Von Hippel-Lindau disease: clinical and genetic investigations in the Netherlands

De ziekte van Von Hippel-Lindau:klinisch en genetisch onderzoek in Nederland (met een samenvatting in het Nederlands)

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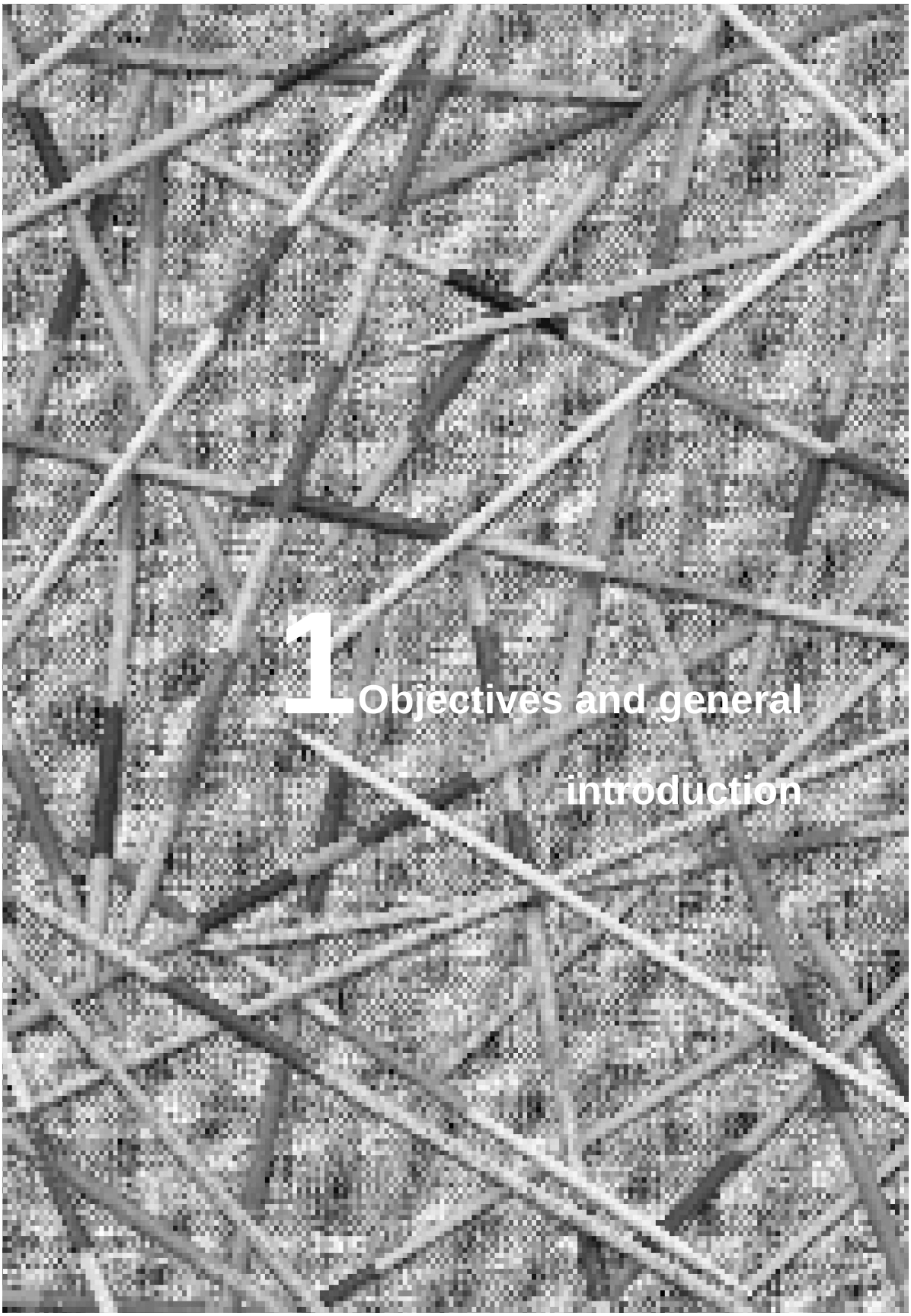

1 Objectives and general introduction

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Objectives and general introduction

This thesis aims to provide strategies for detection, monitoring and treatment of patients with Von Hippel-Lindau (VHL) disease. It also aims to identify and optimise the parameters for both symptomatic and presymptomatic detecting of gene carriers in Dutch VHL families using DNA analysis. First, we describe the main objectives, methods and outline of this thesis.

1.1 Objectives of this thesis

VHL disease is an autosomal dominantly inherited syndrome, predisposing carriers of a germline mutation in the VHL gene to tumours in multiple organs. These tumours include: haemangioblastoma in the retina and central nervous system, renal cell carcinoma, phaeochromocytoma, islet cell tumours of the pancreas, and endolymphatic sac tumours, as well as cysts and cystadenoma in the kidney, pancreas and epididymis (Fig. 1). Most tumours in VHL patients occur multicentric or bilaterally, and manifest at a younger age than in situations without an apparent VHL germline mutation. The germline mutation spectrum is variable and with mutations scattered throughout most of the VHL gene. Although some recurrent mutations have been reported, most families have their own unique germline mutation.

Penetrance of VHL disease in mutation carriers is high. Almost all VHL germline mutation carriers develop one or more VHL-related tumours at an age of 60 years.[1] The most common symptoms include: loss of vision, raised intracranial pressure, neurological deficits, paroxysmal raised blood pressure, and local pain. The median expected survival, based on life table analysis, has been estimated to be 49 years.[2] However, data on the natural history of VHL disease are scarce and derive from studies describing a heterogeneous group of clinically monitored as well as unmonitored VHL patients.[2-6] Accordingly, the mean life expectancy of undetected and untreated VHL patients may be as low as 35 years old. At present, metastases from renal cell carcinoma and neurological complications from cerebellar haemangioblastoma are the most common causes of death in VHL disease. However, intensive radiological and clinical monitoring and advanced operation techniques are likely to contribute to the reduction of both morbidity and mortality.[7-10]

Assuming that 1 in 40,000 people has a hereditary predisposition for this disease, in the Netherlands (with a population of 16 million people) there would be 400 VHL patients. Looking at comparable inherited tumour syndromes (e.g. the Multiple Endocrine Neoplasias Types I and II), we can expect these 400 patients to be found in approximately 40 families.

New families with VHL disease can be found by screening patients with VHL-related tumours and a positive family history for germline mutations. In addition, patients with a VHL related tumour and a negative family history are possible carriers of a VHL germline mutation. Sporadic patients with an autosomal dominant trait and apparently unaffected parents may arise from the following situations: 1) a novel mutation in the sperm or egg cell that produced the child (*de novo* mutation); 2) carriership of mutation in some but not all cells of the body (somatic mosaicism); 3) the disease is not expressed in one of the parents (non-penetrance). When a germline mutation in the VHL gene is found, a new VHL family is identified. Presymptomatic

DNA analysis and identification of carriers of a VHL germline mutations in families then permits physicians to follow the progress of tumour development from a relatively early age and to optimise the time of treatment. Family members who are non-carriers are relieved from the burden of repeated clinical monitoring. Since the necessity for costly and time-consuming surveillance programs can be reduced and monitoring can be directed at those carrying the mutation,[11] the ratio between cost and effectiveness is expected to improve.

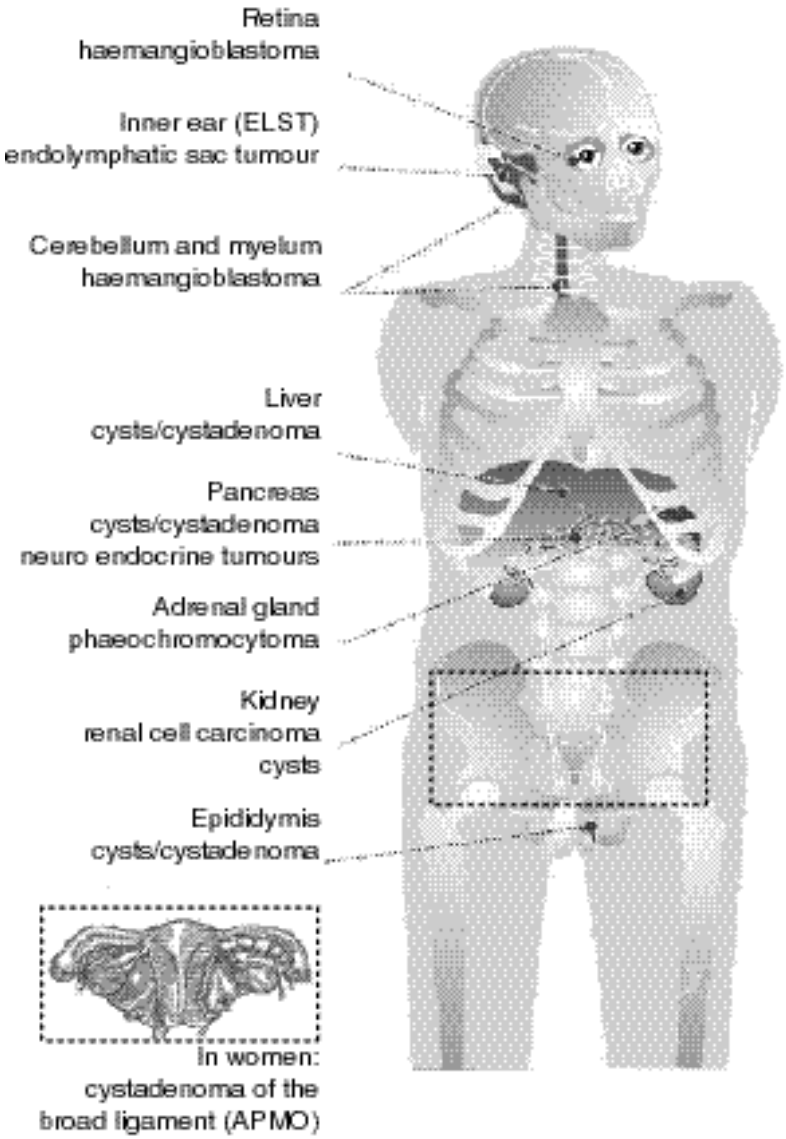

Fig. 1 Clinical manifestations of VHL disease

We realise that there are numerous ethical issues involved in early disease detection. These issues are well descibed in the following principles of Wilson and Jungner:[12] 1) the condition being sought should be an important health problem, for the individual and the community; 2) there should be an acceptable form of treatment for patients with recognisable disease; 3) the natural history of the condition, including its development from latent to declared disease, should be understood; 4) there should be a recognisable latent or early symptomatic stage; 5) there should be a suitable screening test or examination for detecting the disease at the latent or early stage, an this test should be acceptable for the population; 6) the facilities required for diagnosis and treatment of patients revealed by the screening program should be available; 7) there should be an agreed policy on whom to treat as patients; 8) treatment at the presymptomatic, borderline stage of a disease should favourably influence its course and prognosis; 9) the cost of case finding (which would include the cost of diagnosis and treatment) needs to be economically balanced in relation to possible expenditure on medical care as a whole; 10) case finding should be a continuing process, not a "once and for all" project. For reasons also advanced later in this thesis, VHL disease meets, or is anticipated to meet, most of these criteria.

1.1.1 Methods

In order to detect new VHL families and patients, we took several initiatives to increase the amount of persons - possibly affected with VHL disease - referred for DNA diagnosis. Firstly, we asked the Departments of Ophthalmology in the university hospitals for patients with retinal haemangioblastoma. Secondly, we put an appeal in the journal of the Dutch Association for Neurology asking for patients with haemangioblastoma in the central nervous system. Thirdly, we approached approximately 80 medical specialist (including clinical geneticists, internists, endocrinologists, urologists, surgeons, neurosurgeons and paediatricians) with a known interest in hereditary tumour syndromes. Fourthly, we contacted the eight genetic centres in the Netherlands and fifthly, we distributed patient information via the Dutch VHL support group and the Internet.

VHL families and patients, as well as persons suspected of VHL disease, from Dutch, Belgian and Turkish origin were referred to the DNA diagnostic laboratory from the Department of Medical Genetics, UMC Utrecht. DNA analysis for VHL in the Netherlands is also performed in the Department of Clinical Genetics, Rotterdam University Hospital. Blood was taken from every proband of a VHL family and DNA was isolated from peripheral blood samples according to established procedures. DNA analysis included sequencing of the coding region and quantitative Southern blot analysis (complemented by Fluorescence in situ hybridisation analysis when necessary).

Clinical data were collected after patients had signed an informed consent form. Patients were approached via their consulting specialist or clinical geneticist. When patients agreed to participate in the study they were given information about the disease and the research project. In addition, information about the disease and the research project was explained and individualised by genetic assistants during home visits. Some patients were asked if they were interested in becoming a contact person for their family or family branch. These persons were asked to contact family mem-

bers (also at meetings of the Dutch VHL support group) and to distribute informed consent forms. Finally, clinical data were collected from the various hospitals and stored in a database. The protocol for assembling clinical data was approved by the medical ethics committee of the UMC Utrecht.

1.1.2 Summary of objectives

1. To detect VHL families and determine the family-specific germline mutation.
2. To identify presymptomatic family members who carry a VHL germline mutation.
3. To screen for VHL germline mutations in patients with a single VHL-related tumour and without a distinct family history.
4. To improve DNA analysis techniques in identifying germline mutations in families where no mutation could be detected.
5. To collect clinical and genetic data to identify possible genotype-phenotype correlations.
6. To formulate national guidelines for diagnosis and periodic monitoring of VHL patients.

1.1.3 Outline of this thesis

This thesis describes strategies for the detection, follow-up and treatment of patients with VHL disease (chapter 2), and the VHL germline mutation spectrum in classic VHL families and patients suspected for VHL disease (chapter 3).

Chapter 1 is a general introduction to VHL disease. In section 1.1 the main objectives of the study are described. The history of the disease is the subject of section 1.2. Clinical features, including diagnosis, monitoring and treatment of the individual tumours are described in section 1.3. Clinical diagnostic criteria are proposed in section 1.4. Section 1.5 focuses on molecular genetic aspects of the VHL tumour suppressor gene in which germline and somatic mutations of the VHL gene are described, as well as current opinions on genotype-phenotype correlations. An overview of the structure and possible functions of the VHL protein is provided in section 1.6.

Chapter 2 gives an overview of clinical management of VHL. Strategies for the early detection for renal-, adrenal- and pancreatic tumours are described in detail in section 2.1. It addresses the question: *Which methods are suitable for detecting tumours of organs in the upper abdomen in VHL patients?*

In section 2.2 the treatment of renal cell carcinoma is evaluated in ten Dutch patients by clinical and histopathological examination. We addressed the following questions: *What is the morphology of renal cell carcinoma in VHL patients and how aggressive are the tumours? If the choice of treatment (nephrectomy versus nephron-sparing surgery) of renal cell carcinoma depends on the tumour's multi-centricity and growth, what would be the best treatment for a VHL patient with renal cell carcinoma?*

A long-term follow-up of ophthalmological data is analysed in section 2.3 with special attention given to the natural course and results of treatment in patients from six VHL families. We tried to answer the following questions: *Where do ocular haemangioblastoma arise and through which stages of morphology do they proceed?*

What is the optimal treatment of retinal haemangioblastoma, when stage and anatomical position is considered?

The last section (2.4) reports on guidelines for diagnosis and follow-up issued by the Dutch VHL working group. The two principle questions addressed are: *Which persons are eligible for DNA analysis and which persons should be monitored following an annual monitoring protocol?*

The results of VHL germline mutation studies are reported in chapter 3. Section 3.1 gives an overview from 34 germline mutations found in families and patients from different ethnic origin, including their specific phenotypes. Section 3.2 focuses in more detail on deletions found in five of these VHL families. We address the following questions: *What is the mutation spectrum of VHL germline mutations in the Netherlands and how many previously unknown mutations can be detected? Do genotype-phenotype correlations match previously established findings?*

In addition, germline mutations are studied in patients with only haemangioblastoma (section 3.3) or phaeochromocytoma (section 3.4) to address the questions: *What is the genetic epidemiology of sporadic patients with single VHL-related tumours and which patients should undergo DNA analysis? What is the contribution of such cases to the total diagnosis of VHL disease?*

In chapter 4 the principal findings are discussed and summarised in the light of current literature on VHL disease. This chapter also includes a cost-effectiveness analysis of clinical monitoring combined with a discussion of the psychological, ethical and social consequences of early detection of VHL disease.

1.2 History

The German ophthalmologist Eugen von Hippel (1867-1938) is usually credited with the first full description of a retinal vascular abnormality.[13] In 1911 he named this abnormality angiomatosis retinae.[14] This condition had already been reported in 1879,[15] and the microscopic appearance was described in two siblings in 1894.[16] Fifteen years after the report of Von Hippel, the Swedish pathologist Arvid Lindau published a paper describing 40 cases with cystic cerebellar tumours.[17] He associated angiomatosis retinae with cerebellar and spinal haemangioblastomas, and cysts of the kidneys, pancreas and epididymis. Lindau named this syndrome *central nervous system angiomatosis*. Streiff noted in 1951 that French physicians had first reported a probable VHL patient who died with brain and retinal tumours in 1864.[18] The first reports on adrenal involvement in VHL appeared in 1953 and 1959[19,20] and in 1964 Melmon and Rosen published the first major literature review.[21]. Following the leads of Cushing and Bailey they preferred the eponym 'Lindau's disease',[22] although Von Hippel-Lindau disease was already being used by others, first by Davinson et al. in 1936[23], and it was also named Lindau-von Hippel disease by Graig et al. in 1941.[24] Since the 1970s the disease is most commonly called Von Hippel-Lindau disease or VHL.

In 1929, Möller was the first to suggest that VHL disease has an autosomal dominant pattern of inheritance.[25] Segregation analysis confirmed this finding in large families with five and six generations, and in a Hawaiian family with 220 members.[26-28] In addition, it was increasingly recognised that most tumours in VHL patients show typical hereditary features. As well as their familial manifestations, the tumours often occur multiply or bilaterally, and at a young age. In 1988, a genetic locus for the disease was mapped to the short arm of chromosome 3 by linkage studies in nine (including one Dutch kindred) VHL families.[29] Five years later, the VHL tumour suppressor gene was identified following positional cloning studies in VHL families.[30] Subsequently, germline mutations in the VHL gene confirmed the molecular genetic basis of familial inheritance of the disease.

Nowadays, VHL disease is recognised as a hereditary tumour syndrome characterised by predisposition to haemangioblastomas in the retina and central nervous system, renal cell carcinomas, phaeochromocytomas, islet cell tumours of the pancreas, and endolymphatic sac tumours, as well as cyst(adenoma)s in the kidney, pancreas and epididymis (Fig. 1). The most recently discovered VHL-related tumour is the adnexal papillary tumour of probable mesonephric origin (APMO).[31] Sporadic reports of lesions (cysts, cystadenoma, haemangioblastoma/angioma) in cerebrum, liver, spleen, lungs, bladder, omentum, mesocolon, bones and skin have appeared in the literature.[21] Van der Hoeve classified VHL among the phakomatoses,[32] neuro cutaneous syndromes, despite the fact that skin lesions rarely occur in patients with VHL disease.

1.3 Clinical features and management of VHL disease

1.3.1 Ocular haemangioblastoma

Haemangioblastoma are the most common and early tumours in VHL disease.[2,4,33] In VHL patients they may occur in the eye and central nervous system. Ocular haemangioblastoma are considered to be the first manifestation of the disease in 43% of the VHL patients.[2] In the eye the typical lesion is the peripheral retinal haemangioblastoma, a globular reddish tumour with a dilated tortuous feeding artery leading from the optic disc to the tumour and a similar draining vein leading back to the disc.[34] Most ocular haemangioblastoma occur peripherally, 8% occur on the optical disc and 1% at the posterior pole (Fig. 2).[35]

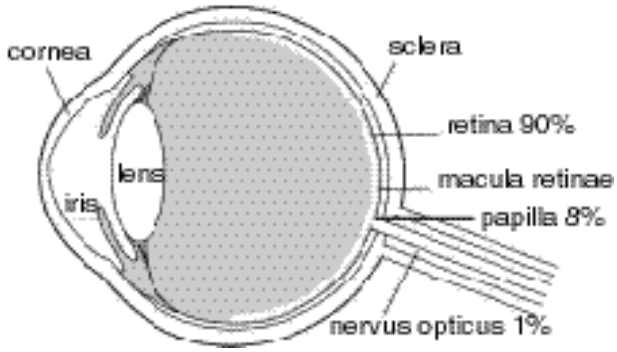

Fig. 2 Sagittal section of the eye, showing the parts most prone to haemangioblastoma (retina, optical disc and the optical nerve). The percentages refer to the relative frequency of tumours found in the corresponding anatomical region.

On histopathological examination, the retinal tumours are seen to be composed of a proliferation of capillaries, and stromal and glial cells, and are identical to the cerebellar haemangioblastoma.[36-38] In 1926 Lindau compared tissue slides from retinal and rhombencephalic cord lesions and identified them as similar manifestations; he called them angiomatosis.[17] The most recent trend is to refer to them as haemangioblastoma instead of the classic - and commonly used - names ‘retinal angioma’ or ‘angiomatosis retinae’.

Peripheral retinal haemangioblastoma generally become symptomatic during the third decade of life. They may cause decrease of visual acuity or induce a visual field defect by retinal exudation, haemorrhages in the vicinity of the tumour, retinal detachment, or wrinkles in the macula.[39] Spontaneous regression can also occur.[40] Recently, Webster et al. studied the natural history in 183 patients from 81 unrelated VHL families.[35] The mean number of tumours in gene carriers was 1.85, and varied between one and 15 lesions. The cumulative probability of incurring visual loss by age 50 years was 35% in all gene carriers, 55% in those diagnosed with retinal tumours, and was significantly worse in patients with symptomatic lesions. 68% of the VHL patients studied were affected by retinal haemangioblastoma, and the tumours were diagnosed at a mean age of 31 years (range 7 to 74 years). Webster et al. observed that the prevalence of retinal tumours did not increase in older VHL patients.[35,41] If there were a lifelong risk of retinal somatic mutation and subsequent ocular haemangioblastoma formation, then the prevalence of ocular tumours would be expected to

increase with age. They therefore suggested that the development of retinal haemangioblastoma may be determined, as in retinoblastoma, at an early age.[35,41]

Diagnosis is made by ophthalmological examination, measuring visual acuity, slit lamp examination, and fundoscopy. If haemangioblastomas are present or suspected in the far periphery of the retina, inspection with a Goldmann three-mirror lens can be performed. Additional fluorescein angiography is sometimes indicated. Many different modes of therapy have been used to treat peripheral retinal haemangioblastomas: diathermic-, xenon-, laser- and cryocoagulation. All have been reported effective depending on the location, size and number of the tumours. Decisions on treatment are often difficult and sometimes only follow-up observations may be justified. Diagnosis and treatment are described in more detail in section 2.3.[34]

1.3.2 Central nervous system haemangioblastoma

Central nervous system (CNS) haemangioblastoma in VHL disease arise preferentially in the cerebellum (~ 75%), medulla oblongata (~ 10%), and spinal cord (~ 15%), but are rare in the cerebrum (1%), see Fig. 3.[4,33,42,43] Tumours located in the cerebellum are mainly found in the hemispheres, or less frequently in the vermis.[33] Spinal tumours are essentially intradural and are most commonly found at the level of cervical and lumbar bulges (Fig. 4).[33]

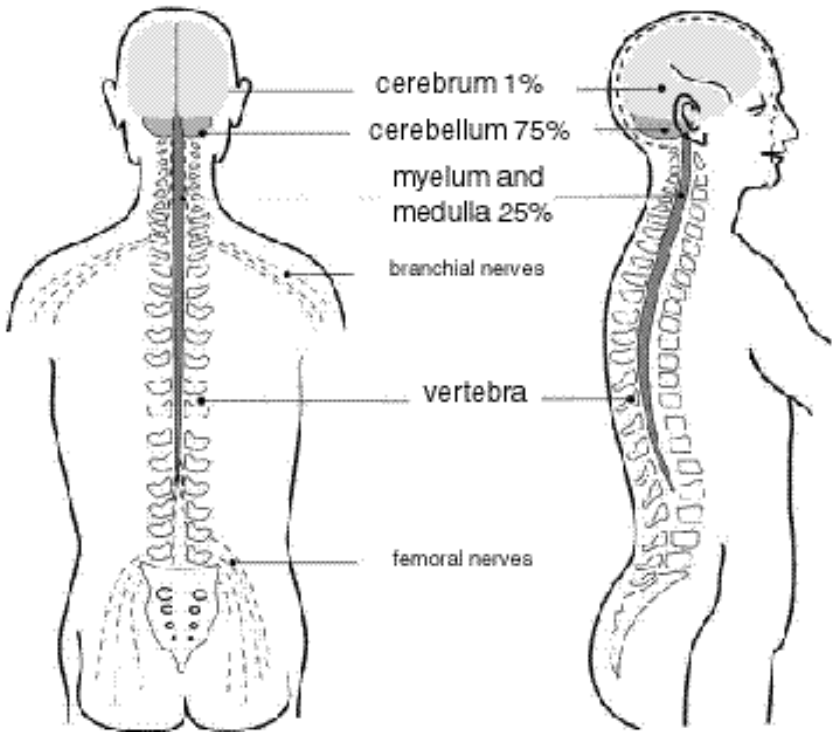

Fig. 3 Dorsal and lateral view of the central nervous system, showing hot spots and relative frequency of occurrence of haemangioblastoma in VHL patients.

Clinical symptoms depend on the site and size of the CNS haemangioblastoma.[5,33,42,44] Headache is the most frequent initial symptom in cerebellar haemangioblastoma. In cases with symptoms at diagnosis, headache, nausea, vomiting, ataxia and dizziness occur most often. In addition, dysarthria, dysgraphia, ptosis, motor deficits, sixth nerve palsy, sensory deficit and impaired hearing have also been described.[42] Pain is the most common symptom in spinal haemangioblastoma and symptoms are related to compression effects.

In approximately 40% of VHL patients, CNS haemangioblastoma is found to be the presenting manifestation of VHL.[33] The mean age at diagnosis is 30 years[2,33,45] Haemangioblastoma are regarded as benign and slow-growing tumours that do not normally invade the surrounding brain. However, complications may arise due to the tumour's tendency to form expanding cysts.[33,37] Cerebellar shift may lead to herniation of the cerebellar tonsils through the foramen magnum and subsequently elevated or even life-threatening intracranial pressure. Hydrocephalus may result in rapid decompensation with papilloedema.[33] Cerebellar haemangioblastoma remain a major cause of morbidity and mortality in VHL.[3-5,33,42,46]

Pathologically, haemangioblastoma are composed predominantly of vascular and stromal cells.[37,47] Allelic losses and mutations of the VHL tumour suppressor gene have been found in stromal cells, suggesting that these cells represent the neoplastic component of a haemangioblastoma.[47,48] Mast cells are often abundant and their presence is helpful in diagnosis.[33] The frequent presence of haemorrhages and cysts make the morphological appearance of the tumours variable.[37] Four macroscopic types of haemangioblastoma can be individualised: 5% are cysts, 60% predominantly cystic, 26% predominantly solid, and 9% solid.[33] The most frequently observed tumour is a small solid lesion within a cystic nidus (Fig. 4).

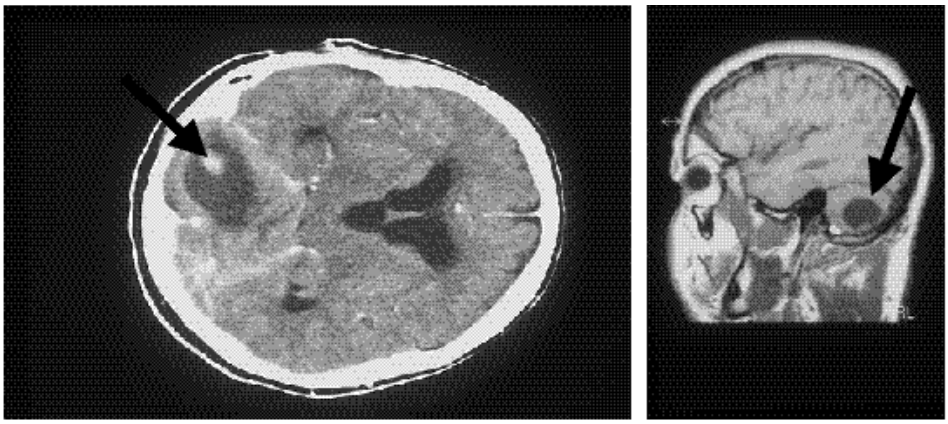

Fig. 4 Comparison of MRI (left) and CT (right) of a typical haemangioblastoma in a VHL patient. Both arrows indicate a cystic lesion with a solid lesion attached to the wall. This lesion is also called nidus (i.e. Latin for nest) since the image resembles a bird's nest with an egg.

The method of choice for early diagnosis and follow-up examination of CNS haemangioblastoma is gadolinium-enhanced magnetic resonance imaging (MRI)[44,49] The tumours appear as a low signal on T1-weighted images and as a high signal on T2-weighted images.

The standard treatment is complete microsurgical removal,[33,42,50] aided if necessary by preoperative embolisation to reduce the tumour's vascularity.[51] The prognosis of VHL patients with CNS haemangioblastoma has improved by atraumatic microsurgery and progress in intensive care, but the possibility of development of other VHL-related tumours is still a major problem.[33] Postoperative mortality is 7-10% and is higher in brainstem localisations.[44,45,49] In cases of spinal haemangioblastoma, paraplegia is the main risk of neurosurgery, especialy when the diagnosis is delayed.[33] Many patients have recurent, difficult operations and CNS haemangioblastoma remain the main cause of death together with renal cel carcinoma.[2,33,45,52]

A new technique, stereotactic radiosurgery, offers the possibility of tackling multiple cerebellar lesions in a single treatment, which is particularly important in VHL patients. Essentially, stereotactic radiosurgery hits the tumour with beams of radiation from hundreds of different angles. Each beam also passes trough normal tissue, but that tissue gets only a very small dose of radiation. Where the beams meet at the target site, the tumour gets the sum of dosages of all the beams, the surgical dose. The recommended surgical dose ranges between 10 and 25 Gray.[10,50,53] Dose escalation above 60 Gray is associated with a significant risk of radiation-associated neurotoxicity.[53] Radiosurgery shrinks or stops the growth of small- or medium-sized (i.e. smaller than 3 cm) solid haemangioblastoma.[10,50,53] Adjoining cysts, however, do not respond to radiosurgery and require later, sometimes repeated, evacuation.[50] Spinal haemangioblastomas are more difficult to target, but with further development of the stereotactic radiosurgery it is anticipated that tumours of the thoracic and lumbar spine will also be treatable with this technology.[10]

Finally, erythrocytosis (or polycythemia) is associated with the production of erythropoietin (Epo) by haemangioblastoma in VHL patients.[21,54-56] Krieg and co-workers[57] demonstrated that preoperative haemoglobin (14.7 vs 13.0 g/L) and haematocrit levels (43.7% vs 39.2%) were higher in haemangioblastoma patients (both VHL and non-VHL) than in a control group of patients with glioblastoma, which are not known to induce erythrocytosis. In addition, in situ hybridisation on snap-frozen haemangioblastomas revealed that stromal cells produced Epo. Since stromal cells are the neoplastic component in haemangioblastoma,[47,48] this suggests that Epo is upregulated due to loss of VHL function.

1.3.3 Renal carcinoma and cysts

In VHL patients, renal lesions can be divided into three different forms with cystic, combined cystic-solid, and solid renal cell carcinoma (Fig. 5).[58] These renal lesions occur multiply, bilaterally and at a relatively young age. While renal cell carcinoma in sporadic patients occurs predominantly in the seventh and eighth decades of life,[59] the mean age of presentation in VHL patients is approximately 36 years.[2,4,60-63] Poston et al.[60] found a mean of 7.8 cystic and 3.0 solid renal lesions in VHL patients (with a mean age at diagnosis of 36 years), which is in agreement with other studies.[5,58]

The prognosis of patients with presymptomatically diagnosed renal tumours is significantly better than that of patients with symptomatic lesions.[8] Renal cysts are usually asymptomatic, whereas renal cell carcinoma may present with haematuria or with back pain. Renal cell carcinoma is the cause of death in 15-50% of VHL patients,[2,5,6] and 30-50% of symptomatic renal cell carcinoma have already metastasised to lymph nodes, liver, bone, lung or brain.[3,21,64,65] Fortunately, nowadays, most renal tumours are detected by periodical monitoring of VHL patients.

Pathologically, renal cell carcinoma is a malignant epithelial tumour of the renal parenchyma and is often found in the renal cortex (Fig. 6). The tumour tissue is frequently crowded with recent and old haemorrhages, necrosis and inflammation, and is surrounded by a pseudocapsule.[66,67] The most common cellular pattern is clear cell carcinoma,[68] arising from cells of the proximal tubuli.[69] Microdissection of material from individual lesions has shown that loss of the wild-type allele and retention of the inherited, mutated VHL allele occurs both in cystic lesions and in renal cell carcinoma.[70] This clearly demonstrates that cysts are precursors for renal cell carcinoma (Fig. 5).

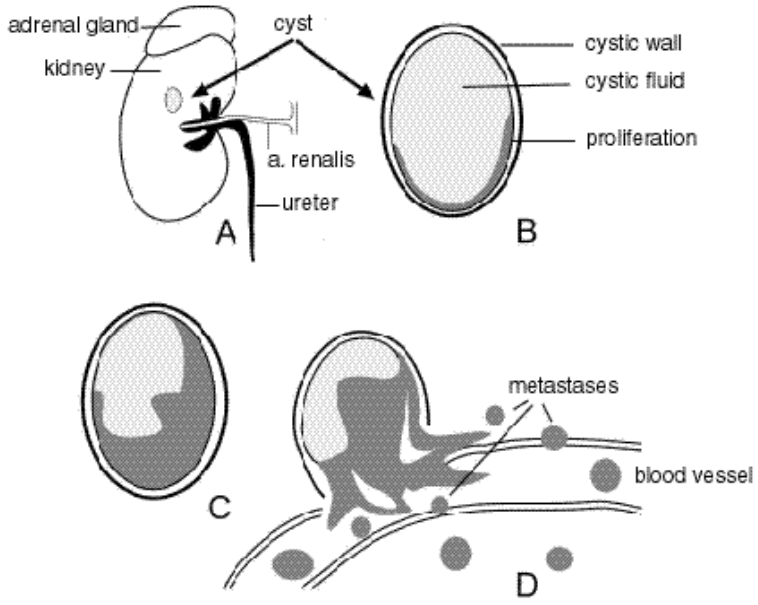

Fig. 5 The developmental stages from a renal cyst to a metastasing renal cell carcinoma. **A** Simple renal cyst; **B** Magnification of the cyst, demonstrating proliferative cells in the cystic wall; **C** Intermediate stage between a cyst and renal cell carcinoma, the tumour is still within a capsule; **D** Renal cell carcinoma breaking through the capsule and metastases migrating via neighbouring blood vessels.

Radiological aspects of kidneys in VHL patients are extensively discussed in section 2.1. Some monitoring protocols recommend yearly alternate CT and ultrasound examinations to reduce both costs and exposure to radiation.[71] In our opinion, careful cross-sectional monitoring with alternate MRI and ultrasound gives the best protection from aggressive renal cell carcinoma in most VHL patients.

Options for treatment of renal lesions in VHL patients are considered in section 2.2. Recommendations range from bilateral nephrectomy to follow-up investigations only. If both kidneys are affected with multiple cysts and tumours, a difficult decision has to be made between radical nephrectomy or nephron-sparing surgery. This decision depends on risk factors (size, progression, capsule involvement and whether the tumour is symptomatic) for metastatic spread. Management of metastatic lesions is a difficult problem, since their response to chemotherapy and radiotherapy is poor. In 1999, the first report of a prospective analysis on treatment of renal cell carcinoma in VHL patients appeared. Walther et al. evaluated 52 VHL patients who were operated on with renal cell carcinoma smaller than 3 cm (group 1) and 44 patients who were operated on with tumours larger than 3 cm (group 2).[63] Median follow-up was five years. In group 1 96% underwent nephron-sparing surgery, and 63% in group 2. The remaining patients underwent a nephrectomy. No metastases occurred in group 1, whereas 25% of the patients in group 2 developed metastatic renal cancer. The authors concluded that using a 3 cm threshold as the upper limit for performing nephron-sparing surgery may help to prevent metastases, and avoid unnecessary renal damage due to frequent surgery, and renal dialysis or transplantation.

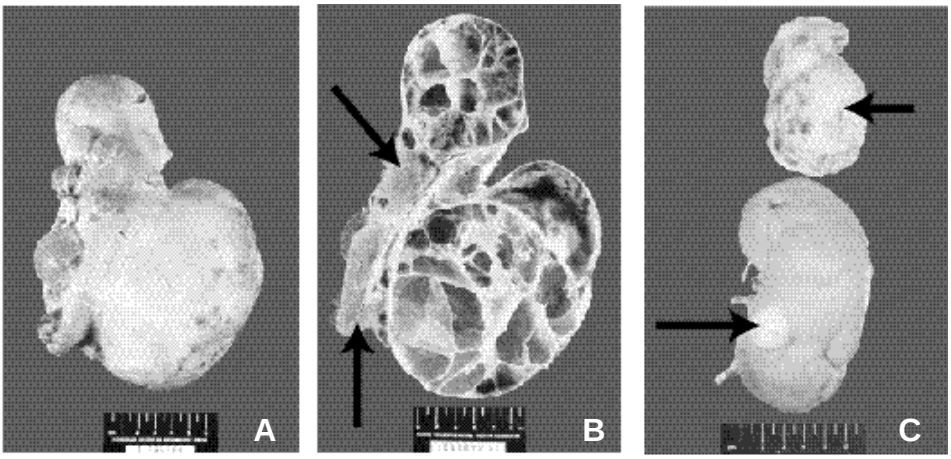

Fig. 6 The devastating effect of symptomatic renal cell carcinoma in the left kidney of a 42 year old male VHL patient (**B** is a section through **A**). **A** and **B** A small rim of normal renal tissue is visible (**B**, arrow). The kidney contained 2 large cystic multiloculated tumours (14 x 10 x 8.5 and 7 x 6.5 x 5 cm) and 8 smaller lesions ranging from 0.6 to 3.0 cm. The large cystic tumours are well encapsulated. In the cortex of the kidney a 0.5 cm vascular tumour was found (not visible in this figure) resembling a haemangioblastoma. Microscopy showed clear cell carcinoma in both large lesions as well as in the vascular tumour.
C Left kidney of a 45 year old female VHL patient. This patient was diagnosed with a symptomatic phaeochromocytoma (small arrow), and on radiological examination a renal lesion (large arrow) measuring ~3 cm was detected. Subsequent angiography indicated that the localisation and vascular pattern made it impossible to perform nephron-sparing surgery. She underwent a nephrectomy and the left adrenal gland was removed in the same session. The lesion measured 26 x 21 mm and was growing into the perinephric fat. A cyst of 9 mm protruded from the tumour, and another cyst of 9 mm was present inside the lesion. The tumour was haemorraghic and had a thin grey capsule. In addition, the renal cortex contained a cyst of 10 x 5 mm (not visible in this figure). The phaeochromocytoma measured 6 x 5 x 4cm.

1.3.4 Phaeochromocytoma

The first reports of phaeochromocytoma in VHL disease appeared in the 1950s.[19,20] This catecholamine-secreting tumour originates from chromaffin cells of the neural crest (paraganglion). Paraganglionic tissue is dispersed from the base of the skull to the pelvic diaphragm. In 10-20% of the cases paraganglioma derive from extra-adrenal neural crest tissues that reside along the aorta (close to the sympathetic and parasympathetic nervous system).[72] The term phaeochromocytoma (paraganglioma medullare) is officially reserved for tumours that arise in the adrenal medulla. However, most reports use the terms phaeochromocytoma and extra-adrenal phaeochromocytoma.

Extra-adrenal paraganglioma have been reported in 12% of VHL patients.[73] The report of a thoracic paraganglioma in 1982 was the first on the occurrence of these tumours outside the abdomen in VHL disease. In 1997 Bender et al.[74] found VHL gene germline mutations in three out of five patients with functioning thoracic ganglioma. Further extra-abdominal paraganglioma in VHL patients have been reported in the carotid body and the sella turcica.[75-78]

Phaeochromocytoma (and paraganglioma) may cause hypertension or paroxysmal unstable blood pressure and symptoms such as headaches (67%), palpitations (44%), chest discomfort (39%), flushing, sweating attacks, postural dizziness, and pallor.[79] In VHL patients, phaeochromocytoma often remain quiescent or produce few symptoms, and investigation may show normal biochemical tests.[80] However, the behaviour of phaeochromocytoma remains unpredictable; biologically inactive lesions may suddenly become dangerous, or benign phaeochromocytoma may become malignant.[81] About 5% of VHL patients die from phaeochromocytoma-induced endogenous catecholamine intoxication, which has also caused fatal pregnancy outcome (for both mother and child).[82-84]

There is strong evidence that the presence or absence of phaeochromocytoma is correlated with the type of VHL germline mutation (see genotype-phenotype correlations, 1.5.6). Phaeochromocytoma may occur in a large range (0-58%) of affected members within a family.[4,5,58,85-88] Beside this clear interfamilial difference, intrafamilial differences have also been observed.

VHL-associated phaeochromocytoma differs from isolated phaeochromocytoma in sporadic patients in having a younger age of onset (on average 19 years earlier), multiple lesions, and a very low proportion of malignant tumours.[89] The mean age at phaeochromocytoma diagnosis is 28 years,[5,89,90] with the youngest reported patient being five years old.[90]

Diagnosis is based on biochemical tests and radiology. Laboratory tests may include serum and urine evaluation of: epinephrines, norepinephrines, nephrines, metanephrines and urinary vanillylmandelic acid.[80,89] Measurement of plasma normetanephrine is the most sensitive test and detects 97% of the tumours in patients.[80] However, this test is not the most suitable for annual monitoring of VHL patients. It requires an intravenous canula in the forearm and patients must rest 15 minutes in a supine position before blood samples can be collected. Measurement of 24-hour urinary norepinephrine excretion is the next most sensitive of the biochemical tests and is less of a burden to the patient. Radiology testing may include ultra-

sound, CT, MRI, and metaiodobenzylguanidine (MIBG) scintigraphy as described in section 2.1.

Adrenalectomy is the standard treatment for phaeochromocytoma. Operative treatment can be considered in symptomatic phaeochromocytoma or if a growing mass in the adrenal gland is present (Fig. 6). Satisfactory results have been reported from laparoscopic removal of adrenal tumours, both in non-VHL patients[91-93] and in VHL patients.[94] Since bilateral tumours develop in 47% of VHL patients with phaeochromocytoma, most patients become dependent on steroids after bilateral adrenalectomy.[95] Enucleation rather than adrenalectomy is therefore recommended by an increasing number of surgeons.[95,96]. Adrenal-sparing surgery is safe, effective and can preserve adrenal function in VHL patients.

1.3.5.1 Pancreatic cysts and cystadenoma

The incidence of pancreatic involvement varies in VHL families from 0-56% of affected family members.[3,5,97-99] In a review of 275 reported cases, 69 (25%) patients had cysts, 4 (1.5%) of which proved to be serous cystadenoma, 14 (5%) cases had islet cell tumours, 2 (0.7%) adenocarcinoma, and 1 patient had an haemangio-endothelioma.[100] Pancreatic lesions may be the only abdominal manifestation in VHL (12%) and may precede any other manifestation[98]. The earliest age of discovery reported is 15 years.[101] Cystadenomas, like cysts, contain serous fluid and are benign in VHL disease.[71,97]

Pancreatic cyst formation is found in 70-72% of VHL patients at autopsy[3,102]. Cysts are often multiple and enlarge the pancreas,[97] or may eventually replace the entire gland.[103] Complications can arise from space-occupying effects (local pain, bile duct obstruction, pancreatitis), while exocrine and endocrine hormonal insufficiency may occur.[21,71,97,99,100,104-107] .

Different imaging techniques, such as ultrasound, MRI, and CT, have comparable diagnostic value.[108] Ultrasound is therefore the method of choice for monitoring programs.[97] However, for identifying smaller solid lesions, CT scanning or MRI may be superior to ultrasound.[58,108] Radiological diagnosis of pancreatic lesions is specified in section 2.1.

Since the disease is usually asymptomatic, conservative measures are considered adequate for cystic lesions.[97,100] However, aggressive resection is mandatory for a solid pancreatic lesion in VHL.[100] Patients with space-occupying cysts have been treated with percutaneous drainage and hypertonic saline sclerosis.[71] Cholestatic jaundice may be treated by endoscopic implantation of a biliary stent,[109] or a biliary bypass.[100]

1.3.5.2 Pancreatic (neuroendocrine) islet cell tumours

Solid islet cell tumours may occur in 5-17% of patients[100,110,111] and may be unrelated to pancreatic cystic disease.[71] Familial islet cell tumours in VHL were first reported in 1979.[99] The mean age at diagnosis of islet cell tumours in VHL patients is 36 years, and many lesions are slow-growing, asymptomatic and do not generally lead to raised pancreatic hormone levels.[71,99,110,111] Immunostaining of 23 pancreatic lesions, which were all non-functional, demonstrated focal positivity for pancreatic polypeptide (6/23), somatostatin (4/23), insulin (2/23) and/or glucagon (1/23) in 35% of the tumours.[112]

Libutti et al.[111] reported that 5 out of 17 VHL patients (from a series of 256 VHL patients) with neuroendocrine tumours were found to have metastatic disease of the pancreas, of whom two died as a result of extensive liver involvement. The size of the primary pancreatic tumour in patients with liver metastasis was significantly larger than in patients without liver metastasis (5 cm vs 2 cm).

Pancreatic islet cell tumours occur more frequently in patients with phaeochromocytoma and may be considered as an additional form of the multiple endocrine neoplastic syndromes.[71,99,110,111] Islet cell tumours arise either from pancreatic islet cells, which are believed to be derived from the neural crest,[113] or from a single neuroendocrine-programmed ectoblast.[114] This might indicate that islet cell tumours share a common origin with phaeochromocytomas.

Ultrasound shows that benign islet cell tumours are usually well demarcated, round or oval, and hypoechoic relative to pancreatic parenchyma. Fat-suppressed and dynamic gadolinium-enhanced MRI is superior to CT in depicting islet cell tumours.[115]

In VHL patients, an organ-sparing strategy consistent with the managing of renal and adrenal tumours, is advised. Libutti et al.[111] have published guidelines for the management of solid pancreatic tumours in VHL patients. Lesions smaller than 1 cm must be monitored every 12 months with CT or MRI. Lesions between 1 and 3 cm are dealt with on a “case by case” assessment. Lesions larger than 3 cm that are symptomatic or functional and lesions that are increasing in size are removed. Intra-operative ultrasound may be useful in identifying focal masses when pancreas-sparing surgery is being considered.

1.3.6 Endolymphatic sac tumours (ELST)

Endolymphatic sac tumours (ELST) are rare neuroectodermal neoplasms in the petrous bone, originating from inner ear structures.[116] It was only recently recognised that ELST is also a manifestation of VHL disease.[117-121] Interestingly, the first patient described by Von Hippel had a tumour of the petrous bone.[13] An assessment after MRI screening revealed the prevalence of ELST in 121 VHL patients to be 11%. This figure may be an underestimate since 65% of these patients had pure tone threshold abnormalities on audiometry, of whom 54% of those affected showed bilateral abnormalities.[120]

Macroscopically, the ELST presents itself as a reddish blue tumour from the labyrinthal compartment of the temporal bone, resembling a haemangioma.[121] Heffner qualified the tumour as a low-grade adenocarcinoma of probable endolymphatic origin.[122] These findings have been questioned by Pollak et al.[121], who found in a VHL patient that this tumour originated from the mucosa of the pneumatic spaces surrounding the jugular bulb and that it was a benign tumour, i.e. papillary adenoma on histopathological examination.

Clinical findings in ELST patients include hearing loss, tinnitus and vertigo.[120] Hearing loss may in fact represent one of the first clinical findings of VHL. ELST may also show symptoms that are related to structures in the cerebello-pontine angle. Involvement of the nervus facialis, causing facial paresis and ataxia or disequilibrium have been reported.[120,123]

Middle ear adenomas (MEA) are the principle tumours in differential diagnosis. ELST and MEA share several features, including histological appearance.[116] However, MEA are restricted to the middle ear and do not normally erode the bone, which makes it possible to distinguish them radiologically.[120,123] Both MRI and CT are used in identifying ELSTs.[120,121]
Although, ELST are not known to metastasise and tumour growth is slow, clinical (audiological tests) and radiological monitoring is indicated.[120] Early detection is crucial because early surgical therapy may prevent hearing deficits from progressing.[116]

1.3.7 Epididymal cysts and cystadenoma

In the epididymis simple cysts and cystadenoma may be encountered, although papillary cystadenoma are thought to represent the VHL specific component. Epididymal cysts, closely resembling renal cell carcinomas were first described by Lindau in 1927.[124] Epididymal tumours have been observed in up to 50% of the males in one VHL family,[5] and bilateral epididymal cystadenoma have been reported in some families.[5,125-128] However, epididymal cysts are relatively common in the normal population, 29% of 40 healthy volunteers had one or more lesions on high-resolution scrotal sonography.[129]

Epididymal lesions are comprised of cuboidal and columnar cells with clear cytoplasm lining tubular and cystic spaces and the intervening stroma is highly vascular.[130] Histopathologically, epididymal cystadenoma resemble metastases from renal cell carcinoma and can be distinguished by immunohistochemistry studies.[130,131] The probable origin of these lesions is from epididymal duct epithelium,[131] that arises from the embryonic mesonephric duct (see also section 1.3.8). A somatic VHL mutation was observed in an epididymal cystadenoma of a non-VHL patient, indicating that VHL gene mutations play a role in the initiation of tumourigenesis.[130] High levels of VEGF mRNA are expressed in the clear cells of epididymal cystadenoma. Leung et al.[132] postulated that elevated VEGF levels may account for cyst formation and also for the vascularised stroma in these lesions.

The lesions may be detected by manual examination or ultrasound. Choyke et al.[133] demonstrated by sonography that 54% of male patients with VHL had a unilateral (33%) or bilateral (67%) solid abnormality in the head of the epididymis suggestive of epididymal cystadenoma. Sonographic appearances ranged from a solid mass with multiple tiny cysts to an almost completely solid mass. Bilateral tumours may lead to oligozoospermia, low ejaculate volume,[128] and may even impair fertility by obstructing the seminiferous ducts.[125,127] Surgery is not known to improve fertility in affected patients.[88] Close clinical monitoring is therefore indicated to enable presymptomatic treatment.

1.3.8 Adnexal papillary tumour of probable mesonephric origin (APMO)

APMO is regarded as the female counterpart of the cystadenoma of the epididymis. In men, the mesonephric duct stays intact and functional, whereas in women, the duct system is a remnant (Fig. 7). APMOs arise from mesonephric (Wolffian) remnants in women and may persist within the broad ligament (epoophoron and paroophoron) or along the wall of the uterus (Gartner's duct) or posterior vagina (Gartner's duct cysts).[134] The APMO is histologically similar to epididymal cystadenoma in VHL disease.[135]

So far, APMO has been reported only as case reports,[31,135-138] including one Dutch patient.[81]. In 1997, a comprehensive study of the literature showed that all cases with APMO described thus far arose in patients with VHL.[135] This association indicates that the APMO may represent a pathognomic visceral manifestation of VHL disease.

