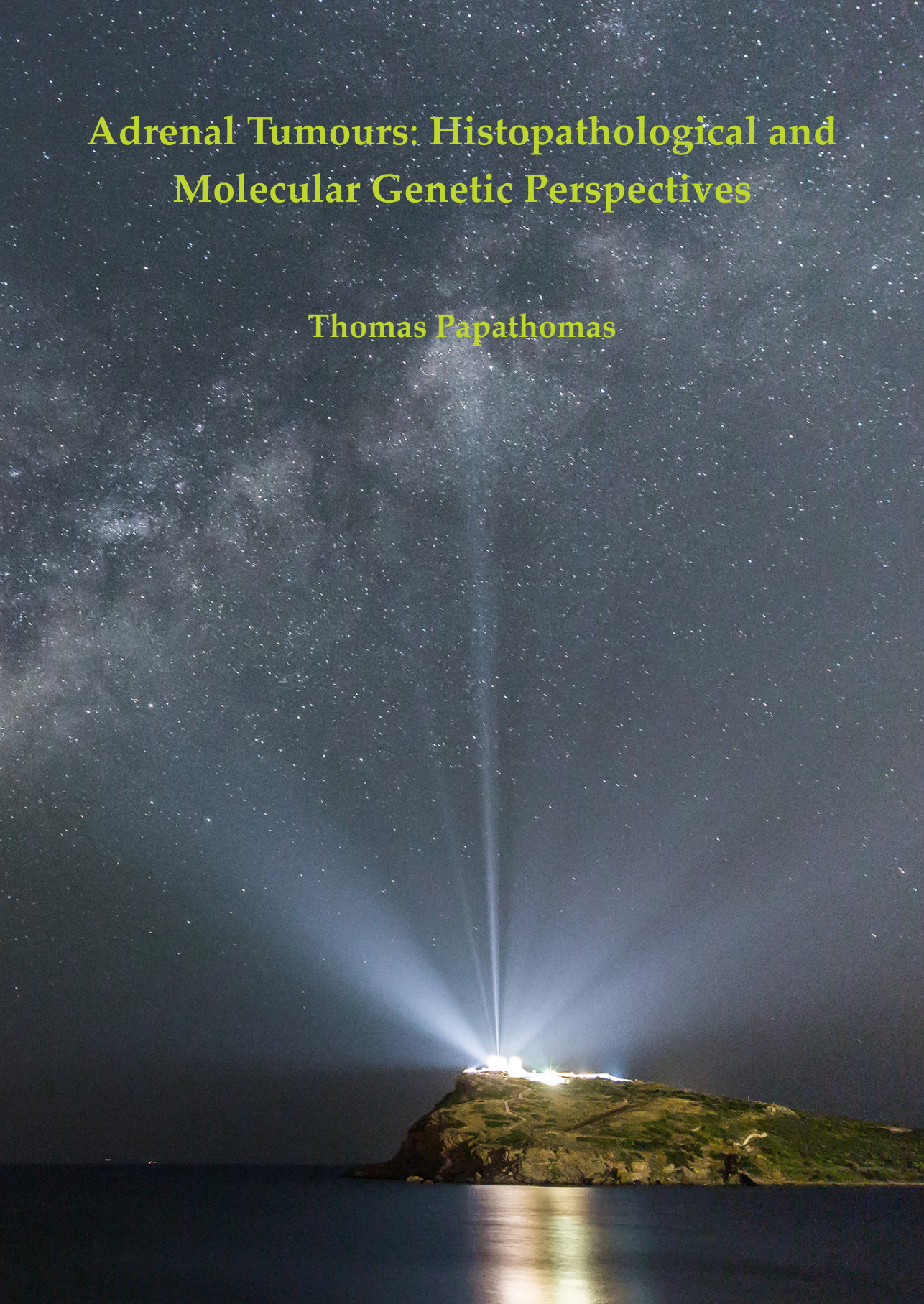
Adrenal Tumours: Histopathological and Molecular Genetic Perspectives

Thomas Papathomas



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Adrenal Tumours: Histopathological and Molecular Genetic Perspectives

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ΤΑ ΘΕΜΕΛΙΑ ΜΟΥ στα βουνά
και τα βουνά σηκώνουν οι λαοί στον ώμο τους
και πάνω τους η μνήμη καίει
άκαυτη βάτος.
Μνήμη του λαού μου σε λένε Πίνδο και σε λένε Άθω.
Ταράζεται ο καιρός
κι απ' τα πόδια τις μέρες κρεμάζει
αδειάζοντας με πάταγο τα οστά των ταπεινωμένων.
Ποιοι, πώς, πότε ανέβηκαν την άβυσσο;
Ποιες, ποιών, πόσων οι στρατιές;
Τ' ουρανού το πρόσωπο γυρίζει κι οι εχθροί μου έφυγαν μακρυά.
Μνήμη του λαού μου σε λένε Πίνδο και σε λένε Άθω.
Εσύ μόνη απ' τη φτέρνα τον άντρα γνωρίζεις
Εσύ μόνη απ' την κόψη της πέτρας μιλάς.
Εσύ την όψη των αγίων οξύνεις
κι εσύ στου νερού των αιώνων την άκρη σύρεις
πασχαλιάν αναστάσιμη!
Αγγίζεις το νου μου και πονεί το βρέφος της Άνοιξης!
Τιμωρείς το χέρι μου και στα σκότη λευκαίνεσαι!
Πάντα πάντα περνάς τη φωτιά για να φτάσεις τη λάμψη.
Πάντα πάντα τη λάμψη περνάς
για να φτάσεις τα ψηλά τα βουνά τα χιονόδοξα.
Όμως τι τα βουνά;
Ποιος και τι στα βουνά;
Τα θεμέλεια μου στα βουνά
και τα βουνά σηκώνουν οι λαοί στον ώμο τους
και πάνω τους η μνήμη καίει
άκαυτη βάτος!

Το Άξιον Εστί

Οδυσσέας Ελύτης
Odysseus Elytis

The Nobel Prize in Literature 1979

To my beloved and adored Marianna

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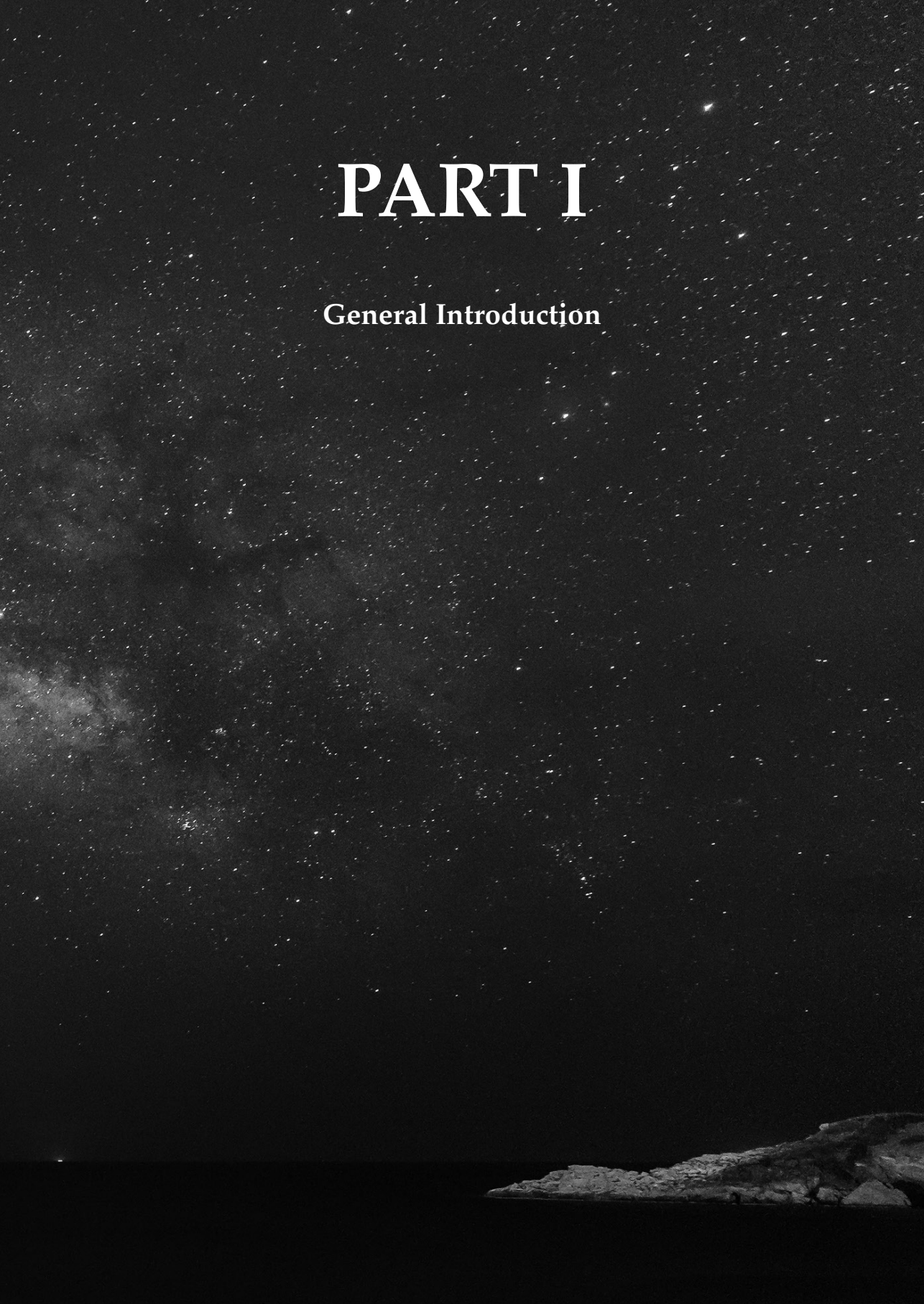

PART I

General Introduction

Chapter 1

Introduction and Scope of the thesis

1.1 THE ADRENAL GLAND AND EXTRA-ADRENAL PARAGANGLIA

The **adrenal gland** comprises two discrete endocrine compartments with distinct embryological origins. The outer, mesodermal-derived, **adrenal cortex** has a characteristic histological and functional zonation (zona glomerulosa, zona fasciculata and zona reticularis) and synthesizes three major classes of steroids i.e. mineralcorticoids, glucorticoids and sex steroids. The inner, neural crest-derived, **adrenal medulla** is populated with chromaffin cells, arranged in discrete nests or short anastomosing cords, and produces catecholamines, which are mediators of the 'fight-or-flight' response. The adrenal medulla is the best understood example of **sympathetic** paraganglia. The latter together with their **parasympathetic** counterparts are **anatomically dispersed neuroendocrine organs**, which are associated with the autonomic nervous system. Although their physiologic role is to release secretory products for endocrine, paracrine, neurotransmitter or neuromodulatory functions in response to neural or chemical stimuli, sympathetic and parasympathetic paraganglia differ not only in their anatomic distribution and the major types of stimuli to which they respond, but also in the tumour types originating from them. **Parasympathetic paraganglia** give rise to tumours almost always of neuroendocrine lineage i.e. extra-adrenal paragangliomas, while **sympathetic paraganglia** give rise to neuroblastomas (NBLs), ganglioneuroblastomas and ganglioneuromas in addition to extra-adrenal paragangliomas (PGLs) and intra-adrenal counterparts i.e. pheochromocytomas (PCCs) [**1-2**].

1.2 INHERITED TUMOUR SYNDROMES INVOLVING THE ADRENAL GLAND AND EXTRA-ADRENAL PARAGANGLIA

According to the **2004 World Health Organization** (**WHO**) classification of tumours of endocrine organs, genetic cancer susceptibility is more frequent in endocrine tumours than in any other class of human neoplasms [**3-5**]. In fact, PCCs and extra-adrenal PGLs carry the highest degree of hereditary susceptibility among all human tumours [**6**]. Recent advances in molecular genetics have expanded the spectrum of disorders associated with tumours originating from the adrenal gland and extra-adrenal paraganglia, including adrenal cortical adenomas (ACAs) and carcinomas (ACCs), primary macronodular adrenal hyperplasia (PMAH), primary pigmented nodular adrenocortical disease (PPNAD), PCCs/PGLs as well as peripheral neuroblastic tumours [**6-23**].

The spectrum of PCC/PGL susceptibility genes is continuously expanding; thus far, several tumour-suppressor genes and oncogenes have been reported, including ***RET***, ***VHL***, ***NF1***, ***SDHA***, ***SDHB***, ***SDHC***, ***SDHD***, ***SDHAF2***, ***FH***, ***MDH2***, ***IDH***, ***TMEM127***, ***MAX***, ***KIF1Bbeta***, ***PHD1/EGLN2***, ***PHD2/EGLN1***, ***EPAS1/HIF2A***, ***HRAS***, ***BRAF, CDKN2A, CDKN2C, GNAS, TP53***, ***BAP1***, ***MEN1, MSH2, ATRX, MET, KMT2D, STAG2, PALB2, STAT3, CREBBP, EP300, SMARCA4, SMARCE1*** and ***ARID1B*** [**6-13, 20-27**]. Germline and/or somatic mutations of the aforementioned genes have been documented to be associated with tumour development [**6-7**, **19**, **28-29**]. Approximately 40% of PCC/PGL patients carry a causal germline mutation, while at least 30% of the remaining 'sporadic' cases harbor a somatic mutation in a predisposing gene [**19**]. In fact, Flynn *et al*. [**27**] showed that 77.5% of cases could be explained by germline or somatic mutations or structural alterations affecting known PCC/PGL genes. Mutations in ***HRAS***, ***ATRX***, ***BRAF, MET***, ***TP53***, ***CDKN2A***, ***CDKN2C, GNAS, IDH***, ***STAG2, PALB2, STAT3, CREBBP, EP300, SMARCA4, SMARCE1*** and ***ARID1B*** have been only reported at the somatic level [**13**, **20-21**, **24-27**, **30-33**] with ***HRAS*** mutations displaying a prevalence of approximately 9% in sporadic paraganglionic tumours and being mutually exclusive with inherited PCC/PGL genes [**13**, **21**, **33**]. In contrast, other somatic variants co-occur with germline mutations e.g. ***ATRX*** with ***SDH-x/VHL/RET/NF1/FH*** mutations [**20-21**, **27**], and ***CDKN2C*** with ***RET*** mutations [**24**]. Given the rarity of reported PCC/PGL cases containing germline ***PHD1/EGLN2***, ***PHD2/EGLN1***, ***MDH2***, ***MSH2***, ***BAP1*** and ***MEN1*** mutations, further studies are warranted to elucidate their exact role in the PCC/PGL pathogenesis [**8-12**, **23, 34-35**].

Despite the fact that hereditary transmission of adrenocortical tumours is less common, the spectrum of genetic alterations associated with adrenocortical *hyperplastic/neoplastic phenotypes and/or syndromes* is continuously expanding. A plethora of genes has been recently revealed at the germline and somatic level: ***TP53*** (Li-Fraumeni syndrome), ***IGF2***/***CDKN1C***/***H19*** (Beckwith-Wiedemann syndrome), **mismatch repair** (**MMR***)* genes: ***MSH2***, ***MSH6***, ***MLH1***, ***PMS2*** (Lynch syndrome), ***APC*** [familial adenomatous polyposis (FAP) syndrome], ***NF1*** [Neurofibromatosis type 1 (NF1)], ***MEN1*** (multiple endocrine neoplasia type 1), ***PRKAR1A*** and ***PRKACB*** (Carney Complex), ***AIP*** (familial isolated pituitary adenoma), ***ARMC5*** (ARMC5 Tumour Syndrome), ***GNAS1*** (McCune-Albright syndrome), ***CYP21*** (congenital adrenal hyperplasia), ***ARMC5***, ***DOT1L, PDE11A***/***PDE8B***, ***EDNRA, MEN1, FH*** and ***GNAS1*** (PMAH), ***PDE11A***/***PDE8B*** (isolated micronodular adrenocortical disease, PPNAD and PMAH), ***PRKACA*** (cortisol-producing ACAs, bilateral adrenocortical hyperplasias and PMAH), ***KCNJ5, ATP1A1, ATP2B3*** and ***CACNA1D*** (aldosterone-producing ACAs), ***CTNNB1*** (non-functioning ACAs), ***CLASP2*** (adrenocortical oncocytomas), ***NR5A1*** and ***FGFR4*** (childhood adrenocortical tumours)

as well as ***CTNNB1***, ***TP53***, ***CDKN2A***, ***RB1***, ***MEN1***, ***ZNRF3***, ***DAXX***, ***TERT***, ***MED12***, ***GNAS***, ***NF2*** and ***PRKAR1A*** (ACCs) [**4**, **14-17, 36-72**].

Germline mutations and structural alterations in ***ALK*** and ***PHOX2B*** genes contribute to inheritable predisposition to NBL [**18**, **73-75**], while as yet unidentified gene(s) predisposing to both Wilms tumour and NBL in a new familial cancer syndromic setting remain to be determined [**76**]. Simultaneous occurrence of peripheral neuroblastic tumours and ACCs in pediatric patients harboring germline ***TP53*** mutations [**77-79**], occurrence of NBL among ***TP53*** p.R337H mutation carriers [**80**] as well as novel evidence adding NBL to the complex repertoire of human cancers influenced by the rs78378222 hypomorphic allele impairing proper termination and polyadenylation of ***TP53*** transcripts [**81**], it appears that ***TP53*** mutations and/or variants might confer susceptibility to this particular pediatric oncogenesis similar to common variations in ***LMO1***, ***BARD1***, ***HACE1*** and ***LIN28B*** [**82-85**]. Inherited loss-of-function ***KIF1Bbeta*** missense mutations were detected both in NBLs and PCCs [**86-87**], while recent experimental evidence suggests that ***KIF1Bbeta*** has NBL tumour-suppressor properties [**88**]. Neuroblastomas arise also in the ***SDHB***- [**89-92**] and ***NF1***-deficient setting [**93**] further expanding the common genetic spectrum in PCC/PGL and NBL tumourigenesis. Along the same lines, PCC/PGL and NBL share both (**1**) a very low mutation load, (**2**) epigenetically inactivated candidate tumour suppressor genes i.e. ***RASSF1A***, ***FLIP***, ***CASP8***, and ***HIC1***, (**3**) an altered expression of death receptors, indicating apoptosis as a deregulated pathway [**13**, **19**].

1.3 NOVEL INSIGHTS INTO THE GENETICS OF PHEOCHROMOCYTOMAS/ PARAGANGLIOMAS AND ADRENAL CORTICAL CARCINOMAS

Advances in pan-genomic analyses have recently improved our understanding of the molecular mechanisms that distinguish different tumour subtypes and identified altered signaling pathways driving tumour initiation and progression [**7**, **19**, **21**, **70**]. Accordingly, these 'OMICs' approaches have refined the molecular classification of PCCs/PGLs and ACCs (**Figures 1** & **2**) and identified deregulated pathways, which could be promising candidates for precision medicine.

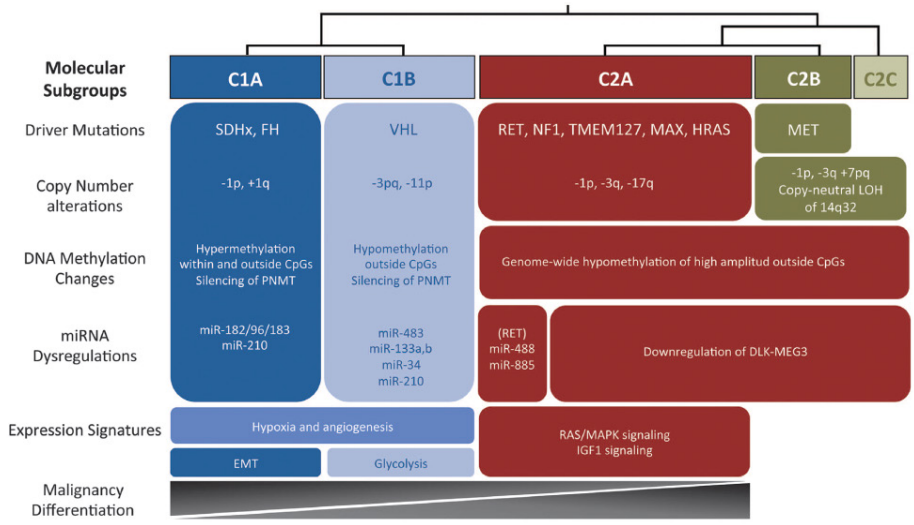

Figure 1. Molecular signatures of PCC/PGL subtypes. A consensus clustering analysis of multi-omics data classifies PCCs/PGLs into five molecular subgroups, each one showing specific genomic alterations and associated clinical characteristics. Adapted by permission from Macmillan Publishers Ltd: Oncogene, **[19]**, 2016.

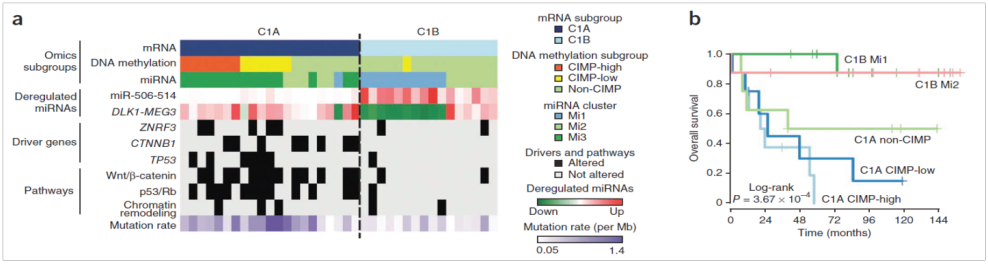

Figure 2. Molecular signatures of ACC subtypes (adapted from **Assié** *et al*. [**70**]): (**a**) consensus clustering analysis of multi-omics data classifies ACCs into two main molecular subgroups (C1A and C1B), each one displaying specific genomic alterations. C1A ACCs can be classified into CIMP-high, CIMP-low and non-CIMP according to methylation profile, while C1B ACCs into Mi1 and Mi2 clusters according to miRNA expression profile; and (**b**) overall survival in ACC molecular subgroups as defined by omics integration.

1.4 PROGNOSTIC PARAMETERS IN ADRENAL CORTICAL CARCINOMAS

The overall prognosis of ACC is poor with a 5-year overall survival varying between 37% and 47% [**94**]. In an effort to refine the molecular classification of ACCs, Assié *et al*. [**70**] identified substantial overlap between the different omics classifications along with two distinct molecular groups with opposite outcome: the C1A subgroup with poor outcome displaying numerous mutations and DNA methylation alterations and the C1B subgroup with good prognosis displaying specific deregulation of two microRNA clusters (**Figure 2**). This particular study [**70**] confirmed previous genome-wide investigations of gene expression in adrenocortical cancer [**95-96**] and further added to the concept of two primary ACC groups with distinctly different outcomes. Apart from assessing the biological aggression in ACCs by molecular methods, routine histology (Weiss score, mitotic grade) as well as IHC (Ki67 Labelling Index, other proliferation-based scoring methods and SF-1) can provide valuable aid in determining prognosis [**97**]. To extend, novel emerging biomarkers, i.e. TOP2A, EZH2 and BARD1, might provide additional prognostic information [**98**].

1.5 AIMS AND OUTLINE OF THE THESIS

Revolutionary breakthroughs, stemming from genetic and genomic studies, have resulted not only in understanding the pathobiology of tumours, but also increased the roles of pathologists in helping to guide patient care. The evolving role of histopathology in tumours originating from the adrenal gland and extra-adrenal paraganglia encompasses (**1**) differential diagnosis, (**2**) prognosis, (**3**) identification of clues to occult hereditary disease and (**4**) triaging of patients for optimal genetic testing by immunohistochemistry (IHC). **The aim of the current thesis was to contribute into this fascinating field of histopathology and hence elucidate various aspects in terms of diagnostics, prognostics and molecular genetics of tumours originating from the adrenal gland and extra-adrenal paraganglia**.

From a histopathological perspective, it is important that endocrine and surgical pathologists are aware of the broad differential diagnostic spectrum, the hereditary susceptibility disorders that are known to be associated with the development of PCCs/PGLs, emphasizing genotype–phenotype correlations in familial PGL syndromes and the role of IHC as a supplementary approach in molecular genetic testing for PCCs/PGLs. Therefore, a comprehensive review is given in **Chapter 2**.

In **Chapter 3**, we investigated interobserver variability among seven expert endocrine pathologists using a web-based virtual microscopy approach in a large multicenter PCC/PGL cohort and we examined the validity of SDHB/SDHA IHC to identify patients with SDH-related PCCs/PGLs and of SDHB IHC as a marker of malignancy. In an effort to elucidate molecular genetic and histopathological aspects of SDH-related neoplasia (**Chapter 4**), we (**1**) reported all currently known *SDH* mutations defining their nature and spectrum; (**2**) analyzed potential genotype–phenotype correlations; (**3**) performed bioinformatics analysis on the functional impact of the reported *SDH* mutations and compared the results with available SDHA and SDHB IHC data; and (**4**) explored the nature of the second hit in all tumours arising in the SDH-deficient setting.

Questions remain unresolved with regard to non-paraganglionic tumours arising in *SDH* mutation carriers concerning their pathogenesis, clinicopathological phenotype, and even causal relatedness to *SDH* mutations. These issues are addressed in **Chapters 5** & **6** by investigating non-paraganglionic tumours affecting patients in the Leiden SDH Mutation Carrier Registry and other European centres.

On the basis of a broad international collaboration to investigate SDH-deficient renal carcinoma, accepted as a provisional entity in the **2013 International Society of Urological Pathology Vancouver Classification**, we aimed to (**1**) identify new cases of SDH-deficient renal carcinoma to further expand knowledge and experience with these carcinomas; (**2**) enable a centralized pathologic review of previously published cases of SDH-deficient renal carcinoma; (**3**) establish the natural history, clinical features, and prognosis of SDH-deficient renal carcinoma; (**4**) establish the risk of germline *SDH* mutation associated with SDH-deficient renal carcinoma; and (**5**) estimate the incidence of SDH-deficient renal carcinoma. **Chapter 7** describes the results of the aforementioned aims.

In **Chapter 8**, we present emerging evidence concerning the adrenocortical tumourigenesis in an effort to elucidate the incompletely understood pathophysiology of this aggressive neoplasia and describe rare subtypes of adrenocortical malignancy elucidating aspects of diagnostic categorization, differential diagnostics and biological behavior. Mutations in the noncoding functional regions of the genome seem to be relevant for the development and/or progression of diverse human neoplasms. In particular, mutations in the promoter of the telomerase reverse transcriptase (*TERT)* gene have recently been shown as novel genetic mechanism underlying telomerase activation in various tumours. In **Chapter 9**, we explored the occurrence and prevalence of hotspot *TERT* promoter mutations in ACCs, PCCs/PGLs and peripheral neuroblastic tumours as well as in human ACC and NBL cell lines.

Ki67 Labelling Index has been proposed in diagnostics, prognostics as well as in guiding treatment decisions in ACC patients. In this context, we developed an open source Galaxy virtual machine application designed for Ki67 hotspot detection in adrenocortical cancer. This is detailed in **Chapter 10**. In **Chapter 11**, we validated the aforementioned application and investigated interobserver variability for Ki67 Labelling Index assessment among endocrine pathologists using a web-based virtual microscopy approach in a multicenter ACC cohort.

In **Chapter 12**, the main findings of the thesis are discussed along with current perspectives about the evolving role of endocrine pathology.

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PART II

Pheochromocytomas & Paragangliomas: Pathology Concepts in Diagnosis and Molecular Genetic Testing

Chapter 2

Paragangliomas: Update on differential diagnostic considerations, composite tumors, and recent genetic developments

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ABSTRACT

Recent developments in molecular genetics have expanded the spectrum of disorders associated with pheochromocytomas (PCCs) and extra-adrenal paragangliomas (PGLs) and have increased the roles of pathologists in helping to guide patient care. At least 30% of these tumors are now known to be hereditary, and germline mutations of at least 10 genes are known to cause the tumors to develop. Genotype–phenotype correlations have been identified, including differences in tumor distribution, catecholamine production, and risk of metastasis, and types of tumors not previously associated with PCC/PGL are now considered in the spectrum of hereditary disease. Important new findings are that mutations of succinate dehydrogenase genes *SDHA*, *SDHB*, *SDHC*, *SDHD*, and *SDHAF2* (collectively "*SDHx*") are responsible for a large percentage of hereditary PCC/PGL and that SDHB mutations are strongly correlated with extra-adrenal tumor location, metastasis, and poor prognosis. Further, gastrointestinal stromal tumors and renal tumors are now associated with *SDHx* mutations. A PCC or PGL caused by any of the hereditary susceptibility genes can present as a solitary, apparently sporadic, tumor, and substantial numbers of patients presenting with apparently sporadic tumors harbor occult germline mutations of susceptibility genes. Current roles of pathologists are differential diagnosis of primary tumors and metastases, identification of clues to occult hereditary disease, and triaging of patients for optimal genetic testing by immunohistochemical staining of tumor tissue for the loss of SDHB and SDHA protein. Diagnostic pitfalls are posed by morphological variants of PCC/PGL, unusual anatomic sites of occurrence, and coexisting neuroendocrine tumors of other types in some hereditary syndromes. These pitfalls can be avoided by judicious use of appropriate immunohistochemical stains. Aside from loss of staining for SDHB, criteria for predicting risk of metastasis are still controversial, and "malignancy" is diagnosed only after metastases have occurred. All PCCs/PGLs are considered to pose some risk of metastasis, and long-term follow-up is advised.

INTRODUCTION

Paragangliomas (PGLs) are tumors arising from paraganglia that are normally distributed along supradiaphragmatic (parasympathetic) nerves in the head, neck, and mediastinum; the pre- and paravertebral sympathetic chains; or sympathetic nerve fibers innervating the pelvic and retroperitoneal organs [1]. They are also occasionally reported outside the usual distribution of sympathetic and parasympathetic paraganglia [2]. A pheochromocytoma (PCC) is an intra-adrenal sympathetic PGL with several distinctive characteristics [3]. All PGLs are derived from chromaffin cells or closely related cells of neural crest origin.

This review presents recent developments in PGLs with regard to genetics, diagnostics, and determination of malignancy. In particular, we focus on morphological variants of extra-adrenal PGLs, unusual anatomic sites of occurrence, composite PGLs/PCCs, and potential immunohistochemical pitfalls with the hope that both endocrine and surgical histopathologists can be aware of the broad differential diagnostic spectrum, which may be site-dependent. In addition, we discuss the hereditary susceptibility disorders that are currently known to be associated with the development of PGLs/PCCs, emphasizing genotype–phenotype correlations in familial PGL syndromes and the role of immunohistochemistry (IHC) as a supplementary approach in molecular genetic testing for PGLs and PCCs.

RECENT ADVANCES IN THE GENETICS OF PARAGANGLIOMAS

Recent advances in genetics, gene expression profiling, and cell biology have led to enormous progress in our understanding of PCC and PGL pathobiology. Given the fact that germline mutations of at least 10 different genes may account for approximately 30–35% of PCC/PGL cases, these tumors could be regarded as a genetic disease [2] Further, although somatic mutations of the same susceptibility genes have until recently been considered uncommon causes of sporadic PCC/ PGL, Burnichon *et al*. [4] have now provided evidence suggesting that NF1 loss of function is a frequent event in the genesis of sporadic tumors. The latter has been reinforced by Welander *et al*. [5] who found that the NF1 gene is themost frequent target of somatic mutations in sporadic PCCs. This may be of particular interest given the fact that NF1-associated PCCs share several distinctive features with sporadic PCCs [6].

The following list encompasses all currently known PCC/PGL susceptibility genes: *RET*, *VHL*, *NF1*, *SDHA*, *SDHB*, *SDHC*, *SDHD*, *SDHAF2*, *FP/TMEM127*, *KIF1Bbeta*, *PHD2/EGLN1*, *MAX*, and *EPAS1/ HIF2A* [7–9]. Germline loss-of-function mutations of *SDHA*, *SDHB*, *SDHC*, *SDHD*, *SDHAF2*, and *PHD2/EGLN1* genes along with mutations affecting the *RET* proto-oncogene and *VHL* and *NF1* tumor suppressor genes have been documented to predispose to the development of both PCCs and extra-adrenal PGLs, with tumor location highly dependent on the specific predisposing gene [2, 10–13]. It has been shown that germline mutations of *TMEM127* and *MAX* confer risks of extra-adrenal PGLs in addition to PCC [14, 15]. In very recent reports, the potential predisposition spectrum of inactivating germline *BRCA1-associated protein-1* (*BAP1*) mutations has been extended to PGL [16], and novel somatic gain-of-function *HIF2A* mutations have been identified in PCC/PGL patients with or without polycythemia [9, 17–22].

Table 1. Paragangliomas as part of cancer susceptibility syndromes.[a]

Syndrome	Gene (chromosome)	Tumor distribution		
		PCC	ea (s) PGLs	ea (p) PGLs
MEN2	RET (10q11.2)	++	−/+	−/+
VHL	VHL (3p25–26)	++	+[b]	+/−
NF1	NF1 (17q11)	+	−/+	−/+
PGL1	SDHD (11q23)	**+/-**	−/+	**++**
PGL2	SDHAF2 (11q12.2)	**-**	**-**	**++**
PGL3	SDHC (1q23.3)	**+/-**	**+/-**	**++**
PGL4	SDHB (1p36.13)[c]	**+**	**++**	**+**
PGL5	SDHA (5p15)	**+**	**++**	**+**
	TMEM127 (2q11)	**++**	**+/-**	**+/-**
	MAX (14q23.3)	**++**	+[d]	−/+
	HIF2A (2p21)[e]	+	++	?
Carney triad	Unknown	+/−	+/++[f]	+/++
Carney–Stratakis dyad or syndrome	SDHB, SDHC, SDHD[g]	+/-	++[h]	**++**

Abbreviations: ea, extra-adrenal; HIF2A, hypoxia-inducible factor 2a; NF1, neurofibromatosis type 1; MAX, MYC associated factor X gene; MEN2, multiple endocrine neoplasia type 2; RET, rearranged in transfection; p, parasympathetic; PCC, pheochromocytoma; PGL, paraganglioma; PGL (1–5), familial paraganglioma syndromes; SDH, succinate dehydrogenase; s, sympathetic; TMEM127, transmembrane protein 127; VHL, von Hippel–Lindau; ++, usual distribution of tumors; +, less common; +/−, rare occurrence; −/+, exceptionally rare; −, absent; ?, currently unknown

[a] Table shows estimated distributions based on information in several references [2,8–11,14,15,17–30]

[b] Abdominal 4 thoracic

[c] In addition to *SDHB* point mutations, large *SDHB* deletion-associated cases presented with PCCs, extra-adrenal PGLs as well as with head and neck PGLs [27]

[d] Approximately 16% of the patients develop extra-adrenal thoracic–abdominal PGLs; the latter appear less common in *TMEM127* and *VHL* mutation carriers [15]

[e] A somatic gain-of-function *EPAS1/HIF2A* mutation has been revealed in almost all reported cases (one germline *EPAS1/HIF2A* mutation) [21], while polycythemia has been observed in 9 of 14 cases [9,17–22]

[f] Approximately half of patients suffered from extra-adrenal PGLs, while six of 79 had PCC; the tumors were about equally distributed in abdomen, thorax, and head and neck region [28]

[g] No mutations or large deletions were identified in the coding regions of the *SDHA*, *SDHB*, *SDHC*, and *SDHD* genes in kindreds of two families [29]

[h] Abdominal (s) PGL > head and neck (p) PGL

Hereditary susceptibility disorders that are well known to be associated with the development of PGLs are multiple endocrine neoplasia type 2 (MEN2), von Hippel–Lindau (VHL) disease, von Recklinghausen neurofibromatosis type 1 (NF1), familial PGL syndromes (PGL 1–4), PGL type 5, Carney–Stratakis dyad, and a newly proposed syndrome of PGL and somatostatinoma associated with polycythemia (**Table 1**) [7, 12, 18, 31]. Carney triad, a syndrome of tumors affecting at least five organs [stomach: gastrointestinal stromal tumor (GIST); lung: chondroma; paraganglia: paraganglioma; adrenal gland: adrenocortical adenoma and PCC; and esophagus: leiomyoma] is a disorder that still has no established etiology [28, 32]. Carney triad is not caused by inactivating mutations of *SDHx* or by activating mutations of *KIT* or *PDGFRA*. Nevertheless, it is generally accepted to be a genetic disorder, possibly caused by somatic mosaicism [28, 33]. It is of interest that GIST, specifically epithelioid gastric GIST in most cases, is a component of both the dyad and the triad. GIST is usually the presenting tumor in Carney triad, whereas PGL is the presenting tumor in Carney–Stratakis dyad (PGL and GIST being the legitimate constituents of this autosomal dominant syndrome [28]. Further, GISTs display SDHB immunonegativity in both Carney– Stratakis dyad and Carney triad despite the absence of *SDHx* mutations in the latter [32]. It has therefore been recommended that Carney–Stratakis syndrome or Carney triad be considered in patients who have GISTS that are immunonegative for SDHB, and that testing for germline *SDHx* mutations be considered in all patients, especially younger individuals, with GISTs that are wild type for *PDGFR* and *KIT* [32, 34, 35]. Germline SDHB mutation is also associated with a type of renal cell carcinoma that can be recognized by SDHB immunonegativity, thus overlapping the VHL disease spectrum [36].

Well-documented genotypic–phenotypic correlations exist for tumors in each of the familial PGL syndromes with respect to distribution (**Table 1**), function, and malignancy. By analyzing a large cohort of previously published carriers of *SDHB*, *SDHC*, and *SDHD* deleterious mutations, Pasini and Stratakis [30] demonstrated the following: (**1**) Approximately one-third of affected *SDHB* mutation carriers have a positive family history, which is low in comparison to *SDHD* (61%) and *SDHC* (62.5%); (**2**) Median age at diagnosis of the first tumor is higher in *SDHC* mutation carriers (38 years) than in patients with *SDHB* (32 years) and *SDHD* (33 years) mutations; (**3**) Multiple tumors represent a frequent manifestation in *SDHD* mutation carriers (79%) in contrast to patients with *SDHB* and *SDHC* mutations presenting with single tumors in 67% and 73% of cases, respectively; (**4**) *SDHD*-affected carriers present more frequently with head and neck PGL only (78%), single or multiple, while PCC (8%) and/or extra-adrenal PGL (1%) are rarely the sole manifestations; (**5**) *SDHC*-affected carriers usually present with head and neck PGLs alone (87%), while PCC (3.3%) and/or abdominal PGL (10%) represent

rare occurrences; (**6**) *SDHB*-affected carriers commonly present with extraadrenal PGLs (53%), mainly abdominal but also thoracic, mediastinal and cervical, while less common manifestations include head and neck PGL only (~20%) as well as PCC alone or associated with PGL (~20%); and (**7**) The prevalence of malignant tumors amongst affected carriers is definitely higher in *SDHB* mutation carriers (41%) in comparison to *SDHD* (5%) and *SDHC* (3%), respectively. Notably, there is an increased risk of malignancy related to extra-adrenal PGL for both patients with *SDHB* mutation (48%) and *SDHD* mutation (17%).

In this context, Heesterman *et al*. [37] showed that asymptomatic carriers of an *SDHD* mutation are at high risk for occult parasympathetic PGL, while the risk is considerably lower for *SDHB* mutation carriers, which is consistent with a lower penetrance of *SDHB* mutations. In order to accurately assess age-related tumor risks and appropriately guide genetic counseling and screening, it has been stressed that estimates of penetrance should optimally be derived from family-based studies that include clinically unaffected mutation carriers so as to avoid upward bias [38, 39].

A very important consideration is that there is a high prevalence of *SDHx* mutations in sporadic extra-adrenal tumors, malignant tumors, and pediatric cases [30]. *SDHD* and *SDHAF2* mutations are associated with a striking parent-oforigin expression phenotype, with tumor development usually seen only on inheritance via the paternal line [7, 27, 38, 39]. However, it has been very recently ascertained that tumor formation can also occur after maternal transmission of *SDHD* mutation [40]. A paternal mode of inheritance has also been reported for MAX-associated tumors [15].

SDHAF2 mutation carriers have presented so far with head and neck PGLs exclusively (no currently known occurrence of extra-adrenal PGL or PCC); a young age at onset; high levels of multifocality; high penetrant phenotype; and benign biologic behavior [27]. In contrast, it appears that *SDHA* mutations are not associated with a particular location nor with a familial presentation, while a low penetrance and a higher median age at onset (median 41 years; mean 40.1 years) seem to characterize these SDHA-related cases [10, 11]. *MAX* mutations are associated with young age at presentation (mean age 34 years), a significant proportion of PCC bilaterality, relatively common occurrence of additional extra-adrenal thoracic-abdominal PGLs following PCC diagnosis (at a median age of 48 y) (**Table 1**), and are likely not associated with a high risk of malignancy [15].

The high prevalence of unsuspected hereditary disease and the genotypic–phenotypic correlations have led to gene-specific recommendations for genetic testing [3, 41, 42].

In addition, the efficiency and accuracy of genetic analysis have been improved as a result of genetic analysis algorithms and new techniques including deletion analysis by multiplex PCR methods such as multiplex ligation-dependent probe amplification (MLPA). Pathologists now play an important role by triaging patients for the most appropriate genetic testing by means of SDHB IHC [41, 43], which can reliably distinguish SDH related cases from other subsets. An algorithm has been developed for molecular genetic testing for PCCs and PGLs based on SDHB IHC results [42]. This supplementary approach awaits further refinement in view of all recent genetic and immunohistochemical developments regarding *SDHA* and *MAX* [8, 11].

DIAGNOSIS

Although PGLs usually exhibit the classic "zellballen" pattern, syncytial-like growth pattern (**Fig. 1**), diffuse architecture, spindle cells, pseudo-acinar appearance, admixture of large and small cells, and extreme cytological atypia (**Fig. 2A–D**) may also be observed [3, 44]. Alterations in the vascular framework and stromal connective tissue may be prominent, causing architectural distortion and further confounding the diagnosis [44]. In this regard, Plaza *et al*. [45] analyzed a series of tumors, localized in the head and neck region and anterior mediastinum, displaying extensive stromal sclerosis/ hyalinization and a pseudo-infiltrative growth pattern. The same authors designated these neoplasms as sclerosing PGLs and discussed their differential diagnostic spectrum as follows [45]:

(**1**) Head and neck sclerosing PGLs:

- metastasis or secondary infiltration by a carcinoma with desmoplastic stroma
- metastatic neuroendocrine carcinomas
- metastatic clear cell renal cell carcinoma
- carcinoma with thymus-like differentiation (CASTLE)
- solitary fibrous tumor arising in the soft tissue of the head and neck
- fibrosarcoma or other type of low-grade fibro- or myofibroblastic spindle cell lesion

(**2**) Mediastinal sclerosing PGLs:

- metastatic carcinoma with desmoplastic stroma
- spindle cell thymoma
- nodular sclerosing Hodgkin lymphoma
- idiopathic sclerosing mediastinitis

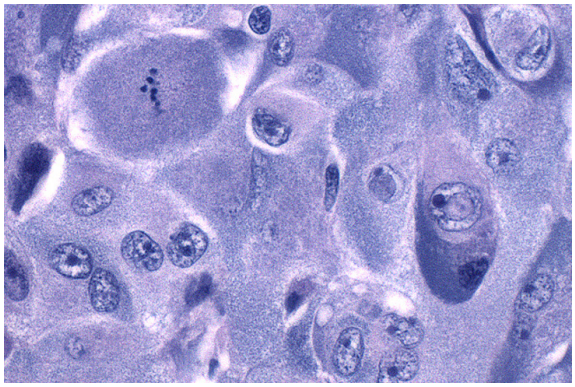

Figure 1. Some PCCs exhibit a syncytial-like growth pattern, intertwined "embracing" tumor cells (right) and, in well-fixed specimens, a mosaic pattern of cells with deeply basophilic and amphophilic cytoplasm. The basophilia is probably caused by abundant chromogranin, which is an acidic protein. Other features in this field are a nuclear pseudoinclusion (right) and a mitosis (left). Mitoses are rare in most PCC and can occur in either large or small tumor cells (H–E, x 40).

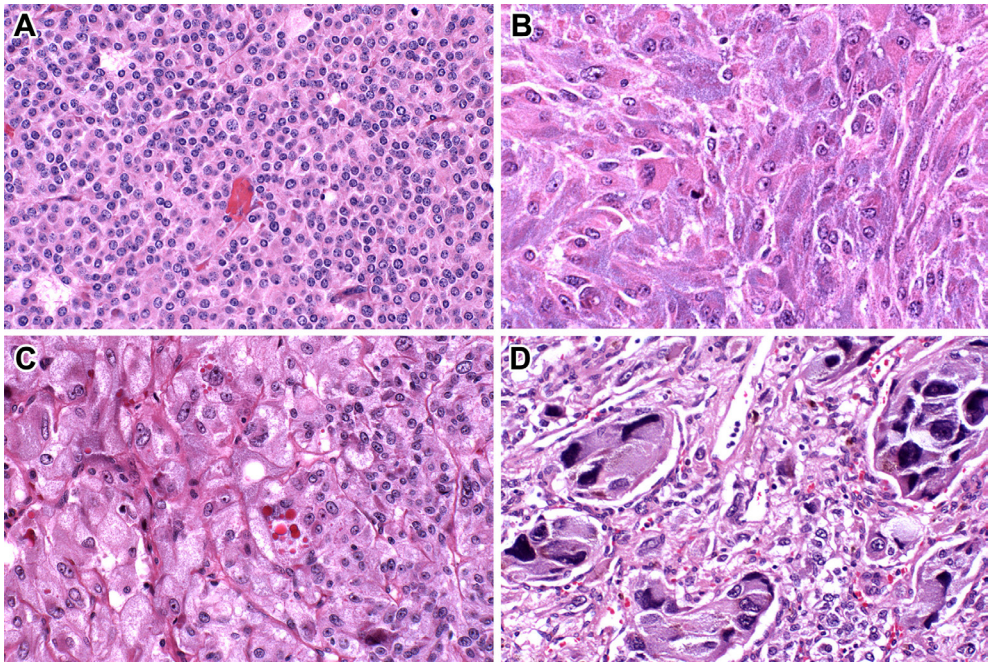

Figure 2. (**A**) Some PCCs exhibit a diffuse growth pattern. (**B**) Spindle cells can be either a dominant or focal component of PCC. (**C**) Discrete patches of cells of different sizes, possibly representing different subclones, often coexist in the same tumor. "Hyaline globules" are seen at the center of this field. (**D**) Extreme cytological atypia and large zellballen, which are usually quite subjective components of the PASS score, are dramatic and unequivocal in this tumor.

Apart from this rare morphological PGL variant, parasympathetic PGLs often demonstrate somewhat clearer cytoplasm and more pronounced "zellballen" pattern than their sympathetic counterparts, but overlap exists between them [3]. According to the 2004 World Health Organization (WHO) classification, extra-adrenal PGLs arising outside the usual distribution of sympathetic and parasympathetic paraganglia include gangliocytic PGLs, PGLs of the cauda equina, and orbital and nasopharyngeal PGLs [2].

Gangliocytic PGLs are distinctive triphasic neoplasms comprising epithelioid endocrine cells, ganglion cell-like elements, and spindled Schwann-like cells in varying proportions. Depending on which constituent predominates, the differential diagnostic considerations vary: carcinoid tumor, paraganglioma, ganglioneuroma, schwannoma, and carcinoma [2]. Okubo *et al*. [46] performed a literature survey using two databases (PubMed and IgakuChuoZasshi), retrieving reports of 192 patients with gangliocytic paraganglioma. The duodenum was confirmed as the most common site of occurrence (90%). Many more tumors than expected extended beyond the submucosal layer, a risk factor for lymph node metastasis [46]. However, patients with gangliocytic paraganglioma have an extremely good prognosis even in the presence of lymph node metastasis [2, 46].

The histopathological diagnosis of PGL may also pose challenges in unexpected anatomic sites. Reported sites include skin, liver, gallbladder/biliary system, inferior vena cava, kidney, ureter, urethra, prostate gland, spermatic cord, vulva, vagina, uterus, ovary, CNS (intracerebral and intracerebellar), orbit, nasal cavity, paranasal sinuses, trachea, tongue, hypoglossal nerve, thyroid gland, and parathyroid gland [47–70]. Two ovarian paragangliomas, one of which was gangliocytic, have been reported to arise in mature cystic teratoma [71, 72].

Given their extreme rarity and the fact that normal paraganglionic tissue has yet to be well characterized in some of these anatomic locations, histopathologists may understandably not consider PGLs or even misinterpret them as metastatic deposits, especially in the familial/syndromic setting, where multifocal PGLs are present. PGLs in unusual anatomic sites have been speculated to arise from ectopic paraganglionic rests or progenitor cells resulting from aberrant migration of neural crest cells during embryogenesis [73]. Some PGLs reported in unusual locations might in fact be incorrect diagnoses. A high index of suspicion, a complete clinicopathological history, radiological workup, and astute use of appropriate immunohistochemical markers are necessary to resolve such diagnostic issues.

From a practical standpoint, hepatic and hepatoid tumors, primary or metastatic carcinomas with endocrine or nonendocrine phenotype, melanoma, alveolar soft-part sarcoma, perivascular epithelioid cell tumor (PEComa), glomus tumors, and other vascular neoplasms should always enter into the differential diagnosis [3]. In addition, specific considerations in differential diagnosis vary according to the anatomic site of involvement:

(**1**) Carotid body paragangliomas: medullary thyroid carcinoma (MTC), hyalinizing trabecular adenoma of the thyroid gland, metastatic neuroendocrine carcinomas (carcinoids), and anaplastic carcinoma [74, 75]
(**2**) Jugulotympanic paragangliomas: middle ear adenoma/ adenomatous neoplasm, meningioma, and schwannoma [44]
(**3**) Vagal paragangliomas: intravagal parathyroid adenoma/ parathyroid hyperplasia/ ectopic parathyroid tissue and similar to the aforementioned differential diagnosis of carotid body paragangliomas [44, 75]
(**4**) Laryngeal paragangliomas: laryngeal atypical carcinoid tumor (moderately differentiated neuroendocrine carcinoma), PEComa, and similar to the aforementioned differential diagnosis of carotid body paragangliomas [44, 75, 76]
(**5**) Pulmonary paragangliomas: bronchial carcinoid tumors, carcinoid tumorlets, and minute meningothelial-like nodules [44]
(**6**) Orbital paragangliomas: alveolar soft-part sarcoma, PEComa, and neuroendocrine tumors (metastatic/ extremely rare primary) [44, 77, 78]
(**7**) Paraganglioma of nasopharynx and nasal cavity: lobular capillary/cavernous hemangioma, nasopharyngeal angiofibroma, sinonasal hemangiopericytoma, sinonasal meningioma, and olfactory neuroblastoma [47, 79]
(**8**) Thyroid paragangliomas: MTC, hyalinizing trabecular adenoma of the thyroid gland, and secondary involvement by extension of carotid body PGL or another cervical PGL [44, 48, 49]
(**9**) Parathyroid paragangliomas: unusual parathyroid adenoma, metastatic PGL to the parathyroid gland, and medullary thyroid carcinoma [50]
(**10**) Ovarian paragangliomas: various neoplasms in the sex cord-stromal and steroid categories (with a nested growth pattern), hepatoid variant of yolk sac tumor, oxyphilic struma ovarii, and hepatoid carcinoma [60]
(**11**) Prostatic paragangliomas/paraganglia: prostatic adenocarcinoma (fused gland growth pattern) [80, 81]
(**12**) Cutaneous paraganglioma: paraganglioma-like dermal melanocytic tumor, epithelioid Spitz nevus, granular cell tumor, hibernoma, cutaneous meningioma, ectopic neural hamartoma, and metastatic PGL to the skin [51, 70, 82, 83]

Another interesting histopathological consideration, expanding the morphological spectrum of extra-adrenal paragangliomas, concerns pigmented PGLs (**Fig. 3A–C**). These are highly unusual tumors with only a few cases thus far published in the literature [73, 84–94]; reported sites of occurrence include the following in decreasing order of frequency: retroperitoneum, uterus, urinary bladder, lumbar spine, vagus nerve; ganglion nodosum and vagal trunk below ganglion nodosum, heart (right and left atrium), mediastinum; and anterior and posterior, orbit and kidney. The presence of pigment, variously classified as lipofuscin, neuromelanin, or true melanin on histochemical and ultrastructural grounds [88], poses additional diagnostic difficulty. In that challenging scenario, caution should be exercised first to exclude the possibility of a metastatic melanoma, while always taking into consideration other relevant (pigmented) tumors, such as melanotic schwannoma, pigmented neurofibroma, meningioma, PEComa, melanotic neuroectodermal tumor of infancy, olfactory neuroblastoma, neuroblastoma, ganglioneuroblastoma, and non-melanocytic tumors with a neuroendocrine phenotype, such as medullary thyroid carcinoma, neuroendocrine neoplasms (carcinoids) of lung and thymus, and pigmented (black) neuroendocrine tumor of the pancreas [77, 88, 89, 95, 96]. Other melanin-pigmented tumors include pigmented dermatofibrosarcoma protuberans (Bednar tumor), clear cell sarcoma of soft tissue, and melanotic Xp11 translocation renal cancer [88, 97]. With regard to primary adrenal pigmented lesions, the differential diagnostic spectrum encompasses primary pigmented nodular adrenocortical disease, incidental pigmented cortical nodule(s), pigmented (black) adrenocortical adenoma, pigmented adrenocortical carcinoma, pigmented pheochromocytoma, primary melanoma, primary PEComa of the adrenal gland with melanin pigment production (not yet reported), and adrenal hematoma with hemosiderin-laden macrophages [44, 98, 99].

IHC can be reliably utilized for differential diagnosis, provided immunohistochemical staining procedures are applied judiciously and with appreciation of potential artifacts [3]. The combination of broad-spectrum neuroendocrine markers, such as chromogranin A (CgA), a member of a family of acidic secretory proteins present in the matrix of catecholamine-containing secretory granules, and synaptophysin, along with S100 protein (and/or GFAP; highlighting the sustentacular cell population) will usually distinguish PGLs from non-neuroendocrine tumors [2, 3]. An additional panel of antibodies is required to rule out other types of neuroendocrine tumors. That panel includes antibodies against tyrosine hydroxylase (TH; the rate-limiting enzyme in catecholamine biosynthesis) and keratin filaments. Both TH immunoreactivity and Pan-Cytokeratin (AE1/AE3) or CAM 5.2 immunonegativity favor a diagnosis of PCC or PGL [3, 100, 101].

