THE MOLECULAR PATHOGENESIS OF PHEOCHROMOCYTOMAS AND PARAGANGLIOMAS

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"By wisdom a house is built, and by
understanding it is established;
by knowledge the rooms are filled with
all precious and pleasant riches."
Proverbs 24, 3-4

To my parents

MOLECULAR PATHOGENESIS OF PHEOCHROMOCYTOMAS AND PARAGANGLIOMAS

Moleculaire pathogenese van pheochromocytomen en paragangliomen

Proefschrift

ter verkrijging van de graad van doctor
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Chapter 1

Introduction

General introduction

General aspects

Paraganglia are small neuroendocrine organs, that usually manifest as anatomically discrete bodies, the parenchymal cells of which are neural crest-derived, and produce catecholamines and various peptides. One group of paraganglia is aligned to the sympathoadrenal and the other to the parasympathetic autonomic nervous system.[1,2] Sympathetic paraganglia are distributed along the pre- and paravertebral sympathetic chains and follow the sympathetic innervations of the pelvic and retroperitoneal organs. Their exact locations are somewhat variable, with the exception of the adrenal medulla and the organs of Zuckerkandl. Parasympathetic paraganglia are almost exclusively located in the region of the cranial and thoracic branches of the glossopharyngeal and vagal nerves. The principal glossopharyngeal paraganglia are the tympanic paraganglia, situated in the wall of the middle ear, and the carotid bodies (Figure 1).

Neoplasms of the neuroendocrine cells found within the sympathetic or parasympathetic paraganglionic axes are designated pheochromocytoma and paraganglioma. The name pheochromocytoma – the Greek synonym of black colored tumor – is derived from the so-called chromaffin reaction, resulting from oxidation of stored catecholamines. Adrenal tumors are usually referred to as pheochromocytomas (because of a positive chromaffin reaction), whereas the extra-adrenal ones are alternatively designated extra-adrenal pheochromocytoma or paraganglioma, the latter name usually being reserved for parasympathetic or nonfunctional (*i.e.*, non catecholamine-secreting) sympathetic tumors. Paraganglioma is also used to describe the parasympathetically aligned tumors. However, since these neoplasms are otherwise indistinguishable, and the chromaffin reaction appeared to be a poor indicator of catecholamine storage, the terminology may be confusing. For this reason, in this thesis, all sympathicoadrenal neoplasms will be referred to as pheochromocytomas. Additional designations (e.g., nonfunctional, extra-adrenal, or malignant) will be used when appropriate. Neoplasms arising from parasympathetic paraganglionic tissues will be referred to as paragangliomas. In the literature, some of the latter may have been referred to as chemodectomas, glomus jugulare, or carotid body tumors.

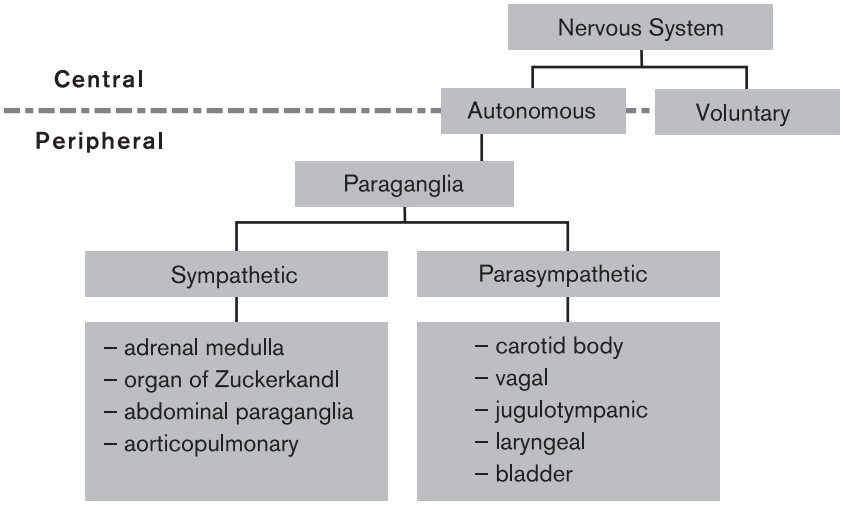

Figure 1 Schematic overview of sympathoadrenal and parasympathetic paraganglioma. Paraganglia are formed by neural crest cells. Fate mapping of the neural crest cells has shown that the trunk neural crest cells that migrate via a so-called ventral pathway contribute to form the sympathetic and parasympathetic (para)ganglia as well as the adrenal medulla. Other domains of the neural crest may contribute as well. Both types of paraganglia are characterized by catecholamine- and peptide-producing secretory cells. Differences in tissue organization and in specific secretory products suggest that they respond to different signals and perform varied functions in different locations.

Pheochromocytoma, tumors of the sympathoadrenal lineage

Pheochromocytoma (PCC) is a rare tumor of chromaffin cells of the sympathetic paraganglionic tissue, most commonly arising from the adrenal medulla. The tumor is usually benign, but may be malignant, with metastases being apparent at first diagnosis or manifesting themselves later, even after clinically disease-free intervals exceeding 15 years.[3,4]

Clinical symptoms

PCC is 'the great mimic' of a variety of conditions, often hampering a successful diagnosis. Most tumors are associated with inappropriate catecholamine secretion, which classically manifests itself with sustained or intermittent hypertension associated with paroxysmal symptoms.[5] Such 'spells' often include headache, sweating, palpitation, tachycardia, and severe anxiety along with epigastric or chest pain. PCC should also be considered if a patient has labile hypertension, or hypertension resistant to antihypertensive therapy.[6] Less commonly, severe hypertensive reactions may occur during incidental surgery, following trauma, exercise, or even micturition in the setting of PCC of the urinary bladder. Orthostatic hypotension is frequent and is probably caused by reduction of intravascular volume following chronic adrenergic stimulation. Correct

diagnosis is important because resection of the tumor dramatically reverses the clinical symptoms and may cure the hypertension.[7] Conversely, a missed or delayed diagnosis may cause considerable morbidity and even mortality.[8-10]

Epidemiology

The annual incidence of PCC is 2 to 8 cases per million population.[11-14] PCCs represent about 5% of incidental adrenal masses[15], and are the cause of hypertension in approximately 0.03 to 0.06% of hypertensive patients.[16,17] From these data, it is estimated that approximately 30-130 cases of PCC are diagnosed in The Netherlands each year. Autopsy series suggest that up to one-third of PCCs remain un-diagnosed throughout life.[18]

With respect to the diagnosis and management of this tumor, it is helpful to remember the 'rule of 10%'. That is, 10% of neoplasms occur in children, 10% of sporadic cases are bilateral, 10% are extra-adrenal, and 10% are malignant.[19-22,23] Also, 10% of PCC patients have a positive family history for associated tumor syndromes, although recent evidence points to a genetic predisposition in up to 24% of apparently sporadic PCC patients.[24] The mnemonic provides only an estimate of these various features of PCC, and there is obviously overlap between the pediatric, familial, and bilateral groups.[25]

Recently, a Swedish study revealed an increased tumor-related mortality after diagnosis of adrenal PCC. For both men and women this mortality was four times higher than for controls. Liver/biliary tract and central nervous system tumors in men, and malignant melanoma and uterine cervical cancer in women, were significantly over-represented.[26]

Associated familial tumor syndromes

The most common familial syndromes that include PCC as an element are the autosomal dominant Multiple Endocrine Neoplasia syndrome types 2A and 2B, and von Hippel-Lindau disease.[19,20,27,28] PCC is also observed in neurofibromatosis type 1, as a component of Carney's triad, and in families associated with paraganglioma and/or extra-adrenal PCC.[29,30,31]

Multiple Endocrine Neoplasia type 2 (MEN 2)

MEN 2 (Sipple syndrome; MIM 171400) is a rare syndrome, with less than 1000 kindreds worldwide.[32] The MEN 2 syndrome is classified into three subtypes: familial medullary thyroid carcinoma (MTC), MEN 2A, and MEN 2B, all characterized by the presence of medullary thyroid carcinoma in nearly 100% of patients. MEN 2A and MEN 2B have an equally increased risk for PCC (occurring in 50% of patients). It is an intriguing and unexplained fact that these patients develop adrenal PCCs but no or hardly any extra-adrenal ones.

MEN 2A is by far the most common form of MEN 2, and is further characterized by parathyroid adenoma in 15%–30% of patients.[33]

Additional features of MEN 2B patients include a marfanoïd habitus and multiple mucosal neuromas.[34] Parathyroid hyperplasia is not associated with MEN 2B. Patients with MEN 2B have a worse prognosis than those with MEN 2A, mainly because MTC is more aggressive and develops earlier in life in MEN 2B patients, compared to MEN 2A patients.[35] The molecular basis of this difference may recently be clarified as MEN 2B MTC exhibit upregulation of gene products that have been previously associated with an increased metastatic potential.[36]

MEN 2 patients have germline mutations in the *RET* proto-oncogene, located at 10q11, which can be identified at 'hot spots' in 97% of individuals with MEN2. *RET* mutations in MEN 2A patients occur predominantly in sequences coding for cysteine residues of the extracellular domain (codons 609, 611, 618, 620, 630, and 634). In contrast, all MEN 2B mutations are located in sequences coding the intracellular domain, with over 95% being located at residue 918 (M918T). Clinically sporadic PCC is rarely associated with germline mutations in the RET proto-oncogene.

Von Hippel-Lindau (VHL) disease

VHL (MIM 193300) disease is a familial tumor syndrome with an autosomal dominant pattern of inheritance. It has an estimated incidence of 2–3 per 100,000 persons.[37] A germline mutation in the VHL gene predisposes carriers to the development of highly vascularized tumors.[38] These tumors include hemangioblastoma in the retina (also referred to as retinal angioma), cerebellum, and spine; renal cell carcinoma (clear cell type); PCC; islet cell tumors of the pancreas; endolymphatic sac tumors; and cysts and cystadenoma in the kidney, pancreas, epididymis, and broad ligament.[39,40] At present, metastatic renal cell carcinoma and neurological complications from cerebellar hemangioblastoma are the most common causes of death. Periodic clinical examination and advanced operation techniques can reduce both morbidity and mortality in patients with VHL disease.[39]

Mutations or deletions in the VHL gene (chromosomal locus 3p25) have been identified in the germline of nearly all tested VHL patients and have in addition been found in about 6% of apparently sporadic PCC.[41-44] Genotype-phenotype correlations have revealed that specific genetic abnormalities can result in four clinical subtypes with different tumor-specific susceptibilities. Of all VHL patients 10-34% develop PCC (VHL type 2), and 96% of these patients harbor missense mutations, as opposed to VHL patients without PCCs (VHL type 1) who frequently harbor deletions or nonsense mutations.45,46 Most PCCs are located in the adrenal gland, but extra-adrenal PCCs are not uncommon. Occasionally, VHL patients also develop PGL (47, unpublished observation).

Neurofibromatosis type 1 (NF1)

NF1 (Von Recklinghausen's disease; MIM 162200), the most common single-gene disorder affecting the human nervous system, is characterized by the occurrence of multiple neurofibromas, by various skin lesions, such as café-au-lait spots, axillary freckling, and cutaneous neurofibromas, and by various other signs and symptoms, such as Lisch nodules of the iris.[48] It is estimated that PCCs develop in about 1-5% of NF1 patients, thus accounting for approximately 5% of all PCCs.[49-53] Patients with NF1 also have an increased incidence of some malignant tumors, including malignant peripheral nerve sheath tumors, and leukemia, particularly juvenile chronic myelogenous leukemia.[54] Like in MEN2 patients, the vast majority of PCCs are located in the adrenal gland.

The *NF1* gene on chromosome 17q11.2 is classified as a tumor suppressor gene; however, the function of neurofibromin, the *NF1* gene product, is not yet fully understood. Molecular genetic testing for mutations in *NF1* is available, but the diagnosis is typically made on a clinical basis. The clinical manifestations of *NF1* and severity of the disease can vary substantially, even among family members carrying the same mutation. There is no apparent relationship between severity of *NF1* and age at diagnosis, birth order, parental age, or environmental factors.[55]

Familial pheochromocytomas

Familial clustering of PCC (MIM 171350) with or without PGL (MIM 16800) has been described in the absence of clinical signs of MEN 2, VHL, or NF1.[56] In the course of our studies, we and others found that most of these patients or families exhibit germline mutations in the genes of the mitochondrial enzyme complex succinate dehydrogenase. Although it is always difficult to prove a negative, unexplained familial clustering of pheochromocytoma still occurs, suggesting a distinct genetic entity.[57]

Malignancy

In reported series, the frequency of malignancy among PCCs has ranged from 5 to 36%.[58-60] These different frequencies may have resulted from selection bias or from different definitions of malignancy. In several studies, local invasion has been regarded as sufficient evidence of malignancy, but most workers accept only distant metastasis as proof of malignancy. Accordingly, about 13% of all PCCs manifest themselves as malignant tumors.[20] Extra-adrenal PCC has been associated with a higher frequency of manifest malignancy,[61] although this has been challenged by some.[62,19] In patients with irresectable, recurrent, or metastatic disease, overall 5-year survival is less than 50%, but some patients survive for many years,[63] with occasional patients living more than 20 years after detection of metastatic disease.[64,65] Overall, there appears to be two distinct subsets of malignant PCC: a group with aggressive behavior leading to early death (within 3 or 4 years) and a group with a far more protracted course of disease reflected in long-term survival (up to 20 or more years).

Treatment

Management of PCC includes establishment of a biochemical diagnosis, preoperative blockade of catecholamine receptors, appropriate imaging, and, if feasible, complete surgical tumor removal. Laparoscopic adrenalectomy is the modality of choice for adrenal pheochromocytomas.[66] If the primary tumor is localized to the adrenal gland and metastases are apparently absent, survival is good, with 94% surviving over 10 years.[63] Most patients become normotensive after surgery, but in some hypertension persist, because of concurrent essential hypertension or residual tumor. The lack of discriminating features of malignancy necessitates life-long follow-up in all instances.

With respect to malignant PCC, pharmacological treatment of the catecholamine excess is mandatory and surgery, radiation therapy, or chemotherapy, or a combination thereof, usually provides palliative benefit.[67,68-76,77,78,79]

Paraganglioma, tumors of the parasympathetic lineage

Paraganglioma (PGL) originates from neural crest-derived chief cells of the paraganglia along the parasympathetic nervous system. The carotid body is the most frequent location (60%), followed by the jugulotympanic paraganglia.[80-82] PGL is slowly growing, highly vascularized, and most often benign, but metastatic spread occurs in 2-19% of patients, depending on tumor location. Malignancy is least common (2–4%) for jugulotympanic tumors, approximately 6% for carotid body tumors, and 16–19% for vagal tumors.[83]

Clinical symptoms

PGL most commonly presents as a painless neck mass, or with symptoms due to compression of the nearby structures (pharyngeal fullness, dysphagia, pain, and coughing). Depending on the anatomic localization, pressure on cranial nerves may cause bradycardia, hoarseness, or hearing loss. Approximately 1% of the patients have symptoms of catecholamine hypersecretion, similar to PCC.

Epidemiology

The annual incidence is estimated at 1:30,000, and the tumors most often become clinically apparent in the fourth or fifth decade.[81]

Etiology

Although most parasympathetic PGLs occur sporadically, there is a positive family history in a considerable minority (10-50%) of patients, with an autosomal dominant mode of transmission and incomplete and age-dependent penetrance.[84-86] Multiple PGLs, and young age of onset are characteristic of familial PGLs, but such features have also been reported in apparently sporadic cases.[87,88] The female predominance in sporadic PGL, the equal sex distribution

among familial patients, and the almost exclusively paternal inheritance pattern in these families, suggests maternal imprinting of the disease gene.[89] It has been demonstrated recently that mutations of the succinate dehydrogenase subunit D (*SDHD*) gene are a causal factor in some PGL families.[90] In addition, mutations of the SDH subunit B (*SDHB*) and subunit C (*SDHC*) have been identified in PGL families.[91,92]

Treatment

There are currently three treatment strategies: surgery (with or without preoperative embolization), radiation therapy, or observation.[93-95] Because tumor growth but also surgery can cause disabling loss of function of nearby structures, preoperative estimation of tumor growth is an important parameter in clinical decision-making. A "wait and scan" approach may thus be the best approach in some instances, although surgical resection remains the mainstay of curative treatment.[96,97] Needless to say, the uncertainty about possible malignant potential may be an important consideration militating against an expectative approach.[98]

Alike, yet distinct

PCCs and PGLs both arise from neuroendocrine cells that have differentiated from a common population of neural crest-derived precursor cells.[99] The tumors share several histopathologic features, to the extent that without clinical data about the location and hormone status of the tumor, the discrimination of PCC and PGL would be very difficult indeed.[100-103] As mentioned previously, concurrence of both PCC and PGL has been described in few patients,[104-107] often in association with mutations in genes of the SDH complex,[108] or with Carneys triad.[109] These concurrences are suggestive of a similar pathogenic pathway in PCC and PGL.

However, as described above, genetic predisposition to PCC or PGL is associated with different hereditary tumor syndromes, and there is a contrast in the frequency of hormone secretion (90% versus 1%, respectively).[110]

Predicting malignancy

Predicting malignant behavior in PCC and PGL is notoriously problematic. The search for markers predictive of malignancy, especially in PCC, has been the object of many studies and has included, among others, studies on hormone secretion, histological features, nuclear volume, DNA ploidy, gene mutations and gene expression.[4,102,111-156] However, although some of the parameters identified in these studies show some prognostic significance, none of them allows the distinction of malignancy on an individual basis.

Table 1 Hereditary Pheochromocytoma.

			Familial		Reference	Apparently sporadic		Reference
			14% (107/738)		[HD, 19, 20, 58, 163, 164]	86% (631/738)		[HD, 19, 20, 58, 163, 164]
MEN2		*RET*	70%	(107)	[HD, 19, 20, 58, 163, 164]	0-5%	(605)	[24, 43, 44, 154, 165, 166, 171, 197]
VHL		*VHL*	14%	(107)	[HD, 19, 20, 58, 163, 164]	2-11%	(558)	[24, 42-44, 166, 197]
NF1		*NF1*	~5%‡	(107)	[HD, 19, 20, 58, 163, 164]	~2%‡		
PGL	PGL1	*SDHD*	~1%^^	(9)	[HD, 158, 169 , 170, 196]	0-4%	(457)	[HD, 24, 166]
	PGL3	*SDHB*	~3%^^	(15)	[91, 167, 168]	4-10%	(379)	[24, 91, 166]
PGL3	PGL3	*SDHC*	0%	(9)	[91, 158]	0%	(47)	[91, 158]
no mutation			3-7%	(16)	[91, 167, 168, 196]	76-88%	(355)	[HD, 24, 166]

¶ 5% in US population and 35% in Dutch population.
HD Dannenberg H. et al., J Clin Oncol., accepted for publication.
‡ Distribution on the basis of 50% de novo mutations.
^^ Familial PCC in which MEN2/VHL/NF1 is ruled out, constitutes ~4% of all familial PCC patients. One-third of these harbors a germline SDHD mutation and 60% harbors a germline SDHB mutation.

Table 2 Hereditary Paraganglioma.

			Familial		Reference	Apparently sporadic		Reference
			10-50% (129/1056)		[84, 85, 89]	50-90% (927/1056)		[84, 85, 89]
MEN2		*RET*	0%	(9)	[158, 161]	0%	(42)	[158, 161]
VHL		*VHL*	*	(2)	[47, 158]	*	–	[47, 158]
NF1		*NF1*	NK‡			NK‡		
PGL	PGL1	*SDHD*	50-100%	(61)	[159, 160, 162]	5-35%¶	(130)	[159, 160, 162]
	PGL3	*SDHB*	20%	(10)	[159]	3%	(37)	[159]
	PGL4	SDHC	~10%	(14)	[92, 157-159]	<2%	(40)	[158, 159]
no mutation			0-10%	(61)	[159, 160, 162]	65-90%	(130)	[159, 160, 162]

* Occasionally, VHL patients develop (and may present with) cervical PGL.
¶ 5% in US population and 35% in Dutch population.
‡ No studies available, but presumably 0%.

Pathogenesis

The hereditary perspective

The recent identification of the genes involved in PCC and PGL predisposition provides a new angle for the understanding of the pathogenesis of these tumors. To date, 1 oncogene (*RET*) and 5 tumor suppressor genes (*VHL, NF1, SDHB, SDHC*, and *SDHD*) are known to be involved in PCC and/or PGL susceptibility. In the course of the studies described in this thesis, germline mutations of genes for members of the succinate dehydrogenase family (*SDHD, SDHB*, and *SDHC*) were identified as causing familial PGL and, less frequently, familial PCC.

As was already known from the literature and as has been studied in greater depth in this thesis, germline mutations are found in nearly all familial tumors and in a considerable subset of apparently sporadic PCCs (Table 1) or PGLs (Table 2).[157-171] Intriguingly however, somatic mutations in each of these genes are uncommon in the truly sporadic examples, with prevalence figures ranging from 0 to about 15%.

Secondary molecular events

The absence or low incidence of somatic mutations in the *RET, VHL, NF1*, and *SDHD, SDHB*, or *SDHC* genes in sporadic tumors, has led us to use a broader approach in the search for clues to PCC and PGL tumorigenesis. Others have studied aberrations in specific genes that, based on new insights from the literature, appeared to be candidate genes. The *c-mos* gene, for example, was screened for mutations in PCCs after PCCs were observed in *c-mos* knockout mice.[172,173] No mutations were found, however. Other genes that have been unsuccessfully screened for mutations include *p16INK4A, endothelin B, RASSF1A*, and *MEN1*.[173-176] From loss of heterozygosity (LOH) studies, losses of chromosomal regions 1p (15-80%),[177-183] 3p (20%),[177,181] 11p,[180,184] 17p (20%),[181,185] and 22q (20-50%)[177,181,183,186] are known to occur frequently in PCC.[179-184,186-188]

LOH analysis did not reveal additional loci of interest in PGL.[189,190] The number of studies of PGLs are, however, limited and include only a few immunohistochemical studies for IGF-II, c-myc, bcl-2, c-jun, and VEGF.[191-195]

Because of the limitations of LOH analysis, such as insufficient and incorrectly mapped markers, we and others have used more sensitive techniques such as comparative genomic hybridization (CGH) and microarray CGH analysis, in order to study chromosomal aberrations in sporadic as well as in inherited PCCs and PGLs.

Aims and outline

Aims

One of the most challenging clinical problems with regard to both PCC and PGL concerns the prediction of malignant potential of the tumor. Metastases, occurring in approximately 13% of PCCs and 5% of PGLs, are the only currently available reliable indicator of malignancy. Metastases may be present at the time of diagnosis or become apparent later, sometimes after disease-free intervals of 25-30 years. This obviates the need for more accurate markers predicting the clinical behavior of these tumors. The genetic mechanisms underlying the pathogenesis of these tumors and the progression towards malignancy are poorly understood. Current evidence indicates that the genes that play a role in the hereditary forms of these tumors are uncommonly affected in their sporadic counterparts. Based on common histologic origin and features, it seems reasonable to hypothesize that the pathogenesis of PCC and PGL may have features in common.

The identification of molecular markers of malignancy would enable physicians to identify patients at risk to develop metastases and to direct follow-up examinations more specifically to these patients. Furthermore, unraveling the tumorigenetic mechanisms underlying tumor development and progression may also help to develop new methods for the treatment of malignant PCC and PGL.

The aims of this thesis, following from the above considerations, are:

- To search for genetic aberrations that distinguish benign from malignant tumors.
- To gain insight into the contribution of germline and somatic mutations of PCC- and PGL associated genes (VHL, SDHD) in the sporadic forms of these tumors.
- To study the pathogenetic relationship between PGL and PCC.

Outline

Previous analyses of the responsible genes of these cancer syndromes have revealed that somatic mutations of the *RET* and *VHL* genes are infrequent in sporadic PCCs.[91,177,196-198] Regarding *VHL* gene alterations, very few have analyzed malignant PCCs so that the clinical significance of the presence or absence of *VHL* alterations with regard to the biology of sporadic PCC has remained unclear. A correlation between somatic *VHL* mutations with nonfavorable prognosis has recently been suggested in a study of renal clear cell carcinoma (RCC), another VHL-related tumor.[199] **Chapter 2** describes the frequency and spectrum of *VHL* alterations in apparently sporadic PCCs in relation to their clinical behavior. In **chapter 3** we examined the value of *SDHD* mutation screening in patients presenting with apparently sporadic and familial PCC. **Chapter 7** shows the results of a similar analysis of sporadic and familial PGL.

The generally low prevalence of somatic mutations in the *RET, VHL, NF1,* and *SDHD, SDHB*, and *SDHC* genes in sporadic tumors, calls for a broader approach in the search for clues to PCC and PGL tumorigenesis. Therefore, we used chromosome CGH to investigate chromosomal copy number changes in 29 sporadic PCCs (**chapter 4**) and 16 PGLs (**chapter 6**).

The results of the former were used as the basis for **chapter 5**. This study reports the preliminary results of a micro array CGH study with a high-resolution array for chromosome 1p. Conventional CGH does not allow specification of the target region, because of its relatively low resolution. The purpose of the study was to narrow down the deleted region at chromosome 1p.

Epidemiological and genetic features suggest that chronic hypoxia can induce PGL. *p53* inactivation is one of the most important tumorigenetic mechanisms, which would enable hypoxic cells to escape from cell cycle arrest and apoptosis. **Chapter 8** describes a study that was undertaken to determine whether **p53** inactivation contributes to PGL tumorigenesis.

Finally, **chapter 9** presents an overview of our current understanding of the molecular pathogenesis of PCC and PGL and offers suggestions for further research.

References

1. Langley JN: The Autonomic Nervous System. Cambridge, UK, Heffer, 1921.
2. Glenner GG, Grimley PM: Tumors of the extra-adrenal paraganglion system (including chemoreceptors). Atlas of Tumor Pathology, Washington DC, Armed Forces Institute of Pathology, 1974.
3. Baba T, Machida K, Ozaki I, Okushima T, Murabayashi S, Kamata Y, Imamura K, Takebe K: A malignant pheochromocytoma with ileus, polyuria and hypercalcemia: a case of recurrence 17 years after the initial operation. *Endocrinol Jpn* 1985, 32:337-345.
4. Schlumberger M, Gicquel C, Lumbroso J, Tenenbaum F, Comoy E, Bosq J, Fonseca E, Ghillani PP, Aubert B, Travagli JP, et al.: Malignant pheochromocytoma: clinical, biological, histologic and therapeutic data in a series of 20 patients with distant metastases. *J Endocrinol Invest* 1992, 15:631-642.
5. Manger WM, Gifford RW: Pheochromocytoma. *J Clin Hypertens* (Greenwich) 2002, 4:62-72.
6. Bravo EL, Tagle R: Pheochromocytoma: state-of-the-art and future prospects. *Endocr Rev* 2003, 24:539-553
7. Geissendorfer R: Beobachtungen bei 4 operierten Phaochromocytomen. Langenbecks *Arch Klin Chir Ver Dtsch Z Chir* 1951, 267:167-173.
8. Schenker JG, Granat M: Phaeochromocytoma and pregnancy--an updated appraisal. *Aust N Z J Obstet Gynaecol* 1982, 22:1-10.
9. Kizer JR, Koniaris LS, Edelman JD, St John Sutton MG: Pheochromocytoma crisis, cardiomyopathy, and hemodynamic collapse. *Chest* 2000, 118:1221-1223.
10. Lee BM, Ti LK: A painful pulsatile abdominal mass in a young man with elevated blood pressures: an unusual presentation of phaeochromocytoma. *Anaesth Intensive Care* 2002, 30:490-494.
11. Fernandez-Calvet L, Garcia-Mayor RV: Incidence of pheochromocytoma in South Galicia, *Spain. J Intern Med* 1994, 236:675-677.
12. Andersen GS, Toftdahl DB, Lund JO, Strandgaard S, Nielsen PE: The incidence rate of phaeochromocytoma and Conn's syndrome in Denmark, 1977-1981. *J Hum Hypertens* 1988, 2:187-189.
13. Stenstrom G, Svardsudd K: Pheochromocytoma in Sweden 1958-1981. An analysis of the National Cancer Registry Data. *Acta Med Scand* 1986, 220:225-232.
14. Beard CM, Sheps SG, Kurland LT, Carney JA, Lie JT: Occurrence of pheochromocytoma in Rochester, Minnesota, 1950 through 1979. *Mayo Clin Proc* 1983, 58:802-804.
15. Barzon L, Sonino N, Fallo F, Palu G, Boscaro M: Prevalence and natural history of adrenal incidentalomas. *Eur J Endocrinol* 2003, 149:273-285.
16. Anderson GH, Jr., Blakeman N, Streeten DH: The effect of age on prevalence of secondary forms of hypertension in 4429 consecutively referred patients. *J Hypertens* 1994, 12:609-615.
17. Omura M, Saito J, Yamaguchi K, Kakuta Y, Nishikawa T: Prospective study on the prevalence of secondary hypertension among hypertensive patients visiting a general outpatient clinic in Japan. *Hypertens Res* 2004, 27:193-202.
18. Sutton MG, Sheps SG, Lie JT: Prevalence of clinically unsuspected pheochromocytoma. Review of a 50-year autopsy series. *Mayo Clin Proc* 1981, 56:354-360.
19. Goldstein RE, O'Neill JA, Jr., Holcomb GW, 3rd, Morgan WM, 3rd, Neblett WW, 3rd, Oates JA, Brown N, Nadeau J, Smith B, Page DL, Abumrad NN, Scott HW, Jr.: Clinical experience over 48 years with pheochromocytoma. *Ann Surg* 1999, 229:755-764; discussion 764-756.
20. Mannelli M, Ianni L, Cilotti A, Conti A: Pheochromocytoma in Italy: a multicentric retrospective study. *Eur J Endocrinol* 1999, 141:619-624.
21. O'Halloran T, McGreal G, McDermott E, O'Higgins N: 47 years of phaeochromocytomas. Ir Med J 2001, 94:200-203.

22. Mathew S, Perakath B, Nair A, Seshadri MS, Shanthly N: Phaeochromocytoma: experience from a referral hospital in southern India. *Aust N Z J Surg* 1999, 69:458-460.
23. Wyszynska T, Cichocka E, Wieteska-Klimczak A, Jobs K, Januszewicz P: A single pediatric center experience with 1025 children with hypertension. *Acta Paediatr* 1992, 81:244-246.
24. Neumann HP, Bausch B, McWhinney SR, Bender BU, Gimm O, Franke G, Schipper J, Klisch J, Altehoefer C, Zerres K, Januszewicz A, Eng C, Smith WM, Munk R, Manz T, Glaesker S, Apel TW, Treier M, Reineke M, Walz MK, Hoang-Vu C, Brauckhoff M, Klein-Franke A, Klose P, Schmidt H, Maier-Woelfle M, Peczkowska M, Szmigielski C: Germ-line mutations in nonsyndromic pheochromocytoma. *N Engl J Med* 2002, 346:1459-1466.
25. Carney JA, Sizemore GW, Sheps SG: Adrenal medullary disease in multiple endocrine neoplasia, type 2: pheochromocytoma and its precursors. *Am J Clin Pathol* 1976, 66:279-290.
26. Khorram-Manesh A, Ahlman H, Nilsson O, Oden A, Jansson S: Mortality associated with pheochromocytoma in a large Swedish cohort. *Eur J Surg Oncol* 2004, 30:556-559.
27. Gimm O: Multiple endocrine neoplasia type 2: clinical aspects. *Front Horm Res* 2001, 28:103-130.
28. Hes FJ, Hoppener JW, Lips CJ: Clinical review 155: Pheochromocytoma in Von Hippel-Lindau disease. *J Clin Endocrinol Metab* 2003, 88:969-974.
29. Kalff V, Shapiro B, Lloyd R, Sisson JC, Holland K, Nakajo M, Beierwaltes WH: The spectrum of pheochromocytoma in hypertensive patients with neurofibromatosis. *Arch Intern Med* 1982, 142:2092-2098.
30. Carney JA: Gastric stromal sarcoma, pulmonary chondroma, and extra-adrenal paraganglioma (Carney Triad): natural history, adrenocortical component, and possible familial occurrence [see comments]. *Mayo Clin Proc* 1999, 74:543-552.
31. Parkin JL: Familial multiple glomus tumors and pheochromocytomas. *Ann Otol Rhinol Laryngol* 1981, 90:60-63.
32. Eng C, Clayton D, Schuffenecker I, Lenoir G, Cote G, Gagel RF, van Amstel HK, Lips CJ, Nishisho I, Takai SI, Marsh DJ, Robinson BG, Frank-Raue K, Raue F, Xue F, Noll WW, Romei C, Pacini F, Fink M, Niederle B, Zedenius J, Nordenskjold M, Komminoth P, Hendy GN, Mulligan LM, et al.: The relationship between specific RET proto-oncogene mutations and disease phenotype in multiple endocrine neoplasia type 2. International RET mutation consortium analysis. *Jama* 1996, 276:1575-1579.
33. Schuffenecker I, Virally-Monod M, Brohet R, Goldgar D, Conte-Devolx B, Leclerc L, Chabre O, Boneu A, Caron J, Houdent C, Modigliani E, Rohmer V, Schlumberger M, Eng C, Guillausseau PJ, Lenoir GM: Risk and penetrance of primary hyperparathyroidism in multiple endocrine neoplasia type 2A families with mutations at codon 634 of the RET proto-oncogene. Groupe D'etude des Tumeurs a Calcitonine. *J Clin Endocrinol Metab* 1998, 83:487-491
34. Lee NC, Norton JA: Multiple endocrine neoplasia type 2B--genetic basis and clinical expression. *Surg Oncol* 2000, 9:111-118.
35. Brandi ML, Gagel RF, Angeli A, Bilezikian JP, Beck-Peccoz P, Bordi C, Conte-Devolx B, Falchetti A, Gheri RG, Libroia A, Lips CJ, Lombardi G, Mannelli M, Pacini F, Ponder BA, Raue F, Skogseid B, Tamburrano G, Thakker RV, Thompson NW, Tomassetti P, Tonelli F, Wells SA, Jr., Marx SJ: Guidelines for diagnosis and therapy of MEN type 1 and type 2. *J Clin Endocrinol Metab* 2001, 86:5658-5671.
36. Jain S, Watson MA, DeBenedetti MK, Hiraki Y, Moley JF, Milbrandt J: Expression profiles provide insights into early malignant potential and skeletal abnormalities in multiple endocrine neoplasia type 2B syndrome tumors. *Cancer Res* 2004, 64:3907-3913.
37. Maher ER, Iselius L, Yates JR, Littler M, Benjamin C, Harris R, Sampson J, Williams A, Ferguson-Smith MA, Morton N: Von Hippel-Lindau disease: a genetic study. *J Med Genet* 1991, 28:443-447.
38. Latif F, Tory K, Gnarra J, Yao M, Duh FM, Orcutt ML, Stackhouse T, Kuzmin I, Modi W, Geil L, et al.: Identification of the von Hippel-Lindau disease tumor suppressor gene. *Science* 1993, 260:1317-1320.
39. Maher ER, Kaelin WG, Jr.: von Hippel-Lindau disease. *Medicine* (Baltimore) 1997, 76:381-391.

40. Melmon KL, Rosen SW: Lindau's Disease. Review of the Literature and Study of a Large Kindred. *Am J Med* 1964, 36:595-617.
41. Stolle C, Glenn G, Zbar B, Humphrey JS, Choyke P, Walther M, Pack S, Hurley K, Andrey C, Klausner R, Linehan WM: Improved detection of germline mutations in the von Hippel-Lindau disease tumor suppressor gene. *Hum Mutat* 1998, 12:417-423.
42. van der Harst E, de Krijger RR, Dinjens WN, Weeks LE, Bonjer HJ, Bruining HA, Lamberts SW, Koper JW: Germline mutations in the vhl gene in patients presenting with phaeochromocytomas. *Int J Cancer* 1998, 77:337-340.
43. Bar M, Friedman E, Jakobovitz O, Leibowitz G, Lerer I, Abeliovich D, Gross DJ: Sporadic phaeochromocytomas are rarely associated with germline mutations in the von Hippel-Lindau and RET genes. *Clin Endocrinol* (Oxf) 1997, 47:707-712.
44. Brauch H, Hoeppner W, Jahnig H, Wohl T, Engelhardt D, Spelsberg F, Ritter MM: Sporadic pheochromocytomas are rarely associated with germline mutations in the vhl tumor suppressor gene or the ret protooncogene. *J Clin Endocrinol Metab* 1997, 82:4101-4104.
45. Zbar B, Kishida T, Chen F, Schmidt L, Maher ER, Richards FM, Crossey PA, Webster AR, Affara NA, Ferguson-Smith MA, Brauch H, Glavac D, Neumann HP, Tisherman S, Mulvihill JJ, Gross DJ, Shuin T, Whaley J, Seizinger B, Kley N, Olschwang S, Boisson C, Richard S, Lips CH, Lerman M, et al.: Germline mutations in the Von Hippel-Lindau disease (VHL) gene in families from North America, Europe, and Japan. *Hum Mutat* 1996, 8:348-357.
46. Chen F, Kishida T, Yao M, Hustad T, Glavac D, Dean M, Gnarra JR, Orcutt ML, Duh FM, Glenn G, et al.: Germline mutations in the von Hippel-Lindau disease tumor suppressor gene: correlations with phenotype. *Hum Mutat* 1995, 5:66-75.
47. Gross DJ, Avishai N, Meiner V, Filon D, Zbar B, Abeliovich D: Familial pheochromocytoma associated with a novel mutation in the von Hippel-Lindau gene. *J Clin Endocrinol Metab* 1996, 81:147-149.
48. North K: Neurofibromatosis type 1. *Am J Med Genet* 2000, 97:119-127.
49. Airewele GE, Sigurdson AJ, Wiley KJ, Frieden BE, Caldarera LW, Riccardi VM, Lewis RA, Chintagumpala MM, Ater JL, Plon SE, Bondy ML: Neoplasms in neurofibromatosis 1 are related to gender but not to family history of cancer. *Genet Epidemiol* 2001, 20:75-86.
50. Friedman JM, Birch PH: Type 1 neurofibromatosis: a descriptive analysis of the disorder in 1,728 patients. *Am J Med Genet* 1997, 70:138-143.
51. Sorensen SA, Mulvihill JJ, Nielsen A: Long-term follow-up of von Recklinghausen neurofibromatosis. Survival and malignant neoplasms. *N Engl J Med* 1986, 314:1010-1015.
52. Zoller ME, Rembeck B, Oden A, Samuelsson M, Angervall L: Malignant and benign tumors in patients with neurofibromatosis type 1 in a defined Swedish population. *Cancer* 1997, 79:2125-2131.
53. Walther MM, Herring J, Enquist E, Keiser HR, Linehan WM: von Recklinghausen's disease and pheochromocytomas. *J Urol* 1999, 162:1582-1586.
54. Gutmann DH: The neurofibromatoses: when less is more. *Hum Mol Genet* 2001, 10:747-755.
55. Huson SM, Compston DA, Clark P, Harper PS: A genetic study of von Recklinghausen neurofibromatosis in south east Wales. I. Prevalence, fitness, mutation rate, and effect of parental transmission on severity. *J Med Genet* 1989, 26:704-711.
56. Cook JE, Urich RW, Sample HG, Jr., Fawcett NW: Peculiar familial and malignant pheochromocytomas of the organs of Zuckerkandl. *Ann Intern Med* 1960, 52:126-133.
57. Woodward ER, Eng C, McMahon R, Voutilainen R, Affara NA, Ponder BA, Maher ER: Genetic predisposition to phaeochromocytoma: analysis of candidate genes GDNF, RET and VHL. *Hum Mol Genet* 1997, 6:1051-1056.
58. van Heerden JA, Roland CF, Carney JA, Sheps SG, Grant CS: Long-term evaluation following resection of apparently benign pheochromocytoma(s)/paraganglioma(s). *World J Surg* 1990, 14:325-329.

59. Scott HW, Jr., Halter SA: Oncologic aspects of pheochromocytoma: the importance of follow-up. *Surgery* 1984, 96:1061-1066.
60. Proye C, Vix M, Goropoulos A, Kerlo P, Lecomte-Houcke M: High incidence of malignant pheochromocytoma in a surgical unit. 26 cases out of 100 patients operated from 1971 to 1991. *J Endocrinol Invest* 1992, 15:651-663.
61. Proye CA, Vix M, Jansson S, Tisell LE, Dralle H, Hiller W: "The" pheochromocytoma: a benign, intra-adrenal, hypertensive, sporadic unilateral tumor. Does it exist? *World J Surg* 1994, 18:467-472.
62. Pommier RF, Vetto JT, Billingsly K, Woltering EA, Brennan MF: Comparison of adrenal and extraadrenal pheochromocytomas. *Surgery* 1993, 114:1160-1165; discussion 1165-1166.
63. John H, Ziegler WH, Hauri D, Jaeger P: Pheochromocytomas: can malignant potential be predicted? *Urology* 1999, 53:679-683.
64. van den Broek PJ, de Graeff J: Prolonged survival in a patient with pulmonary metastases of a malignant pheochromocytoma. *Neth J Med* 1978, 21:245-247.
65. Yoshida S, Hatori M, Noshiro T, Kimura N, Kokubun S: Twenty-six-years' survival with multiple bone metastasis of malignant pheochromocytoma. *Arch Orthop Trauma Surg* 2001, 121:598-600.
66. Bonjer HJ, Sorm V, Berends FJ, Kazemier G, Steyerberg EW, de Herder WW, Bruining HA: Endoscopic retroperitoneal adrenalectomy: lessons learned from 111 consecutive cases. *Ann Surg* 2000, 232:796-803.
67. Mayo-Smith WW, Dupuy DE: Adrenal neoplasms: CT-guided radiofrequency ablation--preliminary results. *Radiology* 2004, 231:225-230.
68. Wang P, Zuo C, Qian Z, Tian J, Ren F, Zhou D: Computerized tomography guided percutaneous ethanol injection for the treatment of hyperfunctioning pheochromocytoma. *J Urol* 2003, 170:1132-1134.
69. Safford SD, Coleman RE, Gockerman JP, Moore J, Feldman JM, Leight GS, Jr., Tyler DS, Olson JA, Jr.: Iodine -131 metaiodobenzylguanidine is an effective treatment for malignant pheochromocytoma and paraganglioma. *Surgery* 2003, 134:956-962; discussion 962-953.
70. Rose B, Matthay KK, Price D, Huberty J, Klencke B, Norton JA, Fitzgerald PA: High-dose 131I-metaiodobenzylguanidine therapy for 12 patients with malignant pheochromocytoma. *Cancer* 2003, 98:239-248
71. Naguib M, Caceres M, Thomas CR, Jr., Herman TS, Eng TY: Radiation treatment of recurrent pheochromocytoma of the bladder: case report and review of literature. *Am J Clin Oncol* 2002, 25:42-44.
72. Kruijtzer CM, Beijnen JH, Swart M, Schellens JH: Successful treatment with paclitaxel of a patient with metastatic extra-adrenal pheochromocytoma (paraganglioma). A case report and review of the literature. *Cancer Chemother Pharmacol* 2000, 45:428-431.
73. Noshiro T, Honma H, Shimizu K, Kusakari T, Watanabe T, Akama H, Shibukawa S, Miura W, Abe K, Miura Y: Two cases of malignant pheochromocytoma treated with cyclophosphamide, vincristine and dacarbazine in a combined chemotherapy. *Endocr J* 1996, 43:279-284.
74. Nakane M, Takahashi S, Sekine I, Fukui I, Koizumi M, Kage K, Ito Y, Aiba K, Horikoshi N, Hatake K, Ishikawa Y, Ogata E: Successful treatment of malignant pheochromocytoma with combination chemotherapy containing anthracycline. *Ann Oncol* 2003, 14:1449-1451.
75. Hartley A, Spooner D, Brunt AM: Management of malignant phaeochromocytoma: a retrospective review of the use of MIBG and chemotherapy in the West Midlands. *Clin Oncol* (R Coll Radiol) 2001, 13:361-366.
76. Tato A, Orte L, Diz P, Quereda C, Ortuno J: Malignant pheochromocytoma, still a therapeutic challenge. *Am J Hypertens* 1997, 10:479-481.
77. Rabii R, Cicco A, Salomon L, Hoznek A, Chopin DK, Abbou CC: Exerese laparoscopique d'une metastase ganglionnaire para-aortique d'un pheochromocytome malin. *Ann Urol* (Paris) 2001, 35:81-83.

78. Khan JH, McElhinney DB, Rahman SB, George TI, Clark OH, Merrick SH: Pulmonary metastases of endocrine origin: the role of surgery. *Chest* 1998, 114:526-534.
79. Chew SL: Recent developments in the therapy of phaeochromocytoma. *Expert Opin Investig Drugs* 2004, 13:1579-1583.
80. Tischler AS: The adrenal medulla and extra-adrenal paraganglia. Functional endocrine pathology. Edited by Kovacs K, Asa SL. Boston, *Blackwell Scientific Publications*, 1990, pp 509-545.
81. Lack EE, Cubilla AL, Woodruff JM: Paragangliomas of the head and neck region. A pathologic study of tumors from 71 patients. *Hum Pathol* 1979, 10:191-218.
82. Batsakis JG: Chemodectomas of the head and neck. Tumors of the head and neck. Clinical and pathological considerations. Edited by Batsakis JG. Baltimore, Williams and Wilkins, 1976, pp 280-288.
83. Lee JH, Barich F, Karnell LH, Robinson RA, Zhen WK, Gantz BJ, Hoffman HT: National Cancer Data Base report on malignant paragangliomas of the head and neck. *Cancer* 2002, 94:730-737.
84. Grufferman S, Gillman MW, Pasternak LR, Peterson CL, Young WG: Familial carotid body tumors: case report and epidemiologic review. *Cancer* 1980, 46:2116-2122.
85. Hodge KM, Byers RM, Peters LJ: Paragangliomas of the head and neck. *Arch Otolaryngol Head Neck Surg* 1988, 114:872-877.
86. van Baars F, Cremers C, van den Broek P, Geerts S, Veldman J: Genetic aspects of nonchromaffin paraganglioma. *Hum Genet* 1982, 60:305-309.
87. Gardner P, Dalsing M, Weisberger E, Sawchuk A, Miyamoto R: Carotid body tumors, inheritance, and a high incidence of associated cervical paragangliomas. *Am J Surg* 1996, 172:196-199.
88. McCaffrey TV, Meyer FB, Michels VV, Piepgras DG, Marion MS: Familial paragangliomas of the head and neck. *Arch Otolaryngol Head Neck Surg* 1994, 120:1211-1216.
89. van der Mey AG, Maaswinkel-Mooy PD, Cornelisse CJ, Schmidt PH, van de Kamp JJ: Genomic imprinting in hereditary glomus tumours: evidence for new genetic theory [see comments]. *Lancet* 1989, 2:1291-1294.
90. Baysal BE, Ferrell RE, Willett-Brozick JE, Lawrence EC, Myssiorek D, Bosch A, van der Mey A, Taschner PE, Rubinstein WS, Myers EN, Richard CW, 3rd, Cornelisse CJ, Devilee P, Devlin B: Mutations in SDHD, a mitochondrial complex II gene, in hereditary paraganglioma. *Science* 2000, 287:848-851.
91. Astuti D, Latif F, Dallol A, Dahia PL, Douglas F, George E, Skoldberg F, Husebye ES, Eng C, Maher ER: Gene mutations in the succinate dehydrogenase subunit SDHB cause susceptibility to familial pheochromocytoma and to familial paraganglioma. *Am J Hum Genet* 2001, 69:49-54.
92. Niemann S, Muller U: Mutations in SDHC cause autosomal dominant paraganglioma, type 3. *Nat Genet* 2000, 26:268-270.
93. Evenson LJ, Mendenhall WM, Parsons JT, Cassisi NJ: Radiotherapy in the management of chemodectomas of the carotid body and glomus vagale. *Head Neck* 1998, 20:609-613.
94. Miller RB, Boon MS, Atkins JP, Lowry LD: Vagal paraganglioma: the Jefferson experience. *Otolaryngol Head Neck Surg* 2000, 122:482-487.
95. Persky MS, Setton A, Niimi Y, Hartman J, Frank D, Berenstein A: Combined endovascular and surgical treatment of head and neck paragangliomas--a team approach. *Head Neck* 2002, 24:423-431.
96. Jansen JC, van den Berg R, Kuiper A, van der Mey AG, Zwinderman AH, Cornelisse CJ: Estimation of growth rate in patients with head and neck paragangliomas influences the treatment proposal. *Cancer* 2000, 88:2811-2816.
97. Sillars HA, Fagan PA: The management of multiple paraganglioma of the head and neck. *J Laryngol Otol* 1993, 107:538-542.
98. Manolidis S, Shohet JA, Jackson CG, Glasscock ME, 3rd: Malignant glomus tumors. *Laryngoscope* 1999, 109:30-34.
99. Le Douarin NM: The Neural Crest. Cambridge, UK, Cambridge University Press, 1982.

