PHEOCHROMOCYTOMAS AND PARAGANGLIOMAS

FROM DNA TO THE DAILY CLINICAL PRACTICE

BART-JEROEN PETRI

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PHEOCHROMOCYTOMAS AND PARAGANGLIOMAS:
FROM DNA TO THE DAILY CLINICAL PRACTICE.

Pheochromocytomen en paragangliomen:
van DNA naar de dagelijkse praktijk

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Chapter 1

General introduction

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Phaeochromocytomas and sympathetic paragangliomas

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General aspects

The neuroendocrine system is a diffuse system in which the nervous system and the hormones of the endocrine glands interact. The neuroendocrine organs of the sympathetic and parasympathetic autonomic nervous system are called paraganglia[1]. These organs usually manifest as anatomically discrete bodies, which derive from the neural crest and produce catecholamines and various peptides. Various localizations of paraganglia in the human body are known, including the adrenal gland, organs of Zuckerkandl, and carotid and aortic bodies[2]. Paraganglia are divided into two functional groups, i.e. the sympathoadrenal and the parasympathetic autonomic nervous system. Sympathetic paraganglia are predominantly located in the prevertebral and paravertebral sympathetic trunks, and along the fibers of the hypogastric plexus, innervating pelvic and retroperitoneal organs. Parasympathetic paraganglia are almost exclusively located in the region of cranial as well as thoracic branches of the of the glossopharyngeal nerves and vagal nerves. The principal glossopharyngeal paraganglia are the tympanic (located in the wall of the middle ear), and the carotid bodies (Figure 1)[3]. Neoplasms of the paraganglia are called pheochromocytomas (PCC), sympathetic and parasympathetic paragangliomas. The name PCC is derived from the Greek synonym "dark colored tumor", because it was first described by Pick as a chromium salt-reactive tumor which lead to dark coloration[4]. PCC are tumors which originate in the adrenal medulla. Sympathetic paragangliomas (sPGL), in the literature often described as extra-adrenal PCC, usually produce catecholamines and occur in the abdominal cavity and the aorticopulmonary bodies, but not in the adrenal medulla. Parasympathetic paragangliomas, also called head and neck paraganglioma, usually do not produce catecholamines and are situated in the wall of the middle ear, along the vagal nerve, and the carotid and jugular bodies. In the literature they are still often referred to as chemodectomas, glomus tumors, or carotid body tumors.

Epidemiology

PCC are rare tumors with a reported incidence of 2-8 per million and it is estimated that annually there are approximately 80 – 130 new cases in The Netherlands[5]. It is estimated that PCC occur in 0.1-0.5% of patients presenting with hypertension, but patients may be completely asymptomatic. Nearly 10% of adrenal masses were eventually shown to be PCC. They are found at autopsy in 0.1%, most often in patients that die suddenly from myocardial infarction or a cerebrovascular incident.

Autopsy series of PCC suggest that approximately 3350% remains undiagnosed throughout life[6]. The peak incidence of PCC occurs within the fourth and the fifth decade, and approximately 10% develops in childhood. The male-to-female ratio in PCC is almost one and there is no racial predilection[7-8].

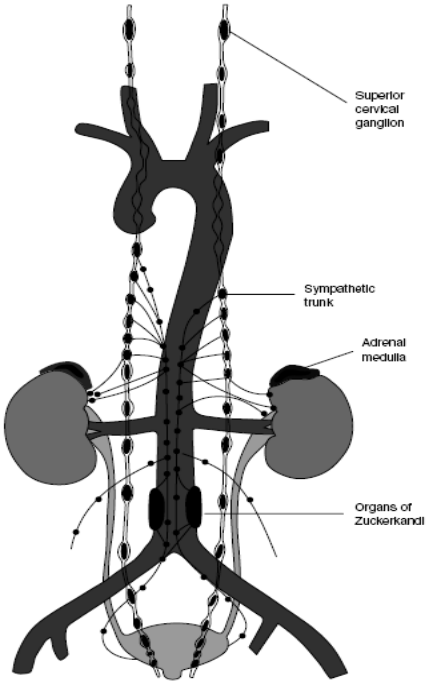

Figure 1. Anatomical distribution of chromaffin tissue

The clinical incidence of PGL is less clear. It is estimated that approximately 10% of the PCC occur extra-adrenal, now called sPGL. The incidence of HNPGL is difficult to estimate. A population-based surgical incidence of 1:1,000,000 was reported in the Netherlands. Higher autopsy incidences of 1/ 3860 and 1/ 13400 were reported for carotid body tumors[9]. Comparison of these incidences suggests that most HNPGL are not clinically recognized or operated[10].

Sporadic pheochromocytoma and paraganglioma

The vast majority of PCC (± 70%) occur in a sporadic setting and are usually diagnosed in individuals aged 40-50 years[11]. PCC can only be regarded as sporadic following exclusion of germline mutations in one of several known PCC candidate genes (see below). Recently, a considerable subset (7.5-24%) of apparently sporadic PCC has been shown to harbor germline mutations in *RET*, *VHL*, *SDHB* and *SDHD* [11-14]. In contrast, the frequency of somatic mutations in these genes in PCC appears low [15-18].

The genetic mechanisms underlying tumorigenesis of truly sporadic PCC are poorly understood. Loss of heterozygosity (LOH) and comparative genomic hybridization studies have revealed involvement of aberrations on chromosome arms 1p, 3p, 3q, 11p, 17p, and 22q, but candidate genes have not yet been identified[19-21].

New insights have shown that there are probably two distinct subgroups of sporadic PCC on the basis of DNA aberrations: one group with loss of 1p and/ or 3q, and another group with loss of 3p with or without concurrent 11p loss. These subgroups could relate to different pathways of tumorigenesis. However, further analysis has to be done[22].

Most PGL and PCC occur sporadically, with between 10% and 50% of parasympathetic PGL and between 10% and 30% of sympathetic PGL and PCC carrying germline mutations in one of several candidate genes (see below). Specifically, in the Netherlands a high proportion of parasympathetic PGL is hereditary due to the occurrence of several founder mutations[10].

Pheochromocytoma, sympathetic paraganglioma, and head and neck paraganglioma in familial setting

Pheochromocytoma in multiple endocrine neoplasia type 2

Multiple endocrine neoplasia type 2 (MEN 2) is a rare autosomal dominant tumor syndrome, with an estimated prevalence of 2.5 per 100,000 in the general population. There are two forms of MEN 2: MEN 2A and MEN 2B. MEN 2A is characterized by medullary thyroid carcinoma (MTC) in all patients, PCC in 50%, and primary hyperparathyroidism caused by parathyroid hyperplasia in approximately 2030% of patients. MEN 2B is also characterized by MTC and PCC, and in addition by mucosal neuromas on the lips and tongue, ganglioneuromatosis in the gastrointestinal tract, and skeletal abnormalities. If PCC is associated with MEN 2, it occurs bilaterally in 50–80% of instances. It is well established that MEN 2 is caused by underlying germline mutations in the *RET* proto-oncogene on chromosome 10q11[23-25]. Familial MTC is a third entity associated with *RET* mutations. When MTC is recognized in four or more family members and other MEN 2-associated tumors have been excluded, a diagnosis of familial MTC is reached[26-27].

Different mutations are found in MEN 2, with a clear genotype–phenotype correlation. Mutations in multiple *RET* gene codons are associated with MEN 2A; mutations of codon 634 occur in 85% of the patients. In both MEN 2A and familial MTC the vast majority of mutations are located in exons 10, 11, 13 and 16 of the *RET* gene[28]. In 95% of patients with MEN 2B a single point mutation is found at codon 918. Therefore, clinical genetic testing of the *RET* gene is usually limited to the above mentioned exons. MEN 2 often manifests relatively early in life, between age 5 and 20 years, usually with MTC, but PCC is the first manifestation in 25% of patients[29-30]. MTC is a potentially curable life-threatening pathology that disseminates relatively early in the course of the disease. Prophylactic total thyroidectomy and total cervical lymphadenectomy at the age of 6 years has been recommended to prevent the occurrence and metastasis of MTC in patients harboring mutations in codons 634, 611, 618, 620, and 891. For patients harboring mutations in codons 918, 883, and 922, thyroidectomy and total cervical lymph node resection is advocated even earlier, before the age of 1 year, as the clinical course of MTC in this syndrome

is particularly aggressive[31-32]. PCCs in MEN 2 are most frequently associated with mutations in codons 634 and 918, but all MEN 2-associated mutations, with the exception of the codon 791 mutation, have been associated with PCC. As 35% of patients have a simultaneous presentation of MTC and PCC, screening for the presence of the latter tumor is indicated before surgical removal of the thyroid. In such circumstances, prior surgical removal of the PCC is necessary to prevent peroperative hypertensive crises[33]. Obviously, when a *RET* gene mutation is found, this has consequences not only for the treatment and follow-up of the patient, but also for the management of his or her family members[27, 34].

Pheochromocytoma in von Hippel–Lindau disease

VHL is an autosomal dominant hereditary tumor syndrome characterized by a large spectrum of benign and malignant tumors. The incidence of VHL is one in 36,000 live births and the disease generally becomes evident between the age of 20 and 40 years[35]. The features of this tumor syndrome are cerebellar hemangioblastoma, retinal angioma, renal cell carcinoma, PCC, renal and pancreatic cysts, neuroendocrine tumors of the pancreas and epididymal tumors[36]. Most symptomatic tumors in VHL will be removed surgically, and PCC must be excluded before removing other tumors because of the high intraoperative risks caused by high levels of circulating catecholamines[37].

Four clinical phenotypes of VHL are known, including type 1, characterized by the above-mentioned tumors without PCC; type 2A and type 2B, characterized by the tumors of type 1 including PCC; and type 2C, which is associated with PCC only. PCC occurs in approximately 10–20% of patients with VHL, but in only 5% is it the presenting manifestation. In 40–80% of those with VHL and PCC, PCCs occur bilaterally. Patients may rarely present with multifocal paraganglial tumors, including sPGL, which may be malignant, and even with head and neck PGL (HNPGL)[7, 38]. Malignancy in VHL is reported in less than 5% of patients. The life expectancy of patients with VHL is reduced, often to less than 50 years, especially in subtypes with hemangioblastoma and renal clear cell carcinoma. Therefore, early screening is important. Magnetic resonance imaging (MRI) of the brain and spinal cord with gadolinium for hemangioblastoma, examination of the retina for retinal angiomas, and MRI or computed tomography (CT) of the abdomen to exclude renal clear cell carcinoma and PCC must be performed.

In patients with VHL, a PCC produces only noradrenaline (norepinephrine), not adrenaline (epinephrine), because these patients lack the phenylethanolamine *N*-methyltransferase enzyme, which catalyses the *N*-methylation of noradrenaline, resulting in the formation of adrenaline. Thus, biochemical analysis of noradrenaline and adrenaline can lead to the diagnosis[7, 39].

VHL is associated with mutations on the *VHL* tumor suppressor gene located on chromosome 3p25 and aberrations are identified in nearly all classical families[35]. More than 300 tumor-specific and germline mutations on the *VHL* gene are known to cause VHL, 36 of which are described in patients with VHL and PCC. These mutations are spread across the gene, which makes screening

of the entire coding region necessary in clinical genetic testing[40-41]. Apart from known families, it should be noted that approximately 20% of those with VHL carry *de novo* mutations, highlighting the need for mutation analysis in patients with apparently sporadic PCC[42]. VHL type 1 is thought to be due to loss of VHL protein function, whereas type 2C is thought to be due to gain of VHL protein function. Co-occurrence of loss and gain of VHL protein function is thought to result in VHL types 2A and 2B[43]. Phenotype–genotype correlation in patients with VHL could tailor clinical attention and surveillance to the organs at risk, and potentially reduce psychological anxiety and the cost of unnecessary investigations[44].

Pheochromocytoma in neurofibromatosis type 1

NF1 or von Recklinghausen's disease is an autosomal dominant genetic disorder with an incidence of approximately one in 3,000 individuals and is associated with the *NF1* gene on chromosome 17q[45-46]. PCCs are identified in approximately 0.1–5.7% of affected patients[47]. When they are examined at autopsy, the prevalence of PCC is slightly higher (3.3–13.0%)[48]. PCCs occur bilaterally in 10% and malignant PCC is identified in 11%, similar to the frequency of malignancy in the general population[47]. Extra-adrenal lesions are reported in 6% of patients[49]. In those with NF1, PCC must be removed surgically.

In clinical practice, diagnosis of NF1 is straightforward, because of the typical multiple neurofibromas and café-au-lait spots. PCC is almost never the first clinical presentation in NF1. If a patient with NF1 presents with hypertension, the usual approach to the investigation for PCC can be used, including biochemical and radiological screening. Although screening is performed, both in a clinical genetic as well as in a research setting, genetic screening for *NF1* mutations is hampered by the fact that this is one of the largest known genes, having 60 exons spanning more than 350 kb of genomic DNA, with the co-occurrence of no fewer than 36 pseudogenes[45, 50].

Pheochromocytoma in pheochromocytoma– paraganglioma syndrome

Recent advances have shown that three of four genes encoding succinate dehydrogenase subunits (*SDHB, SDHC* and *SDHD*) have a role in the development of PCC, sPGL and HNPGL, now called the PCC–PGL syndrome[51]. *SDHA*, the fourth gene of this enzyme complex, is associated with a neurodegenerative disorder known as Leigh syndrome, but until very recently not with the above-mentioned tumours[52].

PGL-1 is linked to the *SDHD* gene, located on chromosome 11q23, with mutations leading to HNPGL and sometimes to PCC[51]. In large groups of apparently sporadic PCC the incidence of *SDHD* mutations varies from 2 to 11% [18, 53], and in apparently sporadic HNPGL this figure is 10–50% [54-56]. Several studies report bilateral PCC in patients with mutated *SDHD*, but overall this is rare[12, 57-58]. In patients with *SDHD* mutations the family history is often inconclusive because

of the complex inheritance pattern. Tumors develop only if mutations are inherited from the paternal side; they do not develop if the mutation is inherited from the mother[59]. This mechanism is known as maternal genomic imprinting. In the Dutch population two frequent founder mutations occur: Asp92Tyr and Leu139Pro.

PGL-3 is linked to the *SDHC* gene located on chromosome 1q21 and worldwide only ten families have been described, all harboring exclusively HNPGL. Two large studies that addressed the role of *SDHC* in PCC have not reported any mutations[12, 60]. PGL-4 is linked to the *SDHB* gene, located on chromosome 1p36. In patients with an *SDHB* mutation, HNPGL, sPGL and adrenal PCC are described; in sPGL, up to 50% of tumors are malignant[7, 61]. *SDHB* mutations were found in 5% of a large series of apparently sporadic HNPGLs, and in 9.5% of apparently sporadic PCCs[61]. The mean age at diagnosis is between the third and the fifth decade[62]. In contrast to PGL-1, an autosomal dominant inheritance without maternal imprinting is observed in PGL-4[63-64]. The susceptibility gene and the possible clinical presentations for PGL-2 have very recently been described and will be covered in the discussion, in addition to the discovery that the *SDHA* gene, too, is related to the occurrence of paraganglial tumors.

Clinical presentation

In clinical practice PCC and sPGL are often considered as great mimickers, because of their wide diversity in presentation (table 1). The vast majority of patients presents with continuously or paroxysmally increased blood pressure, or with episodic signs of flushing or palpitations, although there is a small number that is asymptomatic[7]. In the latter cases the diagnosis is made coincidentally, often in the context of diagnostic imaging procedures for other purposes, nowadays known as incidentalomas[65].

Most patients harboring HNPGL present with complaints in the head and neck region, i.e. swallowing problems, hoarseness, and neurological problems[10]. Rarely, patients present with complaints mentioned in Table 1, because not more than 10% of HNPGL produce catecholamines[55].

Table 1. Frequency of signs and symptoms in patients with pheochromocytoma[7]

	% of patients
Headache	60–90
Palpitations	50–70
Sweating	55–75
Pallor	40–45
Nausea	20–40
Flushing	10–20
Weight loss	20–40
Tiredness	25–40
Psychiatric symptoms (anxiety, panic)	20–40
Hypertension	80–90

As for non-catecholamine-producing PCC or sPGL, for HNPGL the diagnosis is often made coincidentally, in the context of diagnostic imaging procedures for other purposes.

Diagnosis

Biochemical analysis

If a catecholamine-producing tumor (PCC or PGL) is suspected, the diagnosis must be ascertained because of the potentially severe complications. As a result of its high sensitivity, plasma metanephrine/ normetanephrine measurement or 24h-urinary and metanephrine measurement are the preferred initial tests. PCC or PGL can be excluded when this is negative, but if there is a large increase in metanephrine the specificity is close to 100%. As a result of its high sensitivity, 24-h urinary metanephrine measurement is the preferred initial test. PCC or PGL can be excluded when this is negative, but if there is a large increase in metanephrine the specificity is close to 100%. Drugs, such as labetalol or α-methyldopa, influence catecholamine concentrations in both urine and plasma. These may cause increased or decreased levels of catecholamines and metabolites. The next step is imaging for tumor localization, although in the case of an equivocal test result combined with only a marginal increase in plasma metanephrine, plasma catecholamines should be tested for. Again, if this test is positive, there is sufficient evidence of the presence of a tumor. Further imaging studies are needed in all instances of positive biochemical testing[66]. This is summarized in figure 2. All patients presenting HNPGL should be tested as described above, because in rare cases HNPGL can also produce catecholamines, but it is also possible that these patients have developed a PCC simultaneously.

Radiological analysis

Although sensitivity (90–100%) and specificity (70–80%) are similar, the preferred imaging modality for PCC and PGL is MRI rather than CT, because the intravenous contrast used for the latter can provoke catecholamine release[68-69]. However, the mere presence of a mass at MRI cannot distinguish PCC from PGL, or from metastatic lesions. For this determination, [123I] metaiodobenzylguanidine (MIBG) scintigraphy and/or positron emission tomography (PET) and/or 68-Ga-labelled 1,4,7,10-tetraazacyclododecane-1,4,7,10- tetra-acetic acid–Nal3-octreotide receptor PET/CT, a new detection method for neuroendocrine tumors, should be performed[66, 70]. Takano and colleagues[71] compared MRI, MIBG and PET scintigraphy in detecting histologically proven metastatic lesions in 11 patients, and concluded that these techniques would not recognize all metastatic lesions if one of the three techniques were omitted.

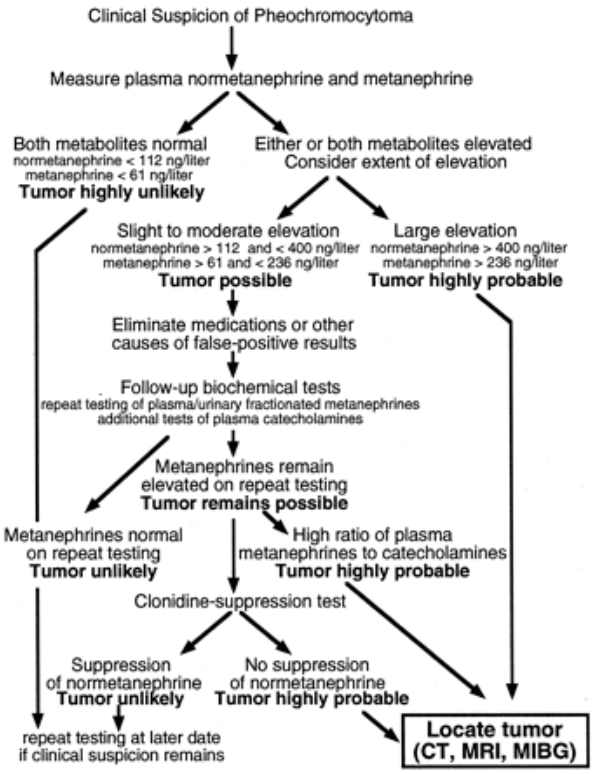

Figure 2. Algorithm for biochemical diagnosis of pheochromocytoma[67]

Genetic testing in pheochromocytoma

The prevalence of germline mutations in *RET, VHL, SDHB* and *SDHD* among patients presenting with apparently non-syndromic PCC appears to be much higher than previously supposed. Mutation analysis has shown rates ranging from 10 to 27% of familial cancer syndrome gene involvement[11-12, 72]. These studies conclude that it is justified to perform genetic testing in every patient presenting with PCC, with or without a positive family history.

Although most inherited PCC presents before the age of 50 years, some tumors occur later. Knowing the incidence of inherited PCC after the age of 50 years and bearing in mind the cost-effectiveness of genetic testing, the present authors suggest that all patients younger than 50 years should be referred for genetic assessment. When patients present with extra-adrenal, bilateral or multiple PCCs, they should always be referred for genetic investigation, regardless of age, because it is known that such presentations frequently occur in the context of a heritable tumor syndrome[57].

There are several reasons to recommend genetic testing. First, there is a clear benefit for the patient, as other tumors that occur in the various tumor syndromes can be screened for and

treated at an early stage. This is especially true for contralateral PCC, which may influence the surgical strategy, or sPGL in other locations. Second, family members can be screened, which may also lead to early recognition of the disease in some of them, and thereby to improved treatment and prognosis of affected family members.

As the number of genes involved in hereditary PCC and PGL syndromes continues to expand, one wonders whether every patient should be tested for all candidate genes, or whether testing algorithms should be developed to limit the cost of such testing. The rarity of *SDHC* abnormalities has already led to the suggestion that this gene should be excluded from genetic testing procedures[73]. A medical assessment with plasma measurement of thyrocalcitonin and carcinoembryonic antigen levels to exclude MTC, and retinal examination to exclude angiomas, is recommended before genetic testing.

Genetic testing should be done for the above-mentioned candidate genes, with the exception of the *NF1* gene, because this disorder can be diagnosed on phenotypic criteria[50]. The *RET* proto-oncogene should be tested for exons 10, 11, 13, 14, 15 and 16, because almost all mutations causing MEN2 occur in these exons[74]. For *VHL, SDHB* and *SDHD* the entire coding region should be tested. Both direct sequencing and single-strand conformation polymorphism analysis can be used to screen for missense mutations and small deletions, but the former technique is preferred because of its higher sensitivity and specificity[75]. It must be noted that direct sequencing cannot detect all genetic abnormalities, such as large deletions, which have been shown with the use of the multiplex ligation dependent probe amplification technique[76]. Denaturing high-performance liquid chromatography is an effective screening tool for the detection of germline mutations in the genes associated with the described tumor syndromes. It is made cost effective by reducing the number of samples for DNA sequencing analysis[77].

Genetic screening and patient follow-up

As more genetic testing will be performed in PCC and PGL, it is probable that increased numbers of patients with hereditary cancer syndromes will be detected at a stage at which they present solely with a PCC or PGL. Once a germline mutation has been found in any of the candidate genes, further screening tailored to the specific syndrome should be performed. Such protocols have been developed recently for MEN2, VHL and the PCC–PGL syndrome.

For patients at risk, imaging is advised on a yearly basis in light of the fact that approximately 10% of PCCs and PGLs do not produce catecholamines[3]. These testing algorithms are also valid for screening for contralateral PCC, recurrent PCC or PGL, and for metastases of malignant PCC or PGL. It is prudent to advise patients to attend hospital in the intervals between screenings if they experience PCC/PGL-related symptoms. Screening protocols for other entities in the various hereditary tumor syndromes are summarized in Table 2.

Table 2. Follow-up and screening regimen for familial pheochromocytomas

	follow up for PCC			screening for other entities		
	biochemical evaluation (1)	imaging procedure (2)	goal	biochemical evaluation (3)	imaging procedure (4)	goal
RET unilateral PCC	every 12 months	every 12 months	contralateral / recurrent PCC	every 12 months	-	MTC, PHP
RET bilateral PCC	every 12 months	every 12 months	recurrent PCC	every 12 months	-	MTC, PHP
VHL unilateral PCC	every 12 months	every 12 months	contralateral/ recurrent/ extra-adrenal PCC/ metastases	-	every 12 months	RCC, HEM, RA
VHL bilateral PCC	every 12 months	every 12 months	recurrent /extra-adrenal PCC/ metastases	-	every 12 months	RCC, HEM, RA
SDHB	every 12 months	every 12 months	metastases	-	-	-
SDHD single PCC	every 12 months	every 12 months	contralateral/ metastases PCC	-	every 12 months	HNPGL
SDHD multiple PCC	every 12 months	every 12 months	recurrent/ metastases PCC	-	every 12 months	HNPGL
Malignant PCC	every 12 months	every 12 months	recurrent/ metastases PCC	-	-	-

PCC, pheochromocytoma; RET, REarranged during Transfection; MTC, medullary thyroid carcinoma; PHP, primary hyperparathyroidism; VHL, von Hippel–Lindau disease; RCC, renal clear cell carcinoma; HAEM, hemangioblastoma; RA, retinal angioma; SDHB, succinate dehydrogenase subunit B; SDHD, succinate dehydrogenase subunit D; HNPGL, head and neck paraganglioma.

Treatment

An adrenalectomy, or complete resection, is the treatment of choice for sporadic or hereditary unilateral PCC or an sPGL. During surgery there is the risk of a life-threatening hypertensive crisis owing to excessive catecholamine production and of compensatory hypotensive episodes. Patients are treated before operation with selective and non-selective α- and β-adrenoceptor antagonists, calcium channel blockers, and/or drugs that inhibit catecholamine synthesis with the intention of preventing such crises[7]. It is usual to administer phenoxybenzamine, a non-competitive α-receptor blocker. If this therapy is not sufficient, β-blocking agents or calcium channel blockers can be added to achieve a reasonable blood pressure before surgery.

Until recently, open adrenalectomy was the standard procedure, but since 1992 laparoscopic adrenalectomy has become the procedure of choice[78]. Several studies have reported superior results with the laparoscopic method[79-82]. The minimally invasive approach is associated with improved patient satisfaction because of reduced postoperative pain and a shorter hospital stay, better cosmesis and a shorter convalescence. Intraoperative hypertensive crises have been less prominent than with open adrenalectomy owing to a clearer view of the vessels and a reduction in intraoperative manoeuvres[83].

As already noted, inherited syndromes such as MEN2, VHL and NF1, must be considered in any patient presenting with bilateral PCCs[57]. Both open total bilateral adrenalectomy and laparoscopic total bilateral adrenalectomy have been described. Again, laparoscopic procedures are now preferred[80, 84-85]. After total bilateral adrenalectomy patients will have permanent adrenal insufficiency,

and will require lifelong steroid replacement with its risk of osteoporosis and hypoandrogenism. These patients are also at risk of Addisonian crises, which affect 25–33%, mostly those with poor treatment compliance[86-87].

Cortex-sparing adrenalectomy has been introduced recently for bilateral PCC. In this procedure a part of the adrenal gland that includes the tumor is resected,

leaving a vascularized part *in situ* to conserve cortical function. Given the close contact between adrenal cortex and medulla, it is likely that medullary tissue will be left behind, resulting in a relatively high chance of disease recurrence[87]. For synchronous bilateral PCC, the larger lesion is resected in total, the smaller one being managed by cortex-sparing adrenalectomy. For metachronous bilateral PCC, the first tumor is resected in total, the second being subjected to the cortex-sparing technique when symptomatic[88]. Peroperative adrenal ultrasonography can be used to discriminate tumors smaller than 3 mm[89]. In a few studies, recurrence rates between 7 and 38% have been described[87, 90-91], with cortical function recovered in 65–92% of patients[90,91].

Postoperative monitoring of the remaining gland for recurrent PCC should be carried out, with biochemical evaluation every 6 months and radiological evaluation at intervals of 1 year, as described previously.

Partial adrenalectomy has evolved to balance the risk of tumor recurrence with that of lifelong steroid replacement after total bilateral adrenalectomy. Several studies have shown that there is better quality of life and lower medical risk after cortex-sparing adrenalectomy[83, 86-87, 90-92]. Another way to avoid lifelong steroid replacement is bilateral adrenalectomy with autotransplantation of medulla-free cortex tissue. Results so far have been poor, but further studies may improve the technique[38]. At present, laparoscopic partial adrenalectomy is the preferred operative technique for the treatment of bilateral PCC.

