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FURTHER DELINEATION OF MOLECULAR ALTERATIONS IN ADRENO-MEDULLARY TUMORS

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Further delineation of molecular alterations in adreno-medullary tumors

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It is not our level of prosperity that makes for happiness but the kinship of heart to heart, and the way we look at the world. Both attitudes lie within our power, so that a man is happy so long as he chooses to be happy, and no one can stop him. (Alexander Solzenitsyn: Cancer Ward)

To cancer patients

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ABSTRACT

Pheochromocytomas, abdominal paragangliomas and neuroblastomas are tumors of the sympathetic nervous system. Anatomically pheochromocytomas and abdominal paragangliomas arise from sympathoadrenal paraganglia, within and outside the adrenal medulla, respectively. On the other hand, neuroblastomas may present both in the adrenal medulla and along the sympathetic trunk. While pheochromocytomas and abdominal paragangliomas are mostly adult cancers originating from chromaffin cells, neuroblastoma is a pediatric tumor, deriving from immature sympathetic nerve cells. During cancer development, neoplastic cells accumulate a perplexing variety of genetic and epigenetic changes, enabling them to escape control mechanisms of proliferation and cell death.

The overall objective of this thesis is to shed further light on the genetic and epigenetic pathways that contribute to the molecular pathogenesis of these tumors.

In **Paper I** promoter specific and genome wide methylation changes were quantitatively assessed in relation to clinical features in a panel pheochromocytomas / abdominal paragangliomas. Based on methylation levels in the tumor suppressor genes, $p16^{INK4A}$, CDH1, DCR2, RARB, RASSF1A, NORE1A, TP73, APC, DAPK1, $p14^{ARF}$ and PTEN, a CpG island methylator phenotype (CIMP) was defined as concerted hypermethylation in three or more genes. A subset of abdominal paragangliomas displayed CIMP phenotype, which was associated with malignant behavior and young age at presentation. This observation raises a prospective for potential benefits of epigenetically acting drugs for a subgroup of young abdominal paraganglioma patients with adverse prognosis. In addition genome wide hypomethylation was seen in tumors as compared to normal adrenal samples providing further support for the general significance of global hypomethylation in neuroendocrine cancers.

In **Paper II** the RIZ1 tumor suppressor in 1p36.2 was assessed as a candidate target for the distal 1p deletions in these tumors. Intragenic LOH and suppressed mRNA expression was detected in a substantial proportion of the tumors. This was not associated, however with *RIZ1* promoter methylation. The recurrent inactivation RIZ1 suggests that this event may be a significant contributing factor to tumorigenesis in pheochromocytomas and abdominal paragangliomas.

NORE1A and RASSF1A are proapoptotic RAS effectors with tumor suppressor functions.

In **paper III** their role was explored in pheochromocytomas and abdominal paraganliomas. Suppressed NORE1A and RASSF1A mRNA levels were detected in tumors compared to normal adrenal medulla. Methylation of the RASSF1A promoter was significantly associated with malignant behavior, while NORE1A methylation was uncommon. The anti-tumorigenic role of NORE1A was functionally investigated in Nore1A transfected PC12 pheochromocytoma cells. Ectopic expression of Nore1a resulted in enhanced apoptosis and impaired colony formation in soft agar suggesting that suppression of NORE1A contributes to the transformed phenotype in these tumors.

In **paper IV** the role of NORE1A was investigated in neuroblastoma tumors and cell lines. Supression of NORE1A mRNA expression was evident in cell lines and tumors, particularly in cases without MYCN amplification. Methylation of the NORE1A promoter was not a characteristic event contrasting RASSF1A, which showed frequent hypermethylation. Transient expression of Nore1a in SK-N-BE(2) cells resulted in enhanced apoptosis and delayed cell cycle progression, suggesting that suppression of NORE1A may contribute to tumorigenesis.

In **Paper V,** to further substantiate the general role of NORE1A and RASSF1A in cancer, we extended the analysis of these molecules to follicular thyroid tumors. Substantially reduced NORE1A mRNA expression was seen in all PAX8-PPARγ positive tumors while RAS mutation and PAX8-PPARγ fusions were mutually exclusive. RASSF1A expression was reduced in the majority of tumors analyzed.

Studies in knock-out models indicate that inactivation of products of the CDKN2A locus, $p16^{INK4A}$ and $p14^{ARF}$, promotes pheochromocytoma development. In **Paper VI**, the involvement of CDKN2A in human pheochromocytomas and abdominal paragangliomas was studied, with regard to promoter methylation, expression and sequence alterations. The $p16^{INK4A}$ promoter was heavily methylated in a subset of paragangliomas, and it was associated with malignant disease. Frequent suppression of $p16^{INK4A}$ mRNA and protein expression was seen in tumors. By contrast no significant methylation or reduced mRNA expression was evident for $p14^{ARF}$. Sequence variations were observed in four tumors including a missense mutation. These results suggest that $p16^{INK4A}$, and not $p14^{ARF}$ is a frequent target of inactivating events in human pheochromocytoma and abdominal paraganglioma.

Key words: pheochromocytoma, abdominal paraganglioma, neuroblastoma, tumor suppressor gene, DNA methylation, gene expression, RIZ1, NORE1A, RASSF1A, CDKN2A

PUBLICATIONS INCLUDED IN THIS THESIS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals (I-VI).

I **Geli J*,** Kiss NB, Karimi M, Lee JJ, Bäckdahl M, Ekström TJ, Larsson C: Global and regional CpG methylation in pheochromocytomas and abdominal paragangliomas: association to malignant behavior
Submitted manuscript

II **Geli J*,** Nord B, Frisk T, Edström Elder E, Ekström TJ, Carling T, Bäckdahl M, Larsson C: Deletions and altered expression of the RIZ1 tumour suppressor gene in 1p36 in pheochromocytomas and abdominal paragangliomas.
International Journal of Oncology, 26:1385-1391, 2005.

III **Geli J*,** Kiss N, Lanner F, Foukakis T, Natalishvili N, Larsson O, Kogner P Höög A, Clark G, Ekström TJ, Bäckdahl M, Farnebo F, Larsson C: The Ras effectors NORE1A and RASSF1A are frequently inactivated in human pheochromocytomas.
Endocr Relat Cancer. 2007 Mar;14(1):125-34.

IV **Geli J*,** Kogner P, Lanner F, Natalishvili N, Juhlin C, Kiss N, Clark GJ, Ekström TJ, Farnebo F, Larsson C: Assessment of NORE1A as a putative tumor suppressor in human neuroblastoma
Submitted manuscript

V Foukakis T, Au A, Wallin G, **Geli J,** Forsberg L, Clifton Bligh R, Robinson B, Lui W-O, Zedenius J, Larsson C: The NORE1A Ras effector is suppressed in follicular thyroid carcinomas with a PAX8-PPARγ fusion.
Journal of Clinical Endocrinology and Metabolism, 2006 Mar;91(3):1143-9.

VI Kiss NB, **Geli J,** Avci C, F Lundberg, Velazquez-Fernandez D, Hashemi J, Weber G, Höög A, Ekström TJ, Bäckdahl M, Larsson C: Methylation of the p16INK4A promoter is associated with malignant behaviour in pheochromocytomas and abdominal paragangliomas
Submitted mansuscript

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OTHER RELATED AND UNRELATED PUBLICATIONS

Lee JJ, **Geli J,** Larsson C, Wallin G, Karimi M, Zedenius J,Höög A, Foukakis T
RASSF1A Inactivation but No Global Hypomethylation in Follicular Thyroid Cancer
Submitted manuscript

Velázquez-Fernández D, Laurell C, **Geli J,** Höög A, Odeberg J, Kjellman M, Lundeberg J, Hamberger B, Nilsson P, Bäckdahl M: Expression profiling of adrenocortical tumors suggests a molecular signature of malignancy.
Surgery 138(6): 1087-1094, 2005

Laurell C, Velazquez-Fernandez D, Enberg U, Juhlin C, **Geli J,** Odeberg J, Kjellman M, Lundeberg J, Larsson C, Hamberger B, Bäckdahl M, Nilsson P: Gene expression profiles allow the molecular classification of adrenocortical tumors.
Submitted manuscript

Jarbo C, Buckley PG, Piotrowski A, Mantripragada KK, Langford CF, Gregory SG, Gimm O, Bäckdahl M, **Geli J,** Larsson C, Westin G, Åkerström G, Dumanski JP: Detailed assessment of chromosome 22 aberrations in sporadic pheochromocytoma using array-CGH.
I*nternational Journal of Cancer, 118:1159-1164, 2006*

Par G, **Geli J,** Kozma N, Varga P, Szekeres-Bartho J: Progesterone regulates IL12 expression in pregnancy lymphocytes by inhibiting phospholipase A2.
American Journal of Reproductive Immunology. 49(1):1-5, 2003

ZhaoY, Kacskovics I, Pan Q, Liberles DA, **Geli J,** Davis SK, Rabbani H, Hammarström L: Artilodactyl IgD: the missing link.
Journal of Immunology. 169(8):4408-4416, 2002

LIST OF ABBREVIATIONS

APC	Adenomatous polyposis of the colon
ARF	Alternative reading frame
BrdU	Bromo-deoxy-uridine
CDH1	Cadherin 1
CDKN2A	Cycline dependent kinase inhibitor 2A gene
CGH	Comparative genomic hybridisation
CIMP	CpG island methylator phenotype
COBRA	Combined bisulfite restriction endonuclease assay
CpG	cytosine-guanine dinucleotides
DAPK1	Death associated protein kinase 1
DCR2	Decoy receptor 2
FLICA	Fluorescent inhibition of caspase activity
FTC	Follicular thyroid carcinoma
LOH	Loss of heterozygosity
LUMA	Luminometric methylation assay
MAPK	Mitogene activated protein kinase
MAX	MYC associated factor X
MEN2A	Multiple endocrine neoplasia type 2A
MST1	Mammalian Ste20-like pro-apoptotic kinase
MYCN	Neuroblatoma MYC oncogene
NF1	Neurofibromatosis type 1
NGF	Neurotrophic growth factor
NORE1	Novel Ras effector 1
PAX8	Paired domain 8 gene
PHOX2B	Paired like homeobox 2B
PI3K	Phosphatidyl-inositol (4,5) 3-kinase
PPARγ	Peroxisome proliferator-activated receptor γ
PTEN	Phosphatase and tensin homolog
RALGDS	RAL guanine nucleotide dissociation stimulator
RARB	Retinoic acid receptor beta
RASSF1	Ras association (RalGDS/AF-6) domain family 1
RB	Retinoblastoma
RIZ	Retinoblastoma interacting zink finger gene
QRT-PCR	Quantitative reverse transcriptase-polymerase chain reaction
SDHB, (C and D)	Succinate dehydrogenase B (C and D) genes
TERT	Telomerase reverse transcriptase
TP53	Tumor protein p53
TP73	Tumor protein 73
Trk A, (B and C)	Nerotorphic tyrosine kinase receptor A, (B and C)
TSG	Tumor suppressor gene
VHL	von Hippel-Lindau disease

INTRODUCTION

GENERAL CANCER GENETICS

Multistep development –accumulation of genetic and epigenetic changes

The emergence and evolution of cancer is a stepwise process, in which each step contributes to the progressive transformation from normal tissue to a highly malignant rapidly growing cell mass, with potentially dismal consequences to the patient (1, 2). Along the way of cancer evolution a number of cellular traits should be acquired for a neoplastic cell to "succeed". These can be summed up in the following major prerequisites: self-sufficiency in growth signals, insensitivity to anti-growth stimuli, disruption of apoptotic (cell death) mechanisms, unlimited replicative potential, angiogenesis and ability to metastasize and invade other tissues (2). Acquisition of these properties is a reflection of genetic and epigenetic alterations sequentially arising during cancer development (Figure 1). A broad variety of structural genetic alterations may occur such as point mutations, deletions, amplifications as well as larger chromosomal alterations (e.g. translocations, inversions and whole chromosomal gains and losses). The term "epigenetic" defines mitotically heritable properties in gene expression pattern that is mediated by mechanisms other than the nucleotide sequence of a gene (3, 4). These alterations may involve a number of fundamental phenomena including DNA methylation, histone modifications, and nucleosome remodelling.

Besides the aforementioned classical genetic and epigenetic alterations yet another "genetic" regulatory mechanism received considerable attention in the scientific community over the past few yeas: RNA interference. RNA interference refers to the endogenous regulation of gene expression by short complementary RNA molecules at the post-transcriptional level. A plethora of experimental evidence support that dysregulation of this machinery plays a significant role in cancer development (5).

The applications of genome-wide analyses on the DNA and transcriptional levels have illustrated the complexity of changes that are associated with cancer development. One goal in cancer research is to extract the events and mechanisms that can explain etiology and be applied for diagnostic, prognostic and therapeutic purposes.

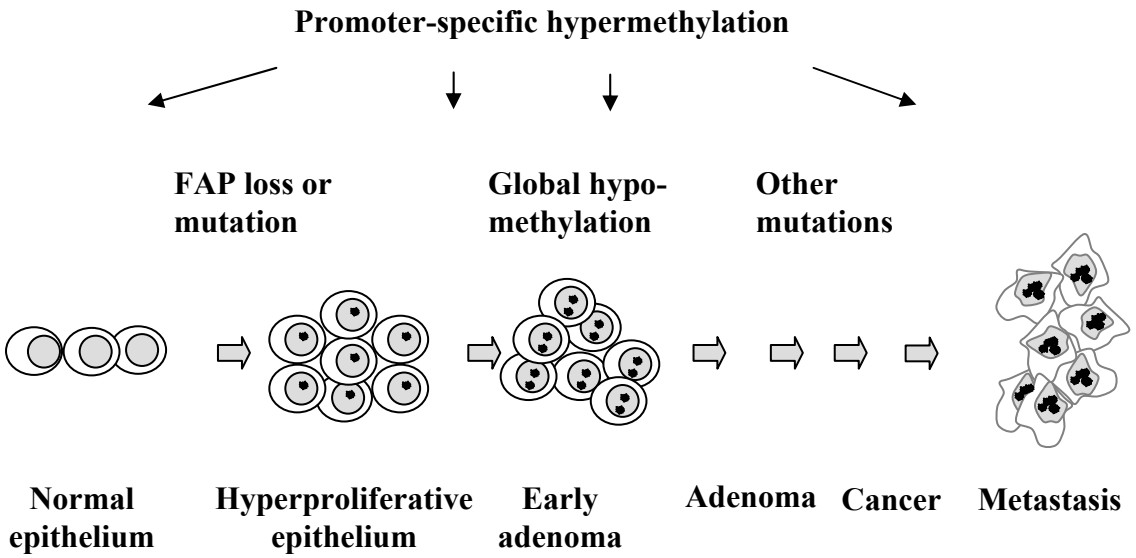

Figure 1.
Multistep development of colorectal cancer as proposed by Vogelstein *et al (6)*. Following inctivation of the FAP tumor suppressor, global hypomethylation occurs in the early adenoma, and is subsequently followed by alterations of other suppressors and oncogenes in the later tumor stages. In addition, the occurrence of promoter-specific hypermethylation is indicated at the top (7).

Oncogenes and Tumor Suppressor Genes

Genes involved in cancer development can be artificially divided into three major categories depending on their function with regard to cell growth and death: tumor suppressor genes, oncogenes and DNA repair genes (8-10).

Oncogenes can be described as the "accelerators" of the cell. Under physiological conditions these genes encode proteins, which enhance proliferation and restrict cell death, such as growth factors or their receptors, signal transducers, transcription factors or apoptosis regulators. Activation of these genes by gain of function alterations leads to excessive proliferative and anti-apoptotic signals that promote cancer development. Examples of underlying genetic events are activating point mutations, translocations and amplifications.

By contrast, tumor suppressor genes (TSGs) are the "brakes" of the cell (11). Proteins encoded by TSGs act as negative cell cycle regulators or promote cell death and differentiation. Their inactivation by loss of function mutations, epigenetic changes, or post-transcriptional mechanisms leads to increased proliferation and / or reduced elimination via cell death. According to Kundson's two hit hypothesis, for inactivation of a TSG, both alleles need to become inactivated (referred to as "two hits") (12). The first hit is often a gene mutation, whereas the second hit commonly involves larger structural changes detectable as

loss of gene copy number or loss of heterozyosity (LOH). In addition, a variety of inactivating alterations are revealed in cancer tissues as exemplified in Figure 2.

DNA methylation in Cancer

DNA methylation is a cardinal tool for the cell to epigenetically regulate gene expression. It occurs at Cytosines that precede a Guanosine commonly referred to as CpG. Overall, CpG dinucleotides are sparse in the human genome, however approximately 40% of mammalian genes exhibit CpG dense regions located mostly in the 5' regions. Methylation of these so-called CpG islands is commonly associated with transcriptional silencing. The majority of CpGs in the genome are sparsely scattered (nonclustered CpGs), mostly in repetitive sequences (e.g., transposons, endogenous retrovirus, repetitive elements). Normally, non-clustered CpG dinucleotides are heavily methylated, which prevents activation and expansion of repetitive elements. By contrast the dinucleotides in CpG islands are usually unmethylated regardless of transcriptional state of the gene (3, 4). Important physiological exceptions exist however, such as the inactive X chromosome, genomic imprinting and age related methylation (13).

Cancer is characterized by an abnormal DNA methylation pattern including two antagonistic features: loss of methylation in non-clustered CpGs and gain of methylation in promoter CpG islands. (14, 15). The previous is commonly referred to as global hypomethylation and the latter as promoter specific hypermethylation. Global hypomethylation is associated with chromosomal instability (16), and activation of retrotransposomal elements (17), which in turn increases mutation frequency. On the other hand, hypermethylation of promoter regions leads to transcriptional suppression, thus serving as an alternative mechanism of tumor suppressor inactivation (4) in addition to classical structural mutations in cancer.

The term CpG island methylator phenotype (CIMP) refers to the presence of concerted hypermethylation in multiple tumor suppressor promoters. After its initial description in colon cancer by Toyota *et al.* in 1999 (18), this molecular phenotype has been recognized in various other tumor types such as those of the gastrointestinal tract (19-22), nervous (23, 24) and haematopoetic systems (25, 26). A crucial question from a clinical perspective is, whether or not a CpG island methylator phenotype affects the natural course of the disease, in other words whether or not it has the potential utility as

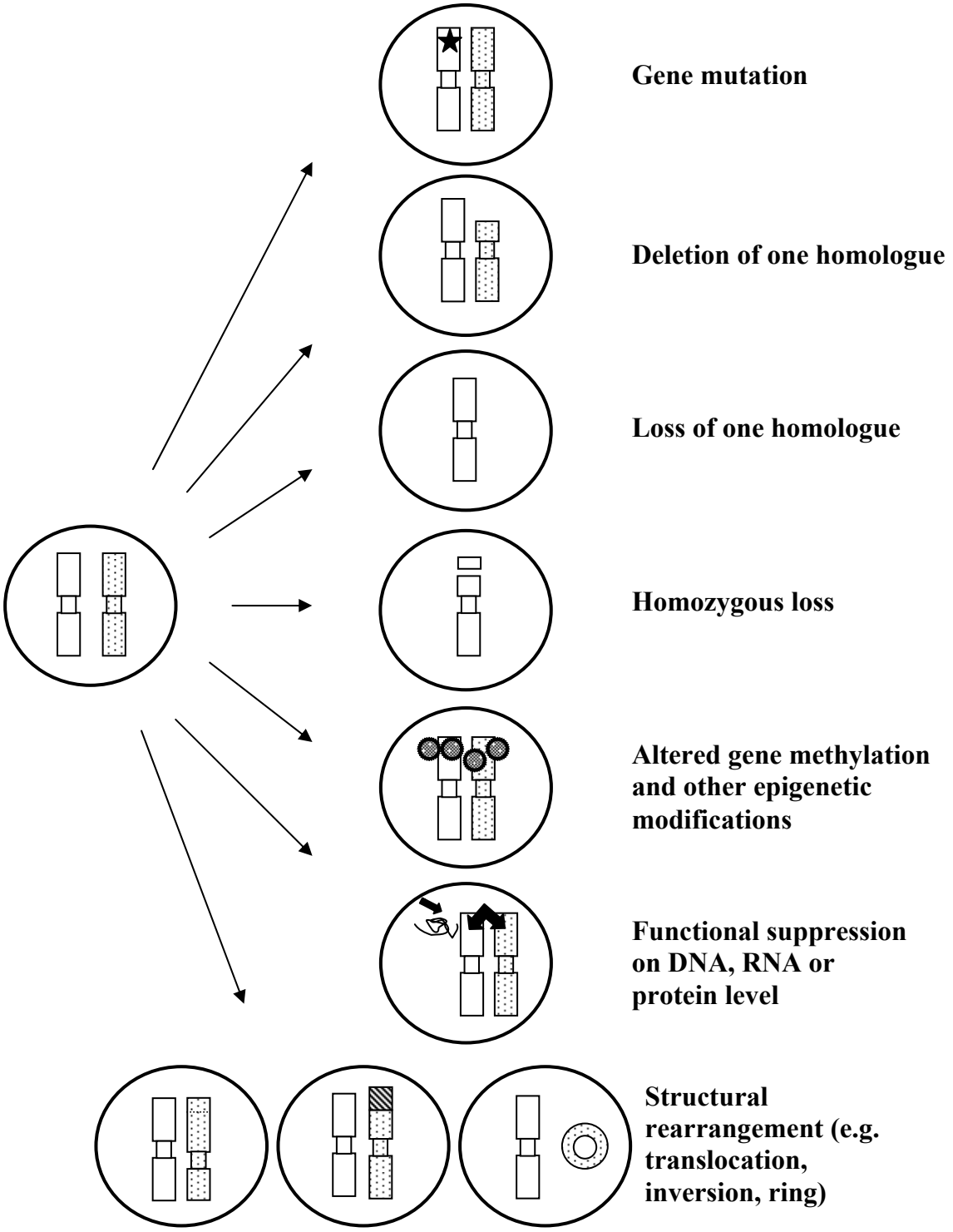

Figure 2.
Examples of genetic and epigenetic alterations, which may contribute to inactivation of tumor suppressor genes. In cancer cells one or more of these changes are often present resulting in classical biallelic inactivation or varying degrees of suppression.

a diagnostic / prognostic or therapeutic marker. Association of CIMP with adverse characteristics is described in some (19, 23, 26-28) albeit not all (29) tumor types examined.

Mechanistically it may reflect a selection for CIMP during malignant transformation (30). However, at present it is unclear whether CIMP is a causative factor or a consequence of the malignification process. As cytosine methylation in CpG dense promoters constitutes a major epigenetic mechanism of transcriptional silencing (13), it is plausible that one of the ways by which CIMP may contribute to the development of neoplastic phenotype is realized through simultaneous transcriptional suppression of multiple genes governing proliferation, cell death and invasion. Since the hallmark of CIMP is excessive methylation of multiple tumor suppressor promoters and DNA methylation is a reversible process, epigenetically acting drugs, such as inhibitors of DNA methylation, appear as alluring candidates for testing as adjuvant treatments in patients with CIMP positive tumors.

PHEOCHROMOCYTOMA AND ABDOMINAL PARAGANGLIOMA

Clinical overview

Pheochromocytomas and abdominal paragangliomas are cathecolamine producing tumors that originate from neural crest derived chromaffin cells of the sympathetic nervous system (31, 32). As outlined in Figure 3 these tumors present in the adrenal medulla (i.e. pheochromocytoma) or paravertebral and paraaortic sympathetic paraganglia (i.e. abdominal paraganglioma). Head and neck paraganglioma is a related entity which, in contrast to abdominal paragangliomas, are mainly of parasympathetic origin and do not secrete catecholamines.

The adrenal medulla and the sympathetic paraganglia are part of the sympathetic nervous system (Figure 4). Their chief physiological function is secretion of catecholamines, which are major stress hormones preparing the organism for stress response. Pheochromocytomas and abdominal paragangliomas may both secret dopamine and noradrenaline. However, adrenaline is produced by pheochromocytomas but not abdominal paragangliomas, since the required enzyme: phenylethanolamine-N-methyltransferase is only present in the adrenal medulla.

The yearly incidence of pheochromocytomas and abdominal paragangliomas is estimated to be 1-2 per million. The real incidence, however, is probably higher as a significant proportion of tumors are found only post-mortem at autopsy (33, 34).