Patients with APMO may present with pain in the lower abdomen.[138] When asymptomatic, the tumour can be detected either during a routine physical exam,[135] as an incidental finding during surgery[137], or using radiological tools.

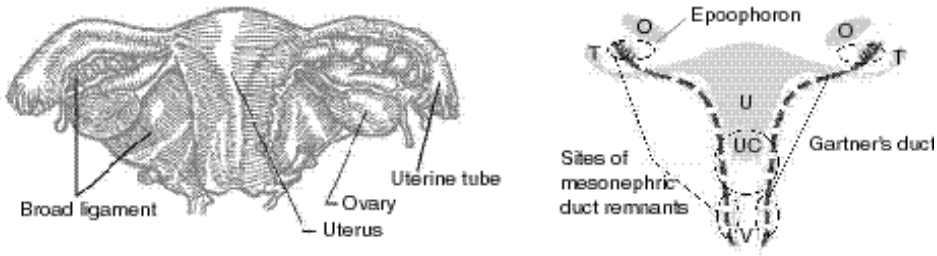

Fig. 7 Ventral view of the female reproductive organs (left) and embryonic tissues (right). The adnexal papillary tumour of probable mesonephric origin (APMO) arises from mesonephric or Gardner's duct remnants and may occur within the broad ligament, along the wall of the uterus or posterior vagina. O, ovary; T, uterine tube; U, uterus; UC, uterine cervix; V, vagina. The drawings were made by Frank W. James and kindly provided by Gale James from the VHL Family Alliance. The figures are based on:
(left) Figure 5.14 in Berek, J.S. et al. (eds) Novak's Gynaecology, Twelfth edition. Baltimore, Williams & Wilkins, 1996: p.100.
(right) Figure 13-16 C in Moore, K.L. (ed.) The Developing Human: Clinically Oriented Embryology, Second edition. Philadelphia, WB Saunders Co., 1977: p.234 and Figure 22.13 in Skandalakis, J.E. & Gray, S.W. (eds.) Embryology for Surgeons, Second edition. Baltimore, Williams & Wilkins, 1994: p.826.

1.3.9 Other lesions

Many other lesions have been reported in VHL patients (see table 1). Typical VHL-related lesions include cysts, cystadenoma, angioma, haemangioma and haemangioblastoma and they have been reported in liver, spleen, adrenal gland, lungs, skin and bones.[3,21,139,140] Haemangioblastoma of the optical nerve has been reported relatively frequently[141-145], and also in further supratentorial locations.[146-149]

In addition, various atypical, non-VHL-related tumours have been reported in VHL patients. Among these lesions are: naevi, café au lait spots, parotid carcinoma, thyroid carcinoma, pituitary adenoma, carcinoid, and testicular germ cell tumour.[4,21,150-155] In the central nervous system epidermoid, ependymoma, astrocytoma, choroid plexus papilloma and neuroblastoma have been reported.[3,42,148,156-159]

One can argue whether all these lesions should indeed be associated with VHL disease. On one hand, the VHL protein is widely expressed in normal human tissues,[160-162] even in organs not at risk for the disease (see 1.6). On the other hand, these lesions may have risen from embryonic VHL-associated residual tissues, or represent metastases.[88] In our opinion, most lesions mentioned in table 1 probably represent coincidental findings.

Table 1 A list of tumours rare in VHL patients

Origin	Type	Reference
Lesions of the CNS	ependymoma	3,4
	astrocytoma	4,157
	meningeoma	4,21
	choroid plexus carcinoma	4,158,163
	neuroblastoma	4,148,159
	syringomyelia	4,164-167
	pituitary haemangioblastoma	152
	epidermoid	156
	neuroectodermal tumour	168
Parotis	carcinoma	150
Lung	cysts	3,4,21
	angioma	3,4
	haemangioblastoma	139
	oat cell carcinoma	165
Kidney	angioma	3
Bladder	haemangioblastoma	21
Adrenal gland	cortical angioma	3
	cortical adenoma	3,21
	cortical hyperplasia	3
Liver	cysts	3,4,21,81
	angioma (haemangioma)	3,4,21
	haemangioblastoma	139,140,169
	carcinoma	4
	adenoma	3,4,21
	hepatocellular carcinoma	165
Spleen	cysts	3,4
	angioma (haemangioma)	4,21
Ovaria	angioma	3
Testes	germ cell tumour	154,155
Endocrine tumours	adenoma of the pituitary gland	4
	thyroid medullary carcinoma	4,151,165
	pancreatic islet cell carcinoma	4
	carcinoid	4,153
Bone	haemangioma	3,21
	cysts	21
Skin	angioma (angioblastoma)	4
	nevus	21
	café au lait spots	21

1.4 Diagnostic criteria in VHL disease

Diagnostic criteria for VHL disease has long been based on the classic review of Melmon and Rosen, published in 1964.[21] These criteria were later modified by various authors.[2,4,7,170] By 1998, VHL disease was considered to be a complex of diagnoses occurring in: (1) typical families with VHL germline mutations; (2) typical families without detectable germline mutations; (3) sporadic cases of VHL disease without germline mutations; (4) sporadic cases of VHL disease and germline mutations; (5) atypical VHL families with germline mutations; (6) patients with a solitary VHL tumour and a germline mutation.[171]

The disease as documented by Lindau in 1926 was defined as an association of cerebellar haemangioblastoma with one or more of the following lesions: retinal haemangioblastoma, spinal cord haemangioblastoma, pancreatic cysts, renal or epididymal abnormalities. However, Melmon and Rosen considered this definition to be incomplete since it failed to consider the hereditary character of the disease. They favoured enlarging the diagnosis of Lindau's disease by including patients with a single lesion of the Lindau complex (haemangioblastoma, pancreatic cysts, renal or epididymal abnormalities), with at least one other family member with a CNS haemangioblastoma.[21] Neumann et al. modified Melmon and Rosen's criteria in 1987[4] such that minimal diagnostic criteria would consist of retinal or CNS haemangioblastoma in a patient and at least one typical VHL lesion in another immediate family member.[88]

Eventually, a clear family history was increasingly valued as an important clinical criterium for diagnosing the disease. Certainly influenced by the rising success rate of detecting VHL gene germline mutations in clinically well-defined VHL families, a shift in clinical diagnosis has taken place in recent decades towards cases with a definite or positive family history. It was therefore suggested that diagnosis of VHL disease can be made in any patient with a single retinal or cerebellar haemangioblastoma, renal cell carcinoma, phaeochromocytoma, or multiple pancreatic cysts, whenever a positive family history is present.[7] However, Maher et al. noted that apparently sporadic cases of VHL disease may be under-ascertained as a result of this requirement.[2] Therefore, a diagnosis can also be made for isolated cases of VHL disease when two or more haemangioblastoma, or a single haemangioblastoma in association with a visceral manifestation, are present in a sporadic patient.[7]

The definition of pathognomic VHL lesions forms another pillar upon which the clinical diagnosis of the disease is based. Which tumours may be regarded as typical VHL lesions? Glenn et al. defined a list of clinical situations leading to suspicion of VHL disease and a evaluation of typical VHL-related tumours (Fig. 1).[170] Recently, bilateral ELSTs and the APMO were added to this list.[7,31]

In conclusion, VHL disease can be diagnosed in an at-risk family member with a typical VHL lesion in combination with a positive family history. However, no unequivocal consensus has been reached for clinical diagnostic criteria in VHL disease. These criteria are discussed in chapter 4.

1.5 Genetics

In 1929, Möller was the first to suggest that VHL disease has an autosomal dominant pattern of inheritance.[25] This means that children of a parent who is a carrier of a mutated VHL gene have a 50% chance of inheriting the disease. Since the disease has a high penetrance,[1,2] a dominant pattern of inheritance can be recognised in the vast majority of VHL families of significant size. However, there are also VHL patients without a family history. These sporadic patients probably represent *de novo* mutations, somatic mosaicism or non-penetrance in one of the parents. A *de novo* mutation is found in 4-15% of the total identified VHL germline mutations.[30,172] Data in the literature on non-penetrance and mosaicism are scarce. Mosaicism has so far been described in two VHL families,[173] and non-penetrance on elder age (> 60 years) is rare.[1,2]

1.5.1 The VHL gene

Genes associated with a particular disease are most commonly found by testing affected family members with DNA markers. This technique is based on the concept that certain chromosomal regions lying in the vicinity of the disease-causing gene segregate in affected family members. This method is called positional cloning, or reverse genetics, in contrast to the candidate gene strategy. In positional cloning the location of the gene in the genome is established on its anticipated function. Using genetic linkage analysis in large VHL families, the VHL gene was localised in 1988, and mapped to chromosome region 3p25-26.[29] Family linkage studies did not show any evidence of genetic heterogeneity.[29,174] Seizinger's group was inspired by Zbar et al.[175], who had found loss of alleles of loci on the short arm of chromosome 3 in renal cell carcinoma, a common manifestation of VHL disease.

Subsequently, tightly linked polymorphic markers in the region 3p25 were identified.[176-179] A decisive step in finding the gene was the identification of large germline deletions in VHL families. Probes from this region were used for pulsed field gel electrophoresis.[180,181] With one specific probe, Richards et al. found germline deletions of 120 en 50 kb in 2 of 91 probands of VHL families.[182] These deletions further refined the localisation of the VHL disease gene to a small region of chromosome 3p25. Latif et al.[30] isolated two genes (called g6 and g7) from the minimally deleted region of 50 kb. They detected mutations in the g7 open reading frame that followed transmission of the disease in VHL families and consequently identified g7 as the VHL disease gene.

The VHL gene covers approximately 14,500 basepairs (bp) of genomic DNA. The full-length messenger RNA is 4,700 bp.[30] The open reading frame is 852 bp long and contains two intragenic start codons (Met1 and Met54). The protein-coding region translated from the first start codon (Met1, nucleotides 214-216) encompasses 639 bp, and is divided into three exons of 340, 123, and 179 bp (Fig. 8). The protein translated from Met54 contains 160 amino acid residues. The two protein products of the VHL mRNA are described in more detail in section 1.6.1. The promoter of the gene was identified in 1995.[183] With the isolation of the 3' untranslated region in 1996, together with the known promoter area, exons and introns, the complete sequence of the human VHL gene was finally identified.[184]

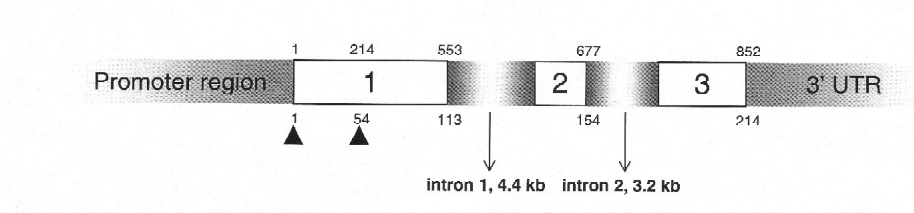

Fig. 8 Overview of the VHL gene, showing the three separate exons, the promoter region and the 3' untranslated region (UTR). The numbers above the exons refer to the numbering of the nucleotides, measured along the open reading frame. The numbers below refer to the codons. The two arrows depict the two start codons (Met1 and Met54). The introns 1 (4.4 kb) and 2 (3.2 kb) are shown (not to scale).

1.5.2 Two-stage mutation model in VHL tumours

The VHL gene is a tumour suppressor gene according to Knudson's 'two-hit' hypothesis: inactivation of both copies of the VHL gene is required for a normal cell to develop into a tumour cell (Fig. 9).[185] In VHL families, germline mutations at the VHL gene are transmitted from affected individuals to their offspring. Patients inherit a mutated germline copy of the VHL gene (the 'first hit') from the affected parent: they are heterozygous for the VHL germline defect. The remaining (normal) copy of the VHL gene is affected by an inactivating event (the 'second hit') at the somatic level: in such a cell, the lack of normal VHL gene product is thought to drive tumourigenesis. The occurrence of the second hit cannot be predicted and may occur at any age. In conclusion, whereas VHL manifests as an autosomal dominant trait, it can be considered as being inherited as a recessive trait at the cellular level.

Evidence for a two-stage model in VHL disease was first delivered by Maher et al. by statistical analysis of single VHL-related tumours and in tumours in VHL patients.[186] They found that the age incidence curves for cerebellar haemangioblastoma and renal cell carcinoma in VHL patients were compatible with a single mutation model in VHL disease, whereas the age incidence curves for patients with either a single cerebellar haemangioblastoma or renal cell carcinoma suggested a two-stage mutation process. The mean age at diagnosis of VHL-related tumours was significantly younger in VHL patients than in sporadic patients. VHL patients with cerebellar haemangioblastoma were diagnosed at a mean age of 29 years and sporadic patients at a mean age of 48 years; similarly patients with renal cell carcinoma were diagnosed at 45 years (VHL patients) and 62 years (sporadic). In 1998, the two-stage model was also confirmed in retinal haemangioblastoma.[187] It was demonstrated that retinal haemangioblastoma not associated with VHL disease occurred at a later age than in VHL patients (48 years vs. 25 years). In addition, the age incidence curve for retinal haemangioblastoma in VHL patients fitted a first-order equation (indicating a single somatic mutation), whereas solitary haemangioblastoma found in sporadic patients fitted a second-order equation, suggesting two separate somatic mutations.

For other tumour suppressor genes (for example APC),[188] it is generally accepted that the second mutation arises independently in many sites. This is also illustrated by studies of genetic changes between primary renal cell carcinoma and metastases. Renal cell carcinoma progression from non-metastatic primary tumours to metastasis is

driven by an accumulation of genetic changes.[189] An evaluation of the clonal relationship between primary and metastatic renal cell carcinoma by comparative genomic hybridisation revealed losses of chromosome regions 3p, 4q, 6q, 8p and 9p.[190] The most common gains were detected for chromosome regions 17q and Xq. 32% of the metastases were genetically almost completely different from the primary tumour. Loss of 3p was detected in 63% of the primary renal cell carcinoma, but in only 25% of the metastases. It was hypothesised that a second copy of 3p can be regained as a result of mitotic reduplication of the remaining allele of 3p.[191] Such a duplication could provide a growth advantage by an elevated expression of a putative oncogene on 3p.

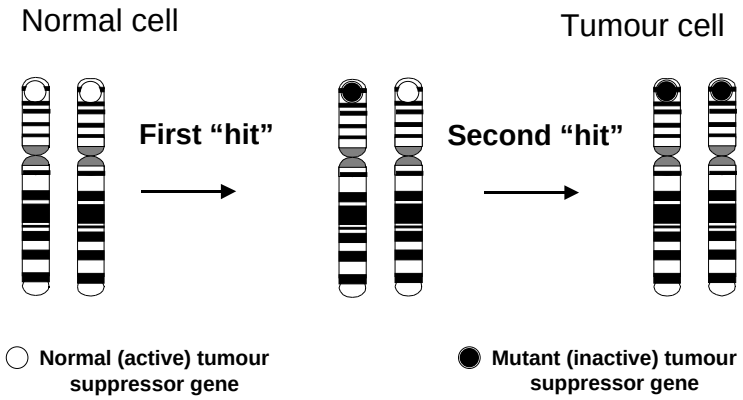

Fig. 9 The two hit model of the VHL tumour suppressor gene. In cells from carriers of a VHL germline mutation one additional somatic hit is required for the initiation of tumourigenesis. In a tumour cell from sporadic patients with a VHL-related tumour two separate somatic hits are involved.

Similar to other tumour suppressor genes, the genotype of each typical VHL tumour in a VHL patient should arise by a loss of the wild-type VHL allele, while maintaining the mutated allele.[192] Since 1993, VHL-associated tumours could also be studied on the molecular genetic level. Loss of heterozygosity (LOH, caused by deletion, non-disjunction, somatic recombination, etc.) at the chromosomal VHL locus (3p) has been demonstrated in: cerebellar and retinal haemangioblastoma,[38,47,193] renal cell carcinoma,[70,175] phaeochromocytoma,[194] pancreatic microcystic adenoma,[195] pancreatic (neuroendocrine) islet cell tumours,[112] and endolymphatic sac tumours.[196]

Tumour types that display frequent chromosome 3 allele loss, but which do not occur in VHL disease (e.g. lung, ovarian, testicular, head and neck, and breast cancers), do not have VHL gene mutations.[197-199] This suggests that several tumour suppressor genes map to chromosome 3p. So far, no other genes apart from the VHL gene, have been isolated from this region.[200]

1.5.3 Detection of mutations in the VHL gene

Methods of detecting germline mutations in VHL patients most commonly include direct sequencing of the coding region of the VHL gene, Southern blot analysis, and fluorescence in situ hybridisation (FISH). It was shown that Southern blot analysis using a control probe was a sensitive method of detecting deletions encompassing the entire VHL gene (see section 3.2). No point mutations, micro deletions or -insertions were found in the first 54 or the last 13 codons of the VHL gene (see also Fig. 12). A hot spot for VHL germline mutations is readily visible in the beginning of exon 3, or more specifically at nucleotide 712/713 (codon 167). The promoter region and 3' UTR have been investigated (also in Dutch VHL families, unpublished data), but failed to reveal any further germline mutations.[201,202]

In VHL families where the above techniques fail to identify a germline mutation, genetic linkage studies with polymorphic markers and intragenic polymorphisms may be of help in diagnosing the disease in at-risk family members. The most informative microsatellite markers are D3S1317, D3S1038, D3S587 and D3S1435, which lie within a 500 kb region of the VHL gene.[172] Intragenic polymorphisms lie at the 5' end of the gene (VHL19A/G),[203] and at the 3'UTR region (VHL1149A/G).[204] A *Taq*I or *Pst*I polymorphism can be identified with Southern blot analysis.[205] Olschwang et al.[206] demonstrated that six microsatellite markers surrounding the VHL gene resulted in an accuracy of risk assessment in 90 of 99 (91%) asymptomatic individuals from 26 VHL families. However, linkage studies cannot be applied in confirming the diagnosis in relatives of sporadic cases or in families where the necessary samples are not available.[8]

1.5.4 Germline mutations in VHL families

Since the identification of the VHL gene in 1993, the success rate of detecting germline mutations has gradually risen to 80% in VHL families in 1996.[86,202,203,207] By the end of 1998 VHL germline mutation analysis in well-defined families had reached a virtually 100% success rate in a study performed by the National Cancer Institute and the University of Pennsylvania in the USA.[173] Several groups reported VHL families where mutation detection failed to identify the disease-causing mutation. However, improvement in mutation detection approaches detected germline mutations in 10 remaining families from Germany and 14 from Italy. In the Netherlands we have also identified VHL germline mutations in all the large - with more than two patients - VHL families (see section 3.1). This supports the concept that VHL is a genetically homogenous disease with clinically well-defined VHL families.

Many different intragenic VHL germline mutations have been detected (Fig. 10 and 12). Most VHL germline mutations are unique to a small number (one or two) of families, suggesting that most of these mutations are of recent origin.[86] Several recurrent VHL mutations were found to occur in multiple, unrelated families (i.e. the possibility of a founder effect was ruled out).[172] Missense mutations are found in 40% of the families with an identified VHL germline mutation, i.e. a mutation that leads to an amino acid substitution in the VHL protein product. Large deletions account for one-third of the germline mutations, of which approximately 30% (or some 10% of all VHL germline mutations) are deletions encompassing the entire gene. Microdeletions

(1-18 bp), insertions (1-8 bp), splice site and nonsense mutations predicted to lead to a truncated protein are found in the rest (i.e. some 27%) of the families.

1.5.5 Somatic mutation spectrum

In non-familial cancers, tumourigenesis is thought to be initiated by independent somatic alteration of both alleles of a tumour suppressor gene (Fig. 9).[192] Somatic VHL gene mutations (57%) and allele loss (98%) are frequent events in clear cell renal cell carcinoma from sporadic patients[198,208,209], but also in central nervous system haemangioblastoma,[210-212] suggesting that the VHL tumour suppressor gene plays a role in their tumourigenesis. Different mutational mechanisms lead to the inactivation of the VHL gene in renal cell carcinoma, including loss of heterozygosity, small intragenic mutations, or hypermethylation.[213-215] However, somatic VHL mutations are uncommon in phaeochromocytoma.[198,216]

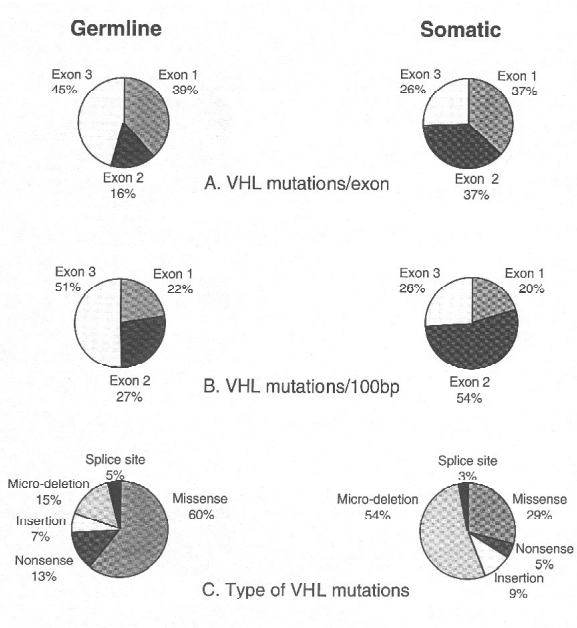

Fig. 10 Graphical presentation of the difference between germline mutations (left) in the VHL gene and somatic mutations (right) found in renal cell carcinoma from sporadic patients. Please note that partial and entire deletions of the VHL gene are not included in these figures.
A Division of 241 germline mutations and 181 somatic mutations over the individual exons (1-3).
B Comparison of germline mutations with somatic mutations per 100 basepairs (bp) for the three VHL gene exons. This illustrates a relatively high frequency of germline mutations in exon 3 and a hot spot for somatic mutations at exon 2.
C Distribution of type of VHL mutation. Missense mutations form the majority of germline mutations, whereas a large proportion of somatic mutations concern micro-deletions.

The distribution of somatic mutations in the VHL gene differs from the distribution of germline mutations (Fig. 10). In contrast to the germline mutations that are mainly concentrated in exons 1 and 3,[86] a relatively large proportion of the somatic mutations is clustered in exon 2.[198,208,209,217-219] Missense mutations represent 60% of the germline mutations and 29% of the somatic mutations in the VHL gene (note that partial and entire deletions of the VHL gene are not included in these figures). Consequently, a relative higher frequency of mutations predicted to result in a truncated protein are found in somatic mutations (71%) compared to germline mutations (40%).

1.5.6 Genotype-phenotype correlations in VHL disease

VHL disease exhibits allelic variation, in that many types of mutations, known as allelic variants, are scattered throughout the VHL gene. From a clinical point of view, VHL disease is also a variable disorder: inter- as well as intrafamilial variability in the clinical expression of the disease is common. A clinical classification of four VHL phenotypes has been proposed, based on the presence or absence of renal carcinoma and phaeochromocytoma (see table 2).[86,203]

Table 2 Classification of VHL disease

Type	CNS haemangioblastoma	Retinal haemangioblastoma	Renal cell carcinoma	Phaeochromocytoma
I	present	present	present	absent
IIA	present	present	absent	present
IIB	present	present	present	present
IIC	absent	absent	absent	present

There is evidence of relationship, to a limited extent, between the specific VHL germline mutation (the 'genotype') and the clinical manifestation of the disease (the 'phenotype'). Accordingly, some interfamilial clinical variability in VHL disease can be explained by differences between the respective VHL germline mutation. In 1996, Zbar et al. noted that 96% of the VHL families with deletions, microdeletions/insertions, splice site, or nonsense mutations did not manifest phaeochromocytoma (VHL type I), while 92% of VHL families with phaeochromocytoma (VHL type II) carried missense mutations in the VHL gene (Fig. 11).[86] However, the recent (1998) improvement in methods detecting deletions of the VHL gene using quantitative Southern blot analysis has revealed that the differences between germline mutations found in VHL types I and II are not that distinct, but still significant (Fig. 11). Missense mutations were found in 69% of the VHL type II families and in 27% of the VHL type I families.[173]

VHL type II is divided into three subtypes (A, B and C, see table 2) Most families have type IIB, a phenotype with renal cell carcinoma. A few specific missense mutations lead either to VHL type IIA, a phenotype without renal cell carcinoma;[203,207,220] or to IIC, a phenotype with only phaeochromocytoma.[82,90,221-227] The monitoring and management of patients may be adjusted based on knowledge of the specific VHL mutation. These genotype-phenotype correlations may have not only clinical significance, but may also indicate functional domain differences within the VHL gene.

In 1999, the three-dimensional structure of the VHL protein was published (see section 1.6.1 for more details).[228] This study provided structural and functional insights into the mechanism by which certain missense mutations lead to a phenotype with

phaeochromocytoma and others to a phenotype without phaeochromocytoma. VHL type I (not associated with phaeochromocytoma) missense mutations frequently map to the beta domain hydrophobic core (Fig. 12), and are thought to cause a complete unravelling of the VHL protein structure.[228] VHL type II (associated with phaeochromocytoma) missense mutations map either to residues that contact Elongin C or to an area that forms the putative substrate for the ubiquitination binding site (see 1.6.2.2).[228] Accordingly, type II mutations are thought to cause mostly local defects and not a total loss of function. For example, the alpha domain contains the most frequently mutated amino acid (Arginine 167), which in most families carrying this mutation results in a phenotype with phaeochromocytoma.[86]

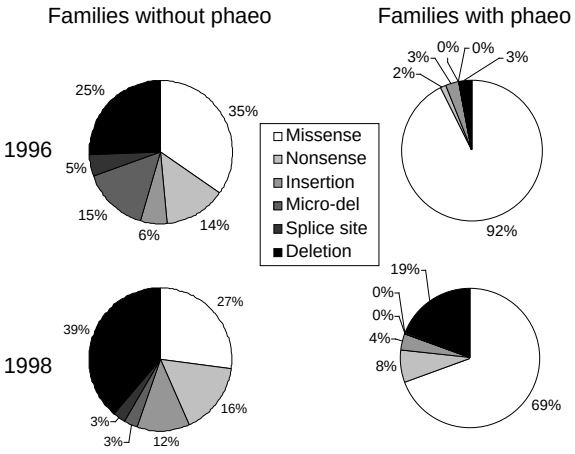

Fig. 11 Mutations found in families with phaeochromocytoma (left) and without phaeochromocytoma (right). The upper pie charts depict the germline mutation spectrum of the VHL gene as reported in 1996;[86] the lower pie charts show an altered mutation spectrum after quantitative Southern blot analysis improved the detection rate of deletions in the VHL gene.[173] Families with phaeochromocytoma have predominantly (specific) missense mutations, whereas families with all types of germline mutations (including missense mutations) have no phaeochromocytoma.

One hypothesis is that the development of phaeochromocytoma may involve a dominant negative effect of the mutant VHL protein. Since VHL has two protein binding sites, a mutant having a defect in only one site (for example, binding to Elongin C) may isolate key components of the biochemical pathway (substrates for ubiquitination) in which VHL is involved.[228] Alternatively, tumourigenesis may be induced by a gain of VHL function in particular cell types.[229] Mutations that lead to a partial loss of function may ensure the viability of certain cell types, due to a residual function of VHL. However, if these explanations were true, then the germline mutation in the VHL gene would be sufficient to cause phaeochromocytoma and a second hit would not be required for tumourigenesis. Apparently, the product of the VHL gene must have multiple and tissue-specific functions (see section 1.6.2).[200]

Nevertheless, patients with identical VHL germline mutations may display different phenotypes, indicating that the issue of genotype-phenotype correlations in VHL is complex. In 1998, evidence was provided that genetic factors (so called modifier genes) and environmental influences play an additional role in the clinical expression of VHL germline mutations, including the occurrence of retinal haemangioblastoma.[230]

First-degree family members (sharing half the genome) showed a significant correlation with regard to the number of ocular haemangioblastoma, in contrast to more distant family members. This suggests that modifying factors are more likely to be shared between closely related family members than between more distant family members. Moreover, individuals with ocular haemangioblastoma had a significantly increased incidence of cerebellar haemangioblastoma and renal cell carcinoma (hazard ratios 2.3 and 4.0, respectively), suggesting that the same modifying factors appear to influence susceptibility to three of these major VHL manifestations.[230]

Environmental factors may also influence genotype-phenotype correlations. A current opinion is that smoking (particularly in males) is correlated with the development of renal cell carcinoma.[231-233] In addition, there is an increased incidence of renal cell carcinoma in certain professions (e.g. among workers exposed to asbestos,[234] trichloroethene,[235] leather workers,[236] fire fighters and painters,[237]) and a correlation has also been found with obesity and hypertension.[232,233]Further evidence for the influence of toxic factors was provided by comparing missense mutations occurring as a germline mutation in the VHL gene and those occurring as somatic mutations in renal cell carcinoma from sporadic patients (Fig. 10). In the latter group 68% of the missense mutations corresponded to transversions, versus 35% for the VHL germline mutations.[219] Since transitions are more common nucleotide substitutions, transversions may indicate the influence of external factors. These findings support the hypothesis of the involvement of environmental factors in the aetiology of sporadic renal cell carcinoma in industrial countries.[231,232,236]

1.5.7 Germline mutations in sporadic patients with VHL-related tumours

In addition to VHL families a diagnosis of VHL disease should also be considered in sporadic patients with a VHL-related tumour (see sections 3.3 and 3.4). The diagnosis of VHL disease is often delayed because patients do not fulfil diagnostic criteria, and the family history may be cryptic or uninformative. In particular, in patients with VHL-related tumours that manifest multiply, bilaterally, familial, or at a young age, a VHL germline mutation may be anticipated.

VHL germline mutations are found in approximately 3% of patients with phaeochromocytoma without family history, and hence isolated tumours.[238] A higher percentage of VHL germline mutation was found in familial phaeochromocytoma (45%) and bilateral tumours.[239] In patients with a single cerebellar haemangioblastoma approximately 10% (2 out of 20) of the patients carried a VHL germline mutation.[211,240] Germline mutations were identified in 3 of 189 (1.6%) unselected renal cell carcinoma patients. Once again, the risk was higher in the following clinical situations of renal cell carcinoma: familial 3 out of 3 (100%); 1 out of 10 patients with bilateral tumours (10%); and patients younger than 50 years old, 1 out of 33 (3%).[241]

1.6 VHL protein (pVHL)

The VHL protein (pVHL) is widely expressed in normal human tissues.[160-162] The pVHL is even expressed in organs not at risk for the disease, suggesting a role for pVHL that goes beyond the organs involved in the disease. Endothelial cells grown in various angiogenic conditions (atherosclerotic lesions, tumour metastasis and inflammatory processes) strongly express pVHL; however, normal endothelial cells do not.[162] In human embryos pVHL was expressed in all three germ layers, with strong expression noted in the central nervous system, kidneys, testis and lung.[242]

The intracellular localisation of the pVHL appears to be related to a novel physiological control mechanism: cell density.[243,244] In sparse cultures, pVHL is mainly found in the nucleus, whereas it can be found in the cytoplasm of more confluent cells. In addition, the subcellular localisation of pVHL is cell-cycle dependent.[245] In G1/G0-phase, pVHL is localised exclusively in the nucleus, whereas the majority of cells in the S-phase show a diffuse cytoplasmatic staining. It was hypothesised that pVHL may need to traffic between the nucleus and the cytoplasm to perform its functions.[246] Lee et al. found that the trafficking between the two cellular components is lost with a deletion of exon 2, but not with deletion of other parts of the VHL gene.

1.6.1 Structure of pVHL

The open reading frame of the VHL gene is unlike that of any known gene. In the N-terminus of this open reading frame there is an acidic pentameric repeat that is somewhat similar to a region of the *Trypanosoma brucei* membrane protein.[30] The corresponding region of the VHL gene is not frequently mutated in VHL families and is not highly conserved between human and rodents.[243,247] In contrast, the C-terminus (downstream) part of pVHL is very similar in human and rodents. Many germline mutations that are thought to lead to a truncated protein occur in the C-terminal part of pVHL,[86] and may therefore impair tumour suppression function. This is supported by the finding that VHL proteins mutated in this C-terminus region, in contrast to wild-type pVHL, are unable to suppress tumour formation when introduced in renal carcinoma cells lacking both copies of the VHL gene.[248,249]

The VHL gene encodes a 213 amino acid protein with a molecular weight of about 28-30 kiloDalton (pVHL30).[248] A second VHL gene product, with an apparent molecular weight of 18 kiloDalton (pVHL18), arises from alternate translation initiation at a second start codon (Met54) within the VHL open reading frame.[250-252] It was ruled out that the VHL18 was a proteolytic fragment of pVHL30, or that pVHL18 arises because of alternative splicing.[251] pVHL30 is predominantly found in the cytosol and to a lesser extent in the nucleus or at the cell membranes.[160,161,248,253] In contrast, pVHL18 is equally distributed between nucleus and cytosol.[251] Both VHL gene products are biologically active. They both bind to the Elongin proteins and they both inhibit the production of hypoxia-inducible proteins, such as vascular endothelial growth factor and glucose transporter-1, when introduced into renal carcinoma cells lacking the wild-type VHL allele.[251] Reintroduction of either pVHL30 or pVHL18 into renal carcinoma cells lacking the wild-type VHL allele suppresses their ability to form tumours in nude mice. This brings us to the function of the VHL protein: *why does loss of function of the VHL gene give rise to tumours?*

1.6.2 Possible functions of pVHL

Based upon its direct role in the initiation of *both* familial and isolated renal tumours (see 1.5.2), VHL is considered to be a 'gatekeeper' gene in renal cells.[254] According to Kinzler and Vogelstein, gatekeepers are genes that directly regulate the growth of tumours by inhibiting growth or death.[254] Each cell type has only one (or a few) gatekeepers. Inactivation of a given gatekeeper leads to a the very specific tissue distribution of cancer. By microdissecting material from individual lesions it has been shown that loss of the wild-type allele and retention of the inherited, mutated VHL allele occurs in both cystic lesions and renal cell carcinoma from VHL patients.[70]

Several studies have addressed the identification of the normal physiological functions of the VHL gene and its protein product by investigating proteins that are capable of binding to pVHL.

1.6.2.1 pVHL and transcription elongation

The interaction of pVHL with the Elongins C and B has provided a possible clue about the normal function of the VHL protein. Based on *in vitro* studies, it has been postulated that pVHL plays a role in regulating the transcription elongation.[243,255-258] Transcription (the process of reading off DNA sequence by a polymerase and creating a corresponding RNA) elongation is mediated by the initiation factor RNA polymerase II. When normal pVHL is present in the cell, binding of pVHL to Elongins C and B (VCB complex), causes RNA polymerase to pause during transcription at several sites along a gene. Most of the tumour-derived mutations destabilise the VCB complex.[255,256,259,260] Failure by pVHL to sequester Elongins C and B may result in interference with elongation control mechanisms, leading to upregulation of specific target genes. At this stage in 1995, it was unknown which gene products where regulated at the transcription level by this mechanism, or how the absence of functional pVHL may have influenced other proteins.

By transfecting rat phaeochromocytoma cells with either wild-type or mutant human pVHL it was demonstrated, for the first time *in vivo*, that regulation of transcript elongation is pVHL-dependent.[261] These two VHL proteins have an opposite effect on regulating the expression of the tyrosine hydroxylase (TH) gene, that is a rate-limiting enzyme in catecholamine synthesis. Whereas the wild-type pVHL leads to a five-fold repression of levels of TH mRNA and protein, a truncated (1-115) pVHL induced a three-fold accumulation of TH mRNA and protein.[261] The investigators demonstrated that in the presence of wild-type pVHL, the TH gene is repressed by inhibition of RNA elongation. During this elongation pause the pVHL is sequestered by the Elongins B and C. In contrast, the mutant pVHL stimulates TH gene expression by increasing the efficiency of TH transcript elongation. Moreover, this study confirmed the anticipated dominant negative activity of pVHL mutants in phaeochromocytoma cells.[261]

1.6.2.2 pVHL and ubiquitination

In 1999 Stebbins et al. studied the three-dimensional structure of the VCB complex by co-expressing the three proteins in *Escherichia coli*.[228] In the structure of this ternary complex, Elongin C binds Elongin B and VHL on two separate binding sites,

whereas Elongin B and VHL do not interact (Fig. 13). The VCB complex binds, in its turn, to Cullin2 (Cul2).[253,260] Elongin C and Cul2 are similar to two yeast proteins, Skp 1 and Cdc53, respectively.[228,253,262] These latter proteins form multi-protein complexes with adapter subunits called F-box proteins that recognise different substrates through specific protein-protein interaction domains.[263] These SCF (i.e. Skp1-Cdc53/Cul1-Fbox) complexes target other proteins in the cell for degradation via a process called ubiquitination. In this process the target is bound by a protein called ubiquitin, that labels a protein to be degraded. The destruction itself takes place inside a proteasome, a protein-digesting complex. Interestingly, Elongin B proved also to harbour an ubiquitin-like structure.[260,264] So far, no other proteins than Elongin C have been found that bind Elongin B.[265]

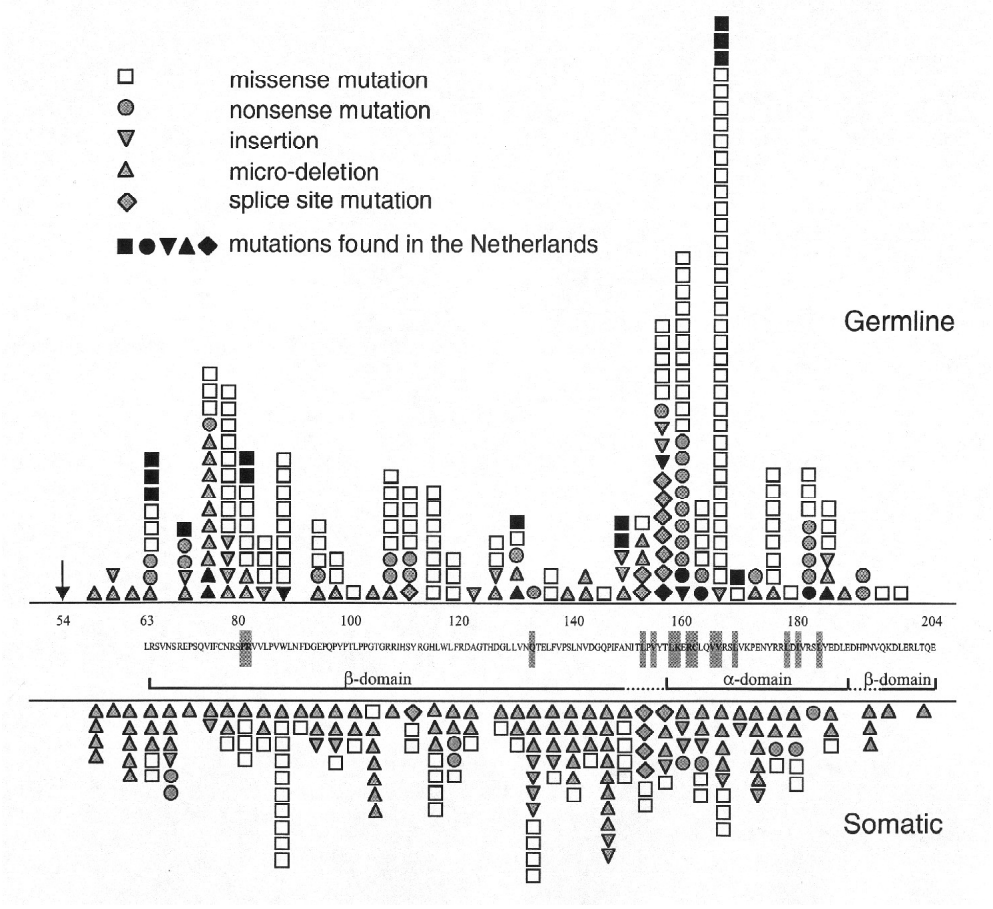

Fig. 12 Spectra of VHL gene mutations (deletions are not shown) related to functional domains. The sequence of the amino acids is depicted (between the lines) with germline mutations (top) and somatic mutations (bottom) in the VHL gene. The germline mutation data derive from VHL families reported by Zbar et al.[86], the somatic mutations derive from various studies on renal cell carcinoma found in sporadic patients.[198,208,209,217-219] The position of each mutation in the coding region is depicted by a symbol representing the specific mutation (see legend). The mutations are pooled per 10 nucleotides. Mutations that are located close to the intron-exon boundaries, for example splice site mutations, are placed in their exon of origin.

The VCB complex targets substrates for degradation. Boxes around the amino acids indicate residues that make contact with Elongin C (Fig. 12). The VHL gene has two functional domains: a domain rich in beta-sheets (beta-domain) and a smaller alpha-helical domain (alpha-domain). The beta domain harbours a spot that binds a, so far unknown, substrate of the VCB complex. A large portion of the alpha-domain and a small portion of the beta-domain interact with Elongin C. Mutations in exon 1 and 2 may affect the binding to substrates by the VCB complex, which are conjugated to ubiquitin for degradation. Mutations in exon 3 would disassemble the VCB complex. In either case, the substrates would no longer be targeted for degradation.[262]

The pVHL has two domains: a ~100 amino acids long beta-sheet domain and a ~35 amino acids long alpha-helical domain (Fig. 12).[228] The so-called SOCS-box (a sequence motif identified in the suppressor of cytokine signaling-1 (SOCS-1) protein sequence motif) in the alpha domain is the key contact to Elongin C.[266] Thus, the alpha domain binds to Elongin C which, in turn, binds to Elongin B and Cul2. The beta domain is thought to be the place where the target protein binds. About half of the mutations found in tumours map to the alpha domain and its specific residues that contact Elongin C, while other mutations affect mainly the beta domain.

Kamura et al. presented further evidence for the role of pVHL in ubiquitination.[267] It was shown that endogenous VHL complex in rat liver also includes Rbx1, an evolutionary conserved protein that interacts with Cullins. Rbx1 regulates SCF to degrade proteins.

However, the substrate that would be degraded by the VCB-Cul2-complex is not yet known. Since VHL disease is characterised by the development of extremely vascularised tumours, factors that are involved in angiogenesis are suitable candidates.

1.6.2.3 Ubiquitination and hypoxia-inducible mRNAs

Possible substrates that are targets for ubiquitination by the VCB complex include mRNA-binding proteins that regulate the stability of hypoxia-inducible factors, such as: vascular endothelial growth factor (VEGF), platelet-derived growth factor-beta, and glucose transporter-1, or the hypoxia-inducible transcription factor HIF-1alpha regulated by ubiquitin-dependent degradation.[262]

In VHL patients, not only haemangioblastoma but also renal cell carcinoma show an abundance of blood vessels. The well-vascularised phenotype of these VHL tumours suggests that inactivation of the VHL gene induces either upregulation of an angiogenic factor or downregulation of an inhibitor of angiogenesis.[268] In 1995, evidence was found that VEGF was upregulated in VHL-associated haemangioblastoma[269] Benjamin and Keshet demonstrated that expression of VEGF in a mouse brain causes blood vessels to proliferate and form a haemangioblastoma-like structure.[270] Overproduction of VEGF mRNA is a feature of many human cancers and has been primarily linked to hypoxia. However, renal cell carcinoma lacking functional pVHL produce high levels of hypoxia-inducible mRNAs, such as the mRNA encoding VEGF, under hypoxic as well as normoxic conditions.[249,271,272] This means that cells lacking pVHL act as though they are deprived of oxygen, whether they are or not.[265]

Hence, the highly vascular nature of VHL-associated neoplasms may be due, in part, to dysregulation of hypoxia-inducible mRNAs following loss of function of the VHL protein. A paradoxical finding in this respect was that a 'knock-out' (with two inactive VHL alleles) mouse model died *in utero* between 10.5 and 12.5 days of gestation, most likely due to an impairment of placental vasculogenesis.[273]

Maxwell and co-workers showed that pVHL binds to two transcription factors, hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha, and targets them for destruction.[274] These proteins are transcription factors that regulate production of mRNAs for hypoxia-inducible factors as VEGF.[275] The alpha-subunits of HIF are rapidly degraded by the proteasome under normal conditions, but are stabilised by hypoxia.[276] In VHL-defective cells, HIF-alpha-subunits are stabilised (i.e. not degraded) in well-oxygenated conditions. When normal pVHL is introduced, oxygen-dependent instability is again restored, leading to degradation of HIF. Maxwell et al. concluded that constitutive HIF activation may underlie the angiogenic phenotype of VHL-related tumours.[274]

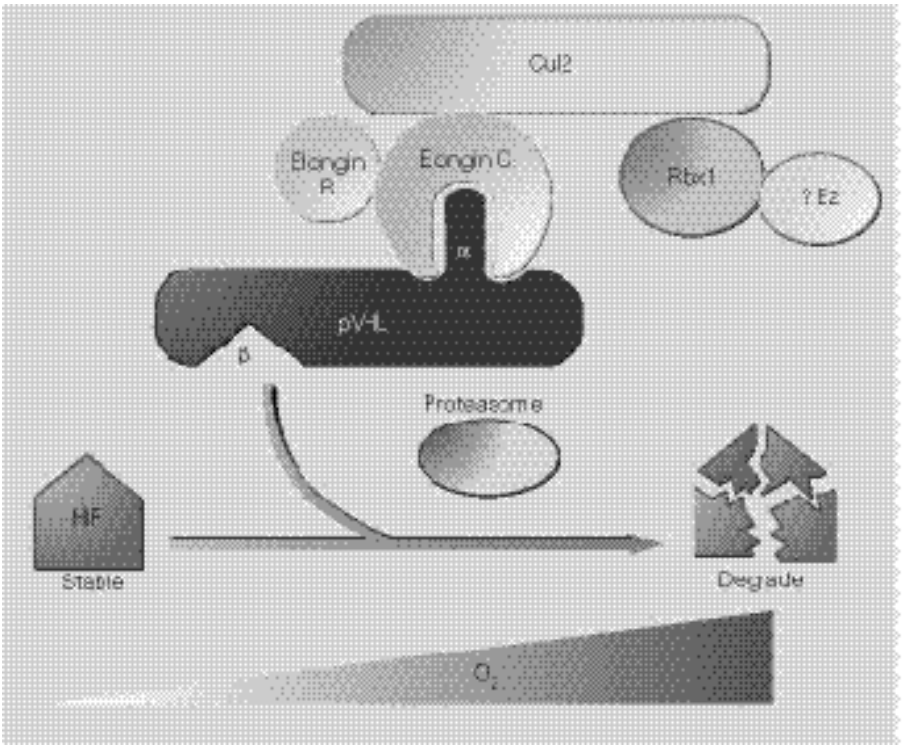

Fig. 13 Molecular basis of VHL disease (Dr. W.G. Kaelin, Jr.), reprinted with permission from Nature[265] copyright (1999) Macmillan Magazines Ltd.
pVHL binds to Elongin C, Elongin B, Cullin2, Rbx1 and E2 (an unknown ubiquitin-conjugating enzyme), and forms a VCB complex. This complex degrades alpha subunits of hypoxia-inducible factor (HIF) in an oxygen-dependent manner. So far, it is unknown whether VCB complex directly or indirectly binds to HIF. Degradation takes place in a proteasome via ubiquitination. Cells that lack pVHL do not degrade HIF in response to oxygen. Normally HIF controls the production of factors that promote formation of blood vessels. In the presence of a defective VCB complex, HIF is not degraded and results in uncontrolled proliferation of blood vessels.

So the simplest model would be one in which pVHL, through its association with Elongin B, Elongin C and Cul2, regulates the stability of HIF-1 and -2[265] (Fig. 13). If these proteins are not degraded, the result is excessive formation of blood vessels. However, important questions remain. Is HIF the substrate of pVHL and does the VCB complex thereby ubiquitinate HIF? Does pVHL bind HIF directly or indirectly and does the VCB complex also bind other proteins? Moreover, how can the VEGF-hypothesis account for the occurrence of cystic lesions in carriers of a VHL germline mutation?

1.6.2.4 Interaction with fibronectin

Ohh et al. showed that pVHL also interacts with fibronectin.[277] Fibronectin is normally found in the extracellular matrix of cells where it can interact with the surface receptors of other cells. Cancer cells characteristically produce low levels of fibronectin and fibronectin matrix involvement in contact inhibition and metastasis has been suggested.[278-280] Fibroblasts from both VHL knock-out mice as wells as tumour cells missing both VHL alleles failed to assemble a fibronectin matrix.[277] Reintroduction of the wild-type pVHL partially corrected this defect. This observation would suggest that the fibronectin interaction of pVHL might also be related to its tumour suppressor activity.[200]

1.6.2.5 Other pVHL-binding proteins

In addition, other proteins have been found that bind or are associated with pVHL, but to which no functional significance could be attributed. These include: VHL binding protein 1 (VBP-1)[281], transcription factor Sp1[282], and protein kinase C (PKC).[283,284] It remains to be elucidated whether these observations indeed represent biologically significant interactions.

1.6.2.6 Other downstream targets of pVHL

pVHL regulates, at least indirectly, the levels of variety of mRNAs other than hypoxia-inducible ones such as VEGF. These include: a glucose transporter (GLUT1), transforming growth factor alpha (TGF alpha), platelet-derived growth factor B (PDGFb) and carbonic anhydrases (CA 9 and 12).[229,271,285] CA9 and CA12 might affect peritumoural pH and thereby indirectly influence tumour growth.

1.6.3 Anticancer therapy

Neovascularisation appears to be one of the crucial steps in a tumour's transition from a small, harmless cluster of mutated cells to a large, malignant growth, capable of spreading to other organs throughout the body.[286,287] Antiangiogenic drugs are being developed that aim to stop new growth and should, in theory, do no harm to blood vessels serving normal tissue in which the vascularisation has stabilised. However, an important disadvantage of antiangiogenic therapy is that it is likely to impact physiological angiogenesis, occurring in processes such as in wound healing and menstruation. Overproduction of VEGF mRNA is a feature of many human cancers and vasculogenesis and is vital for the majority of cancers to thrive.[288,289] Accordingly, manipulation of pVHL and its associated proteins is a potentially productive area for developing anticancer therapies. Although pVHL may be directly responsible for a

rare disease occurring in only ~ 1:40,000 persons, a fundamental understanding of its function may lead to it playing a significant role in the treatment of a much larger group of cancer disorders.

Besides pVHL and its associated binding proteins, other compounds have been proposed for their ability to suppress angiogenesis, particularly in VHL disease. Angiostatin is a potent inhibitor angiogenesis, it reduces tumours to microscopic size and holds them in a dormant state. Angiostatin is a fragment of a larger protein plasminogen, which is not itself antiangiogenic. Thus, when physiological angiogenesis has to be stopped plasminogen is degraded in order to make angiostatin available.[286,287] Additionally, there are two studies being carried out at present on the medical treatment of VHL-related tumours. A team under Dr. William Kaelin of the Dana Farber Cancer Research Institute is proposing to conduct a clinical trial of a new drug designed to inhibit VEGF (see www.vhl.org). Dr. Gross in Jerusalem, Israel, has demonstrated that treatment of mice grafted with paraganglioma from a VHL patient with linomide (quinoline-3-carboxamide) reduced tumour size and weight.[31] Histological examination of the tumours showed marked avascularity and indicated an antiangiogenic effect of the drug. Safe doses of these drugs still have to be determined to enable clinical trials with VHL patients.

