Genetic Mechanisms of Tumourigenesis in von Hippel-Lindau-Associated Tumours with the Emphasis on Capillary Hemangioblastoma

Sebsebe Lemeta

Haartman Institute
Department of Pathology
University of Helsinki
Helsinki, Finland

Finnish Institute of Occupational Health
Health and Work Ability
Helsinki, Finland

Academic dissertation

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Helsinki 2009
To My Brother Tesfu Lemeta
# TABLE OF CONTENTS

**TABLE OF CONTENTS** ............................................................................................................. 3

**LIST OF ORIGINAL PUBLICATIONS** ......................................................................................... 5

**ABBREVIATIONS** ................................................................................................................... 6

**INTRODUCTION** ..................................................................................................................... 7

**REVIEW OF THE LITERATURE** ............................................................................................... 8

**Tumourigenesis** ...................................................................................................................... 8

- Tumour growth ......................................................................................................................... 8
- Oncogenes, tumour suppressor and stability or caretaker genes ............................................... 8
- Familial or inherited cancer syndromes ..................................................................................... 9
- Significance of genetic alterations ........................................................................................... 10
- Epigenetic mechanisms and promoter hypermethylation .........................................................10
- Molecular biology methods and research tools ........................................................................11

**von Hippel-Lindau (VHL) disease and VHL-associated tumours** ......................................... 12

- History of the VHL disease ..................................................................................................... 12
- Capillary hemangioblastoma of the central nervous system ...................................................... 14
  - General features ................................................................................................................... 14
  - Clinical course, diagnosis and treatment .............................................................................. 15
- Pheochromocytoma .................................................................................................................. 17
  - Introduction and genetic characteristics .............................................................................. 17
  - Pheochromocytoma in von Hippel-Lindau disease ............................................................... 17
  - Pheochromocytoma and other inherited tumour syndromes .............................................. 17
  - Sporadic pheochromocytomas ............................................................................................. 18
  - Clinical presentation .............................................................................................................. 18
- Other VHL-associated tumours and lesions ............................................................................ 20
  - Retinal hemangioblastoma .................................................................................................. 20
  - Renal cell carcinoma ............................................................................................................ 20
  - Cysts and other tumour types ............................................................................................. 21

**Genetic mechanisms of the VHL disease and capillary hemangioblastoma** ....................... 22

- VHL gene and protein ............................................................................................................. 22
- Germ-line mutations ............................................................................................................... 25
- Somatic mutations .................................................................................................................. 26
- Genotype - phenotype relations .............................................................................................. 29
- Other candidate tumour suppressor genes ............................................................................... 30
# TABLE OF CONTENTS

AIMS OF THE STUDY ................................................................................................................................. 32

Specific aims ........................................................................................................................................... 32

MATERIALS AND METHODS .................................................................................................................. 33

Patients and clinical data (I, II, III, IV, V) .......................................................................................... 33

Comparative genomic hybridization (II) ................................................................................................. 34

Loss of heterozygosity in capillary hemangioblastoma and pheochromocytoma (III, IV, V) .................. 35

Immunohistochemistry (V) ..................................................................................................................... 36

Analysis of promoter methylation (V) ..................................................................................................... 36

RESULTS .................................................................................................................................................. 38

Clinical study and VHL mutation analysis (I) ........................................................................................ 38

Comparative genomic hybridization in capillary hemangioblastoma (II) .............................................. 38

Loss of heterozygosity in capillary hemangioblastoma and pheochromocytoma (III, IV, V) .............. 39

Immunohistochemistry for ZAC 1 (V) .................................................................................................... 41

Promoter methylation analysis of ZAC 1 gene (V) ................................................................................ 41

DISCUSSION ......................................................................................................................................... 44

VHL disease and prognosis of hemangioblastomas .............................................................................. 44

DNA copy number changes in capillary hemangioblastoma and VHL-associated tumours ............... 45

Loss of heterozygosity in capillary hemangioblastoma and pheochromocytoma ............................... 46

Loss of heterozygosity, immunohistochemistry and promoter hypermethylation at ZAC1 gene ........ 48

CONCLUSIONS ....................................................................................................................................... 50

ACKNOWLEDGEMENTS .......................................................................................................................... 51

REFERENCES ........................................................................................................................................... 54
LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original articles:

I  Niemelä, M., Lemeta, S., Summanen, P., Böhling, T., Sainio, M., Kere, J., Poussa, K., Sankila, R., Haapasalo, H., Käärläinen, H., Pukkala, E., and Jääskeläinen, J.
Long term prognosis of hemangioblastoma of the CNS: Impact of von Hippel-Lindau disease.

II  Lemeta, S., Aalto, Y., Niemelä, M., Jääskeläinen, J., Sainio, M., Kere, J., Knuitila, S., and Böhling, T.
Recurrent DNA sequence copy losses on Chromosomal arm 6q in capillary hemangioblastoma.

III  Lemeta, S., Pylkkänen, L., Sainio, M., Niemelä, M., Saarikoski, S., Husgafvel-Pursiainen, K., and Böhling, T.
Loss of heterozygosity at 6q is frequent and concurrent with 3p loss in sporadic and familial capillary hemangioblastomas.

IV  Lemeta, S., Salmenkivi K., Pylkkänen, L., Sainio, M., Saarikoski, S., Arola, J., Heikkilä, P., Haglund, C., Husgafvel-Pursiainen, K., and Böhling, T.
Frequent loss of heterozygosity at 6q in pheochromocytoma.

V  Lemeta, S., Jarmalaite, S., Pylkkänen, L., Böhling, T., and Husgafvel-Pursiainen, K.
Preferential loss of the non-imprinted allele for the ZAC1 tumour suppressor gene in human capillary hemangioblastoma.

In this doctoral thesis, these original papers are referred to by the Roman numerals indicated above.
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BRCA</td>
<td>Breast cancer gene</td>
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<tr>
<td>CHB</td>
<td>Capillary Hemangioblastoma</td>
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<tr>
<td>CGH</td>
<td>Comparative genomic hybridization</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EPI</td>
<td>Epinephrine</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescent <em>in situ</em> hybridization</td>
</tr>
<tr>
<td>FMTC</td>
<td>Familial medullary thyroid carcinoma</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia inducible factor</td>
</tr>
<tr>
<td>HNPCC</td>
<td>Human nonpolyposis colon cancer</td>
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<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>LATS1</td>
<td>Large tumour suppressor</td>
</tr>
<tr>
<td>LOH</td>
<td>Loss of heterozygosity</td>
</tr>
<tr>
<td>LOT</td>
<td>Lost on transformation</td>
</tr>
<tr>
<td>MEN</td>
<td>Multiple endocrine neoplasia</td>
</tr>
<tr>
<td>MIBG</td>
<td>Metaiodobenzylguanidine</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance image</td>
</tr>
<tr>
<td>MSP</td>
<td>Methylation specific PCR</td>
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<tr>
<td>NE</td>
<td>Norepinephrine</td>
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<tr>
<td>NF</td>
<td>Neurofibromatosis</td>
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<tr>
<td>PCC</td>
<td>Pheochromocytoma</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>RB1</td>
<td>Retinoblastoma gene</td>
</tr>
<tr>
<td>RCC</td>
<td>Renal cell carcinoma</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
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<tr>
<td>SDHB</td>
<td>Succinate dehydrogenase subunit B</td>
</tr>
<tr>
<td>SDHC</td>
<td>Succinate dehydrogenase subunit C</td>
</tr>
<tr>
<td>SDHD</td>
<td>Succinate dehydrogenase subunit D</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
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<tr>
<td>THW</td>
<td>Human transmembrane protein</td>
</tr>
<tr>
<td>TSG</td>
<td>Tumour suppressor gene</td>
</tr>
<tr>
<td>US</td>
<td>Ultrasound</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>VHL</td>
<td>Von Hippel-Lindau</td>
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INTRODUCTION

A century after the rediscovery of Mendel's law and the publication of Boveri's theory of aberrant chromosomes in the origin of cancer in 1914, cancer researchers are now able to trace historical landmarks in the development of cancer genetics. The discovery of oncogenes and tumour suppressor genes took place in the 1980s, and more recently, the sequence for the human genome was completed in 2003 (Balmain 2003; Knudson 2001). The many stimulatory "oncogenes" and inhibitory "tumour suppressor genes" that were identified in human neoplasms in the last decades have greatly increased our understanding of the complexity not only of tumourigenesis but also of normal growth regulatory pathways in different human cell types (Macleod 2000, Nowell 2002). Increasing molecular knowledge has provided a large window of opportunity for both diagnostic and therapeutic purposes (Vogelstein and Kinzler 2004; Vescovi et al. 2006).

The von Hippel Lindau (VHL) disease is a rare, autosomal dominantly inherited disorder which predisposes patients to develop a variety of benign or malignant tumours in different organs, capillary hemangioblastomas (CHB) of the central nervous system (CNS) and retina, renal cell carcinoma (RCC), pheochromocytoma (PCC), pancreatic and endolymphatic cysts. It is believed that the loss or inactivation of the \textit{VHL} gene which is located at 3p25-26 is responsible for the development of these tumours (Gallou et al. 2004; Kaelin 2007). CHBs are highly vascular benign tumours of the CNS, accounting for 1 - 2.5% of all intracranial neoplasms (Böhling et al. 2000; Zbar et al. 1999). They occur either as a sporadic entity or as part of VHL disease (Bahig et al. 2008; Neumann and Bender 1998). In fact, they are the most common manifestations of VHL disease, affecting 60 - 80% of VHL patients. CHB may be single and the only manifestation of the VHL disease, but there are often multiple manifestations in patients with VHL. This disease can strike at virtually any age, from 11 to 71 years (Choyke et al. 1995; Maher 2004). Recently, it was possible to detect a 100% mutation rate in familial cases (Stolle et al. 1998). However, in about half of the sporadic cases, it is possible to demonstrate the inactivation of the \textit{VHL} gene (Bahig et al. 2008; Gläsker et al. 2001; Kanno et al. 1994).

PCC is a rare neuroendocrine tumour of the chromaffin cells that contain catecholamine-containing neurosecretory granules. (Koch et al. 2001). Multiple genetic alterations have been associated with PCC. Most PCCs are sporadic, but they also occur in inherited tumour syndromes. A familial predisposition is seen in patients with VHL disease (Pacak et al. 2001), multiple endocrine neoplasia type II (MEN II) and neurofibromatosis type 1 (NF1), (Brandi et al. 2001; Walther et al. 1999). It has been estimated that 10% of PCCs are familial cases; however, recent studies indicate that this percentage could be as high as 24% (Neumann et al. 2002). PCC occurs in between 10 to 34% of VHL patients (Walther et al. 1999, Maher et al. 1990b; Richard et al. 1994).

The purpose of the present work was to search for genetic alterations, or other mechanisms of inactivation, in addition to the \textit{VHL} gene, that may be important in the development of VHL-associated tumours. Though less dramatic than cure, prevention and early detection are the most promising and feasible means to reduce cancer morbidity and mortality (Vogelstein and Kinzler 2004). This thesis work is based on the view that increasing knowledge on the molecular events underlying tumour development will eventually aid in early detection and in that way lead to improved treatment.
Tumourigenesis

Tumour growth

Tumour is an abnormal growth resulting from uncontrolled, progressive multiplication of cells, serving no physiological function. A tumour may be benign or malignant (Komarova et al. 2003). The major characteristic that differentiates malignant tumours from the benign counterparts is their invasiveness. The cells in malignant tumours differ from benign tumour cells in many ways, at least three to five mutations are required to develop a malignant solid tumour; in contrast only one or two mutations may be needed on the road to a benign tumour (Nowell 2002).

Several lines of evidence indicate that tumourigenesis in humans is a multistep process and that these steps reflect genetic alterations that drive the progressive transformation of a normal human cell into becoming a highly malignant tumour (Hanahan and Weinberg 2000; Knudson 2002; Renan 1993). It has been suggested that the vast catalogue of cancer cell genotypes is a manifestation of six essential alterations in cell physiology that collectively dictate malignant growth; self sufficiency in growth signals, insensitivity to growth inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg 2000).

Oncogenes, tumour suppressor and stability or caretaker genes

Cell proliferation is regulated through connected molecular pathways controlling cell division, differentiation, growth arrest, and apoptosis. Tight control of these events is necessary for the maintenance of homeostasis and involves multiple genes. Deregulation of some of these genes can lead to pathological situations as well as to malignancy. Alterations caused by somatic or germ-line mutations in proto-oncogenes, tumour suppressor genes and stability genes are responsible for tumourigenesis (Vogelstein and Kinzler 2004). After it has mutated, a proto-oncogene becomes activated and this results in a "gain of function" i.e. it becomes an oncogene.

Oncogenic activation can be a result of chromosomal translocations, gene amplifications or from subtle intragenic mutations affecting crucial residues that regulate the activity of the gene product (Balmain 2001; Knudson 1985; Rowley 1983). An activating somatic mutation in one allele of an oncogene is generally sufficient (dominant) to confer a selective growth advantage on the cell. Examples of oncogenes include the MET gene which predisposes to hereditary papillary renal cell carcinoma, and the RET gene which is involved in multiple endocrine neoplasia II. Mutations in K-ras gene occur in lung, ovarian and bladder carcinoma, the N-ras gene mutations are frequent in head and neck cancers (Vogelstein and Kinzler 2004).

Tumour suppressor genes (TSG) are targeted in the opposite way by genetic alterations; mutations reduce or abolish the activity of the gene product, thus causing loss of their functions (Knudson 2002; Vogelstein and Kinzler 2004). This kind of inactivation can arise from missense mutations at locations
that are essential for the protein activity, frame-shift mutations that result in a truncated protein, from deletions or insertions of various sizes, or from epigenetic silencing of the gene. Mutations in other genetic (even epigenetic) alterations affecting both the maternal and paternal alleles of a TSG are generally required in order to confer a selective advantage to the cell. Such a condition may arise through the deletion of one allele via a gross chromosomal event - such as the loss of an entire chromosome or chromosome arm or loss of heterozygosity coupled with an intragenic mutation of the other allele (Elias 1998; Knudson 2002).

A third class of cancer genes called stability genes or caretakers which promote tumourigenesis in a completely different way when mutated. This group includes the mismatch repair (MMR), nucleotide-excision repair (NER) and base-excision repair (BER) genes which are responsible for repairing subtle mistakes made during normal DNA replication or those errors induced by exposure to mutagens. Other instability genes regulate the process involving large portions of chromosomes, such as those responsible for mitotic recombination and chromosomal segregation (Friedberg 2003; Lengauer et al. 1998; Robert 2007b). The XPA and XPC genes for xeroderma pigmentosum, as well as the MSH2 gene for human nonpolyposis colon cancer (HNPCC), are examples of genomic instability genes.

For many years, many investigators have indicated the importance of genetic instability in the clonal evolution of tumour cells and related clinical progression of tumours. However, only a recent profusion of tumourigenic models and discussion articles has led to a higher profile for the debate about the role of genomic instability in tumourigenesis (Sieber et al. 2005). It is now widely accepted that cancer results from the accumulation of mutations in the genes that directly control cell division. However the mechanisms by which these mutations are generated have been the subject of continuing debate. It has been argued that an existing genetic instability is absolutely required for the generation of the multiple mutations that underlie cancer (Lengauer et al. 1998).

Cellular DNA is under constant attack from a variety of sources, including chemicals, ionizing radiation, heat, tumour-inducing viruses, enzymatic errors etc. However genomic stability is maintained by the efficient repair of damage to the bases present in DNA. The stability genes keep genetic alterations to a minimum, and thus, when they became inactivated, mutations in other genes occur at a higher rate (Elias 1998; Friedberg 2003). All genes are potentially affected by the resultant increased rate of mutations but only mutations in oncogenes and tumour-suppressor genes influence the net cell growth and can thereby confer a selective growth advantage onto the mutant cell (Vogelstein and Kinzler 2004).

