The Pathogenesis of Pheochromocytomas: Of Mice and Men

Esther Korpershoek

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The Pathogenesis of Pheochromocytomas: Of Mice and Men

De pathogenese van pheochromocytomen: van muis en mens

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Promotor:
Prof. dr. R.R. de Krijger

Co-promotor:
Dr. W.N.M. Dinjens

Overige leden:
Prof. dr. W.W. de Herder
Prof. dr. L.J. Hofland
Prof. dr. J. Trapman

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Chapter 1

General introduction & outline of this thesis

1.1 Pheochromocytomas and paragangliomas

Pheochromocytomas

Pheochromocytomas are neuro-endocrine tumors that arise from the neural crest derived adrenal medullary chromaffin cells, and produce catecholamines. The first description of a patient with pheochromocytomas was done by Fränkel in 1886[1], but the term pheochromocytoma was invented by the pathologists Ludwig Pick in 1912, after the Greek words *phaios*, meaning dark or dusky, and *chroma*, meaning color, which refers to the dark discoloration of the tumor cells in the chromium-salt reaction.[2]

During embryonic development, cells of the neural crest migrate along pre-programmed pathways, and differentiate into a variety of cell types, such as the intra-adrenal and extra-adrenal chromaffin cells, and the autonomic ganglion cells.[3] The adrenal medulla is composed of chromaffin cells, which are arranged in clusters, enclosed by sustentacular cells and a stromal network (Figure 1). Apart from these structures, the medulla is highly vascularized, and this is also seen in pheochromocytomas. In general, chromaffin cells are thought to store either adrenalin or noradrenalin, but cells containing both catecholamines have been reported in mice.[4] Pheochromocytomas can produce dopamine, adrenalin, noradrenalin, or a combination, depending on their genetic background.[5] Catecholamine production results in sustained, labile or paroxysmal hypertension, and if patients are not treated appropriately, pheochromocytomas will almost always cause fatal cardiovascular events or other devastating complications.[6]

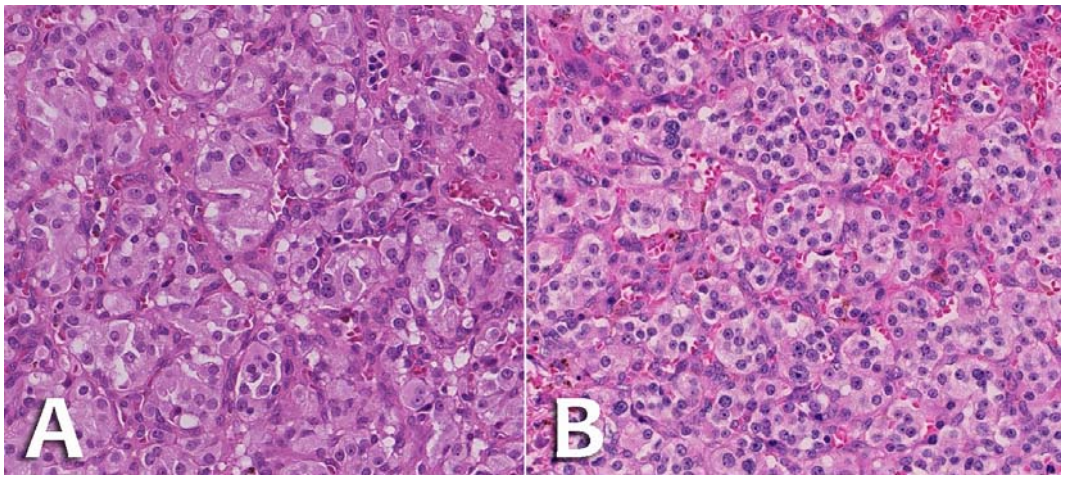

Figure 1. Haematoxylin staining of a pheochromocytoma (A) and a parasympathetic paraganglioma (B).

Pheochromocytomas occur in approximately 1 or 2 per 100,000 adults in the USA per year (0.001-0.002%), but the exact incidence is not precisely known.[7] The true incidence of pheochromocytomas is probably higher (towards 0.05%) as one in twenty cases of the incidentally-found adrenal masses during autopsy, magnetic resonance imaging (MRI), computed tomography (CT), or abdominal ultrasonography, is a pheochromocytoma.[8, 9]

Paragangliomas

Paragangliomas can be subdivided into sympathetic and parasympathetic paragangliomas. Sympathetic paragangliomas occur along the sympathetic trunk, from the proximal aorta to the urinary bladder, and usually produce catecholamines, whereas parasympathetic paragangliomas occur in the head and neck region and generally do not produce catecholamines.[10] Parasympathetic paragangliomas predominantly affect the carotid body, but paragangliomas can also occur in the vagal, jugulotympanic, and laryngeal paraganglia (Figure 2 left). Most frequently, paragangliomas are found in the carotid body. This small organ, which is involved in oxygen sensing, is located in the bifurcation of the carotid artery. Sympathetic paragangliomas can occur along the sympathetic trunk, from the aorta to the bladder (Figure 2 right).[11] The incidence of parasympathetic paraganglioma is approximately 1:1,000,000, whereas the incidence of sympathetic paragangliomas is unknown since tumors have been grouped with pheochromocytomas in the past literature.[12]

1.2 Hereditary syndromes presenting pheochromocytomas and paragangliomas

Pheochromocytomas and paragangliomas can occur sporadically as well as in the context of hereditary syndromes, caused by activating mutations in proto-oncogenes, or inactivating mutations in tumor suppressor genes. Approximately 30% of the pheochromocytomas and up to 50% of the paragangliomas exhibits germline mutations in one of the known susceptibility genes, whereas the remainder are sporadic.[13, 14]

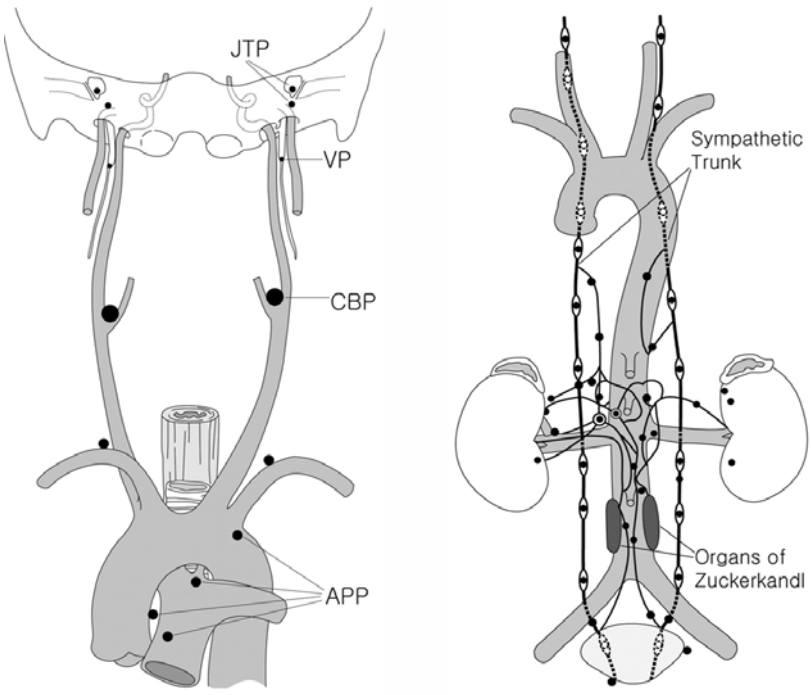

Figure 2. Overview of the distribution of paraganglia of the parasympathetic and sympathetic nerve system. Left; APP = aorticopulmonary paraganglion, CBP = carotid body paraganglion, VP = vagal paraganglia, and JTP = jugulotympanic paraganglia. Right; The sympathetic trunk were sympathetic paraganglia occur, especially in the organs of Zuckerkandl. This overview was adapted from Lee et al.[11]

Multiple Endocrine Neoplasia type 2

Multiple endocrine neoplasia type 2 (MEN2) is one of the syndromes in which pheochromocytoma is a dominant clinical feature. This syndrome is caused by germline mutations in the *RET proto*-oncogene, located on chromosome 10q11. Mutations occur almost exclusively in exon 10, 11 and 16. MEN2 is subdivided into the two clinical subtypes MEN2a and MEN2b. Both variants show a high penetrance of medullary thyroid carcinoma, , as a consequence of which 90% of MEN2 carriers will have a medullary thyroid carcinoma, and 50% will have pheochromocytomas, and only the most common form MEN2a will present with hyperparathyroidism. Pheochromocytomas are bilateral in 85% of cases, and are almost exclusively benign.[15-17] They are characterized by loss of chromosomes 1p and 3q, and mostly have an adrenergic phenotype.[5, 18, 19] Somatic *RET* mutations have also been described in 9% of sporadic pheochromocytomas.[20]

Von Hippel-Lindau disease

Another syndrome that presents with pheochromocytomas at high frequency is von Hippel-Lindau disease (VHL), which is caused by inactivating germline mutations of the *VHL* tumor suppressor gene located on chromosome 3p25.3.[21] There are 4 different subgroups of VHL disease, subdivided according to their clinical presentation (Table 1). Patients with *VHL* mutations also develop clear cell renal cell carcinomas, cerebellar haemangioblastomas, renal and pancreatic cysts, neuroendocrine tumors of the pancreas, and in rare occasions parasympathetic paraganglioma have been described.[22, 23] In keeping with the Knudson 2-hit model, VHL shows inactivating mutations accompanied by loss of the wild type allele. Besides loss of chromosome 3p, pheochromocytomas are also characterized by loss of chromosome 11, and show a more noradrenergic phenotype.[5, 24] Most *VHL*-related pheochromocytomas are benign, but approximately 5% is malignant.[22] Somatic mutations have been found in 4% of sporadic pheochromocytomas.[25]

Table 1. Clinical manifestation per type of VHL disease

Type of VHL disease	*Clinical manifestation*
Type 1	Haemangioblastomas
	Diminished risk of pheochromocytomas
	Renal-cell carcinomas
Type 2A	Haemangioblastomas
	Pheochromocytomas
	Low risk of renal-cell carcinomas
Type 2B	Haemangioblastomas
	Pheochromocytomas
	High risk of renal-cell carcinomas
Type 2C	Pheochromocytomas only

Neurofibromatosis type 1

Neurofibromatosis type 1 is caused by germline mutations in the *NF1* tumor suppressor gene, which is located on chromosome 17q11.2. Worldwide, the incidence is 1 in 2,500 to 1 in 3,000 individuals.[26] Pheochromocytomas occur in 0.1-5.7% of mutation carriers, and are usually benign. Furthermore, 10% of these pheochromocytomas are bilateral and 6% occur outside the adrenal glands, in the abdomen or thorax. Patients are diagnosed on a clinical basis, requiring two of seven easy recognizable characteristics. These features include café-au-lait spots and multiple neurofibromas. Genetic testing for NF1 is almost exclusively performed after phenotypic diagnosis because of the huge size (58 exons) of the *NF1* gene and the presence of several pseudogenes.[27, 28] This laborious sequence analysis of the *NF1* gene is the reason why no studies on somatic *NF1* mutations have been reported.

Pheochromocytoma-Paraganglioma syndrome

The pheochromocytoma-paraganglioma syndrome is caused by germline mutations in subunits of succinate dehydrogenase (SDH), or mitochondrial complex II. Complex II is located in the mitochondrial inner membrane, and couples the oxidation of succinate to fumarate in the Krebs cycle with the electron transport chain. (Figure 3) SDH subunit A (SDHA) is the enzymatically active part of the complex, subunit B (SDHB) transfers the electrons to the other complexes of the electron transport chain, and subunits C and D (SDHC and SDHD) are the anchoring proteins.[29] At first, only mutations in the genes encoding subunits B, C, and D found to be associated with paragangliomas and pheochromocytomas.[14, 30, 31] Recently however, mutations in the genes encoding SDHA and SDH assembly factor 2 (SDHAF2) were described in these tumors, although only in very few patients or families.[32, 33] A database with mutations in the above mentioned *SDH*-related genes is freely available online: http://chromium.liacs.nl/lovd_sdh/home.php.

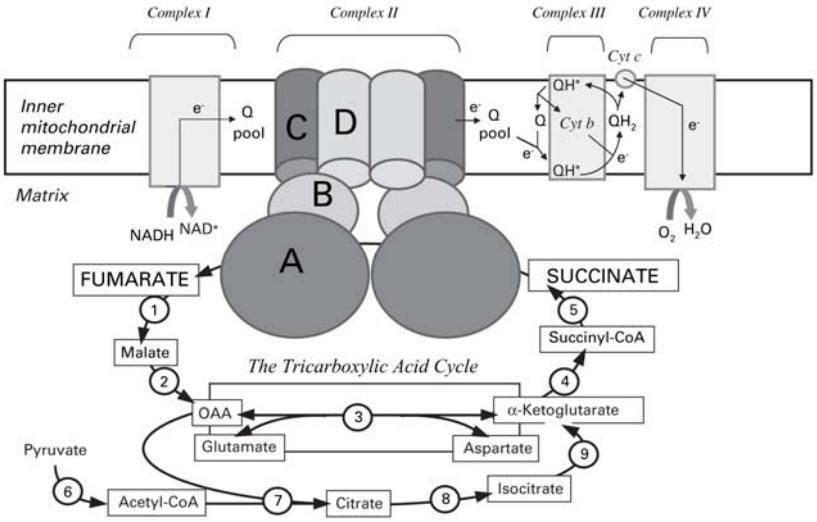

Figure 3. Succinate dehydrogenase is part of the tricarboxic Acid Cycle as well as the electron transport system. [30]

Some decades ago, when the genes causing the pheochromocytoma-paraganglioma syndrome were not identified, already 4 different familial paraganglioma-susceptible loci were recognized through linkage studies (Table 2).[12, 34] The first gene that was shown to be associated with a subtype of this syndrome (PGL-1) was the *SDHD* gene.[35] Soon after this discovery, the genes encoding SDH subunits B and C (*SDHB* and *SDHC*), were associated with two of the other pheochromocytoma-paraganglioma syndromes (PGL-4 and PGL-3 respectively).[36] Last year, the gene responsible for PGL-2 was identified, which was *SDHAF2*. In contrast to the PGL-1, PGL-3, and PGL-4- related genes, SDHAF2 is not a component of mitochondrial complex II. Nonetheless, the protein appeared important for the stabilization and activity of complex II, since it was required for the flavination of SDHA.[37]

Table 2. The pheochromocytoma-paraganglioma hereditary syndromes linked to the causal genes

Syndrome type	*Chromosome*	*Gene*
PGL-1	11q23	*SDHD*
PGL-2	11q13.1	*SDHAF2*
PGL-3	1q21-23	*SDHC*
PGL-4	1p36	*SDHB*

PGL-1; Succinate dehydrogenase D

The first report, describing the association of germline mutations in the *SDHD* gene with the PGL-1 syndrome was by Baysal et al. in the year 2000.[35] *SDHD* is located on chromosome 11q23.1, and is predominantly paternally transmitted, which means that only children that inherit the mutation from the father are at risk for developing tumors.[36] In addition, it has been suggested that combined loss of the wild type allele and a maternal region on chromosome 11p are necessary for tumorigenesis.[38] *SDHD* mutation carriers generally develop parasympathetic paragangliomas (in 89% of cases). Furthermore, pheochromocytomas and sympathetic paragangliomas are seen in 7% and 18% of SDHD patients respectively.[39] Usually, tumors are benign, but approximately 8% present with metastases.[40] Many different mutations have been described, but in the Netherlands 3 founder mutations are known (D92Y, L95P, L139P).[36] Although extremely rare, two somatic *SDHD* mutations have been reported.[41] Genetic alterations that occur in the *SDHD*-related tumors include loss of chromosome 11, and in lesser extend loss of chromosome 1p.[42]

PGL-2; Succinate dehydrogenase assembly factor 2

Until recently, the gene causing the PGL-2 syndrome was unknown. However, in 2009 the gene was identified and was shown to be *SDHAF2*.[37] One *SDHAF2* mutation (G78R) was discovered in a Dutch family, presenting with parasympathetic paragangliomas. In addition, another family with the identical mutation was found in Spain.[32] As is the case

for the *SDHD* gene, *SDHAF2* was maternally imprinted as only the children that inherited the mutation from their father presented with tumors. Neither somatic, nor germline mutations were found in apparently sporadic tumors.[32]

PGL-3; Succinate dehydrogenase C

After mutations in *SDHD* were associated with the PGL-1 locus, the *SDHC* gene was soon linked to the PGL-3 locus (chromosome 1q13).[36] *SDHC* mutation carriers almost exclusively had parasympathetic paragangliomas, but *SDHC* mutations were also reported in a pheochromocytoma and sympathetic paragangliomas. None of the tumors was malignant. Tumors showed loss of the wild type allele, as expected for a tumor suppressor gene.[43-45]

PGL-4; Succinate dehydrogenase B

The PGL-4 causing gene *SDHB*, located on chromosome 1p36.1, was identified in 2001.[36] Germline *SDHB* mutation carriers present predominantly with sympathetic paragangliomas and pheochromocytomas, of which 89% is catecholamine-producing.[46] In smaller numbers, parasympathetic paragangliomas in the head and neck are also seen. Up to 43% of the patients have malignant tumors, which metastasize to the skeleton, lymph nodes, liver, or lungs.[39, 40, 46] Only one patient has been described with a somatic *SDHB* mutation.[47] Many different inactivating *SDHB* mutations have been described. With the exception of one somatic mutation, these are all germline mutations.[47] *SDHB*-related tumors display loss of chromosome 1p as the main chromosomal aberration.[48] Other tumors that also have been associated with *SDHB* mutations are renal cell carcinomas and gastrointestinal stromal tumors.[49, 50]

Succinate dehydrogenase A

The last subunit of mitochondrial complex II is *SDHA*. This tumor suppressor gene, located on chromosome 5p15, was associated with the Leigh syndrome, which is an early-onset progressive degenerative disorder. A few reports exist that have demonstrated different homozygous *SDHA* germline mutations in patients with Leigh syndrome.[51-54] The parents of these patients, who were heterozygous germline carriers, appeared to be healthy and were not reported to have paragangliomas or pheochromocytomas, or clinical signs such as hypertension. A few studies have tried to find *SDHA* mutations in pheochromocytomas, but did not find any, so it was thought that *SDHA* mutations did not exist in these tumors. However, a recent report found an *SDHA*-associated paraganglioma, showing a mutation and loss of the wild type allele. In addition, the tumor cells of the paraganglioma lost SDHA expression.[33] Thus, it appears that *SDHA*, although rarely, is also associated with the occurrence of a paraganglioma.

Carney triad and Carney-Stratakis syndrome

Carney triad as well as Carney-Stratakis syndrome patients both present with paragangliomas and gastrointestinal stromal tumors. Both syndromes are very rare and affect young individuals. The classic Carney triad is manifested by sympathetic paragangliomas, gastrointestinal stromal tumors, and pulmonary chondroma,[55] but later pheochromocytoma, adrenal cortical adenoma, and esophageal leiomyoma were also associated with the syndrome.[56] No (germline) mutations in candidate genes have been linked to the disease. In contrast, the Carney-Stratakis syndrome, which presents solely with gastrointestinal stromal tumors and sympathetic paragangliomas, has been associated with germline mutations in *SDHB*, *SDHC*, and *SDHD*.[57, 58]

TMEM127

The first time *TMEM127* was reported to be involved in the pathogenesis of pheochromocytomas was in 2010. This recently discovered gene, located on chromosome 2q11.2, was mutated in 30% of familial tumors and in 3% of the apparently sporadic pheochromocytomas.[59] TMEM127 seems to act as a tumor suppressor gene, and is now hypothesized to activate mTORC1, which is also a downstream target of RET and the PI3K/Akt signals. Furthermore, mRNA expression profiles tend to cluster with *RET*-related and *NF1*-related tumors.[13]

Rare genes that can cause pheochromocytomas and paragangliomas

Other genes, mutated in the germline, that have been linked to pheochromocytomas and paragangliomas are *Kif1b*β, *MEN1*, and *PHD2*.[60-62] Germline mutations in the *Kif1b*β gene, located on chromosome 1p36.2, which is a chromosomal region deleted very frequently in pheochromocytomas, were discovered in three familial cases that presented with pheochromocytomas. Other neural crest-derived tumors that had *Kif1b*β mutations included neuroblastomas and medulloblastomas and pheochromocytomas.[62, 63] Mutations in the *MEN1* gene, which is located on 11q13, cause the multiple endocrine neoplasia type 1 (MEN1) syndrome. The syndrome is characterized by tumors of the parathyroid gland, pancreatic islets, and anterior pituitary.[16] In less than 1% of the germline mutation carriers a pheochromocytoma is seen.[60] Recently, the first and so far only paraganglioma was reported with a germline *PHD2* mutation. Besides a paraganglioma, the patient had congenital erythrocytosis.[61]

1.3 Pheochromocytoma pathways

One study has tried to link all the major pheochromocytoma and paraganglioma susceptibility genes into one pathway,[64] but according to unsupervised clustering of mRNA expression data of two different studies, there seem to be two different tumor types, involving two different pathways.[65, 66] One cluster includes *VHL* and *SDH*-related tumors that overexpress hypoxia-associated genes (cluster 1). The

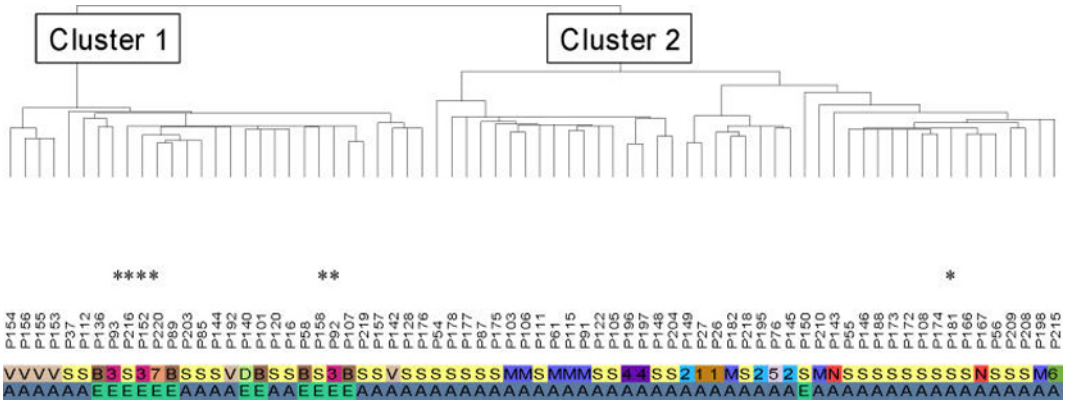

Figure 4. Unsupervised clustering identifies 2 major clusters. Cluster 1 contains the *VHL* (= V) and *SDH* (= B + D)-related tumors, whereas cluster 2 contains the *NF1* (= N) and *RET* (= M)-related tumors. The lower row defines tumor location (A = adrenal, E = extra-adrenal). This figure is adapted from Dahia et al. [65]

other cluster comprises *RET*, *NF1*, and *TMEM127*-related tumors with a more neuronal expression type (cluster 2) (Figure 4).

During normoxia, hypoxia-inducible factor-α subunits (HIF-α) are hydroxylated by prolyl hydroxylases (PHDs), and ubiquitinated by VHL, so they are degraded by the proteasome. During hypoxia, PHDs are not able to hydroxylate HIF-α. As a consequence, HIF-α is not recognized by pVHL, therefore not ubiquitinated, and is not degraded. In contrast, HIF-α forms a stable heterodimer with the HIF-β subunit, and activates genes that stimulate angiogenesis and cell proliferation, and genes that inhibit apoptosis.[67] In cluster-1 pheochromocytomas and paragangliomas, hypoxia is mimicked through inactivating *SDH* or *VHL* gene mutations, a process called pseudo-hypoxia. *SDH* mutations lead to an accumulation of succinate, which suppresses the hydroxylation function of the PHDs, whereas *VHL* mutations result in a defective ubiquitination process. In both cases, HIF-α cannot be degraded and in turn activates the hypoxia pathway and other target genes.[68]

1.4 Mouse models with pheochromocytomas

Knock-in and knock-out mice are proven useful models to investigate the pathogenesis of human tumors. Many different mouse models have been generated, including those that develop pheochromocytomas (Table 3). In many cases, there was no intention to create mice that presented with pheochromocytomas. These accidentally arising pheochromocytomas occurred in mice that were generated to investigate prostate cancer (*Pten*$^{-/-}$), or that were created to investigate the effect of inactivation of some tumor suppressor genes (*Rb*$^{-/-}$, *Nf1*$^{+/-}$, *p18*$^{-/-}$, and *p27*$^{-/-}$) or activation of oncogenes (*RET*$^{Met918Thr/Met918Thr}$) *in vivo*.[69-76]

Pten knock-out mouse models

The *PTEN* gene (phosphatase and tensin homolog deleted from chromosome 10) is a tumor suppressor gene that inhibits the AKT-pathway by converting phosphatidylinositol 3,4,5 triphosphate (PIP3) into phosphatidylinositol 3,4 biphosphate (PIP2). This dephosphorlation in counteracted by PI-3 kinase (PI3K), which converts PIP2 into the active PIP3. Through PI3K multiple pathways are triggered, many of which are associated with cell growth and survival (Figure 5).[77] The *PTEN* gene is one of the most frequently mutated genes in human cancer, but it has never been associated with human pheochromocytomas or paragangliomas.[78, 79] In contrast, several Pten knock-out mouse models have been reported to present with pheochromocytomas at high frequency.[70, 76, 80] In addition, the study of Di Cristofano et al. demonstrated that the main genetic alteration found in their mouse pheochromocytomas was syntenic to a large part of human chromosome 1p, which is the most common chromosomal alteration in human pheochromocytomas.[81]

Nf1 knock-out mouse model

Another model that studied cultured pheochromocytoma cells from *NF*$^{+/n31}$ knock-outs also shows chromosomal loss of syntenic regions to human pheochromocytomas. NF1 negatively regulates the RAS proto-oncogene that activates the AKT pathway, through PI3K (Figure 5).[82] The chromosomal alterations found in the Nf1 knock-outs include loss of mouse chromosome 9, which is homologous to parts of human chromosomes 3p, 3q and 11q, all very common alterations in human pheochromocytomas. In addition, loss of chromosome 4 was also seen at high frequency, which is syntenic to human chromosome 1p.[81, 83] The cultured cells were from the *NF*$^{+/n31}$ knock-out model that developed pheochromocytomas in 20% of the mice spontaneously, and in 87% of the irradiated mice.[73] In addition, the mRNA expression of tumor tissues was compared to healthy control adrenal medulla tissue and it appeared these tumors had a neuronal progenitor expression profile, showing a severe overexpression of *RET*.[84]

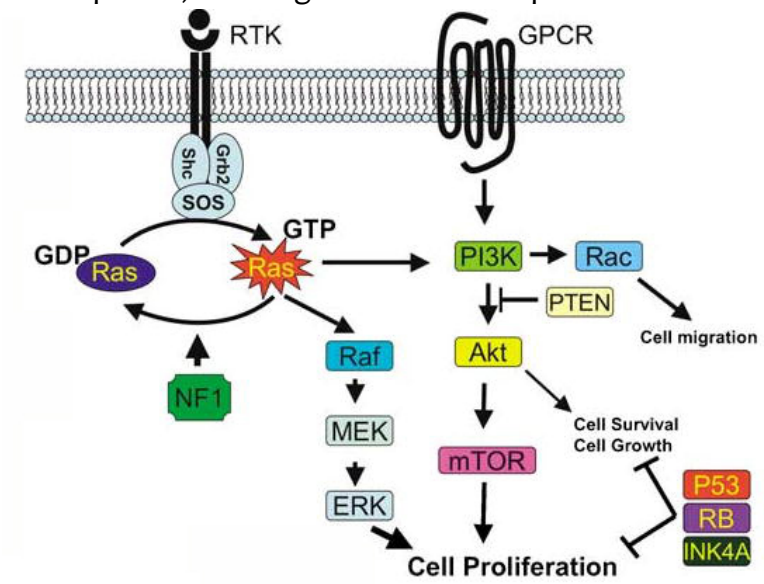

Figure 5. A common pathway linking important pheochromocytoma causing genes in the mouse, such as *Nf1*, *Pten*, and *Rb*. Adapted from Le et al. [82]

Rb knock-out mouse models

The retinoblastoma gene (*Rb*) is another tumor suppressor gene frequently involved in the pathogenesis of different kinds of cancer. Inactivation of the *Rb* gene has been associated with familial and sporadic retinoblastomas, small cell lung carcinomas, and osteosarcomas.[85, 86] Rb functions as an essential regulator of cell cycle progression, but inactivation of Rb has also been associated with chromosomal instability, cancer progression, and activation of angiogenesis.[87] Heterozygous inactivation of *Rb* in a conventional mouse model showed adrenal medulla hyperplasia in approximately half of the mice. In combination with knock-down of the *E2f1* gene, which is also an important factor in cell cycle control, the $Rb^{+/-}$ mice even show adrenal medullary hyperplasia in almost all of the cases.[88] Other studies reported pheochromocytomas in 71% of $Rb^{+/-}$ mice [72], and 55% of $Rb^{+/-}p107^{-/-}$ mice.[69]

$p18^{INK4C}$ and $p27^{Kip1}$ knock-out mouse models

$p18^{INK4C}$ and $p27^{Kip1}$ belong to the family of cyclin-dependent kinase (CDK) inhibitors, thereby modulating the cell cycle progression. CDK inhibitors are classified into two families: the CIP/KIP family members are known to inhibit a variety of cyclin-CDK complexes, whereas the INK4 family members specifically inhibit CDK4/CDK6.[89, 90] All CDK inhibitors result in G1 arrest when overexpressed in transfected.

$p18^{INK4C}$ is described as a tumor suppressor gene in glioblastomas.[91] $p18^{INK4C}$ knock-out mouse models present with organomegaly, and disproportionately enlarged pituitary gland, spleen, and thymus are seen, and also of the adrenal gland.[92] In an additional report, this group also published pheochromocytomas in 8.3% of the $p18^{-/-}$ mice, 23.8% of the $p27^{-/-}$ mice, and in 91.3% of the $p18^{-/-}$- $p27^{-/-}$ double knock-out mice.[71] Another study, using the same *P18* knock-out mice, reported a similar frequency of pheochromocytoma occurrence.[80]

Recently germline mutations in the $P27^{KIP1}$ gene have been associated with a novel type of multiple endocrine neoplasia syndrome type 4.[93] Mouse and rat models with inactivated or mutated $P27^{Kip1}$ also presented with a multiple endocrine neoplasia-like syndrome (MENX). Both the $P27^{Kip1-/-}$ knock-out mice and the $P27^{Kip1}$ mutated rats developed pheochromocytomas, the latter of which in 95% of cases.[94, 95] In addition, the rats also developed sympathetic paragangliomas.[94] These rat pheochromocytomas were investigated for genomic imbalances and approximately 30% of the rats displayed loss of chromosomes 8 and 19.[96] Furthermore, the gene expression profiling was performed on the rat pheochromocytomas and revealed a neural precursor cell-like signature,[97] similar to the pheochromocytomas of the *NF1* knock-out mice described earlier.[84]

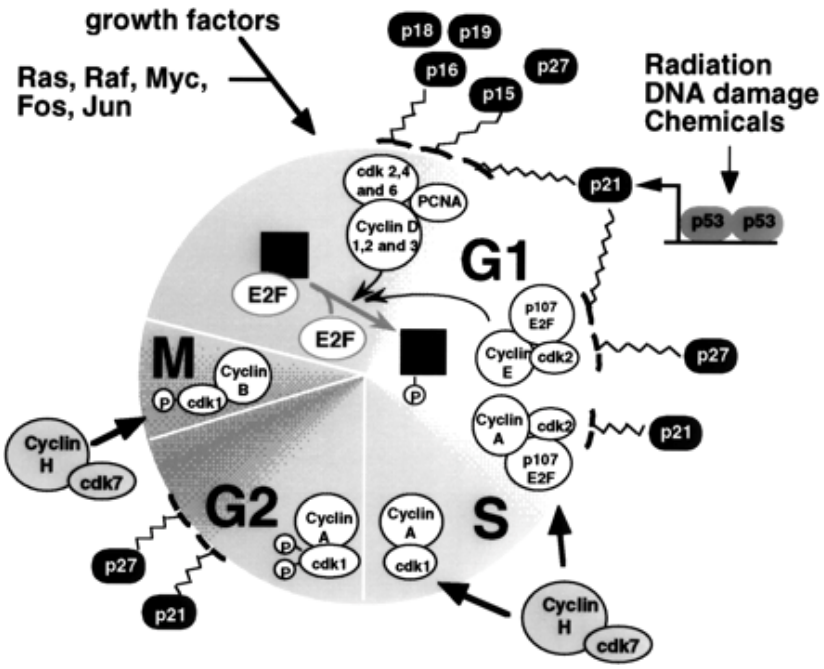

Figure 6. Schematic representation of the cell division showing pathways involving different CDKs and CDK inhibitors during cell division. Adapted from Senderovicz et al.[98]

RET knock-in mouse models

The last model that presents with pheochromocytomas is the RET^{MEN2B} transgenic mouse model.[99] No specific percentage of pheochromocytoma penetrance in this model was mentioned in the report, but besides adrenal tumors, the mice also developed benign neuroglial tumors in the entire sympathetic nervous system, which seem to be histologically identical to human ganglioneuromas.[99]

Table 3. Murine models with pheochromocytomas

Mouse model	AMH	PCC	Metastases	Other affected organs	Reference
$Rb^{+/-}$		71%		Pituitary, thyroid, parathyroid, lung, pancreas	Nikitin et al.[72]
$Rb^{+/-}p107^{-/-}$		4%		Intestine, bone, lymph nodes, ovary, thyroid, lung, testis	Dannenberg et al.[69]
$Rb^{+/-}p130^{+/-}$		55%		Eye, lung	Dannenberg et al.[69]
$Rb^{+/-}$ $Rb^{+/-}E2F1^{+/-}$ $Rb^{+/-}E2F1^{-/-}$	46% 52% 95%			Pituitary, lung, uterine, lymph nodes, gastro, testis, thyroid	Yamasaki et al.[88]
$p18^{Ink4c-/-}$ $p27^{Kip1-/-}$ $p18^{Ink4c-/-}p27^{Kip1+/-}$ $P18^{Ink4c+/-}p27^{Kip1-/-}$ $P18^{Ink4c-/-}p27^{Kip1-/-}$	33% 19% 42% 33% 9%	8% 24% 17% 50% 91%	4% pelvic nerve	Pituitary, thyroid, testis, parathyroid, pancreas, stomach, intestine, lungs	Franklin et al.[71]
$P27^{kip1+/-}$		Yes		Pituitary	Pellagata et al.[95]
$P27^{kip1}$ rat		95%		Pituitary, pancreas, parathyroid, sympathetic paraganglioma (85%)	Fritz et al.[94]
$P27^{kip1;+/CK-}$ $P27^{kip1;CK-/CK-}$	29% 79%			Pituitary, ovarian, lymph nodes, intestine, uterus, liver, breast, harderian	Besson et al.[89]
$Pten^{+/-}$ $p18^{Ink4c-/-}$ $Pten^{+/-}p18^{Ink4c+/-}$ $Pten^{+/-}p18^{Ink4c-/-}$	10% 29% 6% 11%	65% 14% 71% 84%		Pituitary gland, thyroid, prostate, lung, breast	Bai et al.[80]
$Pten^{+/-}$	24%		15% lungs	prostate, breast, salivary gland	You et al.[76]
$Pten^{+/-}$		23%		breast, endometrium, prostate, gastrointestin, lymphoid	Stambolic et al.[75]
$Pten^{+/-}$ $Pten^{+/-}p27^{Kip1-/-}$		100% 100%		Prostate, thyroid, intestine, endometrium, lung	Di Christofano et al.[70]
$Ret^{Met918Thr/Wt}$	16%	2%			Smith-Hicks et al.[74]
$RET^{Met918Thr/Met918Thr}$	100%			Hyperplasia of sympathetic ganglia	Sweetser et al.[99]
$Nf1^{+/-}$ $Nf1^{+/-}$ irradiated		20% 87%			Powers et al.[73]

AMH = adrenal medullary hyperplasia, PCC= pheochromocytoma

1.5 Aims and outline of this thesis

Pheochromocytomas and paragangliomas can occur sporadically, as well as in hereditary syndromes. Up to 30% of the pheochromocytomas and 50% of the paragangliomas are caused by germline mutations in the pheochromocytoma and paraganglioma susceptibility genes. Approximately 10% of the sporadic tumors is malignant, but this frequency can be higher (up to 30%) for *SDHB*-related tumors. Currently, the underlying mechanisms for the pathogenesis of sporadic pheochromocytomas and paragangliomas are poorly understood. Furthermore, there are no predictive markers for malignancy. These issues have been investigated at the molecular level in the first part of this thesis.

Besides the lack of knowledge about predicting malignancy, there are still no curative treatments for progressive disease. In the second part of this thesis, we focused on mouse models that develop malignant pheochromocytomas. These models could provide new insights in the pathogenesis of these tumors and enables us to test new therapeutic strategies.

Although patients with germline mutations in genes that cause pheochromocytoma and paraganglioma susceptible syndromes (*NF1*, *MEN1*, *RET*, *VHL* (VHL type 1, 2A and 2B)) can be recognized by their clinical presentation, patients with germline mutations in other genes (*SDHA*, *SDHB*, *SDHC*, *SDHD*, *SDHAF2*, *TMEM127*, *KIF1B*-β, *VHL* (VHL type 2C)) are not easily recognized, due to incomplete penetration and maternal imprinting for some genes. Genetic testing for all these genes would be a laborious and expensive task. So, in the last part of this thesis we present a method, to determine on what genes germline mutation analysis should be performed.

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

- To search for molecular markers that can distinguish benign from malignant pheochromocytomas and sympathetic paragangliomas.
- To investigate whether mouse models that develop pheochromocytomas can be used as a model for human pheochromocytomas.
- To establish a procedure for the identification of genes to be analyzed in patients with a pheochromocytoma or paraganglioma without obvious syndromal presentation.

Outline

The tumorigenesis of pheochromocytomas in general is poorly understood, particularly of sporadic pheochromocytomas. Furthermore, it is still not clear how these tumors become malignant, or whether they are malignant from onset. In **chapter 2** we show a difference in intra-tumoral molecular heterogeneity between benign and malignant pheochromocytomas and sympathetic paragangliomas, indicating a difference in pathogenesis**.**

Various attempts have been made to distinguish malignant from benign pheochromocytomas and paragangliomas using histological characteristics, or immunohistochemical markers. However, this has not been successful so far. To search for molecular regions that could discriminate benign from malignant tumors, and to obtain insights in the molecular alterations of these tumors, comparative genomic hybridization was performed on benign and malignant sporadic and syndromic pheochromocytomas and sympathetic paragangliomas. The results of the benign series are described in **chapter 3**, and those of the malignant cases in **chapter** 4.

Chapter 5 and **chapter 6** involve studies of a Pten conditional knock-out mouse model and a Pten/Trp53 double knock-out mouse model, using immunohistochemistry and comparative genomic hybridization, to test if they could serve as models for human pheochromocytomas. These mouse models could be used to test different kind of chemotherapies.

Pheochromocytomas can sometimes occur bilaterally, especially in patients with the MEN2 syndrome. Furthermore, sympathetic paragangliomas, previously called extra-adrenal pheochromocytomas, can arise in patients with germline mutations in the *SDH*-genes. In **chapter 7** we screened a series of bilateral pheochromocytomas and sympathetic paragangliomas for mutations in *RET*, *VHL*, *SDHB* and *SDHD*.

Genetic testing is expensive and laborious. The NF1 and MEN2 syndromes can be diagnosed on a clinical basis, so targeted mutation analysis is clear and sometimes even not necessary. However, these syndrome-related patients represent less than 5% of cases. In **chapter 8** we demonstrate that we were able to predict the presence of an *SDH* mutation using SDHB immunohistochemistry.

A recent study described the first *SDHA* mutation causing a paraganglioma. According to this study, the *SDHA*-mutated tumor was immunohistochemically negative for SDHA. In **chapter 9** we investigated the importance of SDHA immunohistochemistry in a large series of pheochromocytomas and paragangliomas.

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Chapter 2

Intra-tumoral heterogeneity in benign and malignant pheochromocytomas and sympathetic paragangliomas

*Esther Korpershoek, *Claudia Stobbe, Francien H van Nederveen, Ronald R de Krijger, Winand NM Dinjens

* These authors should be considered as equal first authors

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Abstract

Pheochromocytomas (PCC) and extra-adrenal sympathetic paragangliomas (sPGL) are catecholamine-producing tumors occurring in the context of hereditary tumor syndromes, with known germline mutations, and as sporadic tumors. The pathogenesis of sporadic PCC and sPGL is poorly understood and little is known about intra-tumoral heterogeneity with respect to molecular aberrations. Since knowledge on intra-tumoral heterogeneity is important for understanding the pathogenesis of these tumors, we investigated 12 benign and 8 malignant PCC and sPGL for loss of heterozygosity (LOH) on DNA extracted from different regions of each tumor and from metastases. LOH markers were selected on chromosomal regions frequently deleted in PCC, including 1p, 3q, 3p, and 11p. Benign tumors were found to have less intra-tumoral heterogeneity (overall 8%) than malignant tumors (overall 23%), with the highest frequencies for 1p36 in the benign tumors (17%) and 1p13 and 3q24 in malignant tumors (both 38%). In addition, differences in LOH patterns were detected between paired primary malignant tumors and their metastases and different LOH patterns were observed in bilateral PCC of a MEN 2 patient. We demonstrate that malignant PCC and sPGL have more intra-tumoral molecular heterogeneity than benign tumors, which suggests that benign and malignant PCC and sPGL have a different pathogenesis.