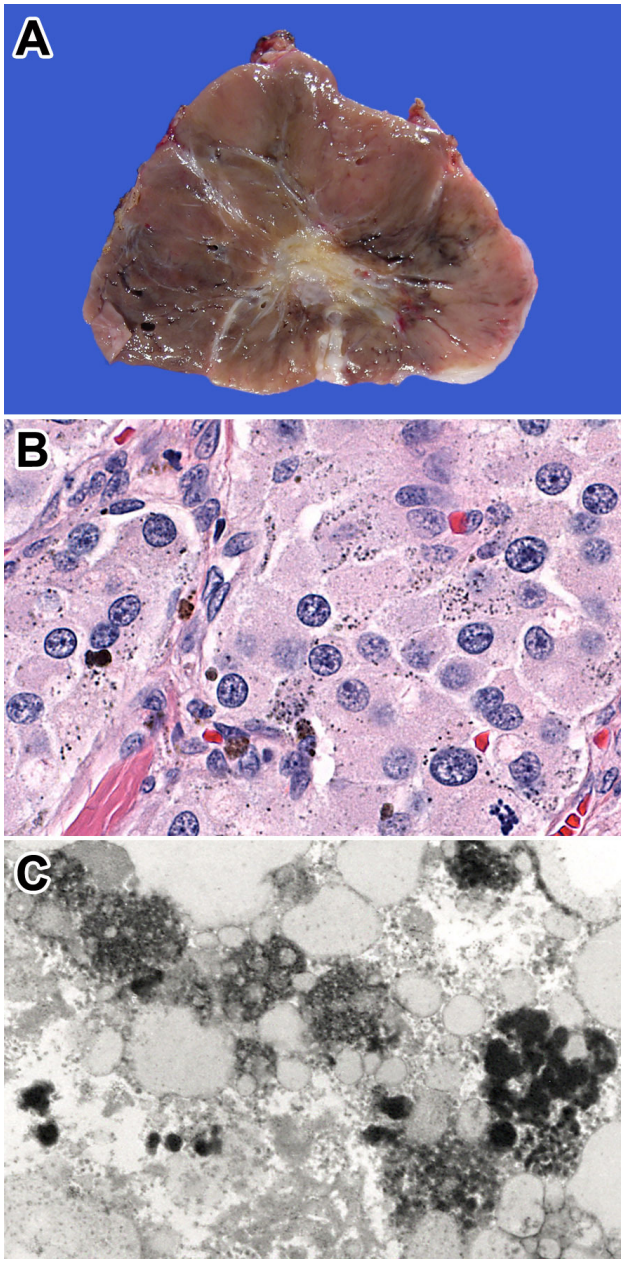

Figure 3. (**A**) Pigmented PGL (unfixed tissue) showing diffuse light brown pigmentation with darker brown patches, corresponding to varying cytoplasmic pigmentation in histologic sections. (**B**) Black granules in the cytoplasm of a pigmented PGL corresponding to lipofuscin-type pigment by electron microscopy. (**C**) Electron micrograph of the pigmented PGL in (**A**) and (**B**), showing lipofuscin pigment and no melanosomes.

Awareness of potential immunohistochemical pitfalls is essential to avoid critical misdiagnosis:

(**1**) Immunostaining for CgA and TH tends to be weaker and more variable in parasympathetic PGLs than in their sympathoadrenal counterparts [3]. Some of these tumors show no staining for either marker (**Fig. 4A** and **B**) [102] and some preferentially express CgB rather than CgA [103]. Synaptophysin can be positive in tumors that are CgA- or CgB-negative, because it is a marker of secretory vesicle membranes rather than matrix. However, it is also a less specific marker.

(**2**) Synaptophysin immunoreactivity should not be used to discriminate PCC/PGL from adrenal cortical tumors, which can show positive immunostaining [104].

(**3**) The presence of S100 protein-positive sustentacular cells has been well documented in MTC [105], while paragangliomalike MTC is a rare MTC variant sharing additional morphologic similarities with PGL [106]. A panel of antibodies, including calcitonin, TTF-1, low-molecular-weight keratins and carcinoembryonic antigen (CEA), is needed for this particular distinction. Sustentacular cells are also displayed in a proportion of pulmonary carcinoids [107, 108], suggesting that their presence and distribution are not reliable criteria for differential diagnosis. In fact, immunohistochemical and cytogenetic studies of both Schwann cells in pediatric neuroblastomas [109, 110] and sustentacular cells in head and neck PGL [111] strongly suggest that these constitute reactive non-neoplastic cell populations are possibly induced or attracted by tumor-derived factors [111–113].

(**4**) TH along with other catecholamine-synthesizing enzymes (dopamine-β-hydroxylase and phenylethanolamine- N-methyltransferase) has been reported in a subset of midgut carcinoids [114]. As for MTC, a panel of antibodies that includes stains for keratins and specific secretory products is required.

(**5**) The distinction between pigmented PGL and melanomas can sometimes be particularly troubling because melanocytes hydroxylate tyrosine for the biosynthesis of melanin [115]. Patients with melanomas can have increased "serum tyrosine hydroxylase activity" [116] and even mildly increased catecholamines. However, the tyrosine hydroxylating activity in melanomas [116] is at least in most cases principally a function of the enzyme tyrosinase (EC1.14.18.1), which is a different enzyme from the tyrosine hydroxylase (TH, EC1.14.16.2) in chromaffin cells. Nonetheless, expression of mRNA encoding TH isoenzyme I, which is one of four splice forms of the TH gene product [117],

has been reported in normal human epidermal melanocytes, together with localization of enzyme protein in the cytosol and melanosomes using IHC, immunofluorescence double staining and immunogold electron microscopy [118]. At least one commonly employed monoclonal TH antibody does not stain melanocytes or melanomas (A.S. Tischler, unpublished), suggesting specific reactivity for TH isoforms expressed in chromaffin cells. Use of control tissues including melanocytes could therefore be helpful in ambiguous cases.

(**6**) Extra-adrenal PGLs are almost always negative for keratins [100]. Nevertheless, cauda equina PGLs have a distinctive cytokeratin profile [44]. Endocrine cells in gangliocytic PGLs are positive for cytokeratins [2], while head and neck PGLs do exceptionally exhibit positivity for CAM 5.2 and pan-cytokeratin AE1 / AE3 [119, 120].

IHC results should be always interpreted in the pertinent morphological and clinical context. In this regard, the differential diagnosis is particularly challenging when individual patients suffer from both PGLs and carcinoids, as can occur in a syndromic setting (NF1 and VHL disease) [3].

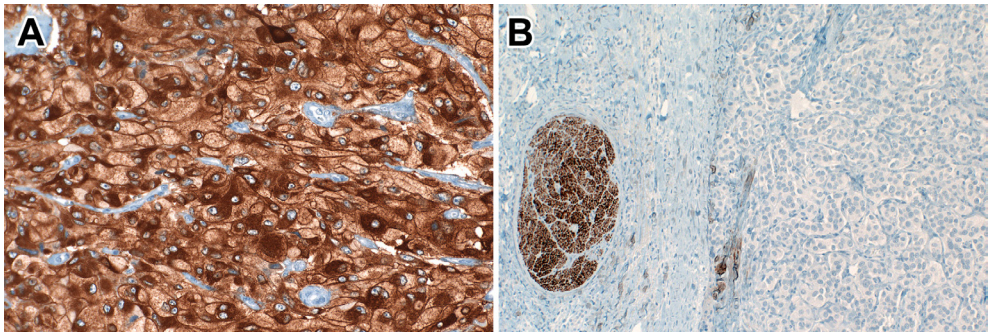

Figure 4. (**A**) IHC for TH is strongly positive in functioning PCC but can be absent in parasympathetic PGL (**B**) (IHC, x 20). (**B**) IHC is negative for TH in this carotid PGL (right). The positive nerve at left serves as an internal positive control. If the patient has recurrent or persistent elevation of metanephrines, the most likely explanation is a second primary tumor, probably associated with an *SDH-x* mutation, rather than a metastasis or local recurrence (IHC, x 10).

COMPOSITE PHEOCHROMOCYTOMAS/PARAGANGLIOMAS

Composite PCC or PGL is a designation for a tumor typically combining features of PCC or PGL with those of ganglioneuroma (GN), ganglioneuroblastoma (GNB), neuroblastoma (NB), or peripheral nerve sheath tumor (PNST) [2]. A cauda equina neoplasm showing ependymal and paraganglionic differentiation has also been described [121]. Other rare

combinations of PCC/PGL with another tumor component do not share a common embryologic ancestry from the neural crest and should best be regarded as collision tumors. These include corticomedullary tumors [122, 123] and a tumor comprising cells of adrenal cortical, medullary, and neural lineages [124]. Metastases of tumor to tumor should always be considered in assessing neoplasms of apparent mixed phenotype. Interesting and potentially challenging examples include composite PCC with intratumoral metastatic squamous cell carcinoma [125] and MTC metastatic to PCC in a MEN2A patient [126]. PCC can also coexist with other separate tumors, either common or rare, in the same gland. Examples of these include adrenocortical adenoma [127, 128] or carcinoma [129] and spindle cell sarcoma with hemangiopericytoma- like features [130].

In an effort to find all previously published cases of composite extra-adrenal PGLs and concomitantly reveal any potential differences from their adrenal counterparts, we conducted a search of the Medline database up to February 2013, using the PubMed interface, as well as reviewing references within relevant articles (**Table 2**). Composite PCCs outnumber composite extra-adrenal PGLs approximately by a ratio of 3:1, while demonstrating a slight female preponderance, in contrast to an equal gender distribution in PGLs. Partners of PCCs included in decreasing order of frequency are the following: GN (**Fig. 5A**), GNB, NB, malignant PNST, and neuroendocrine carcinoma. Ganglioneuroma was found to be the most frequent partner in both composite PCCs (63%) and PGLs (81%). With regard to the anatomic site of occurrence, composite PGLs presented most frequently in the retroperitoneum (48%) followed by the urinary bladder, filum terminale/ cauda equina, mediastinum, and the head and neck region.

Composite PGLs are less often associated with genetic disorders than their adrenal counterparts. In fact, we detected only two cases: a composite PGL-GN arising in a patient with type 1 neurofibromatosis [162] and a composite PGL-NB associated with a succinate dehydrogenase subunit B (*SDHB*) gene deletion [171] in addition to two patients with neuroblastoma and a germline *SDHB* mutation [184–186]. Moreover, we identified three bilateral composite PCC cases associated with NF1 and 13 composite PCC cases combined with NF1 in total (16%); an association highly disproportionate to the overall association of NF1 with ordinary PCC (<1–5%) [2]. This association is consistent with a hypothetical model of tumorigenesis for composite tumors proposed by Kimura *et al*. [113, 146] positing that loss or decrease of neurofibromin may induce proliferation of Schwann cells and sustentacular cells and an increase of neurotrophic factors, resulting in proliferation of ganglionic and PCC cells through autocrine and paracrine loops. Lee *et al*. [187] proposed a unifying model, subsequently refined by

Schlisio *et al*. [188] linking familial pheochromocytoma genes (*NF1, VHL, c-RET*, and *SDH*) through KIF1Bβ to NGF-dependent apoptosis. These genes along with *EglN3* define a single common pathway responsible for elimination of neuronal progenitor cell excess during normal embryological development when growth factors (e.g., NGF) become limiting. Accordingly, inactivating mutations would allow sympathetic neuronal precursors to escape from developmental apoptosis, setting the stage for their neoplastic transformation [189]. Although this model is attractive, there has been as yet no experimental evidence to support a role for a persistent pluripotent precursor in the pathogenesis of composite tumors [2].

Table 2. Comparison of all composite pheochromocytomas versus composite extra-adrenal paragangliomas.

	Composite PCCs	**Composite ea PGLs**
No.	79 [112,124–126,131–161] [a]	27 [86,131,137,159,162–183]
Age	5–82 (majority, 30–70 years)	4–81 (median 48.5 years)
Sex (F:M)	~ 4:3	~ 1:1
Anatomic site	Adrenal	Thirteen retroperitoneum/organ of Zuckerkandl [137,159,162–171] Six urinary bladder [86,172–176] Five filum terminale/conus medullaris region/cauda equine [177–181] Two mediastinum [182,183] One head and neck (carotid body) [131]
Components	50 PCC + GN 15 PCC + GNB 8 PCC + NB 4 PCC + MPNST 1 PCC + neuroendocrine Ca 1 PCC + ganglion cells [b]	22 PGL + GN 4 PGL + NB 1 PGL + GBN
Bilateral/multicentricity	4 (5.06%) [c]	None
Associated genetic conditions	18 (22.78%) NF1 (13/18) VHL (2/18) MEN2A (2/18) MEN2B (1/18)	2 (7.4 %) NF1 (1/2) SDHB (1/2)

Abbreviations: Ca, carcinoma; ea, extra-adrenal; GN, ganglioneuroma; GNB, ganglioneuroblastoma; MPNST, malignant peripheral nerve sheath tumor; NB, neuroblastoma; NF1, neurofibromatosis type 1; PCC, pheochromocytoma; PGL, paraganglioma; MEN2, multiple endocrine neoplasia type 2; VHL, von Hippel–Lindau

[a] References are also cited by Khan et al [154]

[b] Mature ganglion cells in clusters with no visible Schwannian stroma, neuropil or immature neuroblastic components

[c] Three of these patients suffered from neurofibromatosis, while the fourth case is referred to as bilateral and familial

From the perspective of a histogenetic pathway, composite tumors are rare experiments of nature that, at least in some instances, seem to be characterized by transdifferentiation of cells that have already expressed a lineage commitment. Considering the well-established phenotype plasticity of normal and neoplastic chromaffin cells, the direction of differentiation would most likely be from pre-existing pheochromocytoma or paraganglioma cells to neurons [112]. However, of interest are two NB cases displaying chromaffin cell differentiation following different therapeutic modalities [190, 191], albeit not in the context of composite tumors. These two cases highlight the possibility of phenotypic plasticity of either neuroblastoma cells or sympathoadrenal precursors. Significantly, Hedborg *et al*. [192] provided recent evidence suggesting a neuroendocrine maturation pathway with acquisition of chromaffin features apart from the well known NB differentiation toward a sympathetic ganglion / neuronal cell phenotype. In particular, they showed that (**1**) chronic tumor hypoxia is a key microenvironmental factor for neuroblastoma cell differentiation; (**2**) neuroendocrine secretory protein-55 (NESP55) is a reliable marker for this hypoxia-dependent neuronal to neuroendocrine transition; and (**3**) the chromaffin phenotype is the main form of differentiation in stroma-poor tumors, whereas it co-exists with ganglion cell-like differentiation in stroma-rich tumors [192]. These lines of evidence may provide further insight into the intriguing process of histogenesis of composite tumors.

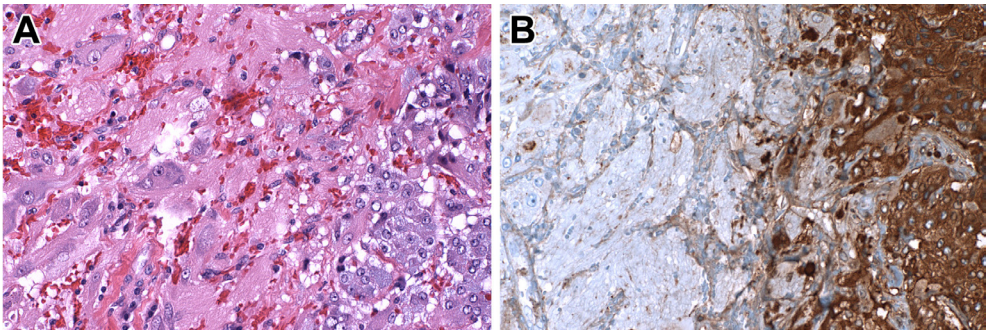

Figure 5. (**A**) Composite adrenal tumor contains tumor types with complete histological features of each. This example contains PCC (**right**) and ganglioneuroma (**left**). (**B**) Differences in pattern of staining for CgA or other markers can help to identify areas of ganglioneuroma or neuroblastoma in composite tumors.

According to the 2004 WHO Classification of Tumors of Endocrine Organs, the diagnosis of composite tumor requires both histoarchitecture and cell populations consistent with an additional type of tumor. Scattered, neuron-like cells occasionally observed in typical PCCs or PGLs are not sufficient for the diagnosis [2]. It is also important not to misinterpret degenerating chromaffin cells or lymphocytes as neuroblasts [135], and

to discriminate maturing neuroblasts from pheochromocytoma cells of the same size [112]. To prevent foci of NB or GNB from being missed in a composite tumor [147], histopathologists should be aware of the IHC expression patterns in these tumors, which recapitulate the staining of their normal counterparts. PCC and PGL cells show strong and diffuse CgA and synaptophysin staining corresponding to the large numbers of secretory granules in their cytoplasm (**Fig. 5B**), whereas NB or GNB show weak and focal staining, often in a linear or punctuate distribution corresponding to portions of axon-like processes where secretory organelles accumulate in neuroblasts and mature neurons [112]. Other immunostains, including bcl-2, CD57, IGF-II, NESP55 [192–195], and RET [147], may provide additional information about lineage subsets and transitional forms. The biological behavior of composite PCCs or PGLs is difficult to define because of the limited number of reported cases, incomplete or absent clinical outcome data, and variability in the nature and extent of each component. When present, a less-differentiated neuronal (NB/GNB) or neuroendocrine component appears to be the usual source of metastases and hence the leading prognostic feature [138, 142, 144, 170]. However, both tumor components occasionally co-exist in metastases or recurrences [133, 134] or even metastasize apart from each other [149]. In one case, a VIP producing metastatic composite tumor causing watery diarrhea–hypokalemia–achlorhydria arose from an apparently pure PCC [196]. Recurrences or metastases did not develop in a substantial percentage of composite PCC/PGL-NB/ GNB [126, 131, 137, 138, 143, 145, 148, 153, 157], but whether this reflects slower progression of NB/GNB in adults than in children or merely a short follow-up remains to be clarified. Composite PCC/PGL-GN tumors have the same prognosis as typical PCCs or PGLs [2]. To our knowledge, only one composite PCC-GN developed metastases, consisting exclusively of the PCC component [141].

Similarly to most tumors with NB/GNB, the clinical behavior of composite PCC-MPNST is dictated by the MPNST component [152] and characterized by aggressive clinical course and poor prognosis [136, 139, 149]. Two cases displayed elements of divergent mesenchymal differentiation, either rhabdomyosarcomatous (Triton tumor) [152] or fibro-, chondro-, osteo-, and angiosarcomatous components [139].

DETERMINATION OF MALIGNANCY

According to the 2004 WHO classification, malignancy of PCCs and extra-adrenal PGLs is defined by the presence of metastases to sites where chromaffin tissue is not normally found in order to prevent misclassification of multicentric primary neoplasms as metastatic [2]. Local invasion was not integrated into the WHO definition [197] because

is a poor predictor of metastases. Further, some PCC/PGLs that metastasize do not show apparent invasion. Nevertheless, extensive local invasion is a potentially lethal manifestation of tumor aggressiveness and, in this context, the Armed Forces Institute of Pathology (AFIP) 2007 Atlas of Tumor Pathology proffered a competing definition of malignancy based on either extensive local invasion or metastasis [44]. Unfortunately, the combined definition obscures the fact that the two types of aggressive behavior may have different mechanistic and genetic underpinnings, as evidenced by their poor correlation, and it potentially confuses efforts to determine the type of risk that a primary tumor presents.

Despite the WHO definition, a pathologist signing out a primary PCC or PGL is often confronted by a clinician's question "Is it malignant?". In 2008, one of the authors of this review proposed that the term "malignant" as applied to a primary tumor should be discarded in order to develop a conceptual framework based on precise definition and stratification of risk. This approach would both eliminate inconsistent terminology and acknowledge the fact that almost all PCC/PGL probably carry some risk of metastasis and therefore should never be signed out as benign [197, 198]. In analogy to melanoma or GIST, the risk of metastasis would be defined by a constellation of parameters [197]. With regard to invasion, classification of PCC/PGL as non-invasive, minimally invasive, or extensively invasive similarly to follicular carcinomas of the thyroid gland would provide a framework for validating preexisting parameters as well as developing reliable new markers in order to optimally guide therapy [197]. It remains to be seen how much acceptance this proposal will receive and how it might evolve.

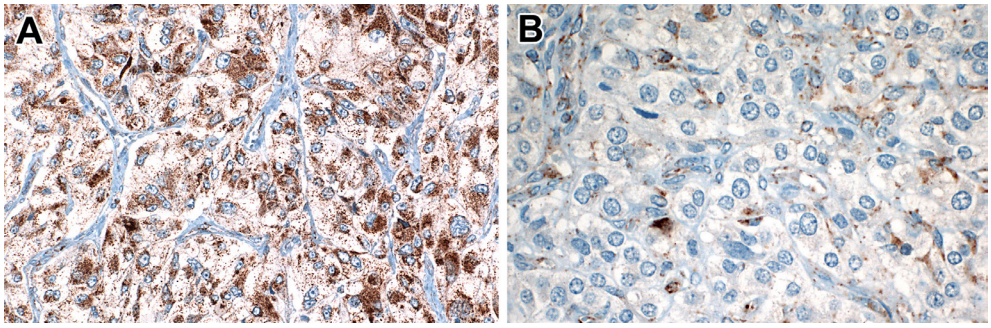

Figure 6. (**A**) IHC for SDHB protein shows granular cytoplasmic staining corresponding to mitochondria in tumor cell cytoplasm in PCC/PGL that do not have *SDH-x* mutations (IHC, x 20). (**B**) IHC for SDHB protein is negative in tumor cell cytoplasm in tumors with *SDH-x* mutations, but is still present in endothelial and sustentacular cells, which serve as internal positive controls (IHC, x 40).

It is generally acknowledged that no single histologic finding alone will reliably predict the risk of metastasis [3]. In response to this, multifactorial scoring systems along with statistical/logistic models have been generated [126, 199–201]. First, a seminal paper by Linnoila *et al*. [126] examined 120 PCCs and extra-adrenal PGLs and identified four parameters most predictive of malignancy (defined by local invasion or metastases): (**1**) extra-adrenal location; (**2**) coarse tumor nodularity; (**3**) confluent tumor necrosis; and (**4**) absence of hyaline globules. Most malignant tumors had two or three of these factors, whereas most of the benign counterparts had only one [126]. In 2002, Thompson [199] proposed the Pheochromocytoma of the Adrenal Gland Scaled Score (PASS) scoring system, which is specific to the adrenal gland and based on 12 histological features, while in 2005, Kimura *et al*. [200] proposed another scoring system for both PCC and extra-adrenal sympathetic PGLs, based not only on histologic parameters but also on immunohistochemical and biochemical characteristics. By incorporating the catecholamine phenotype into the latter system, anatomic site is indirectly given additional weight because extra-adrenal PGLs are almost always noradrenergic [198]. With regard to the validation of the PASS scoring system, results have been conflicting [201–204], while the system proposed by Kimura *et al*. did not yield 100% discrimination [200]. Large tumor size has been found to be a significant independent risk factor in some studies [205] but not in others [199]. The same is true for tumor necrosis [205]. A shortcoming of all scoring systems so far proposed and most parameters individually studied is the absence or incomplete knowledge of how they apply to tumors with different genotypes.

Many IHC markers have been studied as aids to predicting risk of metastasis. As with conventional histology, some markers appear promising but the studies overall are plagued by discrepant results and suffer from incomplete or absent information on tumor genotype. The single marker that appears in the largest number of studies, often with a positive predictive value when high labeling is present, is the proliferation marker Ki67. However, Ki67 labeling is usually very low even in metastases, which are often slow growing and can take years or decades to develop. Further, there is still a need for standardization in terms of a reproducible protocol with a defined cutoff value [3]. Additionally, an IHC-highlighted vascular pattern (CD34 and/or actin) was identified as characteristic of malignant PCCs and related to the overexpression of angiogenesis-related factors [206]. Secondarily, the staining for vascular markers serves to highlight large tumor cell nests and diffuse growth, which were a highly weighted but poorly reproducible [202] component of the PASS score. Other immunohistochemical markers that have been reported to correlate with biological behavior include CD44-S, stathmin, CgB/CgC (C-terminal regions), zinc-finger transcription factor SNAIL alone or

combined with its target Twist, heparanase-1 alone or combined with cyclooxygenase-2 (Cox-2), Cox-2 in combination with galectin-3, and nm-23, Cox-2, c-erbB-2, tenascin, and human telomerase reverse transcriptase (hTERT), the catalytic subunit of the telomerase complex, combined either with telomerase activity or Ki67 [207–222].

To date, the strongest predictors of metastasis are the presence of *SDHB* mutation and extra-adrenal location [223]. These two indicators are strongly correlated with each other because tumors in patients with germline *SDHB* mutations are most often extra-adrenal. *SDHB* mutation also predicts poor outcome for PCC/PGL after metastases have occurred [224]. For pathologists, an important recent development is the finding that immunoreactive SDHB protein is lost in PCC/PGL with *SDHA, SDHAF2, SDHB, SDHC,* or *SDHD* mutations. This presumably results from destabilization and degradation of the SDH complex, which is assembled from four separately coded subunits, when any of those subunits is mutated [42]. Demonstration of SDHB protein loss by IHC therefore serves to triage patients to be tested for any *SDHx* mutation. (**Fig. 6A** and **B**). It has also been reported that SDHB immunonegativity is a marker of adverse outcome in sporadic as well as familial PCC/PGL [225]. Complementary to SDHB IHC is the introduction of SDHA IHC, which is lost together with SDHB in *SDHA*-mutated tumors, but is preserved in tumors with other *SDHx* mutations [11].

Molecular studies including whole-genome array comparative genomic hybridization analysis, expression profiling analysis, and integrative genomic analysis [226–230], seem very promising for both identifying novel markers of malignancy and shedding light on pathogenetic mechanisms of PCC/ PGL [207, 226–231]. In this context, Burnichon *et al*. [230] found a number of genes specifically overexpressed in SDHB tumors (*MMP24, DSP, SIX1, LGR5* and *LAPTM4B*) and an inverse correlation between metastasis suppressor gene *TIP30* and risk of metastasis, suggesting that all these genes be considered as predictive markers of malignancy. Moreover, they confirmed a genetic cause for PCC/PGL in 45.5% of tumors examined, displaying a higher frequency of somatic mutations in *VHL* and *RET* genes than previously reported in sporadic tumors [230]. Recently, Loriot *et al*. [232] identified epithelial to mesenchymal transition as the first pathway conferring specific metastatic properties to SDHB-related PCCs/PGLs, while suggesting that SNAI1/2 nuclear translocation may be used as a histological marker of malignancy for *SDHB*-related tumors.

In addition to testing of cells and tissues, pathologists as well as clinicians should remember that multiple tumors or tumors presenting at an early age suggest hereditary disease. Further, a recent study by Zelinka *et al*. [205] found that patients with metastatic

PCC presented at a younger age than subjects with benign primary tumors, with larger tumors more frequently secreting norepinephrine. In keeping with this, King *et al*. [233] recently showed that patients with metastatic PCC/ PGL, who presented with a primary tumor in childhood/ adolescence, had primary extra-adrenal tumors and harbored *SDHB* mutations. In addition, Eisenhofer *et al*. [234] showed that variations in ages at diagnosis of both PCCs and PGLs were associated with different tumor catecholamine phenotypes, underlying germline mutations and tumor locations. These findings emphasize the need for a coordinated approach combining clinical, biochemical, genetic, and pathology testing as the basis for appropriately treating patients with these challenging tumors.

CONCLUSION

Revolutionary breakthroughs in understanding the biology of PCCs/PGLs have occurred during the past several years. Discoveries of new susceptibility genes and genotype–phenotype correlations have led to the realization that appropriate patient care requires a complete integration of clinical, genetic, biochemical, and pathology findings. In addition to differential diagnosis of primary tumors and metastases, pathologists have important roles in identification of clues to occult hereditary disease and triaging of patients for optimal genetic testing. Diagnostic pitfalls are posed by morphological variants of PCC/PGL, unusual anatomic sites of occurrence, and coexisting tumors of both neuroendocrine and other types in some hereditary syndromes. These pitfalls can be avoided by judicious use of appropriate immunohistochemical stains. A clinicopathologic comment in pathology reports for PCCs and extra-adrenal PGLs can play a critical role in guiding clinicians in order to optimize patient care.

Authors' note: Following acceptance of this manuscript, Toledo *et al*. reported on additional somatic *HIF2A* mutations (c.1591C>AC p.Pro531Thr, c.1591C>TC p.Pro531Ser, c.1592C>TC p.Pro531Leu and c.212C>AC p. Ser71Tyr) occuring in two sporadic ea (s) PGLs and two sporadic PCCs respectively (Toledo *et al*. **In vivo and in vitro oncogenic effects of HIF2A mutations in pheochromocytomas and paragangliomas**. Endocr Relat Cancer 21 May 2013 [PMID: 23533246]). Moreover, Letouzé *et al*. expanded the spectrum of genes predisposing to PCC development to fumarate hydratase (*FH*) gene, given the identification of inactivating germline (c.349G>C p.Ala117Pro) and somatic (c.1043G>C p.Gly348Ala) *FH* mutations by exome sequencing of an apparently sporadic hypermethylated PCC (Letouzé *et al*. **SDH Mutations Establish a Hypermethylator Phenotype in Paraganglioma**. Cancer Cell 10 Jun 2013 Jun [PMID: 23707781]), while Jamilloux *et al*. reported on the first typical MEN1 syndrome (hyperparathyroidism,

pancreatic neuroendocrine tumor, and adrenocortical adenoma) associated with an ea PGL (Jamilloux *et al*. **A MEN1 syndrome with a paraganglioma**. Eur J Hum Genet 19 Jun 2013 [PMID: 23778871]). In addition, Crona *et al*. identified recurrent somatic *H-RAS* mutations along with activation of the RAS/RAF/ERK signaling pathway in PCCs/PGL of sporadic presentation (Crona *et al*. **Somatic Mutations in H-RAS in Sporadic Pheochromocytoma and Paraganglioma Identified by Exome Sequencing**. J Clin Endocrinol Metab 2 May 2013 [PMID: 23640968]).

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Chapter 3

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. 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.

INTRODUCTION

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].

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 dehydrogenaseassociated 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., isbon, 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 × 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 (Nano-Zoomer 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 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).

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 $P < 0.0001$

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

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 (κ = 0.8593) and the lowest between observers 4 and 7 (κ = 0.5318), whereas regarding SDHA immunohistochemistry, the highest agreement was reached between observers 6 and 2/7 (κ = 1.0000) and the lowest between observers 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 κ = 0.7338; SDHA κ = 0.6707) or a three-tiered classification approach (SDHB κ = 0.6543; SDHA κ = 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**.

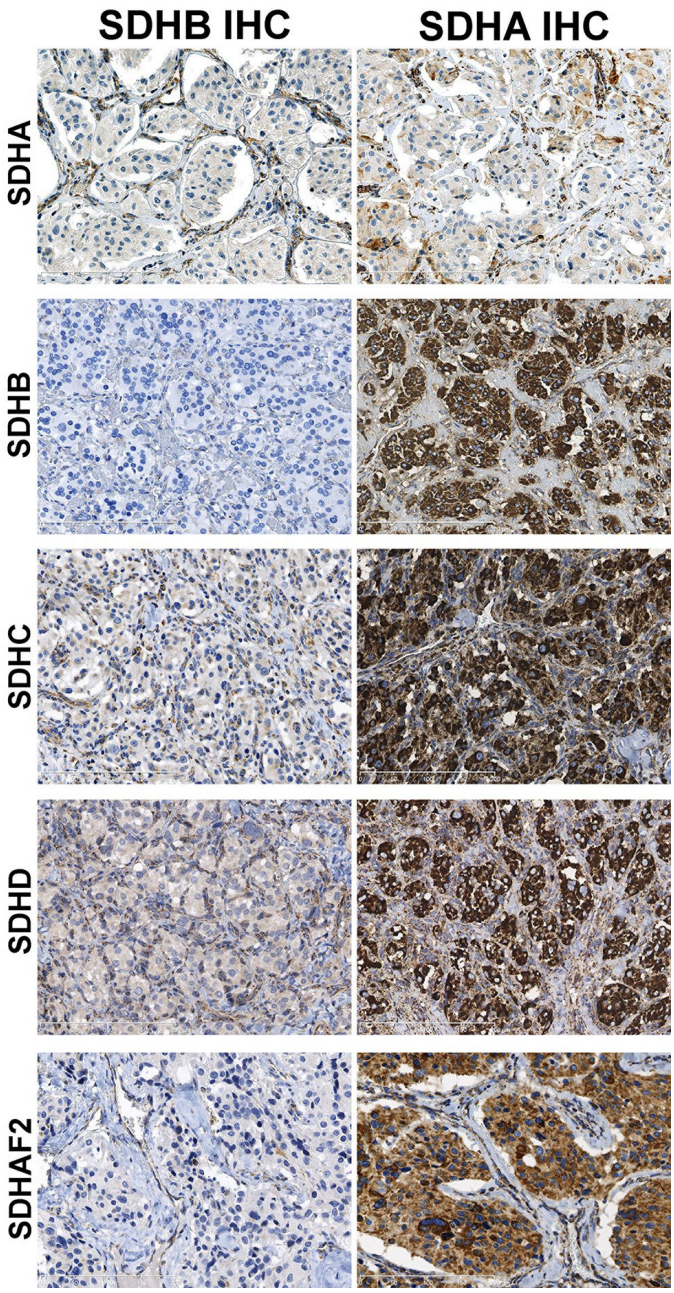

Figure 1. SDHA and SDHB immunohistochemistry in pheochromocytomas/ paragangliomas endowed either with *SDHA* germline mutation displaying loss of SDHA/SDHB protein expression or with *SDHB*, *SDHC*, *SDHD* and *SDHAF2* germline mutations exhibiting loss of SDHB, but intact SDHA expression. Note the granular,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

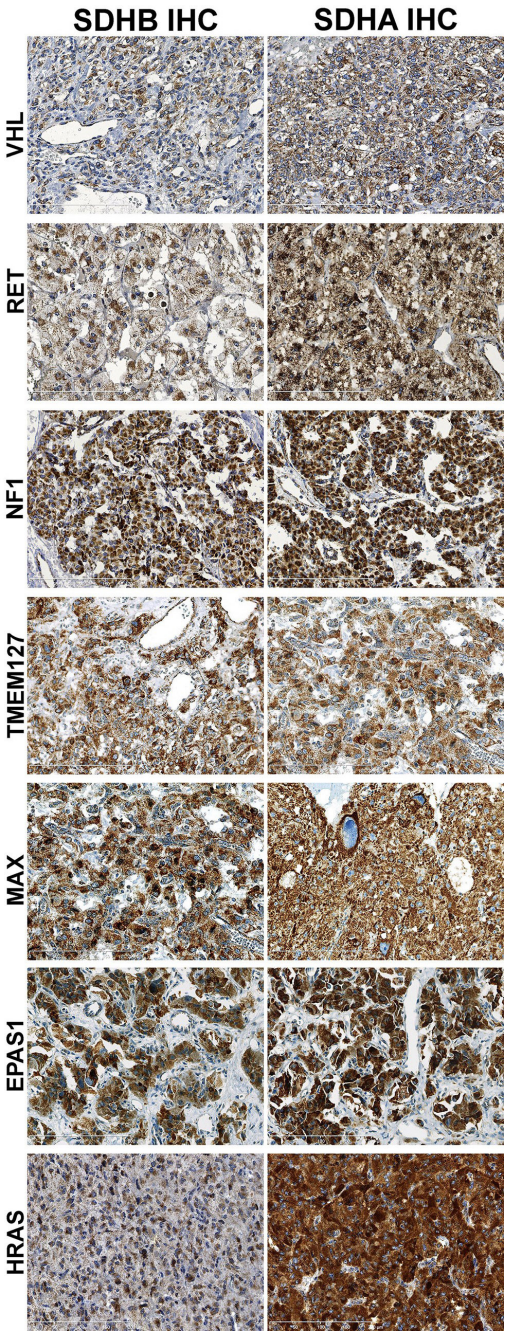

Figure 2. Intact SDHB and SDHA protein expression in non-*SDH-x*-mutated paraganglionic tumors harboring germline or somatic *VHL*, *RET*, *NF1*, *TMEM127*, *MAX*, *EPAS1* and *HRAS* mutations. Note the granular, cytoplasmic staining for SDHA/SDHB in normal cells of the intratumoral fibrovascular network,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

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]

A	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%
***P*val**	*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%
***P*val**	*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: *P*val, *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.

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.

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 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 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 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 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 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].

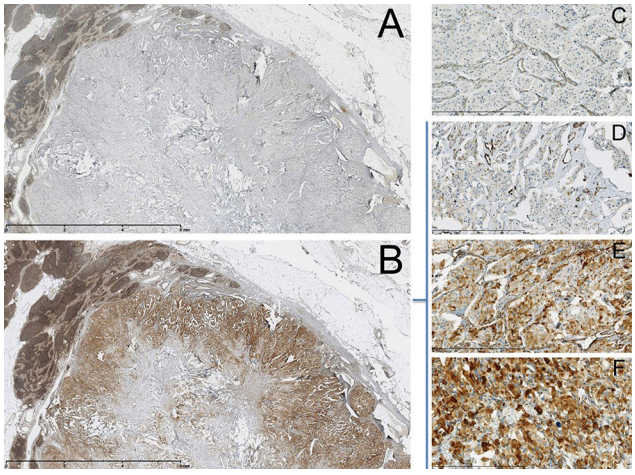

Figure 3. An extra-adrenal paraganglioma harbouring an *SDHA* (c.1534C>T, p.Arg512*) germline mutation, metastatic to a para-aortic lymph node, displaying SDHB immunonegativity (**A**, **C**), but a heterogeneous staining pattern for SDHA (**B**, **D**-**F**): central area (**D**) convincingly negative 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, which serve as internal positive controls.

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 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.

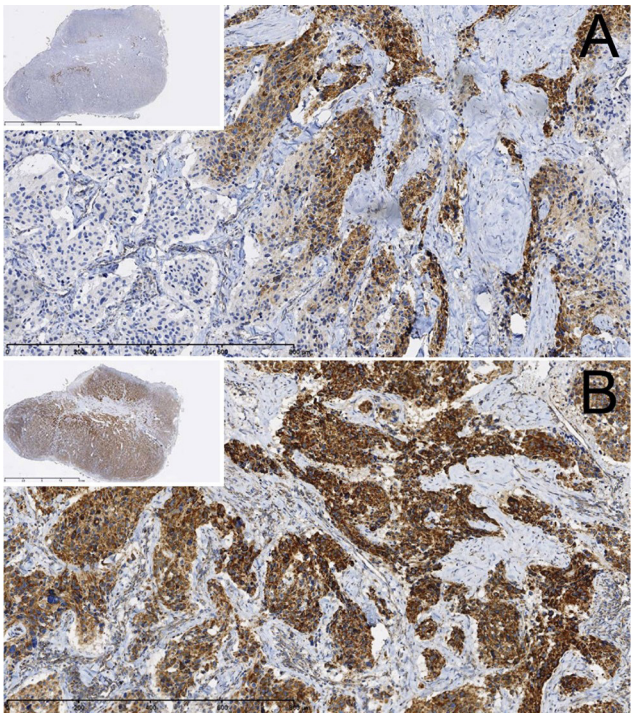

Figure 4. An *SDHAF2*-mutated (c.232G>A, p.Gly78Arg) head and neck paraganglioma showing areas convincingly negative for SDHB and at a lesser extent areas convincingly positive for SDHB (**A**). Three pathologists correctly classified this sample as heterogeneous for SDHB, with the remainder four as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

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

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PART III

Succinate Dehydrogenase (SDH)-related Tumour Spectrum: Clinico-pathological and Molecular Genetic Insights

Chapter 4

Toward an improved definition of the genetic and tumor spectrum associated with *SDH* germ-line mutations

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ABSTRACT

The tricarboxylic acid, or Krebs, cycle is central to the cellular metabolism of sugars, lipids, and amino acids; it fuels the mitochondrial respiratory chain for energy generation. In the past decade, mutations in the Krebs-cycle enzymes succinate dehydrogenase, fumarate hydratase, and isocitrate dehydrogenase have been documented to be causally involved in carcinogenesis. This review is focused on the relationship between *SDH* mutations and the carcinogenic phenotype. The succinate dehydrogenase complex catalyzes the oxidation of succinate to fumarate; mutations in its subunits *SDHA, SDHB, SDHC*, and *SDHD*, and in the assembly factor *SDHAF2*, result in syndromes with distinct tumor types, including pheochromocytoma/paraganglioma, gastrointestinal stromal tumor, and, less often, renal-cell carcinoma and pituitary adenoma. In this study we collected all previously reported *SDH* mutations with the aim of defining their nature and tumor spectrum. In addition, genotype-phenotype correlations as well as mechanisms of biallelic inactivation were analyzed in the *SDH*-deficient setting. Finally, we performed bioinformatics analysis using SIFT, Polyphen2, and Mutation Assessor to predict the functional impact of nonsynonymous mutations. The prediction of the latter was further compared with available SDHA and/or SDHB immunohistochemistry data.

INTRODUCTION

The tricarboxylic acid (TCA) cycle enzyme succinate dehydrogenase (SDH) is a heterotetramer protein complex consisting of four subunits encoded by nuclear genes. These include SDHA and SDHB, which form the catalytic domain, and SDHC and SDHD, which anchor the complex to the inner mitochondrial membrane [1]. The assembly factors, SDHAF1 and SDHAF2, ensure both structural and functional integrity of the complex [2, 3]. SDH, also called mitochondrial complex II, is the only enzyme involved in both the electron transport chain and the TCA cycle, where it catalyzes the oxidation of succinate to fumarate [1]. The TCA cycle is central to the metabolism of sugars, lipids, and amino acids and is a major source of adenosine triphosphate in cells. In addition, the cycle also seems to be involved in tumorigenesis; enzymes of the TCA cycle are involved in the pathogenesis of several tumor types. *SDH* mutations have been involved in the etiopathogeny of pheochromocytomas (PCCs), paragangliomas (PGLs), gastrointestinal stromal tumors (GISTs), renal-cell carcinomas (RCCs), and pituitary adenomas (PAs) [1, 2, 4–9]. In addition, mutations in fumarate hydratase (*FH*), another member of the TCA cycle and which catalyzes the hydration of fumarate to malate, predispose to tumor formation, including RCCs, cutaneous and uterine leiomyomas, and PCCs/PGLs [10, 11]. Finally, isocitrate dehydrogenase (*IDH*), which catalyzes the oxidative decarboxylation of isocitrate, is frequently mutated in specific types of cartilaginous tumors, hematological malignancies, and gliomas [12–14]. The currently known mechanisms underlying tumorigenesis linked to defects in the TCA cycle are well reviewed [15, 16]. Defects in the *SDH*, *FH*, and *IDH* genes inhibit prolyl hydroxylases, leading to decreased hydroxylation of hypoxia-inducible factor-α. This results in activation of the hypoxia pathway, which supports tumor formation by activating angiogenesis, glucose metabolism, cell motility, and cell survival. Furthermore, defects in these enzymes lead to epigenetic alterations through an accumulation of oncometabolites inhibiting α-ketoglutarate-dependent dioxygenases, which are involved in DNA and histone demethylation. In addition to *SDH*-associated tumorigenesis, constitutional complex II deficiencies caused by *SDHA*, *SDHB*, *SDHD*, and *SDHAF1* mutations may also lead to Leigh syndrome, infantile leukodystrophies, and cardiomyopathy [3, 17–19].

In the current review, our aim is to report all currently known *SDH* mutations and define their nature and spectrum in *SDH* related tumors, including PCCs/PGLs, GISTs, RCCs, and PAs, as well as in other unusual tumors arising in *SDH* mutation carriers. We performed bioinformatics analysis using SIFT, Polyphen2, and Mutation Assessor and compared the results with those of SDHA/SDHB mmunohistochemistry (IHC) to predict the functional impact of nonsynonymous mutations. Finally, we explored and report here the nature of the second hit in all tumors arising in the SDH deficiency setting.

MATERIALS AND METHODS

The search for *SDH* mutations was performed using the Leiden Open Variation Database http://www.lovd.nl/3.0/ home) and publications indexed in PubMed (http://www.ncbi.nlm.nih.gov/pubmed) from 2000, the year of the first published mutation in a paraganglioma, to April 2014 [1, 2, 4–6]. The following search terms were used: *SDHA*, *SDHB*, *SDHC*, *SDHD*, and *SDHAF2*, in combination with the tumor type names *pheochromocytomas*, *paragangliomas* (sympathetic and parasympathetic), *gastrointestinal stromal tumors*, *renal-cell carcinomas*, and *pituitary adenomas* [20]. With the aim of predicting the functional impact of the *SDH* mutations on the corresponding proteins, a bioinformatics analysis was performed using the ANNOVAR software [21]. First, the variants found in the literature were checked in the Leiden Open Variation Database, and the genomic positions were obtained using Mutalyzer [20, 22]. Then the variants were annotated using ANNOVAR with the following databases: hg19, RefSeq with HGVS as the nomenclature type, 1000 Genomes 2012APR, NonFlagged dbSNP138, CLINVAR20140211, and COSMIC68 [23–25]. 1000 Genomes 2012APR and NonFlagged dbSNP138, describing only single-nucleotide polymorphisms with a frequency lower than 1%, were used to determine whether the changes were reported as single-nucleotide polymorphisms [25, 26]. CLINVAR20142011 describes the associations between DNA variations and human health, whereas COSMIC68 encompasses somatic mutations in cancers [27, 28]. Finally, the scores of SIFT, Polyphen-2, and Mutation Assessor were calculated by means of the ANNOVAR software to assess the potential functionally damaging impact of the amino acid changes [29–31]. The Consensus Deleteriousness software, which combines information derived from SIFT, Polyphen-2, and Mutation Assessor, was used to predict the functionality of nonsynonymous mutations [32]. In addition, we calculated the Combined Annotation Dependent Depletion score (http://cadd.gs.washington.edu/score), which is another predictor for the pathogenicity of an amino acid change and includes the previous prediction models [33].

RESULTS

Pheochromocytomas and paragangliomas

In total, 445 different germ-line mutations in the *SDH* genes were reported in the literature. Of these, 403 mutations have been described in PCCs/PGLs: 211 (52%) in *SDHB*, 141 (35%) in *SDHD*, 42 (10%) in *SDHC*, 7 (2%) in *SDHA*, and 2 (1%) in *SDHAF2* (**Figure 1**). Missense mutations occurred most frequently in all genes, with the exception of *SDHD*, in which more frameshift mutations were observed (34%)

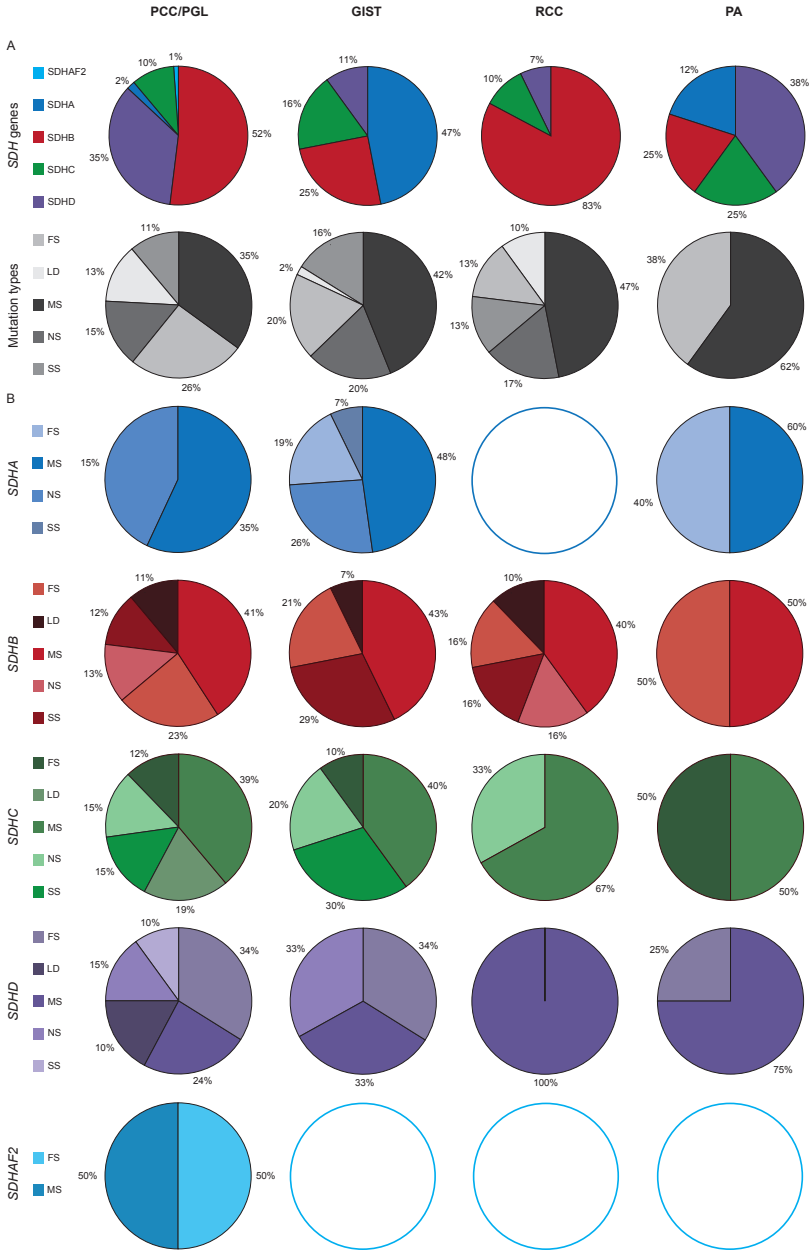

Figure 1 Spectrum and distribution of *SDH* germ-line mutations as reported in *SDH*-deficient tumors including pheochromocytomas (PCCs)/ paragangliomas (PGLs), gastrointestinal stromal tumors (GISTs), renal-cell carcinomas (RCCs) and pituitary adenomas (PAs). (**a**) Percentages of mutations in each *SDH* gene and of the different types of mutations: frameshift deletion/ duplication/insertion (FS), large deletion (LD), missense (MS), nonsense (NS), and splice site (SS). (**b**) Percentages of the different mutation types for each *SDH* gene.

(**Figure 1**). In total, 141 missense mutations (35%) and 104 frameshift mutations (26%) were observed in PCCs/PGLs (**Figure 1**). One somatic missense mutation has been identified in *SDHB* (c.299C>T, p.S100F). Seventy-eight mutations (19%) were described in malignant tumors: 59 (76%) in *SDHB*, 15 (19%) in *SDHD*, and 4 (5%) in *SDHC* (**Figure 2**). In these genes, no hotspot mutations were related to malignant behavior.

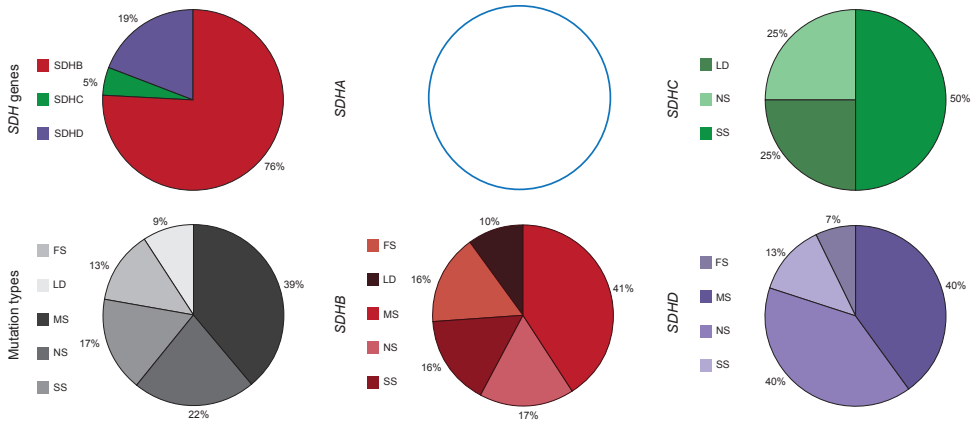

Figure 2 Spectrum and distribution of *SDH* germ-line mutations as reported in malignant pheochromocytoma (PCC)/paragangliomas (PGL). (**a**) Percentages of mutations in each *SDH* gene and of the different types of mutations reported in malignant PCCs/PGLs. (**b**) Percentages of different mutation types identified in malignant tumors for each *SDH* gene.

Gastrointestinal stromal tumors, renal-cell carcinomas, and pituitary adenomas

Fifty-five germ-line mutations were identified in the GISTs, of which 26 (47%) occurred in *SDHA*, 14 (25%) in *SDHB*, 9 (16%) in *SDHC*, and 6 (11%) in *SDHD* (**Figure 1**). Missense mutations occurred most frequently in each gene except *SDHD*, in which frameshift mutations were the most common (34%; **Figure 1**). Thirty mutations were found in RCCs: 25 (83%) in *SDHB*, 3 (10%) in *SDHC*, and 2 (7%) in *SDHD* (**Figure 1**). Most mutations were missense mutations (47%). In addition, eight *SDH* mutations were reported in PAs: three (38%) occurred in *SDHD*, two each (25%) in *SDHB* and *SDHC*, and one (12%) in *SDHA* (**Figure 1**). Five of the PA-related mutations (62%) were missense. Three somatic mutations were observed in *SDHA*: one in a GIST (c.113A>T, p.D38V) and two in one PA (c.725_736del and c.989_990insTA).

Mutations occurring in two or more tumor types

Fifty-three germ-line mutations have been described in more than one tumor type: 29 (55%) occurred in *SDHB*, 10 (19%) in *SDHC*, 10 (19%) in *SDHD*, and 4 (7%) in *SDHA*

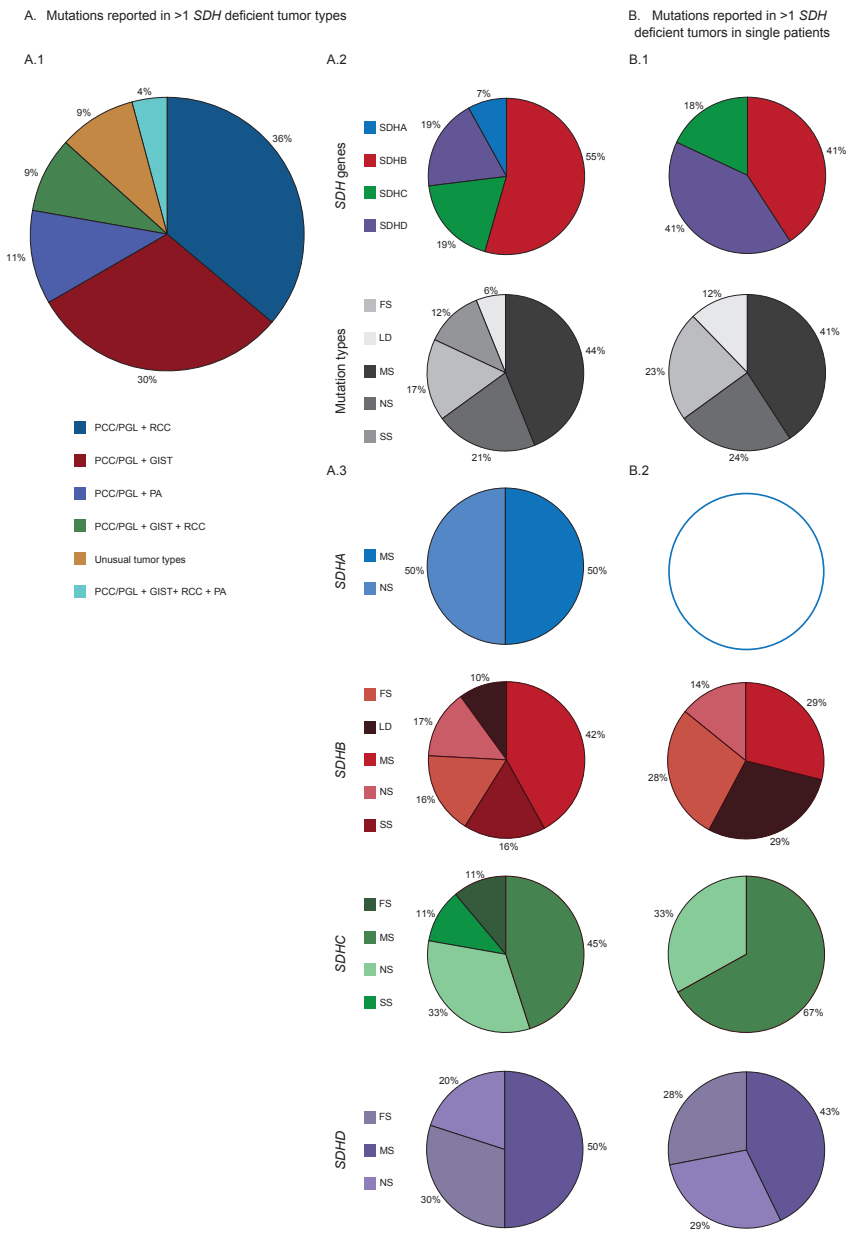

Figure 3 Spectrum and distribution of *SDH* germ-line mutations reported in more than one *SDH*-deficient tumor type. (**a**) Mutations causally related to more than one *SDH*-deficient tumor type: percentages of mutations observed in the different tumor types (A.1); percentages of mutations in each *SDH* gene and of the different types of mutations (A.2); and percentages of the different mutation types for each *SDH* gene (A.3). (**b**) Mutations harbored by single patients suffering from more than one *SDH*-deficient tumor type: percentages of mutations in each *SDH* gene and of the different types of mutations (B.1) and percentages of the different mutation types for each *SDH* gene (B.2).

