Immunohistochemical and Molecular Studies on the Pathogenesis of Pheochromocytomas and Paragangliomas

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ISBN: 978-94-6233-059-7

Cover: Gildeprint
Printed by: Gildeprint

Immunohistochemical and Molecular Studies on the Pathogenesis of Pheochromocytomas and Paragangliomas

Immunohistochemische en moleculaire studies naar de pathogenese van pheochromocytomen en paragangliomen

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof.dr. H.A.P. Pols

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
dinsdag 29 september 2015 om 15.30 uur

door

Lindsey Oudijk
geboren te Boskoop

Erasmus University Rotterdam

Promotiecommissie

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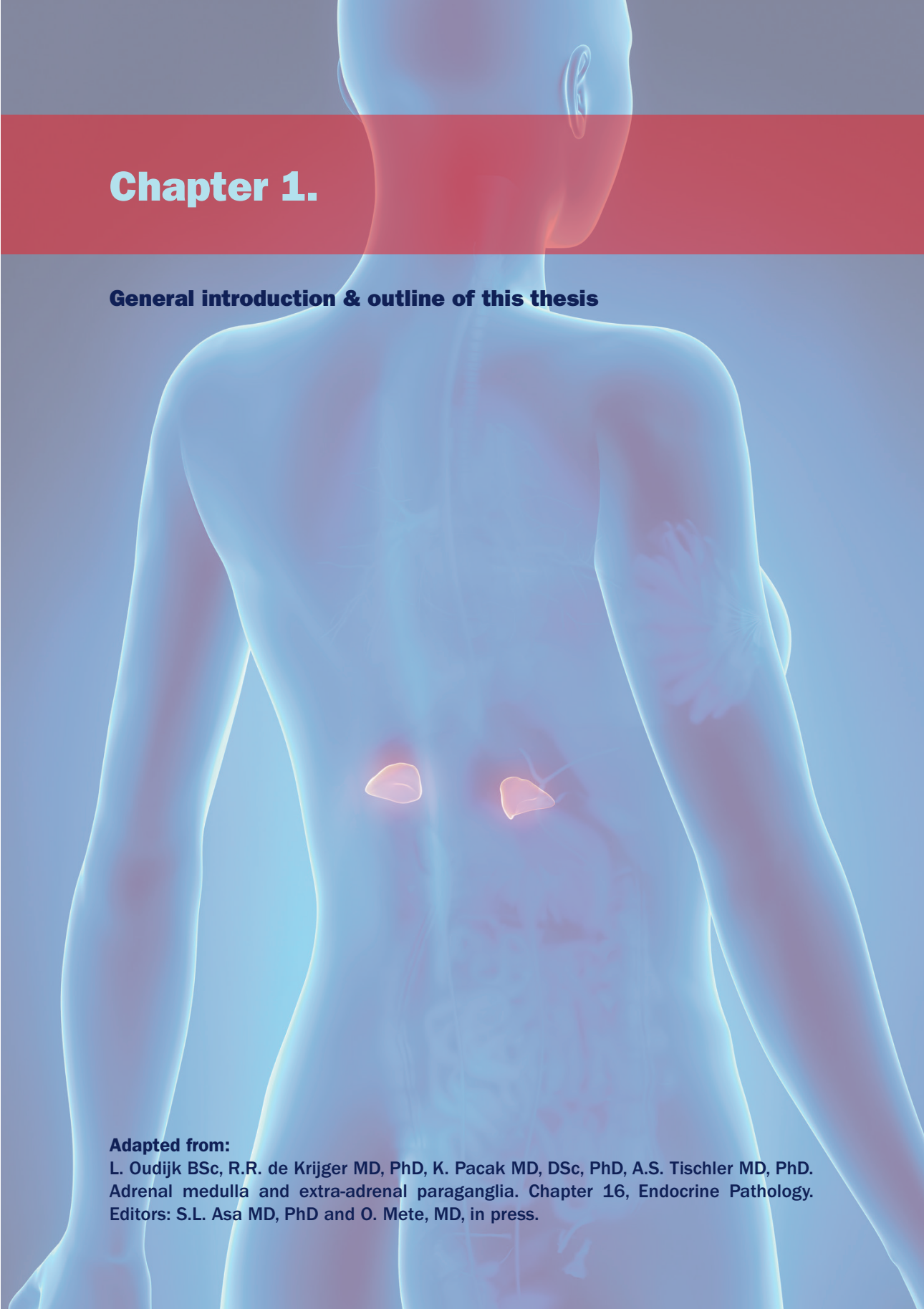
General introduction & outline of this thesis



Adapted from:
L. Oudijk BSc, R.R. de Krijger MD, PhD, K. Pacak MD, DSc, PhD, A.S. Tischler MD, PhD. Adrenal medulla and extra-adrenal paraganglia. Chapter 16, Endocrine Pathology. Editors: S.L. Asa MD, PhD and O. Mete, MD, in press.

1.1 Adrenal medulla and extra-adrenal paraganglia

The normal paraganglia

The paraganglionic system comprises the adrenal medulla together with developmentally related neuroendocrine structures associated with paraxial sympathetic nerve branches throughout the body and with vagus and glossopharyngeal nerve branches in the head and neck. By definition, the adrenal medulla is an intra-adrenal paraganglion and a member of the "sympathetic" or "sympathoadrenal" family of paraganglia (Figure 1 left). In contrast, the cranial nerve-associated paraganglia are called "parasympathetic" (Figure 1 right).

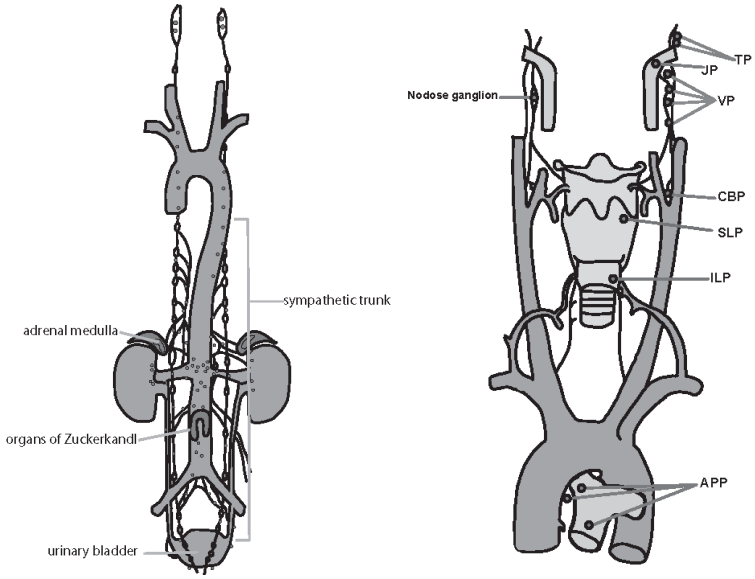


Figure 1. The distribution of the sympathoadrenal (**left**) and parasympathetic (**right**) paraganglia. Aside from the adrenal medulla, the organ of Zuckerkandl, and the carotid bodies, these structures are of microscopic size. APP = aorticopulmonary paraganglia, ILP/SLP = inferior/superior laryngeal paraganglia, CBP = carotid body paraganglion, VP = vagal paraganglia, JP = jugular paraganglion, TP = tympanic paraganglia.

Physiology

The adult adrenal medulla responds to physiological stress signals ('fight or flight' response). Pre-ganglionic sympathetic splanchnic nerve fibers that synapse on chromaffin cells cause exocytosis of catecholamine-containing secretory granules.

Extra-adrenal sympathetic paraganglia are sparsely innervated and are believed to respond mainly to chemical rather than neural stimuli, for example hypoxemia. Most chromaffin cells in the fetus are located in extra-adrenal sites, mainly in the organ of Zuckerkandl. It is thought that fetal chromaffin tissue is involved in the homeostatic maintenance of vascular tone and blood pressure in utero, and in fetal responses to hypoxia (1), with norepinephrine being the predominant catecholamine.(2,3)

Parasympathetic paraganglia are known to function as chemoreceptors. However, only the

carotid body has been extensively studied. The carotid bodies and aortic paraganglia detect low partial pressures of O_2, high CO_2 and low pH, thereby stimulating breathing.(4)

1.2 Pheochromocytomas and paragangliomas

1.2.1 Definition and epidemiology

The World Health Organization classification of endocrine tumors, last updated in 2004, defines pheochromocytoma (PCC) as a neuroendocrine tumor of chromaffin cells arising in the adrenal medulla. This implies that a PCC is an intra-adrenal sympathetic paraganglioma.(5) PCCs are rare tumors with an incidence varying between 2 and 8 per million.(6) The peak age at diagnosis is in the 4^{th}-5^{th} decade of life, but PCCs can occur at any age.(7) Five to ten percent are diagnosed in children.(8) The sex incidence is approximately equal.

Extra-adrenal sympathetic paragangliomas (sPGL) arise from paraganglia distributed along the pre-and paravertebral sympathetic chains and sympathetic nerve fibers innervating viscera including urinary bladder, gallbladder and the intrathoracic area near the base of the heart.(5) sPGL usually arise between 20-50 years of age. In paediatric patients the distribution of intra- and extra-adrenal PGL is almost equal, while in adults only about 10-20% of tumors are extra-adrenal. Sex distribution is generally equal.

Tumors that arise from paraganglia associated with branches of the vagus and glossopharyngeal nerves in the head and neck region and mediastinum are designated parasympathetic PGL (pPGL) and are named by anatomic site of origin. A wide range of terms used for these tumors in the past (e.g. chemodectoma and glomus tumor), should no longer be used.(5) Extra-adrenal pPGL are rare with an incidence of approximately one third of the PCC incidence. Their predominant location is the head and neck region. Approximately 95% of these tumors do not secrete clinically detectable quantities of catecholamines and present as mass lesions.(9) In humans living at high altitude an increase of 10 times in the incidence of carotid PGL has been reported, with a significant female predilection.(10)

Malignant PCC is defined by the 2004 WHO classification as chromaffin cell tumors with the presence of metastases to sites where chromaffin tissue normally should not be found.(5) The same definition applies to extra-adrenal PGL and it distinguishes malignancy from multifocal disease. Metastases of malignant PCC/PGL typically affect the bones, liver, lungs, kidney and lymph nodes. To date, there are no agreed upon indicators of malignancy in the primary tumor. An important obstacle to determine these indicators is the fact that metastasizing PCC/PGL is rare and has a long latency, sometimes up to 20 years.(11) Up to 10% of all PCC and >20% of abdominal PGL give rise to metastases.(12) Pediatric PCC patients have up to a 47% likelihood of metastasis.(13)

1.2.2 Clinical Features

Patients with a PCC often have symptoms and signs related to excess catecholamine production, including hypertension. The classic diagnostic triad of a PCC is severe headache, palpitations and sweating.(14) Myriad other symptoms include paleness, anxiety or other psychological disturbances, nausea, long-standing constipation or pain.(15) These symptoms can occur independently and are not specific, often complicating the correct clinical diagnosis of PCC. Acute and massive release of catecholamines can occur if tumors infarct or are manipulated during surgery (2), causing severe complications including myocardial infarction, arrhythmias, stroke and initially unexplained sudden death. Clinical laboratory testing for the presence of a PCC can be performed by measurement of metanephrines and methoxytyramine either in plasma or urine.(16)

Extra-adrenal abdominal PGL usually present as solitary tumors in adults, however up to one half of paediatric patients develop multiple PGLs. Signs and symptoms may be related to excess catecholamine secretion in up to 70% of sPGL. With the exception of cardiac PGL, pPGL usually do not produce clinically significant quantities of catecholamines and present through their mass effects, often leading to cranial nerve palsies.

1.2.3 Macroscopy

The typical PCC is a solitary, well-circumscribed, rounded intra-adrenal mass. The usual diameter is 3-5 cm, but sizes range to 10 cm or more.(3) An arbitrary cutoff of 1 cm used by some authors to distinguish a hyperplastic nodule from a PCC does not have a scientific basis. The tumors are usually not encapsulated, except by the pre-existing capsule of the adrenal gland itself. Large tumors distort the adjacent adrenal tissue, often to a point where only a thin golden rim of compressed cortex is identifiable after careful searching to confirm the adrenal location. The color of the cut surface ranges from a pale gray, identical to the normal adrenal medulla to a pink gray or tan. The absence of golden yellow distinguishes PCC from most adrenal cortical tumors. Other features that can be seen are a speckled pattern of congestion and focal haemorrhage, or dark purple diffuse hemorrhage. In tumors with larger diameters, secondary central degenerative changes can be identified, including haemorrhage, necrosis or cystic change.(15) Calcification is also sometimes seen. PCCs can extend into adjacent organs or structures such as kidney, liver and sometimes into the inferior vena cava.(3)

1.2.4 Histopathology

Most PCC broadly recapitulate the architectural pattern of the normal adrenal medulla in that the tumor cells tend to be organised in cell nests (Zellballen), trabeculae or an admixture of the two (Figure 2 left). However, there is a great range of both architectural and cytological variation. Architectural variants include large and irregular Zellballen, and diffuse or spindle cell patterns, which are usually less prominent than Zellballen.(17) Mixed

patterns are common. Tumor cell nests can vary markedly in size and shape, and sometimes central degeneration or necrosis is observed. The nests are separated by a rich vascular plexus formed by thin capillaries and/or thicker walled vessels, sometimes with perivascular edema or hyalinization. Sustentacular cells, which are difficult to discern in H&E sections, but readily identified by immunohistochemistry, are present in varying numbers. Foci of chronic inflammation and degenerative changes including fibrosis, hemorrhage, hemosiderin deposition and cystic change can be seen, particularly in large tumors.

PCC are sometimes composed of round to polygonal cells almost indistinguishable from normal chromaffin cells, with finely granular, basophilic to amphophilic cytoplasm and round-to oval nuclei with a stippled 'salt-and-pepper' chromatin pattern. However, there is a wide range of size, shape and nuclear morphology. Although nuclei are inconspicuous in normal chromaffin cells, some PCC have vesicular nuclei with very prominent nucleoli (Figure 2 right). Intranuclear pseudoinclusions are sometimes present. Marked pleomorphism and hyperchromasia may be present. Tumor cell borders may be sharply defined or indistinct. Occasionally, a feature called 'cell embracing' is seen, in which tumor cells partially envelop each other.(3) Both intracytoplasmic and extracellular PAS-positive hyaline globules are often present.

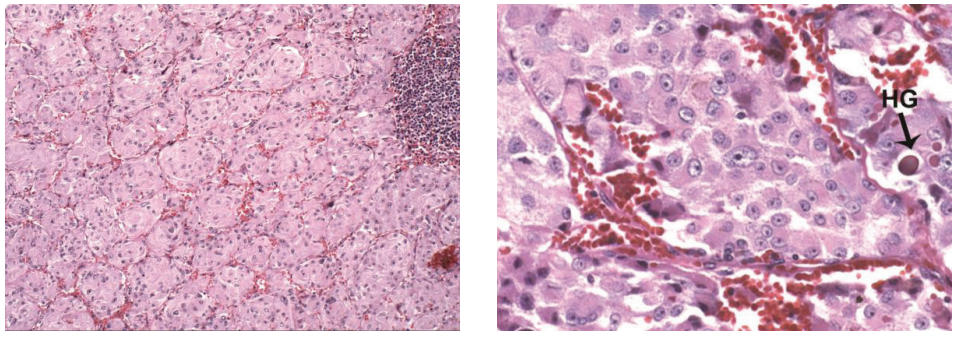


Figure 2. Left: The classic architectural pattern of PCC, showing relatively uniform nests of cells (Zellballen) separated by thin capillaries. A focus of lymphocytes is present at top right. **Right**: PCC cells with prominent nucleoli and amphophilic cytoplasm. HG = hyaline globule.

No single histologic feature is able to predict metastatic potential of PCC/PGL. However, many histologic features and combinations of them have been identified inconsistently in primary tumors that give rise to metastases, including: capsular or vascular invasion, extension into the periadrenal adipose tissue, large and confluent cell nests, necrosis (Figure 3), extreme cytological atypia (Figure 3), nuclear hyperchromasia (Figure 3) diffuse growth, increased cellularity or cellular monotony, tumor spindle cells, increased or atypical mitotic figures and absence of hyaline globules.(5) Many studies have been based on histopathologic criteria to prospectively identify malignant PCC, and multifactorial scoring systems have been proposed to achieve that goal.

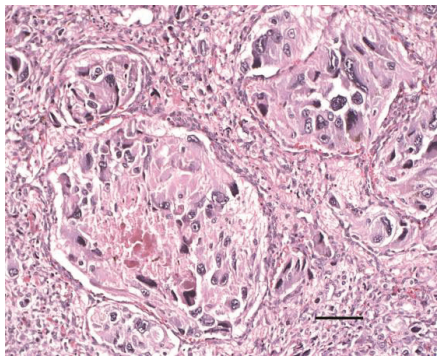


Figure 3. PCC showing giant irregular Zellballen, necrosis, extreme nuclear atypia and nuclear hyperchromasia. Bar = 100 um.

In 1990, Linnoila et al. examined 120 sympathetic PGL and PCC and developed a statistical model to predict malignant behavior. According to their model >70% of the tumors could be classified correctly with a better than 95% probability based on 4 features: extra-adrenal location, coarse nodularity, confluent necrosis and absence of hyaline globules. The majority of malignant tumors had 2 or 3 of those features, while 89% of benign tumors had none or one.(17) Extra-adrenal location was demonstrated as the most powerful predictor in this study.

In 2002, Thompson developed a Pheochromocytoma of the Adrenal Gland Scaled Score (PASS) to separate benign from malignant PCC by histopathological parameters.(18) Fifty malignant and fifty benign PCC were scored for the following features and values (in parentheses): large cell nests (≥ three times the size of Zellballen in a normal paraganglion) or diffuse growth (2), central or confluent tumor necrosis (2), high cellularity (2), cellular monotony (2), tumor cell spindling (2), mitotic figures more than 3 per 10 HPF (2), atypical mitotic figures (2), extension into adipose tissue (2), vascular invasion (1), capsular invasion (1), profound nuclear pleomorphism (1) and nuclear hyperchromasia (1). Vascular invasion is defined by the presence of tumor tissue attached to the wall and/or covered by endothelium in vessels outside the tumor mass or in capsular vessels. Tumor cells in intratumoral vessels do not comprise vascular invasion.(15) All metastatic tumors had a PASS score >4, but 17 of 50 tumors with a score >4 had not metastasized in a follow-up period of 5 years.(18) In a subsequent study by Wu et al. in 2009, five endocrine pathologists investigated the applicability of the PASS and it showed a large inter- and intra-observer variation.(19) Nonetheless, some investigators have found the PASS to be helpful (20) and others not. Importantly, the individual significance of two criteria, necrosis and invasion, has been inconsistent between studies, suggesting that individual parameters might have different predictive values in tumors with different genetic backgrounds.(11)

In 2005, Kimura et al. developed a scoring scale for both PCC and extra-adrenal sympathetic PGL according to histological pattern, cellularity, coagulation necrosis, vascular/capsular

invasion, Ki-67 immunoreactivity and types of catecholamine produced.(21) The tumors were subsequently classified as well, moderately and poorly differentiated types (WD, MD and PD, respectively). Differences between the groups correlated with both metastatic potential and patient survival. However, there is still a considerable proportion of "WD tumors" that metastasize, limiting the practical usefulness of this scoring scale. In 2014, a nationwide survey was performed, using the previously described grading system, which was named the grading system for adrenal pheochromocytoma and paraganlioma (GAPP).(22) The GAPP classification was able to differentiate low-grade malignancies from moderate to high-grade malignancies with different rates of metastases. Combined use of GAPP and SDHB IHC might be useful to predict tumor metastasis and patient prognosis.

1.2.5 Ancillary tools

Immunohistochemistry

Immunostaining for chromogranin A (CgA), distinguishes PCCs from non-neuroendocrine tumors and tumors arising from the adrenal cortex.(23) Staining of PCC for CgA is typically strong and diffuse, such that the diagnosis of PCC should be reconsidered if staining is negative. Synaptophysin is also present but should not be used to distinguish cortical tumors from PCC because immunoreactivity for synaptophysin can be seen in both normal and neoplastic cortical cells.(24) Positive staining for tyrosine hydroxylase (TH) identifies cells that are able to produce catecholamines and can therefore discriminate PCCs from other neuroendocrine tumors metastatic to the adrenal gland.(25) Also useful for that purpose is the fact that PCCs are typically negative for keratins, which are often expressed in pulmonary and gastrointestinal neuroendocrine tumors. The neoplastic chromaffin cells in PCC also express numerous other neuroendocrine markers, including both eutopic and ectopic regulatory peptides. S100 immunohistochemistry will demonstrate sustentacular cells (26), (Figure 4) which can show marked intertumoral and intratumoral heterogeneity in number and distribution.

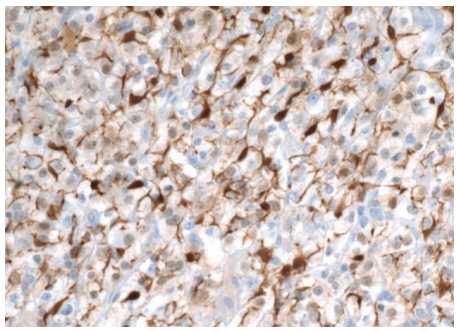


Figure 4. IHC stain for S-100 in a PCC from a patient with neurofibromatosis, showing numerous sustentacular cells at the periphery of Zellballen and interdigitating between tumor cells.

In addition to its use in differential diagnosis, immunohistochemistry now plays an important role as a guide to genetic testing of patients with PCC. SDHB and SDHA are important immunohistochemical markers for that purpose. SDHB protein expression is lost in PCCs with mutations in any of the genes coding for subunits of the succinate dehydrogenase complex: *SDHA*, *SDHB*, *SDHC* or *SDHD*. Immunostaining for SDHA is only negative when there is a mutation in the *SDHA* gene.(27,28) Endothelial cells serve as intrinsic positive controls for both SDHB and SDHA immunohistochemistry, showing granular cytoplasmic staining (Figure 5). Immunohistochemistry might also prove to be useful in screening for *MAX* gene mutations. PCCs with truncating *MAX* mutations are immunohistochemically negative with a MAX C-terminus specific antibody. Nuclear staining of stromal cells serves as an internal positive control.(29)

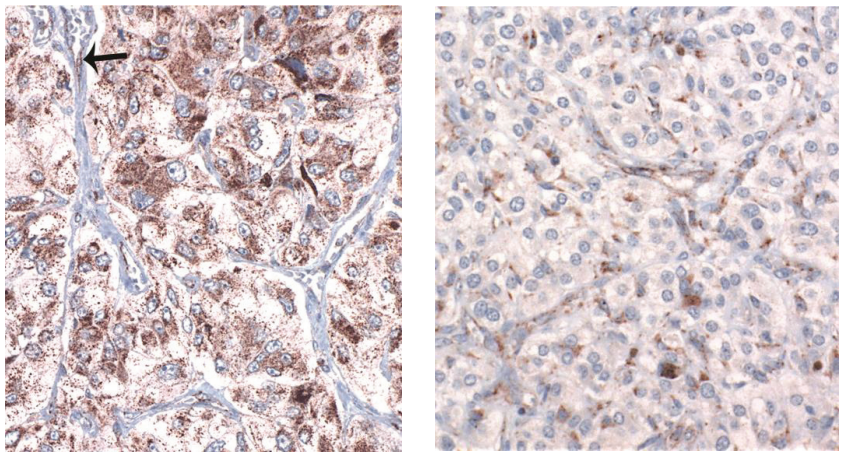


Figure 5. Left: SDHB immunohistochemical stain of a tumor with a wild-type *SDHB* gene. Granular cytoplasmic staining corresponding to the mitochondrial location of the protein is present in the tumor cells and in endothelial cells (arrow), which serve as intrinsic controls. **Right:** SDHB immunohistochemical stain of an *SDHB*-mutated tumor. Immunoreactivity for the protein is present in endothelial cells, which serve as intrinsic controls. Tumor cells are negative.

Molecular

From a molecular standpoint, PCCs with different underlying genotypes roughly segregate into two clusters by gene expression profiling, with characteristic differences in their transcriptomes, signaling pathways and expression of biomarkers. *VHL* and *SDHx* mutations are associated with “pseudo-hypoxic” signalling (‘cluster 1’), while tumors with *RET*, *NF1*, *TMEM127* and *MAX* mutations are associated with alterations of RAS signalling, protein trafficking and a variety of other cell functions (‘cluster 2’).(30,31) Distinct subclusters are identifiable within both cluster 1 and cluster 2.(32) Sporadic tumors or tumors with mutations in one of the other susceptibility genes often cluster in either of the above clusters.(33) These would include the recently discovered somatic mutations of *HRAS* (31)

and *HIF2A* (34).

1.2.6 Genotype-Phenotype and clinicopathological correlations

Major hereditary disorders encompassing PCCs/PGLs in their phenotype are MEN type 2A and 2B, VHL, NF1, familial pheochromocytoma-paraganglioma syndromes type 1-4 (PGL1-4), and Carney-Stratakis syndrome. The associated mutations of the above mentioned hereditary disorders are *RET*, *VHL*, *NF1* and *SDHB*, *SDHAF2*, *SDHC*, *SDHD,* respectively. VHL is divided into types 1 and 2, defined respectively by absence or presence of susceptibility to PCCs and PGLs.(5,35) Some remarkable genotype-phenotype correlations exist for tumors in each of the familial syndromes, as discussed in further detail in the proceeding sections.

Multiple endocrine neoplasia type 2 (MEN2)

The MEN2 syndrome has an autosomal dominant pattern of inheritance caused by missense mutations in the *RET* (Rearranged during Transfection) proto-oncogene located on chromosome 10q11. The estimated prevalence is 2.5 per 100,000.(36) MEN2 syndrome has been subdivided into three clinically distinct varieties: MEN2A, MEN2B and familial medullary thyroid carcinoma (FMTC). MEN2 patients or families show a high penetrance of medullary thyroid carcinoma (MTC) (90%) and bilateral PCCs (50%). Only the most common subtype MEN2A can present with hyperparathyroidism.(37) According to the definition, FMTC patients develop MTC only.(36) PCCs in MEN2 patients are mostly multifocal and bilateral and located in the adrenal medulla.(38) In addition, these PCC typically have a low risk of malignancy (39) and produce both norepinephrine and epinephrine. The majority of MEN2 patients have bilateral diffuse and/or nodular adrenal medullary hyperplasia, representing a precursor of PCC.(5)

Von Hippel-Lindau disease type 2 (VHL)

VHL is an autosomal dominant disorder caused by heterozygous germline mutations in the *VHL* tumor suppressor gene located on chromosome 3p25-26. The incidence of VHL disease is 1 in 36,000 live births.(2) The tumor syndrome occurs when the wild-type *VHL* allele is inactivated, and is characterized by multiple benign and malignant tumors, including cerebellar hemangioblastomas, retinal angiomas, renal clear cell carcinomas and cysts, PCCs, pancreatic serous tumors and cysts and epididymal tumors. VHL disease is subdivided into type 1 (no/rare PCC) and type 2 (with PCC). In 10-26% of VHL type 2 patients a PCC develops with a mean age at diagnosis of 30 years.(2,13) In 40-80% of these patients, the PCCs occur bilaterally, and occasionally are multifocal with abdominal or thoracic PGLs.(40) Of note, 40% of PCC in pediatric patients are caused by *VHL* mutations.(41) PCC in VHL patients typically do not produce epinephrine, but mainly norepinephrine due to the lack of phenylethanolamine N-methyltransferase (PNMT), the enzyme that converts norepinephrine to epinephrine.(2) *VHL*-mutated PCC have been reported to display distinctive microscopic

features consisting of a thick vascular capsule, myxoid and hyalinized stroma, small cells with intermixed small vessels, clear amphophilic cytoplasm, and absence of hyaline globules, nuclear atypia or mitosis.(42)

Neurofibromatis type 1 (NF1)

NF1 or von Recklinghausen's disease is an autosomal dominant disorder, caused by mutations in the *NF1* tumor suppressor gene located on chromosome 17q11.2. However, 30-50% of patients have sporadic *NF1* mutations.(39) The *NF1* gene encodes the neurofibromin 1 protein, which is a negative regulator of the Ras intracellular signalling pathway. The syndrome is frequent, affecting approximately 1 in 3,000 individuals and is characterized by multiple neurofibromas, café-au-lait spots, axillary or inguinal freckling, Lisch nodules of the iris, osseous lesions, optic nerve gliomas, peripheral nerve sheath tumors, gastrointestinal stromal tumors, pancreatic endocrine tumors, leukemia and PCCs.(13,33) However, NF1 is a rare cause of PCC and only 0.1-5.7% of NF1 patients develop these tumors. Diagnosis of an *NF1*-mutated PCC is based on clinical parameters, instead of routine genetic screening, as the latter is rather difficult due to the large number of exons in the *NF1* gene (>50) and the co-occurrence of many pseudogenes. Chromaffin cell tumors occurring in NF1-patients are usually located in the adrenal and typically produce both epinephrine and norepinephrine.(40) Less than 25% of NF1 patients present with bilateral PCC and PCC are diagnosed at a relatively late mean age of 42 years.(39) NF1-associated head and neck PGLs have been reported, but are extremely rare.(43)

Familial pheochromocytoma-paraganglioma syndromes (PCC-PGL syndrome)

Familial PCC-PGL syndromes are caused by germline mutations in *SDHD* (PGL1), *SDHAF2* (PGL2), *SDHC* (PGL3) and *SDHB* (PGL4). All syndromes are inherited in an autosomal dominant pattern, but with varying penetrance. The prevalence of PCC-PGL syndrome is unknown, but PGL1 and PGL4 represent the majority of cases. *SDHB* and *SDHD* mutations are also associated with renal cell carcinoma.(44) Moreover, *SDHB*- and *SDHD*-mutated patients with thyroid carcinoma have been described, although the link between *SDHB* gene mutation and thyroid carcinoma is unproven at present. Mutations in *SDHB*, *SDHC* and *SDHD* can also give rise to Carney-Stratakis syndrome, the familial dyad of PGL and gastrointestinal stromal tumors (GIST). Strikingly, *SDHA* mutations have recently been reported in GIST, but these *SDHA*-mutated patients did not display PGL.(39)

Carney triad

Carney triad was originally described in 1977 and includes PGLs, gastrointestinal stromal tumors (GIST) and pulmonary chondromas.(45) Other tumors such as PCCs, esophageal leiomyomas and adrenocortical adenomas were also shown to be associated with the syndrome.(46) Carney triad is extremely rare and mainly affects young women. About 20%

of the patients have all three tumor types, the remaining have two of the three tumors, usually GIST and pulmonary chondroma. GIST is the most common presenting tumor (75%), followed by the pulmonary chondroma (15%) and paraganglioma (10%).(47) Up to now, no specific germline genetic abnormalities have been detected in Carney triad patients. However, epigenetic inactivation by DNA hypermethylation at the promotor region of *SDHC* was reported in 4 patients with Carney triad. The consequent reduced mRNA and protein levels of SDHC with functional impairment of the SDH complex, is a plausible cause for tumorigenesis.(48) In a study of 37 Carney triad patients with PCC/PGL, sympathetic and parasympathetic PGL were identified in 92% of the cases and 16% displayed a PCC. Multiple PGL were identified in 22% of the patients and bilateral PCC in 3%.(46)

Carney-Stratakis syndrome

Carney-Stratakis syndrome (also known as Carney-Stratakis dyad) is the familial dyad of PGL and GIST. In contrast to Carney triad, the majority of patients with Carney-Stratakis syndrome carry germline mutations in *SDHB*, *SDHC* or *SDHD*. The syndrome is equally common in men and women with an average age at diagnosis of 23 years. GISTs in Carney-Stratakis syndrome patients are multifocal and PGL are multicentric. Carney and Stratakis reported on 11 patients with Carney-Stratakis syndrome-related PCC/PGL and found that all patients had PGL and one patient also had a PCC. Mean age at diagnosis was 33 years. Multiple PGL were seen in 8 of 11 patients, both sympathetic and parasympathetic.(49)

Remarkable genotype-phenotype correlations exist for PCC/PGL with mutations in one of the components of the SDH complex, i.e. *SDHD*, *SDHC*, *SDHB*, *SDHAF2,* and *SDHA*.

SDHD

The *SDHD* gene, located on chromosome 11q23, is a nuclear gene encoding an anchoring subunit of the mitochondrial enzyme succinate dehydrogenase (SDH). In 2000, Baysal et al. discovered *SDHD* as a cause of hereditary PGL syndrome type 1 (PGL1).(50) Mutations in *SDHD* predispose most frequently to benign head and neck PGL and also to sympathetic extra-adrenal PGL and PCC. The penetrance of tumor development is high (86%). However, *SDHD* is maternally imprinted, so tumor development only occurs after paternal transmission of the mutated gene.(51) *SDHD*-mutated patients often develop multiple PGL and mean age at diagnosis is 35 years. Bilateral PCC are extremely rare.(39)

SDHC

SDHC, located on chromosome 1q23, encodes another SDH anchoring subunit and was found to be causative for PGL syndrome type 3 (PGL3) in 2000.(52) Mutations in *SDHC* are rare and mainly associated with benign head and neck PGL, but have also been described in extra-adrenal PGL and PCC.(51) Mean age at diagnosis is 43 years.(39)

SDHB

SDHB is located on chromosome 1p35-36.1, encodes the catalytic iron-sulfur SDH subunit and was linked to PGL syndrome type 4 (PGL4) in 2001.(53) PGL4 is mainly characterized by extra-adrenal sympathetic PGL (52-84%), while head and neck PGL (27-31%) and PCC (18-28%) are less frequently found. The mean age at diagnosis is 33 years and the penetrance of PCC/PGL is relatively low (25-40%).(51) *SDHB* mutations are associated with higher morbidity and mortality compared to mutations in the other *SDHx* genes.(39) Up to 50% of patients with a metastasized PCC or PGL have an *SDHB* mutation.(54)

SDHAF2

SDHAF2, located on chromosome 11q13, encodes a co-factor involved in the correct flavination of SDHA and function of the SDH complex. The locus of this gene associated with PGL syndrome type 2 was already known for more than 10 years (i.e. PGL2 locus) before the specific gene was identified in 2009.(55) Only one pathogenic *SDHAF2* mutation has been found to date, the p.Gly78Arg missense mutation in benign head and neck PGLs in a Dutch and an unrelated Spanish family.(56) Most patients displayed multiple head and neck PGLs. No *SDHAF2*-mutated extra-adrenal PGL or PCC have been reported. Similar to *SDHD*-mutation carriers, *SDHAF2* is maternally imprinted and disease only develops after paternal transmission.(51)

SDHA

SDHA, located on chromosome 5p15, encodes the main catalytic SDH subunit. Homozygous recessive *SDHA* mutations cause Leigh syndrome resulting in encephalopathy, myopathy, developmental retardation and loss of vision and hearing.(57) In 2010, the first association between SDHA and PGL development was identified.(58) Burnichon et al. identified a heterozygous *SDHA* germline mutation together with loss of the wild-type *SDHA* allele in an abdominal PGL. However, *SDHA* mutations in PCC/PGL are extremely rare and have only been described in few cases to date (1 PCC, 3 sPGL and 2 pPGL). The mean age at diagnosis was 40 years and no metastatic or multifocal tumors were identified.(39) These findings suggest that *SDHA* mutations display a reduced penetrance. Recently, the association of a germline *SDHA* mutation and a carotid body PGL in a proband and a pituitary adenoma in her son was described.(59)

In addition, germline mutations in other genes than the *SDHx* genes have been identified in PCC/PGL patients. These genes comprise *TMEM127*, *MAX*, *KIF1B*, *PHD2*, *HIF2A, FH, PHD1*, and *MDH2* will be discussed more in depth now.