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Hanahan and Folkman postulated the idea of an angiogenic "switch" based on the observation that a balance of positive and negative regulatory factors play a role in angiogenesis.[288] The balance of pro-angiogenic and anti-angiogenic factors is important in tumour dormancy and control of micrometastasis. For example, in a small hypoxic tumour that is genetically unstable and exposed to environmental hostility, there will be a strong selection for mutations of genes (such as p53) that lead to resistance to cell death (apoptosis).[289] Subsequently, the apoptosis rate remains high until angiogenic promoting mutations occur (such as VHL), which, in turn, provides the growth advantage of tumours by reducing apoptosis.[289]
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Von Hippel-Lindau disease: strategies in early detection
(renal-, adrenal- and pancreatic masses)

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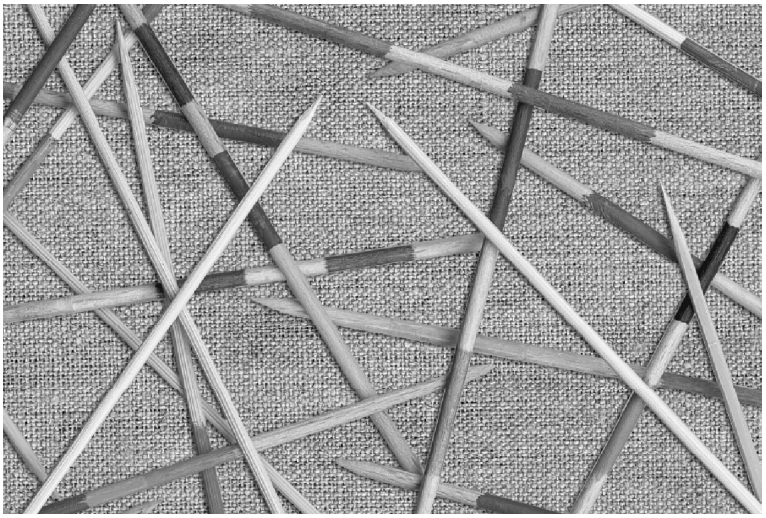

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Abstract

Von Hippel-Lindau (VHL) disease is a hereditary syndrome characterised by a predisposition for bilateral and multicentric retinal haemangioblastoma, haemangioblastoma in the central nervous system, renal cell carcinoma, phaeochromocytoma, islet cell tumours of the pancreas, and endolymphatic sac tumours, as well as cysts in the kidney, pancreas and epididymis. This review focuses on developments in the imaging of renal, adrenal and pancreatic masses in VHL disease. Radiology still has a central place in managing of VHL disease. Radiologists should therefore be aware of the importances of magnetic resonance imaging, computed tomography and ultrasound compared to other radiodiagnostic tools for these three organs. Since a conservative approach to the treatment of VHL lesions is now becoming more widely accepted, ongoing follow-up by careful radiological monitoring with ultrasound and especially magnetic resonance imaging, will play a central role in managing the disease. We also give an overview of recent advances in the molecular biology of VHL disease because the combination of imaging with (presymptomatic) DNA analysis has made early detection and monitoring of lesions possible and led to a reduction of morbidity and mortality.

Introduction

Von Hippel-Lindau (VHL) disease is a hereditary syndrome characterised by predisposition for bilateral and multicentric retinal haemangioblastoma, haemangioblastoma in the central nervous system, renal cell carcinoma, phaeochromocytoma, islet cell tumours of the pancreas, and endolymphatic sac tumours, as well as cysts in the kidney, pancreas and epididymis. VHL disease is a relatively rare disease, with an estimated incidence of between 1:31,000 to 1:53,000 in South Baden (Germany) and East Anglia (Great Britain), respectively.[1-3] The disease is inherited as an autosomal dominant trait with a high penetrance (almost complete by 60 years of age) and variable expression. The basis of familial inheritance of VHL disease is a germline mutation in a tumour suppressor gene, first identified in 1993.[4]

Most tumours in VHL patients show typical hereditary features. The tumours are often multiple or bilateral, and manifest at a young age. The median life expectancy is reduced to 49 years of age. At present, metastasis from renal cell carcinoma and neurological complications from cerebellar haemangioblastoma are the most common causes of death in VHL disease.[5-8] However, the life expectancy has strongly improved over the last years. Intensive radiological and clinical monitoring and advanced operation techniques have contributed to the reduction of both morbidity and mortality.

History

The German ophthalmologist Eugen von Hippel (1867-1938) is usually credited with the first full description of a retinal vascular abnormality.[9] In 1911 he named this abnormality angiomatosis retinae.[10] This condition was already reported in 1879,[11] and the microscopic appearance was described in a brother and sister in 1894.[12] The pathologist Arvid Lindau published a paper in 1926 describing 40 cases with cystic cerebellar tumours.[13] He associated angiomatosis retinae with cerebellar and spinal haemangioblastoma, and cysts of the kidneys, pancreas and epididymis. Lindau named this syndrome *central nervous system angiomatosis*. In 1951 Streif noted that in 1864 French physicians had reported the first probable VHL patient who died with brain and retinal tumours.[14] The initial reports on adrenal involvement in VHL disease appeared in 1953 and 1959 [15,16] Melmon and Rosen published the first major VHL literature review in 1964.[17] They stressed the importance of careful monitoring of the family and gave criteria for the clinical diagnosis of VHL disease.

Richards et al. diagnosed renal carcinoma with intravenous urography and angiography in two asymptomatic members of a VHL family in 1973,[18] and descriptions appeared of radiographic manifestations in various VHL families.[19,20] In 1977 Lee et al. advocated selective renal angiography for all VHL patients.[21] The technique of computed tomography (CT) was introduced in 1972 as head scanner and revolutionised diagnostic medicine of the whole body. Clinical imaging applications of nuclear magnetic resonance increased in the mid 1980's and is known as magnetic resonance imaging or MRI. Filling-Katz et al. reported in 1989 that Gadopentetate dimeglumine (Gd-DTPA) enhanced MRI gave the best results for assessing CNS lesions.[22]

A second revolution in the diagnosis of VHL disease was the identification of the VHL gene in 1993.[4] Presymptomatic DNA analysis and identification of carriers of

VHL germline mutations in families permits the progress of tumour development to be followed from a relatively early age, and optimises the time in which treatment is carried out.

Genetics

In 1988, the VHL gene was localised and mapped to chromosome region 3p25-26 using genetic linkage analysis in large VHL families.[23] A positional cloning strategy subsequently led to the isolation and identification of the VHL gene in 1993.[4] The VHL gene covers approximately 14,500 basepairs (bp) of genomic DNA on chromosome 3. The full-length VHL messenger RNA is a 4,700 bp molecule; an additional mRNA isoform is generated by alternative splicing involving exon 2.[24,25] The open reading frame is 852 bp long and contains two intragenic start codons. The protein-coding region translated from the first start codon (nucleotides 214-216) encompasses 639 bp, and is divided into three exons of 340, 123, and 179 bp (Fig. 1a). The promoter of the gene was identified in 1995.[26] With the isolation of the 3' untranslated region in 1996, together with the known promoter area, exons and introns, the complete sequence of the human VHL gene was identified. [27]

The VHL gene is a tumour suppressor gene according to Knudson's 'two-hit' hypothesis:[28] inactivation of both copies of the VHL gene is required for a normal cell to develop into a tumour cell. Different mutational mechanisms may lead to the inactivation of the VHL gene, including small intragenic mutations, loss of heterozygosity (i.e. deletion of a large part of the gene, or even of the entire VHL gene), or hypermethylation.[29,30]

In VHL families, germline mutations of the VHL gene are transmitted from affected individuals to their offspring. VHL disease has an autosomal dominant pattern of inheritance: children of a parent who is a carrier of a mutated VHL gene, have a 50% chance of inheriting the disease. Patients inherit a mutated germline copy of the VHL gene (the 'first hit') from the affected parent: they are heterozygous for the VHL germline defect. The remaining (normal) copy of the VHL gene is affected by an inactivating event (the 'second hit') at the somatic level: in such a cell, the complete lack of normal VHL gene product is thought to drive tumourigenesis. The moment of the second hit cannot be predicted and may occur at any age. Generally, tumour development in VHL patients occurs between the age of 20 and 40 years.

Structure and possible functions of the VHL protein product

The VHL gene encodes a 213 amino acid protein (pVHL) with a molecular weight of about 30 kiloDalton,[31] of which the normal function is not precisely known. The observation that inactivation of the VHL gene leads to tumour initiation suggests that pVHL plays a crucial role in the control of cellular proliferation in specific tissues, such as the kidney, retina, and adrenal gland.

In addition to the germline mutations identified in families with VHL disease, somatic mutations and/or loss of the VHL gene are frequently observed in sporadic (i.e. non-familial) tumours, including haemangioblastomas and renal cell carcinomas.[25,32] Based upon its direct role in the initiation of *both* familial and sporadic renal tumours, VHL is considered to be a 'gatekeeper' gene in renal cells.[33] According to

Kinzler and Vogelstein,[33] gatekeepers are genes that directly regulate the growth of tumours by inhibiting growth or inhibiting death. Each cell type has only one (or a few) gatekeepers. Inactivation of a given gatekeeper leads to a very specific tissue distribution of cancer. Several studies have addressed the identification of the normal physiological functions of the VHL gene and its protein product. The VHL protein has been shown to associate with at least five other proteins, namely CUL-2,[34] VBP1,[35,36] fibronectin (W.G. Kaelin, personal communication 1997) and the Elongins B and C. The interaction of pVHL with the Elongins has provided a possible clue about the normal function of pVHL. Based on these *in vitro* studies, it has been postulated that pVHL plays a role in regulating the transcription elongation.[37-41] Transcription (the process of converting DNA sequence into corresponding RNA) elongation is mediated by the initiation factor RNA polymerase II. When normal pVHL is present in the cell, binding of pVHL to Elongins B and C causes RNA polymerase to pause during transcription at several sites along a gene. Mutations of the VHL gene lead to absence of normal pVHL. Failure by pVHL to sequester Elongins B and C may result in an ongoing transcription activity. When genes involved in cell cycle control are regulated at the transcription level by this mechanism, the absence of functional pVHL may lead to abnormal cell proliferation.

Interestingly, the region of the VHL gene that encodes the Elongin binding domain (nucleotide 682-781)[39,42] is a hot spot for missense mutations: approximately 70% of VHL families have mutations predicted to disrupt VHL binding to Elongin (Fig. 1a).[43] This suggests that this region is critical for the normal functioning of the VHL protein. However, mutations leading to VHL disease are also found outside the Elongin binding domain, indicating that other regions of the VHL gene encode proteins that have another function.

The VHL protein is widely expressed in normal human tissues.[43a,44] The pVHL is even expressed in organs not at risk for the disease, suggesting a role for pVHL that goes beyond the organs involved in the disease. In human embryos pVHL was expressed in all three germ layers, with strong expression noted in the central nervous system, kidney, testis and lung.[45] The intracellular localisation of pVHL appears to depend on a novel physiological control mechanism: cell density.[41,46] In sparse cultures, pVHL is predominantly present in the nucleus, whereas it can be found in the cytoplasm of more confluent cells. The putative role of the pVHL in transcription elongation (as discussed above) and its ability to localise based on culture conditions suggests an ability to control cellular growth in response to environmental signals.[47] Recently, it was shown that the wild-type (normal) pVHL inhibits the production of hypoxia-inducible mRNAs, such as the vascular endothelial growth factor (VEGF) mRNA, under normoxic conditions.[31,48] In renal tumour-derived cell lines which lack normal pVHL, VEGF mRNA expression is increased.[48] Thus, the highly vascular nature of VHL-associated neoplasms may be due, at least in part, to dysregulation of hypoxia-inducible mRNAs following loss of function of pVHL.

In addition to these biochemical studies, a mouse model has been developed to analyse the function of the VHL gene.[49] Using targeted homologous recombination in murine embryonal stem cells, a so called ‘knock-out’ mouse model was generated for VHL carrying an inactivating mutation at the *Vhl* gene (the murine homologue of the

VHL gene). Heterozygous *Vhl* (+/-) mice survived beyond 15 months of age without evidence of spontaneous disease. However, homozygosity for the *Vhl* 'knock- out' allele leads to embryonic lethality. Apparently, the *Vhl* gene is required for normal embryonic development in the mouse. *Vhl* (-/-) embryos die *in utero* between 10.5 and 12.5 days of gestation, most likely due to an impairment of placental vasculogenesis.[49] This is in contrast to the observations that the wild-type VHL gene results in decreased VEGF levels and that mutations in the VHL gene are associated with richly vascularised VHL lesions.

VHL mutations

Germline mutations in the VHL gene are found in up to 80% of the VHL families.[50] In families with an identified VHL germline mutation, almost 60% have a missense mutation, i.e. a mutation that leads to an amino acid substitution in the VHL protein product. Large deletions account for 20%, and microdeletions, insertions and nonsense mutations are found in another 20%.[43,51] (Note: updated summaries of VHL germline mutations can be found on the World Wide Web at http//www.ncifcrf.gov/kidney and at http://www.uwcm.ac.uk/uwcm/mg/search/120488.html).

A large number of different intragenic (i.e. missense and nonsense mutations, splice defects, micro deletions and insertions) VHL germline mutations has been detected. Most VHL germline mutations are unique to a small number (one or two) of families, suggesting that most of these mutations are of recent origin.[43] However, several recurrent VHL mutations, which occur in multiple, unrelated families, have also been observed (Fig. 1a).

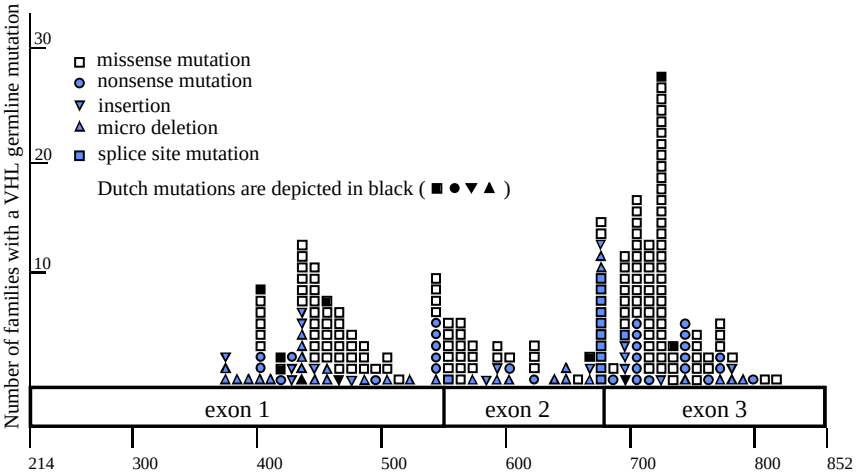

Fig. 1a Germline mutations identified in VHL patients from North America, Japan and Europe (including the Netherlands) are shown. Information was obtained from the World Wide Web page of the National Cancer Institute in Bethesda, USA (www.ncifrcf.gov/kidney) and the Clinical Genetics Centre in Utrecht, the Netherlands (unpublished data). The numbers of the nucleotides are shown along the X-axis, starting at the first start codon (nucleotide 214) and ending at the stop codon (nucleotide 852). VHL germline mutations were found between nucleotides 376 and 811. The number of families with a particular VHL germline mutation are shown along the Y-axis. Each symbol represents one family. Mutations found in Dutch VHL families are shown in black.

Genotype-phenotype correlations in VHL

As illustrated above, VHL disease is heterogeneous at the molecular level: mutations of all types are found, and they are scattered throughout the VHL gene. From a clinical point of view, VHL disease is also a heterogeneous disorder: inter- as well as intrafamilial variability in the clinical expression of the disease is common. Based on the presence or absence of renal carcinoma and phaeochromocytoma in VHL disease, a classification of three VHL phenotypes has been proposed: (1) renal carcinoma without phaeochromocytoma, (2) renal carcinoma with phaeochromocytoma, (3) phaeochromocytoma alone.[43]

There is evidence that, to a certain extent, a relationship exists between the specific VHL germline mutation (the 'genotype') and the clinical manifestation of the disease (the 'phenotype'). Such genotype-phenotype correlations may not only indicate important functional domains of the VHL protein, but may also have clinical significance. Based on the knowledge of the specific VHL mutation, surveillance and management of patients may be adjusted.

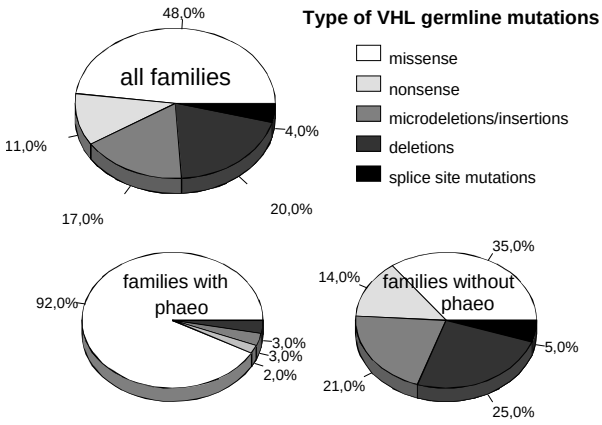

Fig. 1b Genotype-phenotype correlations in VHL disease. The pie charts show the total mutational spectrum and the association of mutations with phaeochromocytoma (based on Zbar et al. [43]).

Interfamilial clinical variability in VHL disease can partly be explained by differences in the family-specific VHL germline mutation. As shown in Figure 1b, almost all families (92%) with phaeochromocytoma (types 2 and 3) have missense mutations in the VHL gene.[43] In VHL families without phaeochromocytoma (type 1), most mutations (65%) are predicted to lead to either constitutional deletion of a VHL allele or synthesis of a truncated VHL protein (Fig. 1b). Zbar et al (1996) identified germline mutations in 300 of 469 VHL families and noted that 96% of the families with either deletions, microdeletions/insertions, splice site, or nonsense mutations were affected by VHL disease without phaeochromocytoma.[43] A few specific missense mutations lead to VHL phenotype with phaeochromocytoma, retinal and CNS haemangioblastoma without renal carcinoma.[52,53]

However, patients with identical VHL germline mutations may display different phenotypes, indicating that the issue of genotype-phenotype correlations in VHL disease is complex. It is likely that other genetic ('modifier' genes) and/or environmental factors (lifestyle, diet, smoking) may play a role in the clinical manifestation of VHL germline mutations.

Renal masses

In VHL patients, renal lesions can be divided into three different forms with cystic, combined cystic-solid and solid renal cell carcinoma (RCC) lesions.[54] Cystic lesions which can be single or multiple, can occur unilaterally but are mostly bilateral. They can grow either slowly or rapidly, but can also involute. The combined cystic-solid lesion, in which the solid (malignant) component gradually increases, may lead in turn to a solid RCC lesion.

RCC may present with haematuria or with back pain. However, most renal tumours are detected as an incidental finding in radiological screening performed for other reasons, while a growing number of renal lesions in patients is found by periodical monitoring of VHL families. If not identified by monitoring, VHL patients with RCC have a shortened life expectancy. At present, RCC is the cause of death in 15-50% of VHL patients,[5-7] and 30-50% of symptomatic RCCs have already metastasised to lymph nodes, liver, bone, lung or brain.[17,55-57] Fortunately, RCC does not occur in every VHL patient (in different VHL families incidence varies between 3% and 63% of the patients)[5,53] nor in every kidney, and RCC does not always have a fatal outcome. These features may be caused by differences in family-specific germline mutation, the timing of origin and nature of the somatic mutation. The somatic mutations may be induced by environmental factors. For example, current opinion is that smoking (particularly in males) is correlated with the development of RCC.[58-60] There is an increased incidence of RCC in certain professions (e.g. among workers exposed to asbestos,[61] trichloroethene,[62] leather workers,[63] fire fighters and painters[64]) and a correlation has been found with obesity and hypertension.[59,60]

The renal lesions have a typical hereditary character, i.e. the lesions occur multiply, bilaterally and at a relatively young age. While sporadic RCC occurs predominantly in the seventh and eighth decades of life,[65] the mean age of presentation in VHL patients is 30 to 36 years.[66-69] However, there is a trend towards detection at younger age, probably as a result of intensification of monitoring. The youngest reported VHL patients with a RCC are 15 and 16 years old.[68,70] Poston et al.[67] found a mean of 7.8 cystic and 3.0 solid renal lesions in VHL patients, which is in agreement with our own observations (unpublished) and other studies.[5,54]

Most RCC in conjunction with VHL disease grow slowly. A radiological study demonstrated that the average increase of diameter in cysts was 0.5 cm/year and that solid lesions grew at an average of 1.6 cm/year.[54] Complex lesions (with cystic and solid parts) appeared to transform to a predominantly solid lesion that continued to grow, while the cystic part of the lesion gradually regressed. More recently, the mean growth rate of RCC in VHL disease was found to be between 0.3 cm (own unpublished data) and 0.5 cm/year.[71] This is comparable to the mean growth rate of sporadic RCC, which was reported to be 0.36 to 0.5 cm/year.[72,73]

Pathologically, RCC is a malignant epithelial tumour of the renal parenchyma and is often found in the renal cortex. The tumour tissue is frequently crowded with recent and old haemorrhages, necrosis and inflammation, and is surrounded by a pseudocapsule.[74,75] The most common cellular pattern is clear cell carcinoma,[76] arising from cells of the proximal tubuli.[77]

Renal cysts in VHL disease can contain lining epithelium exhibiting atypia, and are therefore considered to be premalignant.[57,67,78-80] However, transformation into a solid malignant tumour is rare,[81] Kragel et al. demonstrated that simple cysts in VHL disease arise more commonly from distal tubules, whereas the majority of RCC arises from proximal tubules.[81] Nevertheless, some renal carcinomas do arise from the distal nephron and comparison of markers of tubular differentiation in atypical cysts and RCC supports the progression of atypical cysts to carcinoma.[81]

Furthermore, by microdissecting material from individual lesions it has been demonstrated that loss of the wildtype allele and retention of the inherited, mutated VHL allele occurred both in cystic lesions and in RCC.[82] This clearly demonstrates that cysts are precursors for RCC and that loss of the VHL gene (the second hit in Knudson's theory[28]) occurs early in their development.

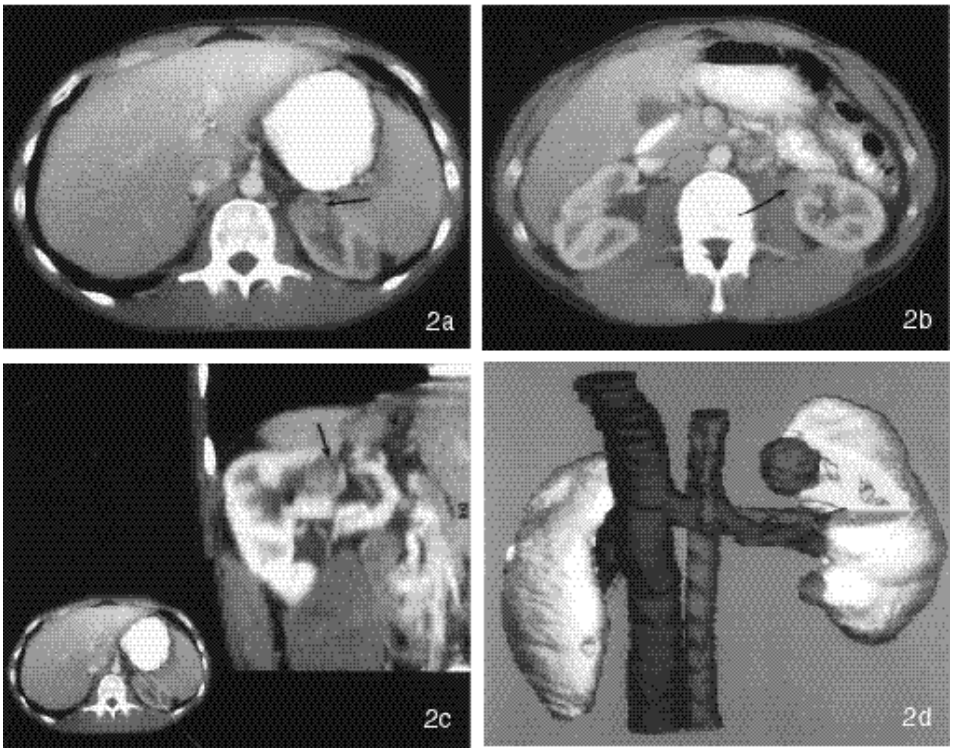

Fig. 2 CT images of the kidneys in the corticomedullary phase after intravenous contrast shows small RCCs in the left kidney of a 30-year old VHL patient. **a** Lesion in the upper pole (arrow) measures nearly 3 cm. **b** Smaller lesion (arrow) in lower pole of the kidney. Both lesions appear to be encapsulated. Right kidney shows a small cortical cyst. **c** Oblique reconstruction and **d** coronal 3D surface rendering show the exophytic lesions emanating from the medial aspect of the kidney, free from the main renal vessels. In the upper pole the renal parenchyma has been partly cut away. The right kidney shows cortical defects caused by cysts (see **b**). Elective surgery could be performed on the left kidney. Encapsulated cystic tumours of 1.8 and 2.6 cm in diameter were removed radically. The larger tumour showed infiltration into the pseudocapsule, but did not penetrate it. Radiological follow-up (four years) showed enlarging cysts in right kidney, but no tumour in left kidney.

Over the past years the treatment of renal lesions in VHL disease has been discussed by many authors.[67,68,71,83-87] Recommendations range from bilateral nephrectomy to follow-up investigations only. If both kidneys are affected with multiple cysts and tumours, a difficult decision has to be made between radical nephrectomy or nephron sparing surgery. If a patient progresses to having a large number of tumours and/or large individual tumours, nephrectomy followed by renal transplantation may be the first option. Bilateral nephrectomy followed by transplantation shortens life expectancy and diminishes the quality of life, although this quality has been improved over the past decade by the use of immunosuppressive therapy.[88,89] Graft 5- year survival rates of 80%, and up to 90% for living donors, have been reported.[88,89] In general, haemodialysis has a less favourable outcome, but this is most likely influenced by selection of patients.[88] Apart from a diminished life expectancy, the quality of life is reduced for dialysis and transplantation patients. Moreover, it has been suggested that immunosuppression accelerates (pre-existing) neoplastic growth.[90]

Nephron sparing surgery is based on maintaining renal function as long as possible, while reducing the risk of metastases [86] The most serious complication is renal atrophy and this has been reported in 11% of nephron sparing surgery.[86] In VHL disease, operative removal of solid RCC and those RCC larger than 3 cm which are still growing has been advocated by several authors.[54,71,72,86] Tumours under this size are more likely to be low grade and less frequently associated with metastases. Cancer specific five- and ten-year survival rates from nephron sparing surgery have been reported to be 100% and 81%, respectively.[68] These results indicate that nephron sparing surgery can provide effective initial- and preventive treatment for VHL patients with RCC.

With the advent of ultrasonography (US) and computed tomography (CT), there has been an increase in detection of small renal masses[91] and monitoring of VHL patients is likely to yield a correspondingly high number of such masses. CT scanning is found to be more reliable than ultrasound in detecting renal masses. Jamis-Dow et al. concluded that CT was more sensitive than US for the detection of small renal masses (particularly those smaller than 1.5 cm).[92] However, a substantial proportion of lesions under 1 cm were not detected with either technique. Nonetheless, US is a critical tool in helping to determine whether a lesion is principally cystic or solid.[93]

Intraoperative colour Doppler US is helpful in characterising deep parenchymal cystic lesions and evaluating larger deep or hilar tumours. [94] This technique minimises blood and renal parenchymal loss and allows safe removal of renal lesions. In particular, patients with many renal lesions benefit from a thorough inspection of the kidney with this technique, before closing.[94]

Thin-section (3 to 5 mm), intravenous contrast-enhanced CT is mandatory, and a helical technique is necessary to ensure that small lesions are not missed.[95] The recent introduction and use of helical or spiral CTscanners offers volumetric imaging of the kidneys in a single breath-hold and can therefore eliminate respiratory misregistration. These scanners can detect and discriminate lesions smaller than 1.0 cm by using overlapping intervals and thin collimation.[96] Bosniak and Rofsky claim that in the general population a normal contrast CT scan of the kidneys using 5 mm sections

rules out a clinically significant renal parenchymal neoplasm.[97] Moreover, CT can exactly localize postoperative defects in patients after nephron sparing surgery.[98] In patients with reduced functioning renal tissue and patients with only one kidney, as often seen in VHL disease, tumour enhancement may be problematic because tissue enhancement is directly related to the level of contrast material in the blood.[97] In such cases gadolinium-enhanced (gd-DTPA) MRI can be of great value, because it provides critical information about lesion vascularity without the risks of intravascular iodinated contrast material.

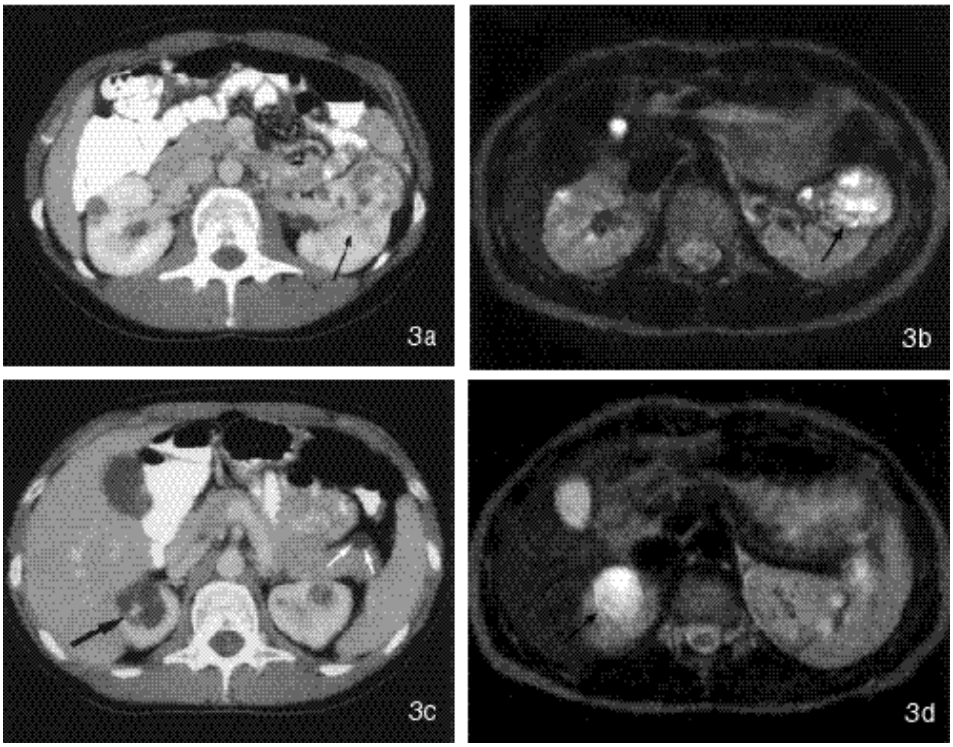

Fig. 3 Comparison of CT and MRI of a RCC in a 27-year old woman with VHL disease. Monitoring was started two years before these imaging results. US showed a left-sided tumour of 3 cm in diameter. **a** CT shows a large cystic tumour in the left kidney (arrow). **b** MRI (TR/TE = 2475/100 ms) shows the pseudocapsule (arrow) better than CT. Note the cortical cysts in the right kidney. Despite the tumour size of 5x5x3 cm, nephron sparing surgery was able to be performed. During operation, major haemorrhage of the wound bed of the left kidney could be treated. A small cystic part of the tumour reached the rim of resection, but enucleation was performed radically. The right kidney shows a more central cystic tumour on CT in **c** and MRI in **d**, which should be removed in the near future. Note the pancreatic cyst in the tail (small arrows) in c.

Imaging of renal masses is also possible with radio-labelled octreotide, which binds to somatostatin receptors of a wide variety of tumours including RCC.[99] An *in vivo* study demonstrated binding of ^{111}In-octreotide to RCC in 43% (3 out of 7 patients). In these three patients 20 of 23 known tumour localisations, predominantly metastases, were clearly visualised. This method may not only provide valuable information on the secondary spread of the tumour but it may also be used to select those patients who could benefit from treatment with somatastatin receptor analogues.[99]

Typically, RCC possess a pseudocapsule,[100] which may play a critical role in the success of nephron sparing surgery. Various imaging techniques have been described to reveal a pseudocapsule of the RCC (Fig. 3). A surrounding radiolucent rim is observed with angiography, a low or high density rim with CT, and a low intensity rim with MRI.[74,75] The sensitivity of CT in detecting pseudocapsules was lower than that of angiography and statistically significant. T2-weighted MRI is superior for visualising a pseudocapsule of RCC and also for providing reliable selection criteria for tumour enucleation.[75,100] The appearance on T2-weighted MRI is related to the fibrous element and compressed renal parenchyma. The inner margins of most pseudocapsules are composed of compressed parenchyma, whereas the outer margins contain reactive hyperplasia of the renal capsule as well as compressed parenchyma.[74] The low-intensity band or rim appears on MRI when the pseudocapsule is thicker than 2 mm. Takahashi et al. mentioned three limitations of the detection of a pseudocapsule by MRI.[75] First, pseudocapsular invasion tends to be observed near the renal hilum, where there are abundant blood vessels. Second, if the tumour is small and located at the upper pole, partial volume averaging may obscure the pseudocapsule. A chemical shift artifact, observed at the interface between the tumour and the perinephric fat, forms a third limitation.[75,100]

Table 1. VHL monitoring recommendations

Test	NIH	Netherlands
Ophthalmoscopy	From infancy, yearly	From age 5 years
Fluorescein angiography	Not routine	When indicated
Physical examination and neurological assessment	From age 2 years, yearly	From age 10 years, yearly
Urinary/blood catecholamines	From age 2 years, every 1-2 years	From age 10 years, yearly
US abdomen	From age 11 years, yearly	From age 10 years, yearly
CT abdomen	From age 20 years, yearly or every other year	-
MRI abdomen (MIBG)	When indicated	When indicated
MRI with gadolinium of cerebellum and myelum	From age 11 years, every two years; after age 60 years, every 3-5 years	From age 15 years, every two years
MRI petrous bones; Audiometry	If hearing loss, tinnitus and/or vertigo*	If hearing loss, tinnitus and/or vertigo*

Information was obtained from Choyke et al.[93] and completed with information kindly provided by Dr. G.M. Glenn from the National Institutes of Health (NIH), Bethesda, Maryland, USA.
* These symptoms may be caused by an endolymphatic sac tumour (ELST) which is associated with VHL disease.[142]

Some monitoring protocols recommend yearly alternate CT and US examinations to reduce both costs and radiation (see Table 1).[93] Other institutes advise that this regime should be complemented by a MRI once every three years. If renal monitoring is performed by CT, spiral 3-D geometry is preferred. Small masses are less likely to be missed by this method. In our opinion, careful cross-sectional monitoring with alternate MRI and US gives the best protection for aggressive RCC in most VHL patients. Both US and MRI can distinguish between solid and cystic masses, but the quality and reliability of MRI is equal or even superior to US in the detection and characterisation of renal masses. The best results are obtained by breath-hold T2-weighted MR images. Others use T1-weighted gadolinium-enhanced MRI with fat suppression,[101] which is useful for the characterisation of renal lesions. Angiography is inadequate for detecting renal lesions,[102] but was predominantly used as preoperative 'road mapping', to identify the renal vascular supply.

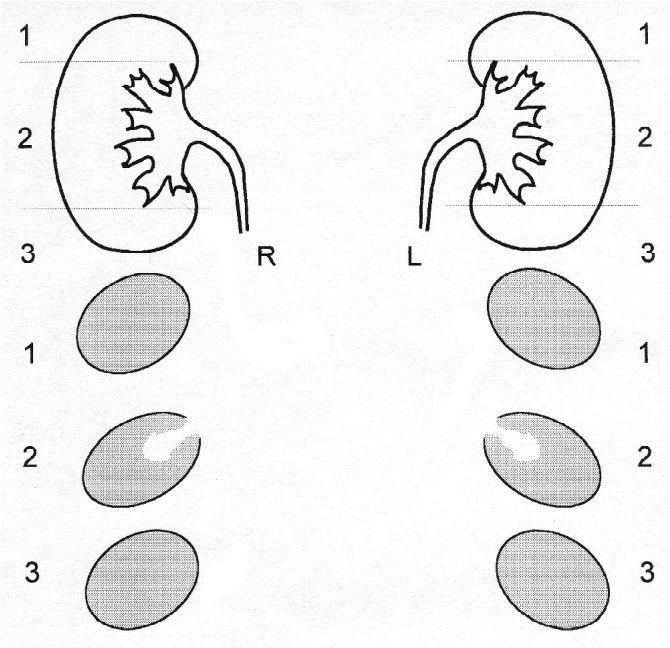

Fig. 4 Schematic diagrams of the kidneys used for monitoring renal lesions. The radiologists are requested

1. to draw lesions on coronal picture and number them. The same number for the same lesion in all following reports should be used and new lesions should be numbered consecutively; 2. to draw lesions on the transversal pictures (1-3) as well and use the same number for each lesion consistently; 3. to give nature of lesions: uncomplicated cyst (c), combined cystic/solid lesion (c/s), solid (s) and indicate whether lesions have a pseudocapsule (pc); 4. to give size of lesions in millimeters and, if possible, growth rate in millimeters per year.

We advise careful radiological monitoring at least once a year, preferably with MRI. Renal lesions can be adequately monitored by documenting the number, size and nature of lesions in a schematic figure of the kidneys (Fig. 4). Examples of cases are shown in Fig. 2, 3, 5 and 6.

Adrenal masses

VHL-associated phaeochromocytoma differs from isolated phaeochromocytoma in having younger age of onset (19 years earlier), multiple lesions, and a very low proportion of malignant tumours [103] The mean age at phaeochromocytoma diagnosis is 27-29 years,[5,103,104] with youngest reported patient of five years old.[104] Phaeochromocytoma may cause palpitations, sweating attacks, hypertension or paroxysmal unstable blood pressure, and headache. In VHL patients, phaeochromocytoma often remain quiescent or produce few symptoms, and investigation may show normal biochemical tests. However, the behaviour of phaeochromocytoma remains unpredictable; biologically inactive lesions may suddenly become dangerous. Benign phaeochromocytoma may also become malignant.[83] About 5% of VHL patients die from phaeochromocytoma-induced endogenous catecholamine intoxication, which has also caused fatal pregnancy outcome.[66,105,106]

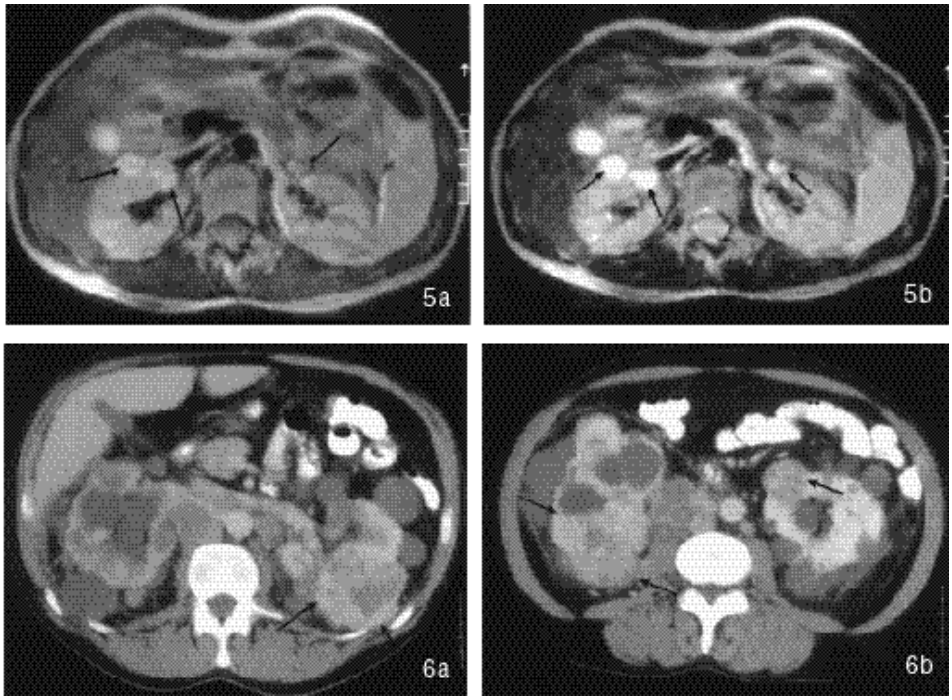

Fig. 5 Transverse MRI of a 27-year old VHL patient and bilateral small renal tumours (arrows). T2-weighted images (**a** TR/TE = 2436/50 and **b** TR/TE = 2436/100) show two combined solid/cystic lesions (arrows) in the right kidney. The one near the hilum is not suited for nephron sparing surgery. Recently, the lesion in the left kidney (arrow) was removed nephron sparingly. Histopathological examination showed a cystic tumour, measuring 2 cm at closer examination, surrounded by an intact pseudocapsule of 1-2 mm thick.

Fig. 6 CT scan of extensive disease of the kidneys in a 45-year old male who was diagnosed in 1966 with retinal haemangioblastoma in another clinic but who unfortunately did not receive periodical monitoring. When he presented with symptoms of back pain in 1996, he already had advanced renal cell carcinoma with metastases in regional lymph nodes, bones and lungs. The kidneys have a polycystic aspect but contain large solid tumours (arrows). This patient could only be treated by palliative radiotherapy on ossal metastasis in the spine and died six months after onset of symptoms.

Phaeochromocytoma occur in 0-58% of patients in VHL families.[5,20,43,54,66,105,107] There is strong evidence that the presence or absence of phaeochromocytoma is correlated with the type of VHL germline mutations (vide supra genotype-phenotype correlations). Beside this clear interfamilial difference, also intrafamilial differences have been observed.

VHL germline mutations are found in approximately 3% of patients with phaeochromocytoma without family history, thus sporadic tumours.[108] A higher percentage of VHL germline mutation was found in familial phaeochromocytoma (45%) and bilateral tumours.[109] This raises the possibility that some VHL mutations might predispose to phaeochromocytoma but be associated with a low risk of other typical VHL lesions.[104,110-115]

Diagnosis is based on biochemical tests and radiology.[103] Laboratory test may include evaluation of serum and urinary norepinephrine, epinephrine, and vanillylmandelic acid. Measurement of 24 hour urinary epinephrine excretion is the most sensitive of the biochemical tests.[103]

Fig. 7 T2-weighted pre-operative MRI of asymptomatic bilateral phaeochromocytoma (arrows) in a 37-year old male with VHL disease. Both kidneys are affected with multiple cysts and slow growing tumours, mostly occurring in places in which surgery is not possible without considerable loss of renal tissue. The largest solid lesion grew from 1.8 cm in 1980 to 2.5 cm in 1996. A difficult decision has to be made between nephrectomy and nephron sparing surgery. If he progresses to having a larger number of tumours, renal transplantation may be the first option, or one could decide to operate nephron sparingly on feasible tumours.

Radiology testing may include US, CT, MRI and metaiodobenzylguanidine (MIBG) scintigraphy. A low sensitivity for US was reported by Neumann (40% vs 90-95% in other reports).[103] In CT scanning, phaeochromocytomas typically enhance after administration of a contrast medium and CT is considered useful for evaluating the adrenal glands and organ of Zuckerkandl, but it is less accurate for investigating ectopic sites.[93] Sensitivity of abdominal CT in VHL disease was reported to be 76%.[103] T2-weighted MRI demonstrates high signal intensity for phaeochromocytoma (Fig. 7) in 95-100% of the cases.[103,116] Most phaeochromocytoma appear markedly hyperintensive to the liver on T2-weighted images, but occasionally phaeochromocytoma may be iso- or hypointense to the liver.[103] One of the new techniques for MRI of the adrenal gland is fat suppression, it reduces cardiac and respiratory motion-induced artifacts, accentuates small differences in tissue contrast, and eliminates chemical shift artifacts.[117] MIBG is about 75-95% sensitive for phaeochromocytoma and is 100% specific, but it may not depict very small lesions.[93,103,116] Preoperative MIBG scintigraphy is also important in localising extra-adrenal phaeochromocytoma.[118] It has been demonstrated that phaeochromocytoma in VHL disease can also occur in the thorax.[118a]

Operative treatment can be considered if a growing mass in the adrenal gland is established. Recently, satisfactory results have been reported from laparoscopic removal of adrenal tumours,[119-121] and in VHL disease (G. Janetschek, personal communication 1997). Enucleation rather than adrenalectomy is recommended by an increasing number of surgeons.[106]

Pancreatic cystic masses

The pancreatic manifestations of VHL disease include simple cysts, diffuse cystosis, cystadenoma, and rarely adenocarcinoma. [83,122] Islet cell tumours are considered separately.[93] The incidence of pancreatic involvement in VHL disease varied from 0-56%.[5,56,122-124] In a review of 275 reported cases,[125] 69 (25%) patients had cysts, 4 (1.5%) of which proved to be serous cystadenoma, 14 (5%) cases had islet cell tumour, two (0.7%) adenocarcinoma, and one patient had an haemangioendothelioma. Pancreatic lesions may be the only abdominal manifestation in VHL disease (12%) and may precede any other manifestation.[122] The earliest age of discovery reported is 15 years.[107]

Cyst formation is found in 70-72% of patients at autopsy studies.[56,126] The cysts, which are lined with a single layer of epithelial cells, are filled with serous fluid and may be haemorrhagic.[127] They can range from several millimeters to 10 cm in diameter (Fig. 3c and 8a).[93] Cysts are often multiple and enlarge the pancreas,[123] or may eventually replace the entire gland.[127] Complications can arise from space-occupying effects (local pain, bile duct obstruction, pancreatitis), but the cysts are mostly asymptomatic, while exocrine and endocrine hormonal insufficiency have been reported in a few cases.[17,93,123-125,128-131] The pancreas may become so replaced with multiple cysts that it becomes nonfunctional, which results in steatorrhea and diarrhea.[93] Insulin-dependent diabetes mellitus has been reported as a complication.[129,130]

Cystadenoma, like cysts, contain serous fluid and are benign in VHL disease.[93,123] Serous cystadenoma is a grape-like cluster of multiple microscopic and macroscopic cysts, separated by thickened walls of stroma.[93,122,129,132] Calcification or a central stellate scar may be seen.[122]

The differential diagnosis of cystic pancreatic lesions in VHL disease includes pseudocysts (Fig. 8b) caused by pancreatitis, pancreatic involvement in polycystic kidney disease and echinococcosis.[123,133] Metastasis from renal cell carcinoma has been described,[134] and has to be considered in the differential diagnosis of solid pancreatic masses. Adenocarcinoma in VHL disease is rare and was first detected by CT in 1979.[5,20]

Different imaging techniques, such as US, MRI and CT, have comparable diagnostic value.[132] US is therefore the method of choice for monitoring programs.[123] However, in finding a small lesion, or identifying a solid lesion, CT scanning or MRI may be superior to US.[54,132] Serous adenoma may appear solid in US because of the multiple acoustic interfaces caused by multiple microscopic cysts.[93] Also, a cystadenoma may be indistinguishable from a cluster of simple cysts, but this has no clinical implications since both are benign lesions[122] (Fig. 8b).

Pancreatic cystadenoma and the long T2 of the serous contents of cysts both result in high signal intensity on T2-weighted images. Gadolinium enhancement may be helpful because of high signal intensity in serous microcystic neoplasm, but it is absent in

cystic lesions. The signal intensity on T1-weighted images is usually low but can vary depending on the presence of haemorrhage.[127]

Asymptomatic pancreas lesions may represent an important feature that may be useful in detecting early expression of the disease. Because the disease is usually asymptomatic, conservative measures are considered adequate for cystic lesions.[123,125] However, aggressive resection is mandatory for a solid pancreatic lesion in VHL disease.[125] Patients with space-occupying cysts have been treated with percutaneous drainage and hypertonic saline sclerosis.[93] Cholestatic jaundice may be treated by endoscopic implantation of a biliary stent,[135] or a biliary bypass.[125]

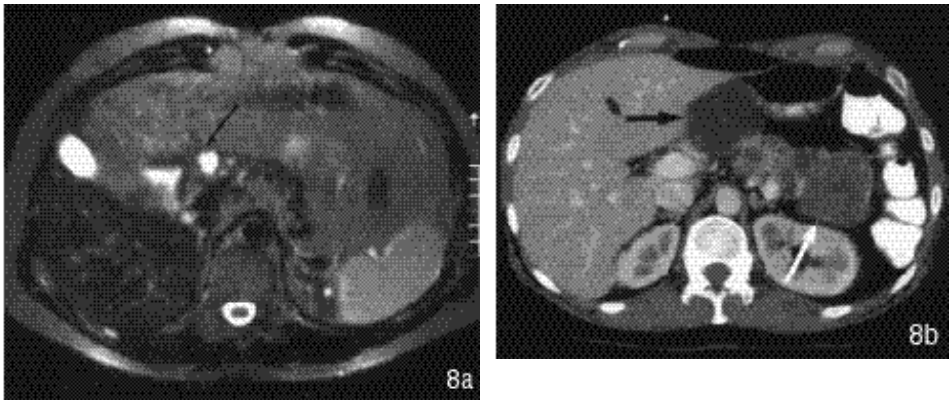

Fig. 8 Pancreatic lesions in VHL disease. a T2-weighted image (TR/TE = 2323/100) shows a small pancreatic cyst (arrow) in a 40-year old male with renal cell carcinoma (not shown). b CT in a 31 years old female with multiple pancreatic cystic lesions in corpus and tail of the pancreas with soft tissue parts, most probably serous cystadenoma (white arrow). On MRI one year before, the lesions had high signal intensity on the T2-weighted images. Despite some mass effect these lesions do not require resection. Moreover, she developed a pseudocystic mass (black arrow) due to a ventriculoperitoneal catheter in this region, inserted after surgery of a cerebellar haemangioblastoma.

Pancreatic islet cell tumours

Familial islet cell tumours in VHL disease were first reported in 1979.[124] Solid islet cell tumours may occur in 5-17% of the patients[125,136] and may be unrelated to pancreatic cystic disease.[93] Many islet cell tumours are slow-growing, asymptomatic and non-functional.[93,124,136] The functional ones most commonly secrete various peptides such as insulin, glucagon and somatostatin.[137] The frequency of malignant islet cell tumours is very low, only case reports have been published,[5,122-126,128,129,136,138] some with metastasis.[123,138]

Pancreatic islet cell tumours occur more frequently in patients with phaeochromocytoma and may be considered as an additional form of the multiple endocrine neoplastic syndromes.[93,124,136] Islet cell tumours arise either from pancreatic islet cells, which are believed to be derived from the neural crest,[139] or from a single neuroendocrine-programmed ectoblast.[137] This might indicate that islet cell tumours share a common origin with phaeochromocytoma.