(**Figure 3a**). Twenty-three (44%) were missense mutations. In total, 19 mutations (36%) were observed in PCCs/PGLs and RCCs, 16 (30%) caused PCCs/PGLs and GISTs, and 6 (11%) were found in PCCs/PGLs and PAs. In addition, five mutations (9%) were reported in PCCs/PGLs, GISTs, and RCCs. Furthermore, five mutations (9%) were identified in at least one causally related tumor type (PCC/PGL, GIST, RCC, and PA) and one unusual tumor type (thyroid carcinoma, uterine myoma, breast cancer, and neuroblastomas), and two mutations (4%) have been identified in the four causally related tumors (**Figure 3a**). Seventeen different mutations—of which seven were missense—that caused multiple causally related tumors (PCCs/PGLs, GISTs, RCCs, and PAs) in single patients were identified: seven (41%) occurred in *SDHB*, seven (41%) in *SDHD*, and three (18%) in *SDHC* (**Figure 3b**).

Mutations occurring exclusively in GISTs, RCCs, or Pas

Of 445 different germ-line mutations reported in the *SDH* genes, 39 (9%) were exclusively found in GISTs, RCCs, or PAs and not in PCCs/PGLs: 24 (61%) occurred in *SDHA*, 8 (21%) in *SDHB*, 5 (13%) in *SDHC*, and 2 (5%) in *SDHD* (**Figure 4a**). In addition, 33 mutations (85%) were observed in GISTs, 5 (13%) in RCCs, and 1 (2%) in PAs (**Figure 4a**). In total, 44% of the mutations reported exclusively in GISTs, RCCs, and PAs were missense (**Figure 4a**).

Mutations associated with tumors occurring rarely in the context of SDH deficiency

Twenty-three different germ-line *SDH* mutations were identified in patients with other tumor types, including thyroid carcinomas, neuroblastomas, and breast cancer, in addition to their PCC/PGL. Of these 23 mutations, 9 (39%) occurred in *SDHB*, 8 (35%) in *SDHD*, 4 (17%) in *SDHC*, and 2 (9%) in *SDHA* (Figure 5a). In total, 35% were missense mutations (**Figure 4b**). Establishing the causal link between these mutations and these tumors was not possible because IHC data were not reported. However, one study reported a patient suffering from a PGL and a testicular seminoma who had an *SDHD* mutation (c.129G>A, p.W43X) and showed loss of the wild-type (WT) allele in the tumor (**Supplementary Table S2** online).

***In silico* analysis of the functional impact of *SDH* mutations**

An *in silico* analysis was performed to predict the functional impact of each *SDH* nonsynonymous mutation. Three scores were assessed: SIFT, Polyphen2, and Mutation Assessor. In addition, the mutations were associated with the immunohistochemical results of the SDHA and SDHB staining because this technique has been proven to reliably predict the presence of an *SDH* mutation: A negative *SDHB* staining is predictive of the presence of a mutation in any *SDH* subunit, with a sensitivity of 100% and a specificity of

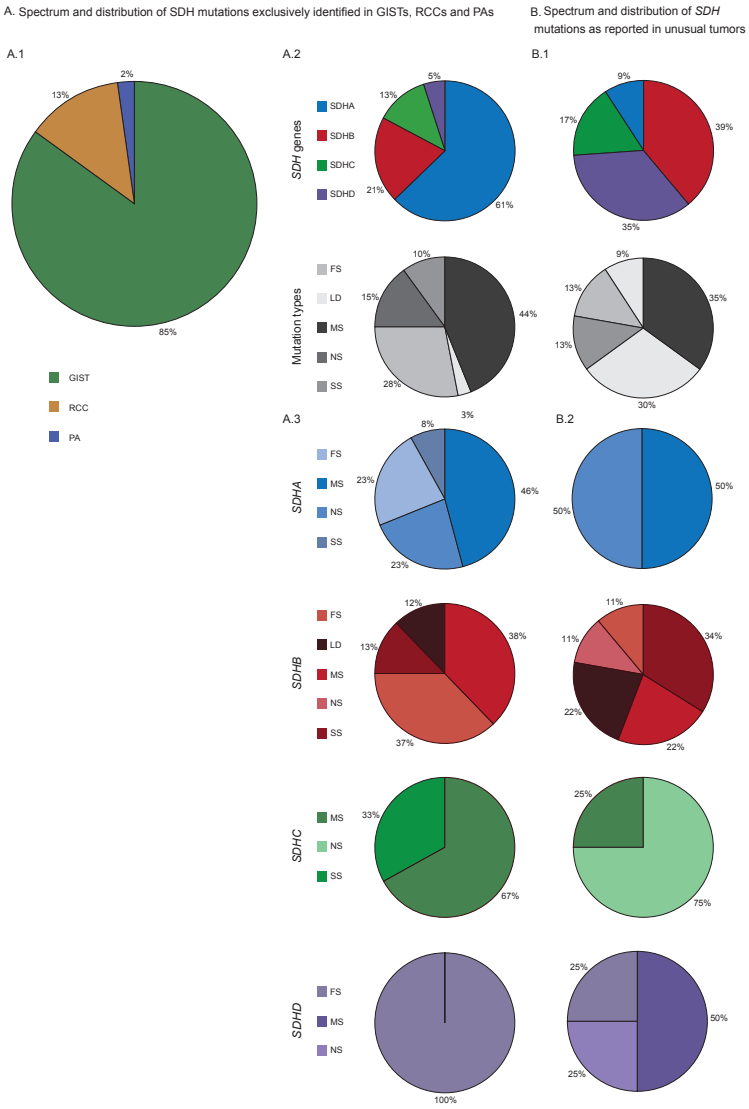

Figure 4 Spectrum and distribution of *SDH* germ-line mutations exclusively identified in gastrointestinal stromal tumors (GISTs), renal-cell carcinomas (RCCs), or pituitary adenomas (PAs) and as reported in tumors occurring rarely in the context of *SDH* deficiency. (**a**) Spectrum and distribution of *SDH* mutations exclusively identified in GISTs, RCCs, and PAs: percentages of mutations as reported only in GISTs, RCCs, and PAs in each of those tumor types (A.1); percentages of the mutations reported in the related tumor types except pheochromocytomas (PCCs)/paragangliomas (PGLs) in each *SDH* gene and of the different types of mutations (A.2); and percentages of the different mutation types identified in the related tumor types except PCCs/PGLs for each *SDH* gene (A.3). (**b**) Spectrum and distribution of *SDH* mutations as reported in tumors occurring rarely in the context of SDH deficiency: percentages of mutations in each *SDH* gene and of the different types of mutations reported in different tumors including thyroid carcinomas and neuroblastomas (B.1), and percentages of different mutation types identified in these different tumors for each *SDH* gene (B.2).

84%, whereas a negative SDHA staining predicts an *SDHA* mutation with a sensitivity of 100% and a specificity of 97% [34, 35]. The IHC results were available for 145 previously reported mutations. In the vast majority (134, or 92%), the IHC pattern was consistent with the prediction obtained by bioinformatics (**Supplementary Table S1** online). A total of 129 mutations were predicted to be damaging to the protein and showed consistent negative SDHA and/or SDHB staining (**Supplementary Table S1** online). The remaining five mutations were predicted to be nondeleterious and showed positive SDHA and/or SDHB staining (**Supplementary Table S1** online). Nevertheless, some exceptions were reported. Two *SDHA* mutations reported in GISTs (c.113A>T, p.Asp38Val and c.1969G>A, p.Val657Ile) were predicted to be nondamaging but showed negative SDHA and SDHB staining (**Table 1**). Moreover, nine mutations predicted to be deleterious had positive SDHA and/or SDHB IHC results, although they were expected to have negative results (**Table 1**). For the mutations showing nonconsistent IHC results, we calculated the Combined Annotation Dependent Depletion [33]. A C score of 10 indicates that the variant is part of the 10% most damaging substitutions in the human genome. Considering as damaging a variant with a C score ≥15 is therefore suggested (http://cadd.gs.washington.edu/info). The Consensus Deleteriousness and the Combined Annotation Dependent Depletion scores reach the same prediction for each mutation.

Second hit

The nature of the second hit has been reported for 85 *SDH* mutations (**Supplementary Table S2** online). Inactivation of the WT allele by loss of heterozygosity (LOH) has been observed 62 times (73%) and by somatic mutations in 12 cases (14%). In addition, eight mutations (9%) co-occurred with retention of heterozygosity, and epigenetic inactivation of the second allele was reported once. A combination of multiple second hits has been found for two *SDHA* mutations: c.91C>T, p.Arg31* and c.1873C>T, p.His625Tyr (**Supplementary Table S2** online).

DISCUSSION

In this review, we present an overview of all currently reported *SDH* mutations and analyze potential genotype–phenotype correlations with regard to the development of neoplasia. In addition, we performed bioinformatics analysis on the functional impact of the reported *SDH* mutations and compared the results with available SDHA and SDHB IHC results. Furthermore, we explored the nature of the second hit observed in tumors arising in both germ-line and somatic *SDH* mutation carriers.

Table 1. *SDH*-related tumors showing discrepancies between *in silico* analysis and SDHA/SDHB Immunohistochemistry.

	Protein subunit	Mutation	Tumor type	SIFT	Polyphen2	Mutation Assessor	CONDEL	CADD (Cscore)	SDHB/ SDHA IHC
1	SDHA*	p.Asp38Val	GIST	Tolerated (0.57)	Benign (0.002)	Low (1.24)	Neutral (0.25)	10.49	Neg/neg [a]
2	SDHA	p.Val657Ile	GIST	Tolerated (0.62)	Benign (0.123)	Low (1.07)	Neutral (0.259)	4.173	Neg/neg [b]
3	SDHA	p.Arg188Trp	GIST	Damaging (0)	Damaging (1.0)	High (5.185)	Deleterious (1.11)	21.1	Neg/pos [c]
4	SDHA	p.Gly260Arg	PCC/PGL	Damaging (0)	Damaging (1.0)	High (5.045)	Deleterious (1.11)	19.19	Pos/pos [d]
5	SDHA	p.Thr273Ile	GIST	Damaging (0)	Damaging (1.0)	High (3.63)	Deleterious (0.977)	18.76	Neg/pos [c, e]
6	SDHA	p.Ala454Glu	GIST	Damaging (0)	Damaging (1.0)	High (3.755)	Deleterious (0.988)	18.37	Neg/pos [c]
7	SDHA	p.His625Tyr	Cecal polyps	Damaging (0)	Damaging (1.0)	High (3.94)	Deleterious (1.003)	20.5	Pos/pos [f]
8	SDHC	p.His127Arg	PTC	Damaging (0)	Damaging (1.0)	High (4.24)	Deleterious (1.029)	20.8	Pos/ [g]
9	SDHD	p.Trp5*	PCC/PGL	—	—	—	—	—	Pos/ [h]
10	SDHD	p.His50Arg	PCC/PGL	Tolerated (0.13)	Damaging (0.804)	Medium (2.62)	Deleterious (0.782)	17.45	Pos/ [i-j]
11	SDHD	p.Asp92Tyr	PA	Damaging (0)	Damaging (1.0)	Medium (3.435)	Deleterious (0.96)	23.6	Pos/pos [k]

The predictions were tabulated with a short description and the corresponding score according to the different tools. (i) SIFT: damaging or tolerated, with a reliability score ranging from 0 to 1; (ii) Polyphen2: damaging or benign, with a score ranging from 0 to 1; (iii) Mutation Assessor, with four scores: high (3.5–5.5), medium (2–3.4), low (1–2), and neutral (<1). The CONDEL score can be neutral (0–0.5) or deleterious (0.5–1), while the CADD score can be deleterious (Cscore >15) or nondeleterious (Cscore <15).

Abbreviations: CADD, Combined Annotation Dependent Depletion; CONDEL, Consensus Deleteriousness software; GIST, gastrointestinal stromal tumor; ihc, immunohistochemistry; neg, negative immunohistochemical result; PTC, papillary thyroid carcinoma; PA, pituitary adenoma; PCC, pheochromocytoma; PGL, paraganglioma; pos, positive immunohistochemical result; SDH, succinate dehydrogenase.

* Somatic mutation; [a] Italiano *et al*. BMC Cancer 2012; [b] Dwight T *et al*. Am J Surg Pathol 2013; [c] Miettinen *et al*. Am J Surg Pathol 2013; [d] Mensenkamp *et al*. Paper presented at ENSAT Meeting, Budapest, Hungary, 22–23 November 2013; [e] Belinsky *et al*. Front Oncol 2013; [f] Dwight T *et al*. J Clin Endocrinol Metab 2013; [g] Gill *et al*. Pathology 2013; [h] Tenorio Jiménez *et al*. Endocr Pathol 2012; [i] van Nederveen *et al*. Lancet Oncol 2009; [j] Sjursen *et al*. Fam Cancer 2013; [k] Papathomas *et al*. Eur J Endocrinol 2014.

Genotype–phenotype correlation analysis

Two genotype–phenotype correlations were highlighted. The first was the high frequency of *SDHB* mutations in malignant PCCs/PGLs: 76% of the mutations identified in malignant cases occurred in *SDHB* (**Figure 1a**). The association between *SDHB* mutations and malignancy has been reported many times, but only a few studies provide mechanistic insights [36–40]. Taking the results of those studies together, the association between malignancy and *SDHB* mutations might be due to a hypermethylator phenotype that was observed in *SDHB*-mutated PCCs/PGLs in particular [37]. This hypermethylation would lead to a high epigenetic silencing of genes that play a role in differentiation and in the epithelial-to-mesenchymal transition [36, 37]. By contrast, *SDHA* mutations seem to show no association with malignancy. This could be because of the small numbers of *SDHA*-mutated cases known to date; only seven different *SDHA* mutations have been identified in PCCs/PGLs, and there is a lack of follow-up of patients with apparently benign disease (**Figure 1a**).

The rarity of *SDHA*-associated PCC/PGL might be related to the low frequency of 5p15 loss seen in PCC and PGL, which is the chromosomal location of *SDHA*. By contrast, PCC and PGL show high frequencies of loss of 1p36 and 11q23, the chromosomal areas where *SDHB* and *SDHD* are located, respectively [6].

The second strong genotype–phenotype correlation concerns the association of GIST with *SDHA*; 47% of the mutations reported in this tumor type occurred in *SDHA* (**Figure 1a**). It was recently shown that *SDHA* mutations occur in ~30% of *SDH*-deficient GISTs. These GISTs occur in patients at an older age and have a lower female-to-male ratio than other *SDH*-deficient malignancies. No differences in histology between *SDHA* WT and mutant GISTs have been discerned [41]. Most *SDH*-deficient GISTs encompass tumors arising in Carney–Stratakis syndrome and the Carney triad, in pediatric GISTs, and in a subset of apparently sporadic *KIT*/*PDGFRA* WT GISTs [41,42]. These GISTs are characterized by unique clinicopathological features and biological properties: (**i**) female preponderance; (**ii**) gastric location (predilection for the distal stomach/antrum); (**iii**) common multifocality; (**iv**) a multinodular/plexiform growth pattern; (**v**) epithelioid cytomorphology, either pure or combined with a spindle-cell component; (**vi**) SDHA and/or SDHB immunonegativity; (**vii**) KIT (and DOG1) immunopositivity despite the lack of *KIT*/*PDGFRA* mutations; (**viii**) metastatic potential (often to lymph nodes); (**ix**) a relatively indolent clinical course, even in the presence of metastatic disease; and (**x**) insensitivity to imatinib [41, 42].

With regard to RCCs and PAs, the number of reported mutations is too low (30 and 10, respectively) to draw reliable conclusions about any potential genotype–phenotype correlations, although it must be noted that 83% of the mutations identified in RCCs occurred in *SDHB* (**Figure 1a**). *SDH*-deficient RCC is a unique subtype of RCC that has been recently accepted as a provisional entity in the 2013 International Society of Urological Pathology Vancouver Classification [43]. *SDH*-deficient RCCs display a slight male predisposition, present as bilateral tumors in 25% of cases, and have stereotypical morphologic features, that is, solid or focally cystic growth, uniform cytology with eosinophilic flocculent cytoplasm, intracytoplasmic vacuolations and inclusions, round to oval low-grade nuclei, and SDHB immunonegativity [44, 45]. SDHA-negative IHC was reported once, but no mutational screening of *SDHA* was performed [44]. Despite the fact that *SDH*-deficient RCC is capable of progression, metastatic disease is rare in the absence of high-grade nuclear atypia or coagulative necrosis [44, 45].

Finally, *SDH*-deficient PAs seem likely to be prolactin-secreting (and/or prolactin-immunopositive) and, to a lesser extent, growth hormone–secreting macroadenomas. They are usually accompanied by a personal history of PCC/PGL and have a slight male predisposition [46, 47].

***In silico* analysis association with SDHA/SDHB IHC**

The prediction of the impact of an *SDH* mutation was associated with available SDHA and/or SDHB IHC data, as SDHA/ SDHB IHC is predictive of the presence of an *SDH* mutation. Indeed, a negative staining for SDHB on IHC indicates a mutation in any of the *SDH* subunits, whereas a negative SDHA staining on IHC exclusively predicts the presence of an *SDHA* mutation. In the majority of cases, bioinformatics analysis, predicting the effect of nonsynonymous mutations, was consistent with the available IHC results (**Supplementary Table S1** online). However, some exceptions were reported. Two previously reported *SDHA* mutations (c.113A>T; p.Asp38Val and c.1969G>A; p.Val657Ile) were predicted as nondeleterious and had a Cscore lower than 15 but were negative for SDHA and SDHB [48, 49] (**Table 1**). The p.Val657Ile mutation was reported in COSMIC, and both mutations have been reported in a sample population: p.Asp38Val; rs34635677; minor allele frequency: T = 0.012/26 and p.Val657Ile; rs6962; minor allele frequency: A = 0.161/351 (dbSNP; http://www.ncbi.nlm.nih.gov/SNP/). These discrepancies could reflect the fact that these variants might affect the epitope recognized by the SDHA antibody, explaining the absence of staining on IHC [48, 49]. However, this is highly unlikely because both SDHA and SDHB are negative on IHC, indicating a deleterious effect of the variant on the protein [48, 49]. Finally, these discrepancies could also be due to staining artifacts. Nonconsistent IHC results were also observed

for 9 mutations predicted as damaging and having a Cscore higher than 15 but having positive IHC results (**Table 1**). Three discrepancies occurred in *SDHA* and showed SDHB-negative and SDHA-positive IHC (c.562C>T; p.Arg188Trp, c.818C>T; p.Thr273Ile, and c.1361C>A; p.Ala454Glu) [41, 50]. Two other *SDHA* mutations (c.778G>A; p.Gly260Arg and c.1873C>T; p.His625Tyr) were positive for SDHB and SDHA on IHC [51, 52]. Another one was in *SDHC* (c.380A>G; p.His127Arg), and the last three were in *SDHD* (c.14G>A; p.Trp5*, c.149A>G; p.His50Arg, and c.274G>T; p.Asp92Tyr) [46, 53–55]. Among these *SDHD* mutations, one resulted in a stop codon (p.Trp5*), which cannot be managed by the prediction tools we used but can be considered to be damaging because the mutation results in a severely truncated protein. This nonsense variant was identified in a patient suffering from Carney–Stratakis syndrome, with the PGL showing positive SDHB on IHC and the GIST presenting with a negative SDHB on IHC, potentially indicating two different second hit events in the *SDHD* gene [54]. Another *SDHD* mutation, p.Asp92Tyr, was reported in two patients affected by PAs; one PA showed an SDHB-negative IHC and the other PA still expressed SDHB [46]. Nevertheless, this variant was observed in many PCCs/PGLs that had corresponding SDHB-negative IHC [34]. Therefore, these few discrepant results might be due to staining artifacts, a wrong interpretation, an undetected mutation, or another mechanism of inactivation.

Although SDHA and SDHB IHC reliably identifies the presence of *SDH* mutations, the previous findings show that positive staining has been observed in tumors with mutations predicted as damaging. Therefore, in addition to IHC, bioinformatics analysis should be used as a tool to support the definition of the pathogenicity of *SDH* variants of unknown significance. Functional studies could provide more proof to identify the pathogenicity of uncertain variants.

Mechanisms of biallelic inactivation

SDH genes function as tumor suppressor genes (TSGs) with LOH as the predominant mechanism of inactivation (and somatic mutation less frequently) of the WT allele (**Supplementary Table S2** online). However, different mechanisms, including biallelic inactivating *SDH* mutations and epigenetic silencing, can coexist (e.g., *SDH*-deficient GISTs) [48, 49, 56–60] (**Supplementary Table S2** online). Notably, two sisters with an *SDHA* mutation (c.91C>T, p.Arg31*) were affected by SDHA/SDHB-immunonegative GISTs; one displayed LOH, whereas the other showed retention of heterozygosity, probably indicating a different mechanism of inactivation, such as promoter methylation of the WT allele [60]. However, an undetected mutation seems to be the most likely mechanism. In addition, haploinsufficient and/or dominant negative effects for the *SDH* genes could be another mechanism of inactivation and have been indicated in

particular cases: (**i**) bilateral adrenal medullary hyperplasia associated with a germline *SDHB* mutation (c.587G>A, p.Cys196Tyr) showing retention of heterozygosity [61]; (**ii** and **iii**) SDHA/SDHB immunonegative GIST and PA in patients with *SDHA* mutations (c.91C>T, p.Arg31*; c.1873C>T, p.His625Tyr) displaying either retention of heterozygosity or paradoxical loss of the mutated *SDHD* allele, respectively [51, 60]; (**iv**) PCCs without loss of the WT *SDHD* allele arising in patients with *SDHA* mutations (c.341A>G, p.Tyr114Cys; c.441delG, p.Gly148Alafs*20) [62]; and (**v**) somatic *SDHD* inactivation associated with consistent reduction of transcript levels in neural crest–derived, neuroendocrine, and gastrointestinal tumors [38, 63]. A recent report showed that somatic *SDHC* hypermethylation might be the cause of the Carney triad, a multitumoral syndrome of unknown etiology affecting five organs: paraganglia (PGL), adrenal gland (adrenocortical adenoma and PCC), lung (chondroma), stomach (GIST), and esophagus (leiomyoma) [64, 65]. This suggests that epigenetic silencing of *SDH* could also be an important mechanism involved in tumor development and could explain SDH deficiency in the absence of germ-line mutations.

Tumorigenesis caused by haploinsufficiency seems unlikely because *SDHD*, *SDHD/H19*, and *SDHB* knockout mice, as well as conditional or inducible tissue-specific *SDHD* knockouts, do not develop tumors or any other genotype-related pathology except for a slight carotid body hyperplasia [66–69] (Judith Favier, personal communication).

Tumor suppressor effect in *SDH*-related tumorigenesis

TSG-related, tissue-specific tumorigenesis may be attributed to a complex interplay, but novel evidence points to a model of TSG loss–driven tumorigenesis, whereas haploinsufficiency might act synergistically with oncogenic signals in a tissue specific manner [70–72]. With regard to *SDH*-related oncogenesis, it has been suggested that the tumor phenotypes could be affected by the size of *SDH* deletions because they can encompass closely located TSGs and compromise their function, which could lead to unusual phenotypes [73, 74]. In addition, some research suggests that germ-line and somatic interactions could account for the clinical variability observed among carriers of an identical *SDH* germ-line mutation, including those within the same family [46, 75–77]. In this context, the occurrence of *SDH*-related tumors is extremely rare in the absence of a germ-line mutation. Indeed, only a few cases have been reported: one extra-adrenal PGL harboring a somatic *SDHB* mutation (c.299C>T, p.Ser100Phe) and a somatic *SDHD* mutation in a PCC (c.242C>T, p.Pro81Leu), both displaying LOH; one PA harboring two somatic *SDHA* mutations (c.725_736del; c.989_990insTA); and tumors from four patients with the Carney triad showing a DNA hypermethylation of *SDHC* [47, 65, 78, 79].

Although familial PCC/PGL syndromes were thought to predispose only to PCCs and PGLs, different tumors such as GISTs, RCCs, and PAs have expanded the tumor spectrum associated with *SDH* mutations [77]. No patient has developed all tumor types, indicating an incomplete penetrance of the syndrome similar to that seen in the Carney triad [64]. To explain the issue of cell specificity, Hoekstra and Bayley [69] pointed toward differences of cell types either in their requirements for adenosine triphosphate, redox agents, and TCA cycle–generated metabolic intermediates during development and/or throughout life or in their response to cancerous transformation. Importantly, both *SDH* mutant PCCs/PGLs as well as GISTs have been recently shown to display a hypermethylator phenotype, exposing a vital interplay among succinate metabolism, epigenetic disruptions, and tissue-specific tumorigenesis [37, 65, 80].

CONCLUSION

Familial PCC/PGL syndromes were initially thought to predispose only to PCCs and PGLs, but several tumors, including GISTs, RCCs, and PAs, have expanded the *SDH*-associated tumor spectrum. Extensive clinical variability can be expected, even among carriers of an identical *SDH* germ-line mutation, with tumor phenotypes being only partially expressed, as in the Carney–Stratakis dyad.

Previous reports of genotype–phenotype correlations showed that *SDHB* mutations are associated with a high risk of malignancy, and *SDHD* mutations are associated with head and neck PGL [81, 82]. Our results show an additional strong genotype– phenotype correlation, as *SDHA* mutations seem to be associated with *SDH*-deficient GISTs.

In addition, the absence of SDHA and/or SDHB expression in tumor tissue on IHC is an indicator of the functional absence of one of the *SDH* genes. Therefore, SDHA/SDHB IHC, along with *in silico* analysis and tumor LOH analysis, seem to be valuable tools in determining the potential pathogenicity of *SDH* variants and therefore have significant implications in the new era of comprehensive next-generation sequencing–based approaches for the analysis of PCC/PGL susceptibility genes. Although questions about some incidental cases remain, based on data from the current comprehensive literature, we demonstrated that the *SDH* genes behave as bona fide TSGs with biallelic inactivation through combinations of germ-line mutations, somatic mutations, LOH, and epigenetic silencing, contributing to a specific spectrum of malignancies.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/gim

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Chapter 5

Non-pheochromocytoma (PCC)/paraganglioma (PGL) tumors in patients with succinate dehydrogenase-related PCC–PGL syndromes: a clinicopathological and molecular analysis

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ABSTRACT

Objective: Although the succinate dehydrogenase (SDH)-related tumor spectrum has been recently expanded, there are only rare reports of non-pheochromocytoma/paraganglioma tumors in *SDHx*-mutated patients. Therefore, questions still remain unresolved concerning the aforementioned tumors with regard to their pathogenesis, clinicopathological phenotype, and even causal relatedness to *SDHx* mutations. Absence of SDHB expression in tumors derived from tissues susceptible to SDH deficiency is not fully elucidated.

Design and methods: Three unrelated *SDHD* patients, two with pituitary adenoma (PA) and one with papillary thyroid carcinoma (PTC), and three *SDHB* patients affected by renal cell carcinomas (RCCs) were identified from four European centers. SDHA/SDHB immunohistochemistry (IHC), *SDHx* mutation analysis, and loss of heterozygosity analysis of the involved *SDHx* gene were performed on all tumors. A cohort of 348 tumors of unknown *SDHx* mutational status, including renal tumors, PTCs, PAs, neuroblastic tumors, seminomas, and adenomatoid tumors, was investigated by SDHB IHC.

Results: Of the six index patients, all RCCs and one PA displayed SDHB immunonegativity in contrast to the other PA and PTC. All immunonegative tumors demonstrated loss of the WT allele, indicating bi-allelic inactivation of the germline mutated gene. Of 348 tumors, one clear cell RCC exhibited partial loss of SDHB expression.

Conclusions: These findings strengthen the etiological association of *SDHx* genes with pituitary neoplasia and provide evidence against a link between PTC and *SDHx* mutations. Somatic deletions seem to constitute the second hit in SDHB-related renal neoplasia, while *SDHx* alterations do not appear to be primary drivers in sporadic tumorigenesis from tissues affected by SDH deficiency.

INTRODUCTION

Familial paraganglioma (PGL) syndromes, caused by *SDHx* (*A, B, C, D, and -AF2*) mutations, are rare syndromes inherited as autosomal dominant traits with *SDHD* and *SDHAF2* mutations being associated with a striking parent of origin phenotypic expression [1]. The *SDHA*/*B*/*C*/D genes encode for the four subunits of succinate dehydrogenase (SDH) or mitochondrial complex II, while the *SDHAF2* gene encodes SDH complex assembly factor 2 (*SDHAF2*) that ensures flavination of SDHA, which is essential for the functional and structural integrity of the SDH complex [2]. Mitochondrial complex II, bound to the inner mitochondrial membrane, is the only enzyme participating both in the tricarboxylic acid/Krebs cycle and oxidative phosphorylation and thereby links deregulation of these cellular functions to tumorigenesis [2].

Although familial PGL syndromes were initially thought to predispose only for pheochromocytoma (PCC) and PGL, other tumor types such as gastrointestinal stromal tumors (GISTs) [2, 3], renal cell carcinomas (RCCs) [4], and pituitary adenomas (PAs) [5, 6] have expanded the *SDHx*-associated tumor spectrum. Several other neoplasms have been reported in *SDHx* mutation carriers including papillary thyroid carcinoma (PTC), medullary thyroid carcinoma, pancreatic neuroendocrine tumor, adrenal cortical adenoma, neuroblastoma (NBL), ganglioneuroma (GN), adenomatoid tumor of the adrenal gland, melanoma, lung cancer, breast carcinoma, oesophageal cancer, rectal and ovarian carcinomas, uterine adenocarcinoma, uterine leiomyoma, testicular seminoma, bladder cancer, meningioma, oligodendroglioma, cecal polyps, and hematolymphoid malignancies [7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19].

However, whether these tumors coincidentally occurred in these patients or are causally related to the *SDHx* germline mutation is largely unknown. Biallelic inactivation of *SDHx* genes has only been reported in six RCC cases [8, 16, 20, 21], two PAs [5, 6], one NBL [14], and one testicular seminoma [15] but was not identified in a PA [7], PTC [16], or small-cell lung carcinoma [8]. Despite the fact that *SDHx* inactivation may contribute to these particular phenotypes, the significance of this contribution remains unclear given the relative lack of studies displaying an increased lifetime risk for these tumors arising in the context of an SDH-deficient state [22].

With regard to *SDHB*-related RCCs, two independent groups have described specific morphological features [11, 23], suggesting a genotype–phenotype association similar to what has been described for other hereditary forms of *VHL-*, *MET-*, *FLCN-*, *FH-*, *MITF-*, *TSC1*/*2-*, and *PTEN*-related renal neoplasia [24]. By contrast, previous reports

of a diverse histopathological spectrum, encompassing oncocytoma [14, 25], clear cell RCC [26, 27], eosinophilic chromophobe RCC [27], papillary RCC (type II) [28, 29, 30], poorly differentiated tumor with a papillary architecture and sarcomatoid areas [11, 31], and angiomyolipoma [32], indicated morphological heterogeneity in *SDHB*-related neoplasia.

In an effort (**i**) to determine whether the occurrence of two PAs was related to *SDHD* mutations, (**ii**) to elucidate PTC as a component of the *SDHx*-related tumor spectrum, and (**iii**) to search for potential genotype–phenotype correlations and clarify the nature of the second hit in *SDHB*-associated renal neoplasia, seven tumors from six *SDHB*- or *SDHD*-mutated patients were meticulously investigated. Moreover, we explored loss of the SDH complex by SDHB immunohistochemistry (IHC) in a large series of 348 tumors of unknown *SDHx* mutational status, including renal tumors, PAs, PTCs, neuroblastic tumors, seminomas, and adenomatoid tumors.

SUBJECTS AND METHODS

Tissue samples

For the case series, archival specimens of tumor and normal formalin-fixed paraffin-embedded (FFPE) tissues were provided by four hospitals from The Netherlands (Erasmus MC, University Medical Center (EMC) and Leiden University Medical Center (LUMC)), Norway (Oslo University Hospital), and Germany (University Medical Center of the Johannes Gutenberg University Mainz). All available cases ($n = 6$) were ascertained from the histopathology archives at each center. Clinical and genetic characteristics of these patients are detailed in **Table 1**. Informed consent was obtained for genetic analysis and access to the clinical data in accordance with institutional guidelines.

The second series included 348 tumors of unknown *SDHx* mutational status diagnosed at Erasmus MC (130 renal tumors (80 clear cell RCCs, 19 papillary RCCs,15 chromophobe RCCs, and 16 oncocytomas), 60 PTCs, 47 peripheral neuroblastic tumors (38 primary tumors and nine metastases), and four composite PCC/GN, 50 seminomas, ten adenomatoid tumors, and 41 PAs (27 GH-, eight PRL-, one FSH-, and one ACTH-secreting and/or positive on IHC as well as four non-functional ones)) and at the Department of Pathology, University Health Network (six PAs (acidophil stem cell adenomas)). These 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.

Table 1. Clinical and molecular characteristics of non-PCC/PGL tumors in *SDHB*/*SDHD*-mutated patients. PCC and/or PGLs were not subjected to genetic analyses but to SDHB/SDHA IHC displaying SDHB immunonegativity and SDHA immunopositivity.

Case *No*	Age [a]/ Sex	Germline *SDH-x* mutation	Tumours observed (age at detection)	SDHB IHC	SDHA IHC	Second hit [b] (LOH)	Status at last follow up/ Age
1	60/ M	***SDHD*** c.274G>T p.Asp92Tyr	HN PGLs **PA** (60yr) PCC (62yr)	Neg	Pos	Pos	AWED/ 68
2	56/ F	***SDHD*** c.274G>T p.Asp92Tyr	HN PGLs (56yr) **PA** (56yr)	Pos	Pos	Neg	68
3	25/ F	***SDHD*** c.14G>A p.Trp5X	CBT (17yr) GJT (17yr) CBT (25yr) **PTC** (25yr) PCCs (26yr) ea PGL (26yr)	Pos	Pos	Neg [c]	27
4 [d]	25/ M	***SDHB*** c.3G>A p.Met1Ile	ea PGL (25yr) **RCC1** (R) (25yr) **RCC2** (R) (25yr) RCC (L) (31yr)	Neg Neg	Pos Pos	Pos Pos	31
5	23/ M	***SDHB*** c.3G>A p.Met1Ile	**RCC** (23yr)	Neg	Pos	Pos	28
6	36/ M	***SDHB*** exon 3 del	GJT (30yr) CBT (34yr) **RCC** (36yr)	Neg	Pos	Pos	46

Abbreviations: AWD, alive with disease; AWED, alive without evidence of disease; CBT, carotid body tumor; ea PGL, extra-adrenal paraganglioma; GJT, glomus jugulare tumor; HN PGL, head and neck paraganglioma; IHC, immunohistochemistry; L, left; NA, not available; PA, pituitary adenoma; PCC, pheochromocytoma; PTC, papillary thyroid carcinoma; R, right; RCC, renal cell carcinoma.

[a] Years at diagnosis of non-PCC/PGL tumor.

[b] None of the evaluated tumors displayed a somatic mutation as a second hit.

[c] Sequencing chromatograms of PTC DNA displayed (i) both the mutated and the WT allele at a ratio 50:50, (ii) no somatic mutation as a second hit, and (iii) retention for a microsatellite marker telomeric to SDHD with LOH analysis. It should be noted that the patient was homozygous (not informative) for two markers centromeric to SDHD (LOH electropherograms not shown).

[d] Non-PCC/PGL tumors of this sibling were tested in Norway.

SDHA/SDHB IHC

All non-PCC/PGL tumors from three *SDHB*- and three *SDHD*-mutated patients were analyzed with SDHA and SDHB IHC. Stainings were performed on 4–5 μm sections of FFPE blocks as described previously [9, 33]. The following primary antibodies against SDHA and SDHB were used: mouse monoclonal 2E3GC12FB2AE2 (Mitosciences, Abcam; 1:500) and rabbit polyclonal HPA002868 (Sigma–Aldrich Corp.; 1:400) respectively. All 348 tumors from the second series were initially analyzed with SDHB IHC (and SDHA IHC in the eventuality of SDHB immunonegativity). If the internal control (granular staining in endothelial cells) was positive, slides were considered suitable. From SDHB immunonegative/SDHA immunopositive tumors, (**i**) the entire *SDHB*, *SDHC*, *SDHD*, and *SDHAF2* coding sequences were assessed for mutations and large intragenic deletions at the germline and somatic level by direct sequencing and multiplex ligation-dependent probe amplification (MLPA) assay with a commercially available kit (SALSA MLPA P226-B2; MRC Holland, Amsterdam, The Netherlands) and (**ii**) loss of heterozygosity (LOH) analysis was performed for polymorphic microsatellite markers flanking the *SDHB*, *SDHC*, *SDHD*, and *SDHAF2* genes.

Mutation screening

DNA isolation from tumors was carried out using standard procedures following manual microdissection (to assess somatic mutations as a potential second hit). All tumor samples were estimated to contain at least 80% neoplastic cells. The full coding sequence, including intron–exon boundaries, of the *SDHD* and *SDHB* genes was screened by direct sequencing in forward and reverse orientation (experimental details available on request).

LOH analysis

LOH analysis was performed for polymorphic microsatellite markers flanking either the *SDHB* or the *SDHD* gene. For this, PCR was performed with fluorescence-labeled primers (Invitrogen; primer sequences are available on request) for 35 cycles with an annealing temperature of 60 *8*C and amplified products analyzed, along with LIZ 500 size standard (Applied Biosystems), using capillary electrophoresis on an ABI 3130-XL genetic analyzer (Applied Biosystems). Data were analyzed using GeneMarker Software (Soft-Genetics LLC, State College, PA, USA).

RESULTS

Case series

Case 1 ► A 60-year-old male with a history of multiple head and neck PGLs (HN PGLs) and a previously diagnosed germline c.274G>T (p.Asp92Tyr) *SDHD* gene mutation presented with a PRL-producing macroadenoma causing bitemporal hemianopsia and was treated with dopamine agonists, subsequently leading to PA shrinkage [10]. Two years later, the patient developed meningitis and liquorrhoea, for which he was treated with antibiotics and underwent a transsphenoidal partial resection of the macroadenoma along with restoration of the sella turcica. The same year, a right-sided PCC was laparoscopically resected. Postoperatively, urinary catecholamine excretion normalized. On the preoperative magnetic resonance imaging (MRI), an additional iodine-123-metaiodobenzylguanidine (MIBG)-negative 7 mm nodule was visualized in the left adrenal gland, suspicious of an adenoma, for which a wait and scan strategy has been followed. Endocrine tests revealed no hormone production by this nodule. Owing to increasing prolactin levels postoperatively and residual tumor on the pituitary MRI, cabergoline therapy was reintroduced leading eventually to normal prolactin levels. Family history was positive for HN PGL, but negative for pituitary tumors. The patient is alive 6 years after the resection of the macroadenoma and PCC and is receiving hormone replacement for hypopituitarism with levothyroxine, hydrocortisone, androgel, and minrin.

Case 2 ► A 56-year-old female with a history of type 2 diabetes mellitus, hypertension, and a nontoxic nodular goiter was referred due to a right-sided vocal cord paralysis on the basis of a vagal PGL [10]. On further evaluation, bilateral carotid body tumors were revealed. Screening MRI along with endocrine tests demonstrated a GH-producing pituitary macroadenoma, which was partially removed by transsphenoidal resection followed by treatment with somatostatin analogs. The diagnosis was confirmed on histopathological grounds (**Figure 1)**. The patient was subsequently shown to harbor a heterozygous germline mutation c.274G>T (p.Asp92Tyr) in the *SDHD* gene. Family history was positive for HN PGL (father, two sisters) and gastric GIST (sister), while negative for pituitary tumors. The patient is alive with intact pituitary function 12 years after surgery.

Case 3 ► A previously reported 25-year-old female with a history of HN PGLs was operated on for a left carotid body tumor and PTC (T1N0 unicentric tumor in the right lobe; **Figure 2**) [34, 35]. One year later, she presented with bilateral PCCs and an intra-abdominal extra-adrenal (ea) PGL and was subsequently shown to harbor a heterozygous

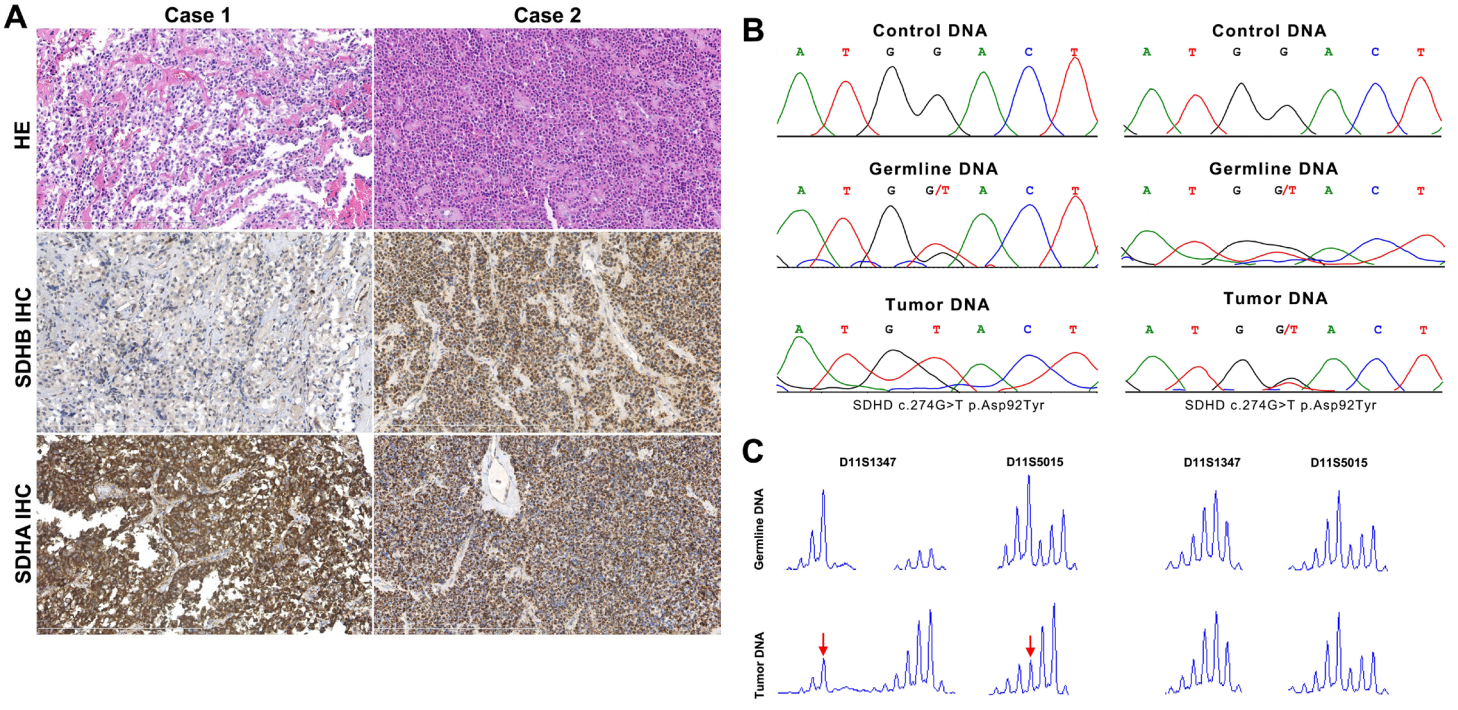

Figure 1. (**A**) H&E staining and SDHB / SDHA IHC in two pituitary adenomas arising in two unrelated patients carrying the *SDHD* c.274G>T (p.Asp92Tyr) mutation: case *no* 1 showed loss of SDHB expression in the neoplastic cells with normal (endothelial) cells serving as positive internal controls, while case *no* 2 retained SDHB labelling; on reticulin stain, the latter revealed breakdown of the reticulin fiber network thereby excluding the possibility of a hyperplastic SDHB ^{pos} lesion (image not shown). Both tumors showed strong positive staining for SDHA. (**B**) Sequencing chromatograms of tumor DNA and healthy germline tissue in cases *no* 1-2: mutational analysis revealed the germline *SDHD* c.274G>T (p.Asp92Tyr) mutation in both the pituitary adenomas and the corresponding normal tissues; only case *no* 1 displayed relative loss of the wild type *SDHD* allele. (**C**) Loss of heterozygosity (LOH) electropherograms of cases *no* 1-2: LOH in the pituitary adenoma (case *no* 1) was confirmed using polymorphic microsatellite markers flanking the *SDHD* locus. The red arrows indicate the allele with relative loss. LOH analysis of the other pituitary adenoma (case *no* 2) exhibited retention of heterozygosity.

germline *SDHD* mutation c.14G>A (p.Trp5X). Although there was suspicion based on imaging studies for metastatic foci affecting the lungs and a peri-pancreatic lymph node, no biopsy was performed in order to confirm and specify the origin of metastases. The patient was lost to follow-up 12 months postoperatively. No other family members were evaluated for *SDHD* mutations or screened for relevant tumors.

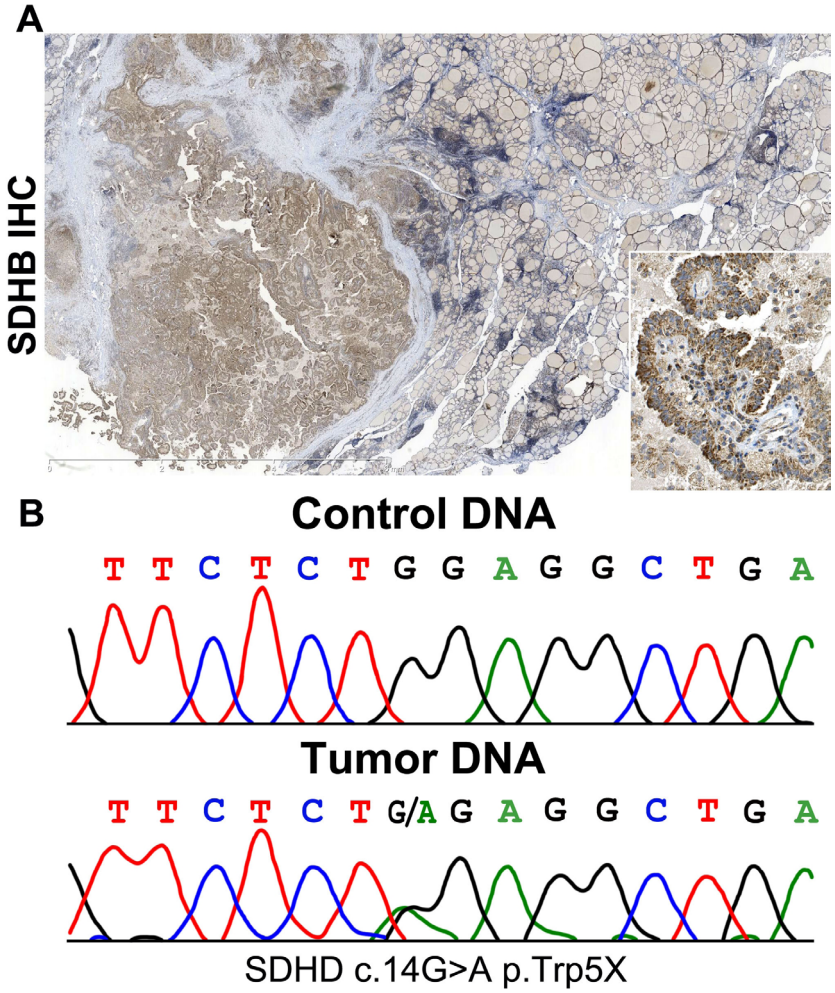

Figure 2. (**A**) SDHB IHC in a papillary thyroid carcinoma arising in an *SDHD*-mutated individual (case *no* 3). At a higher magnification (*inset*), there is strong granular cytoplasmic staining for SDHB. (**B**) Sequencing chromatograms of tumor DNA revealed the germline *SDHD* c.14G>A (p.Trp5X) mutation and displayed 50-to-50 ratio of wild-type versus mutated allele, indicating retention of heterozygosity. Heterozygosity was retained for a microsatellite marker telomeric to *SDHD*, but the patient was homozygous (not informative) for two markers on the centromeric side (LOH electropherograms not shown).

Cases 4 and 5 ► A 25-year-old male (case *no*. 4) presented with periods of headache, throbbing chest pain, sweating, dizziness, and palpitations associated with episodic hypertension. On laboratory investigation, the noradrenaline levels in blood and urine appeared almost 20 times higher than the upper normal limit. Computed tomography (CT) scan of the abdomen revealed a para-aortic mass as well as two large solid tumors in the left kidney with relative homogeneous uptake. MRI scanning of the brain, spine, neck, and thorax showed no abnormalities. The patient underwent a left-sided nephrectomy and resection of the retroperitoneal mass and was subsequently diagnosed with two RCCs measuring 9 cm (RCC1) and 2.8 cm (RCC2) in maximum diameter and an intra-abdominal ea PGL. Histopathologically, the RCCs were originally classified as combined oncocytoma/chromophobe RCC and unclassified RCC respectively. Twenty-four-hour urinary excretion of catecholamines and total metanephrines were postoperatively normalized. *SDHB* sequence analysis revealed a heterozygous germline start codon mutation c.3G>A (p.Met1Ile). An MIBG scan and CT scan performed 6 months postoperatively showed no evidence of recurrent or metastatic disease. However, 6 years postoperatively, a 1.3 cm tumor in the right kidney close to the hilus was treated by open radiofrequency ablation. Identical mutations were identified in both his father and two brothers. One of the latter underwent biochemical and radiological screening for catecholamine-secreting PGLs. Although these investigations were negative for PGLs, the younger sibling (case *no*. 5) suffered from a 2.5 cm renal tumor requiring a right-sided kidney resection at age 23 years. On histopathological grounds, the renal tumor was originally classified as clear cell RCC (Fuhrman grade 2).

Case 6 ► A 47-year-old male was originally diagnosed with a left jugulotympanic PGL at the age of 30 years after a period of unilateral hearing loss and tinnitus. The tumor was surgically resected without major complications. Four years later, a carotid body PGL was detected and treated with radiotherapy. At the age of 36 years, the patient was diagnosed with a renal tumor measuring 13 cm in maximum diameter for which he underwent a left-sided nephrectomy. Histopathologically, the renal tumor was initially diagnosed as eosinophilic chromophobe RCC, while on revision as eosinophilic clear cell RCC (Fuhrman grade 3). Several times hereafter, he suffered from intra-abdominal RCC recurrences and splenic metastasis necessitating surgical resection. Nine years postoperatively, liver metastases were detected on imaging; hence, the patient was treated with radiofrequency ablation and sunitinib. Although being unresponsive to the latter, he is currently alive with disease 11 years postoperatively. On genetic analysis, the patient was subsequently shown to harbor a heterozygous germline *SDHB* mutation (exon 3 deletion). Family history was negative for HN PGL. Results of the SDHA/SDHB IHC, mutation screening, and LOH analysis of non-PCC/PGL tumors (case *no*s 1–6) are detailed in **Table 1**.

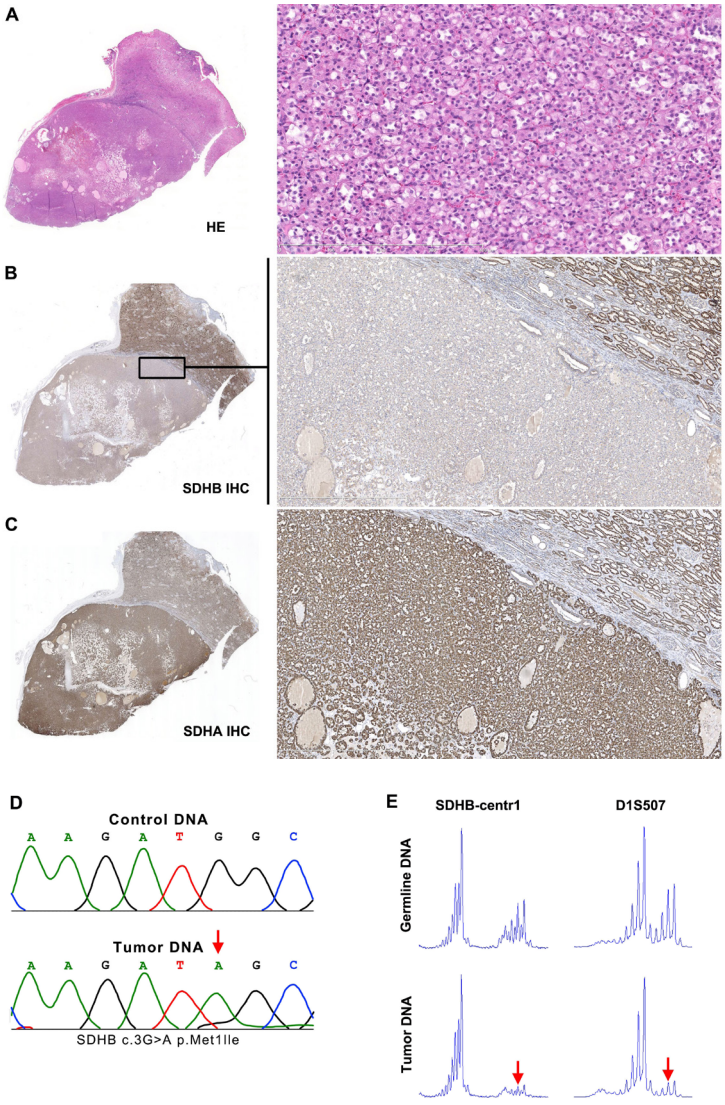

Figure 3. (**A**-**C**) Histological features (H&E staining) and SDHB/SDHA IHC in the RCC of case *no* 5: (**A**) Section of RCC and adjacent non-neoplastic renal parenchyma; the interface with the latter is circumscribed, while there are discrete intratumoral areas of cystic change (*left*). The neoplastic cells are arranged mainly in a nested growth pattern with tubular formations and cytoplasmic inclusions recognized as well (*right*). (**B**) SDHB IHC displaying immunonegativity in the neoplastic cells as opposed to the nontumoral kidney. (**C**) SDHA IHC showing immunopositivity in both neoplastic and non-neoplastic renal compartments. (**D**-**E**) Sequencing chromatogram of tumor DNA and loss of heterozygosity (LOH) electropherogram: (**D**) Sequence of *SDHB* exon 1 in the RCC demonstrating a c.3G>A transversion, leading to a premature stop codon, and relative loss of the wild-type *SDHB* allele. The red arrow indicates the mutation. (**E**) LOH in the RCC was verified using microsatellites linked to the *SDHB* locus. The red arrows indicate the allele with relative loss.