100. Komminoth P, de Krijger RR, Tischler AS: Paraganglia and the Adrenal Medulla. *Endocrine Pathology.* Edited by LiVolsi VA, Asa SL. Philadelphia, Churchill Livingstone, 2002, pp 149-169.
101. McNichol AM: Differential diagnosis of pheochromocytomas and paragangliomas. *Endocr Pathol* 2001, 12:407-415.
102. Chetty R, Pillay P, Jaichand V: Cytokeratin expression in adrenal phaeochromocytomas and extra-adrenal paragangliomas. *J Clin Pathol* 1998, 51:477-478.
103. Fraga M, Garcia-Caballero T, Antunez J, Couce M, Beiras A, Forteza J: A comparative immunohistochemical study of phaeochromocytomas and paragangliomas. *Histol Histopathol* 1993, 8:429-436.
104. Sato T, Saito H, Yoshinaga K, Shibota Y, Sasano N: Concurrence of carotid body tumor and pheochromocytoma. *Cancer* 1974, 34:1787-1795.
105. Campeau RJ, Graves C: Pheochromocytoma associated with prior carotid body tumors (chemodectomas). Detection with I-131 MIBG. *Clin Nucl Med* 1991, 16:511-512.
106. Jensen JC, Choyke PL, Rosenfeld M, Pass HI, Keiser H, White B, Travis W, Linehan WM: A report of familial carotid body tumors and multiple extra-adrenal pheochromocytomas. *J Urol* 1991, 145:1040-1042.
107. Skoldberg F, Grimelius L, Woodward ER, Rorsman F, Van Schothorst EW, Winqvist O, Karlsson FA, Akerstrom G, Kampe O, Husebye ES: A family with hereditary extra-adrenal paragangliomas without evidence for mutations in the von Hippel-Lindau disease or ret genes. *Clin Endocrinol* (Oxf) 1998, 48:11-16.
108. Baysal BE: Hereditary paraganglioma targets diverse paraganglia. *J Med Genet* 2002, 39:617-622.
109. de Jong E, Mulder W, Nooitgedacht E, Taat CW, Bras J: Carney's triad. *Eur J Surg Oncol* 1998, 24:147-149.
110. Zak FG, Lawson W: The paraganglionic chemoreceptor system. Physiology, pathology and clinical medicine. New York, Springer Verlag, 1983.
111. Amberson JB, Vaughan ED, Jr., Gray GF, Naus GJ: Flow cytometric determination of nuclear DNA content in benign adrenal pheochromocytomas. *Urology* 1987, 30:102-104.
112. Hosaka Y, Rainwater LM, Grant CS, Farrow GM, van Heerden JA, Lieber MM: Pheochromocytoma: nuclear deoxyribonucleic acid patterns studied by flow cytometry. *Surgery* 1986, 100:1003-1010.
113. Lai MK, Sun CF, Chen CS, Huang CC, Chu SH, Chuang CK: Deoxyribonucleic acid flow cytometric study in pheochromocytomas and its correlation with clinical parameters. *Urology* 1994, 44:185-188.
114. van der Harst E, de Herder WW, de Krijger RR, Bruining HA, Bonjer HJ, Lamberts SW, van den Meiracker AH, Stijnen TH, Boomsma F: The value of plasma markers for the clinical behaviour of phaeochromocytomas. *Eur J Endocrinol* 2002, 147:85-94.
115. Bamberger CM, Else T, Bamberger AM, Frilling A, Beil FU, Allolio B, Schulte HM: Telomerase activity in benign and malignant adrenal tumors. *Exp Clin Endocrinol Diabetes* 1999, 107:272-275.
116. Barshack I, Goldberg I, Nass D, Olchovsky D, Kopolovic J: CD44 expression in normal adrenal tissue and adrenal tumours. *J Clin Pathol* 1998, 51:52-54.
117. Bornstein SR, Ehrhart-Bornstein M, Gonzalez-Hernandez J, Schroder S, Scherbaum WA: Expression of interleukin-1 in human pheochromocytoma. *J Endocrinol Invest* 1996, 19:693-698.
118. Brown HM, Komorowski RA, Wilson SD, Demeure MJ, Zhu YR: Predicting metastasis of pheochromocytomas using DNA flow cytometry and immunohistochemical markers of cell proliferation: A positive correlation between MIB-1 staining and malignant tumor behavior. *Cancer* 1999, 86:1583-1589.
119. Castilla-Guerra L, Moreno AM, Fernandez-Moreno MC, Utrilla JC, Fernandez E, Galera-Davison H: Expression and prognostic value of c-erbB-2 oncogene product in human phaeochromocytomas. *Histopathology* 1997, 31:144-149

120. Clarke MR, Weyant RJ, Watson CG, Carty SE: Prognostic markers in pheochromocytoma. *Hum Pathol* 1998, 29:522-526.
121. De Fraipont F, Le Moigne G, Defaye G, El Atifi M, Berger F, Houlgatte R, Gicquel C, Plouin PF, Bertagna X, Chabre O, Feige JJ: Transcription profiling of benign and malignant adrenal tumors by cDNA macroarray analysis. *Endocr Res* 2002, 28:785-786.
122. de Krijger RR, van der Harst E, van der Ham F, Stijnen T, Dinjens WN, Koper JW, Bruining HA, Lamberts SW, Bosman FT: Prognostic value of p53, bcl-2, and c-erbB-2 protein expression in phaeochromocytomas. *J Pathol* 1999, 188:51-55.
123. Elder EE, Xu D, Hoog A, Enberg U, Hou M, Pisa P, Gruber A, Larsson C, Backdahl M: KI-67 AND hTERT Expression Can Aid in the Distinction between Malignant and Benign Pheochromocytoma and Paraganglioma. *Mod Pathol* 2003, 16:246-255.
124. Favier J, Plouin PF, Corvol P, Gasc JM: Angiogenesis and vascular architecture in pheochromocytomas: distinctive traits in malignant tumors. *Am J Pathol* 2002, 161:1235-1246.
125. Fogt F, Tomaszewski JE, Vortmeyer AO, Poremba C: Bcl-2 staining in pheochromocytomas. *Hum Pathol* 1999, 30:486-487.
126. Furihata M, Ohtsuki Y: Immunohistochemical characterization of HLA-DR-antigen positive dendritic cells in phaeochromocytomas and paragangliomas as a prognostic marker. *Virchows Arch A Pathol Anat Histopathol* 1991, 418:33-39.
127. Glodny B, Winde G, Herwig R, Meier A, Kuhle C, Cromme S, Vetter H: Clinical differences between benign and malignant pheochromocytomas. *Endocr J* 2001, 48:151-159.
128. Grouzmann E, Gicquel C, Plouin PF, Schlumberger M, Comoy E, Bohuon C: Neuropeptide Y and neuron-specific enolase levels in benign and malignant pheochromocytomas. *Cancer* 1990, 66:1833-1835.
129. Gupta D, Shidham V, Holden J, Layfield L: Prognostic value of immunohistochemical expression of topoisomerase alpha II, MIB-1, p53, E-cadherin, retinoblastoma gene protein product, and HER-2/neu in adrenal and extra-adrenal pheochromocytomas. *Appl Immunohistochem Mol Morphol* 2000, 8:267-274.
130. Hirano Y, Fujita K, Suzuki K, Ushiyama T, Ohtawara Y, Tsuda F: Telomerase activity as an indicator of potentially malignant adrenal tumors. *Cancer* 1998, 83:772-776.
131. Hoffman K, Gil J, Barba J, Liu Z, Pertsemlidis D, Kaneko M, Unger P: Morphometric analysis of benign and malignant adrenal pheochromocytomas. *Arch Pathol Lab Med* 1993, 117:244-247.
132. Januszewicz W, Wocial B, Januszewicz A, Gryglas P, Prejbisz A: Dopamine and dopa urinary excretion in patients with pheochromocytoma--diagnostic implications. *Blood Press* 2001, 10:212-216.
133. Khorram-Manesh A, Ahlman H, Jansson S, Nilsson O: N-cadherin expression in adrenal tumors: upregulation in malignant pheochromocytoma and downregulation in adrenocortical carcinoma. *Endocr Pathol* 2002, 13:99-110.
134. Kinoshita H, Ogawa O, Mishina M, Oka H, Okumura K, Yamabe H, Terachi T, Yoshida O: Telomerase activity in adrenal cortical tumors and pheochromocytomas with reference to clinicopathologic features. *Urol Res* 1998, 26:29-32
135. Koch CA, Vortmeyer AO, Diallo R, Poremba C, Giordano TJ, Sanders D, Bornstein SR, Chrousos GP, Pacak K: Survivin: a novel neuroendocrine marker for pheochromocytoma. *Eur J Endocrinol* 2002, 146:381-388.
136. Kolomecki K, Stepien H, Bartos M, Kuzdak K: Usefulness of VEGF, MMP-2, MMP-3 and TIMP-2 serum level evaluation in patients with adrenal tumours. *Endocr Regul* 2001, 35:9-16.
137. Kubota Y, Nakada T, Sasagawa I, Yanai H, Itoh K: Elevated levels of telomerase activity in malignant pheochromocytoma. *Cancer* 1998, 82:176-179.
138. Kumaki N, Kajiwara H, Kameyama K, DeLellis RA, Asa SL, Osamura RY, Takami H: Prediction of malignant behavior of pheochromocytomas and paragangliomas using immunohistochemical techniques. *Endocr Pathol* 2002, 13:149-156.

139. Lam KY, Lo CY, Wat NM, Luk JM, Lam KS: The clinicopathological features and importance of p53, Rb, and mdm2 expression in phaeochromocytomas and paragangliomas. *J Clin Pathol* 2001, 54:443-448.
140. Liu Q, Djuricin G, Staren ED, Gattuso P, Gould VE, Shen J, Saclarides T, Rubin DB, Prinz RA: Tumor angiogenesis in pheochromocytomas and paragangliomas. *Surgery* 1996, 120:938-942; discussion 942-933.
141. Masmiquel L, Castro-Forns M, de Torres I, Garcia A, Vidal MT, Simo R: Leu-M1 immunoreactivity and phaeochromocytoma. *J Clin Pathol* 1997, 50:168-170.
142. Moreno AM, Castilla-Guerra L, Martinez-Torres MC, Torres-Olivera F, Fernandez E, Galera-Davidson H: Expression of neuropeptides and other neuroendocrine markers in human phaeochromocytomas. *Neuropeptides* 1999, 33:159-163.
143. Nagura S, Katoh R, Kawaoi A, Kobayashi M, Obara T, Omata K: Immunohistochemical estimations of growth activity to predict biological behavior of pheochromocytomas. *Mod Pathol* 1999, 12:1107-1111.
144. Nakada T, Kubota Y, Sasagawa I, Yagisawa T, Watanabe M, Ishigooka M: Remarkably suppressed manganese superoxide dismutase activity in malignant pheochromocytoma. *J Urol* 1995, 153:1787-1790.
145. Ohji H, Sasagawa I, Iciyanagi O, Suzuki Y, Nakada T: Tumour angiogenesis and Ki-67 expression in phaeochromocytoma. *BJU Int* 2001, 87:381-385.
146. Quiros RM, Kim AW, Maxhimer J, Gattuso P, Xu X, Prinz RA: Differential heparanase-1 expression in malignant and benign pheochromocytomas. *J Surg Res* 2002, 108:44-50.
147. Saeger W, Fassnacht M, Reincke M, Allolio B: Expression of HER-2/neu receptor protein in adrenal tumors. *Pathol Res Pract* 2002, 198:445-448.
148. Salmenkivi K, Haglund C, Ristimaki A, Arola J, Heikkila P: Increased expression of cyclooxygenase-2 in malignant pheochromocytomas. *J Clin Endocrinol Metab* 2001, 86:5615-5619.
149. Salmenkivi K, Haglund C, Arola J, Heikkila P: Increased expression of tenascin in pheochromocytomas correlates with malignancy. *Am J Surg Pathol* 2001, 25:1419-1423.
150. Salmenkivi K, Arola J, Voutilainen R, Ilvesmaki V, Haglund C, Kahri AI, Heikkila P, Liu J: Inhibin/activin betaB-subunit expression in pheochromocytomas favors benign diagnosis. *J Clin Endocrinol Metab* 2001, 86:2231-2235
151. Stumvoll M, Fritsche A, Pickert A, Overkamp D: Features of malignancy in a benign pheochromocytoma. *Horm Res* 1997, 48:135-136.
152. Tormey WP, Fitzgerald RJ, Thomas G, Kay EW, Leader MB: Catecholamine secretion and ploidy in phaeochromocytoma. *Int J Clin Pract* 2000, 54:520-523.
153. Unger P, Hoffman K, Pertsemlidis D, Thung S, Wolfe D, Kaneko M: S100 protein-positive sustentacular cells in malignant and locally aggressive adrenal pheochromocytomas. *Arch Pathol Lab Med* 1991, 115:484-487.
154. van der Harst E, de Krijger RR, Bruining HA, Lamberts SW, Bonjer HJ, Dinjes WN, Proye C, Koper JW, Bosman FT, Roth J, Heitz PU, Komminoth P: Prognostic value of RET proto-oncogene point mutations in malignant and benign, sporadic phaeochromocytomas. *Int J Cancer* 1998, 79:537-540.
155. van der Harst E, Bruining HA, Jaap Bonjer H, van der Ham F, Dinjens WN, Lamberts SW, de Herder WW, Koper JW, Stijnen T, Proye C, Lecomte-Houcke M, Bosman FT, de Krijger RR: Proliferative index in phaeochromocytomas: does it predict the occurrence of metastases? *J Pathol* 2000, 191:175-180.
156. Thompson LD: Pheochromocytoma of the Adrenal gland Scaled Score (PASS) to separate benign from malignant neoplasms: a clinicopathologic and immunophenotypic study of 100 cases. *Am J Surg Pathol* 2002, 26:551-566.
157. Niemann S, Muller U, Engelhardt D, Lohse P: Autosomal dominant malignant and catecholamine-producing paraganglioma caused by a splice donor site mutation in SDHC. *Hum Genet* 2003, 113:92-94

158. Bauters C, Vantyghem MC, Leteurtre E, Odou MF, Mouton C, Porchet N, Wemeau JL, Proye C, Pigny P: Hereditary phaeochromocytomas and paragangliomas: a study of five susceptibility genes. *J Med Genet* 2003, 40:e75
159. Baysal BE, Willett-Brozick JE, Lawrence EC, Drovdlic CM, Savul SA, McLeod DR, Yee HA, Brackmann DE, Slattery WH, 3rd, Myers EN, Ferrell RE, Rubinstein WS: Prevalence of SDHB, SDHC, and SDHD germline mutations in clinic patients with head and neck paragangliomas. *J Med Genet* 2002, 39:178-183.
160. Dannenberg H, Dinjens WN, Abbou M, Van Urk H, Pauw BK, Mouwen D, Mooi WJ, de Krijger RR: Frequent germ-line succinate dehydrogenase subunit D gene mutations in patients with apparently sporadic parasympathetic paraganglioma. *Clin Cancer Res* 2002, 8:2061-2066.
161. de Krijger RR, van der Harst E, Muletta-Feurer S, Bruining HA, Lamberts SW, Dinjens WN, Roth J, Heitz PU, Komminoth P: RET is expressed but not mutated in extra-adrenal paragangliomas. *J Pathol* 2000, 191:264-268.
162. Taschner PE, Jansen JC, Baysal BE, Bosch A, Rosenberg EH, Brocker-Vriends AH, van Der Mey AG, van Ommen GJ, Cornelisse CJ, Devilee P: Nearly all hereditary paragangliomas in the Netherlands are caused by two founder mutations in the SDHD gene. *Genes Chromosomes Cancer* 2001, 31:274-281.
163. Noshiro T, Shimizu K, Watanabe T, Akama H, Shibukawa S, Miura W, Ito S, Miura Y: Changes in clinical features and long-term prognosis in patients with pheochromocytoma. *Am J Hypertens* 2000, 13:35-43.
164. Favia G, Lumachi F, Polistina F, D'Amico DF: Pheochromocytoma, a rare cause of hypertension: long-term follow-up of 55 surgically treated patients. *World J Surg* 1998, 22:689-693; discussion 694.
165. Beldjord C, Desclaux-Arramond F, Raffin-Sanson M, Corvol JC, De Keyzer Y, Luton JP, Plouin PF, Bertagna X: The RET protooncogene in sporadic pheochromocytomas: frequent MEN 2-like mutations and new molecular defects. *J Clin Endocrinol Metab* 1995, 80:2063-2068.
166. Gimenez-Roqueplo AP, Favier J, Rustin P, Rieubland C, Crespin M, Nau V, Khau Van Kien P, Corvol P, Plouin PF, Jeunemaitre X: Mutations in the SDHB gene are associated with extra-adrenal and/or malignant phaeochromocytomas. *Cancer Res* 2003, 63:5615-5621.
167. Benn DE, Croxson MS, Tucker K, Bambach CP, Richardson AL, Delbridge L, Pullan PT, Hammond J, Marsh DJ, Robinson BG: Novel succinate dehydrogenase subunit B (SDHB) mutations in familial phaeochromocytomas and paragangliomas, but an absence of somatic SDHB mutations in sporadic phaeochromocytomas. *Oncogene* 2003, 22:1358-1364.
168. Cascon A, Cebrian A, Ruiz-Llorente S, Telleria D, Benitez J, Robledo M: SDHB mutation analysis in familial and sporadic phaeochromocytoma identifies a novel mutation. *J Med Genet* 2002, 39:E64.
169. Cascon A, Ruiz-Llorente S, Cebrian A, Telleria D, Rivero JC, Diez JJ, Lopez-Ibarra PJ, Jaunsolo MA, Benitez J, Robledo M: Identification of novel SDHD mutations in patients with phaeochromocytoma and/or paraganglioma. *Eur J Hum Genet* 2002, 10:457-461.
170. Astuti D, Douglas F, Lennard TW, Aligianis IA, Woodward ER, Evans DG, Eng C, Latif F, Maher ER: Germline SDHD mutation in familial phaeochromocytoma. *Lancet* 2001, 357:1181-1182.
171. Lindor NM, Honchel R, Khosla S, Thibodeau SN: Mutations in the RET protooncogene in sporadic pheochromocytomas. *J Clin Endocrinol Metab* 1995, 80:627-629.
172. Schulz N, Propst F, Rosenberg MP, Linnoila RI, Paules RS, Kovatch R, Ogiso Y, Vande Woude G: Pheochromocytomas and C-cell thyroid neoplasms in transgenic c-mos mice: a model for the human multiple endocrine neoplasia type 2 syndrome. *Cancer Res* 1992, 52:450-455.
173. Eng C, Foster KA, Healey CS, Houghton C, Gayther SA, Mulligan LM, Ponder BA: Mutation analysis of the c-mos proto-oncogene and the endothelin-B receptor gene in medullary thyroid carcinoma and phaeochromocytoma. *Br J Cancer* 1996, 74:339-341.

174. Astuti D, Agathanggelou A, Honorio S, Dallol A, Martinsson T, Kogner P, Cummins C, Neumann HP, Voutilainen R, Dahia P, Eng C, Maher ER, Latif F: RASSF1A promoter region CpG island hypermethylation in phaeochromocytomas and neuroblastoma tumours. *Oncogene* 2001, 20:7573-7577.
175. Aguiar RC, Dahia PL, Sill H, Toledo SP, Goldman JM, Cross NC: Deletion analysis of the p16 tumour suppressor gene in phaeochromocytomas. *Clin Endocrinol* (Oxf) 1996, 45:93-96.
176. Dackiw AP, Cote GJ, Fleming JB, Schultz PN, Stanford P, Vassilopoulou-Sellin R, Evans DB, Gagel RF, Lee JE: Screening for MEN1 mutations in patients with atypical endocrine neoplasia. *Surgery* 1999, 126:1097-1103; discussion 1103-1094.
177. Bender BU, Gutsche M, Glasker S, Muller B, Kirste G, Eng C, Neumann HP: Differential Genetic Alterations in von Hippel-Lindau Syndrome-Associated and Sporadic Pheochromocytomas. *J Clin Endocrinol Metab* 2000, 85:4568-4574.
178. Benn DE, Dwight T, Richardson AL, Delbridge L, Bambach CP, Stowasser M, Gordon RD, Marsh DJ, Robinson BG: Sporadic and familial pheochromocytomas are associated with loss of at least two discrete intervals on chromosome 1p. *Cancer Res* 2000, 60:7048-7051.
179. Mathew CG, Smith BA, Thorpe K, Wong Z, Royle NJ, Jeffreys AJ, Ponder BA: Deletion of genes on chromosome 1 in endocrine neoplasia. *Nature* 1987, 328:524-526.
180. Tsutsumi M, Yokota J, Kakizoe T, Koiso K, Sugimura T, Terada M: Loss of heterozygosity on chromosomes 1p and 11p in sporadic pheochromocytoma. *J Natl Cancer Inst* 1989, 81:367-370.
181. Khosla S, Patel VM, Hay ID, Schaid DJ, Grant CS, van Heerden JA, Thibodeau SN: Loss of heterozygosity suggests multiple genetic alterations in pheochromocytomas and medullary thyroid carcinomas. *J Clin Invest* 1991, 87:1691-1699.
182. Moley JF, Brother MB, Fong CT, White PS, Baylin SB, Nelkin B, Wells SA, Brodeur GM: Consistent association of 1p loss of heterozygosity with pheochromocytomas from patients with multiple endocrine neoplasia type 2 syndromes. *Cancer Res* 1992, 52:770-774.
183. Shin E, Fujita S, Takami K, Kurahashi H, Kurita Y, Kobayashi T, Mori T, Nishisho I, Takai S: Deletion mapping of chromosome 1p and 22q in pheochromocytoma. *Jpn J Cancer Res* 1993, 84:402-408.
184. Vargas MP, Zhuang Z, Wang C, Vortmeyer A, Linehan WM, Merino MJ: Loss of heterozygosity on the short arm of chromosomes 1 and 3 in sporadic pheochromocytoma and extra-adrenal paraganglioma. *Hum Pathol* 1997, 28:411-415.
185. Herfarth KK, Wick MR, Marshall HN, Gartner E, Lum S, Moley JF: Absence of TP53 alterations in pheochromocytomas and medullary thyroid carcinomas. *Genes Chromosomes Cancer* 1997, 20:24-29.
186. Tanaka N, Nishisho I, Yamamoto M, Miya A, Shin E, Karakawa K, Fujita S, Kobayashi T, Rouleau GA, Mori T, et al.: Loss of heterozygosity on the long arm of chromosome 22 in pheochromocytoma. *Genes Chromosomes Cancer* 1992, 5:399-403.
187. Tory K, Brauch H, Linehan M, Barba D, Oldfield E, Filling-Katz M, Seizinger B, Nakamura Y, White R, Marshall FF, et al.: Specific genetic change in tumors associated with von Hippel-Lindau disease. *J Natl Cancer Inst* 1989, 81:1097-1101.
188. Yang KP, Nguyen CV, Castillo SG, Samaan NA: Deletion mapping on the distal third region of chromosome 1p in multiple endocrine neoplasia type IIA. *Anticancer Res* 1990, 10:527-533.
189. Devilee P, van Schothorst EM, Bardoel AF, Bonsing B, Kuipers-Dijkshoorn N, James MR, Fleuren G, van der Mey AG, Cornelisse CJ: Allelotype of head and neck paragangliomas: allelic imbalance is confined to the long arm of chromosome 11, the site of the predisposing locus PGL. *Genes Chromosomes Cancer* 1994, 11:71-78.

190. van Schothorst EM, Beekman M, Torremans P, Kuipers-Dijkshoorn NJ, Wessels HW, Bardoel AF, van der Mey AG, van der Vijver MJ, van Ommen GJ, Devilee P, Cornelisse CJ: Paragangliomas of the head and neck region show complete loss of heterozygosity at 11q22-q23 in chief cells and the flow-sorted DNA aneuploid fraction. *Hum Pathol* 1998, 29:1045-1049.
191. Wang DG, Barros D'Sa AA, Johnston CF, Buchanan KD: Oncogene expression in carotid body tumors. *Cancer* 1996, 77:2581-2587.
192. Wang DG, Johnston CF, Barros D'Sa AA, Buchanan KD: Expression of apoptosis-suppressing gene bcl-2 in human carotid body tumours. *J Pathol* 1997, 183:218-221.
193. Li SL, Goko H, Xu ZD, Kimura G, Sun Y, Kawachi MH, Wilson TG, Wilczynski S, Fujita-Yamaguchi Y: Expression of insulin-like growth factor (IGF)-II in human prostate, breast, bladder, and paraganglioma tumors. *Cell Tissue Res* 1998, 291:469-479.
194. Jyung RW, LeClair EE, Bernat RA, Kang TS, Ung F, McKenna MJ, Tuan RS: Expression of angiogenic growth factors in paragangliomas. *Laryngoscope* 2000, 110:161-167.
195. van Nederveen FH, Dannenberg H, Sleddens HF, de Krijger RR, Dinjens WN: p53 alterations and their relationship to SDHD mutations in parasympathetic paragangliomas. *Mod Pathol* 2003, 16:849-856.
196. Aguiar RC, Cox G, Pomeroy SL, Dahia PL: Analysis of the SDHD gene, the susceptibility gene for familial paraganglioma syndrome (PGL1), in pheochromocytomas. *J Clin Endocrinol Metab* 2001, 86:2890-2894.
197. Eng C, Crossey PA, Mulligan LM, Healey CS, Houghton C, Prowse A, Chew SL, Dahia PL, O'Riordan JL, Toledo SP, et al.: Mutations in the RET proto-oncogene and the von Hippel-Lindau disease tumour suppressor gene in sporadic and syndromic phaeochromocytomas. *J Med Genet* 1995, 32:934-937.
198. Hofstra RM, Stelwagen T, Stulp RP, de Jong D, Hulsbeek M, Kamsteeg EJ, van den Berg A, Landsvater RM, Vermey A, Molenaar WM, Lips CJ, Buys CH: Extensive mutation scanning of RET in sporadic medullary thyroid carcinoma and of RET and VHL in sporadic pheochromocytoma reveals involvement of these genes in only a minority of cases. *J Clin Endocrinol Metab* 1996, 81:2881-2884.
199. Brauch H, Weirich G, Brieger J, Glavac D, Rodl H, Eichinger M, Feurer M, Weidt E, Puranakanitstha C, Neuhaus C, Pomer S, Brenner W, Schirmacher P, Storkel S, Rotter M, Masera A, Gugeler N, Decker HJ: VHL alterations in human clear cell renal cell carcinoma: association with advanced tumor stage and a novel hot spot mutation. *Cancer Res* 2000, 60:1942-1948.

Chapter 2

Von Hippel-Lindau gene alterations in sporadic benign and malignant pheochromocytomas

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Abstract

The Von Hippel-Lindau (*VHL*) gene product has a wide spectrum of tissue-specific functions and specific germline mutations are associated with clinical phenotypes in VHL disease. Particularly missense mutations are correlated with the susceptibility to pheochromocytomas. An association between *VHL* aberrations and prognosis has been suggested in renal clear cell carcinoma, but has not been studied in pheochromocytomas.

We studied the frequency and spectrum of *VHL* alterations in apparently sporadic pheochromocytomas in relation to the clinical behavior in 72 patients, including 48 patients with clinically benign and 24 patients with malignant pheochromocytomas. Single strand conformation polymorphism (SSCP) analysis followed by DNA sequencing, loss of heterozygosity analysis of the *VHL* locus, and immunohistochemistry for VHL protein expression were used to investigate somatic *VHL* gene alterations.

In two patients, one with a malignant tumor, germline mutations were identified in the stop codon. Tumor specific intragenic *VHL* mutations and accompanying loss of heterozygosity were identified in 2 (4.3%) of 47 sporadic benign pheochromocytomas compared to 4 (17.4%) of 23 malignant tumors ($P = 0.064$). Only one of these mutations has been previously described, in a renal clear cell carcinoma. Expression of the VHL protein was observed in all pheochromocytomas. No distinction in the nature of *VHL* alterations between benign and malignant pheochromocytomas and no correlation with histopathologic or clinical features was observed.

We report novel *VHL* mutations in sporadic pheochromocytomas, which are slightly correlated with malignancy. *VHL* mutations may have some impact on the malignant transformation of pheochromocytomas.

The abbreviations used are: VHL, Von Hippel-Lindau; PCC, pheochromocytoma; RCC, renal clear cell carcinoma; LOH, Loss of Heterozygosity; pVHL, VHL protein; MEN2, multiple endocrine neoplasia type 2; NF1, neurofibromatosis type 1, *SDHB*, succinate dehydrogenase subunit B; *SDHD*, succinate dehydrogenase subunit D.

Key words: Von Hippel-Lindau, pheochromocytoma, mutation, Loss of Heterozygosity

Introduction

Von Hippel-Lindau (VHL) disease is characterized by the development of multiple highly vascularized tumors in mesenchymal and neural crest-derived tissues of several organ systems. These concern mostly the central nervous system (hemangioblastoma), eye (retinal angioma), kidney (renal clear cell carcinoma), adrenal medulla (pheochromocytoma), inner ear (endolymphatic sac tumor), and endocrine pancreas (islet cell tumors).[1] In nearly all VHL patients, germline mutations or deletions in the *VHL* gene can be identified.[2,3]

The *VHL* gene codes for a 213 amino acid protein (pVHL), which is involved in regulation of angiogenesis, extracellular matrix formation, and plays a role in the cell cycle.[4-9] As a recessive tumor suppressor gene, *VHL* demonstrates some important additional features, such as allelic heterogeneity resulting in genotype-phenotype correlations, and epigenetic effects.[10-12]

A correlation has been found between the nature and localization of inactivating mutations and the clinical consequences in patients afflicted by VHL disease. Especially the development of pheochromocytoma (PCC) in VHL disease (type II VHL disease) is strongly correlated with missense mutations. These are found in 96% of these families, frequently in the exon 3-encoded α-domain of pVHL.[10-13] PCC may even be the only tumor arising in some individuals with VHL type II disease. It is hypothesized that some retention of pVHL function is necessary in the development of PCC, arising from a dominant-negative effect of mutated pVHL and based on its involvement in the VHL-Elongin C-Elongin B complex.[14]

The majority of PCCs occurs sporadically and the genetic mechanisms underlying their tumorigenesis and progression towards malignancy are poorly understood. At present, one cannot predict which patient will experience progression to metastatic disease, which occurs in approximately 10% of the patients even after a disease free interval of more than 10 years.[15] The search for predictive markers in PCCs has been the aim of many studies and has included, among others, studies on hormone excretion, nuclear volume, DNA ploïdy, gene mutations and expression.[16-27] However, these markers have at best shown a general relation to prognosis and provide no definitive information for the patient.

Since Neumann et al.[28] recently reported germline mutations in 24% of apparently sporadic PCC patients, one can conclude that, in the general population, up to 30% of PCC patients presents with genetic predisposition to this tumor. Previous analyses of the responsible genes of these cancer syndromes revealed that somatic mutations of *RET*, *SDHD*, and *SDHB*, as well as the *VHL* gene are infrequent in sporadic PCCs.[29-34] Regarding *VHL* gene alterations, very few have analyzed malignant PCCs so that the clinical significance of the presence or absence of *VHL* alterations with regard to the biology of sporadic PCC remains unknown. A correlation between somatic *VHL* mutations with nonfavorable prognosis has recently been suggested in renal clear cell carcinoma (RCC), another VHL-related tumor.[35] Somatic *VHL* gene alterations

have recently also been analyzed in MEN2-associated pheochromocytomas to elucidate the pathogenesis of these tumors.[36]

We here examined *VHL* gene alterations using mutational analysis, loss of heterozygosity analysis, and immunohistochemistry for VHL protein expression in a large series of sporadic PCCs, including 48 patients with a clinically benign PCC and 24 patients with a malignant PCC. The results were compared to histopathological features of the tumors.

Materials and Methods

Patients and Tumor Specimens

We collected tumor specimens and clinical data from patients with clinically benign sporadic pheochromocytoma (n=48) and from patients with proven malignant sporadic pheochromocytoma (n=24). All 72 patients had undergone surgery between 1973 and 1997 at several hospitals in The Netherlands, the University Hospital, Lille, France, and the University Hospital Zürich, Switzerland. Two independent pathologists (RRdK, PK) confirmed the diagnosis according to standard histopathologic analysis. Data on histopathologic features including mitotic frequency, tumor size, and the presence of necrosis, angioinvasion, and capsular invasion were obtained from their reports.

A PCC was considered sporadic if neither the patient nor the patients' family (by patient interviews) had clinical characteristics to suggest VHL, MEN II, NF1, or familial pheochromocytoma syndromes. Furthermore, the majority of the patients in this study were previously analyzed for germline *VHL* (n=68) and *RET* (n=50) mutations.[20,37] DNA samples from 7 patients with known germline *VHL* mutations served as positive controls.

Of 24 patients, the tumor was proven to be malignant, determined either by histologically confirmed distant metastases or a positive MIBG scan outside the adrenal area, with persistent postoperative elevation of catecholamine levels, as defined earlier.[37] In 7 of these patients the primary tumor and a metastasis were analyzed. Forty-eight patients had localized disease and so far, after a mean follow-up time of 109 months (range 23 - 248), no metastases have been diagnosed in these patients. Clinical follow-up data were obtained by reviewing medical records. Table 1 summarizes relevant clinical characteristics of the patients.

Table 1 Clinical Characteristics of Patients with Sporadic Pheochromocytoma.

		Benign (N= 48)	**Malignant** (N= 24)
Mean age - years		51,5 ± 14,9	51,2 ± 13,0
Range		14 - 79	23 - 70
Sex - no. (%)	Male	28 (58,3)	12 (50)
	Female	20 (41,7)	12 (50)
Adrenal - no. (%)		45 (93,8)	18 (75)
Bilateral		1	2
Extra-adrenal - no. (%)		3 (6,3)*	6 (25)*
Multifocal		0	0

* All extra-adrenal pheochromocytomas were abdominal tumors

DNA Preparation

Tumor DNA and matched normal DNA from each patient were isolated. DNA from frozen tumor specimens was isolated using the D-5000 Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN) according to the manufacturers' recommendations. DNA extraction from paraffin-embedded tissues and peripheral blood samples was performed by standard detergent-proteinase K lysis, followed by organic extraction and ethanol precipitation as described.[38] Haematoxylin-Eosin staining was performed to assess the amount of tumor tissue in the sections.

Mutation Analysis

To screen the entire open reading frame of the VHL gene, including flanking 3' and 5' untranslated regions and all intron-exon boundaries, we developed 9 primer sets resulting in amplicons of about 200 bp, as necessary for paraffin-derived DNA samples. Primer pairs and PCR conditions were described previously.[39] None of the primer pairs matched to amplify sequences of the processed pseudogene of the VHL gene located at 1q12.[40] PCR amplification of tumor DNA and matched normal DNA was performed in 15 µl reaction mixtures containing 1.5 mM $MgCl_2$, 10 mM Tris-HCl, 50 mM KCl, 0.02 mM dATP, 0.2 mM dGTP, dTTP, dCTP each, 0.8 µCi α32P-dATP (Amersham, Buckinghamshire, UK), 20 pmol of each sense and anti-sense primer, and 1 U Taq DNA polymerase (Amplitaq Gold, Perkin Elmer, Norwalk, CT, USA). Electrophoresis of PCR products was carried out overnight at 8W on non-denaturing gels, containing 8% polyacrylamide (49:1) and 10% (v/v) glycerol. The gels were dried and exposed to X-ray film overnight at –70 °C.

DNA sequencing

Aberrant DNA products from PCR-SSCP analyses were excised from the gels, eluted and reamplified under the above-described conditions. PCR products were then purified using a QIAquick Gel Extraction Kit (Qiagen AG, Basel, Switzerland), and subjected to DNA cycle sequencing in sense and antisense direction using the TaqDyeDeoxy Terminator Cycle

Sequencing kit (Applied Biosystems, Weiterstadt, Germany) and analyzed on an automated DNA sequencer (Applied Biosystems).

Loss of heterozygosity analysis

LOH analyses were performed for tumors with *VHL* mutations when constitutional DNA of the same patient was available. Allelic imbalance of the *VHL* locus was examined at microsatellite loci D3S1110, D3S3525, and by PCR-SSCP of the SNP 462262 and SNP 1678600 loci as described previously.[39] PCR amplification of tumor and normal DNA was performed essentially as described above for SSCP analysis. For markers D3S1110 and D3S3525, PCR products of tumor and normal DNA from each patient were diluted 1:1 in 10 µl loading buffer (95% formamide, 20 mmol/L EDTA, 0.05% xylene cyanol, 0.05% bromophenol blue) and loaded onto a denaturing 6% polyacrylamide gel. Electrophoresis was carried out at 40W for 2.5 hours. The gels were dried and exposed to X-ray films. LOH in a tumor was scored when at least one marker showed allelic loss. Allelic loss was considered to be present when the relative intensities of the allelic signals from the tumor DNA where clearly different from the relative intensities observed in the normal DNA.

VHL expression

Expression of VHL was studied by immunohistochemistry, using a mouse monoclonal antibody directed against human pVHL Ab-1 (Oncogene, Boston, MA, USA) essentially as described.[39] Five µm sections of paraffin-embedded PCC specimens were deparaffinized in xylene and graded ethanol. The antibody was diluted 1:100 in PBS/0.1% Tween, and sections were incubated overnight at room temperature. After washing in PBS, immunoreactivity was visualized with the peroxidase-labeled Stravigen Multilink Kit (Biogenex, San Ramon, CA, USA). Sections were counterstained with Mayer's Haematoxylin and sealed with coverslides. For negative controls, the primary antibodies were replaced by incubation with PBS/BSA 1% instead of the anti-pVHL antibody. Human cerebellum (Purkinje cells) was used as a positive control. To evaluate VHL expression, scores were subdivided in negative, low, and positive expression.

Statistical analysis

An association between the presence of a somatic *VHL* mutation and the dignity of the tumors, histopathologic features or VHL expression levels was calculated by chi-square testing. *P* values < 0.05 were considered statistically significant.

Results

VHL gene mutations in sporadic PCCs

Apart from the known *VHL* germline mutations in the 7 control samples, 2 additional germline mutations were found, involving one patient with a benign and another patient with a malignant tumor. Both mutations were located in the termination codon of the *VHL* gene predicted to result in the elongation of the VHL protein. One mutation involved a frameshift mutation by insertion of a single nucleotide, and a new termination codon appears 128 codons downstream. In the other germline mutation the termination codon changed to a glycine coding sequence followed by 14 other codons before a novel stop codon emerges.

Somatic *VHL* mutations were found in 6 of 70 (8,6%) sporadic PCCs affecting 2 of 47 (4,3%) benign PCCs, and 4 of 23 (17,4%) malignant PCCs ($P = 0.064$). All mutations involved missense mutations. One mutation was identified in exon 1 at codon 57, a highly identical sequence among different species. The other mutations were located in exon 3 in conserved sequences or at the Elongin C or VBP1 binding sites. One of these mutations also affected the termination codon, resulting in a 227 amino acid protein. No distinction in the nature of VHL mutations in benign and malignant PCCs was observed. One mutation was identified in two independent PCCs, one benign and one malignant tumor. This mutation was previously reported in a RCC, but not in PCC. The C712T mutation, associated with familial PCC, was observed in one malignant tumor. Since constitutional DNA from this patient was not available, the possibility of a germline mutation cannot be entirely ruled out which becomes important in the setting of isolated hereditary pheochromocytoma. However, none of the patient's first or second-degree relatives were diagnosed for a neuroendocrine tumor. Thus, most likely, the mutation is somatic in this patient.

All *VHL* mutations are listed in Table 2, showing the histopathological features as well as results of 3p LOH and VHL protein expression. Results of the SSCP and sequence analyses are shown in Figure 1.

Loss of Heterozygosity of the *VHL* gene locus

Constitutional DNA was available from 7 of 8 patients with germline or somatic *VHL* mutations. LOH of at least one of the markers at the *VHL* locus (D3S1110, D3S3525, SNP462265, SNP1678600, and *VHL* exon 1 polymorphism) was observed in 5 of 7 analyzed tumors. Results of tumor 10M and 11M were indicative of LOH (Table 2 and Figure 1). Tumors 3M and 33M remained non-informative for all markers. Examples of LOH results are shown in Figure 1.

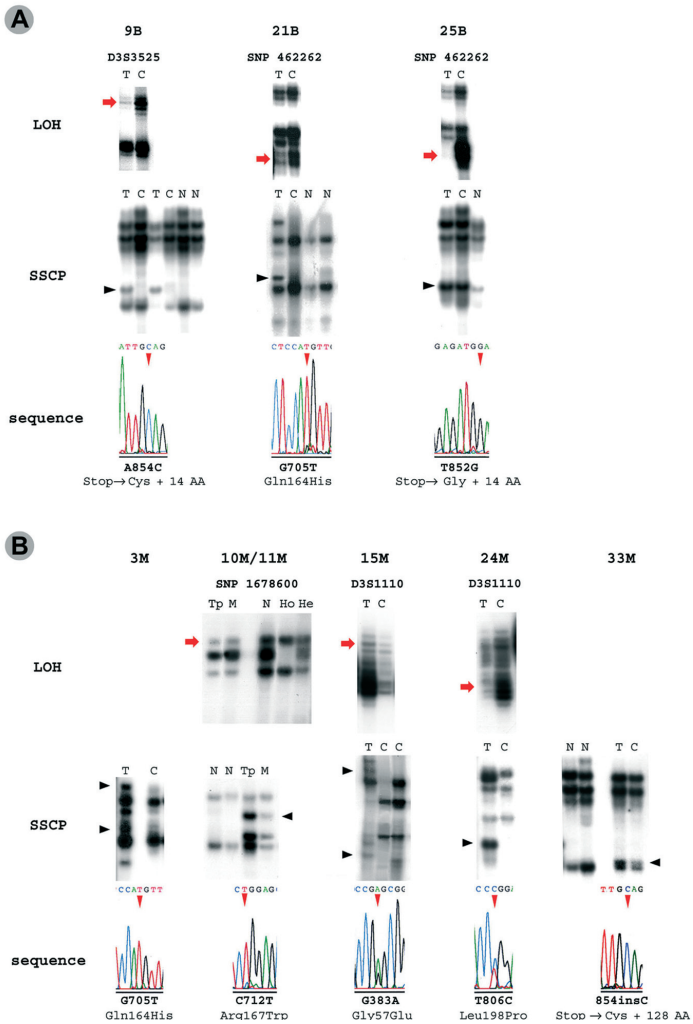

Figure 1 VHL alterations identified in benign and malignant pheochromocytomas (PCCs) by LOH analysis, SSCP analysis and direct sequencing. Of all VHL alterations, LOH and SSCP results of the tumor (T) and constitutional (C) DNA are shown, completed with sequencing results of the aberrant DNA. N = normal control sample. Panel A shows the benign tumors 9B and 21B, which harbor a somatic mutation, while the mutation in tumor 25B was also found in the germline. Panel B shows the 5 malignant PCCs with an intragenic VHL mutation. Tumor 33M exhibits a germline mutation. LOH of tumor 10M was likely on the basis of consistent preferential amplification of one allele at the SNP 1678600 locus in both the primary tumor (pT) and a metastasis (M) of this patient, when compared with a homozygous (Ho) and a heterozygous (He) sample. The top of figures A and B depicts examples of LOH (red arrow indicates deleted allele). Beneath the autoradiographs of PCR-SSCP gels, showing the migration patterns of normal DNA (N) and the mobility shifts produced by aberrant alleles (black arrowheads). The sequencing chromatograms below each autoradiograph demonstrate the alterations (note the red arrowhead to mark the nucleotide change). Since contamination with normal DNA products can occur while excising aberrant DNA products from the SSCP gels before sequencing, some sequence results show both aberrant and normal sequences (15M and 24M).

Table 2 Patient Characteristics and Genetic Findings

							LOH results								Histopathological features		
Patient	Tumor†	Age/Sex	Location	Mutation (cDNA)	Protein change	Origin	D3S1110	D3S3525	SNP 462262	SNP 1678600	IHC pVHL	Size (cm)	Meta-tases	Hormone production	Invasive Growth	Necrosis	Mitoses
1	9B	45/F	A / UL	A854C	X>Cys + 14 AA	somatic	■	■	ni	ni	+	7	–	NA	vascular	yes	n.m.
2	21B	62/F	A / UL	G705T	Gln164His	somatic	■	□	■	■	+/–	7	–	NA	vascular	yes	n.m.
3	25B	49/F	A / UL	T852G	X>Gly + 14 AA	germline	ni	ni	■	■	?	3	–	NA, D	no	no	no
4	*3M*	31/M	A / UL	G705T	Gln164His	somatic	ni	ni	ni	ni	+	nk	lung	NA, A	no	no	n.m.
5	10M	56/F	A / UL	C712T	Arg167Trp	somatic	3p loss by CGH *		■	■		8.5	LN + liver	nk	no	yes	sporadic
	11M		LN	C712T	Arg167Trp		–	–	■	■							
6	*15M*	40/F	A / UL	G383A	Gly57Glu	somatic	ni	ni	■	ni	+	9	lung	nk	capsular	no	frequent
7	24M	49/M	A / UL	T806C	Leu198Pro	somatic	■	■	ni	■	+	5.7	bone	A	no	yes	no
8	33M	nk/F	A / UL	854insC	X>Cys + 128 AA	germline	ni	ni	ni	ni	?	nk	LN	nk	capsular	no	frequent

* Data previously published (Dannenberg et al. 2000)

† B-numbers refer to benign, M-numbers to malignant tumors. Italic style tumor numbers refer to metastases

Abbreviations: M, male; F, female; A, adrenal; UL, unilateral; LN, lymph node metastasis; NA, Noradrenaline; A, Adrenaline; D, Dopamine; ■, LOH; □, no loss; ni, non informative; nk, not known; –, not analyzed; n.m., not mentioned in pathologists' report

Expression of the VHL protein by immunostaining

Staining for pVHL could be evaluated in 57 of 72 PCCs. Diffuse cytoplasmic staining for pVHL either weakly positive or positive, was observed in all benign and malignant tumors. Results for tumors with a *VHL* mutation are listed in Table 2 and examples are shown in Figure 2. There was no correlation between the intensity of VHL immunoreactivity and the *VHL* mutation and/or LOH of the *VHL* locus.

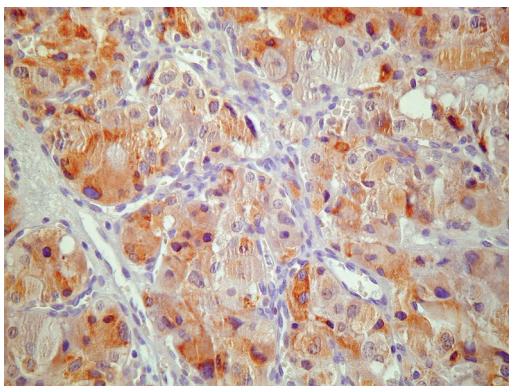

Figure 2 Example of pVHL immunostaining in a benign pheochromocytoma, using a mouse monoclonal antibody directed against human pVHL.

Histopathological and clinical features related to *VHL* aberrations

None of the histopathological features (mitotic frequency, tumor size, presence of necrosis, angioinvasion, capsular invasion) or clinical criteria (age, tumor location) were statistically correlated with the presence or absence of *VHL* mutation, or with the intensity of VHL immunoreactivity. However, all tumors with a *VHL* mutation were of adrenal origin.

Discussion

This study was performed to determine the frequency of *VHL* alterations in a large series of benign and malignant sporadic PCCs and the results were compared with histopathological and clinical data. We observed an increased frequency of *VHL* mutations in malignant PCC as opposed to benign PCC (17,4% *versus* 4.3%) and identified 5 novel mutations, especially 3 stop codon changing mutations. No distinction in the nature of *VHL* mutations in benign and malignant PCCs was observed and all PCCs showed expression of *VHL* at the protein level.

In previous studies on smaller series of PCCs, frequencies of somatic *VHL* mutations were between 0% (0/17 PCCs) and 20% (1/5 PCCs).[32-34] The mutation frequency in benign PCCs in our study corresponds well with results of the largest of these studies by Eng et al.[32], who report 2 somatic *VHL* mutations in 46 truly sporadic PCCs. However, none of the studies performed so far included more than 3 malignant tumors. In the current study, we surveyed 24 malignant sporadic PCCs and observed a higher frequency of *VHL* mutations compared to

the benign tumors. None of the histopathological and clinical features was correlated to the presence or absence of *VHL* mutations. However, all tumors with a mutation were of adrenal origin.

Although the patients in this study lacked a positive family history for any PCC-associated tumor syndrome and the majority of them was already analyzed for germline *RET* and *VHL* mutations, we identified another two germline *VHL* mutations. These may represent *de novo* mutations or chimeric presentation of germline mutations. It is possible that other germline mutations, specifically in the *SDHB, SDHD* genes occurred in our tumors, since Neumann et al.[28] recently reported a *SDHB, SDHD* mutation frequency of 8% in patients with apparently sporadic pheochromocytomas. Excluding these tumors from this study would increase the *VHL* mutation frequency in sporadic PCC.