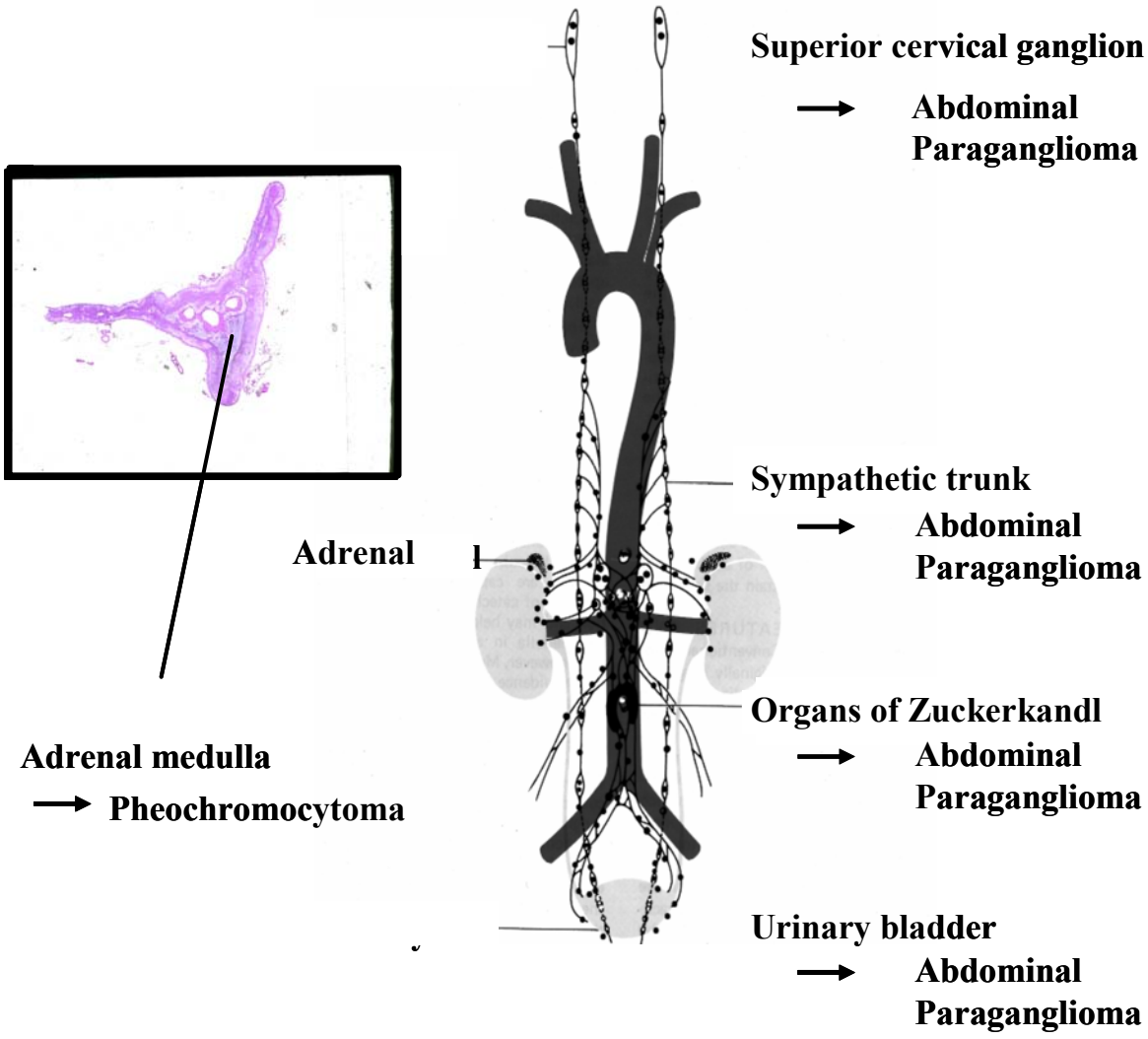

Figure 3.
Schematic illustration of the anatomical locations for pheochromocytoma and abdominal paraganglioma development. Pheochromocytomas occur in the adrenal medulla, which is indicated on the horizontal section of the adrenal, shown to the left. Abdominal paraganglioma develop in sympathetic ganglion, as shown to the right.

The tumors may occur at any age with a peak incidence in the 4th and 5th decades of life. Approximately one out of ten cases present in the paediatric population.

The typical symptoms associated with pheochromocytomas and abdominal paragangliomas are related to excessive catecholamine secretion with resulting hypertension, i.e. paroxysmal headache, palpitations, pallor, sweating and anxiety. However, up to a third of patients may be normotensive. These patients are often not diagnosed, or discovered incidentally when imaging investigations for other reasons are carried out (32, 35).

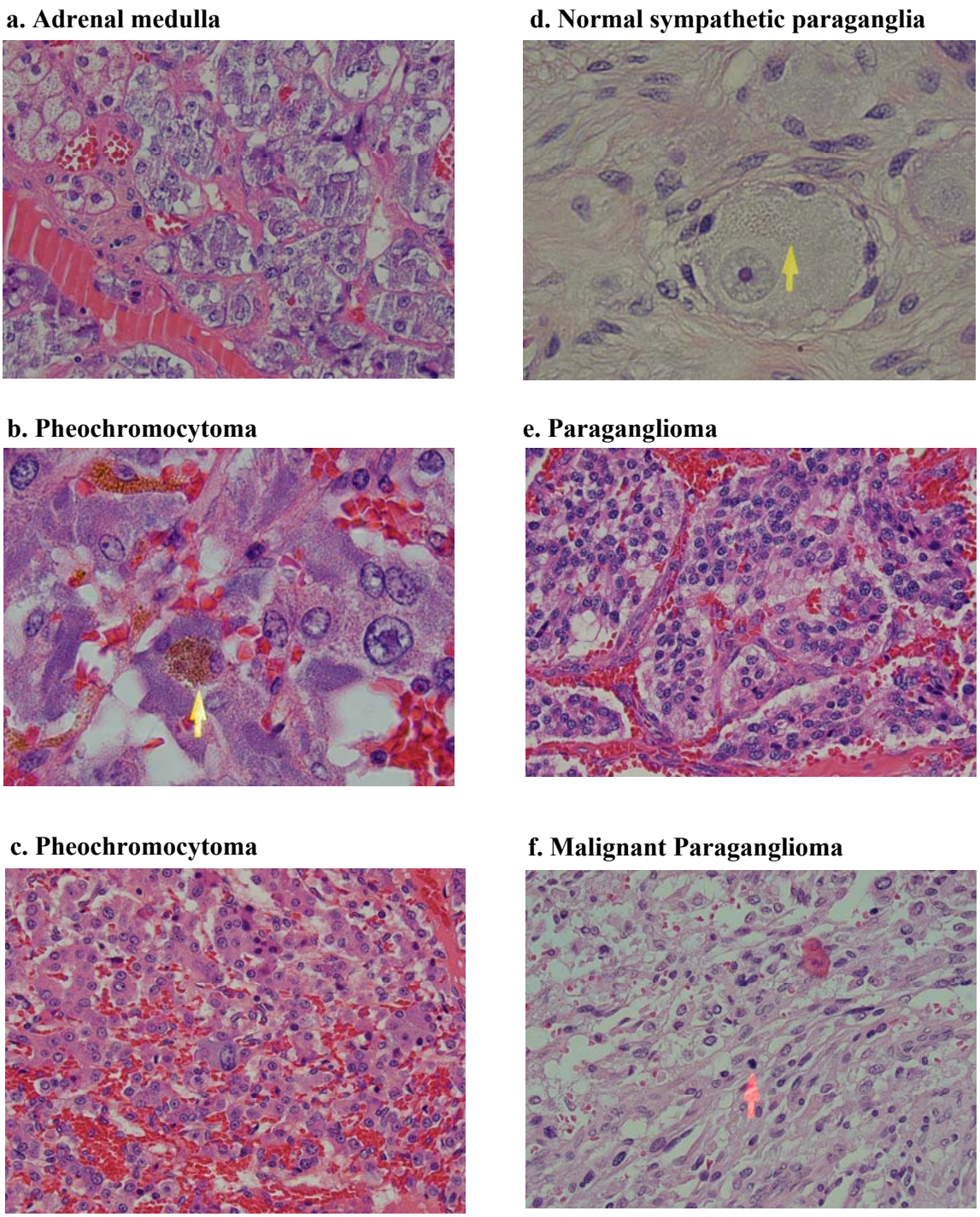

Figure 4.
Photomicrographs of hematoxylin & eosin stained normal adrenal medulla, sympathetic paraganglia, pheochromocytoma and abdominal paraganglioma.
(a) Normal adrenal medulla showing chromaffin cells embedded in fibrovascular stroma. (b and c) Pheochromocytomas exhibiting large pleomorphic nuclei and abundant cytoplasm with chromaffin granules (arrow). The tumors are well vascularised. (d) Sympathetic paraganglia with chief cells (main cell type) surrounded by sustentacular cells. (e and f) Paraganglioma cells are more uniform in appearance and are characteristically arranged in cell nests. A mitotic figure can be seen in (f) (arrow).

Malignant forms of these tumors are relatively sparse. Approximately 10% of pheochromocytomas and 15-35% of abdominal paragangliomas show signs of malignant behaviour. Histopathological distinction between benign and malignant cases is highly challenging as features typically associated with malignancy such as nuclear pleomorphism, mitotic figures, vascular and capsular invasion are commonly seen both in benign and malignant pheochromocytomas and abdominal paragangliomas (36). At present the only absolute proof of malignancy is the presence of distant metastasis (most often in lymph nodes, lungs, liver or bones), which may be seen at the time of initial surgery or be found years after primary tumor removal. Another widely used definition of malignancy is that recommended by the Armed Forces Institute of Pathology (37). This classification, which includes extensive local invasion as an additional criterion for malignancy, was applied in this thesis.

Keeping in mind the difficulty of distinguishing truly benign cases from those presenting only initially as benign and later recurring with metastases, at present, lifelong follow up is recommended for all pheochromocytoma and abdominal paragangliomas patients. With the advent of modern high-throughput molecular techniques an increasing number of novel diagnostic / prognostic and therapeutic biomarkers are discovered. Therefore it is anticipated that in the future, new molecular markers will aid the clinician / pathologist to fine-tune risk stratification of patients, with regard to potential malignant behaviour of these tumors (38, 39). In a number of recent reports several prospective markers have been proposed, such as the proliferation marker Ki-67, the catalytic subunit of the telomerase complex (hTERT), Heat Shock Protein 90 (HSP 90) and Vascular Endothelial Growth Factor (VEGF) (40-43).

Heritable mutations in pheochromocytoma and abdominal paraganglioma

Heritable forms of pheochromocytoma and abdominal paraganglioma have been long recognized, and historically the incidence has been estimated to 10%. In these cases tumors usually present as components of hereditary cancer syndromes such as multiple endocrine neoplasia type 2A (MEN 2A) or type 2B (MEN 2B), von Hippel-Lindau disease (VHL), Neurofibromatosis type 1 (NF1) and familiar paragangliomas (SDHB, SDHD) (Table 1).

These patients harbour constitutional mutations in one of the following genes RET, VHL, NF1, SDHB, SDHC and SDHD (44). The Rearranged during transfection (RET) protooncogene is a thyrosine kinase receptor with important functions during neural development (45). The von Hippel-Lindau (VHL) tumor suppressor has versatile functions that include oxygen-sensing, extracellular matrix assembly, microtubule stabilization and cell-

cycle control (46). The Neurofibromatosis type 1 (NF1) tumor suppressor gene encodes for a protein called neurofibromin. Neurofimbromin is an important negative regulator of the Ras protein (47). The Succinate dehydrogenase proteins B, C and D are components of the mitochondrial complex II, which plays an important role both in the tricarboxylic cycle and in the respiratory chain (48).

Contrasting the traditionally indicated proportion of hereditable disease (around 10 %), concise analyses of susceptibility genes indicate a significantly higher frequency in germ-line alterations in patients with these tumors (44). Mutations in VHL, RET, SDHD and SDHB, may be detected in approximately one quarter of apparently sporadic pheochromocytoma and abdominal paraganglioma patients (44) (Table 1). In resemblance to other familial cancers, younger age at diagnosis, multifocal and extra-adrenal presentation is more prevalent in patients with constitutional predisposing mutation. Presently, genetic testing is recommended for those patients whose clinical presentation is suggestive of hereditary disease, such as a positive family history or an age at presentation younger than 50 years (49). The potential benefit of testing is the implementation of stringent clinical surveillance with regard to recurrences, multifocality and other associated neoplasias, both for patients and family members with proven germ-line mutations.

Somatic mutations in pheochromocytoma and abdominal paraganglioma

Identification of disease genes in familial cancer syndromes often provides substantial insight into the molecular pathogenesis of the sporadic forms of the disease. Thus, somatic inactivation of the VHL tumor suppressor gene is present in the majority of sporadic clear cell renal cell carcinomas. Similarly, somatic RET mutations are commonly detected in medullary thyroid carcinomas (50-55). However, analyses of familial pheochromocytoma cancer genes VHL, (52, 56) RET (56), SDHD, and SDHB (57-62) gave

Table 1. Constitutional mutations associated with pheochromocytoma and paraganglioma

Mutated gene	Location	Associated Syndrome	
RET	10q11.2	Multiple endocrine neoplasia type 2A/B	MEN 2A/B
VHL	3p26-25	von Hippel-Lindau disease	VHL
NF1	17q11.2	Neurofibromatosis type 1	NF1
SDHD	11q23	Familial paraganglioma 1	PGL1
SDHC	1q21	Familial paraganglioma 3	PGL3
SDHB	1p36.1-35	Familial paraganglioma 4	PGL4

Information according to Elder et al. J Surg Oncology. 89(3):193-201, 2005

no indication of frequent involvement of these molecules in sporadic pheochromocytomas or abdominal paragangliomas.

Mutations in widely characterized oncogenes such as H-, K- and N-Ras (63-65), c-mos (66, 67), NMYC (67), CMYC (67), Gs alpha (65), ENRB (66), and tumor suppressor genes like p16 (68) and TP53 (65, 69, 70) are not common in these tumor types. On the other hand, at the protein level, loss of Rb (71), and over-expression of mdm2 (71), and Bcl-2 (72, 73) have been reported.

Thus, despite major advance in unravelling the genetic background of familial cases the molecular pathogenesis of sporadic pheochromocytoma and abdominal paragangliomas remains largely unknown.

Chromosomal alterations in pheochromocytomas and abdominal paragangliomas

Several recent studies attempted to characterize somatic copy number changes in pheochromocytomas and abdominal paragangliomas using comparative genomic hybridisation (CGH) (74-78) or array CGH (79, 80). Deletions in 1p, 3p, 3q, 11p, 11q and 22q as well as gains at 17q and chromosome 19 are common alterations. 1p appears as the earliest and most frequent site for gross losses (Figure 5). Both array CGH and LOH studies suggest that this region harbors several tumor suppressor genes (79-86). Our group has reported three target regions in 1p: one telomeric of *D1S1612* in 1p36.2-pter, one centromeric of *D1S429* in 1cen-p13 and one in the 18cM interval defined by *D1S2134* and *D1S1669* in 1p32 (82). Furthermore, the pattern of copy number changes was highly similar between pheochromocytomas and abdominal paragangliomas, suggesting a common genetic origin (76).

Alterations of chromosome 11 (75, 76) and 17q (74), and in some studies the frequency of overall copy number alterations (74) were higher in malignant samples. The pattern of gross genetic changes in pheochromocytomas from neurofibromatosis knockout mice suggests that the genesis of this neoplasia may be similar across species (78).

Studies of global gene and protein expression

Microarray studies characterizing the transcriptome and proteome in pheochromocytomas and abdominal paragangliomas are scarce (87-92). Comparisons of adrenalin vs. noradrenalin producing tumors suggested distinct molecular pathogenesis characterized by activation of hypoxia driven angiogenic pathways in the latter (87, 89). Distinction between these biochemical subtypes was also confirmed on the proteome level (93). Two recent studies by

Brouwers *et al*. examined molecular differences between malignant and benign pheochromocytomas on transcriptome and protein levels (87, 88). A larger set of genes with various biological functions was identified that could differentiate between benign and malignant cases. This database constitutes the first major resource for mapping the pathways leading to malignancy in these tumors, thus raising the prospect of novel diagnostic / prognostic markers and therapeutic targets. In addition, evaluation of low molecular weight (LMW) protein profiles, generated from serum samples of pheochromocytoma patients, allowed for the identification of a subset of proteins that could discriminate malignant cases from the benign ones (88). Further confirmatory studies exploring the transcriptome and proteome on independent series are awaited.

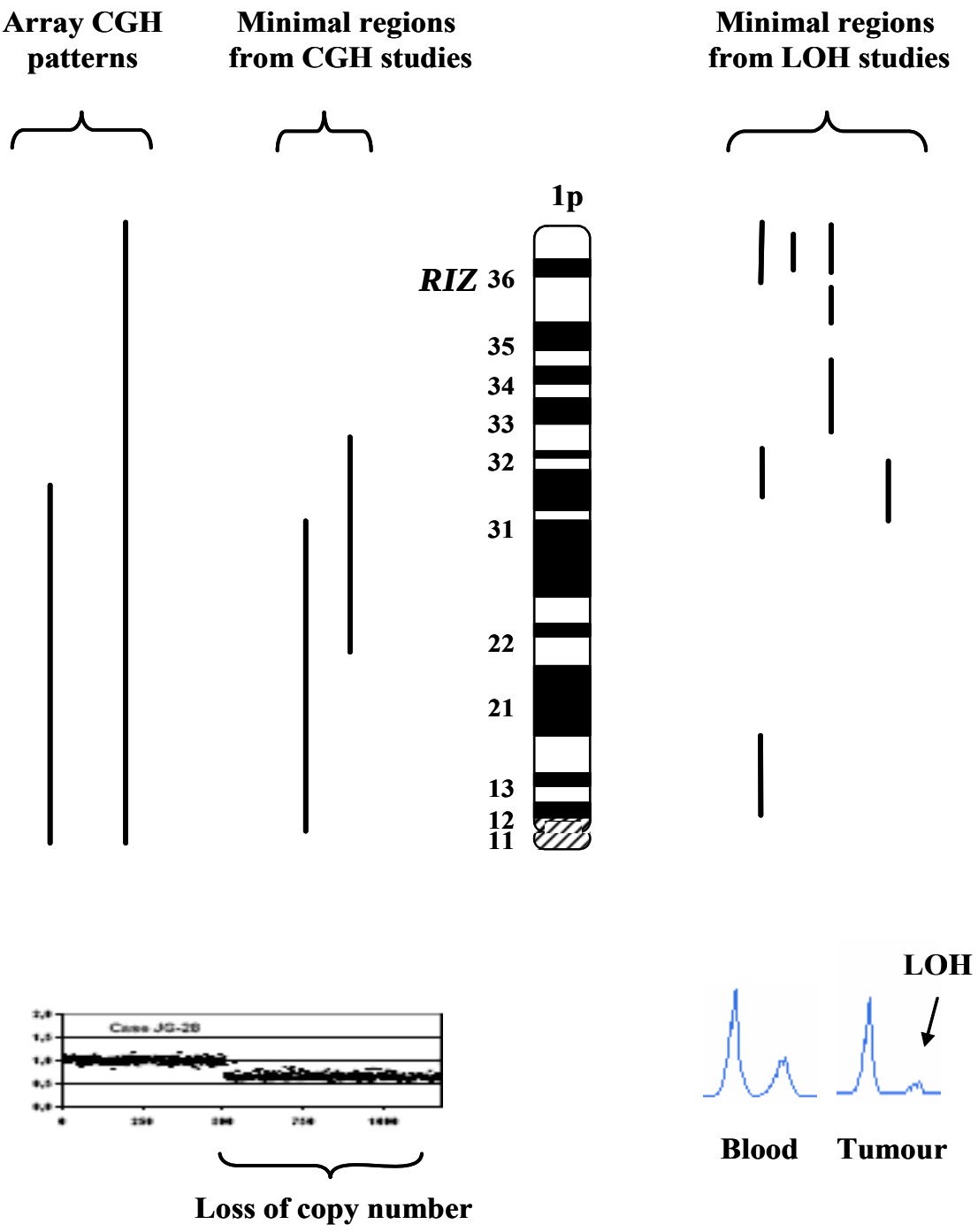

Figure 5.
Overview of 1p loss detected in pheochromocytoma and abdominal paraganglioma using array-CGH, conventional CGH and LOH analyses. Common regions of loss are grossly indicated by vertical bars next to an ideogram of 1p. Examples of a deletion detected by array-CGH and LOH are shown below. Data from (76, 79-82).

NEUROBLASTOMA

Clinical overview

Neuroblastoma is the most common extracranial solid tumor in childhood accounting for about 6% of all pediatric caners (94) (Figure 6). The prevalence is about 1 in 7000 live births, with about 15-20 new cases annually in Sweden (95). Approximately half of the patients are diagnosed by 1 year of age and nearly all by the age of 10 (96).

The tumor probably arises from precursor cells of the peripheral sympathetic nervous system (31, 97, 98) in the adrenal glands or the paravertebral and paraaortic sympathetic tissue situated in the chest, abdomen and pelvis.

Neuroblastoma represents a tumor type with a puzzling disparateness with regard to clinical behaviour. In some cases, especially in infants, the tumors may regress spontaneously or differentiate into more mature entities referred to as ganglioneuroma. On the other hand, most children over the age of 1 year present with extensive or metastatic disease at diagnosis, which entails an overall poor prognosis. Furthermore, within this group, subsets with better or worse outcome can be furtherd stratified. The variable clinical behaviour is strongly associated with the genetic and molecular characteristics of the tumor cells. This has allowed integration of molecular biological features of the tumors into the present risk stratification systems upon which the choice of therapy is based (Table 2). Taken together this has rendered neuroblastoma an exemplary model for successful translation of basic science results into daily clinical practice.

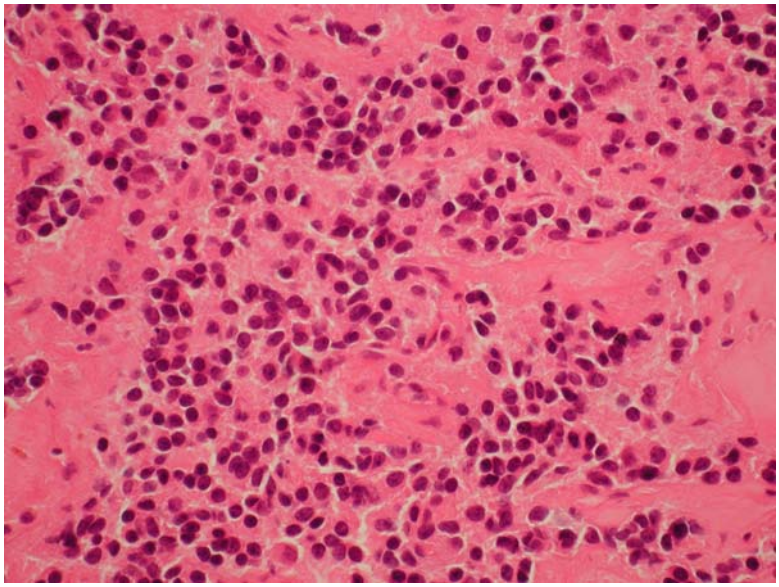

Figure 6.
Photomicrograph of hematoxylin & eosin stained human neuroblastoma. Characteristic “small round blue” cells (arrow).

Major prognostic factors

The stage, which is based on the location, extent of the primary lesion and presence or absence of distant metastases, is the most important prognostic factor in neuroblastoma (99). Age at diagnosis is also an important determinant: children less than 1 year generally have better outcome compared to older patients with equivalent stages (99, 100). Another major predictive factor is the histological appearance of the tumor. The currently used system devised by Shimada *et al* classifies tumors as favourable or unfavourable, depending on the degree of neuroblast differentiation, Schwannian stroma content, mitotic figures, degeneration of nuclei and age at diagnosis (101). According to the classification proposed by the Children's Oncology Group, treatment risk stratification should be based on tumor stage, patients age, and histology, and in addition changes in DNA content and presence or absence of MYCN amplification are incorporated (102) (Table 2).

Germ line genetic alterations in neuroblastoma

Although neuroblastoma occurs in early childhood, it is generally encountered in sporadic cases. Familial cases are rare and appear as a genetically heterogeneous group (103-105). Recently, loss of function germline mutations in PHOX2B, a neurogenesis regulator, have been found in some neuroblastoma patients (106, 107). By contrast, specific somatic genetic and molecular alterations are recurrently recognized in tumor cells and the most important ones are described below and reviewed in (102, 108).

Loss or allelic imbalance of the short arm of chromosome 1

Deletions in 1p are found in approximately one third of neuroblastomas (109-112), and are strongly associated with advanced stage and MYCN amplification. It thus seems likely that one or more tumor suppressor genes are harboured in 1p, which when inactivated in neuroblasts contribute to tumorigenesis. Smallest regions of overlap (SRO) have been mapped by several groups (109-119) and the overall evidence suggests that a prospective neuroblastoma tumor suppressor is located in 1p36. So far, however, no specific gene has been identified as "the wrongdoer". SROs are typically
dozens of megabases large and there is not much support for biallelic inactivation of regional candidate genes. Whole genome array CGH studies have furtherd enlightened the complexity of chromosomal rearrangements (120), identified distinct genetic subgroups (121, 122) and proposed models for progression (123).

Table 2. Proposed Children's Oncology Group risk srtratification schema

Stage	Risk group	Age	Amplification of MYCN	DNA Ploidy	Histology	Other
1	Low					
2A / 2B	Low		No			< 50% resection
	Intermediate		No			< 50% resection
	Intermediate		No			Biopsy only
	High		MYCN			
3	Intermediate	< 1.5 years	No			
	Intermediate	>= 1.5 years	No		Favourable	
	High		MYCN			
	High	>= 1.5 years	No		Unfavourable	
4	High	< 1 year	MYCN			
	Intermediate	< 1 year	No			
	High	1-1.5 years	MYCN			
	High	1-1.5 years		DI = 1		
	High	1-1.5 years			Unfavourable	
	Intermediate	1-1.5 years	No	DI > 1	Favourable	
	High	>= 1.5 years				
4S	Low	< 1 year	No	DI > 1	Favourable	Asymptomatic
	Intermediate	< 1 year	No	DI = 1		
	Intermediate	< 1 year	Missing	Missing	Missing	
	Intermediate	< 1 year	No			Symptomatic
	Intermediate	< 1 year	No		Unfavourable	
	High	< 1 year	MYCN			

Modified from Maris M Lancet (2007;369: 2106-2120)

Amplification of the MYCN oncogene

From a clinical perspective, most important genetic alteration in neuroblastoma is amplification of the MYCN gene locus at chromosomal region 2p24 (124, 125). Approximately 20 % of neuroblastomas carry MYCN amplification (124, 125), which is commonly detected by molecular cytogenetic analyses such as FISH (fluorescence *in situ* hybridization). The alteration has strong prognostic implications. Even in the presence of other favourable factors such as low stage, MYCN amplification strongly predicts aggressive disease and poor outcome. Based on these observations, it can be anticipated that MYCN amplification is of great biological significance. On the molecular level, MYCN forms a heterodimer with MAX which functions as a transcriptional activator, while in the absence of MYCN MAX forms transcriptionally repressive homodimers. Target genes of the MYCN-

MAX complex such as ODC, MCM7 and MRP1 (126, 127) are known to promote cell cycle progression through the G1 phase. Most cases with MYCN amplification also exhibit loss in 1p, however not all tumors with 1p loss have MYCN amplification. This suggests that 1p loss preceeds MYCN amplification in genetic and clinical progression of these tumors (128).

Gain of the long arm of chromosome 17

Gain of chromosome arm 17q is probably the most prevalent gross genetic aberration in neuroblastoma (129-131). It may occur as an unbalanced gain of 17q or more frequently as unbalanced translocation between chromosomes 1 and 17 (132). Since the breakpoints largely vary, it is believed that the important result of 17q alteration is a dosage effect rather than disruption or rearrangement of particular gene(s) (133, 134). The putative gene providing selective growth advantage has not been identified yet, but the apoptosis inhibitor: survivin appears as an attractive candidate (135). As gain of 17q is usually associated with other poor prognostic markers such as MYCN amplification, 1p loss and high stage, its significance as an independent prognostic factor should be furthered delineated.