The VHL gene does not play a pathogenetic role in the development of non-VHL (sporadic) pancreatic endocrine tumours.[140] This is in contrast to some other tumours of the VHL-spectrum (vide supra: Structure and possible functions of the VHL protein product). A locus at chromosome 3p25 centromeric of VHL is frequently lost in these tumours and may harbour a novel pancreatic endocrine tumour suppressor gene. Allelic loss at chromosome 3p25 is thought to be correlated with more clinically advanced disease.[140]

With US, benign islet cell tumours are usually seen to be well demarcated, round or oval, and hypoechoic relative to pancreatic parenchyma. Success rates for insulinoma localisation range from 25-60%, with those for gastrinomas at about 20%.[137] Intraoperative US may be useful in identifying focal masses when pancreas-sparing surgery is being considered, but detection of small lesions is difficult. Most islet cell tumours larger than 2 cm can be visualised by any technique, while those smaller than 2 cm are best seen with bolus-enhanced, contrast medium-enhanced, thin-section dynamic CT.[137] Islet cell tumours show intense enhancement on CT and may contain calcification.[93] As the tumour enlarges, areas of necrosis may be seen. The characteristic arteriographic feature of islet cell tumours is a dense, homogeneous, circumscribed parenchymal blush. Angiography was useful in the past in localising pancreatic islet cell tumours,[93] but has been replaced by CT and MRI. Fat-suppressed and dynamic gadolinium-enhanced MRI are superior to CT in depicting islet cell tumours.[141] Small insulinoma (less than 1.5 cm in diameter) were revealed well on dynamic gadolinium-enhanced, fast low-angle shot (FLASH) images.[141] However, others believe that CT and US are superior to MRI in detecting small islet cell tumours.[137]

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Management of renal cell carcinoma in Von Hippel-Lindau disease

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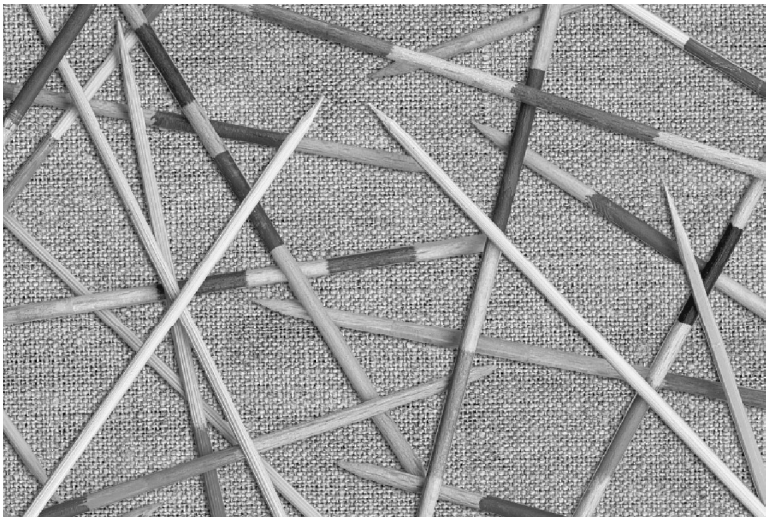

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Abstract

Objective To evaluate nephron sparing surgery (NSS) or radical nephrectomy (RN) for treating renal cell carcinoma (RCC) in patients with Von Hippel-Lindau (VHL) disease.

Patients and methods Between 1976 and 1997 ten patients with RCC from four VHL families, of whom seven were from one family, were studied by clinical and histopathological examination. Prior to 1991 three patients were treated using RN, and thereafter five patients were treated using NSS. Two patients were not operated on.

Results RCCs in our patients showed a slow growth rate (on average 0.3 cm/year) and asymptomatic patients presented with tumours of low-grade malignancy. In all patients, tumours were surrounded by a fibrous pseudocapsule. In five out of seventeen tumours pseudocapsular invasion was observed and three of these five tumours broke through the pseudocapsule. So far, these patients have not shown a less favourable outcome than those without pseudocapsular involvement by tumour growth. Multicentricity of RCC was relatively low (4.6 lesions per kidney). In two of the three RN patients only a single satellite lesion, in the direct vicinity of a RCC, was found in one kidney. Six tumours (1.8-5.5 cm) were enucleated by NSS. During a mean follow-up of 30 months, renal function in these patients was well preserved.

Conclusions In our patients, RCCs grew slowly, were of low grade, had a dense fibrous pseudocapsule and were thus good candidates for NSS.

Introduction

Von Hippel-Lindau (VHL) disease is a hereditary syndrome characterised by predisposition to bilateral and multicentric renal cell carcinomas (RCC), retinal haemangioblastoma, haemangioblastoma in the central nervous system and phaeochromocytoma, as well as cysts in the kidneys, pancreas and epididymis. VHL is a relatively rare disease, with an estimated incidence of between 1:31,000 to 1:53,000 in South Baden (Germany) and East Anglia (Great Britain), respectively.[1-3] The basis of familial inheritance of VHL disease are germline mutations in a tumour suppressor gene, identified in 1993 and located in chromosome region 3p25-26.[4] The disease is inherited as an autosomal dominant trait with a high penetrance and a variable expression. This is well illustrated by RCC, which occurs in different VHL families in between 3% and 63% of the patients.[5,6]

In VHL patients, renal involvement can be divided into three different forms with cystic, combined cystic-solid, and solid RCC lesions.[7] Pathologically, RCC is a malignant epithelial tumour of the renal parenchyma and is often found in the renal cortex. The tumour tissue is often observed to be crowded with recent and old haemorrhages, necrosis and inflammation, and is surrounded by a pseudocapsule. The most common cellular pattern is clear cell carcinoma,[8] which arises from cells of the proximal tubuli.[9]

Presymptomatic identification of carriers of a germline mutation in the VHL gene enables periodic examination. This permits the progress of tumour development to be followed from a relatively early age, and optimises the time in which treatment is carried out. Recommendations for treatment of RCC in VHL patients range from radical nephrectomy (RN), nephron sparing surgery (NSS), to follow-up investigations only.[10-16]

In this study, we report on ten VHL patients with RCC. The treatment of other VHL lesions is not described in this article. Patients operated on before 1991 underwent RN, and those operated on thereafter underwent NSS. The study provides a retrospective analysis of the clinical data of these patients and histopathological pattern of their renal lesions, and how these relate to the surgical treatment used.

Patients and methods

Patients

Between 1976 and 1997, nine RCC patients from three VHL families, were evaluated and treated in the University Hospital of Utrecht. Patients 1-7 were from one large family (Fig. 1, pedigree A), patients 8 and 9 were from two other families (B and C). All these patients underwent an annual monitoring program performed by a multidisciplinary team.[10] An additional patient (10) had been diagnosed with retinal haemangioblastoma in 1966 at another clinic, but unfortunately had not had periodical monitoring. When the patient presented with symptoms of pain in the back in 1996, he already had advanced RCC in both kidneys and metastases in lymph nodes, bones and lungs. He died six months after onset of symptoms. None of the other nine patients studied had metastases of RCC.

All ten patients had a definite VHL family history and fulfilled the VHL criteria.[17,18] Only the patient with metastases (10) presented with symptoms or signs that could be directly associated with RCC. The clinical records of first presentation were not available for one patient (1). Eight other patients (2-9) were asymptomatic and were identified by ultrasound monitoring, with the diagnosis being confirmed by computerised tomography (CT) or magnetic resonance imaging (MRI). Pre-operative angiography was performed in five patients (1, 2, 6, 8 and 9) and spiral CT in two patients (5 and 6) to determine the optimal surgical strategy.

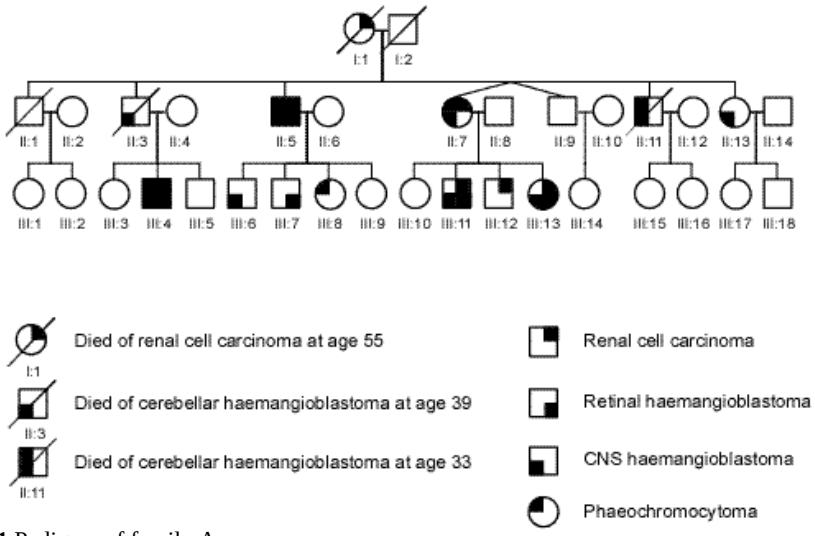


Fig. 1 Pedigree of family A.

Surgery

Three patients were operated on prior to 1991 and underwent RN (1, 2 and 3), five patients were operated on after 1991 and underwent NSS (4, 5, 6, 8 and 9). Two patients were not operated on (7 and 10). In NSS the tumours were enucleated according to the technique described by Novick.[19] After careful radiographic assessment of the size, complexity and progression of the tumour, and following discussion in a multidisciplinary team (general physician, nephrologist, surgeon/urologist and radiologist), renal lesions were electively removed. After median or flank incision, renal artery occlusion was performed with a tourniquet. Intra-operative renal hypothermia was used when clinically indicated; ultrasound examination of the kidney was not applied.

Pathology

Surgical specimens were macroscopically examined, and grossly abnormal tissue as well as random samples were histologically investigated conforming to standard surgical-pathological procedures. Each lesion was characterised as a cystic, solid or combined lesion, and further classified by cell type and architecture (tubular, trabecular, cystic or papillary). Lesions were evaluated for pseudocapsule invasion or breakthrough by tumour tissue and staged according to UICC guidelines.[20]

DNA analysis

High molecular weight DNA of the probands was isolated from peripheral blood according to established procedures. Exons 1, 2 and 3 of the VHL gene and their flanking sequences were amplified using the polymerase chain reaction.[21] The amplified DNA was purified by ultra-low melting point agarose gel electrophoresis. The excised fragments were directly sequenced by the dideoxy-chain termination reaction with a pUC-sequencing kit (Boehringer Mannheim, Mannheim, Germany), using [alpha-^{35}S] dATP (600 Ci/mmol). The amplification primers were used as primer in the sequencing reactions.

Screening for large gene abnormalities was performed by Southern blot analysis. DNA was digested with either *Hind*III or *Eco*RI. After gel electrophoresis and transfer to Hybond-N filters, the genomic DNA was hybridised with a VHL cDNA probe,[4] (kindly provided by I. Kuzmin) according to the manufacturer's instruction, and subsequently rehybridised with PCR products of the respective VHL exons.

Follow-up

Mean follow-up was 30 months (range 21-52 months) for the NSS group and 171 months (97-259 months) for the RN group. Yearly follow-up included physical examination, abdominal radiology (ultrasonography and MRI) and biochemical analysis of blood (including serum creatinine assessment) and urine. Complete follow-up data have been obtained for all patients up to December 1997.

Results

DNA analysis

The results obtained from probands and other patients from families A, B, and C are shown in Table 1 (patients 1-9). A VHL gene mutation has not yet been identified in the proband from family D (patient 10).

Clinical

RCC was detected at a mean age of 32 years (range 24-45 years). Patients included in the presymptomatic monitoring protocol were all diagnosed at a relatively young age (Table 1); five patients from family A (3-7) were diagnosed at a mean age of 25 years (range 24-28 years). Radiologically, lesions showed a mean growth of 0.3 cm a year (range 0.04-0.5 cm). The pre-operative serum creatinine level of five patients (2, 4, 5, 6 and 9) ranged from 0.68-0.89 mg/dl (mean = 0.78 mg/dl). Post-operative serum creatinine levels after one month, ranged from 0.66-1.46 mg/dl (mean = 0.94 mg/dl).

Surgery

Of the ten patients, two were not operated on: patient 10 was not operated on because of advanced RCC with multiple metastases; patient 7 is currently being discussed by the multidisciplinary team and a decision was made recently to enucleate a subcapsular cortical lesion of approximately 3.0 cm in his left kidney (Table 1). This operation has not yet been performed.

Three patients underwent RN and were all treated differently. Patient 1 had a bilateral nephrectomy and has been treated by haemodialysis since 1976. Patient 2 underwent nephrectomy of her left kidney in 1985. During follow-up, only a very small cyst was discovered recently by MRI, but so far she developed no RCC in the contralateral kidney. Patient 3 underwent a left-sided nephrectomy. Six weeks later, he underwent the second nephrectomy and was transplanted in the same session with a cadaveric kidney. Patient 3 was the only patient in the RN group with complications. He developed interstitial cellular rejection in the graft and abundant retroperitoneal inflammatory infiltrate, which had to be drained three times.

Table 1 VHL disease and RCC, surgery

Patient	In pedigree	Mutation	Age	AOD RCC	Surgery	Largest lesion	Compl.	Creat. pre/ post op.
1	A-II-5	V170D	64	42 (1976)	RN L+R	14.0 + 2.5	no	unknown /n.a.
2	A-II-7	“	58	40 (1979)	RN L	2.6	no	0.79/1.46
3	A-III-11	“	35	26 (1988)	RN L+R	2.1 + 1.4	*1	1.36/n.a.
4	A-III-12	“	33	28 (1992)	NSS L	2.6	no	0.86/1.08
5	A-III-8	“	30	24 (1991)	NSS L	5	*2	0.89/0.66
6	A-III-13	“	30	24 (1991)	NSS L	2	no	0.68/0.75
7	A-III-4	“	41	24 (1980)	no	na	na	na
8	B, na	R167Q	42	38 (1993)	NSS L	5.5	no	unknown
9	C, na	deletion exon 1+2	35	31 (1993)	NSS L	2	no	0.70/0.75
10	D, na	unknown	45 (=)	45 (1996)	no	5	*3	na

Na, not applicable; Age, in December 1997; (=), died; AOD, age (and year) of diagnosis of RCC (renal cell carcinoma); RN, radical nephrectomy; NSS, nephron sparing surgery; L, left; R, right; Diameter of largest lesion in cm; Compl., complication: *1, interstitial cellular rejection and retroperitoneal abcess, *2, haemorrhage and urinary tract infection; Creat. pre/post op., creatinine levels in mg/dl pre- and post-operative *3, metastases.

Five patients (4, 5, 6, 8 and 9) were operated on using the NSS procedure and a total of six tumours were enucleated, with two tumours being removed from patient 4. In two patients (5 and 8) the enucleated lesions were larger than 3 cm, measuring 5.0 and 5.5 cm, respectively. In both these patients perirenal fat was also excised, as radiology had demonstrated infiltration into the perirenal tissue. Complications developed only in patient 5 with post-operative urinary tract infection and intra-operative bleeding from the crateriform lesion (haemoglobin concentration dropped from 12.4 to 6.0 g/dl, post-operatively).

Pathology

All lesions had the same appearance (Fig. 2). The tumour cells had slightly pleiomorphic nuclei surrounded by a clear cytoplasm. Mitoses were scarce. The cells formed

solid areas consisting of lobuli with an intervening, fibrovascular lattice-like stroma. Cystic spaces were lined by uni- or multilayered clear cells, identical to those found in the solid parts. Necrosis, haemorrhages and fibrosis also formed part of the lesions. No sarcomatoid, chromophobe, tubulopapillary, medullary or oncocytic lesions were encountered. Due to minimal atypia and scarcity of mitoses, all lesions were considered to be of low grade malignancy. No further grading was done due to the uniform histopathology of the lesions amongst all patients.

Table 2 VHL disease and RCC, clinical-pathological features

Patient	Number (size)	Cysts (size)	Invasion	Break-through	Stage	Satellite lesions
1	L: 2 (7.0 - 14.0)	L: 10 (0.6-3.3)	L: 0/2	0/2	L: T3	L: 1, near RCC
	R: 3 (2.5)	R: 5	R: 0/3	R: 0/3	R: T2	+ several cysts
2	L: 1 (2.6)	L: 1 (1.0)	1/1	1/1	T3	no
3	L: 4 (0.3 - 2.1)	L: 2	L: 1/4 (1.3)	L: 1/4	L: T1	L: 1, near RCC
	R: 1 (1.4)	R: 3 (0.6-1.5)	R: 0/1	R: 0/1	R: T1	
4	L: 2 (1.8 -2.6)	no	2/2	0/2	T1+2	no
5	L: 1 (5.0)	no	0/1	0/1	T2/3	no
6	L: 1 (2.0)	no	0/1	0/1	T1	no
8	L: 1 (5.5)	no	1/1	1/1	T3	no
9	L: 1 (2.0)	no	0/1	0/1	T1	no

Number and size of RCC, in cm; L, left; R, right; Cysts, number and size of cysts; Invasion, invasion of tumour tissue in the pseudocapsule; Breakthrough, tumour tissue breaking through the pseudocapasule; Stage, TNM[20]

Most lesions were surrounded by a fibrous pseudocapsule of varying thickness. Five out of seventeen lesions had pseudocapsular invasion, and four of these tumours were smaller than 3 cm. Three tumours, measuring 1.3-5.5 cm (in patients 2, 3 and 8), invaded surrounding tissue by breaking through the pseudocapsule (Fig. 2c and Table 2). In two patients (1 and 3) some macroscopically invisible cysts and combined lesions were found elsewhere in the removed tissue specimens by microscopic investigation (Fig. 2a and b). These lesions had the same appearance as the larger cysts and solid parts of the grossly visible tumours. Tumours detected in asymptomatic and regularly monitored patients (family A) were small and mostly staged as T1 or T2 (Table 2).

Autopsy of patient 10 revealed that the left kidney contained three peripheral RCC measuring 3-5 cm, with both pseudocapsular breakthrough and renal capsular invasion. The right kidney contained two peripheral and one hilar RCC, with an intrarenal size of 4 cm and vena cava/iliaca communis involvement. Moreover, both kidneys contained multiple cysts lined by clear cells similar to those observed in solid RCCs.

Follow-up

Lesions remaining after removal of the tumour are reported in Table 3. In patients that experienced NSS and in the patient that underwent an unilateral nephrectomy, renal function was well maintained. During the total follow-up time (range 21-259 months), we found an average number of 4.6 tumours per kidney (range 0-14 tumours). Patients with involvement of the pseudocapsule by either invasion or breakthrough did not exhibit a less favourable course than those with tumours entirely surrounded by an intact pseudocapsule.

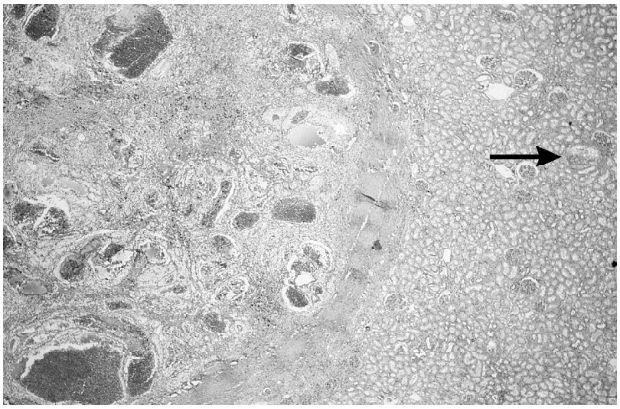
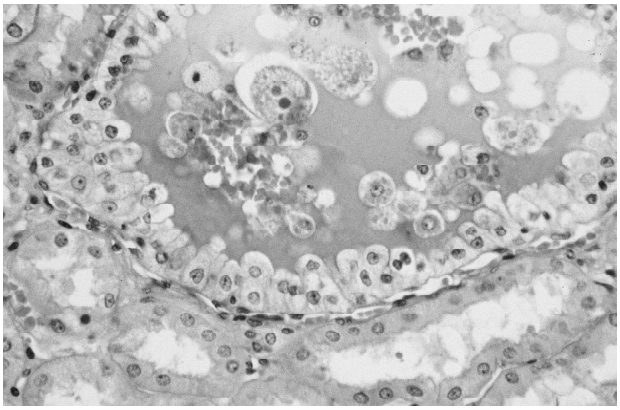
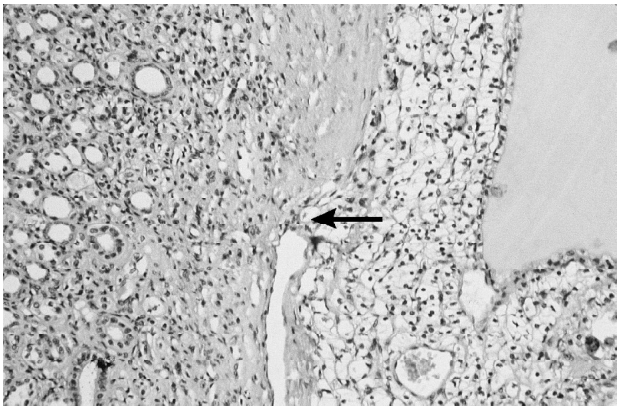


Fig. 2 a Photomicrograph showing a RCC (left) as well as normal renal tissue (right) in patient 1. An intervening fibrous capsule is clearly visible. The adjacent renal tissue shows a satellite lesion (arrow). The tumour shows a microcystic pattern. Most lesions contain erythrocytes, indicating intratumoural haemorrhage and no surgical effects, as the adjacent renal tissue does not contain erythrocytes. H&E, x40.



b Magnification of the satellite lesion show a cyst, lined by multilayered clear cells. H&E, x600.



c Photomicrograph of a RCC from patient 2 showing tumour tissue (right) breaking through the pseudocapsule (arrow). The adjacent renal tissue (left) shows no abnormalities. H&E, x240.

Discussion

Mutation analysis in the VHL families revealed three different genotypes. Regarding RCC, no clear interfamilial differences in phenotypes were found, although this observation is limited by the number of families and patients per family. However, in family A (with the rare V170D mutation), from which seven patients were available for study, considerable variation in both multicentricity and bilaterality of tumours was observed. It is essential to collect material from many more families and patients to be able to form an opinion on genotype/phenotype correlations.

Natural history

If not identified by monitoring, patients with VHL may have a shortened life expectancy, as illustrated by patient I:1 (Fig. 1) from family A and patient 10. At present, RCC is the cause of death in 15-50% of VHL patients.[6,22,23] In symptomatic RCC, 30-50% of the lesions metastasise to lymph nodes, liver, bone, lung or brain.[17,24-26]

Some cysts with lining epithelium show focally irregular hyperplastic features,[10] however transformation into a solid lesion is rare.[7,27,28] Fortunately, RCC does not occur in every VHL patient, nor in every kidney, and RCC does not always have a fatal outcome. We speculate that these features are caused by differences in family-specific VHL germline mutation, and the timing of origin and nature of somatic mutation(s). Such somatic mutations may be induced by environmental factors. In this respect, it is noteworthy that five of the ten patients were smokers, of whom patient 10 was a heavy smoker (Table 3). The current opinion is that smoking is correlated with the development of RCC.[29-31]

Clinical

Most RCC in conjunction with VHL met the criteria of hereditary tumours (multicentric, bilateral and young age of onset) and showed a slow growth rate. Radiological studies demonstrated that the average increase of diameter in cysts was 0.5 cm/year and 0.5-1.6 cm/year in solid lesions.[7,11] Complex lesions (with cystic and solid parts) appeared to transform to a predominantly solid lesion that continued to grow, while the cystic part of lesions gradually regressed.[7]

Pathology

In our cases, most tumours were found to be of low grade malignancy. This may account for the reasonably benign course of the disease. The presence of a pseudocapsule did not necessarily guarantee a favourable course of disease, since invasion and perforation of the capsule was observed in some of our cases. Whether all VHL-associated RCCs are of low grade malignancy cannot be answered by the limited number of cases we have studied to date. A higher grade of malignancy may be present in VHL cases with RCCs showing more progressive growth. In the literature, most RCCs in VHL patients are staged as T1 (75%) or T2 (9-17%).[12,32]

Close microscopical examination of five kidneys removed from our patients only revealed incidental small lesions, mainly in the direct vicinity of the macroscopic visible tumours. Controversially, Walther estimated, by extrapolation of tissue surrounding renal lesions, that the number of microscopic clear cell lesions in an avarage

Table 3 VHL disease and RCC, follow-up

Patient	First lesion + AOD	Sympt.	Creatinine (max.)	Tumour progression	Smoking	Remaining lesions (size)	Total lesions cysts and solid (L+R)
1	RCC (42)	unknown	dialysis	unknown	q (1982)	na	12 + 8
2	RCC + PHAEO (40)	PHAEO	1981-1996: 0.73-0.86 (1.46/1985)	unknown	no	R: 1c (1.0)	2 + 1
3	RCC + RA (26)	no	1988-1996: 0.84-1.47 (4.52/1990)	1978-1988 no screening	q (1989)	n/a	6 +4
4	Renal cysts (28)	no	1988-1996: 0.78-0.88 (1.18/1993)	1993-1996: 0.1 cm/year	q (1982)	L: 1s,3c R: 2c (1.0-2.6)	6 + 2
5	RCC (24)	no	1982-1996: 0.60-0.72 (0.97/1983)	1991-1995: 3.0-5.0 cm (0.5 cm/year)	no	L: 3s,1c/s,4c R: 3s/c,1c (0.5-3.0)	9 + 4
6	HAB (20)	HAB	1988-1996: 0.68-0.82 (0.87/1991)	1991-1996: 1.0-3.0 cm (0.4 cm/year)	no	L: 1s,1c R: 1s,3c (0.5-2.0)	3 + 4
7	RCC and RA (24)	no	1981-1996: 1.13-1.02 (1.21/1993)	1980-1996: 1.8-2.5 cm (0.04 cm/year)	no	L: 6s,2s/c,6c R: 1s/c,3c (1.0-3.0)	14 + 4
8	HAB (30)	no	1993-1994: 1.06-1.18 (1.22/1994)	1993-1996: 4.0-5.5 cm (0.5 cm/year)	no	L: 0 R: 1c (1.0)	1 + 1
9	HAB (29)	no	1993-1995: 0.66-0.82 (0.82/1993)	1993-1996: 2.0-3.0 cm (0.3 cm/year)	5-10/day	L: 0 R: 0 (n/a)	1 + 0
10	RA (15)	yes	unknown	unknown	40/day	L: 3s, m,c	unknown

oma; PHAEO, phaeochromocytoma; HAB, haemangioblastoma in central nervous system; RA, retinal haemangioblastoma; Sympt., presentation with symptoms; Creat., first and last creatinine value during follow-up in mg/dl (maximum value and year); Smoking: q, quit (year of quitting); Remaining lesions after surgery: c, cyst; s, solid; s/c, combined solid/cystic lesion; n/a, not applicable, m, multiple; L, left; R, right; lesions in patients 1-3 are reported in Table 2. During total follow-up time 82 tumours were found in patients 1-9, an average of 4.6 tumours per kidney.

VHL kidney was 1100 cysts (benign and atypical) with clear cell lining and 600 clear cell neoplasms.[33] However, in the following editorial comment it was questioned whether the normal tissue in the area immediately surrounding a lesion is representative of the entire kidney.[33] Extensive tumour formation may occur when inadequate control and treatment is given, as in patient 10. But our general finding of a relatively low number of renal lesions confirms the low frequency reported in other studies.[7,13,15,34] In addition, mild manifestation of renal involvement in VHL is also illustrated by the absence of RCC during long-term follow-up in patient II-13 (Fig. 1), as well as the left kidney of patient 2 and also in three younger patients from family A.

Surgical treatment

If both kidneys are affected with multiple cysts and tumours (as in patients 5 and 7), a difficult decision has to be made between RN or repeated NSS. If further progression of a large number of tumours occurs, RN followed by renal transplantation may be the first option. If a restricted number of lesions is present, NSS is indicated for tumours larger than 3 cm including a rim of normal tissue,[35] since the close surrounding tissue may contain satellite lesions. To determine the surgical strategy, pre-operative angiography for vascular mapping of tumour vessels has a limited role,[36] but spiral CT for locating renal lesions exactly is helpful.

In retrospect, the right kidney of patient 3 would have been an excellent candidate for NSS, with one small, well-encapsulated lesion and three subcapsular cortical cysts (0.6-1.5 cm). Transplantation with a cadaveric kidney was undertaken in the same session because of the patient's fear for dialysis based on his uncle's (patient 1) bad experiences.

Bilateral nephrectomy followed by transplantation shortens life expectancy and diminishes the quality of life, although it has been improved over the past decade by the use of immunosuppressive therapy. Graft 5-year survival rates of between 80% to 90% for kidneys from living donors are reported.[37,38] However, it is suggested that immunosuppression favours the growth of pre-existing cancers.[39] Goldfarb et al. studied the results of renal transplantation after bilateral nephrectomy in 32 VHL patients.[40] Three patients died due to metastatic disease. Their data suggested that for limited disease transplantation can be performed safely soon after nephrectomy, whereas in more advanced cases (pT3 and above) a waiting period of at least two years should be considered, to avoid transplantation in subjects with dormant lesions. In general, haemodialysis has a less favourable outcome than transplantation, but it is greatly influenced by the general health of the patient.[37]

The results with five patients treated by NSS are promising and renal function was preserved in all patients. Large tumours represent more risk at enucleation (as in patient 5) which emphasises the need for periodical examination and excision of tumours before they become too large. For VHL patients, operative removal of solid RCC (larger than 3 cm) showing progressive growth has been advocated by several authors.[7,11,16,41] It was shown that smaller tumours are more likely to be low grade and are less likely to be associated with metastasis. Five- and ten-year cancer-specific survival rates from NSS have been reported to be 100% and 81%, respectively.[12] Our results support the conclusion that NSS can indeed provide effective initial and preventive treatment for VHL patients with RCC.

Conclusions

With a cautious note that seven of the ten patients studied came from the same family, we conclude that RCC in VHL grows slowly and that multicentricity per kidney is relatively low. The majority of tumours had a pseudocapsule and showed little evidence of metastasis. Tumours measuring 1.8-5.5 cm can be safely removed by NSS. However, since smaller tumours may grow progressively and break through the pseudocapsule, we advise removing tumours measuring 2-3 cm, as well as a rim of normal renal tissue. In all affected family members, ongoing follow-up by careful radiological monitoring is required at least once a year.

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Abstract

Von Hippel-Lindau (VHL) disease is an autosomal dominant tumour syndrome caused by germline mutations of the VHL tumour suppressor gene located on chromosome 3p25-26. In VHL disease tumours may occur in fourteen different target organs, including the eye. Retinal angiomas are considered the first manifestation of VHL disease in 43% of cases, and the cumulative probability of developing a retinal angioma in one or both eyes rises during each decade of life, reaching 80% for patients over 80 years old.

Since 1976 patients with VHL disease at the University Medical Centre Utrecht and their at-risk relatives have been screened periodically by a multidisciplinary team. Long term follow-up ophthalmological data were analysed with special attention to natural course and results of treatment. In addition, we looked for a genotype-phenotype correlation. Retinal angiomas were found in all families. In one large family with a missense mutation (V170D) of the VHL gene, in which the complete spectrum of visceral- and CNS features of VHL disease is present, macular-, parapapillary-, optic disc- and ora serrata-angiomas were also found. In general, however, a clear-cut genotype-phenotype correlation could not be found.

Only early detection and treatment of peripheral retinal angiomas can be expected to decrease the percentage of patients with decreased visual acuity. Therefore, early detection and treatment of these tumours is of paramount importance. Ophthalmological monitoring of patients and persons at risk should start as early as possible. In patients with apparently sporadic retinal angiomas it is advisable to perform germline DNA analysis, since the risk of developing VHL disease is high, especially if the angiomas are bilateral, or unilateral and multicentric, or if the patient is young, or if there is a family history suggestive of VHL disease.

Introduction

Von Hippel-Lindau (VHL) disease is an autosomal dominant tumour syndrome, caused by germline mutations of the VHL tumour suppressor gene located on chromosome 3p25-26.[1,2] In VHL disease tumours may occur in fourteen different target organs, including the eye.

In the eye the typical lesion is the so-called peripheral retinal angioma, a globular reddish tumour with a dilated tortuous feeding artery leading from the optic disc to the tumour and a similar draining vein leading back to the disc. It is usually located in the temporal periphery of the retina. Less frequently, the tumour is located elsewhere in the retina, next to or on the optic disc, and even in the orbital portion of the optic nerve.[3]

In the ophthalmic literature, the eponym Von Hippel's disease is used when only the eye is affected. If systemic abnormalities are present the disease is called Von Hippel-Lindau disease,[3,4] after Von Hippel who established retinal angiomatosis as a clinical entity[5] and Lindau who noted the association between retinal angiomatosis, haemangiomatous cysts of the cerebellum and the visceral components of the disease.[6] Von Hippel's disease is considered the first manifestation of VHL disease in 43% of cases, and the cumulative probability of developing a retinal angioma in one or both eyes rises during each decade of life, reaching 80% for patients over 80 years old.[7] On histopathologic examination, the ocular tumours are seen to be composed of a proliferation of capillaries and glial cells, and are identical to the cerebellar haemangioblastoma. Therefore, they should actually be called haemangioblastoma.[8,9]

The natural history of peripheral retinal angiomas is not fully understood; untreated, however, they may eventually cause blindness and loss of the eye.[3] Peripheral retinal angiomas generally become symptomatic during the third decade of life when they cause decrease of visual acuity or a visual field defect as a result of retinal exudation, haemorrhages in the vicinity of the tumour, retinal detachment or macular pucker.[10] Spontaneous regression can also occur.[11] The onset and natural course of angiomas located elsewhere in the eye is not clear and treatment is often unsatisfactory.[3]

Many different modes of therapy have been used to treat peripheral retinal angiomas: diathermic-, xenon-, laser- and cryocoagulation. All have been reported effective depending on the location, size and number of the tumours.[3] However, despite seemingly successful treatment, tumour recurrences are sometimes seen, even after many years, probably because the tumour was only destroyed superficially.[12]

Since 1976 in the University Medical Centre Utrecht VHL patients and their at-risk relatives are screened periodically by a multidisciplinary team. Long term follow-up ophthalmological data were analysed with special attention to the natural course of ophthalmological signs of the disease and results of treatment. In addition, we looked for a correlation between the VHL germline mutations and the ophthalmological manifestations.

Patients and methods

Data of 20 patients from six families were collected from the files. All patients had an annual routine ophthalmological examination consisting of measurement of visual

acuity, slit lamp examination and fundoscopy. If angiomas were present or suspected in the far periphery of the retina, inspection with a Goldmann three-mirror lens was performed. Additional fluorescein angiography was done if micro angiomas were suspected to secure the ophthalmoscopic diagnosis, and usually before treatment of large angiomas, in order to observe the blood supply of the tumour. Peripheral retinal angiomas were treated by xenon- and, later on, by laser coagulation. For small tumours, up to one disc diameter in size, a single treatment of the surface was performed with burns of large size (500 mm), low intensity, and long duration (0.2-0.5 sec). Larger tumours were treated in multiple sessions, starting with treatment of the surrounding retina, avoiding coagulation of the feeder vessels. Retinal detachment caused by retinal angiomas was treated by cryotherapy of the tumours, followed by a scleral buckling procedure. Recurrent retinal detachment was treated by vitrectomy and endolaser coagulation.

Results

The six families with VHL disease are A, B, C, D, E and F. A deletion was found in one family (F) and different missense mutations were found in five families, confirming the diagnosis of VHL disease. In four of the six probands the presenting symptom was cerebellar haemangioblastoma. Age at presentation was between 30 - 44 years. In one of these four probands retinal angiomas were also found. In two probands (C, D) the presenting sign was retinal angioma. Their ages at presentation were 26 and 28 years, respectively. In one of these (D) a cerebellar haemangioblastoma was found; the other proband (C) has the VHL germline mutation, but no other signs of the disease up till now.

In family A one person is suffering from the disease. She has secondary optic atrophy caused by occlusive hydrocephalus because of a cerebellar haemangioblastoma, but no retinal angiomas or other signs of the disease.

In family B two patients have been identified. Four other members of this family have already died because of a CNS tumour. Renal cell carcinoma, medullary haemangioblastoma and pancreas cysts are also present in this family. Follow-up is 12 years. Both patients suffer from cerebellar haemangioblastoma, but only one of them has one angioma in one eye. This was treated successfully with xenon coagulation.

In family C one person is known to suffer from the disease. She was adopted as a baby from Korea. She suffers from a congenital motor nystagmus. At the age of 26 years she experienced a decrease of visual acuity to finger counting in the right eye, in which she appeared to have multiple angiomas with an exudative retinal detachment. After vitrectomy, cryotherapy and scleral buckling procedure, the tumours became fibrotic and the retina was reattached; visual acuity, however, did not recover. Follow-up is three years.

In family D two persons have retinal and cerebellar haemangioblastomas, but no other signs of the disease. Follow-up is 19 years. The retinal angiomas were treated successfully with xenon- and laser therapy. Visual acuity remained normal.

In family E twelve patients suffer from the disease (Fig. 1). Follow-up is 23 years. Another three patients had died before the start of the monitoring program. From the records of the family doctor it appeared that they died of renal cell carci-

noma (patient I-1) at the age of 55 years, and obstructive hydrocephalus caused by a cerebellar haemangioblastoma at the age of 33 and 39 years (patients II-2+4) respectively; no ophthalmological data were recorded. A number of ophthalmological signs and symptoms are seen in patients of family E. The proband (patient II-7), who presented with obstructive hydrocephalus caused by a cerebellar haemangioblastoma, has secondary optic atrophy, but no retinal angiomas at the age of 55 years. He also has pancreas and adrenal cysts. His elder brother (patient II-3) has a small angioma on one optic disc, which has remained unchanged for more than 20 years. Visual acuity is near to normal in this eye. He had bilateral renal carcinomas, and cysts in cerebellum, adrenals and pancreas. His elder sister (patient II-5) has pale optic discs. She was operated for an intra medullary haemangioblastoma Th6-Th7, but has never had signs of raised intracranial pressure. In addition, she has had a unilateral renal carcinoma, bilateral phaeochromocytomas and a unilateral mamma carcinoma.

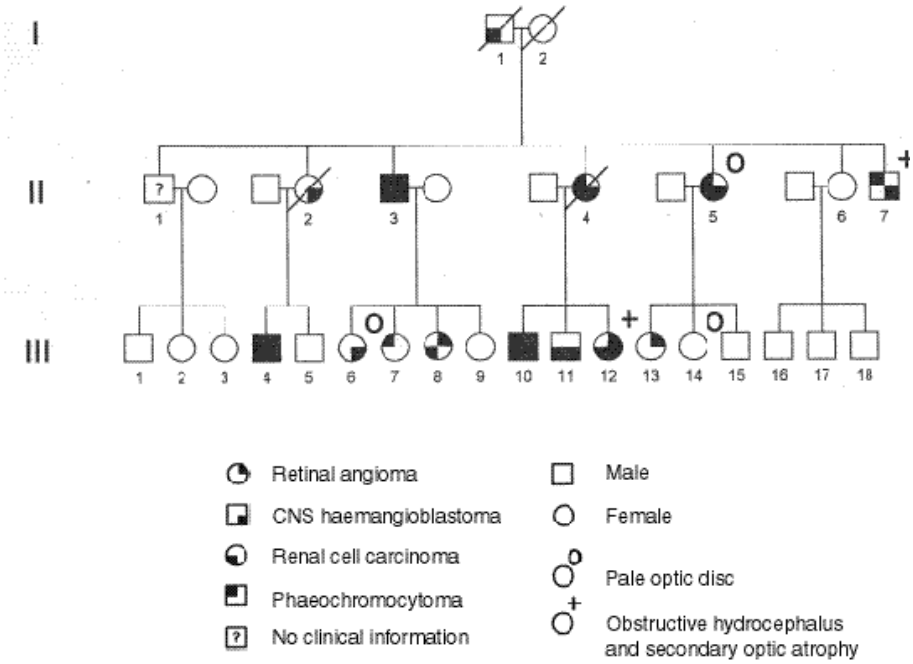

Fig 1. Family tree of family E with VHL disease (missense mutation: V170D)

In the next generation, in patient III-4, a small parapapillary angioma and an ora serrata angioma with a dilated feeder artery and draining vein is present in one eye. The angiomas have never been treated and have not changed for more than 17 years. No new ocular angiomas have developed. He also has cerebellar haemangioblastomas, bilateral phaeochromocytomas, renal carcinomas, pancreatic cysts, liver cysts and medullary haemangioblastomas. Patient III-6 has pale optic discs. She suffers from a small cerebellar haemangioblastoma. Patient III-7 has no ophthalmological signs. She had bilateral phaeochromocytomas. Patient III-8 developed a very small angioma in one eye at the age of 28 years. She also has bilateral renal carcinomas.

Patient III-10 has retinal angiomas in both eyes. One of the tumours originating at the ora serrata was floating in the vitreous cavity. After five years of uneventful follow-up, a retinal detachment developed, that was treated successfully with a scleral buckling procedure and cryotherapy of the tumour. A second retinal detachment was treated successfully by vitrectomy, retinotomy and silicon oil implantation. Visual acuity, however, dropped from 0.8 to finger counting and did not recover. This patient also suffers from bilateral renal carcinomas, phaeochromocytoma and medullary haemangioblastomas. Patient III-11 has no ophthalmological signs. He suffers from a unilateral renal carcinoma and cerebellar haemangioblastoma. Patient III-12 has optic atrophy secondary to obstructive hydrocephalus because of a cerebellar haemangioblastoma and a macular retinal angioma that detached from the retina and is now floating in the vitreous cavity, still attached to the optic disc. The angioma was already detached from the retina when we examined her for the first time. At the age of 27 years new angiomas started to develop in both retinas, including a recurrent angioma in the left macula with exudation in the posterior pole (Fig. 2). After laser coagulation the exudation disappeared. The other angiomas were treated successfully with laser coagulation. This patient also suffered from a haemangioblastoma of the medulla oblongata and bilateral renal carcinomas. Patient III-13 developed retinal angiomas in one eye at the age of 32 years. Family member III-14 has pale optic discs with a normal neurological examination.

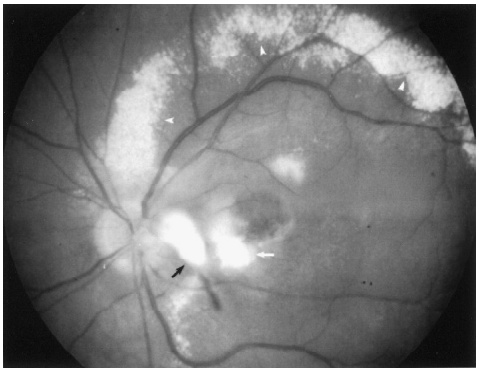

Fig. 2 Posterior pole of left eye of patient E III-12. Angioma attached to optic disc and floating in the vitreous cavity (black arrow). Second angioma in macular area (white arrow), causing exudation along the vascular arcade (arrow heads).

In family F eight patients are suffering from the disease; six patients live in Turkey; two patients are seen more or less regularly in our hospital. Both patients suffer from cerebellar haemangioblastomas and retinal angiomas. In one patient the retinal angiomas caused an exudative retinal detachment that was treated in Turkey.

Discussion

Four of the six probands of the Utrecht families presented with symptomatic cerebellar haemangioblastoma, the other two with retinal angioma. In the literature, cerebellar haemangioblastoma and retinal angioma are given equally frequently as the most common presenting feature of VHL disease.[4,13-16] Symptomatic cerebellar haemangioblastomas usually present in the fourth decade and symptomatic retinal angiomas in the third decade. In monitoring examinations of documented VHL families, however, retinal angioma is the earliest and most frequent manifestation detected.[13,17]

Although most retinal angiomas, both symptomatic and asymptomatic, start to develop in the third decade, which we also found in our patients, it is not unusual to find small lesions in teenagers from affected families, and some lesions have even been documented from birth.[17]

Our patients' case histories clearly reveal the natural course of development of ocular angiomas. A peripheral retinal angioma starts as a small pink or grey spot, the size of a diabetic micro aneurysm. There are no feeder vessels and no well-formed vascular channels within the tumour. Fluorescein angiography therefore gives a picture of a lack of perfusion. This stage 1 tumour can remain unaltered for years. Usually, however, it grows slowly until in stage 2 it shows a small, slightly elevated, red nodule. At this point only the draining vein is prominent. On fluorescein angiography, perfusion and profuse leakage is seen at the site of the angioma (Fig. 3). In stage 3 the angioma attains its classic appearance: an elevated red nodule with prominent feeder vessels (Fig. 4). Exudate can be seen on the angioma and in the macular area. Neovascularisation on the surface causes haemorrhage into the vitreous body. Fluorescein angiography shows profuse leakage, micro aneurysms and capillary dropout in the surrounding capillary bed (Fig. 5) because of ischaemia, caused by the shunting of circulation to the angioma. In addition hyper fluorescence is seen up to several hours after angiography. In stage 4 exudative detachment of part of the retina is seen. In stage 5 total exudative retinal detachment is seen.[18]

Fig. 3 Fluorescein angiographic picture of stage 2 angioma with prominent draining vein (arrow).

Fig. 4 Composite picture of classical stage 3 angiomas (arrows) in the temporal periphery with prominent feeder vessels, leading from and to the optic disc.

Stage 1 carries no risk of immediate exudation or haemorrhage. The only risk lies in its potential for progression to stage 2, but decades may elapse between the two stages. Stage 2 also poses no immediate threat of exudation or haemorrhage, but there is a great risk of progression to stage 3, which may occur within months. Stage 3 is the earliest stage in which exudation and haemorrhage pose a clinical problem. Progression to stage 4 is almost certain to occur within months and there is almost no possibility of stopping the progression to the terminal stage 5. If it is not possible to destroy the tumour(s) and reattach the retina uveitis, cataract and secondary, usually untreatable, glaucoma will eventually develop, necessitating enucleation of the globe.[18]

Most angiomas are present in the temporal periphery. They can, however, also occur in the nasal periphery, in the posterior pole, at the ora serrata, next to or on the optic disc and in the optic nerve.[3,19] In family E, macular-, (Fig. 2), parapapillary-, optic disc- and ora serrata angiomas were found (4 patients) as well as peripheral retinal angiomas. From 1975 to 1987 only 55 cases of optic disc angiomas have been reported, of which 24% were considered part of VHL disease.[4] Angiomas originating at the ora serrata are even more rare. In the series reported by Moore et al. in 1991, however, 11 peripheral retinal angiomas were detected together with five angiomas on the optic disc and one at the ora serrata in 11 patients.[19]

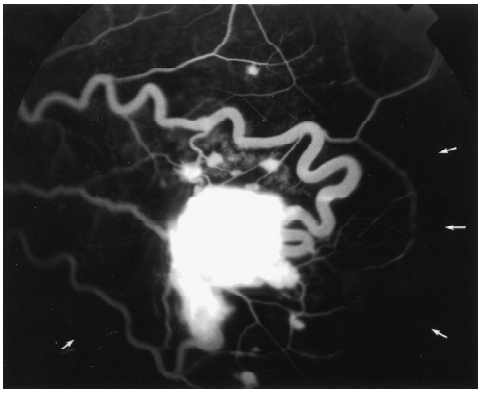

Fig. 5 Fluorescein angiographic picture of classical stage 3 angioma. Profuse leakage from the angioma, micro aneurysms and capillary drop-out in the surrounding capillary bed (arrows).

Angiomas of the optic disc appear as well-circumscribed, reddish-orange, round or oval elevated lesions obscuring part of the optic disc, usually on the temporal side. Less commonly, the lesion is flatter and greyish with poorly defined margins extending from the disc into the retina. Fluorescein angiography reveals that the mass consists of small caliber vessels that fill early during the retinal arterial phase and leak. The most common symptom is visual loss resulting from macular oedema, with or without serous retinal detachment.[15] Intra retinal juxta papillary angiomas appear as a diffuse greyish thickening that obscures the border of the optic disc, often without prominent vascular channels. They are dispersed within the retinal tissue and are not confined to a single layer; they have communication with both choroidal and retinal circulations. They may be confused with papilloedema, papillitis, chorioiditis, choroidal neovascularisation, or choroidal haemangioma.[18] Data concerning age of onset and natural course are lacking. Regarding the findings in our families and in those of Moore et al. in 1991,[19] optic disc-, juxta papillary and ora serrata angiomas may occur more commonly than previously reported. In our series these lesions, together with retinal detachment caused by large peripheral angiomas, are responsible for the decreased visual acuity.

In family E pale optic discs are also found, some are secondary after obstructive hydrocephalus caused by a cerebellar haemangioma, but some are primary in patients without CNS lesions and with no signs of raised intracranial pressure. Up till now no clear explanation could be given for this phenomenon. Since pale optic discs also appeared to occur in a family member without the germline mutation, there is probably no causal relationship with VHL disease. Visual acuity is normal in the persons with pale optic discs.

Regarding the natural history of peripheral retinal angiomas, treatment is indicated from stage 3 on.[3,18] The result of treatment of small stage 3 peripheral angiomas up to 1 disc diameter in size is usually good; tumours of 1 to 1.5 disc diameter in size respond satisfactorily to treatment, but may require repeated treatments and they may recur; large lesions (> 2 disc diameters in size) respond poorly and treatment may result in further tractional detachment of the retina.[18] Histopathologic examination of moderate-sized angiomas following what appeared clinically to be successful laser photo coagulation has revealed residual viable tumour to be present in some cases beneath areas of superficial tissue destruction.[3]

Since the start of our monitoring program, peripheral angiomas were treated with xenon- and, later on, with laser coagulation. No complications of these treatments were seen and visual acuity remained normal. There have been no recurrences in any of our patients thus far.

Ocular angiomas that usually cause a decrease of visual acuity and that are difficult to treat because treatment itself inevitably causes a decrease of visual acuity, are optic disc-, juxta papillary-, macular- and paramacular angiomas. In our families large peripheral, posterior pole and ora serrata angiomas were the cause of decreased visual acuity in 20% of our patients. Coagulation therapy of papillary, juxta papillary and macular angiomas is so unsatisfactory that treatment is not advised before visual acuity drops because of exudation, and even then treatment can be more hazardous than the subsequent natural course.[10] Thus, only early detection and treatment of peripheral angiomas can be expected to reduce the percentage of patients with decreased visual acuity. Early detection and treatment of these peripheral angiomas is therefore of paramount importance. Although it will never be possible to prevent visual loss in all VHL patients, earlier detection of asymptomatic peripheral retinal angiomas should lead to improved visual outcome and, at any rate, a better outcome than that reported in the series in which 36% of patients had visual acuity of less than 0.1 and 11% had enucleation of the globe.[4]

Ocular monitoring of patients and persons at risk should start as early as possible. For ophthalmologists used to examining children, it poses no problem to examine a child annually, starting from birth. For practical purposes, however, and since most retinal angiomas start to develop later in life, ophthalmological examinations may start at the age of five when the child is sufficiently cooperative to detect small peripheral angiomas.

