

GENETIC ASPECTS OF PHEOCHROMOCYTOMA

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We here review the literature on genetics related to pheochromocytoma. About 10 percent of these neuroendocrine tumors are hereditary and are most often associated with multiple endocrine neoplasia type 2 (MEN 2), von Hippel-Lindau disease, and neurofibromatosis type 1 (NF 1). Hereditary tumor syndromes such as the aforementioned ones, are ideal to study the molecular pathogenesis of tumorigenesis as opposed to sporadic tumors in which genetic alterations often merely represent epigenetic tumor progression phenomena. Recent advances in molecular genetics, especially of *RET*, *VHL*, *NF1*, and *SDHD*, helped better understand the pathogenesis of pheochromocytoma. In this paper, we not only summarize key points of genetic discoveries related to pheochromocytoma, but also report in table format all known *RET* germline mutations related to pheochromocytoma.

Key words: Pheochromocytoma – Genetics – RET – VHL – NF1 – LOH

Pheochromocytoma is a neuroendocrine tumor characterized by chromaffin tissue and composed of catecholamine-containing neurosecretory granules. Pheochromocytomas are mostly located in the adrenal medulla, but also in ganglia of the sympathetic nervous system. Pheochromocytoma can cause endocrine hypertension by oversecretion of catecholamines. Such hypertension can be sustained or paroxysmal and may lead to death from cardiovascular or cerebrovascular disease. Our understanding of the pathogenesis of pheochromocytoma has tremendously grown during the last years along with the increasing advances of molecular genetics. Multiple genetic alterations have been found to be associated with pheochromocytomas and these tumors most often occur in a non-inherited, sporadic form. Some pheochromocytomas, however, are inherited and can be subdivided into a variety of groups: multiple endocrine neoplasia type 2 (MEN 2), von Hippel-Lindau (*VHL*) disease,

neurofibromatosis type 1 (*NF 1*), hereditary paraganglioma and *SDHD* gene-related tumors, and hereditary pheochromocytomas of yet unidentified genes (GIMM et al. 2000; BENDER et al. 2000; HANSFORD et al. 2000; JIANG et al. 2000; BAYSAL et al. 2000; NIEMANN et al. 2000; WALTHER et al. 1999a, b; KORF et al. 2000; PACAK et al. 2001; ENG et al. 1999; NILSSON et al. 1999).

In general, genetically predisposed patients are younger at diagnosis of pheochromocytoma compared to patients with sporadic pheochromocytomas (KNUDSON et al. 1972).

Genes that have been implicated in initiation and progression of pheochromocytomas include oncogenes, tumor suppressor genes, and a mitochondrial complex II gene, *SDHD*. Whereas tumor suppressor genes such as *VHL*, are believed to initiate tumorigenesis according to Knudson's two-hit model (KNUDSON et al. 1986), oncogenes such as *RET* may lead to tumor formation by other mechanisms.

Multiple endocrine neoplasia type 2 (MEN 2)

MEN 2 is an autosomal dominant cancer syndrome and divided into three subgroups: 1. MEN 2A, characterized by medullary thyroid carcinoma, pheochromocytoma, and parathyroid hyperplasia/adenoma, 2. MEN 2B, defined by medullary thyroid carcinoma, pheochromocytoma, marfanoid habitus, and multiple mucosal neuromas, and 3. familial medullary thyroid carcinoma. MEN 2 is rare with less than 1000 kindreds worldwide (ENG et al. 1996). Pheochromocytoma in MEN 2 occurs only in MEN 2A and MEN 2B, each with a rate of 50 %. The mean age at diagnosis is about 37 years and in most cases, pheochromocytoma occurs bilaterally and multifo-