Introduction

Pheochromocytomas (PCC) are rare catecholamine-producing neuro-endocrine tumors arising from chromaffin cells of the adrenal medulla. Extra-adrenal sympathetic paragangliomas (sPGL) were previously known as extra-adrenal PCC. sPGL are neuro-endocrine tumors, mostly found in the abdomen, and usually produce catecholamines. PCC and sPGL are histologically indistinguishable and share many clinical features, although sPGL are more frequently malignant than PCC. [1-5]

Most PCC are sporadic but up to 24% are associated with hereditary syndromes such as multiple endocrine neoplasia type 2 (MEN 2 caused by germline mutations in the proto-oncogene *RET*), Von Hippel-Lindau disease (VHL caused by germline mutations in the tumor suppressor gene *VHL*), PCC-PGL syndrome (caused by germline mutations in one of the tumor suppressor genes *SDHB, -C, –D*, or *–AF2*) and neurofibromatosis type 1 (NF1 caused by germline mutations in the *NF1* tumor suppressor gene).[4, 6] The percentage of PCC associated with familial syndromes is higher than previously assumed, as several studies have demonstrated germline mutations in PCC susceptibility genes in apparently sporadic PCC.[7-9]

While loss of chromosome 1p is the most common genetic aberration reported in sporadic PCC[10-13], particular genetic alterations have been demonstrated in syndrome-related PCC.[12, 14, 15] Generally, MEN 2-related PCC show loss of chromosomes 1p and 3q[12, 14], SDHD-related PCC and PGL display loss of chromosome 11[16], and in NF1-related tumors frequent loss of 1p and 17q is found.[12, 17]

Few studies have demonstrated that sporadic PCC have similar genetic aberrations as those found in syndrome-related tumors, such as loss of chromosomes 1p, 3p, 3q, 11p, and 11q.[10-13] Although the results at large of these studies are in agreement with each other, there are also differences. In a previous study, we have shown loss of chromosome 6 in 34% of sporadic PCC[11], while this relatively high percentage was not found in other studies.[10, 12] In addition, Cascon et al reported loss of chromosome 8p in 62% of PCC[10], whereas this high percentage was not observed in other studies.[11-13] These discrepancies could be due to geographical variations and hereditary background of the patients, differences in methods, but can also be the effect of intra-tumoral molecular heterogeneity.

Intra-tumoral molecular heterogeneity has been demonstrated in many different tumors, such as in renal tumors[18], head and neck squamous cell carcinomas[19], lung tumors[20], cervical cancer[21], and meningiomas[22], but has been poorly investigated in PCC. Jarbo et al reported that the ratios of chromosome 22q loss in some PCC samples were higher than expected for a single allele ratio and suggested this could be due to intra-tumoral heterogeneity.[14] Diaz-Cano et al investigated heterogeneity of sporadic and MEN 2-related PCC and adrenal medullary hyperplasia nodules (AMH), by determining the methylation patterns of the Androgen Receptor (*AR*) gene, localized on the X-chromosome.[23] All informative AMH showed concordant inactivation of the same alleles in different nodules from the same adrenal gland, suggesting that these AMH arose from a common progenitor and are clonally-related proliferations. In addition, the authors reported that a high percentage of sporadic PCC, benign as well as malignant, also show intra-tumoral concordant inactivation of the same *AR* alleles, and therefore PCC were considered monoclonal in origin. Apart from this study, no reports exist about intra-tumoral heterogeneity of PCC on the molecular level.

PCC and sPGL are, as most other tumors, considered monoclonal proliferations originating from one transformed cell. Transformation of a normal cell into a tumor cell is caused by the accumulation of mutations, amongst others in oncogenes and tumor suppressor genes. This implies that mutations occurring early in tumorigenesis are present in each individual tumor cell, whereas DNA aberrations that occurred later, during tumor growth, can be present only in a subpopulation of tumor cells. When investigating multiple, macroscopically separated, intra-tumoral regions for DNA aberrations, homogeneity of mutations indicates early aberrations whereas heterogeneity points to later occurrence.

Molecular heterogeneity is important for elucidating the molecular pathogenesis of PCC. In addition, intra-tumoral heterogeneity is important for the interpretation of loss of heterozygosity (LOH) and comparative genomic hybridization (CGH) results in general. Furthermore, heterogeneity of PCC has never been thoroughly investigated. Therefore, we systematically investigated LOH in 4 macroscopically separated parts within each of 12 benign tumors (11 PCC and 1 sPGL) from 11 patients, and 8 malignant tumors (7 PCC and 1 sPGL) and 8 metastases from 8 patients.

Materials and methods

Patients

Nineteen patients with PCC or sPGL were selected, of whom 11 had benign tumors (10 PCC and 1 sPGL) and 8 had malignant tumors (7 PCC and 1 sPGL). All 8 patients with malignant tumors had distant metastases verified by histology. The tumors were retrieved from the archives of the Departments of Pathology of the Erasmus MC, University Medical Center, Rotterdam (15 cases), Maasstad Hospital, Rotterdam (1 case), University Medical Center Utrecht (1 case), Albert Schweitzer Hospital Dordrecht (1 case) and PAMM foundation, Veldhoven (1 case), the Netherlands. The tumors were anonymously used according to the code for adequate secondary use of tissue, code of conduct: "Proper Secondary Use of Human Tissue" established by the Dutch Federation of Medical Scientific Societies (http://www.federa.org).

The benign series included 6 sporadic cases and 5 patients with syndrome-related tumors: two patients with NF1 (clinically determined) and one patient with MEN 2 (*RET* germline
mutation Cys634Arg) of whom both tumors (left and right adrenal) were investigated and 2 patients with the PCC-PGL syndrome (*SDHD* germline mutation Asp92Tyr). One sporadic patient with a benign PCC was also diagnosed with a clear cell renal cell carcinoma (RCC). Although an RCC can be a feature of VHL disease, genetic analysis did not reveal a germline *VHL* mutation. The malignant series was comprised of 7 sporadic PCC, 1 PCC-PGL syndrome-related sPGL (*SDHD* germline mutation Asp92Tyr), and 7 metastases.

The mean patient age at diagnosis was 46 years (range 16-76 yrs) and 54 years (range 36-63 yrs) for the benign and malignant series, respectively. The mean primary tumor diameter was 3.9 cm (range 0.9-6 cm) for the benign series and 8.7 cm (range 0.7-15 cm) for the malignant series. Patient characteristics and clinical data are summarized in table 1.

Table 1. Clinical data of pheochromocytoma and sympathetic paragangliomas patients

	patient	gender	age (years)	other features	location primary tumor	tumor diameter (cm)	tumor weight (gr)	location metastases
benign	F238	F	46	Renal cell carcinoma	a	2	14	-
	F244	M	52	NF1	A	u	U	-
	F247	u	u	-	A	4	22	-
	F249	F	51	MEN2A - bilateral	A	4 / 0.9	23 / 19	-
	F319	F	76	-	A	4	25	-
	F320	F	39	-	A	6	54.3	-
	F321	F	55	-	Ea	6	24	-
	F322	M	16	PCC-PGL syndrome	A	4.5	68.1	-
	F323	F	31	PCC-PGL syndrome	A	u	38	-
	F325	M	29	NF1	A	3	18.8	-
	F327	M	65	-	A	4.5	78	-
malignant	F123	M	62	-	A	5	176	abdominal
	F126	F	63	-	A	10	U	lymph nodes
	F228	F	61	-	A	12	U	lymph nodes
	F235	M	42	-	A	8.5	166	abdominal
	F240	M	61	-	A	u	U	lymph nodes
	F245	F	43	-	A	0.7	23	abdominal
	F299	M	36	-	A	15	U	lymph nodes and liver
	F326	M	62	PCC-PGL syndrome	Ea	10	264	liver

F = female, M = male, a = adrenal, ea = extra-adrenal, u = unknown

DNA preparation

Forty-seven tumor DNA samples were isolated from 4 macroscopically separated areas within the paraffin-embedded tumors of 11 patients, and from 3 areas within the tumor of 1 patient with benign tumors. Matching normal DNA was isolated from paraffin-embedded healthy tissues of other unaffected organs or normal tissue surrounding the tumor. For the malignant tumors an additional DNA sample was isolated from metastatic tissue (Figure 1). In total, 37 tumor DNA samples were isolated from the PCC or sPGL, and metastases of the patients with malignant tumors. From patient 18 only one small primary tumor and 2 metastases were available for investigation, of which 3 tumor DNA samples were isolated. Furthermore, of patient 19 only the primary tumor was available and used to isolate 4 tumor DNA samples.

DNA was isolated using the D-5000 Puregene DNA Isolation kit (Gentra Systems Minneapolis, MN) according to the manufacturer's recommendations. Before and after DNA extraction, HE staining of each section was performed to confirm the percentage of tumor cells in the isolated samples, which was above 80% for proper LOH analysis.

LOH analysis

LOH analysis was performed with 2 different methods, using 10 markers on 5 loci, which have been reported to be frequently lost in PCC. The markers selected were localized on chromosomes 1p13 (D1S252 and D1S2881), 1p36 (D1S2885 and D1S234), 3p13 (D3S3681 and D3S3551), 3q24 (D3S1569 and D3S3694), and 11p13 (D11S4083 and D11S4203).

The first PCR method was carried out using α32P-dATP (Amersham, Buckinghamshire, United Kingdom) and run on a denaturing 6% polyacrylamide gel (Fluka, Neu-Ulm, Germany). In brief, PCR was performed with 1 µl DNA (30-100 ng/ µl)

in a final volume of 15 µl containing 1.5 mM MgC12, 10 mM Tris-HCL, 50 mM KCL, 0.02 mM dATP, 0.2 mM dGTP, dTTP and dCTP, 0.8 µCi α32P-dATP, 15 pmol of each forward and reverse primer and 1 U Taq polymerase (Promega, Madison, WI). PCR was performed for 35 cycles of 95˚C for 30 seconds, 58˚C for 45 seconds, and 1 minute at 72˚C, followed by 1 cycle at 72˚C for 10 minutes. PCR products of tumor and normal DNA were run on a denaturing 6% polyacrylamide gel (Fluka, Neu-Ulm, Germany). After electrophoresis for 2 hours at 65 Watt, the gels were dried and exposed to X-ray films. The results were confirmed by a previously described PCR method[24], using fluorescence-labeled primers (Invitrogen, Paisley, UK) and ABI 3130-XL genetic analyzer (Applied Biosystems, Foster City, CA) for analysis. The results of both methods were analyzed by 2 independent investigators (W.D. and E.K).

Cases were classified as informative when two alleles were found in normal DNA. When relative intensities of allelic signals observed in tumor DNA clearly differed from those in normal DNA this was considered as loss. LOH was further categorized as loss of the lower (or smaller) or upper (or larger) allele. A tumor was considered genetically heterogeneous when LOH was present in at least one but not in all of the samples of the same tumor or if the samples differed in loss of a lower or upper allele.

Results

LOH frequencies

Results of the LOH analysis for each marker in the benign and malignant tumors are shown in tables 2 and 3, respectively. The data is also summarized for both tumor groups in table 4. PCR with a second marker was only performed, if the patient was not informative for the first marker for the investigated locus.

Most frequent losses were found for chromosome 1p occurring in up to 75% (considering 1p13) for both benign and malignant tumors. Loss of chromosome 3q occurred in 45% (5/11) of the benign, and in 63% (5/8) of the malignant tumors. Loss of chromosome 3p was found in 25% (3/12) of the benign and in 38% (3/8) of the malignant tumors. In addition, loss of chromosome 11p was seen in 50% (6/12) of the benign and in 17% (1/6) of the malignant tumors. One PCC (patient 16) of the malignant series did not reveal any LOH.

Notably, two malignant tumors showed homogeneous LOH patterns in the different primary tumor samples, but no LOH in the DNA derived from the metastasis. This occurred in patient 12 for chromosome 3p12 and in patient 17 for chromosome 1p13. The overall LOH frequency did not differ between the benign (44%) and malignant (47%) tumors.

Heterogeneity frequencies

A summary of the intra-tumoral heterogeneity results is shown in Table 4. An example of heterogeneity is illustrated in Figure 2. Tumor DNAs were isolated from macroscopically different parts of the tumor. There was no correlation between histology and molecular heterogeneity, as these macroscopically different parts were histologically similar (Figure 1). Intra-tumoral heterogeneity was found in 42% (5/12) and 75% (6/8) of the benign and malignant tumors, respectively. In the chromosomal regions reported to have high frequencies of LOH in PCC (1p13 and 3q), there was more intra-tumoral heterogeneity

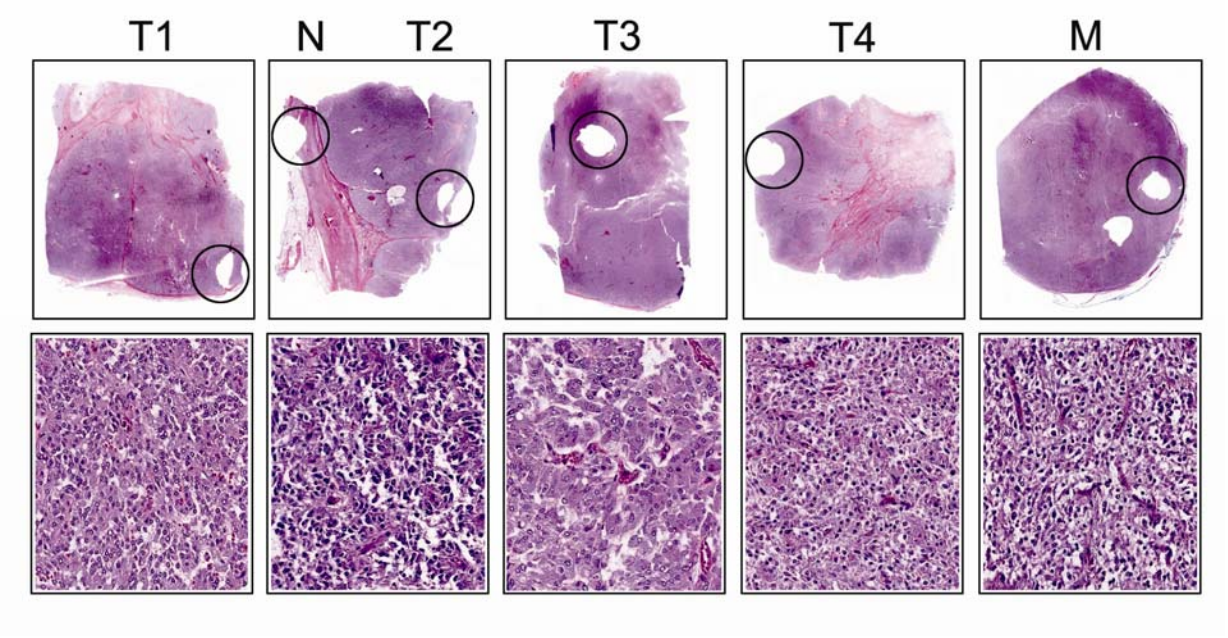

Figure 1. Haematoxylin and Eosin staining of slides of 5 different blocks from the PCC and metastasis of patient 13. From each block one tumor DNA was isolated (T1-T4 and M). Normal DNA (N) was isolated from healthy tissue surrounding the PCC in block T2+N.

present in the malignant tumors (37.5% for both regions) compared with the benign tumors (8.3% for both regions). In contrast, chromosome 11p showed no intra-tumoral heterogeneity in the malignant tumors, compared with 16.7% in the benign tumors. Additionally, the average frequency of intra-tumoral heterogeneity of the malignant tumors (22.5%) was higher than of the benign tumors (10.2%).

Syndrome-related LOH results

Remarkably, both PCCs of the MEN 2A patient (patient 11) revealed different aberrations in chromosomal region 1p13: both tumors, opposing 1p14 allele loss was observed homogeneously in all tumor samples of each tumor (Table 2). In addition, tumor A of patient 11 showed homogeneously loss of chromosome 3q24 in all tumor samples, whereas contralateral tumor B did not display 3q24 LOH in any f the tumor DNA samples (Table 2).

Three patients had PCC-PGL syndrome caused by a germline *SDHD* mutation (localized on chromosome 11). The PCC of 2 PCC-PGL syndrome patients (Table 2, case 8 and 9) showed LOH of chromosome 11p in all tumor samples; the sPGL of the other patient (Table 3, case 19) was not informative for the chromosome 11 marker. In addition, two patients had NF1-related PCC (characterized by loss of chromosome 1p36), which both displayed homogeneous loss of 1p36 in all samples (Table 2, case 2 and 10).

Discussion

The knowledge of intra-tumoral molecular heterogeneity in PCC and sPGL is important for understanding the pathogenesis of these tumors. In addition, heterogeneity can have consequences for interpretation of molecular data. Therefore, we determined the intra-tumoral molecular heterogeneity in a series of 12 benign tumors (11 PCC and 1 sPGL) and 8 malignant tumors (7 PCC and 1 sPGL) by LOH analysis in different areas of the tumors. Molecular intra-tumoral heterogeneity within tumors has been reported in 55% of meningiomas[22], 45% of renal tumors[18], 2.7% of cervical cancer[21], and in 8.8% of

PCC[23]. It is important to emphasize that these frequencies show extreme variation because: 1) the amount of markers or probes used per study are different. The more probes being used, the more heterogeneity is found. In case of Bachtiary et al, who used expression arrays to investigate a series of cervical cancer, a ratio of two internal controls was used to determine intra-tumoral heterogeneity.[21] A ratio of more than 0.90 was considered as high heterogeneity (2.7%), whereas less than 0.10 was regarded as extreme low heterogeneity (2.2%). In contrast, ratios in between had a global lower heterogeneity, so he frequency of heterogeneity will be up to 100%; 2) other studies used only few markers or probes[18,22,23], whose genomic location is of great importance. When probes are chosen in genomic areas that are lost early in pathogenesis, the tumors will show no or little intra-tumoral heterogeneity, whereas probes on chromosomes that are altered later in tumorigenesis will result in high frequencies of heterogeneity; and 3) PCC can occur in several syndromes, such as the MEN 2 syndrome, VHL disease, or the PCC-PGL syndrome.

The PCC in these syndromes have different alterations that occur early in the pathogenesis, such as loss of chromosomes 1p and 3q in MEN 2-related PCC and loss of chromosome 11 in SDHD-related PCC. This could explain why SDHD-related PCC show heterogeneity for markers on chromosome 1p and MEN 2-related PCC show heterogeneity for markers on chromosome 11q in our study. LOH of these chromosomes was most likely a late event in the pathogenesis in both PCC.

Our LOH results, showing heterogeneity in only 1 of the 12 benign tumors, are in concurrence with literature reports since chromosomes 1p13 and 3q are reported as early aberrations in benign sporadic and MEN 2A-related PCC [11,13] and loss of chromosome 1p36 is the most frequent molecular alteration in NF1-related PCC [12,17]. The tumor showing heterogeneous loss of chromosomes 1p13 and 3q24 was an SDHD-related PCC, with consistent identical LOH of 11p in all tumor samples, indicating monoclonal origin. SDHD-related tumors are characterized by bi-allelic inactivation of the *SDHD* gene: the combination of a germline mutation in the *SDHD* gene and loss of the remaining wild type allele (11q23). Therefore, in SDHD-related tumors loss of the wild type chromosome 11q allele is most likely the tumor-initiating event preceding loss of chromosome 1p.

MEN 2A-related PCC are characterized by loss of chromosomes 1p and 3q.[12-14] Our study included one MEN 2A patient with bilateral benign PCC. In both tumors homogeneous loss of chromosome 1p in all tumor samples was found. However, both tumors showed loss of opposing 1p alleles. In addition, loss of chromosome 3q was seen in only one of the bilateral PCC. The PCC of this MEN 2A patient showed inter-tumoral heterogeneity, indicating that these tumors occurred independently.

It is generally accepted that neoplastic cells harbor clonal molecular aberrations and normal cells do not. Tumors, like PCC and sPGL, are composed of neoplastic and normal cells. Consequently, DNA isolated from a - part of - PCC or sPGL is composed of DNA of neoplastic cells and normal cells. With LOH analyses tumor and corresponding normal DNA are compared, so information about loss of DNA within the tumor sample can be obtained, but also information about which allele - or microsatellite marker - is lost. In this study, most tumor DNAs were isolated from parts that consisted of high percentages neoplastic cells, since histology of the tumor blocks was checked before and after DNA isolation. In addition, admixture of high percentages normal cells would mask

Table 2. LOH results of the benign pheochromocytomas and extra-adrenal sympathetic paragangliomas

Locus	Patient	Marker 1				Marker 2			
1p13		T1	T2	T3	T4	T1	T2	T3	T4
	1					x	x	x	x
	2	■	■	■	■	x	x	x	x
	3	▒	▒	▒	▒	x	x	x	x
	4	▒				▒			
	5	NI	NI	NI	NI				
	6					NI	NI	NI	NI
	7	▒	▒	▒	▒	x	x	x	x
	8	▒	▒	▒	▒	x	x	x	x
	9	NI	NI	NI	-	■	■	■	-
	10	■	■	■	■	x	x	x	x
	11A	▒	▒	▒	▒	x	x	x	x
	11B	■	■	■	■	x	x	x	x
1p36		T1	T2	T3	T4	T1	T2	T3	T4
	1								
	2					NI	NI	NI	NI
	3								
	4	■				■			
	5	NI	NI	NI	NI	■	■	■	■
	6	■	■	■	■	x	x	x	x
	7	■	■	■	■	x	x	x	x
	8	■	■	■	■	x	x	x	x
	9	▒	▒	▒	-	x	x	x	x
	10	▒	▒	▒	▒	x	x	x	x
	11A	NI	NI	NI	NI				■
	11B	NI	NI	NI	NI				
3q24		T1	T2	T3	T4	T1	T2	T3	T4
	1					x	x	x	x
	2					x	x	x	x
	3		■		■		▒		▒
	4					x	x	x	x
	5	NI	NI	NI	NI				
	6								
	7	NI	NI	NI	NI	▒	▒	▒	▒
	8	NI	NI	NI	NI	NI	NI	NI	NI
	9	NI	NI	NI	-	■	■	■	-
	10	▒	▒	▒	x	▒	▒	▒	▒
	11A	■	■	■	■	x	x	x	x
	11B					x	x	x	x

Locus	Patient	Marker 1				Marker 2			
3p12		T1	T2	T3	T4	T1	T2	T3	T4
	1					X	x	x	x
	2					X	x	x	x
	3					X	x	x	x
	4					X	x	x	x
	5					X	x	x	x
	6					X	x	x	x
	7	■	■	■	■	X	x	x	x
	8	NI	NI	NI	NI				
	9	▒	▒	▒	-	▒	▒	▒	-
	10	▒	▒	▒	▒	X	x	x	x
	11A					X	x	x	x
	11B					X	x	x	x
11p13		T1	T2	T3	T4	T1	T2	T3	T4
	1	NI	NI	NI	NI	▒	▒	▒	▒
	2	■							
	3					X	x	x	x
	4	▒	▒	▒	▒	X	x	x	x
	5	■	■	■	■	■	■	■	■
	6					X	x	x	x
	7	NI	NI	NI	NI	▒	▒	▒	▒
	8			■	■	X	x	x	x
	9				-	X	x	x	x
	10					X	x	x	x
	11A					X	x	x	x
	11B					X	x	x	x

□ = no LOH, ▒ = loss of upper allele, ■ = loss of lower allele, NI = not informative, x = no data, - = no tissue available, T1-T4 = tumor DNA samples from different areas of the tumor.

Table 3. LOH results of the malignant pheochromocytomas and extra-adrenal sympathetic paragangliomas

Locus	Patient	Marker 1						Marker 2					
1p13		**T1**	**T2**	**T3**	**T4**	**M**	**M2**	**T1**	**T2**	**T3**	**T4**	**M**	**M2**
	12						-	x	x	x	X	X	-
	13	NI	NI	NI	NI	NI	-						-
	14						-	NI	NI	NI	NI	NI	-
	15						-	NI	NI	NI	NI	NI	-
	16						-	x	x	x	x	X	-
	17	NI	NI	NI	NI	NI	-						-
	18		-	-	-			x	-	-	-	X	x
	19	NI	NI	NI	NI	-	-					-	-
1p36		**T1**	**T2**	**T3**	**T4**	**M**	**M2**	**T1**	**T2**	**T3**	**T4**	**M**	**M2**
	12						-	x	x	x	x	X	-
	13						-	x	x	x	x	X	-
	14						-						-
	15						-						-
	16						-						-
	17						-	NI	NI	NI	NI	NI	-
	18		-	-	-			x	-	-	-	X	x
	19					-	-	x	x	x	x	-	-
3q24		**T1**	**T2**	**T3**	**T4**	**M**	**M2**	**T1**	**T2**	**T3**	**T4**	**M**	**M2**
	12						-	x	x	x	x	X	-
	13	NI	NI	NI	NI	NI	-						-
	14						-	x	x	x	x	X	-
	15						-	x	x	x	x	X	-
	16						-	x	x	x	x	X	-
	17						-	NI	NI	NI	NI	NI	-
	18		-	-	-			x	-	-	-	X	x
	19	NI	NI	NI	NI	-	-				-	-	-
3p12		**T1**	**T2**	**T3**	**T4**	**M**	**M2**	**T1**	**T2**	**T3**	**T4**	**M**	**M2**
	12						-	x	x	x	x	X	-
	13	NI	NI	NI	NI	NI	-						-
	14	NI	NI	NI	NI	NI	-						-
	15						-	x	x	x	x	X	-
	16						-	x	x	x	x	X	-
	17						-	x	x	x	x	X	-
	18	NI	-	-	-	NI	NI		-	-	-		
	19	NI	NI	NI	NI	-	-					-	-
11p13		**T1**	**T2**	**T3**	**T4**	**M**	**M2**						
	12						-						
	13						-						
	14						-						
	15	NI	NI	NI	NI	NI	-						
	16						-						
	17						-						
	18		-	-	-								
	19	NI	NI	NI	NI	-	-						

□ = no LOH, ▒ = loss of upper allele, ■ = loss of lower allele, NI = not informative, x = no data, - = no tissue available, T1-T4 = tumor DNA samples from different areas of the tumor, M and M2 = tumor DNA samples of 2 different PCC metastasis.

Table 4. Overview of LOH and intra-tumoral heterogeneity results

Benign tumors	**1p13**	**1p36**	**3q**	**3p**	**11p**
LOH	9/12 (75)	8/12 (67)	5/11 (45)	3/12 (25)	6/12 (50)
intra-tumoral heterogeneity	1/12 (8)	2/12 (17)	1/11 (9)	0/12 (0)	2/12 (17)

Malignant tumors	**1p13**	**1p36**	**3q**	**3p**	**11p**
LOH	6/8 (75)	5/8 (63)	5/8 (63)	3/8 (38)	1/6 (17)
intra-tumoral heterogeneity	3/8 (38)	2/8 (25)	3/8 (38)	1/8 (13)	0/6 (0)

Numbers (in brackets percentages) represent LOH or heterogeneity observed in patients/total patients that were informative.

the detection of aberrations. In our study, all 48 DNA samples of the benign tumor series and in 29 of 37 DNA samples from the malignant series unequivocal LOH was observed with at least one LOH marker. This indicates that these DNA samples were all derived from a homogeneous tumor cell population with little admixture of normal - endothelial or stromal - cells. However, 5 DNA samples from patient 16 and in 3 of 4 samples from patient 19 did not show LOH with 4 and 6 informative markers, respectively. This is probably due to the absence of genomic alterations at these loci, but to confirm this more markers should be tested.

Molecular heterogeneity could also be due to technical artifacts. However, although PCR-based assays can vary in sensitivity between loci, sensitivity is unlikely to differ between samples if the same primers and PCR-conditions were used. The sensitivity of detecting LOH is based on the PCR sensitivity (is a PCR product generated or not), the size difference between the homologous alleles in the individual sample (small differences of e.g. 1 repeat unit, 2 nucleotides, are more difficult to evaluate than large differences) and the percentage of clonal neoplastic cells in the tumor fragment from which the DNA is isolated. But, because the LOH is determined per marker by comparing the tumor DNA with the normal DNA (of the same patient) retrieved from the same FFPE tissue block, PCR efficiency is not influencing the results, as LOH is determined within one locus.

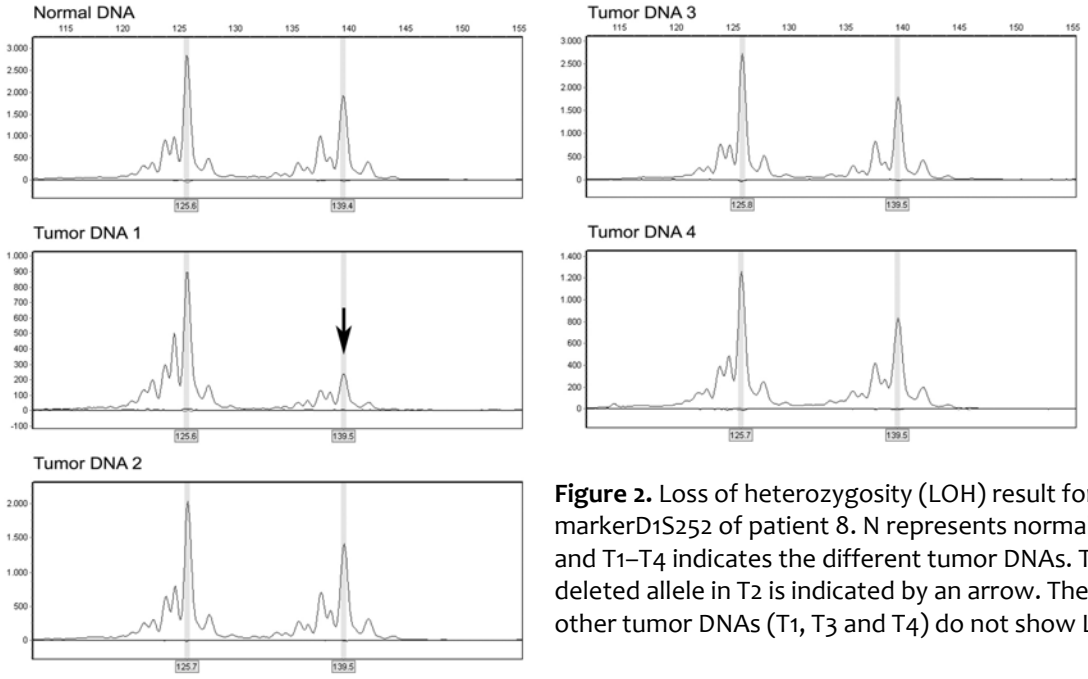

Figure 2. Loss of heterozygosity (LOH) result for markerD1S252 of patient 8. N represents normal DNA and T1–T4 indicates the different tumor DNAs. The deleted allele in T2 is indicated by an arrow. The other tumor DNAs (T1, T3 and T4) do not show LOH.

Previously, tumors were thought to become malignant through a multi-step process, on the cellular level, of accumulation of mutations in benign tumor cells leading to malignant cellular transformation and intra-tumoral heterogeneity.[25] Recently, however, it has been hypothesized that tumors can already be malignant from the onset of tumorigenesis and therefore would be less heterogeneous than thought previously.[25] Genomic aberrations of metastases in association with the primary tumor have been investigated.[12-14] Loss of chromosome 1p is an early event in the pathogenesis of malignant (sporadic) PCC.[10, 11] Our results show LOH of chromosome 1p13 in 75% of malignant tumors, half of those revealing heterogeneity. These results imply that loss of 1p13 is probably not an early event in the pathogenesis of these PCC. Two of these 3 PCC, heterogeneous for the loss of the 1p13 locus, showed homogeneous LOH for chromosome 1p36 or 3p12 and 3q24, which suggests that these tumors are monoclonal proliferations with different genetic backgrounds.

It has been proposed that malignant tumors are generally larger than benign tumors, as was the case in our study. This suggests more cell divisions resulting in a higher chance of genetic aberrations. However, Dannenberg et al. showed that there was no correlation between tumor size and the number of alterations in a comparative genomic hybridization study of benign and malignant PCC.[11] Yet, Dannenberg et al. used a conventional method with a low resolution, so conclusions could only be made firmly about large genomic regions (> 10MB). Furthermore, another more recent study showed more genetic aberrations in malignant tumors compared to benign tumors.[26] Therefore, malignant PCC could also demonstrate molecular heterogeneity at a higher frequency. However, it is very likely that molecular aberrations that occur early in the tumorigenic process will show no heterogeneity in different parts of the tumor. So, the increased frequency of molecular heterogeneity in malignant tumors in our study suggests a different molecular pathogenesis, rather than a bystander effect of the tumor size.

In conclusion, we have investigated intra-tumoral molecular heterogeneity in benign and malignant PCC and found more heterogeneity in malignant tumors in general. Six of the 8 malignant tumors showed intra-tumor heterogeneity for any of the markers studied. The markers on chromosome 1p13 and 3q24, which are lost early in the pathogenesis of benign PCC, result in more heterogeneous patterns in malignant tumors. This indicates that the malignant tumors might have a different molecular tumorigenesis in comparison to benign tumors, probably with losses and/or gains of other genomic regions as early events during pathogenesis. However, 4 of the 12 benign cases also showed intra-tumoral heterogeneity. Therefore, the results of our study also emphasize that caution must be taken when only small areas of a tumor are used for molecular studies. Only molecular aberrations that occurred early in tumorigenesis and have resulted in a pronounced selective growth advantage will be homogeneously present in the tumors. In addition, we demonstrated that intra-tumoral molecular heterogeneity occurred more often in malignant tumors than in benign tumors, suggesting that malignant and benign PCC evolve along different tumorigenic processes. These findings suggest that benign and malignant PCC can be different entities, whereby malignant tumors can be malignant from the onset and do not necessarily evolve from benign tumors.

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Chapter 3

Array-CGH in sporadic benign pheochromocytomas

Francien H. van Nederveen, Esther Korpershoek, Ronald J. deLeeuw, Albert A. Verhofstad†, Jacques W. Lenders, Winand N.M. Dinjens, Wan L. Lam and Ronald R. de Krijger

† deceased

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Abstract

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

Introduction

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

Both syndrome-related PCC and sporadic PCC have been analyzed by comparative genomic hybridization (CGH). Interestingly, PCC from MEN2 patients, NF1 patients and the majority of sporadic PCC show similar genomic aberrations with a characteristic loss of 1p and 3q.[3-5] VHL-related PCC, however, show distinct genetic aberrations consisting of loss of chromosome 3 and 11.[6, 7]

With the introduction of high-resolution array-CGH, it has become technically feasible to study small (submegabase) chromosomal deletions and gains that escaped detection by conventional CGH due to the low resolution. This technique has facilitated the analysis of chromosomes 21 and 22, which were difficult to analyze in conventional CGH. Recently, copy number imbalances affecting chromosome 22 were confirmed by submegabase array-CGH in 44% (29/66) of PCC analyzed, a percentage that had not been described in conventional CGH.[8] In addition, the tiling order of bacterial artificial chromosome (BAC) clones also has the advantage to rule out mismapped clones, and gives precise breakpoint information. A similar array-CGH analysis has been performed on chromosome arm 1p, with 24 samples from hereditary and sporadic PCC, in which breakpoints of chromosome 1p could be identified precisely. These studies illustrate important differences between conventional and array-CGH.[9] To further clarify the pathogenesis of sporadic PCC, we analyzed 36 sporadic benign PCC using a tiling array consisting of 32,433 BAC clones.

Materials and methods

Patients and tumor samples

A series of 40 benign PCC of 40 patients was obtained from the archives of the Departments of Pathology of the Erasmus MC-University Medical Center Rotterdam, Maastricht University, Academic Medical Center Amsterdam, University Medical Center St. Radboud Nijmegen, and University Medical Center Utrecht, The Netherlands and stored at the Erasmus MC tissue bank. Patients with each of the following characteristics were excluded from this study: positive family history of an endocrine hereditary cancer syndrome, evidence of NF1, multiple PCC and/or PGL or the presence of germline mutations. In addition, none of the patients had other tumors related to MEN2, VHL or the PCC-PGL syndrome. After this selection, the study was performed with 36 benign truly sporadic tumors of 36 patients, of which 2 patients with sympathetic PGL. The cohort consisted of 17 females and 19 males. The mean age was 49 years (range 9-76), with an average follow-up of 4.4 years (n=25 patients, 11 patients were lost to follow-up). None of the patients had evidence of metastatic or recurrent disease during follow-up. The mean diameter of the tumors was 5.6 cm (range 2.5-18cm). The clinical data are detailed in table 1. Histology of all tumors was reviewed to confirm the diagnosis of PCC. None of the tumors had adverse histopathological characteristics as published by Thompson [10], supporting the diagnosis of a benign PCC in all cases. Tumor DNA was isolated from fresh frozen tumor tissue, except for 4 tumors in which no frozen tissue was available and DNA was isolated from paraffin embedded archival material. DNA from both fresh frozen and paraffin embedded material was isolated using the D-5000 Puregene DNA Isolation kit (Gentra Systems Minneapolis, MN) according to the manufacturers' recommendations.

Array CGH labeling and hybridization

The submegabase tiling arrays (SMRT) previously described by Ishkanian et al. were used, consisting of 32,433 overlapping BAC clones.[11] Test DNA and pooled reference male DNA (Novagen, Mississauga, Ontario), (300 ng each) were labeled with Cyanine-3 and Cyanine-5 (PerkinElmer, Woodbridge, ON, Canada) respectively, according to a random priming protocol. After 18 hours of random priming the reference and test DNA were combined and 100 µl of Cot-1 DNA (Invitrogen, Burlington, ON, Canada) was added. The mixture was purified using Microcon YM-30columns (Millipore, Mississaga, ON, Canada). The purified mixture was washed with 200 µl of H_2O, and resuspended in 45 µl of DIG easy hybridization solution (Roche, Laval, QC, Canada), containing 20 mg/ml sheared herring sperm (Sigma-Aldrich, Oakville ON, Canada) and 10 mg/ml yeast tRNA (Calbiochem, Mississauga, ON, Canada). The probe was denatured at 85^{o}C for 10 minutes, followed by 60 minutes at 45^{o}C to block repetitive sequences, and subsequently applied in a volume of 43 µl to the slide surface after which cover slips were applied. The slides were incubated at 45^{o}C for 36 hours, washed 4 times 5 minutes in 0.1x saline sodium citrate (SSC), 0,1% SDS at room temperature, and finally rinsed by 0.1x SSC for 5 times and dried by centrifugation.

Array imaging and analysis

Hybridized slides were scanned using a charge-coupled device (CCD) camera system (Applied Precision, Issaquah, WA, USA), and analyzed by SoftWoRx Tracker Spot Analysis

Table 1: clinical data

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

A = adrenal, EA = extra-adrenal, * died, not related to PCC, ** NE=norepinephrine, E=epinephrine, D=dopamine, ACTH= adrenocorticotrope hormone. Bold italic hormones are dominantly produced.

software (Applied Precision). Resultant data was normalized using a stepwise normalization process [12]. Copy number alterations were identified via data visualization using custom software called "SeeGH" (freely available at http://www.flintbox.ca/technology.asp?tech=FB312FB) and loss, normal, and gain probabilities for each clone as determined by a modified hidden Markov model [13, 14]. Data were filtered based on both replicate standard deviation (data points with greater than 0.1 standard deviation removed) and signal to noise ratio (data points with a signal to noise ratio less than 10 removed).

Mutation *analysis*

Mutation analysis was performed on a CGH profile basis. Tumors showing loss of 1p were screened for *SDHB* mutations (n=26), those with loss of 3p for *VHL* mutations (n=11) and those with loss of 11q for *SDHD* mutations (n=10). Because no specific profile is indicative of involvement of the *RET* proto-oncogene, all 35 tumors were tested for *RET* mutations. All exons including the intron-exon boundaries were screened, with the exception of *RET* for which only exons 10, 11, 13 and 16 were investigated. PCR and sequencing conditions have been previously described by Korpershoek et al.[15] Corresponding normal DNA was tested when an alteration was found in the tumor DNA.

Statistical analysis

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

Results

Array CGH

All but 2 tumors included in this study yielded interpretable array results. A frequency plot, adding up percentages of loss and/or gain of each individual BAC clone of all 34 analyzable tumors is shown in figure 1. A representative karyogram of one tumor with highlighted losses and polymorphisms is shown in figure 2. Regions of previously reported natural copy number variation were not included in the analysis of these samples [14]. The commonly observed aberrations in each individual tumor sample are summarized in table 2. Interestingly, there was an overwhelming number of copy number losses compared to copy number gains. In addition, most alterations encompassed whole chromosomes or chromosome arms.

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

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

Loss of 11q was found in 29% (n=10), with loss of the whole arm in 80% of these (n=8). The 2 tumors that had a regional loss showed an overlap from 11q14.3 until the telomeric end of the q-arm. Loss of chromosome 17p was found in 35% (n=12), but no

regional losses were observed. Loss of 21q was observed in 21% (n=7), with one tumor showing a regional loss of 21q22.11 until the telomere. Loss of 21q was shown to be significantly associated with loss of 17p.