Histopathology of the SDHB-associated RCCs

The RCCs from case *no*s 4–6 shared similar morphological features, as illustrated in **Figure 3** and **Supplementary Figures 1** and **2**, see section on **supplementary data** given at the end of this article. All RCCs displayed characteristic eosinophilic appearance and pale eosinophilic and/or bubbly intracytoplasmic inclusions, as described previously [11, 23]. In particular, the neoplastic cells were polygonal with eosinophilic granular cytoplasm and centrally placed nuclei, which were either relatively uniform with inconspicuous nucleoli (RCC1 case *no*s 4 and 5) or pleomorphic with prominent nucleoli (RCC2 case *no*s 4 and 6). Tumor cells were arranged mainly in nested and solid growth patterns with occasional tubular formations. In addition, case *no*. 6 displayed a focal papillary growth pattern (**Supplementary Figure 2**). All tumors were well-circumscribed exhibiting areas of cystic change, which contained eosinophilic material (**Figure 3**). RCC1 case *no*s 4 and 6 showed necrotic areas. All RCCs demonstrated SDHB immunonegativity and SDHA immunoreactivity, as highlighted in **Figure 3** and **Supplementary Figures 1** and **2**.

***SDHx* alterations in unselected tumors of unknown *SDHx* mutational status**

Of the 348 genetically uncharacterized tumors, one clear cell RCC was partly immunonegative for SDHB (1/130 renal tumors, 0.8%; **Table 2**). Histopathologically, the tumor measuring 9.6 cm in maximum diameter comprised low-grade (Fuhrman grade 1) and high-grade areas (up to Fuhrman grade 4) along with sarcomatoid differentiation and extensive areas of necrosis. Although distinctive cytoplasmic inclusions could not be detected, focal vacuolation was present only in high-grade areas. The latter were found to be SDHB neg/SDHA pos compared with the SDHB pos/SDHA pos low-grade tumor component (**Figure 4**). The patient developed lung metastases 3 months following surgical resection, for which he was treated with sunitinib. However, he was unresponsive and eventually succumbed to metastatic disease 5 months postoperatively at age 55 years.

Sequencing and MLPA analysis of the SDHB neg tumor component and normal tissue for *SDHB*, *SDHC*, *SDHD*, and *SDHAF2* genes revealed large intragenic *SDHD* and *SDHAF2* deletions only in the tumor. Being consistent with the latter, LOH analysis revealed LOH both at the *SDHAF2* locus (Fig. 4) and for a microsatellite marker centromeric to *SDHD*, while the patient was homozygous (not informative) for three markers on the telomeric side (data not shown). No germline *SDHx* mutations or large deletions were detected by direct sequencing or MLPA respectively.

Table 2. Immunohistochemical SDHB losses in 348 neoplasms of unknown *SDHx* mutational status.

Tumor type	SDHB immunonegativity
Renal tumors	1/130
Clear cell renal cell carcinoma	1/80
Papillary renal cell carcinoma	0/19
Chromophobe renal cell carcinoma	0/15
Oncocytoma	0/16
Papillary thyroid carcinoma	0/60
Pituitary adenoma	0/47
Neuroblastic tumors	0/47
Composite PCC/GN	0/4
Testicular seminoma	0/50
Adenomatoid tumor	0/10

Abbreviations: GN, ganglioneuroma; PCC, pheochromocytoma

DISCUSSION

Familial PGL syndromes are multiple neoplasia syndromes primarily characterized by the development of PCCs/PGLs. Consistent with the wide expression and the functional role of the *SDHx* genes, it has been suggested that associations with neoplasms other than PCCs/PGLs may exist [36]. In this study, we further strengthen the link between pituitary neoplasia and germline *SDHx* mutations and suggest that the occurrence of PTC in *SDHx* mutation carriers might rather be coincidental.

In accordance with the classical 'two-hit hypothesis' of Alfred Knudson (the combination of an inactivating germline mutation as a first hit and somatic loss of function of the WT allele as a second hit being essential for the tumor development) [37], one of the PAs of an *SDHD* patient displayed somatic allelic deletion at the *SDHD* locus resulting in SDHB immunonegativity (**Figure 1**). This is consistent with Xekouki *et al.* [6] who observed *SDHD* LOH accompanied by decreased SDH enzymatic activity in one GH-secreting PA and preliminary data concerning another relevant case in the SDH-deficient setting [5] (**Supplementary Table 1**, see section on **supplementary data** given at the end of this article). By contrast, the other PA demonstrated retention of *SDHD* heterozygosity, which is in accordance with the positive SDHB expression (**Figure 1**). These findings indicate that this tumor was coincidental and most likely not caused by involvement of the germline *SDHD* mutation. However, *SDHx* gene haploinsufficiency contributing to the tumor formation cannot be ruled out from our analyses. Haploinsufficient effects for the SDHx genes have been suggested in: (**i**) bilateral adrenal medullary hyperplasia associated with a germline SDHB mutation showing retention of heterozygosity [38]; (**ii**) PCCs without loss of the WT *SDHD* allele arising in *SDHD*-mutated patients [39]; and (**iii**) somatic *SDHD* inactivation being accompanied by consistent reduction of transcript

levels in various tumors [40, 41]. Albeit, in mouse models, no indications were obtained for an *SDHB*/*SDHD* haploinsufficient contribution to tumorigenesis [42, 43] (J Favier 2013, personal communication).

An extensive literature search revealed ten reported cases of PAs arising in *SDHD*/*B*/*C*/*A*-mutated patients (**Supplementary Table 1**), with a further 31 PAs co-occurring with PCCs and/or PGLs pointing to a causative association with *SDHx*, *MEN1*, or other yet unidentified predisposing genes [44, 45, 46]. *SDHx*-mutated patients appear to have a characteristic clinical phenotype with PRL-secreting macroadenomas, and to a lesser extent GH-secreting macroadenomas, usually accompanied with a personal history of PCC/PGL, implying that SDH deficiency might promote tumor growth with lactotrophs being more susceptible to this particular deficient state.

Although PTC has been previously suggested as a component of familial PGL syndromes and the risk of thyroid tumor development in *SDHB* mutation carriers has been estimated at 2.5% up to age 70 years [22], a causal role of SDH deficiency in thyroid tumorigenesis has not yet been established. Arguing against such a causal role, our case displayed SDHB immunoreactivity with retention of the WT allele (**Figure 2**) being in accordance with a previously reported PTC [16], a rather small number of reported PTC-affected individuals carrying germline *SDHx* mutations and a lack of family PTC history in four of five cases in total (**Supplementary Table 2**, see section on **supplementary data** given at the end of this article).

According to prior studies from three large multinational and/or multicenter cohorts [22, 35, 47], examining 110, 116, and 358 *SDHB*/*SDHD* mutation carriers respectively, only six thyroid tumors were identified: two PTCs [35], one thyroid PGL [47, 48], as well as three thyroid tumors of unknown histopathology [22]. Given the high PTC incidence rates (incidence of PTC estimated at 7.3/100 000) [49, 50], it is no surprise that thyroid tumors are relatively frequently encountered as incidental findings in patients who are under active surveillance. Taken together, the occurrence of PTCs in the SDH-deficient state may be either coincidental, reflecting the highly prevalent nature of this particular malignancy, or may be less likely attributable to cross talk of SDH- and PTEN-related signaling pathways leading to tissue-specific Cowden syndrome (CS)/CS-like tumorigenesis [51, 52].

With regard to *SDHB*-related renal neoplasia, all RCC cases (case *no*s 4–6) confirm previous histopathological and SDHB immunohistochemical observations [11, 23], highlighting the fact that distinct morphological traits along with SDHB immunonegativity could

aid in screening for SDHB mutations. In addition, LOH seems to constitute the main mechanism of inactivation of the WT allele in these tumors as observed in *SDHx*-related tumorigenesis [40, 53]. Taking into consideration: (**i**) the rarity of SDHB-related RCC cases [8, 11, 14, 16, 21, 22, 23, 25, 26, 27, 28, 29, 30, 31, 32, 35, 54]; (**ii**) the broad distribution of various germline mutations (missense, nonsense, frameshift, and splice site) and large deletions throughout the SDHB gene (**Supplementary Figure 3**, see section on **supplementary data** given at the end of this article); and (**iii**) the fact that mutations within the same codon can generate variable phenotypes (current study, *SDHB* c.3G>A p.Met1Ile; PCC/PGL and/or RCC), previous challenges in establishing genotype–phenotype correlations become conceivable [22].

Interestingly, one clear cell RCC with sarcomatoid dedifferentiation was found to be partly SDHB immunonegative (1/130, 0.8%; **Figure 4**) being in accordance with other SDHB mutational or immunohistochemical studies [31, 55, 56]. Given that the low-grade tumor component retained SDHB expression, this raises the question as to whether this finding is simply a serendipitous secondary event resulting from genomic instability or has an active role in RCC progression. Mechanisms other than germline *SDHx* mutations, including mutations in as yet unidentified susceptibility gene(s) affecting SDH complex assembly/ function, abnormal functional interactions with mitochondrial proteins, and/or epigenetic alterations, could be responsible for loss of protein expression [57, 58]. Another plausible explanation is that HIF1a accumulation due to impaired hydroxylation in hypoxic areas [59] might have resulted in downregulated SDHB expression either in an auto-regulatory loop [60] or through a putative signaling axis involving HIF1a/microRNA-210/iron-sulfur cluster scaffold protein (ISCU) [61].

By analyzing various tumors from 348 patients of unknown *SDHx* mutational status, all except one were found to be SDHB immunopositive. This is consistent with the notion that mutations of hereditary cancer genes may not be directly involved in tumorigenesis of their sporadic counterparts as reflected in the role of fumarate hydratase (FH) in sporadic vs hereditary leiomyomatosis and RCC [62]. Although a high percentage of apparently sporadic PCC/PGL cases is causatively linked to germline mutations [1], this is not the case for other tumor types. In particular, only 5–8% of RCCs, 5% of follicular cell-derived well-differentiated thyroid cancers, < 5% of PAs, 1–2% of testicular germ cell tumors, and NBLs respectively are familial when compared with 30–35% of PCCs/PGLs [1, 24, 63, 64, 65, 66]. With regard to the adenomatoid tumors, there is not only lack of any association with syndromic or genetic conditions but also no example of a typical two-hit mechanisms of TSG inactivation [12].

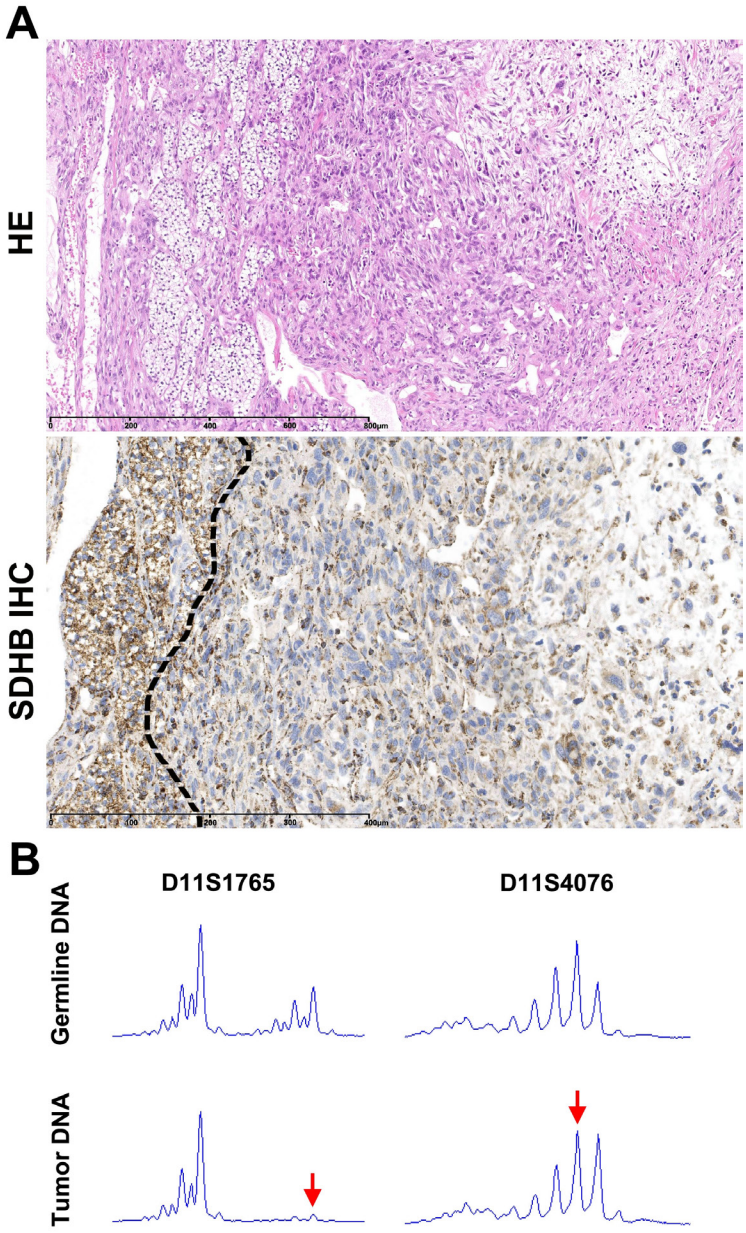

Figure 4. (**A**) H&E staining and SDHB IHC in a clear cell RCC with sarcomatous dedifferentiation. Both low-grade and high-grade tumor components are illustrated with corresponding SDHB immunopositive/immunonegative areas being distinguished by a dotted line; positive staining of normal (endothelial) cells should not be misinterpretd as positive for the neoplastic cells. (**B**) LOH in the SDHB immunonegative component was verified using microsatellites linked to the *SDHAF2* locus. The red arrows indicate the allele with relative loss.

In conclusion, the current study further strengthens the etiological association of *SDHx* genes with pituitary neoplasia, while it does not support a causative link between PTC- and SDH-deficient state. Additionally, we elucidate clinicopathological and genetic aspects of SDHB-related renal neoplasia and report a 0.8% frequency of SDHB immunonegativity in renal tumors, reinforcing the notion that hereditary cancer genes may not always be involved in sporadic tumorigenesis.

Supplementary data: This is linked to the online version of the paper at http://dx.doi.org/10.1530/ EJE-13-0623.

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Chapter 6

Succinate Dehydrogenase (SDH)-Deficient Pancreatic Neuroendocrine Tumor Expands the SDH-Related Tumor Spectrum

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ABSTRACT

Context: Mutations in genes encoding the subunits of succinate dehydrogenase (SDH) can lead to pheochromocytoma/paraganglioma formation. However, *SDH* mutations have also been linked to nonparaganglionic tumors.

Objective: The objective was to investigate which nonparaganglionic tumors belong to the SDH associated tumor spectrum.

Design: This was a retrospective cohort study.

Setting: The setting was a tertiary referral center.

Patients: Patients included all consecutive *SDHA*/*SDHB*/*SDHC* and *SDHD* mutation carriers followed at the Department of Endocrinology of the Leiden University Medical Center who were affected by non-pheochromocytoma/paraganglioma solid tumors.

Main Outcome Measures: Main outcome measures were SDHA/SDHB immunohistochemistry, mutation analysis, and loss of heterozygosity analysis of the involved *SDH*-encoding genes.

Results: Twenty-five of 35 tumors (from 26 patients) showed positive staining on SDHB and SDHA immunohistochemistry. Eight tumors showed negative staining for SDHB and positive staining for SDHA: a pancreatic neuroendocrine tumor, a macroprolactinoma, two gastric gastrointestinal stromal tumors, an abdominal ganglioneuroma, and three renal cell carcinomas. With the exception of the abdominal ganglioneuroma, loss of heterozygosity was detected in all tumors. A prolactinoma in a patient with a germline *SDHA* mutation was the only tumor immunonegative for both SDHA and SDHB. Sanger sequencing of this tumor revealed a somatic mutation (p.D38V) as a likely second hit leading to biallelic inactivation of SDHA. One tumor (breast cancer) showed heterogeneous SDHB staining, positive SDHA staining, and retention of heterozygosity.

Conclusions: This study strengthens the etiological association of *SDH* genes with pituitary neoplasia, renal tumorigenesis, and gastric gastrointestinal stromal tumors. Furthermore, our results indicate that pancreatic neuroendocrine tumor also falls within the SDH-related tumor spectrum.

INTRODUCTION

Mutations in any one of the succinate dehydrogenase (SDH) complex subunits (*SDHA*, *SDHB*, *SDHC*, *SDHD*, and *SDHAF2*) can lead to formation of pheochromocytoma (PCC)/paraganglioma (PGL). Heterozygous germline mutations of *SDHB*, *SDHC*, and *SDHD* cause the well-characterized familial PCC/PGL syndromes known as PGL4, PGL3, and PGL1, respectively [1, 2]. The gene for PGL2 syndrome has been identified as *SDHAF2* (SDH5) [3]. The *SDHA*, *SDHB*, *SDHC*, and*SDHD* genes encode for the four subunits of SDH (mitochondrial complex II), a key respiratory enzyme that links the Krebs cycle and the electron transport chain [4]. The *SDHAF2* gene encodes SDH complex assembly factor 2 (SDHAF2), essential for flavination of the SDHA protein and SDH enzyme activity [2]. If mutations occur in the *SDHA*, *SDHB*, *SDHC*, *SDHD*, or *SDHAF2* genes with corresponding loss of the wild-type allele or a second inactivating mutation, SDHB immunohistochemical staining will become negative [5]. This negative staining for SDHB is now a validated and highly sensitive marker for germline mutations of any of the *SDH* subunits and is a broadly accepted indication of pathogenicity of an *SDH* mutation [6, 7]. In addition, SDHA immunohistochemistry (IHC) is a proven marker for *SDHA* mutations, showing loss of immunoreactivity exclusively in *SDHA*-mutated tumors, whereas non-*SDHA*-mutated tumors, including *SDHB*, *SDHC*, *SDHD*, and *SDHAF2*-mutated cases, show positive SDHA staining [5, 8].

SDHA, *SDHB*, *SDHC*, and *SDHD* mutations have also been linked to gastrointestinal stromal tumor (GIST) [4, 9] and renal-cell carcinoma [10–15]. SDH-deficient renal carcinoma has been accepted as a provisional entity in the 2013 International Society of Urological Pathology Vancouver Classification. Gill *et al*. [16] studied 36 SDH deficient renal carcinomas and showed that these carcinomas had a strong relationship with *SDH* germline mutation. In addition, pituitary adenomas have been reported to be associated with *SDHA*, *SDHB*, *SDHC*, and *SDHD* mutations [12, 17–20]. However, other non-paraganglionic tumors may belong to the *SDH* tumor spectrum. To address this issue, we investigated all non-paraganglionic tumors affecting patients included in the Leiden SDH Mutation Carrier Registry.

SUBJECTS AND METHODS

Subjects

All consecutive *SDHA*, *SDHB*, *SDHC*, and *SDHD* mutation carriers followed at the Department of Endocrinology of the Leiden University Medical Center who were

affected by non-PCC/PGL solid tumors and who gave written informed consent were included. Of the three *SDHA* mutation carriers, one had a non-PCC/PGL tumor. Of the 54 *SDHB* mutation carriers, seven had non-PCC/PGL tumors, of which six were available for investigation. Of the 239 *SDHD* mutation carriers, 22 were affected by non-PCC/PGL tumors. Histological material was unavailable from one patient, and because two additional patients underwent only radiological follow-up, no biopsy or surgically resected material was available. Of the four *SDHC* mutation carriers, one was affected by a non-PCC/PGL tumor. However, this patient did not provide written informed consent and was therefore excluded. In total, 26 patients with 35 non-PCC/PGL tumors were included.

Tissue samples

Archival specimens of tumor and normal formalin-fixed paraffin- embedded tissues were provided by the hospitals where the patients underwent surgery. Clinical and genetic characteristics of the patients are detailed in **Supplemental Table 1**.

SDHA/SDHB IHC

All non-paraganglionic tumors were analyzed with SDHA and SDHB IHC. Formalin-fixed paraffin-embedded tissue sections of 4-μm thickness were stained with commercially available antibodies: mouse monoclonal Ab14715 antibody (1:500 dilution; Mitosciences; Abcam) against SDHA and rabbit polyclonal HPA002868 antibody (1:400 dilution; Sigma-Aldrich Corp) against SDHB. Stainings were performed on an automatic Ventana Benchmark Ultra System (Ventana Medical Systems Inc) using the Ultraview DAB detection system, following heat-induced epitope retrieval with Ventana Cell Conditioning 1 (pH 8.4) at 97°C for 52 and 92 minutes, respectively.

Loss of heterozygosity (LOH) analysis

DNA isolation from SDHB and/or SDHA immunonegative tumors was carried out using standard procedures after manual microdissection. All tumor samples were estimated to contain at least 80% neoplastic cells. LOH analysis of SDHB immunonegative/ SDHA immunopositive tumors was performed using polymorphic microsatellite markers flanking either the *SDHB* (one surrounding a microsatellite located at UCSC chr1: 17,417,100 and D1S507) or the *SDHD* (D11S5015, D11S5017, D11S5019, and D11S1347) gene. Tumor DNA and fluorescently labeled primers (Invitrogen; primer sequences available on request) underwent 35 cycles of PCR at an annealing temperature of 60°C. Amplified products were analyzed, along with LIZ 500 size standard (Applied Biosystems), using capillary electrophoresis on an ABI 3130-XL genetic analyzer (Applied Biosystems). Data were analyzed using GeneMarker Software (Soft- Genetics LLC).

Table 1. Clinicopathological and Molecular Genetic Characteristics of Non-Paraganglionic Tumors Displaying SDHB Immunonegativity or Heterogeneous Immunoexpression Pattern.

Case	Age [a]/ Sex	Germline *SDH* Mutation [¶]	Tumors Observed (Age at Detection, y)	SDHB IHC	SDHA IHC	Second Inactivation Hit [¶¶]	Status at Last Follow-up (Age, y)
1	56/M	*SDHD* p.Asp92Tyr c.274G>T	**pNET** (56) **Oligodendroglioma** (57) GCT (L + R) (56) GVT (R) (56)	Neg Pos	Pos Pos	LOH#	Died (64)
2	61/M [b]	*SDHD* p.Asp92Tyr c.274G>T	**Macroprolactinoma** (61) PCC (R) (61) GJTT (R) (60) GCT (L) (60) GVT (L + R) (60)	Neg	Pos	LOH	AWED (69)
3	38/F [c]	*SDHD* p.Pro81Leu c.242C>T	**Breast cancer** (38) GCT (L) (38)	Heterogeneous [^]	Pos	ROH	Died due to breast cancer (41)
4	55/F	*SDHD* p.Asp92Tyr c.274G>T	**Gastric GIST** (55) GCT (L + R) (50) GVT (L) (50)	Neg	Pos	LOH#	AWED (64)
5	42/M	*SDHB* c.423 + 1G>A	**Abdominal ganglioneuroma** (42) GVT (R) (42)	Neg	Pos	ROH	AWED (50)
6	45/M [d]	*SDHB* c.423 + 1G>A	**RCC L foci1** (45) **RCC L foci2** (45) **RCC R** (45) **Gastric GIST** (45) No PGL	Neg Neg Neg Neg	Pos Pos Pos Pos	LOH LOH LOH LOH	AWED (47)
7	49/F	*SDHA* p.Arg31X c.91C>T	**Macroprolactinoma** (49) GCT (R) (26) GCT (L) (49) **Meningiomas** (49)	Neg Pos	Neg Pos	p.D38V	AWD (meningioma) (65)

Abbreviations: M, male; F, female; pNET, pancreatic neuroendocrine tumor; GCT, glomus caroticum tumor; GVT, glomus vagale tumor; GJTT, glomus jugulotympanicum tumor; RCC, renal cell carcinoma; L, left; R, right; AWD, alive with (non-paraganglionic) disease; AWED, alive without evidence of disease other than head and neck PGL; Pos, positive; Neg, negative.

LOH#, Only one marker (centromeric or telomeric) was informative in each tumor as indicative of LOH. Sanger (direct) sequencing showed loss of the wild-type allele.

[a] Age at diagnosis of non-PCC/PGL tumor.

[b] Patient previously described by Papathomas *et al.* [12].

[c] Patient also carrier of a germline breast cancer 1 (BRCA1) mutation.

[d] Patient previously described by Gill *et al.* [16].

[¶] The germline mutation was documented in all tumors.

[¶¶] Loss of wild-type allele or somatic mutation.

[^] Heterogeneous is defined 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.

Mutation screening

From SDHB immunonegative/SDHA immunopositive tumors without LOH or the lack of informative (centromeric or telomeric) markers, the full coding sequence, including intron exon boundaries, was screened for *SDHD* and *SDHB* mutations at the somatic level either by Sanger (direct) sequencing in forward and reverse orientation or by using an Ion AmpliSeq Custom Panel sequenced on the Ion Torrent Personal Genome Machine (Life Technologies), respectively, as previously described [12, 21]. In addition, Sanger sequencing was also used to confirm the presence of the known mutations in the tumors and to investigate the occurrence of loss of the wild-type allele in all cases with immunonegative SDHB staining.

RESULTS

An overview of the immunohistochemical and sequencing results is shown in **Supplemental Figure 1**. Thirty-five non-paraganglionic tumors from 26 *SDH* mutation carriers were analyzed in the current study (**Supplemental Table 1**). No further analysis was carried out in 25 tumors displaying SDHB and SDHA immunopositivity, with the exception of one GH-producing pituitary adenoma (case 8), because this analysis was conducted previously as reported in Papathomas *et al*. [12]. The 25 SDHB/SDHA immunopositive tumors obtained from 15 *SDHD* and 4 *SDHB* mutation carriers encompassed papillary thyroid carcinoma, melanoma, bladder cancer, endometrial cancer, prostate cancer, testicular cancer, meningioma, basal cell carcinoma, and sebaceous gland carcinoma of the eyelid (**Supplemental Table 1**). The clinicopathological and molecular genetic characteristics of the remaining 10 tumors displaying SDHB immunonegativity (n = 9) or heterogeneous immunoexpression pattern (n = 1) are displayed in **Table 1**. These tumors occurred in seven patients, of which four harbored an *SDHD* germline mutation, two harbored an *SDHB* germline mutation, and one harbored an *SDHA* germline mutation.

In particular, nine tumors showed loss of SDHB expression. Eight of these displayed positive staining for SDHA: a pancreatic neuroendocrine tumor (NET) (case 1; **Figure 1**, A–C), a macroprolactinoma (case 2), an abdominal ganglioneuroma (case 5), two gastric GISTs (cases 4 and 6), and three renal cell carcinomas (case 6). One tumor (case 7) showed loss of SDHB and SDHA expression. Seven of the nine SDHB immunonegative tumors showed LOH for at least one of the microsatellite markers, indicating biallelic inactivation of the given *SDH* gene (**Table 1**). Loss of the wild-type allele was also confirmed by the Sanger sequencing results (**Figure 1**, D and E). Sanger sequencing of the single SDHB/

SDHA-immunonegative macroprolactinoma (case 7) revealed a somatic *SDHA* mutation (p.D38V), along with the germline *SDHA* mutation (p.R31X). In conclusion, eight tumors fulfilled the criteria of biallelic inactivation of the given *SDH* gene (**Table 1**). In contrast, the SDHB immunonegative abdominal ganglioneuroma (case 5) showed retention of heterozygosity (ROH), similar to the single tumor (breast cancer, case 3) exhibiting a heterogeneous SDHB immunoexpression pattern.

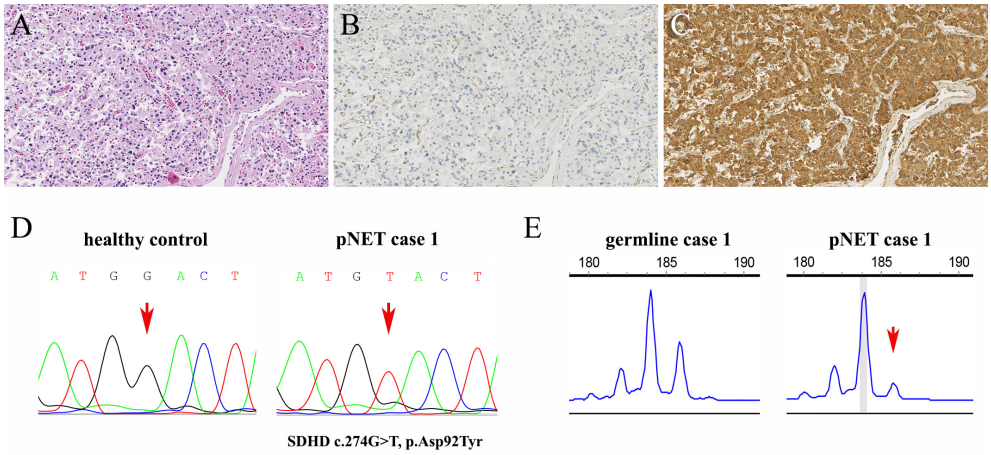

Figure 1. (**A**) Hematoxylin and eosin staining of the pancreatic NET arising in a patient carrying a germline *SDHD* c.274G>T (p.Asp92Tyr) mutation. (**B**) SDHB IHC displaying loss of expression in the neoplastic cells with normal (endothelial) cells serving as positive internal controls. (**C**) SDHA IHC showing immunopositivity in both neoplastic and non-neoplastic cellular compartments. (**D**) Sequencing chromatograms of healthy germline tissue and tumor DNA. Mutational analysis revealed the germline *SDHD* c.274G>T (p.Asp92Tyr) mutation in the pancreatic NET. Note the absence of the wild-type allele indicating LOH. (**E**) LOH Electropherogram. Heterozygosity was lost only for a microsatellite marker (D11S5019) telomeric to the SDHD locus. The red arrows indicate the allele with relative loss. Heterozygosity was retained for a microsatellite marker (D11S5017) centromeric to the SDHD locus, while the patient was homozygous (not informative) for another marker (D11S5015) on the centromeric side (LOH electropherograms not shown). **Abbreviations**: pNET, pancreatic neuroendocrine tumor.

Case 1 originally presented with a pancreatic mass that was eventually diagnosed as a pancreatic NET. An octreotide scan performed to further evaluate the pancreatic mass led to the subsequent detection of head and neck PGLs. Head/neck magnetic resonance imaging (MRI) confirmed the presence of the latter. Genetic analysis identified a germline *SDHD* mutation. The patient's twin sister and brother are both affected by PGLs, as are the father and an uncle (**Figure 2**). After confirmation of the germline *SDHD* mutation, follow-up with urinary analysis for catecholamine excess and head and neck MRIs was initiated. One year later, a brain MRI was performed due to visual field complaints; it

showed a (histologically proven) low grade oligodendroglioma in the right frontal lobe. This resulted in the person being affected by epilepsy seizures. The patient died at the age of 64 years, due to the complications of a pneumosepsis with pleural empyema and left hydropneumothorax. The LOH analysis, together with SDHB immunonegativity, strongly suggests that this tumor is most likely caused by the germline *SDHD* mutation.

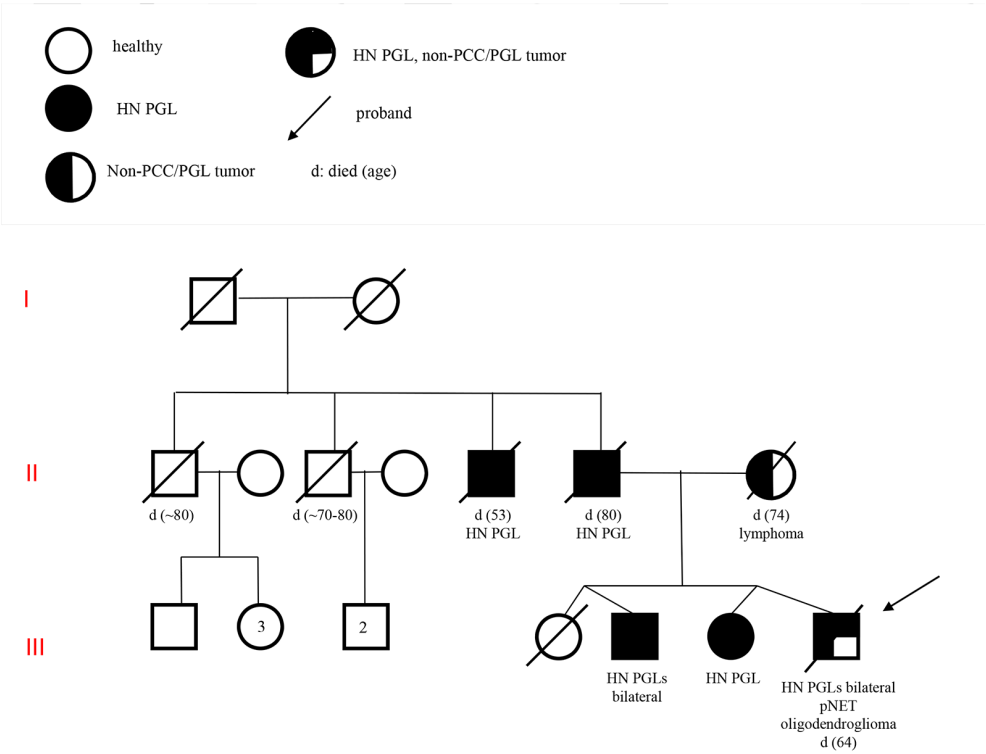

Figure 2. Pedigree from case 1, a patient with a germline *SDHD* (p.Asp92Tyr) mutation with bilateral paragangliomas, a pancreatic NET and an oligodendroglioma. **Abbreviations**: HN PGL, head and neck paraganglioma; pNET, pancreatic neuroendocrine tumor.

DISCUSSION

Our initial immunohistochemical analysis of 35 non-paraganglionic tumors from 26 *SDH* mutation carriers identified and excluded 25 SDHB and SDHA immunopositive tumors. Immunohistochemical and molecular genetic data from eight SDHB immunonegative tumors confirm that a wide range of non-paraganglionic tumors fall within the SDH-related tumor spectrum and suggest that the pancreatic NET may also expand this

spectrum. In particular, we present strong supporting evidence indicating that the pancreatic NET described here very likely arose due to a germline *SDHD* mutation and is therefore a constituent of the *SDHD*-associated tumor spectrum. This is the first report of an association between a germline *SDHD* mutation and pancreatic NET, and as such it expands the spectrum of hereditary pancreatic NETs, previously only attributable to multiple endocrine neoplasia (MEN) type 1, von Hippel-Lindau disease, neurofibromatosis 1, and the tuberous sclerosis complex [22].

The occurrence of a pancreatic NET in a *SDHD* mutation carrier is rare. Of the 239 patients with a germline *SDHD* mutation enrolled in the Leiden SDH Mutation Carrier Registry, only one of 22 patients suffering from a non-paraganglionic tumor was affected by a pancreatic NET. Despite the rarity, this finding might have potential implications for the surveillance of patients with a germline *SDHD* mutation. In The Netherlands, the surveillance protocol for *SDHD* mutation carriers includes urinary analysis for catecholamine excess every 2 years and MRI of the head/neck region every 3 years. Abdominal imaging is only advised when there is evidence of catecholamine excess. The addition of pancreatic NET to the SDH-related tumor spectrum suggests that it might be advisable to amend surveillance protocols, with the addition of standard abdominal imaging studies. Because the latter are not currently included in surveillance protocols in The Netherlands, the possibility that other patients in our registry carry undetected pancreatic NETs cannot be ruled out. However, given the rare occurrence rate in our study, further studies are needed to definitely amend surveillance protocols.

This study also included a patient with a germline *SDHA* mutation and an associated pituitary adenoma. This case, along with an additional case previously described by Papathomas *et al*. [12], suggests an important role for *SDH* mutations in hypophyseal tumorigenesis. These cases, together with the large patient cohort described by Dénes *et al.* [20], not only support a causative role of *SDH* genes in pituitary adenoma formation, but also highlight genotype-phenotype correlations in this fast moving endocrine field. To date, 25 pituitary adenoma cases have been described as occurring in association with confirmed germline *SDH* mutations/variants (**Table 2**) [12, 17–20, 23–26]. Most of these tumors are prolactinomas, nonfunctioning adenomas, or GH-secreting macroadenomas, with variable ages at diagnosis ranging from 15 to 84 years. It is now clear that germline *SDH* mutations are also a component of the familial spectrum of pituitary adenomas comprising familial isolated pituitary adenoma (FIPA) (germline-inactivating aryl hydrocarbon receptor interacting protein [*AIP*] mutations), Carney complex (germline-inactivating *PRKARIA* mutations), MEN type 1 (germline-inactivating *MEN1* mutations), and MEN type 4 (germline-inactivating *CDKN1B* [p27/ KIP1] mutations) [27–29].

Biallelic *SDHA* inactivation has been documented in both paraganglionic tumors and GISTs arising in patients harboring a germline *SDHA* mutation [30]. To the best of our knowledge, this is the first pituitary adenoma with proven biallelic inactivation in a patient with a germline *SDHA* mutation. Dwight *et al*. [19] described a family in which a germline *SDHA* mutation was associated with a PGL in the proband, as well as a pituitary nonfunctioning macroadenoma in the proband's son. SDHA IHC confirmed loss of expression in both tumors. However, biallelic *SDH* inactivation was not detected in the pituitary adenoma; only paradoxical loss of the mutated allele was detected. Dénes *et al*. [20] demonstrated LOH in the pituitary adenomas of three *SDHB* patients, but there was ROH in the pituitary adenomas of two *SDHA*-mutated patients. Gill *et al*. [31] detected two inactivating *SDHA* mutations in a 62-year-old man with a prolactin-producing tumor, but neither of these mutations was present in the germline. In an effort to identify the underlying pathogenic mechanism by which *SDH* mutations lead to pituitary tumor development, Xekouki *et al*. [26] studied the pituitary in *Sdhb* $^{+/-}$ mice and provided evidence that pituitary hyperplasia in SDH-deficient cells may be the initial abnormality in the cascade of events leading to true adenoma formation. These data unravel critical aspects related to hypophyseal pathobiology and further add to the understanding of the tumorigenic process.

In contrast, other tumor types, eg, bladder cancer, melanoma, prostate cancer, and papillary thyroid cancer, retained SDHB/SDHA protein expression, suggesting that these tumors are not part of the SDH-associated tumor spectrum. To extend, the biological nature of heterogeneous breast cancer (case 3) in this particular genetic context (ie, *SDHD* and *BRCA-1*) remains elusive. Along these lines, the SDHB immunonegative abdominal ganglioneuroma (case 5) displayed ROH in the absence of additional mutations, strongly suggesting an alternative mechanism of SDHB protein loss other than loss of genomic regions encompassing the *SDHB* locus and/or a second "exonic" somatic event. An alternative mechanism could be *SDHC* promoter hypermethylation [32, 33]. Nevertheless, a limitation of the current study concerns the lack of methylation analysis for the promoter of *SDHC* gene.

In conclusion, the current study expands the SDH-related tumor spectrum and identifies pancreatic NET as a new component of this spectrum. This study also strengthens the etiological association of *SDH* genes with pituitary neoplasia, renal tumorigenesis, and gastric GISTs as revealed in the Leiden SDH Mutation Carrier Registry. These findings may have implications for the surveillance protocol for patients with a germline *SDHD* mutation. In this context, further studies are warranted to elucidate the role of the disruption of the Krebs cycle in familial and sporadic pancreatic neuroendocrine tumorigenesis.

Table 2. Germline *SDH* Mutations/Variants and Pituitary Adenomas Reported in the Literature.

Case	Age, Y^/Sex	Functional Classification of PA	Germline *SDH* Mutation	Biallelic SDH Inactivation in PA	PGL/PCC	Ref.
1	30/M	Pituitary nonfunctioning macroadenoma	*SDHA* c.1873C>T p.His625Tyr	Paradoxical loss (LOH) of the mutated allele	None	[19]
2	27/M	Pituitary prolactinoma, size NA	*SDHA* c.91C>T p.Arg31X *VHL*** c.589G>A p.Asp197Asn *AIP*, *MEN1*, and *CDKN1B* are not available		PCC	[20]
3	49/F	Pituitary macroprolactinoma	*SDHA* c.91C>T p.Arg31X	p.D38V; somatic mutation as a second hit of biallelic inactivation	Bilateral HN PGL	Present study
4	53/M	Pituitary nonfunctioning macroadenoma	*SDHA* variant c.969C>T p.Gly323Gly†	ROH	Abdominal PGL, Wilms tumor, retroperitoneal liposarcomas, and renal oncocytoma	[20]
5	84/M	Pituitary GH-secreting macroadenoma	*SDHAF2* variant c.-52T>C		HN PGL	[20]
6	33/M¶	Pituitary macroprolactinoma	*SDHB* c.298T>C p.Ser100Pro	LOH	HN PGL	[20]
7	36/F¶	Pituitary macroprolactinoma	*SDHB* c.298T>C p.Ser100Pro		BAH	[20]
8	53/F	Pituitary nonfunctioning macroadenoma	*SDHB* c.587G>A p.Cys196Tyr	LOH	HN PGL	[20]
9	31/F	Pituitary macroprolactinoma	*SDHB* exons 6–8 deletion	LOH		[20]

Case	Age, Y^/Sex	Functional Classification of PA	Germline *SDH* Mutation	Biallelic SDH Inactivation in PA	PGL/PCC	Ref.
10	60/F	Pituitary macroprolactinoma	*SDHB* c.423 + 1G>A		HN PGL	[20]
11	15/NA	Pituitary adenoma NA	*SDHB* c.761insC p.254fsX255		None	[23]
12	71/M#	GH-secreting adenoma	*SDHB* c.689G>A p.Arg230His		Bilateral HN PGL	[26]
13	51/F#	Pituitary microadenoma	*SDHB* c.642 + 1G>A		Metastatic PGL GIST	[26]
14	60/M	Pituitary macroprolactinoma	*SDHC* c.256–257insTTT p.Phe85dup		HN PGL	[18]
15	53/M	Pituitary macroprolactinoma	*SDHC* c.380A>G p.His127Arg		HN PGLs	[20]
16	36/F	Pituitary macroprolactinoma	*SDHD* c.242C>T p.Pro81Leu		HN PGLs	[24]
17	41/M	Pituitary GH-secreting macroadenoma	*SDHD* c.298_301delACTC p.T100fsX133	LOH	HN PGL and ea-PGL Bilateral PCCs	[17]
18	56/F	Pituitary GH-secreting macroadenoma	*SDHD* c.274G>T p.Asp92Tyr	ROH	HN PGLs	[12] *and present study*
19	60/M	Pituitary macroprolactinoma	*SDHD* c.274G>T p.Asp92Tyr	LOH	HN PGLs PCC	[12] *and present study*
20	NA	GH-secreting macroadenoma	*SDHD* c.341A>G p.Tyr114Cys			[25]
21	NA	Pituitary nonfunctioning microadenoma	*SDHD* c.341A>G p.Tyr114Cys			[25]

22	NA	Pituitary nonfunctioning microadenoma	*SDHD* c.341A>G p.Tyr114Cys			[25]
23	NA	Pituitary nonfunctioning microadenoma	*SDHD* c.341A>G p.Tyr114Cys			[25]
24	40/F#	Pituitary macroprolactinoma	*SDHD* c.242C>T p.Pro81Leu		Bilateral PCC	[26]
25	51/F	Pituitary nonfunctioning macroadenoma	*SDHD* c.274G>T p.Asp92Tyr	NA, radiological follow-up	HN PGL	Present study***

Abbreviations: GH, growth hormone; PA, pituitary adenoma; M, male; F, female; VHL, von Hippel-Lindau; AIP, aryl hydrocarbon receptor interacting protein; CDKN1B, cyclin-dependent kinase inhibitor 1B; dup, duplication; HN PGL, head and neck paraganglioma; BAH, bilateral adrenal hyperplasia; NA, not available; ea-PGL, extra-adrenal paraganglioma; fs, frame-shift.

^ Age at diagnosis of the pituitary adenoma.
# Age of diagnosis of the new syndromic association.
¶ These patients were first-degree relatives.
** This variant has been described in polycythemia vera but not in classical von Hippel-Lindau syndrome.
*** One of the excluded patients in the present study, because no biopsy or surgically resected material was available.
† In silico splicing analysis software packages predicted that this variant may create a new splice donor site. RNA was extracted from peripheral blood using PAXgene Blood RNA Kit (PreAnalytiX), but RT-PCR analysis found no evidence of aberrant splicing of the *SDHA* gene. Sequence analysis of DNA extracted from a paraffin-embedded pituitary adenoma sample from this patient showed the presence of this variant with no evidence of loss of the normal allele in the tumor DNA when compared to the peripheral blood DNA. Tissue extracted from the father's nonfunctioning pituitary adenoma (NFPA) did not harbor the variant, whereas it was present in the germline DNA of the mother, suggesting that it is not the cause of NFPA in father and son. Its role in the proband's other tumors is unknown.

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Chapter 7

Succinate Dehydrogenase (SDH)-deficient Renal Carcinoma: A Morphologically Distinct Entity: A Clinicopathologic Series of 36 Tumors From 27 Patients

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ABSTRACT

Succinate dehydrogenase (SDH)-deficient renal carcinoma has been accepted as a provisional entity in the 2013 International Society of Urological Pathology Vancouver Classification. To further define its morphologic and clinical features, we studied a multi-institutional cohort of 36 SDH deficient renal carcinomas from 27 patients, including 21 previously unreported cases. We estimate that 0.05% to 0.2% of all renal carcinomas are SDH deficient. Mean patient age at presentation was 37 years (range, 14 to 76 y), with a slight male predominance (M:F=1.7:1). Bilateral tumors were observed in 26% of patients. Thirty-four (94%) tumors demonstrated the previously reported morphology at least focally, which included: solid or focally cystic growth, uniform cytology with eosinophilic flocculent cytoplasm, intracytoplasmic vacuolations and inclusions, and round to oval low-grade nuclei. All 17 patients who underwent genetic testing for mutation in the SDH subunits demonstrated germline mutations (16 in SDHB and 1 in SDHC). Nine of 27 (33%) patients developed metastatic disease, 2 of them after prolonged follow-up (5.5 and 30 y). Seven of 10 patients (70%) with high-grade nuclei metastasized as did all 4 patients with coagulative necrosis. Two of 17 (12%) patients with low-grade nuclei metastasized, and both had unbiopsied contralateral tumors, which may have been the origin of the metastatic disease. In conclusion, SDH-deficient renal carcinoma is a rare and unique type of renal carcinoma, exhibiting stereotypical morphologic features in the great majority of cases and showing a strong relationship with SDH germline mutation. Although this tumor may undergo dedifferentiation and metastasize, sometimes after a prolonged delay, metastatic disease is rare in the absence of high-grade nuclear atypia or coagulative necrosis.

INTRODUCTION

Loss of immunohistochemical (IHC) staining for succinate dehydrogenase subunit B (SDHB) has been consistently demonstrated in pheochromocytomas/paragangliomas, gastrointestinal stromal tumors (GISTs), renal carcinomas, and pituitary adenomas arising in the setting of germline mutation of *SDHA*, *SDHB*, *SDHC*, *SDHD*, and *SDHAF2* [1–17]. Tumors that show loss of staining for SDHB (indicating disruption of the mitochondrial complex 2 for any reason, not just *SDHB* mutation) have been termed succinate dehydrogenase (SDH) deficient [1]. In addition to absent staining for SDHB, tumors associated with *SDHA* mutation also show loss of staining for SDHA, whereas tumors associated with germline mutation of *SDHB*, *SDHC*, *SDHD*, and *SDHAF2* show positive staining for SDHA [1, 13, 15, 18–20].

Because of their strong syndromic and hereditary basis and distinct natural history, SDH-deficient tumors are important to recognize [1]. To date, 53 patients with renal neoplasms arising in the setting of germline *SDH* mutation have been reported (summarized in **Supplementary Table 1**, **Supplemental Digital Content 1**, http://links.lww.com/PAS/A224) [4, 16, 17, 21–42]. Briefly, 41 cases have been reported arising in the setting of *SDHB* mutation, 5 in the setting of *SDHC* mutation, 3 in the setting of *SDHD* mutation, and none in the setting of *SDHA* mutation. In 4 cases loss of IHC staining for SDHB has been reported without follow-up SDH mutation testing, but all patients with SDH-deficient renal carcinoma who have undergone complete genetic testing to date have been shown to have germline mutation in one of the SDH subunits.

In 2010, we reported that renal carcinomas occurring secondary to *SDH* mutation can be identified by loss of IHC staining for SDHB [12]. In 2011, we reported that SDH-deficient renal carcinomas demonstrate distinctive features that allow them to be recognized prospectively and that this morphology can be used to triage IHC staining for SDHB as a prelude to formal genetic testing [4]. Subsequently SDH-deficient renal carcinoma has been recognized as a provisional entity in the recently published 2013 International Society of Urological Pathology (ISUP) Vancouver Classification of renal tumors [43]. The entity holds provisional status because relatively few cases have been reported, and therefore experience with the morphologic, immunohistochemical, and clinical features, including long-term outcome, has been limited.

We therefore initiated a broad international collaboration to study these tumors, with the following aims: (**1**) To identify new cases of SDH-deficient renal carcinoma to further expand knowledge and experience with these carcinomas. (**2**) To enable a centralized

pathologic review of previously published cases of SDH-deficient renal carcinoma. (**3**) To establish the natural history, clinical features, and prognosis of SDH-deficient renal carcinoma. (**4**) To establish the risk of germline *SDH* mutation associated with SDH-deficient renal carcinoma. (**5**) To estimate the incidence of SDH-deficient renal carcinoma.

METHODS

Case Retrieval and Review

Surgical pathologists with subspecialty interest in urologic pathology or in the pathology of SDH-deficient tumors from 15 institutions in North America, Europe, Asia, and Australia were contacted to submit cases of renal carcinoma occurring in the setting of proven *SDH* mutation or cases suspected to be associated with SDH deficiency on the basis of morphology, IHC, or a personal or family history of paragangliomas or SDH-deficient GIST. Pathologists were provided with detailed morphologic descriptions, photomicrographs, and published papers [4, 12], summarizing the previously reported morphology of SDH-deficient renal carcinomas and were asked to review their files for any cases with compatible morphology. Pathologists were asked to provide either a representative block or 10 to 15 unstained slides for centralized pathology review, IHC, and/or genetic testing. Cases from patients previously reported in any form (patients 41 to 53 in **Supplementary Table 1**, **Supplemental Digital Content 1**, http://links.lww.com/PAS/A224) were also included for review if slides were available, but they were recorded separately to prevent confusion due to double publication of data. For previously published cases, the originating collaborators provided additional clinical follow-up information if available. All submitted cases underwent centralized pathologic review. If the original hematoxylin and eosin sections were unavailable for review (3 cases), the morphologic review was performed by telepathology on scanned whole-slide sections.

Immunohistochemistry

Cases with proven *SDH* mutation or with compatible morphology underwent IHC analysis for SDHB and SDHA, which was performed on whole sections with mouse monoclonal antibodies against SDHB (ABCAM ab14714, clone 21A11, dilution 1 in 100) and SDHA (Mitosciences Abcam MS204, clone 2E, dilution of 1 in 1000), -detailed methods previously described [3, 4, 6, 12, 13, 15, 18]. Cases with definite granular cytoplasmic staining were classified as SDHB/SDHA positive. Cases with absent cytoplasmic staining in the presence of an internal positive control of non-neoplastic cells were classified as negative. If there was negative staining in the neoplastic cells but no internal

positive control in the non-neoplastic cells, the staining was considered indeterminate and repeated. A panel of IHC markers commonly used in urologic pathology (PAX8, AMACR, CD10, c-KIT, AE1/AE3, CK8/ 18, cytokeratin 7, cytokeratin 20, and EMA) was also performed if tissue was available.

MOLECULAR METHODS

DNA Extraction

DNA from formalin-fixed paraffin-embedded (FFPE) tumor tissue was extracted using QIAsymphony DNA Mini Kit (Qiagen, Hilden, Germany) on an automated extraction system (QIAsymphony SP; Qiagen) according to manufacturer's supplementary protocol for FFPE samples (Purification of genomic DNA from FFPE tissue using the QIAamp DNA FFPE Tissue Kit and Deparaffinization Solution). Concentration and purity of isolated DNA was measured using NanoDrop ND-1000 (NanoDrop Technologies Inc., Wilmington, DE). DNA integrity was examined by amplification of control genes in a multiplex polymerase chain reaction (PCR).

Analysis of SDHB Gene Mutation

Mutational analysis of complete CDS and exon-intron junctions of the SDHB gene was performed using PCR and direct sequencing. Briefly, 100 ng DNA was added to a reaction mixture consisting of 12.5 mL of FastStart PCR Master (Roche Diagnostic, Mannheim, Germany), 10 pmol of forward and reverse primers, and distilled water up to 25 mL. The amplification program consisted of denaturation at 95°C for 9 minutes, 35 cycles of denaturation at 95°C for 1 minute, annealing 62°C for 1 minute, and extension at 72°C for 1 minute. The program was terminated by incubation at 72°C for 7 minutes. The PCR products were separated by electrophoresis through a 2% agarose gel.

Successfully amplified PCR products selected for sequencing analysis were purified with magnetic particles Agencourt AMPure (Agencourt Bioscience Corporation, A Beckman Coulter Company; Beverly, MA), both side sequenced using Big Dye Terminator Sequencing kit (Applied Biosystems) and purified with magnetic particles Agencourt CleanSEQ (Agencourt Bioscince Corporation, A Beckman Coulter Company), all according to the manufacturer's protocol. Samples were then run on an automated sequencer ABI Prism 3130xl (Applied Biosystems) at a constant voltage of 13.2 kV for 20 minutes. DNA sequences were compared with the reference sequence (http://www.ncbi.nlm.nih.gov) by the online program BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 1. Clinical, Pathologic, and IHC Features of Previously Unreported SDH-deficient Renal Carcinomas.

	Location	Age	Sex	Size (mm)	Surgery	Stage	ISUP Grade	Necrosis	Status	Follow-up (mo)
1	Right	35	M	75	Partial nephrectomy	2 (T2AN0)	2	No	ANED	3
2	Left	76	F	25	Partial nephrectomy	1 (T1AN0)	2	No	ANED	0
3	Right	32	F	68	Nephrectomy	1 (T1BN0)	2	No	ANED	8
4			M						ANED	
	Right	34		50	Wedge	1 (T1BN0)	2	No	ANED	53
	Left	35		75	Nephrectomy	2 (T2AN0)	4[a]	No	ANED	50
	Left	35		47	Nephrectomy	1 (T1BN0)	2	No	ANED	50
	Left	35		40	Nephrectomy	1 (T1AN0)	2	No	ANED	50
5			M							
	Left	45		50	Nephrectomy	1 (T1BN0)	2	No	ANED	27
	Left	45		41	Nephrectomy	1 (T1BN0)	2	No	ANED	27
	Right	45		7	Wedge	1 (T1AN0)	2	No	ANED	25
6	Left	43	F	38	Nephrectomy	1 (T1AN0)	2	No	ANED	38
7	Right	31	F	35	Partial nephrectomy	1 (T1AN0)	2	No	ANED	1
8	Right	16	M	45	Nephrectomy	1 (T1BN0)	2	No	ANED	1
9	Right	46	M	85	Nephrectomy		3	No	ANED	4
10	Right	30	F	90	Nephrectomy (left kidney mass found, 362mo not resected)	p2 (T2AN0)	2	No	AWD vertebral met 362mo	368[b]
11*			M							
	Left	14					2	No	AUNDS	240
	Right	18					2	No	AUNDS	192
	Right	18					2	No	AUNDS	192
12*	?	44	F				4[a]	Yes	DOD	12
13	Right	57	M	60	Partial nephrectomy (left kidney 70mm mass unresected)	p3 (T3aN0)	2	No	DOD Liver met 4mo	10
14	Right	54	M	28	Partial nephrectomy	1 (T1aN0)	2	No	ANED	5

* Case 12 is the mother of case 11.

