paraganglioma is the result of a combination of factors, including lack of family information, overlap in age distribution between hereditary and sporadic cases, de-novo mutations, incomplete penetrance (*SDHB*), parent-of-origin effects on penetrance (*SDHD*), phenotypic heterogeneity of the disease, and insufficient awareness of clinicians. There is controversy among experts as to whether *RET*, *VHL*, *SDHB*, *SDHC*, and *SDHD* genetic testing should be done in all patients with phaeochromocytoma and paraganglioma. Many experts have advocated that molecular genetic testing should be targeted in patients fulfilling specific clinical criteria.[4,10–12] However, reliable clinical indicators for the presence of *SDHB*, *SDHC*, and *SDHD* germline mutations in patients with phaeochromocytoma and paraganglioma are often absent.

Hidden heredity is most pronounced for patients with apparently sporadic parasympathetic paragangliomas, with up to 34% of cases having a germline mutation in *SDHD*.[13] Clinical indications with high specificity but low sensitivity for the detection of phaeochromocytoma–paraganglioma syndrome (family history of phaeochromocytoma or paraganglioma, multifocal disease, younger age at onset, and malignant tumours) are insufficient for correct diagnosis of the syndrome. The detection of inherited phaeochromocytoma–paraganglioma syndrome is of major importance for patients with phaeochromocytoma and paraganglioma, as well as for their family members, since they are at an increased risk of developing multiple, various, and malignant neoplasms.[4,14–16] Additionally, after identification of an *SDHB*, *SDHC*, or *SDHD* germline mutation, surveillance can be offered to the individual patient with the paraganglionic tumour and to any family members who carry the mutation. Mutation analysis of *SDHB*, *SDHC*, and *SDHD* has been advocated to diagnose phaeochromocytoma–paraganglioma syndrome in all cases of phaeochromocytoma and paraganglioma where there are no clear clinical or family indications for the syndrome.[16] Although *SDH*-mutation carriers will be identified frequently by mutation analysis of all patients with phaeochromocytomas and paragangliomas, most cases will be without mutation, making this genetic-screening strategy a labour-intensive and financially demanding procedure. Phaeochromocytoma–paraganglioma syndrome tumours differ from sporadic phaeochromocytomas and paragangliomas by the presence of *SDHB*, *SDHC*, or *SDHD* mutations, which are, except for a few incidental cases,[17,18] not found in truly sporadic phaeochromocytomas and paragangliomas. Despite this genotypic difference, no reliable phenotypic discrimination between sporadic phaeochromocytomas and paragangliomas, and phaeochromocytoma–paraganglioma syndrome-related tumours, is possible at present. In the present study we determined the value of SDHB immunohistochemistry for discriminating between *SDH*-related and non-*SDH*-related phaeochromocytomas and paragangliomas in large retrospective and prospective series in two different centres.

## Methods

### Patients

Two retrospective series of phaeochromocytomas and paragangliomas were investigated by SDHB immunohistochemistry (Erasmus MC, Rotterdam, Netherlands, 110 cases; Hôpital Européen Georges Pompidou and Hôpital Cochin, Paris, France, 65 cases). These series

consisted of phaeochromocytomas diagnosed at Erasmus MC between 1982 and 2007, and diagnosed at INSERM U970 between 1995 and 2007, and of paragangliomas diagnosed in Erasmus MC between 1993 and 1998, and in INSERM U970 between 1993 and 2008. The series were enlarged with additional germline-mutated *SDHB*, *SDHC* and *SDHD* cases from other centres, with as many different mutations as possible. In total, the series consisted of 175 formalin-fixed and paraffin-embedded (FFPE) tumours (101 phaeochromocytomas, 58 paragangliomas, three metastases, and 13 paraganglionic tumours of unknown location) including 24 *RET*, 29 *VHL*, 12 *NF1*, 34 *SDHB*, 38 *SDHD*, four *SDHC* germline-mutant cases, and 34 sporadic cases.