In HNPGL there are two treatment strategies: surgery (with or without pre-operative embolization) and observation[93-94]. Because tumor growth can cause disabling loss of function of the nearby structures, surgery is the therapy of choice in these cases. In some cases a wait and see policy is the best approach[95-96].

Malignancy

Originally, 10% of all PCCs and sPGLs were thought to be malignant, evidenced by the presence of metastases, predominantly in bone, lungs, liver and lymph nodes. About 50% of the metastases have a synchronous presentation with the primary tumor, whereas 50% occur metachronously[44, 97]. In clinical practice it is not possible to predict whether an apparently sporadic PCC or sPGL is malignant. Although there are still no appropriate markers to differentiate benign from malignant PCC, Elder and colleagues[98] suggest that the combined use of Ki-67/MIB-1 immunohistochemistry and human telomerase reverse transcriptase (*hTERT*) gene expression may become a valuable diagnostic addition for PCC and sPGL. It is known that the risk of malignancy is higher in sPGL than in adrenal PCC, also depending on the presence or absence of mutations in certain susceptibility genes[12, 61]. A Pheochromocytoma of the Adrenal gland Scaled Score (PASS) has been proposed, but the pathological features incorporated into this scale seem to identify only tumors with more aggressive biological behavior[99]. In addition, significant interobserver and intraobserver variation occurs in the assignment of PASS, and so further refinement and validation is necessary[100].

Aims

One of the major problems in PCC management is that the clinical behavior is unpredictable. As described above, the only criterion to call a PCC or PGL malignant is the presence of tumor metastases. There are no current histological, immunohistochemical or molecular markers that can distinguish benign from malignant tumors. Therefore, in the first part of this thesis we investigated several molecular markers of which a potential relationship with malignancy was described in previous work from our group.

In the second part of this thesis we focused on the molecular genotype of subgroups of hereditary PCC, again using CGH and LOH analysis. Specifically, we asked the question whether small precursor lesions, less than 1cm in diameter, in MEN 2 patients would display similar genetic abnormalities as their larger counterparts. Also, we investigated whether adrenal tumors in patients with *SDHD* germline mutations would have a genotype that is comparable with sporadic and MEN 2-related PCC or with PGL from other locations in patients with germline *SDHx* mutations.

The last part of this thesis is devoted to genetic testing of candidate genes in specific subgroups of PCC and PGL patients, i.e. those with multiple or bilateral tumors and children. This is because we hypothesized that, with the advent of new candidate genes, a higher percentage of patients with germline mutations could be found in these groups.

The aims of this thesis, based on the abovementioned issues, are:

- To search for molecular markers which can clarify the pathogenesis of PCC, as well as can distinguish benign from malignant PCC.
- To investigate different subgroups of PCC and reveal mutation frequencies for the various candidate genes.
- To develop algorithms for genetic testing of patients harboring PCC in the context of the abovementioned tumor syndromes

Outline

Chapters 2 and 3 focus on chromosome arm aberrations with a possible link to malignant behavior of PCC. Dannenberg et al described in a conventional CGH study that there could be a link between aberrations on 6q and 17p, on which the *p53* gene is located, and malignancy. We did a comprehensive analysis of both aberrations in chapters 2 and 3 respectively, in an independent large series of sporadic and syndrome-related, malignant and benign PCC.

In chapter 4 we investigated a series of PCC precursor lesions from patients with MEN 2 syndrome, so called adrenomedullary hyperplasia (AMH), to find out if these aberrations carry the same genetic changes as seen in PCC in this syndrome. Such data may serve as a basis for further research into early interventions in patients harboring PCC in MEN 2 syndrome and the possible unwanted effects of catecholamine release (versus the lack of adrenal corticoid hormones).

While HNPGL in patients with germline SDHD mutations have been studied before in our group, little is known about genetic abnormalities in PCC of such patients. Therefore, we performed a conventional CGH analysis of eight adrenal tumors that occurred in association with germline SDHD mutations. The aim of this study was to analyze the pattern of changes and classify these tumors in one of subgroups that we defined previously. An unexpected outcome is discussed in chapter 5.

In the last two decades many studies have focused on mutation frequencies in PCC. New insights have shown that germline mutations in 1 of 6 candidate genes (*RET, VHL, NF1, SDHB, SDHC, SDHD*) are present in approximately 35% of PCC. Therefore, we performed mutation analysis in different subgroups, including bilateral PCC, sPGL, and PCC in children, in which we detected even higher frequencies of mutations and we proposed algorithms to guide the management of PCC patients. We present the PCC in children in chapter 6. In chapter 7 we describe the results of the mutation analysis of bilateral PCC and sPGL.

Chapter 8 presents an overview of the current knowledge of the pathogenesis of hereditary and sporadic PCC, including the differences between malignant and benign PCC. We propose suggestions for future research projects and algorithms for genetic testing in clinical practice to treat and prevent PCC.

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Chapter 2

Genomic analysis of pheochromocytomas: identification of specific DNA copy number changes but exclusion of 6q loss in association with biological behavior

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Endocrine Related Cancer, submitted

Abstract

Pheochromocytomas are rare endocrine neoplasms developing in the adrenal medulla, 10% of which show metastatic disease. To date 25% of pheochromocytomas arise in a familial setting. No reliable histological or molecular parameters are available to predict metastatic disease. Previous genetic studies have identified loss of 1p and 3q as early genetic events and suggested loss of 6q and 17p in association with malignant progression. We recently excluded 17p loss as a marker for malignancy. The aim of this study was to investigate whether 6q loss could discriminate benign and malignant pheochromocytomas and to narrow down the region most frequently involved. Both molecular allelotyping with 12 highly polymorphic 6q markers and comparative genomic hybridization were performed using a series of 68 pheochromocytomas, including 51 benign and 17 malignant pheochromocytomas, of which 53 were sporadic and 15 syndrome-related. Results were correlated with the clinical data of the tumors. Molecular allelotyping revealed that 14/31(45%) pheochromocytomas harbored at least one allelic deletion. No single marker was deleted in more than 21% of the cases and there was no association of 6q loss with metastatic disease. The latter finding was confirmed by CGH analysis. In addition, CGH revealed 1) a significant difference in copy number alterations (9.4 versus 6.1; p=0.004) between malignant and benign pheochromocytomas; 2) a significant difference in copy number alterations between multiple endocrine neoplasia type 2-related and benign sporadic pheochromocytomas (9.2 versus 5.7 ($p<0.0001$)); 3) that losses of 8p and 18p and gains of 5p, 7p, and 12q are more often found in malignant pheochromocytomas ($p \leq 0.047$). In conclusion, our study cannot substantiate earlier findings that 6q loss should be considered a marker for malignancy. Nevertheless, the unmasked DNA copy number changes discriminating benign from malignant pheochromocytomas may point to candidate genes and may help to predict biological behavior.

Introduction

Pheochromocytomas (PCC) represent catecholamine-producing tumors derived from pheochromocytes within the adrenal medulla. PCC occur sporadically (75%) or as part of inherited cancer syndromes, such as multiple endocrine neoplasia type 2 (MEN 2), neurofibromatosis type 1 (NF1), von Hippel-Lindau disease (VHL), or pheochromocytoma-paraganglioma syndromes (PCC-PGL) [1-2]. Histologically confirmed metastases, in places where chromaffin tissue does not normally occur, such as bone, lung, liver, or lymph nodes, are the only accepted sign of malignancy. If only a primary lesion is identified, no reliable markers for malignancy risk assessment are available[3]. Therefore new indicators of malignancy and prognosis are urgently needed.
The genetic mechanisms underlying the tumorigenesis of sporadic PCC are still poorly understood. Only a subset of these tumors harbor germline mutations in the susceptibility genes of the abovementioned syndromes, i.e. the *RET*, *VHL*, *NF1*, *SDHB*, and *SDHD* genes. Loss of heterozygosity (LOH) and comparative genomic hybridization (CGH) studies have reported several putative tumor suppressor gene loci, of which losses of chromosomes 1p and 3q appear early genetic events and loss of 5p, 6q, 17p, and 22q are later events[4-6].
In a previous study, on a series of 29 PCC, we identified loss of 6q and 17p in significant association with malignancy. However, a detailed assessment of chromosome 17p, on which the *p53* tumor suppressor gene is located, revealed no involvement of *p53* in metastatic progression[7].
Losses of chromosome 6q have been detected in many other human neoplasms, including malignant melanoma, carcinomas of the salivary gland, mesothelium, prostate, ovary, stomach, liver and pancreas, and have been implicated in malignancy[8-9]. In a series of 18 PCC, without detectable metastases, Lemeta et al. identified 2 minimal regions of deletion, i.e. 6q14 and 6q23-24 by microsatellite analysis[10]. In order to further substantiate the involvement of 6q loss in the malignant progression of PCC, as well as to identify new DNA copy number changes, we have performed both molecular allelotyping with 12 highly polymorphic 6q markers and comparative genomic hybridization (CGH) using a series of 68 PCC, including 51 benign and 17 malignant PCC, of which 53 were sporadic and 15 syndrome-related. Results were correlated with the clinical data of the tumors.

Material & Methods

Tissue samples and patient information

PCC tissue samples from 68 patients (41 male and 27 female) were available from the archives of the Departments of Pathology, Erasmus MC, Rotterdam (n=18), Radboud UMC, Nijmegen, the Netherlands (n=39), and University Hospital Zurich, Switzerland (n=11). All tumors were collected between 1978 and 2003, and included 51 benign and 17 malignant PCC, of which

the malignant cases had histologically confirmed metastases. The benign group consisted of 38 apparently sporadic and 13 proven syndrome-related PCC (MEN2 n=9, NF1 n=3, SDHB n=3). The malignant group consisted of 15 apparently sporadic PCC and 2 SDHB related PCC. The average patient age was 45.9 ± 14.0 (range 23-79). There were no significant age-related differences between the subgroups.

DNA Extraction

DNA extraction from 55 frozen and 13 paraffin-embedded PCC, as well as from paraffin embedded normal tissue of 32 cases, was performed as described before [4, 9]. Genomic DNA from frozen tissue was isolated by homogenizing approximately 5 mm^3 of each tissue sample prior to proteinase K treatment and DNA purification using the QIAamp DNA mini kit (Qiagen, Hilden, Germany). DNA from paraffin-embedded tumors was isolated from 5-10 μm-thick tissue sections after deparaffinization. DNA quality was checked with agarose gel electrophoresis and quantified by spectrophotometry.

Microsatellite analysis

Thirty-one DNA samples, including 22 benign and 9 malignant PCC, were analyzed for LOH and/or allelic imbalances of 6q using highly polymorphic microsatellite markers, summarized in Table 1. Polymerase chain reaction (PCR) amplification of tumor and normal DNA was performed in reaction mixtures of 15μl. Each reaction contained 50-100ng template DNA, 0.02 mM dATP, 0.2 mM dTTP, dGTP, dCTP each, 0.8μCi α32P-dATP, 20 pmol of each primer, 1.5 mmol/L MgCl2, 10 mmol/L Tris-HCl, 50 mM KCl, and 1 unit Taq DNA polymerase (Amplitaq Gold, Perkin Elmer, Norwalk, CT). An initial denaturation step at 94°C for 5 min was followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 55°C for 60 sec, and extension at 72°C for 60 sec. A final extension step was carried out at 72°C for 10 min. PCR products of tumor and normal DNA from each patient were twice diluted in 5 μl loading buffer (95% formamide, 20 mmol/L EDTA, 0.05% xylene cyanol, 0.05% bromophenol blue) and loaded onto a denaturating 6% polyacrylamide gel. Electrophoresis was carried out at 60W for 1.5 h. The gels were dried and exposed to X-ray film overnight at -70°C. Results were scored by two independent experts.

CGH analysis

CGH was performed with DNA isolated from the 68 PCC samples as previously described [11]. Briefly, 0.2μg tumor DNA was labeled with Spectrum Green-dUTPs (Vysis, Downers Grove, IL) by nick translation (BioNick labeling kit, Life Technologies, Breda, The Netherlands). Spectrum Red-labeled normal and sex-matched reference DNA (Life technologies) was used for co-hybridization.The hybridization mixture consisted of 800ng Spectrum Green-labeled tumor

Table 1. Primers 6q from centromere to telomere

	primer	localisation	Forward 5'-->3'	Reverse 5'-->3'	Size (bp)
1	D6S280	6q12	cagctcacaaatctcattcag	gatatactaatctctgcaagtg	161
2	BJ6Q2	6q21	gctcccactggctagatcac	gggacgattaactctcttgc	150
3	D6S268	6q21	gcagagcaacatactgcctc	gacaatggagtatgcatggtac	121
4	D6S278	6q21	tgctggggttacaggtgtg	ggaggaatgcatccacgtac	130
5	BJ6Q3	6q21	ttgtagtagcagtttgaaactcc	actgagctctgcaatctagaagc	113
6	D6S1594	6q21	ggttcttgactgatccattgc	ccaaaggagcagctaaagtg	160
7	D6S1698	6q21	ggtaatttgactaccccgttc	cacccctcatatatacttgag	150
8	D6S474	6q21	cagggttttccaagagatagac	ctcagccatgctaccagcattcctgc	216
9	D6S261	6q22,1	ccaagagttcaagaccagcc	cctgagtagctggatttatag	152
10	D6S262	6q23,2	ctaagatagccgaatatgcaac	cagatagtggtctaaagagtc	120
11	D6S310	6q24,1	cccttccataatcacaggga	gtgcattagtcagtgttctc	151
12	D6S448	6q25,2	gaaaaacaaacatgcatttctag	ctctgccttcatggagtttac	108

bp basepairs

DNA, 800 ng of Spectrum-Red labeled reference DNA and 15 μg of human Cot-1 DNA (Life Technologies) dissolved in 12 μl of hybridization buffer (50% formamide, 2xSSC, 10% dextran sulfate, pH 7.0). Hybridization was carried out for 3 days at 37ºC to denatured (5 min at 75°C in 70% formamide/2xSSC, pH 7.0) normal male human metaphase spreads (Vysis, Downers Grove, IL). Slides were washed two times at 45ºC for 5 min in 50% formamide/2xSSC, pH 7.0, followed by 5 min in PBS at room temperature. The chromosomes were counterstained with 0.2μg 4,6-diamidino-2-phenylindole per μl Vectashield (Vector laboratories, Burlingame, CA) for identification. Digital images were collected from at least ten metaphases using the Metasystems Image Pro System black and white CCD camera (Altlussheim, Germany) mounted on top of a Leica DMRE fluorescence microscope, equipped with DAPI, Spectrum Green, and Spectrum Red filter sets. The software Metasystems ISIS 4.4.25 program was used to calculate average green-to-red ratio profiles for each chromosome. At least ten observations per autosome and five observations per sex chromosome were included in each analysis. Gains and losses of DNA sequences were defined as chromosomal regions where the mean green-to-red fluorescence ratio was above 1.20 and below 0.80, respectively. Overrepresentations were considered amplifications when the fluorescence ratio values in a subregion of a chromosomal arm exceeded 1.5.

Results

6q microsatellite analysis

We analyzed 31 PCC, including 22 benign and 9 malignant tumors, particularly focusing on the loci 6q21 and 6q23-24, because they have been reported as regions of frequent loss in LOH analysis[10]. Table 2 shows that in 17/31 (55%) cases no LOH of 6q was found. In 6/31 (19%) samples a single region of LOH was found. In 7/31 (23%) samples multiple regions of loss were detected

and in one tumor (3%) loss of the whole arm was identified. These losses were identified at similar frequencies in benign and malignant PCC. An example of the LOH analysis is shown in Figure 1.

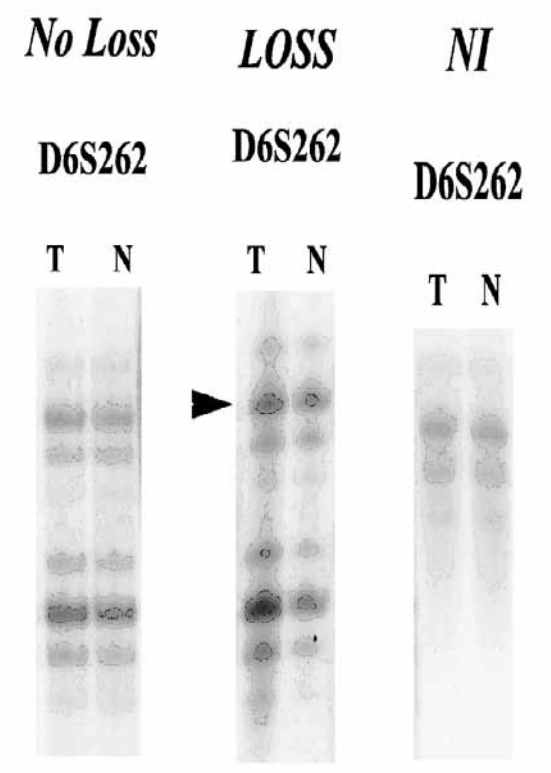

Figure 1. Example of LOH analysis

CGH analysis

Figure 2 summarizes all DNA copy number changes identified in the 68 PCC (for copy number changes in individual patient samples, see supplementary Tables 1 and 2). Genetic aberrations were observed in all tumors and the average number of chromosome arm aberrations per tumor was 6.9 (range 1-24). The high frequencies of 1p and 3q losses in the present set of PCC were in accordance with frequencies found in our previous work and the literature[4-5]. Losses of 6q, 11p, and 18p and gains of 5p, and 12q were exclusively found in sporadic PCC. Comparison of malignant and benign sporadic PCC showed that the former group harbored significantly more alterations, gains, and losses per tumor, i.e. 9.4 versus 6.1, 2.8 versus 1.6, and 6.5 versus 4.6, respectively (supplementary Tables 1 and 2). In addition, losses of 8p and 18p and gains of 5p, 7p, and 12q were significantly associated with malignant behavior (For p-values, see Table 3). In contrast with our previous findings, CGH analysis, as well as microsatellite analysis, of a larger number of PCC revealed that 6q loss is not suitable to predict metastatic disease (Table 2+ 3).

Comparison of benign sporadic and MEN 2-related PCC showed that the latter group harbored a significantly higher number of alterations, gains, and losses per tumor, i.e. 6.1 versus 9.2, 1.6 versus 3.3, and 4.6 versus 5.7, respectively (supplementary Tables 1 and 2). In addition, losses of 3q, 8p, 9p, and 13q as well as gains of 1q, 17q, and 20q were more often observed in the MEN 2-related PCC (Table 3).

Table 3. Comparison between sporadic and familial pheochromocytomas, and between benign and malignant PCC in a contingency table analysis.

	Sporadic	MEN2	NF1	SDHB	P1	Spor Ben2	Spor Mal2	P1
Locus	n=53	n=9	n=3	n=3		n=38	n=15	
Losses								
1p	83	100	100	100	NS	79	93	NS
3p	26	56	0	0	NS	26	27	NS
3q	53	100	100	33	0,051	50	60	NS
6q	26	0	0	0	NS	18	47	NS
8p	19	22	0	33		11	40	0,024
8q	8	44	0	33	0,035	5	13	NS
9p	6	22	0	0	0,026	3	13	NS
11p	21	0	0	33	NS	16	33	NS
13q	19	44	0	0	0,009	16	27	NS
17p	34	11	33	0	NS	32	40	NS
18p	9	0	0	33	NS	3	27	0,047
Gains								
1q	4	44	0	33	0,018	3	7	NS
5p	6	0	0	0	NS	3	13	0,047
7p	9	11	0	0	NS	5	20	0,041
9q	26	33	33	33	NS	24	33	NS
12q	11	0	0	0	NS	5	27	0,041
17q	19	56	33	0	NS	18	20	NS
20q	8	44	0	0	NS	11	0	NS

1Contingency table analysis; NS, not significant
2Spor, sporadic; Ben, benign; Mal, malignant

Discussion

We have performed a comprehensive analysis of chromosome 6q losses and genome-wide copy number changes in a series of 68 PCC with the goal to identify genetic parameters that might discriminate benign from malignant PCC. Despite previous indications our study could not provide evidence that 6q loss is a suitable and reproducible marker for malignancy. Nevertheless, new DNA copy number changes have been unmasked, which showed improved correlation with the biological behavior of PCC.

Table 2. Results of the 6q microsatellite analysis

		Markers	D6S280	BJ6Q2	D6S268	D6S278	BJ6Q3	D6S1594	D6S1698	D6S474	D6S261	D6S262	D6S310	D6S448
		Location	6q12	6q21	6q21	6q21	6q21	6q21	6q21	6q21	6q22,1	6q23,2	6q24,1	6q25,2
		LOH(%)	9%	15%	21%	21%	18%	17%	9%	10%	11%	7%	20%	11%
NRS	sample	m/b	6q loss CGH											
1	F91(5B)	b	-	○	NI	○	NI	NI	○	○	○	NI	NI	○	○
2	F160(N1)	b	-	NI	○	NI	○	○	NI	NI	○	○	○	○	○
3	F161(N2)	b	-	○	NI	○	○	NI	○	○	○	○	○	○	○
4	F179(N20)	b	-	U	U	○	U	U	○	NI	○	U	○	○	○
5	F184(N27)	b	-	○	○	○	○	○	○	NI	NI	-	○	○	○
6	F186(N29)	b	-	○	○	○	NI	○	○	○	○	NI	○	○	○
7	F189(N33)	b	-	○	○	○	NI	○	○	○	○	NI	○	U	U
8	F192(N36)	b	-	U	NI	○	U	U	○	○	○	○	○	U	U
9	F209(N56)	b	ai	NI	○	○	○	○	NI		U	U	○	○	NI
10	F210(N57)	b	ai	○	○	NI	NI	U	○	NI	○	○	○	NI	○
11	F159(32M)	m	+	U	U	○	U	U	NI	NI	U	U	○	U	NI
12	F164(N5)	m	-	U	NI	○	U	NI	NI	○	NI	-	○	○	○
13	F175(N16)	m	-	U	U	○	U	U	NI	○	NI	-	○	○	○
14	F170(N11)	m	-	U	U	○	U	U	NI	○	○	○	○	NI	○
15	F202(N49)	m	-	U	U	NI	U	U	○	○	○	○	○	○	○
16	F207(N54)	m	-	NI	○	○	○	○	U	○	○	○	○	U	○
17	F169(N10)	m	+	NI	○	NI	○	○	○	○	U	NI	○	○	○
18	F7(19M)	m	-	U	●	U	●	●	●	○	U	○	○	○	○
19	F198(N43)	b	-	○	○	●	NI	NI	○	○	○	○	○	○	○
20	F188(N32)	b	-	○	○	○	○	NI	○	NI	●	○	○	○	○
21	F194II(N48)	b	-	NI	○	○	○	○	○	○	●	○	NI	○	○
22	F172(N13)	b	-	○	○	○	NI	○	○	○	○	○	○	●	○
23	F193(N37)	b	*	○	○	○	○	○	○	○	NI	U	○	●	○
24	F5(4M)	m	+	U	U	NI	U	U	●	NI	○	U	○	●	○
25	F88(35B)	b	-	○	○	●	NI	○	○	○	○	NI	○	○	●
26	F86(40B)	b	+	NI	●	●	NI	●	○	○	○	NI	○	○	●
27	F97(50B)	b	-	○	●	○	NI	●	○	○	NI	○	NI	○	○
28	F165(N6)	b	*	●	○	●	○	○	○	●	○	●	NI	●	○
29	F203(N50)	b	-	U	U	●	U	U	○	○	○	●	●	○	○
30	F211(N58)	b	ai	○	○	NI	●	○	●	○	○	○	○	○	○
31	F195(N39)	b	+	●	○	U	NI	●	U	●	U	U	U	NI	●

b benign, m malignant, - no 6q alterations in CGH, + 6q loss in CGH, ai allelic imbalance, * no CGH data known, ○ retention of heterozygosity in LOH analysis, ● loss of heterozygosity in LOH analysis, U unknown, NI not informative.

Loss of chromosome 6q has been detected in many human neoplasms, including malignant melanoma, carcinomas of the salivary gland, ovary, prostate, liver, pancreas, and neuroendocrine tumors, such as endocrine pancreatic tumors, adrenocortical carcinomas, and parathyroid adenomas[8-9]. In a previous CGH study we identified 6q loss in a relatively small series of 29 PCC, and found this alteration to be associated with malignant progression[4]. Two subsequent studies, however, presented contrasting results with respect to involvement of 6q deletions. On the one hand, a CGH study on 21 benign and 20 borderline and malignant PCC revealed that 6q losses (8/41 (19%)) were predominantly associated with non-metastasized tumors[12]. However, on the other hand an LOH study identified a high frequency of overall 6q loss in 13/18 (72%) borderline and malignant PCC[10]. In the latter study, no benign PCC were tested. In addition, classification of PCC as borderline tumors is currently neither supported by the WHO, nor universally accepted on basis of available criteria. Our present investigations using both microsatellite and CGH analysis on a large series of PCC, now provide evidence that 6q deletions are frequently present (45% and 36%, respectively), but do not correlate with malignancy. It is conceivable that loss of 6q could play a role as a late event in PCC progression in general, but more evidence remains required.

Our LOH analysis of 12 highly polymorphic microsatellite loci within the 6q12-25 region revealed LOH in 14/31 (45%) of the PCC, but did not identify common regions of LOH. Moreover, the highest frequency of allelic loss (21%) was detected for two loci at 6q21. This is in contrast with the data of Lemeta et al.[10], who proposed two regions of frequent loss, including 6q14 and 6q23-24. Furthermore, one of their markers showing the highest frequency of loss (33%), D6S268 at 6q21, only showed 21% allelic loss in our study. Remarkably, our LOH data indicate that there are tumors with only single loci of loss and tumors with involvement of multiple loci, which are separated by loci showing retention of heterozygosity. This is also detectable in the data of Lemeta et al.[10]. This rather strange phenomenon has been described before in prostate, bladder, and breast carcinomas[13-14] and might be due to potential multiploidy of chromosome 6, which is difficult to detect in LOH analysis. Other explanations for this uncommon pattern could be caused by genetic instability of this chromosome or it could be a random genetic event, which is not involved in tumorigenesis or clinical behavior of PCC.

The main aim of CGH analysis was a) to validate the results of our previously described LOH study and b) to identify potential correlations of chromosomal alterations with the clinical behavior and syndrome-relatedness of PCC. In the present study, CGH identified 6q deletions in 25/68 (37%) of the PCC and no correlation was found with metastatic disease, in agreement with our microsatellite data. In eight cases, we observed a discrepancy between the CGH and the LOH analysis, identifying loss by LOH but no alterations or allelic imbalances by CGH. This may be due to the fact that not all deletions are picked up in CGH analysis due to its limitations in resolution[7].

