"SDHB/SDHA immunohistochemistry in pheochromocytomas and paragangliomas: a multicenter interobserver variation analysis using virtual microscopy: a Multinational Study of the European Network for the Study of Adrenal Tumors (ENS@T)."

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Abstract

Despite the established role of SDHB/SDHA immunohistochemistry as a valuable tool to identify patients at risk for familial succinate dehydrogenase-related pheochromocytoma/paraganglioma syndromes, the reproducibility of the assessment methods has not as yet been determined. The aim of this study was to investigate interobserver variability among seven expert endocrine pathologists using a web-based virtual microscopy approach in a large multicenter pheochromocytoma/paraganglioma cohort (n=351): (1) 73 SDH mutated, (2) 105 non-SDH mutated, (3) 128 samples without identified SDH-x mutations, and (4) 45 with incomplete SDH molecular genetic analysis. Substantial agreement among all the reviewers was observed either with a two-tiered classification (SDHB κ=0.7338; SDHA κ=0.6707) or a three-tiered classification approach (SDHB κ=0.6543; SDHA κ=0.7516). Consensus was achieved in 315 cases (89.74%) for SDHB immunohistochemistry and in 348 cases (99.15%) for SDHA immunohistochemistry. A...

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SDHB/SDHA immunohistochemistry in pheochromocytomas and paragangliomas: a multicenter interobserver variation analysis using virtual microscopy: a Multinational Study of the European Network for the Study of Adrenal Tumors (ENS@T)

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Despite the established role of SDHB/SDHA immunohistochemistry as a valuable tool to identify patients at risk for familial succinate dehydrogenase-related pheochromocytoma/paraganglioma syndromes, the reproducibility of the assessment methods has not as yet been determined. The aim of this study was to investigate interobserver variability among seven expert endocrine pathologists using a web-based virtual microscopy approach in a large multicenter pheochromocytoma/paraganglioma cohort ($n = 351$): (1) 73 *SDH* mutated, (2) 105 non-*SDH* mutated, (3) 128 samples without identified *SDH-x* mutations, and (4) 45 with incomplete *SDH* molecular genetic analysis. Substantial agreement among all the reviewers was observed either with a two-tiered classification (SDHB $\kappa = 0.7338$; SDHA $\kappa = 0.6707$) or a three-tiered classification approach (SDHB $\kappa = 0.6543$; SDHA $\kappa = 0.7516$). Consensus was achieved in 315 cases (89.74%) for SDHB immunohistochemistry and in 348 cases (99.15%) for SDHA immunohistochemistry. Among the concordant cases, 62 of 69 (~90%) *SDHB-/C-/D-/AF2*-mutated cases displayed SDHB immunonegativity and SDHA immunopositivity, 3 of 4 (75%) with *SDHA* mutations showed loss of SDHA/SDHB protein expression, whereas 98 of 105 (93%) non-*SDH*-x-mutated counterparts demonstrated retention of SDHA/SDHB protein expression. Two *SDHD*-mutated extra-adrenal paragangliomas were scored as SDHB immunopositive, whereas 9 of 128 (7%) tumors without identified *SDH-x* mutations, 6 of 37 (~16%) *VHL*-mutated, as well as 1 of 21 (~5%) *NF1*-mutated tumors were evaluated as SDHB immunonegative. Although 14 out of those 16 SDHB-immunonegative cases were nonmetastatic, an overall significant correlation between SDHB immunonegativity and malignancy was observed ($P = 0.00019$). We conclude that SDHB/SDHA immunohistochemistry is a reliable tool to identify patients with *SDH-x* mutations with an additional value in the assessment of genetic variants of unknown significance. If *SDH* molecular genetic analysis fails to detect a mutation in SDHB-immunonegative tumor, *SDHC* promoter methylation and/or *VHL*/*NF1* testing with the use of targeted next-generation sequencing is advisable.
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Pheochromocytomas and paragangliomas are neural crest-derived neuroendocrine tumors arising from the adrenal medulla and sympathetic/parasympathetic paraganglia, respectively.[1] These carry the highest degree of heritability among human neoplasms. Germline and/or somatic mutations of at least 18 genes (*NF1*, *RET*, *VHL*, *SDHA*, *SDHB*, *SDHC*, *SDHD*, *SDHAF2*, *TMEM127*, *MAX*, *HIF2A*, *KIF1B*, *PHD1*, *PHD2/EGLN1*, *FH*, *HRAS*, *BAP1*, *and MEN1*) are involved in development of the tumors, with ~40% harboring a germline mutation and an additional 25–30% a somatic mutation.[2–4]

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Familial succinate dehydrogenase-related pheochromocytoma/paraganglioma syndromes are caused by *SDHA*, *SDHB*, *SDHC*, *SDHD*, and *SDHAF2* (collectively *SDH-x*) mutations and inherited as autosomal dominant traits.[4] These syndromes predispose not only to pheochromocytomas/paragangliomas, but also to gastrointestinal stromal tumors, renal cell carcinomas, and pituitary adenomas.[5–7] In the vast majority of succinate dehydrogenase-associated tumors, there is also loss of SDHB and/or SDHA protein expression that can be detected by immunohistochemistry.[5–41] In particular, *SDHB*-, *SDHC*-, and *SDHD*-mutated tumors display SDHB immunonegativity but SDHA immunoreactivity, whereas *SDHA*-mutated tumors show negativity for both SDHB and SDHA immunostainings. Gastrointestinal stromal tumors and paragangliomas, associated with Carney triad (the syndromic but nonhereditary association of gastrointestinal stromal

tumor, paraganglioma, pulmonary chondroma, adrenocortical adenoma, and esophageal leiomyoma),[4] show negative staining for SDHB in the absence of *SDH-x* mutations.[29,40] There is provisional evidence that Carney triad-related tumors display somatic hypermethylation of the *SDHC* promoter locus,[42] and therefore negative staining for SDHB may also identify these cases not found by conventional molecular testing.

As loss of SDHB/ SDHA expression is predictive of an underlying *SDH-x* germline mutation,[8,10,11,17,21–24,29,34,39] the role of SDHB/ SDHA immunohistochemistry has been underlined as a supplementary approach in molecular genetic testing especially for pheochromocytomas and paragangliomas.[8,10,11] As Sanger or targeted next-generation sequencing analysis of all pheochromocytoma/paraganglioma susceptibility genes is labor intensive and/or requires clinical molecular diagnostic laboratories,[43–45] it might be prudent to use immunohistochemistry to identify patients with succinate dehydrogenase-related pheochromocytoma/paraganglioma syndromes. In addition, the presence of an *SDHB* mutation is one of the strongest predictors for both metastasis and subsequently poor outcome in pheochromocytomas/paragangliomas.[4] In this context, it has been proposed that a combination of the GAPP (grading system for adrenal phaeochromocytoma and paraganglioma) and SDHB immunohistochemistry might be a valuable aid in the prediction of metastatic disease,[46] further necessitating correct interpretation of SDHB/SDHA immunostainings.

Given the high prevalence of unsuspected hereditary disease, false-positive as well as false-negative evaluations of SDHB/SDHA immunostainings can lead to failure to identify pheochromocytoma/paraganglioma-affected individuals at increased risk for succinate dehydrogenase-related neoplasia, incorrect interpretation of the pathogenicity of genetic variants of uncertain significance, and inappropriate genetic testing. Because studies addressing the issue of interobserver variation for SDHB/SDHA immunohistochemistry in pheochromocytomas/paragangliomas are lacking, we assessed interobserver agreement among practicing expert endocrine pathologists through virtual microscopy in a large multicenter, multinational cohort of genetically well-characterized tumors. Accordingly, we examined the validity of SDHB/SDHA immunohistochemistry to identify patients with succinate dehydrogenase-related pheochromocytomas/paragangliomas and of SDHB immunohistochemistry as a marker of malignancy.