Furthermore, SDHB immunohistochemistry was also done on a prospective series of 45 tumours (six phaeochromocytomas and 39 paragangliomas), for which the *SDH*-gene status was not known beforehand. This prospective series consisted of all paragangliomas diagnosed in Erasmus MC between 2002 and 2008, and all phaeochromocytomas diagnosed in 2008. After the SDHB immunohistochemical results were obtained from this series, *SDH*-gene mutation analysis was done. Detailed information on all investigated cases is shown in the webappendix. Determination of mutation status in these patients and families was done on-site and with the informed consent of the patients. The prospective series was assessed anonymously according to the code for adequate secondary use of tissue code of conduct established by the Dutch Federation of Medical Scientific Societies. Ethical approval for the study was obtained from the institutional review board (CPP Paris-Cochin, January, 2007).

## Procedures

Two different primary antibodies against SDHB were used: mouse monoclonal clone 21A11 (NB600-1366; Novus Biologicals, Littleton, CO, USA; 1:50) and rabbit polyclonal HPA002868 (Sigma-Aldrich Corp; St Louis, MO, USA; 1:500). The antibodies were applied on routine FFPE archival tissues. 4–6 μm sections were cut and mounted on Starfrost Plus (Knittel Gläser; Braunschweig, Germany) glass slides. The sections were deparaffinised, rehydrated, exposed to microwave heating in Tris–EDTA buffer, pH 9·0 or citrate buffer, pH 6·0 at 100°C for 15 min, rinsed in tap water followed by incubation in 3% $H_2O_2$ in PBS for 20 min. The SDHB antibodies were diluted in normal antibody diluent (Klinipath, Duiven, Netherlands) and slides were incubated with 100 μL per slide overnight at 4°C, followed by rinsing in Tris–Tween 0·5%, pH 8·0. Dako ChemMate envision horseradish peroxidase was applied for 30 min (100 μL/slide; Dako envision kit, Glostrup, Denmark), followed by rinsing with phosphate-buffered saline. Diaminobenzidine tetrahydrochloride (100 μL/slide; Dako envision kit) was applied for 5 min twice, after which the slides were rinsed with distilled water. Slides were counterstained with Harris haematoxylin for 1 min, rinsed with tap water, dehydrated, and covered with cover slips. In the negative control reactions, the primary antibodies were omitted from the dilution buffer, which in all instances resulted in a complete absence of staining. Human heart muscle, adrenal gland, liver, and colon tissues were used as positive controls. These tissues showed strong granular staining in the cytoplasm with both antibodies. In phaeochromocytoma and paraganglioma the normal stromal cells of the fibrovascular network surrounding the Zellballen of tumour cells served as an internal positive control for each sample, also showing strong granular cytoplasmatic staining as in the positive control samples.

Pathologists who had no knowledge of the mutation status of the specimens scored the immunohistochemical results from the retrospective series from Rotterdam and Paris independently. The immunohistochemical results of the prospective series were scored by researchers or by pathologists, before mutation analyses were done.

Western blots were done with 50 5-μm sections (approximately 10 mg) cut from five frozen phaeochromocytoma tissue samples from patients with germline mutations in *SDHB* (EX3del), *SDHD* (p.Asp92Tyr), *RET* (p.Cys634Arg), *VHL* (p.Arg64Pro), and *NF1* (clinically determined). Additionally, the same amount of frozen tissue was taken from a lymph node of the patient carrying an *SDHB* mutation, and from a normal adrenal gland. These tissues were transferred into 100 μL 1×Laemmli sample buffer, followed by incubation for 15 min at room temperature. Next, the samples were stirred for 15 s, followed by incubation for 5 min at 100°C. Equal amounts of the samples were then run on a 10% SDS-PAGE gel. After electrophoresis the proteins were transferred to an Immobilon-P Membrane (Millipore, Temecula, CA, USA) and immunoblotted. Both 21A11 and HPA002868 antibodies were used for western blotting and an antibody against β-actin (Sigma-Aldrich; 1:10000) was used as a control for the amount of protein present on the blot.

