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)

SDHB/SDHA Immunohistochemistry in Pheochromocytomas and Paragangliomas:

A Multicenter Interobserver Variation Analysis using Virtual Microscopy

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Abstract

Despite the established role of SDHB and SDHA immunohistochemistry (IHC) as a valuable tool to identify patients at risk for familial succinate dehydrogenase (SDH)-related pheochromocytoma (PCC)-paraganglioma (PGL) 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, multinational cohort of genetically well-characterized paraganglionic tumors comprising 351 samples: **(1) 73 *SDH* mutated (39 *SDHD*, 24 *SDHB*, 4 *SDHA*, 4 *SDHAF2* and 2 *SDHC*), (2) 105 non-*SDH* mutated (37 *VHL*, 25 *RET*, 21 *NF1*, 8 *MAX*, 6 *HIF2A*, 4 *TMEM127* and 4 *HRAS*), (3) 128 without identified SDH-x mutations, and (4) 45 samples with incomplete *SDH* molecular genetic analysis. Substantial agreement among all the reviewers was observed either with a two-tiered classification (SDHB IHC κ=0.7338**; **SDHA IHC κ=0.6707) or a three-tiered classification approach (SDHB IHC κ=0.6543**; **SDHA IHC κ=0.7516). Consensus, defined as agreement at least among 5 out of 7 pathologists, was achieved in 315 cases (89.74%) for SDHB IHC and in 348 cases (99.15%) for SDHA IHC respectively. 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, while 98 of 105 (93%) non-*SDH* mutated counterparts demonstrated retention of SDHA/SDHB protein expression. Of note, two *SDHD*-mutated extra-adrenal paragangliomas were scored as SDHB immunopositive, whereas 8 of 128 (~6%) 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 13 out of the latter were non-metastatic, a significant correlation between SDHB immunonegativity and malignancy was observed (P=0.0002). We conclude that SDHB/SDHA IHC is a reliable tool to identify patients with *SDH* mutations at least in the specialized setting and together with *SDH* molecular genetic analysis should be viewed as complementary tests. In this framework, if *SDH* genetics fails to detect a mutation, *SDHC* promoter methylation and/or *VHL*/*NF1* testing with the use of targeted Next-Generation Sequencing (NGS) is advisable. The presence of discordant cases highlights the need for quality assessment programs regarding not only standardized staining protocols, but also SDHB/SDHA IHC evaluation procedures.**

Introduction

Pheochromocytomas (PCCs) and paragangliomas (PGLs) are neural-crest derived neuroendocrine tumors arising from the adrenal medulla and sympathetic/parasympathetic paraganglia respectively [1]. As carrying the highest degree of heritability among human neoplasms, germline and/or somatic mutations of at least 15 genes (*NF1*, *RET*, *VHL*, *SDHA, SDHB*, *SDHC, SDHD*, *SDHAF2*, *TMEM127*, *MAX, HIF2A*, *KIF1B*, *PHD2, HRAS* **and** *FH*) **are involved in their development with approximately 40% of these tumors harboring a germline mutation and an additional 25-30% a somatic one [2].**

Familial succinate dehydrogenase (SDH)-related PCC/PGL syndromes are caused by *SDHA*, *SDHB*, *SDHC*, *SDHD* and *SDHAF2* (collectively *SDH-x*) mutations and inherited as autosomal dominant traits [3]. These syndromes predispose not only to PCCs/PGLs, but also to gastrointestinal stromal tumors (GISTs), renal cell carcinomas (RCCs) and pituitary adenomas [4-6]. In the vast majority of SDH-associated tumors, there is also loss of SDHB and/or SDHA protein expression, which can be detected by immunohistochemistry (IHC) [4-40]. 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. GISTs and PGLs associated with the Carney Triad (the syndromic but non-hereditary association of GIST, PGL, pulmonary chondroma, adrenocortical adenoma and esophageal leiomyoma) [3] show negative staining for SDHB in the absence of *SDH-x* mutations [28, 39]. There is provisional evidence that Carney Triad-related tumors display somatic hypermethyalation of the SDHC promoter locus [41] and therefore negative staining for SDHB may also identify these cases not found by conventional molecular testing.

Since loss of SDHB/SDHA expression is predictive of an underlying *SDHx* germline mutation [7, 9-10, 16, 20-23, 28, 33, 38], the role of SDHB/SDHA IHC has been underlined as a supplementary approach in molecular genetic testing especially for PCCs and PGLs [7, 9-10]. As Sanger or targeted Next-Generation Sequencing (NGS) analysis of all PCC/PGL susceptibility genes is labor intensive and/or requires clinical molecular diagnostic laboratories [42-44], it might be prudent to use IHC to identify patients with SDH-related PCC/PGL syndromes. In addition, the presence of an *SDHB* mutation is one of the strongest predictors both for metastasis and subsequently poor outcome in PCCs/PGLs [3]. In this context, it has been proposed that a combination of the *grading system for adrenal phaeochromocytoma and paraganglioma* (GAPP) and SDHB IHC might be of valuable aid in the prediction of metastatic disease [45], further necessitating correct interpretation of SDHB/SDHA IHC.

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 PCC/PGL affected individuals at increased risk for SDH-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 IHC in PCCs/PGLs 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 IHC to identify patients with SDH-related PCC/PGL and of SDHB IHC as a marker of malignancy.