Alterations in other loci

Other sites with frequent deletions include 11q, 14q, 18q and 3p (136-139). Cancer genes that appear frequently aberrant in carcinomas such as TP53, Ras and CDKN2A are rarely altered in neuroblastoma (102, 108).

Expression of Trk family members

The Trk family of tyrosine kinases function as membrane receptors of neurotrophic factors relaying developmental signals governing differentiation and cell death during normal neural development (140). Neuroblastomas with favourable prognosis express high levels of TrkA (141, 142). Similarly, TrkC is associated with favourable prognosis (143) whereas high expression of TrkB correlates with aggressive behaviour (144).

Genetic model for neuroblastoma development and progression

Based on the extensive data concerning the genetic alterations observed in neuroblastomas, Brodeur at al proposed a genetic model for disease initiation and progression. According to this model (Figure 7), all neuroblastomas develop from a common precursor, which can essentially follow two paths. One path is characterized by a near triploid karyotype (3N) without structural rearrangements, and with a high TrkA expression ensuring differentiation

or apoptosis, following the presence or absence of its ligand neurotrophic growth factor (NGF). These patients are usually infants with low stage disease and excellent prognosis. Cells following the second path have a near diploid (2N) or near tetraploid (4N) karyotype and are essentially characterized by gross chromosomal rearrangements with gain of 17q being one of the most common. Within this type, two subsets can be distinguished. One subset is characterized by 11q and / or 14q deletion. Patients with these tumors are generally older, with slowly progressive, often fatal disease. The second subset's main feature is loss of 1p with or without MYCN amplification. These tumors frequently express TrkB and its ligand, thereby triggering an autocrine survival loop. Patients with this molecular phenotype present with advance stage, rapidly progressive and usually fatal disease (108).

CANDIDATE GENES STUDIED IN THIS THESIS

The Ras effecors RASSF1A and NORE1A

Ras-GTPases (H-, K- and N-Ras) regulate cell fate decisions via GTP-dependent interaction with a versatile repertoire of downstream effectors. The most extensively studied physiological function of Ras is the relay of proliferative and anti-apoptotic stimuli from cell surface receptors to effector molecules such as Raf and Phosphatidyl-inositol 3 kinase (PI3K) (145). A number of recent studies brought into spotlight yet another Ras effector pathway controlling cell survival (146). By contrast to the previously mentioned cascades, these molecules promote apoptosis and may thus suppress Ras-induced oncogenesis by hampering the cells ability to survive. The Ras association domain family (RalGDS/AF-6) of proteins include 8 genes (RASSF1-8) from which several RASSF isoforms are transcribed (Table 3). An accumulating body of experimental and observational evidence indicates the widespread involvement of the RASSF family in neoplasia (146-149).

Two prominent members of this Ras mediated tumor suppressor pathway are RASSF1A and NORE1A (RASSF5). The RASSF1 gene is located in 3p21.3 (147-149). Loss in 3p is a frequent observation in neuroblastomas and pheochromocytomas

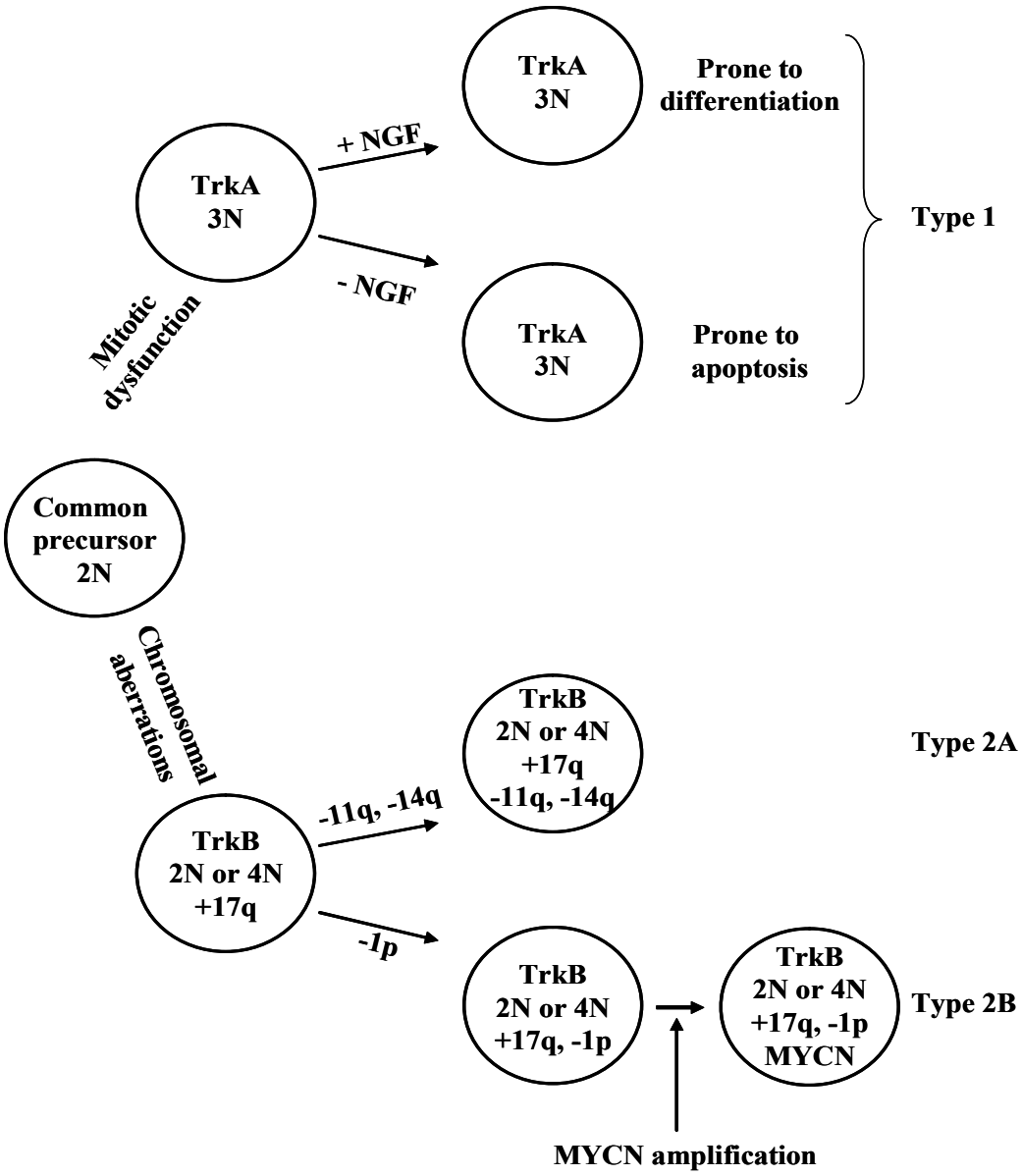

Figure 7.
Genetic model proposed for neuroblastoma development by Brodeur *et al*. (108).

(76, 136, 150). Several isoforms are transcribed from the RASSF1 (Table 3) locus of which RASSF1A has been most extensively characterized. RASSF1A is a *bona fide* tumor suppressor (147-149). Mice deficient of RASSF1A show enhanced susceptibility to tumor development including breast lung and lymphoid neoplasias (151). Moreover RASSF1A inactivation is frequently observed in various human cancers and the most common mechanism of inactivation is promoter hypermethylation (147-149). Point mutations in RASSF1A are rare in human tumors (147-149). RASSF1A is one of the most commonly methylated tumor suppressors in neuroblastomas (150, 152-156). On the other hand studies of RASSF1A in the context of pheochromocytomas and abdominal paragangliomas are sparse. Astuti et al observed frequent allelic loss at

Table 3. Members of the Ras-association domain family (RASSF)

Name	Location	Effect in cancer	Alternative transcripts	Characterized isoforms	Predicted domains for DAG-binding	Ras-assoc.	SARAH
RASSF1	3p21.3	Suppressor	7	RASSF1A	DAG-binding	Ras-assoc.	SARAH
				RASSF1B	-	Ras-assoc.	SARAH
				RASSF1C	-	Ras-assoc.	SARAH
RASSF2	20p13	Suppressor	3	RASSF2A	-	Ras-assoc.	SARAH
RASSF3	12q14.1	Not known	3	RASSF3A	-	Ras-assoc.	SARAH
RASSF4	10q11.21	Suppressor	6	RASSF4A	-	Ras-assoc.	SARAH
RASSF5	1q32.1	Suppressor	4	NORE1A	DAG-binding	Ras-assoc.	SARAH
				NORE1B	-	Ras-assoc.	SARAH
RASSF6	4q13.3	Suppressor	3	RASSF6A	-	Ras-assoc.	SARAH
				RASSF6B	-	Ras-assoc.	SARAH
RASSF7	11p15	Not known	3	RASSF7A	-	Ras-assoc.	-
RASSF8	12p11	Not known	6	RASSF8A	-	Ras-assoc.	-
				RASSF8B	-	Ras-assoc.	-

Information according to van der Weyden and Adams Biochem Biophys Acta 2007
DAG = diacylglycerol; SARAH = Salvador / RASSF / HIPPO interaction

3p.21 as well as methylation of the RASSF1A promoter in 22 % of sporadic pheochromocytomas (150). In a recent report Dammann *et al.* reported almost 50 % RASSF1A methylation in a small series of familial and sporadic pheochromocytomas (157). In both reports, however, promoter methylation was qualitatively characterized entailing a risk of classifying a sample as methylated on the basis of a minor proportion of methylated target, which may not reflect the overall scenario within the tumor sample studied. This is of particular importance when data are analysed in relation to clinico-pathological features such

as malignancy. Therefore we chose to quantitatively assess RASSF1A promoter methylation in a panel of pheochromocytomas / abdominal paragangliomas (paper III) and neuroblastomas (paper IV).

NORE1A is closely related to RASSF1A and the two proteins share nearly 60% similarity. The NORE1 gene locus in chromosomal region 1q32.1 encodes two main isoforms by alternate promoter usage i.e. NORE1A and NORE1B (158) (Figure 8 and Table 3). Data from experimental model systems suggest that loss of NORE1A expression is associated with tumorigenic effects, such as diminished apoptosis and enhanced cell cycle progression (159-161). NORE1A is able to selectively bind to active Ras (162). Furthermore upon interaction with Ras the NORE1A / RASSF1A heterodimer mediates pro-apoptotic signals via recruiting the mammalian Ste20-like pro-apoptotic kinase (MST1) (160, 163, 164) suggesting that NORE1A and RASSF1A are involved in the same Ras dependent pro-apoptotic pathway (Figure 9).

NORE1A expression is frequently suppressed in cancer cell lines as well as primary tumors and the inactivation commonly results from promoter hypermethylation (158, 161, 165-167). On the other hand point mutations in the NORE1A locus or deletions in 1q are rare in human neoplasia (158, 161, 165-167). Observational and experimental data on NORE1A in the context of neural crest derived tumors are scarce. Methylation of NORE1A was rarely detected in a series of neuroblastic tumors (154) yet no study has investigated NORE1A in pheochromocytomas and abdominal paragangliomas. In light of the frequent involvement of the close functional and structural homologue RASSF1A in these tumors we chose to evaluate its "sister" molecule NORE1A in a series of pheochromocytomas / abdominal paragangliomas (paper III) and neuroblastomas (paper IV). Analogously, given the frequent epigenetic inactivation of RASSF1A in thyroid cancer (168, 169) we present the first evidence of the implication of NORE1A in these tumors (paper V).

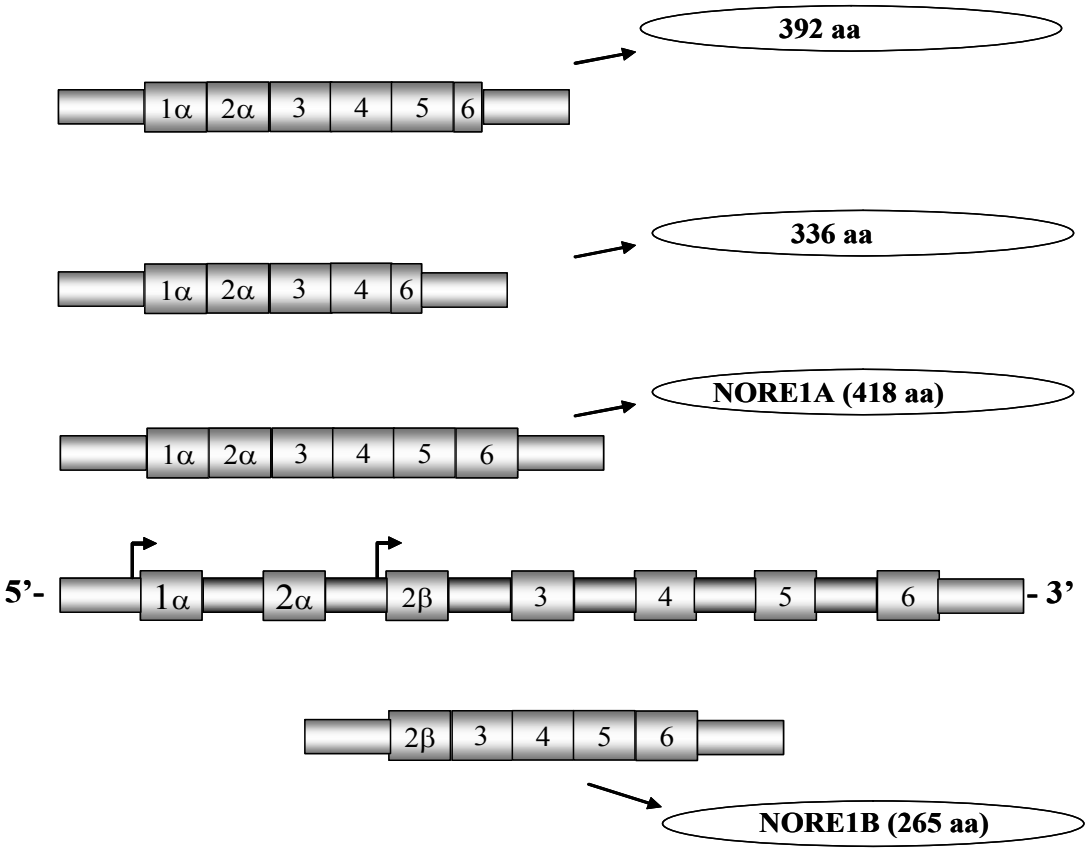

Figure 8.
Schematic illustration of the NORE1 / RASSF5 gene locus in chromosomal region 1q32.1. Four known transcripts are generated by two different promoters and translated into different protein isoforms. The two main characterized isoforms are NORE1A and NORE1B.

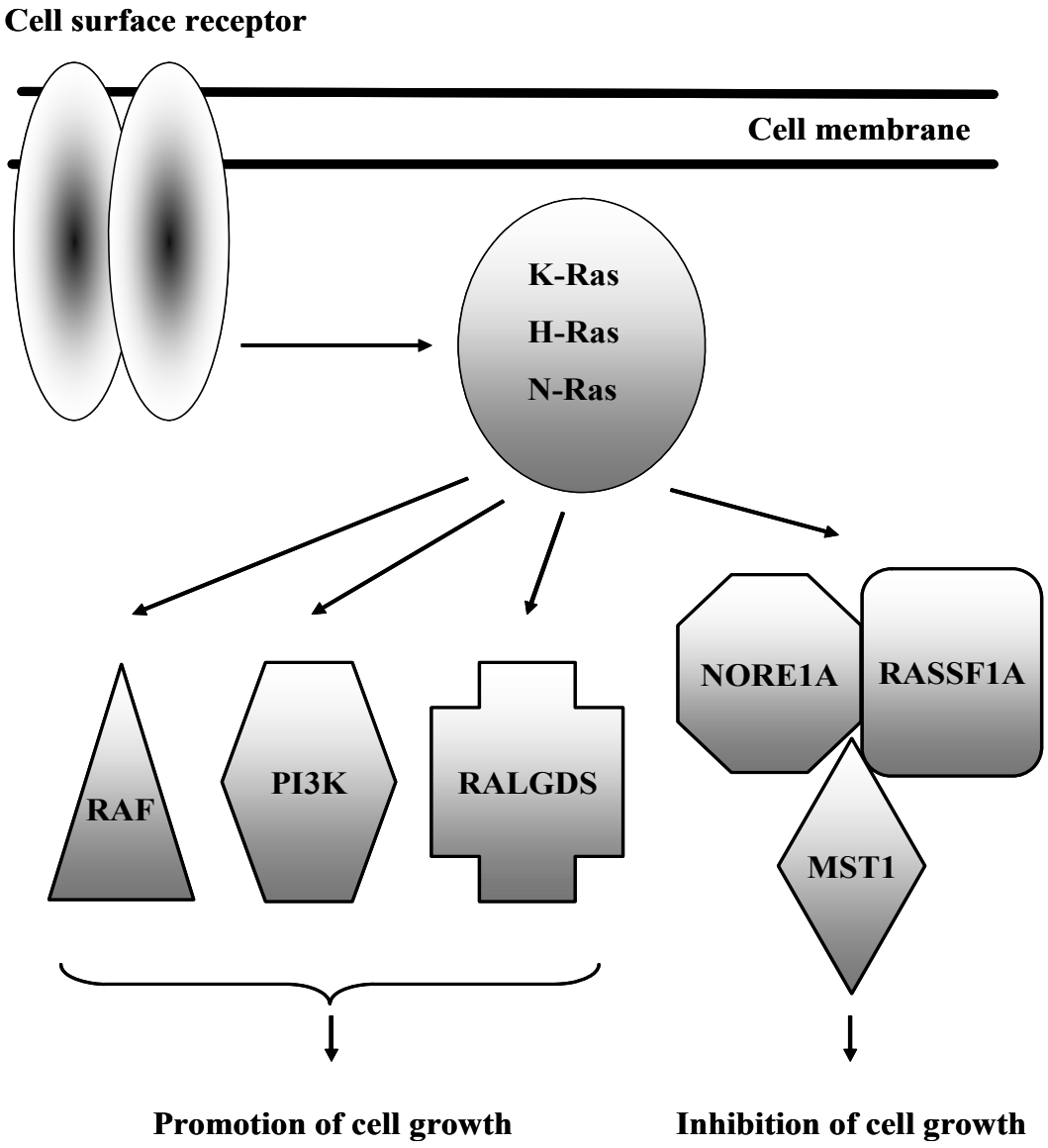

Figure 9.
Ras relays signals from various cell surface receptors to downstream effectors. The effect on cell growth may be versatile depending on the downstream effector pathways activated. Growth enhancing signals may be conveyed via interaction with Raf, PI3K or RALGDS. On the other hand activation of the NORE1A / RASSF1A pathway results in growth inhibitory effect (146).

The RIZ1 tumor suppressor

The Retinoblastoma-interacting zinc finger gene (RIZ), is mapped to 1p36.2 a chromosomal region commonly deleted in various human neuroendocrine tumors (170-172). Two products are encoded by the RIZ gene by means of alternative promoter usage: RIZ1 and RIZ2 (Figure 10). RIZ1 and RIZ2 are identical except for the 5'-end region, corresponding to the PR domain that is present only in RIZ1 (173, 174). RIZ1 belongs to the PR/SET domain family, the members of which are significant players in chromatin-mediated gene expression regulation, both during embryonic development, and in human cancer (173-175). RIZ1 catalyses methylation of the lysine 9 residue in histone H3, which is a modification generally associated with heterochromatin formation and gene silencing (176). In addition RIZ1 can also function as co-activator / co-repressor of nuclear hormone receptors (173, 177). Both RIZ1 and RIZ2 are highly expressed in normal neuroendocrine tissues including the adrenal medulla (170). However, only RIZ1 but not RIZ2, is known to exert tumor suppressive actions (178). Underexpression of RIZ1, but not RIZ2, has been observed in various cancers including colon, breast, pancreas, hepatocellular carcinoma and neuroblastoma (171, 179-182). Epigenetic inactivation by promoter methylation appears to be a common mechanism for RIZ1 silencing (183, 184). In several instances RIZ mutations have been found in cancer (179, 182, 185) albeit not in pheochromocytoma (186). RIZ1 has been implicated in cell cycle arrest and apoptosis induction, as well as suppression of tumor xenograft growth (179, 180). RIZ1 knock-out mice with normal RIZ2 expression develop a broad spectrum of tumors. The most common tumor type is diffuse large B cell lymphoma (185). Given the widespread experimental and observational evidence assigning a tumor suppressive role to RIZ1, as well as its genetic location in distal 1p, commonly implicated in pheochromocytomas and abdominal paragangliomas, we evaluated in paper II RIZ1 as candidate tumor suppressor in these tumors.

The p16 tumor suppressor

The CDKN2A locus at 9p21 is commonly altered in human cancer by point mutations, deletions and promoter methylation (187). The two unrelated proteins p16 and p14 encoded by the gene are both *bona fide* tumor suppressors (188-190). The transcripts $p16^{INK4A}$ and $p14^{ARF}$ have distinct exon 1 (1α and 1β respectively) that are spliced onto the shared exon 2 in differential reading frames resulting in entirely different proteins.

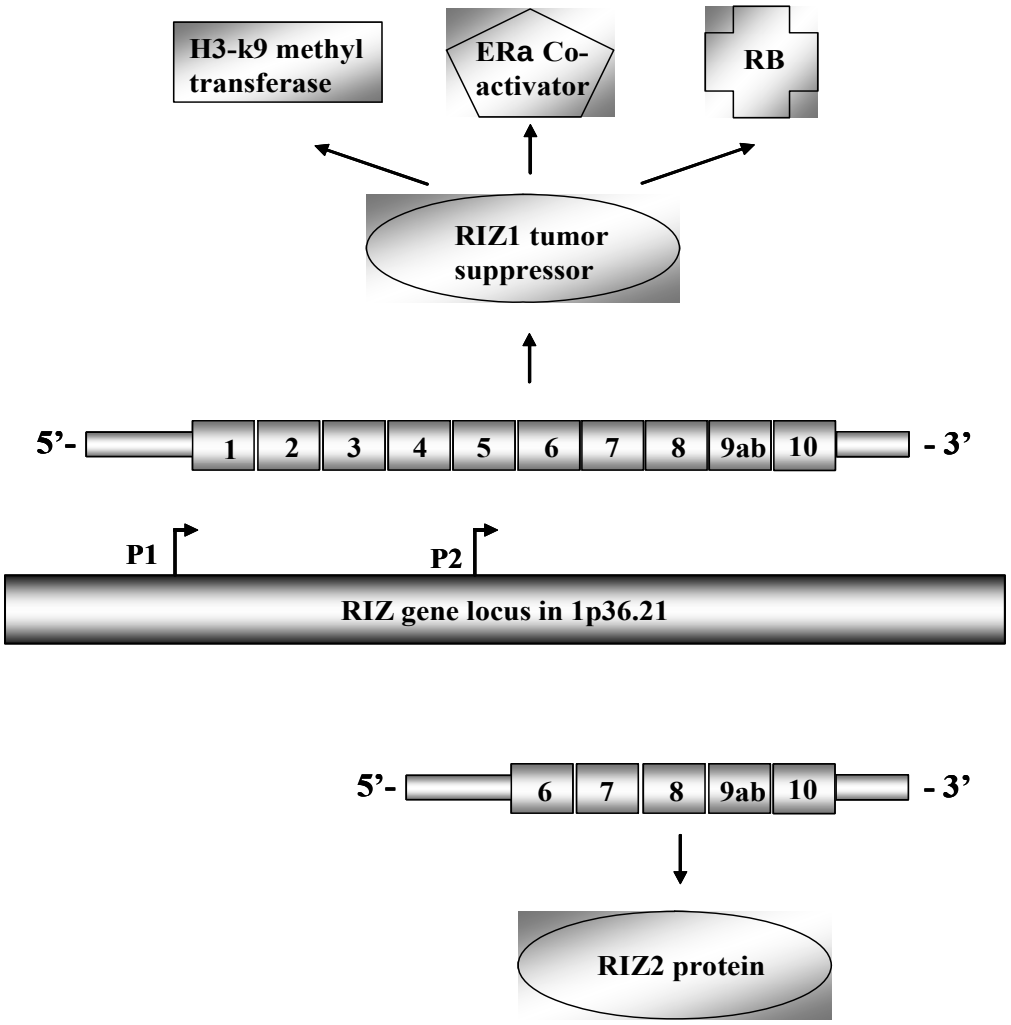

Figure 10.
The RIZ gene locus in 1p36.21 with its encoded products RIZ1 and RIZ2. The RIZ1 tumor suppressor is derived from the P1 promoter and has effects on histone H-3 methylation, estrogen receptor alpha and the retinoblastoma protein as illustrated at the top. RIZ2 is generated from promoter P2 and is suggested to have oncogenic effects.

Transcriptions of exons 1α and 1β are under the control of separate promoters (191, 192) (Figure 11). The proteins p16 and p14 participate in two main cell-cycle control pathways, p16 -Rb and p14 -p53 (193, 194) and generally function as negative regulators of cell growth (195). The p16 protein inhibits phosphorylation of Rb by binding to Cyclin D-cdk4/6 complexes (195). On the other hand p14 inhibits MDM2 mediated degradation of the "genome guardian" p53 (196-198).