TMEM127

TMEM127, located on chromosome 2q11, encodes a transmembrane protein that seems to

function as a negative regulator of the mTOR pathway. In 2010, mutations in this gene were identified in familial and sporadic PCC.(60) Later, mutations were also identified in one patient with multiple head and neck PGL and one patient with a retroperitoneal extra-adrenal PGL and PCC.(61) The inheritance pattern of the *TMEM127* PCC-PGL syndrome is autosomal dominant and the gene acts as a tumor suppressor with tumors displaying LOH of the wild-type *TMEM127* allele. The prevalence of *TMEM127*-mutated PCCs and PGLs seems low, with only 23 reported patients to date. Clinically, the patients are characterized by PCC, frequently bilateral (39%) and a relatively old mean age at diagnosis (43 years) compared to other syndromic PCC/PGL.(39,60,62)

MAX

MYC-associated factor X (*MAX*), located on chromosome 14q23, is a transcription factor that belongs to the basic helix-loop-helix leucine zipper family. In 2011, exome sequencing identified mutations in *MAX* in hereditary PCCs.(29) A paternal transmission of the disease similar to *SDHD* and *SDHAF2* has been suggested. In a recent study by Burnichon et al. the *MAX* gene was sequenced in 1694 PCC/PGL patients and *MAX* germline mutations were found in 1.12% of PCC and sPGL. In addition, this study showed that *MAX*-mutated tumors mainly produce norepinephrine and the median age at diagnosis was 34 years.(63) Of note, 4 sporadic cases with somatic *MAX* mutations were identified as well among the 1694 analyzed cases, with a median age at diagnosis of 47.5 years.

KIF1B

Kinesin family member 1B (*KIF1B*), located on chromosome 1p36, functions as a tumor suppressor that is necessary for neuronal apoptosis.(64) The gene has two splice variants, *KIF1Bα* and *KIF1Bβ*. Schlisio et al. (2008) identified *KIF1Bβ* missense mutations in two PCC patients. The first patient presented with a neuroblastoma at 17 months of age and developed a mature ganglioneuroma and bilateral PCCs at age 22. The patient's paternal grandfather also developed bilateral PCCs at 70 years of age. Germline *KIF1B* mutations have been identified in multiple tumors, including neuroblastoma, ganglioneuroma, leiomyosarcoma and lung adenocarcinoma.(64,65) Transcription analysis revealed that *KIF1B*-mutated PCC cluster with *NF1*- and *RET*-mutated tumors and not with *VHL*- and *SDHx*-mutated tumors.(65)

PHD2/EGLN1

In 2008, a germline mutation in *PHD2* (or *EGLN1*), located on chromosome 1q42, was reported in a 43-year-old female patient with erythrocytosis and recurrent abdominal extra-adrenal PGL.(66) *PHD2* encodes HIF-prolyl hydroxylase 2, an enzyme involved in the degradation of hypoxia-inducible factor alpha (HIF-α). Germline mutations in *PHD2* had previously been reported in patients with erythrocytosis, but had not been associated with

tumors. In 2014, a second patient with a germline *PHD2* mutaton (c.682G>T, p.Ala228Ser) was reported.(67) This patient was diagnosed with polycythemia at age 16, with a left adrenal PCC and 2 PGLs near the left renal artery at age 39 and with a right adrenal PCC and 3 periaortic PGLs at age 60.

HIF2A

In 2012, 2 patients with somatic gain-of-function mutations in hypoxia-inducible factor 2α (*HIF2A*) with multiple PGL, somatostationomas and polycythemia were reported by Zhuang et al.(34) Subsequently, Favier et al. identified a heterozygous *HIF2A* mutation in a PCC from a 24-year-old woman, but without polycythemia, and this was recently confirmed in a large study of PCC and PGL.(68,69) Zhuang et al. and others found that HIF-2α is stabilized in *HIF2A*-mutated PCC/PGL patients leading to increased expression of hypoxia-inducible genes. This 'pseudohypoxic' status seems to be mild compared to that observed in *SDHx*- and *VHL*-related tumors, but more extensive studies are needed.(34,69) Almost concomitantly with this first report on somatic *HIF2A* mutations in 2 PGL patients, a male patient with a germline *HIF2A* mutation with PGL and congenital polycythemia was reported.(70) Subsequently, Pacak et al. introduced the existence of a new hereditary cancer syndrome of PGL and somatostatinomas associated with polycythemia in 4 unrelated female patients.(71)

FH

Inactivation of the tricarboxylic acid cycle component fumarate hydratase (*FH*) has been associated with abnormalities of the cellular activation of hypoxic gene response pathways and DNA methylation. Germline *FH* mutations are classically associated with hereditary cutaneous and uterine leiomyomatosis and renal cell carcinoma.(72) However, in 2013 a 63-year-old female patient with a metastatic PCC and a germline *FH*-mutation was reported.(73) After this first report, four additional cases of predisposition to PCC/PGL caused by *FH* mutations were reported.(74) This study revealed a new role for *FH* in susceptibility to malignant and/or multiple PCC/PGL. Another study recently extended the clinical phenotype associated with *FH* mutations to pediatric PCC.(75) *FH*-mutated PCC and PGL display the same epigenetic (hypermethylated) changes as *SDHx*-related tumors.(74)

PHD1/EGLN2

In 2014, a novel germline *PHD1* (*EGLN2*) mutation causing PCC/PGL and polycythemia was described in a female patient. She presented with a left adrenal PCC at age 14, and later with recurrent PCCs along with metastatic PCC/PGL in a thoracic periaortic lymph node. At age 48 she had multiple and metastatic PCC/PGLs, including 2 lesions found in the right adrenal, one lesion in the aortocaval region, one in the right pelvic area and an urinary bladder tumor confirmed as PGL.(67)

MDH2

In 2015, *MDH2* was identified as a new PCC/PGL susceptibility gene, encoding the mitochondrial malate dehydrogenase enzyme involved in the Krebs cycle. Whole-exome sequencing on tumor DNA from a man age 55 years with multiple malignant PGLs revealed a heterozygous variant, c.429+1G>T of *MDH2*, and the mutation was confirmed with Sanger sequencing (76). A truncating mutation in *MDH2* had only been recorded in neuroblastoma, another neural crest-derive tumor primarily originating in the adrenal gland.

1.3 Aims and outline of this thesis

In the last decades major progress has been made in discovering genes implicated in the syndromic occurrence of PCC and PGL. It's now well established that about 35% of all PCC/PGL are due to germline mutations in one of the genes: *RET*, *VHL*, *NF1*, *SDHA, SDHB, SDHC, SDHD, SDHAF2, TMEM127, MAX, PHD2, HIF2A, KIF1B,* and *FH*. In addition, somatic mutations in *RET*, *VHL*, *NF1*, and *HIF2A* can also be detected in a subset of sporadic PCC/PGL. However, the pathogenesis of sporadic PCC and PGL is currently poorly understood. This issue has been investigated in the first part of this thesis (Chapters 2 and 3) by a candidate gene approach.

SDHB and SDHA immunohistochemistry (IHC) is a valuable tool to identify PCC/PGL patients with mutations in one of the succinate dehydrogenase (*SDHx*) genes. In the second part of this thesis we validated the reproducibility of this assessment method. Moreover, we determined if SDHA IHC could be a valuable tool to guide genetic testing in another tumor type from the *SDHx*-associated tumor spectrum. In addition, we searched for new tools to validate *SDHx* mutations.

A major problem in PCC management remains the lack of predictive markers for malignancy and the lack of curative treatment options for progressive disease. In the last part of this thesis we focused on activation of intracellular pathways that could be targets for therapy and on validation of a prognostic tool for the distinction between benign and malignant PCC.

The aims of this thesis, based on the above-mentioned issues, are:

- To identify new susceptibility genes implicated in the pathogenesis of sporadic PCC/PGL.
- To validate existing and search for new tools to guide genetic testing in patients with *SDHx*-related PCC-PGL syndromes.
- To search for molecular pathways and prognostic tools that can serve as target for therapy in malignant PCC/PGL or that can distinguish benign and malignant PCC/PGL.

Outline

Chapters 2 and 3 focus on two new genes that might play a role in the pathogenesis of sporadic and syndrome-related, malignant and benign PCC. Crona et al identified somatic *HRAS* mutations by exome sequencing in approximately 7% of sporadic PCC/PGL.(31) In **chapter 2** we explored the prevalence of *HRAS* mutations in a large series of PCC and PGL and compared genotype with clinical and pathological characteristics. Hotspot mutations in the promotor of the *TERT* gene have been recently reported in human cancers. In **chapter 3** we explored *TERT* promotor mutations in tumors originating from the adrenal gland and extra-adrenal paraganglia, including PCC and PGL.

SDHA and SDHB IHC are valuable screening methods for the detection of *SDHx*-related PCC and PGL, that can be performed in a routine pathology laboratory. In **chapter 4** we investigated the reproducibility of SDHA and SDHB IHC by assessing the interobserver variability among seven expert endocrine pathologists in a large set of genetically well-characterized PCC/PGL.

In **chapter 5** we investigated if SDHA IHC can also identify *SDHA* mutations in gastrointestinal stromal tumors (GISTs), a tumor type included in the *SDHx*-associated tumor spectrum. SDHB immunostaining is negative in all tumors with a mutation in any of the *SDHx* genes, while it is positive in non-*SDHx*-related tumors. SDHA immunostaining is specifically negative in *SDHA*-mutated tumors. To address whether a same procedure could be applicable to detect patients with germline *SDHD* mutations, we evaluated the efficacy of SDHD IHC in **chapter 6**.

The last part of this thesis is devoted to the identification of markers that could serve as therapeutic targets in PCC/PGL. In **chapter 7** we investigated the immunohistochemical expression of stem cell-associated markers and correlated their expression with genotype and tumor behaviour. In **chapter 8** we explored the expression levels of a variety of mTOR pathway-related proteins in a large set of sporadic and hereditary PCC/PGL, in comparison with clinical and pathological features.

Favier et al. described the use of vascular architecture for the distinction between benign and malignant PCC/PGL (77). In **chapter 9** we validated the use of vascular pattern analysis as a predictive tool for malignancy in a large series of primary PCC/PGL.

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Chapter 2.

H-RAS mutations are restricted to sporadic pheochromocytomas lacking specific clinical or pathological features: data from a multi-institutional series

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J Clin Endocrinol Metab. 2014 Jul;99(7):E1376-80.

Abstract

Context: Somatic or germline mutations in up to 15 disease-causative genes are detectable in up to 50% of pheochromocytoma (PCC) and paraganglioma (PGL) patients. Very recently, somatic *H-RAS* mutations were identified by exome sequencing in around 7% of sporadic PCCs and PGLs, in association with male gender and benign behavior.

Objective: To explore the prevalence of *RAS* mutations in a large cohort of 271 PCC and PGL from a European registry and to compare the genotype with clinical and pathological characteristics of potential clinical interest.

Design: Genetic screening for hotspot mutations in *H-*, *N-* and *K-RAS* genes was performed by means of Sanger sequencing or pyrosequencing methods on tumor DNA in a series of patients with (n=107) or without (n=164) germline or somatic PCC/PGL-related gene mutations.

Results: Overall, *H-RAS* mutations were detected in 5.2% (14/271) of cases which were confined to sporadic PCCs resulting in a prevalence of 10% (14/140) in this cohort. In contrast, no mutations were found in PCC with PCC/PGL-related gene mutations (0/76) or in PGL (0/55) harbouring or not mutations in PCC/PGL susceptibility genes. In this large series, *H-RAS* mutations in PCCs lacked any significant correlation with pathological or basic clinical endpoints.

Conclusions: Somatic *H-RAS* mutations are restricted to a relevant proportion of sporadic PCC. These findings provide the basis to study potential *H-RAS* dependent correlations with long-term outcome data.

Introduction

Pheochromocytoma (PCC) and paraganglioma (PGL) are neural crest-derived tumors, arising from chromaffin cells of the adrenal medulla or from extra-adrenal paraganglia.(1) Approximately 50% of all PCC/PGL patients harbor mutations, either somatic or germline, in one of the 15 disease-causative genes: *SDHA*, *SDHB*, *SDHC*, *SDHD*, *SDHAF2*, *RET*, *VHL*, *NF1*, *MAX*, *TMEM127*, *HIF2A*, *FH*, *KIF1B*, *PHD2*, *IDH*.(2-9) Despite this genetic heterogeneity, the tumors can be divided into 2 groups based on transcription profiling studies. Cluster 1 includes tumors with mutations in *VHL*, *HIF2A* and *SDHx* (*SDHA*, *SDHB*, *SDHC*, *SDHD* and *SDHAF2*) and displays a pseudohypoxic signature; cluster 2 represents tumors with mutations in *RET*, *NF1*, *TMEM127* and *MAX* and displays an activation of kinase signaling pathways (PI3K/AKT/mTOR and RAS/RAF/ERK). Sporadic PCC and PGL can cluster within both groups.(8,10-12)

Recently, somatic *H-RAS* mutations in sporadic PCCs and PGLs were identified by exome sequencing, showing a frequency of 6.9% (13), following a previous report by Yoshimoto and coworkers.(14) These tumors displayed activation of the RAS/RAF/ERK signaling pathway and were associated with male predominance and clinically benign behavior.

RAS is a family of related proteins consisting of H-RAS, K-RAS and N-RAS, which are small GTPases involved in cell growth, proliferation and survival. *RAS* genes belong to the most common mutated genes in human cancer with oncogenic mutation hotspots found in codons 12, 13 and 61. While RAS GTPase signaling is self-limiting due to its intrinsic ability to exchange GTP with GDP mutant RAS protein is defective for this GTP hydrolysis and remains constitutively active.(15) RAS signaling activates the RAF/ERK and PI3K/AKT/mTOR signaling pathways, similar to what has been described in cluster 2 PCCs/PGLs.

In the present multi-institutional study, we aimed to establish the prevalence of *H-RAS* mutations in a cohort of PCCs and PGLs already genotyped for the main PCC/PGL susceptibility genes, and correlated the presence of mutations with major clinical and pathological parameters.

Materials and Methods

Patients selection and characteristics

A series of 271 samples was collected from different institutions participating in the ENS@T network (European Network for the Study of Adrenal Tumors, www.ensat.org), and included 97 cases from Italy, 39 cases from Spain, 126 from the Netherlands and 9 cases from Germany. All cases were genetically characterized for the presence of germline mutations in the *VHL*, *RET*, *NF1*, *MAX*, *SDHAF2*, *SDHA*, *SDHB*, *SDHC*, *SDHD* on either blood or tissue samples, and for the presence of somatic mutations in *VHL*, *RET*, *EPAS1,* and *MAX* on tumor tissue samples when screening for germline mutations was negative. Genetic screening was

performed independently in the enrolling centres as clinical routine work, and methodological conditions are available from the authors upon request. Among this series, 107 cases, 76 PCCs and 31 PGLs, harbored somatic or germline mutations in one of the susceptibility genes mentioned above (Supplemental Figure 1). The residual 140 PCCs and 24 PGLs lacked germline or somatic mutations in the main PCC and PGL susceptibility genes. All except three patients were diagnosed with a single tumor; none of them had family history of the disease. Institutional review board approval was obtained for the study by each of the centers, and informed consent was obtained from all patients.

Clinical variables collected for this study were: gender, age, number of PCC/PGLs, tumor location, tumor size, necrosis, capsular and vascular invasion, mitotic index, as well as presence of metastatic disease. Malignancy was defined as the presence of metastases where chromaffin cells are normally absent. These clinical variables were collected electronically into preformatted forms provided to all contributors, and statistically analyzed in a single center.

DNA extraction and RAS gene mutations

DNA was extracted from either fresh frozen or formalin-fixed, paraffin-embedded specimens, using standard protocols. Sections of each sample were evaluated by a pathologist, and contained at least 80% of tumor cells. All cases, including the series of tumors without known mutations (#164) and those with already known gene mutations (#107) were screened for hotspot mutations in *H-*, *N-* and *K-RAS* genes (exons 2 and 3 for *N-* and *K-* and exons 2, 3 and 4 for *H-RAS*) in three centers (Madrid, Rotterdam and Turin). Either Sanger sequencing (Madrid and Rotterdam) or pyrosequencing (Turin) methods were employed. For direct sequencing, amplified DNA was purified and directly sequenced using an automatic sequencer ABI PRISM TM 3700 (Applied Biosystems. Perkin Elmer, USA). Pyrosequencing method was applied as previously described.(16) PCR and sequencing primers were designed using the PSQ Assay Design Software version 1.0.6 (Biotage AB, Uppsala, Sweden), and sequencing was performed using a PyroGold reagent Kit (Biotage AB) and analyzed using the PSQ-96 MA 2.0.2 software. Primer sequences not already published for both direct sequencing and pyrosequencing are available as Supplemental Table 1.

Statistical analysis

The correlation between *RAS* mutations and known clinical pathological parameters was assessed by Chi-square and Student's t-tests; P=0.05 was set as the level of significance. Statistical analysis was performed using the Graphpad Prism 4 and SPSS software.

Results

H-RAS mutations are restricted to sporadic PCCs

H-RAS mutations were detected in 14 cases, all PCCs without germline or somatic mutations in any of the known PCC/PGL susceptibility genes. In contrast, none of the familial PCCs (n=76) nor any of PGLs (n=55) tested positive for *H-RAS* mutations in the present series. Thereby, the overall prevalence of *H-RAS* mutations in this cohort of PCCs/PGLs was 5% (14/271) with 8% (14/164) considering cases without known mutations only, and 10% (14/140) considering PCCs without known mutations, only. The most common mutation was *H-RAS* p.Q61R (12 cases), the remaining two being one *H-RAS* p.Q61K and one *H-RAS* p.G13R mutation. No mutations were found in *H-RAS* exon 4. The corresponding normal tissue of the 14 mutated cases tested in parallel for exons 2 and 3 was negative, thus showing the somatic nature of *H-RAS* mutations. The prevalence of mutations did not significantly differ among centres performing mutation analysis (9.7%, 9.1% and 12% in Madrid, Turin and Rotterdam, respectively, P=0.92), thus indicating that both methods employed have a similar sensitivity. *N-* and *K-RAS* sequencing did not reveal any disease-causing mutation.

H-RAS mutations in PCCs lack significant clinical or pathological correlations

The major pathological and clinical features of *H-RAS*-mutated PCC were compared with cases without known mutations (Table 1). *H-RAS* mutated PCCs showed heterogeneous morphology, without specific growth patterns or cytological features (Figure 1). A female predominance was observed in our series, and the median age was slightly higher than PCC cases without known mutations, although without reaching statistical significance. With regard to morphological parameters potentially associated with clinical aggressiveness (such as size, multicentricity, presence of vascular and/or capsular invasion and necrosis), *H-RAS*-mutated PCCs were not significantly different from PCCs without known mutations. Although most cases were clinically benign, one case died after local recurrence, another case presented vascular invasion and another a single necrotic focus, thus not excluding that, as for sporadic PCCs in general, a small proportion of *H-RAS*-mutated PCCs might have a potential malignant biological behaviour.

Table 1. Main clinical and pathological features of *H-RAS*-mutated vs wild type PCC.

Parameter	PCC *H-RAS* (#14)	PCC without known mutations (#126)	*P*-value
M/F	5/9	61/65	0.27
Median age (yrs) [range]	59 [38-79]	51 [14-81]	0.08
Median size (mm) [range]	50 [17-90]	53 [11-200]	0.83
Presence of multicentric disease / total	0/14	3/122	1.0
Presence of VI / total	1/13	13/115	1.0
Presence of CI / total	0/14	11/112	0.61
Presence of necrosis / total	1/14	19/112	0.46
Clinically malignant disease / total	1/13*	5/119	0.47

M, male; F, female; VI, vascular invasion; CI, capsular invasion.

*: one patient died of unknown causes and was considered not informative.

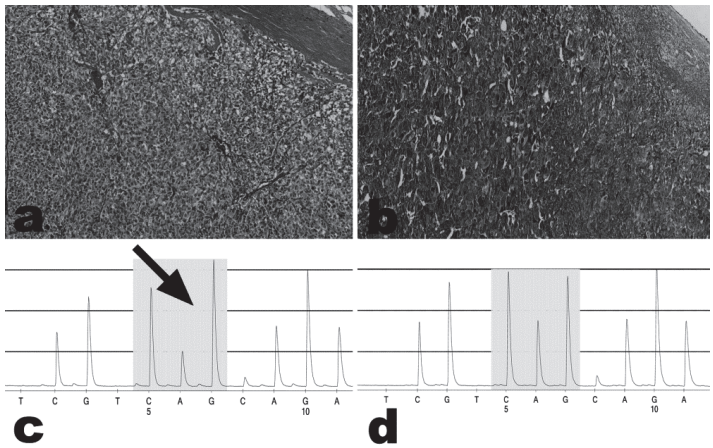

Figure 1. Heterogeneous morphological features of *H-RAS*-mutated PCCs. *H-RAS*-mutated PCC showed heterogeneous cytological and architectural features, with small (**a**) to large (**b**) nests of cells having either scant clear/eosinophilic (**a**) or abundant basophilic (**b**) cytoplasm. Representative pyrograms showing a mutation in *H-RAS* (**c**) as compared to wild type (**d**) normal adrenal tissue: *H-RAS* p.Q61R mutation – c.182A>G substitution – is demonstrated in **c** by a lower peak corresponding to A and a higher peak corresponding to G in the sequence (arrow) as compared to the wild type sequence in **d**.

Discussion

Somatic *H-RAS* gene mutations have been reported very recently in PCCs and PGLs, thus increasing the number of genetic alterations associated with these tumors. In the paper by Crona and co-workers (13), 82 cases were analyzed, including 25 positive for known PCC/PGL susceptibility genes. Four mutated cases were described, 3 PCCs and 1 PGL, all sporadic and devoid of mutations in other susceptibility genes. The patients were all males with a benign clinical course. This prompted us to explore the prevalence of RAS mutations in a cohort of 271 PCC and PGL from the ENS@T registry and to compare the genotype with clinical and pathological characteristics of potential clinical interest. In our series, *H-RAS* was found to be mutated, exclusively among the RAS genes, in 5% of PCC/PGL. All mutated cases were PCCs, clinically sporadic and without mutations in other known PCC/PGL susceptibility genes. Thus, we could confirm the sporadic and the wild type (for other genes) characteristics of *H-RAS* mutated tumors, but not the presence of mutations in PGL, since none of the 55 PGL tested (24 without known mutations and 31 mutated in other genes) was positive. Therefore, the prevalence of *H-RAS* mutations in sporadic PCC without known mutations in other genes should approximate 10% of cases, whereas it seems to be extremely rare (0% in our series) in PGL with the same characteristics.

Clinical and pathological correlations were limited by the small number of *H-RAS* mutated tumors. However, our series showed, differently from the paper by Crona et al., a female predominance and a higher median age (49.5 years in the paper by Crona and co-workers, 59 years in our series). These features, however, were not significantly different from sporadic PCCs without *H-RAS* mutation. Moreover, as described in the four *H-RAS* mutant cases reported so far, the majority of the cases in our series did not show an aggressive clinical course. However, one patient died with local recurrence after 9 years; in addition, in two cases morphological features suspected for a potentially malignant behaviour (presence of vascular invasion or necrosis) were recorded; a third case had a moderate mitotic (3 mitoses in 10 high power fields) index. Moreover, none of the parameters considered was significantly different in the group of *H-RAS*-mutated as compared to wild type PCCs. Therefore, although the occurrence of metastases at the time of diagnosis or during follow up has not been described in these tumors, a long term follow-up is required, especially for patients with clinical predictors of malignancy. Interestingly, the single case associated with local recurrence in our series had a large size, in agreement with previous data in the literature.(17)

On the other hand, our data confirm that *H-RAS* mutations are among the driver pathogenetic alterations in sporadic PCC. It is worth noticing that, among the different types of cancers harbouring somatic RAS mutations, a relevant prevalence of *H-RAS* mutations has been documented in sporadic medullary carcinoma (18), although without a specific association with prognosis. Therefore, both PCC and medullary thyroid carcinoma share a

similar genetic background but, at variance with what described in medullary carcinoma, *RET* mutations in PCC have not been associated to an aggressive behaviour, either if germline (19) or somatic (20).

All the above observations suggest that *H-RAS* mutation testing is of potential impact in sporadic PCC and needs to be validated as a clinically meaningful routine test. In fact, although not significantly associated to a specific clinical behaviour, the value of *H-RAS* genotyping to predict therapeutic responsiveness cannot be excluded until its capability to guide systemic therapeutic approaches will be tested in metastatic *H-RAS* positive PCCs. In conclusion, *H-RAS* mutations are causative for the 10% of PCC without mutations in other known PCC-related genes, and should be considered as part of the routine genetic screening when tumor tissue is available to validate its diagnostic, prognostic or predictive role as a molecular biomarker.

Acknowledgements

We would like to thank Manuel Morente and María Jesús Artiga of the Spanish National Tumor Bank Network (CNIO) for their hard work collecting part of the tumor samples used in this study.

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Supplemental Table 1. Unpublished primer sequences for *RAS* mutation analysis using direct sequencing and pyrosequencing.

Primer name	***Sequence (5'->3')***
Direct sequencing	
HRAS e2 Forward	TGAGGAGCGATGACGGAATA
HRAS e2 Reverse	AGCTGCTGGCACCTGGAC
HRAS e3 Forward	GGAAGCAGGTGGTCATTGAT
HRAS e3 Reverse	GATGTCCTCAAAAGACTTGGTG
HRAS e4 Forward	CTGTCCTCTCTGCGCATGT
HRAS e4 Reverse	GGAGAGGGTCAGTGAGTGCT
NRAS e2 Forward	GTCACACTAGGGTTTTCATT
NRAS e2 Reverse	TCATATTCATCTACAAAGTGGT
NRAS e3 Forward	ACAAGTGGTTATAGATGGTGAAACC
NRAS e3 Reverse	TCCGCAAATGACTTGCTATT
KRAS e2 Forward	GCCTGCTGAAAATGACTGAA
KRAS e2 Reverse	TTGGATCATATTCGTCCACA
KRAS e3 Forward	TAGTAATTGATGGAGAAACCTG
KRAS e3 Reverse	ATTCAATTTAAACCCACCTATA
Pyrosequencing	
HRAS e2 Forward	CTGAGGAGCGATGACGGAATAT
HRAS e2 Reverse	TCTGGATCAGCTGGATGGTCA
HRAS e2 Sequencing	GCACTCTTGCCCACA

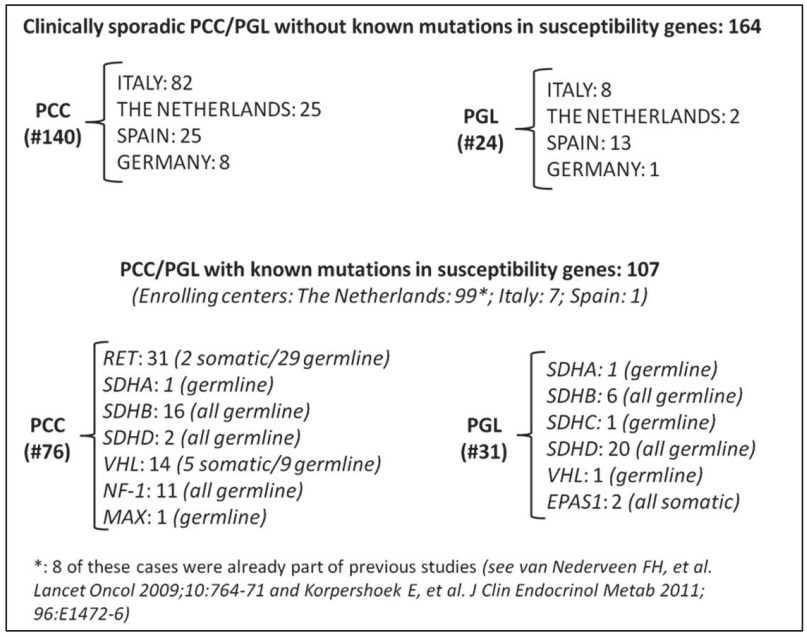
Accession numbers: *H-RAS*: NM_005343; *N-RAS*: NM_002524; *K-RAS*: NM_033360

Supplemental Table 2. Main clinical and pathological characteristics of pheochromocytoma patients with *H-RAS* mutation.

Case	Enrolling center	Sex	Age	Location	Type of secretion	Size mm	VI	CI	Necrosis	Meta's at diagn	*H-RAS* gt	Follow up (months)
1	Italy	F	50	LA	E+NE	80	No	No	No	Absent	Q61K	NED (15)
2	Italy	M	38	RA	E+NE	60	Yes	No	No	Absent	Q61R	NED (18)
3	Italy	F	58	LA	non-secreting	40	No	No	No	Absent	Q61R	NED (25)
4	Italy	M	54	RA	E+NE	17	No	No	No	Absent	Q61R	NED (12)
5	Italy	F	73	RA	E	30	No	No	No	Absent	Q61R	NED (110)
6	Italy	F	47	LA	E+NE	40	No	No	No	Absent	Q61R	NED (42)
7	Italy	M	64	RA	E+NE	19	No	No	No	Absent	Q61R	NED (68)
8	Germany	F	50	na	E	32	No	No	No	Absent	Q61R	NED (3)
9	NL	F	60	LA	E+NE	90	No	No	No	Absent	Q61R	died, local rec. (108)
10	NL	M	62	RA	E+NE	85	No	No	No	Absent	Q61R	NED (72)
11	NL	F	79	Adrenal	E+NE	55	Na	No	No	Absent	Q61R	NED (48)
12	Spain	F	75	na	na	na	No	No	No	Na	Q61R	died, unk cause (12)
13	Spain	M	45	RA	na	50	No	No	No	Absent	Q61R	NED (103)
14	Spain	F	63	RA	non-secreting	65	No	No	No	Absent	Q61R	NED (40)

VI, vascular invasion; CI, capsular invasion; gt, genotype; NL, The Netherlands; M, male; F, female; RA, right adrenal; LA, left adrenal; na: not available; E, epinephrine; NE, norepinephrine; NED: alive, no evidence of disease; unk, unknown.

Supplemental Figure 1. Detailed description of the series analyzed divided into PCC/PGL without and with known mutations in susceptibility genes.



Chapter 3.

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

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Endocr Relat Cancer. 2014 Aug;21(4):653-61.

Funding:
This study was supported by the Seventh Framework Programme (FP7/2007-2013) under grant agreement no. 259735 (ENS@T-Cancer).

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 double-strand 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 1,295,228 C>T and 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 (ETS) 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.(36) By contrast, no mutations have been observed in parafollicular cell-originated medullary thyroid carcinoma (15,21-22,25), while these seem to be extremely rare genetic events in pheochromocytomas (PCCs) and paragangliomas (PGLs).(25,36) 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 *SDHx* 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 5′-GTCCTGCCCCTTCACCTT-3′ and reverse primer 5′-CAGCGCTGCCTGAAACTC-3′. 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 extracted from the ‘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 real-time 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 $2^{-\Delta\Delta CT}$ 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 Fig. 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).

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

Case	Tumor type	Tumor site	Sex	Age	*TERT* mutation	*SDH*-def.	Weiss score	Metastatic disease/site	FU/ 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, and bone	12 mo/ DOD
3	ACC	Adrenal gland	M	42	*C228T*	Yes	8	Yes/liver, lung, and 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[a]	–	Yes/LNs	NA
6	ea PGL	Urinary bladder	M	61	*C228T*	Yes[b]	–	Yes/LNs	226 mo/ AWED
7	GIST	Stomach	F	57	*C250T*	Yes[c]	–	Yes/liver	33 mo/ DOD

ACC, adrenocortical carcinoma; AWED, Alive without evidence of disease; def., deficient; DOD, Dead of disease; ea PGL, extra-adrenal paraganglioma; FU, follow-up; GIST, gastrointestinal stromal tumor; LNs, lymph nodes; mo, months; NA, not available.

[a] 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)

[b] SDHB IHC -/ SDHA + (*SDHB* c.292T>C, p. Cys98Arg)

[c] SDHB IHC -/ SDHA IHC + (*SDHD* c.416T>C, p.Leu139Pro)

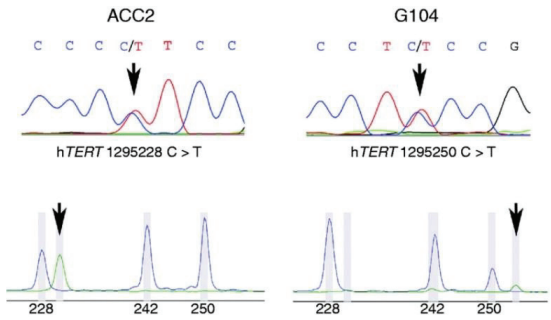

Figure 1. Somatic *TERT* promoter mutations in ACC2 and GIST104 as detected using the Sanger sequencing method (**upper panel**) and confirmed using a SNaPshot assay (**lower panel**). Arrows in the upper panel indicate the *C228T* and *C250T* mutations as displayed in the sequencing chromatograms (from left to right), while arrows in the lower panel indicate the same mutations in the SNaPshot electropherograms.

Enrichment of TERT promoter mutations in SDH-deficient tumors

Given that a subset of PCCs, PGLs, and GISTs is associated with germline *SDHx* 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).

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 the *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 Fig. 2. *TERT* expression in the *TERT* promoter-mutated ACC was approximately half that of the control HEK and SW13 cell lines.