Two studies have been performed on the basis of chromosomal alterations. Firstly, our previously performed CGH study identified statistically significant differences in loss of 6q between malignant and benign PCC[4]. The apparent discrepancy between these previous findings and

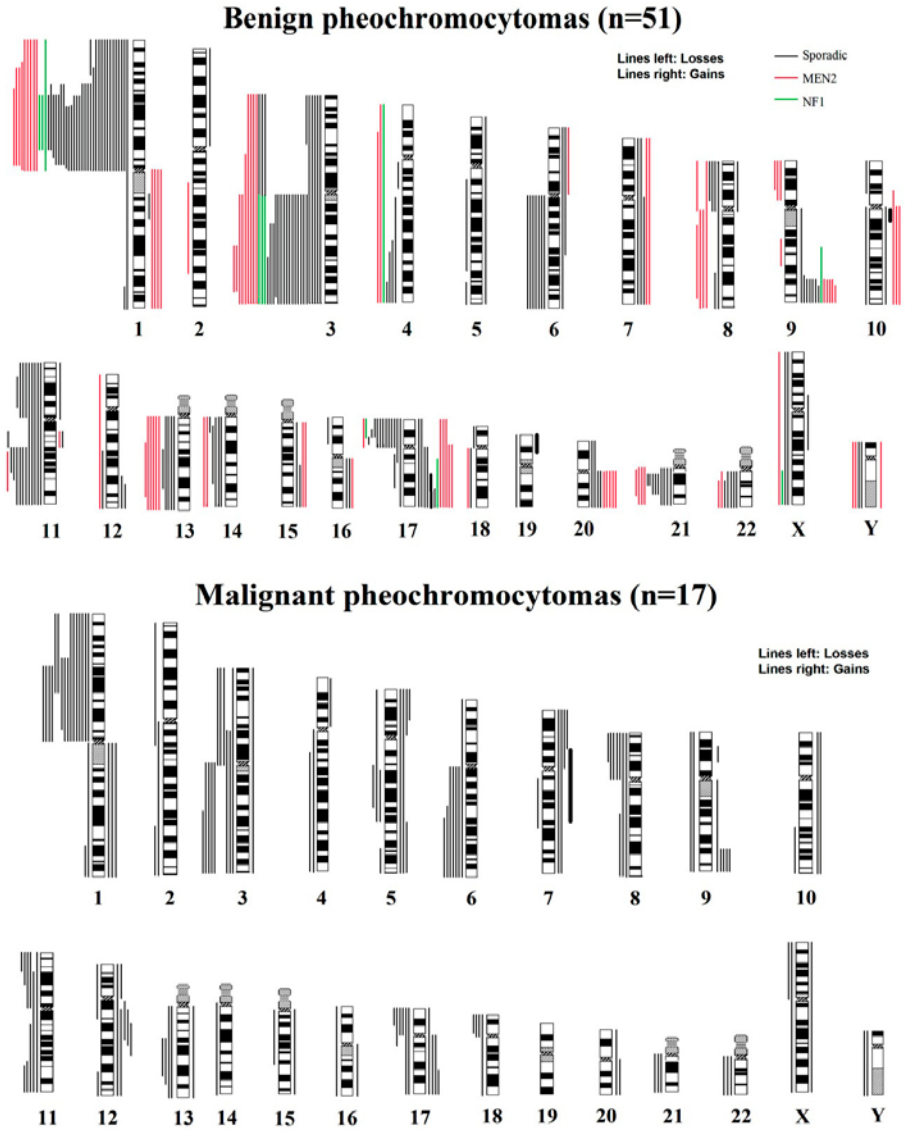

Figure 2. Overview of copy number changes in the 68 studied PCC.

those of the current study may be related to the fact that the current series of PCC cases is much larger and that in our previous study statistical correlation was only marginal (p=0.0368). Secondly, a study performed by August et al.[12] identified a statistically significant difference in copy number changes in malignant versus benign PCC (8.3 versus 4.3) as well as gain of 17q, which occurred significantly more frequent in malignant PCC (p=0.025). Our current data are in perfect accordance with this earlier study, except for the findings on 17q.

Losses of 8p, and 18p, and gains of 5p, 7p, and 12q are statistically associated with metastatic disease. Of these, August et al.[12] already mentioned gain of 12q, but further investigations on this chromosome arm to support a definite correlation remains to be done. The other alterations have not been described before, but indicate a possible relationship with malignancy.

In summary, we have performed a microsatellite and a CGH analysis in a large series of PCC, which reveals that there is no correlation between 6q loss and malignant behavior. In addition, we found several new chromosome arm alterations, including loss of 8p and 18p, and gains of 5p and 7p, which can have a possible association with malignant PCC. We confirmed former conclusions of 12q gain in association with malignancy and that there are much more copy number changes in malignant PCC and MEN 2-related, than in benign PCC.

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Supplementary table 1. Results of the comparative genomic hybridization analysis of the benign samples

pt	DNA	m/b	sp/m2/n/ sdhb	size	# ab	loss	gain	amp	chromosomal aberrations
1	F137	b	sp	4.7	5	4	1	0	1p-, 3p-, 9q+, 11p-, 11q-
2	F141	b	m2	13	7	5	2	0	1p-, 3p-, 3q-, 10q+, 13q-, 17p-, 17q+
3	F142	b	sp	6	5	4	1	0	3p-, 3q-, 9q+, 11p-, 11q-
4	f138	b	sp	5	6	4	2	0	1p-, 11q-, 14q-, 17p+, 17q+,21q-
5	F143	b	sp	5	10	6	4	0	4p-, 4q+, 7p+, 7q+, 9p-, 9q+, 13q-, 15q+, Xp-, Xq-
6	F144	b	sp	9.5	3	2	1	0	1p-, 3q-, 9q+
7	F145	b	sp	6	5	4	1	0	1p-, 3q-, 6q-, 9q+, 10q-
8	F146	b	sp	10	4	4	0	0	1p-, 11q-, 14q-, 21q-
9	F147	b	m2	4	6	4	2	0	1p-, 3p-, 3q-9q+, 14q-, 17q+
10	F149	b	sp	8	15	12	3	0	1p-, 3p-, 3q-, 6q-, 9q+, 10p-, 11p-, 11q-, 13q-, 14q-, 16q+, 17q+, 21q-, Xp-, Xq-
11	F151	b	sp	6	3	3	0	0	1p-, 3q-, 17p-
12	F91	b	sp	4.5	3	3	0	0	1p-, 3q-, Y-
13	F94	b	sp	2.5	8	6	2	0	1p-, 1q+, 4q-, 6q-, 17p-, 17q+, Xp-, Xq-
14	F98	b	sp	3.2	6	5	1	0	1p-, 3p-, 3q-, 10q+, 13q-, 17p-
15	F88	b	sp	5	5	4	1	0	1p-, 3p-, 3q-, 7q+, 11q-
16	F86	b	sp	5	4	3	1	0	1p-, 3q-, 6q-, 11p+
17	F97	b	sp	15	1	1	0	0	8p-
18	F83	b	sp	8	4	4	0	0	1p-, 3q-, 17p-, 17q-
19	F160	b	sdhb	5	2	2	0	0	1p-, 1q-
20	F161	b	sp	2.5	7	1	6	0	7p+, 7q+, 8p+, 15q+, 20p+, 20q+, Y-
21	F162	b	sp	5.5	3	3	0	0	1p-, 3q-, 21q-
22	F166	b	n	12	6	6	0	0	1p-, 3q-, 4p-, 4q-, 17p-, Xq-
23	F167	b	sp	17	4	4	0	0	3p-, 3q-, 11p-, 11q-
24	F168	b	m2	5	10	6	4	0	1p-, 3q-, 8q-, 9q+, 13q-, 17p+, 17q+, 20q+, 21q-, Y-
25	F172	b	n	4.5	4	2	2	0	1p-, 3q-, 9q+, 17q+
26	F173	b	sp	4.5	3	3	0	0	1p-, 3q-, 6q-
27	F178	b	sp	7	5	5	0	0	1p-, 4q-, 6q-, 17p-, 18p-
28	F179	b	m2	4.5	5	4	0	1	1p-, 3q-, 10q+, 12q-, 22q-
29	F181	b	m2	2.5	15	8	7	0	1p-, 1q+, 2q-, 3p-, 3q-, 8p-, 8q-, 9q+, 11q+, 15q+, 17p+, 17q+, 20q+, 21q-,Y-
30	F184	b	m2	5.5	11	7	4	0	1p-, 1q+, 3p-, 3q-, 8q-, 9p-, 11q-, 17p+, 17q+, 20q+, 21q-
31	F185	b	sp	7.5	3	3	0	0	1p-, 3q-, 4q-
32	F186	b	sp	5	11	5	6	0	5p+, 5q+, 7p+, 7q+, 11p-, 11q-, 14q-, 17p-, 20p+, 20q+, 21q-
33	F195	b	sp	13	13	6	7	0	1p-, 3p-, 6q-, 8p-, 8q-, 9q+, 11q+, 12q+, 13q-, 17q+, 20q+, Xp+, Xq+
34	F189	b	sp	8	4	4	0	0	1p-, 3q-, 17p-, 17q-
35	F190	b	sp	3.5	6	6	0	0	1p-, 3p-, 5q-, 11p-, 14q-, 21q-
36	F192	b	sp	2.5	5	4	0	1	1p-, 8p-, 19p+, 21q-, 22q-
37	F194I	b	m2	3	9	3	6	0	1p-, 1q+, 3q-, 6p+, 7p+, 7q+, 10p+, 10q+, 13q-
38	F194II	b	m2	1.1	6	5	1	0	1p-, 3q-, 8p-, 10q+, 21q-, 22q-
39	F191	b	sp	-	3	3	0	0	1p-, 3q-, 17q-

pt	DNA	m/b	sp/m2/n/ sdhb	size	# ab	loss	gain	amp	chromosomal aberrations
40	F197	b	sp	5	4	4	0	0	1p-, 4q-, 5q-, 8q-
41	F198	b	sp	12.5	3	3	0	0	1p-, 17p-, 22q-
42	F199	b	sp	4	6	4	2	0	3p-, 6p+, 6q+, 11p-, 13q-, 17p-
43	F200	b	sp	6.5	6	3	3	0	1p-, 4q-, 6p+, 6q+, 17p-, 17q+
44	F204	b	sp	5.5	13	6	7	0	1p-, 3q-, 9q+, 11q-, 12q+, 13q-, 15q+, 16q+, 17p+, 17q+, 18q-, 20q+, 21q-
45	F203	b	sp	5.4	3	3	0	0	1p-, 3q-, 17p-
46	F205	b	sp	4.6	8	8	0	0	1p-, 1q-, 3q-, 8p-, 11q-, 17p-, 21q-, 22q-
47	F209	b	sp	4	7	7	0	0	1p-, 11p-, 11q-, 16p-, 19p-, 19q-, 22q-
48	F206	b	sp	7	3	2	1	0	2p+, 11p-, 11q-
49	F208	b	sp	8.5	8	7	1	0	1p-, 3q-, 4p-, 4q-, 9q+, 12p-, 12q-, 13q-
50	F210	b	m2	2	14	10	4	0	1p-, 1q+, 3p-, 3q-, 8q-, 9p-, 9q-, 13q-, 14q-, 16q+, 18q-, 20q+, 22q-, Y+
51	F211	b	n	5.6	2	2	0	0	1p-, 3q-

m malignant, b benign, sp sporadic, m2 multiple endocrine neoplasia type 2a, n neurofibromatosis type 1, sdhb succinaatdehydrogenase subtype b, # ab number aberrations, amp amplifacation

Supplementary table 2. Results of the comparative genomic hybridization analysis of the malignant samples.

pt	DNA	m/b	sp/m2/n/ sdhb	size	# ab	loss	gain	amp	chromosomal aberrations
1	F156	m	sp	-	6	4	2	0	1p-, 3q-, 6q-, 9q+, 14q-, 17q+
2	F298	m	sp	15	2	1	1	0	1p-, 9q+
3	F288	m	sp	6	5	5	0	0	1p-, 3q-, 6q-, 17p-, 17q-
4	F140	m	sdhb	-	3	2	1	0	1p-, 9q+, 11p-
5	F5	m	sp	12	8	8	0	0	1p-, 5q-, 6q-, 8p-, 11q-, 17p-, 17q-, 22q-
6	F8	m	sp	11	17	10	7	0	1p-, 1q+, 4q-, 5p+, 5q+, 7p+, 8p-, 8q-, 9p-, 9q-, 12q+, 17p-, 17q+, 18p-, 18q-, 20q+, 21q-
7	`F155	m	sp	8.5	24	11	13	0	1p-, 1q-, 3q+, 5p+, 6q-, 7p+, 7q+, 8p-, 9p+, 9q+, 10p+, 10q+, 11p-, 11q-, 12q+, 13q+, 15q+, 16p-, 17p-, 17q-, 18p-, Xp+, Xq+
8	`F7	m	sp	8	5	3	2	0	1p-, 1q+, 3q-, 13q-, 17q+
9	F346	m	sp	12	24	13	11	0	2p-, 2q-, 3p-, 3q-, 4q-, 5p+, 5q+, 6p-, 6q-, 7p+, 7q+, 8p-, 9p-, 9q-, 10p+, 10q+, 11p-, 11q-, 12p+, 12q+, 13q-, 16q+, 20p+, 20q+
10	F159	m	sp	7.5	7	7	0	0	1p-, 6q-, 8p-, 10q-, 11q-, 13q-, Y-
11	F139	m	sp	18	7	4	3	0	1p-, 3p-, 9q+, 13q-, 17p+, 17q+, Xp-
12	F175	m	sp	-	5	3	2	0	1p-, 3p-, 5q-, 12p+, 12q+
13	F169	m	sp	7	7	5	2	0	1p-, 3p-, 3q-, 6q-, 7p+, 7q+, 11p-
14	F171	m	sp	-	3	3	0	0	1p-, 3p-, 11p-
15	F174	m	sp	-	7	4	3	0	1p-, 3q-, 5p+, 7p+, 12q+, 17p-, 18p-
16	F202	m	sp	-	18	18	0	0	1p-, 1q-, 3p-, 3q-, 5p-, 5q-, 7q-, 8p-, 8q-, 11p-, 11q-, 12p-, 12q-, 15q-, 17p-, 21q-, 22q-, Y-
17	F207	m	sdhb	-	11	10	1	0	1p-, 1q+, 2q-, 3q-, 8p-, 8q-, 18p-, 21q-, 22q-, Xp-, Xq-

m malignant, b benign, sp sporadic, m2 multiple endocrine neoplasia type 2a, n neurofibromatosis type 1, sdhb succinaatdehydrogenase subtype b, # ab number aberrations, amp amplifacation

Chapter 3

Frequent loss of 17p, but no *p53* mutations or protein overexpression in benign and malignant pheochromocytomas

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Abstract

Genetic changes in the tumorigenesis of sporadic pheochromocytomas are poorly understood, and there are no good markers to discriminate benign from malignant pheochromocytomas. p53 is a tumor suppressor gene and aberrations in this gene are frequently found in many tumor types. The role of *p53* in pheochromocytomas tumorigenesis is unclear, with some studies suggesting that p53 mutations can be used to discriminate benign from malignant pheochromocytomas while other studies do not find such an association. Because most of these investigations were hampered by small series of tumors and the use of varying methods, we have performed a comprehensive analysis of p53 aberrations in a large series of pheochromocytomas. Comparative genomic hybridization analysis of 31 benign and 20 malignant tumors showed loss of the p53 locus at chromosome 17p13.1 in 23/51 (45%) cases, and most of these results were confirmed by fluorescence in situ hybridization. Forty-three tumors, including the malignant tumors and the tumors with loss of the p53 locus, were analyzed for p53 mutations in exons 5–8, but none were found. Furthermore, p53 immunohistochemistry on 35 cases revealed strong nuclear p53 expression in only two pheochromocytoma metastases, all other tumors being negative. We conclude that, although there is frequent loss of the p53 locus on 17p, the p53 gene does not appear to play a major role in pheochromocytoma tumorigenesis.

Introduction

Pheochromocytomas (PCC) are neuroendocrine tumors derived from chromaffin cells of the adrenal medulla. Extra-adrenal tumors of chromaffin tissue have recently been renamed sympathetic paragangliomas. PCC are usually benign (90%), but there are no markers that can distinguish the remaining 10% malignant PCC. Histologically confirmed metastases in places where chromaffin tissue does not normally occur, such as in bone, lung, liver, or lymph nodes, are the only accepted sign of malignancy[1].

Whereas most PCC occur sporadically, up to 24% of these tumors appear in familial cancer syndromes, such as multiple endocrine neoplasia 2, Von Hippel–Lindau disease, neurofibromatosis type 1, and pheochromocytomas-paraganglioma syndrome[2]. In some sporadic PCC, genes associated with the inherited tumor syndromes underlie tumorigenesis, but in the majority of sporadic PCC molecular tumorigenesis is poorly understood. Loss of heterozygosity (LOH) and comparative genomic hybridization (CGH) studies performed by us and others have reported allelic losses at 1p, 3pq, 17p, and 22q, but corresponding genes have not been identified[3-6]. Particularly 17p, the chromosome arm where the *p53 gene* is located, is interesting to investigate knowing that aberrations in this gene are implicated in many inherited and sporadic forms of malignancy, such as colon, lung, brain, and breast tumors[7].

The *p53 gene* is functionally involved in guarding the stability of the genome. In case of DNA damage, it will down-regulate the cell cycle and inhibit cell division[8]. Mutations in and inactivation of one or both p53 alleles can promote tumorigenesis, whereas restoration of *p53* function results in tumor regression in in vivo animal models[9]. Over the past decade, several studies have addressed the possible role of *p53* in PCC tumorigenesis. However, in many reports, only few PCC were investigated[10-12]. Five studies, including one from our own group, exclusively described the results of immunohistochemical staining[13-17]. Other studies, examining *p53* mutations with or without concomitant protein expression, included only benign or very few malignant PCC, and generally did not show *p53 gene* mutations[18-20]. In contrast, Yoshimoto et al. described a relatively high frequency of single strand conformation polymorphism (SSCP) analysis abnormalities and p53 gene mutations in multiple and malignant PCC from an Asian population[21].

To examine the involvement of *p53* in PCC development and malignant progression, we set out for CGH analysis of a uniquely large series of well-characterized benign and malignant PCC. We identified chromosome alterations at chromosome 17 by using conventional CGH analysis and confirmed these data by using double-target fluorescence in situ hybridization (FISH) for the *p53 gene* and centromere loci. Subsequently, we used polymerase chain reaction (PCR) and SSCP analysis to detect *p53 gene* mutations in exons 5–8 and immunohistochemistry to visualize nuclear p53 overexpression.

Materials and methods

Tissue Samples

Tissue samples of PCC (n=63 from 48 patients) were collected from the pathology archive of the Departments of Pathology, Erasmus MC, Rotterdam (n=16), Radboud University Nijmegen (n=43), and University Hospital Zurich (n=4). Clinical and tumor data are listed in Table 1. All tumors were collected between 1978 and 2003. All malignant cases had histologically confirmed metastases, but only 12 of the latter are included in this study, because not all were available. There is no overlap between the tissue samples in this study and any of our previous studies, except for six samples that had been used for prior p53 immunostaining[4, 13].

Table 1. Clinical and tumor data of 63 PCC from 48 patients.

Sex (M/F)	19/29	
Average age (years)	45.7	(Range 23-72)
Malignant/benign	18/30	
Metastases	12	
Sporadic cases	39	
Familial cases	8	
*MEN2a	6	
*NF1	2	
Adrenal cases	41	
Extra-adrenal cases	7	(Mal/ben 4/3)
Unilateral cases	43	
Bilateral cases	5	(All MEN2a)

*Indicates that of the 8 familial cases, there are 6 MEN2 cases and 2 NF1 cases.

DNA Extraction

DNA extraction was performed as described before[4, 22]. Genomic DNA from 50 frozen samples was isolated by homogenizing approximately 5mm^3 of each tissue sample prior to proteinase K treatment and DNA purification using the QIAamp DNA mini kit (Qiagen, Hilden, Germany). Genomic DNA from 13 paraffin-embedded tumors was isolated from 5–10mm thick tissue sections after deparaffinization. DNA quality was checked with agarose gel electrophoresis and quantified by spectrophotometry.

CGH

CGH was used to analyze genome-wide DNA copy number imbalances in 51 PCC samples as described previously[23]. This approach uses differentially labeled tumor and 'reference' DNA, which are competitively hybridized to normal metaphase chromosomes. The ratio of the fluorescence intensities detected is indicative of the relative DNA copy number in tumor versus reference DNA.

FISH

Touch preparations of n=29 frozen PCC and 4-mm-thick tissue sections of n=4 routinely fixed, paraffin-embedded tumors were subjected to FISH (n=32) as described previously[22-23]. A combination of centromere 17 (p17H8) and a PAC probe containing the *p53 gene* on chromosome 17p13.1, labeled with digoxigenin and biotin, respectively, was used for hybridization. Digoxigenin was detected by sheep anti-digoxigenin fluorescein Roche) and biotin by two layers of avidin-rhodamine connected by a biotinylated goat anti-avidin antibody (Vector). Probe visualization and nuclear counterstaining were carried out as described for CGH, and slide evaluation and signal scoring to detect DNA copy number gain and losses were performed as described previously[22, 24].

PCR–SSCP

Exons 5–8 of the *p53 gene*, including the exon– intron boundaries, were investigated by PCR–SSCP in n=43 PCC samples. As controls, DNA samples from normal individuals were used. In addition, DNA from the prostate carcinoma cell lines PC-3 and Du-145, and from 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, shown by van Nederveen et al.[25]. The DNA isolated from routine formalin-fixed, paraffin-embedded tissues is highly degraded; therefore, we used small amplicons (<200 bp) with overlapping primers for each exon to maximize chances of detecting mutations whenever they would be present. The primers are listed in Table 2. PCR was performed with 1–3 µl of isolated DNA in a final reaction volume of 15 µl containing, in mmol/l, MgCl2 1.5, dATP 0.02, dGTP 0.2, dTTP 0.2, and dCTP 0.2, [α-32P]dATP 0.8 µCi (Amersham, Buckinghamshire, UK), 20 pmol of each primer, and 0.2U of Taq polymerase (Promega, Madison, WI, USA). PCR was performed for 35 cycles (denaturing at 95°C for 30 sec, annealing at 55°C for 45 sec, and extension at 72°C for 1 min) in a Biometra thermocycler (Biometra, Göttingen, Germany). A final extension was carried out at 72°C for 10 min. PCR products were diluted with loading buffer (95% formamide, 10mmol/l EDTA (pH 8.0), 0.025% bromophenol blue, and 0.025% xylene cyanol), and denatured at 95°C for 10 min. The solution was chilled on ice and 4 µl was loaded on an 8% polyacrylamide gel (acrylamide to bisacrylamide, 49:1) containing 10% glycerol. Electrophoresis was performed at 8W for 16 h at room temperature. Gels were vacuum dried at 80˚C and exposed to X-ray films.

Immunohistochemistry

p53 immunohistochemistry was performed using a monoclonal mouse antibody directed against normal and mutated *p53* (Do-7, Dako, Glostrup, Denmark). Tissue samples of 36 PCC were formalin-fixed and embedded in paraffin. Sections of 4 µm thickness were cut and

mounted on Superfrost Plus slides. The sections were deparaffinized, dehydrated, exposed to microwave heating (in Tris/ EDTA pH 9.0) at 100°C for 15 min, rinsed with tap water followed by incubation in H2O2 30% /PBS 1:10 for 15 min. The *p53* antibody was diluted 1:100 with normal antibody diluents (Klinipath, Duiven, The Netherlands), and slides were incubated with 150 µl per slide for 30 min, followed by rinsing with Tris/ Tween 0.5%, pH 8.0. Dako ChemMate Envision HRP was applied for 30 min (150 µl, Dako envision kit), followed by rinsing with Tris/Tween 0.5%, pH 8.0. Diaminobenzidine tetrahydrochloride (150 µl, Dako envision kit) was applied twice, without rinsing, and rinsed with tap water. Slides were counterstained with Harris haematoxylin for 1 min and rinsed with tap water. Slides were rehydrated and covered. In the negative control reactions, the primary antibodies were omitted from the dilution buffer. A p53-positive breast carcinoma was used as positive control. Scoring of p53 staining was based on a method described by Sinicrope et al.[26]. Two independent observers scored the tumors based on intensity and the percentage of nuclei showing immunostaining. Intensity is scored in three groups: weak=1, moderate=2, and strong=3. Percentages are divided in five groups: <5%=0, 5–25%=1, 25–50%=2, 50–75%=3, and >75%=4. A multiplied score of ≥6 was considered positive staining and a score <6 was considered negative.

Table 2. P53 primer sets for SSCP.

	Forward 5′→ 3′	Reverse 5′→ 3′	Size
Exon 5-I	Cctgactttcaactcttgctc	atgcttgtagatggccatg	158bp
Exon 5-II	Cagctgtgggttgattccac	ctggggaccctgggcaac	176bp
Exon 6-I	Agcctctgattcctcactg	gaccaccacactatgtcga	127bp
Exon 6-II	Ccctcagcatcttatccga	ccactgacaaccaccctt	159bp
Exon 7-I	Aggcgcactggcctcatctt	tccagtgtgatgatggtgagg	141bp
Exon 7-II	Catgtgtaacagttcctgcatg	gcggcaagcagaggctgg	135bp
Exon 8-I	Ccttactgcctcttgcttctc	cttgcggagattctcttcctc	130bp
Exon 8-II	Tggtgcctgtcctgggagag	ctccaccgcttcttgtcct	127bp

Results

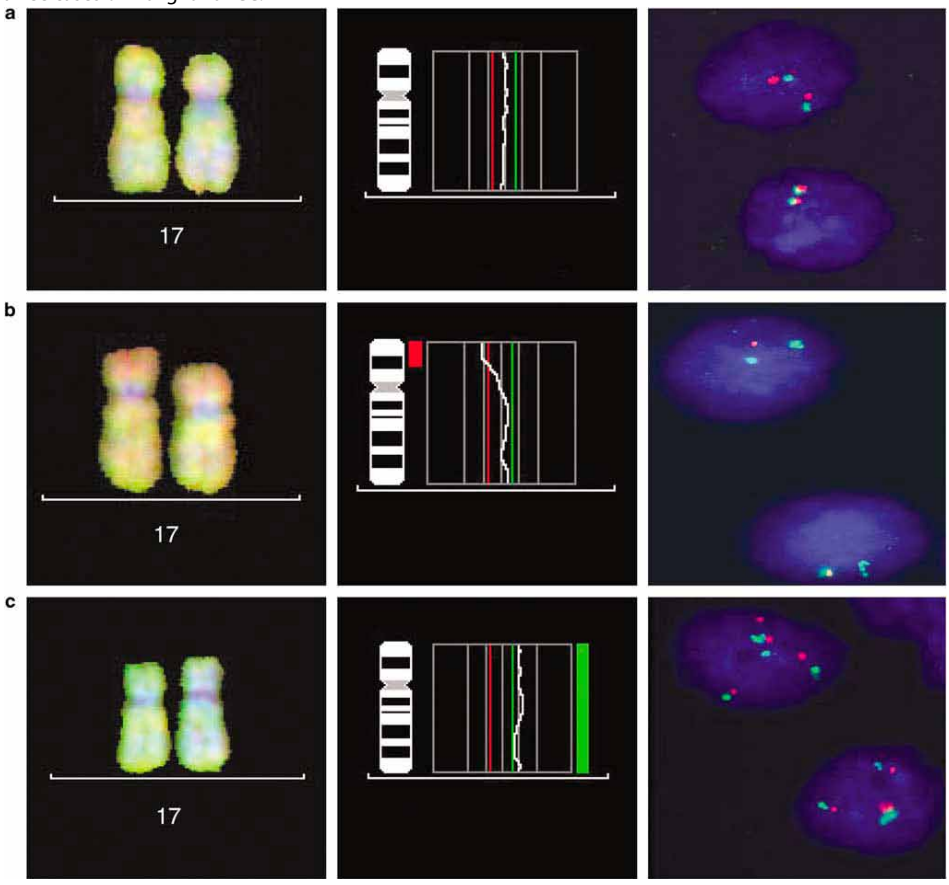
CGH Findings

Table 3 shows the losses and gains on chromosome 17 of the 51 tumor (31 benign and 20 malignant) samples that were examined by CGH. We found CGH abnormalities on chromosome 17 in 29/51 (57%) tumors, including 23/51 (45%) at the *p53 gene* locus at 17p13.1. In 18 of these 23 cases (78%) there was loss, and in 5 of 23 (22%) we found gain of chromosomal material.

