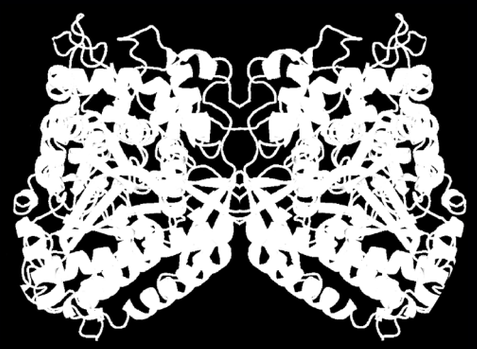

Identifying Genes Involved in Paraganglioma Genesis José Gaal

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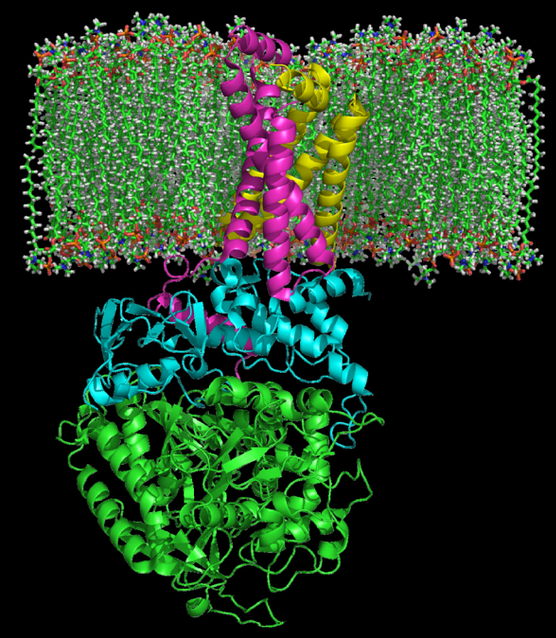

José Gaal

# Identifying Genes Involved in Paraganglioma Genesis

José Gaal

Cover: succinate dehydrogenase protein. Design en layout: Frank van der Panne.
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# Identifying Genes Involved in Paraganglioma Genesis

Het identificeren van genen betrokken bij het ontstaan van paragangliomen

**Proefschrift**

ter verkrijging van de graad van doctor

aan de Erasmus Universiteit Rotterdam

op gezag van de rector magnificus

Prof.dr. H.G. Schmidt

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op

woensdag 10 november 2010 om 15.30

door

**José Gaal**

geboren te Dordrecht.

## Promotiecommissie

| | |
|---|---|
| Promotor: | Prof.dr. R.R. de Krijger |
| Copromotor: | Dr. W.N.M. Dinjens |
| Overige leden: | Prof.dr. R. Fodde |
| | Prof.dr. P. Devilee |
| | Dr. M.M. van den Heuvel-Eibrink |

Dit proefschrift kwam tot stand binnen de afdeling Pathologie van de faculteit der Geneeskunde en Gezondheidswetenschappen van de Erasmus Universiteit Rotterdam. De afdeling maakt deel uit van het Erasmus MC Rotterdam. Het onderzoek is tot stand gekomen met financiele steun van Erasmus MC grant en de Pheo-Para Alliance.

I'm a great believer in luck and I find the harder I work, the more I have of it.

(Thomas Jefferson)

**Contents**

# CHAPTER 1

General introduction and outline of the thesis

## 1.1 The paraganglion system

The paraganglion system is composed of a collection of chromaffin cells that is distributed throughout the body. Embryonically, chromaffin cells arise from the neuroectodermal tissue of the neural crest and are thought to migrate along the innervating nerves or vasculature towards their primordial location to form the paraganglia. (1) The largest paraganglion is the adrenal medulla, an important neuroendocrine organ, which is the body's main source of catecholamines (adrenalin, noradrenalin and dopamine). The adrenal medulla receives input from the sympathetic nervous system through preganglionic fibers upon which it releases its secretions directly into the blood. Besides this adrenal station there are many extra-adrenal paraganglia that are distributed along the body axis and located in the proximity of ganglia of the sympathetic chain or in association with cranial nerves and blood vessels.

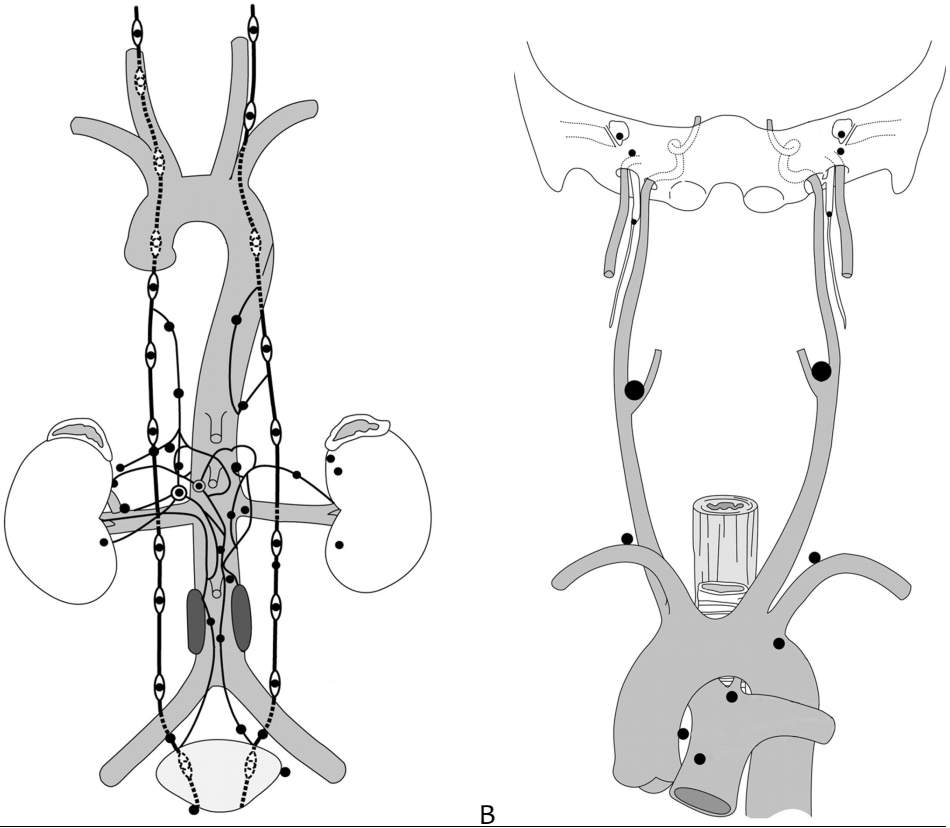


**Figure 1.** Drawings of paraganglion system (adapted from Lee et al. (2)). Drawings show anatomic distribution of healthy paraganglia connected with sympathetic system (**A**) and parasympathetic system (**B**).

These extra-adrenal paraganglia are divided in two major groups: one concerns the sympathetic paraganglia, associated with the orthosympathetic nervous system, which are found from the level of the superior cervical ganglion down the sympathetic trunk and into the pelvis. (3) They include the organs of Zuckerkandl (the source of catecholamines in the early gestational period) around the origin of the mesenteric artery. (4) In the pelvis, the paraganglia are found in association with the inferior hypogastric plexus, the urogenital organs and in the sacral plexus. (5) The second group are the parasympathetic paraganglia, associated with the parasympathetic nervous system, which have a more restricted distribution and are found almost exclusively in association with the thoracic and cranial branches of the glossopharyngeal and vagus nerves. The tympanic paraganglia in the middle ear and the carotid bodies are associated with the glossopharyngeal nerve. The jugular paraganglia of the middle ear, laryngeal paraganglia, subclavian paraganglia and aorticopulmonary and cardio aortic paraganglia at the base of the heart are innervated by the vagus nerve. The most consistent parasympathetic paraganglion is the carotid body that is located at the carotid bifurcation. It is believed to be a chemoreceptor that registers arterial oxygen concentration and transmits sensory signals through a branch of the glossopharyngeal nerve to the central nervous system. (6) Figure 1 shows the anatomic distribution of sympathetic and parasympathetic paraganglia.

**Histology**

Microscopically, all paraganglia have a similar morphologic appearance characterized by well-defined cell nests ("Zellballen"). These are composed of neuroendocrine cells, also called chief cells, type I cells or glomus cells, which are partially or completely surrounded by sustentacular cells. In addition there are connective tissue cells and endothelial cells. Chief cells are polygonal cells with abundant cytoplasm, small membrane bound granules, and small spherical or ovoid pale-staining nuclei with finely stippled chromatin and conspicuous nucleoli. Sustentacular cells, also called satellite cells or type II cells have less conspicuous cytoplasm, are more flattened and are difficult to detect in hematoxylin and eosin (HE) staining. Sustentacular cells are present in parasympathetic and sympathetic paraganglia, but more abundant in parasympathetic paraganglia where the Zellballen are more prominent. (7)

**Hyperplasia**

Criteria that define hyperplasia include increase in weight, size and increment in the percentage or differential count of elongated cells and chief cells. (8-9) Frequently hyperplasia is accompanied with hypertrophy that usually occurs bilaterally and symmetrically. The mechanism is presumably due to chronic hypoxia. Hypertrophy and hyperplasia of vagal, carotid body and aorticopulmonary paraganglia has been described in humans living at high altitude, patients with chronic obstructive pulmonary disease, systemic hypertension, cystic fibrosis, and with cyanotic congenital heart disease. (10-12)

## 1.2 Paragangliomas

Neoplasms of the neuroendocrine cells found within the sympathetic or parasympathetic paraganglionic axes are called paragangliomas. They are different from hyperplasia in that they present proliferation of the chief cells whereas in hyperplasia there is proliferation of the chief and sustentacular cells. (13) Distinct from the adrenal medulla, where nodules smaller than 1.0 cm are defined as nodular medullary hyperplasia and nodules of 1.0 cm or larger are considered pheochromocytomas, in paragangliomas there is no classification based on weight or size.

Based on the location and catecholamine production, paragangliomas are subdivided into parasympathetic and sympathetic paragangliomas. The former are found in the head and neck region, and therefore often named head and neck paragangliomas, and usually do not release catecholamines. The latter are situated along the sympathetic trunk in the abdomen, and usually produce catecholamines. The adrenal tumors are referred to as pheochromocytomas. The distinction between parasympathetic paragangliomas, sympathetic paragangliomas and pheochromocytomas is important because of the implications for associated neoplasms, risk of malignancy, and genetic testing.

**Incidence**

Both parasympathetic and sympathetic paragangliomas occur at very low frequency. They are listed as a rare disease by the office of rare diseases of the National Institute of Health, which means that these tumors affect less than 200,000 people in the US

population. The clinical incidence of parasympathetic paragangliomas is about 1:1,000,000. (14) However in necropsy studies, incidences of 1:3,860 and 1:13,400 were reported. (15) Parasympathetic paragangliomas most often become clinically apparent in the fourth or fifth decade of life. The clinical incidence of sympathetic paragangliomas is not clear, since these tumors have been grouped with pheochromocytomas in the literature. The incidence of pheochromocytomas is about 1:200,000. (16-18) About 90 % of all these tumors occur in adults. Sex distribution is equal, except in children and in patients with thoracic tumors, where males are more affected.

**Clinical presentation**

Patients with parasympathetic paragangliomas present themselves with a painless palpable mass in the neck, or with symptoms due to compression of nearby structures (dysphagia, pain, coughing). Depending on the anatomic localization, pressure on cranial nerves may cause bradycardia, hoarseness or hearing loss. Approximately 5% of the patients have symptoms of catecholamine hypersecretion similar to pheochromocytomas and sympathetic paragangliomas. (19) The vast majority of patients (90%) with sympathetic paragangliomas present with the classic catecholamine excess symptoms of headaches, palpitation, perspiration, pallor, orthostasis and hypertension. (20)

**Histopathology**

Histopathologically, paragangliomas are composed of alveolar groups of cells, called Zellballen, which are embedded in a vascular stroma and demarcated by a fibrous pseudocapsule. Fibrous septa or necrosis may be present. Extensive fibrosis may cause displacement and distortion of tumor nests with loss of the characteristic architecture. The neoplastic chief cells are usually ovoid with centrally located nuclei, finely granular chromatin and indistinct small nucleoli. The cytoplasm is moderate in amount and clear to eosinophilic (Figure 2A). Sustentacular cells, located at the periphery of the Zellballen along the fibrovascular septa, are inconspicuous on routine stained HE sections but are evident on sections stained for S-100 protein (Figure 2B).

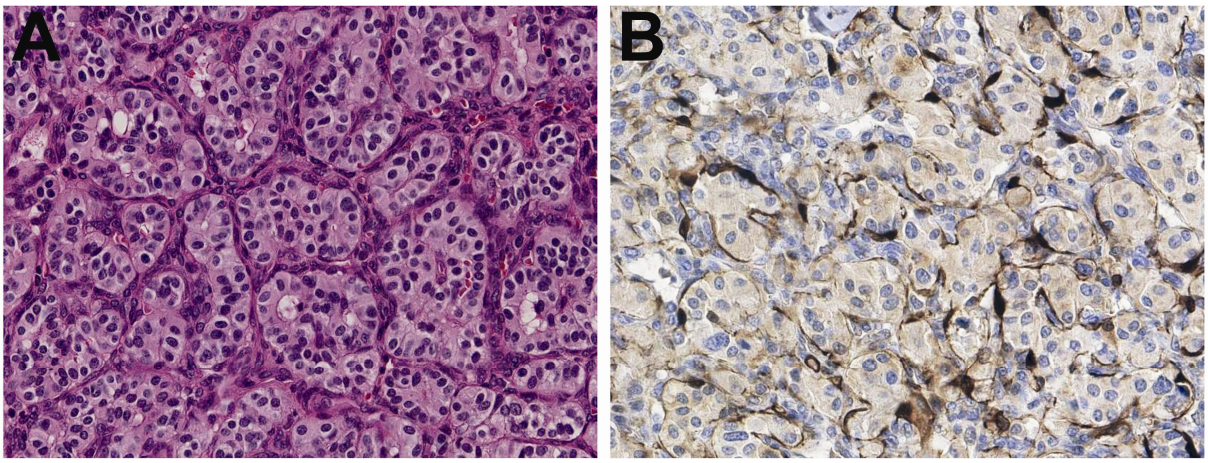


Figure 2. HE staining of paraganglioma (A), S-100 staining: sustentacular cells form a ring around nests of chief cells (B).

**Immunohistochemistry**

Sympathetic and parasympathetic paragangliomas are typically characterized by positivity of the general neuroendocrine markers including synaptophysin, chromogranin A and CD56. (21) Positivity for chromogranin A is usually less intense in parasympathetic paragangliomas. Parasympathetic paragangliomas also express chromogranin B and secretogranin II. (22) As mentioned above, sustentacular cells can be accurately identified with S-100 or glial fibrillary acidic protein. (23)

**Malignancy**

Malignancy is defined as tumor located in tissues where chromaffin cells are not normally present. (24) Frequent sites of metastases are regional lymph nodes, bone, liver, and lung. (25) In familial paraganglioma syndromes, multiple paragangliomas can arise in the same individual and must be distinguished from metastatic disease, which may coexist.

Histopathological studies have not revealed clear criteria that indicate malignant behavior. Many tumors, both benign and malignant show nuclear pleomorphism, capsular and vascular invasion. Molecular markers, such as expression of human telomerase reverse transcriptase and heat shock protein 90 might provide alternative methods for distinguishing malignant from benign paraganglioma. (26-28) Other possible markers of malignancy include tenascin, cyclo-oxygenase2 and VEGF. (29-31) However these studies await further confirmation. Therefore, currently there is no reliable way to distinguish malignant from benign tumors in the absence of metastases. It has been

proposed that, analogous to melanoma, all paragangliomas and pheochromocytomas carry a certain risk of metastasis and therefore it is suggested that the term "malignant", with respect to paragangliomas and pheochromocytomas, should be discarded. (32) The incidence of malignant paragangliomas varies according to the series, from 2-50%. Malignancy is more common in sympathetic paragangliomas (30%–50%) than in pheochromocytomas (10%–15%). (33-34) Because of lack of consensus definition in the literature, there is great variability in malignancy rates.

Patients with metastatic paraganglioma have been reported to have a median survival of about 4.5 years, but tremendous variability is observed. There is one case of a patient with skeletal metastasis who had a 26-year survival period without chemotherapy or radiation treatment. (35)

**Treatment**

For parasympathetic paragangliomas therapeutic options include observation, surgery, and radiation therapy. Surgery is typically difficult (due to the characteristic involvement of intracranial structures) and bloody (due to the highly vascularized nature of the tumor). However, this modality remains a viable option for properly selected patients. Preoperative transarterial embolization does not lead to a significant reduction in intraoperative blood loss. (36) In many cases the devascularisation remains incomplete because of the extensive angioarchitecture and considerable arteriovenous shunting of the lesions. (36) Radiation therapy has demonstrated local control rates equal to that of surgery but without the operative morbidity noted previously. (37) Most recurrences are amenable to radiotherapy. For lesions with bony involvement radiation is the treatment of choice. Newer studies have advocated stereotactic radiosurgery in some patients. (38-39) The management of systemic metastatic disease has been quite variable and site-specific. Multiple authors have reported on systemic therapy using agents such as gemcitabine (Gemzar) and cisplatin, with varying results. (40-42)

### 1.3 Familial paragangliomas and pheochromocytomas

Paragangliomas and pheochromocytomas can occur sporadically and in the context of hereditary syndromes, such as paraganglioma/pheochromocytoma syndrome, von Hippel-Lindau syndrome (VHL), neurofibromatosis type 1 (NF1) and multiple endocrine neoplasia type 2 (MEN2). (43) Table 1 gives an overview of hereditary syndromes associated with paragangliomas and/or pheochromocytomas. Originally, it was suggested that 10% of pheochromocytomas and paragangliomas are hereditary, but advances in molecular genetics over the past decade have shown that germline mutations occur in up to 24% of apparently sporadic pheochromocytomas and paragangliomas. (44-46)

**Table 1.** Syndromes in which paragangliomas and/or pheochromocytomas occur.

| Syndrome | Gene | Chromosome | PCC | sPGL | pPGL |
|---|---|---|---|---|---|
| **PGL1** | *SDHD* | 11q23 | + | + | + |
| **PGL2** | *SDHAF2* | 11q13.1 | - | - | + |
| **PGL3** | *SDHC* | 1q21-23 | rare | rare | + |
| **PGL4** | *SDHB* | 1p36 | + | + | + |
| **VHL** | *VHL* | 3p25-26 | + | + | rare |
| **MEN 2** | *RET* | 10q11.2 | + | rare | rare |
| **NF1** | *NF1* | 17q11 | + | - | - |

PGL1-4: hereditary paraganglioma syndrome 1-4, MEN2: multiple endocrine neoplasia type 2, VHL: von Hippel-Lindau, NF1: neurofibromatosis type 1, PCC: pheochromocytoma, sPGL: sympathetic paraganglioma, pPGL: parasympathetic paraganglioma.

#### Paraganglioma-Pheochromocytoma syndrome

The paraganglioma-pheochromocytoma syndrome is an autosomal dominant syndrome with incomplete penetrance. It is caused by mutations in the tumor suppressor genes *SDHB*, *SDHC*, *SDHD,* and *SDHAF2*, located on 1p36, 1q21, 11q23, 11q13, and 5p15, respectively. (47-50)

SDH, which is the abbreviation for succinate dehydrogenase, is a protein complex that consists of four subunits (A, B, C, and D) and is located at the inner mitochondrial

membrane. It is an important enzyme in both the citric acid cycle and the electron transport chain, where it is known as complex II. SDHA and SDHB serve as catalytic subunits and are anchored to the mitochondrial inner membrane by SDHC and SDHD. SDH catalyzes the oxidation of succinate to fumarate with the reduction of ubiquinone to ubiquinol (Figure 3). (51) Consistent with Knudson's two hit hypothesis for tumorigenesis, a heterozygous germline mutation in *SDHB*, *SDHC*, *SDHD* or *SDHAF2* is associated with somatic loss of the non-mutant allele in the tumor. (52) This makes the complex unstable and susceptible to degradation, which results in complete abolition of SDH enzymatic activity. The malfunction of SDH will cause an accumulation of succinate, which will inhibit the activity of prolyl hydroxylases (PHDs). (53) PHDs are oxygen dependent enzymes that can hydroxylate HIF. The inhibition of PHDs results in prolonged HIF half-life and generates a pseudohypoxic response.

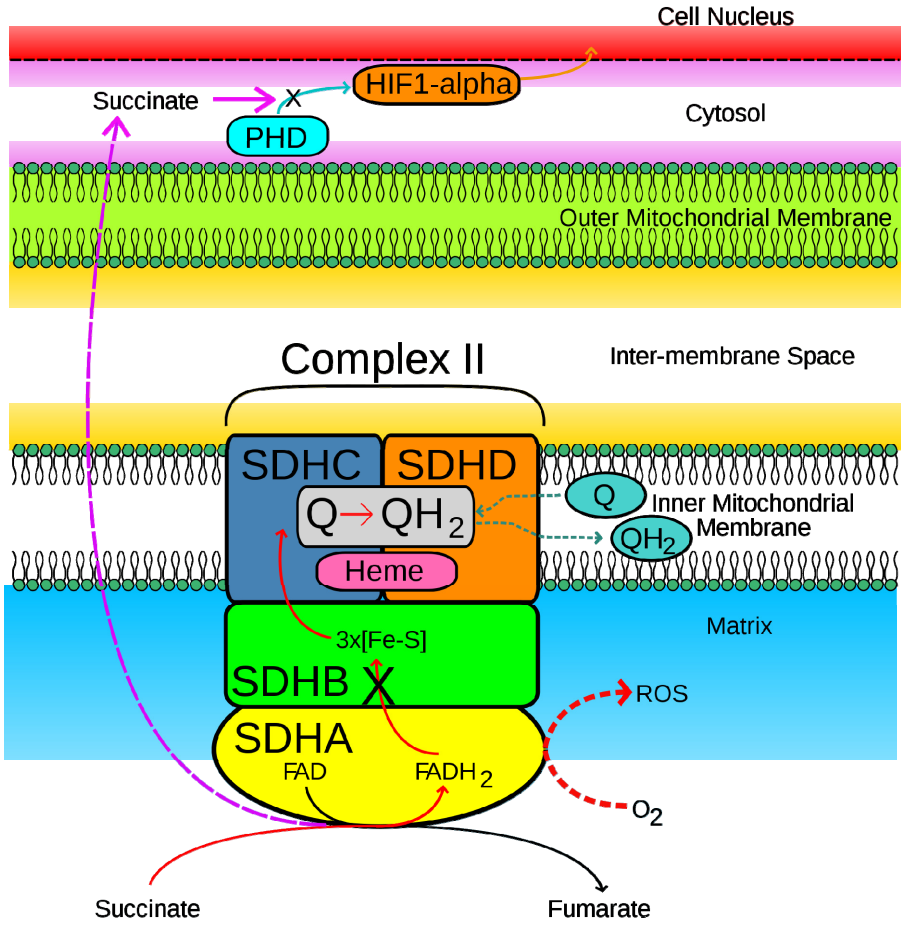


**Figure 3.** SDH catalyzes the oxidation of succinate to fumarate. In case of a mutation succinate will not be converted to fumarate and piles up. Succinate inhibits prolyl hydroxylase (PHD), which is then unable to hydroxylate HIF-1α.

All reported *SDH* allelic variants that give rise to familial PGL syndromes are available online at http://chromium.liacs.nl/lovd_sdh/. (54) Although SDHB, SDHC, SDHD and SDHAF2 are all involved in the same complex, mutations in the different genes result in different phenotypes.

**SDHD (PGL1)**

Germline mutations in *SDHD* were first linked to hereditary paraganglioma in 2000. (48) *SDHD* mutation carriers generally develop parasympathetic paragangliomas (79%) and multiple tumors (74%) that are usually benign. (55) Presently there have been over 100 distinct SDHD mutations described. In The Netherlands most familial parasympathetic paragangliomas are caused by three SDHD founder mutations (p.Asp92Tyr, p.Leu95Pro and p.Leu139Pro). The majority of the mutations constitute protein-truncating or missense mutations that cause loss of function and important reduction in SDH due to disassembly of complex II. *SDHD*-related disease is characterized by maternal imprinting, and as a result generation skipping occurs frequently. (56) Thus, individuals who inheriting a SDHD mutation from mother remain free of paraganglioma, but may still pass on the mutation to their offspring. However there is one case described in the literature of maternal transmission of a SDHD-linked paraganglioma. (57) *SDHD* mutations have also been described in patients with the Carney-Stratakis dyad (paraganglioma and gastrointestinal stromal tumor). (58)

**SDHAF2 (PGL2)**

This gene was discovered in 2009, during my research period. Thus far it has only been described in one Dutch family. (49) In this family, 33 individuals with the SDHAF2 G78R mutation developed parasympathetic paragangliomas. Seven individuals who inherited the mutation from their mother did not develop the disease, which suggests an *SDHD*-like parent of origin-specific inheritance pattern.

**SDHC (PGL3)**

Mutations in *SDHC* are very rare, and mainly associated with parasympathetic paragangliomas. (50, 59-64) However a few pheochromocytomas and sympathetic paragangliomas in patients with *SDHC* mutations have been described. (61, 63) The

prevalence of *SDHC* mutations varied between 0% for patients with sPGL and 4% for patients with HNPGL. (64-65) In patients with a germline SDHC mutation loss of heterozygosity was demonstrated in the tumors, implying that SDHC also behaves as a classical tumor suppressor gene (biallelic inactivation).

**SDHB (PGL4)**

*SDHB* mutations are predominantly associated with the development of sympathetic paragangliomas although occurrence of parasympathetic paragangliomas and pheochromocytomas has been reported as well. (55, 66) *SDHB* mutation carriers have an increased risk to develop malignant disease. (55, 66-67) Malignant paragangliomas are reported in at least 34% of SDHB patients. In addition to paragangliomas and pheochromocytomas, *SDHB* mutations have been associated with renal cell carcinoma and gastrointestinal stromal tumors. (55, 58, 68-70)

**Von Hippel-Lindau syndrome**

Von Hippel-Lindau (VHL) disease is an inherited, autosomal dominant disorder with a prevalence of 2-3 per 100,000. The syndrome is caused by mutations in the *VHL* tumor suppressor gene, which is located on chromosome 3 (3p25-26). (71-72) VHL disease is manifested by hemangioblastomas, clear cell renal cell carcinomas and pheochromocytomas. (73) In addition, a multitude of other rare tumors can occur and even be the sole manifestation of VHL disease. Based upon the likelihood of developing PCC, VHL disease has been divided into types 1 and 2. VHL type 1 families frequently harbor *VHL* deletions or truncation mutations and have a low risk of developing PCC. VHL type 2 families harbor missense mutations and have a high risk of developing PCC. (74-75) Parasympathetic paragangliomas have been described in VHL but are rare. The incidence is around 0.15 percent of all VHL patients. (76) Proof of involvement of the *VHL* gene in these paragangliomas was not given.

VHL- and SDH-related paragangliomas do not differ histologically from each other or from sporadic tumors. Immunohistochemistry for VHL does not facilitate the discrimination of VHL-related and –unrelated tumors. Pheochromocytomas and paragangliomas, either VHL-syndrome related or sporadic, demonstrate positive staining for the VHL protein, which suggests that the antibody also recognizes the mutated VHL protein. (77)

**Neurofibromatosis type 1**

Neurofibromatosis type 1 (NF1), also known as Von Recklinghausen's disease, is an autosomal dominant disorder. It has a prevalence of 1 in 3,000, making it a relatively common condition. Although many cases are inherited, approximately 50% of patients show de novo mutations. (78) NF1 is caused by mutations in the *NF1* gene, located on chromosome 17 (17q11.2). The protein encoded by this gene, called neurofibromin, belongs to a family of GTPase-activating proteins (GAPs). Neurofibromin downregulates the *Ras* oncogene by accelerating the conversion of active Ras-GTP to inactive Ras-GDP; it also regulates adenylyl cyclase activity. (79-80) Due to the fact that the *NF1* gene is one of the largest known genes, with 60 exons spanning more than 350 kb of genomic DNA, routine genetic testing is available only in selected laboratories.

NF1 is characterized by multiple café-au-lait maculae, neurofibromatosis, iris hamartomas, and axillary or inguinal freckling. Diagnosis of NF1 is made on the basis of clinical criteria, requiring the presence of two or more of the following: six or more café-au-lait maculae; two or more neurofibromas; one plexiform neurofibroma; axillary or ingunal freckling; optic gliomas; two or more Lisch nodules; a distinctive osseous lesion; or a first-degree relative with NF1. (81) Other tumors associated with NF1 are optic gliomas, pancreatic endocrine tumors (somatostatinomas) and pheochromocytomas. The latter are rare, with a reported frequency of 0.1-5.7%. (82)

**Multiple endocrine neoplasia type 2**

MEN 2 syndrome has an estimated prevalence of 2.3 per 100,000 in the general population. (83) The syndrome is caused by activating mutations in the *RET* (**Re**arranged during **T**ransfection) proto-oncogene. (84) The *RET* gene is located on chromosome 10q11.2 and codes for the RET protein, which is a member of the receptor tyrosine kinase family. (85) This receptor may activate various signaling pathways, including PI3K/AKT, MAPK, JNK and RAS/ERK pathways. (86) *RET* germline mutations are usually located in exons 10 and 11 (extracellular cysteine-rich region) or in exons 13-16 (intracellular tyrosine kinase domain). (87)

MEN 2 is subdivided into MEN 2A, MEN 2B and familial medullary thyroid carcinoma. MEN 2A, also known as Sipple syndrome, accounts for most cases of MEN 2 syndrome (90%) and is characterized by a combination of medullary thyroid carcinoma in all patients, pheochromocytoma in 50% of patients and hyperparathyroidism resulting from parathyroid hyperplasia or adenoma in 10-20% of patients. (85) Five % of all MEN2 patients have MEN 2B, which has medullary thyroid carcinoma and pheochromocytoma with the same frequencies as MEN 2A but includes additional clinical features such as mucosal neuromas, ganglioneuromatosis of the gastrointestinal tract, and a marfanoid habitus. Familial medullary thyroid carcinoma is characterized by medullary thyroid carcinoma as the sole manifestation of the syndrome. (85) About 50% of patients with MEN 2B have a de novo germline mutation in the *RET* gene. (88) In contrast 6-9% of patients with MEN 2A are thought to have a de novo germline mutation. (89)

Medullary thyroid carcinoma is generally the first manifestation in all MEN 2 subtypes. In MEN2A medullary thyroid carcinoma develops between the ages of 5 and 25 years. In MEN 2B disease onset is usually in the first year of life. Pheochromocytoma is the first manifestation of MEN 2 in 9-27% of patients, is benign in more than 95% of cases and is often bilateral (50% of cases). (90-91)

Pheochromocytomas are found in patients with *RET* mutations in all MEN2-associated codons (except 609, 768, V804M and 891), however they are most often associated with mutations in codon 634. (46, 92-94)

Pheochromocytomas and paragangliomas are also found in the following disorders:

**Multiple endocrine neoplasia type 1**

MEN 1, also known as Wermer syndrome, has an estimated prevalence of 0.15-0.3 per 1000 in the general population. (95) The syndrome is caused by mutations in the *MEN1* gene, which is located on 11q13 and codes for a 610-amino acid protein product, called menin. (96) The exact role of this protein is not fully understood. Tumors from MEN 1 patients often show loss of heterozygosity (LOH) at 11q13. (97) Also, tumors from heterozygous *MEN1* mutant mice exhibit LOH of the wild-type *MEN1* allele, indicating that menin is a bona fide tumor suppressor gene. (98) It is suggested that menin represses the transcriptional activity of junD, which acts as a negative regulator of ras-dependent cell growth and protects cells from p53-dependent senescence and apoptosis. (99-102) In addition to JunD, menin is also known to interact with other transcription factors, including NF-κB, Smad3, p53, and Pem, implicating a general role of menin in regulating transcription. (103) *MEN1* mutations have been found throughout the coding exons of the *MEN1* gene, and no hotspots have been found. (104) Approximately 10% of the mutations arise de novo. (105)

MEN 1 causes combinations of over 20 different endocrine and nonendocrine tumors. Pheochromocytomas occur in less then 1% of MEN1 patients. (92) The classic manifestation of MEN 1 is a combination of parathyroid hyperplasia, pancreatic and/or duodenal endocrine tumors and pituitary adenoma. The clinical diagnosis of MEN1 is made when two of these three endocrine proliferations occur in the same patient. (106)

**Carney triad**

Carney triad is an extremely rare disorder that primarily affects young women (88%). (107) Originally described in 1977, the classic Carney triad includes sympathetic paraganglioma, gastrointestinal stromal tumors (GISTs), and pulmonary chondroma. (108) Pheochromocytoma, adrenal cortical adenoma, and esophageal leiomyoma were later shown to be associated with the syndrome. (109) Carney triad is usually only partially expressed. One-fifth of the patients have all three tumors; the remainder has two of the three tumors, usually GIST and pulmonary chondroma. (109) GIST is usually the

presenting tumor (75%), followed by the pulmonary chondroma (15%) and paraganglioma (10%).

Unlike Carney-Stratakis syndrome (see below) there are no inherited cases of Carney triad. However the triad is generally accepted to be a genetic disorder. The etiology of the syndrome is unknown and positional cloning of the responsible gene is not possible because families are not affected. To date, no coding sequence mutations of *KIT* and *PDGFRA* genes or the *SDHB*, *SDHC*, and *SDHD* genes have been found. The most frequent and largest contiguous change detected by comparative genomic hybridization is deletion of the 1cenq21. (110)

**Carney-Stratakis syndrome**

Carney-Stratakis syndrome is the association of paragangliomas and GISTs. It is a very rare autosomal dominant disorder with incomplete penetrance. It presents at a young age (median age: 19 years) with an apparently equal ratio of male and female patients. The GISTs are multifocal and the paragangliomas are multicentric. Paragangliomas are usually benign, occur without clinical evidence of oversecretion, and arise in the sympathetic nervous system. Germ line mutations in *SDHB*, *SDHC* and *SDHD* genes have been found.

Distinguishing Carney triad and Carney-Stratakis syndrome is difficult in individual patients. However the familial predisposition and paraganglioma as the first presenting tumor in Carney-Stratakis syndrome and the presence of pulmonary chondroma, female predominance and GIST as the first presenting tumor in Carney triad are differentiating features. (111)

In the last few years several other genes were discovered to be associated with pheochromocytomas and paragangliomas, such as SDHA, PHD2, and TMEM127.

**SDHA**

Germline biallelic *SDHA* mutations cause Leigh syndrome, a neurodegenerative disorder, but there are no reports of paraganglioma in parents of *SDHA*-related Leigh syndrome patients, who are presumably heterozygous for *SDHA* mutations. (112-113) However, very recently Burnichon et al described one patient with a sympathetic paraganglioma and a

heterozygous germline *SDHA* mutation, p.Arg589Trp. (114) In this tumor loss of the wild type *SDHA* allele was demonstrated and in addition the tumor cells were negative for SDHA and SDHB immunohistochemically, indicating involvement of SDHA inactivation in this paraganglioma. So *SDHA* should be considered as a new gene causing the paraganglioma-pheochromocytoma syndrome.