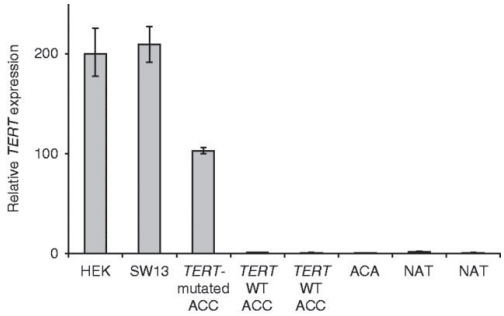

Figure 2. Quantitative real-time *TERT* expression analysis in human HEK and SW13 cell lines, normal adrenocortical tissues (NAT), adrenocortical adenomas (ACAs), and adrenocortical carcinomas (ACCs) with or without (WT) *TERT* promoter mutations. *TERT* expression was measured relative to the housekeeping 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 to be present in diverse human tumors with the highest frequencies in bladder cancer, CNS tumors, melanomas, hepatocellular carcinomas, and myxoid liposarcomas.(10-12,15,17,19-25,41,48) 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 (36,49-50) consistent with previously reported associations in other tumor types.(12,23-25) Interestingly, we found that two ea PGLs of the 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 *SDHx* 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 *SDHx* mutations, including a clinicopathologically and biologically distinctive subset of *KIT/PDGFRA* wild-type GISTs (39,51), poorly and/or un-differentiated NBLs (52), 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.(53-61)

Similarly, a selective combinatorial genetic alteration pattern has been highlighted in various tumor types.(10,12,15-16,20-21,24-25,28,33,62) 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 (10,28,62), 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 respectively (24), while a significant co-occurrence with *CTNNB1*-activating mutations has been reported in hepatocellular carcinomas and adenomas with malignant transformation.(23,63)

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 (17,41,48), meningiomas prone to malignant progression (14), as well as in *CTNNB1*-mutated hepatocellular adenomas associated with the last step of the adenoma–carcinoma transition.(23,63) 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 (64) and similar observations concerning other pediatric embryonal tumors, such as a clinically distinct molecular subtype of medulloblastoma.(15,18,65) 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 ea paraganglia of the 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 in the *TERT* promoter on *SDHx*-related tumorigenesis as well as their prognostic relevance in *SDH*-related tumor types.

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Supplemental Table 1. Probe sequences of the SNaPshot reaction.

Probe	Sequence (5'-> 3')	Size (bp)	Strand	WT	MT
hTERT 1295228	T23 GGCTGGGAGGGCCCGGA	40	sense	**G**	**A/T**
hTERT 1295242	T30 GGAGGGGGCTGGGCCGGG	48	sense	**G**	**A**
hTERT 1295250	T39 CTGGGCCGGGGACCCGG	56	sense	**G**	**A**

WT, wild-type; MT, mutant.

Supplemental Table 2. *TERT* promoter mutation status of individual NBL and ACC cell lines.

Cell lines	Tumor origin	*TERT* promoter mutation
H295	ACC	wild-type
SW13	ACC	wild-type
SJNB-12	NBL (non-NMA)	**C228T**
SJ10 (SJNB-10)	NBL (NMA)	**C228T**
SK-N-BE	NBL (NMA)	**C228T**
KCNR	NBL (NMA)	wild-type
LAN-1	NBL (NMA)	wild-type
LAN-5	NBL (NMA)	wild-type
N206	NBL (NMA)	wild-type
NGP-C4	NBL (NMA)	wild-type
NMB	NBL (NMA)	wild-type
TR-14	NBL (NMA)	wild-type
SH-EP-2/tet2	NBL (non-NMA)	wild-type
SJ1 (SJNB-1)	NBL (non-NMA)	wild-type
SK-N-SH	NBL (non-NMA)	wild-type
SH-SY5Y	NBL (non-NMA)	wild-type
GI-ME-N	NBL (non-NMA)	wild-type
SK-N-AS	NBL (non-NMA)	wild-type

ACC, adrenocortical carcinoma; NBL, neuroblastoma; NMA; *MYCN* gene amplification.

Chapter 4.

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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Modern Pathology. 2015 Jun;28(6):807-21.

Funding:
This study was supported by the Seventh Framework Programme (FP7/2007–2013) under grant agreement no. 259735 (ENS@T-Cancer). Genetic analysis of the Belgian subset was partly supported by the Fonds de la Recherche Scientifique Médicale (FRSM) convention number 3.4.587.08 F (to AP).

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 *SDHx* 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 *SDHx*) mutations and inherited as autosomal dominant traits.(4) These syndromes predispose not only to pheochromocytomas/paragangliomas, but also to gastrointestinal stromal tumors, renal cell carcinomas, and pituitary adenomas.(5-7) In the vast majority of succinate dehydrogenase-associated tumors, there is also loss of SDHB and/ or SDHA protein expression that can be detected by immunohistochemistry.(5-41) In particular, *SDHB*-, *SDHC*-, and *SDHD*-mutated tumors display SDHB immunonegativity but SDHA immunoreactivity, whereas *SDHA*-mutated tumors show negativity for both SDHB and SDHA immunostainings. Gastrointestinal stromal tumors and paragangliomas associated with Carney triad (the syndromic but nonhereditary association of gastrointestinal stromal tumor, paraganglioma, pulmonary chondroma, adrenocortical adenoma, and esophageal leiomyoma (4)), show negative staining for SDHB in the absence of *SDHx* 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 *SDHx* 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.

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

Out of 351 tumor samples, (1) 73 were *SDHx* mutated (39 *SDHD*, 24 *SDHB*, 4 *SDHA*, 4 *SDHAF2*, and 2 *SDHC*), (2) 105 *non-SDHx* 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 *SDHx* genes, and (4) 45 samples with incomplete *SDHx* molecular genetic analysis in terms of either *SDHx* 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, paraffinembedded 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 (NanoZoomer Digital Pathology.view Viewer Software, Hamamatsu Photonics KK).

Participants and Interpretation of Staining Results

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

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

In an effort to simulate widespread adoption of the scoring system as would occur in community practice, no prescoring consensus meeting was organized. In order to imitate clinical practice as much as possible for SDHB/SDHA immunohistochemical interpretations, we selected a large retrospective cohort comprising *SDHx*- and non-*SDHx*-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 *SDHx* mutations vs non-*SDHx* mutations or in *SDHx*-mutated tumors vs counterparts without identified *SDHx* mutations. We used Pearson's $\chi 2$ test to associate (1) SDHB IHC status with biological behavior (ie, benignancy vs malignancy) taking into consideration only concordant cases as well as excluding metastases (n=7) and doubled samples (n=6) (Supplementary Table 2), and (2) *SDHD* mutations and weak diffuse pattern on SDHB immunohistochemistry based on a consolidated call from at least four observers. Two-sided *P*-values of <0.05 were considered statistically significant. Statistical analyses were performed using Analyse-it v2.26 (Analyse-it Software, Leeds, UK).

Results

The interobserver agreement following a two-tiered classification approach (ie, positive and weak diffuse/negative) ranged from moderate to almost perfect for SDHB immunohistochemistry and from fair to perfect for SDHA immunohistochemistry (Table 1). With regard to SDHB immunohistochemistry, the highest agreement was reached between observers 2 and 3 (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 *SDHx* 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 *SDHx* mutations, 119 out of 128 paraganglionic tumors (93%) were scored as SDHB/SDHA immunopositive, whereas the remainder (9 out of 128; 7%) as SDHB immunonegative/SDHA immunopositive. Clinicopathological and genetic data of the latter from four independent centers are detailed in Table 2.

Discordant evaluations of SDHB immunohistochemistry were reported in 5 tumors endowed with *SDHx* (*SDHD*/*SDHB*/*SDHAF2*) mutations, 11 *VHL* and 2 *RET*-mutated tumors, as well as 18 tumors without identified *SDHx* mutations, whereas of SDHA immunohistochemistry concerned 2 *SDHx*-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 *SDHx*-mutated subgroup, whereas the 'weak diffuse' category accounted largely for those in the *SDHx*-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 *SDHx* 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-*SDHx*-mutated tumors or tumors without identified *SDHx* 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).

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

	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.

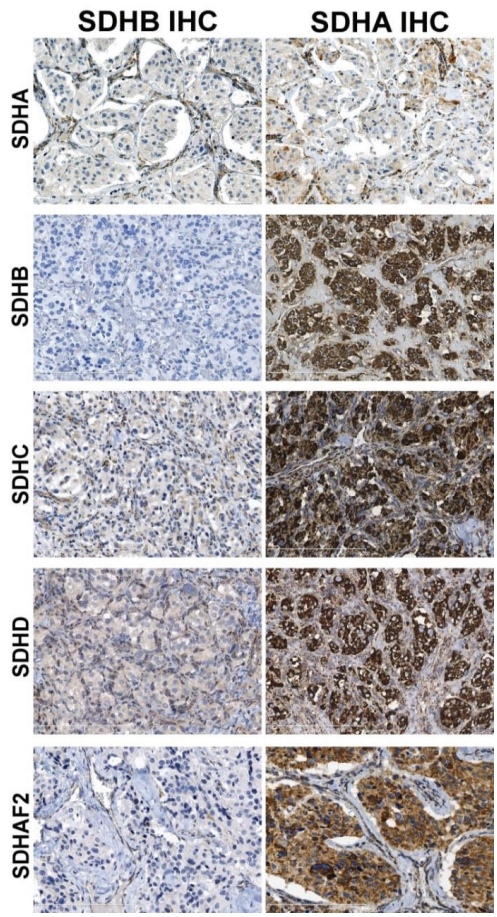

Figure 1. SDHA and SDHB immunohistochemistry in pheochromocytomas/paragangliomas endowed either with *SDHA* germline mutation displaying loss of SDHA/SDHB protein expression or with *SDHB*, *SDHC*, *SDHD* and *SDHAF2* germline mutations exhibiting loss of SDHB, but intact SDHA expression. Note the granular,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

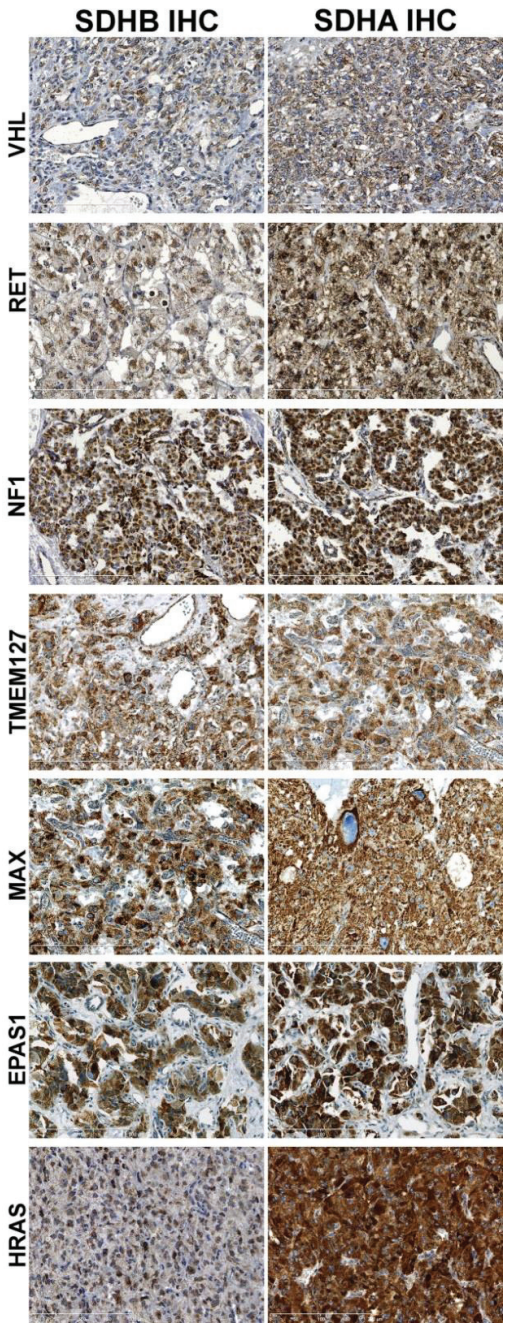

Figure 2. Intact SDHB and SDHA protein expression in non-*SDHx*-mutated paraganglionic tumors harboring germline or somatic *VHL*, *RET*, *NF1*, *TMEM127*, *MAX*, *EPAS1*, and *HRAS* mutations. Note the granular, cytoplasmic staining for SDHA/SDHB in normal cells of the intratumoral fibrovascular network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

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

	Syndromic Presentation						Molecular genetic testing of *PCC/PGL susceptibility genes* [a]								
Sample code	Familial PCC/PGL history	Multiple tumors	Age at diagn	Sex	Tumor type	Dignity	*SDHB*	*SDHD*	*SDHC*	*SDHA*	*SDHAF2*	*SDHAF1*	*VHL*	*TMEM127*	*MAX*
BEL30	No	No	43	F	HN PGL	B	-	-	-	-	-	ND	-	-	-
BEL67	No	No	36	M	HN PGL	B	-	-	-	-	-	ND	-	-	-
DR11	No	No	27	F	HN PGL	B	-	-	-	ND	-	ND	ND	-	-
ITA28	No	No	73	F	HN PGL	B	-	-	-	-	-	ND	-	-	-
DR10[b]	No	Yes	33	F	ea PGL	B	-	-	-	-	-	-	ND	ND	-
BEL66	No	No	15	F	ea PGL	M	-	-	-	-	-	ND	-	-	-
BEL116	No	No	20	M	PCC	B	-	-	-	-	-	ND	-	-	ND
ITA48	No	No	47	F	PCC	B	-	-	-	-	-	ND	-	-	-
FR115[c]	No	No	23	M	PCC	B	-	-	-	-	ND	ND	-	-	-

Abbreviations: B, benign; ea, extra-adrenal; HN, head and neck; F, female; M (Sex), male; M (dignity), malignant; ND, not done; PCC, pheochromocytoma; PGL, paraganglioma; diagn, diagnosis.

[a] *SDHx* 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 *SDHx*/*VHL* large deletions at the germline level without any mutations subsequently detected.

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

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

Abbreviations: Pval, *P*-value $\chi 2$ 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 *SDHx*-mutated tumors that are SDHB immunonegative.

Specificity is defined as the percentage of non-*SDHx*-mutated tumors or tumors without identified *SDHx* mutations that are SDHB immunopositive.

[a] Heterogeneous and noninformative scorings are excluded.

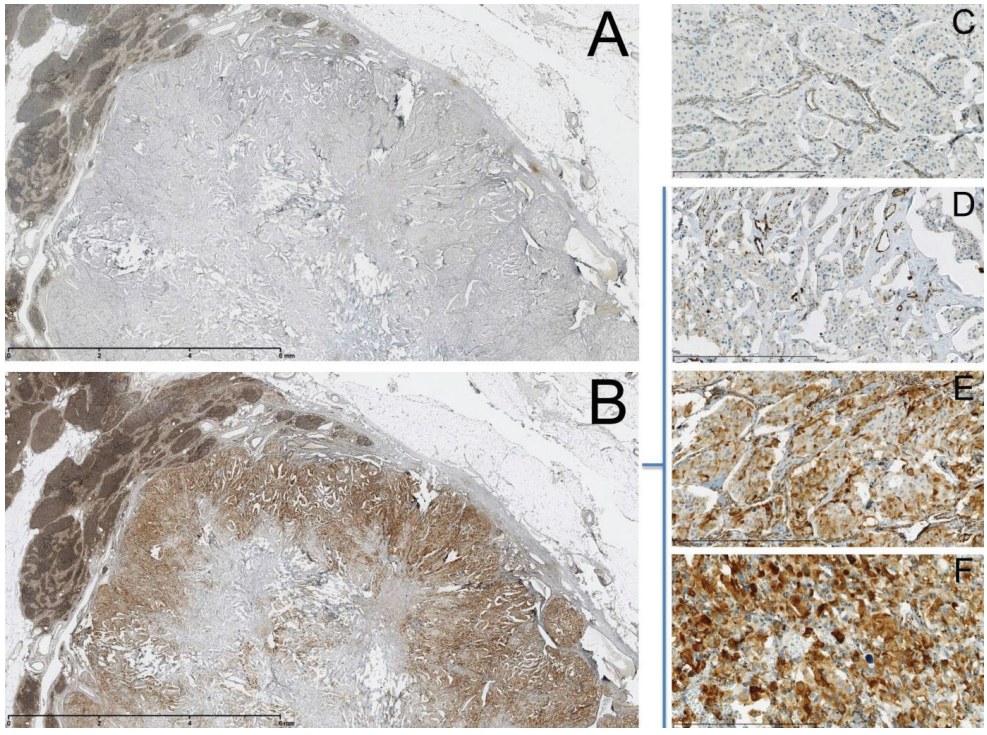

Figure 3. An extra-adrenal paraganglioma harboring an *SDHA* (c.1534C>T, p.Arg512*) germline mutation, metastatic to a para-aortic lymph node, displaying SDHB immunonegativity (**A**, **C**), but a heterogeneous staining pattern for SDHA (**B**, **D-F**): central area (**D**) convincingly negative for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

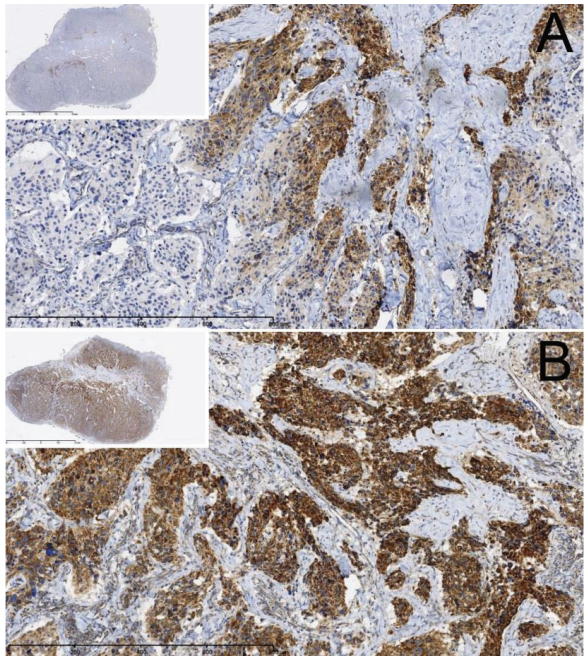

Figure 4. An *SDHAF2*-mutated (c.232G>A, p.Gly78Arg) head and neck paraganglioma showing areas convincingly negative for SDHB and to a lesser extent areas convincingly positive for SDHB (**A**). Three pathologists correctly classified this sample as heterogeneous for SDHB, with the remainder four as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

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 *SDHx* 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 *SDHx* 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* mutations. Nevertheless, these studies lacked either *SDHA*/*SDHAF2* genetic testing (8,11) or screening for large-scale *SDHx* deletions (11) that may account for higher percentages.

Intriguingly, in the present study, eight SDHB-immunonegative tumors were nonmetastatic in the absence of *SDHx* 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 *SDHx*-related tumor, and this 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 *SDHx* 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 *SDHx*-mutated subgroup, whereas the ‘weak diffuse’ category

accounted largely for inconsistencies in the *SDHx*-wild-type and *VHL*-mutated subsets. These could be potentially ascribed to (I) technical variability owing to differences in fixation time, buffered formalin concentrations, and/or age of the formalin-fixed, paraffin-embedded blocks (10,11), (II) biological variability, for example, reduced SDHB protein levels in *VHL*-mutated paraganglionic tumors (65), or even to (III–IV) individual conceptions and experience from specific staining protocols, as has been shown with immunohistochemistry for MMR proteins.(66) Technically suboptimal immunostainings were not unexpectedly encountered given the fact that provided material was derived from several pathology laboratories, each following their own fixation and embedding protocols; highlighting the importance of standardizing preanalytical variables in surgical pathology specimens.(67,68) In contrast to previous studies (10,11) indicating a stronger correlation of weak diffuse staining 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.

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 *SDHx* 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 tumors, *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.

Acknowledgements

AP, SA, and MV acknowledge the contribution of N. Lannoy, A. Mendola, and L. Evenepoel (Clin. Univ. St-Luc/UCL) for genetic analysis and F. Severino (Clin. Univ. St-Luc) for maintenance of the database. Besides Professor A. Mourin (Clin. Univ. St-Luc/ UCL), they are also grateful to all pathologists who contributed tumor samples: Professors M. Delos and B. Weynand and Drs M-C. Nollevaux and C. Fervaille (Cl. Un. De Mont-Godinne, UCL); Drs N. Detrembleur, N. Blétard, and I. Scagnol (CHU Sart-Tilman, Ulg); Drs E. Laterre and G. Beniuga (IPG Gosselies); Professor H. de Raeve and Dr W. Jeuris (OLV, Aalst); Dr V. Duwel (Ziekenhuis KLINA); Drs A. Janssen and S. Talpe (Clniques du Sud-Luxembourg, Arlon); Drs C. Robrechts and J. Bekaert (Imelda Ziekenhuis); Dr R. Duttmann (CHU Brugmann); Dr N. de Saint-Aubain (Institut Bordet); and Drs R. Achten and K. Wouters (Jessa Ziekenhuis). MM, ER, LC, and GN acknowledge the contribution of Dr T. Ercolino for the genetic analysis of the Italian (Florence) samples. GE and GB acknowledge the contribution of Drs C. Pamporaki, R. Därr, S. Richter, and J. Brütting for data collection of the German (Dresden) samples. We thank J. Shukla (Erasmus MC Cancer Institute) for her valuable technical assistance as well as Drs M. Versasky and M. Gomez Morales (Hospital Universitario San Cecilio) for providing one *SDHD*-mutated HNPGL sample.

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Supplementary Information accompanies the paper on Modern Pathology website (http://www.nature.com/ modpathol).

Chapter 5.

SDHA mutations in adult and pediatric wild-type gastrointestinal stromal tumors

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Mod Pathol. 2013;26(3):456-63.

Abstract

Most gastrointestinal stromal tumors (GISTs) harbor oncogenic mutations in *KIT* or platelet-derived growth factor receptor-α (*PDGFRA*). However, a small subset of GISTs lacks such mutations and is termed "wild-type GISTs". Germline mutation in any of the subunits of succinate dehydrogenase predisposes individuals to hereditary paragangliomas and pheochromocytomas. However, germline mutations of the genes encoding SDH subunits A, B, C or D (*SDHA*, *SDHB*, *SDHC* or *SDHD*; collectively *SDHx*) are also identified in GISTs. SDHA and SDHB immunohistochemistry are reliable techniques to identify pheochromocytomas and paragangliomas with mutations in *SDHA*, *SDHB*, *SDHC* and *SDHD*. In this study we investigated if SDHA immunohistochemistry could also identify *SDHA*-mutated GISTs. Twenty-four adult wild-type GISTs and nine pediatric/adolescent wild-type GISTs were analyzed with SDHB and where this was negative, then with SDHA immunohistochemistry. If SDHA immunohistochemistry was negative, sequencing analysis of the entire *SDHA* coding sequence was performed. All nine pediatric/adolescent GISTs and seven adult wild-type GISTs were negative for SDHB immunohistochemistry. One pediatric GIST and three SDHB immunonegative adult wild-type GISTs were negative for SDHA immunohistochemistry. In all four SDHA-negative GISTs a germline *SDHA* c.91C>T transition was found leading to a nonsense p.Arg31X mutation. Our results demonstrate that SDHA immunohistochemistry on GISTs can identify the presence of an *SDHA* germline mutation. Identifying GISTs with deficient SDH activity warrants additional genetic testing, evaluation and follow-up for inherited disorders and paragangliomas.

Introduction

Gastrointestinal stromal tumor (GIST) is the most common mesenchymal tumor of the digestive tract, but accounts for less than 1% of all gastrointestinal neoplasms.(1-4) GISTs are derived from the interstitial cells of Cajal and up to 95% of these tumors express CD117 (KIT).(1,4-6) Approximately one third to half of KIT-negative GISTs stain for DOG-1.(3,7.8) The majority of GISTs arise in the stomach (50-60%) and in the small intestine (30%), other tumor sites include the esophagus, large bowel and rectum (10%).(1,2,4)

Most GISTs (75-80%) harbor oncogenic mutations in KIT (receptor for stem cell factor, SCF) and an additional 7% in platelet-derived growth factor receptor-α (*PDGFRA)*.(2,5,6,9-11) These GISTs respond to targeted imatinib mesylate therapy, which inhibits tyrosine kinase activity.(12,13) A small subset (10%) of GISTs lacking *KIT* or *PDGFRA* mutations are defined as wild-type GISTs.(9) In pediatric patients, 85% of GISTs are KIT/PDGFRA wild-type. These occur mainly in girls, and usually have a clinically indolent course.(4,9,14,15) Unfortunately, wild-type GISTs respond poorly to imatinib.(16) The pathogenetic mechanism in wild-type GISTs is still not clearly understood.

Succinate dehydrogenase (SDH), an enzyme that is involved in the fundamental processes of energy production, participates in both the citric acid cycle and the electron transport chain.(17) SDH functions not only in mitochondrial energy generation, but the genes encoding this enzyme also act as tumor suppressors. SDH consists of the subunits SDHA, SDHB, SDHC and SDHD. Germline mutation in any subunit predisposes individuals to hereditary paragangliomas and pheochromocytomas.(18) In addition, mutations in these genes can also cause GISTs.(9,10,19) The familial dyad of paraganglioma and GIST is also known as Carney-Stratakis syndrome.(10,15) Carney triad describes the association of paragangliomas with GISTs and pulmonary chondromas.(15) GISTs arising in the setting of Carney triad or Carney-Stratakis syndrome are also “wild-type GISTs”.(15)

In previous reports we showed that negative SDHA and SDHB immunohistochemistry reliably identifies pheochromocytomas and paragangliomas caused by germline mutations in *SDHA*, *SDHB*, *SDHC* and *SDHD*.(20,21) In addition, Carney-Stratakis and Carney triad associated and wild-type pediatric GISTs can be recognized by SDHB immunohistochemistry.(17,22)

In this work, we performed SDHA immunohistochemistry on 33 wild-type GISTs, including nine pediatric/adolescent GISTs, in order to investigate whether immunohistochemistry could identify *SDHA*-mutated GISTs.

Materials and Methods

Twenty-four adult wild-type GISTs diagnosed in, or referred to, the Erasmus University Medical Center (Rotterdam, The Netherlands) between 1999 and 2011 were included in this study. In addition, seven pediatric and two adolescent wild-type cases from the files of Prof. M. O'Sullivan, Dublin were included in this study. We considered a GIST as "pediatric" if the tumor was diagnosed below the age of 18 years and "adolescent" if the age at diagnosis was 19 to 25 years. All these GISTs previously resulted negative for *KIT* and *PDGFRA* mutations (*KIT*: exon 8, 9, 11, 13 and 17; *PDGFRA*: exon 12, 14 and 18). Clinico-pathological features of all these cases are shown in Table 1. The tissues were used in accordance with the code of conduct *Proper Secondary Use of Human Tissue* established by the Dutch Federation of Medical Scientific Societies (http://www.federa.org). The Medical Ethical Committee of the Erasmus MC approved the study (MEC-2011-519).

First, all GISTs were analyzed with SDHB immunohistochemistry. If the tumors were SDHB negative, SDHA immunohistochemistry was performed. Immunohistochemical analysis was performed on 4-5µm sections of formalin-fixed paraffin-embedded tumor as previously described.(20,21) Slides were considered suitable if the internal control (granular staining in endothelial cells) was positive.

If a tumor was scored negative with SDHB immunohistochemistry, but stained positive with SDHA, the entire *SDHB*, *SDHC* and *SDHD* coding sequences, including intron-exon boundaries, were analyzed for mutations. If SDHA immunohistochemistry was negative, sequencing analysis of *SDHA* (NM_004168) was performed. The entire *SDHA* coding sequence, including intron-exon boundaries, was analyzed for mutations, taking into account the *SDHA* pseudogenes (NCBI: NR_003263, NR_003264, NR_003265). DNA was isolated according to manufacturer's instructions (Gentra Systems Minneapolis, MN, or AllPrep DNA/RNA Mini Kit, Qiagen).

When a mutation was found in tumor DNA, the presence of the mutation was also investigated in corresponding germline DNA isolated from paraffin-embedded healthy tissue surrounding the tumor. Sequence analysis is a semi-quantitative procedure and supplies some information on the relative amount of the mutated - versus the non-mutated *SDHA* allele. To substantiate the relative presence of the mutated - and non-mutated *SDHA* allele loss of heterozygosity (LOH) analysis was performed in *SDHA*-mutated tumors for a polymorphic microsatellite marker at the *SDHA* locus. For this, polymerase chain reactions (PCRs) were carried out with fluorescence-labeled primers (Invitrogen, Paisley, UK) (primer sequences are available on request) for 35 cycles with an annealing temperature of 60 ºC, and amplified products were analyzed, along with LIZ 500 size standard (Applied Biosystems, Foster City, CA), using capillary electrophoresis on an ABI 3130-XL genetic analyzer (Applied Biosystems). Data were analyzed using GeneMarker software (Soft-Genetics LLC, State College, PA).

Table 1. Clinico-pathological features of adult wild-type and pediatric/adolescent GISTs.

Case	Sex	Age at diagn.	Location	Cell type	Other tumors	*SDH*x mutation analysis
1	F	41	Stomach	Epithelioid	MTC	*SDHA* c.91 C>T*
2	F	53	Stomach	Spindle cell	No	*SDHA* c.91 C>T*
3	F	47	Stomach	Mixed	No	*SDHA* c.91 C>T*
4	M	14	Stomach	Mixed	No	*SDHA* c.91 C>T*
5	F	10	Stomach	Epithelioid	No	-
6	M	15	Stomach	Epithelioid	CT	-
7	F	21	Stomach	Epithelioid	No	-
8	F	12	Stomach	Epithelioid	CT	-
9	F	10	Stomach	Mixed	No	-
10	F	25	Stomach	Epithelioid	No	-
11	F	10	Stomach	Epithelioid	No	-
12	F	18	Stomach	Mixed	No	-
13	M	51	Stomach	Epithelioid	No	-
14	M	57	Stomach	Spindle cell	Adrenal myolipoma	-
15	M	54	Duodenum	Mixed	Duodenal lipoma	-
16	F	69	Stomach	Epithelioid	No	-
17	F	47	Stomach	Epithelioid	No	-
18	M	56	Liver (meta)	Mixed	No	-
19	F	71	Stomach	Spindle cell	No	-
20	F	52	Stomach	Spindle cell	No	-
21	M	61	Stomach	Spindle cell	No	-
22	F	59	Small intestine	Spindle cell	No	-
23	M	48	Small intestine	Spindle cell	No	-
24	F	51	Jejunum	Spindle cell	No	-
25	M	51	Rectum	Epithelioid	No	-
26	M	43	Stomach	Mixed	No	-
27	F	48	Peritoneum (meta)	Spindle cell	Uterus leiomyoma	-
28	M	52	Jejunum	Mixed	No	-
29	F	42	Stomach	Mixed	No	-
30	F	52	Meso small intestine	Mixed	No	-
31	F	76	Stomach	Mixed	No	-
32	F	56	Stomach	Mixed	No	*SDHD* c.416T>C*
33	M	58	Stomach	Mixed	No	-

F, female; M, male; diagn., diagnosis; MTC, medullary thyroid carcinoma; CT, Carney's Triad. *Germline mutation.

Results

Case series

Case 1

A 41-year-old woman was diagnosed with a gastric GIST. Microscopy showed an epithelioid morphology and the tumor cells stained positive for CD117 and DOG-1 during routine diagnostic work-up. Sequencing analysis of exons 8, 9, 11, 13 and 17 of the *KIT* proto-oncogene and exons 12, 14 and 18 of the *PDGFR*A gene did not reveal mutations. In previous research this GIST was tested by SDHB immunohistochemistry.(92) The GIST was immunonegative for SDHB and SDHA (Figure 1). Mutational analysis revealed a germline *SDHA* mutation c.91 C>T leading to p.Arg31X. This patient was homozygous (not informative) for the microsatellite marker, so we could not show LOH, though relative loss of the wild-type allele was seen in the sequencing graph (Figure 2, left panel).

At the age of 45 this patient developed a medullary thyroid carcinoma with local lymph nodes and liver metastases. The medullary thyroid carcinoma was positive for SDHB immunohistochemistry, indicating that the tumor was not caused by mitochondrial complex II disruption. Sequencing analysis of the *RET* gene revealed a mutation p.Met918Thr in the medullary thyroid carcinoma and its metastases, but not in the GIST and the healthy germline tissue of the patient. Somatic mutations are known to occur in up to 40% of sporadic medullary thyroid carcinomas.(23)

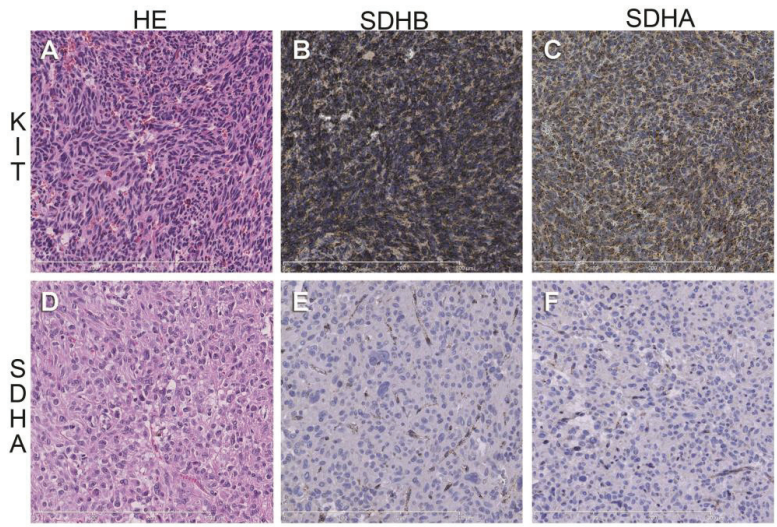

Figure 1. H&E staining and SDHB/SDHA immunohistochemistry. (**A-C**) *KIT*-mutated GIST. (**D-F**) *SDHA*-mutated GIST. (**B**) Strong granular cytoplasmic staining for SDHB and (**C**) SDHA. (**E**) Absent cytoplasmic staining for SDHB and (**F**) SDHA of tumor cells, with positive staining of normal (endothelial) cells.