Mice hemizygously deficient for the tumor suppressor gene Pten are particularly prone to develop unilateral pheochromocytomas, rendering it an applicable model for exploration of this tumor type (199). Furthermore, in Pten-Ink4a/Arf knockout mouse bilateral pheochromocytomas developed with shorter latency after hemizygous Ink4a/Arf inactivation, and in the homozygous form metastasis developement occured

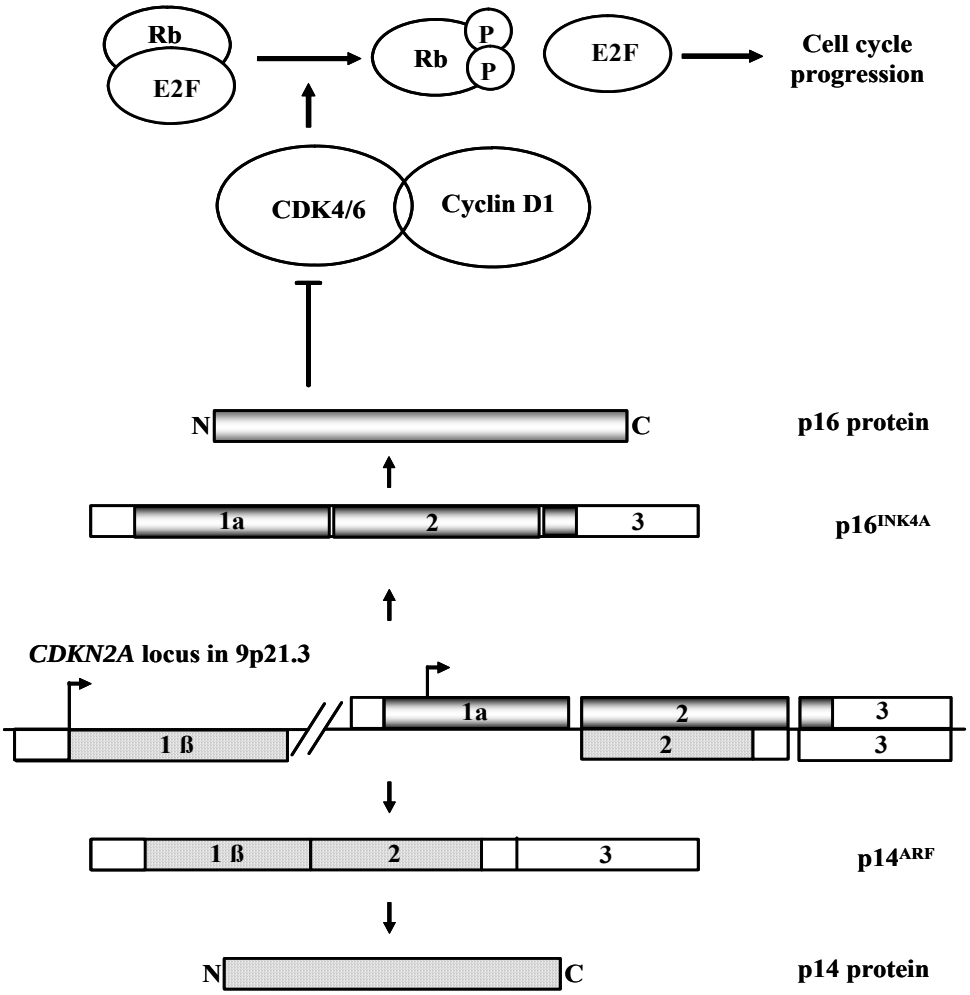

Figure 11.

The CDKN2A locus, its alternative transcripts $p16^{INK4A}$ and $p14^{ARF}$, and their encoded proteins p16 and p14. p16 exerts its growth inhibitory action via the RB pathway by blocking cyclin-dependent kinases CDK4 and CDK6. Normally CDK4 and CDK6 promote cell cycle progression via phosphorylation of the RB protein resulting in the release of the E2F transcription factor.

(199). These findings implicate that loss of p16 and / or p14 contribute to the development of this neoplasia. In human pheochromocytomas loss of heterozygosity at the CDKN2A gene locus in 9p21 has not been observed (68). However $p16^{INK4A}$ promoter methylation was seen in a subset of pheochromocytomas (157).

Alteration of the CDKN2A gene is rare in neuroblastoma (200-204). In neuroblastomas, up-regulation of p16 expression appears to correlate with unfavourable outcome. However, this is not paralleled by alterations in levels of phosphorylated RB, suggesting that p16 may not be functioning properly to regulate the pathway (205). Attenuation of the p14 - MDM2 pathway is frequently found in neuroblastoma cell lines established at relapse and the occurrence p14 methylation has been confirmed in some neuroblastoma tumors (206-208).

AIMS OF THE PRESENT INVESTIGATION

- To explore the occurrence of concerted promoter hypermethylation for multiple tumor suppressor genes in pheochromocytomas and abdominal paragangliomas and its relation to clinical behaviour (paper I).
- To illuminate alterations in global methylation levels in abdominal -paragangliomas and pheochromocytomas and its possible influence on clinico-pathological features (paper I).
- To determine the potential involvement of the RIZ1 tumor suppressor in pheochromocytomas and abdominal paragangliomas tumorigenesis (paper II).
- To shed light on the involvement of the Ras effectors RASSF1A and NORE1A in pheochromocytomas and abdominal paragangliomas (paper III).
- To examine the role of NORE1A in neuroblastoma cell lines and primary tumors (paper IV).
- To explore the involvement of Ras signalling in follicular thyroid cancer with special regards to NORE1A and RASSF1A, and to investigate the possible link between Ras pathway and PAX8-PPRγ rearrangement (paper V).
- To determine alterations in the CDKN2A locus, its transcripts: $p16^{INK4A}$ and $p14^{ARF}$, as well changes in p16 protein expression in order to explore their significance in the pathogenesis pheochromocytomas and abdominal paragangliomas (paper VI).

MATERIALS AND METHODS

Pheochromocytoma and abdominal paraganglioma samples (papers I, II, III and VI)

The patients in this study were operated at the Department of Surgery of the Karolinska University Hospital, Stockholm, Sweden, and subsequently followed up in a polyclinical setting. Most patients are part of a previously published characterization of pheochromocytoma and abdominal paraganglioma patients treated at this hospital (35).

All tissues had been dissected by a pathologist directly after surgery, snapfrozen in liquid nitrogen and stored at -70 ºC. Each sample used in the studies was verified to contain high representation (>70%) of tumor cells by histopathological examination carried out by an experienced endocrine pathologist. Tumor classification followed the criteria of the US Armed Forces Institute of Pathology, according to which malignant cases should exhibit extensive local invasion and/or distant metastasis (37, 38). From a subset of cases, peripheral blood samples were also used for comparative studies. Informed consent was obtained from each patient and the study of the tissue material was approved by the institutional ethical review board.

Neuroblastoma and ganglioneuroma specimens (paper IV)

All patients were diagnosed and treated at the Karolinska University Hospital, Stockholm, Sweden. In each case diagnosis was histopathologically confirmed by a paediatric pathologist, and in addition the routine characterization of tumors included analyses of *MYCN* amplification, 1p loss and DNA-ploidy as reported (111, 117, 209). Tumors were staged according to the International Neuroblastoma Staging System (INSS) (99), and the treatment followed risk-based national and international protocols. Surviving patients were followed at the Department of Paediatric Oncology at the Karolinska University Hospital for a median of 119 months. The samples used for the study had been frozen in connection to surgery and stored at -70 °C. All samples were obtained with informed consent from the patients or their parents, and the study was approved by the ethics committee at the Karolinska University Hospital.

Reference adrenal samples (papers I, II, III and VI)

Extracts from normal adrenal medulla were commercially available. In addition, samples of non-tumor adrenal were obtained with informed consent and ethical approval from patients treated at the Karolinska University Hospital, Stockholm, Sweden.

Thyroid tumor samples (paper V)

The patients with follicular thyroid tumors included in paper V underwent thyroidectomy at the Department of Surgery of the Karolinska University Hospital Stockholm Sweden and were subsequently followed up polyclinically. For histopathological diagnosis the criteria of the WHO classification were applied (210). The tissue samples were collected in direct connection to surgery and verified using the same routine as for pheochromocytoma and abdominal paraganglioma. Using this procedure the tissues were appropriately handled to allow extraction of good quality DNA, RNA and protein.

Established Cell lines (papers II-VI)

HepG2 and Hep3B liver cancer cells served as positive and negative controls, respectively, for determining RIZ1 promoter methylation in paper II. PC12 rat pheochromocyotma cells were used in paper III for functional studies after Nore1a transfection. MCF7 breast cancer cells served as positive control for NORE1A methylation (papers III, IV and V). Seven neuroblastoma cell lines were used for characterization of NORE1A and RASSF1A: SK-N-DZ, SK-N-SH, SK-N-BE(2), SK-N-FI, SK-N-AS, IMR-32, and SH-SY-5Y (paper IV). In paper V cervical carcinoma HeLa cells were used to study the impact of PAX8- PPRγ on NORE1A promoter activity. In paper VI SAOS-2 osteosarcoma cells was applied as positive control and MCF7 cells as negative control for p16 protein expression.

Quantitative Real Time PCR (qRT-PCR) (papers II-VI)

Quantitative real time PCR (qRT-PCR) is and established technique to measure RNA transcript abundance (211). The concept is based on the quantitative detection of PCR products as they accumulate, by means of fluorescent emission using fluorescent probes. The two most commonly utilized chemistries for quantification is SYBR Green and Taqman detection systems of which the latter was used in this study (Figure 12). The experimental procedure starts by isolation of RNA which is converted to cDNA using reverse transcriptase. During the PCR reaction the amount of accumulating PCR products is proportional to the starting amount of target according to the following formula: $X_n=X_0 \times (1+E_x)^n$, where X_0 is initial target abundance, X_n is the amount of PCR product after n cycles, n is the number of PCR cycles and E_x is PCR efficiency.As the fluorescent signal generated during synthesis is proportional to the amount of PCR product it also renders an accurate reflection of the initial target abundance. In practice a parameter called threshold cycle Ct is used which is the number of PCR cycles required to reach a predefined level of fluorescent signal intensity. Ct

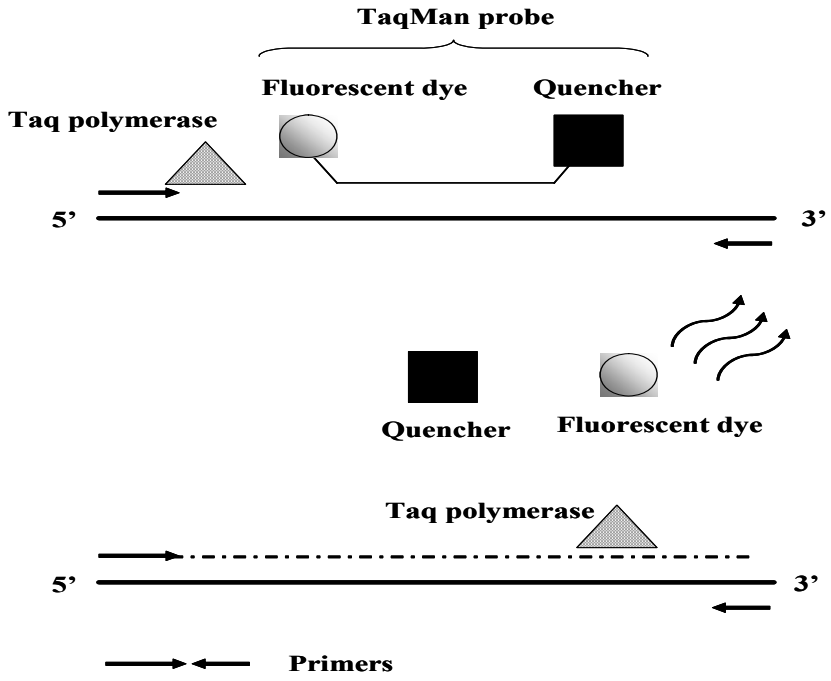

Figure 12.

Quantification of gene expression using qRT-PCR analysis. PCR primers and an oligonucleotide probe (Taqman probe) ensure specificity of the reaction. The Taqman probe carries a fluorescent reporter dye and a quencher dye attached to its 5' and 3' ends, respectively. When the probe is intact, given the steric proximity of the two dyes, the quencher suppresses fluorescence emitted by the reporter. However, once amplification occurs, the probe is degraded by the 5'-3' exonuclease activity of the Taq polymerase, thus the reporter and quencher will no longer be in close proximity. The fluorescent signal is detected by means of a laser integrated in the detector.

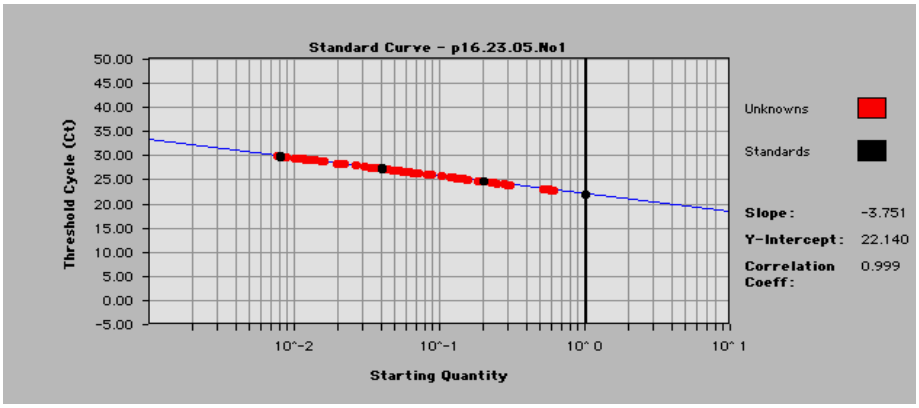

Figure 13.
Standard curve of a qRT-PCR reaction for $p16^{INK4A}$. The slope of the curve indicates the efficiency of the reaction, while the unknown samples (red dots) are quantified in relation to the quantities of the standard curve (black dots).

shows inverse correlation to the starting amount of genetic material. The difference of Ct between different samples provides a direct indication about their relative quantities. An alternative to direct comparison of Ct values is the use of standard curve generated by serial dilutions of a reference sample (Figure 13). This approach carries the advantage of indicating PCR amplification efficiency, which should be constant in the Ct range of the sample panel.

Amplification of an endogenous reference is used to compensate for variations in quantity and quality of RNA used for cDNA synthesis as well as for other experimental biases (such as pipetting errors). The target gene levels are subsequently normalized relative to those of internal reference genes, to allow comparisons between the samples analyzed. However, to achieve proper normalization it is of paramount importance, that the chosen reference gene shows consistent expression across the samples. To achieve this, optimal reference gene(s) are selected from a panel of house-keeping genes and in the analysis the geometrical mean of those with the most stable expression levels is used (212).

In this study, Taqman qRT-PCR was used to assess mRNA expression levels of RIZ-PR and RIZ-CR (paper II), NORE1A and RASSF1A (papers III, IV, V), and $p16^{INK4A}$ as well as $p14^{ARF}$(paper VI).

Sodium bisulfite modification of DNA (papers I-VI)

In higher order eukaryotes, DNA is methylated only at cytosines located 5' to guanosine commonly referred to as CpG. This nucleotide modification has significant regulatory functions particularly when it involves CpG-rich areas known as CpG islands, located in gene promoters. Normally promoter CpG islands are protected from methylation while when methylated it is associated with transcriptional suppression.

Bisulfite treatment is a chemical DNA modification resulting in the conversion of unmethylated cytosine to uracil while methylated cytosines remain intact (Figure 14). This process essentially converts differential methylation patterns into differential sequences which can be accurately detected by further downstream applications such as methylation specific PCR (MSP), combined bisulfite restriction assay (COBRA) and bisulfite Pyrosequencing.

Combined Bisulfite Restriction Assay (COBRA) (papers III, IV and V)

In the COBRA assay restriction enzyme digestion is used to detect methylation dependent sequence differences in PCR products of sodium bisulfite treated DNA. The sequence conversion by bisulfite can lead to the methylation dependent creation of new restriction enzyme sites, alternatively to methylation-dependent retention of pre-existing sites. PCR primers are designed to amplify the interrogated segment regardless of methylation status. Therefore the PCR product contains a mixed population where the fraction that has a newly created or retained restriction site containing a CpG(s) is a direct indication of the proportion of methylation at that site in the original genomic DNA (213) (Figure 14). COBRA analysis was used for the assessment of NORE1A methylation in pheochromocytomas and abdominal

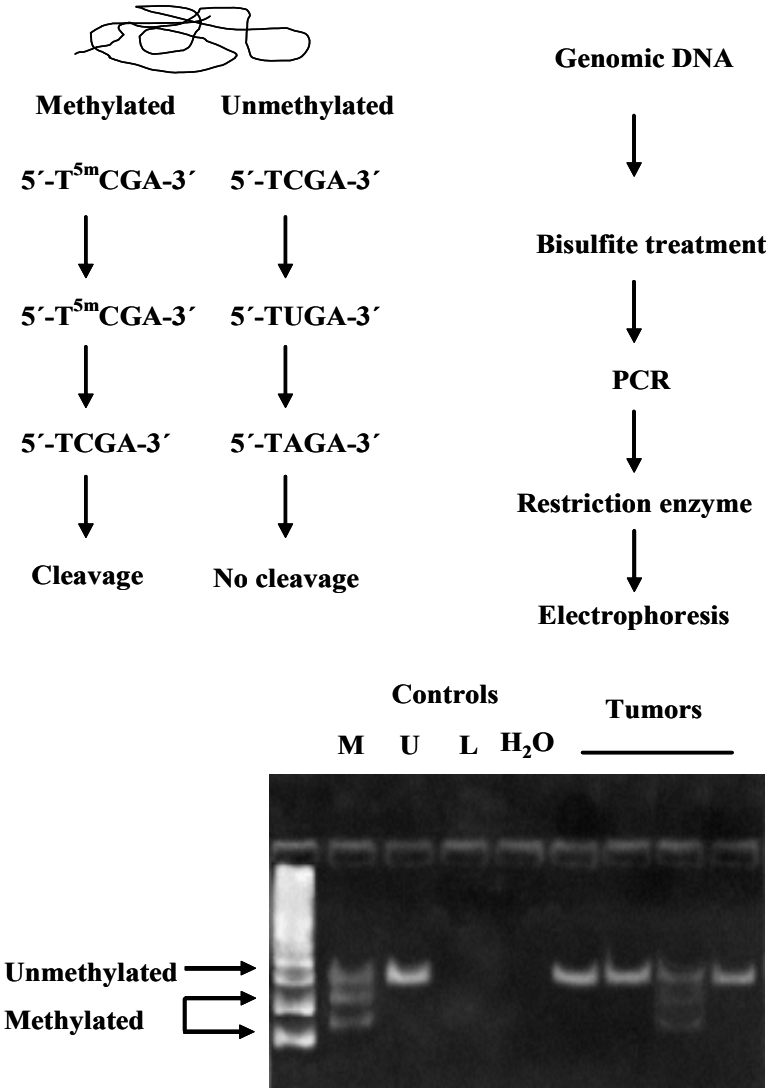

Figure 14.
Schematic illustration of bisulfite DNA modification and COBRA analyses. Bisulfite treated DNA is amplified by PCR using primers that do not distinguish between methylated and unmethylated sequences. The restriction site in the PCR product contains one or more CpGs, which had undergone methylation dependent sequence alteration upon bisulfite treatment. In the example shown, unmethylated alleles have no *Taq*I site and remain uncleaved, while methylated alleles posess *Taq*I site resulting in restriction cleavage. The result is visualized by agarose gel electrophoresis. The methylated control (M) and one of the tumor samples show partial methylation, while unmethylated (U) and blank (L, H_2O) controls as well as three of the tumors are unmethylated.

paragangliomas (paper III), neuroblastomas (paper IV), and follicular thyroid carcinomas and adenomas (paper V).

Methylation Specific PCR (MSP) (papers II and VI)

MSP is an allele specific PCR assay for the differential detection of methylated and unmethylated variants of a given sequence (Figure 15). The assay exploits methylation dependent sequence differences resulting from bisulfite conversion of DNA. Two different primer sets are used: one specific for the methylated allele and one for the unmethylated.

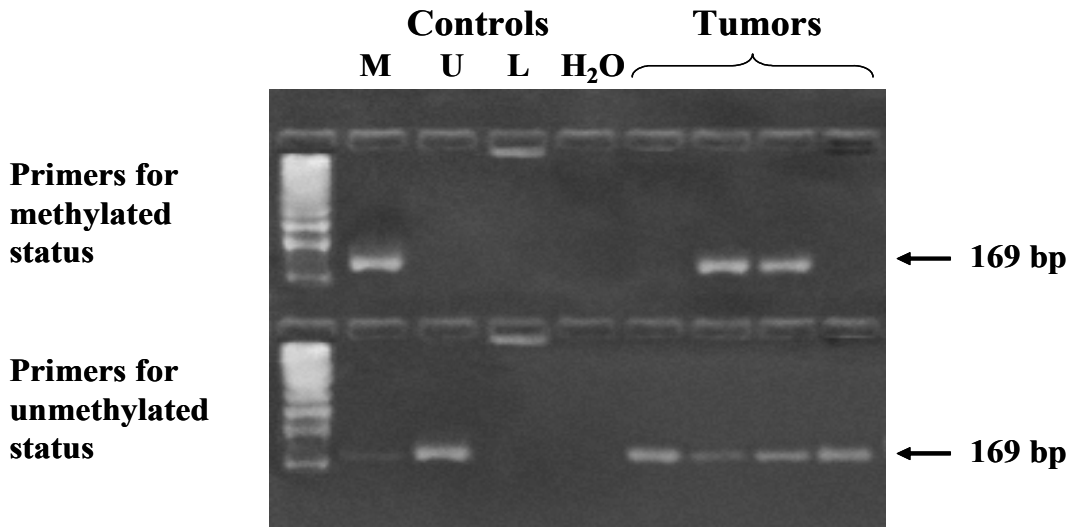

Figure 15.
MSP analyses of the RASSF1A promoter in tumors and controls. After PCR using primers specific for the methylated or unmethylated status the products are visualized in agarose gel. In the example shown, two of the four tumors show partial methylation giving products using both primersets, while the other two tumors are unmethylated. *Sss*I *in vitro* methylated DNA was used as control for methylation (M). Normal bisulfite treated DNA without *Sss*I treatment (U) constitutes the negative controls, while regular DNA (L) and H_20 are blanks.

Optimal primer design is a crucial component of the assay. Primers should be designed such that a CpG is contained at the 3' end. Furthermore, to avoid false positive results (amplification of non-converted DNA with methylation specific primers) the primer should also contain non-CpG Cs.

MSP is one of the most widely used techniques for detection of DNA methylation. It carries the advantage of high sensitivity enabling the methylation detection of miniscule amounts of biological material (214, 215)*)* rendering it particularly useful for prospective diagnostic applications where target amount is a serious limiting factor (such as detection of tumor cells from bodily fluids). On the other hand it is essentially an end point assay i.e. it cannot accurately quantify the proportion of methylated and unmethylated alleles.

Bisulfite Pyrosequencing (papers I, III, IV and VI)

Pyrosequencing is a DNA sequencing method based on sequencing by synthesis. The heart of the technique is the accurate quantitative detection of released pyrophosphate (PPi) after incorporation of each nucleotide. This is achieved by a series of enzymatic reactions using PPi as initial substrate and resulting finally in the generation of light proportional to that of released amount of PPi, thus the number of nucleotides incorporated. Light is detected by a charge coupled device (CCD) and is seen as a peak in the resulting Pyrogram (Figure 16). Single stranded primed DNA is used as template for the nucleic acid synthesis reaction. The

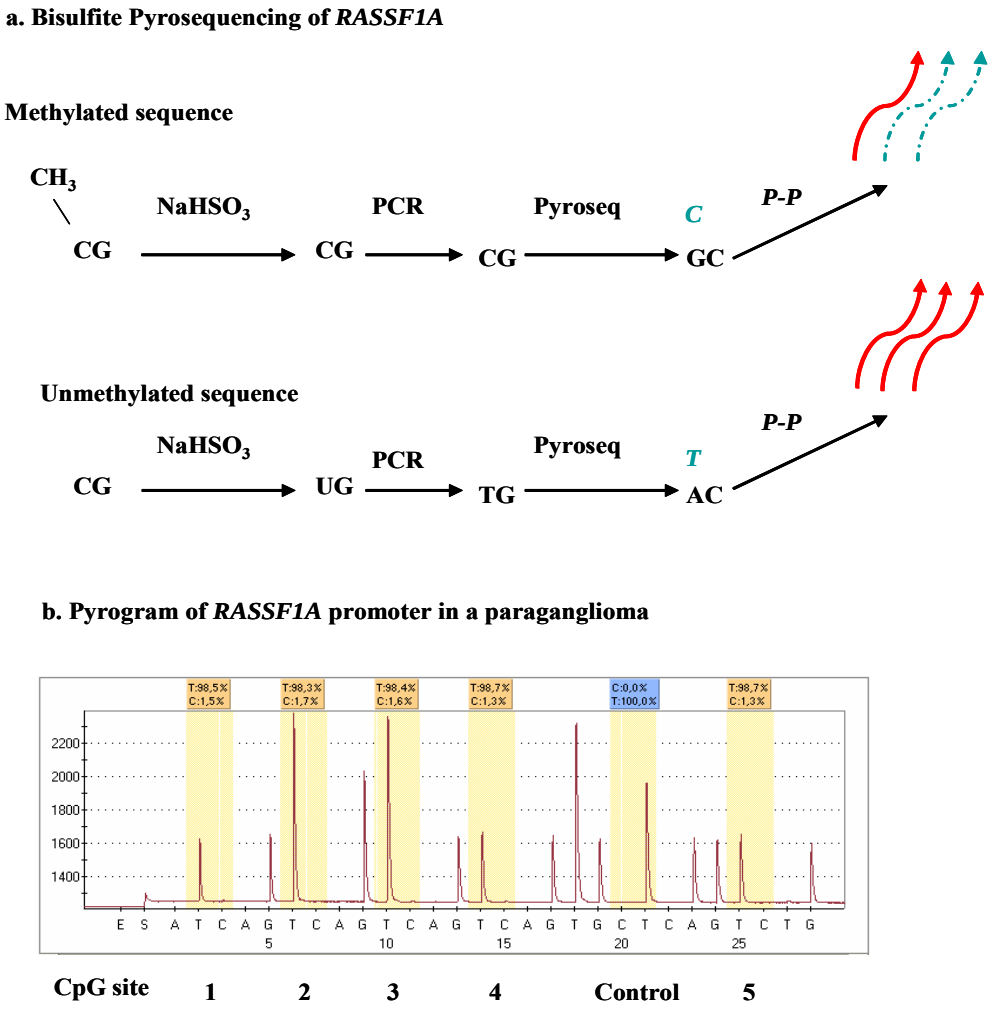

Figure 16.
(a) Schematic illustration of the principles of bisulfite Pyrosequencing. Bisulfite treatment of DNA results in differential conversion of Cytosines (C) to Thymidines (T) depending on the methylation status. During the Pyrosequencing reaction a C is incorporated if the template CpG is methylated while a T is incorporated if the template CpG is unmethylated. For each CpG analysed the ratio of C:T incorporation reflects the proportion of methylated to unmethylated alleles i.e. methylation density.
(b) Pyrogram from quantification of methylation by Pyrosequencing of the RASSF1A promoter. A non-CpG C is used as internal control for efficiency of the bisulfite conversion

addition of nucleotides is performed in a controlled manner one at a time enabling the precise distinction between incorporation of different nucleotides at each template base position.