Materials and methods

Case Selection

A total of 351 paraganglionic tumors from 333 patients of median age 46 years (ranging from 5.5 to 84 years; 56% females) were retrieved from 15 specialized centers from Europe, United States, and Australia: (1) Université catholique de Louvain, Brussels, Belgium (95 samples from 84 patients), (2) Hôpital Européen Georges Pompidou, Paris, France (68 samples from 67 patients), (3) University of Florence, Florence, Italy (40 samples), (4) National Institutes of Health (NIH), Bethesda, MD, USA (24 samples), (5) Klinikum der Universität München, Munich, Germany (20 samples), (6) Radboud University Nijmegen Medical Center, Nijmegen, The Netherlands (18 samples from 17 patients), (7) Instituto Português de Oncologia de Lisboa Francisco Gentil E.P.E., Lisbon, Portugal (15 samples from 12 patients), (8) Hôpital Cochin, Paris, France (13 samples), (9) Jagiellonian University Medical College, Krakow, Poland (12 samples), (10) Technische Universität Dresden, Dresden, Germany (11 samples), (11) San Luigi Gonzaga Hospital and University of Turin, Turin, Italy (11 samples), (12) Erasmus MC Cancer Institute, Rotterdam, The Netherlands (10 samples from 8 patients), (13) University of Sydney, Sydney, Australia (8 samples), (14) Spanish National Cancer Research Centre (CNIO), Madrid, Spain (5 samples), and (15) Hospital Universitario San Cecilio, Granada, Spain (1 sample). Clinical and genetic characteristics of these patients are detailed in Supplementary Tables 1 and 2. Thirty samples (30 out of 351; 8.54%) were considered malignant (Supplementary Table 2) as primary tumors and/or recurrences in the presence of metastatic disease to sites where chromaffin tissue is not normally found[4] or as metastases themselves.

Out of 351 tumor samples, (1) 73 were *SDH-x* mutated (39 *SDHD*, 24 *SDHB*, 4 *SDHA*, 4 *SDHAF2*, and 2 *SDHC*), (2) 105 non-*SDH-x* mutated (37 *VHL*, 25 *RET*, 21 *NF1*, 8 *MAX*, 6 *HIF2A*, 4 *TMEM127*, and 4 *HRAS*), (3) 128 wild-type cases (7 head and neck paragangliomas, 13 extra-adrenal paragangliomas, and 108 pheochromocytomas) that have been tested negative for mutations and large deletions in the *SDH-x* genes, and (4) 45 samples with incomplete *SDH-x* molecular genetic analysis in terms of either *SDH-x* genes or the techniques performed, that is, Sanger sequencing and/or multiplex ligation-dependent probe amplification. A total of 225 samples were analyzed at least for 3 pheochromocytoma/paraganglioma susceptibility genes with 129 and 30 harboring mutations at the germline and somatic level, respectively (Supplementary Table 1). Based on clinical grounds, 19 tumors were considered *NF1*, *RET*, or *VHL* mutated (Supplementary Table 1).

None of these tumor samples have been previously published elsewhere in terms of SDHB/SDHA immunohistochemical investigation and all were anonymously assessed according to the Proper Secondary Use of Human Tissue code established by the Dutch Federation of Medical Scientific Societies (http://www.federa.org). Informed consent was obtained for genetic analysis and access to the clinical data in accordance with institutional

guidelines. The Medical Ethical Committee of the Erasmus MC approved the study.

SDHB/SDHA Immunohistochemistry

Each case was thoroughly reviewed and representative unstained glass slide(s) ($n=$ 147) and/or formalin-fixed, paraffin-embedded block(s) ($n=204$) were selected and further provided for immunohistochemical analysis within a single research setting (Department of Pathology, Erasmus MC Cancer Institute, Rotterdam, The Netherlands) with the following protocol. Slides and formalin-fixed, paraffin-embedded whole-tissue sections of 4 μm thickness were stained with commercially available antibodies: (1) mouse monoclonal Ab14715 antibody (Mitosciences, Abcam, Cambridge, UK; 1:500 dilution) against SDHA and (2) rabbit polyclonal HPA002868 antibody (Sigma-Aldrich, St Louis, MO, USA; 1:400 dilution) against SDHB on an automatic Ventana Benchmark Ultra System (Ventana Medical Systems, Tuscon, AZ, USA) using Ultraview DAB detection system preceded by heat-induced epitope retrieval with Ventana Cell Conditioning 1 (pH 8.4) at 97 °C for 52 and 92 min, respectively. Diaminobenzidine was used as the chromogen.

Telepathology Application

High-resolution, whole-slide images were acquired from 702 SDHB/SDHA immunostainings using a NanoZoomer Digital Pathology System (Hamamatsu Photonics KK, Japan) working at a resolution of 0.23 μm/pixel. The immunostainings were scanned at $\times 40$ magnification and automatically digitized in their proprietary NanoZoomer Digital Pathology Image file format. A quality control was subsequently set to ensure good focus. Between August 2012 and December 2013, digital files were consecutively uploaded in six sets to a server at Erasmus MC through the standard File transfer Protocol with URL http://digimic.erasmusmc.nl/, enabling online worldwide viewing through a virtual microscopy interface (NanoZoomer Digital Pathology.view Viewer Software, Hamamatsu Photonics KK).

Participants and Interpretation of Staining Results

Seven pathologists, including five who had published on SDHB and/or SDHA immunohistochemical assessments and two who had dealt with endocrine pathology on diagnostic and research grounds for many years (AJG, F van N, AST, FT, MV, XM-G, and RRdeK), received: (1) a word file detailing the context and the objectives of the project along with an instructory panel of SDHB/SDHA immunohistochemistry, (2) a Virtual Microscopy (NanoZoomer Digital Pathology) Manual, (3) the corresponding link providing access to the virtual slides of the first set of tumors, and (4) a scoring list to be completed during SDHB/SDHA immunohistochemical evaluations.

All virtual slides were distributed online, reviewed by each observer in a blinded manner without knowledge of the corresponding clinicopathological and genetic data or scores assigned by other pathologists and scored as follows: (1) with regard to SDHB immunohistochemistry: *Positive* as granular cytoplasmic staining displaying the same intensity as internal positive control (endothelial cells, sustentacular cells, lymphocytes); *Negative* as completely absent staining in the presence of an internal positive control; *Weak diffuse* as a cytoplasmic blush lacking definite granularity contrasting the strong granular staining of internal positive control; *Heterogeneous* as granular cytoplasmic staining combined with a cytoplasmic blush lacking definite granularity or completely absent staining in the presence of an internal positive control throughout the same slide; *Noninformative* as completely absent staining in the absence of an internal positive control; and (2) with regard to SDHA immunohistochemistry: *Positive* as granular cytoplasmic staining displaying the same intensity as internal positive control (endothelial cells, sustentacular cells, lymphocytes); *Negative* as completely absent staining in the presence of an internal positive control; *Heterogeneous* as granular cytoplasmic staining combined with a cytoplasmic blush lacking definite granularity or completely absent staining in the presence of an internal positive control throughout the same slide*; Noninformative* as completely absent staining in the absence of an internal positive control.

In an effort to simulate widespread adoption of the scoring system as would occur in community practice, no prescoring consensus meeting was organized. In order to imitate clinical practice as much as possible for SDHB/SDHA immunohistochemical interpretations, we selected a large retrospective cohort comprising *SDH-x*- and non-*SDH-x*-mutated paraganglionic tumors with and without mutations in the remainder pheochromocytoma/paraganglioma-associated genes.