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

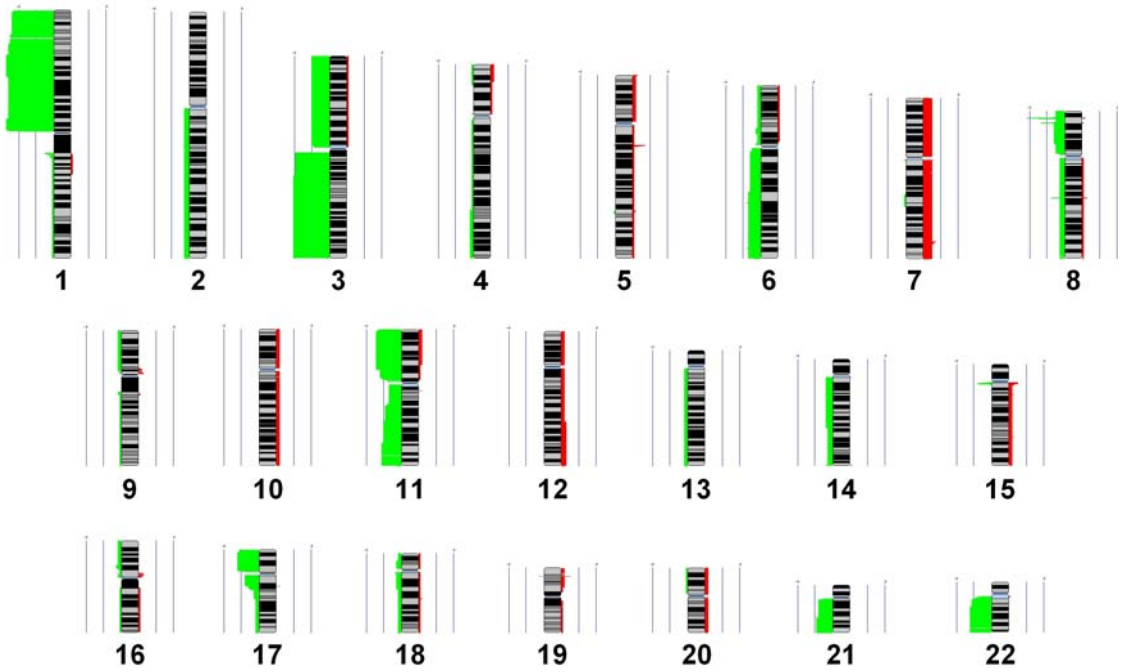

Figure 1. Frequency plot of all 38 PCC. Green lines on the left side indicate loss of BAC clones situated in that area; red lines on right side indicate gain. Blue bars on either side represent 25 and 50%.

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

Mutation analysis

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

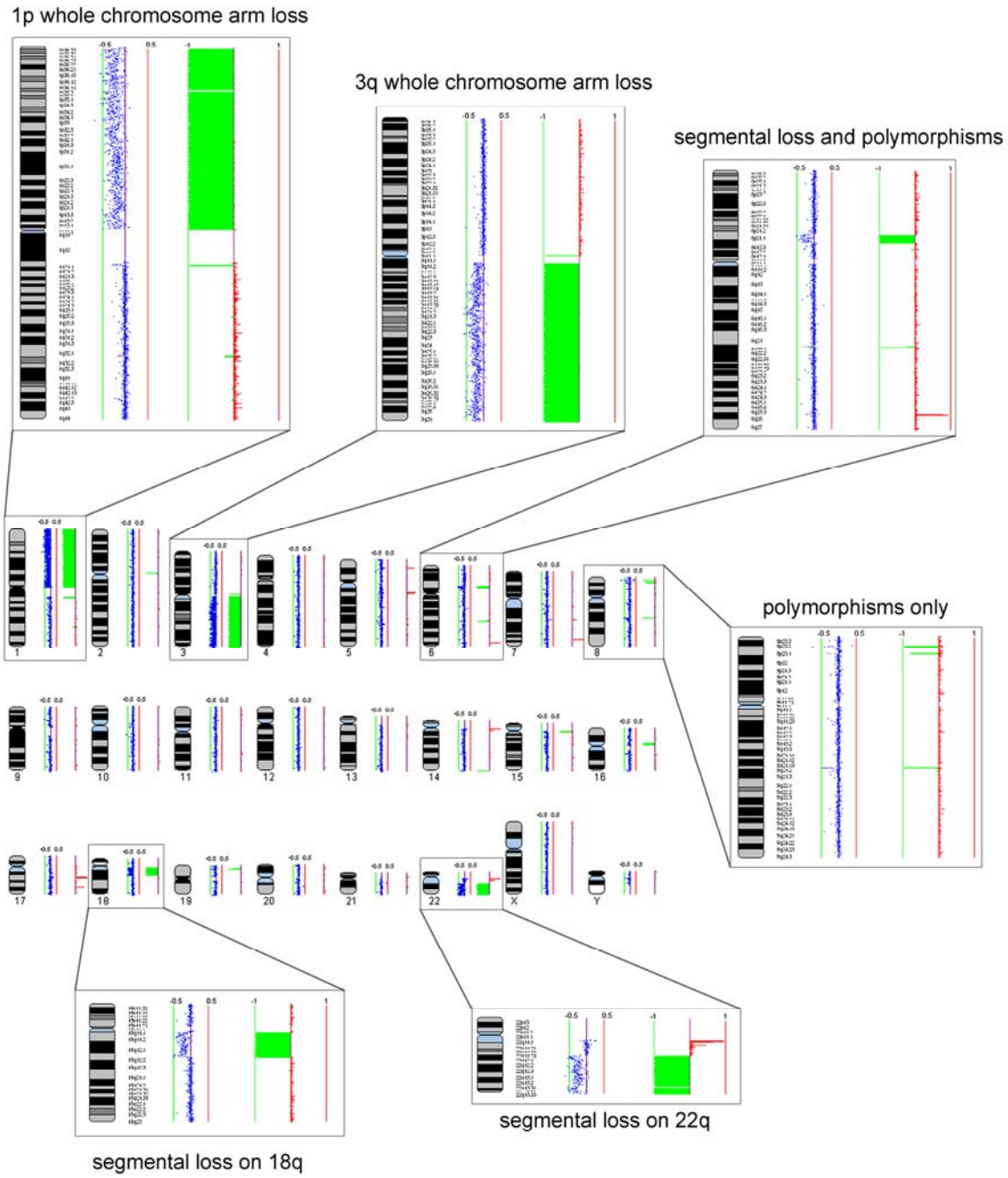

Figure 2. Karyogram of no.28 showing the entire genome with magnified views of chromosomes 1, 3, 6, 8, 18, and 22. Each BAC clone on the array is displayed as a point of representing the segment of the genome covered. The shift of each data point to the left of 0 represents a loss of copy numbers while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

Discussion

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

The majority (76%) of PCC in this analysis of 36 benign sporadic tumors showed loss of 1p, which is comparable to the frequency of loss that has been reported in previous studies.[3-5] Moreover, most PCC in our study (22/36) had loss of the entire short arm of chromosome 1, in contrast to our previous study, where we found regional 1p loss in half of the cases.[9] We speculate that the observed difference with our own previous studies and with series from others is related to the composition of the study group, which in the present study only comprised sporadic cases. In the few cases with partial loss, no minimal region of common loss could be determined, preventing us to speculate on the presence of one region harboring tumor suppressor genes on 1p, which have been postulated by various authors.[9, 16] Still, based on the high frequency of 1p loss in PCC, we support the idea of one or more tumor suppressor genes on this chromosome arm. In most cases 1p loss was accompanied by loss of 3q, which occurred in 62% of all cases with 1p loss, a figure that is comparable with that reported in the literature.[3-5] Four PCC were found with loss of 3q without chromosome 1p loss. These 4 tumors displayed loss of the entire chromosome 3 in combination with loss of the entire chromosome 11 (see below).

Apart from the large group of PCC displaying a $1p^-/3q^-$ genotype, a smaller group of PCC was identified with loss of 3p, which was frequently accompanied by loss of 11p. This pattern of loss has been mentioned previously in PCC from VHL patients, but has so far not been related to a subgroup of sporadic PCC. [6, 7] In order to exclude that this subgroup represented occult VHL disease, we performed mutation analysis of the entire VHL coding region, in which we could not detect germline mutations. However, we found 3 cases showing somatic VHL abnormalities. The p.R161Q and p.A122I VHL mutations have been described previously in an apparently sporadic PCC.[2] The p.G57LfsX59 has never been described before. Although epigenetic silencing of the VHL gene by hypermethylation is not inconceivable, as seen in familial and non-familial renal cell carcinoma, no methylation has been described in PCC. [17] The fact that a subgroup of sporadic PCC, without VHL germline mutations, shows an identical genotype as VHL-related PCC, leads to the suggestion that this group of PCC follows similar pathways of tumorigenesis. Indeed, this might also be the case for MEN2-related PCC and the abovementioned subgroup of sporadic PCC, which have been shown to have similar frequencies of 1p and 3q loss in previous studies. [3-5]

Table 2. Loss of common regions

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

x=loss, reg=regional loss (see text for locations)

In addition to losses affecting chromosomes 1, 3, and 11, we observed the highest frequency of loss in chromosomes 21 and 22, concerning 21% and 35% of all PCC, respectively. Loss of chromosome 21 has so far not been described at this relatively high frequency in benign sporadic PCC. All tumors with 22q loss also displayed 1p loss, and all but one tumor with chromosome 21q loss also revealed 1p loss. Therefore, these regions could be involved in the spectrum of the sporadic and/or MEN2-related PCC. However, as there was only 1 tumor with regional loss of 21q, we cannot draw conclusions with respect to the presence of potential tumor suppressor genes on this chromosomal arm. Previous reports on the loss of chromosome 22 have been based on LOH-analysis and showed loss of chromosomal bands 11.21 to 13.31, or 11.21 alone.[18-20]

Furthermore, in a recent array-CGH study on 66 PCC, copy number alterations of 22q were found in 44%.[18-20] In 8 of these cases (8/29) there was regional loss with a minimal region of common overlap from 22q11.23 until the telomeric end of chromosome 22. One additional interstitial deletion was found from 22q11.23 to 22q12.3. In our analysis we did not find a regional loss concerning chromosome 22q. These findings might indicate the presence of tumor suppressor genes on 22q that could be involved in the pathogenesis of sporadic PCC, however due to the large regions involved, combined with a gene-rich chromosome it is not possible at this time to pinpoint candidate genes.

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

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

Table 3. Mutation analysis results

Patient number	Gene	Mutation → cDNA	Mutation → protein	Hormone**
28	RET	c.1894_1899delGAGCTG	**p.E632_L633del**	NE, E, D
6	RET	*c.2332G>A*	**p.V778I**	NE
12*	RET	*c.2753T>C*	**p.M918T**	NE, E
10	SDHB	*c.299C>T*	**p.S100F**	NE
5	SDHB	*c.487C>T*	*p.S163P* **E**	
29	SDHB	c.487 C>T	*p.S163P*	NE
23	SDHD	c.149A>G	**p.H50R**	NE, E, D
20	VHL	c.169_212delGGG_GCC	**p.G57LfsX60**	-
15	VHL	*[c.364G>A;c.365C>T]*	**p.A122I**	E
13	VHL	*c.482G>A*	**p.R161Q**	E
34	VHL	c.500G>A	**p.R167Q**	-

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

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Chapter 4

Malignant pheochromocytomas show specific genomic alterations in high-resolution array comparative genomic hybridisation

*Esther Korpershoek, *Francien van Nederveen, Ronald J. DeLeeuw, Suzanne van Eeden, Jaqcues W. Lenders, Benno Kuesters, Winand N.M. Dinjens Wan L. Lam, and Ronald R. de Krijger

* These authors should be considered as equal first authors

Submitted

Abstract

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

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

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

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

Introduction

Pheochromocytomas (PCC) and sympathetic paragangliomas (sPGL) are rare neuroendocrine tumors arising from neural crest-derived chromaffin cells. PCC are located in the adrenal medulla, whereas the morphologically related sPGL are located anywhere along the sympathetic chain. Both PCC and sPGL produce catecholamines, leading to a wide range of symptoms.[1, 2] Although the majority of PCC and sPGL are reported to be sporadic, up to 24% of PCC and sPGL can arise in the context of hereditary tumor syndromes.[3] These familial syndromes include: multiple endocrine neoplasia type 2 (MEN 2), Von Hippel-Lindau disease (VHL), neurofibromatosis type 1 (NF1), and PCC-PGL syndrome.[4-6]

Malignancy in PCC and sPGL is defined as the presence of metastases at sites where chromaffin tissue is normally not present, such as lymph nodes, bones or lungs. The risk of malignancy in PCC and sPGL ranges from 10-36%, with the highest percentages of malignant tumors described in sPGL and in patients with *SDHB* germ line mutations.[6-10] Interestingly, other syndrome-related PCC have a low incidence of malignancy.

Attempts have been made to distinguish benign from malignant PCC by histology[11, 12] and immunohistochemistry.[13-15] However, none of the criteria or markers has been proven to be of clinical use. Although syndrome-related and sporadic malignant PCC have been analyzed by comparative genomic hybridisation (CGH) for chromosomal abberations,[16-18] no conclusive regions have been identified in the pathogenesis of malignant PCC.[19] With the introduction of high-resolution array-CGH, it has become technically feasible to study small (submegabase) segmental deletions and gains that escaped detection by conventional CGH due to improved resolution and/or sensitivity. In addition, the tiling order of overlapping bacterial artificial chromosome (BAC) clones also has the advantage to rule out mismapped clones, and gives more detailed breakpoint information. To further clarify the pathogenesis of malignant PCC, we analyzed 16 tumors from 14 patients using a tiling array consisting of 32,433 BAC clones and compared these data with those of a series of benign PCC analyzed with the same platform.[20]

Materials and methods

Patients and tumor samples

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

Array CGH labeling and hybridization

The submegabase tiling arrays (SMRT) previously described by Ishkanian et al. were used, consisting of 32,433 overlapping BAC clones. [22] Test DNA and pooled reference male DNA (Novagen, Mississauga, Ontario), (300 ng each) were labeled with Cyanine-3 and Cyanine-5 (PerkinElmer, Woodbridge, ON, Canada) respectively, according to a random priming protocol. Labeling and hybridization procedures have been previously described.[20]

Array imaging and analysis

Hybridized slides were scanned using a charge-coupled device (CCD) camera system (Applied Precision, Issaquah, WA, USA), and analyzed by SoftWoRx Tracker Spot Analysis software (Applied Precision). Resultant data was normalized using a stepwise normalization process [23]. Copy number alterations were identified via data visualization using custom software called "SeeGH" (freely available at http://www.flintbox.ca/technology.asp?tech=FB312FB) and loss, normal, and gain probabilities for each clone as determined by a modified hidden Markov model.[24, 25] Data

Table 1. Clinical data

patient	sex	age of onset	location primary	diameter	metastases	syndrome	metastasis detection	follow-up
1	M	32	extra-adrenal	21	lymph node, bone	*SDHB* p .Asp161Met fs X14	at diagnosis	DOD (7yr)
2	M	61	adrenal	nk	lymph node	none	2 years	AWD (1yr)
3	F	35	adrenal	9	lymph node	none	at diagnosis	nk
4	F	70	adrenal	12	liver, lymph node	none	1 year	DOD (1yr)
5	F	35	extra-adrenal	3,5	bone	none	5 years	DOD (10yr)
6	F	30	adrenal	7,5	liver, lung	none	4 years	DOD (5yr)
7	M	42	adrenal	7,5	bone	none	nk	nk
8	M	77	adrenal	nk	lymph node	none	2 years	nk
9	F	63	extra-adrenal	nk	lymph node, bone	*SDHB* p.Cys243Ser	nk	DOD (10yr)
10	M	46	extra-adrenal	7,5	lymph node, bone	None	nk	AWD (2yr)
11	M	39	adrenal	15	lymph node, liver	None	at diagnosis	AWD (6yr)
12	M	44	adrenal	nk	gut, peritoneum	None	1 year	AWD (2yr)
13	M	42	adrenal	8,5	bone, lung	None	1 year	AWD (3yr)
14	M	25	adrenal	4	lymph node	*SDHD* p. Leu95Pro	at diagnosis	NED (4yr)

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

were filtered based on both replicate Standard Deviation (SD; data points with greater than 0.1 SD were removed) and signal to noise ratio (data points with a signal-to-noise ratio < 10 were removed).

Comparison of data obtained from benign and malignant PCC

The CGH results obtained in this study were compared with the results of a previous study in which we performed array-CGH on a series of 32 benign PCC, of which 3 samples were not available for this comparison.[20] A straightforward clone-by-clone comparison described previously[26] was performed to determine clones that were differentially altered between the malignant and benign PCC groups. First, the log2 ratios of chromosomal gains and losses were calculated by an algorithm using flexible thresholds based on the SD of the data sets of the specimens. SDs over windows of five consecutive clones were averaged, sliding along the chromosome one clone at a time. Thresholds for gains and losses were defined empirically as 2.5 and 2.5 SD, respectively, using the combined data set. This procedure resulted in sample-dependent detection of genomic alterations with minimal interference of noise from the DNA isolated from formalin-fixed tissue. Subsequently, Fisher's exact test was applied, using SPSS version 15 (SPSS Inc., Chicago, IL) to determine the differentially gained or lost clones in the two tumor groups. P-values less than 0.05 were considered to indicate statistical significance.

Results

Array CGH

All array experiments included in this study yielded interpretable results. A frequency plot, adding up percentages of loss and/or gain of each individual BAC clone of all 14 patients is shown in figure 1. Regions of previously reported natural copy number variation were not included in the analysis of these samples.[25, 27] For the paired tumor/metastasis patients the chromosomal aberrations were mostly overlapping, an example of which is shown in figure 2 (patient 11). The most frequent alterations occurring in at least 38% of PCC/sPGL or more are listed in table 2.

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

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

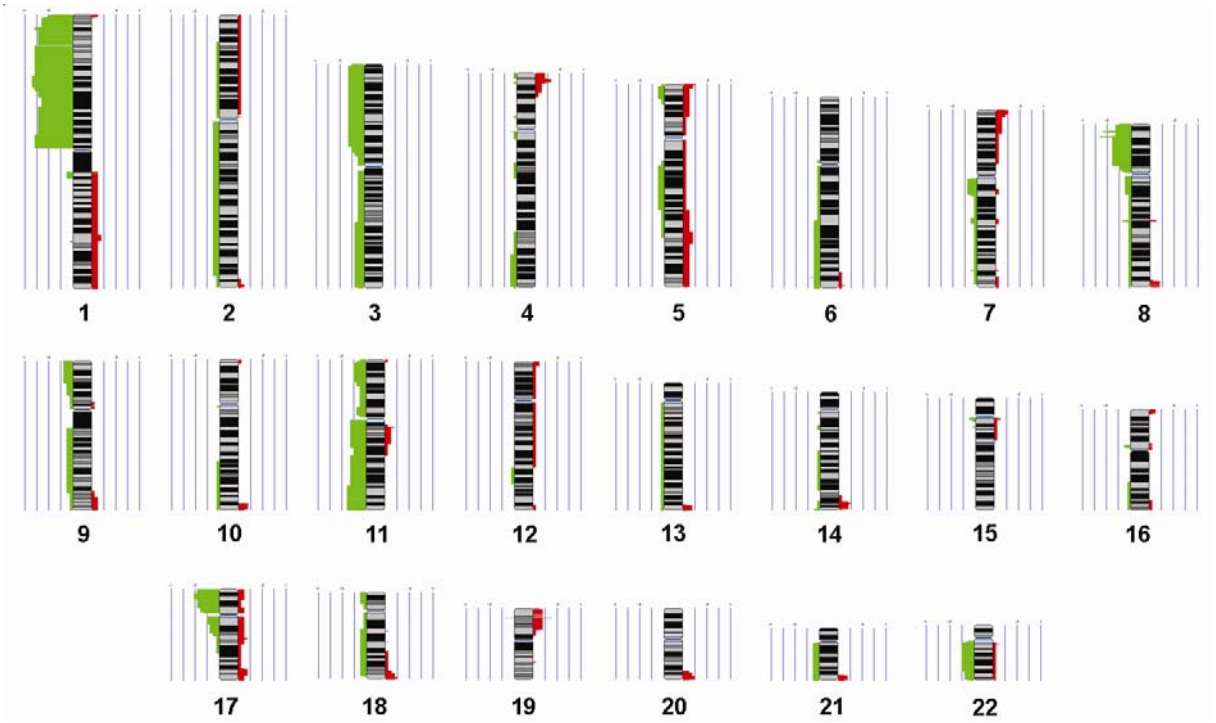

Figure 1. Frequency plot of all 14 malignant PCC. Green lines on the left side indicate loss of BAC clones situated in that area; red lines on the right side indicate gain. Blue bars on either side represent 25, 50, 75, and 100%.

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

In total, array CGH revealed 184 gains or losses of a (part of a) chromosomal arm in the 16 tumors tested. These alterations did not include the numerous small regional losses or gains. These genomic aberrations which seemed to be independent events, occurring usually in a single tumor.

Table 2. CGH results of common alterations

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

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

Comparison of benign and malignant PCC

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

Discussion

Many studies have attempted to identify markers that predict malignant behavior of PCC. However, markers that are useful in everyday practice still have to be discovered. Consequently, a PCC can only be called malignant when metastasis has been demonstrated. Some PCC patients have synchronous metastases, but most patients present with metachronous metastases after a disease-free interval of years to decades.[28] To investigate whether we could find loss or gain of genomic regions that could help predict malignant behavior, array-CGH was performed on a series of 14 proven malignant PCC and 2 corresponding metastases, using a genome-wide submegabase-resolution tiling array (SMRT-array). In this study we have demonstrated that malignant PCC present multiple genomic aberrations, of which loss of chromosome 1p is the most frequent. In addition, loss of chromosomes 8p, 11q and 17p, and gain of chromosomes 4p, 17q, 18q, 19p and 20q were also seen in more than 35% of the tumors. When comparing these data with those obtained in a previously analyzed and published series of benign PCC,[20] there were two regions, 3q and 11p, that were significantly more frequently lost in benign than malignant PCC.

Loss of 1p has been described in benign as well as malignant tumors, as illustrated by one of our previous studies.[17] In that study 84% of the benign and 90% of the malignant PCC showed loss of 1p, which is comparable with our frequency. Sandgren et al showed loss of 1p in 69% of the malignant PCC.[19] This frequency would be much higher when only truly malignant PCC (with proven distant metastasis) would be considered. Another study used loss of heterozygosity analysis to investigate the genetic alterations of a series of PCC, and demonstrated loss of 1p in 67% of malignant PCC,[29] which is also less than our frequency. An additional study also reported a low frequency of 1p loss in malignant PCC of approximately 50%,[16, 19] but they used a different definition for malignancy. The malignant tumors included cases with locally invasive behavior, without the presence of distant metastases. Although loss of 1p seems to be an important step in the pathogenesis of malignant PCC, this high frequency could also be due to the relatively small number of samples studied.

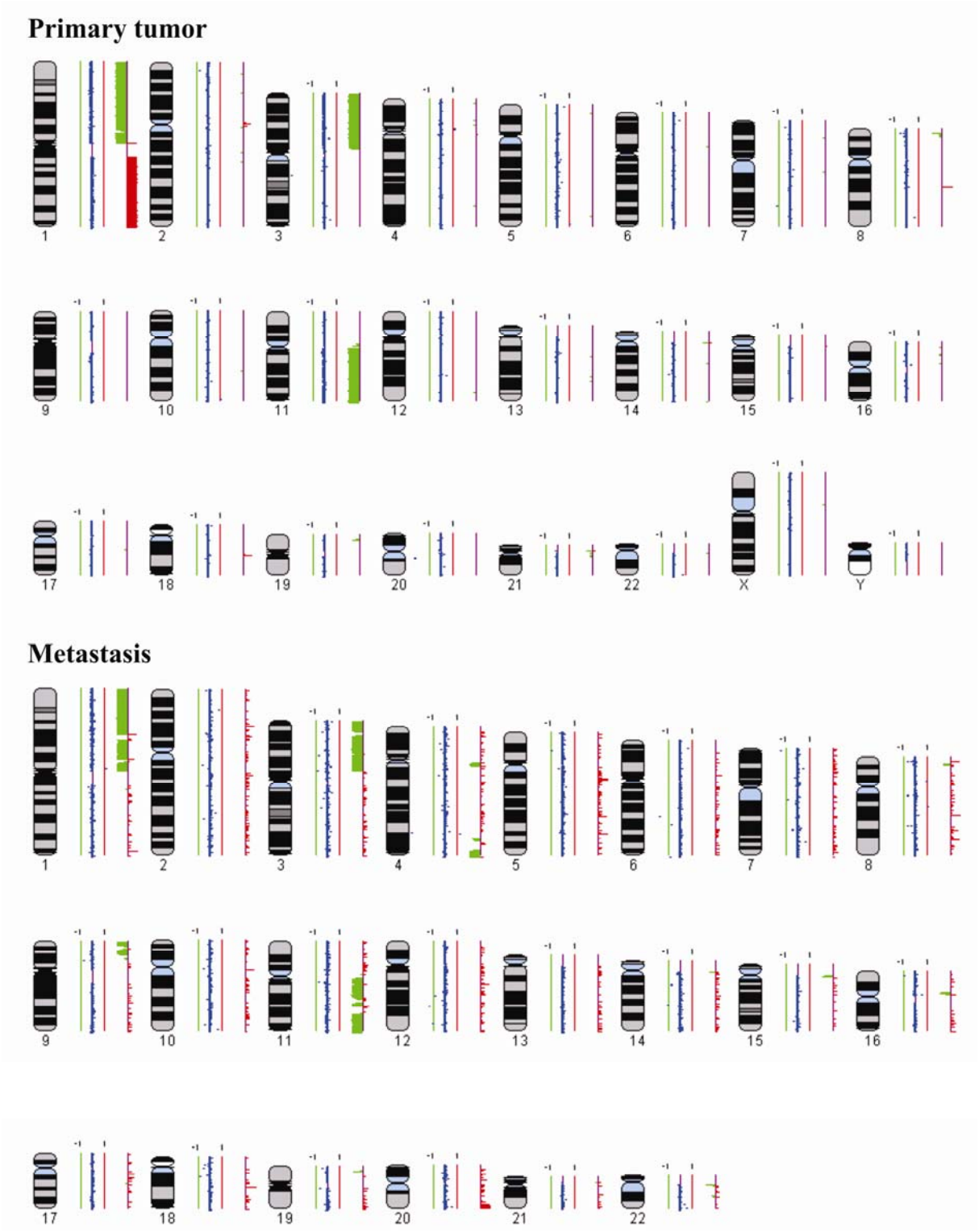

Figure 2. Karyograms of primary tumor and metastasis of patient 11 showing similar losses of chromosomes 1p, 3p, and 11q.

Besides 1p, other chromosomal regions were also affected in malignant PCC. The tumors showed loss of (a part of) 8p as the second most common genetic alteration (56%). This was also demonstrated by other studies, that found loss of 8p in 30% [17], 33% [16] and 20% [16, 19] of malignant PCC. In contrast, at least one other study did not report loss of 8p as a frequent alteration in (malignant) PCC.[18] Again, this could be due to the limited number of malignant PCC used. Other frequent losses observed in the present study included those of 11q and 17p, which had already been correlated with malignant behavior of PCC.[17, 18]

The most frequent gains included 4p, 17q, 18q, 19p and 20q, all of which have been associated with PCC pathogenesis, with the exception of 4p.[16-19] In concurrence, gain of (a part of) 19p was also demonstrated by Edstrom as more frequently present in malignant tumors (PCC and PGL). However, this difference was not significant as there was also 19p gain present in the benign tumors.[18]

Apart from these whole-arm chromosomal gains and losses, many small regional alterations were seen in most investigated tumors, indicating that malignant tumors are in general genetically more instable than benign PCC. In addition, upon diagnosis, malignant PCC are usually larger than benign PCC,[30] indicating that malginant PCC likely went through more cell divisions and therefore theoratically were able to accumulate more genetic alterations. However, Dannenberg et al showed that there was no correlation between tumor size and the number of alterations.[17] Furthermore, recent findings have demonstrated that malignant PCC are genetically more heterogeneous than benign PCC [31], which could be due to the genetic instability of these malignant tumors.

The small regional gains and losses included regions that were affected at high frequency within the studied tumors. One region of interest was 2q14 that showed gain of a 1 Mb region containing the GLI2 gene. Amplification of this proto-oncogene has been discovered initially in gliomas.[32] Roessler et al showed GLI2 involvement in mediating the Sonic hedgehog pathway in a mouse model.[33] Despite its oncogenic potential, GLI2 has never been associated with the malignant behavior of PCC. Waldman et al performed expression arrays on a small series of malignant and benign PCC and did not find GLI2 differentially expressed in the two groups.[34]

Another region of special interest was the telomeric part of chromosome 5p, comprising 13 Mb of gain. Gain of this chromosomal region has been described before as one of the most consistent alterations in non-small cell lung cancer.[35] The region contains multiple genes, including *TRIP13* and *TERT*. TRIP13 is a thyroid hormone receptor, a hormone-dependent transcription factor that regulates a variety of different target genes and seems to be required for recombination and high-order structure during meiosis.[36] Not much is known about the function of TRIP13 in general. Its involvement in the pathogenesis of (malignant) pheochromocytoma is not clear and needs to be further examined. TERT, human telomerase reverse transcritpase, is part of the telomerase

complex. It has been studied as a marker for malignancy in the context of human PCC by Isobe et al [37], who found differences in protein and RNA expression between benign and malignant PCC. However, the series was too small to provide significant differences.

An additional chromosomal region of interest included a 1.2 Mb region of loss of chromosome 8p23, comprising several genes including members of the defensin family (DEFB104A – 107A), *sperm associated antigen 11B* (*Spag11B*), and *HE2*, which is an androgen-dependent epididymis-specific secretory protein. This region has been demonstrated to have high copy number variation, suggesting that the loss might be associated with disease phenotype. [38]

Apart from the demonstration of the most frequent DNA abnormalities in malignant PCC, we also undertook this study with the aim to compare the results with that of a previously published study of benign PCC.[20] For both studies, the same array platform was used and the same analytical methodology was used, so results are entirely compatible and comparable. We demonstrate that there are two chromosomal regions, 3q and 11p, both of which are frequently lost in benign as opposed to malignant PCC. In line with this, the few studies that have investigated malignant PCC for genomic aberrations using CGH also showed 3q loss in less than 25% of malignant tumors.[16-18] The statistically significant difference in loss between benign and malignant PCC in 3q and/or 11p may allow the development of an algorithm to discriminate the two groups of PCC, provided that these data are confirmed in a larger independent study. Interestingly, all 3 cases of malignant PCC with loss of 3q and/or 11p contained an SDHB or SDHD mutation, implying that no sporadic malignant PCC in this study had 3q or 11p loss. Although these findings might be related to the relatively small sample size, they suggests that in patients without an SDHB or SDHD mutation tumors with 3q or 11p loss could be considered as benign.

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

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Chapter 5

Conditional Pten knock-out mice: a model for metastatic Pheochromocytoma

Esther Korpershoek, Anne J.M Loonen, Suzanne Corvers, Francien H. van Nederveen, Jos Jonkers, Xiaoqian Ma, Angelique Ziel-van der Made, Hanneke Korsten, Jan Trapman, Winand N.M Dinjens, Ronald R. de Krijger

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Abstract

Pheochromocytomas (PCC) are neuro-endocrine tumors of the adrenal medulla, that are usually benign, but approximately 10% of patients develop metastases. Malignant PCC can only be diagnosed with certainty if metastases are present. Here we describe adrenal tumors generated in a *Pten* conditional knock-out (KO) mouse model. We characterized the molecular alterations in these tumors and compared them with human PCC. Thirty-two of 41 (78%) male *Psa-Cre;Pten-loxP/loxP* mice presented adrenal tumors that were shown to be PCC by histology and by immunohistochemical staining for enzymes in the catecholamine biosynthetic pathway. In 6 of 17 investigated mice histological and immunohistochemical evidence was obtained for the presence of PCC lung metastases. Array comparative genomic hybridization (CGH) analysis of the primary tumors showed loss of chromosomes 6 and 19, which are syntenic to human 3p and 11q. Another frequent alteration found was gain of chromosome 15, which is syntenic to human chromosome 5. The molecular aberrations in the mouse model corresponded with the alterations found in a subtype of human PCC, suggesting that the PCC of the *Pten* KO mice might be representative of human PCC. The mouse model should allow further studies into the pathogenesis of human malignant PCC and into therapeutic strategies for these tumors.

Introduction

Pheochromocytomas (PCC) are tumors that arise from chromaffin cells of the adrenal medulla. In general, about 10% of human PCC are malignant and metastasize to bone, liver and/or lungs. This frequency is higher in patients with SDHB mutations and/or extra-adrenal primary tumors.[1, 2]

Approximately 25% of human PCC is associated with hereditary syndromes, but the majority of PCC are sporadic.[1, 2] These hereditary syndromes include Multiple Endocrine Neoplasia syndrome type 2 (MEN2), Von Hippel-Lindau disease (VHL) and the PCC - paraganglioma (PGL) syndrome, caused by mutations in RET, VHL, and SDHB and SDHD, respectively. In addition, a small proportion (<2%) of patients with Neurofibromatosis type 1 caused by mutations in NF1 [3], develop PCC as well. So far, it is not clear which genes or molecular mechanisms are responsible for the majority of sporadic PCC.

Many genetically engineered mouse models have been generated to investigate the mechanisms of tumorigenesis. Several mouse models develop PCC at varying frequencies, including 20% of $Nf^{+/-}$ mice [4], 24-100% of $Pten^{+/-}$ mice [4-7], 55% of $Rb^{+/-}$ $p130^{-/-}$ mice [8], 71% of $Rb^{+/-}$ mice [9], and 91% of $p18(Ink4c)^{-/-}$ $p27(Kip1)^{-/-}$.[10] All mouse models described above develop benign PCC, with the exception of the $Pten^{+/-}$ model of You et al [7], showing PCC metastases in the lungs in a small percentage (15%) of mice.

The PTEN tumor suppressor gene is inactivated in many tumors.[11] Its main function is to convert phosphatidylinositol (3,4,5)-triphosphate (PIP-3) into phosphatidylinositol (4,5)-diphosphate (PIP-2), thereby inhibiting the PI3K/AKT pathway. PTEN inactivation results in increasing PIP-3 levels, causing stimulation of cell survival and inhibition of apoptosis through AKT activation.[12] Loss of one PTEN allele was demonstrated in human PCC, but no mutations were found in the remaining allele.[13] The role of Pten in the pathogenesis of human PCC remains therefore unclear. In mouse PCC Pten appears to play an important role, as several studies concerning Pten knock-out (KO) mouse models report the occurrence of PCC, as mentioned above .[5-7, 14]

The most frequent genetic changes in human PCC are losses of chromosome areas 1p and 3q, although losses of chromosomes 3p, 6q, 8p, 11pq, 17p, 22p and gains of chromosomes 5p, 9q, 17q and 19pq have been reported as well.[15-18] It must be noted, however, that most PCC series investigated are relatively small and predominantly composed of benign sporadic PCC. Powers et al. generated PCC cell lines of Nf1 KO mice, which were analyzed by array comparative genomic hybridisation (CGH).[19] The cell lines showed loss of chromosomes 4 and 9, which are syntenic to human chromosome 1p and 3p, and 3q and 11q, respectively. In addition, You et al. [7] investigated PCC in Pten and Ink4a/Arf deficient mice, and also found frequent loss of distal mouse chromosome 4 in a region syntenic to human chromosome 1p. These results imply that (part of) the genetic

mechanisms in the pathogenesis of PCC are similar in humans and these two mouse models.

There are currently no molecular or histopathological markers that can predict whether a human PCC is or will become malignant. In addition, there exists no good treatment for malignant PCC. Therefore, a mouse model of malignant PCC might be of great benefit for the understanding of PCC tumorigenesis and for the development of treatment strategies for PCC. Here, we describe a Pten KO mouse model that presents metastatic PCC. We show that the adrenal tumors were PCC and that the lung lesions were PCC metastases by histological and immunohistochemical analysis. Investigation of PCC in this model by array-CGH showed partial homology to the chromosomal alterations in human malignant PCC.

Materials and methods

Generation of Pten conditional KO mice

The generation of these mice was reported previously.[20] In brief, this mouse model was based on presumed prostate-specific inactivation of Pten, to investigate the pathogenesis of prostate cancer. Cre-recombinase was expressed under the control of the prostate-specific antigen (*PSA*) promoter. FVB mice carrying this *PSA-Cre* construct were crossbred with 129Ola mice carrying the floxed *Pten* alleles. By subsequent breeding of *PSA-Cre;Pten-loxP/+* F1 offspring, biallelic and monoallelic prostate-targeted Pten KO mice were generated.

The mice were sacrificed at various arbitrarily chosen ages and all organs were systematically examined grossly and microscopically for abnormalities. In addition to prostate tumors, the *PSA-Cre;Pten-loxP/loxP* mice often had bilateral adrenal gland tumors, defined as twice the weight of a normal adrenal gland. The latter weight was calculated by taking the average of 16 adrenal glands of PSA-Cre-negative littermates (7-16 months), which was 4mg (range 1.8-7.3 mg). The weight of the adrenal glands of 41 male *PSA-Cre;Pten-loxP/loxP* mice was determined (mean age 11.7 months; range 7-20 months). Twenty-three enlarged adrenal glands and 17 lungs of these male *PSA-Cre;Pten-loxP/loxP* mice were embedded in paraffin. Eleven of the enlarged adrenal glands of *PSA-Cre;Pten-loxP/loxP* mice were frozen. Adrenal glands of a wild type FVB mouse and a *Cre*-negative *PTEN-loxP/loxP* mouse were embedded in paraffin and used as healthy controls for histology and immunohistochemistry.

Histology and immunohistochemistry

To determine the histology of the adrenal tumors, 5 µm sections were cut from 26 paraffin-embedded adrenal glands (n=23 *PSA-Cre;Pten-loxP/loxP*, n=2 *Cre*-negative littermates and n=1 healthy FVB). To investigate the presence of metastases, a minimum

of three step sections of 5 μm of the lungs were cut. The sections were mounted on 3-aminopropyl-triethoxilane (APES)-coated glass slides and stained with hematoxylin and eosin. Immunohistochemistry was performed with primary antibodies against Synaptophysin, S100, Tyrosine Hydroxylase (TH) and Dopamine-β-Hydroxylase (DBH), which were all described to be sufficiently sensitive as well as specific for mice (see table 1). Immunohistochemistry was performed as described before.[21] Because the anti-Synaptophysin is a mouse monoclonal antibody, a preincubation of the primary antibody with the HRP-labeled secondary antibody for 4 h at 4°C was necessary. After incubation, normal mouse serum was added to this antibody mix, followed by an incubation of 1 h at 4°C. After a 15 min microwave treatment of the sections in citric acid buffer (pH 6.0), the antibody against Synaptophysin was allowed to bind overnight at 4°C. Negative controls were performed by omission of the primary antibody. Paraffin-embedded healthy FVB adrenal glands and multi-organ blocks (containing pancreas/ liver/ small and large intestine) were used as controls.

Pten recombination PCR

Tumor DNA was isolated from the 11 frozen adrenal glands and corresponding normal DNA was isolated from mouse-tail tips by standard procedures. Standard PCR was performed with primers Pten-F and Pten-R (Table II) resulting in either a 330 base pairs (bp) product (*Pten* recombination) or a 2200 bp product (no *Pten* recombination).

Single strand conformation polymorphism analysis (SSCP)

SSCP mutation analysis of the genes associated with human PCC, *RET* (exon 10, 11 and 16) *VHL*, *SDHB*, and *SDHD*, was performed on the 11 tumor DNAs (primer sequences are listed in Table II). Normal FVB and normal 129Ola DNA was used as positive controls. Radioactive PCR was performed using standard conditions previously described [22]. PCR products were run on a non-denaturing 6% polyacrylamide gel (Fluka, Neu-Ulm, Germany) for 16 h at 8 W. After electrophoresis, the gels were dried, and exposed to x-ray films.

Array

The high-density whole-genome mouse bacterial artificial chromosome (BAC) microarray slides used for the microarray-based CGH encompassed 2803 unique BAC clones and were produced by the Central Microarray Facility of The Netherlands Cancer Institute in Amsterdam.[23] CGH was performed on DNA of 8 frozen adrenal tumors (*PSA-Cre;Pten-loxP/loxP*).

Array-CGH

DNA labeling was performed by random priming (Bioprime Labeling Kit, Invitrogen, Carlsbad, CA) of 450 ng genomic mouse test and reference DNA with Cy3 and Cy5 (Amersham Biosciences, Roosendaal, the Netherlands) respectively. Briefly, a 150 µl reaction was performed containing 450 ng DNA and a final concentration of 1X Random

Table 1. aCGH analysis of mouse PCC

Mouse	[1]Metastases	Chr. 4	Chr. 6	Chr. 7	Chr. 14	Chr. 15	Chr. 16	Chr. 18	Chr. 19
2M	na					WC +			WC -
3M	Y	63-91Mb -	WC -	38-72Mb -					WC -
4M	Y		WC -					WC -	WC -
5M	N		WC -						
7M	N	101-152Mb -	WC -		WC -	WC +			WC -
8M	N		WC -		WC -		WC -	48-90Mb -	WC -
10M	Y			43-68Mb -		WC +			WC -
17M	na		WC -		WC -			48-90Mb -	

***** na = not available; Y = lung metastases present; N = lung metastases absent; Chr. = mouse chromosome; WC = whole chromosome; + = gain; - = loss

Primers Solution and 23 µM NaCl. After denaturating the DNA for 5 min at 100°C, 15 µl 10X dNTP mix (1 mM dCTP and 2 mM dATP, dTTP and dGTP in TE buffer), 2 µl Cy3-dCTP or Cy5-dCTP (GE healthcare, Buckinghamshire, United Kingdom) and 3 µl Klenow fragment supplied in the kit were added on ice. The reaction was incubated overnight at 37°C.

The Cy3-labeled sample and Cy5-labeled reference DNAs were precipitated together with 125 µg mouse Hybloc (Applied Genetics Laboratories, Melbourne, FL). DNA pellets were redissolved 60 µl hybridization solution (50% formamide, 10% dextran sulfate, 2X SSC, 4% SDS) and 6 µl 1 mg/ml yeast tRNA (Invitrogen). Probe DNA was denaturated for 10 min at 70°C and incubated for 1 h at 37°C before application on the prehybridized BAC microarray slides. The slides were prehybridized with 140 µl hybridization solution containing 400 µg herring sperm DNA (Sigma-Aldrich, St. Louis, MO) and 125 µg mouse Hybloc (Applied Genetics Laboratories) in a small humidity chamber containing paper tissue saturated with 40% formamide and 2X SSC on a rocking table (at 5 rpm) at 37°C for 1 h. After adding the probe DNAs onto the slides, they were incubated in a sealed small humidity chamber with the saturated paper with 40% formamide and 2X SSC for 48 h at 37°C at the rocking table (at 5 rpm). After hybridization, slides were washed for 15 min in 50% formamide, 2X SSC, pH 7.0 at 50°C, and then for an additional 30 min in 2X SSC, 0.1% SDS at 50°C. Finally, the slides were washed for 10 min in PN buffer (0.1M sodium phosphate buffer, 0.1% NP40, pH 8.0), before being dried by spinning in a centrifuge for 3 min at 2000 rpm.