Material and Methods

Case Selection

Three hundred fifty-one paraganglionic tumors from **333** patients of 46 years median age (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, Maryland,

United States of America (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 Table 1**. 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 samples without any *SDHB*-/*C*-/*D*- (*A*-/*AF2*- in part) germline mutations and large deletions (7 head and neck (HN) PGLs, 13 extra-adrenal (ea) PGLs and 108 PCCs) and (**4**) 45 samples with incomplete *SDH-x* molecular genetic analysis in terms either of *SDH-x* genes or the techniques performed i.e Sanger sequencing and/or multiplex ligation-dependent probe amplification (MLPA). Informed consent was obtained for genetic analysis and access to the clinical data in accordance with institutional guidelines.

SDHB/ SDHA Immunohistochemistry

Each case was thoroughly reviewed and representative unstained glass slide(s) (*n*= 147) and/or formalin-fixed paraffin-embedded (FFPE) block(s) (*n*=204) were selected and further provided for immunohistochemical analysis within a single research setting (Department of Pathology, Erasmus MC, Rotterdam, the Netherlands) with the following protocol. Slides and FFPE whole-tissue sections of 4*μ*m 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 Corp, St. Louis, MO, USA.; 1:400 dilution) against SDHB on an automatic Ventana Benchmark Ultra System (Ventana Medical Systems Inc. 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 minutes respectively. Diaminobenzidine was used as the chromogen. No tumor samples have been previously published elsewhere in terms of SDHB/ SDHA IHC investigation and were assessed anonymously according to the Proper Secondary Use of Human Tissue code established by the Dutch Federation of Medical Scientific Societies (http:// www.federa.org). The Medical Ethical Committee of the Erasmus MC approved the study.

Telepathology Application

High-resolution, whole-slide images were acquired from 702 SDHB/SDHA immunostainings using a NanoZoomer Digital Pathology (NDP) System (Hamamatsu Photonics K.K. Japan) working at a resolution of 0.23 μm/pixel. The immunostainings were scanned at a x40 magnification and automatically digitized in their proprietary NDP Image (NDPI) 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 (FTP) in the DMZ with URL: http://digimic.erasmusmc.nl/; enabling online worldwide viewing through a virtual microscopy interface (NDP.view Viewer Software, Hamamatsu Photonics K.K. Japan).

Participants and Interpretation of Staining Results

Nine pathologists, including six who had published on SDHB and/or SDHA IHC assessments and three who had dealt for many years with endocrine pathology both on diagnostic and research grounds, were invited to participate. Accordingly, seven participants (A.J.G, F. van N., A.S.T., F.T., M.V., X.M.-G., R.R.deK.) received: (i) a word file detailing the context and the objectives of the project along with an instructory panel of SDHB/SDHA IHC, (ii) a Virtual Microscopy (NDP) Manual, (iii) the corresponding link providing access to the virtual slides of the first set of tumors, and (iv) a scoring list to be completed during SDHB/SDHA IHC evaluations. All virtual slides were distributed online, reviewed by each observer

in a blinded fashion without knowledge of the corresponding clinicopathological and genetic data or scores assigned by other pathologists and scored as follows:

(i) with regard to **SDHB IHC**
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

(ii) with regard to **SDHA IHC**
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

Of note, 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 IHC interpretations, we selected a large retrospective cohort comprising *SDH-x* and non-*SDH-x* mutated paraganglionic tumors with and without mutations in the remainder PCC/PGL-associated genes.

Statistical Analysis

Interobserver agreement was assessed using kappa (κ) statistics; the strength of the former was evaluated with criteria previously described by Landis and Koch [**46**]. A κ *value <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 defined as agreement at least among 5 out of 7 pathologists reaching the same interpretation on positive, negative/weak diffuse, heterogeneous and non-informative expression for SDHB/SDHA IHC. 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 IHC as a predictive tool, we calculated the 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 SDHB IHC status with biological behavior i.e. benignancy *vs* malignancy and for a potential correlation between *SDHD* mutations and weak diffuse pattern on SDHB IHC; the latter based on a consolidated call from at least four observers. Two-sided *P* values <0.05 were considered statistically significant. Statistical analyses were performed using Analyse-it v2.26 (Analyse-it Software, Ltd. Leeds, United Kingdom).

Results

The interobserver agreement following a two-tiered classification approach i.e. positive and weak diffuse/negative ranged from moderate to almost perfect for SDHB IHC and from fair to perfect for SDHA IHC (**Table 1**). With regard to SDHB IHC, the highest agreement was reached between observer 2 and 3 (κ=0.8593) and the lowest between observer 4 and 7 (κ=0.5318), while regarding SDHA IHC, the highest agreement was reached between observers 6 and 2/7 (κ=1.0000) and the lowest between observer 4 and 5 (κ=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 IHC κ=0.7338; SDHA IHC κ=0.6707) or a three-tiered classification approach (SDHB IHC κ=0.6543; SDHA IHC κ=0.7516). Notably, observer 1 as well as observers 3/4/5 did not score any slide as heterogeneous pattern for SDHB IHC and SDHA IHC respectively.