Statistical Analysis

Interobserver agreement was assessed using κ statistics; the strength of the former was evaluated with criteria previously described by Landis and Koch.[47] A *κ-value* of <0 indicates less than chance agreement, <0.20 is regarded as slight agreement, 0.21–0.40 as fair agreement, 0.41–0.60 as moderate agreement, 0.61–0.80 as substantial agreement, 0.81–0.99 as almost perfect agreement, and 1 indicates perfect agreement. A dichotomous classification was used for the analysis of the pathologists' evaluations (negative/weak diffuse and positive) as well as a three-tiered classification approach (negative/weak diffuse, positive, and heterogeneous). Consensus was

Table 1 Interobserver agreement (κ-values) for SDHA (upper half) and SDHB (lower half) immunohistochemistry

	Observer 1	*Observer 2*	*Observer 3*	*Observer 4*	*Observer 5*	*Observer 6*	*Observer 7*
Observer 1	—	0.7471	0.7471	0.4942	0.5944	0.8557	0.8557
Observer 2	0.7623	—	0.7471	0.4942	0.7972	1.0000	0.8557
Observer 3	0.8561	0.8593	—	0.4942	0.5387	0.8557	0.8557
Observer 4	0.6282	0.6508	0.6819	—	0.3542	0.5672	0.5672
Observer 5	0.7943	0.7998	0.8286	0.5981	—	0.6628	0.6628
Observer 6	0.7199	0.8021	0.7721	0.7276	0.7759	—	1.0000
Observer 7	0.8733	0.6476	0.7923	0.5318	0.6880	0.6621	—

All agreements $P < 0.0001$.

defined as agreement at least among five out of seven pathologists reaching the same interpretation on positive, negative/weak diffuse, heterogeneous, and noninformative expression for SDHB/SDHA immunohistochemistry. Discordant evaluation was defined as at least three observers reporting different SDHB/SDHA expression patterns on the same slide. In order to capture the performance of SDHB immunohistochemistry as a predictive tool, we calculated Youden's J statistic (Youden's index) per pathologist either in tumors harboring *SDH-x* mutations *vs* non-*SDH-x* mutations or in *SDH-x*-mutated tumors *vs* counterparts without identified *SDH-x* mutations. We used Pearson's χ^2 test to associate (1) SDHB IHC status with biological behavior (ie, benignancy *vs* malignancy) taking into consideration only concordant cases as well as excluding metastases ($n = 7$) and doubled samples ($n = 6$) (Supplementary Table 2), and (2) *SDHD* mutations and weak diffuse pattern on SDHB immunohistochemistry based on a consolidated call from at least four observers. Two-sided *P*-values of < 0.05 were considered statistically significant. Statistical analyses were performed using Analyse-it v2.26 (Analyse-it Software, Leeds, UK).

Results

The interobserver agreement following a two-tiered classification approach (ie, positive and weak diffuse/negative) ranged from moderate to almost perfect for SDHB immunohistochemistry and from fair to perfect for SDHA immunohistochemistry (Table 1). With regard to SDHB immunohistochemistry, the highest agreement was reached between observers 2 and 3 ($\kappa = 0.8593$) and the lowest between observers 4 and 7 ($\kappa = 0.5318$), whereas regarding SDHA immunohistochemistry, the highest agreement was reached between observers 6 and 2/7 ($\kappa = 1.0000$) and the lowest between observers 4 and 5 ($\kappa = 0.3542$). All agreements were highly significant ($P < 0.0001$). Substantial agreement among all the reviewers was observed either with a two-tiered classification (SDHB $\kappa = 0.7338$; SDHA $\kappa = 0.6707$) or a three-tiered classification approach (SDHB $\kappa = 0.6543$; SDHA $\kappa = 0.7516$). Notably, observer 1 as well as observers 3/4/5 did not score any slide as heterogeneous pattern for SDHB and SDHA immunohistochemistry respectively.

Consensus among pathologists was achieved in 348 cases (99.15%) for SDHA immunohistochemistry and in 315 cases (89.74%) for SDHB immunohistochemistry, respectively. Out of 69 tumor samples with *SDHB*/*SDHC*/*SDHD*/*SDHAF2* mutations, 62 (89.85%) displayed SDHB immunonegativity and SDHA immunopositivity, whereas 3 of 4 with *SDHA* mutations (75%) showed loss of SDHA/SDHB protein expression (Figure 1). Two *SDHD*-mutated extra-adrenal paragangliomas (c.274G>T p.Asp92Tyr and c.405delC p.Phe136Leufs*32) were scored as SDHB immunopositive by 5 observers and as immunonegative (weak diffuse) by the other observers (observers 2/5).

All tumors harboring *RET*, *TMEM127*, *HIF2A*, and *HRAS* mutations, 31 of 37 *VHL*-mutated tumors (83.7%), and 20 of 21 *NF1* mutated-tumors (95.2%) displayed retention of SDHB/SDHA expression (Figure 2). Six benign *VHL*-mutated pheochromocytomas (6 out of 37; ~16%) and one malignant *NF1*-mutated extra-adrenal paraganglioma (1 out of 21; ~5%) were evaluated as SDHB immunonegative (*VHL*: by all observers (3 cases), 6 observers (1 case), and 5 observers (2 cases); *NF1*: by 6 observers (1 case)) in the absence of *SDH-x* mutations in four of these cases (two examined at the germline, one at the germline and somatic, and one at the somatic level). Data on the exact mutations were available only in four cases (*VHL* p.Ser80Asn, p.Arg161*, p.Arg167Gln, and *NF1* p.Trp561*).

In the absence of *SDH-x* mutations, 119 out of 128 paraganglionic tumors (93%) were scored as SDHB/SDHA immunopositive, whereas the remainder (9 out of 128; 7%) as SDHB immunonegative/SDHA immunopositive. Clinicopathological and genetic data of the latter from four independent centers are detailed in Table 2.

Discordant evaluations of SDHB immunohistochemistry were reported in 5 tumors endowed with *SDH-x* (*SDHD*/*SDHB*/*SDHAF2*) mutations, 11 *VHL*- and 2 *RET*-mutated tumors, as well as 18 tumors without identified *SDH-x* mutations, whereas of SDHA immunohistochemistry concerned 2 *SDH-x*-mutated tumors (*SDHA*-/*SDHD*-) and 1 *NF1*-mutated tumor.

