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MOLECULAR PATHOLOGY OF PHEOCHROMOCYTOMA AND PARAGANGLIOMA DEVELOPMENT

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MOLECULAR PATHOLOGY OF PHEOCHROMOCYTOMA AND PARAGANGLIOMA DEVELOPMENT

Moleculaire pathologie van het ontstaan van pheochromocytomen en paragangliomen

Proefschrift

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Those who know cannot explain, those who don’t know cannot understand

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Tumours of the paraganglia and the adrenal medulla

Tumours of the adrenal medulla have been described since the beginning of the 20th century, when clinicians first discovered autopsy cases with paroxysmal hypertension and bilateral adrenal tumours. The first propsed name for these tumours was phäochromocytom, derived from the Greek phaios (dark), chroma (colour), and cytoma (tumour). This name was given because of the dark discoloration of the lesions in the chromium-salt reaction, as described by Pick, a german pathologist, in 1912. From this time the tumours have been studied by many clinicians, such as Sipple, who described Sipple's syndrome, that was later named multiple endocrine neoplasia type 2 (see text below). With the advancements in pathology, the field of molecular pathology has developed. Not only are tumours classified according to location and histology, but with molecular techniques tumours they can be subdivided according to their (DNA) aberrations. The known characteristics of pheochromocytomas and paragangliomas, from histology to genetics and molecular pathology are described in this chapter.

Paraganglia

Paraganglia are small aggregates of neural crest-derived cells divided into sympathetic and parasympathetic paraganglia. The sympathetic paraganglia are located along the sympathetic trunk that is situated along the proximal aorta reaching down to the abdominal aorta and urinary bladder.[1]

One of these paraganglia is distinct in young infants, and is designated the organ of Zuckerkandl.[2] The adrenal medulla is also part of the sympathetic paraganglion system, which results from small primitive cells (sympathicoblasts) migrating into the adrenal cortex, also described as invasion, to form the definitive adrenal. [3] The parasympathetic paraganglia are located in the head and neck region, with locations aligned to the parasympathetic nervous system, but also in close relation to vascular structures. The nervous system-associated paraganglia are the jugulotympanic and vagal paraganglia. An association with major vessels is found in the carotid body paraganglia and aorticopulmonary paraganglia. In 2004, the WHO has redefined tumours arising from paraganglia into three groups. [4] The term pheochromocytoma

(PCC) is only used for tumours of the adrenal medulla, which usually but not always produce catecholamines, including epinephrin, norepinephrin, and dopamine, which are subsequently metabolised and excreted in the urine. All other tumours arising outside the adrenal and originating from sympathetic paraganglia are called sympathetic paragangliomas (sPGL). Again, these tumours usually produce catecholamines. sPGL are referred to as extra-adrenal PCC in many studies, and constitute about 10-20% of the PCC population.[5] Finally, tumours arising from parasympathetic paraganglia, mostly in the head and neck area, are designated parasympathetic paragangliomas (pPGL). In contrast to the previous two groups, these tumours do not produce hormones, except for a minority of 1% to 3%.

All tumours derived from chromaffin cells (PCC, sPGL and pPGL) are histologically and immunohistochemically similar, although some variations can be seen between the sPGL and pPGL on the one hand, and PCC on the other hand (Figure 1). The annual incidence of pPGL is 1:30,000 and of PCC is 0.5-9:1,000,000. [6-8] The incidence of sPGL is not clear, especially since these tumours have been grouped with PCC in the literature.

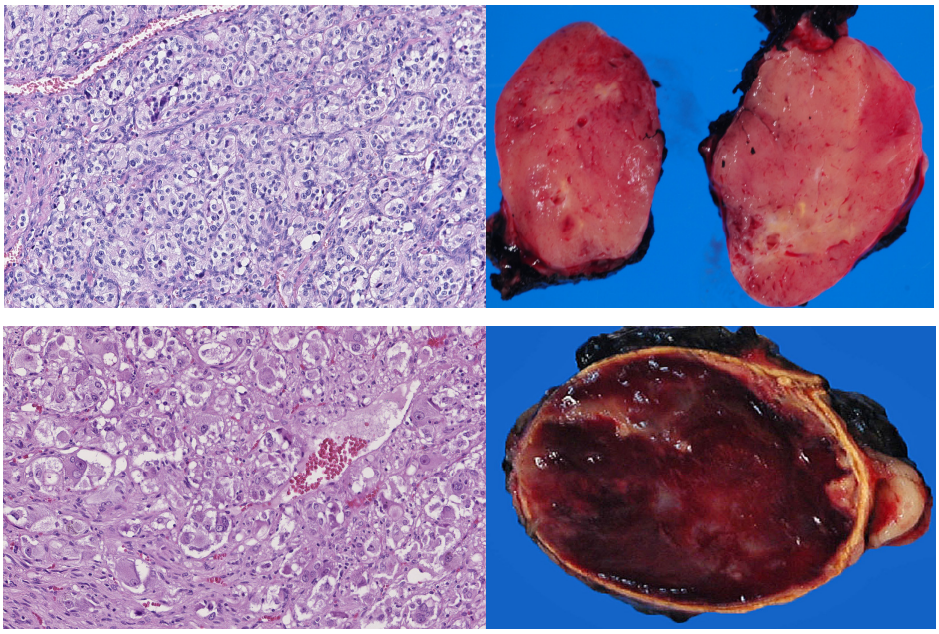

Figure 1: Examples of a sPGL (upper panels) and PCC (lower panels)

Histopathology

PCC and PGL have a similar histological appearance, with chromaffin cells in small nests, the so-called “Zellballen”, surrounded by sustentacular cells and small vessels. pPGL and sPGL usually exhibit small Zellballen and are more eosinophilic, whereas PCC are usually basophilic with more abundant cytoplasm, which is granular. Nuclear pleomorphism is present to a certain degree in most tumours and should not be taken as an indication of malignancy (Figure 2). Some pPGL may be very sclerotic and highly vascularized, to the extent that the lesional chromaffin cells are difficult to identify in mechanically disrupted excisional biopsies without additional immunohistochemical studies. The chromaffin cells are positive for neuro-endocrine markers, with synaptophysin and chromogranin A being the most commonly used. The sustentacular cells are positive for S100 and although they are characteristic of PCC and PGL, they are a non-neoplastic component of PCC and PGL. [9] Almost all PCC and PGL are devoid of significant keratin staining, which may be used in cases where a differential diagnosis exists with a carcinoma.

Generally, it has been stated that in order to diagnose an adrenal lesion as a PCC, it should have a diameter of at least 1cm. [10] Below this threshold a lesion is called adrenomedullary hyperplasia. However, this distinction is rather arbitrary, since it is not based on biological criteria or behaviour. Indeed, our own preliminary data show that in patients with multiple endocrine neoplasia type 2 (MEN 2) adrenomedullary lesions smaller and greater than 1cm in diameter show the same clinical characteristics and genetic abnormalities (Petri et al., unpublished observations).

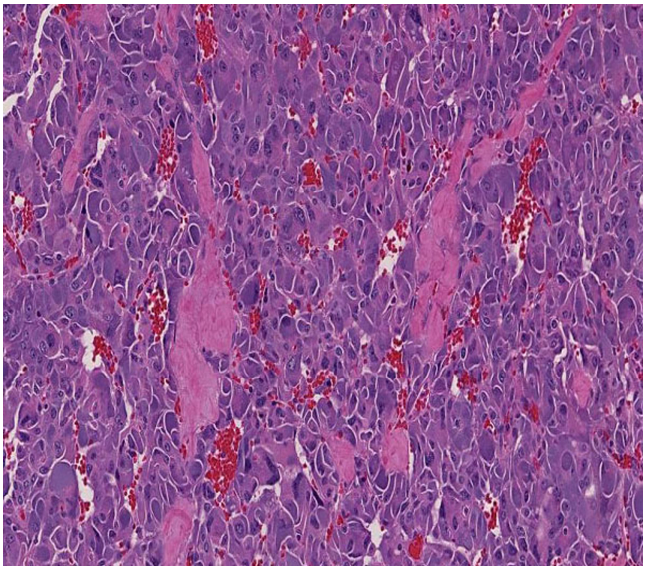

Figure 2: Example of nuclear pleomorphism in PCC

Genetics

Originally, the rule of 10 was applied to PCC. This implied that 10% of PCC were considered to be extra-adrenal (which are now called sPGL), 10% occurred in children (which is still more or less true), 10% were bilateral and 10% were hereditary. This frequency for hereditary tumours has also been quoted for head and neck PGL. However, with the discovery of a number of candidate genes, responsible for various tumour syndromes, and the advent of systematic genetic screening of various populations of PCC and PGL patients, it has now become clear that the percentage of patients harbouring germ line mutations has been underestimated and appears to be between 25% and 35%. [11-13] The exact percentage depends on the specific patient group investigated (only PCC patients, only PGL patients or all PCC/PGL patients) and on geographical variations, where founder mutations can have a high impact. Specifically, a founder effect is known for von Hippel-Lindau (*VHL*) gene mutations in the German Black Forest region [14], for succinate dehydrogenase subunit D (*SDHD*) gene mutations in The Netherlands [15], and for *SDHB* gene mutations in Italy.[16]

MULTIPLE ENDOCRINE NEOPLASIA

This syndrome is divided into MEN 1 and MEN 2. The latter can be divided into MEN 2A and MEN 2B. Recently, a MEN-related-syndrome has been described with mutations in the *CDKN1B* gene, with a MEN 1-like phenotype, but the occurrence of this syndrome and the finding of PCC have only been documented in animal models, not in humans.[17]

MEN 1 is due to mutations in the *menin* tumour-suppressor gene (TSG), located on chromosome 11q13. The occurrence of PCC in this syndrome is very rare, with only 7 patients with mutations in the *menin* gene described in the literature, as reviewed by Schussheim et al. [18]

The incidence of MEN 2 is not known, however it is estimated to be 1.25-7.5/10,000,000. [4] MEN 2 is due to activating mutations in the *RET* proto-oncogene, located on chromosome 10q11, and is subdivided into 2 clinically distinct syndromes designated MEN 2A and MEN 2B. The localisation of mutations is indicative of the subtype of the MEN 2-syndrome. In MEN 2A mutations are mainly found in the exons coding for the extra-cellular domain (exons 10 and 11 of the RET-gene), although exon 13-15 can be involved in individual cases. Functional changes due to these mutations are ligand-independent dimerisation of the RET-receptors, with subsequent activation of the RET-signalling pathway. MEN 2A is clinically defined by the presence of parathyroid hyperplasia, C-cell hyperplasia/medullary thyroid carcinoma (MTC) and PCC. The risk of MTC in MEN 2A patients is 100%, whereas the risk for PCC is 50%. The disease can present early in life, usually with MTC, but can also manifest as a hypertensive crisis due to a PCC. [19] PCC occur in about 50% of all patients with MEN 2, and are frequently bilateral. [20, 21] In addition, as described by Carney et al., most contralateral adrenal glands show either diffuse or nodular hyperplasia, which represents a precursor for PCC. [10, 22] It should be noted that absence of a family history is not sufficient to exclude diagnosis of MEN 2A, since *de novo* germ line mutations have been reported in 10% of patients.

MEN 2B is also due to activating mutations in the *RET* proto-oncogene, with mutations in the kinase domain of the receptor (coded by exon 16 of the *RET* gene, p.M918T in 95% of cases). The functional consequences of this mutation are diverse with loss of kinase inhibition, dimerisation, and autophosphorylation in the absence of

substrate binding to the receptor, all leading to aberrant RET signalling. [23] Clinically, the syndrome is largely similar to MEN 2A, with the exception of parathyroid hyperplasia. Striking in this syndrome is the occurrence of mucosal ganglioneuromas, giving a peculiar facial appearance, and skeletal deformities as described by Carney et al.[24] De novo mutations causing MEN 2B syndrome occur frequently, in up to 50% of patients.[25] In addition to germline mutations, somatic *RET* mutations in MTC and PCC have been described (the exon 16 p.M918T), but do not increase the risk for other tumours. For all MEN 2 patients, the occurrence of malignant PCC is rare. The occurrence of PGL in the context of MEN 2 is extremely rare and only few cases have been described. [26]

VON HIPPEL-LINDAU DISEASE

This disease has an incidence ranging from 1:36,000 to 1:39,000 [27, 28] and has been subdivided into VHL type 1 and VHL type 2 (2a, 2b and 2c). No PCC develop in VHL type 1, but a considerable frequency of PCC (16-30%) has been reported in VHL type 2 mutation carriers. Overall, PCC develop in 10-20% of patients with VHL disease, and usually in a bilateral mode. [20, 21] Chromaffin tumours in VHL disease are almost always PCC, but rare sPGL and pPGL have been described. [29, 30]

The most frequently encountered tumours in VHL type 2a patients are haemangioblastomas and PCC. VHL type 2b causes the same tumours as in the spectrum of VHL type 2a, with addition of clear cell renal cell carcinoma. Other rare tumours that occur in VHL disease are cystic adenomas and endocrine tumours of the pancreas, papillary cystadenomas of the broad ligament and epididymis as well as endolymphatic sac tumours. In VHL type 2c there is isolated familial PCC. VHL disease is due to mutations in the *VHL* TSG, located on 3p25. De novo mutations occur in about 20% of clinically detected VHL patients. Therefore, not only family history, but also the clinical presentation is important in PCC patients. The vast majority of VHL-related PCC is benign. [31] In addition to germ line mutations, somatic *VHL* mutations in PCC have been described.

The VHL protein is involved in the regulation of HIF degradation in the presence of normal oxygen tension. Under hypoxic conditions HIF-1α is hydroxylated by prolyl hydroxylases, which allows recognition by the VHL E3 ligase complex, by which HIF-

1α is ubiquitinated. The majority of *VHL* mutations described in PCC are missense mutations that abrogate binding of HIF to the E3 ligase complex and thus the degradation of HIF. [32] (Shown in figure 3, right side of the pathway)

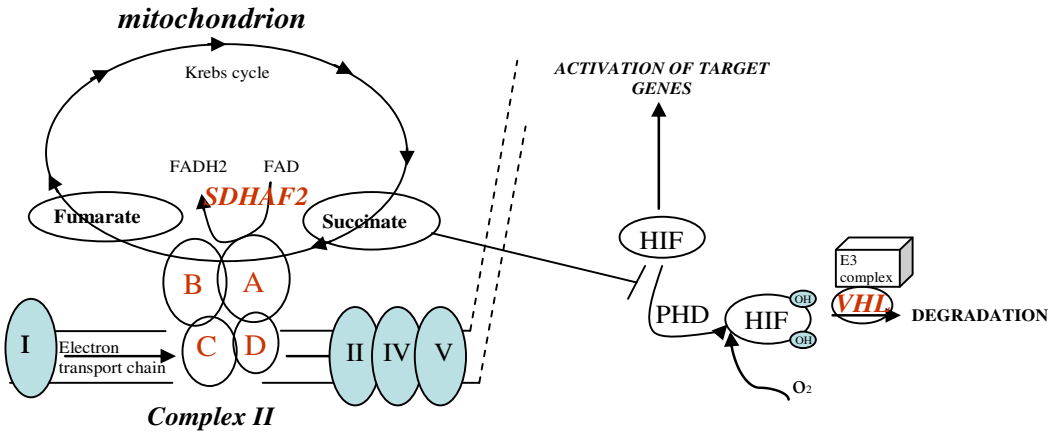

Figure 3: Schematic drawing of hypoxia pathway, incorporating 5 of the currently known PGL and PCC susceptibility genes. The left part of the figure shows the mitochondrion with Krebs cycle and electron transport chain. Succinate is converted to fumarate by active SDHA and SDHB. SDHC and SDHD are anchoring proteins in the mitochondrial membrane, and act in the electron transport chain. SDHAF2 acts as a cofactor with FAD for the flavination of SDHA. Succinate, when entering the cytoplasm, suppresses PHD function.

The right part of the figure shows circulating HIF that can activate target genes. HIF is regulated by oxygen tension and PHD, forming hydroxylated HIF. This hydroxylation provides the recognition signal that enables HIF to be captured by VHL to the E3 complex and to degrade the complex.

Abbreviations: HIF: hypoxia-inducible factor, PHD: prolyl hydroxylase, SDH (A,B,C, D and F2): succinate dehydrogenase (subunit A, B,C, D and complex assembly factor 2). FAD: flavin adenine dinucleotide.

NEUROFIBROMATOSIS

In neurofibromatosis type 1 (NF1; also known as von Recklinghausen's disease) there is a small proportion of patients (up to 5%) with PCC. Compared to the previously described PCC-susceptibility syndromes, this syndrome is frequent, affecting 1:3,000 individuals. The hallmarks of this syndrome are the occurrence of multiple neurofibromas and the presence of café-au-lait pigmentations and Lisch nodules of the iris. However, the spectrum is much more diverse, including gastro-intestinal stromal tumours (GIST), central nervous system malignancies and an increased risk of breast cancer in young women. [33, 34] NF1 is caused by mutations in the *neurofibromin* or *NF1* TSG located on chromosome 17q11. The pattern of inheritance is autosomally dominant. In addition, almost half of the germ line mutations arise de novo. In the literature, a subset of apparently sporadic PCC has been investigated, of which one patient turned out to have NF1 after genetic screening. Additional examination of this patient confirmed the diagnosis of NF1 clinically. [35] There is no relationship between the type of *neurofibromin* mutation and the occurrence of PCC in NF1 patients. [36] Thus far, no somatic mutations in PCC of NF1 patients have been described. Malignancy in NF1-related PCC is reported in about 10%.

PCC-PGL SYNDROME

The spectrum of the PCC-PGL syndrome is expanding, but is predominantly characterized by the presence of one or more PCC, pPGL and sPGL. Originally, 4 loci, named PGL1 to PGL4, had been linked to the occurrence of PGL. All of these loci have now been shown to contain a tumour suppressor gene, which is responsible for this syndrome. Three of the 4 genes involved code for three of four subunits of the succinate-ubiquinone oxidoreductase complex II of the aerobic transport chain and the tricarboxylic acid cycle. PGL1 corresponds with *SDHD* on chromosome 11q23, which is one of the integral membrane proteins of the complex II. PGL3 corresponds with *SHDC* on 1q21, and is also an anchoring protein. PGL4 corresponds with *SDHB* on 1p36, the iron sulphur protein that functions as a catalytic domain. The fourth subunit of the SDH complex, SDHA, is not related to the occurrence of PCC or PGL, but instead homozygous mutations cause a rare

neurodegenerative disorder, known as Leigh syndrome. [37] Recently, the PGL2 locus on 11q13 was linked to *SDHAF2 (*also described as *SDH5),* and codes for succinate dehydrogenase complex assembly factor 2, a highly conserved cofactor of flavin adenine dinucleotide (FAD). [38] The biological effect of all *SDH*-related gene mutations is the accumulation of succinate due to loss of function of the complex (in SDHB, C and D), or as lack of flavination of SDHA due to loss of the cofactor SDHAF2. The accumulation of succinate prevents hypoxia inducible factor (HIF) hydroxylation, leading to accumulation of HIF. This process is similar to the pathogenesis of *VHL* mutations, also leading to HIF accumulation, causing a pseudohypoxic state (See figure 3).

The penetrance of the PCC-PGL syndrome depends on the gene involved and increases with age. [39] In addition, it appears to be influenced by environmental factors such as the oxygen tension of the patients' habitat. [40] The frequency of *SDHx* germ line mutations in PGL and PCC, and consequently the penetrance of the syndrome, has long been underestimated. This is partly because of the fact that PGL are slow-growing tumours, which do not come to clinical attention readily. Furthermore, the *SDHD* gene appears to be maternally imprinted, which leads to generation skipping, masquerading the hereditary component. [41] Therefore, a significant subset of apparently sporadic PGL has germ line mutations in the genes involved in the PCC-PGL syndrome. [12, 13, 42]

The PCC-PGL syndrome due to *SDHD* mutations is mainly characterized by multiple pPGL in combination with PCC and sPGL. Other tumours do not appear to occur in the context of *SDHD* mutations. *SDHB* germ line mutations are frequently involved in sPGL but have also been described in PCC and pPGL. Also, *SDHB* patients have been described in the literature with pPGL and renal cell carcinoma. [43, 44] Mutations in the gene encoding for SDHC are infrequently found. [45-48] Only a small subset of pPGL patients has been described with *SDHAF2* mutations, and no PCC have been found with either somatic or germ line mutations of the *SDHAF2* gene. [49] Recently the Carney-Stratakis syndrome has been described, where there is co-occurrence of PGL, or in one case PCC, and gastrointestinal stromal tumours (GIST) in families with germ line mutations in *SDHB, C and D*. [50, 51]

Malignancy in the *SDHx*-related PCC and PGL is more frequent than in the aforementioned syndromes. Especially in *SDHB*-related PCC and sPGL the risk of having metastasis at initial presentation or developing metastasis during follow-up is at least threefold higher than in sporadic or other syndrome-related PCC and sPGL. [52-54] Also, *SDHB* germ line mutations in pPGL give an increased risk of malignant behaviour, as described by Boedeker et al. in a large series of pPGL. [55] A single case of a malignant sPGL in a patient with a germ line *SDHC* mutation has been described. [48] Malignancy in patients with *SDHD* germ line mutations has been described, and a possible correlation has been found with the "Dutch founder mutation" (D92Y) of the *SDHD* gene. All 5 malignant SDHD-related tumours described by Havekes et al. were PGL, including one sPGL, and 4 pPGL that harboured the D92Y mutation. [56] Somatic mutations in the *SDHx* genes are rare, with only 2 somatic mutations described, one *SDHD* mutation in a PCC and one *SDHB* mutation in a sPGL. [57, 58] In patients with Cowden-like syndrome, characterized by breast, thyroid and endometrial carcinomas germ line mutations in *SDHB* and *SDHD* have been reported, although no PCC or PGL are reported in these patients so far. [59]

MALIGNANCY

Determining malignancy in both PGL and PCC has proven to be very difficult on pure histological criteria. Presently, according to the WHO, the only criterion to call a PCC or PGL malignant is the presence of a tumour metastasis [4]. However, in the newest AFIP fascicle there is an additional criterion for the diagnosis of malignancy in the form of extensive locally invasive growth. [60] Given the fact, that certain syndromes are characterized by multiple PGL or PCC, a metastasis is defined as the presence of chromaffin tissue in organs where normally no chromaffin tissue is present. The most common sites for metastases are liver, lungs, bone and lymph nodes.

HISTOPATHOLOGY

Determining malignancy based on histology of the tumour has not been very predictive and reproducible so far. [61-64] Initially it was Linnoila, who proposed 4 criteria that were associated with malignancy in a study of 120 PCC: extra-adrenal

location, coarse nodularity of the tumour, confluent necrosis, and the absence of hyaline globules. Of these, extra-adrenal location has now been supported by the frequent occurrence of *SHDB* germline mutations in sPGL. A more recent strategy for the prediction of PCC and sPGL behaviour is that by Thompson, who took 12 histopathological criteria (diffuse growth/large nests, tumour necrosis, high cellularity, cellular monotony, tumour cell spindling (even if focal), >3/10 HPF mitoses, atypical mitoses, tumour ingrowth in fat, capsular penetration, vascular invasion, profound nuclear pleomorphism, and nuclear hyperchromasia). If present, the first 8 criteria would score 2 points, the latter 4 score 1 point, adding up to a maximum of 20. Tumours with 4 points or more would be more likely to display aggressive behaviour. Although a few papers have supported the use of this so-called Pheochromocytoma of the Adrenal gland Scoring Scale (PASS), a more recent multi-centre study has indicated that there is a very high interobserver variation in the application of the PASS, which may lead to both false-positive and false-negative diagnoses. Meanwhile, a new study has been published with a similar approach as the PASS, adding the MIB1 labelling index to a number of histopathological and clinical criteria, and dividing the tumours over a three-tired grading system (well/moderately/poorly differentiated). Also this study awaits further confirmation. [61]

IMMUNOHISTOCHEMISTRY

Many attempts have been made to find immunohistochemical differences in benign and malignant PCC, pPGL and sPGL, but none have been applicable in the daily practice of routine immunohistopathology thus far. A major drawback in many of these studies is the low number of tumours investigated, the limited follow-up of cases, and the fact that authors did not always adhere to the strict criteria of malignancy, defined as the presence of chromaffin tissue in organs where normally no chromaffin tissue is present. The group of Salmenkivi have published a series of potential markers of malignancy, including tenascin, cyclo-oxygenase 2, and vascular endothelial growth factor. [65-68] However, these studies await further confirmation. Another promising set of markers consists of proteins involved in telomere maintenance. Several groups have shown that hTERT expression, determined by

RT-PCR, as well as expression of heat-shock protein 90 (HSP90) and telomerase activity are associated with malignant behaviour of PCC. [69]

MOLECULAR MARKERS

Due to the fact that no predictive markers could be identified by conventional histopathology or immunohistochemistry, molecular approaches have been used to elucidate both the molecular pathogenesis of PCC and PGL and the differences between benign and malignant tumours. Initially, loss of heterozygosity (LOH) studies have been carried out that showed that there were losses of chromosomal arms 1p, 3p, 3q, 17p, and 22q. [70] These studies were succeeded by whole genome analyses with the use of comparative genomic hybridization (CGH), which allows investigating gains and losses in all chromosomal regions in one experiment. The initial disadvantage of this technique was the limited resolution, which was later improved by high-resolution tiling bacterial artificial chromosome (BAC)-array CGH (see Chapter 2 and 3). Also the technique of SNP profiling, using high density microarrays is a promising thechnique, but no large cohorts have been studied yet. With conventional CGH we and others confirmed the abovementioned losses that had been described by LOH. [71, 72] In addition, we found frequent loss of 6q and 11q as well as gain of 9q and 17q. Of all losses and gains, loss of 6q and 17p appeared to be associated with malignant behaviour of PCC. (Petri et al. unpublished observations). In studies by Dannenberg et al. it was shown that chromosomal aberrations in pPGL were clearly different from the chromosomal aberrations in PCC, where pPGL revealed only limited number of aberrations per tumour, mainly concerning loss of 11q, corresponding with the location of *SDHD*. [42, 73]

Aims and outline

A major problem that remains in the understanding of the clinical behaviour of PCC is our lack of knowledge of the exact pathogenesis of these tumours. As explained in the introduction, there are currently no clinical, histological, immunohistochemical or even molecular criteria, which distinguish benign from malignant PCC. Therefore, this problem has been investigated in the first part of this thesis both by a genome-wide approach and by a candidate gene approach, partially on the basis of relevant chromosomal regions that appeared from the genome-wide analysis.

Over the past decade the main progress that has been booked in PCC and PGL research is their frequent hereditary basis in the context of various tumour syndromes. This is especially the case for the PCC-PGL syndrome, caused by the *SDHx* genes, from which the genotype-phenotype correlations and the spectrum of this syndrome are only partially known. Another issue that has become important is whether all PCC and PGL patients should have genetic testing all candidate genes. Now that the number of candidate genes has increased to seven (*RET, VHL, NF1, SDHB, SDHC, SDHD, SDHAF2*), and the overall frequency of germ line mutations appears to be 25-25% this would be costly, laborious and and technically demanding. Therefore, these various aspects of the PCC-PGL syndrome are addressed in the second part of this thesis.

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

- To search for genome-wide aberrations in truly sporadic PCC, with the aim to elucidate the pathogenesis of PCC in general, as well as to compare apparently benign with proven malignant tumours
- To test whether abnormalities in known candidate genes can distinguish benign and malignant PCC and PGL.
- To analyse the spectrum of clinical presentation of the PCC-PGL syndrome and to find cost-effective markers to identify and distinguish syndromal PCC and PGL

Outline

Chapter 2 will focus on sporadic PCC, with a genome-wide analysis of DNA aberrations in these tumours. Previous research has elucidated part of the aberrations in PCC, but series had been "polluted" with syndromic cases. Also, with the development of high-resolution tiling arrays small aberrations in the genome can be detected that could have been missed by the conventional CGH in the past. [74] This is followed by the analysis of malignant PCC with the same technique in chapter 3.

Many genes are known to be involved in malignant behaviour of various tumour types. In bladder cancer, endometrial carcinoma and breast cancer a gene located on 17p, the *p53* gene, is known to be overexpressed in malignant tumours. Also, cellular stress such as DNA damage or hypoxia induces *p53*. [75] As mentioned above, PCC and PGL are tumours that have a hypoxic or pseudohypoxic state, with a possible upregulation of the p53 protein. For other tumour types, e.g. high-grade primary brain tumours, a gene on chromosome 10, called *PTEN*, is a well-known TSG involved in malignant tumours. The latter gene is also known to be involved in PCC tumorigenesis in mouse models for PCC, and is therefore an interesting candidate gene in the pathogenesis of human malignant PCC.[76] These genes are discussed in chapter 4 and 5.

The last part of this thesis is devoted to characteristics of SDH-related tumours, both PCC and PGL. In chapter 6 we discuss the occurrence of *SDHD* germ line mutations in PCC. In the course of our analysis we came across an unusual and so far unique case of a somatic *SDHB* mutation in an sPGL, which is described in chapter 7. Finally, chapter 8 describes a screening method based on SDHB immunohistochemistry, which is a fast and cost-effective method that can be performed in a routine pathology laboratory, for the detection of SDH-related PCC, sPGL and pPGL. Chapter 9 will focus on SDHB immunohistochemistry and the follow-up in a large cohort.

Chapter 10 presents an overview of the current knowledge in the pathogenesis of hereditary and sporadic PCC and PGL. Future prospects for PCC and PGL research, including the possibility to distinguish benign from malignant tumours are discussed in the context of remaining gaps of knowledge.

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CHAPTER 2, ARRAY-COMPARATIVE GENOMIC HYBRIDIZATION IN SPORADIC BENIGN PHEOCHROMOCYTOMAS

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ABSTRACT

Pheochromocytomas (PCC) are catecholamine-producing tumors arising from the adrenal medulla that occur either sporadically or in the context of hereditary cancer syndromes, such as multiple endocrine neoplasia type 2 (MEN2), von Hippel-Lindau disease (VHL), neurofibromatosis type 1, and the PCC-paraganglioma (PGL) syndrome. Conventional comparative genomic hybridization (CGH) studies have shown loss of 1p and 3q in the majority of sporadic and MEN2-related PCC, and 3p and 11p loss in VHL-related PCC. The development of a submegabase tiling resolution array enabled us to perform a genome-wide high-resolution analysis of 36 sporadic benign PCC. The results show that there are two distinct patterns of abnormalities in these sporadic PCC, one consisting of loss of 1p with or without concomitant 3q loss in 20/36 cases (56%), the other characterized by loss of 3p with or without concomitant 11p loss in 11/36 (31%). In addition, we found loss of chromosome 22q at high frequency (35%), as well as the novel finding of high frequency chromosome 21q loss (21%). We conclude that there appear to be two subgroups of benign sporadic PCC, one of which has a pattern of chromosomal abnormalities that is comparable to PCC from patients with MEN2 and the other that is comparable to the PCC that arise in patients with VHL disease. In addition, genes on 21q and 22q might play a more important role in PCC pathogenesis than had been assumed thus far.

INTRODUCTION

Pheochromocytomas (PCC) are rare neuro-endocrine tumors arising from the adrenal medulla. Similar tumors arise from extra-adrenal chromaffin tissues, and are now referred to as sympathetic paragangliomas. (Baguet, et al. 2004) These tumors produce catecholamines, causing paroxysmal or sustained hypertension in the majority of patients. The elevated blood pressure can cause myocardial and cerebral infarctions, leading to morbidity and mortality. In up to 25% of PCC the tumors occur in the context of 4 hereditary tumor syndromes, including multiple endocrine neoplasia type 2 (MEN2), Von Hippel-Lindau disease (VHL), the pheochromocytoma-paraganglioma syndrome (PCC-PGL), and Neurofibromatosis type 1 (NF1). (Neumann, et al. 2002) Patients with MEN2 have mutations of the *RET* oncogene located at 10q11.21, the *VHL* tumor suppressor gene is located on 3p25.3, the *SDHD* gene in PGL-PCC patients is located on 11q23.1 and the related *SDHB* gene, also involved in the PCC-PGL syndrome is located on 1p36.13. Finally, the *NF1* gene is located on 17q11.2. In the remaining 75% of sporadic PCC somatic mutations of these 5 genes play a minor role, and the pathogenesis of these tumors is largely unknown.