Consensus among pathologists was achieved in 348 cases (99.15%) for SDHA IHC and in 315 cases (89.74%) for SDHB IHC respectively. Sixty-two of 69 tumor samples endowed with *SDHB*/*C*/*D*/*AF2* mutations displayed SDHB immunonegativity and SDHA immunopositivity, while 3 of 4 with SDHA mutations showed loss of SDHA/ SDHB protein expression (**Figure 1**). Two *SDHD*-mutated ea-PGLs (c.274G>T p.Asp92Tyr and c.405delC p.Phe136Leufs*32) were scored as SDHB immunopositive by 5 observers, while as immunonegative (weak diffuse) by the remainder ones (observers 2/5) respectively. Discordant evaluations of SDHB and SDHA IHC were reported in 5 tumors carrying *SDH-x*- (*SDHD*-, *SDHB*- and *SDHAF2*-) mutations and 2 *SDHA*-/*SDHD*-mutated ones respectively. All tumors harboring *RET*, *TMEM127*, *HIF2A* and *HRAS* mutations as well as 31 of 37 *VHL* mutated- and 20 of 21 *NF1* mutated-tumors displayed retention of SDHB/SDHA expression **(Figure 2**). Six benign *VHL*-mutated PCCs (6 out of 37; ~16%) and one malignant *NF1*-mutated ea-PGL (1 out of 21; ~5%) were evaluated as SDHB imunonegative [*VHL*: by all observers (3 cases), 6 observers (1 case), 5 observers (2 cases); *NF1*: by 6 observers (1 case)] in the absence of *SDH-x* mutations in four of these cases (two examined only at the germline, one both at the germline and somatic, and one only at the somatic level) . Data on the exact mutations were available in four cases (*VHL* p.Ser80Asn, p.Arg161*, p.Arg167Gln; *NF1* p.Trp561*).

Likewise, 8 out of 128 (6.25 %) paraganglionic tumors were scored as SDHB immunonegative/ SDHA immunopositive in the absence of *SDH-x* mutations. Clinicopathological and genetic data of these eight cases from three independent centers are detailed in **Table 2**. Discordant evaluations of SDHB IHC were additionally observed in 18 tumors without identified *SDH-x* mutations, 11 *VHL*- and 2 *RET*-mutated tumors, whereas of SDHA IHC concerned one *NF1*-mutated tumor. The classification of stainings as 'non-informative' and 'heterogeneous' represented the major reasons for SDHA/SDHB IHC discrepancies in the *SDH-x* mutated subgroup, while the 'weak diffuse' category accounted largely for those in tumors without identified *SDH-x* mutations and *VHL*-mutated subsets.

The association between the predicted SDH genetic status with SDHB IHC was investigated for each observer. The sensitivity of this approach, defined as the percentage of *SDH-x* mutated tumors which are SDHB immunonegative, ranged from 83.58% to 98.57% (mean 94.23%). The specificity, defined as the percentage either of non-SDH-x mutated tumors or tumors without identified *SDH-x* mutations which 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=0.0002), while no association could be shown between the *SDHD* mutations and the weak diffuse pattern on SDHB IHC (P=0.1490).

Discussion

Immunohistochemistry has notably revolutionized the practice of endocrine pathology during the last decade [47]. In parallel with recent advances in molecular genetics, IHC has been shown to detect various types of molecular alterations i.e. *BRAF V600E* mutation in papillary thyroid carcinomas [47], *PTEN* mutations in various neoplastic thyroid lesions [48], beta-catenin (*CTNNB1*) mutations in cribriform-morular variant of papillary thyroid carcinoma, undifferentiated carcinomas of the thyroid gland and adrenocortical carcinomas [47, 49-50], *TP53* mutations as

well as mutations in *mismatch repair* (*MMR*) genes i.e. *MLH1*, *MSH2*, *MSH6* and *PMS2* in adrenocortical carcinomas [50-52], *HRPT2* mutations in parathyroid carcinomas and hyperparathyroidism-jaw tumor syndrome-related adenomas [47, 53], *PRKAR1A* mutations in Carney complex-associated tumors [54-56], *SDH*-, *FH*- as well as *MAX* deleterious- mutations in PCCs/PGLs [7, 9-10, 57-58].

Loss of SDHB protein expression is seen in PCCs/PGLs either harboring a mutation in any of the *SDH* genes or with somatic hypermethylation of the SDHC promoter region [41], whereas loss of both SDHB and SDHA immunoreactivity is demonstrated only in the context of an *SDHA* mutation [7-19]. In agreement with previous studies [7, 9-10, 16-19], *SDHB*-/*C*-/*D*- and *SDHA*- mutated tumors displayed the aforementioned 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 6 benign *VHL*-mutated PCCs and 1 malignant *NF1*-mutated ea-PGL were evaluated as SDHB immunonegative. The latter contrasts previous observations in 37 PCCs/PGLs and 14 PCCs endowed with *VHL* [7, 10] and *NF1* mutations [7, 9] respectively. Albeit, by using a mouse monoclonal (21A11) SDHB antibody at a low concentration (1 in 1000), Gill et al. [9] 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 [7]. Along the same lines, loss of SDHB protein expression has been recently displayed in a subset of *NF1*-mutated PCC/PGLs (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 4 of these 7 cases occurring in the *VHL*- and *NF1*- deficient setting.

To further extend upon earlier observations [7, 10], 8 of 128 (6.25%) tumors without identified SDH-x mutations were evaluated as SDHB immunonegative (Table 2). van Nederveen et al. [7] and Castelblanco et al. [10] reported on 9 cases (6 out of 53; 11% & 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 [7, 10] or screening for large-scale *SDH-x* deletions [10], which may account for higher percentages. Intriguingly, in the present study, seven SDHB immunonegative tumors in the absence of SDH-x mutations were non-metastatic bearing a close resemblance to the Carney-triad-associated counterparts in terms of SDHB IHC and biologic behavior [3, 28, 59]. Because somatic hypermethylation of *SDHC* was not investigated, the intriguing possibility that the aforementioned tumors represented cases of Carney-Triad could not be assessed. Nevertheless, as shown herein, SDHB IHC status overall is strongly correlated with the clinical behavior of PCC/PGL further strengthening the role of SDHB IHC as a prognostic marker [60].