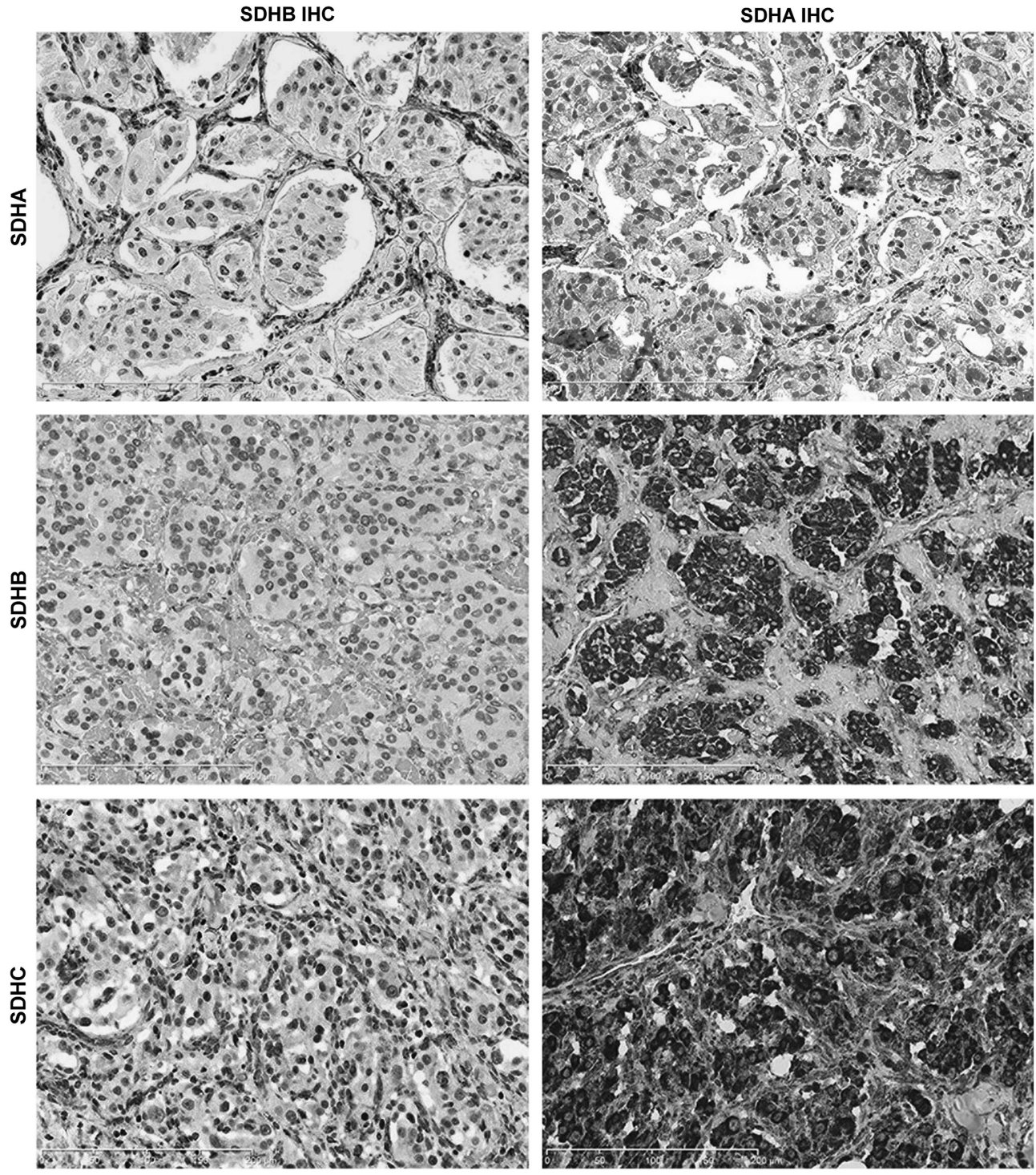

Figure 1 SDHA and SDHB immunohistochemistry in pheochromocytomas/paragangliomas endowed either with *SDHA* germline mutation displaying loss of SDHA/SDHB protein expression or with *SDHB*, *SDHC*, *SDHD*, and *SDHAF2* germline mutations exhibiting loss of SDHB, but intact SDHA expression. Note the granular, cytoplasmic staining for SDHA/SDHB in normal cells of the intratumoral fibrovascular network that serve as internal positive controls.

The classification of stainings as 'noninformative' and 'heterogeneous' represented the major reason for SDHB/SDHA immunohistochemical discrepancies in the *SDH-x*-mutated subgroup, whereas the 'weak diffuse' category accounted largely for those in the *SDH-x*-wild-type and *VHL*-mutated subsets.

The association between the predicted SDH genetic status and SDHB immunohistochemistry

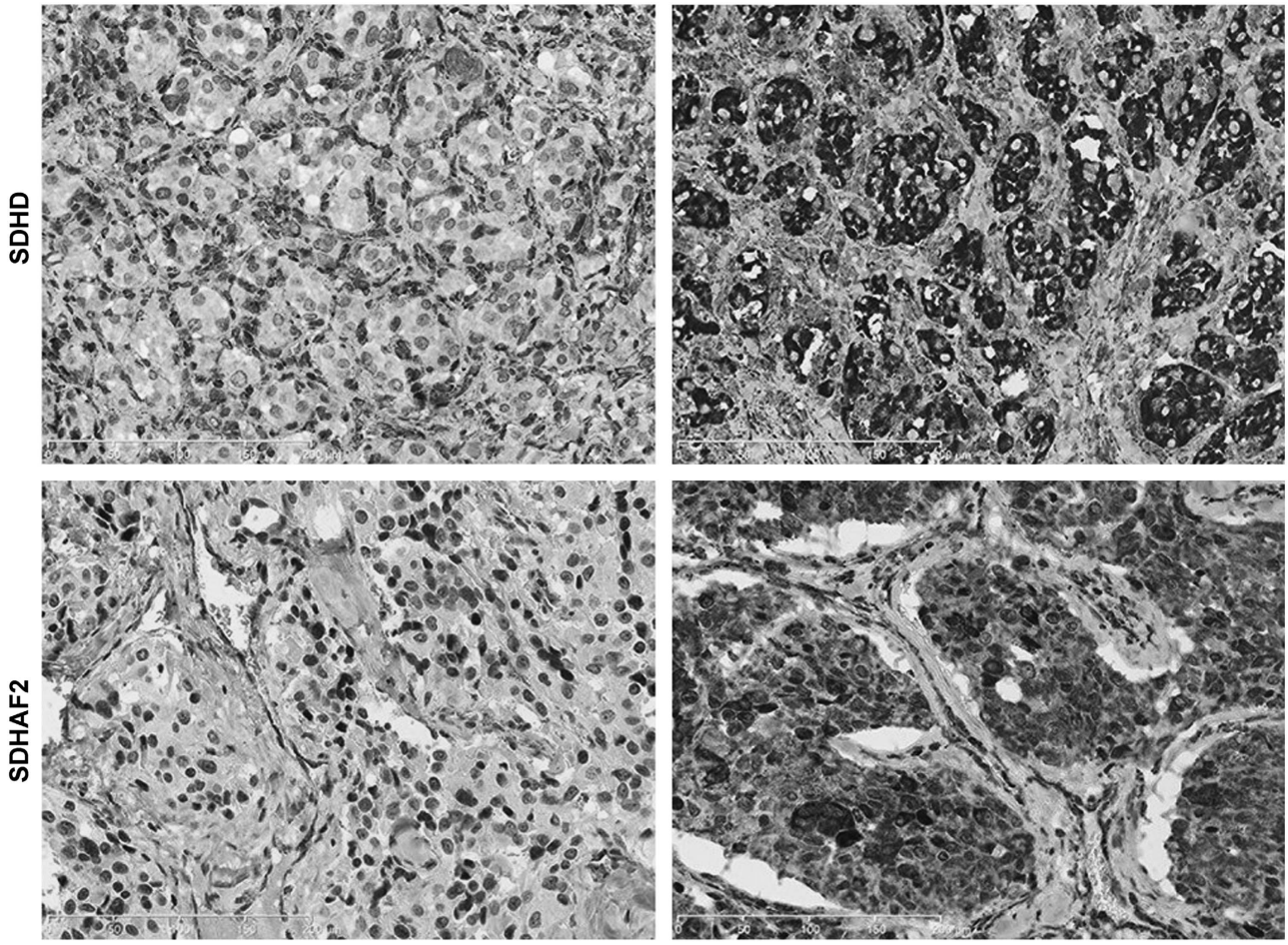

Figure 1 Continued.

was investigated for each observer. The sensitivity of this approach, defined as the percentage of *SDH-x* mutated tumors that are SDHB immunonegative, ranged from 83.58 to 98.57% (mean 94.23%). The specificity, defined as the percentage of either non-*SDH-x*-mutated tumors or tumors without identified *SDH-x* mutations that are SDHB immunopositive, varied between 74.03 and 96.11% (mean 84.35%) as well as 83.06 and 92.91% (mean 86.67%), respectively. Observer 1 was the best predictor with a Youden's index of 0.880 and 0.860 (Table 3). A significant correlation was observed between SDHB immunonegativity and malignancy ($P = 0.00019$). No association could be shown between the *SDHD* mutations and the weak diffuse pattern on SDHB immunohistochemistry ($P = 0.1490$).