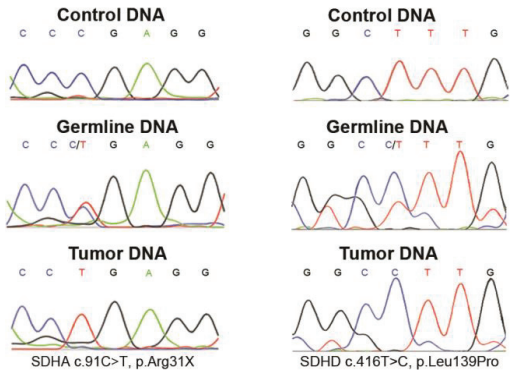

Figure 2. Left panel: chromatogram of *SDHA*-mutated GIST. *SDHA* mutant allele and wild-type allele are shown in germline DNA, and c.91C4T transition leading to a nonsense p.Arg31X mutation in tumor DNA. In addition, tumor DNA only shows the mutant allele, demonstrating loss of the wild-type allele and indicating bi-allelic *SDHA* inactivation (bona fide tumor-suppressor gene). **Right panel**: chromatogram of *SDHD*-mutated GIST. *SDHD*-mutant allele and wild-type allele are shown in germline DNA and c.416T4C transition leading to a missense p.Leu139Pro mutation in tumor DNA.

Cases 2 and 3

Two sisters (respectively 47 and 53-year-old) were both diagnosed with a GIST located in the stomach. Microscopy of the GIST of sister 1 mainly showed a mixed epithelioid and spindle cell morphology. Tumor cells stained positive for DOG-1 and CD34 and were partly positive for CD117 on routine diagnostic work-up. The tumor of sister 2 showed a spindle cell CD117- and CD34-positive GIST. In both GISTs no mutations were found in exons 8, 9, 11, 13 and 17 of the *KIT* gene and exons 12, 14 and 18 of the *PDGFRA* gene. Tumor cells of both tumors were immunonegative for SDHB and SDHA. Immunohistochemical stainings for CD117 and SDHA of both tumors are shown in the two bottom panels of figure 3. Mutational analysis of tumor and germline DNA revealed in both sisters the same *SDHA* mutation c.91 C>T leading to p.Arg31X. However, relative loss of the wild-type *SDHA* allele was only seen in sister 1 and not in sister 2 in the sequence (figure 3, middle panel). This was confirmed by the LOH analysis, which only showed LOH in the tumor of sister 1 (two upper panels of figure 3).

Case 4

A 14-year-old boy was diagnosed with a gastric GIST. Histology showed spindle and epitheloid morphology and CD117 was positive in routine diagnostics. Staining for SDHB and SDHA were negative (not shown). The tumor was defined as wild-type, since no mutations were found in exons 8, 9, 11, 13 and 17 of *KIT* and exons 12, 14 and 18 of *PDGFRA.* Mutational analysis revealed a germline *SDHA* mutation c.91 C>T (p.Arg31X) with no relative loss of the wild-type allele. Indeed, LOH analysis showed no LOH in the tumor (figure 3).

General findings

All nine pediatric/adolescent GISTs were negative for SDHB by immunohistochemistry. Of the 24 adult wild-type GISTs, seven resulted immunonegative for SDHB (29%). SDHA immunohistochemistry was performed on all SDHB immunonegative GISTs. One out of nine pediatric/adolescent GISTs (11%) and three out of 24 adult wild-type GISTs (13%) were negative for SDHA immunohistochemistry. Four adult GISTs and eight pediatric/adolescent GISTs were negative for SDHB, but positive for SDHA by immunohistochemistry. Sequence analysis of these GISTs revealed a germline *SDHD* missense mutation c.416T>C in one adult tumor leading to a p. Leu139Pro. Figure 2 (right panel) shows the sequencing chromatograms of this *SDHD* mutation. Sequencing analysis of the remaining eleven GISTs revealed neither mutations nor LOH in *SDHB*, *SDHC* or *SDHD*.

Sequencing analysis of *SDHA* performed on the four GISTs negative for SDHA immunohistochemistry showed the same *SDHA* nonsense c.91C>T mutation (p.Arg31X) in all four. Powerplex16 analysis showed that our four *SDHA*-mutated patients did not share the same alleles (except from the two sisters who showed an overlap of some alleles), excluding contamination (data not shown).

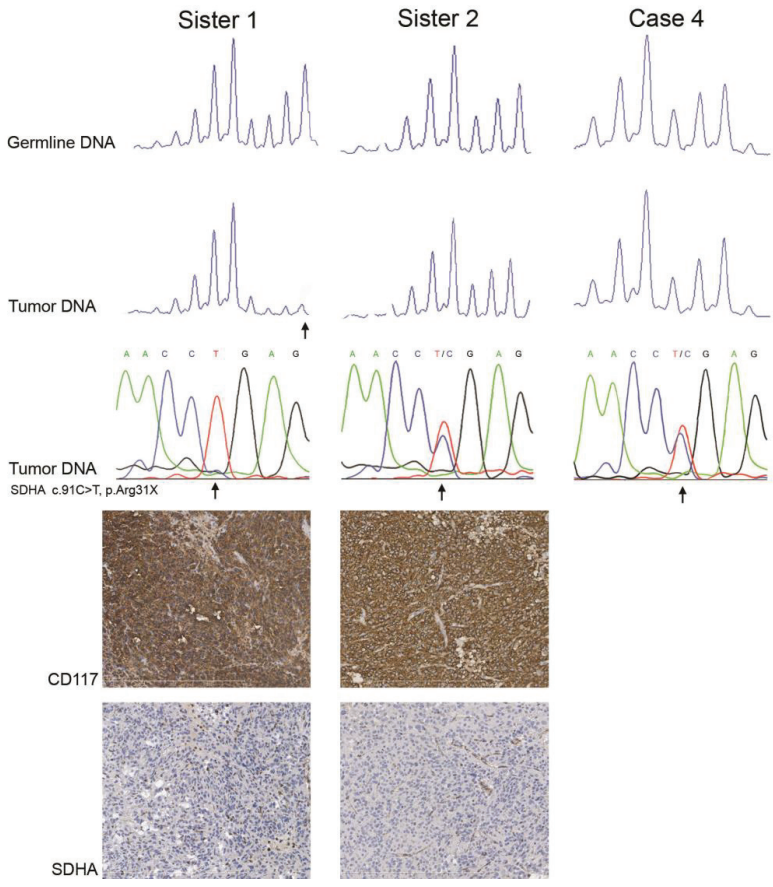

Figure 3. Three upper panels: LOH Electropherograms. Sister 1 shows LOH for a microsatellite marker on the centromeric side of *SDHA*. The arrow indicates the allele with relative loss. Sister 2 and case 4 show no LOH. Middle panel: sequencing chromatograms of tumor DNA. *SDHA* p.Arg31X due to c.91C>T. Arrows indicate the mutation. The chromatogram of sister 1 reveals predominantly the mutant allele, while there is no relative loss of the wild-type *SDHA* allele in sister 2 and in case 4. **Two bottom panels**: CD117 and SDHA immunohistochemistry in the tumors of both sisters. Strong positive staining for CD117 and absent staining for SDHA of tumor cells, with positive SDHA staining of normal (endothelial) cells.

Discussion

The precise role of *SDHA* as a tumor suppressor gene in oncogenesis is poorly understood.(24) Oncogenic *SDHA* mutations have been described in paragangliomas and recently in GISTs.(9,21,24) Burnichon et al. detected LOH at the *SDHA* locus in 4.5% of a large series of paragangliomas and pheochromocytomas.(24) Loss of the wild-type allele of *SDHA* in a tumor from a patient with a germline inactivating mutation in *SDHA* indicates that complete loss of SDHA function accompanies tumor formation. In the present study we found the same *SDHA* (p.Arg31X) mutation in 1 of 9 (11%) pediatric/adolescent wild-type GISTs and in 3 of 24 (13%) adult wild-type GISTs. This inactivating *SDHA* mutation can be detected by SDHA immunohistochemistry, since the tumor cells show absent SDHA staining in the presence of positive staining of internal control normal (endothelial) cells. All four identified *SDHA* mutations were demonstrated to be present in the germline.

Due to the fact that we found the same *SDHA* germline mutation in four cases, the possibility of a founder mutation was considered. Three of our investigated patients were from the Netherlands and the fourth was from the United Kingdom. In addition, in an Italian patient the same p.Arg31X was described recently.(9) Also, Nannini et al. (25) and Wagner et al. (26) identified the *SDHA* p.Arg31X mutation (amongst others) in wild-type and SDH-deficient GISTs, respectively. The frequency of *SDHA* mutations within the SDH-deficient GISTs (36%) of Wagner et al. is slightly higher, but in accordance with our frequency of *SDHA*-mutated GISTs that show a negative staining of SDHB (25%). The occurrence of the *SDHA* p.Arg31X mutation in three different countries (Italy, UK and The Netherlands) renders a founder mutation less likely and might suggest a hotspot mutation. The relatively high percentage (12%) of *SDHA* mutations found in the 33 wild-type GISTs in the present study may be due to the small sample size and due to patient selection bias, since Erasmus MC is a tertiary referral center.

Interestingly, the *SDHA* p.Arg31X mutation has also been identified in a Dutch healthy control group (0.3%).(21) However, as the mutation causes a truncated protein and three of the four *SDHA*-mutated tumors showed loss of the wild-type allele, according to Knudson's two-hit hypothesis, the p.Arg31X mutation seems to be involved in the pathogenesis of the GISTs. It is possible that the mutation is present in healthy controls because of low penetrance of tumor development in *SDHA*-mutation carriers. In our two *SDHA*-mutated patients without LOH, there is probably a different mechanism responsible for tumor formation, such as inactivation of the wild-type *SDHA* allele by a somatic mutation or promoter methylation of the wild-type *SDHA* gene.

Based on previous findings and our present ones of *SDHA* mutations in wild-type GISTs, we recommend testing for germline mutations of *SDHA* in all patients diagnosed with wild-type GISTs that are negative for SDHA by immunohistochemistry.(9)

The link between paragangliomas, pheochromocytomas and GISTs has been established in

the Carney-Stratakis syndrome and Carney triad.(10,15) In Carney-Stratakis syndrome *SDHB*, *SDHC* and *SDHD* mutations have been described.(10) In Carney triad no mutations in *SDH* have been found.(27) It has been shown that GISTs from patients with Carney-Stratakis syndrome or Carney triad and pediatric GISTs are SDHB negative by SDHB immunohistochemistry.(17,22,28) Janeway et al. found germline mutations in *SDHB*, *SDHC* and *SDHD* in 6 of 38 wild-type GISTs, but they also found loss of SDHB protein expression in wild-type GISTs without identifiable mutations in *SDHB*, *SDHC* or *SDHD*.(28) This could mean that loss of function of the SDH complex, even without an *SDH* mutation or deletion, contributes to the pathogenesis of wild-type GISTs. However, the absent SDHB expression in their series might be also due to *SDHA* mutations, for which they did not perform mutational analysis. *SDH* germline mutations were neither found in 66 SDHB immunonegative wild-type GISTs investigated by Miettinen et al.(19) However, the mutational analysis was not performed on all SDH-deficient GISTs in their series, not all the exons of *SDHB*, *SDHC* and *SDHD* were analysed and again no mutational analysis of *SDHA* was performed.

In accordance with Janeway et al. (28) and Miettinen et al. (19), we did not find a mutation in *SDHB*, *SDHC* or *SDHD* in three adult GISTs which were immunonegative for SDHB, but positive for SDHA in the present study, but we did find a *SDHD* mutation in one of the cases. Moreover, we did not find any *SDHx* mutations in 8 of 9 pediatric/adolescent GISTs, even though they were all negative for SDHB immunohistochemistry. Possible explanations for the absence of associated *SDHx* mutations or deletions are mutations in other genes affecting the SDH complex or epigenetic modifications leading to decreased mRNA expression of one of the subunits of the complex. However, we did not compare mRNA expression of *SDHB*, *SDHC* and *SDHD* between the SDHB immunonegative and -positive cases in our study. In addition, we did not investigate large intragenic deletions of *SDHB*, *SDHC* and *SDHD* in our samples, which can be detected by multiplex ligation-dependent probe amplification analysis. Therefore, large genetic aberrations in the *SDHx* genes cannot be categorically excluded.

As mentioned before, wild-type GISTs respond poorly to the tyrosine kinase inhibitor Imatinib. The finding that loss of function of the SDH complex plays a role in a subset of wild-type GISTs, could be a new focus for treatment. Moreover, identifying GISTs with deficient SDH activity in patients warrants additional genetic testing, evaluation and follow-up for Carney triad, Carney-Stratakis syndrome and paragangliomas.(19) Imatinib targets the constitutively active tyrosine kinase in GISTs with oncogenic mutations in KIT or PDGFRA.(12) However, the mechanism by which inactivation of one of the subunits of SDH leads to tumorigenesis is still unexplained. Studies suggest that activation of the hypoxic/angiogenic pathway plays a role.(29,30) Possibly, pharmacological agents that target the hypoxia pathway or its downstream targets (such as VEGF, GLUT1, IGF2) could be used as new treatment options.

In conclusion, germline *SDHA* mutations are causal for pediatric/adolescent and adult wild-type GISTs in a subset of patients in our series. SDHA immunohistochemistry can be used to detect GISTs with an *SDHA* mutation and we recommend testing for germline *SDHA* mutations in all patients with SDHA immunonegative GISTs. Recognition of *SDH*-mutated GISTs by SDHB and SDHA immunohistochemistry is important for prognosis, treatment and follow-up.

Acknowledgements

We thank Dr. Jouko Lohi for the contribution of materials and clinical data of a case, and Frank van der Panne for assistance with preparing the histology figures.

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Chapter 6.

SDHD immunohistochemistry: a new tool to validate *SDHx* mutations in pheochromocytoma/paraganglioma

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J Clin Endocrinol Metab. 2015 Feb;100(2):E287-91.

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Funding:
The work leading to these results has received funding from the Agence Nationale de la Recherche (ANR-2011-JCJC-00 701 MODEOMAPP), the Programme Hospitalier de Recherche Clinique grant COMETE 3 (AOM 06 179) and from the European Union Seventh Framework.

Abstract

Context: Pheochromocytomas (PCC) and paragangliomas (PGL) may be caused by a germline mutation in 12 different predisposing genes. We previously reported that immunohistochemistry is a useful approach to detect patients harboring *SDHx* mutations. SDHA immunostaining is negative in *SDHA*-mutated tumors only, while SDHB immunostaining is negative in samples mutated on all *SDHx* genes. In some cases of *SDHD* or *SDHC*-mutated tumors, a weak diffuse SDHB labeling has however been described.
Objective: Here, we addressed whether the same procedure could be applicable to detect patients with germline *SDHD* mutations, by testing two new commercially available anti-SDHD antibodies.
Design and Methods: We performed a retrospective study on 170 PGL/PCC in which we investigated SDHD and SDHB expression by immunohistochemistry.
Results: *SDHx*-mutated PGL/PCC showed a completely negative SDHB staining (23/27) or a weak cytoplasmic background (4/27). Unexpectedly, we observed that SDHD immunohistochemistry was positive in *SDHx*-deficient tumors and negative in the other samples. Twenty-six of 27 *SDHx* tumors (including the 4 weakly stained for SDHB) were positive for SDHD. Among non-*SDHx* tumors, 138/143 were positive for SDHB and negative for SDHD. Five cases showed a negative immunostaining for SDHB but were negative for SDHD.
Conclusion: Our results demonstrate that a positive SDHD immunostaining predicts the presence of an *SDHx* gene mutation. Because SDHB negative immunostaining is sometimes difficult to interpret in case of background, the addition of SDHD positive immunohistochemistry will be a very useful tool to predict or validate *SDHx* gene variants in PGL/PCC.

Introduction

Pheochromocytomas (PCC) and paragangliomas (PGL) are neuroendocrine tumors in which the contribution of genetics has a major impact with around 40% of inherited forms and 12 susceptibility genes identified.(1) These include the *RET* proto-oncogene, *VHL*, *NF1*, *MAX*, *TMEM127*, the four subunits of succinate dehydrogenase: *SDHA*, *SDHB*, *SDHC* and *SDHD*, and SDH assembly protein, *SDHAF2*, *HIF2A* (2,3), and finally *FH* (4). Specific genotype-phenotype correlations have been established. For instance, it is now well established that the presence of a germline mutation in the *SDHB* gene increases the risk of malignancy (5,6) and is associated with poor prognosis and reduced survival.(7) Hence, it is important to identify the mutated gene both for the follow-up of affected patients and for the genetic counseling to their family members. Algorithms have been designed to orientate genetic tests depending on the clinical phenotype of index cases.(8,9) These algorithms, however, are not always efficient enough as *SDHB* mutations carriers for example, may have an apparently sporadic presentation at first diagnosis. Moreover, establishing the pathogenicity of variants of unknown significance is frequently needed and requires functional validation that completes in silico predictions.

In the absence of efficient chemotherapeutic treatments, the gold standard treatment of PGL/PCC is still the surgical removal of tumors, which are then systematically evaluated in the departments of pathology. In that context, developing biological assays using immunohistochemistry (IHC) to predict the mutation status of a patient is the most realistic approach for routine clinical practice. We have previously established that evaluating SDHB protein expression by IHC is a sensitive and specific tool to detect patients with germline *SDHA*, *SDHB*, *SDHC* or *SDHD* gene mutations.(10) SDHB protein is lost in all *SDHx* mutated tumors, whatever the mutated gene. SDHB positive immunostaining, characterized by a mitochondrial-specific granular labelling is specifically detected in the other inherited forms and in sporadic tumors. However, in some cases (particularly in *SDHD*-mutated tumors), a weak diffuse signal can be observed (11), which should be considered as negative but may be misleading for nonexperts. Similarly, some sporadic cases with no SDHB staining have been described. SDHB immunohistochemistry is thus a predicting criterion with a specificity of 84%. SDHA immunohistochemistry allows detecting *SDHA*-related tumors. It is negative in *SDHA*-mutated PGL/PCC, but positive in *SDHB*-, *SDHC*- and *SDHD*-related tumors.(12) Similar results were reported for SDHB and SDHA IHC in *SDH*-related gastro-intestinal stromal tumors (13,14), and kidney cancers.(15) Finally, MAX immunohistochemistry is efficient for detecting tumors due to truncating *MAX* mutations, but not to missense *MAX* mutations.(16,17)

The purpose of the present study was to evaluate the efficacy of SDHD immunohistochemistry using two novel commercially available SDHD-antibodies, with the original aim to specifically detect patients with *SDHD* germline mutations.

Patients and Methods

Patients

We performed a retrospective study on a series of 170 tumors, which included 136 pheochromocytomas, 26 sympathetic paragangliomas and 2 metastasis. 164 tumors were collected by the COMETE network from patients operated in two referral centers in Paris (Hôpital Européen Georges Pompidou and Hôpital Cochin) and 6 from the archives of the Department of Pathology of the Erasmus MC Cancer Institute, Erasmus MC, University Medical Center (Rotterdam, the Netherlands). The series contained tumors with different germline mutations including 16 *NF1*, 13 *RET*, 28 *VHL*, 13 *MAX*, 1 *TMEM127*, 3 *SDHA*, 13 *SDHB*, 3 *SDHC*, 8 *SDHD* and 72 sporadic cases (see Supplemental Table 1 for genetic and clinical data). The procedures used for PGL/PCC diagnosis and genetic testing were in accordance with institutional guidelines. Genetic testing was performed for all PGL/PCC predisposing genes. Tumors without mutations are classified as sporadic cases. Somatic analysis of *SDHB*, *SDHC* and *SDHD* genes was performed by Sanger sequencing and QMPSF (Quantitative Multiplex PCR of Short fluorescent Fragments) as previously described.(18,19)

Ethics Statement

Informed signed consent for germline and somatic DNA analysis was obtained from each patient recruited by the COMETE network, and the study was formally approved by an institutional review board (IRB) [Comité de Protection des Personnes (CPP) Ile de France III, June 2012]. The Dutch tissues were used in accordance with the code of conduct Proper Secondary Use of Human Tissue established by the Dutch Federation of Medical Scientific Societies (http://www.federa.org).

Immunohistochemistry

Four to 6 µm sections of formalin-fixed and paraffin-embedded archival tissues were cut and mounted on Superfrost Plus glass slides. The sections were deparaffinized and rehydrated and heat-mediated antigen retrieval was performed using Tris-EDTA at pH 9 for 45 minutes after H_2O_2 treatment. After blockade of unspecific sites in goat serum for 30 minutes, anti-SDHD (HPA045727, Sigma-Aldrich Corp; 1:50 or sc-67 195, SantaCruz; 1:50) or anti-SDHB (HPA002868, Sigma-Aldrich Corp; 1:500) antibodies were applied for 1 hour at room temperature. The biotinylated secondary anti-rabbit antibody (Vector Lab; 1:400) was applied for 45 minutes and the revelation was assessed using histogreen kit (Vector Laboratories, EUROBIO/ABCYS, Les Ulis, France). Nuclear Fast Red counterstaining was performed before rehydrating and mounting of slides in Eukitt media (Sigma-Aldrich). Negative control was performed by omitting the primary antibody. Acquisitions were performed using Leica DM400B microscope, with x40 objective.

Slides were analyzed blindly, by two independent observers (M.M and J.F). SDHB was scored

as completely negative, weak diffuse (considered as negative), or weak mitochondrial (considered as positive). SDHD was considered as either positive or negative.

Results

SDHD and SDHB immunostainings were analyzed in all 170 tumors (Supplemental Table 1). SDHB IHC revealed a granular mitochondrial staining in 138/143 of non-*SDHx* tumors, including one *VHL*-mutated tumor with a weak signal that was however considered positive because of its specific localization in the mitochondria. Five non-*SDHx* samples showed a completely negative staining for SDHB: 3 *VHL*, 1 *NF1* and 1 sporadic. In three of these tumors (2 *VHL* and 1 *NF1*), we were able to obtain tumor DNA and thus searched for somatic mutations or large deletions in *SDHB*, *SDHC*, and *SDHD* genes without identifying any. SDHA immunostaining was positive in these five samples (data not shown). All *SDHx*-mutated PCC and PGL were scored as negative for SDHB staining (n=27), although four tumors had a weak diffuse cytoplasmic background (1 *SDHD*, 3 *SDHC*). The sensitivity and specificity of SDHB immunohistochemistry can thus be estimated using different criteria: 1) if only a completely negative staining is considered, the sensitivity is 85% and the specificity is 96% while 2) if both completely negative and weak diffuse signals are associated with *SDHx*-mutated status, the sensitivity is then 100% and the specificity 97%.

Very surprisingly, we observed the opposite result for SDHD immunohistochemistry, which was performed using two different polyclonal antibodies. SDHD protein was indeed not detected in *RET*, *NF1*, *VHL*, *MAX*, *TMEM127* nor in sporadic tumors, while both antibodies led to a positive immunostaining (although not typically mitochondrial) in all types of *SDHx*-mutated tumors (Figure 1). Twenty-six of 27 (96%) *SDHx* tumors were positive for SDHD (using the HPA045727 Sigma-Aldrich antibody). The only SDHD negative tumor in that group was from a patient carrying a punctual *SDHD* gene mutation (c.274G>T, p.Asp92Tyr), which was clearly negative for SDHB immunostaining. It is worth noting that SDHD was positive in the four *SDHx* tumors where the weak diffuse staining was observed after SDHB IHC (Figure 2A). Among the 143 tumors with a germline mutation in a non-*SDHx* predisposing gene (n=71) or with no identified mutation (n=72), 138 (97%) were negative for SDHD IHC with Sigma-Aldrich antibody and five were positive (1 *MAX*, 1 *VHL* and 3 sporadic). These five tumors were SDHB-positive. The six tumors that had a weak (1 *VHL*) or negative (1 sporadic, 1 *NF1* and 3 *VHL*) SDHB immunostaining were negative for SDHD (Figure 2B). In order to establish whether these observations were due to differences in SDHD transcription, we analyzed SDHD expression in previously published transcriptome data obtained from 188 PGL/PCC (19), which revealed very comparable levels of expression in all cases, except *SDHD*-mutated tumors. They displayed reduced *SDHD* mRNA levels, probably because of the haploinsufficiency caused by loss of heterozygosity at the SDHD locus (Supplemental Figure 1).

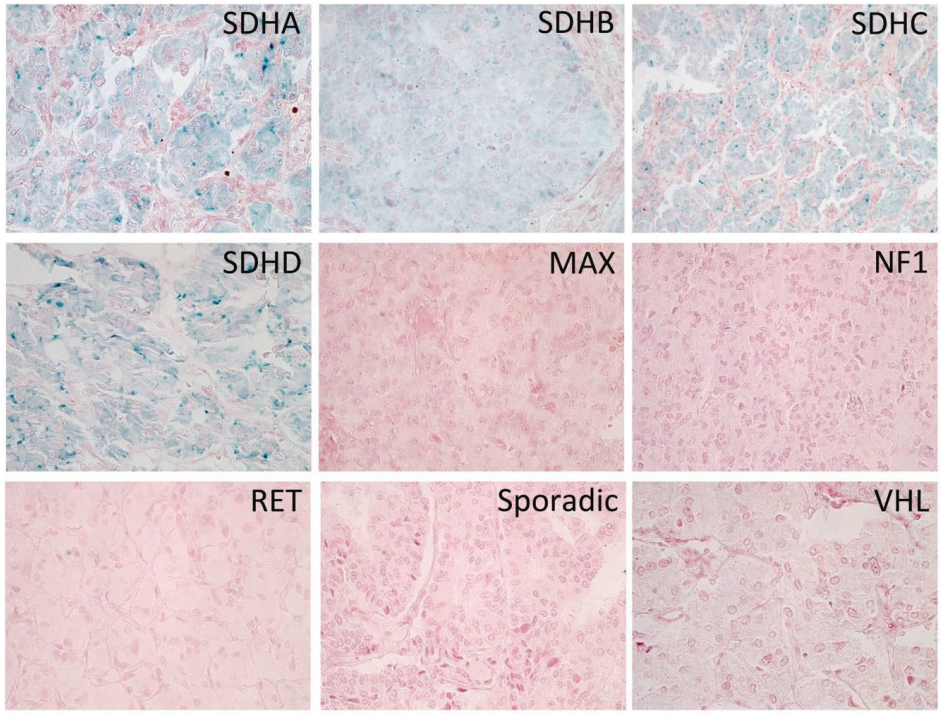

Figure 1. SDHD immunohistochemistry in PGL/PCC with various genetic backgrounds. SDHD immunostaining reveals a positive labeling in germline *SDHx*-mutated patients (*SDHA*, *SDHB*, *SDHC,* and *SDHD*) and a negative staining in the other type of mutations (*MAX*, *NF1*, *RET*, *VHL*) or in sporadic cases.

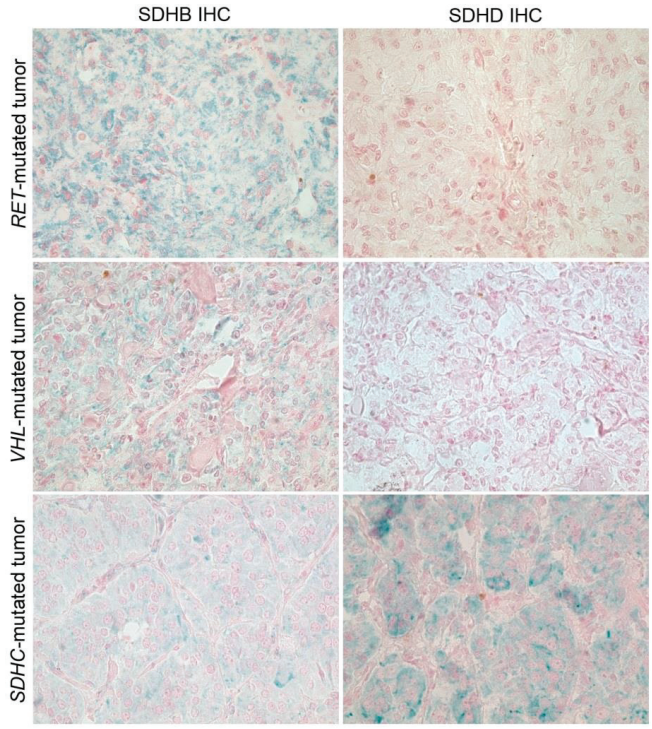

Figure 2. Contribution of SDHD immunohistochemistry to the genetic diagnosis in case of ambiguous SDHB immunostaining. Although SDHB IHC is often easy to interpret in case of clear granular staining (upper lane, *RET*-mutated PCC), it may lead to weak staining or background that may be difficult to distinct from each other, as illustrated with a *VHL*-mutated tumor showing a weak staining (middle lane) or in an *SDHC*-mutated tumor with a strong background for SDHB immunohistochemistry. In these cases the respective negative and positive SDHD IHC allows to validate the genetics diagnosis.

Discussion

In the current study, we first evaluated SDHD immunohistochemistry in an attempt to predict *SDHD* mutational status in a large cohort of inherited or sporadic PGL/PCC. Unexpectedly, we observed that in contrast with SDHB (10), which is lost in all *SDHx*-mutated tumors, and with SDHA, which is specifically lost in *SDHA*-mutated tumors (12), SDHD protein was not detected in non-*SDHx* related PGL/PCC, while its immunostaining was positive in *SDHx*-deficient ones. Surprisingly enough, SDHD immunolabeling was positive in the adjacent adrenal cortex of all PCC in which it could be evaluated, independently of the mutational status (Supplemental Figure 2). One explanation to apprehend this puzzling result is based on the fact that SDHB and SDHA protein expression are both very low in PGL or PCC as compared with normal adrenal for instance, even in non-*SDHx* related tumors. One can therefore suspect that SDHD and SDHC proteins are also expressed at low levels in these tumors. SDHD is an anchorage subunit of succinate dehydrogenase, and the antibodies used in this study are directed against an epitope that is localized within a transmembrane domain of the SDHD protein (Supplemental Figure 3). It is therefore likely that such an epitope would be masked when the SDHD protein is included in the active complex. Hence, in normal cells that express large amounts of *SDHx* genes, the presence of an excess of SDHD protein, would lead to coexistence of the SDHD subunit included in the complex or not, allowing the detection of free protein by the antibody. In contrast, in *SDHx* wild-type PGL/PCC cells, the reduced basal *SDHx* expression would lead to a complete integration of all SDHD proteins within the complex, making the immunostaining negative. Finally, in *SDHx*-mutated tumor cells, the disruption of the complex would release the epitope, leading to the positive labeling we observed, which did not appear as a mitochondrial granular labeling, but rather as a cytoplasmic diffuse signal, sometimes associated with patchy accumulation.

Although unanticipated, this result is nevertheless of a true clinical interest for the identification of *SDHx*-related PGL/PCC. SDHB IHC is indeed a very efficient tool to predict the *SDHx* mutational status but it is nevertheless sometimes problematic to interpret, in particular for nonspecialists. For instance, the internal positive control (in general endothelial cells) is not always positive. Moreover, SDHx-deficient tumors (and in particular *SDHD* or *SDHC*-mutated ones) may also present a weak to strong background that makes the diagnosis difficult. In this study, four *SDHx* tumors displayed such a background (1 *SDHD* and 3 *SDHC*). All were clearly positive for SDHD. On the contrary, the Warburg effect, which is particularly strong in *VHL*-related tumors (20), may lead to a very weak SDHB immunostaining, even in the absence of an *SDHx* gene mutation (10,21). In these perplexing situations, the use of the SDHD “reverse” IHC will constitute a most valuable tool that will comfort the diagnosis of analyzed tumor samples.

The question of identifying patients with *SDHx*-related disease is of true clinical interest, in particular for the follow-up of affected patients, who may be predisposed to multiple tumors

or to metastatic disease in case of *SDHB* mutation. Several approaches are being evaluated to develop easy-to-use and specific tools to allow such predictions. Recently, Lendvai et al. reported the quantification of succinate-to-fumarate ratios by mass spectrometry, as such a predictive tool.(22) This approach is of great interest as it is quantitative, and can be performed in tumor tissue of patients. Immunohistochemistry requires that formalin-fixed paraffin-embedded tissue is available, but is then feasible in any pathology department. Before being transferred to routine clinical practice, the SDHD IHC described here will have to be validated in an independent validation series, with different observers, as the interpretation of immunohistochemical data may vary between observers and experimental procedures. This first study however suggests that there is an almost perfect interobserver agreement (Supplemental Table 1).

Ultimately, SDHD IHC could potentially be added to the IHC algorithm of PGL/PCC pathological analyses (Supplemental Figure 4). Although SDHD IHC by itself is not 100% specific or sensitive, it produces a labeling that mirrors SDHB immunostaining and will provide a strong complement to this procedure, ruling out any doubt that may persist regarding the *SDHx* mutational status.

Acknowledgements

We express our gratitude to Prs Pierre-François Plouin and Xavier Bertagna from the COMETE Network as well as Nirubiah Thurairajasingam and Caroline Travers for technical assistance. We thank Dr Rossella Libé and Dr Frédérique Tissier for their contribution to this study.

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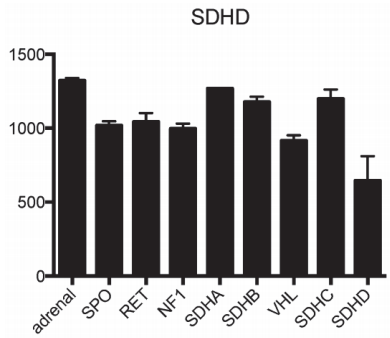
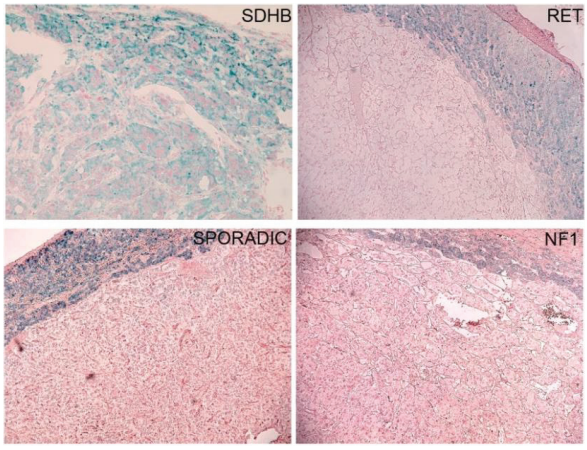
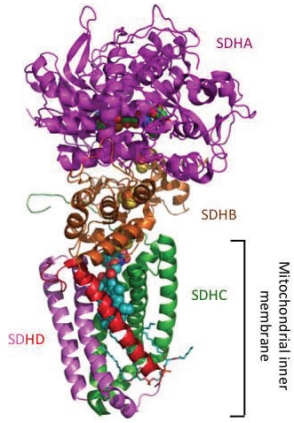
Supplemental Table 1. Clinical data and immunohistochemical results: http://press.endocrine.org/doi/suppl/10.1210/jc.2014-1870.