Confirmation of CGH Data by FISH

FISH analysis on 29 touch preparations and 4 paraffin-embedded tissue sections confirmed the CGH results at the p53 locus. We found abnormalities in the FISH analysis in 16/33 (48%) cases, including 14/33 (42%) cases with loss of the p53 locus and 2/33 (6%) with gain of the p53 locus. There was almost complete concordance between the FISH and CGH data for those samples that were analyzed with both techniques, except for the benign cases 13, 14, 15, and 17 in which there was loss in the FISH analysis but not in the CGH and benign cases 16, 18, 19, and 21 in which we observed gain in the CGH but not in the FISH analysis. The results are shown in Tables 3 and 4 and a representative result of CGH and FISH analysis is shown in Figure 1.

Figure 1. Representative results of chromosome 17 CGH analysis confirmed by FISH of the p53 locus in three cases of malignant PCC.



(a) Malignant PCC (case 15), not showing loss or gain in CGH and FISH analysis. (b) Malignant PCC (case 8) with loss of 17p in the CGH analysis, including the p53 gene locus, also showing loss in FISH. (c) Malignant PCC (case 13) showing gain of chromosome 17 in the CGH analysis and multiple p53 gene loci by FISH.

PCR–SSCP analysis

PCR–SSCP analysis was performed on 43 samples to examine sequence abnormalities in exons 5–8 of the *p53 gene*. No band shifts were found at SSCP, indicating that there were no mutations in n=17 benign and n=26 malignant pheochromocytomas, as our positive control yielded the expected results. The results are shown in Tables 3 and 4.

Immunohistochemistry

Immunohistochemical staining was performed on 36 samples to analyze whether there was overexpression of the p53 protein. Two of 36 samples (6%) showed overexpression (score≥6), whereas all other samples, including the corresponding primary tumor of one of these two metastases, were negative (score≤5) (Figure 2). One of these two positive samples was available for SSCP analysis, but did not show a band shift, nor did we find loss in the CGH analysis of the other primary tumor. The results are summarized in Tables 3 and 4.

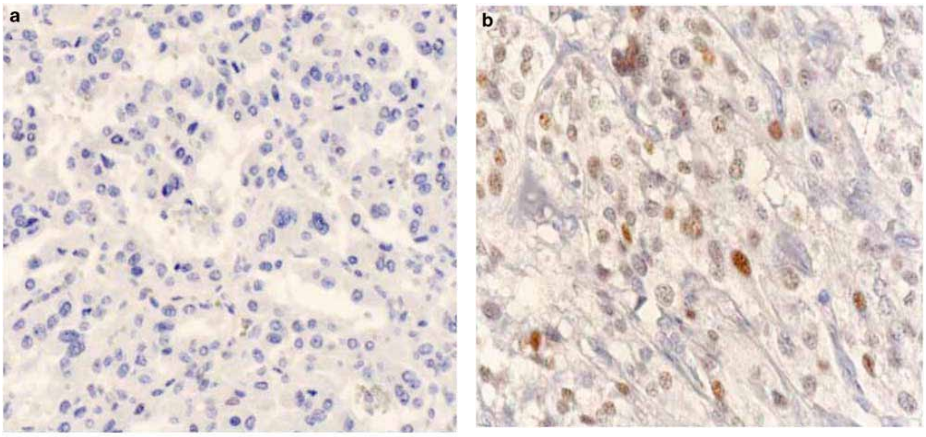


Figure 2. Representative results of p53 immunohistochemistry in malignant PCC. (a) Primary tumor of a malignant PCC (case 6) not showing nuclear overexpression of p53 protein (score 0). (b) Malignant PCC (case 7) showing the corresponding metastasis of the primary tumor shown in (a) with positive nuclear immunostaining for p53 (score 6).

Discussion

In this study, we have performed a comprehensive analysis of a large series of benign and malignant PCC to detect abnormalities in *p53* at the DNA and protein level using a variety of techniques. Although we found a loss rate of 45% in CGH analysis at the p53 locus, which was confirmed by FISH analysis, we could not detect gene mutations in the *p53* exons 5–8, nor did

Table 3. Results of CGH analysis, FISH, SSCP analysis and immunohistochemistry in benign PCC

samples	patients	characteristics	CGH 17p	FISH p53	SSCP	IH
1	1	F/62 sp/adr/u	●	●	○	○
2	2	M/30 sp/adr/u	●	●	○	-
3	3	F/41 sp/adr/u	●	●	○	-
4	4	F/24 n/adr/u	●	●	○	-
5	5	M/66 sp/adr/u	●	-	○	-
6	6	M/49 m/adr/bi	●	-	○	○
7	7	M/42 sp/adr/u	●	-	○	○
8	8	M/47 sp/ea/u	●	-	○	-
9	9	F/69 sp/adr/u	●	-	○	-
10	10	F/37 sp/adr/u	●	-	○	○
11	11	M/41 sp/adr/u	●	-	-	-
12	12	F/30 sp/adr/u	●	-	○	○
13	13	F/56 sp/ea/u	○	●	-	-
14	14	M/41 sp/adr/u	○	●	○	-
15	15	F/33 sp/adr/u	○	●	○	○
16	16	M/72 m/adr/bi l	○○	○	-	○
17		ll	○	●	○	○
18	17	M/40 m/adr/u	○○	○	○	-
19	18	M/53 m/adr/bi l	○○	○	○	-
20		ll	○	-	-	○
21	19	F/33 sp/adr/u	○○	○	-	○
22	20	F/44 sp/adr/u	○	○	-	○
23	21	M/24 m/adr/u	○	○	-	○
24	22	M/58 sp/adr/u	○	○	-	○
25	23	F/53 n/adr/u	○	○	-	○
26	24	M/53 sp/adr/u	○	-	○	-
27	25	F/59 sp/adr/u	○	○	-	○
28	26	F/49 sp/adr/u	○	○	-	○
29	27	F/48 sp/adr/u	○	○	-	-
30	28	F/24 m/adr/bi l	○	○	-	-
31		/27 ll	○	-	-	○
32	29	F/59 m/adr/bi	-	-	-	○
33	30	M/43 sp/adr/u	-	-	-	○

In CGH analysis, ○ represents 'no gain or loss,' ● is 'loss', and ○○ means 'gain' of chromosome 17. In FISH analysis, ○ is 'retention of heterozygosity,' ● means 'loss of heterozygosity,' and ○○ represents 'gain' of the p53 locus on chromosome 17. In SSCP, ○ means no mutation. In immunohistochemistry, ○ represents 'no overexpression' and ● means p53 protein overexpression. In all four techniques, — means that these samples are not examined. M =male; F=female; sp=sporadic; m=men; n=neurofibromatosis; adr=adrenal; ea= extra-adrenal; u=unilateral; bi=bilateral; LN=lymph node.

we find a high frequency of p53 protein overexpression by immunohistochemistry, with only two metastases showing nuclear immunostaining. This leads us to suggest that p53 abnormalities apparently do not appear to play a major role in the tumorigenesis of benign and malignant PCC, except for a possible minor role in metastatic progression. *p53* abnormalities thus cannot be used for the distinction between benign and malignant PCC.

Table 4. Results of CGH analysis, FISH, SSCP analysis and immunohistochemistry in malignant PCC.

samples	patients	characteristics	CGH 17p	FISH p53	SSCP	IH
1	1	F/47 sp/adr/u	●	●	○	○
2	2	F/65 sp/adr/u	●	●	○	○
3	3	F/70 sp/adr/u	●	●	○	○
4	4	F/56 sp/adr/u	●	-	○	-
5		meta	-	-	○	○
6	5	F/30 sp/ea/u	-	●	-	○
7		Meta intercost I	○	-	○	●
8		Meta intercost II	●	●	○	-
9	6	M/70 sp/adr/u	-	-	-	○
10		Meta LN	●	●	○	-
11	7	F/53 sp/ea/u	-	-	-	○
12		Meta	○	-	○	○
13	8	F/27 sp/adr/u	○○	○○	○	○
14	9	F/32 sp/adr/u	○	-	○	-
15		Meta LN I	○	○	○	○
16		Meta LN II	○	-	○	-
17	10	M/32 sp/ea/u	○	○	○	○
18	11	M/36 sp/adr/u	○	○	○	○
19		Meta LN	-	-	○	○
20	12	F/23 sp/adr/u	○	○	○	-
21		Meta bone	-	-	○	●
22	13	F/62 sp/adr/u	○	○	○	○
23	14	M/42 sp/adr/u	○	-	○	-
24	15	M/42 sp/adr/u	-	-	○	-
25	16	F/39 sp/adr/u	-	-	○	○
26		/49 meta LN	○	-	○	-
27	17	F/45 sp/adr/u	○	-	○	-
28		Meta LN	-	○	-	-
29	18	F/32 sp/adr/u	-	-	○	-
30		Meta liver	○	-	○	-

In CGH analysis ○ represents "no gain or loss", ● is "loss", and ○○ means "gain" of chromosome 17. In FISH-analysis ○ is "retention of heterozygosity", ● means "loss of heterozygosity", and ○○ represents "gain" of the p53 locus on #17. In SSCP ○ means no mutation. In immunohistochemistry ○ represents "no overexpression" and ● means p53-protein overexpression. In all four techniques – means that these samples are not examined. M= male; F= female; sp=sporadic; m=MEN; n=Neurofibromatosis; adr=adrenal; ea= extra-adrenal; u=unilateral; bi=bilateral; Meta=metastasis; LN=Lymph Node.

Until now, the molecular mechanisms underlying sporadic PCC tumorigenesis have largely remained elusive. Because of its frequent involvement in carcinogenesis, *p53* has been the topic of several investigations, employing a range of techniques. However, most of these studies were limited to a single technique or considered only small numbers of tumors. Our study is the first to specifically address loss of 17p, including the *p53* locus, by conventional CGH analysis. This analysis, which we confirmed by FISH, was performed in the context of a genome-wide CGH study, which is the topic of a separate paper. In four cases, we observed a discrepancy between the CGH and the FISH analysis, identifying loss by FISH but no alterations by CGH. This may

be due to the fact that not all deletions are picked up in CGH analysis due to its limitations in resolution[23]. The observed loss rate of 45% is in accordance with our previous work, where we found 31% loss in a series of 29 PCC, and with LOH analyses performed by others, which showed loss rates between 18 and 24%[4, 19, 27]. In five PCC (10%), gain of 17p was found in CGH, although copy number gain could only be confirmed in two instances by FISH analysis. This may most probably be due to heterogeneity in the tumors or may be partly due to the PCC being rather hypodiploid.

In the majority of cases in which we found loss of 17p at the CGH analysis and in almost all malignant PCC, we performed mutation analysis of exons 5–8 using SSCP analysis, which allows the detection of over 90% of mutations. However, we did not find a single mutation in our analysis, which is in accordance with several previous studies[18-19]. However, two Asian studies reported 83% (5/6) and 40% (4/10) p53 mutations, respectively[11, 21]. The reason for the discrepancy between these two groups of studies is not known, but may be related to geographical factors. Alternatively, we cannot entirely exclude the fact that mutations are present in other parts of the p53 gene, but it is known that more than 95% of mutations occur in exons 5–8[7].

In normal cells, nuclear p53 expression is usually below the detection level of immunohistochemistry. Mutant *p53*, because of its longer half-life, and cellular stress can induce overexpression, resulting in positive nuclear immunostaining. In the present study, we did not find a single positive case in the entire group of primary PCC. It must be noted that we employed very strict criteria before a tumor was scored as positive (see Materials and methods for scoring system). This is based on the notion that for *p53* to play a major role in tumorigenesis, whether in PCC or in other tumors, a large proportion of cells should show moderate or strong immunostaining to be considered physiologically relevant. In addition, in our experience, no *p53* mutations, for example in intestinal or breast tumors, are found if only weak or focal staining is present (Dinjens, personal communication). In many of the studies on p53 immunohistochemistry in pheochromocytomas that have been published so far, a variety of scoring systems have been used, usually yielding a low percentage of cases that were considered positive. Whenever cases were considered positive, these were frequently malignant PCC or metastases thereof, which is in line with our finding of two metastases showing p53 overexpression. The corresponding primary tumors, however, were negative.

Taken together, we have found a relatively high frequency of loss at the *p53 gene* locus on chromosome 17p, without concomitant *p53* mutations or signs of protein overexpression by immunohistochemistry. These results were similar between PCC and sympathetic paragangliomas. This suggests that *p53* does not appear to play a major role either in tumorigenesis or in the clinical behavior of human PCC. Because of the relatively high frequency of 17p loss, one may hypothesize the existence of other tumor suppressor genes on this chromosomal arm, which may be involved in PCC. An example of these is described in patients with breast cancer and endocrine pancreatic tumors[28-29]. However, it cannot be excluded that loss of 17p is the result of stochastic accumulation of genetic abnormalities in PCC.

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Chapter 4

Adrenal medullary hyperplasia in MEN2 syndrome is a precursor lesion for pheochromocytoma

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Abstract

PURPOSE: For decades, an arbitrary distinction has been made between adrenal medullary hyperplasia (AMH) and pheochromocytoma (PCC). AMH is diagnosed as an adrenal medullary proliferation with a size <1 cm, a lesion >1 cm as PCC. AMH and PCC are frequently diagnosed in MEN2 patients and their distinction is based on the assumption that they are two unrelated clinical entities. However, the biological relationship between AMH and PCC is unknown. In this study we investigated the molecular relationship between AMH and PCC, which could have impact on diagnosis and treatment.

MATERIALS AND METHODS: In 19 AMHs and 13 PCCs from 18 proven MEN2 patients, genomic aberrations were determined by loss of heterozygosity (LOH) analysis for chromosomal regions 1p13, 1p36, 3p, and 3q.

RESULTS: Molecular aberrations were found in all cases of AMH indicating that these are clonal neoplastic proliferations. Identical genomic aberrations were found in AMHs and PCCs, at similar frequencies. These results indicate that AMHs should be regarded as PCC precursor lesions.

CONCLUSION: Identical molecular aberrations are present in AMHs and PCCs and they occur at similar frequencies. These results indicate that AMHs are not hyperplasias and in clinical practice they should be regarded as PCCs.

Introduction

Pheochromocytomas (PCCs) are tumors that originate from adrenal medulla chromaffin cells and occur with an incidence of 1-2 per 100,000. Most PCCs are benign and only in about 10% of cases malignancy is found. The clinical impact of PCCs is far beyond the issue of malignancy since the vast majority of PCCs produce catecholamines, which can cause severe life-threatening situations such as myocardial infarctions and cerebrovascular accidents. Up to 25% of PCCs occur in the context of hereditary cancer syndromes. These include multiple endocrine neoplasia type 2 (MEN 2) caused by germ line activating *RET* gene mutations, von Hippel-Lindau (VHL) disease due to inactivating *VHL* gene mutations, neurofibromatosis type 1 (NF1) as a result of *NF1* gene inactivation, and the recently discovered pheochromocytoma-paraganglioma (PCC-PGL) syndrome resulting from inactivation of the *succinate dehydrogenase (SDH) -B, -C, -D* or -AF genes[1-3]. Syndrome-related PCCs are most frequently found in MEN 2, an autosomal dominant disorder, caused by germ line mutations in the *RET* proto-oncogene[4]. Fifty percent of MEN 2 patients develop PCC, which in up to 80% of patients occur bilaterally. Half of the bilateral lesions are diagnosed synchronously, but contralateral PCC can occur after an interval of many years[3]. The *RET* proto-oncogene encodes a transmembrane receptor tyrosine kinase involved in regulation of cell proliferation and apoptosis. Activating mutations in *RET* transform this proto-oncogene into an oncogene with constitutive activation of the receptor as a result. The activating germ line mutation in the *RET* gene in MEN2 patients is a prerequisite though insufficient to develop PCC and additional somatic molecular aberrations have to occur in adrenal medullary cells to initiate PCC development[5-6].

Despite increased research on PCCs in the last decades, relatively little is known about the molecular tumorigenesis of this neoplasm. An arbitrary distinction is made, based on the diameter of the lesion, between adrenal medullary hyperplasia (AMH), which is considered a benign, and generally non-catecholamine-producing lesion, and PCC, which is usually catecholamine-producing and potentially malignant. The definition of AMH as a nodular lesion of the adrenal medulla with a size smaller than 1 cm in diameter, has been put forward more than 3 decades ago by Carney et al.[7]. In contrast, PCC have been defined as catecholamine-producing lesions of the adrenal medulla with a size ≥ 1 cm in diameter. More recently, the World Health Organization has adopted this size criterion in their definition: adrenal medullary nodules smaller than 1 cm in diameter should be considered as AMH, while nodular lesions ≥ 1 cm must be classified as PCC. Most AMH cases are found in known hereditary PCC syndrome patients as a result of surveillance. Non-hereditary AMH is very rarely found and generally only diagnosed after catecholaminergic symptoms mimicking PCC[8-11].

The current therapy of choice for unilateral catecholamine-producing PCC in MEN2 patients is total unilateral adrenalectomy. In case contralateral PCC occurs later in life, cortex-sparing adrenalectomy is preferred. If a patient presents with bilateral PCC, the larger PCC will be resected by adrenalectomy and the smaller will be treated by cortex-sparing adrenalectomy. In general, it is

preferred to perform cortex-sparing adrenalectomy on one side rather than both sides, because of the increased recurrence risk in the latter situation[4, 12-13].

In sporadic as well as in hereditary PCC, especially in MEN2, early genetic aberrations are loss of chromosomal arms 1p (82-86%), 3p (24-31%), and 3q (41-52%)[14-15]. Moley et al. investigated chromosome 1p by loss of heterozygosity (LOH) analysis and found loss in 9/9 PCCs from MEN2 patients[16]. The high frequencies of these chromosomal aberrations indicate that they might be causally related to PCC tumorigenesis, but tumor suppressor genes on these chromosomes are yet still to be found[14-17]. Data on possible molecular aberrations in AMH have not been reported to date. Because the distinction between AMH and adrenal PCC solely on the basis of the size of the lesion appears arbitrary, the aim of the present study was to identify the possible relationship between AMH and PCC on the basis of the molecular abnormalities in these lesions. For this purpose, 32 adrenal lesions from 18 MEN2 patients, comprising 19 histologically proven AMHs and 13 PCCs, were investigated for loss of chromosomes 1p13, 1p36, 3p and 3q by LOH analysis using highly polymorphic microsatellite markers.

Material & Methods

Tumor samples

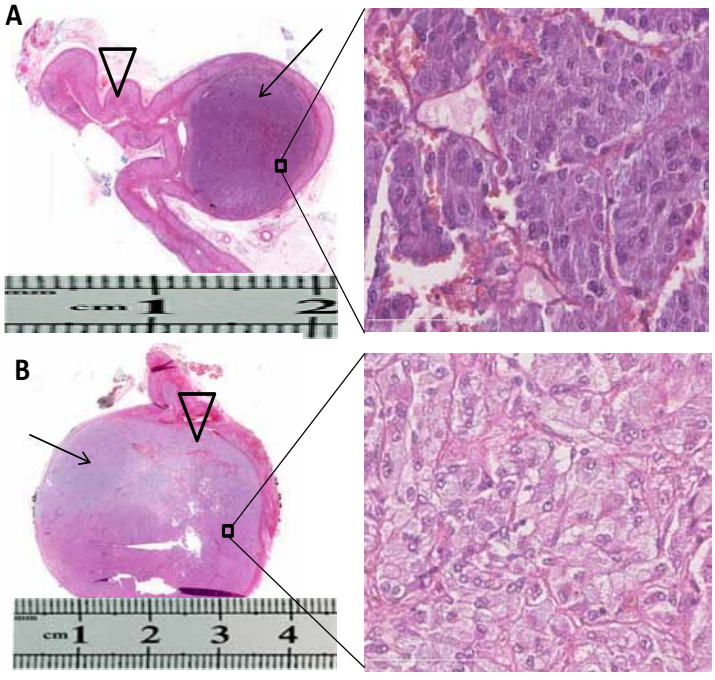
For several decades, at the Erasmus MC, the therapy of choice for MEN2 patients with elevated catecholamine levels or enlarged adrenals has been bilateral adrenalectomy. In the resected adrenals nodular medullary lesions of variable size were found. For the diagnosis of these lesions the guidelines of the World Health Organization were used: AMH is a nodular lesion of the adrenal medulla with a diameter of less than 1cm whereas nodular lesions with diameter of ≥ 1cm are considered PCCs. Thirty-two histologically proven nodular adrenal medullary lesions, comprising 19 AMHs and 13 PCCs, from 18 patients from 10 different MEN2 families, all with a known germ line *RET* gene mutation, were investigated (table 1). All patients had undergone total thyroidectomy because of risk for medullary thyroid carcinoma (MTC). Fourteen patients underwent bilateral and 4 unilateral adrenalectomy. All adrenal tumors were benign and were collected between 1982 and 2005. The routine formalin fixed and paraffin-embedded (FFPE) tissue specimens were retrieved from the files of the Department of Pathology (Erasmus MC), following approval of the experimental design and protocols by the institutional Medical Ethics Committee. From each case lesional and normal DNA was isolated from FFPE tissues by manual microdissection of tissue fragments comprising more than 80% lesional cells and 100% normal cells, respectively (Figure 1). The isolated tissue fragments were digested by standard detergent-proteinase K treatment and DNA was obtained after phenol/chloroform extraction and ethanol precipitation. Relevant characteristics and clinical data are summarized in table 1.

Table 1. Clinical and tumor data of 32 tumors from 18 MEN2 patients.

Pt	Family	M/F	m/b	Age	Mutation RET	L(cm)	R(cm)	MTC	PHP
1	A	F	b	20	pCys 634 Arg	7	0.4	+	-
2	A	M	b	55	"	0.7	-	+	+
3	B	F	b	32	pCys 634 Arg	-	0.7	+	+
4	B	M	b	42	"	0.6	0.4	+	+
5	B	F	b	18	"	0.2	0.7	+	+
6	B	F	b	24	"	0.9	0.7	+	-
7	B	F	b	50	"	<1	<1	+	-
8	C	F	b	52	pCys 634 Arg	2	0.8	+	-
9	C	F	b	38	"	4	1.6	+	+
10	C	F	b	38	"	0.8	-	+	-
11	C	F	b	51	"	0.9	4	+	+
12	D	F	b	29	pCys 634 Arg	4	0.8	+	+
13	E	F	b	29	pCys 620 Arg	5	3	+	-
14	F	M	b	26	pCys 634 Arg	0.8	3	+	+
15	G	M	b	23	pCys 634 Arg	2.5	0.6	+	-
16	H	F	b	51	pCys 611 Tyr	-	1.5	+	-
17	I	F	b	34	pMet 918 Thr	0.2	0.8	+	-
18	J	F	b	25	pCys 634 Arg	4.2	2	+	+

Pt patient; M/F Male/Female; m/b malignant/benign; Age years; L tumor size in left adrenal gland; R tumor size in right adrenal gland; MTC medullary thyroid carcinoma; PHP primary hyperparathyroidism.

Figure 1. Submacroscopic and microscopic examples of adrenal medullary hyperplasia (A) and pheochromocytoma (B)



Arrow heads: normal adrenal tissue; Arrow figure A: AMH, arrow figure B: PCC

LOH analysis

All DNA samples were analyzed for allelic imbalances of chromosomes 1p13, 1p36, 3p and 3q using highly polymorphic microsatellite markers, summarized in table 2. Polymerase chain reaction (PCR) amplification of paired lesional and normal DNA was performed in reaction mixtures of 15µl. Each reaction contained 50-100ng template DNA, 0.02mM dATP, 0.2mM dTTP, dGTP, dCTP each, 0.8µCi α32P-dATP, 20pmol of each primer, 1.5mM MgCl2, 10mM Tris-HCl, 50mM KCl, and 1 unit Taq DNA polymerase (Amplitaq Gold, Perkin Elmer, Norwalk, CT). An initial denaturation step at 94°C for 5 minutes was followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 55°C for 60 seconds, and extension at 72°C for 60 seconds. A final extension step was carried out at 72°C for 10 minutes. PCR products of tumor and normal DNA from each patient were diluted 1:1 in 10µl loading buffer (95% formamide, 20mM EDTA, 0.05% xylene cyanol, 0.05% bromophenol blue) and loaded onto a denaturating 6% polyacrylamide gel. Electrophoresis was carried out at 60W for 90 minutes. The gels were dried and exposed to X-ray film overnight at -80°C. Results were scored by two independent investigators.

Table 2. Microsatellite markers, chromosomal localization, primer sequences and approximate product sizes of the eight markers in 4 regions.

	Marker	localization	Forward 5'-->3'	Reverse 5'-->3'	Size (bp)
1	D1S252	1p13.1	AGCTTTTTACTCTTAACCTATTCAT	TCATTAATACACATGTTCTCTGC	107
2	D1S2881	1p13.2	ATCTGCCAGCCCATGA	CAAACAGAGCCTGGCA	188
3	D1S2885	1p36.11	ATCTGCCAGCCCATGA	CAAACAGAGCCTGGCA	127
4	D1S234	1p36.11	GCCCAGGAGGTTGAGG	CTGTAATTCAAGCCTGCCTT	142
5	D3S3681	3p12.3	GTGAGAACCATTTGGGGCAG	CCCTGACAGATAGCTCGCC	226
6	D3S3551	3p13	AACGGAGTTCCCACATAAA	CTTTTGTCTGTATTGCTAATGAACC	142
7	D3S3694	3q23	AGTGTCCATCAACATGGG	GAATCCTGTTATTTGTGCGA	162
8	D3S1569	3q24	GCACCTTGGCTTACCTTCTA	CTTTAAGAACCTTTCAACTGTCC	234

bp base pairs

Results

Nineteen AMHs and 13 PCCs from 18 MEN2 patients were investigated for LOH on chromosomal regions 1p13, 1p36, 3p12-13 and 3q23-24. In all cases there was sufficient DNA to perform LOH analysis on the 4 different loci with 2 highly polymorphic markers per locus. Representative LOH and retention of heterozygosity (ROH) results are shown in figure 1 and summarized in tables 3 and 4. In each of the investigated 19 AMHs and 13 PCCs at least one marker demonstrated unequivocal LOH indicating that the DNA was retrieved from a high percentage of lesional cells. LOH (of at least one marker per locus) in the AMH cases was found for 1p13 in 9/12 (75%), for 1p36 in 9/13 (69%), for 3p12-13 in 12/16 (75%) and for 3q23-24 in 10/13 (77%) of the informative cases. In the PCCs, LOH was detected at 1p13 in 13/13 (100%), 1p36 in 9/9 (100%), 3p12-13 in 4/9 (44%) and at 3q23-24 in 11/11 (100%) of informative tumors. From 14 patients, bilateral lesions

Table 3. Results of LOH analyses

					1p13.1	1p13.2	1p36.11	1p36.11	3p12.3	3p13	3q23	3q24
pt	family	left/ right	size(cm)	tumor	D1S252	D1S2881	D1S2885	D1S234	D3S3681	D3S3551	D3S3694	D3S1569
1	A	L	7.0	PCC	■↓	x	x	x	NI	x	NI	x
		R	0.4	AMH		x		x	NI	■↑	NI	x
2	A	L	0.7	AMH	■↑	x	■↑		■↓	■↑	■↓	■↑
3	B	R	0.7	AMH			■↓		■↓		■↓	
4	B	L	0.6	AMH	■↑	x	x	x	x	■↑	x	■↑
		R	0.4	AMH	■↑	x	x	x	x	x	x	
5	B	L	0.2	AMH		NI			■↑	■↑	■↓	■↑
		R	0.7	AMH		NI				■↑		
6	B	L	0.9	AMH		■↓	■↓	■↓		■↓	NI	
		R	0.7	AMH	■↓	■↓		■↓		■↓	NI	
7	B	L	u	AMH						■↑		
		R	u	AMH						■↑		
8	C	L	2.0	PCC	■↑	■↑	x	x	x	x	NI	■↓
		R	0.8	AMH		■↑	x	x		■↓	NI	■↑
9	C	L	4.0	PCC	■↓		x	NI		■↓	■↑	■↓
		R	1.6	PCC	■↓		■↓	NI		■↑	■↑	■↓
10	C	L	0.8	AMH		NI	■↓				■↓	■↑
11	C	L	0.9	AMH	■↑		NI				■↓	
		R	4.0	PCC	■↓		NI					■↑
12	D	L	4.0	PCC	■↑	x	■↑	■ ↓		■↑	■↓	■↓
		R	0.8	AMH		x		■↓			■↓	x
13	E	L	5.0	PCC	NI	■↓	x	■↑	NI			■↓
		R	3.0	PCC	NI	■↓	■↓	■↑	NI	■↑	■↑	■↑
14	F	L	0.8	AMH	NI	x	x	x	NI	■↑	x	x
		R	3.0	PCC	NI	■↑	x		NI		■↓	x
15	G	L	2.5	PCC	■↓	■↑					■↑	■↑
		R	0.6	AMH	■↑	■↓		■↑			■↓	■↓
16	H	R	1.5	PCC		■↓		■↑			NI	
17	I	L	0.2	AMH		NI	x	■↓		NI	NI	NI
		R	0.8	AMH		NI	■↓	■↓		NI	NI	NI
18	J	L	4.2	PCC	■↓	■↑	NI	■↓			■↑	NI
		R	2.0	PCC	■↑	■↓	NI	■↑			■↓	NI

Pt patient; L left adrenal gland; R right adrenal gland; PCC pheochromocytoma; AMH adrenal medullary hyperplasia

■↑	loss upper allele	NI	not informative
■↓	loss lower allele	x	no data
	no loss	u	Unknown

were investigated and in 12 of these cases the paired proliferations demonstrated different LOH profiles (cases 7 and 17 demonstrated an identical LOH pattern in the bilateral lesions). A total of 128 loci were investigated (4 loci in 32 samples) and only from 15 loci (1p13 in case 14L; 1p36 in case 4L, 4R, 8L, 8R, 9L and 14L; 3p12-13 in case 1L, 4R and 8L; 3q23-24 in case 1L, 1R, 14L. 17L and 17R) no information on possible LOH could be obtained. The lack of LOH information from both markers in each of these 15 loci was due to the fact that the markers were non-informative or no data were obtained. Out of 256 LOH analyses performed 73 (29%) did not yield an analyzable result, even after repeated testing. Included in these results are 12 patients that were non-informative for 17 microsatellite markers. Examples are shown in figure 2.