Familial or inherited cancer syndromes

The past decade has witnessed the elucidation of the specific genetic bases of numerous inherited predispositions. Hereditary cancer syndromes comprise approximately 5 - 10% of diagnosed carcinomas. They are caused by mutations in specific genes; carriers of mutations in these genes are at an increased risk of developing cancer at young age (Apetosos et al. 2008).

The identification of susceptibility genes for specific types of cancer provided the necessary information for the complete characterization of inherited cancer syndromes. The close observation of carrier families has significantly enriched our knowledge on distinct phenotypical features, age of onset and survival rates for each syndrome, and provide the possibility to further understand the molecular bases of hereditary cancer (Eng et al. 2001; Fostira et al 2007).
Significance of genetic alterations

In 1914, Theodor Boveri first hypothesised that tumour growth is based on a particular, incorrect chromosome combination which is the cause of the abnormal growth characteristics passed on to daughter cells (reviewed in (Balmain 2001). More than half a century later, in 1971, Knudson and co-workers carried out an epidemiological study on retinoblastoma and postulated that “two hits” are required for the complete inactivation of a TSG, suggesting that cancer predisposition results from inheritance of a specific mutation in a suppressor gene, but that the development of tumours requires subsequent somatic alterations that causes loss of the wild type copy of the same gene (Knudson 1971). Examples of TSG include the $TP53$ gene, which is mutated in more than 50% of human cancers; the $RB1$ gene that predisposes to retinoblastoma; $VHL$ gene that is involved capillary hemangioblastoma (CHB), Renal cell carcinoma (RCC), pheochromocytoma (PCC), the $BRCA1$ and $BRCA2$ genes that confer susceptibility towards breast and ovarian cancer, and the $NF1$ gene, predisposing to neurofibromas (Vogelstein and Kinzler 2004).

Additionally, chronic inflammation has been proposed as a risk factor for a variety of epithelial cancers. An inflammatory response, accompanied by the generation of free radicals, is capable of both directly damaging DNA and enhancing the genetic instability of the affected cells (Dobrovolskaia and Kozlov 2005).

Epigenetic mechanisms and promoter hypermethylation

Interestingly, in addition to the extensively studied current dogma that cancer is predominantly a genetic disease, recent data have also highlighted the contribution of epigenetic events (Baylin et al. 2000). Heritable changes that do not depend on changes in the DNA sequence but rather on the pattern of gene expression are called epigenetic changes. They may affect the expression of the gene or the properties of its product (Read and Strachan 1999). This change in gene expression involves the methylation of DNA in promoter regions of the genes. DNA methylation is a crucial epigenetic modification of the genome that is involved in regulating many cellular processes, including genomic imprinting and chromosome stability (Robertson 2005). Changes in methylation patterns in the promoter regions of crucial genes can alter gene expression. Since the methylation rate is thought to be faster than the genetic mutation rate, epigenetic alterations may be more likely to initiate neoplasms rather than genetic mutations (Baylin et al. 2000) Epigenetic changes, such as aberrant promoter methylation and loss of imprinting, have been identified as early and ubiquitous alterations in human cancer. Promoter hypermethylation of tumour suppressor genes is recognized as an alternative to genetic alterations in disrupting gene function. They can also be tissue- and tumour-type specific (Belinsky 2004; Feinberg et al. 2006; Jones and Baylin 2002).

In tumour suppressor gene inactivation, genetic and epigenetic mechanisms may act in parallel; bi-allelic inactivation may occur via a combination of mutational and epigenetic events, following the principle of the Knudson two-hit hypothesis of tumourigenesis (Jones and Laird 1999).

In the human genome, DNA methylation is restricted to cytosines of CpG (cytosine-phosphoguanine) dinucleotides which are often clustered into CpG-rich regions known as CpG islands. CpG islands are
defined as regions of the genome which are about 1kb long and are not only CpG rich, but generally G/C rich as well. These islands are usually found at the 5′ end of genes, the region often associated with sites where the transcription of DNA into RNA begins i.e, the promoter region (Bird 1992; Laird and Jaenisch 1994; Laird 2005). In the bulk of the genome, about 80% of the CpG dinucleotides that are not associated with CpG islands are heavily methylated. In contrast, the dinucleotides in CpG islands, especially those associated with gene promoters, are usually unmethylated, whether or not the gene is being transcribed (Bird 2002; James and Stephen 2003).

DNA molecules can be altered covalently by the attachment of methyl groups to cytosine bases, this modification of genomic DNA can be as important as a mutation in shutting down tumour suppressor genes. More specifically, when CpG methylation occurs in the vicinity of the gene promoter, it can cause repression of transcription of the associated gene (Robert, 2007b). A growing number of cancer genes that harbour dense methylation in normally unmethylated promoter CpG islands are becoming recognised, this hypermethylation can contribute to loss of gene function. In many tumours, it has been discovered that the function of a tumour suppressor gene is abrogated by hypermethylation of the promoter region, e.g VHL (renal cell carcinoma, capillary hemangioblastoma), BRCA1 (in breast and ovarian cancer), BP16 (in most lymphoma) (Baylin et al. 2000; Jones and Baylin 2002). Properly established and maintained DNA methylation patterns are essential for mammalian development and for normal functioning of the adult organism (Mayer et al. 2000.; Merlo et al. 2006; Panning B 1996; Robertson 2005). Imprinted genes are particularly intriguing examples of epigenetic modification. Their expression is controlled by patterns of methylation that differ according to the parental origin of the gene. When either the imprinting mechanism malfunctions or the parental origin is not as expected, then a pathogenic loss of function or inappropriate expression can occur in intact genes (Arima et al. 2005; Read and Strachan 1999).

**Molecular biology methods and research tools**

Important advanced methods have been developed and used to clarify the puzzle of tumour formation and growth. The development of cytogenetic methods was crucial in understanding chromosomal aberrations as visualised by Boveri under the microscope (reviewed in (Balmain 2001). Subsequently, multiple cytogenetic and molecular genetic methods have been developed. Comparative genomic hybridization (CGH) was the first efficient approach for scanning the entire genome for variations in DNA copy number. CGH is a powerful molecular cytogenetic method for detecting DNA sequence copy number changes throughout the genome in tumors; it helps to analyze the total genome for loss or gain of genetic material in a single experiment (Kallioniemi et al. 1992; Kallioniemi et al. 1994). Additional methods such as fluorescence in situ hybridization (FISH) can also be used to determine the copy number associated with a given ratio level, and recently a further method development called Array CGH has been implemented using a wide variety of techniques (Pinkel and Albertson 2005). Array comparative genomic hybridization (array CGH), is a method designed for identifying genomic regions with copy number aberrations (Pinkel et al. 1998; Pinkel and Albertson 2005). The development of high density arrays consisting of tens of thousands of DNA targets spanning the entire human genome has enabled precision mapping of the boundaries of genetic alterations throughout the genome in a single experiment (Barrett et al. 2004; Bignell et al. 2004).
The cytogenic discovery of germ-line aberrations, together with the use of restriction fragment length polymorphism (RFLPs), led to the cloning of several hereditary cancer genes (Knudson 2001). Chromosomal locations commonly deleted in tumour cells can be characterised by loss of heterozygosity. Inactivation (by mutation or methylation) of one copy of a TSG may be followed by other mechanisms that facilitate loss of the other gene copy. These mechanisms depend on LOH at the TSG locus, and may involve mitotic recombination, loss of a chromosomal region that harbors the target gene, inappropriate chromosomal segregation (nondisjunction), or loss of a more defined chromosomal region. Repeated LOH occurring in a given chromosomal region in a number of independently arising tumours often indicates the presence of a TSG in that region (Read and Strachan 1999; Weinberg 2007).

von Hippel-Lindau (VHL) disease and VHL-associated tumours

History of the VHL disease

In 1872, the first reports of apparently cystic, neoplastic lesions involving several organs, particularly the retina were published, describing angiomatous lesions of the retina, which were sometimes associated with identical lesions in the cerebellum (Melmon 1964, Jakson 1872). However, in 1894, it was Treacher Collins, who detailed his histological observations on bilateral lesions in the retinas and stated that the site of the lesions was in the vascular structures. He was the first to have access to tissues from a brother and sister, and described the angiomatous nature of the retinal lesion (Collins 1894). In 1904 a German ophthalmologist, von Hippel, presented clinical data gathered from two brothers. Unaware of Collin's work and lacking tissue for study, at that time he was unable to assign a known cause to this disease. However, seven years later, having obtained tissue from one of his patients and now being aware of Collins' work, von Hippel concluded that the primary lesion in the retina was a tumour and called the disease "angiomatosis retinae" (von Hippel 1904).

Later, several case reports were published about the findings of angiomatous retinæ in patients who additionally suffered brain tumours and visceral lesions. Arvid Lindau, a Swedish pathologist from Lund, was the first to be credited with these critical observations. He collected the data on forty such cases, twenty-four from the literature and sixteen of his own, and reviewed the literature on over 200 cases. In his monograph in 1926, Lindau brought together into one coherent entity the retinal, cerebral, and visceral components of this disease, and noted that the condition is heritable and used the term "Central nervous system angiomatosis" (Lindau 1927). Lindau's findings were rapidly accepted by Cushing and Bailey, who in 1928 re-named the vascular central nervous system (CNS) tumours as capillary hemangioblastoma (CHB) and used the eponym "Lindau's disease" (Cushing and Bailey 1928). Later scattered clinical reports of families with several affected members confirmed the association of CHB of CNS, renal and pancreatic cysts, pheochromocytomas (PCC), renal cell carcinomas (RCC) and epididymal cytadenomas. In 1964 Melmon and Rosen summarized this data and described a large family with this disease and codified the term "von Hippel-Lindau" (VHL) disease (Melmon and Rosen 1964).
The nosology of VHL disease has been extended through an improved understanding of the lesions of the CNS, delineation of renal and pancreatic involvement, and the associated risk of PCCs, e.g. its adrenal and extra-adrenal nature. Computed tomography (CT) and ultrasound (US) greatly helped in the identification of affected members, and later, magnetic resonance image (MRI) became a key method for screening cerebellum and spine (Fill et al. 1979; Jennings et al. 1988). Numerous cytogenetic and molecular studies identified deletions of 3p as a hallmark of VHL tumours, but it was not until 1988 than the gene was mapped to the short arm of chromosomal region 3p25-26 (Seizinger et al. 1988). Later, in 1993, the gene was isolated by positional cloning, then after many different germ-line and somatic VHL mutations had been identified as causes of VHL disease, the gene was demonstrated to be a TSG (Latif et al. 1993).

The identification of a pathogenic germ-line VHL gene mutation provides a specific and reliable basis for the diagnosis of VHL -disease. The key indications for the molecular test are indicated in Table 1. This has improved the diagnosis and clinical management of the disease, and also provided insights into the pathogenesis of sporadic VHL-associated tumours (Maher 2004) (Table 1). However if molecular diagnosis is not possible, less sensitive clinical diagnostic criteria need to be employed. The clinical diagnostic criteria for VHL disease were proposed by Melmon and Rosen (Melmon and Rosen 1964). Thus if there is the presence of a confirmed family history of VHL disease, the finding of a single typical VHL tumour in an at-risk relative enables a clinical diagnosis of VHL disease to be made. However, in isolated cases without a family history, the presence of two or more CHBs or one CHB and visceral manifestations are required for the diagnosis (Maher et al. 1991). Relatively few patients exhibit all the manifestations of VHL disease. About 50% of VHL patients will only display one manifestation of the disease (Latif et al. 1993; Maher 2004).

The onset of VHL disease is variable and depends on the expression of the disease within the individual and within the family and the intensity with which asymptomatic lesions are sought. It may present in childhood or at old age but the usual age of symptomatic presentation is in the 2nd or 3rd decades of life, and penetrance is considered to be complete by the age 60. Retinal lesions generally occur first, the mean age at symptomatic diagnosis is 24.5 years; for CHBs 33 years is the most common age for presenting with features (Bahig et al. 2008; Bender et al. 2001).

Molecular genetic analyses enable an early diagnosis of VHL disease in patients who do not satisfy conventional clinical diagnostic criteria (Maher 2004). On average, the diagnosis of VHL disease is made 4.5 years after the onset of symptoms (Maddock et al. 1996). However, early detection and followup are essential for adequate management of this syndrome and it is of considerable importance for genetic counselling as well as for the detection of treatable, usually unsuspected lesions, such as RCC, that may cause early death. Similarly, early detection makes a difference to the prognosis of both PCC and retinal CHB which need early treatment to avoid severe complications. In addition, there are obvious consequences for relatives who will require genetic counselling and screening for occult lesions (Filling-Katz et al. 1991). Before comprehensive screening surveys became routine, the median survival of patients with the VHL disease was less than 50 years with the main cause of death being complications linked to RCC and CNS hemangioblastomas (Maddock et al. 1996; Richard et al. 1998)
Table 1. Key criteria for molecular genetic tests.

<table>
<thead>
<tr>
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<th>Criteria</th>
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<tr>
<td>1</td>
<td>A patient with classic VHL disease (meeting clinical diagnostic criteria) and/or first-degree family members</td>
</tr>
<tr>
<td>2</td>
<td>An individual from a family in which a germ-line VHL gene mutation has been identified (presymptomatic test)</td>
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<tr>
<td>3</td>
<td>A VHL-suspected patient, <em>i.e.</em>: a multicentric tumours in one organ b. bilateral tumours c. two organ systems affected d. one VHL-associated tumour at a young age (<em>i.e.</em> &lt;50 years for CHB and PCC and &lt;30 years for RCC)</td>
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<tr>
<td>4</td>
<td>A patient from a family with CHB, RCC or PCC only</td>
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**Capillary hemangioblastoma of the central nervous system**

**General features**

Capillary hemangioblastomas are highly vascular benign tumours of the central nervous system. They are rare, accounting for only 1 - 2.5% of all intracranial neoplasms (Böhling et al. 2000; Oberstrass et al. 1996; Surawicz et al. 1999; Zbar et al. 1999). CHBs occur either as a sporadic entity or as part of VHL disease; they are the most common manifestations of VHL disease, affecting 60 - 80% of all patients (Bahig et al. 2008; Conway et al. 2001; Maddock et al. 1996; Neumann and Bender 1998). They can arise anywhere along the craniospinal axis, but the cerebellum is the most frequent site, about 44 -72% of the tumours are localised in the cerebellum, followed by 13 - 50% in the spinal cord, and 10 -25% in the brain stem. With respect to the spinal tumours, the majority are localised in the thoracic part, followed by cervical, and to the lesser extent in the lumbar area (Böhling et al. 2000; Choyke et al. 1995; Filling-Katz et al. 1991; Maher 2004).

CHBs may be the single and only manifestation of the VHL disease, but the tumours are often multiple in patients with VHL, involving both posterior fossa and the spinal cord (Couch et al. 2000; Wanebo et al. 2003). CNS tumours in VHL may arise at virtually any age from 11 to 71 years, but the average age of presentation for CHB is 33 years (Bouhey et al. 1990). Approximately 30% of all patients with CHB have the VHL disease and the mean age of 33 years at diagnosis of those with VHL disease is considerably younger as compared with sporadic cases (48 years) (Bahig et al. 2008; Maher et al. 1990b; Neumann et al. 1989; Richard et al. 1994b).