Both syndrome-related PCC and sporadic PCC have been analyzed by comparative genomic hybridization (CGH). Interestingly, PCC from MEN2 patients, NF1 patients and the majority of sporadic PCC show similar genomic aberrations with a characteristic loss of 1p and 3q. (Cascon, et al. 2005; Dannenberg, et al. 2000; Edstrom, et al. 2000) VHL-related PCC, however, show distinct genetic aberrations consisting of loss of chromosome 3 and 11. (Hering, et al. 2006; Lui, et al. 2002)

With the introduction of high-resolution array-CGH, it has become technically feasible to study small (submegabase) chromosomal deletions and gains that escaped detection by conventional CGH due to the low resolution. This technique has facilitated the analysis of chromosomes 21 and 22, which were difficult to analyze in conventional CGH. Recently, copy number imbalances affecting chromosome 22 were confirmed by submegabase array-CGH in 44% (29/66) of PCC analyzed, a percentage that had not been described in conventional CGH. (Jarbo, et al. 2005) In addition, the tiling order of bacterial artificial chromosome (BAC) clones also has the

advantage to rule out mismapped clones, and gives precise breakpoint information. A similar array-CGH analysis has been performed on chromosome arm 1p, with 24 samples from hereditary and sporadic PCC, in which breakpoints of chromosome 1p could be identified precisely. These studies illustrate important differences between conventional and array-CGH. (Aarts, et al. 2006) To further clarify the pathogenesis of sporadic PCC, we analyzed 36 sporadic benign PCC using a tiling array consisting of 32,433 BAC clones.

MATERIALS AND METHODS

Patients and tumor samples

A series of 40 benign PCC of 40 patients was obtained from the archives of the Departments of Pathology of the Erasmus MC-University Medical Center Rotterdam, Maastricht University, Academic Medical Center Amsterdam, University Medical Center St. Radboud Nijmegen, and University Medical Center Utrecht, The Netherlands and stored at the Erasmus MC tissue bank. Patients with each of the following characteristics were excluded from this study: positive family history of an endocrine hereditary cancer syndrome, evidence of NF1, multiple PCC and/or PGL or the presence of germline mutations. In addition, none of the patients had other tumors related to MEN2, VHL or the PCC-PGL syndrome. After this selection, the study was performed with 36 benign truly sporadic tumors of 36 patients, of which 2 patients with sympathetic PGL. The cohort consisted of 17 females and 19 males. The mean age was 49 years (range 9-76), with an average follow-up of 4.4 years (n=25 patients, 11 patients were lost to follow-up). None of the patients had evidence of metastatic or recurrent disease during follow-up. The mean diameter of the tumors was 5.6 cm (range 2.5-18cm). The clinical data are detailed in table 1. Histology of all tumors was reviewed to confirm the diagnosis of PCC. None of the tumors had adverse histopathological characteristics as published by Thompson (Thompson 2002), supporting the diagnosis of a benign PCC in all cases. Tumor DNA was isolated from fresh frozen tumor tissue, except for 4 tumors in which no frozen tissue was available and DNA was isolated from paraffin embedded archival material. DNA from both fresh frozen and paraffin embedded material was isolated using the D-5000 Puregene DNA Isolation kit (Gentra Systems Minneapolis, MN) according to the manufacturers' recommendations.

Array CGH labeling and hybridization

The submegabase tiling arrays (SMRT) previously described by Ishkanian et al. were used, consisting of 32,433 overlapping BAC clones. (Ishkanian, et al. 2004) Test DNA and pooled reference male DNA (Novagen, Mississauga, Ontario), (300 ng each) were labeled with Cyanine-3 and Cyanine-5 (PerkinElmer, Woodbridge, ON,

Canada) respectively, according to a random priming protocol. After 18 hours of random priming the reference and test DNA were combined and 100 µl of Cot-1 DNA (Invitrogen, Burlington, ON, Canada) was added. The mixture was purified using Microcon YM-30columns (Millipore, Mississaga, ON, Canada). The purified mixture was washed with 200 µl of H_2O, and resuspended in 45 µl of DIG easy hybridization solution (Roche, Laval, QC, Canada), containing 20 mg/ml sheared herring sperm (Sigma-Aldrich, Oakville ON, Canada) and 10 mg/ml yeast tRNA (Calbiochem, Mississauga, ON, Canada). The probe was denatured at 85^0C for 10 minutes, followed by 60 minutes at 45^0C to block repetitive sequences, and subsequently applied in a volume of 43 µl to the slide surface after which cover slips were applied. The slides were incubated at 45^0C for 36 hours, washed 4 times 5 minutes in 0.1x saline sodium citrate (SSC), 0,1% SDS at room temperature, and finally rinsed by 0.1x SSC for 5 times and dried by centrifugation.

Array imaging and analysis

Hybridized slides were scanned using a charge-coupled device (CCD) camera system (Applied Precision, Issaquah, WA, USA), and analyzed by SoftWoRx Tracker Spot Analysis software (Applied Precision). Resultant data was normalized using a stepwise normalization process (Khojasteh, et al. 2005). Copy number alterations were identified via data visualization using custom software called "SeeGH" (freely available at http://www.flintbox.ca/technology.asp?tech=FB312FB) and loss, normal, and gain probabilities for each clone as determined by a modified hidden Markov model (Chi, et al. 2004; Shah, et al. 2006). Data were filtered based on both replicate standard deviation (data points with greater than 0.1 standard deviation removed) and signal to noise ratio (data points with a signal to noise ratio less than 10 removed).

Mutation analysis

Mutation analysis was performed on a CGH profile basis. Tumors showing loss of 1p were screened for *SDHB* mutations (n=26), those with loss of 3p for *VHL* mutations (n=11) and those with loss of 11q for *SDHD* mutations (n=10). Because no specific

profile is indicative of involvement of the *RET* proto-oncogene, all 35 tumors were tested for *RET* mutations. All exons including the intron-exon boundaries were screened, with the exception of *RET* for which only exons 10, 11, 13 and 16 were investigated. PCR and sequencing conditions have been previously described by Korpershoek et al. (Korpershoek, et al. 2007). Corresponding normal DNA was tested when an alteration was found in the tumor DNA.

Statistical analysis

Fisher's exact test was applied, using SPSS version 11.5. P values < 0.05 were considered to indicate statistical significance.

Table 1: clinical data

patient number	m/f	age	location	diameter (cm)	weight (gr)	Follow-up (months)	Hormone ***
1	m	43	A	-	42	72	***NE***, E, D
2	M	65	A	18	458	60	***NE***, E, D
3	F	70	A	2.5	-	60	E
4	M	59	A	13	240	48	***E***, NE
5	M	63	A	5	67	3	E
6	F	40	A	7	180	9*	NE
7	F	38	A	8	123	60	NE
8	M	29	A	12	710	37	-
10	F	25	EA	5	-	264	NE
11	M	24	A	2.5	-	72	***NE***, E
12	M	67	A	5.5	70	84	***NE***, E
13	M	24	A	6	66	4	E
14	M	46	A	7	246	4	NE
15	F	32	A	6	32	108	E
16	M	46	A	11.5	340	-	-
17	F	65	A	6	49	-	-
18	M	50	A	9	260	24	NE
19	M	56	A	16	-	12	-
20	M	43	A	7	79	-	-
21	F	63	A	4	-	-	-
22	M	53	EA	9	193	24	NE
23	F	52	A	97	93	84	NE, E, D
24	F	24	A	7	-	36	***NE***, E, D
25	F	70	A	3.5	234	-	E
26	F	70	A	8.5	137	-	-
27	M	78	A	6.5	50	-	-
28	M	41	A	4.2	-	2	NE, E, D
29	F	74	A	7	20	12	NE
30	M	29	A	4	-	30	ACTH
31	M	40	A	7	-	-	-
32	F	64	A	4.5	450	24	NE, E
33	M	9	A	10	-	18	-
34	F	48	A	4.8	-	-	-
36	F	26	A	7	-	-	ACTH
37	F	60	A	5	-	132	-
38	F	76	A	4	-	-	NE

A= adrenal

EA=extra-adrenal

* died, not related to PCC

** NE= norepinephrine, E=epinephrine, D=dopamine, ACTH=adrenocorticotrope hormone. Bold italic hormones are dominantly produced

RESULTS

Array CGH

All but 2 tumors included in this study yielded interpretable array results. A frequency plot, adding up percentages of loss and/or gain of each individual BAC clone of all 34 analyzable tumors is shown in figure 1. A representative karyogram of one tumor with highlighted losses and polymorphisms is shown in figure 2. Regions of previously reported natural copy number variation were not included in the analysis of these samples (Shah et al. 2006). The commonly observed aberrations in each individual tumor sample are summarized in table 2. Interestingly, there was an overwhelming number of copy number losses compared to copy number gains. In addition, most alterations encompassed whole chromosomes or chromosome arms.

In general, loss of 1p was found in 76% (26/34) of cases, where 88% (23/26) of these showed loss of the entire p-arm. Three tumors showed regional loss, consisting of 1p12-1p13.3, 1p31.3-1p36.33, and 1p12-1p35.1. Loss of 3q was observed in 59% (n=20) of the 34 tumors. No regional losses were observed. Loss of 1p and additional 3q loss was shown to be significantly associated ($p<0.05$).

Chromosome 3p loss was seen in 32% (n=11) of the tumors. In addition, eight of these tumors concordantly showed loss of chromosome 11p. Chromosome 3p loss was significantly associated with chromosome 11p loss ($p<0.05$).

Loss of 11q was found in 29% (n=10), with loss of the whole arm in 80% of these (n=8). The 2 tumors that had a regional loss showed an overlap from 11q14.3 until the telomeric end of the q-arm. Loss of chromosome 17p was found in 35% (n=12), but no regional losses were observed. Loss of 21q was observed in 21% (n=7), with one tumor showing a regional loss of 21q22.11 until the telomere. Loss of 21q was shown to be significantly associated with loss of 17p.

Finally, loss of chromosome 22 was found in 35% (n=12) with no regional losses. Interestingly, one tumor displayed a high negative Log_2Ratio, suggesting more than just a single copy loss of that region of the chromosome. However, with additional LOH analysis of several polymorphic markers in that region no homozygous deletion could be identified (data not shown).

Because of the association between 1p and 3q loss on the one hand and the association between 3p and 11p loss on the other hand, there appear to be two distinct groups of PCC. The first group (n=20) encompassed tumors showing 1p and/or 3q loss, without having concurrent 3p loss. The second smaller group (n=11) showed loss of 3p with or without concomitant loss of 11p. In addition, there was a limited number of PCC (n=3) that revealed no losses of the previously mentioned chromosomal regions (1p, 3p). One of these tumors showed gain of chromosomes 15 and 20. The second tumor had loss of the chromosomes 17 and 19, and gain of chromosome 7. The third tumor showed loss of chromosomes 11, 17 and 21.

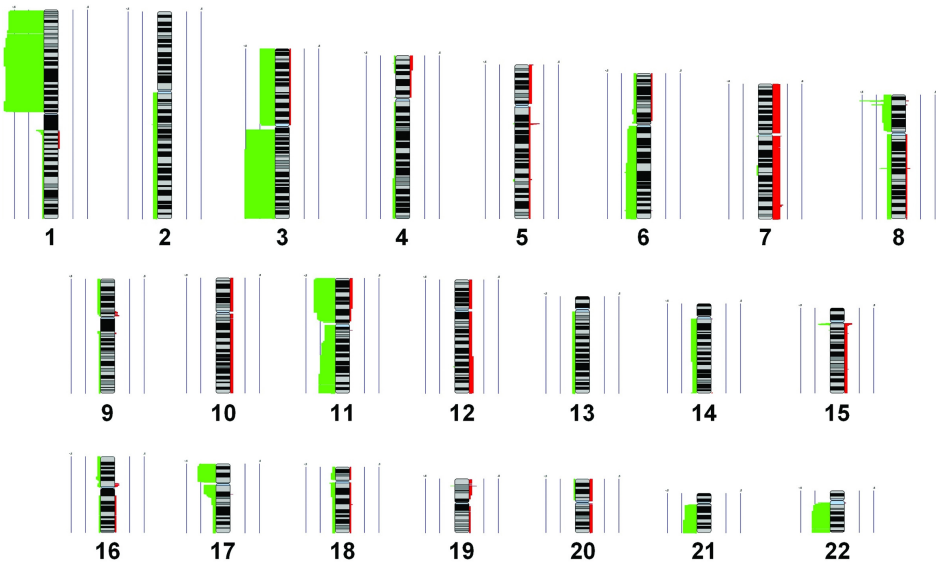

Figure 1: Frequency plot of all 38 PCC. Green lines on left side indicate loss of BAC clones situated in that area; red lines on right side indicate gain. Blue bars on either side represent 25 and 50%.

Mutation analysis

Sequence analysis of the four PCC susceptibility genes revealed mutations in 7 tumors, of which 3 occurred in *RET*, 1 in *SDHB* and 3 in *VHL* (Table 3). Analysis of corresponding germline DNA confirmed that 6 of the mutations were somatic. Corresponding germline DNA was not available from patient 12 with the *RET* p.M918T mutation. With the exception of the p.H50R polymorphism, no alterations were found in *SDHD*. Furthermore, two additional polymorphisms were found in *SDHB* which were both p.S163P (Table 3).

The patients with the *RET* mutations all had mainly norepinephrine overproduction, of the four patients somatic *VHL* mutations only two had available information on hormone production, both of these tumors showed only epinephrine overproduction.

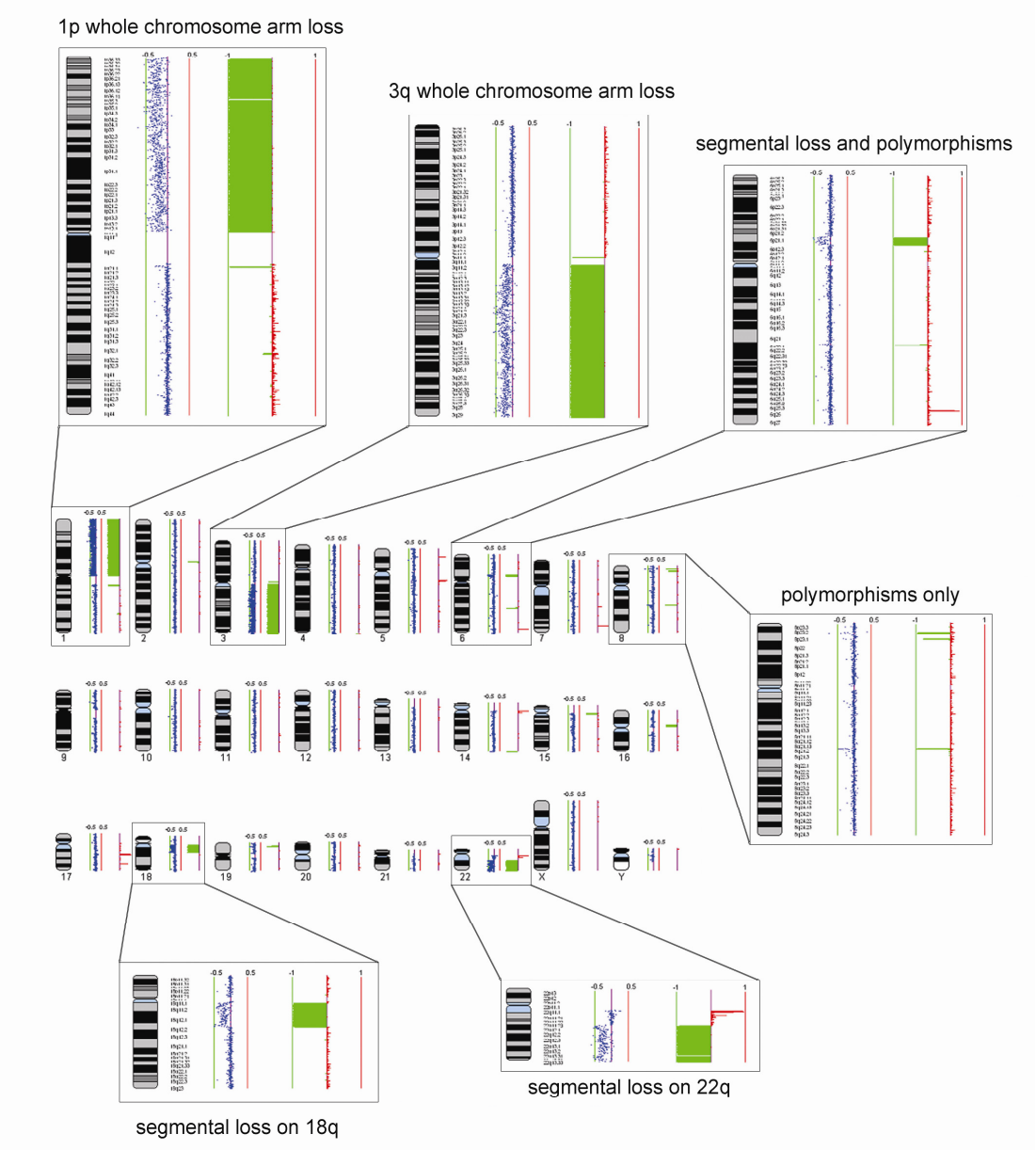

Figure 2: Karyogram of no. 28 showing the entire genome with magnified views of chromosomes 1, 3, 6, 8, 18, and 22. Each BAC clone on the array is displayed as a point representing the segment of the genome covered. The shift of each data point to the left of 0 represents a loss of copy numbers while a shift to the right represents a gain in copy numbers. The bars to the left and right of CGH data represent Log2 ratios of -0.5 and +0.5, respectively. To the right of the CGH data, hidden Markov model probabilities are displayed. Probabilities range from-1 (100% probability of copy number loss, green) to +1 (100% probability of copy number gain, red). Well-known polymorphisms are evident throughout the genome and are apparent in the magnified views of chromosomes 6 and 8.

Table 2: Loss of common regions

Patient number	1p	3p	3q	11p	11q	17p	21q	22q
1	*x*		*x*		*reg*	*x*	*x*	*x*
2	*reg*							*x*
3	*x*		*x*					*x*
4	*reg*	*x*	*x*			*x*	*x*	*x*
6	*reg*			*x*		*x*	*reg*	
7	*x*							*x*
8	*x*		*x*	*x*	*x*			
10	*x*							
12	*x*		*x*				*x*	
13	*x*	*x*	*x*	*x*				
14	*x*			*x*	*reg*			
16	*x*			*x*	*x*			
17	*x*	*x*	*x*	*x*				
19	*x*					*x*	*x*	*x*
20	*x*	*x*	*x*	*x*				
22	*x*	*x*	*x*			*x*		*x*
23	*x*		*x*			*x*	*x*	
25	*x*	*x*	*x*					*x*
26	*x*					*x*		*x*
27	*x*		*x*			*x*		
28	*x*		*x*					*x*
29	*x*		*x*					
30	*x*			*x*				
31	*x*		*x*			*x*		*x*
32	*x*		*x*			*x*		
15		*x*	*x*	*x*	*x*			
18		*x*		*x*				
24		*x*	*x*	*x*	*x*			
33		*x*	*x*	*x*	*x*			
34		*x*	*x*	*x*	*x*			
21						*x*		
37				*x*	*x*	*x*	*x*	

X=loss, reg-regional loss (see text for locations)

Table 3: Mutation analysis results

Patient number	Gene	Mutation→ cDNA	Mutation→protein	Hormone **
28	*RET*	c. 1894_1899 del GAGCTG	**p.E632_L633del**	NE, E, D
6	*RET*	c.2332G>A	**p.V778I**	NE
12*	*RET*	c.2753T>C	**p.M918T**	NE, E
10	*SDHB*	c.299C>T	**p.S100F**	NE
5	*SDHB*	c.487C<T	*p.S163P*	E
29	*SDHB*	c.487 C<T	*p.S163P*	NE
23	*SDHD*	c.149A>G	**p.H50R**	NE, E, D
20	*VHL*	c.169_212delGGG_GCC	**p.G57LfsX60**	-
15	*VHL*	[c.364G>A;c.365C>T	**p.A122I**	E
13	*VHL*	c.482G>A	**p.R161Q**	E
34	*VHL*	c.500G>A	**pR167Q**	-

Mutations are in bold, polymorphism in italic. *It is not known whether this mutation was gerrmline or somatic. All other mutations were somatic. ** NE= norepinephrine, E=epinephrine, D=dopamine

DISCUSSION

This study represents the first comprehensive analysis of a large series of sporadic benign PCC using a genome-wide submegabase-resolution tiling array (SMRT-array). On the basis of DNA aberrations we could distinguish 2 distinct subgroups of PCC, one with loss of 1p and/or 3q, representing more than 56% of all PCC investigated, and a second, smaller, group with loss of 3p with or without concurrent 11p loss, representing 32% of these PCC. These findings may relate to the different pathways of tumorigenesis in PCC.

The majority (76%) of PCC in this analysis of 36 benign sporadic tumors showed loss of 1p, which is comparable to the frequency of loss that has been reported in previous studies. (Cascon et al. 2005; Dannenberg et al. 2000; Edstrom et al. 2000) Moreover, most PCC in our study (22/36) had loss of the entire short arm of chromosome 1, in contrast to our previous study, where we found regional 1p loss in half of the cases. (Aarts et al. 2006) We speculate that the observed difference with our own previous studies and with series from others is related to the composition of the study group, which in the present study only comprised sporadic cases. In the few cases with partial loss, no minimal region of common loss could be determined, preventing us to speculate on the presence of one region harboring tumor suppressor genes on 1p, which have been postulated by various authors. (Aarts et al. 2006; Geli, et al. 2005) Still, based on the high frequency of 1p loss in PCC, we support the idea of one or more tumor suppressor genes on this chromosome arm. In most cases 1p loss was accompanied by loss of 3q, which occurred in 62% of all cases with 1p loss, a figure that is comparable with that reported in the literature. (Cascon et al. 2005; Dannenberg et al. 2000; Edstrom et al. 2000) Four PCC were found with loss of 3q without chromosome 1p loss. These 4 tumors displayed loss of the entire chromosome 3 in combination with loss of the entire chromosome 11 (see below).

Apart from the large group of PCC displaying a $1p^{-}/3q^{-}$ genotype, a smaller group of PCC was identified with loss of 3p, which was frequently accompanied by loss of 11p. This pattern of loss has been mentioned previously in PCC from VHL patients, but has so far not been related to a subgroup of sporadic PCC. (Hering et al. 2006;

Lui et al. 2002) In order to exclude that this subgroup represented occult VHL disease, we performed mutation analysis of the entire VHL coding region, in which we could not detect germline mutations. However, we found 3 cases showing somatic VHL abnormalities. The p.R161Q and p.A122I VHL mutations have been described previously in an apparently sporadic PCC. (Neumann et al. 2002) The p.G57LfsX59 has never been described before. Although epigenetic silencing of the VHL gene by hypermethylation is not inconceivable, as seen in familial and non-familial renal cell carcinoma, no methylation has been described in PCC. (Prowse, et al. 1997) The fact that a subgroup of sporadic PCC, without VHL germline mutations, shows an identical genotype as VHL-related PCC, leads to the suggestion that this group of PCC follows similar pathways of tumorigenesis. Indeed, this might also be the case for MEN2-related PCC and the abovementioned subgroup of sporadic PCC, which have been shown to have similar frequencies of 1p and 3q loss in previous studies. (Cascon et al. 2005; Dannenberg et al. 2000; Edstrom et al. 2000)

In addition to losses affecting chromosomes 1, 3, and 11, we observed the highest frequency of loss in chromosomes 21 and 22, concerning 21% and 35% of all PCC, respectively. Loss of chromosome 21 has so far not been described at this relatively high frequency in benign sporadic PCC. All tumors with 22q loss also displayed 1p loss, and all but one tumor with chromosome 21q loss also revealed 1p loss. Therefore, these regions could be involved in the spectrum of the sporadic and/or MEN2-related PCC. However, as there was only 1 tumor with regional loss of 21q, we cannot draw conclusions with respect to the presence of potential tumor suppressor genes on this chromosomal arm. Previous reports on the loss of chromosome 22 have been based on LOH-analysis and showed loss of chromosomal bands 11.21 to 13.31, or 11.21 alone. (Khosla, et al. 1991; Shin, et al. 1993; Tanaka, et al. 1992) Furthermore, in a recent array-CGH study on 66 PCC, copy number alterations of 22q were found in 44%. (Khosla et al. 1991; Shin et al. 1993; Tanaka et al. 1992) In 8 of these cases (8/29) there was regional loss with a minimal region of common overlap from 22q11.23 until the telomeric end of chromosome 22. One additional interstitial deletion was found from 22q11.23 to 22q12.3. In our analysis we did not find a regional loss concerning chromosome 22q. These findings might indicate the presence of tumor suppressor genes on 22q that

could be involved in the pathogenesis of sporadic PCC, however due to the large regions involved, combined with a gene-rich chromosome it is not possible at this time to pinpoint candidate genes.

Apart from the 31 PCC that could be fitted in either of the two groups already mentioned, there were 3 tumors that did not have losses in 1p, 3p, or 3q. No common pattern could be derived from these 3 tumors, although it is interesting to note that 2 of these presented with chromosomal gains. It is of relevance to note that none of these were from an extra-adrenal location, as these 2 PGL presented with a CGH pattern that fitted well with that of the PCC.

Taken together, the predominant chromosomal abnormalities found in this genome-wide array-CGH study of 36 benign sporadic PCC concern losses of various chromosomal arms, most notably 1p, 3p, 3q, 11p, 11q, 17p, 21q, and 22q. By contrast, we observed no consistent gain of any chromosomal region. Furthermore, we could not confirm abnormalities of other chromosomes that have been suggested in the literature, such as aberrations of chromosomes 2 and 16. (Dahia, et al. 2005) In addition, there appear to exist two different groups of benign sporadic PCC, each of them characterized by a specific genotypic pattern of chromosomal loss: a predominant form showing a 1p/3q- genotype, which can also be found in MEN2-related PCC; and a minor form showing a 3p-/11p- genotype, which can also be found in VHL-related PCC. Apart from this, the high frequency of loss of 21q and 22q indicates that these chromosomal arms might also be important in the pathogenesis of benign sporadic PCC.

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CHAPTER 3, MALIGNANT PHEOCHROMOCYTOMAS SHOW SPECIFIC GENOMIC ALTERATIONS IN HIGH-RESOLUTION ARRAY COMPARATIVE GENOMIC HYBRIDISATION

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ABSTRACT

Background

Pheochromocytomas (PCC) are rare endocrine tumors arising in the adrenal medulla that metastasize in 10 to 30 percent of sporadic cases. Metastases can occur up to several decades after the development of the primary tumor. Furthermore, there are no reliable criteria that can predict the potential malignant behavior of a PCC.

Materials and Methods

To obtain more detailed information about the genomic alterations in malignant PCC, we have performed high-resolution comparative genomic hybridization on 16 tumors from 14 patients, using a tiling array consisting of 32,433 BAC clones, enabling us to observe sub-megabase deletions or amplifications.

Results

CGH revealed loss of chromosome 1p in 94% malignant PCC as the most frequent aberration, with loss of 8p in 9 of the 16 (56%) of the tumors as second most frequent abnormality. Gain was most frequently seen in chromosomal regions 4p, 17q, 18q, 19p and 20p (all in 38% of cases).

Conclusion

The results of our CGH study show that malignant PCC show distinct and specific genomic alterations, which differ from benign PCC, that predominantly display combined loss of 1p and 3q or combined loss of 3p and 11p. These data suggest that genomic alterations are different between benign and malignant PCC, and could be used for the development of diagnostic tests.

INTRODUCTION

Pheochromocytomas (PCC) and sympathetic paragangliomas (sPGL) are rare neuroendocrine tumors arising from neural crest-derived chromaffin cells. PCC are located in the adrenal medulla, whereas the morphologically related sPGL are located anywhere along the sympathetic chain. Both PCC and sPGL produce catecholamines, leading to a wide range of symptoms (Lenders, et al. 2005; Timmers, et al. 2008). Although the majority of PCC and sPGL are reported to be sporadic, up to 24% of PCC and sPGL can arise in the context of hereditary tumor syndromes (Neumann, et al. 2002). These familial syndromes include: multiple endocrine neoplasia type 2 (MEN 2), Von Hippel-Lindau disease (VHL), neurofibromatosis type 1 (NF1), and PCC-PGL syndrome (Benn, et al. 2006; Dannenberg, et al. 2005; Gimenez-Roqueplo, et al. 2003).

Malignancy in PCC and sPGL is defined as the presence of metastases at sites where chromaffin tissue is normally not present, such as lymph nodes, bones or lungs. The risk of malignancy in PCC and sPGL ranges from 10-36%, with the highest percentages of malignant tumors described in sPGL and in patients with *SDHB* germ line mutations (Amar, et al. 2007; Brouwers, et al. 2006; Gimenez-Roqueplo et al. 2003; Neumann, et al. 2004; Van Nederveen, et al. 2006). Interestingly, other syndrome-related PCC have a low incidence of malignancy.

Attempts have been made to distinguish benign from malignant PCC by histology (Kimura, et al. 2005; Thompson 2002) and immunohistochemistry (de Krijger, et al. 1999; Salmenkivi, et al. 2003; van der Harst, et al. 2002). However, none of the criteria or markers has been proven to be of clinical use. Although syndrome-related and sporadic malignant PCC have been analyzed by comparative genomic hybridisation (CGH) (Cascon, et al. 2005; Dannenberg, et al. 2000; Edstrom, et al. 2000), no conclusive regions have been identified in the pathogenesis of malignant PCC. With the introduction of high-resolution array-CGH, it has become technically feasible to study small (submegabase) chromosomal deletions and gains that escaped detection by conventional CGH due to the low resolution. In addition, the tiling order of bacterial artificial chromosome (BAC) clones also has the advantage to rule out mismapped clones, and gives precise breakpoint information. To further

clarify the pathogenesis of malignant PCC, we analyzed 16 tumors from 14 patients using a tiling array consisting of 32,433 BAC clones and compared these data with those of a series of benign PCC analyzed with the same platform.

MATERIALS AND METHODS

Patients and tumor samples

A series of 16 tumors from 14 patients was obtained from the archives of the Departments of Pathology of the Erasmus MC-University Medical Center Rotterdam, University Medical Center St. Radboud Nijmegen, and Amsterdam Medical Center, The Netherlands and stored at the Erasmus MC tissue bank. One sample was obtained from Kantonsspital Zürich, Switzerland. All tumors were tested for the presence of *SDHB* and *SDHD* mutations using primers and PCR and sequencing conditions described preciously (Korpershoek, et al. 2007). Three patients had mutations, one had a germline *SDHD* mutation (p.Leu95Pro) and two had a *SDHB* mutation (p.Asp161MetfsX14 and p.Cys243Ser). In addition, there were no clinical signs for the presence of MEN 2, VHL or NF1 syndrome in these patients. The cohort consisted of 5 females and 9 males. The mean age was 46 years (range 25-77), with an average follow-up of 4.6 years (n=11 patients, 3 patients were lost to follow-up). Five patients died of disease during follow-up. Only one patient had no residual disease during 4 years of follow-up. The mean diameter of the tumors was 9.5 cm (range 3.5-21cm). The clinical data are detailed in table 1. Histology of all tumors was reviewed to confirm the diagnosis of PCC or PCC metastasis. Tumor DNA was isolated from fresh frozen tissue (n=12), and paraffin embedded archival material (n=4). The tumor cell content of samples used for DNA extraction was at least 70%. DNA from both fresh frozen and paraffin embedded material was isolated using the D-5000 Puregene DNA Isolation kit (Gentra Systems Minneapolis, MN) according to the manufacturers' recommendations.

Array CGH labeling and hybridization

The submegabase tiling arrays (SMRT) previously described by Ishkanian et al. were used, consisting of 32,433 overlapping BAC clones. (Ishkanian, et al. 2004) Test DNA and pooled reference male DNA (Novagen, Mississauga, Ontario), (300 ng each) were labeled with Cyanine-3 and Cyanine-5 (PerkinElmer, Woodbridge, ON, Canada) respectively, according to a random priming protocol. Labeling and

hybridization procedures have been previously described (van Nederveen, et al. 2009).