Figure 2. LOH example.

LOH results of patient 18 for marker D1S234. Arrow heads point to relative deleted alleles. Note relative loss of the upper allele for T1 (18R) and relative loss of the lower allele for T2 (18L). N = patient 18 normal DNA.

Table 4. Overall LOH results AMHs vs. PCCs

Locus	AMH (%)	PCC (%)
1p13	75	100
1p36	69	100
3p	75	44
3q	77	100

PCCs Pheochromocytomas; AMHs adrenal medullary hyperplasias.

Discussion

AMHs and PCCs are adrenal medullary proliferations, frequently found in MEN2 patients, considered unrelated, and discriminated by lesional size (smaller or ≥ 1cm, respectively). Because AMHs generally do not produce catecholamines whereas PCCs do, and that PCCs can progress to malignancy, treatment of AMH and PCC is different. As a result, PCCs are almost always resected, whereas AMHs are only subject to follow-up.

The diagnostic distinction between AMH and PCC solely on the basis of the size of the lesion is biologically questionable. Therefore, we investigated in the present study the possible molecular relationship between MEN2-related AMHs and PCCs. Guided by chromosomal aberrations, frequently found in PCC, deletions of chromosomal regions 1p13, 1p36, 3p12 and 3q24 in PCCs and AMHs were compared. In all investigated AMH cases at least one molecular aberration (LOH) was found indicating that AMH lesions are clonal and (pre)neoplastic proliferations.

Genetic abnormalities in PCC have been studied extensively over the last decades, although understanding of the molecular pathogenesis of this tumor is still limited. DNA-based analyses, including LOH and comparative genomic hybridization (CGH), have identified several distinct chromosomal regions frequently lost in PCC, including chromosomes 1p, 3p, and 3q. In particular, loss of 1p has been suggested to be an early event and occurs at a frequency of 82-86% of PCC[14-19]. Although chromosomal regions of minimal overlapping loss have been described at 1p13 and 1p36, relevant tumor suppressor genes have not been detected to date[17]. This is also the case for potential genes on 3p and 3q. There appear to be slightly differing patterns of DNA loss for sporadic and MEN2-related PCC on the one hand, and *VHL*- and *SDH*-related PCC on the other hand, the latter having more abnormalities of chromosomes 3p and 11[20-23].

In the present study a high frequency of loss at 1p13, 1p36, 3p12, and 3q24 was found both in the 19 AMH and 13 PCC cases, strongly suggesting a molecular relationship between AMH and PCC. In 12 out of 14 bilateral cases the genomic aberrations were different in paired lesions indicating that the bilateral proliferations are independent entities. The observed LOH frequencies in PCCs are comparable to several published series[14-16]. The percentage of loss of 1p and 3q is slightly less in the AMH series than in the PCC, which may be related to the accumulation of genetic abnormalities during tumor progression in the latter group. However, the differences are small and they may also be related to chance. The latter may also be the case for the difference observed for loss of 3p between AMH (75%) and PCCs (44%). However, these results could indicate that AMH with loss of chromosome 3p will less likely progress to PCC.

Our findings of a molecular relationship between AMH and PCC may have implications for the treatment of MEN2 patients, as they develop bilateral PCCs in up to 80% of cases[24]. The currently recommended treatment in MEN2 patients is unilateral resection of the affected adrenal gland and bilateral adrenalectomy in cases where there are synchronous tumors. In the case of bilateral resection, one of these is preferably performed in a cortex-sparing manner, to prevent morbidity and mortality due to adrenocortical insufficiency[25]. From the data presented in this study, it

appears that future surgical treatment in MEN2 patients may need to be more "aggressive". Not only PCCs need to be operated upon, but also AMHs could be considered for surgical removal. Such an approach may have the advantage of allowing cortex-sparing surgery, which also may lead to a lower risk of developing bilateral PCC than thus far reported[12]. Another advantage of early surgery for AMH is the fact that they are often asymptomatic, which gives a lower chance for pre- and intra-operative hypertensive crises.

In summary, we have shown that AMHs in the context of MEN2-syndrome have identical genomic aberrations as PCCs, at similar frequencies, and should thus be regarded as PCC precursor lesions. In addition, the finding of clonal molecular aberrations in AMHs indicates that these lesions are not hyperplasias but small nodular pheochromocytomas. By definition, hyperplasias are non-clonal proliferations of normal cells, i.e. cells without molecular aberrations.

As a consequence of our findings, we propose that the size criterion for the distinction between AMH and PCC should be abolished, at least in the case of MEN2 syndrome, but we speculate that this will also be the case for non-MEN2 related AMH. Furthermore, our results could have implications for the treatment of MEN2 patients, as these very small lesions (AMHs) should be regarded as precursor lesions for PCC and can be treated with total or cortex-sparing adrenalectomy. Our results aid in our understanding of PCC tumorigenesis and may alter the surgical approach in MEN2 patients with synchronous or metachronous adrenomedullary tumors.

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Chapter 5

Molecular taxonomy of pheochromocytomas and paragangliomas

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Pheochromocytomas (PCC) and paragangliomas (PGL) are rare tumors with a combined annual clinical incidence of 40 per million[1-3]. The tumors arise from neural crest derived chromaffin cells in the adrenal medulla and in sympathetic paraganglioma (sPGL) and parasympathetic paraganglia (pPGL). PCC and PGL are histologically and immunohistochemically similar tumors with no major discriminating morphological features. In 2004 the World Health Organization (WHO) has redefined this group of related tumors from the adrenal medulla and paraganglia into three categories: pheochromocytomas are tumors of the adrenal medulla, which usually, but not always, produce catecholamines, including epinephrine, norepinephrine, and dopamine. All other tumors arising outside the adrenal gland and originating from sympathetic paraganglia are called sPGL and tumors arising from parasympathetic paraganglia are designated pPGL. sPGL usually produce catecholamines and in many studies they are designated as extra-adrenal PCC. pPGL occur predominantly in the head and neck region and rarely produce catecholamines[4].

Originally 10% of PCC and PGL were considered to occur in an inherited context, but more detailed studies during the last decade have revealed that about 30% of these tumors are familial[5-6]. To date, germline mutations in 9 genes (RET, VHL, NF1, SDHA, -B, -C, -D, SDHAF2, TMEM127) have been described associated with familial PCC and PGL. Multiple endocrine neoplasia type 2 is caused by germline mutations of the RET proto-oncogene and characterized by medullary thyroid carcinoma in association with adrenal PCC and primary hyperparathyroidism. The VHL syndrome is an autosomal dominantly inherited tumor syndrome caused by de VHL gene. Patients with VHL syndrome have predisposition to develop retinal and central nervous system hemangioblastomas, clear cell renal cell carcinomas, adrenal PCC and sPGL, pancreatic cysts and islet cell tumors, cystadenomas of the epididymis, and endolymphatic sac tumors. Neurofibromatosis type 1 is associated with mutations in the NF1 gene and patients in this syndrome can develop adrenal PCC. The PCC–PGL syndrome is caused by mutations in subunits of the mitochondrial complex II, also known as succinate dehydrogenase (SDH). All four SDH genes have been implicated in the occurrence of pPGL. While SDHC and SDHA has only infrequently been described and exclusively in the context of head and neck PGL, SDHB and SDHD are also associated with sPGL and adrenal PCC. Recently, mutations in SDHAF2 and TMEM127 have been described in association with families harboring pPGL[5-9]. Somatic mutations in the RET and VHL gene are found in <1% and 5%, respectively of sporadic PCC but all other genes involved in inherited PCC and PGL do not play major roles in sporadic tumors[10]. The causative genes involved in sporadic PCC and PGL remain largely unknown[11].

Several genomic aberration studies on syndrome related and sporadic PCC and PGL were performed. Comparative genomic hybridization studies revealed that PCCs from MEN2 and NF1 patients have characteristic loss of chromosome 1p and 3q as do the majority of sporadic PCC. VHL related PCC, however, showed distinct genetic aberrations with combined loss of chromosomes 3 and 11[12].

Dannenberg et al, Edstrom et al and August et al. performed a whole genome microarray CGH study on PCC and sPGL[13-15]. Edstrom et al revealed that in PCC and sPGL the most frequent losses

on chromosome arms 1p (83%), 3q (39%), 11p (17%), 3p (17%), 4q (17%), and 11q (13%) were found. Gains were seen predominantly on 19p (26%), 19q (26%), 17q (17%), and 16p (9%), and in one tumor 20q was amplified. In sPGL, the most frequent losses were found on chromosome arms 1p (82%), 3q (45%), 11p (45%), 3p (36%), 4q (27%), and 11q (18%). Gains were seen predominantly on chromosome arms 19p (55%), 11q (36%), 16p (27%), 17q (27%), and 19q (18%). In this study with PCC and sPGL an average of copy number changes of 5.3 (range 0-14) was seen[15]. For August et al the most important aim of the study was the characterization of chromosomal aberrations on CGH in PCC en sPGL. In this study the most frequent losses were found on chromosome arms 1p (75.6%) and 3q (44%) followed by losses of 22q (29.2%), 11q (22%), 6q (19.5%), 11p (17%) and 21q (17%). Gains were observed predominantly on 17q (36.6%), 20q (22%), 7p, 9q and 12q (all 14.6%). Among extra-adrenal PCC, chromosomal losses on 1p (4/6), 1q (3/6) 6q (2/6) and 11p (2/6) were most frequent. In this study with PCC and sPGL an average of copy number changes of 6.2 (range 0-18) was seen[13]. Dannenberg et al had seen that the most common chromosome arm copy number changes included losses of chromosomes 1p11-p32 (86%), 3q (52%), 6q (34%), 3p, 17p (31% each), and 11q (28%), and gains of chromosome 9q (38%) and 17q (31%). In this study with PCC and sPGL an average of copy number changes of 6.2 (range 0-18) was seen. Concluding that in these studies no major differences in the patterns and amounts of copy number changes nor in the minimal regions of involvement between PCC and sPGL were seen[14].

There are two whole genome microarray CGH studies done in pPGL. Dannenberg et al: performed the first comprehensive, genome-wide analysis of chromosomal aberrations in sporadic and familial parasympathetic PGLs. The results indicate that DNA copy number changes are infrequent in these tumors and that tumor suppressor genes on chromosome 11 may play a critical role in the tumorigenesis of familial PGLs. The average number of copy number changes in this study was 1.8[16]. Sevilla et al. also concludes that that DNA copy number changes are infrequent in PGL. Further, PGLs without any chromosomal aberrations were mostly sporadic, whereas frequent deletions on chromosomes 1 and 11 occurred in germline mutation carriers. The average number of copy number changes was 1.9[17].

These results indicate that the WHO classification of chromaffin tumors in PCC and sPGL and pPGL, primarily based on the combination of histology and tumor location, is by and large accompanied by tumor-type specific molecular aberrations. In the PCC-PGL syndrome, caused by germ line mutations in one of the SDH related genes, sPGL and pPGL occur and occasionally PCC are found. We wondered whether the PCCs within this syndrome are molecularly more similar to non-PCC-PGL syndrome related PCC or to PCC-PGL syndrome related PGL. Therefore we investigated by CGH 8 histologically proven (adrenal) PCCs from 8 different PCC-PGL syndrome patients all with an SDHD germ line mutation (Table 1). Patients were available from the archives of the Departments of Pathology, Erasmus MC, Rotterdam (n=4), Radboud UMC, Nijmegen, the Netherlands (n=1), Leiden University Medical Center (n=2), and Amsterdam Medical Center (n=1). All tumors were collected between and 1996 and 2008.

Table 1 summarizes the genome wide chromosome arm aberrations and the average number of chromosome arm aberrations per tumor of the 8 PCC occurred in cases of PCC-PGL syndrome subtype 1 , identified by CGH. We found chromosome arm aberrations in 6/8 PCC and all tumors with copy number changes show loss of the 11q region. In only one tumor loss of 1p and 3q loss is seen, but in this tumor also the 11q region is lost. the average number of chromosome arm aberrations per tumor was 2.5 (range 0-5).

From the CGH results it is evident that adrenal PCCs in SDHD germ line mutated PCC-PGL syndrome patients have a PGL and not a PCC molecular aberration profile. This indicates that molecularly adrenal PGLs exist. Whether PCC and adrenal PGLs have a common progenitor cell but are different with regard to the oncogenic transformation pathways, or whether these tumors have a different cell type of origin remains to be established.

In summary: Syndromic and non-syndromic PCC and PGL can be classified by their genomic aberrations. This molecular tumor taxonomy largely resembles the different syndromes and the WHO classification which is based on the combination of tumor location and histology. However exceptions do occur as exemplified in the current study in which PCC in SDHD germ line mutated PCC-PGL syndrome patients were found to have a PGL aberration pattern. The molecular aberration profiles in PCC and PGL can currently already be used in the diagnostics of syndrome related tumors. It can be envisaged that these molecular profiles can be indicators for prognosis and response to future (personalized) treatments.

Table 1. Overview of mutations and chromosome arm aberrations in 8 SDHD related pheochromocytomas.

tumor	Mutation SDHD	CGH aberrations
1	D92Y	-
2	D92Y	3q-, regional 11q-
3	D92Y	11pq-
4	L95P	1p-, 2q-, 3pq-, regional 9p-, 11pq-
5	D92Y	regional 11q-, 17p+, regional 17q+
6	D92Y	5p+, 8pq+, 11pq-, 17p-, Y-
7	D92Y	-
8	D92Y	2p+, 5p+, 11pq-

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Chapter 6

Frequent genetic changes in childhood pheochromocytomas

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Abstract

Pheochromocytomas (PCCs) are rare catecholamine producing tumors of the adrenal gland which may also occur elsewhere in the abdomen and are then called paragangliomas. A proportion of PCC occur in hereditary cancer syndromes, including multiple endocrine neoplasia Type 2 (MEN2), caused by mutations in the *RET* proto-oncogene, von Hippel–Lindau (VHL) disease, caused by *VHL* gene abnormalities, and the pheochromocytoma–paraganglioma (PCC–PGL) syndrome, caused by mutations in SDHB and SDHD. Since a proportion of PCCs occurs in children we hypothesized that germline mutations in *RET*, *VHL*, *succinate dehydrogenase subunit B* (*SDHB*), and *subunit D* (*SDHD*) occur more frequently in the pediatric age range. From our single-institution collection of PCCs, we have selected 10 cases that occurred in individuals up to 18 years of age at diagnosis. In these, we have performed mutation analysis on normal and tumor tissues for exons 10, 11, and 16 of *RET* and for the entire coding sequence of *VHL*, *SDHB*, and *SDHD*. The 10 patients include 7 boys and 3 girls, with an average age of 15.5 years (range 9–18 years). Two patients had germline *RET* exon 11 mutations (C634R) and 1 patient had an R64P germline mutation in the *VHL* gene. In the remaining 7 patients there was one patient from a family fulfilling the clinical criteria for VHL disease. All tumors were benign (average follow-up: 12 years) and were located in the adrenals. From our findings we conclude that (a) a large proportion (40%) of pediatric PCC patients is diagnosed in the context of inherited cancer syndromes, and (b) candidate gene analysis appears to be indicated to detect germline mutations.

Introduction

Pheochromocytomas (PCCs) are catecholamine-producing tumors of chromaffin cells in the adrenal medulla which may also occur in extra-adrenal locations and are then called paragangliomas (PGLs). Such sympathetic PGLs should be distinguished from head and neck PGLs, which have a parasympathetic origin, do not usually produce catecholamines, and have a different genetic background[1-2]. PCCs were originally called "the 10% tumor" for the frequency of malignancy, familial occurrence, and the proportion of tumors presenting during childhood. However, several studies have challenged these percentages, and it is obvious now that the frequency of familial occurrence, that is, the proportion of PCC that is inherited, may be up to 25%[3].

There are several heritable (endocrine) tumor syndromes, which all have an autosomal dominant mode of inheritance, that include PCC: von Hippel–Lindau disease (VHL), caused by abnormalities in the VHL tumor-suppressor gene; multiple endocrine neoplasia Type 2 (MEN2), caused by activating point mutations in the *RET* proto-oncogene; and the newly described PCC–PGL syndrome, caused by mutations in one of three genes encoding succinate dehydrogenase subunits (*SDHB*, *SDHC*, and *SDHD*). The incidence of VHL and MEN2 is approximately the same, 1 of 36,000, whereas this incidence is not yet known for the PCC–PGL syndrome[4].

Until now, relatively little attention has been paid to PCC occurring during childhood and adolescence, with only a few studies describing larger series of patients, predominantly from a clinical point of view[5-8]. None of these studies has systematically studied molecular genetic abnormalities in the above-mentioned genes. Therefore, we have undertaken such an analysis in a single-institution-derived group of 10 patients under 18 years that presented with adrenal-based PCCs, covering the entire coding region of *VHL*, *SDHB*, and *SDHD*, and exons 10, 11, and 16 of *RET*.

Material and methods

Patients

Tissue specimens were retrieved from the archives of the Department of Pathology of the Erasmus MC (Rotterdam), following approval of the experimental design and protocols by the Medical Ethical Committee. Ten PCCs were selected for mutation analysis for RET, VHL, SDHB, and SDHD, without knowing the patient or family history (for patient details see table 1).

Table 1. Clinical details and results of mutation analysis in pediatric PCC patients

	sample	age at operation	sex	side	size(cm)	weight	mutation	syndrome
1	F18	17	M	R	6	60	none	
2	F30	14	M	R	3.2	20	none	
3	F31	15	M	R	8x4.4x4	80	none	
4	F41 R	16	M	R	3	17	RET C634R	MEN2A
5	F41 L			L	3	15	RET C634R	MEN2A
6	F44	18	M	U	4x2.5	U	none[a]	
7	F68 L	9	M	L	4x2.8	U	none	
8	F68 R			R	1.7x1.3	U	none	
9	F80 L	18	F	L	3x2.5	U	RET C634R	MEN2A
10	F80 R			R	3x3	U	RET C634R	MEN2A
11	F95 L	13	F	L	7x3x1.5	U	none	U[aaa]
12	F95 R			R	5.5x5x2.5	U	none	
13	F107	17	M	R	2.5x1.5x1.5	U	none[aa]	VHL[aa]
14	F119	18	F	L	3	23	VHL R64P	VHL

NOTE: U = unknown; M = male; F = female; size has been indicated as greatest tumor diameter or alternatively in two or three dimensions, as recorded. A In this patient an exon 5 TCT to CCT base change was found in SDHB, leading to an S163P amino acid change, which is presently considered as a polymorphism. [aa]This patient and his family fulfilled all clinical criteria for VHL disease, including clear-cell renal cell carcinoma and two hemangioblastomas in addition to the PCC in the index patient, but no VHL mutation was detected because of the inferior DNA quality of this sample. [aaa]This patient presented with hemihypertrophy, multinodular adrenocortical hyperplasia, and fibroadenoma of the breast, in addition to bilateral PCC.

Tissue Preparation

Tumor DNA and matching normal DNA were isolated from paraffin embedded tissue. DNA isolation was performed using Puregene (Gentra, Minneapolis,MN) according to the manufacturer's instructions.

Denaturating Gradient Gel Electrophoresis (DGGE)

Polymerase chain reaction (PCR) using genomic DNA as template was carried out in a 50 µL mixture of 1x PCR buffer (Perkin Elmer Europe, Rotkreuz, Switzerland) containing 10 to 400 ng of template DNA, 200µM of each intron based primer (TABLE 1), and 1 µL Taq polymerase (Ampli Taq Gold, Perkin Elmer Europe). After a hot start of 7 min at 95◦C, a "touch-down" procedure was used consisting of denaturation for 60 sec at 95◦C, annealing for 60 sec at temperatures decreasing from 60 to 55◦C during the first 11 cycles (with 0.5◦C decremental steps in cycles 2 to 11), and ending with an extension step at 72◦C for 60 sec. Ten cycles with an annealing temperature of 55◦C and 15 cycles with an annealing temperature of 45◦C followed with extension times of 90 sec. After a final extension for 10 min at 72◦C, heteroduplex formation was induced by initial denaturation for 10 min at 98◦C followed by incubations at 55◦C for 30 min and 37◦C for 30 min. For DGGE, 10 µL of the PCR product in 3 µL Ficoll-based loading buffer were loaded onto 10%

polyacrylamide gels containing a urea-formamide gradient in 0. 5×Tris-acetate TAE-EDTA. The amplicons were electrophoresed at 60◦C and 100 V for 16 h. DNA strands were visualized using silver staining as described previously[9].

Single-Strand Conformation Polymorphism (SSCP) Analysis

PCR amplification of tumor DNA was performed with 10 to 100 ng DNA in a final volume of 15μL containing 1.5mM MgCl2, 10mM Tris-HCl, 50mM KCl, 0.02 mM dATP, 0.2 mM dGTP, dTTP, and dCTP, 0.8 μCi α32P-dATP (Amersham, Buckinghamshire, United Kingdom), 15 pmol of each forward and reverse primer (TABLE 2), and 3U Taq polymerase (Ampli Taq Gold, Perkin Elmer Europe). PCR was performed for 35 cycles of 95◦C for 30 sec, 55◦C for 45 sec, and 1 min at 72◦C, followed by 1 cycle at 72◦Cfor 10 min. PCR products were electrophoresed overnight at 8W on a nondenaturing gel, containing 8% polyacrylamide ([49:1] Fluka, Neu-Ulm, Germany) and 10% glycerol (v/v). After electrophoresis, the gel was dried and exposed to an X-ray film.

DNA Sequencing

All samples demonstrating aberrant patterns were sequenced. PCR was performed in a final volume of 50 μL under identical conditions as the previous PCR, except that this mix contained 0.2mMdNTPs instead of 0.02mMdATPs and 0.8 μCi α32P-dATP. The PCR products were purified using Nucleospin® Extract II (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. The purification was followed by a sequence reaction using the BigDye® Terminator V3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). Two microliters of Termination Ready Reaction mix, 2 μL of 5 × Sequencing buffer, 1 μL forward or reverse primer (13.2 pM), and 14 μL of deionized water was added to 1 μL purified PCR product. The cycle sequencing program was performed for 25 cycles of 96◦C for 10 sec, 50◦C for 5 sec, and 4 min for 60◦C. This was followed by a precipitation step, adding 13 μL deionized water, 3 μL 3MNaAc (pH 5.2), and 64 μL ethanol (100%) to the sequence reaction product and incubating overnight at room temperature before centrifuging 20 min. at 14,000 rpm. After washing the samples with ethanol 70%, centrifuging them 10 min at 14,000 rpm, the pellets were resuspended in 20 μL formamide (Applied Biosystems, Warrington, UK). Products were analyzed on the ABI Prism 3100 genetic analyzer (Applied Biosystems).

Table 2. Primers used for mutation analysis.

Locus	forward 5'→3'
RET10	GCGCCCCAGGAGGCTGAGTG
RET11	CTCTGCGGTGCCAAGCCTCA
RET16	cgcccgccgcgcccgcgcccggcccgccgcccccgcccgaaataataaaAGGGATAGGGCCTGGGCTTC
VHL1a	gcgcgcgAGCGCGTTCCATCCTCTAC
VHL 1b	gcgcgGCGGAGAACTGGGACGAG
VHL2	cgcccgccgcgcccgcgcccggcccgccgcccccgcccgaaataataaaCTTTAACAACCTTGCTT
VHL3	gcgcgTTCCTTGTACTGAGACCCTAGT
SDHB1	GAGCGACCTCGGGGTTAAG
SDHB2	cgcccgccgcgcccgcgcccggcccgccgcccccgcccgAGCTGAGATGTGTGAACTTT
SDHB3	cgcccgccgcgcccgcgcccggcccgccgcccccgcccGGAACTTTACATAAATACCACTGGA
SDHB4	GATTCCGGATATGGGTGAG / cgcccgcccgcccgcgcccggcccgccgcccccgcccgaaatataaaT
SDHB5	gcgcgTGATGATGGAATCTGATCC
SDHB6	cgcccgccgcgcccgcgcccggcccgccgcccccgcccgaaataataaaCCTCTCTTTTCTCCCCATAC
SDHB7	gcgcgAGCTAATCATCCCTGGTTT
SDHB8	cgcccgccgcgcccgcgcccggcccgccgcccccgcccgGTGGGTTTTCCCTTTCAGTT
SDHD1	cgcccgccgcgcccgcgcccggcccgccgcccccgcccgGTGGGTTTTCGACCTTGAGCCCTCAGGAACG
SDHD2	GATCATCCTAATGACTCTTTCC
SDHD3	ATCTGGTCCTTTTTG
SDHD4	GATTTTTTCTTTTTCT

Results

Patients and Tissues

All patient characteristics have been summarized in Table 1. In our series of 10 patients, representing 6.4% of all cases of PCC in our institution over a 17-year period, 70% were in boys, 40% of patients presented with bilateral tumors, and 40% had mutations in RET or VHL (see below) or fulfilled the clinical criteria for VHL, leading to a diagnosis of MEN2A or VHL disease in two patients each. In contrast, there were neither extra-adrenal tumors nor tumors with malignant behavior, after an average follow-up of 12 years, in this group of patients.