Previous studies of genotype-phenotype correlations in VHL disease have shown an association between deletions/protein-truncating mutations and a low risk of PCC. Moreover, predisposition to PCC in VHL patients is correlated with missense mutations. This suggests that some retention of function of mutant pVHL is required for PCC development. Concordant with findings in VHL-related PCC, most of the mutations in our series represented missense mutations, and were located within highly conserved sequences or in interacting sites of the exon 3-encoded α-domain of pVHL. Interestingly, three different mutations were identified in the termination codon of the gene. These mutations, two substitutions and one insertion of single base pares, result in 227- and 341-residue pVHL mutants, respectively. It is known that many mutant mRNAs are subject to degradation, especially mutants with altered termination codon sites.[41,42] Although we did not proof the presence of stable cDNA mutants, we observed pVHL immunoreactivity in these tumors, pointing towards the presence of mutant pVHL in the cytoplasm. In all PCCs harboring a *VHL* mutation and in which LOH analysis could be performed, allelic loss of the *VHL* gene region was demonstrated, suggesting biallelic inactivation. Altogether, these findings further support the hypothesis that dysregulation without complete loss of pVHL function is necessary for PCC tumorigenesis.

Apart from mutation and gene loss, inactivation of the *VHL* gene can occur by promoter hypermethylation. This mechanism has been reported to be frequently present in RCC.[10,43] Promoter hypermethylation blocks the gene transcription and as a result no protein will be expressed. We demonstrated pVHL expression by immunohistochemistry in all investigated PCCs. Together with our LOH results, these findings suggest that complete *VHL* silencing by promoter hypermethylation in sporadic PCC is unlikely and does not largely contribute to PCC development.

Brauch and colleagues showed that somatic *VHL* alterations in RCC are associated with advanced tumor stage.[35] Although *VHL* alterations in PCC are far more infrequent, our data show a similar trend. In both tumor types, the VHL-related counterparts tend to be more indolent[44,45] and the *VHL* mutations frequently affect the Elongin C binding domain either

by truncating mutations (RCC) or missense mutations (PCC). Presymptomatic screening of family relatives of VHL patients mostly improves early diagnosis and resection of VHL-related PCC compared to the sporadic tumors and, therefore, may prevent malignant degeneration. Secondly, the paucity of malignant progression in VHL patients might be because mutations might prevent progression to malignancy if present at a critical period of adrenal chromaffin-cell development or cell cycling. Currently, there are no good explanations for the role of mutant pVHL or pVHL dysfunction in the susceptibility of malignancy. Changes in the physical coupling of cells to their microenvironment are proposed to be important in tumor invasion and metastasis. Tumor cell adhesion to the extracellular matrix through integrins is thought to be a critical step in metastasis. Recently, it was shown that pVHL is required for the proper assembly of fibronectin into the extracellular matrix.[5,46] Furthermore, some pVHL mutants associated with increased risk of PCC in VHL disease, have been shown to impair fibronectin matrix assembly while they retain the ability to downregulate HIF.[47] Thus, one explanation for the role of mutant pVHL in progression to malignancy, could be the loss of tumor cell adhesion to the extracellular matrix. This, among other critical steps in metastasis, could make tumor cells more prone to aggressive behavior.

In conclusion, we report a trend towards an increased *VHL* mutation frequency in malignant PCC compared to benign PCC. Newly identified mutations in the *VHL* gene and the observation of pVHL immunoreactivity in these tumors support the perception that residual pVHL function is essential in PCC tumorigenesis. Further analysis needs to establish the precise functional abrogations of the pVHL mutants.

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References

1. Maher ER and Kaelin WG, Jr. von Hippel-Lindau disease. *Medicine (Baltimore)* 1997;76(6):381-91.
2. Stolle C, Glenn G, Zbar B, Humphrey JS, Choyke P, Walther M, Pack S, Hurley K, Andrey C, Klausner R and Linehan WM. Improved detection of germline mutations in the von Hippel-Lindau disease tumor suppressor gene. *Hum Mutat* 1998;12(6):417-23.
3. Vortmeyer AO, Huang SC, Pack SD, Koch CA, Lubensky IA, Oldfield EH and Zhuang Z. Somatic point mutation of the wild-type allele detected in tumors of patients with VHL germline deletion. *Oncogene* 2002;21(8):1167-70.
4. Ivanov SV, Kuzmin I, Wei MH, Pack S, Geil L, Johnson BE, Stanbridge EJ and Lerman MI. Down-regulation of transmembrane carbonic anhydrases in renal cell carcinoma cell lines by wild-type von Hippel-Lindau transgenes. *Proc Natl Acad Sci USA* 1998;95(21):12596-601.
5. Ohh M, Yauch RL, Lonergan KM, Whaley JM, Stemmer-Rachamimov AO, Louis DN, Gavin BJ, Kley N, Kaelin WG and Iliopoulos O. The von Hippel-Lindau tumor suppressor protein is required for proper assembly of an extracellular fibronectin matrix. *Mol Cell* 1998;1(7):959-68.
6. Pause A, Lee S, Lonergan KM and Klausner RD. The von Hippel-Lindau tumor suppressor gene is required for cell cycle exit upon serum withdrawal. *Proc Natl Acad Sci U S A* 1998;95(3):993-8.
7. Pause A, Peterson B, Schaffar G, Stearman R and Klausner RD. Studying interactions of four proteins in the yeast two-hybrid system: structural resemblance of the pVHL/elongin BC/hCUL-2 complex with the ubiquitin ligase complex SKP1/cullin/F-box protein. *Proc Natl Acad Sci U S A* 1999;96(17):9533-8.
8. de Paulsen N, Brychzy A, Fournier MC, Klausner RD, Gnarra JR, Pause A and Lee S. Role of transforming growth factor-alpha in von Hippel--Lindau (VHL)(-/-) clear cell renal carcinoma cell proliferation: a possible mechanism coupling VHL tumor suppressor inactivation and tumorigenesis. *Proc Natl Acad Sci USA* 2001;98(4):1387-92.
9. Kondo K and Kaelin WG, Jr. The von Hippel-Lindau tumor suppressor gene. *Exp Cell Res* 2001;264(1):117-25.
10. Herman JG, Latif F, Weng Y, Lerman MI, Zbar B, Liu S, Samid D, Duan DS, Gnarra JR, Linehan WM and Baylin SB. Silencing of the VHL tumor-suppressor gene by DNA methylation in renal carcinoma. *Proc Natl Acad Sci U S A* 1994;91(21):9700-4.
11. Neumann HP and Bender BU. Genotype-phenotype correlations in von Hippel-Lindau disease. *J Intern Med* 1998;243(6):541-5.
12. Webster AR, Richards FM, MacRonald FE, Moore AT and Maher ER. An analysis of phenotypic variation in the familial cancer syndrome von Hippel-Lindau disease: evidence for modifier effects. *Am J Hum Genet* 1998;63(4):1025-35.
13. Chen F, Kishida T, Yao M, Hustad T, Glavac D, Dean M, Gnarra JR, Orcutt ML, Duh FM, Glenn G, Green J, et al. Germline mutations in the von Hippel-Lindau disease tumor suppressor gene: correlations with phenotype. *Hum Mutat* 1995;5(1):66-75.
14. Stebbins CE, Kaelin WG, Jr. and Pavletich NP. Structure of the VHL-ElonginC-ElonginB complex: implications for VHL tumor suppressor function. *Science* 1999;284(5413):455.
15. Morikawa T, Suzuki M, Unno M, Endo K, Katayose Y and Matsuno S. Malignant pheochromocytoma with hepatic metastasis diagnosed 10 years after a resection of the primary incidentaloma adrenal lesion: report of a case. *Surg Today* 2001;31(1):80-4.
16. Tippett PA, West RS, McEwan AJ, Middleton JE and Ackery DM. A comparison of dopamine and homovanillic acid excretion, as prognostic indicators in malignant phaeochromocytoma. *Clin Chim Acta* 1987;166(2-3):123-33.
17. Helman LJ, Cohen PS, Averbuch SD, Cooper MJ, Keiser HR and Israel MA. Neuropeptide Y expression distinguishes malignant from benign pheochromocytoma. *J Clin Oncol* 1989;7(11):1720-5.

18. Nativ O, Grant CS, Sheps SG, O'Fallon JR, Farrow GM, van Heerden JA and Lieber MM. Prognostic profile for patients with pheochromocytoma derived from clinical and pathological factors and DNA ploidy pattern. *J Surg Oncol* 1992;50(4):258-62.
19. Kubota Y, Nakada T, Sasagawa I, Yanai H and Itoh K. Elevated levels of telomerase activity in malignant pheochromocytoma. *Cancer* 1998;82(1):176-9.
20. van der Harst E, de Krijger RR, Bruining HA, Lamberts SW, Bonjer HJ, Dinjes WN, Proye C, Koper JW, Bosman FT, Roth J, Heitz PU and Komminoth P. Prognostic value of RET proto-oncogene point mutations in malignant and benign, sporadic phaeochromocytomas. *Int J Cancer* 1998;79(5):537-40.
21. de Krijger RR, van der Harst E, van der Ham F, Stijnen T, Dinjens WN, Koper JW, Bruining HA, Lamberts SW and Bosman FT. Prognostic value of p53, bcl-2, and c-erbB-2 protein expression in phaeochromocytomas. *J Pathol* 1999;188(1):51-5.
22. van der Harst E, Bruining HA, Jaap Bonjer H, van der Ham F, Dinjens WN, Lamberts SW, de Herder WW, Koper JW, Stijnen T, Proye C, Lecomte-Houcke M, Bosman FT, et al. Proliferative index in phaeochromocytomas: does it predict the occurrence of metastases? *J Pathol* 2000;191(2):175-80.
23. Dannenberg H, Speel EJ, Zhao J, Saremaslani P, van Der Harst E, Roth J, Heitz PU, Bonjer HJ, Dinjens WN, Mooi WJ, Komminoth P and de Krijger RR. Losses of chromosomes 1p and 3q are early genetic events in the development of sporadic pheochromocytomas. *Am J Pathol* 2000;157(2):353-9.
24. Gupta D, Shidham V, Holden J and Layfield L. Prognostic value of immunohistochemical expression of topoisomerase alpha II, MIB-1, p53, E-cadherin, retinoblastoma gene protein product, and HER-2/neu in adrenal and extra-adrenal pheochromocytomas. *Appl Immunohistochem Mol Morphol* 2000;8(4):267-74.
25. Salmenkivi K, Arola J, Voutilainen R, Ilvesmaki V, Haglund C, Kahri AI, Heikkila P and Liu J. Inhibin/activin betaB-subunit expression in pheochromocytomas favors benign diagnosis. *J Clin Endocrinol Metab* 2001;86(5):2231-5.
26. van der Harst E, de Herder WW, Bruining HA, Bonjer HJ, de Krijger RR, Lamberts SW, van de Meiracker AH, Boomsma F, Stijnen T, Krenning EP, Bosman FT, Kwekkeboom DJ. [(123)I]metaiodobenzylguanidine and [(111)In]octreotide uptake in begnign and malignant pheochromocytomas. *J Clin Endocrinol Metab* 2001;86(2):685-93.
27. Koch CA, Vortmeyer AO, Diallo R, Poremba C, Giordano TJ, Sanders D, Bornstein SR, Chrousos GP and Pacak K. Survivin: a novel neuroendocrine marker for pheochromocytoma. *Eur J Endocrinol* 2002;146(3):381-8.
28. Neumann HP, Bausch B, McWhinney SR, Bender BU, Gimm O, Franke G, Schipper J, Klisch J, Altehoefer C, Zerres K, Januszewicz A, Eng C, et al. Germ-line mutations in nonsyndromic pheochromocytoma. *N Engl J Med* 2002;346(19):1459-66.
29. Gimm O, Armanios M, Dziema H, Neumann HP and Eng C. Somatic and occult germ-line mutations in SDHD, a mitochondrial complex II gene, in nonfamilial pheochromocytoma. *Cancer Res* 2000;60(24):6822-5.
30. Astuti D, Latif F, Dallol A, Dahia PL, Douglas F, George E, Skoldberg F, Husebye ES, Eng C and Maher ER. Gene mutations in the succinate dehydrogenase subunit SDHB cause susceptibility to familial pheochromocytoma and to familial paraganglioma. *Am J Hum Genet* 2001;69(1):49-54.
31. Aguiar RC, Cox G, Pomeroy SL and Dahia PL. Analysis of the SDHD gene, the susceptibility gene for familial paraganglioma syndrome (PGL1), in pheochromocytomas. *J Clin Endocrinol Metab* 2001;86(6):2890-4.
32. Eng C, Crossey PA, Mulligan LM, Healey CS, Houghton C, Prowse A, Chew SL, Dahia PL, O'Riordan JL, Toledo SP, Smith DP, Maher ER, et al. Mutations in the RET proto-oncogene and the von Hippel-Lindau disease tumour suppressor gene in sporadic and syndromic phaeochromocytomas. *J Med Genet* 1995;32(12):934-7.

33. Hofstra RM, Stelwagen T, Stulp RP, de Jong D, Hulsbeek M, Kamsteeg EJ, van den Berg A, Landsvater RM, Vermey A, Molenaar WM, Lips CJ and Buys CH. Extensive mutation scanning of RET in sporadic medullary thyroid carcinoma and of RET and VHL in sporadic pheochromocytoma reveals involvement of these genes in only a minority of cases. *J Clin Endocrinol Metab* 1996;81(8):2881-4.
34. Bender BU, Gutsche M, Glasker S, Muller B, Kirste G, Eng C and Neumann HP. Differential Genetic Alterations in von Hippel-Lindau Syndrome-Associated and Sporadic Pheochromocytomas. *J Clin Endocrinol Metab* 2000;85(12):4568-74.
35. Brauch H, Weirich G, Brieger J, Glavac D, Rodl H, Eichinger M, Feurer M, Weidt E, Puranakanitstha C, Neuhaus C, Pomer S, Brenner W et al. VHL alterations in human clear cell renal cell carcinoma: association with advanced tumor stage and a novel hot spot mutation. *Cancer Res* 2000;60(7):1942-8.
36. Koch CA, Huang SC, Zhuang Z, Stolle C, Azumi N, Chrousos GP, Vortmeyer AO and Pacak K. Somatic VHL gene deletion and point mutation in MEN 2A-associated pheochromocytoma. *Oncogene* 2002;21(3):479-82.
37. van der Harst E, de Krijger RR, Dinjens WN, Weeks LE, Bonjer HJ, Bruining HA, Lamberts SW and Koper JW. Germline mutations in the vhl gene in patients presenting with phaeochromocytomas. *Int J Cancer* 1998;77(3):337-40.
38. Bot FJ, Sleddens HF and Dinjens WN. Molecular assessment of clonality leads to the identification of a new germ line TP53 mutation associated with malignant cystosarcoma phyllodes and soft tissue sarcoma. *Diagn Mol Pathol* 1998;7(6):295-301.
39. Wijnhoven BP, Lindstedt EW, Abbou M, IJzendoorn Y, de Krijger RR, Tilanus HW and Dinjens WN. Molecular genetic analysis of the von Hippel-Lindau and human peroxisome proliferator-activated receptor g tumor-suppressor genes in adenocarcinomas of the gastroesophageal junction. *Int J Cancer* 2001;96(6).
40. Bradley JF and Rothberg PG. Processed pseudogene from the von Hippel-Lindau disease gene is located on human chromosome 1. *Diagn Mol Pathol* 1999;8(2):101-6.
41. Frischmeyer PA, van Hoof A, O'Donnell K, Guerrerio AL, Parker R and Dietz HC. An mRNA surveillance mechanism that eliminates transcripts lacking termination codons. *Science* 2002;295(5563):2258-61.
42. Cassan M and Rousset JP. UAG readthrough in mammalian cells: Effect of upstream and downstream stop codon contexts reveal different signals. *BMC Mol Biol* 2001;2(1):3.
43. Prowse AH, Webster AR, Richards FM, Richard S, Olschwang S, Resche F, Affara NA and Maher ER. Somatic inactivation of the VHL gene in Von Hippel-Lindau disease tumors. *Am J Hum Genet* 1997;60(4):765-71.
44. Neumann HP, Berger DP, Sigmund G, Blum U, Schmidt D, Parmer RJ, Volk B and Kirste G. Pheochromocytomas, multiple endocrine neoplasia type 2, and von Hippel-Lindau disease. *N Engl J Med* 1993;329(21):1531-8.
45. Wilson RA and Ibanez ML. A comparative study of 14 cases of familial and nonfamilial pheochromocytomas. *Hum Pathol* 1978;9(2):181-8.
46. Davidowitz EJ, Schoenfeld AR and Burk RD. VHL Induces Renal Cell Differentiation and Growth Arrest through Integration of Cell-Cell and Cell-Extracellular Matrix Signaling. *Mol Cell Biol* 2001;21(3):865-74.
47. Hoffman MA, Ohh M, Yang H, Klco JM, Ivan M and Kaelin WG, Jr. von Hippel-Lindau protein mutants linked to type 2C VHL disease preserve the ability to downregulate HIF. *Hum Mol Genet* 2001;10(10):1019-27.

Chapter 3

Clinical characteristics of pheochromocytoma patients with germ line mutations in *SDHD*

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Abstract

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

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

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

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

Introduction

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

Familial PCC is inherited as an autosomal dominant trait alone or as a component of the multiple endocrine neoplasia type 2 syndrome, von Hippel-Lindau disease, or neurofibromatosis type 1. The remaining 90 percent of PCCs is classified as sporadic or nonsyndromic. However, Neumann et al. recently reported the presence of germ line mutations in 24% of a large series of apparently sporadic PCC patients.[3] One can thus conclude that, in the general population, more than 24% of PCC patients has a genetic predisposition to this tumor. This recent improvement in recognizing predisposition to PCCs is caused by the finding of germ line mutations in succinate dehydrogenase subunit D (*SDHD*), in patients with familial and apparently sporadic PCC. The *SDHD* gene was initially identified as a susceptibility gene for the autosomal dominant familial parasympathetic paraganglioma syndrome (PGL1; MIM 168000).[4] The gene encodes the small subunit (cybS) of cytochrome b in the mitochondrial enzyme complex II (succinate-ubiquinone oxidoreductase), and plays an important role in both the citric acid cycle and the aerobic respiratory chain.[5] It has been demonstrated that germ line mutations in *SDHC* (succinate dehydrogenase subunit C) and *SDHB* (succinate dehydrogenase subunit B), encoding two other components of complex II, also predispose to hereditary paraganglioma (PGL4 and PGL3, respectively).[6,7]

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

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

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

Patients and Methods

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

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

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

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

Table 1 Clinical Characteristics of 126 patients.

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

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

DNA preparation and SSCP Analysis

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

The entire open reading frame of the *SDHD* gene and all exon-intron boundaries were investigated with PCR primers and conditions as described previously.[4] PCR amplification of tumor DNA and matched normal DNA was performed in 15 µl reaction mixtures containing 1.5 mM $MgCl_2$, 10 mM Tris-HCl, 50 mM KCl, 0.02 mM dATP, 0.2 mM dGTP, dTTP, dCTP each, 0.8 µCi α32P-dATP (Amersham, Buckinghamshire, UK), 20 pmol of each sense and anti-sense primer, and 1 U Taq DNA polymerase (Amplitaq Gold, Perkin Elmer, Norwalk, CT, USA). The amplification profile consisted of an initial denaturation step at 94°C for 5 min, 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. Electrophoresis of PCR products was carried out overnight at 8W on non-denaturing gels, containing 8% polyacrylamide (49:1) and 10% (v/v) glycerol. For the exon 4 amplicons, electrophoresis was performed on

8% polyacrylamide gel without glycerol for 6 hours at +4°C and 20W. The gels were dried and exposed to X-ray film overnight at –70°C. DNA samples from 3 PGL patients with known germ line *SDHD* mutations D92Y, L95P (both exon 3), and L139P (exon 4) served as positive controls.

DNA sequencing

For each variant pattern identified by SSCP analysis, two independent genomic DNA samples from the patient's tumor were amplified for direct sequencing with the original primer pair. These PCR products were bi-directionally sequenced using Applied Biosystems Taq DyeDeoxy terminator cycle sequencing (Baseclear, Leiden, The Netherlands).

Statistics

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

Results

Identification of *SDHD* gene mutations

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

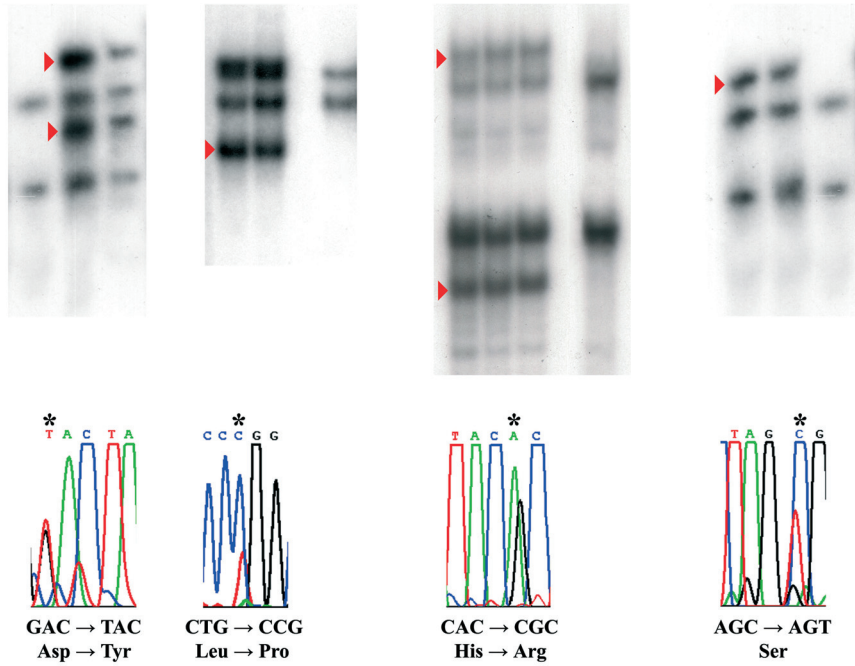

Figure 1 SDHD alterations identified in PCC patients by SSCP analysis and direct sequencing. Tumor (T) and corresponding germ line (N) DNA of the patients are shown. C = normal control sample. The autoradiographs show the migration patterns of normal DNA and the mobility shifts produced by aberrant alleles (red arrowheads). The figure shows the two pathogenic mutations, D92Y and L95P in patient A and C, the H50R variant in the primary tumor and a lung metastasis (M) of patient D, and the S68S polymorphism. The sequencing chromatograms below each autoradiograph show the exact nucleotide alterations. Note: when comparing SSCP fragment intensities, LOH of the wild type SDHD alleles is present in the D92Y and L95P samples, but not in S68S and H50R cases.

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

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

The H50R variant, which is likely a rare polymorphism, but possibly increases PCC susceptibility,[20] was present in the germ line DNA of 1 (0.8%) patient (D). This 32-year-old patient presented with both an extra-adrenal and an adrenal PCC, but had no additional tumors during 7 years of follow-up.

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

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

Patients' characteristics associated with *SDHD* mutations

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

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

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

Table 2 Overview of SDHD mutations.

		Freq. (%)	Freq. (%)	Freq. (%)	2001 Freq. (%)	2001 Freq. (%)	2002 Freq. (%)	2002 Freq. (%)	2002 Freq. (%)	2002 Freq. (%)	2003 Freq. (%)
TUMOR TYPE											
Adrenal		**496**	**384**	**109**	**28**	**20**	**44**	**241**	**9**	**18**	**24**
Sporadic		4/432	3/350	1/82‡	24	19	36	3/241¶	9	0/12	0/9
		(0.9)	(0.9)	(1.2)				(1.2)		H50R/ G12S	G12S
Bilateral		14	10	4						5	5
Familial	PCC only	1/5	1/5		1/4	1					
	PGL	1	1							1	
*malignant** (sporadic)*		*33*	*9*	*24*		*4*	*5*				
Extraadrenal		**4/55** (7.3)	**4/41** (9.8)	**14**			**6**	**4/22¶** (18)	**0/4** H50R	**2**	**0/7** G12S
malignant				9			3				
benign				5			3				
Multifocal		**8/17 (47)**	**6/14 (43)**	**2/3 (67)**				**4/8 (50)**		**1/3 (33)**	**1/3 (33)**
Sporadic	PCC	4/11	4/10	0/1 H50R				4/8			2
	PCC + PGL	1/2	1	1/1				?		1	
Familial	PCC + PGL	3/4 (75)	2/3 (67)	1/1						1/2	1/1
TOTAL		**17/565 (3.0)**	**14/439 (3.2)**	**3/126 (2.4)**	**1/28 (3.6)**	**20**	**50**	**11/271 (4.1)**	**13**	**1/23 (4.3)**	**1/34 (2.9)**

Discussion

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

Genetic screening is still under considerable debate as unnecessary screening has an undesirable psychological impact on the patients and is not cost-effective. One should therefore carefully report on indications that favor genetic testing.[21] So far, studies have recognized the fact that *SDHD* mutations are associated with extra-adrenal PCC,[22] but the clinical relevance of *SDHD* mutation screening has been poorly discussed. A careful review of all current data indicates that specific subgroups of PCC patients could be considered for genetic screening of *SDHD*. These include patients presenting with multifocal tumors (PCC and/or PGL) independent of their family history of PCC/PGL (50% harbor *SDHD* mutations) and patients presenting

with an extra-adrenal PCC (7% harbor *SDHD* mutations). Regarding patients presenting with a sporadic adrenal PCC, the overall likelihood of germ line *SDHD* mutations is only 1%. However, younger age of onset (≤ 35 years) or a family history of PCC or PGL in these patients are two features that increase the likelihood of a mutation to 6.3% (based on this study and Neumann et al.[3]) and 16.7%, respectively. Screening of patients presenting at age 35 and younger will identify at least 72% of patients with germ line *SDHD* mutations. Screening in apparently sporadic patients older than 35 years and without a family history of PCC/PGL, as well as in patients with sporadic bilateral PCC seems redundant since mutations in these patients are extremely rare (< 1%).

To justify genetic screening, testing for *SDHD* mutations should help to improve early diagnosis, prognosis or influence treatment. In PCC patients, early detection is the key factor to reduce morbidity and mortality and identification of patients that are prone to develop multiple tumors may improve early detection. It is thus of interest to consider to what extent *SDHD* mutation screening contributes to early diagnosis in PCC patients. Most patients that appear to harbor an *SDHD* mutation present with multiple tumors, so that the risk of additional tumors is already evident and surveillance will be adjusted. In these cases, genetic screening is of interest to clarify the genetic cause of the disease, or to identify positive family members. Additionally, in some patients presenting with multiple abdominal foci on MRI, MIBG or octreotide scintigraphy, mutation screening may help to differentiate between lymph node metastases and multiple independent synchronous tumors.

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

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

Table 3 Presenting diagnosis and follow-up of patients with a germ line SDHD mutation, in this study and in the literature.

Patient	Age	Presentation	Family history	Follow up	SDHD mutation
This Study					
A	27	unilateral adrenal PCC	–	para-aortic PCC after 25 yrs	D92Y
B	38	mediastinal PCC, bilateral CBT	PGL	para-aortic & aortico-pulm. PCC	D92Y
C	25	multiple para-aortic PCC	–	CBT	L95P
D	32	adrenal malignant PCC, para-aortic PCC	–	–	H50R*
Astuti et al. (2001)					
E (family)	13-23	unilateral or bilateral adrenal PCC, para-aortic PCC	CBT	?	S32fs
Neumann et al. (2002) / Gimm et al. (2000)					
F	39	unilateral adrenal PCC	–	CBT	IVS1+2T>G
G	33	thoracic and para-aortic PCC	–	?	R38X
H‡	5, <20, <30, <30, 31-40, 41-50, 59	multiple PCC	–	majority of patients develops additional tumors, either (extra-adrenal PCC) or PGL	W5X, C11X, A13fs, R38X, D92Y, Q121X
I‡		multiple PCC	–		
K‡		multiple PCC	–		
L‡		unilateral adrenal PCC	–		
M‡		unilateral adrenal PCC	–		
N‡		extra-adrenal PCC	–		
O‡		extra-adrenal PCC	–		
P‡		extra-adrenal PCC	–		
R‡		extra-adrenal PCC	–		
Cascon et al. (2002)					
S	40	Para-aortic and bilateral CBT	PCC and PGL	?	W43X
Bauters et al. (2003)					
T	30	juxtarenal PCC, cervical PGL	PCC and/or PGL	?	IVS2-1G>T

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

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

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

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

patients, will hopefully contribute to improving appropriate genetic screening for patients who are at risk of developing multiple PCCs and PGLs.

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References

1. Kenady DE, McGrath PC, Sloan DA, et al: Diagnosis and management of pheochromocytoma. *Curr Opin Oncol* 9:61-67, 1997.
2. Walther MM, Keiser HR, Linehan WM: Pheochromocytoma: evaluation, diagnosis, and treatment. *World J Urol* 17:35-39, 1999.
3. Neumann HP, Bausch B, McWhinney SR, et al: Germ-line mutations in nonsyndromic pheochromocytoma. *N Engl J Med* 346:1459-1466, 2002.
4. Baysal BE, Ferrell RE, Willett-Brozick JE, et al: Mutations in SDHD, a mitochondrial complex II gene, in hereditary paraganglioma. *Science* 287:848-851, 2000.
5. Hirawake H, Taniwaki M, Tamura A, et al: Characterization of the human SDHD gene encoding the small subunit of cytochrome b (cybS) in mitochondrial succinate-ubiquinone oxidoreductase. *Biochim Biophys Acta* 1412:295-300, 1999.
6. Astuti D, Latif F, Dallol A, et al: Gene mutations in the succinate dehydrogenase subunit SDHB cause susceptibility to familial pheochromocytoma and to familial paraganglioma. *Am J Hum Genet* 69:49-54, 2001
7. Niemann S, Muller U: Mutations in SDHC cause autosomal dominant paraganglioma, type 3. *Nat Genet* 26:268-270, 2000.
8. Sato T, Saito H, Yoshinaga K, et al: Concurrence of carotid body tumor and pheochromocytoma. *Cancer* 34:1787-1795, 1974.
9. Astuti D, Douglas F, Lennard TW, et al: Germline SDHD mutation in familial phaeochromocytoma. *Lancet* 357:1181-1182, 2001.
10. Cascon A, Ruiz-Llorente S, Cebrian A, et al: Identification of novel SDHD mutations in patients with phaeochromocytoma and/or paraganglioma. *Eur J Hum Genet* 10:457-461, 2002.
11. Gimm O, Armanios M, Dziema H, et al: Somatic and occult germ-line mutations in SDHD, a mitochondrial complex II gene, in nonfamilial pheochromocytoma. *Cancer Res* 60:6822-6825, 2000.
12. Perren A, Barghorn A, Schmid S, et al: Absence of somatic SDHD mutations in sporadic neuroendocrine tumors and detection of two germline variants in paraganglioma patients. *Oncogene* 21:7605-7608, 2002.
13. Kytola S, Nord B, Elder EE, et al: Alterations of the SDHD gene locus in midgut carcinoids, Merkel cell carcinomas, pheochromocytomas, and abdominal paragangliomas. *Genes Chromosomes Cancer* 34:325-332, 2002.

14. Aguiar RC, Cox G, Pomeroy SL, et al: Analysis of the SDHD gene, the susceptibility gene for familial paraganglioma syndrome (PGL1), in pheochromocytomas. *J Clin Endocrinol Metab* 86:2890-2894, 2001.
15. Bauters C, Vantyghem MC, Leteurtre E, et al: Hereditary phaeochromocytomas and paragangliomas: a study of five susceptibility genes. *J Med Genet* 40:e75, 2003.
16. Dannenberg H, Dinjens WN, Abbou M, et al: Frequent germ-line succinate dehydrogenase subunit D gene mutations in patients with apparently sporadic parasympathetic paraganglioma. *Clin Cancer Res* 8:2061-2066, 2002.
17. Baysal BE, Willett-Brozick JE, Lawrence EC, et al: Prevalence of SDHB, SDHC, and SDHD germline mutations in clinic patients with head and neck paragangliomas. *J Med Genet* 39:178-183, 2002.
18. Baysal BE: Hereditary paraganglioma targets diverse paraganglia. *J Med Genet* 39:617-622, 2002.
19. Taschner PE, Jansen JC, Baysal BE, et al: Nearly all hereditary paragangliomas in the Netherlands are caused by two founder mutations in the SDHD gene. *Genes Chromosomes Cancer* 31:274-281, 2001.
20. Cascon A, Ruiz-Llorente S, Cebrian A, et al: G12S and H50R variations are polymorphisms in the SDHD gene. *Genes Chromosomes Cancer* 37:220-221, 2003.
21. American Society of Clinical Oncology policy statement update: genetic testing for cancer susceptibility. *J Clin Oncol* 21:2397-2406, 2003.
22. Eng C, Kiuru M, Fernandez MJ, et al: A role for mitochondrial enzymes in inherited neoplasia and beyond. *Nat Rev Cancer* 3:193-202, 2003.
23. Benn DE, Croxson MS, Tucker K, et al: Novel succinate dehydrogenase subunit B (SDHB) mutations in familial phaeochromocytomas and paragangliomas, but an absence of somatic SDHB mutations in sporadic phaeochromocytomas. *Oncogene* 22:1358-1364, 2003.
24. Cascon A, Cebrian A, Ruiz-Llorente S, et al: SDHB mutation analysis in familial and sporadic phaeochromocytoma identifies a novel mutation. *J Med Genet* 39:E64, 2002.
25. Young AL, Baysal BE, Deb A, et al: Familial malignant catecholamine-secreting paraganglioma with prolonged survival associated with mutation in the succinate dehydrogenase B gene. *J Clin Endocrinol Metab* 87:4101-4105, 2002.
26. Renard L, Godfraind C, Boon LM, et al: A novel mutation in the SDHD gene in a family with inherited paragangliomas--implications of genetic diagnosis for follow up and treatment. *Head Neck* 25:146-151, 2003.

Chapter 4

Losses of chromosomes 1p and 3q are early genetic events in the development of sporadic pheochromocytomas

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Abstract

Despite several loss of heterozygosity studies, a comprehensive genomic survey of pheochromocytomas is still lacking. To identify DNA copy number changes, which might be important in tumor development and progression and which may have diagnostic utility, we evaluated genetic aberrations in 29 sporadic adrenal and extra-adrenal pheochromocytomas (19 clinically benign tumors and 10 malignant lesions). Comparative genomic hybridization (CGH) was performed using directly fluorochrome-conjugated DNA extracted from frozen (16) and paraffin-embedded (13) tumor tissues. The most frequently observed changes were losses of chromosomes 1p11-p32 (86%), 3q (52%), 6q (34%), 3p, 17p (31% each), 11q (28%), and gains of chromosomes 9q (38%) and 17q (31%). No amplifications were identified and no differences between adrenal and extra-adrenal tumors were detected. Progression to malignant tumors was strongly associated with deletions of chromosome 6q (60% vs. 21% in clinically benign lesions, $P = 0.0368$) and 17p (50% vs. 21%). Fluorescence *in situ* hybridization confirmed the CGH data of chromosomes 1p, 3q, and 6q, and revealed aneuploidy in some tumors.

Our results suggest that the development of pheochromocytomas is associated with specific genomic aberrations, such as losses of 1p, 3q, and 6q and gains of 9q and 17q. In particular, tumor suppressor genes on chromosomes 1p and 3q may be involved in early tumorigenesis, and deletions of chromosomes 6q and 17p in progression to malignancy.

Introduction

Pheochromocytomas (PCCs) represent neuroendocrine tumors derived from pheochromocytes within and outside the adrenal medulla. PCCs usually produce catecholamines and, as a result of inappropriate hormone secretion, can cause life-threatening disorders, such as myocardial infarction and cerebrovascular hemorrhage.

To date no reliable clinical or histopathological markers are available to distinguish benign from malignant PCCs. Metastases, occurring in approximately 10% of the tumors, are the only convincing sign of malignancy. They may already be present at the time of diagnosis, or occur only after lag phases, as long as 25-30 years.[1] Such a situation calls for more accurate markers that can predict the clinical behavior of these tumors.

The genetic mechanisms underlying the tumorigenesis of PCCs are poorly understood. Because of the known association with the inherited tumor syndromes Multiple Endocrine Neoplasia type 2 (MEN2), Von Hippel-Lindau disease (VHL) and Neurofibromatosis type 1 (NF1)[2-4], PCCs have been investigated for involvement of the responsible *RET*, VHL, and NF1 genes, respectively.[5-9] However, only a subset of sporadic lesions harbors somatic mutations in these genes, suggesting that they do not play a prominent role in PCC tumorigenesis.[10-13] Furthermore, no mutations have been detected in the c-mos proto-oncogene and the p16 tumor suppressor gene (TSG) in benign and malignant lesions.[14,15] Conflicting results exist with regard to the frequency of *TP53* gene alterations in PCCs as well as for the association of c-*erb*B-2 expression with malignancy.[16-19] Other oncogenes that have been found to be overexpressed in PCCs, include c-myc, c-fos, and bcl-2.[19,20]

Several LOH studies provided evidence for involvement of TSGs on chromosomes 1p, 3p, 11p, 17p, and 22q.[21-25] However, the impact of these data is limited, since small series of sporadic PCCs were analyzed and often no correlation with clinical data was presented. Moreover, the vast majority of the genome remaines unexamined. Here, we performed comparative genomic hybridization (CGH) analysis on 29 apparently sporadic PCCs, including 19 benign and 10 malignant lesions, in order to identify chromosomal alterations which may be important in tumor development and behavior and might have diagnostic utility. Additionally, fluorescence *in situ* hybridization (FISH) experiments were carried out to independently confirm some of the CGH data.

Materials and Methods

Patients and tumor samples

Altogether, tumors of 29 patients with an apparently sporadic adrenal (n=25) or extra-adrenal (n=4) PCC were analyzed, including 19 clinically benign and 10 proven malignant lesions (with metastases). A pheochromocytoma was considered sporadic if there was no family history of MEN2, VHL, or its constituent tumors and there were no other signs of MEN 2, VHL, or NF1 recorded by the clinician. Furthermore, previous analyses did not reveal germline mutations in the *RET* and VHL gene in these tumors[10,11] (Dannenberg et al., unpublished data). The average age of the patients was 50.3 years (range: 23-79) and the mean size of the tumors was 7.1 ± 4.6 cm for benign lesions and 11.0 ± 4.7 cm for malignant lesions. Nine of 19 benign and 7 of 10 malignant tumors were from female patients. Except for two lymph node metastases, all specimens were primary tumors. Clinical follow-up was obtained in all benign cases with an average follow-up period of 7.6 years. Relevant characteristics of all patients are listed in Table 1.

DNA Extraction

Genomic DNA from 16 frozen tumors was isolated using the D-5000 Puregene DNA Isolation kit (Gentra Systems, Minneapolis, MN). DNA extraction from 13 formalin-fixed, paraffin-embedded samples was performed by standard detergent-proteinase K lysis, followed by phenol/chloroform extraction and ethanol precipitation, as described elsewhere.[26]

CGH and Digital Image Analysis

CGH was carried out as previously described.[26] One µg of high molecular weight tumor DNA was labeled with Spectrum Green-dUTPs (Vysis, Downers Grove, IL) by nick translation (BioNick kit, Life Technologies, Basel, Switzerland). Archival tumor DNA with fragment sizes of < 1 kb was labeled with a platinum/D-Green complex (D-Green-ULS) using the Kreatech Universal Linkage System (ULS) D-Green labeling kit (kindly provided by Kreatech Morwell Diagnostics GmbH, Zürich, Switzerland), according to the manufacturer's recommendations. Briefly, 800 ng of tumor DNA and 1.6 µl of D-Green-ULS, adjusted to a total volume of 20 µl was incubated at 65°C for 20 minutes. To reduce background signals, the ULS-labeled samples were purified using Qiagen nucleotide removal columns (Qiagen GmbH, Hilden, Germany).

The hybridization mixture consisted of 200 ng Spectrum (or D-) Green-labeled tumor DNA, 200 ng Spectrum Red-labeled sex matched normal reference DNA (Vysis) and 10-20 µg human Cot-1 DNA (Life Technologies) dissolved in 10 µl of hybridization buffer (50% formamide, 10% dextran sulfate, 2x SSC, pH 7.0). Hybridization to normal metaphase spreads (Vysis) took place for 3 days at 37°C.

Digital images were collected from 6 to 7 metaphases using a Photometrics cooled CCD camera (Microimager 1400; Xillix Technologies, Vancouver, Canada). The QUIPS software program (Vysis) was used to calculate average green-to-red ratio profiles of at least four observations per autosome and two observations per sex chromosome 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. Because of some false positive results at chromosomes 1p32-pter, 16p, 19, and 22 found in normal tissues, gains of these known G-C-rich regions were excluded from all analyses.

FISH Analysis

To validate CGH data independently, touch preparations of four frozen PCCs were subjected to fluorescence *in situ* hybridization (FISH) analysis using a chromosome 1-specific centromere probe (pUC1.77) in combination with an 1p22-31-specific YAC probe (929-E-1) (kindly provided by S. Franke, Center for Human Genetics, University of Leuven, Belgium and J.R. Testa, Fox Chase Cancer Center, Philadelphia, USA), or a repeat probe (p1-79), mapping to the 1p36 region.[27-29] Chromosomes 3 and 6 were examined using a chromosome 3 (pα3.5) and 6 (p308) centromere probe, in combination with a 3q26-specific (*pancpin*) and 6q22-specific (cCI6-44) probe, respectively.[30-33] Cell processing, probe labeling, *in situ* hybridization, and detection of the hybridized signals were performed as recently described.[34] For each tumor, hybridization signals of 100 interphase nuclei were scored. Numerical chromosomal aberrations were considered an aneusomy when the percentage of nuclei showing an abnormal number of centromere signals exceeded 30%. The presence of only half of the locus specific signals compared to the number of centromere signals in more than 30% of the nuclei was interpreted as a deletion.

Statistical Analysis

To calculate the statistical significance of differences in genomic changes between benign and malignant tumors, we used contingency table analysis, which was also used to analyze the relationship between genomic alterations and sex. Regression analysis was carried out to compare the number of chromosomal aberrations with tumor size.

Results

Overview of CGH Findings

Fig. 1A summarizes all DNA copy number changes identified in the 29 evaluated sporadic PCCs. Genetic alterations were observed in all PCCs, and the average number of chromosome arm aberrations per tumor was 6.3 ± 4.4 (range: 1-21). Chromosomal losses (mean: 4.3, range: 1-12) were more frequent than gains (mean: 1.9, range: 0-13) and no amplifications could be detected (Table 1). The average number of chromosomal alterations was marginally associated with malignancy (5.3 ± 2.7 vs. 8.2 ± 6.1), principally due to a higher frequency of gains. 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%). Representative examples of CGH results are shown in Fig. 1B.

Genetic Changes in Relation to Clinical Parameters

Ten of 29 PCCs fell into the category of malignant tumors, since regional or distant metastases occurred in these patients. In these lesions, losses of chromosome 6q were more often observed as compared to clinically benign lesions (60% vs. 21%, $P = 0.0368$). To a lesser degree, losses of 17p were also related to metastatic disease (50% vs. 21%), whereas deletions of chromosome 3p and 3q were preferably associated with benign tumors (42% vs. 10% and 63% vs. 30%, respectively). Although the malignant tumors were significantly larger in size (11.0 ± 4.7 cm vs. 7.1 ± 4.6 cm, $P = 0.0470$) and demonstrated a slightly higher number of chromosome arm copy number changes, there was no correlation between the number of genomic aberrations and the tumor size. No differences were seen between adrenal and extra-adrenal PCCs.

Additionally, a statistically significant correlation was found between the number of chromosomal alterations and female gender ($P = 0.0196$), predominantly due to a higher frequency of gains of different genomic regions.

Table 1 Patient Characteristics and Genetic Findings.

				CGH results					FISH results			
				Number of changes				IHC				
No.	Age/ Sex	Size (cm)	Location	All	Gain	Loss	Chromosomal changes of chromosomes 1, 3, 6, 9, and 17	p53*	1p22†	1p36†	3q26†	6q22†
Benign PCCs												
1	63/F	4.7	EA	4	1	3	1p11-31–, 3p–, 9q33-qter+					
2	29/F	18	L	6	3	3	1p11-31–, 3p–, 9q33-qter+, 17pq+					
3	49/M	13	BI	7	2	5	1p–, 3pq–, 17p–, 17q+					
4	34/M	6	L	4	0	4	3pq–, 9q33-qter+		di/tetra	di/tetra	mon/di	di/tetra
5	75/F	5	EA	6	2	5	1p11-32–, 17pq+		4-2	4-2	tetra	tetra
6	52/F	5	R	9	4	2	9q32-34+					
7	43/F	3	R	3	1	2	1p11-32–, 3q–, 9q32-qter+					
8	42/M	6	L	5	1	4	1p11-32–, 3q–, 6q–, 9q32-qter+		2-1/4-2	di/tetra	2-1/4-2	2-1/4-2
9	53/M	nk	L	4	0	4	1p11-31–					
10	27/F	11	L	6	2	4	1p–, 3pq–, 9q32-qter+, 17q+					
11	72/F	nk	R	14	2	12	1p11-32–, 3pq–, 6q–, 9q33-qter+, 17q+					
12	43/M	6	R	3	0	3	1p11-31–, 3q21-qter–, 17p–					
13	42/M	4.5	R	3	0	3	1p11-35–, 3q–	–				
14	45/F	2.5	L	7	2	5	1p11-31–, 6q–, 17q22-qter+	–				
15	66/M	3.2	L	6	1	5	1p–, 3pq–, 17p–	–				
16	79/M	5	R	5	1	4	1p11-35–, 3pq–	–				
17	51/F	5	L	3	0	3	1p11-32–, 3q–, 6q–	–				
18	57/M	15	nk	1	0	1		–				
19	47/M	nk	EA	4	0	4	1p–, 3q–, 17pq–	–				
Malignant PCCs												
20	49/F	4	Me., LN	5	2	3	1p11-31–, 3q–, 6q–, 9q33-qter+, 17q22-qter+	+				
21	36/M	nk	nk	1	0	1	1p11-21–					
22	43/F	nk	Me., LN	5	0	5	1p–, 3q–, 6q–, 17pq–		2-1	2-1	2-1	2-1
23	36/M	21	EA	3	1	2	1p11-31–, 9q32-qter+					
24	70/F	16	L	6	0	6	1p31-pter–, 6q–, 17p–	–				
25	65/F	11	R	13	6	7	1p11-31–, 9pq11-33–, 17p–, 17q23-qter+	+				
26	56/F	8.5	L	21	13	8	1p–, 3p–, 3q+, 6q–, 9pq+, 17pq–	+				
27	23/F	8	nk	4	2	2	1p11-32–, 3q–, 17q+	–				
28	62/F	12	L	16	9	7	6q–	–				
29	49/M	7,5	R	8	0	8	1p–, 6q22-qter–, 17p–	–				

* Data previously published (ref. 20).

† In combination with the chromosome-specific centromere probe.

Abbreviations: M, male; F, female; L, left adrenal gland; R, right adrenal gland; BI, bilateral adrenal localization; EA, extra-adrenal localization; Me., metastasis; LN, lymph node; nk, not known; n, no detectable changes; mon, monosomy; di, disomy; tetra, tetrasomy; 4-2, tetrasomy with only two copies (loss) of the specific region; 2-1, disomy with allelic loss of the specific region.