Our data reinforce the notion that IHC is a valid tool to identify patients at risk for familial SDH-related PCC/PGL syndromes, whereas occasionally this might be difficult even in a specialized setting (Table 3). In support of the latter, two ea-PGLs endowed with missense and frameshift *SDHD* mutations were scored as SDHB immunopositive by five observers similar to what has been previously reported on an ea-PGL harboring a nonsense *SDHD* mutation (c.14G>A p.Trp5*) in the context of Carney Stratakis syndrome (CSS) [30]. Given that the latter developed an additional GIST exhibiting SDHB immunonegativity [30], while identical missense and nonsense *SDHD* mutations in other tumors destabilized the protein leading to absence of expression [4, 7], it is tempting to postulate that either the second hit in the *SDHD* gene resulted in an inactive SDH-complex with preservation of antigenicity or this could be alternatively ascribed to an erroneous interpretation. Paradoxically, one papillary RCC arising in a patient with a germline missense *SDHC* mutation (c.3G>A p.M1I) and harboring somatic loss of heterozygosity (LOH) of the *SDHC* locus displayed SDHB immunopositivity [35] further adding to those rare familial cases characterized by disparity between molecular genetic aberrations of a tumor suppressor gene and retention of protein expression [61]. Of note, every pathologist missed at least 1 *SDH-x*-related tumor, of which *SDHD* counterparts most frequently, necessitating SDHD IHC as a potential complementary tool to SDHB IHC to identify *SDHD*-mutated patients [62]. Taken together, SDHB IHC and *SDH-x* genetic analysis should be viewed as complementary tests. In the eventuality of

strong clinical suspicion, despite retention of SDHB expression, follow-up mutational analysis should be considered.

The relatively 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 [63]. Albeit, the classification of stainings as 'non-informative' and 'heterogeneous' represented the major reasons for SDHA/SDHB IHC discrepancies in the *SDH-x*-mutated subgroup, while the 'weak diffuse' category accounted largely for inconsistencies in the *SDH-x* wild-type and *VHL*-mutated subsets. These could be potentially ascribed to (i) technical variability owing to differences in fixation time, buffered formalin concentrations and/or age of the FFPE blocks [9-10], (ii) biological variability e.g. reduced SDHB protein levels in VHL-mutated PGLs [64] or even to (iii-iv) individual conceptions and experience from specific staining protocols, as has been shown with IHC for MMR proteins [65]. 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.

In contrast to previous studies [9-10] indicating a stronger correlation of weak diffuse pattern with *SDHD* mutations, we could not significantly reinforce this particular association. Moreover, SDHB and/or SDHA IHC may not always be an all-or-none phenomenon. In particular, two *SDHAF2*- and *SDHA*-mutated tumors displayed a heterogeneous expression pattern (Fig. 3 & 4) being consistent with previous observations concerning SDHB IHC in a pituitary adenoma harboring an *SDHD* germline mutation [36]. Along the same lines, heterogeneous expression patterns have been reported both with MMR protein IHC in Lynch syndrome and PTEN IHC in Cowden syndrome respectively [66-68]. The biologic nature of heterogeneous tumors in these genetic contexts is currently unknown [36, 66-68]. Because of potential misinterpretation of heterogeneous IHC for SDHB and/or SDHA protein loss, *SDH* genetic testing is recommended when confronted with such cases.

In addition to a comprehensive NGS-based strategy for the analysis of multiple PCC/PGL susceptibility genes [42-44], several algorithms have been proposed as a targeted approach to genetic testing in clinical practice [7, 69-71]. 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 IHC in determining the functionality of such variants, alone or in an integrated approach with *in-silico* analysis [72] and/or Western blot analysis, SDH enzymatic assay and mass spectrometric-based measurements of ratios of succinate:fumarate and other metabolites [73-75].

In the current study, we conclude that SDHB/SDHA IHC appears to be a reliable tool to identify patients with *SDHA*/*-B*/*-C*/*-D*/*-AF2* mutations with an additional utility to evaluate the pathogenicity of *SDH* variants of unknown significance in the new NGS era. A heterogeneous pattern of SDHB IHC has to be followed by *SDH-x* molecular genetic testing, while a SDHB immunonegative subset of *VHL*- and *NF1*-mutated paraganglionic tumors challenges the issue of specificity for SDHB IHC. Hence, if *SDH-x* genetics fails to detect a mutation, *SDHC* promoter methylation and/or *VHL*/*NF1* testing with the use of targeted NGS is advisable. Our findings highlight the need for quality assessment programs regarding not only standardized staining protocols, but also SDHB/SDHA IHC evaluation procedures. In a prospective setting, with standardized tissue fixation combined with a locally fine-tuned immunohistochemical staining protocol, the sensitivity and specificity of the SDHA/SDHB IHC can be improved.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