Regardless of location, CHBs have the same histological characteristics, which relate to Lindau`s original concept that the disease is an "angiomatosis of the central nervous system" (Choyke et al. 1995; Lindau 1927). Macroscopically, CHBs appear as well-defined and highly vascular tumours, often cystic but they contain a solid nodule. Histologically they are composed of two major components, *i.e.* stromal and endothelial, and furthermore CHBs contain pericytes and scattered mast cells (Böhling et al. 1987; Choyke et al. 1995). The stromal cells are believed to be the true neoplastic cells of CHB (Vortmeyer et al. 1997). There are several reports which have associated CHBs with secondary polycythaemia in about
20% of cases; moreover it has been shown that CHBs harbour cells with erythropoietin-like immunoreactivity (Bahig et al. 2008; Böhling et al. 1987; Horton et al. 1991; Munt et al. 1992).

Clinical course, diagnosis and treatment

Clinical course. Although considered histologically as benign, CHBs may cause significant neurological deficits and are associated with a serious mortality rate. The symptoms associated with CHBs depend on tumour location, number and size, and are caused by "mass effect", either by the tumour itself or an accompanying cyst or oedema (Bahig et al. 2008; Gläsker et al. 1999; Maddock et al. 1996). Patients with CHBs of the brain usually present with a longer history of minor neurological symptoms which in many cases, are followed by sudden exacerbation necessitating immediate neurosurgical intervention (Neumann et al. 1989). These tumours may be life-threatening because of large cystic components in the posterior fossa or they may cause paraplegia if they are localised in the spinal cord.

Symptomatic tumours grow faster than asymptomatic tumours, whereas symptomatic cysts grew faster than the CHBs associated with them (Filling-Katz et al. 1991; Gläsker 2005). It has been claimed that CHBs display several periods of tumour growth separated by periods of arrested growth, though many untreated tumours may remain the same size for years (Wanebo et al. 2003). Tumours in the cerebellar hemispheres evoke manifestations due to increased intracranial pressure, and thus headache is the most frequent presenting symptom. Other typical signs of cerebellar dysfunction are gait ataxia, dysmetria and nystagmus. The classical polycythaemia, secondary to cerebellar CHB, occurs in about 20% of cases. In severe cases, the tumour can lead to occlusive hydrocephalus with headache, nausea, vomiting and eventually coma and death. Tumours located in the spinal cord may evoke pain which is local and can be pinpointed by the patient, sensory loss, spinal ataxia, incontinence and paresis. Spinal CHBs are more frequently related to VHL than CHBs with an infratentorial localization, (Boughey et al. 1990; Choyke et al. 1995; Gläsker 2005; Maher and Kaelin 1997; Richard et al. 1998). Many individuals with symptomatic tumours also exhibit asymptomatic lesions. Brainstem tumours may manifest through symptoms such as orthostatic hypotension and they have a slightly higher likelihood of being symptomatic than cerebellar or spinal tumours, perhaps due to the constraints of the craniocephal junction (Filling-Katz et al. 1991). When comparing patients with familial VHL to patients with sporadic tumours, familial cases have multifocal lesions more often than their sporadic counterparts. However, no significant differences between sporadic and hereditary CHBs are noted in the radiological features, or in the duration of symptoms (Neumann et al. 1989; Neumann et al. 1992).

Diagnosis. The method of choice for early diagnosis and follow up examination of CHB is MRI (Figure 1.). MRI scanning has the advantages of being more sensitive and posing less radiation risk as compared to computer tomography (Maddock et al. 1996; Resche et al. 1993). Usually the diagnosis can be established by MRI because of the typical appearance of the tumour, MRI displaying a typically bright enhancing mass, clearly delineated from the surrounding brain or spinal cord tissue (Bahig et al. 2008; Baker et al. 2000). Although the detection of an asymptomatic CHB of the CNS does not necessarily mean intervention is inevitable, early detection can facilitate patient management and thus VHL disease patients and at-risk relatives may be offered MRI scans every 24 - 36 months (Maher 2004). Angiography reveals a highly vascular tumour blush and it can be performed preoperatively to demonstrate the feeding vessels (Malis 2002; Spetzger et al. 1996; Van Velthoven et al. 2003). In rare
cases, where the diagnosis of CHBs is not clearly visualized by MRI and a history of VHL, angiography may remain a useful diagnostic tool (Malis 2002; Richard et al. 1998; Van Velthoven et al. 2003).

Since 50% of all CNS CHBs require emergency neurosurgical treatment, early identification of this tumour would avoid such situations. All VHL patients and their at-risk relatives (unless excluded by molecular testing) should be offered regular surveillance starting from childhood; ophthalmic examination should be initiated within the first 2 years of life and MRI after 10 years of age (Filling-Katz et al. 1991). Improved surveillance, earlier diagnosis of lesions by modern imaging and laboratory studies, improvements in treatment, and increased knowledge of this disease have improved the prognosis and reduced the complications related to these tumours (Lamiell et al. 1989; Maher et al. 1990b; Neumann et al. 1992).

Treatment. The primary treatment is the surgical removal of the tumours. Total neurosurgical excision with the use of a high power microscope remains the curative treatment of symptomatic CHBs and often has to be done as emergency treatment (Neumann et al. 1992). In VHL patients, CHBs often grow at several sites simultaneously and new lesions can arise with time. Furthermore, the growth pattern can be irregular and unpredictable; therefore the indication for surgery in asymptomatic cases is still a matter of debate. However, there is a general consensus for surgical treatment of symptomatic CHBs (Lonser et al. 2003c). There have been some suggestions that asymptomatic tumours showing radiographic progression should be operated, since preoperative symptoms are usually not reversible whereas surgery is associated with low morbidity (Gläsker 2005).

Due to the potential morbidity associated with resection of multiple craniospinal CHBs, for small or medium sized CHBs (< 30mm), external-beam radio therapy and gamma knife radio-surgery can be used to arrest the progression of symptoms, by forcing the tumours to shrink or stop growing (Chakraborti et al. 1997; Chandler and Friedman 1994; Niemelä et al. 1996). Although microsurgery has considerably improved post-operative results, multifocal tumour development and recurrence remain a serious problem in the clinical management of VHL patients. Due to their susceptibility to tumour growth, patients suffering from VHL are recommended to undergo follow-up MRI investigation at one year intervals, and those without CNS manifestation at two years intervals (Russell and Theis 2003).

Figure 1. MRI detection of capillary hemangioblastoma tumours. A. Cerebellum. B. Brain stem. C. Spine (arrow).
Pheochromocytoma

Introduction and genetic characteristics

Pheochromocytoma is a rare neuroendocrine tumour of the chromaffin cells that contain catecholamine-containing neurosecretory granules. PCC mostly arises in the adrenal medulla but also in ganglia of the sympathetic nervous system. The term extra-adrenal (paraganglioma) has been widely used to describe tumours of the paraganglion system localized extra-adrenally, in the retro-peritoneum, pelvis, thorax, and adjacent to major vessels of parenchymal organs. These two types often share the same clinical course and are histologically equivalent (Koch et al. 2001; Neumann et al. 2002b).

Multiple genetic alterations have been associated with PCC. Most PCCs are sporadic, but they can also occur in inherited tumour syndromes. A familial predisposition is seen in patients with VHL disease (Pacak et al. 2001), multiple endocrine neoplasia type II (MEN II) and neurofibromatosis type 1 (NF1), (Brandi et al. 2001; Eng et al. 1996; Walther et al. 1999). Germ-line mutations in three of the succinate dehydrogenase subunit genes (SDHD, SDHB and SDHC) have been shown to be responsible for the familial occurrence of adrenal and extra-adrenal (paraganglioma) PCCs (Maher and Eng 2002). It has been estimated that approximately 10% of PCCs are familial cases; however, recent studies indicate that the percentage could be as high as 24% (Neumann et al. 2002).

Pheochromocytoma in von Hippel-Lindau disease

Pheochromocytoma occurs in 10 to 34% of VHL patients, and it is the presenting manifestation in about 5% of the cases. The mean age at diagnosis is 28 years and in about 50% of the cases, the tumours are bilateral (Maher et al. 1990b; Richard et al. 1994b; Walther et al. 1999).

VHL disease has been divided into four subtypes based on the central role of PCC. VHL-type 1 is without PCC, and VHL-type 2 presents with PCC. A distinct subtype of type 2 patients (type 2C) will develop PCC only, without any other manifestation of VHL disease (Table 3). More than 300 VHL germ-line mutations have been identified, and those associated with PCCs are nearly all of the missense type (Brauch et al. 1995a; Chen et al. 1995; Hust et al. 2003; Neumann et al. 2002a; Zbar et al. 1996).

About 15% of patients with VHL-associated PCCs have large germ-line deletions detected by Southern blot analysis (Richards et al. 1993). A mutation hotspot has been described at codon 167 (nt 712/713) in exon 3 (Chen et al. 1995; Crossey et al. 1994). "Founder effects", such as those present in the Black Forest region in Southern Germany with the missense mutation occurring at codon 98 (Tyr98His), may explain regional prevalence rates (Neumann 1993; Neumann et al. 1993).

Pheochromocytoma and other inherited tumour syndromes

Multiple endocrine neoplasia 2 is an autosomal dominant tumour syndrome and it is divided into three subgroups, MEN 2A, MEN 2B and familial medullary thyroid carcinoma (FMTC). PCCs occur only in MEN 2A and MEN 2B, each with a rate of 50%. All patients with MEN 2 have germ-line mutations in the RET proto-oncogene (10q11.2) (Brandi et al. 2001; Eng et al. 1996). The prevalences of MEN 2 and VHL are
similar, 1 in 35 000 individuals, but mutations predisposing to PCC have a greater penetrance in MEN 2 in comparison to VHL. The mean age at diagnosis is about 37 years and in most cases PCCs occur bilaterally and multifocally (Koch et al. 2002; Maddock et al. 1996; Ponder 1999).

Approximately 1% of the patients diagnosed with PCC have NF1 disease. NF1 is the most common familial cancer syndrome predisposing to PCC. The disease is caused by germ-line mutations in the \( \text{NF1} \) gene, located on 17q11.2 (Huson et al. 1988; Riccardi 1991). PCC in patients with NF1 occurs at a later age as compared to occurrences in MEN2 and VHL disease. The mean age at diagnosis is during the fifth decade, and in fact onset before the age of 20 years is uncommon (Knudson and Strong 1972). About 22% of NF1 patients with PCC have multiple or bilateral tumours, less than 12% of the PCCs in NF1 patients are metastatic, and extra-adrenal PCCs in patients with NF1 are rare (Gutmann 1997; Howe et al. 1993; Koch et al. 2001; Walther et al. 1999).

Paraganglioma is a genetically heterogeneous condition; it arises in extra-adrenal chromaffin tissue, such as the organ of Zuckerkandl. The genetic foundations of this disorder remain largely unknown. The most common tumour site is the carotid body. In some of these tumours, a germ-line mutation in \( \text{SDHD} \) has been identified. The \( \text{SDHD} \) gene is located on chromosome 11q23, \( \text{SDHC} \) at 1q21, and \( \text{SDHB} \) at 1p36 (Astuti et al. 2001; Baysal et al. 2000). Recent studies suggest that germ-line \( \text{SDHD} \) and \( \text{SDHB} \) mutations are an important cause for familial and isolated PCCs (Maher and Eng 2002; Neumann et al. 2002).

### Sporadic pheochromocytomas

Hereditary tumours and their sporadic counterparts often share the same genetic background. Although the etiology of most inherited forms of PCCs is rather well documented, little is known about the etiology of sporadic tumours. Mutations of those genes that harbour germ-line mutations in familial cases account for only 10 -15 % of somatic mutations in sporadic PCCs (Bar et al. 1997; Bender et al. 2000; Brauch et al. 1997; Koch et al. 2002a). Somatic intragenic \( \text{VHL} \) mutations are infrequent, seen only in about 8% of sporadic PCCs. Similarly, somatic \( \text{RET} \) mutations are also uncommon, only found in about 10 - 20% of cases (Eng et al. 1995; Hofstra et al. 1996; Januszewicz et al. 2000). Gutmann and co workers described a somatic mutation of \( \text{NF1} \) in 7/20 (35%) sporadic PCCs suggesting that \( \text{NF1} \) inactivation may be involved in the pathogenesis of non-familiar PCCs (Gutmann 1994). Furthermore, mutation analyses of \( \text{SDHD} \) and \( \text{SDHB} \) in sporadic PCCs have revealed only a single somatic mutation (Astuti et al. 2001; Gimm et al. 2000). Recently (Neumann et al. 2002) identified germ-line mutations in \( \text{RET} \), \( \text{VHL} \), \( \text{SDHB} \), \( \text{SDHC} \), \( \text{SDHD} \) in up to 24% of studied patients with apparently sporadic PCCs and they suggested that among all PCCs, more than 24% may be attributed to one of those six genes. However, allele losses on chromosome 1p, 3p and 17p, 22q are also common findings in familial and sporadic PCCs (Bender et al. 2000; Benn et al. 2000; Vargas et al. 1997). A recent cytogenetic analysis indicated frequent loss of 6q in sporadic PCCs (Dannenberg et al. 2000).

### Clinical presentation

Pheochromocytomas are rare, in Finland only about 10 - 15 cases are diagnosed annually. PCCs can occur at any age, but the peak age at diagnosis is the fifth decade of life. In general, genetically
predisposed patients are younger at diagnosis and have more often multifocal tumours compared to patients with sporadic PCCs (Lack 1997).

Hypertension is the most common clinical sign in all patients with PCC, this kind of hypertension can be sustained or paroxysmal and may lead to death from cardiovascular or cerebrovascular disease. It has been estimated that only about 0.1 - 0.3% of hypertensive patients have an underlying PCC (Bravo 1994). The most typical presentation of a PCC is a paroxysmal hypertensive crisis characterized by severe hypertension, diaphoresis, headaches and tachycardia or arrhythmia. Hypertensive episodes can be spontaneous or associated with physical exercise, surgical operation, defecation (Sutton et al. 1981).

The clinical symptoms of PCCs derive from the catecholamines they secrete and from their relative stimulation of alpha or beta-adrenergic receptors. Catecholamines are normally produced by sympathetic nerves and by the adrenal medulla. High catecholamine levels are not specific for PCC, because sometimes PCCs do not secrete enough catecholamines to produce signs and symptoms. The secretion may also be episodic and between episodes, levels of catecholamines may be normal (Bravo 1994; Eisenhofer et al. 1999; Gerlo and Sevens 1994; Pacak et al. 2001).

Diagnosis of PCC usually requires biochemical evidence of excessive catecholamine production by the tumour, which usually is achieved from measurements of catecholamines or catecholamine metabolites (metanephrines, normetanephrines and vanillylmandelic acid) in urine or plasma. Measurements of plasma levels of normetanephrine and metanephrine have higher sensitivity and specificity than the other biochemical tests for the diagnosis of both familial and sporadic PCC (Bravo 1994; Gerlo and Sevens 1994). In familial PCC, periodic screening can lead to early stage detection before symptoms and signs, at a point when tumours are small and are not secreting large amounts of catecholamines (van der Harst et al. 2002). Plasma concentrations of normetanephrine greater than 2.5 pmol/ml or metanephrine levels greater than 1.4 pmol/ml (more than 4 and 2.5 fold above the upper reference limits) indicate a PCC with 100% specificity (Bahig et al. 2008; Lenders et al. 2002; Pacak et al. 2001). The tumour can be anatomically localized using CT, MRI, or metaiodobenzylguanidine (MIBG) scanning (Berglund et al. 2001; Pacak et al. 2001). Positron emission tomography (PET) has been also used to visualize primary and metastatic tumours (Pacak et al. 2001).