Table 1
Germline mutations of *RET* associated with pheochromocytoma

Exon	Codon	Base pair change	Amino acid change
10	609	TGC to TAC	Cys to Tyr
		TGC to CGC	Cys to Arg
	611	TGC to TAC	Cys to Tyr
		TGC to TGG	Cys to Trp
	618	TGC to GGC	Cys to Gly
		TGC to TGA	Cys to Stop
		TGC to TAC	Cys to Tyr
		TGC to TTC	Cys to Phe
		TGC to AGC	Cys to Ser
		TGC to TCC	Cys to Ser
		TGC to CGC	Cys to Arg
		TGC to TTC	Cys to Phe
	620	TGC to GGC	Cys to Gly
		TGC to CGC	Cys to Arg
		TGC to TCC	Cys to Ser
		TGC to TAC	Cys to Tyr
11	631	GAC to TAC	Cys to Tyr
	634	TGC to TTC	Cys to Phe
		TGC to GGC	Cys to Gly
		TGC to CGC	Cys to Arg
		TGC to TCC	Cys to Ser
		TGC to TAC	Cys to Tyr
		TGC to AGC	Cys to Ser
		TGC to TGG	Cys to Trp
13	790	TTG to TTT	Leu to Phe
		TTG to TTC	Leu to Phe
14	804	GTG to ATG	Val to Met
	804	GTG to GCG	Val to Ala
	806	TAC to TGC	Tyr to Cys
15	883	GCT to TTT	Ala to Phe
16	918	ATG to ACG	Met to Thr

cally (HOWE et al. 1993). These clinical and pathological observations are related to the genetic origin of MEN 2-associated pheochromocytoma. All patients with MEN 2 have germline mutations in the *RET* proto-oncogene, located on chromosome 10q11.2. This gene comprises 21 exons with 6 so-called “hot spot” exons, i.e. exons that harbor mutations in more than 97% of patients with MEN 2. *RET* encodes a receptor tyrosine kinase, whose ligands are glial cell line-derived neurotrophic factor (GDNF) and neurturin (PONDER et al. 1999). GDNF is a member of the transforming growth factor (TGF)- β family. *RET* activation by GDNF appears to occur via a membrane-bound protein, GFR α , which seems to function as the ligand-binding domain of the ligand-receptor complex (JHANG et al. 2000; JING et al. 1996; TRUPP et al. 1996). The normal function of *RET* is largely unknown. It is expressed in neural crest-derived tissues such as the chromaffin and parafollicular C-cells, and it may play a role in kidney and gastrointestinal neuronal development (SCHUCHHART et al. 1994).

More than 85% of patients with MEN 2A have mutations in codon 634, exon 10 of *RET*. Analyzing exons 10, 11, and 16 of *RET* identifies over 95% of known mutations causing MEN 2. Pheochromocytoma in MEN 2 has been reported with certain *RET* mutations (see Table 1). The result of many of these missense mutations is (constitutive) *RET* dimerization at steady state, and hence activation of downstream signal transduction (SANTORO et al. 1995; ASAI et al. 1995). The risk for development of pheochromocytoma is higher in codon 634 mutations than in other exon 10 and 11 mutations (MULLIGAN et al. 1994; SCHUFFENECKER et al. 1994). Although patients with *RET* germline mutations develop hyperplasia of the parafollicular C-cells and adrenal medulla, the exact mechanism(s) of tumor initiation remain unknown. However, we investigated 9 pheochromocytomas from patients with MEN 2A and found that duplication of the mutant *RET* allele or loss of the wild-type allele can cause tumorigenesis (HUANG et al. 2000, Figure 1). Of interest in this context is that recent studies on *RET* double mutations and sequence alterations in the germline of patients with MEN 2 did not report a more aggressive phenotype. In these reports, however, the *RET* double mutation or sequence alter-