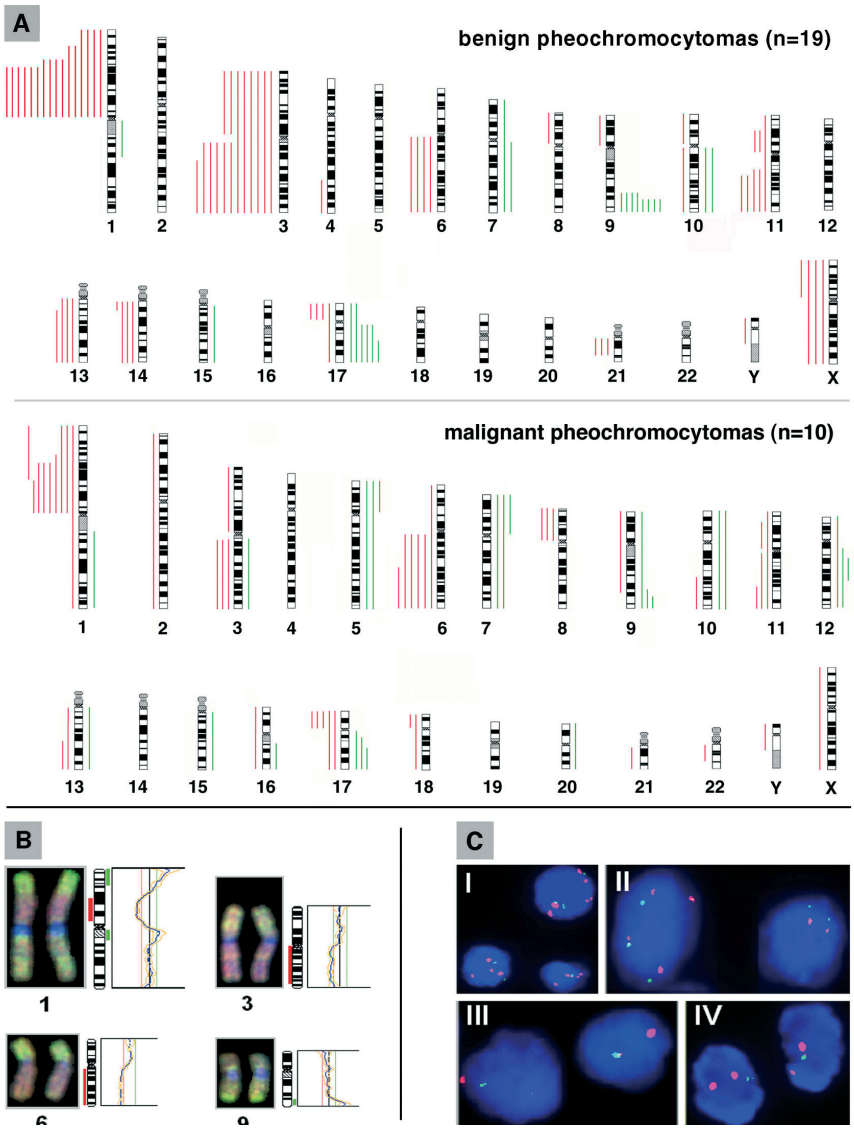

Figure 1 Summary of all DNA copy number changes detected by CGH in 19 benign and 10 malignant sporadic PCCs (A). The vertical green lines on the right side of the chromosome ideograms indicate gains, the red on the left losses of the corresponding chromosomal region. (B) Individual representative examples of CGH digital images (left) and fluorescent ratio profiles (right) illustrating genomic alterations of chromosomes 1 (loss of 1p11-32 with gain of the C-G rich telomeric region), 3 (loss of 3q) and 6 (loss of 6q) and 9 (gain of 9q33-34). (C) Results of FISH analysis. I and II: interphase touch preparations of tumor number 8 analyzed using a centromeric probe for chromosome 1 (red signals) in combination with a 1p22-31-specific probe (green signals) (I) or a 1p36-specific probe (II), demonstrating aneuploidy of this tumor with loss of the 1p22-31 locus (I) but not of the 1p36 locus (II). III: monosomy of chromosome 3 in case 4. VI: diploid, malignant tumor, showing the two copies of centromere 6 (red signals) and only one copy of the 6q22-specific locus (green signal), indicating loss of 6q.

Comparison of CGH and FISH

FISH analysis confirmed the CGH results of chromosomes 1p, 3q, and 6q in 4 tumors that showed chromosomal imbalances as detected by CGH (Table 1). Two of the 4 tumors presented with a partial loss of chromosome 1p (1p11-32). In one case, FISH analysis proved the loss of this region and the retention of 1p36. However, the other tumor appeared to have a complete loss of chromosome 1p, which could be expected, since the 1p32-pter region is known to generate some false positive results in CGH.

In addition, the 3 benign lesions revealed a tetrasomy in the major population of cells for all chromosomes analyzed. In contrast, the malignant tumor turned out to be diploid. In two of the three aneuploid lesions a subpopulation of diploid tumor cells (as demonstrated by two centromere signals and only one locus specific signal per nucleus) could also be detected. Examples of FISH results are shown in Figure 1C.

Discussion

Our genome-wide survey of genetic alterations in sporadic benign and malignant PCCs identified specific aberrations associated with tumor initiation and progression, which might be useful in clinical diagnosis. Our results highlight several novel chromosomal regions that may harbor genes critically involved in the pathogenesis of sporadic PCCs. A high frequency of losses of chromosomes 1p and 3q in the benign tumors suggests that they are important genetic events in early tumorigenesis. Deletions of chromosome 6q were strongly associated with metastatic disease and thus may help to define the malignant potential of PCCs.

The most commonly encountered chromosomal aberrations in our series of 29 sporadic PCCs involved 1p, 3q, 9q, 6q, 3p, 17p, 17q, and 11q. These findings support and extend the hypothesis of Koshla *et al.*[22] that multiple genes are involved in the pathogenesis of these tumors. Although LOH studies have shown loss of heterozygosity at 1p, 3p, 11q, 17p, and 22q in syndrome-related and sporadic PCCs[21-25], the here found losses of 3q and 6q and gains of 9q and 17q have not been reported previously. In contrast to a previous LOH analysis[25], we observed only one tumor with loss of 22q. Possible explanations for this discrepancy are the limited sensitivity of CGH for small losses (< 10 Mb) and the fact that G-C-rich chromosomal regions, like those in chromosome 22, are known to yield false positive CGH results.

Others[21-24] and we found a very high incidence of 1p loss in PCCs, suggesting that it might be an important tumorigenic event. Deletions of chromosome 1p are common in several other human neoplasms, including adrenocortical carcinomas and neurectodermally derived tumors, such as neuroblastomas.[35, 36] The smallest common region of overlap as detected by CGH in our study involves the 1p22-32 region. Because of known false positive CGH results at 1p32-pter, we verified the CGH data by FISH using a probe mapping to 1p36. Our observations

indicate that some PCCs indeed exhibit a partial loss of chromosome 1p. This correlates well with the results of others[23,37] who found evidence for a potential PCC tumor suppressor locus in the middle of the short arm of chromosome 1. There are multiple candidate TSGs in this region, including *TGF*β*-R3*, *p18INK4C*, and *PTPRF/LAR*, none of which has been proven to be relevant in PCC tumorigenesis. One should also consider the possibility that more than a single TSG on 1p contributes to the development of PCCs.

Another interesting finding of our study is the frequent loss of chromosome 3q in PCCs, which is more often encountered in benign than in malignant tumors. This might point to diverging pathways in the development of benign and malignant PCCs, although it is known that in malignant tumors duplications of chromosome arms can occur.[38] In that case, LOH will not be detected by CGH. However, it is certainly conceivable that loss of chromosome 3q is an early genetic event in the pathogenesis of PCCs. Thus far, frequent allelic loss of chromosome 3q has been demonstrated only in osteosarcoma, with a putative TSG localized to a region between 3q26.2 and 3q26.3.[39] The possible relationship of this candidate TSG for PCC development however, is not clear.

Data on the genetic events that determine the malignant potential of PCCs are scarce. We observed a strong association of losses of chromosome 6q with advanced disease stage. Frequent allelic imbalances at 6q have also been reported in other malignancies[40,41] and appear to be related to poor prognosis or metastatic disease in certain tumors.[42,43] Although these data are suggestive of the existence of several TSGs at 6q, none has been identified so far. In addition, underrepresentations of chromosome 17p were often observed in metastasizing lesions. 17p13 contains the *TP53* gene, the TSG most commonly involved in human carcinogenesis. Fourteen of 29 PCCs evaluated in the present study have previously been immunohistochemically investigated for TP53 accumulation.[20] Comparison of these results with our CGH results (Table 1), indicates that *TP53* alterations may play a role in a subset of these tumors. Involvement of another TSG on 17p, however, cannot be ruled out and one candidate gene could be the putative TSG at the medulloblastoma locus 17p13.3.[44] Interestingly, three malignant PCCs exhibited simultaneous gains of chromosomes 5p, 7p and 12q, similar to findings for malignant endocrine pancreatic tumors.[45]

In conclusion, our results indicate that the development of PCCs is associated with specific genetic alterations, of which deletions of chromosome 1p (1p22-32) and 3q are early genetic events. Losses involving chromosome 3q (minimal overlapping region, 3q21-qter) and 6q (6q22-qter) and overrepresentations of chromosome 9q (9q33-34) and 17q point to new regions that may contain PCC TSGs and oncogenes in addition to the previously reported TSG loci 1p, 3p, 11q, 17p, and 22q.

Further studies are required to narrow down the critical regions in each identified chromosome and to characterize the putative genes involved in tumor initiation and progression of sporadic PCCs.

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References

1. Schlumberger M, Gicquel C, Lumbroso J, Tenenbaum F, Comoy E, Bosq J, Fonseca E, Ghilani PP, Aubert B, Travagli JP, Gardet P, Parmentier C: Malignant pheochromocytoma: clinical, biological, histologic and therapeutic data in a series of 20 patients with distant metastases. *J Endocrinol Invest* 1992, 15:631-642.
2. Ponder BAJ, Smith D: The MEN2 syndromes and the role of the ret proto-oncogene. *Adv Cancer Res* 1996, 70:179-222.
3. Neumann HPH, Lips CJM, Hsia YE, Zbar B: Von Hippel-Lindau syndrome. *Brain Path* 1995, 5:181-193.
4. Bader JL: Neurofibromatosis and cancer. Ann NY Acad Sci 1986, 486:57-65.
5. Mulligan LM, Kwok JB, Healey CS, Elsdon MJ, Eng C, Gardner E, Love DR, Mole SE, Moore JK, Papi L, et al: Germ-line mutations of the RET proto-oncogene in multiple endocrine neoplasia type 2A. *Nature* 1993, 363:458-460
6. Latif F, Tory K, Gnarra J, Yao M, Duh FM, Orcutt ML, Stackhouse T, Kuzmin I, Modi W, Geil L, Schmidt L, Zhou F, Li H, Wei MH, Chen F, Glenn G, Choqke P, Walther MM, Weng Y, Duan DR, Dean M, Glavac D, Richards FM, Crossey PA, Ferguson-Smith MA, Le Paslier D, Chumakov I, Cohen D, Chinault CA, Maher ER, Linehan WM, Zbar B, Lerman MI: Identification of the von Hippel-Lindau tumour suppressor gene. *Science* 1993, 260:1317-1320.
7. Richards FM, Webster AR, McMahon R, Woodward ER, Rose S, Maher ER: Molecular genetic analysis of von Hippel-Lindau disease. *J Int Med* 1998, 243:527-533.
8. Crossey PA, Richards FM, Foster K, Green JS, Prowse A, Latif F, Lerman MI, Zbar B, Affara NA, Ferguson-Smith MA, Maher ER: Identification of intragenic mutations in the von Hippel-Lindau disease tumor suppressor gene and correlation with disease phenotype. *Hum Mol Genet* 1994, 3:1303-1308.
9. Ponder B: Human Genetics. Neurofibromatosis gene cloned. *Nature* 1990, 346:703-704.
10. Harst E van der, Krijger RR de, Bruining HA, Lamberts SWJ, Bonjer HJ, Dinjens WNM, Proye C, Koper JW, Bosman FT, Roth J, Heitz PU, Komminoth P: Prognostic value of *RET* proto-oncogene point mutations in malignant and benign, sporadic phaeochromocytomas. *Int J Cancer* 1998, 79:537-540.
11. Harst E van der, Krijger R.R. de, Dinjens WNM, Weeks LE, Bonjer HJ, Bruining HA, Lamberts SWJ, Koper JW: Germline mutations in the *vhl* gene in patients presenting with phaeochromocytomas. *Int J Cancer* 1998, 77:337-340.
12. Eng C, Crossey PA, Mulligan L, Healey CS, Houghton C, Prowse A, Chew SL, Dahia PLM, O'Riordan JLH, Toledo SPA, Smith DP, Maher ER, Ponder BAJ: Mutations in the *RET* proto-oncogene and the von Hippel-Lindau disease tumour suppressor gene in sporadic and syndromic phaeochromocytomas. *J Med Genet* 1995, 32:934-937.

13. Gutmann DH, Geist RT, Rose K, Wallin G, Moley JF: Loss of neurofibromatosis type I (NF1) gene expression in pheochromocytomas from patients without NF1. *Genes Chromosomes Cancer* 1995, 13:104-109.
14. Eng C, Foster KA, Healey CS, Houghton C, Gayther SA, Mulligan LM, Ponder BAJ: Mutation analysis of the c-mos proto-oncogene and the endothelin-B receptor gene in medullary thyroid carcinoma and phaeochromocytoma. *Br J Cancer* 1996, 74:339-341.
15. Aguiar RCT, Dahia PLM, Sill H, Toledo SPA, Goldman JM, Cross NCP: Deletion analysis of the p16 tumour suppressor gene in phaeochromocytomas. *Clin Endocrinol* 1996, 45:93-96.
16. Herfarth KK, Wick MR, Marshall HN, Gartner E, Lum S, Moley JF: Absence of TP53 alterations in pheochromocytomas and medullary thyroid carcinomas. *Genes Chromosomes Cancer* 1997, 20:24-29.
17. Yoshimoto T, Naruse M, Zeng Z, Nishikawa T, Kasajima T, Toma H, Yamamori S, Matsumoto H, Tanabe A, Naruse K, Demura H: The relatively high frequency of p53 gene mutations in multiple and malignant phaeochromocytomas. *J Endocrinol* 1998, 59:247-255.
18. Castilla-Guerra L, Moreno AM, Fernández-Moreno MC, Utrilla JC, Fernández E, Galera-Davison H: Expression and prognostic value of c-*erb*B-2 oncogene product in human phaeochromocytomas. *Histopathol* 1997, 31:144-149.
19. Krijger RR de, Harst E van der, Ham F van der, Stijnen T, Dinjens WNM, Koper JW, Bruining HA, Lamberts SWJ, Bosman FT: Prognostic value of p53, bcl-2, and c-*erb*B-2 protein expression in phaeochromocytomas. *J Pathol* 1999, 188:51-55.
20. Goto K, Ogo A, Yanase T, Haji M, Ohashi M, Nawata H: Expression patterns of the c-myc gene in adrenocortical tumors and pheochromocytomas. *J Clin Endocrinol Metab* 1990, 70:353-357.
21. Tsutsumi M, Yokota J, Kakizoe T, Koiso K, Sugimura T, Terada M: Loss of heterozygosity on chromosomes 1p and 11p in sporadic pheochromocytoma. *J Natl Cancer Inst* 1989, 81:1097-1101.
22. Khosla S, Patel VM, Hay ID, Schaid DJ, Grant CS, Van Heerden JA, Thibodeau SN: Loss of heterozygosity suggests multiple genetic alterations in pheochromocytomas and medullary thyroid carcinomas. *J Clin Invest* 1991, 87:1691-1699.
23. Moley JF, Brother MB, Fong CT, White PS, Baylin SB, Nelkin B, Wells SA, Brodeur GM: Consistent association of 1p loss of heterozygosity with pheochromocytomas from patients with multiple endocrine neoplasia type 2 syndrome. *Cancer Res* 1992, 52:770-774.
24. Vargas MP, Zhuang Z, Wang C, Vortmeyer A, Linehan WM, Merino MJ: Loss of heterozygosity on the short arm of chromosomes 1 and 3 in sporadic pheochromocytoma and extra-adrenal paraganglioma. *Hum Pathol* 1997, 28:411-415.
25. Tanaka N, Nishisho I, Yamamoto M, Miya A, Shin E, Karakawa K, Fujita S, Kobayashi T, Rouleau GA, Mori T, Takai S: Loss of heterozygosity on the long arm of chromosome 22 in pheochromocytoma. *Genes Chromosomes Cancer* 1992, 5:399-403.
26. Richter J, Jiang F, Gorog JP, Sartorius G, Egenter C, Gasser TC, Moch H, Mihatsch MJ, Sauter G: Marked genetic differences between stage pTa and stage pT1 papillary bladder cancer detected by comparative genomic hybridization. *Cancer Res* 1997, 57:2860-2864.
27. Cooke HJ, Hindley J: Cloning of human satellite III DNA: different components are on different chromosomes. *Nucleic Acids Res* 1979, 6:3177-3197.
28. Lee WC, Balsara B, Liu Z, Jhanwar SC, Testa JR: Loss of heterozygosity analysis defines a critical region in chromosome 1p22 commonly deleted in human malignant mesothelioma. *Cancer Res* 1996, 56:4297-4301.
29. Buroker N, Bestwick R, Haight G, Magenis RE, Litt M: A hypervariable repeated sequence on human chromosome 1p36. *Hum Genet* 1987, 77:175-181.

30. Waye JS, Willard HF: Chromosome specificity of satellite DNAs: short- and long-range organization of a diverged dimeric subset of human alpha satellite from chromosome 3. *Chromosoma* 1989, 97:475-480.
31. Jabs EW, Wolf SF, Migeon BR: Characterization of a cloned DNA sequence that is present at centromeres of all human autosomes and the X chromosome and shows polymorphic variation. *Proc Natl Acad Sci U S A* 1984, 81:4884-4888.
32. Ozaki K, Nagata M, Suzuki M, Fujiwara T, Miyoshi Y, Ishikawa O, Ohigashi H, Imaoka S, Takahashi E, Nakamura Y: Isolation and characterization of a novel human pancreas-specific gene, pancpin, that is down-regulated in pancreatic cancer cells. *Genes Chromosomes Cancer* 1998, 22:179-185.
33. Saito S, Okui K, Tokino T, Oshimura M, Nakamura Y: Isolation and mapping of 68 RFLP markers on human chromosome 6. *Am J Hum Genet* 1992, 50:65-70.
34. Görtz B, Roth J, Speel EJM, Krähenmann A, De Krijger RR, Matias-Guiu X, Muletta-Feurer S, Rütimann K, Saremaslani P, Heitz PU, Komminoth P: MEN 1 gene mutations in sporadic adrenocortical lesions. *Int J Cancer* 1999, 80: 373-379.
35. Zhao J, Speel EJM, Muletta-Feurer S, Rütimann K, Saremaslani P, Roth J, Heitz PU, Komminoth P: Analysis of genomic alterations in sporadic adrenocortical lesions: Gain of chromosome 17 is an early event in adrenocortical tumorigenesis. *Am J Pathol* 1999, 155:1039-1045.
36. Fong CT, Dracopoli NC, White PS, Merrill PT, Griffith RC, Housman DE, Brodeur GM: Loss of heterozygosity for the short arm of chromosome 1 in human neuroblastomas: correlation with N-myc amplification. *Proc Natl Acad Sci U S A* 1989, 86:3753-3757.
37. Yang KP, Nguyen CV, Castillo SG, Samaan NA: Deletion mapping on the distal third region of chromosome 1p in multiple endocrine neoplasia type IIA. *Antioncogene Res* 1990, 10:527-534.
38. White VA, McNeil BK, Horsman DE: Acquired homozygosity (isodisomy) of chromosome 3 in uveal melanoma. *Cancer Genet Cytogenet* 1998, 102:40-45.
39. Kruzelock RP, Murphy EC, Strong LC, Naylor SL, Hansen MF: Localization of a novel tumor suppressor locus on human chromosome 3q important in osteosarcoma tumorigenesis. *Cancer Res* 1997, 57:106-109.
40. Gaidano G, Hauptschein RS, Parsa NZ, Offit K, Rao PH, Lenoir G, Knowles DM, Chaganti RS, Dalla-Favera R: Deletions involving two distinct regions of 6q in B-cell non-Hodgkin lymphoma. *Blood* 1992, 80:1781-1787.
41. Bell DW, Jhanwar SC, Testa JR: Multiple regions of allelic loss from chromosome arm 6q in malignant mesothelioma. *Cancer Res* 1997, 57:4057-4062.
42. Healy E, Belgaid C, Takata M, Harrison D, Zhu NW, Burd DA, Rigby HS, Matthews JN, Rees JL: Prognostic significance of allelic losses in primary melanoma. *Oncogene* 1998, 16:2213-2218.
43. Speel EJM, Richter J, Moch H, Egenter C, Saremaslani P, Rütimann K, Zhao J, Barghorn A, Roth J, Heitz PU, Komminoth P: Genetic differences in endocrine pancreatic tumor subtypes detected by comparative genomic hybridization. *Am J Pathol* 1999, 155:1787-1794.
44. Cogen PH, Daneshvar L, Metzger AK, Duyk G, Edwards MS, Sheffield VC: Involvement of multiple chromosome 17p loci in medulloblastoma tumorigenesis. *Am J Hum Genet* 1992, 50:584-589.
45. Terris B, Meddeb M, Marchio A, Danglot G, Flejou JF, Belghiti J, Ruszniewski P, Bernheim A: Comparative genomic hybridization analysis of sporadic neuroendocrine tumors of the digestive system. *Genes Chrom Cancer*, 1998, 22:50-56.

Chapter 5

Microarray-based CGH of sporadic and syndrome-related pheochromocytomas using a 0.1-0.2 Mb BAC array spanning chromosome 1p

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Abstract

Pheochromocytomas (PCC) are relatively rare neuroendocrine tumors, mainly of the adrenal medulla. They arise sporadically or occur secondary to inherited cancer syndromes, such as multiple endocrine neoplasia type 2 (MEN2), von Hippel-Lindau disease (VHL), or neurofibromatosis type 1 (NF1). Loss of 1p is the most frequently encountered genetic alteration, especially in MEN2-related and sporadic PCC. Previous studies have revealed three regions of common somatic loss on chromosome arm 1p using chromosome-based CGH and LOH analysis. In order to investigate these chromosomal aberrations with a higher resolution and sensitivity, we performed microarray-based CGH with 13 sporadic and 11 syndrome-related (10 MEN2A-related and 1 NF1-related) tumors. The array consisted of 642 overlapping bacterial artificial chromosome (BAC) clones mapped to 1p11.2-p36.33. Chromosomal deletions on 1p were detected in 18 of 24 cases (75%). Among 9 tumors with partial 1p loss, the deleted region was restricted to 1cen-1p32.3 in 6 cases (25%), indicating a region of genetic instability. The consensus regions of deletion in this study involved 1cen-1p32.3 and 1p34.3-1p36.33. In conclusion, these data strongly suggest that chromosome 1p is the site for multiple tumor suppressor genes, although the potential candidate genes *CDKN2C* and *PTPRF/LAR* are not included in these regions.

Introduction

Pheochromocytoma (PCC) is a relatively rare tumor, arising in the neural crest-derived catecholamine- and peptide-producing secretory cells, mainly of the adrenal medulla. Approximately 10-25% of all tumors present as a component of inherited cancer syndromes, such as multiple endocrine neoplasia type 2 (MEN2), von Hippel-Lindau disease (VHL), hereditary paraganglioma (PGL) or neurofibromatosis type 1 (NF1). These familial forms of PCC have been associated with germ line mutations in *RET* (10q11), *VHL* (3p25), *SDHB* (1p36.13) or *SDHD* (11q23), and *NF1* (17q11), respectively (Xu et al., 1992; Latif et al., 1993; Mulligan et al., 1993b; Astuti et al., 2001a; Astuti et al., 2001b). However, PCC most often occurs sporadically. The genetic background of sporadic tumors is poorly understood, since only a small subset displays somatic mutations in the genes associated with familial disease: 0-5% in *RET*, 4-10% in *SDHB*, 0-4% in *SDHD*, 2-11% in *VHL*, and ~2% in *NF1* (reviewed in Dannenberg et al., 2003).

Previous studies have indicated the presence of tumor suppressor genes at chromosomes 1p, 3p, 5p, 11p, 17p, and 22q (Khosla et al., 1991; Shin et al., 1993; Vargas et al., 1997; Dannenberg et al., 2000; Edstrom et al., 2000; Lui et al., 2002). Loss of the short arm of chromosome 1 has been found in more than 80% of MEN2-related and sporadic tumors. However, 1p deletions are much less frequent in VHL-related tumors (~15%), indicating the existence of at least two different genetic pathways in the tumorigenesis of PCC (Bender et al., 2000; Lui et al., 2002).

Three regions of possible tumor suppressor loci on 1p have been identified so far (Figure 1). The first region encompasses the telomeric part of 1p, with a region at 1p36 that is not only involved in PCC (Moley et al., 1992; Vargas et al., 1997; Benn et al., 2000; Edstrom Elder et al., 2002), but also in other endocrine tumors, including medullary thyroid carcinoma (Khosla et al., 1991; Mulligan et al., 1993a; Marsh et al., 2003) and parathyroid adenoma (Williamson et al., 1997; Valimaki et al., 2002), as well as in tumors of neural crest origin such as neuroblastoma (Bauer et al., 2001; Ejeskar et al., 2001; White et al., 2001). As shown in figure 1, fine structure mapping of deletions in this telomeric region suggested at least three putative tumor suppressor gene loci within 1pter-1p34.3 (Benn et al., 2000). In the telomeric part of 1p, the *SDHB* gene, located at 1p36.13, is the only gene known to be involved in PCC tumorigenesis (Astuti et al., 2001a). However, only a small subset of PCC with 1p loss exhibits a germ line *SDHB* mutation.

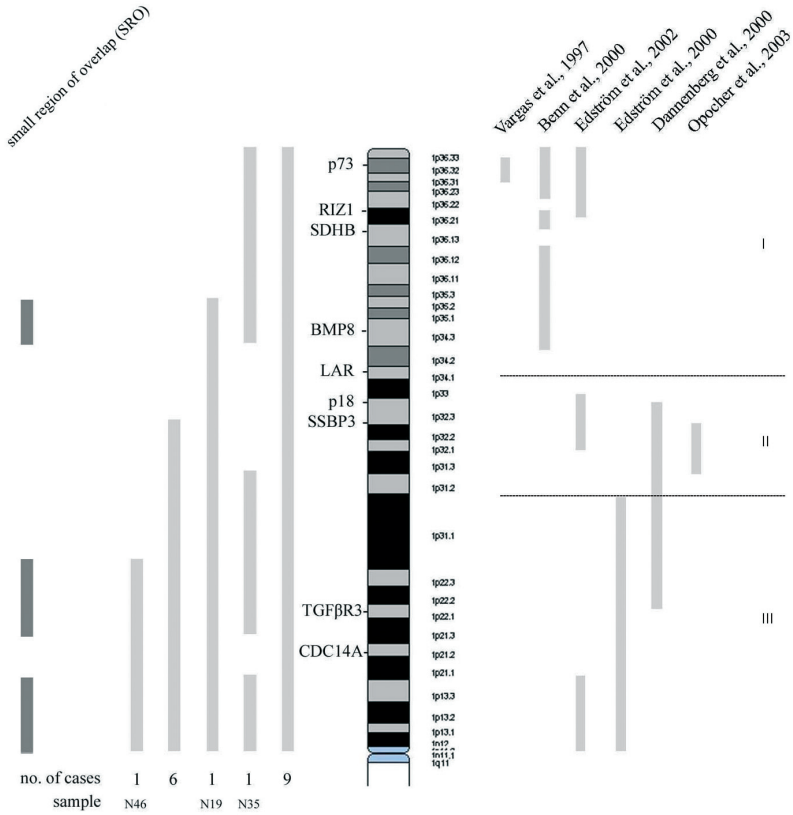

Figure 1 Minimal regions of 1p loss in pheochromocytomas identified in the current array CGH study, compared with the minimal regions reported previously by others. Nine tumors showed complete loss of chromosome arm 1p (cases N9.I, N9.II, N16, N27.I, N27.II, N55, N57, Zb3, 8M). Case N35 showed three regions of deletions: 1p36.33-1p34.3, 1p31.3-1p21.3, and 1p21.1-1cen. The deleted region in tumor N19 was restricted to a region centromeric of 1p35.2. A more proximal breakpoint was found in 6 tumors (cases N13, N14, N23, N28, N38, and N43) at 1cen-1p32.3. Case N46 showed the most proximal breakpoint in cen-1p31.1. Based on these data, the consensus regions of deletion are defined to 1cen-1p32.3 and 1p34.3-1p36.33.

The second and third target region, identified using chromosome-based comparative genomic hybridization (CGH), consist of the centromeric part of 1p with minimal regions located in 1p22-p32 and 1cen-1p31 (Dannenberg et al., 2000; Edstrom et al., 2000). Further genotyping resulted in the partition of this region into two smaller regions: a proximal region centromeric of D1S429 (1cen-1p13) and a more distal interval at 1p32 (Edstrom Elder et al., 2002; Opocher et al., 2003).

These regions are still rather large and include many genes, which complicates the identification of candidate tumor suppressor genes. In addition, chromosome-based CGH is a laborious technique with limited sensitivity for small chromosomal aberrations (5-10 Mb)

(Forozan et al., 1997). Hybridization to an array of mapped sequences instead of metaphase chromosomes could overcome these limitations (Pollack et al., 1999; Beheshti et al., 2002). Recently, high-throughput and high-resolution detection of chromosomal alterations has been made possible through the use of bacterial artificial chromosome (BAC) microarray-based comparative genomic hybridization (array CGH) (Cai et al., 2002; Albertson and Pinkel, 2003). This assay is capable of rapidly detecting single copy number changes at thousands of loci in a single experiment, with a resolution determined by the map distance between the arrayed clones and by the length of the cloned DNA segments.

In this study, a complete coverage array of chromosome arm 1p was used, consisting of 642 overlapping BAC clones. Together, the arrayed clones span approximately 121 Mb of DNA, resulting in a resolution of 0.1-0.2 Mb (Ishkanian et al., 2004; Garnis et al., 2005; Henderson et al., 2005). This will facilitate the identification of candidate tumor suppressor genes by providing precise information on the location of deletion boundaries. Moreover, array CGH may reveal additional small or homozygous deletions that are not detectable by LOH analysis or chromosome-based CGH. A panel of 13 sporadic and 11 syndrome-related (10 MEN2A-related and 1 NF1-related) PCC was screened for DNA sequence copy number alterations to identify the regions on 1p that are likely involved in the development of these tumors.

Materials and Methods

Patients and Tumor samples

A series of 24 PCC samples of 22 patients was studied. In 9 of these patients PCCs occurred as part of a neuroendocrine hereditary cancer syndrome: 8 patients were diagnosed with MEN2A, and 1 patient was diagnosed with NF1. Tumors of 13 patients with apparently sporadic PCC were analyzed, including 2 proven malignant lesions. The average age of the patients was 46 years (range: 24-72). Nine of 22 benign and 2 malignant tumor samples were from female patients. None of the 13 sporadic patients and their families had clinical signs of MEN2, VHL or familial PCC/PGL. The clinical data are detailed for each case in Table 2.

Tumors of which a large amount high molecular weight tumor DNA was available, were selected from a previous CGH study based on the following considerations: (1) the likelihood of finding 1p aberrations, either copy number changes or homozygous deletions, for which we included all MEN2-related and NF1-related PCC and sporadic PCC, (2) the likelihood of finding small changes, for which we selected tumors with normal 1p chromosome arms as analyzed by conventional CGH, (3) the assessment of exact breakpoints, for which we included tumors with known partial 1p deletions (Dannenberg et al., 2000; Speel et al., manuscript in preparation).

DNA Extraction

Genomic DNA from 23 frozen tumors was isolated using the D-5000 Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN) according to the manufacturers' recommendations. DNA extraction from 1 formalin-fixed, paraffin-embedded tumor (8M) was performed by standard detergent-proteinase K lysis, followed by phenol/chloroform extraction and ethanol precipitation, as described elsewhere (Richter et al., 1997).

BAC Array Construction

A minimal overlapping tiling set of 642 human BAC clones spanning chromosome arm 1p (1p11.2 to 1p36.33) was selected from the RPCI-11 library based on their location on the human physical map (http://www.genome.wustl.edu). The map location of 395 (62%) sequenced clones was verified using the University of California at Santa Cruz Biotechnology Human Genome Browser (http://www.genome.ucsc.edu). The identity of each BAC DNA sample was confirmed based on its *Hin*dIII fingerprint. The complete clone list is publicly available at http://www.bccrc.ca/cg/1pArray.html.

Table 1 Primers for PCR-SSCP analysis of PTPRF/LAR and CDKN2C in pheochromocytoma.

Primer	Length (bp)		Sequence
PTPRF/LAR exon 21	171	Forward:	5' - CTCACCAgCCTCCCTTCTg - 3'
		Reverse:	5' - ggCACACAggCCATAggAg - 3'
PTPRF/LAR exon 25	176	Forward:	5' - TCAgCCTgCCCTgCTCATC - 3'
		Reverse:	5' - AgAgATATgCACTggggAAAg - 3'
PTPRF/LAR exon 26	207	Forward:	5' - AgTATgTCCCCACTTTgTCC - 3'
		Reverse:	5' - CCTCTgggTggCACTgTAC - 3'
PTPRF/LAR exon 30	207	Forward:	5' - TCCCCTATTCCTTCTCACCTg - 3'
		Reverse:	5' - CgTTATggACACACTCAATgC - 3'
PTPRF/LAR exon 31	247	Forward:	5' - ATgTACCCCACCCACCTTTC - 3'
		Reverse:	5' - gCCTCATgCAgTgggTCTg - 3'
CDKN2C exon 2	201	Forward:	5' - ACgACTAATTCATCTTTTCCTg - 3'
		Reverse:	5' - ACTTgTTTTCCCCACCTCTC - 3'
CDKN2C exon 3a	238	Forward:	5' - AggATTCTACCATTTCTACTTC - 3'
		Reverse:	5' - CTTTggCAgCCAAgTgCAAg - 3'
CDKN2C exon 3b	270	Forward:	5' - CAAgCTgATgTTAACATCgAg - 3'
		Reverse:	5' - TgATAAAgTAgAggCAACgTg - 3'
SSBP3 exon 1	206	Forward:	5' - AAggAgTTCCAgggCgATg - 3'
		Reverse:	5' - gCCACTTgCAAAATAgggCT - 3'
SSBP3 exon 2	149	Forward:	5' - CCTTTCCCTCTCTCTCTTTg - 3'
		Reverse:	5' - gTCAAgAAATTggATgCTgg - 3'
SSBP3 exon 3	149	Forward:	5' - CTCCTCTAAgCTgCTTCTgC - 3'
		Reverse:	5' - CCCAgggCTCACAACAAAAC - 3'
SSBP3 exon 4	205	Forward:	5' - CTTgCCCTTgTgTACACAAg - 3'
		Reverse:	5' - gAAgTTAgAgCTATCCAACCC - 3'

Primer	Length (bp)	Sequence	
SSBP3 exon 5	165	Forward:	5' - TTTggCCACTAACTCTCTgC - 3'
		Reverse:	5' - TACAAggTggAgCACAAgAC - 3'
SSBP3 exon 6	182	Forward:	5' - TAACCTgCCTCTACTgTCTg - 3'
		Reverse:	5' - TTTCCTCTgCgggCTCTCTg - 3'
SSBP3 exon 7	155	Forward:	5' - AgCAgCTCCTCAgCTTgTg - 3'
		Reverse:	5' - ggAgCCCCTCCTggCATC - 3'
SSBP3 exon 8	217	Forward:	5' - gATgCCAggAggggCTCC - 3'
		Reverse:	5' - gCATggAAACAggAgAgAAg - 3'
SSBP3 exon 9	143	Forward:	5' - TCTCATAgACTgACCCTTgg - 3'
		Reverse:	5' - ggAAgACCTggACTCTgAg - 3'
SSBP3 exon 10	148	Forward:	5' - CACCgCTTggAgTTTACAgC - 3'
		Reverse:	5' - TCCCCTgACAAACACCACC - 3'
SSBP3 exon 11	121	Forward:	5' - CACTCAgTgACTCTgACgTg - 3'
		Reverse:	5' - ACgTggCTAgACATgCAATg - 3'
SSBP3 exon 12	177	Forward:	5' - CATgTTCgTTACCACCgTgg - 3'
		Reverse:	5' - TCCAggTCTCTCTggCAAC - 3'
SSBP3 exon 13	184	Forward:	5' - TTAATCACCCCgACTTgTgg - 3'
		Reverse:	5' - TTTCCCCTCAgCTgATgAAg - 3'
SSBP3 exon 14	287	Forward:	5' - AggACATgTCCgAgTgTgAg - 3'
		Reverse:	5' - TgAggCACAgAgCTggCT - 3'
SSBP3 exon 15	130	Forward:	5' - gTggAAgAgCAAgCAAgAAAC - 3'
		Reverse:	5' - AACAACCTgCCCACACAgATg - 3'
SSBP3 exon 16	225	Forward:	5' - ATCTgTgTGGGCAggTTgTTg - 3'
		Reverse:	5' - AACTTgTTAggAAAgAgTCCC - 3'
SSBP3 exon 17	168	Forward:	5' - CTCAgTTCTgTgCAgTCACC - 3'
		Reverse:	5' - TgCAATgCCTgCTCTCTCAg - 3'

BAC DNA was digested with *Mse*I and amplified using a linker-mediated PCR protocol as described previously (Garnis et al., 2004). The amplified DNA was dissolved in a 20% DMSO solution to a concentration of 1 µg/µl, was denatured by boiling for 10 minutes, and re-arrayed for robotic printing. Each BAC clone was printed in triplicate using a VersArray ChipWriter Pro (BioRad, Mississauga, ON) with Stealth Micro Spotting Pins (Telechem/ArrayIT SMP2.5, Sunnyvale, CA) onto amine-coated slides (Telechem, Sunnyvale, CA). To allow normalization of the hybridization signal intensities between dyes, 48 linker-mediated PCR-amplified male human genomic DNA samples (Novagen, Madison, WI) were spotted in triplicate on the array. The DNA was covalently bonded to the slides by baking and UV cross-linking. Slides were washed to remove unbound DNA.

Array Comparative Genomic Hybridization

Test and reference DNA (200 ng each) were labeled separately with Cyanine 5 and Cyanine 3 dCTPs, respectively, as previously described (Garnis et al., 2004). The labeled DNA probes

were combined, denatured, and annealed with 100 µg Cot-1 DNA in a final volume of 28 µl of DIG Easy hybridization solution (Roche, Laval, Que) containing 70 µg of sheared herring sperm DNA (Sigma-Aldrich, Oakville, ON), and 350 µg of yeast tRNA (Calbiochem, La Jolla, CA). The probes were incubated for 1 h at 37°C to allow blocking of the repetitive sequences. The BAC array slides were prehybridized with 28 µl DIG Easy hybridization solution (Roche) containing 1% BSA, and 70 µg of sheared herring sperm DNA for 1 h at 45°C. The probe mixture was applied to the array area and hybridized for 36 h at 45°C. After hybridization, slides were washed five times for 5 minutes each in 0.1xSSC/0.1% SDS with agitation at room temperature, then rinsed five times in 0.1xSSC, and dried by centrifugation.

Hybridized slides were imaged using a charge-coupled device-based imaging system (Arrayworx eAuto, API, Issaquah, WA). Images were analyzed using the Softworx array analysis program (API, Issaquah, WA) to determine signal intensities for each dye. A scale factor based on the signal intensities of the 48 human genomic DNA control spots was used to normalize spot signal data for each channel. The average signal ratios and standard deviations for each triplicate spot set were calculated and displayed as a plot of the normalized Cyanine5/Cyanine3 log2 signal ratio versus relative tiling path position of the BAC clones. Arrays were initially subjected to normal versus normal hybridizations for quality control (Veltman, et al. 2003). Spots that exhibited signal ratios outside of three standard deviations from 0 in these experiments were discarded, resulting in the establishment of a ±0.2 log2 signal ratio threshold for defining regions of copy number increase and decrease.

PCR-SSCP analysis for mutations in *CDKN2C, PTPRF/LAR,* and *SSBP3*

To screen for mutations of *CDKN2C*, *PTPRF/LAR*, and *SSBP3*, we used single strand conformation polymorphism (SSCP) analysis in a panel of 20 PCCs, including 19 tumors of which CGH-array data were obtained. Primer pairs (Table 1) were selected in order to analyse the tyrosine kinase domains of *PTPRF/LAR*, the entire open reading frame of *CDKN2C*, and al exons of *SSBP3*. PCR amplification of tumor DNA was performed with standard PCR procedures in 15 µl reaction mixtures containing 1.5 mM $MgCl_2$, 10 mM Tris-HCl, 50 mM KCl, 0.02 mM dATP, 0.2 mM dGTP, dTTP, dCTP each, 0.8 µCi α32P-dATP (Amersham, Buckinghamshire, UK), 20 pmol of each sense and anti-sense primer, and 1 U Taq DNA polymerase (Amplitaq Gold, Perkin Elmer, Norwalk, CT, USA). Electrophoresis of PCR products was carried out overnight at 8W on non-denaturing gels, containing 8% polyacrylamide (49:1) and 10% (v/v) glycerol. To obtain optimal SSCP patterns, glycerol percentage and room temperature were adjusted for *PTPRF/LAR* exon 25 and exon 31 and *CDKN2C* exon 3 (0% glycerol and 4˚C room temperature). The gels were dried and exposed to X-ray film overnight at –70 °C.

Results

Overall, chromosomal deletions of 1p were detected in 18 of 24 cases (75%), including 9 of 10 MEN2A-related PCC (90%) and 8 of 13 sporadic PCC (62%), and the single NF1 case that was analyzed. In 4 sporadic and 2 MEN2A-related tumors no 1p copy number changes were found. Of the 18 tumors with 1p deletions, 9 showed a whole arm deletion (7 MEN2A-related PCC and 2 sporadic PCC), which was in 14/24 (58%) of cases consistent with previous chromosome-based CGH analysis (shown in Table 2). No evident homozygous deletions and no gains were found in these tumors. The bilateral tumors from two patients with MEN2A (case N9I - II, and N27I - II) all showed complete loss of chromosome arm 1p.

The remaining 9 tumors showed partial loss of 1p, permitting more detailed mapping of the target regions (Figure 1). Tumor N19 showed a clear breakpoint distal in the studied contig, involving a region of 1p loss centromeric of BAC RP11-84A19 (GenBank accession number AL354919) at 1p35.2. A more proximal breakpoint at BAC RP11-514E9 was found in 6 tumors (25%). Based on the deletions identified in these tumors (cases N13, N14, N23, N28, N38, and N43), the target region was mapped to 1cen-1p32.3. Tumor N35 showed three specific regions of deletion: 1p36.33-1p34.3 (BAC RP11-421C4 (AC026283) to RP11-593A21), 1p31.3-1p21.3 (BAC RP11-182I10 (AL6065170) to RP11-31O12), and 1p21.1-1cen (BAC RP11-771H6 to RP11-114O18 (AC019250)). A more proximal breakpoint in the centromeric region was identified in tumor N46 at BAC RP11-30M11 (AC027606). Representative results are shown in Figure 2. Altogether, array CGH analysis was consistent with previous conventional CGH analysis and allowed the identification of exact breakpoints. Again, no additional small or homozygous deletions were detected.

PCR-SSCP of *PTPRF/LAR, CDKN2C*, and *SSBP3* did not show any abnormalities, which is shown in Table 2.

Discussion

The high frequency of 1p loss in MEN2A-related and sporadic PCC, but not in VHL-related PCC, indicates that inactivation of tumor suppressor loci on this chromosome arm is a specific genetic event in the molecular pathogenesis of these PCC. Several minimal regions of overlapping deletions, identified by chromosome-based CGH and LOH analysis, have been reported so far, including a telomeric region at 1p36 (Vargas et al., 1997; Benn et al., 2000; Edstrom Elder et al., 2002), a more proximal region at 1p32 (Dannenberg et al., 2000; Opocher et al., 2003), and a centromeric region at 1cen-1p13 (Edstrom Elder et al., 2002). In our study, we used array CGH in order to investigate these regions of chromosomal loss with a higher resolution and sensitivity.

Nine of 18 PCCs with 1p deletion exhibited loss of the whole 1p arm, which was not always to be expected on the basis of previous CGH analysis of these tumors. In all these cases the deletion found with array CGH extended to the centromeric and telomeric regions of 1pas compared to the results of conventional CGH analysis. Centromic and telomeric regions of chromosomes are known for false copy number gains in conventional CGH analyses, which is probably the best explanation for the usually slight discrepancies.

Nine tumors showed partial deletions of 1p, and based on these results we mapped the smallest regions of overlap (SRO) at 1cen-1p21.1, 1p21.3-1p31.1, and 1p34.3-1p35.2, since tumors N35 and N46 exhibited smaller regions of loss at the centromeric part, and tumors N19 and N35 indicated a smaller telomeric part (Figure 1). However, the accurate mapping of chromosomal loss in a series of tumors to define a common region of interest is greatly confounded by technical artifacts, genetic instability and intertumor heterogeneity (Devilee et al., 2001). Therefore, to include all candidate genes, one might also define larger regions, such as a region centromeric of 1p32.3, and a telomeric region at 1p34.3-1p36.33. These regions can be defined from 6 tumors showing the same breakpoint at 1p32.3 and tumor N35 with 3 very specific deletions, one at 1p34.3-1pter.

In our study, we did not detect any additional small deletions or homozygous deletions compared to previously performed chromosome-based CGH. This may imply that such small changes are rare in PCC, although LOH studies suggest the commonness of small regions of loss (Edstrom Elder et al., 2002; Opocher et al., 2003).

In neuroblastoma, loss of 1p36 is associated with an unfavorable outcome (Caron et al., 1996). The *TP73* gene, a *TP53* homologue that induces apoptosis and inhibits cell growth, was originally hypothesized as the candidate tumor suppressor for neuroblastoma, since *TP73* mapped to 1p36.3. PCC have not been investigated for *TP73* mutations, and PCR-SSCP analysis revealed only two missense mutations in 140 neuroblastomas, one somatic and one germ line (Ichimiya et al., 2001). These data indicate that *TP73* may not act as a tumor suppressor in neuroblastoma, except when such a role results from either a gene-dosage effect or transcriptional silencing of the remaining allele.

Recently, mutations in the gene for succinate dehydrogenase subunit B (*SDHB*), located at 1p36.13, have been identified in hereditary paraganglioma (PGL), a related neuroendocrine disease (Astuti et al., 2001a). *SDHB* encodes a subunit of the mitochondrial complex II that is involved in the Krebs cycle and the aerobic respiratory chain (Baysal et al., 2001). Germ line mutations in *SDHB*, *SDHC*, *SDHD*, three subunits of the mitochondrial complex II, not only cause susceptibility to head and neck PGL (Baysal et al., 2002), but *SDHB* and *SDHD* also play a role in a small subset of hereditary and sporadic PCC (Neumann et al., 2002; Astuti et al., 2003). However, Benn et al. did not find any somatic *SDHB* mutations in the remaining allele of 11 sporadic PCC that showed LOH at 1p36 (Benn et al., 2003). The mechanisms of predisposition to PCC have not been elucidated, but may include dysregulation of hypoxia-

Table 2 Patient and Tumor Characteristics

Patient	Tumor	Sex/ Age	Diagnosis	Location	Dignity	* Genomic changes of chromosome 1	Array CGH results	CDKN2C	PTPRF/ LAR	SSBP3
1	N9.I	M / 72	MEN2Aa; bilat.b PCC	Adrenal	Benign	1p 12-35-	1p-	N	N	N
	N9.II	M / 72		Adrenal	Benign	1p 11-34-	1p-	N	N	N
2	N23	M / 40	MEN2A	Adrenal	Benign	1p13-34-	1cen -1p32.3	N	N	N
3	N27.I	M / 53	MEN2A; bilat. PCC	Adrenal	Benign	1p13-32-, 1q+	1p-	N	N	
	N27.II	M / 53		Adrenal	Benign	1p11-33-	1p-	N	N	
4	N38	F / 24	MEN2A; bilat. PCC	Adrenal	Benign	1p13-34-	1p-	N	N	N
5	N55	F / 36	MEN2A	Adrenal	Benign	1p-	1p-	N	N	
6	N57	M / 40	MEN2A	Adrenal	Benign	1p-, 1q+	1p-	N	N	N
7	**Zb3**	**M / 49**	**MEN2A; bilat. PCC**	**Adrenal**	**Benign**	**1p-**	**1p-**			
8	**Zb7**	**F / 50**	**MEN2A; bilat. PCC**	**Adrenal**	**Benign**	**N**	**N**			
9	N13	F / 53	NF1c	Adrenal	Benign	1p21-31-, (1p36+)	1cen -1p32.3	N	N	N
10	N2	M / 24	sporadic	Adrenal	Benign	N	N	N	N	N
11	N8	M / 58	sporadic	Adrenal	Benign	N	N	N	N	N
12	N14	M / 41	sporadic	Adrenal	Benign	1p21-31-	1cen -1p32.3	N	N	N
13	N16	F / 32	sporadic	Meta LNd	Malignant	1p 13.2-32 -	1p-	N	N	N
14	N19	F / 41	sporadic	Adrenal	Benign	1p13-32-	1cen-1p35.2	N	N	N
15	N28	F / 56	sporadic	Extra-Adrenal	Benign	1p21-32-	1cen -1p32.3	N	N	N
16	N35	F / 49	sporadic	Adrenal	Benign	1p13.1-31.3- 1p 34.2-pter-	1cen-1p21.1 1p21.3-1p31.3 1p34.3-1pter	N	N	N
17	N43	M / 30	sporadic	Adrenal	Benign	1p13.3-31.3-	1cen -1p32.3	N	N	N
18	N44	F / 37	sporadic	Adrenal	Benign	N	N	N	N	N
19	N46	F / 62	sporadic	Adrenal	Benign	1p 12-31.1-	1p31.1	N	N	N
20	**50B**	**M / 57**	**sporadic**	**Adrenal**	**Benign**	**N**	**N**			
21	**8M**	**F / 65**	**sporadic**	**Adrenal**	**Malignant**	**1p11-31–**	**1cen -1p32.3**			**N**
22	**Zb4**	**M / 34**	**sporadic**	**Adrenal**	**Benign**	**N**	**N**			**N**

* As detected by conventional CGH. Bold rows represent previously published CGH data (Dannenberg et al., 2000).
a MEN2A = multiple endocrine neoplasia type 2A, bbilat. = bilateral tumors, cNF1 = neurofibromatosis type 1, dMeta LN = lymph node metastasis
N = Normal

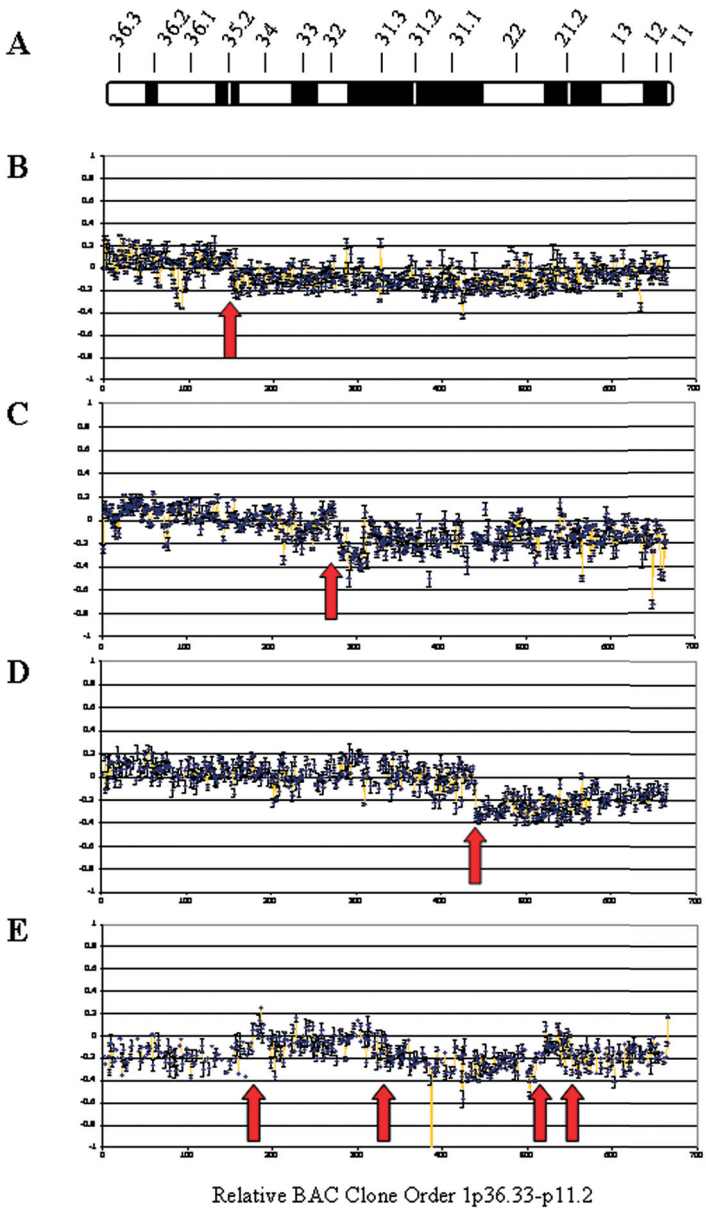

Figure 2 Detection of copy number alterations on chromosome 1p in pheochromocytoma samples by microarray-based CGH.