Table 1. Clinical data

patient	sex	age of oneset	location primary	diameter	metastases	syndrome	metastasis detection	follow-up
1	M	32	extra-adrenal	21	lymph node, bone	*SDHB* p .Asp161Metfs X14	at diagnosis	DOD (7yr)
2	M	61	adrenal	nk	lymph node	none	2 years	AWD (1yr)
3	F	35	adrenal	9	lymph node	none	at diagnosis	nk
4	F	70	adrenal	12	liver, lymph node	none	1 year	DOD (1yr)
5	F	35	extra-adrenal	3,5	bone	none	5 years	DOD (10yr)
6	F	30	adrenal	7,5	liver, lung	none	4 years	DOD (5yr)
7	M	42	adrenal	7,5	bone	none	nk	nk
8	M	77	adrenal	nk	lymph node, omentum	none	2 years	nk
9	F	63	extra-adrenal	nk	lymph node, bone	*SDHB* p.Cys243Ser	nk	DOD (10yr)
10	M	46	extra-adrenal	7,5	lymph node, bone	None	nk	AWD (2yr)
11	M	39	adrenal	15	lymph node, liver	None	at diagnosis	AWD (6yr)
12	M	44	adrenal	nk	gut, peritoneum	None	1 year	AWD (2yr)
13	M	42	adrenal	8,5	bone, lung	None	1 year	AWD (3yr)
14	M	25	adrenal	4	lymph node	*SDHD* p. Leu95Pro	at diagnosis	NED (4yr)

nk: not known
DOD: Died of disease
AWD: Alive with disease
NED: No evidence of disease

Array imaging and analysis

Hybridized slides were scanned using a charge-coupled device (CCD) camera system (Applied Precision, Issaquah, WA, USA), and analyzed by SoftWoRx Tracker Spot Analysis software (Applied Precision). Resultant data was normalized using a stepwise normalization process (Khojasteh, et al. 2005). Copy number alterations were identified via data visualization using custom software called "SeeGH" (freely available at http://www.flintbox.ca/technology.asp?tech=FB312FB) and loss, normal, and gain probabilities for each clone as determined by a modified hidden Markov model (Chi, et al. 2004; Shah, et al. 2006). Data were filtered based on both replicate Standard Deviation (SD; data points with greater than 0.1 SD removed) and signal to noise ratio (data points with a signal-to-noise ratio < 10 removed). Comparison of data obtained from benign and malignant PCC. The CGH results obtained in this study were compared with the results of a previous study in which we performed array-CGH on a series of 32 benign PCC, of which 3 samples were not available for this comparison. (van Nederveen et al. 2009) A straightforward clone-by-clone comparison described previously (van Dekken, et al. 2006) was performed to determine clones that were differentially altered between the malignant and benign PCC groups. First, the $\log_2$ ratios of chromosomal gains and losses were calculated by an algorithm using flexible thresholds based on the SD of the data sets of the specimens. SDs over windows of five consecutive clones were averaged, sliding along the chromosome one clone at a time. Thresholds for gains and losses were defined empirically as 2.5 and 2.5 SD, respectively, using the combined data set. This procedure resulted in sample-dependent detection of genomic alterations with minimal interference of noise from the DNA isolated from formalin-fixed tissue. Subsequently, Fisher's exact test was applied, using SPSS version 15. (SPSS Inc., Chicago, IL) to determine the differentially gained or lost clones in the two tumor groups. P-values less than 0.05 were considered to indicate statistical significance.

RESULTS

Array CGH

All arrays included in this study yielded interpretable results. A frequency plot, adding up percentages of loss and/or gain of each individual BAC clone of all 14 patients is shown in figure 1. Regions of previously reported natural copy number variation were not included in the analysis of these samples (Shah et al. 2006). For the paired tumor/metastasis patients the chromosomal aberrations were mostly overlapping, an example of which is shown in figure 2 (patient 11). The most frequent alterations occurring in at least 38% of PCC/sPGL or more are listed in table 2.

The known hereditary PCC with an *SDHB* gene mutation (patients 1 and 9) both showed loss of the *SDHB* locus on 1p36, and the PCC with an *SDHD* gene mutation (patient 14) showed loss of the *SDHD* gene locus at 11q23. Interestingly, the profiles of the 2 *SDHB*-related PCC showed no overlap of genomic aberrations apart from loss of the whole arm of 1p. The *SDHD*-related PCC showed multiple genomic aberrations, both losses and gains, involving numerous chromosomes, including loss of 3q and 11p.

Loss of 1p was the most frequent genomic aberration, seen in 15 of the 16 PCC investigated (94%), of which 11 showed loss of the entire chromosomal arm. The smallest region of overlap in this series of malignant PCC was from 1p34.3 to 1p31.1. The second most frequent alteration was loss of chromosome 8p, seen in 9 of the 16 tumors (56%), of which 6 showed loss of the entire chromosome 8p. As the third most frequent genomic alteration, loss of both chromosome 11q and 17p was found in half of the PCC. In 4 tumors, loss of both these chromosomal arms was demonstrated. The other cases showed loss of either 11q or 17p. Loss of the entire chromosomal 17p was seen in all cases, whereas regional loss of chromosome 11q was shown in 3 tumors.

The most frequently observed gains involved chromosomes 4p, 17q, 18q, 19p and 20q, which occurred in 6 of the 16 PCC (38%) for all previously mentioned chromosomes.

In total, CGH revealed 184 gains or losses of a (part of a) chromosomal arm in the 16 tumors tested. These alterations did not include the numerous small regional losses

or gains. These genomic aberrations seemed to be independent events, occurring usually in a single tumor.

Comparison of benign and malignant PCC

There were only 2 chromosomal regions showing statistically significant differences between benign (n=29) and malignant (n=14) tumors. Both involved areas of loss and concerned 3q and 11p. In 3q as well as in 11p clones distributed over the entire arm reached statistical significance, with the highest levels in 3q21.1 ($p<0.001$) and 11p11.2 ($p<0.0035$). There were 6 benign PCC (21%), which did not have loss of 3q or 11p and there were 3 malignant PCC (21%) with loss of 3q and/or 11p. This difference (79% versus 21% for loss of 3q/11p) was statistically significant ($p<0.05$).

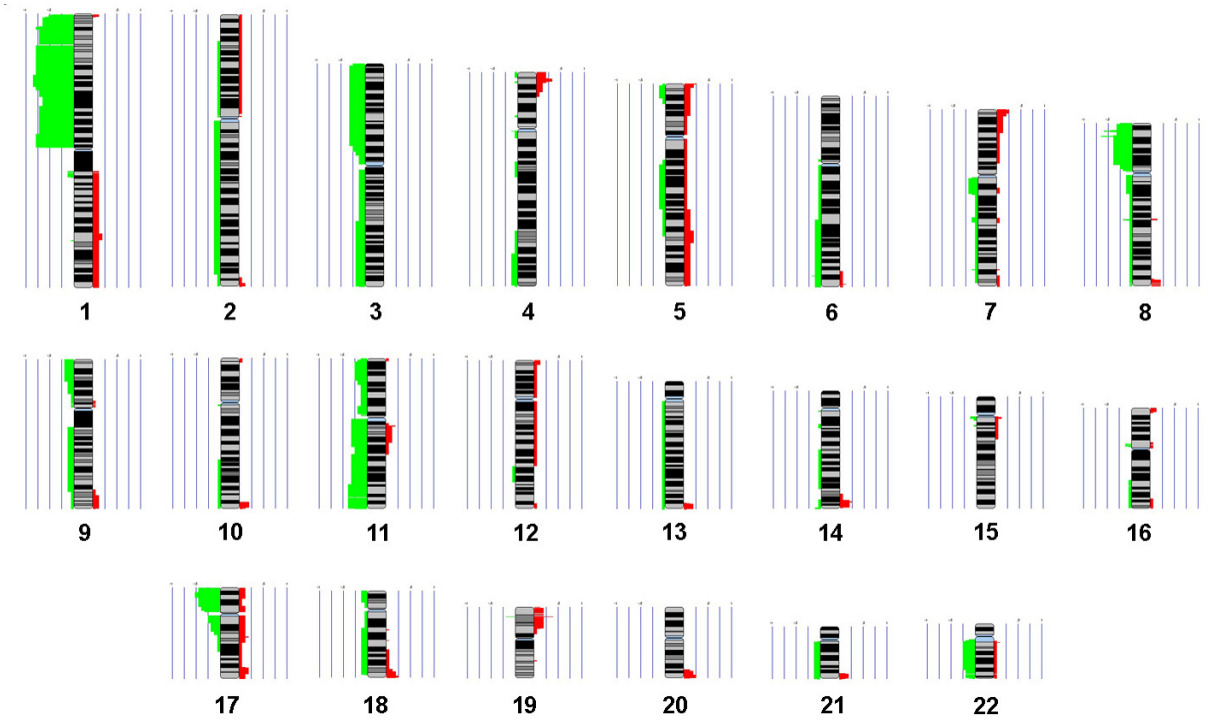

Figure 1: Frequency plot of all 14 malignant PCC. Green lines on left side indicate loss of BAC clones situated in that area; red lines on right side indicate gain. Blue bars on either side represent 25, 50, 75 and 100%.

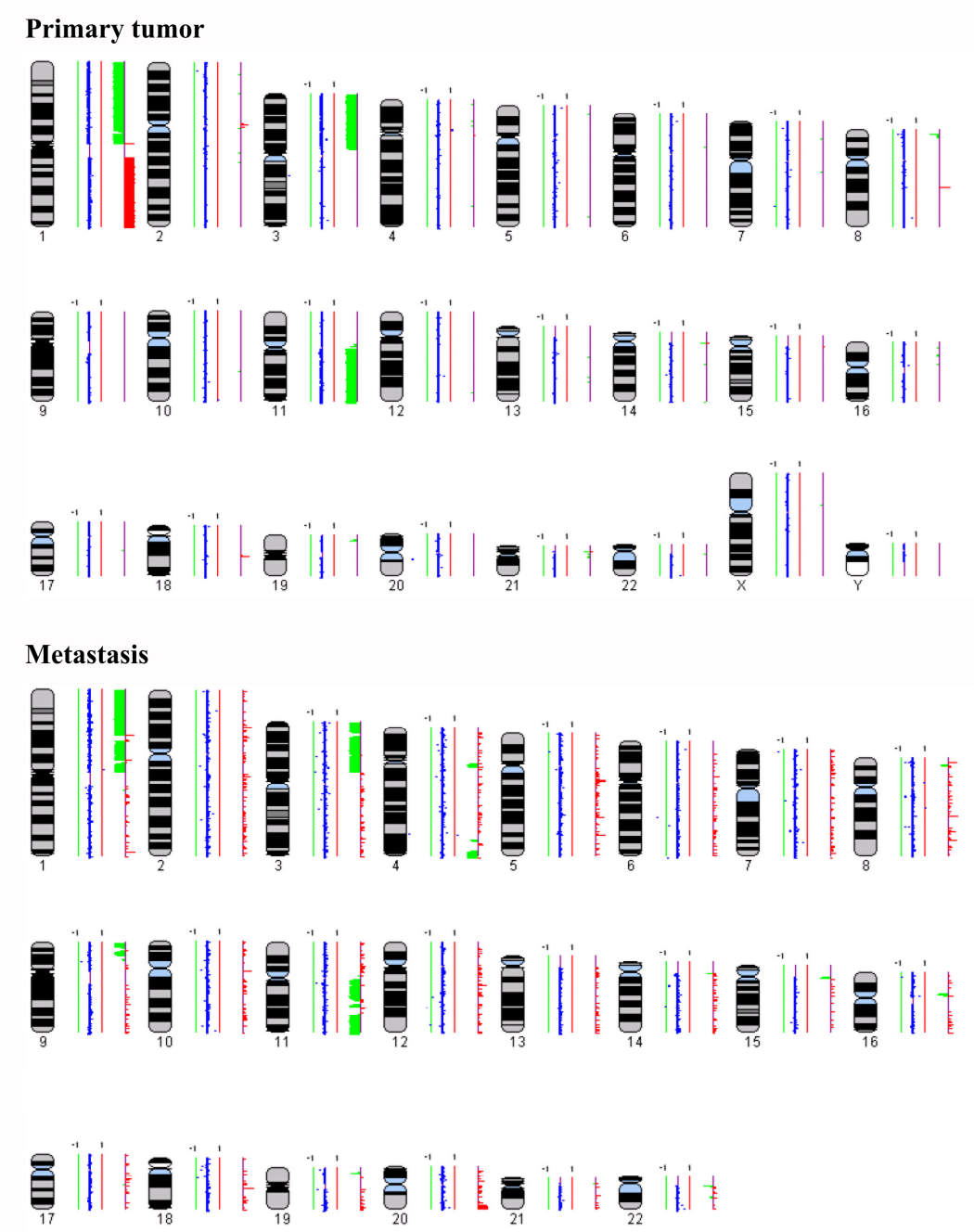

Figure 2: Karyograms of primary tumor and metastasis of patient 11 showing similar losses of chromosomes 1p, 3p and 11q.

Table 2. CGH results of common alterations

Tumor	Chromosome														
	1p	3p	4p	7q	8p	11p	15q	17p	17q	18q	19p	19q	20q	21q	22q
1	L	-	-	-	-	L	-	-	-	-	-	-	-	-	-
2	-	-	-	-	L	-	-	L	-	RG	-	-	-	L	-
3	L	L	-	-	L	-	-	L	-	RG	G	-	-	-	-
4	RL	-	TG	-	L	-	RG	L	L+G	TG	L+G	-	TG	TG	L
5A	L	-	-	RL	-	-	-	-	-	-	-	-		-	-
5B	L	G	G	L+G	-	-	L+G	L	G	G	L+G	L+G	L+G	L+G	L+G
6	L	L	-	RL	-	L	L	L	L	-	L	L	RL	-	L
7	L	-	TG	-	L	G	RG	L	L+G	RG	G	RG	G	TG	G
8	RL	-	-	RL	TL	-	RL	L	-	-	TL	TL	-	-	L
9	L	-	-	-	L	-	-	-	-	-	-	-	-	L	L
10	RL	-	TG	-	L	L	-	RG	G	TG	G	L+G	RG	TG	-
11A	L	L	-	-	TL	-	-	-	-	-	TL	-	-	-	-
11B	L	L	-	-	-	-	-	-	-	-	-	-	TG	-	-
12	L	-	TG	L	-	-	-	L	-	RL	-	-	-	-	L
13	L	-	-	-	-	-	L+G	-	-	L	-	-	-	-	-
14	RL	L	TG	-	-	L	RG	RG	-	-	L+G	L+G	RG	RG	G

L: Loss of entire chromosomal arm
RL: Regional loss
TL: Loss of telomeric region
G: Gain of entire chromosomal arm
RG: Regional gain
TG: Gain of telomeric region

DISCUSSION

Many studies have attempted to come up with markers that predict malignant behavior of PCC. However, markers that are useful in everyday practice still have to be discovered. Consequently, a PCC can only be called malignant when a metastasis has been demonstrated. Some PCC patients have synchronous metastases, but most patients present with metachronous metastases after a disease-free interval of one or more decades (Goldstein, et al. 1999). To investigate whether we could find loss or gain of genomic regions that could help predicting malignant behavior, we have performed array-CGH on a series of 14 proven malignant PCC and 2 corresponding metastases, using a genome-wide submegabase-resolution tiling array (SMRT-array). In this study we have demonstrated that malignant PCC present multiple genomic aberrations, of which loss of chromosome 1p is the most frequent. In addition, loss of chromosomes 8p, 11q and 17p, and gain of chromosomes 4p, 17q, 18q, 19p and 20q were also seen in more than 35% of the tumors. When comparing these data with those obtained in a previously analyzed and published series of benign PCC, there were two regions, 3q and 11p, that were more frequently lost in benign than malignant PCC.

Loss of 1p has been described in benign as well as malignant tumors, as illustrated by a previous study we performed (Dannenberg et al. 2000). In that study 84% of the benign and 90% of the malignant PCC showed loss of 1p. Another study used loss of heterozygosity analysis to investigate the genetic alterations of a series of PCC, and demonstrated loss of 1p in 67% of malignant PCC (Edstrom, et al. 2002), which is less than our frequency. However, this could be due to the small number of malignant tumors investigated. An additional study also reported a low frequency of 1p loss in malignant PCC (50%), but a different definition for malignancy was used, including cases with locally invasive behavior (Cascon et al. 2005). Although loss of 1p seems to be an important step in the pathogenesis of malignant PCC, our high frequency could also be due to the relatively small number of samples studied.

Besides 1p, other chromosomal regions were also affected in malignant PCC. The tumors showed loss of (a part of) 8p as the second most common genetic alteration (56%). Loss of 8p was also demonstrated by other studies, who found loss of 8p in

30% (Dannenberg et al. 2000) and 33% (Cascon et al. 2005) of malignant PCC. In contrast, at least on other study did not report loss of 8p as a frequent alteration in (malignant) PCC (Edstrom et al. 2000). Again, this could be due to the limited number of malignant PCC used. Other frequent losses observed in the present study included those of 11q and 17p, which had already been correlated with malignant behavior of PCC (Dannenberg et al. 2000; Edstrom et al. 2000).

The most frequent gains included 4p, 17q, 18q, 19p and 20q, all of which have been associated with PCC pathogenesis, with the exception of 4p (Cascon et al. 2005; Dannenberg et al. 2000; Edstrom et al. 2000). In concurrence, gain of (a part of) 19p was also demonstrated by Edstrom as more frequently present in malignant tumors (PCC and PGL). However, this difference was not significant as there was also 19p gain present in the benign tumors. (Edstrom et al. 2000)

Apart from these whole-arm chromosomal gains and losses, many small regional alterations were seen in most investigated tumors, indicating that malignant tumors are genetically very instable, whereas benign PCC in general are not. This could be due to the size of malignant PCC, which are usually larger than benign PCC (Shen, et al. 2004), and therefore could gather more genetic alterations. However, Dannenberg et al showed that there was no correlation between tumor size and the number of alterations (Dannenberg et al. 2000). Furthermore, recent findings have demonstrated that malignant PCC are genetically more heterogeneous than benign PCC (unpublished observations), which could be due to the genetic instability of these malignant tumors.

Apart from the demonstration of the most frequent DNA abnormalities in malignant PCC, we also undertook this study with the aim to compare the results with that of a previously published study of benign PCC (van Nederveen et al. 2009). For both studies, the same array platform was used and the same analytical methodology was used, so results are entirely compatible and comparable. We demonstrate that there are two chromosomal regions, 3q and 11p, both of which are frequently lost in benign as opposed to malignant PCC. In line with this, the few studies that have investigated malignant PCC for genomic aberrations using CGH also showed 3q loss in less than 25% of malignant tumors (Cascon et al. 2005; Dannenberg et al. 2000; Edstrom et al. 2000). The statistically significant difference in loss between benign versus malignant

PCC in 3q and/or 11p may allow the development of an algorithm to discriminate the two groups of PCC, provided that these data are confirmed in a larger independent study. Interestingly, all 3 cases of malignant PCC with loss of 3q and/or 11p contained an *SDHB* or *SDHD* mutation, implying that no sporadic malignant PCC in this study had 3q or 11p loss. Although this finding might be related to the relatively small sample size, this suggests that after mutation analysis tumors with 3q or 11p loss could be considered as benign.

In conclusion, our CGH results have shown that malignant PCC display specific genomic alterations. These alterations included loss of 1p and 8p, and gain of 4p, 17q, 18q, 19p and 20q, which seem to occur almost exclusively in malignant PCC. In addition, malignant PCC show infrequent loss of 3q and 11p. These data suggests that a diagnostic test might be developed to predict malignant behavior of PCC.

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CHAPTER 4, *P53* ALTERATIONS AND THEIR RELATIONSHIP TO *SDHD* MUTATIONS IN PARASYMPATHETIC PARAGANGLIOMAS

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ABSTRACT

Experimental and observational evidence suggests that chronic hypoxic stimulation can induce parasympathetic paraganglioma. This is emphasized by the identification of germline mutations in genes of the mitochondrial succinate dehydrogenase enzyme complex II in hereditary paraganglioma. Because of inactivating mutations in the succinate dehydrogenase subunit B (*SDHB*), C (*SDHC*), D (*SDHD*) gene, the paraganglia undergo a chronic hypoxic stimulus leading to proliferation of paraganglionic cells. Hypoxia is a known inducer of p53 up-regulation, which triggers cell cycle arrest and apoptosis. Inactivation of the p53 pathway, by gene mutation or by MDM2 overexpression, would enable cells to escape from cell cycle arrest and apoptosis and could contribute to tumorigenesis.

To determine whether p53 inactivation plays a role in paraganglioma tumorigenesis, we investigated a series of 43 paragangliomas of 41 patients (of whom 24 patients harbored a germline SDHD mutation) for mutations in *p53* exons 5-8 by PCR-SSCP. In addition, these tumors were investigated for p53 and MDM2 protein expression by immunohistochemstry, and the results were compared with clinical data and the presence of *SDHD* mutations.

No aberrations in *p53* exons 5-8 were found. The immunohistochemical experiments showed nuclear p53 expression in 15 tumors. Three tumors were positive for MDM2 were also positive that were also positive for p53. There was no correlation between p53 and MDM2 expression and clinical data or *SDHD* status. Given the fact that hypoxia induces p53 expression and regarding the absence of *p53* mutations, these results suggest that p53 inactivation does not play a major role in the tumorigenesis of hereditary and sporadic paragangliomas.

INTRODUCTION

Parasympathetic paragangliomas (PGL; OMIM #168000) originate from neural crest-derived chief cells in the paraganglia. The tumors occur mostly in the head and neck region, with the carotid body being the most frequent location of paragangliomas, followed by the jugulotympanic paraganglia. The tumors are slowly growing, highly vascularized, and mostly benign, but metastatic spread is found in ~10% of patients (reviewed in [1]).

A positive family history is present in 10 to 50% of the patients [2-4], but genetic predisposition may also be present in 8 to 32% of isolated patients.[5, 6] Genetic predisposition to parasympathetic paraganglioma was recently revealed by the identification of germline mutations in subunit D of the mitochondrial succinate dehydrogenase enzyme complex II (*SDHD*) in familial paraganglioma patients.[7] Since then, mutations in other subunits, B (*SDHB*) and C (*SDHC*) of complex II have also been found to predispose to paraganglioma development.[8, 9] Co-occurrence of parasympathetic paragangliomas and their sympathoadrenal counterpart pheochromocytomas, and association with Carney's syndrome and neurofibromatosis type 1 (NF1) has been described.[10-13]

Apart from mutations in succinate dehydrogenase enzyme complex II, little is known about the pathogenetic mechanisms underlying paraganglioma development. By comparative genomic hybridization, we previously detected that loss of chromosome 11 is the only recurrent chromosomal aberration in parasympathetic paragangliomas, particularly in familial paragangliomas.[14] Overall DNA copy number changes are infrequent, which is in concordance with the benign and slow-growing nature of these tumors. Flow cytometric analyses revealed DNA aneuploïdy in 21 - 50% of the tumors, which was not predictive of malignant behavior or decreased survival.[15-17] A few immunohistochemical studies have suggested a paracrine/autocrine role for IGF-II, c-myc, bcl-2, and c-jun in paraganglioma pathogenesis.[18-21]

The mitochondrial succinate dehydrogenase enzyme complex II is involved in the citric acid cycle and the aerobic respiratory chain.[22] A complete loss of complex II enzymatic activity, due to inactivating mutations in the *SDHB*, *SDHC*, or *SDHD* gene and loss of heterozygosity (LOH) of the corresponding wild type allele, leads to a

high expression of hypoxic-angiogenic responsive genes like vascular endothelial growth factor (VEGF) and endothelial PAS domain protein 1 (EPAS1/HIF2α).[23, 24] The fact that cellular hypoxia stimulates paraganglioma development is further suggested by a markedly increased incidence of carotid body paragangliomas in people living permanently under hypoxic conditions (at high altitude or due to chronic obstructive pulmonary disease).[25-27] Cellular stress such as DNA damage or hypoxia induces *p53* [28], after which MDM2 is upregulated to serve as a negative feedback for *p53*. Induction of the tumor suppressor gene *p53* results in cell cycle arrest at the G0/G1 boundary, but when *p53* is mutated, control of cell proliferation is lost. Cells with mutated *p53* have a growth advantage compared to the surrounding cells and this can contribute to tumor formation. Obviously, paraganglioma cells escape from hypoxia-induced cellular senescence. One of the mechanisms to circumvent the hypoxia-induced cellular senescence is the inactivation of p53. In numerous tumor types p53 inactivation is caused by mutation in the *p53* gene itself or by MDM2 overexpression.[29, 30] The MDM2 protein targets p53 for proteasomal degradation and is as such involved in the perturbation of p53 function.[31, 32] There is strong evidence that *p53* mutation and MDM2 overexpression are mutually exclusive in most tumors and represent two alternative mechanisms to inactivate suppression of cell growth.

In paragangliomas, investigations on *p53* alterations are scarce and especially molecular analysis is lacking.[33-35] These data prompted us to determine the expression of p53 and MDM2 in a series of hereditary and sporadic paragangliomas. In addition, *p53* exons 5-8 were investigated for mutations by PCR-SSCP.

METHODS

Patients and tumor samples. From our archival files, we randomly selected 43 parasympathetic paragangliomas from 41 patients, diagnosed between 1987 and 2000 at the Erasmus Medical Center (Erasmus MC) Rotterdam, The Netherlands (see Table 1). Of these patients, 24 were female and 17 were male. The mean age was 42 years (range 20-74 years) and 17 patients (41%) had a positive family history. *SDHD* mutation analysis had been performed previously in all patients and germline mutations were found in 24 (59%) patients: 16 patients had the Dutch founder mutation D92Y, 6 patients harbored the L95P mutation, and in 2 patients the L139P mutation was found.[6] Table 1 summarizes all relevant clinical characteristics of the 41 paraganglioma patients evaluated for p53/MDM2 alterations in this study.

DNA isolation. DNA was isolated from both frozen (n=7) or paraffin (n=36) embedded tissues. Tissue regions consisting of at least 80% neoplastic cells were selected from H&E stained sections. These regions were manually dissected from (deparaffinized) unstained consecutive sections. White blood cell pellets from healthy volunteer blood donors and cell pellets from cultured tumor cells were used as controls. Dissected tissue fragments and the cell pellets were digested overnight at 56 ºC in 200 µL digestion buffer containing 10 µL Proteinase K (20 µg/µL), 50 mmol/L Tris-HCL (pH 8.0), 100 mmol/L EDTA and 0.5% sodium dodecyl sulfate. DNA was extracted by phenol-chloroform and precipitated with ethanol. Pellets were dissolved in 10mM Tris-HCL (pH 7.8).

PCR-SSCP. Exons 5 to 8 of the *p53* gene, including the exon-intron boundaries, were investigated by PCR-SSCP. As controls, DNA samples from normal individuals were used. In addition, DNA from the prostate carcinoma cell lines PC-3 and Du-145, and the colorectal carcinoma cell lines Colo-320 and HT-29, with known *p53* mutations in exons 5, 6, 7 and 8, respectively, served as positive controls. The DNA isolated from routine formalin-fixed and paraffin-embedded tissues is highly degraded, therefore we used small amplicon (<200bp) PCR to investigate exons 5-8 of the *p53* gene. All 4 exons were amplified in 2 fragments each, as recently

described.[36] PCR was performed in 15 µl reaction volume consisting of (per 50 µl): 1 Unit Taq DNA polymerase (Promega, Madison, WI, USA.), 1.5 mM $MgCl_2$, 200 ng of each primer, 0.2 mM dGTP, dTTP, dCTP, 0.02 mM dATP, 2.5 µCi α-^{32}P-dATP, and approximately 100 ng of DNA. Temperatures for amplification were 95 ºC for 30 seconds, 55 ºC for 45 seconds, and 72 ºC for 45 seconds. These steps were repeated for 35 cycles followed by a final extension at 72 ºC for 10 minutes. The PCR product was diluted with an equal amount of loading buffer (95% formamide, 10 mM EDTA pH 8.0, 0.025% bromophenol blue and 0.025% xylene cyanol) and denaturated at 95 ºC for 5 minutes. The solution was chilled on ice and 4 µl was loaded on a 8% polyacrylamide gel (acrylamide to bisacrylamide 49:1) containing 10% glycerol. Electrophoresis was performed at 8W for 16 hours at room temperature. Gels were vacuum dried at 80 ºC and exposed to X-ray films.

Immunohistochemistry. Five µm sections of paraffin-embedded tumors were mounted onto amino-alkyl-silane (AAS)-coated slides and deparaffinized. Subsequently, slides were washed twice in 100 percent alcohol, incubated for 20 min in 3 percent H_2O_2 in methanol, and rinsed with tap water. A microwave antigen retrieval method (15 min in citrate buffer, pH 6, at 600 W) was used, followed by incubation for 15 min in 10 percent normal goat serum (Dako, Glostrup, Denmark). Do7 anti-p53 monoclonal antibody (Dako) was used at a dilution of 1:50 for 30 minutes at room temperature and the MDM2 monoclonal antibody 1B10 (Novocastra laboratories, Newcastle upon Tyne, UK) was used at a dilution of 1:25 for 30 minutes at room temperature, both followed by biotinylated goat-anti-multilink and streptavidin-biotin peroxidase complex (both undiluted; Lab Vision Corporation, Fremont, CA, USA). Visualization was achieved by diaminobenzidine tetrahydrochloride (Fluka, Neu-Ulm, Germany) with 3 percent H_2O_2 for 7 min.
In the negative control reactions, the primary antibodies were omitted from the dilution buffer (phosphate-buffered saline with 5 per cent bovine serum albumin). A *p53*-positive esophageal adenocarcinoma and an MDM2-positive breast carcinoma were used as positive controls. Staining of p53 and MDM2 was assessed according to the method described by Sinicrope *et al.*[37] This method is based on the percentage of positive tumor cells and the staining intensity. A score of 0 to 4 was

assigned according to the percentage of positively stained tumor cells: 0 = positive staining in < 5%; 1 = >5 - 25%; 2 = >25 - 50%; 3 = >50 – 75% and 4 = >75%. These results are multiplied by the staining intensity score of the tumor cells: 1 = negative - weak; 2 = moderate and 3 = strong staining. A multiplied score of 6 or more is regarded as positive staining and a score below 6 as negative.

Statistics. Correlations between *p53* and MDM2 alterations and *SDHD* mutation status or clinical features were tested by use of the chi-square test or an unpaired t-test. *P* values less than 0.05 were considered statistically significant.

TABLE 1. Clinical Characteristics of 42 Paraganglioma Patients Evaluated for *p53/MDM2* Alterations

CHARACTERISTIC	ALL PATIENTS (N=41)	PATIENTS WITH *SDHD* GENE MUTATION † (N=24)	PATIENTS WITH WILDTYPE *SDHD* GENE † (N=17)	*P* VALUE *
Patient				
Sex - no. (%)				NS
Male	17	11 (65)	6 (35)	
Female	24	13 (52)	11 (46)	
Mean Age of Onset - yr (± SE)	42.8 (± 13.3)	41.2 (± 13.1)	44.3 (± 13.7)	NS
Mean Follow-up Time - months (range)	53 (1 - 218)	64 (5 - 136)	38 (1 - 218)	
Family History - no. (%)				< .0001
Positive	17	17 (71)		
Negative	24	7 (29)	17 (100)	
Tumor focality - no. (%)				.003
Single Paraganglioma	19	5 (25)	14 (74)	
Recurrence	6	3	3	
Bilateral Carotid Body	7	6 (86)	1 (14)	
Multiple	12	10 (83)	2 (17)	
With sympathoadrenal tumors	2	2 (100)		
Not known	1	1 (100)		

† Data published previously (see ref. 6).

* We used the chi-square test to compare all variables except mean age at onset, for which we used the Mann Whitney U-test.

NS = statistically not significant

RESULTS

PCR-SSCP analysis.

PCR products of *p53* exon 5-8 could be obtained from all 43 tumor/normal DNA samples. By SSCP analysis, no aberrations were found in the 43 tumor samples, whereas the 4 different *p53* mutations in the tumor cell lines were clearly identified with the applied SSCP conditions. Figure 1 shows an example of a PCR-SSCP normal pattern of PGL samples and a band shift of a positive control (PC-3). This cell line contained a C deletion in codon 138 of the *p53* gene.

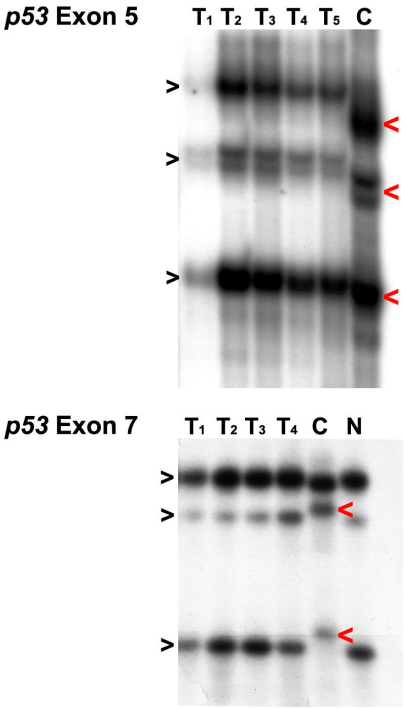

Figure 1. Examples of SSCP analysis of *p53* exon 5 and exon 7 in parasympathetic paragangliomas. The autoradiographs of the PCR-SSCP gel show the migration patterns of tumor (T) and normal (N) DNA and the mobility shifts (red arrowheads) produced by aberrant control samples (C) of the positive controls PC3 (exon 5) and Colo-320 (exon 7).

p53/MDM2 protein expression and association with *SDHD* mutations.

Of 43 paragangliomas, p53 immunoreactivity was detected in 15 tumors (35%) of 13 patients. Three tumors (7%) from different patients showed concurrent MDM2 expression, leaving the majority (n=28, 65%) of the tumors negative for both p53 and MDM2. Immunoreactivity of p53 and MDM2 was observed both in the nucleus and the cytoplasm. Also, p53 positivity was observed in tumor and stromal cells in all these cases. Figure 2 shows examples of positive and negative staining of p53 and MDM2.

From a patient with bilateral carotid body tumors, one tumor was p53-positive whereas the other tumor was p53-negative. A vagal and a carotid body tumor of another patient both showed the same expression pattern (p53+/MDM2–). Of the 13 patients with a p53-positive paraganglioma, 9 had a single paraganglioma, 4 of which recurred after resection. The other 4 paragangliomas with detectable p53 were from patients with bilateral or multiple tumors. There was no correlation between p53/MDM2 status and tumor focality or tumor location.

