TUMOR MARKERS IN PHEOCHROMOCYTOMAS

Kaisa Salmenkivi

Department of Pathology
Haartman Institute
University of Helsinki
Finland

Academic dissertation

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Supervised by

Docent Johanna Arola, M.D., Ph.D.
Department of Pathology,
Haartman Institute,
University of Helsinki

and

Docent Päivi Heikkilä, M.D., Ph.D.
Department of Pathology,
Haartman Institute,
University of Helsinki

Reviewed by

Professor Ylermi Soini, M.D., Ph.D.
Department of Pathology
University of Oulu

and

Docent Timo Sane, M.D., Ph.D.
Department of Endocrinology
Helsinki University Central Hospital

Opponent

Professor Veli-Matti Kosma, M.D., Ph.D.
Department of Pathology
University of Tampere

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To my family

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to in the text by their Roman numerals. Some unpublished data are also presented.

I Salmenkivi K, Heikkilä P, Haglund C, Louhimo J, Arola J: Lack of histologically suspicious features, proliferative activity, and p53 expression suggests benign diagnosis in pheochromocytomas. *Histopathology*, in press, 2003

II Salmenkivi K, Arola J, Voutilainen R, Ilvesmäki V, Haglund C, Kahri AI, Heikkilä P, Liu J: Inhibin/activin βB-subunit expression in pheochromocytomas favors benign diagnosis. *J Clin Endocrinol Metab* 86: 2231-2235, 2001

III Salmenkivi K, Heikkilä P, Liu J, Haglund C, Arola J: VEGF in 105 pheochromocytomas: enhanced expression correlates with malignant outcome. *APMIS*, in press, 2003

IV Salmenkivi K, Haglund C, Arola J, Heikkilä P: Increased expression of tenascin in pheochromocytomas correlates with malignancy. *Am J Surg Pathol* 25: 1419-1423, 2001

V Salmenkivi K, Haglund C, Ristimäki A, Arola J, Heikkilä P: Increased expression of cyclooxygenase-2 in malignant pheochromocytomas. *J Clin Endocrinol Metab* 86: 5615-5619, 2001

ABBREVIATIONS

ALAAD	aromatic L-amino acid decarboxylase
ACTH	adrenocorticotropic hormone
bFGF	basic fibroblast growth factor
CAT	catecholamine
CDK	cyclin-dependent kinase
CDKI	cyclin-dependent kinase inhibitor
cDNA	complementary deoxyribonucleic acid
CEA	carcinoembryonic antigen
COX	cyclooxygenase
CT	computed tomography
DA	dopamine
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EGF	epidermal growth factor
EMA	epithelial membrane antigen
EPI	epinephrine
FMTC	familial medullary thyroid carcinoma
FSH	follicle-stimulating hormone
HPF	high power field
kb	kilobase
kDa	kilodalton
LOH	loss of heterozygosity
MEN	multipple endocrine neoplasia
MIBG	metaiodobenzylguanidine
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MVD	microvessel density
NCAM	neural cell adhesion molecule
NE	norepinephrine
NF	neurofibromatosis
NK	natural killer
NSAID	non-steroidal anti-inflammarory drug
NSE	neuron-specific enolase
PAS	periodic acid-Schiff
PASS	pheochromocytoma of the adrenal gland scaled score
PDGF	platelet-derived growth factor
Rb	retinoblastoma
SDHA	succinate dehydrogenase subunit A
SDHB	succinate dehydrogenase subunit B

SDHC	succinate dehydrogenase subunit C
SDHD	succinate dehydrogenase subunit D
TGF-α	transforming growth factor α
TGF-β	transforming growth factor β
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VHL	von Hippel-Lindau

ABSTRACT

Introduction:
Pheochromocytomas are uncommon tumors arising from the human sympathoadrenal system. It is extremely difficult to distinguish malignant pheochromocytomas from benign ones. Classical features of malignancy, such as hyperchromasia and pleomorphism are commonly observed and can not be used in the distinction. Traditionally only metastasized tumor is considered malignant. Good prognostic markers have not been available.

Materials and Methods:
In our search for new prognostic markers, we evaluated a large set of adrenal and extraadrenal pheochromocytomas. Tumors were considered malignant only if histologically or radiologically proven metastases were present, except one that invaded extensively to a spinal vertebral body. Tumors without metastases but having any of the histologically suspicious features, i.e. more than 5 mitoses/10 high-power fields, confluent tumor necrosis, or vascular or capsular invasion, were evaluated as a separate group, here called borderline tumors. We analyzed, in addition to histological features, the proliferative activity and the microvessel density, p53, p21, inhibin βA and -βB, VEGF, tenascin and COX-2 expression.

Results:
All malignant tumors showed at least one histologically suspicious feature, and indeed, most of them several. Proliferative activity was clearly higher in metastasized pheochromocytomas. p53 immonohistochemical overexpression was found in two malignant tumors, whereas all benign and borderline adrenal tumors were negative. p21 immunostaining was mostly negative. All malignant tumors were negative or only weakly positive for inhibin βB. VEGF, tenascin, and COX-2 were overexpressed in malignant tumors.

Conclusions:
These studies suggest that proliferative activity, inhibin/activin βB, VEGF, tenascin, and COX-2, together with the histological features, can benefit when evaluating the behavior of a pheochromocytoma.

INTRODUCTION

A catecholamine-secreting tumor arising from the chromaffin cells of the sympathoadrenal system was first termed pheochromocytoma by Poll in 1905. The term refers to the dusky (pheo) color (chromo) of the cut surface of the tumor when exposed to dichromate (Poll, 1905). The first successful resection of a pheochromocytoma was done by Dr. C. H. Mayo in 1927 (Mayo, 1927). Since those times, the understanding and management of pheochromocytomas has improved enormously. Pheochromocytomas most commonly arise from the adrenal medulla. Extraadrenally located pheochromocytomas are called paragangliomas, and arise from the paraganglion system. Although these tumors are rare, they are of great clinical importance because of the catecholamines they secrete. Hypertension is the clinical hallmark of a pheochromocytoma, and can be potentially fatal. The clinical diagnosis is established by the demonstration of elevated levels of catecholamines or their metabolites (Bravo, 1994). The tumor can be localized anatomically by using CT, MRI, MIBG, or PET scanning. Patients with a functional pheochromocytoma need a preoperative α- and β-blockade, but the treatment of choice is surgical resection, if the disease is limited (Kopf et al., 2001).

An adrenal pheochomocytoma is usually a rounded, gray-white, firm tumor 3 to 5 cm in diameter. When larger, they can adhere to adjacent structures, although still not metastasizing. Extraadrenally located tumors mimic adrenal tumors macroscopically and microscopically. A pheochromocytoma is relatively easy to diagnose histologically, and can be confirmed by neuroendocrine markers, such as chromogranin A (Lack, 1997). Most pheochromocytomas are benign. However, approximately 10% of these tumors metastasize. The distinction between a benign and malignant tumor based on histological features is very difficult, if not impossible. Classically only metastasized tumors are considered malignant for certain. Necrosis, vascular or capsular invasion, and high mitotic count have been associated with more malignant disease (Linnoila et al., 1990; van der Harst et al., 2000). Several attempts have been made to find markers that would predict the future behavior of an unmetastasized pheochromocytoma, but thus far no definite markers for malignancy have been found.

The present study was designed to find markers that would predict the malignant potential in pheochromocytomas. The five separate publications gathered together in this thesis examine markers associated with different steps in the tumorigenesis. The aim is to improve the histopathological diagnosis of a benign or a malignant pheochromocytoma.

REVIEW OF THE LITERATURE

1. Sympathoadrenal system

1.1 The adrenal gland

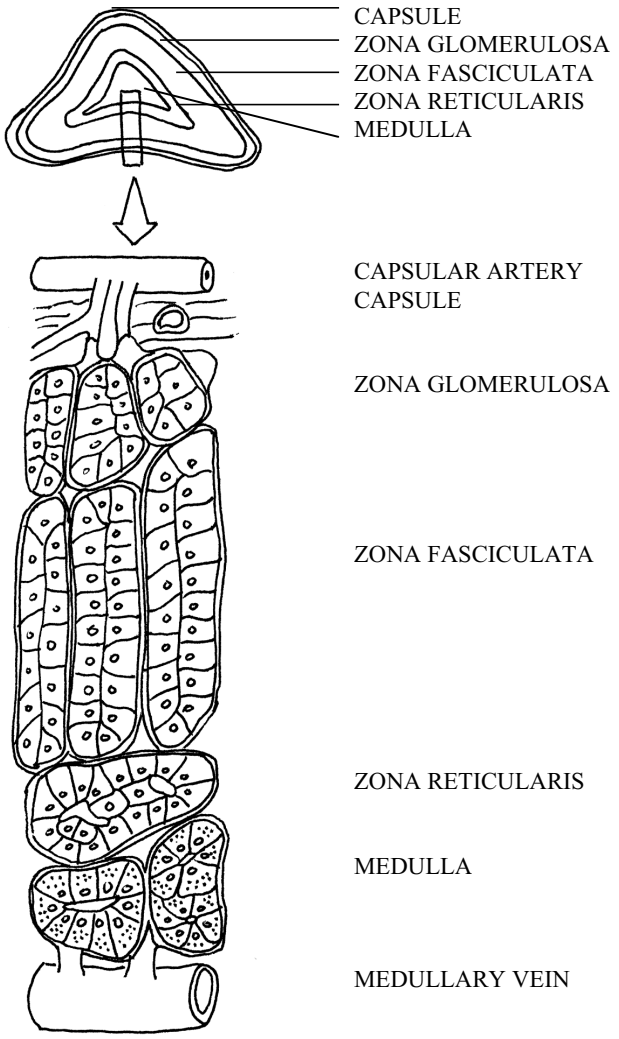

Figure 1. Schematic diagram of the adrenal gland (modified from Gartner et al, 2001).

The paired adrenal glands are a composite of two endocrine organs, cortex and medulla, located in the retroperitoneum, superomedial to the kidneys. The adrenal glands are surrounded by a connective tissue capsule containing large amounts of adipose tissue. The adrenal cortex is of mesodermal origin, while the medulla is neuroectodermal. Precursor cells from the neural crest migrate from primitive spinal ganglia to form the primitive sympathetic nervous system dorsal to the aorta. Some cells migrate further alongside large blood vessels that penetrate the encapsulated adrenal cortex to enter the adrenal primordium and pass among the fetal cortical cells. At birth, the medulla comprises a central, thin core of small neuroblasts and larger pheochromoblasts, arranged in irregularly sized clumps. At birth the combined average weight of the glands is about 20 g. Postnatally the provisional cortex collapses, so that the weight of each gland decreases to about 5 g within 2 weeks. The weight then remains constant for 2 years, and gradually increases to the adult total weight of about 13 g (Carney, 1997).

The adrenal cortex is subdivided histologically into three zones. The outermost layer is the zona glomerulosa, which synthesizes and secretes the mineralocorticoid hormones, principally aldosterone. The synthesis of these hormones is stimulated by angiotensin II. In the middle zona fasciculata produces glucocorticoid hormones, cortisol and corticosterone, stimulated by ACTH. The innermost layer is the zona reticularis, which synthesizes and secretes adrogens and small amounts of glucocorticoids. The secretion of both is stimulated by ACTH (Gartner and Hiatt, 2001).

The adult adrenal medulla comprises two populations of parenchymal cells: chromaffin cells producing catecholamines, epinephrine (EPI) and norepineprine (NE), and sympathetic ganglion cells, scattered throughout the connective tissue and often associated with a nerve. The chromaffin cells are arranged in clusters or short trabeculae, supported by fibrovascular stroma and sustentacular cells. Small groups of cortical cells can also be found within the medulla (Carney, 1997).

The chromaffin cells contain granules that stain intensely with chromaffin salts. These granules contain catecholamines, transmitters produced by postganglionic cells of the sympathetic nervous system. Catecholamines are secreted in response to stimulation by preganglionic sympathetic (cholinergic) splanchnic nerves. When the stimulus is derived from an emotional source, secretion of NE predominates; and when the stimulus is physiological, secretion of EPI is predominates. EPI has a role in controlling cardiac output, heart rate, and in increasing blood flow through organs, while NE has little effect on these. NE nevertheless elevates blood pressure by vasocontraction.

1.2 The paraganglion system

The paraganglion system includes the adrenal medulla, the organ of Zuckerkandl, carotid body, vagal body, and small groups of cells associated with cervical, thoracic, and abdominal sympathetic ganglia. The sympathetic paraganglia are associated with the peripheral sympathetic nervous system, predominantly as anatomically discrete bodies close

to the prevertebral and paravertebral sympathetic ganglia, and in connective tissue in or near the walls of the pelvic organs. Parasympathetic paraganglia are located along the cervical and thoracic branches of the vagus and glossopharyngeal nerves (Komminoth et al., 2002). Paraganglia are mostly composed of neuroendocrine cells (chief or type I cells) that are oval or round and contain neurosecretory granules that store catecholamines. The spindle-shaped sustentacular cells (satellite or type II cells) are located at the periphery of the neuroendocrine cells (Whalen et al., 1992). With the exception of the adrenal medulla, the organs of Zuckerkandl, and the carotid bodies, paraganglia are normally less than 1 mm in dimension (Komminoth et al., 2002).