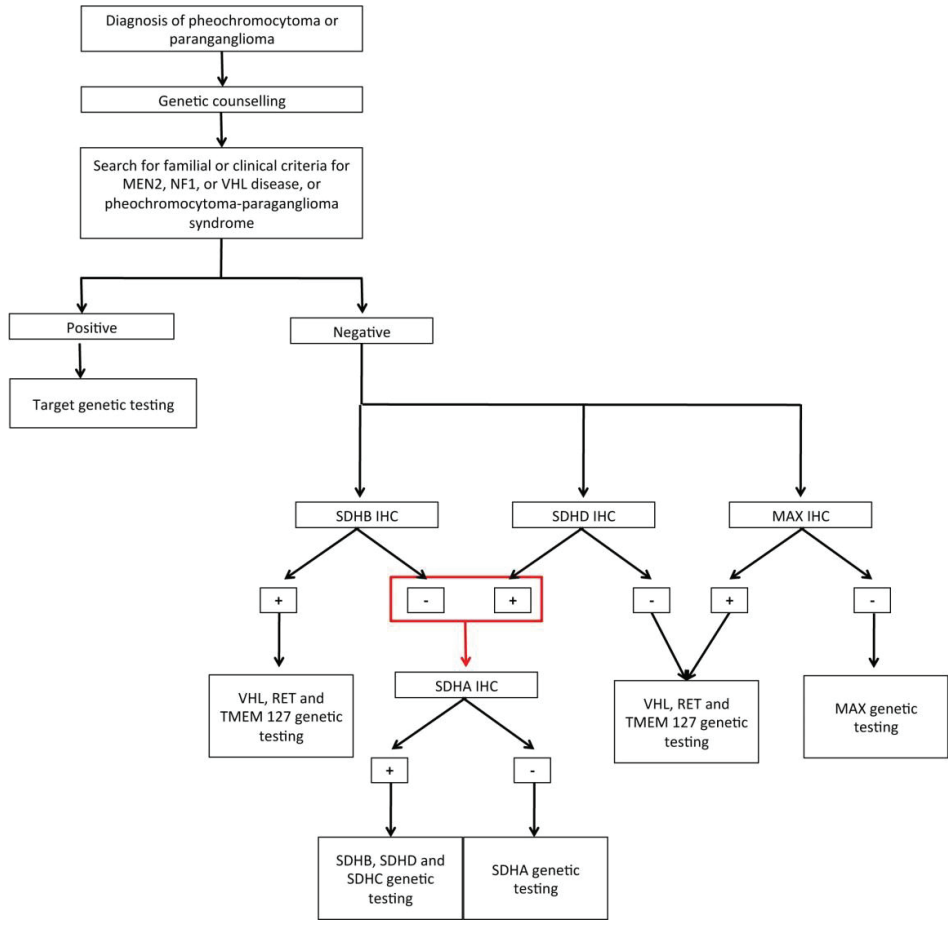
Supplemental Figure 1. SDHD mRNA levels in PGL/PCC with different genotype. Mean relative expression values are shown for normal adrenal tissues (n=2), sporadic (SPO) PGL/PCC (n=71), and tumors harboring either germline or somatic mutations in *RET* (n=15), *NF1* (n=33), *VHL* (n=38), *SDHA* (n=1), *SDHB* (n=17), *SDHC* (n=2), and *SDHD* (n=3) genes. Graphs were generated form transcriptome data generated and described elsewhere (18).



Supplemental Figure 2. SDHD immunohistochemistry in the adjacent adrenal cortex. SDHD immunohistochemistry is positive in the adjacent adrenal cortex of all types of PCC, independently of the mutated gene. For non-*SDHx* tumors such as *RET*, *NF1* or sporadic tumors, the tumor is negative but the adrenal cortex is still positive. However, in *SDHx* tumors such as *SDHB*, the tumor and the cortex are both positive.



Supplemental Figure 3. Three dimensional SDH structure of Escherichia coli succinate dehydrogenase (PDB : 2WS3). The localization of the corresponding epitope sequence recognized by the SDHD antibody in the SDH complex is shown in red.



Supplemental Figure 4. Algorithm for genetic counseling, including immunohistochemical analyses.

Chapter 7.

Immunohistochemical expression of stem cell markers in pheochromocytomas/paragangliomas is associated with *SDHx* mutations

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Eur J Endocrinol. 2015 Jul;173(1):43-52.

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Funding:
The research leading to these results received funding from the Seventh Framework Programme (FP7/2007-2013) under grant agreement no. 259735 (ENSAT-CANCER) and from the Wilhelm Sander Stiftung (to F. Beuschlein). C.M. Neuhofer received a short visit grant (reference number 5228) from the European Science Foundation, European Network for the Study of Adrenal Tumours (ESF-ENS@T).

Abstract

Objective: Pheochromocytomas (PCCs) are neuroendocrine tumors that occur in the adrenal medulla, whereas paragangliomas (PGLs) arise from paraganglia in the head, neck, thorax, or abdomen. In a variety of tumors, cancer cells with stem cell-like properties seem to form the basis of tumor initiation because of their ability to self-renew and proliferate. Specifically targeting this small cell population may lay the foundation for more effective therapeutic approaches. In the present study, we intended to identify stem cells in PCCs/PGLs.

Design: We examined the immunohistochemical expression of 11 stem cell markers (SOX2, LIN28, NGFR, THY1, PREF1, SOX17, NESTIN, CD117, OCT3/4, NANOG, and CD133) on tissue microarrays containing 208 PCCs/PGLs with different genetic backgrounds from five European centers.

Results: SOX2, LIN28, NGFR, and THY1 were expressed in more than 10% of tumors, and PREF1, SOX17, NESTIN, and CD117 were expressed in <10% of the samples. OCT3/4, NANOG, and CD133 were not detectable at all. Double staining for chromogranin A/SOX2 and S100/SOX2 demonstrated SOX2 immunopositivity in both tumor and adjacent sustentacular cells. The expression of SOX2, SOX17, NGFR, LIN28, PREF1, and THY1 was significantly associated with mutations in one of the succinate dehydrogenase (*SDH*) genes. In addition, NGFR expression was significantly correlated with metastatic disease.

Conclusion: Immunohistochemical expression of stem cell markers was found in a subset of PCCs/PGLs. Further studies are required to validate whether some stem cell-associated markers, such as SOX2, could serve as targets for therapeutic approaches and whether NGFR expression could be utilized as a predictor of malignancy.

Introduction

Pheochromocytomas (PCCs) are catecholamine-producing neural crest-derived tumors of the adrenal medulla. Paragangliomas (PGLs) are closely related to PCCs and arise from paraganglia of the head and neck or of the sympathetic trunk.(1)

Although the majority of PCCs/PGLs occur sporadically, about one-third of these tumors develop as a result of germline mutations.(2,3) So far, 16 genes are known to be associated with PCCs/PGLs: *SDHA*, *SDHB*, *SDHC*, *SDHD* and *SDHAF2* (together *SDHx*), *VHL*, *RET*, *NF1*, *TMEM127*, *MAX, KIF1B,* and *PHD2*, as well as the recently identified *HIF2A* (2,4-6), *HRAS (7)*, *FH* (8), and *PHD1* (9). The identification of distant metastases is still the only proof of malignancy in PCCs/PGLs, and because treatment options are limited, finding an appropriate strategy poses a clinical challenge.(10) A better mechanistic understanding of tumorigenesis, proliferation and malignant behavior is therefore warranted.

Many tumors, including PCCs and PGLs, are known to be composed of a variety of cells with different functional properties that are likely caused by an increasing number of genetic alterations.(11) However, according to the cancer stem cell theory, tumor heterogeneity could also result from stem cell-like cancer cells (SCCs), which provide the very basis of cellular tumorigenesis.(12,13) Physiologically, stem cells are defined by three functional properties: i) proliferation, including self-renewal and asymmetric cell division; ii) differentiation to maintain organ function; and iii) homeostatic control to balance between self-renewal and differentiation.(14) Adult stem cells are multipotent or designated progenitors and can differentiate into only a limited number of cell types. In contrast to their non-pathogenic counterparts, homeostatic control is lost in SCCs, which results in extensive uncontrolled proliferation and allows self-renewal and the generation of various subtypes of cells. This in turn leads to tumor heterogeneity. To date, cells with stem cell properties have been identified in various tumor types.(15-17) Whether SCCs derive from malignant cancer cells that acquire stem cell characteristics or from somatic progenitor cells that turn malignant is not well understood yet.(12) However, tumor hypoxia could contribute to this conversion (18), and both processes might play a role.

Stem cells are believed to account for only ~0.1% of a tumor's total mass but are thought to be responsible for most of its proliferation and the malignant properties. In addition to identifying SCCs for prognostic purposes, specifically targeting this small cell population may lay the foundation for more effective therapeutic approaches.(12,19) To investigate whether stem cells or stem cell signaling can be found in PCCs/PGLs, we chose candidate genes that were previously reported to be associated with stem cells or SCCs. We examined the immunohistochemical expression of the most promising embryonic, hematopoietic, neural, and mesenchymal stem cell (MSC) markers in a large series of PCCs and PGLs. Finally, we correlated stem cell marker expression with genetic background and tumor behavior.

Subjects and Methods

Candidate marker selection

A list of relevant progenitor markers identified in stem cells and SCCs was generated by a literature search and was examined by immunohistochemistry (IHC) on tissue microarrays (TMA). These candidate SCC markers included: the embryonic stem cell (ESC) markers *LIN28, OCT3/4, SOX2,* and *NANOG;* the hematopoietic stem cell (HSC) markers *SOX17, PROMININ (CD133), c-KIT (CD117)*, and *THY1*; the neural progenitor marker *NESTIN*; and the MSC markers *NGFR* and *PREF1 (DLK1)*.

Patients and tumor samples

IHC was performed on PCCs/PGLs from 216 patients from five European centers (81 from France, 60 from Italy, 48 from Spain, 20 from Germany, and 7 from The Netherlands). Patient characteristics were collected on the basis of the European Network for the Study of Adrenal Tumors (ENS@T) registry (www.ensat.org). Sample and data collection was approved by the local ethical committees of partaking centers, and all of the patients provided written informed consent.

For immunohistochemical analysis, five TMAs were constructed using an ATA-27 Automated Tissue Microarrayer (Beecher Instruments, Sun Prairie, WI, USA). For each case, two areas of tumor tissue were selected and marked on a representative hematoxylin and eosin (H&E)-stained slide. Tissue cylinders with a diameter of 1 mm were punched from the representative areas of the 'donor' block and brought into the 'recipient' paraffin block at predefined coordinates. Normal liver, kidney, placenta, adrenal cortex, and adrenocortical carcinomas were included in the TMAs as internal controls. Moreover, whole sections of four normal adrenal glands were used as a control.

Eight tumors were excluded from further analyses (because of insufficient clinical data in two cases and six tissue core dropouts on the TMA slides). A summary of the clinicopathological characteristics of the remaining 208 PCC/PGL patients analyzed in the present study is provided in Table 1. For two patients, the primary tumor and corresponding metastasis were included, and for one patient, only metastatic tumor tissue was available. Both the germline and somatic DNA of all tumors except nine were genetically analyzed for mutations in *SDHA*, *SDHB*, *SDHC*, *SDHD*, *SDHAF2*, *VHL*, *RET*, *NF1*, *MAX*, *TMEM127,* and *HRAS*.

Table 1. Patient characteristics.

Baseline characteristic	n=208	%
Gender		
Male	91	43.7
Female	117	56.3
Age (median 45 years)		
<45	97	46.6
≥45	111	53.4
Genotype (pheochromocytoma)	**166**	**100**
VHL germline/somatic	14/3	8.4/1.8
RET germline/somatic	29/3	17.5/1.8
NF1 germline/somatic	8/4	4.8/2.4
MAX germline/somatic	4/1	2.4/0.6
TMEM127 germline	2	1.2
SDHB germline	1	0.6
SDHD germline	2	1.2
HRAS somatic	4	2.4
Non-mutated	83	0.5
Not examined	8	4.8
Genotype (abdominal paraganglioma)	**21**	**100**
VHL germline/somatic	1/1	4.8/4.8
SDHB germline	7	33.3
SDHC germline	2	9.5
SDHD germline	3	14.3
Non-mutated	6	28.6
Not examined	1	4.8
Genotype (head and neck paraganglioma)	**17**	**100**
SDHB germline	5	29.4
SDHD germline	10	58.8
Non-mutated	2	11.8
Genotype (metastasis)	**3**	**100**
SDHB germline	2	66.7
Non-mutated	1	33.3
Genotype (unknown localization)	**3**	**100**
RET germline	1	33.3
SDHD germline	2	66.7
Malignant potential		
Non-metastatic	193	92.8
Metastatic	13	6.3
Not available	2	0.9

Immunohistochemistry

IHC for OCT3/4, SOX2, SOX17, LIN28, NANOG, CD133, CD117, NGFR, NESTIN, THY1, and PREF1 was performed on 4-5 µm sections that were cut from the TMAs. The sections were deparaffinized, rehydrated, and exposed to heat-induced epitope retrieval; they were then incubated in 3% H_2O_2 in PBS for 20 min. The primary antibody specifications and experimental conditions are shown in Supplementary Table S1, see section on supplementary data given at the end of this article.

For SOX2, SOX17, NANOG, LIN28, and PREF1, a biotinylated rabbit anti-goat secondary antibody was used, and for THY1, a biotinylated goat anti-rabbit antibody was used. After 30 min of incubation with the secondary antibody, the slides were rinsed in PBS. Then, Avidin Biotin Complex solution (ABC, Vectastain ABC Kit, Burlingame, CA, USA, no. PK-6100) was applied for 30 min at room temperature. For CD133, Dako ChemMate Envision HRP rabbit-mouse was applied for 30 min (Dako Envision Kit, Glostrup, Denmark). Slides were again rinsed in PBS, and bound antibody complex was visualized with DAB (3'3'Diaminobenzidine, Dako Envision Kit), which was applied twice for 5 min each, after which the slides were washed with distilled water, dehydrated, counterstained with hematoxylin, and coverslipped using permanent mounting medium. For PREF1 and THY1, the counterstaining was performed by incubating the slides for 13 min at 60°C with methylgreen (Vector Laboratories, no. H-3402).

Testicular embryonic carcinoma/CIS was used as a positive control for all of the markers. Normal endothelial cells served as an internal positive control for SOX17 (see Fig. 1), as did human pancreas tissue for CD133.

Staining for CD117, NESTIN, and NGFR was performed with a BenchMark XT automated immunostainer (Ventana Medical Systems, Tucson, AZ, USA). The positive control tissues were human kidney for NESTIN and colon for CD117 and NGFR. In PCCs/PGLs, the fibrovascular network surrounding the tumor cell nests served as an internal positive control for NESTIN and NGFR, whereas mast cells served as an internal positive control for CD117 (see Fig. 1).

SOX2/S100 and SOX2/chromogranin A double staining were performed on whole sections of tumors that corresponded to the positive cores to determine if the S100-expressing sustentacular cells co-expressed SOX2. This protocol is available upon request. 3-Amino-9-ethyl-carbazole (A5754; Sigma)/H_2O_2 was used for nuclear red SOX2 staining, and Fast Blue/naphthol AS-MX phosphate (F3378 and N500; Sigma) was used for cytoplasmic blue S100/CgA staining.(20)

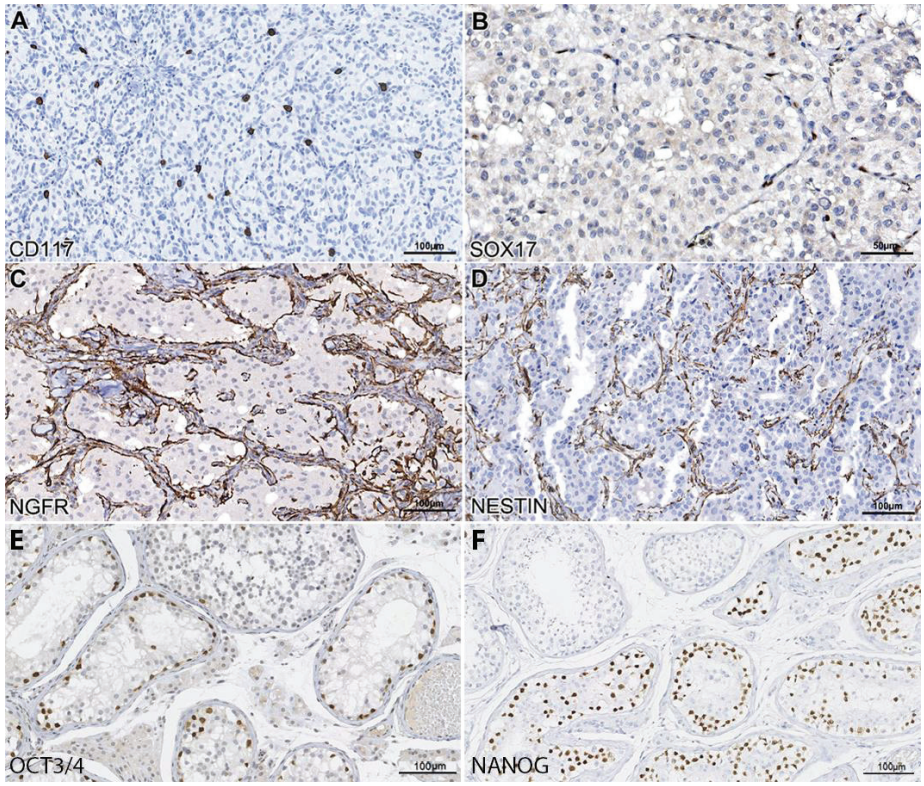

Figure 1. Immunohistochemical staining of human positive control tissue. Representative images showing CD117 staining of mast cells (**A**), SOX17 staining of normal endothelial cells (**B**), staining of the fibrovascular network surrounding the tumor cell nests for NGFR (**C**) and NESTIN (**D**), and staining of testicular embryonic carcinoma for OCT3/4 (**E**) and NANOG (**F**).

Scoring of TMAs

An overview of the scoring (nuclear, cytoplasmic or membranous) is provided in Supplementary table S1, see section on supplementary data given at the end of this article. If positive staining was present, a quantity score of 1-3 was given to each core (1=<10% of cells; 2=10-50% of cells, and 3=>50% of cells). For THY1 and PREF1, staining intensity was scored in three grades (1=weak, 2=moderate, and 3=strong) and multiplied by the quantity score to establish a final score that ranged from 0 to 9. The resultant score was classified as negative (0), weak (1-2, or 1+), moderate (3-4, or 2+), or strong (6-9, or 3+). The scoring system was established with an expert endocrine pathologist (RdK) and carried out by three observers (L.O., C.N. and T.P.) for each marker. A consensus score was reached in case of discrepancy. The highest score of the paired cores was taken into consideration.

Statistical analysis

All immunohistochemical quantity scores of 1 to 3 were considered as positive for the statistical analysis. Because of background staining, negative and weak scores of THY1 and PREF1 were considered negative, whereas moderate and strong THY1 and PREF1 staining was considered positive for the statistical analysis. Associations of immunohistochemical expression between markers as well as those of marker expression vs clinical and pathological parameters were analyzed using Fisher's exact test in Stata version 11 (Stata Corp., College Station, TX, USA). FDR values and two-sided *P*-values of <0.05 were considered statistically significant.

Results

Immunohistochemistry

TMAs containing 208 cases of PCC/PGL (including three metastases) were analyzed for the ESC markers LIN28, OCT3/4, SOX2, and NANOG, the HSC markers SOX17, CD133, CD117, and THY1, the neural progenitor marker NESTIN, the neural crest stem cell (NCSC), and the MSC markers NGFR and PREF1/DLK1. No OCT3/4, CD133, or NANOG expression was seen in any of the cores. The scores and percentages of the other investigated markers are displayed in Table 2, and they are exemplified in Fig. 2 for SOX2. Figure 3 provides an overview of the investigated markers. SOX2-positive cells were found in 12% (25/210) of samples, and in most cases in <10% of the cells. SOX2-positive nuclei were seen in both sustentacular cells as well as in PCC/PGL cells, and this was confirmed by double staining with S100 and chromogranin-A (CgA) (Figs 2 and 4). Nuclear expression of the ESC marker LIN28 was seen in 15% (31/209) of cases, whereas cytoplasmic LIN28 expression was seen in 24% (51/209) of samples, with co-occurrence in 24 cases (40% of all LIN28 positive cores). NGFR expression was shown in 19% (39/210), with an equal distribution of the weak, moderate, and strong expression subgroups. In one patient, from whom both primary and metastatic tumor tissue was included in the TMA, the primary tumor showed nuclear NGFR staining in 10-50% of the tumor cells, while NGFR was negative in the metastatic lesion. Moderate PREF1 staining and NESTIN staining was demonstrated in 5% (10/207) and 3% (7/208) of cases, respectively. The lowest frequencies of expression were seen for the HSC markers SOX17, which was expressed in 2% (5/210) of cases, and *CD117*, which was expressed in 3% (6/10) of cases. The HSC marker THY1 was expressed at a much higher frequency, with positivity in 16% (33/208) of samples. It is important to mention that no positive stem cell marker expression could be seen in the chromaffin cells of any of the four normal adrenal medullary tissues analyzed. However, we did see some SOX2-positive sustentacular cells in the normal adrenals.

Correlations among the investigated stem cell markers by immunohistochemistry

The correlation of marker expression was assessed by the Fischer's exact test (see Supplementary table S2, see section on supplementary data given at the end of this article). Significant relationships were observed between SOX2 and cytoplasmic/nuclear LIN28 (P=0.006/P<0.001), CD117 (P=0.002), and THY1 (P=0.02). Cytoplasmic/nuclear LIN28 expression correlated with SOX17, PREF1 (P<0.001), and NGFR (P=0.001) expression. Furthermore, NGFR expression correlated with PREF1 (P=0.01) and THY1 (P=0.001) expression. THY1 therefore in turn was synergistically expressed with SOX2 (P=0.02), nuclear LIN28 (P<0.001), NGFR (P=0.001), and PREF1 (P=0.048). PREF1 expression correlated significantly with all other markers except for NESTIN and CD117, and SOX17 correlated significantly with all markers except for NESTIN and NGFR.

Associations between stem cell marker expression and clinical pathological features

The correlation of the expression of each stem cell marker was tested with metastatic behavior, tumor size, and genotype. The expression of stem cell markers SOX2, SOX17, NGFR, LIN28, PREF1 and THY1 positively correlated with an *SDHx* mutation status (all P<0.01; Table 3). In *RET*-mutated tumors, there was an association with the absence of THY1 expression (P=0.033). Overall, tumor size did not correlate with stem cell marker expression. Detailed analyses revealed a significant inverse relation between stem cell marker expression and tumor size for CD117 (P=0.003) and a tendency for an inverse relation for SOX2 and nuclear LIN28 (P=0.08 each; Fig. 5A, B, and C). NGFR expression was significantly associated with malignancy (P=0.039; Fig. 5D), whereas a tendency was observed for THY1 expression (P=0.09).

Table 2. Immunohistochemical expression of stem cell markers in pheochromoctyomas/ paragangliomas.

Marker		0	1+	2+	3+	Sum of pos samples (1-3+)	% of pos samples
SOX2		185	21	2	2	25	12
LIN28C	ESC[1]	158	16	25	10	51	24
LIN28N		178	26	5	0	31	15
NGFR	NCSC[2], MSC[3]	171	14	14	11	39	19
PREF1[4]	MSC	167	30	10	0	40	19
NESTIN	Neural progenitor	201	0	7	0	7	3
SOX17		205	3	1	1	5	2
CD117	HSC[5]	204	6	0	0	6	3
THY1[4]		125	50	25	8	83	40

0, negative; 1+, weak or <10% of cells; 2+, moderate or 10-50% of cells; 3+, strong or >50% of cells; pos, positive; C, cytoplasmic; N, nuclear.

[1]Embryonic stem cell, [2]Neural crest stem cell, [3]Mesenchymal stem cell, [4]For PREF1 and THY1, 1+ represents a weak resultant score (staining intensity multiplied by the quantity score), 2+ represents a moderate resultant score, and 3+ represents a strong resultant score, [5]Hematopoietic stem cell.

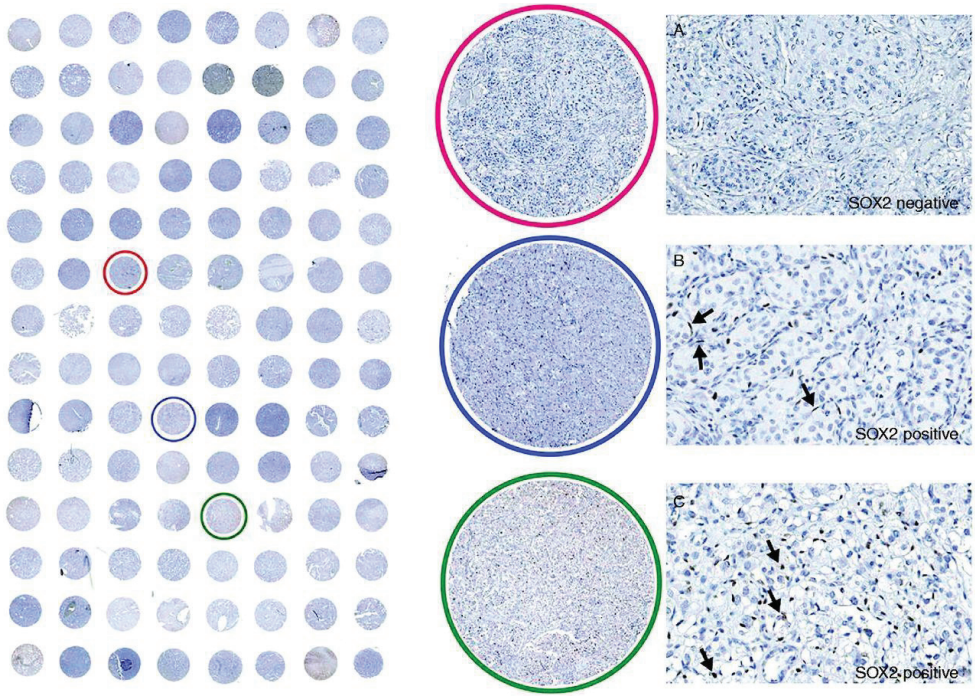

Figure 2. TMA and the expression of SOX2 in human PCCs/PGLs. The upper core is negative for SOX2 (**A**), the central core displays SOX2-positive sustentacular cells (arrows) (**B**), and the lower core displays SOX2-positive tumor cells (arrows) (**C**).

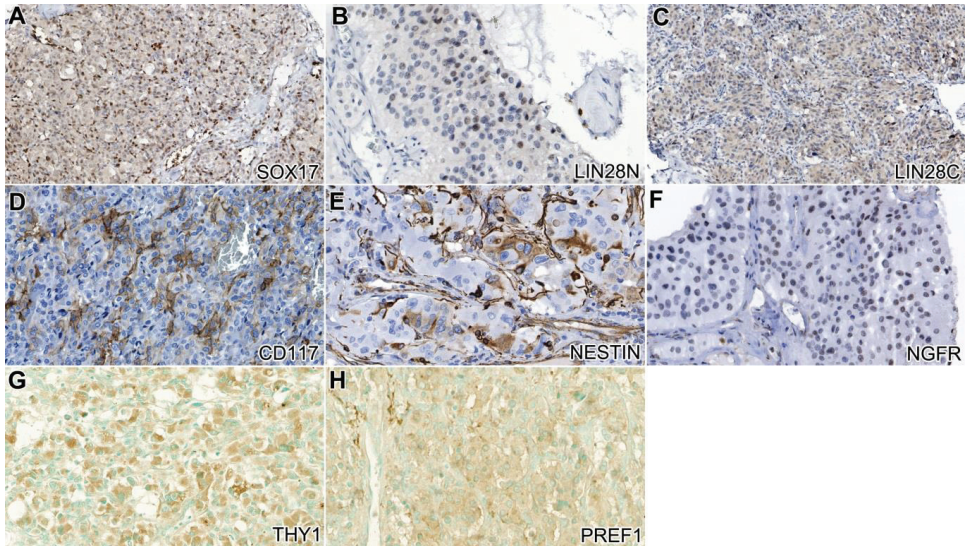

Figure 3. The expression of stem cell markers in human PCCs/PGLs. Representative images showing the expression of nuclear SOX17 (**A**), nuclear (**B**) and cytoplasmic (**C**) LIN28, membranous CD117 (**D**), cytoplasmic NESTIN (**E**), nuclear NGFR (**F**), cytoplasmic THY1 (**G**), and cytoplasmic PREF1 (**H**). Magnification 40x.

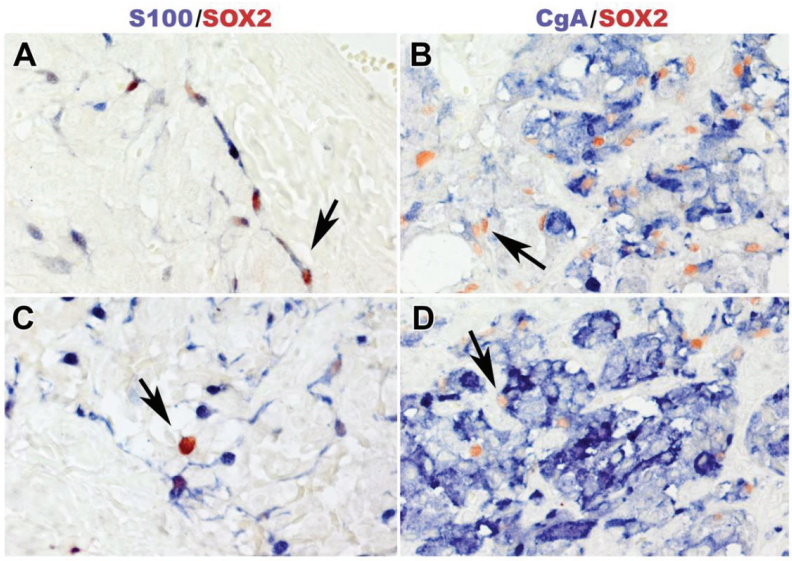

Figure 4. Immunohistochemical double staining for S100/SOX2 (A and C) and chromogranin A/SOX2 (B and D). The arrows in (A) and (B) indicate SOX2-positive sustentacular cells, with positive cytoplasmic staining for S100 (**A**) and negative cytoplasmic staining for CgA (**B**). The arrows in (C and D) indicate SOX2-positive tumor cells, with negative cytoplasmic staining for S100 (**C**) and positive cytoplasmic staining for CgA (**D**). Magnification 40x.

Table 3. Association between stem cell marker expression and *SDHx* mutation status.

	SOX2			SOX17			NGFR			LIN28C[4]			LIN28N[5]			PREF1			THY1		
Genotype	pos[2]	neg[3]	*% pos*	pos	neg	% pos	pos	neg	% pos	pos	neg	% pos	pos	neg	% pos	pos	neg	% pos	pos	neg	% pos
SDHx[1]	11	22	33	4	29	12	13	20	39	21	12	64	22	11	67	12	21	36	29	4	88
other	14	152	8	1	165	0.6	22	144	13	28	138	17	9	157	5	25	138	15	50	114	30
P	<0.001			0.006			0.002			<0.001			<0.001			0.015			<0.001		

[1]*SDHB/SDHC/SDHD*-mutated tumor; [2]Positive; [3]Negative; [4]Cytoplasmic; [5]Nuclear.

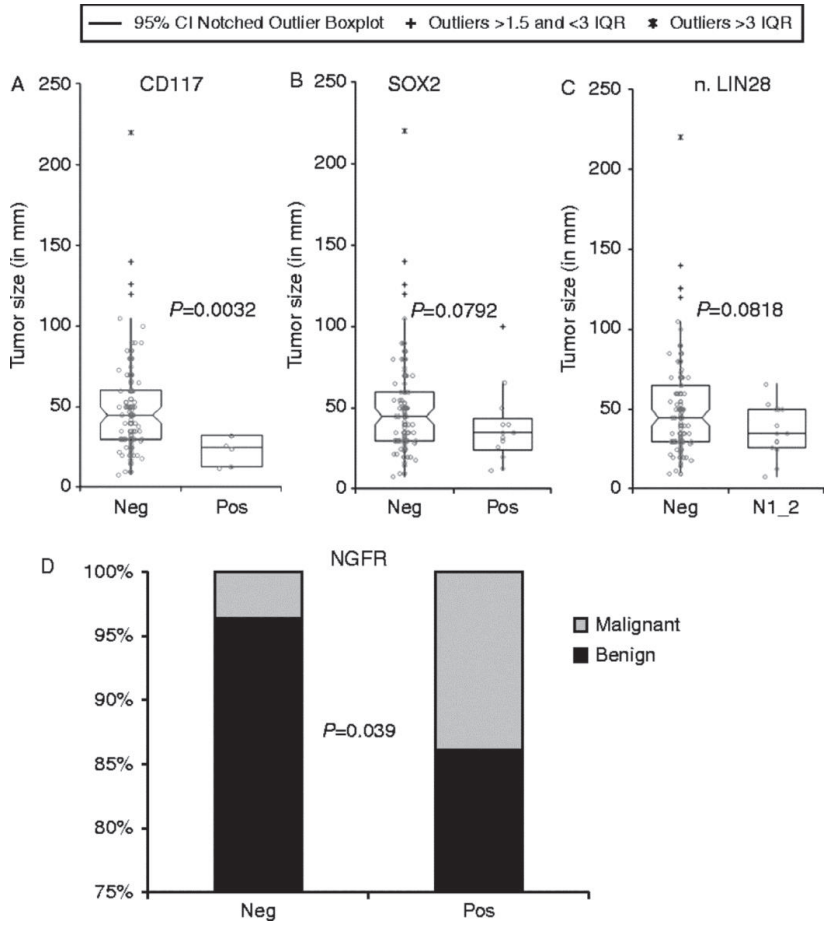

Figure 5. The correlation of immunohistochemical expression of CD117 (**A**), SOX2 (**B**), and nuclear LIN28 (**C**), with tumor size in mm. Horizontal bar: median; skewed vertical bar: 95% CI. 50% of the samples are included in the depicted boxplot. (**D**) Non-metastatic/metastatic disease vs NGFR expression.

Discussion

Stem cell markers have not yet been systematically studied in PCCs/PGLs. Given the accumulating evidence to support the presence of SCCs in other endocrine tumors (21), we investigated stem cell marker expression in a large series of PCCs/PGLs by IHC analysis for the potential association between stem cell phenotype, genotype and/or metastatic behavior. Whereas *OCT3/4* and *NANOG,* which take part in a molecular network that has been shown to induce pluripotency in somatic cells (22), were not expressed in any PCCs/PGLs, all other markers were detectable at variable frequencies. The present findings are consistent with those of Looijenga et al. (23), who demonstrated OCT3/4

immunonegativity in 36 PCCs/PGLs that they examined, whereas the study by Alexander et al.(24) showed strong diffuse cytoplasmic OCT4 expression in 30 PCCs. Although this discrepancy might be attributable to the employment of different antibodies or IHC techniques, the present findings in our large, multicenter cohort argue against the assertion that the cytoplasmic OCT4 staining pattern can be regarded as a stem cell marker in PCCs/PGLs.