Because hypoxia is known to be present in *SDHD*-mutated paraganglionic cells and hypoxia is known to stimulate *p53* transcription, leading to cell cycle arrest and apoptosis, abrogation of the *p53* pathway could especially be expected in *SDHD*-mutated paragangliomas. However, p53 positivity was present in 6 of 25 (24%) tumors with an *SDHD* mutation and 9 of 18 (50%) tumors without an *SDHD* mutation were positive for p53. Similarly, MDM2 positive staining was found in 1 patient with an *SDHD* mutation.

By calculating the significance of the correlation of p53 expression with sex, family history, tumor focality (follow-up), site of the tumor and *SDHD* germline status, none of these parameters was significantly associated with absence of p53 immunoreactivity. Results of p53 and MDM2 immunotyping and correlations with tumor and patient characteristics are shown in Table 2.

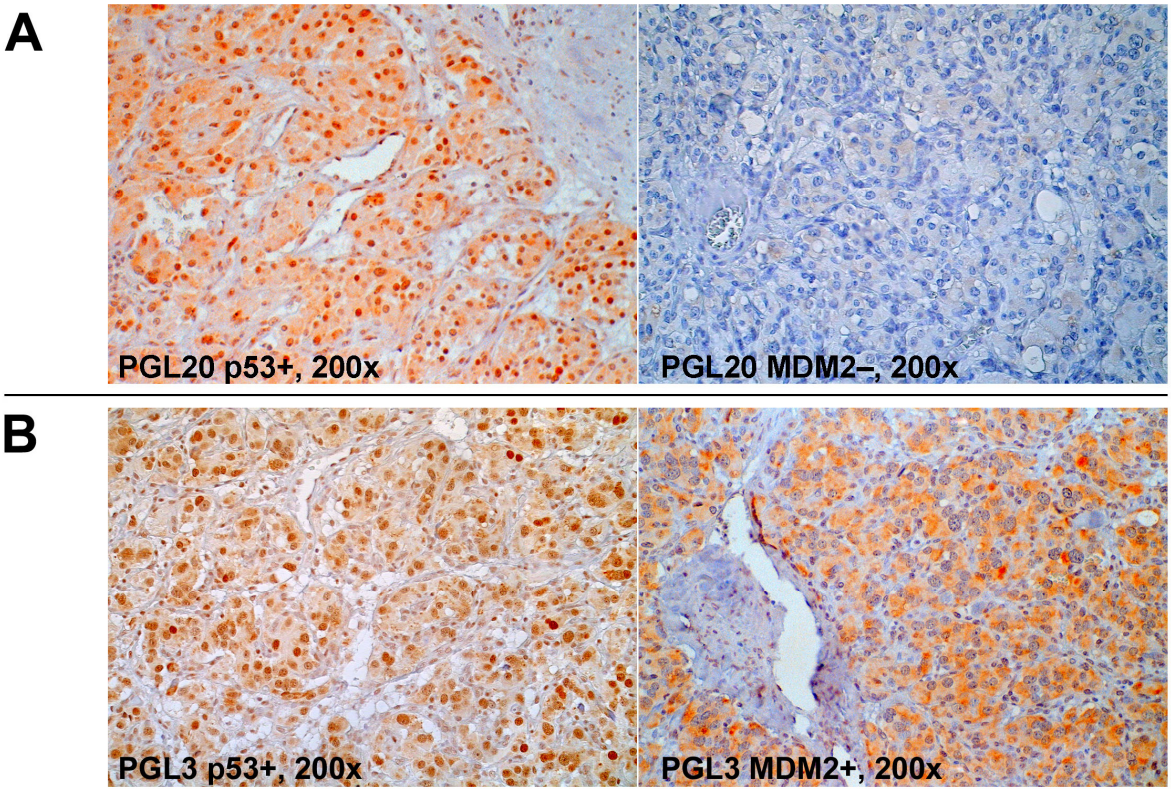

Figure 2. Immunohistochemical staining of p53 and MDM2 in parasympathetic paragangliomas using the anti-p53 monoclonal antibody Do7 and MDM2 monoclonal antibody 1B10, respectively.

Panel A shows positive p53 staining (left) of tumor and stromal cells in PGL20, a mediastinal paraganglioma of a patient with a negative family history and no germline *SDHD* mutation. MDM2 expression is absent in PGL20 (right).

Panel B: PGL3, a vagal paraganglioma of a patient with multiple paragangliomas, a positive family history and a germline D92Y SDHD mutation. Tumor and stromal cells stain positive for *p53*, whereas MDM2 staining is mainly present in the tumor cells.

Note the nuclear and cytoplasmic staining of p53 in both paragangliomas, and the cytoplasmic presence of MDM2 in the tumor cells of PGL3.

TABLE 2. P53/MDM2 Immunophenotype and Correlations with the Patients' Characteristics

CHARACTERISTIC		ALL TUMORS (N=43)	IMMUNOPHENOTYPE P53 + / MDM2 - (N=12)	P53 + / MDM2 + (N=3)	P53 - / MDM2 - (N=28)	*P*-VALUE ¶
Patient						
Sex						NS
Male	(n=17)	18	4	1	13	
Female	(n=24)	25	8	2	15	
Mean Age of Onset - yr (± SE)		42.5 (± 13.4)	39.2 (± 11.7)	47.7 (± 19.1)	43.1 (± 13.6)	NS
Mean Follow-up Time - months (range)		53 (1 - 218)	61 (3 - 160)	58 (2 - 142)	50 (1 - 218)	NS
Family History - no. (%)						NS
Positive		18	4	0	14 (78)	
Negative		25	8	3	14 (56)	
Tumor focality - no (%)						NS
Single Paraganglioma	(n=19)	19	7	3	9 (47)	
(Recurrences)		6	4	1	1	
Bilateral Carotid Body	(n= 8)	9	2		7 (78)	
Multiple	(n=12)	13	3		10 (77)	
With sympathetic PGL	(n= 2)	2			2 (100)	
Site of the tumor - no. (%)						NS
Carotid Body		22	4	1	17	
Vagal Nerve		6	2	1	3	
Tympanic Nerve		6	3	1	2	
Jugular Nerve		5	1		4	
Mediastinal		3	2		1	
Spinal cord		1			1	
***SDHD* germline - no. (%)**						NS
Mutated	**(n=24)**	**25**	**5**	**1**	**19 (76)**	
D92Y	(n=16)	17	5		12	
L95P	(n= 6)	6		1	5	
L139P	(n= 2)	2			2	
Normal	**(n=17)**	**18**	**7**	**2**	**9 (50)**	

¶ The P values are for comparison of p53– tumors with all p53+ tumors and resulted from Chi-square tests.
NS = statistically not significant

DISCUSSION

Experimental and observational evidence indicates that chronic hypoxic stimulation is involved in the tumorigenesis of paraganglioma. Hypoxia is a well known inducer of *p53* which in turn results in cell cycle arrest or apoptosis, a mechanism that is abrogated in most, if not all, cancers. The present study was undertaken to investigate the possible involvement of *p53* in the development of parasympathetic paragangliomas with and without *SDHD* mutations, using immunohistochemical assessment of p53 and MDM2 expression, and mutation analysis of *p53* exon 5-8.

Fifteen of the 43 investigated paragangliomas (35%) showed nuclear and cytoplasmic p53 immunoreactivity. MDM2 staining was observed in 3 tumors (7%) which were simultaneously positive for p53. We found a p53/MDM2 concordance of 75%, similar to that described in breast and colorectal carcinoma.[38, 39] p53 immunoreactivity was more frequent in paragangliomas without *SDHD* mutations (50%) than in paragangliomas with *SDHD* mutations (24%), although this was not statistically significant (P= 0.08).

Under normal conditions, the p53 concentration in cells is low and cannot be detected by immunohistochemistry. By cellular stress the concentration of p53 can rise, and hence be detected by immunohistochemistry.[40, 41] In addition, mutant p53 has often a longer half-life than wild type p53 and can be detected immunohistochemically.[42, 43] However, there is no direct correlation between *p53* mutation and immunohistochemical p53 overexpression.[32, 44] The immunohistochemical detection of p53 expression in 15 paragangliomas indicates increased wild type p53 expression or the presence of mutant p53. However, no aberrations in exons 5-8 of the *p53* gene were found by PCR-SSCP. It is known from the literature that more than 95% of *p53* mutations are found in exons 5-8 [29], but we cannot exclude the presence of mutations outside this region. In addition, the mutation detection efficiency of PCR-SSCP is not 100% and mutations could remain undetected, although all 4 different control *p53* mutations were identified by the procedure used. Despite this, we consider our molecular results as strong indication that *p53* mutations do not contribute to paraganglioma tumorigenesis. Moreover, the observation of p53 immunoreactivity in tumor and stromal cells suggests hypoxia

rather than gene mutation as the cause of p53 expression. Inactivation of *p53* in tumors is often the result of the combination of a mutant *p53* allele and 17p allele loss. In several molecular studies no 17p loss in paragangliomas has been found.[14, 45] This is in accordance with the observed absence of *p53* mutations in these tumors. A recent investigation has shown that the increase in p53 during hypoxia is not accompanied by a parallel rise in MDM2.[40] If p53 is active in the p53-expressing paragangliomas this implies that the tumorigenic mechanism in these tumors overrules the tumor suppressor capacity of wild type p53. In accordance with this concept paragangliomas are very slowly growing tumors.

MDM2 overexpression in tumors with wild type p53 accumulation has also been described in bladder, testicular, esophageal, and laryngeal carcinoma and in acute lymphoblastic leukemia.[46-50] As suggested in the literature, the concomitant expression of MDM2 and p53 proteins indicates inactive p53, implying that p53 is inactive in the 3 paragangliomas with MDM2 expression in this study. In the remaining 12 p53 positive paragangliomas, p53 could be active, although inactivation of p53 by other proteins like viral oncogenes or cellular proteins can not be excluded.[51]

In 28 (65%) of the investigated paragangliomas, besides the absence of *p53* mutations, no p53 expression was detected. This could point to a *p53*-independent tumorigenic pathway. Nineteen of these 28 tumors have an *SDHD* gene mutation resulting in cellular hypoxia. Obviously, hypoxia in these tumors does not lead to p53 upregulation. However, there are more ways to perturb the *p53* pathway during tumor development in addition to the commonly seen *p53* gene mutations or MDM2 overexpression. These include loss of the ability to stabilize p53, through mechanisms such as loss of ARF or inactivation of kinases, inappropriate localization of p53, and inactivation of downstream mediators of p53 such as Apaf-1 or Bax.[52, 53] Many cancers with wild type *p53* show loss of the p14ARF protein resulting in destabilization of p53.[54] This loss is often the result of p14ARF locus deletion, but in paragangliomas loss of chromosomal region 9p has not been observed.[14, 45] Also, in a case report of 2 brothers with paraganglioma no allele loss nor mutations in *p53* and the 9p gene p16INK4A were found. More than 8 years after radiotherapy a

recurrence appeared to have a *p53* as well as a p16INK4A mutation and the authors suggest that these mutations may have resulted from the therapy.[55]

In summary, our data indicate that p53 is expressed in at least 35% of paragangliomas independent of *SDHD* gene status and not caused by *p53* gene mutations. Abrogation of the *p53* tumor surveillance mechanism by MDM2 overexpression is detected in a small subset (7%) of these tumors, which is also not associated with *SDHD* gene mutations. Further experiments need to clarify the mechanisms by which paragangliomas escape from apoptotic signals.

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CHAPTER 5, *PTEN* GENE LOSS BUT NO MUTATION, IN BENIGN AND MALIGNANT PHEOCHROMOCYTOMAS

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ABSTRACT

Mutations of the "phosphatase and tensin homologue deleted on chromosome 10" (*PTEN/MMAC1*) gene have been associated with a variety of human cancers, including prostate cancer, glioblastoma, and melanoma. The gene is thought to be one of the most frequently mutated tumour suppressor genes and inactivation of *PTEN* is associated with disease progression and angiogenesis. High vascularization and resistance to chemo- and radio-therapy are two well-established features of phaeochromocytomas (PCCs). Furthermore, benign and malignant PCCs are found in several *PTEN* knockout mouse models. This study therefore evaluated whether inactivation of PTEN may be involved in the tumourigenesis of PCC in man and whether *PTEN* abnormalities may help to define the malignant potential of these tumours. Tumour and germline DNA was analysed from 31 patients with apparently sporadic PCC, including 14 clinically benign and 17 malignant tumours, for loss of the *PTEN* gene locus, mutations in the *PTEN* gene, and for PTEN protein expression by immunohistochemistry. Loss of heterozygosity (LOH) analysis showed loss of *PTEN* in four malignant tumours (40%) and in one benign tumour (14%). However, no mutations of *PTEN* were observed. Immunohistochemistry showed no correlation with clinical behaviour and/or LOH status. The results indicate that inactivation of the *PTEN/MMAC1* gene may play a minor role in the development of malignant phaeochromocytomas.

INTRODUCTION

Phaeochromocytomas (PCCs) are rare catecholamine-producing tumours of the adrenal medulla, which may also infrequently occur at extra-adrenal sites. Approximately 10% of these tumours follow an aggressive course, characterized by poor survival. To date, no discriminating markers exist for the distinction of benign from malignant PCC. In previous studies, we and others evaluated the prognostic value of many histological, immunohistochemical, and molecular markers, focusing on the differences between benign and malignant PCC [1-10]. Although some of these studies showed a statistically significant correlation, none of the markers tested thus far has diagnostic utility.

Whereas the majority of PCCs are sporadic, up to 24% of the tumours may occur in the context of hereditary cancer syndromes, including multiple endocrine neoplasia type 2 (MEN2), von Hippel-Lindau (VHL) disease, and the phaeochromocytoma-paraganglioma syndrome [11]. The first two syndromes are caused by activating mutations in the *RET* proto-oncogene and inactivating mutations or deletions of the *VHL* tumour suppressor gene, respectively. Previous studies have shown that the *RET* and *VHL* genes do not play a significant role in the pathogenesis of sporadic PCC [7][12][13]. The phaeochromocytoma-paraganglioma syndrome is caused by inactivating mutations in the succinate dehydrogenase genes *SDHD* and *SDHB*. Again, these genes appear not to play a major role in sporadic PCC [14].

PTEN (phosphatase and tensin homologue deleted on chromosome 10), also known as *MMAC1* (mutated in multiple advanced cancers), is a recently identified tumour suppressor gene that is responsible for Cowden syndrome and other autosomal dominant disorders such as Bannayan-Riley-Ruvucalba syndrome and the Proteus and Proteus-like syndromes. In Cowden syndrome, multiple hamartomatous and tumourous lesions occur, including neuroendocrine tumours of the skin. Apart from its role in hereditary syndromes, *PTEN* inactivation has been shown in a series of sporadic human cancers, including glioblastomas of the central nervous system, endometrial carcinoma, prostatic adenocarcinoma, and melanoma [15][16]. With the

exception of endometrial carcinoma, PTEN inactivation has been related to more advanced tumour stages [17][18]. The *PTEN* gene encodes a protein phosphatase, which has an antagonistic role to protein tyrosine kinases such as the RET protein. Loss of PTEN function results in increased expression of the hypoxia-inducible transcription factor HIF-1α, leading to increased VEGF expression and highly vascularized tumours, identical to the situation with loss of VHL function [19]. Mice with heterozygous *PTEN* mutations and conditional *PTEN* knockout mice are known to develop various neoplasms including multiple and also malignant PCCs [20-22]. These observations indicate that *PTEN* might be a candidate tumour suppressor gene involved in PCC tumourigenesis and led us to hypothesize that loss of PTEN function may play a role in PCC development, particularly in progression to malignancy.

The aim of this study was to investigate whether the *PTEN* gene plays a role in PCC tumourigenesis and whether analysis of this gene can help to distinguish benign from malignant PCC. We evaluated 31 clinically benign (n = 14) and malignant (n = 17), apparently sporadic PCCs for loss of the *PTEN* locus by LOH using highly polymorphic microsatellite markers located within the *PTEN* gene. In addition, *PTEN* mutations were investigated by performing polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP) analysis of all exons including the exon-intron boundaries. Finally, we performed immunohistochemistry using a specific PTEN antibody to determine expression at the protein level.

MATERIALS AND METHODS

We evaluated 31 apparently sporadic PCCs, from 31 patients, including 14 clinically benign and 17 malignant (ie metastasized) tumours. All specimens were retrieved from the files of the Department of Pathology (Erasmus MC and University of Zürich) following approval of the experimental design and protocols by the Medical Ethics Committee. None of the patients had a personal or family history suggestive of familial phaeochromocytoma, (familial) paraganglioma, VHL disease, MEN2 syndrome, or neurofibromatosis type 1 (NF1). Mutation analysis for the *RET* and *VHL* genes had been performed previously and revealed one germline *RET* mutation in a patient with a benign PCC, and one germline *VHL* mutation in a patient with bilateral PCCs, one of which was malignant [7][12]. *SDHD* mutation analysis was performed in 13 benign and 16 malignant tumours but no mutations were found.

Tumour and normal DNAs were isolated from either frozen (n = 9, all but one benign tumour) or paraffin-embedded (n = 22) tissues or peripheral blood leukocytes by standard detergent-proteinase K lysis, followed by phenol/chloroform extraction and ethanol precipitation. Only tissues with more than 80% tumour cells were included in this study. Relevant patient characteristics and clinical and histopathological data are summarized in Table 1.

Loss of heterozygosity (LOH) analysis

In 25 (14 malignant and 11 benign) of 31 PCCs, loss of the *PTEN* locus was investigated with the microsatellite markers D10S2491 and AFMa086wg9. The forward primers were 5′-labelled with either HEX or 6-FAM fluorescent dyes. Fragment size analysis was performed with the 3100 Genetic Analyzer, Applied Biosystems/Hitachi and Gene-Scan software (Applied Biosystems, Foster City, CA, USA). Cases were classified as informative when two distinct alleles of similar intensity were found in the normal DNA. LOH was defined as present when an allele peak signal from tumour DNA was reduced by at least 50% compared with the corresponding normal DNA. Microsatellite instability (MSI) was defined as a shift of the allele pattern in the tumour DNA compared with the normal DNA (Figure 1).

Table 1. Clinical Characteristics of 25 Pheochromocytomas Evaluated for LOH

dignity	age	sex	diameter (cm)	metastases	site	background	D10S2491	AFMa068wg	IHC PTEN
Benign	62	female	4,7	-	extraadrenal	sporadic	NI	NI	2
Benign	39	male	6	-	adrenal	sporadic	NI	NI	x
Benign	50	female	5	-	adrenal	**MEN2**	ROH	ROH	1
Benign	42	male	6	-	adrenal	sporadic	ROH	ROH	2
Benign	52	male	-	-	adrenal	sporadic	NI	-	2
Benign	66	male	3,2	-	adrenal	sporadic	LOH	NI	x
Benign	65	male	6	-	adrenal	sporadic	NI	NI	x
Benign	49	female	5	-	adrenal	sporadic	ROH	NI	2
Benign	57	male	15	-	adrenal	sporadic	ROH	NI	1-2
Benign	13	female	5,5	-	bilateral	sporadic	ROH	NI	2
Benign	36	male	21	-	extraadrenal	sporadic	ROH	ROH	1-2
Malignant	46	female	6,5	bone, pleura, bone marrow	bilateral	sporadic	NI	NI	1
Malignant	70	female	16	liver, para-aortic	adrenal	sporadic	ROH	NI	0-1
Malignant	31	male	7,5	para-aortic	bilateral	**VHL**	LOH	NI	2-1
Malignant	65	female	11	paraaortic, bone, liver, bone marrow	adrenal	sporadic	NI	NI	2
Malignant	65	female	-	metastases	-	sporadic	NI	NI	2
Malignant	23	female	8	lymph node, bone	extraadrenal	sporadic	MSI	ROH	1
Malignant	65	male	-	lymph node	bilateral	sporadic	LOH	NI	x
Malignant	49	male	8	bone	adrenal	sporadic	NI	NI	1-2
Malignant	56	male	6	lymph node	extraadrenal	sporadic	MSI	LOH	x
Malignant	54	male	18	lymph node	extraadrenal	sporadic	LOH	LOH	x
Malignant	42	male	7,5	bone	adrenal	sporadic	ROH	ROH	2
Malignant	66	male	12	lymph node	adrenal	sporadic	ROH	NI	2
Malignant	29	female	18	pleura	adrenal	-	NI	ROH	1
Malignant	70	female	5	bone	extraadrenal	sporadic	ROH	NI	0

PTEN Immunohistochemistry:1: weak or absent staining; 2: Strong cytoplasmic staining; x: negative internal control
IHC = immunohistochemistry; ROH = retention of heterozygosity, LOH = loss of heterozygosity; NI = not informative; - = unknown
VHL = von Hippel-Lindau disease; MEN2 = multiple endocrine neoplasia type 2

PCR-SSCP analysis

The entire coding region and splice sites of the *PTEN* gene were screened for mutations according to previously published protocols [23]. Polymerase chain reaction (PCR) fragments were amplified from 100 ng of tumour DNA using 11 primer pairs. Primer sequences, product length, and PCR conditions were essentially as published previously. PCR amplification was performed for 35 cycles in a total volume of 50-µl reaction mixture containing 0.2 mmol/l dATP, dTTP, dGTP, dCTP; 20-50 pmol each of sense and antisense primers; 1.5 mmol/l Mg^{2+}; 10 mmol/l Tris-HCl; 50 mM KCl; and 1 Unit of Taq DNA polymerase (AmpliTaq Gold, Perkin Elmer, Norwalk, CT, USA).

For the SSCP analysis, 10 µl of PCR products were diluted 1 : 1 in stop buffer (95% formamide, 20 mM EDTA, 0.05% xylene cyanol, 0.05% bromophenol blue), heat-denatured at 96 °C for 10 min, and quickly chilled in a liquid nitrogen bath before loading onto non-denaturing 0.8 mm-thick 6% polyacrylamide gels (29 : 1 acrylamide: bisacrylamide; BioRad, Glattbrugg, Switzerland) containing 5% glycerol. Electrophoresis was carried out using sequencing gel electrophoresis apparatus (Gibco BRL, Life Technologies; Zürich, Switzerland) at 35 W for 6 h at room temperature. The DNA was visualized by silver staining as previously described [24].

Abnormal bands from PCR-SSCP analysis were excised from additionally prepared SSCP polyacrylamide gels, stained with Sybr Green I nucleic acid (Molecular Probes, Eugene, OR, USA), placed in 100 µl of 1× Tris EDTA buffer (pH 8.0), and incubated for 120 min at 95 °C to elute DNA. An aliquot (3-5 µl) of the supernatant was used as PCR template for 35 further PCR cycles as detailed above to yield PCR products predominantly harbouring the mutated appropriate *PTEN* sequence; 40 µl of re-amplified DNA sequences and PCR products showing heteroduplex formation in the electrophoresis assay were agarose gel-purified using the QIAquick Gel Extraction Kit (Qiagen, Basel, Switzerland), alcohol-precipitated after adding 20 µg of glycogen (Boehringer-Mannheim), and resuspended in 12 µl of 10 mM Tris buffer (pH 8.0). The DNA concentration of purified PCR products was estimated by comparing the band intensities of 2 µl sample DNA and the quantified DNA molecular weight marker

pUCBM21/HpaII, DraI, HindIII (Boehringer-Mannheim) in an ethidium bromide-stained agarose gel electrophoresis.

DNA sequences of 30 ng PCR products were determined in sense and antisense directions by fluorescence-based dideoxy terminator cycle sequencing using the TaqDyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems, Weiterstadt, Germany) followed by gel electrophoresis, data collection, and analysis on an automated DNA sequencer (model 373A, Applied Biosystems).

Immunohistochemistry

PTEN immunohistochemistry was performed on 25 paraffin-embedded tumours with the monoclonal anti-human PTEN antibody 6H2.1 (Cascade Bioscience, Winchester, MA, USA). This antibody was raised against the last 100 C-terminal amino acids of PTEN. Four-micrometre sections were cut and mounted on Superfrost Plus slides. Immunohistochemistry was performed as described previously by Perren *et al* [25]. The staining was assessed for both nuclear and cytoplasmic staining of tumour cells and the score was divided into two categories: (1) weak or absent staining, and (2) strong cytoplasmic staining. Non-neoplastic nerves served as an internal control.

Statistical analysis

Fisher's exact test was applied, using SPSS version 11.5, to compare the results of the LOH analysis and the results of the immunohistochemical staining in comparison with the clinical behaviour of the PCCs. *p* values less than 0.05 were considered to indicate statistical significance.

RESULTS

LOH analysis

Of 25 tumours analysed with the intragenic marker D10S2491, 17 were informative. Four tumours showed LOH: three of these were malignant and one was benign. Of the three malignant tumours, one was extra-adrenal and two were adrenal, both in patients with bilateral tumours. All patients with LOH were males, with a mean age of 50 years. LOH analysis with AFMa086wg identified eight informative tumours, two of which showed LOH. Both of these tumours were malignant. One tumour with LOH of D10S2491 also showed LOH of AFMa086wg. This malignant PCC was extra-adrenal and 18 cm in size. The other three PCCs showing LOH with D10S2491 were not informative with AFMa086wg. In two PCCs, both malignant, the LOH pattern of D10S2491 showed an aberrant pattern compared with the normal DNA from the same patients, which was interpreted as microsatellite instability (MSI). Taken together, five of 25 tumours showed LOH for at least one of the intragenic polymorphic markers, including four malignant PCCs and one benign PCC. If the uninformative cases are excluded, 40% (4/10) of malignant PCCs and 14% (1/7) of benign PCCs showed LOH. Examples of the LOH analysis are shown in Figure 1.

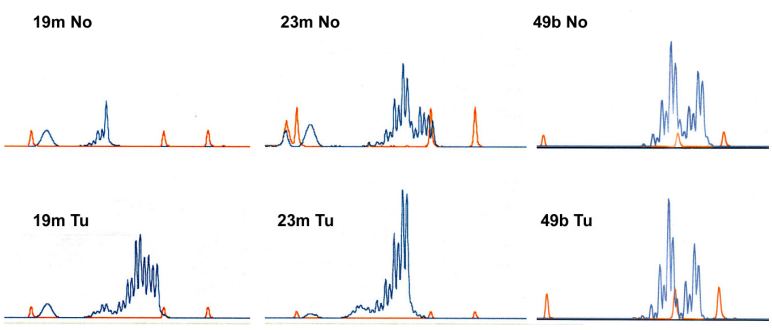

Figure 1. Example of MSI (19M) as shown by an aberrant broad peak not matching the normal DNA. LOH in 23M, the second peak has decreased more than 50% matching LOH. No LOH of 49B, with identical peaks in tumour and normal DNA. No=normal, Tu=tumour.

PCR-SSCP analysis

All 31 PCCs were screened for *PTEN* mutations, analysing all 11 exons of the gene. The DNA was of good quality and could be analysed in all instances, except for three tumours, where one exon each did not yield an adequate result. There was one malignant PCC with an SSCP band shift in exon 1. However, upon sequence analysis, only a wild-type sequence was found and no mutation could be detected. No other evidence of band shifts as an indicator of mutations was found. In all experiments, positive control specimens with known mutations were used, which yielded the expected results.

Immunohistochemistry

All 25 tumours that were analysed by immunohistochemistry had also been analysed for the previously mentioned LOH markers. Staining could not be assessed in six cases due to negativity of the internal control. Nine of the remaining 19 tumours showed positive staining, including five benign and four malignant tumours. Four of these PCCs were not informative in LOH analysis and five showed no loss. A second group consisted of four tumours (two benign and two malignant) with focal staining, as shown in Figure 2. Of these, one tumour was not informative, one tumour showed LOH, and two showed no loss. Weak or absent staining was found in the remaining six tumours. One of these six tumours showed MSI, four showed no loss, and one was not informative. No correlation could be found between immunohistochemical staining and LOH status and/or clinical behaviour ($p > 0.05$ for both comparisons). These data are summarized in Table1.

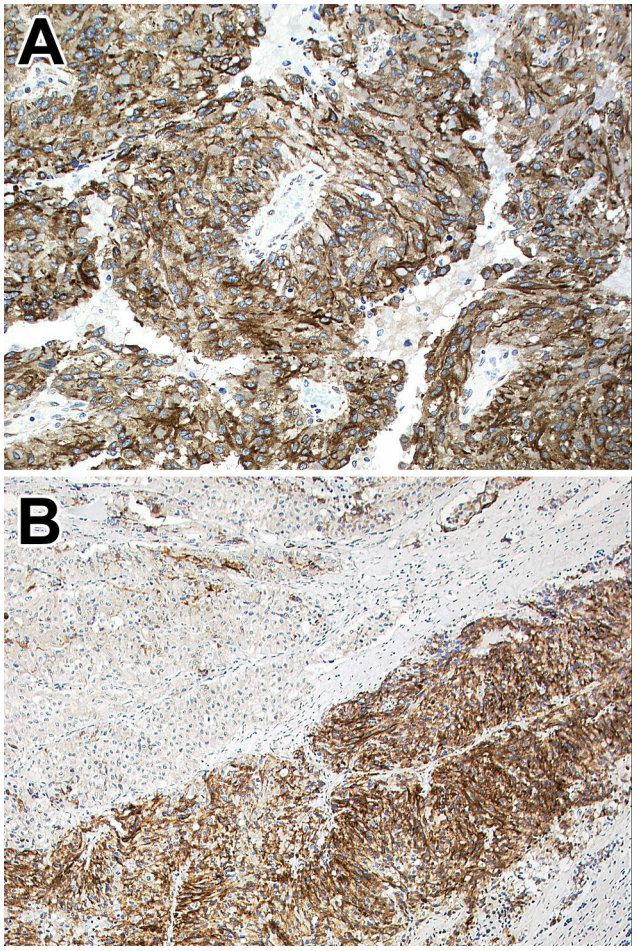

Figure 2. Immunohistochemical staining of PCC with the monoclonal anti-human PTEN antibody 6H2.1. A: Diffuse strong cytoplasmatic staining in a malignant PCC; B: Focal positive staining in another malignant PCC.

DISCUSSION

The molecular mechanisms underlying the tumourigenesis of PCC are poorly understood. The existence of inherited neuroendocrine tumour syndromes suggests the involvement of a specific cell-signalling pathway in the constituent tumours. However, none of the known pathways has been proven to be significantly involved in PCC tumourigenesis. Few studies have been published in which neuroendocrine tumours, especially PCC, were investigated for alterations in the *PTEN/MMAC1* tumour-suppressor gene [26-28]. The present study represents the first combined molecular and immunohistochemical analysis to compare the clinical behaviour of PCC with the presence or absence of *PTEN* mutations, LOH, and protein expression. We showed LOH for *PTEN* intragenic polymorphic markers in 29% of malignant PCCs, whereas in the group of benign PCCs, LOH was found in 9%. However, these findings were not supported by decreased PTEN protein expression in the (malignant) PCCs with LOH. An explanation for the higher frequency of LOH in malignant PCCs could be that such tumours are genetically unstable and have an inherently higher frequency of chromosomal loss, as is supported by our previous comparative genomic hybridization studies [29]. Another explanation for genetic instability is the recent discovery of up-regulation of hypoxia-inducible factor 1α (HIF1α) in at least a subset of PCCs with *VHL, SDHB*, or *SDHD* mutations [30]. The up-regulation of HIF1α leads to down-regulation of mismatch repair genes, such as *MSH2* and *MSH6* [31]. These genes are known to be involved in both hereditary and sporadic carcinomas. In addition to the down-regulation, *MSH2* was found to be inactivated in 36% of 25 analysed tumours, indicating that these genes could also be involved in genetic instability, and hence the pathogenesis of PCC [28].

Although only a limited number of tumours could be analysed and PTEN expression is reduced or absent in a subset of tumours, it appears that PTEN may play a minor role in the tumourigenesis of malignant PCC.

Our results are comparable to a study of sporadic metastasized melanomas in which these tumours showed LOH of 10q23 in 32%. Immunohistochemical data revealed weak PTEN immunoreactivity of which almost half had LOH for the *PTEN* region [32].

Identical to our study, no *PTEN* mutations were found. Comparable data were found in a study of PTEN protein expression in breast carcinogenesis [25]. Both studies suggested that a mechanism of epigenetic silencing of *PTEN* might be involved. Recently, promoter hypermethylation has been shown to be the most important mechanism of *PTEN* inactivation in breast cancer [33]. In addition, in a series of 13 sporadic and 12 MEN2-related PCCs, *PTEN* methylation was found in two tumours (one sporadic and one MEN2). This finding could indicate that hypermethylation indeed occurs in a small subset of PCCs. Unfortunately, LOH status or immunohistochemistry was not performed [28].