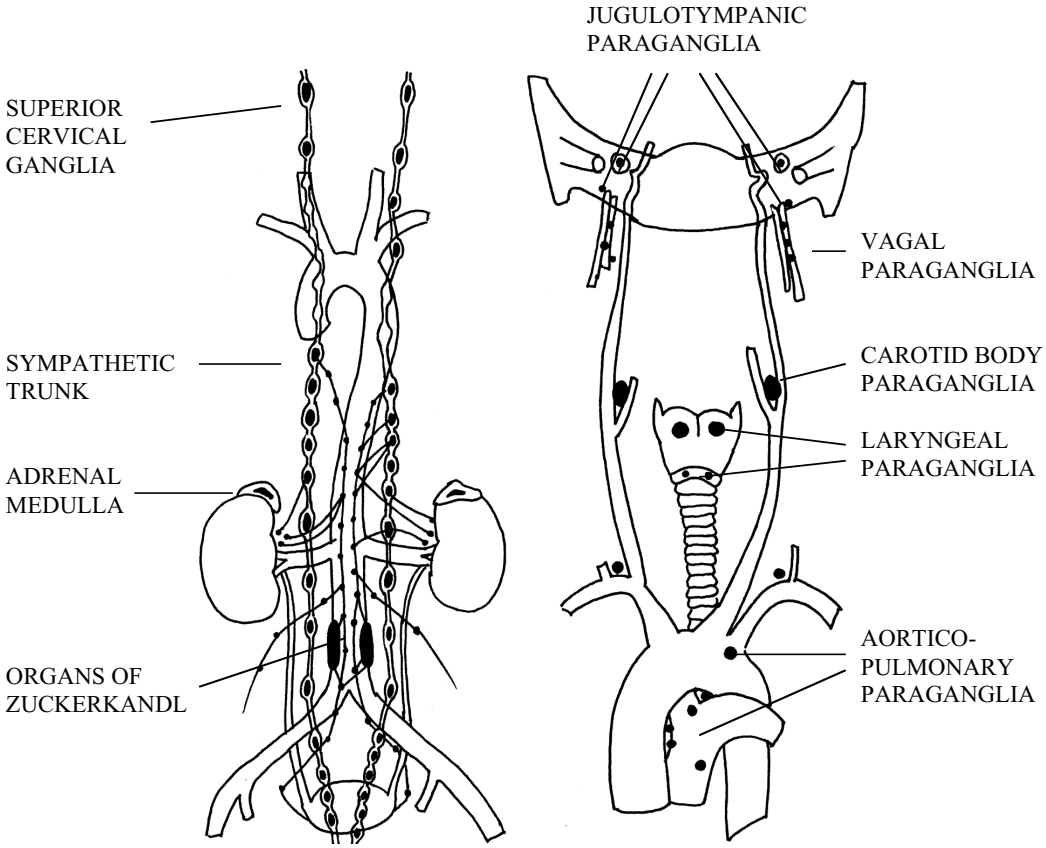

Figure 2. Anatomic distribution of sympathetic and parasympathetic paraganglia (modified from Lack, 1997).

2. Pheochromocytomas

Pheochromocytomas are rare tumors of chromaffin cells most commonly arising from the adrenal medulla. In Denmark, the average annual incidence is 1.9 cases per million population (Andersen et al., 1986), and in Sweden it is 2.1 per million (Stenstrom and Svardsudd, 1986). We have now evaluated the number of pheochromocytoma diagnoses made in 1996-2000 in five University Hospitals in Finland. Based on these figures, the annual incidence in Finland is about 10 to 15 new cases annually. Pheochromocytomas can occur at any age, but the peak age at diagnosis is in the fifth decade of life (Lack, 1997). Most studies report a slight predilection for females (Goldstein et al., 1999; Melicow, 1977; Proye et al., 1992).

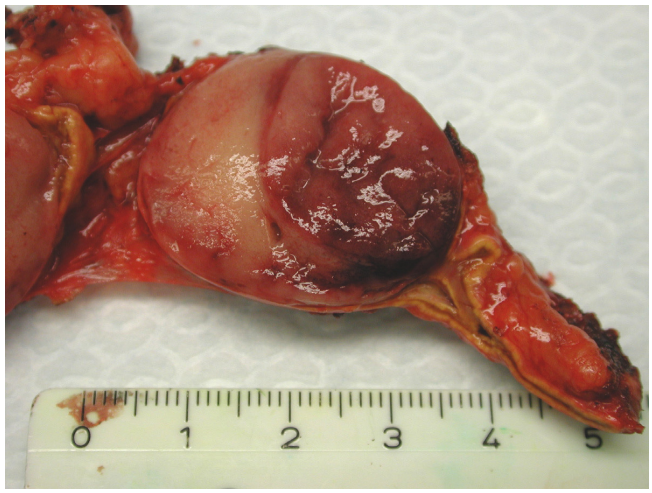

Figure 3. Adrenal pheochromocytoma.

Extraadrenally located pheochromocytomas are called paragangliomas, and arise from the paraganglion system. More than 90% of the extraadrenal sympathetic paragangliomas occur in the retroperitoneum, and 30-50% of these are in the vicinity of the organ of Zuckerkadl (Altergott et al., 1985; Melicow, 1977). Parasympathetic paragangliomas are most common in jugular and tympanic paraganglia, followed by carotid body, vagal, and aortic paraganglia (Komminoth et al., 2002). These tumors are often multicentric. Although most paragangliomas are sporadic, familial cases do occur.

2.1 Genetic basis and associated syndromes

Approximately 90% of pheochromocytomas occur in the noninherited, sporadic form. The genetic defect has been identified in several major groups of familial sydromes, such as von Hippel-Lindau (VHL) disease caused by germline mutation in *VHL* gene, neurofibromatosis type 1 (NF1) caused by germline mutations in *NF1* gene, multiple endocrine neoplasia type II (MEN II) syndrome caused by germline mutations in *RET* gene, or hereditary paraganglioma caused by germline mutations in the *SDHB*, *SDHC*, or *SDHD* genes (Astuti et al., 2001; Baysal et al., 2000; Niemann and Muller, 2000).

2.1.1 von Hippel-Lindau disease

When a patient presents with a pheochromocytoma as the primary manifestation and has a family history, the *VHL* gene is a possible cause (Pacak et al., 2001). VHL disease is a tumor predisposition syndrome characterized by the presence of retinal angiomas, hemangioblastomas of the cerebellum and spinal cord, renal cell carcinomas, angiomatous or cystic lesion of the kidney, pancreas, epididymis, and pheochromocytomas (Böhling et al., 2000; Couch et al., 2000; Singh et al., 2001). The inheritance of VHL is autosomal-dominant with high age-dependent penetrance (Singh et al., 2001). The *VHL* gene is a tumor suppressor gene located in chromosome 3p25-26 (Latif et al., 1993). The type of germline mutations of the *VHL* gene causes different types of clinical manifestations. Phenotypes are based on the absence (type 1) or presence (type 2) of pheochromocytoma (Koch et al., 2002). Pheochromocytomas are found in 10% (Horton et al., 1976) to 21% (Neumann et al., 1993) of patients with VHL. Most patients with VHL-associated pheochromocytomas have missense mutations (Van der Harst et al., 1998). Metastatic pheochromocytoma is rare in von Hippel-Lindau disease, as well as is all other familial syndromes (Neumann et al., 1993).

2.1.2 Neurofibromatosis type 1

Neurofibromatosis type 1 (NF1, von Recklinghausen's disease type I) is one of the most common autosomal-dominant diseases in humans, the prevalence being 1 in 2500 to 3300. The *NF1* is a tumor-supressor gene located in chromosome 17q11.2 (Stacey and Kazlauskas, 2002). Patients with neurofibromatosis develop plexiform neurofibromas, café au lait spots, as well as many other lesions, including pheochromocytomas (Walther et al., 1999). The incidence of pheochromocytomas in Swedish neurofibromatosis patients has been reported to be about 6% (Zoller et al., 1997). Less than 10% of NF1 patients with pheochromocytoma have bilateral tumors, and less than 12% of the pheochromocytomas in patients with NF1 are metastatic (Walther et al., 1999).

2.1.3 Multiple endocrine neoplasia type II

MEN II is an autosomal dominant syndrome, which has at least three distinct variants: FMTC (familial medullary thyroid carcinoma), MEN IIA, and MEN IIB (Brandi et al., 2001), all caused by germline mutation in the *RET* gene (Eng et al., 1996; Mulligan et al., 1993). *RET* proto-oncogene is specifically expressed in neural crest-derived cells, such as the calcitonin-producing C cells in the thyroid gland and the catecholamine-producing chromaffin cells in the adrenal gland (Takahashi et al., 1985). *RET* is located on chromosome 10q11.2 and encodes a receptor tyrosine kinase, RET protein (Takahashi, 2001). The prevalence of MEN II and VHL disease is similar, i.e. 1 in 35 000 individuals being affected (Koch et al., 2002). MEN IIA was first described in 1961 by Sipple, and is defined by the occurrence of thyroid medullary carcinoma, pheochromocytoma, and hyperparathyroidism (Hansford and Mulligan, 2000; Sipple, 1961). MEN IIB represent about 5% of all MEN II cases. It is defined by the presence of thyroid medullary carcinoma, pheochromocytoma, and associated abnormalities including mucosal neuromas, abnormal corneal nerve fibers, and marfanoid habitus (Carney et al., 1976). 30-50% of patients with MEN IIA or MEN IIB develop pheochromocytomas, which can be bilateral, as in up to 80% of the cases (Nguyen et al., 2001).

2.1.4 Hereditary paragangliomas

The genetic basis of paragangliomas is still largely unknown, although recent analyses have revealed several possible susceptibility chromosomal changes for these tumors: germline mutations in *SDHD* (the succinate-ubiquinone oxidoreductase subunit D gene) at 11q23 (Baysal et al., 2000), *SDHC* at 1q21 (Niemann and Muller, 2000), and *SDHB* at 1p36 (Astuti et al., 2001). *SDHD*, *SDHC*, *SDHB*, and *SDHA* each encode a subunit of the mitochrodrial respiratory chain complex II, which is important for the aerobic respiratory chain of eukaryotic cell mitochrondria (Baysal et al., 2001). The majority of hereditary paragangliomas with germline mutation in *SDHD* demonstrate LOH at 11q23, indicating a loss-of-function status, which is commonly seen in tumor-suppressor gene-related disorders (van Schothorst et al., 1998).

2.1.5 Sporadic pheochromocytomas

Table 1. The frequency of mutations in *RET*, *VHL*, and *SDHD/C/B* in sporadic pheochromocytomas (Koch et al., 2002).

Gene	Frequency
RET	<10%
VHL	<8%
SDHD	<7%
SDHC	0%
SDHB	4%

Allele losses on chromosomes 1p, 3p, 3q, 17p, and 22q are common in hereditary and non-hereditary pheochromocytomas, as indicated by comparative hybridization and microsatellite analyses (Bender et al., 2000; Khosla et al., 1991; Tsutsumi et al., 1989; Vargas et al., 1997). The frequency of mutation in *RET*, *VHL*, and *SDHD*/*C*/*B* in sporadic pheochromocytomas is shown in Table 1. Recently, Neumann et al. (2002) identified germline mutations in *RET*, *VHL*, *SDHB*, or *SDHD* in up to 24% of 271 patients with apparently sporadic pheochromocytoma in mean age of 25 years.

2.2 Clinical presentation

The clinical symptoms of pheochromocytomas derive from the catecholamines they secrete and from their relative stimulation of alpha- or beta-adrenergic receptors. As catecholamines are normally produced by sympathetic nerves and by the adrenal medulla, high catecholamine levels are not specific to pheochromocytoma. The secretion is often episodic, and sometimes the secretion is not high enough to produce typical signs and symptoms or positive test results (Pacak et al., 2001). Pheochromocytomas, unlike the normal adrenal medulla, are not innervated and catecholamine release is not initiated by neural impulses. Direct pressure, changes in tumor blood flow, and some chemicals and drugs may initiate catecholamine release (Landsberg and Young, 1992). Most pheochromocytomas secrete predominantly NE, many tumors produce both NE and EPI, while pure EPI production is more rare (Ito et al., 1992; Kimura et al., 1992). Pheochromocytomas from patients with MEN II produce EPI, whereas tumors from VHL patiens produce almost exlusively NE (Eisenhofer et al., 1999). Extraadrenal pheochromocytomas, with the occacional exception of tumors in organ of Zuckerkandl, typically secrete only NE (Landsberg and Young, 1992).

Hypertension is the clinical hallmark of a pheochromocytoma, and can be potentially fatal. Only about 0.1-0.3% of hypertensive patients have an underlying pheochromocytoma, however. Hypertension is sustained in half of the patients, it is paroxysmal in about a third, and absent in approximately a fifth (Bravo, 1994). The most typical presentation of a pheochromocytoma is a paroxysmal hypertensive crisis characterized by severe hypertension, diaphoresis, headaches and tachycardia, or arrhythmia. Hypertensive episodes are not provoked by psychological stress or anxiety, but can be spontaneous or associated with physical exercise, surgical operations, defecation, palpitation, or certain drugs that lower the blood pressure (Sutton et al., 1981). A variety of symptoms have been reported to occur in patients with pheochromocytomas (Table 2). Myocardial infarction is a common complication of these patients (Cohen and Dent, 1984; Liao et al., 2000). Another frequent complication is a stroke caused by cerebral infarction, intracranial hemorrhage, or embolism (Thomas et al., 1966).

Many patients have orthostatic hypotension, which probably reflects the reduced plasma volume resulting from high levels of circulating catecholamines. Metabolic alterations are common in patients with pheochromocytoma. Weight loss is also usual. Elevated plasma glucose level is associated with a low plasma level of insulin. Hypercalcemia may reflect associated hyperparathyroidism, particularly in familial cases. Cushing syndrome secondary to ectopic production of corticotrophin has often been found in patients with pheochromocytoma (Landsberg and Young, 1992; Sane, 2000).

Table 2. Frequency of symptoms in 100 patients with pheochromocytoma (Landsberg and Young, 1992).

Symptom		Symptom		Symptom	
Headache	80	Chest pain	19	Tinnitus	3
Excessive perspiration	71	Dyspnea	19	Dysartria	3
Palpitation	64	Flushing or warmth	18	Gagging	3
Pallor	42	Numbness	11	Bradycardia	3
Nausea	42	Blurring of vision	11	Back pain	3
Tremor or trembling	31	Tightness of throat	8	Coughing	1
Weakness or exhaustion	28	Dizziness or faintness	8	Yawning	1
Nervousness or anxiety	22	Convulsiones	5	Syncope	1
Epigastric pain	22	Neck-shoulder pain	5	Unsteadiness	1
		Extremity pain	4		
		Flank pain	4		

Extraadrenal pheochromocytomas can be functional or nonfunctional. Less than a third of the patients with extraadrenal pheochromocytomas have an excess of tumoral catecholamines, and less than 5% of carotid body tumors have been shown to produce and secrete catecholamines (Erickson et al., 2001). Functional tumors usually present classical symptoms similar to pheochromocytomas and tend to be smaller than nonfuntional tumors. Patients with nonfunctional tumors generally present with signs and symptoms that result from the compression of adjacent structures (Whalen et al., 1992).

Most pheochromocytomas are benign neoplasms. However, certain features of the patients or of the tumors have suggested an association with malignant disease. Higher age at the time of resection (van der Harst et al., 2000), sporadic occurrence (van der Harst et al., 2000), and larger tumor size (Clarke et al., 1998; Linnoila et al., 1990; Medeiros et al., 1985; van der Harst et al., 2000) are believed to be more common in patients with malignant pheocromocytoma. Some studies have nevertheless suggested that age is of no significance (John et al., 1999; Plouin et al., 1997). Except for two studies (Goldstein et al., 1999; Pommier et al., 1993), malignat tumors are reported to be more commonly located extraadrenally (John et al., 1999; Linnoila et al., 1990; Mornex et al., 1992; van der Harst et al., 2000). Some studies have reported that male gender is more common in patients with

malignant tumors (Clarke et al., 1998; Linnoila et al., 1990), while others have not (van der Harst et al., 2000).