In patients with apparently sporadic retinal angiomas it is advisable to perform germline DNA analysis, since the risk of developing VHL disease is more than 40%,[7] especially if the angiomas are bilateral, or unilateral and multicentric, or if the patient is young, or if the family history is suggestive of VHL disease.

Four different clinical types of VHL disease are distinguished at the moment (Table 1).[20-22] Families A to D and F suffer from the clinical type 1. Family E suffers from the clinical type 2B. Although clinical types of VHL disease can be distinguished, a relation to a specific genotype has not yet been found. A clear-cut genotype-phenotype correlation is not very likely, because in addition to the germline mutation also somatic mutations in the homologous allele, which can be manifold, are necessary to bring out the ophthalmological spectrum of signs of VHL disease.

Table 1. Classification of VHL disease

Type	CNS haemangioblastoma	Retinal haemangioblastoma	Renal cell carcinoma	Phaeo-chromocytoma
I	present	present	present	absent
IIA	present	present	absent	present
IIB	present	present	present	present
IIC	absent	absent	absent	present

Patients in all our families developed peripheral retinal angiomas. In one family with the missense mutation V170D optic disc-, juxta papillary-, macular- and ora serrata tumours were also found. In general, however, no clear-cut genotype-phenotype correlation could be determined in our families with VHL disease.

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Von Hippel Lindau disease: guidelines for diagnosis and clinical monitoring

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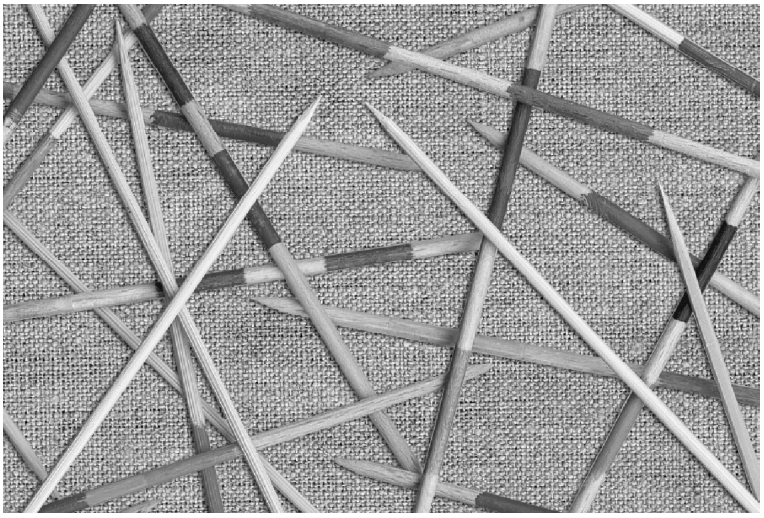

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Abstract

Von Hippel-Lindau (VHL) disease is an autosomal dominant inherited syndrome presenting with tumours in various organs. The Dutch VHL Working Group presents guidelines for DNA testing and clinical monitoring, to enhance early detection and treatment of VHL patients in the Netherlands.

Diagnosis of VHL disease is justified in patients presenting with a typical VHL tumour and a positive family history. In addition, patients with a VHL-related tumour and a negative family history may have VHL disease. Diagnosis of VHL disease can be confirmed by molecular genetic analysis of the VHL gene and is informative in virtually all VHL families. A patient with confirmed or suspected VHL disease should be referred for genetic counselling.

A protocol for clinical monitoring of VHL disease is presented and is recommended for: carriers of a VHL germline mutation; members of VHL families with an unknown familial mutation; members of VHL families who decline testing of the familial mutation; patients suspected of having VHL disease, but who do not have a VHL mutation.

Introduction

Von Hippel-Lindau (VHL) disease is an autosomal dominant inherited disorder with a high penetrance and is characterised by tumours occurring in various organs. The most frequent lesions are haemangioblastoma in retina and central nervous system (CNS), renal cell carcinoma, phaeochromocytoma, cyst and cystadenoma in kidneys, pancreas and epididymis, and the endolymphatic sac tumour (ELST) in the inner ear.[1,2] The minimal birth incidence has been estimated at 1:36,000.[3]

In order to support early detection and treatment of VHL patients in the Netherlands, these recommendations, based on international guidelines, for DNA diagnosis and periodic, clinical monitoring have been proposed by the National VHL Working Group.[4,5] We expect early detection and treatment of complications of VHL disease, especially from haemangioblastoma and renal cell carcinoma, will reduce morbidity and mortality in VHL patients.

Diagnostic criteria

Clinical For diagnosing VHL disease in a patient, both clinical manifestations and family history are important. Typical tumours that are associated with VHL disease are: haemangioblastoma (retinal or CNS), phaeochromocytoma, renal cell carcinoma, ELST and multiple pancreatic cysts.[1,6] Multiple pancreatic cysts are specific for VHL disease because they are rare in the normal population.[7] In contrast, renal or epididymal cysts occur more often in the normal population. In the presence of a positive family history, VHL disease can be diagnosed in a patient with a typical VHL tumour.[1,8] In the absence of a VHL family history, two or more haemangioblastoma, or a haemangioblastoma combined with a further typical VHL tumour are required.[1]

Molecular genetics The gene that, in mutated form, is responsible for the disease was identified in 1993.[9] The gene is located on chromosome 3 (3p25-26) and is a tumour suppressor gene accordingly to Knudson's two hit theory.[10] Inactivation (mutation) of both alleles is thought to drive a normal cell into a tumour cell. In all cells of VHL patients a germline (inherited) mutation is present in one allele (the first hit). The second hit is a somatic mutation in the remaining allele.

With molecular genetic analysis of DNA obtained from lymphocytes from peripheral blood, a germline mutation is detected in virtually all well-defined VHL families.[11] Many different mutations are found in the VHL gene. Between 60-70% of these mutations are point mutations, or micro-deletions and -insertions in the coding region of the VHL gene. In the other families, the VHL gene is partially or fully deleted. Mutation analysis comprises direct sequencing, Southern blotting and Fluorescence In Situ Hybridisation (FISH).[11] In addition, VHL germline mutations are found in patients with apparently sporadic haemangioblastoma (10%),[12,13] renal cell carcinoma (1.6%),[14] and phaeochromocytoma (3-9%).[15,16]

Natural history

VHL disease is characterised by inter- and intrafamilial variation in expression of the disease. This means that both manifestations and the age at which symptoms occur may differ not only between families but also within a family. Two phenotypes are distinguished in VHL disease: families without phaeochromocytoma (VHL type I)

and those with (VHL type II). Mutations that are associated with a loss of function VHL protein are correlated with a phenotype without phaeochromocytoma. In contrast, families with phaeochromocytoma have predominantly specific missense mutations.[22] In view of the variability of expression of the disease, it was suggested that, as well as the family-specific VHL gene mutation, external factors (environment and lifestyle) and modifier genes also play a role.[23]

Penetrance of the disease is high, almost all carriers of VHL germline mutation develop one or more VHL-related tumours at an age of 60 years.[4] The mean prevalences of the various tumours are depicted in Figure 1.[1,4,6,17-21] Generally, tumours occur at a relative young age in VHL patients. This age is however dependent on the intensity of clinical monitoring of asymptomatic lesions. The mean age at diagnosis of the separate tumours are: retinal haemangioblastoma 25 years (range 1-68 years), phaeochromocytoma 28 years (10-56 years), cerebellar haemangioblastoma 30 years (11-78 years) and renal cell carcinoma 36 years (15-69 years).[4,5,17,24] Most VHL patients die from the consequences of cerebellar haemangioblastoma or renal cell carcinoma.[4,17]

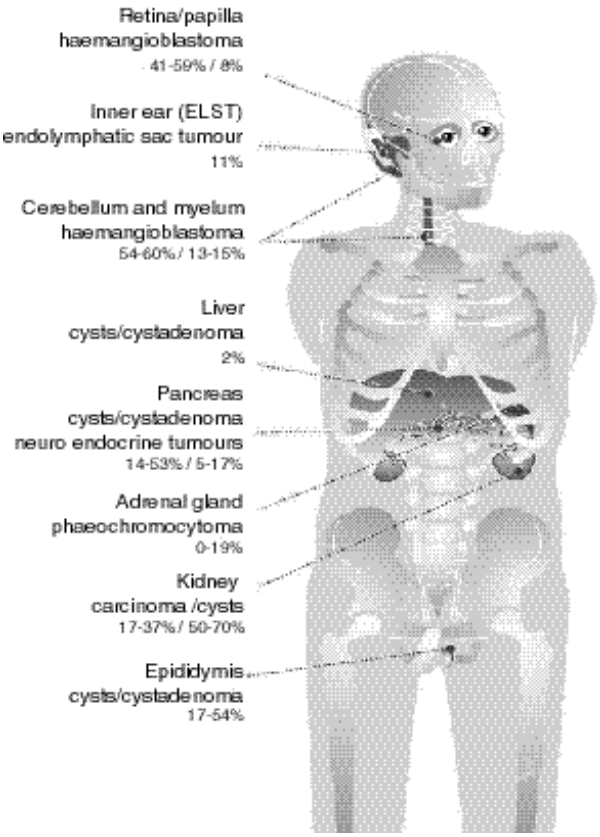

Fig. 1 A survey of the most frequent tumours in patients with VHL disease. The percentages are derived from studies in large populations of VHL patients (n= 43-554).[1,4,6,17-21]

Dutch guidelines

DNA diagnosis

Clinical monitoring (Table 1) and DNA analysis are indicated for any patient suspected of having VHL disease (Figure 2). DNA analysis in minors is only possible after consulting a clinical geneticist. The assessment of a VHL germline mutation has consequences for confirmation of the clinical diagnosis or presymptomatic diagnosis in family members. Diagnosis is confined to presymptomatic DNA analysis only for persons from families with an identified VHL gene mutation.

DNA analysis for VHL disease is carried out by the Department of Medical Genetics at the UMC Utrecht and the Department of Clinical Genetics in the University Hospital Rotterdam. An application for DNA testing should meet the following requirements: 1) an informed consent procedure should proceed any application; 2) a completed application form (including clinical data and family tree); 3) three heparine-tubes with 10 ml blood each (two tubes for DNA-diagnostics and one for FISH).

Table 1 VHL protocol for periodic clinical monitoring

Investigation	Age	Frequency
Obtaining patient's history	From 10 years old	annually
Physical examination, blood pressure	"	"
Biochemical blood tests[2,5]	"	"
24 h-urine tests (catecholamines and metanefrines)[a]	"	"
Ophthalmological examination	From 5 years old	annually
Upper abdominal ultrasound	From 10 years old	annually
MRI (with gadolinium) cerebellum en myelum	From 15 years old	biannually[b]
MRI upper abdomen	When indicated[c]	
MRI inner ear	When indicated[d]	
Audiogram	When indicated[d]	
Neurological examination	When indicated	

[a] Assess separately
[b] Radiosurgical techniques are being developed that enable presymptomatic treatment of solid cerebellar haemangioblastoma, which may justify more frequent monitoring for these tumours.
[c] When an MRI of the myelum is made every two years, it is recommended the upper abdominal organs be imaged simultaneously. In this way the upper abdomen is monitored with ultrasound and MRI in alternate years.
[d] When an Endolymphatic Sac tumour (ELST) is suspected; i.e. hearing loss/deafness, tinnitus, or vertigo.

A presymptomatic DNA test can only be requested by a clinical geneticist. A test to confirm the clinical (symptomatic) diagnosis can be requested by a clinical geneticist or the attending physician. It is recommended that a clinical geneticist be consulted before requesting a DNA test. Firstly, a genetic diagnosis not only has consequences for the applicant, but also for his family members. Secondly, since a mutation

Fig. 2 Guidelines for DNA diagnosis and periodic monitoring of patients with a VHL tumour.

is not found in all VHL families, proper interpretation of the test result requires consultation between the attending physician and clinical geneticist. This has particular consequences for a patient in which the diagnosis of VHL disease is considered, but no mutation is detected.

Periodic clinical monitoring (Table 1): *Who are eligible?*

1. carriers of a VHL germline mutation;
2. first- and second-degree family members in a VHL family without an identified germline mutation;
3. first- and second-degree family members that decline a DNA test;
4. patients (and first-degree family members) with a typical VHL tumour and features that suggest the presence of a germline mutation (i.e.: young age at diagnosis, bilateral or multiple tumours).

Assuming that early detection, periodic monitoring and treatment of VHL patients lead to a better prognosis, giving information to family members is of utmost importance. Family members of VHL patients may not be contacted directly. An informative brochure (distributed via the patient or informed family members) can provide information to persons at risk for VHL disease and they can be advised to seek genetic counselling for themselves. In this way they can choose whether they (or their offspring) want to be tested. Patients can turn to the Dutch VHL support group for information and advice on social issues (see: www.vhl.org).

Fig. 2 Guidelines for DNA diagnosis and periodic monitoring of patients with a VHL tumour.

* At the moment, a VHL germline mutation is found in virtually all families that meet clinical diagnostic criteria. If no germline mutation is found in the presence of a positive family history, patients and first- and second-degree family members should be monitored according to the protocol. In patients who meet clinical diagnostic criteria and in patients persistently suspected of having VHL disease, we advocate submitting the patients and their first-degree relatives to regular monitoring. In the absence of a VHL family history, a once-only investigation following the protocol is recommended. If this investigation reveals no further typical VHL tumours, the diagnosis of VHL disease is unlikely.

Acknowledgement

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Germline mutations in the Von Hippel-Lindau (VHL) gene

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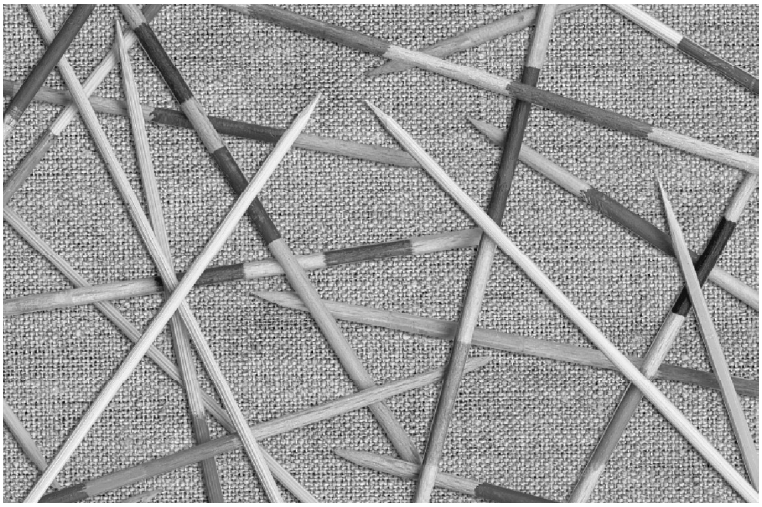

In preparation

Abstract

Introduction Von Hippel-Lindau (VHL) disease is a complex, autosomal, dominant inherited disorder, variably presenting with retinal and cerebellar haemangioblastoma, renal cell carcinoma, phaeochromocytoma and endolymphatic sac tumours. Cysts and cystadenoma may develop in kidney, pancreas and epididymis. Germline mutations in the VHL tumour suppressor gene are found in most of the families fulfilling the clinical diagnostic criteria of VHL disease.

Objective To summarise the results of mutation analysis of the VHL gene in familial and sporadic cases of VHL disease diagnosed in the Netherlands.

Patients and methods Familial (n=25) and sporadic (n=7) VHL patients, as well as sporadic patients (n=2) with VHL-related tumours, not fulfilling current diagnostic criteria for VHL disease, were investigated by direct sequencing of the coding region, quantitative Southern blot analysis and Fluorescence in Situ Hybridisation (FISH) of the VHL gene.

Results We report 34 VHL germline mutations, including eight novel germline mutations in the open reading frame of the VHL gene. Analyses of genotype-phenotype correlations were consistent with previous reports. In nine sporadic patients with a VHL germline mutation we could identify four de novo VHL gene mutations. In four of the nine sporadic patients the parents were not available for testing. One of the nine patients shared a VHL germline mutation with a clinically unaffected parent (age 77 years) suggesting non-penetrance of VHL disease. Family histories of VHL in two other families were suggestive for reduced penetrance of VHL germline mutations.

Conclusions These results indicate that at least 12% of the germline mutations in the VHL gene occur de novo. Germline mutations are found in patients not fulfilling the currently accepted diagnostic criteria for VHL disease. Absence of VHL symptoms in carriers of VHL germline mutations indicate reduced penetrance and has implications for genetic counselling.

Introduction

Von Hippel-Lindau (VHL) disease is an autosomal, dominantly inherited disorder. Current estimates of the prevalence of VHL germline mutation carriers range between two and three per 100.000 persons.[1-3] A germline mutation in the VHL gene predisposes carriers for haemangioblastoma in the central nervous system and retina, and for renal cell carcinoma, phaeochromocytoma, endolymphatic sac tumours, and cysts and cystadenoma in kidney, pancreas and epididymis.[4,5] VHL disease is characterised by multiple, richly vascularised tumours that may occur at a young age, i.e. between 20 and 40 years.[6,7] The penetrance of the disease appears almost complete by the age of 60 years and the median expected survival is 49 years.[2,7] However, early detection of tumours by intensive radiological and clinical screening, together with advanced operation techniques are likely to reduce both morbidity and mortality in VHL disease.[5,8-10] Early identification is now facilitated by presymptomatic detection of VHL germline mutations.

With a positive family history, VHL disease can be diagnosed in a patient with at least one typical VHL-related tumour.[5,11] Typical VHL-related tumours include retinal and cerebellar haemangioblastoma, renal cell carcinoma, and phaeochromocytoma.[5] Endolymphatic sac tumours and multiple pancreatic cysts suggest a positive carrier status (in the presence of a positive VHL family history), since they are uncommon in the general population.[5,12] In contrast, renal and epididymal cysts occur more frequently in the general population and alone they are unreliable indicators of the carrier status.[13] Without a family history, VHL disease can be diagnosed when two or more retinal or cerebellar haemangioblastomas or a single haemangioblastoma in combination with a typical visceral lesion are present in a sporadic patient.[5]

A genetic locus for the disease was mapped to the short arm of chromosome 3 by linkage studies,[14] and the VHL tumour suppressor gene was identified in 1993 by positional cloning.[4] Subsequently, more than 300 VHL germline mutations have been reported world-wide.[15,16] Using direct sequencing of the coding region and quantitative Southern blot analysis, a detection rate of 100% was reported in well-defined VHL families.[17] VHL germline mutations of all types are scattered over the VHL gene and also include entire gene deletions.

Recently, it was demonstrated that the VHL protein (pVHL) plays a role in the degradation (via a process called ubiquitination) of hypoxia-inducible proteins, possibly including vascular endothelial growth factor (VEGF).[18-20] Excessive blood vessel formation may occur when these proteins are not properly degraded.[21] pVHL fulfils its function by binding to other proteins called Elongin C, Elongin B, and Cullin2.[18]

Analysis of the structure of pVHL also enables the study of genotype-phenotype correlations. The disease has been divided in two phenotypes: families without (VHL type I) and with phaeochromocytoma (VHL type II).[15,17] Mutations in patients with VHL type I group are most commonly found in the beta domain of the pVHL and are predicted to lead to a loss of function. This beta domain probably binds the target proteins for ubiquitination.[18] In contrast, most mutations in patients with VHL type II (i.e. specific missense mutations) are located in the alpha domain and allow a residual ability to bind Elongin C.[18] It was suggested that specific missense mutations would have a dominant negative effect by sequestering key components of the ubiquitin

pathway. Missense mutations are present in 69% of VHL type II families and 27% of VHL type I families.[17] Most VHL type II families have renal cell carcinoma (type IIB), but some do not (type IIA).[22] A phaeochromocytoma-only phenotype (type IIC) is associated with specific missense mutations.[23] Intrafamilial variability indicates that other genetic ('modifier' genes) and/or environmental factors are involved in the clinical manifestations of VHL gene germline mutations.[24].

We report a survey of VHL germline mutations and their associated phenotypes found in families and sporadic patients with VHL disease, as well as sporadic patients with a VHL-related tumour (but not fulfilling the current diagnostic criteria for VHL disease) diagnosed in the Netherlands.

Patients and methods

Patients

Familial VHL patients and patients with at least one VHL-related tumour were referred for DNA testing by clinical geneticists, internists, neurologists, neurosurgeons, and ophthalmologists between January 1985 and August 1999. Detailed family histories were obtained for all carriers of a VHL germline mutation as well as clinical information from medical and pathological reports.

The patient population consisted of 25 VHL patients with well-documented family histories and seven VHL patients (4, 9, 16, 17, 21, 23 and 32) without a family history but who met the diagnostic criteria of sporadic VHL disease. We also included two patients (6 and 10) with a VHL germline mutation who do not fulfil the current VHL diagnostic criteria. The success rates of finding germline mutations in such sporadic patients with VHL-related tumours, e.g. central nervous system haemangioblastoma,[25] renal cell carcinoma [26], and phaeochromocytoma [27] are reported elsewhere. Most of these patients presented with a young age of onset, and/or with multicentric or bilateral manifestations.

DNA analysis

DNA of probands was extracted from 10 ml peripheral blood samples according to established procedures. Exons 1, 2 and 3 of the VHL gene and their flanking sequences were amplified using PCR, using oligonucleotides according to Gnarra et al.[28] The flanking sequences included 90 nucleotides upstream of the start codon (the first nucleotide of the coding region is 214) and 45 nucleotides downstream of the stop codon. The nucleotides are numbered according to Latif et al. (Genbank accession number L15409).[4] PCR products were purified and subjected to sequence analysis using an ABI 377 automated sequencer. When a missense mutation was found, DNAs of 50 non-VHL patients were used as control samples to investigate the possibility of a polymorphism. Probands with the same germline mutation were haplotyped with a panel of polymorphic markers linked to the VHL gene (D3S651, D3S656, D3S1038, D3S1304, D3S1317, and D3S1537) to study common ancestry.

Screening for structural rearrangements, including deletions, was performed by Southern blot analysis. DNA was digested with *Eco* RI,[4] and with an *Eco* RI / *Ase* I double digest.[17] After gel electrophoresis and transfer to Hybond-N filters, the genomic DNA was hybridised with the VHL *g7*-cDNA probe.[4] After detection of aberrant fragments the DNA was digested with at least two other restriction enzymes to

exclude the possibility of polymorphisms affecting an *Eco* RI or *Ase* I site. A human beta globin gene probe was used as an internal control to enable comparison of signal intensities in the case of apparently normal hybridisation patterns.[29] Additionally, exon-specific probes generated by PCR amplification of exons 1, 2, and 3 were hybridised to the same filters, for further delineation of the abnormalities.

Deletions encompassing the entire VHL gene were confirmed by FISH analysis on lymphocytes from affected individuals. FISH analysis was carried out on metaphase chromosome spreads according to established procedures [30]. The VHL cosmid-11 probe was labelled by nick translation with biotin-14-dATP. After precipitation of the labelled probe in the presence of Cot-1 DNA, pre-annealing was performed to block repetitive sequences. The final concentration of the probe was 15 ng/ml. Hybridisation of the denatured probe onto the denatured metaphase chromosomes was carried out overnight at 37˚C. Each slide was mounted with 15 ml antifade medium (Vectashield, Brunschwig) containing DAPI. Microscopic analysis of images was performed using a CytoVision, Applied Imaging.

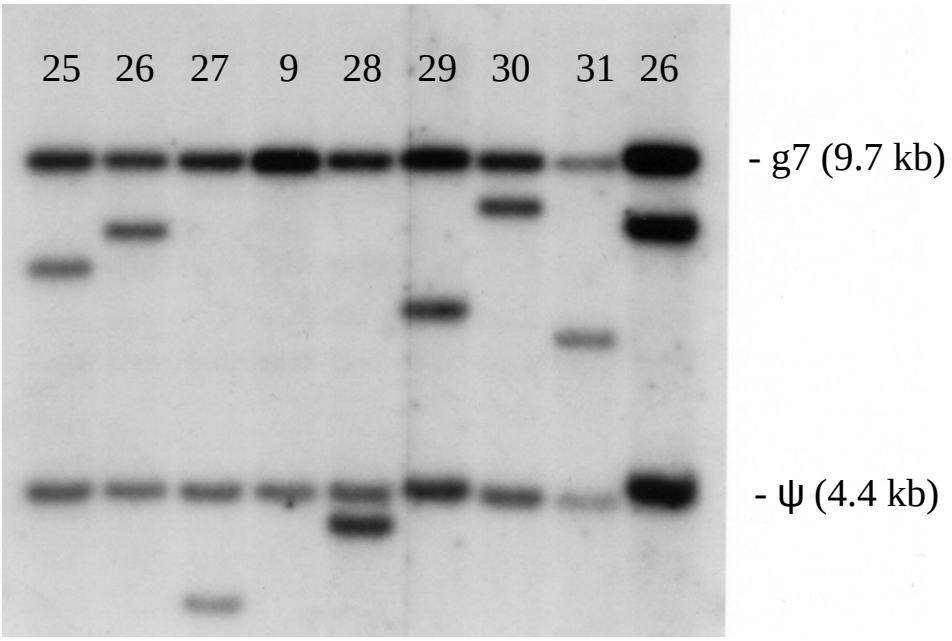

Fig.1 Southern blot analysis of eight patients with a partial VHL gene deletion.
Numbers of the probands are depicted above the lanes and correspond with the numbers of Table 1. Genomic DNA (7 mg) was digested with an *Eco* RI and *Ase* I double digest and hybridised with ^{32}P labelled probes specific for the VHL gene (g7 cDNA, upper band). The lower band represents a VHL pseudogene (ψ), located on chromosome 1.[34]
Samples with a rearrangement in the VHL gene exhibit a less intense g7 band and an abnormal migrating band. The abnormal migrating band shows different lengths for all patients with a partial deletion, except for lane 2 and 9 (both members from family 26). Patient 9 is a carrier of a missense mutation and can be regarded as a normal control in this analysis.

Results

Mutation analysis of the VHL gene revealed a total of 34 variants. Sequence analysis revealed 13 missense mutations, three nonsense mutations, four microdeletions, three insertions, and one splice site mutation (Table 1). None of the missense mutations was found in a control panel of 50 non-VHL patients. Quantitative Southern blot analysis showed eight gene rearrangements (Fig. 1), and two entire VHL gene deletions (five of these ten are described in more detail in a manuscript in preparation). Since the gene rearrangements showed unique banding patterns on Southern blot analysis, no further investigation for shared haplotypes was undertaken. FISH analysis in patients with deletions encompassing the entire VHL gene showed a single fluorescent signal, whereas two signals at 3p were present on the chromosomal pairs of healthy family members.

32 of the 34 germline mutations in the VHL gene were found in patients with familial or sporadic VHL disease. The remaining mutations were identified in two sporadic patients (6 and 10) with a VHL-related tumour, but who did not meet the diagnostic criteria for VHL disease. A missense mutation (P81S) was found in a 47-year old woman (6) with a solitary cerebellar haemangioblastoma (diagnosed at 44 years). The P81S mutation was not identified in four relatives (father, son, sister and aunt; aged 77, 17, 43 and 64 years respectively), who were without signs of VHL-related lesions when clinically screened. Another missense mutation (V166A) was found in a 15-year old girl (10) with bilateral phaeochromocytoma diagnosed at age 11 years and a negative VHL family history.

In four (9, 10, 21 and 23) of the nine sporadic patients the VHL germline mutation was not identified in either of their parents; while the father of patient 6 shared the germline mutation with his daughter, but has no VHL-related tumours at the age of 77 years. The parents of four other sporadic patients were not available for DNA analysis. Patient 4 is an adopted child with bilateral retinal haemangioblastoma. Patient 16 (37 years old), has a negative family history for VHL disease; neither parents (~70 years old) nor seven sibs (25-50 years old) were reported to have VHL-related tumours. Patient 17 (59 years old) has a negative family history; his parents are deceased and he has no children. Patient 32 (29 years old) has seven relatives (brother, parents and great-parents) who underwent ophthalmological screening but were all negative for VHL-related ophthalmological lesions.

Table 1 Genotypes and phenotypes of the reported patients and families.
Fam, family number; Code, unique family registration number; Mutation, VHL germline mutation (consequence of point mutations); Origin: D, Dutch; B, Belgian; T, Turkish; K, Kosovarian;Prev. reported, previously reported and reference; Pts, number of patients with an identified germline mutation in the VHL gene; rHAB, retinal haemangioblastoma; cHAB, central nervous system haemangioblastoma; RCC, renal cell carcinoma and (renal cysts); Phaeo, phaeochromocytoma; PC, pancreatic cysts; ELST, patients suspected (deafness, tinnitius, or vertigo) for an endolymphatic sac tumour; APMO, adnexal papillary tumour of probable mesonephric origin; other, (if in more than one VHL mutation carrier, number of patients).
No, novel germline mutation in the VHL gene; sporadic, sporadic patient and parents not tested (except for case 6, see text); *de novo*, sporadic patient and tested parents do not share the VHL germline mutation; *, patients not fulfilling clinical diagnostic VHL criteria; part. del, partial deletion of the VHL gene that has not been precisely characterised; na, not applicable.

Fam	Code	Mutation (Consequence)	Origin	Case	Prev. reported	Pts	rHAB	cHAB	RCC	Phaeo	PC	Other VHL-related tumours
1	U3	404G>C (R64P)	D	Familial	[31]	6	0	0	1(0)	2	0	carotis paraganglioma
2	U49	407C>T (S65L)	D	Familial	[32]	1	0	1	1(1)	0	1	epididymal cyst
3	H2221	407C>G (S65W)	D	Familial	[32]	1	1	1	1(1)	0	1	
4	U25	421G>A (E70K)	D	Sporadic	[25]	1	1	0	0(0)	0	0	
5	#12	452G>A (S80N)	B	Familial	[32]	1	1	0	0(0)	0	0	
6	U24	454C>T (P81S)	D	Sporadic*	[32]	5	0	1	0(0)	0	0	
7	H1554	601G>T (V130F)	D	Familial	No	6	4	3	2(4)	0	3	
8	U30	665C>T (I151T)	D	Familial	No	1	0	1	1(0)	0	0	
9	H9282	667A>C (T152P)	D	*de novo*	No	1	0	1	0(1)	0	1	epididymal cyst
10	H9944	710T>C (V166A)	D	*de novo**	No	1	0	0	0(0)	1	0	
11	U4	713G>A (R167Q)	D	Familial	[32]	5	3	3	2(3)	1	1	ELST(2), APMO
12	H2229/10903	713G>A (R167Q)	D	Familial	[32]	5	2	2	1(0)	4	0	ELST
13	U1/H1011	722T>A (V170D)	D	Familial	[32]	14	6	9	7(8)	4	5	ELST (2), APMO, hepatic cyst (5)
14	#6	694C>T (R161X)	T	Familial	[32]	1	1	0	0(1)	0	1	
15	G1	703C>T (Q164X)	D	Familial	[32,33]	1	0	1	1(0)	0	0	epididymal cyst
16	U50	761C>A (S183X)	B	Sporadic	[32]	1	1	1	0(0)	0	0	
17	U40	440delTCT	D	Sporadic	[32]	1	0	1	1(0)	1	0	epididymal cyst
18	H2034	440delTCT	D	Familial	"	3	2	1	2(0)	0	0	
19	U86	606 CC>A	D	Familial	No	2	1	0	0(1)	0	1	
20	#10	777 del G	B	Familial	No	1	0	1	0(1)	0	0	
21	U47	474 insA	D	*de novo*	No	1	1	1	1(0)	0	0	
22	H1003	685 insT	D	Familial	[32]	13	6	2	7(6)	0	4	pancreatic carcinoma
23	U19	699 insG	D	*de novo*	No	1	1	1	0(0)	0	0	
24	U22	677 -2 A>C	D	Familial	[32]	1	1	1	1(1)	0	1	
25	H2257	del ex.1	K	Familial	na	2	1	2	1(0)	1	0	epididymal cyst
26	U61	del ex.1	D	Familial	na	2	2	0	0(0)	0	0	epididymal cyst, neurofibromatosis
27	H7951	del ex.3	D	Familial	na	1	1	1	1(1)	0	1	
28	H10034	del ex.1	D	Familial	na	12	7	8	7(0)	0	1	hepatic carcinoma
29	U2	del ex.1+2	T	Familial	na	3	2	3	1(1)	0	2	hepatic cyst
30	U15	del ex.1	D	Familial	na	6	3	5	1(0)	0	2	ELST, APMO, hepatic cyst
31	U51	del ex.1	B	Familial	na	1	1	1	0(0)	0	1	
32	#9	partial del	B	Sporadic	na	1	1	0	0(1)	0	1	
33	U23	del VHL	B	Familial	na	5	1	4	0(1)	0	2	ELST(2), APMO

Clinical manifestations corresponded with VHL type I (without phaeochromocytoma) in 27 families. Different types of germline mutations were identified in these families, including eight missense mutations (30%), three nonsense mutations, three microdeletions, three insertions, one splice site mutation, seven partial deletions, and two entire VHL gene deletions. Phaeochromocytoma were present (VHL type II) in seven families, five of which had (specific) missense mutations (71%), one a microdeletion, and one a partial deletion of the VHL gene. The frequency of missense mutations was almost significantly different (Fisher's exact test, $p = 0.057$) between VHL types I and II. Renal cell carcinoma occurred in six of the seven families with phaeochromocytoma (VHL type IIB). One sporadic 15-year old patient (number 10) had only phaeochromocytoma diagnosed at age 11 (VHL type IIC).

In addition to the P81S carriers, other families in this study suggest a reduced penetrance of VHL germline mutations. In family 1 with the R64P mutation, two patients had bilateral phaeochromocytoma (14 and 29 years), one renal cell carcinoma (48 years) and one a paraganglioma (29 years). Two other closely related carriers (55 and 34 years) of the R64P mutation had no symptoms after clinical screening so that the age of the first carrier would also suggest non-penetrance. Patient 4 (E70K), aged 25 years, only has retinal haemangioblastoma. Patient 5 (S80N), aged 15 years, only has retinal haemangioblastoma and his father, who died from cerebellar haemangioblastoma aged 37 years, was his only relative with a VHL-related lesion.

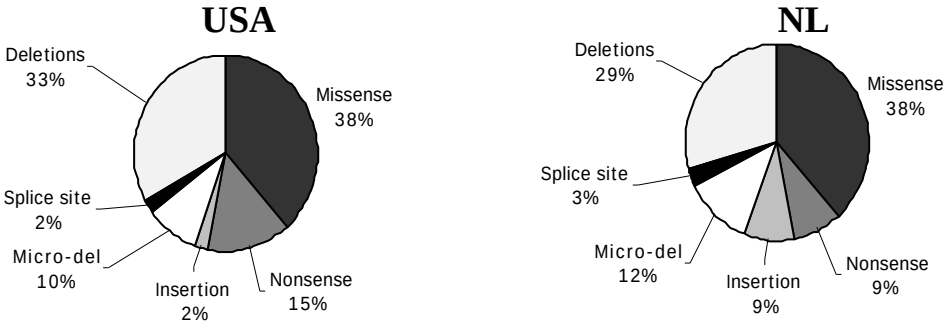

Fig. 2 Comparison of germline mutations in the VHL gene reported in the USA [17] and in this study, the Netherlands (NL).

Discussion

We identified VHL germline mutations in 32 patients with familial and sporadic VHL disease and in two sporadic patients with a VHL-related tumour not fulfilling the currently accepted diagnostic VHL criteria. The mutation spectrum did not differ significantly from that reported in a comparable study (Fig. 2).[17] We identified two unrelated families with the R167Q mutation. Codon 167 is a hotspot for mutations in the VHL gene (Fig. 3) and has a structurally central role in the functional domain of the VHL protein that binds Elongin C.[18] In addition, we confirmed another recurrent VHL germline mutation (440 del TCT), which has been decribed at least ten times worldwide.[15,17,32] Apparently, the presence of a tandem repeat (TCTTCT) renders this sequence susceptible to the deletion of a TCT triplet. Haplotype analysis with six polymorphic markers ruled out the possibility of common ancestry for both mutations.

This study identified eight novel germline mutations and 10 deletions (Table 1). Since the exact breakpoints of the deletions were not investigated in either the literature or this study, it remains obscure whether the deletions resemble reported ones. Recently, a new technique, 'long PCR', was developed that facilitates the detection of deletion breakpoints.[35] The authors successfully sequenced deletion breakpoints in nine unrelated patients from VHL families, after a germline deletion was demonstrated with long PCR.

Four of the six missense mutations identified in the 5'-end of exon 1 of the VHL gene were associated with a mild phenotype or reduced penetrance (Table 1 and Fig. 3). In this study a P81S mutation presented in an apparently sporadic 44 year old patient with a solitary cerebellar haemangioblastoma. Four other closely related carriers (17, 43, 62 and 77 years) have no VHL-related tumours so far. Particularly, the cases of two older patients suggest reduced penetrance of the P81S germline mutation.

The P81S mutation has been reported four times previously: (1) in an isolated German patient with a full-blown VHL tumour spectrum (i.e. cerebellar and spinal HAB, renal cell carcinoma, and renal, pancreatic and epididymal cysts); (2) in a 34-year-old American patient with HAB-only; (3) in 35-year-old American patient with retinal haemangioblastoma and islet cell tumour of the pancreas, the father is the only other relative with a VHL-related tumour and had a phaeochromocytoma; (4) in an isolated Japanese patient with multiple HABs and a renal cell carcinoma.[15,17,36] Confirmation of reduced penetrance will have implications for genetic counselling in these families and contrasts with the previous observation that penetrance of the disease is almost complete by the age of 60 years.[2,7]

In addition, the R64P, E70K and S80N mutations possibly predispose to reduced penetrance. Since these missense mutations are present within the first part of the translated area of the VHL gene, they could have a mild effect on the VHL protein. In contrast, the two missense mutations in the Serine65 residue were associated with more severe phenotypes. Serine65 is mutated more frequently and has so far been described in seven families world-wide.[32] This may indicate that this amino acid is located within an important functional domain of the VHL gene.

We identified four VHL germline mutations as definitely *de novo* in nine sporadic patients. The families of three further sporadic VHL patients were suggestive for *de novo* occurrence of the mutations. We estimate that *de novo* mutations in the VHL gene in the Netherlands therefore occur in least 12% (4/34) to 21% (7/34) of the identified cases of VHL disease. Data in the literature on *de novo* mutations are scarce. VHL germline mutations are detected. In 4-15% of the identified VHL germline mutations, the mutation was found in patients without a family history,[4,37] and may represent de novo mutations or incomplete penetrance. Richards et al. studied 106 families with known VHL gene mutations, of whom 23 families were presumed to have a *de novo* mutation (22%). A *de novo* mutation in the VHL gene was identified in 16 sporadic patients (15%), while in the remaining seven cases the parents could not be tested.[37] It is conceivable that only a small proportion of *de novo* mutations are recognised, since sporadic patients with a single VHL-related lesion are not routinely tested for VHL germline mutations.

25, 26, 28, 30, 31

29

27

32

33, 34

Fig. 3 VHL germline mutations world-wide and in the Netherlands

VHL germline muations were detected both in sporadic patients with two or more typical VHL lesions as well as in sporadic patients with a single VHL-related lesion. An alternative explanation for the occurrence of sporadic patients with a single VHL-related tumour may be that they represent mosaicism and therefore have a milder phenotype. Mosaicism has so far been described in only two VHL families.[17] Moreover, independent somatic mutations may occur by chance and cause a single typical VHL-related lesion in a sporadic patient. The success rates of finding VHL germline mutations in sporadic patients with a single VHL-related lesion, e.g central nervous system haemangioblastoma, phaeochromocytoma and renal cell carcinoma, are respectively 10%, 3% and 1.6%.[25-27] Most of the sporadic patients with a VHL germline mutation present with a young age of onset, and/or with multicentric or bilateral manifestations.

In conclusion, we recommend genetic screening for VHL germline mutations not only for patients who meet the clinical diagnostic criteria, but also for sporadic patients with a VHL-related tumour who do not meet the classic diagnostic VHL criteria. These patients should be screened particularly when they present at a young age of onset, or with multicentric or bilateral tumours. We have presented evidence for non-penetrance of VHL germline mutations and found that a significant proportion of germline mutations in the VHL gene concern *de novo* mutations. Because the costs of DNA analysis are relatively low, the molecular genetic analysis of the VHL gene is readily feasible, and the vast majority of VHL gene mutations can be detected, we strongly recommend that sporadic patients should be analysed for VHL germline mutations. Especially, since each identified proband enables genetic counselling and clinical management for VHL disease of at risk family members.

Fig. 3 VHL germline mutations in the Netherlands (in black) and world-wide (deletions are not shown).[15,32] The position of each mutation in the coding region is depicted by a symbol representing the specific mutation (see caption). The mutations are pooled per 10 nucleotides. Mutations that are located close to the intron-exon boundaries, for example splice site mutations, are placed in their exon of origin. A hotspot for VHL germline mutations is readily visible in the beginning of exon 3, or more specific at nucleotide 712/713 (codon 167). Note that mutations in the VHL gene are restricted to an area between nucleotide 376 (codon 55) and nucleotide 820 (codon 202). The solid bars below represent genomic deletions found in probands 25-34, the dotted lines characterise the possible extent of the deletions.

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Genotype-phenotype correlations in families with deletions in the von Hippel-Lindau (VHL) gene

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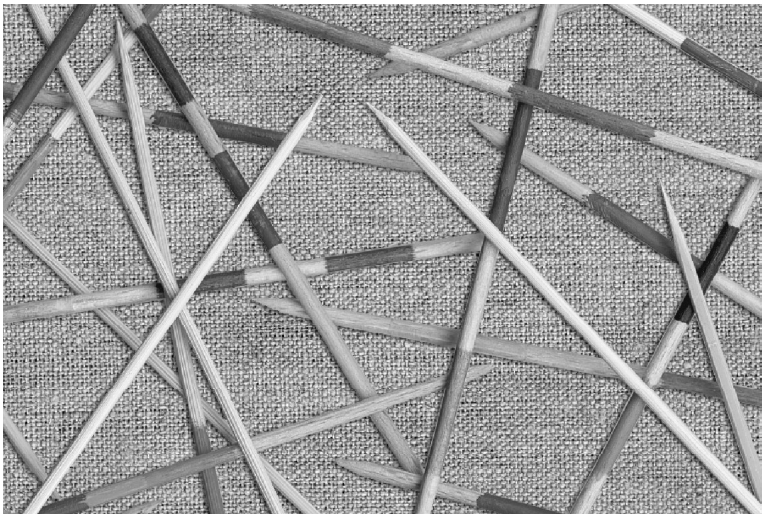


Submitted

Abstract

Von Hippel-Lindau (VHL) disease is a hereditary tumour syndrome characterised by predisposition for bilateral and multi-centric haemangioblastoma in the retina and central nervous system, phaeochromocytoma, renal cell carcinoma, as well as cysts in the kidney, pancreas and epididymis.

We describe five families where direct sequencing of the coding region of the VHL gene failed to identify the family-specific mutation. Further molecular analysis revealed deletions involving the VHL gene in each of these families. In four families partial deletions of one or more exons were detected by Southern blot analysis. In the fifth family, FISH analysis demonstrated deletion of the entire VHL gene.

Our results show that (quantitative) Southern blot analysis is a sensitive method for detecting germline deletions of the VHL gene that should be implemented in routine DNA diagnosis for VHL disease. In addition, our data support the previously established observation that families with a germline deletion have a low risk for phaeochromocytoma. Furthermore, families with a full or partial deletion of the VHL gene display a phenotype with a preponderance of central nervous system haemangioblastoma.

Introduction

Von Hippel-Lindau (VHL) disease is a hereditary tumour syndrome characterised by predisposition for bilateral and multi-centric haemangioblastoma in the retina and central nervous system, phaeochromocytoma, renal cell carcinoma, as well as cysts in the kidney, pancreas and epididymis, and endolymphatic sac tumours. VHL disease is a relatively rare disorder, with an estimated birth incidence of 1/36,000.[1] The basis of familial inheritance of the disease is a germline mutation in the VHL tumour suppressor gene, located in chromosome region 3p25-26 and identified in 1993.[2] The disease is inherited as an autosomal dominant trait with a high penetrance. A genotype-phenotype correlation has been described for the presence of phaeochromocytoma, but not for other VHL-related tumours.[3]

Germline mutations are found in up to 100%.[4] of the families fulfilling the clinical VHL criteria.[5,6] Missense, nonsense, splice site mutations, and microdeletions and -insertions, are detected in approximately two-thirds of these families.[3,4,7] In one-third of the VHL families, large deletions (4 - 380 kb) are found.[3,4,7] Such deletions are demonstrated by Southern blot analysis,[4] pulsed field gel electrophoresis,[8,9] or fluorescent in situ hybridisation (FISH).[10,11]

Detection of germline mutations in VHL families allows diagnosis of the disease, and also at an early or presymptomatic stage. Identification of carrier status avoids the inconvenience of intensive clinical surveillance of non-carriers. Carriers of the mutated VHL gene can be monitored closely and given the appropriate treatment.

We describe five families where direct sequencing of the coding region of the VHL gene failed to identify the family specific mutation. However, further molecular analysis revealed large deletions involving the VHL gene in each of these families. Evaluation of clinical features in these families suggests that VHL gene deletions result in a disease phenotype characterised by an absence of phaeochromocytoma and a high incidence of haemangioblastoma.

Patients and methods

Patients

The five families (A, B, C, D and E) described here were referred to the Department of Medical Genetics, UMC Utrecht, for germline mutation analysis in the VHL gene. The patients were clinically examined in the university hospitals of Utrecht, Groningen, Leuven and Gent, and in the Merwede Hospital, Dordrecht. (Table 1). Clinical monitoring included annual ophthalmoscopy, yearly alternate Magnetic Resonance Imaging (MRI) and ultrasound of the abdomen, and - in variational frequencies - MRI of the central nervous system (CNS).[12] All probands fulfilled the clinical diagnostic criteria; i.e. in the presence of a positive family history, a diagnosis of VHL disease can be made by the identification of a single retinal or cerebellar haemangioblastoma, renal cell carcinoma, or phaeochromocytoma, in an at-risk individual.[5,6]

DNA analysis

High molecular weight DNA was isolated from peripheral blood samples according to established procedures. Exons 1, 2 and 3 of the VHL gene and their immediately flanking sequences were amplified by PCR, using oligonucleotides according to Gnarra

et al. 1994.[13] The flanking sequences included 90 nucleotides upstream of the second start codon in exon 1 and 45 nucleotides downstream of the stop codon in exon 3. Amplification products were purified and subjected to automated sequence analysis on an ABI 377. The amplification primers were used as primers in the sequencing reactions.

Screening for structural rearrangements, including gross deletions, was performed by Southern blot analysis. DNA was digested with *Eco* RI alone,[2] and with an *Eco* RI / *Ase* I double digest.[4] To confirm the results two other restriction enzymes, *Hind* III en *Stu* I were used (see Fig. 1 for restriction map). After gel electrophoresis and transfer to Hybond-N filters, the genomic DNA was hybridised with the VHL *g7*-cDNA probe,[2] according to the manufacturer's instruction. The human beta globin gene was used as an internal control.[14] Additionally, exon-specific probes generated by PCR amplification of exons 1, 2, and 3 were hybridised to the same filters.

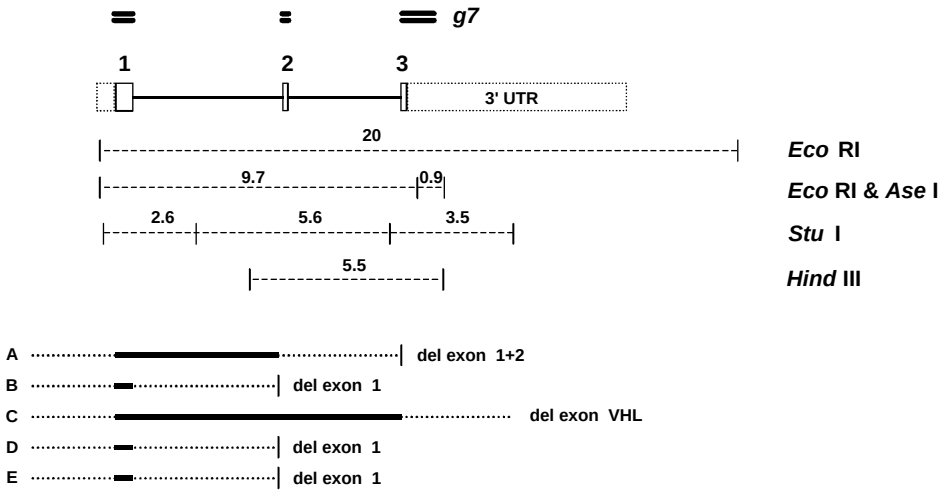


Fig. 1 Genomic organisation of the VHL gene (to scale), including the 5'- and 3' untranslated regions (UTR), the VHL *g7* probe and a restriction map of enzymes used in this article. Numbers refer to size of restriction fragments in kb (= 1000 base pairs). The solid bars below represent genomic deletions found in families A-E, encompassing the exons indicated. The dotted lines characterise the possible extent of the deletions.

FISH

FISH analysis was carried out on metaphase chromosome spreads according to established procedures.[10] The VHL cosmid-11 probe was labelled by nick translation with biotin-14-dATP. After precipitation of the labelled probe in the presence of Cot-1 DNA, pre-annealing was performed to block repetitive sequences. The final concentration of the probe was 15 ng/ml. Hybridisation of the denatured probe onto the denatured metaphase chromosomes was carried out overnight at 37˚C. Each slide was mounted with 15 ml antifade medium (Vectashield, Brunschwig) containing DAPI. Microscopic analysis of images was performed using a CytoVision, Applied Imaging.

Results

Clinical manifestations

No phaeochromocytoma occurred in any of the 34 clinically well-monitored patients in the five families studied (Table 1). Other visceral VHL-related manifestations included: three patients with renal cell carcinoma, two with renal cysts, six with pancreatic cysts and two with ovarian cysts. Five patients had symptoms associated with an endolymphatic sac tumour (i.e. hearing loss, tinnitus or vertigo), however, magnetic resonance imaging did not show tumours in the posterior fossa in these patients. One patient in family E had neurofibromatosis.

CNS as well as retinal haemangioblastoma were found in four of the five families: in the retina in 17 patients (50%), and in the central nervous system in 28 patients (82%).