**PHD2**

Prolyl hydroxylase domain (PHD) proteins play a major role in regulating the hypoxia-inducible factor (HIF) that induces expression of genes involved in angiogenesis, erythropoiesis, and cell metabolism, proliferation, and survival. Germ line mutations in the *PHD2* gene have been reported in patients with familial erythrocytosis. Only one case of a patient with erythrocytosis and recurrent paraganglioma with a *PHD2* mutation is described. (115) The His374Arg *PHD2* mutation found in this patient affects PHD2 function and stabilizes HIF-α proteins. In addition, there was loss of heterozygosity of the *PHD2* locus in the tumor, suggesting that *PHD2* can act as a tumor-suppressor gene. (115)

**TMEM127**

Recently the transmembrane-encoding gene *TMEM127* on chromosome 2q11 was identified as a new pheochromocytoma susceptibility gene. (116) In a cohort of 103 samples, truncating germ line *TMEM127* mutations were found in approximately 30% of familial tumors and about 3% of apparently sporadic pheochromocytomas. The wild-type allele was deleted in tumor DNA, suggesting a classic mechanism of tumor suppressor gene inactivation.

In vitro gain-of-function and loss-of-function analyses indicate that TMEM127 is a negative regulator of mTOR. (116) mTOR, also known as FK506 binding protein 12-rapamycin associated protein 1 (FRAP1), is a serine/threonine protein kinase that regulates cell growth, cell proliferation, cell motility, cell survival, protein synthesis, and transcription. (117)

## 1.4 HIF pathway

The key pathway in the development of paragangliomas seems to be the HIF pathway. HIFs (Hypoxia inducible factors) are transcription factors that respond to decreases in oxygen. HIF is comprised of three HIF-α and one HIF β –subunits and its physiological function is to promote adaptation of cells to low oxygen by inducing neovascularization and glycolysis. (118-119)

Under normoxic conditions HIF-1α is hydroxylated by prolyl-hydroxylases. This hydroxylation is oxygen-dependent and makes HIF-1α a target for polyubiquitination by the E3 ubiquitin ligase. (120-123) After polyubiquitination HIF-1α will be degraded by proteases. Since prolyl-hydroxylase utilizes oxygen as a co-substrate it is inhibited in hypoxic conditions. When HIF-1α is not hydroxylated it will not be ubiquitinated and accumulates. It could then bind to HIF-1β and is able to form the HIF complex, which can induce the transcription of hypoxia-inducible genes such as erythropoietin and vascular endothelial growth factor (VEGF). (124-125) Succinate also inhibits prolyl-hydroxylase action. (126) As stated above, mutations in *SDHB*, *SDHC*, *SDHD* and *SDHAF2* result in a complete abolition of SDH enzymatic activity. The abnormal SDH function induces an accumulation of succinate, which will inhibit prolyl-hydroxylase, which in its turn is unable to hydroxylase HIF. This is also called pseudo hypoxia. Figure 4 shows the HIF pathway in normoxic and (pseudo)hypoxic conditions.

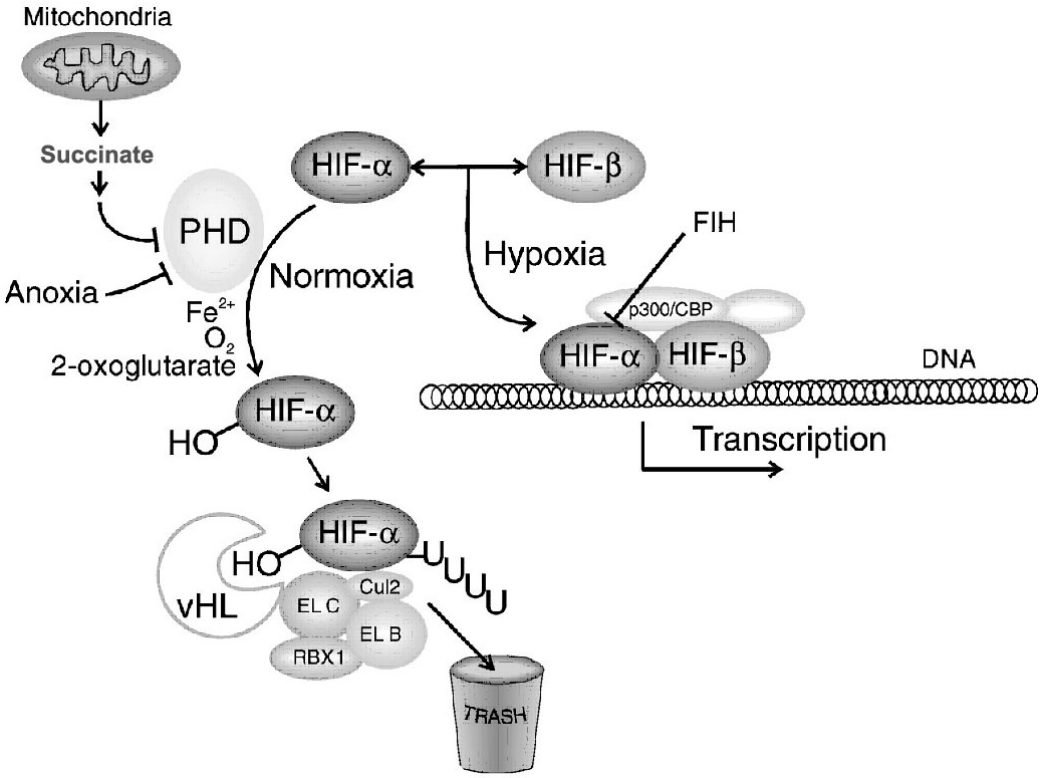


**Figure 4.** HIF pathway in normoxic and (pseudo)hypoxic conditions

## 1.5 Aims and outline of this thesis

Presently there are ten genes (*RET*, *VHL*, *NF1*, *SDHA, SDHB*, *SDHC*, *SDHD*, *SDHAF2*, *PHD2 and TMEM127*) known to cause paragangliomas and pheochromocytomas. A remaining problem is whether all paragangliomas- and pheochromocytoma patients should have genetic testing of all these genes. The overall frequency of germ line mutations is high (25-35%), but genetic testing of all candidate genes would be costly, laborious and technically demanding. This problem has been investigated in the first part of this thesis.
The genetic background of a large proportion of paragangliomas is determined, however about 50% of all paragangliomas are not caused by mutations in one of the nine genes mentioned above. In the second part of this thesis we searched for other candidate genes involved in paraganglioma development.

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

- To search for a method to distinguish paragangliomas and pheochromocytomas caused by *SDH* mutations from those unrelated to SDH.
- To search for other candidate genes causing paragangliomas.

**Outline**

Distinguishing paragangliomas and pheochromocytomas of the different syndromes is difficult. Immunohistochemistry for VHL does not facilitate the discrimination of tumors obtained from VHL patients or those unrelated to VHL disease. Paragangliomas and pheochromocytomas, either VHL-related or sporadic, demonstrate positive staining for the VHL protein, which suggests that the antibody also recognizes a mutated VHL protein. (77) In pheochromocytomas the same applies to RET immunohistochemistry, which has been shown to have an increased expression in a variety of hereditary and sporadic pheochromocytomas. (127) In Chapter 2 we investigated if distinction between pheochromocytomas and paragangliomas with different genetic background could be made using a SDHB immunohistochemistry.

Immunohistochemistry of the *SDHA* related tumor described by Burnichon et al showed SDHB protein loss, but also SDHA protein loss. In contrast, *RET*, *NF1*, *SDHB*, and *SDHD*-

related tumors were immunohistochemically positive for SDHA. (114) These results suggest that SDHA immunohistochemistry would be an adequate technique to diagnose *SDHA*-mutated pheochromocytomas and paragangliomas. We therefore determined the significance of SDHA immunohistochemistry for identifying patients with *SDHA* mutations in chapter 3.

Recently, other tumors have been described to be associated with *SDH* mutations. For instance, renal cell carcinoma is observed in *SDHB* mutation carriers. *SDHB*, *SDHC*, and *SDHD* germline mutations have also been found in patients with the dyad of paraganglioma and gastrointestinal stromal tumors, also called the Carney-Stratakis syndrome. (58) It appears that certain other tumors may be involved in the PCC-PGL syndrome. Since SDHB immunohistochemistry is a reliable technique to identify pheochromocytomas and paragangliomas caused by mutations in *SDHB*, *SDHC* and *SDHD* (as shown in chapter 2), it is to be expected that others tumors caused by *SDH* mutations, could be diagnosed by SDHB immunohistochemistry as well. In Chapter 4 we investigated gastrointestinal stromal tumors in which SDHB immunohistochemistry could also be a diagnostic tool.

The *VHL* gene is a bona fide tumor suppressor gene with biallelic inactivation contributing to tumor formation. However, in parasympathetic paragangliomas occurring in VHL disease biallelic inactivation of the *VHL* gene was not demonstrated. In chapter 5 we studied the biallelic inactivation of *VHL* in parasympathetic paraganglioma.

It has been shown that isocitrate dehydrogenase-1 (*IDH1*) carrying an arginine at codon 132 dominantly inhibits wild type IDH1 activity through the formation of catalytically inactive heterodimers. In cultured cells with forced expression of mutant IDH1, a reduced formation of α-ketoglutarate and increased levels of HIF-1α were seen. The rise in HIF-1α levels was reversible by an alpha ketoglutarate derivative. (128) HIF-1α levels were higher in human gliomas harboring an *IDH1* mutation than in tumors without a mutation. In conclusion, IDH1 appears to function as a tumor suppressor that contributes to tumorigenesis, through induction of the HIF1 pathway. Since HIF1 pathway activation also

plays a role in paragangliomas we investigated sporadic paragangliomas for IDH1 and IDH2 mutations in chapter 6.

A germline mutation in the *SDHAF2* gene is to date only found to be mutated in one large Dutch kindred with parasympathetic paragangliomas. (49) In chapter 7 we aimed to identify *SDHAF2* mutations in sporadic paraganglioma and pheochromocytoma patients to assess the clinical genetic significance of SDHAF2.

Biallelic germline mutations in *succinate dehydrogenase assembly factor 1* (*SDHAF1*) have recently been found in two families with mitochondrial complex II deficiency. (129) Similar to *SDHAF2*, *SDHAF1* is essential for SDH assembly but does not physically associate with the complex in vivo. In chapter 8 we investigated whether SDHAF1 mutations could be involved in the pathogenesis of paragangliomas and pheochromocytomas.

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# CHAPTER 2

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

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**Abstract**

Pheochromocytomas and paragangliomas are neuro-endocrine tumors that occur sporadically and in several hereditary tumor syndromes, including the pheochromocytoma–paraganglioma syndrome. This syndrome is caused by germline mutations in succinate dehydrogenase B (*SDHB*), C (*SDHC*), or D (*SDHD*) genes. Clinically, the pheochromocytoma–paraganglioma syndrome is often unrecognized, although 10–30% of apparently sporadic pheochromocytomas and paragangliomas harbor germline *SDH*-gene mutations. Despite these figures, the screening of pheochromocytomas and paragangliomas for mutations in the *SDH* genes to detect pheochromocytoma–paraganglioma syndrome is rarely done because of time and financial constraints. We investigated whether SDHB immunohistochemistry could effectively discriminate between *SDH*-related and non-*SDH*-related pheochromocytomas and paragangliomas.

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

SDHB protein expression was absent in all 102 pheochromocytomas and paragangliomas with an *SDHB*, *SDHC*, or *SDHD* mutation, but was present in all 65 paraganglionic tumors related to multiple endocrine neoplasia type 2, von Hippel–Lindau disease, and neurofibromatosis type 1. 47 (89%) of the 53 pheochromocytomas and paragangliomas with no syndromic germline mutation showed SDHB expression. The sensitivity and specificity of the SDHB immunohistochemistry to detect the presence of an *SDH* mutation in the prospective series were 100% (95% CI 87–100) and 84% (60–97), respectively.

Pheochromocytoma–paraganglioma syndrome can be diagnosed reliably by an immunohistochemical procedure. *SDHB*, *SDHC*, and *SDHD* germline mutation testing is indicated only in patients with SDHB-negative tumors.

## Introduction

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

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

With the exception of the NF1 syndrome, where the cutaneous café-au-lait spots are characteristic,[8] patients with inherited pheochromocytomas and paragangliomas often go without clinical detection. In large published series of patients with pheochromocytomas and paragangliomas, it has been shown that 25–30% of patients have an inherited form and 12% of patients with an apparently sporadic pheochromocytoma and paraganglioma have unexpected germline mutations in *VHL*, *SDHB*, or *SDHD* genes.[3,7–9] The underdiagnosis of patients with inherited pheochromocytoma and paraganglioma is the result of a combination of factors, including lack of family information, overlap in age distribution between hereditary and sporadic cases, de-novo mutations, incomplete penetrance (*SDHB*), parent-of-origin effects on penetrance (*SDHD*), phenotypic heterogeneity of the disease, and insufficient awareness of clinicians. There is controversy among experts as to whether *RET*, *VHL*, *SDHB*, *SDHC*, and *SDHD* genetic testing should be done in all patients with pheochromocytoma and paraganglioma. Many experts have advocated that molecular genetic testing should be targeted in patients fulfilling specific clinical criteria.[4,10–12] However, reliable clinical indicators for the presence of *SDHB*, *SDHC*, and *SDHD* germline mutations in patients with pheochromocytoma and paraganglioma are often absent.

Hidden heredity is most pronounced for patients with apparently sporadic parasympathetic paragangliomas, with up to 34% of cases having a germline mutation in *SDHD*.[13] Clinical indications with high specificity but low sensitivity for the detection of pheochromocytoma–paraganglioma syndrome (family history of pheochromocytoma or paraganglioma, multifocal disease, younger age at onset, and malignant tumors) are insufficient for correct diagnosis of the syndrome. The detection of inherited pheochromocytoma–paraganglioma syndrome is of major importance for patients with pheochromocytoma and paraganglioma, as well as for their family members, since they are at an increased risk of developing multiple, various, and malignant neoplasms.[4,14–16] Additionally, after identification of an *SDHB*, *SDHC*, or *SDHD* germline mutation, surveillance can be offered to the individual patient with the paraganglionic tumor and to any family members who carry the mutation. Mutation analysis of *SDHB*, *SDHC*, and *SDHD* has been advocated to diagnose pheochromocytoma–paraganglioma syndrome in all cases of pheochromocytoma and paraganglioma where there are no clear clinical or

family indications for the syndrome.16 Although *SDH*-mutation carriers will be identified frequently by mutation analysis of all patients with pheochromocytomas and paragangliomas, most cases will be without mutation, making this genetic-screening strategy a labour-intensive and financially demanding procedure.

Pheochromocytoma–paraganglioma syndrome tumors differ from sporadic pheochromocytomas and paragangliomas by the presence of *SDHB*, *SDHC*, or *SDHD* mutations, which are, except for a few incidental cases,[17,18] not found in truly sporadic pheochromocytomas and paragangliomas. Despite this genotypic difference, no reliable phenotypic discrimination between sporadic pheochromocytomas and paragangliomas, and pheochromocytoma–paraganglioma syndrome-related tumors, is possible at present. In the present study we determined the value of SDHB immunohistochemistry for discriminating between *SDH*-related and non-*SDH*-related pheochromocytomas and paragangliomas in large retrospective and prospective series in two different centers.

## Methods

### Patients

Two retrospective series of pheochromocytomas and paragangliomas were investigated by SDHB immunohistochemistry (Erasmus MC, Rotterdam, Netherlands, 110 cases; Hôpital Européen Georges Pompidou and Hôpital Cochin, Paris, France, 65 cases). These series consisted of pheochromocytomas diagnosed at Erasmus MC between 1982 and 2007, and diagnosed at INSERM U970 between 1995 and 2007, and of paragangliomas diagnosed in Erasmus MC between 1993 and 1998, and in INSERM U970 between 1993 and 2008. The series were enlarged with additional germline-mutated *SDHB*, *SDHC* and *SDHD* cases from other centers, with as many different mutations as possible. In total, the series consisted of 175 formalin-fixed and paraffin-embedded (FFPE) tumors (101 pheochromocytomas, 58 paragangliomas,three metastases, and 13 paraganglionic tumors of unknown location) including 24 *RET*, 29 *VHL*, 12 *NF1*, 34 *SDHB*, 38 *SDHD*, four *SDHC* germline-mutant cases, and 34 sporadic cases.

Furthermore, SDHB immunohistochemistry was also done on a prospective series of 45 tumors (six pheochromocytomas and 39 paragangliomas), for which the *SDH*-gene status was not known beforehand. This prospective series consisted of all paragangliomas diagnosed in Erasmus MC between 2002 and 2008, and all pheochromocytomas diagnosed in 2008. After the SDHB immunohistochemical results were obtained from this series, *SDH*-gene mutation analysis was done. Detailed information on all investigated cases is shown in the Supplemental table 1. Determination of mutation status in these patients and families was done on-site and with the informed consent of the patients. The prospective series was assessed anonymously according to the code for adequate secondary use of tissue code of conduct established by the Dutch Federation of Medical Scientific Societies. Ethical approval for the study was obtained from the institutional review board (CPP Paris-Cochin, January, 2007).

**Procedures**

Two different primary antibodies against SDHB were used: mouse monoclonal clone 21A11 (NB600-1366; Novus Biologicals, Littleton, CO, USA; 1:50) and rabbit polyclonalHPA002868 (Sigma-Aldrich Corp; St Louis, MO, USA;1:500). The antibodies were applied on routine FFPE archival tissues. 4–6 μm sections were cut and mounted on Starfrost Plus (Knittel Gläser; Braunschweig, Germany) glass slides. The sections were deparaffinised, rehydrated, exposed to microwave heating in Tris–EDTA buffer, pH 9·0 or citrate buffer, pH 6·0 at 100°C for 15 min, rinsed in tap water followed by incubation in 3% H2O2 in PBS for 20 min. The SDHB antibodies were diluted in normal antibody diluent (Klinipath, Duiven, Netherlands) and slides were incubated with 100 μL per slide overnight at 4°C, followed by rinsing in Tris–Tween 0·5%, pH 8·0. Dako ChemMate envision horseradish peroxidase was applied for 30 min (100 μL/slide; Dako envision kit, Glostrup, Denmark), followed by rinsing with phosphatebuffered saline. Diaminobenzidine tetrahydrochloride (100 μL/slide; Dako envision kit) was applied for 5 min twice, after which the slides were rinsed with distilled water. Slides were counterstained with Harris haematoxylin for 1 min, rinsed with tap water, dehydrated, and covered with cover slips. In the negative control reactions, the primary antibodies were omitted from the dilution buffer, which in all instances resulted in a complete absence of staining. Human heart muscle, adrenal gland, liver, and colon tissues were used as positive controls. These

tissues showed strong granular staining in the cytoplasm with both antibodies. In pheochromocytoma and paraganglioma the normal stromal cells of the fibrovascular network surrounding the Zellballen of tumor cells served as an internal positive control for each sample, also showing strong granular cytoplasmatic staining as in the positive control samples. Pathologists who had no knowledge of the mutation status of the specimens scored the immunohistochemical results from the retrospective series from Rotterdam and Paris independently. The immunohistochemical results of the prospective series were scored by researchers or by pathologists, before mutation analyses were done.

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

To test whether absence of immunohistochemical staining for SDHB in the tumors correlated with decreased SDH enzyme activity, SDH enzyme histochemistry was done according to Pearse19 with minor modifications. Cryostat sections from the same tumor samples used for western blotting were incubated at 37°C for 1 h with an SDHenzyme substrate solution (containing 8·3 mmol/L $NaH_2PO_4.H_2O$, 33·3 mmol/L $Na_2HPO_4.2H_2O$, 41·7 mmol/L $Na_2C_4H_4O_4$, 2·5 mol/L Nitroblue terazolium (N-6876, Sigma-Aldrich), 0·22 mmol/L $AlCl_2.6H_2O$, 0·13 mM $CaCl_2$, 25 mM $Na_2HCO_3$, and 0·17 mmol/L Phenazine methosulfate (P9625, Sigma-Aldrich). After rinsing in water twice, the slides were

incubated at 4°C for 15 min in formaline-macrodex solution (containing 10 mL 37% formaldehyde, 10 mL 1% CaCl2, 80 mL macrodex [Pharmalink, Stockholm, Sweden]). After rinsing the slides in water again three times, the slides were mounted with imsolmount (Klinipath, Duiven, Netherlands) and covered with cover slips. Snap frozen healthy triceps muscle tissue was used as a positive control. As negative controls, sections from the same tumor tissues were incubated in buff er from which nitroblue terazolium was omitted. Mutation analyses for *RET*, *VHL*, *SDHB*, *SDHC*, and *SDHD* genes of the series of 175 retrospective tumors were done previously.[4,20] For these analyses, DNA was retrieved from FFPE tumor and normal tissues or from peripheral blood, in the period from 1993 until 2008. DNA was isolated using described and standard procedures, and mutation analyses were done with or without pre-screening by single-strand conformation polymorphism analysis (SSCP) followed by direct, in-house, or commercial (Baseclear, Leiden, Netherlands) sequencing of PCR products.[13,20,21]

Mutation analyses of the additional samples from other centers were done by sequencing on site and verified at Erasmus MC and INSERM U970. Mutation analysis of all 34 sporadic cases was done by direct sequencing of the open reading frames, including the exon–intron boundaries, of the *SDHB*, *SDHC*, and *SDHD* genes.[4] The prospective series of 45 tumors was also investigated for *SDHB*, *SDHC*, and *SDHD* mutations by direct sequencing of the open reading frames including all exon–intron boundaries as described previously.[20] Additionally, this series was investigated for the presence of large genomic deletions in the *SDH* genes by multiplex ligation-dependent probe amplification (MLPA) assay with a commercially available kit (SALSA MLPA P226; MRC Holland, Amsterdam, Netherlands).

**Statistical analysis**

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

**Results**

Immunohistochemical staining was done on all 220 tumor samples. Of these tumors, 102 had a germline *SDH* mutation (36 *SDHB*, five *SDHC* and 61 *SDHD*) and all were negative for SDHB immunohistochemistry (figure 1A–C). In four *SDH*-mutated tumors (*SDHB* p.Cys98Arg and p.Pro197Arg, and *SDHD* p.Asp92Tyr and c.169_169+9delTGTATGTTCT) a weak and diff use cytoplasmic SDHB immunoreactivity was seen in the tumor cells, clearly distinct from the strong speckled pattern present in normal cells of the intratumoral fibrovascular network (figure 1C). However, independent tumor samples with the same mutation (*SDHB* p.Pro197Arg and *SDHD* p.Asp92Tyr) were clearly negative for SDHB immunostaining. Therefore, this weak diff use cytoplasmic staining in the tumor cells was considered to be a non-specific background artifact and scored as negative. 65 tumors had a germline mutation in *RET* (24 cases), *VHL* (29 cases), or *NF1* (12 cases, diagnosed pheno typically), and all showed expression of SDHB by immunohistochemistry (figure 1D–F). In the remaining 53 tumors, of which six tumors were SDHB-negative, no germline mutation in the *RET*, *VHL*, *SDHB*, *SDHC*, or *SDHD* genes was seen, nor was any *NF1* gene involvement detected. A summary of the results is listed in table 1 and comprehensive information on tumor characteristics, including type of mutation and results is presented in the supplemental table 1. In the prospective series, sensitivity and specificity were 100% (95% CI 87–100) and 84% (60–97), respectively. Table 2 shows that there was a highly significant association between the SDHB immunohistochemistry test result and the absence or presence of an *SDH* mutation ($p<0{\cdot}0001$; Fisher's exact test). SDHB immunohistochemistry done on cryostat sections from three pheochromocytomas, two with an *SDHD* mutation and one with a *RET* mutation, gave results comparable to FFPE tissue sections: speckled staining patterns in the normal cells and an absence of staining in *SDHD*-mutated tumor cells. This comparable SDHB immunoreactivity pattern on FFPE and frozen tissues is an additional indication for the specificity of the immunohistochemistry results. The decreased expression of SDHB protein in both *SDHB*-mutated and *SDHD*-mutated tumors was confirmed by western blotting (figure 2A). Additionally, the absence of SDH enzyme activity was determined by enzyme histochemistry. The *SDHB*-related and *SDHD*-related tumors showed no SDH activity, except for the normal cells of the intratumoral fibrovascular network, which showed

strong staining (figure 2B). By contrast, strong SDH enzyme activity was present in the triceps muscle tissue and the *RET*-related tumor tissue (figure 2C).

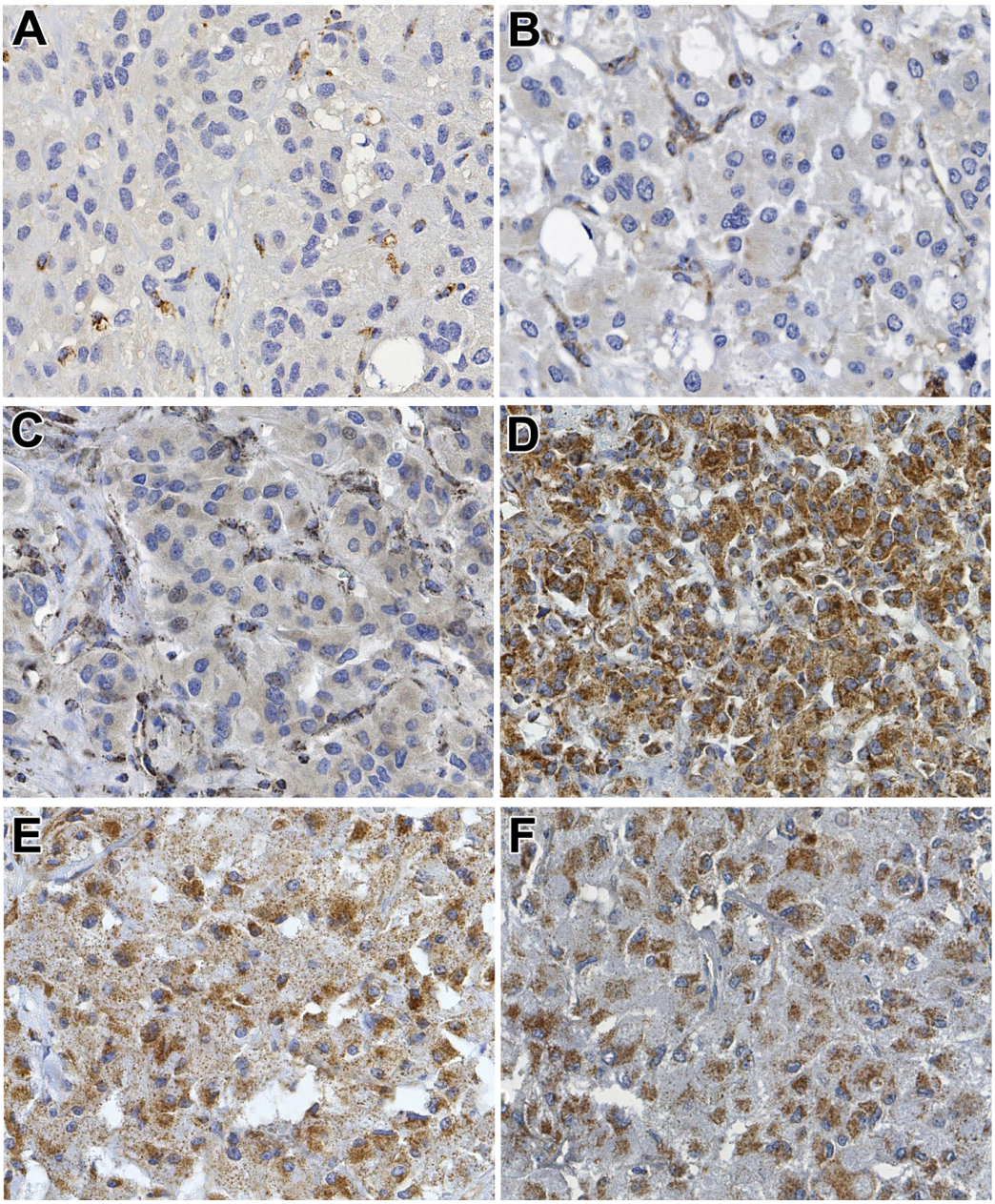


**Figure 1.** SDHB immunohistochemistry on paragangliomas and pheochromocytomas.

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

**Table 1. Clinical data and SDHB immunohistochemistry (IHC) related to the various syndromes.**

| Syndrome | Number | Gene mutated | Gender M/F | Age range (mean) | PCC | PGL | SDHB IHC positive | SDHB IHC negative |
|---|---|---|---|---|---|---|---|---|
| NF1 | 12 | *NF1* | 3/9 | 29-67 (44.2) | 12 | 0 | 12 | 0 |
| MEN2 | 24 | *RET* | 8/16 | 18-76 (35.6) | 24 | 0 | 24 | 0 |
| VHL | 29 | *VHL* | 12/13 (4 U) | 7-62 (25.6) | 21 (3U) | 5 | 29 | 0 |
| PCC-PGL | 36 | *SDHB* | 13/12 (11 U) | 10-63 (34.6) | 11 (7U) | 18 | 0 | 36 |
| PCC-PGL | 5 | *SDHC* | 2/3 | 15-47 (30.6) | 0 | 5 | 0 | 5 |
| PCC-PGL | 61 | *SDHD* | 25/35 (1 U) | 16-72 (40.9) | 5 (3U) | 53 | 0 | 61 |
| Sporadic | 53 | none | 17/34 (2 U) | 12-79 (49.3) | 34 (1U) | 18 | 47 | 6 |

NF1: neurofibromatosis type 1, MEN2: multiple endocrine neoplasia type 2, VHL: von Hippel-Lindau, PCC-PGL: pheochromocytoma-paraganglioma, U: unknown.

**Table 2. SDHB IHC test results according to subgroups within SDH-related and Non-SDH related tumors.**

| | | | SDHB IHC | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Series | Group | Gene | No. of tumors | Negative | Positive | Sensitivity | 95% CI | Specificity | 95% CI |
| Retrospective | SDH-related | SDHB | 34 | 34 | 0 | 100% | 90-100% | | |
| | | SDHC | 4 | 4 | 0 | 100% | 40-100% | | |
| | | SDHD | 38 | 38 | 0 | 100% | 91-100% | | |
| | Non-SDH related | RET | 12 | 0 | 12 | | | 100% | 74-100% |
| | | VHL | 24 | 0 | 24 | | | 100% | 86-100% |
| | | NF1 | 29 | 0 | 29 | | | 100% | 88-100% |
| | | Sporadic | 34 | 3 | 31 | | | 91% | 76-98% |
| Prospective | SDH-related | | 26 | 26 | 0 | 100% | 87-100% | | |
| | Non-SDH related | | 19 | 3 | 16 | | | 84% | 60-97% |

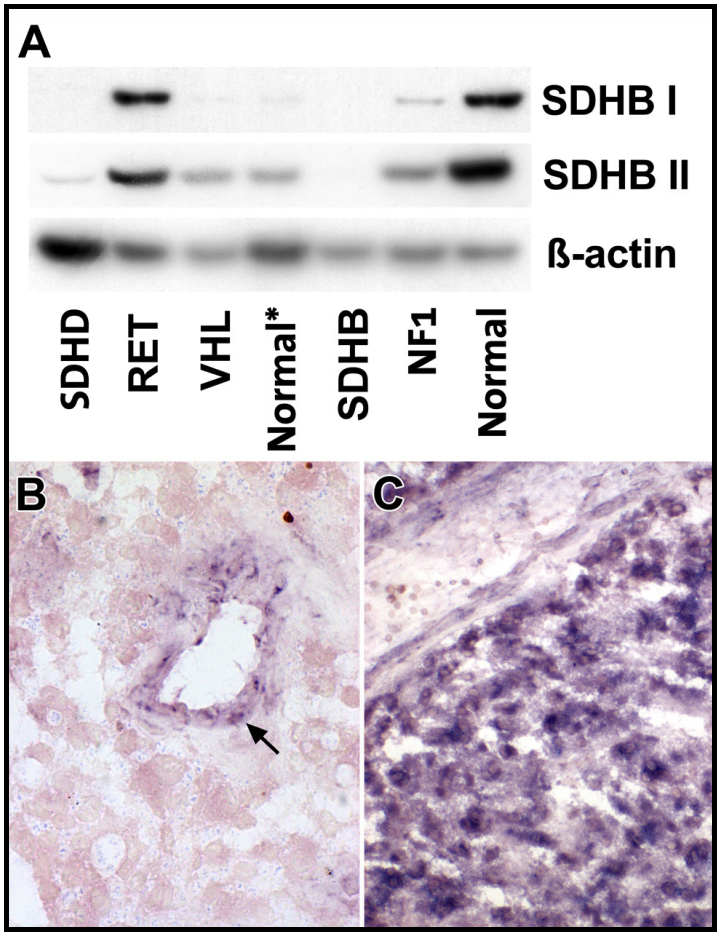


**Figure 2.** Western blotting and enzyme histochemical results.

A) Western blot result with SDHB antibodies from Novus biologicals NB600-1366 (SDHB I) and Sigma HPA002868 (SDHB II) and β-actin of PCC with different mutations. SDHB case: *SDHB* exon 3 deletion; SDHD case: *SDHD* p.Asp92Tyr missense mutation; RET case: *RET* p.Cys634Arg missense mutation; VHL case *VHL* p.Arg64Pro missense mutation; NF1 case: clinically NF1. *Normal is a lysate from a lymph node from the patient with the *SDHB* mutation and Normal is a lysate from a healthy adrenal gland.

SDH-enzyme histochemistry results. B) loss of SDH activity in tumor cells of a PCC with a *SDHD* p.Asp92Tyr mutation, but retained activity in the normal cells of the intratumoral fibro-vascular network (arrow), C) strong SDH activity in tumor and normal cells of a PCC with a *RET* p.Cys634Arg mutation.