Array-CGH data analysis

After hybridization, the slides were scanned with a ScanArray Express HT (Perkin Elmer Life Science, Boston, MA). The spot intensities were measured with GenePix Pro 5.1 software (Axon Instruments, Leusden, The Netherlands). Furthermore we used Excel 2000 and the aCGH-smooth application (http://www.few.vu.nl/~vumarray/acghsmooth.html) to analyse the normalized data. Spots outside the 20% confidence interval of the average of the duplicates were excluded from the analyses. To detect all chromosomal imbalances the default settings of aCGH-smooth were used except for $\lambda = 8.5$.

Results

Tumors in PTEN KO mice

At the time of sacrifice all mice generally appeared to be in good condition. There were no apparent effects of the PCC. Apart from prostate tumors and microscopic abnormalities of the prostate, that have been described elsewhere, there were no other grossly apparent tumors except the adrenal tumors described herein [20]. Thirty-two of 41 (78%) male *PSA-Cre;Pten-loxP/loxP* mice presented with adrenal tumors: 30% of mice aged 7-9 months (n = 10; average PCC weight 9 mg), 88% of mice aged 10-14 months (n = 17; average PCC weight = 74 mg) and 100% of the mice of 15-16 months (n = 14; average PCC weight = 123 mg). In contrast, none of our *PSA-Cre* negative littermates (n=16, age 4-18 months), show adrenal tumors or tumors of other organs. Of the 17 lungs investigated of the *PSA-Cre;Pten-loxP/loxP* mice, six showed small tumors (35%). Two lung tumors occurred in mice of 10-14 months, the other 4 occurred in mice of 15-16 months.

Histology and Immunohistochemistry

In hematoxylin and eosin stained sections, the adrenal tumors were composed of large tumor cells with ample basophilic cytoplasm and slightly polymorphic nuclei, very similar to the aspect of normal adrenal medullary cells in mice or in humans and of human PCC cells (Figure 1A-C). The lungs of 6 *PSA-Cre;Pten-loxP/loxP* mice showed multiple small clusters of cells (ranging from 2 to 50 cells) mostly peripherally located in the lung and morphologically identical to the tumor cells in the primary tumors.

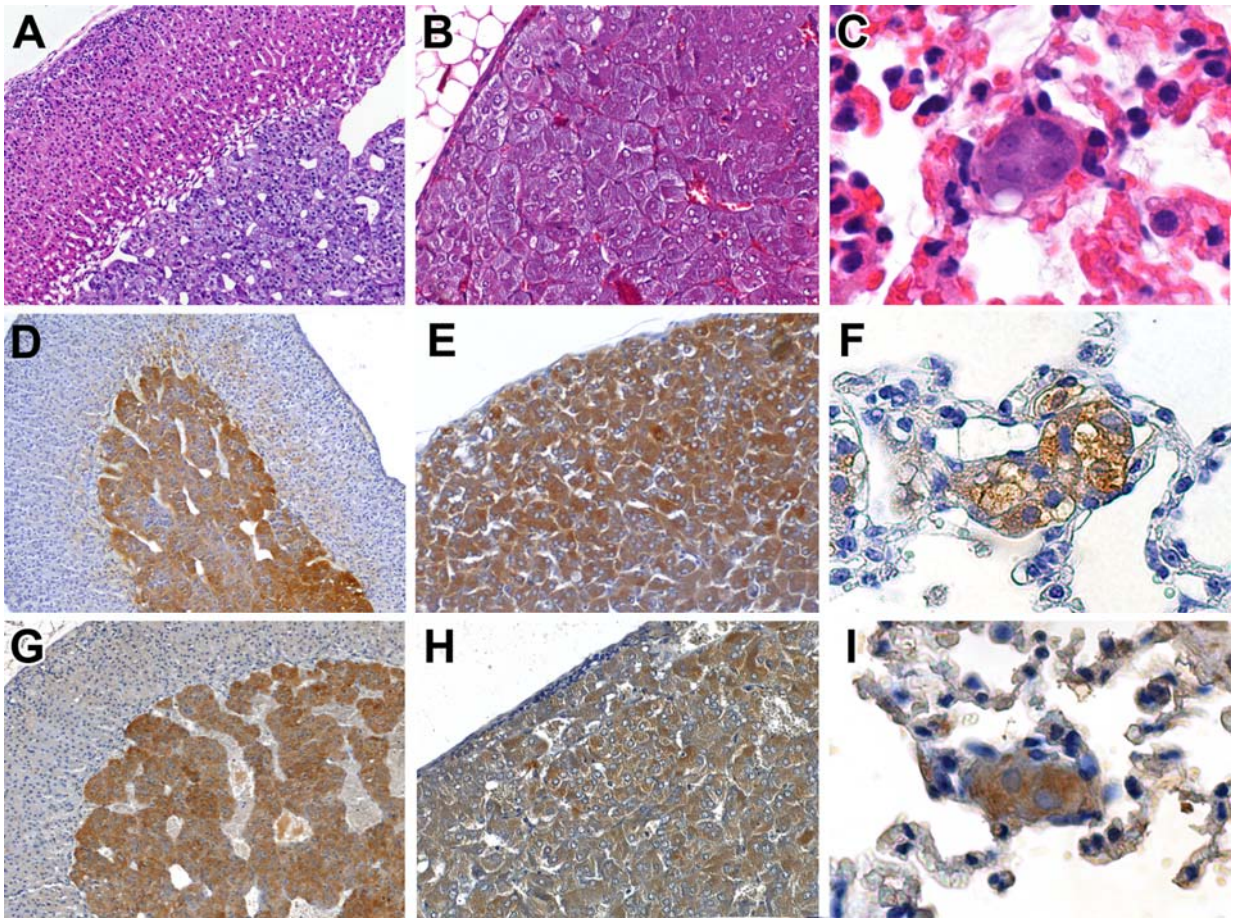

Figure 1. Haematoxylin and eosin staining of (A) normal adrenal gland, (B) PCC, (C) PCC metastasis. The normal adrenal gland was obtained from a *Cre-* negative mouse of 11 months and the PCC and metastasis were from a *PSA-Cre;Pten-loxP/loxP* mouse of 16 months. Immunohistochemical staining with antibody against tyrosine of (D) normal adrenal gland, (E) PCC, and (F) metastasis. Positive immunohistochemical staining with antibody against DBH is seen for normal adrenal gland (G), PCC (H), and metastasis (I).

To confirm that the adrenal tumors were indeed PCC, and that the clusters of cells within the lungs originated from these adrenal tumors, we showed that all adrenal tumors and lung tumors expressed Synaptophysin (data not shown), TH (Figure 1D-F) and DBH (Figure 1G-I). In addition, the PCC showed S100 positive cells, compatible with sustentacular cells, evenly distributed throughout the tumor tissue. The lung metastases were all negative for S100 staining (data not shown). To exclude the presence of prostate metastases, we stained prostate tumors of four *Pten* KO mice with antibodies against TH (Figure 2) and DBH (data not shown). The immunohistochemical stainings did not reveal positive cells in the prostate tumors for both adrenal medulla specific antibodies.

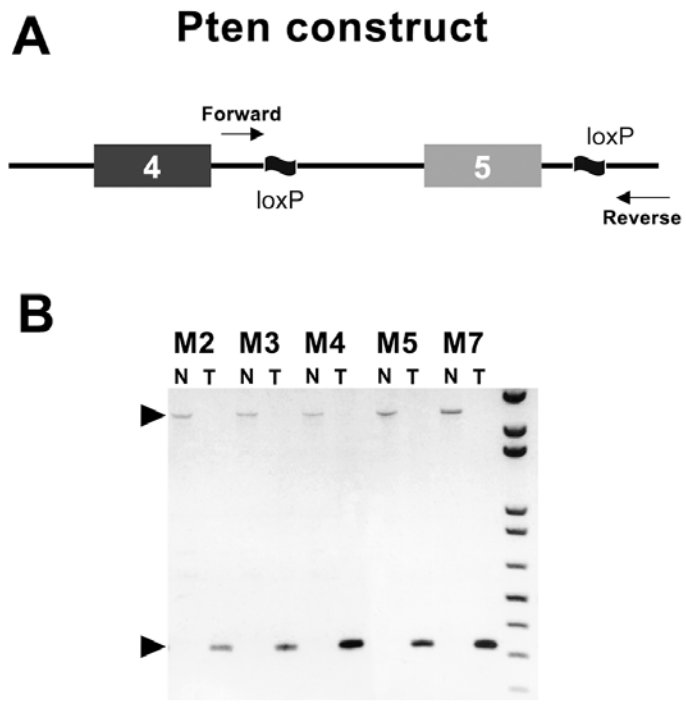

Figure 2. (A) Prostate-specific recombination was mediated by Cre recombinase expression driven by a *PSA* promoter, which results in the deletion of exon 5 after activation of the *PSA* promoter. (B) The result of the *Pten* PCR shows that recombination has occurred in tumour, but not in normal DNA. Arrowheads indicate the 2700 bp unrecombined PCR product and the 330 bp recombined PCR product.

Molecular Analyses

PCR was performed with primers flanking the *loxP* sites of *Pten* exon 5 and found that all 11 tumor DNA samples examined, showed recombination of the Pten gene (Figure 2). Furthermore, we performed SSCP analyses of the 11 tumor DNA samples, which did not show mutations in *Ret* (exon 10, 11 and 16), and the entire coding sequence and the intron-exon boundaries of *Vhl*, *Sdhb* and *Sdhd*.

CGH

The results of the array CGH analysis on DNA isolated from the PCC of 8 *PSA-Cre;Pten-loxP/loxP* mice are summarized in Table III. Six of the 8 PCC showed loss of chromosome 6, including 2 mice that presented lung metastases. In addition, six PCC showed loss of chromosome 19 (Figure 3), of which 4 coincided with loss of chromosome 6. Other frequent chromosomal alterations included loss of chromosome 14 (n=3 PCC), gain of chromosome 15 (n=3 PCC) and loss of whole (n=1) or part of (n=2) chromosome 18.

Discussion

In this study we have investigated the adrenal tumors and lung metastases of male *Pten* conditional KO mice. First, we showed that these adrenal tumors are PCC and that the tumors in the lungs are PCC metastases. Second, we have demonstrated that recombination of *Pten* occurred in all PCC, indicating that *Pten* is involved in the tumorigenesis of these PCC. Third, the CGH analyses revealed loss of chromosome 6 and 19 as the most frequent alterations. These chromosomes are syntenic to human chromosomes 3p and 11q respectively, which are frequently lost in human PCC. Therefore, this mouse model is potentially of value for understanding the pathogenesis and for treatment of human malignant PCC.

The *Pten* conditional KO mice were initially generated to investigate the pathogenesis of prostate cancer. To achieve prostate specificity, Cre-recombinase was expressed under the control of a prostate-specific androgen-regulated antigen (PSA) promoter, which in humans is exclusively prostate specific [20, 24]. However, these mice not only develop prostate cancer, but also PCC at high frequency. Although the mouse genome lacks a *PSA* homologue, other factors might contribute to activation of Cre expression.

The increasing frequency of PCC in the elder Pten mice suggests that a second hit may be necessary for the development of PCC in these mice. Di Cristofano et al. [6] report the occurrence of PCC in 100% of *Pten*$^{+/-}$ mice at age 9-16 months. This higher frequency compared to our study could be due to our definition of PCC, which was determined as twice the weight of a normal adrenal gland. It should be noted at this point that a size criterion for PCC is arbitrary, as it is for the distinction between hyperplasia and PCC in humans. Another study of *Pten*$^{+/-}$ KO mice described PCC in 24% (14 of 59 mice), at an average age of 42 weeks. [7]

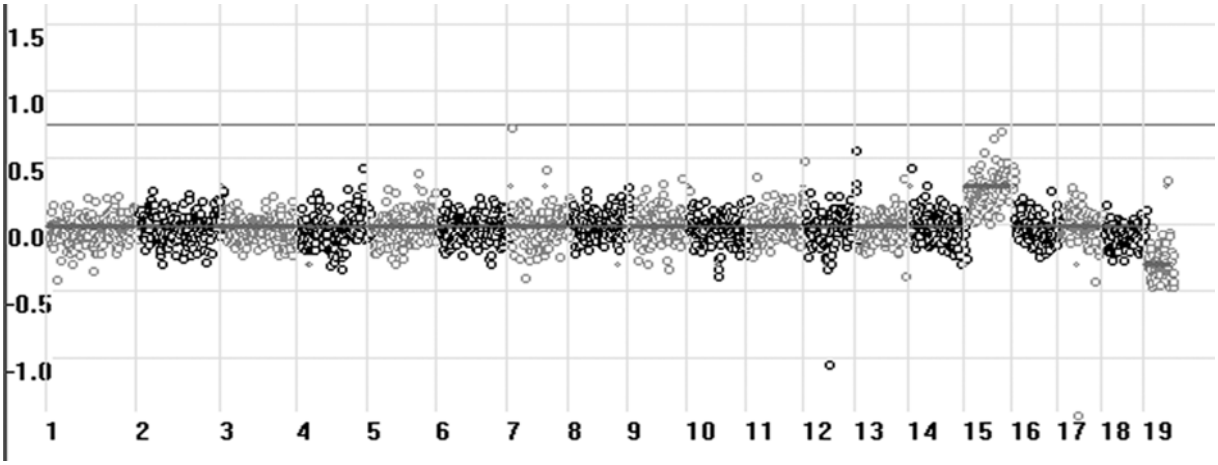

Figure 3. Example of a mouse PCC CGH result, analysed by the aCGH-smooth application, that shows gain of chromosome 15 and loss of chromosome 19.

This frequency is considerably less than found in our study, but in that study adrenal tumors were only included when the diameter was more than 2.5 mm in size. However, our results are in the same range as the results of Bai et al. [5], who reported the presence of PCC in 65% of 22 *Pten*$^{+/-}$ mice of 6-14 months. In addition, differences in PCC frequency could also be due to the genetic background of the mice studied.

A few reports show that a heterogeneous background sometimes results in more frequent occurrence of tumors.[25, 26] The high numbers of PCC that occur in our study are not due to the FVB/Ola129 background, as no *PSA-Cre* negative mice had adrenal tumors, which also have the FVB/Ola background.

Loss of PTEN function results in phosphorylation and activation of AKT, promoting cell survival, growth and proliferation.[11] Although no *PTEN* mutations have been found in human PCC, loss of the *PTEN* gene [13] and increased expression of total AKT and pAKT have been reported in human PCC [27], indicating a role for the AKT pathway in the pathogenesis of human PCC. The relation between PTEN and PCC pathogenesis is confirmed by the tumor specific *Pten* recombination in our conditional KO mice. This is in accordance with various other mouse models, which have demonstrated PCC occurrence concordant with loss of at least one *Pten* allele.[5-7, 14]

A recent report suggested a functional link between PTEN and SDHB.[28] Mutations in the human *SDHB* gene cause the PGL-PCC syndrome, in which sympathetic PGL and PCC occur that metastasize in approximately half of the patients.[29, 30] The report showed that patients with Cowden and Cowden-like syndromes without *PTEN* mutations had germline mutations in the *SDHB* gene. However, these mutations have also been described as polymorphisms in other studies.[31, 32]

It is not very likely that the human PCC susceptibility genes *RET*, *VHL* and *SDHD* are involved in the pathogenesis of these mouse PCC, as most mouse models concerning these genes do not develop PCC.[33-35] In accordance, our SSCP mutation analyses of *Sdhb*, *Sdhd*, *Vhl* and *Ret* did not reveal any alterations. This SSCP analysis does not entirely exclude the possibility of genetic abnormaltities in these genes, as the sensitivity is in the range of 80-90%. In addition, these genes could be inactivated by epigenetic mechanisms, such as methylation. However, such mechanisms have not been shown to play a role in PCC tumorigenesis.

The lung tumors of the mice of our study showed PCC lung metastases as indicated by histology and immunoreactivity for DBH and TH of the lung lesions. While synaptophysin is a general neuro-endocrine marker that can also be found in non-tumoral endocrine cells native to the lung, TH is highly specific for catecholamine-producing cells, of which PCC are composed. We did not find any TH staining in the prostate carcinomas, nor in the lung outside the metastases (data not shown). Together, these findings provide evidence that the lung tumors we observed are not PCC metastases.

PCC lung metastases in a mouse model have been described only once by You et al.[7] However, there are several essential differences between our model and that of You et al. Firstly, the frequency of metastases in our study was at least twice as high. This may be related to the genetic constitution of the mice, the age of the mice investigated, or to the extent to which the lungs have been investigated. It must be added with regard to the metastases, that we cannot exclude a higher frequency, as some lung lesions were very small and others might have been missed. In addition, metastases might have been present in body parts that have not been investigated in that much detail. Secondly, in the conventional KO mouse model by You et al. the additive effect of homozygous *InkA/Arf* deletion and heterozygous *Pten* inactivation is necessary for the occurrence of PCC metastases, in contrast to our conditional KO mice. Thus, in the former mouse model the additive effect of two genes is necessary for malignant behaviour in the study of You et al. Third, our *Pten* KO mice have biallelic inactivation of *Pten*, whereas the mice of You et al have inactivation of only one allele. In addition, the *Ink4A/Arf-Pten* mice and our conditional *Pten* KO mice display distinct genomic profiles, which resemble genomic profiles found in human PCC, which can also be separated into various subcategories.

The CGH results of the PCC from 8 *Pten* conditional KO mice revealed loss of chromosomes 6 and 19 as the most common chromosomal aberrations. Mouse chromosome 6 carries regions syntenic to human chromosomal regions 7q21-35, 7p11-15, 4q22, 2p12-14, 3p25-26, 22q11 and 12p11-13. Of these regions, loss of chromosome 3p is a frequent genetic alteration in a subset of human PCC.[15-17] The *VHL* gene, which is involved in a large subset of hereditary PCC, is located on human chromosome 3p25. Although the CGH results of the *Pten* KO mice show loss of the *Vhl* allele, no mutations were found in the remaining allele. It is therefore unlikely that *Vhl* is involved in the pathogenesis of these mouse PCC. Mouse chromosome 19 has regions syntenic to human chromosomal regions 11q13, 9p23-24, 10q23-25. Loss of chromosome 11q is another genetic alteration that occurs frequently in human PCC, especially in *Sdhd* and *Vhl* related PCC.[16, 36] However, mutation analysis of both genes in the PCC of the *Pten* KO mice did not reveal mutations. Furthermore, there are no human PCC susceptibility genes known that are located on the chromosomal regions that are syntenic to mouse chromosome 19.

Another frequent genetic alteration present in mouse PCC was gain of chromosome 15. Chromosome 15 is syntenic to human chromosome 5p13-15, 8q22-24, 22q13.1-3 and 12q12-13. Gain of chromosome 5 was described previously by Dannenberg et al.[16] in malignant human PCC and was also seen in our recent array CGH experiments on a large subset of malignant human PCC (van Nederveen et al, unpublished data). One candidate gene on chromosome 15 in this region is *Tert* which is upregulated in human malignant PCC.[37, 38] Therefore, the *Tert* gene might be the target of gain in the malignant *Pten* KO mouse PCC.

Approximately 85% of human PCC show loss of 1p.[16, 17] In accordance, two studies have shown loss of chromosome 4 in mouse PCC, which is syntenic to a large region of human chromosome 1p.[7, 19] We did not find loss of chromosome 4, with the exception of two tumors that showed loss of only small non-overlapping regions of chromosome 4. Our recent array-CGH study on a large series of human PCC (Van Nederveen et al, unpublished observations) indicates that malignant PCC have a very low rate of 1p loss, in contrast to sporadic benign PCC. Therefore, it seems that other molecular alterations might be responsible for the pathogenesis of malignant PCC. This could explain the absence of loss of chromosome 4 in our mouse PCC, which often presents as malignant PCC.

In summary, we have investigated a mouse model that develops malignant PCC with frequent lung metastases. The molecular aberrations found by array-based CGH are different from other studies, but do parallel some of the molecular alterations found in human PCC. Therefore, we conclude that this mouse model could be useful for the study of human malignant PCC tumorigenesis and might allow studies of novel drugs that target these tumors.

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Chapter 6

Accelerated induction of malignant pheochromocytomas in a Pten/Trp53 KO mouse model

Esther Korpershoek, Nanne K. Kloosterhof, Angelique Ziel van der Made, Hanneke Korsten, Lindsey Oudijk, Jan Trapman, Winand N.M. Dinjens, and Ronald R. de Krijger

Submitted

Abstract

Pheochromocytomas (PCC) are neuro-endocrine tumors of the adrenal medulla, which are usually benign. Approximately 10% of PCC patients develop metastases, but this can increase up to 32% in succinate dehydrogenase subunit b-mutated tumors. The reliable diagnosis of malignant PCC can only be made after identification of a metastasis. To study the effect of mono- and bi-allelic *Trp53* inactivation on the PCC of Pten KO mice, we investigated the adrenals of all 236 mice with conditional mono- and bi-allelic inactivation of *Trp53* and *Pten*, under the control of the *PSA* promoter. The adrenal weight was determined for all mice. In a proportion of these mice, immunohistochemistry for tyrosine hydroxylase and dopamine beta-hydroxylase was performed on the adrenals (n = 48) and corresponding lungs (n = 28). Finally, to examine what genomic alterations occurred in the PCC, comparative genomic hybridization (CGH) was performed (n = 14). The histological and immunohistochemical results confirmed that the adrenal tumors were PCC. Inactivation of one or both alleles of *Trp53* resulted in an earlier tumor occurrence in the $Pten^{loxP/loxP}$ mice as well as in $Pten^{loxP/+}$ mice. In addition, lung metastases were found in up to 67% of mice, even in mice without *Trp53* inactivation. The CGH results showed that the most frequent alterations were loss of chromosome 19 (86%) and gain of chromosome 15 (71%). The PCC of the present *Pten*/*Trp53* KO mouse model display malignant behavior at high frequency and could therefore be a suitable model for the study of the pathogenesis of human malignant PCC and might allow the development of therapeutic strategies for these tumors.

Introduction

In humans, pheochromocytomas (PCC) are relatively rare tumors that usually produce catecholamines, such as adrenalin or noradrenalin.[1] This overproduction causes high blood pressure, which in some cases may be fatal, through myocardial infarction or stroke.[2] PCC arise from the adrenal medulla and occur sporadically as well as in the context of hereditary syndromes. These include the multiple endocrine neoplasia syndrome type 2 (MEN2), von Hippel-Lindau disease (VHL), Neurofibromatosis type 1 (NF1), and the pheochromocytoma-paraganglioma (PCC-PGL) syndrome.[3] Most PCC are benign, but approximately 10% of sporadic cases present with metastases. This percentage can be up to 32% in patients with the PCC-PGL syndrome, especially in patients that have a germline mutation in the *succinate dehydrogenase subunit b* gene.[4, 5] Currently, there are no markers that can predict the clinical behavior of PCC. Also, apart from surgical resection, there is no curative treatment.

There are many distinct knock-out mouse models that have been shown to develop almost exclusively benign PCC. Genes that were silenced in these models included *Nf*, *Rb1*, *p130*, *p18*(*Ink4c*), *p27*(*Kip1*), and *Pten*.[6-11] Only two studies have reported mice with lung metastases. These included the *Pten* KO mice of You et al., that displayed metastases in a small percentage (15%),[12] and our previous study in which we reported a conditional *Pten* KO mouse model that also presented with lung metastasis in 35% of the mice of 10 months or older.[13]

The *PTEN* gene is a tumor suppressor gene that is involved in the pathogenesis of many tumor types, such as prostate and breast cancer, and belongs to the most frequently mutated genes in human cancer. Although mouse models that involve the inactivation of the *Pten* gene develop PCC, human PCC have never been associated with *PTEN* mutations.[14] Nevertheless, the genomic alterations found in the PCC of *Pten* KO mice show similarities with the genomic alterations found in human PCC.[12, 13]

Another gene that belongs to the most frequently mutated genes in human cancer is the *TP53* gene, which has been associated with aggressive tumor behavior, including local invasion and metastasis.[15] *Trp53* KO mouse models display many different tumors, of which malignant lymphomas and osteosarcomas are seen most frequently.[16, 17] Another study reported highly aggressive medulloblastomas in a combined *Rb* and *Trp53* conditional KO mouse model.[18]

In the present study we have investigated the effect of inactivation of the *Trp53* gene (on a *Cre-loxP* basis, under the control of the PSA promoter) on the behavior of PCC in single or double *Pten* KO mice. Parameters investigated included the frequency of metastases, the age of tumor presentation, size (weight) of the tumors, and chromosomal alterations. So, to investigate whether these mice could be a suitable model for the study of metastatic PCC, we have investigated the PCC of a *Pten*/*Trp53* double KO mouse model, using the *PSA-Cre;Pten*$^{loxP/loxP}$ KO mice from our previous study [13] and a *Trp53*$^{loxP/loxP}$ strain that originally was created by Marino et al.[18]

Materials and Methods

Generation of the Pten/Trp53 mice

The generation of the *Pten*$^{loxP/loxP}$ and *Trp53*$^{loxP/loxP}$ *mice* was described previously.[18, 19] *Rb*$^{loxP/loxP}$*;Trp53*$^{loxP/loxP}$ FVB mice of Marino et al. were crossbred with the *Pten*$^{loxP/loxP}$ mice to obtain *Pten*$^{loxP/+}$*; Rb*$^{loxP/loxP}$*;Trp53*$^{loxP/+}$ offspring. This F1 offspring was inbred to obtain *Pten*$^{loxP/loxP}$*;Trp53*$^{loxP/loxP}$ mice, that were crossbred with the previously reported *PSA-Cre+/-* mice.[19] The *PSA-Cre;Pten*$^{loxP/+}$*;Trp53*$^{loxP/+}$ offspring was interbred with *Pten*$^{loxP/loxP}$*;Trp53*$^{loxP/loxP}$ to obtain the six genotypes investigated (Table 1). Mice were housed according to institutional guidelines, and procedures were carried out in compliance with the standards for use of laboratory animals. A total of 236 mice were sacrificed at arbitrarily chosen ages and all organs were systematically investigated macroscopically and microscopically for abnormalities. In addition, the weight of the adrenals was determined for all mice. Adrenal glands that weigh 8 mg (twice the weight of a healthy adrenal) or more were considered as tumor-containing. Mice were genotyped using tail DNA and PCR was performed with forward and reverse primers for *Pten* and *Trp53* (primer sequences available on request). Forty-eight adrenal tumors and 28 corresponding lungs of mice of different genotypes were available for immunohistochemical investigation (Supplementary table 1).

Immunohistochemistry

Forty-eight formalin-fixed paraffin-embedded (FFPE) adrenals of 25 *PSA-Cre;Pten*$^{loxP/loxP}$, 8 *PSA-Cre; Pten*$^{loxP/loxP}$*;Trp53*$^{loxP/+}$, and 15 *PSA-Cre; Pten*$^{loxP/loxP}$*;Trp53*$^{loxP/loxP}$ were investigated with immunohistochemistry according to the previously described method with markers for tyrosine hydroxylase (TH) and dopamine-β-hydroxylase (DBH).[13] In addition, immunohistochemistry with an antibody for succinate dehydrogenase subunit B (Rabbit polyclonal HPA002686; Sigma-Aldrich, Saint Louis, MO, USA; 1/250) was performed according to procedures reported previously.[20] Furthermore, 28 corresponding lungs were histologically and immunohistochemically (TH and DBH) investigated for the presence of PCC metastases. For each tumor and lung, negative controls were performed by omission of the primary antibody. Lungs were systematically investigated for metastases by three step sections of 5 µm at intervals of at least 10 µm. The adrenals of Cre-negative littermates were used as positive controls.

Table 1. Frequency of pheochromocytomas in mice of different ages and genotypes

Genotype	2 months	4-5 months	7-8 months	11-12 Months	15 months	18 months
Pten$^{loxP/loxP}$	0 / 7	0 / 10	0 / 9	9 / 10 (90%)	7 / 7 (100%)	-
Pten$^{loxP/loxP}$;Trp53$^{loxP/+}$	0 / 2	1 / 10 (10%)	11 / 12 (92%)	12 / 12 (100%)	8 / 8 (100%)	-
Pten$^{loxP/loxP}$;Trp53$^{loxP/loxP}$	0 / 7	2 / 10 (20%)	27 / 29 (93%)	-	-	-
Pten$^{loxP/+}$	0 / 2	0 / 11	0 / 10	2 / 8 (25%)	1 / 4 (25%)	3 / 5 (60%)
Pten$^{loxP/+}$;Trp53$^{loxP/+}$	0 / 2	0 / 7	0 / 5	2 / 6 (33%)	3 / 3 (100%)	3 / 3 (100%)
Pten$^{loxP/+}$;Trp53$^{loxP/loxP}$	0 / 3	0 / 10	0 / 9	7 / 9 (78%)	5 / 5 (100%)	-
Total	0 / 23	2 / 58 (3.4%)	38 / 75 (51%)	32 / 45 (71%)	24 / 27 (89%)	6 / 8 (75%)

The columns contain the number of mice presenting with PCC per total number of mice, and the corresponding percentages are between brackets.

Table 2. Adrenal weights at different ages per genotype

Genotype	2 months	SD	4-5 months	SD	7-8 months	SD	11-12 months	SD	15 months	SD	18 months	SD
$Pten^{loxP/loxP}$	3.8 mg	1.1	5.0 mg	1.1	5.0 mg	1.3	44.0 mg	50.0	122 mg	93.5	-	
$Pten^{loxP/loxP};Trp53^{loxP/+}$	6.3 mg	0.9	5.9 mg	3.6	17.5 mg	11.1	225 mg	183	326 mg	209	-	
$Pten^{loxP/loxP};Trp53^{loxP/loxP}$	3.7 mg	1.4	8.6 mg	9.2	38.0 mg	99.3	-		-		-	
$Pten^{loxP/+}$	4.0 mg	1.3	3.5 mg	0.6	2.8 mg	0.7	6.0 mg	4	72.7 mg	142	26.8 mg	44.4
$Pten^{loxP/+};Trp53^{loxP/+}$	3.8 mg	1.2	3.5 mg	1.4	3.3 mg	1.7	158 mg	377	210 mg	310	338 mg	286
$Pten^{loxP/+};Trp53^{loxP/loxP}$	3.6 mg	0.9	3.0 mg	1.1	3.6 mg	1.0	72.7 mg	117	163 mg	126	-	

SD = standard deviation

Comparative Genomic Hybridization

Of the 14 mice of which snap-frozen PCC tissue was available, DNA was isolated using the Gentra Puregene Tissue Kit (Qiagen, Venlo, the Netherlands) according to manufacturer's procedures. Labelling, hybridization conditions and methods were described previously.[13] For hybridization, high-density whole-genome mouse bacterial artificial chromosome microarray slides produced by the Central Microarray Facility of The Netherlands Cancer Institute in Amsterdam were used.[21] Slide scanning was performed with the ScanArray Express HT (Perklin Elmer Life Science, Boston, MA). Spot intensities were measured with GenePix Pro 5.1 software (Axon Instruments, Leusden, the Netherlands). Excel 2000 and the aCGH-smooth application (http://www.few.vu.nl/~vumarray/acgsmooth.htm) were used to analyze the normalized data. The default settings of aCGH-smooth were used, except for $\lambda = 8.5$.

Results

Trp53 inactivation leads to an earlier presentation and a higher frequency of PCC

All average adrenal weights and tumor percentages per age group and genotype are listed in Table 1. PCC occurred from the age of 11 months onwards in the $Pten^{loxP/loxP}$ and the $Pten^{loxP/+}$ mice. In contrast, if *Trp53* was inactivated in the $Pten^{loxP/loxP}$ mice (homozygously as well as heterozygously), PCC occurred already at the age of 4 to 5 months. Both the $Pten^{loxP/loxP};Trp53^{loxP/+}$ and the $Pten^{loxP/loxP};Trp53^{loxP/loxP}$ mice displayed a nearly full penetrance at the age of 7-8 months (92% and 93% respectively). The main difference between the two genotypes was that all $Pten^{loxP/loxP};Trp53^{loxP/loxP}$ mice had to be sacrificed at the age of 8 months, because of severe health problems due to extremely fast growing salivary gland tumors, whereas the $Pten^{loxP/loxP};Trp53^{loxP/+}$ stayed healthy until the age of 15 months. $Pten^{loxP/+}$ mice showed the first PCC at 11-12 months, similar to the $Pten^{loxP/loxP}$, but at a much lower frequency (33% versus 90%). In addition, heterozygous and homozygous *Trp53* inactivation in the $Pten^{loxP/+}$ mice resulted in larger tumors that occurred at a higher frequency compared to the $Pten^{loxP/+}$ mice.

Immunohistochemistry

Forty-eight primary tumors were immunohistochemically stained for TH and DBH, to confirm the tumors were PCC. All tumor cells stained positive for TH and DBH, as well as the positive controls, which included the adrenals of Cre-negative mice. SDHB immunohistochemistry was positive for all mouse PCC investigated (Figure 1).

Presence of lung metastases

In total, 28 lungs of 3 different genotypes were available for investigation of the presence of metastases (Supplementary table 1). Mouse lungs were examined by histology and immunohistochemistry with markers for TH and DBH. Metastases typically encompassed few cells, usually approximately 5 to 10 cells with a maximum of 25 cells (illustrated in Figure 1). Generally, up to 5 metastases were seen scattered throughout the lungs. Both *Pten*$^{loxP/loxP}$ and *Pten*$^{loxP/loxP}$;*Trp53*$^{loxP/+}$ mice displayed PCC metastases in 67% of mice (8 in 12 mice, and 2 in 3 mice, respectively). The *Pten*$^{loxP/loxP}$;*Trp53*$^{loxP/loxP}$ showed lung metastases in 61.5% of mice.

Comparative Genomic Hybridization

CGH was performed on 14 mouse tumors from four genotypes. An overview of the CGH results is shown in Table 3 and a typical example of the result is shown in figure 2. The most frequent aberrations were complete loss of chromosome 19 (in 85.7% of mice) and gain of (parts of) chromosome 15 (in 71.4% of mice). Furthermore, loss of (parts of) chromosome 14 and chromosome 6 (in 42.9% and 35.7% of mice, respectively) were demonstrated as the second and third most frequent alterations.

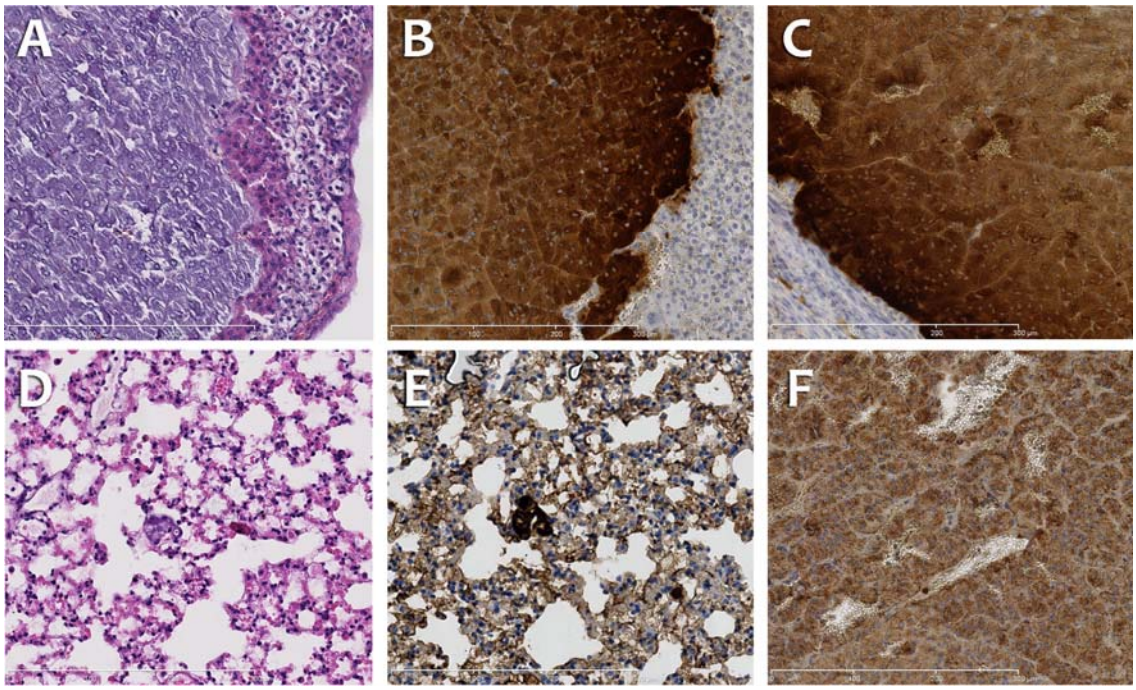

Figure 1. Haematoxylin and eosin staining of normal adrenal gland (A) and lung metastasis (D); TH immunohistochemistry of normal adrenal gland (B), PCC (C), and lung metastasis (E); and SDHB immunohistochemistry of a PCC.

Table 3 . Overview of genomic alterations

Mouse #	Genotype	Chr. 2	Chr. 4	Chr. 6	Chr. 7	Chr. 8	Chr. 9	Chr. 13	Chr. 14	Chr. 15	Chr. 16	Chr. 19
3	$Pten^{loxP/loxP}$								WC -	62-103Mb +		
4	$Pten^{loxP/loxP}$									WC +		WC -
6	$Pten^{loxP/loxP}$	42-181Mb +		27-150Mb -	79-153Mb -				WC -			
10	$Pten^{loxP/loxP}$			WC -	51-153MB -							WC -
13	$Pten^{loxP/loxP};Trp53^{loxP/+}$								WC -	WC +		WC -
14	$Pten^{loxP/loxP};Trp53^{loxP/+}$									WC +		WC -
15	$Pten^{loxP/loxP};Trp53^{loxP/+}$					30-61Mb +				88-103Mb +		WC -
30	$Pten^{loxP/loxP};Trp53^{loxP/+}$									WC +		WC -
31	$Pten^{loxP/loxP};Trp53^{loxP/+}$				112-153Mb -					WC +		WC -
32	$Pten^{loxP/loxP};Trp53^{loxP/+}$					WC +			WC -	WC +	WC +	WC -
33	$Pten^{loxP/loxP};Trp53^{loxP/+}$									WC+		WC -
18	$Pten^{loxP/loxP};Trp53^{loxP/loxP}$			WC -	100-153Mb -		WC -	WC -	0-104Mb -			WC -
28	$Pten^{loxP/loxP};Trp53^{loxP/loxP}$			WC -						WC +		WC -
29	$Pten^{loxP/+};Trp53^{loxP/loxP}$		0-18Mb -	WC -			WC -		WC -			WC -

WC = whole chromosome, - = loss, + = gain.

Discussion

In this study, we have shown that the *Pten*/*Trp53* KO mouse model presents malignant PCC at a high frequency, that display genomic alterations similar to those found in our previous study, and which are comparable with the alterations found in a subtype of human PCC.

The *PTEN* gene belongs to the most frequently mutated genes in human cancer. The main function of PTEN is to convert phosphatidylinositol (3,4,5)-triphosphate to phosphatidylinositol (4,5)-diphosphate, thereby inhibiting the activation of AKT and its down-stream targets.[22] To investigate the effect of *PTEN* inactivation in tumorigenesis, many *Pten* KO mouse models have been generated.[8, 12, 13, 19, 23-26] In the conventional *Pten* KO mouse models, *Pten* nullizygosity leads to embryonic lethality, whereas *Pten* heterozygotes present with many different tumors, including prostate cancer and pheochromocytomas.[12, 26] To investigate the exact effect of *PTEN* inactivation in the prostate, a prostate-specific conditional mouse model was generated using the Cre-lox system under the control of the PSA-promoter.[19] The $Pten^{loxP/loxP}$ KO mice developed invasive prostate carcinomas and pheochromocytomas, of which the earliest pheochromocytomas occurred in mice aged 7 to 9 months. In the present study, the $Pten^{loxP/loxP}$ mice presented the first PCC in the mice aged 11 to 12 months. This difference is because age groups of the present study were chosen differently compared to the previous study. In the present study, mice were not sacrificed at the age of 9 months. Furthermore, both the previous 10 to 14 month old mice and the present 11 to 12 month old mice show PCC at identical frequency (in 90% of cases).