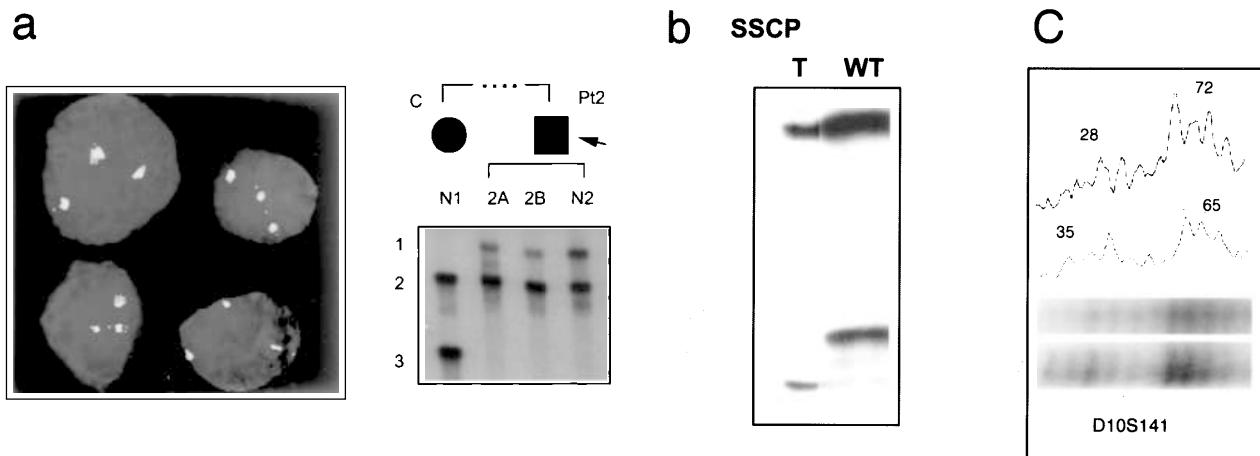


Figure 1

- a.** Duplication of mutant *RET* in trisomy 10 in MEN 2-related pheochromocytoma. On the left side, pheochromocytoma tumor has been analyzed by FISH. Three yellow-green signals in the red tumor cells are shown, indicating trisomy 10. On the right side, linkage analysis shows that allele number 2 is the inherited mutant *RET* allele. This mutant *RET* allele number 2 demonstrates strong intensity in lanes 2A and 2B (both pheochromocytoma) compared to lanes N1 (blood DNA from the patient's cousin "C") and lane N2 (blood DNA from the patient "Pt2"), indicating allelic imbalance between mutant and wild-type *RET* which turned out to occur in a ratio of 2:1 by phosphorimage densitometry (modified from Huang/Koch: Cancer Res 2000)
- b.** Single-strand conformation polymorphism analysis of the same MEN 2-related pheochromocytoma. T, tumor with *RET* mutation in exon 10; WT, wild-type normal tissue without *RET* mutation in exon 10. T shows a shift in the gel electrophoresis from the "normal, wild-type germline", indicating a mutation in this specific exon (10) of *RET*. Note that only the mutant allele is shown in the bands of T.
- c.** Loss of heterozygosity (LOH) analysis with polymorphic marker D10S141 and phosphorimage densitometry of the same tumor and genomic blood DNA. In the tumor (T), only one allele is shown, whereas N (genomic blood DNA) shows both alleles. Confirmatory phosphorimage densitometry analysis shows a ratio of the respective alleles, indicating LOH of this pheochromocytoma specimen

ation occurred on the same allele, thus leaving one intact *RET* wild-type allele which may exert protective neutralizing effects (KOCH et al. 2000; BARTSCH et al. 2000; TESSITORE et al. 1999). This supports our recent findings of pheochromocytoma tumorigenesis by a dominant effect of mutant *RET*.

Patients with MEN 2B usually have a more aggressive phenotype and more than 95% of these patients have a single methionine to threonine substitution at codon 918 in exon 16 of *RET*, the tyrosine kinase domain (MULLIGAN et al. 1995; ENG et al. 1994; CARLSON et al. 1994; GORDON et al. 1998). This may be related to the fact that mutations in exon 16 affect the tyrosine kinase catalytic site in the intracellular domain of the protein (ENG et al. 1994; CARLSON et al. 1994; HOFSTRA et al. 1996). Authorities recommend screening for pheochromocytoma

toma to begin at age 6, since pheochromocytoma has been described in 10- to 12-year old children (GAGEL et al. 1988; JADOUL et al. 1989). Genetic testing for *RET* mutations is readily available (see [HTTP://ENDOCRINE.MDACC.TMC.EDU](http://ENDOCRINE.MDACC.TMC.EDU); Hoppner W, Institute of Hormone and Fertility Research, Hamburg, Germany; Mayo Clinic, Rochester, MN).