2.3 Diagnosis

The diagnosis of pheochromocytoma is established by the demonstration of elevated levels of free catecholamines (NE and EPI) or their metabolites (normetanephrines, metanephrines, and vanillylmandelic acid) in blood or urine (Bravo, 1994). Analysis of 24-h collection of urinary metanephrines and normetanephrines provide the greatest sensitivity and specificity. Negative plasma free metanephrines virually exclude pheochromocytoma (Lenders et al., 2002). Van der Harst et al. (2002) found basal plasma catecholamine levels to be elevated in 81 of 87 (93%) patients. The tumors having normal catecholamine levels were less than 1 cm in size in four cases, and all but one were asymptomatic. Pacek et al. (2001) at the National Institutes of Health (Bethesda, Maryland, USA) conclude that plasma concentrations of normetanephrine greater than 2.5 pmol/mL or metanephrine levels greater that 1.4 pmol/mL (more than 4- and 2.5-fold above the upper reference limits) are an indication of a pheochromocytoma with 100% specificity. Chromogranin A concentrations in plasma have been found to be elevated in pheochromocytoma patients (O'Connor, 1984).

The prognostic significance of specific secretion patterns is still being debated. Dopamine (DA) secretion has been reported to be indicative of malignancy (John et al., 1999; Proye et al., 1992; Proye et al., 1994). In a large study by van der Harst et al. (2002), elevated NE, DA, and aromatic L-amino acid decarboxylase (ALAAD) levels occurred more frequently in malignant pheochromocytomas, and the values were significantly higher than in benign tumors. In contrast, the ratio EPI/EPI + NE was lower in malignant pheochromocytomas.

Once the diagnosis has been biochemically confirmed, the tumor can be localized anatomically using computed tomography (CT), magnetic resonance imaging (MRI) or metaiodobenzylguanidine (MIBG) scanning. MIBG scintigraphy is superior to CT as far as specificity is concerned, but CT finds better the exact anatomical location of the tumor (Berglund et al., 2001). CT has good sensitivity (93-100%) for detecting adrenal pheochromocytoma, while MRI is superior for detecting extraadrenal tumors (Pacak et al., 2001). Both imaging methods have poor specificity, however (Maurea et al., 1993). MIBG is especially useful for detecting recurrent or metastatic tumors. Scintigraphy after the admistration of radiolabeled ocreotide has had only limited success (Pacak et al., 2001). 6-[^{18}F]fluorodopamine positron emission tomography (PET) can be used in cases with clinical symptoms and signs suggestive for pheochromocytoma, and when the results of biochemical tests are positive, but conventional imaging studies unable to locate the tumor (Pacak et al., 2001). The performance of an echocardiogram is recommended to ensure that chronic exposure to high levels of circulating catecholamines have not resulted in dilated cardiomyopathy (Bravo, 1994).

2.4 Treatment

The most important aspect in the preoperative treatment is the control of blood pressure. α-blockade with phenoxybenzamine (Dibenzyran®) is commonly used at an initial dose of 10 mg twice a day. The dosage is increased up to 40 mg three times daily until the blood pressure does not exceed 160/90 mmHg (Bravo, 1994). 10-80 mg of β-blocker propranolol per day may be given to control possible tachycardia, which is often associated with α-blocade (Sane, 2000). The operative treatment of a pheochromocytoma has great potential for complications because of the excessive release of catecholamines. Treatment with metyrosine (Demser®) reduces the tumor stores of catecholamines and decreases the need for intraoperative medication. However, it is used only in very severe cases (Pacak et al., 2001). During the operation, nitroprusside should be administered to control the hypertensive episodes (Klingler et al., 2001).

Primary surgical resection is the treatment of choice if the disease is limited at the time of diagnosis (Kopf et al., 2001). If the disease has progressed, surgery has to be considered for debulking and palliative treatment (Mundschenk et al., 1998). Open surgery with abdominal or posterior approach has previously been the treatment of choice (Proye et al., 1992; van Heerden et al., 1982), but after the development of minimally invasive techniques, laparoscopic adrenalectomy is an option for majority of patients (Kercher et al., 2002; Kopf et al., 2001). Laparoscopic adrenalectomy is associated with less postoperative pain, a shorter stay in hospital, fewer complications, and more rapid recovery than the open surgery (Jacobs et al., 1997). Adrenal-sparing resection is an option for bilateral pheochromocytomas, occurring commonly with inherited disorders (Kopf et al., 2001). Pheochromocytoma found during pregnancy is very rare, but dangerous and potentially fatal. In a review of 30 246 pregnant women at the Mayo Clinic, only two pheochromocytomas were found (Harrington et al., 1999). The timing of surgery in the pregnant patient with a pheochromocytoma depends on the medical control of hypertension, tumor size, likelihood of malignancy, and stage of pregnancy (Brunt, 2001).

The first choice of treatment in malignant pheochromocytoma is the surgical removal of the tumor and metastases, if possible. If the tumor gains ^{131}I-labelled MIBG, it can be used to decrease the tumor bulk and to suppress the growth (Sane, 2000). Chemotherapy is reserved for unresectable tumors without sufficient response to ^{131}I-MIBG (Kopf et al., 2001). Hartley et al. (2001) consider chemotherapy a more active modality than previously thought, as MIBG uptake may increase after chemotherapy. The treatment with ocreotide is still under evaluation. Lamarre-Cliche et al. (2001) suggested that slow-release octreotide is only of limited value in the long-term treatment of patients with malignant pheochromocytomas.

2.5 Histopathology

Adrenal pheochromocytoma is usually a rounded, single mass with distortions of the gland. Small tumors can be surrounded by the cortex, while identification of an adrenal remnant can be difficult with extremely large tumors. Most pheochromocytomas measure 3 to 5 cm in diameter, with a wide range from 1 to 10 cm or more. In cross-section, the tumor is gray-white to tan, firm, usually sharply circumscribed, and may apper encapsulated. Areas of hemorrhage or degenerative changes, such as fibrosis or cystic alterations, can sometimes be seen (Lack, 1997). Pheochromocytomas can grow adherent to adjacent structures such as kidney or liver. Occasional extending into the inferior vena cava or even into the right atrium has been reported (Rötker et al., 1996).

Microscopically pheochromocytomas usually have a trabecular or alveolar pattern with distinct nests of cells ("zellballen"), or a mixture of both (Linnoila et al., 1990). A spindle cell pattern is rare and usually not prominent throughout the tumor (Linnoila et al., 1990; Remine et al., 1974). The nuclei of the tumor cells can contain pseudoinclusions, and also pleomorphia and hyperchromasia are frequently present. The cytoplasm of the pheochromocytoma cells is often lightly basophilic and finely granular. Lipid degeneration can mimic adrenal cortical neoplasm (Unger et al., 1990). Oncocytic appearance has also been reported (Li and Wenig, 2000). Some cells may contain intracytoplasmic hyaline globules which are periodic acid-Schiff (PAS) positive and resistant to diastase predigestion. The cytoplasm typically contains neurosecretory granules, which can be demonstrated with Grimelius stain (Vassallo et al., 1971). Melanin-like pigment has also been reported in some pheochromocytomas (Chetty et al., 1993).

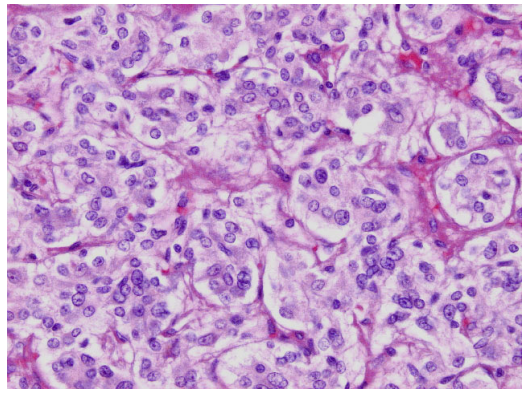

Figure 4. Adrenal pheochromocytoma showing typical nests of cells and sustentacular cells.

Extraadrenally located pheochromocytomas, paragangliomas, macroscopically and microscopically resemble adrenal tumors, and there is no reliable pattern to discriminate between them. Paragangliomas from different sites are also histologically similar. Some tumors show extensive hemorrhage or cystic degeneration. Microscopically these tumors have a trabecular arrangement with anastomosing cords of tumor cells. Some may have a diffuse or alveolar pattern (Lack, 1997). There may be considerable nuclear pleomorphism and even occasional mitotic figures. Intracytoplasmic hyaline globules can be found in some tumors (Lack, 1997).

Classical histological features of malignancy, such as hyperchromasia and pleomorphism are commonly observed in pheochromocytomas and therefore can not be used to distinguish malignant tumors from benign ones. It has been commonly accepted that the biologic behavior of a pheochromocytoma cannot be predicted on the basis of macroscopic or microscopic features. However, certain histological features have been associated with malignancy. Necrosis, vascular invasion, and extensive capsular invasion have been reported to correlate with malignancy (Linnoila et al., 1990; van der Harst et al., 2000). Recently, in a large study of 50 benign and 50 histologically malignant (33 developed pathologically documented metastatic disease) pheochromocytomas, Thompson (2002) introduced a Pheochromocytoma of the Adrenal Gland Scaled Score (PASS) to separate benign tumors from malignant ones (Thompson, 2002). Similar to the Weiss score, used to evaluate the malignancy of adrenal cortical neoplasms, PASS scores several histological features (Table 3). Tumors having PASS ≥4 are believed to behave more aggressively.

Table 3. Pheochromocytoma of the Adrenal Gland Scoring Scale (PASS) (Thompson, 2002).

Feature	Score if present
Large nest or diffuse growth (>10% of tumor volume)	2
Central (middle of large nests) or confluent necrosis (not degenerative change)	2
High cellularity	2
Cellular monotony	2
Tumor cell spindling (even if focal)	2
Mitotic figures >3/10HPF	2
Atypical mitotic figure(s)	2
Extension into adipose tissue	2
Vascular invasion	1
Capsular invasion	1
Profound nuclear pleomorphism	1
Nuclear hyperchromasia	1
Total	20

Analysis of DNA patterns with flow cytometry is used to characterize tumor cell populations at various stages of the cell proliferation cycle. Pheochromocytomas with a diploid DNA pattern have been reported to behave in a benign manner, whereas non-diploid pattern associated with tumor recurrence or metastases (Nativ et al., 1992). However, it has been suggested that flow cytometry is not a reliable predictor of malignancy in pheochromocytomas (Brown et al., 1999).

2.6 Immunohistochemical diagnosis

Adrenal medullary cells and extraadrenal paraganglionic cells and their tumors typically exhibit positivity for a variety of neuroendocrine markers, such as chromogranin A. Chromogranins are major proteins in the neurosecretory granules of neuroendocrine cells and sympathetic nerves. The highest concentration is found in the adrenal medulla. The synthesis of chromogranins is regulated by many different factors, including steroid hormones. They are thought to stabilize the soluble portion of neurosecretory granules by interaction with adenosine triphosphate and catecholamines. Chromogranins are released into the serum after splanchnic stimulation. Antibodies to chromogranin A label most normal neuroendocrine cells and their corresponding neoplasms. The chromogranin A clone LK2H10 was derived from human pheochromocytoma (Leong et al., 1999a). Chromogranin A is expressed in more than 95% of pheochromocytomas. The immunoreactivity of normal cells is generally more intense than that of neoplastic cells (DeLellis and Shin, 2002).

Synaptophysin is a membrane glycoprotein of presynaptic vesicles (Jahn et al., 1985), showing reactivity with neuronal presynaptic vesicles of the brain, spinal cord, retina, neuromuscular junctions and small vesicles of adrenal medulla and pancreatic islets. Antibody to synaptophysin stains specifically neuronal, adrenal and neuroepithelial tumors, including adrenal and extraadrenal pheochromocytomas, pancreatic islet cell tumors, thyroid medullary carcinoma, carcinoid tumors, and pituitary/parathyroid adenomas. Other neural tumors also demonstrate positivity with this antibody (Leong et al., 1999c).

Neuron-specific enolase (NSE) is a glycolytic isoenzyme specifically detected in neurons and neuroendocrine cells, and their corresponding neoplasms (Wick et al., 1983). NSE is highly sensitive, but its specificity is low. When used for the identification of neuroendocrine differentation, it must be used in a panel with more specific markers. CD56, the neural cell adhesion molecule (NCAM), was found when cell surface molecules that contribute to cell-cell interactions during neural development were being searched (Rutishauer et al., 1988). In the normal adrenal gland, the zona glomerulosa, the medulla, as well as the pheochromocytomas, are positive for CD56 (Komminoth et al., 1995). CD56 is also a marker for natural killer (NK) cells, and the current major application of CD56 is the diagnosis of NK and NK-like T-cell lymphoma (Chan, 1997).

Both adrenal and extraadrenal pheochromocytomas may also contain serotonin (80%), leu- and met-encephalin (70%), neuropeptide Y (64%), substance P (36%), and calcitonin (21%)

(Grignon et al., 1991; Salim et al., 1993). Sustentacular cells of a pheochromocytoma as well as of a normal adrenal medulla are positive for S-100 protein (Komminoth et al., 2002). S-100 has been demonstrated in a wide variety of normal and neoplastic tissues. It is a useful marker in the identification of melanoma, Langerhans' cell histiocytosis, chondroid tumors, and peripheral nerve tumors (Leong et al., 1999b).

It may be difficult to differentiate a pheochromocytoma from an adrenocortical carcinoma, or from a metastatic carcinoma, such as renal cell carcinoma, hepatocellular carcinoma, or other metastatic adenocarcinoma. This distinction can be facilitated by using certain immunohistochemical stainings (Table 4). A melanoma marker MelanA (MART-1, A103) is positive in adrenocortical carcinomas, but negative in other carcinomas as well as in pheochromocytomas. Pheochromocytomas are usually cytokeratin-negative. Other epithelial markers, such as EMA and CEA, are typically negative (DeLellis and Shin, 2002). In conclusion, if an adrenal tumor histologically resembles a pheochromocytoma, chromogranin A positivity is sufficient for diagnosing it as a pheochromocytoma. If in doubt, synaptophysin may help. If both of these remain negative, it is possible that the tumor is a cortical carcinoma or a metastatic tumor.