**Discussion**

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

Of the 220 independent tumors analysed, 102 had a germline *SDH* mutation (36 *SDHB*, five *SDHC*, and 61 *SDHD*), and all were negative for SDHB immunostaining. 65 tumors had a germline mutation in *RET* (24 cases), *VHL* (29 cases) or *NF1* (12 cases, diagnosed phenotypically), and all showed expression of SDHB by immunohistochemistry. In the remaining 53 tumors no germline mutation in the *RET*, *VHL*, *SDHB*, *SDHC*, or *SDHD* gene, nor *NF1* gene involvement was detected, but six tumors were negative for SDHB immunostaining. The absence of SDHB protein in these six tumors might be caused by *SDH* mutations escaping detection by the DNA sequencing and MLPA methods used (eg, deleterious mutations in untranslated, intronic, or promoter regions of the genes, which were not investigated), or by epigenetic silencing of *SDH* genes. In two of these six patients without *SDH* mutations, but with SDHB immunohistochemistry-negative tumors, the clinical information was indicative of pheochromocytoma–paraganglioma syndrome: one patient had a family history of paraganglioma and one patient suffered from multiple paragangliomas (supplemental table 1). Furthermore, three of the four other SDHB-negative tumors without *SDH*-gene mutations were diagnosed at a young age (supplemental table 1; cases 179A, 180B, and 220C), indicating possible germline involvement. A negative *SDH* genetic testing in association with negative SDHB immunohistochemistry could indicate the possibility of a pheochromocytoma or paraganglioma hereditary syndrome, and we recommend that the patient be followed up in the same way as for a proven pheochromocytoma or paraganglioma hereditary syndrome. There is a highly significant association between the SDHB

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

The reliability of the immunohistochemical results on FFPE tumor specimens is also indicated by the similar results obtained with two different antibodies, applied on three different tumor series in two different laboratories (the retrospective series in Rotterdam and Paris, and prospective series in Rotterdam), and the concordant results obtained on cryostat sections, in western blotting, and by SDH-enzymehistochemistry. Our results show that in tumor cells with various mutations (*SDHB*; 15 different missense, two different nonsense, six different frameshift, three different exon deletions, three mutations probably affecting splicing), *SDHC*; two different missense, one nonsense, and two exon deletions, and *SDHD*; five different missense, two different nonsense, three different frameshift, and three mutations probably affecting splicing, no immunoreactive SDHB protein could be detected. These results are in accordance with preliminary findings by Douwes-Dekker and colleagues,[22] who reported generally decreased diffuse cytoplasmic SDHB expression in 11 *SDHD*-related (two different *SDHD* mutations) paragangliomas and strong granular expression in sporadic tumors and normal cells. Additionally, Dahia and colleagues[23] reported comparable decreased SDHB expression in five *SDHB*-related, one *SDHD*-related, and six *VHL*-related pheochromocytomas. However, in the present study we were able to discriminate *VHL*-related tumors from *SDH*-related pheochromocytoma and paraganglioma on the basis of SDHB immunohistochemistry, which could be the result of differences in the applied immunohistochemistry procedure or tissue processing. The differences in SDHB protein concentrations are probably not the result of differences in transcriptional efficiency, since there are indications that SDHB mRNA concentrations do not parallel SDHB protein abundance.[23] Additionally, it has been shown previously that, whatever SDH subunit is mutated, be it anchorage (SDHC and SDHD) or catalytic (SDHB), inactivation of an *SDH* gene induces a complete abolition of SDH enzyme activity in the tumor, suggesting a conformational change or a

destabilisation and a subsequent proteolysis of the complex II.[7,22,24] Furthermore, Lima and colleagues25 showed by crystallography the severe structural consequences on the SDHB protein of five clinically validated *SDHB* missense mutations. Cervera and colleagues[26] recently obtained evidence that three missense-mutated SDHB proteins can reach the mitochondrion and localise normally, although two of three missense-mutated SDHB proteins showed decreased expression by western blotting compared with the wild-type protein. These results match with the recent evidence that most rare missense variants in genes are deleterious.[27]

In the present study four tumors, positive for SDHB immunostaining, harboured non-synonymous polymorphisms (*SDHB* p.Ala3Gly, p.Arg11His, p.Ser163Pro, and *SDHD* p.His50Arg) without concomitant pathogenic *SDH*-gene mutation, indicating that these variants are indeed neutral polymorphisms.[15,28] Biallelic inactivation of the *SDHB*, *SDHC*, or *SDHD* gene has been reported in *SDH*-related tumors.[17,24,29] Our results indicate that mutations in *SDHB*, *SDHC*, or *SDHD* lead to the same phenotypic consequence in the tumors–ie, the absence of immunoreactive SDHB protein. Such observations have already been described for mutations in complex I genes, which were shown to affect the assembly and stability of both the whole complex I and other mitochondrial complexes, such as complex III.[30] The observed absence of SDHB immunoreactivity in all *SDH*-mutated tumors, shown by immunohistochemistry in both FFPE and frozen tumor tissues, and by western blotting after denaturing gel electrophoresis, with both a monoclonal antibody generated against cow SDHB and an affinity-isolated polyclonal antiserum against a recombinant carboxyterminal part of human SDHB, provides strong evidence that no functional SDHB protein is present in *SDH*-mutated tumors. As previously reported in other mitochondrial disorders, it is therefore likely that altered assembly or complex stability is the first consequence of *SDH* gene mutations, as opposed to catalytic site dysfunction. It confirms the accuracy of immunological approaches for the diagnosis of mitochondrial diseases.[31] By use of our applied procedure, patients with pheochromocytoma–paraganglioma syndrome with an apparently sporadic presentation can be detected by SDHB immunohistochemistry on paragangliomas and pheochromocytomas. Additionally, it can be speculated that the syndromic involvement of tumors that have recently been described in relation with paragangliomas, such as

gastrointestinal stromal tumors in the Carney–Stratakis dyad and familial renal-cell carcinomas, could also be detected by SDHB immunohistochemistry.[29,32] In actual fact, tissue from one of these germline *SDHB* mutated renal-cell carcinomas was available for study, and this tumor seemed to be negative for SDHB expression (data not shown).

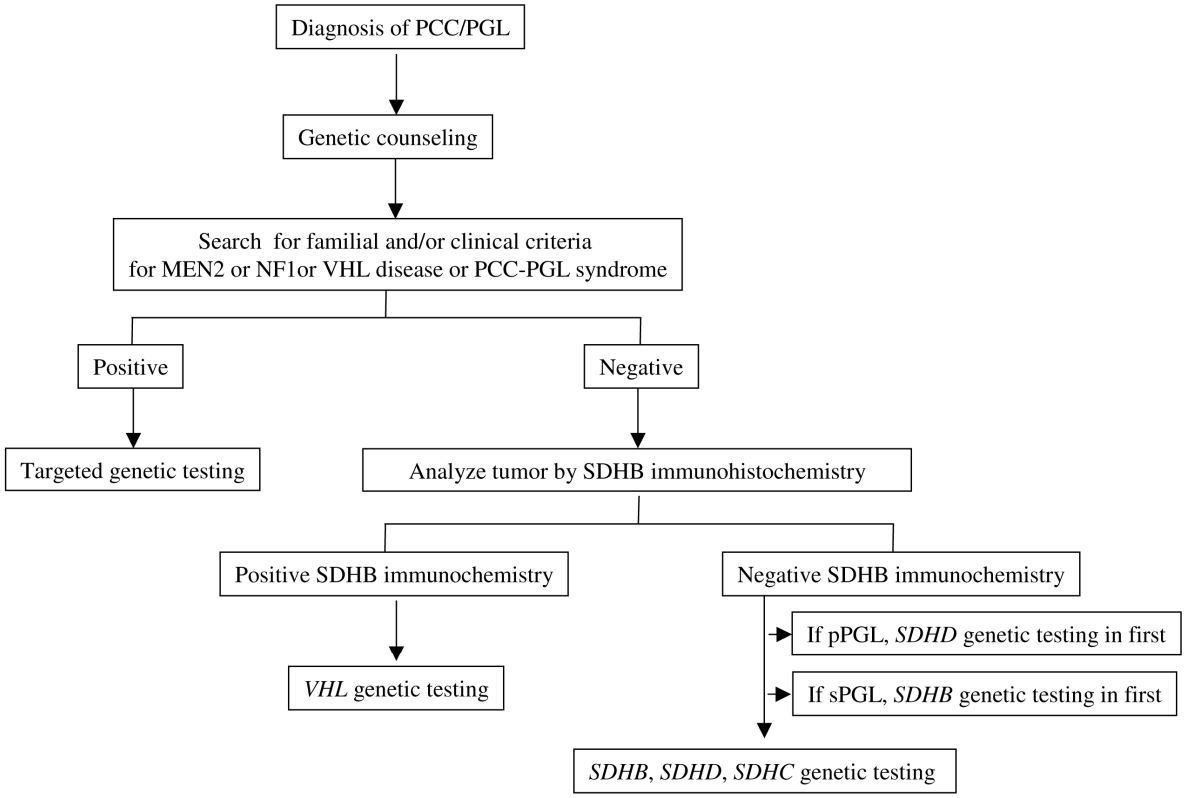


**Figure 3.** Suggested algorithm for molecular genetic testing for PCC and PGL.

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

As for Lynch syndrome diagnostics, where the testing of tumors usually starts with immunohistochemistry for mismatch repair gene products, SDHB immunohistochemistry could have an important role in the future genetic testing of pheochromocytomas and paragangliomas (figure 3).[33] Because of the simplicity of the standard immunohistochemical procedure and data interpretation, the immunohistochemistry test could easily be applied in diagnostic pathology services worldwide. It is technically and

financially feasible to routinely test all pheochromocytoma and paraganglioma for SDHB expression, in particular in the absence of familial or clinical indications for a specific form of inherited pheochromocytoma or paraganglioma. Our results show that *SDHB*, *SDHC*, and *SDHD* germline mutation testing is indicated only when tumors are immunohistochemically negative for SDHB expression. Obviously, our proposed diagnostic test can only be done after patients have been operated on and tumor tissue is available for study. The effect that our test will have on patient management is unclear, since international controversy exists regarding preoperative and postoperative genetic testing, and the effect on patient management. Nonetheless, by routinely doing SDHB immuno histochemistry, hereditary syndromes caused by germline mutations in *SDHB*, *SDHC*, or *SDHD* could be identified with a high degree of reliability.

**Supplementary table 1.** Patient's characteristics and SDHB immunohistochemistry results.

| | Sex M/F | Age at diagnosis (years) | Tumor site[2] | Benign/ malignant | Gene mutated | Mutation type[3] | Mutation | Mutation protein | SDHB IHC[4] |
|---|---|---|---|---|---|---|---|---|---|
| 1A | M | 32 | APGL | U | *SDHB* | FS | c.481delG | p.Asp161MetfsX14 | 0 |
| 2A | F | 25 | APGL | U | *SDHB* | MS | c.299C>T | p.Ser100Phe | 0 |
| 3A | F | 63 | APGL | U | *SDHB* | MS | c.727C>A | p.Cys243Ser | 0 |
| 4A | F | 12 | APGL | U | *SDHB* | LD | Ex3del | U | 0 |
| 5A | U | U | U | U | *SDHB* | Splice | c.72+1G>T | IVS1+1G>T | 0 |
| 6A | U | U | U | U | *SDHB* | MS | c.590C>G | p.Pro197Arg | 0 |
| 7A | U | U | U | U | *SDHB* | MS | c.292C>T | p.Cys98Arg | 0 |
| 8A | U | U | U | U | *SDHB* | MS | c.590C>G | p.Pro197Arg | 0 |
| 9A | U | U | U | U | *SDHB* | MS | c.380C>A | p.Ile127Asn | 0 |
| 10A | M | 14 | U | U | *SDHB* | MS | c.137G>A | p.Arg46Gln | 0 |
| 11A | U | U | U | U | *SDHB* | FS | c.502insC | p.Gln168ProfsX11 | 0 |
| 12A | U | U | PCC | U | *SDHB* | NS | c.268C>T | p.Arg90X | 0 |
| 13A | U | U | PCC | U | *SDHB* | MS | c.418G>T | p.Val140Phe | 0 |
| 14A | U | U | PCC | U | *SDHB* | NS | c.343C>T | p.Arg115X | 0 |
| 15A | U | U | PCC | U | *SDHB* | MS | c.689G>A | p.Arg230His | 0 |
| 16A | U | U | PCC | U | *SDHB* | MS | c.587G>A | p.Cys196Tyr | 0 |
| 17A | M | 25 | APGL | U | *SDHB* | MS | c.395A>C | p.His132Pro | 0 |
| 18A | M | 60 | APGL | U | *SDHB* | MS | c.395A>C | p.His132Pro | 0 |
| 19B | F | 33 | APGL | B | *SDHB* | Splice | c.200+1G>A | IVS2+1G>A | 0 |
| 20B | M | 59 | PCC | M | *SDHB* | MS | c.203G>A | p.Cys68Tyr | 0 |
| 21B | M | 36 | APGL | B | *SDHB* | FS | c.591del | p.Ser198AlafsX22 | 0 |
| 22B | F | 20 | PCC | B | *SDHB* | FS | c.166_170del | p.Pro56TyrfsX5 | 0 |
| 23B | M | 29 | APGL | M | *SDHB* | MS | c.127G>C | p.Ala43Pro | 0 |
| 24B | F | 21 | PCC | B | *SDHB* | MS | c.758G>A | p.Cys253Tyr | 0 |
| 25B | M | 43 | APGL | M | *SDHB* | LD | Ex3_8del | U | 0 |
| 26B | F | 54 | PCC | M | *SDHB* | MS | c.137G>A | p.Arg46Gln | 0 |
| 27B | F | 34 | HHPGL | B | *SDHB* | MS | c.763A>G | p.Lys255Glu | 0 |
| 28B | M | 39 | APGL | M | *SDHB* | FS | c.620-621delTG | p.Leu207ArgfsX14 | 0 |
| 29B | M | 28 | APGL | M | *SDHB* | LD | Ex1del | U | 0 |
| 30B | F | 10 | APGL | B | *SDHB* | FS | c.713del | p.Phe238SerfsX10 | 0 |
| 31B | F | 47 | APGL | B | *SDHB* | Splice | c.540+2T>C | IVS5+2T>C | 0 |
| 32B | M | 28 | PCC | M | *SDHB* | MS | c.137G>A | p.Arg46Gln | 0 |
| 33B | M | 28 | Metastasis (abdominal ganglia) | M | *SDHB* | MS | c.137G>A | p.Arg46Gln | 0 |
| 34B | F | 56 | PCC | M | *SDHB* | MS | c.689G>A | p.Arg230His | 0 |
| 35C | M | 48 | HHPGL | U | *SDHB* | MS | c.649C>G | p.Arg217Gly | 0 |
| 36C | F | 20 | HHPGL | U | *SDHB* | Splice | c.200+1G>A | IVS2+1G>A | 0 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 37A | F | 15 | APGL | U | *SDHC* | NS | c.126G>A | p.Trp42X | 0 |
| 38A | M | 36 | HHPGL | U | *SDHC* | MS | c.214C>T | p.Arg72Cys | 0 |
| 39B | F | 16 | TPGL | B | *SDHC* | LD | Ex2del | U | 0 |
| 40B | M | 47 | APGL | M | *SDHC* | LD | Ex3del | U | 0 |
| 41C | F | 39 | HHPGL | U | *SDHC* | MS | c.397C>T | p.His127Tyr | 0 |
| 42A | F | 25 | PCC | B | *SDHD* | MS | c.274G>T | p.Asp92Tyr | 0 |
| 43A | M | 16 | PCC | B | *SDHD* | MS | c.274G>T | p.Asp92Tyr | 0 |
| 44A | F | 31 | PCC | B | *SDHD* | MS | c.274G>T | p.Asp92Tyr | 0 |
| 45A | M | 41 | HHPGL | B | *SDHD* | FS | c.277delT | p.Ile93Tyrfs X42 | 0 |
| 46A | F | 33 | HHPGL | B | *SDHD* | Splice | c.170-1G>T | IVS2-1G>T | |
| 47A | F | 18 | U | U | *SDHD* | FS | c.94_95d elTC | p.Ala32IlefsX 35 | 0 |
| 48A | U | U | U | U | *SDHD* | NS | c.342T>A | p.Tyr114X | 0 |
| 49A | F | 72 | PCC | U | *SDHD* | MS | c.274G>T | p.Asp92Tyr | 0 |
| 50A | M | 36 | HHPGL | B | *SDHD* | MS | c.284T>C | p.Leu95Pro | 0 |
| 51A | M | 39 | HHPGL | B | *SDHD* | MS | c.416T>C | p.Leu139Pro | 0 |
| 52A | F | 43 | HHPGL | B | *SDHD* | MS | c.274G>T | p.Asp92Tyr | 0 |
| 53A | F | 20 | HHPGL | B | *SDHD* | MS | c.274G>T | p.Asp92Tyr | 0 |
| 54A | F | 62 | HHPGL | B | *SDHD* | MS | c.284T>C | p.Leu95Pro | 0 |
| 55A | M | 43 | HHPGL | B | *SDHD* | MS | c.274G>T | p.Asp92Tyr | 0 |
| 56A | F | 44 | HHPGL | B | *SDHD* | MS | c.274G>T | p.Asp92Tyr | 0 |
| 57A | F | 42 | HHPGL | B | *SDHD* | MS | c.274G>T | p.Asp92Tyr | 0 |
| 58A | M | 48 | HHPGL | B | *SDHD* | MS | c.274G>T | p.Asp92Tyr | 0 |
| 59A | F | 44 | HHPGL | B | *SDHD* | MS | c.284T>C | p.Leu95Pro | 0 |
| 60A | M | 36 | HHPGL | B | *SDHD* | MS | c.284T>C | p.Leu95Pro | 0 |
| 61A | M | 43 | HHPGL | B | *SDHD* | MS | c.274G>T | p.Asp92Tyr | 0 |
| 62A | M | 43 | HHPGL | B | *SDHD* | MS | c.274G>T | p.Asp92Tyr | 0 |
| 63A | M | 44 | HHPGL | B | *SDHD* | MS | c.274G>T | p.Asp92Tyr | 0 |
| 64A | M | 70 | APGL | B | *SDHD* | MS | c.274G>T | p.Asp92Tyr | 0 |
| 65A | M | 28 | HHPGL | B | *SDHD* | MS | c.284T>C | p.Leu95Pro | 0 |
| 66A | M | 41 | HHPGL | B | *SDHD* | MS | c.284T>C | p.Leu95Pro | 0 |
| 67A | M | 56 | HHPGL | B | *SDHD* | MS | c.274G>T | p.Asp92Tyr | 0 |
| 68A | M | 34 | HHPGL | B | *SDHD* | MS | c.274G>T | p.Asp92Tyr | 0 |
| 69A | F | 41 | HHPGL | B | *SDHD* | MS | c.274G>T | p.Asp92Tyr | 0 |
| 70A | F | 29 | HHPGL | B | *SDHD* | MS | c.274G>T | p.Asp92Tyr | 0 |
| 71A | F | 36 | HHPGL | B | *SDHD* | MS | c.416T>C | p.Leu139Pro | 0 |
| 72A | F | 36 | HHPGL | B | *SDHD* | MS | c.416T>C | p.Leu139Pro | 0 |
| 73A | F | 57 | U | B | *SDHD* | MS | c.284T>C | p.Leu95Pro | 0 |
| 74A | F | 45 | HHPGL | B | *SDHD* | MS | c.284T>C | p.Leu95Pro | 0 |
| 75A | F | 47 | HHPGL | B | *SDHD* | MS | c.274G>T | p.Asp92Tyr | 0 |
| 76A | F | 40 | HHPGL | B | *SDHD* | MS | c.284T>C | p.Leu95Pro | 0 |
| 77A | F | 40 | HHPGL | B | *SDHD* | MS | c.274G>T | p.Asp92Tyr | 0 |
| 78A | M | 62 | PCC | B | *SDHD* | MS | c.274G>T | p.Asp92Tyr | 0 |
| 79B | M | 45 | APGL | B | *SDHD* | NS | c.64C>T | p.Arg22X | 0 |
| 80C | M | 20 | HHPGL | B | *SDHD* | FS | c.116delC | p.Pro39Leuf sX37 | 0 |
| 81C | F | 50 | HHPGL | U | *SDHD* | MS | c.209G>T | p.Arg70Met | 0 |
| 82C | F | 50 | HHPGL | U | *SDHD* | MS | c.209G>T | p.Arg70Met | 0 |
| 83C | M | 26 | HHPGL | B | *SDHD* | MS | c.274G>T | p.Asp92Tyr | 0 |
| 84C | F | 34 | HHPGL | B | *SDHD* | MS | c.274G>T | p.Asp92Tyr | 0 |
| 85C | M | 26 | HHPGL | B | *SDHD* | MS | c.274G>T | p.Asp92Tyr | 0 |
| 86C | F | 58 | HHPGL | B | *SDHD* | MS | c.274G>T | p.Asp92Tyr | 0 |
| 87C | F | 22 | HHPGL | B | *SDHD* | MS | c.274G>T | p.Asp92Tyr | 0 |
| 88C | F | 35 | HHPGL | B | *SDHD* | MS | c.274G>T | p.Asp92Tyr | 0 |
| 89C | F | 35 | HHPGL | B | *SDHD* | MS | c.274G>T | p.Asp92Tyr | 0 |
| 90C | F | 54 | HHPGL | U | *SDHD* | MS | c.274G>T | p.Asp92Tyr | 0 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 91C | F | 25 | HHPGL | B | *SDHD* | MS | c.274G>T | p.Asp92Tyr | 0 |
| 92C | F | 51 | HHPGL | B | *SDHD* | MS | c.274G>T | p.Asp92Tyr | 0 |
| 93C | F | 40 | HHPGL | B | *SDHD* | MS | c.274G>T | p.Asp92Tyr | 0 |
| 94C | F | 40 | HHPGL | B | *SDHD* | MS | c.274G>T | p.Asp92Tyr | 0 |
| 95C | F | 37 | HHPGL | U | *SDHD* | MS | c.274G>T | p.Asp92Tyr | 0 |
| 96C | F | 47 | HHPGL | U | *SDHD* | MS | c.284T>C | p.Leu95Pro | 0 |
| 97C | M | 51 | HHPGL | U | *SDHD* | MS | c.284T>C | p.Leu95Pro | 0 |
| 98C | M | 53 | HHPGL | B | *SDHD* | MS | c.416T>C | p.Leu139Pro | 0 |
| 99C | M | U | APGL | U | *SDHD* | MS | c.439G>A | p.Val147Met | 0 |
| 100C | F | 54 | HHPGL | U | *SDHD* | Splice | c.52+3G>A | IVS1+3G>A | 0 |
| 101C | M | 38 | HHPGL | U | *SDHD* | MS | c.284T>C | p.Leu95Pro | 0 |
| 102C | M | 35 | HHPGL | U | *SDHD* | Splice | c.169_169+9delTGTATGTTCT | U | 0 |
| 103A | F | 39 | PCC | B | *NF1* | ND* | | | 1 |
| 104A | F | 47 | PCC | B | *NF1* | ND* | | | 1 |
| 105A | F | 61 | PCC | B | *NF1* | ND* | | | 1 |
| 106A | M | 52 | PCC | B | *NF1* | ND* | | | 1 |
| 107A | M | 29 | PCC | B | *NF1* | ND* | | | 1 |
| 108A | F | 63 | PCC | B | *NF1* | ND* | | | 1 |
| 109A | M | 33 | PCC | B | *NF1* | ND* | | | 1 |
| 110A | F | 67 | PCC | B | *NF1* | ND* | | | 1 |
| 111B | F | 37 | PCC | B | *NF1* | ND* | | | 1 |
| 112B | F | 33 | PCC | B | *NF1* | ND* | | | 1 |
| 113B | F | 38 | PCC | B | *NF1* | ND* | | | 1 |
| 114B | F | 32 | PCC | B | *NF1* | ND* | | | 1 |
| 115A | F | 38 | PCC | B | *RET* | MS | c.1900T>C | p.Cys634Arg | 1 |
| 116A | F | 25 | PCC | U | *RET* | MS | c.1900T>C | p.Cys634Arg | 1 |
| 117A | F | 51 | PCC | M | *RET* | MS | c.1900T>C | p.Cys634Arg | 1 |
| 118A | M | 29 | PCC | B | *RET* | MS | c.1900T>C | p.Cys634Arg | 1 |
| 119A | M | 41 | PCC | U | *RET* | NS | c.1894_1899delgagctg | p.Glu632_Leu633del | 1 |
| 120A | M | 35 | PCC | B | *RET* | MS | c.1900T>C | p.Cys634Arg | 1 |
| 121A | M | 31 | PCC | B | *RET* | ND* | | | 1 |
| 122A | F | 26 | PCC | B | *RET* | ND* | | | 1 |
| 123A | F | 65 | PCC | B | *RET* | MS | c.1900T>C | p.Cys634Arg | 1 |
| 124A | F | 20 | PCC | B | *RET* | MS | c.1900T>C | p.Cys634Arg | 1 |
| 125A | M | 21 | PCC | B | *RET* | MS | c.1900T>C | p.Cys634Arg | 1 |
| 126A | F | 32 | PCC | B | *RET* | MS | c.1900T>C | p.Cys634Arg | 1 |
| 127A | F | 35 | PCC | U | *RET* | ND* | | | 1 |
| 128A | M | 70 | PCC | B | *RET* | MS | c.1900T>C | p.Cys634Arg | 1 |
| 129A | M | 26 | PCC | B | *RET* | MS | c.1900T>C | p.Cys634Arg | 1 |
| 130A | F | 38 | PCC | B | *RET* | MS | c.1900T>C | p.Cys634Arg | 1 |
| 131A | M | 23 | PCC | B | *RET* | MS | c.1900T>C | p.Cys634Arg | 1 |
| 132B | F | 18 | PCC | B | *RET* | MS | c.1900T>C | p.Cys634Arg | 1 |
| 133B | F | 37 | PCC | B | *RET* | MS | c.1901_1902GC>TG | p.Cys634Leu | 1 |
| 134B | F | 29 | PCC | B | *RET* | MS | c.2753T>C | p.Met918Thr | 1 |
| 135B | F | 76 | PCC | B | *RET* | MS | c.1597G>T | p.Gly533Cys | 1 |
| 136B | F | 27 | PCC | B | *RET* | MS | c.1900T>C | p.Cys634Arg | 1 |
| 137B | F | 44 | PCC | B | *RET* | MS | c.2647_2648GC>TT | p.Ala883Phe | 1 |
| 138B | F | 18 | PCC | B | *RET* | MS | c.1900T>C | p.Cys634Arg | 1 |
| 139A | M | 55 | PCC | B | *VHL* | MS | c.403G>C | p.Gly144Gln | 1 |
| 140A | F | 62 | PCC | B | *VHL* | MS | c.705G>T | p.Gln164His | 1 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 141A | F | 18 | PCC | B | *VHL* | MS | c.403G>C | p.Arg64Pro | 1 |
| 142A | F | 32 | PCC | B | *VHL* | MS | c.364_365 GC>AT | p.Ala122Ile | 1 |
| 143A | M | 7 | PCC | U | *VHL* | MS | c.403G>C | p.Arg64Pro | 1 |
| 144A | U | U | PCC | U | *VHL* | FS | c.343insA A | p.His115Asnf sX23 | 1 |
| 145A | U | U | U | U | *VHL* | MS | c.482G>A | p.Arg161Gln | 1 |
| 146A | U | U | U | U | *VHL* | MS | c.357C>G | p.Phe119Leu | 1 |
| 147A | U | U | PCC | U | *VHL* | ND* | | | 1 |
| 148A | M | 12 | U | U | *VHL* | MS | c.250G>T | p.Val84Leu | 1 |
| 149A | F | 49 | PCC | B | *VHL* | MS | c.403G>C | p.Arg64Pro | 1 |
| 150A | F | 39 | PCC | B | *VHL* | MS | c.430G>C | p.Gly144Gln | 1 |
| 151A | M | 31 | PCC | B | *VHL* | MS | c.403G>C | p.Arg64Pro | 1 |
| 152A | M | 26 | PCC | B | *VHL* | MS | c.188T>C | p.Leu63Pro | 1 |
| 153A | M | 24 | PCC | B | *VHL* | MS | c.403G>C | p.Arg64Pro | 1 |
| 154A | F | 23 | HHPGL | U | *VHL* | MS | c.403G>C | p.Arg64Pro | 1 |
| 155B | M | 15 | PCC | B | *VHL* | MS | c.533T>C | p.Leu178Pro | 1 |
| 156B | F | 15 | PCC | B | *VHL* | MS | c.500G>A | p.Arg167Gln | 1 |
| 157B | F | 20 | APGL | B | *VHL* | MS | c.467A>G | p.Tyr156Cys | 1 |
| 158B | F | 7 | APGL | B | *VHL* | MS | c.482G>A | p.Arg161Gln | 1 |
| 159B | M | 26 | PCC | B | *VHL* | MS | c.460C>T | p.Pro154Ser | 1 |
| 160B | M | 26 | APGL | B | *VHL* | MS | c.460C>T | p.Pro154Ser | 1 |
| 161B | M | 31 | PCC | B | *VHL* | LD | Ex1_3del | U | 1 |
| 162B | F | 36 | APGL | M | *VHL* | LD | Ex3del | U | 1 |
| 163B | F | 19 | PCC | B | *VHL* | MS | c.292T>C | p.Tyr98His | 1 |
| 164B | F | 16 | PCC | B | *VHL* | MS | c.500G>A | p.Arg167Gln | 1 |
| 165B | M | 17 | PCC | B | *VHL* | MS | c.467A>G | p.Tyr156Cys | 1 |
| 166B | F | 10 | PCC | B | *VHL* | MS | c.290C>T | p.Pro97Leu | 1 |
| 167B | M | 25 | PCC | B | *VHL* | MS | c.500G>A | p.Arg167Gln | 1 |
| 168A | F | 70 | PCC | M | NONE | | | | 1 |
| 169A | M | 48 | PCC | B | NONE | | | | 1 |
| 170A | M | 49 | PCC | B | NONE | | | | 1 |
| 171A | M | 63 | PCC | M | NONE | | | | 1 |
| 172A | F | 79 | PCC | B | NONE | | | | 1 |
| 173A | F | 38 | PCC | B | NONE | | | | 1 |
| 174A | F | 64 | PCC | U | NONE | | | | 1 |
| 175A | F | 62 | PCC | U | NONE | | | | 1 |
| 176A | F | 40 | PCC | U | NONE | | | | 1 |
| 177A | F | 62 | PCC | U | NONE | | | | 1 |
| 178A | F | 42 | PCC | U | NONE | | | | 1 |
| 179A | M | 12 | PCC | B | NONE | | | | 0 |
| 180B | M | 27 | APGL | B | NONE | | | | 0 |
| 181B | F | 27 | PCC | M | NONE | | | | 0 |
| 182B | F | 40 | PCC | B | NONE | | | | 1 |
| 183B | M | 17 | PCC | B | NONE | | | | 1 |
| 184B | F | 53 | PCC | B | NONE | | | | 1 |
| 185B | M | 47 | PCC | B | NONE | | | | 1 |
| 186B | M | 40 | PCC | M | NONE | | | | 1 |
| 187B | F | 46 | Metast asis | M | NONE | | | | 1 |
| 188B | F | 37 | APGL | B | NONE | | | | 1 |
| 189B | F | 39 | PCC | M | NONE | | | | 1 |
| 190B | F | 49 | PCC | B | NONE | | | | 1 |
| 191B | F | 26 | APGL | M | NONE | | | | 1 |
| 192B | F | 26 | APGL | M | NONE | | | | 1 |
| 193B | M | 62 | PCC | B | NONE | | | | 1 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 194B | F | 41 | PCC | B | NONE | 1 |
| 195B | M | 57 | PCC | B | NONE | 1 |
| 196B | F | 44 | PCC | B | NONE | 1 |
| 197B | F | 47 | PCC | B | NONE | 1 |
| 198B | F | 63 | Metastasis | M | NONE | 1 |
| 199B | F | 66 | PCC | B | NONE | 1 |
| 200B | M | 59 | PCC | B | NONE | 1 |
| 201B | M | 45 | PCC | B | NONE | 1 |
| 202C | M | 67 | HHPGL | B | NONE | 1 |
| 203C | F | 55 | HHPGL | B | NONE | 1 |
| 204C | F | 45 | HHPGL | B | NONE | 1 |
| 205C | F | 57 | HHPGL | B | NONE | 1 |
| 206C | F | 47 | HHPGL | B | NONE | 1 |
| 207C | F | 57 | HHPGL | B | NONE | 1 |
| 208C | F | 51 | U | B | NONE | 1 |
| 209C | F | 71 | HHPGL | B | NONE | 1 |
| 210C | M | 56 | HHPGL | U | NONE | 1 |
| 211C | F | 71 | HHPGL | U | NONE | 1 |
| 212C | F | 57 | PCC | U | NONE | 1 |
| 213C | F | 54 | PCC | U | NONE | 1 |
| 214C | M | 43 | PCC | U | NONE | 1 |
| 215C | U | U | PCC | U | NONE | 1 |
| 216C | U | U | PCC | U | NONE | 1 |
| 217C | F | 45 | PCC | U | NONE | 1 |
| 218C | F | 74 | HHPGL | B | NONE | 0 |
| 219C | M | 31 | HHPGL | B | NONE | 0 |
| 220C | M | 45 | HHPGL | B | NONE | 0 |

[1] The tumors included a retrospective series from the Erasmus MC (A) and the INSERM U970 (B) and a prospective series also from the Erasmus MC (C).
[2] The total series of tumors was comprised of abdominal PGL (APGL), pheochromocytoma (PCC), head and neck paraganlioma (HHPGL), thoracic PGL (TPGL), metastasis and tumors of unknown location (U).
[3] Mutations encompassed frame shift (FS), missense (MS), nonsense (NS), and splice site (Splice) mutations and large (exon) deletions (LD). The syndrome of some patients was determined on clinical grounds, so no mutation data were available (ND*). In addition, some tumors did not harbor any mutation and were sporadic (NONE).
[4] The scoring of the SDHB immunohistochemistry was positive (1) or negative (0).
Throughout the entire table unknown data is abbreviated as U.

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# CHAPTER 3

**SDHA immunohistochemistry to detect *SDHA* mutations in Paragangliomas and Pheochromocytomas**

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Submitted

**Abstract**

**Context:** The pheochromocytoma-paraganglioma syndrome is caused by mutations in three of four genes coding for succinate dehydrogenase subunits. Regarding succinate dehydrogenase, it was thought until just recently that pheochromocytomas and paragangliomas exclusively exhibit *SDHB*, *SDHC*, and *SDHD* mutations. In addition, SDHAF2, which is required for flavination of SDHA, was found to be mutated in one Dutch family with parasympathetic paragangliomas. Recently, Burnichon et al recently demonstrated a causal germ line *SDHA* mutation in an abdominal paraganglioma. This *SDHA*-mutated tumor showed a negative staining for SDHA in the tumor cells, whereas non-*SDHA*-mutated tumors were immunohistochemically positive for SDHA. The significance of SDHA immunohistochemistry for identifying patients with *SDHA* mutations has not yet determined. Therefore we investigated the SDHA immunohistochemistry-based distinction between *SDHA*, *SDHB*, *SDHC*, and *SDHD*-mutated (from here on collectively called *SDHx*) and non-*SDHx*-mutated pheochromocytomas and paragangliomas.

**Material and methods:** We investigated a series of 224 sporadic and syndrome-related pheochromocytomas and paragangliomas for SDHA expression. Sequence analysis of *SDHA* was performed on all tumors that were immunohistochemically negative for SDHA. In addition, sequence analysis on *SDHA* was performed on 15 SDHA immunohistochemically positive tumors.

**Results:** Four SDHA immunohistochemically negative tumors were found. All four tumors showed a c. 91C>T *SDHA* gene mutation (NCBI: NM_004168), leading to a truncated protein (p. Arg31X). Sequence analysis of the tumor DNAs displayed almost entirely the mutation, indicating loss of the wild-type SDHA allele. This was confirmed by LOH analysis. Sequence analysis of a 15 SDHA immunohistochemically positive tumors revealed no mutations of *SDHA*.