Malignant pheochromocytoma is characterized by metastatic deposits of primary tumor tissue outside the site of origin. The prevalence of malignant pheochromocytoma in familial syndromes varies depending on the familial syndrome and the follow-up period. In MEN 2- and *VHL*-related pheochromocytomas, about 25% of tumors are reported as malignant on up to 25 year follow-up (WALTHER et al. 1999a; CARNEY et al. 1976; NEUMANN et al. 1993; WILSON et al. 1978; PADBERG et al. 1992; KOCH et al., unpublished data).

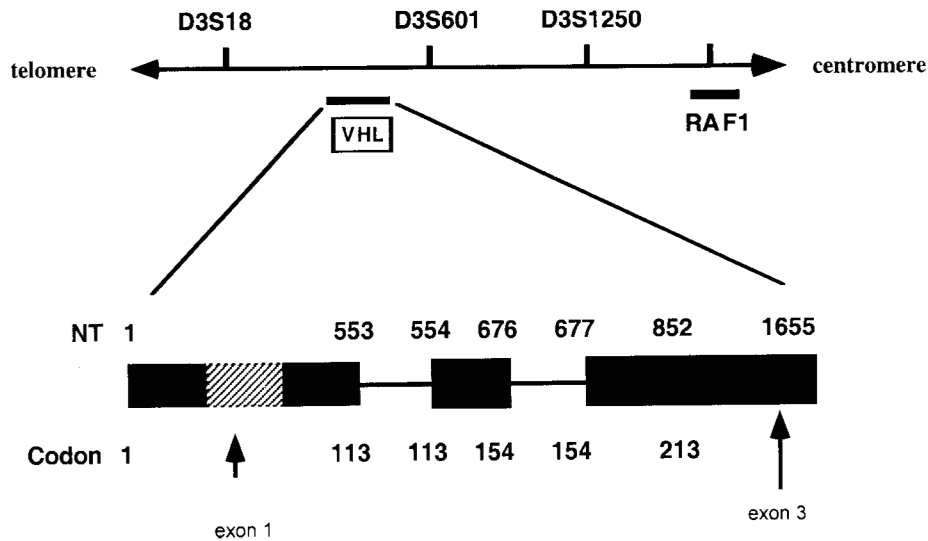


Figure 2
Genomic organization of the VHL gene showing the three exons (modified from Pacak 2000)

Pheochromocytoma in von Hippel Lindau disease type 2

VHL disease is an autosomal dominant inherited tumor syndrome with pheochromocytoma (*VHL* type 2) or without pheochromocytoma (*VHL* type 1). Based on the *VHL* classification system, the most common form of *VHL* disease, *VHL* type 1, is characterized by a predisposition to develop retinal angiomas, central nervous system hemangioblastomas and clear cell renal cell carcinomas. *VHL* type 2B is the second most common form of *VHL* disease and is characterized by development of *VHL* type 1 tumors and pheochromocytomas. *VHL* type 2A is a form of *VHL* disease characterized by a predisposition to develop pheochromocytomas without renal cell carcinoma, and infrequent hemangioblastomas and retinal angiomas. Germline mutations in the *VHL* gene responsible for each of these three *VHL* phenotypes have been identified and catalogued (see [HTTP://WWW.NCIFCRF.GOV/RESEARCH/KIDNEY](http://www.ncifcrf.gov/research/kidney); ZBAR et al. 1996). Pheochromocytoma occurs in 10 to 34% of *VHL* patients (WALTHER et al. 1999a; MAHER et al. 1990; RICHARD et al. 1994) and is the presenting manifestation in about 5% of cases. The mean age at diagnosis is 28 years and in about 50% of cases tumors are

bilateral (RICHARD et al. 1994; KOCH et al., unpublished).