Table 1. Genotypes and phenotypes

Fam	Age (mean)	Origin	Deletion	Pts	Number of patients with a VHL-related manifestation				
					Phaeo	RCC	cHAB	rHAB	Other
A (2)	16-37 (31)	Turkish	exon 1+2	5	0	2	4	2	Pancreatic cysts (2), bilateral renal cysts (1)
B (15)	20-80 (49)	Dutch	exon 1	20	0	1	17	12	Pancreatic cysts (2), multiple ovarian cysts (1)
C (23)	47-72 (57)	Belgian	exon 1-3	5	0	0	5	0	Pancreatic cysts (1), ovarian cyst and renal cyst (1)
D (51)	46-? (46)	Belgian	exon 1	2	0	0	2	1	Pancreatic cysts (1)
E (61)	31-60 (46)	Dutch	exon 1	2	0	0	0	2	Neurofibromatosis (1)
Total	16-80 (47)			34	0	3	28	17	

Fam, family (unique identification number); Age, range of current age in years of affected family members; Deletion, germline mutation; Pts, number of clinically affected family members; Phaeo, phaeochromocytoma; RCC, renal cell carcinoma; cHAB, central nervous system haemangioblastoma; rHAB, retinal haemangioblastoma

Germline mutations in the VHL gene

In family A direct sequencing did not reveal a germline mutation, neither was linkage analysis with highly polymorphic markers informative (data not shown). Southern blot analysis after *Eco* R1 digestion, with the g7 probe and an internal control probe, demonstrated an extra band above the 20 kb normal fragment in the proband. To further characterise the putative genetic alteration, Southern blot analysis was repeated using a panel of restriction enzymes, of which *Hind* III showed an aberrant fragment segregating with the disease (Fig. 2). Hybridisation of the *Hind* III blot with radiolabelled PCR products of exons 1, 2 and 3 demonstrated a deletion of exon 1 and 2. This deletion was confirmed by digesting DNA with the enzymes *Bam* HI, *Ksp* 632I and *Bgl* II, that have restriction sites in exons 1, 2 and 3, respectively. Bam HI revealed a diminished intensity of the exon 1 specific band, and *Ksp* 632I demonstrated

an extra band, also suggesting loss of a restriction site. *Bgl* II showed a normal banding pattern. This finding was also confirmed with *Stu* I restriction enzyme that yields fragments of the three separate VHL exons. On Southern blot analysis diminished band intensity was seen for exons 1 and 2, and a normal intensity for the fragment containing exon 3 (data not shown).

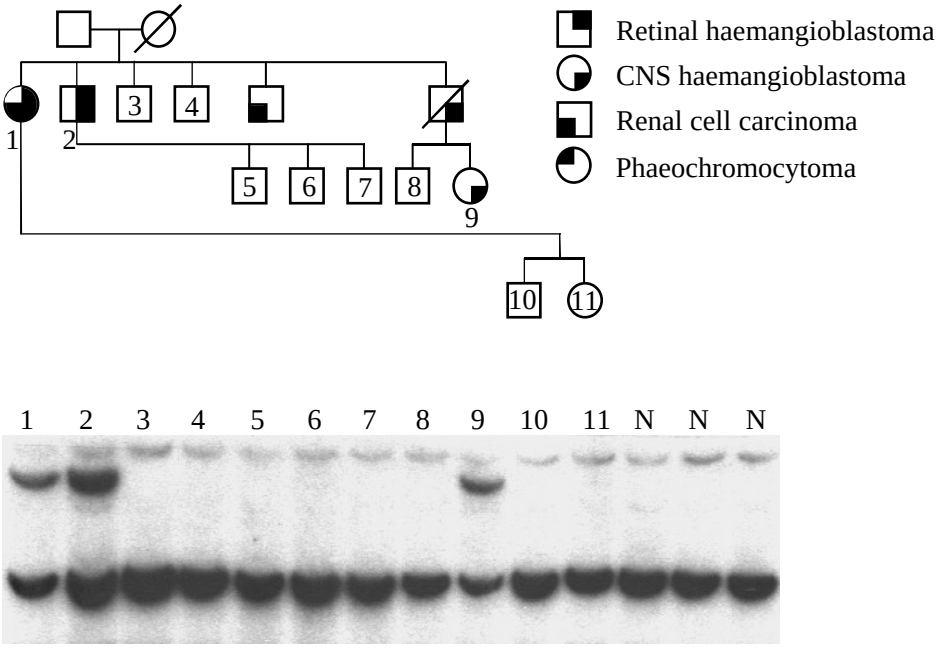


Fig. 2 Analysis of the segregation of an aberrant fragment with the disease.
Numbers of the persons tested of family A correspond with the Southern blot analysis lanes. Genomic DNA of 11 family members and of three control persons was digested with *Hind* III and revealed an aberrant banding pattern in three affected family members.

Consequently, Southern blot analysis was also performed in four additional families where no mutations in the VHL gene had been detected by direct sequencing. In families B and D, Southern blot analysis using *Eco* RI and hybridisation with the *g7* probe, generated an aberrant restriction fragment (Fig. 3). This aberrant restriction fragment was recognised by probes representing exons 2 and 3. However, hybridisation with the exon 1 probe resulted in a normal banding pattern, indicating a deletion encompassing exon 1.

In the proband from family C, six different restriction enzymes (*Eco* RI, *Eco* RI / *Ase* I double-digestion, *Hind* III, *Stu* I, *Dra* I and *Pvu* II) consistently revealed a diminished band intensity of the VHL band compared to the beta globin control probe. This indicated the presence of a deletion encompassing the entire VHL gene. Indeed, FISH analysis with the cos-11 probe demonstrated loss of signal of one of the two VHL alleles in three patients from this family, but not in an unaffected family member (Fig. 4).

In family E, we noticed that *Eco* RI, as well as *Hind* III and *Stu* I did not result in abnormalities on Southern blots. As recently described by Stolle et al. (1998), the resolution of Southern blot analysis for the VHL gene is improved by using an *Eco* RI / *Ase* I double-digestion. When we subjected all five families to the latter method, aberrations could be seen, as expected, in families A, B, C and D (Fig. 3). Surprisingly, the proband from family E showed an altered restriction fragment. Further analysis revealed a deletion of exon 1.

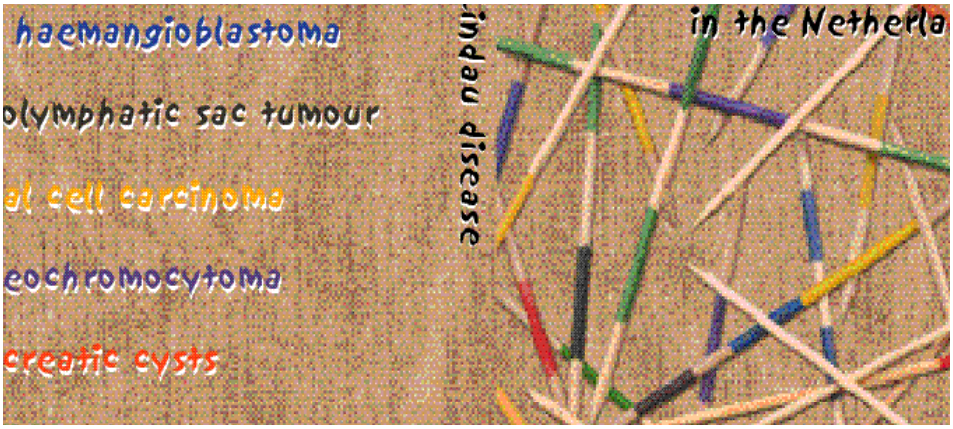


Fig. 3 Constitutional VHL gene deletions identified by Southern blot analysis in five families. Genomic DNA from family A-E and control (N) digested with *Eco* RI (lane 1-6) and with *Eco* RI / *Ase* I (lane10-15) was hybridised with the *g7* probe and a beta-globin control probe. The lambda-x-Hind III marker (lane 8) shows fragment sizes in kb (= 1000 base pairs). Aberrant bands are indicated with arrows. The bands marked with an asterisk (*) might represent a restriction site polymorphism or partially digested DNA.

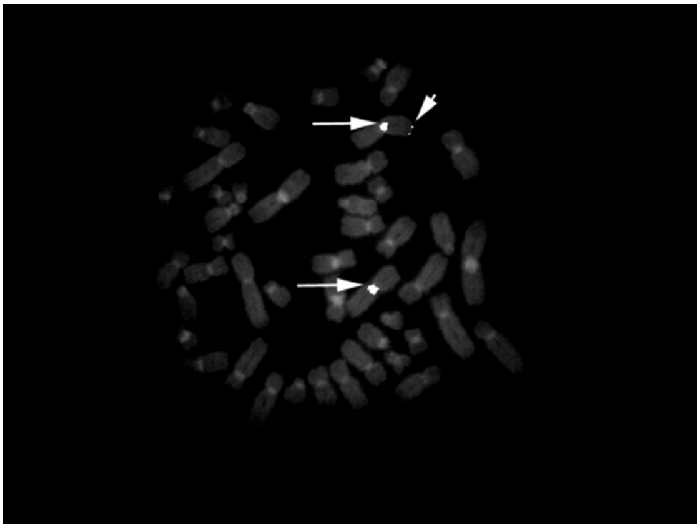


Fig. 4 Detection of a deletion encompassing the entire VHL gene by FISH analysis in family C. A loss of signal from one of the two VHL alleles (short arrow) was detected in a patient from family C. The long arrows represent the centromeric probe of chromosome 3.

Discussion

Detection of VHL gene deletions

We describe five kindreds with germline deletions of the VHL gene. In four families partial deletions removing one or more exons were readily detected by Southern blot analysis. In a fifth family, FISH analysis demonstrated deletion of the entire VHL gene We found that these deletions are involved in 28% (5/18) of the VHL families that were referred to our department (unpublished data). Although in three families (B, D and E) the deletion involved exon 1 only, differences in the restriction fragment patterns generated by Southern blot analysis indicated that the deletions were distinct and had different breakpoints. Our results indicate that Southern blot analysis (and FISH when necessary) should be implemented in routine diagnostic screening protocols for VHL gene mutations. The *Eco* RI / *Ase* I double-digestion hybridised with *g7* cDNA and a control probe is becoming the method of choice in screening for large deletions in the VHL gene.[4] Southern blot analysis using *Eco* RI only is a less sensitive method of detecting VHL gene deletions, as illustrated in family E and two cases in the study by Stolle et al. 1998.[4] Each of these cases had rearrangements detectable by *Eco* RI / *Ase* I digestion that were not found after *Eco* RI digestion. The *Eco* RI / *Ase* I double-digestion has a high resolution because it isolates exactly the coding region of the VHL gene. To further delineate the molecular nature of the deletion, the enzymes *Bam* HI, *Ksp* 632I and *Bgl* II, which have restriction sites in exons 1, 2 and 3, respectively, may be applied as well as hybridisation of Southern blot analysis with probes for the individual exons of the VHL gene.

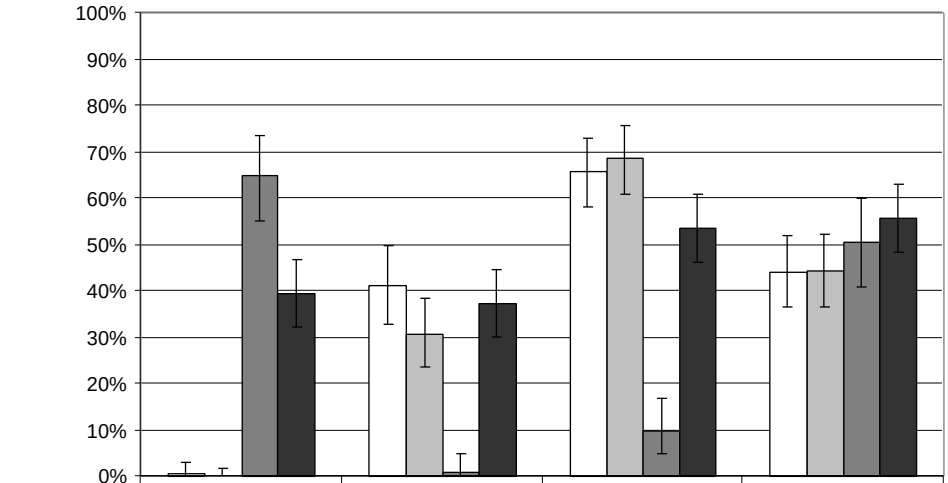
When Southern blot analysis of the VHL gene, using *Eco* RI alone or the *Eco* RI / *Ase* I double-digestion, demonstrates a diminished band intensity, FISH analysis should confirm the deletion of one VHL allele. The presence of large deletions may also be revealed in studies involving highly polymorphic short-tandem repeat (STR)-markers. Deletions encompassing polymorphic marker loci will result in loss of specific alleles and reduced intensities for observed alleles.

Genotype-phenotype correlations

Phaeochromocytoma The five families were affected with various VHL-related tumours, except for phaeochromocytoma. This relationship between genotype and the VHL phenotype has been described in several studies.[3,4,7,15,16] Families with a deletion or a mutation that predicts a truncated VHL protein are predominantly associated with a disease phenotype without phaeochromocytoma (VHL type I). Whereas, 69% of families with phaeochromocytoma (VHL type II) is associated with specific missense mutations.[4] It is hypothesised that phaeochromocytoma may arise from a dominant-negative effect of VHL proteins, based on the involvement of VHL in the multi-protein VCB (VHL-Elongin C-Elongin B) complex that may target proteins for degradation (via a process called ubiquitination).[17] Structural analysis of this complex revealed that VHL has two protein-binding sites. A mutant (type II) having a defect in only one site may exert a dominant-negative effect by sequestering key components of the ubiquitin pathway.[17,18] However, the dominant negative model would implicate that one ‘hit’ may be sufficient for the initiation of phaeochromocytoma tumourigenesis. This hit will probably concern a specific missense mutation, that occurs either in the

germline or as a somatic mutation. Since oncogenesis is a multi-step process, other genes must also be involved in tumourigenesis, otherwise every carrier of such a missense mutation would develop phaeochromocytoma. In addition, one would expect also very young patients with phaeochromocytoma.

In contrast, mutations found in families without phaeochromocytoma (type I) are predicted to cause a complete unravelling of the VHL structure.[17] This genotype-phenotype correlation was confirmed in a comparison of phenotypes of our families with those reported in the literature.[15,16] with deletions and missense mutations associated with VHL types I and II (Fig. 5). The absence of phaeochromocytoma in our families may be explained by the presence of deletions that are unlikely to result in dominant-negative VHL proteins.



	Phaeo	RCC	cHAB	rHAB
Deletions	1%	41%	66%	44%
Missense I	0%	31%	69%	44%
Missense IIa	65%	1%	10%	50%
Missense IIb	39%	37%	54%	56%

Fig. 5 The frequency of patients with four types of VHL tumours associated with their genotype. This figure shows pooled data of genotypes and phenotypes in families from the present study, and in families studied by Glavac et al. and Chen et al.[15,16] Phaeo: phaeochromocytoma; RCC: renal cell carcinoma; cHAB: central nervous system haemangioblastoma; rHAB: retinal haemangioblastoma. The missense mutation T505C (Tyr98His) was the only missense mutation that we included in the missense type IIa group. More missense type IIa mutations could still be hidden in the missense IIb group. Data from the three studies were pooled, but we excluded those deletion patients from our study since they differed significantly ($p < 0.001$) in their incidence of RCC compared to the two other studies.

Renal cell carcinoma Our families showed a relatively low frequency of renal cell carcinoma (9%), compared to other studies (41%).[15,16] We therefore did not include our deletion patients in Fig. 5. Renal cell carcinoma in VHL patients occur at a mean age of 36 years,[19] and the mean age, as well as the median age, of the VHL patients we studied was 47 years. The mean age of the patients in the two comparison articles was

not reported. It was hypothesised that renal lesions develop as a consequence of several structural aberrations such as large deletions, nonsense, splice and frame shift mutations, and insertions.[16] So far, a low frequency of renal cell carcinoma has only been reported in families (VHL type 2A) with specific missense mutations.[20] (Fig. 5).

The above suggests that the relationship between germline mutation and renal cell carcinoma in VHL appears to be rather complex. Apart from chance, the relatively low frequencies of renal cell carcinoma reported in our clinically well-monitored families could be due to other factors. Like retinal haemangioblastoma in VHL patients, modifier genes.[21] or external factors may contribute to a renal cell carcinoma risk (e.g. smoking is associated with a higher risk).[22-24]

Haemangioblastoma So far, the risks of CNS haemangioblastoma in VHL disease have not been correlated with allelic heterogeneity. Our deletion families exhibited a phenotype with a preponderance of CNS haemangioblastoma (Table 1). This prompted us to investigate whether phenotypes of families with VHL gene deletions differ from families with other VHL gene germline mutations (Fig. 5). With respect to the incidence of CNS haemangioblastoma, we noted that families with deletions did not significantly differ from other types of VHL gene germline mutations; except for families with missense mutations, who exhibited a low frequency of CNS haemangioblastoma. On a closer look, we identified that this relatively low frequency of CNS haemangioblastoma in families with missense mutations was caused by a specific subset of missense mutations, i.e. type IIa (Fig. 5). Thus, VHL deletion families show a significantly (Chi-square 85, $p < 1x10^{-10}$) higher incidence of CNS haemangioblastoma compared to type IIa missense mutations. Apparently, VHL IIa mutations are not only associated with a low risk for renal cell carcinoma, but also for CNS haemangioblastoma.

In contrast to CNS haemangioblastoma, the risk for retinal haemangioblastoma is comparable for all kinds of VHL gene germline mutations. Since retinal and CNS haemangioblastoma are histopathologically identical and both arise from stromal cells.[25,26] one would also expect a relatively low frequency of retinal haemangioblastoma in families with type IIa mutations. However, this was clearly not the case. These two findings (the high frequency of CNS haemangioblastoma in VHL deletion families compared to type IIa missense mutations, whereas the risk of retinal haemangioblastoma is similar for both groups) tempted us to speculate upon possible explanations.

Assuming that type IIa missense mutations result in a high frequency of phaeochromocytoma, the same mutations seem to have the opposite effect on the incidence of CNS haemangioblastoma. However, apart from considering the different functional effects of VHL mutations, it is clear that other factors, including tissue-specific differences may also play a role. For instance, stromal cells in the retina could require a different level of functional VHL protein to maintain cellular homeostasis than stromal cells in the CNS. Also, the multi-functional VHL protein may be implicated in different cellular pathways in the retina and the CNS. Moreover, there is evidence that modifier genes play a role in the aetiology of retinal haemangioblastoma.[21] and this could be similar for other target tissues in VHL disease.

Interestingly, in family C the deletion of the entire VHL gene is associated with a phenotype with a preponderance of CNS haemangioblastoma. Given that deletions of the entire VHL gene represent true null alleles, this family supports the manifestation of haemangioblastoma occurring when the VHL gene is in the hemizygous state. Although complete VHL gene deletions occur in approximately 9% of VHL families.[4] no clinical details have been published for complete gene deletion families. Additional studies embodying carefully executed clinical analysis of patients with entire VHL gene deletions are required to test our hypothesis.

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Cryptic Von Hippel-Lindau disease: Germline mutations in haemangioblastoma-only patients

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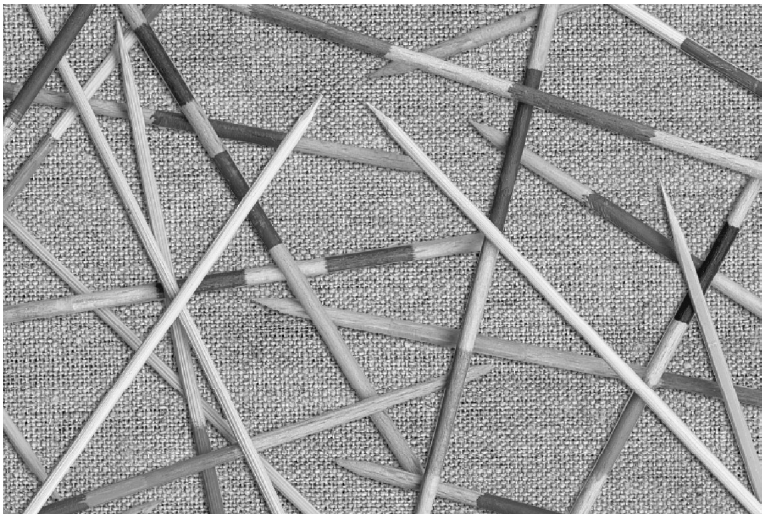


Submitted

Abstract

Objective In addition to families with Von Hippel-Lindau (VHL) disease, sporadic patients with haemangioblastoma (HAB) in the central nervous system have also been found to carry VHL germline mutations. Carriers of such a mutation and their relatives have a risk of developing multiple tumours. We investigated the frequency of VHL germline mutations in HAB-only patients.

Patients and methods 84 patients with a single HAB (23 Dutch, 61 UK) and four with multiple HAB (two Dutch, two UK), with no clinical or radiological evidence of VHL disease, were studied by direct sequencing of the coding region and quantitative Southern blotting.

Results A VHL germline mutation was found in three of 84 (3.6%) single HAB patients. A germline VHL mutation was detected in a 44-year old woman with a solitary cerebellar HAB, as well as in four clinically unaffected close relatives, and in two single HAB cases presenting at ages 29 and 36 years. Germline VHL mutations were detected in two of four cases with multiple HAB.

Conclusions VHL gene mutation analysis should be offered to all HAB patients younger than 50 years. Further data is required to evaluate the detection rate in late-onset cases. The low detection rate in patients with multiple HAB may indicate the presence of somatic mosaicism or additional HAB susceptibility genes.

Introduction

Haemangioblastomas (HAB) are non-metastasising tumours of the central nervous system and account for about 2% of all intracranial tumours.[1] HAB arise preferentially in the cerebellum (~ 75%), medulla and spinal cord (~ 25%).[2] HAB in the cerebrum are rare.[3] HAB are regarded as benign on their histopathological characteristics and do not normally invade the surrounding brain. However, complications may arise due to the tendency of HAB to form expanding cysts, leading to elevated or even life-threatening intracranial pressure.[2] They are composed predominantly of vascular and stromal cells.[2,4] The frequent presence of haemorrhages and cysts means the tumours vary in morphological appearance.[2] Four types of HAB can be recognised macroscopically: 5% are cysts, 60% predominantly cystic, 26% predominantly solid, and 9% solid.[5]

The standard treatment is complete microsurgical removal,[5-7] aided if necessary by preoperative embolisation to reduce the tumour's vascularity.[8] Stereotactic radiosurgery shrinks or stops the growth of small- or medium-sized HAB.[7,9,10] Adjoining cysts, however, do not respond to radiosurgery and require later and sometimes repeated evacuation.[7]

HAB may occur in sporadic form or as a manifestation of Von Hippel-Lindau (VHL) disease.[11] VHL disease is an autosomal dominant tumour syndrome with an estimated birth incidence of approximately 1:36,000.[12] The disease is characterised by a predisposition to bilateral and multifocal tumours. The most common tumours in VHL disease are HAB in the central nervous system and retina, clear cell carcinoma in the kidney, phaeochromocytoma in the adrenal gland, endolymphatic sac tumours in the inner ear, as well as cysts in the kidney, pancreas and epididymis. In the presence of a positive family history, a diagnosis of VHL disease can be made by the identification of a single retinal or cerebellar HAB, renal cell carcinoma, phaeochromocytoma, or multiple pancreatic cysts in an at-risk individual.[13,14] In isolated cases of VHL disease, two or more HABs, or a single HAB in association with a visceral manifestation are required.[14]

The basis of familial inheritance of VHL disease is a germline mutation in the VHL tumour suppressor gene, first identified in 1993 and located in chromosome region 3p25.[15] In both VHL disease and sporadic HAB, allelic losses and mutations of the VHL tumour suppressor gene affecting stromal cells have been found, suggesting that stromal cells represent the neoplastic component of a HAB.[4,16] In addition, it was demonstrated that vascular endothelial growth factor (VEGF) is upregulated in stromal cells as a consequence of mutations in the VHL gene.[17]

VHL disease demonstrates variable expression, age-dependent penetrance, and a low but consistent new mutation rate.[12,18] The diagnosis of VHL disease should be considered in all patients with a HAB, as early recognition of a predisposition to develop further HAB and other tumours (e.g. renal cell carcinoma) may reduce morbidity and mortality. The diagnosis of 'new mutation' VHL cases is frequently delayed because at least two typical manifestations are required, whereas molecular genetic diagnosis of VHL disease offers the potential to detect subclinical cases of VHL disease in sporadic patients with a single HAB. VHL disease demonstrates complex genotype-phenotype correlations. Most VHL gene mutations predispose to HAB,

but specific missense mutations may cause high or low risks for renal cell carcinoma or phaeochromocytoma.[19-23] In addition, rare missense mutations may produce a phaeochromocytoma-only phenotype.[24-26] This suggests that specific VHL gene mutations might cause a HAB-only phenotype.

To investigate the genetic epidemiology of HAB in the central nervous system, we performed an international multicentre study of patients with single HAB and multiple HAB without evidence of VHL disease (i.e. HAB-only). As the mean age of VHL patients with HAB is significantly younger than that for sporadic cases, 29 vs 48 years[27] (or 33.5 vs 43.6 years[28]), we directed our study towards younger patients with single HAB as these present the most difficult diagnostic problems in clinical practice.

Patients and methods

Patients

We investigated two groups of patients with HAB-only in the central nervous system. Group 1 consisted of 61 UK and 23 Dutch HAB patients with a single HAB. These cases were ascertained with the help of neurosurgeons, neurologists, internists and clinical geneticists. In addition to DNA analysis, all patients underwent clinical examinations for detection of VHL associated tumours (ophthalmological examination and abdominal sonography or MRI) with negative findings. Group 2 consisted of four patients with multiple HABs, but no other evidence of VHL disease (i.e. absence of further VHL-related tumours) on clinical screening and radiological screening. All patients had histopathologically confirmed HAB at operation.

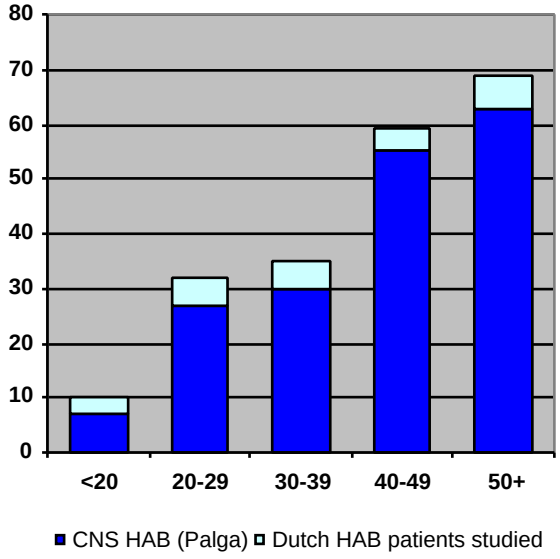


Fig. 1 Number of patients and age at diagnosis for:
182 patients with haemangioblastoma (HAB) in central nervous system (CNS) reported in the National Dutch Pathological Archive (*Palga*), between 1973 and 1996.
23 Dutch patients in this study with a single HAB, between 1996 and 1999.
The x-axis represents the age at diagnosis, the y-axis represents the number of patients with haemangioblastoma

Age at diagnosis

Between 1973 and 1996 a total of 182 HAB patients were reported to the National Dutch Pathological Archive (Palga). Figure 1 shows the age at diagnosis of Palga patients as well as studied patients. Details of age distribution of a previous population based cohort of UK HAB patients have been reported previously.[27]

In 1996, guidelines were distributed via the Dutch newsletter for neurologists on screening all patients with a HAB for a VHL germline mutation. The Dutch patients in the present study were referred for DNA diagnosis from 1996 to 1999, and the mean age at diagnosis was 37.5 years (range 14-71 years). Compared to unselected cases, the age distribution in the UK as well as in the Dutch cases in this study was biased towards an earlier age at onset.

DNA analysis

High molecular weight DNA of the probands was isolated from peripheral blood according to established procedures. Exons 1, 2 and 3 of the VHL gene and their immediately flanking sequences were amplified using the polymerase chain reaction.[29] The flanking sequences included 90 nucleotides upstream of the start codon (the first nucleotide of the coding region is 214) and 45 nucleotides downstream of the stop codon. The nucleotides are numbered according to Latif et al. (Genbank accession number L15409).[15] PCR products were purified and subjected to sequence analysis using either an ABI automated sequencer or the dideoxy-chain termination reaction with a pUC-sequencing kit (Boehringer Mannheim, Mannheim, Germany), using g-^{35}S dATP (600 Ci/mmol). The amplification primers were used as primers in the sequencing reactions. In the UK cases, intragenic mutations were also sought by Single-strand conformation polymorphism (SSCP).

Screening for genetic rearrangements and deletions was performed by Southern blot analysis, or a novel PCR-based deletion assay (Dow et al. in preparation). In Southern blot analysis DNA was digested with *Eco* RI alone or with *Eco* RI and *Ase* I double digest. After gel electrophoresis and transfer to Hybond-N filters, the genomic DNA was hybridised with the VHL g7-cDNA probe,[15] according to the manufacturer's instruction. Quantitative Southern blotting was performed by hybridising genomic DNA with the VHL g7-cDNA probe and with a beta-globin probe, to detect deletions encompassing the entire VHL gene.[30]

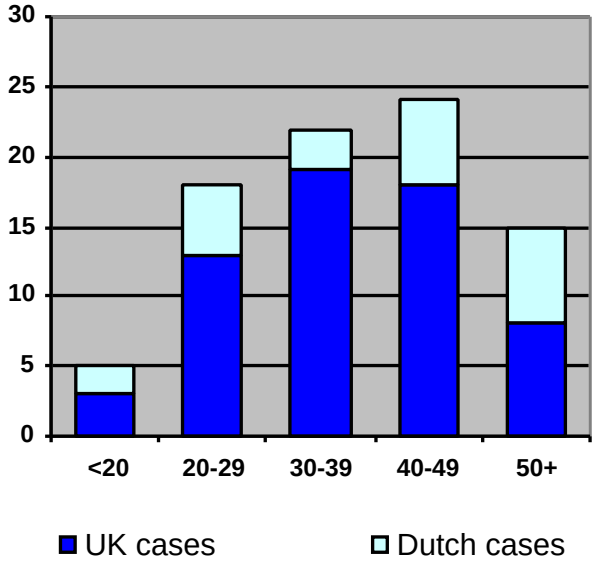


Fig. 2 Distribution of single HAB patients by age at diagnosis.
dark = UK
light = Dutch
The x-axis represents the age at diagnosis, the y-axis represents the number of patients with haemangioblastoma

Results

Single HAB

84 patients with single HAB in the central nervous system (23 Dutch, 61 UK) were investigated. The age distribution of the studied patients is shown in Figure 2. VHL germline mutations were identified in three patients (see Table 1). The incidence of a VHL germline mutation in single HAB patients younger than 30 years was 4.3% (1/23), 30-39 years was 4.5% (1/22), 40-49 years was 4.2% (1/24) and age 50 years or older was 0% (0/15).

Details of the three germline mutations identified were:

(a) a C to T transition at nucleotide 454 was detected in a 47-year old woman (D24) with a single cerebellar HAB, diagnosed at an age of 44 years (Fig. 3). This missense mutation leads to a change of Proline to Serine at codon 81 (P81S) in the VHL protein. DNA analysis of other close relatives revealed that four clinically unaffected first and second degree relatives (age 17-77 years) were also carriers of a P81S germline mutation;

(b) a 7 basepair frameshift mutation (del 582 GACACAC) was detected in a 29-year old patient (B1) with a single cerebellar HAB with no family history and no evidence of VHL disease on clinical and radiological screening. However, she subsequently developed pancreatic cysts at age 34 years;

(c) a large germline deletion was identified by Southern blot analysis in a 36-year old woman (B2) with a single cerebellar HAB but no other features of VHL disease.

Two patients with a single HAB developed some additional features of VHL disease during the study: one patient (B1) with a VHL gene mutation (del 582 GACACAC) developed pancreatic cysts (see above) and one patient (B3) developed renal cell carcinoma aged 44 years following a cerebellar HAB at age 40 years, but no VHL mutation was identified.

Table 1 Summary of results

Pat	Age	Clinical features		Fam Hist	Mutation	Previously reported
		HAB	Other			
D24	44	Solitary cerebellar	-	-	P81S	([21,30,31])
B1	29	Solitary cerebellar	Subsequent pancreatic cysts	-	del 582 GACACAC	No
B2	36	Solitary cerebellar	-	-	Deletion	n/a
B5	40	Cerebellar & medullary	-	-	Deletion	n/a
B6	44	Multiple spinal	-	-	Deletion	n/a

Pat, patient's unique identification number; Age, age at diagnosis, in years; HAB, type and origin of haemangioblastoma; Clinical features, Other, further VHL associated manifestations after clinical screening; Fam Hist, family history, further VHL associated manifestations in family members of proband; Mutation, VHL germline mutation; Previously reported, whether the mutation has been published (references); n/a, not applicable.

Multiple HABs

Four patients with multiple HABs (two Dutch and two UK) and without additional VHL-related tumours were analysed for VHL germline mutations. Deletions were detected in two patients: (a) a male (B5) with one cerebellar and one medullary HAB at age 40 years; and (b) a male (B6) with multiple spinal HABs at age 44 years. Germline mutations were not identified in the two Dutch patients (D13 and D32) with both cerebellar and spinal HABs (ages 44 and 66 years).

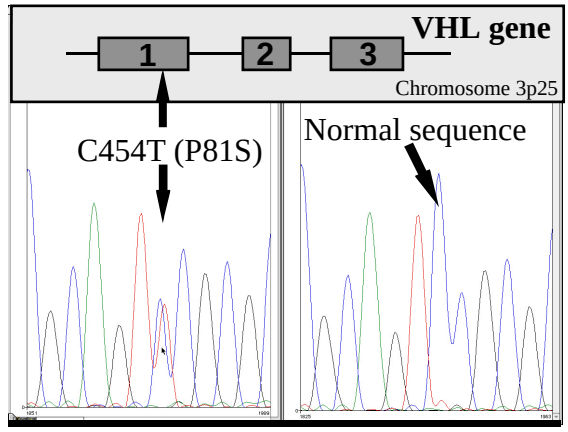
Discussion

We found that the overall risk for finding a VHL germline mutation in a population of 84 patients with a single HAB in the central nervous system and no further features of VHL disease at the time of diagnosis was approximately 4%. In clinical studies of sporadic patients with a HAB it was suggested that a substantial proportion of HAB could be associated with VHL disease upon more detailed examination; i.e. 23% to 34.3% was found to be afflicted with VHL disease.[6,28] Molecular genetic analysis of the VHL gene indicated that sporadic patients with a HAB have a risk of a VHL gene germline mutation of approximately 10%.[32,33] The lower detection rate in our cases, despite use of more sensitive methods of VHL gene analysis, is presumably related to better clinical and radiological screening prior to entry into our study. The identification of a VHL germline mutation has important implications for the risk of further tumours and for the risk of VHL disease in relatives.

Statistical analysis of the age at onset of HAB in VHL disease and non-VHL cases (based on clinical criteria) is consistent with a one- and two-hit tumourigenesis model as predicted by the Knudson hypothesis.[27] Mean age at diagnosis of cerebellar HAB in VHL disease is younger than in sporadic cases (29 years versus 48 years, respectively) so we anticipated a higher incidence of unsuspected VHL gene mutations in early onset cases. Older patients with VHL gene mutations would be more likely to manifest other evidence of VHL disease and so be excluded from our study. Thus, although our results were broadly compatible with this hypothesis, the identification of germline VHL gene mutations in two of a group of 46 patients aged 30-49 years with a single HAB and no clinical or radiological features of VHL disease suggests that molecular genetic analysis should be employed in all single HAB patients younger than 50 years. For older onset patients the frequency of germline mutations is likely to be less and larger research-based studies are required to define the risks more precisely.

A major strength of the present study was the use of recently developed techniques to detect large germline deletions. Prior to the introduction of these techniques, there was a VHL germline mutation detection rate (with Southern blotting and sequencing of the coding region) of approximately 80% in known VHL families.[22] However, methods to detect large deletions (e.g. quantitative Southern blotting) have significantly increased the detection rate, reaching 100% in proven familial VHL disease.[30] Therefore a conservative estimate of the mutation detection sensitivity of the strategy used in this study would be in the order of 95%. Although other studies of sporadic patients with a HAB have used less sensitive techniques,[32-34] it is of interest that Oberstrass et al. detected a germline mutation in 2 of 20 patients (aged 18 and 40

years) with HAB of the central nervous system (although no data were available about a possible family history of VHL disease).[32] Decker et al.[34] reported a 29-year-old patient with recurrent spinal HAB, a negative family history of VHL disease and a *de novo* frameshift VHL gene exon 2 mutation. However, this case differed from any of those in our study because a renal mass and pancreatic and renal cysts were detected on clinical screening. In a series of 18 sporadic patients with a HAB, Olschwang et al.[33] found a missense mutation in two patients (42 and 56 years of age) without clinical investigations revealing any evidence of VHL disease. The latter case would only have been detected by also screening single HAB patients aged over 50 years for a VHL gene mutation.



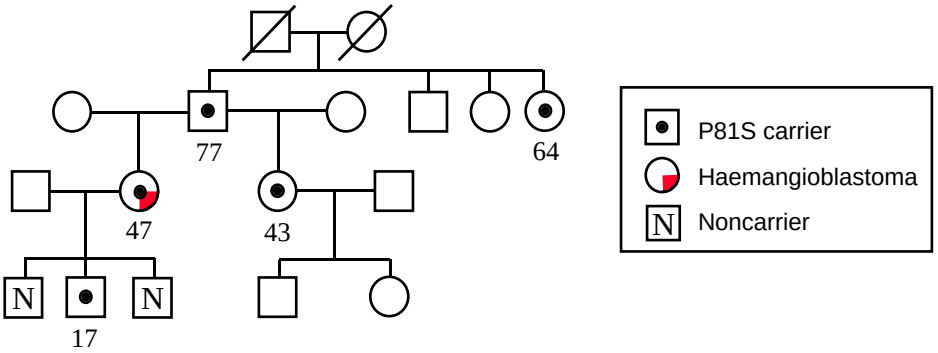


Fig 3. Sequence analysis showing the P81S mutation (left) and normal sequence (right). Below, the pedigree of patient D24, depicting four clinically unaffected relatives with the VHL germline mutation P81S and their ages .

Although specific missense VHL mutations may cause a phaeochromocytoma-only phenotype,[24-26] we did not find unequivocal evidence for VHL gene mutations that would predispose to a HAB-only phenotype. However, the VHL germline mutation (P81S) that was found in a 47-year old woman with a solitary HAB and in four clinically unaffected family members was associated with an unusually low penetrance within this family. To reduce the possibility of a genetic polymorphism, 50 non-VHL patients were sequenced. Sequence analysis of exon 1 demonstrated that all persons were homozygous for nucleotide 545C (data not shown). Phenotypic expression in VHL disease is influenced by allelic heterogeneity, stochastic events and genetic modifiers.[35]

The P81S mutation has been reported four times previously: 1) in an isolated German patient with a full-blown VHL tumour spectrum (i.e. cerebellar and spinal HAB, renal cell carcinoma, and renal, pancreatic and epididymal cysts); 2) in a 34-year-old American patient with HAB-only; 3) in 35-year-old American patient with retinal haemangioblastoma and islet cell tumour of the pancreas, the father is the only other relative with a VHL-related tumour and had a phaeochromocytoma; 4) in an isolated Japanese patient with multiple HABs and a renal cell carcinoma.[21,30,31] These findings suggest that P81S mutation carriers in the family are also at risk of renal cell carcinoma. Moreover, only one of the P81S carriers had affected family members, which may imply that this missense mutation has a low penetrance.

Interestingly, we did not find VHL germline mutations in two of the four patients with multiple HABs. As the presence of two or more retinal or cerebellar HAB satisfies the strict diagnostic criteria for VHL disease,[12,14] this was an unexpected finding in the light of the high sensitivity of the mutation detection methods used, and could perhaps indicate additional HAB susceptibility gene(s). Alternative explanations would include a mutation in part of the VHL gene not analysed (e.g. regulatory domain) or somatic mosaicism. In this context it is interesting, that the two multiple HAB patients with germline mutations had the earliest age at onset and the patients with later onset may be mosaic and so have a milder phenotype or represent phenocopies (independent mutation events giving rise to various HAB could also be expected by chance). Although mosaicism has so far been described in only two VHL families,[30] it is frequent, for example, in neurofibromatosis type 2.[36]

We have demonstrated that VHL gene mutation analysis facilitates the management of sporadic patients with HAB and should be performed in patients younger than 50 years with a single HAB even if there is no other clinical or radiological evidence of VHL disease. Although the detection rate in older patients should be lower, we suggest that such patients need to be studied on a research basis, using the latest mutation detection strategies to define cost–benefit consequences for molecular genetic analysis of this group of patients.

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Absence of VHL germline mutations in patients with phaeochromocytoma-only:
implications for clinical management

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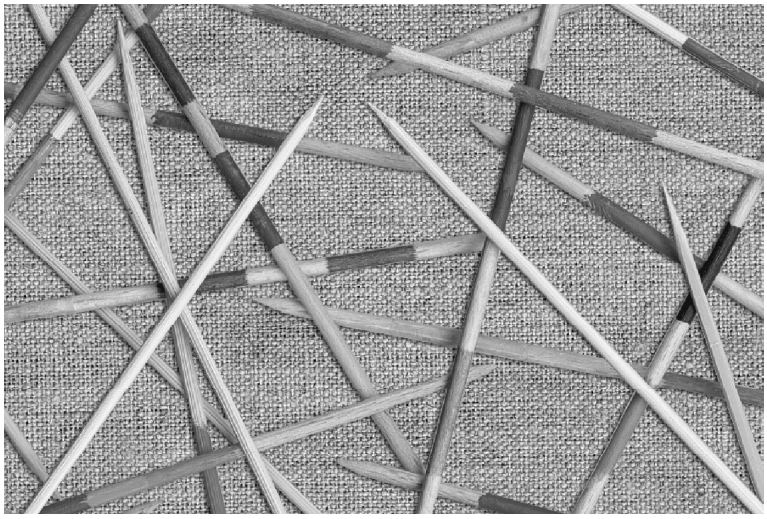

Abstract

Objective Phaeochromocytoma may occur in sporadic forms or as a manifestation of Von Hippel-Lindau (VHL) disease. Germline mutations in the VHL gene are detected in virtually all well-defined families. In addition to VHL families, sporadic patients with VHL-related tumours have been found to carry germline mutations in the VHL gene. Because carriers of a VHL germline mutation and their relatives risk developing multiple tumours, we investigated the frequency of VHL germline mutations in patients with phaeochromocytoma-only.

Patients and methods Between 1996 and 1999, 24 probands (14 with solitary tumours, 7 with multiple, bilateral or recurrent tumours and 3 with familial phaeochromocytoma) were investigated. Mutation screening of the VHL gene was performed by direct sequencing of the coding region and quantitative Southern blot analysis.

Results VHL germline mutations were not found in any proband of the solitary phaeochromocytoma group (mean age at diagnosis 49 years, range 17-70 years), nor in any of the multiple phaeochromocytoma (mean age at diagnosis 37 years, range 19-64 years) or familial phaeochromocytoma groups.

Conclusions VHL germline mutations were not found in 24 probands with phaeochromocytoma, even when features suggesting a germline mutation (early onset, multiple, recurrent, bilateral or familial tumours) were present. However, the absence of VHL germline mutations in these phaeochromocytoma patients may indicate the possible occurrence of somatic mosaicism or the presence of additional phaeochromocytoma susceptibility genes. Since mutation analysis of the VHL gene detects germline mutations in virtually all well-defined VHL families, we conclude that annual clinical monitoring for further VHL-related tumours in patients with phaeochromocytoma and without a VHL germline mutation should not be recommended.

Introduction

Phaeochromocytoma are neuro-endocrine tumours arising from chromaffin cells in the medulla of the adrenal gland. They may also occur in the ganglia of the autonomic nervous system at extra-adrenal sites and in this case are called paraganglioma. Functioning tumours usually secrete the catecholamines norepinephrine and epinephrine, and may cause palpitations, chest discomfort, sweating attacks, hypertension or paroxysmal unstable blood pressure, and headache.

Diagnosis is based on biochemical tests and radiology. Laboratory tests may include evaluation of catecholamines in serum as well as in urine.[1,2] Measurement of plasma normetanephrine and 24-hour urinary norepinephrine excretion are the most sensitive tests.[2] Radiology testing may include ultrasound (US), computed tomography (CT), magnetic resonance imaging (MRI) and metaiodobenzylguanidine (MIBG) scintigraphy. T2-weighted MRI demonstrates high signal intensity for phaeochromocytoma in 95-100% of the cases.[1,3] MIBG is sensitive for 75-95% of phaeochromocytoma and is 100% specific, but may not depict very small lesions.[1,3,4]

Phaeochromocytoma may occur both in sporadic and in familial forms. Familial manifestation of phaeochromocytoma may occur in hereditary neoplastic syndromes including Von Hippel-Lindau (VHL) disease, multiple endocrine neoplasia type IIA and IIB (MEN IIA and MEN IIB) and neurofibromatosis type I (NF-I). In 1993, a study by Neumann showed, upon more detailed examination, that 19 of 82 patients (23%) with phaeochromocytoma had either VHL disease (19%) or MEN II (4%).[1] Pooled data from three more recent studies[5-7] indicated that 13 of 133 patients with phaeochromocytoma (10%) were familial cases. These cases could be associated with either VHL disease (n=1), MEN IIA (n=1), MEN IIB (n=5), NF-I (n=3) or genuine familial phaeochromocytoma (n=3).

VHL disease is an autosomal dominant tumour syndrome with an estimated birth incidence of 1/36,000.[8] The disease is characterised by a predisposition for bilateral and multi-centric tumours. The most common tumours in VHL disease are haemangioblastoma in the central nervous system and retina, clear cell carcinoma in the kidney, and phaeochromocytoma in the adrenal gland. VHL patients are enrolled on an annual screening programme to enable early detection (and treatment) of these tumours. The basis of familial inheritance of VHL disease is a germline mutation in the VHL tumour suppressor gene located in chromosome region 3p25-26.[9]

The mean age at diagnosis in VHL patients with phaeochromocytoma is approximately 28 years,[1,10,11] with the youngest reported patient being five years old.[11] In VHL patients, phaeochromocytoma often remain quiescent or produce few symptoms, and biochemical tests may reveal normal results. [2] However, the behaviour of phaeochromocytoma remains unpredictable: biologically inactive lesions may suddenly become dangerous, benign phaeochromocytoma may become malignant.[12] About 5% of VHL patients die from phaeochromocytoma-induced endogenous catecholamine intoxication, which has also caused fatal pregnancy outcome (for the mother and/or the child).[13-15]

Adrenalectomy is the standard treatment for phaeochromocytoma. Satisfactory results have been reported from laparoscopic removal of adrenal tumours,[16-18] and also in VHL patients.[19] Since bilateral tumours develop in 47% of VHL patients with

phaeochromocytoma, most patients become steroid-dependent upon bilateral adrenalectomy.[20] Enucleation rather than adrenalectomy is therefore recommended by an increasing number of surgeons.[20,21] Adrenal-sparing surgery is safe, effective and can preserve adrenal function in VHL patients.

Since the identification of the VHL gene in 1993, studies of the disease have intensified and clinical studies have suggested that the number of phaeochromocytoma patients associated with a hereditary neoplastic syndrome has diminished from 23% in 1993 to 10% in 1998.[1,5-7] We think that the putative pool of phaeochromocytoma patients possibly affected by VHL disease has decreased as the number of VHL families being identified has increased in the past few years. We therefore investigated the prevalence of VHL germline mutations in 24 probands with phaeochromocytoma-only, with the purpose of identifying new cases with VHL disease.

Patients and methods

Patients

Between 1996 and 1999, 24 patients with phaeochromocytoma-only were referred for VHL mutation analysis by endocrinologists, internists and clinical geneticists. In addition to DNA analysis, all patients underwent clinical examination for detection of VHL-associated tumours (opthalmological examination and abdominal sonography or MRI). At the time of referral, clinical screening revealed no evidence that any of the patients had VHL disease. Cases 22, 23 and 24 had other family members with phaeochromocytoma but none of the probands' family members exhibited other VHL-related lesions. All patients had been operated for phaeochromocytoma, later confirmed histopathologically.

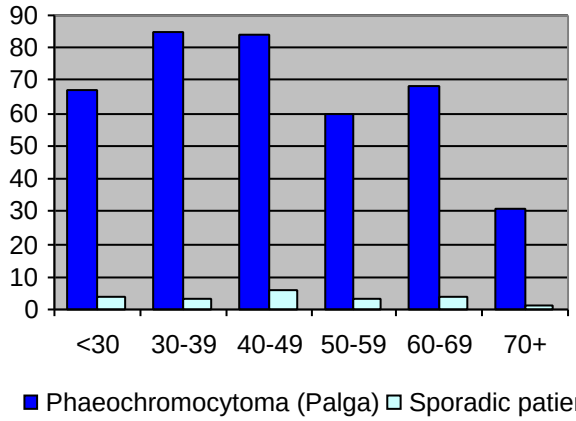

Fig. 1 Number of patients and age at diagnosis for:
395 patients with phaeochromocytoma reported in the National Dutch Pathological Archive (Palga), between 1973 and 1996.
21 sporadic patients in this study with a one or more phaeochromocytoma, between 1996 and 1999.
The x-axis represents the age at diagnosis, the y-axis represents the number of patients with phaeochromocytoma

The Dutch Pathological Archive (Palga)

Between 1973 and 1996, a total of 395 phaeochromocytoma patients were reported in the Dutch Pathological Archive (Fig. 1). The mean age at diagnosis of these patients was 46 years. Figure 1 demonstrates that the patients studied (mean age at diagnosis 45 years, range 17-70 years) are a representative selection of phaeochromocytoma patients by age at diagnosis.

DNA analysis

High molecular weight DNA of the probands was isolated from peripheral blood according to established procedures. Exons 1, 2 and 3 of the VHL gene and their immediately flanking sequences were amplified using the polymerase chain reaction (PCR), using oligonucleotides according to Gnarra et al.[22] The flanking sequences included 90 nucleotides upstream of the start codon (the first nucleotide of the coding region is 214) and 45 nucleotides downstream of the stop codon. The nucleotides are numbered according to Latif et al. (Genbank accession number L15409).[9] PCR products were purified and subjected to sequence analysis using an ABI 377 automated sequencer. The amplification primers were used as primers in the sequencing reactions.

Screening for genetic rearrangements and deletions was performed by Southern Blot analysis. DNA was digested with *Eco* RI alone or with *Eco* RI and *Ase* I double digest. After gel electrophoresis and transfer to Hybond-N filters, the genomic DNA was hybridised with the VHL *g7*-cDNA probe,[9] (kindly provided by I. Kuzmin, Frederick, MD, USA) according to the manufacturer's instruction. Quantitative Southern blotting was performed by hybridising genomic DNA with the VHL *g7*-cDNA probe and with a beta-globin probe (kindly provided by C. Stolle, Philadelphia, PA, USA), to detect deletions encompassing the entire VHL gene.

Results and discussion

We found no VHL germline mutations in all the studied probands with phaeochromocytoma-only (table 1). However, our study included at least eight cases that are associated with a potentially higher risk for a hereditary tumour syndrome. These eight cases were the three patients with bilateral phaeochromocytoma (age 24, 28, 30 years), patient number 8 with a solitary tumour at an early age (17 years), patient number 21 with recurrent tumours at an early age (19 years), and three cases of familial phaeochromocytoma.

VHL-associated phaeochromocytoma differ from sporadic phaeochromocytoma in having multiple or bilateral tumours and an average manifestation two decades earlier (27 years versus 46 years, respectively).[1] Based on Knudson's two-hit theory, patients with a germline mutation in a tumour suppressor gene are predisposed to multi-centric tumours that are likely to manifest at a younger age than in sporadic patients (since the first hit has already taken place).[23] Therefore, familial phaeochromocytoma and also childhood cases show a tendency to multi-centricity and recurrence of tumours. Consequently, patients and at risk family members have to undergo life-long surveillance. It is important to ascertain whether a patient has an associated hereditary syndrome in order to monitor not only phaeochromocytoma, but also further lesions associated with that disease.