[a] Tumor showed areas of high-grade transformation in direct continuity with lower-grade areas.

[b] At the time of presentation with metastatic disease the patient was found to have a metachronous tumor in the left kidney (not biopsied). Therefore the vertebral metastasis may represent either a delayed metastasis from the original primary or metastasis from metachronous disease.

ANED indicates alive no evidence of disease; AUNDS, alive with unknown disease status; AWD, alive with disease; DOD, dead of disease.

Table 1. *(continued).*

	Mutation	SDHB	SDHA	PAX8	AMACR	CD10	c-KIT	EMA	CK7	CK20	AE1/AE3	CK8/18
1	*SDHB* [c.137G>A,pArg46Gln]	Neg	Pos	Pos	Neg	Focal	Neg	Focal	Neg	Neg	Neg	Neg
2	*SDHB* [c.725G>A,p.Arg242His]	Neg	Pos	Pos	Pos	Neg	Neg	Focal	Neg	Neg	Neg	Neg
3	*SDHB* [c.423+1G>A] Splice	Neg	Pos	Pos	Neg	Pos	Neg	Pos	Neg	Neg	Neg	Neg
4	*SDHB* exon 3 deletion											
		Neg	Pos	Pos	Focal	Focal	Neg	Focal	Neg	Neg	Neg	Neg
		Neg	Pos	Pos	Neg	Focal	Neg	Focal	Neg	Neg	Neg	Neg
		Neg	Pos									
		Neg	Pos									
5	*SDHB* [c.423+1G>A] Splice											
		Neg	Pos	Pos	Pos	Neg	Neg	Focal	Neg	Neg	Neg	Neg
		Neg	Pos	Pos	Pos	Focal	Neg	Focal	Neg	Neg	Pos	Pos
		Neg	Pos	Pos	Pos	Neg	Neg	Focal	Neg	Neg	Neg	Neg
6		Neg	Pos	Pos	Pos	Neg	Neg	Focal	Neg	Neg	Neg	Neg
7	*SDHB* [c.338G>A, p.Cys113Tyr]	Neg	Pos	Pos	Pos	Pos	Focal	Focal	Neg	Neg	Neg	Neg
8		Neg	Pos	Pos	Neg	Focal	Neg	Focal	Neg	Neg	Neg	Neg
9		Neg	Pos	Pos	Pos	Focal	Neg	Focal	Neg	Neg	Neg	Neg
10	*SDHB* [c.423+1G>A] Splice	Neg	Pos	Pos	Pos	Neg	Neg	Focal	Neg	Neg	Focal	Focal
11*												
		Neg	Pos	Pos	Neg	Focal	Neg	Focal	Neg	Neg	Neg	Neg
		Neg	Pos									
		Neg	Pos									
12*		Neg	Pos	Focal	Pos	Neg	Neg	Pos	Neg	Neg	Pos	Pos
13	*SDHB* [c.749C>A, p.Thr250Lys]	Neg	Pos	Pos	Pos	Focal	Neg	Pos	Neg	Neg	Pos	Pos
14		Neg	Pos	Focal	Focal	Focal	Neg	Focal	Focal	Neg	Pos	Pos

Table 2. Clinical, Molecular, and IHC Details of Previously Published Patients With Material Available for Pathologic Review.

	Prev. Pub.	Location	Age	Sex	Size (mm)	Surgery	Stage	ISUP Grade	Necrosis	Follow-up (mo)	Status
15	[16]	Bilateral	27	M	41	Biopsy of met only	Stage 4	3	Yes	30	DOD
16	[4,12]	Right	21	F	22	Wedge	1 (T1ANO)	2	No	84	ANED
17	[4,12]	Left	28	M	29	Nephrectomy	1 (T1A)	2	No	48	ANED
18	[4,12]	Left	22	M	100	Nephrectomy	Stage 4	4	Yes	12	DOD
19	[4,37,42]	Left	58	F	78	Nephrectomy	2 (T2AN0)	2	No	24	ANED
20	[17]	Right	22	F	65	Nephrectomy	1 (T1BN0)	2	No	160	ANED
21	[41]			M		Nephrectomy			No		
		Left	25		90	Nephrectomy	2 (T2AN0)	2	No	72	ANED
		Left	25		28	Nephrectomy	1 (T1AN0)	3	No	72	ANED
		Right	31		13	Radiofrequency ablation	1 (T1AN0)	2	No	0	ANED
22	[41]	Right	23	M	25	Nephrectomy	1 (T1AN0)	2	No	60	ANED
23	[41]	Left	36	M	130	Nephrectomy	2 (T2BN0)	3	No	132	AWD Spleen met 66 mo, liver met 10mo
24	[43]		40	M			4	3	No		Adrenal metastasis
25	[43]		35	M				3	Yes		Retroperitoneal node metastasis
26	[43]		44	M			4	3	No		Adrenal metastasis
27	[43]	Right	59	F	70		2 (T2AN0)	2	No		AUNDS

ANED indicates alive with no evidence of disease; AUNDS, alive with unknown disease status; AWD, alive with disease; DOD, dead of disease.

Table 2. *(continued).*

	Germline Mutation	SDHB	SDHA	PAX8	AMACR	CD10	c-KIT	EMA	CK7	CK20	AE1/AE3	CK8/18
15	*SDHB* c.88delC	Neg	Pos	Pos	Neg	Focal	Neg	Pos	Focal	Neg	Pos	Pos
16	*SDHB* c.268C>T	Neg	Pos	Pos	Neg	Neg	Neg	Focal	Neg	Neg	Neg	Neg
17	*SDHB* c.166-170 delCCTCA	Neg	Pos	Pos	Pos	Focal	Neg	Focal	Neg	Neg	Focal	Neg
18	*SDHB* c.423+1G>A	Neg	Pos	Pos	Focal	Focal	Neg	Pos	Pos	Neg	Pos	Pos
19	*SDHB* c.72+1G>T	Neg	Pos	Pos	Pos	Focal	Neg	Neg	Neg	Neg	Neg	Neg
20	*SDHC* c.380A>G	Neg	Pos	Pos	Neg	Neg	Neg	Focal	Neg	Neg	Neg	Neg
21	*SDHB* c.3G>A											
		Neg	Pos									
		Neg	Pos									
		Neg	Pos									
22	*SDHB* c.3G>A	Neg	Pos	Pos	Pos	Focal	Neg	Focal	Neg	Neg	Neg	Neg
23	*SDHB* exon 3 deletion	Neg	Pos	Pos	Pos	Focal	Neg	Focal	Neg	Neg	Neg	Neg
24		Neg	Pos									
25		Neg	Pos									
26		Neg	Pos									
27		Neg	Pos									

Incidence Assessment

To assess the incidence of SDH-deficient renal carcinoma in an unselected population, the computerized database of the Department of Anatomical Pathology Royal North Shore Hospital, Sydney, Australia was searched for all primary renal neoplasms resected between 1998 and 2013, with material available in archived FFPE blocks (excluding consultation cases). Similar assessments were made using the database of renal tumors, collected between 2000 and 2013 in the Department of Pathology and Laboratory Medicine of the Calgary Laboratory Services and University of Calgary, Alberta, Canada and for the tumors collected between 2003 and 2013 in the renal tumor registry at the Department of Pathology, Charles University, Pilsen, Czech Republic. The original slides were reviewed explicitly in search of cases with morphology considered compatible with proven cases of SDH-deficient renal carcinoma.

Representative areas of each tumor from the Royal North Shore Hospital cohort were also marked for tissue microarray (TMA) construction. The TMA was constructed with duplicate 1-mm-thick cores of neoplastic tissue from all available cases, and this TMA was evaluated by IHC for SDHB.

RESULTS

Clinical Features

We identified 21 previously unreported SDH-deficient renal carcinomas from 14 patients. The clinical and IHC features are summarized in **Table 1**. Briefly, the mean age at presentation with a renal tumor was 39.8 years (range, 14 to 76 y; median 43.5 y), with a slight male predominance (M:F=1.3:1). At presentation all tumors with known size and stage were confined to the kidney, with an average size of 51mm (range, 7 to 90 mm). The mean follow-up from initial presentation was 55 months (4.6 y) with a range of 0 to 368 months (30.7 y). Three of the 14 patients (21%) were known to have developed metastatic disease. One of these patients died 12 months after presentation (stage at presentation unknown). The other 2 patients with metastasis had unbiopsied neoplasms in the contralateral kidney, which were identified at the time of presentation with metastasis. One of these patients developed liver metastasis, proven by fine-needle aspiration, 4 months after partial nephrectomy and died of disease 10 months after surgery. The other patient developed vertebral metastases, confirmed by core biopsy, 362 months after nephrectomy and is alive with disease 368 months (30.7 y) after the initial presentation.

Fifteen SDH-deficient renal carcinomas from 13 previously published patients [4, 12, 16, 17, 40, 42] were available for central pathologic review (summarized in **Table 2**, with updated survival data). These cases also showed a male predisposition (M:F=2.3:1) but otherwise demonstrated similar demographic features with mean age at initial presentation of 33.8 years. Two of these patients died of metastatic disease (at 12 and 30 mo, respectively), and 1 patient was alive with metastatic disease 132 months (11 y) after presentation. Three other previously reported patients were also known to have developed metastatic disease (2 to the adrenal gland, 1 to retroperitoneal lymph node) but lacked further follow-up. When both the previously reported (**Table 2**) and novel patients (**Table 1**) were combined, the mean age at first presentation was 37 years (range, 14 to 76 y). There was a slight male predominance (M:F=1.7:1). There were 4 patients with multifocal tumors in the same kidney, and bilateral neoplasms were present in 7 of 27 (26%) patients. The incidence of synchronous or metachronous GIST and pheochromocytoma/paraganglioma as well as the family history of renal carcinoma, GIST, and pheochromocytoma/ paraganglioma are presented in **Table 3**. Briefly, 4 of 27 (15%) patients also had SDH-deficient GISTs, and 4 of 27 (15%) patients developed paragangliomas. Five patients (19%) had first-degree relatives with renal carcinoma and 1 patient a second-degree relative. There were 5 first-degree and 2 second-degree relatives with pheochromocytoma/paraganglioma and 1 first-degree relative with SDH-deficient GIST. Two patients also had incidental small renal angiomyolipomas resected. The angiomyolipoma from patient 6 was 10mm in diameter, and the angiomyolipoma from patient 9 was 3mm in diameter. The angiomyolipoma from patient 9 was available for IHC and demonstrated positive staining for SDHB. Neither patient with angiomyolipomas was known to have tuberous sclerosis complex.

Pathologic Features

Centralized pathologic review was undertaken on 36 available SDH-deficient renal carcinomas from 27 patients. Macroscopic descriptions of the tumors were not always detailed or available, but in all cases with gross description, the tumors were characterized as well circumscribed with a tan to red cut surface. Some of the tumors were noted to demonstrate cystic change. Although this cystic change was sometimes striking (**Figure 1A**), this was not a constant feature, and the majority of tumors were solid (**Figure 1B**). Histologically, the dominant morphology was as previously described [4] and was found at least focally in 34 tumors from 24 patients. This morphology is illustrated in **Figures 2–5**, and whole-slide scanned images from all tumors are available for review at http://www.cancerdxpathology.org.au. Briefly, the tumors were well circumscribed or demonstrated coarse lobulation, with a pushing border sometimes associated with a pseudocapsule (**Figures 2A**, **B**).

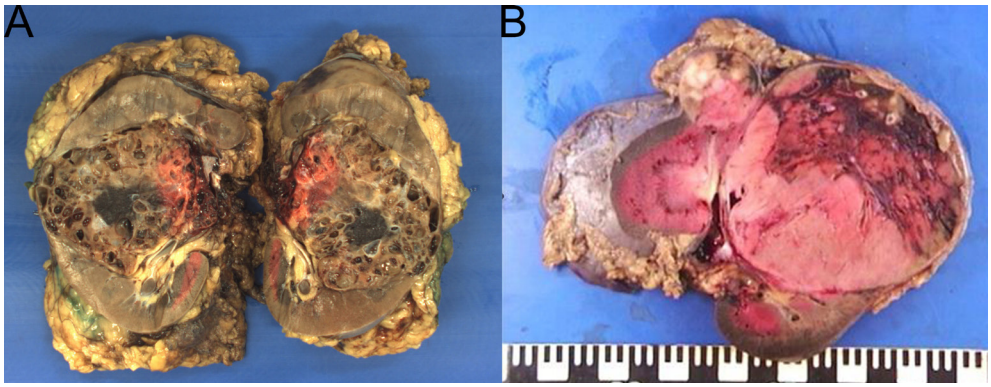

Figure 1. Although many of the tumors demonstrated cystic change, which was often profound (**A**, 85mm tumor from the right kidney of patient 9), this was not a constant finding, and some neoplasms were solid (**B**, 2 solid tumors, 90 and 28mm, from the left kidney of patient 21).

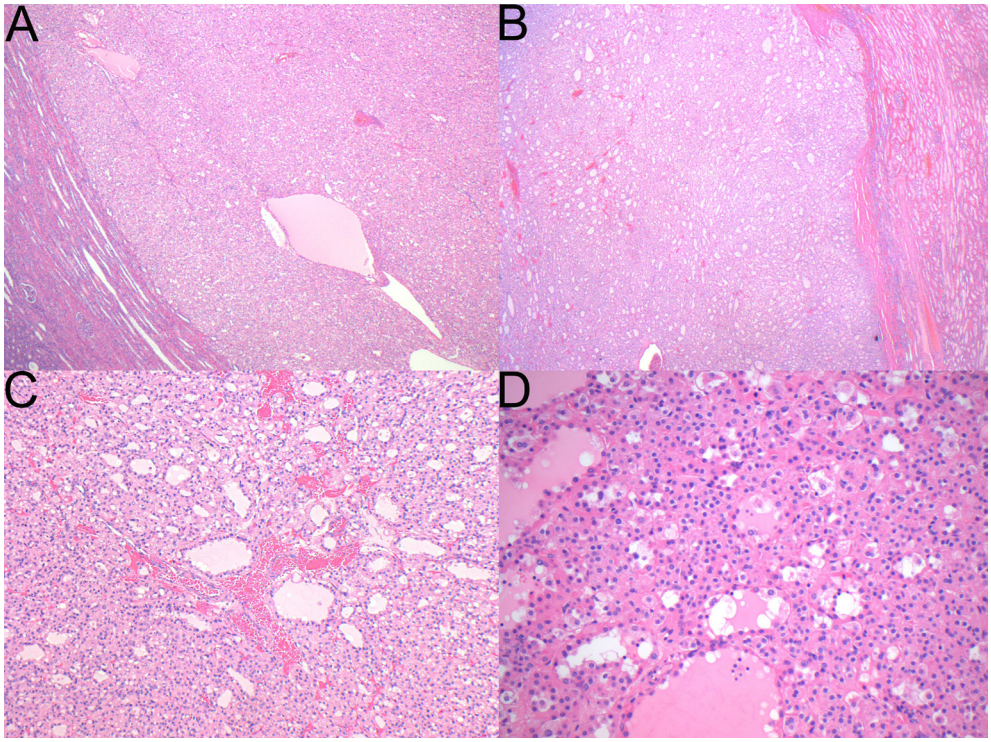

Figure 2. The tumors were well circumscribed (**A**) and only occasionally separated from the adjacent kidney by a pseudocapsule (**B**). **C** and **D**, Cystic change was commonly appreciated histologically, and the cystic spaces contained pale eosinophilic fluid (hematoxylin and eosin).

Cystic change in the form of microcysts and macrocysts was commonly appreciated histologically, and these cysts usually contained pale eosinophilic fluid (**Figures 2C**, **D**). In a few tumors the stroma showed areas of prominent myxoid change or hyalinization. The neoplastic cells were cuboidal to oval with round nuclei and inconspicuous nucleoli, consistent with an ISUP nucleolar (nuclear) grade 2 in 26 cases (**Figure 3**). The nuclei were grade 3 in 7 cases and grade 4 in 3 cases (all of which demonstrated at least focal sarcomatoid change). In most tumors the nuclear chromatin commonly had a dispersed quality reminiscent of cells with neuroendocrine differentiation. The cell borders were sometimes indistinct. The cytoplasm was eosinophilic or flocculent but not truly oncocytic (**Figure 3**). Tumor cells demonstrated a variably solid or nested architecture, and sometimes nests of tumor cells surrounded cystic spaces imparting a pseudoglandular appearance.

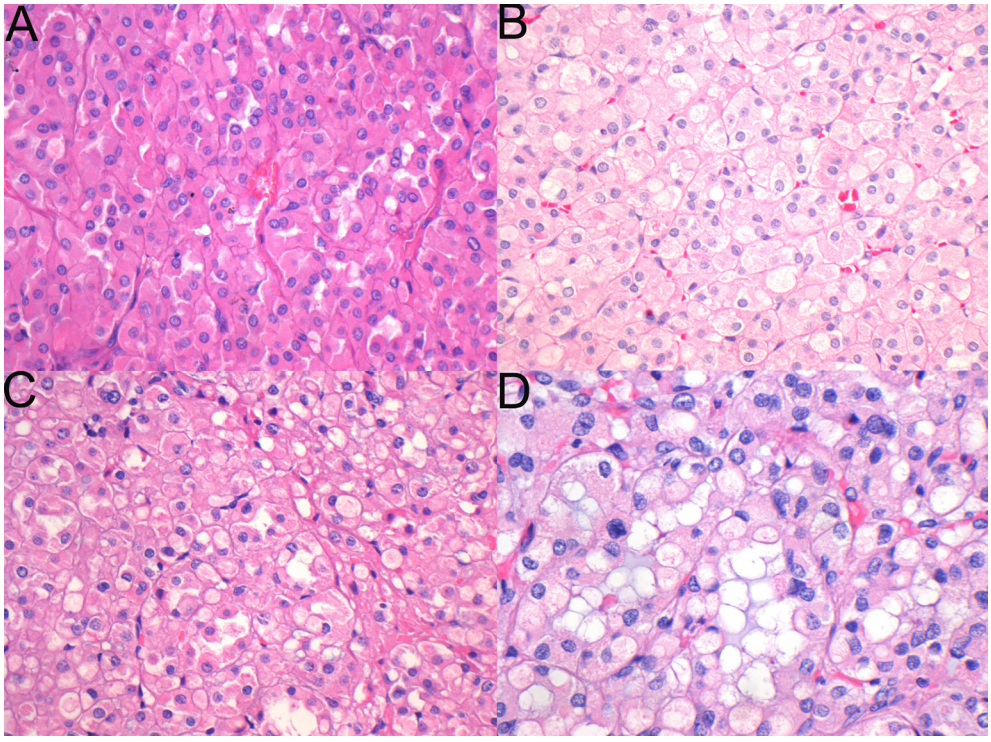

Figure 3. The tumor cells had eosinophilic cytoplasm but lacked the granularity associated with true oncocytes. In some cases the eosinophilic cytoplasm was dense (**A**), but in most cases (**B** and **C**) it had a pale and wispy, almost flocculent, appearance. In some tumors (**D**) the combinations of flocculent cytoplasm and frequent intracytoplasmic inclusions imparted a bubbly appearance to many of the tumor cells (hematoxylin and eosin).

The most constant and distinctive histologic feature was the presence of cytoplasmic vacuoles and inclusion like spaces (**Figures 3C**, **D**). These contained either pale eosinophilic fluid or flocculent material. In most cases, these inclusions were readily identified throughout the tumor, but in some cases, particularly in those with higher-grade nuclei, these cytoplasmic inclusions were subtle and were only identified focally after a thorough search of multiple sections (**Figure 5**). Non-neoplastic tubules or glomeruli were frequently entrapped at the periphery of the neoplasm (**Figure 4**). Intratumoral mast cells were commonly highlighted with c-KIT IHC but were not appreciable as a conspicuous finding on routine hematoxylin and eosin sections. Allowing for the secondary effects of the tumor, the adjacent non-neoplastic kidney was normal, and no dysplastic or precursor lesions were identified in the adjacent renal parenchyma.

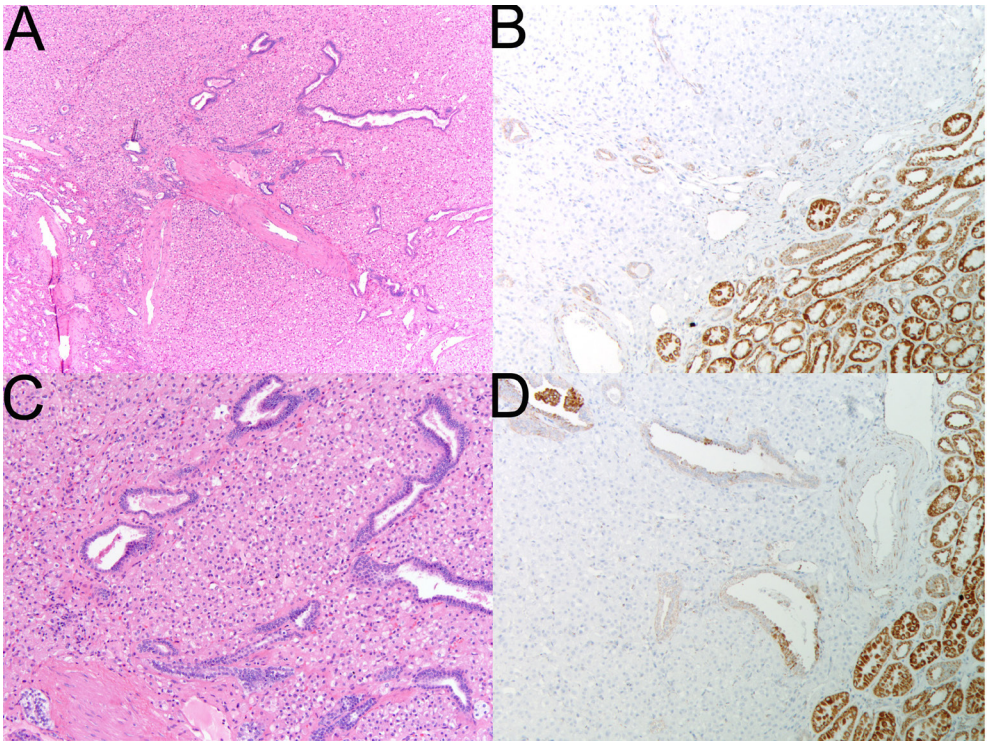

Figure 4. Serial sections stained with hematoxylin and eosin (**A** and **C**) and SDHB IHC (**B** and **D**). Frequently entrapped benign tubules were noted at the edge of the tumors. SDHB IHC demonstrates positive staining in the internal controls (including the entrapped benign tubules) but all the neoplastic cells are negative.

In the 5 tumors with ISUP nucleolar (nuclear) grade 3 nuclei, which were still recognizable as SDH-deficient renal carcinomas, in addition to prominent nucleoli, the neoplastic cells in the higher-grade areas acquired darker and coarser chromatin and more dense eosinophilic (rather than flocculent) cytoplasm. The nuclei in these areas were about 2 times larger than the nuclei in low-grade areas and demonstrated oval to slightly elongated shape, with irregular nuclear outlines. In some areas these tumors lost their nested architecture and commonly grew as solid sheets, occasionally with a very focal abortive papillary architecture.

Three cases demonstrated frank sarcomatoid transformation, with ISUP nucleolar (nuclear) grade 4. The sarcomatoid areas were composed of pleomorphic spindled cells essentially indistinguishable from other high-grade sarcomatoid renal carcinomas. In 2 of the cases with sarcomatoid change, the sarcomatoid areas were in direct continuity with areas showing the stereotypical low-grade morphology (including ISUP nucleolar [nuclear] grade 2 nuclei), indicating true dedifferentiation rather than the existence of a different tumor type. In the other case with areas of sarcomatoid transformation, the entire tumor was high-grade (either grade 3 or grade 4 nuclei). However, even in this case intracytoplasmic inclusions, albeit subtle, were identified after a search of multiple slides.

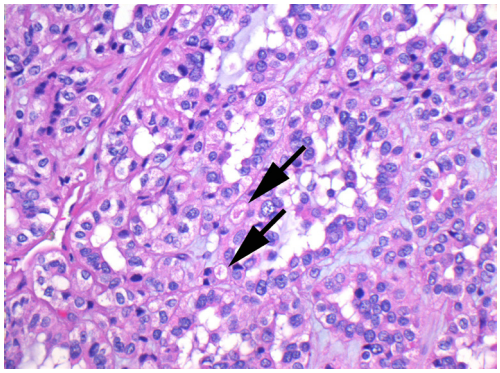

Figure 5. In this case with higher-grade nuclear features and early dedifferentiation, the intracytoplasmic inclusions are more subtle (arrows) and were identified only after a careful search (hematoxylin and eosin).

Although fibrosis, hyalinization, and hemorrhage were not uncommon, true coagulative necrosis was only found in 4 tumors—all ISUP nucleolar (nuclear) grade 3 or 4.

Only 2 of 36 (6%) cases lacked any areas with typical morphologic features or cytoplasmic inclusions and would not have been recognizable as SDH-deficient renal carcinomas on

the basis of morphology. These cases, illustrated in **Figure 6**, were previously reported by Miettinen *et al*. [42] and identified by screening a large cohort by IHC rather than triaging IHC on the basis of morphology [42]. In 1 case, the morphology was that of a typical clear cell renal carcinoma, ISUP nucleolar (nuclear) grade 3. In this case, only 1 block was available for review. In the second case, the morphology was in keeping with papillary renal carcinoma type 2, ISUP nucleolar (nuclear) grade 3. In this case, 4 blocks were available for review, all of which demonstrated similar histology.

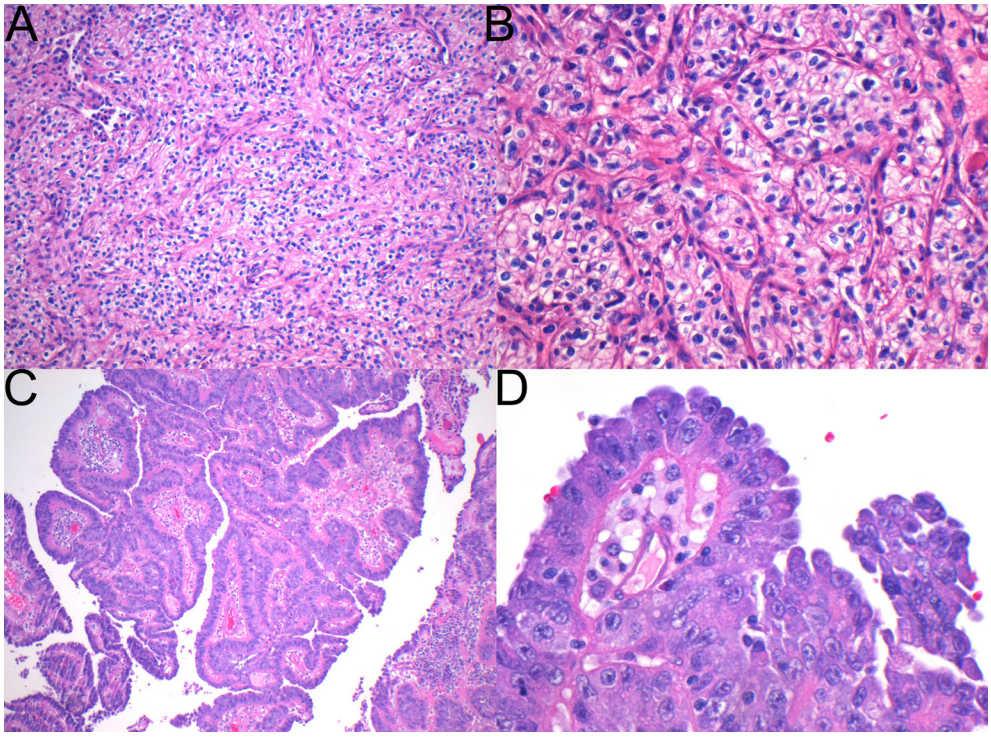

Figure 6. Cases with variant morphology. One case demonstrated morphology reminiscent of conventional clear cell renal carcinoma of ISUP nucleolar (nuclear) grade 3 (**A** and **B**). **C** and **D**, A second case demonstrated a papillary architecture with prominent nucleoli, reminiscent of type 2 papillary renal carcinoma (hematoxylin and eosin).

Immunohistochemistry

All cases demonstrated negative staining for SDHB in all neoplastic cells (which was considered an inclusion criterion for the study). All cases also showed preserved positive staining for SDHA. At least focal positive staining for PAX8 was found in all cases. All but 1 case (96%) demonstrated at least focal reactivity for EMA, which was often quite limited, in some cases involving <1% of neoplastic cells, and commonly restricted to the apical border of cells. Only 3 of 25 cases (12%) demonstrated positive staining for

CK7, and this staining was focal in 2 cases. Immunoreactivity for other markers was not specific. It is noteworthy that 68% of the cases demonstrated completely negative staining for all cytokeratins. IHC for c-KIT was negative in 96% of cases but did highlight scattered intratumor mast cells in many tumors.

Genetic Testing

Of the previously reported cases, 9 had undergone germline molecular testing and were found to harbor a pathogenic mutation in *SDHB* (8 cases) or *SDHC* (1 case)—mutation data previously reported [4, 8, 12, 16, 17, 40]. Of the previously unpublished cases, genetic testing was performed for SDHB in 8 patients, and in all of them a pathogenic germline mutation was identified. That is all 17 patients with SDH-deficient renal carcinoma who have undergone testing were found to harbor a germline mutation of 1 of the components of the mitochondrial complex 2 (16 *SDHB*, 1 *SDHC*, and none in *SDHA* or *SDHD*).

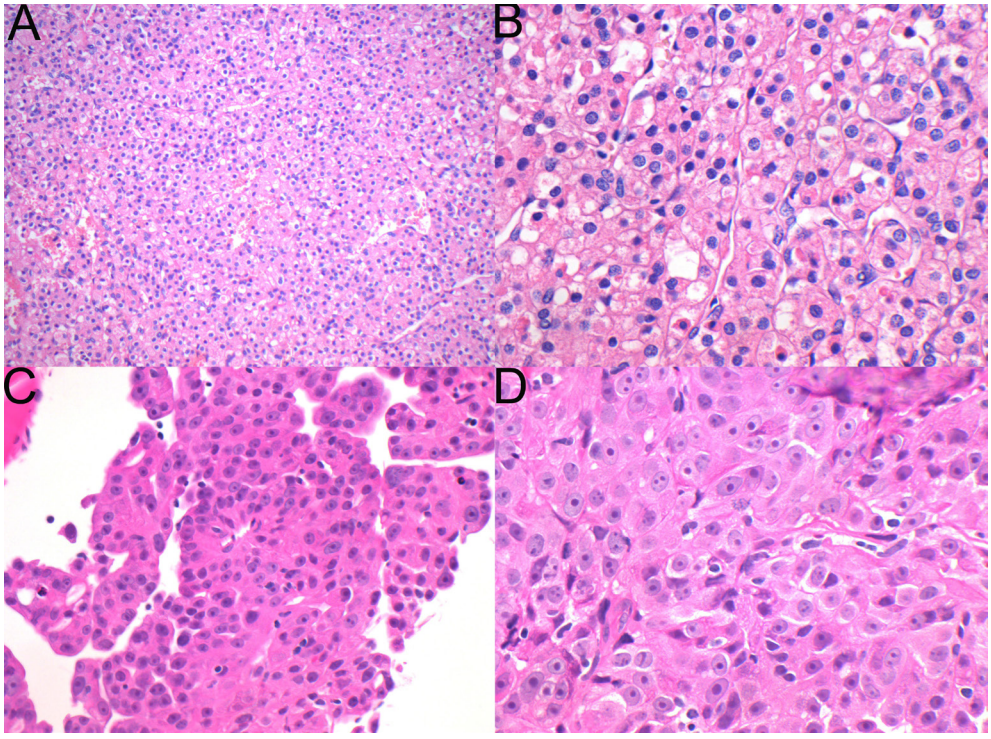

Figure 7. Representative photomicrographs from the primary tumor (**A** and **B**) and the vertebral metastasis (**C** and **D**) of case 10. The primary tumor demonstrated stereotypical low-grade features with ISUP nucleolar (nuclear) grade 2. In the metastasis documented 30 years later, the tumor demonstrated high-grade nuclear features but still showed negative staining for SDHB. As the patient had a contralateral renal tumor, which was unbiopsied at the time of metastatic disease, this may represent spread from a second primary tumor (hematoxylin and eosin).

Morphologic Predictors of Metastasis

A total of 9 patients with pathologic material available for histologic review developed metastatic disease (6 previously reported and 3 new patients). Four of these patients died of metastatic disease at a mean of 18 months after initial presentation, all of whom had an ISUP nucleolar (nuclear) grade of 3 or 4 at presentation, and 3 of whom had coagulative necrosis. The other patient with coagulative necrosis was known to have metastatic disease but had no further follow-up information available. Two patients were alive with metastatic disease, 132 months (11 y) and 368 months (30.7 y) after initial presentation. One of these 2 patients showed increased cytologic atypia and an ISUP nucleolar (nuclear) grade 3 but lacked frank sarcomatoid change and subsequently developed biopsy-proven metastases in the spleen at 66 months (5.5 y) and the liver at 108 months (9 y) after initial presentation. The other patient showed only typical low-grade features in the initial resection with an ISUP nucleolar (nuclear) grade of 2, but then developed biopsy proven vertebral metastasis 30 years later. The metastasis showed increased cytologic atypia with an ISUP nucleolar (nuclear) grade 3 and an abortive papillary architecture but lacked sarcomatoid differentiation (**Figure 7**). Importantly, at the time of diagnosis of the metastasis, this patient was found to have a solid tumor on diagnostic imaging in her contralateral kidney. Unfortunately, this tumor was not biopsied or resected, and the origin of the metastasis, either from the original SDHB tumor or from the metachronous neoplasm in the contralateral kidney, could not be established with certainty. Both patients with exclusively variant morphology (illustrated in **Figure 6**) developed metastatic disease, but no further follow-up information was available.

Estimated Incidence

The review of consecutive unselected cases from the Department of Anatomical Pathology, Royal North Shore Hospital, Sydney, Australia identified 420 renal neoplasms. None of these tumors demonstrated morphologic features of SDH-deficient renal carcinoma, and IHC for SDHB, performed on a TMA, was positive in all cases, suggesting that the incidence in a truly unselected group of primary renal carcinomas is <1 in 420 (0.2%). The database from the Rockyview Hospital (Calgary Laboratory Services and University of Calgary) included 1750 in-house resected renal tumors. All renal neoplasms, reported as "unclassified" or "oncocytic" were reviewed, and 2 cases were identified on the basis of morphology, with an estimated overall incidence of 0.1%. The morphologic review of the renal tumor registry at the Department of Pathology, Charles University, Pilsen, Czech Republic, identified only 1 case from 2004 locally resected tumors, with an estimated incidence of 0.05%.

DISCUSSION

SDH-deficient renal carcinoma has recently been accepted as a provisional entity in the 2013 ISUP Vancouver Classification. However, reflecting its rarity, published experience with this tumor has been limited. To substantiate its distinctive morphologic and clinical features, the prognosis, and the genetic associations of SDH-deficient renal carcinoma and to estimate its incidence, we evaluated a multi-institutional cohort of 36 SDH-deficient renal carcinomas from 27 patients, including 21 previously unreported cases.

This study confirmed that the previously reported distinctive morphologic features of SDH-deficient renal carcinoma are highly specific for the diagnosis. That is, all the cases with the typical morphology demonstrated negative staining for SDHB. Therefore morphology should be considered the primary screening test to identify SDH-deficient renal carcinoma in routine practice. However, we caution that the study was not intended or designed to demonstrate that all renal carcinomas arising in the context of SDH mutation will show this morphology. That is many cases reported in this series were first identified primarily on the basis of morphology, and only selected cases with compatible morphology then underwent screening IHC. Therefore, there may be a selection bias in this series toward cases with typical morphologic features. It is therefore worthnoting that 2 (6%) cases from this series (both identified by IHC screening of large cohorts) lacked this distinctive morphology, and in other cases (particularly those with high ISUP nuclear [nucleolar] grade) this morphology was only a focal finding and may not be appreciated in routine clinical practice. Therefore, in addition to performing SDHB IHC on cases with compatible morphology, regardless of age or clinical features, we would also recommend that screening IHC be considered for other cases with suggestive clinical features (for example, multifocality, onset at a young age, or a personal or family history of renal carcinoma, pheochromocytoma/paraganglioma, gastric GIST, or pituitary adenoma).

We would estimate the true incidence of SDH-deficient renal carcinoma as being 0.05% to 0.2% of unselected renal neoplasms. In the local case series from Australia (Sydney), we found no morphologically or immunohistochemically compatible cases in 420 consecutive unselected renal tumors screened both by morphology and IHC. Similarly, only 1 and 2 cases were identified in large population-based cohorts of 2004 and 1750 consecutive renal carcinomas, respectively, in institutions from Europe (Pilsen) and North America (Calgary), which were screened by morphology. A limiting factor in the 2 latter series was the lack of systematic IHC for SDHB and SDHA, which could have potentially detected additional cases, particularly any with variant morphology. However, identification of

the cases in these cohorts was based on the recognition of an unusual morphology and routine IHC, in the setting of large centralized uropathology practices with experienced genitourinary pathologists. Thus the estimated incidence derived from 3 institutions from different continents was similar and ranged from 0.05% to 0.2%. These results are also in keeping with the recently reported data by Miettinen *et al*. [42] who performed IHC on 711 renal carcinomas and 64 oncocytomas and found that only 4 cases (0.5%) demonstrated loss of staining for SDHB. The low incidence of SDH deficiency in renal carcinomas is similar to the low incidence reported in pituitary adenomas (0.3%) [15] and contrasts to the high incidence found in pheochromocytoma/paraganglioma (3% in adrenal pheochromocytomas and up to 40% in extra-adrenal paranganglioma) [6] and significant incidence in gastric GIST (5% to 7.5%) [3,10]. Therefore, although it has been recommended that all pheochromocytomas and all paragangliomas as well as gastric GISTs with compatible morphology for SDH-deficient GIST undergo screening IHC for SDHB [1, 6, 18], it is unlikely to be cost-effective or practical to screen all renal carcinomas with SDHB IHC.

This study confirmed that classical low-grade tumors showing typical histologic features and an ISUP nucleolar (nuclear) grade 2 are usually cured by excision alone. Of the 9 patients who developed metastatic disease, in only 2 did the primary tumor demonstrate exclusively low-grade features with an ISUP nucleolar (nuclear) grade 2. Importantly, by the time of metastasis, both of these patients had developed a contralateral renal neoplasm, which had not been resected or biopsied. Therefore the metastasis may have arisen from the metachronous tumors, which may have been of higher grade and not from the primary low-grade SDH-deficient renal tumor.

We note that SDH-deficient renal carcinoma may undergo dedifferentiation including sarcomatoid transformation, and cases with high-grade nuclei commonly metastasize. In fact, metastatic disease developed in 7 of 10 patients with ISUP nucleolar (nuclear) grade 3 or 4 nuclei or variant morphology. Although hemorrhage, fibrosis, and hyalinization were relatively common, only 4 tumors demonstrated true coagulative necrosis. Given that all 4 of these metastasized (and 3 were confirmed dead of disease), it is likely that coagulative necrosis is an adverse prognostic indicator.

Given the low risk for metastatic disease and the high incidence of bilateral tumors in 7 of 27 (26%) patients, our findings support nephron-sparing surgery for patients with low-grade tumors. Although there is insufficient evidence to recommend adjuvant treatment, patients with high-grade neoplasms (variant morphology, sarcomatoid change, coagulative necrosis, or high ISUP nucleolar [nuclear] grade) should be considered at

high risk for metastasis, and consideration should be given to more radical treatments in these patients. We note that in 2 patients metastasis occurred >5 years after the initial presentation, and therefore extended (if not lifelong) follow-up is required for late recurrences, as well as metachronous disease and other syndromic manifestations of germline *SDH* mutation (GIST, paraganglioma, pituitary adenoma) [1]. The differential diagnosis of SDH-deficient renal carcinoma, which includes oncocytoma and chromophobe renal carcinoma is limited, and we consider loss of staining for SDHB as definitive confirmation of the diagnosis. Although SDHB IHC is not widely available, the morphologic features of typical SDH-deficient renal carcinoma, such as uniform low-grade morphology in the great majority of cases, flocculent (rather than truly oncocytic) cytoplasm, cytoplasmic vacuoles, lack of distinct cell borders, negative staining for c-KIT, and commonly negative or focal cytokeratin reactivity, are important clues to the diagnosis.

In our series of SDH-deficient renal carcinomas, germline mutations were identified in all 17 patients who underwent genetic testing. This is similar to the findings in SDH-deficient paragangliomas and pituitary adenomas, where the presence of negative staining for SDHB almost always signifies germline mutation of one of the components of the mitochondrial complex 2 (*SDHA, SDHB, SDHC, SDHD, SDHAF2*), rather than being due solely to somatic inactivation [1]. In fact, we are aware of only 2 cases of SDH-deficient paraganglioma and 1 case of SDH-deficient pituitary adenoma in which double-hit SDH inactivation has occurred in the absence of germline mutation [15, 44, 45]. It is possible that our series is subject to a referral bias because patients with known mutation or personal or family histories of syndrome-related tumors were more likely to be recognized and included in this study. However, our findings suggest that, similar to paraganglioma and pituitary adenoma, it is likely that most, perhaps almost all, SDH-deficient renal carcinomas will be associated with germline mutation of one of the SDH genes. Therefore, the diagnosis of SDH-deficient renal carcinoma can be considered an absolute indication for germline SDH mutation testing. No clear-cut genotype- phenotype correlations have emerged, although it is interesting to note in this series that 4 unrelated patients who developed renal carcinoma all harbored the same *SDHB* [c.423+1G>A] splice site mutation and that 2 of the patients with this mutation developed multifocal disease.

Although SDH-deficient renal carcinoma shows an extremely strong correlation with germline *SDH* mutation, we believe that IHC remains a phenotype test rather than a genotype test, and it is likely that not all SDHB IHC–negative tumors will be shown to have *SDH* mutations using current technology. Therefore, as we have previously

stated in the setting of paraganglioma [6], we do not believe that specialized consent or formal genetic counseling would ordinarily be required before IHC is performed. This is analogous to IHC for DNA mismatch repair proteins being used to triage patients with colorectal cancer for genetic testing for Lynch syndrome wherein there is now a trend toward universal screening, and most jurisdictions do not require genetic counseling before screening IHC is performed.

To date, no mutations in *SDHA* have been reported in association with renal carcinoma, but given that loss of staining for SDHA identifies both paragangliomas and GISTs associated with germline *SDHA* mutation [1, 18–20, 46–48], we would recommend that IHC for SDHA also be performed in SDH-deficient renal carcinoma to assist in triaging genetic testing for *SDHA* mutation.

The extremely high rate of germline mutation in the SDH subunits in SDH renal carcinoma is different to that found in SDH-deficient GIST, in which approximately 30% of cases are associated with *SDHA* mutation, and 10% to 20% of cases are associated with mutations in the other SDH subunits (*SDHB, SDHC*, or *SDHD*), leaving the mechanism of SDH deficiency uncertain in up to half of cases [18, 19, 46–48]. It is noteworthy that some patients with SDH-deficient GIST but without germline mutation were found to have the Carney Triad (the nonhereditary but syndromic association of SDH-deficient GIST, paraganglioma, and pulmonary chondroma) [3]. It is therefore possible that some patients with SDH-deficient renal carcinoma may be syndromic, even if no germline mutations are identified. From a practical point, because longterm follow-up is required due to the possibility of late metastasis, we would also recommend long-term follow-up for other syndromic manifestations (pheochromocytoma/ paraganglioma, GIST, pulmonary chondroma, or pituitary adenoma), regardless of whether or not a germline mutation is identified. In fact, although there may have been a selection bias toward recognizing patients with syndromic disease, we note that in our series 30% of patients also developed either paraganglioma or SDH-deficient GISTs—a particularly striking association given the relative rarity of these tumors.

In conclusion, SDH-deficient renal carcinoma represents a distinct and rare renal neoplasm, which is defined by loss of IHC staining for SDHB. Because of its rarity, it is impractical to perform reflex screening IHC on all renal cancers. However, the great majority of SDH-deficient renal tumors (94% in this series) demonstrated typical appearances at least focally and were recognized by their uniform low-grade cytology, cytoplasmic vacuoles, eosinophilic or flocculent (rather than truly oncocytic) cytoplasm, focal cystic change, and solid to lobulated growth with peripherally entrapped renal

tubules. In tumors exhibiting low-grade nuclear features with ISUP nucleolar (nuclear) grade 2, metastasis is unusual but can occur even after a prolonged period. SDH-deficient renal carcinoma may be associated with high ISUP nucleolar (nuclear) grade, coagulative necrosis or sarcomatoid transformation, in which case the development of metastatic disease is much more likely. SDH-deficient renal carcinomas are commonly multifocal and with prolonged follow-up, bilateral tumors can be identified in up to 26% of patients. To date, all reported cases have been associated with germline mutations of the *SDH* genes.

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PART IV

Evolving Concepts & Novel Tumourigenic Mechanisms in Neoplasms originating from the Adrenal Gland and Extra-adrenal Paraganglia

Chapter 8

Adrenocortical neoplasia: evolving concepts in tumorigenesis with an emphasis on adrenal cortical carcinoma variants

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ABSTRACT

Adrenocortical carcinoma (ACC) is a rare, heterogeneous malignancy with a poor prognosis. According to WHO classification 2004, ACC variants include oncocytic ACCs, myxoid ACCs and ACCs with sarcomatous areas. Herein, we provide a comprehensive review of these rare subtypes of adrenocortical malignancy and emphasize their clinicopathological features with the aim of elucidating aspects of diagnostic categorization, differential diagnostics and biological behavior. The issue of current terminology, applied to biphasic tumors with pleomorphic, sarcomatous or sarcomatoid elements arising in adrenal cortex, is also discussed. We additionally present emerging evidence concerning the adrenal cortical tumorigenesis and the putative adenoma–carcinoma sequence as well.

INTRODUCTION

Adrenocortical carcinoma (ACC) is a rare heterogeneous malignancy with an incidence of 0.7–2.0 cases per million population per year [1]. Although the diagnosis of malignancy is easy in most cases, mainly due to advanced stage at presentation, strictly intra-adrenal tumors must be evaluated for malignant potential [2]. In this regard, several multiparametric scoring systems, including the Hough scoring system, the Weiss scoring system, the Van Slooten scoring system and the Weiss revisited index [3, 4], and, also recently, diagnostic algorithms, such as the stepwise discriminate diagnostic system and the simplified diagnostic algorithm [5, 6], have been generated. The latter takes into account the presence of a disrupted reticulin framework, which constitutes the first step of this diagnostic approach, as being the single most sensitive feature of malignancy. Other parameters include mitotic count >5⁄50 HPF, presence of necrosis and venous invasion [6].

Table 1. Weiss system for assessing malignant potential of adrenocortical neoplasms [2, 3].

Histological features to be evaluated
Diffuse architecture (greater than one third of the tumor)
Clear cells comprising 25% or less of the tumor
High nuclear grade (grade 3 or 4 according to Fuhrman criteria)
Mitotic rate ≥ 6 per 50 high-power fields
Atypical mitotic figures
Necrosis
Venous invasion
Sinusoidal invasion
Capsular invasion

The presence of three or more criteria highly correlates with malignant behavior

Currently, the Weiss scoring system is the most popular among multiparametric scoring systems, owing to its reliability and relative simplicity (**Table 1**) [1, 7]. Nevertheless, the Weiss system suffers particular limitations including (**1**) lack of reproducibility of several criteria, (**2**) "borderline" tumors with a Weiss score of 2 or 3, (**3–4**) oncocytic and myxoid variants of adrenocortical tumors and (**5**) pediatric adrenocortical neoplasms [2, 7]. The latter are frequently characterized by a limited malignant potential especially in children<5 years of age, despite the presence of impressively atypical histologic features (**Figure 1**), which could be attributed either to a low stage upon presentation or to a putative origin from a cell of the fetal adrenal cortex [8].

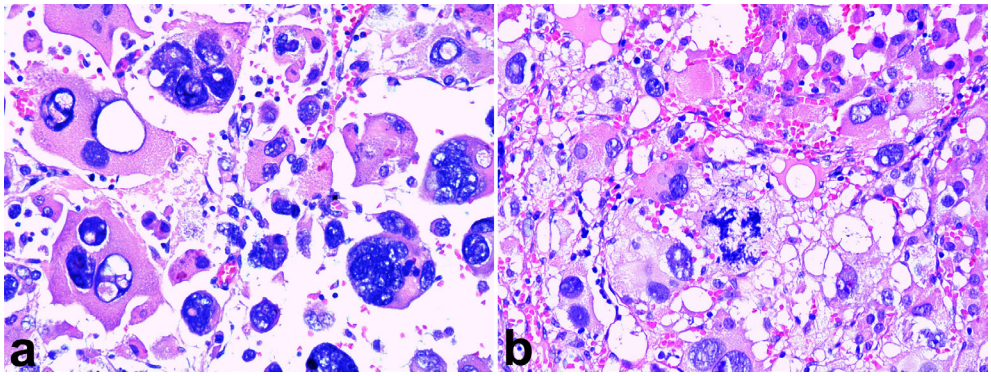

Figure 1. Two areas from a pediatric adrenal cortical neoplasm, weighing 88 g. Large, multinucleated tumor giant cells, with hyperchromatic nuclei and nuclear pseudoinclusions in a few, among smaller tumor cells displaying high nuclear/cytoplasmic ratio and hyperchromatic nuclei (**a**). Tumor cells with moderately atypical nuclei and an atypical mitotic figure near the center of the field (**b**). Despite these worrisome histological features, this neoplasm had a benign biological behavior.

The objectives of this review are (**1**) to present emerging evidence concerning the adrenocortical tumorigenesis in order to address the issue of an adenoma–carcinoma sequential progression as well as to further elucidate the incompletely understood pathophysiology of this aggressive neoplasia and (**2**) to describe in detail the rare variants of ACC to increase awareness of their clinicopathological features and the potential diagnostic challenges.

PART I. HISTOGENESIS OF CONVENTIONAL ADRENOCORTICAL CARCINOMAS

A hotly debated issue is whether or not adenomas progress stepwise to carcinoma. Given (**1**) the differences between benign and malignant tumors in terms of transcriptome and genetic alterations, (**2**) the epidemiology of adrenal incidentaloma and ACC and (**3**) the low incidence of progression (5–25%) of clinically inapparent adrenal masses to hyperfunction or an increase in size by at least 1 cm coupled with (**4**) the lack of clear evolution from benign, non-secreting adenoma to ACC in untreated cases [9, 10], it seems that multistep progression from benign to malignant is unlikely for adrenal cortical neoplasms.

However, not all evidence points in that direction. Fewer genetic aberrations were found in adenomas than in carcinomas. Most (72–86%) distinct alterations seen in the adenomas

were also present in carcinomas [11, 12]. Furthermore, the positive correlation between the number of alterations detected by comparative genomic hybridization (CGH) and tumor size [11, 13] supports the notion of an adenoma–carcinoma sequence. Additional evidence in favor of such a sequence derives from X-chromosome inactivation pattern analysis which shows monoclonal ACCs along with ACAs of polyclonal or monoclonal origin. Allelotyping with microsatellites and a few reported cases provide support for an adrenal adenoma–carcinoma sequence [14–20].

With regard to the latter, Bernard *et al.* [17] reported on an intriguing case of a localized adrenal neoplasm composed of two different components exhibiting different pathological features: a central zone of a Weiss score (5) and a peripheral zone of a Weiss score (0). The former zone displayed complete LOH at the *TP53* (17p13.3) gene locus, uniparental disomy at the 11p15 locus, overexpression of the IGF-II gene and numerous CGH changes, with gains at chromosomes 4, 5p, 10, 12p, 16p, 19 and 20 and losses at chromosomes 2 and 11. The outer zone did not show any of these genetic abnormalities. A similar case was presented by Trezzi *et al.* [18] as a dedifferentiated adrenal cortical neoplasm comprising a sharply outlined, hemorrhagic, central nodule (ACC) and an outer ring zone (ACA), while Gaujoux *et al.* [19] included one heterogeneous ACT in a small series consisting of an ACC component (Weiss score 6) developed within an ACA component (Weiss score 1). The malignant component exhibited diffuse cytoplasmic and focal nuclear β-catenin accumulation and harbored a β-catenin gene (*CTNNB1*) mutation, whereas the benign component showed only focal cytoplasmic staining and no such mutation. No *TP53* somatic mutation was found in the ACC and ACA components. Similarly, Schmitt *et al.* [20] presented an ACA case showing frank heterogeneity both by means of H&E staining (atypia, hyperchromasia, nucleoli and atypical mitoses) and immunohistochemistry, including perinuclear dot-like immunoreactivity for IGF-II, nuclear staining for CDK4 and Ki67 labeling index > 5%. These particular areas within the adenoma were interpreted as small foci of malignant transformation, in accordance with a van Slooten index > 8.

Whether the fact that different scoring systems yield different diagnoses in individual ACTs [20, 21] points to a multistep process remains to be clarified. It is tempting to speculate that in a process of clonal evolution, one dominant clone may overtake neighboring subclones to an extent that the ACA component is no longer visible.

Recent evidence has suggested multistep tumorigenesis within the group of ACCs [9]. In particular, transcriptome analysis has established an ACC subclassification into two distinct subgroups with different outcomes [22–24], consistent with the notion that ACC

survival and outcome vary greatly mainly due to different molecular defects modulating the tumor phenotype and, to a lesser extent, to tumor stage at diagnosis [25]. However, ACCs in the poor prognosis group displayed more advanced disease, subsequently confounding the issue of whether these subgroups correspond to distinct stages of the same disease or to distinct ACC types. Statistical independence of transcriptome-based prognostic information from stage seems to argue in favor of the latter [26].