**Conclusion:** The results of this study demonstrate that SDHA immunohistochemistry on (para)sympathetic paragangliomas and pheochromocytomas, can reveal the presence of *SDHA* germ line mutations with 100%sensitivety and 100%specificity and can be used in a cost-effective algorithm to screen all pheochromocytoma and paraganglioma patients.

## Introduction

Pheochromocytomas and paragangliomas are rare tumors that originate from neural crest-derived chromaffin cells. (1) The intra-adrenal tumors are called pheochromocytomas whereas similar extra-adrenal tumors are called paragangliomas. Based on location and catecholamine production paragangliomas are subdivided into parasympathetic and sympathetic paragangliomas.

Paragangliomas occur sporadically and in the context of inherited tumor syndromes, amongst which the pheochromocytoma-paraganglioma syndrome. (2) This syndrome is caused by mutations in three of the four genes coding for succinate dehydrogenase subunits. Succinate dehydrogenase, also known as complex II, is involved in the citric acid cycle and electron transport chain and is composed of four subunits: SDHA, SDHB, SDHC and SDHD. (3) At the beginning of this century *SDHD* gene involvement in paragangliomas was discovered. (4) The association of the *SDHB* and *SDHC* genes with paraganglioma was found soon after *SDHD* and more recently, SDHAF2, a flavination protein of SDHA, was found to be mutated in head and neck paragangliomas. (5-7) Surprisingly, no genetic link between *SDHA* and paragangliomas was established and it was thought that *SDHA* was only involved in Leigh syndrome. (8-11) Recently however, Burnichon et al identified a heterozygous germline *SDHA* mutation (p.Arg589Trp) in a patient with an abdominal paraganglioma. (12)

Identifying patients with *SDHB*, *SDHC* and *SDHD* mutations is possible by SDHB immunohistochemistry. (13) Not only *SDHB*-related tumors, but also *SDHC* and *SDHD*-related tumors are immunohistochemically negative for SDHB. The *SDHA*-related tumor described by Burnichon et al showed not only loss of SDHB protein expression, but also loss of SDHA protein expression immunohistochemically. In contrast *RET*, *NF1*, *SDHB* and *SDHD*-related tumors were immunohistochemically positive for SDHA. (12) These results suggest that SDHA immunohistochemistry would be an adequate technique to diagnose *SDHA*-mutated pheochromocytomas and paragangliomas. Therefore, we determined the significance of SDHA immunohistochemistry for the identification of patients with *SDHA* mutations.

## Materials and Methods

*Patients and tumor samples*

A series of 224 tumors (145 pheochromocytomas, 16 sympathetic paragangliomas, and 63 parasympathetic paragangliomas) were available for this study. Of these tumors, 167 were retrieved from the pathology archives of the Erasmus MC (Rotterdam, The Netherlands), 26 tumors from the Radboud University Nijmegen Medical Center (Nijmegen, The Netherlands), 10 from the University Hospital of Lille (Lille, France), 8 from the Leiden University Medical Center (Leiden, the Netherlands), and there were 13 tumors from various other Dutch and foreign centres. Of the 145 pheochromocytomas, 98 were sporadic and 47 were syndrome-related tumors (21 MEN2A, 1 MEN2B, 15 NF1, 3 SDHD, 7 VHL). Of the 63 parasympathetic paragangliomas, 44 were sporadic and 18 were syndrome-related tumors (1 SDHB, 1 SDHC, 16 SDHD), and 1 tumor had a somatic *IDH1* mutation. (14). In addition, of the 16 sympathetic paragangliomas, 13 occurred sporadically and 3 were syndrome-related (2 SDHB, 1 SDHD). Clinical data of all patients are shown in Supplementary table 1. The tumors were anonymously used according to the code for adequate secondary use of tissue, code of conduct: "Proper Secondary Use of Human Tissue" established by the Dutch Federation of Medical Scientific Societies (http://www.federa.org).

*Immunohistochemistry*

Immunohistochemistry was performed for SDHA and SDHB, using a 1/100 dilution for the SDHA antibody ab14715 (Abcam, Cambridge, United Kingdom) and a 1/500 for the SDHB antibody HPA002868 (Sigma-Aldrich, St. Louis, MO). The antibodies were applied on routine formalin-fixed and paraffin-embedded archival tissues, processed as described previously (13). Slides containing liver, heart, large intestine, and pancreas tissue were used as positive controls. Negative controls were performed by omission of the primary antibody. Tumors were scored negative when the normal endothelial cells surrounding the tumor cells stained positive (internal positive control) and the tumors cells were negative as previously described.(13) The immunohistochemical results were evaluated by two independent researchers (RdK and EK).

*Sequence analysis*

Sequence analysis of *SDHA* was performed on all tumors that were immunohistochemically negative for SDHA (primers available on request). In addition, sequence analysis was performed on 15 SDHA positive tumors. Tumor DNA was isolated according to manufacturer's instructions (Gentra Systems Minneapolis, MN). The entire *SDHA* coding sequence, including intron-exon boundaries, was analyzed for mutations, taking into account the *SDHA* pseudogenes (NCBI: NR_003264 and NR_003265). These pseudogenes are highly homologous to the *SDHA* gene, and even contain parts of *SDHA* intron sequences. When a mutation was demonstrated in the tumor DNA, germline DNA of the same patient was also tested for the presence of the mutation. Germline DNA was isolated from histologically confirmed paraffin-embedded healthy tissue surrounding the tumor. To discriminate between the functional *SDHA* gene and the two *SDHA* pseudogenes, amplicons for sequence analysis were chosen containing at least 2 nucleotide differences between the functional gene and the pseudogenes. By this we were able tot demonstrate that the mutations are present in the functional *SDHA* gene and are not derived from one of the pseudogenes.

*Loss of heterozygosity (LOH)*

Two microsatellite markers (one telomeric and one centromeric of *SDHA*) on chromosome 5p15 were selected for LOH analysis of the *SDHA* gene locus. Primers are available on request. LOH was performed on the tumor and normal DNA from patients presenting with SDHA-negative tumors. The analysis was performed by a previously described PCR method, using fluorescence-labeled primers (Invitrogen, Paisley, UK) and ABI 3130-XL genetic analyzer (Applied Biosystems, Foster City, CA) for analysis. (15) In addition, sequencing of the non-coding SNP (rs6878087) within intron 2 of *SDHA* was used for LOH analysis.

## Results

*Immunohistochemistry*

SDHA immunohistochemistry of the 224 tumors revealed four tumors (2%) with SHDA-negative tumor cells with positive internal control of the endothelial cells (Figure 1A). These four SDHA immunohistochemically negative tumors included one pheochromocytoma (patient 133), one thoracic sympathetic paraganglioma (patient 161), one vagal parasympathetic paraganglioma (patient 164), and one carotid body parasympathetic paraganglioma (patient 190). SDHB immunohistochemistry was also performed on these pheochromocytomas and paragangliomas and showed absence of SDHB protein in all four tumors.

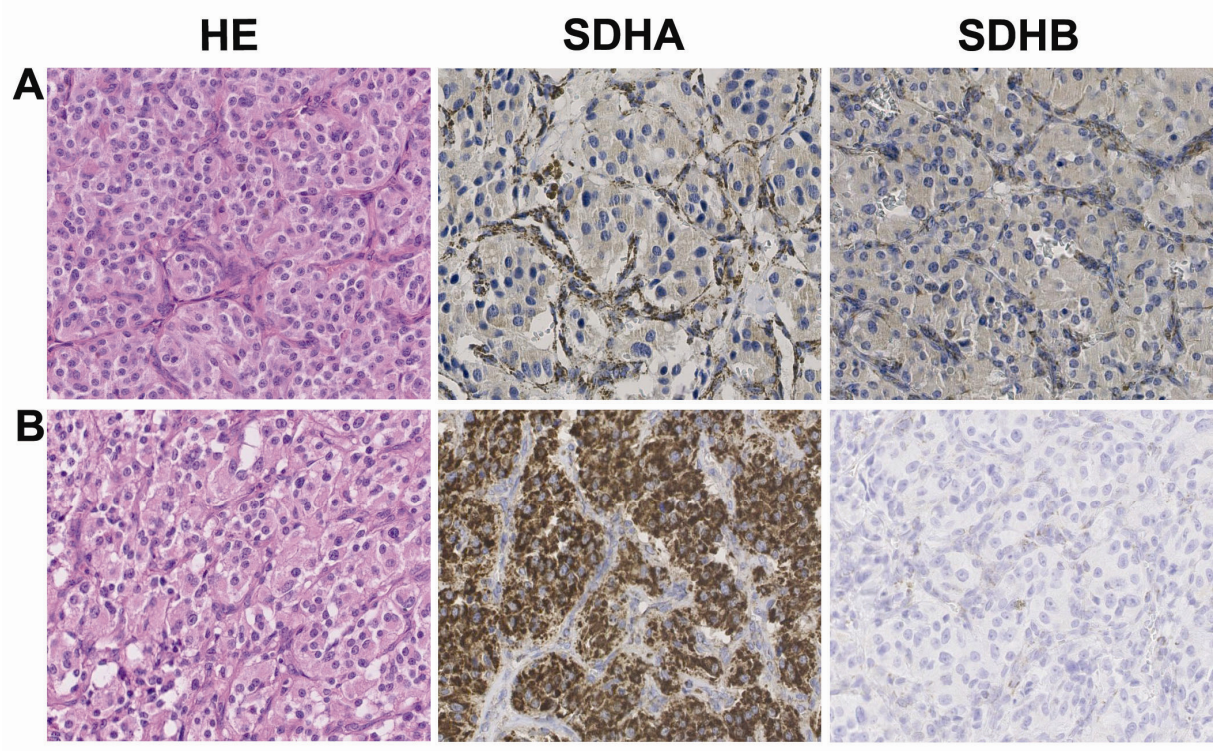


**Figure 1.**

A) *SDHA*-mutated paraganglioma. Hematoxylin and eosin staining showing classical Zellballen, separated by fibrovascular stroma. SDHA immunohistochemistry showing negative tumor cells. Note the positive internal control of the fibrovascular network. SDHB immunohistochemistry is also negative similar to the SDHA immunohistochemistry. B) *SDHB*-mutated paraganglioma. SDHA expression is present in the tumor cells, whereas SDHB expression is absent.

*Mutation analysis*

Mutation analysis of *SDHA* was performed on the four SDHA immunohistochemically negative tumors, and all tumors showed a c. 91C>T *SDHA* gene mutation (NCBI: NM_004168), leading to a truncated protein (p. Arg31X). Analysis of the corresponding germline DNAs, isolated from formalin–fixed paraffin-embedded normal tissues indicated that all four mutations are present in the germline (Figure 2A). The sequence analysis of the tumor DNAs displayed almost entirely the mutation, indicating loss of heterozygosity (LOH) of the wild-type allele.

*Loss of Heterozygosity analysis*

LOH was performed with two markers, of which the centromeric marker was informative. Three of the 4 tumors samples showed loss of heterozygosity (Figure 2B). The pattern of loss differed in these three patients. The fourth tumor sample was not informative. LOH analysis using the SNP was not contributory as the SNP was homozygous in all four patients.

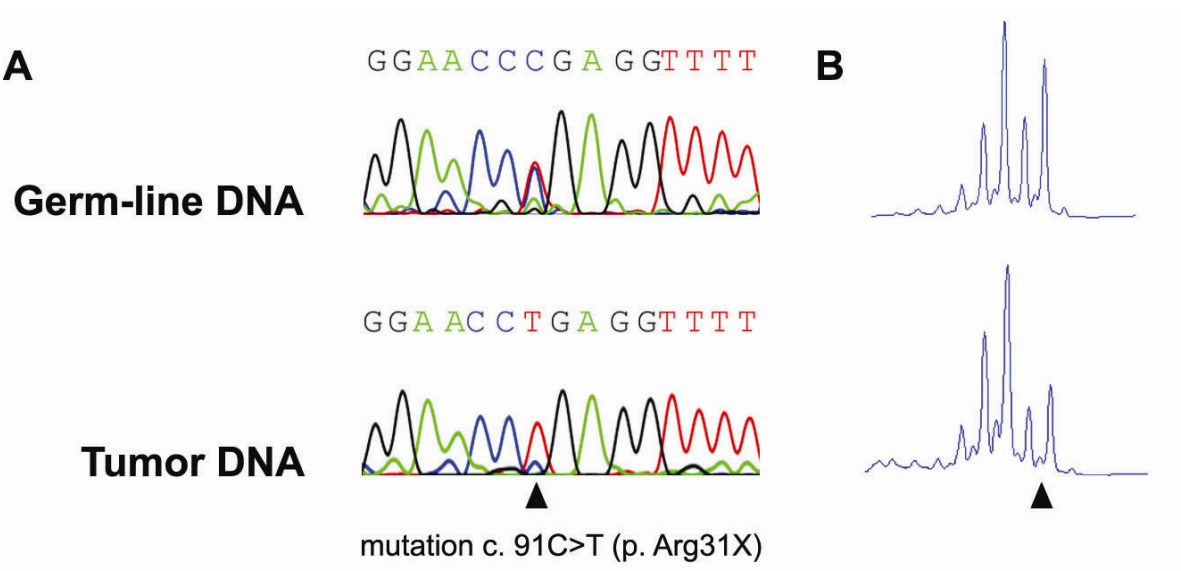


**Figure 2.**

A) Sequence chromatogram. Arrow shows p. Arg31X due to c. 91C>T mutation. The chromatogram of the tumor revealed predominantly the mutant allele, indicating relative loss of the wild type SDHA allele. B) Electrophoretogram demonstrating loss of heterozygosity, with loss of the larger allele.

**Discussion**

Regarding mitochondrial complex II, it was thought until just recently that only *SDHB*, *SDHC*, and *SDHD* mutations cause pheochromocytomas and paragangliomas. However, Burnichon et al demonstrated a causal germ line *SDHA* mutation in an abdominal paraganglioma. (12) This *SDHA*-mutated tumor showed negative immunohistochemical staining for SDHA in the tumor cells, whereas non-SDHA-related tumors were immunohistochemically positive for SDHA. In the present study we investigated a series of 224 sporadic and syndrome-related pheochromocytomas and paragangliomas for SDHA expression and found four negative tumors (2%).

Sequence analysis of the SDHA-negative tumors revealed a novel c. 91C>T *SDHA* mutation, which was found in the tumor DNA as well as germ line DNA of all four patients. The c. 91C>T *SDHA* mutation results in a translation stop (p. Arg31X) and has never been described before. In accordance with the Knudson's two-hit hypothesis, the sequence analyses showed loss of the wild type allele of all four tumors. Loss of the SDHA locus was confirmed by LOH analysis in three of the four SDHA immunohistochemically negative tumors. The fourth tumor appeared to be non-informative for the used LOH markers. This indicates that *SDHA* can act as a bona fide tumor suppressor gene.

All other 220 tumors were positive for SDHA immunohistochemically, including all syndrome-related tumors, which is in accordance with the study of Burnichon et al, who found SDHA expression in *RET*, *VHL*, *SDHB*, and *SDHD*-mutated tumors. (12) Recently, we demonstrated that SDHB, SDHC, and SDHD-related tumors all show loss of SDHB expression immunohistochemically. (13) It was suggested that absence of functional SDHC or SDHD results in impairment of complex II formation and degradation of SDHB. In accordance with this explanation are the current results of absence of SDHB expression in *SDHA*-mutated tumors. However, it is remarkable that in *SDHB*- and *SDHD*-mutated tumors SDHA immunohistochemical expression is present. Obviously the SDHA subunit remains intact and SDHA protein expression remains detectable in the absence of SDHB expression and in the absence of complex II. The mechanism behind this phenomenon is

unknown. Nevertheless, tumors immunohistochemically negative for SDHA expression are very likely to be caused by SDHA mutations.

Burnichon et al analyzed 202 tumors with BAC array CGH and found nine tumors with LOH on chromosome 5p15, encompassing the *SDHA* locus. Of the 202 tumors only one had an *SDHA* mutation (1%). In accordance, in the present study we found four *SDHA*-mutated tumors (2%). Since *SDHA* mutations seem to be extremely rare, and all our patients had the same mutation and were born in the Netherlands, we suspected the patients to be (distantly) related. Only limited pedigree information of these four patients was available. No relatives with pheochromocytomas and/or paragangliomas, or with symptoms suggestive for the presence of these tumors were known in all four families. We could not investigate family members for the mutation, so it is theoretically possible that these mutations are de novo mutations. However, it appears unlikely that all four identical mutations have arisen as de novo mutations. Therefore we hypothesize that these mutations are either the result of a founder effect or as a hot spot mutation. Although uncommon, hot spot mutations in tumor suppressor genes can occur, as in the *APC* or the *P53* gene. Our attempts to prove or disprove a founder mutation were not conclusive, as the markers used were not informative or too distant from the SDHA gene. To unravel a possible founder effect, familial relatedness will be determined by haplotype analysis of the *SDHA* locus in the four patients with the same *SDHA* mutation.

The results of this study demonstrate that an inexpensive and straightforward investigation, SDHA immunohistochemistry, on (para)sympathetic paragangliomas and pheochromocytomas, can reveal the presence of *SDHA* germ line mutations with great specificity and sensitivity. In the absence of familial or clinical indications for a specific form of inherited pheochromocytoma or paraganglioma it could be important to perform simple, quick and cheap immunohistochemical analyses for SDHB and SDHA in pheochromocytomas and paragangliomas to detect potential inherited cases (Figure3).

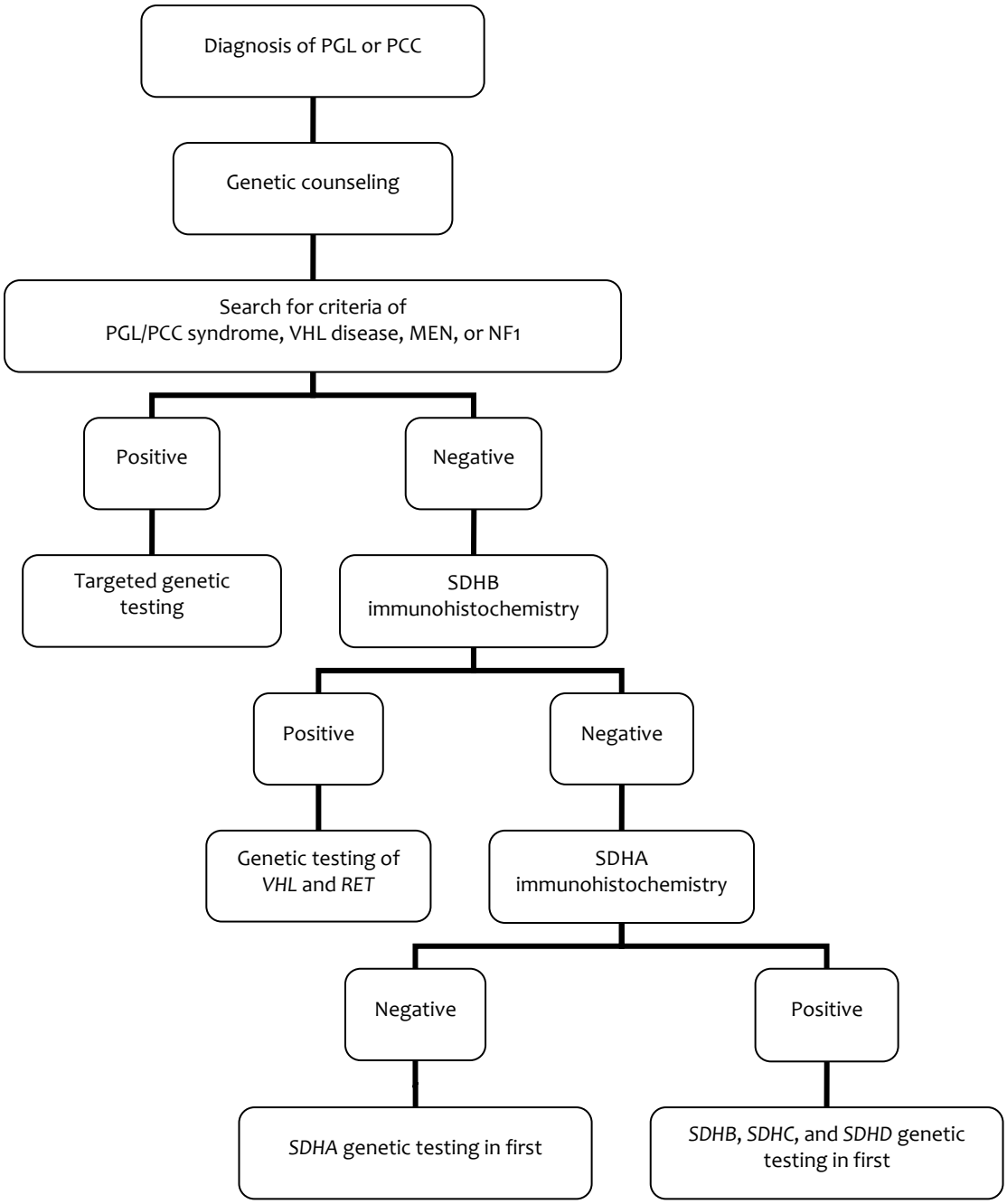


**Figure 3.**

Suggested algorithm for molecular genetic testing for pheochromocytomas and paragangliomas.

PCC: pheochromocytoma, PGL:paraganglioma.

Supplementary table 1. Clinical data of all patients.

| Patient | PCC/PGL | gender | age | geneticbackground | SDHA IHC | SDHB IHC |
|---|---|---|---|---|---|---|
| 1 | PCC | m | 36 | sporadic | positive | positive |
| 2 | PCC | f | 46 | sporadic | positive | positive |
| 3 | PCC | f | 70 | sporadic | positive | positive |
| 4 | PCC | m | 31 | VHL | positive | positive |
| 5 | PCC | f | 23 | sporadic | positive | positive |
| 6 | PCC | f | 65 | sporadic | positive | positive |
| 7 | PCC | m | 48 | sporadic | positive | positive |
| 8 | PCC | m | 17 | sporadic | positive | positive |
| 9 | PCC | f | 29 | MEN2A | positive | positive |
| 10 | PCC | f | 47 | NF1 | positive | positive |
| 11 | PCC | m | 55 | VHL | positive | positive |
| 12 | PCC | f | 61 | NF1 | positive | positive |
| 13 | PCC | m | 33 | sporadic | positive | positive |
| 14 | PCC | m | 58 | sporadic | positive | positive |
| 15 | PCC | f | 25 | sporadic | positive | positive |
| 16 | PCC | f | 62 | sporadic | positive | positive |
| 17 | PCC | m | 14 | sporadic | positive | positive |
| 18 | PCC | m | 15 | sporadic | positive | positive |
| 19 | PCC | f | 40 | sporadic | positive | positive |
| 20 | PCC | f | 63 | NF1 | positive | positive |
| 21 | PCC | f | 54 | sporadic | positive | positive |
| 22 | PCC | f | 20 | MEN2A | positive | positive |
| 23 | PCC | f | 32 | MEN2A | positive | positive |
| 24 | PCC | m | 42 | MEN2A | positive | positive |
| 25 | PCC | m | 55 | MEN2A | positive | positive |
| 26 | PCC | f | 40 | sporadic | positive | positive |
| 27 | PCC | m | 53 | sporadic | positive | positive |
| 28 | PCC | m | 33 | NF1 | positive | positive |
| 29 | PCC | f | 40 | sporadic | positive | positive |
| 30 | PCC | f | 40 | NF1 | positive | positive |
| 31 | PCC | f | 35 | sporadic | positive | positive |
| 32 | PCC | m | 67 | sporadic | positive | positive |
| 33 | PCC | f | 39 | VHL | positive | positive |
| 34 | PCC | f | 62 | sporadic | positive | positive |
| 35 | PCC | m | 68 | sporadic | positive | positive |
| 36 | PCC | f | 67 | NF1 | positive | positive |
| 37 | PCC | m | 59 | sporadic | positive | positive |
| 38 | PCC | m | 26 | NF1 | positive | positive |
| 39 | PCC | m | 43 | sporadic | positive | positive |
| 40 | PCC | m | 9 | sporadic | positive | positive |
| 41 | PCC | f | 45 | sporadic | positive | positive |
| 42 | PCC | m | 64 | sporadic | positive | positive |
| 43 | PCC | m | 41 | sporadic | positive | positive |
| 44 | PCC | m | 65 | sporadic | positive | positive |
| 45 | PCC | f | 48 | sporadic | positive | positive |
| 46 | PCC | f | 61 | sporadic | positive | positive |
| 47 | PCC | f | 27 | sporadic | positive | positive |
| 48 | PCC | f | 34 | sporadic | positive | positive |
| 49 | PCC | f | 29 | MEN2A | positive | positive |

| | | | | | | |
|---|---|---|---|---|---|---|
| 50 | PCC | m | 26 | MEN2A | positive | positive |
| 51 | PCC | f | 24 | MEN2A | positive | positive |
| 52 | PCC | f | 50 | MEN2A | positive | - |
| 53 | PCC | m | 54 | sporadic | positive | positive |
| 54 | PCC | f | 51 | sporadic | positive | positive |
| 55 | PCC | m | 24 | VHL | positive | positive |
| 56 | PCC | f | 38 | MEN2A | positive | positive |
| 57 | PCC | m | 23 | MEN2A | positive | positive |
| 58 | PCC | m | 45 | sporadic | positive | positive |
| 59 | PCC | f | 51 | MEN2A | positive | positive |
| 60 | PCC | f | 50 | sporadic | positive | positive |
| 61 | PCC | f | 45 | sporadic | positive | positive |
| 62 | PCC | f | 49 | sporadic | positive | positive |
| 63 | PCC | m | 65 | sporadic | positive | positive |
| 64 | PCC | m | 66 | sporadic | positive | positive |
| 65 | PCC | f | 39 | NF1 | positive | positive |
| 66 | PCC | f | 75 | sporadic | positive | positive |
| 67 | PCC | f | 34 | MEN2B | positive | positive |
| 68 | PCC | m | 59 | sporadic | positive | positive |
| 69 | PCC | m | 63 | sporadic | positive | positive |
| 70 | PCC | m | 48 | sporadic | positive | positive |
| 71 | PCC | f | 18 | VHL | positive | positive |
| 72 | PCC | m | 63 | sporadic | positive | positive |
| 73 | PCC | f | 79 | sporadic | positive | positive |
| 74 | PCC | f | 71 | sporadic | positive | positive |
| 75 | PCC | m | 29 | sporadic | positive | positive |
| 76 | PCC | f | 35 | sporadic | positive | - |
| 77 | PCC | f | x | sporadic | positive | positive |
| 78 | PCC | f | x | sporadic | positive | positive |
| 79 | PCC | f | 56 | sporadic | positive | positive |
| 80 | PCC | f | 31 | sporadic | positive | positive |
| 81 | PCC | f | 59 | MEN2A | positive | positive |
| 82 | PCC | f | 24 | NF1 | positive | positive |
| 83 | PCC | f | 45 | sporadic | positive | negative |
| 84 | PCC | f | 30 | sporadic | positive | positive |
| 85 | PCC | m | 41 | sporadic | positive | positive |
| 86 | PCC | m | 43 | sporadic | positive | positive |
| 87 | PCC | f | 41 | sporadic | positive | positive |
| 88 | PCC | f | 41 | MEN2A | positive | positive |
| 89 | PCC | m | 40 | MEN2A | positive | positive |
| 90 | PCC | m | 53 | MEN2A | positive | positive |
| 91 | PCC | f | 69 | sporadic | positive | positive |
| 92 | PCC | m | 53 | sporadic | positive | positive |
| 93 | PCC | f | 48 | sporadic | positive | positive |
| 94 | PCC | f | 68 | sporadic | positive | positive |
| 95 | PCC | f | 33 | sporadic | positive | - |
| 96 | PCC | m | 30 | sporadic | positive | positive |
| 97 | PCC | m | 41 | NF1 | positive | positive |
| 98 | PCC | f | 30 | sporadic | positive | positive |
| 99 | PCC | f | 50 | sporadic | positive | positive |
| 100 | PCC | m | 44 | NF1 | positive | positive |

| | | | | | | |
|---|---|---|---|---|---|---|
| 101 | PCC | f | 62 | sporadic | positive | positive |
| 102 | PCC | f | 42 | sporadic | positive | positive |
| 103 | PCC | x | 61 | sporadic | positive | positive |
| 104 | PCC | m | 24 | sporadic | positive | - |
| 105 | PCC | m | 42 | sporadic | positive | positive |
| 106 | PCC | f | 25 | MEN2A | positive | positive |
| 107 | PCC | m | 52 | NF1 | positive | positive |
| 108 | PCC | f | 43 | sporadic | positive | positive |
| 109 | PCC | f | 51 | MEN2A | positive | positive |
| 110 | PCC | m | 50 | sporadic | positive | positive |
| 111 | PCC | m | 39 | sporadic | positive | positive |
| 112 | PCC | m | 35 | MEN2A | positive | positive |
| 113 | PCC | f | 60 | sporadic | positive | positive |
| 114 | PCC | f | 39 | sporadic | positive | positive |
| 115 | PCC | m | 16 | SDHD | positive | negative |
| 116 | PCC | f | 31 | SDHD | positive | negative |
| 117 | PCC | m | 29 | NF1 | positive | positive |
| 118 | PCC | m | 62 | SDHD | positive | negative |
| 119 | PCC | m | 21 | MEN2a | positive | positive |
| 120 | PCC | m | 7 | VHL | positive | positive |
| 121 | PCC | m | 44 | sporadic | positive | positive |
| 122 | PCC | f | 31 | sporadic | positive | - |
| 123 | PCC | m | 56 | sporadic | positive | positive |
| 124 | PCC | f | 43 | MEN2A | positive | positive |
| 125 | PCC | m | 28 | sporadic | positive | negative |
| 126 | PCC | m | 42 | sporadic | positive | positive |
| 127 | PCC | f | 57 | sporadic | positive | positive |
| 128 | PCC | f | 56 | sporadic | positive | positive |
| 129 | PCC | m | 31 | sporadic | positive | positive |
| 130 | PCC | m | 61 | sporadic | positive | positive |
| 131 | PCC | f | 53 | NF1 | positive | positive |
| 132 | PCC | f | 88 | sporadic | positive | positive |
| 133 | PCC | f | 48 | sporadic | negative | negative |
| 134 | PCC | f | 77 | sporadic | positive | positive |
| 135 | PCC | m | 47 | sporadic | positive | positive |
| 136 | PCC | m | 38 | VHL | positive | positive |
| 137 | PCC | f | 57 | sporadic | positive | positive |
| 138 | PCC | f | 69 | sporadic | positive | positive |
| 139 | PCC | m | 43 | sporadic | positive | positive |
| 140 | PCC | m | 62 | sporadic | positive | positive |
| 141 | PCC | f | 59 | NF1 | positive | positive |
| 142 | PCC | m | 28 | sporadic | positive | positive |
| 143 | PCC | f | 21 | sporadic | positive | negative |
| 144 | PCC | m | x | sporadic | positive | positive |
| 145 | PCC | f | x | sporadic | positive | positive |
| 146 | sPGL | f | 53 | sporadic | positive | positive |
| 147 | sPGL | m | 25 | SDHD | positive | negative |
| 148 | sPGL | m | 54 | sporadic | positive | - |
| 149 | sPGL | m | 79 | sporadic | positive | positive |
| 150 | sPGL | f | 43 | sporadic | positive | positive |
| 151 | sPGL | f | 49 | sporadic | positive | negative |

| | | | | | | |
|---|---|---|---|---|---|---|
| 152 | sPGL | f | 35 | sporadic | positive | - |
| 153 | sPGL | f | 30 | sporadic | positive | negative |
| 154 | sPGL | f | 56 | sporadic | positive | positive |
| 155 | sPGL | f | 63 | SDHB | positive | negative |
| 156 | sPGL | m | 30 | sporadic | positive | positive |
| 157 | sPGL | m | 48 | sporadic | positive | positive |
| 158 | sPGL | f | 12 | SDHB | positive | negative |
| 159 | sPGL | m | 40 | sporadic | positive | positive |
| 160 | sPGL | m | 61 | sporadic | positive | positive |
| 161 | sPGL | f | 55 | sporadic | negative | negative |
| 162 | PGL | f | 33 | sporadic | negative | negative |
| 163 | PGL | m | 39 | sporadic | positive | negative |
| 164 | PGL | m | 44 | sporadic | positive | negative |
| 165 | PGL | m | 42 | sporadic | positive | positive |
| 166 | PGL | f | 14 | sporadic | positive | negative |
| 167 | PGL | m | 34 | sporadic | positive | negative |
| 168 | PGL | f | 33 | sporadic | positive | positive |
| 169 | PGL | f | 67 | sporadic | positive | positive |
| 170 | PGL | f | 29 | sporadic | positive | positive |
| 171 | PGL | f | 62 | sporadic | positive | negative |
| 172 | PGL | f | 36 | SDHD | positive | negative |
| 173 | PGL | f | 53 | SDHD | positive | negative |
| 174 | PGL | f | 53 | sporadic | positive | positive |
| 175 | PGL | m | 25 | SDHD | positive | negative |
| 176 | PGL | f | 33 | SDHD | positive | negative |
| 177 | PGL | m | 26 | SDHD | positive | negative |
| 178 | PGL | f | 39 | SDHC | positive | negative |
| 179 | PGL | f | 24 | SDHD | positive | negative |
| 180 | PGL | f | 55 | sporadic | positive | positive |
| 181 | PGL | f | 35 | sporadic | positive | negative |
| 182 | PGL | f | 57 | SDHD | positive | negative |
| 183 | PGL | m | 45 | sporadic | positive | positive |
| 184 | PGL | f | 57 | sporadic | positive | positive |
| 185 | PGL | f | 39 | SDHD | positive | negative |
| 186 | PGL | f | 57 | sporadic | positive | positive |
| 187 | PGL | m | 45 | sporadic | negative | negative |
| 188 | PGL | f | 61 | IDH1 | positive | positive |
| 189 | PGL | f | 23 | sporadic | positive | positive |
| 190 | PGL | f | 50 | SDHD | positive | negative |
| 191 | PGL | f | 22 | SDHD | positive | negative |
| 192 | PGL | f | 53 | SDHD | positive | negative |
| 193 | PGL | m | 73 | sporadic | positive | positive |
| 194 | PGL | f | 32 | sporadic | positive | negative |
| 195 | PGL | m | 63 | sporadic | positive | positive |
| 196 | PGL | f | 36 | SDHD | positive | negative |
| 197 | PGL | m | 52 | sporadic | positive | positive |
| 198 | PGL | m | 48 | SDHB | positive | negative |
| 199 | PGL | m | 34 | SDHD | positive | negative |
| 200 | PGL | m | 20 | sporadic | positive | negative |
| 201 | PGL | x | x | sporadic | positive | positive |
| 202 | PGL | m | 56 | sporadic | positive | positive |

| | | | | | | |
|---|---|---|---|---|---|---|
| 203 | PGL | m | 38 | sporadic | positive | negative |
| 204 | PGL | m | 51 | SDHD | positive | negative |
| 205 | PGL | f | 57 | sporadic | positive | positive |
| 206 | PGL | f | 37 | SDHD | positive | negative |
| 207 | PGL | m | 57 | sporadic | positive | negative |
| 208 | PGL | m | 48 | SDHD | positive | negative |
| 209 | PGL | f | 35 | sporadic | positive | - |
| 210 | PGL | f | 27 | sporadic | positive | negative |
| 211 | PGL | x | x | sporadic | positive | positive |
| 212 | PGL | x | x | sporadic | positive | - |
| 213 | PGL | f | 27 | sporadic | positive | negative |
| 214 | PGL | f | 25 | sporadic | positive | negative |
| 215 | PGL | m | 61 | sporadic | positive | negative |
| 216 | PGL | x | x | sporadic | positive | negative |
| 217 | PGL | x | x | sporadic | positive | negative |
| 218 | PGL | x | x | sporadic | positive | negative |
| 219 | PGL | x | x | SDHAF2 | positive | negative |
| 220 | PGL | x | x | SDHAF2 | positive | negative |
| 221 | PGL | x | x | SDHAF2 | positive | negative |
| 222 | PGL | x | x | SDHAF2 | positive | negative |
| 223 | PGL | x | x | SDHAF2 | positive | negative |
| 224 | PGL | x | x | sporadic | positive | positive |

PCC: pheochromocytoma, sPGL: extra-adrenal sympathetic paraganglioma, PGL: paraganglioma, x:unknown

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# CHAPTER 4

**SDHB immunohistochemistry: a useful tool in diagnosis of Carney-Stratakis and Carney triad gastrointestinal stromal tumors.**

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**Abstract**

Mutations in the tumor suppressor genes *SDHB*, *SDHC*, and *SDHD* (or collectively *SDHx)* cause the inherited paraganglioma syndromes, characterized by pheochromocytomas and paragangliomas. However, other tumors have been associated with *SDHx* mutations, such as gastrointestinal stromal tumors (GISTs) in Carney-Stratakis syndrome. Previously we have shown that SDHB immunohistochemistry is a reliable technique for the identification of pheochromocytomas and paragangliomas caused by *SDHx* mutations. We hypothesized that GISTs in patients with *SDHx* mutations would be negative immunohistochemically for SDHB as well.