Table 4. Immunohistochemical markers in the differential diagnosis of a pheochromocytoma (Arola et al., 2000; DeLellis and Shin, 2002).

Tumor type	Chromo-granin A	Synapto-physin	MelanA	Cyto-keratin	EMA	CEA	Inhibin α
Pheochromocytoma	+	+	-	-	-	-	-
Adrenocortical ca	-	+/-	+	-/+	-	-	+/-
Renal cell ca	-	-	-	+	+	-	-
Hepatocellular ca	-	-	-	+	+/-	+	-
Metastatic adenoca	-	-	-	+	+	+	-

+, positive; +/-, mostly positive; -/+, mostly negative; -, negative

2.7 Prognosis

Most pheochromocytomas are benign, slowly growing tumors. The percentage of malignant tumors has been reported to range from 2.4% (Melicow, 1977) to 26% (Proye et al., 1992). Benign, surgically operated pheochromocytomas have a 5-year survival rate of over 95%, and the recurrence rate is less than 10% (Klingler et al., 2001). The surgical mortality is 0% to 4% in experienced hands (Gagner et al., 1997; van Heerden et al., 1982). Pheochromocytomas can metastasize via lymphatic or hematogenous pathways, and the most common metastatic sites are lymph nodes, bone, lung, and liver (Lack, 1990). The 5-

year survival of patients with malignant pheochromocytoma is about 44%, but some patients have lived up to 20 years or longer (Remine et al., 1974; Yoshida et al., 2001; Järveläinen and Viikari, 2001). The length of the follow-up is debatable, but a follow-up of at least 5 years is recommended (Klingler et al., 2001).

Several attempts have been made to find markers that would predict the future behavior of an unmetastasized pheochromocytoma. Loss of S-100 positive sustentacular cells is reported to correlate with unfavorable prognosis in adrenal (Unger et al., 1991) and extraadrenal pheochromocytomas (Achilles et al., 1991; Montresor et al., 1994), though the relationship between S-100-positive cells and clinical course is believed to be dependent on tumor weight (Clarke et al., 1998). C-met, bFGF, cathepsin B, cathepsin D, and collagenase are strongly expressed in pheochromocytomas, but they are not predictive of outcome (Clarke et al., 1998). Bcl-2, the first antiapoptosis gene identified, is variably expressed, and is not significant either (Clarke et al., 1998). Leu-M1 expression is frequently seen in pheochromocytomas, but seems to be useless in predicting malignant behavior and to be influenced mainly by tumor size (Masmiquel et al., 1997). Linnoila et al. (1988) have reported that certain neuropeptides (encephalins, somatostatin, pancreatic polypeptide, VIP) are expressed less frequently in malignant extraadrenal pheochromocytomas. Neuropeptide Y was found in only four out of 11 malignant pheochromocytomas, while nine benign tumors were negative (Helman et al., 1989). c-erbB-2 expression has been suggested to be higher in malignant sporadic cases of pheochromocytoma (Castilla-Guerra et al., 1997; de Krijger et al., 1999). Kubota et al. (1998) reported elevated telomerase activity in three malignant pheochromocytomas, while no telomerase activity was found in benign tumors (n = 16) nor in normal adrenal medulla (n = 16). Despite these numerous studies, no definite markers for malignancy have yet been found.

3. Tumorigenesis

3.1 General remarks

Tumorigenesis is a multistep process reflecting genetic alterations that drive the progressive transformation of normal cells into cancer cells. Hanahan and Weinberg suggest that a cancer is a manifestation of six essential alterations in cell physiology that collectively dictate malignant growth: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg, 2000). They propose that these capabilities are shared by perhaps all types of human tumors.

3.2 Growth stimulation

3.2.1 Cell cycle

A malignant tumor is almost always monoclonal, derived from one altered cell. It is more common that cancer occurs in organs where cells replicate fast, such as skin, gastrointestinal tract, and blood. Cell replication is controlled by chemical factors in the microenvironment, which either stimulate or inhibit cell proliferation. The cell growth cycle consists of G_1 (presynthetic), S (DNA synthesis), G_2 (premitotic), and M (mitotic) phases (Figure 5). Quiescent cells are in a physiologic state called G_0.

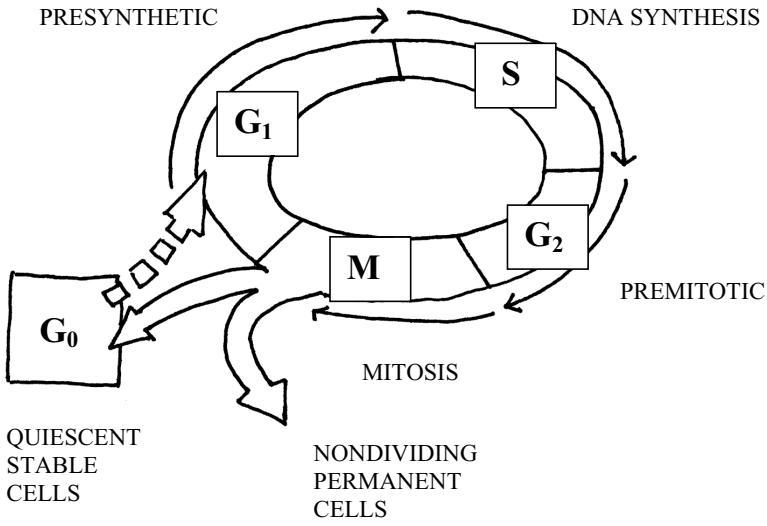

Figure 5. The cell cycle (modified from Cotran et al. 1999).

3.2.2 Growth factors

Normal human cells carry an intrinsic, cell-autonomous program that limits their multiplication. Normal cells require mitogenic growth signals before they can proliferate, while tumor cells generate many of their own growth signals. Most common strategies for achieving growth signal autonomy are alterations of extracellular growth signals, of transcellular tranducers of these signals, and of intracellular circuits that translate these signals into action (Hanahan and Weinberg, 2000). Many cancer cells create a positive feedback signaling loop, termed autocrine stimulation, by synthesizing growth factors to

which they are responsive themselves. For example, the production of PDGF (platelet-derived growth factor) and TGFα (tumor growth factor α) by glioblastomas and sarcomas, respectively (Fedi et al., 1997).

Many tumors overexpress growth factor receptors, which may enable the neoplastic cell to become hyperresponsive to ambient levels of growth factors that normally would not trigger proliferation (Fedi et al., 1997). E.g. HER2/neu receptor is upregulated in stomach and mammary carcinomas (Slamon et al., 1987). The SOS-Ras-Raf-MAPK cascade plays a central role in the downstream cytoplasmic circuitry that receives and processes the signals emitted by ligand-activated growth factor receptors. In about 25% of human tumors, Ras proteins are structurally altered, enabling them to release a flux of mitogenic signals into cells (Medema and Bos, 1993).

3.2.3 Proliferation activity

The growth capacity of a tumor can be assessed by counting the mitoses, or by immunohistochemistry using Ki-67 or MIB-1 proliferative activity markers. Ki-67 is a large protein of approximately 395 kDa, encoded for by almost 30 000 base pairs. Ki-67 protein appears in mid-to-late G1 phase and increases as the cell progresses through S phase to reach maximum expression during G2 phase. Ki-67 is absent in resting (G0) cells. During mitosis, the Ki-67 protein undergoes phosphorylation and dephosphorylation; it is susceptible to proteases, and its expression is regulated by proteolytic pathways (Schluter et al., 1993). The protein is located in the nucleus, and changes its location within the nucleus during mitosis (Endl and Gerdes, 2000). Ki-67 is essential for cell proliferation, since its removal by antisense nucleotides prevents cell proliferation (Schluter et al., 1993). The exact function of Ki-67 is still unclear (Brown and Gatter, 2002). The original Ki-67 antibody has served as a prototype for other antibodies which also identify epitopes of Ki-67 protein, e.g. MIB-1 (Cattoretti et al., 1992), MIB-5 (Gerlach et al., 1997), and TEC-3 (Scholzen and Gerdes, 2000).

Antibodies against the Ki-67 protein are practical tools for the assessment of cell proliferation in cancer research, as well as in diagnostics. In breast cancer, most studies show a strong correlation with clinical outcome. In a large study of 462 primary breast carcinomas, Beck et al. (1995) demonstrated reduced overall survival and a disease-free period with tumors having higher proliferative activity measured with MIB-1. On the other hand, some tumors show little correlation between Ki-67 and patient survival, e.g. colon cancer (Kubota et al., 1992). In endocrine tumors, proliferative activity measured by MIB-1 or Ki-67 has been shown to be a predictor of malignancy in pituitary adenomas (Shibuya et al., 1992), thyroid malignancies (Katoh et al., 1995), parathyroid carcinomas (Abbona et al., 1995), pancreatic endocrine tumors (Pelosi et al., 1996), and adrenocortical carcinomas (Goldblum et al., 1993). Van der Harst et al. (2000) reported that 50% of malignant pheochromocytomas (n = 37) had a proliferative index greater than 2.5%, while all benign

ones (n = 99) had less. Similar results were reported by Brown et al. (1999) and Ohji et al. (2001), but the number of tumors investigated was smaller.

3.3 Growth inhibition

It is assumed that growth inhibitory signals originate outside the cell and use receptors, signal transducters, and cell cycle and nuclear transcription regulators to accomplish growth inhibition. The tumor suppressor genes encode different components of this growth inhibitory pathway. Many growth-inhibitory signals are funneled through the retinoblastoma protein (pRb), which blocks proliferation when in a hypophosphorylated state (Weinberg, 1995). One of the most documented signaling molecules, transforming growth factor-β (TGFβ), prevents the phosphorylation that inactivates pRb and, in doing so, blocks advance through G1 (Moses et al., 1990). TGFβ receptor, and its signal tranducers Smad 2 and 4, are often mutated in pancreatic and colon cancer (Derynck et al., 2001).

3.3.1 Inhibins/activins

Inhibins and activins are glycoproteins of the TGF-β superfamily of growth and differentiation factors. They were isolated and characterized as gonadal-derived regulators of FSH (follicle-stimulating hormone) synthesis and secretion. Several studies have shown that the development of resistance to TGFβ by tumor cells represents a key event in the progression to malignancy. Inhibins consist of an α subunit linked to either a βA-(inhibin A) or a βB-(inhibin B) subunit, whereas activins are heterodimers of βA and βB, or homodimers of either one (Ying, 1988). Inhibin/activin βA and βB mature subunits share 63% identity at the amino acid level (Brown et al., 2000). The genes for the α- and the βB-subunit are located on the long (q) arm of chromosome 2, while the gene for the βA-subunit is on short arm (p) of chromosome 7 (Barton et al., 1989). Inhibin/activin α-, βA- and βB-subunits are expressed in gonadal tissues (Ying, 1988) and in a wide range of extragonadal sites including pituitary, bone marrow, kidney, spinal cord, brain, adrenal and placenta (De Jong et al., 1990; Meunier et al., 1988; Voutilainen et al., 1991).

Inhibin α-subunit is a tumor suppressor gene with gonadal and adrenal specificity based on the results from transgenic mouse models, in which deficiency of the inhibin α-subunit was associated with tumorigenesis (Matzuk et al., 1992). In the adrenal gland, strong immunoreactivity for the inhibin α-subunit is found mainly in the inner zones of the cortex, whereas zona glomerulosa and medulla are immunohistochemically negative (Arola et al., 2000; McCluggage et al., 1998; Munro et al., 1999). Diffuse immunostaining of all three cortical zones has been reported for both βA- and βB-subunits (Munro et al., 1999; Spencer et al., 1992), but no positivity was found in the medulla (Spencer et al., 1992). We have previously shown that most adrenocortical tumors are positive for the inhibin α-subunit, while pheochromocytomas are negative (Arola et al., 2000).

3.3.2 Cyclin-dependent kinases

Cyclin-dependent kinases (CKD) are key regulators of cell cycle progression. Their activity is controlled through modulation of expression, specific phosphorylation/ dephosphorylation, and interaction with other proteins, such as CDK inhibitors (CDKI) (Grana and Reddy, 1995; Pines, 1995). p21 (also known as WAF1, CIP1, or SDI1) was the first CDKI to be isolated (Gu et al., 1993; Harper et al., 1993; Xiong et al., 1992). The p21 protein product prevents the cycling cells to passage from the G_1 phase to the S phase by blocking the cyclin/cyclin-dependent kinase complex activity. p21 mediates p53-induced cell cycle arrest resulting from DNA damage. This arrest is important in the process of DNA repair, or switch to apoptosis (el-Deiry et al., 1994). p21 is regulated by wild-type p53, although p53-independent pathways have been proposed (Parker et al., 1995). BRCA1-mediated cell cycle arrest operates throught p21 expression (Somasundaram et al., 1997). In addition, p21 protein possesses many other tumor suppressive properties (Chen et al., 1995).

p21 is commonly altered in human malignancies, and inverse expression of p21 and p53 has been reported in breast carcinoma (Wakasugi et al., 1997). In patients with ovarian carcinoma, a combination of p21-positive and p53-negative was a better independent indicator of prognosis and survival than either p21 or p53 alone (Geisler et al., 2001). However, Yoshimoto et al. (1998) found immunohistochemical staining of p21 only in scattered pheochromocytoma cells, and in contrast to p53, p21 was poorly expressed despite the presence of mutated p53. In the rat pheochromocytoma neural cell line (PC12), p21 enhanced neuronal differentation, but did not inhibit cell death at levels capable of inducing permanent growth arrest (Erhardt and Pittman, 1998).

3.4 Limitless replicative potential

The limitless replicative potential of a tumor cell is thought to be a phenotype that is acquired during tumor progression. The key component of the capability for unlimited replication is telomere maintenance, evident in virtually all types of malignant cells (Shay and Bacchetti, 1997). Telomeres are the ends of chromosomes and they are composed of several thousand repeats of a short sequence element. During the cell cycle, 50-100 bp of telomeric DNA from the ends of every chromosome are lost. This progressive shortening eventually causes the inability to protect the ends of chromosomal DNA. The unprotected chromosomal ends participate in end-to-end chromosomal fusions, resulting in the death of the cell (Counter et al., 1992). Telomere maintainance is evident in all types of malignant cells (Shay and Bacchetti, 1997). Most of them upregulate the telomerase enzyme expression, while the remainder have invented a way of activating a mechanism, termed ALT (alternative lengthening of telomeres) (Neumann and Reddel, 2002). The telomeres of the malignant cells are maintained at a length above a critical threshold, and this permits the unlimited replication of these cells (Hanahan and Weinberg, 2000). Telomerase activity in tumorous tissue has been found in a variety of

carcinomas (Kim et al., 1994). In pheochromocytomas, the telomerase activity has been suggestive of malignancy (Kubota et al., 1998).