To test whether absence of immunohistochemical staining for SDHB in the tumours correlated with decreased SDH enzyme activity, SDH enzyme histochemistry was done according to Pearse[19] with minor modifications. Cryostat sections from the same tumour samples used for western blotting were incubated at 37°C for 1 h with an SDHenzyme substrate solution (containing 8·3 mmol/L $NaH_2PO_4$. $H_2O$, 33·3 mmol/L $Na_2HPO_4.2H_2O$, 41·7 mmol/L $Na_2C_4H_4O_4$, 2·5 mol/L Nitroblue terazolium (N-6876, Sigma-Aldrich), 0·22 mmol/L $AlCl_2.6H_2O$, 0·13 mM $CaCl_2$, 25 mM $Na_2HCO_3$, and 0·17 mmol/L Phenazine methosulfate (P9625, Sigma-Aldrich). After rinsing in water twice, the slides were incubated at 4°C for 15 min in formaline-macrodex solution (containing 10 mL 37% formaldehyde, 10 mL 1% $CaCl_2$, 80 mL macrodex [Pharmalink, Stockholm, Sweden]). After rinsing the slides in water again three times, the slides were mounted with imsolmount (Klinipath, Duiven, Netherlands) and covered with cover slips. Snap frozen healthy triceps muscle tissue was used as a positive control. As negative controls, sections from the same tumour tissues were incubated in buffer from which nitroblue terazolium was omitted.

Mutation analyses for *RET*, *VHL*, *SDHB*, *SDHC*, and *SDHD* genes of the series of 175 retrospective tumours were done previously.[4,20] For these analyses, DNA was retrieved from FFPE tumour and normal tissues or from peripheral blood, in the period from 1993 until 2008. DNA was isolated using described and standard procedures, and mutation analyses were done with or without pre-screening by single-strand conformation polymorphism analysis (SSCP) followed by direct, in-house, or commercial (Baseclear, Leiden, Netherlands) sequencing of PCR products.[13,20,21] Mutation analyses of the additional samples from other centres were done by sequencing on site and verified at Erasmus MC and INSERM U970. Mutation analysis of all 34 sporadic cases was done by direct sequencing of the open reading frames, including the exon–intron boundaries, of the *SDHB*, *SDHC*, and *SDHD* genes.[4]

The prospective series of 45 tumours was also investigated for *SDHB*, *SDHC*, and *SDHD* mutations by direct sequencing of the open reading frames including all exon–intron boundaries as described previously.[20] Additionally, this series was investigated for the presence of large genomic deletions in the *SDH* genes by multiplex ligation-dependent probe amplification (MLPA) assay with a commercially available kit (SALSA MLPA P226; MRC Holland, Amsterdam, Netherlands).

### Statistical analysis

Patients were grouped on the basis of the presence and absence of an *SDH* mutation, and sensitivity and specificity of the SDHB immunohistochemistry to detect an *SDH* mutation were determined. Within the prospective series we tested for associations between SDHB immunohistochemistry test result and *SDH* mutation status using Fisher's exact test. 95% CI were calculated using the exact binomial method. Analyses were done with STATA, version 10.0.

### Role of the funding source

None of the sponsors had any role in study design, data collection, analysis, interpretation of the data, or the writing of this article. FHN, JG, EK, JF, APGR, RRK, and WNMD had access to the raw data. The corresponding author had full access to all the data and the final responsibility to submit the manuscript for publication.

## Results

Immunohistochemical staining was done on all 220 tumour samples. Of these tumours, 102 had a germline *SDH* mutation (36 *SDHB*, five *SDHC* and 61 *SDHD*) and all were negative for SDHB immunohistochemistry (figure 1A–C). In four *SDH*-mutated tumours (*SDHB* p.Cys98Arg and p.Pro197Arg, and *SDHD* p.Asp92Tyr and c.169_169+9delTGTATGTTCT) a weak and diffuse cytoplasmic SDHB immunoreactivity was seen in the tumour cells, clearly distinct from the strong speckled pattern present in normal cells of the intratumoral fibrovascular network (figure 1C). However, independent tumour samples with the same mutation (*SDHB* p.Pro197Arg and *SDHD* p.Asp92Tyr) were clearly negative for SDHB immunostaining. Therefore, this weak diffuse cytoplasmic staining in the tumour cells was considered to be a non-specific background artifact and scored as negative. 65 tumours had a germline mutation in *RET* (24 cases), *VHL* (29 cases), or *NF1* (12 cases, diagnosed pheno typically), and all showed expression of SDHB by immunohistochemistry (figure 1D–F). In the remaining 53 tumours, of which six tumours were SDHB-negative, no germline mutation in the *RET*, *VHL*, *SDHB*, *SDHC*, or *SDHD* genes was seen, nor was any *NF1* gene involvement detected. A summary of the results is listed in table 1 and comprehensive information on tumour characteristics, including type of mutation and results is presented in the webappendix.