In an effort to further elucidate the molecular genetics of the poor-outcome ACC group, Ragazzon *et al.* [25] identified three subgroups by using unsupervised clustering analysis: (**1**) p53 group, encompassing all tumors with a *TP53* mutation, (**2**) β-catenin group, containing all tumors with β-catenin nuclear staining, and (**3**) x group, with neither p53 nor β-catenin altered pathways, but enriched in cell cycle and metabolism genes. Moreover, IGF-II overexpression has been shown in approximately 90% of ACCs, with paternal unidisomy and loss of imprinting being the underlying mechanisms accounting for this markedly elevated expression in familial ACC cases associated with Beckwith–Wiedemann Syndrome, while additional mechanisms of transcriptional regulation are likely to be involved in sporadic ACCs [9, 23]. In this context, Ragazzon *et al.* [25] did refer to widespread IGF-II overexpression among both subgroups, highlighting its importance in adrenocortical tumorigenesis, but concomitantly suggesting a role for additional genomic alterations promoting the development of the most aggressive tumors [25, 27]. Prior to IGF-II overexpression, it appears that ACC loses nephroblastoma overexpressed (nov) expression [28], in line with the finding of a significantly reduced novH expression correlated with the acquisition of a malignant tumoral phenotype [29].

Several lines of evidence suggest that the activation of the Wnt/β-catenin signaling pathway is important in adrenocortical oncogenesis [19, 25, 30–37]. In fact, it has been demonstrated that this activation, as assessed by abnormal nuclear and/or cytoplasmic β-catenin accumulation with or without somatic activating *CTNNB1* mutations, is involved both in benign and malignant adrenocortical tumors [19, 31–33]. It is of particular interest to note that *CTNNB1* mutations (**1**) were detected only in macronodules of primary pigmented nodular adrenocortical disease cases, suggesting that these are secondary genetic events contributing to the nodular development of the disease and potentially implying a more aggressive phenotype [19, 34], (**2**) were related to a specific phenotype of larger and non-secreting ACAs, indicating a less differentiated state [33], and (**3**) were associated with decreased overall and disease-free survival in ACCs, suggesting a specific effect on tumor biology in terms of progression towards a more aggressive phenotype within the group of ACCs [25, 35]. Altogether, these observations, along with data stemming from an experimental mouse model [30], do not completely

clarify whether the activation of Wnt/β-catenin signaling pathway is an early or a late event in the development of adrenal neoplasms.

PART II. ADRENOCORTICAL CARCINOMA VARIANTS

Oncocytic adrenocortical neoplasms

Oncocytic adrenocortical neoplasms (OAN) represent a rare group of neoplasms with approximately 115 cases reported in the literature [38–44]. Kakimoto *et al.* [38] first described this particular histological variant in 1986, which was followed by numerous reports either in the form of single cases or of small series, with the largest one comprising 13 cases [39].

Accurate classification of OAN is important. Given that OANs display (**1**) cells with eosinophilic cytoplasm, clear cell population as a rule consisting of less than 25% of the tumor volume, (**2**) high-grade nuclear atypia in at least a subgroup of neoplastic cells and (**3**) almost always diffuse architecture and since these inherent histological parameters do not confer a poor prognosis [45], applying the Weiss system to this subset of adenocortical neoplasms will lead to a diagnosis of ACC, which is not appropriate given the benign behavior of most of these lesions.

Table 2. Lin–Weiss–Bisceglia (LWB) system for diagnostic categorization of oncocytic adrenocortical neoplasms [42].

Major criteria
Mitotic count >5 per 50 high-power fields
Atypical mitoses
Venous invasion
Minor criteria
Size >10 cm and/or weight >200 g
Necrosis
Sinusoidal invasion
Capsular invasion

The presence of one major criterion indicates malignancy, one to four minor criteria present indicates uncertain malignant potential, and the absence of all major and minor criteria is indicative of benign biological behavior.

Bisceglia and colleagues [46] developed criteria for the classification of oncocytic adrenocortical tumors (**Table 2**). These were based on parameters of the Weiss system

and constituted the Lin–Weiss–Bisceglia (LWB) system: (**1**) major criteria (a mitotic rate of more than 5 mitoses per 50 high-power fields, any atypical mitoses or venous invasion), (**2**) minor criteria [large size (>10 cm and/or > 200 gr), necrosis, capsular invasion or sinusoidal invasion] and (**3**) definitional criteria (predominantly cells with eosinophilic-granular cytoplasm, high nuclear grade and diffuse architectural pattern). The latter are not used as they are present in all subgroups of pure oncocytic neoplasms and are not determinants of biological behavior. According to the proposed working rules, the presence of any one of the major criteria indicates malignancy (oncocytic adrenocortical carcinoma), and the presence of one to four minor criteria is indicative of uncertain potential (borderline oncocytic neoplasm of uncertain malignant potential) (**Figure 2**), while the absence of all major and minor criteria indicates benign behavior (adrenocortical oncocytoma) [46]. In addition, Bisceglia *et al*. [45] proposed categories for oncocytic adrenocortical tumors as follows: (**1**) pure oncocytic tumor, if a tumor is exclusively or almost entirely composed (greater than 90%) of oncocytic cells, (**2**) mixed oncocytic tumor, when a clear cell component is also present (ranging from 10% to 50%), and (**3**) ordinary adrenocortical tumor with focal oncocytic changes, if the oncocytic component is not a predominant one (less than 50% of the tumor mass).

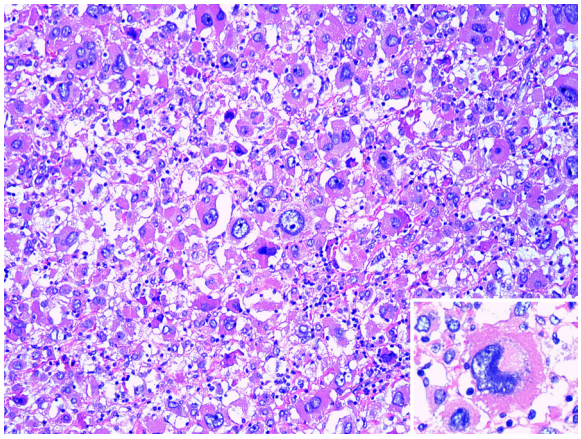

Figure 2. Section of an oncocytic adrenocortical neoplasm, weighing >200 g; in the presence of only one minor criterion (according to the Lin–Weiss–Bisceglia classification), a diagnosis of borderline oncocytic neoplasm of uncertain malignant potential was finally rendered. Diffuse growth pattern of tumor cells, characteristically displaying abundant eosinophilic, granular cytoplasm and at least focal high nuclear atypia. Marked degree of nuclear pleomorphism (inset). These would lead to a Weiss score 3, probably overestimating the biological behavior of this lesion.

It is critical to extensively sample an oncocytic adrenocortical neoplasm for several reasons. First, one should discriminate a pure oncocytic tumor either from an ordinary

adrenocortical tumor with focal oncocytic changes or its conventional counterpart of the compact cell type in order not to incorrectly apply the Weiss system and accordingly inadequately estimate its biological behavior [45]. Second, an oncocytic tumor can only be labeled as pure after quantifying each individual component (oncocytic and clear) since pure and mixed oncocytic tumors do not seem to share similar clinical outcome [45]. In this regard, immunohistochemistry using antimitochondrial antibodies can support the recognition of oncocytic differentiation and help to accurately quantify the oncocytic component. Of note, adrenocortical cells are, by nature, rich in mitochondria and therefore positive, so only strong, diffuse and finely granular staining should be taken as indicative of oncocytic differentiation, as suggested by Wong *et al*. [39] with respect to mES-13 staining. Moreover, the differential diagnostic spectrum of OANs also includes the oncocytic variant of pheochromocytoma, the granular cell variant of clear cell renal cell carcinoma, the eosinophilic variant of chromophobe renal cell carcinoma and the hepatocellular carcinoma [45, 46].

In an effort to further delineate and elucidate this subtype of adrenocortical neoplasia, Wong *et al*. [39] provided a comprehensive review and retrospectively applied the LWB criteria to all reported cases with sufficient information, along with 13 previously unpublished cases. Of note, they validated the effectiveness of the LWB criteria in predicting malignant potential and provided preliminary evidence supporting the concept of a more favorable prognosis for oncocytic adrenocortical carcinomas in comparison with their conventional counterparts. Moreover, they found approximately 30% of OANs to be functional while emphasizing the frequency of small oncocytes in several cases of their series [39], accordingly including in their view of the oncocyte not only cells with abundant granular eosinophilic cytoplasm.

Myxoid adrenocortical neoplasms

In keeping with the current notion that myxoid extracellular matrix is a histological feature that can be found in both physiological and pathological conditions, non-neoplastic (e.g. myxedema) as well as neoplastic (benign/malignant epithelial or mesenchymal tumors) [47], myxoid change has been recognized rarely also in neoplasms of adrenal cortical origin. The myxoid substance has been shown to be Alcian blue positive and negative or focally weak positive for PAS stain and mucicarmine. In particular, there have been thus far 46 reports of myxoid adrenocortical neoplasms (MANs) [48–65]: 17 myxoid adrenocortical adenomas [49–58], 2 borderline MANs [48, 50] and 27 myxoid adrenocortical carcinomas [48, 49, 59–65]. These cases were either published as isolated case reports or were presented in small series, with the largest ones each consisting of 14 cases [48, 49].

The reported age range of MANs (46 cases) is 16 to 82 years, with a mean and a median age of 51 and 51.5 years, respectively. In general, cases in females slightly outnumber those in males (26:20). Hormone hypersecretion occurs frequently in these tumors; only 13 out of 42 cases were non-functioning (eight adenomas, one borderline and four carcinomas), whereas five patients had biochemical evidence of hormone production (two adenomas, one borderline and two carcinomas). The remaining 24 cases with an available endocrine evaluation presented with clinical evidence of hormone overproduction: 16 with Cushing-related symptoms, 4 with Conn syndrome, 2 with symptoms due to cortisol and androgens, 1 with gynecomastia and breast pain and 1 with virilization (4 adenomas and 20 carcinomas). Although it had been suggested that the functional status could be associated with the presence of myxoid material, non-functioning tumors displaying a higher percentage of myxoid areas and being lipid depleted [61], no definite conclusions can be reached on morphological grounds. In fact, eight cases showing a myxoid area > 70% were hormonally active [48–50, 62, 63], whereas another eight cases with a similar myxoid content were non-functional [48, 51, 53–55, 57, 59, 60]. The proportion of myxoid change varied in all reported cases from 5% to approximately 100% of the total area. Interestingly, neurofilament (NF) expression has been recently found to be related more to the extent of myxoid changes and, therefore, suggested as a potential characteristic of myxoid ACCs with a predominant (> 70%) myxoid component [48]. This, along with the fact that the expression of neuroendocrine/neuronal markers such as NF and CD56 was mostly restricted to the myxoid areas of conventional ACCs with focal myxoid change, seems to imply a peculiar association between NF expression and myxoid change [48].

An important point is the distinction of myxoid borderline/malignant ACTs into two subgroups as proposed by Papotti *et al*. [48]: myxoid ACCs and conventional ACCs with focal myxoid changes. This is based on a different type of myxoid change, growth pattern, cell size, nuclear atypia and cell cytoplasm. Myxoid ACCs are usually characterized by (**1**) extensive myxoid areas separated from conventional ACC areas, if present, (**2**) trabecular/microacinar growth pattern, (**3**) small, uniform cell size, (**4**) mild nuclear atypia and (**5**) scant, eosinophilic cell cytoplasm, whereas conventional ACCs with focal myxoid changes are characterized by (**1**) focal myxoid areas (< 20% of the total area) always merging with conventional ACC areas, (**2**) solid/diffuse growth pattern, (**3**) large, heterogenous cell size, (**4**) moderate to high nuclear atypia and (**5**) abundant, granular eosinophilic cell cytoplasm. Nevertheless, two cases of the former subset showed overlapping features such as solid growth pattern and pleomorphism as well. In addition, the same investigators [48] suggested that only the former subgroup actually belongs to the distinctive myxoid variant of ACTs, with the latter potentially representing the result of myxoid degenerative changes (**Figure 3**). Whether or not these subgroups have a distinct biologic behavior needs to be further elucidated.

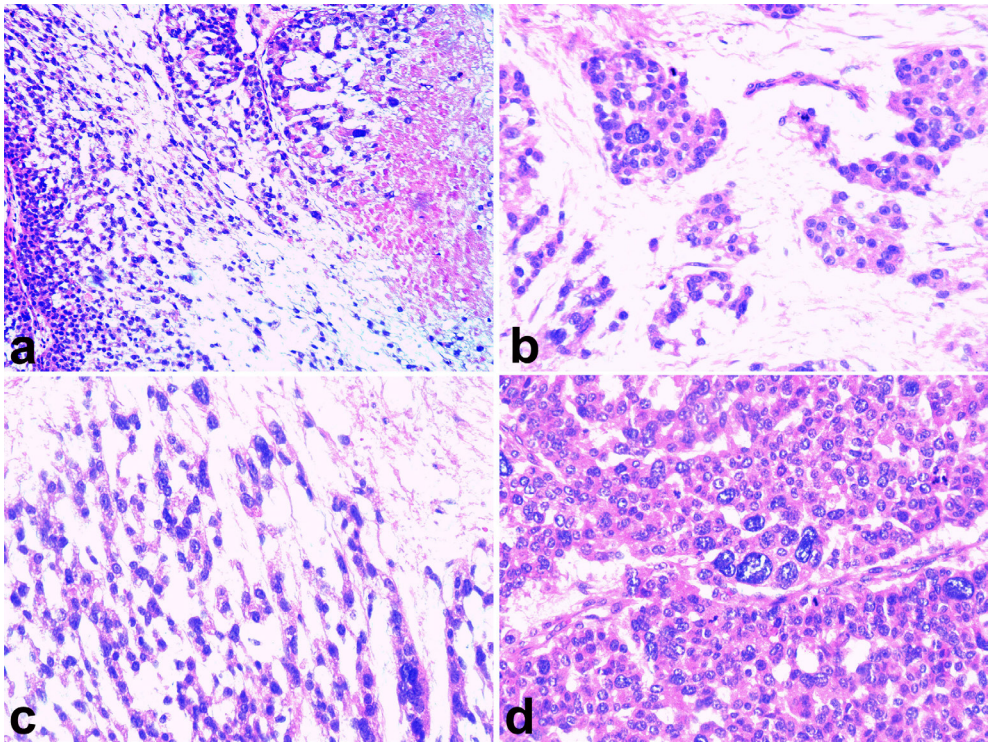

Figure 3. Section of a myxoid adrenocortical carcinoma showing a necrotic area at the right of the field (**a**). Tumor cells are arranged in a small nested (**b**) or trabecular/cord-like (**c**) growth pattern focally displaying marked nuclear atypia (**b–c**). Area of conventional adrenocortical carcinoma (60–70% of total area in histologic sections); neoplastic cells are arranged in an interanastomosing trabecular pattern showing severe nuclear atypia near the center of the field (**d**).

In the histopathological assessment of these lesions, it is critical first to exclude potential morphological mimics, including metastatic tumors affecting the adrenal gland (breast carcinomas, salivary or skin adnexal carcinomas of adenoid cystic type and neuroendocrine tumors) [48] as well as primary retroperitoneal neoplasms with myxoid areas (chordomas, myxomas, lipomas or liposarcomas, leiomyomas or leiomyosarcomas, benign or malignant nerve sheath tumors, extraskeletal myxoid chondrosarcomas, gastrointestinal stromal tumors and myxoid malignant fibrous histiocytomas) [49, 50, 63]. The presence of typical foci of adrenocortical differentiation, along with an immunohistochemical profile similar to that of the conventional ACT, provides valuable aid in excluding these mimics [48, 50, 51]. In addition, accurate assessment of malignant potential in any given tumor of the myxoid variant is important. In this regard, the issue of whether the Weiss scoring system is adequate to reliably predict malignancy in MANs is currently being questioned because:

(**1**) A MAN case lacking morphological signs of malignancy (Weiss score 1/clear cell cytoplasm present in less than 25% of the tumor cells) and diagnosed as myxoid borderline ACT [59] subsequently did demonstrate an aggressive clinical behavior (peritoneal spread), with a dismal outcome 68 months following the initial diagnosis [48]. Another such tumor of uncertain malignant potential (Weiss score 1/clear cell cytoplasm present in less than 25% of the tumor cells) has been recently described by the same group, with no evidence of recurrence or metastasis 9 months following surgical resection. Both cases were characterized by the same extent of myxoid area (90%), growth pattern, Weiss score and low Ki67 labeling index (3% and 4%, respectively). Interestingly, the latter case had an intact reticulin network as opposed to the former, which displayed stromal framework disruption as confirmed by the reticulin stain [48].

(**2**) Another MAN of borderline malignancy (average mitoses 3/10 HPFs, mild to moderate nuclear pleomorphism, cells with ample predominantly eosinophilic cytoplasm, occasional clear cells and a tumor weight of 94 g) did not recur or metastasize after a follow-up period of 18 months [50]. In addition, four out of six myxoid ACAs reported in a series by Brown *et al.* [49] were large (≥ 6 cm), showed focal capsular invasion and a mitotic activity of ≥ 1/10 HPFs in the absence of recurrence or metastatic disease after a mean follow-up period of 12 years. It should be stressed that 9 out of 17 myxoid ACAs were relatively large (≥ 6 cm), a feature further challenging the prediction of a benign clinical course [49, 50, 54–57].

(**3**) Some of the Weiss score parameters were hard to assess in the myxoid areas, while others could be identified in a variable percentage according to observations made by Papotti *et al*. [48] in the subset of myxoid ACCs. In particular, diffuse growth pattern was never a feature, atypia was minimal or absent, and small vessel invasion was less apparent due to myxoid areas in contrast to necrosis, "dark" cell cytoplasm and mitotic figures which were the only readily apparent criteria. In keeping with this, Hsieh *et al*. [62] reported on a myxoid ACC showing an extensive myxoid area (80% of the tumor area) of Weiss score (3) and a nonmyxoid component of Weiss score (6); the latter consisted of three growth patterns (trabecular, solid and clear cell), each exhibiting different Weiss score parameters. Moreover, Brown *et al*. [49] noted that mitoses, when present, were generally less frequent in myxoid areas, while extensive extracapsular involvement was a feature only of carcinomas.

Adrenocortical carcinomas with a sarcomatous or sarcoma-like component

According to WHO classification 2004, oncocytic ACCs, myxoid ACCs and ACCs with sarcomatous areas are regarded as rare variants of ACC [66]. Whether the latter variant

should be designated carcinosarcoma or sarcomatoid carcinoma, as previously suggested by Sturm and colleagues [67] in agreement with the WHO terminology applied to biphasic tumors with pleomorphic, sarcomatous or sarcomatoid elements arising in other organs [68], is still unsettled. Nevertheless, nine cases of this unusual variant have been previously reported [67, 69–76], with only four corresponding to true carcinosarcomas [69, 72–74], consistent with the concept that carcinosarcomas are tumors composed of morphologically malignant epithelial and specialized mesenchymal elements, easily recognizable histologically as forms of mesenchymal malignancy resembling osteosarcoma, chondrosarcoma, rhabdomyosarcoma, angiosarcoma or liposarcoma (**Figure 4**) [77]. Adrenal carcinosarcomas, like their counterparts in other organ systems, were mainly characterized by a rhabdomyosarcomatous component (three out of four) [69, 73, 74], while only one case was characterized by a mixture of osteosarcoma and chondrosarcoma [72]. The remaining five biphasic cases without heterologous elements did have a spindle cell component [67, 70, 71, 75, 76], with three out of five also showing variably giant cells in the sarcomatous component [67, 70, 75].

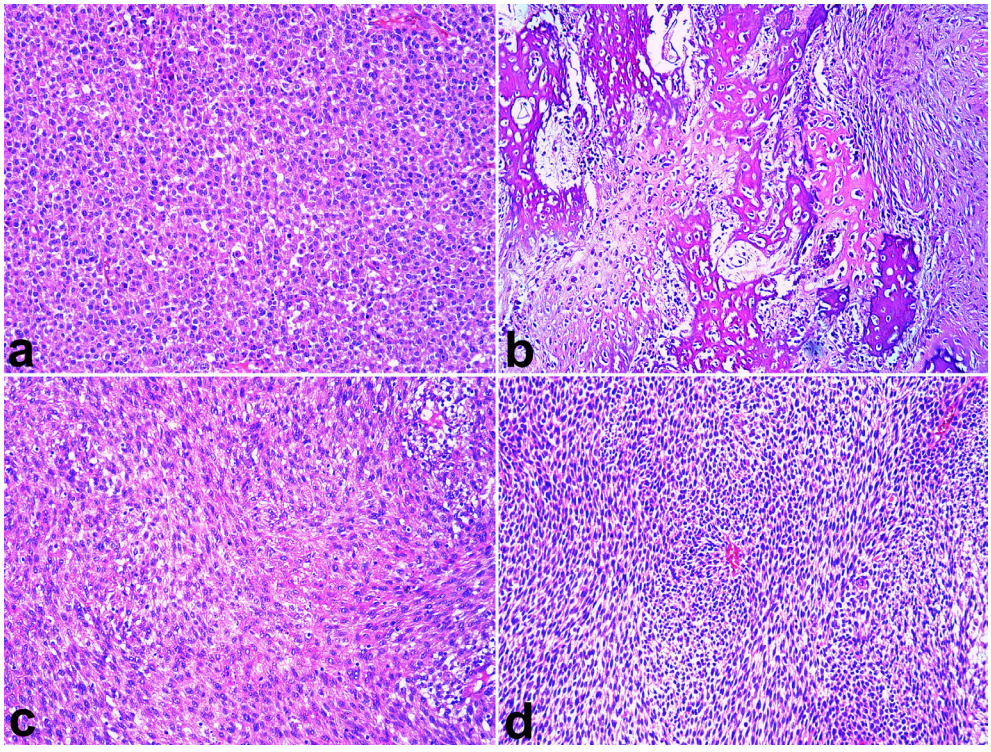

Figure 4. Sections of an adrenocortical carcinosarcoma comprising a well-differentiated carcinomatous component (**a**) and a sarcomatous component demonstrating osteosarcomatous differentiation (**b**) along with areas of uniform spindle cells arranged in a fascicular (**c**) or herringbone (**d**) growth pattern.

Another unique malignant biphasic neoplasm arising as a primary tumor of the adrenal gland is the adrenocortical blastoma. This functional (virilizing) malignant tumor has been reported in an infant, and it consisted of a mixture of immature epithelial and mesenchymal elements, along with slit-like spaces partially lined by primitive epithelial cells, focally recapitulating the morphology of the normal embryologic development of the adrenal cortex [78]. There have been no additional cases reported, and therefore, the question whether this neoplasm is indeed restricted to the pediatric age group [79] similar to pleuropulmonary blastoma (virtually restricted) but in contrast to pulmonary blastoma [77] remains unresolved.

Of interest, only two out of four carcinosarcomas were functional, presenting with clinical signs either of hyperaldosteronism or of virilization [72, 73], whereas all adrenocortical sarcomatoid carcinomas were nonfunctioning [67, 70, 71, 75, 76].

Although the limited number of reported cases does not allow any definite conclusion, adrenocortical carcinosarcomas and sarcomatoid carcinomas have similar clinical outcome (postoperative survival ranging from 2 days to 12 months; median 6 months) [69] and therefore have a worse prognosis than their conventional counterparts [67]. This dramatically aggressive behavior may be attributed not only to advanced stage at presentation but also in part to inherent biological properties potentially deriving by progression (dedifferentiation) from a pre-existing better differentiated ACC, in analogy to the undifferentiated (anaplastic) carcinoma of the thyroid gland [66, 80]. This endows cells with migratory and invasive properties, epithelial-mesenchymal transition, stemness, prevention of apoptosis and senescence, and it contributes to immunosuppression and notably confers resistance to chemotherapy and immunotherapy [81].

Although various theories have been proposed with regard to the histogenesis of carcinosarcomas, namely (**1**) the composition tumor theory (paradoxically requiring a nonmalignant non-epithelial component as a reactive proliferative response induced by the epithelial component via paracrine secretion), (**2**) the collision or biclonal tumor theory (a collision between two synchronous, histogenetically independent, biclonal tumors), (**3**) the conversion tumor theory (neoplastic transformation within a monoclonal tumor recapitulating the naturally occurring event of conversion of epithelial to mesenchymal cells during embryogenesis) [81] and (**4**) the combination or divergent tumor theory (deriving from a common monoclonal stem cell precursor) [82–85], molecular genetic evidence of monoclonality supports the single pluripotential stem-cell-divergence hypothesis and the epithelial-to-mesenchymal transition as well [83–85]. With regard to ACCs with sarcomatous areas, evidence emerging from the immunophenotypical

profile of the sarcomatous component (focal retaining of Melan-A, synaptophysin and calretinin positivity) [69] and the presence of transitional (intermingled) zones between carcinomatous and sarcomatous components [67, 70, 73] seem to imply such a putative transition. Differences of the mitotic count in the distinct elements [7/10 HPF (epithelioid component) versus 30/10 HPF (pleomorphic/spindle cell component)] [69] and the presence only of the sarcomatous component either in large veins into the periadrenal fat [67] or in well-established metastases [71, 74] are consistent with a dedifferentiation process.

Aberrant phenotypical differentiation observed in this particular variant includes both neural and melanocytic differentiation (NSE, S100 and HMB-45 immunoreactivity) [70, 71]. Although melanocytic differentiation, suggestive of a divergent evolutionary pathway, has been reported in carcinosarcomas of other anatomic locations [86–89], pathologists should always keep in mind to first exclude the possibilities of a metastatic melanoma, a collision tumor consisting of an ACC and a primary or metastatic melanoma (not yet reported) and a primary perivascular epithelioid cell tumor [90]. Other differential diagnostic considerations include composite pheochromocytoma with a malignant peripheral nerve sheath tumor component (the latter with or without divergent differentiation into mesenchymal elements such as rhabdomyo-, osteo-, chondro-, angio- and/or fibro-sarcomatous components) [91, 92], metastatic carcinoma with sarcomatous or sarcoma-like areas and metastatic neoplasm with sarcomatoid elements other than carcinomas (melanoma or germ cell tumor), as well as primary retroperitoneal sarcomas [69]. Given that adrenal biphasic tumors, in contrast to those arising in epithelial organs, are unlikely to express cytokeratins while focal smooth muscle differentiation is a common finding, thorough sampling of the resected tumor is of critical importance in order to reveal the co-existent carcinomatous component and subsequently prove the adrenal origin on immunohistochemical grounds [67, 69].

Since ACCs with sarcomatous areas are highly aggressive tumors and a sarcomatous or sarcomatoid component seems to be a predictor of shorter survival in ACC, the question arises whether the Weiss scoring system should be applied to this extremely rare ACC variant. In fact, ACCs with sarcomatous areas regroup the majority of Weiss histological criteria [67]. In this setting, Sturm and colleagues [67] have proposed that a sarcomatous or sarcomatoid component should be well circumscribed and represent at least 10% of the tumor bulk to establish this unusual diagnosis. Nevertheless, the sarcomatous or sarcomatoid component is not included in the Weiss system, further confounding this issue when dealing with a biphasic tumor demonstrating a predominant non-carcinomatous component [67, 69]; in such cases, the criterion of diffuse architecture (greater than 1/3) is being obviously challenged.

CONCLUSION

In summary, we discussed the issue of multistep tumorigenesis in adrenocortical neoplasia and provided an overview of the ACN variants in order to make histopathologists aware of the clinicopathological spectrum. It is important to correctly characterize their biological potential, which is occasionally not an easy task for myxoid and oncocytic adrenocortical tumors. In this context, applying the Weiss scoring system on the latter would definitely tilt the diagnostic balance towards malignancy in a similar way as pediatric ACNs (pathologically malignant but benign-behaving tumors) [8], whereas underdiagnosis could potentially be the case with regard to the myxoid ones. In this setting, even the multistep tumorigenic processes are hard to take apart and analyze, while evidence for a distinct biological behavior is either preliminary [39] or unestablished [48] due to the small number of reported cases, limited follow-up period, insufficient data and heterogeneity. Whenever pathologists report on these variants singly or as part of larger series, it is recommended to note the diagnostic subcategory, as has been previously suggested (pure oncocytic tumor/mixed oncocytic tumor/ordinary adrenocortical tumor with focal oncocytic changes, myxoid ACCs/conventional ACCs with focal myxoid changes and carcinosarcoma/ sarcomatoid carcinoma) [39, 48].

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Chapter 9

Telomerase reverse transcriptase promoter mutations in tumors originating from the adrenal gland and extra-adrenal paraganglia

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ABSTRACT

Hotspot mutations in the promoter of the telomerase reverse transcriptase (*TERT*) gene have been recently reported in human cancers and proposed as a novel mechanism of telomerase activation. To explore *TERT* promoter mutations in tumors originating from the adrenal gland and extra-adrenal paraganglia, a set of 253 tumors (38 adrenocortical carcinomas (ACCs), 127 pheochromocytomas (PCCs), 18 extra-adrenal paragangliomas (ea PGLs), 37 head and neck PGLs (HN PGLs), and 33 peripheral neuroblastic tumors) was selected along with 16 human neuroblastoma (NBL) and two ACC cell lines to assess *TERT* promoter mutations by the Sanger sequencing method. All mutations detected were confirmed by a SNaPshot assay. Additionally, 36 gastrointestinal stromal tumors (GISTs) were added to explore an association between *TERT* promoter mutations and SDH deficiency. *TERT* promoter mutations were found in seven out of 289 tumors and in three out of 18 human cell lines; four *C228T* mutations in 38 ACCs (10.5%), two *C228T* mutations in 18 ea PGLs (11.1%), one *C250T* mutation in 36 GISTs (2.8%), and three *C228T* mutations in 16 human NBL cell lines (18.75%). No mutation was detected in PCCs, HN PGLs, neuroblastic tumors as well as ACC cell lines. *TERT* promoter mutations preferentially occurred in a SDH-deficient setting ($P = 0.01$) being present in three out of 47 (6.4%) SDH-deficient tumors vs zero out of 171 (0%) SDH-intact tumors. We conclude that *TERT* promoter mutations occur in ACCs and ea PGLs. In addition, preliminary evidence indicates a potential association with the acquisition of *TERT* promoter mutations in SDH-deficient tumors.

INTRODUCTION

Telomerase is a ribonucleoprotein complex consisting of the telomerase reverse transcriptase (TERT) catalytic subunit and the telomerase RNA component. Telomerase is responsible for the addition of telomeric repeats at the end of linear eukaryotic chromosomes, thereby maintaining the telomere length [1]. Telomeres have two major functions in normal cells [1-2]. First, they function to protect chromosome ends from being recognized as DNA doublestrand breaks by the DNA repair machinery that can result in fusion of chromosome ends and gross chromosomal alterations. Secondly, telomeres prevent 3'-DNA shortening during cell division that can trigger cellular senescence.

In cancer cells, which display uncontrolled proliferation, maintenance of telomeres is crucial to prevent senescence induction. As a consequence, tumor cells frequently show activation of mechanisms that protect telomeres and confer cellular immortalization. In over 90% of cases, tumor cells display constitutive telomerase activation [2]. While there exists evidence that telomerase activity is regulated at various levels including epigenetic mechanisms [3-4], posttranslational modification [5-6], or nuclear translocation [7] of TERT, upregulation of TERT at the transcriptional level, via the inappropriate binding of transcription factors such as c-myc to the core promoter region [3, 8-9], appears to be the primary mechanism yielding telomerase activation.

Consistent with this, recent studies in melanoma have demonstrated that activation of telomerase via transcriptional TERT upregulation can be caused by mutations in the core promoter region of *TERT* (Chr5) with 1 295 28 C>T, 1 295 250 C>T being the two most frequent mutation hotspots [10-11]. Both mutations result in novel binding motifs for E-twenty-six transcription factors. This results in enhanced transcription of TERT, demonstrating a novel mechanism contributing to telomerase activation in human cancer [10-11]. Similarly, other studies have revealed *TERT* promoter mutations at varying site-specific frequencies in conjunctival melanoma, non-melanoma skin cancer, bladder cancer, CNS tumors, thyroid tumors, soft-tissue sarcomas, neuroblastomas (NBLs), hepatocellular carcinomas, renal cell carcinomas (RCCs), mesotheliomas, oral cavity carcinomas, and endometrial and ovarian clear cell carcinomas as well as gastrointestinal tract tumors [12-34].

The prevalence of *TERT* promoter mutations in follicular cell-derived thyroid cancer indicated that these mutations may be important in endocrine tumorigenesis [20-22, 25]. Consistent with this prevalence, four independent research groups illustrated that more aggressive thyroid cancer subtypes were enriched for these mutations [20-22, 25]. With

regard to adrenocortical carcinomas (ACCs), a frequency of 12% has been recently shown in a single cohort [35]. By contrast, no mutations have been observed in parafollicular cell-originated medullary thyroid carcinoma [15, 21-22, 25, 36], while these seem to be extremely rare genetic events in pheochromocytomas (PCCs) and paragangliomas (PGLs) [25, 35]. In the current study, we examined the presence of these mutations in tumor types originating from the adrenal gland and extra-adrenal paraganglia including ACCs, PCCs, extra-adrenal (ea)- and head and neck- (HN-) PGLs, as well as peripheral neuroblastic tumors. Given that *TERT* promoter mutations occur preferentially in specific genetic backgrounds in various tumors, any association with SDH-deficient status in PCCs, PGLs, and gastrointestinal stromal tumors (GISTs) was explored.

SUBJECTS AND METHODS

Tumor tissue samples and cell lines

A total of 253 formalin-fixed and paraffin-embedded (FFPE) tumors were selected, including 38 ACC samples (Erasmus MC, Rotterdam, The Netherlands: 35 primary tumors, two recurrences, and one metastasis), 127 PCCs/18 ea PGLs/37 HN PGLs (Erasmus MC, Rotterdam, The Netherlands: 167 cases; UMC St Radboud, Nijmegen, The Netherlands: 12 cases; and Birmingham, UK: three cases), and 33 peripheral neuroblastic tumors (Erasmus MC, Rotterdam, The Netherlands: 15 NBLs, eight ganglioneuroblastomas, and ten ganglioneuromas). Tumors with mutations in the *SDH-x* genes, such as *SDHA, SDHB, SDHC, SDHD,* and *SDHAF2*, display loss of immunohistochemical staining for SDHB [37-38]. Given that loss of SDHB expression reflects SDH deficiency [39], we will collectively use the term 'SDH deficient' for tumors displaying SDHB immunonegativity. As SDH deficiency also defines a subset of GISTs similar to the SDH-related PCC/PGL subgroup, an additional series of 36 GISTs was examined to explore the relationship between *TERT* promoter mutations and SDH deficiency in a non-endocrine tumor type.

All tumor samples 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. Human NBL cell lines: SJNB-12, SJ10 (SJNB-10), SK-N-BE, KCNR, LAN-1, LAN-5, N206, NGP-C4, NMB, TR-14, SH-EP-2/tet2, SJ1 (SJNB-1), SK-N-SH, SH-SY5Y, GI-ME-N, and SK-N-AS as well as human ACC cell lines NCI-H295 (source: ATCC (CRL-2128); method of authentication: STR profiling; passage number: P7) and SW13 (source: ATCC (CCL-105); method of authentication: STR profiling; passage number: P2) were also included in the analysis. The NBL cell lines have been originally obtained from the

NCI and are molecularly well characterized/established in the field of NBL research [40]. These cell lines were grown from the original clones and used after < 35 passages; all have been checked for molecular characteristics in our departmental research laboratory.

DNA isolation and *TERT* promoter mutation analysis

DNA isolation from tumors was carried out using standard procedures following manual microdissection of all tumor samples to ensure a > 80% neoplastic cell content. Standard PCR was performed to amplify a 163 bp fragment of the *TERT* promoter region, covering all previously described mutations (*C228T*, *CC229TT*, *CC242TT*, and *C250T*, corresponding to nucleotide positions -124, -125, -138, and -145 from the translational start site (UCSC: chr5 nt 1 295 104)), using forward primer 50-GTCCTGCCCCTTCACCTT-30 and reverse primer 50-CAGCGCTGCCTGAAACTC-30. Subsequently, PCR products were used as templates for direct sequencing using the BigDye Terminator V3.1 cycle sequencing kit (Applied Biosystems). Products were analyzed on the ABI Prism 3130 Genetic Analyzer (Applied Biosystems).

TERT promoter mutations were confirmed by a SNaPshot assay using the ABI Prism SNaPshot Multiplex Kit (Applied Biosystems) as described previously [41]. In brief, after the multiplex SNaPshot reaction, the products were treated with shrimp alkaline phosphatase to remove excess dideoxynucleotide triphosphates, and subsequently were labeled and separated in a 25-min run on 36-cm-long capillaries in an automatic sequencer (ABI Prism 3130 Genetic Analyzer, Applied Biosystems). GeneScan Analysis Software, version 3.7 (Applied Biosystems) was used for data analysis. All experimental conditions are available on request. Probe sequences of the SNaPshot reaction are given in **Supplementary Table 1**, see section on **supplementary data** given at the end of this article.

SDHB/SDHA immunohistochemistry, mutation screening, and loss of heterozygosity analysis

SDH (immunohistochemistry (IHC) and/or mutation) status was known for 218 PCCs, ea PGLs, HN PGLs, and GISTs. To investigate the SDH status of the ACC samples included in the current study, these samples were arranged in a tissue microarray (TMA) format along with additional adrenocortical adenomas (ACAs), normal adrenal tissue, and control tissue samples (38 ACC, 17 ACA, five normal adrenal tissue, and 12 control tissue samples) using an automated TMA constructor (ATA-27 Beecher Instruments, Sun Prairie, WI, USA) available at the Department of Pathology, Erasmus MC. For each tumoral case, representative areas were selected and marked on a hematoxylin and eosin-stained slide. Accordingly, two tissue cores with a diameter of 1 mm were

Table 1. Clinicopathological and genetic data of patients with *TERT* promoter-mutated tumors.

Case *No*	Tumor Type	Anatomic Site	Sex	Age	*TERT* promoter mutation	*SDH*-deficient	Weiss score	Metastatic Disease/ Site	Follow-up/ Status
1	ACC	Adrenal gland	F	50	*C228T*	No	5	Yes/ liver	9 mo/ DOD
2	ACC	Adrenal gland	M	51	*C228T*	No	6	Yes/ liver, lung, bone	12 mo/ DOD
3	ACC	Adrenal gland	M	42	*C228T*	Yes	8	Yes/ liver, lung, LNs	2 mo/ DOD
4	ACC	Adrenal gland	F	58	*C228T*	No	7	None	105 mo/ AWED
5	ea PGL	Urinary bladder	M	46	*C228T*	Yes *	-	Yes/ LNs	NA
6	ea PGL	Urinary bladder	M	61	*C228T*	Yes **	-	Yes/ LNs	226 mo/AWED
7	GIST	Stomach	F	57	*C250T*	Yes ***	-	Yes/ liver	33 mo/DOD

Abbreviations: ACC, adrenocortical carcinoma; AWED, Alive without evidence of disease; DOD, Dead of disease; ea PGL, extra-adrenal paraganglioma; GIST, gastrointestinal stromal tumor; LN, lymph nodes; NA, not available
* SDHB IHC -/ SDHA IHC – as previously published in *Korpershoek et al. J Clin Endocrinol Metab 96 E1472-1476* (non-informative on mutational analysis due to poor DNA quality)
** SDHB IHC -/ SDHA + (*SDHB* c.292T>C p. Cys98Arg)
*** SDHB IHC -/ SDHA IHC + (*SDHD* c.416T>C p.Leu139Pro)

extracted from 'donor' block and brought into the 'recipient' paraffin block at predefined coordinates. SDHA and SDHB immunostaining procedures were performed on 4–5 μm TMA sections with a mouse monoclonal Ab14715 antibody (Mitosciences, Abcam, Cambridge, UK; 1:500 dilution) against SDHA and a 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). If the internal control (granular staining in endothelial cells) was positive, slides were considered as informative. From SDHB-immunonegative/SDHA-immunopositive ACCs, (**i**) the entire *SDHA*, *SDHB*, *SDHC*, *SDHD*, and *SDHAF2* coding sequences were assessed at the germline and somatic levels for mutations using an Ion AmpliSeq Custom Panel that was sequenced on the Ion Torrent Personal Genome Machine (PGM; Life Technologies) on 10 ng FFPE tumor DNA according to the manufacturer's protocols. In short, libraries were made using the Ion AmpliSDefault 2.0 Library Kit. Template was prepared using the Ion OneTouch Template Kit and sequencing was performed with the Ion PGM Sequencing 200 Kit v2.0 on an Ion 316v2 chip. Data were analyzed using the Torrent Suite Software, version 3.6.2 (Life Technologies). Annotation of variant calls was performed with Annovar (http://www.openbioinformatics. org/annovar/; [42]) and facilitated using an in-house galaxy platform/server on which Annovar wrapper was installed [43-46]. The variants with a read frequency higher than 10%, not known as common polymorphisms according to 1000G2012 April and ESP6500, non-synonymous with a minimum of five forward/reverse variant reads and 100 total depth reads were retained as interesting ones (mutations) (sequences of all primers and probes are available upon request); and large intragenic deletions using multiplex ligation-dependent probe amplification (MLPA) assay were analyzed using a commercially available kit (SALSA MLPA P226-B2; MRC Holland, Amsterdam, The Netherlands) and (**ii**) loss of heterozygosity (LOH) analysis was performed for polymorphic microsatellite markers flanking the *SDHB*, *SDHC*, *SDHD*, and *SDHAF2* genes as described previously [47].

RNA extraction and TERT expression analysis by quantitative real-time PCR

Total mRNA was extracted from human primary adrenal tissue (one ACC harboring a TERT promoter mutation, two ACCs without *TERT* promoter mutation, one ACA, and two normal adrenocortical tissue samples) or cell pellets (HEK and SW13 cell lines) using TRIzol reagent (Invitrogen Life Technologies) and the RNA-containing supernatant was purified using RNeasy spin columns (Qiagen Benelux B.V.). First-strand cDNA synthesis was performed on 200 ng total RNA using qScript cDNA Supermix (Quanta Biosciences, Gaithersburg, MD, USA), followed by *TERT*-specific and hypoxanthine phosphoribosyltransferase 1 (HPRT1)-pre-amplification using PerfeCTa PreAmp SuperMix (Quanta Biosciences). The PreAmp product was diluted and used to assess

human telomerase expression in all samples by quantitative realtime PCR in triplicate using TaqMan (Applied Biosystems) gene expression assays. *TERT* (*TERT* Hs00972656_m1) was measured relative to *HPRT* (*HPRT1*) expression. The relative amount of RNA was calculated by the 2-ΔΔCT method. Fold changes in gene expression were determined by comparing expression levels of tumor tissue or cell lines with normal adrenocortical tissue. No RNA was available to test the remaining tumors endowed with the *C228T* and *C250T* mutations.

STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS (IBM SPPS Statistics, version 20) on a series of 218 tumors (PCCs/PGLs/GISTs) of known SDH status. Fisher's exact test was used to determine the relationships between the presence of a *TERT* promoter mutation and SDH deficiency. Statistical differences were considered to be significant when the *P* value is < 0.05.

RESULTS

Prevalence of *TERT* promoter mutations in various human tumors and cell lines

TERT promoter mutations were found in seven out of 289 tumors investigated with *C228T* being the most frequent substitution. There were four *C228T* mutations in 38 ACCs (10.5%), two *C228T* mutations in 18 ea PGLs (11.1%), and one *C250T* mutation in 36 GISTs (2.8%). Clinicopathological and genetic data of these patients are given in **Table 1** in detail, while representative somatic *TERT* promoter mutations (*C228T* and *C250T*) detected both by the Sanger sequencing method and a SNaPshot assay are displayed in **Figure 1**. Out of seven, six *TERT* promoter-mutated tumors were metastatic (**Table 1**). Although three out of four mutation-positive ACCs were characterized by highly aggressive biological behavior, we could not perform proper survival analysis due to the limited number of these cases. Mutations were not detected in any of the 127 PCCs, 37 HN PGLs, and 33 peripheral neuroblastic tumors. The *TERT* promoter mutation *C228T* was found in three out of 16 (18.8%) human NBL cell lines (SJNB-10, SJNB-12, and SK-N-BE), while no mutations were present in the two ACC cell lines (**Supplementary Table 2**, see section on **supplementary data** given at the end of this article).

Enrichment of *TERT* promoter mutations in SDH-deficient tumors

Given that a subset of PCCs, PGLs, and GISTs is associated with germline *SDH-x* mutations

and/or loss of SDHB immunoexpression (collectively known as SDH-deficient tumors) and three out of 47 (6.4%) SDH-deficient tumors harbored a *TERT* promoter mutation, we analyzed the relationship between the SDH-deficient status and the presence of *TERT* promoter mutations. It has been demonstrated that *TERT* promoter mutations occur preferentially in SDH-deficient tumors (6.4 vs 0%; $P = 0.01$).

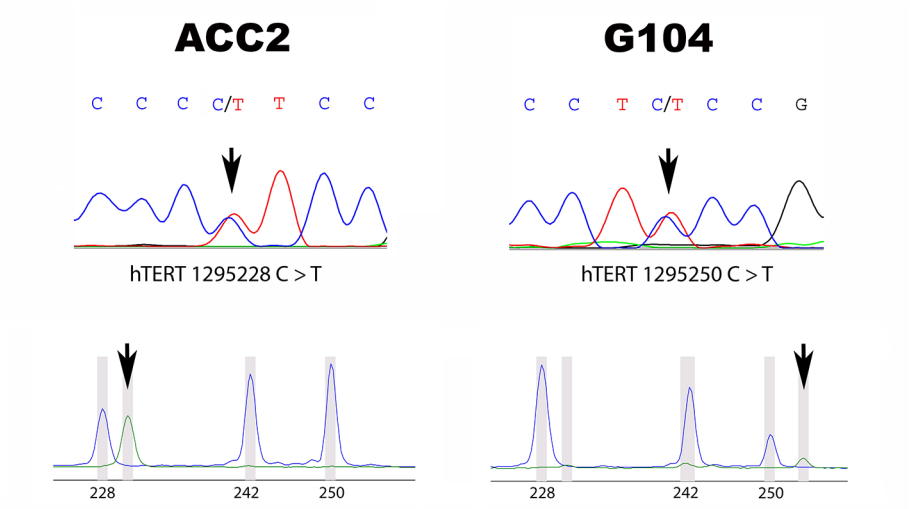

Figure 1. Somatic *TERT* promoter mutations in ACC2 and G104 as detected by Sanger sequencing (upper panel) and confirmed by a SNaPshot assay (lower panel). Arrows in the upper panel indicate the *C228T* and *C250T* mutations as displayed in the sequencing chromatograms (from left to right), while arrows in the lower panel indicate the same mutations in the SNaPshot electropherograms.

Loss of SDHB expression in *TERT* promoter-mutated ACCs

Out of 55 adrenocortical tumor samples, one ACC harboring a TERT *C228T* mutation was SDHB immunonegative/ SDHA immunopositive. SDHB/SDHA IHC was re-performed on whole-tissue sections in all four *TERT* promoter-mutated ACCs and accordingly confirmed the aforementioned finding. Mutational analysis did not reveal any pathogenic germline or somatic *SDHB/C/D/AF2* mutations, while large intragenic *SDHB*, *SDHD*, and *SDHAF2* deletions were detected only at the somatic level. Being consistent with the latter, LOH analysis revealed LOH both at the *SDHAF2* and *SDHD* loci and for a microsatellite marker telomeric to *SDHB* gene.

Role of *TERT* promoter mutation in gene expression

To determine as to whether this mutation resulted in increased *TERT* expression, quantitative RT-PCR was performed on a single *TERT* promoter-mutated ACC for which frozen material was available. Significant *TERT* expression was detected in the promoter-

mutated ACC, while the non-mutated ACCs demonstrated very low to negligible *TERT* expression similar to that detected in normal adrenocortical tissue as shown in **Figure 2**. *TERT* expression in the *TERT* promoter-mutated ACC was approximately half that of the control HEK and SW13 cell lines.

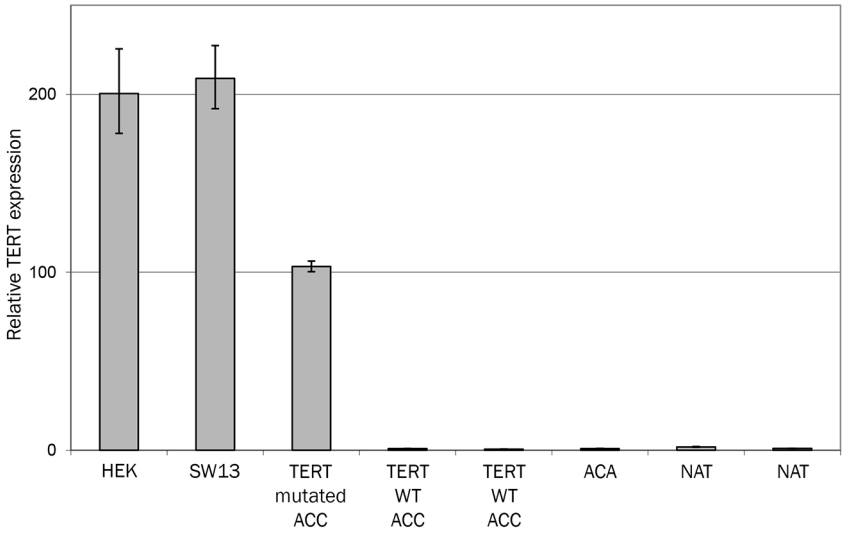

Figure 2. Quantitative real-time *TERT* expression analysis in human HEK and SW13 cell lines, normal adrenocortical tissues (NATs), adrenocortical adenoma (ACA), and adrenocortical carcinomas (ACCs) with or without *TERT* promoter mutations.*TERT* expression was measured relative to the housekeeping *HPRT* gene with fold changes normalized to expression in human adrenocortical tissue for all samples.

DISCUSSION

TERT promoter mutations have recently been shown as a novel genetic mechanism underlying telomerase activation and present in diverse human tumors with the highest frequencies in bladder cancer, CNS tumors, melanomas, hepatocellular carcinomas, and myxoid liposarcomas [10-12, 15, 17, 19-25, 27, 41]. In this study, we expanded the spectrum of *TERT* promoter-mutated tumors to ACCs, ea PGLs, and GISTs, while adding *TERT* promoter mutations to other mechanisms of *TERT* mRNA upregulation in adrenocortical tumorigenesis [35, 48-49] consistent with previously reported associations in other tumor types [12, 23-25].

Interestingly, we found that two ea PGLs of urinary bladder harboring *TERT* promoter mutations were SDH-deficient tumors. Other tumors that have been linked to SDH deficiency are GISTs [39]. To further explore a potential association between the

presence of these mutations and SDH deficiency, a series of 36 GISTs were examined and subsequently revealed one *SDHD*-mutated GIST containing a *TERT* promoter mutation. This prompted us to examine the SDH status of the *TERT* promoter-mutated ACCs. Despite the fact that this latter tumor type has never been associated with SDH deficiency, we showed loss of SDHB expression in one of the aforementioned ACCs, but without any germline *SDH-x* pathogenic mutations or gross deletions detected. This finding further extends the spectrum of tumors displaying loss of SDHB and/or SDHA expression in the absence of causative *SDH-x* mutations, including a clinicopathologically and biologically distinctive subset of KIT/PDGFRA WT GISTs [39, 50], poorly and/or un-differentiated NBLs [51], and a clear cell RCC with sarcomatous dedifferentiation [47].

Although only a small subset of SDH-deficient ea PGLs and GISTs harbored a *TERT* promoter mutation, the latter did occur exclusively in the SDH-deficient setting. As all SDH-deficient *TERT* promoter-mutated tumors were clinically aggressive, these observations may reflect that *TERT* promoter mutations can cooperate in SDH-deficient cells to support an enhanced tumor progression. Whether or not the latter could be attributed to telomerase-mediated extension of telomeres extending the lifespan of mutated clones, conferring them infinite proliferation potential as well as enabling the accumulation of additional genetic alterations, and/or to other non-canonical functions interfering with extra-telomeric tumor-promoting pathways remains to be elucidated [36, 52-59].

Similarly, a selective combinatorial genetic alteration pattern has been highlighted in various tumor types [10, 12, 15-16, 20-21, 24-25, 28, 33, 60]. In CNS tumors, *TERT* promoter mutations mostly occur in (**i**) tumors with EGFR amplification, (**ii**) *IDH* WT tumors, (**iii**) almost all tumors with concurrent total chromosome 1p and 19q loss and *IDH1*/*IDH2* mutations, and (**iv**) *IDH1*/*IDH2*-mutated tumors exhibiting oligodendroglial morphologies [12, 15-16]. Similar to the previously reported coexistence with *BRAF*-activating mutations or with concomitant *BRAF* and *CDKN2A* alterations in melanomas [10, 28, 60], two independent groups displayed a preferential occurrence of *TERT* promoter mutations in *BRAF V600E* mutation positive papillary thyroid carcinomas [21, 25], while Landa *et al*. [20] observed a significant co-occurrence of *TERT* mutations with *BRAF* and *RAS* mutations in poorly differentiated thyroid carcinomas and anaplastic thyroid carcinomas. In bladder cancer and mesotheliomas, *TERT* promoter mutations were frequently associated with inactivating mutations in the TP53/RB1 signaling pathway [33] and tumor suppressor *CDKN2A* gene inactivation [24] respectively, while a significant co-occurrence with *CTNNB1*-activating mutations has been reported in hepatocellular carcinomas and adenomas with malignant transformation [23, 61].

In this study, all *TERT* promoter-mutated tumors except one appeared to be metastatic (**Table 1**); this being in accordance with previous studies demonstrating that these mutations are more highly prevalent in advanced forms of particular malignancies, including follicular cell-derived thyroid cancer, melanoma, and primary glioblastoma [10, 15, 20-22, 25]. By contrast, *TERT* promoter mutations occur as an early genetic event in bladder tumorigenesis [17, 27, 41], meningiomas prone to malignant progression [14], as well as in *CTNNB1*-mutated hepatocellular adenomas associated with the last step of the adenoma–carcinoma transition [23, 61]. In this context, *BRAF V600E*-mutated papillary thyroid carcinomas, which are more aggressive than their *BRAF* WT counterparts [21], are preferentially enriched for *TERT* promoter mutations [21, 25].

TERT promoter mutations seem to be present in NBLs at low frequencies (~9%; two out of 22; [15]). NBLs are characterized by high expression and/or amplification of NMYC, the neuronal equivalent of c-myc. A direct binding of NMYC to the *TERT* promoter has not been established. In this study, *TERT* promoter mutations were not detected in any peripheral neuroblastic tumor being consistent with the data stemming from a recent whole-genome sequencing project for NBLs [62] and similar observations concerning other pediatric embryonal tumors, such as a clinically distinct molecular subtype of medulloblastoma [15, 18, 63]. Nevertheless, three human NBL cell lines harbored *TERT* promoter mutations indicating that lack in tumor samples could be attributed either to decreased sensitivity of the technique owing to the presence of normal cells or to the inclusion of other peripheral neuroblastic tumor types, such as ganglioneuroblastomas and/or ganglioneuromas.

In summary, this study demonstrates that *TERT* promoter mutations occur, albeit rarely, in tumors originating from the adrenal cortex (ACCs) and extra-adrenal paraganglia of urinary bladder. Their absence in PCCs and HN PGLs indicates that these seem unlikely to be critical genetic events in their development and/or progression. In addition, it provides preliminary evidence of a potential association with the acquisition of *TERT* promoter mutations in a subset of aggressive SDH-deficient tumors. Further studies are warranted to elucidate this connection and to provide mechanistic insights into the effects of these gain-of-function mutations at the *TERT* promoter on *SDH-x*-related tumorigenesis as well as their prognostic relevance in SDH-related tumor types.