3.5 Apoptosis

The ability of a tumor to expand in size is determined by the rate of cell proliferation, and by extend of cell distraction, e.g. apoptosis and necrosis. Acquired resistance to apoptosis is believed to be a hallmark of most types of cancer (Hanahan and Weinberg, 2000). Commitment to apoptosis in response to cytotoxic agents and diverse physiological cues is governed by proteins of the Bcl-2 family. These members have either proapoptotic (Bax, Bak, Bid, Bim) or antiapoptotic (Bcl-2, Bcl-XL, Bcl-W) function, and act in part by governing mitochondrial death signaling by releasing cytochrome C, a potent catalyst of apoptosis (Green and Reed, 1998). The p53 protein can elicit apoptosis by upregulating the expression of Bax in response to sensing DNA damage. Bax in turn stimulates the cytochrome C release from mitochondria (Hanahan and Weinberg, 2000).

3.5.1 p53

The *p53* tumor-supressor gene has been reported to be the most common target for genetic alteration found in human malignancies (Hollstein et al., 1991). p53 protein is thought to serve as a crital gatekeeper against the formation of cancer. In normal cells the activation of p53 by hypoxia or by DNA-damaging agents leads to cell cycle arrest in G_1 boundary and induction of DNA repair, by transcriptional up-regulation of target genes such as *p21*(Marx, 1993). If DNA repair fails, p53-induced activation of the *Bax* gene promotes apoptosis. However, in cells with mutations or loss of *p53*, DNA damage does not induce DNA repair or cell cycle arrest, and genetically damaged cells can proliferate and give rise to malignant neoplasms.

The *p53* gene is located on chromosome 17p13.1 and compasses 16-20 kb of DNA (McBride et al., 1986; Miller et al., 1986). The product of *p53* gene is a 393-amino-acid nuclear phosphoprotein, which weighs about 53 kDa. It was first discovered as a cellular protein in 1979 because it co-immunoprecipitated with anti-T antibodies from extracts of SV40-transformed cells (Lane and Crawford, 1979; Linzer and Levine, 1979). Mutant p53 has a longer half-life than wild-type p53, and can therefore be detected by immuno-histochemisty (Martinez et al., 1991). Mutation of the *p53* gene and immunohistochemical overexpression of its protein product p53 have been detected in a wide range of tumors, including epithelial, mesenchymal, hematopoietic, and lymphoid neoplasms, as well as in tumors of the central nervous system (Chang et al., 1993; Hollstein et al., 1991). The loss of heterozygosity at 17p shown in a number of pheochromocytoma cases suggests some role of p53 in the tumorigenesis of adrenal tissues (Khosla et al., 1991). A few studies on p53 expression in pheochromocytomas have been published (Dahia et al., 1995; de Krijger et al., 1999; Herfarth et al., 1997; Lam et al., 2001; Lin et al., 1994; Reincke et al., 1996; Wang et al., 1995; Yoshimoto et al., 1998).

3.6 Angiogenesis

Oxygen and nutrients are essential for cell function and survival. Most cells in a tissue need to reside within 100 μm of a capillary blood vessel. To grow beyond 1 to 2 mm in diameter, a tumor needs neovascularisation because hypoxia induces apoptosis by activation of p53. New blood vessels have a dual effect on tumor growth: perfusion supplies oxygen and nutrients, and new endothelial cells stimulate the growth of adjacent tumor cells by secreting polypeptides, such as insulin-like growth factors, platelet-derived growth factor (PDGF), and interleukin-1 (Cotran et al., 1999). Angiogenesis is essential also for metastasis.

In order to progress in size, incipient tumors must develop angiogenic abilities. Vascular endothelial growth factor (VEGF) is one of the best known angiogenesis promoting factors (Ferrara and Henzel, 1989; Gospodarowicz et al., 1989). However, tumor cells not only produce angiogenic factors, but also induce antiangiogenic molecules. Early in their growth, most tumors don't induce angiogenesis, until the angiogenic switch terminates the vascular quiescence. Tumors appear to activate this switch by changing the balance of angiogenic inducers and inhibitors (Hanahan and Folkman, 1996). A typical angiogenesis inhibitor is thrombospondin-1, which binds to CD36, a transmembrane receptor on endothelial cells (Bull et al., 1994). Thrombospondin-1 is positively regulated by the p53 tumor suppressor protein in some cell types. Consequently, loss of p53 funcion, which occurs in most human cancers, can cause thrombospondin-1 levels to fall (Dameron et al., 1994).

3.6.1 Vascular endothelial growth factor (VEGF)

Many solid tumors produce several growth factors with angiogenic properties to sustain the neovascularization. VEGF is a monodimeric protein identified as a mitogen for endothelial cells in vitro and as an angiogenesis-promoting factor in vivo (Ferrara and Henzel, 1989; Gospodarowicz et al., 1989). VEGF was originally found to be secreted by glioma cells in glioma-associated brain edema (Bruce et al., 1987). The human *VEGF* gene is located on chromosome 6p21.3. Five human isoforms of 121, 145, 165, 189, and 206 amino acids are generated as a result of alternative splicing from *VEGF* gene (Houck et al., 1991; Park et al., 1993). All VEGF isoforms interact with cell surface receptors, VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1), which are selectively expressed in the endothelium (de Vries et al., 1992; Shibuya et al., 1990).

In normal human adult tissues, the highest levels of VEGF are found in the epithelial cells of lung alveoli, renal glomeruli, and adrenal cortex (Berse et al., 1992). It contributes to the vascular remodeling that occurs during the ovarian cycle and embryonic implantation (Shweiki et al., 1993). Deregulated VEGF expression contributes to the etiology of several

diseases that are characterized by abnormal angiogenesis like diabetes mellitus (Hovind et al., 2000), psoriasis (Bhushan et al., 1999), and rheumatoid arthritis (Cho et al., 2000).

VEGF has an important role in tumor angiogenesis, and it has been identified in many human malignancies, such as gliomas (Plate et al., 1992), kidney and bladder carcinoma (Brown et al., 1993a), gastrointestinal tract adenocarcinoma (Brown et al., 1993b; Tanigawa et al., 1997), breast cancer (Brown et al., 1995), Kaposi's sarcoma (Cornali et al., 1996), and lung adenocarcinoma (Shibusa et al., 1998). It has also been shown that the levels of VEGF are often higher in malignant tumors than in normal tissues (Dobbs et al., 1997; Guidi et al., 1996). In esophageal carcinoma, VEGF expression correlates both to the depth of invasion and to the lymph node metastasis (Uchida et al., 1998). The median survival time of the patients with VEGF-positive pancreatic cancer has been reported to be significantly shorter compared to patiens with VEGF-negative tumors (Ikeda et al., 1999).

3.6.2 Microvessel density

The microvessel density (MVD) in the areas of most intensive neovascularisation has been suggested to be an independent predictor of metastatic disease (Weidner et al., 1991) and to associate with tumor aggressivenss (Weidner, 1995; Weidner and Folkman, 1996). However, MVD does not indicate the degree of angiogenesis nor the functional status of the tumor neovasculature (Eberhard et al., 2000; Hlatky et al., 2002), nor does it reflect the angiogenic dependence of a tumor. The microvessel density can be assessed by counting the microvessels that have been stained immunohistochemically with CD34. The CD34 antigen is a 110 kD transmembrane protein of unknown function. It was primarily identified on human myeloid leukemia cells (Civin et al., 1984), later noted on human hematopoietic precursors (Andrews et al., 1986). CD34 is expressed in endothelial cells and endothelial tumors, and has been used as an endothelial marker (Fina et al., 1990). Also many mesenchymal and nerve-sheath tumors express high levels of CD34 (Weiss and Nickoloff, 1993).

3.7 Invasion and metastasis

In order to cause cancer-related morbidity and mortality, a tumor must be able to invade and to metastasize. The metastatic process consists of a series of steps which must be successfully completed. First a primary tumor must develop neovascularization to support its metabolic needs. These new blood vessels also provide an escape route, a process called intravasation (Wyckoff et al., 2000). Tumor cells must survive in the circulation and extravasate into the new site, where they must initiate and maintain growth to form pre-angiogenic micrometastases. And finally, this growth must be sustained by angiogenesis for macroscopic metastasis (Chambers et al., 2002).

3.7.1 Matrix metalloproteinases

Tumor cells must interact with the extracellular matrix (ECM) at several stages in the metastatic cascade. Invasion of the ECM can be resolved into several steps: detachment of the tumor cells from each other, attachment to matrix components, degradation of ECM, and migrarion of the tumor cells. Matrix metalloproteinases (MMP) are a family of zinc-dependent endopeptidases collectively capable of degrading essentially all matrix components. Up to date 21 members of the human MMP gene family are known (Vihinen and Kähäri, 2002). A high expression of certain MMPs related to tumor invasion capacity has been shown in several cancers, such as laryngeal (Cazorla et al., 1998), esophageal (Ohashi et al., 2000), and bladder carcinoma (Kanayama et al., 1998). MMPs can also be used as markers to predict tumor recurrence in many cancer types. E.g. high expression levels of MMP-2 in ovarian cancer cells are shown to predict tumor recurrence (Westerlund et al., 1999). The expression of some MMPs predicts both lymph node and hematogenous metastasis. Certain MMPs also may play a role in tumor vascularization (Vihinen and Kähäri, 2002).

3.7.2 Tenascin

Matricellular proteins, such as tenascin, interact with matrix proteins, cell surface receptors, growth factors, or cytokines that interact, in turn, with the cell surface. These proteins share the ability to disrupt cell-matrix interactions. Tenascin (tenascin-C) is a large glycoprotein of ECM with a unique six-armed multidomain macromolecular structure (Schenk and Chiquet-Ehrismann, 1994). The term “tenascin” (from the latin *tenere* = to hold and *nascere* = to be born) was proposed by Chiquet-Ehrismann et al. (1986). Tenascin is synthesized by fibroblasts, and it is believed to have a role in cell adhesion and motility, guidance along cell migration pathways, shedding of epithelial cells from the surface, promotion of cell growth, tissue modeling, and demarcation of tissue boundaries (Inaguma et al., 1988). Tenascin is expressed in various tissues during embryogenesis and growth. During adult life its expression is induced during wound healing and inflammatory processes (Chiquet-Ehrismann et al., 1986; Mackie et al., 1988).

Originally, tenascin was found to be a stromal marker of malignancy in breast cancer (Mackie et al., 1987), later verified by others (Jahkola et al., 1996). However, increased expression has been reported in various other tumors, such as colon (Iskaros et al., 1997), lung (Kusagawa et al., 1998), and prostate (Xue et al., 1998), and in the stroma of some benign leasions. In addition, increased tenascin expression has been found to correlate with a higher grade of astrocytic brain tumors (Kim et al., 2000). As tenascin molecule is made up of epidermal growth factor (EGF)-like repetitions, which could bind to EGF receptors of tumor cells, it is suggested that tenascin may play a role in tumor invasion and metastasis (Jones et al., 1988).

3.7.3 Cyclooxygenase-2 (COX-2)

Cyclooxygenase (COX) is the key enzyme in the conversion of arachidonic acid to prostaglandins. Elevated COX-2 is suggested to promote cancer growth by two mechanisms. First, COX-2 derived prostaglandins might act on the malignant epithelial cells, and lead to changes in key regulatory genes that cause resistance to apoptosis by elevating the levels of the anti-apoptotic protein Bcl-2. These prostaglandins might also enhance cell migration and invasion that is associated with elevated levels of several MMPs (Tsujii and DuBois, 1995; Tsujii et al., 1997). Secondly, COX-2 derived prostaglandins stimulate tumor-associated angiogenesis (Tsujii et al., 1998). COX-2 might also modify tumor growth by limiting the ability of fibroblasts to support neovascularization within the microenvironment of a tumor (Williams et al., 2000).

Two *COX* genes have been characterized, *COX-1* and *COX-2*, and they share over 60% homology at the amino acid level (Dubois et al., 1998). Two isoforms of COX enzyme exist (Herschman, 1994). COX-1 is constitutively expressed in most normal human tissues and usually its expression is not regulated. *COX-2* encodes a 71-kDa protein which is usually absent in most normal cells, but upregulated in inflammation, reproduction, and many epithelial cancers (Taketo, 1998a; Taketo, 1998b). COX-2 can be highly induced in response to cell activation by hormones, proinflammatory cytokines, growth factors, and tumor promoters (Dubois et al., 1998; Vane et al., 1998). Recently, Chandrasekharan et al. (2002) indentified COX-3 and PCOX-1a which are made from the *COX-1* gene but retain intron 1 in their mRNAs. Inhibition of COX-3 is thought to represent a central mechanism by which acetaminophen and other analgesic/antipyretic drugs suppress pain and possibly fever.

Several epidemiological studies have demonstrated a reduced cancer risk associated with the prolonged use of high doses of non-steroidal anti-inflammatory drugs (NSAID) (Giardiello et al., 1995; Thun et al., 1993). The best known target of NSAIDs is COX-2, the expression of which is increased in 85-90% of human colorectal adenocarcinomas (Williams et al., 1999a). In colorectal cancers, COX-2 has been found in epithelial tumor cells, inflammatory cells, vascular endothelium, and fibroblasts (Sheehan et al., 1999), whereas in sporadic colorectal adenomas COX-2 is expressed predominantly by interstitial macrophages (Chapple et al., 2000). There is clinical evidence that COX-2 inhibitors can reduce intestinal polyp burden in patients with an inherited predisposition to colorectal carcinoma, called famial adenomatous polyposis (FAP) (Oshima et al., 1996). In addtion to colorectal cancers (Eberhart et al., 1994; Sano et al., 1995; Sheehan et al., 1999), high COX-2 expression is also found in upper gastrointestinal tumors (Ristimäki et al., 1997; Wilson et al., 1998). Patients with adenocarcinoma arising from a Barrett's esophagus and high COX-2 expression in the tumor are more likely to develop distant metastases and local recurrences. Their survival is also significantly reduced (Buskens et al., 2002). Increased expression has also been described in several other human malignancies including lung carcinoma (Wolff et al., 1998), hepatocellular carcinoma (Shiota et al., 1999), pancreatic carcinoma (Tucker et al., 1999), squamous cell carcinoma of the head and neck (Chan et al., 1999), prostate adenocarcinoma (Gupta et al., 2000b), retinoblastoma (Karim et al., 2000), and urinary

bladder carcinoma (Ristimäki et al., 2001). In human breast cancer, the COX-2 expression is associated with angiogenesis, lymph node metastasis, and apoptosis (Costa et al., 2002).