Discussion

Immunohistochemistry has revolutionized the practice of endocrine pathology during the last decade. In parallel with recent advances in molecular genetics, immunohistochemistry has been shown to detect various types of molecular alterations, that is, *BRAF V600E* mutation in papillary thyroid carcinomas,[48] *PTEN* mutations in various neoplastic thyroid lesions,[49] *CTNNB1* mutations in cribriform-morular variant of papillary thyroid carcinoma, undifferentiated carcinomas of the thyroid gland and adrenocortical carcinomas,[48,50,51] *TP53* mutations as well as mutations in *mismatch repair* (*MMR*) genes such as *MLH1*, *MSH2*, *MSH6*, and *PMS2* in adrenocortical carcinomas,[51–53] *HRPT2* mutations in parathyroid carcinomas and hyperparathyroidism-jaw tumor syndrome-related adenomas,[48,54] *PRKAR1A* mutations in Carney complex-associated tumors,[55–57] and *SDH-*, *FH-* as well as *MAX* deleterious-mutations in pheochromocytomas/paragangliomas.[8,10,11,58,59]

Loss of SDHB protein expression is seen in pheochromocytomas/paragangliomas either harboring a mutation in any of the *SDH* genes or with somatic hypermethylation of the *SDHC* promoter region,[42] whereas loss of both SDHB and SDHA immunoreactivity is demonstrated only in the context of an *SDHA* mutation.[8–20] In agreement with previous studies,[8,10,11,17–20] *SDHB-/C-/D-* and *SDHA*-mutated tumors displayed the aforementioned

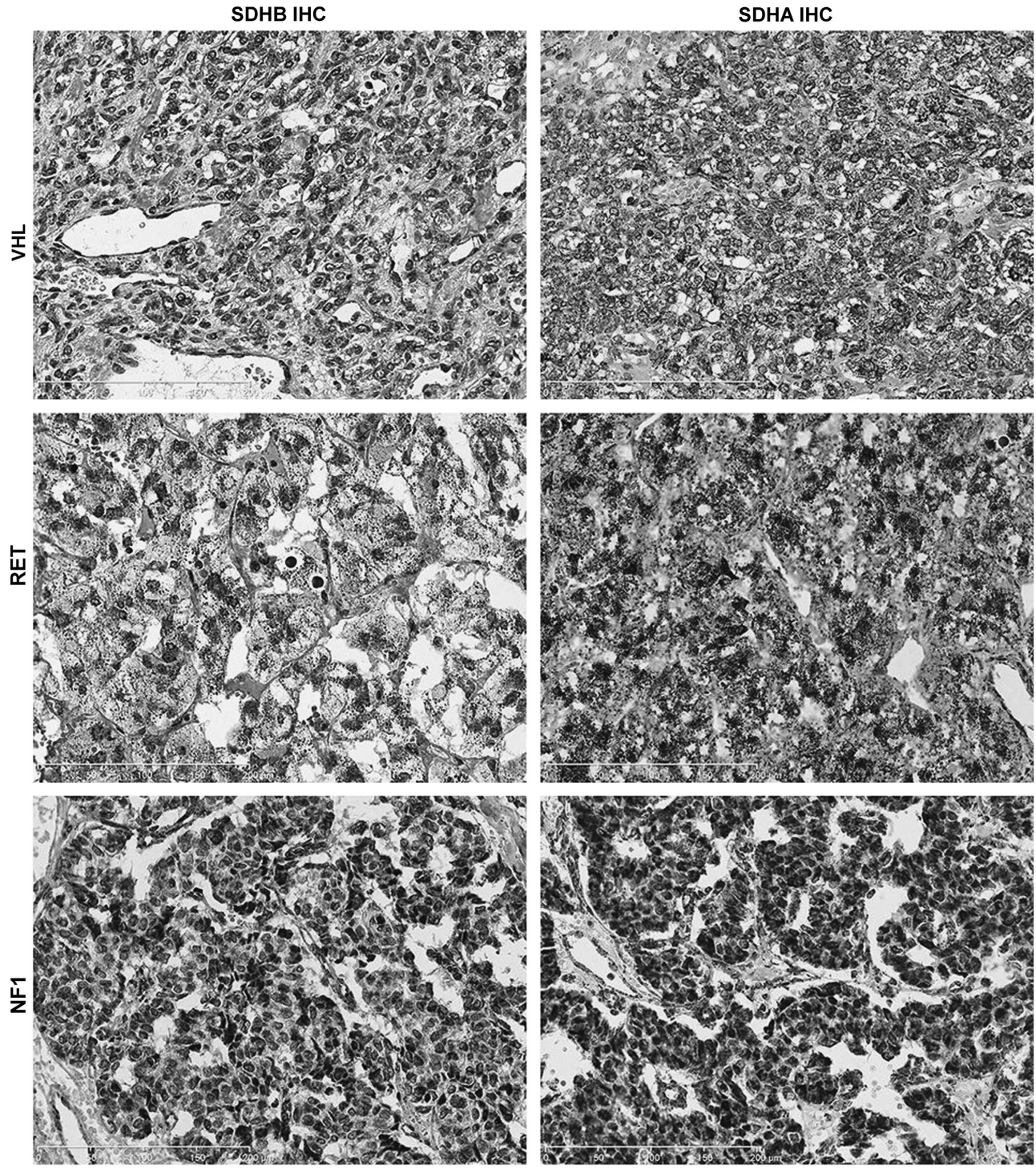

Figure 2 Intact SDHB and SDHA protein expression in non-*SDH-x*-mutated paraganglionic tumors harboring germline or somatic *VHL*, *RET*, *NF1*, *TMEM127*, *MAX*, *EPAS1*, and *HRAS* mutations. Note the granular, cytoplasmic staining for SDHA/SDHB in normal cells of the intratumoral fibrovascular network that serve as internal positive controls.

immunoexpression patterns with *SDHAF2*-mutated counterparts showing SDHB immunonegativity and SDHA immunopositivity. Notably, all tumors harboring *RET*, *TMEM127*, *HIF2A*, and *HRAS* mutations displayed retention of SDHB/SDHA expression, whereas six benign *VHL*-mutated pheochromocytomas and one malignant *NF1*-mutated extra-adrenal paraganglioma were evaluated as SDHB immunonegative. The latter contrasts previous observations in 37 pheochromocytomas/paragangliomas