References

1. Tischler AS. Paraganglia. In *Histology for Pathologists*, (4th edn), Mills SE (ed). Lippincott Williams & Wilkins: Philadelphia, 2012; 1277–1299.
2. Dahia PL. Pheochromocytoma and paraganglioma pathogenesis: learning from genetic heterogeneity. *Nat Rev Cancer* 2014;**14:**108-119.
3. Papathomas TG, de Krijger RR, Tischler AS. Paragangliomas: update on differential diagnostic considerations, composite tumors, and recent genetic developments. *Semin Diagn Pathol* 2013;**30:**207-223.
4. Papathomas TG, Gaal J, Corssmit EP, *et al*. Non-pheochromocytoma (PCC)/paraganglioma (PGL) tumors in patients with succinate dehydrogenase-related PCC-PGL syndromes: a clinicopathological and molecular analysis. *Eur J Endocrinol* 2013;**170:**1-12.
5. Belinsky MG, Rink L, von Mehren M. Succinate dehydrogenase deficiency in pediatric and adult gastrointestinal stromal tumors. *Front Oncol* 2013;3:117.
6. Gill AJ, Hes O, Papathomas T, *et al*. Succinate dehydrogenase (SDH)-deficient renal carcinoma: A morphologically distinct entity: A clinicopathologic series of 36 tumors from 27 patients. *Am J Surg Pathol* 2014 in press
7. van Nederveen FH, Gaal J, Favier J, *et al*. An immunohistochemical procedure to detect patients with paraganglioma and phaeochromocytoma with germline SDHB, SDHC, or SDHD gene mutations: a retrospective and prospective analysis. *Lancet Oncol* 2009;**10:**764-771.
8. Burnichon N, Rohmer V, Amar L, *et al*. The succinate dehydrogenase genetic testing in a large prospective series of patients with paragangliomas. *J Clin Endocrinol Metab* 2009;**94:**2817-2827.
9. Gill AJ, Benn DE, Chou A, *et al*. Immunohistochemistry for SDHB triages genetic testing of SDHB, SDHC, and SDHD in paraganglioma-pheochromocytoma syndromes. *Hum Pathol* 2010;**41:**805-814.

10. Castelblanco E, Santacana M, Valls J, *et al*. Usefulness of negative and weak-diffuse pattern of SDHB immunostaining in assessment of SDH mutations in paragangliomas and pheochromocytomas. *Endocr Pathol* 2013;**24:**199-205.

11. Martins RG, Nunes JB, Máximo V, *et al*. A founder SDHB mutation in Portuguese paraganglioma patients. *Endocr Relat Cancer* 2013;**20:**L23-26.

12. Sjursen W, Halvorsen H, Hofsli E, *et al*. Mutation screening in a Norwegian cohort with pheochromocytoma. *Fam Cancer* 2013;**12:**529-535.

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13. Lefebvre S, Borson-Chazot F, Boutry-Kryza N, *et al*. Screening of mutations in genes that predispose to hereditary paragangliomas and pheochromocytomas. *Horm Metab Res* 2012;**44:**334-338.

14. Wang CP, Chen TC, Chang YL, *et al*. Common genetic mutations in the start codon of the SDH subunit D gene among Chinese families with familial head and neck paragangliomas. *Oral Oncol* 2012;**48:**125-129.

15. Millar AC, Mete O, Cusimano RJ, *et al*. Functional Cardiac Paraganglioma Associated with a Rare SDHC Mutation. *Endocr Pathol* 2014 in press

16. Korpershoek E, Favier J, Gaal J, *et al*. SDHA immunohistochemistry detects germline SDHA gene mutations in apparently sporadic paragangliomas and pheochromocytomas. *J Clin Endocrinol Metab* 2011;**96:**E1472-1476.

17. Burnichon N, Brière JJ, Libé R, *et al*. SDHA is a tumor suppressor gene causing paraganglioma. *Hum Mol Genet* 2010;**19:**3011-3020.

18. Welander J, Garvin S, Bohnmark R, *et al*. Germline SDHA mutation detected by next-generation sequencing in a young index patient with large paraganglioma. *J Clin Endocrinol Metab* 2013;**98:**E1379-1380.

19. Mason EF, Sadow PM, Wagner AJ, *et al*. Identification of succinate dehydrogenase-deficient bladder paragangliomas. *Am J Surg Pathol* 2013;**37:**1612-1618.

20. Wagner AJ, Remillard SP, Zhang YX, *et al*. Loss of expression of SDHA predicts SDHA mutations in gastrointestinal stromal tumors. *Mod Pathol* 2013;**26:**289-294.

21. Miettinen M, Killian JK, Wang ZF, *et al*. Immunohistochemical loss of succinate dehydrogenase subunit A (SDHA) in gastrointestinal stromal tumors (GISTs) signals SDHA germline mutation. *Am J Surg Pathol* 2013;**37:**234-240.

22. Dwight T, Benn DE, Clarkson A, *et al*. Loss of SDHA expression identifies SDHA mutations in succinate dehydrogenase-deficient gastrointestinal stromal tumors. *Am J Surg Pathol* 2013;**37:**226-233.

23. Oudijk L, Gaal J, Korpershoek E, *et al*. SDHA mutations in adult and pediatric wild-type gastrointestinal stromal tumors. *Mod Pathol* 2013;**26:**456-463.

24. Italiano A, Chen CL, Sung YS, *et al*. SDHA loss of function mutations in a subset of young adult wild-type gastrointestinal stromal tumors. *BMC Cancer* 2012;**12:**408.

25. Pantaleo MA, Astolfi A, Urbini M, *et al*. Analysis of all subunits, SDHA, SDHB, SDHC, SDHD, of the succinate dehydrogenase complex in KIT/PDGFRA wild-type GIST. *Eur J Hum Genet* 2014;**22:**32-39.