TP53 is the most frequently mutated gene in human cancer. It acts as a tumor suppressor gene and regulates different cellular functions, especially cellular senescence and apoptosis. In a normal cell, p53 is suppressed by E3 ubiquitin ligase MDM2 (mouse double minute 2), which will bind to p53 and ubiquinate p53 for degradation.[15, 27] In a stressed cell, p53 will be activated. Recently, mutated *TP53* has been associated with cell migration and invasion, suggesting that p53 inactivation can contribute to a tumor's metastatic potential. In the present study, the heterozygous and homozygous inactivation of *Trp53* resulted in an earlier tumor presentation in the *Pten* KO mice. In contrast, the percentage of PCC metastases was not elevated in the $Pten^{loxP/loxP}$;$Trp53^{loxP/+}$

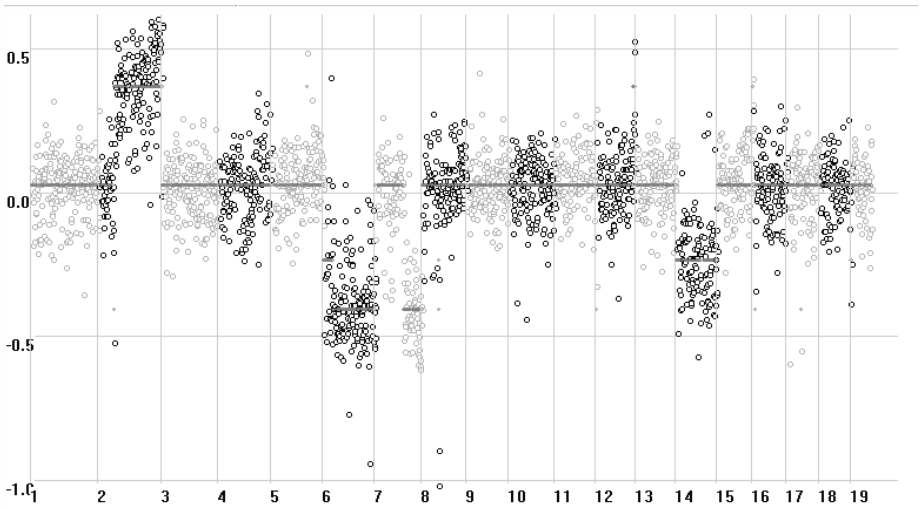

Figure 2. An example of a CGH result of mouse number 6, displaying gain of chromosome 2, loss of whole chromosome 6 and 14, and a part of chromosome 7.

or *Pten*$^{loxP/loxP}$;*Trp53*$^{loxP/loxP}$ KO mice, compared to that of the *Pten*$^{loxP/loxP}$ KO mice. The *Pten*$^{loxP/loxP}$;*Trp53*$^{loxP/loxP}$ mice developed severe health problems, so they had to be sacrificed at the age of 8 months. We speculate that metastasizing cells did not have enough time to reach the lungs in these mice. Nonetheless, the frequency of metastases was considerably higher in the three KO mouse genotypes of the present study compared to the *Pten* KO mice of the previous study.[13] There is no clear explenation for this phenomenon, as the present and previous mouse models both have an FVB/Ola129 background. It has been reported previously that crossing different mouse strains can increase tumor susceptibility,[23, 28, 29] so we assume that the present *Pten*$^{loxP/loxP}$ mice are more susceptible to lung metastases as a result of the mixture of genetic backgrounds, but these are not different in the former and present *Pten*$^{loxP/loxP}$ mice. Also, the evaluation method of investigating the mouse lungs for metastases was identical in both studies.

Genetic alterations have been investigated by comparative genomic hybridization (CGH) in 2 *Pten* KO mouse models.[12, 13] The first study investigated the PCC of (conventional) heterozygous KO mice and found loss of chromosome 4 as the most frequent genetic alteration.[12] In contrast, in our previous study, we found almost no loss of chromosome 4 in our (conditional) *Pten* KO mice, but found loss of chromosome 6 and 19 as the main chromosomal alterations.[13] In the present study, we performed CGH on 14 PCC of 4 different genotypes. The most frequent aberration found was loss of chromosome 19 and gain of chromosome 15. The frequency of loss of chromosome 19 was similar to that of the previous study (86% and 75% respectively). Chromosome 19 is syntenic to human chromosome 11q13, which is associated with *SDHD*-related and *VHL*-related PCC. Gain of chromosome 15 occurred considerably more often in PCC of the present model and was not associated with a specific genotype. The higher frequency of gain of chromosome 15 could be due to more malignant behavior of the tumors of the present mice, as they displayed lung metastases more frequently than the previous model. The presence of more chromosomal gains in malignant tumors compared to benign lesions has also been demonstrated in human malignant PCC.[30, 31] As mentioned previously, mouse chromosome 15 is syntenic to human chromosome 5p13-15, 8q22-24, 22q13,1-3, and 12q12-13, of which 5p has been associated with the pathogenesis of human malignant PCC (personal observations of a large series of human malignant PCC).

Approximately 10% of human PCC presents with metastases, but this frequency is higher in SDHB-related PCC, which develop metastases in 32% of cases.[4] In a previous study on human PCC we showed that SDHB immunohistochemistry could predict the presence of an *SDHB*, *SDHC*, or *SDHD* mutation.[20] PCC that had a mutation in one of these genes showed negative SDHB staining of the tumor cells. In the present study, we performed SDHB immunohistochemistry on the PCC of the *Pten* and *Trp53* KO mice, and found only tumors with positive tumor cells. Therefore, it is highly unlikely that succinate hydrogenase (mitochondrial complex II) is involved in the pathogenesis of these mouse tumors. This is in accordance with our previous findings, as *Sdhb* or *Sdhd* mutations were not found in the *Pten*$^{loxP/loxP}$ mice.[13]

In conclusion, we have investigated 236 mouse adrenal glands from six different genotypes involving heterozygous en homozygous knock-down of *Pten* and *Trp53* for the presence of PCC, by determining adrenal weights and histological confirmation. Our results demonstrate that tumors occurred at much earlier ages in the *Pten*$^{loxP/loxP}$;*Trp53*$^{loxP/loxP}$ and the *Pten*$^{loxP/lox}$;*Trp53*$^{loxP/+}$ mice, compared to the *Pten*$^{loxP/loxP}$

mice. Tumors and lung metastases were immunohistochemically positive for TH and DBH expression, which confirms that they are of adrenal medulla origin. The fact that this mouse model is highly malignant is also illustrated by the CGH patterns that display gain of chromosome 15 at a high frequency. This mouse model has higher metastatic potential and is therefore more suitable for the investigation of malignant PCC compared to our previous *Pten* KO mouse model, regardless of the inactivation of *Trp53*.

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Supplementary table 1. Presence of PCC lung metastases

	Mouse #	adrenal weight (mg)	age (months)	lung metastasis
$Pten^{loxP/loxP}$	1	32	10	Y
	2	41.4	12	N
	3	20.5	12	Y
	4	133	12	Y
	5	32.1	12	Y
	6	140	12	Y
	7	9.6	12	N
	8	26.2	15	N
	9	66.4	15	Y
	10	103	15	Y
	11	80.3	15	Y
	12	69.7	15	N
$Pten^{loxP/loxP};Trp53^{loxP/+}$	13	286	11	Y
	14	117	12	Y
	15	71	15	N
$Pten^{loxP/loxP};Trp53^{loxP/loxP}$	16	40.5	7	Y
	17	8.0	7	N
	18	21.8	7	Y
	19	27.5	8	Y
	20	33.7	8	N
	21	18.0	8	N
	22	27.3	8	Y
	23	18.1	8	N
	24	46.8	8	Y
	25	38.2	8	Y
	26	15.1	8	Y
	27	34.8	8	Y
	28	55.6	11	N

Chapter 7

CANDIDATE GENE MUTATION ANALYSIS IN BILATERAL ADRENAL PHEOCHROMOCYTOMA AND SYMPATHETIC PARAGANGLIOMA

*Esther Korpershoek, *Bart-Jeroen Petri, Francien H. van Nederveen, Winand N.M. Dinjens, †Albert A. Verhofstad, Wouter W. de Herder, Sonja Schmid, Aurel Perren, Paul Komminoth, Ronald R. de Krijger

* These authors should be considered as equal first authors
† deceased

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Abstract

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

Introduction

Pheochromocytomas (PCC) are rare catecholamine-producing tumors that arise from chromaffin cells of the adrenal medulla, but can also occur outside the adrenal in the abdomen, and are then called sympathetic paragangliomas (sPGL).[1] Pheochromocytoma-associated syndromes include multiple endocrine neoplasia type 2 (MEN2), von Hippel-Lindau disease (VHL), neurofibromatosis-1 (NF1) and the familial pheochromocytoma - paraganglioma (PCC-PGL) syndrome.[2] MEN2 is characterized by medullary thyroid carcinoma (MTC) in association with PCC and has three clinical variants, MEN2A, familial medullary thyroid carcinoma (FMTC), and MEN2B.[3] The syndrome is caused by germline mutations of the RET proto-oncogene, which are mostly (80-96%) found in RET exons 10, 11 and 16.[3, 4] Somatic RET mutations have also been found in sporadic PCC affecting exons 10, 11 and 16.[5]

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

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

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

Although knowledge about genotype-phenotype relationships has improved for germline RET, VHL, SDHB and SDHD mutations, only few studies have addressed mutations of these PCC causing genes in specific series of bilateral PCC and/or sPGL. In addition, there are no studies that have compared tumor tissue and corresponding normal tissue for the detection of somatic mutations. To determine the mutation frequency for each of these four candidate genes in a large subset of patients, we have

selected 33 patients with bilateral PCC and 26 patients with sPGL, to screen for germline and somatic mutations in RET exon 10, 11, 13, 14, 15 and 16, and in all exons of VHL, SDHB and SDHD genes. In addition, we discuss the significance of SDHB and SDHD sequence abnormalities.

Materials and Methods

Patients

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

Tissue preparation

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

Denaturating Gradient Gel Electrophoresis (DGGE)

PCR with genomic DNA as template was carried out in a 50 µl mixture of 1X PCR buffer (Perkin Elmer Europe, Rotkreuz, Switzerland) containing 10 to 400 ng of template DNA, 200µM of each intron-based primer (Table 1) and 1 µl Taq polymerase (Ampli Taq Gold, Perkin Elmer Europe). After a hot start of 7 min at 95oC, a 'touch-down' procedure was used consisting of denaturation for 60 s at 95oC, annealing for 60 s at temperatures decreasing from 60 to 55oC during the first 11 cycles (with 0.5oC decremental steps in cycles 2 to 11), and ending with an extension step at 72oC for 60 s. Ten cycles with an annealing temperature of 55oC and 15 cycles with an annealing temperature of 45oC followed with extension times of 90 s. After a final extension for 10 min at 72oC, heteroduplex formation was induced by initial denaturation for 10 min at 98oC followed by incubations at 55oC for 30 min and 37oC for 30 min. For DGGE, 10 µl of the PCR product in 3 µl Ficoll-based loading buffer were loaded onto 10% polyacrylamide gels containing a urea-formamide gradient in 0,5x Tris-acetate-EDTA (TEA). The amplicons were

Table 1. Primers used for mutation analysis

	Forward 5' → 3'	Reverse 5' → 3'	Product size (bp)	Mutation analysis method
RET 10	GCGCCCCAGGAGGCTGAGTG	cgcccgccgcgccccgcgcccgtcccgccgcccccgcgTGGTGGTCCCGGCCGCC	225	DGGE
RET 11	CTCTGCGGTGCCAAGCCTCA	cgcccgccgcgccccgcgcccggcccgccgcccccgcccgGAGTAGCTGACCGGGAAGGC	262	DGGE
RET 13	CTCAAGCAGCATCGTCTTTG	AAGGGAGAAAGAGGGAGAAC	328	SSCP
RET 14	GGTTCAAGAGAAAGCTGAGG	CTAGAGTGTGGCATGGTGG	524	SEQ
RET 15	CTCTGCTGGTCACACCAGG	CGGTATCTTTCCTAGGCTTC	296	SSCP
RET 16	cgcccgccgcgcccgcgcccggcccgccgccccgcccgaaataataaaAGGGATAGGGCCTGGGCTTC	ACCCCAAGAGAGCAACACCC	224	DGGE
VHL 1a	gcgcgcgAGCGCGTTCCATCCTCTAC	cgcccgccgcgccccgcgcccggcccgccgccccgcccgAGGGCCGTACTCTTCGAC	255	DGGE
VHL 1b	gcgcgGCGGAGAACTGGGACGAG	cgcccgccgcgccccgcgcccggcccgccgccccgcccgGCTTCAGACCGTGCTATCGT	418	DGGE
VHL 2	cgcccgccgcgcccgcgcccggcccgccgccccgcccgaaataataaaCTTTAACAACCTTGCTT	cgtcccgcGTCTATCCTGTACTTACCAC	266	DGGE
VHL 3	gcgcgTTCCTTGTACTGAGACCCTAGT	cgcccgccgcgccccgcgcccggcccgccgccccgcccgAGCTGAGATGAAACAGTGTAAGT	316	DGGE
SDHB 1	GAGCGACCTCGGGGTTAAG	GCTTTCCTGACTTTTCCCTC	157	SSCP
SDHB 2	cgcccgccgcgcccgcgcccggcccgccgccccgcccgTTTTTCCTTTTTGTGAACTTT	AAGCATGTCCCTAAATCAAA	265	DGGE
SDHB 3	cgcccgccgcgcccgcgcccggcccgccgccccgcccGGAACTTTACATAAATACCACTGGA	cgCTATCAGCTTTGGCCAGC	242	DGGE
SDHB 4	cgcccgccgcgcccgcgcccggcccgccgccccgcccgaaataataaaTGATTCCGGATATGGGTGAG	cgtcccgcCCCCCATGCAAATAAAAACA	256	DGGE
SDHB 5	gcgcgTGATGATGGAATCTGATCCT	cgcccgccgcgccccgcgcccggcccgccgccccgcccgaaataataaaCAGATTGAAACAATAAATAGGGA	261	DGGE
SDHB 6	cgcccgccgcgcccgcgcccggcccgccgccccgcccgaaataataaaCCTCTCTTTTCTCCCCATAC	cgCAGCAATCTATTGTCCTCTTG	252	DGGE
SDHB 7	gcgcgAGCTAATCATCCCTGGTTTT	cgcccgccgcgccccgcgcccggcccgccgccccgcccgaaataataaaTTGTGAGCACATGCTACTTC	270	DGGE
SDHB 8	cgcccgccgcgcccgcgcccggcccgccgccccgcccgGTGGGTTTTCCCTTTCAGTT	cgcccgccgcgccccgcgcccggcccgccgccccgcccgGTGGGTTTTCCCTTTCAGTT	361	DGGE
SDHD 1	cgcccgccgcgcccgcgcccggcccgccgccccgcccgaaataataaaTGACCTTGAGCCCTCAGGAACG	gcgcgTCAGGGTGGGAAGACCCCT	153	DGGE
SDHD 2	GATCATCCTAATGACTCTTTCC	AGCAGCAGCGATGGAGAGAA	168	SSCP
SDHD 3	cgcccgccgcgcccgcgcccggcccgccgccccgcccgCTTTTATGAATCTGGTCCTTTTTG	gcgcgCAACTATATTTGGAATTGCTATAC	245	DGGE
SDHD 4	cgcccgccgcgcccgcgcccggcccgccgccccgcccgTGATGTTATGATTTTTTCTTTTTCT	gcgcgCAATTCTTCAAAGTATGAAGTCA	269	DGGE

electrophoresed at 60oC and 100 V for 16 h. DNA strands were visualized using silver staining as described previously.[19]

Single Strand Conformation Polymorphism (SSCP) analysis

PCR amplification of tumor DNA was performed with 10 to 100 ng DNA in a final volume of 15 µl containing 1.5mM $MgCl_2$, 10mM Tris-HCl, 50mM KCl, 0.02mM dATP, 0.2mM dGTP, dTTP, and dCTP, 0.8µCi α32P-dATP (Amersham, Buckinghamshire, United Kingdom), 15 pmol of each forward and reverse primer (Table 1), and 3 U Taq polymerase (Ampli Taq Gold, Perkin Elmer Europe). PCR was performed for 35 cycles of 95°C for 30 s, 55°C for 45 s, and 1 min at 72°C, followed by 1 cycle at 72°C for 10 min. PCR products were electrophoresed overnight at 8 W on a non-denaturing gel, containing 8% polyacrylamide ((49:1) Fluka, Neu-Ulm, Germany) and 10% glycerol (v/v). After electrophoresis, the gel was dried and exposed to an X-ray film.

DNA sequencing

All samples demonstrating aberrant patterns in the DGGE or SSCP analysis were sequenced. PCR was performed in a final volume of 50 µl under identical conditions as the previous PCR, except that this mix contained 0.2mM dNTPs instead of 0.02mM dATPs and 0.8µCi α32P-dATP. The PCR products were purified using nucleospin®Extract II (Macherey-Nagel, Düren, Germany) according to the manufacturers instructions. The purification was followed by a sequence reaction using the Bigdye® Terminator V3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). Two µl of Termination Ready Reaction mix, 2 µl of 5x Sequencing buffer, 1 µl forward or reverse primer (13.2 pM), and 14 µl of deionised water was added to 1 µl purified PCR product. The cycle Sequencing program was performed for 25 cycles of 96oC for 10 s, 50oC for 5 s and 4 min for 60oC. This was followed by a precipitation step, adding 13 µl deionised water, 3 µl 3M NaAc (pH 5.2) and 64 µl ethanol (100%) to the sequence reaction product and incubating overnight at room temperature before centrifuging 20 min at 14.000 rpm. After washing the samples with ethanol 70%, centrifuging them 10 min at 14.000 rpm, the pellets were resuspended in 20 µl formamide (Applied Biosystems, Warrington, UK). Products were analyzed on the ABI Prism 3100 genetic analyzer (Applied Biosystems).

Table 2. Summary of results of bilateral PCC mutation carriers

Patient	Sex [1]	Age (year)	b/m	Gene	Nucleotide Change Mutation/ *Polymorphism**	Amino Acid Change *Polymorphism**	Family	Follow up (Years)
F6	m	31	b	*VHL*	CGC → CCC	R64P	A	21
F28	m	29	b	RET	TGC → CGC	C634R		
				SDHB	*CAC → CCC**	*A6A**		
				SDHD	*CAC → CGC**	*H50R**	D	26
F37	f	32	b	RET	TGC → CGC	C634R	B	24
F38	m	42	b	*RET*	TGC → CGC	C634R	B	22
F39	f	42	b	*RET*	TGC → CGC	C634R	C	24
F41	m	16	b	*RET*	TGC → CGC	C634R		
				SDHD	AGC → AGT*	S68S*	D	21
F42	f	29	b	*RET*	TGC → CGC	C634R	D	24
F65	m	u	b	*RET*	TGC → CGC	C634R	E	u
F78	f	29	b	*RET*	TGC → CGC	C620R		2
F79	m	26	b	*RET*	TGC → CGC	C634R	E	14
F80	f	18	b	*RET*	TGC → CGC	C634R	B	14
F82	f	24	b	*RET*	TGC → CGC	C634R	B	u
F84	f	50	b	*RET*	TGC → CGC	C634R	B	11
F87	m	24	b	VHL	CGC → CCC	R64P	A	11
F89	f	38	b	*RET*	TGC → CGC	C634R	C	12
F92	f	51	b	*RET*	TGC → TAC	C611Y	-	12
F102	f	27	b	*RET*	ATG → ACG	M918T	-	10
F141	m	49	b	*RET*	TGC → TGG	C634W	-	u
F147	f	27	b	*RET*	ATG → ACG	M918T	-	u
F165	f	59	b	*RET*	TGC → TAC	C611Y	-	17
F168	m	72	b	*RET*	TGC → TAC	C611Y	-	10 †
F184	m	53	b	*RET*	TGC → TAC	C634Y	F	2 †
F188	f	25	b	*SDHD*	GAC → TAC	D92Y	-	38
F194	f	24	b	*RET*	TGC → TAC	C634Y	F	9

Results

Bilateral adrenal PCC

The series of bilateral adrenal PCC encompassed 31 patients with benign PCC and 2 patients with malignant PCC. The results of the mutation analysis are summarized in table 2. Twenty-one patients (8 male, 13 female) with germline mutations in the RET proto-oncogene were identified in this group (average age 37 years), of which 15 were located in exon 11 (codon 634), 4 in exon 10 (codon 611 and 620) and 2 in exon 16 (codon 918) (Figure 1). No mutations were found in RET exons 13, 14 and 15. All of the RET mutation carriers had benign PCC. After mutation analyses was performed, it was revealed that 12 patients with RET mutations belonged to 4 families (with 5, 3, 2 and 2 patients, see table 2). We did not have clinical information about familial occurrence in 4 of the RET mutation-positive cases. The mutation analysis showed 2 patients with R64P germline mutations in exon 1 of the VHL tumor suppressor gene, which both had no metastasis.

These patients belonged to the same family. In addition, one D92Y mutation was found in exon 3 of the SDHD gene. Thus, if we consider each family as a single entity, we would have 14 index patients with bilateral PCC, in whom we found 12 RET mutations, 1 VHL mutation and 1 SDHD mutation.

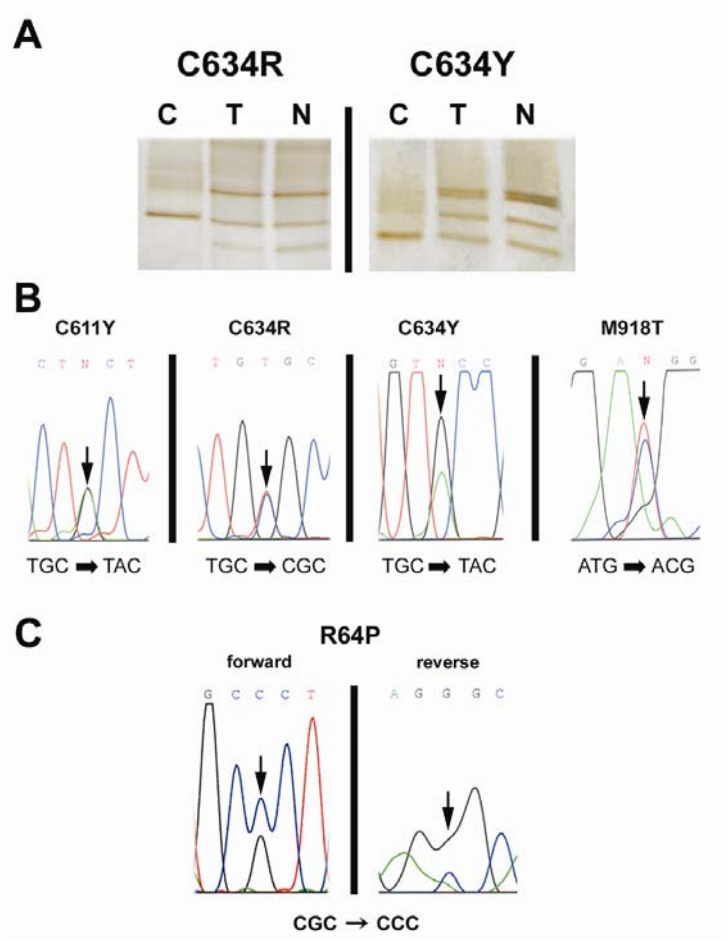

Figure 1. (A) DGGE patterns of healthy control (C), tumor (T), and normal (N) DNA of patients carrying a germline *RET* C634R or C634 Y mutation. (B) Sequence result of four of the six germline *RET* mutations detected by DGGE mutation analysis: C611Y, C634R, and M918T. (C) Forward and reverse sequence of tumor sample carrying the germline *VHL* R64P mutation found in a patient with bilateral PCC

None of the patients with bilateral adrenal PCC had mutations in SDHB. However, 3 heterozygous polymorphisms were found in SDHB or SDHD in two patients harboring a RET C634R mutation. These included A6A in exon 1 of SDHB, and H50R and S68S in exon 2 and 3 of SDHD respectively. On one occasion, SDHB A6A and SDHD H50R were present in the same patient (Table 3).

Three patients had metachronous appearance of their bilateral PCC. These patients included one with a C634Y mutation who developed PCC with a 3 year interval, and two patients that did not have mutations in RET, VHL, SDHB or SDHD, which developed PCC with a 4 and 6 year interval. Twenty-seven of the patients with bilateral PCC had synchronous PCC.

Sympathetic PGL

The sPGL included 11 benign and 15 malignant tumors. Results are summarized in table 2. Mutation analysis of RET and VHL did not show any abnormalities in the 26 patients with sPGL. In contrast, four aberrations were detected in SDHB exon 4, 5 and 7. The first aberration revealed a novel mutation, S100F in exon 4, which was not present in normal DNA of the same patient. The sPGL of this 25-year-old female patient was localized in the bladder. The second aberration represented a deletion of one nucleotide (c. 481del G),

which was also present in corresponding normal DNA and results in a truncated protein (p.D161fsX14). The third variant pattern of SDHB, in exon 5, was identified as a heterozygous germline S163P substitution, which is described as a rare polymorphism.[20] The fourth SDHB aberration represented the heterozygous mutation C243S in exon 7, which was also present in corresponding normal DNA (Figure 2).

Mutation analysis of the SDHD gene showed four abnormal patterns, of which two represented previously described germline mutations (D92Y and L95P). The SDHD D92Y mutation was found in a 52-year-old woman, who had a bilateral adrenal PCC 27 years earlier, which is in our bilateral series as well (Table 2, patient F188). The L95P mutation was found in a patient with sPGL at multiple abdominal spots. The other two patterns appeared to be the H50R and S68S polymorphisms (Table 3). There were too few tumors with mutations in this series to draw any conclusions on genotype-phenotype relationships.

Discussion

It has been reported that patients with RET or VHL germline mutations often present with bilateral adrenal PCC [1, 15] and that part of the germline SDHB or SDHD carriers develop sPGL [13, 17, 21]. However, detailed information about the frequencies of these mutations in patients with bilateral adrenal PCC or sPGL is limited. In addition, there have been virtually no studies based on tumor tissue, which allows the detection of somatic mutations in addition to germline mutations. In the present study we have investigated tumor and corresponding normal tissue from a unique series of bilateral PCC and sPGL, predominantly from The Netherlands, for germline and somatic mutations in the above-mentioned PCC susceptibility genes. We found 12 RET, 1 VHL and 1 SDHD germline mutations in the 23 unrelated patients and families with bilateral PCC, and 3 SDHB and 2 SDHD mutations in the series of 26 sPGL.

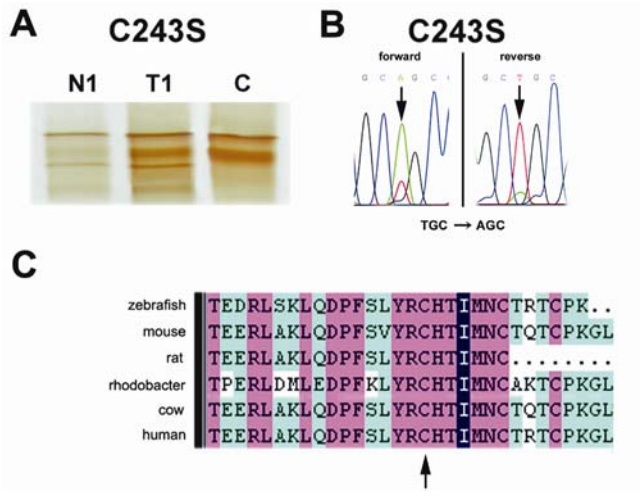

Figure 2. (A) Altered *SDHB* DGGE pattern in tumor (T1) and corresponding normal (N1) DNA from patient F207 compared with healthy control DNA (C). Note the identical aberrant pattern in both tumor and normal DNA compared with healthy control DNA pattern, indicating germline aberration. (B) Patient F207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

The 12 RET mutations encompassed 3 mutations in codon 611 (C611Y), 1 in codon 620 (C620R), 6 in codon 634 (n=4 C634R, n=1 C634Y, n=1 C634W), and 2 in codon 918 (M918T). All of these mutations have been reported previously.[10, 22, 23] Since other RET mutations have been described in exon 13, 14 and 15 [4] we performed additional SSCP mutation analyses for these exons on the PCC that were negative for mutations in RET exon 10, 11 or 16 or VHL, but no mutations were found. Two patients with bilateral PCC showed the same germline VHL mutation (R64P), and because both patients appeared to be related, they were counted as one patient in our series. These cases were previously reported by van der Harst et al.[24] The patient with the SDHD D92Y germline mutation wil be discussed below.

Mutation analysis of the 26 sPGL showed two novel germline (p.D161fsX14 and C243S) and one novel somatic variant in SDHB (S100F) and two germline mutations in SDHD (D92Y and L95P). The SDHB p.D161fsX14 variant was found in a patient with lung metastases. Since this mutation results in a truncated protein and loss of the wild-type allele was seen, we consider this abberation as a pathogenic mutation. In addition, loss of the wild-type allele was seen in the tumor DNA (data not shown). The novel SDHB C243S variant was seen in a patient with bone metastasis. We consider the SDHB C243S variant as a pathogenic germline mutation, because loss of the wild-type allele was seen in the sequence analysis results in our study (Figure 2). Second, SDHB C243 is highly conserved throughout many species. Third, two studies report patients who developed a malignant PCC, and had a SDHB mutation affecting amino acid R242 (R242H), which is also highly conserved.[13, 25] The somatic SDHB S100F variant appeared to be a mutation as well, as loss of the wild-type allele was seen (van Nederveen et al. 2007). In addition, this amino acid was conserved throughout many species. Mutations in SDHB S100 have also previously been described by Pollard et al.[26] in a patient with PGL and Neumann et al.[13] reported a patient with an sPGL who had a SDHB C101Y mutation.

Two polymorphisms were found in SDHB: A6A and S163P. The A6A polymorphism was found in one patient (2% of all patients) with an sPGL. A6A was previously reported as a polymorphism with a prevalence of 4% (NCBI: rs2746462), which is in concurrence with our data. The other SDHB polymorphism found in our study was S163P (2% of all patients), which was found in a patient with a sPGL. The frequency in our series was in the same range as the 2.3% S163P substitutions found by Cascon et al. in a healthy control population.[20]

Two previously described germline SDHD mutations were found in the sPGL group, D92Y and L95P.[21] Both mutations are known as Dutch founder mutations in head and neck paragangliomas.[27] The patient with the D92Y mutation also had an adrenal bilateral PCC 27 years earlier, which were surgically removed and included in our bilateral PCC series. The L95P mutation was found in a patient with a benign PCC, and after 12 years of follow-up, the patient was alive and well. Both patients are previously described by Dannenberg et al.[21]

Two polymorphisms were observed in SDHD, H50R in two patients and S68S in one. Since the frequency of the H50R substitution in our series (4% of all patients) is comparable to the frequency that has been reported in the literature for the normal population, we considered it a polymorphism.[28, 29] The SDHD S68S variant was seen in two patients (one with sPGL, one with bilateral PCC) and has previously been described as a polymorphism.[20]

Interestingly, the SDHB and SDHD mutation frequency found in this study is considerably lower (12% for SDHB including the somatic mutation and 8% for SDHD) than that reported in the study of Amar et al. which were 29% for SDHB and 12% for SDHD.[10] This difference might be explained on the basis of geographical variation in mutation frequency, as has become evident from comparative studies.[30] Another study reported a mutation range of 30% to 41% of SDHB mutations in a series of catecholamine-producing PGL.[31] This high mutation frequency could be due to a sample bias, as all PGL were malignant, whereas only approximately half of our sPGL were malignant. In addition, the technique used in our investigation cannot be used to detect all genetic changes in these candidate genes, i.e. it is not suitable for detecting large deletions, which have been demonstrated in VHL, SDHB and SDHD.[32-34]

The nucleotide alterations of SDHB and SDHD discussed above are thought to be polymorphisms (SDHB A6A and S163P; SDHD H50R and S68S), because they have also been demonstrated in healthy controls. It is striking though, that of the 5 patients with either a SDHB mutation or polymorphism, 3 patients also had a polymorphism in SDHD (Table 2 and 3). In our study, one patient with an sPGL harbored the SDHB S163P polymorphism and the SDHD H50R. In addition, the sPGL with the SDHB C243S mutation also showed the SDHD S68S polymorphism. To test whether there was a relation between SDHB and SDHD polymorphisms and PCC development, we screened an additional series of 89 normal DNA samples of patients with adrenal PCC for the SDHB S163P and SDHD H50R polymorphism. However, we did not find additional patients with both S163P and H50R, suggesting that there is no relationship between the polymorphisms and the development of adrenal PCC (data not shown). For sPGL this could not be assessed, due to the limited number of specimens available. Interestingly, SDHB and SDHD polymorphisms were recently demonstrated at increased frequency in patients with familial medullary thyroid carcinoma.[35]

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

In our study we found 52% RET and 4% VHL and 4% SDHD germline mutations in 33 patients with bilateral PCC and 12% SDHB and 8% SDHD mutations in 26 patients with sPGL. Amar et al. recently described 41% VHL (n=17), 27% RET (n=11), and 7% (n=3) SDHD germline mutations in 41 patients with bilateral adrenal PCC.[10] In addition these authors found 7% VHL (n=4), 29% SDHB (n=17) and 12% SDHD (n=7) mutations in the 58 patients with sPGL.[10] These results are not entirely in concurrence with our data. This occurrence is most likely due to geographical differences, as most of our patients were from the Netherlands, whereas all of the patients of Amar et al. were from France. In addition, geographical differences of mutation frequencies of the PCC susceptibility genes between France, Germany, and Italy have recently been shown by Giminez-Roqueplo et al. [30] In addition, the technique used in this investigation cannot be used to detect all

genetic changes in these candidate genes, i.e. it is not suitable for detecting large deletions, which have been demonstrated in VHL, SDHB and SDHD.[32-34]

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

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Chapter 8

A routine immunohistochemical procedure for the detection of paraganglioma and pheochromocytoma patients with germline *SDHB*, -C, or -*D* gene mutations: a retrospective and prospective analysis

*Francien H. van Nederveen, *José Gaal, *Judith Favier, Esther Korpershoek, Rogier A. Oldenburg, Elly M.C.A. de Bruyn, Hein F.B.M. Sleddens, Pieter Derkx, Julie Riviere, Hilde Dannenberg, Bart- Jeroen Petri, Paul Komminoth, Karel Pacak, Wim C.J. Hop, Patrick J. Pollard, Massimo Mannelli, Jean-Pierre Bayley, Aurel Perren, Stephan Niemann, †Albert A. Verhofstad, Adriaan P. de Bruïne, Eamonn R. Maher, Frédérique Tissier, Tchao Méatchi, Cécile Badoual, Jérôme Bertherat, Laurence Amar, Despoina Alataki, Eric Van Marck, Francesco Ferrau, Jerney François, Wouter W. de Herder, Mark-Paul F.M. Vrancken Peeters, Anne van Linge, Jacques W.M. Lenders, Anne-Paule Gimenez- Roqueplo, ♠Ronald R. de Krijger, and ♠Winand N.M. Dinjens.

* These authors should be considered as equal first authors
♠ These authors should be considered as equal last authors

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Abstract

Background
Pheochromocytomas (PCCs) and paragangliomas (PGLs) are neuro-endocrine tumors that occur sporadically and in several hereditary tumor syndromes, including the pheochromocytoma-paraganglioma (PCC-PGL) syndrome. This syndrome is caused by germline mutations in *succinate dehydrogenase B* (*SDHB*), *C* (*SDHC*), or *D* (*SDHD*) genes. Clinically, the PCC-PGL syndrome is often unrecognized although 10 to 30% of apparently sporadic PCCs and PGLs harbor germline *SDH*-gene mutations. Despite these figures, screening of all PCCs and PGLs for mutations in the *SDH*-genes to detect the PCC-PGL syndrome is rarely performed due to the workload and financial constraints.

Methods
Immunohistochemistry for SDHB was performed on 220 tumors. Two retrospective series of 175 PCCs and PGLs with known germline mutation status for PCC/PGL susceptibility genes were investigated. In addition, a prospective series of 45 PCCs and PGLs was investigated for SDHB immunostaining followed by *SDHB*, *-C*, and *-D* mutation testing.

Findings
SDHB protein expression was absent in all PCCs and PGLs with an *SDHB*, *-C*, or *-D* mutation and present in all MEN2-, VHL-, and NF1-related paraganglionic tumors. In addition, 47 of 53 (89%) PCCs and PGLs, in which no syndromic germline mutation was identified, showed SDHB expression. The sensitivity and specificity of the SDHB IHC to detect the presence of an *SDH*-mutation in the prospective series are 100% (95% CI: 87-100%) and 84% (95% CI: 60-97%), respectively.

Interpretation
Our results demonstrate that the PCC-PGL syndrome can be diagnosed reliably by an immunohistochemical procedure. Only in patients with SDHB-negative tumors *SDHB*, *- C*, and *-D* germline mutation testing is indicated. SDHB immunohistochemistry on PCCs and PGLs could improve the diagnosis of PCC-PGL syndrome, which is important for surveillance of PCC and PGL patients and their family members.

Introduction

Pheochromocytomas (PCCs) and paragangliomas (PGLs) are rare, usually benign, highly vascularised tumors that both originate from neural crest-derived chromaffin cells. The term PCC is reserved for intra-adrenal tumors whereas similar extra-adrenal tumors are called PGL. PGLs are subdivided in sympathetic PGLs (sPGLs) and parasympathetic PGLs (pPGLs) depending on their location and catecholamine production. pPGLs are located in the head and neck region, and usually do not produce catecholamines, whereas sPGLs are situated along the sympathetic trunk in the abdomen and usually produce catecholamines.[1]

PCCs and PGLs occur sporadically and in the context of several inherited tumor syndromes including multiple endocrine neoplasia type 2 (MEN2, with *RET* gene germline mutations), von Hippel-Lindau (VHL) disease (caused by germline mutations in the *VHL* gene), neurofibromatosis type 1 (NF1, with NF1 gene germline mutations) and the PCC-PGL syndrome (with germline mutations in the genes encoding the succinate dehydrogenase (SDH) subunits B, C, or D).[2, 3] The latter syndrome is characterized by the familial occurrence of PGLs and/or PCCs, at a generally younger age, and often by multifocal disease with increased risk of recurrence and increased frequency of malignancy in the case of SDHB mutations.[4] The PCC-PGL syndrome is the most frequent hereditary condition with manifestation of PGLs and is caused by germline mutations in the SDHB, -C, or -D genes. These genes encode three of four subunits of mitochondrial complex II, the succinate-ubiquinone oxidoreductase enzyme located at crossroads between the mitochondrial aerobic electron transport chain and the tricarboxylic acid cycle.[5] Recent studies demonstrated that SDH inactivation induces angiogenesis and tumorigenesis through the inhibition of hypoxia-inducible factors (HIF)-prolyl hydroxylase.[6] The SDHB, -C, and -D genes are bona fide tumor suppressor genes as biallelic inactivation is found in PCCPGL syndrome tumors (inherited inactivating germline mutation and acquired inactivating mutation of the corresponding wild type allele in the tumor).[7]

With the exception of the NF1 syndrome where the cutaneous café-au-lait spots are characteristic,[8] inherited PCC and PGL patients often escape clinical detection. In large published series of patients with PCC and/or PGL, it has been demonstrated that 25-30% of the patients have an inherited form and 12% of the patients with an apparently sporadic PCC and/or PGL have unexpected germline mutations in *VHL*, SDHB, or -D genes.[3, 7-9] The underdiagnosis of inherited PCC and PGL patients is the result of a combination of factors including lack of family information, overlap in age distribution between hereditary and sporadic cases, de novo mutations, incomplete penetrance (SDHB), parent of origin effects on penetrance (SDHD), phenotypic heterogeneity of the disease and insufficient awareness of clinicians. Controversy exists among experts whether *RET*, *VHL*, SDHB, -C, and -D genetic testing should be performed in all PCC and PGL patients. Many experts have advocated that molecular genetic testing should be targeted in patients fulfilling specific clinical criteria.[4, 10-12] However, reliable clinical indicators for the presence of SDHB, -C, and -D germline mutations in PCC and PGL patients are often lacking.

Hidden heredity is most pronounced for apparently sporadic pPGL patients, with up to 34% of cases having a germline mutation in SDHD.[13] Clinical indications with high specificity but low sensitivity for the detection of PCC-PGL syndrome (family history of PCC or PGL, multifocal disease, younger age and malignant tumors) are insufficient for

correct diagnosis of the syndrome. Detection of the inherited PCC-PGL syndrome is of major importance for PCC and PGL patients as well as for their family members since they are at increased risk for development of multiple, various and malignant neoplasms.[4, 14-16] In addition, after identification of an SDHB, -C, or -D germline mutation, surveillance can be offered to the individual patient with the paraganglionic tumor as well as to family members carrying the mutation. Mutation analysis of SDHB, -C, and -D has been advocated to diagnose the PCC-PGL syndrome in all PGL and PCC cases where there are no clear clinical or family indications for the syndrome.16 Although SDH-mutation carriers will be identified frequently by mutation analysis of all PCC and PGL patients, the majority of cases will be without mutation, making this genetic screening strategy a labor intensive and financially demanding procedure. PCC-PGL syndrome tumors differ from sporadic PCCs and PGLs by the presence of SDHB, -C, or -D mutations, which are, except for a few incidental cases,[17, 18] not found in truly sporadic PGL and PCC. Despite this genotypic difference, no reliable phenotypic discrimination between sporadic PCC and PGL, and PCC-PGL syndrome related tumors, is possible to date. In the present study we determined the value of SDHB immunohistochemistry (IHC) for the discrimination between SDH- and non-SDH-related PCCs and PGLs on large retrospective and prospective series in 2 different centers.

Methods

Patients and tumor samples

Two retrospective series of PCCs and PGLs were investigated by SDHB IHC (Erasmus MC: 110 cases, INSERM U970: 65 cases). These series consisted of PCCs diagnosed at Erasmus MC between 1982 and 2007 and diagnosed at INSERM U970 between 1995 and 2007, and of PGLs diagnosed in Erasmus MC between 1993-1998 and in INSERM U970 between 1993 and 2008. The series were enlarged with additional germline mutated *SDHB*, *-C* and *-D* cases from other centres, with as many different mutations as possible. In total, the series consisted of 175 formalin-fixed and paraffin embedded (FFPE) tumors (101 PCCs, 58 PGLs, 3 metastases, and 13 paraganglionic tumors of unknown location) including 24 *RET*, 29 *VHL*, 12 *NF1*, 34 *SDHB*, 38 *SDHD*, 4 *SDHC* germline mutant cases and 34 sporadic cases.
Furthermore, SDHB IHC was performed on a prospective series of 45 tumors (6 PCCs and 39 PGLs), from which the SDH-gene status was not known beforehand. This prospective series consisted of all PGL diagnosed in Erasmus MC between 2002 and 2008 and all PCC diagnosed in 2008. After the SDHB immunohistochemical results were obtained from this series, *SDH*-gene mutation analysis was performed. Detailed information on all investigated cases is presented in the Web Appendix Table1. The retrospective series (175 cases) consisted of 110 cases investigated in Rotterdam (70 tumors from Erasmus MC and 40 tumors obtained from national and international collaborators) and 65 cases investigated in Paris collected by the COMETE network. Mutation status in these patients/families was performed on-site previously with informed consent of the patients. The prospective series consisted of 45 cases from The Netherlands investigated in Rotterdam and were anonymously used according to the code for adequate secondary use of tissue, code of conduct: "Proper Secondary Use of Human Tissue" established by

the Dutch Federation of Medical Scientific Societies (http://www.federa.org). Ethical approval for the study was obtained from the institutional review board (CPP Paris-Cochin, January 2007).