Although most PCCs are benign, about 10% of them may be malignant, and cause metastases. In MEN 2 and VHL related PCCs, about 25% are reported as being malignant over a 25 year follow-up period. At present, there is no definite histological marker which can differentiate between benign and malignant PCCs (Salmenkivi et al. 2001). It is believed that a better understanding of the molecular biology of PCCs by identification of the genes involved in the tumourigenesis will help surveillance, early diagnosis and the development of more effective treatment modalities (Pacak et al. 2001). Studies show that genetic testing for RET, VHL, NF1, SDHD, SDHB and SDHC gene mutations does represent a useful means to diagnose those PCCs that have occurred as the first manifestation of these hereditary diseases. The authorities recommend screening for PCCs to be started at the age of 6, since PCC has been described in children as young as 10 to 12 years old (Neumann et al. 1993).

The definitive treatment for PCC is surgical resection of the tumour, but surgery has a great risk for complications because of excessive release of catecholamines from the tumour which can lead to hypertensive crisis, stroke, arrhythmias, or myocardial infarction. Preoperatively patients must thus undergo pharmacological blockade of catecholamine synthesis and receptors (Kercher et al. 2002; Okamoto et al. 1996; Pacak et al. 2001; Walther et al. 1999; Walther et al. 2000). Recurrent PCC develops in 20% of patients with VHL; in MEN2 the prevalence is as high as 33%.
The principles of management of malignant PCC include pharmacological control of symptoms and tumour mass reduction by surgical resection of the primary tumour and metastases, followed by radionuclide therapy with or without chemotherapy (Eisenhofer et al. 2004; Hartley et al. 2001). Hepatic resection should be considered for local liver metastases; however less invasive techniques such as arterial embolisation or chemoembolization, cryoablation, and radiofrequency ablation will provide a transient response (Pacak et al 2001). Treatment with high dose I-MIBG results in an objective tumour response, stabilization of disease and a reduction in catecholamine secretion. Chemotherapy can be used alone or in combination with radionuclide therapy, particularly when there is extensive residual disease or poor uptake and response to radionuclide treatment (Joel et al. 2008).

Other VHL- associated tumours and lesions

Retinal hemangioblastoma

Retinal capillary hemangioblastomas are the most frequent and earliest detected lesions associated with VHL disease and they are histologically identical to the CHBs of the CNS (Grossniklaus et al. 1992; Webster et al. 1999). The lesions are usually peripheral in the retina or near the optic disc or both. A dilated feeding artery and vein is typically present, and microaneurysms can be seen (Dollfus et al. 2002). They are frequently multiple and bilateral, about 60% of VHL patients exhibit retinal CHB. The mean age at diagnosis is 25 years, retinal CHBs have been diagnosed in 1 - 2 year old infants, but this is rare, only 5% of the cases are diagnosed in children younger than 10 years of age (Bahig et al. 2008; Maher et al. 1990b; Moskowitz et al. 2005; Niemelä et al. 2000).

They are usually asymptomatic if they are in peripheral location, but if they become large or if they are located centrally, they can cause profound vision loss. Both peripheral and central angiomas can cause exudative and tractional retinal detachment, and hemorrhage as they enlarge, leading to blindness (Russell and Theis 2003; Wittebol-Post et al. 1998). Ophthalmoscopy allows identification of most retinal tumours. Routine use of fluorescein angiography can be used for detecting peripheral and optic nerve lesions. It is recommended that screening examination with dilated fundoscopy should be done in a VHL patient at least once a year, starting at age 1 year (Neumann and Wiestler 1991).

Early diagnosis and treatment can prevent visual loss or blindness. The treatment consists of aggressive use of laser photocoagulation or cryotherapy since most peripheral retinal tumours do respond to treatment. Irreversible glaucoma associated with severe pain can be an indication for enucleation (Martz 1991; Singh et al. 2001). The visual prognosis is frequently poor in patients with VHL disease, about 25% will experience permanent visual loss (Moskowitz et al. 2005).

Renal cell carcinoma

Renal cell carcinoma occurs predominantly as a sporadic disease, but to a lesser extent also as part of the VHL disease. RCC rarely is found as a first manifestation of VHL disease, since eye and CNS findings usually precede the discovery of renal involvement. However, RCC has been reported to develop in up to 45% of patients with VHL disease (Gnarra et al. 1993; Maher et al. 1990b). VHL-associated RCC is frequently multicentric and occurs at a younger age than in the sporadic forms: the mean age at
diagnosis is 39 years, 20 years younger than is the case for the sporadic RCCs (Bahig et al. 2008; Richard et al. 1994b; Richard et al. 1998). About 50 -70% of patients eventually develop renal cysts, but these rarely produce significant renal impairment (Richards et al. 1998; Richards FM 1998). The lifetime risk of clear cell RCC is >70%, and the incidence of metastasis of RCC in VHL patients reported to be 20%, usually at the sites in the liver, lung and bones. RCC is the most common cause of death in VHL disease (Maher et al. 1990b; Neumann 1987).

Usually RCCs are asymptomatic, but occasionally the more advanced cases of RCC can present with haematuria, and flank pain; thus serial imaging of the kidneys is useful for early diagnosis in VHL patients. Due to the absence of early clinical symptoms and the importance of early detection, diagnosis during presymptomatic screening has the potential to enhance overall outcome. Renal US and contrast-enhanced MRI are useful in helping to determine whether a lesion is cystic or solid. CT allows detection and quantification by size and number of RCC and cysts (DrRussell R Lonser MD and K Libutti MD 2003; Lonser et al. 2003a). Angiography is not as sensitive as the other techniques for the detection of RCC, and it is used only for preoperative screening to identify the renal vascular supply prior to partial nephrectomy (Goldfarb 1998).

Previously the presence of bilateral renal tumours has been taken as an indication for bilateral nephrectomy and kidney transplantation. However, recently nephron-sparing surgery has been recommended for carcinomas that have a maximum diameter of 3 cm. Renal sparing resection is designed to reduce the risk of metastasis while still preserving kidney function. For patients and at-risk relatives under regular surveillance, renal tumours should be detected at an early symptomatic stage and followed until they are approximately 3 cm in size (Walther et al. 1995; Walther et al. 1999).

Cysts and other tumour types

Endolymphatic sac tumours are slow growing, low grade papillary adenocarcinomas, rare in the general population but associated with VHL disease in up to 10% of patients, and they are frequently bilateral. Though they are mostly asymptomatic, they may evoke partial or even hearing loss, tinnitus, a sense of disequilibrium and facial paresis (Manski et al. 1997). They are highly vascular, and often erode or expand the surrounding temporal bone. Histologically they differ from CHBs but are similar to the papillary cystadenoma of the epididymis. They are not known to metastasize but are locally aggressive. CT and MRI of the internal auditory canals are the recommended radiological examination techniques for diagnosis of endolymphatic sac tumours (Blamires et al. 1992). Surgery is curative for completely excised tumours, and the preoperative level of hearing is usually preserved (Lonser et al. 2003b).

Epididymal cystadenomas are benign, seen in approximately 25 - 60% of men with VHL disease. They can be multiple and bilateral, firm and easily palpable, typically appearing in the teenage years (Wernert et al. 1986). They are characteristically asymptomatic, but sometimes the patient may report a hard, smooth "pebble" in the scrotum. Infertility due to azospermia has been reported (Lamiell et al. 1989). The diagnosis is made by palpation and confirmed by MRI and US. Since the lesion is benign and typically asymptomatic, they are managed conservatively.

Broad ligament cystadenomas, papillary cystadenomas in the broad ligament have occasionally been reported but these may go unrecognised in many women with VHL. The mean age at presentation and the true frequency are unknown. The earliest age at which this tumour has been diagnosed is 16 years, but it was reported that the usual age of onset is between 22 and 46 years. It can be diagnosed by
CT-imaging or US, MRI. The tumours are grossly and histologically similar to epididymal cystadenomas. Since they are benign and typically asymptomatic, they can be managed conservatively (Funk and Heiken 1989; Lonser et al. 2003b).

**Pancreatic cysts**, there is frequent involvement of these cysts in VHL disease, in most cases multiple cysts occur, which are rarely of functional significance, and they vary in size from several millimetres to over 10cm. (Choyke et al. 1995; Libutti et al. 1998; Libutti et al. 2000; Maher and Kaelin 1997). They are benign, generally asymptomatic, and become evident only when they cause bile duct obstruction. Periodic imaging of asymptomatic individuals is important for diagnosis. Most commonly detected with CT and US; once this cyst is identified with CT, MRI can be used to confirm the diagnosis. If they are asymptomatic then followup is recommended; however, if they are symptomatic, the specific approach to treatment, is determined by location and size of the cyst > 3cm.

**Genetic mechanisms of the VHL disease and capillary hemangioblastoma**

Adaptation to decreased oxygen tension is a fundamental requirement of all living organisms. At the molecular level, this adaptation to hypoxia involves induction of hypoxia responsive genes such as erythropoietin (Epo) and vascular endothelial growth factor (VEGF), the former to increase blood oxygen carrying capacity and the latter to increase blood delivery. New blood vessel formation or angiogenesis is also an adaptive response to cell and tissue hypoxia (Kaelin 2005; Shweiki et al. 1992). Tumour angiogenesis that is required for tumour growth is recognised as a response to hypoxia. Solid tumour growth is dependent upon the angiogenesis in the tumour induced by angiogenic factors such as VEGF. Vascularisation provides the tumour with nutrients and oxygen as well as the ability to establish metastasis via the circulatory system (Levy et al. 1997; White et al. 1995).  VEGF, also known as vascular permeability factor, is a potent angiogenic factor, which is regulated by hypoxia in vitro and in vivo (Leung et al. 1989). Many tumour cell lines have been demonstrated to constitutively overexpress VEGF (Levy et al. 1997).

**VHL gene and protein**

The human VHL gene located at 3P25-26 and contains 856bp in three exons. It encodes a protein that contains 213 amino acids with no significant homology to any other known proteins (Figure 2) (Duan et al. 1995). Depending on the site of the translation initiation codon, it encodes two proteins of different size (pVHL19 and pVHL30). Both isoforms appear to retain tumour suppressor activity, and the term "pVHL" is used to refer to both isoforms generically since their function is similar (Duan et al. 1995; Kim and Kaelin 2004).

Tumours linked to VHL inactivation are often highly vascular and overproduce angiogenic factors such as VEGF (Böhling et al. 1996). VEGF and Epo are normally induced under conditions where there is inadequate oxygenation. However, cells lacking pVHL constitutively over-produce hypoxia-inducible mRNAs and the restoration of pVHL function results in down-regulation of hypoxia-inducible mRNAs in the
presence of oxygen. Thus overproduction of hypoxia-inducible gene expression is a hallmark of pVHL-defective cells (Iliopoulos et al. 1996; Kim and Kaelin 2004).

Several studies have revealed that a cellular transcription factor, Elongin (SIII), is a functional target of the pVHL. Elongin (SIII) comprises a transcriptional active subunit (A) and two regulatory subunits Elongin (B and C). pVHL can compete with elongin A for binding to elongins B and C, thereby inhibiting elongin/SIII activity (Duan et al. 1995; Kibel et al. 1995). pVHL, when bound to elongin B and elongin C, interacts with a fourth protein called Cul2, (Aso et al. 1995; Kibel et al. 1995; Kishida et al. 1995; Zimmer et al. 2004). These pVHL/elongin/Cul2 (VCB) complexes interact with a protein called Rbx1, a complex target protein destined for polyubiquitination (these enzymes are often referred to as ubiquitin ligases) and hence the presence of the polyubiquitin tail serves as a signal or flag for the substrate to be degraded (Maina et al. 2005; Maynard and Ohh 2005). In another words, the VCB complex targets substrates for intracellular degradation via a process called ubiquitination. In this process, the target is bound by a protein called ubiquitin that labels a protein for degradation. The destruction takes place inside the proteasome, a protein-digesting complex (Ohh et al. 1998).

The VHL gene product, pVHL, has multiple functions, but the one most extensively studied relates to the regulation of the transcription factor called hypoxia-inducible factor (HIF). Many of the genes that are regulated by hypoxia including VEGF and EPO are under the control of hypoxia-inducible factor. HIF is made up of two non-identical subunits, the α and β subunits, and it binds to specific DNA sequences (Kaelin 2007b; Kim and Kaelin 2004). The HIF β is a stable protein, whereas HIF α protein is highly unstable if oxygen is present, thus the formation of an active HIF heterodimer is restricted to hypoxic conditions. Recently it was reported that cells lacking pVHL fail to degrade HIF α subunits in the presence of oxygen, thus providing an explanation for the expression of HIF target genes in pVHL-defective cells (Cockman et al. 2000; Iliopoulos et al. 1996; Kaelin 2007b; Kim and Kaelin 2004; Maxwell et al. 1999).

The VEGF gene is one of the possible target genes regulated by HIF (Maxwell et al. 1999). Moreover it was shown that the region of pVHL that binds to elongins B and C is frequently mutated in VHL disease, evidence that tumour suppression by VHL is linked to its ability to inhibit elongin /SIII in vivo (Duan et al. 1995; Kibel et al. 1995; Maxwell 2005). When hypoxia-inducible proteins are not properly degraded, an excessive blood vessel formation may occur. Thus in cells lacking wild-type pVHL, degradation of HIF is impaired and cells behave as if they were deprived of oxygen (Michelle et al 2008).
**Figure 2.** Function of wild-type and mutant pVHL in a simplified model which illustrates targeting of HIF-α for degradation by the pVHL E3 ubiquitin ligase complex (modified from Michelle et al 2008)

(A) In normoxia, key proline residues of HIF-α are hydroxylated by PHDs (prolyl hydroxylase domain-containing proteins). The functioning of PHDs is dependent on the presence of molecular oxygen. This hydroxylation process enables recognition of HIF-α by the pVHL/E3 ubiquitin ligase complex. HIF-α is polyubiquitinated and subsequently degraded by the 26S proteasome.

(B) In normoxia, VHL gene mutations in the β or α domain can disrupt the binding of pVHL to HIF-α and/or to the other components of the E3 ubiquitin ligase complex. Stabilized HIF-α dimerizes with HIF-1β. Co-activators are recruited and the HIF complex can activate the transcription of target genes under normoxic conditions. Ub, ubiquitin.
Germ-line mutations

Inactivation of the \textit{VHL} gene is believed to be responsible for the development of both sporadic and familial cases of CHB (Figure 3, table 2) (Choyke et al. 1997; Fearon 1997; Goldsmith and Thomas 1999; Maher et al. 1991). Most individuals with the disorder inherit a germ-line mutation of the gene from an affected parent and a normal (wild type) gene from the unaffected parent (Latif et al. 1993; Maher et al. 1990b; Neumann and Wiestler 1991). A germ-line mutation of the \textit{VHL} gene is present in all the cells of the affected individuals who inherit the genetic trait. However, only those cells that undergo a deletion or mutation of the remaining wild type allele in susceptible target organs develop tumours (Russell and Theis 2003). The first hit (germ-line mutation) is typically a localised intragenic mutation (e.g., point mutation, microdeletion, etc.) and the second hit is a somatic DNA alteration which is acquired during the patient’s lifetime and is present only in tumour tissue. Such secondary events are frequently large deletions or mitotic recombination events resulting in LOH. (Gläsker et al. 2001; Kaelin 2004). So far germ-line mutations of the \textit{VHL} gene have been reported in >500 VHL kindreds and the success for detecting a Germ-line mutation in cases expected to carry one has been 100% (Corless et al. 1997; Crossey et al. 1993; Maher 2004; Tse et al. 1997; Zbar et al. 1996; Zbar et al. 1999). The improvement in detection of germ-line \textit{VHL} mutations is related to the use of quantitative Southern blotting and fluorescence \textit{in situ} hybridization. Though also direct sequencing, LOH and hypermethylation studies have been conducted (Herman et al. 1994; Stolle et al. 1998). Currently close to 800 different \textit{VHL} germ-line mutations of different types are known (Beroud et al. 2000).