(A) Ideogram of chromosome arm 1p.

(B-E) Array CGH copy number profiles of representative cases. The relative position of each BAC clone (represented by a dot in the graph) is given along the x-axis, ranging from 1p36.33 to 1p11.2. Data are plotted as normalized Cy5/Cy3 log2 fluorescence ratios, representing tumour to normal copy number ratios, of the triplicate spots for each BAC clone along the y-axis. A log2 ratio of -0.2 indicates a single-copy deletion.

(B) Profile for tumor N19 showing a breakpoint in 1p35.2-1pcen.

(C) The deleted region in tumor N28 was mapped to 1p32.3-1pcen.

(D) The deletion in tumor N46 was restricted to a region centromeric of 1p31.1.

(E) In tumor N35, there are three deleted regions, including 1p36.33-1p34.3, 1p31.3-1p21.3, and 1p21.1-1cen, which have been indicated by arrows.

responsive genes and impairment of mitochondria-mediated apoptosis (Maher and Eng, 2002). Whether the *SDHB* gene plays a role in the tumorigenesis of the screened sporadic PCC samples remains to be investigated.

Another candidate gene in the telomeric region of 1p is *BMP8*, which is located at 1p34.2. Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β (TGF-β) super-family and have been shown to play a role in cell proliferation and differentiation in bone. However, TGF-β2, TGF-β3, and BMP4 may also be involved in chromaffin cell development, since these molecules are highly expressed in the neural crest-derived sympathoadrenal progenitor cells and their migratory pathways during embryonic development (Huber et al., 2002). Neutralization of these molecules by antibodies interfered with the proliferation of immigrated adrenal chromaffin cells, but did not affect the chromaffin phenotype, indicating that TGF-β2, TGF-β3, and BMP4 are not essential for chromaffin cell differentiation.

Aberrations in BMP signaling have recently been implicated as a cause of human cancer. BMP4 appeared to have some typical tumor suppressive properties, since BMP4 treatment eliminated the tumorigenic potential of an undifferentiated human cancer cell line (Nishanian et al., 2004). This loss of tumorigenicity was accompanied by an increase in apoptosis, alterations in cell cycle profile, and an increase in cell size. Identification of the downstream targets of BMP4-mediated tumor suppression by Affymetrix Gene chips showed the induction of many genes of the Wnt signaling cascade.

In prostate cancer, BMP2 expression was significantly decreased compared to benign prostate tissue (Horvath et al., 2004). Also, loss of BMP2 expression correlated with progression to a more aggressive phenotype. Waite et al. found an interesting link between BMP2 and the tumor suppressor gene *PTEN* (Waite and Eng, 2003). The breast cancer cell line MCF-7 showed a decrease in cellular proliferation after BMP2 treatment. BMP2 exposure increased PTEN protein levels in a time- and dose-dependent manner. This rapid increase was caused by inhibition of PTEN degradation due to decreased association of PTEN with two proteins in the ubiquitin degradation pathway. In mice, dual inactivation of *PTEN* and *INK4a/ARF* tumor suppressor genes resulted in the development of PCC, whereas the classical PCC susceptibility genes *RET*, *VHL*, and *NF1* remained intact (You et al., 2002). Possibly, loss of BMP2 plays a role in PCC tumorigenesis by modulating PTEN activity.

Involvement of BMP signaling proteins in neuroendocrine development and human cancers, make *BMP8* an interesting candidate gene. However, the role of BMP8 in neuroendocrine tumorigenesis is not clear yet.

Two other candidate tumor suppressor genes on the short arm of chromosome 1 are *PTPRF/LAR* and *CDKN2C*. *PTPRF/LAR* specifically suppresses the biological activities of RET-MEN2A by reducing its kinase activity (Qiao et al., 2001), however it is located outside our consensus region. We could also exclude *CDKN2C* as a candidate, which is an important finding, considering the occurrence of PCC in *CDKN2C* knockout mice (Franklin et al., 2000).

Indeed, we could not detect any mutations in the *CDKN2C* gene or in the tyrosine kinase domains of the *PTPRF/LAR* gene in a panel of 20 PCC (Table 2).

Interestingly, 6 tumors showed a chromosomal breakpoint at the exact same BAC clone in 1p32.3. At this position the *SSBP3* gene is located, encoding single-stranded DNA-binding protein 3 (Castro et al., 2002). This protein was identified as a member of the same family as *SSBP2*, the target of disruption caused by chromosomal translocation in the leukemic cell line ML3 (within a D5S672-D5S620 interval at 5q13.3). *SSBP3* may be involved in transcription regulation of the α2(I)-collagen gene where it binds to the single-stranded polypyrimidine sequences in the promoter region. Localization at chromosomal breakpoints and the DNA-binding activity suggest that members of the *SSBP* family are capable of potential tumor suppressor activity by gene dosage or other epigenetic mechanisms. Whether the breakpoint identified in those 6 tumors reflects some kind of selection for a particular change in gene expression or an underlying genetic instability remains to be investigated, although we could not detect any mutation in *SSBP3* by PCR-SSCP analysis.

Recently, transforming growth factor-β receptor type III (*TGFBR3*) has been identified as a candidate tumor suppressor in one of three melanoma susceptibility loci at chromosome band 1p22 (Walker et al., 2004). TGFBR3, a TGF-β-binding glycoprotein that exists in both a membrane-bound and a soluble form, may serve as a receptor accessory molecule in TGF-β signaling. Mutations in TGF-β-binding proteins may contribute to disturbances in the regulation of cell differentiation and proliferation and lead to oncogenic transformation. However, no coding mutations of TGFBR3 could be detected in 1p22-linked melanoma kindreds (Walker et al., 2004). Melanoma cell lines showed significantly less allelic 1p loss compared to fresh tumors, indicating that the *TGFBR3* gene may not be involved in cellular alterations necessary for the in vitro propagation of melanoma cells. The identified melanoma susceptibility regions at 1p22 are located within the deleted 1p region found in our study, suggesting the involvement of the same unidentified tumor suppressor genes in both melanoma and PCC.

The *CDC14A* gene is also located in the centromeric target region, at 1p21.2 (Wong et al., 1999). Human Cdc14A is a dual-specificity phosphatase that has been shown to be a critical regulator of the centrosome-duplication cycle (Mailand et al., 2002). Down-regulation of endogenous Cdc14A by short inhibitory RNA duplexes (siRNA) induced mitotic defects including impaired centrosome separation and failure to undergo productive cytokinesis. Deregulated Cdc14A might be involved in multi-step tumorigenesis by abolishing the centrosome-division cycle, which may lead to chromosome missegregation and chromosomal instability (Mailand et al., 2002). Mutational screening of a panel of 136 cDNAs from tumor cell lines, revealed a 48-bp in-frame deletion in the *CDC14A* gene in a breast carcinoma cell line (Wong et al., 1999). Loss of expression of the wild type allele in this cell line supports the possibility that *CDC14A* may be a tumor suppressor gene that is targeted for inactivation during tumorigenesis.

In conclusion, our results indicate that loss of 1cen-1p32.3 and 1p34.3-1p36.33 are frequent genetic events in the development of sporadic and MEN2A-associated PCC. The candidate genes *CDKN2C* and *PTPRF/LAR* can be excluded from these regions. Loss of the same regions on 1p in different kinds of neuroendocrine cancers supports the hypothesis that one or more genes in this region may function as a gatekeeper of neuroendocrine proliferation. More studies with larger sample size are required to narrow down the critical regions and to identify candidate genes.

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References

1. Albertson DG, Pinkel D. 2003. Genomic microarrays in human genetic disease and cancer. *Hum Mol Genet* 12 Spec No 2:R145-152.
2. Astuti D, Douglas F, Lennard TW, Aligianis IA, Woodward ER, Evans DG, Eng C, Latif F, Maher ER. 2001a. Germline SDHD mutation in familial phaeochromocytoma. *Lancet* 357:1181-1182.
3. Astuti D, Latif F, Dallol A, Dahia PL, Douglas F, George E, Skoldberg F, Husebye ES, Eng C, Maher ER. 2001b. Gene mutations in the succinate dehydrogenase subunit SDHB cause susceptibility to familial pheochromocytoma and to familial paraganglioma. *Am J Hum Genet* 69:49-54.
4. Astuti D, Hart-Holden N, Latif F, Lalloo F, Black GC, Lim C, Moran A, Grossman AB, Hodgson SV, Freemont A Ramsden R, Eng C, Evans DG, Maher ER. 2003. Genetic analysis of mitochondrial complex II subunits SDHD, SDHB and SDHC in paraganglioma and phaeochromocytoma susceptibility. *Clin Endocrinol* 59:728-733.
5. Bauer A, Savelyeva L, Claas A, Praml C, Berthold F, Schwab M. 2001. Smallest region of overlapping deletion in 1p36 in human neuroblastoma: a 1 Mbp cosmid and PAC contig. *Genes Chromosomes Cancer* 31:228-239.
6. Baysal BE, Rubinstein WS, Taschner PE. 2001. Phenotypic dichotomy in mitochondrial complex II genetic disorders. *J Mol Med* 79:495-503.
7. Baysal BE, Willett-Brozick JE, Lawrence EC, Drovdlic CM, Savul SA, McLeod DR, Yee HA, Brackmann DE, Slattery WH, 3rd, Myers EN, Ferrell RE, Rubinstein WS.2002. Prevalence of SDHB, SDHC, and SDHD germline mutations in clinic patients with head and neck paragangliomas. *J Med Genet* 39:178-183.
8. Beheshti B, Park PC, Braude I, Squire JA. 2002. Microarray CGH. *Methods Mol Biol* 204:191-207.
9. Bender BU, Gutsche M, Glasker S, Muller B, Kirste G, Eng C, Neumann HP. 2000. Differential Genetic Alterations in von Hippel-Lindau Syndrome-Associated and Sporadic Pheochromocytomas. *J Clin Endocrinol Metab* 85:4568-4574.

10. Benn DE, Croxson MS, Tucker K, Bambach CP, Richardson AL, Delbridge L, Pullan PT, Hammond J, Marsh DJ, Robinson BG. 2003. Novel succinate dehydrogenase subunit B (SDHB) mutations in familial phaeochromocytomas and paragangliomas, but an absence of somatic SDHB mutations in sporadic phaeochromocytomas. *Oncogene* 22:1358-1364.
11. Benn DE, Dwight T, Richardson AL, Delbridge L, Bambach CP, Stowasser M, Gordon RD, Marsh DJ, Robinson BG. 2000. Sporadic and familial pheochromocytomas are associated with loss of at least two discrete intervals on chromosome 1p. *Cancer Res* 60:7048-7051.
12. Cai WW, Mao JH, Chow CW, Damani S, Balmain A, Bradley A. 2002. Genome-wide detection of chromosomal imbalances in tumors using BAC microarrays. *Nat Biotechnol* 20:393-396.
13. Caron H, van Sluis P, de Kraker J, Bokkerink J, Egeler M, Laureys G, Slater R, Westerveld A, Voute PA, Versteeg R. 1996. Allelic loss of chromosome 1p as a predictor of unfavorable outcome in patients with neuroblastoma. *N Engl J Med* 334:225-230.
14. Castro P, Liang H, Liang JC, Nagarajan L. 2002. A novel, evolutionarily conserved gene family with putative sequence-specific single-stranded DNA-binding activity. *Genomics* 80:78-85.
15. Dannenberg H, Komminoth P, Dinjens WN, Speel EJ, de Krijger RR. 2003. Molecular genetic alterations in adrenal and extra-adrenal pheochromocytomas and paragangliomas. *Endocr Pathol* 14:329-350.
16. Dannenberg H, Speel EJ, Zhao J, Saremaslani P, van Der Harst E, Roth J, Heitz PU, Bonjer HJ, Dinjens WN, Mooi WJ, Komminoth P, de Krijger RR. 2000. Losses of chromosomes 1p and 3q are early genetic events in the development of sporadic pheochromocytomas. *Am J Pathol* 157:353-359.
17. Devilee P, Cleton-Jansen AM, Cornelisse CJ. 2001. Ever since Knudson. *Trends Genet* 17:569-573.
18. Edstrom E, Mahlamaki E, Nord B, Kjellman M, Karhu R, Hoog A, Goncharov N, Teh BT, Backdahl M, Larsson C. 2000. Comparative genomic hybridization reveals frequent losses of chromosomes 1p and 3q in pheochromocytomas and abdominal paragangliomas, suggesting a common genetic etiology. *Am J Pathol* 156:651-659.
19. Edstrom Elder E, Nord B, Carling T, Juhlin C, Backdahl M, Hoog A, Larsson C. 2002. Loss of heterozygosity on the short arm of chromosome 1 in pheochromocytoma and abdominal paraganglioma. *World J Surg* 26:965-971.
20. Ejeskar K, Sjoberg RM, Abel F, Kogner P, Ambros PF, Martinsson T. 2001. Fine mapping of a tumour suppressor candidate gene region in 1p36.2-3, commonly deleted in neuroblastomas and germ cell tumours. *Med Pediatr Oncol* 36:61-66.
21. Forozan F, Karhu R, Kononen J, Kallioniemi A, Kallioniemi OP. 1997. Genome screening by comparative genomic hybridization. *Trends Genet* 13:405-409.
22. Franklin DS, Godfrey VL, O'Brien DA, Deng C, Xiong Y. 2000. Functional collaboration between different cyclin-dependent kinase inhibitors suppresses tumor growth with distinct tissue specificity. *Mol Cell Biol* 20:6147-6158.
23. Garnis C, Coe BP, Ishkanian A, Zhang L, Rosin MP, Lam WL. 2004. Novel regions of amplification on 8q distinct from the MYC locus and frequently altered in oral dysplasia and cancer. *Genes Chromosomes Cancer* 39:93-98.
24. Garnis C, Campbell J, Davies JJ, Macaulay C, Lam S, Lam WL. 2005. Involvement of multiple developmental genes on chromosome 1p in lung tumorigenesis. *Hum Mol Genet* 14:475-482.
25. Henderson LJ, Coe BP, Lee EH, Girard L, Gazdar AF, Minna JD, Lam S, MacAulay C, Lam WL. 2005. Genomic and gene expression profiling of minute alterations of chromosome arm 1p in small-cell lung carcinoma cells. *Br J Cancer* 92:1553-1560.
26. Horvath LG, Henshall SM, Kench JG, Turner JJ, Golovsky D, Brenner PC, O'Neill GF, Kooner R, Stricker PD, Grygiel JJ, Sutherland RL. 2004. Loss of BMP2, Smad8, and Smad4 expression in prostate cancer progression. *Prostate* 59:234-242.

27. Huber K, Combs S, Ernsberger U, Kalcheim C, Unsicker K. 2002. Generation of neuroendocrine chromaffin cells from sympathoadrenal progenitors: beyond the glucocorticoid hypothesis. *Ann N Y Acad Sci* 971:554-559.
28. Ichimiya S, Nimura Y, Kageyama H, Takada N, Sunahara M, Shishikura T, Nakamura Y, Sakiyama S, Seki N, Ohira M, Kaneko Y, McKeon F, Caput D, Nakagawara A. 2001. Genetic analysis of p73 localized at chromosome 1p36.3 in primary neuroblastomas. *Med Pediatr Oncol* 36:42-44.
29. Ishkanian AS, Malloff CA, Watson SK, DeLeeuw RJ, Chi B, Coe BP, Snijders A, Albertson DG, Pinkel D, Marra MA, Ling V, MacAulay C, Lam WL. 2004. A tiling resolution DNA microarray with complete coverage of the human genome. *Nat Genet* 36:299-303.
30. Khosla S, Patel VM, Hay ID, Schaid DJ, Grant CS, van Heerden JA, Thibodeau SN. 1991. Loss of heterozygosity suggests multiple genetic alterations in pheochromocytomas and medullary thyroid carcinomas. *J Clin Invest* 87:1691-1699.
31. Latif F, Tory K, Gnarra J, Yao M, Duh FM, Orcutt ML, Stackhouse T, Kuzmin I, Modi W, Geil L. 1993. Identification of the von Hippel-Lindau disease tumor suppressor gene. *Science* 260:1317-1320.
32. Lui WO, Chen J, Glasker S, Bender BU, Madura C, Khoo SK, Kort E, Larsson C, Neumann HP, Teh BT. 2002. Selective loss of chromosome 11 in pheochromocytomas associated with the VHL syndrome. Oncogene 21:1117-1122.
33. Maher ER, Eng C. 2002. The pressure rises: update on the genetics of phaeochromocytoma. *Hum Mol Genet* 11:2347-2354.
34. Mailand N, Lukas C, Kaiser BK, Jackson PK, Bartek J, Lukas J. 2002. Deregulated human Cdc14A phosphatase disrupts centrosome separation and chromosome segregation. *Nat Cell Biol* 4:317-322.
35. Marsh DJ, Theodosopoulos G, Martin-Schulte K, Richardson AL, Philips J, Roher HD, Delbridge L, Robinson BG. 2003. Genome-wide copy number imbalances identified in familial and sporadic medullary thyroid carcinoma. *J Clin Endocrinol Metab* 88:1866-1872.
36. Moley JF, Brother MB, Fong CT, White PS, Baylin SB, Nelkin B, Wells SA, Brodeur GM. 1992. Consistent association of 1p loss of heterozygosity with pheochromocytomas from patients with multiple endocrine neoplasia type 2 syndromes. *Cancer Res* 52:770-774.
37. Mulligan LM, Gardner E, Smith BA, Mathew CG, Ponder BA. 1993a. Genetic events in tumour initiation and progression in multiple endocrine neoplasia type 2. *Genes Chromosomes Cancer* 6:166-177.
38. Mulligan LM, Kwok JB, Healey CS, Elsdon MJ, Eng C, Gardner E, Love DR, Mole SE, Moore JK, Papi L. 1993b. Germ-line mutations of the RET proto-oncogene in multiple endocrine neoplasia type 2A. *Nature* 363:458-460.
39. Neumann HP, Bausch B, McWhinney SR, Bender BU, Gimm O, Franke G, Schipper J, Klisch J, Altehoefer C, Zerres K Januszewicz A, Eng C, Smith WM, Munk R, Manz T, Glaesker S, Apel TW, Treier M, Reineke M, Walz MK, Hoang-Vu C, Brauckhoff M, Klein-Franke A, Klose P, Schmidt H, Maier-Woelfle M, Peczkowska M, Szmigielski C, Eng C; Freiburg-Warsaw-Columbus Pheochromocytoma Study Group. 2002. Germ-line mutations in nonsyndromic pheochromocytoma. *N Engl J Med* 346:1459-1466.
40. Nishanian TG, Kim JS, Foxworth A, Waldman T. 2004. Suppression of Tumorigenesis and Activation of Wnt Signaling by Bone Morphogenetic Protein 4 in Human Cancer Cells. *Cancer Biol Ther* 3:667-675.
41. Opocher G, Schiavi F, Vettori A, Pampinella F, Vitiello L, Calderan A, Vianello B, Murgia A, Martella M, Taccaliti A, Mantero F, Mostacciuolo ML. 2003. Fine analysis of the short arm of chromosome 1 in sporadic and familial pheochromocytoma. *Clin Endocrinol* 59:707-715.
42. Pollack JR, Perou CM, Alizadeh AA, Eisen MB, Pergamenschikov A, Williams CF, Jeffrey SS, Botstein D, Brown PO. 1999. Genome-wide analysis of DNA copy-number changes using cDNA microarrays. *Nat Genet* 23:41-46.

43. Qiao S, Iwashita T, Furukawa T, Yamamoto M, Sobue G, Takahashi M. 2001. Differential effects of leukocyte common antigen-related protein on biochemical and biological activities of RET-MEN2A and RET-MEN2B mutant proteins. *J Biol Chem* 276:9460-9467.
44. Richter J, Jiang F, Gorog JP, Sartorius G, Egenter C, Gasser TC, Moch H, Mihatsch MJ, Sauter G. 1997. Marked genetic differences between stage pTa and stage pT1 papillary bladder cancer detected by comparative genomic hybridization. *Cancer Res* 57:2860-2864.
45. Shin E, Fujita S, Takami K, Kurahashi H, Kurita Y, Kobayashi T, Mori T, Nishisho I, Takai S. 1993. Deletion mapping of chromosome 1p and 22q in pheochromocytoma. *Jpn J Cancer Res* 84:402-408.
46. Valimaki S, Forsberg L, Farnebo LO, Larsson C. 2002. Distinct target regions for chromosome 1p deletions in parathyroid adenomas and carcinomas. *Int J Oncol* 21:727-735.
47. Vargas MP, Zhuang Z, Wang C, Vortmeyer A, Linehan WM, Merino MJ. 1997. Loss of heterozygosity on the short arm of chromosomes 1 and 3 in sporadic pheochromocytoma and extra-adrenal paraganglioma. *Hum Pathol* 28:411-415.
48. Veltman JA, Fridlyand J, Pejavar S, Olshen AB, Korkola JE, DeVries S, Carroll P, Kuo WL, Pinkel D, Albertson D, Cordon-Cardo C, Jain AN, Waldman FM. 2003. Array-based comparative genomic hybridization for genome-wide screening of DNA copy number in bladder tumors. *Cancer Res* 63:2872-2880.
49. Waite KA, Eng C. 2003. BMP2 exposure results in decreased PTEN protein degradation and increased PTEN levels. *Hum Mol Genet* 12:679-684.
50. Walker GJ, Indsto JO, Sood R, Faruque MU, Hu P, Pollock PM, Duray P, Holland EA, Brown K, Kefford RF, Trent JM, Mann GJ, Hayward NK. 2004. Deletion mapping suggests that the 1p22 melanoma susceptibility gene is a tumor suppressor localized to a 9-Mb interval. *Genes Chromosomes Cancer* 41:56-64.
51. White PS, Thompson PM, Seifried BA, Sulman EP, Jensen SJ, Guo C, Maris JM, Hogarty MD, Allen C, Biegel JA, Matise TC, Gregory SG, Reynolds CP, Brodeur GM. 2001. Detailed molecular analysis of 1p36 in neuroblastoma. *Med Pediatr Oncol* 36:37-41.
52. Williamson C, Pannett AA, Pang JT, Wooding C, McCarthy M, Sheppard MN, Monson J, Clayton RN, Thakker RV. 1997. Localisation of a gene causing endocrine neoplasia to a 4 cM region on chromosome 1p35-p36. *J Med Genet* 34:617-619.
53. Wong AK, Chen Y, Lian L, Ha PC, Petersen K, Laity K, Carillo A, Emerson M, Heichman K, Gupte J, Tavtigian SV, Teng DH. 1999. Genomic structure, chromosomal location, and mutation analysis of the human CDC14A gene. *Genomics* 59:248-251.
54. Xu W, Mulligan LM, Ponder MA, Liu L, Smith BA, Mathew CG, Ponder BA. 1992. Loss of NF1 alleles in phaeochromocytomas from patients with type I neurofibromatosis. *Genes Chromosomes Cancer* 4:337-342.
55. You MJ, Castrillon DH, Bastian BC, O'Hagan RC, Bosenberg MW, Parsons R, Chin L, DePinho RA. 2002. Genetic analysis of Pten and Ink4a/Arf interactions in the suppression of tumorigenesis in mice. *Proc Natl Acad Sci U S A* 99:1455-1460.

Chapter 6

Differential loss of chromosome 11q in familial and sporadic parasympathetic paragangliomas detected by comparative genomic hybridization

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Abstract

Parasympathetic paragangliomas represent neuroendocrine tumors arising from chief cells in branchiomeric and intravagal paraganglia, which share several histological features with their sympathetic counterpart sympathoadrenal paragangliomas. In recent years, genetic analyses of the familial form of parasympathetic paraganglioma have attracted considerable interest. However, the majority of paragangliomas occurs sporadically and it remains to be determined whether the pathogenesis of sporadic paraganglioma resembles that of the familial form. Furthermore, data on comparative genetic aberrations are scarce. In order to provide fundamental cytogenetic data on sporadic and hereditary parasympathetic paragangliomas, we performed comparative genomic hybridization (CGH) using directly fluorochrome-conjugated DNA extracted from 12 frozen and 4 paraffin-embedded tumors. The CGH data were extended by loss of heterozygosity analysis of chromosome 11q.

DNA copy number changes were found in 10 (63%) of 16 tumors. The most frequent chromosomal imbalance involved loss of chromosome 11. Six of 7 familial tumors and 2 of 9 sporadic tumors showed loss of 11q (86% *vs.* 22%, P= 0.012). Deletions of 11p and 5p were found in 2 of 9 sporadic tumors. We conclude that overall DNA copy number changes are infrequent in parasympathetic paragangliomas compared to sympathetic paragangliomas and that loss of chromosome 11 may be an important event in their tumorigenesis, particularly in familial paragangliomas.

The abbreviations used are: CGH, comparative genomic hybridization; PGL, paraganglioma; PCC, pheochromocytoma; LOH, loss of heterozygosity; TSG, tumor suppressor gene; *SDHD*, succinate dehydrogenase complex II subunit D.

Introduction

Parasympathetic paragangliomas (PGLs) are rare, highly vascularized tumors, originating from neural crest-derived chief cells of paraganglia in the head and neck region. They share many histological features with sympathoadrenal PGLs, including pheochromocytomas (PCCs). Metastases of PGLs are uncommon, but may emerge in lymph nodes, lung, and liver. Dependent on the anatomic location the tumor can cause serious symptoms like dysphagia, bradycardia, and hearing loss. Because of this and in view of the risk of progression to malignancy, surgical resection of PGLs is often required.

The carotid body and jugulotympanic paraganglia are the most common sites of origin of parasympathetic PGLs, followed by vagal, laryngeal, and aorticopulmonary paraganglia.[1,2] Although most parasympathetic PGLs occur sporadically, there is a positive family history in a considerable minority (10-50%) of cases.[3] Predominance in females, multiple PGLs, and young age of onset are characteristic of familial PGLs, but such features have also been reported in sporadic cases.[4,5] Co-occurrence of parasympathetic PGLs and PCCs, and occurrence in Carney's syndrome and neurofibromatosis type 1 (NF1) has been described.[6-9]

Flow cytometric analyses revealed DNA aneuploidy in 21 - 50% of parasympathetic PGLs, which did not predict of malignant behavior or decreased survival.[10-12] A few immunohistochemical studies have suggested a paracrine/autocrine role for IGF-II, c-myc, bcl-2, and c-jun in PGL pathogenesis.[13-15]

In attempts to clarify the genetic mechanisms underlying the development of parasympathetic PGLs, the inherited form of this tumor has so far been the main subject of investigation. Linkage analysis and loss of heterozygosity (LOH) studies in unrelated families provided evidence for the existence of two distinct PGL susceptibility genes. The putative *PGL1* gene (11q23) and the *PGL2* gene (11q13.1) are both thought to be tumor suppressor genes (TSGs) and maternally imprinted.[16-20] Recently, a third, not maternally imprinted gene was demonstrated to cause PGL in a German family (*PGL3*).[21]

Although substantial progress has thus been made in the identification of genetic changes involved in the development of hereditary PGL, comparative data on genomic changes in sporadic and familial parasympathetic PGL are not available and it remains to be clarified whether these tumors develop along the same genetic pathways. To characterize cytogenetic alterations, we investigated 9 sporadic and 7 familial parasympathetic PGLs by comparative genomic hybridization (CGH) analysis. In addition, LOH analysis was carried out to confirm CGH results.

Materials and Methods

Patients and tumor samples

Sixteen benign tumors from 14 patients with parasympathetic PGLs, diagnosed between 1992 and 1996, were studied. The average age of the patients (9 female) at first presentation was 37 years (range: 29-55) and the mean size of the tumors was 2.5 ± 1.2 cm. Information on family history and other tumors or relevant conditions was obtained by reviewing medical charts and by interviewing all patients, after an average follow-up period of 7.8 years (94 months). Six of 14 patients had a positive family history for parasympathetic PGL and 8 patients had sporadic PGL. A PGL was considered sporadic when there were no first or second-degree relatives known with a parasympathetic PGL. Clinical data are summarized in Table 1.

Table 1 Clinical Characteristics of 57 Paraganglioma Patients Evaluated for SDHD Mutations.

Characteristic	All Patients (N=57)	Patients with Positive Family History† (N=19)	Patients with Negative Family History† (N=38)	*P* Value*
Patient				
Sex - no. (%)				0,56
Male	21 (37)	8 (42)		13 (34)
Female	36 (63)	11 (58)		25 (66)
Mean Age of Onset - yr (+ SE)	42.4 (+ 12.4)	37.3 (+ 8.2)	45.0 (+ 14.1)	0,04
Mean Follow-up Time - months (range)	52 (1 - 218)	57 (1 - 166)	49 (1 - 218)	0,56
Tumor focality - no. (%)				< 0.0001§
Single Paraganglioma	30 (53)	2 (10)	28 (74)	
Recurrence	*7 (12)*		*7 (18)*	
Bilateral Carotid Body	10 (17)	7 (37)	3 (8)	
Multiple	17 (30)	10 (53)	7 (18)	
Site of tumor - no. (%)	(N= 105)	(N= 51)	(N= 54)	
Carotid Body	55 (52)	33 (64)	22 (41)	0,17
Vagal Nerve	17 (16)	8 (16)	9 (17)	
Tympanic Nerve	15 (14)	3 (6)	12 (22)	0,035
Jugular Nerve	13 (13)	6 (12)	7 (13)	
Mediastinal	4 (4)	1 (2)	3 (5)	
Spinal cord	1 (1)	0 (0)	1 (2)	

† Family history data as reported at the time of the original interview.
* We used the chi-square test to compare all variables except mean age at onset and mean follow-up time, for which we used an unpaired t-test.
§ The P value is for the comparison of single PGL with bilateral and multiple PGL.

DNA Extraction

Genomic DNA from 12 frozen tumors was isolated using the D-5000 Puregene DNA Isolation kit (Gentra Systems, Minneapolis, MN). DNA extraction from 4 formalin-fixed, paraffin-embedded samples was performed by standard detergent-proteinase K lysis, followed by phenol/chloroform extraction and ethanol precipitation, as described elsewhere.[22] Only tumors with > 80% tumor cell content were included in this study.

CGH and Digital Image Analysis

CGH was carried out as described.[22] In brief, 1 µg of tumor DNA was labeled with Spectrum Green-dUTPs (Vysis, Downers Grove, IL) by nick translation (BioNick kit, Life Technologies, Basel, Switzerland). The hybridization mixture consisted of 200 ng Spectrum Green-labeled tumor DNA, 200 ng Spectrum Red-labeled sex matched normal reference DNA (Vysis) and 10-20 µg human Cot-1 DNA (Life Technologies) dissolved in 10 µl of hybridization buffer (50% formamide, 10% dextran sulfate, 2x SSC, pH 7.0). Hybridization to normal metaphase spreads (Vysis) took place for 3 days at 37°C. Slides were washed at 45°C three times for 10 minutes in 50% formamide/2x SSC and two times in 2x SSC. The chromosomes were counterstained with 4,6-diamidino-2-phenylindole in anti-fade solution for identification.

Digital images were collected from 6 to 7 metaphases using a Photometrics cooled CCD camera (Microimager 1400; Xillix Technologies, Vancouver, Canada). The QUIPS software program (Vysis) was used to calculate average green-to-red ratio profiles of at least four observations per autosome and two observations per sex chromosome 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. Because of some false positive results at chromosomes 1p32-pter, 16p, 19, and 22 found in normal tissues, gains of these known G-C-rich regions were excluded from all analyses.

LOH Analysis

To validate CGH data independently, 12 PGLs of 11 patients of whom normal DNA was available, were analyzed for allelic imbalances of the 11q23 locus using two microsatellite markers D11S1347 and D11S1986 (Research Genetics, Huntsville, AL).[19] Polymerase chain reaction (PCR) amplification of tumor and germline DNA was performed in reaction mixtures of 50 µl. Each reaction contained 50-100 ng template DNA, 0.2 mmol/L dATP, dTTP, dGTP, dCTP, 20-50 pmol of each primer, 1.5 mmol/L Mg2+, 10 mmol/L Tris-HCl, 50 mmol/L 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 s, annealing at 55 °C (for marker D11S1986 at 52 °C) for 60 s, and extension at 72 °C for 60 s. A final extension

step was carried out at 72 °C for 10 min. PCR products of tumor and normal DNA from each patient were diluted 1:1 in 10 µl loading buffer (95% formamide, 20 mmol/L EDTA, 0.05% xylene cyanol, 0.05% bromophenol blue) and loaded onto a denaturing 6% polyacrylamide gel. Electrophoresis was carried out at 40W for 2.5 hours. The DNA was visualized by silver staining and evaluated as described previously.[23] Allelic loss was considered to be present when the intensity of the signal from one allele was significantly reduced in the tumor DNA when compared with normal DNA by direct visualization. Since PGLs are known to contain admixed normal tissue (sustentacular cells and supportive fibrovascular stroma), a weak band of the lost allele was accepted in the determination of LOH.

Statistical Analysis

Chi-square test for nominal variance was used to calculate the statistical significance of differences in genomic changes between familial and sporadic tumors.

Table 2 Genetic Findings and Correlations to the Patients' Characteristics.

		SDHD Gene Alteration				
		All Missense	D92Y	L95P	No Alteration	
Characteristic		(N=32)	(N=22)	(N=9)	(N=25)	*P*-Value ¶
Patient - no. (%)						
Sex						0,38
Male	(n=21)	14	10	4	7	
Female	(n=36	18	12	5	18	
Mean Age of Onset - yr (+ SE)		40.3 (+ 11.4)	38.6 (+ 9.9)	44.8 (+ 14.1)	44.9 (+ 14.2)	0,21
Tumor focality - no (%)						0,0001
Single Paraganglioma	(n=30)	8	5	3	22	
Bilateral Carotid Body	(n=10)	9	7	2	1	
Multiple	(n=17)	15*	10	4	2	
Family history - no. (%)						< 0.0001
Positive	**(n=19)**	**19***	**13**	**5**		
Single Paraganglioma	(n=2)	2		2		
Bilateral Carotid Body	(n=7)	7	6	1		
Multiple	(n=10)	10*	7	2		
Negative	**(n=38)**	**13**	**9**	**4**	**25**	
Single Paraganglioma	(n=28)	6	5	1	22	
Recurrence		*4*	*3*	*1*	*3*	
Bilateral Carotid Body	(n=3)	2	1	1	1	
Multiple	(n=7)	5	3	2	2	

* Includes the patient harboring the L139P mutation.

¶ The P values resulted from chi-square tests to compare all variables except mean age of onset, for which we used an unpaired t-test. All P values are for the differences between patients with and without a missense mutation.

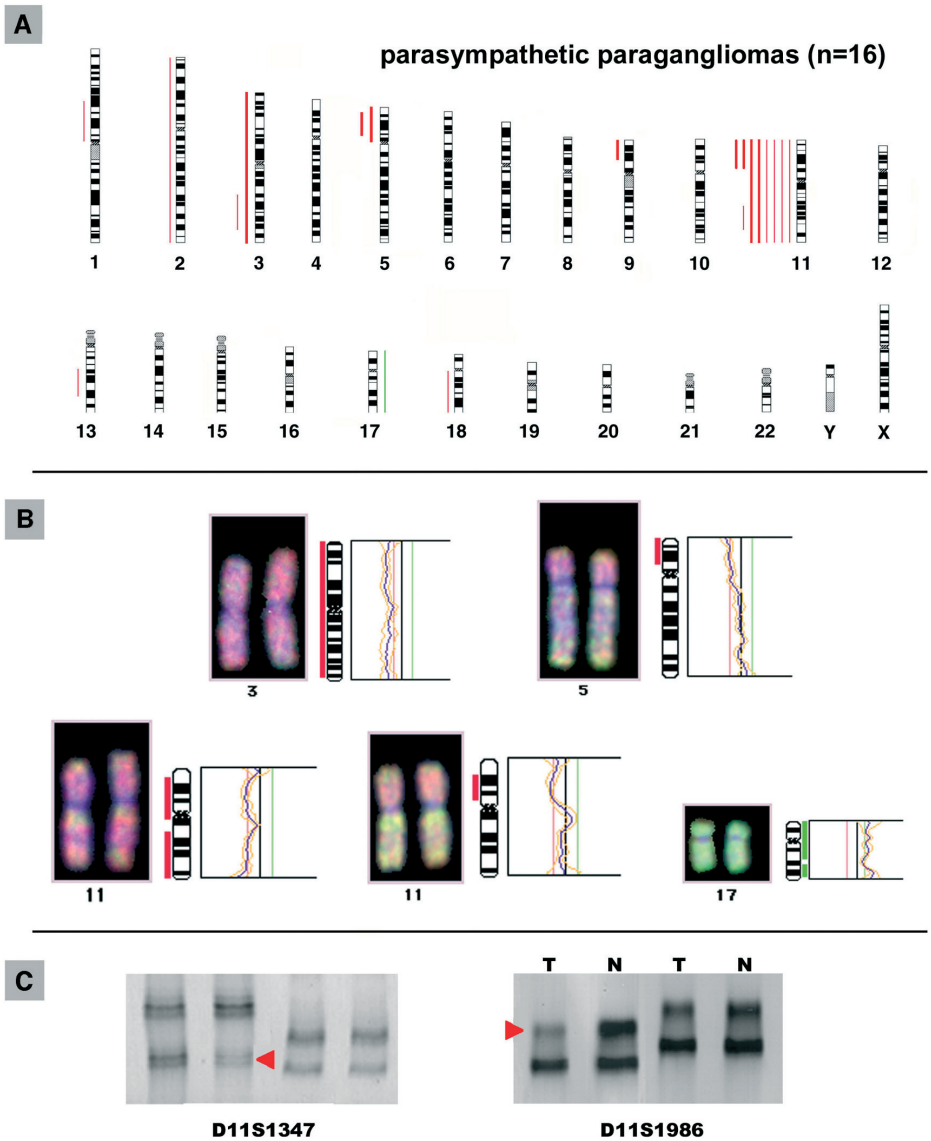

Figure 1 (A) Summary of all DNA copy number changes detected by CGH in 16 (7 familial and 9 sporadic) parasympathetic PGLs. The vertical green lines on the right side of the chromosome ideograms indicate gains, the red lines on the left side indicate losses of the corresponding chromosomal region. Findings in sporadic PGLs are indicated as solid bars. (B) Individual examples of CGH digital images (left) and fluorescent ratio profiles (right) illustrating genomic alterations of chromosome 3 (loss), 5 (loss of 5p), 11 (loss of the the entire chromosome and of 11p), and 17 (gain). (C) Example of LOH analysis of a familial PGL, showing a band markedly diminished in tumoral (T) DNA in comparison with normal (N) DNA (allelic loss) in 2 microsatellite markers at 11q23, D11S1986 and D11S1347 (red arrowheads).

Results

Overview of CGH Findings

Fig. 1A summarizes all DNA copy number changes identified in 16 parasympathetic PGLs. Genetic alterations were observed in 10 (63%) of 16 PGLs, and the average number of chromosome arm aberrations per tumor was 1.75 ± 1.69 (range: 0-5). With the exception of one gain of chromosome 17, all aberrations implied chromosomal losses and no amplifications were identified (Table 1). The most common chromosome arm copy number change was loss of chromosome 11. Six tumors showed loss of the entire chromosome, one tumor exhibited loss of 11q, and two tumors had a deletion of 11p. Losses of chromosome 11q were strongly associated with familial PGL (86% versus 22% in sporadic PGL) and appeared to be statistically significant (P= 0.012). Other genomic imbalances were rare; one gain was found on chromosome 17 (1/16), and losses involved 3q and 5p (both 2/16), 1p, 3p, 9p, 13q, and 18q (1/16 each). Both tumors with loss of 11p also exhibited loss of 5p and appeared to be sporadic tumors. Representative examples of CGH results are shown in Fig. 1B.

Comparison of CGH and LOH Results

Twelve tumors of 11 patients of whom normal DNA was available were examined by microsatellite analysis. We could confirm the CGH results of chromosome 11q in all tumors that were analyzed and detected LOH of this region in one additional tumor without apparent loss of 11q in CGH analysis (Table 1). Altogether, most deletions of chromosome 11q were found in familial PGLs (6/7 familial PGLs vs. 2/9 sporadic PGLs). Representative examples of LOH results are shown in Fig. 1C.

Discussion

This study represents the first comprehensive, genome-wide analysis of chromosomal aberrations in sporadic and familial parasympathetic PGLs. Our results indicate that DNA copy number changes are infrequent in these tumors and that TSGs on chromosome 11 may play a critical role in the tumorigenesis of familial PGLs. Sporadic PGLs may develop along different genetic pathways.

Cancer, as a genetic disease, is believed to arise from an accumulation of genetic aberrations that promote clonal selection of cells with increasingly aggressive behavior. It is proposed that most cancer cell genotypes demonstrate essential alterations in cell physiology. This is reflected by the finding of multiple chromosomal imbalances in the majority of human cancers. However, our results indicate that DNA copy number changes are infrequent in

parasympathetic PGLs. This is consistent with their characteristically slow growth rate and benign behavior and indicates that some cell-signaling mechanisms, generally disrupted in cancer cells, may still be intact in PGL tumor cells.

In a considerable proportion of PGLs, we found a normal DNA copy number profile. To exclude the possibility of false negative results, we included in our analysis only samples with at least > 80% tumor cells. Nonetheless, it should be borne in mind that the lower limit of CGH-based detection is about 10 Mb. Indeed, we found additional loss of 11q by LOH analysis in one tumor.

Only one PGL in this study showed a DNA copy number gain. This gain could not be related to one of the proteins found to be upregulated in PGLs.[13-15] Therefore, in parasympathetic PGL, upregulation of growth stimulating factors is probably caused by other events, such as genetic mutations or rearrangements.

Loss of 11q was the most common chromosomal aberration in our series of PGLs, with a remarkable difference in incidence between familial and sporadic tumors (86% vs. 22%, respectively. P= 0.012). This may point towards distinct tumorigenic pathways in these subgroups of PGLs.

Co-occurrence of parasympathetic PGLs and sympathoadrenal PGLs (including PCCs) has been described.[6,8] Since these tumors share many histological characteristics, they may be thought to result from similar genetic changes. However, comparison of CGH data of benign parasympathetic and sympathoadrenal PGLs shows that these types are genetically different. In benign sympathoadrenal PGLs, genetic changes are much more frequent and 11q losses are less frequent compared to the parasympathetic PGLs in this study.[24,25] Yet, it remains to be tested whether the same TSG(s) on 11q are involved in the tumorigenesis of both parasympathetic PGLs and PCCs.

The long arm of chromosome 11 contains 2 well-defined critical regions that each harbors a putative PGL disease gene, *PGL1* at 11q23 and *PGL2* at 11q13.[16,18,26] Recently, Baysal and co-workers detected germline mutations of the mitochondrial succinate dehydrogenase complex II subunit d *(SDHD)* gene (11q23) cosegregating with tumor occurrence in PGL families, indicating that this is the putative *PGL1* gene.[27] The mitochondrial complex II is an important enzyme complex in the aerobic respiratory chains of mitochondria. Loss of function of this complex may cause cellular hypoxia and increased superoxide levels. Chronic hypoxia is known to be an important cause of carotid body hyperplasia and conceivably plays a role in tumor initiation and progression of carotid body PGLs. In this respect, it is of interest that two of 9 sporadic PGLs showed loss of 5p, the gene locus of another component (the flavoprotein subunit) of the mitochondrial succinate dehydrogenase complex II.[28] These tumors also demonstrated loss of 11p, but not of 11q. This may imply that in the pathogenesis of some sporadic PGLs other components of the mitochondrial complex II are involved in their tumorigenesis.

In conclusion, our study demonstrates that chromosomal imbalances are infrequent in parasympathetic PGLs compared to their sympathetic counterpart, PCC. We observed a high frequency of 11q loss in familial but not in sporadic PGL, pointing towards differences in the genetic evolution of familial and sporadic PGLs. Genetic changes on chromosome 11p and 5p may be involved in sporadic PGLs.

More genetic data are needed to assess the concept and aspects of genetic differences between familial and sporadic parasympathetic PGL. Further analysis of the candidate TSG *SDHD* and other genes of the mitochondrial complex II is required to determine their involvement in the development of these tumors.

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References

1. E. E. Lack, A. L. Cubilla and J. M. Woodruff: Paragangliomas of the head and neck region. A pathologic study of tumors from 71 patients. *Hum Pathol* 1979, 10:191-218.
2. A. S. Tischler The adrenal medulla and extra-adrenal paraganglia. In*:* K. Kovacs and S. L. Asa (eds.), Functional endocrine pathology, Vol. pp. 509-545. Boston: *Blackwell Scientific Publications*, 1990.
3. A. G. van der Mey, P. D. Maaswinkel-Mooy, C. J. Cornelisse, P. H. Schmidt and J. J. van de Kamp: Genomic imprinting in hereditary glomus tumours: evidence for new genetic theory [see comments]. *Lancet* 1989, 2:1291-4.
4. P. Gardner, M. Dalsing, E. Weisberger, A. Sawchuk and R. Miyamoto: Carotid body tumors, inheritance, and a high incidence of associated cervical paragangliomas. *Am J Surg* 1996, 172:196-9.
5. T. V. McCaffrey, F. B. Meyer, V. V. Michels, D. G. Piepgras and M. S. Marion: Familial paragangliomas of the head and neck. *Arch Otolaryngol Head Neck Surg* 1994, 120:1211-6.
6. J. C. Jensen, P. L. Choyke, M. Rosenfeld, H. I. Pass, H. Keiser, B. White, W. Travis and W. M. Linehan: A report of familial carotid body tumors and multiple extra-adrenal pheochromocytomas. *J Urol* 1991, 145:1040-2.
7. J. W. Pritchett: Familial concurrence of carotid body tumor and pheochromocytoma. *Cancer* 1982, 49:2578-9.
8. L. M. DeAngelis, M. B. Kelleher, K. D. Post and M. R. Fetell: Multiple paragangliomas in neurofibromatosis: a new neuroendocrine neoplasia. *Neurology* 1987, 37:129-33.
9. J. A. Carney: Gastric stromal sarcoma, pulmonary chondroma, and extra-adrenal paraganglioma (Carney Triad): natural history, adrenocortical component, and possible familial occurrence [see comments]. *Mayo Clin Proc* 1999, 74:543-52.
10. L. Barnes and S. R. Taylor: Vagal paragangliomas: a clinical, pathological, and DNA assessment. *Clin Otolaryngol* 1991, 16:376-82.