Mutation Analysis

The results from the mutation analysis of RET exons 10, 11, and 16, as well as the entire coding region of VHL (3 exons), SDHB (8 exons), and SDHD (4 exons) are summarized in table 1 as well. In three of these we found a germline RET or VHL mutation present in both normal and tumor DNA of these patients. In one patient the family history convincingly indicated the presence of VHL disease, although this could not be confirmed in the normal and PCC tumor tissue of this patient on account of the inferior DNA quality. Furthermore, one patient showed an S163P alteration in

reverse 5′→3′	size(bp)	method
cgcccgccgcgcccgcgcccgtcccgccgcccccgcgTGGTGGTCCCGGCCGCC	225	DGGE
cgcccgccgcgcccgcgcccggcccgccgcccccgcccgGAGTAGCTGACCGGGAAGGC	262	DGGE
ACCCCAAGAGAGCAACACCC	224	DGGE
cgcccgccgcgcccgcgcccggcccgccgcccccgcccgAGGGCCGTACTCTTCGAC	255	DGGE
cgcccgccgcgcccgcgcccggcccgccgcccccgcccgGCTTCAGACCGTGCTATCGT	418	DGGE
cgtcccgcGTCTATCCTGTACTTACCAC	266	DGGE
cgcccgccgcgcccgcgcccggcccgccgcccccgcccgGCTTCAGACCGTGCTATCGT	316	DGGE
GCTTTCCTGACTTTTCCCTC	157	SSCP
AAGCATGTCCCTAAATCAAA	265	DGGE
cgCTATCAGCTTTGGCCAGC	242	DGGE
cgtcccgcCCCCCATGCAAATAAAAACA	256	DGGE
cgcccgccgcgcccgcgcccggcccgccgcccccgcccgaaataataaaCAGATTGAAACAATAAATAGGGA	261	DGGE
cgCAGCAATCTATTGTCCTCTTG	252	DGGE
cgcccgccgcgcccgcgcccggcccgccgcccccgcccgaaataataaaTTGTGAGCACATGCTACTTC	270	DGGE
cgcccgccgcgcccgcgcccggcccgccgcccccgcccgGTGGGTTTTCCCTTTCAGTT	361	DGGE
cgcccgccgcgcccgcgcccggcccgccgcccccgcccgaaataataaa / gcgcgTCAGGGTGGGAAGACCCCT	153	DGGE
AGCAGCAGCGATGGAGAGAA	168	SSCP
cgcccgccgcgcccgcgcccggcccgccgcccccgcccgCTTTTATGA / gcgcgCAACTATATTTGGAATTGCTATAC	245	DGGE
cgcccgccgcgcccgcgcccggcccgccgcccccgcccgTGATGTTAT / gcgcgCAATTCTTCAAAGTATGAAGTCA	269	DGGE

the SDHB gene, which was interpreted as a polymorphism, based on findings in the literature and our own investigations (data not shown). Although all tumors that were available for this study ($n = 14$) yielded sufficient DNA for analysis, 35% of amplicons tested did not give an analyzable result. The result of the SSCP analysis of VHL exon 1 of patient F119 and the sequence result of the alteration in exon 5 of SDHB in patient F44 are shown in Figure 1A and 1B, respectively.

Discussion

In this study we have shown that in a small series of childhood PCCs up to 40% of patients have familial disease as evidenced by the presence of germline mutations in RET and VHL. This finding indicates that routine molecular analysis and a thorough family history are essential in patients presenting with PCC under the age of 18.

The clinical findings of our patient series are generally in good agreement with the larger series of childhood PCC patients that have been published[5-8]. There is a male predominance, with 60–75% of patients being boys, compatible with a figure of 70% in this study. Secondly, childhood PCCs are frequently bilateral, ranging from 25% to 55% in the literature, in agreement with our finding of 40% bilateral tumors. There is less concordance on extra-adrenal occurrence, with one study in agreement with our results, reporting no extra-adrenal PCC, whereas there are two

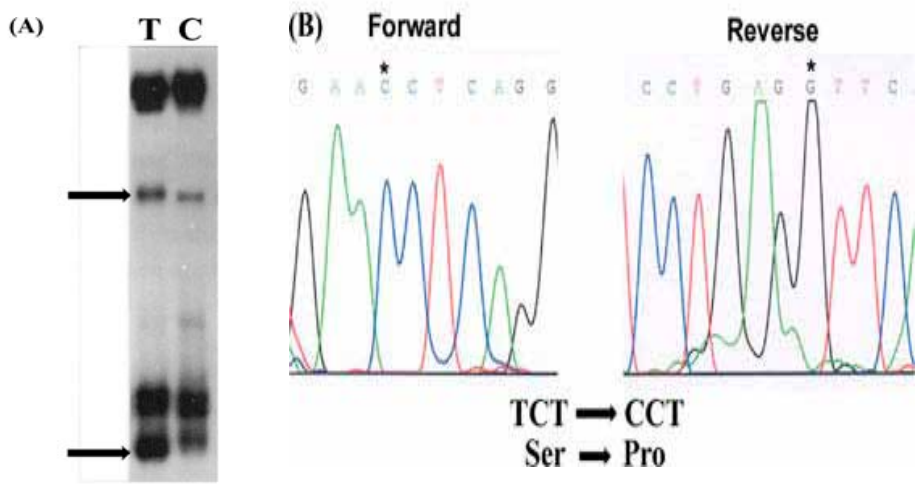

Figure 1. (A) Single strand conformational polymorphism analysis, showing comparison of the banding pattern of VHL exon 1 between tumor (T) and control (C) sample in patient F119. Clearly, the tumor sample shows the presence of two aberrant bands (arrows). Upon sequence analysis, a mutation in codon 64 was detected, leading to an amino acid change from arginine to proline. **(B)** Forward (left) and reverse (right) sequence analysis results from SDHB exon 5 in patient F44, showing a TCT to CCT change, indicated by an asterisk, leading to an amino acid change from serine to proline. Because this change occurred at equal frequency in normal individuals and PCC patients, this alteration is regarded as a polymorphism, and not as a tumor-related mutation.

studies that found 8% and 18% of such localization. Finally, the issue of malignancy in childhood PCC is difficult to assess in the various published studies, as it is not always entirely clear what criteria for malignancy have been applied. In our opinion, only PCCs with histologically proven metastases can be classified as malignant, although an exception could be made for cases where elevated catecholamine levels persist after primary tumor resection and are supported by a positive metaiodobenzylguanidyl scan in an area of the body where chromaffin cells do not normally occur. Applying the above criteria, we did not find any malignant case, after an average follow-up of 12 years. Although there are recent attempts to develop histological criteria that might indicate malignancy, these studies await further confirmation[10-11].

There are only few case studies that have reported on mutation analysis of *RET*, *VHL*, or the *SDH*-genes in childhood PCC[12-13]. In one, an MEN2A family was identified when a father presented with a PCC first, and 18 years later his son had a medullary thyroid carcinoma (MTC)[12]. In the other study, a *de novo VHL* germline mutation was unexpectedly discovered in an incidentally found adrenal PCC in a 10-year-old boy[13]. In the present study, we report two MEN2A patients, both with a C634R mutation in RET exon 11, and both presenting with bilateral PCCs. Although all tests were performed without knowledge of the clinical patient history, it was revealed after genetic analysis that both patients came from MEN2A families that had already been recognized. It is interesting to note that the particular mutation in these patients has been associated

with a more aggressive phenotype of MTC, although such prognostic information is lacking for PCC behavior[14]. Further, we found one patient with an exon 1 *VHL* mutation, who also came from a known VHL family. One other patient also came from a family with a clinical history that was compatible with VHL, presenting with two hemangioblastomas and a clear-cell renal cell carcinoma in addition to the PCC. Because of the relatively inferior DNA quality, however, we could not obtain a result for all VHL exons tested in this patient, which may have led to our failure to detect a VHL mutation. Alternatively, there may have been other *VHL* gene abnormalities, such as a large deletion, which we may have missed with our technique, although VHL patients with PCCs usually have missense mutations. Finally, there was one patient in whom we found a missense alteration in exon 5 of SDHB. Although this alteration represents an amino acid change in this codon, the same change is found in 2.3% of a control population in the literature, which is comparable to the frequency that we have found in a series of 150 PCCs in our laboratory (unpublished data), favoring the idea that this change should be considered a polymorphism[15]. Apart from these patients with known tumor syndromes, there was one girl who presented with metachronous bilateral PCCs at the ages of 13 and 17 years, who also had several other benign tumors or tumor-like conditions, including multinodular adrenocortical hyperplasia, a fibroadenoma of the breast, as well as hemihypertrophy. This patient has been extensively discussed in a separate case report, to which the reader is referred[16].

In conclusion, mutation analysis of the most relevant candidate genes presently available, including *VHL*, *RET*, *SDHB*, and *SDHD*, reveals a mutation frequency of 40% in a small series of childhood PCCs, which strongly favors mutation screening in each childhood PCC patient in a prospective fashion. The presence of other tumors or congenital abnormalities in such patients should be registered as well, to facilitate investigations into other disease causing genes.

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Chapter 7

Candidate gene mutation analysis in bilateral adrenal pheochromocytoma and sympathetic paraganglioma

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Abstract

Pheochromocytomas (PCCs) are rare tumors that arise from chromaffin tissue in the adrenal medulla, but can also occur in the abdomen outside the adrenals and are then called sympathetic paragangliomas (sPGLs). According to the literature, between 15 and 25% of apparently sporadic adrenal PCC and sPGL are caused by germline mutations in RET, von Hippel–Lindau disease (VHL), succinate dehydrogenase subunit B (SDHB), or subunit D (SDHD). However, few studies have addressed the mutation frequency of these candidate genes in selected subgroups of PCC and sPGL, such as bilateral adrenal PCC or extra-adrenal sPGL, and none have looked at somatic mutations by analyzing tumor tissue. Therefore, we have investigated the occurrence of germline and somatic mutations in RET, VHL, SDHB, and SDHD in comparatively large series of bilateral adrenal PCC(n=33 patients) and sPGL (n=26 patients), with the aim of determining the mutation frequency of each of these genes and to establish a genetic testing algorithm. Twenty-one RET, two VHL germline, and one SDHD mutations were found in the patients with bilateral adrenal PCC. In sPGL, one novel SDHB germline and one novel SDHB somatic mutation were observed. In addition, two SDHD germline mutations were found. We conclude that germline RET mutations are predominantly found in bilateral PCC, and that somatic and germline SDHB and SDHD mutations usually occur in sPGL, which has practical consequences for genetic testing algorithms. We suggest that sequential mutation analysis should be directed first at RET, followed by VHL and SDHD for patients with bilateral adrenal PCC at diagnosis, and at SDHB and SDHD for patients with sPGL.

Introduction

Pheochromocytomas (PCC) are rare catecholamine producing tumors that arise from chromaffin cells of the adrenal medulla, but can also occur outside the adrenal in the abdomen, and are then called sympathetic paragangliomas (sPGL)[1]. Pheochromocytoma-associated syndromes include multiple endocrine neoplasia type 2 (MEN2), von Hippel–Lindau disease (VHL), neurofibromatosis- 1, and the familial pheochromocytoma– paraganglioma (PCC–PGL) syndrome[2]. MEN2 is characterized by medullary thyroid carcinoma (MTC) in association with PCC and has three clinical variants, MEN2A, familial medullary thyroid carcinoma (FMTC), and MEN2B[3]. The syndrome is caused by germline mutations of the RET proto-oncogene, which are mostly (80–96%) found in RET exons 10, 11, and 16[3-4]. Somatic RET mutations have also been found in sporadic PCC affecting exons 10, 11, and 16[5].

The VHL syndrome is an autosomal dominantly inherited tumor syndrome, with a prevalence of 2–3 per 100,000 individuals[6]. Patients with VHL syndrome have predisposition to develop retinal and central nervous system hemangioblastomas, clear cell renal cell carcinomas, PCC, pancreatic cysts and islet cell tumors, cystadenomas of the epididymis, and endolymphatic sac tumors[7]. Both germline and somatic VHL mutations have been found in PCC, which include missense, nonsense, splice-site mutations, and small intragenic and large deletions, but most VHL patients with PCC have missense mutations [2, 7-8].

The PCC–PGL syndrome is caused by mutations in subunits of the mitochondrial complex II, also known as succinate dehydrogenase (SDH), which is involved in the electron transport chain and the Krebs cycle. SDH consists of a flavoprotein (SDHA), an iron–sulfur protein (SDHB), and two anchoring membranespanning polypeptides (SDHC and SDHD)[9]. Apart from SDHA, which is related to a rare neurodevelopmental disorder called Leigh syndrome, all three SDH genes have been implicated in the occurrence of PGL. While SDHC has only infrequently been described and exclusively in the context of head and neck PGL, SDHB and SDHD are also associated with abdominal (sympathetic) PGL and adrenal PCC. SDHB mutation carriers present predominantly with sPGL, often with a malignant phenotype, although adrenal PCC and head and neck PGL may occur. SDHD mutation carriers present more head and neck PGL and, at a lower frequency, adrenal PCC and abdominal sPGL, which are almost always benign[10-14].

In VHL disease and the MEN2 syndrome, PCC often have a bilateral adrenal presentation and are occasionally found at extra-adrenal sites[1, 15-16]. In contrast, patients with a germline SDHB mutation present with extra-adrenal catecholamine-producing tumors in 50%of cases. Germline SDHD mutation carriers also develop extra-adrenal catecholamine-producing tumors (sPGL), and present with bilateral adrenal PCC[10, 12-13, 17-18].

Although knowledge about genotype–phenotype relationships has improved for germline RET, VHL, SDHB, and SDHD mutations, only few studies have addressed mutations of these PCC-causing genes in specific series of bilateral PCC and/or sPGL. In addition, there are no studies that have compared tumor tissue and the corresponding normal tissue for the detection of

somatic mutations. To determine the mutation frequency for each of these four candidate genes in a large subset of patients, we have selected 33 patients with bilateral PCC and 26 patients with sPGL, to screen for germline and somatic mutations in RET exons 10, 11, 13, 14, 15, and 16, and in all exons of VHL, SDHB, and SDHD genes. In addition, we discuss the significance of SDHB and SDHD sequence abnormalities.

Material and methods

Patients

Tissue specimens were retrieved from the archives of the Department of Pathology of the Erasmus MC (Rotterdam, The Netherlands), The University Medical Center St Radboud (Nijmegen, The Netherlands), and the University Hospital Zürich (Zürich, Switzerland) following approval of the experimental design and protocols by the Erasmus MC Medical Ethical Committee. These are all tertiary referral centers for endocrine tumor syndromes. A series of 33 bilateral PCCs and 26 sPGLs was selected for mutation analysis of RET exons 10, 11, 13–16 and the entire coding sequence of VHL, SDHB, and SDHD. Of the 33 patients with bilateral PCC, 2 patients had metastases and 31 patients had no metastases. Twenty-six patients with sPGL were selected, of which 15 patients had metastases and 11 patients had no metastases. All samples were coded, so that patient identity was unknown to the investigator. However, a set of clinical data corresponding to the tumor samples was available for further analysis. Throughout this paper, the extraadrenal catecholamine-producing tumors from the abdomen are designated sPGL. Malignancy was defined as the presence of (distant) metastases at sites where chromaffin tissue is not normally present.

Tissue preparation

Initially, mutation analysis was carried out on tumor DNA. Corresponding normal DNA was used to determine whether a mutation was also present in the germline. DNA was isolated from paraffin-embedded tissues or snap-frozen tissues whenever available, using Puregene (Gentra, Minneapolis, MN, USA), according to manufacturers' instructions. The exclusive presence of tumor tissue was confirmed by making control slides prior to DNA extraction. Positive controls from patients with known mutations and negative controls from normal individuals were included in all experiments.

Denaturating gradient gel electrophoresis(DGGE)

PCR with genomic DNA as template was carried out in a 50 ml mixture of 1X PCR buffer (Perkin–Elmer Europe, Rotkreuz, Switzerland) containing 10–400 ng template DNA, 200 µM of each intron-based primer (Table 1), and 1 µl Taq polymerase (Ampli Taq Gold, Perkin–Elmer Europe). After a hot start of 7 min at 95 °C, a 'touch-down' procedure was used consisting of denaturation for 60 s at 95 °C, annealing for 60 s at temperatures decreasing from 60 to 55 °C during the first 11 cycles (with 0.5 °C decremental steps in cycles 2–11), and ending with an extension step for 60 s at 72 °C. Ten cycles with an annealing temperature of 55 °C and 15 cycles with an annealing temperature of 45 °C followed with extension times of 90 s. After a final extension for 10 min at 72 °C, heteroduplex formation was induced by initial denaturation at 98 °C for 10 min followed by incubations at 55 °C for 30 min and 37 °C for 30 min. For DGGE, 10 ml PCR product in 3 µl Ficoll-based loading buffer were loaded onto 10% polyacrylamide gels containing a urea–formamide gradient in 0.5X Tris–acetate–EDTA (TEA). The amplicons were electrophoresed at 60 °C and 100 V for 16 h. DNA strands were visualized using silver staining as described previously[19].

Single-strand conformation polymorphism (SSCP) analysis

PCR amplification of tumor DNA was performed with 10–100 ng DNA in a final volume of 15 µl containing 1.5 mM MgCl2, 10 mM Tris–HCl, 50 mM KCl, 0.02 mM dATP, 0.2 mM dGTP, dTTP and dCTP, 0.8 µCi α32P-dATP (Amersham), 15 pmol of each forward and reverse primer (Table 1), and 3 U Taq polymerase (Ampli Taq Gold, Perkin–Elmer Europe). PCR was performed for 35 cycles at 95 °C for 30 s, 55 °C for 45 s, and 72 °C for 1 min, followed by 1 cycle at 72 °C for 10 min. PCR products were electrophoresed overnight at 8 W on a nondenaturing gel, containing 8% polyacrylamide ((49:1) Fluka, Neu-Ulm, Germany) and 10% glycerol (v/v). After electrophoresis, the gel was dried and exposed to an X-ray film.

DNA sequencing

All samples demonstrating aberrant patterns in the DGGE or SSCP analysis were sequenced. PCR was performed in a final volume of 50 µl under identical conditions as the previous PCR, except that this mix contained 0.2 mM dNTPs instead of 0.02 mM dATPs and 0.8 µCi α32P-dATP. The PCR products were purified using nucleospin Extract II (Macherey- Nagel, Düren, Germany) according to manufacturers' instructions. The purification was followed by a sequence reaction using the Bigdye Terminator V3.1cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). To 1 µl purified PCR product, 2 µl Termination Ready Reaction mix, 2 µl of 5X sequencing buffer, 1 µl forward or reverse primer (13.2 pM), and 14 µl deionized water were added. The cycle Sequencing program was performed for 25 cycles at 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min. This was followed by a precipitation step, adding 13 µl deionized water, 3 ml of 3 M NaAc

Table 1. Primers used for mutation analysis

Locus	forward 5'→3'
RET10	GCGCCCCAGGAGGCTGAGTG
RET11	CTCTGCGGTGCCAAGCCTCA
RET13	CTCAAGCAGCATCGTCTTTG
RET14	GGTTCAAGAGAAAGCTGAGG
RET15	CTCTGCTGGTCACACCAGG
RET16	cgcccgccgcgccccgcgcccggcccgccgcccccgcccgaaataataaaAGGGATAGGGCCTGGGCTTC
VHL1a	gcgcgcgAGCGCGTTCCATCCTCTAC
VHL 1b	gcgcgGCGGAGAACTGGGACGAG
VHL2	cgcccgccgcgccccgcgcccggcccgccgcccccgcccgaaataataaaCTTTAACAACCTTGCTT
VHL3	gcgcgTTCCTTGTACTGAGACCCTAGT
SDHB1	GAGCGACCTCGGGGTTAAG
SDHB2	cgcccgccgcgccccgcgcccggcccgccgcccccgcccgAGCTGAGATGTGTGAACTTT
SDHB3	cgcccgccgcgccccgcgcccggcccgccgcccccgcccGGAACTTTACATAAATACCACTGGA
SDHB4	GATTCCGGATATGGGTGAG / cgcccgcccgccccgcgcccggcccgccgcccccgcccgaaatataaaT
SDHB5	gcgcgTGATGATGGAATCTGATCC
SDHB6	cgcccgccgcgccccgcgcccggcccgccgcccccgcccgaaataataaaCCTCTCTTTTCTCCCCATAC
SDHB7	gcgcgAGCTAATCATCCCTGGTTT
SDHB8	cgcccgccgcgccccgcgcccggcccgccgcccccgcccgGTGGGTTTTCCCTTTCAGTT
SDHD1	cgcccgccgcgccccgcgcccggcccgccgcccccgcccgGTGGGTTTTCGACCTTGAGCCCTCAGGAACG
SDHD2	GATCATCCTAATGACTCTTTCC
SDHD3	ATCTGGTCCTTTTTG
SDHD4	GATTTTTTCTTTTTCT

(pH 5.2), and 64 µl ethanol (100%) to the sequence reaction product and incubating overnight at room temperature before centrifuging for 20 min at 14 000 r.p.m. After washing the samples with 70% ethanol and centrifuging them for 10 min at 14 000 *g*, the pellets were resuspended in 20 µl formamide (Applied Biosystems, Warrington, UK). Products were analyzed on the ABI Prism 3100 genetic analyzer (Applied Biosystems).

Results

Bilateral adrenal PCC

The series of bilateral adrenal PCC encompassed 31 patients with benign PCC and 2 patients with malignant PCC. The results of the mutation analysis are summarized in Table 2. Twenty-one patients (8 males and 13 females) with germline mutations in the RET proto-oncogene were identified in this group (average age 37 years), of which 15 were located in exon 11 (codon 634), 4 in exon 10 (codons 611 and 620), and 2 in exon 16 (codon 918; Fig. 1). No mutations were found in RET exons 13, 14, and 15. All of the RET mutation carriers had benign PCC. After mutation

reverse 5′→3′	size(bp)	method
cgcccgccgcgccccgcgcccgtcccgccgcccccgcgTGGTGGTCCCGGCCGCC	225	DGGE
cgcccgccgcgccccgcgcccggcccgccgcccccgcccgGAGTAGCTGACCGGGAAGGC	262	DGGE
AAGGGAGAAAGAGGGAGAAC	328	SSCP
CTAGAGTGTGGCATGGTGG	524	SEQ
CGGTATCTTTCCTAGGCTTC	296	SSCP
ACCCCAAGAGAGCAACACCC	224	DGGE
cgcccgccgcgccccgcgcccggcccgccgcccccgcccgAGGGCCGTACTCTTCGAC	255	DGGE
cgcccgccgcgccccgcgcccggcccgccgcccccgcccgGCTTCAGACCGTGCTATCGT	418	DGGE
cgtcccgcGTCTATCCTGTACTTACCAC	266	DGGE
cgcccgccgcgccccgcgcccggcccgccgcccccgcccgGCTTCAGACCGTGCTATCGT	316	DGGE
GCTTTCCTGACTTTTCCCTC	157	SSCP
AAGCATGTCCCTAAATCAAA	265	DGGE
cgCTATCAGCTTTGGCCAGC	242	DGGE
cgtcccgcCCCCCATGCAAATAAAAACA	256	DGGE
cgcccgccgcgccccgcgcccggcccgccgcccccgcccgaaataataaaCAGATTGAAACAATAAATAGGGA	261	DGGE
cgCAGCAATCTATTGTCCTCTTG	252	DGGE
cgcccgccgcgccccgcgcccggcccgccgcccccgcccgaaataataaaTTGTGAGCACATGCTACTTC	270	DGGE
cgcccgccgcgccccgcgcccggcccgccgcccccgcccgGTGGGTTTTCCCTTTCAGTT	361	DGGE
cgcccgccgcgccccgcgcccggcccgccgcccccgcccgaaataataaa / gcgcgTCAGGGTGGGAAGACCCCT	153	DGGE
AGCAGCAGCGATGGAGAGAA	168	SSCP
cgcccgccgcgccccgcgcccggcccgccgcccccgcccgCTTTTATGA / gcgcgCAACTATATTTGGAATTGCTATAC	245	DGGE
cgcccgccgcgccccgcgcccggcccgccgcccccgcccgTGATGTTAT / gcgcgCAATTCTTCAAAGTATGAAGTCA	269	DGGE

analyses was performed, it was revealed that 12 patients with RET mutations belonged to four families (with 5, 3, 2, and 2 patients; see Table 2). We did not have clinical information about familial occurrence in four of the RET mutation-positive cases. The mutation analysis showed two patients with R64P germline mutations in exon 1 of the VHL tumor suppressor gene and both had no metastasis. These patients belonged to the same family. In addition, one D92Y mutation was found in exon 3 of the SDHD gene. Thus, if we consider each family as a single entity, we would have 14 index patients with bilateral PCC, in whom we found 12 RET mutations, 1 VHL mutation, and 1 SDHD mutation. None of the patients with bilateral adrenal PCC had mutations in SDHB. However, three heterozygous polymorphisms were found in SDHB or SDHD in two patients harboring a RET C634R mutation. These included A6A in exon 1 of SDHB, and H50R and S68S in exons 2 and 3 of SDHD respectively. On one occasion, SDHB A6A and SDHD H50R were present in the same patient (Table 3). Three patients had metachronous appearance of their bilateral PCC. These patients included one with a C634Y mutation who developed PCC with a 3-year interval, and two patients who did not have mutations in RET, VHL, SDHB, or SDHD, which developed PCC with a 4- and 6-year interval. Twenty-seven of the patients with bilateral PCC had synchronous PCC.

Table 2. Summary of results of bilateral pheochromocytoma mutation carriers

pt	DNA	Sex[a]	Age(years)	b/ m[aa]	gene	Nucleotide change mutation/ polymorphismc[aaa]	Amino acid change[aaa]	family	follow up(Years)
1	F6	m	31	b	VHL	CGC→CCC	R64P	A	21
2	F28	m	29	b	RET	TGC→CGC	C634R	-	
					SDHB	CAC→CCC	A6A	-	
					SDHD	CAC →CGC	H50R	D	26
3	F37	f	32	b	RET	TGC→CGC	C634R	B	24
4	F38	m	42	b	RET	TGC→CGC	C634R	B	22
5	F39	f	42	b	RET	TGC→CGC	C634R	C	24
6	F41	m	16	b	RET	TGC→CGC	C634R	-	
					SDHD	AGC→AGT	S68S	D	21
7	F42	f	29	b	RET	TGC→CGC	C634R	D	24
8	F65	m	u		RET	TGC→CGC	C634R	E	U
9	F78	f	29	b	RET	TGC→CGC	C620R	-	2
10	F79	m	26	b	RET	TGC→CGC	C634R	E	14
11	F80	f	18	b	RET	TGC→CGC	C634R	B	14
12	F82	f	24	b	RET	TGC→CGC	C634R	B	U
13	F84	f	50	b	RET	TGC→CGC	C634R	B	11
14	F87	m	24	b	VHL	CGC→CCC	R64P	A	11
15	F89	f	38	b	RET	TGC→CGC	C634R	C	12
16	F92	f	51	b	RET	TGC→TAC	C611Y	-	12
17	F102	f	27	b	RET	ATG→ACG	M918T	-	10
18	F141	m	49	b	RET	TGC→TGG	C634W	-	U
19	F147	f	27	b	RET	ATG→ACG	M918T	-	U
20	F165	f	59	b	RET	TGC→TAC	C611Y	-	17
21	F168	m	72	b	RET	TGC→TAC	C611Y	-	10†
22	F184	m	53	b	RET	TGC→TAC	C634Y	F	2†
23	F188	f	25	b	SDHD	GAC→TAC	D92Y	-	38
24	F194	f	24	b	RET	TGC→TAC	C634Y	F	9

†, Deceased. [a], male; f, female. [aa], benign; m, malignant; u, unknown. [aaa] Nucleotide and amino acid changes representing polymorphisms are given in italics.