AIMS OF THE STUDY

The general aim of this study was to find molecular markers that would predict the behavior of a pheochromocytoma. For this purpose, we collected a large set of adrenal and extraadrenal pheochromocytomas. Nonmetastasized tumors were grouped into benign and borderline tumors based on their histological features. We examined these tumors to see whether certain markers would help in the diagnosis of benign or malignant pheochromocytoma.

The specific aims of the study were:

- to assess the histopathological features of 105 pheochromocytomas (I)
- to evaluate the growth capacity of these tumors (I)
- to study the role of apoptosis by evaluating the expression p53 (I)
- to study the value of growth inhibition caused by p21 (I) and inhibin/activin βB-subunit (II)
- to evaluate the role of VEGF and angiogenesis (III)
- to study the role of tenascin and COX-2 in invasion and metastasis (IV and V)

MATERIALS AND METHODS

1. Clinical material (Study I-V)

Table 5. Tumor material in original publications.

Study	I	II	III	IV	V
Time period	1976-2001	1985-1999	1976-2001	1976-2000	1976-2001
No. of patients	97	80	97	95	85
No. of normal adrenals	0	7	6	5	5
No. of pheochromocytomas	105	83	105	103	92
Adrenal	69	56	69	68	63
Extraadrenal	36	27	36	35	29
Metastases	0	5	0	0	6
Syndrome associated	19	12	19	19	16
Sporadic	86	71	86	84	76

This study was approved by the Ethics Committee of the Department of Internal Medicine, Helsinki University Central Hospital (no. 88/2000). All tissue specimens were obtained at operations performed at Helsinki University Central Hospital in 1976-2001. Normal adrenal glands were from patients undergoing nephrectomy for a renal tumor. The tissue specimens were dissected, and visible medullary parts were carefully separated within 0.5 h if used for mRNA analysis. Tissues were fixed in buffered 10% formalin or Bouin's fixation fluid (n = 13). Samples were routinely processed for light microscopic study, and stained with hematoxylin-eosin or with van Gieson stain. Each tumor had been diagnosed as part of the routine diagnostic procedures, but all cases were re-evaluated by two investigators (KS and PH) for the present study. There was no discrepancy between the two investigators in classifying the tumors. The tissue material used in each study is shown in Table 5. The weight or size of the tumors was scored as follows: 1 = <30 g weight or ≤4.0 cm diameter, 2 = 30-100 g weight or 4.1-7.9 cm diameter, 3 = >100 g weight or ≥8.0 cm diameter (Linnoila et al., 1990). The following features suggestive of malignancy were evaluated from the tumors: mitotic count per 10 HPFs, necrosis, vascular, or capsular invasion. Tumors that did not exhibit any of these features were evaluated as benign, the rest were considered borderline tumors. Only metastasized or extensively invasive tumors were considered malignant.

The clinical data collected from each patient included age at the time of diagnosis, gender, associated syndromes, catecholamine secretion, location and size of the tumor, and the clinical outcome of the disease. The actual clinical data were checked at the end of November 2000, and the survival data at the end of January 2002. The survival data and the

cause of death were obtained from the Population Registry of Finland. The numbers of cases included in each study are given in Table 5.

2. Immunohistochemistry (Study I-V)

Sections of 4-µm were cut from paraffin-embedded blocks, deparaffinized in xylene, and rehydrated in a series of graded alcohols. The endogenous peroxidase activity was blocked with hydrogen peroxide in methanol for 30 min, and unspecific binding sites were blocked in blocking solution (1.5:100 normal horse serum in PBS) for 15 min. Antibodies (Tables 6 and 7) were applied overnight in PBS at +4°C or room temperature (see original publications I-V). The detections were performed using the Vectastain ABC Kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions, and sections were lightly counterstained with hematoxylin. To exclude the effect of possible endogenous biotin on immunohistochemical staining, biotin blocking (Avidin-biotin blocking kit, Vector Laboratories, Burlingame, CA, USA) was performed in at least one sample of each diagnostic group before the addition of the primary antibody. Sections fixed in Bouin's fixative were excluded from the Ki-67 analysis. All antibodies were monoclonal. In inhibin/activin βA and βB immunostaining, omission of primary antibodies and staining with nonimmunized mouse IgG_2 and (in the case of βA-subunit) with the primary antibody preabsorbed with a 7-fold molar excess of recombinant human activin A, were used as negative controls. Recombinant activin B was not available for preabsorbtion. Omission of primary antibody was used as a negative control in all other immunohistochemical stainings. In COX-2 immunostaining, specificity of the antibody was determined by preadsorption of the primary antibody with human COX-2 control peptide (10µg/ml; Cayman Chemical Co, Ann Arbor, MI, USA) for 1 h at room temperature before the staining.

Table 6. Antibodies and their sources.

Antibody	Source	Study
Ki-67	Immunotech, Marseille, France	I
p53	Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA	I
p21	Novo Castra Laboratories, Newcastle upon Tyne, UK	I
Inhibin/activin βA	MCA950S, Serotec Ltd, Oxford, UK	II
Inhibin/activin βB	MCA1661, Serotec Ltd, Oxford, UK	II
VEGF	MAB293, clone 26503.11, R&D systems, Minneapolis, MN, USA	III
CD34	347660, Becton Dickinson Immunocytometry Systems, San Jose, CA, USA	III
Tenascin	clone DB7, Locus Oy, Helsinki, Finland	IV
COX-2	160112, Cayman Chemical Co., Ann Arhor, MI, USA	V

Table 7. Dilutions, antigen retrieval, and categories used.

Antibody	Dilution	Antigen retrieval	Categories used
Ki-67	1:500	microwave	0-5%, 6-10%, 11-20%, 21-100%
p53	1:300	microwave	0-9% = no overexpression, ≥10% = overexpression
p21	1:30	microwave	0-9% = no overexpression, ≥10% = overexpression
Inhibin/activin βA	1:50	microwave	0%,1-10%, 11-50%, 51-100%
Inhibin/activin βB	1:50	microwave	0%,1-10%, 11-50%, 51-100%
VEGF	1:1000	Target Retrieval Solution	0%,1-10%, 11-50%, 51-100%
CD34	1:100	microwave	microvessels/mm^2
Tenascin	1:2000	trypsin	0-4%, 5-25%, 26-50%, 51-100%
COX-2	1:200	microwave	0%, 1-19%, 20-49%, 50-100%

2.1 Microvessel density (Study III)

The microvessel density was assessed in three nonoverlapping areas of highest neovascularization at high power (x300; 0.32 mm^2), and reported per one mm^2. The areas of highest neovascularization were found most frequently at the margins of the tumors, but could occur anywhere in the tumor. Vessel lumens were not necessary for a structure to be defined as a microvessel (Weidner et al., 1991). Any red-brown-staining endothelial cell or endothelial-cell cluster that was clearly separete from adjacent microvessel was considered a single, countable microvessel.

3. Northern blot analysis (Study II, III, V)

The number of tumors studied with Northern blot analysis in each study is shown in the original publications II, III, and V. For Study II inhibin/activin βB-subunit messenger RNA (mRNA) was detected by Northern blots hybridized with two radiolabeled oligonucleotides complementary to different regions of human inhibin/activin βB mRNA. The sequences were 5'-GTG GAA GGA GGA GGC AGA GCC GGG GAC CCC-3' and 5'-GGG CAC GTC CCG CTT GAC GAT GTT GTA CTC-3' complementary to nucleotides 864-893 and 1002-1031 of the βB-subunit mRNA, respectively (GenBank accession no. M13437) (Mason et al., 1986). The mouse ribosomal 28S RNA complementary to DNA was used as a loading control (Arnheim, 1979). The oligonucleotides and 28S complementary DNA were labeled as previously described (Liu et al., 1994).

For Study III RNA isolation and Northern blot hybridization were performed as described previously (Liu et al., 1994). A 30-mer oligonucleotide probe for VEGF mRNA was synthesized at the Institute of Biotechnology, University of Helsinki. The sequence was 5'-GTA CTC CTG GAA GAT GTC CAC CAG GGT CTC-3' corresponding to nucleotides 182-211 of the human VEGF gene (GenBank accesion no NM003376). The ribosomal 28S RNA was used as a loading control.

For Study V human COX-2 and β-actin cDNAs were labeled using [α-^{32}P]deoxy-CTP (NEN Life Science Products, Boston, MA, USA) and Prime-a-Gene kit (Promega Corp., Madison, WI, USA) (Ristimäki et al., 1994). Probes were purified with nick columns (Pharmacia, Uppsala, Sweden) and used at 1 x 10^6 cpm/ml. Hybridizations were performed at 60°C for 16 h in ExpressHyb Hybridization solution (CLONTECH Laboratories, Inc., Palo Alto, CA, USA).

4. Western blot analysis (Study V)

For COX-2, Western blotting samples were homogenized in radioimmunoprecipitation assay buffer [150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, and 50 mM Tris (pH 8.0)] supplemented with Complete mini protease inhibitor cocktail tablets (Roche Diagnostics GmbH, Mannheim, Germany). Proteins (100 μg) were transferred electrophoretically to Hybond-C membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK), and nonspecific binding was blocked by TBS-NP40-5% low-fat dry milk solution overnignt at 4°C. For immunodetection, the membranes were incubated with the monoclonal COX-2 antibody (1:1000) for 1h. Membranes were washed three times in TBS-NP40, and incubated with sheep antimouse antibodies conjugated to horseradish peroxidase (1:2000, ECL Western blotting analysis system, Amersham Pharmacia Biotec) for 1h.

5. Statistical analysis (Study I-V)

The κ statistic was used to examine the interobserver variation in the scoring of the staining. The analysis of variance was used to determine the statistical significance of the Ki-67 immunohistochemistry, the mitosis count, the size, and the age in Study I. Statistical significances of p53 and p21 were calculated with χ^2-test, which was also used to analyze the significance of the gender. Statistical analysis of the RNA and immunohistochemical results in Studies II, IV and V was performed using the Mann-Whitney test. The correlation between inhibin/activin βB-subunit immunoreactivity and RNA expression in Study II was analyzed by Spearman's test. The statistical significance in VEGF immunohistochemisty in Study III and the significance of the histological features in Study I was calculated with Fisher´s exact test. Analysis of variance was used to determine the statistical significance of microvessel density. The level of significance in all studies was chosen as $p < 0.05$.

RESULTS

1. Clinical and histopathological data (Study I)

Table 8. Clinical features and tumor characteristics of 105 pheochromocytomas from 97 patients.

	Benign		**Borderline**		**Malignant**	
	Adrenal	Extra	Adrenal	Extra	Adrenal	Extra
Total no. of tumors	37	23	29	8	3	5
Sex						
Female	22	10	20	4	0	1
Male	12	11	9	4	3	4
Age (yr)						
Female						
Mean	48.1	50.2	46.8	47.8	-	46.0
Range	25-73	27-72	17-82	32-62	-	
Male						
Mean	52.2	47.9	44.6	48.8	44.0	42.0
Range	35-69	21-69	17-64	20-65	18-69	31-51
Associated syndromes						
von Hippel-Lindau	2		3		0	
Neurofibromatosis 1	1		1		1	
MEN II	7		1		0	
Follow-up (months)						
Mean	84.2	50.3	96.7	58.0	100.3	52.6
Range	18-295	0-96	15-295	0-131	0-197	11-84
Left adrenal	17		11		0	
Right adrenal	20		18		3	
Tumor size						
<30g or <4cm	21	14	4	6	1	0
30-100g or 4.1-7.9cm	12	9	17	2	0	4
>100g or >8cm	3	0	8	0	2	1
Unknown	1					
Number of mitoses						
Mean	0.8	1.0	2.1	1.5	5.7	7.4
Range	0-4	0-4	0-13	0-5	3-11	0-12

Adrenal, adrenal pheochromocytoma; extra, extraadrenal pheochromocytoma

The clinical data are summarized in Table 8. Five patients, three women and two men, had a bilateral tumors. One woman had a bilateral adrenal pheochromocytoma and a new

adrenal tumor 21 years later. One man had one tumor in the right carotid body and one in the retroperitoneum. Two of these patients with bilateral tumors had MEN II and one had von Hippel-Lindau disease. The secretion of either metanephrines or normetanephrines was above the reference limit in all adrenal pheochromocytoma patients on whom data were available.

Pheochromocytomas were regarded as malignant in case of histologically or radiologically proven distant metastatic lesions, and in one patient with extensive invasion into the spinal vertebral body**.** Three primary tumors were located in the right adrenal. Five tumors were extraadrenal, two were in the retroperitoneum, one in the aortic bifurcation, one in the carotid body, and one in the right upper abdomen. All primary tumors had at least one histologically suspicious feature, and, indeed, five out of eight displayed three or more of these suspicious features (Table 9). Two of them had neither confluent necrosis nor vascular or capsular invasion, but their mitosis frequency was high, over 10/HPF.

Twenty-nine adrenal and eight extraadrenal tumors without metastases, but having any of the histologically suspicious features, i.e. over five mitoses / 10 HPF, confluent tumor necrosis, vascular or capsular invasion (Linnoila et al., 1990), were evaluated as a separate group, and are here called borderline tumors. Thirty-seven adrenal and 23 extraadrenal pheochromocytomas had none of these histologically suspicious features and were considered benign (Table 9).

Table 9. Number of histologically suspicious features (over 5 mitoses / 10 HPFs, tumor necrosis, vascular or capsular invasion) in benign, borderline, and malignant pheochromocytomas.

No. of features	**0**	**1**	**2**	**3**	**4**
No. of tumors					
Benign					
Adrenal (n = 37)	37	0	0	0	0
Extraadrenal (n = 23)	23	0	0	0	0
Borderline					
Adrenal (n = 29)	0	19	8	2	0
Extraadrenal (n = 8)	0	6	2	0	0
Malignant					
Adrenal (n = 3)	0	1	1	1	0
Extraadenal (n = 5)	0	1	0	3	1

Statistically, gender or age had no significant difference in adrenal ($p = 0.08$, $p = 0.78$, respectively) and in extraadrenal tumors ($p = 0.62$, $p = 0.72$, respectively). The size of both adrenal ($p = 0.04$) and extraadrenal ($p < 0.01$) malignant tumors was significantly larger. The number of mitoses was significantly higher in borderline ($p = 0.01$) and malignant ($p < 0.01$) adrenal tumors than in benign ones. In extraadrenal tumors, this difference was

evident in malignant tumors ($p < 0.01$), but not in borderline ones. When borderline and malignant tumors were compared, the number of histologically suspicious features was not significant in adrenal tumors, but a difference was found in extraadrenal ones ($p = 0.01$). When malignant tumors were compared to both benign and borderline ones, however, a statistical difference in histopathological features was found in both adrenal ($p = 0.02$) and extraadrenal tumors ($p < 0.01$).