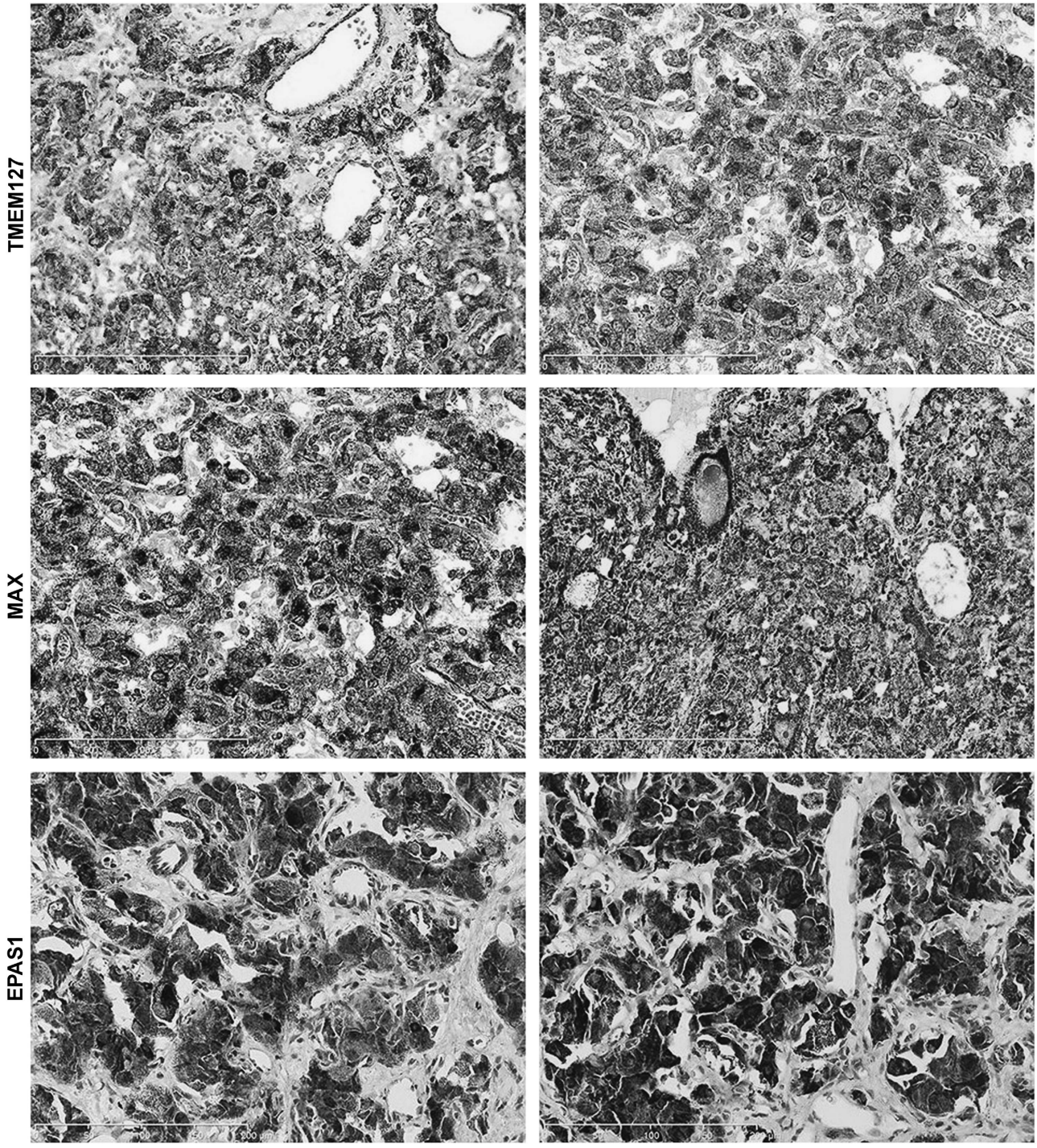

Figure 2 Continued.

and 14 pheochromocytomas endowed with *VHL*[8,11] and *NF1* mutations,[8,10] respectively. By using a mouse monoclonal (21A11) SDHB antibody at a low concentration (1 in 1000), Gill *et al*[10] suggested that *VHL*-associated tumors could be classified as negative or weak diffuse rather than positive as demonstrated by a high concentration approach of two SDHB antibodies.[8] In accordance, loss of SDHB protein expression has been recently displayed in a subset of *NF1*-mutated paraganglionic tumors (J Favier 2014, personal communication). The remote possibility of a double mutant, potentially explaining the SDHB immunonegativity by an additional *SDH-x* mutation, was ruled out in four of these seven cases occurring in the *VHL*- and *NF1*-deficient setting.

To further expand earlier observations,[8,11] 9 of 128 (7%) tumors without identified *SDH-x* mutations

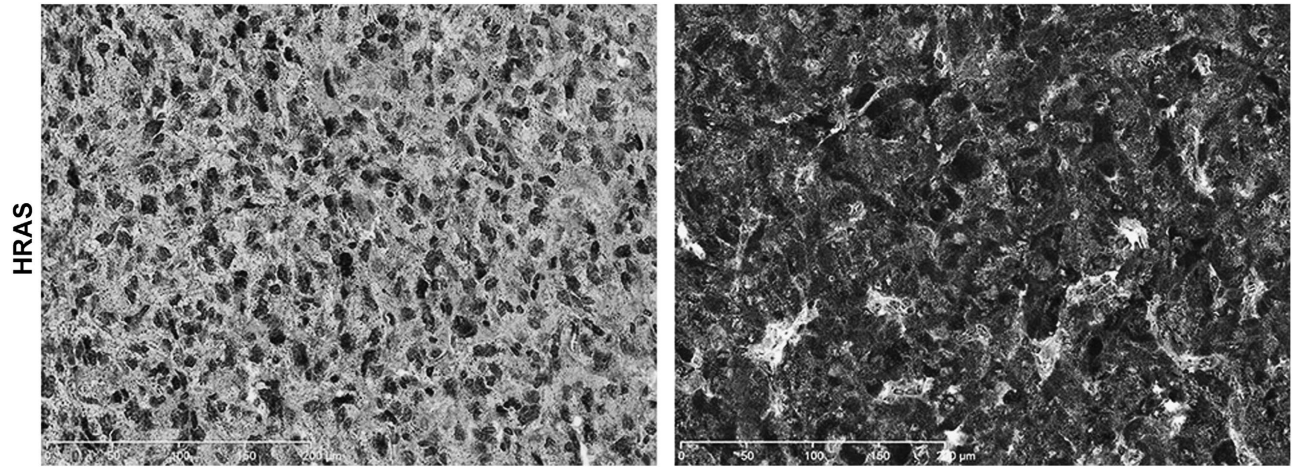

Figure 2 Continued.

Table 2 Clinicopathological and genetic data of patients with SDHB-immunonegative paraganglionic tumors in the absence of *SDH-x* mutations

	Syndromic presentation						*Molecular genetic testing of PCC/PGL susceptibility genes* [a]								
Sample code	*Familial PCC/ PGL history*	*Multiple tumors*	*Age at presentation*	*Sex*	*Tumor type*	*Dignity*	*SDHB*	*SDHD*	*SDHC*	*SDHA*	*SDHAF2*	*SDHAF1*	*VHL*	*TMEM127*	*MAX*
BEL 30	No	No	43	F	HN PGL	B	—	—	—	—	—	ND	—	—	—
BEL 67	No	No	36	M	HN PGL	B	—	—	—	—	—	ND	—	—	—
DR 11	No	No	27	F	HN PGL	B	—	—	—	ND	—	ND	ND	—	—
ITA 28	No	No	73	F	HN PGL	B	—	—	—	—	—	ND	—	—	—
DR 10[b]	No	Yes	33	F	EA PGL	B	—	—	—	—	—	—	ND	ND	—
BEL 66	No	No	15	F	EA PGL	M	—	—	—	—	—	ND	—	—	—
BEL 116	No	No	20	M	PCC	B	—	—	—	—	—	ND	—	—	ND
ITA 48	No	No	47	F	PCC	B	—	—	—	—	—	ND	—	—	—
FR115[c]	No	No	23	M	PCC	B	—	—	—	—	ND	ND	—	—	—

Abbreviations: B, benign; EA, extra-adrenal; F, female; HN, head and neck; M, male; M, malignant; ND, not done; PCC, pheochromocytoma; PGL, paraganglioma.
[a]*SDH-x* genes have been tested both for point mutations and large deletions at the germline level with DR10 and ITA48 also investigated at the somatic level.
[b]Tested for *FH* at the germline and *EPAS1* at the somatic level without any mutations subsequently detected.
[c]Tested for *RET* mutations as well for *SDH-x*/*VHL* large deletions at the germline level without any mutations subsequently detected.