In contrast to the *RET* proto-oncogene, the *VHL* gene is a tumor suppressor gene. Tumor formation in tumor suppressor gene-related neoplasms usually occurs by the “two-hit” model, that is inactivation of the second, wild-type allele by various mechanisms (KNUDSON et al. 1986). More than 300 *VHL* germline mutations have been identified and 36 of them are associated with pheochromocytoma (NEUMANN et al. 2001). The *VHL* tumor suppressor gene is located on chromosome 3p25-26. Its cloned coding sequence is represented in three exons (Figure 2). About 15% of patients with *VHL*-associated pheochromocytoma have large germline deletions detected by Southern blot analysis. Another 2% of patients have larger deletions detected by Southern pulsed field gel electrophoresis (LATIF et al. 1993; RICHARDS et al. 1993; Yao et al. 1993). Most patients with *VHL*-associated pheochromocytoma have missense mutations (CROSSEY et al. 1994; VAN DER HARST et al. 1998; RITTER et al. 1996). A mutation hotspot has been described at codon 167 in exon 3 (equivalent to nt 712/713) which accounts for about 9% of patients (WALTHER et al. 1999a; CROSSEY et al. 1994; Chen et al. 1995). Genotype-

specific *VHL* phenotypes have been reported (WALTHER et al. 1999a; ATUK et al. 1998). “Founder effects” may explain regional prevalence rates, e.g. the Black Forest area in Southern Germany with the missense mutation tyrosine to histidine at codon 98 (Tyr98His) and subsequent high risk of pheochromocytoma (NEUMANN et al. 1993; BRAUCH et al. 1995; GROSS et al. 1996). The *VHL* gene product forms a stable complex with the highly conserved transcription elongation factors elongin B and elongin C, factors that regulate RNA polymerase II elongation. Formation of this heterotrimeric complex with elongin B and C appears to be the tumor suppressor function of the *VHL* gene, since the majority of tumor-predisposing mutations of *VHL* disrupt the formation of this complex (DUAN et al. 1995; NEUMANN et al. 1995; ASO et al. 1995). Elongin A is required to inhibit the processing of RNA polymerase II, allowing cell processivity of transcription. The *VHL* gene product and elongin A compete for binding to elongin B and C via a short shared sequence motif. This sequence which is found in the third exon of *VHL* is highly mutated in *VHL* disease.

Pheochromocytoma in neurofibromatosis type 1

NF1 is the most common familial cancer syndrome predisposing to pheochromocytoma. It affects about one in 4000 individuals. The risk of pheochromocytoma in *NF1*, however, is small, about 2% (RICCARDI et al. 1991; HUSON et al. 1998). *NF1* is inherited as an autosomal dominant trait with variable expression. 50% of patients have new mutations. Pheochromocytoma in patients with *NF1* occurs at a later age than in MEN 2 and *VHL* disease. The mean age at diagnosis is in the fifth decade. Onset before age 20 years is uncommon (KNUDSON et al. 1972). About 22% of *NF1* patients with pheochromocytoma have multiple and/or bilateral tumors. Extraadrenal pheochromocytomas in patients with *NF1* are rare (about 6%) in contrast to patients with *VHL* disease (about 30%) and with MEN 2 (about 13%) (WALTHER et al. 1999b; HOWE 1993; CARNEY et al. 1976; NEUMANN et al. 1993; KALF et al. 1982; RICHARDS et al. 1994; SATO et al. 1988; ATUK et al. 1979; DELLELIS et al. 1976; WEBB et al. 1980; CARNEY et al. 1978; ROSENTHAL et al.

1936; LICHTENSTEIN et al. 1949; VISSER et al. 1975; SAMAAAN et al. 1988; TISHERMAN et al. 1993; CANCE et al. 1985; GOSSET et al. 1999; LIPS et al. 1981).

The *NF1* gene is a tumor suppressor gene mapping to chromosome 17q11.2. It was isolated in 1990 (VISKOCIL et al. 1990; WALLACE et al. 1990; CAWTHON et al. 1990). Because of the large gene size (11 kb of coding sequence extending over 300 kb of genomic DNA), mutation analysis has been difficult (in only about 15% of patients mutations are identified). Patients with *NF1* associated pheochromocytoma show loss of the wild type allele (XU et al. 1992; GUTMANN et al. 1994). Neurofibromin, the *NF1* gene product, bears homology to the *RAS*/GTPase activating protein (GAP) (BALLESTER et al. 1990). P21-*RAS*/GAP increases the rate of intrinsic GTP hydrolysis in the small G proteins, the *RAS* genes, thereby mediating the return of the G protein switch to the “off” GDP-bound form. It can therefore decrease (or control) signal transduction via the *RAS* pathways, thereby perhaps acting as a tumor suppressor. This notion is supported by the fact that inactivating mutations in the *NF1* gene are mainly found in the *RAS*/GAP homology region.