Four GISTs from patients with Carney-Stratakis syndrome and six from patients with Carney triad were investigated by SDHB immunohistochemistry. Five GISTs with *KIT* or *PDGFRA* gene mutations were used as controls. In addition, SDHB immunohistochemistry was performed on 42 apparently sporadic GISTs. In cases where the SDHB immunohistochemistry was negative, mutational analysis of *SDHB*, *SDHC*, and *SDHD* was performed.

All GISTs from patients with Carney-Stratakis syndrome and Carney triad were negative for SDHB immunohistochemically. In one patient with Carney-Stratakis syndrome a germline *SDHB* mutation was found (p.Ser92Thr). The five GISTs with a *KIT* or *PDGFRA* gene mutation were all immunohistochemically positive for SDHB. Of the 42 sporadic tumors, one GIST was SDHB-negative. Mutational analysis of this tumor did not reveal an *SDHx* mutation. All SDHB-negative GISTs were located in the stomach, had an epithelioid morphology and had no *KIT* or *PDGFRA* mutations.

We demonstrate that Carney-Stratakis syndrome- and Carney triad-associated GISTs are negative by immunohistochemistry for SDHB in contrast to *KIT* or *PDGFR*A mutated GISTs and a majority of sporadic GISTs. We suggest GIST type 2 should be tested for SDHB immunohistochemically. In case of negative SDHB staining in GISTs Carney-Stratakis syndrome or Carney triad should be considered and appropriate clinical surveillance should be instituted.

**Introduction**

Succinate dehydrogenase (SDH) is an enzyme complex that catalyses the oxidation of succinate to fumarate in the citric acid cycle and participates in the electron transport chain. SDH is located in the mitochondrial inner membrane and consists of four nuclear encoded subunits: the flavoprotein SDHA, the iron-sulfur protein SDHB, and the integral membrane proteins SDHC, and SDHD. (1)

Mutations in the different subunits result in very different disorders. Mutations in *SDHA* cause Leigh syndrome, a rare inherited neurometabolic disorder characterized by degeneration of the central nervous system. (2) Mutations in the tumor suppressor genes *SDHB*, *SDHC*, *SDHD* from here on collectively referred to as *SDHx*, occur in patients with the pheochromocytoma-paraganglioma syndrome. (3) Diverse additional tumors have been associated with *SDHx* mutations, including gastrointestinal stromal tumors (GISTs), renal cell carcinomas, renal oncocytomas and, rarely, papillary thyroid carcinomas, neuroblastomas and seminomas. (4-8)

Of these tumors, GISTs occur most frequently in patients with *SDHx* mutations. The majority of sporadic GIST are caused by mutations in the *KIT* and *PDGFRA* genes whereas 4 different *SDHB* mutations, 2 *SDHC* mutations and one *SDHD* mutation have been identified in patients with the familial dyad of paraganglioma and GIST, also known as the Carney-Stratakis syndrome.(7) The Carney triad is similar to Carney-Stratakis syndrome, but includes pulmonary chondromas and is apparently infrequently inherited; *SDHx* mutations have not been described In the Carney triad.(9)

In a previous report we showed that SDHB immunohistochemistry is a reliable technique to identify pheochromocytomas and paragangliomas caused by mutations in *SDHB*, *SDHC* and *SDHD*. (10) It seemed likely that other tumors in patients with *SDHx* mutations would be negative for SDHB immunohistochemistry as well. In this study we performed SDHB immunohistochemistry on GISTs that occurred as a component of the Carney-Stratakis syndrome and the Carney triad. Also we performed SDHB immunohistochemistry on a series of apparently sporadic GISTs.

## Material and methods

### Tumor samples

Four GISTs from patients with the Carney-Stratakis syndrome and six GISTs from patients with Carney triad were available for this study. Distinguishing Carney triad and Carney-Stratakis syndrome is difficult in individual patients. In this study we used the familial predisposition and paraganglioma as the first presenting tumor in Carney Stratakis syndrome and the presence of pulmonary chondroma, female predominance and GIST as the first presenting tumor in Carney triad as differentiating features. (11) As a control group we used 5 GISTs with a mutation in *KIT* or *PDGFRA*. GIST diagnosis was made based on histology and verified immunohistochemically using DOG1 (RM-9132-R7) antibody (Thermo Scientific, Cheshire, UK; 1:50) and CD117, c-kit (A4502) antibody (DAKO, Heverlee, Belgium; 1:25).

In addition we investigated a series of 42 formalin-fixed paraffin-embedded sporadic GISTs that were diagnosed in Erasmus MC between 2001 and 2009. These samples were anonymously used according to the code for adequate secondary use of tissue, code of conduct: "Proper Secondary Use of Human Tissue" established by the Dutch Federation of Medical Scientific Societies (http://www.federa.org).

### Immunohistochemistry

All GISTs were investigated by SDHB immunohistochemistry as previously described (10), using the rabbit polyclonal antibody HPA002868 (Sigma-Aldrich Corp, St. Louis, MO; 1:500). Immunoreactivity was scored independently by two observers (JG and RRdK) who were blinded to all clinical, pathological and molecular data. Slides with a granular staining in the tumor cell cytoplasm were scored as positive. Slides in which the tumor cells were negative or showed diffuse cytoplasmatic staining, but with granular staining in endothelial cells (internal control) were scored as negative. Samples lacking the internal positive control were considered non-informative and were repeated.

Mutational analysis

Following SDHB immunohistochemical evaluation, *SDHx*-gene mutational analysis was performed on the tumors with negative SDHB immunohistochemistry. A region containing at least 70% tumor cells was micro-dissected from the tumor block and DNA was isolated using the Puregene DNA isolation kit (Qiagen, Minneapolis, USA) according to the manufacturer's protocol. No tumor DNA was available of one sample and germ-line DNA was used instead. Mutational analysis was performed by direct sequencing of the open reading frames, including the exon-intron boundaries of the *SDHB*, *SDHC*, and *SDHD* genes. In addition, sequence 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 was performed on the SDHB immunonegative tumors.

**Results**

There was uniform agreement between the two observers in classifying tumors as SDHB-positive or SDHB-negative. Four GISTs from patients with the Carney-Stratakis syndrome were negative for SDHB by immunohistochemistry (Figure 1A). By contrast, all *KIT* or *PDGFRA* mutated GISTs were SDHB-positive (Figure 1B and Figure 1C). In one of the Carney-Stratakis syndrome patients, an *SDHB* germ-line mutation (p. Ser92Thr) was present. In the other three patients no mutations in *SDHB*, *SDHC*, and *SDHD* were found. The six GISTs from patients with Carney triad were negative for SDHB by immunohistochemistry (Figure 1D). However, no mutations in *SDHB*, *SDHC* and *SDHD* were found. The GISTs in the Carney-Stratakis syndrome and Carney triad all had an epithelioid morphology. In contrast, the *KIT* and *PDGFR*A mutated tumors had a spindled morphology. The clinical details of the Carney-Stratakis syndrome and Carney-triad patients are summarized in Table 1.

**Table 1.** Details of the Carney-Stratakis syndrome and Carney-triad patients

| | Sex | Age at diagnosis | Location | Cell type | Other tumors | *SDHx* mutation |
|---|---|---|---|---|---|---|
| **CSS** | | | | | | |
| 1 | M | 18 | Stomach | Epithelioid | No | Yes |
| 2 | M | 10 | Stomach | Epithelioid | No | No |
| 3 | M | 50 | Stomach | Epithelioid | PGL | No |
| 4 | F | U | Stomach | Epithelioid | PGL, angiolipoma | No |
| **CT** | | | | | | |
| 1 | F | 12 | Stomach | Epithelioid | No | No |
| 2 | F | 41 | Stomach | Epithelioid | PGL | No |
| 3 | F | 25 | Stomach | Epithelioid | No | No |
| 4 | F | 13 | Stomach | Epithelioid | No | No |
| 5 | F | 28 | Stomach | Epithelioid | Adrenal adenoma | No |
| 6 | M | 51 | Stomach | Epithelioid | chondroma | No |

CSS: Carney-Stratakis syndrome; CT: Carney triad; *SDHx*: *SDHB*, *SDHC*, and *SDHD* gene; U: unknown, PGL: paraganglioma, GIST: gastrointestinal stromal tumor.

Among the 42 sporadic GISTs, one GIST (2%) from a 41-year-old woman, located in the stomach, was negative for SDHB immunohistochemically. She developed a medullary thyroid carcinoma at age 45 with local lymph node and liver metastasis. The medullary thyroid carcinoma was positive with SDHB immunohistochemistry. Microscopy of the GIST showed an epithelioid morphology and the tumor cells were positive for CD117 and DOG1. Mutational analysis revealed no mutations in *SDHB*, *SDHC*, *SDHD* and *SDHAF2* genes. Sequencing analysis of exons 8, 9, 11, 13 and 17 of the *KIT* proto-oncogene and exons 12, 14 and 18 of the *PDGFRA* gene did not reveal mutations.

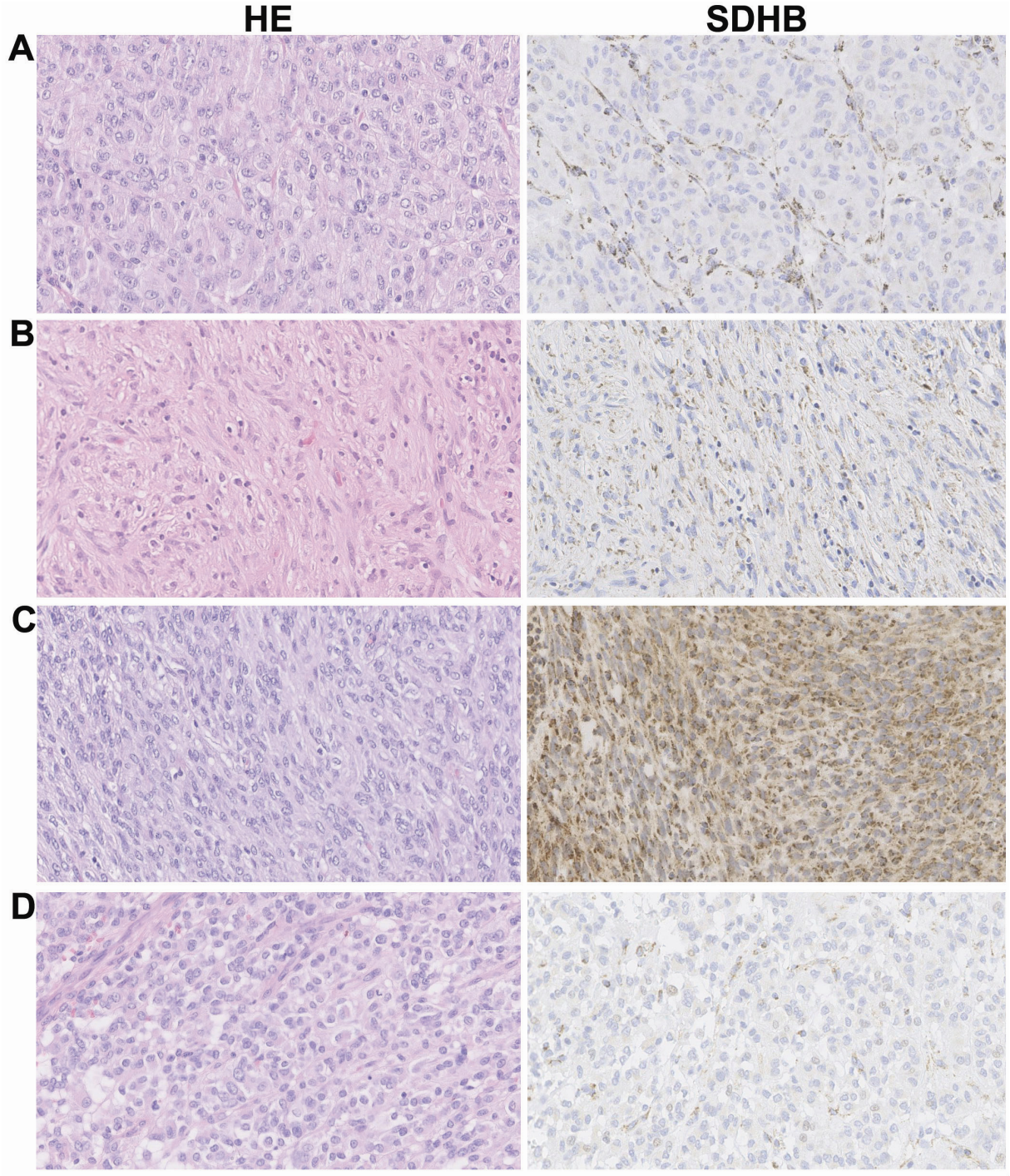


**Figure 1.**

H&E staining and SDHB immunohistochemistry: A) Gastrointestinal stromal tumor from a patient with the Carney-Stratakis syndrome and negative immunostaining for SDHB. The endothelial cells serve as an internal positive control. B) Gastrointestinal stromal tumor with a *PDGFRA* mutation. All cells show granular staining. C) Gastrointestinal stromal tumor with a *KIT* mutation. All cells show granular staining. D) Gastrointestinal stromal tumor from a patient with Carney triad and negative immunostaining for SDHB.

**Discussion**

In pheochromocytomas and paragangliomas absence of SDHB staining is an indicator of Complex II disruption caused by *SDHB*, *SDHC*, or *SDHD* mutations. (10) In this study, a GIST from a patient with Carney-Stratakis syndrome had a proven germ-line *SDHB* mutation, which confirms our hypothesis that GISTs caused by mutations in *SDHB*, *SDHC*, and *SDHD* would be negative with SDHB immunohistochemistry.

*SDHB* mutations are also described in different types of renal tumors. (6, 8, 12), usually clear cell renal cell carcinomas, but the mutations have also been found in oncocytomas, eosinophilic chromophobe renal cell carcinomas and papillary renal cell carcinomas. Previously, we reported negative SDHB immunohistochemistry in a renal cell carcinoma (10). This suggests that all tumor types in patients with *SDHx* mutations are characterized by absent SDHB staining.

The GISTs from Carney-Stratakis syndrome and Carney triad patients in our study were negative for SDHB by immunohistochemistry, results that are concordant with a recent publication of Gill et al (2010), in which are described tumors from five Carney triad patients with negative SDHB immunohistochemistry (13). However they did not describe any Carney-Stratakis syndrome patients. We found that mutations in *SDHB*, *SDHC*, and *SDHD* were absent in all but one SDHB immunonegative GIST, which came from a Carney-Stratakis syndrome patient. In addition we found no proof of loss of heterozygosity in these tumors samples (results not shown). In a previous study of pheochromocytomas and paragangliomas, we found six tumors with negative SDHB immunohistochemistry, but lacking *SDHB*, *SDHC*, and *SDHD* mutations (11%). (10, 14) This may be due to a less than 100% sensitivity of the technique of sequence analysis or to the fact that we did not perform systematic multiplex ligation-dependent probe amplification in all our samples, thus probably missing up to 10% of genetic abnormalities present. However, it is also possible that epigenetic changes or other genes affect complex II, and that mutations in such additional genes might result in disruption of complex II and subsequently in negative SDHB immunohistochemistry. The question therefore remains whether absent SDHB immunostaining implies the presence of *SDHx* mutations in GISTs, as we have

shown with more than 85% sensitivity in pheochromocytomas and paragangliomas. Although the mechanism of tumorigenesis of the SDHB immuno negative GIST is unknown, several studies have shown that VEGF and HIF1α are relatively overexpressed in GISTs (15-16), as is the case in SDHx mutated paragangliomas.
The negative SDHB immunohistochemistry in Carney-Stratakis syndrome and Carney triad GISTs implies that complex II is degraded. However the mammalian mitochondria contain 1100 proteins of which nearly 300 are uncharacterized, so it is highly likely that epigenetic abnormalities of SDH genes or pathogenic mutations or functional abnormalities of other mitochondrial proteins both drive tumorigenesis and account for SDHB negativity in Carney-stratakis syndrome and Carney triad related GISTs.

Among the 42 apparently sporadic GISTs we studied, one tumor (2%) was negative for SDHB by immunohistochemistry. This finding is in agreement with Gill et al who found that 3% (3/101) of sporadic GISTs in their series was negative for SDHB immunohistochemically (13). Interestingly, our patient developed a medullary thyroid carcinoma four years after diagnosis of the GIST. This combination of GIST and medullary thyoid carcinoma has been described previously in a patient with multiple endocrine neoplasia type 2A (17). In our case we found a somatic mutation in *RET* in the medullary thyroid carcinoma (results not shown). In addition, SDHB immunohistochemistry was positive in this medullary thyroid carcinoma, indicating that the tumor was not caused by complex II disruption.

Gill et al divided GISTs into two broad groups: GIST type 1, which includes most GISTs and occurs mainly in adults and *KIT* or *PDGFRA* mutant tumors. Type I tumors usually show spindled morphology. GIST type 2 occurs predominantly in children and young adults. Type II tumors show an epithelioid morphology, occur exclusively in the stomach and are never associated with *KIT* or *PDGFRA* mutations. (13) The one apparently sporadic SDHB negative GIST in our study, although occurring in an adult, was located in the stomach and had an epithelioid morphology, in line with the observations by Gill et al.

In conclusion, we have demonstrated that Carney-Stratakis syndrome and Carney triad GISTs are negative for SDHB by immunohistochemistry in contrast to *KIT* or *PDGFRA*

mutated GISTs and the majority of apparently sporadic GISTs which are immunopositive. Our findings suggest that absent SDHB immunostaining in GISTs has a high likelihood of syndromic implications for either Carney-Stratakis syndrome or the Carney triad. Consequently, *SDHx* mutational analysis and clinical surveillance for the development of paragangliomas and pulmonary chondromas should be instituted.

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# CHAPTER 5

**PARASYMPATHETIC PARAGANGLIOMAS ARE PART OF THE VON HIPPEL-LINDAU SYNDROME.**

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**ABSTRACT**

Von Hippel-Lindau (VHL) disease, caused by germline mutations in the *VHL* gene, is a hereditary tumor syndrome manifested by hemangioblastomas, clear cell renal cell carcinomas and pheochromocytomas. In addition, a multitude of other rare tumors, amongst which parasympathetic paragangliomas, can occur and even be the sole manifestation of VHL disease. The *VHL* gene is a bona fide tumor suppressor gene with biallelic inactivation contributing to tumor formation. However, in parasympathetic paragangliomas occurring in VHL disease biallelic inactivation of the *VHL* gene has not been demonstrated to date.

Here we present two VHL patients with head and neck paraganglioma. Apart from germline *VHL* mutations, no additional mutations were found in the paraganglioma-related tumor suppressor genes *SDHB*, *SDHC* and *SDHD*. Analysis of paraganglioma tissue revealed loss of the *VHL* wild type allele in both tumors, indicating that in these tumors biallelic *VHL* gene inactivation occurred, which probably contributed to the tumorigenesis of the paragangliomas. These findings indicate that parasympathetic paragangliomas in VHL disease, although rare, are part of the syndrome and related to *VHL* gene inactivation. Clinicians should be aware of the potential occurrence of parasympathetic paragangliomas in VHL disease.

## INTRODUCTION

Von Hippel-Lindau disease is a rare hereditary tumor syndrome, with various manifestations. Clinically there are large differences between affected families in the spectrum of tumors. Therefore, VHL disease has been subdivided into 2 subtypes, VHL type 1 and VHL type 2. In VHL type 1, patients frequently harbor *VHL* deletions or truncating mutations and usually present with hemangioblastomas and clear cell renal cell carcinomas , but develop very rarely pheochromocytomas, whereas VHL type 2 patients usually harbor missense mutations and do have pheochromocytomas (1,2). Although hemangioblastomas, clear cell renal cell carcinomas and pheochromocytomas are the hallmarks of VHL disease, a multitude of other more rare tumors can occur in the context of this syndrome, e.g. endocrine tumors of the pancreas, endolymphatic sac tumors of the inner ear, papillary cystadenomas of the epididymis and broad ligament and paragangliomas (3).

Paragangliomas are neuroendocrine tumors, which are subdivided into parasympathetic and sympathetic paragangliomas, based on their location and catecholamine production (4). Parasympathetic paragangliomas are found in the head and neck region, and usually do not release catecholamines. Sympathetic paragangliomas are situated along the sympathetic trunk in the abdomen, and usually produce catecholamines. Although the majority of parasympathetic paragangliomas occur sporadically (5), a subset of these tumors occur due to mutations in the succinate dehydrogenase (*SDH*) *B*, *C* and, *D* genes (6-8). In several reports parasympathetic paragangliomas have been reported in the context of VHL disease (9-15). However, no molecular evidence was presented for the involvement of the *VHL* gene in these parasympathetic paragangliomas. In the present study the involvement of the *VHL* gene in two non-catecholamine-producing head and neck paragangliomas in VHL disease was investigated.

## MATERIAL AND METHODS

*Subjects and tumor samples*

Recently, Boedeker et al described twelve patients with hereditary non-*SDHx* parasympathetic paragangliomas and eleven of these patients had a germline *VHL* mutation (15). In these eleven patients different *VHL* mutations were found (eight missense, one nonsense, one frame-shift, and one deletion). The parasympathetic paragangliomas from 2 patients in this study were available for analysis.

Case 1 concerned a 23-year-old female, who presented with a tumor on the left side of the neck at routine check-up because she was a carrier of the p.Arg64Pro *VHL* mutation, which has been described before (15). No other tumors were found in this patient, but family history revealed pheochromocytomas and clear cell renal cell carcinomas.

Case 2 was a 7-year-old boy, diagnosed with a malignant sympathetic paragangliomas in the retro-peritoneum with regional lymph node metastases, who presented with a parasympathetic paragangliomas of the left carotid body 8 years later. Genetic testing revealed the *VHL* p.Tyr98His mutation, which has also been described previously (15). In addition hemangioblastomas and pheochromocytomas were found in family members.

To assess the biochemical phenotype of these tumours plasma and urine concentrations of catecholamines were measured in both patients.

*Molecular analysis*

DNA was isolated from formalin fixed paraffin embedded (FFPE) material from the two parasympathetic paragangliomas. A region of at least 80% tumor cells was micro-dissected and DNA was isolated using the Puregene DNA isolation kit (Gentra, Minneapolis, USA) according to manufacturer's protocol. In case 1 normal DNA was isolated from FFPE lymph node tissue and in case 2 from blood cells. The samples were anonymously used according to the code for adequate secondary use of tissue, code of conduct: "Proper Secondary Use of Human Tissue" established by the Dutch Federation of Medical Scientific Societies (http://www.federa.org).

Mutation analysis of normal DNA was performed by direct sequencing of *VHL*, *SDHB*, *C*, and *D* gene. In addition, the samples were investigated for the presence of large genomic

deletions in the *SDH* genes by multiplex ligation-dependent probe amplification (MLPA) assay.

Mutation analysis of tumor DNA was performed by direct sequencing of the *VHL* gene (only the region surrounding codon 64 and codon 98 was investigated). To rule out other underlying genetic causes of the parasympathetic paragangliomas we also performed mutation analysis by direct sequencing of the *SDHB*, *SDHC* and *SDHD* genes, with primers previously described (16). In addition SDHB immunohistochemistry was carried out (17). LOH analysis was performed for 3 microsatellite loci near the VHL gene. For this, polymerase chain reactions were carried out with fluorescence-labeled primers (Invitrogen, Paisly, UK) (table 1) for 28 cycles with an annealing temperature of 60°C, and amplified products were analyzed, along with LIZ 500 Size Standard (Applied biosystems, Foster City, USA), using capillary electrophoresis on an ABI 3130-XL Genetic Analyzer. Data was analyzed using GeneMarker software (SoftGenetics LLC, State college, PA, USA). In addition, array-CGH was performed as previously described on one of the tumor DNA samples (case 1) (18). Slides containing triplicats of ~3,500 BAC clones were produced at Leiden University Medical Center.

**RESULTS**

*Clinical characteristics and Pathology*

Both tumors were non-catecholamine-releasing carotid body tumors. Histopathologically the tumors consisted of nests of round to polygonal cells with a characteristic 'Zellballen' pattern surrounded by a fine fibrovascular stroma (Figure 1A). The tumor cells were positive for chromogranin A. S100 immunohistochemistry (IHC) revealed staining of sustentacular cells. The anatomical locations of the tumors, the histopathological appearance, the immunohistochemical expression pattern and the lack of increased serum levels of catecholamines led to a solid diagnosis of parasympathetic paragangliomas. Additional SDHB IHC showed positive staining in tumor cells of both cases (Figure 1B), indicating normal function of the SDH enzyme and absence of mutations in one of the SDHX genes (17).

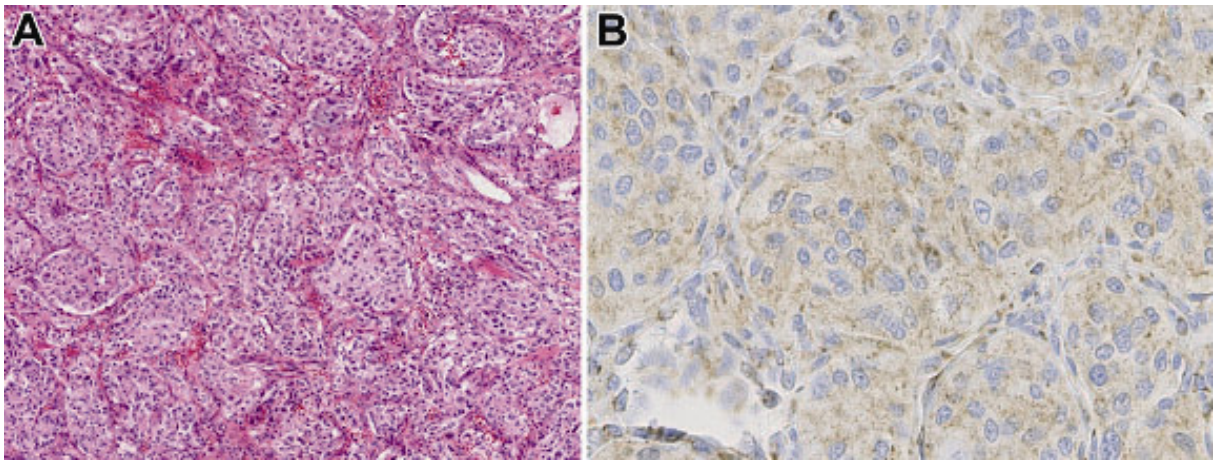


**Figure 1.**

A) Hematoxylin and eosin staining of paraganglioma composed of classical Zellballen, separated by a delicate fibrovascular stroma. B) SDHB IHC, showing positive tumor cells.

*Mutation analysis*

Sequence and MLPA analyses of normal DNA revealed no mutations in the *SDHB*, *C*, and *D* genes. Direct sequencing of the *VHL* gene on normal and tumor derived DNA revealed a G→C transition in codon 64 leading to p.Arg64Pro in case 1. In case 2 a T→C transition in codon 98, leading to p.Tyr98His, was found. The chromatogram of both tumor DNAs revealed predominantly the mutant allele, indicating loss of the wild type *VHL* allele in the tumor cells (Figure 2A). The residual signal for the wild-type allele is most likely derived from normal cells within the tumor sample.

Additional mutation analysis of tumor DNA revealed no somatic mutations in the *SDHB*, *SDHC* and *SDHD* gene.

*LOH analysis*

Next to sequencing analysis of the VHL gene, loss of one *VHL* allele in the parasympathetic paragangliomas was confirmed through analysis of the polymorphic microsatellites: D3S1597, D3S1435, and D3S1263 within the VHL gene locus. The paraganglioma of case 1 showed LOH for all three markers and in case 2 LOH was found with markers D3S1597 and D3S1263. Case 2 was homozygous (not informative) for marker D3S1435. Figure 2B shows the results of LOH analysis.

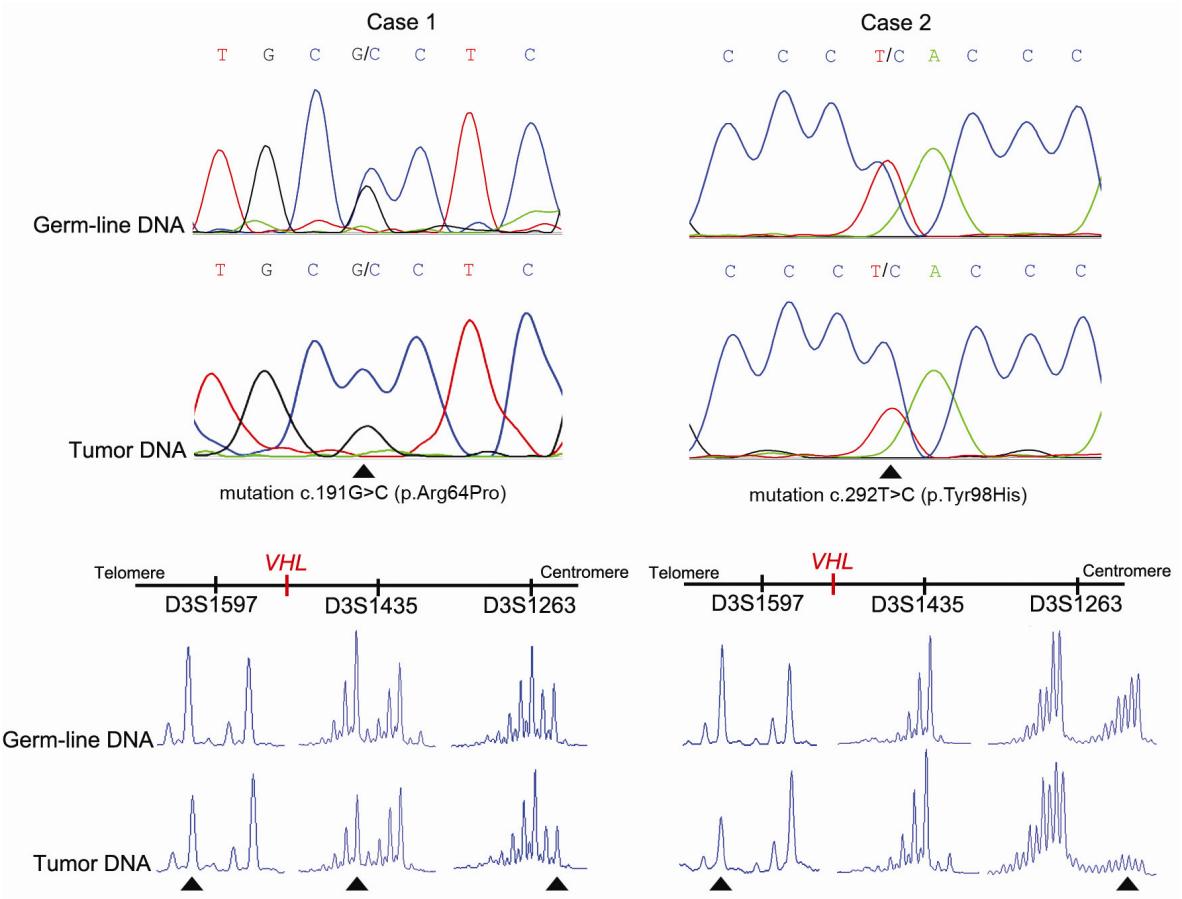


**Figure 2.**

**Top:** Sequencing chromatograms, Case 1: showing p.Arg64Pro due to c. 191 G→C (NM_000551). Case 2: showing the p.Tyr98His due to c.292 G→C (NM_000551). The chromatograms of the tumors revealed in both cases predominantly the mutant allele, indicating relative loss of the wild type *VHL* allele.

**Bottom:** Electrophoretograms demonstrating loss of heterozygosity (LOH). Case 1 showed LOH for all three markers. In case 2, the paraganglioma showed LOH for markers D3S1597 and D3S1263 and was homozygous (not informative) for marker D3S1435. (Arrows indicate alleles with relative loss)

*Array CGH*

Array CGH of case 1 showed loss of chromosome 3p and chromosome 11, as well as loss of the centromeric part of chromosome 1 (SDHB region not included), and partial loss of chromosomes 14, 21 and 22. (Result not shown)

**DISCUSSION**

The presence of parasympathetic paragangliomas in patients with VHL disease is rare and thus far 16 cases have been described in the literature (9-15). Although this incidence of parasympathetic paragangliomas in VHL disease (0.005) is larger than the population frequency (0.00001 to 0.00002) it is still not a foregone conclusion whether parasympathetic paragangliomas is a bona fide part of the VHL syndrome (15). In the present study we show that in two VHL disease-related parasympathetic paragangliomas the *VHL* gene is bi-allelically inactivated by the combination of a *VHL* germline mutation and loss of the wild type allele. Therefore, our results suggest that these parasympathetic paragangliomas arose by the absence of functional VHL protein and as such are part of the VHL syndrome.