11. E. R. Sauter, L. H. Hollier, J. S. Bolton, J. L. Ochsner and A. Sardi: Prognostic value of DNA flow cytometry in paragangliomas of the carotid body. *J Surg Oncol* 1991, 46:151-3.
12. A. G. van der Mey, C. J. Cornelisse, J. Hermans, J. L. Terpstra, P. H. Schmidt and G. J. Fleuren: DNA flow cytometry of hereditary and sporadic paragangliomas (glomus tumours). *Br J Cancer* 1991, 63:298-302.
13. S. L. Li, H. Goko, Z. D. Xu, G. Kimura, Y. Sun, M. H. Kawachi, T. G. Wilson, S. Wilczynski and Y. Fujita-Yamaguchi: Expression of insulin-like growth factor (IGF)-II in human prostate, breast, bladder, and paraganglioma tumors. *Cell Tissue Res* 1998, 291:469-79.
14. R. W. Jyung, E. E. LeClair, R. A. Bernat, T. S. Kang, F. Ung, M. J. McKenna and R. S. Tuan: Expression of angiogenic growth factors in paragangliomas. *Laryngoscope* 2000, 110:161-7.
15. D. G. Wang, C. F. Johnston, A. A. Barros D'Sa and K. D. Buchanan: Expression of apoptosis-suppressing gene bcl-2 in human carotid body tumours. *J Pathol* 1997, 183:218-21.
16. B. E. Baysal, E. M. van Schothorst, J. E. Farr, P. Grashof, D. Myssiorek, W. S. Rubinstein, P. Taschner, C. J. Cornelisse, B. Devlin, P. Devilee and C. W. Richard, 3rd: Repositioning the hereditary paraganglioma critical region on chromosome band 11q23. *Hum Genet* 1999, 104:219-25.
17. P. Heutink, E. M. van Schothorst, A. G. van der Mey, A. Bardoel, G. Breedveld, J. Pertijs, L. A. Sandkuijl, G. J. van Ommen, C. J. Cornelisse, B. A. Oostra and et al.: Further localization of the gene for hereditary paragangliomas and evidence for linkage in unrelated families. *Eur J Hum Genet* 1994, 2:148-58.
18. E. C. Mariman, S. E. van Beersum, C. W. Cremers, P. M. Struycken and H. H. Ropers: Fine mapping of a putatively imprinted gene for familial non-chromaffin paragangliomas to chromosome 11q13.1: evidence for genetic heterogeneity. *Hum Genet* 1995, 95:56-62.
19. E. M. van Schothorst, J. C. Jansen, A. F. Bardoel, A. G. van der Mey, M. J. James, H. Sobol, J. Weissenbach, G. J. van Ommen, C. J. Cornelisse and P. Devilee: Confinement of PGL, an imprinted gene causing hereditary paragangliomas, to a 2-cM interval on 11q22-q23 and exclusion of DRD2 and NCAM as candidate genes. *Eur J Hum Genet* 1996, 4:267-73.
20. P. Devilee, E. M. van Schothorst, A. F. Bardoel, B. Bonsing, N. Kuipers-Dijkshoorn, M. R. James, G. Fleuren, A. G. van der Mey and C. J. Cornelisse: Allelotype of head and neck paragangliomas: allelic imbalance is confined to the long arm of chromosome 11, the site of the predisposing locus PGL. *Genes Chromosomes Cancer* 1994, 11:71-8.
21. S. Niemann and U. Muller: Mutations in SDHC cause autosomal dominant paraganglioma, type 3 [In Process Citation]. *Nat Genet* 2000, 26:268-70.
22. J. Richter, F. Jiang, J. P. Gorog, G. Sartorius, C. Egenter, T. C. Gasser, H. Moch, M. J. Mihatsch and G. Sauter: Marked genetic differences between stage pTa and stage pT1 papillary bladder cancer detected by comparative genomic hybridization. *Cancer Res* 1997, 57:2860-4.
23. P. Komminoth, E. Kunz, O. Hiort, S. Schroder, X. Matias-Guiu, G. Christiansen, J. Roth and P. U. Heitz: Detection of RET proto-oncogene point mutations in paraffin-embedded pheochromocytoma specimens by nonradioactive single-strand conformation polymorphism analysis and direct sequencing. *Am J Pathol* 1994, 145:922-9.
24. E. Edstrom, E. Mahlamaki, B. Nord, M. Kjellman, R. Karhu, A. Hoog, N. Goncharov, B. T. Teh, M. Backdahl and C. Larsson: Comparative genomic hybridization reveals frequent losses of chromosomes 1p and 3q in pheochromocytomas and abdominal paragangliomas, suggesting a common genetic etiology. *Am J Pathol* 2000, 156:651-9.
25. H. Dannenberg, E. J. Speel, J. Zhao, P. Saremaslani, E. van Der Harst, J. Roth, P. U. Heitz, H. J. Bonjer, W. N. Dinjens, W. J. Mooi, P. Komminoth and R. R. de Krijger: Losses of chromosomes 1p and 3q are early genetic events in the development of sporadic pheochromocytomas. *Am J Pathol* 2000, 157:353-9.

Chapter 7

Frequent germline *SDHD* gene mutations in patients with apparently sporadic parasympathetic paraganglioma

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Abstract

Purpose: Recently, familial paraganglioma was shown to be caused by mutations in the gene encoding succinate dehydrogenase subunit D (*SDHD*). However, the prevalence of *SDHD* mutations in apparently sporadic paraganglioma is unknown. We studied the frequency and spectrum of germline and somatic *SDHD* mutations in patients with parasympathetic paraganglioma.

Experimental design: We studied 57 unselected patients who developed parasympathetic paragangliomas (n=105 tumors) and who were treated between 1987 and 1999 at the Erasmus Medical Center, Rotterdam, The Netherlands. Thirty-eight (67%) of these patients (n=51 tumors) lacked a family history of parasympathetic paraganglioma. We used conformation-dependent gel electrophoresis and sequence determination analysis of germline and tumor DNA to identify *SDHD* mutations. We compared the clinical and molecular characteristics of sporadic and hereditary paragangliomas.

Results: Three different *SDHD* germline mutations were identified in 32 (56%) of the 57 patients. These included 19 (100%) of the 19 patients with familial paraganglioma, but also 13 (34%) of the 38 patients with apparently sporadic paraganglioma. All three mutations were characterized as missense mutations (D92Y, L95P and L139P) in highly conserved regions of the *SDHD* gene and were not observed in 200 control alleles. No somatic mutations were found.

Conclusions: Germline mutations of the *SDHD* gene are present in a significant number of patients with apparently sporadic parasympathetic paraganglioma. Somatic *SDHD* mutations do not play a significant role in the sporadic form of this tumor. Genetic testing for *SDHD* germline mutations should be considered for every patient presenting with this tumor, even if a personal or family history of paraganglioma is absent, in order to allow appropriate clinical management.

Introduction

Parasympathetic paragangliomas are highly vascularized, slow growing tumors of parasympathetic ganglia, mostly of the head and neck region. The annual incidence is estimated at 1: 30,000, and the tumors typically present in the fourth or fifth decade.[1,2] The tumor most commonly presents as a painless neck mass, or with symptoms due to compression and damage of the surrounding structures. Depending on the anatomic localization, cranial nerve damage may cause bradycardia, hoarseness, or hearing loss. Approximately 1% of the tumors produces catecholamines. Progression to malignancy occurs in 2-10% of cases and can only be determined by detection of metastatic spread, particularly to lymph nodes and lungs. There are currently three treatment strategies: surgery, radiation therapy, or observation.[3] Because tumor growth as well as surgery can cause disabling loss of function, preoperative estimation of tumor growth is an important parameter influencing clinical management. A "wait and scan" approach is thus often considered, although surgical resection remains the mainstay of the curative treatment of parasympathetic paragangliomas.[4,5]

Familial occurrence is observed in a significant minority (approximately 20%) of cases, with an autosomal dominant mode of transmission with incomplete and age-dependent penetrance.[6-8] The female predominance in sporadic paraganglioma with equal sex distribution in familial patients, and an almost exclusively paternal inheritance pattern in these families, suggests maternal imprinting of the disease gene.[9] Clinical features that suggest a genetic predisposition in a given patient include bilateral or multiple paragangliomas and an unusually young age (before the third decade) at presentation. Linkage analysis and LOH[2] studies in unrelated and multigenerational families with hereditary paraganglioma (PGL; MIM number 168,000) have provided evidence for the existence of two distinct PGL susceptibility genes on chromosome 11: *PGL1* at 11q23 and *PGL2* at 11q13, both of which are thought to be maternally imprinted.[10,11] Recently, the succinate dehydrogenase subunit D (*SDHD*) gene, which maps to the *PGL1* locus, has been implicated as the putative disease gene, because inactivating germline *SDHD* mutations have been detected in PGL families.[12] *SDHD* germline mutations were recently described also in a few cases of familial phaeochromocytoma and to date only one sporadic mutation has been reported.[13]

The *SDHD* gene, which comprises four exons and three introns extending over 19 kb, encodes a 159 amino acid protein. This protein constitutes the small subunit (cybS) of cytochrome b in the mitochondrial enzyme complex II (succinate-ubiquinone oxidoreductase), and plays an important role in both the citric acid cycle and the aerobic respiratory chain.[14] Recently, it has been demonstrated that germline mutations in *SDHC* (succinate dehydrogenase

[2] The abbreviations used are: LOH, loss of heterozygosity; PGL, hereditary paraganglioma; SDHD, succinate dehydrogenase subunit D; SSCP, single strand conformation polymorphism; SNP, single nucleotide polymorphism; RET, rearranged during transfection; MTC, medullary thyroid carcinoma.

subunit C) and *SDHB* (succinate dehydrogenase subunit B), encoding two other components of comlex II, also predispose to hereditary PGL.[15,16]

Whether genetic alterations of the *SDHD* gene play a role in the pathogenesis of sporadic parasympathetic paraganglioma is presently unknown. Also, the prevalence of germline *SDHD* mutations among apparently sporadic paraganglioma patients has not been studied. Further knowledge of the contribution and nature of gene mutations involved in paraganglioma tumorigenesis could provide early diagnosis and allow accurate genetic counseling in affected families. Therefore, we assessed the frequency and type of *SDHD* mutations in an unselected series of patients with parasympathetic paragangliomas, treated at our hospital, and including both familial and apparently sporadic cases.

Materials and Methods

Patients

Individuals, who received a diagnosis of parasympathetic paraganglioma between May 1987 and December 1999, were identified by retrospectively reviewing the records of the Pathology Department at the Erasmus Medical Center (Erasmus MC), Rotterdam, The Netherlands. Of 89 paraganglioma patients eligible to participate, specimens and constitutional DNA from 57 patients were available for retrieval and testing. Information on medical and family histories was obtained by direct interviews and by review of the medical charts. The presence of multiple tumors was assessed by review of the radiology reports of somatostatin-receptor scintigraphy (SRS) and/or magnetic resonance imaging (MRI). From 48 patients, SRS and/or MRI reports were available (41 patients (72%) underwent SRS). Of the 9 patients without such information, 4 had histopathologic diagnoses of bilateral or multiple tumors. The average follow-up period was 52 months (range 1-218), with no significant difference between familial and apparently sporadic patients.

A total of 105 parasympathetic paragangliomas was observed in the 57 patients, of which 78 tumors and matched normal tissues were available for analysis. After coupling of the clinical information to the pathology specimen, both patient information and DNA samples were anonymized in order to preserve the confidentiality of the patients and in accordance with the Erasmus MC guidelines for studies involving patient data and tissues. A tumor was considered sporadic if the patient did not know any relative with paraganglioma.

Control DNA consisted of 100 encoded peripheral blood samples, from persons residing in the same geographic area, randomly selected from the blood bank (Bloedbank ZWN Rotterdam, The Netherlands).

In addition, 13 paragangliomas from 13 patients residing in other geographic areas (from hospitals in the south of The Netherlands and Belgium) were investigated for exon 3 *SDHD* mutations.

Clinical database

A collective database of clinical and molecular features was prepared. The date of the patient's first biopsy or resection with a histopathologic diagnosis of parasympathetic paraganglioma was recorded as the date of diagnosis. The age of onset was defined as the age at which the patient first experienced symptoms. Three patients (two women and one man) were related to one another, and a further two patients belonged to another family. For each patient, we recorded the place of birth, age of onset and diagnosis, clinical history, the patient's family history with respect to parasympathetic PGL, the laterality/multifocality of the tumors, and the presence of any metastases.

DNA preparation and SSCP Analysis

Fresh frozen or formalin-fixed, paraffin-embedded tumor and normal tissues from all patients, including 78 of the 105 tumors, were retrieved from the archives of the Pathology Department of the Erasmus MC, Rotterdam, The Netherlands. Paraffin blocks containing tumor and normal tissues were obtained from 13 patients residing in other areas (kindly provided by Prof. Dr. E. Van Marck, Department of Pathology, University Hospital Antwerp, Belgium; Dr. G. van Lijnschoten, Society for Pathological Anatomy and Medical Microbiology (PAMM), Eindhoven, The Netherlands; and by Dr. A.P. de Bruïne, Department of Pathology, University of Maastricht, The Netherlands).

From each specimen, regions containing at least 80% of neoplastic cells were selected. DNA from 12 fresh frozen tumors was isolated using the D-5000 Pure gene DNA isolation kit (Gentra Systems, Minneapolis, MN, USA) according to the manufacturers' recommendations. DNA from 66 paraffin-embedded tumors and all normal tissues was extracted by standard detergent-proteinase K lysis, followed by phenol/chloroform extraction and ethanol precipitation, as described previously.[17]

The entire open reading frame of the *SDHD* gene, and all exon-intron boundaries were investigated with the PCR primers previously described.[12] PCR amplification of tumor DNA and matched normal DNA was performed in 15 µl reaction mixtures containing 1.5 mM $MgCl_2$, 10 mM Tris-HCl, 50 mM KCl, 0.02 mM dATP, 0.2 mM dGTP, dTTP, dCTP each, 0.8 µCi α32P-dATP (Amersham, Buckinghamshire, UK), 20 pmol of each sense and anti-sense primer, and 1 U Taq DNA polymerase (Amplitaq Gold, Perkin Elmer, Norwalk, CT, USA). The amplification profile consisted of an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 45 sec, annealing at 55 for 60 sec, and extension at 72 °C for 60 sec. A final extension step was carried out at 72 °C for 10 min. Electrophoresis of PCR products was carried

out overnight at 8W on non-denaturing gels, containing 8% polyacrylamide (49:1) and 10% (v/v) glycerol. For the exon 4 amplicons, electrophoresis was performed on 8% polyacrylamide gel without glycerol for 6 hours at +4 °C and 20W. The gels were dried and exposed to X-ray film overnight at –70 °C.

DNA sequencing

For each variant pattern identified by SSCP analysis, two independent genomic DNA samples were amplified for direct sequencing or the aberrant PCR product was cloned and re-amplified with the original primer pair. These products were bidirectionally sequenced using Applied Biosystems Taq DyeDeoxy terminator cycle sequencing (Baseclear, Leiden, The Netherlands).

Statistics

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

Results

Patients' characteristics

The characteristics of the patients and their tumors are shown in Table 1. The group of 19 patients with a positive family history of PGL showed a roughly equal sex distribution (42% male *versus* 58% female), while there was a female predominance in patients without a family history of PGL (34% male *versus* 66% female). The mean age at onset was earlier in patients with a positive family history ($P = 0.04$). The majority of patients with a single paraganglioma tumor had no family history and bilateral carotid body tumors and multiple tumors were generally associated with a positive family history ($P < 0.0001$).

The carotid body was the most common site of origin (52%) and comprised 33 (64%) of 51 tumors in familial PGL patients. Tympanic nerve paragangliomas occurred more frequently in apparently sporadic patients ($P = 0.035$), other sites being equally distributed between the two groups.

None of the patients had evidence of metastatic disease at the end of our study. From the 57 patients, only one young patient (1,8%) had evidence of a malignant paraganglioma, when tumors cells were detected in one lymph node after total resection of a carotid body tumor. After 141 months of follow-up time she is alive and well. Seven patients developed a recurrence after resection of a single paraganglioma, which was either a tympanic, jugular or vagal tumor. None of these patients had a positive family history.

Table 1 Clinical Characteristics of 57 Paraganglioma Patients Evaluated for SDHD Mutations.

Characteristic	All Patients (N=57)	Patients with Positive Family History† (N=19)	Patients with Negaitive Family History† (N=38)	*P* Value *
Patient				
Sex - no. (%)				0,56
Male	21 (37)	8 (42)	13 (34)	
Female	36 (63)	11 (58)	25 (66)	
Mean Age of Onset - yr (+ SE)	42.4 (+ 12.4)	37.3 (+ 8.2)	45.0 (+ 14.1)	0,04
Mean Follow-up Time - months (range)	52 (1 - 218)	57 (1 - 166)	49 (1 - 218)	0,56
Tumor focality - no. (%)				< 0.0001§
Single Paraganglioma	30 (53)	2 (10)	28 (74)	
Recurrence	*7 (12)*		*7 (18)*	
Bilateral Carotid Body	10 (17)	7 (37)	3 (8)	
Multiple	17 (30)	10 (53)	7 (18)	
Site of tumor - no. (%)	(N= 105)	(N= 51)	(N= 54)	
Carotid Body	55 (52)	33 (64)	22 (41)	0,17
Vagal Nerve	17 (16)	8 (16)	9 (17)	
Tympanic Nerve	15 (14)	3 (6)	12 (22)	0,035
Jugular Nerve	13 (13)	6 (12)	7 (13)	
Mediastinal	4 (4)	1 (2)	3 (5)	
Spinal cord	1 (1)	0 (0)	1 (2)	

† Family history data as reported at the time of the original interview.
* We used the chi-square test to compare all variables except mean age at onset and mean follow-up time, for which we used an unpaired t-test.
§ The P value is for the comparison of single PGL with bilateral and multiple PGL.

Identification of *SDHD* gene mutations

SSCP analysis revealed 4 different aberrant patterns, which were present in 49 (63%) of 78 paraganglioma specimens. In each instance, the same SSCP variant was also found in the germline DNA of the patient (totaling 33 of 57 patients; 58%). By sequence analysis, the aberrant patterns, all located in exon 3 and 4, were found to correspond to 3 different missense *SDHD* mutations and one single nucleotide polymorphism (SNP). All three missense mutations were located in highly conserved regions and were absent from the 200 control alleles. The specific D92Y missense mutation was observed in 22 patients, the L95P mutation in 9 patients and the L139P mutation in one patient. Altogether, germline *SDHD* missense mutations were identified in 32 (56%) of 57 patients.

The SNP, which does not result in an amino acid substitution in the *SDHD* protein, involved a change in codon 68 (AGC → AGT). This sequence alteration was also observed in one of the 200 control alleles and therefore is regarded as an uncommon polymorphism, apparently not associated with hereditary PGL.

Examples of SSCP analysis and sequence determination in patients with *SDHD* missense mutations are shown in Figure 1A.

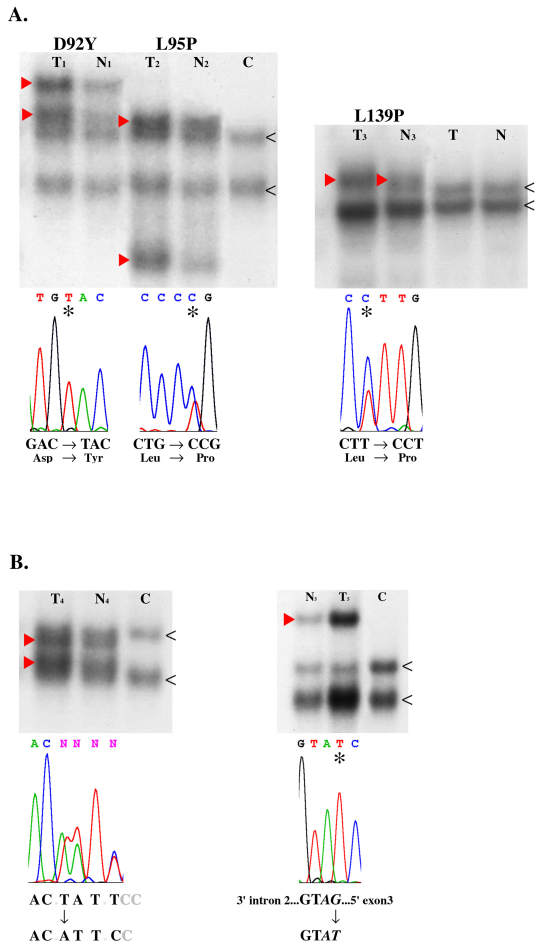

Figure 1 SDHD alterations identified in PGL patients by Single-Strand Conformation Polymorphism (SSCP) analysis and direct sequencing. From all SDHD alterations, SSCP patterns of the tumor (T) and germline (N) DNA are shown. C = normal control sample. Panel A shows the three missense mutations, D92Y, L95P, and L139P, respectively. Panel B shows 2 new mutations found in 5 of 13 patients residing in other geographic areas. On the left, the frame-shift mutation, caused by deletion of the first thymidine in codon 93, and resulting in a premature stop codon in exon 4. On the right, the splice-site mutation (ag → at) at the boundary of intron 2 and exon 3. The autoradiographs of PCR-SSCP gels show the migration patterns of normal DNA (black arrowheads) and the mobility shifts produced by aberrant alleles (red arrowheads). The sequencing chromatograms below each autoradiograph show the alterations (note the substituted nucleotide marked by an asterisk). In samples that were cloned before sequencing, the normal sequence is absent, whereas in direct sequenced samples both normal and aberrant sequences are present.

Patients' characteristics associated with *SDHD* mutations

A germline *SDHD* mutation was associated with a positive family history for PGL in 19 (59%) of 32 patients, but with a negative family history in 13 (41%) of 32 patients (Table 2). The mean age of onset in patients harboring a germline missense mutation was 40.3 years (± 11.4) compared to 44.9 years (± 14.2) in patients without a *SDHD* mutation, which is not a significant difference. The difference in age of onset did also not differ significantly among the specific mutations. Eighty-eight percent of patients without a *SDHD* mutation had a single paraganglioma tumor, whereas in patients with *SDHD* aberrations bilateral and multiple paragangliomas were most common (75%).

From 7 patients with recurrency of a single paraganglioma, 4 patients harbored a germline *SDHD* mutation. We did not detect a germline *SDHD* mutation in the patient with metstatic spread in a lymph node.

***SDHD* mutations in patients residing in other geographic areas**

To further investigate whether the recurrent mutations could be regarded as founder mutations, we analyzed 13 patients from hospitals in the south of The Netherlands and Belgium for exon 3 mutations. Five (38%) of these patients harbored a germline *SDHD* mutation in exon 3, including the Dutch founder mutation D92Y (3 patients), a mutation resulting in a change of a splice site (5' intron 2-…gt**at**-3'exon 3), and a frame shift mutation, caused by a deletion of the first thymidine in codon 93 and resulting in a premature stop codon in exon 4 (Figure 1B).

Table 2 Genetic Findings and Correlations to the Patients' Characteristics.

Characteristic		All Missense (N=32)	D92Y (N=22)	L95P (N=9)	No Alteration (N=25)	*P* Value ¶
Patient - no. (%)						
Sex						0,38
Male	(n=21)	14	10	4	7	
Female	(n=36)	18	12	5	18	
Mean Age of Onset - yr (+ SE)		40.3 (+ 11.4)	38.6 (+ 9.9)	44.8 (+ 14.1)	44.9 (+ 14.2)	0,21
Tumor focality - no (%)						0,0001
Single Paraganglioma	(n=30)	8	5	3	22	
Bilateral Carotid Body	(n=10)	9	7	2	1	
Multiple	(n=17)	15*	10	4	2	
Family history - no. (%)						< 0.0001
Positive	**(n=19)**	**19***	**13**	**5**		
Single Paraganglioma	(n=2)	2		2		
Bilateral Carotid Body	(n=7)	7	6	1		
Multiple	(n=10)	10*	7	2		
Negative	**(n=38)**	**13**	**9**	**4**	**25**	
Single Paraganglioma		6	5	1	22	
Recurrence		*4*	*3*	*1*	*3*	
Bilateral Carotid Body		2	1	1	1	
Multiple		5	3	2	2	

* Includes the patient harboring the L139P mutation.

¶ The P values resulted from chi-square tests to compare all variables except mean age of onset, for which we used an unpaired t-test. All P values are for the differences between patients with and without a missense mutation.

Discussion

Paraganglioma is the only well-documented manifestation of the hereditary PGL syndrome, in which *SDHD* gene mutations apparently represent an early and essential pathogenic event [12]. We identified *SDHD* mutations in 32 cases (56%) of a cohort of 57 unselected patients. This supports the prediction that the *SDHD* gene has an important role in the pathogenesis of parasympathetic paraganglioma. All *SDHD* mutations identified in this study were present in the patients' germline DNA, also in over 30% of patients where the family history was negative for PGL. These data indicate that family history is a poor indicator of the risk of hereditary PGL, and that screening for germline *SDHD* mutations is clinically relevant in all patients presenting with parasympathetic paragangliomas.

SSCP screening for the presence of mutations revealed 4 different aberrant patterns. Upon sequencing, three aberrations proved to be missense mutations (D92Y, L95P and L139P), whereas the remaining one was a silent substitution SNP. These mutations could theoretically

be clinically unimportant polymorphisms. However, we regard the missense mutations found in this study as pathogenic, since they occur at highly conserved residues of the *SDHD* protein, they were absent from 200 control alleles and finally, the D92Y and L95P mutation, have been reported independently as pathogenic in familial PGL.[12,18]

All *SDHD* mutations identified in our study were present in the germline DNA; no somatic *SDHD* mutations were found. Recently, one somatic *SDHD* mutation was described in a phaeochromocytoma.[13] These results imply that the involvement of the *SDHD* gene is restricted to inherited cases of paraganglioma.

It is of interest that only three different *SDHD* mutations were found in the 32 patients with mutated *SDHD*, with two of them, D92Y and L95P comprising 97 percent of mutations detected in this study. This indicates that either these mutations are ancient or that the affected persons are more closely related than known. Information on places of birth in this cohort of patients is suggestive of a founder effect, although some of the patients originate from other European and non-European countries. Baysal and colleagues (2000) previously reported the specific D92Y mutation in a Dutch founder population.[12,19] To further investigate whether this founder mutation occurs in patients from other geographic areas, we analysed 13 patients from hospitals in the south of The Netherlands and Belgium for exon 3 mutations. Except the Dutch founder mutation, we found also two new mutations, supporting evidence for founder effects.

The finding of the same germline *SDHD* mutations in familial and sporadic cases points most likely to reduced penetrance of the mutated *SDHD* gene, although the occurrence of *de novo* hot spot mutations can not completely be ruled out. Mutational and haplotype analysis of patients and family members would provide additional information on the origin of the mutations.

In all of the 19 familial PGL patients a *SDHD* mutation was detected, indicating high mutation detection sensitivity for SSCP analysis. However, in the apparently sporadic paraganglioma patients, we cannot rule out the possibility that *SDHD* mutations escaped detection by SSCP.[20] Furthermore, mutations can be present in the *SDHD* gene outside the screened sequences. It is known that gene mutations in the untranslated regions can severely affect RNA stability. Finally, in some apparently sporadic patients, a germline mutation in another gene may cause PGL. Recently, germline mutations in the *SDHC and SDHB* genes have been demonstrated to cause autosomal dominant PGL syndromes as well.[15,16]

As shown, a positive family history of PGL is the main parameter associated with *SDHD* gene mutations (19 of 19 patients), followed by the presence of bilateral or multiple paragangliomas (24 of 27 patients). Importantly, in patients without a family history of PGL, bilateral or multiple tumors, but not early age at onset, pointed towards a germline *SDHD* gene mutation. In the 28 patients presenting with a single paraganglioma tumor and lacking a family history of PGL, 6 germline *SDHD* mutations were found (21%). No clinical parameter was significantly correlated with any of the three mutations, but the study size precludes the detection of small differences.

These results show that clinical and patient data have limited value in indicating germline *SDHD* mutations. Comparable results were reported for apparently sporadic medullary thyroid carcinoma (MTC) patients, which appeared to have germline *RET* gene mutations in about 10% of cases.[21] The authors show that, in spite of this low percentage, the genetic analysis of all apparently sporadic MTC patients is cost-effective.

Genetic counseling on the basis of DNA linkage analysis in PGL families has previously been described by Oosterwijk et al.[22] Our results indicate that screening of all paraganglioma patients may further affect counseling strategies, since the detection of germline *SDHD* mutations in apparently sporadic patients is likely to improve early diagnosis of PGL, which will support adequate and appropriate clinical follow-up and management. On the other hand, we have no data on the lifetime risk of paragangliomas in people harboring a germline *SDHD* mutation. More data are needed in order to allow a valid estimate of the risk of parasympathetic paraganglioma in patients with *SDHD* mutations. However, our data do support the conclusion that *SDHD* mutation screening should be considered in every patient presenting with this tumor.

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References

1. Lack, E. E., Cubilla, A. L., and Woodruff, J. M. Paragangliomas of the head and neck region. A pathologic study of tumors from 71 patients, *Hum Pathol 10:* 191-218, 1979.
2. Mariman, E. C., van Beersum, S. E., Cremers, C. W., van Baars, F. M., and Ropers, H. H. Analysis of a second family with hereditary non-chromaffin paragangliomas locates the underlying gene at the proximal region of chromosome 11q, *Hum Genet* 91: 357-361, 1993.
3. Evenson, L. J., Mendenhall, W. M., Parsons, J. T., and Cassisi, N. J. Radiotherapy in the management of chemodectomas of the carotid body and glomus vagale, *Head Neck* 20: 609-613, 1998.
4. Sillars, H. A. and Fagan, P. A. The management of multiple paraganglioma of the head and neck, *J Laryngol Otol* 107: 538-542, 1993.

5. Jansen, J. C., van den Berg, R., Kuiper, A., van der Mey, A. G., Zwinderman, A. H., and Cornelisse, C. J. Estimation of growth rate in patients with head and neck paragangliomas influences the treatment proposal, *Cancer* 88: 2811-2816, 2000.
6. Hodge, K. M., Byers, R. M., and Peters, L. J. Paragangliomas of the head and neck, *Arch Otolaryngol Head Neck Surg* 114: 872-877, 1988.
7. van Baars, F., Cremers, C., van den Broek, P., Geerts, S., and Veldman, J. Genetic aspects of nonchromaffin paraganglioma, *Hum Genet* 60: 305-309, 1982.
8. Grufferman, S., Gillman, M. W., Pasternak, L. R., Peterson, C. L., and Young, W. G., Jr. Familial carotid body tumors: case report and epidemiologic review, *Cancer* 46: 2116-2122, 1980.
9. van der Mey, A. G., Maaswinkel-Mooy, P. D., Cornelisse, C. J., Schmidt, P. H., and van de Kamp, J. J. Genomic imprinting in hereditary glomus tumours: evidence for new genetic theory, *Lancet* 2: 1291-1294, 1989.
10. van Schothorst, E. M., Jansen, J. C., Bardoel, A. F., van der Mey, A. G., James, M. J., Sobol, H., Weissenbach, J., van Ommen, G. J., Cornelisse, C. J., and Devilee, P. Confinement of PGL, an imprinted gene causing hereditary paragangliomas, to a 2-cM interval on 11q22-q23 and exclusion of DRD2 and NCAM as candidate genes, *Eur J Hum Genet* 4: 267-273, 1996.
11. Mariman, E. C., van Beersum, S. E., Cremers, C. W., Struycken, P. M., and Ropers, H. H. Fine mapping of a putatively imprinted gene for familial non-chromaffin paragangliomas to chromosome 11q13.1: evidence for genetic heterogeneity, *Hum Genet* 95: 56-62, 1995.
12. Baysal, B. E., Ferrell, R. E., Willett-Brozick, J. E., Lawrence, E. C., Myssiorek, D., Bosch, A., van der Mey, A., Taschner, P. E., Rubinstein, W. S., Myers, E. N., Richard, C. W., 3rd, Cornelisse, C. J., Devilee, P., and Devlin, B. Mutations in SDHD, a mitochondrial complex II gene, in hereditary paraganglioma, *Science* 287: 848-851, 2000.
13. Gimm, O., Armanios, M., Dziema, H., Neumann, H. P., and Eng, C. Somatic and occult germ-line mutations in SDHD, a mitochondrial complex II gene, in nonfamilial pheochromocytoma, *Cancer Res.* 60: 6822-6825, 2000.
14. Hirawake, H., Taniwaki, M., Tamura, A., Amino, H., Tomitsuka, E., and Kita, K. Characterization of the human SDHD gene encoding the small subunit of cytochrome b (cybS) in mitochondrial succinate-ubiquinone oxidoreductase, *Biochim Biophys Acta* 1412: 295-300, 1999.
15. Niemann, S. and Muller, U. Mutations in SDHC cause autosomal dominant paraganglioma, type 3, *Nat Genet* 26: 268-270, 2000.
16. Astuti, D., Latif, F., Dallol, A., Dahia, P. L., Douglas, F., George, E., Skoldberg, F., Husebye, E. S., Eng, C., and Maher, E. R. Gene mutations in the succinate dehydrogenase subunit SDHB cause susceptibility to familial pheochromocytoma and to familial paraganglioma, *Am J Hum Genet* 69: 49-54, 2001.
17. Bot, F. J., Sleddens, H. F., and Dinjens, W. N. Molecular assessment of clonality leads to the identification of a new germ line TP53 mutation associated with malignant cystosarcoma phyllodes and soft tissue sarcoma, *Diagn Mol Pathol* 7: 295-301, 1998.
18. Taschner, P. E., Jansen, J. C., Baysal, B. E., Bosch, A., Rosenberg, E. H., Brocker-Vriends, A. H., van Der Mey, A. G., van Ommen, G. J., Cornelisse, C. J., and Devilee, P. Nearly all hereditary paragangliomas in the Netherlands are caused by two founder mutations in the SDHD gene, *Genes Chromosomes Cancer* 31: 274-281, 2001.
19. van Schothorst, E. M., Jansen, J. C., Grooters, E., Prins, D. E., Wiersinga, J. J., van der Mey, A. G., van Ommen, G. J., Devilee, P., and Cornelisse, C. J. Founder effect at PGL1 in hereditary head and neck paraganglioma families from the Netherlands, *Am J Hum Genet* 63: 468-473, 1998.
20. Sheffield, V. C., Beck, J. S., Kwitek, A. E., Sandstrom, D. W., and Stone, E. M. The sensitivity of single-strand conformation polymorphism analysis for the detection of single base substitutions, *Genomics* 16: 325-332, 1993.

21. Wiench, M., Wygoda, Z., Gubala, E., Wloch, J., Lisowska, K., Krassowski, J., Scieglinska, D., Fiszer-Kierzkowska, A., Lange, D., Kula, D., Zeman, M., Roskosz, J., Kukulska, A., Krawczyk, Z., and Jarzab, B. Estimation of risk of inherited medullary thyroid carcinoma in apparent sporadic patients, *J Clin Oncol* 19: 1374-1380, 2001.

22. Oosterwijk, J. C., Jansen, J. C., van Schothorst, E. M., Oosterhof, A. W., Devilee, P., Bakker, E., Zoeteweij, M. W., and van der Mey, A. G. First experiences with genetic counselling based on predictive DNA diagnosis in hereditary glomus tumours (paragangliomas), *J Med Genet* 33: 379-383, 1996.

Chapter 8

p53 alterations and their relationship to *SDHD* mutations in parasympathetic paragangliomas

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Abstract

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

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

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

Keywords: paraganglioma; *p53*; MDM2; immunohistochemistry; PCR-SSCP

Introduction

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

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

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

The mitochondrial succinate dehydrogenase enzyme complex II is involved in the citric acid cycle and the aerobic respiratory chain.[22] A complete loss of complex II enzymatic activity, due to inactivating mutations in the *SDHB*, *SDHC*, or *SDHD* gene and loss of heterozygosity (LOH) of the corresponding wild type allele, leads to a high expression of hypoxic-angiogenic responsive genes like vascular endothelial growth factor (VEGF) and endothelial PAS domain protein 1 (EPAS1/HIF2α).[23,24] The fact that cellular hypoxia stimulates paraganglioma development is further suggested by a markedly increased incidence of carotid body paragangliomas in people living permanently under hypoxic conditions (at high altitude or due to chronic obstructive pulmonary disease).[25-27] Cellular stress such as DNA damage or hypoxia induces *p53*,[28] after which MDM2 is upregulated to serve as a negative feedback for *p53*. Induction of the tumor suppressor gene *p53* results in cell cycle arrest at the G0/G1 boundary, but when *p53* is mutated, control of cell proliferation is lost. Cells with mutated *p53* have a

growth advantage compared to the surrounding cells and this can contribute to tumor formation. Obviously, paraganglioma cells escape from hypoxia-induced cellular senescence. One of the mechanisms to circumvent the hypoxia-induced cellular senescence is the inactivation of p53. In numerous tumor types p53 inactivation is caused by mutation in the *p53* gene itself or by MDM2 overexpression.[29,30] The MDM2 protein targets p53 for proteasomal degradation and is as such involved in the perturbation of p53 function.[31,32] There is strong evidence that *p53* mutation and MDM2 overexpression are mutually exclusive in most tumors and represent two alternative mechanisms to inactivate suppression of cell growth.

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

Methods

Patients and tumor samples

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

DNA isolation

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

Table 1 Clinical Characteristics of 42 Paraganglioma Patients Evaluated for p53/MDM2 Alterations

Characteristic	All Patients (N=41)	Patients with SDHD Gene Mutation† (N=24)	Patients with Wildtype SDHD Gene† (N=17)	*P* Value *
Patient				
Sex - no. (%)				NS
Male	17	11 (65)	6 (35)	
Female	24	13 (52)	11 (46)	
Mean Age of Onset - yr (+ SE)	42.8 (+ 13.3)	41.2 (+ 13.1)	44.3 (+ 13.7)	NS
Mean Follow-up Time - months (range)	53 (1 - 218)	64 (5 - 136)	38 (1 - 218)	
Family History - no. (%)				< .0001
Positive	17	17 (71)		
Negative	24	7 (29)	17 (100)	
Tumor focality - no. (%)				.003
Single Paraganglioma	19	5 (25)	14 (74)	
Recurrence	*6*	*3*	*3*	
Bilateral Carotid Body	7	6 (86)	1 (14)	
Multiple	12	0 (83)	2 (17)	
With sympathoadrenal tumors	2	2 (100)		
Not known	1	1 (100)		

PCR-SSCP

Exons 5 to 8 of the *p53* gene, including the exon-intron boundaries, were investigated by PCR-SSCP. As controls, DNA samples from normal individuals were used. In addition, DNA from the prostate carcinoma cell lines PC-3 and Du-145, and the colorectal carcinoma cell lines Colo-320 and HT-29, with known *p53* mutations in exons 5, 6, 7 and 8, respectively, served as positive controls. The DNA isolated from routine formalin-fixed and paraffin-embedded tissues is highly degraded; therefore we used small amplicon (<200bp) PCR to investigate exons 5-8 of the *p53* gene. All 4 exons were amplified in 2 fragments each, as recently described.[36] PCR was performed in 15 µl reaction volume consisting of (per 50 µl): 1 Unit Taq DNA polymerase (Promega, Madison, WI, USA.), 1.5 mM $MgCl_2$, 200 ng of each primer, 0.2 mM dGTP, dTTP, dCTP, 0.02 mM dATP, 2.5µCi α-32P-dATP, and approximately 100 ng of DNA. Temperatures for amplification were 95 °C for 30 seconds, 55 °C for 45 seconds, and 72 °C for 45 seconds. These steps were repeated for 35 cycles followed by a final extension at 72 °C for 10 minutes. The PCR product was diluted with an equal amount of loading buffer (95% formamide, 10 mM EDTA pH 8.0, 0.025% bromophenol blue and 0.025% xylene cyanol) and denaturated at 95 °C for 5 minutes. 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 hours at room temperature. Gels were vacuum dried at 80 °C and exposed to X-ray films.

Immunohistochemistry

Five µm sections of paraffin-embedded parasympathetic paragangliomas were mounted onto amino-alkyl-silane (AAS)-coated slides and deparaffinized. Subsequently, test and control slides of each tumor were washed twice in 100 percent alcohol, incubated for 20 min in 3 percent H_2O_2 in methanol, and rinsed with tap water. A microwave antigen retrieval method (15 min in citrate buffer, pH 6, at 600 W) was used, followed by incubation for 15 min in 10 percent normal goat serum (Dako, Glostrup, Denmark). Do7 anti-p53 monoclonal antibody (Dako) was used at a dilution of 1:50 for 30 minutes at room temperature and the MDM2 monoclonal antibody 1B10 (Novocastra laboratories, Newcastle upon Tyne, UK) was used at a dilution of 1:25 for 30 minutes at room temperature, both followed by biotinylated goat-anti-multilink and streptavidin-biotin peroxidase complex (both undiluted; Lab Vision Corporation, Fremont, CA, USA). Visualization was achieved by diaminobenzidine tetrahydrochloride (Fluka, Neu-Ulm, Germany) with 3 percent H_2O_2 for 7 min.

Negative controls of the tumors were prepared by omission of the dilution buffer (phosphate-buffered saline with 5 per cent bovine serum albumin). A *p53*-positive esophageal adenocarcinoma and an MDM2-positive breast carcinoma were used as positive controls.

Staining of p53 and MDM2 was assessed according to the method described by Sinicrope *et al*.[37] This method is based on the percentage of positive tumor cells and the staining intensity. A score of 0 to 4 was assigned according to the percentage of positively stained tumor cells: 0 = positive staining in < 5%; 1 = >5 - 25%; 2 = >25 - 50%; 3 = >50 - 75% and 4 = >75%. These results are multiplied by the staining intensity score of the tumor cells: 1 = negative - weak; 2 = moderate and 3 = strong staining. A multiplied score of 6 or more is regarded as positive staining and a score below 6 as negative.

Statistics

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

Results

PCR-SSCP analysis

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

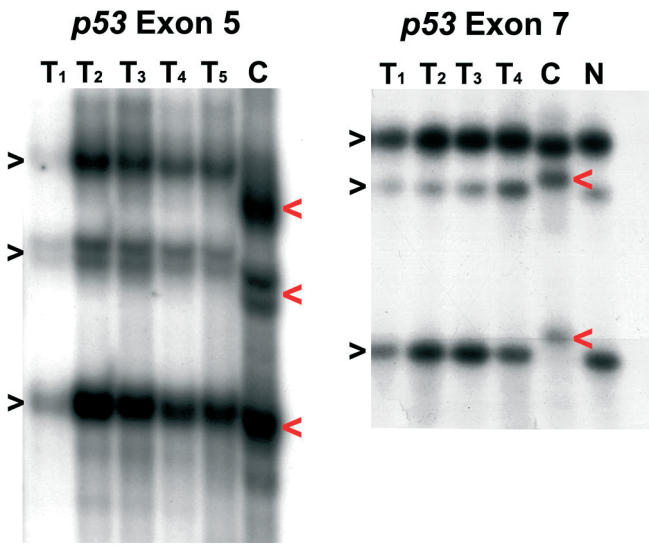

Figure 1 Examples of SSCP analysis of p53 exon 5 and exon 7 in parasympathetic paragangliomas. The autoradiographs of the PCR-SSCP gel show the migration patterns of tumor (T) and normal (N) DNA and the mobility shifts (red arrowheads) produced by aberrant control samples (C) of the positive controls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

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

Of 43 paragangliomas, p53 immunoreactivity was detected in 15 tumors (35%) of 13 patients, whereas no p53 or MDM2 immnopositivity was detected in the control sections. Three tumors (7%) from different patients showed concurrent MDM2 expression, leaving the majority (n=28, 65%) of the tumors negative for both p53 and MDM2. Immunoreactivity of p53 and MDM2 was observed both in the nucleus and the cytoplasm. Also, p53 positivity was observed in tumor and stromal cells in all these cases. Figure 2 shows examples of positive and negative staining of p53 and MDM2.

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

Because hypoxia is known to be present in *SDHD*-mutated paraganglionic cells and hypoxia is known to stimulate *p53* transcription, leading to cell cycle arrest and apoptosis, abrogation of the *p53* pathway could especially be expected in *SDHD*-mutated paragangliomas. However, p53 positivity was present in 6 of 25 (24%) tumors with an *SDHD* mutation and 9

of 18 (50%) tumors without SDHD mutations were positive for p53. Similarly, MDM2 positive staining was found in 1 patient with an *SDHD* mutation.

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

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

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

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

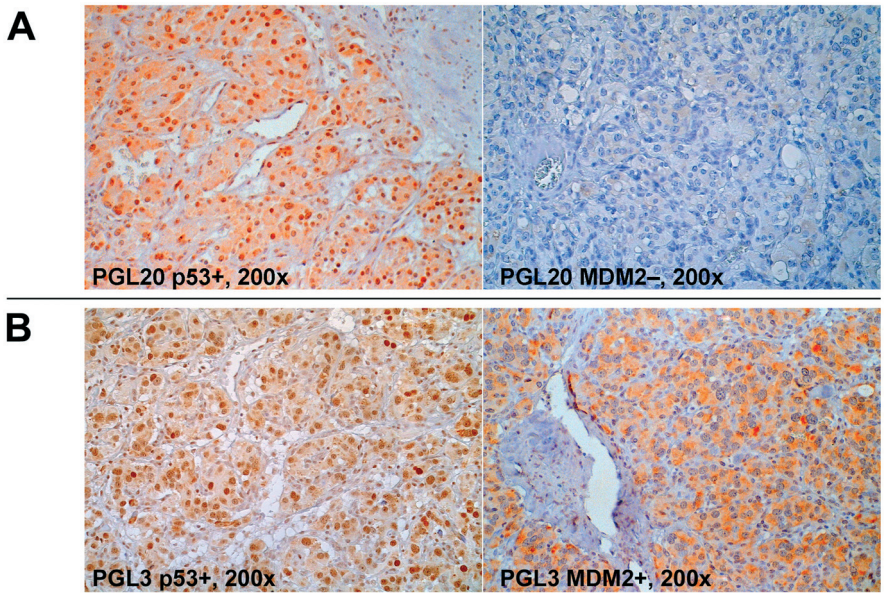

Figure 2 Immunohistochemical staining of p53 and MDM2 in parasympathetic paragangliomas using the anti-p53 monoclonal antibody Do7 and MDM2 monoclonal antibody 1B10, respectively.
Panel A: shows positive p53 staining (left) of tumor and stromal cells in PGL20, a mediastinal paraganglioma of a patient with a negative family history and no germline *SDHD* mutation. MDM2 expression is absent in PGL20 (right).
Panel B: PGL3, a vagal paraganglioma of a patient with multiple paragangliomas, a positive family history and a germline D92Y SDHD mutation. Tumor and stromal cells stain positive for *p53*, whereas MDM2 staining is mainly present in the tumor cells. Note the nuclear and cytoplasmic staining of p53 in both paragangliomas, and the cytoplasmic presence of MDM2 in the tumor cells of PGL3.

Discussion

Experimental and observational evidence indicates that chronic hypoxic stimulation is involved in the tumorigenesis of paraganglioma. Hypoxia is a well-known inducer of *p53*, which in turn results in cell cycle arrest or apoptosis, a mechanism that is abrogated in most cancers. The present study aimed to investigate the involvement of *p53* in the development of parasympathetic paragangliomas with or without *SDHD* mutations, using immunohisto-chemical assessment of p53 and MDM2 expression, and mutation analysis of *p53* exon.[5-8]

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

Under normal conditions, the p53 concentration in cells is low and cannot be detected by immunohistochemistry. By cellular stress the concentration of p53 can rise, and hence be detected by immunohistochemistry.[40,41] In addition, mutant p53 has often a longer half-life than wild type p53 and can be detected immunohistochemically.[42,43] However, there is no direct correlation between *p53* mutation and immunohistochemical p53 overexpression.[32,44] The immunohistochemical detection of p53 expression in 15 paragangliomas indicates increased wild type p53 expression or the presence of mutant p53. However, no aberrations in exons 5-8 of the *p53* gene were found by PCR-SSCP. It is known from the literature that more than 95% of *p53* mutations are found in exons 5-8,[29] but we cannot exclude the presence of mutations outside this region. In addition, the mutation detection efficiency of PCR-SSCP is not 100% and mutations could remain undetected, although all 4 different control *p53* mutations were identified by the procedure used. Despite this, we consider our molecular results as strong indication that *p53* mutations do not contribute to paraganglioma tumorigenesis. Moreover, the observation of p53 immunoreactivity in tumor and stromal cells suggests hypoxia rather than gene mutation as the cause of p53 expression. Inactivation of *p53* in tumors is often the result of the combination of a mutant *p53* allele and 17p allele loss. In several molecular studies no 17p loss in paragangliomas has been found.[14,45] This is in accordance with the observed absence of *p53* mutations in these tumors. A recent investigation has shown that the increase in p53 during hypoxia is not accompanied by a parallel rise in MDM2.[40] If p53 is active in the p53-expressing paragangliomas this implies that the tumorigenic mechanism in these tumors overrules the tumor suppressor capacity of wild type p53. In accordance with this concept paragangliomas are very slowly growing tumors.