26. Belinsky MG, Rink L, Flieder DB, *et al*. Overexpression of insulin-like growth factor 1 receptor and frequent mutational inactivation of SDHA in wild-type SDHB-negative gastrointestinal stromal tumors. *Genes Chromosomes Cancer* 2013;**52:**214-224.

27. Janeway KA, Kim SY, Lodish M, *et al*. Defects in succinate dehydrogenase in gastrointestinal stromal tumors lacking KIT and PDGFRA mutations. *Proc Natl Acad Sci U S A* 2011;**108:**314-318.

28. Gaal J, Stratakis CA, Carney JA, *et al*. SDHB immunohistochemistry: a useful tool in the diagnosis of Carney-Stratakis and Carney triad gastrointestinal stromal tumors. *Mod Pathol* 2011;**24:**147-151.

29. Celestino R, Lima J, Faustino A, *et al*. A novel germline SDHB mutation in a gastrointestinal stromal tumor patient without bona fide features of the Carney-Stratakis dyad. *Fam Cancer* 2012;**11:**189-194.

30. Tenorio Jiménez C, Izatt L, Chang F, *et al*. Carney Stratakis syndrome in a patient with SDHD mutation. *Endocr Pathol* 2012;**23:**181-186.

31. Gill AJ, Lipton L, Taylor J, *et al*. Germline SDHC mutation presenting as recurrent SDH deficient GIST and renal carcinoma. *Pathology* 2013;**45:**689-691.

32. Gill AJ, Pachter NS, Chou A, *et al*. Renal tumors associated with germline SDHB mutation show distinctive morphology. *Am J Surg Pathol* 2011;**35:**1578-1585.

33. Gill AJ, Pachter NS, Clarkson A, *et al*. Renal tumors and hereditary pheochromocytoma-paraganglioma syndrome type 4. *N Engl J Med* 2011;**364:**885-886.

34. Paik JY, Toon CW, Benn DE, *et al*. Renal carcinoma associated with succinate dehydrogenase B mutation: a new and unique subtype of renal carcinoma. *J Clin Oncol* 2014;**32:**e10-13.

35. Malinoc A, Sullivan M, Wiech T, *et al*. Biallelic inactivation of the SDHC gene in renal carcinoma associated with paraganglioma syndrome type 3. *Endocr Relat Cancer* 2012;**19:**283-290.

36. Xekouki P, Pacak K, Almeida M, *et al*. Succinate dehydrogenase (SDH) D subunit (SDHD) inactivation in a growth-hormone-producing pituitary tumor: a new association for SDH? *J Clin Endocrinol Metab* 2012;**97:**E357-E366.

37. Dwight T, Mann K, Benn DE, *et al*. Familial SDHA mutation associated with pituitary adenoma and pheochromocytoma/paraganglioma. *J Clin Endocrinol Metab* 2013;**98:**E1103-1108.

38. Gill AJ, Toon CW, Clarkson A, *et al*. Succinate Dehydrogenase Deficiency Is Rare in Pituitary Adenomas. *Am J Surg Pathol* 2014;**38:**560-566.

39. Gill AJ, Chou A, Vilain R, *et al*. Immunohistochemistry for SDHB divides Gastrointestinal Stromal Tumors (GISTs) into two distinct types. Am J Surg Pathol 2010;**34:**636-644.

40. Williamson SR, Eble JN, Amin MB, *et al*. Succinate dehydrogenase-deficient renal cell carcinoma: detailed characterization of 11 tumors defining a unique subtype of renal cell carcinoma. *Mod Pathol* 2014;DOI: 10.1038/modpathol.2014.86.

41. Haller F, Moskalev EA, Faucz FR, *et al*. Aberrant DNA hypermethylation of SDHC: a novel mechanism of tumor development in Carney triad. *Endocr Relat Cancer* 2014;**21:**567-577.

42. Rattenberry E, Vialard L, Yeung A, *et al*. A comprehensive next generation sequencing-based genetic testing strategy to improve diagnosis of inherited pheochromocytoma and paraganglioma. *J Clin Endocrinol Metab* 2013;**98:**E1248-1256.

43. McInerney-Leo AM, Marshall MS, Gardiner B, *et al*. Whole exome sequencing is an efficient and sensitive method for detection of germline mutations in patients with phaeochromcytomas and paragangliomas. *Clin Endocrinol (Oxf)* 2014;**80:**25-33.

44. Crona J, Verdugo AD, Granberg D, *et al*. Next-generation sequencing in the clinical genetic screening of patients with pheochromocytoma and paraganglioma. *Endocr Connect* 2013;**2:**104-111.

45. Kimura N, Takayanagi R, Takizawa N, *et al*. Pathologic grading for predicting metastasis in phaeochromocytoma and paraganglioma. *Endocr Relat Cancer* 2014;**21:**405-414.

46. Landis JR, Koch GG. The measurement of observer agreement for categorical data. *Biometrics* 1977;33:159-174.

47. Chan JK, Ip YT, Cheuk W. The utility of immunohistochemistry for providing genetic information on tumors. *Int J Surg Pathol* 2013;**21:**455-475.

48. Barletta JA, Bellizzi AM, Hornick JL. Immunohistochemical staining of thyroidectomy specimens for PTEN can aid in the identification of patients with Cowden syndrome. *Am J Surg Pathol* 2011;**35:**1505-1511.

49. Gaujoux S, Grabar S, Fassnacht M, *et al*. β-catenin activation is associated with specific clinical and pathologic characteristics and a poor outcome in adrenocortical carcinoma. *Clin Cancer Res* 2011;**17:**328-336.