were evaluated as SDHB immunonegative (Table 2). Van Nederveen *et al*[8] and Castelblanco *et al*[11] reported on 9 cases (6 out of 53; 11% and 3 out of 19; 15.7%) displaying loss of SDHB expression in the absence of *SDHB*, *SDHC*, *SDHD*, *VHL*, or *RET* mutation. Nevertheless, these studies lacked either *SDHA*/*SDHAF2* genetic testing[8,11] or screening for large-scale *SDH-x* deletions[11] that may account for higher percentages. Intriguingly, in the present study, eight SDHB-immunonegative tumors were nonmetastatic in the absence of *SDH-x* mutations (Table 2), bearing a close resemblance to the Carney triad-associated counterparts in terms of SDHB immunohistochemistry and biologic behavior.[4,29,60] Because somatic hypermethylation of *SDHC* was not investigated, the possibility that the aforementioned tumors represented cases of Carney triad could not be assessed. Nevertheless, as shown herein, SDHB immunohistochemical status overall is strongly correlated with the clinical behavior of pheochromocytoma/paraganglioma, further strengthening the role of SDHB immunohistochemistry as a prognostic marker.[46,61]

Our data reinforce the notion that immunohistochemistry is a valid tool to identify patients at risk for familial succinate dehydrogenase-related pheochromocytoma/paraganglioma syndromes, although occasionally this might be difficult even in a specialized setting (Table 3). Exemplifying the latter, two extra-adrenal paragangliomas with missense and frameshift *SDHD* mutations were scored as SDHB immunopositive by five observers. Similar discrepancy has been previously reported for an extra-adrenal paraganglioma harboring a nonsense *SDHD* mutation (c.14G>A p.Trp5*) in a patient with Carney Stratakis syndrome.[31] Given that the patient additionally developed an SDHB-immunonegative gastrointestinal stromal tumor[31] and that identical

Table 3 Associating predicted SDHB IHC status either with *SDH-x*-mutated *vs* non-*SDH-x*-mutated status (A) or with *SDH-x*-mutated *vs* *SDH-x*-wild-type status (B)[a]

	Observer 1	*Observer 2*	*Observer 3*	*Observer 4*	*Observer 5*	*Observer 6*	*Observer 7*
A							
Sensitivity	95.71%	98.57%	94.44%	93.22%	98.57%	95.52%	83.58%
Specificity	92.30%	77.66%	90.00%	74.03%	82.35%	78.02%	96.11%
PPV	89.33%	75.00%	87.17%	67.07%	79.31%	76.19%	93.33%
NPV	96.96%	98.76%	95.74%	95.06%	98.82%	95.94%	90.00%
Pval	$P<0.0001$	$P<0.0001$	$P<0.0001$	$P<0.0001$	$P<0.0001$	$P<0.0001$	$P<0.0001$
Youden's index	0.880	0.762	0.844	0.672	0.809	0.735	0.796
B							
Sensitivity	95.71%	98.57%	94.44%	93.22%	98.57%	95.52%	83.58%
Specificity	90.47%	83.06%	87.70%	84.55%	83.73%	84.21%	92.91%
PPV	84.81%	76.66%	81.92%	74.32%	77.52%	78.04%	86.15%
NPV	97.43%	99.03%	96.39%	96.29%	99.03%	96.96%	91.47%
Pval	$P<0.0001$	$P<0.0001$	$P<0.0001$	$P<0.0001$	$P<0.0001$	$P<0.0001$	$P<0.0001$
Youden's index	0.860	0.816	0.821	0.777	0.823	0.797	0.764

Abbreviations: Pval, *P*-value χ^2 test; PPV, positive predictive value; NPV, negative predictive value.
Youden's index is defined as sensitivity+specificity-1. The higher the Youden's index, the better the prediction.
Sensitivity is defined as the percentage of *SDH-x-mutated tumors* that are SDHB immunonegative Specificity is defined as the percentage of *non-SDH-x-mutated tumors* or *tumors without identified SDH-x mutations* that are SDHB immunopositive.
[a]Heterogeneous and noninformative scorings are excluded.

missense and nonsense *SDHD* mutations in other tumors have led to absence of SDHB expression,[5,8] it is possible that either the second hit in the *SDHD* gene in the paraganglioma resulted in an inactive succinate dehydrogenase complex with preservation of antigenicity or that the interpretation was erroneous. Of note, every pathologist in the current study missed at least one *SDH-x*-related tumor, and these most frequently involved mutations in *SDHD.* This suggests SDHD immunohistochemistry as a potential complementary tool to SDHB immunohistochemistry to identify *SDHD*-mutated patients.[62] Further adding to those rare familial cases characterized by disparity between molecular genetic aberrations of a tumor suppressor gene and retention of protein expression,[63] one papillary renal cell carcinomas arising in a patient with a germline missense *SDHC* mutation (c.3G>A p.M1I) and harboring somatic loss of heterozygosity of the *SDHC* locus paradoxically displayed SDHB immunopositivity.[36] Taken together, SDHB immunohistochemistry and *SDH-x* genetic analysis should be viewed as complementary tests. In cases of strong clinical suspicion, follow-up mutational analysis should be considered despite retention of SDHB expression.

The good level of reproducibility in the current study may either reflect a high level of experience with scoring SDHB/SDHA immunostainings among expert endocrine pathologists or be attributable in part to the fact that very precise scoring guidelines were provided. Accordingly, it would be essential to provide such guidelines in clinical reporting templates[64] as well as to guide development of algorithms for computer-assisted diagnostics in a digital pathology perspective. The classification of stainings as 'non-informative' and 'heterogeneous' represented the major reason for SDHA/SDHB immunohistochemical discrepancies in the *SDH-x*-mutated subgroup, whereas the 'weak diffuse' category accounted largely for inconsistencies in the *SDH-x*-wild-type and *VHL*-mutated subsets. These could be potentially ascribed to (1) technical variability owing to differences in fixation time, buffered formalin concentrations, and/or age of the formalin-fixed, paraffin-embedded blocks,[10,11] (2) biological variability, for example, reduced SDHB protein levels in *VHL*-mutated paraganglionic tumors,[65] or even to (3–4) individual conceptions and experience from specific staining protocols, as has been shown with immunohistochemistry for MMR proteins.[66] Technically suboptimal immunostainings were not unexpectedly encountered given the fact that provided material was derived from several pathology laboratories, each following their own fixation and embedding protocols; highlighting the importance of standardizing preanalytical variables in surgical pathology specimens.[67,68]

In contrast to previous studies[10,11] indicating a stronger correlation of weak diffuse pattern with *SDHD* mutations, we could not significantly reinforce this particular association. Moreover, SDHB and/or SDHA immunohistochemistry may not always be an all-or-none phenomenon. In particular, two *SDHA*- and *SDHAF2*-mutated tumors displayed a heterogeneous expression pattern (Figures 3 and 4) being consistent with previous observations concerning SDHB immunohistochemistry in a pituitary adenoma harboring an *SDHD* germline mutation.[37] Along the same lines, heterogeneous expression patterns have been reported both with MMR protein immunohistochemistry in Lynch syndrome and PTEN immunohistochemistry in Cowden syndrome.[49,69,70] The biologic nature of heterogeneous tumors in these genetic contexts is currently