Bisulfite Pyrosequencing uses PCR products of bisulfite treated DNA as template (Figure 16). Prior to Pyrosequencing the PCR primers amplify the target sequence without discriminating between templates according to original methylation status (i.e. no CpG is located 3' in the primer). Thus the PCR products contain a mixture of methylated and unmethylated alleles. As bisulfite treatment results in methylation dependent conversion of cytosines to tymidines, the ratio of C/T (or A:G if sequenced from the reverse strand) accurately reflects the proportion of methylated to unmethylated alleles at the CpG site analysed. Generally, a single assay can

cover between 4-20 CpG sites and determine methylation density at each of them. Given the quantitative nature of bisulfite Pyrosequencing it offers the possibility of stratifying biological samples according to their level of methylation of a particular CpG site.

Bisulfite Pyrosequencing was used for assessing methylation density of the following tumor suppressor gene promoters: CDH1, DCR2, RARB, TP73, APC, DAPK1 and PTEN (paper I) RASSF1A (paper III and IV), NORE1A (paper IV), $p16^{INK4A}$ and $p14^{ARF}$ (paper VI). Furthermore, bisulfite Pyrosequencing was used for assessing methylation at LINE-1 repetitive elements as a surrogate measure of global methylation (paper I).

Luminometric Methylation Assay (LUMA) (paper I)

LUMA is a novel method for quantitative assessment of genome-wide methylation. It quantifies methylation density at CCGG restriction sites across the genome and uses it as a surrogate indicator of global methylation (Figure 17). The technique incorporates cleavage by methylation-sensitive restriction enzymes and the quantitative detection of generated "overhangs" by Pyrosequencing. Genomic DNA is separately cleaved with two combinations of restriction enzymes *Hpa*II + *Eco*RI and *Msp*I + *Eco*RI. *Hpa*II and *Msp*I are isoschizomers i.e. they recognize the same restriction site CCGG, but whereas *Hpa*II cuts only if the restriction sequence is unmethylated, *Msp*I is able to cleave regardless of methylation status. Thus the extent of *Hpa*II / *Msp*I digestion gives a direct indication of the degree of methylation at the CCGG sites across the genome. The degree of cleavage is quantified by Pyrosequencig. Since digestions by the two isoschizomers are run in separate reactions digestion by *Eco*RI is used as internal control. LUMA analysis was used for the assessment of global methylation in pheochromocyotmas and abdominal paragangliomas (paper I).

Mutation detection by sequencing (papers I, V and VI)

Sanger sequencing is based on template and primer dependent DNA synthesis reaction aimed to reveal the nucleotide order in a given DNA stretch. As opposed to PCR only one primer is used. In addition to the four deoxynucleotide bases fluorescently labelled dideoxynucleotides are added to the reaction mix. These nucleotides have the capacity to incorporate into the new DNA strand but block further elongation. Subsequently the sequence reaction mix is size separated in gel or capillary based systems. The four different labelling reveal the base and the size of the fragment reflects the base position. The sequence is then presented as peaks in

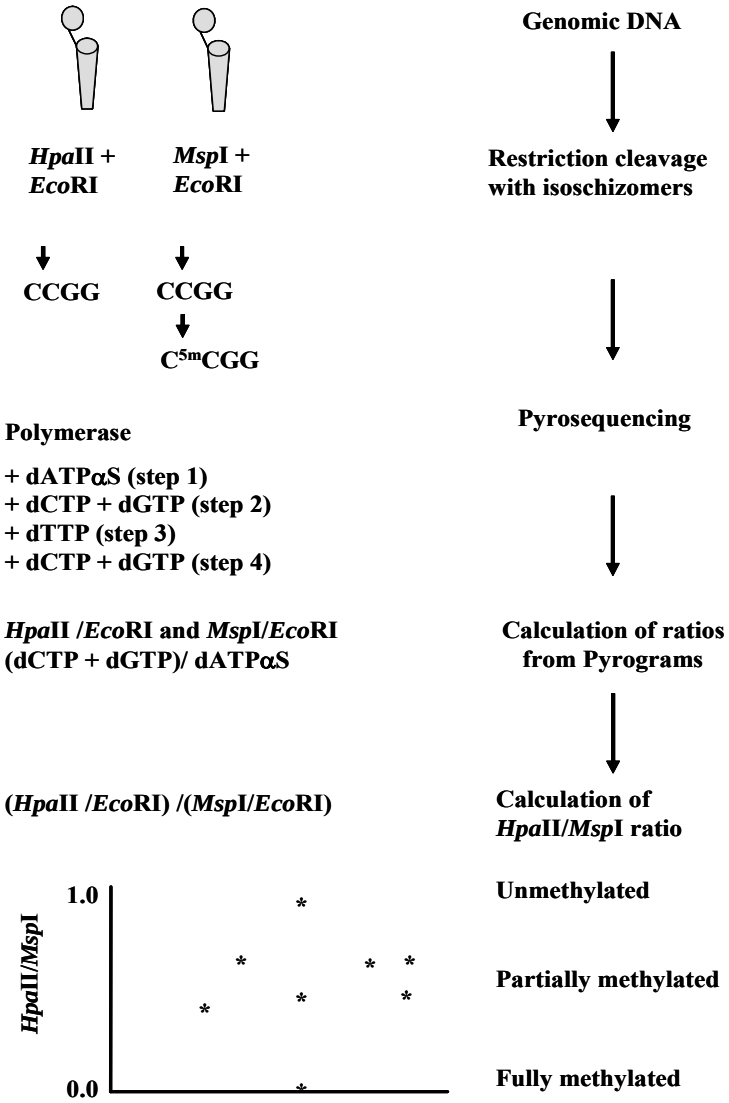

Figure 17.
The principles of LUminometric Methylation Assay (LUMA) for quantification of genome-wide methylation at CCGG sites. After restriction cleavage with methylation sensitive isoschizomer enzymes and quantification of cleavage by Pyrosequencing, *Hpa*II/*Msp*I ratios are calculated and used as relative indicators of methylation levels.

the resulting chromatogram (Figure 18). To firmly identify a mutation, different controls are recommended such as reverse and forward sequencing, confirmatory restriction cleave and exclusion of the alteration in the normal population. Sequencing was used in papers I, V and VI for the assessment of BRAF, RAS and $p16^{INK4A}$ genes respectively.

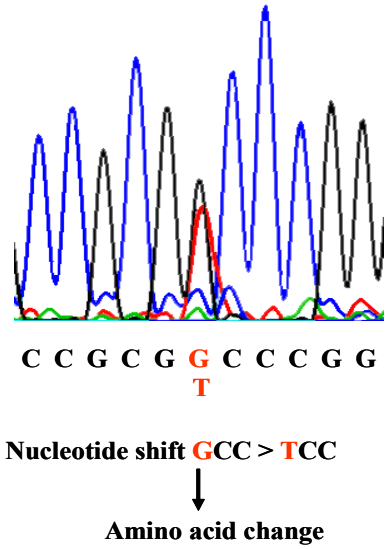

Figure 18.
Sequencing chromatogram obtained from capillary analysis illustrating a hypothetical sequence alteration involving a heterozygous base shift from G to T with a predicted missense aminoacid alteration.

Loss of Heterozygosity (LOH) (paper II)

Loss of heterozygosity (LOH) analysis is used to detect allelic imbalance (AI), a DNA dosage disparity between the maternal and paternal alleles of a given target region when comparing tumor and constitutional DNA. It may occur as a result of various genetic alterations, such as unbalanced translocation, deletion, or whole chromosomal loss. Commonly, single nucleotide polymorphisms or highly polymorphic microsatelite markers are used for the detection of LOH/AI. It is essential that the maternal and paternal alleles of a given polymorphic microsatellite marker are of different lengths enabling distinction between them by size. If the two alleles are of the same size the marker is called non-informative. Constitutional and tumor DNA is used for the amplification of markers by fluorescently (or radioactively) labelled primers annealing to flanking regions. Today the PCR products are size separated in an automated sequencer and quantified by means of fluorescent emission using Gene Scan software. As exemplified in Figure 5, alleles of different lengths appear as separate peaks in the program. LOH/AI is detected by visual inspection and by calculation of ratios between peaks in the constitutional vs. the tumor DNA. This technique was used in paper II for the detection of LOH in the RIZ locus at 1p36 in pheochromocytomas and abdominal paragangliomas.

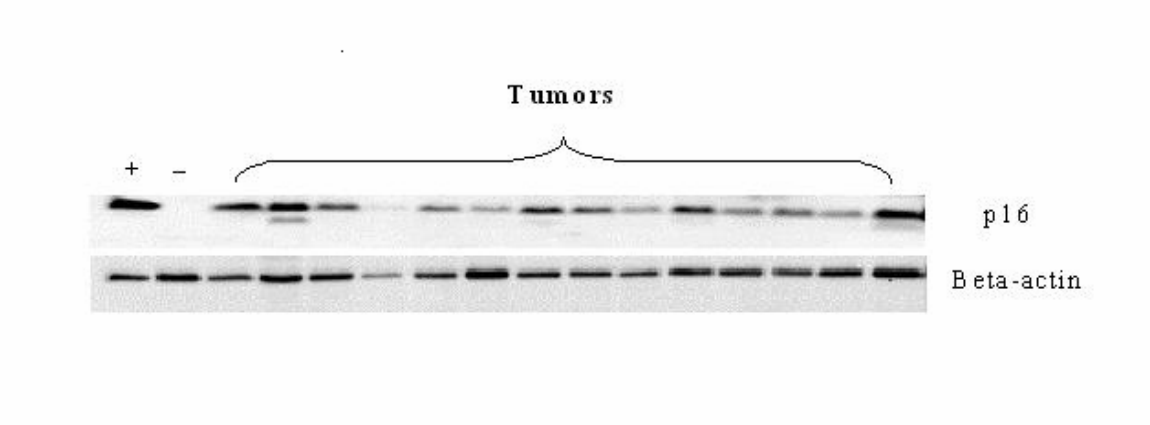

Figure 19.
Results from Western blot analyses of the p16 protein in pheochromocytomas and abdominal paragangliomas. Beta-actin was used as loading control**.**

Western blot analysis (papers III, V and VI)

Western blotting is a method for specific protein detection in tissue lysates. As a first step the mixed protein population from tissue extracts are electrophoretically size separated on SDS polyacrylamid gel and subsequently transferred onto a membrane (typically nitrocellulose). The target protein is then detected by means of a specific antibody and subsequent colorimetric or radiographic detection of antibody binding. The assay reveals information about the proteins size and amount (Figure 19).

Western blot analyses were used for detecting NORE1A (papers III and V) and for evaluating p16 protein expression (paper VI).

Fluorochrome inhibition of Casepases (FLICA) (papers III and IV)

Caspases play a pivotal role as executioners in apoptotic cell death. FLICA is a fuorescently labelled, cell permeable small molecule that covalently binds to the catalytic site of activated caspases. Because FLICA is covalently coupled to the enzyme, it is retained in the cell while any unbound reagent is washed out. As FLICA is a pan-caspase inhibitor it does not give further indication of which apoptotic pathway is activated. Sulforhodamine-FLICA assay was used to determine the fraction of apoptotic cells after transfection with Nore1a in PC-12 pheochromocytoma cells (paper III) and SK-N-BE(2) neuroblastoma cells (paper IV). Analyses were done by flowcytometry. The green fluorescent cell population (transfected cells, Figure 20) was assessed for red fluorescence (FLICA) representing the apoptotic subpopulation of transfected cells.

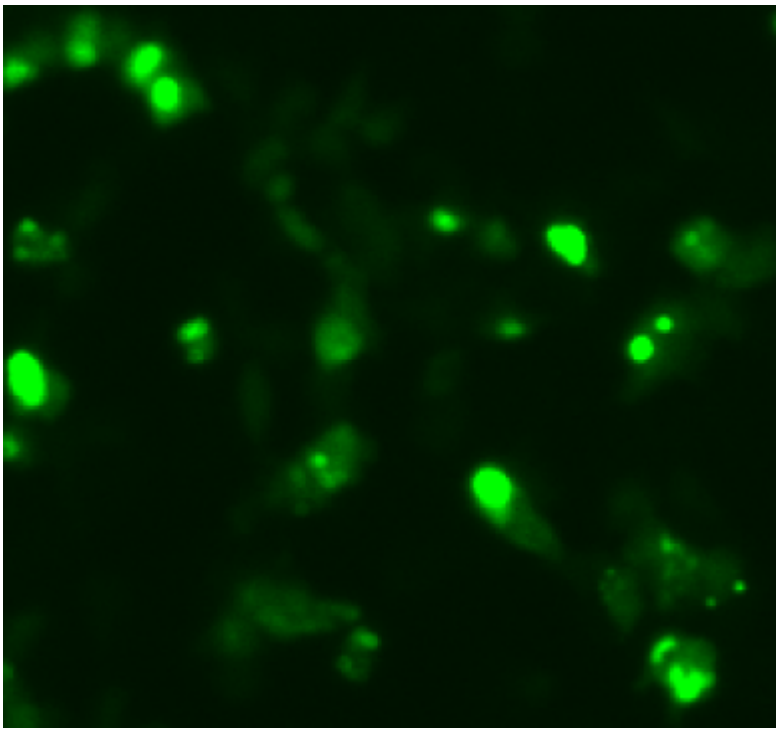

Figure 20.
Fluorescent live cell imaging showing successful transfection of SK-N-BE(2) cells with the GFP~Nore1a construct.

Bromodeoxyuridine (BrdU) incorporation assay (paper IV)

BrdU is a thymidine analogue, which can be incorporated into the newly synthesizing DNA strand during S phase. Incorporated BrdU is then stained with fluorescently labelled anti-BrdU antibodies and quantified with flow cytometry. The proportion of cells showing BrdU incorporation after short term "pulse" labelling reflects thus the population fraction entering and progressing through S phase. BrdU incorporation assay was utilized in paper IV to assess the effect of ectopic Nore1a expression on cell cycle progression from G1 to S phase in SK-N-BE(2) neuroblastoma cells.

Soft agar assay (paper III)

Neoplastic transformation of cells is associated with a number of phenotypic changes such as loss of contact inhibition and anchorage independence. Normal cells fail to grow when suspended in a viscous matrix. However, when transformed they acquire the ability to grow without attachment to a solid phase i.e. in an anchorage independent fashion. In general there is a reasonably good correlation between the level of anchorage independent growth of tumor cells (number and size of colonies formed in soft agar) and their *in vivo* tumorigenic potential.

Soft agar assay was used in paper III to study the effect of Nore1a transfection on anchorage independent growth in PC-12 cells.

Luciferase reporter assay (paper V)

The Luciferase reporter system assesses the effect of regulatory signals on transcirptional activity of the target gene. The target gene promoter (in this case NORE1A) is cloned into a plasmid upstream of the luciferase gene. Luciferase is an enzyme that catalyses a luminescent reaction in the presence of luciferin and ATP. An increased promoter activity leads to increased transcription of luciferase, resulting finally in increased light emission, which can be quantified by a luminometer. In paper V, the effect of PAX8, PPARγ and PAX8-PPARγ on NORE1A promoter activity was studied.

Definition of CIMP and Z score (paper I)

For each sample and promoter an average methylation density was calculated based on the methylation densities at the individual CpG-s as measured by bisulfite Pyrosequencing. Tumors demonstrating mean methylation density above the range observed in normal tissue in 3 or more promoters were classified as CIMP. To enable accurate assessment of concerted methylation at multiple promoters as a continuous variable, Z score analysis was used (216) including promoters whereby significant hypermethylation was detected relative to normal tissue in DCR2, RASSF1A, NORE1A, $p16^{INK4A}$, RARB, CDH1, and APC. As a first step promoter specific Z score was computed using the given formula: (mean CpG methylation density of the assessed promoter for each sample - mean methylation density of the promoter for the tumor panel)/SD of methylation density of the promoter. Then for each case a mean Z-score was calculated based on promoter specific Z-scores, and used subsequently as a single measure characterizing methylation density. Thus a mean Z-score > 0 reflects an overall promoter methylation density higher than the population average.

RESULTS AND DISCUSSION

ALTERATIONS IN GLOBAL AND PROMOTER SPECIFIC METHYLATION LEVELS IN PHEOCHROMOCYTOMAS AND ABDOMINAL PARAGANGLIOMAS (PAPER I)

CpG island methylator phenotype in abdominal paragangliomas

CpG island methylator phenotype (CIMP) refers to the concordant occurrence of consistently increased methylation in multiple genes. It is a molecular phenotype described in a number of neoplasias and often associated with distinct clinical and pathological features (30). This study sought to explore the occurrence and relevance of CIMP in pheochromocytomas and abdominal paragangliomas. To this aim promoter methylation of a set of tumors suppressor genes was quantitatively determined and evaluated in relation to clinical characteristics with focus on malignant behaviour.

Fourteen primary tumors (6 malignant, 8 benign) and two distant metastases showed hypermethylation at one or more of the following promoters: RASSF1A, NORE1A, $p16^{INK4A}$, RARB, DCR2, CDH1, APC. Cut-off value for hypermethylated status was set above methylation levels observed in normal adreno-medullary controls. Thus methylation above 10% was chosen as a cut-off value for hypermethylated status of all genes, with the exception of DCR2, for which 30% was used as a cut-off. Analyses of the TP73, DAPK1, PTEN and $p14^{ARF}$ genes only revealed background levels of methylation comparable to that observed in non-neoplastic tissue.

Concerted hypermethylation of 3 or more promoters defined as CIMP was confined to a group of 5 primary paragangliomas, 4 of which were malignant, while none of the pheochromocytomas displayed this molecular phenotype. Three primary paragangliomas with CIMP phenotype gave rise to distant metastases and one showed tumor recurrence on the contralateral side after primary surgery. Thus CIMP was significantly associated with malignant disease ($p=0.005$). This finding was accurately mirrored by Z score analyses where a preponderance of malignant cases was observed among tumors with a mean Z-score >0 ($p=0.02$).

Association of CIMP with adverse clinical features, a finding also described in other types of neoplasias (19, 23, 26-28) may reflect a selection for this phenotype in the malignification process. It is yet to be unravelled whether CIMP is a causative factor or a consequence of malignant transformation. Given that promoter hypermethylation is generally associated with transcriptional silencing it is possible that one of the mechanisms by which CIMP contributes to tumorigenesis is realized through simultaneous suppression of multiple tumor suppressors governing cellular growth and death. Alternatively CIMP may be associated with other underlying genetic alterations driving oncogenesis and /or controlling the methylation machinery.

The two paraganglioma distant metastases included in the study displayed a similar pattern of promoter hypermethylation as their primary counterparts suggesting that promoter hypermethylation is a relatively early event occurring prior to metastasis development. This concept requires confirmation on larger sets of matched primary tumors and metastases.

Significantly younger age of presentation was detected in CIMP cases compared to those without CIMP ($p<0.007$, mean: 29 vs. 52 years), while overall no difference in age at presentation was shown between malignant and benign cases. Interestingly the single CIMP positive benign case presented with paraganglioma at a young age of 14 years. Younger age of presentation is a typical feature of familial cancers. In addition, several reports described an increased cancer prevalence amongst first-degree relatives of patients with CIMP colon lesions (217, 218). Although based on a limited number of observations, it can be speculated that a CIMP phenotype is characteristic of patients with malignant abdominal paraganglioma occurring at young age. This could in turn follow from a constitutional mutation in a predisposing gene. Therefore detailed assessment of cancer incidence in the pedigrees of CIMP paragangliomas should be addressed, together with sequencing of known predisposing genes (Table 1).

Finally given that by definition CIMP is characterized by hypermethylation of multiple tumor suppressor gene promoters, epigenetically acting drugs such as DNA methyl transferase inhibitors, may be highly pertinent candidates for assessment as adjuvant therapy in CIMP malignant paragangliomas.

Global methylation in pheochromocytomas and abdominal paragangliomas

Global hypomethlation is another form of epigenetic alteration commonly but not unanimously observed in human cancers (14, 219). Here we have explored global methylation alterations and their relevance in pheochromocytomas and abdominal paragangliomas. Bisulfite Pyrosequencing of LINE-1 elements, used as a surrogate marker for global methylation, revealed genome-wide

hypomethylation in tumors compared to normal adrenal samples ($p<0.02$ 0.632 vs. 0.674). This observation is in agreement with findings in other neuroendocrine tumors (220). However, when applying a different methodology, which essentially assesses methylation density at CCGG restriction sites across the genome (LUMA), no such difference could be demonstrated. This further underlines that interrogation of different sequences as surrogate markers for global methylation explore distinct components of genome-wide methylation, which may result in slightly divergent findings. However, there was an overall correlation between global methylation values obtained by LUMA (expressed as *Hpa*II / *Msp*I ratios) and LINE-1. No differences were revealed in genome wide methylation in relation to clinio-pathological tumor features or presence or absence of CIMP, suggesting that alterations in global methylation levels are more of a general feature of chromaffin cell tumor development.

INVOLVMENT OF THE RIZ1 TUMOR SUPPRESSOR IN PHEOCHROMOCYTOMAS AND ABDOMINAL PARAGANGLIOMAS (PAPER II)

Somatic losses involving the short arm of chromosome 1 are one of the common gross genetic alterations observed in pheochromocytomas and abdominal paragangliomas (76, 79-81, 86). Although no target gene has been defined, it is believed that inactivation of one or more tumor suppressor genes in 1p significantly contributes to the tumorigenic process in these neoplasias. Further exploration of the regions in 1p using LOH identified 1p36.2-pter as one of the minimally deleted regions (82). The RIZ1 tumor suppressor is a product of the RIZ gene in 1p36.21 and represents an appealing candidate target for the distal 1p deletions in these tumors.

In paper II, a set of 11 abdominal paragangliomas (4 benign, 7 malignant) and 18 pheochromocytomas (14 benign, 4 malignant) was assessed for somatic deletions of the RIZ gene using LOH analysis. mRNA expression levels of RIZ1 were quantified by Taqman qRT-PCR. Furthermore, RIZ1 promoter methylation status was evaluated utilizing MSP.

Frequent LOH of the RIZ locus were observed in the tumors. More specifically: 10 of the 16 informative cases (62%) displayed intragenic LOH including 8 out of 12 pheochromocytomas (67%) and 2 out of 4 paragangliomas (50%). RIZ1 showed frequent suppression at the mRNA level as compared to that observed in a pool of normal adrenals (mean 0.6 vs. 1.0 $p<0.001$). This was true both for tumors with and without LOH in the RIZ locus. On the other hand, no

significant decrease has been observed when quantifying total RIZ (RIZ1 and RIZ2) mRNA expression using primers and probes specific for the CR domain common to the two transcipts. These findings may indirectly imply that a decrease in RIZ1 is paralelled by an increase in RIZ2 levels –a phenomenon already observed in leukaemic cells (221).

Promoter methylation of RIZ1 is a common mechanism leading to transcriptional suppression (183, 184) therefore we tested whether it also was the case in pheochromocytomas and abdominal paragangliomas. However, no promoter methylation was detected indicating that promoter hypermethylation is unlikely to be the underlying cause of the frequent expressional silencing. RIZ structural mutations are not common in pheochromocytomas (186). Therefore other mechanisms such as histone modification or regulatory mechanisms at the transcriptional or posttranscriptional levels may be responsible for the RIZ1 underexpression observed.

Given that the RIZ gene maps to a minimal region of deletion observed in pheochromocytomas and abdominal paragangliomas and considering the frequent transcriptional suppression of RIZ1, we propose that RIZ1 is one target tumor suppressor gene in 1p36.2 involved in the development in these neoplasias. Further substantiation of this concept is needed by functional studies in pheochromocytoma cells.

EVALUATION OF NORE1A AND RASSF1A IN PHEOCHROMOCYTOMAS AND ABDOMINAL PARAGANGLIOMAS (PAPER III)

Pheochromocytomas frequently show RASSF1A inactivation by promoter methylation or deletion (150, 157). NORE1A, a functional collaborator and structural homologue to RASSF1A , is frequently suppressed in various neoplasias (158, 159, 161, 165, 167). However its role has not been invetigated in the context of pheochromocytomas and abdominal paragangliomas. This study aimed to explore the potential involvement of RASSF1A and NORE1A in these tumors.

Frequent transcriptional suppression of NORE1A and RASSF1A in pheochromocytomas and abdominal paragangliomas

Suppressed NORE1A and RASSF1A mRNA levels were observed in tumors compared to normal adreno-medullary samples ($p<0.001$). No significant correlations were detected between mRNA levels and the various clinico-pathological characteristics. Suppression of the tumor suppressive

Ras effectors NORE1A and RASSF1A in pheochromocytomas and abdominal paragangliomas suggests their involvement in the neoplastic process. Lack of correlation between NORE1A/RASSF1A mRNA expression levels and the various clinico-pathological features connote a more general role for these molecules in tumorigenesis that is not confined to a particular subgroup of patients.

Recurrent promoter hypermethylation of RASSF1A but not NORE1A

RASSF1A promoter was frequently hypermethylated in tumors and higher methylation levels were observed in malignant samples compared to benign ones ($p<0.05$). Both matched pairs of metastasis and primary tumor showed high RASSF1A methylation density. These findings are in accordance with recent reports showing common occurrence of RASSF1A methylation in these tumors (150, 157). On the other hand NORE1A promoter methylation is an uncommon event in these tumors. Only one sample showed weak partial methylation detected by COBRA.

Tumor suppressor function of Nore1a in pheochromocytoma cells

Ectopic expression of NORE1A in cell lines with lost endogenous expression is associated with reduced proliferation, enhanced apoptosis, and impaired anchorage independent growth (158, 159, 161, 165, 167). To inquire whether suppression of NORE1A expression affects the propensity of pheochromocytoma cells to undergo apoptosis we have assessed apoptotic rate in Nore1a transfected PC12 cells using FLICA. Increased apoptotic rate was detected amongst transfected cells, indicating that loss of NORE1A expression in pheochromocytomas may impair the cells ability to undergo apoptosis, an observation that was also suggested by Vos *et al*. (161).