50. Ragazzon B, Libé R, Gaujoux S, *et al*. Transcriptome analysis reveals that p53 and {beta}-catenin alterations occur in a group of aggressive adrenocortical cancers. *Cancer Res* 2010;**70:**8276-8281.

51. Waldmann J, Patsalis N, Fendrich V, *et al*. Clinical impact of TP53 alterations in adrenocortical carcinomas. *Langenbecks Arch Surg* 2012;**397:**209-216.

52. Raymond VM, Everett JN, Furtado LV, *et al*. Adrenocortical carcinoma is a lynch syndrome-associated cancer. *J Clin Oncol* 2013;**31:**3012-3018.

53. Gill AJ. Understanding the Genetic Basis of Parathyroid Carcinoma. *Endocr Pathol* 2014;**25:**30-34.

54. Kim MJ, Choi J, Khang SK, *et al*. Primary intraosseous melanotic schwannoma of the fibula associated with the Carney complex. *Pathol Int* 2006;**56:**538-542.

55. Zembowicz A, Knoepp SM, Bei T, *et al*. Loss of expression of protein kinase a regulatory subunit 1alpha in pigmented epithelioid melanocytoma but not in melanoma or other melanocytic lesions. *Am J Surg Pathol* 2007;**31:**1764-1775.

56. Gaujoux S, Tissier F, Ragazzon B, *et al*. Pancreatic ductal and acinar cell neoplasms in Carney complex: a possible new association. *J Clin Endocrinol Metab* 2011;**96:**E1888-1895.

57. Castro-Vega LJ, Buffet A, De Cubas AA, *et al*. Germline mutations in FH confer predisposition to malignant pheochromocytomas and paragangliomas. *Hum Mol Genet* 2014;**23:**2440-2446.

58. Comino-Méndez I, Gracia-Aznárez FJ, Schiavi F, *et al*. Exome sequencing identifies MAX mutations as a cause of hereditary pheochromocytoma. *Nat Genet* 2011;**43:**663-667.

59. Carney JA. Carney triad. *Front Horm Res* 2013;**41:**92-110.

60. Blank A, Schmitt AM, Korpershoek E, *et al*. SDHB loss predicts malignancy in pheochromocytomas/sympathethic paragangliomas, but not through hypoxia signalling. *Endocr Relat Cancer* 2010;**17:**919-928.

61. Witkowski L, Carrot-Zhang J, Albrecht S, *et al*. Germline and somatic SMARCA4 mutations characterize small cell carcinoma of the ovary, hypercalcemic type. *Nat Genet* 2014;**46:**438-443.

62. Menara M, Badoual C, Bertherat J, *et al*. SDHD immunohistochemistry: A new tool to confirm SDHx mutations in pheochromocytoma/paraganglioma; 23-24 Nov 2012; Madrid, Spain. 11th Scientific Meeting of ENS@T: Madrid; 2012.

63. Mete O, Tischler AS, de Krijger R, et al. Protocol for the examination of specimens from patients with pheochromocytomas and extra-adrenal paragangliomas. *Arch Pathol Lab Med* 2014;**138**:182-188.

64. Dahia PL, Ross KN, Wright ME, *et al*. A HIF1alpha regulatory loop links hypoxia and mitochondrial signals in pheochromocytomas. *PLoS Genet* 2005;**1:**72-80.

65. Klarskov L, Ladelund S, Holck S, *et al*. Interobserver variability in the evaluation of mismatch repair protein immunostaining. *Hum Pathol* 2010;**41:**1387-1396.

66. Watson N, Grieu F, Morris M, *et al*. Heterogeneous staining for mismatch repair proteins during population-based prescreening for hereditary nonpolyposis colorectal cancer. *J Mol Diagn* 2007;**9:**472-478.

67. Barletta JA, Bellizzi AM, Hornick JL. Immunohistochemical staining of thyroidectomy specimens for PTEN can aid in the identification of patients with Cowden syndrome. *Am J Surg Pathol* 2011;**35:**1505-1511.

68. Garg K, Broaddus RR, Soslow RA, *et al*. Pathologic scoring of PTEN immunohistochemistry in endometrial carcinoma is highly reproducible. *Int J Gynecol Pathol* 2012;**31:**48-56.

69. King KS, Pacak K. Familial pheochromocytomas and paragangliomas. *Mol Cell Endocrinol* 2014;**386:**92-100.

70. Welander J, Söderkvist P, Gimm O. Genetics and clinical characteristics of hereditary pheochromocytomas and paragangliomas. *Endocr Relat Cancer* 2011;**18:**R253-276.

71. Jafri M, Maher ER. The genetics of phaeochromocytoma: using clinical features to guide genetic testing. *Eur J Endocrinol* 2012;**166:**151-158.

72. Kircher M, Witten DM, Jain P, *et al*. A general framework for estimating the relative pathogenicity of human genetic variants. *Nat Genet* 2014;**46:**310-315.

73. Canu L, Rapizzi E, Zampetti B, *et al*. Pitfalls in genetic analysis of pheochromocytomas/paragangliomas-case report. *J Clin Endocrinol Metab* 2014;**99:**2321-2326.

74. Lendvai N, Pawlosky R, Bullova P, *et al*. Succinate-to-fumarate ratio as a new metabolic marker to detect the presence of SDHB/D-related paraganglioma: initial experimental and ex vivo findings. *Endocrinology* 2014;**155:**27-32.