In the prospective series, sensitivity and specificity were 100% (95% CI 87–100) and 84% (60–97), respectively. Table 2 shows that there was a highly significant association between the SDHB immunohistochemistry test result and the absence or presence of an *SDH* mutation ($p<0{\cdot}0001$; Fisher's exact test).

SDHB immunohistochemistry done on cryostat sections from three phaeochromocytomas, two with an *SDHD* mutation and one with a *RET* mutation, gave results comparable to FFPE tissue sections: speckled staining patterns in the normal cells and an absence of staining in *SDHD*-mutated tumour cells. This comparable SDHB immunoreactivity pattern on FFPE and frozen tissues is an additional indication for the specificity of the immunohistochemistry results. The decreased expression of SDHB protein in both *SDHB*-mutated and *SDHD*-mutated tumours was confirmed by western blotting (figure 2A). Additionally, the absence of SDH enzyme activity was determined by enzyme histochemistry. The *SDHB*-related and *SDHD*-related tumours showed no SDH activity, except for the normal cells of the intratumoral fibrovascular network, which showed strong staining (figure 2B). By contrast, strong SDH enzyme activity was present in the triceps muscle tissue and the *RET*-related tumour tissue (figure 2C).

## Discussion

The results of this study show that SDHB immunohistochemistry on routine FFPE paragangliomas and phaeochromocytomas can reveal the presence of *SDHB*, *SDHC*, and *SDHD* germline mutations with a high degree of reliability. The absence of SDHB staining in tumour cells was found irrespective of whether *SDHB*, *SDHC*, or *SDHD* is mutated, and regardless of the type of mutation, whether missense, nonsense, splice site, or frameshift. The SDHB protein-expression results obtained by immunohistochemistry using both SDHB antibodies (Sigma mouse monoclonal 21A11 and Novus rabbit polyclonal HPA002868) were the same. Either antibody might be used for the immunohistochemical detection of SDHB.

Of the 220 independent tumours analysed, 102 had a germline *SDH* mutation (36 *SDHB*, five *SDHC*, and 61 *SDHD*), and all were negative for SDHB immunostaining. 65 tumours had a germline mutation in *RET* (24 cases), *VHL* (29 cases) or *NF1* (12 cases, diagnosed phenotypically), and all showed expression of SDHB by immunohistochemistry. In the remaining 53 tumours no germline mutation in the *RET*, *VHL*, *SDHB*, *SDHC*, or *SDHD* gene, nor *NF1* gene involvement was detected, but six tumours were negative for SDHB immunostaining. The absence of SDHB protein in these six tumours might be caused by *SDH* mutations escaping detection by the DNA sequencing and MLPA methods used (eg, deleterious mutations in untranslated, intronic, or promoter regions of the genes, which were not investigated), or by epigenetic silencing of *SDH* genes. In two of these six patients without *SDH* mutations, but with SDHB immunohistochemistry-negative tumours, the clinical information was indicative of phaeochromocytoma–paraganglioma syndrome: one patient had a family history of paraganglioma and one patient suffered from multiple paragangliomas (webappendix). Furthermore, three of the four other SDHB-negative tumours without *SDH*-gene mutations were diagnosed at a young age (webappendix; cases 179A, 180B, and 220C), indicating possible germline involvement. A negative *SDH* genetic testing in association with negative SDHB immunohistochemistry could indicate the possibility of a phaeochromocytoma or paraganglioma hereditary syndrome, and we recommend that the patient be followed up in the same way as for a proven phaeochromocytoma or paraganglioma hereditary syndrome. There is a highly significant association between the SDHB immunohistochemistry test result and the absence or

presence of an *SDH* mutation. The SDHB immunohistochemical test has a high sensitivity and specificity for the presence of an *SDH* mutation. The possibility that in the six SDHB-negative tumours without identified *SDH* gene mutations the mutations escaped detection would mean that the sensitivity and specificity of SDHB immunohistochemistry for the detection of phaeochromocytoma–paraganglioma syndrome is even higher than estimated here.