Functionally, PTEN is known to down-regulate the phosphoinositol-3′-kinase (PI3k)/Akt pathway. From a mechanistic point of view, loss of PTEN function results in increased PIP-3 levels and in Akt hyperexpression, leading to increased cell survival and proliferation [34]. In contrast, PTEN up-regulation leads to decreased Akt expression, and hence cell cycle arrest and apoptosis, which could protect against carcinogenesis [35].

Surprisingly, we did not find any mutation in the entire coding region of the *PTEN* gene. We cannot entirely exclude the presence of *PTEN* mutations, as mutations can escape detection by SSCP. Technical problems, such as preferential PCR amplification of the wild-type allele, or low detection sensitivity on DNA derived from archival tissue, cannot be excluded as a possible explanation for the negative results. This explanation seems unlikely, however, since the positive control samples gave consistently positive results on the *PTEN* mutations tested. Also, all the samples tested were composed of more than 80% tumour cells. Furthermore, it is known that the *PTEN* gene may be inactivated by promoter hypermethylation, and this has been proven in PCC [28][33].

A previously performed immunohistochemical study of PTEN in neuroendocrine tumours suggested a correlation between loss of protein expression and tumour progression [26]. The two PCCs analysed in that study were benign and showed strong immunoreactivity. In our present study, however, no clear correlation between tumour progression and PTEN immunoreactivity could be seen. This difference might

result from the small sample size of the study by Wang *et al*, since only two tumours had been analysed [26].

In patients with PTEN-associated syndromes, there is an increased risk of breast, thyroid, and endometrial neoplasia, but not of PCC [35]. The finding of PCC in *PTEN* gene knockout mice, however, supports the idea that *PTEN* may be involved in the tumourigenesis and malignant behaviour of PCC. In mice with homozygous *Ink4a/ARF* mutations in combination with a heterozygous *PTEN* mutation, the finding of bilateral, malignant phaeochromocytomas at a young age is remarkable. Mutation analysis of *RET, VHL*, and *NF1* was negative. However, other mouse models of RET and NF1 may develop PCCs [36][37]. This supports the idea that more than one pathway is involved in the development of PCC.

We have shown that LOH at the *PTEN* locus occurs in a minority of PCCs and more frequently in malignant PCCs. In this small series, LOH was not accompanied by *PTEN* mutation or aberrant expression at the protein level. Nevertheless, the *PTEN* gene, and its related pathway, may play a role in PCC tumourigenesis, a suggestion that is supported by some of the available PCC mouse models.

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CHAPTER 6, CLINICAL CHARACTERISTICS OF PHEOCHROMOCYTOMA PATIENTS WITH GERM LINE MUTATIONS IN *SDHD*

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ABSTRACT

Purpose: We examined the value of *SDHD* mutation screening in patients presenting with apparently sporadic and familial pheochromocytoma for the identification of *SDHD*-related pheochromocytomas.

Patients and Methods: This retrospective study involved 126 patients with adrenal or extra-adrenal pheochromocytomas, including 24 patients with a family history of MEN2, VHL, NF1, or paraganglioma (PGL). Conformation-dependent gel electrophoresis and sequence determination analysis of germ line and tumor DNA were used to identify *SDHD* alterations. The clinical and molecular characteristics of sporadic and hereditary tumors were compared. We reviewed the literature and compared our results with those from previously published studies.

Results: Pathogenic germ line *SDHD* mutations were identified in 3 patients: 2 (2.0%) of the 102 apparently sporadic pheochromocytoma patients and 1 patient with a family history of PGL. These patients presented with multifocal disease (2 of 3 multifocal patients) or with an adrenal tumor (1 of 82 patients). In the literature, mutations are mostly found in patients ≤ 35 years old or presenting with multifocal or extra-adrenal disease. All patients with an *SDHD* mutation developed extra-adrenal tumors (pheochromocytomas or paragangliomas) at presentation or during follow up.

Conclusion: *SDHD* gene mutations in patients presenting with apparently sporadic adrenal pheochromocytoma are rare. We recommend *SDHD* mutation screening for patients presenting with (1) a family history of pheochromocytoma or PGL, (2) multiple tumors, (3) isolated adrenal or extra-adrenal PCC and age ≤ 35 years. Analysis of *SDHD* can also help to distinguish synchronous primary tumors from abdominal metastases.

INTRODUCTION

Pheochromocytoma (PCC) is a neuroendocrine tumor, usually arising in the adrenal medulla. Despite its low incidence, the diagnosis of PCC is considered in many clinical situations, since catecholamine secretion by the tumor causes a wide range of symptoms. Furthermore, rapid establishment of the diagnosis is important to prevent life-threatening complications, whereas surgical resection of the tumor is curative in the majority of patients.[1,2]

Familial PCC is inherited as an autosomal dominant trait alone or as a component of the multiple endocrine neoplasia type 2 syndrome, von Hippel-Lindau disease, or neurofibromatosis type 1. The remaining 90 percent of PCCs is classified as sporadic or nonsyndromic. However, Neumann et al. recently reported the presence of germ line mutations in 24% of a large series of apparently sporadic PCC patients.[3] One can thus conclude that, in the general population, more than 24% of PCC patients has a genetic predisposition to this tumor. This recent improvement in recognizing predisposition to PCCs is caused by the finding of germ line mutations in succinate dehydrogenase subunit D (*SDHD*), in patients with familial and apparently sporadic PCC. The *SDHD* gene was initially identified as a susceptibility gene for the autosomal dominant familial parasympathetic paraganglioma syndrome (PGL1; MIM 168,000).[4] The gene encodes the small subunit (cybS) of cytochrome b in the mitochondrial enzyme complex II (succinate-ubiquinone oxidoreductase), and plays an important role in both the citric acid cycle and the aerobic respiratory chain.[5] It has been demonstrated that germ line mutations in *SDHC* (succinate dehydrogenase subunit C) and *SDHB* (succinate dehydrogenase subunit B), encoding two other components of complex II, also predispose to hereditary paraganglioma (PGL4 and PGL3, respectively).[6,7]

Because PCCs and parasympathetic paragangliomas (PGLs) both develop from neural-crest derived tissue, and co-occurrence of both tumors is reported,[8] analysis of *SDHD* as a susceptibility gene for sporadic PCC was performed in seven previous studies, with mutation rates between 0 and 17%.[3,9-15] These results are somewhat inconclusive and contradictory, especially with respect to whether *SDHD* mutation screening is appropriate for all PCC patients or only for a specific subset of these

patients. To determine appropriate indications for genetic screening is clinically important because of psychological and financial implications.

As for parasympathetic PGL,[16,17] screening for *SDHD* mutations in PCCs can be clinically important if it identifies patients who are at risk for developing multiple tumors. Screening potentially improves appropriate follow-up and early diagnosis of multiple tumors. In addition, it would be important to screen first-degree relatives in order to identify family members who are predisposed and should undergo biochemical and radiographic monitoring for the development of component tumors.

To establish whether screening for *SDHD* mutations is of value for all PCC patients, we evaluated a series of 126 patients with sporadic and syndrome-related PCCs. We also performed a comparative review of the literature to compare our results with those from previously published studies.

PATIENTS AND METHODS

We collected tumor specimens and normal tissues together with the clinical data of 126 patients with adrenal or extra-adrenal PCC, including 89 patients with clinically benign PCC and 37 patients with a proven malignant tumor. All 126 patients had undergone surgery between 1973 and 2001 at several hospitals in The Netherlands, the University Hospital, Lille, France, and the University Hospital Zürich, Switzerland. Patients investigated by Perren et al.[12] were excluded from this study. The diagnosis of the tumors was confirmed according to standard histopathologic analysis. Clinical (follow-up) data were obtained by review of medical records.

Malignancy was determined either by histologically confirmed distant metastases or a positive MIBG scan outside the adrenal area, with persistent postoperative elevation of catecholamine levels. Ninety-eight patients had localized disease and so far, after a mean follow-up time of 136 months (range 11 - 336), no metastases have been diagnosed in these patients.

A PCC was considered sporadic if the patient did not harbor a germ line mutation specific for MEN2 and VHL and the patient's personal and family histories were not suggestive of NF1, familial PCC or hereditary PGL. Information on medical and family histories was obtained by review of the medical records. The presence of multiple tumors was assessed by review of the pathology reports and the radiology reports of octreotide scintigraphy and/or magnetic resonance imaging (MRI).

A total of 144 primary PCCs (adrenal and extra-adrenal) were observed in the 126 patients, of which 134 primary tumors and matched normal tissues were available for analysis. In 14 of these patients the primary tumor and a metastasis were analyzed. After coupling of the clinical information to the pathology specimen, both patient information and DNA samples were anonymized in accordance with the Erasmus MC guidelines for studies involving patient data and tissues. A collective database of clinical and molecular features was prepared. Patients were classified by presenting diagnosis and genetic background. For each patient, we recorded the age at diagnosis, clinical history, genetic background of the tumor, hormonal activity, the laterality/multifocality of the tumors, and the presence of metastases. Table 1 summarizes relevant clinical characteristics of the patients.

In addition, clinical data from PCC patients and their *SDHD* status were extracted from the literature and were compared with our results. We also assessed whether genetic testing would have had impact on clinical decision-making and follow-up.

Table 1. Clinical Characteristics of 126 Patients with Sporadic or Familial Pheochromocytoma

		Benign (N= 89)	**Malignant** (N= 37)	**All** (N=126)
Mean age - years		46.9 ± 14.8	43.7 ± 15.2	46.2 ± 14.8
Range		13 - 79	23 - 70	13 - 79
Sex - no.	Male	44	15	59
	Female	45	22	67
Adrenal - no.		**83**	**26**	**109**
Sporadic		*60*	*22*	***82***
	Bilateral	2	2	4
Familial		*21*	*2*	***23***
	MEN2	10		10
	VHL	7	2	9
	NF1	4		4
Extra-adrenal - no.		**5**	**9**	**14**
Multifocal		**1**	**2**	**3**
Sporadic	PCC only		1	1
	PCC + PGL		1	1
Familial	PCC + PGL	1		1

Abbreviations: MEN 2, multiple endocrine neoplasia type 2; VHL, von Hippel-Lindau; NF1, neurofibromatosis type 1.

DNA preparation and SSCP Analysis

Fresh frozen or formalin-fixed, paraffin-embedded tumor and normal tissues from all patients, including 134 of the 144 tumors, were retrieved from the archives of the Pathology Departments of the above-mentioned hospitals. Haematoxylin-Eosin staining was performed to assess the amount of tumor tissue in the sections. DNA from fresh frozen tumors was isolated using the D-5000 Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN, USA) according to the manufacturers' recommendations. DNA extraction from paraffin-embedded tumor and normal tissues or peripheral blood samples was performed by standard detergent-proteinase K lysis, followed by phenol/chloroform extraction and ethanol precipitation.

The entire open reading frame of the *SDHD* gene and all exon-intron boundaries were investigated with PCR primers and conditions as described previously.[4] PCR amplification of tumor DNA and matched normal DNA was performed in 15 µl reaction mixtures containing 1.5 mM $MgCl_2$, 10 mM Tris-HCl, 50 mM KCl, 0.02 mM dATP, 0.2 mM dGTP, dTTP, dCTP each, 0.8 µCi α^{32}P-dATP (Amersham, Buckinghamshire, UK), 20 pmol of each sense and anti-sense primer, and 1 U Taq DNA polymerase (Amplitaq Gold, Perkin Elmer, Norwalk, CT, USA). The amplification profile consisted of an initial denaturation step at 94ºC for 5 min, followed by 35 cycles of denaturation at 94ºC for 45 sec, annealing at 55ºC for 60 sec, and extension at 72ºC for 60 sec. A final extension step was carried out at 72ºC for 10 min. Electrophoresis of PCR products was carried out overnight at 8W on non-denaturing gels, containing 8% polyacrylamide (49:1) and 10% (v/v) glycerol. For the exon 4 amplicons, electrophoresis was performed on 8% polyacrylamide gel without glycerol for 6 hours at +4ºC and 20W. The gels were dried and exposed to X-ray film overnight at –70ºC. DNA samples from 3 PGL patients with known germ line *SDHD* mutations D92Y, L95P (both exon 3), and L139P (exon 4) served as positive controls.

DNA sequencing

For each variant pattern identified by SSCP analysis, two independent genomic DNA samples from the patient's tumor were amplified for direct sequencing with the original primer pair. These PCR products were bi-directionally sequenced using

Applied Biosystems Taq DyeDeoxy terminator cycle sequencing (Baseclear, Leiden, The Netherlands).

Statistics

Correlations between a specific *SDHD* mutation and clinical features were tested by use of the chi-square test or an unpaired t-test. *P* values less than 0.05 were considered statistically significant.

RESULTS

Identification of SDHD gene mutations

SSCP analysis revealed 4 different aberrant patterns, which were present in the tumors and germ line DNA of 8 patients. We did not detect any somatic *SDHD* gene alterations in our series of 134 tumors from 126 patients. By sequence analysis, the aberrant patterns, located in exons 2 and 3, were identified as the pathogenic mutations D92Y and L95P, and polymorphisms H50R and S68S. D92Y, and H50R have been described in PCC patients previously,[3,12] whereas L95P has only been reported in patients with PGL so far.[18] Tumors from patients with D92Y and L95P showed loss of heterozygosity (LOH) of the wild type allele, whereas no LOH was observed in the 4 tumors with the S68S mutation. From the patient with the H50R variant, the adrenal PCC and a lung metastasis did not exhibit LOH, whereas the extra-adrenal tumor was found to have loss of 11q by CGH analysis (manuscript in preparation). Examples of SSCP analysis, the LOH observed herein, and the sequence determination of the *SDHD* missense mutations are shown in Figure 1.

The specific D92Y missense mutation, known as a Dutch founder mutation,[19] was observed in 2 Dutch patients. Patient A (Table 3), a 27-year old woman, presented with an apparently sporadic adrenal PCC and later developed a second primary tumor, i.c. an extra-adrenal PCC after 25 years. Patient B had a family history of PGL and presented with a mediastinal catecholamine-producing tumor at age 38. SRS imaging revealed a carotid body tumor and the patient developed multiple extra-adrenal PCCs during the first year of follow-up.

The L95P mutation was found in patient C (25-years old) with extra-adrenal PCCs at multiple abdominal spots, which was suspect of malignancy. Histopathological examination did not prove the presence of tumor surrounded by pre-existent lymphoid tissue. On SRS imaging, the patient also appeared to have bilateral carotid body tumors. After 12 years of follow-up, the patient is alive and well.

The H50R variant, which is likely a rare polymorphism, but possibly increases PCC susceptibility[20], was present in the germ line DNA of 1 (0.8%) patient (D). This 32-

year-old patient presented with both an extra-adrenal and an adrenal PCC, but had no additional tumors during 7 years of follow-up.

The S68S polymorphism was observed in 4 (3.2%) patients, including 3 patients with adrenal PCC and one patient with an extra-adrenal tumor.

Altogether, pathogenic *SDHD* mutations were identified in 2 (2.0%) of 102 apparently sporadic patients and in the one patient with a family history of PGL. No mutations were found in patients with MEN2, VHL, or NF1.

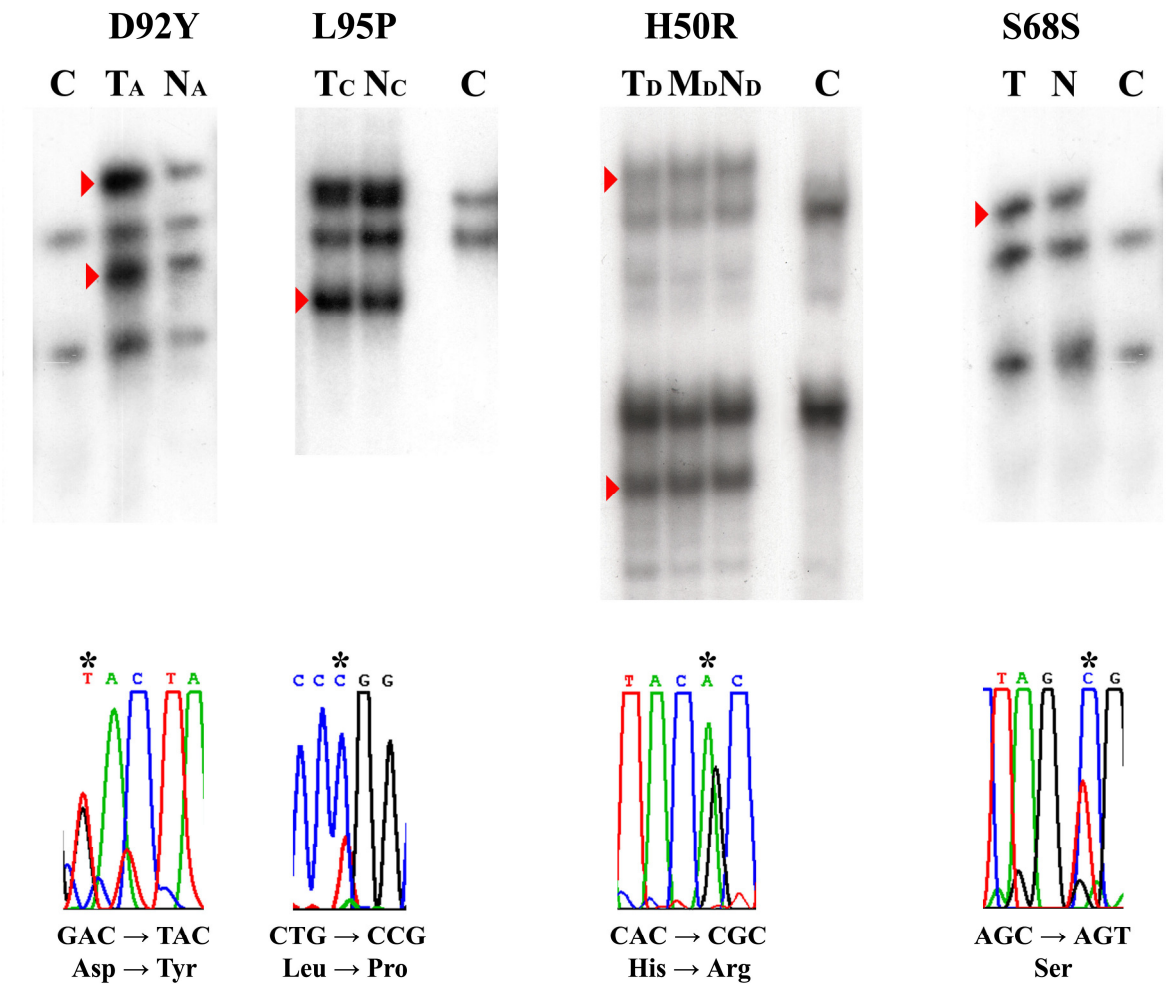

Figure 1. *SDHD* alterations identified in PCC patients by Single-Strand Conformation Polymorphism (SSCP) analysis and direct sequencing. From all *SDHD* alterations, SSCP patterns of the tumor (T) and corresponding germ line (N) DNA are shown. C = normal control sample. The autoradiographs of PCR-SSCP gels show the migration patterns of normal DNA and the mobility shifts produced by aberrant alleles (red arrowheads). The figure shows the two pathogenic mutations, D92Y and L95P, in patient A and C (Table 3), respectively, the H50R variant in the adrenal tumor and a lung metastasis (M) of patient D, and the silent S68S polymorphism.

Note: when comparing SSCP fragment intensities in the tumor samples, LOH of the wild type *SDHD* alleles is present in the D92Y and L95P samples. No LOH is present in S68S and H50R cases. The sequencing chromatograms below each autoradiograph show the alterations (note the substituted nucleotide marked by an asterisk).

Patients' characteristics associated with SDHD mutations

Two of the 3 patients with a germ line *SDHD* mutation presented with multifocal disease. One patient presented with a single adrenal PCC, but this patient also developed an extra-adrenal PCC during follow-up. The mean age of onset in patients harboring a germ line mutation was 30 years (range: 25-38) compared to 47 years (range 13-79) in patients without a *SDHD* mutation (*P*= 0.032).

Table 2 shows all publications that report on *SDHD* mutation analysis in PCC, including the number of mutations and the relevant clinical characteristics. These studies included 412 apparently sporadic and 27 familial PCC patients, either with or without PGL. Altogether, germ line *SDHD* mutations were found in 11 (2.7%) of 412 apparently sporadic patients and in 3 (33%) of 9 patients with a family history of PGL and/or PCC in which MEN2, VHL or NF1 was excluded. Only one somatic mutation was found, P81L, which is also known as germ line mutation in some PGL families.[11] Mutations were not found in MEN2-, VHL-, or NF1-related PCCs, but were observed in 1 of 5 PCC families. The majority (10/14) of the *SDHD* mutations were observed in patients presenting with an extra-adrenal PCC or with multiple tumors. Again, all patients with an *SDHD* mutation and presenting with a sporadic adrenal tumor developed one or more extra-adrenal tumors (including PGL) during follow-up.

Comparing our data with those from the literature reveals similar clinical features that indicate the likelihood of identifying an *SDHD* mutation in PCC patients. Overall, these include multifocal presentation (8/17, 47%), extra-adrenal location (4/55, 7.3%), or family history of (extra-adrenal) PCC or PGL (4/10, 40%). Twelve (72%) of the 17 patients with a mutation were 35-years old or younger and 15 (88%) of the 17 patients presented at age 40 or younger. In patients presenting with an adrenal tumor, a younger age of onset (≤ 35 years) increased the likelihood of a *SDHD* mutation (6.3% *versus* 1.1% in total subpopulation, based on this study and Neumann et al.[3]). Table 3 shows all PCC patients with a *SDHD* mutation, including their presenting diagnosis and follow-up.

Table 2. Presenting Diagnosis and Follow-Up of Patients With a Germline *SDHD* Mutation in this Study and in the Literature

Patient	Age (years)	Presentation	Family History	Follow-Up	*SDHD* Mutation
This study					
A	27	Unilateral adrenal PCC	—	Para-aortic PCC after 25 years	D92Y
B	38	Mediastinal PCC, bilateral CBT	PGL	Para-aortic and aortico-pulm, PCC	D92Y
C	25	Multiple para-aortic PCC	—	CBT	L95P
D	32	Adrenal malignant PCC, para-aortic PCC	—	—	H50R
Astuti et al[6] (2001)					
E (family)	13-23	Unilateral or bilateral adrenal PCC, para-aortic PCC	CBT	?	S32fs
Neumann et al[3] (2002)/Glmm et al[11] (2000)			—		
F	39	Unilateral adrenal PCC	—	CBT	IVS1+2T > G
G	33	Thoracic and para-aortic PCC	—	?	R38X
H‡	5	Multiple PCC	—		
I‡	< 20	Multiple PCC	—		
K‡	< 20	Multiple PCC	—		
L‡	< 30	Unilateral adrenal PCC	—	Majority of patients develop additional tumors, either extra-adrenal PCC or PGL	W5X, C11X, A13fs, R38X, D92Y, Q121X
M‡	< 30	Unilateral adrenal PCC	—		
N‡	< 30	Extra-adrenal PCC	—		
O‡	31-40	Extra-adrenal PCC	—		
P‡	41-50	Extra-adrenal PCC	—		
R‡	59	Extra-adrenal PCC	—		
Cascon et al[10] (2002)					
S	40	Para-aortic and bilateral CBT	PCC and PGL	?	W43X
Bauters et al[15] (2003)					
T	30	Juxtarenal PCC, cervical PGL	PCC and/or PGL	?	IVS2-1G > T

Abbreviations: CBT, carotid body tumor; PCC, pheochromocytoma; PGL, paraganglioma.
*Pathogenicity of this mutation is uncertain.
‡Data of these patients are not assigned per individual patient.

Table 3. Overview of *SDHD* Mutation Analysis in Sporadic and Syndrome-Related Pheochromocytoma in this Study and the Literature

	Frequency of *SDHD* Gene Mutations																					
	Total		Other Studies		This Study		Astuti et al[6] 2001		Aguiar et al[14] 2001		Kytölä et al[13] 2002		Neumann et al[3] 2002*		Perren et al[12] 2002		Cascon et al[10] 2002		Bauters et al[15] 2003	
Tumor Type	Frequency	%	Frequency	%	Frequency	%	Frequency	%	Frequency	%	Frequency	%	Frequency	%	Frequency	%	Frequency	%	Frequency	%
Adrenal	496		384		109		28		20		44		241		9		18		24	
Sporadic	4/432	0.9	3/350	0.9	1/82‡	1.2	24		19		36		3/241§	1.2	9		0/12 H50R/G12S		0/9 G12S	
Bilateral	14		10		4												5		5	
Familial																				
PCC only	1/5		1/5				1/4		1											
PGL	1		1														1			
Malignant† (sporadic)	33		9		24				4		5									
Extra-adrenal	4/55	7.3	4/41	9.8	14						6		4/22§	18	0/4 H50R		2		0/7 G12S	
Malignant					9						3									
Benign					5						3									
Multifocal	8/17	47	6/14	43	2/3	67							4/8	50			1/3	33	1/3	33
Sporadic PCC	4/11		4/10		0/1 H50R								4/8						2	
PCC + PGL	1/2		1		1/1								?				1			
Familial																				
PCC + PGL	3/4	75	2/3	67	1/1												1/2		1/1	
Total	17/565	3.0	14/439	3.2	3/126	2.4	1/28	3.6	20		50		11/271	4.1	13		1/23	4.3	1/34	2.9

NOTE. Number of mutations refers to pathogenic mutations. Rare, possibly nonpathogenic variants (G12S and H50R) are denoted separately and not regarded as mutations. The frequency of mutations is only denoted if mutations were found in a specific set of tumors (eg, one mutation was found in our series of 82 isolated sporadic adrenal PCCs [one of 82]). The frequency of mutations is also depicted in % directly next to the absolute numbers. If no mutations were found, only the number of tumors analyzed is shown. Variants G12S and H50R are not counted as pathogenic mutations in this table. Their occurrence is denoted separately, for example, in the study by Cascon et al[10] in two isolated sporadic adrenal tumors. Numbers in the table may not reflect the numbers from the published articles, as some sample series contained nonpheochromocytoma patients as a result of different selection criteria.

Abbreviations: PCC, pheochromocytoma; PGL, paraganglioma.

*Including study of Gimm et al, 2000.

†Malignancy was in some studies operationally defined as local infiltration of adjacent tissues and/or metastatic disease.

‡Patient developed an extra-adrenal PCC after 25 years.

§Four of these seven patients developed PGL during follow-up.

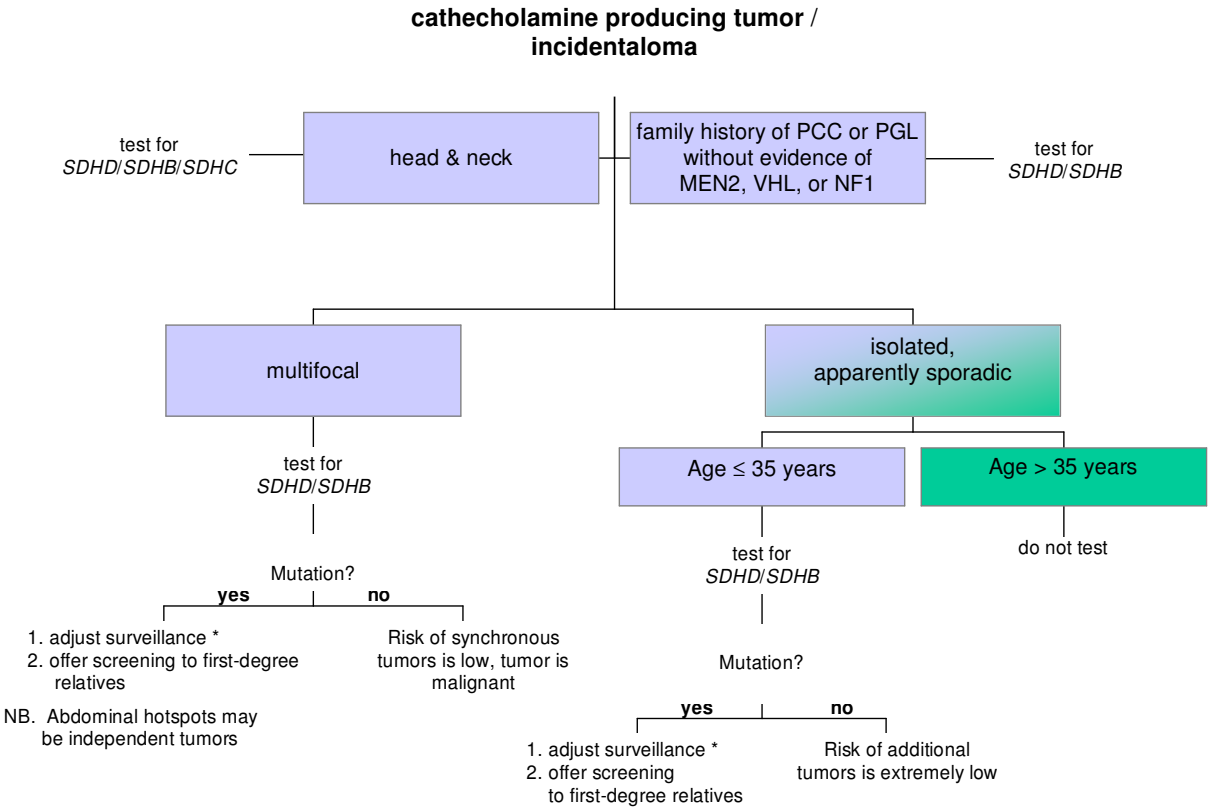

Figure 2: Decision tree for SDHD/SDHB genetic screening in pheochromocytoma (PCC) patients. MEN 2, multiple endocrine neoplasia; VHL, von Hippel-Lindau disease; NF1, neurofibromatosis type 1. (*) Surveillance should be supplemented with periodic ultrasonographic examination of the neck or cervical magnetic resonance imaging (MRI) for detection of PGL. For the detection of extra-adrenal pheochromocytoma, an MRI or paravertebral sympathetic chain is recommended. Alternatively, [^{125}I] metaoidobenzylguanidine, octreotride scintigraphy, or positron emission tomography scanning can be performed.

DISCUSSION

This investigation of *SDHD* alterations in 126 pheochromocytoma (PCC) patients underlines specific clinical features, including multifocal presentation of the tumor, younger age of onset (≤ 35 years), and a family history of extra-adrenal PCC or PGL, that increase the likelihood of identifying an *SDHD* mutation in these patients.

Genetic screening is still under considerable debate as unnecessary screening has an undesirable psychological impact on the patients and is not cost-effective. One should therefore carefully report on indications that favor genetic testing.[21] So far, studies have recognized the fact that *SDHD* mutations are associated with extra-adrenal PCC,[22] but the clinical relevance of *SDHD* mutation screening has been poorly discussed. A careful review of all current data indicates that specific subgroups of PCC patients could be considered for genetic screening of *SDHD*. These include patients presenting with multifocal tumors (PCC and/or PGL) independent of their family history of PCC/PGL (50% harbor *SDHD* mutations) and patients presenting with an extra-adrenal PCC (7% harbor *SDHD* mutations). Regarding patients presenting with a sporadic adrenal PCC, the overall likelihood of germ line *SDHD* mutations is only 1%. However, younger age of onset (≤ 35 years) or a family history of PCC or PGL in these patients are two features that increase the likelihood of a mutation to 6.3% (based on this study and Neumann et al.[3]) and 16.7%, respectively. Screening of patients presenting at age 35 and younger will identify at least 72% of patients with germ line *SDHD* mutations. Screening in apparently sporadic patients older than 35 years and without a family history of PCC/PGL, as well as in patients with sporadic bilateral PCC seems redundant since mutations in these patients are extremely rare (< 1%).

To justify genetic screening, testing for *SDHD* mutations should help to improve early diagnosis, prognosis or influence treatment. In PCC patients, early detection is the key factor to reduce morbidity and mortality and identification of patients that are prone to develop multiple tumors may improve early detection. It is thus of interest to consider to what extent *SDHD* mutation screening contributes to early diagnosis in PCC patients. Most patients that appear to harbor an *SDHD* mutation present with multiple tumors, so that the risk of additional tumors is already evident and

surveillance will be adjusted. In these cases, genetic screening is of interest to clarify the genetic cause of the disease, or to identify positive family members. Additionally, in some patients presenting with multiple abdominal foci on MRI, MIBG or octreotide scintigraphy, mutation screening may help to differentiate between lymph node metastases and multiple independent synchronous tumors.

Mutations are infrequent in patients presenting with apparently sporadic isolated PCC (up to 6.3% in patients ≤ 35 years old), which does not favor genetic testing in these patients. However, an *SDHD* mutation specifically identifies patients that are prone to develop additional PCC or PGL tumors, a reason to target a specific follow-up strategy to these patients alone. At least 60% (5/8, Table 2; Table 3) of isolated patients with germ line *SDHD* mutations developed metachronous primary tumors (PCC or PGL), and also Neumann et al.[3] estimated a 20-30% likelihood of the subsequent development of a parasympathetic PGL. One reason to extend the *SDHD* screening to patients that present at age 40 or younger is the fact that it will identify almost 90% of patients with germ line *SDHD* mutations, instead of 72% when the cutoff age is 35. This will decrease the likelihood of a mutation, but is certainly defendable in the light of the relatively low burden of the disease and the importance for early diagnosis and treatment. Since PCC patients remain in follow-up because of the risk of malignancy, the follow-up management in PCC patients with *SDHD* mutation needs complementation. Periodic physical and ultrasonographic examinations of the neck or cervical MRI can be performed to detect PGL. Furthermore, we propose MRI imaging of the paravertebral sympathetic chain for the surveillance of (extra-adrenal) PCC. Alternatively, MIBG or octreotide scintigraphy can be used.