In our TMA series, SOX2, LIN28, SOX17, NGFR, and THY1 appeared to be frequently co-expressed and were all significantly associated with *SDHx* mutation status, which possibly suggests interdependence or a common regulatory mechanisms. In fact, SOX2 and LIN28 co-expression is in line with the known function of SOX2 as a direct binding partner of LIN28A in a nuclear protein-protein complex that thereby modulates LIN28A activity in ESCs and induced pluripotent stem cells (iPSCs).(25) Nuclear SOX2 expression was the highest and was found in 12% of the PCC/PGL samples. Double staining for S100/SOX2 (sustentacular cells) and for chromogranin A/SOX2 (tumor cells) identified SOX2 expression not only in sustentacular cells within normal adrenal glands, but also in tumor cells of PCCs. So far, sustentacular cells have been considered to be non-neoplastic cells (26,27), but the precise origin and nature of this cell type in PCCs/PGLs has not yet been fully clarified. Histological studies have described varying ratios of sustentacular cells in metastatic PCCs as compared to locally growing PCCs (28,29), which suggests their importance for tissue homeostasis in the normal adrenals and their possible role in PCC neoplastic/metastatic potential. Interestingly, in the anterior pituitary gland, S100/SOX2 co-expressing folliculo-stellate cells have been identified and have been proposed to be pluripotent adult stem cells.(21) There are rare reports of sellar neoplasms that are assumed to originate from folliculo-stellate cells (30) and of a distinctive neoplasm with a suggestive derivation from sustentacular cells of the adrenal (26), both of which support the notion that folliculo-stellate cells may become neoplastic. However, in the present cohort, a correlation between SOX2 expression and clinical features or metastatic behavior could not be found. This could be a result of the relatively low numbers of malignant tumors in our TMAs.

Although PCCs and PGLs originate from the neural crest, we could not identify a significant expression of CD133, which has previously been described as a hematopoietic, neural, and cancer stem cell marker.(31) The absence of NESTIN expression in the TMA samples is not unexpected, seeing as NESTIN is commonly found in stem cells of the central nervous system and is not routinely expressed in tissues of the sympathetic nervous system.

Stem cell markers of the hematopoietic system are well established. Because these markers are widely used to screen for progenitor cells in other tissues, we utilized SOX17, CD117, and THY1 in our screening. Of these, we found only THY1 to be expressed in PCCs/PGLs. THY1 (CD90) is commonly used in cell sorting protocols to enrich for hematopoietic (32) and other stem cells, such as progenitor cells of the liver.(33) Its role in oncogenesis is still unclear, but it is found in a variety of cell types and seems to play a role in a large number of cellular

processes.(34) Because of the complex functional background of THY1, the interpretation of THY1 positivity in PCCs/PGLs is difficult, and further research is warranted.

PREF1 is considered to be an MSC marker because of its inhibitory role on adipose tissue differentiation through MEK/ERK signaling.(35) PREF1 was detectable only at very low levels, so it therefore does not seem to be a major player. In contrast, NGFR (p75 low affinity) displayed the second highest expression levels of all of the markers tested. NGFR has been described as being a potent MSC marker (36) and has been found to be expressed, for example, in the progenitor cells of human salivary glands.(37) In the present study, NGFR expression was significantly more often associated with malignant PCCs/PGLs (5/12, or 42%) as compared to non-metastatic PCCs/PGLs (31/194, or 16%). Of interest, Loriot et al. (38) described how activation of the epithelial-mesenchymal transition process might play a critical role in *SDHB*-metastatic PCCs/PGLs, which further addresses the mesenchymal marker NGFR as a marker of interest. The fact that NGFR was also expressed in apparently benign tumor cores could account for the limited specificity of the marker, but on the other hand, it could also highlight the general problem of defining non-metastatic disease in PCC/PGL patients. Certainly, taken into account the limited sample size of metastatic cases in the present series, further studies are needed in order to properly assess NGFR before it can be claimed that it is a potential diagnostic or therapeutic molecular marker that indicated malignancy in these tumors in any given genetic context.

In conclusion, we performed IHC on TMAs from 208 tumors, and we found stem cell marker-positive cells in a subset of PCCs/PGLs. Interestingly, stem cell marker expression was associated with mutations in one of the succinate dehydrogenase (SDH) genes. In addition, NGFR expression was significantly correlated with metastatic disease. Further studies are required to validate if any of these markers could serve as targets for future therapies or as predictors of malignancy.

Acknowledgements

The authors would like to thank the ESF-ENS@T (European Science Foundation, European Network for the Study of Adrenal Tumours) for supporting this cooperative project with a 'Short Visit Grant'. This funding has made an exchange visit possible and thus facilitated and strengthened this cooperation between the Department of Pathology of the Erasmus MC Rotterdam and the Endocrine Research Unit of the Klinikum der Universität München (LMU).

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Supplemental Table 1. Antibodies and experimental conditions.

Antigen	Species	Clone	Cat #	Source	Pretreatment	Dilution	Incubation	Scoring
SOX2	Goat pAb		AF2018	R&D Systems	Tris-EGTA pH 9.0	1:250	2 hr RT	N
SOX17	Goat pAb		GT15094	Neuromics	Tris-EGTA pH 9.0	1:3000	2 hr RT	N
OCT3/4	Mouse mAb	C-10	sc5279	Santa Cruz	Tris-EGTA pH 9.0	1:350	2 hr RT	N
LIN28	Goat pAb		AF3757	R&D Systems	Tris-EGTA pH 9.0	1:600	2 hr RT	C & N
Nanog	Goat pAb		AF1997	R&D Systems	Tris-EGTA pH 9.0	1:400	2 hr RT	N
CD133	Mouse mAb	AC133	130-090-422	MACS Miltenyi Biotec	FLEX Low pH Target retrieval (Dako) in a PT Module (Dako)	1:100	1 hr RT	M
NGFR	Rabbit pAb		HPA004765	Sigma-Aldrich	CC1 (64 min)	1:800	32 min 37°C	N
Nestin	Rabbit pAb		HPA026111	Sigma-Aldrich	CC1 (64 min)	1:8000	32 min 37°C	C
CD117	Rabbit mAb	YR145	117R-16	Cell Marque	CC1 (64 min)	1:200	32 min 37°C	C
Thy1	Rabbit pAb	H-110	sc-9163	Santa Cruz	Sodium citrate pH 6.0	1:100	12 hr 4°C	C
Pref1	Goat pAb	C-19	sc-8624	Santa Cruz	Sodium citrate pH 6.0	1:1000	12 hr 4°C	C
S100	Rabbit pAb		Z0311	Dako	Tris-EGTA pH 9.0	1:3200	12 hr 4°C	C
CgA	Rabbit pAb		A0430	Dako	Tris-EGTA pH 9.0	1:1600	12 hr 4°C	C

pAb, polyclonal antibody; mAb, monoclonal antibody; RT, room temperature; N, nuclear; C, cytoplasmic; M , membranous.

Supplemental Table 2. Relationships among the investigated stem cell markers (Fisher's exact).

Marker	LIN28C	LIN28N	NGFR	PREF1	NESTIN	SOX17	CD117	THY1
SOX2	*P*=0.006	*P*<0.001	NS	*P*<0.001	NS	NS	*P*=0.002	*P*=0.015
LIN28C	**X**	*P*<0.001	*P*=0.001	*P*<0.001	NS	*P* =0.001	NS	NS
LIN28N	*P*<0.001	**X**	*P*<0.001	*P*<0.001	NS	*P*=0.002	NS	*P*<0.001
NGFR	*P*=0.001	*P*<0.001	**X**	*P*=0.01	NS	NS	NS	*P*=0.001
PREF1	*P*<0.001	*P*<0.001	*P*= 0.01	**X**	NS	*P*=0.005	NS	*P*=0.048
NESTIN	NS	NS	NS	NS	**X**	NS	NS	NS
SOX17	*P*=0.001	*P*=0.002	NS	*P*=0.005	NS	**X**	NS	NS
CD117	NS	NS	NS	NS	NS	NS	**X**	NS

C, cytoplasmic; N, nuclear; NS, non-significant.

Chapter 8.

mTORC1 complex is significantly over-activated in *SDHx*-mutated paragangliomas

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Submitted

Funding:
The research leading to these results has received funding from the Seventh Framework Programme (FP7/2007-2013) under grant agreement no. 259735. LO received a support from the European Science Foundation (ESF) within the framework of the ESF activity 'European Network for the Study of Adrenal Tumors (ENSAT) (Exchange Grant 4202).

Abstract

We aimed at exploring the activation pattern of mTOR pathway in sporadic and hereditary pheochromocytomas (PCC) and paragangliomas (PGL). A total of 178 PCC and 44 PGL, already characterized for the presence of germline mutations in *VHL*, *RET*, *NF-1*, *MAX*, *SDHA*, *SDHB*, *SDHC*, *SDHD* and somatic mutations in *VHL*, *RET, HRAS* and *MAX*, were included into five TMAs and tested using immunohistochemistry for mTOR and Rictor and the phosphorylated forms of mTOR, p70S6K, AMPK, AKT, 4E-BP1, S6 and Raptor. The positive correlation among most of the molecules investigated proved the functional activation of the mTOR pathway in PCC/PGL. Total mTOR, p-S6K and p-S6 and mTORC1-associated molecules p-Raptor and p-AMPK were all significantly over-expressed in PGLs rather than in PCCs, and in head and neck rather than in abdominal locations. None of the markers, except the low expression of p-mTOR, was associated to malignancy. Concerning genotype-to-phenotype correlations, Cluster 1 had higher total mTOR, p-Raptor and p-S6 expression than Cluster 2 PCC/PGL. In contrast, p-mTOR and mTORC2-associated molecule Rictor were significantly over-expressed in Cluster 2 tumors. Within Cluster 1, molecules active in the mTORC1 complex were significantly over-expressed in *SDHx*- as compared to *VHL*-mutated tumors. In summary, the mTOR pathway is activated in a high proportion of PCC/PGLs, with a preferential over-activation of mTORC1 complex in PGLs of the head and neck and/or harbouring *SDHx* mutations.

Introduction

Pheochromocytomas (PCCs) and paragangliomas (PGLs) are neuroendocrine tumors arising from chromaffin cells of the adrenal medulla or of paraganglia in the head and neck region or along the sympathetic trunk. PCC and PGL can be either familial or sporadic. Germline mutations in the *SDHA*, *SDHB*, *SDHC*, *SDHD*, *SDHAF2* (together *SDHx*), *VHL*, *RET*, *NF1*, *TMEM127*, *MAX* or the most recently identified *HIF2A* are identified in about 40% of PCC/PGL patients.(1) Somatic mutations in *RET*, *VHL*, *MAX* and *HIF2A* genes are also reported in 17% of sporadic tumors. Moreover, recent reports identified somatic *NH1* and *H-RAS* mutations in 22-26% and 5-7% of sporadic PCCs/PGLs, respectively.(2-5) Although the disease is the perfect example of genetic heterogeneity, two main transcriptomic signatures have been evidences. The first one, named cluster 1, is enriched with *VHL*-, *SDHx*- and *FH*-mutated tumors, and shares a pseudohypoxic profile. The second one, named cluster 2, groups tumours related to mutations in *RET*, *NF1*, *TMEM127* and *MAX*, and involves a kinase pathway.(1) The first integrative genomic study, recently published, demonstrated the crucial role of predisposing mutations as being the main drivers of PCC/PGLs.(6)

The mTOR pathway is of great interest since it functionally interacts with genes whose alterations characterize both PCC/PGL clusters. In fact, several cancer models demonstrated that the components of the mTOR pathway have signalling interactions with RET, TMEM127, MAX, NF1 and VHL gene products as well as with the succinate dehydrogenase complex. The mTOR protein is a kinase acting downstream in the phosphoinositide-3-kinase (PI3K)/AKT signalling pathway and forms two multiprotein complexes, named mTORC1 (sensitive to rapamycin) and mTORC2 (resistant to rapamycin). The mTORC1 complex is activated by diverse stimuli, such as growth factors, nutrients, oxygen availability, energy and stress signals in order to control cell growth, proliferation and survival, whereas mTORC2 regulates the cytoskeleton function and is generally insensitive to nutrients and energy signals.(7) Hence, the mTOR pathway has been reported to be de-regulated in several human tumors, including - among others - neuroendocrine ones.(8-11) In PCCs, altered expression of mTOR-pathway molecules (phosphorylated forms of AKT and the mTOR downstream effector S6) has been documented in small series.(12-13) Moreover, total mTOR protein was investigated in a larger series of PCC and PGL apparently with a very low proportion of tumours (5 out of 100 cases) showing mTOR expression.(14) However, despite incomplete evidence of mTOR activation in PCC/PGL tumor tissues, therapeutic strategies selectively inhibiting mTOR have been tested both in vitro and in vivo. In fact, everolimus, a clinically used mTOR inhibitor, proved to be effective, although partially, in patients with progressive malignant PCCs/PGLs (15), whereas the dual inhibition of both mTORC1 and mTORC2 complexes has shown to be highly effective in PCC primary cell cultures and the MTT cell line.(16)

The present study was therefore designed to explore the activation pattern of mTOR signalling pathway in a large series of sporadic and hereditary PCC/PGL, in order to check its relation to clinical, pathological and genetic features.

Materials and Methods

Case series

A total of 222 genetically well-characterized PCCs and PGLs were included in the study from the databases of the following centres: Department of Pathology, Erasmus MC, Rotterdam, The Netherlands (7 cases); the Spanish National Cancer Research Centre (CNIO) and ISCIII Center for Biomedical Research on Rare Diseases (CIBERER), Madrid, Spain (41 cases); INSERM, UMR970, Paris-Cardiovascular Research Center (PARCC)) and Biological Resources Center and Tumor Bank Platform, Hôpital européen Georges Pompidou (BB-0033-00063, 75015 Paris, France (78 cases); the Department of Experimental and Clinical Biomedical Sciences , University of Florence, Florence, Italy (51 cases) and the Division of Pathology, Department of Oncology, University of Turin at San Luigi Hospital, Orbassano, Turin, Italy (45 cases). Institutional review board approval was obtained for the study by each of the centers, and informed consent was obtained from all patients. The overall series included 178 PCC and 44 PGL. Fourteen cases were metastatic. The genetic characterization in all cases for the presence of germline mutations in the *VHL*, *RET*, *MAX*, *TMEM127*, *SDHA*, *SDHB*, *SDHC*, *SDHD* and *FH* and of somatic mutations in *VHL*, *RET* and *MAX* was performed in the enrolling centres as clinical routine work. The presence of *NF1* mutations was determined in cases with clinically suspected neurofibromatosis type 1 (i.e. presence of neurofibromata and skin spots). Methodological conditions are available from the authors upon request. Moreover, *HRAS* mutations were investigated in this series in a recent study by some of the present authors.(5) The baseline characteristics of the included samples are summarized in Table 1.

Table 1. Baseline characteristics of patients.

Baseline characteristic	n=222
Gender	
Male	98
Female	124
Age	
< mean 45	103
≥ mean 45	119
PCC genotype	177
VHL germline/somatic	14/3
RET germline/somatic	30/3
NF1 germline/somatic	6/4
MAX germline/somatic	5/1
TMEM127 germline	2
SDHB germline	2
SDHD germline	3
HRAS somatic	6
No mutation found	98
EA PGL genotype	21
VHL germline/somatic	1/1
NF1 germline	1
SDHB germline	6
SDHC germline	2
SDHD germline	2
No mutation found	8
HNPGL genotype	22
SDHB germline	5
SDHD germline	13
*SDHx** germline	1
No mutation found	3
META genotype	2
SDHB germline	1
FH germline	1
Behaviour	
Non-metastatic	208
Metastatic	14

PCC, pheochromocytoma; EA PGL, extra-adrenal paraganglioma; HNPGL, head&neck paraganglioma; meta, metastasis; **SDHB* immunonegative, but no *SDHB*/*SDHC*/*SDHD*/*SDHAF2* mutation identified with Sanger sequencing.

Immunohistochemistry

Five tissue micro arrays were prepared at the Erasmus University Medical Centre and at the University of Turin for immunohistochemical analysis using the ATA-27 Automated Tissue Microarrayer (Beecher Instruments, Sun Prairie, WI, USA) or the semi-automated Quick-RAYTM tissue arrayer (Bio-Optica, Milan, Italy). For each case, two samples of tumor tissue were selected from a representative hematoxylin and eosin-stained slide, and tissue cylinders with a diameter of 1 mm were punched from the representative areas of the

'donor' block and brought into the 'recipient' paraffin block.

All cases included on the five TMAs were analysed by means of immunohistochemistry using the following antibodies: mTOR (rabbit monoclonal, 7C10, diluted 1:50, Cell Signaling), phospho-mTOR (rabbit monoclonal, 49F9, Ser2448, diluted 1:100; Cell Signaling Tech, Beverly, MA), phospho-p70S6K (mouse monoclonal, 1A5, Thr389, diluted 1:400; Cell Signaling), phospho-AMPK (rabbit monoclonal, 40H9, Thr172, diluted 1:100; Cell Signaling), phospho-AKT (rabbit monoclonal, 736E11, Ser473, diluted 1:40; Cell Signaling), phospho-4E-BP1 (rabbit monoclonal, diluted 1:300; Cell Signaling), p-S6 (rabbit polyclonal, Ser 235/236, diluted 1:400; Cell Signaling), Rictor (rabbit monoclonal, diluted 1:100, Cell Signaling) and phospho-Raptor (rabbit polyclonal, diluted 1:100; Cell Signaling). Immunoreactions were revealed by means of a biotin-free, dextran-chain detection system (Envision, Dako, Glostrup, Denmark) and developed using diaminobenzidine (DAB) as the chromogen. For all antibodies, immunohistochemical staining was scored in each core by multiplying the most prevalent staining intensity (0=negative, 1=weak, 2=moderate, 3=strong) and the quantity of staining (0-100%) giving a final IHC score (IHS) from 0 to 300. The mean score of the two cores for each tumor was recorded for subsequent statistical correlations. All TMAs were evaluated by one of us (LO); moreover, random slides or cases with equivocal staining were assessed at a multihead microscope by two observers (LO and MV) to uniform the staining interpretation or reach a consensus.

Statistical analysis

The association between immunohistochemical findings, known clinical and pathological parameters and genotype was assessed by non-paired Student's t test. The Spearman test was used to analyse the correlation index among the expression of markers. The level of significance was set at $P<0.05$. Statistical analysis was performed using the GraphPad Prism 4 (GraphPad Software, Inc., San Diego, CA).

Results

The mTOR pathway is activated in PCCs/PGLs

The functional activation of the mTOR pathway in the series analyzed was demonstrated by the positive correlation among most of the molecules investigated (Table 2). Total mTOR protein expression was positively associated to p-S6K, p-S6, p-AKT, p-Raptor and p-AMPK expression (Spearman's correlation coefficient R: >0.3). The specific functional activation of the mTORC1 complex was strengthened by the reciprocal correlation of p-Raptor (which couples with mTOR in the mTORC1 complex) and both p-S6K and p-S6, and by the positive correlation of p-AMPK (which specifically interacts with the mTORC1 complex) with p-S6K, p-S6, p-AKT and p-Raptor.

By contrast, Rictor (which couples with mTOR in the mTORC2 complex) was correlated with p-AKT, only. Phospho-mTOR protein, which represents the activated form of mTOR and interacts with both mTORC1 and mTORC2 complexes, was not significantly associated to a specific molecule, except for p-AKT.

Table 2. Reciprocal correlations among markers investigated.

	p-mTOR	**p-S6K**	**p-S6**	**p-AKT**	**p-Raptor**	**Rictor**	**p-AMPK**	**p-4EBP1**
mTOR	R: 0.1019 *P*=0.1347	R: 0.39 *P*<0.0001	R: 0.45 *P*<0.0001	R: 0.34 *P*<0.0001	R: 0.37 *P*<0.0001	R: 0.11 *P*=0.09	R: 0.51 *P*<0.0001	R: 0.23 *P*=0.001
p-mTOR	-	R: -0.02 *P*=0.82	R: 0.02 *P*=0.72	R: 0.34 *P*<0.0001	R: 0.04 *P*=0.59	R: 0.21 *P*=0.002	R: 0.15 *P*=0.02	R: 0.07 *P*=0.32
p-S6K	-	-	R: 0.42 *P*<0.0001	R: 0.18 *P*=0.01	R: 0.40 *P*<0.0001	R: -0.01 *P*=0.89	R: 0.50 *P*<0.0001	R: 0.29 *P*<0.0001
p-S6	-	-	-	R: 0.19 *P*=0.006	R: 0.47 *P*<0.0001	R: 0.03 *P*=0.66	R: 0.41 *P*<0.0001	R: 0.22 *P*=0.001
p-AKT	-	-	-	-	R: 0.16 *P*=0.02	R: 0.38 *P*<0.0001	R: 0.38 *P*<0.0001	R: 0.18 *P*=0.008
p-Raptor	-	-	-	-	-	R: 0.00 *P*=0.94	R: 0.32 *P*<0.0001	R: 0.13 *P*=0.06
Rictor	-	-	-	-	-	-	R: 0.16 *P*=0.02	R: 0.16 *P*=0.02
p-AMPK	-	-	-		-	-	-	R: 0.28 *P*<0.0001

The mTORC1 complex is over-expressed in PGLs

Molecules active in the mTOR pathway were differentially expressed in PCCs as compared to PGLs. Total mTOR, p-S6K and p-S6 were all significantly over-expressed in PGLs than in PCCs. The mTORC1-associated molecules (p-Raptor and p-AMPK) showed the same profile. By contrast, p-mTOR and the mTORC2-associated molecule Rictor were over-expressed in PCCs (Table 3). When comparing tumor location, head and neck PGLs displayed a significantly higher expression of mTOR, p-S6K, p-S6, p-AMPK and p-Raptor as compared with abdominal PCC/PGLs (*P*<0.0001 for all markers). This association retained statistical significance restricting the analysis to extra-adrenal and head and neck PGLs, only. Phospho-4EBP1 expression did not show significant differences between tumor type (PCC/PGL) and tumor location (abdominal/head and neck). None of the markers was significantly associated to the presence of malignant behaviour, except for p-mTOR which showed a higher mean IHS in benign cases. When comparing mean age at diagnosis, the expression of p-mTOR and Rictor was higher in older patients (using the median age of 45 years as the cut off), while the expression of p-AMPK and p-4EBP1 was higher in younger patients. Finally, any of the investigated markers was significantly associated to patients' gender.

Table 3. Correlation of mTOR pathway molecules with clinical and pathological characteristics.

	mTOR		p-mTOR		p-S6K		p-S6		p-AKT		p-Raptor		Rictor		p-AMPK		p-4EBP1	
	mean ± SE	*P*	mean ± SE	*P*	mean ± SE	*P*	mean ± SE	*P*	mean ± SE	*P*	mean ± SE	*P*	mean ± SE	*P*	mean ± SE	*P*	mean ± SE	*P*
PCC	42.0 ± 4.7		91.1 ± 5.9		30.7 ± 3.8		14.0 ± 2.6		144.7 ± 6.1		82.8 ± 6.1		155.0 ± 6.3		45.9 ± 6.0		38.8 ± 5.1	
PGL	101.9 ± 16.5	<0.001	42.0 ± 9.1	<0.001	53.3 ± 6.9	0.001	42.3 ± 8.8	<0.001	130 ± 10.9	0.25	144.2 ± 16.2	<0.001	110.1 ± 12.3	<0.001	89.5 ± 15.6	0.004	57.3 ± 12.9	0.17
EAPGL	36.9 ± 12.0		44.3 ± 13.7		31.7 ± 9.7		13.5 ± 5.3		114.0 ± 15.8		81.0 ± 17.9		122.1 ± 20.2		52.4 ± 18.4		74.3 ± 20.1	
HNPGL	163.9 ± 23.5	<0.001	39.8 ± 12.3	0.72	75.0 ± 7.3	0.001	69.7 ± 14.2	<0.001	145.2 ± 14.6	0.19	204.5 ± 19.4	<0.001	98.1 ± 14.0	0.55	125.0 ± 22.7	0.015	41.1 ± 16.0	0.16
non-mets	54.7 ± 5.5		84.2 ± 5.4		34.8 ± 3.3		18.8 ± 2.8		143.7 ± 5.5		93.0 ± 6.1		148.0 ± 5.9		55.0 ± 6.0		40.4 ± 4.8	
mets	35.8 ± 11.6	0.86	35 ± 11.4	0.02	37.3 ± 11.9	0.60	28.6 ± 18.6	0.82	114.6 ± 16.8	0.16	130.4 ± 29.4	0.24	144.6 ± 28.0	0.75	42.3 ± 19.5	0.66	78.6 ± 27.9	0.33
Female	51.7 ± 7.0		88.1 ± 7.3		34.2 ± 4.3		13.7 ± 2.8		136.9 ± 7.1		88.2 ± 7.8		150.2 ± 7.9		54.1 ± 8.0		39.3 ± 6.2	
Male	56.0 ± 7.9	0.65	72.7 ± 7.4	0.16	35.9 ± 4.6	0.87	26.7 ± 5.2	0.13	148.0 ± 7.9	0.38	104.4 ± 9.3	0.19	144.6 ± 8.4	0.64	54.3 ± 8.4	0.80	47.3 ± 7.7	0.31
age<mean	54.9 ± 7.8		67.4 ± 7.2		39.7 ± 5.0		26.0 ± 4.9		134.0 ± 7.8		105.8 ± 9.5		134.4 ± 8.6		68.6 ± 9.5		51.9 ± 7.8	
age>mean	52.5 ± 7.0	0.98	93.1 ± 7.3	0.01	30.8 ± 3.9	0.24	13.7 ± 3.0	0.35	148.8 ± 7.2	0.11	86.2 ± 7.5	0.19	159.5 ± 7.5	0.02	41.7 ± 6.8	0.04	34.9 ± 5.9	0.04

PCC, pheochromocytoma; PGL, paraganglioma; EAPGL, extra adrenal paraganglioma; HNPGL, head and neck paraganglioma; mets, metastatic.

***mTOR pathway activation is associated with specific genotypes*.**

The expression of the mTOR markers tested across the diverse PCC/PGL susceptibility genes and in cases with no germline or somatic mutations detected (n=109) was heterogeneous (Table 4). Highest levels of expression of mTOR, pS6K, pS6, p-Raptor and p-AMPK were detected in *SDHx*-mutated tumors. By contrast, *TMEM127*-mutated cases had very low protein expression levels of all markers. Cases with no known mutations showed expression levels for each marker generally close to the mean levels of the overall series. P-4EBP1 and p-AKT were the only markers that lacked any significant association with tumor genotype. For statistical comparison, all tumors from patients with known mutations in one of the PCC/PGL susceptibility genes were arbitrarily grouped into clusters: Cluster 1 included *SDHx*, *FH*- and *VHL*-mutated tumors (n=53), whereas Cluster 2 included *NF1*, *RET*, *TMEM127*, *MAX* and *H-RAS* PCC/PGL (n=57). Cluster 1 tumors had significantly higher total mTOR, p-Raptor and p-S6 expression than Cluster 2 PCC/PGL. In contrast, p-mTOR and Rictor expression were significantly higher in Cluster 2 tumors as compared to Cluster 1 tumors. Among genes in Cluster 2, a significant difference was observed between *MAX* and *H-RAS* for p-mTOR expression (*P*=0.0170), and between *RET* and *H-RAS* for p-S6K expression (*P* =0.0128). More interestingly, within Cluster 1, *VHL*- and *SDHx*-mutated cases showed significantly different mTOR pathway profiles. Molecules active in the mTORC1 complex (p-AMPK and p-Raptor) and mTOR itself were significant over-expressed whereas p-mTOR expression was reduced in *SDHx*- as compared to *VHL*-mutated tumors (Figure 1).

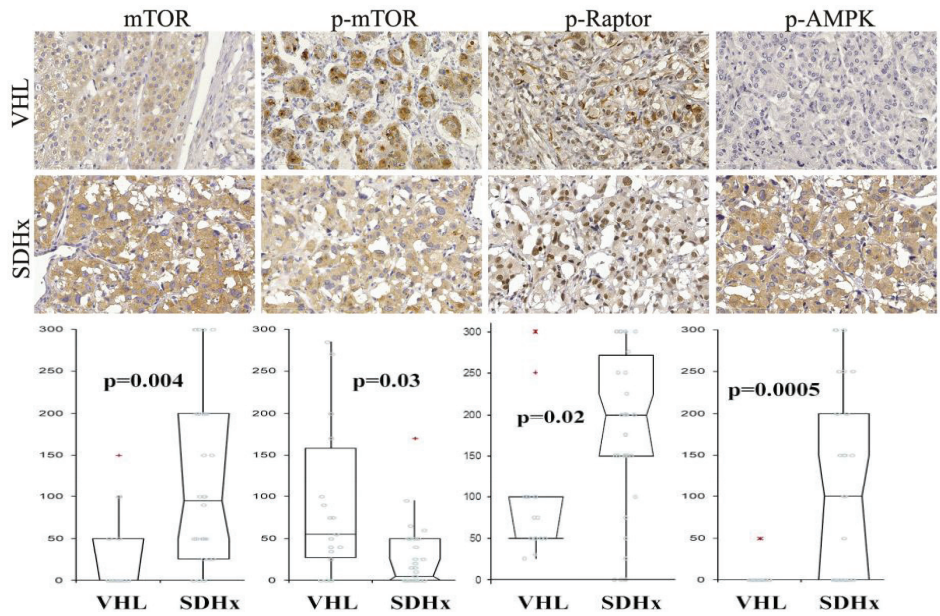

Figure 1. Representative immunohistochemical pictures (**above**) and boxplots (**below**) showing the differential expression of mTOR-pathway molecules in *VHL* as compared to *SDHx*-mutated tumors.

Table 4. Correlation of mTOR pathway components with genotype.

	mTOR		p-mTOR		p-S6K		p-S6		p-AKT		p-Raptor		Rictor		p-AMPK		p-4EBP1	
	mean ± SE	*P*	mean ± SE	*P*	mean ± SE	*P*	mean ± SE	*P*	mean ± SE	*P*	mean ± SE	*P*	mean ± SE	*P*	mean ± SE	*P*	mean ± SE	*P*
H-RAS (#6)	58.33 ± 23.86		166.67 ± 28.07		5.0 ± 3.16		10.0 ± 6.32		183.3 ± 30.73		73.3 ± 29.20		153.33 ± 18.65		33.33 ± 24.72		40.83 ± 20.02	
MAX (#6)	30.0 ± 30.0		37.0 ± 13.38		18.0 ± 7.84		0.0		130.0 ± 48.99		43.0 ± 28.27		100.0 ± 25.0		10.0 ± 10.0		108.33 ± 49.20	
NF1 (#10)	45.91 ± 20.64		73.18 ± 16.79		21,36 ± 5.80		20 ± 11.06		159.1 ± 25.95		102.3 ± 23.94		137.27 ± 28.99		31.82 ± 13.94		45.45 ± 31.23	
RET (#33)	41.29 ± 12.64		110.61 ± 13.90		61.56 ± 11.28		16.6 ± 7.23		142.6 ± 15.62		90.0 ± 16.58		167.58 ± 14.55		103.03 ± 19.09		48.28 ± 12.76	
TMEM127 (#2)	50.0 ± 50.0		12.5 ± 12.5		10 ± 0		0.0		25.0 ± 25.0		7.5 ± 7.5		50.0 ± 50.0		25.0 ± 25.0		0.0	
SDHX (#35)	118.09 ± 18.60		24.24 ± 6.36		62.58 ± 7.74		49.47 ± 10.80		128.9 ± 10.64		180.0 ± 16.71		110.29 ± 13.23		108.82 ± 18.58		54.57 ± 12.58	
FH (#1)	0.0		0.0		0.0		0.0		100.0		100.0		300.0		0.0		0.0	
VHL (#17)	26.32 ± 10.38		90.0 ± 20.88		29,11 ± 8.90		28.16 ± 11.24		114.2 ± 16.30		87.1 ± 16.32		128.16 ± 17.98		5.26 ± 3.62		60.26 ± 22.51	
NO MUT* (#109)	36.09 ± 7.05	0.002	85.43 ± 10.55	<0.001	18.48 ± 4.05	<0.001	13,80 ± 5.92	0.003	149.81 ± 7.51	0.12	75.65 ± 7.14	<0.001	137.5 ± 10.04	0.031	26.09 ± 8.48	<0.001	26.91 ± 8.32	0.33
CLUSTER1 (#53)	86.83 ± 14.01		45.29 ± 9.3		51.24 ± 6.32		42.16 ± 8.16		124.15 ± 9.09		149.15 ± 13.74		116.06 ± 10.81		73.08 ± 13.97		40.83 ± 20.02 ±	
CLUSTER2 (#57)	43.36 ± 8.95	0.012	99.39 ± 10.26	<0.001	41.88 ± 7.24	0.21	14.45 ± 4.69	<0.001	144.82 ± 11.88	0.18	83.60 ± 11.48	<0.001	150.18 ± 11.03	0.018	71.05 ± 12.64	0.9	108.33 ± 49.20	0.65

* *P* value as compared to cases with any type of mutation.

Discussion

The mTOR signalling pathway in PCC/PGLs has attracted research interest because cluster 2 PCC/PGLs are associated with a de-regulation of this pathway and components of the mTOR pathway have signalling interactions with SDHx and VHL gene products (i.e. cluster 1 PCC/PGLs) as well. Therefore, the use of drugs targeting the mTOR pathway has been considered suitable in PCC/PGL patients.

In this study, we investigated the immunohistochemical expression of mTOR-signalling components in a very large series of PCC/PGLs. We correlated the expression of a variety of markers acting in the mTOR pathway with major clinical data and genotype of the tumors. Although a few studies have investigated the protein expression of single or various components of the mTOR pathway.(12-14) in this setting, a comprehensive assessment of all key members of this intracellular signalling cascade in a genetically well-characterized set of PCC/PGLs has not been performed. Examining the entire population, a substantial activation of the mTOR pathway emerged by the positive correlation between mTOR protein expression and its down and upstream regulators, with special reference to those acting in the mTORC1 complex. Our data are partly in contrast with the findings by Pinato (14), who found a very low expression of mTOR and AKT in a series of PCC and PGL. However, in the present study the protein expression data were supported by the integrated analysis of several molecules active in the same pathway which were all consistent and significantly correlated with each other. Phosphorylated-mTOR was not directly correlated with mTOR and other proteins active in the mTORC1 complex except for p-AKT. These findings are probably related to the fact that the phosphorylated form of mTOR is also active in the mTORC2 complex, thus its detection at the tissue level is the consequence of more complex stimuli.