\textit{VHL} mutations are extremely heterogeneous in both their type and position, and are distributed widely throughout the coding sequence, sparing the first 54 and the last 13 codons. Germ-line \textit{VHL} mutations may be divided into three groups (Table 3), (i) large deletions (including deletions encompassing the entire gene), detectable in about 25 - 40% of patients, (ii) intragenic missense mutations in about 30% of the patients, and (iii) microdeletions (1 - 18 bp) and other protein truncating mutations (nonsense, frame shift insertions of 1 -8 bp, splice site mutations) detected in approximately 30% of cases (Frederik et al. 2003; Maher 2004; Richard et al. 1994; Friedrich et al. 2001).

By studying different families from different continents and countries (North America, Europe and Japan) it was possible to detect a common germ-line mutation and to compare the effects of identical germ-line mutations in different populations. Codon 167 mutations were present in VHL families of all nationalities tested. These mutations produced similar phenotypes in Caucasian and Japanese families (Zbar et al. 1996). Missense mutations at nucleotide 712 (codon 238) represent a mutational hot spot found in different unrelated families and mutations at this site are associated with a high risk of PCC (Chen et al. 1995). Another "hot-spot" site identified in the German study is a mutation at nucleotide 505T>C (the Black Forest - founder mutation), that is associated with a high risk for PCC but without RCC (Brauch et al. 1995a). However not all missense mutations predispose to PCCs; according to a Japanese study, mutations found in 22 VHL patients without PCC consisted of 11 missense mutations (Clinical Research Group for VHL in Japan, 1995).

Although most index cases with VHL disease have a positive family history of VHL, some do not and may represent de novo cases. Cases without a family history of VHL may or may not have a germ-line mutation in the VHL tumour-suppressor gene. Patients diagnosed without a family history of the disease have been reported in as many as 23% of the kindreds with VHL.
First-generation diagnoses may result from a new mutation occurring during oogenesis or spermatogenesis in the parent. Another possibility is that the seemingly unaffected parent is mosaic for the disease. Mosaicism is defined as the presence in an individual of at least two cell lines differing in genotype and arising from a single zygote (Austin and Hall 1992). Depending on the stage of development and the cell in which the mutation occurred, the individual may be unaffected or may suffer a clinical disease (Zlotogora 1998). The finding of mosaicism has important implications both in counselling family members and in risk assessment for disease development in the individual with VHL mosaicism (Sgambati et al. 2000; Zlotogora 1998).

**Figure 3.** Functional domains of the VHL protein and distribution of germ-line mutations (from VHL database). Hotspots for mutations are readily visible at amino acids 67–117 HIF binding domain and 157–170 [Elongin C (EloC) binding domain]. (Modified from Richard 2002).

**Somatic mutations**

In sporadic tumours, tumourigenesis is thought to be initiated by a somatic alteration of both alleles of a TSG responsible for the corresponding hereditary tumour syndromes. As opposed to the situation in the heritable disease where the first hit occurs in the germ-line, in sporadic tumours, both the first and second hit occur somatically (i.e, after conception) (Gläsker et al. 2001; Kaelin 2004). Combining all the investigated data together, it has been possible to exhibit a mutation of the VHL gene in only 20% - 50% of sporadic tumours (Gläsker et al. 2001; Zbar et al. 1999). The intragenic distribution of somatic VHL mutations is similar to that of germ-line mutations, except that somatic mutations are relatively more frequent in exon 2 (Foster et al. 1994; Gnarra et al. 1994).

Somatic mutations of the VHL gene have been documented in sporadic CHBs in up to 30% cases (Brauch et al. 1995a; Kanno et al. 1994; Olschwang et al. 1998). LOH at the VHL gene locus has been detected in 52% of the stromal cell component of sporadic CHBs (Lee JY 1998). However, hypermethylation of VHL in sporadic CHB has not been detected to date (Kaelin 2004). Somatic VHL mutations and allele loss can be detected in up to 60% of clear cell RCC, and in most cases there is loss of the remaining VHL allele (Decker et al. 1997; Kondo and Kaelin 2001). Furthermore transcriptional
silencing by promoter hypermethylation occurs in RCC cell lines in 10% to 20% of sporadic primary clear cell RCC (Herman et al. 1994; Prowse et al. 1997). No differences were exhibited in the mutation frequency comparing early and advanced stages of RCC (Maher and Kaelin 1997). However somatic \textit{VHL} mutations are uncommon in sporadic PCCs, less than 8% of cases display a \textit{VHL} mutation (Eng et al. 1995; Kanno et al. 1994; Koch et al. 2002b). The mutations that have been detected are missense mutations, suggesting that specific mutations are required to instigate PCC, analogous to the missense mutations predominantly associated with the development of PCC \textit{VHL} in type 2 disease.
**Table 2.** Summary of genetic alterations of VHL-associated tumours, with gene inactivation by epigenetic mechanism also indicated.

<table>
<thead>
<tr>
<th>Author</th>
<th>No of samples</th>
<th>Mutations %</th>
<th>Methods used</th>
<th>LOH %</th>
<th>Methylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crosse et al. 1994</td>
<td>94/-</td>
<td>59/-</td>
<td>SSCP/ Heteroduplex</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Herman et al. 1994</td>
<td>-/26</td>
<td>-/19</td>
<td>Methyl</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Kanno et al. 1994</td>
<td>-/13</td>
<td>-/20</td>
<td>SSCP/ Direct sequencing</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Brauch et al. 1995</td>
<td>-/62</td>
<td>-/3</td>
<td>SSCP</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Chen et al. 1995</td>
<td>114/-</td>
<td>75/-</td>
<td>SSCP/ Dideoxy sequencing</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Clinical Research Group for VHL in Japan 1995</td>
<td>45/-</td>
<td>57/-</td>
<td>SSCP/ Southern blotting</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Oberstrass et al. 1996</td>
<td>-/20</td>
<td>10/40</td>
<td>SSCP/ Heteroduplex/Direct sequencing</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Zbar et al. 1996</td>
<td>469/-</td>
<td>63/-</td>
<td>SSCP/ DGGE/ Dideoxy sequencing</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Prowse et al. 1997</td>
<td>-/15</td>
<td>-/46</td>
<td>SSCP/ Direct sequencing</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tse et al 1997</td>
<td>3/5</td>
<td>100/40</td>
<td>SSCP/ Direct sequencing</td>
<td>66/40</td>
<td>NI</td>
</tr>
<tr>
<td>Lee et al. 1998</td>
<td>-/20</td>
<td>-/52</td>
<td>SSCP/ Direct sequencing</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Olschwang et al. 1998</td>
<td>92/18</td>
<td>67/11</td>
<td>DGGE/ Direct sequencing/Southern blotting</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Stolle et al. 1998</td>
<td>93/-</td>
<td>100/-</td>
<td>Quantitative Southern blotting/ FISH/ Direct sequencing</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Webster et al. 1999</td>
<td>183/-</td>
<td>88/-</td>
<td>SSCP/ Southern blotting/Direct sequencing</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Niemelä et al. 2000</td>
<td>8/21</td>
<td>75/-</td>
<td>Direct sequencing</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Yoshida et al. 2000</td>
<td>-/240</td>
<td>-/50</td>
<td>SSCP/ Direct sequencing/Southern blotting/Methyl/LOH</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Gläsker et al. 2001</td>
<td>29/13</td>
<td>94/23</td>
<td>SSCP/ LOH/Southern blotting</td>
<td>62/50</td>
<td></td>
</tr>
<tr>
<td>Gijtenbeek et al. 2002</td>
<td>7/16</td>
<td>86/31</td>
<td>Direct sequencing</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

SSCP = single strand confirmation polymorphism; DGGE = denaturing gradient gel electrophoresis; FISH = fluorescent *in situ* hybridization; LOH = loss of heterozygosity; Methyl = Gene promoter methylation analysis; - = not included or studied; + = detected; NI = no information.
**Genotype - phenotype relations**

As many as 50% of patients in VHL families may exhibit only one manifestation of the syndrome (Maher et al. 1991; Neumann and Wiestler 1991). Some individuals in the same family may present with a life-threatening disease with multiple tumours and suffer a considerable reduction of lifespan, whereas other patients have only few manifestations of the disease with no impairment of life (Catapano et al. 2005; Lamiell et al. 1989). Several studies have been performed to determine the nature and distribution of the mutations responsible for VHL disease, furthermore to correlate the mutations with neoplastic manifestations, and clear genotype-phenotype correlations have emerged.

Interfamilial differences in the presence of PCC are well recognized in VHL disease. The frequency of different mutation classes has been compared between kindreds with and without PCCs, and the type of VHL mutation appears to correlate with the clinical manifestations of the disease. Missense mutations are more frequent among families affected by PCC than in those families in which PCC has not appeared (Friedrich 2001).

Thus, studies have classified VHL families into two types based on the presence or absence of PCC, VHL type 1 without PCC, and VHL type 2 with PCC (Table 3). The types of mutations responsible for VHL type 1 differed from those responsible for VHL type 2. Micro-deletions/insertions, nonsense mutations or deletions, splice site mutations and mutations that lead to a complete loss of the VHL gene product are associated with VHL type 1. In contrast, most of the mutations identified for VHL type 2 were missense point mutations.

Mutations found in codon 238 account for most of the mutations identified for VHL type 2, and are strongly predictive of a predisposition to develop PCC (Chen et al. 1996; Linehan et al. 1995; Neumann and Wiestler 1991; Neumann and Bender 1998; Zbar 1995). However, not all missense mutations are associated with a high risk for PCCs only. Some PCC-associated missense mutations are also associated with a high risk for RCC. The intragenic nucleotide (nt)c. 712 C>T mutation is associated with a high risk of both PCC and RCC (Green et al. 1986). On the contrary, the nt 505 T>C mutation is associated with a high frequency of PCCs but not RCCs (Brauch et al. 1995a; Glenn et al. 1991). Brauch and co-workers also described a missense mutation at nt 505 as a founder effect in VHL families of the Black Forest region where there is a predominance of PCC (Brauch et al. 1995a; Brauch et al. 1995b).

VHL type 2 is further subdivided into type 2A, where patients develop PCC without RCC, and type 2B, where patients develop both PCC and RCC. Furthermore, it has been noted that some families with VHL disease have an increased risk of PCC without the other classical signs of VHL disease and this type is called 2C (Chen et al. 1996; Crossey et al. 1995; Neumann and Bender 1998; van der Harst et al. 2002) (Table 3). Recently it has been demonstrated that there is a higher prevalence of RCC in patients with partial germ-line VHL deletions relative to complete deletions (Maranchie et al. 2004). Gallou and co-workers proposed that mutations leading to truncated protein (MLTP) are more likely to promote renal tumourigenesis, and confirmed a key role for MLTP and large rearrangements in conferring genetic susceptibility to renal lesions in VHL patients (Gallou et al. 2004). Yoshida et al. also found MLTP to be a risk factor for developing RCC in a Japanese-Asian VHL population (Yoshida et al. 2000).

The detection of a mutation in a proband allows the identification of mutation carriers among family members who do not yet exhibit any clinical manifestation of VHL. The prognosis for the lifetime risk of PCC can be estimated by determination of the underlying mutation even if there is no family history of
VHL. Relatives may benefit from pre-symptomatic detection of elevated tumour susceptibility, followed by regular surveillance for tumour development. Relatives who do not inherit the mutation will be spared anxiety and the need for regular monitoring. The standard care is for patients or family members to receive genetic counselling before and after undergoing molecular testing.

Table 3. Characteristics of different types of VHL disease and types of mutations related to them (Brauch et al. 1995a; Chen et al. 1996; Hes et al. 2003; Neumann et al. 2002a; Zbar et al. 1996).

<table>
<thead>
<tr>
<th>Type of VHL disease</th>
<th>Mutation type</th>
<th>Clinical Manifestation¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 1</td>
<td>Microdeletion (1 -18bp), insertion (1-8 bp) Large deletion (entire gene), splice site and nonsense mutation</td>
<td>CHB, RCC but not PCC</td>
</tr>
<tr>
<td>Type 2A</td>
<td>Missense mutation (Amino acid substitution)</td>
<td>CHB, PCC but not RCC</td>
</tr>
<tr>
<td>Type 2B</td>
<td>Missense mutation (Amino acid substitution)</td>
<td>CHB, PCC and RCC</td>
</tr>
<tr>
<td>Type 2C</td>
<td>Missense mutation (Amino acid substitution )</td>
<td>PCC only</td>
</tr>
</tbody>
</table>

¹ CHB, Capillary hemangioblastoma; RCC, Renal cell carcinoma; PCC, Pheochromocytoma.

Other candidate tumour suppressor genes

In tumour suppression, genetic and epigenetic mechanisms may act in parallel; bi-allelic inactivation may occur via a combination of mutational and epigenetic events. With respect to the epigenetic mechanisms, promoter hypermethylation has been widely recognized as an alternative to genetic alterations as a way of disrupting gene function (Jones and Laird 1999; Jones and Baylin. 2002).

Previous studies have revealed loss or inactivation of the VHL gene in approximately 20% to 50% of sporadic CHBs (Kanno et al 1994; Lee et al 1998; Tse et al. 1997). A CGH-study showed DNA losses on 6q, in addition to those on 3p in sporadic CHBs (Sprenger et al 2001). Moreover in other VHL-associated tumours particularly in RCCs losses of 6q have been reported (Gronwald et al 1997, Bissing et al. 1999). These findings imply that chromosome 6q may harbour genes important in the development of VHL-associated tumours. To study this further, among the candidate genes located at 6q we selected ZAC1, THW and LATS genes, because these genes had been shown to be frequently deleted in many tumours. Moreover they possess a similar mechanism of inactivation with VHL, since hypermethylation is one of the inactivating mechanism for VHL-associated tumours.

ZAC1 was isolated by Abdollahi and co-workers (Abdollahi et al. 1997) in a search for expression of genes lost in an in vitro model of cell transformation. The ZAC1 gene encodes a zinc finger protein, and it
is an imprinted gene expressed only from the paternal allele with the maternal allele silenced by promoter region hypermethylation (Arima et al. 2005; Piras et al. 2000). Imprinted genes are expressed from one allele according to their parent of origin, and many such genes are essential in mammalian embryogenesis (Arima et al. 2005).

Functionally the ZAC1 gene product possesses the same characteristic as p53. ZAC1 exhibits tumour suppressor activity, and inhibits tumour cell proliferation through the induction of both apoptotic cell death and G1 arrest (Spengler et al. 1997; Varrault et al. 1998). Thus ZAC1 displays antiproliferative properties through pathways known to be central to the activity of p53. ZAC1 is located at 6q24-25, a chromosomal region which has been shown to be deleted in many solid tumours, such as ovarian cancer (Foulkes et al. 1993), breast cancer (Bilanges et al. 1999), melanoma (Millikin et al. 1991), and squamous cell carcinoma of head and neck (Koy 2004).

ZAC1 is expressed both in adult and fetal tissues in human pituitary gland, kidney, adrenal gland, uterus, ovary, mammary gland, liver, whole brain, and spinal cord. In the adult brain, the most intense signals were observed in the occipital lobe and cerebral cortex (Varrault et al. 1998). In mouse brain, high levels of Zac1 expression were determined early in brain development, but in cerebellum, levels of Zac1 mRNAs were maintained in Purkinje cells (Valente and Auladell 2001; Alam et al. 2005; Ciani et al. 2003; Valente et al. 2005). An alteration in the expression of the imprinted genes is one of the most common changes seen in cancers. Imprinted genes, including ZAC1, function as tumour suppressor genes suggesting a direct link between loss of imprinting, either by epigenetic changes or chromosomal deletion, and failure of tumour suppressor mechanisms. Hypermethylation of DNA for the 5’ regulatory region of ZAC1 was recently detected in several cancer cell lines and primary tumours, and this directly evoked loss of gene expression (Kamikihara et al. 2005).