Although the majority of patients presenting with multiple tumors have *SDHD* mutations, a considerable number of multifocal patients lacks a germ line *SDHD* mutation. These patients may harbor a mutation in *SDHB*.[23] Patients with *SDHB* mutations present more frequently with PCCs (mostly extra-adrenal),[3,6,23-25] and *SDHD* carriers present more frequently with PGL,[16,17] but these studies also show that similar features (multifocal presentation, family history of PGL or PCC, extra-adrenal location, or age of onset ≤ 35 years) indicate the presence of a germ line

mutation. Therefore, when genetic testing is appropriate, both *SDHD* and *SDHB* genes should be investigated simultaneously.

Genetic testing can be offered to first-degree relatives of patients with a germ line mutation. For appropriate genetic counseling, an estimation of the penetrance and the lifetime risk on PCC and PGL is important. Unfortunately, data on penetrance of the disease or the lifetime risk on PCC or PGL are only poorly established with regard to *SDHD* and *SDHB* germ line mutations. Examination of available data suggests that the family history of more than 60% of apparently sporadic patients with mutations becomes positive after screening of asymptomatic carriers in their families.[3] Follow-up in asymptomatic carriers should probably be proposed at 5 to 10 years of age[26] and should comprise of physical and ultrasonographic examinations of the neck and exclusion of catecholamine hypersecretion.

When patients have multiple tumor locations at presentation or during follow up, it can be difficult to distinguish independent primary tumors from metastases or recurrent disease. In patient A (Table 3), we can regard the second lesion as a primary tumor based on the location of the tumors, the absence of pre-existent lymph node tissue and the otherwise clinically benign behavior. The finding of an *SDHD* mutation indicates the presence of a second primary tumor in this patient. Patient C was suspected to have a malignant tumor, because of multiple extra-adrenal abdominal spots observed with MIBG scintigraphy. Again, the absence of preexistent lymphoid tissue in combination with the presence of a germ line *SDHD* mutation is suggestive of synchronous para-aortic PCCs.

Patient D harbored the H50R variant, which is shown to occur in 2.8% of apparently healthy individuals, indicating that H50R is a non-pathogenic variant. However, our patient presented with two independent tumors, as indicated by the absence of preexistent lymphoid tissue and shown by completely different CGH profiles of the two tumors (data not shown, manuscript in preparation). Furthermore, the relatively young age of onset in this patient als suggests the existence of a genetic predisposition. Interestingly, the extra-adrenal tumor showed loss of 11q by CGH analysis, whereas the adrenal tumor and a lung metastasis did not reveal LOH of the *SDHD* locus (Figure 1). Although we could not exclude a germ line mutation in other genes, e.g. *SDHB*, H50R may also act as a low penetrance mutation in this patient.

In summary, early detection of PCCs is important to improve prognosis and can be achieved by the appropriate follow-up management in patients at risk. *SDHD* mutation analysis specifically identifies patients that are susceptible to develop multiple PCCs and PGLs. Since surveillance is already continued in most PCC patients because of the risk of malignancy, an adjusted surveillance strategy needs to be targeted to mutation positive patients. The subsequent identification of mutation carriers in family members will further improve early detection of PCC and PGL. We have demonstrated that *SDHD* gene mutations in patients with apparently sporadic, adrenal PCC are rare, and therefore, screening for *SDHD* mutations in these patients is redundant. However, *SDHD* mutation screening is appropriate for patients presenting with a family history of PCC or PGL, multiple tumors, or isolated adrenal or extra-adrenal PCC and age ≤ 35 years. The results of this study, correlating *SDHD* mutations with clinical features of PCC patients, will hopefully contribute to improving appropriate genetic screening for patients who are at risk of developing multiple PCCs and PGLs.

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CHAPTER 7, SOMATIC MUTATION IN AN EXTRAADRENAL PHEOCHROMOCYTOMA

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To the Editor:

Up to 25 percent of pheochromocytomas, catecholamine producing tumors located along the sympathetic nervous system including the adrenals, occur in hereditary tumor syndromes that include Von Hippel-Lindau Disease (VHL gene) [1], Multiple Endocrine Neoplasia Type 2 (RET gene), Neurofibromatosis Type 1 (NF1 gene) and the Pheochromocytoma-Paraganglioma syndrome (PCC-PGL syndrome (SDHB and SDHD genes)). These last 2 genes are also correlated with extra-adrenal pheochromocytomas. [2, 3] To date, except for one sporadic SDHD mutation, only germline mutations in SDHB and D have been described, even in reported mutations in these genes in apparently sporadic pheochromocytomas and paragangliomas. [4, 5]

We present a case of a 25-year-old woman with an extra-adrenal pheochromocytoma in the wall of her urinary bladder. Mutation analysis of the pheochromocytoma candidate genes RET, VHL, SDHB and SDHD was performed in tumor and normal DNA. A single aberration was found: an SDHB 433C>T transition in tumor DNA but not in the patient's normal DNA (Figure 1). This finding was confirmed by allelotyping the DNA samples, and repeating the entire procedure starting from isolating DNA from tumor and normal tissue. This somatic SDHB gene mutation results in a serine to phenylalanine (S100F) substitution. Functional consequences of the S100F mutation can be anticipated because of the large physical differences between the two amino acids: an uncharged polar side chain (S) is substituted by a nonpolar side chain (F). Additionally, the region of the SDHB gene including the S100F mutation is highly conserved at the protein level. In addition, an SDHB gene germline missense mutation of the S100 neighboring amino acid (C101Y) has been described in a patient with an extra-adrenal pheochromocytoma.(1)

From the sequence analysis of the tumor DNA it is apparent that the mutated allele is in excess of the wild type allele (Figure 1D), indicating amplification of the mutated allele or loss of wild type allele. Comparative genomic hybridization, loss of heterozygosity of the SDHB locus, and chromosome 1p fluorescent in situ

hybridization all demonstrated loss of one 1p allele. These findings point to the biallelic inactivation of SDHB in this tumor: mutation of one SDHB allele and loss of the second SDHB allele. In addition, we found absence of SDHB expression in tumor cells, indicating complete loss of SDHB function (Figure 1E).

We would suggest that the somatic S100F mutation played a causal role in the tumorigenesis of the extra-adrenal pheochromocytoma. This finding indicates that the SDHB gene not only plays a role in the pathogenesis of a subset of inherited pheochromocytoma but also can be involved in a subset of truly sporadic pheochromocytomas.

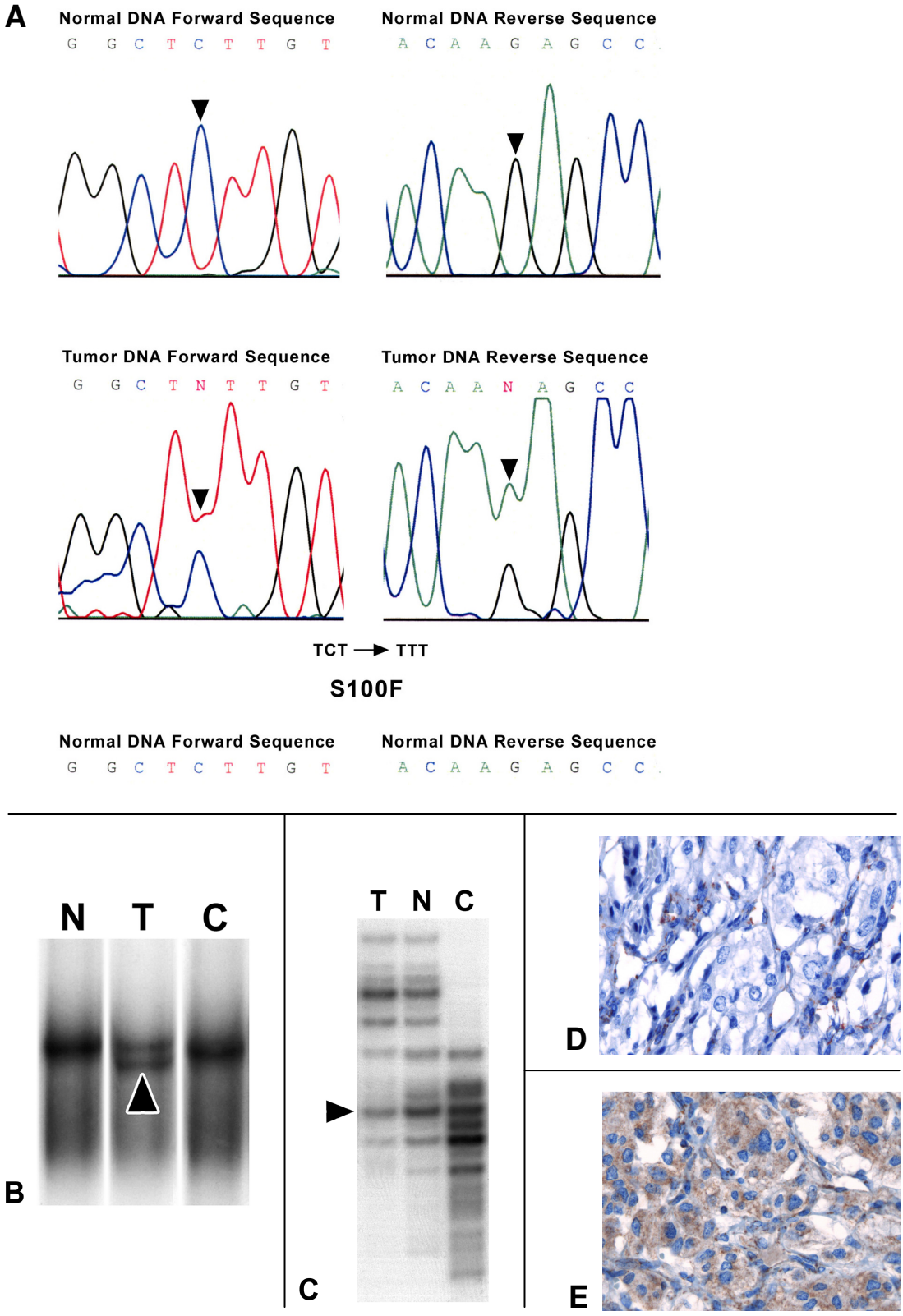
A
Normal DNA Forward Sequence
G G C T C T T G T
Normal DNA Reverse Sequence
A C A A G A G C C
Tumor DNA Forward Sequence
G G C T N T T G T
Tumor DNA Reverse Sequence
A C A A N A G C C
TCT → TTT
S100F
Normal DNA Forward Sequence
G G C T C T T G T
Normal DNA Reverse Sequence
A C A A G A G C C
N T C
B
T N C
C
D
E

Figure 1 A: Denaturing gradient gel electrophoresis (DGGE) analysis pattern of the patient's normal (N) and tumor (T) compared to control (C) DNA. Note the aberrant migration pattern in tumor DNA (arrowhead).

Figure 1 B: The autoradiograph of the polymerase chain reaction–single strand conformation polymorphism (PCR-SSCP) gel shows the migration patterns and the mobility shift produced by aberrant tumor (T) DNA compared to the patient's normal (N) DNA.

Figure 1 C: Loss of Heterozygosity (LOH) autoradiograph of chromosome 1p with a marker in proximity to the SDHB gene showing relative loss of tumor (T) DNA (arrowhead) compared to the patient's normal (N) DNA.

Figure 1 D: Sequence analysis of SDHB exon 4 of tumor and normal DNA with the relative sequence signal intensities at position 433 of the wildtype nucleotide C and the substituted nucleotide T, indicating relative loss of the wild type allele in the tumor DNA.

Figure 1 E: Immunohistochemical staining for SDHB. Note the negative tumor cells surrounded by positive endothelial cells with speckled staining.

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CHAPTER 8, AN IMMUNOHISTOCHEMICAL PROCEDURE TO DETECT PATIENTS WITH PARAGANGLIOMA AND PHAEOCHROMOCYTOMA WITH GERMLINE *SDHB*, *SDHC*, or *SDHD* GENE MUTATIONS: A RETROSPECTIVE AND PROSPECTIVE ANALYIS

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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 NaH2PO4.H2O, 33·3 mmol/L Na2HPO4.2H2O, 41·7 mmol/L Na2C4H4O4, 2·5 mol/L Nitroblue terazolium (N-6876, Sigma-Aldrich), 0·22 mmol/L AlCl2.6H2O, 0·13 mM CaCl2, 25 mM Na2HCO3, 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).

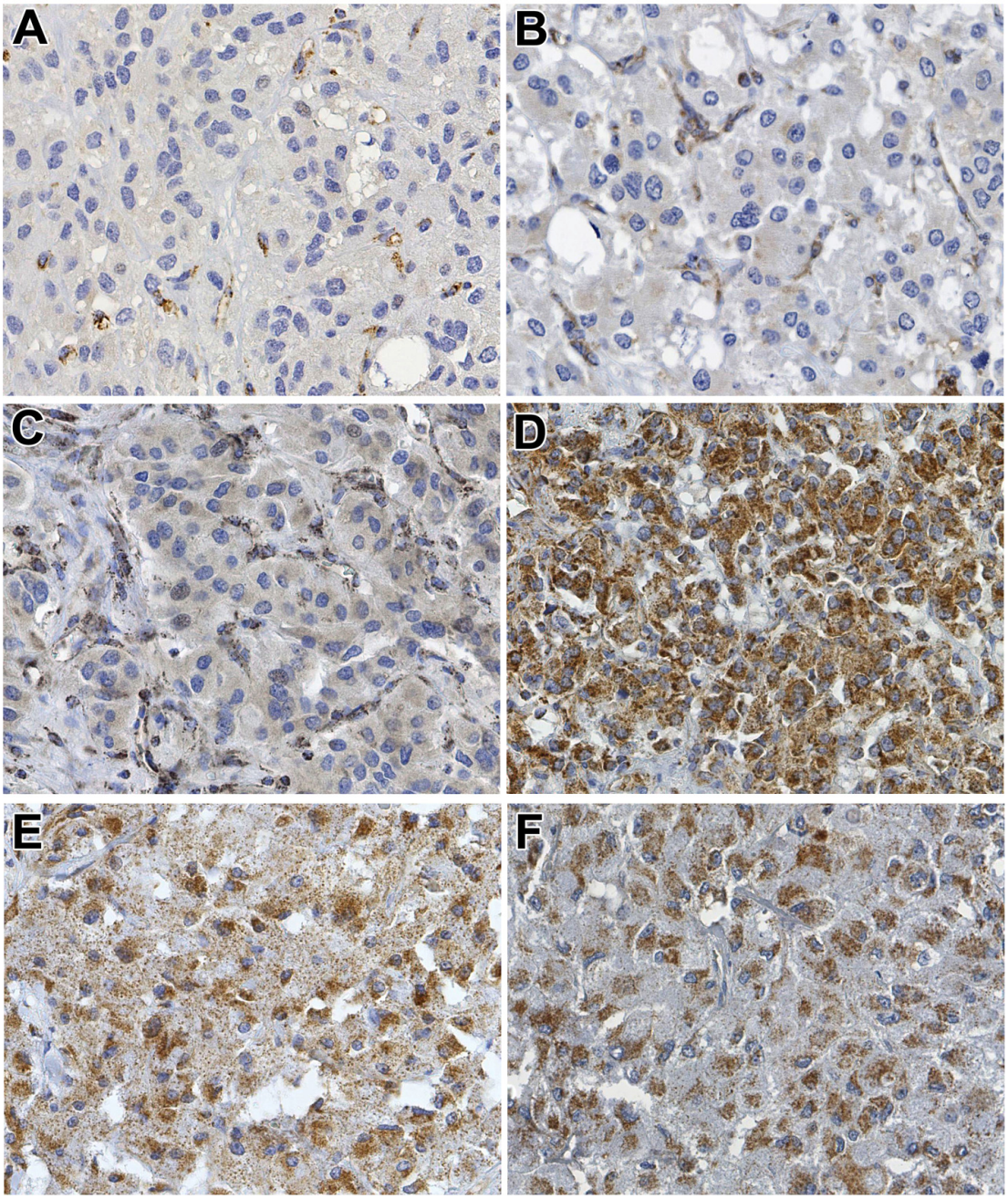

Figure 1. SDHB immunohistochemistry on paragangliomas and pheochromocytomas.

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.

Series	Group	Gene	No. of tumors	SDHB IHC		Sensitivity	95% CI	Specificity	95% CI
				negative	positive				
Retro-spective	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%

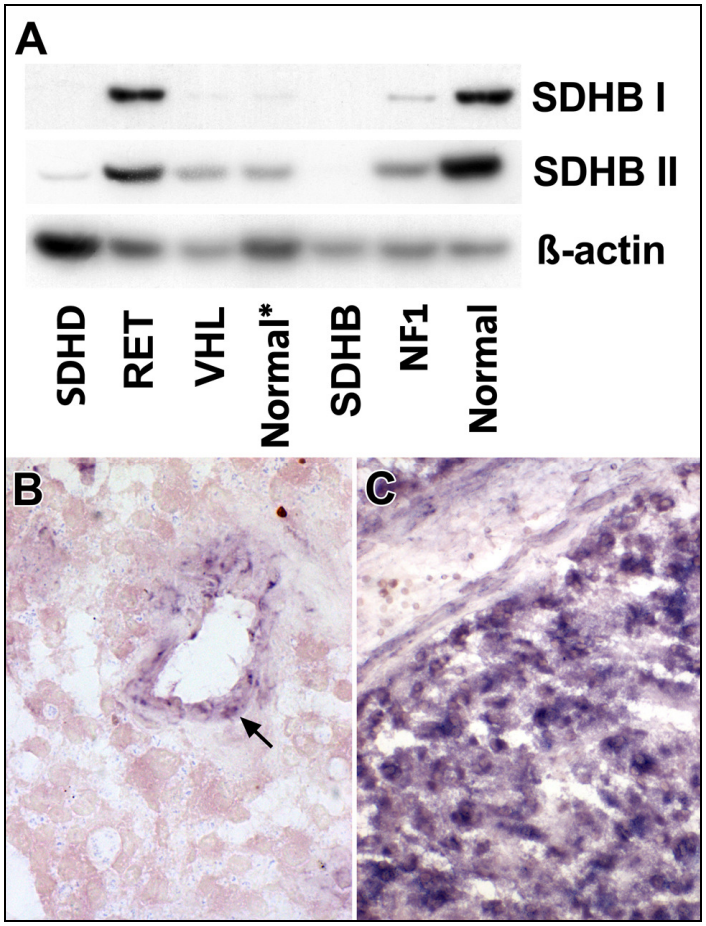

Figure 2. Western blotting and enzyme histochemical results.

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).

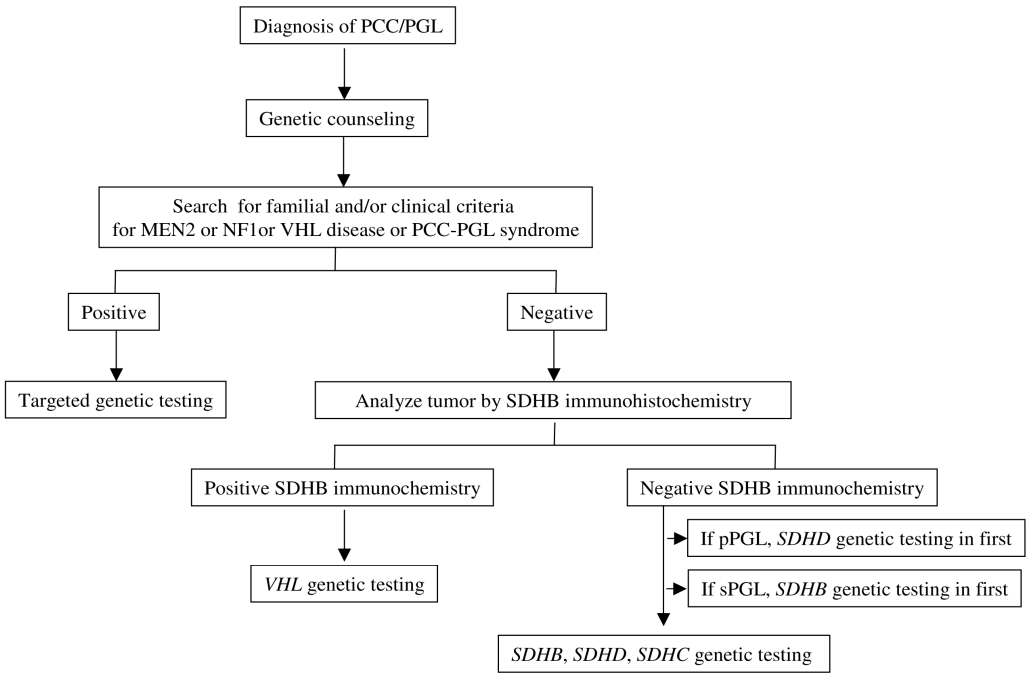

Figure 3. Suggested algorithm for molecular genetic testing for PCC and PGL.

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.

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CHAPTER 9, SDHB PREDICTS MALIGNANCY IN PHEOCHROMOCYTOMAS/ SYMPATHETIC PARAGANGLIOMAS, BUT NOT THROUGH HYPOXIA SIGNALLING

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ABSTRACT

Prediction of malignant behaviour of pheochromocytomas/sympathetic paragangliomas (PCC/PGL) is very difficult if not impossible on a histopathological basis. In a familial setting, it is well known that SDHB-associated PCC/PGL very often metastasize. Recently, loss of SDHB immunohistochemistry was shown to be an excellent indicator of the presence of an *SDH* germline mutation in PCC/PGL. SDHB loss is believed to lead to tumour formation by activation of hypoxia signals.

To clarify the potential use of SDHB immunohistochemistry as a marker of malignancy in PCC/PGL and its association with hypoxia signalling we examined SDHB, Hif-1α and its target, CA-9 expression on protein level using immunohistochemistry on a tissue micro array on a series of 126 familial and sporadic tumours. Survival data was available for 66 patients.

SDHB expression was lost in 12 of 99 evaluable tumours. *SDHB* germline mutations were present in 5 patients, absent in 4 patients and unknown in 3 patients. Loss of SDHB expression was not associated with increased hypoxia signalling as detected by HIF-1α staining or CA-9 staining. Loss of SDHB expression was associated with an adverse outcome.

The lack of correlation of SDHB loss with hypoxia signals argues against the current hypoxia hypothesis. We suggest SDHB protein loss as a marker of adverse outcome both in sporadic and in familial PCC/PGL.

INTRODUCTION

Pheochromocytomas are rare tumours of neural crest-derived chromaffin cells. Patients can become clinically symptomatic due to uncontrolled secretion of catecholamines. Most tumours arise in the adrenal gland, but about 10% are localized in extraadrenal tissue and are called sympathetic paragangliomas (DeLellis *et al.* 2004). About 30% of these tumours occur in familial tumour syndromes (Tischler 2008) (Komminoth 2009) including neurofibromatosis type 1 (NF1), von Hippel-Lindau disease (VHL), multiple endocrine neoplasia type 2 (MEN2) and SDH-syndromes (Dluhy 2002).

The underlying molecular mechanisms leading to pheochromocytomas/sympathetic paragangliomas are not fully understood. Several studies suggest that hypoxia signalling may play a central role: *VHL* inactivation leads to hypoxia signalling by inhibiting Hif-1α degradation (Maxwell *et al.* 1999). This mechanism is supported by expression analysis of pheochromocytomas: on the RNA level, Eisenhofer et al described activation of hypoxia-driven angiogenic pathways in VHL syndrome tumours (Eisenhofer *et al.* 2004). Hif-1α was not in the list of upregulated genes, possibly because it is regulated on protein rather than on expression levels. Dahia et al describe a hypoxia-induced expression profile in *VHL* and *SDH*-induced pheochromocytomas/paragangliomas (Dahia *et al.* 2005). By gain- and loss-of-function analyses they additionally suggested that the link between hypoxia signals (via pVHL) and mitochondrial signals (via SDH) is mediated by Hif-1α. Succinate accumulating due to *SDH* mutations can inhibit the degradation of Hif-1α as do *VHL* mutations. This is thought to cause upregulation of Hif-targets leading to tumorigenesis (Dahia *et al.* 2005) (Selak *et al.* 2005) (Selak *et al.* 2006) in SDH-associated pheochromocytomas/paragangliomas. However, other results point towards other mechanisms than hypoxia signalling: VHL mutants leading to the VHL 2c phenotype consisting exclusively of pheochromocytomas retain their ability to downregulate Hif (Hoffman *et al.* 2001). In C. elegans, a subset of genes dysregulated in vhl mutants is not normalized in vhl/hif-1 double mutants (Bishop *et al.* 2004). Failure of developmental apoptosis may be the hypoxia-independent

mechanism of pheochromocytoma pathogenesis (Lee *et al.* 2005), possibly with a regulatory loop including Hif (Maxwell 2005).

In the familial setting, it is well known that SDHB-associated pheochromocytomas/paragangliomas very often lead to metastases (Timmers *et al.* 2007), sometimes many years after resection (Maier-Woelfle *et al.* 2004). Prediction of malignancy in sporadic tumours is an unsolved problem; the only definite evidence of malignancy is the detection of metastases. The “Pheochromocytoma of the adrenal gland scaled score (PASS)”, a morphological scoring system to identify more aggressive tumours (Thompson 2002) has not proved to be useful due to great interobserver variability in a recent study (Wu *et al.* 2009). An increased risk of malignancy seems to be indicated by Ki-67 proliferation indices greater than 2% or 3%, but this is of limited clinical use (August *et al.* 2004) (Kimura *et al.* 2005), (Strong *et al.* 2008) (van der Harst *et al.* 2000). Recently, loss of SDHB immunohistochemistry was shown to be an excellent indicator of the presence of an *SDH* germline mutation in pheochromocytomas and paragangliomas (van Nederveen *et al.* 2009). Its potential use for predicting biological behaviour is unknown.

To clarify the potential use of SDHB immunohistochemistry as a marker of malignancy and its association with hypoxia signalling we decided to examine SDHB, Hif-1α and its target, CA-9 expression on protein level on a series of familial and sporadic pheochromocytomas and sympathetic paragangliomas and to correlate the results with survival data.

MATERIALS AND METHODS

Patients and tumour specimens

All pheochromocytomas and sympathetic paragangliomas analysed in the Institute of Surgical Pathology, University Hospital Zurich in the years from 1975 to 2006 were included.

Clinical data and follow up information were extracted retrospectively from patient charts. A questionnaire enquiring about tumour relapse or progression was sent to family doctors.

	Total	**Men**	**Women**	**Unknown**
Patients	115	62 (53.9%)	46 (40.0%)	7 (6.1%)
Multifocal	19 (16.5%)	11 (9.6%)	7 (6.1%)	1 (0.9%)
Follow-up available	66 (57.4%)	35 (30.4%)	31 (27.0%)	0 (0%)
Tumour-related death	15 (13.0%)	5 (4.3%)	3 (2.6%)	7 (6.1%)
No tumour-related death/alive	59 (51.3%)	30 (26.1%)	29 (25.2%)	0 (0%)
Follow-up				
Range (month)	2 – 291	5 - 291	2 - 188	-
Mean (month)	78,39	77.4	79.52	-
Median (month)	55,5	52	71	-
Syndromic patients				
NF-1	1 (0.9%)	1 (0.9%)	0 (0%)	0 (0%)
VHL	5 (4.3%)	1 (0.9%)	3 (2.6%)	1 (0.9%)
MEN2	6 (5.2%)	3 (2.6%)	3 (2.6%)	0 (0%)
SDHB	5 (4.3%)	4 (3.5%)	1 (0.9%)	0 (0%)
Any syndrome	17 (14.8%)	10 (8.7%)	7 (6.1%)	0 (0%)
Localization (primary tumour)				
Unknown	3 (2.6%)	2 (1.7%)	1 (0.9%)	0 (0%)
Adrenal	76 (66.1%)	37 (32.2%)	32 (27.8%)	7 (6.1%)
Extraadrenal	33 (28.7%)	22 (19.1%)	11 (9.6%)	0 (0%)

Table 1. Clinical characteristics of 115 PCC patients

Tumour Specimens

We analyzed a total number of 126 tumour specimens from 115 patients with pheochromocytoma and extraadrenal sympathetic paraganglioma (62 males, 46 females, sex could not be evaluated in 7 patients). The available paraffin specimens comprised 118 primary tumours and 8 metastases. Of 3 patients, only tissue from metastatic sites was available. Of the primary tumours, 33 were of extraadrenal localization (men: 22; women: 11) and 76 originated from the adrenal medulla (men: 37; women: 32; not specified: 7). Information about the localization was not available in 3 tumours (figures do not add up to 118, the number of primary tumours mentioned on the previous page). A tissue micro array (TMA) comprising these 126 tissues was constructed as described (Bubendorf *et al.* 2001).

Syndromic patients

One male patient suffered from NF-1 disease clinically, five patients from VHL disease (4 with proven *VHL*-mutation, one clinical VHL disease with multiple bilateral clear cell renal cell carcinomas in addition to the pheochromocytoma (1 man, 3 women, 1 not specified)) and 6 patients from MEN 2 (proven *RET*-mutation (3 male, 3 female)) (table 4). An overview of patients and follow up data is given in table 1.

Immunohistochemistry

The analysis was performed on 4 µm sections from the tissue micro array, which was stained with antibodies against CA-9, Hif-1α, CD34 and SDHB. The immunohistochemical staining for the antigens was performed on automated staining systems (CA-9 on Bond Refine, Vision BioSystems Ltd., Newcastle Upon Tyne, UK; Hif-1α on Bond Refine, Vision BioSystems Ltd., Newcastle Upon Tyne, UK; CD34 on Ventana BenchMark, Ventana Medical Systems, Tucson, Arizona). For SDHB staining the slides were pre-treated by microwave heating in Tris/EDTA buffer, pH 9.0 at 100^0C for 40 min or citrate buffer, pH 6.0 for 15 min. After rinsing in tap water followed by incubation in 3% H_2O_2 in PBS for 15 minutes the SDHB antibody was incubated overnight at 4°C. The presence of tumour tissue was verified by synaptophysin and H&E stainings in all punch cylinders.

The following antibodies were used: CA-9 polyclonal antibody ab15086 (Abcam, Cambridge, UK), dilution 1:200; Hif-1α monoclonal antibody ab16066 (Abcam Limited, Cambridge, UK; dilution 1:500 and SDHB rabbit polyclonal HPA002868 (Sigma-Aldrich Corp, St. Louis, MO) dilution 1:250; CD34 clone QBEND / 10 (MCAP 547, Serotec, MorphoSys, Oxford, UK), dilution 1:800.

Visualization was accomplished using the avidin-biotin-complex (ABC) method leading to a brown staining signal. As controls, for SDHB and CD34 endothelial cells served as internal positive control, for CA-9 normal liver tissue, for Hif-1α glioblastoma tissue was used.

Cytoplasmic and / or membranous staining was scored positive for CA-9. For Hif-1α nuclear and cytoplasmic staining were separately evaluated. Depending on the intensity of staining a semiquantitative scoring system was used, comprising strongly positive, weakly positive and negative immunoreactivities. Tumours with less than 5% positive tumour cells were scored as negative.

SDHB was scored as positive if the cytoplasm showed a strong dot-like positivity. We categorized the tumour as negative if the cytoplasm was negative in the presence of internal positive control in endothelial cells. Tumours with homogeneous faint cytoplasmic staining were scored negative (van Nederveen *et al.* 2009).

Microvessel density was calculated by counting all vessels of each TMA cylinder. A correction factor was used in case the cylinder did not completely consist of tumour tissue. The number of vessels per square millimetre was calculated. Examples of immuohistochemical stainings are given in figure 2.

SDHB Mutation Analysis

Germline mutation analysis of the SDH genes was performed on peripheral blood after obtaining informed consent in patients with negative SDHB immunohistochemistry. Where no non-neoplastic tissue was available, mutation analysis was performed in tumour tissue.

DNA was extracted from peripheral blood using the Puregene kit (GentraSystems, Minneapolis, MN) according to the manufacturer's instructions. DNA was extracted from paraffin tissue as described (Maier-Woelfle *et al.* 2004). Mutation analysis was performed by denaturing gradient gel electrophoresis (DGGE)-based mutation

analysis as described (Maier-Woelfle *et al.* 2004). PCR reactions were repeated for all samples with abnormal banding patterns followed by cycle sequencing.