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

In 28 (65%) of the investigated paragangliomas, besides the absence of *p53* mutations, no p53 expression was detected. This could point to a *p53*-independent tumorigenic pathway. Nineteen of these 28 tumors have an *SDHD* gene mutation resulting in cellular hypoxia. Obviously, hypoxia in these tumors does not lead to p53 upregulation. However, there are more ways to perturb the *p53* pathway during tumor development in addition to the commonly seen *p53* gene mutations or MDM2 overexpression. These include loss of the ability to stabilize p53, through mechanisms such as loss of ARF or inactivation of kinases, inappropriate localization of p53, and inactivation of downstream mediators of p53 such as Apaf-1 or Bax.[52,53] Many cancers with wild type *p53* show loss of the p14ARF protein resulting in destabilization of p53.[54] This loss

is often the result of p14ARF locus deletion, but in paragangliomas loss of chromosomal region 9p has not been observed.[14,45] Also, in a case report of 2 brothers with paraganglioma neither allele loss nor mutations in *p53* and the 9p gene p16INK4A were found. More than 8 years after radiotherapy a recurrence appeared to have a *p53* as well as a p16INK4A mutation and the authors suggest that these mutations may have resulted from the therapy.[55]

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

References

1. Lee JH, Barich F, Karnell LH*, et al.* National Cancer Data Base report on malignant paragangliomas of the head and neck. *Cancer* 2002; 94**:** 730-737.
2. Grufferman S, Gillman MW, Pasternak LR, Peterson CL and Young WG. Familial carotid body tumors: case report and epidemiologic review. *Cancer* 1980; 46**:** 2116-2122.
3. Hodge KM, Byers RM and Peters LJ. Paragangliomas of the head and neck. *Arch Otolaryngol Head Neck Surg* 1988; 114**:** 872-877.
4. van Baars F, Cremers C, van den Broek P, Geerts S and Veldman J. Genetic aspects of nonchromaffin paraganglioma. *Hum Genet* 1982; 60**:** 305-309.
5. Baysal BE, Willett-Brozick JE, Lawrence EC*, et al.* Prevalence of SDHB, SDHC, and SDHD germline mutations in clinic patients with head and neck paragangliomas. *J Med Genet* 2002; 39**:** 178-183.
6. Dannenberg H, Dinjens WN, Abbou M*, et al.* Frequent germ-line succinate dehydrogenase subunit D gene mutations in patients with apparently sporadic parasympathetic paraganglioma. *Clin Cancer Res* 2002; 8**:** 2061-2066.
7. Baysal BE, Ferrell RE, Willett-Brozick JE*, et al.* Mutations in SDHD, a mitochondrial complex II gene, in hereditary paraganglioma. *Science* 2000; 287**:** 848-851.
8. Astuti D, Latif F, Dallol A*, et al.* Gene mutations in the succinate dehydrogenase subunit SDHB cause susceptibility to familial pheochromocytoma and to familial paraganglioma. *Am J Hum Genet* 2001; 69**:** 49-54.
9. Niemann S and Muller U. Mutations in SDHC cause autosomal dominant paraganglioma, type 3. *Nat Genet* 2000; 26: 268-270.
10. Jensen JC, Choyke PL, Rosenfeld M*, et al.* A report of familial carotid body tumors and multiple extra-adrenal pheochromocytomas. *J Urol* 1991; 145: 1040-1042.
11. Pritchett JW. Familial concurrence of carotid body tumor and pheochromocytoma. *Cancer* 1982; 49: 2578-2579.
12. DeAngelis LM, Kelleher MB, Post KD and Fetell MR. Multiple paragangliomas in neurofibromatosis: a new neuroendocrine neoplasia. *Neurology* 1987; 37: 129-133.
13. Carney JA. Gastric stromal sarcoma, pulmonary chondroma, and extra-adrenal paraganglioma (Carney Triad): natural history, adrenocortical component, and possible familial occurrence. *Mayo Clin Proc* 1999; 74: 543-552.

14. Dannenberg H, de Krijger RR, Zhao J*, et al.* Differential loss of chromosome 11q in familial and sporadic parasympathetic paragangliomas detected by comparative genomic hybridization. *Am J Pathol* 2001; 158: 1937-1942.
15. Barnes L and Taylor SR. Vagal paragangliomas: a clinical, pathological, and DNA assessment. *Clin Otolaryngol* 1991; 16: 376-382.
16. Sauter ER, Hollier LH, Bolton JS, Ochsner JL and Sardi A. Prognostic value of DNA flow cytometry in paragangliomas of the carotid body. *J Surg Oncol* 1991; 46: 151-153.
17. van der Mey AG, Cornelisse CJ, Hermans J, Terpstra JL, Schmidt PH and Fleuren GJ. DNA flow cytometry of hereditary and sporadic paragangliomas (glomus tumours). *Br J Cancer* 1991; 63: 298-302.
18. Wang DG, Barros D'Sa AA, Johnston CF and Buchanan KD. Oncogene expression in carotid body tumors. *Cancer* 1996; 77: 2581-2587.
19. Jyung RW, LeClair EE, Bernat RA*, et al.* Expression of angiogenic growth factors in paragangliomas. *Laryngoscope* 2000; 110: 161-167.
20. Li SL, Goko H, Xu ZD*, et al.* Expression of insulin-like growth factor (IGF)-II in human prostate, breast, bladder, and paraganglioma tumors. *Cell Tissue Res* 1998; 291: 469-479.
21. Wang DG, Johnston CF, Barros D'Sa AA and Buchanan KD. Expression of apoptosis-suppressing gene bcl-2 in human carotid body tumours. *J Pathol* 1997; 183**:** 218-221.
22. Hirawake H, Taniwaki M, Tamura A, Amino H, Tomitsuka E and Kita K. Characterization of the human SDHD gene encoding the small subunit of cytochrome b (cybS) in mitochondrial succinate-ubiquinone oxidoreductase. *Biochim Biophys Acta* 1999; 1412: 295-300.
23. Gimenez-Roqueplo AP, Favier J, Rustin P*, et al.* The R22X mutation of the SDHD gene in hereditary paraganglioma abolishes the enzymatic activity of complex II in the mitochondrial respiratory chain and activates the hypoxia pathway. *Am J Hum Genet* 2001; 69: 1186-1197.
24. Gimenez-Roqueplo AP, Favier J, Rustin P*, et al.* Functional consequences of a SDHB gene mutation in an apparently sporadic pheochromocytoma. *J Clin Endocrinol Metab* 2002; 87: 4771-4774.
25. Saldana MJ, Salem LE and Travezan R. High altitude hypoxia and chemodectomas. *Hum Pathol* 1973; 4: 251-263.
26. Pacheco-Ojeda L, Durango E, Rodriquez C and Vivar N. Carotid body tumors at high altitudes: Quito, Ecuador, 1987. *World J Surg* 1988; 12: 856-860.
27. Chedid A and Jao W. Hereditary tumors of the carotid bodies and chronic obstructive pulmonary disease. *Cancer* 1974; 33: 1635-1641.
28. Greenblatt MS, Bennett WP, Hollstein M and Harris CC. Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Res* 1994; 54: 4855-4878.
29. Hollstein M, Sidransky D, Vogelstein B and Harris CC. p53 mutations in human cancers. *Science* 1991; 253: 49-53.
30. Deb SP. Function and dysfunction of the human oncoprotein MDM2. *Front Biosci* 2002; 7: d235-243.
31. Kubbutat MH, Jones SN and Vousden KH. Regulation of p53 stability by Mdm2. *Nature* 1997; 387: 299-303.
32. Haupt Y, Maya R, Kazaz A and Oren M. Mdm2 promotes the rapid degradation of p53. *Nature* 1997*; 387: 296-299*.
33. Lam KY, Lo CY, Wat NM, Luk JM and Lam KS. The clinicopathological features and importance of p53, Rb, and mdm2 expression in phaeochromocytomas and paragangliomas. *J Clin Pathol* 2001; 54: 443-448.
34. Welkoborsky HJ, Gosepath J, Jacob R, Mann WJ and Amedee RG. Biologic characteristics of paragangliomas of the nasal cavity and paranasal sinuses. *Am J Rhinol* 2000; 14: 419-426.
35. Cheng L, Leibovich BC, Cheville JC*, et al.* Paraganglioma of the urinary bladder: can biologic potential be predicted? *Cancer* 2000; 88: 844-852.

36. van der Sijp JR, van Meerbeeck JP, Maat AP*, et al.* Determination of the molecular relationship between multiple tumors within one patient is of clinical importance. *J Clin Oncol* 2002; 20: 1105-1114.
37. Sinicrope FA, Ruan SB, Cleary KR, Stephens LC, Lee JJ and Levin B. bcl-2 and p53 oncoprotein expression during colorectal tumorigenesis *Cancer Res* 1995; 55: 237-241.
38. Elkablawy MA, Maxwell P, Williamson K, Anderson N and Hamilton PW. Apoptosis and cell-cycle regulatory proteins in colorectal carcinoma: relationship to tumour stage and patient survival. *J Pathol* 2001; 194: 436-443.
39. McCann AH, Kirley A, Carney DN*, et al.* Amplification of the MDM2 gene in human breast cancer and its association with MDM2 and p53 protein status. *Br J Cancer* 1995; 71: 981-985.
40. Koumenis C, Alarcon R, Hammond E*, et al.* Regulation of p53 by hypoxia: dissociation of transcriptional repression and apoptosis from p53-dependent transactivation. *Mol Cell Biol* 2001; 21: 1297-1310.
41. Ashcroft M, Taya Y and Vousden KH. Stress signals utilize multiple pathways to stabilize p53. *Mol Cell Biol* 2000; 20: 3224-3233.
42. Baas IO, Mulder JW, Offerhaus GJ, Vogelstein B and Hamilton SR. An evaluation of six antibodies for immunohistochemistry of mutant p53 gene product in archival colorectal neoplasms. *J Pathol* 1994; 172: 5-12.
43. Gannon JV, Greaves R, Iggo R and Lane DP. Activating mutations in p53 produce a common conformational effect. A monoclonal antibody specific for the mutant form. *Embo J* 1990; 9: 1595-1602.
44. Battifora H. p53 immunohistochemistry: a word of caution. *Hum Pathol* 1994; 25: 435-437.
45. Devilee P, van Schothorst EM, Bardoel AF*, et al.* Allelotype of head and neck paragangliomas: allelic imbalance is confined to the long arm of chromosome 11, the site of the predisposing locus PGL. *Genes Chromosomes Cancer* 1994; 11: 71-78.
46. Lianes P, Orlow I, Zhang ZF*, et al.* Altered patterns of MDM2 and TP53 expression in human bladder cancer. *J Natl Cancer Inst* 1994; 86: 1325-1330.
47. Zhou M, Yeager AM, Smith SD and Findley HW. Overexpression of the MDM2 gene by childhood acute lymphoblastic leukemia cells expressing the wild-type p53 gene. *Blood* 1995; 85: 1608-1614.
48. Riou G, Barrois M, Prost S, Terrier MJ, Theodore C and Levine AJ. The p53 and mdm-2 genes in human testicular germ-cell tumors. *Mol Carcinog* 1995; 12: 124-131.
49. Pruneri G, Pignataro L, Carboni N*, et al.* MDM-2 oncoprotein overexpression in laryngeal squamous cell carcinoma: association with wild-type p53 accumulation. *Mod Pathol* 1997; 10: 785-792.
50. Soslow RA, Altorki NK, Yang G, Xie D and Yang CS. mdm-2 expression correlates with wild-type p53 status in esophageal adenocarcinoma. *Mod Pathol* 1999; 12: 580-586.
51. Pise-Masison CA, Radonovich M, Sakaguchi K, Appella E and Brady JN. Phosphorylation of p53: a novel pathway for p53 inactivation in human T-cell lymphotropic virus type 1-transformed cells. *J Virol* 1998; 72: 6348-6355.
52. Soengas MS, Capodieci P, Polsky D*, et al.* Inactivation of the apoptosis effector Apaf-1 in malignant melanoma. *Nature* 2001; 409: 207-211.
53. Rampino N, Yamamoto H, Ionov Y*, et al.* Somatic frameshift mutations in the BAX gene in colon cancers of the microsatellite mutator phenotype. *Science* 1997; 275: 967-969.
54. Sherr CJ and Weber JD. The ARF/p53 pathway. *Curr Opin Genet Dev* 2000; 10: 94-99.
55. Guran S and Tali ET. p53 and p16INK4A mutations during the progression of glomus tumor. *Pathol Oncol Res* 1999; 5: 41-45.

Chapter 9

State of the Art
Discussion and Perspectives

Adapted from:

Dannenberg H, Komminoth P, Dinjens WNM, Speel EJM, de Krijger RR. Molecular genetic alterations in adrenal and extra-adrenal pheochromocytoma and paraganglioma. *Endocrine Pathology 14(4):329-250, 2003*

Many studies have aimed to identify the key genetic events involved in the pathogenesis of pheochromocytoma (PCC) and paraganglioma (PGL), but a comprehensive review of these data is lacking. Recent findings of CGH and LOH studies have provided new data of substantial interest in this respect. This concluding chapter aims to present and discuss a comprehensive overview of the currently available data concerning the molecular pathogenesis of PCC and PGL.

Generally, three approaches have been used in the study of the molecular pathogenesis of sporadic PCC and PGL. These include the investigation of the genes involved in the inherited tumors (chapters 2, 3, and 7), the search for important secondary genetic events (chapters 4, 5, 6, and 8), and the search for markers of malignancy (chapters 2, and 4).

The Hereditary Perspective

RET

RET is especially expressed in all neural-crest derived tissues, and is known to be involved in kidney and enteric nervous system development.[1-3] As a receptor tyrosine kinase, RET activates a variety of intracellular signaling pathways, including the RAS/ERK, phosphatidylinositol 3-kinase (PI3K)/AKT, and phospholipase C pathways (Figure 2; reviewed in several recent surveys[4-6]). However, the tissue-specific activation of these pathways is currently unclear.

Somatic mutations of *RET* are observed in 15% of benign adrenal PCC but in only 3% of the malignant tumors; mutations of RET-ligands are very rare.[7-19] Together with the observation of hyperplastic stages in nearly all MEN2-related PCCs and their remarkably benign behavior, this suggests that RET overactivation confers a growth advantage to cells of the adrenal medulla, but does not seem to be necessary for the further progression of these tumors. Interestingly, overexpression of RET is observed in the majority (50-70%) of sporadic PCCs that do not harbor somatic mutations, amplifications, or rearrangements of RET. This indicates that RET has a significant role in the tumorigenesis of most, if not all, sporadic PCCs, either by autocrine or paracrine stimulation.[1,20-22] No doubt, a more precise understanding of the molecular basis of RET signaling in pheochromocytes will contribute to the clarification of the role of RET over expression in PCC tumorigenesis.

Most extra-adrenal PCC and PGL do express RET, as would be expected on the basis of their common embryological origin.[21,23] However, it appears unlikely that RET overexpression or mutations occur in these tumors.

VHL

Somatic *VHL* mutations are present in a small subset (2-4%) of sporadic PCCs13, 24, 25, and are found with slightly increased frequency (17%) in malignant examples.[26] The mutations

are usually accompanied by loss of the wild-type allele, indicating biallelic inactivation. The observed VHL protein (pVHL) immunoreactivity in such tumors, points towards the presence of mutant pVHL in the cytoplasm.[27] pVHL immunoreactivity is also commonly observed in tumors with LOH of 3p25 in the absence of mutations, indicating that *VHL* promoter hypermethylation, unlike in renal cell cancer, is uncommon in PCCs.[28,29] Altogether, these findings support the hypothesis that deregulation of pVHL, but without complete loss of its functions, can drive PCC tumorigenesis.

pVHL interacts in a tissue-specific manner with many cellular proteins and is involved in the regulation of angiogenesis and extracellular matrix formation, and plays a role in the cell cycle (Figure 2; reviewed in Ivan et al.30). One of the best-known functions of pVHL is its ability to downregulate hypoxia-inducible factors HIF1α and HIF2, thereby exerting its influence on angiogenesis. Abrogation of this function has been proven to play a role in renal cell carcinoma.31-33 However, pVHL mutants associated with PCC susceptibility (R64P, V84L, F119S, L188V) retain the ability to downregulate HIF and cyclin D1 but are defective in the promotion of fibronectin matrix assembly.[34,35] One study showed a role for pVHL in microtubule stabilization and provided evidence that only point mutations that predispose to haemangioblastoma and PCC (but not renal cell carcinoma) disrupt pVHL's microtubule-stabilizing function.[36] These studies raise the possibility that VHL-related PCCs, and a small subset of sporadic PCCs feature deregulation of microtubule stabilization or fibronectin matrix assembly, rather than deregulation of HIF or loss of pVHL-mediated suppression of cyclin D1. Further investigations of PCC cell lines should clarify this.

As discussed in chapter 2 of this thesis, an increased frequency of VHL mutations in malignant PCC as compared to benign PCC is difficult to explain regarding the usually benign behavior of VHL-related PCC. It needs to be pointed out that the difference in mutation frequency was not statistically significant and that no analogous studies have been performed to confirm such findings.

Despite the absence of *VHL* alterations in most sporadic PCCs, the pVHL signaling pathway might well be involved as upstream or downstream targets of pVHL may be affected.

To our knowledge, the VHL protein has never been studied in PGL, but aberrations are unlikely, since mutations have not been found (personal observations).

NF1

NF1 is transmitted with an autosomal dominant mode of inheritance, apparently as a result of a single loss-of-function allele of the *NF1* gene. The *NF1* tumor suppressor gene encodes a GTP-ase-activating protein, neurofibromin, which functions primarily as a *ras* inhibitor. Thus, loss of NF1 affects the same signaling pathway (RAS) as does overexpression of RET (Figure 1). Complete loss of neurofibromin has been demonstrated in all NF1-related PCCs, in 33-50% of MEN 2-related PCCs and in 25% of sporadic PCCs.[37,38] NF1 alterations have not been

studied in PGL, and there are no clear indications that NF1 is involved in the pathogenesis of these tumors.

SDHD/SDHC/SDHB

The genetic cause of familial PGL [39-41] was identified only recently, when Baysal and colleagues identified germline mutations in the succinate dehydrogenase subunit D (*SDHD*) gene in five unrelated PGL families.[42] *SDHD* encodes one of the four nuclear-encoded polypeptides that form the mitochondrial complex II. The complex can be subdivided into two main components: a membrane-extrinsic catalytic component composed of a FAD-binding flavoprotein (SDHA) and an iron-sulfur protein (SDHB); and an hydrophobic component composed of the large (SDHC; cybL) and small (SDHD; cybS, membrane anchor protein) subunits of cytochrome b. In contrast to the other components of complex II, the function of cytochrome b is not completely understood.

Germline mutations in succinate dehydrogenase subunit A (*SDHA*) cause early-onset progressive neurodegeneration (Leigh syndrome), but mutations in *SDHB*, *SDHC*, and *SDHD* predispose to tumors of sympathetic and parasympathetic paraganglionic tissue (reviewed in Baysal et al.[43]). Germline mutations in one of these genes are found in virtually all familial PGL patients, in 8-35% of apparently sporadic PGL patients, and in almost all patients suffering both from PGL and PCC.[44-52] In contrast, patients with adrenal PCC rarely harbor germline SDH mutations and if such a mutation is found, these patients will also develop extra-adrenal PCC and/or PGL.[53-55] Of the patients with both PCC and PGL, those presenting with PGL are more likely to harbor an *SDHD* mutation and those presenting with PCC are more likely to have a germline *SDHB* mutation (Table 1 and 2). Furthermore, *SDHB* mutations cosegregate with a high risk for malignancy or recurrence.[54]

Somatic mutations of the SDH genes are rarely (if ever) found in PGL, PCC, or other neuroendocrine tumors.[48-50,56-59] Thus, involvement of complex II seems to be limited to PGL and a small subset of (extra-adrenal) PCCs, leaving unexplained the pathogenesis of about half of the truly sporadic PGLs.

The SDH enzyme complex II is involved in the citric acid cycle and the aerobic respiratory chain.[60, 61] Loss of complex II enzymatic activity in PCC and PGL is accompanied by a high expression of hypoxic-angiogenic responsive genes such as vascular endothelial growth factor (VEGF) and endothelial PAS domain protein 1 (EPAS1/HIF2α).[62,63] Together with the observation of increased PGL incidence in people living permanently under hypoxic conditions,[64] this suggests that hypoxia is of major importance in PGL tumorigenesis. However, a cause-effect relationship between the loss of complex II and the expression of hypoxic-angiogenic factors remains to be experimentally demonstrated. Also, additional functions of complex II may exist, analogous to cytochrome c, the release of which from the mitochondrial intermembrane space results in apoptosis.[65]

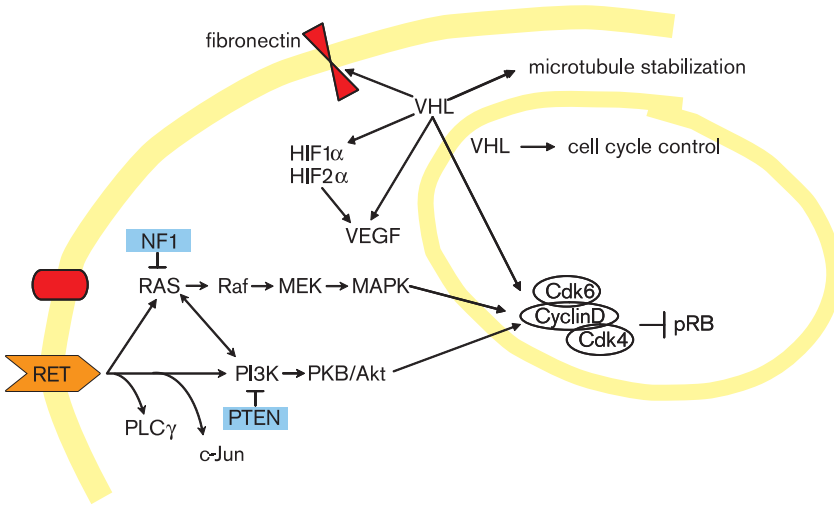

Figure 1 Intracellular signaling pathways mediated by RET, NF1 and VHL.

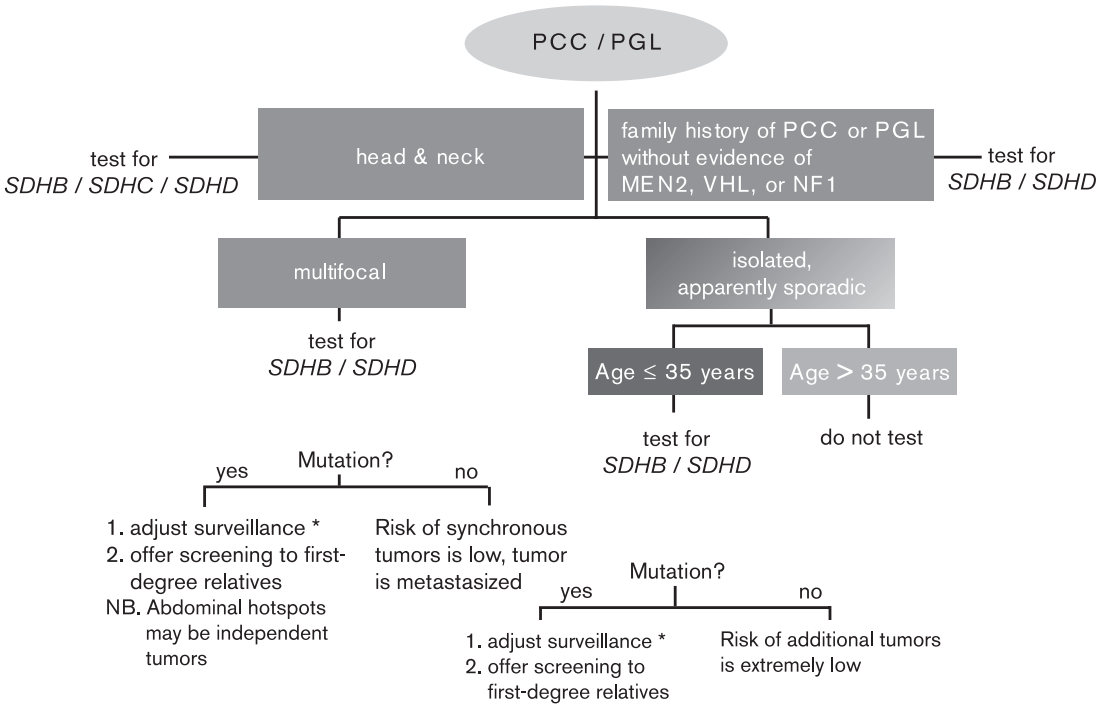

Figure 2 Decision tree for SDHD/SDHB genetic screening in PCC patients

* Surveillance should be supplemented with periodical ultrasonographic examination of the neck or cervical MRI for detection of PGLs. For the detection of extra-adrenal PCC, an MRI of paravertebral sympathetic chain is recommended. Alternatively, MIBG or octreotide scintigraphy can be performed.

Screening for mutation carriers

Screening for germline mutations in PCC or PGL patients and family members is clinically important since it improves early diagnosis of syndrome-related tumors. Indications for *VHL* and *RET* screening include (I) family history of VHL or MEN 2; (II) apparently sporadic multiple PCC, especially bilateral adrenal PCC; (III) solitary sporadic PCC in young patients (age < 50).[66] In the latter group, VHL mutations are detected in 3-11% of patients; 0-5% of patients carry *RET* mutations.[7,15, 53] Because *NF1* analysis is technically difficult, the diagnosis of NF1 remains (sufficiently) dependent on clinical features.

Based on current data (see also chapter 3, chapter 7) we propose a decision tree for the identification of SDH-related PCC and PGL (Chapter 3, Figure 2). Whereas all PGL patients are eligible for genetic screening of *SDHB*/*SDHC*/*SDHD*, specific subgroups of PCC patients should be considered for screening of *SDHB*/*SDHD*. These include patients presenting with (I) a family history of (extra-adrenal) PCC and/or PGL (50% harbor *SDHD* mutations); (II) multiple tumors (38% carry *SDHD* mutation); (III) solitary adrenal or extra-adrenal PCC and age ≤ 35 years (7% harbor *SDHD* mutations).[55]

Secondary Molecular Events

Loss of 17p and *p53* mutations

Loss of 17p might point to the involvement of *p53*, which is possibly the most commonly mutated gene in human cancer.[67] However, aberrant expression of p53 is observed only in ~8% of PCCs, with a slight increase in malignant ones, and mutations of *p53* are rare.[68-80] Interestingly, 2 of the 4 studies of Japanese and Chinese patients report a relatively high frequency of p53 mutations in PCCs, indicating that there may be differences related to geographical or ethnic factors.

In PGLs, which usually do not exhibit LOH of 17p, expression of p53 is more frequently observed, but again in the absence of *p53* mutations.[81] However, again, a study of PGLs of Japanese and Chinese patients revealed, over-expression of MDM2, but not of p53, suggesting geographical or ethnic influence on secondary genetic events.[73] Since hypoxia may induce p53 expression and *p53* mutations are rare, current data suggest that *p53* inactivation does not play a major role in the pathogenesis of PCC or PGL. Occasionally, *p53* inactivation may however feature in the pathogenesis and progression of PCC and PGL.[82]

Loss of Rb

The retinoblastoma (Rb) protein is an important suppressor of the cell cycle by binding to, and thus blocking the function of E2F transcription factors. A large number of anti-proliferative signals converge onto the Rb protein. Disruption of this cell cycle control mechanism is seen

in the majority of human cancers, often by abrogation of Rb function. Thusfar, only two studies investigated loss of the Rb gene product in PCCs.[73,76] By immunohistochemistry, loss of Rb expression was seen in 40-70% of adrenal PCCs, again slightly more often in malignant tumors. Extra-adrenal PCCs and PGLs have loss of Rb immunoreactivity in only 25% of cases, also more commonly (50%) in the malignant ones.

ras family

The *ras* genes encode guanine-nucleotide binding proteins that function in transduction of mitogenic signals from a variety of growth factor receptors, e.g. RET, EGFR. In about 25% of human tumors, ras proteins (h-, k-, and n-ras) are structurally altered, thereby constitutively releasing growth-stimulating signals. Although mitogenic ras signaling is involved in MEN2-, NF1-related, and sporadic PCCs, *ras* genes themselves are not mutated or rearranged in these tumors.[83-85]

myc family

The myc family constitutes a group of transcription factors, n-myc, c-myc, and l-myc. The *c-myc* oncogene is located on chromosome 8q24. As a transcription factor, binding of myc oncoprotein to DNA-specific regions of the genome regulates expression and activity of at least three major groups of genes: those controlling proliferation, differentiation, and apoptosis.[86] c-myc is constitutively expressed in normal adrenal medulla and in benign PCCs and PGLs, but increased *c-myc* mRNA levels have been observed in most malignant PCCs.[87-90] No amplifications or rearrangements of *n-myc*, *c-myc*, or *l-myc* have been found in PCCs.[89,91] Thus, a possible role, if any, for *c-myc* appears to be limited to the malignant progression of PCCs. Interestingly, n-myc (2p24.3) amplification occurs in a subgroup of neuroblastomas, tumors with a sympathoadrenal origin similar to PCCs. N-myc overexpression is a major determinant of the clinical course of neuroblastomas.[92, 93]

Telomerase

During normal somatic cell division, telomeres shorten progressively. This telomere shortening ultimately results in potent antiproliferative signaling and complete cessation of proliferative activity, and cells enter the senescent phase. In contrast, immortalized and carcinoma cells show no loss of telomere length during cell division. Telomerase is a ribonucleoprotein complex that synthesizes the repetitive TTAGGG sequence of telomeric DNA, thus maintaining telomere length and preventing the senescence-type of growth arrest. The increase in telomerase activity (TA) that accompanies most neoplastic and many preneoplastic conditions may permit the emergence of a population of immortalized cells, thereby facilitating the subsequent accumulation of genetic mutations. By using a PCR-based TRAP (telomeric repeat amplification protocol) assay, six studies show that increased levels of TA are present in 60% of malignant PCCs.

Detectable levels of telomerase reverse transcriptase (hTERT) mRNA were detected in 80% of malignant PCCs.94-100 In contrast, low or undetectable TA or hTERT was observed in the large majority of benign PCCs. Only 4/61 (7%) of apparently benign tumors showed increased TA levels, raising the possibility that these tumors had a malignant potential. Indeed, one of these tumors metastasized 47 months after initial therapy.[96]

Genome wide analyses

Comparative genomic hybridization (CGH), a genome-wide screening method to detect DNA copy number changes, revealed frequent losses of chromosomal region 1p and 3q in over 80% of sporadic and MEN2-related PCCs.[101,102] These deletions are usually accompanied by several other genomic alterations, such as losses of 17p or 13q and gains of 9q33-qter and 17q. In contrast, VHL-related PCCs showed a low frequency of 1p deletion (17%), but often exhibited loss of 3p (the VHL locus) accompanied by losses of (the short arm of) chromosome 11.[103]

The high frequency of 1p loss in MEN2A-related and sporadic PCC, but not in VHL-related PCC, indicates that inactivation of tumor suppressor loci on this chromosome arm is a specific genetic event in the molecular pathogenesis of these PCC. In order to narrow down the regions of interest and to identify candidate tumors suppressor genes, the short arm of chromosome 1 has been the subject of several studies. Several small regions of overlap (SRO) have been reported so far, including a telomeric region at 1p36 104-106, a more proximal region at 1p32 106,107, and a centromeric region at 1cen-1p13.[106] In aggregate, these data would suggest that 1p contains more than one critical PCC suppressor gene. Most studies found a common breakpoint at 1p32, indicating either a fragile site or the location of a PCC suppressor gene in this defined region.[101,102, 106-108]

However, one has to be careful in interpreting SROs, as this may be confounded by deficient LOH detection, genetic instability and intertumor heterogeneity or result from erroneous interpretation of the data.[109] It might be impossible to causally link an LOH event observed in a single tumor to the inactivation of one specific locus among several other loci. Accordingly, relevant genes may reside away from the SRO. This imposes an intrinsic limitation on the usefulness of mapping the extent of LOH events to delineate the location of the targeted gene.

So far, only the *SDHB* gene, mapped to 1p36.13 and encoding one of the four subunits of the mitochondrial complex II, is known to be involved in a small subset of PCC/PGL families.[49] Several other genes have been suggested as candidate PCC suppressor genes, including *p73* 110, *RIZ1* (1p36.21) 111, *TGFβR3* 112,113, *CDC14A* (1p21.2) 114,115, *SSBP3* and *BMP8* (1p34.2). Chapter 5 of this thesis discusses these candidate genes in more detail.

P18INK4C and *PTPRF/LAR* form two interesting candidate genes, as *p18INK4C* knockout mice develop PCC 116 and *PTPRF/LAR* is able to reverse RET overactivation.[117,118] However, our array-based CGH study suggests that at best, these two are involved in a subset of PCC as they usually reside outside the regions of overlap.

We found a correlation between malignancy and losses of 6q 101 or 8p (Speel EJM, manuscript in preparation). Also, a combination of 5p, 7p, and 12q gains was found in a considerable subset of malignant PCCs (Dannenberg et al.,[101] Speel EJM, manuscript in preparation). Thus, it appears that several independent molecular alterations can drive progression towards malignancy in PCC.

CGH studies have revealed a similar pattern of aberrations in adrenal and most extra-adrenal PCCs, but a different profile in PGLs and PCCs with SDH mutations. PGLs usually exhibit few chromosomal aberrations, and especially deletions of 1p and 3q (in combination) are uncommon. The only frequent aberration in PGL, loss of chromosome 11(q), occurs mainly in the familial tumors (86% *vs.* 22% in apparently sporadic PGL), which corresponds with the occurrence of mutations in *SDHD*.[119]

Further studies are needed to elucidate possible relations between the abovementioned chromosomal alterations and the gene expression patterns in benign and malignant PCCs and PGLs. Nevertheless, based on the literature, a picture emerges in which 3 major types of PCC/PGL tumorigenesis can be recognized, each with its specific chromosomal aberrations (Figure 3).

Together with current data on cancer gene expression studies, a model of (sporadic) PCC tumor initiation and progression can be proposed (Figure 4). In considering such a model, one must realize that distinct initiation and progression steps may not arise in a specific sequence. In addition, recent reports on expression profiling suggest that solid tumors may be malignant from the onset, rather than evolving from a benign neoplastic precursor lesion.[120-122] Cancer is also known to be the result of an accumulation of genetic abnormalities and a minimal set of acquired pathophysiological mechanisms is needed for a tumor cell to metastasize.[123] Genome-wide analyses in PCCs suggest such an accumulation, as malignant tumors tend to have more genomic aberrations than the clinically benign ones. Array technology and comparative studies on a well-defined set of PCC or PGL may shed more light on the progression of these tumors.

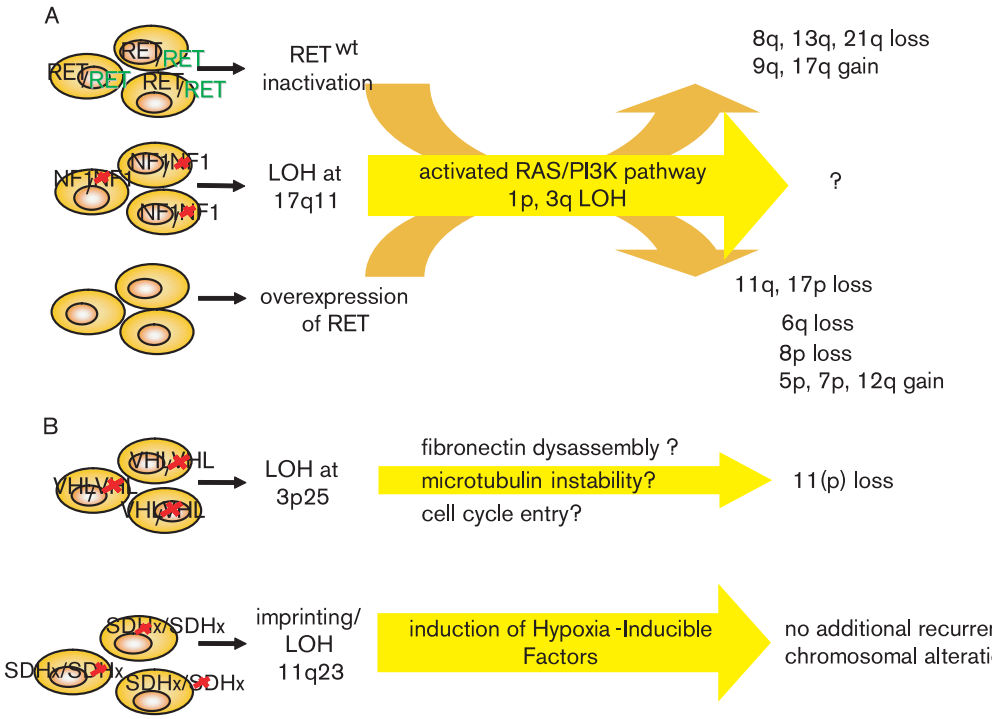

Figure 3 Major types of pheochromocytoma and paraganglioma, based on comparative genomic hybridization (CGH) studies. A) MEN2-, NF1- and sporadic PCCs develop via a similar pathogenetic mechanism: RAS/PI3K activation. These tumors share common losses of chromosomal regions 1p (>80%) and 3q (>50%), although the three tumor types also have differential aberrations. MEN2 tumors have more chromosomal changes than NF1 or sporadic tumors. B) VHL related PCCs develop via different a mitogenic pathway and exhibit low frequency of 1p losses (17%), but are associated with loss of 11p (86%). C) Tumors with mutations in SDH, whether cervically or abdominally located, only show recurrent loss of 11q; loss of 1p is seen when SDHB is involved. [Refs. 141-144 and Speel EJM et al., manuscript in preparation]

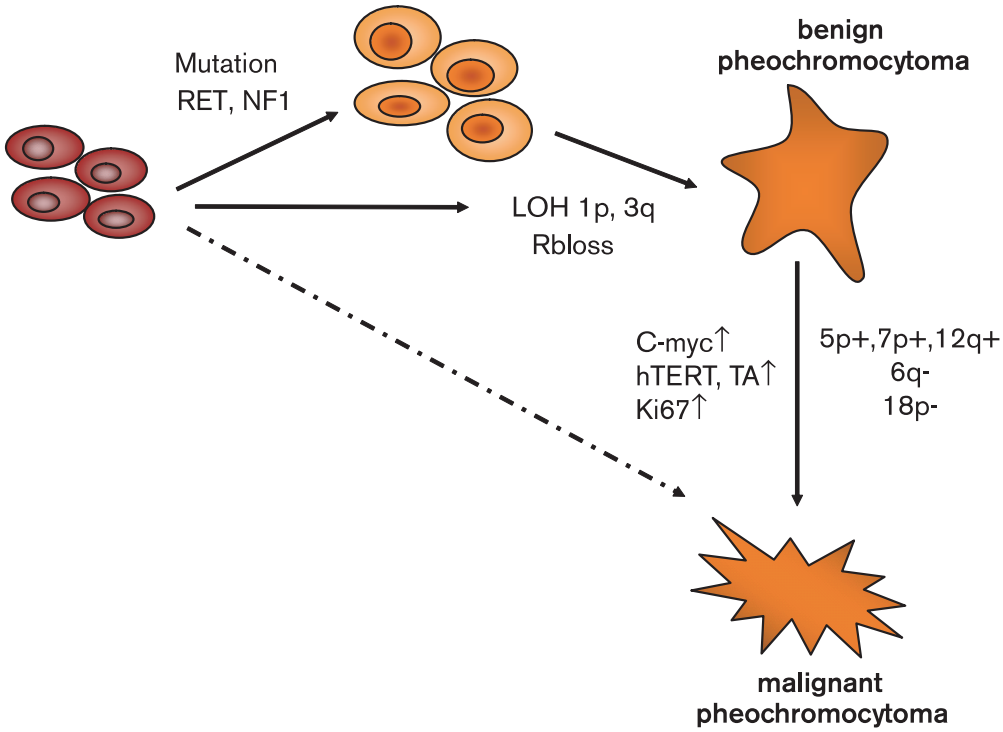

Figure 4 Proposed model of pheochromocytoma tumorigenesis. MEN2- and NF1-related are associated with adrenomedullary hyperplasia, which may also precede sporadic PCCs. Adrenomedullary hyperplasia arises from a genetic event (e.g. RET or NF1 mutation) that alters cell proliferation by RAS/PI3K activation. When accompanied by LOH of 1p and 3q, and other genetic events (e.g. loss of Rb expression), progression to pheochromocytoma occurs. The aguired capability of limitless replicative potential (hTERT↑), increased proliferation and oncogenic mutations (e.g. c-myc and/or oncogenes at 5p, 7p and 12q) is associated with malignancy. Distinct initiation and progression steps need not arise in a specific sequence. In addition, PCC cells may have malignant potential from the onset.

Markers of Malignancy

At present, the most promising markers of malignancy in PCC as well as PGL are Ki67/MIB-1, TA, and hTERT. Ki67/MIB-1 is an indicator of proliferation rate and correlates with tumor progression and malignancy in many tumors. At least 50% of malignant PCCs and PGLs have increased Ki67 index (> 2.5%), whereas the vast majority of clinically benign PCCs exhibit a Ki67 index of less than 1%. The combined analysis of Ki-67/MIB-1, hTERT and TA provides currently the most sensitive and specific tool to identify PCC patients that are at risk of developing recurrent or metastatic disease.[94,97,124]

On the other hand, the recently identified chromosomal regions 6q, 8p, 5p, 7p and 12q provide additional markers of progressive disease, but the responsible genes remain to be

identified (Figure 4). Interestingly, Gimenez-Roqueplo *et al.* found that mutations in the *SDHB* gene are associated with malignancy in PCC. 54 In our era of liquid nitrogen storage of tumor samples, standard analysis of Ki67/MIB1 and TA is to be strongly recommended.

Perspectives

The well characterized hereditary basis and the unique functional nature of PCC and PGL provides a useful framework that offers advantages for establishing the pathways of tumorigenesis and malignancy. Such findings may have relevance for understanding the basis of other more common malignancies where similar frameworks are not available. Reversely, comparative investigations of corresponding genomic aberrations in different types of neuroendocrine tumors will facilitate the identification of PCC/PGL target genes. For example, neuroblastoma, medullary thyroid carcinoma, and parathyroid tumors should be implicated in (LOH and mutational) studies to search for PCC suppressor genes on 1p.

According to Hanahan and Weinberg, the genetic pathways leading to cancer can be portrayed as six essential alterations in cell physiology: self-sufficiency in growth signals, insensitivity to anti-growth signals, sustained angiogenesis, limitless replicative potential, evading apoptosis, and tissue invasion and metastasis.[123] By applying our knowledge of PCC tumorigenesis to this concept, one realizes how much ongoing research is needed to elucidate important questions. For decades, researchers have cherished the notion that identification of the relevant pathways leading to PCC will provide tools for the development of new (drug-mediated) therapies targeted to specific pathways. In this respect, the identification of the mechanisms by which adrenomedullary and paranglionic chief cells acquire the six hallmarks of cancer provides such potential. The success of this will require well-designed and coordinated multi-center studies.[125]

Murine models

Genetically engineered mouse models have been used to study the mechanisms underlying the carcinogenesis of a wide variety of human cancers. Until recently, such a model has been lacking for PCC.[126-128] A considerable number of mouse and rat models, used for the study of elementary tumorigenic mechanisms, develop PCCs (Table 3).[129] Some animal knockout and transgenic models resemble hereditary syndrome-related PCC in humans,[130-132] and some of the animal models reveal new starting points for human PCC research. The identification of secondary genetic alterations in those murine PCCs may reveal aberrations in chromosomal regions syntenic to regions that are involved in human PCC, which may provide additional tools for the study of the pathogenetic mechanisms underlying PCC in man.[133,134]

Table 1 Overview of mouse models.

Genotype	Adrenal appearance in mice (%): hyperplasia	PCC	malignant PCC	Reference
$Rb^{+/-}$		71%		Nikitin AY *et al.*, 1999
	46%	–	–	Williams BO *et al.*, 1994
$Rb^{-/-}p130^{-/-}$		55%		Dannenberg JH *et al.*, 2004
$Rb^{+/-}E2F1^{-/-}$	95%			Yamasaki L *et al.*, 1998
$p18(Ink4c)^{-/-}$	33%	8%		
$p21(Waf1)^{-/-}$	–	–		
$p18(Ink4c)^{-/-}p21(Waf1)^{-/-}$	92%			
$p27(Kip1)^{-/-}$	19%	24%		
$p18(Ink4c)^{-/-}p27(Kip1)^{-/-}$	9%	91%	4%	Franklin DS *et al.*, 2000
$PTEN^{+/-}$	23%			Stambolic V *et al.*, 2000
		24%		You MJ *et al.*, 2002
$PTEN^{+/-}p27(Kip1)^{-/-}$		100%		Di Cristofano A *et al.*, 2001
$RET^{Met918Thr/WT}$	16%	2%		Smith-Hicks CL *et al.*, 2000
$RET^{Met918Thr/Met918Thr}$	100%	–		Sweetser DA *et al.*, 1999
$NF1^{+/-}$		20%		Powers JF *et al.*, 2000
c-mos (transgenic mice)	+	58%		Schultz N *et al.*, 1992

Micro array CGH and expression profiling

Over 1500 publications have described the use of CGH to analyze the patterns of nonrandom copy number alterations in cancer, but very few of the responsible genes have been identified. This is partly due to the limitations of current methods for mapping alterations. CGH analysis only detects large deletions (> 5Mb) and LOH analysis is often limited by inaccurately mapped, and insufficient numbers of markers. One of the applications that can facilitate fine mapping of genetic alterations is the high-resolution CGH or DNA array, which allows high resolution mapping of genetic alterations in chromosomal regions such as 1p, 3q, 8p, 5p, 7p, and 12q, and thus facilitates identification of minimal deleted regions and potential genes of interest.[135-138] Furthermore, our understanding of pathogenetic mechanisms in PCC and PGL tumorigenesis will increase significantly by gene expression profiling and the identification of pathways involved in their pathogenesis. Especially, the comparison of benign versus malignant tumors, primary versus metastatic lesions, or tumors that respond to palliative therapy with those that do not, will elucidate gene products and pathways involved in key aspects of their behavior.

Expression profiling wil provide enormous amounts of data that are often difficult to interpret. Effective analysis methods will be of utmost importance to select the genes of interest from the muddle of information. Also, the data should be screened for and compared with previously known pathways involved. As has been shown for other endocrine tumors 139, the

genes of interest may include hormones that modulate normal hormonal activity, or growth factors that are implicated in normal development of fetal adrenal glands and paraganglia (Table 4).

Table 2. Gene products involved in neural crest cell formation.

trunk neural crest formation:	BMP4, BMP7
emigration:	Slug, RhoB, N-Cadherin
migration promotors:	fibronectin, laminin, tenascin, collagens, proteoglycans, HNK-1
migration inhibitors:	ephrins
differentiation:	GAP-43, SCG10, NGF, IL-6, Activin A, GNDF, Phox2a, IGF2
sensory neurons -	neurogenin
sympathetic and parasympathetic neurons -	Mash-1
adrenergic to cholinergic conversion -	LIF
cholinergic neurons -	BMP2
glial differentiation -	GGF (neuregulin)
melanocytes -	endothelin 3
adrenomedullary -	glucocorticoides

Towards a new definition of PCC and PGL?

Future studies need to reveal the association of PCC and PGL in hereditary PGL caused by SDH mutations. We have shown that patients with such mutations very rarely develop adrenal PCC, but develop multiple extra-adrenal PCC and PGL. This raises the question whether SDH-related extra-adrenal PCC in fact may originate from cells with parasympathetic differentiation. Although the question may seem of academic interest only, the answer may have clinical implications regarding prognosis.[54] Preliminary CGH-based studies show that indeed, SDH-related PCC exhibit a PGL-like profile of chromosomal aberrations (Dinjens WNM, unpublished data).

Conclusions

From human and animal studies of PCC and PGL, a picture emerges with involvement of at least three different signaling pathways (Figure 3, 4). Activation of the RAS/PI3K and losses of 1p and 3q are of major importance in MEN2-related, NF1-related and sporadic PCC. VHL-related PCC develop via a different pathogenetic mechanism, unrelated to 1p loss, but related to alterations on chromosomes 3 (*VHL*) and 11.

The pathogenesis of adrenal and extra-adrenal tumors of the sympathetic paraganglionic system is different from parasympathetic PGLs, as is evident from different genetic predisposition and different genomic aberrations. In the latter, hypoxia induced by abrogation of mitochondrial complex II function or by living under constant hypoxic conditions is of major importance,

whereas complex II dysfunction is infrequently involved in PCC development. In contrast to PGL, only specific subgroups of PCC should be considered for SDH gene analysis. PCCs with SDH mutations may in fact originate from cells with parasympathetic differentiation.