Sympathetic PGL

The sPGL included 11 benign and 15 malignant tumors. The results are summarized in Table 2. Mutation analysis of RET and VHL did not show any abnormalities in the 26 patients with sPGL. In contrast, three aberrations were detected in SDHB exons 4, 5, and 7. The first aberration revealed a novel mutation, S100F in exon 4, which was not present in normal DNA of the same patient. The sPGL of this 25-year-old female patient was localized in the bladder. The second variant pattern of SDHB, in exon 5, was identified as a heterozygous germline S163P substitution, which is described as a rare polymorphism[20]. The third SDHB aberration represented the heterozygous mutation C243S in exon 7, which was also present in the corresponding normal DNA (Fig. 2). Mutation analysis of the SDHD gene showed four abnormal patterns, of which two represented previously described germline mutations (D92Y and L95P). The SDHD D92Y mutation was found

Table 3. Clinical data and mutation analysis results of sympathetic paragangliomas (sPGL)

pt	DNA	Sex[a]	Age (years)	b/ m[aa]	gene	Nucleotide change mutation/ polymorphismc[aaa]	Amino acid change[aaa]	Location sPGL	Location metastasis	follow up (years)
1	F4	f	39	m		-	-	Abdomen	Lung, Lymph node	4†
2	F5	f	70	m		-	-	u	u	1†
3	F7	f	23	m		-	-	Abdomen	Bone	6†
4	F10	m	41	m		-	-	Bladder	Lymph node	6†
5	F12	m	25	m	SDHD	CTG→CCG	L95P	Abdomen	Lymph node	11
6	F13	m	64	m		-	-	Abdomen	Lung, Brain	2†
7	F15	m	42	m		-	-	Abdomen	Lung	u
8	F29	f	62	b		-	-	u	-	u
9	F54	m	56	b	SDHB	TCT→CCT[aaa]	S163P[aaa]	Thorax	-	13†
					SDHD	CAC→CGC[aaa]	H50R[aaa]			
10	F81	f	55	b		-	-	Thorax	-	11
11	F83	m	47	b		-	-	Abdomen	-	u
12	F88	m	79	b		-	-	Abdomen	-	5†
13	F122	f	43	b		-	-	Abdomen	-	u
14	F138	f	70	b		-	-	Abdomen	-	u
15	F140	u	u	m		-	-	Abdomen	Lung	5
16	F160	f	25	b	SDHB	TCT→TTT	S100F	Bladder	-	23
17	F170	f	35	m		-	-	Abdomen	Bone	10†
18	F185	f	56	b		-	-	Abdomen	-	10
19	F188	f	52	b	SDHD	GAC→TAC	D92Y	Abdomen	-	38
20	F207	f	63	m	SDHB	TGC→AGC	C243S	Abdomen	Abdomen, Bone	10†
					SDHD	AGC→AGT[aaa]	S68S[aaa]			
21	F209	m	30	b		-	-	Abdomen	-	5
22	F218	u	u	m		-	-	u	Bone	u
23	F219	u	u	m		-	-	u	Liver	u
24	F220	u	u	m		-	-	Abdomen	Lymph node	u
25	F221	u	u	m		-	-	u	u	u
26	F222	u	u	m		-	-	u	u	u

[a]m, male; f, female; u, unknown. [aa]b,benign; m, malignant; u, unknown. [aaa]Nucleotide and amino acid changes representing polymorphisms are given in italics. The somatic mutation is depicted in bold.
†Deceased.

in a 52-year-old woman, who had a bilateral adrenal PCC, 27 years earlier, which is in our bilateral series as well (Table 2, patient F188). The L95P mutation was found in a patient with sPGL at multiple abdominal spots. The other two patterns appeared to be the H50R and S68S polymorphisms (Table 3). There were very few tumors with mutations in this series which prevented us from drawing any conclusions on genotype–phenotype relationships.

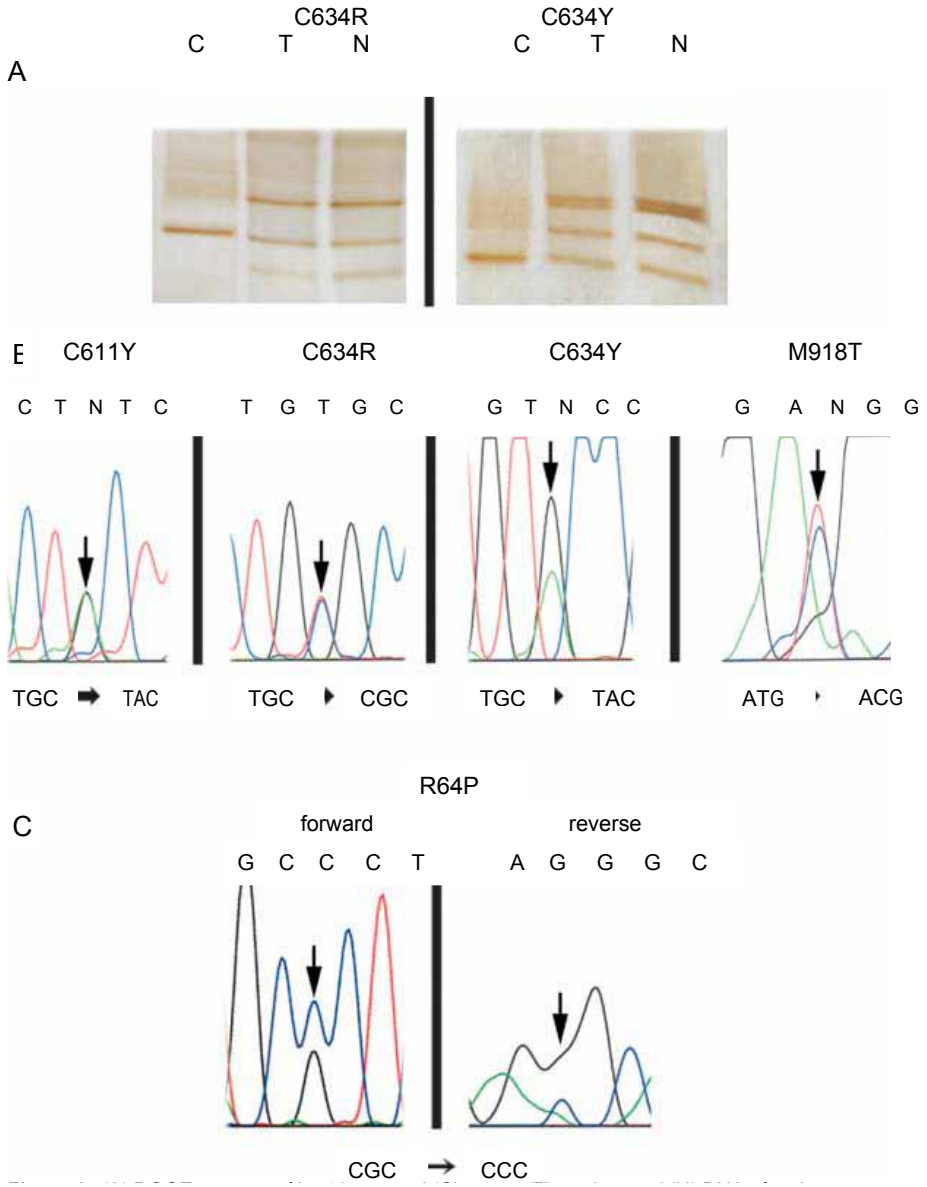

Figure 1. (A) DGGE patterns of healthy control (C), tumor (T), and normal (N) DNA of patients carrying a germline RET C634R or C634Y mutation. (B) Sequence results of four of the six germline RET mutations detected by DGGE mutation analysis: C611Y, C634R, C634Y, and M918T. (C) Forward and reverse sequence of tumor sample carrying the germline VHL R64P mutation found in a patient with bilateral PCC.

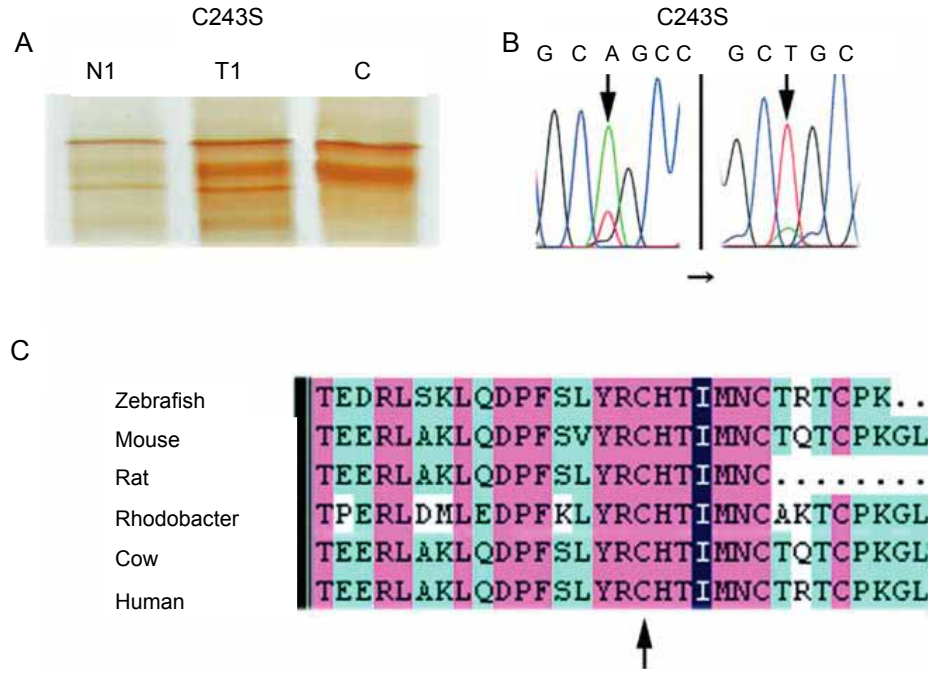

Figure 2. (A) Altered SDHB DGGE pattern in tumor (T1) and corresponding normal (N1) DNA from patient F207 compared with healthy control DNA (C). Note the identical aberrant pattern in both tumor and normal DNA compared with healthy control DNA pattern, indicating germline aberration. (B) Patient F207 tumor DNA sequence result (forward and reverse) of the germline SDHB C243S mutation. Note the relative loss of the T nucleotide compared with the A nucleotide (in the forward sequence; arrow), indicating loss of the wild-type allele (with the A nucleotide) in the tumor. (C) Alignment of SDHB amino acid sequence (NCBI: NM_003000) of a variety of species. The arrow points to the highly conserved C243.

Discussion

It has been reported that patients with RET or VHL germline mutations often present with bilateral adrenal PCC[1, 15], and that part of the germline SDHB or SDHD carriers develop sPGL[8, 13, 17] . However, detailed information about the frequencies of these mutations in patients with bilateral adrenal PCC or sPGL is limited. In addition, there have been virtually no studies based on tumor tissue, which allows the detection of somatic mutations in addition to germline mutations. In the present study, we have investigated tumor tissue and the corresponding normal tissue from a unique series of bilateral PCC and sPGL, predominantly from the Netherlands, for germline and somatic mutations in the above-mentioned PCC susceptibility genes. We found 12 RET, 1 VHL, and 1 SDHD germline mutations in the 23 unrelated patients and families with bilateral PCC, and 2 SDHB and 2 SDHD mutations in the series of 26 sPGLs.

The 12 RET mutations encompassed three mutations in codon 611 (C611Y), one in codon 620 (C620R), six in codon 634 (n=4 C634R, n=1 C634Y, n=1 C634W), and two in codon 918 (M918T).

All of these mutations have been reported previously[10, 21-22]. Since other RET mutations have been described in exons 13, 14, and 15[4], we performed additional SSCP mutation analyses for these exons on the PCC which were negative for mutations in RET exons 10, 11, or 16 or VHL, but no mutations were found. Two patients with bilateral PCC showed the same germline VHL mutation (R64P), and because both patients appeared to be related, they were counted as one patient in our series. These cases were previously reported by van der Harst et al.[23]. The patient with the SDHD D92Y germline mutation will be discussed below.

Mutation analysis of the 26 sPGLs showed one novel germline and one novel somatic variant in SDHB (C243S and S100F) and two germline mutations in SDHD(D92Y and L95P). The novel SDHB C243S variant was seen in a patient with bone metastasis. We consider the SDHB C243S variant as a pathogenic germline mutation, firstly because loss of the wild-type allele was seen in the sequence analysis results in our study (Fig. 2). Secondly, SDHB C243 is highly conserved throughout many species. Thirdly, two studies report patients who developed a malignant PCC and had a SDHB mutation affecting amino acid R242 (R242H),which is also highly conserved[13, 24]. The somatic SDHB S100F variant appeared to be a mutation as well, as loss of the wild-type allele was seen[25]. In addition, this amino acid was conserved throughout many species. Mutations in SDHB S100 have also previously been described by Pollard et al. in a patient with PGL, and Neumann et al. reported a patient with an sPGL who had a SDHB C101Y mutation[13, 26].

Two polymorphisms were found in SDHB: A6A and S163P. the A6A polymorphism was found in one patient (2% of all patients) with an sPGL. A6A was previously reported as a polymorphism with a prevalence of 4% (NCBI: rs2746462), which is in concurrence with our data. The other SDHB polymorphism found in our study was S163P (2% of all patients), which was found in a patient with an sPGL. The frequency in our series was in the same range as the 2.3%S163P substitutions found by Cascon et al. in a healthy control population[20]. Two previously described germline SDHD mutations were found in the sPGL group, D92Y and L95P[27]. Both mutations are known as Dutch founder mutations in head and neck paragangliomas [28]. The patient with the D92Y mutation also had an adrenal bilateral PCC, 27 years earlier, which were surgically removed and included in our bilateral PCC series. The L95P mutation was found in a patient with a benign PCC, and after 12 years of follow up, the patient was alive and well. Both patients are previously described by [27].Two polymorphisms were observed in SDHD, H50R in two patients and S68S in one. Since the frequency of the H50R substitution in our series (4% of all patients) is comparable with the frequency that has been reported in the literature for the normal population, we considered it a polymorphism[29-30]. The SDHD S68S variant was seen in two patients (one with sPGL and one with bilateral PCC) and has previously been described as a polymorphism[20].

Interestingly, the SDHB and SDHD mutation frequencies found in this study are considerably lower (8% for SDHB including the somatic mutation and 8% for SDHD) than that reported in the study of Amar et al. which were 29% for SDHB and 12% for SDHD[10]. This difference might be explained on the basis of geographical variation in mutation frequency, as has become evident from comparative studies[31]. Another study reported a mutation range of 30–41% of

SDHB mutations in a series of catecholamine-producing PGLs[32]. This high mutation frequency could be due to a sample bias, as all PGLs were malignant, whereas only approximately half of our sPGLs were malignant. In addition, the technique used in our investigation cannot be used to detect all genetic changes in these candidate genes, i.e. it is not suitable for detecting large deletions, which have been demonstrated in VHL, SDHB, and SDHD[33-35].

The nucleotide alterations of SDHB and SDHD discussed above are thought to be polymorphisms (SDHB A6A and S163P; SDHD H50R and S68S), because they have also been demonstrated in healthy controls. Though, it is striking that out of four patients with either a SDHB mutation or polymorphism, three patients also had a polymorphism in SDHD (Tables 2 and 3). In our study, one patient with an sPGL harbored the SDHB S163P polymorphism and the SDHD H50R. In addition, the sPGL with the SDHB C243S mutation also showed the SDHD S68S polymorphism. To test whether there was a relationship between SDHB and SDHD polymorphisms and PCC development, we screened an additional series of 89 normal DNA samples of patients with adrenal PCC for the SDHB S163P and SDHD H50R polymorphisms. However, we did not find additional patients with both S163P andH50R, suggesting that there is no relationship between the polymorphisms and the development of adrenal PCC (data not shown). For sPGL, this could not be assessed, due to the limited number of specimens available. Interestingly, SDHB and SDHD polymorphisms were recently demonstrated at increased frequency in patients with familial medullary thyroid carcinoma[36].

In most published series, usually only germline mutations in SDHB and SDHD have been found, especially since most studies have addressed germline DNA only. In this study, we have chosen to perform mutation analysis on DNA from tumor tissue and the corresponding normal tissue, in order not to miss somatic mutations. We found only a single somatic mutation, in the SDHB gene, in our group of 58 patients, although it must be noted that the likelihood of finding such mutations in bilateral PCC is probably low. In our previous studies, we have shown that somatic mutations in RET and VHL occur at a low, but not insignificant, frequency. In contrast, somatic SDHB and SDHD mutations are so far very rare. As such, mutation analysis directed at the detection of somatic mutations in these candidate genes appears not warranted.

In our study, we found 52% RET, 4% VHL, and 4% SDHD germline mutations in 33 patients with bilateral PCC, and 8% SDHB and 8% SDHD mutations in 26 patients with sPGL. Amar et al. recently described 41% VHL (nZ17), 27% RET (nZ11), and 7% SDHD (nZ3) germline mutations in 41 patients with bilateral adrenal PCC[10]. In addition, these authors found 7% VHL (nZ4), 29% SDHB (nZ17), and 12% SDHD (nZ7) mutations in 58 patients with sPGL. These results are not entirely in concurrence with our data. This occurrence is most likely due to geographical differences, as most of our patients were from the Netherlands, whereas all of the patients of Amar et al. were from France[10]. In addition, geographical differences in mutation frequencies of the PCC susceptibility genes between France, Germany, and Italy have recently been shown by Gimenez-Roqueplo et al.[31]. In addition, the technique used in this investigation cannot be used

to detect all genetic changes in these candidate genes, i.e. it is not suitable for detecting large deletions, which have been demonstrated in VHL, SDHB, and SDHD[20, 34-35].

In summary, we have performed mutation analysis on the PCC susceptibility genes RET, VHL, SDHB, and SDHD in a series of bilateral adrenal PCC and sPGL. The bilateral adrenal PCC showed only germline RET, VHL, or SDHD mutations and the sPGL only germline and somatic SDHB or germline SDHD mutations. Our results imply that it is advantageous to first test patients with bilateral PCC for mutations in RET, although, based on previous literature, simultaneous testing for VHL and SDHD appears justified, while patients with sPGL should be first tested for mutations in the SDHB and SDHD genes. In addition, the finding of rare somatic SDHB gene mutations indicates that mutation analysis of tumor DNA should always be considered when germline mutations are not found.

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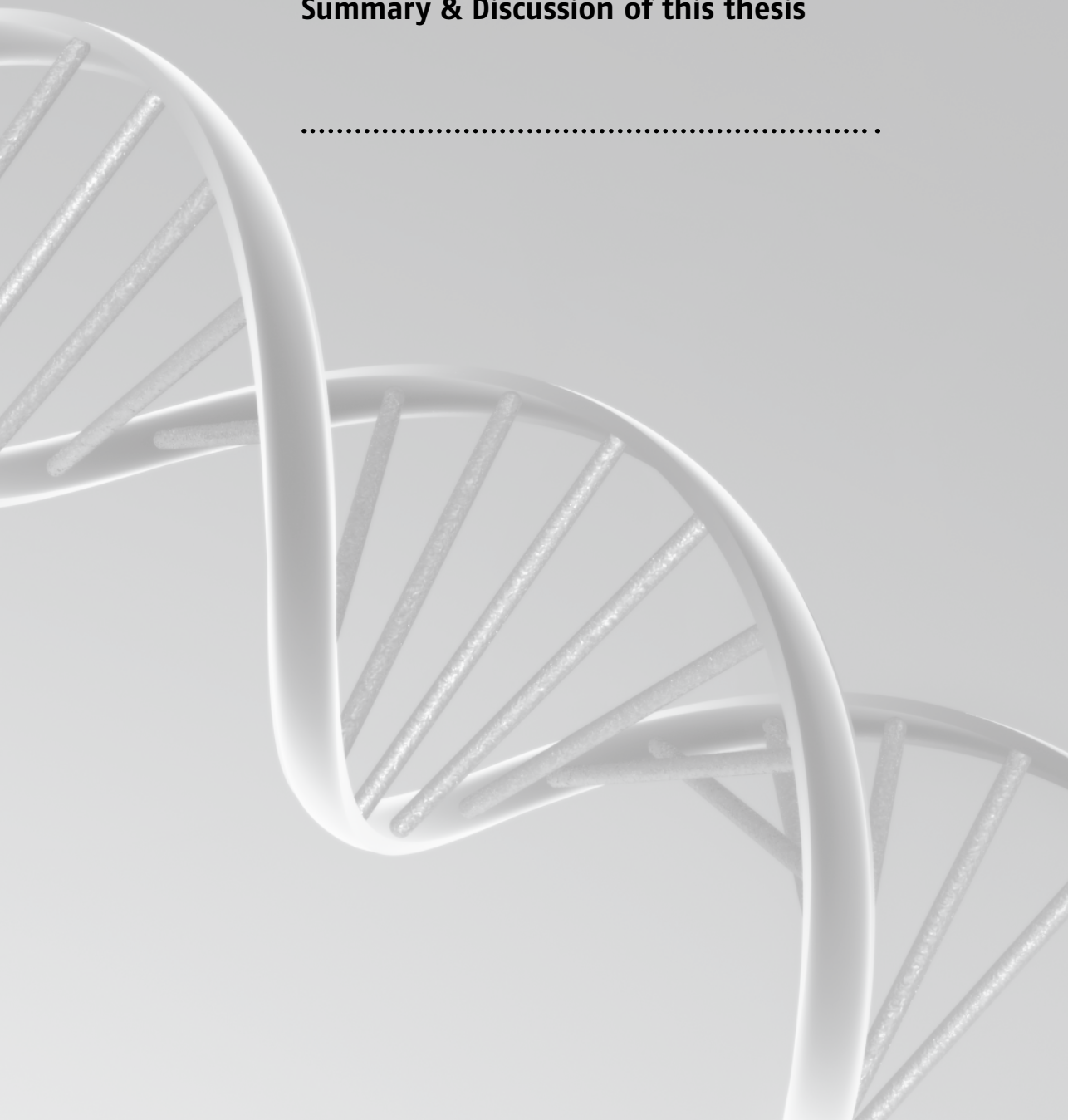
Chapter 8

Summary & Discussion of this thesis



Malignancy

In clinical practice, it is not possible to predict whether a primary pheochromocytoma (PCC) or sympathetic paraganglioma (sPGL) is malignant. Presently, the only criterion for malignancy in PCC and sPGL is the presence of tumor metastasis or extensive locally invasive growth of PCC[1-2]. It is known that the frequency of malignant behavior in sPGL is higher than in PCC, which is mostly related to mutations in the SDHB gene[3-5]. Furthermore a Pheochromocytoma of the Adrenal gland Scaled Score (PASS) has been proposed, in which the incorporated histopathological features identify tumors with more aggressive biological behavior[6]. But, significant interobserver and intraobserver variation occurs in the assignment of PASS, and so further refinement and validation is necessary[7]. There are no suitable immunohistochemical differences between benign and malignant PCC. Salmenkivi et al. suggested some potential markers of malignancy, including tenascin, cyclo-oxygenase 2, and vascular endothelial growth factor[8-10]. Bjorklund et al. suggested stathmin as a marker for malignancy[11]. These studies await further confirmation in larger series. Also the high expression of hTERT (human telomerase reverse transcriptase) as well as heat-shock protein 90 and telomerase activity were suggested to be associated with malignant behavior, but these studies did not always adhere to the strict criteria of malignancy and were also performed on low numbers of tumors[12].

Furthermore, molecular approaches have been performed to clarify the differences between malignant and benign PCC. In former CGH and LOH studies from our group, in which we investigated 29 tumors, we found frequent loss of 6q and 11q as well as gain of 9q and 17q[13]. Of all losses and gains, loss of 6q and 17p appeared to be the strongest in their association with malignancy[13]. In chapter 2 we have performed a detailed microsatellite and CGH analysis in 68 PCC to confirm the relationship between 6q and malignancy in a larger group. However this study reveals that there is no correlation between 6q loss and malignant behavior. But, we found several new chromosome arm alterations, including loss of 8p and 18p, and gains of 5p and 7p, which might have a possible association with malignant PCC. In chapter 3 we also performed a comprehensive, more detailed analysis of 17p in a larger series of benign and malignant PCC to detect abnormalities in p53 in relationship with malignant behavior at the DNA and protein level using CGH, FISH, SSCP and IHC. Although we found a loss rate of 45% in CGH analysis at the *p53* locus, which was confirmed by FISH analysis, we could not detect gene mutations in *p53* exons 5–8, nor did we find a high frequency of p53 protein overexpression by immunohistochemistry. Only two metastases showed nuclear immunostaining leading us to suggest that *p53* abnormalities do not appear to play a major role in the tumorigenesis of benign and malignant pheochromocytomas, except for a possible minor role in metastatic progression. Abnormalities in *p53* thus cannot be used for the distinction between benign and malignant pheochromocytomas. Further analysis of the newly revealed copy number changes should be done in future experiments on sufficiently large series of tumors. In the before mentioned

studies presented in this thesis we have shown that malignant PCC show more copy number changes than benign PCC, probably due to genetic instability of malignant tumors.
Another study from our group has shown that both benign and malignant PCC may show heterogeneous molecular abnormalities in various parts of the tumor and that such heterogeneity occurs more extensively in malignant tumors, compromising the results of CGH analyses[14].
Another approach to the differentiation between benign and malignant PCC is the use of RNA expression platforms. This technique has great potential for classifying tumors according to their RNA expression profile. Waldmann et al. revealed a more than twofold difference in expression between benign and malignant PCCs in 132 genes: 19 were up-regulated and 113 were down-regulated. Expression differences of six genes (calsequestrin, NNAT, neurogranin, secreted protein acidic and rich in cysteine (SPARC), EGR2, and MAOB) were confirmed by RT-PCR in 25 PCCs. IHC for calsequestrin revealed an overexpression in malignant PCCs (7/10 versus 1/10, P=0.03)[15]. Preliminary data of our own study of 54 PCC revealed 113 genes, which were differentially expressed between malignant and benign samples. However, further investigations are necessary to provide an accurate profile to predict clinical behavior.

Tumorigenesis

Conventional comparative genomic hybridization (CGH) and loss of heterozygosity (LOH) analysis, as described in chapters 2 and 3, reveals patterns of nonrandom copy number alterations. These experiments were done to narrow down the region most frequently involved, with the final goal to identify responsible genes to get better informed about the pathogenesis and clinical behavior of these tumors. There are several disadvantages of these techniques, including the limited resolution of CGH, only detecting large deletions (> 5 Mb). LOH analysis is often hampered by inaccurately mapped, and insufficient numbers of markers. Initially, to unravel tumorigenesis in pheochromocytomas (PCC), LOH studies were performed showing losses of 1p, 3p, 3q, 17p, and 22q, which were subsequently corroborated by whole genome analysis by CGH, which allows investigating gains and losses in all chromosomal regions in one experiment[13, 16-17]. It has been suggested that losses of 1p and 3q are early aberrations in PCC development, and the remaining aberrations are thought to be late events[13]. We and others have confirmed the abovementioned losses with CGH, which had been initially found by LOH (chapters 2 and 3).
Subsequently, in chapter 4, we investigated a large series of PCC precursor lesions from patients with MEN 2 syndrome, so called adrenomedullary hyperplasia (AMH) to find out if AMH carry the same genetic changes as seen in PCC. We have shown that AMH in the context of MEN 2-syndrome has identical genomic aberrations as PCCs, at similar frequencies, and should thus be regarded as PCC precursor lesions. In addition, the finding of clonal molecular aberrations in AMH indicates that these lesions are not hyperplasias but small nodular pheochromocytomas.

Furthermore, our results could have implications for the treatment of MEN2 patients, as these very small lesions (AMHs) should be regarded as precursor lesions for PCC and can be treated with total or cortex-sparing adrenalectomy. Our results aid in our understanding of PCC tumorigenesis and may alter the surgical approach in MEN2 patients with synchronous or metachronous adrenomedullary tumors.