Statistical analysis

The statistical analysis was performed with SPSS version 16.0.1 (SPSS® software, Chicago, Illinois, USA). We used two-sided Pearson's Chi-square test to analyze dependence of the data. Kaplan-Meier curves were used for demonstration of survival. P values < 0.05 were considered to indicate statistical significance.

Ethics

The study was approved by the local ethical committee (Kantonale Ethikkommission, StV 37-2006.)

RESULTS

Follow up

Follow up data was available for 66 patients (57.4%). The survival data ranged from 2 to 291 months (mean: 78.39 months; median: 55.5 months). In 15 patients (13.0%) death was caused by the tumour (5 men, 3 women, 7 not specified) (table 1).

CA-9 Immunohistochemistry

Tumours of 111 patients (96.5%) could be evaluated for CA-9 protein expression by immunohistochemistry. 15 (13.5%) of these showed a strong positivity (11 male, 4 female), 12 (10.8%) were weakly positive (6 male, 4 female, 2 without details about sex). 84 of 111 (75.7%) tumours showed no CA-9 staining (42 male, 37 female, sex of 5 patients unknown).

Hif-1α Immunohistochemistry

Hif-1α could be evaluated in 114 patients (99.1%). Nuclear staining was negative in 110 of 114 (96.5%) patients (60 male, 43 female, sex of 7 patients unknown), only 4 of 114 (3.5%) patients showed nuclear staining (2 male, 2 female).

Regarding cytoplasmic staining, 6 of 114 (5.3%) showed a strong positive staining (4 male, 1 female; sex of 1 patient unknown) and 14 of 114 (12.3%) patients were weakly positive (6 male, 4 female, sex of 4 patients unknown) whereas the vast majority of patients (94 of 114 (82.5%) were negative (52 male, 40 female, sex of 2 patients unknown).

SDHB Immunohistochemistry

SDHB immunohistochemistry could be evaluated in 99 patients (86.1%). 12 of 99 (12%) patients were SDHB negative (10 male, 2 female). 87 of 99 (87%) were weakly or strongly positive (41 male, 39 female, 7 unknown). Follow up was available for 60 of these 99 patients including 9 of the 12 SDHB immunonegative patients.

11 of the 12 (92%) SDHB negative tumours were localized outside the adrenal gland (i.e. extraadrenal sympathetic paragangliomas). 3 of the 12 (25%) SDHB negative

tumours showed a strong positivity in the CA-9 staining, 1 (8%) was weakly positive for CA-9 and 8 (67%) were negative (not significant in cross tabulation).
None of the SDHB negative patients showed a strong nuclear positivity for Hif-1α, all 12 were negative for Hif-1α in the nucleus. Two (17%) SDHB negative patients showed a strong cytoplasmic positivity for Hif-1α, one (8%) was weakly positive and 9 (75%) were negative for Hif-1α.

Mutation status in SDHB negative tumours

In 2 brothers an *SDHB* germline mutation was already known. Mutation analysis of the *SDHB*, *SDHC* and *SDHD* genes was performed in all remaining 10 patients. Informative results for SDHB could be obtained in 7 patients, whereas in the remaining 3 patients mutation analysis could not be performed due to degraded DNA. *SDHB* mutations were absent in 4 of these 7 patients. In 3 patients we detected previously unknown *SDHB* mutations (Table 2). The remaining patients were tested for *SDHC* and *SDHD* mutations. *SDHC* mutations were absent in all 3 informative patients and *SDHD* mutations were absent in all 5 informative patients (table 2).

Patient N°	Sex	Syndrom	Known SDHB mutation	SDHB Mutation (retrospectively determined)	Tumor	Organ	Localization	multifocal
1	M	No		All ex neg	primary tumour	abdominal ea	paraaortal	no
4	M	SDHB		Del 632G in Exon 5	metastasis	abdominal ea	pararenal	no
10	M	No		All ex neg. (tu+nnt)	primary tumour	abdominal ea	pararenal	no
11	M	SDHB		C. 307insC/ p.103 Trp fs	primary tumour	abdominal ea	paraaortal	no
17	M	No		Ex 4/6 neg. remaining n.a. (tu)	primary tumour	Adrenal gland		no
22	M	No		Ex 2/3/4/5/7 neg, 8 n.a. (tu)	primary tumour	abdominal ea	paraaortal	yes
33	F	SDHB		del 632 G in Exon 5	primary tumour	abdominal ea	pararenal	no
77	M	SDHB	H132P		primary tumour	abdominal ea	paraaortal	no
78	M	SDHB	H132P		metastasis	abdominal ea	paraaortal	yes
90	F	No		All ex neg. (tu)	metastasis	mediastinal	other	yes
119	M	No		n.a.	primary tumour	abdominal ea	paraaortal	no
80	M	No		n.a.	primary tumour	abdominal ea	paraaortal	yes

Table 2: Clinical characteristics of SDHB immunonegative tumors *SDHB,* succinate dehydrogenase subunit B; *del,* deletion; *ins*, insertion; *ea*, extraadrenal; *n.a.,* not assessable; *tu*, tumour; *nnt*, non neoplastic tissue

Microvessel density

In 109 evaluable cases the number of vessels in the area of the cylinder (0.28mm^2), corrected for non-neoplastic portions if present, ranged from 13.26 to 4046.69 (mean 564.41, median 431.55). Tumours were subdivided into quartiles according to their number of vessels. The first quartile ranged from 13 to 535 vessel section per mm^2 the second from 548 to 1083 vessel section per mm^2, the third from 1268 to 1530 vessel section per mm^2 and the fourth from 1641 to 4046 vessel sections per mm^2.

Statistical analysis

CA-9 correlated significantly with both nuclear (p=0.001) and cytoplasmic (p< 0.000) Hif-1α reactivity but not with SDHB (p=0.241). Nuclear Hif-1α did not correlate significantly with cytoplasmic Hif-1α (p=0.643) and SDHB (p=0.726). Cytoplasmic Hif-1α did not correlate with SDHB (p=0.399).

Survival analysis was performed for all immunohistochemical markers. The survival of patients with SDHB immunonegative tumours was significantly adverse compared to SDHB positive tumours (p<0.0001) (figure1). No survival difference between a weak and strong SDHB positivity was found. An extraadrenal localisation of the primary tumour was also associated with a shortened tumour specific survival (p=0.005). The survival depending on hypoxia markers CA-9 or Hif-1α offered no significant trend.

Increased microvessel density correlated significantly with CA-9 (p=0.000) and SDHB (p =0.017) but did not reach statistical significance with Hif-1α in the nucleus (p=0.192) or Hif-1α in the cytoplasm (p=0.496) or tumour size. An overview over the correlation results is given in table 3.

	HIF – 1α nucleus	**HIF – 1 α cytoplasma**	**SDHB**	**Number of vessels**	**Localization**
CA-9	**0.001**	**0.000**	0.241	**0.000**	0,708
HIF – 1 α nucleus		0.643	0.555	0.192	0.291
HIF – 1 α cytoplasma			0.399	0.496	0.174
SDHB				**0.017**	**0.000**

Table 3: Correlations of immunohistochemistry and number of vessels p-values; bold numbers indicate statistical significance

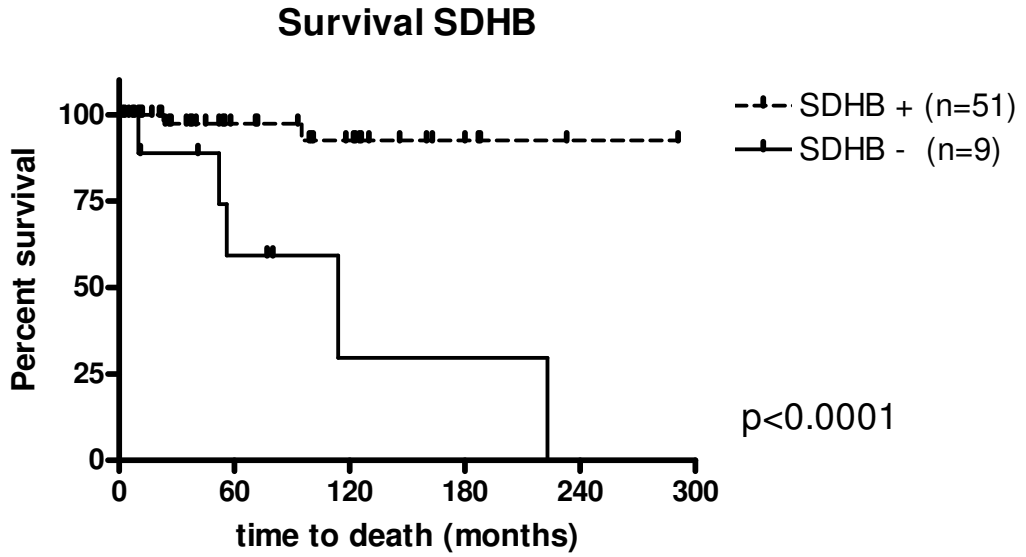

Figure 1: Kaplan-Meier curve depicting percentage of tumour-specific survival for PCC/PGL with (upper curve) and without (lower curve) SDHB immunoreactivity

Patient N°	Syndrom	Sex	Organ	SDHB	Hif-1α	CA-9
40	NF-1	m	adrenal gland	1	1	0
28	RET(MEN2)	m	adrenal gland	1	0	0
39	RET(MEN2)	m	adrenal gland	1	0	0
60	RET(MEN2)	m	adrenal gland	1	0	0
61	RET(MEN2)	f	adrenal gland	1	0	0
71	RET(MEN2)	f	adrenal gland	1	0	0
117	RET(MEN2)	f	adrenal gland	1	0	0
5	SDHB	m	abdominal ea	n.a.	0	0
11	SDHB	m	abdominal ea	0	1	1
33	SDHB	f	abdominal ea	0	0	0
77	SDHB	m	abdominal ea	0	0	0
78	SDHB	m	abdominal ea	0	0	0
21	VHL	m	adrenal gland	n.a.	0	n.a.
54	VHL	f	adrenal gland	1	1	1
115	VHL	f	adrenal gland	1	1	0
126	VHL	uk	adrenal gland	1	0	0
42	VHL	f	adrenal gland	1	0	0

0, no staining; *1*, positive staining; *n.a*, not assessable; *ea*, extraadrenal; *uk,* unknown

Table 4: Results of immunohistochemical hypoxia-stainings in familial tumours.

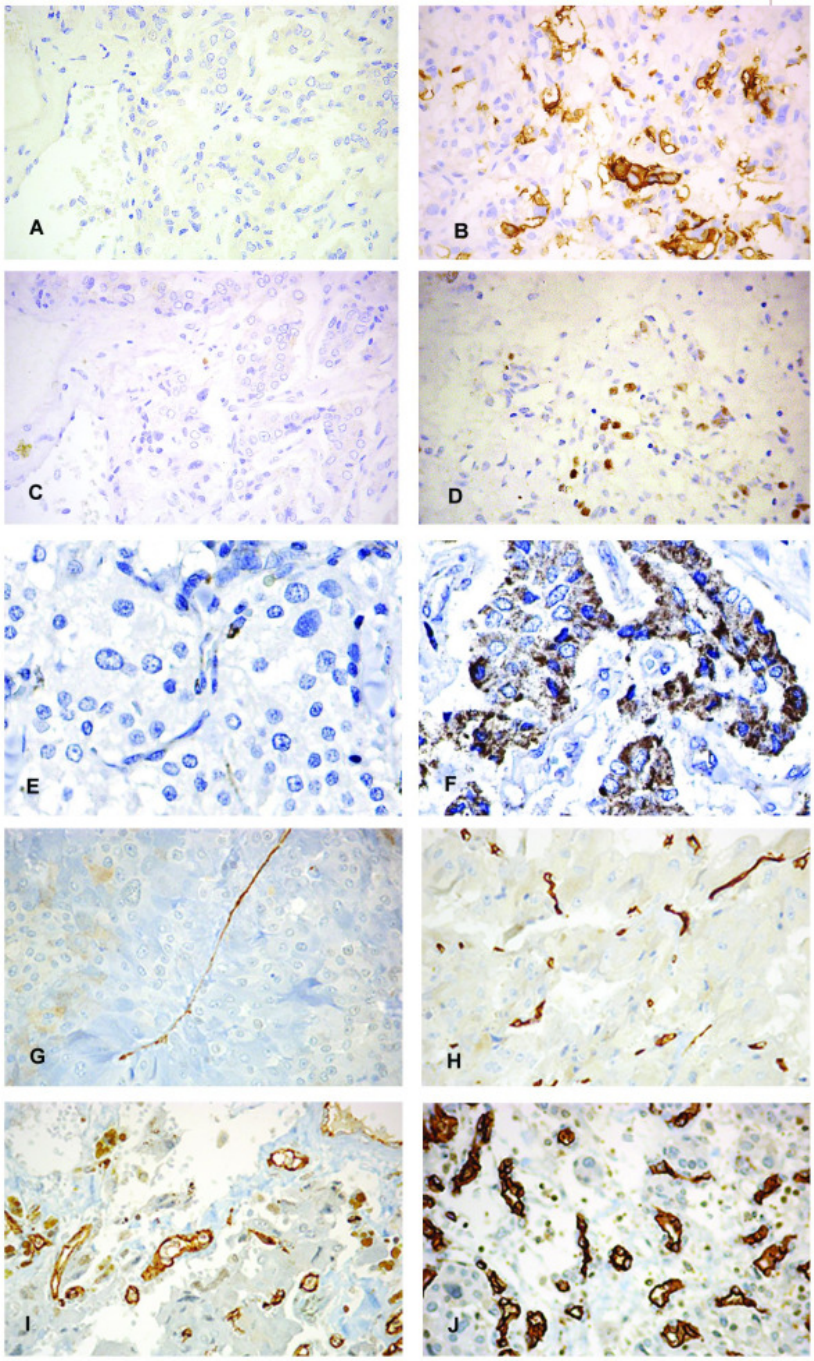

Figure 2: Immunohistochemistry of PCC/PGL A: negative for CA-9; B: positive for CA-9; C: nuclear negativity for HIF-1α; D: nuclear positivity for HIF-1α; E: negative for SDHB (positive signal in endothelial cells); F: positive for SDHB (note granular cytoplasmic staining); G: first quartile MVD; H: second quartile MVD; I: third quartile MVD; J: fourth quartile MVD

DISCUSSION

Prediction of malignant behaviour of pheochromocytomas is very difficult if not impossible on a histopathological basis. A proposed morphological scoring scheme did not prove useful in a recent analysis due to large interobserver variablility (Thompson 2002), (Kimura *et al.* 2005), (Wu *et al.* 2009). The present survival analysis on 60 unselected pheochromocytoma/sympathetic paraganglioma patients with follow up revealed SDHB immunohistochemistry as a very powerful prognostic marker. The survival of patients with SDHB immunonegative tumours was significantly worse than of patients with SDHB positive tumours. Five of 9 patients with SDHB immunonegative tumours and available follow up died of their tumour, 3 of whom had an *SDHB* germline mutation. A tumour-related death did not occur in the 3 strongly SDHB positive tumours and only twice in the 46 weakly SDHB positive tumours with available follow up. A possible explanation of these findings is that the SDHB negative tumours arise in patients with the SDHB-associated pheochromocytoma/paraganglioma syndrome. Indeed, we could demonstrate a *SDHB* germline mutation in 5 of the 9 immunonegative patients we were able to examine. We were unable to analyse 3 immunonegative patients at least in the majority of SDHB exons. We cannot exclude the presence of other genomic *SDHB* alterations such as deletions in the remaining 4 patients. However, we think these patients could suffer in part from sporadic tumours. Only one of these patients suffered from multifocal tumours and SDHB germline deletions are described in up to 30% of SDHB kindreds (McWhinney *et al.* 2004), (Tischler 2008). Complete loss of granular SDHB immunopositivity has been shown as a predictor of SDH germline mutation with a positive predictive value of at least 92% and a negative predictive value of 100%. (van Nederveen *et al.* 2009) In our series, both tumours with a known SDH germline mutation stained negative for SDHB immunohistochemistry, confirming the high negative predictive value of a positive SDHB staining. From the clinical point of view our data also stresses the role of localisation of the tumour. In our series there was a strong correlation of extraadrenal localization with poor prognosis and SDHB germline mutation, which is in line with previous findings.(Amar *et al.* 2005),

(Maier-Woelfle *et al.* 2004), (Lenders *et al.* 2005), (Neumann *et al.* 2002), (Tischler 2008).

To examine the possible link between SDHB immunostaining, which is indicative of enzymatic activity of the SDH complex (van Nederveen *et al.* 2009) with hypoxia signalling, we also examined Hif-1α expression and its hypoxia target CA-9 by immunohistochemistry. Surprisingly we detected hypoxia signals only in a small minority of tumours. There was no association of SDHB loss with CA-9 and Hif-1α expression. This indicates that other mechanisms than hypoxia signals are involved in the genesis of SDHB-associated and most sporadic pheochromocytomas. The two hypoxia markers Hif-1α and CA-9 showed a strong correlation with each other, arguing against technical problems in identifying hypoxia signals. In contrast to SDHB expression, hypoxia signals were of no prognostic significance in our series. We did not detect an association of hypoxia signals with a specific syndrome in the 17 familial tumours included. An overview is given in table 4. Only 2 of 17 tumours from patients with familial syndromes were strongly positive for Hif-1α and CA-9 including one tumour of an SDHB patient and VHL patient each. These results argue against the current hypoxia hypothesis as the mechanism leading to pheochromocytomas/paragangliomas (Eisenhofer *et al.* 2004, Maher& Eng 2002), even in the setting of the VHL syndrome. Therefore other hypotheses of tumorigenesis seem to be of more importance in pheochromocytomas/paragangliomas, such as apoptosis and inhibition of microtubule stabilisation (Lee *et al.* 2005), (Hergovich *et al.* 2003).

As a potential biological read-out of hypoxia signals we examined the microvessel density. As opposed to Rooijens et al. (Rooijens *et al.* 2004) we did not find a correlation of microvessel density and survival. However a high number of microvessels correlated with the presence of the hypoxia marker CA-9 and absence of SDHB staining but not with Hif-1α. This might indicate that hypoxia induces a higher number of microvessels, but not necessarily via Hif-1α.

In summary we suggest SDHB protein loss as a marker of adverse outcome both in sporadic and in familial pheochromocytomas/paragangliomas. Inclusion of this marker in the assessment of pheochromocytomas/paragangliomas might be

mandatory for 2 reasons: First for direction of molecular genetic testing towards the SDH genes in the case of absent staining, second as a prognostic marker. We suggest to include also patients with sporadic SDHB negative tumours in more stringent follow up protocols.

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CHAPTER 10, GENERAL DISCUSSION AND FUTURE PERSPECTIVES

From past to present;

The discovery of the succinate dehydrogenase subunit genes

Families with paragangliomas had long been recognized, but until the end of the previous century, no causative genes were identified. For a long time, four chromosomal regions had been implied in the pathogenesis of such tumours, including PGL1, located on 11q23, PGL2, located on 11q13, PGL3 located on 1q21, and PGL4, located on 1p36. The first gene to be pinpointed, on 11q23, was *SDHD*, encoding one of the anchoring proteins of complex II of the electron transport chain, which is also involved in the Krebs cycle in the transformation of succinate into fumarate. [1] The initial paper reported the *SDHD* gene involved in a family with hereditary head and neck PGL. The discovery of the *SDHD* gene led to large scaled screening of patients with PGL and PCC, and in both groups PCC and PGL patients germ line *SDHD* mutations were discovered.[2, 3] Soon thereafter, genes encoding two of the other three SDH subunit proteins were found to correspond to the loci for PGL3 and PGL4 (SDHC and SDHB, respectively) [4, 5]. Subsequently, the vast majority of familial cases of PGL and a significant subset of apparently sporadic head and neck PGL were shown to be caused by germ line mutations in *SHDB, SDHC, or SDHD*. [5-7] Especially in the Netherlands the percentage of hereditary cases was high, and two *SDHD* founder mutations (D92Y and L95P) were found in the Dutch pPGL population.[8] In other countries, founder effects have been discovered as well, predominantly for *SDHB*. [9-12] All three genes have an autosomal dominant mode of inheritance, in the context of which maternal imprinting has been suggested for *SDHD*. [13-15] However, one patient with pPGL was described by Pigny et al. resulting from maternal transmission of the *SDHD* gene. [16] Interestingly, the fourth subunit of the mitochondrial complex II, SDHA, is not involved in the pathogenesis of PGL, but instead causes a lethal neurodegenerative syndrome called Leigh syndrome in case of homozygous mutations. [17] With the very recent discovery of the *SDHAF2* gene, the gene in the remaining PGL2 locus has been identified. *SDHAF2* is involved in the flavination of SDHA, and is therefore also linked to mitochondrial complex II.[18] As in patients with *SDHD* mutations tumour development is only paternally transmitted. So far, only 2 families have been

described, both harbouring the same mutation in a highly conserved region of *SDHAF2*. All of these patients presented with pPGL, and no germ line or somatic mutations were discovered in sPGL and PCC.[19]

Now that *SDHB, C* and *D* mutations are known for almost a decade, the tumour spectrum of the PCC-PGL syndrome is beginning to unravel. This spectrum now encompasses several independently reported renal cell carcinomas, all related to *SDHB*-germ line mutations. [15, 20, 21] Two of these tumours have been tested with SDHB immunohistochemistry, and were found to be negative, supporting their SDH-related pathogenesis.[22] It should be noted that various renal tumour types have been associated with the PCC-PGL syndrome, including clear cell renal cell carcinomas, oncocytomas, and a tumour that could not readily be classified according to the current WHO criteria. Another tumour type related to PCC-PGL syndrome is gastrointestinal stromal tumour (GIST). The Carney-Stratakis syndrome is characterized by familial clustering of both pPGL and GIST. In this syndrome germ line *SDHB, C* and *D*-mutations have been found, and loss of the corresponding wild type alleles was demonstrated in pPGL and in GIST. [23, 24]

Genotype-phenotype analysis

Over the last 5 years several large series of PGL and PCC patients have been systematically screened for mutations in the abovementioned *SDH* genes as well as for mutations in VHL, RET and NF1. [15, 25, 26] This has largely been done by direct sequence analysis of blood-derived DNA, although some studies have also looked at paired normal and tumour-derived DNA. For this approach it is crucial to select areas with at least 70% tumour cells for tumour-DNA isolation. Investigating tumours has the advantage that somatic mutations can also be detected.[3, 27, 28] For the *SDH* genes it has been shown that somatic mutations are extremely rare. No *de novo* germ line mutations have been found thus far. Furthermore, some groups have used multiplex ligation-dependent probe amplification, with which large deletions, sometimes encompassing multiple exons, have been detected in a small subset (6-9% for *SHDB* and 3-6% for *SDHD*) of patients.[15, 29, 30] More recently, denaturing high-performance liquid chromatography has been introduced as an alternative to direct sequencing, because this technique has been claimed to have a higher

sensitivity, and the costs and intensity of labour are significantly less than conventional sequencing. [31]

Using mutation analysis it has been shown that virtually all familial cases of head and neck PGL carry *SDH* germ line mutations. [32] The frequency with which PGL and PCC occur and the age-related penetrance vary between *SDHB* and *SDHD* mutation carriers. In *SDHD* mutation carriers the lifetime risk for pPGL is 80-90%, with less than 10% of the patients being younger than 10 years, reaching 90% of patients having a tumour at 70 years of age. The risk of tumour development depends on the type of mutation, causing absence of the protein, stable protein or a protein lacking structural integrity. In patients with *SDHD* mutations leading to a stable protein the penetrance is lower than the other groups in the first 3 decades, after which the penetrance increases and reaches the same levels of those with unstable protein by the age of 70. The risk of PCC development depends on the type of mutation in *SDHD,* where the group of patients with predicted unstable protein has a penetrance of 50% by age 40. This is in sharp contrast to the mutations predicted to have stable protein, with a lifetime risk of less than 10%. The occurrence of pPGL in *SDHB* patients is relatively low, 34% by the age of 60. In contrast *SDHB* mutations cause a high risk for PCC and sPGL, as high as 60 to 70% by the age of 60. [15, 25, 33]

Genetic testing and immunohistochemical pre-screening

With the discovery of the *SDH* genes and the well known *VHL, RET* and *NF1* genes involved in the pathogenesis of PCC and PGL, there are now 7 candidate genes that may have germ line mutations, together representing up to 35% of PGL and PCC cases. On the one hand, the high percentage of hereditary cases warrants genetic testing in every patient, although some have advocated testing only patients below the age of 45 or 50 years. [34-36] On the other hand, systematic genetic screening for 7 genes is time-consuming, laborious and expensive. Because neurofibromatosis type 1 is usually diagnosed clinically, and no mutations have been described in apparently sporadic PCC or PGL, no genetic screening is indicated for this gene. [37, 38] For the remaining 6 genes, several groups have tried to develop algorithms for genetic testing. The use of SDHB immunohistochemistry presented in Chapter 8 is an important addition to the dilemma of genetic testing as it may form the first step of

the testing algorithm. [22] Also, it can aid in the distinction between polymorphisms and true mutations. Since the *SDHB* gene is a bona fide tumour suppressor gene, fulfilling Knudson's two hit concept, both *SDHB* alleles have to be either mutated or lost for complete tumour suppressor inactivation. Biallelic inactivation of *SDHx* genes is generally achieved by point mutation of one allele and deletion of the other. This will lead to absence of protein staining, as shown in chapter 8. In case of a polymorphism, 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. An interesting unpublished observation is the finding of minimal cytoplasmic background staining in a small subset of *SDHD*-mutated tumours. This was also observed in another immunohistochemical study of pPGL. [39] As discussed in chapter 8, scoring of SDHB immunohistochemistry is based on staining intensity of the endothelium compared to the tumour cells, and therefore these (*SDHD*-related) tumours are considered negative. Enzyme histochemistry for SDH can also be performed if frozen tumour tissue is available. This method will show whether SDH enzyme activity is decreased or retained. In SDHB immunonegative tumours no SDH activity will be found in the tumour cells, but non-neoplastic cells show staining.

In contrast, most PCC with proven *RET* mutations show a more intense SDHB immunostaining than normal adrenal medulla and the organ of Zuckerkandl. SDH activity assessed by enzyme histochemistry is also strongly positive in these tumours. This finding is hard to explain based on the current pathway-knowledge of the *RET* oncogene, without a known functional link to the SDH-complex. However, it is supported by the high protein expression levels for mitochondrial complex I, II (SDHB and SDHA), III and IV in tumours with *RET* mutations, as described by Favier et al. [40] Therefore, increased SDHB protein could be an explanation for more intense immunohistochemical staining of PCC with *RET* mutations. Since immunohistochemistry is relatively cheap compared to genetic screening by sequencing and MLPA it would be important to find more immunohistochemical markers that can discriminate between the various syndromes. Interestingly, SDHB immunohistochemistry has been published in 2 *SDHAF2* tumours, where the authors found a more "speckled staining" compared to the *SDHD* and *SDHB* related tumours,

although no internal control such was described. [39] However, in a small series of pPGL from patients with germ line *SDHAF2* mutations the tumour cells were negative (Gaal et al, unpublished observations).

Other immunohistochemical markers, including antibodies to SDHC and SDHD, could be investigated in an attempt to discriminate patients with *SDHD* and *SDHC* mutations from patients with *SDHB* mutations. In the SDHB immunopositive group RET immunohistochemistry could be of additional value to detect RET-overexpression in patients with MEN 2. Although this approach has been suboptimal in the past, many improvements have been made in the antibodies as well as in antigen retrieval methods, and this should be tested. *VHL*–related tumours display upregulation of HIF, which might be detected by immunohistochemistry. Especially, since this (pseudo)hypoxic state has not been observed in MEN 2-related PCC this could be of discriminative use. [40, 41] It should be noted that SDHB and other potential immunohistochemical markers have strong implications for a hereditary background of tumours and preferably should be part of an integral approach including genetic counseling.

Testing strategies based on the current knowledge

As discussed above, SDHB immunohistochemistry can be of help in the strategy for genetic testing, provided that tumour tissue is available. When SDHB immunostaining is negative, the probability of an *SDHx* mutation is high, and therefore mutation analysis for *SDHB*, *SDHC*, and *SDHD* should be performed. As shown in figure 1, *SDHD* is the first gene to be analysed in pPGL, whereas *SDHB* is the first gene to analysed in sPGL. If SDHB immunostaining is positive, for pPGL chances are low that it concerns a hereditary tumour, but *VHL* (and for the sake of completeness *RET*) mutation analysis can be performed. In sPGL and PCC, however, the likelihood of *VHL* or *RET* mutations is much higher and these two genes should be tested. Based on the prevalence in the literature and our own clinical experience a scheme is proposed for screening, shown in figure 1. This scheme is different from a recent study by Erlic et al. [36] that is based on the European-American PCC group encompassing 989 apparently nonsyndromic patients. In this study the different geographical differences have not been taken into

account. Based on overall literature, most of our proposed scheme is in concordance with a scheme designed by the the Pheochromocytoma and paraganglioma RESearch Support Organization (PRESSOR) workgroup. [42]

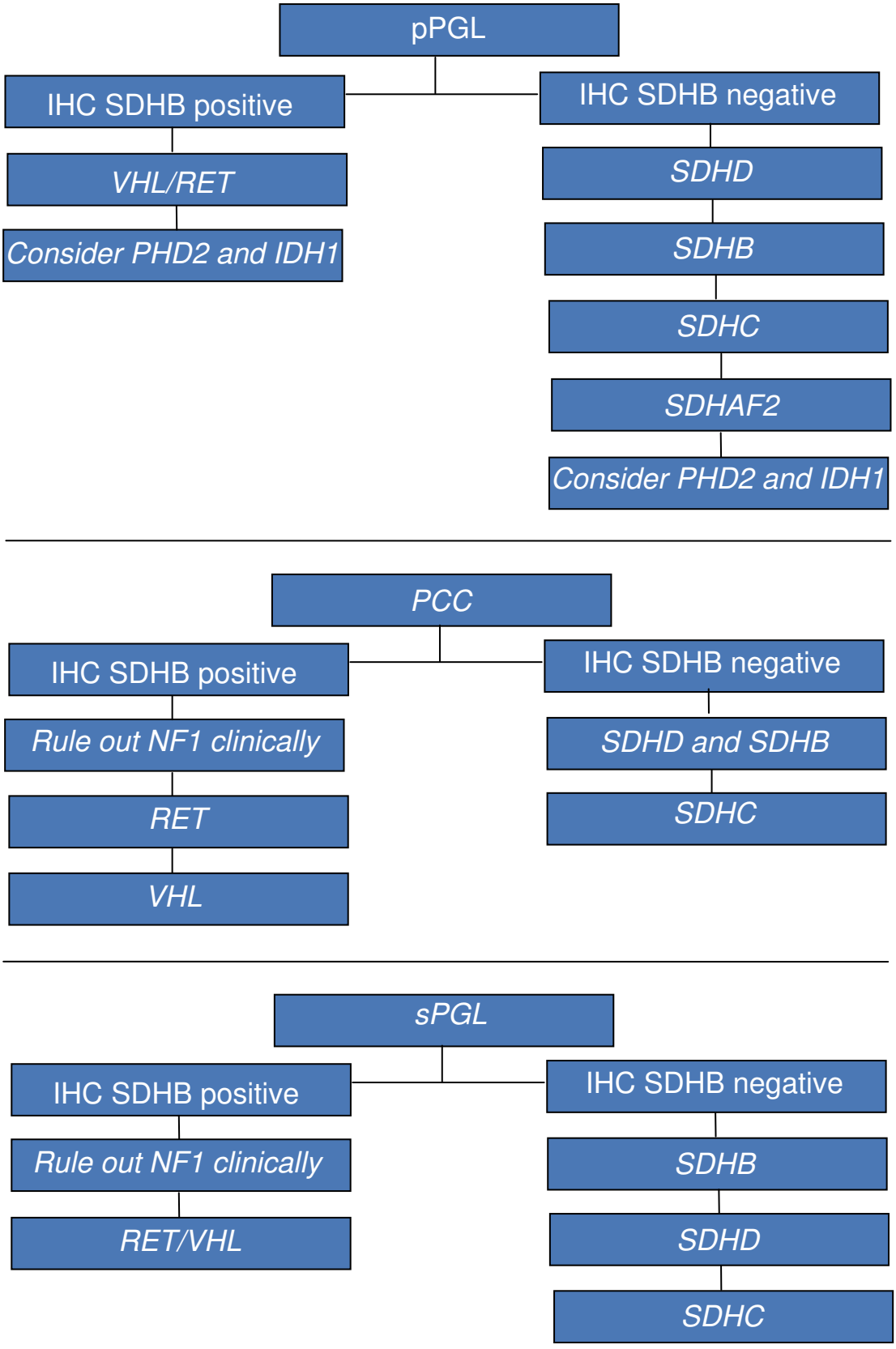

Figure 1: Proposed genetic screening strategy for pPGL, PCC and sPGL, with the use of SDHB immunohistochemistry.