The reliability of the immunohistochemical results on FFPE tumour specimens is also indicated by the similar results obtained with two different antibodies, applied on three different tumour series in two different laboratories (the retrospective series in Rotterdam and Paris, and prospective series in Rotterdam), and the concordant results obtained on cryostat sections, in western blotting, and by SDH-enzyme histochemistry. Our results show that in tumour cells with various mutations (*SDHB*; 15 different missense, two different nonsense, six different frameshift, three different exon deletions, three mutations probably affecting splicing), *SDHC*; two different missense, one nonsense, and two exon deletions, and *SDHD*; five different missense, two different nonsense, three different frameshift, and three mutations probably affecting splicing, no immunoreactive SDHB protein could be detected. These results are in accordance with preliminary findings by Douwes-Dekker and colleagues,[22] who reported generally decreased diffuse cytoplasmic SDHB expression in 11 *SDHD*-related (two different *SDHD* mutations) paragangliomas and strong granular expression in sporadic tumours and normal cells. Additionally, Dahia and colleagues[23] reported comparable decreased SDHB expression in five *SDHB*-related, one *SDHD*-related, and six *VHL*-related phaeochromocytomas. However, in the present study we were able to discriminate *VHL*-related tumours from *SDH*-related phaeochromocytoma and paraganglioma on the basis of SDHB immunohistochemistry, which could be the result of differences in the applied immunohistochemistry procedure or tissue processing.

The differences in SDHB protein concentrations are probably not the result of differences in transcriptional efficiency, since there are indications that SDHB mRNA concentrations do not parallel SDHB protein abundance.[23] Additionally, it has been shown previously that, whatever SDH subunit is mutated, be it anchorage (SDHC and SDHD) or catalytic (SDHB), inactivation of an *SDH* gene induces a complete abolition of SDH enzyme activity in the tumour, suggesting a conformational change or a destabilisation and a subsequent proteolysis of the complex II.[7,22,24] Furthermore, Lima and colleagues[25] showed by crystallography the severe structural consequences on the SDHB protein of five clinically validated *SDHB* missense mutations. Cervera and colleagues[26] recently obtained evidence that three missense-mutated SDHB proteins can reach the mitochondrion and localise normally, although two of three missense-mutated SDHB proteins showed decreased expression by western blotting compared with the wild-type protein. These results match with the recent evidence that most rare missense variants in genes are deleterious.[27]

In the present study four tumours, positive for SDHB immunostaining, harboured non-synonymous polymorphisms (*SDHB* p.Ala3Gly, p.Arg11His, p.Ser163Pro, and *SDHD* p.His50Arg) without concomitant pathogenic *SDH*-gene mutation, indicating that these variants are indeed neutral polymorphisms.[15,28]

Biallelic inactivation of the *SDHB*, *SDHC*, or *SDHD* gene has been reported in *SDH*-related tumours.[17,24,29] Our results indicate that mutations in *SDHB*, *SDHC*, or *SDHD* lead to the same phenotypic consequence in the tumours–ie, the absence of immunoreactive SDHB protein. Such observations have already been described for mutations in complex I genes, which were shown to affect the assembly and stability of both the whole complex I and other mitochondrial complexes, such as complex III.[30] The observed absence of SDHB immunoreactivity in all *SDH*-mutated tumours, shown by immunohistochemistry in both FFPE and frozen tumour tissues, and by western blotting after denaturing gel electrophoresis, with both a monoclonal antibody generated against cow SDHB and an affinity-isolated polyclonal antiserum against a recombinant carboxyterminal part of human SDHB, provides strong evidence that no functional SDHB protein is present in *SDH*-mutated tumours. As previously reported in other mitochondrial disorders, it is therefore likely that altered assembly or complex stability is the first consequence of *SDH* gene mutations, as opposed to catalytic site dysfunction. It confirms the accuracy of immunological approaches for the diagnosis of mitochondrial diseases.[31]

By use of our applied procedure, patients with phaeochromocytoma–paraganglioma syndrome with an apparently sporadic presentation can be detected by SDHB immunohistochemistry on paragangliomas and phaeochromocytomas. Additionally, it can be speculated that the syndromic involvement of tumours that have recently been described in relation with paragangliomas, such as gastrointestinal stromal tumours in the Carney–Stratakis dyad and familial renal-cell carcinomas, could also be detected by SDHB immunohistochemistry. [29,32] In actual fact, tissue from one of these germline *SDHB* mutated renal-cell carcinomas was available for study, and this tumour seemed to be negative for SDHB expression (data not shown).