More recently, improved techniques, including high-resolution tiling bacterial artificial chromosome array CGH analysis gave an even more detailed assessment of chromosomal alterations due to its better resolution. Aarts et al. performed such analysis on chromosome arm 1p, and revealed that 1pcen-1p32.3 and 1p34.3-1p36.33 loss are frequent genetic events in the development of sporadic and MEN 2A-associated PCC, although corresponding candidate genes were not identified[18]. In addition, van Nederveen et al., using a genome wide submegabase-resolution tiling array on a large series of sporadic PCC, could distinguish two distinct subgroups of PCC. One with loss of 1p and/or 3q, representing more than 56% of all benign PCC investigated, which can also be found in MEN 2-related PCC. A second smaller group with loss of 3p with or without concurrent 11p loss, representing 32% of PCC, which also can be found in VHL-related PCC[19]. These results indicate that sporadic PCC could develop through different pathways, as shown in their different hereditary counterparts.

In chapter 5 we performed a conventional CGH analysis of eight adrenal tumors that occurred in association with germline SDHD mutations. The aim of this study was to analyze the pattern of changes and classify these tumors in one of subgroups that we defined previously. Interestingly, we found DNA alterations identical to those that are described in head and neck paragangliomas (HNPGL). This leads us to the conclusion, that while these tumors were located in the adrenal gland and thus should be called PCC, from a clinical as well as a molecular point of view, rather should be classified as paragangliomas.

Apart from being used to distinguish PCC for their clinical behavior, RNA expression arrays may shed light on tumorigenesis. Dahia et al did one of the earlier studies on PCC using this platform.[20]. They described how SDH- and VHL-related PCC are connected to a *HIF1α* regulatory loop (Figure 1). A part of sporadic tumors segregated with these SDH- and VHL-related PCC, essentially supporting our CGH data.

Familial tumor syndromes

The prevalence of germline mutations in *RET, VHL, NF1, SDHB, SDHC* and *SDHD* among patients presenting with PCC appears to be much higher than previously supposed. Fifteen years ago, it was thought that approximately 10% of these tumors occur in a familial setting. The occurrence of PCC and sPGL in the setting of MEN 2, VHL, and NF1 is known for some time now, but in the last decade, an important percentage of cases of familial PCC, but especially of sPGL and HNPGL has been attributed to germline mutations in *SDHB*, *SDHC*, and *SDHD*[21-22]. Even more

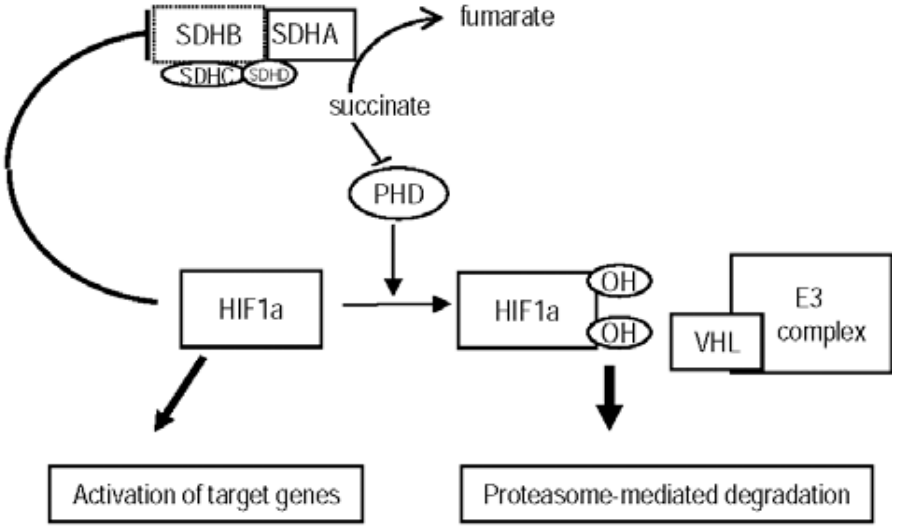


Figure 1. Proposed model of HIF1a and SDHB interrelationship. HIF1a downregulates SDHB, which leads to complex II dysfunction. High succinate levels resulting from loss of complex II, in turn, inhibit prolyl hydroxylase (PHD) activity [19]. Non-hydroxylated HIF1a is resistant to VHL-mediated targeting for degradation and can therefore activate downstream genes, such as angiogenic factors. "E3 complex" indicates the E3 ubiquitin ligase complex for which VHL is the substrate recognition factor20.

recently, mutations in *SDHA* and *SDHAF2* have been found in some PCC and PGL patients[23-24]. *SDHAF2* appears to be the gene located in the PGL2 candidate region and has been described in two families with HNPLG, but not in a large series of PCC[23]. *SDHA* had been known only for its homozygous mutations in the context of Leigh syndrome, a lethal neurodegenerative disorder, but has now been found as a heterozygous mutation in a patient with an sPGL[25].

Screening on a large series of apparently sporadic PCC for *RET*, *VHL*, *SDHD*, and *SDHB* mutations has been done, initially by SSCP, and later mostly with direct sequence analysis. This has revealed mutation frequencies up to 35%. We found 8% *VHL*, *SDHB*, and *SDHD* mutations in 136 apparently sporadic PCC[26]. Bryant et al. had found that 11% of patients had a *VHL* mutation, 5% a *RET* mutation, 4% a *SDHD* mutation, and 4% an *SDHB* mutation in a group of apparently sporadic PCC[27]. Mannelli et al. found 32.1% mutations in 501 consecutive patients with PCC[28]. In chapter 6 we investigated different subgroups, including PCC in children, patients with bilateral PCC, and sPGL. In 10 pediatric PCC from 10 patients, two patients had germline *RET* exon 11 mutations (C634R) and 1 patient had an R64P germline mutation in the *VHL* gene. In the remaining 7 patients there was one patient from a family fulfilling the clinical criteria for VHL disease. All tumors were benign and were located in the adrenals. From our findings we conclude that a large proportion (40%) of pediatric PCC patients is diagnosed in the context of inherited cancer syndromes. In a subsequent nationwide study an even higher percentage of germline mutations was found, with 21 of 30 patients that could be analyzed showing mutations in *RET*, *VHL*, *SDHB*, or *SDHD* (Boerman et al. unpublished observations).

Furthermore, in chapter 7 we have performed mutation analysis on the PCC susceptibility genes *RET*, *VHL*, *SDHB*, and *SDHD* in a series of bilateral adrenal PCC and sPGL. The bilateral adrenal PCC showed only germline *RET*, *VHL*, or *SDHD* mutations (61%) and the sPGL only germline and somatic *SDHB* or germline *SDHD* mutations (15%). From the studies described in chapter 6 and chapter 7, including the studies described above, it appears justified to offer genetic testing to every patient presenting with PCC, with or without a positive family history.

Genetic testing in pheochromocytomas

Although most inherited PCC present before the age of 50 years, some tumors occur later[29]. Knowing the incidence of inherited PCC after the age of 50 years and bearing in mind the cost-effectiveness of genetic testing, some have advocated that all patients younger than 50 years should be referred for genetic assessment. In addition the high percentage of genetic aberrations justifies genetic testing in every patient. When patients present with extra-adrenal, bilateral or multiple PCCs, they should always be referred for genetic investigation, regardless of age, because it is known that such presentations frequently occur in the context of a heritable tumor syndrome. There are several reasons to recommend genetic testing. First, there is a clear benefit for the patient, as other tumors that occur in the various tumor syndromes can be screened for and treated at an early stage. This is especially true for contralateral PCC, which may influence the surgical strategy, or sPGL in other locations. Secondly, family members can be screened, which may also lead to early recognition of the disease in some of them, and thereby to improved treatment and prognosis of affected family members.

As more genetic testing will be performed in PCC and PGL patients, it is likely that increased numbers of patients with hereditary cancer syndromes will be detected at a stage at which they present with a PCC or PGL only. Once a germline mutation has been found in any of the candidate genes, further screening tailored to the specific syndrome should be performed. Fortunately, such protocols have been developed during recent years for MEN 2, VHL, and the PCC-PGL syndrome. These testing algorithms are also valid for screening for contralateral PCC, recurrent PCC or PGL, and for metastases of malignant PCC or PGL. It is prudent to advise patients to come to the hospital in the intervals between screenings if they experience PCC/PGL-related symptoms, which are described in chapter 1.

During the development of testing algorithms for patient presenting with PCC, a study was done to determine the value of SDHB immunohistochemistry for discriminating between SDH-related and non SDH-related PCC and PGL[30]. The results of this study show that SDHB immunohistochemistry can indicate the presence of *SDHB*, *SDHC*, and *SDHD* germline mutations with a sensitivity of 85% and a specificity of 100%. 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. A study performed by Gill et al. does support these results[31]. These findings indicate

that before genetic testing SDHB immunostaining should be done and the results of this cost-sparing test will lead to a decreased number of genes that must be tested. Testing algorithms for sporadic PCC, bilateral PCC, and sPGL are shown in Figure 13, respectively.

As described above, the spectrum of PCC/sPGL-susceptible genes is increasing. Recently, *isocitrate dehydrogenase type 1 and 2, SDHAF2*, *TMEM127,* and *PHD2* mutations have been described in association with PGL and/or PCC, and sPGL[32-35]. However, only few patients have been described with these mutations, mostly PGL. Bearing in mind the cost-effectiveness of genetic testing, these genes are currently not included in the testing algorithms.

Consequences of positive or negative tests as described in figure 1-3 are always resection of the tumor. Follow up and screening regimen for each syndrome are summarized in table 1.

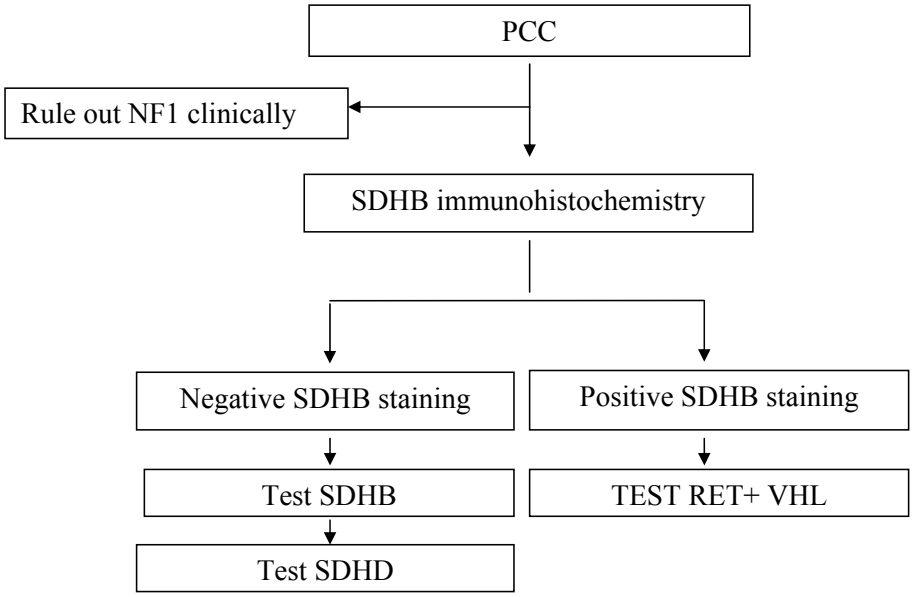


Figure 1. Flow chart for genetic testing in patients with a unilateral, apparently sporadic pheochromocytoma (PCC). NF, neurofibromatosis; RET, REarranged during Transfection; VHL, von Hippel–Lindau disease; SDHB, succinate dehydrogenase subunit B; SDHD, succinate dehydrogenase subunit D

Overview and future prospects

To date there is still no effective test to predict malignancy. Several markers have been identified, but these are not suitable for clinical practice yet. Reproducibility and studies on larger series are lacking. New genome-based tools, including gene expression arrays and other detailed assessments of the whole genome have to be improved to develop a feasible test for predicting the clinical behavior of PCC. These techniques could also be used for revealing the pathogenesis of PCC, although the understanding of pathways and the involved genes is expanding rapidly in

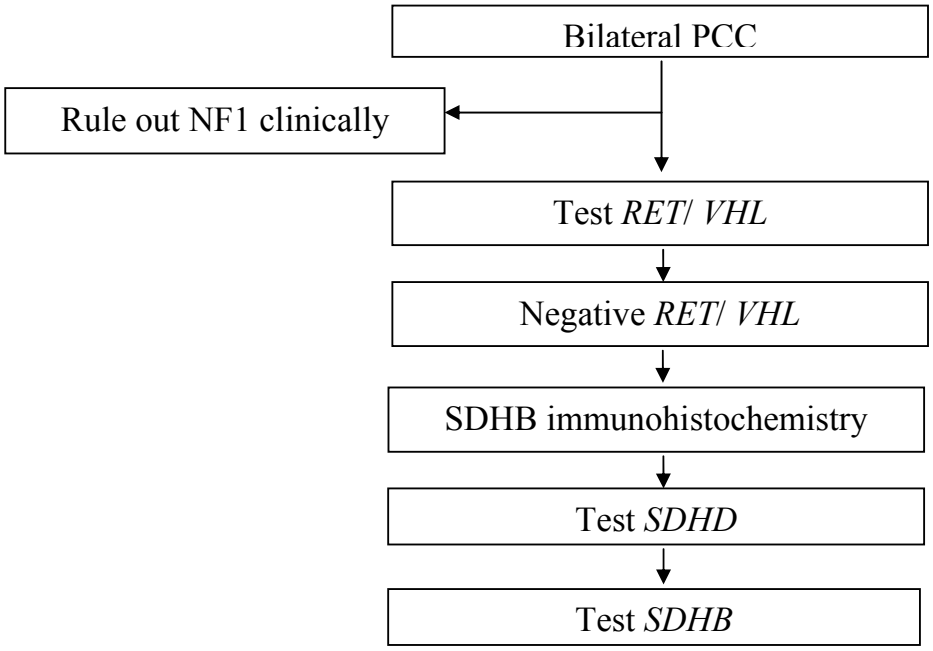


Figure 2. Flow chart for genetic testing in patients presenting with bilateral pheochromocytoma (PCC). NF, neurofibromatosis; RET, REarranged during Transfection; VHL, von Hippel–Lindau disease; SDHB, succinate dehydrogenase subunit B; SDHD, succinate dehydrogenase subunit D

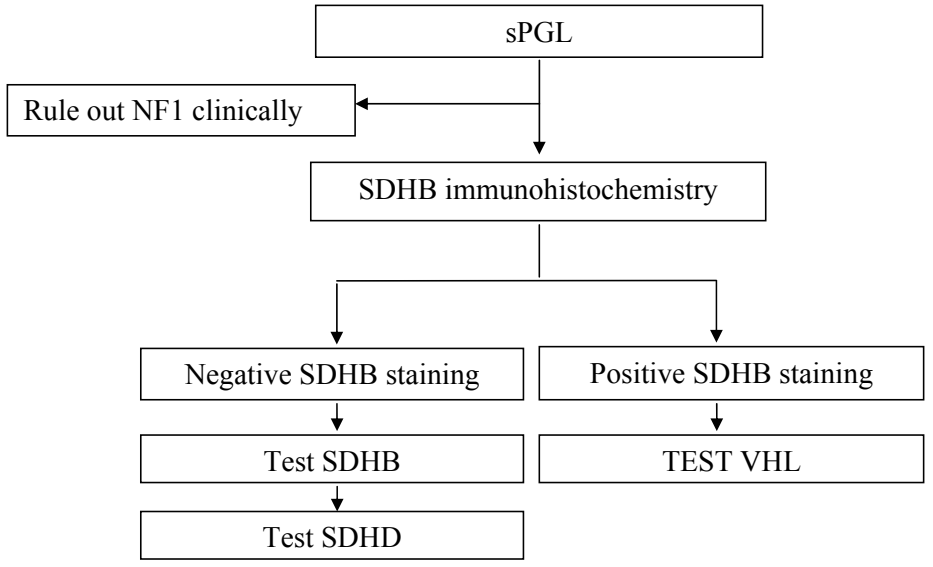


Figure 3. Flow chart for genetic testing in patients harboring sympathetic paraganglioma (sPGL). SDHB, succinate dehydrogenase subunit B; SDHD, succinate dehydrogenase subunit D; VHL, von Hippel–Lindau disease

the last decade. In clinical practice physicians should realize that PCC and PGL occur more often in the setting of hereditary cancer syndromes than previously thought. Immunohistochemistry for the SDHB protein and genetic testing for *RET, VHL, SDHB, SDHC,* and *SDHD* mutations in patients should become routine and such patients should be referred to a clinical geneticist. All patients and family members with proven mutations should be entered into a standardized screening protocol. The preferred treatment of PCC and PGL is surgical resection.

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Chapter 9

Samenvatting

Curriculum vitae

Publication list

Samenvatting

Pheochromocytomen (PCC) zijn relatief zeldzame neuro-endocriene tumoren. Eén van de grootste problemen in het management van PCC is dat het klinisch gedrag van deze tumoren onvoorspelbaar is. Het enige criterium dat gehanteerd wordt voor maligniteit is de aanwezigheid van metastasen. Er zijn momenteel geen histologische, immunohistochemische of moleculaire markers die goedaardige van kwaadaardige tumoren kunnen onderscheiden. Daarnaast is er weinig bekend over de ontstaanswijze van deze tumoren. In grote array-CGH studies zijn er onlangs verschillende patronen ontdekt waarin zich genen bevinden die mogelijk zouden kunnen leiden tot het ontstaan van PCC. Zo is er een groep PCC, met name sporadische en multipele endocriene neoplasie type 2 (MEN 2) gerelateerde PCC, die afwijkingen laten zien op chromosoomarm 1p en of 3q. Een tweede groep laat met name chromosoomarmafwijkingen zien op 3p met, of zonder 11p en q. Deze tumoren gaan gepaard met het Von Hippel Lindau (VHL) syndroom en succinaatdehydrogenase (SDH) gerelateerde tumoren. Echter, welke genen precies betrokken zijn is niet bekend.

Er is in de laatste tien jaar gebleken dat er een veel groter percentage van PCC familiair voorkomt dan tot dan toe werd aangenomen. Naast de reeds bekende familiaire tumorsyndromen, zoals MEN 2, het VHL syndroom en patiënten met neurofibromatose type 1 (NF1), zijn ongeveer 10 jaar geleden de SDH genen ontdekt waardoor het percentage van familiair voorkomende PCC ongeveer van 10% naar 25% is gestegen. Ook de zich steeds ontwikkelende diagnostische technieken hebben het percentage doen stijgen naar ongeveer 35%. Er zijn in het laatste jaar enkele nieuwe genen, zoals *IDH type 1* en *2*, *SDHAF2*, *TMEM127*, en *PHD2* ontdekt die mogelijk betrokken zijn bij het familiair voorkomen van deze tumoren. Het verhoogde percentage van familiaire PCC heeft consequenties voor het klinisch beleid dat wordt gevoerd bij patiënten met PCC. Bij iedere patiënt met PCC is het gerechtvaardigd om een familiaire component uit te sluiten, dan wel aan te tonen. Hierdoor kan er vroeger gescreend worden op deze potentieel gevaarlijke tumor bij overige familileden.

Het doel van dit proefschrift, gebaseerd op de bovengenoemde ontwikkelingen, is:

1. Het zoeken naar moleculaire markers die de pathogenese van PCC kunnen verduidelijken, alsmede het vinden van moleculaire markers om het klinisch gedrag van PCC in een vroeger stadium te kunnen voorspellen.
2. Het identificeren van verschillende subgroepen van PCC en het bepalen van mutatiefrequenties van de verschillende kandidaatgenen.
3. Het ontwikkelen van algoritmes voor genetische testen bij patiënten met PCC om bovengenoemde tumorsyndromen te identificeren en om PCC of recidief PCC in een vroeg stadium te diagnosticeren.

In het eerste deel van het proefschrift hebben we, naar aanleiding van een onderzoek van Dannenberg et al., die een conventioneel CGH onderzoek hebben gedaan waaruit blijkt dat er een verband zou kunnen bestaan tussen chromosoomarmafwijkingen op 6q en 17p en

maligniteit, een meer uitgebreider analyse gedaan naar chromosoomarm 6q en kwaadaardig gedrag van PCC. In hoofdstuk 2 wordt een LOH analyse beschreven met 12 6q markers en CGH, uitgevoerd op 68 PCC, inclusief 51 benigne en 17 maligne PCC, waarvan 53 sporadische en 15 syndroom gerelateerde PCC. Uit de LOH analyse bleek dat 14/31 (45%) van de PCC ten minste één allelische deletie had, maar geen enkele marker liet meer dan 21% verlies zien. Concluderend kan gezegd worden dat er geen aanwijzingen zijn voor betrokkenheid van 6q bij de pathogenese van PCC. In deze studie werd er geen verband aangetoond van 6q verlies met gemetastaseerd PCC. Deze laatste bevinding werd bevestigd door CGH analyse op de 68 tumoren.

In hoofdstuk 3 wordt er een uitgebreide analyse beschreven van de p53 genlocatie, gelegen op chromosoomarm 17p met de betrekking op een mogelijke relatie met maligne gedrag van PCC. Er werd een CGH analyse van 31 maligne en 20 benigne tumoren gedaan waaruit bleek dat het verlies van de p53 locus op chromosoom 17p13.1 in 23/51 (45%) gevallen voorkomt. Deze resultaten werden bevestigd met behulp van fluorescentie in situ hybridisatie. Alle kwaadaardige tumoren en de tumoren met verlies van de p53 locus werden geanalyseerd op p53 mutaties in exonen 5-8, maar ook hierin werden geen afwijkingen gevonden. Daarnaast werd p53 immunohistochemie op de tumoren uitgevoerd, waaruit bleek dat er in slechts twee metastasen van PCC sterke nucleaire p53 expressie was; alle andere tumoren waren negatief. concluderen dat, hoewel er regelmatig verlies van de p53 locus op 17p voorkomt, kunnen we concluderen dat het p53-gen waarschijnlijk geen belangrijke rol speelt bij het ontstaan van PCC.

Het tweede deel van dit proefschrift is met name gericht op het moleculaire genotype van de subgroepen van de eerder genoemde erfelijke PCC, dit wederom met behulp CGH en LOH analyse. In hoofdstuk 4 hebben we ons de vraag gesteld of precursorlaesies, laesies kleiner dan 1 cm in diameter, de zogenaamde adrenale medullaire hyperplasie (AMH), bij de MEN 2 patiënten gelijke genetische afwijkingen hebben als hun grotere "broertjes", de PCC in MEN 2 patiënten. We onderzochten 19 PCC en 13 AMH van 18 genetisch bewezen MEN2 patiënten. Door middel van LOH analyse werden de chromosomale regio's 1p13, 1p36, 3p, en 3q onderzocht, lokalisaties op het genoom die in het overgrote deel van MEN 2 PCC afwijkend zijn. In alle gevallen van AMH werden afwijkingen gevonden op de onderzochte locaties en deze kwamen in gelijke aantallen voor als in de PCC bij het MEN 2 syndroom. Deze resultaten geven aan dat AMH in de klinische praktijk beschouwd moeten worden als PCC.

In hoofdstuk 5 werden patiënten bestudeerd die bekend zijn met het pheochromocytoom-paraganglioom (PCC-PGL) syndroom subtype 1, en die naast een hoofd hals paraganglioom (PGL) ook een PCC hebben ontwikkeld. Het doel van deze studie was om het patroon van chromosoomarmveranderingen te analyseren en deze tumoren te classificeren in een van de subgroepen die eerder werden gedefinieerd. We hebben daarom een conventionele CGH analyse van acht PCC die een bewezen kiembaan succinaat dehydrogenase subtype D (SDHD) mutatie hadden en kwamen tot de conclusie dat deze PCC exact hetzelfde patroon met afwijkingen, namelijk verlies van 11q, laten zien als familiaire PGL. Dit zou kunnen wijzen op een derde

pathway waarin PCC kunnen ontstaan. Daarnaast zouden deze afwijkingen kunnen passen bij gemetastaseerd PGL. Deze bevindingen hebben echter (nog) geen klinische consequenties.

Het laatste deel van dit proefschrift is gewijd aan het genetisch testen van kandidaatgenen in specifieke subgroepen van PCC en PGL patiënten. Dat wil zeggen: multipele PCC, extra-adrenale PCC, bilaterale PCC en PCC bij kinderen. De hypothese was dat er in deze subgroepen een substantieel hoger percentage met genmutaties zou voorkomen dan in de enkelvoudige adrenale PCC. In hoofdstuk 6 onderzochten we 14 PCC van 10 kinderen en testten we de eerder beschreven kandidaatgenen. Hieruit bleek dat in deze groep de mutatiefrequentie 40 % was. Dit was aanmerkelijk hoger dan het op dat moment bekende percentage van 25%. Dit heeft geleid tot het genetisch testen van alle kinderen die een PCC ontwikkelen.

In hoofdstuk 7 worden de resultaten beschreven van de mutatieanalyse van de bilaterale PCC en extra-adrenale PCC. We onderzochten 33 patiënten met bilaterale PCC en 26 patiënten met extra-adrenale PCC. Het bleek dat er bij patiënten met een bilateraal PCC een zeer hoge kans op familiaire belasting is, met name bij patiënten met het MEN 2 syndroom. Bij de extra-adrenale tumoren kwamen er met name *SDHx* mutaties voor, dit echter in een verwacht percentage. Met de conclusies van deze studie werden flowdiagrammen gemaakt om het genetisch testen van patiënten met een PCC zo efficiënt mogelijk te laten verlopen.

Tenslotte geeft hoofdstuk 8 een overzicht van de huidige kennis van de pathogenese van erfelijke en sporadische PCC, tevens een inzicht in de mogelijkheid tot het differentiëren tussen benigne en maligne PCC. Daarnaast zijn er suggesties voor toekomstig onderzoek gedaan en worden er voorstellen gedaan voor genetische testschema's die iedere clinicus kan gebruiken om elke patiënt met PCC optimaal te behandelen, te vervolgen en tijdig te diagnosticeren.

Curriculum Vitae

Bart-Jeroen Petri werd geboren op 8 maart 1977 te Vlaardingen. In 1996 behaalde hij het gymnasiumdiploma aan de Vlaardingse Openbare Scholengemeenschap (VOS) in Vlaardingen. Aansluitend startte hij met de studie geneeskunde aan de Erasmus Universiteit in Rotterdam. In het kader van het doctoraalexamen werd een onderzoeksstage verricht op de afdeling Pathologie van het Erasmus MC (Prof.dr. R.R. De Krijger/ Dr. W.N.M. Dinjens) Met dit onderzoek zette hij de eerste stappen in de totstandkoming van dit proefschrift. Na het doctoraalexamen werd dit onderzoek vervolgd op dezelfde afdeling, nu in samenwerking met de afdeling Heelkunde (Prof. dr. C.H.J. Van Eijck) van het Erasmus MC. In het kader van dit onderzoek was hij voor een half jaar verbonden aan de afdeling Pathologie van het Labor für Endokrinpathologie (Prof.dr. Komminoth en Dr. A. Perren) van het Univeritätsspital te Zürich, Zwitserland. Het artsexamen werd behaald in 2006 en van juli 2006 tot januari 2008 was hij werkzaam als arts-onderzoeker op de eerder genoemde afdelingen, via een door MRACE gesubsidieerd project van het Erasmus MC. Gedurende twee jaar deed hij onderzoek naar de pathogenese en de genetische achtergrond van pheochromocytomen en paragangliomen . Van juli 2007 tot en met oktober 2007 was hij werkzaam als AGNIO op de afdeling Heelkunde van het Erasmus MC (Prof.Dr. J.N.M. IJzermans). In januari 2008 begon hij aan de opleiding tot chirurg in het Erasmus MC (Opleiders Prof.dr. J.N.M. IJzermans & Prof.dr. J.J.B. van Lanschot). Sinds januari 2010 is hij werkzaam als AIOS chirurgie in het Maasstad Ziekenhuis te Rotterdam (Opleider Dr. E. van der Harst).

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