To date, hTERT in combination with Ki67/MIB-1 and TA are the most reliable determinants of malignant behavior. Malignancy can be acquired through several mechanisms associated with loss of chromosomal region 6q or 18p, or gains of 5p, 7p, and 12q. Modern molecular techniques may help to elucidate mechanisms of PCC and PGL progression in order to identify more and better markers and to provide new therapeutic targets. The rarity of the tumor and the resulting fragmented nature of studies, typically involving small numbers of patients, represent limiting factors to the development of effective treatment and of markers of malignancy. Such development is being facilitated by the availability of new genomics-based tools, but ultimately, success of such approaches depends on studies involving large numbers of patients, stringently collected clinical data and tumor samples, and interdisciplinary collaborations among multiple centers.

References

1. Takaya K, Yoshimasa T, Arai H, Tamura N, Miyamoto Y, Itoh H, Nakao K: Expression of the RET proto-oncogene in normal human tissues, pheochromocytomas, and other tumors of neural crest origin, *J Mol Med* 1996, 74:617-621.
2. Nakamura T, Ishizaka Y, Nagao M, Hara M, Ishikawa T: Expression of the ret proto-oncogene product in human normal and neoplastic tissues of neural crest origin, *J Pathol* 1994, 172:255-260.
3. Schuchardt A, D'Agati V, Larsson-Blomberg L, Costantini F, Pachnis V: Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret, *Nature* 1994, 367:380-383.
4. van Weering DH, Bos JL: Signal transduction by the receptor tyrosine kinase Ret, *Recent Results Cancer Res* 1998, 154:271-281.
5. Takahashi M: The GDNF/RET signaling pathway and human diseases, *Cytokine Growth Factor Rev* 2001, 12:361-373.
6. Santoro M, Melillo RM, Carlomagno F, Fusco A, Vecchio G: Molecular mechanisms of RET activation in human cancer, *Ann N Y Acad Sci* 2002, 963:116-121.
7. Bar M, Friedman E, Jakobovitz O, Leibowitz G, Lerer I, Abeliovich D, Gross DJ: Sporadic phaeochromocytomas are rarely associated with germline mutations in the von Hippel-Lindau and RET genes, *Clin Endocrinol* (Oxf) 1997, 47:707-712.
8. Woodward ER, Eng C, McMahon R, Voutilainen R, Affara NA, Ponder BA, Maher ER: Genetic predisposition to phaeochromocytoma: analysis of candidate genes GDNF, RET and VHL, *Hum Mol Genet* 1997, 6:1051-1056.
9. Rodien P, Jeunemaitre X, Dumont C, Beldjord C, Plouin PF: Genetic alterations of the RET proto-oncogene in familial and sporadic pheochromocytomas, *Horm Res* 1997, 47:263-268.
10. Marsh DJ, Zheng Z, Arnold A, Andrew SD, Learoyd D, Frilling A, Komminoth P, Neumann HP, Ponder BA, Rollins BJ, Shapiro GI, Robinson BG, Mulligan LM, Eng C: Mutation analysis of glial cell line-derived neurotrophic factor, a ligand for an RET/coreceptor complex, in multiple endocrine neoplasia type 2 and sporadic neuroendocrine tumors, *J Clin Endocrinol Metab* 1997, 82:3025-3028.

11. Lindor NM, Honchel R, Khosla S, Thibodeau SN: Mutations in the RET protooncogene in sporadic pheochromocytomas, *J Clin Endocrinol Metab* 1995, 80:627-629.
12. Komminoth P, Roth J, Muletta-Feurer S, Saremaslani P, Seelentag WK, Heitz PU: RET proto-oncogene point mutations in sporadic neuroendocrine tumors, *J Clin Endocrinol Metab* 1996, 81:2041-2046.
13. Eng C, Crossey PA, Mulligan LM, Healey CS, Houghton C, Prowse A, Chew SL, Dahia PL, O'Riordan JL, Toledo SP, et al.: Mutations in the RET proto-oncogene and the von Hippel-Lindau disease tumour suppressor gene in sporadic and syndromic phaeochromocytomas, *J Med Genet* 1995, 32:934-937.
14. Dahia PL, Toledo SP, Mulligan LM, Maher ER, Grossman AB, Eng C: Mutation analysis of glial cell line-derived neurotrophic factor (GDNF), a ligand for the RET/GDNF receptor alpha complex, in sporadic phaeochromocytomas, *Cancer Res* 1997, 57:310-313.
15. Brauch H, Hoeppner W, Jahnig H, Wohl T, Engelhardt D, Spelsberg F, Ritter MM: Sporadic pheochromocytomas are rarely associated with germline mutations in the vhl tumor suppressor gene or the ret protooncogene, *J Clin Endocrinol Metab* 1997, 82:4101-4104.
16. Beldjord C, Desclaux-Arramond F, Raffin-Sanson M, Corvol JC, De Keyzer Y, Luton JP, Plouin PF, Bertagna X: The RET protooncogene in sporadic pheochromocytomas: frequent MEN 2-like mutations and new molecular defects, *J Clin Endocrinol Metab* 1995, 80:2063-2068.
17. van der Harst E, de Krijger RR, Bruining HA, Lamberts SW, Bonjer HJ, Dinjes WN, Proye C, Koper JW, Bosman FT, Roth J, Heitz PU, Komminoth P: Prognostic value of RET proto-oncogene point mutations in malignant and benign, sporadic phaeochromocytomas, *Int J Cancer* 1998, 79:537-540.
18. Yoshimoto K, Tanaka C, Hamaguchi S, Kimura T, Iwahana H, Miyauchi A, Itakura M: Tumor-specific mutations in the tyrosine kinase domain of the RET proto-oncogene in pheochromocytomas of sporadic type, *Endocr J* 1995, 42:265-270.
19. Eng C, Smith DP, Mulligan LM, Nagai MA, Healey CS, Ponder MA, Gardner E, Scheumann GF, Jackson CE, Tunnacliffe A, et al.: Point mutation within the tyrosine kinase domain of the RET proto-oncogene in multiple endocrine neoplasia type 2B and related sporadic tumours, *Hum Mol Genet* 1994, 3:237-241.
20. Le Hir H, Colucci-D'Amato LG, Charlet-Berguerand N, Plouin PF, Bertagna X, de Franciscis V, Thermes C: High levels of tyrosine phosphorylated proto-ret in sporadic phenochromocytomas, *Cancer Res* 2000, 60:1365-1370.
21. Edstrom E, Frisk T, Farnebo F, Hoog A, Backdahl M, Larsson C: Expression analysis of RET and the GDNF/GFRalpha-1 and NTN/GFRalpha-2 ligand complexes in pheochromocytomas and paragangliomas, *Int J Mol Med* 2000, 6:469-474.
22. Matias-Guiu X, Colomer A, Mato E, Cuatrecasas M, Komminoth P, Prat J, Wolfe H: Expression of the ret proto-oncogene in phaeochromocytoma. An in situ hybridization and northern blot study, *J Pathol* 1995, 176:63-68.
23. de Krijger RR, van der Harst E, Muletta-Feurer S, Bruining HA, Lamberts SW, Dinjens WN, Roth J, Heitz PU, Komminoth P: RET is expressed but not mutated in extra-adrenal paragangliomas, *J Pathol* 2000, 191:264-268.
24. Hofstra RM, Stelwagen T, Stulp RP, de Jong D, Hulsbeek M, Kamsteeg EJ, van den Berg A, Landsvater RM, Vermey A, Molenaar WM, Lips CJ, Buys CH: Extensive mutation scanning of RET in sporadic medullary thyroid carcinoma and of RET and VHL in sporadic pheochromocytoma reveals involvement of these genes in only a minority of cases, *J Clin Endocrinol Metab* 1996, 81:2881-2884.
25. Bender BU, Gutsche M, Glasker S, Muller B, Kirste G, Eng C, Neumann HP: Differential Genetic Alterations in von Hippel-Lindau Syndrome-Associated and Sporadic Pheochromocytomas, *J Clin Endocrinol Metab* 2000, 85:4568-4574.
26. Dannenberg H, De Krijger RR, Van Der Harst E, Abbou M, Y IJ, Komminoth P, Dinjens WN: Von Hippel-Lindau gene alterations in sporadic benign and malignant pheochromocytomas, *Int J Cancer* 2003, 105:190-195.

27. Los M, Jansen GH, Kaelin WG, Lips CJ, Blijham GH, Voest EE: Expression pattern of the von Hippel-Lindau protein in human tissues, *Lab Invest* 1996, 75:231-238.
28. Herman JG, Latif F, Weng Y, Lerman MI, Zbar B, Liu S, Samid D, Duan DS, Gnarra JR, Linehan WM, et al.: Silencing of the VHL tumor-suppressor gene by DNA methylation in renal carcinoma, *Proc Natl Acad Sci U S A* 1994, 91:9700-9704.
29. Clifford SC, Prowse AH, Affara NA, Buys CH, Maher ER: Inactivation of the von Hippel-Lindau (VHL) tumour suppressor gene and allelic losses at chromosome arm 3p in primary renal cell carcinoma: evidence for a VHL-independent pathway in clear cell renal tumourigenesis, *Genes Chromosomes Cancer* 1998, 22:200-209.
30. Ivan M, Kaelin WG, Jr.: The von Hippel-Lindau tumor suppressor protein, *Curr Opin Genet Dev* 2001, 11:27-34.
31. Gnarra JR, Tory K, Weng Y, Schmidt L, Wei MH, Li H, Latif F, Liu S, Chen F, Duh FM, et al.: Mutations of the VHL tumour suppressor gene in renal carcinoma, *Nat Genet* 1994, 7:85-90.
32. Turner KJ, Moore JW, Jones A, Taylor CF, Cuthbert-Heavens D, Han C, Leek RD, Gatter KC, Maxwell PH, Ratcliffe PJ, Cranston D, Harris AL: Expression of hypoxia-inducible factors in human renal cancer: relationship to angiogenesis and to the von Hippel-Lindau gene mutation, *Cancer Res* 2002, 62:2957-2961.
33. Clifford SC, Cockman ME, Smallwood AC, Mole DR, Woodward ER, Maxwell PH, Ratcliffe PJ, Maher ER: Contrasting effects on HIF-1alpha regulation by disease-causing pVHL mutations correlate with patterns of tumourigenesis in von Hippel-Lindau disease, *Hum Mol Genet* 2001, 10:1029-1038.
34. Hoffman MA, Ohh M, Yang H, Klco JM, Ivan M, Kaelin WG, Jr.: von Hippel-Lindau protein mutants linked to type 2C VHL disease preserve the ability to downregulate HIF, *Hum Mol Genet* 2001, 10:1019-1027.
35. Zatyka M, da Silva NF, Clifford SC, Morris MR, Wiesener MS, Eckardt KU, Houlston RS, Richards FM, Latif F, Maher ER: Identification of cyclin D1 and other novel targets for the von Hippel-Lindau tumor suppressor gene by expression array analysis and investigation of cyclin D1 genotype as a modifier in von Hippel-Lindau disease, *Cancer Res* 2002, 62:3803-3811.
36. Hergovich A, Lisztwan J, Barry R, Ballschmieter P, Krek W: Regulation of microtubule stability by the von Hippel-Lindau tumour suppressor protein pVHL, *Nat Cell Biol* 2003, 5:64-70.
37. Gutmann DH, Geist RT, Rose K, Wallin G, Moley JF: Loss of neurofibromatosis type I (NF1) gene expression in pheochromocytomas from patients without NF1, *Genes Chromosomes Cancer* 1995, 13:104-109.
38. Xu W, Mulligan LM, Ponder MA, Liu L, Smith BA, Mathew CG, Ponder BA: Loss of NF1 alleles in phaeochromocytomas from patients with type I neurofibromatosis, *Genes Chromosomes Cancer* 1992, 4:337-342.
39. Grufferman S, Gillman MW, Pasternak LR, Peterson CL, Young WG: Familial carotid body tumors: case report and epidemiologic review, *Cancer* 1980, 46:2116-2122.
40. Hodge KM, Byers RM, Peters LJ: Paragangliomas of the head and neck, *Arch Otolaryngol Head Neck Surg* 1988, 114:872-877.
41. van der Mey AG, Maaswinkel-Mooy PD, Cornelisse CJ, Schmidt PH, van de Kamp JJ: Genomic imprinting in hereditary glomus tumours: evidence for new genetic theory [see comments], *Lancet* 1989, 2:1291-1294.
42. Baysal BE, Ferrell RE, Willett-Brozick JE, Lawrence EC, Myssiorek D, Bosch A, van der Mey A, Taschner PE, Rubinstein WS, Myers EN, Richard CW, 3rd, Cornelisse CJ, Devilee P, Devlin B: Mutations in SDHD, a mitochondrial complex II gene, in hereditary paraganglioma, *Science* 2000, 287:848-851.
43. Baysal BE, Rubinstein WS, Taschner PE: Phenotypic dichotomy in mitochondrial complex II genetic disorders, *J Mol Med* 2001, 79:495-503.

44. Taschner PE, Jansen JC, Baysal BE, Bosch A, Rosenberg EH, Brocker-Vriends AH, van Der Mey AG, van Ommen GJ, Cornelisse CJ, Devilee P: Nearly all hereditary paragangliomas in the Netherlands are caused by two founder mutations in the SDHD gene, *Genes Chromosomes Cancer* 2001, 31:274-281.
45. Cascon A, Cebrian A, Ruiz-Llorente S, Telleria D, Benitez J, Robledo M: SDHB mutation analysis in familial and sporadic phaeochromocytoma identifies a novel mutation, J *Med Genet* 2002, 39:E64.
46. Astuti D, Douglas F, Lennard TW, Aligianis IA, Woodward ER, Evans DG, Eng C, Latif F, Maher ER: Germline SDHD mutation in familial phaeochromocytoma, *Lancet* 2001, 357:1181-1182.
47. Baysal BE, Willett-Brozick JE, Lawrence EC, Drovdlic CM, Savul SA, McLeod DR, Yee HA, Brackmann DE, Slattery WH, 3rd, Myers EN, Ferrell RE, Rubinstein WS: Prevalence of SDHB, SDHC, and SDHD germline mutations in clinic patients with head and neck paragangliomas, *J Med Genet* 2002, 39:178-183.
48. Dannenberg H, Dinjens WN, Abbou M, Van Urk H, Pauw BK, Mouwen D, Mooi WJ, de Krijger RR: Frequent germ-line succinate dehydrogenase subunit D gene mutations in patients with apparently sporadic parasympathetic paraganglioma, *Clin Cancer Res* 2002, 8:2061-2066.
49. Astuti D, Latif F, Dallol A, Dahia PL, Douglas F, George E, Skoldberg F, Husebye ES, Eng C, Maher ER: Gene mutations in the succinate dehydrogenase subunit SDHB cause susceptibility to familial pheochromocytoma and to familial paraganglioma, *Am J Hum Genet* 2001, 69:49-54.
50. Benn DE, Croxson MS, Tucker K, Bambach CP, Richardson AL, Delbridge L, Pullan PT, Hammond J, Marsh DJ, Robinson BG: Novel succinate dehydrogenase subunit B (SDHB) mutations in familial phaeochromocytomas and paragangliomas, but an absence of somatic SDHB mutations in sporadic phaeochromocytomas, *Oncogene* 2003, 22:1358-1364.
51. Cascon A, Ruiz-Llorente S, Cebrian A, Telleria D, Rivero JC, Diez JJ, Lopez-Ibarra PJ, Jaunsolo MA, Benitez J, Robledo M: Identification of novel SDHD mutations in patients with phaeochromocytoma and/or paraganglioma, Eur J Hum Genet 2002, 10:457-461.
52. Bauters C, Vantyghem MC, Leteurtre E, Odou MF, Mouton C, Porchet N, Wemeau JL, Proye C, Pigny P: Hereditary phaeochromocytomas and paragangliomas: a study of five susceptibility genes, *J Med Genet* 2003, 40:e75.
53. Neumann HP, Bausch B, McWhinney SR, Bender BU, Gimm O, Franke G, Schipper J, Klisch J, Altehoefer C, Zerres K, Januszewicz A, Eng C, Smith WM, Munk R, Manz T, Glaesker S, Apel TW, Treier M, Reineke M, Walz MK, Hoang-Vu C, Brauckhoff M, Klein-Franke A, Klose P, Schmidt H, Maier-Woelfle M, Peczkowska M, Szmigielski C: Germ-line mutations in nonsyndromic pheochromocytoma, *N Engl J Med* 2002, 346:1459-1466.
54. Gimenez-Roqueplo AP, Favier J, Rustin P, Rieubland C, Crespin M, Nau V, Khau Van Kien P, Corvol P, Plouin PF, Jeunemaitre X: Mutations in the SDHB gene are associated with extra-adrenal and/or malignant phaeochromocytomas, *Cancer Res* 2003, 63:5615-5621.
55. Dannenberg H, van Nederveen FH, Abbou M, Verhofstad AA, Komminoth P, de Krijger RR, Dinjens WNM: Clinical characteristics of pheochromocytoma patients with germline mutations in SDHD, *J Clin Oncol* 2004, accepted for publication.
56. Gimm O, Armanios M, Dziema H, Neumann HP, Eng C: Somatic and occult germ-line mutations in SDHD, a mitochondrial complex II gene, in nonfamilial pheochromocytoma, *Cancer Res* 2000, 60:6822-6825.
57. Aguiar RC, Cox G, Pomeroy SL, Dahia PL: Analysis of the SDHD gene, the susceptibility gene for familial paraganglioma syndrome (PGL1), in pheochromocytomas, *J Clin Endocrinol Metab* 2001, 86:2890-2894.
58. Kytola S, Nord B, Elder EE, Carling T, Kjellman M, Cedermark B, Juhlin C, Hoog A, Isola J, Larsson C: Alterations of the SDHD gene locus in midgut carcinoids, Merkel cell carcinomas, pheochromocytomas, and abdominal paragangliomas, *Genes Chromosomes Cancer* 2002, 34:325-332.

59. Perren A, Barghorn A, Schmid S, Saremaslani P, Roth J, Heitz PU, Komminoth P: Absence of somatic SDHD mutations in sporadic neuroendocrine tumors and detection of two germline variants in paraganglioma patients, *Oncogene* 2002, 21:7605-7608.
60. Ackrell BA: Progress in understanding structure-function relationships in respiratory chain complex II, FEBS Lett 2000, 466:1-5.
61. Hederstedt L: Structural biology. Complex II is complex too, *Science* 2003, 299:671-672.
62. Gimenez-Roqueplo AP, Favier J, Rustin P, Rieubland C, Kerlan V, Plouin PF, Rotig A, Jeunemaitre X: Functional consequences of a SDHB gene mutation in an apparently sporadic pheochromocytoma, *J Clin Endocrinol Metab* 2002, 87:4771-4774.
63. Gimenez-Roqueplo AP, Favier J, Rustin P, Mourad JJ, Plouin PF, Corvol P, Rotig A, Jeunemaitre X: The R22X mutation of the SDHD gene in hereditary paraganglioma abolishes the enzymatic activity of complex II in the mitochondrial respiratory chain and activates the hypoxia pathway, *Am J Hum Genet* 2001, 69:1186-1197.
64. Rodriguez-Cuevas S, Lopez-Garza J, Labastida-Almendaro S: Carotid body tumors in inhabitants of altitudes higher than 2000 meters above sea level, *Head Neck* 1998, 20:374-378.
65. Liu X, Kim CN, Yang J, Jemmerson R, Wang X: Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c, *Cell* 1996, 86:147-157.
66. Hes FJ, Lips CJ, van der Luijt RB: Molecular genetic aspects of Von Hippel-Lindau (VHL) disease and criteria for DNA analysis in subjects at risk, *Neth J Med* 2001, 59:235-243.
67. Khosla S, Patel VM, Hay ID, Schaid DJ, Grant CS, van Heerden JA, Thibodeau SN: Loss of heterozygosity suggests multiple genetic alterations in pheochromocytomas and medullary thyroid carcinomas, *J Clin Invest* 1991, 87:1691-1699.
68. Lin SR, Lee YJ, Tsai JH: Mutations of the p53 gene in human functional adrenal neoplasms, *J Clin Endocrinol Metab* 1994, 78:483-491.
69. Dahia PL, Aguiar RC, Tsanaclis AM, Bendit I, Bydlowski SP, Abelin NM, Toledo SP: Molecular and immunohistochemical analysis of P53 in phaeochromocytoma, *Br J Cancer* 1995, 72:1211-1213.
70. Reincke M, Wachenfeld C, Mora P, Thumser A, Jaursch-Hancke C, Abdelhamid S, Chrousos GP, Allolio B: p53 mutations in adrenal tumors: Caucasian patients do not show the exon 4 “hot spot” found in Taiwan, *J Clin Endocrinol Metab* 1996, 81:3636-3638.
71. Herfarth KK, Wick MR, Marshall HN, Gartner E, Lum S, Moley JF: Absence of TP53 alterations in pheochromocytomas and medullary thyroid carcinomas, *Genes Chromosomes Cancer* 1997, 20:24-29.
72. Yoshimoto T, Naruse M, Zeng Z, Nishikawa T, Kasajima T, Toma H, Yamamori S, Matsumoto H, Tanabe A, Naruse K, Demura H: The relatively high frequency of p53 gene mutations in multiple and malignant phaeochromocytomas, *J Endocrinol* 1998, 159:247-255.
73. Lam KY, Lo CY, Wat NM, Luk JM, Lam KS: The clinicopathological features and importance of p53, Rb, and mdm2 expression in phaeochromocytomas and paragangliomas, *J Clin Pathol* 2001, 54:443-448.
74. Yana I, Nakamura T, Shin E, Karakawa K, Kurahashi H, Kurita Y, Kobayashi T, Mori T, Nishisho I, Takai S: Inactivation of the p53 gene is not required for tumorigenesis of medullary thyroid carcinoma or pheochromocytoma, *Jpn J Cancer Res* 1992, 83:1113-1116.
75. de Krijger RR, van der Harst E, van der Ham F, Stijnen T, Dinjens WN, Koper JW, Bruining HA, Lamberts SW, Bosman FT: Prognostic value of p53, bcl-2, and c-erbB-2 protein expression in phaeochromocytomas, *J Pathol* 1999, 188:51-55.
76. Gupta D, Shidham V, Holden J, Layfield L: Prognostic value of immunohistochemical expression of topoisomerase alpha II, MIB-1, p53, E-cadherin, retinoblastoma gene protein product, and HER-2/neu in adrenal and extra-adrenal pheochromocytomas, *Appl Immunohistochem Mol Morphol* 2000, 8:267-274.

77. Adleff V, Racz K, Toth M, Varga I, Bezzegh A, Glaz E: p53 protein and its messenger ribonucleic acid in human adrenal tumors, *J Endocrinol Invest* 1998, 21:753-757.
78. Hagemeyer O, Gabius HJ, Kayser K: Paraganglioma of the lung--developed after exposure to nuclear radiation by the Tschernobyl atomic reactor accident? *Respiration* 1994, 61:236-239.
79. Cheng L, Leibovich BC, Cheville JC, Ramnani DM, Sebo TJ, Neumann RM, Nascimento AG, Zincke H, Bostwick DG: Paraganglioma of the urinary bladder: can biologic potential be predicted? *Cancer* 2000, 88:844-852.
80. Wang DG, Johnston CF, Anderson N, Sloan JM, Buchanan KD: Overexpression of the tumour suppressor gene p53 is not implicated in neuroendocrine tumour carcinogenesis, *J Pathol* 1995, 175:397-401.
81. van Nederveen FH, Dannenberg H, Sleddens HF, de Krijger RR, Dinjens WN: p53 alterations and their relationship to SDHD mutations in parasympathetic paragangliomas, *Mod Pathol* 2003, 16:849-856.
82. Guran S, Tali ET: p53 and p16INK4A mutations during the progression of glomus tumor, *Pathol Oncol Res* 1999, 5:41-45.
83. Moul JW, Bishoff JT, Theune SM, Chang EH: Absent ras gene mutations in human adrenal cortical neoplasms and pheochromocytomas, *J Urol* 1993, 149:1389-1394.
84. Moley JF, Brother MB, Wells SA, Spengler BA, Biedler JL, Brodeur GM: Low frequency of ras gene mutations in neuroblastomas, pheochromocytomas, and medullary thyroid cancers, *Cancer Res* 1991, 51:1596-1599.
85. Lin SR, Tsai JH, Yang YC, Lee SC: Mutations of K-ras oncogene in human adrenal tumours in Taiwan, Br J Cancer 1998, 77:1060-1065.
86. Pelengaris S, Khan M, Evan G: c-MYC: more than just a matter of life and death, *Nat Rev Cancer* 2002, 2:764-776.
87. Wang DG, Johnston CF, Marley JJ, Phenix KV, Atkinson AB, Russell CF, Buchanan KD: Expression of the apoptosis-suppressing gene BCL-2 in pheochromocytoma is associated with the expression of C-MYC, *J Clin Endocrinol Metab* 1997, 82:1949-1952.
88. Liu J, Voutilainen R, Kahri AI, Heikkila P: Expression patterns of the c-myc gene in adrenocortical tumors and pheochromocytomas, *J Endocrinol* 1997, 152:175-181.
89. Goto K, Ogo A, Yanase T, Haji M, Ohashi M, Nawata H: Expression of c-fos and c-myc proto-oncogenes in human adrenal pheochromocytomas, *J Clin Endocrinol Metab* 1990, 70:353-357.
90. Wang DG, Barros D'Sa AA, Johnston CF, Buchanan KD: Oncogene expression in carotid body tumors, *Cancer* 1996, 77:2581-2587.
91. Moley JF, Wallin GK, Brother MB, Kim M, Wells SA, Jr., Brodeur GM: Oncogene and growth factor expression in MEN 2 and related tumors, *Henry Ford Hosp Med J* 1992, 40:284-288.
92. Schwab M, Ellison J, Busch M.et a: Enhanced expression of the human gene N-myc consequent to amplification of DNA may contribute to malignant progression of neuroblastoma, *Proc. Natl Acad. Sci. U. S. A.* 1984, 81:4940-4944
93. Seeger RC, Brodeur GM, Sather H, et a: Association of multiple copies of the N-myc oncogene with rapid progression of neuroblastomas, *New Engl. J. Med.* 1985, 313:1111-1116.
94. Elder EE, Xu D, Hoog A, Enberg U, Hou M, Pisa P, Gruber A, Larsson C, Backdahl M: KI-67 AND hTERT Expression Can Aid in the Distinction between Malignant and Benign Pheochromocytoma and Paraganglioma, *Mod Pathol* 2003, 16:246-255.
95. Hirano Y, Fujita K, Suzuki K, Ushiyama T, Ohtawara Y, Tsuda F: Telomerase activity as an indicator of potentially malignant adrenal tumors, *Cancer* 1998, 83:772-776.
96. Hirano Y, Nobata S, Takahashi H, Kageyama S, Sudoko H, Ushiyama T, Suzuki K, Fujita K: Histologically benign but telomerase positive adrenal pheochromocytoma, *Int J Urol* 2002, 9:697-699.
97. Bamberger CM, Else T, Bamberger AM, Frilling A, Beil FU, Allolio B, Schulte HM: Telomerase activity in benign and malignant adrenal tumors, *Exp Clin Endocrinol Diabetes* 1999, 107:272-275.

98. Kinoshita H, Ogawa O, Mishina M, Oka H, Okumura K, Yamabe H, Terachi T, Yoshida O: Telomerase activity in adrenal cortical tumors and pheochromocytomas with reference to clinicopathologic features, *Urol Res* 1998, 26:29-32
99. Kubota Y, Nakada T, Sasagawa I, Yanai H, Itoh K: Elevated levels of telomerase activity in malignant pheochromocytoma, *Cancer* 1998, 82:176-179.
100. Lehnert H, Mundschenk J, Hahn K: Malignant pheochromocytoma, *Front Horm Res* 2004, 31:155-162
101. Dannenberg H, Speel EJ, Zhao J, Saremaslani P, van Der Harst E, Roth J, Heitz PU, Bonjer HJ, Dinjens WN, Mooi WJ, Komminoth P, de Krijger RR: Losses of chromosomes 1p and 3q are early genetic events in the development of sporadic pheochromocytomas, *Am J Pathol* 2000, 157:353-359.
102. Edstrom E, Mahlamaki E, Nord B, Kjellman M, Karhu R, Hoog A, Goncharov N, Teh BT, Backdahl M, Larsson C: Comparative genomic hybridization reveals frequent losses of chromosomes 1p and 3q in pheochromocytomas and abdominal paragangliomas, suggesting a common genetic etiology, *Am J Pathol* 2000, 156:651-659.
103. Lui WO, Chen J, Glasker S, Bender BU, Madura C, Khoo SK, Kort E, Larsson C, Neumann HP, Teh BT: Selective loss of chromosome 11 in pheochromocytomas associated with the VHL syndrome, *Oncogene* 2002, 21:1117-1122.
104. Vargas MP, Zhuang Z, Wang C, Vortmeyer A, Linehan WM, Merino MJ: Loss of heterozygosity on the short arm of chromosomes 1 and 3 in sporadic pheochromocytoma and extra-adrenal paraganglioma, *Hum Pathol* 1997, 28:411-415.
105. Benn DE, Dwight T, Richardson AL, Delbridge L, Bambach CP, Stowasser M, Gordon RD, Marsh DJ, Robinson BG: Sporadic and familial pheochromocytomas are associated with loss of at least two discrete intervals on chromosome 1p, *Cancer Res* 2000, 60:7048-7051.
106. Edstrom Elder E, Nord B, Carling T, Juhlin C, Backdahl M, Hoog A, Larsson C: Loss of heterozygosity on the short arm of chromosome 1 in pheochromocytoma and abdominal paraganglioma, *World J Surg* 2002, 26:965-971.
107. Opocher G, Schiavi F, Vettori A, Pampinella F, Vitiello L, Calderan A, Vianello B, Murgia A, Martella M, Taccaliti A, Mantero F, Mostacciuolo ML: Fine analysis of the short arm of chromosome 1 in sporadic and familial pheochromocytoma, *Clin Endocrinol* (Oxf) 2003, 59:707-715.
108. Yang KP, Nguyen CV, Castillo SG, Samaan NA: Deletion mapping on the distal third region of chromosome 1p in multiple endocrine neoplasia type IIA, *Anticancer Res* 1990, 10:527-533.
109. Devilee P, Cleton-Jansen AM, Cornelisse CJ: Ever since Knudson, *Trends Genet* 2001, 17:569-573.
110. Yang A, Walker N, Bronson R, Kaghad M, Oosterwegel M, Bonnin J, Vagner C, Bonnet H, Dikkes P, Sharpe A, McKeon F, Caput D: p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours, *Nature* 2000, 404:99-103.
111. Carling T, Du Y, Fang W, Correa P, Huang S: Intragenic allelic loss and promoter hypermethylation of the RIZ1 tumor suppressor gene in parathyroid tumors and pheochromocytomas, *Surgery* 2003, 134:932-939; discussion 939-940.
112. Copland JA, Luxon BA, Ajani L, Maity T, Campagnaro E, Guo H, LeGrand SN, Tamboli P, Wood CG: Genomic profiling identifies alterations in TGFbeta signaling through loss of TGFbeta receptor expression in human renal cell carcinogenesis and progression, *Oncogene* 2003, 22:8053-8062.
113. Walker GJ, Indsto JO, Sood R, Faruque MU, Hu P, Pollock PM, Duray P, Holland EA, Brown K, Kefford RF, Trent JM, Mann GJ, Hayward NK: Deletion mapping suggests that the 1p22 melanoma susceptibility gene is a tumor suppressor localized to a 9-Mb interval, *Genes Chromosomes Cancer* 2004, 41:56-64.
114. Wong AK, Chen Y, Lian L, Ha PC, Petersen K, Laity K, Carillo A, Emerson M, Heichman K, Gupte J, Tavtigian SV, Teng DH: Genomic structure, chromosomal location, and mutation analysis of the human CDC14A gene, *Genomics* 1999, 59:248-251.

115. Mailand N, Lukas C, Kaiser BK, Jackson PK, Bartek J, Lukas J: Deregulated human Cdc14A phosphatase disrupts centrosome separation and chromosome segregation, *Nat Cell Biol* 2002, 4:317-322.
116. Franklin DS, Godfrey VL, O'Brien DA, Deng C, Xiong Y: Functional collaboration between different cyclin-dependent kinase inhibitors suppresses tumor growth with distinct tissue specificity, *Mol Cell Biol* 2000, 20:6147-6158.
117. Yang T, Martignetti JA, Massa SM, Longo FM: Leukocyte common-antigen-related tyrosine phosphatase receptor: altered expression of mRNA and protein in the New England Deaconess Hospital rat line exhibiting spontaneous pheochromocytoma, *Carcinogenesis* 2000, 21:125-131.
118. Qiao S, Iwashita T, Furukawa T, Yamamoto M, Sobue G, Takahashi M: Differential effects of leukocyte common antigen-related protein on biochemical and biological activities of RET-MEN2A and RET-MEN2B mutant proteins, *J Biol Chem* 2001, 276:9460-9467.
119. Dannenberg H, de Krijger RR, Zhao J, Speel EJ, Saremaslani P, Dinjens WN, Mooi WJ, Roth J, Heitz PU, Komminoth P: Differential loss of chromosome 11q in familial and sporadic parasympathetic paragangliomas detected by comparative genomic hybridization, *Am J Pathol* 2001, 158:1937-1942.
120. Kang Y, Siegel PM, Shu W, Drobnjak M, Kakonen SM, Cordon-Cardo C, Guise TA, Massague J: A multigenic program mediating breast cancer metastasis to bone, *Cancer* Cell 2003, 3:537-549.
121. Liotta LA, Kohn EC: Cancer's deadly signature, *Nat Genet* 2003, 33:10-11.
122. van 't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AA, Mao M, Peterse HL, van der Kooy K, Marton MJ, Witteveen AT, Schreiber GJ, Kerkhoven RM, Roberts C, Linsley PS, Bernards R, Friend SH: Gene expression profiling predicts clinical outcome of breast cancer, *Nature* 2002, 415:530-536.
123. Hanahan D, Weinberg RA: The hallmarks of cancer, Cell 2000, 100:57-70.
124. van der Harst E, Bruining HA, Jaap Bonjer H, van der Ham F, Dinjens WN, Lamberts SW, de Herder WW, Koper JW, Stijnen T, Proye C, Lecomte-Houcke M, Bosman FT, de Krijger RR: Proliferative index in phaeochromocytomas: does it predict the occurrence of metastases? *J Pathol* 2000, 191:175-180.
125. Eisenhofer G, Lenders JW, Pacak K: Biochemical diagnosis of pheochromocytoma, Front Horm Res 2004, 31:76-106.
126. Jacks T, Shih TS, Schmitt EM, Bronson RT, Bernards A, Weinberg RA: Tumour predisposition in mice heterozygous for a targeted mutation in Nf1, *Nat Genet* 1994, 7:353-361.
127. Smith-Hicks CL, Sizer KC, Powers JF, Tischler AS, Costantini F: C-cell hyperplasia, pheochromocytoma and sympathoadrenal malformation in a mouse model of multiple endocrine neoplasia type 2B, *Embo J* 2000, 19:612-622
128. Kawai K, Iwashita T, Murakami H, Hiraiwa N, Yoshiki A, Kusakabe M, Ono K, Iida K, Nakayama A, Takahashi M: Tissue-specific carcinogenesis in transgenic mice expressing the RET proto-oncogene with a multiple endocrine neoplasia type 2A mutation, *Cancer Res* 2000, 60:5254-5260.
129. Tischler AS, Powers JF, Alroy J: Animal models of pheochromocytoma, *Histol Histopathol* 2004, 19:883-895
130. Schulz N, Propst F, Rosenberg MP, Linnoila RI, Paules RS, Kovatch R, Ogiso Y, Vande Woude G: Pheochromocytomas and C-cell thyroid neoplasms in transgenic c-mos mice: a model for the human multiple endocrine neoplasia type 2 syndrome, *Cancer Res* 1992, 52:450-455.
131. Crabtree JS, Scacheri PC, Ward JM, Garrett-Beal L, Emmert-Buck MR, Edgemon KA, Lorang D, Libutti SK, Chandrasekharappa SC, Marx SJ, Spiegel AM, Collins FS: A mouse model of multiple endocrine neoplasia, type 1, develops multiple endocrine tumors, *Proc Natl Acad Sci U S A* 2001, 98:1118-1123.
132. Nikitin AY, Juarez-Perez MI, Li S, Huang L, Lee WH: RB-mediated suppression of spontaneous multiple neuroendocrine neoplasia and lung metastases in Rb+/- mice, *Proc Natl Acad Sci U S A* 1999, 96:3916-3921.
133. Powers JF, Schelling K, Brachold JM, Tsokas P, Schayek H, Friedman E, Tischler AS: High-level expression of receptor tyrosine kinase Ret and responsiveness to Ret-activating ligands in pheochromocytoma cell lines from neurofibromatosis knockout mice, *Mol Cell Neurosci* 2002, 20:382-389.

134. You MJ, Castrillon DH, Bastian BC, O'Hagan RC, Bosenberg MW, Parsons R, Chin L, DePinho RA: Genetic analysis of Pten and Ink4a/Arf interactions in the suppression of tumorigenesis in mice, *Proc Natl Acad Sci U S A* 2002, 99:1455-1460.
135. Pinkel D, Segraves R, Sudar D, Clark S, Poole I, Kowbel D, Collins C, Kuo WL, Chen C, Zhai Y, Dairkee SH, Ljung BM, Gray JW, Albertson DG: High resolution analysis of DNA copy number variation using comparative genomic hybridization to microarrays, *Nat Genet* 1998, 20:207-211.
136. Clark J, Edwards S, Feber A, Flohr P, John M, Giddings I, Crossland S, Stratton MR, Wooster R, Campbell C, Cooper CS: Genome-wide screening for complete genetic loss in prostate cancer by comparative hybridization onto cDNA microarrays, *Oncogene* 2003, 22:1247-1252.
137. Cai WW, Mao JH, Chow CW, Damani S, Balmain A, Bradley A: Genome-wide detection of chromosomal imbalances in tumors using BAC microarrays, *Nat Biotechnol* 2002, 20:393-396.
138. Albertson DG: Profiling breast cancer by array CGH, *Breast Cancer Res Treat* 2003, 78:289-298
139. Asa SL, Ezzat S: The pathogenesis of pituitary tumours, *Nat Rev Cancer* 2002, 2:836-849.

Nederlandse samenvatting

Pheochromocytomen en paragangliomen zijn relatief zeldzame neuroendocriene tumoren. Ze delen een aantal belangrijke histopathologische kenmerken, maar de associatie met erfelijke tumor syndromen is in de meeste gevallen verschillend. Voor een gerichtere follow-up van patiënten met deze tumoren, is er behoefte aan accurate markers die het klinische gedrag van deze tumoren voorspellen. Omdat kanker een ziekte van het genoom is, zou een dergelijke marker van genetische aard kunnen zijn. De studies die beschreven worden in dit proefschrift beoogden een beter begrip van de pathogenese en progressie van pheochromocytomen en paragangliomen, en het identificeren van moleculaire markers die clinici in staat kunnen stellen om die patiënten te identificeren die at risk zijn, en zodoende de follow-up specifiek te richten op deze patiënten.

De genen de betrokken zijn bij predispositie voor het ontstaan van pheochromocytomen en paragangliomen, bieden startpunten voor het begrijpen hun pathogenese. Daarom onderzochten we het *VHL* en *SDHD* gen op mutaties in zowel kiembaan als tumor DNA van patiënten. Kiembaan of somatische *VHL* mutaties komen voor in respectievelijk 8% en 4% van de sporadische pheochromocytomen. We vonden een licht verhoogde frequentie van somatische *VHL* mutaties in maligne pheochromocytomen in vergelijking met de benigne tumoren (17,4% *vs.* 4.3%). Tevens werden in deze studie een aantal nog niet eerder beschreven missense mutaties gevonden. De aard van de *VHL* mutaties was niet verschillend voor benigne of maligne pheochromocytomen; ook toonden alle tumoren expressie van *VHL* op eiwit niveau. Verschillende studies hebben aangetoond dat kiembaan *SDHD* mutaties zelden voorkomen bij patiënten die zich presenteren met een pheochromocytoom, in tegenstelling tot patiënten die zich presenteren met een paraganglioom. *SDHD* mutatie analyse identificeert ook de patiënten die at risk zijn voor het ontwikkelen van multipele (extra-adrenale) pheochromocytomen en paragangliomen. Somatische *SDHD* mutaties komen niet of nauwelijks voor. Gebaseerd op studies in dit proefschrift en andere, komen we tot de aanbeveling om screening op SDH mutaties te verrichten bij patiënten die zich presenteren met (0) paragangliomen, (1) een familielid met paragangliomen of pheochromocytomen, (2) multipele tumoren, (3) een geïsoleerd (extra-adrenaal) pheochromocytoom en jonge leeftijd (≤ 35 jaar). *SDHD* mutatie analyse kan ook helpen bij het onderscheiden van patiënten met multipele primaire tumoren of een gemetastaseerde tumor.

De afwezigheid of lage frequentie van somatische mutaties in de genen *RET*, *VHL*, *NF1* en *SDHB*, *SDHD*, of *SDHC*, vraagt om een algemenere benadering in het ontrafelen van de tumorigenese van pheochromocytomen en paragangliomen. In een eerste poging om secundaire genetische veranderingen te identificeren, analyseerden we beide typen tumoren met behulp van *comparative genomic hybridization* (CGH). Deze studies toonden aan dat de meeste

adrenale en extra-adrenale pheochromocytomen een vergelijkbaar patroon van genomische veranderingen heeft. Paragangliomen tonen echter een ander profiel. Paragangliomen hebben gewoonlijk maar weinig chromosomale afwijkingen en in het bijzonder gecombineerde deleties van de chromosoom armen 1p en 3q worden nauwelijks gezien. De enige frequent voorkomende afwijking in paragangliomen, verlies van chromosoom 11(q), treedt vooral op in de familiaire tumoren (86% *vs* 22% in klinisch sporadische PGL). Dit blijkt ook te corresponderen met het voorkomen van mutaties in *SDHD* (11q23). We rapporteren verlies van de chromosomale regio's 1p en 3q in meer dan 80% van de sporadische en MEN2-gerelateerde pheochromocytomen. Deze deleties gaan meestal vergezeld van meerdere andere genomische veranderingen, zoals verlies van chromosoom arm 17p en 13q en extra kopieën van 9q33-qter en 17q. De specifiek hoge frequentie van 1p verlies in MEN2-gerelateerde en sporadisch pheochromocytomen (en niet in VHL-gerelateerde tumoren) geeft aan dat inactivatie van één of meerdere tumor suppressor genen op 1p een pathogene rol speelt. Verder suggereren onze onderzoeksresultaten dat verschillende moleculaire veranderingen, zoals verlies van 6q of duplicaties van een combinatie van 5p, 7p en 12q betrokken zouden kunnen zijn bij de progressie naar maligniteit in pheochromocytomen. Gebaseerd op de verschillende CGH profielen van pheochromocytomen en paragangliomen, kan men deze tumoren beschouwen als verschillende entiteiten. Dit ondanks hun ontstaan uit een gemeenschappelijke *precursor cell*. Voor het nauwkeuriger bepalen van de betrokken chromosomale regio, maakten we gebruik van een 0.1-0.2 Mb BAC array die chromosoom arm 1p geheel bevat. Het gebied waarin hoogstwaarschijnlijk de kandidaat tumor suppressor genen gezocht moeten worden, reduceerden we hiermee tot drie kleinere regio's: 1cen-1p21.1, 1p21.3-1p31.1 en 1p34.3-1p35.2. In onze studie werden geen additionele kleine deleties of homozygote deleties gedetecteerd, wat suggereert dat dergelijke kleine veranderingen zeldzaam zijn in pheochromocytomen. Tot op heden zijn alleen in het *SDHB* gen (gelokaliseerd op 1p36.13 en coderend voor een van de subunits van het mitochondriale succinaat dehydrogenase eiwitcomplex) pathogene mutaties gevonden, in een klein aantal families met pheochromocytomen en paragangliomen. Verscheidene andere genen op 1p zijn genoemd als kandidaat tumor suppressor genen, waarvan $p18^{INK4C}$ en *PTPRF/LAR* de meest interessante kandidaten lijken te zijn. Echter, mutaties hebben wij een subset van onze tumoren niet gedetecteerd en ook onze array-CGH resultaten suggereren dat deze genen hoogstens in een kleine groep pheochromocytomen een rol spelen.

Chronische hypoxie is een factor het ontstaan van paragangliomen maar is ook een bekende oorzaak van p53 overexpressie, welke op zijn beurt de cel cyclus remt en apoptose induceert. Derhalve kan worden gehypothetiseerd dat *p53* inactivatie de mogelijkheid biedt aan paraganglioomcellen om te ontsnappen aan dit cellulaire controle mechanisme. We bepaalden daarom de eiwit expressie van p53 en MDM2 in een serie erfelijke en sporadische paragangliomen en toonden aan dat wildtype p53 tot expressie komt in ten minste 35% van de paragangliomen, onafhankelijk van de *SDHD* status en in afwezigheid van *p53* gen mutaties.

Gelijktijdige overexpressie van MDM2, wat geassocieerd is met uitschakeling van het *p53* tumor controle mechanisme, werd gedetecteerd in 7% van deze groep; ook hierbij ontbrak een associatie met *SDHD* mutaties. Gegeven het feit dat hypoxie p53 expressie induceert en gezien de afwezigheid van *p53* mutaties, suggereren deze resultaten dat disfunctie van p53 geen grote rol speelt in de tumorigenese van erfelijke en sporadische paragangliomen.

Uit dierproeven en studies met humane tumoren wordt duidelijk dat zeker drie signaal transductie routes betrokken zijn bij het ontstaan van pheochromocytomen of paragangliomen. Activering van RAS/PI3K en verlies van de chromosoom armen 1p en 3q spelen een belangrijke rol in het ontstaan van sporadische en MEN2- en NF1-gerelateerde pheochromocytomen. VHL-gerelateerde pheochromocytomen ontstaan via andere pathogene mechanismen, onafhankelijk van 1p verlies, maar gerelateerd aan afwijkingen op chromosoom 3 (*VHL*) and 11.

Paragangliomen ontstaan door mutaties in één van de SDH genen. Secundaire genetische veranderingen in deze groep tumoren zijn tot op heden vrijwel onbekend. Uitschakeling van het *p53* tumor controle mechanisme lijkt geen rol van betekenis te spelen.

Ki67/MIB-1, in combinatie met hTERT en TA, zijn tot op heden de meest betrouwbare determinanten van maligne tumor gedrag in pheochromocytomen en paragangliomen. In pheochromocytomen komt maligne tumorgedrag waarschijnlijk tot stand door mechanismen die geassocieerd zijn met verlies van de chromosomale regio's 6q of 18p, of duplicatie van 5p, 7p, and 12q. Moderne moleculaire technieken zullen verder bijdragen aan het ophelderen van mechanismen maligne ontaarding van pheochromocytomen en paragangliomen, het identificeren van bruikbare genetische markers en het vinden van nieuwe therapeutische targets.

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Curriculum Vitae

Hendrika Dannenberg werd geboren op 14 maart 1976 te Rijssen. In 1994 behaalde zij het VWO diploma aan de Jacobus Fruytier scholengemeenschap te Apeldoorn. Aansluitend startte zij met de studie geneeskunde aan de Rijksuniversiteit Maastricht. Na de propedeuse vervolgde zij de studie aan de Erasmus Universiteit Rotterdam. In het kader van het doctoraal examen werden 2 onderzoeksstages ondernomen. Hierbij verrichte zij onderzoek op het gebied van decubitus preventie bij intensive care patienten op de Afdeling Chirurgie, Intensive Care (Prof. dr. H.A. Bruining) van het Erasmus Medisch Centrum Rotterdam. Een tweede onderzoek werd verricht bij de Afdeling Pathologie (dr. R. R. de Krijger, dr. W. N. M. Dinjens). Na het doctoraal examen werd laatstgenoemde onderzoek vervolgd met een werkbezoek aan de Afdeling Pathologie, Labor für Endokrinopathologie (Prof. dr. P. Komminoth), van het UniversitätsSpital Zürich, Zwitserland. Het artsexamen behaalde zij in 2002.

Vanaf april 2002 was zij werkzaam als arts-onderzoeker op de afdeling Pathologie, op een door de Vanderes Stichting gesubsidieerd project. Gedurende deze periode werd het promotieonderzoek naar de moleculaire pathogenese van pheochromocytomen vervolgd. In dit kader verrichtte zij gedurende 3 maanden onderzoek bij Dr. W. Lam aan de afdeling Cytogenetics and Developmental Biology, BC Cancer Research Centre, Vancouver, Canada.

Na enige tijd als AGNIO chirurgie werkzaam te zijn geweest in het Medisch Specturm Twente te Enschede, is ze in september 2004 begonnen met de opleiding Heelkunde in het Deventer Ziekenhuis.