Several putative tumour suppressor genes are located in the vicinity of 6q23-24, including the newly described THW (Human Transmembrane Protein) gene, which has been shown to be expressed in brain, kidney, liver, pancreas, adrenal glands, uterus and prostate (Hildebrandt et al. 2000). It is located between 6q16-23, and LOH of the THW gene has been detected in cell lines derived from melanoma, pancreas, breast, prostate, cervical and colon cancer (Hildebrandt et al. 2000; Miele et al. 2000). Recently (Attardi et al. 2000) reported identification of a murine homolog of the THW gene referred to as PERP. These two genes, THW and PERP share 90% homology in the amino acids in their coding regions. PERP was found to be expressed in a p53-dependent manner (Hildebrandt et al. 2001).

Another tumour suppressor gene located at 6q24-25 is the LATS1 (Large Tumour Suppressor1) gene, which has been mapped to chromosome 6q24-25. Loss of heterozygosity for LATS1 has been observed in ovarian, cervical, and breast cancers. Morinaga and co-workers did not detect any mutation in breast cancer samples in the LATS1 coding region, but pointed to the possibility that LATS1 may be inactivated by promoter methylation (Morinaga et al. 2000). LATS1 can suppress tumourigenesis by regulating cell proliferation and modulating cell survival (St John et al. 1992; Xia et al. 2002).
AIMS OF THE STUDY

Despite the major role of the VHL gene, a full understanding of mechanism of tumourigenesis of VHL-associated tumours is still lacking. The objective of this study was to search for genetic alterations, or other mechanisms in addition to VHL inactivation, which may be important for the development of these tumours. In particular, the focus was on identifying these events in the sporadic forms of capillary hemangioblastoma and pheochromocytoma.

Specific aims

With regard to the more detailed objectives, this study sought

- to assess the prevalence of VHL disease in patients suffering from CHB by performing detailed clinical and other necessary examinations, and further, to study the rate of germ-line mutations of the VHL gene.
- to characterize DNA copy number changes in addition to those at 3p25-26 which may be of importance in the development of CHBs by comparative genomic hybridization.
- to study further those chromosomal loci that were frequently affected by DNA copy number changes and therefore of potential importance in the tumourigenesis of CHBs. Moreover the aim was to identify candidate genes residing on these particular chromosomal sites.
- to investigate if the same chromosomal areas are affected in PCC.
- to study in detail the role of the ZAC1 gene, located in the chromosomal area identified in the previous studies.
MATERIALS AND METHODS

Patients and clinical data (I, II, III, IV, V)

In all, 110 CHB patients were collected from the files of the Department of Neurosurgery, Helsinki University Central Hospital (HUCH), fulfilling the main three criteria: primary operation for CHBs of the CNS between 1953 and 1993, histological verification of solid tumour, and CHB ascertained at histological re-examination by a neuropathologist. The studies were approved by the Ethics Committee of Helsinki University Central Hospital. These 110 CHB patients consisted of 50 men and 60 women. We collected all available data and examined records of all hospitals involved for evidence of VHL. Before the follow-up examination, a detailed inquiry asking about their illnesses, hospital admissions and operations was sent to patients. The follow-up time started at the date of the primary operation and ended at death (49/110 patients) or on August 31, 1998. In 61 of 66 living patients, a detailed clinical examination was performed in between 1991-1998 to search for signs of VHL disease. This included a family history of VHL, a physical, neurological and ophthalmologic examination including indirect ophthalmoscopy, enhanced MRI (1.0 T) of the head and upper cervical spine, and furthermore a CT of the upper abdomen was performed.

Detailed pedigree analysis was constructed for 35 CHB patients with their first and second degree relatives, by interviewing the patients or using the files of public registration center (PRC) and the parish records. These 35 patients with their 471 relatives were linked with the files of the Finnish cancer registry to search for VHL related neoplasms.

Mutation analysis of VHL

The VHL gene was analysed for germ-line mutations by direct sequencing from all living patients including 4 cases with clinically clear VHL, and 11 suspect patients, with CHB and internal organ cysts and 25 patients with apparently sporadic CHB. High molecular weight DNA was extracted from peripheral blood leucocytes using the QiA Amp Blood kit (QiAGEN Ltd., Cambridge UK). The three exons of the VHL gene were sequenced, including the exon splice sites. The primers and the PCR conditions used were as described in Chen et al. (1995) with slight modifications. We also used a reverse primer for the exon 1, SS1, 5' GCGGTAGAGGGCTTCAGACCGTG-3'. PCR products were purified with the QiA Quick PCR purification kit (Qiagen, Hilden, Germany), and the sequencing reactions were done using the ABI PRISM DYE Terminator sequencing kit (Perkin-Elmer Applied Biosystems Division, Foster City, CA), and were analyzed on the ABI 373 A sequencer. Both strands were sequenced.

These patients and the findings of this study served as the basis for further studies.
Comparative genomic hybridization (II)

All CHB specimens included in this study had been removed during surgical procedures conducted in the Department of Neurosurgery, University of Helsinki. In all cases, tumour samples had been fixed in 4% phosphate buffered formaldehyde immediately after removal, and transported to the Department of Pathology, University of Helsinki, where the tissue was processed by standard methods and finally embedded in paraffin. From each tumour the diagnosis had been set as part of routine diagnostic procedures, but all the cases were re-evaluated for the present study. Altogether we collected 22 tumour samples from 22 individuals, all were histologically typical CHBs, 18 were from the cerebellum and 4 from the medulla. Five of the 22 patients had VHL disease, with the diagnosis being based on mutation analysis and clinical characterization (1). Follow-up information was collected and in some patients new lesions had been observed. DNA was extracted from the paraffin-embedded sections as described elsewhere (Miller et al. 1998) with a slight modification. Genomic DNA from peripheral blood lymphocytes from a healthy donor was also extracted according to standard procedures and used as a reference in the CGH analysis. CGH was performed as described previously (el-Rifai et al. 1997; Kallioniemi et al. 1992; Kallioniemi et al. 1994).

Briefly, tumour and reference DNA were labeled differentially with fluorochrome-conjugated dCTP and dUTP (DuPont, Boston, MA, USA) and Texas-red-conjugated dCTP and dUTP (DuPont) by nick translation to obtain fragments ranging from 600 to 2000 base pairs. The hybridization mixture consisting of equal amounts of tumour and reference DNA was co-hybridized competitively to a normal metaphase slide prepared from a lymphocyte cell culture of a normal healthy individual. After hybridization and washes, the chromosomes were counterstained with DAPI (blue) and slides were mounted with an antifading medium. The hybridizations were analyzed using an Olympus fluorescence microscope (Olympus Optical Co. LTD., Japan) and the ISIS digital image analysis system (MetaSystems GmbH, Altlussheim, Germany) based on an integrated high-sensitivity monochrome charge coupled device camera and automated CGH analysis software. Three-color images (red for reference DNA, Green for tumour DNA, and blue for counterstaining) were acquired from 7 - 10 metaphases per sample. An increase in the DNA copy number was visualised by the intensity of green (hybridized tumour DNA), whereas a decrease appeared as a red colour (reference DNA). Chromosomal regions were interpreted as being over-represented when the corresponding ratio exceeded 1.17 (gains) or under-represented (losses) when the ratio was less than 0.85. Inverse labeling-CGH was performed (tumour DNA labeled with Texas-red-conjugated and reference DNA with FITC-conjugated dCTP and dUTP) to confirm the alteration. Imbalances were scored only when present in both hybridizations (CGH and inverse CGH). All findings were confirmed using a confidence interval of 99% with 1% error probability. CGH is able to detect losses up to 2 - 5 MB.
MATERIALS AND METHODS

Loss of heterozygosity in capillary hemangioblastoma and pheochromocytoma (III, IV, V)

Tissue samples for 15 capillary hemangioblastomas were collected from the files of the Department of Pathology, University of Helsinki (Study III). The set included 11 sporadic and 4 VHL- associated cases. Nine of the 15 tumours were part of both previous CGH and present LOH studies.

DNA was extracted from histologically characterized capillary hemangioblastomas, from which adjacent normal tissues were carefully removed, and from the corresponding peripheral blood samples using standard methods, proteinase-K digestion and phenol/chloroform purification followed by ethanol precipitation. Tumour and non-tumour DNA were amplified by the polymerase chain reaction (PCR), and LOH analysis was performed with 22 micro-satellite markers (Research Genetics, Inc, Huntsville, AL) covering chromosome 6q and 16 spanning 3p (III). The markers were selected to ensure a comprehensive representation of 6q and 3p. The names and the localization of the markers are based on the unified database (UDB) (http://bioinformatics.weizmann.ac.il/udb). PCR was carried out under the following conditions: 1x PCR buffer (10 mM Tris-HCl, 1.5 mM MgCl₂, 50 mM KCl, 0.1% Triton X-100); 200 mM dGTP, dATP and dTTP; 2 µM dCTP; 0.7 µCi of [³²P] dCTP (3000 Ci/mmol); 10 ng of each primer; 100 ng of genomic DNA template; and 0.5 units of Dynazyme II polymerase (Finnzymes, Espoo, Finland) in a volume of 10 µl. PCR amplification started with 5 min at 95°C, then 35 cycles for 1 min at 95°C, 1 min at 52-55°C, 1min at 72°C, followed by elongation for 8 min at 72°C. The amplified samples were cooled down quickly and stored at 4°C. Heat-denatured PCR products were subjected to electrophoresis in 6% polyacrylamide gel containing 7.7 M urea. After electrophoresis, the gels were transferred to 3mm Whatman paper, dried and then exposed by autoradiography to X-ray film.

All PCR amplifications with unclear results were repeated. LOH was scored when an allelic band was absent or clearly reduced in density compared to the corresponding band in normal DNA in repeated experiments. All of the 15 cases were informative for more than one marker studied (a marker was considered non-informative when the normal tissue of the patient was homozygous with respect to this marker) (III).

LOH was studied also in study IV where paraffin-embedded tumour tissue samples from 18 surgically removed PCCs, fixed in 10% phosphate-buffered formaldehyde were collected from the archives of the Department of Pathology, Helsinki University Central Hospital. The set included one VHL- associated and 17 sporadic tumours, based on family history and clinical findings (III). All were located in the adrenal gland. Nine of the PCC were benign, and another nine were borderline tumours, i.e. tumours without metastases but having any of the histologically suspicious features, such as more than 5 mitoses/10 high power fields, confluent tumour necrosis, vascular or capsular invasion (Salmenkivi et al. 2001). Sixteen of the cases were women, and two were men, with an age range of 26 – 72 years. For statistical comparisons, student's t-test (Microsoft Excel, Windows 2003) was used.

DNA was extracted from histologically well-characterized paraffin sections, after careful removal of adjacent normal tissue. Normal DNA was obtained from non-tumourous tissue samples, removed during the surgical procedures performed on these patients. DNA extraction from the tumour tissue and the corresponding normal tissue samples were performed by standard methods as in the previous LOH study on CHB (III). Tumour and non-tumour DNA were amplified by the polymerase chain reaction (PCR), and LOH analysis was performed with 22 micro-satellite markers (Research Genetics, Inc, Huntsville, AL).
covering chromosome 6q, the markers were selected to ensure a comprehensive representation of the 6q region, as described in detail in study III.

PCR conditions were as indicated above and elsewhere (III, IV). All PCR amplifications with unclear results were repeated. LOH was scored when an allele band showed 70%-100% reduction in signal density in the autoradiogram for one of the alleles of heterozygous tumour DNA, as compared to the corresponding band in normal DNA. Allelic imbalance (AI) was defined as an alteration constantly seen as a decrease in signal density or uneven signal intensity for a specific allelic band in the tumour DNA in repeated experiments. All of the cases were informative for more than one of the markers studied (a microsatellite marker was considered non-informative when the patient was homozygous with respect to this marker) (IV).

Study V included 8 sporadic and 2 VHL-associated cases as judged/diagnosed by their positive family history and instrumental investigation results. Previously, both had been part of our LOH studies and showed LOH at VHL gene area at 3p25-26 (III, IV). Six of these tumours had also been included in our previous CGH and LOH studies. DNA was extracted from the tumours and the corresponding peripheral blood samples as described above and in the studies (III, IV).

Tumour and non-tumour DNA was amplified by polymerase chain reaction (PCR) and LOH analysis was performed with 6 microsatellite markers spanning the ZAC gene area, and we also included 3 markers at 6q23-24 which were also part of the previous study and had been reported as a minimal deleted area (III). The markers were selected to ensure a comprehensive representation of 6q23-25, including ZAC specific markers. The names and the localization of the markers are based on the database UDB (unified data base) as indicated above (III, IV). Similar PCR conditions as described were also used for this study. All PCR amplifications with unclear results were repeated. All of the 10 CHBs were informative for more than one marker studied (a marker was considered non-informative when the normal tissue of the patient was homozygous with respect to this marker) (V).

**Immunohistochemistry (V)**

An immunohistochemical analysis was performed to study ZAC expression in CHBs. Immunostaining of these tumour tissue sections was performed using the avidin-biotin-peroxide complex method (Ultra vision, Lab Vision Inc, Fermont, CA, USA). Antigen retrieval was performed by incubation of the slides in 10mM sodium citrate buffer (pH 6.0) for 10 minutes at 95 °C. The rabbit anti-human ZAC1 antiserum (H-253, Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) was used at a 1:100 dilution with overnight incubation. Normal rabbit serum served as a negative control. Sections from normal cerebellum, pituitary adenomas and breast carcinomas, all known to express ZAC1, were used as positive controls.

**Analysis of promoter methylation (V)**

Methylation-specific PCR (MSP); (Herman et al. 1996) was used for the analysis of the methylation pattern in the 5’ regulatory region of the imprinted ZAC1 gene. DNA (1 µg) from 10 primary CHB, and also white blood cells from the same patients and 2 specimens from normal brain tissues were exposed
to bisulphite modification as described previously (Jarmalaite et al. 2003). There was either 14 h exposure at 50°C with sodium metabisulfite (Sigma) for DNA extracted from paraffin-embedded tissues or 16 h from frozen specimens. Bisulphite-treated DNA was amplified by PCR with two sets of primers designed for methylated (M) and unmethylated (U) sequences of ZAC 1 gene 5’ region (GenBank accession number AL109755, nucleotides 305-439). PCR product was analysed in non-denaturing 7.5% polyacrylamide gels. The imprinted status of the gene was seen as an equivalent MSP product in both reactions with the primers for M and U sequences. The predominant presence of methylated DNA in MSP was recorded as a loss of an unmethylated allele. Bisulphite-modified DNA from healthy donors was used as a negative control and in vitro methylated DNA (SssI methylase, New England BioLabs) as a positive control for loss of unmethylated allele of ZAC 1. To verify the sensitivity of MSP analysis, DNA from cancer cell lines (MDA-MB-453 and SKOV-3) with an established degree of methylation in the region (Abdollahi et al. 2003) was used in our study. Water controls were always included. The specificity of MSP was tested by direct sequencing of the PCR product from M and U reactions with automated sequencer (Applied Biosystems) (V).
RESULTS

Clinical study and VHL mutation analysis (I)

All available clinical data gathered during the median follow-up time of 14 years (range 0.3 - 36 years) together with a detailed clinical examination 61/110 patients, family history 93/110 patients, detailed pedigree 35/110 patients, mutation analysis 40/110 patients, and linkage to the files of FCR (35 CHB patients and 471 relatives) made it possible to differentiate three patient groups, (a) 14 VHL patients (b) 13 patients with a single CHB and visceral cysts, but no other VHL manifestations and (c) 83 apparently sporadic CHBs. From the unrelated 14 VHL patients discovered, six (43%) had a family history of VHL and eight (57%) apparently had a new mutation. Ten of them had multiple CHBs of the CNS, while four had a single CHB only (I)

In 11 of the 14 VHL patients, CHBs of the CNS were the first manifestation of the disease. After the primary operation 12 of the 14 VHL patients survived at least two years, nine of them died at a median age of 47 (range 26 - 66) years. RCC was a major cause of mortality, where five died of RCC at a median age of 49 (range 26 - 66) (I). The linkage of CHB patients and their relatives to the FCR files to identify VHL-related tumours did not reveal new families. Our analysis revealed a germ-line missense mutation in exon one (c. 278 G>C and c. 293 A>G) in two of the four VHL patients studied, but none of the other 36 patients (I).