2. p53 (Study I)

p53 overexpression was considered present, if 10% or more of the tumor cells showed immunopositivity. All benign (n = 37) as well as borderline (n = 29) adrenal pheochromocytomas showed less than 10% p53 expression. Only one malignant adrenally located tumor overexpressed p53, however, indicating a statistically significant difference in p53 overexpression between benign and borderline adrenal tumors compared to malignant ones ($p < 0.01$). The staining was more heterogenous in extraadrenal tumors, and no statistical differences were found.

3. p21 (Study I)

Only one extraadrenally located benign pheochromocytoma (n = 60) overexpressed p21, disclosing the expected nuclear staining in more than 10% of the tumor cells. In the borderline group (n = 37) one adrenal pheochromocytoma expressed p21 in 15% of the tumor cells. All malignant tumors showed less than 10% p21 positivity. Therefore, p21 expression was not significantly different in these tumor groups.

4. Ki-67 (Study I)

All benign and 18 out of 21 borderline adrenal pheochromocytomas (85.7%) had at the most 5% immunopositivity for Ki-67 (Table 10). Also, 12 out of 23 (52.2%) benign extraadrenal pheochromocytomas and five out of eight (62.5%) borderline extraadrenal tumors showed at the most 5 % Ki-67 positivity. Two malignant adrenal tumors had 17% and 13% positivity. Unfortunately one malignant tumor was fixed in Bouin's fluid and was therefore rejected from Ki-67 staining. Proliferative activity in extraadrenal malignant tumors was more heterogenous, as one of these tumors had only 1% positivity, two had 10%, and two over 21% positivity. Statistically a significant difference was found between malignant and benign adrenal ($p < 0.01$) as well as extraadrenal ($p < 0.01$) pheochoromocytomas.

Table 10. Scoring of immunoreactivity for Ki-67.

Immunoreactivity	0-5%	6-10%	11-20%	21-100%	Total
No. of tumors					
Benign					
Adrenal	33	0	0	0	33
Extraadrenal	12	9	2	0	23
Borderline					
Adrenal	18	2	1	0	21
Extraadrenal	5	2	1	0	8
Malignant					
Adrenal	0	0	2	0	2
Extraadrenal	1	2	0	2	5

5. Inhibin/activin (Study II)

5.1 Normal adrenal gland

Strong positive inhibin/activin βB-subunit immunostaining was found in the normal adrenal medulla, whereas the cortex was negative. The immunohistochemical distribution and intensity of the staining were similar in all normal adrenal grands (n = 7). Normal medulla was immunonegative for inhibin/activin βA, while weak diffuse positivity was seen in the cortex, mainly in the inner zones. Northern blot analysis revealed a predominant 4.8-kb inhibin/activin βB-subunit transcript in all normal adrenal medullary samples (n = 4).

5.2 Pheochromocytomas

Twenty-seven out of 30 (90%) benign adrenal pheochromocytomas showed strong or moderate immunoreactivity for the inhibin/activin βB-subunit, whereas all seven malignant tumors were negative or only weakly positive (Table 11). Most borderline adrenal tumors (17 out of 23, 74%) showed strong or moderate immunopositivity. The profile of the βB-subunit immunoreactivity was slightly different in extraadrenal tumors, as 10 out of 17 (59%) benign and four out of six (67%) borderline extraadrenal tumors showed no or only weak reactivity. One of the five metastases expressed moderate immunopositivity, whereas the rest were negative or only weakly positive for the βB-subunit. Immunohistochemistry with the βA-subunit-specific antibody revealed mostly negative results. Only three out of ten benign extraadrenal pheochromocytomas were weakly positive.

Table 11. Scoring of immunoreactivity for the inhibin/activin βB-subunit.

Immunoreactivity	Negative 0%	Weak 1-10%	Moderate 11-50%	Strong 51-100%	Total
No. of tumors					
Benign					
Adrenal	2	1	7	20	30
Extraadrenal	7	3	5	2	17
Borderline					
Adrenal	2	4	4	13	23
Extraadrenal	4	0	1	1	6
Malignant					
Adrenal	1	2	0	0	3
Extraadrenal	0	4	0	0	4

Statistically, a significant difference was found in immunohistochemical inhibin/activin βB-subunit expression between benign and malignant adrenal tumors ($p = 0.006$) as well as between borderline and malignant adrenal tumors ($p = 0.027$). Statistical differences were not found between any other groups.

Northern blot analysis showed an inhibin/activin βB-subunit transcript in all pheochromocytomas studied ($n = 18$). The relative βB-subunit mRNA levels varied considerably in the different tumors, as well as in the normal adrenal medullary samples. No statistical differences in the βB-subunit mRNA levels among the different tumor groups were found.

6. VEGF and microvessel density (Study III)

6.1 Normal adrenal gland

In normal adrenal gland ($n = 6$) weak and patchy cytoplasmic VEGF positivity was seen only in the zona reticularis of the cortex. Medullary cells were negative in all samples. Northern blot analysis revealed VEGF mRNA in all adrenal samples studied.

6.2 Pheochromocytomas

Most benign adrenal pheochromocytomas (26 of 37, 70.3%) expressed VEGF only weakly, or were totally negative (Table 12). In benign extraadrenal tumors, only 34.8% (8 of 23) were negative or weakly positive. In borderline adrenal tumors, the immunohistochemical expression of VEGF was enhanced, as 41.4% (12 of 29) were moderately and 20.7% (6 of

29) strongly positive. Half of the extraadrenal borderline tumors showed moderate (2 of 8, 25.0%) or strong (2 of 8, 25.0%) immunopositivity. All malignant adrenal and extraadrenal tumors (n = 8) were strongly (5 of 8, 62.5%) or moderately (3 of 8, 37.5%) positive. Two metastases of adrenal tumors showed weak and one moderate immunopositivity, while two metastases of the extraadrenal tumors had moderate and one had strong positivity. Statistically, a significant difference was found in immunohistochemical VEGF expression between benign and malignant adrenal pheochromocytomas ($p = 0.013$) as well as between benign and borderline adrenal tumors ($p = 0.025$).

Table 12. Scoring of immunoreactivity for VEGF in 105 pheochromocytomas.

Immunoreactivity	**Negative** (<1%)	**Weak** (1-10%)	**Moderate** (11-50%)	**Strong** (51-100%)	**Total**
No. of tumors					
Benign					
Adrenal	10	16	9	2	37
Extraadrenal	2	6	5	10	23
Borderline					
Adrenal	7	4	12	6	29
Extraadrenal	1	3	2	2	8
Malignant					
Adrenal	0	0	1	2	3
Extraadrenal	0	0	2	3	5

Northern blot analysis revealed VEGF mRNA in all pheochromocytomas (n = 22) and metastases (n = 3). The mean of relative intensities of the autoradiographic signal was higher in borderline tumors (n = 6) compared to benign adrenal ones (n = 13). However, RNA was available from only one malignant adrenal and two extraadrenal tumors, so the number of tumors is too small for statistical analysis.

The mean microvessel density (MVD) varied greatly in the different tumor groups, and ranged from 105 to 828/mm^2. There was no significant association between vessel counts and tumor groups. VEGF expression did not correlate with MVD in these tumors either.

7. Tenascin (Study IV)

7.1 Normal adrenal gland

In normal adrenal gland (n = 5) patchy tenascin immunopositivity was seen mostly in sinusoids of the zona reticularis, and to a lesser extent in sinusoids of the zona fasciculata. Tenascin expression was also seen in the walls of some vascular vessels. All samples of normal medulla stained negative.

7.2 Pheochromocytomas

In the tumor samples, tenascin positivity was seen in the stroma surrounding the tumor cells. No cytoplasmic staining was seen in any of the tumor cells. Only the stromal staining within the tumor was interpreted and given as an area percentage of positive stromal staining. The staining surrounding the tumor as well as the staining in larger vessel walls was excluded.

Most benign pheochromocytomas (26 of 37, 70%) were negative or expressed tenascin only weakly (Table 13). The staining profile in benign extraadrenal pheochromocytomas was slighty different; 52% of the tumors were negative or only weakly positive. In borderline pheochromocytomas the staining was enhanced, as 46% of the adrenal and 50% of the extraadrenal tumors showed moderate or strong immunopositivity. Malignant adrenal pheochromocytomas expressed tenascin either strongly (2 of 3, 67%) or moderately (1 of 3, 33%). Two of four malignant extraadrenal tumors showed strong staining, whereas two expressed tenascin weakly. Only the primary tumors were analyzed for tenascin expression. Statistically, a significant difference was found between benign and malignant adrenal tumors ($p = 0.034$), as well as between borderline and malignant adrenal tumors ($p = 0.023$). No statistical difference was found between benign and borderline pheochromocytomas, nor between any of the extraadrenal pheochromocytoma groups.

Table 13. Scoring of immunoreactivity for tenascin.

Immunoreactivity	**Negative** <5%	**Weak** 6-25%	**Moderate** 26-50%	**Strong** >51%	**Total**
No. of tumors					
Benign					
Adrenal	14	12	4	7	37
Extraadrenal	1	11	6	5	23
Borderline					
Adrenal	6	9	11	2	28
Extraadrenal	1	3	2	2	8
Malignant					
Adrenal	0	0	1	2	3
Extraadrenal	0	2	0	2	4

8. COX-2 (Study V)

8.1 Normal adrenal gland

The cortex of the normal adrenal glands (n = 5) was positive for COX-2 as detected by immunohistochemistry using the monoclonal antibody. In contrast, no COX-2 immunoreactivity was evident in the medulla of the normal adrenals. Northern blot revealed COX-2 mRNA in a normal adrenal medullary, as well as in a cortical sample.

8.2 Pheochromocytomas

Most benign adrenal pheochromocytomas (27 of 36, 75%) showed no or only weak immunopositivity for COX-2 (Table 14). Extraadrenally located tumors stained differently, as most of them (15 of 18, 83%) showed either moderate or strong positivity. Borderline adrenal tumors stained as benign ones, as most of them showed no (6 of 24, 25%) or only weak (13 of 24, 54%) immunopositivity. Extraadrenally located borderline tumors were mostly strongly (3 of 6, 50%) or moderately (2 of 6, 33%) positive. All malignant pheochromocytomas, regardless of their primary location, showed strong (6 of 8, 75%) or moderate (2 of 8, 25%) COX-2 immunoractivity. The expression was weak in all the metastases (n = 3) from the adrenal tumors, whereas metastases (n = 3) from the extraadrenal tumors exhibited either moderate or strong immunoreactivity. The immunohistochemical expression was confirmed by Western blotting, which revealed a 79-kDA band corresponding to COX-2 protein in each tumor. Statistically a significant difference was found in immunohistochemical COX-2 expression between benign and malignant adrenal tumors ($p = 0.014$) as well as between borderline and malignant adrenal tumors ($p = 0.012$).

Table 14. Scoring of immunoreactivity for COX-2.

Immunoreactivity	**Negative** (0%)	**Weak** (1-19%)	**Moderate** (20-49%)	**Strong** (50-100%)	**Total**
No. of tumors					
Benign					
Adrenal	8	19	5	4	36
Extraadrenal	2	1	2	13	18
Borderline					
Adrenal	6	13	3	2	24
Extraadrenal	0	1	2	3	6
Malignant					
Adrenal	0	0	1	2	3
Metastases	0	3	0	0	3
Extraadrenal	0	0	1	4	5
Metastases	0	0	1	2	3

Northern blot analysis showed low levels of COX-2 mRNA in most pheochromocytomas studied (n = 11), even though one tumor in each diagnostic group (benign, borderline, malignant, and metastasis) did not show any transcript. The relative intensities of the autoradiographic signals ranged from 0-0.090 (median, 0.051) in nonmetastasized pheochromocytomas, and from 0-0.184 (median, 0.060) in metastasized pheochromocytomas.

9. Summary table

Table 15. Summary of all molecular markers suggesting benign, borderline, or malignant nature of a pheochromocytoma. The agreement between the pathologists were either good or very good in all the studies when κ statistics was used to examine the interobserver variation in the scoring of the staining.

Immuno-reactivity	**p53** (≥10%)	**p21** (≥10%)	**Ki-67** (≥6%)	**inh βB** (>10%)	**VEGF** (>10%)	**Tenascin** (>25%)	**COX-2** (≥20%)
No. of tumors							
Adrenal							
Benign	0/37	0/37	0/33	27/30	11/37	11/37	9/36
Borderline	0/29	1/29	3/21	17/23	18/29	13/28	5/24
Malignant	1/3	0/3	2/2	0/3	3/3	3/3	3/3
Extraadrenal							
Benign	6/23	1/23	11/23	7/17	15/23	11/23	15/18
Borderline	3/8	0/8	3/8	2/6	4/8	4/8	5/6
Malignant	1/5	0/5	4/5	0/4	5/5	2/4	5/5

DISCUSSION

Pheochromocytomas are uncommon, mostly benign, sympathoadrenal tumors. However, approximately 10% of these tumors metastasize, and cause morbidity as well as mortality. The diagnosis and the treatment of pheochromocytomas are well established, but the main problem for clinicians and pathologists is to predict the future behavior of the tumor. Thus far, no definite marker for malignancy, other than metastatic growth, has been found. In our studies we have focused on searching for markers that would facilitate the diagnosing of benign or malignant pheochromocytoma.