Furthermore we examined the effect of ectopic Nore1a expression in PC12 cells, on the ability to proliferate in soft agar. Substantial reduction in the number of colonies was detected after Nore1a transfection, implying that Nore1a under-expression may be a significant contributing factor to the neoplastic growth of pheochromocytoma cells. Similar observation was reported by Vos *et al*. 2003 in A549 lung cancer cells (161).

ASSESSMENT OF NORE1A IN NEUROBLASTOMAS (PAPER IV)

Altered NORE1A mRNA expression in neuroblastoma cell lines and tumors.

Previous observations by others indicated the involvement of RASSF1A in neuroblastomas (23, 150, 152-156). Therefore, we proceeded to assess the role of NORE1A the "sister" molecule to

RASSF1A. In agreement with data obtained in other cancer cell lines, miniscule or absent NORE1A mRNA levels were observed in the 7 neuroblastoma cells lines studied (SK-N-DZ, SK-N-SH, SK-N-BE(2), SK-N-FI, SK-N-AS, IMR-32, and SH-SY-5Y). Primary tumors showed considerable variation in this respect, ranging from absent expression to expression levels comparable to that found in normal adult adrenal tissue. Tumors with different prognostic characteristics were compared with regard to NORE1A mRNA expression. Neuroblastomas displayed significantly lower expression compared to ganglioneuromas ($p<0.02$ Mann-Whitney U Test). Amongst neuroblastomas, reduced NORE1A mRNA expression was observed in tumors with normal *MYCN* copy numbers compared to samples with MYCN amplification ($p<0.005$), in tumors without 1p loss compared to those with 1p loss ($p<0.005$), and in tumors from non-high risk patients compared to those from high-risk-patients ($p<0.005$). Furthermore tumors classified according to INSS as stage 1, 2, 3 or 4S expressed lower levels of NORE1A compared to stage 4 neuroblastomas ($p<0.02$). Whether MYCN or other genes in 1p have a direct or indirect regulatory link to NORE1A remains to be further defined.

NORE1A promoter methylation is rare while RASSF1A methylation is common in neuroblastomas

In light of the frequent methylation of the NORE1A promoter in other tumor types (158, 161, 165, 167) we assessed whether it is a common mechanism of NORE1A suppression in neuroblastomas. No NORE1A promoter methylation was detected in neuroblastoma cells with the exception of SK-N-BE(2) which showed weak partial methylation. Neither was NORE1A methylation seen in primary tumors by COBRA. These results were in agreement with data obtained by bisulfite Pyrosequencing whereby only background levels of methylation were detected at the 13 CpG sites analysed.

By contrast, RASSF1A promoter methylation was commonly found both *in vitro* and *in vivo*. All seven neuroblastoma cell lines showed high RASSF1A promoter methylation density (>90%). Neuroblastoma tumors exhibited higher RASSF1A methylation density than ganglioneuromas ($p<0.03$). Amongst the 27 neuroblastomas high methylation density was observed in tumors with normal MYCN copy numbers compared to those with MYCN amplification ($p<0.04$), as well as in non high-risk neuroblastomas compared to high-risk tumors ($p<0.04$). Furthermore, higher RASSF1A methylation was seen in neuroblastomas without 1p loss compared to those with 1p loss ($p<0.04$), and in lower stage tumors compared to stage 4 neuroblastomas ($p<0.03$). Within the MYCN amplified group of tumors older patients showed a tendency towards higher levels of RASSF1A promoter methylation ($p<0.05$ $R=0.81$ Spearman Rank Order Correlations). These

observation endorses previous data by others indicating common occurrence of RASSF1A hypermethylation in neuroblastomas.

5-AzaC or TSA treatment cannot reverse suppression of NORE1*A* mRNA expression in neuroblastoma cells

Besides promoter methylation other means of epigenetic silencing such as histone deacetylation may be considered for NORE1A transcriptional silencing. To this end we determined whether treatment with the histone deacetylase inhibitor TSA could restore NORE1A mRNA expression in neuroblastoma cells. However, Trichostatin (TSA) resulted in no significant increase in NORE1A mRNA expression in neuroblastoma cells. Although we did not detect methylation in the NORE1A promoter, inhibition of DNA methylation by 5-AzaCytidine (5-AzaC) may theoretically lead to enhanced NORE1A transcription by secondary mechanisms. This was experimentally addressed by assessing NORE1A mRNA expression after treatment of neuroblastoma cell lines with 5-AzaC, which resulted in no significant induction of NORE1A mRNA expression.

Increased apoptotic rate and delayed cell cycle progression in Nore1a transfected neuroblastoma cells

Usig FLICA, a 2-fold increase in the apoptotic population was detected in GFP~Nore1a transfected SK-N-BE(2) cells compared to cells merely transfected with GFP (47% vs. 23% apoptotic cells). Furthermore, GFP~Nore1a transfected cells showed a 2.5 fold reduction in the proportion of actively cycling cells compared to cells transfected with GFP alone (24% vs. 9%). These observations are in harmony with data obtained by others in various cancer cells (161) and suggest that impairment of NORE1A expression enhance neoplastic growth of neuroblastoma cells via reduction of apoptosis and disruption of cell cycle control.

ALTERATIONS OF THE RAS PATHWAY IN FOLLICULAR THYROID TUMORS (PAPER V)

Activation of the RAS oncogenic cascade constitutes and important alternative pathway in thyroid tumorigenesis. In this study in addition to mutation screening for the H-, K-, and N-RAS genes we have assessed the involvement of the negative RAS effectors: NORE1A and RASSF1A in follicular thyroid tumors. The tumors have been characterized concerning the presence or absence of a rearrangement of the PPARγ gene locus. These rearrangements commonly results from a

translocation between chromosomes 2 and 3 leading to the creation of a PAX8- PPARγ fusion gene with tumor promoting properties (222).

Frequent and distinct occurrence of RAS mutations and PPARγ rearrangements

Direct DNA sequencing of exons 1 and 2 of H-, K-, and N-RAS lead to mutation identification in 5 of 32 follicular tumors analysed. All mutations have been previously characterized for their effect on RAS activity, and in each case only one allele was affected i.e. mutations were heterozygous. There was no overlap between tumors with RAS mutation and samples harbouring a PPARγ rearrangement and this observation is in accordance with data from other groups (223).

Suppressed NORE1A mRNA levels in thyroid tumors with PPARγ rearrangements

Taqman qRT-PCR was used to measure NORE1A and RASSF1A mRNA expression in a set of 25 follicular thyroid carcinomas (FTC), seven matched normal thyroid specimens and 8 follicular thyroid adenomas (FTA). Significantly reduced NORE1A mRNA levels were observed in FTC-s harbouring PPARγ rearrangements (five PAX8-PPARγ + and one CREB3L2- PPARγ + case) compared to normal thyroid samples ($p<0.001$ unpaired t test). On the other hand no reduction was detected in FTC-s without PPARγ translocation. Interestingly the only adenoma with PPARγ rearrangement also displayed NORE1A suppression. Reduced mRNA levels were well reflected by lower NORE1A protein levels as demonstrated on selected samples by Western blot analysis. To clarify whether methylation in the NORE1A promoter could account for the mRNA under-expression demonstrated in here, COBRA analysis was performed, which revealed no methylation.

No dirrect effect of PAX8-PPARγ on NORE1A promoter activity

Luciferase transactivation assay in HeLa cells did not demonstrate any change in NORE1A promoter activity upon transfection with PAX8-PPARγ. Thus no direct mechanistic link could be established between PAX8-PPARγ and NORE1A. It is plausible that NORE1A suppression by PAX8-PPARγ is realized indirectly through yet unidentified players. Alternatively tissue specific co-factors may be required for transactivation.

Reduced RASSF1A mRNA expression in tumors compared to normal tissue

Significantly reduced RASSF1A mRNA levels were revealed in FTC-s and FTA-s compared to normal thyroid tissue. On the other hand no expressional difference was seen with regard PPARγ rearrangement status or RAS mutations. This supports a more general role for RASSF1A in the tumorigenic process, not restricted to different subgroups by differential molecular pathway

activation. RASSF1A suppression in both adenomas and cancers suggests that this process is a comparatively early during neoplastic transformation.

INVOLVMENT OF THE CDKN2A LOCUS IN PHEOCHROMOCYTOMAS AND ABDOMINAL PARAGANGLIOMAS (PAPER VI)

Experimental studies in knock out mouse models have shown that inactivation of products of the CDKN2A gene locus, $p16^{INK4A}$ and $p14^{ARF}$ facilitates the development of pheochromocytoma, especially the bilateral and malignant forms of the disease. The objective of this study was to assess the role of CDKN2A in human pheochromocytomas and abdominal paragangliomas with regard to promoter methylation, expression and sequence alterations. $p16^{INK4A}$ and $p14^{ARF}$ were studied in a panel of 57 tumor samples from 55 patients.

Hypermethylation of $p16^{INK4A}$ but not the $p14^{ARF}$ promoter-association with malignant behaviour

Seven tumor samples from five patients (9%) showed extensive $p16^{INK4A}$ methylation by bisulfite Pyrosequencing. MSP analyses confirmed the data whereby $p16^{INK4A}$ methylation was only detected in the 7 tumors indicated. Similarly high levels of methylation were observed in the two pairs of matched primary tumors and metastases. Promoter methylation was significantly associated with malignant disease ($p = 0.0043$) with six of the 7 cases exhibiting $p16^{INK4A}$ methylation being malignant tumors and matching metastases. This may suggest a role of $p16^{INK4A}$ methylation in the malignification process. Confirmation on larger tumor sets and functional studies are awaited to ascertain this concept. Alternatively it is plausible that a more general dysregulation of the methylation machinery is induced by yet unidentified somatic structural gene alterations in a subset of malignant cases. Importantly, all methylated cases were classified as paragangliomas, which further underlines distinctive molecular features between pheochromocytomas and paragangliomas.

In contrast to $p16^{INK4A}$ only very low levels of $p14^{ARF}$ methylation were seen in the tumors, which was within the range of that measured in normal controls.

Suppression of $p16^{INK4A}$ but not the $p14^{ARF}$ expression-association with malignant behaviour

Concerning $p16^{INK4A}$ and $p14^{ARF}$ mRNA transcript abundance, no significant difference has been detected between tumors and normal adrenals as measured by Taqman qRT-PCR. However, when comparisons were made amongst tumors with different clinical features significantly lower $p16^{INK4A}$ mRNA levels were revealed in malignant samples compared to benign ones ($p<0.05$). Generally, transcriptional suppression of a negative cell cycle regulator such as $p16^{INK4A}$ is considered as an event favoring neoplastic growth. On the other hand, no such relative under-expression was observed for $p14^{ARF}$. To evaluate whether altered mRNA expression also translated to the protein level p16 Western analysis was performed. Overall 16 tumors exhibited lost or reduced p16 protein expression as compared to that measured in normal adrenals (<50%), which in all cases was reflected by decreased $p16^{INK4A}$ mRNA levels. In 33 tumors, the level was scored unaltered (50-150%), while in 7 tumors the expression was increased (>150%).

$p16^{INK4A}$ sequence variants and mutations

Direct sequencing of the coding region and flanking sequences of $p16^{INK4A}$ revealed sequence alterations in four benign tumors (11%). In one case a missense mutation A57V, was observed resulting from a substitution GCC > GTC in exon 2. This alteration has been previously reported as a germ line mutation as well as in a few cases of haematological and other malignancies (224). Furthermore, three tumors harboured a known SNP, A148T, which has been reported as a predisposing alteration in to malignant melanoma, breast and lung cancers (225, 226). No sequence variations were detected in exons 1 and 3, nor their flanking splice junctions.

CONCLUSIONS

- An epigenetic phenotype, CIMP, was identified in abdominal paragangliomas showing strong association with malignant behaviour and young age at presentation. The findings raise a prospective for potential therapeutic benefits of epigenetically acting drugs for a subgroup of young abdominal paraganglioma patients with adverse prognosis (paper I).
- Global hypomethylation observed in pheochromocytomas / abdominal paragangliomas bares no correlation to clinical features and appears as a general neoplastic phenomenon (paper I).
- Recurrent inactivation of the tumor suppressor *RIZ1* suggests that this event may be an important contributing factor to the neoplastic process in pheochromocytomas / abdominal paragangliomas (paper II)
- Suppression of the antitumorigenic Ras effectors NORE1A and RASSF1A occurs frequently in pheochromocytoma / paraganglioma. RASSF1A but not NORE1A promoter methylation is a common event in these tumors, the previous also showing good correlation with malignant behaviour. Functional evidence suggests a role for Nore1a suppression in these tumors during the neoplastic process (paper III).
- NORE1A mRNA levels were associated with prognostic indicators such as MYCN amplification, 1p loss, and INSS staging. Contrasting the frequent occurrence of RASSF1A methylation, hypermethylation of the NORE1A promoter is an uncommon event these tumors. Therefore other mechanisms are likely to account for the frequent suppression seen at the mRNA level. In addition, functional evidence suggests an antitumorigenic role of NORE1A in human neuroblastoma (paper IV).
- PAX8-PPARγ translocations and Ras mutations are non-overlapping events in follicular thyroid neoplasia. Restriction of NORE1A suppression to PPARγ rearranged tumors suggests a link between Ras and PPRγ as major oncogenic pathways in follicular thyroid tumors (paper V).
- $p16^{INK4A}$, and not $p14^{ARF}$ is a frequent target of inactivating events in human pheochromocytoma and abdominal paraganglioma. Notably, hypermethylation of the $p16^{INK4A}$ promoter is significantly associated with malignancy and metastasis in these tumors (paper VI).

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REFERENCES

1. Green, D. R. and Evan, G. I. A matter of life and death. Cancer Cell, *1:* 19-30, 2002.
2. Hanahan, D. and Weinberg, R. A. The hallmarks of cancer. Cell, *100:* 57-70, 2000.
3. Bird, A. DNA methylation patterns and epigenetic memory. Genes Dev, *16:* 6-21, 2002.
4. Feinberg, A. P. and Tycko, B. The history of cancer epigenetics. Nat Rev Cancer, *4:* 143-153, 2004.
5. Bartel, D. P. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell, *116:* 281-297, 2004.
6. Vogelstein, B., Fearon, E. R., Hamilton, S. R., Kern, S. E., Preisinger, A. C., Leppert, M., Nakamura, Y., White, R., Smits, A. M., and Bos, J. L. Genetic alterations during colorectal-tumor development. N Engl J Med, *319:* 525-532, 1988.
7. Baylin, S. B. and Ohm, J. E. Epigenetic gene silencing in cancer - a mechanism for early oncogenic pathway addiction? Nat Rev Cancer, *6:* 107-116, 2006.
8. Hoeijmakers, J. H. Genome maintenance mechanisms for preventing cancer. Nature, *411:* 366-374, 2001.
9. Land, H., Parada, L. F., and Weinberg, R. A. Cellular oncogenes and multistep carcinogenesis. Science, *222:* 771-778, 1983.
10. Teh, B. T., Larsson, C., and Nordenskjold, M. Tumor suppressor genes (TSG). Anticancer Res, *19:* 4715-4728, 1999.
11. Kinzler, K. W. and Vogelstein, B. Cancer-susceptibility genes. Gatekeepers and caretakers. Nature, *386:* 761, 763, 1997.
12. Knudson, A. G., Jr. Mutation and cancer: statistical study of retinoblastoma. Proc Natl Acad Sci U S A, *68:* 820-823, 1971.
13. Jones, P. A. and Takai, D. The role of DNA methylation in mammalian epigenetics. Science, *293:* 1068-1070, 2001.
14. Feinberg, A. P. and Vogelstein, B. Hypomethylation distinguishes genes of some human cancers from their normal counterparts. Nature, *301:* 89-92, 1983.
15. Goelz, S. E., Vogelstein, B., Hamilton, S. R., and Feinberg, A. P. Hypomethylation of DNA from benign and malignant human colon neoplasms. Science, *228:* 187-190, 1985.
16. Jones, P. A. and Baylin, S. B. The fundamental role of epigenetic events in cancer. Nat Rev Genet, *3:* 415-428, 2002.
17. Yoder, J. A., Walsh, C. P., and Bestor, T. H. Cytosine methylation and the ecology of intragenomic parasites. Trends Genet, *13:* 335-340, 1997.
18. Toyota, M., Ahuja, N., Ohe-Toyota, M., Herman, J. G., Baylin, S. B., and Issa, J. P. CpG island methylator phenotype in colorectal cancer. Proc Natl Acad Sci U S A, *96:* 8681-8686, 1999.
19. Brock, M. V., Gou, M., Akiyama, Y., Muller, A., Wu, T. T., Montgomery, E., Deasel, M., Germonpre, P., Rubinson, L., Heitmiller, R. F., Yang, S. C., Forastiere, A. A., Baylin, S. B., and Herman, J. G. Prognostic importance of promoter hypermethylation of multiple genes in esophageal adenocarcinoma. Clin Cancer Res, *9:* 2912-2919, 2003.
20. Shen, L., Ahuja, N., Shen, Y., Habib, N. A., Toyota, M., Rashid, A., and Issa, J. P. DNA methylation and environmental exposures in human hepatocellular carcinoma. J Natl Cancer Inst, *94:* 755-761, 2002.

21. Toyota, M., Ahuja, N., Suzuki, H., Itoh, F., Ohe-Toyota, M., Imai, K., Baylin, S. B., and Issa, J. P. Aberrant methylation in gastric cancer associated with the CpG island methylator phenotype. Cancer Res, *59:* 5438-5442, 1999.
22. Ueki, T., Toyota, M., Sohn, T., Yeo, C. J., Issa, J. P., Hruban, R. H., and Goggins, M. Hypermethylation of multiple genes in pancreatic adenocarcinoma. Cancer Res, *60:* 1835-1839, 2000.
23. Abe, M., Ohira, M., Kaneda, A., Yagi, Y., Yamamoto, S., Kitano, Y., Takato, T., Nakagawara, A., and Ushijima, T. CpG island methylator phenotype is a strong determinant of poor prognosis in neuroblastomas. Cancer Res, *65:* 828-834, 2005.
24. Li, Q., Jedlicka, A., Ahuja, N., Gibbons, M. C., Baylin, S. B., Burger, P. C., and Issa, J. P. Concordant methylation of the ER and N33 genes in glioblastoma multiforme. Oncogene, *16:* 3197-3202, 1998.
25. Garcia-Manero, G., Daniel, J., Smith, T. L., Kornblau, S. M., Lee, M. S., Kantarjian, H. M., and Issa, J. P. DNA methylation of multiple promoter-associated CpG islands in adult acute lymphocytic leukemia. Clin Cancer Res, *8:* 2217-2224, 2002.
26. Roman-Gomez, J., Jimenez-Velasco, A., Agirre, X., Castillejo, J. A., Navarro, G., Calasanz, M. J., Garate, L., San Jose-Eneriz, E., Cordeu, L., Prosper, F., Heiniger, A., and Torres, A. CpG island methylator phenotype redefines the prognostic effect of t(12;21) in childhood acute lymphoblastic leukemia. Clin Cancer Res, *12:* 4845-4850, 2006.
27. Abe, M., Westermann, F., Nakagawara, A., Takato, T., Schwab, M., and Ushijima, T. Marked and independent prognostic significance of the CpG island methylator phenotype in neuroblastomas. Cancer Lett, *247:* 253-258, 2007.
28. Ward, R. L., Cheong, K., Ku, S. L., Meagher, A., O'Connor, T., and Hawkins, N. J. Adverse prognostic effect of methylation in colorectal cancer is reversed by microsatellite instability. J Clin Oncol, *21:* 3729-3736, 2003.
29. Shaw, R. J., Hall, G. L., Lowe, D., Bowers, N. L., Liloglou, T., Field, J. K., Woolgar, J. A., and Risk, J. M. CpG island methylation phenotype (CIMP) in oral cancer: Associated with a marked inflammatory response and less aggressive tumour biology. Oral Oncol, 2007.
30. Issa, J. P. CpG island methylator phenotype in cancer. Nat Rev Cancer, *4:* 988-993, 2004.
31. Hoehner, J. C., Hedborg, F., Eriksson, L., Sandstedt, B., Grimelius, L., Olsen, L., and Pahlman, S. Developmental gene expression of sympathetic nervous system tumors reflects their histogenesis. Lab Invest, *78:* 29-45, 1998.
32. Elder, E. E., Elder, G., and Larsson, C. Pheochromocytoma and functional paraganglioma syndrome: no longer the 10% tumor. J Surg Oncol, *89:* 193-201, 2005.
33. Stenstrom, G. and Svardsudd, K. Pheochromocytoma in Sweden 1958-1981. An analysis of the National Cancer Registry Data. Acta Med Scand, *220:* 225-232, 1986.
34. Sutton, M. G., Sheps, S. G., and Lie, J. T. Prevalence of clinically unsuspected pheochromocytoma. Review of a 50-year autopsy series. Mayo Clin Proc, *56:* 354-360, 1981.
35. Edstrom Elder, E., Hjelm Skog, A. L., Hoog, A., and Hamberger, B. The management of benign and malignant pheochromocytoma and abdominal paraganglioma. Eur J Surg Oncol, *29:* 278-283, 2003.
36. McNicol, A. M. Histopathology and immunohistochemistry of adrenal medullary tumors and paragangliomas. Endocr Pathol, *17:* 329-336, 2006.
37. Lack, E. Tumors of the adrenal gland and extra-adrenal paraganglia. *In:* Armed Forces Institute of Pathology. Washington DC, 1997.

38. Eisenhofer, G., Bornstein, S. R., Brouwers, F. M., Cheung, N. K., Dahia, P. L., de Krijger, R. R., Giordano, T. J., Greene, L. A., Goldstein, D. S., Lehnert, H., Manger, W. M., Maris, J. M., Neumann, H. P., Pacak, K., Shulkin, B. L., Smith, D. I., Tischler, A. S., and Young, W. F., Jr. Malignant pheochromocytoma: current status and initiatives for future progress. Endocr Relat Cancer, *11:* 423-436, 2004.
39. de Krijger, R. R., van Nederveen, F. H., Korpershoek, E., and Dinjens, W. N. New developments in the detection of the clinical behavior of pheochromocytomas and paragangliomas. Endocr Pathol, *17:* 137-141, 2006.
40. Boltze, C., Lehnert, H., Schneider-Stock, R., Peters, B., Hoang-Vu, C., and Roessner, A. HSP90 is a key for telomerase activation and malignant transition in pheochromocytoma. Endocrine, *22:* 193-201, 2003.
41. Boltze, C., Mundschenk, J., Unger, N., Schneider-Stock, R., Peters, B., Mawrin, C., Hoang-Vu, C., Roessner, A., and Lehnert, H. Expression profile of the telomeric complex discriminates between benign and malignant pheochromocytoma. J Clin Endocrinol Metab, *88:* 4280-4286, 2003.
42. Elder, E. E., Xu, D., Hoog, A., Enberg, U., Hou, M., Pisa, P., Gruber, A., Larsson, C., and Backdahl, M. KI-67 AND hTERT expression can aid in the distinction between malignant and benign pheochromocytoma and paraganglioma. Mod Pathol, *16:* 246-255, 2003.
43. Favier, J., Plouin, P. F., Corvol, P., and Gasc, J. M. Angiogenesis and vascular architecture in pheochromocytomas: distinctive traits in malignant tumors. Am J Pathol, *161:* 1235-1246, 2002.
44. Neumann, H. P., Bausch, B., McWhinney, S. R., Bender, B. U., Gimm, O., Franke, G., Schipper, J., Klisch, J., Altehoefer, C., Zerres, K., Januszewicz, A., Eng, C., Smith, W. M., Munk, R., Manz, T., Glaesker, S., Apel, T. W., Treier, M., Reineke, M., Walz, M. K., Hoang-Vu, C., Brauckhoff, M., Klein-Franke, A., Klose, P., Schmidt, H., Maier-Woelfle, M., Peczkowska, M., and Szmigielski, C. Germ-line mutations in nonsyndromic pheochromocytoma. N Engl J Med, *346:* 1459-1466, 2002.
45. Zbuk, K. M. and Eng, C. Cancer phenomics: RET and PTEN as illustrative models. Nat Rev Cancer, *7:* 35-45, 2007.
46. Roe, J. S. and Youn, H. D. The positive regulation of p53 by the tumor suppressor VHL. Cell Cycle, *5:* 2054-2056, 2006.
47. Ballester, R., Marchuk, D., Boguski, M., Saulino, A., Letcher, R., Wigler, M., and Collins, F. The NF1 locus encodes a protein functionally related to mammalian GAP and yeast IRA proteins. Cell, *63:* 851-859, 1990.
48. Baysal, B. E., Rubinstein, W. S., and Taschner, P. E. Phenotypic dichotomy in mitochondrial complex II genetic disorders. J Mol Med, *79:* 495-503, 2001.
49. Bryant, J., Farmer, J., Kessler, L. J., Townsend, R. R., and Nathanson, K. L. Pheochromocytoma: the expanding genetic differential diagnosis. J Natl Cancer Inst, *95:* 1196-1204, 2003.
50. Clifford, S. C., Prowse, A. H., Affara, N. A., Buys, C. H., and Maher, E. R. Inactivation of the von Hippel-Lindau (VHL) tumour suppressor gene and allelic losses at chromosome arm 3p in primary renal cell carcinoma: evidence for a VHL-independent pathway in clear cell renal tumourigenesis. Genes Chromosomes Cancer, *22:* 200-209, 1998.
51. Eng, C., Crossey, P. A., Mulligan, L. M., Healey, C. S., Houghton, C., Prowse, A., Chew, S. L., Dahia, P. L., O'Riordan, J. L., Toledo, S. P., and et al. Mutations in the RET proto-oncogene and the von Hippel-Lindau disease tumour suppressor gene in sporadic and syndromic phaeochromocytomas. J Med Genet, *32:* 934-937, 1995.