The *VHL* gene, located on chromosome 3p25, is generally accepted to be a classic tumor suppressor gene, where, according to Knudson's two-hit model, bi-allelic inactivation is contributing to tumorigenesis. In VHL disease the first hit is a *VHL* gene germline inactivating mutation, present in one allele in every nuclear cell in the body. The second hit is a somatic DNA alteration in the remaining wild type *VHL* allele, acquired during the patient's life and as a consequence is only present in the tumor cells. In the present study of two parasympathetic paragangliomas cases we found a *VHL* p.Arg64Pro germline mutation in case 1 and a *VHL* p.Tyr98His germline mutation in case 2. The sequence chromatograms of both tumors revealed a predominance of the mutant allele, indicating loss of the wild type allele, which was subsequently confirmed by LOH analysis with microsatellite markers D3S1597, D3S1435, and D3S1263. This combination of a germline mutation in the *VHL* gene and the somatic inactivation of the wild-type *VHL* allele in the

tumor is consistent with the two hit model of tumorigenesis. Both *VHL* germline missense mutations found in this study are considered to be pathogenic since these mutations have been previously reported also in other VHL families (19,20). Interestingly, Hes et al described a family with a *VHL* p.Arg64Pro germline mutation presenting with clear cell renal cell carcinomas and pheochromocytomas and one family member with a parasympathetic paragangliomas (14).

The chromosomal aberrations of VHL-related pheochromocytomas are well characterized, showing loss of chromosome 3p and/or 11p as the most frequent events in these tumors (21,22). Parasympathetic paragangliomas on the other hand show few aberrations, with loss of chromosome 11 being the most frequent abnormality (23). This is in accordance with the pathogenesis of parasympathetic paragangliomas in general, which is frequently related to mutations in one of the *SDHX* tumor-suppressor genes. The finding of chromosome 3p loss in case 1, however, is more indicative of VHL-related pathogenesis.

In both investigated cases, direct sequencing and MLPA analysis of the *SDHB*, *SDHC* and *SDHD* genes in normal and tumor DNA revealed no mutations. In addition, SDHB immunostaining was positive in both cases, indicating that the SDHB protein is present in the tumor cells. According to our recent study this strongly suggests that there is no *SDHB*, *C*, or *D* involvement in these tumors, as no tumors with positive SDHB immunostaining carried mutations in any of these genes (17). These results make the co-occurrence of a *VHL* germline mutation and an *SDHX* mutation unlikely.

VHL disease has been subdivided into 2 subtypes, VHL type 1 and VHL type 2. In VHL type 1, patients frequently harbor *VHL* deletions or truncating mutations and usually present with hemangioblastomas and clear cell renal cell carcinomas, but develop very rarely pheochromocytomas, whereas VHL type 2 patients usually harbor missense mutations and do have pheochromocytomas (1). Hull et al described a patient with VHL disease having a parasympathetic paragangliomas and a pheochromocytomas, and a family history of hemangioblastomas (9). Of the eleven VHL patients described by Boedeker et al, eight patients or relatives of these patients had a pheochromocytomas and seven of them harbored a missense mutation (15). These and our findings indicate that parasympathetic paragangliomas are part of the tumor spectrum of type 2 VHL disease.

The prevalence of parasympathetic paragangliomas in VHL disease is 0.005 (15). This frequency is considered too low to systematically screen for parasympathetic paragangliomas in VHL syndrome patients. Depending on the anatomical location, parasympathetic paragangliomas can cause various symptoms like palpable mass in the neck, dysphagia, bradycardia, and hearing loss. A similar surveillance protocol for endolymphatic sac tumors, where a CT and MRI of internal auditory canals are performed after onset of symptoms (24), should be considered for parasympathetic paragangliomas in VHL patients.

In conclusion, our results indicate that the tumor spectrum in VHL disease (especially in VHL type 2 disease) includes parasympathetic paragangliomas. Clinicians treating VHL patients or having them under surveillance should be aware of the potential presence of parasympathetic paragangliomas in VHL disease.

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# CHAPTER 6

**Isocytrate dehydrogenase mutations are rare in pheochromocytomas and paragangliomas.**

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**Abstract**

Context: Paragangliomas and pheochromocytomas are neuroendocrine tumors that occur sporadically and in the context of inherited tumor syndromes including hereditary paraganglioma-pheochromocytoma syndrome and von Hippel-Lindau disease (VHL). The paraganglioma-pheochromocytoma syndrome is caused by germline-inactivating mutations in the mitochondrial succinate dehydrogenase (SDH) genes SDHB, SDHC, SDHD, or SDHAF2, and VHL is the result of inactivating VHL gene mutations. In SDH- and VHL-related paraganglioma and pheochromocytoma, hypoxia-inducible factor (HIF) stabilization has been described as the causal oncogenic event. Recently, HIF activation has also been found in glioblastoma multiforme, as the result of somatic mutational inactivation of the isocitrate dehydrogenase (IDH) type 1 or type 2 enzymes. These findings suggest that inactivating IDH1 and IDH2 mutations might also play a role in paraganglioma and pheochromocytoma tumorigenesis, especially in non-SDH- or non-VHL-related tumors.

Design: We investigated 365 pheochromocytomas and paragangliomas, including 269 sporadic tumors without SDH or VHL gene mutations, for mutations in IDH1 and IDH2. Only codons 132 and 172 were screened because these are the ones exclusively involved.

Results: In one of 131 paragangliomas, a somatic heterozygous IDH1 p.Arg132Cys mutation was detected in a sporadic carotid paraganglioma diagnosed in a 61-yr-old woman. No mutations were found in 234 pheochromocytomas.

Conclusion: IDH mutations are very rare in paragangliomas and pheochromocytomas and do not appear to play an important role in oncogenic HIF activation known to be present in these tumors.

## Introduction

**P**heochromocytomas and paragangliomas are neuroendocrine tumors that occur sporadically and as part of several inherited tumor syndromes, including multiple endocrine neoplasia type 2 with germline mutations in the *RET* proto-oncogene, von Hippel-Lindau disease (VHL) with germline mutations in the *VHL* gene, neurofibromatosis type 1 (NF1) with germline mutations in the *NF1* gene, and the hereditary paraganglioma-pheochromocytoma syndrome with germline mutations in the succinate dehydrogenase (*SDH*) *-B*, *-C*, *-D*, or *-AF2* gene (1, 2). The *SDH* genes encode mitochondrial proteins that comprise the SDH enzyme, which catalyzes the oxidation of succinate into fumarate in the tricarboxylic acid cycle. Inactivation of SDHB, -C, -D, or -AF2 proteins ultimately leads to succinate accumulation, which impairs hypoxia inducible factor (HIF) hydroxylation by prolyl-4-hydroxylases and consequently the degradation of HIF under normoxic conditions (3). The VHL protein also has an effect on HIF via its role in the ubiquitination and degradation of the α-subunits of HIF (4).

Recently, somatic heterozygous mutations in residue Arg132 of isocitrate dehydrogenase 1 (IDH1) and in the analogous residue Arg172 in IDH2 were found in the vast majority of glioblastoma multiforme and in smaller subsets of other glial tumors, prostate cancer, and B-acute lymphoblastic leukemia (5). IDH1 and IDH2 catalyze the oxidative decarboxylation of isocitrate to _-ketoglutarate, which is a tricarboxylic acid cycle metabolite that serves as an essential cosubstrate for prolylhydroxylase activity. Recently, evidence was obtained that IDH1 functions as a tumor suppressor with the heterozygous missense mutation of residue Arg132 acting as a dominant negative: mutant IDH1 protein complexes with the wild-type protein, rendering the complex enzymatically inactive (6). Inactivation of IDH1 (or IDH2) leads to a decreased_-ketoglutarate level, a decreased HIF hydroxylation and, therefore, results in HIF accumulation (6). This effect is hypothesized to result from prolylhydroxylase inhibition and inappropriate HIF stabilization in a pseudohypoxic condition.

Recent genome-wide expression microarray analyses have confirmed a common pattern of a high stimulation of the genes involved in the hypoxia-angiogenesis pathway as well as a HIF-2_ overexpression in *VHL*- and *SDH*related pheochromocytomas and paragangliomas (4). Whereas excess succinate and fumarate inhibit prolylhydroxylases,

α-ketoglutarate is essential for the hydroxylation activity. Because somatic mutations in *SDH* genes were rarely found in pheochromocytomas (7) and paragangliomas and because *IDH* mutations, which result in overexpression of HIF and induction of tumor angiogenesis, are present in various types of cancer, we hypothesized that *IDH* mutations could play a role in pheochromocytomas and paragangliomas, especially in sporadic cases.

## Materials and Methods

### Tissue samples

Aseries of 253 formalin-fixed and paraffin-embedded tumors (132 pheochromocytomas and 121 paragangliomas), including 96 inherited (12 *SDHB*, 1 *SDHC*, 51 *SDHD*, 13 *RET*, 14 *VHL* and 5 *NF1*) and 157 sporadic cases were tested for *IDH1* and *IDH2* mutations. The samples were anonymously used according to the code for adequate secondary use of tissue, code of conduct: "Proper Secondary Use of Human Tissue" established by the Dutch Federation of Medical Scientific Societies (http://www.federa.org). DNA was isolated from paraffin-embedded material from a region of more than 70% tumor cells, using the PuregeneDNAisolation kit (Gentra, Minneapolis,MN)according to the manufacturer's protocol. In addition, tumor DNA was extracted from 112 sporadic frozen tumors (102 pheochromocytomas and 10 paragangliomas) collected by the COMETE network, diagnosed as previously described (8, 9) at Hoˆ pital europe´en Georges Pompidou and atHoˆ pital Cochin (Paris, France) and directly sequenced for Arg132 IDH1 and Arg172 IDH2 mutations. The study was approved by an institutional review board (CPP Paris Cochin, January 2007).

### Mutation analysis

PCR and sequencing (SEQ) primers corresponding to *IDH1* exon 4, which encodes codon 132, and *IDH2* exon 4, which encodes codon 172, were designed: *IDH1*, forward (PCR), CTCCTGATGAGAAGAGGGTTG; reverse (PCR), TGGAAATTTCTGGGCCATG; and forward (SEQ), GGCACGGTCTTCAGAGAAGC; reverse (SEQ), TGCAAAATCACATTATTGCC; *IDH2*, forward (PCR), TGGAACTATCCGGAACATCC; reverse (PCR), AGTCTGTGGCCTTGTACTGC;

and forward (SEQ), ACATCCTGGGGGGGACTGTC; and reverse (SEQ), GACAAGAGGATGGCTAGGCG. PCR was performed in 96-well formats in 15-_l reaction volumes containing 7.6 _l H2O, 3.0 _l 5X colorless Gotaq flexi buffer, 0.9_l25mMMgCl2,1_l each of forward and reverse primer, 0.3 _l deoxynucleotide triphosphates, and 0.2 _l 5 U/_l Gotaq (Promega, Madison, WI). PCR conditions were as follows: 35 cycles of 95 C for 30 sec, 58 C for 45 sec, and 72 C for 45 sec, followed by 72 C for 10 min. Cycle sequencing was performed using BigDye Terminator v3.1 sequencing kit, following the manufacturer's protocol. Sequencing products were analyzed on the 3130XL Genetic analyzer (Applied Biosystems, Foster City,CA). Sequence traces were analyzed using Mutation Surveyor software (SoftGenetics, State College, PA). In case an *IDH1* or *IDH2* mutation was found, the patient's germlineDNAwas isolated from formalin-fixed and paraffin-embedded tissue subsequently, and mutation analysis was performed using the same methods as described above for tumor DNA. Mutation analysis for *RET*, *VHL*, *SDHB*, *-C*, *-D*, and *-AF2* of this series was performed previously (8,10). NF1 was clinically determined. The absence of an *SDH* mutation was also checked by SDHB immunochemistry as described elsewhere (11).

**Results**

From all 365 tumors, reliable forward and reverse DNA sequencing results were obtained from the amplified fragments of both genes. No mutations in *IDH1* or *IDH2* were found in any of the 234 pheochromocytomas (31 inherited and 203 sporadic cases) or in the 65 *SDH*- or*VHL*-related paragangliomas analyzed.

Among the 66 sporadic paragangliomas (with no mutations identified in the paraganglioma-pheochromocytoma susceptibility genes and with positive SDHB immunostaining indicating absence of *SDH* gene mutations) (11), the IDH1 p.Arg132Cys mutation was detected in a single carotid paraganglioma diagnosed in a 61-yr-old woman. The presence of the mutation was confirmed by repeating the DNA extraction from the tumor and the direct sequencing of *IDH1*. The mutation appeared to be heterozygous, indicating that no loss of the *IDH1* wildtype allele in the tumor cells occurred. The mutation was absent in the patient's germline DNA (Fig. 1).

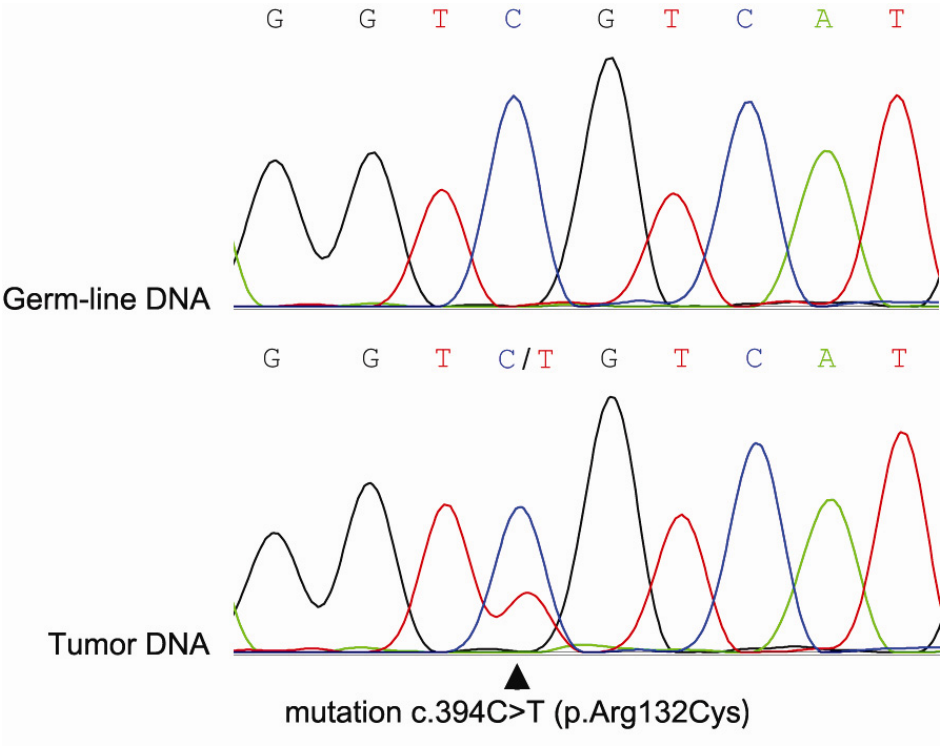


**Figure 1.** Sequence chromatogram of *IDH1.* Mutation c.394 C3T (p.Arg132Cys) is only seen in tumor DNA (*bottom*) and not in patient's germ-line DNA (*top*) (NM_005896).

Histologically, the appearance of this *IDH1*-mutated paraganglioma is similar to*SDH*-related paragangliomas. The *IDH1*-mutated paraganglioma was composed of nests of cells surrounded by an extensive fibrovascular network. The tumor cells demonstrated SDHB expression as determined by immunohistochemistry (Fig. 2).

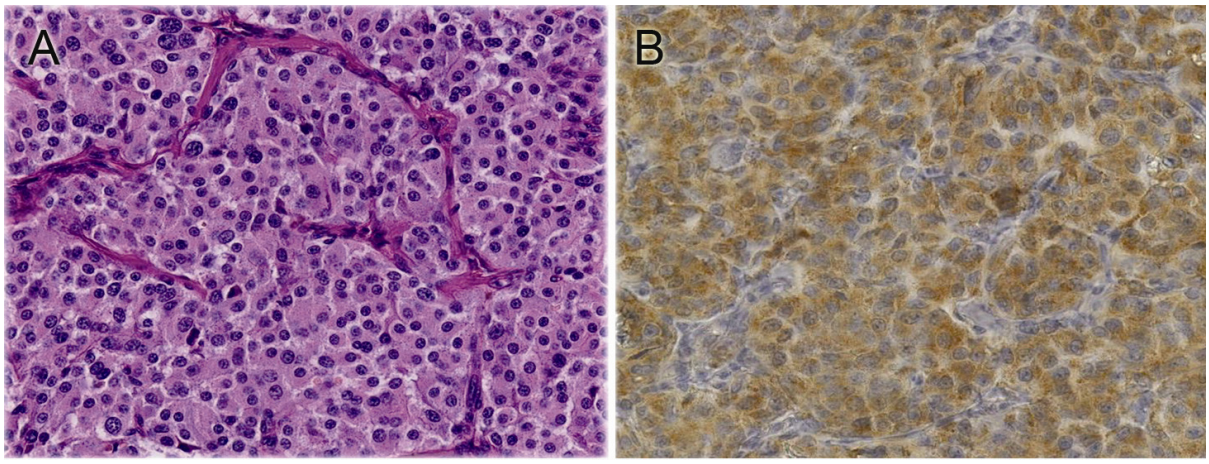


**Figure 2.** Microscopy of the *IDH1*-mutated paraganglioma.

A, Hematoxylin and eosin staining of paraganglioma composed of nests of tumor cells, separated by a fibrovascular stroma. B, SDHB immunohistochemistry, showing positive tumor cells.

An indium-111-pentetreotide scintigraphy was performed and revealed no other paraganglioma locations. Family history of paraganglioma or pheochromocytoma was negative. In addition, no glioblastoma multiforme, other glial tumors, or B-acute lymphoblastic leukemia were diagnosed in this patient.

**Discussion**

In the present study on 365 paragangliomas and pheochromocytomas, one somatic IDH1 p.Arg132Cys mutation in a sporadic carotid paraganglioma was found. In this tumor, no mutations in *SDHB*, *SDHC*, *SDHD*, *SDHAF2*, and *VHL* were found. In addition, the presence of SDHB protein expression in the tumor cells indicates lack of SDH involvement in this tumor (11).

The investigated DNA of the *IDH1*-mutated paragangliomas was isolated from a tissue fragment comprised of a high percentage (70%) of tumor cells. The heterozygous aspect of the sequence electropherogram indicated that there was no loss of the *IDH1* wild-type allele in the tumor cells. This result is in agreement with the previously described lack of loss of the wild-type *IDH* allele observed in mutated gliomas and the recent demonstration of a dominant negative effect of the p.Arg132His *IDH1* mutation(6).

To date there are six types of IDH1 mutations found at codon Arg132 (p.Arg132His, p.Arg132Cys, p.Arg132Ser, p.Arg132Gly, p.Arg132Leu, and p.Arg132Val). In IDH1-mutated glioblastoma multiforme, the most common mutation is p.Arg132His (88.2%) followed by p.Arg132Cys (4.3%) (12). Interestingly, p.Arg132Cys mutations in IDH1 have been reported to occur at higher frequencies in histological subtypes of glioma (13), in astrocytomas of Li-Fraumeni patients (14), and in patients with acute myeloid leukemia (15). In addition, an association has been suggested between the IDH1 p.Arg132Cys and mutations in the *P53* gene (14, 16). These results indicate that IDH1 p.Arg132Cys mutations, compared with the more frequent p.Arg132His mutations, are present in distinct histological and molecular (sub-)types of tumors. However, we obtained no evidence for the presence of a *P53* mutation in theIDH1p.Arg132Cys mutated paraganglioma by TP53 immunohistochemistry, *P53* exon 4-9 mutation analysis, and *P53* locus loss of heterozygosity analysis (results not shown).

Inactivation of *IDH*, *SDH*, fumarate hydratase (*FH*), *or VHL* genes causes different types of cancer such as glioblastoma multiforme, paraganglioma/pheochromocytoma, renal cell carcinoma, or hemangioblastomas, respectively (12, 17, 18). Despite this clinical heterogeneity, there is evidence for shared mechanisms of tumorigenesis. *IDH*, *SDH*, and *FH* genes all encode mitochondrial metabolic enzymes. Mutational inactivation of either of these genes leads to the inhibition of prolylhydroxylase activity via either the accumulation of Krebs cycle organic acids, such as succinate and fumarate (19), or by the reduction of α-ketoglutarate levels (6). Inhibition of prolylhydroxylase leads to HIF stabilization and, as a consequence, to activation of the hypoxia inducible-angiogenesis pathway. In tumors caused by *VHL* gene mutations, HIF is also stabilized because the mutantVHLprotein is not able to exert its normal function in HIF ubiquitination (20). Although inactivation of VHL and SDHB/D may disrupt similar HIF-dependent and HIF-independent signaling pathways, their effects on target gene expression and on glycolysis are not identical (21). Also, there are many other oxygenases dependent on α-ketoglutarate, and therefore other tumor mechanisms besides HIF could be responsible for tumorigenesis in *IDH*-mutated tumors.

Finally, our data were quite surprising and suggest that hitherto unknown tissue-specific mechanisms would explain an occurrence of neural tumors due to dominant negative heterozygous mutations in genes encoding for isocitrate dehydrogenases and of paragangliomas caused by recessive mutations in genes encoding for SDHs.

The somatic IDH1 p.Arg132Cys mutation in the sporadic paraganglioma has probably played a critical role in the tumorigenesis, but our results demonstrate that mutations at codon Arg132 in IDH1 and at codon Arg172 in IDH2 in paragangliomas and pheochromocytomas are infrequent.

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# CHAPTER 7

**_SDHAF2_ (PGL2) mutations in paraganglioma and pheochromocytoma**

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**Abstract**

**Background:** Paragangliomas and phaeochromocytomas are neuroendocrine tumours associated frequently with germline mutations of SDHD, SDHC, and SDHB. Previous studies have shown the imprinted SDHAF2 gene to be mutated in a large Dutch kindred with paragangliomas. We aimed to identify SDHAF2 mutation carriers, assess the clinical genetic significance of SDHAF2, and describe the associated clinical phenotype.

**Methods:** We undertook a multicentre study in Spain and the Netherlands in 443 apparently sporadic patients with paragangliomas and phaeochromocytomas who did not have mutations in SDHD, SDHC, or SDHB. We analysed DNA of 315 patients for germline mutations of SDHAF2; a subset (n=200) was investigated for gross gene deletions. DNA from a group of 128 tumours was studied for somatic mutations. We also examined a Spanish family with head and neck paragangliomas with a young age of onset for the presence of SDHAF2 mutations, undertook haplotype analysis in this kindred, and assessed their clinical phenotype.

**Findings:** We did not identify any germline or somatic mutations of SDHAF2, and no gross gene deletions were noted in the subset of apparently sporadic patients analysed. Investigation of the Spanish family identified a pathogenic germline DNA mutation of SDHAF2, 232G-->A (Gly78Arg), identical to the Dutch kindred.

**Interpretation:** SDHAF2 mutations do not have an important role in phaeochromocytoma and are rare in head and neck paraganglioma. Identification of a second family with the Gly78Arg mutation suggests that this is a crucial residue for the function of SDHAF2. We conclude that SDHAF2 mutation analysis is justified in very young patients with isolated head and neck paraganglioma without mutations in SDHD, SDHC, or SDHB, and in individuals with familial antecedents who are negative for mutations in all other risk genes.

## Introduction

Paragangliomas of the head and neck are generally benign tumours that can give rise to substantial morbidity due to compromised function of major blood vessels and cranial nerves of the neck and skull base. Phaeochromocytomas are related closely to these cancers, sharing the neuroectodermal origin of the parasympathetic paragangliomas, but they affect the adrenal medulla and—as sympathetic paragangliomas—intra-abdominal and thoracic paraganglia. Clinical presentation of phaeochromocytomas is usually accompanied by hypertension, sweating, and palpitations due to tumour-derived catecholamine excess. Phaeochromocytomas can be aggressive and metastatic, especially in cases of extra-adrenal localisation.

Germ line mutations in genes encoding succinate dehydrogenase—*SDHD, SDHC*, or *SDHB* (formerly known as *PGL1, PGL3*, and *PGL4*)—are a frequent cause of paragangliomas of the head and neck and phaeochromocytomas, accounting for 30–54% of all cases. (1 and 2) The major catalytic subunit of the succinate dehydrogenase complex, SDHA, has not been linked to these tumours. Succinate dehydrogenase has a central role in cellular energy metabolism as both a mitochondrial tricarboxylic acid (TCA) cycle enzyme and as the complex II component of the electron transport chain. Since identification of *SDHD* in 2000, (3) the role of TCA enzymes as tumour suppressors has broadened, with mutations of fumarate hydratase reported as the cause of hereditary leiomyomatosis and renal-cell cancer, (4) and somatic mutations of isocitrate dehydrogenase genes noted in glioblastoma. (5)The *SDHAF2* gene (formerly known as *PGL2* or *SDH5*) encodes succinate dehydrogenase complex assembly factor 2 (SDHAF2), a highly evolutionarily conserved cofactor of flavin adenine dinucleotide (FAD). (6) SDHAF2 has a role in flavination of SDHA, and correct flavination of this subunit is essential for a fully functional succinate dehydrogenase complex. Loss of SDHAF2 results in loss-of-function of succinate dehydrogenase and a reduction in stability of the enzyme complex, leading to diminished amounts of all subunits.[6]

In a large Dutch kindred with paragangliomas of the head and neck, (6) *SDHAF2* carried a missense cDNA mutation, 232G→A (Gly78Arg), in a conserved region, resulting in complete loss of both flavination of SDHA and activity of the succinate dehydrogenase complex. This *SDHAF2* gene mutation also showed a striking parent-of-origin expression phenotype, with onset of tumour development only on inheritance via the paternal line. Kindreds containing several family members with head and neck paraganglioma without identified mutations in known susceptibility genes are scarce. However, we have identified a Spanish family in which all three daughters presented with paragangliomas of the head and neck at a young age.

In the first part of this multicentre study, we sought to assess the role of mutations of *SDHAF2* in a large cohort of patients with paragangliomas of the head and neck and phaeochromocytomas and to ascertain the proportion of these tumours that can be accounted for by mutation of *SDHAF2*. In the second part of this study, our objective was to ascertain whether the cancers in the Spanish family are attributable to mutations of *SDHAF2*. Moreover, by haplotype analysis both of this family and of the Dutch kindred described previously, (6) we aimed to assess the level of relatedness between the two families. Here, we use head and neck paraganglioma to describe paragangliomas with locations in the neck—including the carotid body, the vagal body, and jugulotympanic regions—and any other area of the head or neck. We use phaeochromocytoma to describe tumours of the adrenal medulla and of sympathetic paraganglia of the abdomen and thorax.

## Methods

### Patients

We selected patients with paragangliomas of the head and neck and phaeochromocytomas who we had screened previously for mutations of succinate dehydrogenase subunits (*SDHD, SDHC*, and *SDHB*). In most cases, full

deletion analysis had also been undertaken. Since not all patients were screened exhaustively for all relevant enzyme subunits, a small proportion (2·5%) could have carried a succinate dehydrogenase gene mutation or deletion. This estimate is based on known frequencies of mutations and deletions and the number of individuals with incomplete screening. (1 and 2) All patients were index cases with no known familial antecedents, and therefore they were diagnosed with cancer with an apparently sporadic presentation.

We obtained written informed consent from all patients for DNA testing according to protocols approved by local ethics committees for every participating centre. Tumour samples were investigated anonymously at the Department of Pathology, Erasmus MC, Rotterdam, Netherlands, according to the code of conduct—Proper Secondary Use of Human Tissue—established by the Dutch Federation of Medical Scientific Societies. We obtained written informed consent from every living individual represented in the pedigree, which has not been modified.

**Procedures**

We obtained germ line DNA extracted from whole blood by standard techniques. DNA for somatic analysis was obtained from resected tumour tissue samples, which were fixed in formalin and embedded in paraffin.

We sequenced all four coding exons of the *SDHAF2* gene, undertook denaturing high-performance liquid chromatography (dHPLC), and did multiplex-PCR deletion analysis, as described previously, (7) with specifically designed primers (details available on request). When initial screening of one cohort by dHPLC for the specific *SDHAF2* Gly78Arg mutation indicated no new mutation carriers, we did full sequencing of all four exons of *SDHAF2* in every patient. We established population frequencies of potentially pathogenic variants—identified in either the Netherlands or Spain—in panels of 200–300 healthy blood donors from the appropriate country. We undertook deletion analysis in patients selected on the basis of availability of sufficient high-quality DNA. For mutation analysis of tumour

material, we isolated DNA from areas with at least 80% tumour cells. Owing to the short fragment length of DNA retrieved from tumours available as routine formalin-fixed and paraffin-embedded specimens, we redesigned PCR primers to allow amplification of small fragments (details available on request). Because tumour samples generally contain fully normal germ line DNA, we regard these samples as part of the general cohort, while they also serve to address the role of somatic mutations of *SDHAF2*. Mutation nomenclature follows Human Genome Variation Society guidelines.

For family analysis and haplotyping, we undertook sequencing of *SDHAF2* and did dHPLC as described above. The Dutch patients who underwent haplotyping were from the kindred described previously.(6, 8, 9 and 10) We did microsatellite haplotype analysis of the chromosome 11q13 region by standard protocols, with informative markers.

**Statistical analysis**

We analysed data with SoftGenetics package Gene Marker 1.6 (SoftGenetics, State College, PA, USA) and Cyrillic (Cyrillic Software, Wallingford, UK). We processed 610-Quad Beadchips (Illumina, Eindhoven, Netherlands) according to the manufacturer's recommendations, and we analysed findings with the Beadstudio package (Illumina), PLINK, (11) and Haploview. (12) We calculated haplotype frequencies with fastPHASE (13) in HapMap populations and 866 Dutch individuals. The Human610-Quad BeadChip array (Illumina) we used in this analysis uses tagging single-nucleotide polymorphisms (SNPs). These provide coverage of 89% in a European population at an $r^2$ of 0·8. Haplotypes were formed with informative SNPs between rs545230 and rs7947046, shared between the Spanish and Dutch families and the control population of 866 Dutch individuals, all typed on the same array. The HapMap population data were based on Illumina HumanHap650K Beadchips, and SNPs common to both platforms were used to derive haplotypes.

**Role of the funding source**

The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, writing of the report, or in the decision to submit for publication. JPB, HPMK, AC, MLS, JS, EK, WD, AHG, HJLMT, LHH, AKH, and MR had access to raw data. The funding source had no access to raw data. The corresponding author had full access to all data and had final responsibility for the decision to submit for publication.

**Results**

To undertake efficient mutation scanning, we brought together patients in whom mutations of the *SDHB, SDHC*, and *SDHD* genes had previously been excluded partly or entirely. We analysed 315 individuals for germline mutations of *SDHAF2* in genomic DNA, and 200 of these were further analysed for gross gene deletions (123 Spanish and 77 Dutch patients). An additional 100 phaeochromocytoma and 28 head and neck paraganglioma patients from the Netherlands (for whom tumour DNA was available) were analysed for somatic mutations. This strategy allowed us to undertake comprehensive assessment of 443 patients for point mutations, gross deletions, and somatic mutations of *SDHAF2*. Table 1 contains details of the mutation analysis for all patients, and table 2 presents a clinical summary of the patient cohorts.

**Table 1.** Diagnosis and previous genetic screening of patient cohorts

| | Type of DNA | Paraganglioma of head and neck (n=201) | Phaeochromocytoma (n=242) | Sequencing | | | Deletion analysis | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | SDHB | SDHC | SDHD | SDHB | SDHC | SDHD |
| Leiden 1 | Genomic | 42 | 9 | Y | Y | Y | Y | Y | Y |
| Leiden 2 | Genomic | 6 | 4 | Y | N | Y | Y | Y | Y |
| Leiden 3 | Genomic* | 46 | 0 | Y | Y | Y | Y | Y | Y |
| Oviedo | Genomic | 48 | 1 | Y | Y | Y | Y | Y | Y |
| Madrid† | Genomic | 11 | 112 | Y | Y | Y | Y | Y | Y |
| Rotterdam | Tumour‡ | 28 | 100 | Y | Y | Y | N | N | N |
| Nijmegen 1a | Genomic | 0 | 7 | Y | Y | Y | 4§ | Y | Y |
| Nijmegen 1b | Genomic | 0 | 9 | Y | Y | 4§ | N | N | N |
| Nijmegen 2a | Genomic | 3 | 0 | Y | Y | Y | N | N | N |
| Nijmegen 2b | Genomic | 17 | 0 | N | N | Y | N | N | N |

Y=analysis done. N=analysis not done. Cohorts are defined by centre of origin and the common genetic testing regimen. * 24 samples originated in Nijmegen. † *RET* and *VHL* mutations also excluded in patients with adrenal phaeochromocytoma (n=97). ‡ 27 samples originated in Nijmegen. § Number of patients analysed.

**Table 2.** Clinical summary of patient cohorts

| | Phaeochromocytoma (n=219) | Extra-adrenal paraganglioma (n=23) | HN-PGL: location | | | | Diagnosis | |
|---|---|---|---|---|---|---|---|---|
| | | | Carotid body (n=103)* | Vagal body (n=19)* | Jugulotympanic (n=100)* | Other location/not specified (n=9) | Radiological (n=98) | Histological (n=338) |
| Leiden | 8 | 5 | 65 | 13 | 43 | 7 | 54 | 81 |
| Rotterdam | 97 | 3 | 16 | 3 | 12 | 0 | 0 | 128 |
| Nijmegen | 16 | 0 | 6 | 0 | 16 | 0 | 21 | 16 |
| Madrid | 97 | 15 | 6 | 0 | 3 | 2 | 8 | 115 |
| Oviedo | 1 | 0 | 10 | 3 | 26 | 0 | 16 | 23 |

* Numbers of diagnoses and tumours do not necessarily correspond owing to the occurrence of several tumours in patients with paragangliomas of the head and neck (HN-PGL).

Although all currently affected *SDHAF2* mutation carriers have head and neck paraganglioma, (6) mutations of *SDHAF2* might also give rise to pheochromocytoma. 242 cases of phaeochromocytoma were available and were analysed for germline mutations of *SDHAF2* by sequencing. No mutations were identified in any case of phaeochromocytoma. 201 cases of head and neck paraganglioma were also available for screening. All head and neck paraganglioma cases were found to be negative for germline mutations of *SDHAF2*.

Three Dutch patients carried a variant in a poorly conserved region of the 3'untranslated region of the SDHAF2 gene (*12C>T), close to exon 4 (table 3). The presence of this variant in healthy blood donors (4/204) indicates that it is not specific to paragangliomas but is a rare non-pathogenic polymorphism. Part or whole gene deletions arise at a rate of around 5% in succinate dehydrogenase genes. (1 and 14) We screened 200 patients for large deletions affecting SDHAF2 by multiplex-PCR, which would allow detection of deletions at an allele frequency of 1/400 (0·25%). Although we could have detected deletions at a fairly low frequency, none was identified.