As for Lynch syndrome diagnostics, where the testing of tumours usually starts with immunohistochemistry for mismatch repair gene products, SDHB immunohistochemistry could have an important role in the future genetic testing of phaeochromocytomas and paragangliomas (figure 3).[33] Because of the simplicity of the standard immunohistochemical procedure and data interpretation, the immunohistochemistry test could easily be applied in diagnostic pathology services worldwide. It is technically and financially feasible to routinely test all phaeochromocytoma and paraganglioma for SDHB expression, in particular in the absence of familial or clinical indications for a specific form of inherited phaeochromocytoma or paraganglioma. Our results show that *SDHB*, *SDHC*, and *SDHD* germline mutation testing is indicated only when tumours are immunohistochemically negative for SDHB expression. Obviously, our proposed diagnostic test can only be done after patients have been operated on and tumour tissue is available for study. The effect that our test will have on patient management is unclear, since international controversy exists regarding preoperative and postoperative genetic testing, and the effect on patient management. Nonetheless, by routinely doing SDHB immunohistochemistry, hereditary syndromes caused by germline mutations in *SDHB*, *SDHC*, or *SDHD* could be identified with a high degree of reliability.

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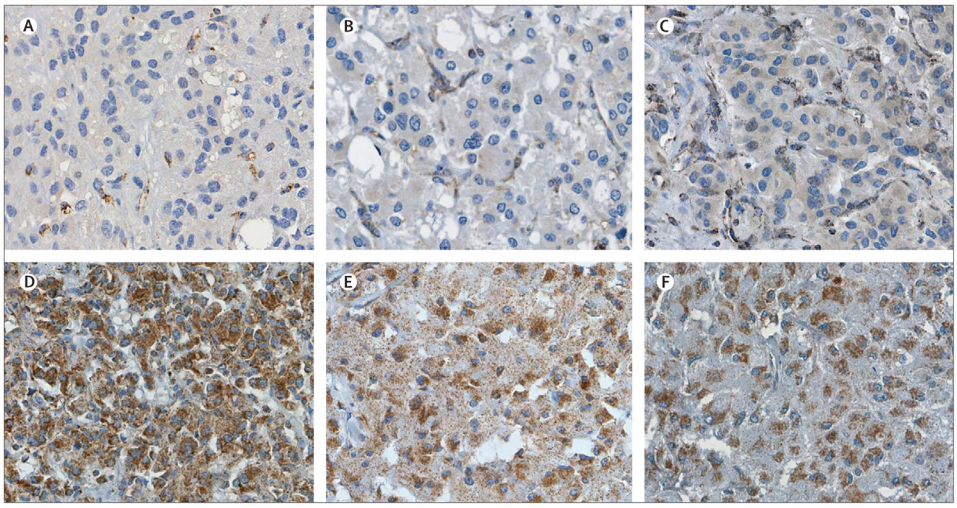


**Figure 1. SDHB immunohistochemistry**

Phaeochromocytoma and paraganglioma SDHB immunohistochemistry. (A) Phaeochromocytoma with *SDHB* mutation. (B) Paraganglioma with *SDHC* mutation. (C) Paraganglioma with *SDHD* mutation. (D) Phaeochromocytoma with *VHL* mutation. (E) Phaeochromocytoma with *RET* mutation. (F) Phaeochromocytoma from a patients with NF1 (clinical diagnosis). Note: Strong speckled SDHB immunostaining in non-*SDH* mutated tumours (D, E, and F). Absence of SDHB immunostaining in the tumour cells of *SDHB*, *SDHC*, and *SDHD*-mutated tumours, with positive staining in the normal cells of the intratumoral fibrovascular network (A, B, and C). In the *SDHD*-mutated tumour (C) diffuse cytoplasmic background staining is seen, clearly distinct from the staining of the intratumoral fibrovascular network.