When trying to compare the patterns of expression of all molecules investigated with major clinical and pathological parameters, it was clearly evident that PCC and PGL have opposite profiles of activation. In fact, mTORC1-active molecules and mTOR itself were over-expressed in PGLs as compared to PCCs. This same profile was observed in head and neck PGLs when compared to extra-abdominal tumors. By contrast, mTORC2 protein Rictor and p-mTOR were over-expressed in PCCs as compared to PGLs, but failed to be significantly different when comparing head and neck versus extra-abdominal PGLs. With regard to other clinical or pathological parameters, all markers failed to show relevant associations. Phospho-mTOR was significantly over-expressed in non-metastatic cases, a finding which is in agreement with what observed by Ghayee and coworkers (13) in a smaller series, but this association is more probably the result of the higher expression in PCC cases observed in our entire population. Hence, we might argue that the mTOR pathway is expressed in both benign and malignant PCC/PGLs and mTOR inhibition might be a successful therapy target in malignant PCC/PGL cases which need medical treatment for disease control.

A further aim of this study was to extensively explore the association between mTOR activation status and genotype of PCC/PGL cases. The hypothesis of specific genetic-driven activation profiles of the mTOR pathway in PCC/PGLs partly stemmed from previous observations by some of the present authors on the association between mTOR activation and Ret mutational status in medullary thyroid carcinoma.(11) This hypothesis was also partly sustained by Pinato and co-workers who found, although at very low levels - as commented above - a preferential expression of mTOR and AKT in *SDHx*-mutated tumors.(14)

Comparing genes grouped into the two major molecular clusters, namely *SDHx* and *VHL* in cluster 1 and *NF1*, *RET*, *TMEM127*, *MAX* and *H-RAS* in cluster 2 PCC/PGL, it was strongly evident that mTORC1 complex molecules (including p-S6, p-Raptor and mTOR itself) were over-expressed in Cluster 1 tumors, whereas phospho-mTOR and Rictor were over-expressed in Cluster 2 tumors. Moreover, it is worth to notice that restricting the analysis to Cluster 1 a significant over-expression of some of the above mentioned molecules (p-Raptor and mTOR itself) together with p-AMPK (all belonging to the mTORC1 complex) was observed in *SDHx*- as compared to *VHL*-mutated tumors. These findings overall suggest two major issues: first, that although grouped into major molecular clusters, PCC and PGL with different genetic profiles are characterized by specific and more heterogeneous intracellular signalling activation patterns; second, that if the genetic landscape of tumors is a major responsible for mTOR activation in PCC/PGL, the hypothetical strategy of mTOR-targeting therapies in PCC/PGL should take into consideration not only the biological behaviour of tumors but also their genetic characteristic to be of clinical meaningfulness.

In summary, our data show that the mTOR pathway is activated in a relevant proportion of PCC/PGLs, with a preferential over-expression of mTORC1 complex proteins in PGLs of the head and neck and/or harbouring *SDHx* mutations.

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Chapter 9.

Vascular pattern analysis for the prediction of clinical behaviour in pheochromocytomas and paragangliomas

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PLoS One. 2015 Mar 20;10(3):e0121361.

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Abstract

Pheochromocytomas (PCCs) are neuroendocrine tumors arising from chromaffin cells of the adrenal medulla. Related tumors that arise from the paraganglia outside the adrenal medulla are called paragangliomas (PGLs). PCC/PGLs are usually benign, but approximately 17% of these tumors are malignant, as defined by the development of metastases. Currently, there are no generally accepted markers for identifying a primary PCC or PGL as malignant. In 2002, Favier et al. described the use of vascular architecture for the distinction between benign and malignant primary PCC/PGLs. The aim of this study was to validate the use of vascular pattern analysis as a test for malignancy in a large series of primary PCC/PGLs.

Six independent observers scored a series of 184 genetically well-characterized PCCs and PGLs for the CD34 immunolabeled vascular pattern and these findings were correlated to the clinical outcome. Tumors were scored as malignant if an irregular vascular pattern was observed, including vascular arcs, parallels and networks, while tumors with a regular pattern of short straight capillaries were scored as benign. Mean sensitivity and specificity of vascular architecture, as a predictor of malignancy was 59.7% and 72.9%, respectively. There was significant agreement between the 6 observers (mean κ=0.796). Mean sensitivity of vascular pattern analysis was higher in tumors >5 cm (63.2%) and in genotype cluster 2 tumors (100%).

In conclusion, vascular pattern analysis cannot be used in a stand-alone manner as a prognostic tool for the distinction between benign and malignant PCC, but could be used as an indicator of malignancy and might be a useful tool in combination with other morphological characteristics.

Introduction

Pheochromocytomas (PCCs) are neuroendocrine tumors derived from chromaffin cells of the adrenal medulla. Related tumors that arise from the paraganglia outside the adrenal medulla are called paragangliomas (PGLs). Paragangliomas can be further classified as parasympathetic or sympathetic, depending on their origin. Parasympathetic PGLs are mainly located in the head and neck, and usually do not secrete catecholamines, unlike PCCs and sympathetic PGLs (located in the thorax, abdomen and pelvis) that usually produce adrenaline or noradrenaline.(1) About 40% of PCCs and PGLs carry a germline mutation in one of the following genes: *SDHA*, *SDHB*, *SDHC*, *SDHD* (referred to as *SDHx*), *RET*, *VHL*, *NF1*, *TMEM127*, *MAX*, *SDHAF2,* and the recently identified gene *FH* (for review, see (2)). In addition, somatic mutations of *RET*, *VHL*, *NF1*, *MAX*, *HIF2A,* and *HRAS* can be detected in a further 25-30% of these tumors.(3-6) *SDHx* and *VHL*-related PCC/PGLs are referred to as 'cluster 1' tumors, following microarray gene expression studies, while 'cluster 2' includes the tumors with *RET*, *NF1*, *TMEM127*, and *MAX* mutations.(7,8)

Ten to 17% of PCCs and sympathetic PGLs are malignant, in which cases the prognosis of patients is poor, and treatment is basically palliative. Malignancy in PCC/PGL is defined by the 2004 World Health Organization classification as a chromaffin cell tumor with the presence of metastases to sites where chromaffin tissue should normally not be found (principally bones, liver, lymph nodes and lungs).(9,10) This definition distinguishes malignancy from multifocal disease. Local invasive growth of the tumor into other organs or major blood vessels, as has been reported to predict malignant behavior in other tumor types, does not fulfill the definition of malignancy in PCC/PGL. The diagnosis of malignancy is clear-cut if metastatic lesions are present, but to date, no molecular or histologic markers exist to predict if a primary PCC/PGL has metastatic potential. Metastases can occur after a long latency period, sometimes more than 10 or even 20 years after diagnosis of the primary tumor.(11)

Currently, clinical risk factors for metastasis in patients with PCC/PGLs include tumor location, genotype, and size.(12) Patients with *SDHB*-related PCC/PGLs, extra-adrenal tumors or a primary tumor size over 5 cm have a higher risk to develop metastatic disease. In general, the risk is highest for *SDHB*-mutated tumors (at least 30%), which are usually extra-adrenal and relatively large. Many studies have tried to find biomarkers to differentiate between benign and malignant PCC/PGL, such as the Ki67 labeling index, human telomerase reverse transcriptase (hTERT) or CD44 expression.(13,14) However, none of these markers allows a definite diagnosis of malignancy in PCC/PGL. Also, histologic criteria such as vascular invasion, mitotic activity, or cellular atypia cannot be used to definitely differentiate tumors with the potential to metastasize. The Pheochromocytoma of the Adrenal gland Scaled Score (PASS) was the first scoring system for the diagnosis of PCCs, which combined histopathological features to distinguish between benign and malignant tumors.(15)

However, the reproducibility of the PASS has not been established.(16) Recently, Kimura et al. proposed the combination of GAPP (Grading of Adrenal Phaeochromocytoma and Paraganglioma) classification and SDHB immunohistochemistry for the prediction of metastasis in PCCs.(17)

PCCs and PGLs are highly vascularized tumors, even though there is a lot of variation in the vascular architecture of individual tumors. In 2002, we described the use of the vascular architecture for the distinction between benign and malignant PCC/PGLs.(18) We observed that malignant PCCs displayed a peculiar vascular pattern, which was not found in benign tumors. However, this published series was small and therefore the vascular pattern analysis was not implemented in routine PCC/PGL diagnostics. In the present study we determined, in a multi-center international collaboration, the vascular pattern of 184 genetically well-characterized PCCs and PGLs. The results of these analyses were correlated to the clinical behavior of the tumors. This allows determining the role of vascular pattern analysis in the initial pathological work-up of PCC and PGL patients.

Materials and methods

We analyzed 88 paraffin-embedded tumors collected from 72 PCC/PGL patients collected from the archives of the Department of Pathology of the Erasmus MC Cancer Institute, Erasmus MC, University Medical Center (Rotterdam, the Netherlands) and 96 tumors from 87 patients collected from Paris by the French COMETE network from patients operated in two referral centers in Paris (Hôpital Européen Georges Pompidou and Hôpital Cochin).

The series comprised 59 malignant tumors and 125 benign samples. As defined by the World Health Organization (WHO) in 2004 (19), and by the international guidelines of the Endocrine Society (20) diagnosis of malignancy was based on the presence of a metastasis at a site were chromaffin cells are usually absent. A summary of the clinicopathological characteristics of the tumors is provided in Table 1. The Dutch tissues were used in accordance with the code of conduct Proper Secondary Use of Human Tissue established by the Dutch Federation of Medical Scientific Societies (http://www.federa.org). The study obtained the institutional review board approval (Medical Ethical Committee from Erasmus MC University Medical Center, Rotterdam, The Netherlands) and patients gave their informed written consent. For French tissues, informed signed consent was obtained from each patient recruited by the COMETE network, and the study was formally approved by an institutional review board (Comité de Protection des Personnes (CPP) Ile de France III, June 2012).

Table 1. Clinicopathological characteristics.

Mutated gene	*No. of tumors*	*Sex M/F*	*Age range (yr; mean)*	*PCC*	*PGL*	*Meta*	*B/M*	*Tumor size (mm; mean)*	*FU (yr; mean)*
***VHL* germline**	20	13/7	7–50 (22.3)	16	4	0	17/3	25–80 (47.8)	9.2
***VHL* somatic**	7	2/5	17–56 (37.1)	5	2	0	4/3	18–110 (62)	10.83 (N = 1D)
***RET* germline**	24	4/20	16–76 (38.3)	24	0	0	24/0	9–110 (32.3)	9.97 (N = 1D)
***RET* somatic**	2	1/1	49–62 (55.5)	2	0	0	2/0	NA	7.5
***NF1* germline**	13	4/8 (1U)	26–65 (40)	13	0	0	13/0	25–70 (48.3)	7.75
***NF1* somatic**	5	4/1	37–73 (49.6)	4	0	1	3/2	60–80 (38.3)	7.6
SDHA	2	1/1	32	1	1	0	2/0	60–72 (66)	0.5
SDHB	20	9/11	10–63 (36.6)	8	10	2	5/14 (1U)	40–140 (90.9)	9.83 (N = 4D)
SDHC	1	0/1	16	0	1	0	1/0	45	NA
SDHD	8	8/0	16–62 (36)	2	6	0	4/4	20–100 (51.3)	15.83
TMEM127	2	0/2	44	2	0	0	2/0	NA	15
***HRAS* somatic**	1	0/1	61	1	0	0	1/0	NA	9 (D)
FH	1	0/1	63	0	0	1	0/1	40	10.5 (D)
SPOR	78	37/41	9–79 (48.6)	61 (2U)	10	5	46/32	7–130 (60.5)	7.20 (N = 1D)

SPOR, sporadic; M, male; F, female; U, unknown; PCC, pheochromocytoma; PGL, paraganglioma; Meta, metastasis; B, benign; M, malignant; FU, follow-up; D, died.

Immunohistochemical analysis was performed by staining endothelial cells with a CD34 monoclonal antibody on 4-6 µm sections of formalin-fixed paraffin-embedded tumors.

For French slides, the anti-CD34 (IM0786, Beckman Coulter) was used at a 1:500 dilution. After deparaffinization, rehydration and H_2O_2 treatment, tissues were blocked in goat serum for 30 minutes and the primary antibody was applied at room temperature for 1h. The biotinylated secondary anti-rabbit antibody (Vector Lab; 1:400) was applied for 45 minutes and the revelation was assessed using histogreen kit (Vector Laboratories, EUROBIO/ABCYS, Les Ulis, France). Slides were counterstained with Nuclear Fast red and coverslipped.

For Dutch samples, a 1:75 dilution of the CD34 monoclonal antibody (clone QBEnd/10; Neomarkers) was used, together with a protocol based on the Ventana BenchMark Ultra System (Ventana Medical Systems, Inc, Tucson, AZ). After deparaffinization, slides were submitted to heat-induced epitope retrieval in Ventana Cell Conditioning 1 (pH 8.4) at 99°C for 64 minutes. Endogenous peroxidase was blocked using 3% hydrogen peroxide. The diluted CD34 antibody was applied and incubated for 32 minutes at 36°C. The stains were developed using 3,3'-diaminobenzidine (DAB) as chromogene. Finally, the slides were counterstained in haematoxylin, washed in water, dehydrated through graded alcohol and coverslipped. In all cases, negative control experiments were performed by omitting the primary antibody.

Based on the results from the study by Favier et al., a tutorial (training set) with representative immunohistochemical images of vascular patterns related to benign and malignant tumors (see supplementary figure 1-4) was distributed in advance among the 6 observers (1 research scientist (JF) and 5 trained pathologists (CB, RK, FN, FT, AT)). After this, the immunohistochemical images of the 184 tumors were independently evaluated by the 6 observers. All tumors had to be scored into 4 groups according to their vascular architecture: benign (B+), probably benign (B-), probably malignant (M-) and malignant (M+).

Statistical analysis

To calculate the interobserver agreement the Kappa test was performed. The Chi-square test was used to associate the prediction of the observers with clinicopathological characteristics of the samples, i.e. clinical behavior, tumor size, genotype. Two-sided *P*-values <0.05 were considered as statistically significant. Statistical tests were performed using Analyse-it v2.26 (Analyse-it Software, Ltd. Leeds, United Kingdom).

Results

The vascular architecture of 184 PCC/PGL tumors was revealed by labeling endothelial cells using CD34 immunohistochemistry. Tumors were blindly scored as "probably benign" (B-), "certainly benign" (B+), "probably malignant" (M-), or "certainly malignant" (M+) according to their vascular pattern by six expert pathologists. If the pattern was distributed regularly throughout the whole tissue section, consisting mostly of short, straight vascular segments, tumors were designated as benign. In contrast, if a discontinuous distribution of blood vessels (i.e. highly vascularized zones adjacent to avascular areas) and vascular structures forming arcs, parallels and networks could be identified, tumors were scored as malignant (Figure 1 and Supplemental Figures 2-4).

The interobserver agreement (Kappa agreement)

The interobserver agreements between all 6 observers after sorting the tumors into 4 groups (B+, B-, M-, M+) ranged from a fair to moderate agreement (Table 2). The highest agreement was reached between observer 2 and 3 (κ=0.596) and the lowest between observer 2 and 5 (κ=0.351).

When the tumors were sorted into 2 groups (B+/- vs M+/-) the Kappa agreement was substantial to almost perfect (Table 3). Observer 1 and 4 displayed the highest agreement (κ=0.897) and observer 2 and 5 the lowest ((κ=0.725).

All agreements were highly significant, with *P*<0.0001.

Vascular architecture as a prognostic tool

For each observer, the association between the predicted clinical outcome with the true benign or malignant status of the tumor was investigated. The specificity of this approach, defined as the percentage of benign cases correctly predicted as benign, ranged from 55.3%-79.8% (mean 72.9%). The sensitivity, defined as the percentage of malignant cases correctly predicted as malignant, ranged from 49.2% - 71.9% (mean 59.7%). Observer 1 was the best predictor, with a Youden index of 0.493 (Table 4).

Overall, 35 of 59 (sensitivity is 59%) malignant PCCs were scored as malignant by the majority (i.e. $\geq$4 of 6) of the observers. For benign tumors, there were 94 of 124 (specificity is 76%) tumors that were correctly predicted to be benign. In 13 benign tumors all observers

scored the tumor as malignant.

Taking tumor size into consideration, malignant tumors were significantly more often greater than 5 cm compared to benign tumors (*P*=0.0233). Mean sensitivity of vascular architecture as a prognostic tool in tumors >5 cm was 63.2% (range 50.0% – 78.3%), while in smaller tumors (<5 cm) the mean sensitivity was 57.5% (range 30.58% - 76.8%). Mean specificity was 66.3% (range 50.0% - 73.5%) and 80.8% (range 71.7% - 85.7%) in tumors >5 cm and <5 cm, respectively.

After splitting up the tumors according to genotype cluster (i.e. cluster 1: *SDHx*- and *VHL*-related PCC/PGL; cluster 2: *RET*-, *NF1*-, and *TMEM127*-related tumors), malignant tumors (n=24) clustered more often in genotype cluster 1 (*P*<0.0001), compared to benign tumors (n=33). Mean sensitivity of vascular pattern analysis in genotype cluster 1 tumors was 52.3% (range 33.3% - 68.2%) and the mean specificity 69.1% (range 57.1% - 78.8%). Mean sensitivity in cluster 2 tumors (n=52, of which 49 benign and 3 malignant) was 100% (range 100% - 100%) and the mean specificity 77.4% (range 57.58% - 57.8%).

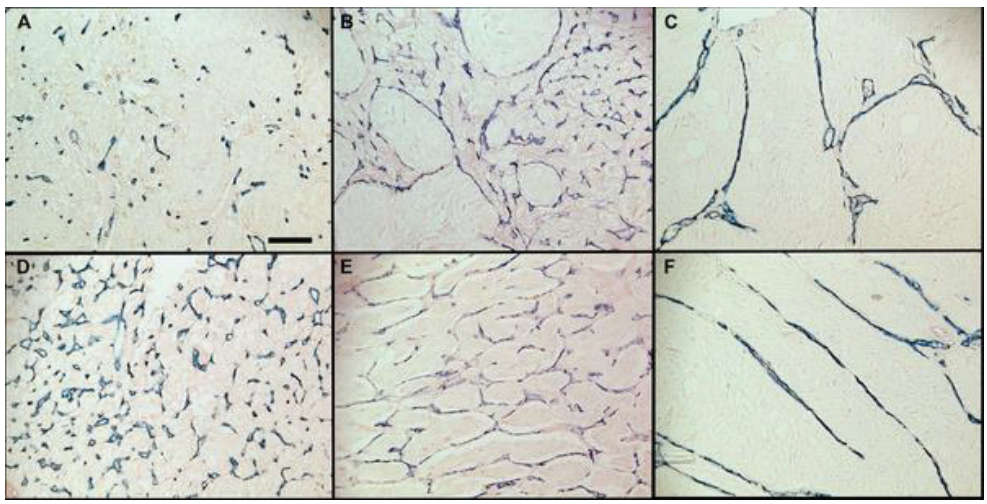

Figure 1. Vascular architecture in PCC/PGLs. Immunostaining of blood vessels with anti-CD34 reveals a homogenously distributed vascular pattern in benign tumors (**A**, **D**), while malignant tumors display irregularity (**B**) and vascular structures forming arcs (**C**), networks (**E**) and parallels (**F**). All panels are at the same magnification. Scale bar = 100μm.

Table 2. Interobserver agreement (Kappa test) of benign, probably benign, probably malignant and malignant tumors (4 groups).

	Observer 1	*Observer 2*	*Observer 3*	*Observer 4*	*Observer 5*
Observer 2	0.546				
Observer 3	0.570	0.596			
Observer 4	0.554	0.525	0.552		
Observer 5	0.424	0.351	0.432	0.471	
Observer 6	0.429	0.393	0.459	0.397	0.430

All agreements *P*<0.0001.

Table 3. Interobserver agreement (Kappa test) of (probably) benign versus (probably) malignant tumors (2 groups).

	Observer 1	*Observer 2*	*Observer 3*	*Observer 4*	*Observer 5*
Observer 2	0.852				
Observer 3	0.849	0.842			
Observer 4	0.897	0.803	0.831		
Observer 5	0.779	0.725	0.747	0.785	
Observer 6	0.783	0.765	0.773	0.788	0.727

All agreements $P<0.0001$.

Table 4. Associating predicted benign/malignant call with TRUE benign/malignant status**.**

	Observer 1	Observer 2	Observer 3	Observer 4	Observer 5	Observer 6
Sensitivity	69.5%	50.8%	50.9%	66.1%	71.9%	49.2%
Specificity	79.8%	74.0%	74.1%	77.4%	55.3%	76.6%
PPV	62.1%	48.4%	48.3%	58.2%	44.6%	50.0%
NPV	84.6%	75.8%	76.1%	82.8%	79.7%	76.0%
Pval	8.3E-11	0.0009	0.0012	1.1E-08	0.0008	0.0005
Youden	0.493	0.248	0.250	0.435	0.272	0.258

Sensitivity is defined as the percentage of TRUE malignant cases correctly predicted as malignant.

Specificity is defined as the percentage of TRUE benign cases correctly predicted as benign.

Pval: *P*-value Chi-square test.

PPV: positive predictive value.

NPV: negative predictive value.

Youden is defined as sensitivity+specificity-1. The higher the Youden, the better the prediction.

Discussion

Currently it is still not possible to state with certainty whether a primary PCC or PGL is benign or malignant. The diagnosis of malignancy depends on the occurrence of metastases, which is rare and can have a latency of many years.(11) Considering the concept that induction of angiogenesis is necessary for tumor growth and metastases, and because PCCs are highly vascularized tumors, we analyzed the vascular pattern of 184 PCC/PGL, immunohistochemically stained with anti-CD34 antibody. Mean sensitivity and specificity of vascular architecture, as a predictor of malignancy was 59.7% and 72.9%, respectively. There was significant agreement between the 6 observers (mean κ=0.796). Mean sensitivity of vascular pattern analysis was higher in tumors >5 cm (63.2%) and in genotype cluster 2 tumors (100%).

The rationale of this study is based on a previous study where we observed an abnormal and discontinuous vascular pattern, including vascular arcs, parallels and networks in 9 of 9 malignant PCCs.(18) On the contrary, 9 of 10 benign tumors in that series showed a homogenous pattern of short straight capillaries. The biological signification of these differences in vascular pattern between benign and metastatic PCC/PGL is unclear. Whether the irregularity in the vascular architecture of malignant tumors corresponds to different steps in the angiogenic process or to a specific growth pattern of tumor cells is difficult to apprehend in the context of the actual study. It may anyhow have an influence on the

oxygenation status of tumor cells and may reflect a capacity of malignant cells to resist to hypoxia. Since the initial cohort of 19 tumors was small (and of incomplete genotyping, at that time), we validated vascular pattern analysis in a larger cohort, fully characterized at the molecular level, and with multiple observers. In the current study, 35 of 59 (59%) malignant PCCs were scored as malignant by a majority of the observers. In contrast, 94 of 124 (76%) benign tumors were correctly predicted to be benign. We could not confirm the high sensitivity of 100% using vascular pattern analysis for the distinction between benign and malignant PCC, as reported in the initial study.

The Pheochromocytoma of the Adrenal gland Scaled score (PASS) was developed in 2002 by Thompson, to distinguish benign from malignant PCC by histopathological parameters.(15) The following features (with values) were scored: large cell nests or diffuse growth (2), central or confluent tumor necrosis (2), high cellularity (2), cellular monotony (2), tumor cell spindling (2), mitotic figures more than 3 per 10 HPF (2), atypical mitotic figures (2), extension into adipose tissue (2), vascular invasion (1), capsular invasion (1), profound nuclear pleomorphism (1) and nuclear hyperchromasia (1). Each histopathological feature of the PASS score was given a value of 1 or 2 and these values are summed up. Of the 50 metastatic tumors investigated in that study, all had a PASS score of more than 4. However, the study also reported that 17 of the 50 benign tumors had a score of more than 4 as well (with a follow-up period of 5 years).(15) Hence, an inappropriately high proportion of tumors were suggested to have malignant potential. This overestimation of malignant tumors is something we also encountered in the present study, although our specificity was higher (76% versus 66%).

Later, in a study by Wu et al. in 2009, five endocrine pathologists investigated the applicability of the PASS and it showed a large inter- and intra-observer variation.(16) In contrast, we found a substantial interobserver agreement (mean κ=0.796) among 6 observers recognizing vascular patterns, indicating that the vascular pattern classification system used in our study is a much less complicated tool. However, because of low sensitivity and specificity the system is unlikely to be useful for stratifying risk of metastasis. Despite this disappointing outcome, it is of interest that several of the vascular patterns might actually provide means to enhance recognition of histologic parameters scored in the PASS including diffuse growth (Fig 1C and 1F) and large cell nests (Fig 1B). The latter are also a component of the risk stratification system proposed by Kimura et al.(17)

Many studies have reported the evaluation of angiogenesis in PCC/PGL by examining the microvascular density (MVD). Most of these studies suggest an increase in MVD in malignant versus benign tumors.(21-24) However, the quantification of MVD appeared not to be a reliable predictor of malignancy, which is confirmed by other studies reporting the absence of a statistical association between microvascular counts and malignancy.(25) MVD counting can be based on the number, length or branching of the vessels, which will definitely influence the results. To our knowledge, our initial study (18) and the present study are the

only ones focusing on the difference in vascular architecture between benign and malignant PCC/PGL, instead of blood vessel counting.

Although the immunohistochemical stainings were performed in two different medical university centers, there was no bias due to the origin of the section, which was also demonstrated by the kappa test.

Twenty-four clinically benign tumors (corresponding to 23 patients) in the present series were scored as malignant by ≥4 observers (Supplemental table 1). Metastasis can occur up to 20 or even 30 years after the occurrence of a primary pheochromocytoma. Hence, we have addressed whether the follow-up was sufficient to clearly establish the benign status of these tumors. For eight tumors (7 patients), we had no follow-up data (most corresponded to surgeries performed at least 25 years ago). One patient has died due to unrelated disease, and one (scored as malignant by 5 observers) actually became metastatic recently. For the 15 other patients, median follow-up was of 10 ± 6.4 years (range 2.3-19). Hence, although the follow-up is quite important for these patients, we cannot exclude that some of these patients will develop a metastatic form of their disease in the future.

In particular, 13 benign PCCs of 12 patients were scored as malignant by all 6 observers, so attention should be paid to the follow-up of these patients. The corresponding cases included 4 *RET* (2 germline and 1 somatic mutation), 2 *VHL* (1 germline and 1 somatic mutation) and 6 sporadic PCCs.

In summary, we tested vascular architecture as a prognostic tool in 184 PCC/PGL after CD34 immunolabeling. Tumors were scored as malignant if a discontinuous vascular pattern was observed, including vascular arcs, parallels and networks, while tumors with a uniform pattern of short straight capillaries were scored as benign. There was significant agreement between observers, indicating that there is a real variance between the different vascular patterns. However, the mean sensitivity, defined as the percentage of TRUE malignant cases correctly predicted as malignant, was only 59.7%. Accordingly, vascular pattern analysis cannot be used in a stand-alone manner as a prognostic tool for the distinction between benign and malignant PCCs. However, understanding the mechanisms responsible for the development of different patterns might provide new information on tumor biology and ultimately prove to have clinical value.

Acknowledgements

We express our gratitude to Prs. Pierre-François Plouin and Xavier Bertagna for the COMETE Network.

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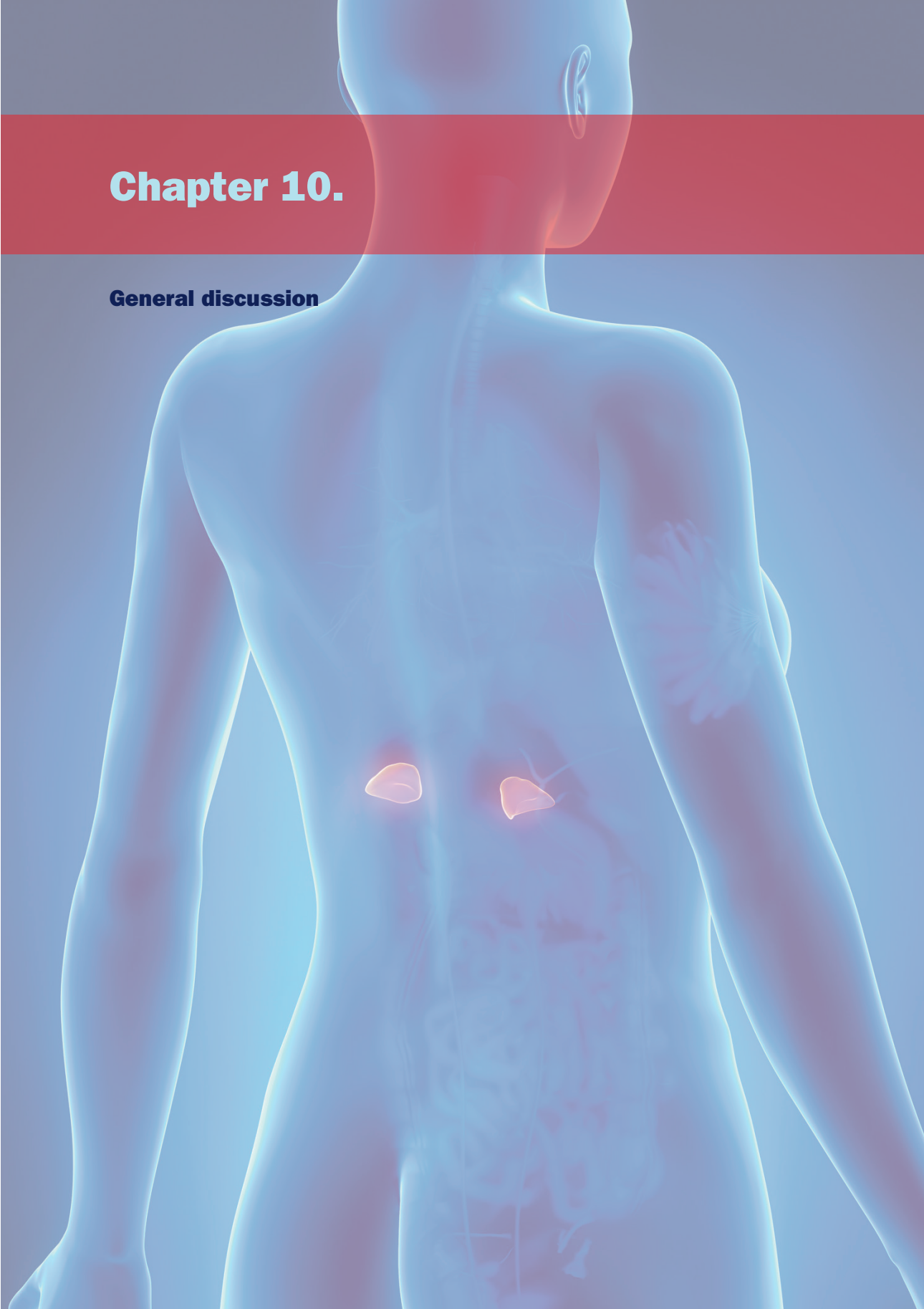
Chapter 10.

General discussion



Genetics and SDHA/SDHB/SDHD immunohistochemistry

Pheochromocytomas and paragangliomas carry the highest degree of heritability in human cancers.(1) In 2010, when I started doing research, nine susceptibility genes had been discovered, but in the last few years at least eight additional genes have been identified, suggesting that the list of genes has not been completed yet. Approximately 40% of pheochromocytomas and paragangliomas develop as a result of germline mutations (2,3) (Figure 1). At present, 17 susceptibility genes have been identified: *VHL*, *RET*, *NF1*, *SDHA*, *SDHB*, *SDHC*, *SDHD,* and *SDHAF2* (together *SDHx*), *TMEM127*, *MAX*, *KIF1B*, *PHD2,* and the recently identified genes *HIF2A* (2,4-6), *HRAS* (7), *FH* (8), *PHD1* (9), and *MDH2 (81)*. The remaining 60% of tumors without germline mutations are thought to occur sporadic. In at least one-third of these sporadic cases a somatic mutation in *RET*, *VHL*, *NF1*, *MAX*, or *HIF2A* can be identified.(10)

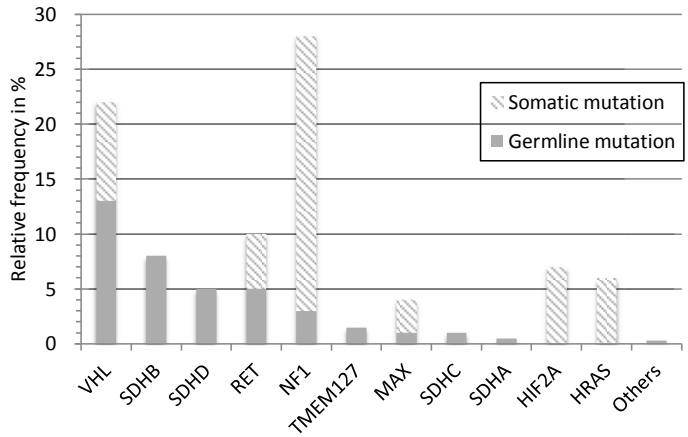


Figure 1. Relative frequencies of germline or somatic gene mutations in pheochromocytomas and paragangliomas. The 'others' category refers to *KIF1B*, *PHD2* and *FH*.

In **chapter 2** we found an incidence of 8% of somatic *HRAS* mutations in 164 sporadic pheochromocytomas and paragangliomas. Clinical and pathological correlations like gender, median age at diagnosis, and tumor behavior were not significantly different between *HRAS*-mutated tumors and sporadic pheochromocytomas without *HRAS* mutation. In contrast, Crona et al. described a male predominance and benign clinical behavior in four *HRAS*-mutated sporadic pheochromocytomas and paragangliomas.(7) However, these correlations were limited by the small number of tumors with *HRAS* mutations in both studies. We recommend that *HRAS* mutation screening should be performed as part of routine genetic screening in pheochromocytomas and paragangliomas without germline mutations in one of the susceptibility genes. Thus we might be able to collect a sufficient number of tumors to validate the role of *HRAS* as diagnostic, prognostic and/or therapeutic molecular biomarker.