**Table 3.** Summary of identified mutations and polymorphisms

| | Designation | cDNA | Protein | dbSNP | Allele frequency |
|---|---|---|---|---|---|
| HN-PGL | Mutation | 232G>A | Gly78Arg | 184955586 | 0·00 |
| PC | SNP | 139A>G | Met47Val | 184955585 | 0·00 |
| HN-PGL | SNP | *12C>T | – | 184955589 | 0·01 |
| HN-PGL | SNP | 261–42G>A | – | rs879647 | ND |
| HN-PGL | SNP | 260+23T>C | – | 184955584 | ND |
| PC | SNP | *8T>C | – | 184955590 | ND |
| PC | SNP | 192A>G | – | 184955588 | ND |
| PC | SNP | 260+11A>G | – | 184955587 | ND |

HN-PGL=paraganglioma of the head and neck. PC=phaeochromocytoma. SNP=single-nucleotide polymorphism. ND=not done.

Somatic mutations are rare in genes of succinate dehydrogenase subunits, but some have been described. (15 and 16) Sequence analysis of 128 available samples identified one

variant, 139A>G (Met47Val), in exon 2 of *SDHAF2* in both a phaeochromocytoma and in germline DNA of the same patient, indicating that this change was not a somatic mutation (table 3). The variant is rare since it was not identified in 200 healthy blood donors. Methionine and valine are both aminoacids with non-polar side chains, and the methionine 47 residue is poorly conserved across species eg, valine is the usual aminoacid at this position in yeast. Data of SIFT (17) and PolyPhen (18) analyses both suggest that this mutation is non-pathogenic. No further variants were detected.In the mid 1990s, a Spanish family with a young age of onset of paraganglioma came to our attention (figure 1). In 1994, a 20-year-old woman (III:1) received an initial diagnosis of goitre with dyspnoea. Imaging showed a mass in the thyroid, and after complete surgical removal and histopathological analysis of the tumour, the diagnosis was amended to intrathyroid paraganglioma. She subsequently developed bilateral paragangliomas of the carotid body, removed in 1999 and 2005. The patient is currently disease-free at age 35 years. In 1999, a left carotid body tumour was detected in this woman's 23-year-old sister (III:2). After preoperative embolisation and surgical removal of the cancer, histopathological analysis confirmed the diagnosis. In 2001, a right-sided carotid body tumour was diagnosed and removed. Then in 2004, two new cervical masses were detected, morphologically compatible with vagal paragangliomas. Because of the location, surgical removal was not possible, and the tumours were treated with radiotherapy. The patient is being followed-up annually and, currently, no further tumour growth has been seen. A third sister (III:3) was diagnosed with a jugulotympanic paraganglioma in 2004, at age 21 years. The tumour has not been operated on and she is currently seen every 6 months. Finally, in 2007, the proband's father (II:1) was diagnosed with bilateral carotid paraganglioma by MRI and arteriography, at age 59 years. He is clinically asymptomatic and has not undergone surgery to date.

All family members had previously tested negative for both mutations and deletions of succinate dehydrogenase genes. The exclusive head and neck paraganglioma phenotype of this family indicated a possible link to the *SDHAF2* gene. Sequencing of the gene in generations II and III led to identification of a pathogenic mutation in exon 2, 232G>A (Gly78Arg), identical to that described previously in the Dutch *SDHAF2* kindred. (6, 8-10) The mutation cosegregated with disease and was inherited via the male line (figure 1).

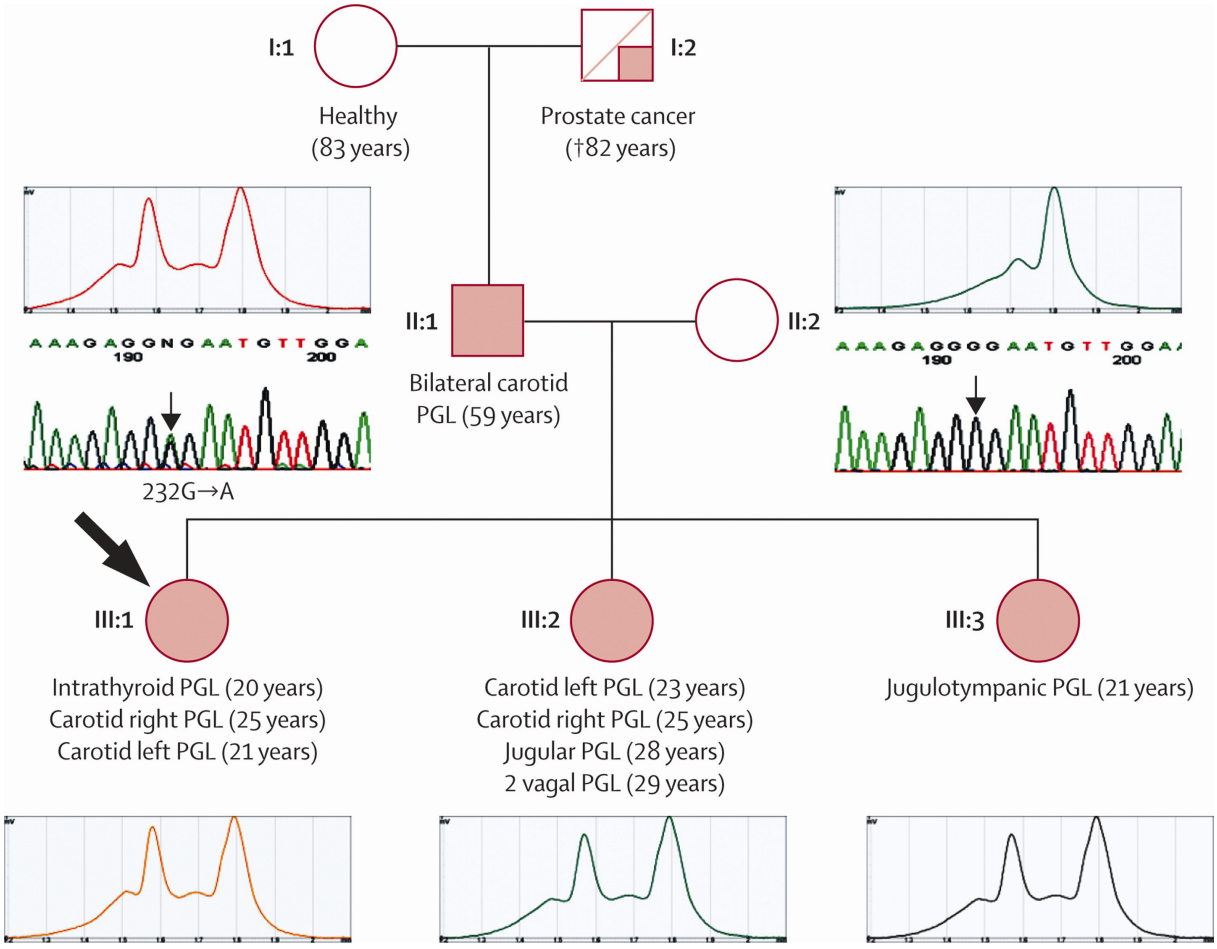


**Figure 1. Pedigree of the Spanish family**

The proband is indicated by the large black arrow. dHPLC chromatograms corresponding to wild-type and mutated sequence are shown, together with sequencing profiles of the parents. The cDNA mutation, (232G>A) is indicated by the small arrow. dHPLC results were confirmed in the daughters by sequence analysis. Ages of onset are in parentheses. PGL=paraganglioma.

Identification of an identical mutation within these two families indicated a possible mutual ancestor, so we used haplotype analysis to ascertain whether they shared a common genetic origin. A shared rare haplotype surrounding the mutation would suggest a single founder mutation, identical by descent, whereas a mutation on a shared frequent haplotype would suggest chance concurrence, if unsupported by further genetic or historical evidence of relatedness. Initial haplotyping with microsatellite markers was inconclusive (figure 2A), so we increased resolution with a high-density SNP array of 610 000 SNPs. Ten patients from the Dutch kindred and all five members of the

Spanish family were analysed and found to share a 400 kb haplotype that included the *SDHAF2* mutation (figure 2B).

**A**

| | | Spanish family | | | | | | | | | | Dutch family |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Marker | Physical location | II:1 | | II:2 | | III:1 | | III:2 | | III:3 | | |
| D11S4174 | 45214592 | 276 | 276 | 278 | 278 | 276 | 278 | 276 | 278 | 276 | 278 | 276/282 |
| D11S4109 | 47744219 | 186 | 194 | 194 | 194 | 186 | 194 | 186 | 194 | 186 | 194 | 186/196 |
| D11S1983 | 58201625 | 214 | 250 | 222 | 242 | 214 | 222 | 214 | 222 | 214 | 242 | 223/231 |
| D11S956 | 58252793 | 251 | 251 | 257 | 255 | 251 | 257 | 251 | 257 | 251 | 255 | 251/255 |
| D11S1765 | 60535120 | 231 | 241 | 249 | 235 | 231 | 249 | 231 | 249 | 231 | 235 | 235 |
| D11S4076 | 61119670 | 150 | 154 | 156 | 148 | 150 | 156 | 150 | 156 | 150 | 148 | 157 |
| D11S1883 | 63130299 | 247 | 247 | 253 | 251 | 247 | 253 | 247 | 253 | 247 | 251 | 247 |
| D11S913 | 65692736 | 218 | 218 | 220 | 222 | 218 | 220 | 218 | 220 | 218 | 220 | ND |
| D11S4136 | 69324892 | 187 | 185 | 177 | 183 | 187 | 177 | 187 | 177 | 187 | 177 | 183/185 |

**B**

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Spanish family | A | C | A | A | G | G | G | G | A | G | G | A | G | A | A | C | C | A | A | C | A | A | A | G | G | A | A | A | A | G | A |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 |
| Dutch family | A | C | A | A | G | G | G | G | A | G | G | A | G | A | A | C | C | A | A | C | A | A | A | G | G | A | A | A | A | G | A |
| | | | | | | | | | | | | | | | | | | | | | | | | | | Mutation | | | | | |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 |
| Dutch population | A | C | A | A | G | G | G | G | A | G | G | A | G | A | A | C | C | A | A | C | A | A | A | G | G | A | A | A | G | A |

**Figure 2.** Microsatellite haplotype analysis of chromosome 11 region

(A) In the Spanish family, the haplotype indicated in dark red is shared by all affected members. In the Dutch kindred, markers with two alleles indicated are discordant between patients, showing the border of the disease haplotype. The marker allele sizes shaded in dark red are the alleles for these markers which are shared by all patients and are therefore the disease haplotype (ND=not done). The *SDHAF2* gene is located between markers D11S1765 and D11S4076. (B) The shared 400 kb haplotype consisted of 30 SNPs (from 1 [rs545230] to 30 [rs7947046]). The location of the mutation is between SNP 26 and 27 and is indicated by a dark red shaded "A".

We calculated the frequency of this haplotype in a dataset of 866 individuals of Dutch origin typed with the same array and in three southern European populations (Spanish [Basque region], French, and Italian) from the HGDP-CEPH (Human Genome Diversity Project–Centre d'Etude du Polymorphisme Humain) panel.[19] These datasets show that the relevant haplotype is the most common in both the Dutch (19%) and southern

European populations (17–22%), thus providing little evidence for relatedness of the two families. Analysis of genome-wide SNPs with the identity-by-state binomial test (pairwise population concordance in PLINK) showed that members of the Spanish family were no more closely related to the Dutch kindred than to 15 randomly selected Dutch controls (data not shown).

**Discussion**

More than 290 separate mutations of the paraganglioma-related succinate dehydrogenase genes have been described. (20) In analyses of patients with paragangliomas of the head and neck and phaeochromocytomas, 30–54% were reported to carry a mutation. (1 and 2) Our analysis did not record any germline or somatic mutations, or gross germline deletions, of the *SDHAF2* gene in patients with paragangliomas of the head and neck and phaeochromocytomas. Pedigree analysis of a Spanish family with paragangliomas of the head and neck showed a pathogenic mutation in *SDHAF2* leading to an aminoacid substitution (Gly78Arg), which was identical to a variant noted previously in a Dutch kindred.

The absence of additional Gly78Arg mutation carriers and other mutations of the *SDHAF2* gene in our series is remarkable, especially since cohorts were drawn from the same populations as the two kindreds. The rarity of *SDHAF2* mutations is puzzling in view of the important role of the other subunits of succinate dehydrogenase, and could be related to specific constraints on the protein or to particular interactions with SDHA. Although the current analysis includes only two European populations, the known distribution of mutations of succinate dehydrogenase suggests that the frequency of *SDHAF2* mutations will not be significantly higher in other populations.

Our analysis of germline DNA for mutations of *SDHAF2* indicates that germline mutations have a very limited role in initiation of paragangliomas of the head and neck and phaeochromocytomas. The role of somatic mutation of genes of TCA-cycle enzymes has been established firmly, with identification of very frequent somatic mutations of isocitrate dehydrogenase genes in gliobastoma. (5) However, somatic mutations are rare

in succinate dehydrogenase subunit genes. (15 and 16) Our sequencing analysis of this paraganglioma-related gene was very extensive, since no similar study of somatic mutations in the *SDHD, SDHC*, and *SDHB* genes has been published to date. The absence of any germline deletions in genomic DNA and point mutations in tumour DNA led us to conclude that tumours were very unlikely to carry specific gross deletions and, therefore, these were not analysed. Studies of tumour DNA for regions showing loss of heterozygosity might indicate the presence of cryptic mutations, but because many regions in phaeochromocytomas and paragangliomas are known to show loss of heterozygosity in the absence of mutations in candidate genes, this approach was not pursued.

The point variant identified in cDNA of tumours, 139A→G (Met47Val), was not noted in 200 healthy blood donors, indicating that it is not a common polymorphism. No indication of loss of the wild-type allele was recorded (data not shown). The nature of the aminoacid change and the fact that the methionine 47 residue is poorly conserved led us to conclude that this variant is non-pathogenic.

The Spanish family described here is the second currently known to be linked to the *SDHAF2* gene, and follows the description of a loss-of-function mutation in the *SDHAF2* gene in a large Dutch kindred with paragangliomas of the head and neck. (6) No known family history suggests a link between these two kindreds, and haplotype analysis produced no clear evidence of relatedness. These data indicate that Gly78Arg is probably a recurrent, rather than a founder, mutation. Identification of the Gly78Arg mutation for a second time suggests that this residue is important to the function of SDHAF2; this area has yet to be investigated.

The phenotype of the Spanish family is very similar to the Dutch kindred, with all affected patients having paragangliomas of the head and neck, (6) and currently with no known occurrence of phaeochromocytomas. All paraganglioma-related SDH subunit genes have been associated with phaeochromocytomas. (16, 21-23) Mutations of *SDHC* and *SDHD* genes, which encode transmembrane subunits, result in a greater frequency of paragangliomas of the head and neck, whereas the only catalytic subunit gene related to

paragangliomas of the head and neck and phaeochromocytomas (*SDHB*) leads most typically to phaeochromocytoma. Why should mutation of SDHAF2, a protein associated with a catalytic subunit, result in a phenotype most usually associated with transmembrane subunits? Although mutation analysis of study cohorts included just over 240 cases of phaeochromocytoma, no changes in *SDHAF2* were identified, suggesting that if mutation of *SDHAF2* can lead to development of phaeochromocytoma, it must be a rare event.

Identification of an interaction between SDHAF2 and SDHA was unexpected, because mutations of *SDHA* have only previously been reported in Leigh syndrome, (24) a genetically and phenotypically heterogeneous mitochondrial deficiency disorder. SDHA is the only subunit of succinate dehydrogenase that has not been linked to paragangliomas of the head and neck and phaeochromocytomas, but since the *SDHAF2* gene was reported, *SDHA* mutation screening should now be considered in families of patients with paragangliomas of the head and neck when all other genes have been excluded.

The *SDHAF2* gene mutation shows an imprinted or parent-of-origin expression phenotype, with tumour development inherited paternally, as seen previously in *SDHD*-related paraganglioma. (6 and 25) The mutation in the Spanish family was inherited via the father, although the size of this family precludes any confirmation of the expression phenotype in the Dutch kindred. Current evidence suggests that the parent-of-origin expression phenotype cannot be accounted for by a simple imprinted-gene hypothesis.[3] An alternative mechanism has been proposed, which includes an additional tumour-modifier gene that is itself imprinted. (26)

The Dutch *SDHAF2* kindred shows a highly penetrant phenotype, (6) which is seen broadly in the Spanish family described here. The imprinted phenotype, and consequent possibility of maternal transmission obscuring familial antecedents, should be taken into account when considering genetic screening. This will be counterbalanced by the highly penetrant phenotype in most mutation carriers. Therefore, one can reasonably expect there may be clear familial antecedents where patients have any knowledge of family medical history.

In conclusion, our findings suggest that neither germline nor somatic *SDHAF2* mutations lead to development of phaeochromocytoma. Mutation of *SDHAF2* is a rare cause of head and neck paraganglioma. Genetic analysis of *SDHAF2* should be considered in head and neck paraganglioma patients with familial antecedents, and in individuals with a young age of onset and no mutations in *SDHD, SDHC*, or *SDHB*. However, ease and cost of screening might help to decide whether this gene is included with current genes in routine clinical diagnostic screening of cases of paragangliomas of the head and neck and phaeochromocytomas.

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# CHAPTER 8

## NO EVIDENT ROLE FOR SDHAF1 MUTATIONS IN PARAGANGLIOMAS AND PHEOCHROMOCYTOMAS

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**Abstract**

Functional deficiency of the mitochondrial complex II contributes to paraganglioma (PGL) and pheochromocytoma (PCC) development. The PGL-PCC syndrome, a condition characterized by the nearly exclusive development of PGL and PCC, is caused by germline inactivating mutations in the genes encoding the complex II subunits succinate dehydrogenase (SDH) B, C or D. Recently, germline mutations in two other complex II associated genes, *SDHA* and *SDHAF2*, were found associated with PGL and PCC. In addition, biallelic germline mutations in the *SDHAF2*-related gene *SDHAF1* have recently been described in two families with mitochondrial complex II deficiency. These results indicate that inactivating *SDHAF1* mutations lead to complex II deficiency and as such potentially can contribute to PGL and PCC development. Therefore, mutation analysis was performed with direct sequencing of the coding region of the *SDHAF1* gene in 38 PGL and 86 PCC. No *SDHAF1* mutations were found in these tumors. Our results indicate that although *SDHAF1* inactivation leads to complex II deficiency, *SDHAF1* mutations do not seem to play a role in the pathogenesis of PGL and PCC.

## Introduction

A recent study reported biallelic germline mutations in *succinate dehydrogenase assembly factor 1* (*SDHAF1*), causing mitochondrial complex II deficiency and leading to infantile leukoencephalopathy (1). This study was the first to describe SDHAF1 as a protein targeted to the mitochondria and associated with the succinate dehydrogenase enzyme complex (SDH, or complex II). SDHAF1 appeared to be essential for SDH biogenesis, although without physical association with complex II in vitro.

Complex II deficiency is also known as a cause of the development of pheochromocytomas (PCC) and paragangliomas (PGL). For a decade, it is known that germline inactivating mutations in three out of four complex II subunit genes (*SDHB*, *SDHC*, and *SDHD*) cause the hereditary PCC-PGL syndrome. Recently, this syndrome has also been linked to germline mutations in the *SDHAF1*-related gene *SDHAF2* (2). Even more recently, a germline mutation in the *SDHA* gene has been linked to PGL (3).

The PCC-PGL syndrome presents almost exclusively PGL and PCC, with the rare exception of the occurrence of gastrointestinal stromal tumors and renal cell carcinomas (4-9). The complex II related genes *SDHA, SDHB*, *SDHC, SDHD*, and *SDHAF2* are bona fide tumor suppressor genes with PGL and PCC generation after biallelic inactivation, generally by the combination of an inactivating germline mutation in one of the genes and a somatic "second hit" in the corresponding wildtype allele, leading to complete abolishment of complex II enzyme activity.

PGL and PCC are neuroendocrine tumors that occur along the sympathetic chain and are histologically indistinguishable. Parasympathetic PGL (pPGL) are found in the head and neck region and are usually biochemically silent, whereas extra-adrenal sympathetic PGL (sPGL) occur in the abdomen and usually produce catecholamines. PCC are tumors that arise in the adrenal medulla and also produce catecholamines (10). PCC and PGL occur also in other hereditary syndromes, such as the Multiple Endocrine Neoplasia 2 syndrome (MEN2, caused by mutations in *RET*), Von Hippel Lindau disease (VHL, caused by mutations in *VHL*), and neurofibromatosis type 1 (NF1, caused by mutations in *NF1*). Mutations in these genes, including the *SDH*-genes, occur in approximately 50% of the PGL (11) and 25% of the PCC (12, 13), whereas the remaining tumors are considered sporadic.

Recently, mutations in succinate dehydrogenase assembly factor 1 (*SDHAF1*) were found in two families, of whom affected members show severely decreased SDH-activity. Also, the affected individuals suffered from infantile leukoencephalopathy, having psychomotor regression as the major symptom, which is also described in patients with homozygous (14) or compound heterozygous *SDHA* mutations (15). Because the *SDHA* gene has now been associated with PGL and there is a strong correlation between complex II deficiency and development of PGL and PCC we hypothesized that *SDHAF1* mutations, could be responsible for a subpopulation of PCC and PGL. Therefore, we performed mutation analysis of the entire coding region of *SDHAF1* in a series of 38 PGL and 86 PCC, including 34 and 58 sporadic tumors, respectively.

## Materials and Methods

### *Patients*

One hundred and twenty-four tumors were selected from the archives of the Department of Pathology of the Erasmus MC University Medical Center, Rotterdam, the Radboud University Medical Center Nijmegen, the Netherlands, and the University Hospital Zürich, Switzerland. The tumors were anonymously used according to the code for adequate secondary use of tissue, code of conduct: "Proper Secondary Use of Human Tissue" established by the Dutch Federation of Medical Scientific Societies (http://www.federa.org). The series of 38 PGL consisted of 32 tumors located in the head and neck (PGL), 5 tumors in the abdomen (sPGL), and 1 sPGL metastasis. In addition, the PGL series included 4 *SDHD*-related tumors, and 34 PGL that occurred sporadically which included 3 tumors without SDHB expression and without an identified *SDH*-gene mutation as previously reported (16). Furthermore, the investigated series consisted of 86 PCC, of which 58 occurred sporadically and 28 had a mutation in *RET* (n=10), *VHL* (n=8), *SDHB* (n=2), *SDHD* (n=4), or *NF1* (n=4). The 4 NF1 patients and 1 of the MEN 2 patients were determined clinically. DNA was isolated from paraffin embedded tumor tissue and, when available, normal tissue using standard procedures previously reported (17).

### *SDHB immunohistochemistry*

Immunohistochemistry was performed as previously described (16) on tumors of which paraffin embedded material was available.

*Sequence analysis*

Sequence analysis was performed for the entire coding region of the *SDHAF1* gene. Because of short DNA fragment length in old paraffin blocks 5 PCR primer combinations were used that produced overlapping PCR products. PCR conditions were are previously reported (17) and primer sequences are listed in table 1.

**Table 1.** Primer sequences

| | **Forward 5'> 3'** | **Reverse 5'> 3'** |
|---|---|---|
| Part 1 | cgttcgctgagcgtctctg | atgctgccggaactctgc |
| Part 2 | ctgtaccgcgatctgctg | gtacaggtactcgatgcgcag |
| Part 3 | gagtgcgggcagagttcc | catggcggtggcgtg |
| Part 4 | tgcgcatcgagtacctgtacc | ggggttccttggactgtcg |
| *Part 5* | agctgcagctgctacgctc | gagccgaactcgctcgat |

**Results**

*Sequence Analysis*

Sequence analysis did not reveal *SDHAF1* mutations in 37 (s)PGL, 1 metastasis of sPGL, and 86 PCC. In 10 tumors a silent c.333C>G substitution (NCBI: NM_001042631: p.Arg111Arg) (Figure 1) was present. This DNA sequence variant was found in 3 PGL and 7 PCC, in 9 cases in a heterozygous and in 1 case in a homozygous fashion (frequency of variant G allele 11/248=4.4%; Supplementary Table 1). This variant was found both in the tumor and corresponding normal DNA samples. In the 9 c.333C>G heterozygous cases no indication for loss of heterozygosity (LOH) of the *SDHAF1* gene in these tumors was obtained from the DNA sequences (Figure 1). The c.333C>G variant appeared to be present at a comparable frequency (6.7%) in the DNA of 82 population-matched healthy individuals.

In addition, the *SDHAF1* sequence results of all 124 samples demonstrated deviation of the reference DNA (NCBI: NM_001042631) for polymorphism c.269C>G (p.Ser90Cys;

rs7249826), indicating that the reference genomic sequence contains an infrequent polymorphism. However, the SDHAF1 mRNA (NM_001042631.1) and protein (NM_001036096) sequences did not contain the polymorphism.

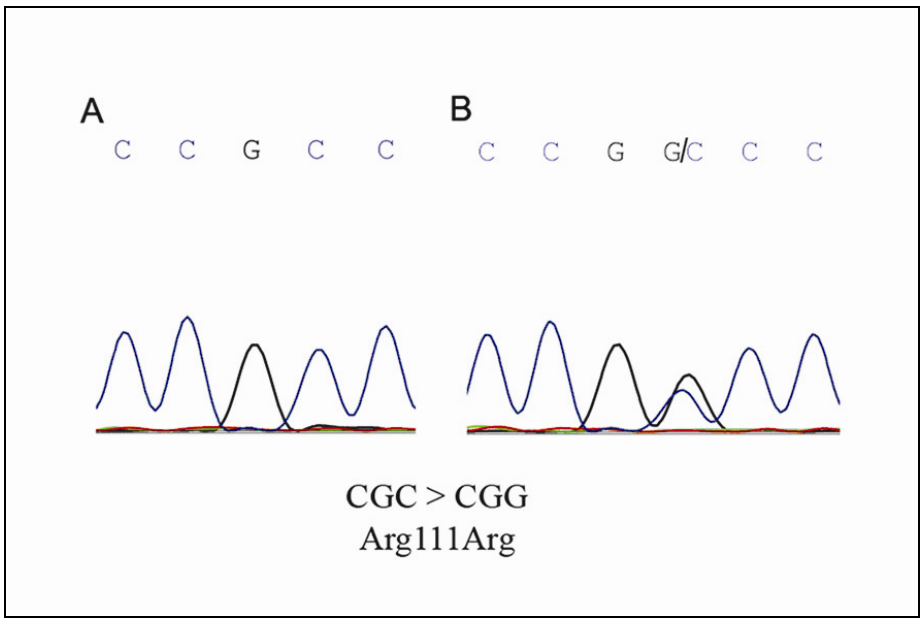


**Figure 1.** Part of *SDHAF1* sequence of two different tumors showing (A) no alteration and (B) the heterozygous polymorphism c.333C>G (NCBI: NM_001042631). Note the equal intensity of the C and G allele indicating no LOH.

*SDHB immunohistochemistry*

Of the 84 tumors investigated by SDHB immunohistochemistry, 24 appeared to be negative, including all two investigated *SDHB* mutant and all five investigated *SDHD* mutant cases. Two SDHB negative tumors displayed the *SDHAF1* c.333C>G substitution. Yet, the other tumors showing the c.333C>G alteration were immunohistochemically positive for SDHB. Therefore, it seems there is no correlation between SDHB immunostaining and the presence of the *SDHAF1* c. 333C>G polymorphism.

**Discussion**

MEN 2, VHL and NF1 syndromes present with PCC and PGL, but are also characterized by other tumors, such as medullary thyroid carcinomas in MEN 2, renal cell carcinomas in VHL and neurofibromas in NF1 (18). In contrast, the PGL-PCC syndrome, caused by mutations in the mitochondrial complex II associated genes *SDHA*, *SDHB*, *SDHC*, *SDHD* and *SDHAF2*, presents nearly exclusively PGL and PCC (4-9). Inactivation of any of these *SDH* genes, including the recently discovered *SDHAF1*, leads to complex II deficiency.

Approximately 50% of PGL and 25% of PCC are caused by mutations in the known PGL and PCC susceptibility genes, but for the remaining (sporadic) tumors the pathogenesis is still unknown (11-13). Because of the association of *SDHAF1* gene with complex II (or succinate dehydrogenase) expression and activity, we have investigated a series of 124 sporadic PGL and PCC, including 92 sporadic tumors, for mutations in the *SDHAF1* coding sequence. The only alteration found was a silent p.Arg111Arg variant present in 3 PGL and 7 PCC and also present in the patient matched constitutional DNA. In addition, the *SDHAF1* variant was found at comparable frequency in a healthy control population. Also, no loss of heterozygosity of the wildtype allele was seen in the heterozygous tumors. Furthermore, the alteration was found in syndrome-related and apparently sporadic patients. Therefore, the variant was considered as a polymorphism.

*SDHAF1* mutations have recently been associated with severely reduced complex II activity. In fact, complex II activity was almost undetectable in fibroblasts and muscle cells of patients with homozygous *SDHAF1* mutations (1). Previously we showed that PCC and PGL with *SDHB*, *SDHC*, or *SDHD* mutation lack SDHB protein expression and as a consequence are negative for succinate dehydrogenase activity (16).

Furthermore, absence of SDHB protein expression and loss of complex II activity was recently also demonstrated in an sPGL of the first described SDHA-mutation carrier. In addition, the PGL from three Dutch *SDHAF2* patients appeared to be negative for SDHB expression as well (Bayley, Korpershoek, unpublished observations). These results indicate that functional absence of SDHB, SDHC, SDHD, SDHAF2 or SDHAF1 leads to absence of complex II activity and most likely also to absence of SDHB protein expression. Therefore, it can be anticipated that *SDHAF1* mutations, when excisting, will be preferentially present in immunohistochemically SDHB negative tumors. However, no

*SDHAF1* mutations were found in 24 SDHB negative PGL and PCC nor in the remaining 100 investigated tumors. In addition, no correlation between the SDHB expression and the *SDHAF1* c. 333C>G polymorphism was seen, as the polymorphism was present in SDHB positive and negative tumors.

Although we did not find mutations in *SDHAF1*, it is possible that the gene is inactivated through other mechanisms e.g. by promoter hypermethylation. In addition, large genomic deletions could be involved in inactivation of the *SDHAF1* gene, which escape detection by direct sequencing. Although not investigated, we regard these possibilities for *SDHAF1* inactivation as unlikely, because methylation and large deletions of the *SDH*-related genes appear to play only a minor role (19-22). In addition, potential decreased SDHAF1 expression can be the result of transcriptional, translational and/or post-translational mechanisms including expression of specific miRNAs inhibiting SDHAF1 expression, however data on these possibilities are lacking.

In summary, a large series of 124 PGL and PCC was screened for germline and somatic mutations in the *SDHAF1* gene. Except for the silent variant c.333C>G (p.Arg111Arg) no genetic aberrations were found. These results suggest that *SDHAF1* mutations do not play a major role in the pathogenesis of PGL or PCC.

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# General discussion

SDHB and SDHA immunohistochemistry: screening for mutation carriers in pheochromocytomas and paragangliomas.

A significant proportion (about 35%) of paragangliomas and pheochromocytomas are due to germline mutations in several different genes. Recognizing the genetic background is important because of the implications for associated neoplasms, risk for malignancy, and family members. There are ten different genes known to cause paragangliomas (*SDHA*, *SDHB*, *SDHC*, *SDHD*, *SDHAF2*, *VHL*, *NF1*, *RET*, *PHD2*, and *TMEM127*). (1-8) Discrimination between the tumors caused by mutations in the different genes is not possible on histological grounds. In addition, immunohistochemistry was not helpful until recently: in pheochromocytomas a VHL antibody was used to investigate if VHL-related pheochromocytomas had a different expression pattern than pheochromocytomas caused by other mutations than *VHL*. However, VHL was expressed in both VHL-related and sporadic tumors, which suggests that the antibody also recognizes the mutated VHL protein. (9) It seems that the same applies to RET, for which commercially available antibodies have not yielded consistent results. The wild type RET protein is often overexpressed in nonhereditary pheochromocytoma. (10-11) However, large studies comparing RET immunohistochemical expression in *RET*-mutated and non-*RET*-mutated pheochromocytomas are nonexistent.

Because neurofibromatosis type 1 is usually diagnosed clinically, and no mutations have been described in apparently sporadic paragangliomas or pheochromocytomas, genetic testing is not indicated for this gene. For the remaining genes several groups have tried to develop algorithms for genetic testing to identify which patients should be genetically tested, and to determine the order in which genes should be tested. (12-13) Depending on age, number of tumors, location of the tumor, and family history, it is decided which genes tot test first. We have shown in chapter 2 that SDHB immunohistochemistry could discriminate paragangliomas caused by mutations in *SDHB*, *SDHC*, and *SDHD* and tumors which are caused by mutations in other genes (*VHL*, *RET*). Subsequently, in chapter 3 we have shown that SDHA immunohistochemistry is useful to detect paragangliomas and pheochromocytomas with *SDHA* mutations. Although these immunohistochemical studies are promising so far, important issues remain: the sensitivity and specificity of

these tests should be determined in larger independent cohorts, the cost-effectiveness should be studied, and the best SDHA and SDHB antibodies and immunohistochemical methods should be determined.

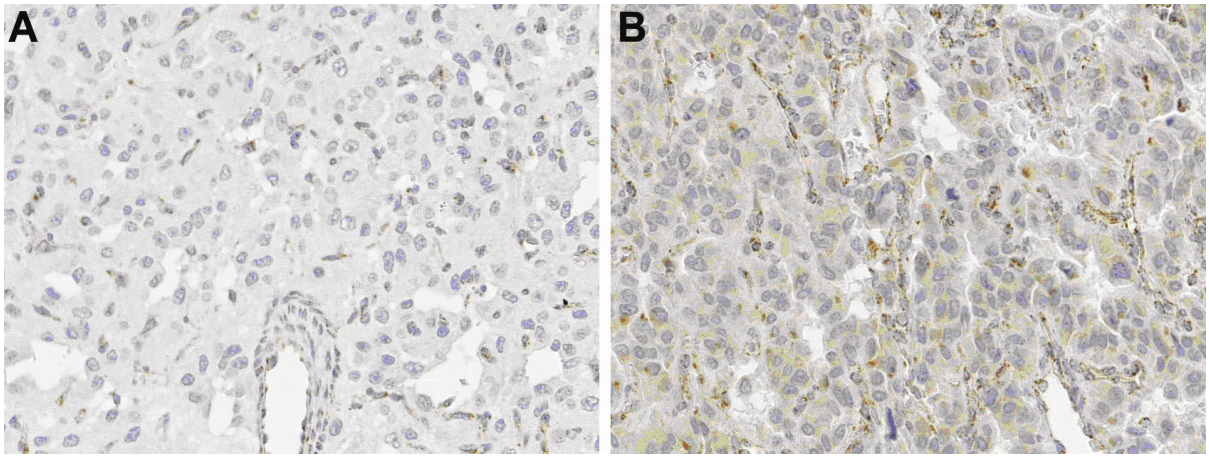


**Figure 1.** SDHB immunohistochemically negative paragangliomas. A) without background staining. B) with background staining.