SUPPLEMENTARY DATA

This is linked to the online version of the paper at http://dx.doi.org/10.1530/ ERC-13-0429.

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PART V

Digital Pathology Application in Determining Prognosis in Adrenocortical Cancer.

Chapter 10

Automated Selection of Hotspots (ASH): enhanced automated segmentation and adaptive step finding for Ki67 hotspot detection in adrenal cortical cancer

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ABSTRACT

Background: In prognosis and therapeutics of adrenal cortical carcinoma (ACC), the selection of the most active areas in proliferative rate (hotspots) within a slide and objective quantification of immunohistochemical Ki67 Labelling Index (LI) are of critical importance. In addition to intratumoral heterogeneity in proliferative rate i.e. levels of Ki67 expression within a given ACC, lack of uniformity and reproducibility in the method of quantification of Ki67 LI may confound an accurate assessment of Ki67 LI.

Results: We have implemented an open source toolset, Automated Selection of Hotspots (ASH), for automated hotspot detection and quantification of Ki67 LI. ASH utilizes NanoZoomer Digital Pathology Image (NDPI) splitter to convert the specific NDPI format digital slide scanned from the Hamamatsu instrument into a conventional tiff or jpeg format image for automated segmentation and adaptive step finding hotspots detection algorithm. Quantitative hotspot ranking is provided by the functionality from the open source application ImmunoRatio as part of the ASH protocol. The output is a ranked set of hotspots with concomitant quantitative values based on whole slide ranking.

Conclusion: We have implemented an open source automated detection quantitative ranking of hotspots to support histopathologists in selecting the 'hottest' hotspot areas in adrenocortical carcinoma. To provide wider community easy access to ASH we implemented a Galaxy virtual machine (VM) of ASH which is available from http://bioinformatics.erasmusmc.nl/wiki/Automated_Selection_of_Hotspots.

Virtual Slides: The virtual slide(s) for this article can be found here: http://www.diagnosticpathology.diagnomx.eu/ vs/13000_2014_216

BACKGROUND

Adrenal cortical carcinoma (ACC) is a rare type of endocrine malignancy with an estimated incidence of 0.7–2.0 cases per million population per year and a poor overall prognosis [1]. According to recent evidence from the European Network for the Study of Adrenal Tumors (ENS@T) ACC study group, the resection status and the Ki67 labelling index (LI) in both localized and advanced ACC [2, 3] constitute the most relevant prognostic parameters [4]. In this regard, it has been suggested that the histopathology report should include Ki67 LI along with confirmation of the adrenocortical origin on immunohistochemical grounds, Weiss score and resection status [4]. Importantly, Ki67 LI has been integrated in treatment flow charts for ACC patients with either tumor amenable to radical resection or advanced disease [4].

Taken together, the production of accurate and reproducible Ki67 LIs remains a key issue and main responsibility of pathologists. It should be recognized that various factors, such as pre-analytical, analytical, interpretation, scoring, and data analysis, might affect Ki67 LI [5]. Given the biological heterogeneity of Ki67 immunostaining across tumor specimens [5, 6], the area of slide read has been controversial for Ki67 LI assessment e.g. in breast cancer [5, 7]. According to the European Society of Neuroendocrine Tumors (ENETS), the mitotic count and the Ki67 LI should be assessed in areas with the highest proliferating activity (hotspots) in order to determine the proliferation grade in gastroenteropancreatic neuroendocrine tumors (GEP-NETs) [8]. As far as ACCs are concerned, there is not only lack of studies addressing the issues of a potential biological heterogeneity of Ki67 staining and interobserver variation, but also different methods of objective quantification of the Ki67 proliferative index.

In routine diagnostic practice, representative areas of slides are manually selected by histopathologists using visual examination of whole mount Ki67-immuostained slides at a low magnification. Of note, this process might lack reproducibility and affect the Ki67 LI [5]. Since digitized immunohistochemical (IHC) stained tissue sections have become amenable to the application of computerized image analyses, two independent groups have developed either a hybrid clustering approach for the detection of Ki67 hotspots in whole tumor slide images [9] or a simplified computerized method for hotspot detection in digitized IHC slides [10]. In this context, we developed Automated Selection of Hotspots (ASH) to provide clinical labs with the ability to determine the most active areas in proliferative rate within a slide and subsequently quantitate Ki67 LI using a desktop PC without requiring extensive bioinformatics support. ASH uses Galaxy [11] as a simple graphical user interface and to join the components of ASH into

an analytical workflow for hotspot detection and this, Galaxy is contained in a VMware virtual machine (VM) [12] which ensures that the system is platform independent. The use of VM technology has been highlighted by Nocq *et al*. [13], to improve the usability of next generation sequencing software by simply sharing entire installations.

We believe that this is the first time that Galaxy-VM has been used to deliver single user (on a personal computer) or as a multi-user (on a server) hotspot detection software with the same easy access via the Galaxy graphical user interface (GUI).

METHOD

ASH is delivered as a virtual machine which consists of 3 classes: NDPI Segmentation, Adaptive Step Finding and Reporting Visualization (**Figure 1**). NDPI Segmentation used previously described NDPI splitter [14] to split the input image files into A × B matrix followed by a step shift of 1/2 split image and quantitation Ki67 in all images using ImmunoRatio [15], implemented in ASH. This preprocessing step provides an initial quantitative ranking of all blocks from which the top 10 are used to focus in on the actual 'hotspot' fields. To ascertain the exact hotspot positions on the image we develop an Adaptive Step Finding class to adaptively determine the shifting step size, and trade-off between the hotspot detection resolution and system complexity. This Adaptive Step Finding class uses three of the same functions (Image shifting, ImmunoRatio and Ranking) that are used by NDPI Segmentation (**Figure 1**), however in this class eight blocks are created around the region selected by NDPI Segmentation (**Figure 2**). The rectangle area is shifted by a step size shrunk 50% every adaptive loop. ASH provides an end to end workflow for hotspot detection using the functionality of a Galaxy GUI to provide the user with a simple data upload and html style reporting environment.

Implementation

The application is for digital images obtained on the Hamamatsu NanoZoomer Digital Pathology (NDP) System (Hamamatsu Photonics K.K. Japan), in their proprietary NDP Image (NDPI) file format. NDPI image segmentation using NDPI-splitter is available from [16]. Quantitation of segmented blocks with ImmunoRatio is available from [17]. For image processing, analysis, and visualization, we adopted OpenCV [18]. The ASH software tool is developed on the Ubuntu 12.04 [19] Linux operating system, as a Galaxy application [11] and is distributed as a VMware virtual machine [12] for a Windows user.

The detection of hotspots uses adaptive step finding methodology which has been utilized in engineering for many years [20] and extensively evaluated and validated [21]. Experimental evaluation has demonstrated the effectiveness of the adaptive step size [22] and the adaptive step finding method applied in ASH has the same functionality. The selection of the step size is critical both from the point of view of computational efficiency and detection performance.

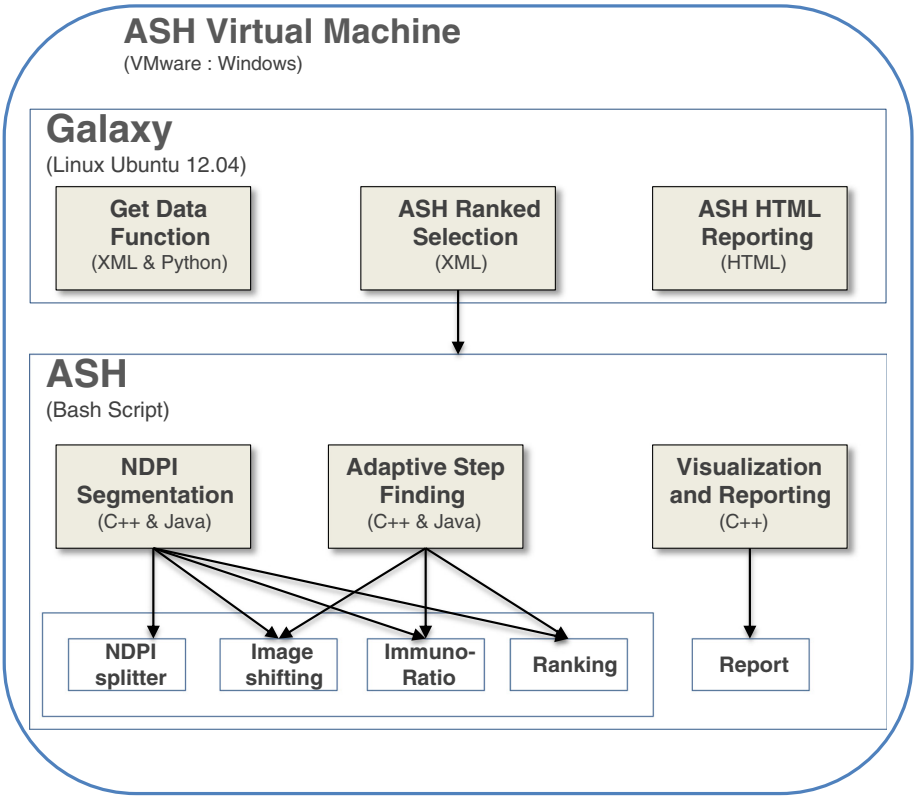

Figure 1. Software architecture overview: the ASH virtual machine contains both the ASH image analysis and the graphical user interface provided by Galaxy. ASH image analysis, NDPI segmentation and Adaptive Step finding components use three of the same methods.

To simplify the use of ASH, we have implemented a Galaxy within the same virtual machine (VM) to provide a standardized graphical user interface (GUI) for accessing, running and visualizing ASH. Galaxy is an open, webbased platform [23] and developed tools to upload image files, to analyse the files by ASH in batch mode and to deliver a html report of the selected image with the quantitative ranking of the hotspots displayed

in that image. All components and dependencies were created into a VMware virtual machine (VM) [12] which is an environment that is used like any physical computer [24] but also shared by download. The entire virtual machine is usually contained in a few files on the host computer (the physical machine that the virtual machine is running on). This means that all the dependency's required by ASH, including NDPI splitter, ImmunoRatio, openCV and Galaxy, are replaced by just having VMware installed.

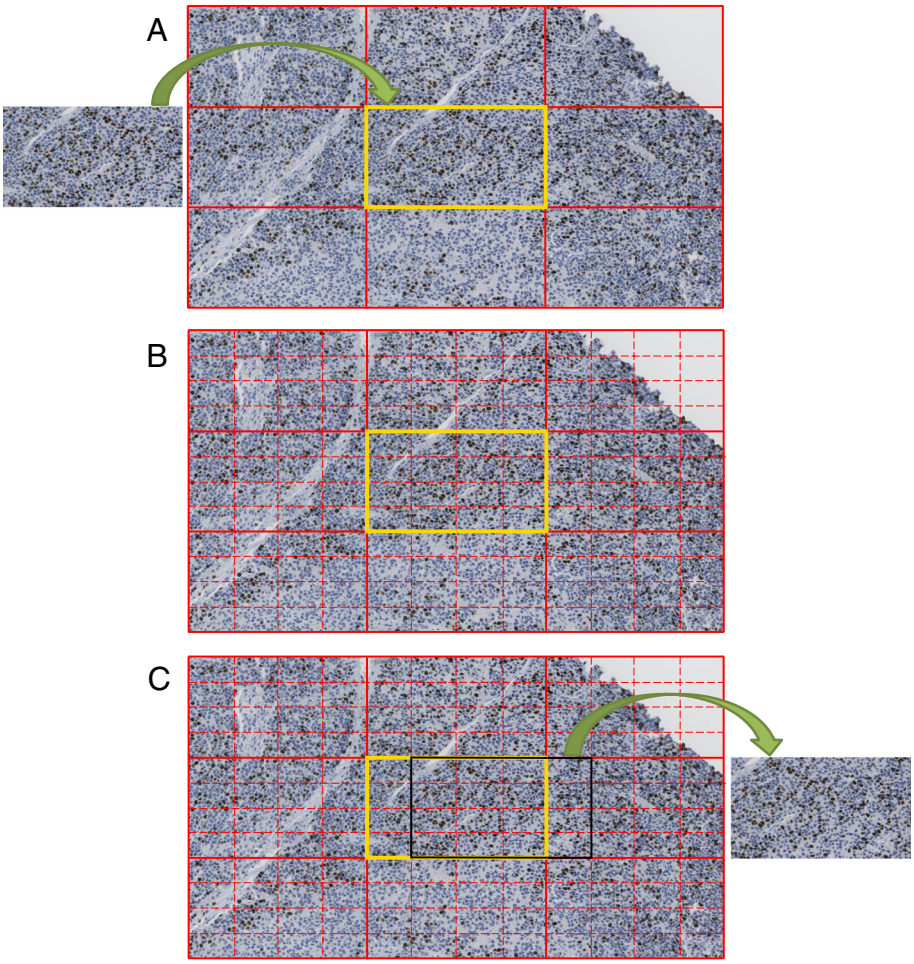

Figure 2. Adaptive step finding. This figure represents ¼ step shift analysis; (**A**) Eight neighboring images are generated around each of the top ten ImmunoRatio images (left hand side) obtained from the segmentation step of ASH; (**B**) this 3 × 3 image is divided into totally 81 image blocks by step shifting ¼ and the ImmunoRatio is for each block; (**C**) image with highest ImmunoRatio among these 81 images is outlined (black) and displayed on the right hand side.

RESULTS

Automated selection of hotspots

The overall work flow for the image analysis outlined in **Figure 3** includes the main classes developed for ASH which include NPI Segmentation, Adaptive Step Finding and Visual Reporting.

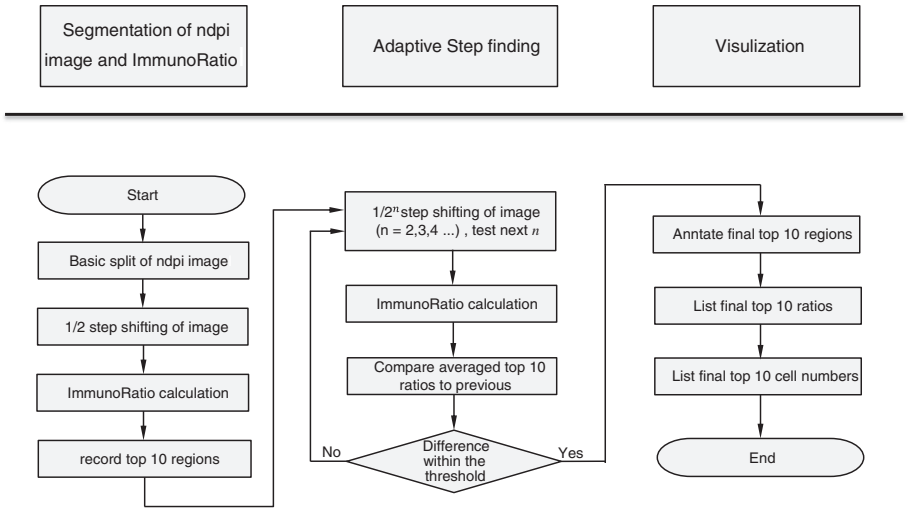

Figure 3. Image analysis workflow. The process is divided into the classes, NDPI Segmentation, Adaptive step finding and visual reporting.

NDPI segmentation

In the first class, the NDPI Segmentation, the whole digital image scanned from Hamamatsu NanoZoomer is first divided with the NDPI splitter (**Figure 3**). NDPI splitter processes the basic split of the image from a single (100 K × 100 K pixel) NDPI image into thousands of smaller (2 K × 1 K pixels) images known as image blocks. Step shifting of ½ the size of an image block is performed to provide overlapping blocks, in order to scan more area and improve ImmunoRatio detection resolution. For the primary selection of hotspots, a ranked list of these image blocks is determined based on the quantitation, using ImmunoRatio, of each block (**Figure 4**). "Step shifting" is illustrated in **Figure 2** as well, while the black block moving from the yellow block indicates a 1/4 step shifting. ImmunoRatio provides quantitative image analysis of estrogen receptor (ER), progesterone receptor (PR), and Ki67 immunostained tissue sections [15]. In our software, the ImmunoRatio result is ranked and used to determine the hotspot areas.

The whole scanned image is segmented with NDPI splitter, as shown from the left upper image to the right upper image in **Figure 4**.

Based on the split images, we shift them by 1/4 of the side length, as shown from the right upper image to the bottom image in **Figure 4**. After the successful creation of JPEG images from the NDPI files, we adopt ImmunoRatio to calculate the IR% per block of the image, and rank the top 10 IR% image blocks.

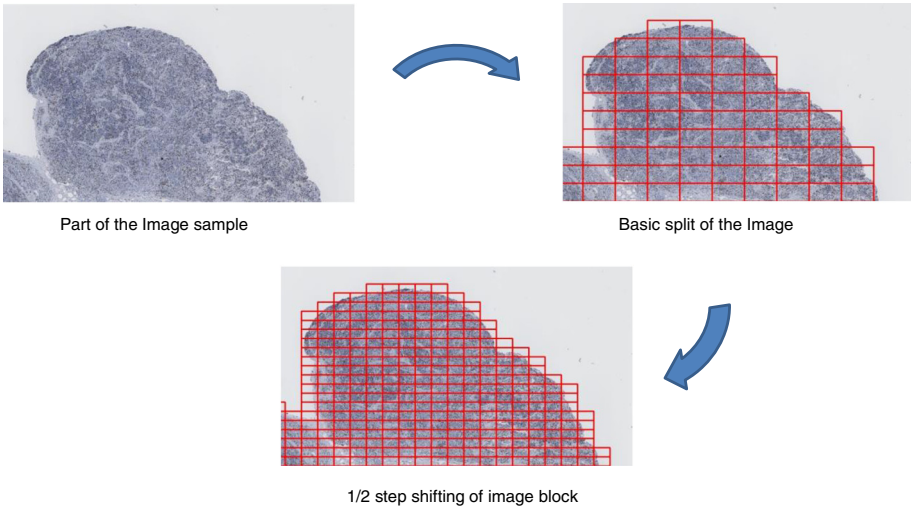

Figure 4. NDPI Segmentation: the image is segmented using followed by step shifting of these blocks by ½ their size prior to quantitation.

Adaptive step finding

In this part, a smaller step finding procedure is applied to the top 10 images, regions of interest, obtained from the previous segmentation, ImmunoRatio and ranking procedure. The initial iteration uses 1/2 of shifting step from last iteration followed by more sensitive steps, such as 1/4 step (**Figure 2**) to precisely select the appropriate region of interest. Subsequently, the averaged top 10 ratios of current iteration are compared to the previous top ten ratios. The Step Finding procedure stops when the slope of Immuno-Ratio to block number (as shown in **Figure 5B**) within a preset threshold of 0.01.

Visualization and reporting

In this part, we annotate the final top 10 regions in the original image and generate a report to list final top 10 ratios and their corresponding locations. **Figure 6** shows an annotated image with Top 10 ImmunoRatio regions marked with red rectangles.

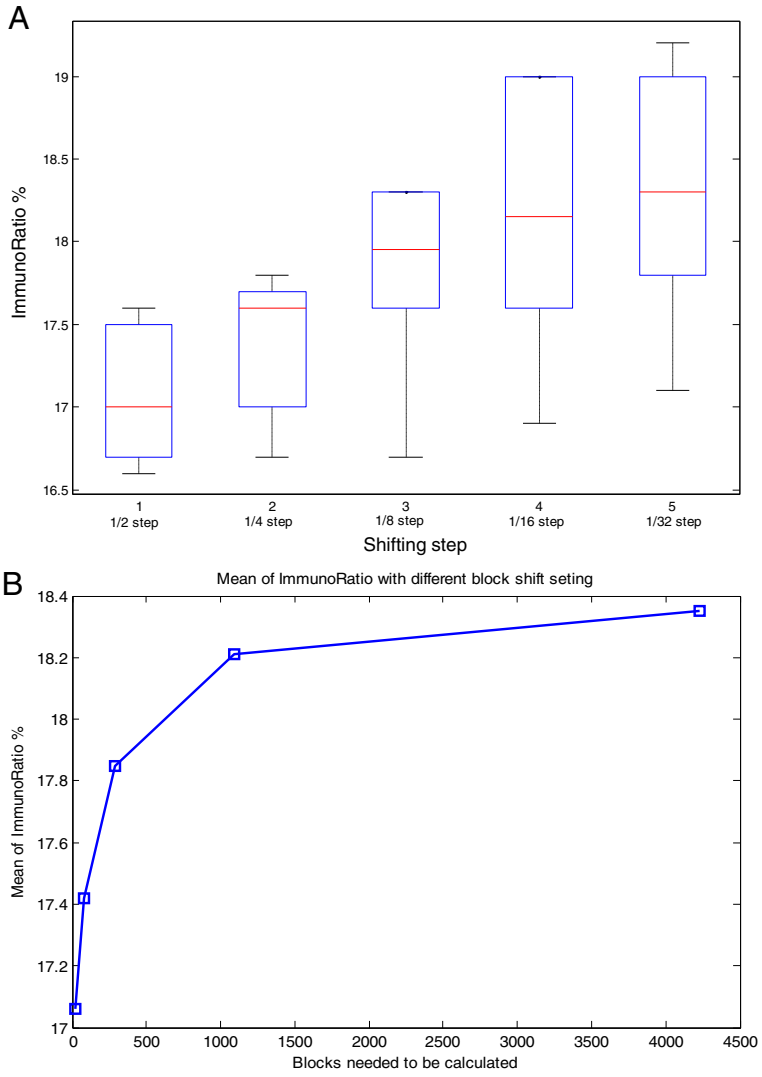

Figure 5. The effect of (A) step size on ImmunoRatio % and (B) the blocks need to calculate these step sizes. The average value as determined by ImmunoRatio (red line).

Optimization of adaptive step selection

To determine the effect of step size of the performance of ASH, we calculated the averaged ImmunoRatio as the step size was decreased from 1/2 to 1/32 (**Figure 5A**). The averaged ImmunoRatio increases when step decreases from 17.07% to a maximum of 18.35% (**Figure 5** and **Table 1**). The step size and its corresponding ImmunoRatio, block number, and processing time are indicated in **Table 1**. **Figure 2** shows an example with 1/4 step shifting and its 81 (9×9) blocks. The more blocks are calculated, the more

chances to obtain the block with higher ImmunoRatio. Decreasing the step size from 1/2 to 1/32 requires a nonlinear increase in the number of blocks that must be calculated from 25 blocks up to 4225 blocks with an increase in average calculation time increase of >150 fold (i.e. from 25 seconds to about 1 hour per image) using a single core on an Intel Xeon X5650 CPU.

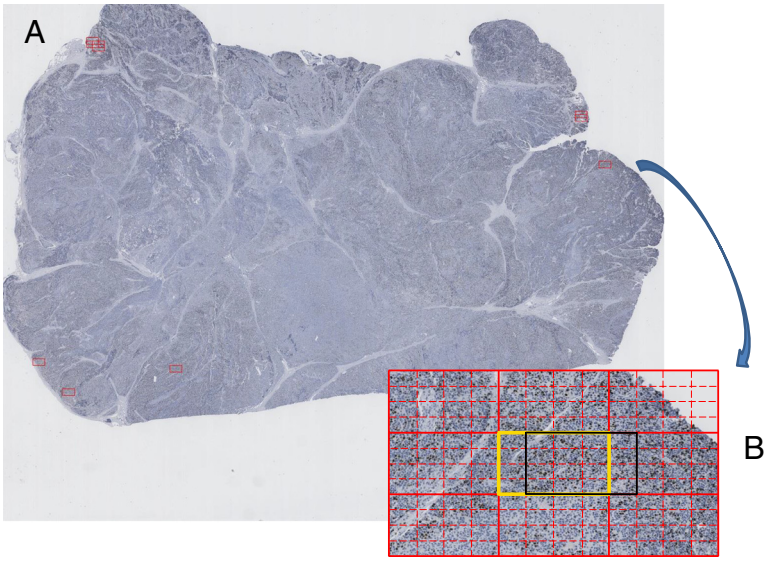

Figure 6. Hotspot Reporting (**A**) The original image input for ASH analysis is overlaid with the hotspots (red rectangles). (**B**) The inset image shows the output from the adaptive step finding algorithm and the black box is the part that is displayed on the main image in (**A**) as a red rectangle.

Table 1. Optimization of ASH: The effect of step size on the performance of ASH was determined by as the average % ImmunoRatio (IR%), the number of blocks (# blocks) and the time in seconds to completion (Time) at decreasing step size intervals (Step size).

Step size	IR (%)	# blocks	Time (sec)
1/2	17.06	25	25
1/4	17.42	81	82
1/8	17.85	289	291
1/16	18.25	1089	1097
1/32	18.35	4225	4254

Validation of quantitative hotspot detection

Adaptive step finding has been utilized in engineering for many years [20] and extensively evaluated and validated by [21]. In [22], experimental evaluation demonstrates the effectiveness of the adaptive step size, while the adaptive step finding method applied

in ASH had the same functionality. We have tested ASH in a set of >60 whole-slide digitally-scanned ACC images and in comparison with manual assessment labelling index assessment achieved a strong correlation (rho > 0.8, $p = 0$) as calculated with Spearman rank order metric (publication in progress).

DISCUSSION

There are many commercial image analysis products such as AQUA [25], Genie (Aperio) [26], TissueStudio (Definiens) [27], InForm (PerkinElmer) [28] which are capable of high quality image processing and Ki67 quantitation, which are cited in other studies and are not freely available for comparative testing. Whilst there are several open source image analysis tools (e.g. ImageJ [29], ImmunRatio [17]) and multiple custom built in house applications (e.g. Seedlink [9]) and our requirements included that the applications be open source and that it could provide hotspot detection and quantitative Ki67 scoring in a desktop application. Thus, we developed ASH, an open source, open access, application using Galaxy-VM technology, to support histopathologists in determining the most active areas in proliferative rate within a slide based on Ki67 LI staining. Additionally, since ASH was developed in a Galaxy environment, the currently segmentation and quantitation methods can be easily supplemented or replaced, in the central ASH application (by the authors) or by a user (in their local ASH instance), with improved methods developed by other research teams.

We implemented an overlapping block creation method, Step Shifting, since NDPI splitter is only capable of splitting an image and not generating overlapping blocks and to support our Adaptive Step Finding method which has been utilised in multiple engineering projects over many years [20-22].

When we shift the image block by different steps, we can see that the averaged ImmunoRatio increases when step decreases. Therefore, we developed an adaptive step finding technique to obtain the tradeoff between hotspot detection resolution and processing time. Whilst the accuracy of the ImmunoRatio % per image block improves, there is an increased cost for calculation time. Optimal calculation time to accuracy ratio occurs at 1/16 step size with ~1000 block based on the time to calculate one block is 1.0069 s based on a single core on an Intel Xeon X5650 processor.

Seedlink, a hybrid clustering method [9], that provides the users with automatic identification of hotspots is comparable to ASH with respect to usability and output.

Seedlink requires a post-processing step to determine true hotspots from the false positive hotspots to ensure accurate determination of Ki67, whilst ASH provide a ranked set of regions for from which the user can include or reject as part of the quantitation of Ki67. Thus ASH simplifies the decision making process by integrating the visualization of the detected hotspots with the quantitation of detected hotspots as a single output in the Galaxy-VM GUI.

Since different types of colored pollutions and colour interferences sometimes cause trouble to the hotspot detection, Adobe photoshop or an alternative program enabling pathologists to delete parts of the scanned image i.e. artifacts created during slide production, will improve the accuracy of the hotspot detection. Whilst we have tested ASH in a training set it is clear that there are 'inactive' areas apparently with 'low' Ki67 Labelling index. Hence it is more prudent to compare automated selected hot spot areas versus hot spot areas as selected by pathologists and further studies are warranted to confirm our findings in a lager cohort.

Galaxy provides the user with a simple GUI to apply ASH using only standard web browser. Galaxy provides the remote access for ASH, so people can benefit from the higher processing speed and larger storage space than a local computer. To ensure that ASH is available to individual researchers and/or pathologists as well as those who are supported by a bioinformatics team, we have implemented this Galaxy as a VMware-VM. The combination of Galaxy in a VM provides a multi-user environment in which users can analyse their images in a password protected user specific space, but with the additional functionality of Galaxy and the capability to share any of the data, analysis and results. The current Galaxy-VM has been implemented to run using 1 CPUs, but can be scaled up by resetting the VM once installed to run more CPUs (see project website for help documentation).

CONCLUSIONS

We have developed ASH, an open source Galaxy virtual machine application designed for Ki67 LI hotspot detection support, aimed at both individual and large diagnostic laboratories, who have little bioinformatics experience or support. ASH is designed to assist pathologists and accelerate the time-consuming Ki67 hotspot selection procedure, enhance the detection resolution and eventually lead to improved reproducible Ki67 LI reporting. Prior to image processing, pathologists should initially exclude with an interface tool various artifacts, such as tissue folds, intrinsic/extrinsic pigmentation

(deposit artifacts), necrotic areas, etc. ASH delivers a ranked list of hotspots as a combination of images and quantitative values for each hotspot detected, based on the Adaptive step finding algorithm [20-22] developed as part of ASH. The selection of the step size is critical both from the point of view of computational efficiency and detection performance and although we have successfully tested ASH in a training set of whole-slide digitally-scanned ACC images, further studies are warranted in to confirm its efficiency with a larger ACC set.

Availability and requirements

Project name: Automated Selection of Hotspots (ASH)

Project home page: http://bioinformatics.erasmusmc.nl/ wiki/index.php/Automated_Selection_of_Hotspots which has a Galaxy VM instance of ASH.

Operating system(s): Windows, Linux (Ubuntu 12.04).

Programming language: C++, Bash, Java.

Requirements: VM ware player, Hamamatsu SDK, JAI 1.1.3, JAI Image IO 1.1, Ant, Deep Zoom. License: GNU GPL version 3 [30].

Ethical approval

These ACCs 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) and the Medical Ethical Committee of the Erasmus MC, Rotterdam, The Netherlands, approved the study.

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28. InForm: PerkinElmer, 940 Winter Street, Waltham, Massachusetts 02451, USA [http://www.perkinelmer.com/CMSResources/Images/44-144380PRD_inForm.pdf]
29. ImageJ: Image Processing and Analysis in Java [http://imagej.nih.gov/ij/ docs/index.html]
30. Free Software Foundation: A Quick Guide to GPLv3 – GNU Project – Free Software Foundation (FSF) [http://www.gnu.org/licenses/quick-guide-gplv3.html]

Chapter 11

An International Ki67 Reproducibility Study in Adrenal Cortical Carcinoma

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ABSTRACT

Despite the established role of Ki67 labeling index in prognostic stratification of adrenocortical carcinomas and its recent integration into treatment flow charts, the reproducibility of the assessment method has not been determined. The aim of this study was to investigate interobserver variability among endocrine pathologists using a web-based virtual microscopy approach. Ki67-stained slides of 76 adrenocortical carcinomas were analyzed independently by 14 observers, each according to their method of preference including eyeballing, formal manual counting, and digital image analysis. The interobserver variation was statistically significant ($P < 0.001$) in the absence of any correlation between the various methods. Subsequently, 61 static images were distributed among 15 observers who were instructed to follow a category-based scoring approach. Low levels of interobserver ($F = 6.99$; $F_{crit} = 1.70$; $P < 0.001$) as well as intraobserver concordance ($n = 11$; Cohen k ranging from -0.057 to 0.361) were detected. To improve harmonization of Ki67 analysis, we tested the utility of an open-source Galaxy virtual machine application, namely Automated Selection of Hotspots, in 61 virtual slides. The software-provided Ki67 values were validated by digital image analysis in identical images, displaying a strong correlation of 0.96 ($P < 0.0001$) and dividing the cases into 3 classes (cutoffs of 0%-15%-30% and/or 0%-10%-20%) with significantly different overall survivals ($P < 0.05$). We conclude that current practices in Ki67 scoring assessment vary greatly, and interobserver variation sets particular limitations to its clinical utility, especially around clinically relevant cutoff values. Novel digital microscopy–enabled methods could provide critical aid in reducing variation, increasing reproducibility, and improving reliability in the clinical setting.

INTRODUCTION

Adrenocortical carcinoma (ACC) is a rare endocrine malignancy with a poor overall prognosis and an estimated incidence of 0.7 to 2 cases per million [1]. When confronted with this tumor, pathologists are expected to provide the Weiss score, the status of resection margins, and prognosticators including the Weiss score, mitotic grade, and Ki67 labeling index (LI) and, if diagnostically challenging, confirm its adrenocortical origin on immunohistochemical grounds [2, 3]. It has been shown [4] that ACCs can be subdivided using a variety of methods including the mitotic frequency into low grade (≤ 20 mitoses/50 high-power fields) and high grade (> 20 mitoses/50 high-power fields) [5], Stereoidogenic Factor-1 immunohistochemistry [6, 7], and other proliferation based scoring methods such as phosphohistone H3–specific immunohistochemistry [8].

According to recent data generated by the European Network for the Study of Adrenal Tumors (ENS@T) ACC study group [9, 10], the resection status and the Ki67 LI in both localized and advanced ACCs constitute the most relevant prognostic parameters [2]. In accordance, Duregon *et al*. [8] demonstrated that Ki67 LI is the most powerful tool in terms of prognostic stratification. In addition to its emerging value as a critical determinant of prognosis, Ki67 LI has been recently integrated in treatment flow charts for adrenocortical cancer patients suffering from tumors either amenable to radical resection or at advanced presentation. Accordingly, thresholds of 10%, 20%, and 30% seem to be crucial in therapeutic decisions, including adjuvant mitotane, radiotherapy of the tumor bed, and combination therapy of mitotane and 3 cycles of cisplatin, respectively [1, 2].

The standardized assessment of Ki67 LI is important and remains a key issue and responsibility of histopathologists. Nevertheless, various factors, such as pre-analytical, analytical, interpretation, scoring, and data analysis, might affect the Ki67 LI [11]. In particular, lack of uniformity and consistency in quantification [12] as well as intratumoral heterogeneity of proliferation [5, 11, 13, 14] might limit its assessment. In this context, we have implemented an open-source toolset, namely Automated Selection of Hotspots (ASH) aiming at improved accuracy and reproducibility of reporting of the Ki67 LI [15].

In the present study, we determined the interobserver variability for Ki67 LI and examined the current practices among expert endocrine pathologists in a multicenter cohort of conventional ACCs using virtual microscopy. The impact of various parameters, that is, readout technique of preference in diagnostics, selected fields for evaluation, and estimated total number of cells on Ki67 assessment was further investigated. Moreover, we evaluated the variability of Ki67 LI around clinically relevant cutoffs [1, 2], and

validated the efficiency of ASH as compared with the human independent selection of hotspot areas.

MATERIALS AND METHODS

Case Selection and Ki67 (MIB1) Immunohistochemistry

A total of 101 conventional ACCs were collected from 4 specialized centers from Europe and United States: (**1**) San Luigi Gonzaga Hospital and University of Turin, Turin, Italy (25 samples), (**2**) Erasmus MC Cancer Institute, Rotterdam, The Netherlands (12 samples), (**3**) University of Wisconsin School of Medicine and Public Health (5 samples), and (**4**) University of Michigan Health System (59 samples). Borderline/atypical adrenocortical neoplasms and ACC variants (oncocytic, myxoid, and sarcomatoid) were not included in the present study. Each case was thoroughly reviewed and representative unstained glass slide(s) were selected and provided for immunohistochemical analysis within a single center (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 a commercially available antibody: mouse monoclonal MIB1 M7240 antibody (1:400 dilution; Dako, Glostrup, Denmark) against Ki67 on an automatic Ventana Benchmark Ultra System (Ventana Medical Systems Inc., Tucson, AZ) using Ultraview DAB detection system preceded by heat-induced epitope retrieval with Ventana Cell Conditioning 1 (pH 8.4) at 97°C for 52 minutes. Diaminobenzidine was used as the chromogen. All cases 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. Cases displaying artifactual intratumoral variation in labeling were excluded by use of Ki67-labeled mitotic figures as internal positive controls.

Digital Pathology Application

High-resolution, whole-slide images were acquired from all Ki67 (MIB1)-stained slides using a NanoZoomer Digital Pathology (NDP) System (Hamamatsu Photonics K.K., Japan) working at a resolution of 0.23 μm/pixel. The immunostains were scanned at x 40 magnification and automatically digitized in their proprietary NDP Image (NDPI) file format. Between October 2013 and March 2014, digital files were consecutively uploaded in 1 set 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 (NDP.view Viewer Software; Hamamatsu Photonics K.K.).

Participants and Interpretation of Staining Results

In the first round (**Supplemental Digital Content 1**, http://links.lww.com/PAS/A325), 14 observers, among which 11 expert endocrine pathologists (R.V.L., L.E., V.N., O.M., S.L.A., X.M.-G., T.J.S., K.S., F.T., F.H.v.N., and R.R.d.K.) and 3 residents (T.G.P., E.D., and J.T) received: (**i**) an email detailing the objectives of the project and clearly stating that only nuclear staining (plus mitotic figures that are stained by Ki67) should be incorporated into the Ki67 score defined as the percentage of positively stained cells among the total number of malignant cells scored with staining intensity being of no relevance [11], (**ii**) the corresponding link providing access to the virtual slides, and (**iii**) a scoring list to be completed during Ki67 immunohistochemical evaluations.

All virtual slides were distributed online, reviewed by each observer in a blinded manner without knowledge of the corresponding clinicopathologic data or scores assigned by other pathologists. In particular, participants were asked to assess (**i**) the Ki67 LI based on (**ii**) the method of their preference/practice in diagnostics (visual estimation, formal manual count, or digital image analysis [DIA]) reporting on (**iii**) the estimated total number of cells and (**iv**) the selected fields for evaluation, that is, hot spot area(s) or average score across the section, or average score across the section adding hot spot area(s).

Twenty-five cases were excluded from the analysis due to suboptimal staining, poor scan quality, and fixation artifacts. The remaining tumors from 76 patients of a mean age of 47.6 years (ranging from 8 to 85 y; 1.17 female:male ratio) comprised 62 primary tumors, 6 recurrences, and 8 metastases. Thirty-four patients died of the disease, whereas 42 are alive with or without evidence of disease. The latter are currently in follow-up at various institutions with a mean of 34.27 months (range, 1wk to 169 mo).

In the second round of assessment performed 9 months later (**Supplemental Digital Content 1**, http://links.lww.com/PAS/A325), 61 static images (.JPG files) were circulated among 15 observers, including 11 expert endocrine pathologists (R.V.L., L.E., O.M., S.L.A., T.J.S., K.S., M.V., A.S.T., A.J.G., F.H.v.N., and R.R.d.K.) and 4 residents (T.G.P., E.P., E.D., and J.T). These images were selected as the most active areas based on an automated approach [15]. The participants were instructed to follow a category-based evaluation of the Ki67 LI on the basis of visual estimation without performing formal manual count or DIA.

Statistical Analysis

Interobserver variability using either virtual microscopy (first evaluation) or visual estimation on static images (second evaluation) as well as differences in the type of assessment was assessed with analysis of variance (ANOVA) single factor. To evaluate intraobserver agreement, Cohen k was performed after conversion of the Ki67 values of the initial numerical assessment into categorical variables. With regard to automatically selected areas, we compared computerized counts based on ImmunoRatio and DIA, respectively, in identical images using Pearson correlation [17]. The correlation between human independent selection and software selection of hotspot areas was examined with Spearman rank order correlation. To compare the results of Ki67 assessment with the overall survival, Kaplan-Meier curves were plotted and *P*-values were calculated using the log rank test. The level of significance was set at $P < 0.05$. All other statistical analyses were performed using SPSS software (SPSS version 21; SPSS Inc., Chicago, IL).

RESULTS

Interobserver Variation in KI67 LI Assessment

Seventy-six cases were initially analyzed displaying statistically significant variance between 14 observers (ANOVA, $F = 10.43$; $F_{crit} = 1.73$; $P < 0.001$) (**Figure 1**). Differences in current practices concerning the Ki67 LI assessment are highlighted in **Figure 2**. Of the 14 observers, 8 preferred formal manual counting, 4 visual estimation, and 2 DIA (ImageJ software, 1.47v; Wayne Rasband, NIH and KS400 image analysis software, version 3.0; Carl Zeiss Vision GmbH). With regard to the residents, 2 used formal manual count and 1 DIA (KS400 image analysis software, version 3.0; Carl Zeiss Vision GmbH). The overall agreement was not affected by different levels of experience in endocrine pathology (data not shown). No statistical significance was found between the different methods of assessment (ANOVA, $P = 0.079$), except between visual estimation and formal manual count (t test, $P = 0.014$). Kaplan-Meier curves based on the overall survival were plotted against 0%-15%-30% cutoffs (**Figure 3**).

Impact of Visual Estimation on Variation

Given the large variation observed in the initial Ki67 assessment, we decided to reduce potential complexities by using visual estimation and following a category-based approach in 61 predetermined images. In this context, the variation remained statistically significant between 15 observers (ANOVA, $F = 6.99$; $F_{crit} = 1.70$; $P < 0.001$). To evaluate interobserver concordance, ASH maximum values were utilized as "gold standard" and transformed into categorical variables. The highest levels of concordance were

achieved within the lowest range of Ki67 values, that is, 0% to 10% (**Figure 4**). Likewise, the overall agreement was not affected by different levels of experience in endocrine pathology (data not shown). To assess intraobserver concordance, we transformed those numerical values of the initial assessment into categorical variables. A very low degree of concordance was detected for every observer ($n = 11$) (**Supplemental Digital Content 2**, http://links.lww.com/PAS/A326) with the majority having a higher score on visual estimation of predetermined images (**Figure 5**).

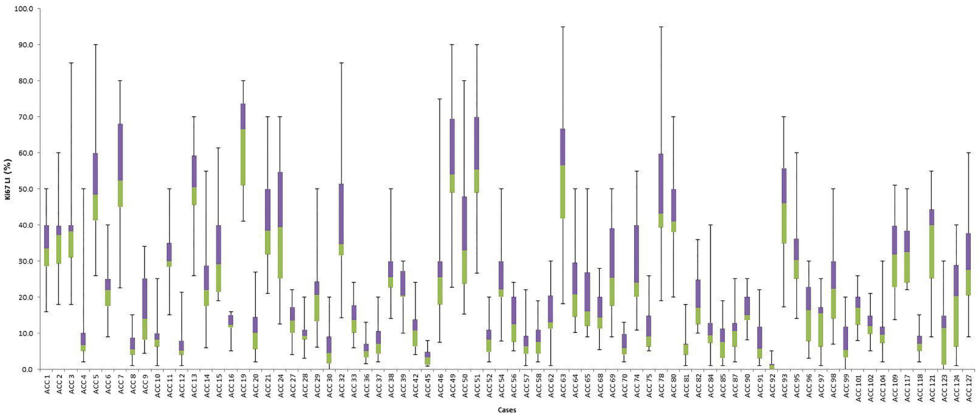

Figure 1. Ki67 LI determined by 14 observers on 76 virtual slides with various methods of assessment. Ki67 was quantified as percentage of positive immunoreactive tumor cells against total tumor cells and was expressed as mean.

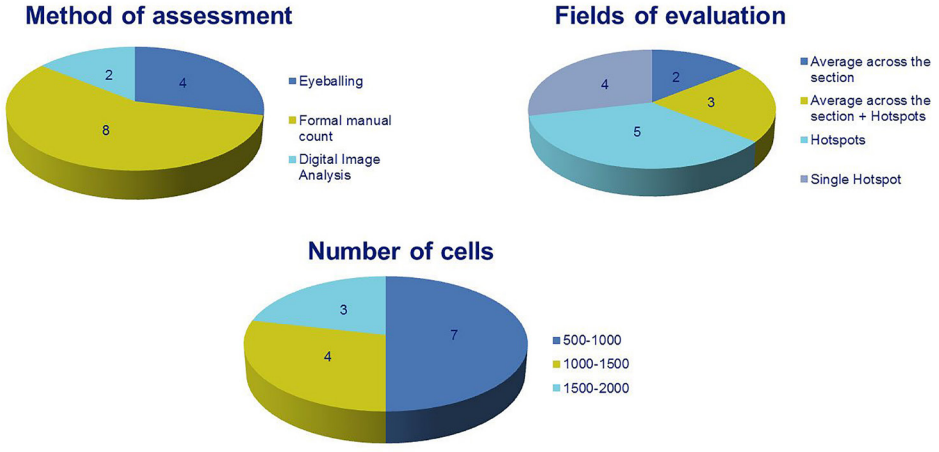

Figure 2. Observers' evaluation as referred to the method of assessment, fields of evaluation, and total number of cells utilized in the count.

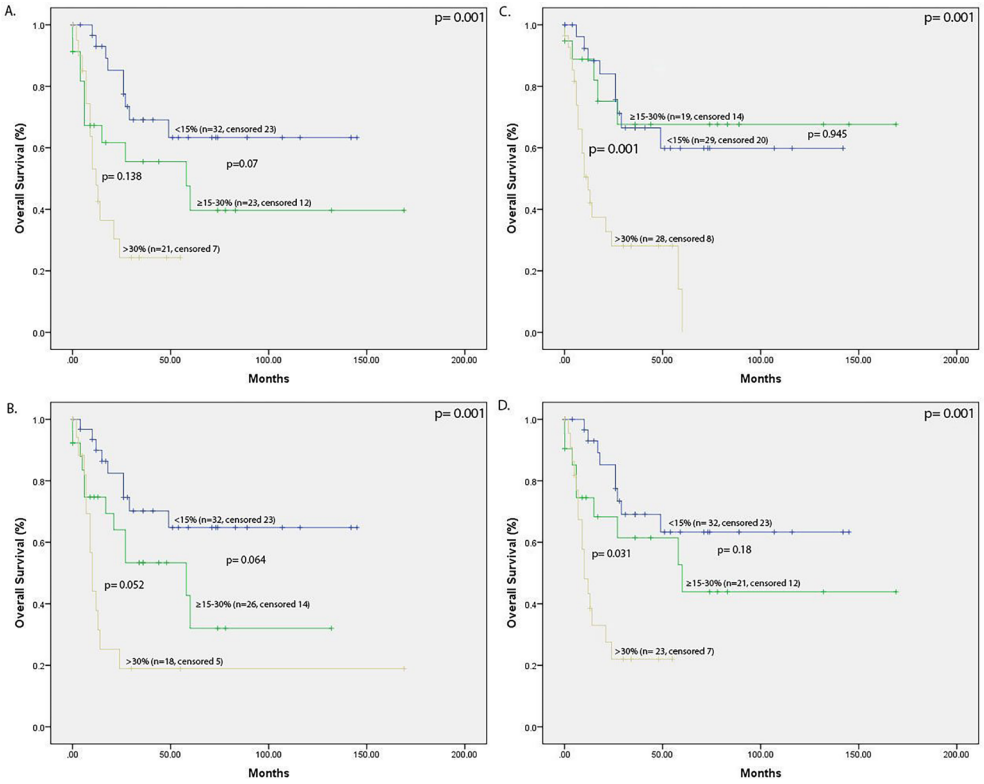

Figure 3. Overall survival for DIA-MC performers (**A**), best performer (**B**), eyeballers (**C**), and all pathologists (**D**) using 0%-15%-30% as cutoffs.

ASH: **A Virtual Microscopy–enabled Assessment of Ki67 LI**

After software assessment, 15 of 76 cases were excluded due to artifacts interfering with the analysis. To verify its applicability in the remaining 61 cases, we determined the degree of concordance (**i**) between computerized counts as provided by the software (ImmunoRatio) and by DIA (KS400 image analysis software, version 3.0; Carl Zeiss Vision GmbH) in identical images (n = 610; 10 images as selected by the ASH per virtual slide); and (**ii**) between computerized counts as provided by the software (ImmunoRatio) and as generated by human independent selection (DIA) in different images displaying the highest Ki67 expression (n = 61; 1 image per virtual slide). To this end, an observer (T.G.P.) selected 10 hotspot areas by visual estimation on a virtual microscopy interface and subsequently performed DIA (KS400 image analysis software, version 3.0; Carl Zeiss Vision GmbH). In this setting, strong correlations of 0.96 and 0.84 were detected, respectively ($P < 0.001$). From a clinical standpoint, we determined whether software-provided Ki67 values could divide the cases into 3 classes with significantly different

overall survivals. In fact, when overall survival Kaplan-Meier curves were plotted against 0%-15%-30% and/or 0%-10%-20% cutoffs (**Figure 6**), overall comparisons were statistically significant ($P < 0.05$).

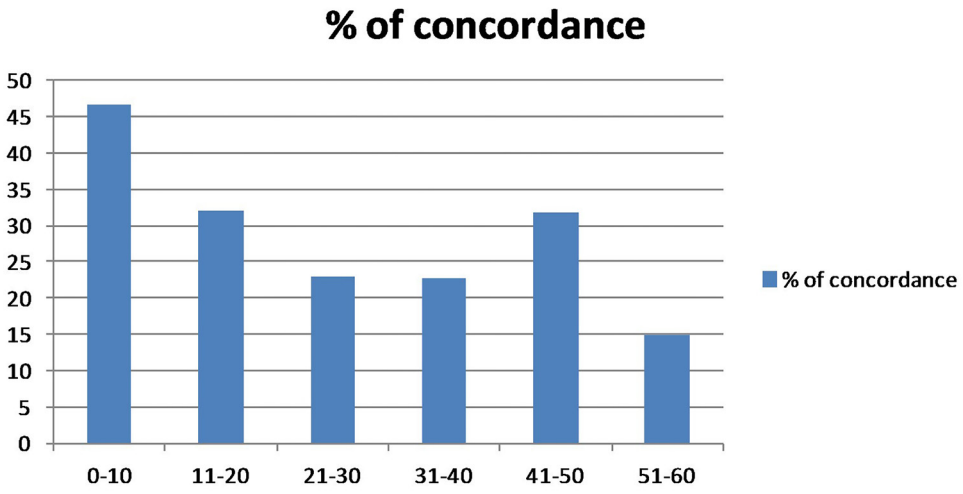

Figure 4. Levels of concordance between observers following a category-based Ki67 scoring by visual estimation.

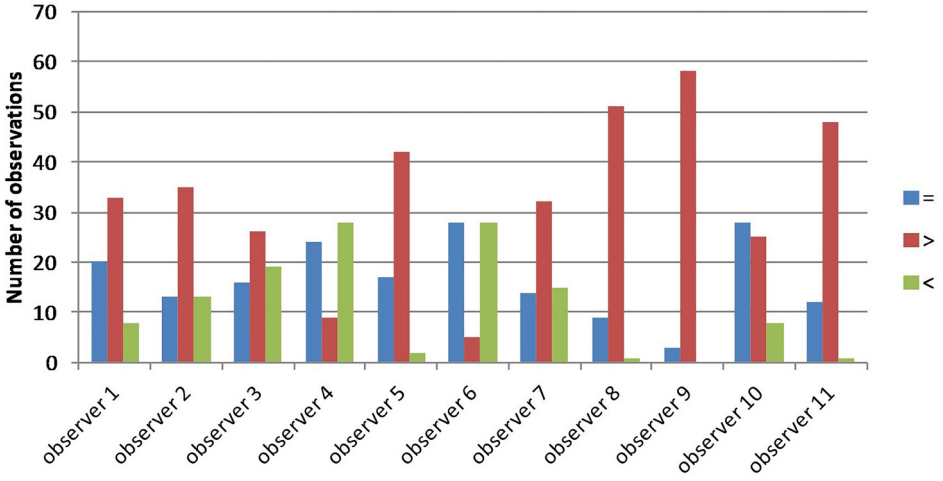

Figure 5. Intraobserver concordance of 11 observers participating both in numerical and category-based assessment of the Ki67 LI (= , equal; >, higher; <, lower score on visual estimation of predetermined images).

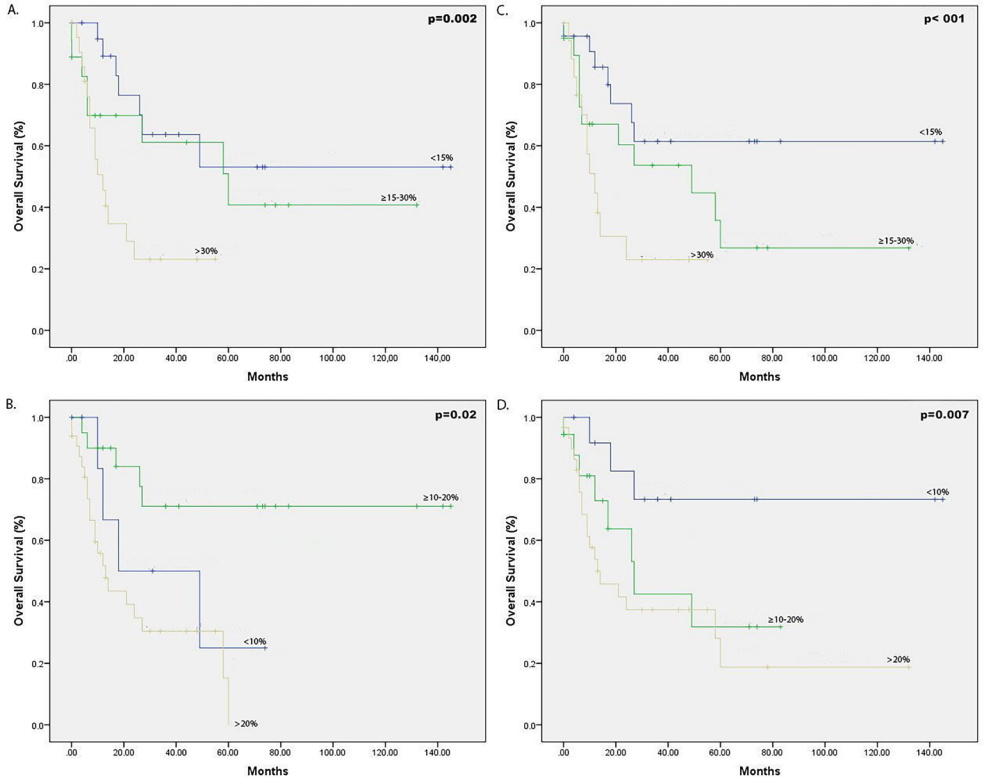

Figure 6. Overall survival determined by pathologists using 0%-15%-30% (**A**) and 0%-10%-20% (**B**) cutoffs compared with the software (ASH) cutoff ranges of 0%-15%-30% (**C**) and 0%-10%-20% (**D**), respectively.

DISCUSSION

Ki67 immunohistochemistry has been integrated in routine pathology practice not only in diagnostics, that is, grading and tumor classification, diagnosis of intraepithelial neoplasia, and assessment of malignant potential, but also as a prognostic and predictive biomarker. With regard to ACCs, it has been proposed in diagnostics [18, 19], prognostics [8–10, 20], and in guiding treatment decisions [1, 2]. The current study highlights the need for standardized use of the Ki67 LI discouraging visual estimation and verifies the applicability of ASH in Ki67 assessment.

A large variation was noted among 14 observers in Ki67 index determination using a virtual microscopy interface. Because of the stringent centralized staining protocol, all participants were seeing the same slides. The variation therefore could not be explained

by technical issues and had to be attributed to different practices with respect to interpretation and scoring such as area(s) of slide read, total number of cells in fields of evaluation, and methods of assessment [11]. In support of the last, we still observed significant levels of variation even when reducing complexities by estimating Ki67 LI levels in preselected areas and following a category-based approach using visual estimation. This is consistent with studies in breast carcinomas using a tissue microarray platform [21] as well as in gastroenteropancreatic neuroendocrine tumors using predetermined images [12].