Comparative genomic hybridization in capillary hemangioblastoma (II)

Of the 22 CHBs studied, chromosomal imbalances were revealed in six tumour samples (5 cerebellar and 1 medullary) by CGH (CGH profiles, Figure 4). Of these, five occurred in sporadic tumours and one in a tumour from a VHL patient, whereas no aberrations were detected in the remaining 16 tumours. The most frequent change was a loss on chromosome 6 seen in five of the 22 (23%) cases, with a minimal overlapping region at 6q. Additionally losses on chromosome 3 were seen in two of the cases with the concomitant loss of 6q. In one case, loss on chromosome 8 was detected. No gains were detected in this study (Figure 5).
RESULTS

Figure 4. Comparative genomic hybridization profiles of the cases with losses on chromosome 6. The centre line is the baseline ratio (1.0) and those to the left and right indicate the cut-off values (0.85 and 1.17). (II)

Figure 5. Summary of DNA copy number changes detected by comparative genomic hybridization in 22 capillary hemangioblastomas. Losses are marked on the left side of the chromosome diagrams. Each line illustrates the affected region of the chromosome in a single sample.

Loss of heterozygosity in capillary hemangioblastoma and pheochromocytoma (III, IV, V)

Altogether 15 cases of capillary hemangioblastomas were investigated for LOH in the 6q and 3p regions with a comprehensive set of microsatellite markers. LOH was observed with at least one marker in all 15 cases.

Allelic losses of 6q were detected in 11 of the 15 tumours (III). Nine of these 11 cases were sporadic tumours, with the other two being VHL-associated. In the sporadic tumours, the prevalence of 6q LOH was thus 83%. In the VHL-associated tumours, 6q LOH was seen in 2 out of the 4 tumours included in the study. Four tumours (T3, T6, T7, and T8) had displayed DNA loss on the long arm of chromosome 6 in the previous CGH study (II); in 3 of those, 6q LOH was also detected. The result allowed us to define a minimal deleted region that was located between the markers D6S250 and D6S1705. Nine (82%) of the
informative cases showed LOH with at least one of the markers (D6S250, D6S1703 or D6S1705), (Figure 6). These markers spanned a 3 Mb region on 6q23-24 (III).

With microsatellite markers for 3p, LOH was found in 14 of the 15 CHB cases (III), (Figure 6). The only case in which 3p LOH could not be detected was a VHL-associated tumour, in which LOH on 6q was demonstrated with several markers. Allelic losses on 3p were detected in several different regions. LOH on 3p was detected also in both tumours (T3 and T6) in which 3p and 6q loss had previously been shown by CGH (II). Altogether, all 5 tumours negative in the CGH analysis were positive in the LOH analysis. Moreover, two tumours negative in the CGH analysis (T9 and T10) displayed LOH concomitantly at 6q and 3p. Four tumours (2 VHL-associated and 2 sporadic) showed LOH only at 3p, and one VHL-associated tumour at 6q only. Concomitant LOH of 6q and 3p was detected on 10 of the 15 cases. These 10 cases with LOH detected on both sites included nine sporadic tumours and one VHL-associated tumour (III).

![Figure 6](image)

**Figure 6.** Illustration of LOH detected with the microsatellite markers D6S1698, 6q, D3S1038, 3p in two tumours (T) as compared to (B) peripheral blood or normal tissue in CHB. The horizontal lines indicate the alleles, and the arrows show the location of the missing alleles (III).

In study IV, a total of 18 cases of PCC were investigated for LOH at 6q with a comprehensive set of microsatellite markers. In particular, we focused on the region 6q23-24, using a total of nine markers. In the first set of analyses, allele loss of 6q was detected with at least one microsatellite marker in 12 of the 18 tumours. All these 12 cases were sporadic PCCs, and the only VHL-associated tumour did not show LOH. Thus, for the sporadic cases, the prevalence of 6q LOH was somewhat higher 12/17.

For benign PCCs, LOH at 6q was observed in six out of nine cases when the allelic imbalance for the ZAC 1 specific markers in one tumour (T14) was taken into account. From the tumours classified as borderline, 7/9 exhibited LOH (IV).

Previously, we found a minimal deleted region between markers D6S250 and D6S1705 in CHB (III). In study IV, 6/18 of the PCCs showed LOH or AI (allelic imbalance) with at least one of the markers for that region (D6S250, D6S1703 or D6S1705, D6S978) spanning a 3 Mb stretch on 6q23-24. Another frequently affected region was 6q14, where 9/18 tumours showed LOH or AI with at least one of the markers studied (D6S1275, D6S1557, D6S280). Four tumours showed LOH in both of these two regions (IV).
RESULTS

We further focused the LOH search using six microsatellite markers specific for the ZAC1 gene, one of the tumour suppressor genes (TSGs) located in 6q23-24 region. For those markers, four of the cases showed LOH, and two AI. Summing the results obtained with all markers for 6q23-24 region, LOH or AI was observed in 9/18 of the tumours in this series of PCCs. In addition there were two other regions that showed frequent LOH (39%), i.e. 6q12 and 6q21-22. All in all, taking into account the whole set of current LOH data, the results revealed that 13 of the PCCs showed allele loss of 6q (IV).

In study V, the LOH analysis of a series of 10 CHBs carried out with the microsatellite markers D6S308, D6S310, D6S311, D6S978, D6S314, D6S409 (6q23-25) indicated a high overall prevalence of LOH at the ZAC1 gene region in CHBs 6/10. Five out of 8 sporadic tumours investigated and one of the two VHL-associated ones exhibited allele loss in the ZAC1 region (V).

Table 4 summarizes the LOH data in combination with our previous CHG results on 6q alterations in the same CHBs (II, III, IV). With respect to the tumours negative in the present analysis for ZAC1 LOH, three (T3, T8, T12) exhibited 6q alterations in the CGH analysis (II), with T3 and T8 showing 6q loss (III). In contrast, tumour T14, a familial case, remained negative as had been the case in the earlier analyses. In summary, according to our combined data, we identified 6q alterations in all of the sporadic CHBs and in one of the two VHL-associated cases; i.e., altogether, 6q was affected in 10/11 of the tumours investigated (V).

**Immunohistochemistry for ZAC 1 (V)**

A reduced expression of ZAC1 was observed by immunohistochemistry in stromal cells of all CHBs studied IV. In contrast, endothelial cells of the vessels showed a clear positive staining reaction. ZAC1 was clearly expressed in Purkinje cells in cerebellar tissue adjacent to the tumour, a finding not previously reported for human brain tissue. In other cerebellar tissue, ZAC1 expression was weak but still recognizable compared to the tumour tissue. A clear positive staining was seen within the tumour cells of meningiomas, whereas the gliomas exhibited variable ZAC1 expression. Positive signal was also seen in all high grade (glioblastoma) tumour cells. The staining varied in low grade tumours, with no positivity detected in 2 low grade astrocytomas. In general, the IHC results for ZAC1 expression indicated a strongly reduced expression in all 6/6 of the CHB tumours analyzed, which is in accordance with the results from the LOH and methylation analyses (V).

**Promoter methylation analysis of ZAC 1 gene (V)**

To determine which allele (i.e. the maternal, imprinted one or the paternal, expressed one) was affected by the allele losses found in CHB (V), the methylation status of the 5' region of the ZAC1 gene was investigated using MSP assay. Concurrently to the imprinted status of the gene, MSP analysis detected both methylated and unmethylated sequences (50% - 50%) in leukocyte DNA from healthy controls and from patients with CHB. In contrast, a predominance of the methylated, i.e. imprinted, ZAC1 allele was detected in the majority of the CHBs 9 out of 10 tumours. For these tumours, the MSP amplification product from the U allele was negligible; it was incomparably weaker than that from leukocyte DNA,
indicating preferential loss of the transcribed allele in CHB tumours. Two specimens of normal DNA extracted from cerebellar tissue were also analysed for methylation status and both M and U alleles of the ZAC promoter region were detected in one of the cerebellar tissue samples, while no reproducible results were obtained from the other sample (V).

In parallel, the methylation status for ZAC 5’ promoter region in four primary glioma cell lines was also analysed. One of the glioma cell line exhibited both M and U alleles (i.e., the regular imprinting status), whereas only the U (transcribed) DNA sequence was present in two cell lines. The fourth cell line showed the M allele only. In CHBs, an absence of the U sequence for the ZAC1 promoter region was found to occur concurrently with ZAC allele loss 6/8 (V). When the 6q DNA losses identified in the CGH study (II) were taken into account, almost all tumours showed this concurrency of allele loss and absence of an unmethylated ZAC sequence 9/10. The exception was tumour T12, in which no deviation from the 50-50% wild type balance was observed with MSP, and furthermore no LOH was detected in ZAC1 region (V).
**Table 4.** Summary of LOH data in CHB with six microsatellite markers specific for the ZAC1 gene, previous LOH data on 6q23-24, data on promoter methylation of ZAC gene by methylation specific PCR and immunohistochemical (IHC) staining of ZAC protein expression in capillary hemangioblastomas (V), also includes data from studies II and III.

<table>
<thead>
<tr>
<th>Cases</th>
<th>Allele Loss at 6q</th>
<th>Loss of unmethylated ZAC allele</th>
<th>IHC staining</th>
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<tr>
<td></td>
<td>LOH on ZAC markers⁷</td>
<td>Previous 6q Loss data²</td>
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<tr>
<td><strong>VHL-associated</strong></td>
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<td>T13</td>
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na, not analysed (no DNA or tissue available)

■ Positive result
□ Negative result
● Negative for ZAC expression by IHC

¹ Data from study (V)
² Data from study (II, III)
Since the characterization of VHL disease and, later, after the discovery of the VHL gene, many investigators have investigated cytogenetic changes, germ-line and somatic mutation, gene promoter hypermethylation and LOH in order to determine the mutation rate, the loss or inactivation of the gene in VHL-associated tumours both in familial and sporadic cases. Moreover, dozens of studies revealed the functional properties of the VHL protein which helped in understanding the molecular mechanism of the development not only of VHL-associated tumours, but also how tumours grow in general.

In accordance with the Knudson two-hit model (Knudson 1971), biallelic VHL inactivation is a common tumourigenic mechanism in the development in both sporadic and familial cases of these tumours. Individuals with VHL disease carry one wild-type and one inactivated VHL allele. Germ-line inactivation of the VHL gene initiates VHL hereditary cancer syndromes, and somatic mutations of this gene have also been linked to the development of both familial and sporadic tumours (Kaelin 2004). In this thesis work, VHL-associated tumours, particularly capillary hemangioblastomas (CHB) and pheochromocytomas (PCC) were investigated using an array of various molecular biological techniques including direct sequencing of the VHL gene, CGH, LOH, promoter hypermethylation and IHC in an attempt to characterize the mechanism involved in the development of these tumours.

VHL disease and prognosis of hemangioblastomas

Melmon and Rosen proposed the clinical diagnostic criteria for VHL disease (Maher et al. 1991; Melmon and Rosen 1964; Neumann 1987). In accordance with the classical clinical definition of VHL, our results (I) revealed three patient groups (i) definite VHL (14) cases, where a patient has a family history or a germ-line mutation of VHL and has developed CHBs of CNS or retina or has suffered other VHL-related neoplasms; and (ii) suspect VHL (13) cases, patients with no family history who developed one or more CHB of the CNS or retina and visceral cysts. The rest, the majority, with a single CHB were classified as sporadic cases (83 cases) several studies have revealed that the sites of CHBs differ between VHL and non-VHL patients (Filling-Katz et al. 1991; Julow et al. 1994; Maher et al. 1990a; Resche et al. 1993). In our study, about half of the 14 VHL patients developed CHBs at brain stem or spinal sites, whereas only 5% and 4% of the non-VHL patients had a single CHB in either of these two locations CHB (I).

The average life-expectancy of VHL patients has been reported to be 40 - 50 years (Maher et al. 1996) and there is data that RCC is the leading cause of death of VHL patients; in our series after the primary operation, 12 of the 14 VHL patients survived at least two years, nine of them died at a median age of 47 (range 26 - 66) years. In our patients, RCC was noted to be the major cause of mortality, five died of RCC at median of 49 (range 26 - 66) years (I). Inactivation of the VHL gene has been reported to cause tumour and tumour-like lesions in more than five different organs and systems; however, organ involvement can vary considerably among families and has been shown to correlate with the underlying germ-line alteration (Russell and Theis 2003).

In our mutation analysis, we found a germ-line mutation in exon one in two of the four VHL patients studied, but in none of the other 36 patients (I). We used a direct sequencing method which is known to detect about 75% of VHL mutations (Brauch et al. 1995b; Zbar et al. 1996), both mutations were
missense mutations known to be associated with PCCs (Chen et al. 1996), which were identified in the family of one VHL patient. However, clinically we observed retinal CHBs in eight patients (5/8 bilateral), RCC in 10 patients which in six cases were bilateral, and PCCs in two patients (one bilateral), visceral cysts in a minority of cases (I). In accordance with our result, (Rasmussen et al. 2006) used direct sequencing to identify the germ-line mutation in 5/6 of definite VHL cases but none in the possible (suspect) VHL and sporadic cases.

Recently, by using more sensitive methods, it was possible to detect a 100% inactivation rate in familial cases of CHB (Zbar et al. 1999). However, using those same methods, only up to 20 - 50% of sporadic CHBs were shown to be inactivated in the VHL gene area (Gläsker et al. 2001). These results warrant further study, and hopefully will encourage other research teams to seek for other chromosomal sites which may be of interest in the development of sporadic cases in particular and CHBs in general.

**DNA copy number changes in capillary hemangioblastoma and VHL-associated tumours**

To seek and identify potentially new chromosomal areas that may be important in helping to understand the molecular mechanism behind the development of these tumours a CGH study was performed. The study revealed chromosomal imbalances in 6 tumour samples; no aberrations could be detected in 16 tumours. The most frequently detected loss was the loss on chromosomal arm 6q, seen in 5 (23%) of the 22 CHBs. Among those 6 cases with chromosomal imbalance, 5 were classified as sporadic and one was a VHL patient (II). The study also revealed losses of chromosome 3 in two of the cases, but concomitantly with the loss of 6q (II). When i started this work 6q losses was not known to be related with CHBs, but, later in accordance with our results, in the study by Sprenger where specifically sporadic CHBs were investigated by CGH, a high rate (70%) of chromosome 3 loss was found, but frequent chromosomal aberrations including those on chromosome 6 (50%), were also detected (Sprenger et al. 2001). Additionally Gijtenbeek studied sixteen sporadic and seven familial CHBs where loss of chromosome 3 in 69% and chromosome 6 losses were identified in 44% of the tumours studied (Gijtenbeek et al. 2002).