In chapter 2 we described diffuse cytoplasmatic background staining, which is clearly distinct from the granular positive staining, as non-specific. Figure 1 shows a negative tumor with and without background staining. A recent article described that paragangliomas or pheochromocytomas caused by *SDHB* mutations do not have this background staining, but that *SDHC*- and *SDHD*-related paragangliomas do have this background staining. (14) Further research is needed to investigate whether this background staining is really non-specific or not. After validation of the aforementioned immunohistochemical method a new screening algorithm for molecular genetic testing of patients with paraganglioma and pheochromocytoma might be in place, were SDHB and SDHA immunohistochemistry play an important role (figure 2).

SDHAF2 is required for flavination of SDHA and therefore also for SDH activity and stability and inactivation of *SDHAF2* renders the SDH complex more susceptible to degradation. It is to be expected that the *SDHAF2*-related tumors would have a highly similar protein expression profile to tumors caused by mutations in *SDHB*, *SDHC* and *SDHD*, including negative SDHB immunostaining. Hao et al described that the levels of all four SDH subunits were significantly decreased in the *SDHAF2* mutant. The residual SDHA level

was higher than that of the other subunits, but much of it was in the soluble fraction, unassociated with the SDH complex. (5) Therefore it can be anticipated that SDHA immunohistochemistry on tumors from patients with *SDHAF2* mutations will show negative or diffuse cytoplasmatic SDHA and SDHB expression.

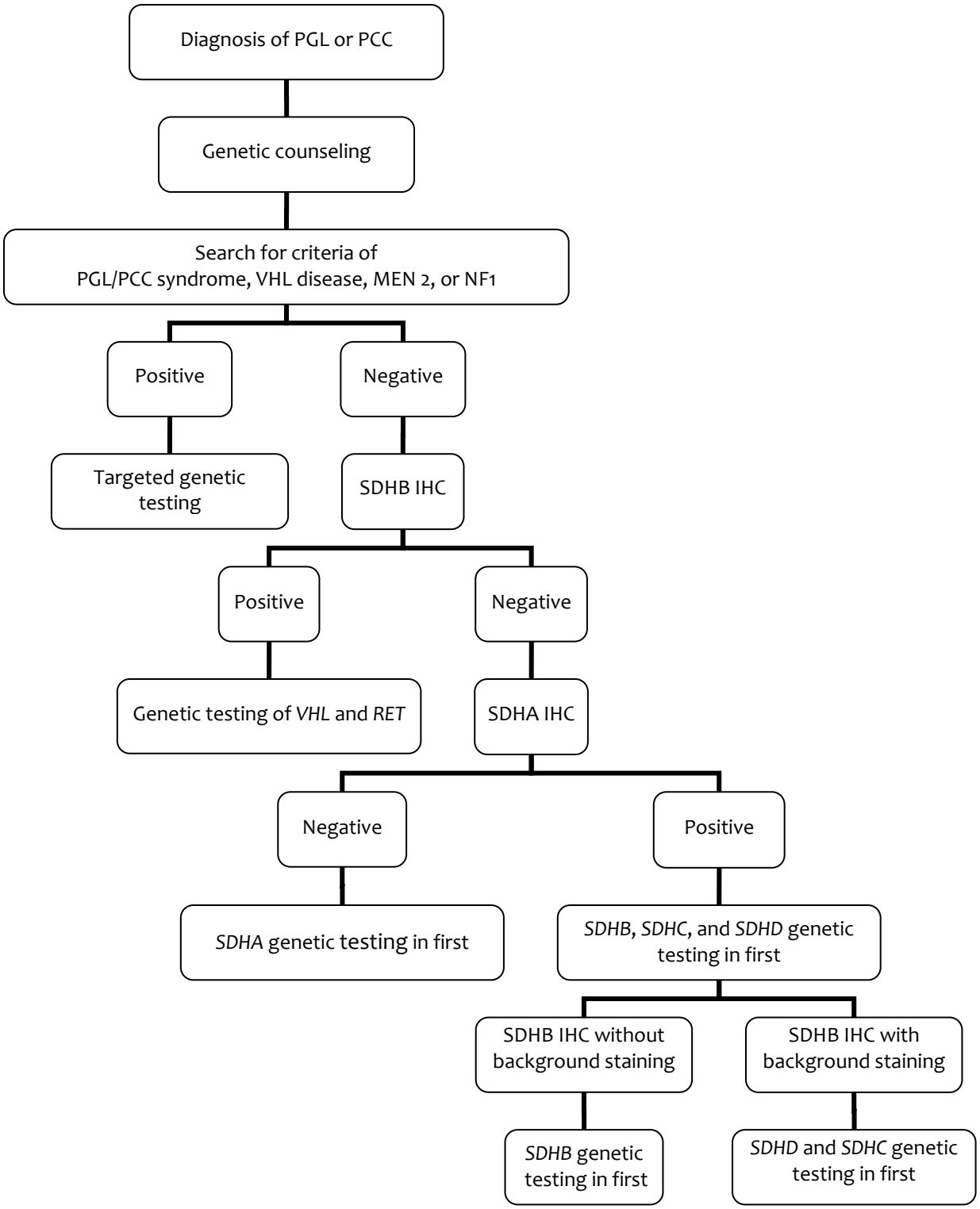


**Figure 2.** Proposed genetic screening strategy for paragangliomas and pheochromocytomas, with the use of SDHB and SDHA immunohistochemistry. PGL: paraganglioma, PCC: pheochromocytoma, VHL: von Hippel-Lindau, MEN 2: multiple endocrine neoplasia type 2, NF1: neurofybromatosis type 1, IHC: immunohistochemistry.

SDHB and SDHA immunohistochemistry: applicable to other tumors?

In chapter 3 we have shown that Carney-Stratakis syndrome- and Carney triad-related GISTs are also negative by SDHB immunohistochemistry. However we found that mutations in *SDHB*, *SDHC*, and *SDHD* were absent in all but one SDHB immunonegative GIST, which came from a Carney-Stratakis syndrome patient. In chapter 2 we found six pheochromocytomas and paragangliomas with negative SDHB immunohistochemistry, but lacking *SDHB*, *SDHC*, and *SDHD* mutations. It is possible that we missed mutations, since the sensitivity of direct sequencing is not 100%. In addition we did not investigate the UTR-, promoter-, and deep intronic regions. However, it is also conceivable that epigenetic changes in *SDHx* genes or other genes affect complex II, and mutations in such additional genes might result in disruption of complex II and subsequently in negative SDHB immunohistochemistry. Although the mechanism of tumorigenesis of the SDHB immunonegative GISTs is unknown, several studies have shown that VEGF and HIF1α are relatively overexpressed in GISTs (15-16), as is the case in *SDHx*-mutated paragangliomas. The recent discovery of an *SDHA* mutation in a paraganglioma by Burnichon et al and the four additional mutations described in chapter 3 might explain a part of the SDHB negative cases without *SDHB*, *SDHC* and *SDHD* mutations. In fact, one of the *SDHA* mutants described in chapter 3 is one of the six SDHB immunohistochemically negative paragangliomas without *SDHB*, *SDHC*, and *SDHD* mutations described in chapter 2. Therefore, SDHA immunohistochemistry should be performed on these Carney-Stratakis syndrome- and Carney triad-related GISTs.

Succinate dehydrogenase mutations have not only been described in paragangliomas, pheochromocytomas, and gastrointestinal stromal tumors, but several additional tumors have been associated with *SDHx* mutations. *SDHB* mutations have been found in various types of renal tumors. The most frequently reported SDHB-associated renal tumors are clear cell renal cell carcinomas, but oncocytomas, eosinophilic chromofobe renal cell carcinomas and papillary renal cell carcinomas are also described. Ricketts et al investigated 68 patients with features of non-syndromic inherited RCC for mutations in *FH*, *SDHB*, *SDHC*, or *SDHD* and described three patients (one with familial RCC and two with bilateral RCC) with a germline *SDHB* (p.Arg46X, p.Arg46Gln and p.Arg11His) mutation. (17)

Vanharanta et al. described one patient with a clear cell RCC and a mother who had a paraganglioma, both having a germline *SDHB* (p.Arg27X) mutation. In addition, they described 2 patients from the same family with both a paraganglioma and an RCC (of solid histology) with a germline *SDHB* (p.Ser239TyrfsX8) mutation.(18) The same *SDHB* (p.Ser239TyrfsX8) mutation was found by Neumann et al in two patients belonging to one family with clear cell RCC, while tumor tissue showed loss of the wild type allele.(19) Srirangalingam et al. described one patient with an abdominal paraganglioma and a metastatic type II papillary RCC in a retrospective case-series of 32 patients with *SDHB* mutations.(20) This patient had a p.TRP47X mutation. A renal oncocytoma has been described in a patient with a germline *SDHB* (p.Trp200Cys) mutation. (21) Recently, the lifetime risk for the development of any of these renal tumors in *SDHB* or *SDHD* mutation carriers was estimated to be 14% and 8%, respectively. (22)

Possible associations between *SDHB* mutations and other tumors, including neuroblastomas, papillary thyroid carcinomas, and seminomas have also been described. (19, 23-25) Table 1 gives an overview of all possible *SDHx*-related tumors other than pheochromocytoma and paraganglioma. It is conceivable that these tumors caused by *SDHx* mutations, could be diagnosed by SDHB immunohistochemistry similar to paragangliomas, pheochromocytomas and gastrointestinal stromal tumors. In fact, in chapter 2, one renal cell carcinoma from a patient with an *SDHB* germline mutation was investigated, which had no SDHB expression immunohistochemically.

**Table 1.** Previously reported tumors associated with *SDHx* mutations

| Tumor | Gene | Mutation | Amino acid change | Reference |
|---|---|---|---|---|
| GIST | *SDHB* | 72+1G>T | | Pasini |
| GIST | *SDHB* | 423+1G>C | | Pasini |
| GIST | *SDHB* | 45_46insCC | Thr16ProfsX62 | Pasini |
| GIST | *SDHB* | Large deletion | | Pasini |
| GIST | *SDHC* | 43C>T | Arg15X | Pasini |
| GIST | *SDHC* | 405+1G>A | | Pasini |
| GIST | *SDHD* | 57delG | Leu20CysfsX66 | Pasini |
| PTC | *SDHB* | 194T>C | Leu65Pro | Neumann |
| PTC | *SDHD* | 14G>A | TRP5X | Neumann |
| RCC | *SDHB* | 713-716delTCTC | Ser239TyrfsX8 | Neumann |
| RCC (papillary) | *SDHB* | 141G>A | Trp47X | Srirangalingam |
| RCC (clear cell) | *SDHB* | 79C>T | Arg27X | Vanharanta |
| RCC | *SDHB* | 713-716delTCTC | Ser239TyrfsX8 | Vanharanta |
| RCC clear cell | *SDHB* | 136C>T | Arg46X | Ricketts |
| RCC | *SDHB* | 137G>A | Arg46Gln | Ricketts |
| RCC eosinopfilic chromophobe | *SDHB* | 32G>A | Arg11His | Ricketts |
| Oncocytoma/ RCC (chromophobe) | *SDHB* | 3G>A | Met1Ile | Henderson |
| Oncocytoma | *SDHB* | 600G>T | Trp200Cys | Henderson |
| neuroblastoma | *SDHB* | Large deletion | | Armstrong |
| neuroblastoma | *SDHB* | Exon 1 deletion | | Cascon |
| Seminoma | *SDHD* | 129G>A | W43X | Galera-Ruiz |

GIST: gastro intestinal stromal tumor; PTC: papillary thyroid carcinoma; RCC: Renal cell carcinoma

SDHB and SDHA immunohistochemistry: other purposes

Since *SDHA*, *SDHB*, *SDHC*, *SDHD*, and *SDHAF2* genes are bona fide tumor suppressor genes, fulfilling Knudson's two hit concept, both alleles have to be either mutated or lost for complete tumor suppressor inactivation. (26) Biallelic inactivation of the *SDHx* genes is generally achieved by a point mutation in one allele and deletion of the other. (27) This will lead to absence of protein staining as shown in chapters 2 and 4. In case of polymorphisms, normal intact protein will be produced. Examples of polymorphisms are S163P in *SDHB* and H50R in *SDHD*, showing positive immunohistochemical staining, suggestive of an intact SDHB and SDHD protein, respectively. Therefore, SDHB immunohistochemistry could aid in the distinction between polymorphisms and true pathogenic mutations.

*SDHA* mutations were previously thought to be only associated with Leigh syndrome, a neurodegenerative disorder causing epilepsy, psychomotor retardation, and tetraspasticity. (28-30) Immunoblot analyses on an *SDHA*-mutated Leigh patient were performed with the patient's fibroblast mitochondria in which the decrease of complex II activity was also observed. (31) There was a marked decrease in the steady-state level of the SDHA and SDHB protein subunits in the fibroblast mitochondrial fraction of the patient compared with the control, confirming our immunohistochemical results. However, Parfait et al found no difference in the level of SDHC between patient and control. (31) Because there are different genes causing Leigh syndrome, a possible role for SDHB or SDHA immunohistochemistry might exist in Leigh syndrome patients caused by *SDHA* mutations. In addition, biallelic germ-line mutations in the *SDHAF2*-related gene *SDHAF1* have recently been described infantile leukoencephalopathy. This mutation caused SDH deficiency and therefore it is conceivable that patients with this mutation could also be identified with SDHB or SDHA immunohistochemistry.

**Tumorigenesis and the different susceptibility genes**

The exact mechanism of tumorigenesis of paragangliomas and pheochromocytomas is not known. Interestingly, the transcription signature of the various syndromic forms of pheochromocytomas shows similarities, even though the mutations are present in distinct genes. In CGH studies, sporadic pheochromocytomas (mutations of the known candidate genes *RET*, *VHL*, *SDHB* and *SDHD* were excluded) could be classified into two main groups with different genetic profiles: one profile resembling that of VHL-related pheochromocytomas (loss of 3p and 11p) and one with a profile resembling that of MEN2-related pheochromocytomas (loss of 1p and 3q). (32) In addition, with RNA expression arrays, Dahia et al found a regulatory loop, linking all hereditary and sporadic tumors to hypoxia. Based on unsupervised clustering they found two clusters of tumors: one with *SDH*- and *VHL*-related tumors together with part of the sporadic tumors and another cluster with *RET*- and *NF1*-related tumors and the remaining sporadic tumors. (33) Thus, it appears that paragangliomas from different genetic background have a similar pathway leading to tumor formation, given the similar RNA and protein expression profile. The pathogenesis of sporadic paragangliomas and pheochromocytomas is thought to result from similar pathways as their hereditary counterparts.

HIF pathway

Succinate dehydrogenase plays two key roles: one, as a component of the citric acid cycle, by converting succinate into fumarate; and two, by serving as a source of electrons for mitochondrial respiration, as complex II of the electron transport chain. As mentioned in the introduction, inactivation of succinate dehydrogenase (by mutations of *SDHx* genes) results in the accumulation of succinate. This is believed to result in aberrant regulation of prolyl hydroxylase and mitochondrial respiratory chain complexes that are thought to play a central role in oxygen sensing. (34)

In preliminary experiments, we investigated the expression pattern of a series of hypoxia pathway-related proteins (HIF1α, HIF2 α, VEGF, PHD1, PHD2, PHD3, SDHA, SDHB, IGF2, and GLUT1), in parasympathetic paragangliomas of different genetic background and compared them with sporadic cases. We included one tumor from a patient with an *SDHA* mutation, ten with *SDHB*, one with *SDHC*, ten with *SDHD*, 11 with *SDHAF2*, and two with a *VHL* mutation and ten from patients with sporadic paragangliomas. Interestingly, expression of HIF1α and HIF2α and their downstream proteins was similar in parasympathetic paragangliomas from different genetic background and in sporadic parasympathetic paragangliomas.

Another citric acid cycle enzyme, fumarate hydratase (*FH*) also acts as tumor suppressor gene. Germ line mutations in *FH* predispose individuals to leiomyomas and renal cell cancer. (35) *FH*-deficient tumors accumulate fumarate and, to a lesser extent, succinate. (36) In situ analyses showed that these tumors also have over-expression of HIF1α. (36) So in addition to succinate, fumarate also causes stabilization of HIF1α by inhibition of prolyl hydroxylase. Next to the known paraganglioma pheochromocytoma susceptibility genes (*SDHA*, *SDHB*, *SDHC*, *SDHD*, *SDHAF2*, *VHL*, and to a lesser extent *PHD2* and *IDH1*) other genes (like *FH*) play a role in the same pathway. Figure 3 shows a schematic drawing of the hypoxia pathway incorporating the currently known paraganglioma susceptibility genes. It could be useful to perform snp arrays or whole genome sequencing on well-characterized sporadic tumors to identify candidate genes involved in the genesis of sporadic paragangliomas.

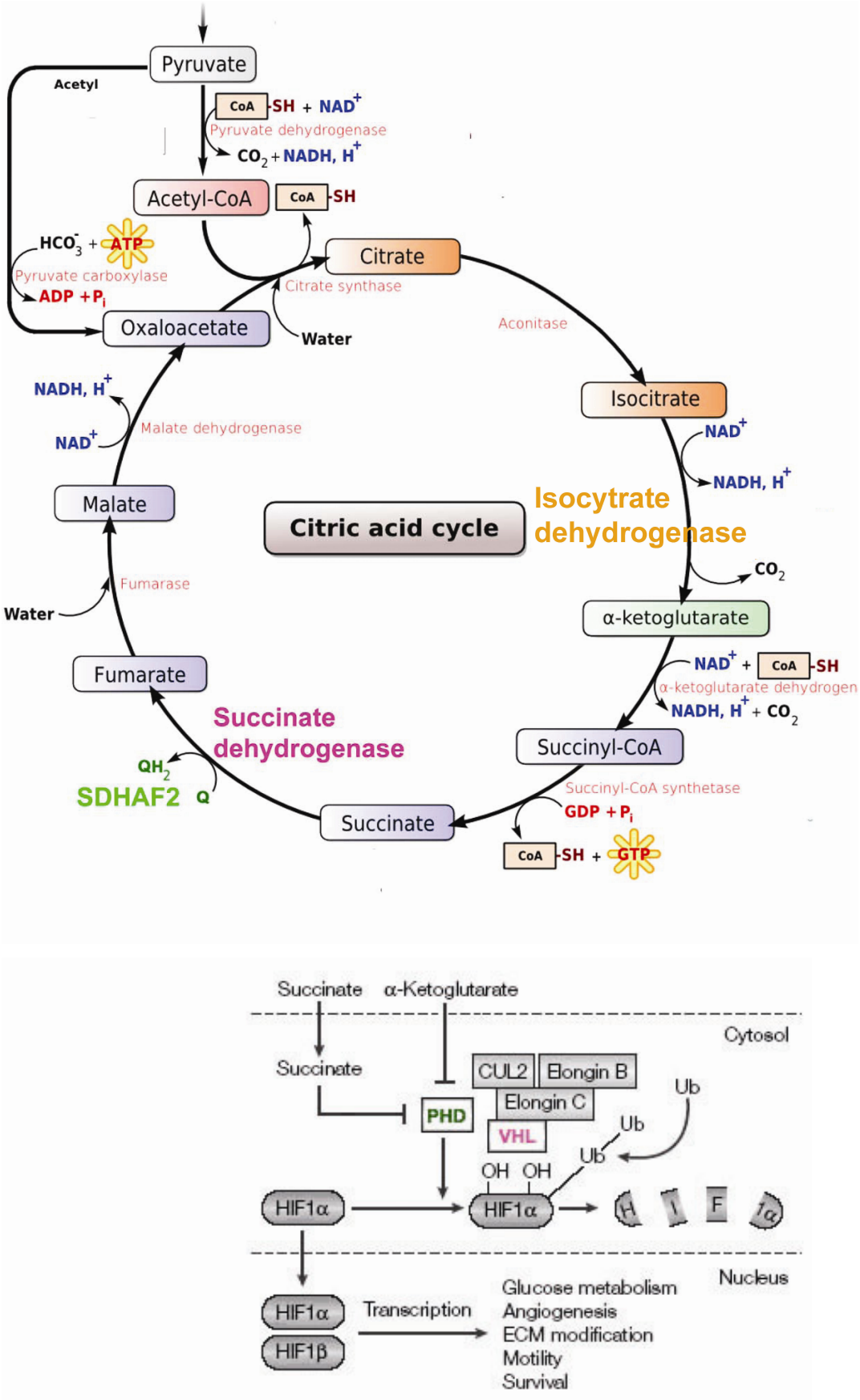


**Figure 3.** Schematic drawing of hypoxia pathway, incorporating all of the known paraganglioma susceptibility genes. The upper part shows the mitochondrion with the citric acid cycle. Succinate

is converted to fumarate by succinate dehydrogenase. SDHAF2 acts as a cofactor with FAD for the flavination of SDHA. The lower part of the figure shows that accumulated succinate, because of succinate dehydrogenase (SDH) inhibition, can leave the mitochondria and inhibit prolyl hydroxylase (PHD) activity in the cytosol. Consequently, hypoxia-inducible factor 1α (HIF1α) is not hydroxylated and can escape polyubiquitylation (Ub-Ub, mediated by the von Hippel-Lindau protein (pVHL) ubiquitin-ligase complex that includes cullin 2 (CUL2), elongin B and elongin C) and degradation even under normoxic conditions. HIF1α then translocates to the nucleus where, together with HIF1β, it forms an active HIF complex that induces the expression of genes that support tumour growth and spreading, and might decrease apoptosis.

Reactive oxygen species

One of the unresolved controversies surrounding cellular effects of succinate dehydrogenase loss is the involvement of reactive oxygen species (ROS). It is thought that when succinate-ubiquinone activity is inhibited, electrons that would normally transfer through the SDHB subunit to the ubiquinone pool are instead transferred to $O_2$ to create ROS. In agreement with this model ROS accumulation in SDH mutants was shown in a mev-1(kn1) mutant of *Caenorhabditis elegans* that harbored a homozygous inactivating missense *SDHC* mutation, which displayed a premature aging phenotype as a result of increased superoxide levels. (37) In addition, hamster cell lines carrying heterozygous *Sdhc* mutations have been shown to have increased ROS production rates. (38)

ROS arising from complex III trigger HIF-1α stabilization during hypoxia. It is possible that increased ROS production at complex II, caused by mutations in *SDHx*, could activate HIF by mimicking the hypoxia pathway. In fact Guzy et al described that inhibition of SDHB, increases normoxic ROS production, increases HIF-1α stabilization in a ROS-dependent manner, and increases growth rates in vitro and in vivo without affecting hypoxia-mediated activation of HIF-α. (39) Deletion of *SDHB* in yeast leads to increased production of ROS, which is accompanied by stabilization of HIF. (40)

So, maybe both succinate and ROS accumulate in the mitochondria following mutations in the succinate dehydrogenase (SDH) subunit genes and cause HIF stabilization (Figure 4).

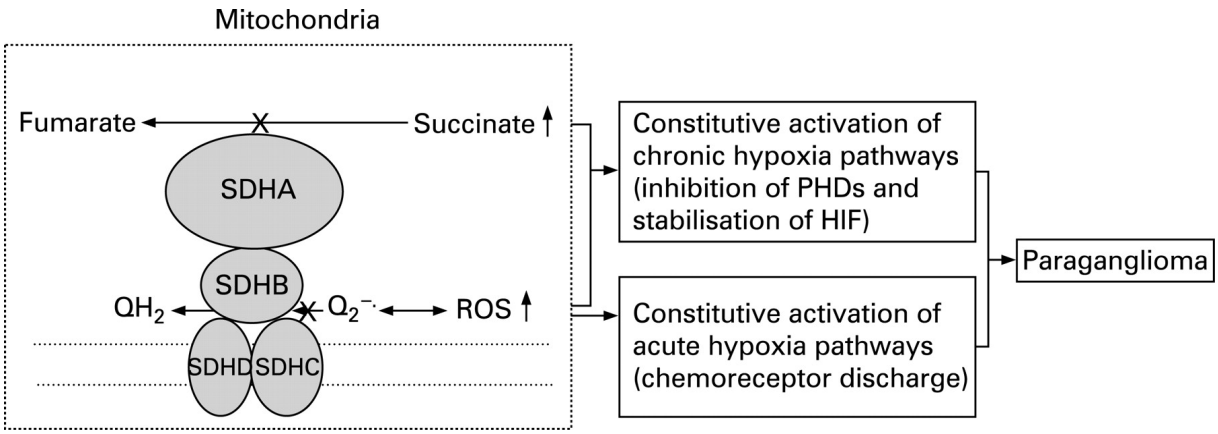


**Figure 4.** Model for the pathogenesis of hereditary paraganglioma (adapted from Baysal et al (41)). Both succinate and reactive oxygen species (ROS) accumulate in the mitochondria following mutations in the succinate dehydrogenase (SDH) subunit genes. These substrates presumably transit into the cytoplasm, constitutively stimulate hypoxia sensing and signaling pathways, and lead to paraganglioma formation. Acute hypoxia sensing occurs within seconds of oxygen deprivation and leads to stimulation of ventilation by an electrochemical mechanism. Chronic hypoxia sensing leads to a transcriptional response orchestrated by the hypoxia inducible factors (HIF).

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# Samenvatting

Paragangliomen zijn zeldzame neuroendocriene tumoren. Er worden twee verschillende typen paragangliomen onderscheiden. 1) sympatische paragangliomen, welke geassocieerd zijn met het sympatische deel van het autonome zenuwstelsel en welke catcholamines produceren. 2) parasympatische paragangliomen, welke geassocieerd zijn met het parasympatische deel van het autonome zenuwstelsel en zelden catecholamines produceren. Sympatische paragangliomen komen voornamelijk voor in borst- en buikholte met als voornaamste locatie het bijniermerg. Deze tumoren noemen we feochromocytomen. Parasympathische paragangliomen komen voornamelijk voor in het hoofd hals gebied met als voornaamste locatie de carotis body, welke geleden is in de vork van de grote halsslagader.

Via genetisch familieonderzoek zijn veel van de verantwoordelijke mutaties geïdentificeerd die betrokken zijn bij het ontstaan van erfelijke paragangliomen. Het betreft mutaties in genen van succinaat dehydrogenase, dat een onderdeel vormt van de intracellulaire ademhalingsketen die zich bevindt in de mitochondria. Naast mutaties in de 4 genen (SDHA, SDHB, SDHC en SDHD) die coderen voor succinaat dehydrogenase zijn mutaties gevonden in SDHAF2, VHL, PHD, RET, NF1 en TMEM127.

Het onderscheid tussen paragangliomen met verschillende genetische achtergrond is belangrijk, omdat het risico op andere tumoren en de kans op metastasering verschillend is. Echter alle feochromocytomen en paragangliomen zien er microscopisch hetzelfde uit. In hoofdstuk 2 hebben we laten zien dat we onderscheid kunnen maken tussen tumoren ontstaan door mutaties in een van de SDH genen en tumoren die ontstaan zijn door mutaties in VHL, RET en NF1 en sporadische tumoren met behulp van een immunohistochemische kleuring tegen SDHB. SDH gerelateerde tumoren laten geen SDHB eiwitexpressie zien terwijl de andere tumoren wel SDHB eiwit expressie hebben. In hoofdstuk 3 hebben we aangetoond dat een immuunhistochemische kleuring tegen SDHA, SDHA gerelateerde tumoren onderscheid kan maken tussen van de andere SDH gerelateerde tumoren. Deze resultaten betekenen dat SDHA samen met SDHB immunohistochemie een rol kan spelen in het genetisch onderzoek van patiënten met feochromocytomen en paragangliomen.

Niet alleen patiënten met feochromocytomen en paragangliomen hebben mutaties in succinaat dehydrogenase. Er zijn ook andere tumoren, zoals Gastrointetinale stroma tumoren (GISTen), beschreven met SDHB, SDHC en SDHD mutaties. Deze mutaties zijn beschreven in tumorsyndromen waarbij paragangliomen en GISTen samen voorkomen (Carney-Stratakis syndroom en Carney triad). In hoofdstuk 4 hebben we laten zien dat SDHB immunohistochemie ook GISTen in het kader van Carney-Stratakis syndroom en Carney triad kan onderscheiden van GISTen veroorzaakt door mutaties in KIT en PDGFRA.

VHL is een tumorsuppressorgen. Dit betekent dat er twee hits nodig zijn voor tumor vorming. 1) een kiembaan mutatie op het ene allel en 2) een somatische deletie van het andere allel. VHL mutaties in parasympatische paragangliomen zijn zeer zeldzaam (0.9%) en tot op heden was biallelische inactivatie niet aangetoond. In hoofdstuk 5 hebben we de biallelische inactivatie van VHL aangetoond in 2 parasympatische paragangliomen.

Van ongeveer 50% van patiënten met paragangliomen is de genetische achtergrond bekend. De overige 50% hebben zogenoemde sporadische tumoren (genetische oorzaak onbekend). Er zijn aanwijzingen dat mogelijk een pseudo-registratie van zuurstofgebrek (pseudo-hypoxie) signaalroutes activeert die cellen stimuleren tot celdeling. Niet alleen SDH, VHL en PHD spelen een rol in deze signaalroutes, maar ook vele andere eiwitten spelen een rol in deze signaalroutes.
Isocitraat dehydrogenase-1 (*IDH1*) somatische mutaties zijn beschreven in glioblastomen. Door deze mutatie is er vermindering van α-ketoglutaraat waardoor ook de pseudo hypoxie signaalroutes worden geactiveerd. Gezien deze overlap in de signaalroute hebben we in hoofdstuk 6 onderzocht of paragangliomen ook IDH mutatie hebben. Slechts 1 van de paragangliomen bleek een IDH1 mutatie te hebben.

SDHAF2 mutaties zijn gevonden in een Nederlandse familie met parasympatische paragangliomen. In hoofdstuk 7 hebben we onderzocht of SDHAF2 mutaties ook voorkomen in sporadische paragangliomen. Het bleek dat SDHAF2 mutatie slechts een kleine rol speelt in sporadische paragangliomen.

In veel succinaat dehydrogenase gerelateerde genen zijn mutaties gevonden (SDHA, SDHB, SDHC, SDHD en SDHAF2). SDHAF1 mutaties zijn beschreven in twee families met succinaat dehydrogenase deficiëntie. Net zoals aan SDHAF2 is SDHAF1 essentieel voor de vorming van het succinaat dehydrogense complex. Onderzoek (beschreven in hoofdstuk8) wijst echter uit dat er geen mutaties van SDHAF1 zijn in paragangliomen.

# Appendices

**List of publications**

**Gaal J**, Stratakis CA, Carney JA, Ball ER, Korpershoek E, Lodish MB, Levy I, Xekouki P, van Nederveen FH, den Bakker MA, O'Sullivan M, Dinjens WN, de Krijger RR. **SDHB immunohistochemistry: a useful tool in the diagnosis of Carney-Stratakis and Carney triad gastrointestinal stromal tumors.** Mod Pathol. 2010 Oct 1.

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**Gaal J**, Burnichon N, Korpershoek E, Roncelin I, Bertherat J, Plouin PF, de Krijger RR, Gimenez-Roqueplo AP, Dinjens WN. **Isocitrate dehydrogenase mutations are rare in pheochromocytomas and paragangliomas.** J Clin Endocrinol Metab. 2010 Mar;95(3):1274-8.

**Gaal J**, van Nederveen FH, Erlic Z, Korpershoek E, Oldenburg R, Boedeker CC, Kontny U, Neumann HP, Dinjens WN, de Krijger RR. **Parasympathetic paragangliomas are part of the Von Hippel-Lindau syndrome**. J Clin Endocrinol Metab. 2009 Nov;94(11):4367-71.

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Boedeker CC, Erlic Z, Richard S, Kontny U, Gimenez-Roqueplo AP, Cascon A, Robledo M, de Campos JM, van Nederveen FH, de Krijger RR, Burnichon N, **Gaal J**, Walter MA, Reschke K, Wiech T, Weber J, Rückauer K, Plouin PF, Darrouzet V, Giraud S, Eng C, Neumann HP. **Head and neck paragangliomas in von Hippel-Lindau disease and multiple endocrine neoplasia type 2.** J Clin Endocrinol Metab. 2009 Jun;94(6):1938-44.

**PhD Portfolio**

**Summary of PhD training and teaching**

| | |
|---|---|
| **Name PhD student:** José Gaal<br>**Erasmus MC Department:** Pathology<br>**Research School:** Erasmus Postgraduate School Molecular Medicine (MolMed) | **PhD period:** 1st march 2008 to 31st march 2010<br>**Promotor(s):** Prof. dr. Ronald R. de Krijger<br>**Supervisor:** Dr. Winand N.M. Dinjens |

**1. PhD training**

| | Year | Workload (Hours/ECTs) | |
|---|---|---|---|
| **General courses** | | | |
| - Academic writing for PhD students | 2010 | 32h | 1.14 |
| **Specific courses** | | | |
| - The course molecular Diagnostics IV | 2009 | 16h | 0.57 |
| - The Partek Training Course | 2009 | 16h | 0.57 |
| - Basis onderwijs pathologie; oncologie | 2010 | 16h | 0.57 |
| **Seminars and workshops** | | | |
| - USCAP seminars | 2010 | | |
| - Photoshop CS3 Workshop | 2010 | 7h | 0.25 |
| **Presentations** | | | |
| - A routine immunohistochemical procedure for the detection of Paraganglioma and Pheochromocytoma patients with germline *SDHB*, *-C*, or *-D* gene mutations, 22nd European congress of pathology, Florence It | 2009 | 40h | 1.43 |
| **Poster presentations** | | | |
| - Parasympathetic paragangliomas in a patient with von Hippel-Lindau disease, 2nd International symposium on pheochromocytoma (ISP), Cambridge UK | 2008 | 40h | 1.43 |
| - Parasympathetic paragangliomas are part of the von Hippel-Lindau syndrome, 22nd European congress of pathology, Florence It | 2009 | 40h | 1.43 |
| - **A routine immunohistochemical procedure for the detection of Paraganglioma and Pheochromocytoma patients with germline *SDHB*, *-C*, or *-D* gene mutations** , Dutch Pathology Society annual meeting, Zeist NL | 2009 | 40h | 1.43 |
| - SDHB immunohistochemistry: a useful tool for genetic testing of non-c-kit and non-PDGFRα mutated gastro intestinal stromal tumors, 14th Molecular Medicine Day, Rotterdam NL | 2010 | 40h | 1.43 |

| | | | |
|---|---|---|---|
| **(Inter)national conferences**<br>- 2nd International symposium on pheochromocytoma (ISP), Cambridge UK<br>- 22nd European congress of pathology, Florence It<br>- United-states & Canadian Academy of pathology (USCAP) Annual meeting, Washington USA | 2008<br>2009<br>2010 | 40 h<br>40h<br>40h | 1.43<br>1.43<br>1.43 |
| **Other**<br>Hubert Wolfe award,<br>Endocrine Pathology society, Washington USA | 2009 | | |
| **2. Teaching** | | | |
| **Supervising practicals and excursions, Tutoring**<br>- Histology and histopathology of endocrine organs; 2nd year students Medicine<br>- Congenital heart disease; 1st year students Medicine<br>- Pathology of lungcarcinoma; 2nd year students Medicine<br>- Development, anatomy and pathology of the placenta | 2008-2009<br>2008-2009<br>2009<br>2008-2009 | | |