Six other studies have also investigated the genetic epidemiology of (familial) phaeochromocytoma (Table 2).[24-29] 21 VHL germline mutations but no RET gene mutations were found in a total of 225 observations. However, we would like to comment on two of the VHL gene mutations found in one study.[27] First, the P25L mutation is probably not a disease-causing mutation. So far, no VHL germline mutations have been described in VHL families upstream of the start site located at codon 54.[30,31]

Second, the R64P mutation, found in two related patients (uncle and nephew) should be considered as one case of familial phaeochromocytoma. Consequently, the presence of three patients with a VHL germline mutation in 66 sporadic patients with solitary phaeochromocytoma (i.e. 5%)[27] is in better agreement with the other studies (table 2).[24,25,29] Joint data demonstrate that seven out of 17 cases with familial phaeochromocytoma (41%) had a VHL germline mutation.[26,28,29] In addition, case records have been published of VHL germline (missense) mutations in familial phaeochromocytoma.[13,32] These cases suggested a distinct VHL phenotype, i.e. VHL type IIC.

Table 1 Age at diagnosis and type of phaeochromocytoma

Pat	Unr	Age	Type	RET
1	6	56	Solitary	-
2	7	65	Solitary	-
3	12	38	Solitary	
4	35	42	Solitary	-
5	42	43	Solitary	-
6	48	70	Solitary	
7	56	35	Solitary	
8	66	17	Solitary	
9	67	49	Solitary	-
10	68	53	Solitary	-
11	70	66	Solitary	
12	73	62	Solitary	
13	76	49	Solitary	
14	79	43	Solitary	
15	20	28	Bilateral	-
16	43	24	Bilateral	-
17	59	30	Bilateral	-
18	60	42	Multiple	
19	62	52	Multiple	-
20	11	64	Recurrent	
21	37	19	Recurrent	-
22	9	64,?,?	Familial, three brothers	-
23	80	57,59	Familial, mother and daughter	
24	31	23,32,44	Familial and bilateral, two brothers and uncle	-

Pat, patient number; Unr, unique identification number; Age, age at diagnosis; RET, germline muation in the RET proto-oncogene.
13 of 23 probands were screened for RET mutations and were all negative (-)
Mean age at diagnosis in solitary phaeochromocytoma is 49 years; in the bilateral, multiple and recurrent group it is 37 years old.

Nearly all the mutations (20 out of 21) identified in phaeochromocytoma patients from these six reports concerned missense mutations. A hot spot was located at and around codon 167 of the VHL gene, confirming previously established genotype-phenotype correlations in VHL disease.[30,31] There is evidence that the presence or absence of phaeochromocytoma is correlated with the type of VHL germline mutations.[30,31] It was suggested that especially specific missense mutations would lead to phaeochromocytoma by a dominant negative effect of the mutated VHL protein.[33] However, besides this clear interfamilial difference, intrafamilial differences have also been observed, suggesting that genetic or environmental modifiers play a role in the manifestation of VHL disease.[34]

Table 2 Comparative studies analysing the genetic epidemiology of patients with phaeochromocytoma-only.

Study	Patients	n	VHL	Mutation (age)	RET
Bar	sporadic	24	0	-	0
Brauch	sporadic	62	2 (3%)	codon167 (33), splice site codon 155 (63)	0
Giraud	sporadic	11	0	-	n.t.
v/d Harst	sporadic	68	6 (9%)	P25L (38)[a], L63P (26), R64P (24)[b], G144Q (39), I147T (58)	n.t.
Hes	sporadic	14	0	-	0 (6/14 tested)
Bar	bilateral	3	1 (33%)	R161Q (13)	0
Giraud	bilateral	5	4 (80%)	Y98H, L129P, R167Q, V170G (m=17.5)[c]	n.t.
Woodward	bilateral	2	1 (50%)	R167W (?)	0
Woodward	multiple [d]	6	0	-	0
Hes	multiple	7	0	-	0 (5/7 tested)
Crossey	familial	3	2 (67%)	V81L, R167Q	0
Giraud	familial	6	2 (33%)	P97L, R167Q	n.t.
Woodward	familial	8	3 (38%)	S80G, R161Q, R167W	0
Hes	familial	3	0	-	0 (2/3 tested)
Total		**225**	**21**		**0**

This table includes patients with phaeochromocytoma from our study and those reported in the literature.[24-29]
Patients, type and (n)umber of patients studied; VHL, number of patients with a VHL germline mutation; RET, RET proto-oncogene germline mutation (exons 10 and 11 tested and sometimes also exons 13 and 16); n.t., not tested;
P25L[a], so far, no VHL germline mutations have been described in VHL families upstream of codon 54; R64P[b], found in two related (uncle and nephew) patients;
(m=17.5)[c], mean age at diagnosis was 17.5 years; multiple phaeo[d] were all cases of multiple extra-adrenal or adrenal phaeochromocytoma with a family history of neuro-ectodermal tumours.

The fact that we did not detect VHL mutations in our phaeochromocytoma patients was to be expected given the relatively low number investigated. VHL germline mutations are detected in virtually all well-defined VHL families.[31] A conservative estimate of the mutation detection sensitivity of the strategy used in this study would therefore be in the order of 95%. Moreover, since most VHL germline mutations in phaeochromocytoma patients concern missense mutations, these are not likely to be missed easily. This would suggest that as well as familial phaeochromocytoma associated with VHL disease (approximately 41%), there is also room for familial cases with a distinct genetic basis. From this point of view, our case 24 is of major interest, since it shows multiple features (i.e. young age at diagnosis, bilateral and familial tumours) that would indicate a germline mutation. This type of family would be an appropriate candidate for a genome-wide screening to identify candidate genes associated with the inheritance of phaeochromocytoma. Alternative explanations would include a mutation in part of the VHL gene not analysed (e.g. regulatory domain) or somatic mosaicism. Although mosaicism has so far been described in only two VHL families.[31]

We have demonstrated that sporadic patients with solitary phaeochromocytoma and even those exhibiting aspects normally regarded as caused by a hereditary mutation (early onset, multiple, recurrent, bilateral or familial tumours) could not be associated with VHL germline mutations. However, we still advise genetic screening for VHL mutations in all cases with possibly hereditary features since the costs are relatively low, the molecular genetic analysis of the VHL gene is readily feasible and the vast majority of VHL mutations can be detected. Annual clinical screening for further VHL-related tumours in patients with phaeochromocytoma-only and a negative test for VHL germline mutations seems not recommended. Since each identified proband may provide appropriate clinical management for possibly affected family members, molecular testing is likely to be cost-effective.[25,35] Moreover, the absence of germline VHL gene mutations in cases showing strong hereditary features may indicate the occurrence of somatic mosaicism for VHL mutations or, alternatively, suggest that other phaeochromocytoma susceptibility loci exist.

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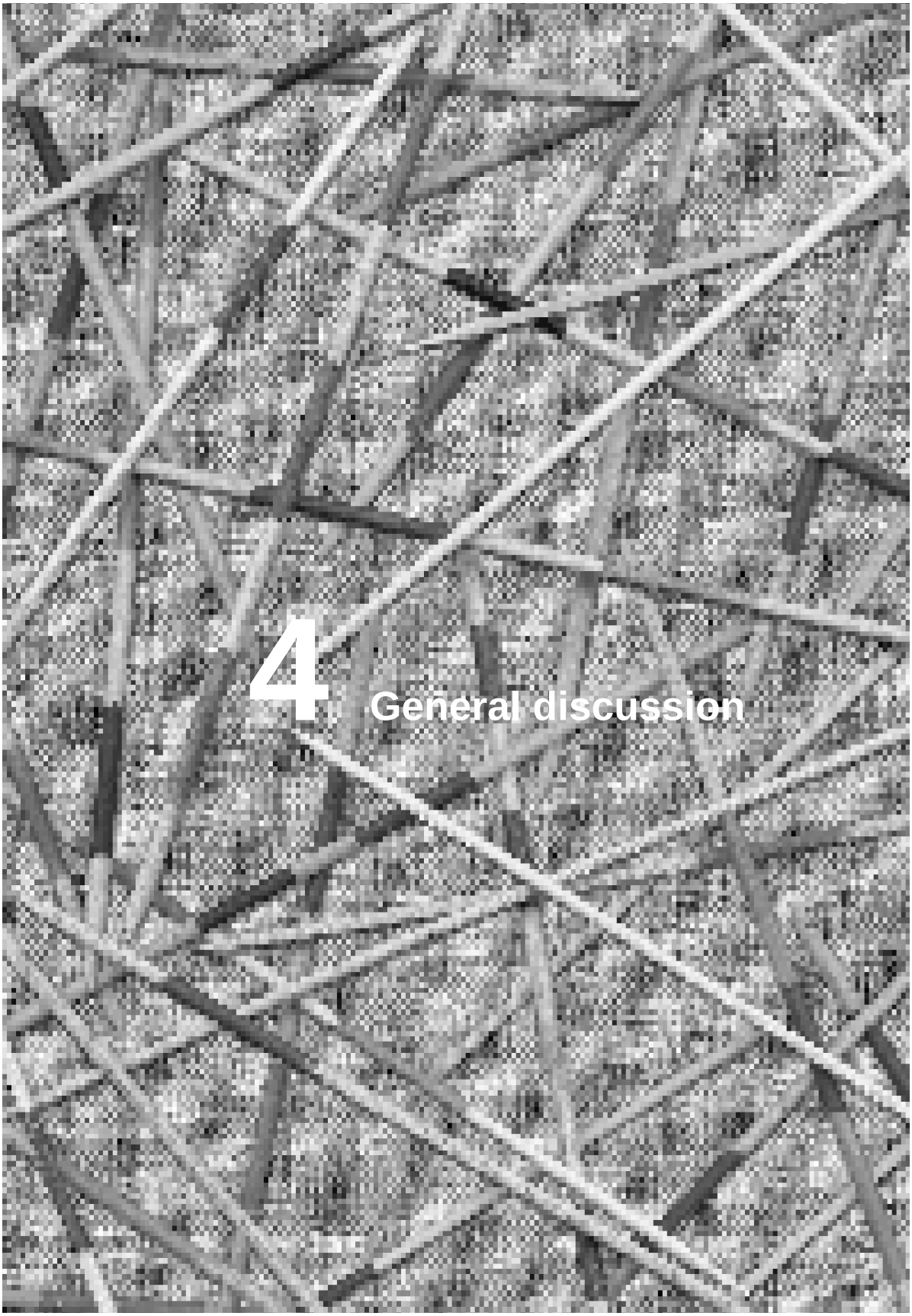

4 General discussion

Contents

General discussion

Following the identification of the VHL tumour suppressor gene in 1993, studies on VHL disease intensified. The studies outlined in this thesis have focused on the clinical and genetic aspects of VHL families and of sporadic patients with VHL-related tumours in the Netherlands. These studies are summarised and discussed in the light of reports in the literature. Since one of the main objectives of this research is to identify as many carriers of germline mutations in the VHL gene as possible, we also discuss the efficiency of methods used in detecting patients. In addition, psychological, social, political and ethical issues are addressed, as well as the financial and legal repercussions for persons identified as being at risk for a hereditary cancer syndrome.

4.1 Summary of clinical aspects of VHL disease

In chapter 2 the management of two organs (kidneys and eyes) in VHL disease has been considered in detail and the radiological monitoring in VHL patients for the upper abdominal VHL-related organs is described. The clinical and radiological aspects of the remaining VHL-related organs are reviewed in chapter 1. The last section deals with guidelines for the diagnosis and clinical monitoring of VHL patients in the Netherlands.

Studies on the natural history of a complex disorder such as VHL disease depend on clinical data gathered from long and intensive studies of VHL patients monitored according to an uniform clinical surveillance protocol. In the Netherlands, the relatively small numbers of such VHL patients has hampered these studies. It is important that, over the coming years, we continue collecting clinical data on as many VHL patients as possible, in order build up a sufficiently large data collection to permit valid conclusions on the complex manifestations and natural history of VHL disease within our own country. Given that VHL disease is a complex disorder, it can not be assumed that the phenotypical expression will be identical in all countries. Different environmental and genetic modifiers may play different roles in different countries. However, multi-centered European studies should be also initiated to increase the number of VHL patients investigated. Moreover, it is important that the Dutch studies are as complete as possible to permit valid comparisons with trans-European studies and to provide an as comprehensive as possible identification of VHL families. In this context, the natural history of retinal haemangioblastoma and renal cell carcinoma in a limited series of Dutch VHL patients are reported in detail. In addition, the management of CNS haemangioblastomas is considered as these present a pressing problem in clinical practice.

4.1.1 Ocular haemangioblastoma

In section 2.3, the results of long-term follow-up in Dutch patients (as long as 23 years for some patients) are reported. Ophthalmological data are described with special attention to the natural course of development of ocular haemangioblastoma. Five stages of development of ocular haemangioblastoma are distinguished and illustrated by fluorescein angiographic pictures. Although the limited number of VHL patients available unfortunately hampers in depth studies on additional aspects of the natural history - such as age of onset, the frequency of visual impairment, etc -, one of the

main conclusions was that isolated occurrence of haemangioblastomas, particularly when associated with young age, multicentricity or bilateralism, is a strong indication for carrying out DNA diagnosis (see 4.2).

The mean age at diagnosis of tumours for VHL mutation carriers is lower than in (sporadic) patients who do not carry a germline mutation (Table 1). Retinal haemangioblastoma is the first tumour manifestation in many cases of VHL disease and this may be the sole manifestation for some time. Solitary retinal haemangioblastoma can also occur in the absence of VHL disease and has an estimated prevalence of 1:110,000 persons.[1] This is in contrast to other typical VHL tumours that occur more commonly as isolated forms than as part of VHL disease. Webster et al. provided two possible explanations, with reference to Knudson's hypothesis*, for the relatively low frequency of solitary retinal haemangioblastoma. Firstly, the risk that an ocular cell is affected with two independent somatic hits is likely to be lower than in other VHL associated organs, because the number of susceptible cells in the eye is lower. Secondly, the critical period for tumourigenesis may be limited to early retinal development and growth, as in retinoblastoma, whereas it may be life-long in other organs (as illustrated by the old age of sporadic patients in which renal cell carcinoma occur, see section 1.3.3). Since the frequency of solitary retinal haemangioblastoma is rarer than VHL associated haemangioblastoma, we recommend performing VHL germline mutations analysis in all sporadic patients.

Further, recommendations are provided in section 2.3 for the annual monitoring and treatment of ocular lesions in VHL patients. We stress that only early detection and treatment of peripheral retinal haemangioblastoma can be expected to decrease the percentage of patients with impaired visual acuity. Ophthalmological monitoring (and subsequent treatment) of patients and persons at risk should therefore start as early as possible. Since the prevalence of retinal tumours does not increase in older VHL patients,[1,4] it should be questioned whether all carriers of a VHL germline mutation should undergo similar ocular monitoring. Younger patients (e.g. younger than 30 years) may be monitored more frequently (e.g. twice a year), whereas older patients (e.g. older than 60 years) could be monitored every two years. In addition, reduced probability of new haemangioblastoma formation in older gene-carriers may also allow us to reassure haemangioblastoma-free VHL patients in the clinic.[5]

Tumour	VHL	Sporadic
Retinal haemangioblastoma[1,5,6]	24 years	31 years
CNS haemangioblastoma[6-10]	29-33 years	44-48 years
Renal cell carcinoma[6,11-17]	36 years	60-70 years
Phaeochromocytoma[18-20]	28 years	47 years

Table 1 Age at diagnosis of solitary VHL-related tumours in sporadic patients and carriers of a VHL germline mutation.

* In 1999, the two-hit model was demonstrated in ocular lesions of VHL patients with markers flanking the VHL gene. Two research groups independently confirmed, by microdissection of ocular haemangioblastoma, that a somatic deletion of the VHL gene locus (the second hit) took place in stromal cells.[2,3] This demonstrated that stromal cells (as in CNS haemangioblastoma, see 1.3.2) represent the true neoplastic component in these tumours.

4.1.2 CNS haemangioblastoma

Despite the fact that many haemangioblastoma grow little or not at all for years, CNS haemangioblastoma remain responsible for considerable morbidity and mortality in VHL disease and many patients require emergency treatment, especially in cases of delayed diagnosis.[8] The standard treatment for symptomatic lesions is complete microsurgical removal. The treatment of asymptomatic cerebellar haemangioblastoma however, remains controversial. There are clinics in the Netherlands that do not perform radiological monitoring of the CNS in VHL patients. In their opinion, radiological monitoring using MRI is too expensive and has no consequences for treatment since many neurosurgeons will not operate on asymptomatic lesions. Although proponents of monitoring asymptomatic haemangioblastomas are most commonly aware that the monitoring of CNS lesions has no therapeutical consequences, these clinicians feel reassured when such potentially life-threatening tumours can be closely monitored.

A consensus for the monitoring interval of CNS lesions in VHL patients does not exist in the literature. Some recommendations are: 1) every two years from the age of 11 years and every three-five years after an age of 60 years (NIH);[21] 2) every three years from mid-teens to an age of 50 years and there after every five years (Cambridge);[6] 3) after the age of 20 years, at least once per decade.[19] This illustrates the variability in the choice in frequencies of monitoring for CNS related haemangioblastoma. This difference in opinion is also present within the Dutch VHL working group. At present, it is not possible to propose a single uniform standard for this component of clinical monitoring in VHL disease.

The multidisciplinary team at the UMC Utrecht recently agreed that CNS imaging could be combined with the monitoring of the upper abdominal organs. Since this single procedure evaluates both the CNS and the upper abdominal components of VHL disease, patients in our hospital are now given a double MRI appointment every two years. In conjunction with ultrasound, this protocol monitors the kidneys, adrenal glands, pancreas and liver of VHL patients alternately using ultrasound and MRI.

In my opinion, clinical research is, next to reassurance of the attending physician, a second argument to perform radiological monitoring of CNS lesions. Radiological data can be studied to further evaluate the natural history of CNS haemangioblastoma in VHL patients. These data could, for example, favour presymptomatic treatment in some clinical situations. It is conceivable, that a neurosurgeon would operate on an asymptomatic lesion when MRI indicates the presence of a developing hydrocephalus due to herniation of the cerebellar tonsils through the foramen magnum. Moreover, the possibility of treating small solid lesions presymptomatically with radiosurgery may justify radiological follow-up of cerebellar lesions in the near future. Chang et al.[22] are already treating asymptomatic haemangioblastomas in VHL patients with radiosurgery if the lesions are larger than 5 mm and when the tumours are observed in a setting of other progressive symptomatic tumours, or if there is evidence of progressing in serial imaging studies. It is evident that this aspect, above all other facts of VHL monitoring, gives the largest problems to define an uniform national monitoring program. In the mean time, the proponents and opponents of CNS monitoring in VHL disease have agreed to disagree and each centre will proceed according to their own vision.

4.1.3 Renal cell carcinoma

We give recommendations for the management of renal lesions in VHL patients in section 2.2. If both kidneys are affected with multiple cysts and renal cell carcinomas, a difficult decision has to be made between radical nephrectomy and nephron-sparing surgery (Fig.1). This decision depends on risk factors (size, progression, capsule involvement and whether the tumour is symptomatic) for metastatic spread. Management of metastatic lesions is a difficult problem, since response to chemotherapy and radiotherapy is poor.

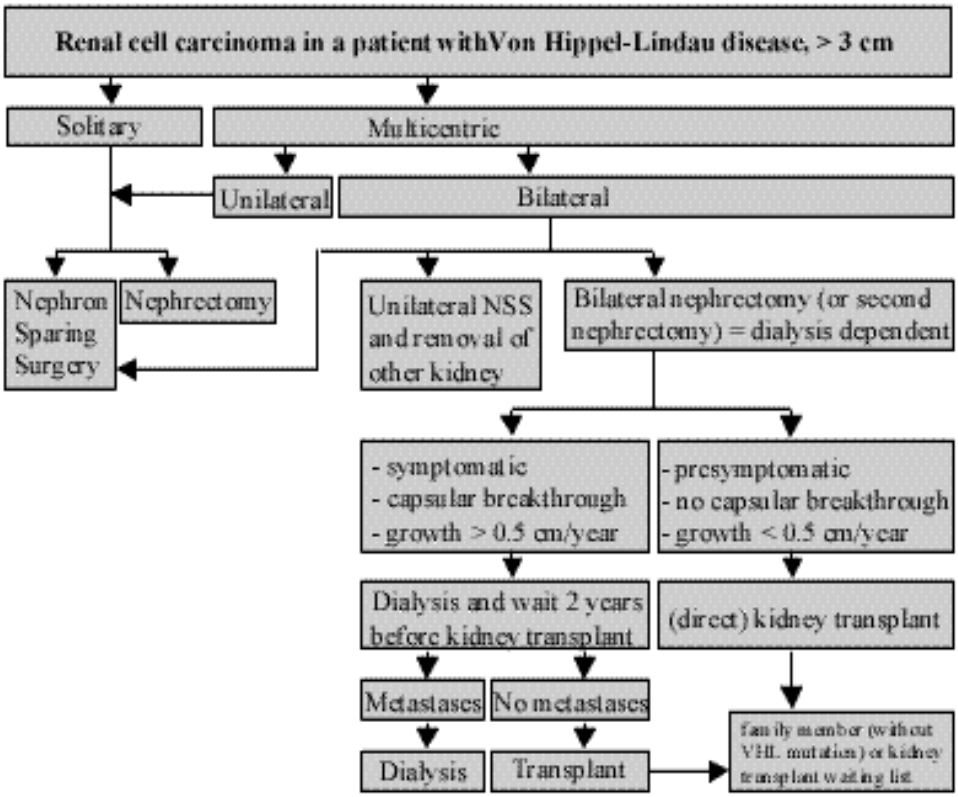

Fig. 1 Flow-chart for VHL patients with renal cell carcinoma larger than 3 cm. In the first part of this scheme decisions for treatment are made by a multidisciplinary team consisting of an urologist (or surgeon), a nephrologist, a radiologist, and an oncologist (or internist). Once the patient becomes dependent on dialysis, we advise attending physicians to follow the flow chart.

Metastases in VHL patients with renal cell carcinoma have until recently been associated with a seemingly critical tumour size of 7 cm.[17] However, an arbitrary 3 cm surgical threshold has been postulated by many authors. In 1999, the first report of a prospective analysis on treatment of renal cell carcinoma in VHL patients appeared and supported the 3 cm threshold.[16] The presence of metastases was demonstrated in eight of the 17 patients with tumours larger than 6 cm, in three of 27 VHL patients with tumours of 3-6 cm, but in none of the 52 studied patients with tumours smaller

than 3 cm. However in our study, five out of 17 lesions had pseudocapsular invasion (and four of these tumours were smaller than 3 cm). Breakthrough was observed in three of the five tumours that measured 1.3, 2.6 and 5.5 cm respectively. It is important to note that our observations of breakthrough were made on pathological specimens following kidney removal and there is no guarantee that we could have made these observations *in vivo*. In practice, there is a problem that the resolution of even the most refined imaging techniques will probably not permit detection of breakthrough in such small (e.g. 1.3 cm) renal lesions. We anticipate that pseudocapsular breakthrough of renal cell carcinoma is associated with a higher risk for metastases. Our results indicate that the 3 cm guideline should be validated in a larger set of intensively studied patients. In addition, it should be studied whether a correlation exists between a VHL germline mutation and (late or early) breakthrough. Meanwhile, from a pragmatic point of view, we should like to advocate that attending medical specialists follow the surgical threshold of 3 cm in VHL patients.

Monitoring renal lesions

In the literature an annual CT scan is recommended to monitor renal lesions in VHL patients.[6,19,21] For reasons of possible increased radiation susceptibility - advanced elsewhere in this chapter - the VHL working group subjectively prefers alternate monitoring of renal lesions using MRI and ultrasound. Although it should be stated that the combination of these methods has not been demonstrated to be cost-effective. To reduce costs, abdominal surveillance might be performed using only ultrasound. However, the use of ultrasound alone, in the opinion of the VHL working group, would not constitute an optimal monitoring procedure. Firstly, pseudocapsular breakthrough, which is important to initiate surgical treatment of renal cell carcinoma, cannot be detected by ultrasound. Secondly, MRI gives better insight into the exact location of a renal lesion; this may lead to an early operation when growth of the tumour towards blood vessels or renal pyelum is anticipated.

Renal cell carcinoma and fibrous pseudocapsule

We concluded that, in our patients, renal cell carcinomas grew slowly, were of low grade, and had a dense fibrous pseudocapsule. They are thus good candidates for nephron-sparing surgery. The fibrous pseudocapsule has been described as a typical feature of renal cell carcinoma in VHL patients.[17,23,24] The fibrous pseudocapsule may be a critical differential feature between renal cell carcinomas arising via a VHL germline mutation and those arising via other mutational mechanisms. Consequently, less stringent surgical procedures, such as nephron-sparing surgery, may be generally indicated in VHL patients.

We consider that at least two mechanisms are responsible for the fibrous capsule formation. Firstly, fibrous tissue formation may be the result of an inflammatory reaction caused by haemorrhage in the cysts. These haemorrhages in tumours and cysts may well be caused by abundant and vulnerable neovascularisation. Secondly, an enlarging renal cell carcinoma may lead to compression of renal stroma and disappearance of normal parenchyme tissue. This leaves the resistant connective tissue skeleton, which normally supports the renal tubuli.

Recently, further evidence was presented for the slow growth and relatively late metastasis of VHL-induced renal cell carcinoma. The urokinase-type plasminogen activator system mediates proteolysis of the extracellular matrix and is therefore important for tumour cell invasion, metastases and angiogenesis. In this system, plasminogen is converted by urokinase-type plasminogen activator into the proteolytic enzyme plasmin. Los et al. demonstrated that the VHL gene plays a role in the regulation of this system.[25] They demonstrated that inactivation of the VHL gene results in a decreased overall urokinase activity. As a result proteolysis of the extracellular matrix is hampered. In the presence of wild-type VHL, urokinase is upregulated and plasminogen activator inhibitor-1 is downregulated. This might explain, to a certain extent, the clinical observations that: (1) in VHL patients with renal cell carcinoma, metastases are rarer than in sporadic patients with renal cell carcinoma, and (2) at initial diagnosis, renal cell carcinoma can be very large without evidence of metastases. However, since the VHL gene is frequently involved both in renal cell carcinoma of sporadic patients as well as VHL patients, it remains difficult to explain these differences in biological behaviour.

Multi-centric renal lesions

By extrapolation of tissue surrounding renal tumours, the number of microscopic lesions in an average VHL kidney was estimated at 1100 cysts and 600 clear cell neoplasms.[26] Close microscopical examination of five kidneys in our study only revealed incidental small lesions, mainly in the direct vicinity of the macroscopical visible studies. We therefore referred to them as satellite lesions, indicating that the tissue surrounding renal lesions is not representative for the entire kidney. A relatively low number of renal lesions per kidney is also in agreement with other studies.[13,19,27,28]

Multi-centricity can either arise from independent somatic mutations or multiple lesions can originate from a common initial tumour. Loss of heterozygosity (LOH) of the VHL chromosome region has been demonstrated in both cystic lesions and renal cell carcinoma by microdissecting material from individual lesions.[29] This strongly suggests that cysts and microcysts are precursors for renal cell carcinoma, but leaves unanswered whether the somatic mutation has occurred independently at many sites or singly followed by dispersal. A more detailed molecular analysis of either the pattern of LOH or the somatic mutation would distinguish between the two possibilities.

We hypothesise that three phenomena may play a role in the origin of satellite lesions in patients with renal cell carcinoma. Firstly, the paracrine effect of large and enduring renal cell carcinoma produces factors such as vascular endothelial growth factor (VEGF), which has been demonstrated to be the key tumour angiogenesis factor in VHL.[30] Secondly, satellite lesions may be the consequence of metastasis in the same kidney. Thirdly, tumour growth may lead to compression of the directly surrounding renal tissue, followed by dilation of tubuli and may thereby promote the formation of microcysts with proliferation of the lining cells. Therefore, satellite lesions are more likely to be found only in the close vicinity of expanding renal lesions. An increased rate of cell division is associated with a higher rate of errors in DNA and chromosome replication, leading to further mutations and the possibility of the renal lesion acquiring a more malignant character.

Interestingly, this same phenomena is also observed in retinal haemangioblastoma, where small secondary lesions appear near areas of detached or treated retina.[4] The authors suggested that new retinal lesions are most likely to occur when retinal or endothelial or vascular cells are mitotically active and susceptible to mutation, such as in the developing retina or areas that turned ischaemic through detachment or ablative treatment.

4.1.4 Clinical management of VHL patients: organisation and recommendations

One of the most important challenges in the management of VHL disease is to establish an intercentre co-operation between medical specialists who advise VHL patients and their families. Some doctors tend to keep patients as a curiosity for themselves since they exhibit 'such an interesting syndrome'. However, not all clinicians may be aware of the complex factors involved in the disease. Nowadays, some VHL patients and families are still monitored and treated by individual doctors. This has resulted in unnecessary morbidity and even mortality. Examination of our database showed that predominantly patients that had not been regularly monitored died of VHL-related tumours in the last four years. These involved four patients who died of complications from renal cell carcinoma, and a patient who, although intensively monitored over the past 25 years, died as a result of complications following a surgical procedure. It is clear that well-organised, multidisciplinary teams following national and international guidelines, with access to reliable databases for patient status, will guarantee the best result in the management of patients and families with VHL disease.

Collaboration on a rare disease in a small country may avoid individual institutes following their own protocol for patient care. A central organisation, led by independent representatives from various institutes, can define and secure a uniform clinical management of VHL patients as well as lead to nationally structured research projects. We therefore initiated a national VHL working group in 1996. The six goals of this working group are: (1) to draft guidelines for diagnosis of the disease, DNA testing, and periodical clinical monitoring; (2) to provide attending physicians with uniform information; (3) to provide co-ordination of multidisciplinary teams in regional VHL centres; (4) to establish a central registration of clinical and genetic data; (5) to stimulate research; and (6) to raise funds for periodical clinical monitoring. At the moment we are in contact with some 80 doctors from eleven different medical fields. A board will be selected (preferably with representation of all the medical specialists involved in VHL) with three representatives from each of the eight university hospitals. A smaller board takes care of the day-to-day organisation.

However, we anticipate some problems in the continuity of clinical surveillance of VHL patients. Firstly, the responsibility for guaranteeing the continuity in periodical clinical monitoring places a heavy burden on the attending physician.[31] Secondly, continuation of periodic monitoring cannot be guaranteed if the attending physician departs, or if there is loss of funds or facilities, conclusion of short-term research programmes, or if patients move house. Such factors disrupt the continuity of health care, are disturbing to the families, and may lead to patients and families feeling frustrated or neglected. The working group therefore contacted the Netherlands Foundation for the Detection of Hereditary Tumours (STOET - *Stichting Opsporing Erfelijke*

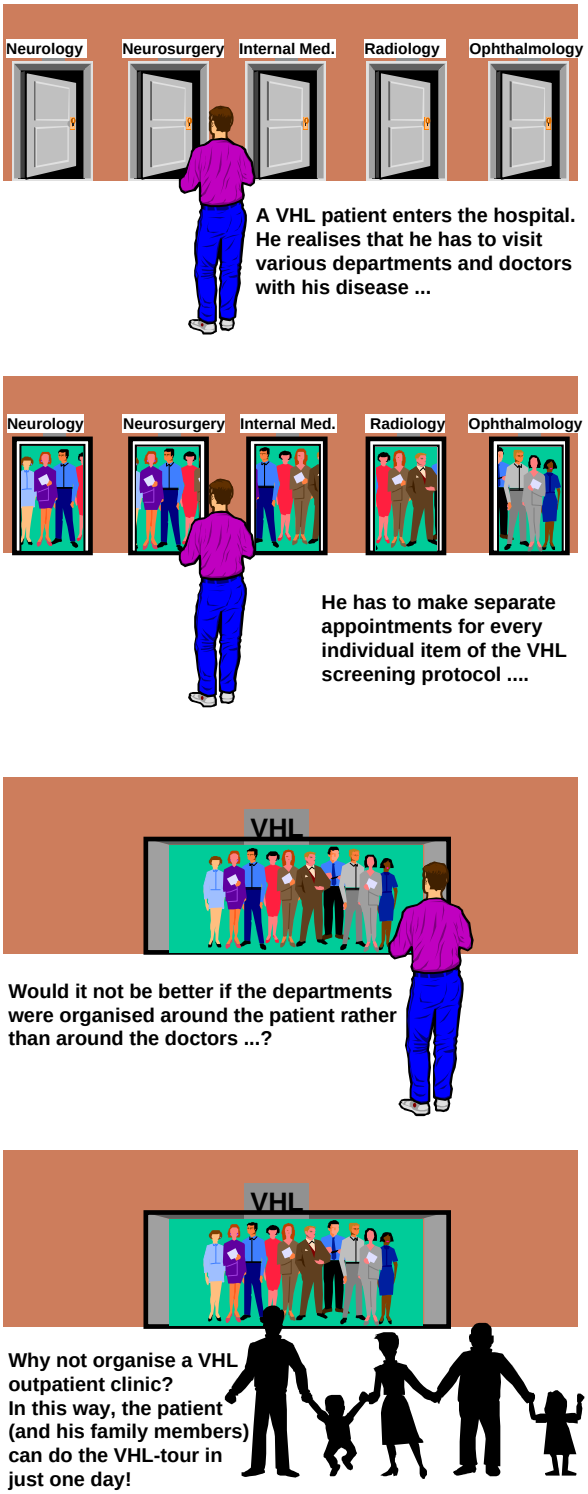

Fig. 2 Clinical monitoring of VHL patients, a cartoon adapted from Dr. G. Oppocher of the University of Padua, Italy (reproduced with permission).

Tumoren) which maintains a register of families with inherited tumours. This organisation has the following objectives: (1) to improve the surveillance of family members; (2) to guarantee the continuity of periodical clinical examination; (3) to serve general practitioners and specialists by advising them about matters related to diagnosis, treatment, and monitoring procedures as well as referral of subjects to clinical genetic centres for genetic counselling; and (4) to collect data as a basis for scientific investigations.[32] Another option is that members of the working group themselves carry out the above mentioned tasks.

In the UMC Utrecht, the annual clinical monitoring for VHL patients and their affected family members is organised in a production line fashion. In one day the patients complete the entire 'VHL tour' along various medical specialists, radiological monitoring, and blood and urine tests (Fig. 2). A central administrator books appointments approximately a year in advance. Within a month the families return for the clinical test results. This multidisciplinary, centrally organised approach has now been adopted by five of the eight Dutch university clinics.

4.2 Molecular genetic analysis of VHL disease

Germline mutations of the VHL gene have been identified in families and patients who meet the clinical diagnostic criteria as well as in sporadic patients with a solitary VHL-related tumour. Since the costs of DNA analysis of the VHL gene are relatively low, molecular genetic analysis of the VHL gene is readily feasible and the vast majority of VHL gene mutations can be and have been detected in the Netherlands. Every patient suspected of having VHL disease should, in my opinion, be tested. In this section the results of case findings using VHL germline analysis are compared to the clinical diagnostic criteria and the eligibility of patients for DNA analysis. In addition, genotype-phenotype correlations are discussed.

4.2.1 Diagnostic criteria for VHL disease and implications for case finding

Diagnosis of VHL disease follows two routes: the classic pedigree analysis method and VHL gene mutation analysis. However, for pedigree analysis, family history data may be incomplete and the results of clinical screening are age-dependent.[6,27,33] Moreover, the value of the clinical data for pedigree analysis depends on family size and penetrance of the disease, and the reliability and extent of clinical monitoring. Therefore, the assessment of a VHL gene germline mutation must be considered as the gold standard in carrier detection. Since DNA analysis detects mutations in virtually all 'well defined' VHL families,[34] clinical diagnostic criteria are in themselves only significant for classic VHL patients or families that decline DNA testing. In the few remaining cases with patently obvious VHL disease where the germline mutation has not been found and linkage studies are not informative, all family members at risk have to be monitored annually.

In order to set diagnostic criteria for a complex disorder like VHL disease we need to be aware of circular reasoning. Firstly, clinical diagnostic criteria are defined and then, using these criteria, germline mutations are found with varying success rates for different manifestations of VHL disease. Subsequently, these findings may be used to adjust the clinical diagnostic criteria for VHL disease. In order to study this in the Dutch VHL population, the probands referred for DNA analysis were divided into groups based on existing clinical diagnostic criteria (see Table 2, Fig. 3). Since these criteria were drawn up retrospectively, after identification of the VHL gene, they are likely to have been influenced by case findings of VHL germline mutations. We discern six types of patient categories:

Familial cases

1. VHL germline mutations are consistently detected in 100% of families with more than two affected family members, with multiple or single VHL-related tumours. This finding agrees the clinical diagnostic criteria of Maher and Kaelin,[35] who described how VHL disease could be diagnosed in patients with a typical VHL-related lesion in combination with a positive family history.

2. VHL germline mutations are NOT detected in ALL cases in which only two first-degree relatives exhibit VHL-related tumours. This observation suggests that a VHL family history requires sometimes more than two family members or alternatively, a multiple VHL-related tumour spectrum in two close relatives. However, in such clinical situations persons should always be tested for a VHL germline mutation.

3. VHL germline mutations are found in varying success-percentages in families with two or more first-degree relatives exhibiting a single type of VHL related tumour. However, next to the type of tumour, factors such as age of onset, multi-centricity and number of affected family members, are important indicators for the presence of VHL germline mutations. It is generally accepted that VHL germline mutations are found in up to 100% of families with haemangioblastomas, since these families meet the VHL diagnostic criteria.[35] Variable percentages are reported in familial renal cell carcinoma (0-100%),[17,36,37] and in phaeochromocytoma families (33-67%).[38-40]

Sporadic cases

4. In ALL sporadic patients exhibiting a clear multi-organ expression of VHL-related tumours a VHL germline mutation is identified. This finding agrees with the clinical diagnostic criteria for sporadic patients of Maher and Kaelin.[35]

5. In sporadic patients with a single type but multiple sites of VHL-related tumours the success rate for identifying a VHL germline mutation depends partly on the tumour type, but also on features such as: age at diagnosis, multi-centricity and bilateralism. For example, in patients with multiple CNS haemangioblastoma however, who meet the clinical diagnostic criteria for isolated patients[35], a VHL germline mutation is NOT always identified. Examples of finding VHL germline mutations are described in sections 3.3 and 3.4 for patients with multiple CNS haemangioblastoma (50%) and multiple phaeochromocytoma (0-80%). Moreover, 10% of the patients with bilateral renal cell carcinoma is a carrier of a VHL germline mutation.[17]

6. In sporadic patients with a single VHL-related tumour the success rate for identifying a VHL germline mutation is generally low (i.e. <5%, see sections 3.3 and 3.4). However as described in section 4.1.1, retinal haemangioblastoma form an exception. Identification of a VHL germline mutation in sporadic patients with a single VHL-related tumour depends not only on the tumour type, but also on the age at diagnosis. We agree with the members of the Dutch VHL working group that the cut-off point for 'young age at diagnosis' should be 50 years for CNS haemangioblastoma and phaeochromocytoma. In contrast, renal cell carcinoma requires a much younger age at diagnosis (<30 years), since this tumour is relatively common in the general population at older age. These age differences are based on studies that reported the age at diagnosis of VHL-related tumours both in sporadic patients without germline mutations as well as in proven carriers of a VHL gene mutation (Table 2). In category 6 we would also like to include the sporadic patients with a single VHL-related tumour and a less specific VHL-related tumour such as a renal cyst.

Although, this classification is an arbitrary way of categorising patients, it generally reflects the chance of finding a germline mutation for each type of patient. Taking into account that VHL germline mutations have been identified in situations varying from classic VHL families to sporadic patients with a history suggesting just one or some features of VHL disease, clinical diagnostic criteria must be neither too restrictive nor too open-ended. It therefore remains questionable whether unambiguous clinical diagnostic criteria can be defined at all for this tumour syndrome. We suggest treating each patient suspected of having VHL disease, according to the six categories above, with an open mind and performing: (1) an extensive pedigree analysis, (2) DNA analysis, and (3) clinical screening for further VHL-related tumours.

Table 2 Case findings of VHL disease by molecular genetic analysis in the Department of Medical Genetics, UMC Utrecht, 1994-1999

Cat	Clinical situation	n	Mutation	+	-	rHAB	cHAB	RCC	Phaeo	PC	Other
1	VHL (n>2)	10	10 (100%)	36	53						
2	VHL (n=2)	6	4 (67%)	6	2						
			+				m	+			sister with cHAB and RCC
			+			+	+			+	father with multiple cHAB
			+			b+m					mother with bilateral + multiple rHAB
			+			m					father with cHAB
			-				+				father with RCC
			-			+	m				sister with rHAB
3	Phaeo	4	-	0	4				f		
4	VHL (n=1)	7	6 (86%)	6	11						
			+			m	m				
			+				m	+	+		epididymis cyst
			+			m	m	+			
			+			b+m	+				
			+			+				+	renal cysts
			+				m			+	renal cysts
			-			m	+				
5	cHAB	4	-	0	4		m				
	rHAB	1	+ (100%)	0	1	b+m					
	Phaeo	7	-	0	7				m		
6	rHAB	1	-	0	1	s					
	cHAB	26	1 (4%)	5	30		s				
	cHAB	1	-	0	1		s				grandmother died of RCC aged 82 years
	RCC	1	-	0	1			s			age at diagnosis 6 years

Fig. 3 Germline mutation analysis of the VHL gene in probands referred to the Department of Genetics in the UMC Utrecht. The number of probands is depicted on the y-axis and the year of referral on the x-axis. The probands are divided into four groups: patients or families that met the clinical diagnostic criteria (VHL: n>2, n=2, n=1) and cases that did not meet clinical diagnostic criteria (Miscellaneous); this groups includes patients with a solitary haemangioblastoma, probands with phaeochromocytoma only, etc.

Moreover, the discussion about establishing definite criteria for diagnosing VHL disease has often been confused by the criteria for eligibility for VHL germline mutation analysis. In my opinion, the question of 'which persons should be tested for mutation analysis of the VHL gene', is far more important than somewhat arcane discussions on what constitutes the perfect combination of clinical criteria for a VHL diagnosis. We consider all the conditions listed in table 3, and defined by our six criteria eligible, for DNA analysis.

Clinical situations leading to suspicion of VHL disease	
Family history of VHL disease	Bilateral and multi-centric renal cysts
Retinal haemangioblastoma	Phaeochromocytoma (paraganglioma)
CNS haemangioblastoma	- familial
- familial	- bilateral
- multi-centric	- diagnosis at age 50 years or younger
- diagnosis at age 50 years or younger	Bilateral ELSTs
Renal cell carcinoma (type clear cell or non-papillary)	Multiple pancreatic cysts
- younger than 30 years	Multiple pancreatic islet cell tumours
- bilateral or multi-centric	Bilateral or multi-centric epididymal cystadenomas
- familial	Bilateral or multi-centric APMO

Table 3 Clinical situations leading to suspicion of VHL disease
CNS, central nervous system; ELST, endo-lymphatic sac tumour; APMO, adnexal papillary cystadenoma of probable mesonephric origin.This table is based on recommendations from Glenn et al.[41]

Finally, we discuss the numbers of tested patients with and without a VHL germline mutation in table 2. When we pool sporadic and familial VHL patients and their first and second degree relatives from the Utrecht data (categories 1, 2 and 4), 48 of the 111 persons tested have a VHL germline mutation (43%), which is in a reasonable concordance with an autosomal dominant inheritance. The remaining clinical situations (categories 3, 5 and 6) lead to five identified VHL germline mutation carriers in 67 persons (8%). These figures indicate that procedures for VHL germline analysis in VHL disease are effective in detecting carriers of a VHL germline mutation. In section 4.2.5 VHL disease is compared in this respect to other hereditary cancers and in section 4.3.5 cost-effectiveness of screening for VHL germline mutations are discussed.

An estimate of the prevalence of VHL disease in the Netherlands

By 1999, 34 probands with a germline mutation in the VHL gene had been reported after a joint effort by the two VHL DNA diagnostic centres in Utrecht and Rotterdam (section 3.1). Exclusion of seven Belgian and Turkish families leaves 27 'Dutch families'. At the moment, at least 15 known VHL families have not yet been subjected to DNA analysis and six families are in progress. This indicates that the provisional number of detected VHL families in the Netherlands is approximately 48.

However, a more difficult problem is how to extrapolate the number of families to an estimated number of VHL germline mutation carriers. An indication that the number of mutation carriers detected is proportional to the amount of time invested in investigating each family is indicated by the four families referred at the start of this study in 1996 (Fig. 3). These four extensively monitored families comprised 35 patients, including persons 'said to be affected', giving a mean number of nine patients per family. However, these families probably represent a selection bias. During the last four years we have detected 15 new families with a mean number of almost four patients per family (range 1-12). This is almost certainly a more realistic determination of the average number of carriers in each per family. If we extrapolate the average of four to the total of 48 known families we come to a total of approximately 200 patients.

It is reasonable to assume that the chance of clinically diagnosing a family is directly related to the number of affected patients present in the family. As a result we are very unlikely to detect entirely new families with a large number of patients (i.e. larger than four), but may detect many more families with just one or two patients. However, it is difficult, perhaps impossible, to define what number of new families still need to be detected. An interesting aspect is that all seven families with just one single affected person (defined at the moment of diagnosis) were associated with *de novo* mutations, either on the basis of DNA diagnosis or family history (see section 3.1). These observations suggest a relatively high mutation frequency, which will require further future investigations.

However, the estimate of 200 Dutch persons carrying a VHL germline mutation is probably too low. Firstly, there will still be unidentified VHL families, despite the fact that health care is well organised in the Netherlands and well-defined large families are not likely to be missed. Secondly, the mean number of four patients per family is most likely an underestimate. Thorough genealogical research in families of identified VHL carriers should result in a higher number of mutation carriers detected per family. These considerations lead us to give a conservative estimate of a total number of VHL germline mutation carriers in the Netherlands at 250. With a population of 16 million people this would give a prevalence of 1:64,000; which is lower than estimates made in areas in Germany and England that vary between 1:31,000 and 1:53,000.[42-44]. This aspect requires also further investigations.

4.2.2 Molecular genetic implications of sporadic patients with VHL-related tumours

We describe several clinical situations that exhibit features suggesting a germline mutation, such as multi-centric, bilateral, or familial tumours, but that are not associated with a VHL germline mutation. These situations may provide evidence for additional VHL-related-tumour-susceptibility genes, or alternatively, mutations in parts of the VHL gene not investigated (e.g. promoter area).

The absence of VHL germline mutations in all phaeochromocytoma patients, particularly in those diagnosed at a young age, or with multiple/bilateral tumours, suggest the presence of additional phaeochromocytoma susceptibility genes. Moreover, four families with phaeochromocytoma, including one family displaying bilateral

tumours that also manifest at a young age, open the possibility of investigating whether indeed other (familial) phaeochromocytoma susceptibility loci are involved. Another, but less likely explanation is, that these patients are somatic mosaics for VHL mutations. Either of these two explanations would also account for the absence of a germline mutation in the VHL gene in patients with multiple CNS haemangioblastoma, or those who were diagnosed at a young age.

Candidates genes may be found by studying genes that are involved in the biochemical pathway of the VHL protein. So far, only the role of the putative tumour suppressor gene Cullin2 (Cul2) has been investigated in the pathogenesis of phaeochromocytoma.[45] Elongin C, Elongin B, Rbx1, etc. have not been studied yet. Cul2 is part of the VCB complex (described in the introduction) that plays a role in the ubiquitination of proteins. Only one of 26 studied tumours showed a hemizygous deletion of the Cul2 gene, and it was suggested that this gene does not play a major role in the tumourigenesis of phaeochromocytoma. In addition, candidate genes may be found using the same method as for the VHL gene. The group that linked the VHL gene to chromosome region 3p25-26 was inspired by Zbar et al., who found loss of alleles of loci on 3p in renal cell carcinoma, a common manifestation of VHL disease.[46] Other candidate loci (in the 3p14.2 and 3p21.2-p21.3 regions) for tumour suppressor genes involved in renal cell carcinoma were also identified on the short arm of chromosome 3.[47] However, none of these genes have been cloned yet and hence they provide no useful information for the attending physician of such a patient.

***De novo* VHL germline mutations**

In section 3.1 we identified four *de novo* VHL germline mutations (12%). Although, not all parents could be tested, the lack of clinical expression of the parents and other close relatives led us to conclude that the occurrence of *de novo* mutations in the VHL gene in the Netherlands could be as high as 21%. However, as advanced in the previous section, sporadic patients are likely to be an under-diagnosed group of VHL patients and this is one of the many unknown factors preventing conversion of the number of *de novo* mutations in our sample group into a VHL mutation frequency for the Netherlands.

Diagnosis of VHL disease may be hampered in sporadic patients by lack of additional symptoms of VHL-related tumours. It is conceivable that a relatively low number (n =25) of *de novo* mutations has been reported in the literature so far,[48-50] since most sporadic patients with VHL-related tumours remain unrecognised. However, seven of nine of our sporadic patients with a VHL mutation met the clinical diagnostic criteria for VHL disease, and four of these patients exhibited a clear multiorgan expression of VHL disease. Alternatively, sporadic patients with VHL-related tumours may be mosaic, although mosaicism has so far been described in only two instances.[34]. Another possibility is that these patients represent phenocopies. However, we think that it is very unlikely that independent somatic mutation events giving rise to multiple VHL-related tumours in one patient could be expected to occur by chance (see 4.2.5). On the assumption that, averaged over time, *de novo* mutations replace the disease genes lost through natural selection, there is a simple relationship between the rate at which natural selection is removing disadvantageous genes, the

rate at which *de novo* mutations creates them, and their frequency in the population.[51] We think this model has three implications: VHL patients are reproductively less efficient because of: (1) early death in some instances, prior to completion of families; (2) diminished male or female fertility, because of epididymal lesions or adnexal papillary tumours of probable mesonephric origin (APMO); and (3) an unwillingness to pass the disease on to the next generation. This last consideration is likely to be that with the highest impact. In any case our observation of a high *de novo* mutation frequency is compatible with these arguments.

4.2.3 Clinical implications of DNA analysis

Section 4.2.1. clearly illustrates that VHL germline mutations are identified in all classic VHL families as well as in classic sporadic VHL patients. Consequently, is not necessary to test patients with patently obvious VHL disease, except for possible probands. DNA analysis can therefore be restricted to relatives with less distinctive expression of VHL disease (e.g. a solitary renal cell carcinoma) and/or to young relatives at risk for the disease, in order to establish presymptomatic (or prenatal) diagnosis and prevent unnecessary monitoring.