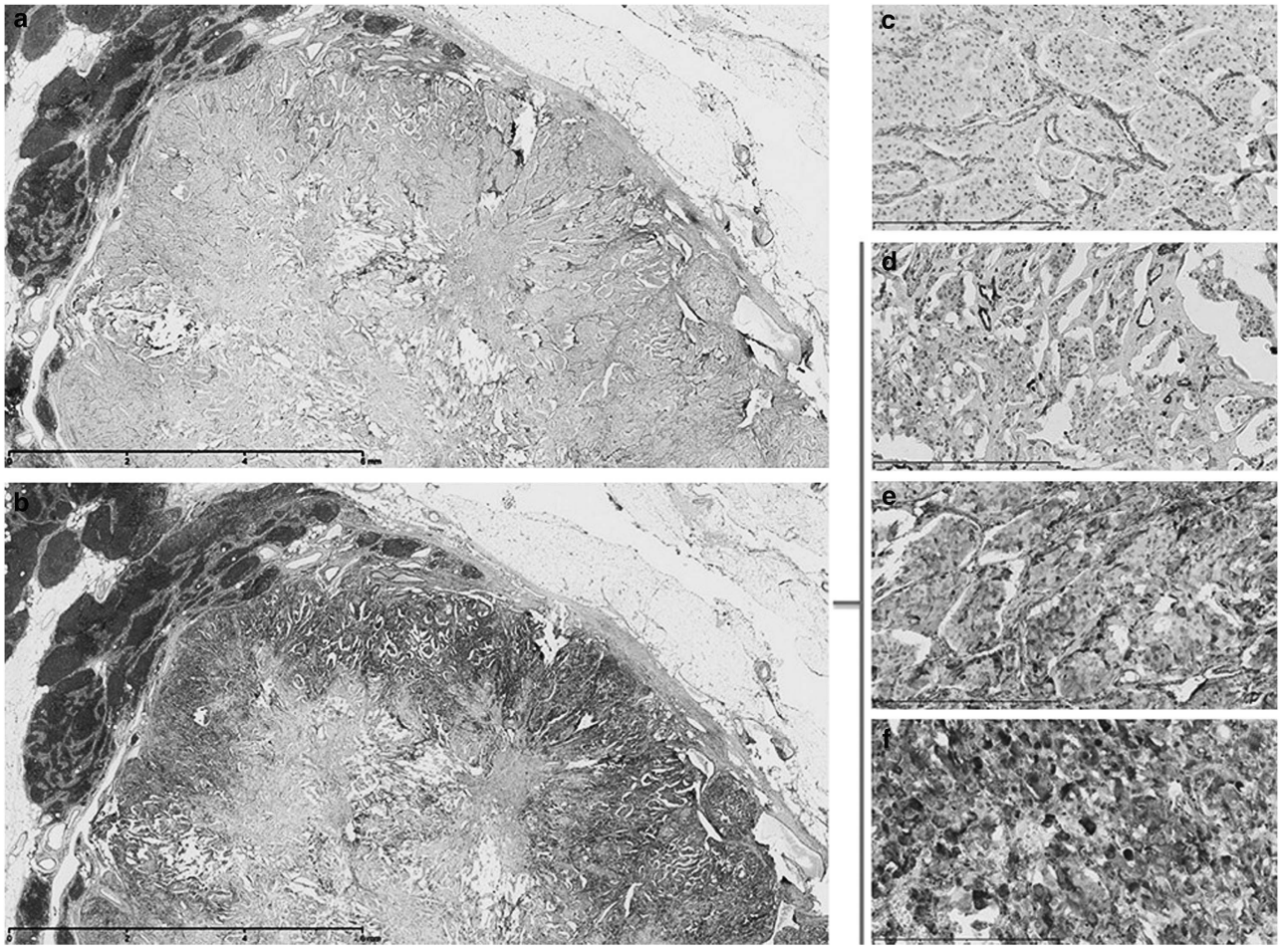

Figure 3 An extra-adrenal paraganglioma harboring an *SDHA* (c.1534C>T, p.Arg512*) germline mutation, metastatic to a paraaortic lymph node, displaying SDHB immunonegativity (**a**, **c**), but a heterogeneous staining pattern for SDHA (**b**, **d–f**): central area (**d**) convincingly negative for SDHA, peripheral areas (**f**) convincingly positive for SDHA, and transitional zones (**e**) in between exhibiting cells with intact SDHA expression intermingled with cells with absent SDHA expression. Three pathologists correctly classified this sample as heterogeneous for SDHA, with the remainder four observers as positive for SDHA. Note the granular, cytoplasmic staining for SDHA/ SDHB in normal cells of the intratumoral fibrovascular network that serve as internal positive controls.

unknown.[37,49,69,70] Because of potential misinterpretation of heterogeneous patterns for SDHB and/or SDHA protein loss, *SDH* genetic testing is recommended when confronted with such cases.

In addition to a comprehensive next-generation sequencing-based strategy for the analysis of multiple pheochromocytoma/paraganglioma susceptibility genes,[43–45] several algorithms have been proposed as a targeted approach to genetic testing in clinical practice.[8,71–74] In this rapidly expanding field, the importance of assessing the pathogenicity of a 'variant of unknown significance' has become a major and complex problem facing diagnostic laboratories. Our data further strengthen the role of SDHB/SDHA immunohistochemistry in determining the functionality of such variants, alone or in an integrated approach with *in silico* analysis[75,76] and/or western blot analysis, succinate dehydrogenase enzymatic assay, and mass spectrometric-based measurements of ratios of succinate/fumarate and other metabolites.[77–79]

In the current study, we conclude that SDHB/ SDHA immunohistochemistry represents a reliable tool to identify patients with *SDH-x* mutations with an additional utility to evaluate the pathogenicity of *SDH* variants of unknown significance in the new next-generation sequencing era. A heterogeneous SDHB and/or SDHA immunoexpression pattern has to be followed by *SDH* molecular genetic testing, although a SDHB-immunonegative subset of *VHL*- and *NF1*-mutated paraganglionic tumors challenges the issue of specificity for SDHB immunohistochemistry. Hence, if *SDH* genetics fails to detect a mutation in SDHB-immunonegative tumor, *SDHC* promoter methylation and/or *VHL*/*NF1* testing with the use of targeted next-generation sequencing is advisable. Our findings highlight the need for quality assessment programs regarding not only standardized staining protocols, but also SDHB/SDHA immunohistochemical evaluation procedures. In a prospective setting, with standardized tissue fixation combined with a locally fine-tuned

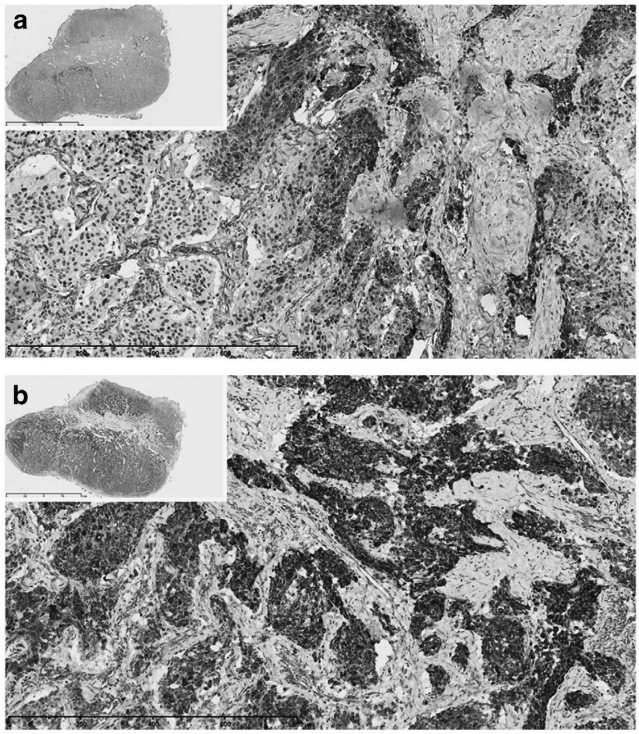

Figure 4 An *SDHAF2*-mutated (c.232G>A, p.Gly78Arg) head and neck paraganglioma showing areas convincingly negative for SDHB and to a lesser extent areas convincingly positive for SDHB (**a**). Three pathologists correctly classified this sample as heterogeneous for SDHB, with the remainder four as negative for SDHB, whereas all observers scored it as SDHA immunopositive (**b**). Note the granular, cytoplasmic staining for SDHB in normal cells of the intratumoral fibrovascular network that serve as internal positive control.

immunohistochemical staining protocol, the sensitivity and specificity of the SDHA/SDHB immunohistochemistry can be improved.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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