Malignant pheochromocytomas in patients with *NF1* are rarely reported (KALFF et al. 1982; OKADA et al. 1984).

Hereditary paragangliomas

Paragangliomas are tumors arising in extra-adrenal chromaffin tissue such as the organ of Zuckerkandl. The most common tumor site is the carotid body, a chemoreceptive organ that senses oxygen levels in the blood. The genetic basis of this disorder remains largely unknown, although recent analyses of families with paraganglioma revealed two possible chromosomal loci for this tumor, one on chromosome 11q13 and the other on chromosome 11q23. In some of these tumors, germline mutations in *SDHD*, a mitochondrial complex II gene, have been identified (BAYSAL et al. 2000). *SDHD* is located on chromosome 11q23 and encodes the small subunit of cytochrome b in the succinate-ubiquinone oxidoreductase complex (mitochondrial complex II). This enzyme complex is important for the aerobic respiratory chain of eukaryotic cell mitochondria. The *SDHD* gene comprises three introns and four exons.

Non-familial, sporadic pheochromocytoma

The underlying genetic basis for tumorigenesis of sporadic pheochromocytomas remains to be elucidated as does the search for molecular markers that can distinguish between benign and malignant pheochromocytomas.

Oftentimes, investigators search apparently sporadic tumors for somatic mutations of genes that have been identified as causes for familial cancer syndromes such as *RET*, *VHL*, and *NF1*. This approach led to a new defined non-familial pheochromocytoma type related to germline mutations of *SDHD* (GIMM et al. 2000). However, the percentage of somatic mutations of genes that are known to be responsible for familial cancer syndromes, is low in non-familial forms of the relevant tumor. In sporadic pheochromocytomas, somatic mutations of the *VHL* gene are uncommon with a prevalence of about 8% (BENDER et al. 2000; HOFSTRA et al. 1996; BAR et al. 1997; ENG et al. 1995; BRAUCH et al. 1997; CROSSEY et al. 1995). Somatic mutations of *RET* in sporadic pheochromocytomas are also uncommon with about 10 to 20% (ENG et al. 1995; LINDOR et al. 1995; BELDJORD et al. 1995; BENDER et al. 2000; JANUSZEWICZ et al. 2000; KOMMINOTH et al. 1996; CHEW et al. 1995; QUADRO et al. 1994), with the codon 918 somatic *RET* mutation (MEN 2B mutation) as the one most commonly found, i.e. in up to 10% of cases (ENG et al. 1995; LINDOR et al. 1995; CHEW et al. 1995; QUADRO et al. 1994; THIBODEAU et al. 1994). Somatic mutations in *NF1* have also been described in sporadic pheochromocytomas including a finding of reduced or absent *NF1* gene expression in seven of 20 non-*NF1* pheochromocytomas (GUTMANN et al. 1994) suggesting that *NF1* inactivation can be involved in the pathogenesis of non-familial pheochromocytomas.

Somatic and occult germline mutations in *SDHD* have been detected in 4 of 18 apparently sporadic pheochromocytoma and paraganglioma tumors (GIMM et al. 2000).

Allele losses on chromosome 1p, 3p and 17p are common findings in familial and non-familial pheochromocytomas (BENDER et al. 2000; KHOSLA et al. 1991; VARGAS et al. 1997; TSUTSUMI et al. 1989; MOLEY et al. 1992; BENN et al. 2000; DANENBERG et al. 2000; MATHEW et al. 1987; MULLI-

GAN et al. 1993). However, most of these allele losses are not clearly involved in tumorigenesis of pheochromocytomas but rather in tumor progression, therefore representing more likely “epigenetic” phenomena. One can implicate an accumulation of mutations in several genes in both familial and sporadic pheochromocytoma. However, the specific role/function of some of these genes remains to be elucidated i.e. details of how mutations in different biochemical pathways might interact with each other to produce tumorigenesis. Future analyses of pheochromocytomas with microarray techniques and proteomics may help answer these questions.

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