Immunohistochemistry (IHC)

Two different primary antibodies against SDHB were used: mouse monoclonal clone 21A11 (NB600- 1366, Novus Biologicals, Littleton, CO; 1:50) and rabbit polyclonal HPA002868 (Sigma-Aldrich Corp, St. Louis, MO; 1:500). The antibodies were applied on routine FFPE archival tissues. Four to 6µm sections were cut and mounted on Superfrost Plus glass slides. The sections were deparaffinized, rehydrated, exposed to microwave heating in Tris/EDTA buffer, pH 9.0 or citrate buffer, pH 6.0 at 100°C for 15 min, rinsed in tap water followed by incubation in 3% H_2O_2 in PBS for 20 minutes. The SDHB antibodies were diluted in normal antibody diluent (Klinipath, Duiven, The Netherlands) and slides were incubated with 100µl per slide overnight at 4°C, followed by rinsing in Tris/Tween 0·5%, pH 8·0. Dako ChemMate envision HRP was applied for 30 minutes (100µl/slide, Dako envision kit, Glostrup, Denmark), followed by rinsing with PBS. Diaminobenzidine tetrahydrochloride (100µl/slide, Dako envision kit, Glostrup, Denmark) was applied for 5 minutes twice, after which the slides were rinsed with distilled water. Slides were counterstained with Harris Haematoxylin for 1 minute, rinsed with tap water, dehydrated, and covered with cover slips. In the negative control reactions, the primary antibodies were omitted from the dilution buffer, which in all instances resulted in complete absence of staining. Human heart muscle, adrenal gland, liver, and colon tissues were used as positive controls. These tissues showed strong granular staining in the cytoplasm with both antibodies. In PCC and PGL the normal stromal cells of the fibro-vascular network surrounding the Zellballen of tumor cells served as an internal positive control for each sample, also showing strong granular cytoplasmatic staining as in the positive controle samples.

Pathologists who had no knowledge of the mutation status of the specimens scored the IHC results from the retrospective series from Rotterdam and Paris independently. The IHC results of the prospective series were scored by researchers or by pathologists, before mutation analysis was performed.

Western blotting

Fifty sections of 5µm were cut (approximately 10mg) from 5 frozen PCC tissues of patients with germline mutations in *SDHB* (EX3del), *SDHD* (p.Asp92Tyr), *RET* (p.Cys634Arg), *VHL* (p.Arg64Pro), and *NF1* (clinically determined). In addition, the same amount of frozen tissue was taken from a lymph node of the SDHB patient, and from a normal adrenal gland. These tissues were transferred into 100µl 1X Laemmli sample buffer, followed by incubation for 15 minutes at room temperature. Next, the samples were stirred for 15 seconds, followed by incubation for 5 minutes at 100°C. Equal amounts of the samples were then run on a 10% SDS-PAGE gel. After electrophoresis the proteins were transferred to an Immobilon-P Membrane (Millipore, Temecula, CA) and immunoblotted. Both 21A11 and HPA002868 antibodies were used for Western blotting and an antibody against β-actin (Sigma- Aldrich, Corp, St. Louis, MO; 1:10,000) was used as a control for the amount of protein present on the blot.

Enzyme histochemistry

To test whether absence of immunohistochemical staining for SDHB in the tumors correlated with decreased SDH-enzyme activity, SDH-enzyme histochemistry was performed according to Pearse with minor modifications.19 From the *RET*-, *SDHB*-, and *SDHD*-mutated frozen tumor samples also used for the Western blotting experiments cryostat sections were incubated at 37°C for 1 hour with an SDHenzyme substrate solution (containing 8·3mM NaH2PO4.H2O, 33·3mM Na2HPO4.2H2O, 41·7mM Na2C4H4O4, 2·5M Nitroblue terazolium (N-6876, Sigma-Aldrich Corp, St. Louis, MO), 0·22mM AlCl2.6H2O, 0·13mM CaCl2, 25mM Na2HCO3, and 0·17mM Phenazine methosulfate (P 9625, Sigma-Aldrich Corp, St. Louis, MO). After rinsing in water twice, the slides were incubated at 4°C for 15 minutes in formaline-macrodex solution (containing 10ml 37% formaldehyde, 10ml 1% CaCl2, 80ml Macrodex). After rinsing the slides in water again for three times, the slides were mounted with imsolmount and covered with cover slips. Snap frozen healthy triceps muscle tissue was used as a positive control. As negative controls sections from the same tumor tissues were incubated in buffer from which Nitroblue terazolium was omitted.

Mutation analysis

Mutation analyses for *RET*, *VHL*, *SDHB*, *-C*, and *-D* genes of the series of 175 retrospective tumors were performed previously.[4, 20] For these analyses DNA was retrieved from FFPE tumor- and normal tissues or from peripheral blood, in the period from 1993 till 2008. DNA was isolated using described and standard procedures and mutation analyses were performed with or without pre-screening by single strand conformation polymorphism analysis (SSCP) followed by direct, in house or commercial (Baseclear, Leiden, The Netherlands), sequencing of PCR products.[13, 20, 21] Mutation analyses of the additional samples from other centers were performed by sequencing on site and verified at

Table 1. Clinical data and SDHB immunohistochemistry related to the various syndromes

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

Nf1: neurofibromatosis type 1, MEN2: multiple neoplasia type 2, VHL: von Hippel-Lindau, PCC-PGL: pheochromocytoma –paraganglioma, U: unknown

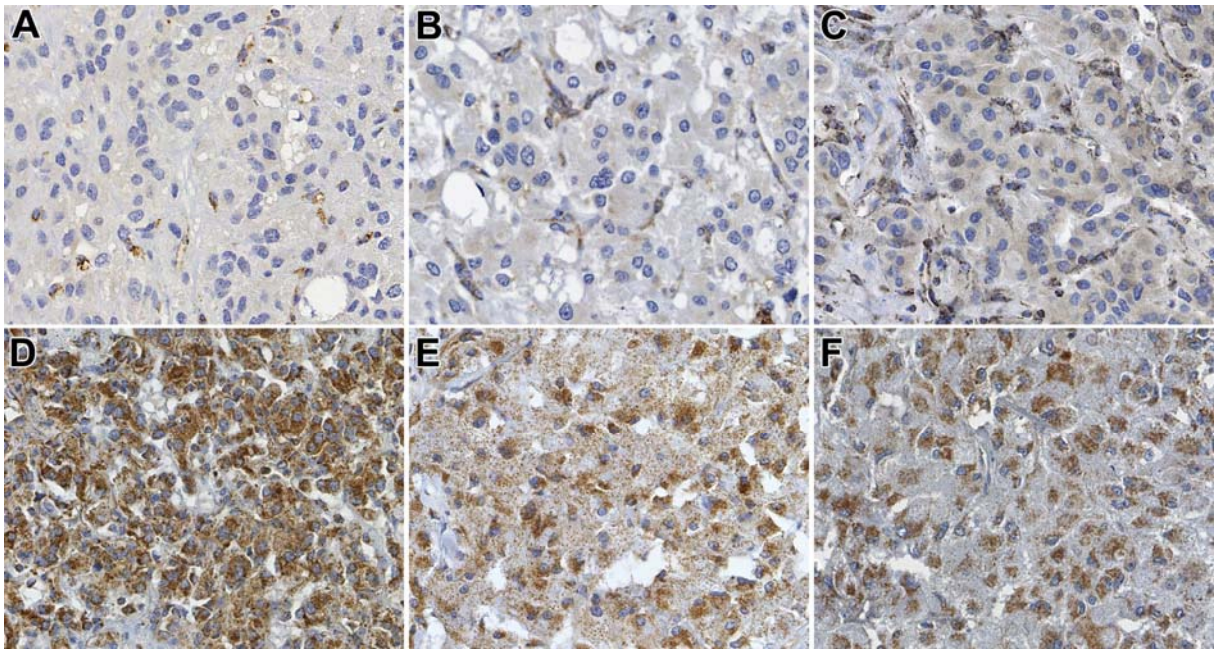

Figure 1. Pheochromocytoma and paraganglioma SDHB immunohistochemistry. (A) Pheochromocytoma with *SDHB* mutation. (B) Paraganglioma with *SDHC* mutation. (C) Paraganglioma with *SDHD* mutation. (D) Pheochromocytoma with *VHL* mutation. (E) Pheochromocytoma with *RET* mutation,. (F) Pheochromocytoma from a patient with NF1 (clinical diagnosis). Note: Strong speckled SDHB immunostaining in non-*SDH* mutated tumors (D, E, and F). Absence of SDHB immunostaining in the tumour cells of *SDHB*, *SDHC*, and *SDHD*-mutated tumors, with positive staining in the normal cells of the intra-tumoral fibrovascular network.

Erasmus MC and INSERM U970. On all 34 sporadic cases mutation analysis was performed by direct sequencing of the open reading frames, including the exon-intron boundaries, of the *SDHB*, *SDHC*, and *SDHD* genes.[4]

The prospective series of 45 tumors was also investigated for *SDHB*, *-C*, and *-D* mutations by direct sequencing of the open reading frames including all exon – intron boundaries as described previously.20 In addition, this series was investigated for the presence of large genomic deletions in the SDH genes by multiplex ligation-dependent probe amplification (MLPA) assay with a commercially available kit, SALSA MLPA P226 (MRC Holland, Amsterdam, The Netherlands).

Statistical analysis

Patients were grouped on the basis of the presence and absence of an SDH mutation and sensitivity and specificity of the SDHB IHC to detect an *SDH* mutation were determined. Within the prospective series we tested for association between SDHB IHC test result and *SDH* mutation status using Fishers exact test. Ninety-five percent confidence intervals (95% CI) were calculated using the exact binominal method.

Results

Immunohistochemical staining was performed on all 220 tumor samples (two retrospective series from Rotterdam and Paris of in total 175 cases and a series of 45 prospective cases from Rotterdam). Of these tumors 102 had a germline *SDH*-mutation

(36 *SDHB*, 5 *SDHC* and 61 *SDHD*) and all were negative for SDHB IHC (Fig. 1A-C). In 4 *SDH*-mutated tumors (*SDHB* p.Cys98Arg and p.Pro197Arg, and *SDHD* p.Asp92Tyr and c.169_169+9delTGTATGTTCT) a weak and diffuse cytoplasmic SDHB immunoreactivity was observed in the tumor cells, clearly distinct from the strong speckled pattern present in normal cells of the intratumoral fibro-vascular network (Fig. 1C). However, independent tumor samples with the same mutation (*SDHB* p.Pro197Arg and *SDHD* p.Asp92Tyr) were clearly negative for SDHB immunostaining. Therefore this weak diffuse cytoplasmic staining in the tumor cells was considered as a non-specific background artifact and scored as negative. Sixty-five tumors had a germline mutation in *RET* (24 cases), *VHL* (29 cases) or *NF1* (12 cases, diagnosed phenotypically) and all demonstrated expression of SDHB by IHC (Fig. 1D-F). In the remaining 53 tumors no germline mutation in the *RET*, *VHL*, *SDHB*, *-C*, or *-D* gene nor *NF1* gene involvement was detected of which 6 tumors were SDHB-negative. A summary of the results is listed in Table 1 and comprehensive information on tumor characteristics and results is presented in the Web Appendix Table 1.

In the prospective series, sensitivity and specificity are 100% (95% CI: 87-100%) and 84% (95% CI: 60-97%), respectively. Table 2 shows that there is a highly significant association between the SDHB IHC test result and the absence/presence of an *SDH* mutation (P<0·0001; Fisher exact test).

SDHB IHC performed on cryostat sections from 3 PCCs, two with a *SDHD* and one with a *RET* mutation, yielded results comparable to FFPE tissue sections: speckled staining patterns in the normal cells and absence of staining in *SDHD*-mutated tumor cells. This comparable SDHB immunoreactivity pattern on FFPE and frozen tissues is an additional indication for the specificity of the IHC results. The decreased expression of SDHB protein in both *SDHB*- and *SDHD*-mutated tumors was confirmed by Western blotting (Fig. 2A). In addition, the absence of SDH-enzyme activity was determined by enzyme histochemistry. The *SDHB*- and *SDHD*-related tumors showed no succinate dehydrogenase activity, except for the normal cells of the intratumoral fibro-vascular network, which showed strong staining (Fig. 2B). In contrast, strong SDH-enzyme activity was present in the triceps muscle tissue and the *RET*-related tumor tissue (Fig. 2C).

Table 2. SDHB immunohistochemistry results according to subgroups within *SDH*-related and non-*SDH*-related tumors.

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

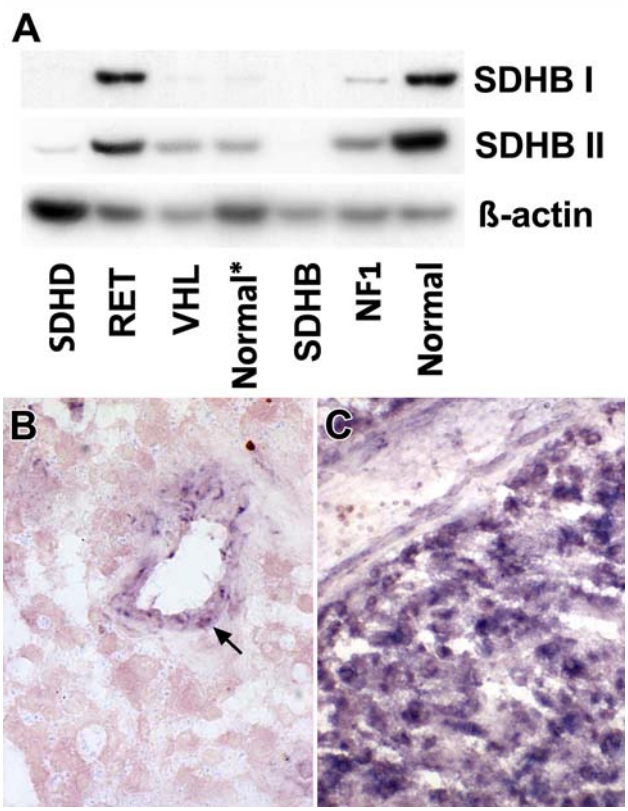

Figure 2. Western blot result with SDHB antibodies from Novus Biologicals NB600-1366 (SDHB I) and Sigma HPA002868 (SDHB II) and β-actin of pheochromocytoma with different mutations. SDHB caseL *SDHB* exon 3 deletion; SDHD case: *SDHD* p.Asp92Tyr missense mutation; RET case: *RET* p.Cys634Arg missense mutation; VHL case: *VHL* p.Arg64Pro missense mutation; NF1 case: clinically determined NF1. *Normal is a lysate from a lymph node from the patient with the *SDHB* mutation. Other normal is a lysate from a healthy adrenal gland. SDH-enzyme histochemistry result: (B) loss of SDH activity in tumor cells of a pheochromocytoma with an *SDHD* p.Asp92Tyr mutation, but retained activity in the normal cells of the intra-tumoral fibrovascular network (arrow); (C) strong SDH activity in tumor and normal cells of a pheochromocytoma with a *RET* p.Cys634Arg mutation.

Discussion

The results of this study demonstrate that an inexpensive and straightforward investigation, SDHB IHC on routine FFPE PGLs and PCCs, can reveal the presence of *SDHB*, *-C*, and *-D* germline mutations with great reliability. Absence of SDHB staining in the tumor cells was found irrespective whether *SDHB*, *-C*, or *-D* is mutated and regardless of the type of mutation, whether missense, nonsense, splice site, or frameshift. The SDHB protein expression results obtained by IHC using both SDHB antibodies (Sigma mouse monoclonal 21A11 and Novus rabbit polyclonal HPA002868) were comparable. Either antibody may be used for immunohistochemical detection of SDHB.

Of the 220 independent tumors 102 had a germline *SDH*-mutation (36 *SDHB*, 5 *SDHC* and 61 *SDHD*) and all were negative for SDHB immunostaining. Sixty-five tumors had a germline mutation in *RET* (24 cases), *VHL* (29 cases) or *NF1* (12 cases, diagnosed phenotypically) and all demonstrated expression of *SDHB* by IHC. In the remaining 53 tumors no germline mutation in the *RET*, *VHL*, *SDHB*, *-C*, or *-D* gene nor *NF1* gene involvement was detected but 6 tumors were negative for SDHB immunostaining. The absence of SDHB protein in these 6 tumors might be caused by *SDH* mutations escaping detection by the DNA sequencing and MLPA methods used (e.g deleterious mutations in not investigated untranslated, intronic, or promoter regions of the genes) or by epigenetic silencing of *SDH* genes. In 2 of these 6 patients without *SDH* mutations but with SDHB IHC-negative tumors the clinical information was indicative of PCC-PGL syndrome: one patient had a family history of PGL and one patient suffered from multiple PGLs (Web Appendix Table 1 cases 219C and 181B, respectively). Furthermore, 3 of the 4

other SDHB-negative tumors without *SDH*-gene mutation were diagnosed at young age (Web Appendix Table 1 cases 179A, 180B and 220C) indicating possible germline involvement. A negative *SDH* genetic testing in association with a negative SDHB IHC should indicate the possibility of a PCC/PGL hereditary syndrome and we recommend to follow up the patient the same as for proven PCC/PGL hereditary syndrome. There is a highly significant association between the SDHB IHC test result and the absence/presence of an SDH mutation. The SDHB immunohistochemical test has a high sensitivity and specificity for the presence of an *SDH* mutation, which is in the prospective series 100% (95% CI: 87-100%) and 84% (95% CI: 60-97%), respectively. The possibility that in the 6 SDHB negative tumors without identified *SDH* gene mutations the mutations escaped detection would mean that the sensitivity and specificity of SDHB IHC for detection of PCC-PGL syndrome is even higher.

The reliability of the immunohistochemical results on FFPE tumor specimens is indicated furthermore by the similar results obtained with 2 different antibodies, applied on 3 different tumor series in 2 different laboratories (retrospective series Rotterdam and Paris and prospective series Rotterdam), and the concordant results obtained on cryostat sections, in Western blotting and by SDH-enzyme histochemistry. Our results demonstrate that in tumor cells with various *SDHB* (15 different missense, 2 different nonsense, 6 different frameshift, 3 different exon deletions, 3 mutations probably affecting splicing), *SDHC* (2 different missense, 1 nonsense, and 2 exon deletions) and *SDHD* (5 different missense, 2 different nonsense, 3 different frameshift and 3 mutations

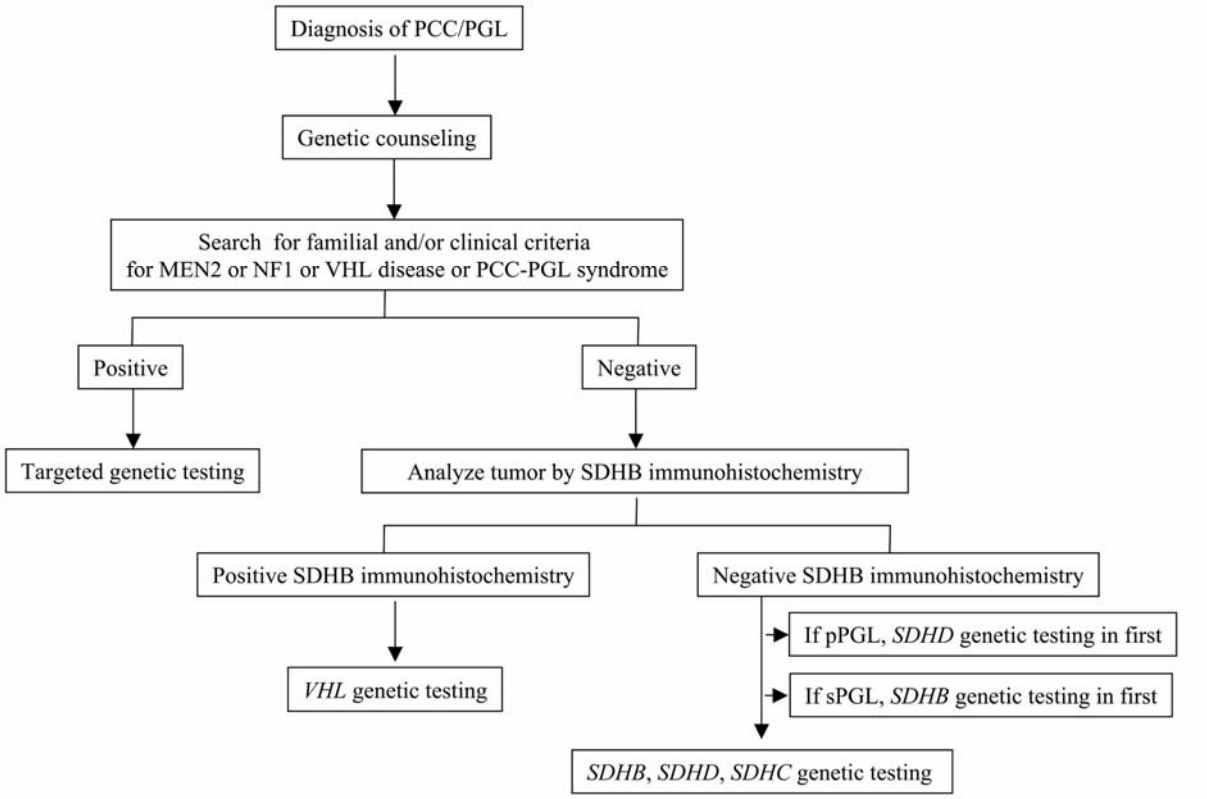

Figure 3. The presence of familial or clinical criteria for a pheochromocytoma- or paraganglioma-associated inherited disease should lead to targeted genetic testing. In the absence of criteria, SDHB immunohistochemistry is indicated. A positive SDHB immunohistochemistry result should lead to *VHL* and *RET* genetic testing, a negative SDHB immunohistochemistry result should lead to *SDH* (*SDHD*, *SDHB*, *SDHC*) genetic testing, starting with *SDHD* in cases of head and neck paraganglioma starting with *SDHB* cases of thoracic-abdominal or pelvic paraganglioma.

probably affecting splicing) mutations, no immunoreactive SDHB protein could be detected. These results are in accordance with preliminary findings by Douwes-Dekker et al. who reported generally decreased, diffuse cytoplasmic, SDHB expression in 11 SDHD-related (2 different *SDHD* mutations) PGL and strong granular expression in sporadic tumors and normal cells.[22] In addition, Dahia et al. previously reported comparable decreased SDHB expression in five SDHB-, one SDHD- and six *VHL*-related PCC.[22] However, in the present study we were able to discriminate *VHL*-related tumors from SDH-related PCC and PGL on the basis of SDHB IHC, which can be the result of differences in the applied IHC procedure and/or tissue processing.

The differences in SDHB protein levels are probably not resulting from differences in transcriptional efficiency since there are indications that SDHB mRNA levels do not parallel SDHB protein abundance.[23] In addition, it has been previously shown that, whatever SDH subunit is mutated, anchorage (SDHC and SDHD) or catalytic (SDHB), inactivation of an *SDH* gene induces a complete abolition of succinate dehydrogenase enzyme activity in the tumor suggesting a conformational change and/or a destabilization and a subsequent proteolysis of the complex II.[7, 22, 24] Moreover, Lima et al demonstrated by crystallography the severe structural consequences on the SDHB protein of 5 clinically validated *SDHB* missense mutations.[25] Furthermore, Cervera et al recently obtained evidence that 3 missense-mutated SDHB proteins can reach the mitochondrion and localize normally, although 2 of 3 missense-mutated SDHB proteins showed decreased expression by Western blotting compared to the wild-type protein.[26] These results match with the recent evidence that most rare missense variants in genes are deleterious.[27]

In the present study 4 tumors, positive for SDHB immunostaining, harbored nonsynonymous polymorphisms (*SDHB* p.Ala3Gly, p.Arg11His, p.Ser163Pro, and *SDHD* p.His50Arg) without concomitant pathogenic SDH-gene mutation, indicating that these variants are indeed neutral polymorphisms.[15, 28] Biallelic inactivation of the SDHB, -C or -D gene has been reported in SDH-related tumors.[17, 24, 29] Our results indicate that biallelic inactivation of *SDHB*, *-C*, or *-D* all lead to the same phenotypic consequence, i.e. the absence of immunoreactive SDHB protein. Such observations have already been described for mutations in complex I genes, which were demonstrated to affect the assembly and/or stability of both the whole complex I and other mitochondrial complexes, such as complex III.[30] The observed absence of SDHB immunoreactivity in all SDH-mutated tumors, demonstrated by IHC in both FFPE- and frozen tumor tissues and by Western blotting after denaturing gel electrophoresis, with both a monoclonal antibody generated against cow SDHB and an affinity isolated polyclonal antiserum against a recombinant carboxyterminal part of human SDHB, provides strong evidence that no functional SDHB protein is present in SDH-mutated tumors. As previously reported in other mitochondrial disorders, it is thus likely that altered assembly or complex stability is the first consequence of SDH gene mutations, as opposed to catalytic site dysfunction. It confirms the accuracy of immunological approaches for the diagnosis of mitochondrial diseases.[31]

By our applied procedure, PCC-PGL syndrome patients with an apparently sporadic presentation can be detected by SDHB IHC on PGLs and PCCs. In addition, it can be speculated that syndromic involvement of tumors that have recently been described in relation with PGLs, such as gastrointestinal stromal tumors (GISTs) in the Carney-Stratakis dyad and familial renal cell carcinomas (RCCs), could also be detected by SDHB IHC.[29, 32]

Actually, tissue of one of these germline *SDHB* mutated RCCs was available for study and this tumor appeared to be negative for SDHB expression (result not shown).
As for Lynch syndrome diagnostics where testing of tumors usually starts with IHC for mismatch repair gene products, SDHB IHC can play a major role in future genetic testing of PCCs and PGLs (for testing algorithm see Figure 3).[33] Because of the simplicity of the standard immunohistochemical procedure as well as of the data interpretation, the IHC test can easily be applied in diagnostic pathology services worldwide. So it will be technically and financially feasible to routinely test all PCC and PGL for SDHB expression, in particular in absence of familial and/or clinical indications for a specific form of inherited PCC or PGL. Our results demonstrate that it is indicated to perform *SDHB*, *-C*, and *-D* germline mutation testing only when tumors are immunohistochemically negative for SDHB expression. Obviously our proposed diagnostic test can only be performed after patients have been operated and tumor tissue is available for study. The influence our test will have on patients management is yet unclear since international controversy exists regarding pre- and postoperative genetic testing and the effect on patient management. In conclusion, by routinely performed SDHB IHC on PCC and PGL hereditary syndromes caused by germline mutations in *SDHB*, *SDHC*, or *SDHD* can be identified with great reliability.

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

Patient[1]	Sex M/F	Age at diagnosis (years)	Tumor site[2]	Benign/ malignant	Gene mutated	Mutation type[3]	Mutation nucleotide	Mutation protein	SDHB IHC[4]
1A	M	32	APGL	U	SDHB	FS	c.481delG	p.Asp161MetfsX14	0
2A	F	25	APGL	U	SDHB	MS	c.299C>T	p.Ser100Phe	0
3A	F	63	APGL	U	SDHB	MS	c.727C>A	p.Cys243Ser	0
4A	F	12	APGL	U	SDHB	LD	Ex3del	U	0
5A	U	U	U	U	SDHB	Splice	c.72+1G>T	IVS1+1G>T	0
6A	U	U	U	U	SDHB	MS	c.590C>G	p.Pro197Arg	0
7A	U	U	U	U	SDHB	MS	c.292C>T	p.Cys98Arg	0
8A	U	U	U	U	SDHB	MS	c.590C>G	p.Pro197Arg	0
9A	U	U	U	U	SDHB	MS	c.380C>A	p.Ile127Asn	0
10A	M	14	U	U	SDHB	MS	c.137G>A	p.Arg46Gln	0
11A	U	U	U	U	SDHB	FS	c.502insC	p.Gln168ProfsX11	0
12A	U	U	PCC	U	SDHB	NS	c.268C>T	p.Arg90X	0
13A	U	U	PCC	U	SDHB	MS	c.418G>T	p.Val140Phe	0
14A	U	U	PCC	U	SDHB	NS	c.343C>T	p.Arg115X	0
15A	U	U	PCC	U	SDHB	MS	c.689G>A	p.Arg230His	0
16A	U	U	PCC	U	SDHB	MS	c.587G>A	p.Cys196Tyr	0
17A	M	25	APGL	U	SDHB	MS	c.395A>C	p.His132Pro	0
18A	M	60	APGL	U	SDHB	MS	c.395A>C	p.His132Pro	0
19B	F	33	APGL	B	SDHB	Splice	c.200+1G>A	IVS2+1G>A	0
20B	M	59	PCC	M	SDHB	MS	c.203G>A	p.Cys68Tyr	0
21B	M	36	APGL	B	SDHB	FS	c.591del	p.Ser198AlafsX22	0
22B	F	20	PCC	B	SDHB	FS	c.166_170del	p.Pro56TyrfsX5	0
23B	M	29	APGL	M	SDHB	MS	c.127G>C	p.Ala43Pro	0
24B	F	21	PCC	B	SDHB	MS	c.758G>A	p.Cys253Tyr	0
25B	M	43	APGL	M	SDHB	LD	Ex3_8del	U	0
26B	F	54	PCC	M	SDHB	MS	c.137G>A	p.Arg46Gln	0
27B	F	34	HHPGL	B	SDHB	MS	c.763A>G	p.Lys255Glu	0
28B	M	39	APGL	M	SDHB	FS	c.620-621delTG	p.Leu207ArgfsX14	0
29B	M	28	APGL	M	SDHB	LD	Ex1del	U	0
30B	F	10	APGL	B	SDHB	FS	c.713del	p.Phe238SerfsX10	0
31B	F	47	APGL	B	SDHB	Splice	c.540+2T>C	IVS5+2T>C	0
32B	M	28	PCC	M	SDHB	MS	c.137G>A	p.Arg46Gln	0
33B	M	28	Metastasis (abdominal ganglia)	M	SDHB	MS	c.137G>A	p.Arg46Gln	0
34B	F	56	PCC	M	SDHB	MS	c.689G>A	p.Arg230His	0
35C	M	48	HHPGL	U	SDHB	MS	c.649C>G	p.Arg217Gly	0
36C	F	20	HHPGL	U	SDHB	Splice	c.200+1G>A	IVS2+1G>A	0
37A	F	15	APGL	U	SDHC	NS	c.126G>A	p.Trp42X	0
38A	M	36	HHPGL	U	SDHC	MS	c.214C>T	p.Arg72Cys	0
39B	F	16	TPGL	B	SDHC	LD	Ex2del	U	0
40B	M	47	APGL	M	SDHC	LD	Ex3del	U	0
41C	F	39	HHPGL	U	SDHC	MS	c.397C>T	p.His127Tyr	0
42A	F	25	PCC	B	SDHD	MS	c.274G>T	p.Asp92Tyr	0
43A	M	16	PCC	B	SDHD	MS	c.274G>T	p.Asp92Tyr	0
44A	F	31	PCC	B	SDHD	MS	c.274G>T	p.Asp92Tyr	0
45A	M	41	HHPGL	B	SDHD	FS	c.277delT	p.Ile93TyrfsX42	0
46A	F	33	HHPGL	B	SDHD	Splice	c.170-1G>T	IVS2-1G>T	
47A	F	18	U	U	SDHD	FS	c.94_95delTC	p.Ala32IlefsX35	0
48A	U	U	U	U	SDHD	NS	c.342T>A	p.Tyr114X	0

49A	F	72	PCC	U	SDHD	MS	c.274G>T	p.Asp92Tyr	0
50A	M	36	HHPGL	B	SDHD	MS	c.284T>C	p.Leu95Pro	0
51A	M	39	HHPGL	B	SDHD	MS	c.416T>C	p.Leu139Pro	0
52A	F	43	HHPGL	B	SDHD	MS	c.274G>T	p.Asp92Tyr	0
53A	F	20	HHPGL	B	SDHD	MS	c.274G>T	p.Asp92Tyr	0
54A	F	62	HHPGL	B	SDHD	MS	c.284T>C	p.Leu95Pro	0
55A	M	43	HHPGL	B	SDHD	MS	c.274G>T	p.Asp92Tyr	0
56A	F	44	HHPGL	B	SDHD	MS	c.274G>T	p.Asp92Tyr	0
57A	F	42	HHPGL	B	SDHD	MS	c.274G>T	p.Asp92Tyr	0
58A	M	48	HHPGL	B	SDHD	MS	c.274G>T	p.Asp92Tyr	0
59A	F	44	HHPGL	B	SDHD	MS	c.284T>C	p.Leu95Pro	0
60A	M	36	HHPGL	B	SDHD	MS	c.284T>C	p.Leu95Pro	0
61A	M	43	HHPGL	B	SDHD	MS	c.274G>T	p.Asp92Tyr	0
62A	M	43	HHPGL	B	SDHD	MS	c.274G>T	p.Asp92Tyr	0
63A	M	44	HHPGL	B	SDHD	MS	c.274G>T	p.Asp92Tyr	0
64A	M	70	APGL	B	SDHD	MS	c.274G>T	p.Asp92Tyr	0
65A	M	28	HHPGL	B	SDHD	MS	c.284T>C	p.Leu95Pro	0
66A	M	41	HHPGL	B	SDHD	MS	c.284T>C	p.Leu95Pro	0
67A	M	56	HHPGL	B	SDHD	MS	c.274G>T	p.Asp92Tyr	0
68A	M	34	HHPGL	B	SDHD	MS	c.274G>T	p.Asp92Tyr	0
69A	F	41	HHPGL	B	SDHD	MS	c.274G>T	p.Asp92Tyr	0
70A	F	29	HHPGL	B	SDHD	MS	c.274G>T	p.Asp92Tyr	0
71A	F	36	HHPGL	B	SDHD	MS	c.416T>C	p.Leu139Pro	0
72A	F	36	HHPGL	B	SDHD	MS	c.416T>C	p.Leu139Pro	0
73A	F	57	U	B	SDHD	MS	c.284T>C	p.Leu95Pro	0
74A	F	45	HHPGL	B	SDHD	MS	c.284T>C	p.Leu95Pro	0
75A	F	47	HHPGL	B	SDHD	MS	c.274G>T	p.Asp92Tyr	0
76A	F	40	HHPGL	B	SDHD	MS	c.284T>C	p.Leu95Pro	0
77A	F	40	HHPGL	B	SDHD	MS	c.274G>T	p.Asp92Tyr	0
78A	M	62	PCC	B	SDHD	MS	c.274G>T	p.Asp92Tyr	0
79B	M	45	APGL	B	SDHD	NS	c.64C>T	p.Arg22X	0
80C	M	20	HHPGL	B	SDHD	FS	c.116delC	p.Pro39LeufsX37	0
81C	F	50	HHPGL	U	SDHD	MS	c.209G>T	p.Arg70Met	0
82C	F	50	HHPGL	U	SDHD	MS	c.209G>T	p.Arg70Met	0
83C	M	26	HHPGL	B	SDHD	MS	c.274G>T	p.Asp92Tyr	0
84C	F	34	HHPGL	B	SDHD	MS	c.274G>T	p.Asp92Tyr	0
85C	M	26	HHPGL	B	SDHD	MS	c.274G>T	p.Asp92Tyr	0
86C	F	58	HHPGL	B	SDHD	MS	c.274G>T	p.Asp92Tyr	0
87C	F	22	HHPGL	B	SDHD	MS	c.274G>T	p.Asp92Tyr	0
88C	F	35	HHPGL	B	SDHD	MS	c.274G>T	p.Asp92Tyr	0
89C	F	35	HHPGL	B	SDHD	MS	c.274G>T	p.Asp92Tyr	0
90C	F	54	HHPGL	U	SDHD	MS	c.274G>T	p.Asp92Tyr	0
91C	F	25	HHPGL	B	SDHD	MS	c.274G>T	p.Asp92Tyr	0
92C	F	51	HHPGL	B	SDHD	MS	c.274G>T	p.Asp92Tyr	0
93C	F	40	HHPGL	B	SDHD	MS	c.274G>T	p.Asp92Tyr	0
94C	F	40	HHPGL	B	SDHD	MS	c.274G>T	p.Asp92Tyr	0
95C	F	37	HHPGL	U	SDHD	MS	c.274G>T	p.Asp92Tyr	0
96C	F	47	HHPGL	U	SDHD	MS	c.284T>C	p.Leu95Pro	0
97C	M	51	HHPGL	U	SDHD	MS	c.284T>C	p.Leu95Pro	0
98C	M	53	HHPGL	B	SDHD	MS	c.416T>C	p.Leu139Pro	0
99C	M	U	APGL	U	SDHD	MS	c.439G>A	p.Val147Met	0
100C	F	54	HHPGL	U	SDHD	Splice	c.52+3G>A	IVS1+3G>A	0
101C	M	38	HHPGL	U	SDHD	MS	c.284T>C	p.Leu95Pro	0
102C	M	35	HHPGL	U	SDHD	Splice	c.169_169+9delTGTATGTTCT	U	0

103A	F	39	PCC	B	NF1	ND*			1
104A	F	47	PCC	B	NF1	ND*			1
105A	F	61	PCC	B	NF1	ND*			1
106A	M	52	PCC	B	NF1	ND*			1
107A	M	29	PCC	B	NF1	ND*			1
108A	F	63	PCC	B	NF1	ND*			1
109A	M	33	PCC	B	NF1	ND*			1
110A	F	67	PCC	B	NF1	ND*			1
111B	F	37	PCC	B	NF1	ND*			1
112B	F	33	PCC	B	NF1	ND*			1
113B	F	38	PCC	B	NF1	ND*			1
114B	F	32	PCC	B	NF1	ND*			1
115A	F	38	PCC	B	RET	MS	c.1900T>C	p.Cys634Arg	1
116A	F	25	PCC	U	RET	MS	c.1900T>C	p.Cys634Arg	1
117A	F	51	PCC	M	RET	MS	c.1900T>C	p.Cys634Arg	1
118A	M	29	PCC	B	RET	MS	c.1900T>C	p.Cys634Arg	1
119A	M	41	PCC	U	RET	NS	c.1894_1899delgagctg	p.Glu632_Leu633del	1
120A	M	35	PCC	B	RET	MS	c.1900T>C	p.Cys634Arg	1
121A	M	31	PCC	B	RET	ND*			1
122A	F	26	PCC	B	RET	ND*			1
123A	F	65	PCC	B	RET	MS	c.1900T>C	p.Cys634Arg	1
124A	F	20	PCC	B	RET	MS	c.1900T>C	p.Cys634Arg	1
125A	M	21	PCC	B	RET	MS	c.1900T>C	p.Cys634Arg	1
126A	F	32	PCC	B	RET	MS	c.1900T>C	p.Cys634Arg	1
127A	F	35	PCC	U	RET	ND*			1
128A	M	70	PCC	B	RET	MS	c.1900T>C	p.Cys634Arg	1
129A	M	26	PCC	B	RET	MS	c.1900T>C	p.Cys634Arg	1
130A	F	38	PCC	B	RET	MS	c.1900T>C	p.Cys634Arg	1
131A	M	23	PCC	B	RET	MS	c.1900T>C	p.Cys634Arg	1
132B	F	18	PCC	B	RET	MS	c.1900T>C	p.Cys634Arg	1
133B	F	37	PCC	B	RET	MS	c.1901_1902GC>TG	p.Cys634Leu	1
134B	F	29	PCC	B	RET	MS	c.2753T>C	p.Met918Thr	1
135B	F	76	PCC	B	RET	MS	c.1597G>T	p.Gly533Cys	1
136B	F	27	PCC	B	RET	MS	c.1900T>C	p.Cys634Arg	1
137B	F	44	PCC	B	RET	MS	c.2647_2648GC>TT	p.Ala883Phe	1
138B	F	18	PCC	B	RET	MS	c.1900T>C	p.Cys634Arg	1
139A	M	55	PCC	B	VHL	MS	c.403G>C	p.Gly144Gln	1
140A	F	62	PCC	B	VHL	MS	c.705G>T	p.Gln164His	1
141A	F	18	PCC	B	VHL	MS	c.403G>C	p.Arg64Pro	1
142A	F	32	PCC	B	VHL	MS	c.364_365GC>AT	p.Ala122Ile	1
143A	M	7	PCC	U	VHL	MS	c.403G>C	p.Arg64Pro	1
144A	U	U	PCC	U	VHL	FS	c.343insAA	p.His115AsnfsX23	1
145A	U	U	U	U	VHL	MS	c.482G>A	p.Arg161Gln	1
146A	U	U	U	U	VHL	MS	c.357C>G	p.Phe119Leu	1
147A	U	U	PCC	U	VHL	ND*			1
148A	M	12	U	U	VHL	MS	c.250G>T	p.Val84Leu	1
149A	F	49	PCC	B	VHL	MS	c.403G>C	p.Arg64Pro	1
150A	F	39	PCC	B	VHL	MS	c.430G>C	p.Gly144Gln	1
151A	M	31	PCC	B	VHL	MS	c.403G>C	p.Arg64Pro	1
152A	M	26	PCC	B	VHL	MS	c.188T>C	p.Leu63Pro	1
153A	M	24	PCC	B	VHL	MS	c.403G>C	p.Arg64Pro	1
154A	F	23	HHPGL	U	VHL	MS	c.403G>C	p.Arg64Pro	1
155B	M	15	PCC	B	VHL	MS	c.533T>C	p.Leu178Pro	1
156B	F	15	PCC	B	VHL	MS	c.500G>A	p.Arg167Gln	1

157B	F	20	APGL	B	VHL	MS	c.467A>G	p.Tyr156Cys	1
158B	F	7	APGL	B	VHL	MS	c.482G>A	p.Arg161Gln	1
159B	M	26	PCC	B	VHL	MS	c.460C>T	p.Pro154Ser	1
160B	M	26	APGL	B	VHL	MS	c.460C>T	p.Pro154Ser	1
161B	M	31	PCC	B	VHL	LD	Ex1_3del	U	1
162B	F	36	APGL	M	VHL	LD	Ex3del	U	1
163B	F	19	PCC	B	VHL	MS	c.292T>C	p.Tyr98His	1
164B	F	16	PCC	B	VHL	MS	c.500G>A	p.Arg167Gln	1
165B	M	17	PCC	B	VHL	MS	c.467A>G	p.Tyr156Cys	1
166B	F	10	PCC	B	VHL	MS	c.290C>T	p.Pro97Leu	1
167B	M	25	PCC	B	VHL	MS	c.500G>A	p.Arg167Gln	1
168A	F	70	PCC	M	NONE				1
169A	M	48	PCC	B	NONE				1
170A	M	49	PCC	B	NONE				1
171A	M	63	PCC	M	NONE				1
172A	F	79	PCC	B	NONE				1
173A	F	38	PCC	B	NONE				1
174A	F	64	PCC	U	NONE				1
175A	F	62	PCC	U	NONE				1
176A	F	40	PCC	U	NONE				1
177A	F	62	PCC	U	NONE				1
178A	F	42	PCC	U	NONE				1
179A	M	12	PCC	B	NONE				0
180B	M	27	APGL	B	NONE				0
181B	F	27	PCC	M	NONE				0
182B	F	40	PCC	B	NONE				1
183B	M	17	PCC	B	NONE				1
184B	F	53	PCC	B	NONE				1
185B	M	47	PCC	B	NONE				1
186B	M	40	PCC	M	NONE				1
187B	F	46	Metastasis (abdominal ganglia)	M	NONE				1
188B	F	37	APGL	B	NONE				1
189B	F	39	PCC	M	NONE				1
190B	F	49	PCC	B	NONE				1
191B	F	26	APGL	M	NONE				1
192B	F	26	APGL	M	NONE				1
193B	M	62	PCC	B	NONE				1
194B	F	41	PCC	B	NONE				1
195B	M	57	PCC	B	NONE				1
196B	F	44	PCC	B	NONE				1
197B	F	47	PCC	B	NONE				1
198B	F	63	Metastasis (Right psoas)	M	NONE				1
199B	F	66	PCC	B	NONE				1
200B	M	59	PCC	B	NONE				1
201B	M	45	PCC	B	NONE				1
202C	M	67	HHPGL	B	NONE				1
203C	F	55	HHPGL	B	NONE				1
204C	F	45	HHPGL	B	NONE				1
205C	F	57	HHPGL	B	NONE				1
206C	F	47	HHPGL	B	NONE				1
207C	F	57	HHPGL	B	NONE				1

208C	F	51	U	B	NONE	1
209C	F	71	HHPGL	B	NONE	1
210C	M	56	HHPGL	U	NONE	1
211C	F	71	HHPGL	U	NONE	1
212C	F	57	PCC	U	NONE	1
213C	F	54	PCC	U	NONE	1
214C	M	43	PCC	U	NONE	1
215C	U	U	PCC	U	NONE	1
216C	U	U	PCC	U	NONE	1
217C	F	45	PCC	U	NONE	1
218C	F	74	HHPGL	B	NONE	0
219C	M	31	HHPGL	B	NONE	0
220C	M	45	HHPGL	B	NONE	0

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

Chapter 9

SDHA immunohistochemistry detects germline *SDHA* gene mutations in apparently sporadic paragangliomas and pheochromocytomas

*Esther Korpershoek, *Judith Favier, *José Gaal, Nelly Burnichon, Bram van Gessel, Lindsey Oudijk, Cécile Badoual, Noémie Gadessaud, Annabelle Venisse, Jean-Pierre Bayley, Marieke F. van Dooren, Wouter W. de Herder, Frédérique Tissier, Pierre-François Plouin, Francien H. van Nederveen, #Winand N.M. Dinjens, #Anne-Paule Gimenez-Roqueplo, and #Ronald R. de Krijger

* These authors should be considered as equal first authors
# These authors should be considered as equal last authors

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Abstract

Context: Pheochromocytoma-paraganglioma syndrome is caused by mutations in *SDHB*, *SDHC*, and *SDHD*, encoding subunits of succinate dehydrogenase, and in *SDHAF2*, required for flavination of SDHA. A recent report described a patient with an abdominal paraganglioma, immunohistochemically negative for SDHA, and identified a causal germline mutation in *SDHA*.