52. Eng, C., Smith, D. P., Mulligan, L. M., Nagai, M. A., Healey, C. S., Ponder, M. A., Gardner, E., Scheumann, G. F., Jackson, C. E., Tunnacliffe, A., and et al. Point mutation within the tyrosine kinase domain of the RET proto-oncogene in multiple endocrine neoplasia type 2B and related sporadic tumours. Hum Mol Genet, *3:* 237-241, 1994.
53. Foster, K., Crossey, P. A., Cairns, P., Hetherington, J. W., Richards, F. M., Jones, M. H., Bentley, E., Affara, N. A., Ferguson-Smith, M. A., and Maher, E. R. Molecular genetic investigation of sporadic renal cell carcinoma: analysis of allele loss on chromosomes 3p, 5q, 11p, 17 and 22. Br J Cancer, *69:* 230-234, 1994.
54. Gnarra, J. R., Tory, K., Weng, Y., Schmidt, L., Wei, M. H., Li, H., Latif, F., Liu, S., Chen, F., Duh, F. M., and et al. Mutations of the VHL tumour suppressor gene in renal carcinoma. Nat Genet, *7:* 85-90, 1994.
55. Herman, J. G., Latif, F., Weng, Y., Lerman, M. I., Zbar, B., Liu, S., Samid, D., Duan, D. S., Gnarra, J. R., Linehan, W. M., and et al. Silencing of the VHL tumor-suppressor gene by DNA methylation in renal carcinoma. Proc Natl Acad Sci U S A, *91:* 9700-9704, 1994.
56. Hofstra, R. M., Stelwagen, T., Stulp, R. P., de Jong, D., Hulsbeek, M., Kamsteeg, E. J., van den Berg, A., Landsvater, R. M., Vermey, A., Molenaar, W. M., Lips, C. J., and Buys, C. H. Extensive mutation scanning of RET in sporadic medullary thyroid carcinoma and of RET and VHL in sporadic pheochromocytoma reveals involvement of these genes in only a minority of cases. J Clin Endocrinol Metab, *81:* 2881-2884, 1996.
57. Aguiar, R. C., Cox, G., Pomeroy, S. L., and Dahia, P. L. Analysis of the SDHD gene, the susceptibility gene for familial paraganglioma syndrome (PGL1), in pheochromocytomas. J Clin Endocrinol Metab, *86:* 2890-2894, 2001.
58. Astuti, D., Douglas, F., Lennard, T. W., Aligianis, I. A., Woodward, E. R., Evans, D. G., Eng, C., Latif, F., and Maher, E. R. Germline SDHD mutation in familial phaeochromocytoma. Lancet, *357:* 1181-1182, 2001.
59. Astuti, D., Latif, F., Dallol, A., Dahia, P. L., Douglas, F., George, E., Skoldberg, F., Husebye, E. S., Eng, C., and Maher, E. R. Gene mutations in the succinate dehydrogenase subunit SDHB cause susceptibility to familial pheochromocytoma and to familial paraganglioma. Am J Hum Genet, *69:* 49-54, 2001.
60. Astuti, D., Morris, M., Krona, C., Abel, F., Gentle, D., Martinsson, T., Kogner, P., Neumann, H. P., Voutilainen, R., Eng, C., Rustin, P., Latif, F., and Maher, E. R. Investigation of the role of SDHB inactivation in sporadic phaeochromocytoma and neuroblastoma. Br J Cancer, *91:* 1835-1841, 2004.
61. Gimm, O., Armanios, M., Dziema, H., Neumann, H. P., and Eng, C. Somatic and occult germ-line mutations in SDHD, a mitochondrial complex II gene, in nonfamilial pheochromocytoma. Cancer Res, *60:* 6822-6825, 2000.
62. Kytola, S., Nord, B., Elder, E. E., Carling, T., Kjellman, M., Cedermark, B., Juhlin, C., Hoog, A., Isola, J., and Larsson, C. Alterations of the SDHD gene locus in midgut carcinoids, Merkel cell carcinomas, pheochromocytomas, and abdominal paragangliomas. Genes Chromosomes Cancer, *34:* 325-332, 2002.
63. Lin, S. R., Tsai, J. H., Yang, Y. C., and Lee, S. C. Mutations of K-ras oncogene in human adrenal tumours in Taiwan. Br J Cancer, *77:* 1060-1065, 1998.
64. Moul, J. W., Bishoff, J. T., Theune, S. M., and Chang, E. H. Absent ras gene mutations in human adrenal cortical neoplasms and pheochromocytomas. J Urol, *149:* 1389-1394, 1993.

65. Yoshimoto, K., Iwahana, H., and Itakura, M. Relatively good prognosis of multiple endocrine neoplasia type 2B in Japanese: review of cases in Japan and analysis of genetic changes in tumors. Endocr J, *40:* 649-657, 1993.
66. Eng, C., Foster, K. A., Healey, C. S., Houghton, C., Gayther, S. A., Mulligan, L. M., and Ponder, B. A. Mutation analysis of the c-mos proto-oncogene and the endothelin-B receptor gene in medullary thyroid carcinoma and phaeochromocytoma. Br J Cancer, *74:* 339-341, 1996.
67. Moley, J. F., Wallin, G. K., Brother, M. B., Kim, M., Wells, S. A., Jr., and Brodeur, G. M. Oncogene and growth factor expression in MEN 2 and related tumors. Henry Ford Hosp Med J, *40:* 284-288, 1992.
68. Aguiar, R. C., Dahia, P. L., Sill, H., Toledo, S. P., Goldman, J. M., and Cross, N. C. Deletion analysis of the p16 tumour suppressor gene in phaeochromocytomas. Clin Endocrinol (Oxf), *45:* 93-96, 1996.
69. Dahia, P. L., Aguiar, R. C., Tsanaclis, A. M., Bendit, I., Bydlowski, S. P., Abelin, N. M., and Toledo, S. P. Molecular and immunohistochemical analysis of P53 in phaeochromocytoma. Br J Cancer, *72:* 1211-1213, 1995.
70. Herfarth, K. K., Wick, M. R., Marshall, H. N., Gartner, E., Lum, S., and Moley, J. F. Absence of TP53 alterations in pheochromocytomas and medullary thyroid carcinomas. Genes Chromosomes Cancer, *20:* 24-29, 1997.
71. Lam, K. Y., Lo, C. Y., Wat, N. M., Luk, J. M., and Lam, K. S. The clinicopathological features and importance of p53, Rb, and mdm2 expression in phaeochromocytomas and paragangliomas. J Clin Pathol, *54:* 443-448, 2001.
72. Clarke, M. R., Weyant, R. J., Watson, C. G., and Carty, S. E. Prognostic markers in pheochromocytoma. Hum Pathol, *29:* 522-526, 1998.
73. Wang, D. G., Johnston, C. F., Marley, J. J., Phenix, K. V., Atkinson, A. B., Russell, C. F., and Buchanan, K. D. Expression of the apoptosis-suppressing gene BCL-2 in pheochromocytoma is associated with the expression of C-MYC. J Clin Endocrinol Metab, *82:* 1949-1952, 1997.
74. August, C., August, K., Schroeder, S., Bahn, H., Hinze, R., Baba, H. A., Kersting, C., and Buerger, H. CGH and CD 44/MIB-1 immunohistochemistry are helpful to distinguish metastasized from nonmetastasized sporadic pheochromocytomas. Mod Pathol, *17:* 1119-1128, 2004.
75. Cascon, A., Ruiz-Llorente, S., Rodriguez-Perales, S., Honrado, E., Martinez-Ramirez, A., Leton, R., Montero-Conde, C., Benitez, J., Dopazo, J., Cigudosa, J. C., and Robledo, M. A novel candidate region linked to development of both pheochromocytoma and head/neck paraganglioma. Genes Chromosomes Cancer, *42:* 260-268, 2005.
76. Edstrom, E., Mahlamaki, E., Nord, B., Kjellman, M., Karhu, R., Hoog, A., Goncharov, N., Teh, B. T., Backdahl, M., and Larsson, C. Comparative genomic hybridization reveals frequent losses of chromosomes 1p and 3q in pheochromocytomas and abdominal paragangliomas, suggesting a common genetic etiology. Am J Pathol, *156:* 651-659, 2000.
77. Lui, W. O., Chen, J., Glasker, S., Bender, B. U., Madura, C., Khoo, S. K., Kort, E., Larsson, C., Neumann, H. P., and Teh, B. T. Selective loss of chromosome 11 in pheochromocytomas associated with the VHL syndrome. Oncogene, *21:* 1117-1122, 2002.
78. Powers, J. F., Tischler, A. S., Mohammed, M., and Naeem, R. Microarray-based comparative genomic hybridization of pheochromocytoma cell lines from neurofibromatosis knockout mice reveals genetic alterations similar to those in human pheochromocytomas. Cancer Genet Cytogenet, *159:* 27-31, 2005.

79. Aarts, M., Dannenberg, H., deLeeuw, R. J., van Nederveen, F. H., Verhofstad, A. A., Lenders, J. W., Dinjens, W. N., Speel, E. J., Lam, W. L., and de Krijger, R. R. Microarray-based CGH of sporadic and syndrome-related pheochromocytomas using a 0.1-0.2 Mb bacterial artificial chromosome array spanning chromosome arm 1p. Genes Chromosomes Cancer, *45:* 83-93, 2006.
80. Jarbo, C., Buckley, P. G., Piotrowski, A., Mantripragada, K. K., Benetkiewicz, M., Diaz de Stahl, T., Langford, C. F., Gregory, S. G., Dralle, H., Gimm, O., Backdahl, M., Geli, J., Larsson, C., Westin, G., Akerstrom, G., and Dumanski, J. P. Detailed assessment of chromosome 22 aberrations in sporadic pheochromocytoma using array-CGH. Int J Cancer, *118:* 1159-1164, 2006.
81. Benn, D. E., Dwight, T., Richardson, A. L., Delbridge, L., Bambach, C. P., Stowasser, M., Gordon, R. D., Marsh, D. J., and Robinson, B. G. Sporadic and familial pheochromocytomas are associated with loss of at least two discrete intervals on chromosome 1p. Cancer Res, *60:* 7048-7051, 2000.
82. Edstrom Elder, E., Nord, B., Carling, T., Juhlin, C., Backdahl, M., Hoog, A., and Larsson, C. Loss of heterozygosity on the short arm of chromosome 1 in pheochromocytoma and abdominal paraganglioma. World J Surg, *26:* 965-971, 2002.
83. Khosla, S., Patel, V. M., Hay, I. D., Schaid, D. J., Grant, C. S., van Heerden, J. A., and Thibodeau, S. N. Loss of heterozygosity suggests multiple genetic alterations in pheochromocytomas and medullary thyroid carcinomas. J Clin Invest, *87:* 1691-1699, 1991.
84. Moley, J. F., Brother, M. B., Fong, C. T., White, P. S., Baylin, S. B., Nelkin, B., Wells, S. A., and Brodeur, G. M. Consistent association of 1p loss of heterozygosity with pheochromocytomas from patients with multiple endocrine neoplasia type 2 syndromes. Cancer Res, *52:* 770-774, 1992.
85. Mulligan, L. M., Gardner, E., Smith, B. A., Mathew, C. G., and Ponder, B. A. Genetic events in tumour initiation and progression in multiple endocrine neoplasia type 2. Genes Chromosomes Cancer, *6:* 166-177, 1993.
86. Shin, E., Fujita, S., Takami, K., Kurahashi, H., Kurita, Y., Kobayashi, T., Mori, T., Nishisho, I., and Takai, S. Deletion mapping of chromosome 1p and 22q in pheochromocytoma. Jpn J Cancer Res, *84:* 402-408, 1993.
87. Brouwers, F. M., Elkahloun, A. G., Munson, P. J., Eisenhofer, G., Barb, J., Linehan, W. M., Lenders, J. W., De Krijger, R., Mannelli, M., Udelsman, R., Ocal, I. T., Shulkin, B. L., Bornstein, S. R., Breza, J., Ksinantova, L., and Pacak, K. Gene expression profiling of benign and malignant pheochromocytoma. Ann N Y Acad Sci, *1073:* 541-556, 2006.
88. Brouwers, F. M., Petricoin, E. F., 3rd, Ksinantova, L., Breza, J., Rajapakse, V., Ross, S., Johann, D., Mannelli, M., Shulkin, B. L., Kvetnansky, R., Eisenhofer, G., Walther, M. M., Hitt, B. A., Conrads, T. P., Veenstra, T. D., Mannion, D. P., Wall, M. R., Wolfe, G. M., Fusaro, V. A., Liotta, L. A., and Pacak, K. Low molecular weight proteomic information distinguishes metastatic from benign pheochromocytoma. Endocr Relat Cancer, *12:* 263-272, 2005.
89. Eisenhofer, G., Huynh, T. T., Pacak, K., Brouwers, F. M., Walther, M. M., Linehan, W. M., Munson, P. J., Mannelli, M., Goldstein, D. S., and Elkahloun, A. G. Distinct gene expression profiles in norepinephrine- and epinephrine-producing hereditary and sporadic pheochromocytomas: activation of hypoxia-driven angiogenic pathways in von Hippel-Lindau syndrome. Endocr Relat Cancer, *11:* 897-911, 2004.
90. Elkahloun, A. G., Powers, J. F., Nyska, A., Eisenhofer, G., and Tischler, A. S. Gene expression profiling of rat pheochromocytoma. Ann N Y Acad Sci, *1073:* 290-299, 2006.

91. Huynh, T. T., Pacak, K., Wong, D. L., Linehan, W. M., Goldstein, D. S., Elkahloun, A. G., Munson, P. J., and Eisenhofer, G. Transcriptional regulation of phenylethanolamine N-methyltransferase in pheochromocytomas from patients with von Hippel-Lindau syndrome and multiple endocrine neoplasia type 2. Ann N Y Acad Sci, *1073:* 241-252, 2006.
92. Takahashi, M., Yang, X. J., McWhinney, S., Sano, N., Eng, C., Kagawa, S., Teh, B. T., and Kanayama, H. O. cDNA microarray analysis assists in diagnosis of malignant intrarenal pheochromocytoma originally masquerading as a renal cell carcinoma. J Med Genet, *42:* e48, 2005.
93. Brouwers, F. M., Glasker, S., Nave, A. F., Vortmeyer, A. O., Lubensky, I., Huang, S., Abu-Asab, M. S., Eisenhofer, G., Weil, R. J., Park, D. M., Linehan, W. M., Pacak, K., and Zhuang, Z. Proteomic profiling of von Hippel-Lindau syndrome and multiple endocrine neoplasia type 2 pheochromocytomas reveals different expression of chromogranin B. Endocr Relat Cancer, *14:* 463-471, 2007.
94. Grovas, A., Fremgen, A., Rauck, A., Ruymann, F. B., Hutchinson, C. L., Winchester, D. P., and Menck, H. R. The National Cancer Data Base report on patterns of childhood cancers in the United States. Cancer, *80:* 2321-2332, 1997.
95. Gustavsson G, L. F., Pihkala U, de Verdier B, Heyman M, Tysell L Childhood Cancer in Nordic countries. Nordic Society of pediatric haematology and oncology, Aalborg, 2000.
96. Brodeur G, M. a. M. J. M. in Principles and Practice of Pediatric Oncology 895-937, 2002.
97. Hoehner, J. C., Gestblom, C., Hedborg, F., Sandstedt, B., Olsen, L., and Pahlman, S. A developmental model of neuroblastoma: differentiating stroma-poor tumors' progress along an extra-adrenal chromaffin lineage. Lab Invest, *75:* 659-675, 1996.
98. Edsjo, A., Holmquist, L., and Pahlman, S. Neuroblastoma as an experimental model for neuronal differentiation and hypoxia-induced tumor cell dedifferentiation. Semin Cancer Biol, *17:* 248-256, 2007.
99. Brodeur, G. M., Pritchard, J., Berthold, F., Carlsen, N. L., Castel, V., Castelberry, R. P., De Bernardi, B., Evans, A. E., Favrot, M., Hedborg, F., and et al. Revisions of the international criteria for neuroblastoma diagnosis, staging, and response to treatment. J Clin Oncol, *11:* 1466-1477, 1993.
100. Breslow, N. and McCann, B. Statistical estimation of prognosis for children with neuroblastoma. Cancer Res, *31:* 2098-2103, 1971.
101. Shimada, H., Chatten, J., Newton, W. A., Jr., Sachs, N., Hamoudi, A. B., Chiba, T., Marsden, H. B., and Misugi, K. Histopathologic prognostic factors in neuroblastic tumors: definition of subtypes of ganglioneuroblastoma and an age-linked classification of neuroblastomas. J Natl Cancer Inst, *73:* 405-416, 1984.
102. Maris, J. M., Hogarty, M. D., Bagatell, R., and Cohn, S. L. Neuroblastoma. Lancet, *369:* 2106-2120, 2007.
103. Kushner, B. H., Gilbert, F., and Helson, L. Familial neuroblastoma. Case reports, literature review, and etiologic considerations. Cancer, *57:* 1887-1893, 1986.
104. Maris, J. M., Kyemba, S. M., Rebbeck, T. R., White, P. S., Sulman, E. P., Jensen, S. J., Allen, C., Biegel, J. A., and Brodeur, G. M. Molecular genetic analysis of familial neuroblastoma. Eur J Cancer, *33:* 1923-1928, 1997.
105. Shojaei-Brosseau, T., Chompret, A., Abel, A., de Vathaire, F., Raquin, M. A., Brugieres, L., Feunteun, J., Hartmann, O., and Bonaiti-Pellie, C. Genetic epidemiology of neuroblastoma: a study of 426 cases at the Institut Gustave-Roussy in France. Pediatr Blood Cancer, *42:* 99-105, 2004.

106. Mosse, Y. P., Laudenslager, M., Khazi, D., Carlisle, A. J., Winter, C. L., Rappaport, E., and Maris, J. M. Germline PHOX2B mutation in hereditary neuroblastoma. Am J Hum Genet, *75:* 727-730, 2004.
107. Trochet, D., Bourdeaut, F., Janoueix-Lerosey, I., Deville, A., de Pontual, L., Schleiermacher, G., Coze, C., Philip, N., Frebourg, T., Munnich, A., Lyonnet, S., Delattre, O., and Amiel, J. Germline mutations of the paired-like homeobox 2B (PHOX2B) gene in neuroblastoma. Am J Hum Genet, *74:* 761-764, 2004.
108. Brodeur, G. M. Neuroblastoma: biological insights into a clinical enigma. Nat Rev Cancer, *3:* 203-216, 2003.
109. Bauer, A., Savelyeva, L., Claas, A., Praml, C., Berthold, F., and Schwab, M. Smallest region of overlapping deletion in 1p36 in human neuroblastoma: a 1 Mbp cosmid and PAC contig. Genes Chromosomes Cancer, *31:* 228-239, 2001.
110. Maris, J. M., Guo, C., Blake, D., White, P. S., Hogarty, M. D., Thompson, P. M., Rajalingam, V., Gerbing, R., Stram, D. O., Matthay, K. K., Seeger, R. C., and Brodeur, G. M. Comprehensive analysis of chromosome 1p deletions in neuroblastoma. Med Pediatr Oncol, *36:* 32-36, 2001.
111. Martinsson, T., Sjoberg, R. M., Hedborg, F., and Kogner, P. Deletion of chromosome 1p loci and microsatellite instability in neuroblastomas analyzed with short-tandem repeat polymorphisms. Cancer Res, *55:* 5681-5686, 1995.
112. White, P. S., Thompson, P. M., Seifried, B. A., Sulman, E. P., Jensen, S. J., Guo, C., Maris, J. M., Hogarty, M. D., Allen, C., Biegel, J. A., Matise, T. C., Gregory, S. G., Reynolds, C. P., and Brodeur, G. M. Detailed molecular analysis of 1p36 in neuroblastoma. Med Pediatr Oncol, *36:* 37-41, 2001.
113. Caron, H. Allelic loss of chromosome 1 and additional chromosome 17 material are both unfavourable prognostic markers in neuroblastoma. Med Pediatr Oncol, *24:* 215-221, 1995.
114. Ejeskar, K., Sjoberg, R. M., Abel, F., Kogner, P., Ambros, P. F., and Martinsson, T. Fine mapping of a tumour suppressor candidate gene region in 1p36.2-3, commonly deleted in neuroblastomas and germ cell tumours. Med Pediatr Oncol, *36:* 61-66, 2001.
115. Hogarty, M. D., Liu, X., Guo, C., Thompson, P. M., Weiss, M. J., White, P. S., Sulman, E. P., Brodeur, G. M., and Maris, J. M. Identification of a 1-megabase consensus region of deletion at 1p36.3 in primary neuroblastomas. Med Pediatr Oncol, *35:* 512-515, 2000.
116. Maris, J. M., White, P. S., Beltinger, C. P., Sulman, E. P., Castleberry, R. P., Shuster, J. J., Look, A. T., and Brodeur, G. M. Significance of chromosome 1p loss of heterozygosity in neuroblastoma. Cancer Res, *55:* 4664-4669, 1995.
117. Martinsson, T., Sjoberg, R. M., Hallstensson, K., Nordling, M., Hedborg, F., and Kogner, P. Delimitation of a critical tumour suppressor region at distal 1p in neuroblastoma tumours. Eur J Cancer, *33:* 1997-2001, 1997.
118. Spieker, N., Beitsma, M., Van Sluis, P., Chan, A., Caron, H., and Versteeg, R. Three chromosomal rearrangements in neuroblastoma cluster within a 300-kb region on 1p36.1. Genes Chromosomes Cancer, *31:* 172-181, 2001.
119. White, P. S., Maris, J. M., Beltinger, C., Sulman, E., Marshall, H. N., Fujimori, M., Kaufman, B. A., Biegel, J. A., Allen, C., Hilliard, C., and et al. A region of consistent deletion in neuroblastoma maps within human chromosome 1p36.2-36.3. Proc Natl Acad Sci U S A, *92:* 5520-5524, 1995.
120. Mosse, Y. P., Greshock, J., Weber, B. L., and Maris, J. M. Measurement and relevance of neuroblastoma DNA copy number changes in the post-genome era. Cancer Lett, *228:* 83-90, 2005.

121. Vandesompele, J., Speleman, F., Van Roy, N., Laureys, G., Brinskchmidt, C., Christiansen, H., Lampert, F., Lastowska, M., Bown, N., Pearson, A., Nicholson, J. C., Ross, F., Combaret, V., Delattre, O., Feuerstein, B. G., and Plantaz, D. Multicentre analysis of patterns of DNA gains and losses in 204 neuroblastoma tumors: how many genetic subgroups are there? Med Pediatr Oncol, *36:* 5-10, 2001.
122. Wang, Q., Diskin, S., Rappaport, E., Attiyeh, E., Mosse, Y., Shue, D., Seiser, E., Jagannathan, J., Shusterman, S., Bansal, M., Khazi, D., Winter, C., Okawa, E., Grant, G., Cnaan, A., Zhao, H., Cheung, N. K., Gerald, W., London, W., Matthay, K. K., Brodeur, G. M., and Maris, J. M. Integrative genomics identifies distinct molecular classes of neuroblastoma and shows that multiple genes are targeted by regional alterations in DNA copy number. Cancer Res, *66:* 6050-6062, 2006.
123. Bilke, S., Chen, Q. R., Whiteford, C. C., and Khan, J. Detection of low level genomic alterations by comparative genomic hybridization based on cDNA micro-arrays. Bioinformatics, *21:* 1138-1145, 2005.
124. Brodeur, G. M., Seeger, R. C., Schwab, M., Varmus, H. E., and Bishop, J. M. Amplification of N-myc in untreated human neuroblastomas correlates with advanced disease stage. Science, *224:* 1121-1124, 1984.
125. Seeger, R. C., Brodeur, G. M., Sather, H., Dalton, A., Siegel, S. E., Wong, K. Y., and Hammond, D. Association of multiple copies of the N-myc oncogene with rapid progression of neuroblastomas. N Engl J Med, *313:* 1111-1116, 1985.
126. Norris, M. D., Bordow, S. B., Haber, P. S., Marshall, G. M., Kavallaris, M., Madafiglio, J., Cohn, S. L., Salwen, H., Schmidt, M. L., Hipfner, D. R., Cole, S. P., Deeley, R. G., and Haber, M. Evidence that the MYCN oncogene regulates MRP gene expression in neuroblastoma. Eur J Cancer, *33:* 1911-1916, 1997.
127. Shohet, J. M., Hicks, M. J., Plon, S. E., Burlingame, S. M., Stuart, S., Chen, S. Y., Brenner, M. K., and Nuchtern, J. G. Minichromosome maintenance protein MCM7 is a direct target of the MYCN transcription factor in neuroblastoma. Cancer Res, *62:* 1123-1128, 2002.
128. Fong, C. T., Dracopoli, N. C., White, P. S., Merrill, P. T., Griffith, R. C., Housman, D. E., and Brodeur, G. M. Loss of heterozygosity for the short arm of chromosome 1 in human neuroblastomas: correlation with N-myc amplification. Proc Natl Acad Sci U S A, *86:* 3753-3757, 1989.
129. Abel, F., Ejeskar, K., Kogner, P., and Martinsson, T. Gain of chromosome arm 17q is associated with unfavourable prognosis in neuroblastoma, but does not involve mutations in the somatostatin receptor 2(SSTR2) gene at 17q24. Br J Cancer, *81:* 1402-1409, 1999.
130. Bown, N., Cotterill, S., Lastowska, M., O'Neill, S., Pearson, A. D., Plantaz, D., Meddeb, M., Danglot, G., Brinkschmidt, C., Christiansen, H., Laureys, G., Speleman, F., Nicholson, J., Bernheim, A., Betts, D. R., Vandesompele, J., and Van Roy, N. Gain of chromosome arm 17q and adverse outcome in patients with neuroblastoma. N Engl J Med, *340:* 1954-1961, 1999.
131. Bown, N., Lastowska, M., Cotterill, S., O'Neill, S., Ellershaw, C., Roberts, P., Lewis, I., and Pearson, A. D. 17q gain in neuroblastoma predicts adverse clinical outcome. U.K. Cancer Cytogenetics Group and the U.K. Children's Cancer Study Group. Med Pediatr Oncol, *36:* 14-19, 2001.
132. Savelyeva, L., Corvi, R., and Schwab, M. Translocation involving 1p and 17q is a recurrent genetic alteration of human neuroblastoma cells. Am J Hum Genet, *55:* 334-340, 1994.