Interestingly, other VHL-associated tumours have also been revealed losses on chromosomal arm 6q. Dannenberg studied 29 PCCs by CGH and reported among several other changes that there was 34% 6q loss in 34 % of of cases. Similarly, they reported loss of 3p in 31% of the tumours (Dannenberg et al. 2000). Other VHL-associated tumours, particularly RCCs, have also been studied by CGH and by other cytogenetic studies; where losses of 6q have been reported in 14–28% of the cases (Bissig et al. 1999; Gronwald et al. 1997; Maloney et al. 1991). However loss of 6q is not explicitly related only to VHL-associated tumours, loss of 6q has also been reported in several benign and malignant CNS tumours, including meningioma (Khan et al. 1998), ependymoma (Readon et al. 1999), glioblastoma (Kim et al. 1995) and some other gliomas (Kros et al. 1999).

In our series, it was observed that loss of 6q did not affect the clinical behaviour of the tumour (II). A new CHB developed only in one of the patient with a tumour harbouring a 6q loss, and this patient had VHL disease. The other 4 cases with loss of 6q were sporadic tumours, and no recurrences have occurred in these patients during the follow-up period. It may be too early to draw any firm
conclusion, however, according to the findings of this study, loss of 6q in CHB does not appear to be an indicator of an aggressive phenotype. Instead, it seems to point to an inactivation of a so-far unidentified gene located at 6q that may be involved in the tumourigenesis of VHL-associated tumours.

Additionally, we found chromosome 3 loss in two of the 22 tumours (II), CGH detects losses in a range of 2 - 5 Mb, thus it may not sensitive enough to detect small losses (Kallioniemi et al. 1992; Kallioniemi et al. 1994). The VHL tumour suppressor gene has been mapped at 3p25—p26, and the initiation of tumourigenesis is believed to occur as a result of mutational inactivation of both alleles of the VHL gene. Interestingly, different previous studies related to sporadic CHB showed loss or inactivation of the VHL gene up to 27 - 52% (Bender et al. 2000; Kanno et al. 1994; Oberstrass et al. 1996; Prowse et al. 1997; Lee et al. 1998) also suggesting the existence of other inactivating or tumourigenic mechanisms. Thus, our result provides support for the belief that some other chromosomal regions, probably 6q, are involved in the tumourigenesis of CHBs.

This new insight is in accordance with the growing new knowledge and hypothesis for the development of VHL-associated sporadic tumours. Since inactivation of VHL gene predisposed patients to CHBs, clear cell RCC and PCCs, a close relationship between the tumourigenesis of these tumours is to be expected. The brief summary of the frequencies of aberrations detected in these three tumour types, as indicated by the above findings from recent CGH and other studies highlights the importance of both chromosome 3 and 6 losses, and suggests that the three VHL-associated tumour types may possess a similar pathway of tumourigenesis.

Loss of heterozygosity in capillary hemangioblastoma and pheochromocytoma

In the studies following the CGH findings, a series of LOH studies were performed in both CHBs and PCCs separately. Detection of allelic loss on a specific chromosomal region in a given tumour type is one important way to discover regions of TSG important for tumour pathogenesis. The study of 15 CHBs for LOH on chromosome 6q and 3p (III), revealed LOH at 6q in 73% of the cases. From these tumours, the majority (82%) occurred in sporadic cases, in VHL cases, the frequency was 50% (III).

As the studies of Morita revealed on RCC (Morita et al. 1991), we also observed LOH over large regions of the chromosomal arm of 3p, At 3p LOH was detected in 93% of tumours and 67% of the cases showed 6q and 3p losses concurrently (III). As in the CHBs, the study of the PCC (IV) also revealed LOH on 13/18 (72%) of PCCs examined, including tumours classified as benign or borderline, showing allele loss at 6q (IV). Similar to the situation with the CHBs, which were characterised by a minimal deleted region at 6q 23 - 24, it was also able to characterize two commonly affected regions at 6q in PCC. One of them was located at 6q14, where (9/18) tumours displayed LOH or allelic imbalances, and another at 6q23-24 where, when taking also the ZAC1 specific markers into account, 9 out of 18 PCCs showed LOH or AI.

As described above, two genetic events or "hits" are thought to occur to the two alleles of the TSG; the first hit being a germ-line mutation, and the "second hit" usually a deletion in the somatic cells (Knudson 1971; Knudson 1995), and the impact of the size of this deletion is thought to be critical since
other TSGs are located nearby. Following the two hit theory, Gläsker et al. (Gläsker 2005) mapped the deletion size of the predicted somatic deletion of the "second hit" from 16 different VHL-associated lesions taken from the same patient. It was claimed that the deletion size was highly variable, ranging from short deletions around the \textit{VHL} gene to a complete loss of chromosome 3. The size of the somatic deletion did not correlate with the site of the germ-line mutation, the affected organ, or the type or biological behavior of the tumour, even preneoplastic cystic structures may harbor entire loss of chromosome 3, suggesting that loss of \textit{VHL} gene function alone was not immediately causative for neoplastic growth (Gläsker 2005).

In this study, concomitant LOH on 6q and 3p was observed in the majority (67%) of the CHBs (III). This observation was even more common in sporadic CHBs, where 82% of the sporadic CHBs displayed LOH on both 3p and 6q. This suggests that tumourgenesis in most CHBs and in the sporadic cases in particular, may be dependent on the inactivation of genes located in both 6q and 3p. In support of these results, Gijtenbeek et al. (2002) reported their findings suggesting that the molecular mechanisms underlying sporadic and familial hemangioblastomas may be different. In their study, 3p loss was more common in sporadic CHBs than previously reported, pointing to a role in the tumourgenesis of sporadic tumours. Thus, our CGH and LOH results combined with the above mentioned studies, suggest that 3p in addition to 6q is a characteristic site of allelic loss in CHBs.

As indicated above, in addition to CHBs, loss of 6q has also been observed in most other tumour types occurring in VHL disease, including sporadic and VHL-associated RCC (Alimov et al. 2000; Bissig et al. 1999; Morita et al. 1991; Thrash-Bingham et al. 1995), PCCs (Dannenberg et al. 2000), endocrine pancreatic tumours (Barghorn et al. 2001) where losses of 6q are involved in the malignant progression of sporadic endocrine pancreatic tumours, as well as in other tumour types (Acevedo et al. 2002; Hansen et al. 2002; Miyakawa et al. 2000). However, since CHBs are classified as benign tumours (Gläsker 2005), these findings in our CHB series may suggest that 6q allelic losses are early events in tumour development, and accordingly no differences in clinical behaviour would be expected to be associated with LOH on 6q (III). Moreover, three of the four VHL-associated tumours were located in the medulla, and in one of these LOH of 6q was detected, showing that allelic losses at 6q are not specific to cerebellar CHBs (III). Some work, (Theile et al. 1996; Wan et al. 1999) has provided convincing evidence for the importance of 6q in the process of tumourigenicity, i.e. the transfer of a normal chromosome 6 into breast and ovarian carcinoma cell lines suppressed their tumourigenic potential. Similar to our study, these above authors analyzed the cell lines for the occurrence of LOH, and they indicated the region 6q23-25 as the area for a putative TSG.

Interestingly, some studies not only showed 6q LOH in general, but also a minimal deleted region at 6q14 and 6q23-24, suggesting that the genes located in this area are likely to contribute to the pathogenesis and malignant behavior of these tumours (Barghorn et al. 2001; Foulkes et al. 1993a). In accordance with these findings, we also observed minimal deleted regions both at 6q14 and 6q23-24 in our series of PCC tumours (IV). Moreover, Tibletti et al. showed 6q abnormalities in benign, borderline, and malignant tumours of ovarian surface epithelium. The authors suggest that, at least in part of the cases, some of the borderline tumours may have arisen from benign tumours, and, again, some malignant cases may have evolved from tumours of borderline malignancy (Tibletti et al. 2001). They further suggested that the genes located on 6q may play a role in the early events of tumourigenesis, which is in good agreement with our findings in CHBs (III).
In agreement with the result of the above study, we found a slightly higher frequency (7/9) of allele loss at 6q in borderline in comparison with benign (6/9) PCC tumours (IV). In addition, the LOH positive tumours tended to be somewhat larger in size, even though, no clinical differences were observed (IV), similar to the results of ovarian tumours (Tibiletti et al. 2001). In summary, these various sets of data indicate that these regions may contain one or more of the TSGs involved in the tumourigenesis of PCCs as has been proposed for some other tumour types. Nevertheless, it remains an open question whether the same gene(s) may be involved in each tumour type.

Loss of heterozygosity, immunohistochemistry and promoter hypermethylation at ZAC1 gene

In the above investigations, a series of studies on VHL-associated tumours, in particular CHBs and PCCs were carried out (I - IV). These studies revealed frequent genetic alterations on chromosome 6q including the candidate TSG LOT-1 gene, also called ZAC1, located at 6q24 -25. Further, we focused our attention on clarifying the importance of the functional loss of this suppressor gene in the tumourigenesis of CHBs.

Promoter hypermethylation of tumour suppressor genes is recognized as an alternative to genetic alterations in disrupting gene function (Belinsky 2004; Feinberg et al. 2006; Jones and Laird 1999). Hypermethylation of DNA in the 5' regulatory region of ZAC1 was also detected in several cancer cell lines and primary tumours, and it directly caused loss of gene expression (Cvetkovic et al. 2004; Kamikihara et al. 2005). In our study, a methylated ZAC1 promoter sequence was predominant in the tumours; i.e. we detected only the methylated sequence in the ZAC1 promoter in all but one (9/10) of the CHBs.

During tumour suppression, genetic and epigenetic mechanisms may act in parallel, (Jones and Laird 1999). In the case of an imprinted tumour suppressor gene such as ZAC1, one allele is not expressed due to imprinting, and therefore an alteration - genetic or epigenetic - of the other, expressed allele is sufficient to achieve gene inactivation. In accordance with such a situation, in our LOH study the majority (5/8) of the sporadic CHBs were positive for ZAC1 LOH, and one of the two familial cases also exhibited ZAC1 LOH (V). By combining data from our earlier studies (I - IV) on the same set of CHBs, we found that the rest of the studied 10 tumours either exhibited LOH at 6q23-24, or were shown to display 6q CGH loss, with the exception one tumour. Thus our result showed a predominance of the methylated promoter allele of ZAC1 in a series of capillary hemangioblastomas and concurrent LOH or 6q loss in all but one of the CHBs investigated (V).

Loss of expressed allele has also been reported in glioma, another CNS tumour. Selective loss (6/6 cases of LOH) of paternally expressed alleles on chromosome 19q has been described in oligodendrogliomas (Sanson et al. 2002). We included four glioma cell lines in our analysis of ZAC1 methylation. One of them showed loss of the unmethylated allele, two exhibited loss of imprinting with only the unmethylated ZAC1 promoter sequence being present, and one was a wild type with both types of ZAC1 alleles detected. In contrast to the situation with CHBs, in gliomas, ZAC1 alterations appeared to be random, possibly influenced by additional factors. Our findings suggest that ZAC1 has different functions in the pathogenesis of CHBs and gliomas (V).
Our IHC analysis detected limited expression of ZAC1 in adult brain tissues, and a weak expression was found in cerebellum but ZAC1 was strongly expressed in Purkinje cells (V). Accordingly, Varrault et al. reported weak expression of ZAC in different brain areas including cerebellum (Varrault et al. 1998). However, expression of Zac1 in Purkinje cells was observed in the brains of adult mice (Ciani et al. 2003). Interestingly, a similar pattern of expression has also been shown for VHL. Zac1 is abundant in Purkinje cells but, only a weak staining was observed in the molecular layer of the cerebellum (Maartje and Gerard 1996; Varrault et al. 2006). As previously indicated, loss of VHL functions leads to stabilization of HIF, and overproduction of HIF-inducible proteins, and this promotes tumour vascularisation (Maxwell 2005). Recently, significant HIF-1α-dependent suppression of Zac1 production was detected in mouse fibroblasts exposed to nickel or hypoxia, while in HIF-1α deficient cells, Zac1 expression was totally lost (Salnikow et al. 2003), which when combined with data from the present study may point to a possible involvement of ZAC1 in HIF-controlled cellular responses to hypoxia, or in the VHL/HIF-associated pathway to carcinogenesis. In summary, the results of the five studies included in this PhD thesis and other studies described above point to the involvement of not only one particular gene, VHL, but to roles for other TSGs in CHB and PCC, two VHL-associated tumours. This work points to the presence of other pathways that may play an important role in tumourigenesis of VHL-associated tumours, particularly in sporadic cases where there is limited or no VHL gene inactivation.
CONCLUSIONS

These studies were intended to clarify the mechanism of tumourigenesis of VHL-associated tumours. Initially, 120 consecutive patients operated for capillary hemangioblastoma were collected, and, after clinical and radiological examination, 14 definitely VHL patients, 13 suspect, and 93 apparently sporadic cases were identified. In VHL gene sequence analysis, we found a VHL germline mutation in two out of four familial cases, but none of the suspect or sporadic cases displayed any mutations. Our result and other reports on sporadic CHBs point to the involvement of other genes in the development of these tumours.

The screening of the whole chromosomal area of 22 sporadic and familial cases of CHBs by CGH revealed 27% DNA copy number losses. In the six cases with DNA copy number changes, five occurred in sporadic cases with the other being a VHL patient, in these patients, the most recurrent finding was loss of chromosomal arm 6q (23%). Apparently, loss of 6q may be important for the development of sporadic tumours, and may possibly also occur in some of familial cases.

A LOH analysis of 15 CHBs of both sporadic and familial origin revealed that a majority of the cases displayed 6q LOH; most of the cases were sporadic, only two of them were familial. Importantly this work characterized a minimal deleted region at 6q23 -24 apparently involved in CHB development. However, clinically no tumour-characteristic changes could be exhibited. This result provided a new insight into our understanding of the mechanism behind the development of these tumours, and opened a new direction for the further studies included in this work.

The search for other regions of importance not only revealed losses in CHBs but also in PCCs, where again a very high frequency of 6q LOH was discovered. Moreover, similar to our findings in CHBs, PCCs also exhibited a high frequency (50%) of LOH at 6q23-24 area. On the other hand, both borderline and benign tumours of PCCs showed a high frequency of LOH at 6q, thus 6q is probably not a characteristic differentiative marker for borderline and benign tumours, since both types showed a similarly high frequency of LOH.

We hypothesized that the ZAC1 gene might be involved in CHB formation based on the observed high frequency of inactivation occurring either via chromosomal or allelic deletions at this candidate TSG gene area of 6q23-24. In accordance with the imprinted gene status and thus an initial transcriptional silencing of maternal allele characteristics, the final study of the present series revealed a predominance of the methylated ZAC1 sequence in 90% of CHBs studied. In parallel, 60% of LOH was exhibited in ZAC gene area. In agreement with the above result, immunohistochemistry also revealed a strongly reduced expression of ZAC1 in CHBs.

Finally, as an overall conclusion, the current observations strongly propose involvement of 6q losses in the tumourigenesis of VHL-associated tumours in both PCCs and CHBs. Moreover, the work demonstrated the inactivation of the imprinted ZAC1 tumour suppressor gene through loss of the expressed allele, a novel finding particularly in CHBs. Collectively the studies included in this thesis work highlight the importance of the ZAC1 gene in the tumourigenesis of sporadic CHBs. These data also imply that the two types of VHL-associated tumours may share other common molecular features in their tumourigenesis, in addition to VHL gene mutations.
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