Although visual estimation has been suggested as an acceptable method of assessment on expert diagnostic [13] and/or research grounds [22–24], our findings further reinforce the notion that this readout technique is subjective, inaccurate, and thus unreliable [12, 21, 25, 26]. Importantly, low levels of concordance were revealed around categorical cutoff values recently proposed in ACCs. This is in keeping with Tang *et al*. [12] who reported significant discordance among 18 observers, which was sufficient to alter the final grade of the majority of 45 neuroendocrine tumors. Whether such discordances could be solely ascribed to the method of assessment or partly to parameters residing in the realm of cognitive psychology [22] remains uncertain.

The aforementioned data challenge the clinical applicability of clinically relevant cutoffs in ACCs. In accordance with Polley *et al*. [21] and Mengel *et al*. [27], Beuschlein *et al*. [9] suggested that Ki67 LI variability is to be expected in ACCs at different clinical centers highlighting the issue of interlaboratory variation due to pre-analytical and analytical parameters [21, 27]. In this setting, rigorous methods in tissue preparation, that is, fixation, processing, and generation of uniform sections, would seem to be important. Interlaboratory variables at play, for example, variation affecting controlled conditions, variability in microtomes used, and differences in the temperature of the formalin-fixed paraffin-embedded blocks, might have affected the thickness of the immunostained sections in the current study. In addition to the interlaboratory variation, interobserver and intraobserver variation [12, 22, 28, 29] and tumor heterogeneity of Ki67 expression levels [13, 14, 30, 31] seem to add further levels of complexity to the issue of reproducibility, thereby hampering its clinical utility. This issue was emphasized by the International Ki67 in Breast Cancer Working Group [11] that was unable to reach a consensus in the absence of harmonized methodology with respect to ideal thresholds that could be useful in clinical routine practice. Accordingly, they recommended that cutoffs for prognosis, prediction, and monitoring should be applied only if the results from local practice have been validated against the respective ones in studies that have defined these particular cutoffs [11, 21].

Various approaches have been developed to obtain standardized Ki67 scoring. These include efforts to reduce interlaboratory variation by calibrating to a common scoring method using a web-based tool [32] and efforts to reduce interobserver and intraobserver variation by either selecting the most representative tumor areas based on an automated approach [15, 33, 34] or providing a software-automated quantitation of Ki67 LI [16, 35–37]. In the setting of computerized image analysis, we verified the applicability of a digital microscopy– enabled method for assessment of Ki67 expression in adrenocortical cancer. The novel approach of software-selected areas aims not only to reduce the interobserver variation, but also to characterize Ki67 levels of heterogeneity in primary tumors, recurrences, and metastases.

User interaction is recommended before virtual slide analysis to ensure that areas leading to miscalculations, that is, intrinsic and extrinsic pigmentation (deposit artifacts), necrotic areas, tissue folds, etc., are excluded [15]. In this series, excluding certain tissue regions was not sufficient to avoid serious miscalculations with regard to 15 cases (15/76, 20%) that were subsequently excluded from the analysis, calling into question potential clinical actions based on such cases. Future efforts should focus on software amendments to overcome technical shortcomings in addition to improving methods of scoring.

In conclusion, current practices in Ki67 scoring assessment vary greatly, and interobserver variation sets particular limitations to the clinical utility of Ki67 LI, especially around clinically relevant cutoff values, in adrenocortical cancer. Our results highlight the need for standardization and suggest that visual estimation should be strongly discouraged as a readout technique, while computerized DIA seems to provide a reliable alternative. To drive forward harmonization of Ki67 analysis, we have previously developed and now validated an opensource Galaxy virtual machine application, namely ASH. Given certain pre-analytical and analytical concerns, quality assurance schemes, that is, standardized tissue fixation along with fine-tuned immunohistochemical staining protocols, are expected to additionally increase reproducibility and reliability of the Ki67 LI in endocrine pathology practice.

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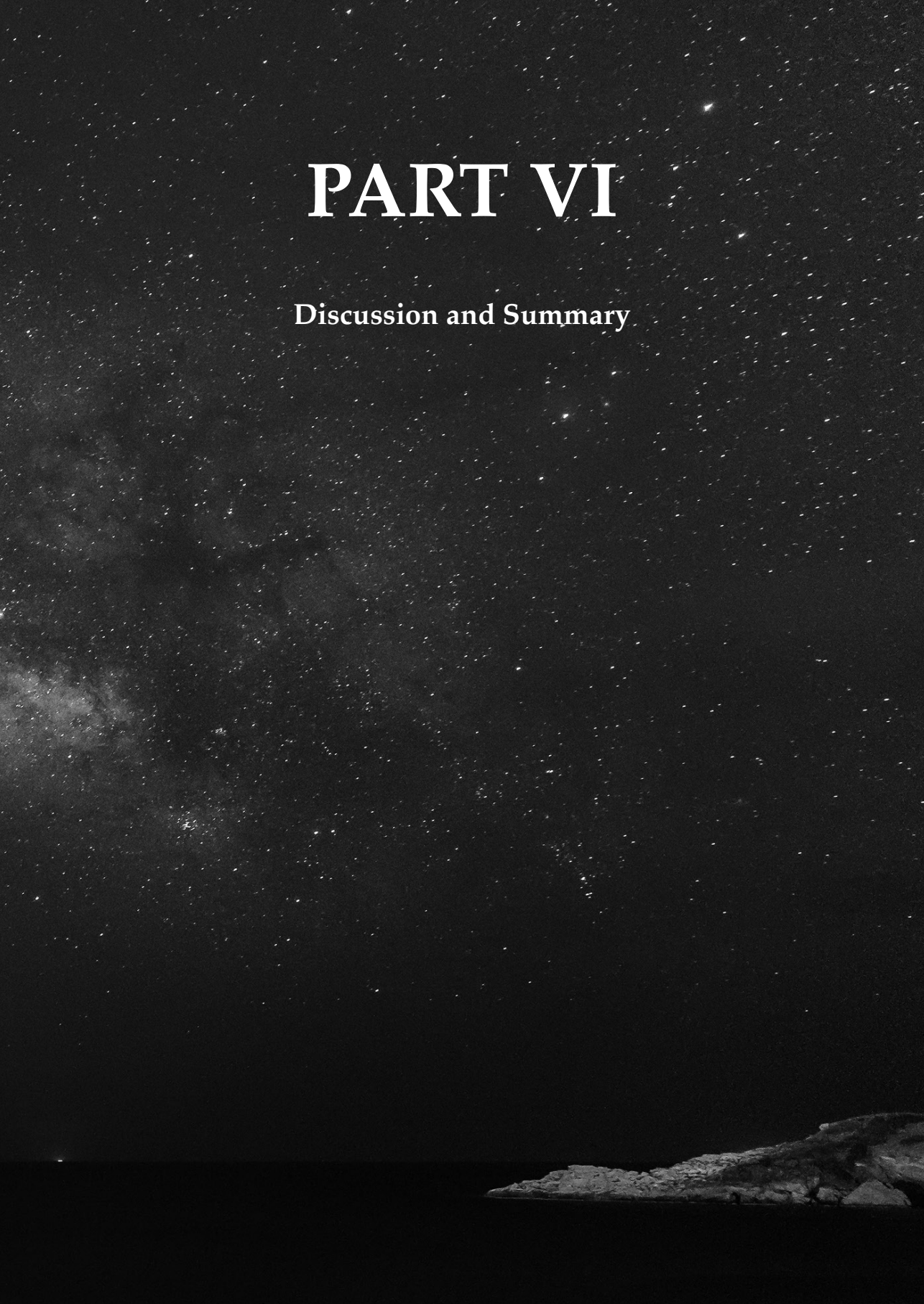
PART VI

Discussion and Summary



Chapter 12

General Discussion

The current thesis aims at contributing to the evolving landscape of endocrine pathology practice and hence elucidates various aspects in terms of diagnostics, prognostics and molecular genetics of tumours originating from the adrenal gland and extra-adrenal paraganglia.

All endocrine-associated medical specialists should be aware of the full phenotypic spectrum along with genotype-phenotype correlations concerning the following endocrine-related genetic syndromes i.e. **MEN1**, **MEN2**, **MEN4**, **NF1**, **VHL syndrome**, **Carney Complex**, **Carney-Stratakis syndrome**, **Carney triad**, **Hereditary PGL syndromes** (or **SDH-related PCC-PGL syndromes**), **Familial PCC syndromes** (or **MAX**- and **TMEM127**-related **PCC-PGL** syndromes), **Polycythemia PGL syndromes** (or **EPAS1/HIF2a-related PCC/PGL syndromes**), **HLRCC syndrome**, **Beckwith-Wiedemann syndrome**, **Lynch Syndrome**, **Li-Fraumeni syndrome**, **ARMC5 Tumour Syndrome**, **McCune-Albright syndrome**, **FAP syndrome**, **PHTS/ CS**, **familial non-medullary thyroid cancer syndromes**, **Pendred syndrome**, **Werner syndrome**, **FIPA**, and **HPT-JT syndrome** [1-18].

In this setting, we tried to elucidate the SDH-related tumour spectrum highlighting GISTs, renal cell carcinomas, pituitary adenomas and pancreatic neuroendocrine tumours as tumour components in addition to PCCs/PGLs [19-20]. Along the same lines, we analyzed genotype-phenotype correlations and displayed (**1**) high frequency of *SDHB* mutations in malignant PCCs/PGLs; (**2**) association of GIST with *SDHA*; approximately 50% of the mutations reported in this tumour type occurred in *SDHA* gene; (**3**) unique clinicopathological features and biological properties in SDH-deficient GISTs i.e. GISTs arising in Carney–Stratakis syndrome and the Carney triad, pediatric GISTs, and a subset of apparently sporadic KIT/PDGFRA WT GISTs; (**4**) particular clinicopathological parameters along with high frequency of *SDHB* mutations in SDH-deficient renal carcinoma; and (**5**) a characteristic clinical phenotype of pituitary adenomas [21].

With regard to the clinical presentations of PCCs/PGLs arising in various genetic backgrounds, relevant data are summarized in **Tables 1** & **2**. Although various neoplasms and/or related conditions, such as entero-pancreatic neuroendocrine tumours, pituitary adenomas, renal tumours, GISTs as well as polycythemia, do arise within different genetic syndromes, it remains elusive whether particular dysregulated pathways e.g. defects in the oxygen sensing pathway (PHD2-VHL-HIF-axis) for polycythemia [22-25], or inherently susceptible tissues could explain such co-occurences in response to cancerous transformation [26]. Differences in phenotypic expression of mutations involving identical loci, even within a familial context, are also poorly understood [21].

Table 1. Clinical presentations of pheochromocytomas and paragangliomas associated with germline mutations.

Gene	Mutation Rate (%)!	Predominant Tumour Site	Tumour number	Major Non-Paraganglionic Tumours & other Related Features
VHL	7	PCC>PGL [1-3]	multiple	Retinal and CNS Hemangioblastoma, Clear Cell Renal Cell Carcinoma & Renal Cysts, Pancreatic Neuroendocrine Tumour and Pancreatic Cyst, Endolymphatic SAC Tumours, Papillary Cystadenoma of the Epididymis/ Broad Ligament and Mesosalpinx [4]
RET	6	PCC>PGL [1-3]	multiple	Medullary Thyroid Carcinoma, Multiglandular Parathyroid Disease, Hirschsprung disease, Marfanoid habitus, Mucocutaneous Neuromas, Gastrointestinal Ganglioneuromatosis, Cutaneous Lichen Amyloidosis [5]
NF1	3	PCC>PGL [1-3]	single	Neurofibroma, Malignant Peripheral Nerve Sheath Tumour, Gastrointestinal Stromal Tumour, Optic Nerve and other Brain Stem Glioma, Neuroendocrine tumour (somatostatinoma) of the periampullary duodenum, cafe-au-lait spots and axillary or inguinal freckling, Lisch nodules, dysplasia of sphenoid bone or long bones, kyphoscoliosis, cerebral arteriopathy, pulmonary artery stenosis, learning disabilities [6-7]
SDHA $$$	<1	PGL>PCC [1-3]	single	Gastrointestinal Stromal Tumour, SDH-deficient Renal Carcinoma?, Pituitary Adenoma [11-12]
SDHB $$$	10	PGL>PCC [1-3]	multiple	Gastrointestinal Stromal Tumour, SDH-deficient Renal Carcinoma, Pituitary Adenoma [11]
SDHC $$$	1	PGL>PCC [1-3]	multiple	Gastrointestinal Stromal Tumour, SDH-deficient Renal Carcinoma, Pituitary Adenoma [11]
SDHD	9	PGL>PCC [1-3]	multiple	Gastrointestinal Stromal Tumour, SDH-deficient Renal Carcinoma, Pituitary Adenoma, Pancreatic Neuroendocrine Tumour? [11, 13]
SDHAF2	<0.1	PGL>PCC [14-17]	multiple	None Reported [11]
FH	1	PCC>PGL [18-20]	single #	Cutaneous and Uterine Leiomyomas and/or Leiomyosarcomas?, Type 2 Papillary Renal Cell Carcinoma; spectrum of renal tumours ranging from Type 2 Papillary to Tubulo-Papillary to Collecting-duct Renal Cell Carcinoma [21]
MDH2	<1	PGL? [22]	? #	None Reported *
TMEM127	1	PCC>PGL [17, 27-29]	multiple	Clear Cell Renal Cell Carcinoma [30-31]
MAX	1	PCC>PGL [32-33]	multiple	Renal Oncocytoma? [33-34]
KIF1B	<1	PCC? [35-36]	?	Neuroblastoma? [35-37]
EPAS1/ HIF2a (s ### >>g)	1	PGL~PCC [Table 2: Ref 38-48]	multiple	Polycythemia with markedly elevated erythropoietin (EPO) levels, Duodenal Somatostatinoma [Table 2: Ref 38-48]

PHD1/ EGLN2	<1	PCC/PGL [49]	? #	Polycythemia with borderline or mildly elevated erythropoietin (EPO) levels [49]
PHD2/ EGLN1	<1	PGL~PCC [49-50]	? #	Polycythemia with borderline or mildly elevated erythropoietin (EPO) levels? [49-50]
BAP1	<1?	PGL? [51]	? #	Uveal Melanoma, Malignant Mesothelioma, Cutaneous Melanoma, Atypical Spitz Tumours / Naevi, Basal Cell Carcinoma, Renal Cell Carcinoma [52-53]
MEN1	<1?	PCC [54-56] > PGL [57]	single #	Parathyroid Adenoma/Hyperplasia, Entero-Pancreatic Neuroendocrine Tumour (Gastrinoma > Non-Functioning / PPoma > Insulinoma > Glucagonoma / VIPoma), Pituitary Adenoma (Prolactinoma > Somatotrophinoma > Corticotrophinoma / Non-Functioning), Adrenal cortical tumour, Brochopulmonary Neuroendocrine Tumour, Thymic Neuroendocrine Tumour, Gastric Neuroendocrine Tumour, Angiofibromas, Collagenomas, Lipomas, Meningiomas [58]
MSH2	<1?	PCC? [59]	? #	germline *MMR* gene mutations are associated with Lynch syndrome; the tumour spectrum encompasses colorectal cancers, gynecological (endometrial and ovarian) cancers, gastric cancer, small bowel cancer, urinary tract cancers and other LS-associated extracolonic cancers i.e. CNS tumours (glioblastomas), sebaceous neoplasms as well as pancreatic, prostate and breast cancers [60]
KMT2D (s>>g)	<1?	PCC [61-62]	? #	germline *KMT2D* mutations are associated with Kabuki syndrome, a rare multiple congenital anomaly syndrome, characterized by distinctive facial features, global developmental delay, intellectual disability and cardiovascular and musculoskeletal abnormalities [63] $

Abbreviations: (s) somatic; (g) germline
! combined data for prevalence in PCC and PGL (Favier *et al*. Nat Rev Endocr 2014)
$$$ approximately 10% of Carney Triad patients, suffering from multifocal gastrointestinal stromal tumours, chondromas and/or paragangliomas, harbour germline variants in the ***SDHA, SDHB*** or ***SDHC*** genes **[8]**; therefore being allelic to Carney-Stratakis dyad/syndrome (***SDHA/B/C/D*** germline mutations). Other causative defects concern aberrant DNA hypermethylation of ***SDHC*** **[9]**. Nevertheless, Carney Triad is generally regarded as a genetic disorder of no established etiology, possibly caused by somatic mosaicism **[1, 10]**.
based on the following estimations: (**1**) ***FH***: unilateral paraganglionic tumours (n=6) > PCC/PGL (n=2); (**2**) ***MDH2***: 1 case affected by ea-PGLs (thoracic/retroperitoneal); (**3**) ***PHD1/EGLN2***: 1 case affected by PCC/PGL; (**4**) ***PHD2/EGLN1***: 2 cases affected by multiple paraganglionic tumours and 1 case by a single tumour; (**5**) ***BAP1***: 1 case affected by an extra-adrenal (thoracic : pericardial) PGL; (**6**) ***MEN1***: unilateral PCCs (n=10) & HN-PGL (n=1); (**7**) ***MSH2***: 1 case affected by unilateral PCC; (**8**) ***KMT2D***: 14 PCCs (14/99) over 0/58 PGLs
* one truncating ***MDH2*** mutation (p.E153*) is recorded in The Catalogue of Somatic Mutations in Cancer (COSMIC; http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/). The mutation was found in a neuroblastoma **[22]**
indicating low level constitutional mosaicism
$ paraganglionic tumours are not features of this disorder

Table 2. Polycythemia paraganglioma syndrome (Phenotype MIM number: 603349): Clinicopathological and Mutational Data of All Reported Cases in the Literature.

References	HIF2A mutation Somatic (s) or Germline (g)	Polycythemia	Extra-adrenal (s) PGL Single (s) or Multiple (m)	PCC Unilateral (u) or Bilateral (b)	Duodenal Somatostatinoma(s)
Toyoda *et al.* Pediatrics 2014	s	+ congenital	+ m	-	-
Buffet *et al.* JCEM 2014	s	+ congenital	+ s	-	-
	s	+	+ m	+ u	+
Welander *et al.* Endocr Relat Cancer 2014	s	-	-	+ u	-
	s	-	-	+ u	-
	s *	-	-	+ u	-
	s *	-	-	+ u	-
	g	-	-	+ u	-
	g	-	-	+ u	-
Toledo *et al.* Endocr Relat Cancer 2013	s	-	-	+ u	-
	s	-	-	+ u	-
	s	-	+ m	-	-
	s	-	+ m	-	-
Comino-Mendez *et al.* Hum Mol Genet 2013	s	+	+ m	+ u	-
	s	-	+ m	+ b	-
	s	+	+ m	+ u	-
	s	+	+ m	+ u	-
	s	-	-	+ u	-
	s	-	-	+ u	-
	s	-	+ m	-	-
	g	-	-	+ u	-
	g	-	-	+ u	-

Lorenzo *et al.* J Mol Med (Ber) 2013	g **	+ congenital	+ m	-	-
Taieb *et al.* JCEM 2013	s	+ congenital	+ m	+ b	-
Favier *et al.* N Engl J Med 2012	s	-	-	+ u	-
Pacak *et al.* J Clin Oncol 2013 & Zhuang *et al.* N Engl J Med 2012 ***	s	+ congenital	+ m	-	+
	s	+ congenital	+ m	-	+
	s	+	+ m	-	?
Yang *et al.* Blood 2013	s	+ congenital	+ m	-	+
	s	+ congenital	+ m	-	+

* Unknown if present at the germline level
** Inherited by an apparently unaffected parent
*** Two overlapping cases

Underlying pathogenetic mechanisms e.g. pathogenic mutations affecting genomic territories, such as coding and noncoding functional regions, and/or epigenetic and copy-number aberrations, seem to provide valuable insights into tumourigenesis and progression both of endocrine and/or endocrine-related cancer [9, 27-36]. In this setting, we explored the occurrence of hotspot mutations in the promoter of the telomerase reverse transcriptase (*TERT*) gene in tumours originating from the adrenal gland and extra-adrenal paraganglia i.e. adrenocortical carcinomas (ACCs), PCCs/PGLs and peripheral neuroblastic tumours along with human neuroblastoma (NBL) and ACC cell lines [26]. Although *TERT* promoter mutations occurred rarely in ACCs and extra-adrenal PGLs of the urinary bladder, a potential association with the acquisition of *TERT* promoter mutations in a subset of aggressive SDH-deficient tumours has been additionally displayed [26]; further reinforcing the notion that mutations in functional non-coding genomic regions might be relevant for tumour progression in particular genetic contexts [27].

Together with awareness of the various tumoural spectra and genotype-phenotype correlations, identification of clues to occult hereditary disease might have important implications both for the patients and their families in terms of further genetic testing and active surveillance programs.

Particular tumour combinations should raise the suspicion i.e. (**1**) **paraganglionic tumours** and **entero-pancreatic neuroendocrine tumours** in NF1 and Polycythemia PGL syndrome (duodenal somatostatinoma) as well as VHL syndrome, MEN1 syndrome and PGL-1 (*SDHD*-associated) syndrome (pancreatic neuroendocrine tumour) [19, 37-40]; (**2**) **paraganglionic tumours and GISTs** arising in the *SDH*- and *NF1*-deficient state [21, 41-42]; (**3**) **paraganglionic tumours and renal neoplasms** arising in the *SDH*-, *FH*-, *TMEM127*-, *VHL*- and *MAX*- deficient setting [21, 43-46]; (**4**) **paraganglionic tumours and pituitary adenomas** in patients harbouring germline *MEN1* and *SDH-x* mutations [19-21, 47]; (**5**) **paraganglionic tumours and adrenal cortical adenomas** in Carney Triad; a genetic disorder, possibly caused by somatic mosaicism and/or SDH-x defects i.e. aberrant DNA hypermethylation of *SDHC* and/or germline variants in the *SDHA*, *SDHB* or *SDHC* genes, as recently shown in [8, 30], and characterized by a constellation of tumours affecting at least five organs i.e. **stomach**: GIST; **lung**: chondroma; **adrenal medulla**/ **extra-adrenal paraganglia**: PCC/PGL; **adrenal cortex**: adrenal cortical adenoma; and **esophagus**: leiomyoma [7-8, 30, 48-49]; and (**6**) **paraganglionic tumours and peripheral neuroblastic tumours** either in different anatomic sites (*KIF1b, SDHB, SDHC*) or within the setting of a composite tumour (*NF1, VHL, MEN2A, MEN2, SDHB*) [8, 48-51].

From a histopathological perspective, pathologists should be aware of the (**1**) MEN2- and TMEM127-related adrenal gland pathology i.e. bilateral and/or multicentric micro- and macro-PCCs as opposed to VHL counterparts i.e. a thick vascular capsule, small- to medium-sized tumour cells with amphophilic clear cytoplasm and numerous interspersed small vessels [52-55]; (**2**) clinico-pathological characteristics of SDH-deficient GIST [56-57]; and (**3**) pathological features of FH- and SDH-deficient RCC [58-60]. With regard to the latter, we elucidated its clinicopathological aspects by substantiating distinctive morphologic and clinical features as well as genetic associations [58]. This is defined by SDHB immunonegativity and should be recognized by its uniform low-grade cytology, cytoplasmic vacuoles, eosinophilic or flocculent cytoplasm, focal cystic change, and solid to lobulated growth with peripherally entrapped renal tubules [58].

Approximately 12% of PCC/PGL patients with a non-syndromic presentation and without a familial history do have hereditary PCC/PGL, with such an apparently sporadic presentation mostly attributed to germline *SDH-x* mutations [6]. In addition, around 10-15% of PCC/PGL patients can develop metastatic disease to sites where chromaffin tissue is not normally found i.e. bone, liver, lungs and lymph nodes [5], while 10%-70% of PCC/PGL patients with metastatic disease have been reported to be *SDHB* mutation carriers at the germline level [6].

Triaging of patients for optimal genetic testing is critical by performing the appropriate IHC i.e. (**1**) SDHB/SDHA/SDHD IHC to evaluate for SDH deficiency in PCCs/PGLs as parts of Hereditary PGL syndromes (or SDH-related PCC-PGL syndromes) [61-64]; (**2**) 5-hydroxymethylcytosine (5-hmC)/ S-(2-succinyl) cysteine (2SC) IHC to evaluate for FH deficiency in PCCs/PGLs and accordingly identify patients harboring *FH* germline mutations [65]; and (**3**) a MMR panel by IHC (MLH1, MSH2, MSH6, PMS2) to evaluate for MMR deficiency in PCC [66] and ACC [11] as a component of the Lynch syndrome. In addition to highlighting SDH-, FH- and MMR-deficiency [11, 61-66], IHC has been shown to detect various molecular alterations i.e. *BAP1* and *MAX* deleterious mutations in PCCs/PGLs [67-68] as well as *TP53* and *CTNNB1* mutations in ACCs [69-71].

Despite the fact that the availability of genetic testing and other molecular analyses of PCCs/PGLs at an ever-decreasing cost might overshadow the need for pathology input [72], immunohistochemical characterization stills remains an important integrated component of algorithms to guide either a sequential genetic testing by Sanger sequencing or a biological validation of the variants identified by the analysis of all PCC/PGL susceptibility genes by NGS (**Figure 1**) [6]. The role of histopathologists is critical in terms of correct immunohistochemical interpretation, given that false-positive and false-

negative evaluations can result in (**1**) failure to identify PCC/PGL affected individuals at increased risk for SDH-/FH-/MAX-related neoplasia, (**2**) incorrect interpretation of the pathogenicity of genetic variants of uncertain significance, (**3**) inappropriate genetic testing as well as (**4**) inaccurate information on prognosis [21, 63].

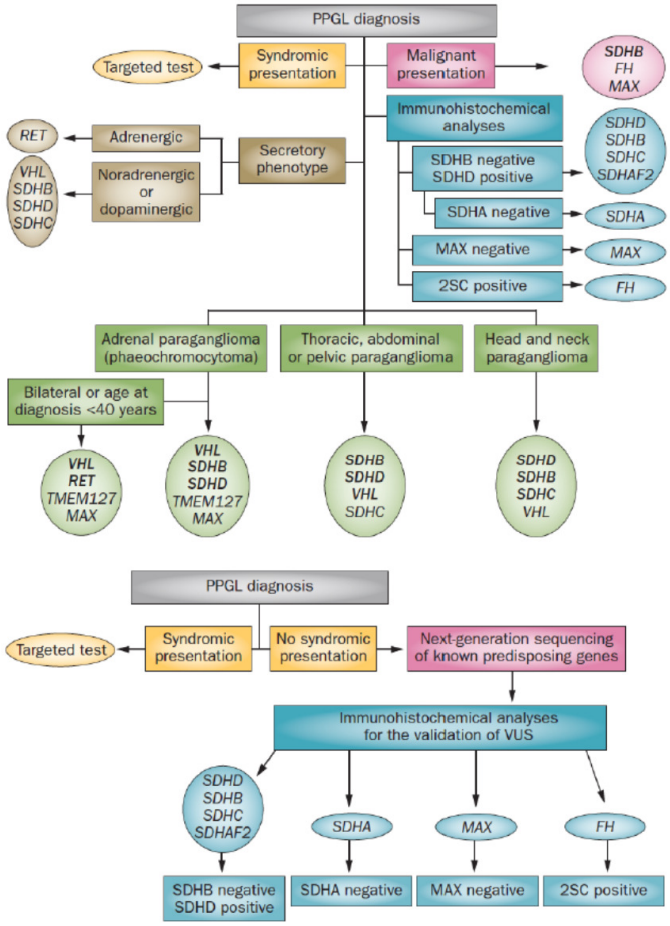


Figure 1. Algorithms for PCC/PGL genetic testing by Sanger sequencing or by Next-Generation sequencing. Adapted by permission from Macmillan Publishers Ltd: Nat Rev Endocrinol, [**6**], 2015.

In this context, we determined the reproducibility of SDHB/SDHA IHC using a web-based virtual microscopy approach and displayed a good level of reproducibility among seven expert endocrine pathologists [63]. To extend, in the vast majority of *SDH-x* mutated cases, accompanied by SDHB and/or SDHA immune expression data as reported in the literature (134 out of 145; 92%), the IHC pattern was consistent with

the prediction obtained by *in silico* analysis (three scores were initially assessed: SIFT, Polyphen2, and Mutation Assessor; for those *SDH* mutations showing nonconsistent IHC results, we calculated the *C*ombined *A*nnotation *D*ependent *D*epletion [CADD]) [21]. This accordingly indicates that SDHB/SDHA IHC, in addition to *in silico* analysis, should be used as a tool to support the definition of the pathogenicity of *SDH* variants of unknown significance.

Further efforts to maintain and update pre-existing databases, i.e. **Leiden Open Variation Database** (**LOVD**) http://www.lovd.nl/3.0/home, **The Catalogue of Somatic Mutations in Cancer** (**COSMIC**) http://cancer.sanger.ac.uk/cosmic, **The IARC TP53 Database** http://p53.iarc.fr and/or **MMR Gene Unclassified Variants Database** http://www.mmruv.info, will contribute into a solid foundation for further understanding of the disease, e.g. frequency and significance of variants, interactions of somatic/germline variants and/or mutations, phenotypic expression and associated tumoural spectra, but also as a resource for all healthcare professionals involved in endocrine and/or endocrine related cancer [73-77].

Additionally to its emerging value as a critical determinant of prognosis in ACC [78-80], Ki67 labelling index has been recently integrated in treatment flow charts for adrenocortical cancer patients suffering from tumours either amenable to radical resection or at advanced presentation [81-82]. Accordingly, its inaccurate quantification on immunohistochemical grounds might have important prognostic and therapeutic implications. In this setting, we performed a rigorous evaluation of the inherent pitfalls which attend the quantitative assessment of Ki67 labelling index in ACCs using a web-based virtual microscopy approach. Of note, we documented inter- and intra-observer variation [83], with the former challenging the clinical applicability of clinically relevant cut-offs as previously proposed in ACCs [78-82]. To this end, novel digital microscopy-enabled methods, i.e. an open source Galaxy virtual machine application namely Automated Selection of Hotspots [83-84], could provide critical aid in reducing variation, increasing reproducibility and improving reliability in the clinical setting.

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Chapter 13

SUMMARY

An overview of the present knowledge with regard to inherited tumour syndromes involving the adrenal gland and extra-adrenal paraganglia, novel insights into the genetics of pheochromocytomas (PCCs)/ paragangliomas (PGLs) and adrenocortical carcinomas (ACCs) as well as prognostic parameters in adrenocortical cancer is given in **Chapter 1**.

Chapter 2 is a comprehensive review on recent advances in the genetics of PCCs/ PGLs highlighting the hereditary susceptibility disorders associated with the PCC/ PGL development, genotype–phenotype correlations in familial PGL syndromes and the role of immunohistochemistry (IHC) as a supplementary approach in molecular genetic testing for PCCs/ PGLs. The morphological variants of extra-adrenal PGLs, unusual anatomic sites of occurrence, composite PGLs/PCCs, potential immunohistochemical pitfalls and determination of malignancy are also discussed.

In **Chapter 3**, we conclude that SDHA/SDHB IHC represents a reliable tool to identify patients with *SDH* mutations and display a good level of reproducibility among expert endocrine pathologists through virtual microscopy in a large multicenter/multinational cohort of genetically well-characterized PCCs/PGLs. A SDHB-immunonegative subset of *VHL*- and *NF1*-mutated tumours challenges the issue of specificity for SDHB IHC, while another subset of paraganglionic tumours (7%) is evaluated as SDHB immunonegative in the absence of *SDH* mutations. Therefore, if SDH genetics fails to detect a mutation in any SDHB-immunonegative tumour, VHL/NF1 testing with the use of targeted NGS and/or SDHC promoter methylation analysis is advisable. Given that SDHB IHC status overall is strongly correlated with the clinical behavior of PCC/PGL, the role of SDHB IHC as a prognostic marker has been accordingly strengthened.

In **Chapter 4**, bioinformatics analysis, predicting the effect of non-synonymous *SDH* mutations, is consistent with available SDHA and SDHB IHC results. Taken together with LOH as the predominant mechanism of SDH inactivation, SDHA/SDHB IHC and *in silico* analysis seem to be valuable tools in determining the pathogenicity of *SDH* variants of unknown significance with important implications in the new NGS era. Genotype-phenotype correlations have been detailed in SDH-related tumours i.e. PCCs/PGLs, gastrointestinal stromal tumours (GISTs), renal carcinomas and pituitary adenomas, as follows: (**1**) high frequency of *SDHB* mutations in malignant PCCs/PGLs; (**2**) association of GIST with *SDHA*; approximately 50% of the mutations reported in this tumour type occurred in *SDHA* gene; (**3**) unique clinicopathological features and biological properties

in SDH-deficient GISTs i.e. GISTs arising in Carney–Stratakis syndrome and the Carney triad, pediatric GISTs, and a subset of apparently sporadic KIT/PDGFRA WT GISTs; (**4**) particular clinicopathological parameters along with high frequency of *SDHB* mutations in SDH-deficient renal carcinoma; and (**5**) a characteristic clinical phenotype of pituitary adenomas.

In **Chapters 5** & **6**, we investigate non-paraganglionic tumours arising in *SDH* mutation carriers from the Leiden SDH Mutation Carrier Registry and other European centres. In this regard, the SDH-related tumour spectrum has been expanded highlighting pancreatic neuroendocrine tumour (NET) as a new member. Although we strengthen the etiological association of *SDH* genes with pituitary neoplasia, renal tumourigenesis and gastric GISTs, a causative link between papillary thyroid carcinoma (PTC), melanoma, prostate and bladder cancer cannot be supported. To extend, we reinforce the notion that hereditary cancer genes may not always be involved in sporadic tumourigenesis.

In **Chapter 7**, we elucidate clinicopathological aspects of SDH-deficient renal carcinoma by substantiating its distinctive morphologic and clinical features, genetic associations as well as prognosis and estimating its incidence. In this regard, SDH-deficient renal carcinoma represents a distinct and rare renal neoplasm, which is defined by SDHB immunonegativity. Owing to its rarity, with a true incidence estimated as being 0.05% to 0.2% of unselected renal neoplasms, it is impractical to perform screening IHC on all renal cancers. Nevertheless, the great majority demonstrates typical appearances at least focally and are recognized by their uniform low-grade cytology, cytoplasmic vacuoles, eosinophilic or flocculent cytoplasm, focal cystic change, and solid to lobulated growth with peripherally entrapped renal tubules. In tumours exhibiting low-grade nuclear features with International Society of Urological Pathology (ISUP) grade 2, metastasis is unusual but can occur even after a prolonged period. SDH-deficient renal carcinoma may be associated with high ISUP grade, coagulative necrosis or sarcomatoid transformation, in which case the development of metastatic disease seems much more likely. SDH-deficient renal carcinomas are commonly multifocal and with prolonged follow-up, bilateral tumours can be identified in up to 26% of patients. To date, all reported cases have been associated with germline *SDH* mutations.

In **Chapters 8 & 9**, we discuss emerging evidence concerning the adrenocortical tumourigenesis and in this context we explore the occurrence and prevalence of mutations in the promoter of the telomerase reverse transcriptase (*TERT*) gene; noncoding functional genomic region potentially relevant for human tumourigenesis. Accordingly, we demonstrate that hotspot *TERT* promoter mutations occur, albeit rarely,

in tumours originating from the adrenal cortex (ACCs) and extra-adrenal paraganglia of the urinary bladder. Their absence in PCCs, head and neck PGLs and peripheral neuroblastic tumours indicates that these seem unlikely to be critical genetic events in their development and/or progression. Preliminary evidence of a potential association with the acquisition of *TERT* promoter mutations in a subset of aggressive SDH-deficient tumours is additionally provided.

In **Chapter 10**, we developed an open source Galaxy virtual machine application, namely Automated Selection of Hotspots, designed for Ki67 hotspot detection in adrenocortical cancer. In **Chapter 11**, we validate the aforementioned application and investigate interobserver variability for Ki67 Labelling Index assessment among endocrine pathologists using a web-based virtual microscopy approach in a multicenter ACC cohort. Current practices in Ki67 scoring assessment vary greatly with inter-observer variation setting particular limitations to the clinical utility of the Ki67 Labelling Index, especially around clinically relevant cut-off values. The need for standardization is highlighted indicating that visual estimate should be strongly discouraged as a readout technique, while computerized digital image analysis can provide a reliable alternative.

The general discussion in **Chapter 12** describes the main findings reported in this dissertation related to current perspectives about the evolving role of endocrine pathology.

SAMENVATTING

Een overzicht van de huidige kennis over erfelijke tumor syndromen van de bijnier en extra-adrenale paraganglia, nieuwe inzichten in de genetica van feochromocytoom (FCC)/ paraganglioom (PGL) en bijnierschorscarcinoom (BSC) en prognostische parameters van de adrenocorticale kanker, wordt in het **Hoofdstuk 1** weergegeven.

Hoofdstuk 2 betreft een overzicht van de recente ontwikkelingen in de genetica van FCC/PGL, met nadruk op de erfelijke aandoeningen in samenhang met FCC/PGL ontwikkeling, de genotype-fenotype correlaties in familiaire PGL syndromen en de rol van immunohistochemie (IHC) als surrogaat marker voor moleculair-genetische tests voor FCC/PGL. De morfologische varianten van extra-adrenale PGL, de zeldzame anatomische locaties waar ze voorkomen, zogenaamde composiet PGL/FCC, potentiële immunohistochemische valkuilen en analyse van maligniteit worden ook besproken.

In **Hoofdstuk 3** concluderen we dat SDHA/SDHB IHC een betrouwbaar hulpmiddel is voor de identificatie van patiënten met *SDH* mutaties en goed reproduceerbaar is onder pathologen bij gebruik van virtuele microscopie in een grote groep van genetisch goed gekarakteriseerde FCC/PGL. Er is echter een groep van *VHL*- en *NF1*-gemuteerde tumoren die immunonegatief is en ook een groep van immunonegatieve tumoren (7%) die negatief zijn voor SDHB mutaties. Daarom is het bij immunonegatieve tumoren en afwezigheid van *SDH* mutaties aan te raden om mutatie analyse voor *VHL* en *NF1* uit te voeren of *SDHC* promoter hypermethylatie na te gaan. Aangezien SDHB IHC sterke correlatie vertoont met het klinisch gedrag FCC/PGL, werd de rol van SDHB IHC als prognostische marker verder onderbouwd.

In **Hoofdstuk 4**, is bio-informatica analyse, die de gevolgen van niet-synonieme *SDH* mutaties voorspelt, consistent met de resultaten van SDHA en SDHB IHC. In samenhang met LOH als belangrijkste mechanisme van geïnactiveerd *SDH*, blijkt dat SDHA/SDHB IHC en *in silico analyse* betrouwbare hulpmiddelen zijn om de pathogene eigenschappen van DNA varianten te bepalen. Genotype-fenotype correlaties werden geanalyseerd in met SDH samenhangende tumoren, d.w.z. FCC/PGL, gastro-intestinale stromale tumor (GIST), renale carcinomen en hypofyse-adenomen: (1) hoge frequentie van *SDHB* mutaties in maligne FCC/PGL, (2) associatie van GIST met *SDHA*: ongeveer 50% van de mutaties in dit tumortype komt voor in het *SDHA* gen, (3) unieke clinicopathologische kenmerken en biologische eigenschappen in SDH-deficiente GIST, zoals GIST in het kader van het Carney-Stratakis syndroom en de Carney triade, pediatrische GIST en een subgroep van sporadische KIT/PDGFRA WT GIST, (4) bijzondere clinicopathologische

parameters en hoge mutatie frequentie van *SDHB* in SDH-deficiente renale carcinomen, en (5) een kenmerkend klinisch fenotype van het hypofyse-adenoom.

In de **Hoofdstukken 5 & 6**, onderzochten we niet paraganglionaire tumoren die zich voordoen in *SDH* mutatiedragers uit de Leiden SDH Mutation Carrier Registry en andere Europese Centra. Het spectrum van tumoren in de context van SDH kon worden uitgebreid met pancreatische neuro-endocriene tumoren (NET) als nieuw lid. Hoewel de etiologische associatie van *SDH* genen met tumoren van de hypofyse en de nier en met GIST van de maag kan worden bevestigd, kan geen oorzakelijk verband met papillair schildkliercarcinoom (PSC), melanoom, prostaat- en blaaskanker gelegd worden. Verder onderbouwen we het standpunt dat erfelijke kanker genen niet altijd verantwoordelijk zijn voor sporadische tumorgenese.

In **Hoofdstuk 7**, helderen we de clinicopathologische aspecten van het SDH-deficiente renaal carcinoom op door het documenteren van zijn specifieke morfologische en klinische kenmerken, de genetische associaties, de prognose en de schatting van de frequentie. Het SDH-deficiente renaal carcinoom betreft een aparte en zeldzame niertumor, die gekenmerkt wordt door immunonegativiteit voor SDHB. Wegens de zeldzaamheid, met een frequentie die geschat wordt op 0,05% tot 0,2% van ongeselecteerde niertumoren, is het niet mogelijk om immuunhistochemische screening met SDHB uit te voeren op alle nierceltumoren. Niettemin toont het grootste deel van deze tumoren typische kenmerken, ten minste focaal, en ze kunnen worden herkend door hun uniforme laaggradige cytologie, cytoplasmatische vacuolen, eosinofiele of vlokkige cytoplasma, focale cysteuze verandering, en solide tot gelobde groei met perifeer ingevangen renale tubuli. In tumoren met laaggradige nucleaire veranderingen overeenkomend met graad 2 in de International Society of Urological Pathology (ISUP), zijn metastasen ongewoon maar kunnen voorkomen bij langdurige follow-up. SDH-deficient renaal carcinoom kan geassocieerd zijn met een hoge ISUP graad, coagulatieve necrose of sarcomatoide verandering, en in dit geval is het ontstaan van metastatische ziekte waarschijnlijker. De SDH-deficiente renale carcinomen zijn gewoonlijk multifocaal en bij langdurige follow-up kunnen bilaterale tumoren geïdentificeerd worden in 26% van de patiënten. Tot op heden werden alle gevallen veroorzaakt door kiembaan *SDH* mutaties.

In **Hoofdstukken 8 & 9** bespreken we adrenocorticale tumorgenese en in dit kader onderzoeken we het voorkomen en de prevalentie van mutaties in de promotor van het gen telomerase reverse transcriptaseremmers (*TERT*): een niet-coderend functioneel genoomgebied dat potentieel relevant is voor tumorigenese bij de mens. We tonen aan dat hotspot *TERT* promotor mutaties zich voordoen, ofschoon zeldzaam, in tumoren die

ontstaan uit de bijnierschors (BSC) en extra-adrenale paraganglia van de urineblaas. Hun afwezigheid in FCC, hoofd-hals PGL en perifere neuroblastaire tumoren wijst erop dat *TERT* mutaties waarschijnlijk niet cruciaal zijn in de ontwikkeling en/of progressie van deze tumoren. Preliminair bewijs van een potentiële rol van *TERT* promotor mutaties in een subgroep van agressieve SDH-deficiente tumoren wordt bovendien verschaft.

In **Hoofdstuk 10** hebben we een applicatie in open source Galaxy ontwikkeld, met name Automated Selection of Hotspots, ontworpen voor Ki67 hotspot detectie in bijnierschorscarcinoom. In **Hoofdstuk 11** valideren we deze applicatie en onderzoeken we de interobserver variabiliteit voor de analyse van de Ki67 Labelling Index bij endocriene pathologen door het gebruik van een virtuele microscopie benadering in een groep BSC. De huidige methoden van Ki67 labelling index analyse variëren sterk, met als gevolg dat de interobserver variatie de klinische bruikbaarheid sterk beperkt, vooral in relatie tot klinisch afkapwaarden. De behoefte aan standaardisatie wordt benadrukt, waarbij zogenaamde "eyeballing" sterk moet worden ontmoedigd, terwijl de geautomatiseerde digitale beeldanalyse een betrouwbaar alternatief kan opleveren.

De algemene bespreking in **Hoofdstuk 12** beschrijft de hoofdbevindingen die in dit proefschrift beschreven worden in relatie tot de huidige ontwikkelingen in de endocriene pathologie.

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2013 Pathology and underlying Genetics of Inheritable Human Cancer and Developmental Defects (Symposium & Practicum), LUMC
2012 Molecular Diagnostics VII, Erasmus MC
2012 Bioinformatics Workshop "Browsing Genes and Genomes with UCSC", Erasmus MC
2012 Course Genetics for Dummies, Erasmus MC
2012 Photoshop & Illustrator CS5 Workshop, Erasmus MC
2012 Basic Introduction Course on SPSS, Erasmus MC

Presentations

2015 The evolving role of histopathology in tumors originating from the adrenal gland and extra-adrenal paraganglia. Centre for Endocrinology, Diabetes, and Metabolism (CEDAM, University of Birmingham), Birmingham, United Kingdom

2014 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 Tumours** (**ENS@T**) 13th Scientific Meeting of ENS@T, Nice, France

2013 *TERT* promoter mutations in tumors originating from the adrenal gland and extra-adrenal paraganglia. 12th Scientific Meeting of ENS@T, Budapest, Hungary

2013 Current status: **ENS@T Ki67 immunohistochemistry project**. 12th Scientific Meeting of ENS@T, Budapest, Hungary

2013 Current status: **ENS@T ACC Tissue Microarray (TMA) project**. 12th Scientific Meeting of ENS@T, Budapest, Hungary

2012 Toward an improved definition of the tumor spectrum associated with *SDH-x* germline mutations. Josephine Nefkens Institute (JNI) lecture, Rotterdam, The Netherlands

2012 Current status: **ENS@T ACC Tissue Microarray (TMA) project**. 11th Scientific Meeting of ENS@T, Madrid, Spain

2011 SDHB immunohistochemistry confirms SDH involvement in malignancies in *SDH-x* mutated patients, but does not appear to be a screening tool in the general population. 10th Scientific Meeting of ENS@T, Duiven, The Netherlands

2011 A unified way of assessing malignancy in adrenocortical tumours, fact or fiction? European Expert Group on Adrenal Gland Pathology, Rotterdam, The Netherlands

Conferences/ Scientific Meetings

2015 14th Scientific Meeting of ENS@T, Munich, Germany
2014 13th Scientific Meeting of ENS@T, Nice, France
2013 12th Scientific Meeting of ENS@T, Budapest, Hungary
2013 4th International Symposium on Adrenal Cancer, Paris, France
2012 11th Scientific Meeting of ENS@T, Madrid, Spain
2012 LUMC Paraganglioma Mini-Symposium, Leiden, The Netherlands
2012 ENS@T-CANCER General Assembly Meeting, Barcelona, Spain
2012 16th Molecular Medicine Day, Rotterdam, The Netherlands
2011 10th Scientific Meeting of ENS@T, Duiven, The Netherlands
2011 International Symposium on Pheochromocytoma and Paraganglioma, Paris, France
2011 European Expert Group on Adrenal Gland Pathology, Rotterdam, The Netherlands
2011 ENS@T-CANCER Kick-Off Meeting, Rotterdam, The Netherlands
2011 3rd International Adrenal Cancer Symposium, Würzburg, Germany

Awards & Travel Grants

2015 Endocrine Pathology Society Hubert Wolfe Award 2014 (United States and Canadian Academy of Pathology: Endocrine Pathology Society annual meeting, Boston, Massachusetts, USA)

2014 The European Network for the Study of Adrenal Tumors (ENS@T) 2014 Travel Grant Award (13th Scientific Meeting of ENS@T, Nice, France)

Other Activities

2015 WHO Classification of Tumours of Endocrine Organs, **4th edition** Invited Author in **4-1**: **Adrenal cortical carcinoma** T.J. Giordano (Resp.), G.P. Chrousos, A. Kawashima, C.A. Koch, L.J. Medeiros, M.J. Merino, **T.G. Papathomas**, M. Papotti, H. Sasano, L.M. Weiss
5-1: **Pheochromocytoma** A.S. Tischler (Resp.), A. Kawashima, N. Kimura, P. Komminoth, **T.G. Papathomas**, L.D. Thompson, F. Tissier, M.D. Williams, W.F. Young Jr.
5-6: **Extra-adrenal paraganglioma** (**A**: **Head & Neck Paragangliomas** & **B**: **Sympathetic Paragangliomas**) N. Kimura (Resp.), C. Capella, R.A. DeLellis, J. Epstein, A.J. Gill, A. Kawashima, C.A. Koch, P. Komminoth, A. Lam, M.J. Merino, O. Mete, **T.G. Papathomas**, P. Sadow, L.D. Thompson, A.S. Tischler, M.D. Williams, W.F. Young Jr.

Teaching

2016 Scenario 32 Cancer Pathology Tutorials; 2nd year medical students, Guy's Campus, **King's College London**, London, United Kingdom

2014 Adrenal Gland & Extra-adrenal Paraganglia in HIPON: **An International Educational Project** entitled "ICT eModules on HistoPathology: a useful online tool for students, researchers and professionals" supported and co-financed by the Lifelong Learning Program of the Education, Audiovisual and Culture Executive Agency (EACEA) of the Commission of the European Union

2013 Histology and Histopathology of endocrine organs; 2nd year medical students, **Erasmus MC Cancer Institute**, Rotterdam, The Netherlands

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The author of this thesis was born 19 November 1980. He graduated from the 14th Lyceum of Thessaloniki (Greece) in 1998 and Medical Faculty, Aristotle University of Thessaloniki in 2004, respectively. In 2005, he started a residency programme in Pathology at the 1st Department of Pathology, National & Kapodistrian University of Athens (Greece), which was paused after seven months in order to commence a new post as a research associate under the supervision of Prof. dr. Andreas C. Lazaris. In 2007, he continued his training in Pathology at the Hippokration General Hospital of Thessaloniki and subsequently completed 4-years of training in Histopathology. Just prior to entering the last year of Cytopathology training in Greece, he decided to take on a PhD project (2011-2014) under the supervision of Prof. dr. Ronald R. de Krijger and Dr. Winand N.M. Dinjens at the Department of Clinical Pathology, Erasmus MC Cancer Institute, Rotterdam (The Netherlands). This project was funded by the European Union Seventh Framework Programme and was part of a broad collaboration within the ENS@T-Cancer consortium. In 2015, he was presented the Endocrine Pathology Society Hubert Wolfe Award 2014 (USCAP Annual Meeting, Boston, MA, USA) and was invited as an author of the WHO Classification of Tumours of Endocrine Organs, 4th edition. Since 2014, he has been working as a Clinical Fellow at the Department of Pathology, King's College Hospital, London (United Kingdom) and is aiming to become a Fellow of the Royal College of Pathologists.

On July 17th, 2016, he will marry the love of his life Dr Marianna Rifouna.

PUBLICATIONS

1. Jouinot A*, Assié G*, Libe R**, Fassnacht M**, **Papathomas T**, Barreau O, de la Villeon B, Faillot S, Hamzaoui N, Neou M, Perlemoine K, Rene-Corail F, Rodriguez S, Sibony M, Tissier F, Sbiera S, Ronchi C, Kroiss M, Korpershoek E, de Krijger R, Waldmann J, Quinkler M, Haissaguerre M, Tabarin A, Chabre O, Sturm N, Luconi M, Mantero F, Mannelli M, Cohen R, Kerlan V, Touraine P, Barrande G, Groussin L, Bertagna X, Baudin E, Amar L, Beuschlein F, Clauser E, Coste J, Bertherat J; for the European Network for the Study of Adrenal Tumors. **DNA methylation is an independent prognostic marker of survival in adrenocortical cancer** 2016 *submitted*

2. **Papathomas TG***, Duregon E*, Korpershoek E, Restuccia DF, van Marion R, Cappellesso R, Sturm N, Rossi G, Coli A, Zucchini N, Stoop H, Oosterhuis W, Ventura L, Volante M, Fassina A, Dinjens WNM, Papotti M*, de Krijger RR*. **Sarcomatoid Adrenocortical Carcinoma**: **A Comprehensive Pathological, Immunohistochemical and Targeted Next-Generation Sequencing Analysis** 2016 *submitted*

3. **Papathomas TG***, Pucci E*, Giordano TJ, Lu H, Duregon E, Volante M, Papotti M, Lloyd RV, Tischler AS, van Nederveen FH, Nose V, Erickson L, Mete O, Asa SL, Turchini J, Gill AJ, Matias-Guiu X, Skordilis K, Stephenson TJ, Tissier F, Feelders RA, Smid M, Nigg A, Korpershoek E, van der Spek PJ, Dinjens WNM, Stubbs AP, de Krijger RR. **An International Ki67 Reproducibility Study in Adrenal Cortical Carcinoma. The American Journal of Surgical Pathology 2016** 40(4):569-576

4. Korpershoek E, Koffy D, Eussen BH, Oudijk L, **Papathomas TG**, van Nederveen FH, Belt EJT, Franssen GJH, Restuccia DFJ, Krol NMG, van der Luijt RB, Feelders RA, Oldenburg RA, van IJcken WFJ, de Klein A, de Herder WW, de Krijger R, Dinjens WNM. **Complex MAX rearrangement in a family with malignant pheochromocytoma, renal oncocytoma and erythrocytosis. The Journal of Clinical Endocrinology & Metabolism 2016** 101(2):453-460

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STELLINGEN

Behorende bij het proefschrift

Adrenal Tumours: Histopathological and Molecular Genetic Perspectives

1. Germline *SDH* mutations expand the familial spectrum of pituitary adenomas comprising familial isolated pituitary adenoma (FIPA), Carney complex, MEN type 1 and MEN type 4. (**dit proefschrift**)
2. Pancreatic neuroendocrine tumour falls within the succinate dehydrogenase (SDH)-related tumour spectrum. (**dit proefschrift**)
3. SDH-deficient renal carcinoma is a rare and unique type of renal carcinoma, exhibiting stereotypical morphologic features in the great majority of cases and displaying a strong relationship with germline *SDH* mutation. (**dit proefschrift**)
4. Telomerase reverse transcriptase (*TERT*) promoter mutations occur, albeit rarely, in adrenal cortical carcinomas and extra-adrenal paragangliomas of the urinary bladder. (**dit proefschrift**)
5. Novel digital microscopy-enabled methods could provide critical aid in determining accurately the Ki67 labelling index in adrenocortical cancer and as such reducing variation, increasing reproducibility and improving reliability in the clinical setting. (**dit proefschrift**)
6. The complete genomic landscape of pheochromocytoma/paraganglioma is mainly driven by distinct germline and/or somatic mutations in susceptibility genes and reveals different molecular entities, characterized by a set of unique genomic alterations. (**Castro-Vega** *et al.* **Nat Commun 2015**)
7. The SDH mutation database offers a valuable tool and resource for clinicians involved in the treatment of patients with paraganglioma-pheochromocytoma, clinical geneticists needing an overview of current knowledge, and geneticists and other researchers needing a solid foundation for further exploration of both these tumour syndromes and SDHA-related phenotypes. (**Bayley** *et al.* **BMC Med Genet 2005**)
8. The location of mutations in the *TERT* promoter, rather than the coding region of the gene, creating additional binding sites for transcription factors, represents also a novel mechanism of genetic activation in cancer. (**Vinagre** *et al.* **Nat Commun 2013**)
9. There are in fact two things, science and opinion; the former begets knowledge, the latter ignorance. (**Hippocrates**)
10. The task of science is to stake out the limits of the knowable, and to center consciousness within them. (**Rudolf Virchow**)
11. He who is not contented with what he has, would not be contented with what he would like to have. (**Socrates**)

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