133. Lastowska, M., Cotterill, S., Bown, N., Cullinane, C., Variend, S., Lunec, J., Strachan, T., Pearson, A. D., and Jackson, M. S. Breakpoint position on 17q identifies the most aggressive neuroblastoma tumors. Genes Chromosomes Cancer, *34:* 428-436, 2002.
134. Lastowska, M., Cotterill, S., Pearson, A. D., Roberts, P., McGuckin, A., Lewis, I., and Bown, N. Gain of chromosome arm 17q predicts unfavourable outcome in neuroblastoma patients. U.K. Children's Cancer Study Group and the U.K. Cancer Cytogenetics Group. Eur J Cancer, *33:* 1627-1633, 1997.
135. Islam, A., Kageyama, H., Takada, N., Kawamoto, T., Takayasu, H., Isogai, E., Ohira, M., Hashizume, K., Kobayashi, H., Kaneko, Y., and Nakagawara, A. High expression of Survivin, mapped to 17q25, is significantly associated with poor prognostic factors and promotes cell survival in human neuroblastoma. Oncogene, *19:* 617-623, 2000.
136. Ejeskar, K., Aburatani, H., Abrahamsson, J., Kogner, P., and Martinsson, T. Loss of heterozygosity of 3p markers in neuroblastoma tumours implicate a tumour-suppressor locus distal to the FHIT gene. Br J Cancer, *77:* 1787-1791, 1998.
137. Guo, C., White, P. S., Weiss, M. J., Hogarty, M. D., Thompson, P. M., Stram, D. O., Gerbing, R., Matthay, K. K., Seeger, R. C., Brodeur, G. M., and Maris, J. M. Allelic deletion at 11q23 is common in MYCN single copy neuroblastomas. Oncogene, *18:* 4948-4957, 1999.
138. Takita, J., Hayashi, Y., Takei, K., Yamaguchi, N., Hanada, R., Yamamoto, K., and Yokota, J. Allelic imbalance on chromosome 18 in neuroblastoma. Eur J Cancer, *36:* 508-513, 2000.
139. Thompson, P. M., Seifried, B. A., Kyemba, S. K., Jensen, S. J., Guo, C., Maris, J. M., Brodeur, G. M., Stram, D. O., Seeger, R. C., Gerbing, R., Matthay, K. K., Matise, T. C., and White, P. S. Loss of heterozygosity for chromosome 14q in neuroblastoma. Med Pediatr Oncol, *36:* 28-31, 2001.
140. Barbacid, M. Neurotrophic factors and their receptors. Curr Opin Cell Biol, *7:* 148-155, 1995.
141. Nakagawara, A., Arima, M., Azar, C. G., Scavarda, N. J., and Brodeur, G. M. Inverse relationship between trk expression and N-myc amplification in human neuroblastomas. Cancer Res, *52:* 1364-1368, 1992.
142. Nakagawara, A., Arima-Nakagawara, M., Scavarda, N. J., Azar, C. G., Cantor, A. B., and Brodeur, G. M. Association between high levels of expression of the TRK gene and favorable outcome in human neuroblastoma. N Engl J Med, *328:* 847-854, 1993.
143. Ryden, M., Sehgal, R., Dominici, C., Schilling, F. H., Ibanez, C. F., and Kogner, P. Expression of mRNA for the neurotrophin receptor trkC in neuroblastomas with favourable tumour stage and good prognosis. Br J Cancer, *74:* 773-779, 1996.
144. Nakagawara, A., Azar, C. G., Scavarda, N. J., and Brodeur, G. M. Expression and function of TRK-B and BDNF in human neuroblastomas. Mol Cell Biol, *14:* 759-767, 1994.
145. Frame, S. and Balmain, A. Integration of positive and negative growth signals during ras pathway activation in vivo. Curr Opin Genet Dev, *10:* 106-113, 2000.
146. Feig, L. A. and Buchsbaum, R. J. Cell signaling: life or death decisions of ras proteins. Curr Biol, *12:* R259-261, 2002.
147. Agathanggelou, A., Cooper, W. N., and Latif, F. Role of the Ras-association domain family 1 tumor suppressor gene in human cancers. Cancer Res, *65:* 3497-3508, 2005.
148. Dammann, R., Schagdarsurengin, U., Seidel, C., Strunnikova, M., Rastetter, M., Baier, K., and Pfeifer, G. P. The tumor suppressor RASSF1A in human carcinogenesis: an update. Histol Histopathol, *20:* 645-663, 2005.
149. van der Weyden, L. and Adams, D. J. The Ras-association domain family (RASSF) members and their role in human tumourigenesis. Biochim Biophys Acta, 2007.

150. Astuti, D., Agathanggelou, A., Honorio, S., Dallol, A., Martinsson, T., Kogner, P., Cummins, C., Neumann, H. P., Voutilainen, R., Dahia, P., Eng, C., Maher, E. R., and Latif, F. RASSF1A promoter region CpG island hypermethylation in phaeochromocytomas and neuroblastoma tumours. Oncogene, *20:* 7573-7577, 2001.
151. Tommasi, S., Dammann, R., Zhang, Z., Wang, Y., Liu, L., Tsark, W. M., Wilczynski, S. P., Li, J., You, M., and Pfeifer, G. P. Tumor susceptibility of Rassf1a knockout mice. Cancer Res, *65:* 92-98, 2005.
152. Banelli, B., Gelvi, I., Di Vinci, A., Scaruffi, P., Casciano, I., Allemanni, G., Bonassi, S., Tonini, G. P., and Romani, M. Distinct CpG methylation profiles characterize different clinical groups of neuroblastic tumors. Oncogene, *24:* 5619-5628, 2005.
153. Harada, K., Toyooka, S., Maitra, A., Maruyama, R., Toyooka, K. O., Timmons, C. F., Tomlinson, G. E., Mastrangelo, D., Hay, R. J., Minna, J. D., and Gazdar, A. F. Aberrant promoter methylation and silencing of the RASSF1A gene in pediatric tumors and cell lines. Oncogene, *21:* 4345-4349, 2002.
154. Lazcoz, P., Munoz, J., Nistal, M., Pestana, A., Encio, I., and Castresana, J. S. Frequent promoter hypermethylation of RASSF1A and CASP8 in neuroblastoma. BMC Cancer, *6:* 254, 2006.
155. Wong, I. H., Chan, J., Wong, J., and Tam, P. K. Ubiquitous aberrant RASSF1A promoter methylation in childhood neoplasia. Clin Cancer Res, *10:* 994-1002, 2004.
156. Yang, Q., Zage, P., Kagan, D., Tian, Y., Seshadri, R., Salwen, H. R., Liu, S., Chlenski, A., and Cohn, S. L. Association of epigenetic inactivation of RASSF1A with poor outcome in human neuroblastoma. Clin Cancer Res, *10:* 8493-8500, 2004.
157. Dammann, R., Schagdarsurengin, U., Seidel, C., Trumpler, C., Hoang-Vu, C., Gimm, O., Dralle, H., Pfeifer, G. P., and Brauckhoff, M. Frequent promoter methylation of tumor-related genes in sporadic and men2-associated pheochromocytomas. Exp Clin Endocrinol Diabetes, *113:* 1-7, 2005.
158. Hesson, L., Dallol, A., Minna, J. D., Maher, E. R., and Latif, F. NORE1A, a homologue of RASSF1A tumour suppressor gene is inactivated in human cancers. Oncogene, *22:* 947-954, 2003.
159. Aoyama, Y., Avruch, J., and Zhang, X. F. Nore1 inhibits tumor cell growth independent of Ras or the MST1/2 kinases. Oncogene, *23:* 3426-3433, 2004.
160. Khokhlatchev, A., Rabizadeh, S., Xavier, R., Nedwidek, M., Chen, T., Zhang, X. F., Seed, B., and Avruch, J. Identification of a novel Ras-regulated proapoptotic pathway. Curr Biol, *12:* 253-265, 2002.
161. Vos, M. D., Martinez, A., Ellis, C. A., Vallecorsa, T., and Clark, G. J. The pro-apoptotic Ras effector Nore1 may serve as a Ras-regulated tumor suppressor in the lung. J Biol Chem, *278:* 21938-21943, 2003.
162. Vavvas, D., Li, X., Avruch, J., and Zhang, X. F. Identification of Nore1 as a potential Ras effector. J Biol Chem, *273:* 5439-5442, 1998.
163. Praskova, M., Khoklatchev, A., Ortiz-Vega, S., and Avruch, J. Regulation of the MST1 kinase by autophosphorylation, by the growth inhibitory proteins, RASSF1 and NORE1, and by Ras. Biochem J, *381:* 453-462, 2004.
164. Ortiz-Vega, S., Khokhlatchev, A., Nedwidek, M., Zhang, X. F., Dammann, R., Pfeifer, G. P., and Avruch, J. The putative tumor suppressor RASSF1A homodimerizes and heterodimerizes with the Ras-GTP binding protein Nore1. Oncogene, *21:* 1381-1390, 2002.
165. Chen, J., Lui, W. O., Vos, M. D., Clark, G. J., Takahashi, M., Schoumans, J., Khoo, S. K., Petillo, D., Lavery, T., Sugimura, J., Astuti, D., Zhang, C., Kagawa, S., Maher, E. R., Larsson, C., Alberts, A. S., Kanayama, H. O., and Teh, B. T. The t(1;3)

breakpoint-spanning genes LSAMP and NORE1 are involved in clear cell renal cell carcinomas. Cancer Cell, *4:* 405-413, 2003.

166. Hesson, L., Bieche, I., Krex, D., Criniere, E., Hoang-Xuan, K., Maher, E. R., and Latif, F. Frequent epigenetic inactivation of RASSF1A and BLU genes located within the critical 3p21.3 region in gliomas. Oncogene, *23:* 2408-2419, 2004.
167. Tommasi, S., Dammann, R., Jin, S. G., Zhang Xf, X. F., Avruch, J., and Pfeifer, G. P. RASSF3 and NORE1: identification and cloning of two human homologues of the putative tumor suppressor gene RASSF1. Oncogene, *21:* 2713-2720, 2002.
168. Schagdarsurengin, U., Gimm, O., Hoang-Vu, C., Dralle, H., Pfeifer, G. P., and Dammann, R. Frequent epigenetic silencing of the CpG island promoter of RASSF1A in thyroid carcinoma. Cancer Res, *62:* 3698-3701, 2002.
169. Xing, M., Cohen, Y., Mambo, E., Tallini, G., Udelsman, R., Ladenson, P. W., and Sidransky, D. Early occurrence of RASSF1A hypermethylation and its mutual exclusion with BRAF mutation in thyroid tumorigenesis. Cancer Res, *64:* 1664-1668, 2004.
170. Buyse, I. M., Shao, G., and Huang, S. The retinoblastoma protein binds to RIZ, a zinc-finger protein that shares an epitope with the adenovirus E1A protein. Proc Natl Acad Sci U S A, *92:* 4467-4471, 1995.
171. Fang, W., Piao, Z., Buyse, I. M., Simon, D., Sheu, J. C., Perucho, M., and Huang, S. Preferential loss of a polymorphic RIZ allele in human hepatocellular carcinoma. Br J Cancer, *84:* 743-747, 2001.
172. He, L., Yu, J. X., Liu, L., Buyse, I. M., Wang, M. S., Yang, Q. C., Nakagawara, A., Brodeur, G. M., Shi, Y. E., and Huang, S. RIZ1, but not the alternative RIZ2 product of the same gene, is underexpressed in breast cancer, and forced RIZ1 expression causes G2-M cell cycle arrest and/or apoptosis. Cancer Res, *58:* 4238-4244, 1998.
173. Huang, S., Shao, G., and Liu, L. The PR domain of the Rb-binding zinc finger protein RIZ1 is a protein binding interface and is related to the SET domain functioning in chromatin-mediated gene expression. J Biol Chem, *273:* 15933-15939, 1998.
174. Canote, R., Du, Y., Carling, T., Tian, F., Peng, Z., and Huang, S. The tumor suppressor gene RIZ in cancer gene therapy (review). Oncol Rep, *9:* 57-60, 2002.
175. Rea, S., Eisenhaber, F., O'Carroll, D., Strahl, B. D., Sun, Z. W., Schmid, M., Opravil, S., Mechtler, K., Ponting, C. P., Allis, C. D., and Jenuwein, T. Regulation of chromatin structure by site-specific histone H3 methyltransferases. Nature, *406:* 593-599, 2000.
176. Kim, K. C., Geng, L., and Huang, S. Inactivation of a histone methyltransferase by mutations in human cancers. Cancer Res, *63:* 7619-7623, 2003.
177. Abbondanza, C., Medici, N., Nigro, V., Rossi, V., Gallo, L., Piluso, G., Belsito, A., Roscigno, A., Bontempo, P., Puca, A. A., Molinari, A. M., Moncharmont, B., and Puca, G. A. The retinoblastoma-interacting zinc-finger protein RIZ is a downstream effector of estrogen action. Proc Natl Acad Sci U S A, *97:* 3130-3135, 2000.
178. Liu, L., Shao, G., Steele-Perkins, G., and Huang, S. The retinoblastoma interacting zinc finger gene RIZ produces a PR domain-lacking product through an internal promoter. J Biol Chem, *272:* 2984-2991, 1997.
179. Chadwick, R. B., Jiang, G. L., Bennington, G. A., Yuan, B., Johnson, C. K., Stevens, M. W., Niemann, T. H., Peltomaki, P., Huang, S., and de la Chapelle, A. Candidate tumor suppressor RIZ is frequently involved in colorectal carcinogenesis. Proc Natl Acad Sci U S A, *97:* 2662-2667, 2000.
180. Jiang, G., Liu, L., Buyse, I. M., Simon, D., and Huang, S. Decreased RIZ1 expression but not RIZ2 in hepatoma and suppression of hepatoma tumorigenicity by RIZ1. Int J Cancer, *83:* 541-546, 1999.

181. Poetsch, M., Dittberner, T., and Woenckhaus, C. Frameshift mutations of RIZ, but no point mutations in RIZ1 exons in malignant melanomas with deletions in 1p36. Oncogene, *21:* 3038-3042, 2002.
182. Sakurada, K., Furukawa, T., Kato, Y., Kayama, T., Huang, S., and Horii, A. RIZ, the retinoblastoma protein interacting zinc finger gene, is mutated in genetically unstable cancers of the pancreas, stomach, and colorectum. Genes Chromosomes Cancer, *30:* 207-211, 2001.
183. Du, Y., Carling, T., Fang, W., Piao, Z., Sheu, J. C., and Huang, S. Hypermethylation in human cancers of the RIZ1 tumor suppressor gene, a member of a histone/protein methyltransferase superfamily. Cancer Res, *61:* 8094-8099, 2001.
184. Tokumaru, Y., Nomoto, S., Jeronimo, C., Henrique, R., Harden, S., Trink, B., and Sidransky, D. Biallelic inactivation of the RIZ1 gene in human gastric cancer. Oncogene, *22:* 6954-6958, 2003.
185. Steele-Perkins, G., Fang, W., Yang, X. H., Van Gele, M., Carling, T., Gu, J., Buyse, I. M., Fletcher, J. A., Liu, J., Bronson, R., Chadwick, R. B., de la Chapelle, A., Zhang, X., Speleman, F., and Huang, S. Tumor formation and inactivation of RIZ1, an Rb-binding member of a nuclear protein-methyltransferase superfamily. Genes Dev, *15:* 2250-2262, 2001.
186. Carling, T., Du, Y., Fang, W., Correa, P., and Huang, S. Intragenic allelic loss and promoter hypermethylation of the RIZ1 tumor suppressor gene in parathyroid tumors and pheochromocytomas. Surgery, *134:* 932-939; discussion 939-940, 2003.
187. Pomerantz, J., Schreiber-Agus, N., Liegeois, N. J., Silverman, A., Alland, L., Chin, L., Potes, J., Chen, K., Orlow, I., Lee, H. W., Cordon-Cardo, C., and DePinho, R. A. The Ink4a tumor suppressor gene product, p19Arf, interacts with MDM2 and neutralizes MDM2's inhibition of p53. Cell, *92:* 713-723, 1998.
188. Baylin, S. B., Herman, J. G., Graff, J. R., Vertino, P. M., and Issa, J. P. Alterations in DNA methylation: a fundamental aspect of neoplasia. Adv Cancer Res, *72:* 141-196, 1998.
189. Esteller, M., Corn, P. G., Baylin, S. B., and Herman, J. G. A gene hypermethylation profile of human cancer. Cancer Res, *61:* 3225-3229, 2001.
190. Rocco, J. W. and Sidransky, D. p16(MTS-1/CDKN2/INK4a) in cancer progression. Exp Cell Res, *264:* 42-55, 2001.
191. Mao, L., Merlo, A., Bedi, G., Shapiro, G. I., Edwards, C. D., Rollins, B. J., and Sidransky, D. A novel p16INK4A transcript. Cancer Res, *55:* 2995-2997, 1995.
192. Quelle, D. E., Zindy, F., Ashmun, R. A., and Sherr, C. J. Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. Cell, *83:* 993-1000, 1995.
193. Serrano, M., Hannon, G. J., and Beach, D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. Nature, *366:* 704-707, 1993.
194. Sharpless, N. E. and DePinho, R. A. The INK4A/ARF locus and its two gene products. Curr Opin Genet Dev, *9:* 22-30, 1999.
195. Serrano, M. The tumor suppressor protein p16INK4a. Exp Cell Res, *237:* 7-13, 1997.
196. Tao, W. and Levine, A. J. P19(ARF) stabilizes p53 by blocking nucleo-cytoplasmic shuttling of Mdm2. Proc Natl Acad Sci U S A, *96:* 6937-6941, 1999.
197. Weber, J. D., Taylor, L. J., Roussel, M. F., Sherr, C. J., and Bar-Sagi, D. Nucleolar Arf sequesters Mdm2 and activates p53. Nat Cell Biol, *1:* 20-26, 1999.
198. Zhang, Y. and Xiong, Y. Mutations in human ARF exon 2 disrupt its nucleolar localization and impair its ability to block nuclear export of MDM2 and p53. Mol Cell, *3:* 579-591, 1999.

199. You, M. J., Castrillon, D. H., Bastian, B. C., O'Hagan, R. C., Bosenberg, M. W., Parsons, R., Chin, L., and DePinho, R. A. Genetic analysis of Pten and Ink4a/Arf interactions in the suppression of tumorigenesis in mice. Proc Natl Acad Sci U S A, *99:* 1455-1460, 2002.
200. Castresana, J. S., Gomez, L., Garcia-Miguel, P., Queizan, A., and Pestana, A. Mutational analysis of the p16 gene in human neuroblastomas. Mol Carcinog, *18:* 129-133, 1997.
201. Diccianni, M. B., Chau, L. S., Batova, A., Vu, T. Q., and Yu, A. L. The p16 and p18 tumor suppressor genes in neuroblastoma: implications for drug resistance. Cancer Lett, *104:* 183-192, 1996.
202. Iolascon, A., Giordani, L., Moretti, A., Tonini, G. P., Lo Cunsolo, C., Mastropietro, S., Borriello, A., and Ragione, F. D. Structural and functional analysis of cyclin-dependent kinase inhibitor genes (CDKN2A, CDKN2B, and CDKN2C) in neuroblastoma. Pediatr Res, *43:* 139-144, 1998.
203. Kawamata, N., Seriu, T., Koeffler, H. P., and Bartram, C. R. Molecular analysis of the cyclin-dependent kinase inhibitor family: p16(CDKN2/MTS1/INK4A), p18(INK4C) and p27(Kip1) genes in neuroblastomas. Cancer, *77:* 570-575, 1996.
204. Takita, J., Hayashi, Y., Kohno, T., Yamaguchi, N., Hanada, R., Yamamoto, K., and Yokota, J. Deletion map of chromosome 9 and p16 (CDKN2A) gene alterations in neuroblastoma. Cancer Res, *57:* 907-912, 1997.
205. Omura-Minamisawa, M., Diccianni, M. B., Chang, R. C., Batova, A., Bridgeman, L. J., Schiff, J., Cohn, S. L., London, W. B., and Yu, A. L. p16/p14(ARF) cell cycle regulatory pathways in primary neuroblastoma: p16 expression is associated with advanced stage disease. Clin Cancer Res, *7:* 3481-3490, 2001.
206. Carr, J., Bell, E., Pearson, A. D., Kees, U. R., Beris, H., Lunec, J., and Tweddle, D. A. Increased frequency of aberrations in the p53/MDM2/p14(ARF) pathway in neuroblastoma cell lines established at relapse. Cancer Res, *66:* 2138-2145, 2006.
207. Gonzalez-Gomez, P., Bello, M. J., Lomas, J., Arjona, D., Alonso, M. E., Aminoso, C., Lopez-Marin, I., Anselmo, N. P., Sarasa, J. L., Gutierrez, M., Casartelli, C., and Rey, J. A. Aberrant methylation of multiple genes in neuroblastic tumours. relationship with MYCN amplification and allelic status at 1p. Eur J Cancer, *39:* 1478-1485, 2003.
208. Slack, A., Chen, Z., Tonelli, R., Pule, M., Hunt, L., Pession, A., and Shohet, J. M. The p53 regulatory gene MDM2 is a direct transcriptional target of MYCN in neuroblastoma. Proc Natl Acad Sci U S A, *102:* 731-736, 2005.
209. Frostad, B., Martinsson, T., Tani, E., Falkmer, U., Darnfors, C., Skoog, L., and Kogner, P. The use of fine-needle aspiration cytology in the molecular characterization of neuroblastoma in children. Cancer, *87:* 60-68, 1999.
210. Hedinger Histological Typing of Thyroid tumors. . In: the WHO International Histological Classification of Tumors. , *Berlin: Springer-Verlag*, 1998.
211. Nolan, T., Hands, R. E., and Bustin, S. A. Quantification of mRNA using real-time RT-PCR. Nat Protoc, *1:* 1559-1582, 2006.
212. Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., and Speleman, F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol, *3:* RESEARCH0034, 2002.
213. Xiong, Z. and Laird, P. W. COBRA: a sensitive and quantitative DNA methylation assay. Nucleic Acids Res, *25:* 2532-2534, 1997.
214. Herman, J. G., Graff, J. R., Myohanen, S., Nelkin, B. D., and Baylin, S. B. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci U S A, *93:* 9821-9826, 1996.

215. Sun, D., Zhang, Z., Van do, N., Huang, G., Ernberg, I., and Hu, L. Aberrant methylation of CDH13 gene in nasopharyngeal carcinoma could serve as a potential diagnostic biomarker. Oral Oncol, *43:* 82-87, 2007.
216. Konishi, K., Shen, L., Wang, S., Meltzer, S. J., Harpaz, N., and Issa, J. P. Rare CpG island methylator phenotype in ulcerative colitis-associated neoplasias. Gastroenterology, *132:* 1254-1260, 2007.
217. Frazier, M. L., Xi, L., Zong, J., Viscofsky, N., Rashid, A., Wu, E. F., Lynch, P. M., Amos, C. I., and Issa, J. P. Association of the CpG island methylator phenotype with family history of cancer in patients with colorectal cancer. Cancer Res, *63:* 4805-4808, 2003.
218. Ricciardiello, L., Goel, A., Mantovani, V., Fiorini, T., Fossi, S., Chang, D. K., Lunedei, V., Pozzato, P., Zagari, R. M., De Luca, L., Fuccio, L., Martinelli, G. N., Roda, E., Boland, C. R., and Bazzoli, F. Frequent loss of hMLH1 by promoter hypermethylation leads to microsatellite instability in adenomatous polyps of patients with a single first-degree member affected by colon cancer. Cancer Res, *63:* 787-792, 2003.
219. Estecio, M. R., Gharibyan, V., Shen, L., Ibrahim, A. E., Doshi, K., He, R., Jelinek, J., Yang, A. S., Yan, P. S., Huang, T. H., Tajara, E. H., and Issa, J. P. LINE-1 Hypomethylation in Cancer Is Highly Variable and Inversely Correlated with Microsatellite Instability. PLoS ONE, *2:* e399, 2007.
220. Choi, I. S., Estecio, M. R., Nagano, Y., Kim do, H., White, J. A., Yao, J. C., Issa, J. P., and Rashid, A. Hypomethylation of LINE-1 and Alu in well-differentiated neuroendocrine tumors (pancreatic endocrine tumors and carcinoid tumors). Mod Pathol, *20:* 802-810, 2007.
221. Sasaki, O., Meguro, K., Tohmiya, Y., Funato, T., Shibahara, S., and Sasaki, T. Altered expression of retinoblastoma protein-interacting zinc finger gene, RIZ, in human leukaemia. Br J Haematol, *119:* 940-948, 2002.
222. Kroll, T. G., Sarraf, P., Pecciarini, L., Chen, C. J., Mueller, E., Spiegelman, B. M., and Fletcher, J. A. PAX8-PPARgamma1 fusion oncogene in human thyroid carcinoma [corrected]. Science, *289:* 1357-1360, 2000.
223. Nikiforova, M. N., Lynch, R. A., Biddinger, P. W., Alexander, E. K., Dorn, G. W., 2nd, Tallini, G., Kroll, T. G., and Nikiforov, Y. E. RAS point mutations and PAX8-PPAR gamma rearrangement in thyroid tumors: evidence for distinct molecular pathways in thyroid follicular carcinoma. J Clin Endocrinol Metab, *88:* 2318-2326, 2003.
224. Soufir, N., Avril, M. F., Chompret, A., Demenais, F., Bombled, J., Spatz, A., Stoppa-Lyonnet, D., Benard, J., and Bressac-de Paillerets, B. Prevalence of p16 and CDK4 germline mutations in 48 melanoma-prone families in France. The French Familial Melanoma Study Group. Hum Mol Genet, *7:* 209-216, 1998.
225. Debniak, T., Scott, R. J., Huzarski, T., Byrski, T., Rozmiarek, A., Debniak, B., Gorski, B., Cybulski, C., Medrek, K., Mierzejewski, M., Masojc, B., Matyjasik, J., Zlowocka, E., Teodorczyk, U., Lener, M., Klujszo-Grabowska, E., Nej-Wolosiak, K., Jaworowska, E., Oszutowska, D., Szymanska, A., Szymanska, J., Castaneda, J., van de Wetering, T., Suchy, J., Kurzawski, G., Oszurek, O., Narod, S., and Lubinski, J. CDKN2A common variant and multi-organ cancer risk--a population-based study. Int J Cancer, *118:* 3180-3182, 2006.
226. Puig, S., Malvehy, J., Badenas, C., Ruiz, A., Jimenez, D., Cuellar, F., Azon, A., Gonzalez, U., Castel, T., Campoy, A., Herrero, J., Marti, R., Brunet-Vidal, J., and Mila, M. Role of the CDKN2A locus in patients with multiple primary melanomas. J Clin Oncol, *23:* 3043-3051, 2005.