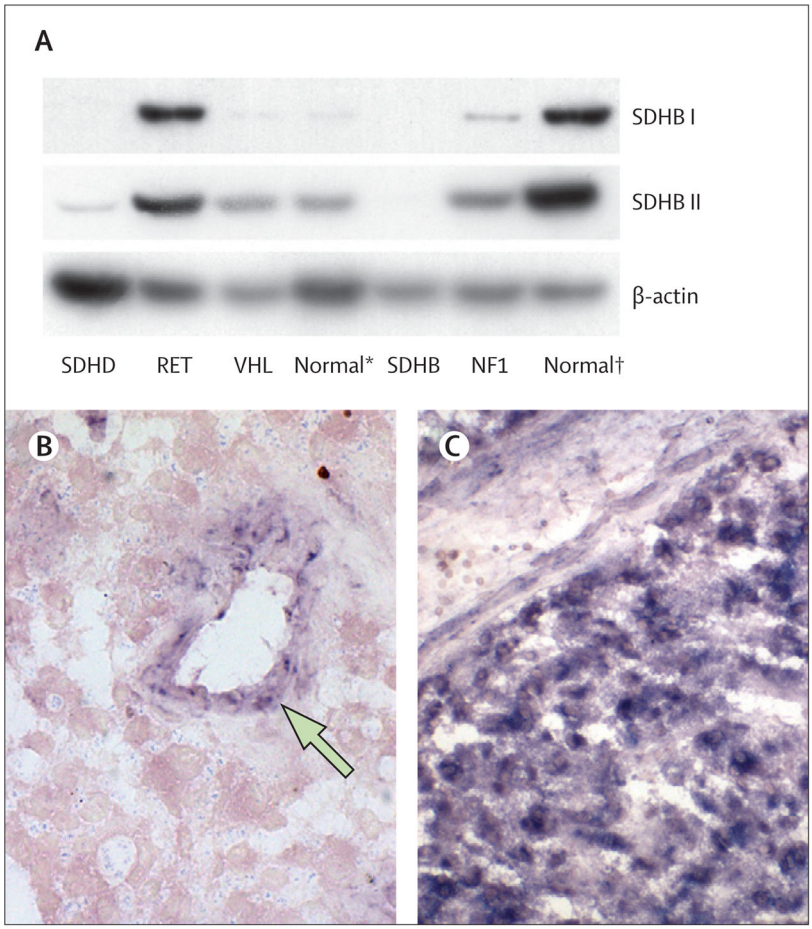


**Figure 2. Western blotting and enzyme histochemical results**

(A) Western blot result with SDHB antibodies from Novus Biologicals NB600-1366 (SDHB I) and Sigma HPA002868 (SDHB II) and β-actin of phaeochromocytoma with different mutations. SDHB case: *SDHB* exon 3 deletion; SDHD case: *SDHD* p.Asp92Tyr missense mutation; RET case: *RET* p.Cys634Arg missense mutation; VHL case: *VHL* p.Arg64Pro missense mutation; NF1 case: clinically defined NF1. *Normal is a lysate from a lymph node from the patient with the *SDHB* mutation. †Normal is a lysate from a healthy adrenal gland. SDH-enzyme histochemistry results: (B) loss of SDH activity in tumour cells of a phaeochromocytoma with a *SDHD* p.Asp92Tyr mutation, but retained activity in the normal cells of the intratumoral fibrovascular network (arrow); (C) strong SDH activity in tumour and normal cells of a phaeochromocytoma with a *RET* p.Cys634Arg mutation.