Familial succinate dehydrogenase (*SDH*)-related pheochromocytomas or paragangliomas are caused by *SDHA*, *SDHB*, *SDHC*, *SDHD* or *SDHAF2* mutations. The *SDHA*, *SDHB*, *SDHC,* and *SDHD* genes code for subunits of succinate-ubiquinone oxidoreductase (SDHx), which is both a component of the citric acid cycle and the aerobic electron transport chain (complex II).(11-15) A mutation in one of these four genes is presumed to lead to disassembly of the complex and degradation of the individual proteins.(11) The *SDHAF2* gene encodes succinate dehydrogenase complex assembly factor 2, which is required for the flavination of SDHA and SDH function and stability.(14,16) In the *SDHx*-related tumor syndrome not only pheochromocytomas and paragangliomas develop, but also gastrointestinal stromal tumors (GISTs), renal cell carcinomas (RCCs) and pituitary adenomas.(17-19) In a recent study it was shown that noncoding regions of the *SDHD* gene are frequently mutated across many tumor types.(20) *SDHD* promoter mutations (C>T) occurred exclusively in melanoma samples and were associated with reduced *SDHD* gene expression and poor prognosis. In that same study, *TERT* promoter mutations constituted the most significant hotspot in 7 cancer types.(20) *TERT* promoter mutations lead to increased activity of the telomerase enzyme, which prevents the telomeres of chromosomes from getting shorter. In **chapter 3** we explored the presence of *TERT* promoter mutations in pheochromocytomas, paragangliomas and GISTs. We have found two paragangliomas of the urinary bladder with a C228T *TERT* promoter mutation and one GIST with a C250T *TERT* promoter mutation. All three of these tumors were SDH-deficient, suggesting a possible association between the presence of *TERT* promoter mutations and SDH deficiency.

SDHB immunohistochemistry can be used to identify *SDHx*-associated tumors, as there is loss of the normal SDHB protein expression, exhibiting a typical granular staining pattern by immunohistochemistry in *SDHA*, *SDHAF2*, *SDHB*, *SDHC* and *SDHD*-mutated tumors. Non-*SDHx*-related tumors are immunohistochemically positive for SDHB.(11) In addition, SDHA immunohistochemistry can identify *SDHA*-mutated pheochromocytomas, as only *SDHA*-mutated tumors are immunonegative for both SDHA and SDHB (21), while *SHDB*, *SDHC*, *SDHD* or *SDHAF2*-mutated tumors are immunonegative for SDHB, but immunopositive for SDHA. Since the *SDHA*, *SDHB, SDHC, SDHD,* and *SDHAF2* genes are bona fide tumor suppressor genes, both alleles have to be mutated or lost for complete inactivation. This biallelic inactivation of *SDHx* genes is usually achieved by a germline mutation of one allele and loss of heterozygosity (LOH) of the wild-type allele (22), leading to absence of protein staining.

In **chapter 5** it is presented that SDHA immunohistochemistry can also be used to identify *SDHA*-mutated GISTs. We found the same *SDHA* p.Arg31X mutation in 1 of 9 pediatric and 3 of 24 adult wild-type GISTs that showed absent SDHA staining of the tumor cells, but positive SDHA staining of control (endothelial) cells. GISTs are most often caused by oncogenic mutations in *KIT* and *PDGFRA* genes, but a subset of these tumors is associated with germline mutations in *SDHA*, *SDHB*, *SDHC* or *SDHD.*(23) Therefore, a link has been

established between paragangliomas, pheochromocytomas and GISTs. The dyad of paraganglioma and GIST is known as Carney-Stratakis syndrome, while Carney triad describes the association of paragangliomas with GISTs and pulmonary chondromas.(24) It has been shown that GISTs from patients with Carney-Stratakis syndrome or Carney triad are SDHB immunonegative.(25-27) Also, SDH (Complex II) activity, determined by a functional assay, was substantially reduced in two GISTs without an *SDHx* mutation or deletion.(28) In Carney-Stratakis syndrome germline *SDHB*, *SDHC,* and *SDHD* mutations have been described (29), but in Carney triad no *SDHx* mutations have been found, leaving the SDH-deficient tumor phenotype unexplained.(30) However, Killian et al. recently demonstrated *SDHC* epimutation, evidenced by highly focal *SDHC* promoter CpG island hypermethylation and transcriptional silencing in five of eight GISTs from Carney triad patients.(31) This points to *SDHC* epimutation as the molecular aberration for SDH complex inactivation in a considerable part of Carney triad patients. We showed in chapter 5 that SDHA immunohistochemistry can specifically detect *SDHA*-mutated GISTs. SDHA is the most frequently mutated subunit of the SDH complex in GISTs with *SDHx* mutations (47%).(22) Altogether, this suggests that SDHB and SDHA immunohistochemistry could be used to detect any *SDHx*-associated tumor type.

The role of SDHB and SDHA immunohistochemistry on tumor tissue is important to preselect genes for genetic testing. Mutation analysis of all known pheochromocytoma and paraganglioma susceptibility genes is still expensive and labor intensive. Moreover, *SDHB*-mutated tumors have a higher incidence of malignancy, so it is important to identify these patients.(32) In view of all this, it is important that the SDHB and SDHA immunostaining is interpreted correctly. In the majority of cases, interpretation of SDHB and SDHA staining is straightforward, as can be inferred from the high degree of concordance between multiple observers. However, a weak diffuse cytoplasmic SDHB staining pattern has been described in *SDHD*-related tumors (12,33), making it more difficult to conclude whether these tumors are immunonegative. Also, heterogeneous staining for both SDHB and SDHA can be present, 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” (**chapter 4**). In both cases, interobserver variation is high and may lead to false positive or false negative diagnoses. *SDHx* mutation analysis is recommended when SDHB and/or SDHA immunohistochemistry shows a weak diffuse or heterogeneous staining pattern, to prevent misinterpretation regarding the *SDH* mutation status of the tumor. Interestingly, another procedure was recently described that provides a complement to SDHB immunohistochemistry of pheochromocytomas and paragangliomas (**chapter 6**). Initially, SDHD immunohistochemistry was evaluated to detect patients with *SDHD* germline mutations. In contrast to SDHB immunohistochemistry, SDHD immunostaining was positive in *SDHx*-mutated tumors, while it was not detected in non-*SDHx*-related pheochromocyomas or paragangliomas. The SDHD subunit of the SDH complex

is anchored in the mitochondrial membrane, together with SDHC. The SDHD antibodies that were used in the study were directed against an epitope that is located within the transmembrane domain of the SDHD protein. Therefore, we hypothesize that this epitope is masked when the SDHD protein is present in the active SDH complex, as is the case in non-*SDHx*-related tumors. However, in *SDHx*-mutated tumor cells, the disruption of the SDH complex leads to release of the epitope making it accessible to immunolabelling by the SDHD antibody. As described above, some tumors show a weak diffuse cytoplasmic SDHB staining, that makes the interpretation more difficult. In this situation the use of SDHD immunohistochemistry can be a valuable tool, as the SDHD staining will be positive in *SDHx*-mutated tumors. We suggest that SDHD immunohistochemistry should be added to the algorithm of pheochromocytoma and paraganglioma pathological analyses (Figure 2).

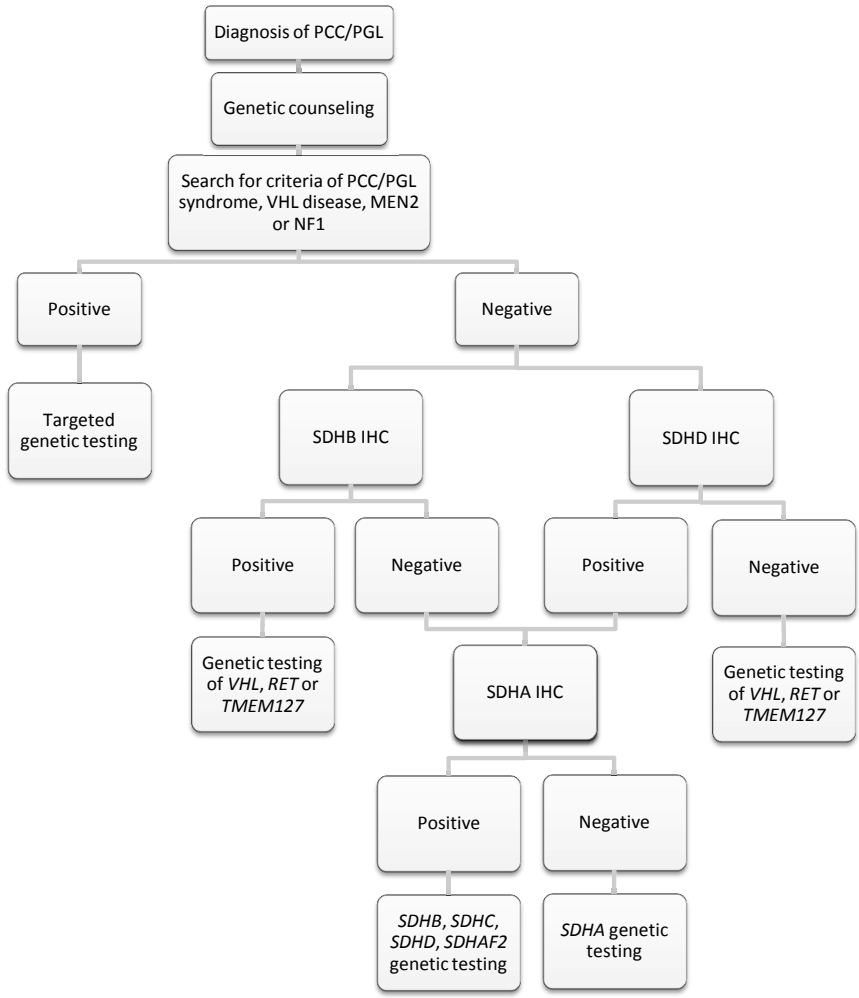


Figure 2. Algorithm for genetic counselling of pheochromocytomas (PCC) and paragangliomas (PGL) based on SDHB, SDHA, and SDHD immunohistochemistry.

Molecular pathways

Pheochromocytomas and paragangliomas cluster in distinct groups based on the transcription profile investigated by mRNA expression analysis. The first cluster includes *VHL*-, *SDHx*-, *PHD2*-, and *HIF2A*-related tumors, and also includes about 30% of sporadic cases.(34-36) The second cluster contains *RET*-, *NF1*, *KIF1B*-, *TMEM127*- and *MAX*-mutated tumors, and also about 70% of sporadic tumors.(34,37-40) These two mRNA clusters appear to display distinct routes to the molecular pathogenesis of pheochromocytomas and paragangliomas (figure 3).

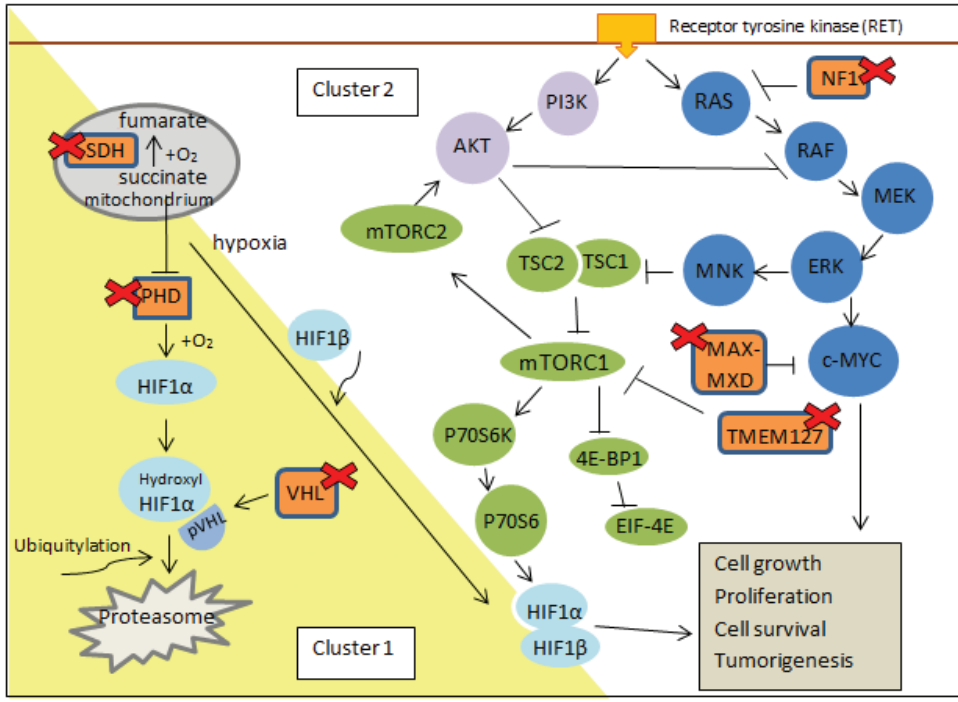


Figure 3. Overlap between cluster 1 and cluster 2 signaling pathways, affected by PCC and PGL promoting gene mutations (✕). Cluster 1 (yellow panel): SDHx mutations lead to a decreased activity of prolyl hydroxylase (PHD) which normally prepares HIF-1α for proteasomal degradation via a VHL-dependent protein. Stabilized HIF-1α promotes tumorigenesis. Cluster 2 (white panel): growth factors can activate receptor tyrosine kinases (for example RET) resulting in PI3K activation and also in RAS/RAF/ERK-pathway activation. mTORC1 promotes the phosphorylation and activation of various proteins including p70S6. Activated p70S6 induces cell growth, proliferation, cell survival, and tumorigenesis.
---| inhibitory effect, ---> stimulatory effect.

Cluster 1

Signaling pathways of cluster 1 pheochromocytomas and paragangliomas are associated with Krebs cycle modulation of hypoxia signaling. Normal cells respond to low cellular oxygen levels (hypoxia) by inducing the transcription of several hypoxia-inducible factors (HIFs) involved in angiogenesis, metabolism and cell growth.(41,42) The active form of HIF consists of an alpha (either HIF1a or HIF2a) and beta subunit, and HIF function is regulated by hydroxylation and proteasomal degradation.(43-45) Cluster 1 pheochromocytoma and paraganglioma genes are directly or indirectly involved in these processes. Pseudohypoxia occurs when the HIF pathway becomes active under normoxia.(46) Mutations of *VHL*, *SDHx*, *HIF2A* or *FH* are associated with pseudohypoxic signatures in pheochromocytomas.(1) Rarely, other genes coding for enzymes involved in the Krebs cycle are mutated, such as *PHD1/2* or *IDH1/2* (8,47), and very recently a mutation in malate dehydrogenase 2 (*MDH2*)

has been identified.(81) However, a proportion of pseudohypoxic pheochromocytomas does not have mutations in any of the abovementioned genes, so it is conceivable that other enzymes related to the hypoxia pathway might be aberrant in these tumors.

Cluster 2

Pheochromocytomas and paragangliomas of cluster 2 display activation of the PI3K/AKT and RAS/RAF/ERK signaling pathways.(48) Phosphoinositide-3-kinase (PI3K) is activated by the binding of growth factors to receptor tyrosine kinases, including RET. PI3K activates AKT, which in turn leads to activation of mTORC1 by inhibiting the TSC1/TSC2 suppression of mTORC1. After activation of the multiprotein complex mTORC1, downstream effectors of the pathway are phosphorylated/activated, including ribosomal protein phospho-p70 S6 kinase (p70S6K).(49) This downstream effector regulates mRNA translation and is a stimulator of several oncogenic proteins such as c-Myc, HIF1α, VEGF, IGF2, and cyclin D.(50) Activation of receptor tyrosine kinases by growth factors not only activates the PI3K/AKT pathway, but also the RAS/RAF/ERK pathway, leading to activation of mTORC1. Gain of function mutations of the proto-oncogene *RET* have been associated with abnormal activation of both pathways.(51,52) Mutations in the tumor suppressor gene *NF1* lead to constitutive activation of RAS and mTORC1, as the NF1 protein normally inactivates RAS.(53) In addition, the tumor suppressor gene *TMEM127* is associated with mTORC1 downregulation, so mutations in this gene enhance mTORC1 activity.(54) Finally, mutations in the tumor suppressor gene *MAX* result in the dysregulation of the MYC–MAX–MXD1 network, and this signalling pathway seems closely related to the mTOR pathway.(40,48)

Although it might appear from the above discussion that completely separate, mutually exclusive, pathways are active in cluster 1 and cluster 2 pheochromocytomas and paragangliomas, there are overlapping signaling interactions. mTOR can regulate HIF, and there is also crosstalk between HIF and MYC.(55,56) In cluster 1 tumors stabilized and activated HIF1a is linked to tumorigenesis, while aberrant mTORC1 activation leads to increased synthesis of HIF1a in cluster 2 tumors.(48) So, the mTOR pathway appears to play an important role in the pathogenesis of pheochromocytomas and paragangliomas. In **chapter 8** we explored the protein expression of all key members of the mTOR signaling pathway in 222 pheochromocytomas and paragangliomas of different genetic background. We found that the mTOR pathway was expressed in an important part of the tumors, demonstrated by a positive correlation among the proteins that were investigated. Moreover, the mTORC1 complex proteins were preferentially expressed in head and neck paragangliomas and/or *SDHx*-mutated tumors. Our findings suggest that the mTORC1 complex functions as a point of convergence in both cluster 1 and cluster 2 pheochromocytomas and paragangliomas. This makes mTOR-targeting therapy an interesting strategy, and both tumor behaviour and genetic background should be taken into

consideration when this therapy option is investigated. The mTOR inhibitor Everolimus (RAD001) did not demonstrate a major clinical benefit in four patients with malignant pheochromocytomas.(57) However, this inhibitor targets only mTORC1, and later on Giubellino et al. suggested that both mTORC1 and mTORC2 complexes should be inhibited, as mTORC1 inhibitors have been associated with an Akt upregulatory loop in the mTOR pathway.(58,59) Giubellino et al. showed that the dual mTORC1/2 inhibitors AZD8055 and Torin-1 could block proliferation, as well as cell migration, in pheochromocytoma primary cell cultures and in the MTT cell line.(59) In another study, the antitumor activity of a dual mTORC1/2 inhibitor PP242 was evaluated in a pheochromocytoma PC12 cell tumor model.(60) These results showed that treatment with PP242 dramatically inhibited tumor growth, and increased tumor cell apoptosis. In addition, PP242 significantly downregulated VEGF expression in the xenograft tumor model. VEGF is a key target for inhibiting angiogenesis in pheochromcytomas and paragangiomas.(61) However, the results of the study are limited, as PC12 cells do not truly reproduce the pathogenesis of malignant pheochromocytomas in vivo. Therefore, PP242 treatment should be further investigated in clinical trials.

Malignancy and identifying targets for treatment

Malignant pheochromocytomas or paragangliomas are defined by the 2004 WHO classification as chromaffin cell tumors with the presence of metastases to sites where chromaffin tissue normally should not be found.(62) Metastases most frequently affect lymph nodes, liver, kidney, lungs or bones. To date, there are no agreed upon indicators of malignancy in the primary tumor and all patients require lifelong follow-up. Risk factors for metastasis include tumor location, genotype, biochemical profile, and size.(63) In general, risk is low for pheochromocytomas (~10%), higher for extra-adrenal abdominal paragangliomas and highest for tumors with *SDHB* mutations (at least 30%), which are usually extra-adrenal and often large. Currently, not only predictive markers for malignancy are lacking, but there is also no effective long-term or curative treatment for metastatic pheochromocytomas and paragangliomas. Transient responses are sometimes achieved with tyrosine kinase inhibitors or conventional chemotherapy. In the latter category, the combination of cyclophosphamide, vincristine, and dacarbazine (CVD), has historically been used most extensively, showing 79% hormonal and tumor response with minimal side effects, but little or no improvement in survival. In some patients, treatment failure is heralded by “explosive” accelerated growth of previously indolent tumors.(64) A possible explanation for tumor progression is the acquisition of additional genetic mutations. Another possible explanation for this acquired resistance to therapy may be the presence of a small minority of cells in the tumor called cancer stem cells.(106) According to the cancer stem cell theory, these cells are often resistant to treatment and provide the very basis of cellular tumorigenesis.(65,66) Specifically targeting these stem cell-like cancer cells in malignant

pheochromocytomas and paragangliomas might improve our therapeutic approach.(65,67)

In order to investigate if stem cells or stem cell signaling can be found in pheochromocytomas and paragangliomas, we examined the immunohistochemical expression of eleven embryonic, hematopoietic, neural and mesenchymal stem cell markers in a large set of these tumors in **chapter 7**. The selection of markers was based on a list of relevant progenitor markers identified in embryonic and somatic stem cells, as well as stem cell-like cancer cells, generated by a literature search. There are more markers that could be evaluated, but our list included the best known markers and/or markers that are related to the nervous system, as pheochromocytomas and paragangliomas are neural crest-derived tumors. In addition, a reliable antibody should be available. In our series, SOX2, LIN28, SOX17, NGFR, and THY1 were frequently co-expressed in the same tumor and their expression was associated with the presence of mutations in one of the *SDHx* genes. In addition, NGFR expression was significantly more often associated with malignant pheochromocytoma or paraganglioma (42%) compared to non-metastatic ones (16%), though the number of metastatic tumors in our series was limited (n=12).
The mesenchymal marker NGFR in this context is an interesting one, as the process of epithelial to mesenchymal transition (EMT) has been described to be implicated in metastatic properties of pheochromocytomas and paragangliomas.(68) EMT is a normal process in embryonic development, but it can be reactivated in cancer cells which then acquire migratory and invasive potential.(69) The activation of specific transcription factors leads to the loss of epithelial markers and to the gain of features of a mesenchymal phenotype. In two studies, protein overexpression of the EMT markers Twist1 and Snail1 was correlated with malignancy of pheochromocytoma and paraganglioma.(70,71) In the study by Loriot et al. specific nuclear translocation of Snail1/2 protein expression was observed in *SDHB*-metastatic pheochromocytomas and paragangliomas.(68) In addition, they performed transcriptomic profiling of EMT in 188 tumors, in which most *SDHB*-metastatic samples clustered together. The latter suggests that EMT is differently regulated in this subtype of tumors. A possible explanation for the participation of Twist1 and Snail1 in *SDHB*-related tumors is that these factors are induced by hypoxia, a pathway that is activated in *SDHx*- and *VHL*-related tumors, as described above.(72,73) So, NGFR was identified in our study as a marker of malignancy. However, NGFR expression has not yet been investigated in metastatic *SDHB*-mutated pheochromocytomas harboring an EMT-phenotype. Therefore, this should be studied, and our findings should be validated in another, larger set of (metastatic) pheochromocytomas and paragangliomas.
Another finding in **chapter 7**, was the identification of SOX2 in both sustentacular cells and pheochromocytoma tumor cells demonstrated by double-staining for S100/SOX2 and Chromogranin A/SOX2, respectively. Interestingly, in the anterior pituitary gland S100/SOX2 co-expressing folliculo-stellate cells, which are the sustentacular cells of this organ, have also

been identified.(74) In the pituitary these cells are proposed to be pluripotent adult stem cells. To be able to prove that these markers may identify the stem cell component in pheochromocytomas, additional experiments are warranted. This can be achieved by demonstrating the expression of some of these stem cell markers in spheres generated from pheochromocytoma cell lines. Another possible experiment would be the use of flow cytometry to identify sub-populations of cells in pheochromocytomas and paragangliomas with Hoechst 33342 DNA dye. This method is based on the passive uptake of the dye by live cells. Stem cells and early progenitors have a Hoechst low fluorescence because of efficient efflux capacity via ATP-Binding Cassette (ABC) transporters.(75) It would also be relevant to see if this identified side population enriched in cells positive for stem cell-associated factors could expand clonally into spheres in vitro.

As mentioned before, no single histologic feature is able to predict metastatic potential of pheochromocytomas and paragangliomas. Several studies have focussed on histologic features and combinations of them to identify malignant behavior.(76-78) The most recent described scoring system (GAPP classification) uses histological pattern, cellularity, coagulation, necrosis, vascular/capsular invasion, Ki-67 immunoreactivity, biochemical profile, combined with SDHB immunohistochemistry.(79) However, these histologic criteria cannot be used to definitely differentiate between benign and malignant pheochromocytomas and paragangliomas. Another study had described earlier that malignant pheochromocytomas display a peculiar irregular vascular pattern, which is not present in benign tumors.(80) The authors found a sensitivity of 100% using vascular architecture as a predictive marker, as they observed an abnormal and discontinuous vascular pattern, including vascular arcs, parallels and networks in 9 of 9 malignant PCCs. In **chapter 9** we validated this vascular pattern analysis in a larger cohort of 59 malignant and 124 benign pheochromocytomas in a multiobserver study. We found a mean sensitivity of vascular architecture as a predictor of malignancy of 59.7%. This suggests that vascular pattern analysis cannot be used as a single prognostic feature for distinguishing malignant tumors from benign, but should be combined with other criteria. However, there was significant agreement among the six observers in our study, indicating that there is a variance between the vascular patterns. If the variance is real, the biological background of the different vascular patterns is not clear. It would be interesting to investigate how these different vascular patterns develop and what exact role they play in tumor biology. Possible explanations could be that the irregular vascularization in malignant tumors corresponds to different steps in the process of angiogenesis, or that it represents a specific growth pattern of malignant tumor cells.

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Appendices

Samenvatting

Pheochromocytomen zijn zeldzame neuroendocriene tumoren die ontstaan vanuit de chromaffiene cellen in het bijniermerg. De meeste pheochromocytomen produceren catecholamines, zoals adrenaline en noradrenaline. Paragangliomen zijn verwante tumoren, maar ontstaan voornamelijk uit chromaffiene cellen in de borst- en buikholte of in het hoofd-hals gebied. De afgelopen jaren is er grote vooruitgang geboekt bij het ontdekken van genen die een rol spelen bij het ontstaan van pheochromocytomen en paragangliomen. We weten nu dat circa 40% van deze tumoren wordt veroorzaakt door een erfelijke kiembaanmutatie in één van de volgende genen: *SDHA*, *SDHB*, *SDHC*, *SDHD*, *SDHAF2*, *TMEM127*, *MAX*, *EGLN1*,*HIF2A*, *KIF1B* of *FH*. Bovendien kan er ook bij een deel van de tumoren een niet-erfelijke, somatische mutatie in het *RET*, *VHL*, *NF1* of *HIF2A* gen gevonden worden. Echter, de pathogenese van sporadische pheochromocytomen en paragangliomen zonder mutaties in één van de bovengenoemde genen is nog niet duidelijk.

Met de techniek van exoomsequencing zijn bij circa 7% van de sporadische pheochromocytomen en paragangliomen somatische mutaties in het *HRAS* gen gevonden. In **hoofdstuk 2** hebben wij laten zien dat *HRAS* mutaties daadwerkelijk in een kwantitatief belangrijk deel van de sporadische pheochromocytomen aanwezig zijn (10%). Echter, wij vonden geen significante correlatie tussen het genotype en klinische of pathologische kenmerken van tumoren met een *HRAS* mutatie.

In **hoofdstuk 3** hebben we gekeken naar de aanwezigheid van *TERT* promotor mutaties in pheochromocytomen en paragangliomen. Het enzym telomerase is in verschillende soorten humane kankers geactiveerd en op die manier beschermt het de telomeren van de chromosomen. Wij vonden *TERT* promotor mutaties in twee paragangliomen, die bovendien ook deficiënt waren voor het SDH enzym. Dit suggereert dat er een associatie bestaat tussen het ontstaan van *TERT* promotor mutaties in SDH-deficiënte tumoren.

SDHA en SDHB immunohistochemie spelen een belangrijke rol in de routine diagnostiek van het pathologie laboratorium bij het selecteren van patiënten met een mutatie in een van de *SDH* genen. *SDHx*-gerelateerde tumoren laten geen expressie van het SDHB eiwit zien, waarbij de *SDHA*-gerelateerde tumoren tevens geen SDHA eiwitexpressie hebben. Niet-*SDHx*-gerelateerde tumoren daarentegen hebben wel SDHB en SDHA eiwitexpressie. In **hoofdstuk 4** hebben we gekeken naar de overeenstemming tussen zeven endocriene pathologen bij het voorspellen van de *SDHx*-mutatie status van pheochromocytomen en paragangliomen aan de hand van SDHB en SDHA immunohistochemie. Voor beide kleuringen was de overeenstemming zeer hoog, namelijk voor de SDHB kleuring 90% en voor de SDHA kleuring 99%. Dit betekent dat de kleuringen bruikbaar zijn in de dagelijkse praktijk van het pathologie laboratorium. In **hoofdstuk 5** hebben we aangetoond dat een immunohistochemische kleuring tegen SDHA niet alleen pheochromocytomen met een kiembaanmutatie in het *SDHA* gen kan aantonen, maar ook gastro-intestinale stromacel

tumoren (GISTen). Dit laatstgenoemde tumortype maakt samen met pheochromocytomen en paragangliomen deel uit van het *SDHx*-geassocieerde tumorspectrum. Inmiddels is duidelijk dat immunohistochemie voor SDHA en SDHB ons in staat stelt om van alle tumoren die bij patiënten met *SDHx* mutaties voorkomen, vast te stellen of zij in het kader van dit syndroom ontstaan, of dat dit een toevallige samenloop van omstandigheden is. Om in staat te zijn om specifiek patiënten met *SDHD* mutaties te identificeren, hebben wij in **hoofdstuk 6** gekeken of dit mogelijk is met behulp van SDHD immunohistochemie. Tot onze verbazing bleek de SDHD immunokleuring positief te zijn in *SDHx*-gerelateerde tumoren, dit in tegenstelling tot de SDHB immunokleuring die juist verlies van SDHB eiwitexpressie toont in geval van een *SDHx*-mutatie. In niet-*SDHx*-gerelateerde tumoren is de SDHD kleuring negatief. Dit resultaat betekent dat SDHD immunohistochemie een aanvulling kan zijn op de SDHB immunokleuring, als deze laatste moeilijk te interpreteren is door bijvoorbeeld achtergrondaankleuring. Een verklaring voor de onverwachte bevindingen zou kunnen zijn dat de epitoop van het SDHD eiwit in de normale situatie gemaskeerd is door de configuratie van het actieve SDH enzymcomplex, zoals het geval is bij niet-*SDHx*-gerelateerde tumoren. Echter, in *SDHx*-gemuteerde tumoren leidt de ontregeling van het SDH complex tot een demaskering van de epitoop, zodat deze wel bereikbaar wordt voor immunokleuring met het SDHD antilichaam.

Een groot probleem vormt nog steeds het gebrek aan voorspellende markers die onderscheid kunnen maken tussen benigne en maligne pheochromocytomen/ paragangliomen. De diagnose van een maligne pheochromocytoom kan dan ook pas gesteld worden op het moment dat er uitzaaiingen in lymfklieren, longen, lever of botten voorkomen. Tevens is de pathogenese van maligne tumoren nog niet duidelijk en bestaat er geen curatieve behandeling voor progressieve ziekte. Volgens de stamceltheorie staan stamcellen aan de basis van tumorgenese. In **hoofdstuk 7** hebben wij een serie benigne en maligne pheochromocytomen en paragangliomen onderzocht op de aanwezigheid van stamcelmarker eiwitexpressie. In deze studie was de eiwitexpressie van SOX2, SOX17, NGFR, LIN28, PREF1 en THY1 significant geassocieerd met tumoren die een *SDHx*-mutatie hebben. Bovendien was de expressie van NGFR significant gecorreleerd met gemetastaseerde tumoren. Verder onderzoek is nodig om te valideren of een van deze stamcelmarkers als target voor therapie kan worden gebruikt en of NGFR als voorspeller voor maligniteit kan dienen. In **hoofdstuk 8** hebben we gekeken naar de expressie van verschillende eiwitten uit de mTOR signaalcascade. We weten dat deze cascade een belangrijke rol speelt in de pathogenese van pheochromocytomen en paragangliomen met verschillende genetische achtergronden. Door middel van immunohistochemie hebben we aangetoond dat de mTOR signaalcascade actief is in een belangrijk deel van de pheochromocytomen en paragangliomen. Met name in hoofd-hals paragangliomen en tumoren met *SDHx*-mutaties was er activatie van het MTORC1 eiwitcomplex. Mogelijk kunnen medicijnen die ingrijpen op de mTOR signaalcascade effectief zien bij de behandeling van deze tumoren.

In 2002 is er een studie gedaan waarin analyse van het vaatpatroon beschreven wordt als methode om onderscheid te maken tussen benigne en maligne pheochromocytomen. Er werd gebruik gemaakt van CD34 immunohistochemie om de endotheelcellen van bloedvaten aan te kleuren. De auteurs vonden in maligne tumoren een onregelmatig vaatpatroon, met vasculaire bogen, parallellen en netwerken, terwijl het vaatpatroon in benigne tumoren regelmatig was met korte, rechte capillairen. In **hoofdstuk 9** hebben wij deze studie gevalideerd op een grotere serie van 184 pheochromocytomen en paragangliomen. Wij konden de hoge sensitiviteit van 100% uit de eerdere studie niet bevestigen, maar vonden een sensitiviteit van 59.7%, wanneer analyse van het vaatpatroon als prognostische marker gebruikt wordt. Dit betekent dat deze methode niet op zichzelf staand kan worden toegepast om onderscheid te maken tussen benigne en maligne pheochromocytomen en paragangliomen. Echter, in combinatie met andere prognostische markers, zou analyse van het vaatpatroon wel bijdragend kunnen zijn.

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Curriculum Vitae

Lindsey Oudijk werd geboren op 26 oktober 1989 te Boskoop. In 2008 behaalde ze haar VWO diploma (cum laude), waarna zij startte met de studie Geneeskunde aan de Erasmus Universiteit te Rotterdam. Aan het einde van haar eerste studiejaar liep zij als student 3 dagen mee op de afdeling Pathologie in het kader van een beroepsoriëntatiestage. Daar ontmoette zij Prof.dr. R.R. de Krijger en kwam er een samenwerking tot stand waarbij Lindsey als student wetenschappelijk onderzoek verrichtte parallel aan haar studie Geneeskunde. Het eerste artikel ging over klinisch-pathologische correlaties en immunohistochemie in myofibromen, maar daarna richtte het onderzoek zich op de pathogenese van pheochromocytomen en paragangliomen. In 2011 ging het ENS@T-CANCER consortium van start, een Europees netwerk dat onderzoek doet naar bijniertumoren. Binnen dit project kwam een er een promotieplek vrij waar Lindsey voor in aanmerking kwam. Na het behalen van haar Bachelor of Science in 2011 en het eerste jaar van de Master Geneeskunde, deed zij van 1 maart 2012 t/m 1 april 2014 fulltime promotieonderzoek op de afdeling Pathologie van het Erasmus MC. Deel van dit onderzoek is gedaan op de afdeling Pathologie van het Universitaire Ziekenhuis San Luigi Gonzaga in Turijn, Italië (Prof.dr. M. Volante), via een beurs van de European Science Foundation. In april 2014 begon zij aan haar coschappen en zij hoopt haar studie Geneeskunde medio 2016 af te ronden. Daarna wil zij graag starten met de opleiding Pathologie in Rotterdam.