75. Richter S, Peitzsch M, Rapizzi E, *et al*. Krebs Cycle Metabolite Profiling for Identification and Stratification of Pheochromocytomas/Paragangliomas due to Succinate Dehydrogenase Deficiency. *J Clin Endocrinol Metab* 2014 *in press*

Figure Legends

Figure 1. SDHA and SDHB IHC in PCCs/PGLs 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, which serve as internal positive controls.

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

Figure 3. An ea PGL harbouring an *SDHA* (c.1534C>T, p.Arg512*) germline mutation, metastatic to a para-aortic lymph node, showing 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**) inbetween exhibiting cells with intact SDHA expression intermingled with cells with absent SDHA expression. Note the granular, cytoplasmic staining for SDHA/SDHB in normal cells of the intratumoral fibrovascular network, which serve as internal positive controls. Three pathologists correctly classified this sample as heterogeneous for SDHA, with the remainder four observers as positive for SDHA.

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

Table 1. Interobserver agreement (% upper half) and κ values (lower half) for SDHB and SDHA IHC

	Observer 1	**Observer 2**	**Observer 3**	**Observer 4**	**Observer 5**	**Observer 6**	**Observer 7**
SDHB IHC							
Observer 1	-	89.82%	94.28%	84.31%	91.34%	87.78%	95.54%
Observer 2	0.7623	-	93.88%	84.16%	90.91%	90.85%	85.54%
Observer 3	0.8561	0.8593	-	86.29%	92.68%	89.87%	92.38%
Observer 4	0.6282	0.6508	0.6819	-	82.10%	87.71%	81.17%
Observer 5	0.7943	0.7998	0.8286	0.5981	-	89.71%	87.65%
Observer 6	0.7199	0.8021	0.7721	0.7276	0.7759	-	85.99%
Observer 7	0.8733	0.6476	0.7923	0.5318	0.6880	0.6621	-
SDHA IHC							
Observer 1	-	99.43%	99.43%	98.86%	98.85%	99.71%	99.71%
Observer 2	0.7471	-	99.43%	98.86%	99.42%	100.00%	99.71%
Observer 3	0.7471	0.7471	-	98.86%	98.56%	99.71%	99.71%
Observer 4	0.4942	0.4942	0.4942	-	97.99%	99.14%	99.14%
Observer 5	0.5944	0.7972	0.5387	0.3542	-	99.13%	99.14%
Observer 6	0.8557	1.0000	0.8557	0.5672	0.6628	-	100.00%
Observer 7	0.8557	0.8557	0.8557	0.5672	0.6628	1.0000	-

All agreements $p<0.0001$

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

	Syndromic Presentation						Molecular genetic testing of *PCC/PGL susceptibility genes**									SDHB IHC	SDHA IHC
Sample code	Familial PCC/PGL history	Multiple tumors	Age at presentation	Sex	Tumor Type	Dignity	*SDHB*	*SDHD*	*SDHC*	*SDHA*	*SDHAF2*	*SDHAF1*	*VHL*	*TMEM127*	*MAX*	Neg	Pos
BEL 30	No	No	43	F	HN PGL	B	-	-	-	-	-	ND	-	-	-	5/7	7/7
BEL 67	No	No	36	M	HN PGL	B	-	-	-	-	-	ND	-	-	-	7/7	7/7
DR 11	No	No	27	F	HN PGL	B	-	-	-	ND	-	ND	ND	-	-	6/7	7/7
ITA 28	No	No	73	F	HN PGL	B	-	-	-	-	-	ND	-	-	-	6/7**	7/7
DR 10 ^	No	Yes	33	F	ea PGL	B	-	-	-	-	-	-	ND	ND	-	7/7	7/7
BEL 66	No	No	15	F	ea PGL	M	-	-	-	-	-	ND	-	-	-	7/7	7/7
BEL 116	No	No	20	M	PCC	B	-	-	-	-	-	ND	-	-	ND	7/7	7/7
ITA 48	No	No	47	F	PCC	B	-	-	-	-	-	ND	-	-	-	5/7	7/7

* *SDH-x* genes have been tested both for point mutations and large gross deletions at the germline level with DR10 and ITA48 also investigated at the somatic level

** one non-informative call

^ tested as well for FH at the germline and EPAS1 at the somatic level without any mutations subsequently detected

Abbreviations: ea, extra-adrenal; F, female; HN, head and neck; IHC, immunohistochemistry; M, male; ND, not done; Neg, negative; PCC, pheochromocytoma; PGL, paraganglioma; Pos, positive.

Table 3. Associating predicted SDHB IHC status either with *SDH-x* mutated *vs*. non-*SDH-x* mutated * status (**Table 3I**) or with *SDH-x* mutated *vs*. *SDH-x* wild-type status (**Table 3II**) **

TABLE 3I	Observer 1	Observer 2	Observer 3	Observer 4	Observer 5	Observer 6	Observer 7
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

TABLE 3II	Observer 1	Observer 2	Observer 3	Observer 4	Observer 5	Observer 6	Observer 7
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

* including *RET*-, *VHL*-, *NF1*-, *TMEM127*-, *MAX*-, *HIF2A*- and *HRAS*-mutated tumors

** Heterogeneous and noninformative calls are excluded

Sensitivity is defined as the percentage of ***SDH-x mutated tumors*** which are SDHB immunonegative
Specificity is defined as the percentage of ***non-SDH-x mutated tumors*** or ***tumors without identified SDH-x mutations*** which are SDHB immunopositive

Pval: p-value Chi-square 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