1. Histopathology

Although the histologic diagnosis of a pheochromocytoma is relatively easy to make, the distinction between benign and malignant tumor based on histological grounds alone is not easy, if not impossible. Necrosis, vascular invasion, and extensive local invasion may indicate malignant behavior in pheochromocytomas (Linnoila et al., 1990; van der Harst et al., 2000). Thompson evaluated 50 histologically malignant adrenal pheochromocytomas, 33 of them metastasizing, and 50 histologically benign, and reported that malignant tumors more frequently demonstrated diffuse growth, necrosis, high cellularity, cellular monotony, tumor cell spindling, mitotic figures >3/10 HPF, atypical mitoses, vascular, capsular, or local invasion, pleomorphism, and hyperchromasia (Thompson, 2002). However, Thompson's scoring method can be quite laborious in practice and may not be reproducible. Also, the potential drawback of this scoring method is the lack of an intermediate category (undetermined malignant potential, low grade malignant tumor, uncertain malignant potential). In our study, only metastasized tumors were considered malignant. Tumors with confluent necrosis, over 5 mitoses/10 HPF, vascular or capsular invasion, were evaluated separately, and called borderline tumors. All malignant tumors displayed at least one of the suspicious features, most of them several. However, it is important to point out that no single histologic feature is diagnostic for malignancy. Perhaps an intermediate category is needed also in pheochromocytomas, as is the case in many other tumors, such as neuroendocrine tumors of the lung, bladder neoplasms, or ovarian tumors.

2. Growth capacity

The growth capacity of a tumor is essential for malignancy. It can be assessed by counting the mitoses in a certain area of a tumor, but it is a challenging and slightly unreliable method. Mitotic figures can be difficult to interpret due to overstaining, poor sectioning, inadequate fixation, and pycnotic nuclei. Indeed, mitotic count has been reported rather unreliable in predicting the malignancy of pheochromocytomas (Linnoila et al., 1990). In

our study we found a significantly higher number of mitoses in malignant tumors. However, proliferative activity should not be measured by merely counting the mitoses, but by resorting to immunohistochemical evaluation with Ki-67 or MIB-1. A reliable distinction can be made between apoptotic and mitotic nuclei by using Ki-67. A few studies on proliferative activity in pheochromocytomas have been done. Clarke et al. (1998) studied 23 benign and 10 malignant pheochromocytomas, and reported 5% proliferative activity in the malignant ones. Nagura et al. (1999) suggest that a high MIB-1 index, over 2%, is associated with malignancy. Van der Harst et al. (2000) studied a large number of malignant (n = 37) and benign (n = 99) pheochromocytomas and reported that none of the benign, but 50% of the malignant ones had a proliferative index greater than 2.5%. In our material of 105 pheochromocytomas, proliferative activity was also significantly higher in malignant tumors, as six out of seven malignant pheochromocytomas had over 6% immunohistochemical positivity of Ki-67. Thus, our study further emphasizes the value of Ki-67 immunohistochemistry in predicting the malignancy of pheochromocytomas.

3. Growth inhibition

Inhibins and activins are members of the TGFβ superfamily of growth and differentiation factors (Risbridger et al., 2001). Two previous studies have documented the presence of inhibin/activin β-subunits in the adrenal gland. Immunoreactivities for βA- and βB-subunits have been reported in the adrenal cortex (Munro et al., 1999; Spencer et al., 1992), whereas the medulla was negative. In our study, weak diffuse immunopositivity for the inhibin/activin βA-subunit was seen in the cortical tissue, mainly in the inner zones, leaving medullary cells negative. However, the normal adrenal medulla was strongly positive for the inhibin/activin βB-subunit, whereas the cortex was negative. The differences between our results and the previous ones may be explained by different specificities of the antibodies used. Previous studies have been done with polyclonal antibodies, while we used monoclonal ones. Most benign adrenal (27 of 30, 90.0%) and borderline adrenal (17 of 23, 73.9%) pheochromocytomas also showed strong or moderate immunostaining for the inhibin/activin βB-subunit, whereas all seven malignant tumors, regardless of their location, had only weak or no immunoreactivity. It seems that pheochromocytomas lose their potential to express inhibin/activin βB-subunit when they become malignant. The staining in extraadrenally located tumors was more heterogenous, as four of six borderline tumors and seven of 17 benign tumors were immunohistochemically negative for the inhibin/activin βB-subunit. So, in the case of extraadrenal tumors, the lack of βB-subunit expression is not a reliable sign of malignancy.

Cyclin-dependent kinases are major regulators of the cell cycle. p21 cyclin-dependent kinase inhibitor is commonly altered in human malignancies. Its immunohistochemical expression has been reported to correlate with a good prognosis in some epithelial tumors

(el-Deiry et al., 1994; Gomyo et al., 1997). In our study, however, over 10% immunohistochemical expression of p21 was seen only in one benign extraadrenal and in one borderline extraadrenal pheochromocytoma. Thus, it is suggested that p21 does not have any significant role in pheochromocytomas.

4. Apoptosis and p53

p53 tumor-suppressor gene was initially found to be essential for the DNA-damage ckeckpoint in the cell cycle. Activation of normal *p53* by DNA damaging agents stimulates the transcription of several genes that mediate the two major effects of p53: cell-cycle arrest and induction of DNA repair, and apoptosis. In cells with loss or mutation of *p53*, DNA damage does not induce cell cycle arrest or apoptosis, and hence genetically damaged cells proliferate, and eventually give rise to malignant tumors. Over 50% of human tumors contain mutations in the *p53* tumor-suppressor gene. Nonmutant (wild-type) p53 protein is rapidly degraded and not detectable in most tumors, while mutant p53 is stable and is associated with many solid tumors. The possible role of p53 in pheochromocytomas has also been studied by other investigators. De Krijger et al. (1999) found a significantly higher frequency of p53 protein expression in malignant pheochromocytomas (n = 29) than in benign ones (n = 85). However, Gupta et al. (2000a) found no differences in p53 expression in 30 benign and 20 malignant pheochromocytomas. Dahia et al. (1995) found no mutation of the *p53* gene in 25 pheochromocytomas, and only one of the tumors showed over 10% immunohistochemical expression. In our study, all benign and borderline adrenal tumors were negative, but one malignant adrenal tumor overexpressed p53. Although p53 was found statistically significant for malignancy ($p < 0.01$), definite conclusions cannot be drawn because of the limited number of malignant adrenal tumors. In extraadrenally located tumors the staining was more heterogenous, and statistically significant differences were not found.

The role of other apoptotic markers, such as apoptosis inhibiting Bcl-2 and apoptosis-inducing Bax, in the malignancy of pheochromocytomas has been studied by others, but further investigations are needed. De Krijger et al. (1999) studied 29 malignant and 85 benign pheochromocytomas, and suggested that overexpression of Bcl-2 is involved in the pathogenesis of pheochromocytomas. Kanauchi et al. (2002) demonstrated Bcl-2 and Bax RNA expression in 20, appearently benign pheochromocytomas.

5. Angiogenesis

Tumor growth over the size of 2-3 mm^2 is possible only if sufficient angiogenesis occurs to maintain nutrition and oxygen support. The development of new blood vessels is stimulated by angiogenic cytokines, secreted by tumor cells. The most important angiogenic cytokine, VEGF, stimulates endothelial cell growth and promotes angiogenesis (Folkman et al., 1989). It has been widely reported that the levels of VEGF are often

higher in malignant tumors than in normal tissues (Dobbs et al., 1997; Guidi et al., 1996). Malignant cells produce VEGF in response to hypoxia and inflammation, or if they have undergone genetic changes (Bouck et al., 1996). Several studies have shown that overexpression of VEGF is associated with the grade of angiogenesis, determined by measurements of the microvessel density (MVD) (Toi et al., 2001). We found strong or moderate expression in all malignant pheochromocytomas, and negative or weak expression in benign adrenal tumors. Normal adrenal medulla was negative. MVD nevertheless varied greatly in the different tumor groups and did not correlate with malignancy. These findings support the hypothesis which suggests that the overall angiogenesis phenotype is determined by a balance between pro- and antiangiogenic factors. For example, not all tumors expressiong VEGF are highly angiogenic. Potent negative regulators of angiogenesis, thrombospondins 1 and 2, are significantly associated with a favorable prognosis, and the combined status of thrombospondins and VEGF is a more precise indicator of prognosis than their individual status (Oshika et al., 1998). The role of thromobospondins in the angiogenesis and pathogenesis of pheochromocytomas is yet to be determined.

6. Invasion and metastasis

The interaction between extracellular matrix and tumor cells is important in tumor invasion and metastasis. As tenascin is believed to have an active role in epithelial-mesenchymal interactions, the overexpression of tenascin may facilitate tumor cell invasion during tumor progression. Increased tenascin expression has been demonstrated in many malignant tumors. In small axillary node-negative breast carcinomas, the expression of tenascin at the invasion border has been found to be a prognostic factor for both local breast cancer recurrence and distant metastasis (Jahkola et al., 1998; Jahkola et al., 1996). Its expression has also been demonstrated to be markedly increased in the stroma surrounding the invasive nests of squamous cancer cells of the uterine cervix, but not around pseudoinvasion or invaginating papillae of abnormal squamous epithelium (Iskaros and Koss, 2000). Opposite views have also been expressed, as Pilch et al. (1999) reported that tenascin-positive cervical carcinoma patients had a significantly better prognosis than tenascin-negative patients. In this study we found weak or negative tenascin expression in most of the benign pheochromocytomas, whereas all malignant adrenal tumors were strongly or moderately positive. It seems that tenascin expression is upreguated in the neoplastic events of adrenal medulla, and that tenascin plays some role in invasion and metastasis in pheochromocytomas. Previously Yoshida et al. (1999) found tenascin in the cytoplasm of invasive laryngeal cancer cells, suggesting that these cancer cells could themselves produce and secrete tenascin. However, most other reports do not support this theory (Howeedy et al., 1990; Koukoulis et al., 1991; Pilch et al., 1999). We found tenascin positivity exclusively in the stroma, whereas no expression was observed in the tumor cells. Thus, pheochromocytoma cells are unlikely capable of producing or secreting tenascin.

Overexpression of COX-2, and high concentrations of prostaglandins, have been associated with several types of human cancer. During cancer progression, prostaglandins

can mediate several steps, including cell proliferation (Hanif et al., 1996), apoptosis (Sheng et al., 1998), modulation of the immune system (Hla et al., 1993), and angiogenesis (Tsujii et al., 1998). COX-2 has been most throughly studied in colorectal cancers (Dubois et al., 1998; Taketo, 1998b; Williams et al., 1999b). COX-2 overexpression in colorectal cancers has been shown to correlate with larger size, more advanced Dukes stage, and is particularly evident in tumors with lymph node metastasis (Sheehan et al., 1999). Cells that express COX-2 have also been shown to be more invasive than human colon cancer cells that do not express COX-2 (Tsujii et al., 1997). Furthermore, Ristimäki et al. (2001) reported that in transitional cell bladder carcinoma, COX-2 immunoreactivity is most prominent in invading cells. Enhanced hematogenous metastasis of human colorectal cancer (Tomozawa et al., 2000) and lymphatic invasion and metastasis of human gastric carcinoma (Murata et al., 1999) are shown to be associated to COX-2 overexpression. In our study we have shown that most of the benign pheochromocytomas are negative or only weakly immunopositive, whereas malignant tumors exhibited strongly enhanced COX-2 immunopositivity. Interestingly, four metastases expressed weaker immunopositivity than their primary tumors, while in two cases the immunopositivity remained strong. The expression of COX-2 was more enhanced in extraadrenal tumors than in adrenal ones. 83.3% of the extraadrenally located pheochromocytomas and 20.8% of the adrenal ones that showed capsular or vascular invasion, confluent tumor necrosis, or over five mitoses per 10 HPFs, expressed COX-2 moderately or strongly. It is possible that some of the tumors evaluated as borderline may in fact have had malignant potential, but were without metastases at the time of operation, and were thus treated curatively by surgery. The strong COX-2 expression in extraadrenal pheochromocytomas might have two explanations. Firstly, extraadrenally located tumors tend to behave more aggressively (Linnoila et al., 1990). Secondly, extraadrenal tumors lack the special hormonal environment of the adrenal gland, and this may also influence the COX-2 expression.

CONCLUSIONS

These studies have focused on seaching new markers that would predict the future behavior of a pheochromocytoma. For this purpose we have collected 105 pheochromocytomas, 69 of them adrenal and 36 extraadrenal. Eight tumors were malignant, 37 were called borderline tumors and had histologically suspicious features: over 5 mitoses/10 HPF, necrosis, capsular or vascular invasion, but had not metastasized. Sixty tumors were benign. All malignant tumors showed at least one of the histologically suspicious features, five of them displayed three or more of these features. Malignant tumors were larger, but the age and gender of the patients did not differ significantly. The proliferative activity measured with Ki-67 was higher in malignant tumors than in benign ones in both adrenal and extraadrenal pheochromocytomas. p53 was overexpressed in two malignant tumors, while p21 expression did not correlate with malignancy.

The inhibin/activin βB-subunit was strongly expressed in normal adrenal medullary cells. Strong or moderate staining was seen in 90% of the benign adrenal pheochromocytomas and 74% of the borderline ones, whereas the malignant tumors were mostly negative. The expression was more heterogenous in extraadrenal tumors. Evidently, adrenal tumors lose their potential to express inhibin/activin βB when they become malignant.

VEGF was not found immunohistochemically in normal adrenal medulla. All malignant pheochromocytomas, regardless of their primary location, had strong or moderate VEGF immunoreactivity, while most (70.3%) benign adrenal pheochromocytomas were either negative or only weakly positive. In borderline tumors the expression was enhanced. So, VEGF could be used as an immunohistochemical marker for those adrenal tumors that might behave in a more benign manner. The microvessel count varied greatly in all tumor groups, and no statistical differences were found.

Our results also show that tenascin expression was enhanced in malignant adrenal pheochromocytomas, whereas the expression was weak or totally negative in 70% of the benign adrenal tumors. The staining profile was more heterogenous in borderline and extraadrenal tumors.

Strong COX-2 expression was found in malignant pheochromocytomas, whereas most benign (75%) and borderline (79%) adrenal tumors expressed COX-2 only weakly or not at all. In extraadrenally located tumors the expression was enhanced also in benign and borderline tumors. Normal adrenal medulla did not show any COX-2 immunopositivity. Based on these results, strong COX-2 expression points to malignancy in adrenal pheochromocytomas.

Table 16. Conclusion of the immunostainings.

	Ki-67	Inh βB	VEGF	Tenascin	COX-2
Normal medulla		⇧	negative	negative	negative
Benign	⇩	⇧	⇩	⇩	⇩
Malignant	⇧	⇩	⇧	⇧	⇧

In conlusion, these studies suggest that proliferative activity, inhibin/activin βB, VEGF, tenascin, and COX-2 can facilitate the diagnosing of benign, borderline, or malignant pheochromocytoma (Table 16). These immunohistochemical stainings are easy to perform in everyday pathological practice, and they offer easy and practical tools for evaluating the malignant potential of a pheochromocytoma. However, immunohistochemical markers should always be considered together with histological features as well as clinical data.

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