With regard to conventional screening without available tumour histology and immunohistochemistry it appears that for pPGL, at least in The Netherlands, but probably also elsewhere, *SDHD* is the first gene to be tested (and the known founder mutations as a first step), followed by *SDHB* and *SDHC*. In the few familial cases that remain negative, despite mutation analysis and MLPA, *SDHAF2* testing should be considered. Also, in rare cases germ line *VHL* mutations can be found in PGL. In these patients a family history of PCC and other VHL-related tumours is usually present. [43, 44] Finally, in the literature there is one patient described with a PGL in the context of the MEN 2 syndrome. However, there was no histological confirmation of this PGL.

For PCC the algorithm for genetic testing is different from PGL. As mentioned previously, NF1 is detected clinically, and therefore this syndrome is not included in the genetic testing algorithm. Many PCC occur in the context of MEN 2, followed by VHL. Especially in patients with bilateral PCC the frequency of mutations in the *RET* gene, followed by mutations in the *VHL* gene is significant. [45, 46] A proportion of PCC is due to germ line mutations in *SDHD* and *SDHB*. Although rare, PCC can also occur in the context of *SDHC*. [47] The biochemical profile of catecholamine production of PCC can be of help, since PCC in VHL-patients lack the enzyme phenylethanolamine N-methyltransferase (PNMT) and therefore are predominantly noradrenergic. [48] Until now, no *SDHAF2*-related PCC have been described, so screening for this mutation is not indicated, with current knowledge. [19]

The group of sPGL is more similar to PGL than PCC. This similarity concerns not only histology, but also in the genetic background that is more related to the *SDH* genes than the *RET* and *VHL* background that is most frequently found in PCC. The difference between sPGL to PCC is remarkable because of their common sympathetic derivation and clinical presentation of hormone excretion, in contrast to pPGL. Most hereditary sPGL are due to mutations in *SDHB* and *SDHD*. If these genes are excluded the other known susceptibility genes *SDHC, RET* and *VHL* should be analysed is this order. As in PCC, *SDHAF2* mutations are not associated with sPGL, based on current insight.

Malignancy

The frequency of malignant behaviour in sPGL is higher than in their parasympathetic and adrenal medullary counterparts. This is mostly related to the high frequency of *SDHB* mutations, but studies also have found a higher frequency of malignant behaviour of patients with sPGL without germ line *SDHB* mutations.[15, 49-51] A higher frequency of malignancy is also suggested in *SDHD*-related tumours, especially associated with the Dutch founder mutations, D92Y. [52]

In the study described in chapter 9 it is suggested that negative SDHB-immunohistochemistry in PCC is negatively correlated to survival. The SDHB immunonegative cases only showed an *SDHB* mutation in 3 of the 7 patients investigated by sequence analysis. In the remaining 4 patients additional mutation analysis was performed on *SDHD* and *SDHC,* but no mutations were detected. This could be due to large mutations, not detected by sequence analysis. Another possible explanation for the mutation negative, immunonegative tumours is that the tumours arise in patients with mutations in genes involved in the mitochondrial II stabilisation and/or activation, similar to *SDHAF2*. It has been shown in the literature that other enzymes in the Krebs cycle are potential tumour suppressor genes, as shown for fumarate hydratase in leiomyoma and leiomyosarcoma.[53] Therefore further analysis of the SDH-pathway could improve the understanding of the malignant behaviour in sPGL and *SDHB*-related tumours. Up to date, no other reliable markers have been introduced to aid in the assessment of biological behaviour of PCC and PGL.

Other PCC and PGL genes

As described above, the spectrum of PCC and PGL susceptibility genes is expanding, while most of the currently known genes are associated with the Krebs cycle. (Figure 2) Recently, isocitrate dehydrogenase type 1 (*IDH1*), another enzyme involved in the Krebs cycle, was coined as a candidate gene for PGL, but was only found to be somatically mutated in one case of pPGL in a series of 269 investigated PCC and PGL. [54] Also, another potential susceptibility gene in a small subset of pPGL is *PHD2*. Thus far only one patient with erythrocytosis and paraganglioma has been described with a proven *PHD2* mutation. [55] On the p arm of chromosome 1 a

putative tumour-suppressor gene (TSG) is located, KIF1Bβ. A missense mutation was found in 2 PCC, but one of the tumours showed retention of the wild-type allele, contradicting Knudson's 2-hit hypothesis of TSGs. [56] Finally, a study using linkage analysis in PCC has found two loci for familial PCC at 2cen and 16p13. Up to date these loci have not been described by other groups, and no genes have been identified for these loci. [57] Finally a recently discovered gene related to complex II is *SDHAF1*, encoding a new LYR-motif protein. So far this is only described in infantile leukoencephalopathy with defective succinate dehydrogenase, but no mutations in PCC or PGL have been observed so far. [58]

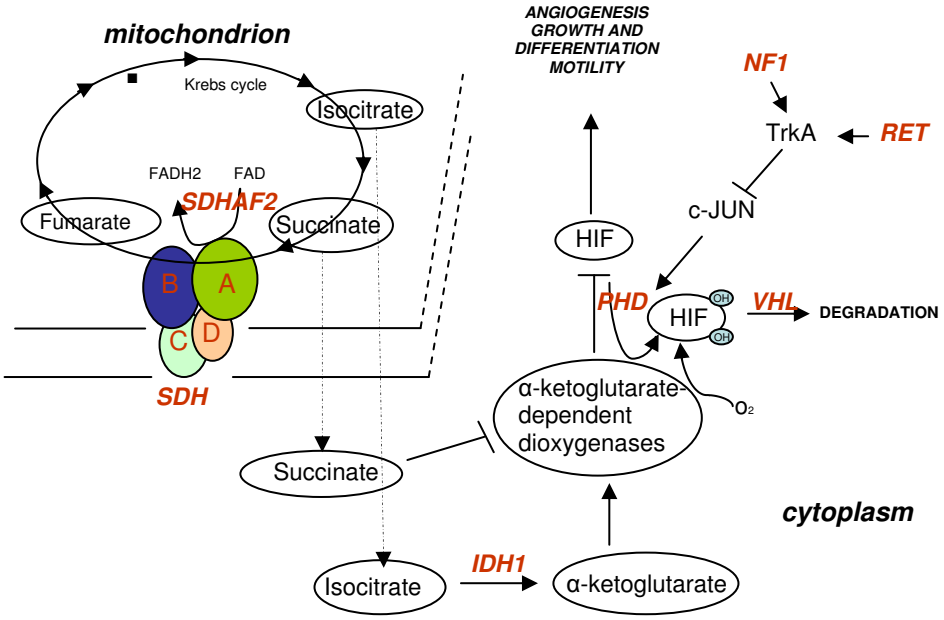

Figure 2: Schematic (drawing of hypoxia) pathway, incorporating all of the currently known PGL and PCC susceptibility genes. The left part of the figure shows the mitochondrion with Krebs cycle and electron transport chain. Succinate is converted to fumarate by active SDHA and SDHB. SDHC and SDHD are anchoring proteins in the mitochondrial membrane, and act in the electron transport chain. SDHAF2 acts as a cofactor with FAD for the flavination of SDHA. Succinate, when entering the cytoplasm, suppresses PHD function.

The right part of the figure shows circulating HIF that can activate target genes. HIF is regulated by oxygen tension and PHD, forming hydroxylated HIF. This hydroxylation provides the recognition signal that enables HIF to be captured by VHL to the E3 complex and to degrade the complex.

The interactions of NF1 and RET are through TrkA signaling, suppressing c-jun. PHD is activated by c-jun and if TrkA is activated, this will indirectly suppress PHD function, resulting in HIF accumulation and activation of target genes.

Abbreviations: HIF: hypoxia-inducible factor, PHD: prolyl hydroxylase, SDH (A,B,C, D and AF2): succinate dehydrogenase (subunit A, B,C, D and complex assembly factor 2). FAD: flavin adenine dinucleotide. NF1: neurofibromatosis type 1, RET: rearranged during transfection, IDH1: isocitrate dehydrogenase type 1, c-jun: jun oncogene, TrkA: nerve growth factor receptor.

Mouse models

Over the years several mouse models have been developed to study PCC tumourigenesis. Some have been developed on the basis of TSGs and oncogenes that appeared relevant in humans, such as the NF1 and RET- mouse models. [59] [60] The mouse model for *SDHD,* however, does not show PCC or PGL

development.[61] Other mouse models developed for the study of other malignancies coincidentally also developed PCC. One of these mouse models is the *Pten*$^{+/-}$ mouse, developing PCC in 24-100%, but also frequent PCC develop in *Rb*$^{+/-}$*p130*$^{-/-}$ mice, *Rb*$^{+/-}$ mice and *p18*(*Ink4c*)$^{-/-}$*p27*(*Kip1*)$^{-/-}$ mice. [62-65] In our lab a conditional *Pten* knockout mouse model was developed, with *Pten* under control of the *prostate specific antigen* promoter, for the study of prostatic adenocarcinoma. Subsequently, these mice were shown to develop PCC at high frequency (78%), which were metastatic to the lungs in 35%. [66] These mouse models are of potential value for understanding the pathogenesis, and for treatment, of human (malignant) PCC. However, no *Pten* mutations have been found in human PCC, as shown in chapter 5.

From present to future: the use of array-platforms

The use of array-comparative genomic hybridization (array-CGH), as described in chapter 2 and 3, has confirmed the molecular aberrations in PCC, previously reported in LOH and conventional CGH studies. In contrast to most previous studies, this research was performed on sporadic PCC, in which mutations of the known candidate genes *RET, VHL, SDHB* and *SDHD* had been excluded. Interestingly, as described in chapter 2, the genomic aberrations found in the sporadic tumours, could be classified into 2 main groups, one with a profile resembling that of VHL-related PCC (loss of 3p and 11p) and one with a profile resembling that of MEN 2-related PCC (loss of 1p and 3q). [67] Therefore the development of sporadic tumours is thought to involve the same pathways as their hereditary counterparts. In comparison to benign PCC, malignant PCC show more extensive copy-number variation, suggestive of genetic instability in these tumours.

Another whole-genome approach is the use of RNA expression arrays. This technique has great potential for classifying tumours according to their RNA expression profile. Well-known studies on solid tumours are the large breast cancer studies, where the different expression profiles cluster patients to similarity in tumour types. (Reviewed in [68]) This specific clustering allows researchers to select differentially expressed genes that could predict clinical behaviour of the various subgroups. The first comprehensive study on PCC using this platform was done by Dahia et al. [41]. In this study a regulatory loop was found, linking hereditary and sporadic tumours all to hypoxia. Based on unsupervised clustering Dahia et al. found 2 clusters of tumours, one with *SDH* and *VHL* related tumours together with part of the sporadic tumours, and another cluster the *RET* and *NF1* related tumours and the remaining sporadic tumours. This finding matches our hypothesis of truly sporadic tumours resembling the hereditary tumours, based on our array-CGH study in chapter 2.

A few studies have been performed to find differentially expressed genes between benign and malignant PCC and sPGL. [48, 69-71] The study by Brouwers et al. highlighted the limitations of statistical analysis on relatively small tumour sets, showing a spectrum of differentially expressed genes, depending on the statistical

analysis used. RNA expression platforms generally analyse a large number of genes, leading to a pool of information, due to the biological heterogeneity of the tumours analysed. This heterogeneity makes it difficult to separate coincidentally up- or downregulated genes from the truly pathogenic genes. Sample size has to be increased to reduce the problem of biological variability. Also, several other factors than biological variability play a role in the analysis, such as bias due to sample quality for RNA integrity and sample handling during the process. This can lead to bias of isolation-date, hybridization-date, or reflect differences due to specimen handling and differences in storage in different institutes. Most studies have published large sets of genes that are differentially up or downregulated, but none have been proven to provide a profile accurate enough to predict clinical behaviour. Only minor overlap is found in the studies in the gene-sets predicting malignant behaviour.[69, 71] Problems mainly occur due to annotation problems. Threfore, part of the gene signatures from different studies could be driven by genes and annotations represented in the platforms used, combined with the other variables mentioned, such as specimen quality and handling, rather than reflecting true biological differences.[72]

Preliminary data of our own study of 54 PCC (43 benign and 11 malignant tumours with proven metastases), confirmed the difficulties in the analysis of a small dataset. Using the standardized Affymetrix HU133 Plus 2.0 platform arrays were performed. A scan-date bias (batch-effect) was detected and removed, and subsequent we performed an ANOVA analysis with which we selected genes that were differentially "expressed" between malignant and benign samples. In this analysis 113 genes were differentially expressed in this series between benign and malignant tumours. (Figure3).

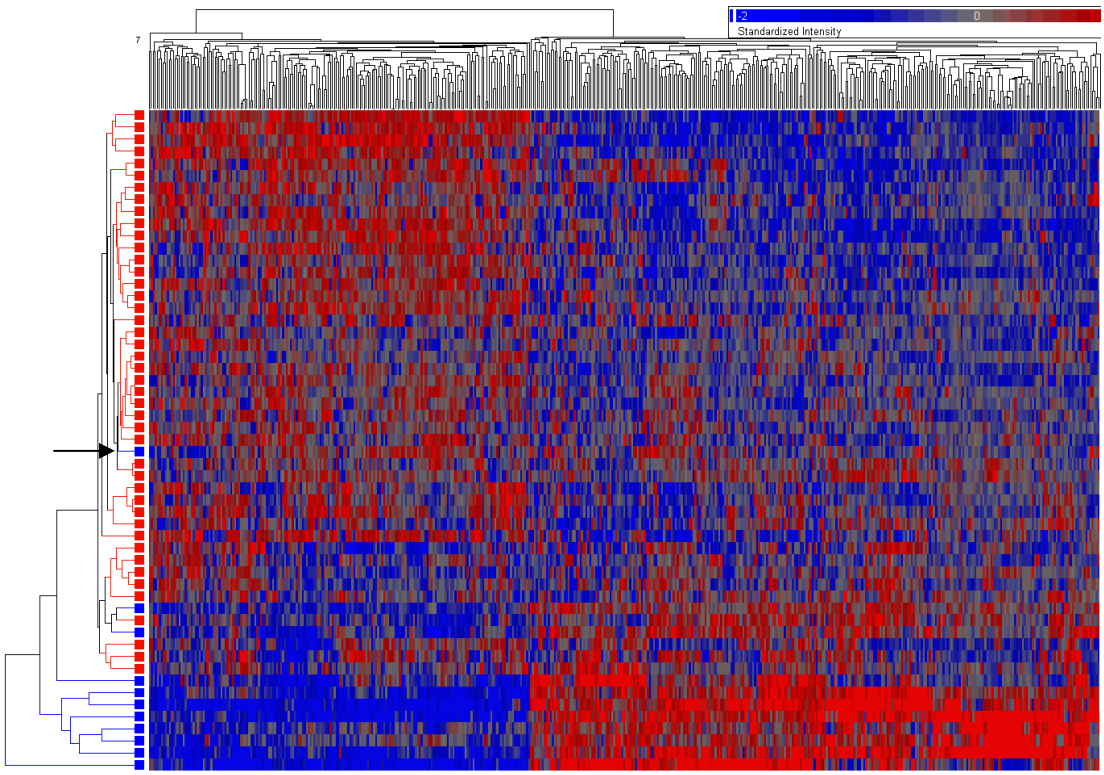

Figure 3: Unsupervised analysis of PCC. Blue boxes are malignant cases.

Using significant analysis of microarray data (SAM) of *RET* related tumours against *SDH*-related tumours we found similar results as described in the studies by Eisenhofer et al., with a strong difference in *PNMT*-expression between these tumours.[48] As shown in figure 3, representing unsupervised clustering of the samples, most of the malignant tumours (blue boxes on the left side of the panel) differ from the group of benign tumours. A correlation plot of the same samples is shown in figure 4. One outlier in this unsupervised analysis is important to note (arrow in figure 3, star in figure 4). This is a non-syndromic patient with a small cluster of chromaffin cells in a sinus of a neighbouring lymph node. No evidence of recurrent disease has been found thus far, indicating a possible seeding during operation, with possible misclassification as a consequence. This raises the question of “circulating“ tumour cells in lymph nodes and their interpretation, which also constitutes a problem in other solid tumours. In the group of upregulated genes the *survivin* gene was already described in the literature to be expressed in PCC. This gene is indicated in progression of pancreatic endocrine tumours, and therefore an interesting gene for further research. [73] Other genes that are known to be

expressed in endocrine malignancies have been found, of which *somatostatin receptor 2*, *SSTR2,* is an interesting marker not only with regard to differentiation but also as a target gene for therapeutic purposes. SSTR2 is known as a target for somatostatin based therapy, a well-known therapy for endocrine malignancies. [74] Although there are some interesting genes in our series, combining these data with 2 series from our international collaborators will improve quality, and will enable us to exclude the biological differences not truly related to malignancy. Finally, enlarging the set will improve the statistical power to predict biological behaviour.

In conclusion, the understanding of hereditary and sporadic PCC and PGL has expanded rapidly in the last decade. All PGL loci have been mapped and parts of the mechanisms of these TSGs are understood. The pathogenesis of the sporadic tumours is thought to result from similar pathways as their hereditary counterpart, as found in Array-CGH and RNA expression profiling. Unfortunately, predicting malignant behaviour in a simple test has not been feasible so far, but with the potential power of combined RNA-expression array studies could pinpoint new targets for future analysis are likely to be pinpointed.

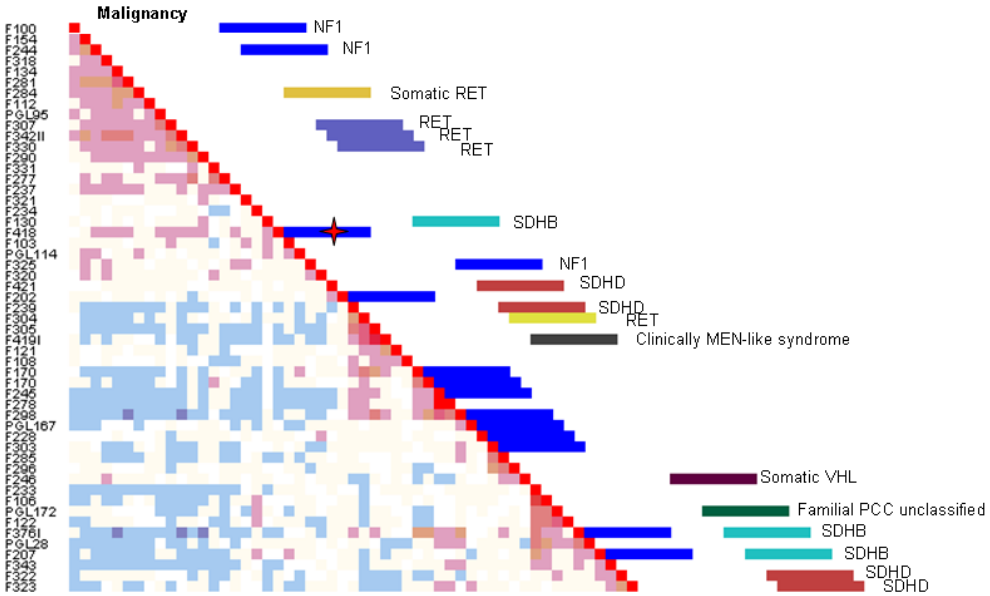

Figure 4: Correlation plot of PCC, first row after correlation plot indicates the malignant samples in blue, second row indicates the known mutations or clinical presentation.

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SAMENVATTING

Pheochromocytomen (PCC) zijn relatief zeldzame tumoren van het bijniermerg. Deze tumoren zijn afkomstig van de neurale lijst, net als paragangliomen (PGL) welke gelokaliseerd zijn in het hoofd-halsgebied en langs de parasympatische grensstreng. Histomorfologisch zijn PCC en PGL vrijwel identiek, maar is pathogenese van deze tumoren verschillend, hoewel er enige overlap bestaat. In de afgelopen jaren is er toenemend inzicht gekomen in de verschillende genen welke verantwoordelijk zijn voor het ontstaan van PCC en PGL. Ook is de herkenning van kiembaanafwijkingen bij patiënten met deze tumoren verbeterd, waarbij in patiënten met PGL nu in meer dan 50% van de gevallen een kiembaanmutatie wordt gevonden en in patiënten met PCC in 25% een kiembaanmutatie wordt aangetroffen.

De genen welke betrokken zijn bij PCC zijn het *RET* oncogen en de tumorsuppressorgenen *VHL, NF1, SDHB, SDHC en SDHD*. Bij PGL zijn met name *SDHB, SDHC* en *SDHD* betrokken bij het ontstaan van de tumoren, maar worden in een klein percentage ook *VHL*-kiembaanmutaties aangetroffen. Zeer recent is ook *SDHAF2* geïdentificeerd, een tumorsupressorgen dat betrokken is bij een kleine groep van familiaire PGL.

Tot op heden zijn er geen adequate markers gevonden die het gedrag van PCC en PGL voorspellen. De erfelijke achtergrond kan in beperkte mate een voorspelling geven over het klinische gedrag van de tumoren. Bij patiënten met een kiembaanmutatie in het *RET*-gen is de kans op metastasering zeer gering, in tegenstelling tot kiembaanmutaties in het *SDHB*-gen waar er juist een hoge kans is op metastasering.

De tumoren die ontstaan zonder kiembaanafwijkingen hebben op DNA niveau dezelfde afwijkingen als de groep van erfelijke PCC, waarbij er een groep wordt gezien met afwijkingen vrijwel identiek aan de tumoren van patiënten met een kiembaan *RET* mutatie, en een groep met DNA afwijkingen identiek aan tumoren van patiënten met een kiembaan *VHL* mutatie. In een klein percentage van deze groep tumoren van patiënten zonder kiembaanafwijkingen werden ook tumor specifieke (somatische) mutaties gevonden in het *VHL, RET* en in het *SDHB* gen. In maligne PCC worden andere afwijkingen gezien dan in de benigne PCC. Hierbij valt op dat naast verlies van DNA materiaal in de maligne tumoren ook vaak winst wordt gezien

van delen van chromosomen. Maligne PCC hebben gemiddeld ook meer DNA afwijkingen dan de benigne PCC.

Er is een sterke relatie tussen zuurstoftekort (hypoxie) en het voorkomen van PGL. Deze relatie is aangetoond bij bewoners van het hooggebergte, die vaker PGL ontwikkelen dan mensen op zeeniveau. Chronische hypoxie is een bekende oorzaak van P53 overexpressie, welke op zijn beurt de celcyclus remt en apoptose induceert. De hypothese was dat de PGL mogelijk een *p53* inactivatie hebben, waardoor de tumoren kunnen groeien zonder het signaal te krijgen om in apoptose te gaan. Deze hypothese bleek niet te kloppen, aangezien er geen mutaties in het *p53* gen werden gevonden en normaal (wildtype) p53 tot expressie komt in de meeste onderzochte PGL.

In muismodellen met mutaties in *PTEN* ontwikkelen de muizen ook regelmatig PCC. *PTEN* is geassocieerd met meerdere vormen van kanker, en inactivatie van het gen is vaak geassocieerd met progressie van tumoren. Derhalve is er gekeken naar PTEN eiwit expressie en *PTEN* genmutaties in menselijke benigne en maligne PCC. Er werd verlies gevonden van het gebied dat codeert voor het *PTEN* gen, waarbij er in de maligne PCC verlies was in 40% van de onderzochte tumoren, en in de benigne PCC verlies was in 14% van de tumoren. Mutaties in het *PTEN* gen werden niet gevonden. Op PTEN-eiwitniveau kon geen verschil worden aangetoond tussen benigne en maligne tumoren. Hierdoor kon worden gesteld dat inactivatie van *PTEN* bij mensen geen grote rol speelt bij het ontstaan van PCC.

Zoals reeds beschreven speelt *SDHD* een rol in het ontstaan van PCC. Om te onderzoeken hoe vaak mutaties in *SDHD* nu voorkomen werd mutatie analyse verricht op een grote serie PCC. Hierbij is gevonden dat, in tegenstelling tot PGL, er in PCC relatief weinig *SDHD* mutaties voorkomen. Deze kleine groep patiënten met een PCC en *SDHD* mutatie heeft meer risico op het ontwikkelen van meerdere tumoren en kan ook op relatief jonge leeftijd (<35 jaar) al tumoren hebben. Derhalve wordt op basis van deze studie aanbevolen om een selectieve groep patiënten met PCC te screenen voor *SDHD* mutaties. Criteria hiervoor zijn: positieve familie-anamnese voor PCC of PGL, meerdere PCC of PGL bij de patiënt, of een leeftijd jonger dan 35 jaar.

Somatische mutaties in een van de SDH genen zijn zeer zeldzaam. In hoofdstuk 7 wordt de eerste en tot nu toe enige somatische *SDHB* mutatie bij een extra-adrenale PCC beschreven. Om aan te tonen dat een *SDHB, C* of *D* mutatie werkelijk de oorzaak is van het ontstaan van een PCC of PGL kan worden gekeken of het eiwit nog in de tumor aanwezig is. In het geval van een mutatie in *SDHB, C* of *D* en een verlies van het eiwit is de kans groot dat de mutatie inderdaad de oorzaak is van het ontstaan van de tumor. De tumor met de somatische *SDHB* mutatie toonde geen eiwitexpressie meer voor SDHB. Naast deze tumor werden er nog 219 tumoren met en zonder bekende mutaties onderzocht middels immunohistochemie om te kijken naar het SDHB eiwitniveau. In alle tumoren met een bekende *SDHB, C* of *D* mutatie was er geen eiwit aanwezig in de tumorcellen van PCC en PGL. Bij patiënten met een *VHL* mutatie, een *RET* mutatie of een *NF1* mutatie was het SDHB eiwit wel aantoonbaar in de tumorcellen. Ook in een prospectieve serie werd in de meeste SDHB eiwit-negatieve tumoren ook een *SDH*-mutatie gevonden. Daarmee is de immunohistochemische test voor SDHB eiwit 100% sensitief en 84% specifiek, en kan deze zeer goed als aanvullende test gebruikt worden bij patiënten met PCC en PGL die genetisch gescreened worden.

Naast het genetisch screenen van patiënten met PCC en PGL kan SDHB immunohistochemisch onderzoek ook mogelijk een voorspellende waarde hebben voor het gedrag van PCC en PGL. Opvallend genoeg was er geen duidelijke relatie tussen SDHB eiwitverlies en aanwezigheid van eiwitten gerelateerd aan hypoxie.

Nieuwe technieken, zoals-RNA expressie arrays, geven een nieuwe impuls om te zoeken naar het onderscheid tussen benigne en maligne PCC en PGL. Niet alleen gedrag, maar ook genetische achtergrond en tumorovereenkomsten zullen in de toekomst kunnen worden bepaald op basis van de RNA-expressie profielen. Alhoewel het kenmerkende profiel nog niet beschreven is, deels ook door verschillen in platforms en annotaties van genen, is er een belangrijke toekomst voor het ontwikkelen van profielen van benigne en maligne tumoren, om in de toekomst patiënten adequate behandeling en follow-up te geven.

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DANKWOORD

PFFFFFF……Da's nog het moeilijkste. Uiteraard uren, weken, maanden zitten wikken en wegen wat er in het meest gelezen deel van dit pheochromafiele boekje moest komen. Mijn eerste idee was alleen BEDANKT! Maar aangezien je niet per letter maar per pagina betaalt kan ik er maar beter wat van maken. Er zijn heel veel mensen belangrijk geweest voor dit werk, maar je moet ergens beginnen en ergens eindigen, dus bij deze een dankwoord FFF-stijl. (Voor de mensen die weten waar de FFF voor staat, die zijn bij deze bedankt!)

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José, jij ook bedankt voor je spontaniteit, you're next! Ook dank aan Bart-Jeroen en Hilde.

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CURRICULUM VITAE

Francien Heleen van Nederveen kwam ter wereld op 8 november 1978 te Schiedam. In 1997 behaalde ze haar VWO diploma, waarna er gestart werd met de opleiding Geneeskunde aan de Erasmus Universiteit te Rotterdam. Het afstudeeronderzoek was het eerste echte onderdeel op de afdeling pathologie, waar gekeken werd naar *P53* mutaties in paragangliomen. Na het doorlopen van de co-schappen, welke wederom werd afgesloten bij de pathologie, is het artsendiploma (*cum laude*) behaald. Na een korte uitstap naar de interne geneeskunde, is Francien op 1 april 2004 getart met de opleiding pathologie te Rotterdam. Dit opleidingstraject was dankzij het KWF en ZonMW uit te breiden naar een AGIKO constructie, waarbij het onderzoek voor dit proefschrift na 1 jaar na de start van de opleiding kom worden voortgezet. Deel van dit onderzoek is gedaan in het British Columbia Cancer Research Center, Vancouver, British Columbia, Canada, waarna er in Rotterdam werd verder gewerkt. Tijdens dit schrijven is Francien in de laatste fase van de opleiding pathologie. Binnen de pathologie is er door haar een voorliefde ontwikkeld voor de endocriene- en haematopathologie, welke in de nabije toekomst verfijnd zullen worden.

Erasmus MC
Universitair Medisch Centrum Rotterdam

PhD Portfolio

Summary of PhD training and teaching

Name PhD student: Francien H. van Nederveen Erasmus MC Department: Pathology Research School:Erasmus Postgraduate School Molecular Medicine (Molmed)	PhD period:1st april 2005 -11th june 2010 Promotor(s): Prof. dr. J.W. Oosterhuis Supervisor: Dr. R.R. de Krijger

1. PhD training

	Year	Workload (Hours/ECTS)
General courses		
- Erasmus MC summer school; How to write a medical paper	2004	1 ECT
Specific courses (e.g. Research school, Medical Training)		
- Basis onderwijs pathofysiologie; oncologie	2007	1 ECT
- Basis onderwijs pathofysiologie; immunologie en ontsteking	2008	1 ECT
- European Confederation of Neuropathological Societies; *Tumours of the CNS and its Covering,* Amsterdam	2005	1ECT
- Spotfire course	2006	1 ECT
Seminars and workshops		
- 3rd workshop of innovative mouse models, Leiden	2005	1 ECT
- Coupeavonden Pathologie	2005-2010	1 ECT
- USCAP seminars; Neuropathology, advanced molecular pathology, thyroid and parathyroid pathology, neuroendocrine tumors including dermatologic manifestations	2007	16 hr/ 0,57 ECT
- Symposium NFI (grensvlakken van de klinische (obductie) pathologie	2006	4,5 hr/ 0,16 ECT
Presentations		
- The possibility to distinguish pheochromocytomas and paragangliomas with SDHD mutations using CGH. Dutch Pathology Society Annual Meeting, Ede	2005	1 ECT
- Somatic SDHB mutation in an extraadrenal pehochromocytoma. Pheochromocytoma; First international conference, Bethesda, USA	2005	1 ECT

- High resolution array CGH eveals differences in benign and malignant sporadic pheochromocytomas. Dutch Pathology Society Annual Meeting, Ede	2006	1 ECT
- Precursor lesions in endocrine pathology: criteria, molecular concepts and clinical significance; Precursor lesions of the adrenal gland. Interim meeting, European Society of Pathology, Ioannina	2006	1 ECT
-		
- Benign and malignant sporadic pheochromocytomas: Recent insights in their molecular development. AACR, "Future leaders new directions symposium", Los Angeles, USA.	2007	1 ECT
- Update on phechromocytomas and paragangliomas: recommendations for practicing pathologists, European Society of Pathology, Interim Meeting, Barcelona, Spain	2008	
Poster presentations		
- PTEN gene loss but no mutations in benign and malignant pheochromocytomas. Molecular medicine day, Rotterdam	2006	1 ECT
- Array CGH in benign sporadic pheochromocytomas, Molecular medicine day, Rotterdam	2007	1 ECT
- Gene expression analysis in benign and malignant pheochromocytomas, looking for hallmarks of malignancy. Dutch Pathology Society annual meeting, Ede	2007	1 ECT
- The predictive value of SDHB immunohistochemistry in hereditary pheochromocytomas and paragangliomas. Erasmus MC science day for residents, Rotterdam, ***award best poster***	2008	1 ECT
- The value of SDHB immunohistochemistry in hereditary phaeochromocytomas and paragangliomas, Pheochromocytoma; Second international conference, 2008, Cambridge, U.K.	2008	1 ECT
(Inter)national conferences		
United-states& Canadian Academy of pathology (USCAP) Annual meeting, San Diego, USA	2007	1 ECT
	2007	1 ECT
American association for cancer research (AACR) Annual meeting, Los Angeles, USA	2008	1 ECT

OECI Pathobiology workshop, Cluj, Romania Pheochromocytoma; First international conference, Bethesda, USA 9th congress of the European Skull Base Society, Rotterdam, The Netherlands, 2009	2005 2009	1 ECT 1 ECT
Other René Vogels-stipendium Hubert Wolfe award, Endocrine Pathology society, San Diego	2004 2007	

2. Teaching

	Year	**Workload (Hours/ECTS)**
Supervising practicals and excursions, Tutoring		
- Pathology of adrenal disease; 1st year students Medicine	2006-2010	4 ECT
- Graft-versus-host disease; 2nd year students Medicine	2008-2009	2 ECT