Objective: In this study we evaluated the significance of SDHA immunohistochemistry in the identification of new patients with *SDHA* mutations.

Setting: This study was performed in the Erasmus MC in Rotterdam (the Netherlands), and the Université Paris Descartes in Paris (France).

Methods: We investigated 316 pheochromocytomas and paragangliomas for SDHA expression. Sequence analysis of *SDHA* was performed on all tumors that were immunohistochemically negative for SDHA, and on a subset of tumors immunohistochemically positive for SDHA.

Results: Six tumors were immunohistochemically negative for SDHA. Four tumors from Dutch patients showed a germline c.91C>T *SDHA* gene mutation (p.Arg31X). Another tumor (from France) carried a germline *SDHA* missense mutation c.1753C>T (p.Arg585Trp). Loss of the wild-type *SDHA* allele was confirmed by LOH analysis. Sequence analysis of 35 SDHA immunohistochemically positive tumors did not reveal additional *SDHA* mutations.

Conclusions: Our results demonstrate that SDHA immunohistochemistry on paraffin-embedded tumors can reveal the presence of *SDHA* germline mutations and allowed the identification of *SDHA*-related tumors in at least 3% of patients affected by apparently sporadic (para)sympathetic paragangliomas and pheochromocytomas.

Introduction

Pheochromocytomas and paragangliomas are rare tumors that originate from neural crest-derived cells.[1] Intra-adrenal tumors are called pheochromocytomas whereas similar extra-adrenal tumors are called paragangliomas. Based on location, paragangliomas are subdivided into parasympathetic and sympathetic paragangliomas and are classified as functional or non-functional, depending on their catecholamine production.

Succinate dehydrogenase, also known as mitochondrial complex II, is involved in the citric acid cycle and electron transport chain and is composed of four subunits: SDHA, SDHB, SDHC and SDHD.[2] Previously, *SDHAF2*, *SDHB*, *SDHC* and *SDHD* mutations have been associated with paragangliomas and pheochromocytomas.[3-7] Initially no genetic link between SDHA and paragangliomas could be established and *SDHA* mutations were only known to be involved in Leigh syndrome.[8-11] However, we recently identified the first heterozygous germline *SDHA* mutation (p.Arg589Trp), associated with an abdominal paraganglioma.[12]

Patients with *SDHB*, *SDHC* and *SDHD* mutations can be identified using SDHB immunohistochemistry, as their tumors are immunohistochemically negative for SDHB.[13] The *SDHA*-related tumor we described was also immunohistochemically negative for SDHB and, in addition, lacked expression of SDHA. In contrast, *RET*, *NF1*, *SDHB* and *SDHD*-related tumors were uniformly immunohistochemically positive for SDHA.[12] These results suggested that SDHA immunohistochemistry might be an appropriate and efficient technique to diagnose new *SDHA*-mutated pheochromocytomas and paragangliomas. The aim of this study was to validate the usefulness of SDHA immunohistochemistry in the identification of patients with *SDHA* mutations.

Methods

Patients and tumor samples

This study included a series of 316 tumors (202 pheochromocytomas, 43 sympathetic paragangliomas, 65 parasympathetic paragangliomas, and six metastases) diagnosed between 1978 and 2009. Of these tumors, 167 came from the archives of the Erasmus MC (Rotterdam, The Netherlands), 92 were collected by the COMETE Network (Paris, France), and the remaining tumors came from various Dutch and foreign centers. Of the 202 pheochromocytomas, 129 were apparently sporadic and 73 were syndrome-related tumors. Of the 65 parasympathetic paragangliomas, 40 were apparently sporadic and 24 were syndrome-related tumors due to germline mutations in different susceptibility genes, and one tumor had a somatic *IDH1* mutation.[14] In addition, of the 43 sympathetic paragangliomas, 24 occurred sporadically and 19 were syndrome-related. Four metastases were seen in patients with a sporadic presentation and two in patients with an *SDHB* mutation. Clinical data of all patients is shown in Supplemental Table 1. The tumors were used anonymously, in accordance with the code of conduct: "Proper Secondary Use of Human Tissue" established by the Dutch Federation of Medical Scientific Societies (http://www.federa.org). The French study was formally approved by the institutional review board (CPP Paris-Cochin, January 2007). DNA was isolated from

anonymous healthy subjects, consisting of Dutch blood donors (227 for exon 2; 116 for exon 13) and normal French volunteers (119 for exon 2; 370 for exon 13).

Immunohistochemistry

Immunohistochemistry was performed for SDHA and SDHB, using a 1/1000 dilution of the SDHA monoclonal antibody ab14715 (Abcam, Cambridge, United Kingdom) and a 1/500 dilution of the SDHB polyclonal antibody HPA002868 (Sigma-Aldrich, St. Louis, MO). The antibodies were applied to routine formalin-fixed and paraffin-embedded archival tissues, processed as described previously.[13] Tumors received a negative score if the non-tumorous cells from the fibrovascular network surrounding the tumor cells stained positive (internal positive control) and the tumor cells were negative as previously described.[13] Tumors were scored as positive if the tumor cells had the same intensity as internal positive-control cells. Following these guidelines, no equivocal cases were seen and there were no discrepancies between observers. The immunohistochemistry results were evaluated by two independent observers: RdK and EK in Rotterdam or JF and NG in Paris.

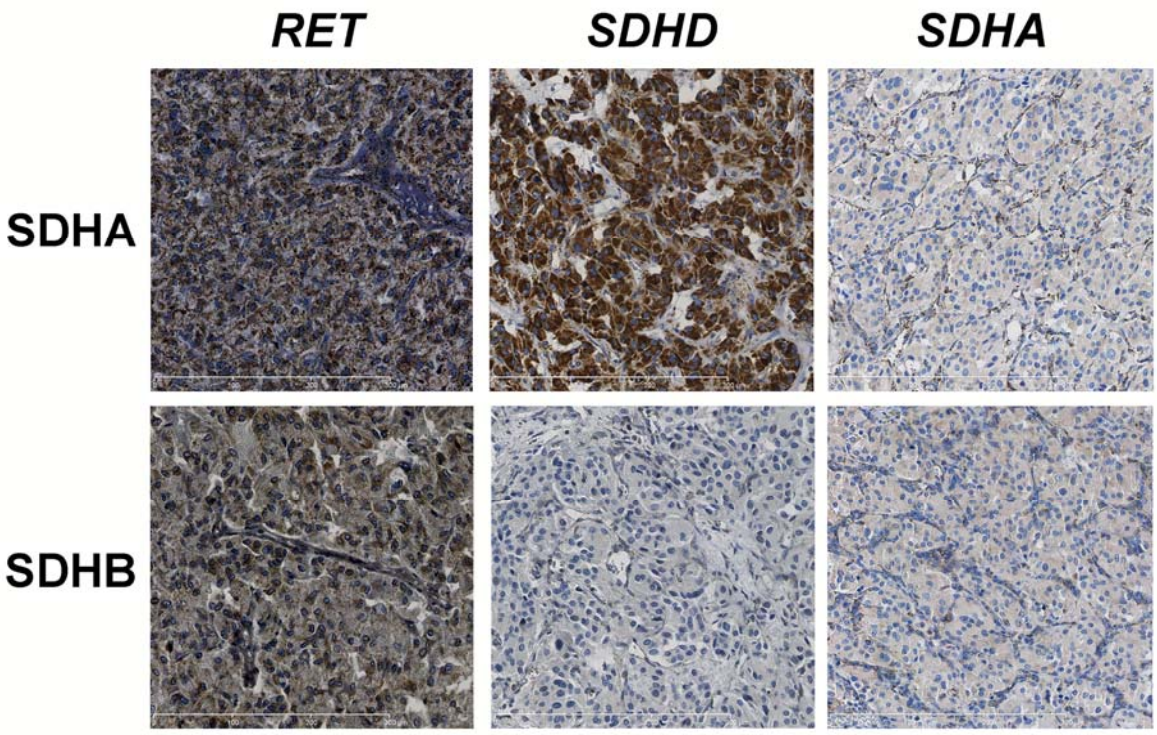

Figure 1. Upper panel: SDHA immunohistochemistry showing positive staining in a *RET*- and *SDHD*- mutated tumor. The *SDHA*-mutated tumor displays negative staining of the tumor cells while the fibrovascular stromal cell staining is positive. Lower panel: SDHB immunohistochemistry shows positive immunohistochemical staining in the *RET*-related tumor, whereas the *SDHD*- and *SDHA*-mutated tumors are negative.

Sequence analysis
Sequence analysis of *SDHA* (NM_004168) was performed on all tumors immunohistochemically negative for SDHA (primers available on request) and on 35 SDHA immunohistochemically positive tumors (21 Dutch, 14 French). DNA was isolated according to the manufacturer's instructions (Gentra Systems Minneapolis, MN, or AllPrep DNA/RNA Mini Kit, Qiagen). The entire *SDHA* coding sequence, including intron-exon boundaries, was analyzed for mutations, taking into account the *SDHA* pseudogenes (NCBI: NR_003263, NR_003264, NR_003265). When a mutation was demonstrated in the tumor DNA, germline DNA of the same patient was also tested, isolated from paraffin-embedded, histologically normal tissue surrounding the tumor or from leukocytes.

Loss of heterozygosity (LOH)
A microsatellite marker, located at position 1,004,307 – 1,004,351 bp on chromosome 5 (UCSC; February 2009 GRCh37/hg19 Assembly), was selected for LOH analysis of the *SDHA* gene (primers are available on request). LOH was performed on tumor and normal DNA from patients presenting with SDHA-negative tumors, as described previously, using fluorescence-labeled primers (Invitrogen, Paisley, UK) and ABI 3130-XL genetic analyzer (Applied Biosystems, Foster City, CA) for analysis. [15]

Results

Immunohistochemistry
SDHA immunohistochemistry of the 316 tumors revealed seven tumors with SDHA-negative tumor cells (Figure 1), including the previously described *SDHA*-mutated paraganglioma (patient 297).[12] The other six SDHA immunohistochemically negative tumors included one pheochromocytoma (patient 132), one abdominal paraganglioma (patient 291), one malignant bladder sympathetic paraganglioma (patient 146), one thoracic sympathetic paraganglioma (patient 161), one vagal parasympathetic paraganglioma (patient 162), and one carotid body parasympathetic paraganglioma (patient 187). As expected, SDHB immunohistochemistry was negative in all seven tumors. None of these tumors harbored a mutation in the *SDHB*, *SDHC*, or *SDHD* genes.

Mutation analysis
Mutation analysis of *SDHA* was performed on five of the six new SDHA immunohistochemically-negative tumors. In one case, mutation analysis could not be performed due to an insufficient DNA quality. Four tumors from Dutch patients showed a novel c.91C>T *SDHA* gene mutation (NCBI: NM_004168), leading to a truncated protein (p.Arg31X). One French patient harbored a novel c.1753C>T mutation leading to a missense change (p.Arg585Trp). Pseudogenes differ by at least two nucleotides from the *SDHA* gene within each of the amplicons, confirming that these two new mutations did occur in the *SDHA* gene. Corresponding germline DNA, isolated from FFPE normal tissue (four Dutch patients) or from leukocytes (one French patient) confirmed the presence of the germline mutation in all five patients (Figure 2). The sequence chromatogram of the tumor DNAs displayed the mutation almost exclusively, indicating loss of the wild-type allele (Figure 2c). Among the healthy control population, both novel mutations were identified in respectively 2/692 (c.91C>T; 0.3%, only present in Dutch controls) and 1/972 (c.1753C>T; 0.1%) alleles of healthy subjects.

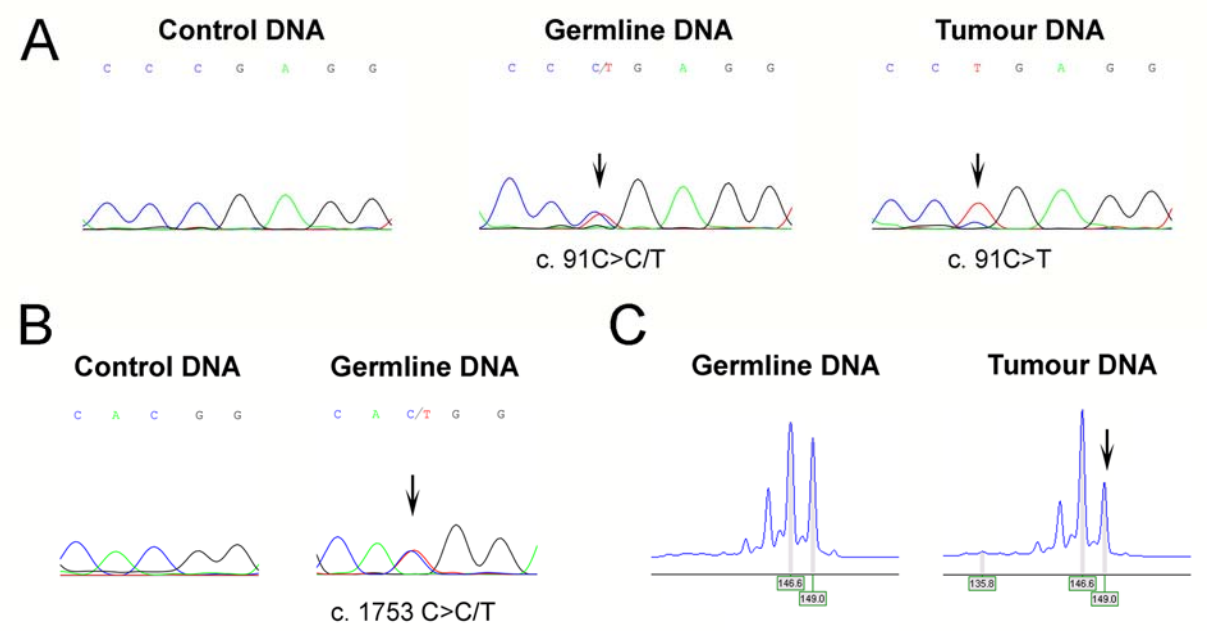

Figure 2. A) *SDHA* sequence electropherogram showing a c.91C>T (p.Arg31X) mutation present in germline and tumor DNA of patient 161, but not in control DNA. The *SDHA* sequence electropherogram of the tumor DNA revealed predominantly the mutated allele (T), indicating relative loss of the wild type SDHA allele. B) Sequence electropherogram displaying the c.1753C>T (p.Arg585Trp) mutation in the germline DNA of patient 291 compared to a control DNA. C) Microsatellite electropherogram demonstrating loss of heterozygosity, with loss of the larger allele (149, indicated by the arrow) in tumor DNA compared to germline DNA.

Loss of heterozygosity analysis

Four of the 6 patients were heterozygous for the marker alleles and all corresponding tumor DNAs showed loss of heterozygosity (Figure 2). The Dutch p.Arg31X patients did not share the same alleles, providing no evidence of relatedness. In addition, expression of the mutant allele was confirmed by RT-PCR (supplementary data file 1).

Discussion

In the present study we investigated a series of 316 apparently sporadic and syndrome-related pheochromocytomas and paragangliomas for SDHA protein expression and found a further six negative tumors (2.2% overall). These were exclusively present in apparently sporadic cases, yielding a percentage of 3% in this group (six additional SDHA negative tumors out of 198 apparently sporadic cases).

Sequence analysis of the SDHA-negative tumors revealed two novel *SDHA* mutations (c.91C>T (n=4) and c.1753C>T (n=1)), which were found in the tumor DNA as well as in corresponding germline DNA of the affected patients. In accordance with Knudson's two-hit hypothesis, all SDHA immunohistochemically negative *SHDA*-mutated tumors showed loss of the wild type allele. This confirms that *SDHA*, as we described previously, acts as a bona fide tumor suppressor gene.[12]

Interestingly, both *SDHA* mutations found in this study were also identified in a healthy control group. The p.Arg31X occurred in 0.3% of the control cases, which is 10 fold lower than that observed in the apparently sporadic tumor group (3%). However, the p.Arg31X mutation appears to be unequivocally involved in the pathogenesis of these tumors as the mutation leads to a truncated protein, and all *SDHA*-mutated tumors show loss of the wild type allele, causing loss of SDHA expression. In addition, the p.Arg585Trp mutation was also found in one in 972 alleles in a healthy control group. Hence, the occurrence of these mutations in healthy controls suggests a low penetrance of paragangliomas in patients with *SDHA* mutations, which could be putatively explained by the rarity of loss of the 5p15 (*SDHA*) locus.[12] Furthermore, none of the affected *SDHA* mutation-carriers that we identified had a family history of the disease, comparable to most of the newly diagnosed *SDHB* mutation carriers presenting with a paraganglioma.[16] Therefore, the majority of germline *SDHA* mutation-carriers in the "normal healthy" population will most likely not develop the disease.

Negative SDHA staining was expected in the tumors with the c.91C>T, as this mutation leads to a truncated SDHA protein. In contrast, the c.1753C>T missense mutation does not lead to a truncated protein, but the SDHA staining was also negative. This could be due to a conformational change of the mutated SDHA protein destroying the antigenic epitope for the antibody. The SDHA antibody used was developed against cow complex II so was not directed against a specific peptide. To determine if SDHA is present, but is not recognized by the used antibody, additional SDHA antibodies directed against other SDHA epitopes should be used.

All other tumors (n = 309) were immunohistochemically positive for SDHA, in accordance with our previous results, which showed SDHA expression in *RET*, *VHL*, *SDHB*, and *SDHD*-mutated tumors.[12] In addition, 35 apparently sporadic tumors with positive immunohistochemical SDHA staining were analyzed for *SDHA* gene mutations, but none were found. Since the SDHA antibody does not cross-react with other proteins, it is unlikely that false positive staining occurred.

We recently, we demonstrated that *SDHB*, *SDHC*, and *SDHD*-related tumors all show loss of SDHB immunohistochemical expression, whereas *RET*, *VHL*, *NF1*, and *TMEM127* related-tumors were immunohistochemically SDHB-positive. [13, 17] It was suggested that absence of functional SDHC or SDHD leads to impairment of complex II formation and degradation of SDHB. The current results, showing absence of SDHB expression in *SDHA*-mutated tumors, are in accordance with this explanation. In contrast, while *SDHB*, *SDHC* and *SDHD*-related tumors were immunohistochemically negative for SDHB, these tumors showed positive staining for SDHA. These findings suggest that the SDHB protein is degraded when the complex is disrupted while the SDHA protein remains intact.

In conclusion, this study provides additional evidence that *SDHA* is a bona fide tumor suppressor gene responsible for a significant number of genetically-determined paragangliomas and pheochromocytomas (3% of apparently sporadic tumors). Although the number of identified mutation carriers is still low, current observations suggest that *SDHA* mutations are not associated with a particular paraganglia location, nor with a familial presentation. The results of this study show that, in the absence of familial or clinical indications for a specific form of inherited pheochromocytoma or paraganglioma, SDHA immunohistochemistry on tumor tissue can detect patients carrying germline *SDHA* mutations.

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Supplementary table 1. Patient clinical data and immunohistochemistry results

Patient	PCC/PGL	Gender	Age	M/B	Location	Genetic background	SDHA IHC
1	PCC	M	36	M	A	sporadic	positive
2	PCC	F	46	M	A	sporadic	positive
3	PCC	F	70	M	A	sporadic	positive
4	PCC	M	31	B	A	VHL	positive
5	PCC	F	23	M	A	sporadic	positive
6	PCC	M	48	M	A	sporadic	positive
7	PCC	M	17	B	A	sporadic	positive
8	PCC	F	29	B	A	MEN2A	positive
9	PCC	F	47	B	A	NF1	positive
10	PCC	M	55	B	A	VHL	positive
11	PCC	F	61	B	A	NF1	positive
12	PCC	M	33	B	A	sporadic	positive
13	PCC	M	58	B	A	sporadic	positive
14	PCC	F	25	B	A	sporadic	positive
15	PCC	F	62	B	A	sporadic	positive
16	PCC	M	14	B	A	sporadic	positive
17	PCC	M	15	B	A	sporadic	positive
18	PCC	F	40	B	A	sporadic	positive
19	PCC	F	63	B	A	NF1	positive
20	PCC	F	54	B	A	sporadic	positive
21	PCC	F	20	B	A	MEN2A	positive
22	PCC	F	32	B	A	MEN2A	positive
23	PCC	M	42	B	A	MEN2A	positive
24	PCC	M	55	B	A	MEN2A	positive
25	PCC	F	40	B	A	sporadic	positive
26	PCC	M	53	B	A	sporadic	positive
27	PCC	M	33	B	A	NF1	positive
28	PCC	F	40	B	A	sporadic	positive
29	PCC	F	40	B	A	NF1	positive
30	PCC	F	35	B	A	sporadic	positive
31	PCC	M	67	B	A	sporadic	positive
32	PCC	F	39	B	A	VHL	positive
33	PCC	F	62	B	A	sporadic	positive
34	PCC	M	68	B	A	sporadic	positive
35	PCC	F	67	B	A	NF1	positive
36	PCC	M	59	B	A	sporadic	positive
37	PCC	M	26	B	A	NF1	positive
38	PCC	M	43	B	A	sporadic	positive
39	PCC	M	9	B	A	sporadic	positive
40	PCC	F	45	B	A	sporadic	positive
41	PCC	M	64	x	A	sporadic	positive
42	PCC	M	41	B	A	sporadic	positive
43	PCC	M	65	B	A	sporadic	positive
44	PCC	F	48	x	A	sporadic	positive
45	PCC	F	61	B	A	sporadic	positive
46	PCC	F	27	x	A	sporadic	positive
47	PCC	F	34	x	A	sporadic	positive
48	PCC	F	29	B	A	MEN2A	positive
49	PCC	M	26	B	A	MEN2A	positive
50	PCC	F	24	x	A	MEN2A	positive
51	PCC	F	50	B	A	MEN2A	positive
52	PCC	M	54	x	A	sporadic	positive

53	PCC	F	51	B	A	sporadic	positive
54	PCC	M	24	B	A	VHL	positive
55	PCC	F	38	B	A	MEN2A	positive
56	PCC	M	23	B	A	MEN2A	positive
57	PCC	M	45	B	A	sporadic	positive
58	PCC	F	51	B	A	MEN2A	positive
59	PCC	F	50	B	A	sporadic	positive
60	PCC	F	45	B	A	sporadic	positive
61	PCC	F	49	B	A	sporadic	positive
62	PCC	M	65	B	A	sporadic	positive
63	PCC	M	66	B	A	sporadic	positive
64	PCC	F	39	B	A	NF1	positive
65	PCC	F	75	B	A	sporadic	positive
66	PCC	F	34	B	A	MEN2B	positive
67	PCC	M	59	B	A	sporadic	positive
68	PCC	M	63	B	A	sporadic	positive
69	PCC	M	48	B	A	sporadic	positive
70	PCC	F	18	B	A	VHL	positive
71	PCC	M	63	M	A	sporadic	positive
72	PCC	F	79	B	A	sporadic	positive
73	PCC	F	71	B	A	sporadic	positive
74	PCC	M	29	B	A	sporadic	positive
75	PCC	F	35	B	A	sporadic	positive
76	PCC	F	x	x	A	sporadic	positive
77	PCC	F	x	x	A	sporadic	positive
78	PCC	F	56	M	A	sporadic	positive
79	PCC	F	31	M	A	sporadic	positive
80	PCC	F	59	B	A	MEN2A	Positive
81	PCC	F	24	B	A	NF1	Positive
82	PCC	F	45	M	A	sporadic	Positive
83	PCC	F	30	M	A	sporadic	Positive
84	PCC	M	41	B	A	sporadic	Positive
85	PCC	M	43	B	A	sporadic	Positive
86	PCC	F	41	B	A	sporadic	Positive
87	PCC	F	41	B	A	MEN2A	Positive
88	PCC	M	40	B	A	MEN2A	Positive
89	PCC	M	53	B	A	MEN2A	positive
90	PCC	F	69	B	A	sporadic	positive
91	PCC	M	53	B	A	sporadic	positive
92	PCC	F	48	B	A	sporadic	positive
93	PCC	F	68	B	A	sporadic	positive
94	PCC	F	33	B	A	sporadic	positive
95	PCC	M	30	B	A	sporadic	positive
96	PCC	M	41	B	A	NF1	positive
97	PCC	F	30	B	A	sporadic	positive
98	PCC	F	50	B	A	sporadic	positive
99	PCC	M	44	B	A	NF1	positive
100	PCC	F	62	x	A	sporadic	positive
101	PCC	F	42	x	A	sporadic	positive
102	PCC	x	61	M	A	sporadic	positive
103	PCC	M	24	x	A	sporadic	positive
104	PCC	M	42	M	A	sporadic	positive
105	PCC	F	25	x	A	MEN2A	positive
106	PCC	M	52	B	A	NF1	positive
107	PCC	F	43	M	A	sporadic	positive

108	PCC	F	51	x	A	MEN2A	positive
109	PCC	M	50	B	A	sporadic	positive
110	PCC	M	39	M	A	sporadic	positive
111	PCC	M	35	B	A	MEN2A	positive
112	PCC	F	60	B	A	sporadic	positive
113	PCC	F	39	B	A	sporadic	positive
114	PCC	M	16	B	A	SDHD	positive
115	PCC	F	31	B	A	SDHD	positive
116	PCC	M	29	B	A	NF1	positive
117	PCC	M	62	M	A	SDHD	positive
118	PCC	M	21	B	A	MEN2a	positive
119	PCC	M	7	x	A	VHL	positive
120	PCC	M	44	B	A	sporadic	positive
121	PCC	F	31	M	A	sporadic	positive
122	PCC	M	56	M	A	sporadic	positive
123	PCC	F	43	M	A	MEN2A	positive
124	PCC	M	28	B	A	sporadic	positive
125	PCC	M	42	B	A	sporadic	positive
126	PCC	F	57	M	A	sporadic	positive
127	PCC	F	56	M	A	sporadic	positive
128	PCC	M	31	B	A	sporadic	positive
129	PCC	M	61	M	A	sporadic	positive
130	PCC	F	53	B	A	NF1	positive
131	PCC	F	88	x	A	sporadic	positive
132	PCC	F	48	x	A	sporadic	negative
133	PCC	F	77	x	A	sporadic	positive
134	PCC	M	47	B	A	sporadic	positive
135	PCC	M	38	B	A	VHL	positive
136	PCC	F	57	x	A	sporadic	positive
137	PCC	F	69	B	A	sporadic	positive
138	PCC	M	43	B	A	sporadic	positive
139	PCC	M	62	B	A	sporadic	Positive
140	PCC	F	59	B	A	NF1	positive
141	PCC	M	28	x	A	sporadic	positive
142	PCC	F	21	B	A	sporadic	positive
143	PCC	M	x	B	A	sporadic	positive
144	PCC	F	x	x	A	sporadic	positive
145	sPGL	F	53	M	Abdomen	sporadic	positive
146	sPGL	M	41	M	Abdomen	sporadic	negative
147	sPGL	M	25	M	Abdomen	SDHD	positive
148	sPGL	M	54	B	X	sporadic	positive
149	sPGL	M	79	B	Abdomen	sporadic	positive
150	sPGL	F	43	B	Abdomen	sporadic	positive
151	sPGL	F	49	M	X	sporadic	positive
152	sPGL	F	35	M	X	sporadic	positive
153	sPGL	F	30	M	Abdomen	sporadic	positive
154	sPGL	F	56	B	Abdomen	sporadic	positive
155	sPGL	F	63	M	Abdomen	SDHB	positive
156	sPGL	M	30	B	Abdomen	sporadic	positive
157	sPGL	M	48	x	Thorax	sporadic	Positive
158	sPGL	F	12	x	Abdomen	SDHB	Positive
159	sPGL	M	40	x	Abdomen	sporadic	positive
160	sPGL	M	61	x	Bladder	sporadic	positive
161	sPGL	F	55	B	Thorax	sporadic	negative
162	pPGL	F	33	B	Vagus	sporadic	negative

163	pPGL	M	39	B	Carotis	sporadic	positive
164	pPGL	M	44	B	Carotis	sporadic	positive
165	pPGL	M	42	B	Carotis	sporadic	positive
166	pPGL	F	14	B	Carotis	sporadic	positive
167	pPGL	M	34	B	Tympanicus	sporadic	positive
168	pPGL	F	33	B	Thyroid	sporadic	positive
169	pPGL	F	67	B	Tympanicus	sporadic	positive
170	pPGL	F	29	B	Tympanicus	sporadic	positive
171	pPGL	F	62	B	Carotis	sporadic	positive
172	pPGL	F	36	B	Carotis	SDHD	positive
173	pPGL	F	53	B	Carotis	SDHD	positive
174	pPGL	F	53	B	Vagus	sporadic	positive
175	pPGL	M	25	B	Carotis	SDHD	positive
176	pPGL	F	33	B	Carotis	SDHD	positive
177	pPGL	M	26	B	Carotis	SDHD	positive
178	pPGL	F	39	B	Carotis	SDHC	positive
179	pPGL	F	24	B	Carotis	SDHD	positive
180	pPGL	F	55	B	Tympanicus	sporadic	positive
181	pPGL	F	35	B	Carotis	sporadic	positive
182	pPGL	F	57	B	Carotis	SDHD	positive
183	pPGL	M	45	B	Dura	sporadic	positive
184	pPGL	F	57	B	Tympanicus	sporadic	positive
185	pPGL	F	39	B	Jugularis	SDHD	positive
186	pPGL	F	57	B	Tympanicus	sporadic	positive
187	pPGL	M	45	B	Carotis	sporadic	negative
188	pPGL	F	61	B	Carotis	IDH1	positive
189	pPGL	F	23	B	Carotis	sporadic	positive
190	pPGL	F	50	B	Carotis	SDHD	positive
191	pPGL	F	22	B	Jugularis	SDHD	positive
192	pPGL	F	53	B	Carotis	SDHD	positive
193	pPGL	M	73	B	Tympanicus	sporadic	positive
194	pPGL	F	32	B	x	sporadic	positive
195	pPGL	M	63	B	x	sporadic	positive
196	pPGL	F	36	B	x	SDHD	positive
197	pPGL	M	52	B	Carotis	sporadic	positive
198	pPGL	M	48	B	Carotis	SDHB	positive
199	pPGL	M	34	B	Carotis	SDHD	positive
200	pPGL	M	20	B	Jugularis	sporadic	positive
201	pPGL	X	x	B	Tympanicus	sporadic	positive
202	pPGL	M	56	B	Carotis	sporadic	positive
203	pPGL	M	38	B	Jugularis	sporadic	positive
204	pPGL	M	51	B	Carotis	SDHD	positive
205	pPGL	F	57	B	tympanicus	sporadic	positive
206	pPGL	F	37	B	Carotis	SDHD	positive
207	pPGL	M	57	B	x	sporadic	positive
208	pPGL	M	48	B	x	SDHD	positive
209	pPGL	F	35	B	Carotis	sporadic	positive
210	pPGL	F	27	B	Carotis	sporadic	positive
211	pPGL	x	x	B	x	sporadic	positive
212	pPGL	x	x	B	x	sporadic	positive
213	pPGL	F	27	B	x	sporadic	positive
214	pPGL	F	25	B	Carotis	sporadic	positive
215	pPGL	M	61	B	Carotis	sporadic	positive
216	pPGL	x	x	B	Carotis	sporadic	positive
217	pPGL	x	x	B	x	sporadic	positive

218	pPGL	x	x	B	x	sporadic	positive
219	pPGL	x	x	B	x	SDHAF2	positive
220	pPGL	x	x	B	x	SDHAF2	positive
221	pPGL	x	x	B	x	SDHAF2	positive
222	pPGL	x	x	B	x	SDHAF2	positive
223	pPGL	x	x	B	x	SDHAF2	positive
224	pPGL	x	x	B	Carotis	sporadic	positive
225	Met	M	46	M	lymph node	sporadic	positive
226	Met	F	27	M	Abdomen	sporadic	positive
227	Met	F	38	M	Abdomen	SDHB	positive
228	Met	M	28	M	Abdomen	SDHB	positive
229	Met	F	46	M	Abdomen	sporadic	positive
230	Met	F	63	M	psoas	sporadic	positive
231	PCC	F	33	B	A	NF1	positive
232	PCC	F	33	B	A	NF1	positive
233	PCC	F	32	B	A	NF1	positive
234	PCC	F	32	B	A	NF1	positive
235	PCC	F	38	B	A	NF1	positive
236	PCC	F	27	M	A	sporadic	positive
237	PCC	F	44	B	A	sporadic	positive
238	PCC	F	39	M	A	sporadic	positive
239	PCC	M	40	M	A	sporadic	positive
240	PCC	F	40	B	A	sporadic	positive
241	PCC	F	36	B	A	sporadic	positive
242	PCC	F	29	B	A	sporadic	positive
243	PCC	F	28	B	A	sporadic	positive
244	PCC	F	44	B	A	RET	positive
245	PCC	F	18	B	A	RET	positive
246	PCC	F	18	B	A	RET	positive
247	PCC	F	76	B	A	RET	positive
248	PCC	F	18	B	A	RET	positive
249	PCC	F	31	B	A	SDHB	positive
250	PCC	F	20	B	A	SDHB	positive
251	PCC	M	15	B	A	VHL	positive
252	PCC	F	10	B	A	VHL	positive
253	PCC	F	10	B	A	VHL	positive
254	PCC	M	31	B	A	VHL	positive
255	PCC	M	45	B	A	sporadic	positive
256	PCC	M	46	x	A	sporadic	positive
257	PCC	M	62	B	A	sporadic	positive
258	PCC	M	63	M	A	sporadic	positive
259	PCC	F	44	B	A	TMEM127	positive
260	PCC	F	37	B	A	NF1	positive
261	PCC	M	53	M	A	sporadic	positive
262	PCC	F	41	B	A	sporadic	positive
263	PCC	F	53	B	A	sporadic	positive
264	PCC	F	49	B	A	sporadic	positive
265	PCC	F	47	B	A	sporadic	positive
266	PCC	F	66	B	A	sporadic	positive
267	PCC	M	57	B	A	sporadic	positive
268	PCC	M	59	B	A	sporadic	positive
269	PCC	M	47	B	A	sporadic	positive
270	PCC	M	21	B	A	sporadic	positive
271	PCC	M	24	B	A	sporadic	positive
272	PCC	M	27	B	A	sporadic	positive
273	PCC	M	24	B	A	sporadic	positive

274	PCC	M	28	M	A	SDHB	positive
275	PCC	F	54	M	A	SDHB	positive
276	PCC	F	44	B	A	TMEM127	positive
277	PCC	M	26	B	A	VHL	positive
278	PCC	M	15	B	A	VHL	positive
279	PCC	M	17	B	A	VHL	positive
280	PCC	M	25	B	A	VHL	positive
281	PCC	F	16	B	A	VHL	positive
282	PCC	M	17	B	A	sporadic	positive
283	PCC	F	27	B	A	sporadic	positive
284	PCC	F	27	B	A	sporadic	positive
285	PCC	F	68	B	A	sporadic	positive
286	PCC	M	31	B	A	sporadic	positive
287	PCC	M	27	B	A	sporadic	positive
288	pPGL	M	60	B	Carotis	SDHD	positive
289	pPGL	F	33	B	Carotis	sporadic	positive
290	sPGL	M	24	M	Abdomen	sporadic	positive
291	sPGL	M	27	B	Abdomen	sporadic	negative
292	sPGL	F	37	B	Abdomen	sporadic	positive
293	sPGL	F	15	x	Abdomen	sporadic	positive
294	sPGL	F	24	B	Abdomen	sporadic	positive
295	sPGL	F	45	B	Abdomen	sporadic	positive
296	sPGL	M	46	B	Abdomen	sporadic	positive
297	sPGL	F	32	B	Abdomen	SDHA	negative
298	sPGL	F	47	B	Abdomen	SDHB	positive
299	sPGL	M	16	B	Abdomen	SDHB	positive
300	sPGL	M	39	M	Abdomen	SDHB	positive
301	sPGL	M	43	M	Abdomen	SDHB	positive
302	sPGL	M	39	M	Abdomen	SDHB	positive
303	sPGL	M	36	B	Abdomen	SDHB	positive
304	sPGL	F	22	B	Abdomen	SDHB	positive
305	sPGL	M	32	B	Abdomen	SDHD	positive
306	sPGL	M	45	B	Abdomen	SDHD	positive
307	sPGL	M	26	B	Abdomen	VHL	positive
308	sPGL	M	20	B	Abdomen	VHL	positive
309	sPGL	M	20	B	Abdomen	VHL	positive
310	sPGL	F	26	M	Abdomen	sporadic	positive
311	sPGL	F	26	M	Abdomen	sporadic	positive
312	sPGL	F	15	B	Thorax	sporadic	positive
313	sPGL	M	26	B	Carotis	SDHD	positive
314	sPGL	M	26	B	Carotis	SDHD	positive
315	sPGL	F	59	B	Sigmoid	sporadic	positive
316	sPGL	F	16	B	Thorax	SDHC	positive

PCC = pheochromocytoma, sPGL = extra-adrenal sympathetic paraganglioma, pPGL = para-sympathetic paraganglioma, Met = metastasis, x = unknown, B = benign, M = malignant, A = adrenal

Supplementary Table 2. Clinical data patients with SDHA immunochemically negative tumors

Patient	Gender	Age	Primary tumor	Metastases	Status	Follow-up	Producing
132	f	48	PCC – adrenal	no	Alive	nk	NA
146	m	41	sPGL – bladder	local lymph nodes	Alive	3 yr ('89)	NA, A
161	f	55	sPGL – thorax	no	Alive	7 yr ('99)	nk
162	f	33	pPGL – vagal	no	Alive	0.5 yr ('94)	nk
187	m	45	pPGL – carotid	no	Deceased *	nk	nk
291	m	27	sPGL – abdomen	no	Alive	nk	NA
297	F	32	sPGL – abdomen	No	Alive	nk	NA

Age = age at diagnosis, PCC = pheochromocytoma, sPGL = extra-adrenal sympathetic paraganglioma, pPGL = parasympathetic paraganglioma, no = no metastases present at diagnosis, * = dead not related to PGL, yr = years, between brackets was the last year of contact, nk = not known, NA = noradrenaline, A = adrenaline

Supplementary data. RT-PCR procedure and results

RT-PCR method

RT-PCR was performed to determine whether the mutant allele was expressed at the RNA level. RNA was isolated from the paraffin-embedded tumor tissue of the *SDHA*-related patients using the RNeasy FFPE kit (Qiagen Benelux, Venlo, the Netherlands) according to manufacturer's instructions. RT-PCR was performed with intron-spanning *SDHA*-specific primers (forward: gcggcaacagcagacatgtc, reverse: cctcttgttcccatcaacagtg) using the Access RT-PCR System (Promega, Madison, WI) according to manufacturer's instructions. This was followed by sequence analysis using the RT-PCR primers described above.