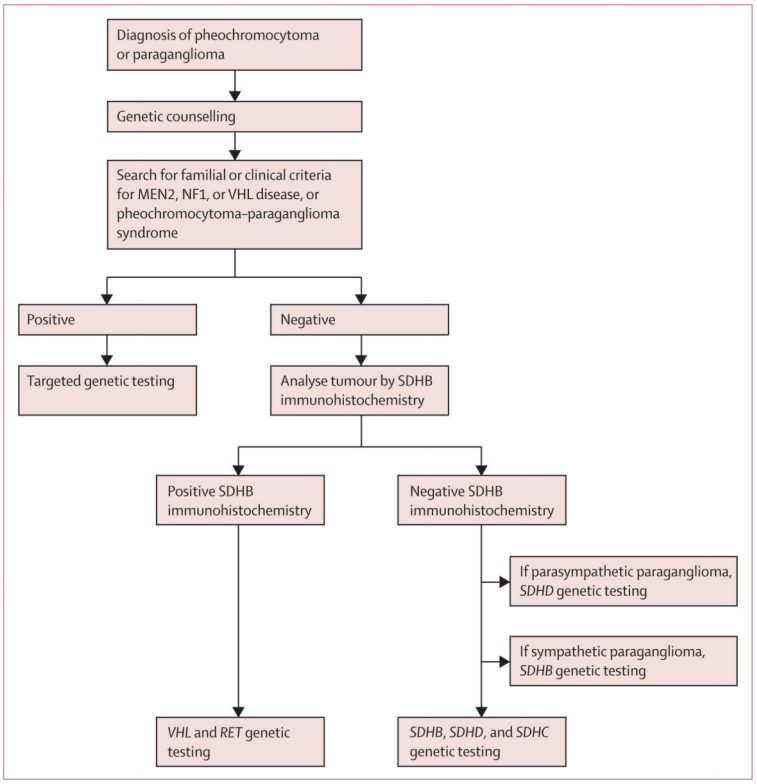


**Figure 3. Suggested algorithm for molecular genetic testing for phaeochromocytoma and paraganglioma**

The presence of familial or clinical criteria for a phaeochromocytoma-associated or paraganglioma-associated inherited disease should lead to targeted genetic testing. In the absence of criteria, SDHB immunohistochemistry is indicated. A positive SDHB immunohistochemistry result should lead to *VHL* and *RET* genetic testing, a negative SDHB immunohistochemistry should lead to *SDH* (*SDHD*, *SDHB*, and *SDHC*) genetic testing, starting with *SDHD* in cases of head and neck paraganglioma or starting with *SDHB* in cases of thoracic–abdominal or pelvic paraganglioma.

**Table 1**

Clinical data and SDHB immunohistochemistry related to various syndromes

| | Number | Gene mutated | Sex (male/female) | Age range (years; mean) | Phaeochromocytoma | Paraganglioma | SDHB immunohistochemistry positive | SDHB immunohistochemistry negative |
|---|---|---|---|---|---|---|---|---|
| NF1 | 12 | *NF3* | 3/9 | 29–67 (44·2) | 12 | 0 | 12 | 0 |
| MEN2 | 24 | *RET* | 8/16 | 18–76 (35·6) | 24 | 0 | 24 | 0 |
| VHL | 29 | *VHL* | 12/13 (4 U) | 7–62 (25·6) | 21 (3U) | 5 | 29 | 0 |
| Phaeochromocytoma–paraganglioma | 36 | *SDHB* | 13/12 (11 U) | 10–63 (34·6) | 11 (7U) | 18 | 0 | 36 |
| Phaeochromocytoma–paraganglioma | 5 | *SDHC* | 2/3 | 15–47 (30·6) | 0 | 5 | 0 | 5 |
| Phaeochromocytoma–paraganglioma | 61 | *SDHD* | 25/35 (1 U) | 16–72 (40·9) | 5 (3U) | 53 | 0 | 61 |
| Sporadic | 53 | None | 17/34 (2 U) | 12–79 (49·3) | 34 (1U) | 18 | 47 | 6 |

NF1=neurofibromatosis type 1. MEN2=multiple endocrine neoplasia type 2. VHL=von Hippel–Lindau disease. U=unknown.

**Table 2**

SDHB immunohistochemistry test results according to subgroups within SDH-related and non-SDH-related tumours

| | Number of tumours | SDHB immunohistochemistry negative | SDHB immunohistochemistry positive | Sensitivity (95% CI) | Specificity (95% CI) |
|---|---|---|---|---|---|
| **Retrospective** | | | | | |
| SDH-related | | | | | |
| *SDHB* | 34 | 34 | 0 | 100% (90–100) | ·· |
| *SDHC* | 4 | 4 | 0 | 100% (40–100) | ·· |
| *SDHD* | 38 | 38 | 0 | 100% (91–100) | ·· |
| Non-SDH related | | | | | |
| *RET* | 12 | 0 | 12 | ·· | 100% (74–100) |
| *VHL* | 24 | 0 | 24 | ·· | 100% (86–100) |
| *NF3* | 29 | 0 | 29 | ·· | 100% (88–100) |
| Sporadic | 34 | 3 | 31 | ·· | 91% (76–98) |
| **Prospective** | | | | | |
| SDH-related | 26 | 26 | 0 | 100% (87–100) | ·· |
| Non-SDH related | 19 | 3 | 16 | ·· | 84% (60–97) |