RT-PCR result

To determine whether the *SDHA*-mutant allele (c.91T) was also expressed in the tumor, RNA was isolated from FFPE tumor tissues of four *SDHA*-mutation carriers. Only one tumor yielded RNA of sufficient quality to be reliably analyzed and showed a clear expression of the allele with the p.Arg31X mutation (see figure).

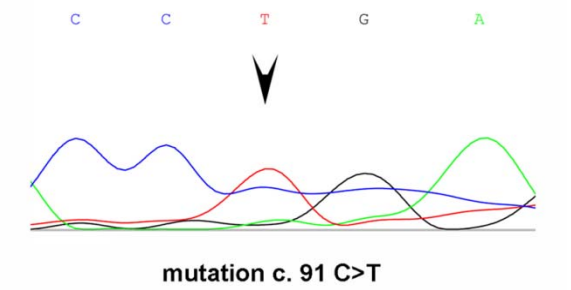

Figure legend. *SDHA* sequence electropherogram showing that the *SDHA* c.91C>T mutation, indicated by the arrow, is normally expressed at the RNA level.

General discussion

Discussion

Discriminating malignant from benign tumors

The prediction of malignant behavior of pheochromocytomas and paragangliomas remains elusive. A tumor can only be diagnosed malignant with certainty if distant metastases are found. An apparently benign tumor should always be considered potentially malignant since metastases can occur decades after the diagnosis of the primary tumor.[1, 2] There are clinical signs, such as tumor location or co-occurrence of other tumors, which can indicate a higher chance of malignant behavior. For instance, an extra-adrenal sympathetic paraganglioma in the abdomen will metastasize more often compared to a pheochromocytoma.[3] In contrast, parasympathetic paragangliomas, which occur in the head and neck region, are mostly benign.[4] Another indicator for malignant behavior is the presence of mutations in certain genes. For example, patients with a germline *SDHB* mutation will have a higher chance of metastases, compared to patients with a germline *RET* mutation, that usually do not present malignant pheochromocytomas.[5-8]

Some studies have tried to identify immunohistochemical markers, such as p53, RET, BCL-2, or histological features to be able to predict malignant behavior, but all were inconclusive.[9-11] Other studies have focused on molecular differences by comparative genomic hybridization. Our studies on genomic alterations (chapters 3 and 4) revealed distinct genetic alterations for benign and malignant sporadic pheochromocytomas and paragangliomas. The benign tumor group could be subdivided into two groups: 1) displayed loss of chromosome 1p and/or loss of chromosome 3q, without loss of chromosome 3p, 2) showed loss of chromosome 3p with or without concomitant loss of chromosome 11p. The tumors of group 1 showed genetic alterations that were similar to those found in *RET*-mutated tumors, whereas the alterations found in tumors of group 2 were similar to those found in *VHL*-related tumors.[12, 13] Malignant tumors showed genomic alterations comparable to those found in the benign series, but at different frequencies. Loss of chromosome 1p was seen most frequently, whereas loss of chromosome 3q was much less frequent in the malignant tumors. When the results of our CGH studies of the benign and malignant tumors were compared, two chromosomal areas were significantly different between the benign and malignant tumor groups. The first alteration was loss of chromosome 3q21.1, which occurred significantly more frequently in the benign tumors ($p<0.001$), and the second was loss of chromosome 11p11.2, which was also more present in the benign group ($p<0.0035$). In addition, the malignant tumors showed more gains compared to the benign group. The presence of more gains in the malignant series is in accordance with previous literature.[12-15] Although our results suggest that we can discriminate benign and malignant tumors by their molecular alterations of chromosomes 3q and 11p, this was not reported in other studies.[12-15] These discrepancies could be due to the limited sample sizes of these studies, but could also be caused by differences in techniques, use of different (conventional) arrays, or due to intra-tumoral heterogeneity.

The latter is found in benign as well as malignant pheochromocytomas, but at a much higher frequency in the malignant tumors as we showed in Chapter 2. This suggests that malignant tumors are more heterogeneous and have a different pathogenesis

compared to benign tumors. In addition, it indicates that molecular aberrations found in genomic studies (comparative genomic hybridization and loss of heterozygosity), are not necessarily representative of the whole tumor. In fact, the aberration could only be present in that part of the tumor, from which the DNA isolation was done. In future, such studies should isolate DNA preferably from a relative large area of the tumor to circumvent detection of molecular aberrations present in only a subpopulation of the tumor cells (intra-tumoral heterogeneity). The differences in intra-tumoral heterogeneity and chromosomal alterations between benign and malignant tumors suggest that they reflect two independent neoplastic entities.

Several studies have focused on the differences in mRNA expression patterns between benign and malignant pheochromocytomas.[16-18] The study of Suh et al.[17] showed that many genes are differentially expressed between malignant and benign pheochromocytomas, including those that are involved in signal transduction, transcription, protein synthesis, and electron transport. Ten diagnostically applicable, significantly differing genes were listed that could discriminate malignant from benign pheochromocytomas at the mRNA level. However, frozen tissue is necessary to perform these tests, which is not always available. It would be much easier and more suitable for diagnostic use if these genes could be tested by immunohistochemistry, on formalin-fixed paraffin-embedded tumor tissue. The markers of the study of Suh et al. should be tested on an independent validation set of benign and malignant tumors by immunohistochemistry. An example of such an immunohistochemical test for the prediction of the biological behavior of esophageal adenocarcinoma was recently reported by Fitzgerald et al.[19] They showed that an immunohistochemical test with 4 markers on formalin-fixed paraffin-embedded tumors enabled them to predict the survival of patients with esophageal cancer.

Another study investigated the differences between 10 benign and 9 malignant pheochromocytomas by looking at the vascular architecture of the tumors, as angiogenesis is a critical step in tumor growth and metastatic invasion.[20] The authors used immunohistochemical markers CD34 and vascular smooth muscle actin (α-actin) to stain the endothelial and smooth muscle cells of the blood vessel wall, respectively. It appeared that there was a clear difference in vascular pattern between the two groups. The benign pheochromocytomas showed regularly distributed, short and straight vascular segments, whereas the malignant tumors showed much longer segments, of irregular length, and showed a lower vessel density. Also, the malignant tumors were characterized by three-dimensional flattened bags. The results of the study suggest that the analysis of the vascular architecture of pheochromocytomas might allow distinguishing malignant tumors from benign. However, the sample size of the study was relatively low, so the series has to be expanded in future studies. In addition, the markers should be tested prospectively.

Mouse and human pheochromocytomas: similar pathways?

There are many mouse models that develop pheochromocytomas. Most of these mouse models involve the inactivation of genes that are directly or indirectly involved in the regulation of the cell cycle.[21-31] However, in most cases these genes appear not to be involved in the pathogenesis of human pheochromocytomas. Nonetheless, there are many similarities between mouse and human pheochromocytomas.

The genes *Rb*, *p18*, *p27*, *Pten*, *Nf1*, and *Ret*, which are involved in the pathogenesis of mouse pheochromocytomas, are closely or directly involved in cell cycle regulation. *Rb* has the capability to arrest cells in G1 by inhibiting E2F transcription factors, but also through stabilization of p27.[32] Besides its role in controlling the G1-S phase transition of the cell cycle, *Rb* is also involved in the control of cellular differentiation during embryogenesis and in adult tissues, in regulation of apoptotic cell death and angiogenesis, and in the preservation of chromosomal stability. *p27(Kip1)* encodes a cell cycle inhibitor that inhibits cyclin-dependent kinases, which initiate the G1 to S phase transition.[33] Germline *p27* mutations in humans have recently been associated with a novel type of neuro-endocrine neoplasia type 4.[33] In contrast, *Pten*, *Nf1*, and *Ret* seem to be more upstream in the pathways regulating the cell cycle, and appear related, as they all activate or inhibit Ras. Ras stimulates the cell division by activating the MEK and ERK pathways.[34] This is illustrated in figure 1.

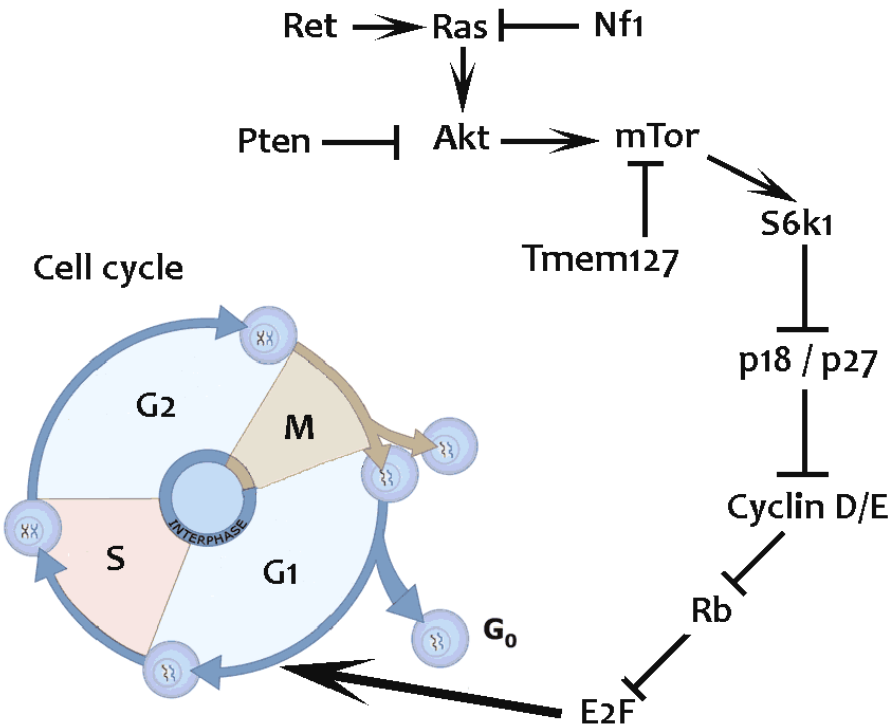

Figure 1. Simplified overview of all genes associated with mouse pheochromocytomas in one common pathway.[35-38]

Pheochromocytomas of *Ret* transgenic rats show 200 up-regulated genes of which 18 appeared implicated in the differentiation of neural crest cells into precursor cells of the sympathoadrenal cell lineage from which adrenal medullary cells are derived.[39] The pheochromocytomas of *Nf1* KO mice also show overexpression of genes that are up-regulated in neural stem cells or progenitors.[40] The difference in up-regulated genes between the two studies is probably (at least in part) due to the two different species of the studies. Additionally, although *Ret* and *Nf1* appear to be in the same pathways, they have different target genes.

In humans, *RET* and *NF1*-mutated tumors cluster in one group, but do not cluster with *SDH* or *VHL*-related tumors. The *RET* and *NF1*-mutated tumors displayed a neural progenitor expression profile, whereas the *SDH* and *VHL*-mutated tumors showed a (pseudo)hypoxia profile.[41, 42] Recently germline mutations in the *TMEM127* gene were associated with pheochromocytomas.[43] TMEM127 appeared to negatively regulate mTOR, which is an important downstream target of AKT. AKT is inhibited by PTEN and, although *PTEN* mutations have not been found in human pheochromocytomas, *Pten* inactivation is involved in mouse pheochromocytomas. So it appears that a common pathways are involved in the pheochromocytomas of the *Pten* KO mice and the human *TMEM127*-related pheochromocytomas. However, it must be noted that only few *TMEM127*-mutated pheochromocytomas were malignant (5%), whereas almost two-third of the *Pten*-related mouse tumors were malignant .[44]

Differentially expressed genes in genetically different tumors: a common pathway?

As previously mentioned, human pheochromocytomas and paragangliomas cluster in distinct groups. A few studies have investigated mRNA expression patterns, and have compared tumors with different hereditary backgrounds. One report investigated 76 catecholamine-producing pheochromocytomas (n = 63) and sympathetic paragangliomas (n = 13), which had *VHL*, *SDHB*, *SDHD*, *NF1*, and *RET* mutations, or occurred sporadically. Unsupervised clustering resulted in two main clusters: cluster 1 included the *VHL* and *SDH*-related tumors, and cluster 2 contained the *RET* and *NF1*-related tumors. The sporadic cases occurred at similar frequencies in both clusters. The *VHL* and *SDH*-related tumors displayed up-regulation of genes involved in angiogenesis, such as HIF1a, and down-regulation of genes coding for components of the electron transport chain. In contrast, *RET*-mutated tumors showed up-regulation of genes of the RAS and MAP kinase pathways, having a more neural profile in general.[41] Another study also examined mRNA profiles from pheochromocytoma and paraganglioma of different hereditary backgrounds, including tumors with germline *SDHB*, *SDHC*, *SDHD*, *VHL*, *RET*, and *NF1* mutations.[42] Unsupervised clustering also resulted in distinct groups in which *RET* and *NF1*-related tumors formed a cluster, and *VHL* and *SDH*-mutated tumors formed another cluster. In addition, the tumors were also used to perform unsupervised clustering based on the expression of genes that are involved in glycolysis. This is a way for the cell to generate energy without oxygen, but it is less efficient than through the citric acid cycle, for which oxygen is necessary. The switch of a tumor cell to glycolysis as its main energy source is also called the Warburg effect.[45] The unsupervised clustering of all tumors resulted in 3 groups, of which the first included solely *VHL* cases. The second group was subdivided into two clusters. The first cluster contained most *SDH* tumors (n = 18), one *RET*, one *VHL,* and two *NF1* cases, and the second cluster included most *RET* and *NF1* cases,

and 3 of the 17 *SDHB* tumors. Thus, it appears that *VHL* and *SDH* mutated tumors share similar pathways in general, but differ in the use of glycolysis as the main energy source. It even appears that *NF1* and *RET*-related tumors have more similarities with *SDH*-related tumors regarding the Warburg effect, as glycolysis-related genes were less upregulated compared to the *VHL*-related tumors.

Because of the rarity of pheochromocytomas and paragangliomas, it is difficult to collect large series, which can be easily done for common cancers like breast cancer or colon cancer. It is even more difficult to have high numbers of cases with mutations in specific genes. These high numbers are essential to investigate the pathogenesis of these tumors in more detail. If sample size increases, investigation of genotype-phenotype correlations would be much easier. Therefore, as occurs more and more over the last years, it is important that research groups join forces and become less competitive and share samples and data.

If sample size would increase, we might be able to explain why SDHB mutation carriers develop metastases more often, whereas patients that have a germline mutation in one of the other genes encoding complex II subunits (*SDHA*, *SDHC*, *SDHD*, and *SDHAF2*) present with metastases to a much lesser extent (Chapter 9).[6, 46, 47]

In general, it is remarkable that mutations in genes encoding apparently unrelated proteins or enzymes can cause pheochromocytomas and paragangliomas. This suggests the presence of a common pathway, which is illustrated in figure 3.

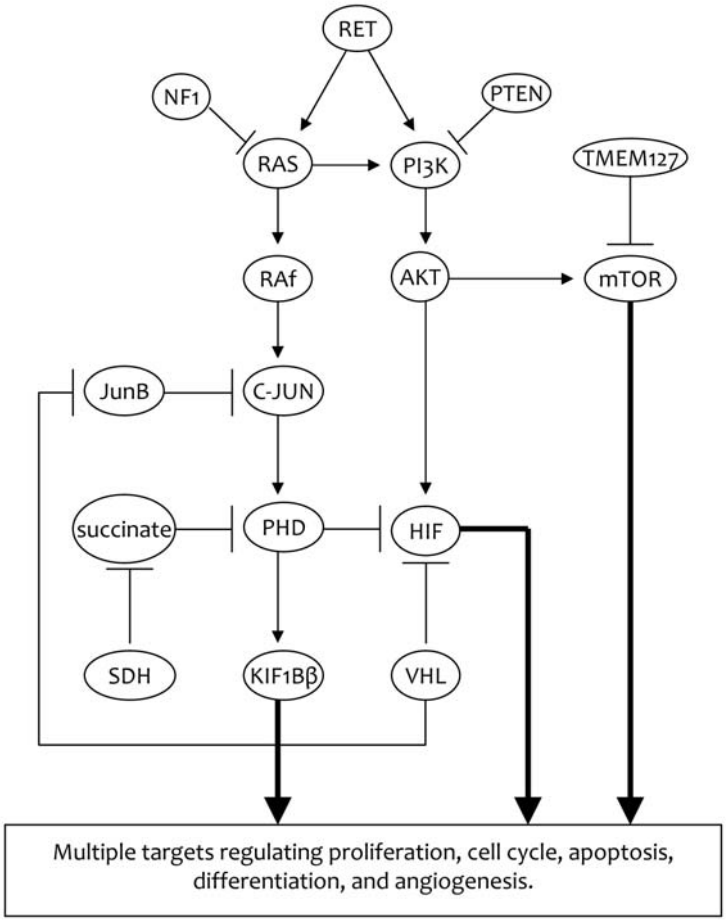

Figure 3. A common pathway for the pheochromocytoma and paraganglioma susceptibility genes.

Selecting genes for mutation analysis: SDHA and SDHB immunohistochemistry

Approximately 25-30% of pheochromocytomas and 50% of paragangliomas is caused by germline mutations in genes that cause various hereditary syndromes. These genes include *RET*, *VHL*, *NF1*, *SDHA*, *SDHAF2*, *SDHB*, *SDHC*, *SDHD*, *TMEM127*, and *KIF1B*-β.[6, 43, 46, 48, 49] Besides pheochromocytomas and paragangliomas, other tumors occur in patients with germline mutations in one of these genes. For example, germline mutation carriers of the *RET* oncogene usually present first with medullary thyroid carcinoma, and *VHL* germline mutation carriers frequently develop retinoblastomas or renal cell carcinomas.[50, 51] In addition, patients with neurofibromatosis type 1 are characterized by café-au-lait spots.[52] So, the co-occurrence of other tumors can indicate which gene is affected in the patient. However, the pheochromocytoma or paraganglioma could also be the presenting tumor in these patients. Another indication for which gene is mutated could be the location of the primary tumor. For instance, extra-adrenal sympathetic paragangliomas appear to occur more frequently in *SDHB* germline mutation carriers, whereas parasympathetic paragangliomas occur more frequently in *SDHD* mutation carriers.[8] However, because there are many exceptions to this rule, related to reduced penetrance, and the occurrence of de novo mutations, these clinical features can only be used as indicators for gene testing.

SDHB-related tumors are malignant at a higher frequency compared to tumors that are caused by other genes.[53] Therefore, it is important to identify these patients, also in view of their family members. However, mutations in the other pheochromocytoma and paraganglioma susceptibility genes can also cause malignant tumors, although at low frequency. To perform mutation analysis on all these genes is expensive and laborious, and is not necessary as we have shown in Chapter 8. Using SDHB immunohistochemistry on the tumor, we can preselect genes to test, as *SDHA*, *SDHAF2*, *SDHB*, *SDHC*, and *SDHD*-mutated tumors are immunohistochemically negative and non-*SDH*-related tumors stain positive. Furthermore, we could separate *SDHA*-related tumors from non-*SDHA*-related tumors, as only the *SDHA*-mutated tumors stain negative for SDHA (Chapter 9). Taking into account the tumor location, co-occurrence of other tumors than pheochromocytomas and paragangliomas, and immunohistochemistry a genetic screening algorithm could be proposed (Figure 3).

The fact that *SDHA*, *SDHAF2*, *SDHB*, *SDHC* and *SDHD*-mutated pheochromocytomas and paragangliomas are negative for SDHB immunohistochemistry is quite surprising. The negative staining of the tumor cells suggests that mitochondrial complex II, or at least SDHB, is degraded when an *SDH* mutation occurs. However, some *SDH*-related tumors showed a moderate cytoplasmic SDHB staining, such as an *SDHD* mutated tumor with an D92Y mutation (Chapter 8, figure 1c), making it more difficult to conclude whether such a tumor was truly negative. However, since the staining was not as strong and granular as the healthy cells of the surrounding stromal network, it was considered as background staining and the tumor was scored negative. In addition, the Western blotting results of a tumor with an identical *SDHD* mutation (D92Y) showed almost no expression of SDHB (Chapter 8, figure 2a). The weak SDHB expression that was still seen in the western blotting result was most likely derived from the non-tumoral cells of the stromal network.

In contrast to SDHB immunohistochemistry, SDHA staining is positive in the *SDH*- and non-*SDH*-mutated tumors with the exception of *SDHA*-mutated tumors.

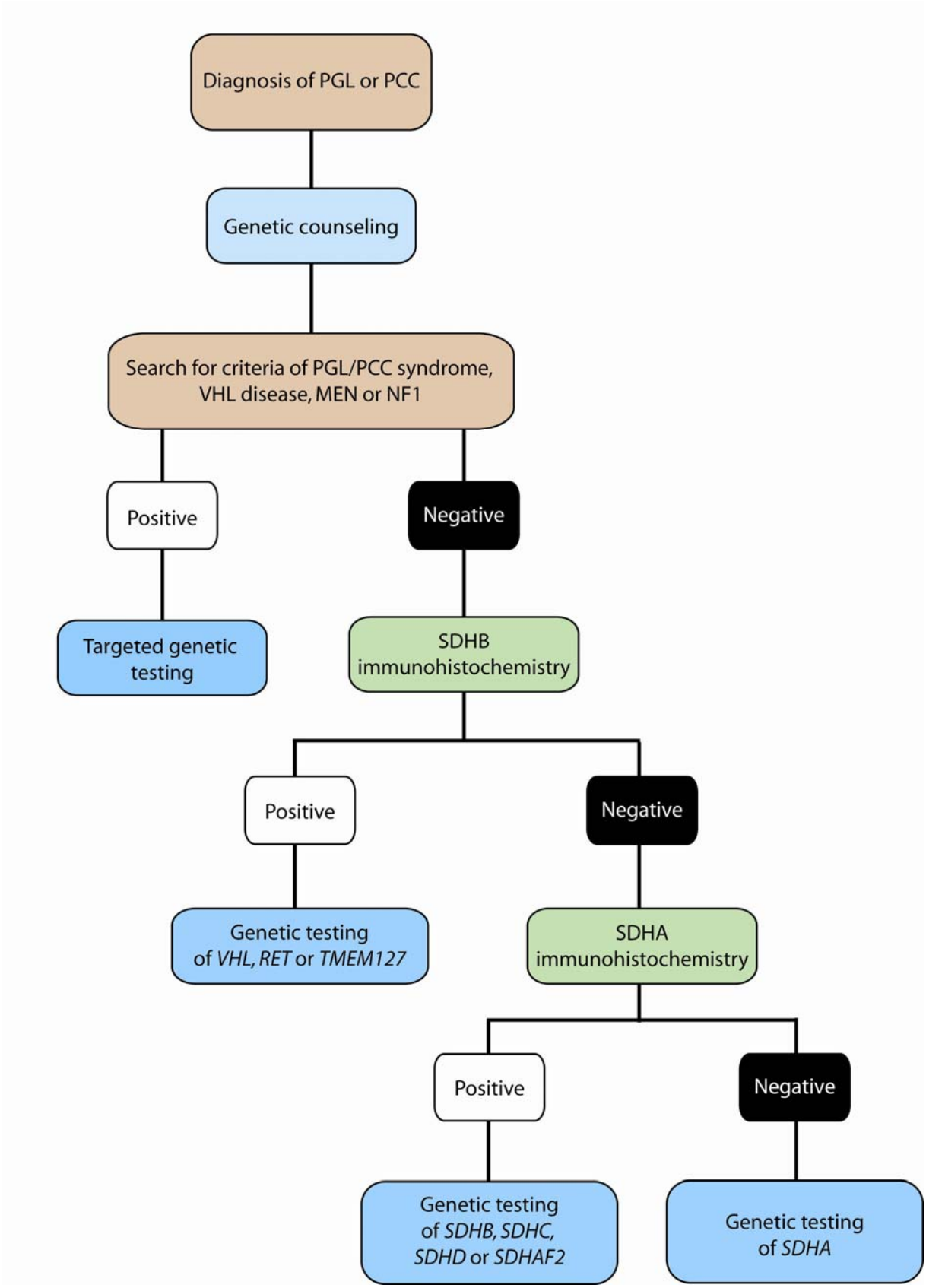

Figure 3. Suggested algorithm for molecular genetic testing of pheochromocytomas and paragangliomas based on SDHB and SDHA immunohistochemistry results. PCC = pheochromocytoma, PGL = paraganglioma.

Thus, it appears that the SDHA subunit of mitochondrial complex II is not degraded in all other tumors. However, there is a difference in staining pattern between the non-*SDH*-related compared to the *SDH*-related tumors. The positive SDHA staining of non-*SDH* related tumors is cytoplasic,strong and speckled (granular), whereas the *SDHAF2*, *SDHB*, *SDHC*, and *SDHD*-mutated tumors show a strong homogeneous diffuse cytoplasmic staining without a clear granular pattern (Figure 4b). To examine whether SDHA is still localized in the mitochondria, we performed immunofluorescence analysis with antibodies against SDHA, SDHB, and Cox VI (Figure 4). Preliminary data shows that SDHA staining is not exclusively found in the mitochondria in the tumor cells of *SDHB*, *SDHC* and *SDHD*-mutated tumors, but is present throughout the entire cytoplasm. We have shown in Chapter 8 that complex II is not enzymatically active in *SDH*-mutated tumors, but these SDHA immunohistochemistry results demonstrate that SDHA is still present in the tumor cells.

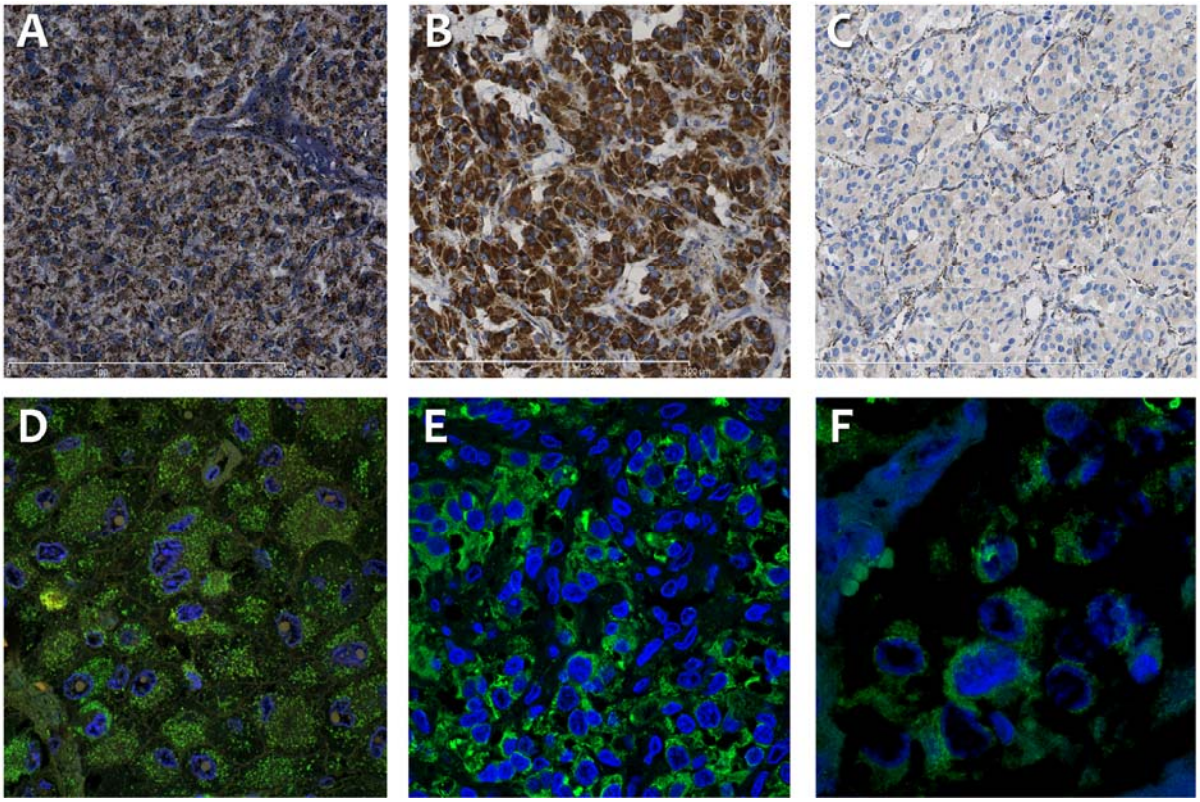

Figure 4. SDHA immunohistochemical staining of a sporadic tumor (A), an *SDHD*-related tumor (B), and an *SDHA*-related tumor (C). Note the granular staining of the sporadic tumor, compared to the homogeneous staining of the *SDHD*-related tumor. The *SDHA*-mutated tumor cells are completely negative, in contrast to the positive granular staining of the healthy stromal cells surrounding the tumor cells. This is also illustrated by SDHA immunofluorescence staining of a sporadic tumor (D), an *SDHD*-mutated tumor (E), and a magnification of the *SDHD*-mutated tumor (F).

The Arg31X SDHA mutation: a Dutch founder?

Mutations in *SDHA* have just recently been associated with paraganglioma formation.[48] Subsequently, we too, have found *SDHA* mutations in paragangliomas and pheochromocytomas, as described in Chapter 9. All of the *SDHA* mutations found in the Dutch paragangliomas and pheochromocytomas were c.91C>T (p.Arg31X), whereas the French *SDHA* mutation was a c.1753C>T (p.Arg585Trp). A recent study reported that The Netherlands has a higher prevalence of *SDHAF2* and *SDHD* mutations compared to other parts of Europe. Probably due to geopolitical reasons the Dutch *SDHD*-mutated gene did not spread far, as was also the case for the *VHL* mutations in the Black forest in Germany.[54, 55] *SDHA* mutations have been discovered just recently, so the prevalence is not known.[48, 56] To investigate whether the seemingly unrelated patients of our study (chapter 9) were related we tried to genotype these patients, using microsatellite markers near the *SDHA* gene. The patients did not share marker alleles, which could be due to the marker, which was located outside the haploblock in which the *SHDA* gene is located. To determine if these patients are distantly related, common polymorphisms within the gene should be searched and checked. Difficulties related to the presence of 3 *SDHA* pseudogenes have forced us to use cloning techniques, which allow us to selectively look into the functional (and mutated) *SDHA* gene. Using the p.Arg31X mutation as a marker for the functional *SDHA* gene, it is certain that all polymorphisms that have been found are present in the mutated allele. If these polymorphisms are present in all patients with the SDHA p.Arg31X mutation, we will be able draw conclusions about whether this is probably a Dutch founder mutation.

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Samenvatting

Pheochromocytomen zijn zeldzame neuronendocriene tumoren die ontstaan vanuit het bijniermerg en meestal catecholamines produceren. Paragangliomen zijn tumoren die verwant zijn aan pheochromocytomen. Paragangliomen kunnen in twee typen worden verdeeld; 1) parasympathische paragangliomen komen in het hoofd-hals gebied voor en producerende slechts zelden catecholamines, 2) sympathische paragangliomen komen voor langs de grensstreng van de aorta tot de blaas en produceren in het algemeen catecholamines. Pheochromocytomen en paragangliomen komen, in combinatie met andere tumoren, in verschillende tumorsyndromen voor, zoals het multiple endocrine neoplasia syndroom type 2 (veroorzaakt door *RET* mutaties), neurofibromatose type 1 (veroorzaakt door *NF1* mutaties), en de ziekte van von Hippel-Lindau (veroorzaakt door *VHL* mutaties). Daarnaast bestaat er ook het pheochromocytomen-paraganglioom syndroom, wat gekenmerkt wordt door het voorkomen van uitsluitend pheochromocytomen en paragangliomen. Dit syndroom wordt veroorzaakt door kiembaan mutaties in genen die geassocieerd zijn met mitochondriaal complex II (*SDHAF2*, *SDHB*, *SDHC*, *SDHD*). Mutaties in andere genen zijn beschreven, zoals in *IDH1*, *SDHA*, *TMEM127*, KIF1Bβ, maar zijn zeer zeldzaam.

Ongeveer 10% van de sporadische pheochromocytomen en paragangliomen is maligne, waarbij metastasen voornamelijk in de longen, lever en botten voorkomen. Er zijn nog steeds geen markers gevonden, die onderscheid kunnen maken tussen benigne en maligne tumoren. Ook is de pathogenese van deze tumoren nog niet duidelijk. In **hoofdstuk 2** hebben wij laten zien dat er intra-tumorale heterogeniteit bestaat in zowel benigne als maligne tumoren, maar dat de maligne tumoren een veel hoger percentage moleculaire heterogeniteit vertonen dan de benigne tumoren. Dit suggereert dat beide groepen een andere pathogenese hebben en dat de maligne tumoren zelfs al vanaf het begin van hun ontstaan maligne van aard zijn.

In **hoofdstuk 3** hebben wij laten zien dat benigne sporadisch pheochromocytomen moleculaire afwijkingen vertonen die ook in de syndromale tumoren zijn gevonden. De tumoren zijn op geleide van hun chromosomale afwijkingen in 2 groepen te verdelen; tumoren met verlies van chromosoom 1p en/of 3q (MEN2 profiel), en tumoren met chromosoom 3p met of zonder verlies van chromosoom 11p verlies (VHL profiel). In **hoofdstuk 4** hebben we aangetoond dat maligne pheochromocytomen veel meer chromosomale afwijkingen laten zien dan benigne. In het bijzonder werden er veel vaker geamplificeerde chromosomale regio's gevonden.

Er zijn meerdere muismodellen beschikbaar voor de bestudering van pheochromocytomen, maar een muismodel dat metastaserende pheochromocytomen oplevert is zeer zeldzaam. In **hoofdstuk 5** beschrijven wij een *Pten* conditioneel knock-out muismodel waarbij één derde van de muizen van 10 maanden en ouder zich presenteert met longmetastasen. We hebben in deze studie door middel van bijniermerg-specifieke immuunhistochemische markers aangetoond dat de long tumortjes afkomstig zijn van pheochromocytomen. Het *Pten* gen is in deze muizen geïnactiveerd door middel van het Cre-lox systeem, waarbij Cre door middel van de PSA (prostaat specifiek antigeen) promotor tot expressie wordt gebracht. Blijkbaar komen androgenen in het bijniermerg voor die zorgen voor de recombinatie van het *Pten* gen wat wij hebben kunnen bevestigen. Ook hebben we laten zien dat de chromosomale afwijkingen in de pheochromocytomen van deze muizen overeen komen met een deel van de genetische afwijkingen die gevonden worden in pheochromocytomen van de mens.

Het *TP53* tumorsuppressorgen is één van de meest frequent betrokken genen bij kanker bij de mens. *TP53* mutaties worden vooral vaak gezien in tumoren die invasief gedrag vertonen of al gemetastaseerd zijn. P53 inactivatie in combinatie met andere genen in verschillende muismodellen heeft laten zien dat de tumoren die ontstaan een agressief karakter hebben. De tumoren ontstaan eerder, groeien sneller en vertonen vaker invasief gedrag. In **hoofdstuk 6** hebben wij de pheochromocytomen van zes verschillende muizengenotypen bestudeerd, die inactivatie van één of beide allelen van *Trp53* en *Pten* hadden. Ook deze keer was het inactivatie systeem gebaseerd op het Cre-lox systeem, waarbij Cre-expressie weer onder controle van de PSA promotor stond. De longen werden weer systematisch onderzocht op de aanwezigheid van metastasen die afkomstig waren van pheochromocytomen met behulp van immuunhistochemische markers. Ook werd het gewicht van bijnieren bepaald voor al deze groepen, waarbij gesproken werd van een pheochromocytoom wanneer het gewicht van de bijnier meer dan 2x het gewicht van een normale bijnier was. Uit de resultaten bleek dat de tumoren bij inactivatie van *Trp53* inderdaad veel eerder ontstonden, dan wanneer alleen *Pten* was geïnactiveerd. Daarnaast bleek dat in bijna tweederde van de muizen maligne pheochromocytomen werden gevonden, die metastaseerden naar de longen. De chromosomale afwijkingen die gevonden werden in de primaire tumoren kwamen grotendeels overeen met de eerder gevonden afwijkingen in de tumoren van de *Pten* knock-out muizen. Omdat deze muizen in een nog hogere frequentie metastasen lieten zien lijkt dit model zeer geschikt voor de bestudering van maligne pheochromocytomen.

Pheochromocytomen en sympathische paragangliomen produceren catecholamines die voor een sterk verhoogde bloeddruk kunnen zorgen, wat zelfs tot de dood kan leiden. Daarnaast kunnen deze tumoren zich goedaardig presenteren, maar zelfs tientallen jaren na de verwijdering van de primaire tumor kunnen metastasen ontstaan. Omdat kiembaan mutaties in bepaalde genen een hogere kans geven op maligne gedrag van de tumor, is het belangrijk de patiënt genetisch te testen. Daarnaast zijn er syndromen die veroorzaakt worden door kiembaan mutaties in bekende genen, die naast pheochromocytomen ook andere maligne tumoren kunnen vertonen. Het is daarom voor alle patiënten met een pheochromocytoom of paraganglioom, en voor hun familieleden, belangrijk om zich genetisch te laten testen. Er is een aantal genen, dat op mutaties onderzocht kan worden, en selectie van deze genen kan op basis van klinische kenmerken worden gedaan. Zo laten patiënten met kiembaan *RET* en *VHL* mutaties in hoge aantallen bilaterale pheochromocytomen zien. Dit in tegenstelling tot mensen met kiembaan *SDHB* of *SDHD* mutaties, die vaker sympathische paragangliomen ontwikkelen. In **hoofdstuk 7** hebben we een serie bilaterale pheochromocytomen en een serie extra-adrenale sympathische paragangliomen onderzocht op het voorkomen van mutaties in *SDHB*, *SDHD*, *VHL* en *RET*. De groep van bilaterale pheochromocytomen liet vooral kiembaan mutaties in *RET* zien, en in mindere mate in *VHL*, terwijl de groep sympatische paragangliomen alleen *SDHB* en *SDHD* mutaties liet zien. Daarbij werd een nooit eerder beschreven somatische *SDHB* mutatie gevonden. De resultaten bevestigden dat bij bilaterale pheochromocytomen eerder aan *RET* en *VHL* mutaties gedacht moet worden, terwijl bij een sympathisch paraganglioom beter mutatie analyse van *SDHB* en *SDHD* gedaan zou kunnen worden.

Ondanks dat deze klinische kenmerken gerichter genetisch testen iets makkelijker maakt zijn er nog teveel tumoren die een sporadisch voorkomen hebben, maar toch een kiembaan mutatie in één van de bekende genen blijkt te hebben. Daarom zou een

methode die deze selectie zou vergemakkelijken wenselijk zijn. Daarnaast is het ook erg belangrijk patiënten te identificeren die een grotere kans hebben op het ontstaan van een maligne tumor, zoals het geval is bij mensen met een *SDHB* kiembaan mutatie. In **hoofdstuk 8** beschrijven we een eenvoudige en relatief goedkope methode om een eerste selectie van mogelijke kandidaat-genen te kunnen maken die getest kunnen worden. Hierbij konden we met behulp van SDHB immuunhistochemie onderscheid maken tussen tumoren met een *SDHAF2*, *SDHB*, *SDHC* en *SDHD* mutatie, en tumoren die niet SDH-gerelateerd zijn.

Tot voorkort werden *SDHA* mutaties alleen met een niet-tumor syndroom geassocieerd, het Leigh syndroom. Pas zeer recentelijk werd de eerste *SDHA* mutatie aangetoond in een sympatisch paraganglioom van een jonge vrouw zonder familie geschiedenis van de ziekte. Met behulp van SDHA immuunhistochemie werd een negatieve aankleuring in de tumorcellen van het paraganglioom aangetoond, terwijl alle niet-*SDHA* geassocieerde tumoren wel positief aankleurde voor SDHA. In **hoofdstuk 9** hebben we laten zien dat alleen *SDHA* gemuteerde tumoren negatieve kleuring gaven bij SDHA immuunhistochemie, terwijl tumoren met een andere genetische achtergrond een positieve aankleuring gaven, inclusief de *SDHB*, *SDHC*, *SDHD* en *SDHAF2*-gemuteerde tumoren. Deze resultaten hebben ertoe geleid dat SDHB en SDHA immuunhistochemie steeds frequenter toegepast wordt in de diagnostiek ter voorselectie voor de te testen genen.

List of Publications

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