In clinically less well-defined situations of patients with VHL-related tumours, DNA analysis plays an important role to confirm (or to reject) the diagnosis of VHL disease. The studies described in sections 3.3 and 3.4 indicate that a relatively small number of VHL germline mutations may be found in the population of sporadic patients with an apparently solitary VHL-related tumour. For example, before the identification of the VHL gene it was suggested that a substantial proportion of sporadic patients with CNS haemangioblastoma could be associated with VHL disease. Upon more detailed examination 23% to 34.3% was found to be afflicted with VHL disease.[10,52] Our findings, based on DNA diagnosis of 4% indicate that, the clinical criteria used at that time by Neumann and Richard were not as critically defined as the current situation is. However, periodic clinical monitoring is warranted for the small group of patients without a VHL germline mutation, but with features suggesting the presence of a germline mutation (e.g. one type of multiple VHL-related tumours). In families with only one type of hereditary VHL-related lesions and without a VHL germline mutation, periodic monitoring should at least be focussed on the tumour concerned. In all other patients with apparently solitary VHL-related tumours and without a VHL germline mutation, outgrowth of the tumour needs to be regularly controlled. In these latter cases once-only clinical screening combined with molecular genetic analysis of the VHL gene is probably sufficient to rule out VHL disease.

Detection of the family-specific VHL germline mutation can identify persons at risk for the disease. This is illustrated well by the following case from section 3.2. In a large classic VHL family, a 55 year old female was originally considered to be a VHL patient when diagnosed with a renal cell carcinoma. Since this tumour predominantly occurs in the seventh and eighth decade of life,[53] a person with a renal cell carcinoma in a VHL family and diagnosed at a relatively young age would certainly be suspected of having VHL disease. However, Southern blot analysis demonstrated that this patient did not carry the deletion associated with VHL in her family. Consequently, she could be released from the annual clinical monitoring protocol. One should

keep in mind that renal cell carcinoma is a relatively common malignancy. Kidney cancer accounts for 3% of adult malignancies, and the world incidence is increasing at an annual rate of 2%.[54] We feel this case history establishes that VHL diagnosis should be confirmed, wherever possible, by molecular genetic analysis and particularly in situations where the patient concerned exhibits only a limited spectrum of VHL features.

4.2.4 Genotype-phenotype correlations in VHL disease: do they exist?

Our studies support the well-established observation in the literature that there is a relationship between the presence of a VHL germline mutation and the occurrence of one or more VHL-related tumours. In chapter 3 (sections 3.1 and 3.2) however, we provided evidence for reduced or even non-penetrance of some VHL germline mutations. Two missense mutations (P81S and R64P), that were not associated with a genetic polymorphism by screening 50 non-VHL patients, suggested non-penetrance in some older carriers of the VHL germline mutation concerned. In four earlier reported cases of the P81S mutation a higher penetrance is described,[34,55,56] and may indicate the presence of modifying genes that influence the expression of VHL disease. In my opinion however, these observations of reduced or even non-penetrance of VHL germline mutations have no substantial implications for the clinical practice; carriers of such a mutation can not be relieved from clinical surveillance of VHL-related tumours. At most, the frequency of monitoring can be lowered at an older age.

Earlier observations suggested that almost all families with phaeochromocytoma were associated with missense mutations. We confirmed the recent observation that missense mutations were found in 30% of the VHL type I (without phaeochromocytoma) families and in 71% of the VHL type II (with phaeochromocytoma) families, which is significantly higher than the proportion of missense mutations (~40%) in all VHL germline mutations. Apparently, germline VHL mutations that are associated with a loss of function, such as partial deletions or micro deletions, also predispose carriers of such a mutation to the occurrence of phaeochromocytoma. We hypothesise that, with respect to the dominant negative model - proposed for the association between specific missense mutations and the occurrence of phaeochromocytoma - one ‘hit’ may be sufficient for the initiation of phaeochromocytoma tumourigenesis. This hit will probably concern a specific missense mutation, which occurs either in the germline or as a somatic mutation. Since oncogenesis is a multi-step process, other genes must also be involved in tumourigenesis, otherwise every carrier of such a missense mutation would develop phaeochromocytoma. In addition, one would expect also very young patients with phaeochromocytoma. The involvement of other genes is also supported by: (1) the absence of VHL germline mutations in some patients with multiple, bilateral or familial phaeochromocytoma, and (2) the low frequency of somatic VHL mutations in sporadic patients with solitary phaeochromocytoma, in contrast to renal cell carcinoma and CNS haemangioblastoma (Fig. 4).

We conclude that there is no apparent simple relationship between a germline mutation in the VHL gene and the manifestation (age of onset and type) of VHL-related tumours. Since the germline mutation in the VHL gene is most commonly

defined as the genotype, we would question whether it is appropriate to speak of genotype-phenotype correlations, especially since it is now appreciated that many genetic events and environmental factors are involved in the mechanism of oncogenesis. Moreover, these observations do not enable the conversion of the risk for specific VHL-related tumours - associated with a particular VHL germline mutation - to individualised monitoring of VHL disease.

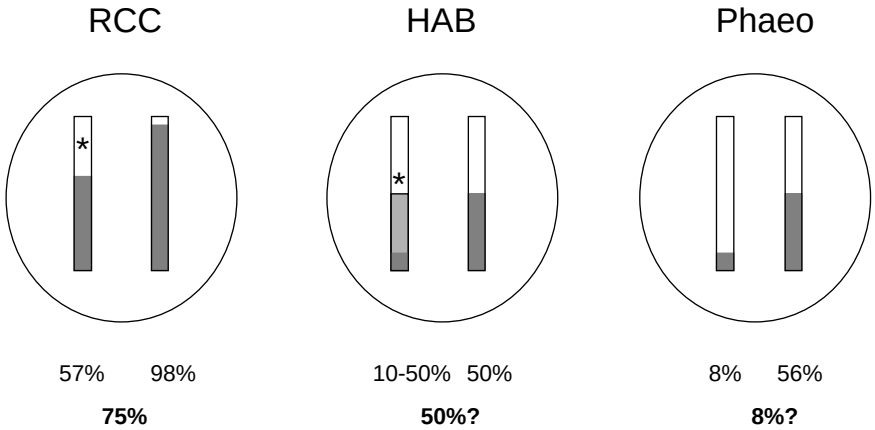

Fig. 4 Somatic mutations in solitary VHL-related tumours from sporadic (non-VHL) patients. Left-hand bars represent the frequency of somatic mutations in the VHL gene, bars on the right represent the frequency of loss of the wild-type allele (i.e. loss of heterozygosity). The frequencies in bold represent the (minimal) percentages in which both VHL alleles are inactivated. RCC, renal cell carcinoma;[57] HAB, CNS haemangioblastoma;[58-60] Phaeo, phaeochromocytoma.[61,62] In sporadic RCC and HAB, hypermethylation (*) plays a role in 19-33% of the tumours.[62,63]

External risk factors for the development of VHL-related tumours

VHL patients display both inter- as well as intrafamilial differences in manifestations of renal tumours. This variation may be caused by differences in the timing of origin, and nature of the somatic mutation. In turn, these somatic mutations may be induced by environmental factors, e.g. smoking. A current opinion is that smoking (particularly in males), obesity, hypertension and certain professions are correlated with the development of renal cell carcinoma (see 1.5.6). In this respect, it may be coincidence that in a family living near a nuclear power station, we observed early (<30 years) and aggressive (metastases) renal cell carcinoma in seven members of a VHL family with twelve patients. However, no further data are available, either in our study or the literature, to substantiate the effects of smoking and/or nuclear radiation on the induction of tumours in patients with VHL disease. We recommend collecting more data on past smoking habits as well as the working and living conditions of VHL patients, in order to investigate putative variances in multi-centricity, aggressiveness, and age of onset of renal cell carcinoma. These data should be compared to the manifestations of renal cell carcinoma in VHL patients who do not smoke and who have different working and living conditions. In retrospect, it is a pity that we did not make use of the opportunity to question our patients in a consisting fashion as to their smoking habits and other environmental influences.

Radiation risk

We advocate annual monitoring of VHL lesions with MRI and ultrasound rather than CT (section 2.1). Ionising X-rays emitted by CT may mutate the DNA of the second (non-mutated) VHL allele. However, there are no data available showing that exposed carriers of a germline mutation in the VHL gene develop tumours earlier than non-exposed patients. So far, there are only limited data available on tumourigenic radiosensitivity in cancer-prone genetic conditions. A review of epidemiological, clinical and experimental data suggested a ten times greater-than-normal risk after radiation in heterozygous patients with breast cancer.[64] It is prudent, in my opinion, to regard VHL as a disease where carriers of a VHL germline mutation have an increased risk for tumours, since tumourigenesis may be initiated with only one somatic hit. However, in view of the multi-step mechanism in tumourigenesis, it is still an open question whether there is a linear dose-effect relationship between radiation and tumourigenesis via induced mutations.[65]

4.2.5 Comparison of VHL disease with other hereditary cancers

At first glance, VHL disease represents an extraordinary form of hereditary cancer. Since VHL disease is classically defined as a composite of different tumours in different locations, by definition, a sporadic VHL patient can only arise from an inherited mutation. We think that screening procedures for germline mutations in VHL disease are going to have a relatively larger effect in reducing morbidity and early mortality than for other forms of hereditary cancer, in particular hereditary breast cancer (HBC) and hereditary colorectal cancer, including hereditary non-polyposis colorectal cancer (HNPCC). In the latter conditions, the chances of detecting a germline mutation in the respective disease gene(s) is generally much smaller than in a sporadic VHL patient. Although, in absolute quantities the morbidity and lethality prevented by DNA diagnosis for HBC and HNPCC is larger than for VHL disease. The DNA diagnostic load is several orders of magnitudes higher and the identified number of germline mutations relative to all patients investigated much lower. In addition, there are several other factors that should be taken into account when comparing VHL disease to other hereditary cancer susceptibilities.

Firstly, VHL disease is a disorder characterised by tumours in many different organs. In contrast, in hereditary breast cancer and hereditary colorectal cancer, tumours mostly occur in a single, major target organ. The sporadic or non-inherited counterparts of these tumours occur at a relatively high frequency in the general population. Consequently, there are many families in which a high incidence of breast or colorectal cancer is, in fact, due to chance clustering of sporadic tumours. In many cases it is difficult, if not impossible, to distinguish such families from families with a hereditary predisposition. Because the individual VHL-related tumours are far less common, sporadic patients with a solitary VHL-related tumour are more easily distinguished from patients with VHL disease who either display multiple tumours in various organs or have a positive family history for VHL-related tumours. Moreover, sporadic cases of classic VHL disease arising from somatic mutations of both VHL alleles in multiple target organs are extremely unlikely to occur.

Secondly, VHL disease is a genetically homogeneous disorder with respect to well-defined VHL patients, whereas it is well known that HBC and HNPCC display genetic heterogeneity. No matter how strictly one defines the eligibility criteria for molecular genetic analysis of HBC and HNPCC families, or how extensively mutation analysis is carried out, BRCA1/BRCA2 and MLH1/MSH2 germline mutations will probably never be detected in 100% of the affected families.

Thirdly, a major difference between mutation studies of the VHL gene and of those for HBC and HNPCC is that VHL analysis is facilitated by the small size of the gene, allowing direct sequencing to detect alterations. Moreover, large deletions (which represent a major proportion of the mutations) can be efficiently identified. In contrast, BRCA1, BRCA2, as well as the genes responsible for HNPCC, are large, complex genes, which preclude comprehensive mutation analysis by sequencing in a routine diagnostic setting, at least for the time being. Usually, less sensitive, pre-screening methods are used for the analysis of these genes, resulting in a lower detection rate.

In short, DNA diagnostic procedures are much more likely to make significant inroads into combating VHL disease in it’s entirety within the Dutch population than is the case for many other inherited tumours.

4.3 Detecting persons at risk for VHL disease

In the Netherlands there has been an estimated 30-fold increase in cancer-related genetic counselling over the last eleven years.[66] The rising number of cancer genetics referrals could be due to an increasing awareness of doctors and patients. On the other hand, in some hospitals and areas in the Netherlands hereditary cancer may have been under-recognised in the past and suggests that information on hereditary cancer has not been adequately provided or 'consumed'.[66]

In order to detect new families and patients possibly affected with VHL disease, we took several initiatives (see 1.1.1). The five lines resulted in an increase in the number of probands with VHL disease as well as sporadic patients with VHL-related tumours referred to the Department of Medical Genetics in Utrecht (Fig. 3). The request to the Departments of Ophthalmology resulted in the referral of one VHL family. The appeal in the journal of the Dutch Association for Neurology resulted in the referral of 14 probands with haemangioblastoma in the central nervous system, of whom two had multiple tumours. The 80 medical specialists and the eight genetic centres resulted in the referral of 12 VHL families, seven sporadic patients with VHL disease, 17 patients with haemangioblastoma in the central nervous system (of whom two had multiple tumours), 24 patients with phaeochromocytoma, one with renal cell carcinoma, and one with retinal haemangioblastoma. Moreover, the genetic centres are in contact with at least another 15 VHL families that have not undergone DNA analysis so far. Via information on the Internet, three VHL families contacted the Dutch VHL support group and asked for DNA analysis.

A drawback in the detection of new VHL patients was the varied co-operation of their doctors. We encountered difficulties in reaching patients through their attending physicians or clinical geneticists. Much time and effort was invested in convincing some doctors of the purpose of this study. A few were opposed to approaching patients, others to the methods used for collecting or storing the clinical and genetic data. We also experienced difficulties in collecting clinical data on VHL patients. For example, some institutes were not willing to hand over data upon the patients' individual informed consent or they charged for sending copies of the patients' clinical notes. In contrast, the patients themselves were favourably disposed to the research project. People at risk for the disease came forward, on their own initiative, at meetings of the VHL support group or via the information found on the Internet. Most (97%) of the patients we approached to sign the informed consent form kindly co-operated and gave us permission to retrieve their clinical data. In the last phase of the study, the collecting and storing of data was speeded up with the help of two genetic assistants.

4.3.1 Evaluation of methods of detecting persons at risk for VHL disease

Individuals with a predisposition for an inherited tumour syndrome such as VHL disease are most likely to benefit from early identification of the disease followed by periodic clinical monitoring. Potentially life-threatening tumours can be removed at an early stage and may improve both prognosis and life expectancy. These advantages of early identification are the ones most commonly mentioned, but these should be weighed against the negative consequences of approaching patients. An active ap-

proach in detecting persons at risk for VHL disease raises the problem of intruding into their privacy and provoking feelings of threat. However, there is the problem of the responsibility and liability of doctors performing insufficient or incomplete investigations, and/or treatment of identified VHL patients and persons at risk for the disease.

Active versus passive methods for patient detection

An active approach towards discovering new patients in a population is likely to result in a higher number of detected patients than a passive approach. But an active or even aggressive approach could result in emotional problems for persons at risk for the disease (see 4.3.2). Patients and relatives are confronted with the disease in all its aspects. For example, they are faced with the death of their parents and close relatives, the fear of losing other relatives and especially their children, or developing life-threatening tumours themselves.[66] This burden is well expressed by the Dutch saying: "one suffers most from the suffering one fears" (*men lijdt het meest, van het lijden dat men vreest*).

A passive approach is likely to result in more undiagnosed patients and in a higher rate of morbidity and mortality among VHL patients. One advantage is, however, that persons at risk for the disease are not offended or threatened by doctors and researchers convincing them to undergo clinical and genetic tests. This non-directive approach leaves persons at risk for VHL free to make their own choice, *if* and *when* they are prepared to consult their doctor.

The starting point in solving this dilemma is, in my opinion, that the motivation and initiative for both DNA analysis and clinical monitoring should come from the person at risk. Therefore VHL families and persons at risk for the disease should be provided with clear oral and written information about the clinical symptoms and signs, complications, treatment, prognosis and inheritance of the disease in order to prevent both patient and doctor delaying a request for diagnosis or treatment.[67] Persons should be reached by non-threatening media such as information booklets handed over by family members, general practitioners or medical specialists, and via the Internet (www.vhl.org). In addition, doctors should be provided with information on the disease and on hereditary forms of cancer in general. This approach of spreading information as much as possible will certainly take more time, but it is likely to be more effective in the end, and it also keeps a balance between the over-active approach to persons at risk and the doctors' responsibility to guarantee health care.

Family support groups

The Dutch VHL support group provides patients and their relatives with information from experts in the field, emotional support (companionship in distress), and organises discussions of common interests (about ethical, legal and social aspects, such as employment and insurance) guided by a psychologist. The Dutch support group was founded in 1996, following the American VHL Family Alliance that was the first to be initiated in 1993. The VHL Family Alliance provides information for families and physicians about this disorder and local self-help support groups for families affected with VHL. Volunteers, mostly families living with VHL, health professionals, and interested friends, run the Alliance.

Most of the information on VHL disease is now published and edited by various family support groups. Local family support chapters exist in many states in the USA, and more are being formed. Local chapter meetings are scheduled periodically in several states. International VHL Support Organisations have been established in Australia, New Zealand, Britain, Canada, Denmark, Germany, France, Italy, Belgium and the Netherlands. The American VHL Family Alliance is in touch with nearly 6,000 affected people in 27 countries, the latest additions being from Brazil and Nepal.

4.3.2 Detection of VHL patients and presymptomatic persons; psychological distress

Hereditary diseases in general and hereditary forms of cancer in particular, harbour more far-reaching consequences than other 'apparently ordinary' diseases. Genetic testing not only provides information of carriership to the person tested, but also affects family members that may or may not want to receive this information. So far, no studies have been undertaken to investigate the psychological consequences of gene testing in VHL disease. However, in many respects multiple endocrine neoplasia type 2A (MEN 2A) is comparable to VHL disease and it may be reasonable to assume that many of the patients responses described for MEN 2A will also be applicable to VHL disease.[68,69] In addition, data are available from studies on other hereditary forms of cancer such as breast and ovarian cancer, and familial polyposis.[70-74]

Data from MEN 2A studies suggest that some VHL patients undergoing presymptomatic DNA testing will experience increased levels of psychological distress (F. Grosfeld, personal communication). In particular, elevated pre-test anxiety, poor socio-economical background, lack of information, young age, individual tendency towards anxiety and depression, and a defensive style of coping, are risk factors for psychological complaints. Caution is therefore warranted towards a directive approach in offering DNA tests, since this is also likely to reach persons that are more vulnerable to psychological distress.

In MEN 2 patients an unfavourable test outcome resulted in anxiety and depression but also relief.[68] Anxiety and depression may come from concern about how the test outcome may affect the future of the carrier or their family.[71,75] The feeling of relief derives from the benefits of this outcome.[70,71,73] The options of close clinical monitoring and preventive treatment help to reassure carriers that they had made the right decision.

A favourable test leads, in most applicants and partners, to relief, especially because of the health benefits for their offspring.[68] On the other hand, a favourable test may also lead to worry, some non-carriers may feel guilty ('survivors' guilt) and isolated from their families.[76] In addition, some applicants may have planned their lives accordingly, acting as if they in fact have the disease (e.g. with low expectations for a career). A favourable test outcome can be difficult for them to accept. Suddenly, the applicant has to reject his or her old philosophy of life (living in fear of the disease) and has to build up a new concept of life, without the threat of the disease.[77] In addition, they face the same responsibilities as any other healthy member of society. This may cause depression or even suicidal behaviour.

4.3.3 Prenatal and infantile DNA analysis

Since curative treatment is impossible in VHL, arising tumours should be treated electively but preventive (bilateral) removal of organs such as kidney, adrenal glands or eyes, is not a desirable option in VHL patients. Therefore, the detection of VHL carriership brings a life-long and close monitoring for multiple and recurrent tumours. Understandably most parents wish to have an unaffected child.

At present, there are technical possibilities for prenatal diagnosis, i.e. chorion biopsy, amniocentesis or pre-implantation genetic diagnosis (PGD) following in vitro fertilisation (IVF).[78] When future parents decide to perform chorion biopsy or amniocentesis and the result indicates VHL carriership of the foetus, they can choose to terminate the pregnancy. When parents decide to have PGD performed, the embryos can be checked for carriership before implantation. Recently, the Dutch National Health Council has decided that inclusion criteria for PGD are, in principle, similar to accepted medical indications for prenatal diagnostics (with the exception of tests based on maternal age).[79] Since VHL is a Mendelian inherited disease this opens the door towards PGD. So far, one patient has requested PGD in the Netherlands (and one in Belgium).

After the Sixth International Workshop on multiple endocrine neoplasia (MEN) and VHL in 1997, in Noordwijkerhout, the Netherlands, an opinion poll was held about PGD among 76 medical professionals (Table 4).[80] The results showed that both prenatal and presymptomatic diagnosis remain controversial or may suggest that the participants are unaware of the pros and cons of PGD. In my opinion, this issue has to be solved in individual encounters between parents, clinical geneticists and attending physicians.

In VHL patients, tumours, especially retinal haemangioblastoma, may occur at an early age. Symptomatic lesions, even leading to unilateral or bilateral blindness have been reported before the age of five years old. (Third International VHL symposium, Paris, France, personal communication).[6,81] Early detected haemangioblastoma in the eye are an outstanding example of how presymptomatic and preventive treatment of VHL disease should work. Because of the chance of early disease symptoms manifesting in the eye, genetic testing of young people with a high risk of VHL disease must be carried out as early as possible, in order to be fully prepared to treat the disease if it occurs. Unfortunately, this position is not supported by every clinical genetic centre in this country. One can not refrain from observing that this is a position which would not be supported in the US, where the chance of legal action against medical practice failing to carry a timely DNA diagnosis is much higher than in the Netherlands.

4.3.4 Financial and legal consequences

Unfortunately, some VHL patients have difficulty in obtaining insurance, applying for a job, or getting a mortgage. Legal and financial issues involved in cancer genetics differ between countries. The Dutch situation described here is adapted, with permission, from Dr. R.H. Sijmons.[66] DNA testing for hereditary disorders is restricted by national law to the non-commercial clinical genetics centres. The health insurance companies cover the costs of clinical genetic services, as well as periodic monitoring

Table 4 An opinion poll* on pre-implantation genetic diagnosis (PGD) and in vitro fertilisation (IVF).

'If a specific mutation in the VHL gene is present in an affected family member, at what age would you advise having DNA analysis performed in his/her offspring?'	
5%	prenatally
15%	at birth
36%	between 1 and 5 years
15%	between 5 and 10 years
8%	at the start of the periodic clinical examination
18%	at an age that the children can make their own choice (puberty)
3%	if symptoms are present

'Would you advise IVF and PGD?'	
42%	'never'
32%	'yes, for MEN 1, and MEN 2, as well as VHL
13%	'yes, only for MEN 1 and VHL (because MEN 2 may be cured)'
13%	'yes, only for VHL

*This opinion poll was held among 76 medical professionals after the Sixth International Workshop on Multiple Endocrine Neoplasia (MEN) and VHL in 1997, in Noordwijkerhout, the Netherlands.

and prophylactic surgery in individuals with a high genetic cancer risk. Because of a self-imposed restriction, these companies do not ask for disclosure of the DNA test results when selling life insurance or disability insurance, unless the amounts to be covered by a policy are higher than average (respectively, Euro 150.000 and Euro 30.000). Employers are prohibited by law from asking their new employees for information on genetic diseases.

As the knowledge about the genetic basis of disorders grows, so does the potential for discrimination in health insurance coverage. Insurance companies with genetic information could convert current risks for certain customers into demands for higher contributions from persons with a predisposition to a hereditary disease. In addition, they could also deny health insurance to such persons and thereby limit their own risk. The fear of discrimination has other undesirable effects. People may be unwilling to participate in family investigations, including DNA diagnosis, and to share information about their genetic status with their attending doctors or family members because of concerns about misuse of this information.

Genetic information has already been used in discrimination. In the early seventies, some insurance companies denied coverage and charged higher rates to African Americans carrying the gene for sickle cell anaemia.[82] This has also been reported in American patients with Huntington's disease.[83] In a study from Georgetown University 22% of the people with a known genetic condition in the family indicated that they had been refused health insurance coverage because of their genetic status, whether they were affected or not.[82]

We consider using genetic information, to exclude persons from insurance coverage, represents genetic discrimination. In 1995, the National Action Plan on Breast Cancer and the working group on Ethical, Legal, and Social Implications of the Humane Genome Project drew up recommendations to protect people against genetic discrimination. Insurance providers should be prohibited from: (1) using genetic information to deny or limit any coverage; (2) establishing differential rates or premium payments based on genetic information; (3) requesting or requiring collection or disclosure of genetic information; (4) releasing genetic information without prior written authorisation of the individual.[82] VHL disease should be considered, along with many other inherited disorders, in preventing genetic discrimination and protecting the interests of these patients concerning insurance and work (see also 4.2.4).

4.3.5 Cost-effectiveness of early detection

Genetic screening of individuals presenting with clinical features suggesting VHL disease facilitates confirmation of the diagnosis, accurate genetic counselling and clinical monitoring of at risk family members. The necessity for costly and time-consuming monitoring programs can be reduced and surveillance directed at those carrying the mutation.[84] Assuming that the aim of a screening test is to detect a genetic disease and allow therapeutic intervention that reduces morbidity and mortality, the real 'cost' of the test represents the total cost of treating the disease and ultimately saving a life (i.e. the 'cost-benefit' of the test).[85] 'Cost-benefit' is a term derived from the financial sector. In a medical context the term 'cost-effectiveness' is preferred, because the outcomes are seen not only from a financial point of view, but also from looking at the consequences that accumulate for an individual and society.[86,87]

Beforehand, we would like to state that it is exceedingly difficult to calculate costs and effectiveness of early detection and we emphasise that further investigations are required. In order to calculate the cost-effectiveness of detecting patients with VHL disease by DNA analysis, we start from the 100 VHL patients that have been identified so far (section 3.1). Although we report a disease prevalence in the Netherlands of ~ 1:64,000, in this model we assume that a prevalence of 1:40,000. The hypothetical Dutch VHL population would therefore comprise 400 patients. We estimate that the remaining 300 patients are identified in 200 first- and 800 second-degree family members who are at 50% and 25% risk for the disease, respectively. This results in an estimated population of 1100 patients (100 + 200 + 800) to be monitored for VHL disease each year, while only 400 persons would, in fact, have VHL disease. The cost of annual VHL monitoring is estimated at Euro 525 per patient and DNA analysis could consequently save Euro 367,500 each year (Table 5).

At the moment, the actual costs are likely to be much higher, because symptomatic patients needing expensive and extensive treatment are still being diagnosed at an advanced stage of the disease. Moreover, it seems likely that identification of VHL carriership by DNA analysis (cost of test is Euro 600) is not only cost-effective but may also save lives. We recommend that clinical criteria for DNA analysis, as defined in section 4.2.1, should therefore include both patients meeting the VHL diagnostic criteria as well as sporadic patients with solitary VHL-related tumours, in order to identify as many VHL mutation carriers as possible.

Table 5 Technical assessment of clinical monitoring of persons at risk for VHL

'Test'	**Costs in Euro**	**'Test'**	**Costs in Euro**
Consultations		Radiological monitoring**	
Ophthalmologist	35	MRI CNS and abdomen	390
Neurologist	80	Ultrasound abdomen	70
Internist	80		
	195		230
Biochemical urine tests	75	Blood tests**	25
ureum		blood count	
creatinin		creatinin	
VMA		ureum	
norepinephrine		Na, K, Cl, P	
metanephrine			
adrenaline			
noradrenaline			
Total costs of annual clinical monitoring		525	
Costs of monitoring 1100 persons		577,500/year	
Costs of monitoring 400 persons		210,000/year	
Savings		367,500/year	

This table contains figures from the UMC Utrecht and may not be representative for other Dutch hospitals.
* The cost for a biochemical test is calculated in points representing approx. Euro 0.8 (Euro 1 ~ NLG 2.20).
** An MRI of the CNS (including spine) is made every two years, and also depicts the upper abdominal organs. Therefore, ultrasound and MRI are used alternately each year in monitoring VHL patients.

4.4 Future perspectives

From the VHL families identified, we need to collect detailed clinical data of long and extensively monitored patients with complete DNA analyses. With these clinical data we could investigate:

- the natural history of VHL disease,
- new entities (VHL-related tumours) within VHL disease,
- genotype-phenotype correlations,
- cost-effectiveness rates of early detection and periodic clinical monitoring.

The identified VHL families should be investigated using pedigree analysis to detect further carriers of germline mutations in the VHL gene. Importantly, this would allow us to make a more reliable estimate of the prevalence of VHL disease in the Netherlands.

VHL disease seems to be a genetically homogeneous disorder from the perspective of large and well-defined families, but this leaves unanswered which molecular genetic mechanism accounts for the patients and families having some VHL characteristics but who do not carry a germline mutation in the VHL gene. Therefore, data on families that are affected by one of the tumours from the VHL spectrum, and in particular those suggesting a germline mutation (with multi-centric, bilateral, familial tumours, or a young age at diagnosis), should be collected. These families should be investigated by linkage studies in order to identify other candidate genes that are involved in the tumourigenesis of VHL-related lesions.

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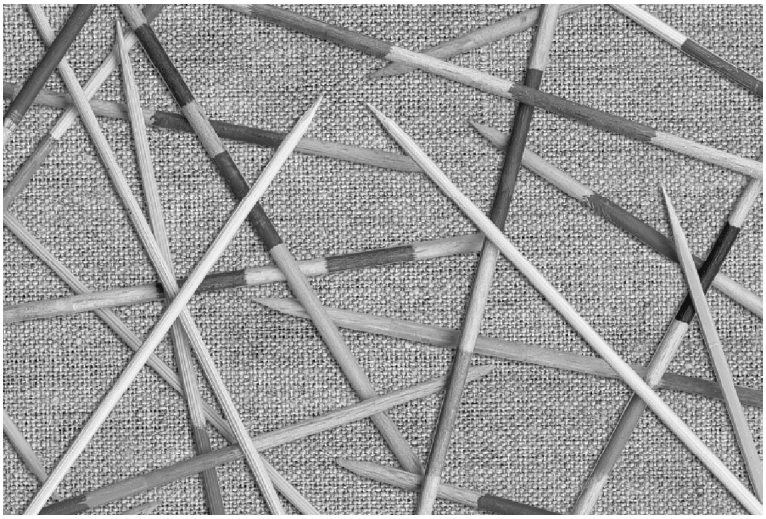
Summary



Chapter 1, objectives and general introduction

Chapter 1 is the introduction to this thesis and describes the main objectives and methods. It provides an overview of the history of VHL disease, as well as the clinical and molecular genetic aspects of the disease. In addition, the VHL protein and its possible functions are reviewed.

Clinical picture

Von Hippel-Lindau (VHL) disease is an autosomal, dominant inherited tumour syndrome. A germline mutation in the VHL gene predisposes carriers to tumours in multiple organs. These tumours may include haemangioblastoma in the retina and central nervous system (CNS), renal cell carcinoma, phaeochromocytoma, islet cell tumours of the pancreas, and endolymphatic sac tumours (ELST), as well as cysts and cystadenoma in the kidney, pancreas, epididymis and broad ligament. The estimated prevalence of the disease varies between 1:31,000 and 1:53,000 persons. The disease is named after the German ophthalmologist Eugen von Hippel, who described retinal haemangioblastoma in 1904, and the Swedish pathologist Arvid Lindau who associated retinal and CNS haemangioblastoma with cysts of the kidneys, pancreas and epididymis in 1926. Most tumours in VHL patients are multicentric or bilateral, and manifest at a younger age than in patients without a VHL germline mutation. The mutation spectrum is heterogeneous, with mutations scattered throughout most of the VHL gene. Although some recurrent mutations have been reported, most families have their own unique germline mutation.

Penetrance of VHL disease is high, most carriers of a VHL germline mutation develop one or more tumours by the age of 60 years. The most common symptoms include: loss of vision, raised intracranial pressure, neurological deficits, paroxysmal raised blood pressure and local pain. The median expected survival, based on life table analysis, has been estimated at 49 years. At present, metastases from renal cell carcinoma and neurological complications from cerebellar haemangioblastoma are the most common causes of death in VHL disease. However, it is anticipated that intensive radiological and clinical monitoring, and advanced operation techniques will reduce both morbidity and mortality.

Objectives of this thesis

The main objective of this thesis is to identify patients and families with VHL disease by molecular genetic analysis. New families with VHL disease can be found by screening patients with VHL-like tumours (with a positive or a negative family history) for germline mutations. Identification of a VHL germline mutation confirms the clinical diagnosis. Presymptomatic DNA analysis and identification of carriers of VHL germline mutations in families then permits tumour development to be followed from a relative early age, and optimises the time at which treatment is carried out. Since clinical surveillance can be specifically directed towards carriers of a VHL germline mutation, the cost-effectiveness of annual monitoring is expected to improve. Moreover, tested individuals are no longer uncertain regarding their risk for developing the disease and family members who are non-carriers are relieved of the burden of repeated clinical monitoring.

Patients and methods

In order to detect new VHL families and patients, we took several initiatives to increase the number of persons possibly affected with VHL disease being referred for DNA diagnosis. Firstly, we asked the Departments of Ophthalmology in the university hospitals for patients with retinal haemangioblastoma. Secondly, we put an appeal in the journal of the Dutch Association for Neurology asking for patients with haemangioblastoma in the central nervous system. Thirdly, we approached approximately 80 medical specialist (including clinical geneticists, internists, endocrinologists, urologists, surgeons, neurosurgeons and paediatricians) with a known interest in hereditary tumour syndromes. Fourthly, we contacted the eight genetic centres in the Netherlands and fifthly, we distributed patient information via the Dutch VHL support group and the Internet.

DNA analysis for VHL in the Netherlands is performed at the Department of Medical Genetics, UMC Utrecht and in the Department of Clinical Genetics, Rotterdam University Hospital. DNA analysis included sequencing of the coding region and quantitative Southern blot analysis, complemented by Fluorescence in situ hybridisation (FISH) analysis when necessary. Clinical data were collected after patients had signed an informed consent form.

Summary of objectives

1. To detect VHL families and determine the family-specific germline mutation.
2. To identify presymptomatic relatives who carry a VHL germline mutation.
3. To screen for VHL germline mutations in patients with a single VHL-related tumour and without a distinct family history.
4. To improve DNA analysis techniques in identifying germline mutations in families where no mutation could be detected.
5. To collect clinical and genetic data to identify possible genotype-phenotype correlations.
6. To formulate national guidelines for diagnosis and periodic monitoring of VHL patients.

Chapter 2, clinical investigations

This chapter focuses on clinical aspects of VHL disease and describes radiological techniques (2.1) and guidelines for diagnosis and monitoring of the disease (2.4). Two organs that are involved in VHL disease are discussed in more detail, the kidney (2.2) and the eye (2.3).

Imaging of renal-, adrenal- and pancreatic masses

Section 2.1 reviews developments in the imaging of renal, adrenal and pancreatic masses in VHL disease. The imaging of other organs involved in VHL disease is described in chapter 1. Radiological imaging may favour early detection and monitoring of VHL-related lesions and is likely to lead to a reduction of morbidity and early mortality. Ongoing follow-up by careful radiological monitoring with ultrasound and especially MRI (magnetic resonance imaging) plays a central role in managing the disease.

We advocate annual monitoring of VHL lesions with MRI and ultrasound rather than CT (Computed Tomography). Ionising X-rays emitted by CT may mutate the DNA of the second (non-mutated) VHL allele. It is prudent to regard VHL as a disease where carriers of a VHL germline mutation have an increased risk for tumours, since tumourigenesis may be initiated with only one somatic hit.

Management of renal cell carcinoma

Since renal cell carcinoma occur often multiply and bilateral in carriers of a VHL germline mutation, a choice has to be made between careful radiological monitoring, nephron-sparing surgery and nephrectomy. This decision depends on size, growth and biological behaviour of renal tumours.

Renal cell carcinoma in our patients showed a slow growth rate (on average 0.3 cm/year) and asymptomatic patients presented with tumours of low-grade malignancy. In all patients, a fibrous pseudocapsule surrounded tumours. In five of 17 tumours, pseudocapsular invasion was observed and three of the five tumours had broken through the pseudocapsule. These patients did not show a less favourable outcome than those without pseudocapsular involvement by tumour growth. Multicentricity of renal cell carcinoma was relatively low (4.6 lesions per kidney). In two of the three patients that underwent a nephrectomy, only a single satellite lesion, in the direct vicinity of a renal cell carcinoma, was found in one kidney. Six tumours (1.8-5.5 cm) were enucleated by nephron-sparing surgery. During a mean follow-up of 30 months, renal function in these patients was well preserved. We concluded that, in our patients, renal cell carcinoma grew slowly, were of low grade, had a dense fibrous pseudocapsule and were hence good candidates for nephron-sparing surgery.

Ocular haemangioblastoma

Haemangioblastoma are the most common and early occurring tumours in VHL disease. In the eye the typical lesion is the peripheral retinal haemangioblastoma. Most ocular haemangioblastoma occur peripherally and 8% occur on the optical disc.

We describe long-term follow-up, ophthalmological data from 20 patients from six families, with special attention to the natural course of ocular haemangioblastoma. Five stages of the natural course of development of ocular haemangioblastoma are discerned and illustrated by fluorescein angiographic pictures. The patient from family C is an example of the ability of DNA analysis to find cases of VHL disease with a negative family history. After five years of extensive clinical monitoring, retinal haemangioblastoma are still the only manifestations of VHL disease. We advocate that only early detection and treatment of peripheral retinal haemangioblastoma can be expected to decrease the percentage of patients with impaired visual acuity. Since ocular haemangioblastoma are early tumours in carriers of a VHL germline mutation, ophthalmological monitoring (and subsequent treatment) of VHL patients and persons at risk should start as early as possible.

Guidelines for diagnosis and monitoring

The Dutch VHL Working group presents guidelines to enhance the early detection and treatment of VHL patients in the Netherlands.

For diagnosing VHL disease in a patient, both clinical manifestations and family history are important. Typical tumours that are associated with VHL disease are: retinal and CNS haemangioblastoma, phaeochromocytoma, renal cell carcinoma, ELST and multiple pancreatic cysts. Multiple pancreatic cysts are specific for VHL disease because they are rare in the normal population. In contrast, renal or epididymal cysts occur more often in the normal population. In the presence of a positive family history, VHL disease can be diagnosed in a patient with a typical VHL tumour. In the absence of a VHL family history, two or more haemangioblastoma, or a haemangioblastoma combined with a further typical VHL tumour are required.

Clinical diagnosis of VHL disease can be confirmed by molecular analysis of the VHL gene and is informative in virtually all classic VHL families (families with multiple tumours) and classic sporadic VHL-patients (individuals with multiple VHL-related tumours).

Guidelines for clinical monitoring of VHL patients are presented. This monitoring protocol is recommended for carriers of a VHL germline mutation; members of VHL families with an unknown familial mutation; members of VHL families who decline testing for the familial mutation; and patients suspected of having VHL disease but without a VHL germline mutation.

Chapter 3, genetic investigations

Chapter 3 focuses on the clinical genetic and molecular genetic aspects of VHL disease. Section 3.1 describes VHL germline mutations and section 3.2 focuses on five families with a germline deletion of the VHL gene. Sections 3.3 and 3.4 report on case findings of VHL germline mutations in sporadic patients with a single type of VHL-related tumours.

VHL-germline mutations in the Netherlands

In the DNA laboratories of Utrecht and Rotterdam, VHL germline mutations were detected in 25 familial and seven sporadic VHL patients. We also identified VHL-germline mutations in two sporadic patients with VHL-related tumours who did not fulfil the current diagnostic criteria for VHL disease. Analyses of genotype-phenotype correlations were consistent with previous reports. Our study shows that *de novo* mutations represent at least 12% - and potentially 21% - of the germline mutations detected in the VHL gene in the present series, and provides evidence for non-penetrance and reduced penetrance of VHL germline mutations.

Five families with VHL germline deletions

We describe four families with partial deletions removing one or more exons of the VHL gene and one family with a deletion of the entire VHL gene. The deletions were detected by Southern blot analysis. In the fifth family, FISH analysis confirmed the deletion of the entire VHL gene. Our results showed that (quantitative) Southern blot analysis is a sensitive method for detecting germline deletions of the VHL gene and should be implemented in routine DNA diagnostics for VHL disease. Germline deletions in the studied patients were associated with a low risk for phaeochromocytoma and a preponderance of CNS haemangioblastoma.

Patients with CNS haemangioblastoma-only

We report on the frequency of VHL germline mutations in 88 patients from the United Kingdom (n=63) and the Netherlands (n=25), with only CNS haemangioblastoma. A VHL germline mutation was found in three (3.6%) of 84 sporadic patients with a single haemangioblastoma and in two (50%) of the four sporadic patients with multiple haemangioblastoma. We concluded that VHL gene mutation analysis should be offered to all haemangioblastoma patients younger than 50 years. Further data are required to evaluate the detection rate in late-onset cases. The fact that we did not find a VHL gene germline mutation in two of the four patients with multiple haemangioblastoma may indicate, next to coincidence, the presence of additional haemangioblastoma susceptibility genes or alternatively, somatic mosaicism.

Patients and families with phaeochromocytoma-only

We investigated the frequency of VHL germline mutations in Dutch patients with a phaeochromocytoma-only phenotype. A total of 24 probands (14 with solitary, seven with multiple, bilateral or recurrent, and three with familial phaeochromocytoma), were tested by molecular genetic analysis of the VHL gene. VHL germline mutations were not found in any of these probands with phaeochromocytoma, even when features suggesting a germline mutation - such as early onset, multiple, recurrent, bilateral or familial tumours - were present. However, the absence of VHL germline mutations in the phaeochromocytoma families may indicate the presence of additional phaeochromocytoma susceptibility genes. Since mutation analysis of the VHL gene detects germline mutations in virtually all well-defined VHL families, we conclude that annual clinical monitoring for further VHL-related tumours in patients with phaeochromocytoma and without a VHL germline mutation should not be recommended.

Chapter 4, discussion

In this chapter the principal findings of this research are discussed. This chapter focuses on the implications of the study and also provides conclusions and recommendations on some clinical and genetic aspects of VHL disease.

Carriers of a VHL germline mutation are predisposed for developing multiple tumours that often manifest at a relatively early age. In order to prevent both patient and doctor delay in the diagnosis of VHL disease, persons at risk for the disease as well as doctors should be provided with clear oral and written information about the clinical and genetic aspects of the disease. In addition, an intercentre co-operation should be established between the medical specialists involved to prevent unnecessary morbidity and mortality in patients with VHL disease. Multidisciplinary teams following national and international guidelines should guarantee the best results in the management of patients with VHL disease. A national VHL working group, led by a multidisciplinary board representing various institutes, has been established in the Netherlands to ensure uniform clinical management of VHL patients and families, as well as to carry out structural research projects. Clinical monitoring should be primarily organised around those VHL patients who have tested positive for a VHL germline mutation. To diminish the burden of frequent clinical surveillance, monitoring should

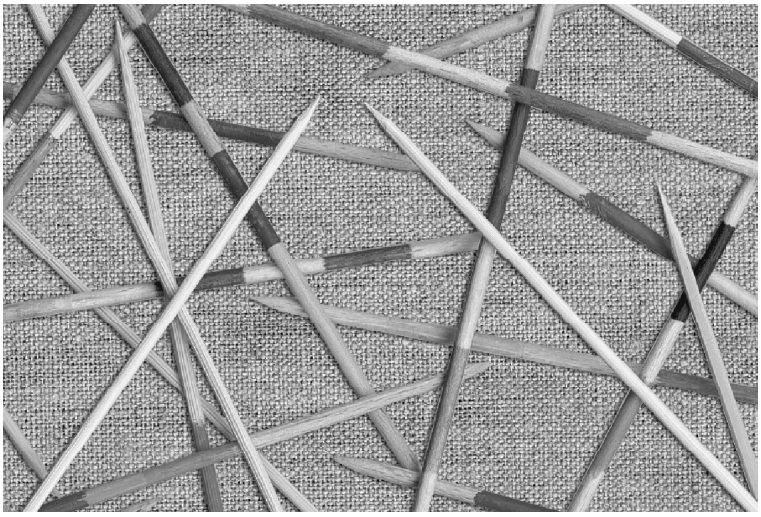
be organised in a production line fashion so that all the necessary tests can be carried out during one hospital visit.

Although VHL germline mutations are identified in 100% of the classic families and patients with VHL disease, we report here on patients and families who exhibit some VHL characteristics but who do not carry a germline mutation in the VHL gene. Patients with multicentric, bilateral or familial VHL-related tumours and without a VHL germline mutation could play a role in identifying genes that are involved in their specific tumourigenesis. We demonstrate genotype-phenotype correlations for some tumours in VHL disease, but there appears to be no simple relationship between a germline mutation in the VHL gene and the manifestation of VHL-related tumours. For example, there is intrafamilial variability in the age of onset and the manifestation of different types of VHL-related tumours. There is evidence that genetic factors (so called modifier genes) and environmental influences play an additional role in the clinical expression of VHL germline mutations. Furthermore, we provide evidence of reduced penetrance and non-penetrance of certain VHL germline mutations.

It is not possible to accurately define the prevalence of VHL disease in the Netherlands from this study because of particular selection biases and incompleteness of information. However, our rough estimate of 1:64,000 will almost certainly prove to be higher and, interestingly, *de novo* mutations occur in a considerable proportion of all the families we studied. We present reasons why this group of patients is underrepresented in our series and we demonstrate that *de novo* mutations represent at least 12% - and potentially 21% - of the VHL germline mutations detected in the Netherlands. In addition, we illustrated that VHL germline mutations can be identified in sporadic patients with VHL-related tumours who do not meet the current diagnostic criteria. These findings emphasise the importance of screening sporadic patients with one or more typical VHL-related tumours for germline mutations in the VHL gene. We suggest treating each patient suspected of having VHL disease, according to six categories we define here, with an open mind and performing: (1) an extensive pedigree analysis, (2) clinical screening for further VHL-related tumours, and (3) DNA analysis. Moreover, clinical situations leading to a suspicion of VHL disease should be eligible for DNA analysis in order to confirm, or exclude, the diagnosis of the disease since: (1) carriers of a VHL germline mutation and their relatives have a risk of developing multiple tumours; (2) molecular genetic analysis is readily feasible and identifies virtually all the classic VHL families and patients.

A principal finding of this study is that the early detection of VHL families and patients using molecular genetic analysis is effective, assuming that annual monitoring and timely treatment leads to a better prognosis for VHL patients. However, regarding the early detection of VHL patients, we observe that: (1) there is insufficient evidence of an improved quality of life or a longer life span; (2) there is no reliable analysis of the cost-effectiveness; and (3) the psychological consequences have not been studied sufficiently. These three observations should provide a basis for further clinical investigations. More extensive genetic research is indicated for clinical situations suggesting the presence of VHL disease, but without a VHL germline mutation.

Samenvatting



Hoofdstuk 1, doelstellingen en algemene inleiding

Dit hoofdstuk bevat de inleiding van dit proefschrift en beschrijft de doelstelling en methoden van het onderzoek. Tevens komen hierin de geschiedenis en klinische en genetische aspecten van het ziektebeeld aan de orde.

Ziektebeeld

De ziekte van Von Hippel-Lindau (VHL) is een erfelijke vorm van kanker waarbij verschillende organen kunnen zijn aangedaan. De ziekte is vernoemd naar de Duitse oogarts Eugen von Hippel die in 1904 vaatrijke afwijkingen van het netvlies beschreef en naar de Zweedse patholoog Arvid Lindau die deze oogtumoren in 1926 associeerde met tumoren in de kleine hersenen en de buik. Schattingen van de prevalentie van de ziekte lopen uiteen tussen 1:31.000 en 1:53.000. Bij mensen met een erfelijke aanleg voor deze ziekte kunnen hemangioblastomen (vaatrijke tumoren) ontstaan in het cerebellum (kleine hersenen), myelum (ruggenmerg) en retina (netvlies). Daarnaast worden ook de volgende gezwellen tot de typische VHL-tumoren gerekend: niercelcarcinomen (tumoren in de nier), feochromocytomen (tumoren in de bijnier), tumoren in het binnenoor en cysten in nieren, pancreas (alvleesklier), lever, en geslachtsorganen.

De meest voorkomende complicaties worden veroorzaakt door: hemangioblastomen van het netvlies (netvliesloslating, verminderd gezichtsvermogen of blindheid), hemangioblastomen van de kleine hersenen en/of ruggenmerg (gevoels- en/of krachtsverlies en/of pijn door verdrukking van kleine hersenen of zenuwen), tumoren in het binnenoor (oorsuizen en doofheid), niercelcarcinomen (lokale pijn), feochromocytomen (aanvalsgewijs verhoogde bloeddruk, met complicaties voor hart, nieren en hersenen) en pancreastumoren (onder andere: galwegobstructie, ontsteking van de pancreas en spijsverteringsproblemen). De meeste VHL-patiënten overlijden aan de gevolgen van niercelcarcinomen of van hemangioblastomen in het cerebellum. Door een ruimte-innemend gezwel in de kleine hersenen kan een gedeelte van het cerebellum inklemmen in het grote achterhoofdsgat en stijgt de hersendruk. De (levens)bedreigende gevolgen van niercelcarcinomen bestaan uit uitzaaiingen en infiltratie in het omliggende weefsel. Het niercelcarcinoom is, vanwege de zojuist genoemde eigenschappen, de enige kwaadaardige tumor die bij VHL-patiënten voorkomt. De overige VHL-tumoren worden als goedaardig bestempeld, maar kunnen desalniettemin (levens)bedreigend zijn.

De gemiddelde leeftijd waarop de ziekte zich manifesteert is voor retinale hemangioblastomen 25 jaar, feochromocytomen 28 jaar, cerebellaire hemangioblastomen 30 jaar en voor niercelcarcinomen 36 jaar. De gemiddelde levensverwachting van een onbehandelde VHL-patiënt wordt geschat op 49 jaar. Op 60-jarige leeftijd ontwikkelt bijna iedereen met een erfelijke aanleg voor de ziekte wel één of meer typische VHL-tumoren. De verwachting is dat vroegtijdige opsporing, periodiek klinisch onderzoek en behandeling van VHL-tumoren, vooral van hemangioblastomen en niercelcarcinomen, zowel ziekteverschijnselen als sterfte van VHL-patiënten zullen doen afnemen. Voor de operatieve behandeling van de meeste VHL-tumoren zijn nog geen definitieve richtlijnen opgesteld. Momenteel wordt in Boston (USA) onderzoek verricht naar medicijnen die de groei van tumoren bij VHL-patiënten kunnen afremmen of mogelijk zelfs kunnen verhinderen.

Erfelijkheid

Het menselijk lichaam is opgebouwd uit levende eenheden die we cellen noemen. Elke cel heeft een kern waarin zich 23 paren chromosomen bevinden. Eén chromosoom van zo'n paar is afkomstig van de vader en één van de moeder. De chromosomen bevatten de informatie voor alle erfelijke eigenschappen. Elk afzonderlijk chromosoom van een paar bevat informatie over dezelfde eigenschappen. De aanleg voor elke eigenschap is dus dubbel aanwezig.

Op de chromosomen liggen genen die, ieder voor zich, de informatie bevatten voor één erfelijke eigenschap. De genetische informatie is opgeslagen in DNA-moleculen. Het DNA van een bepaald gen codeert voor een eiwit, dat een specifieke functie in de cel vervult. Genen kunnen beschadigd raken. Men noemt dit een mutatie. Een gemuteerd gen werkt niet goed meer. Dit betekent dat eiwitten niet of slechts gedeeltelijk, hun specifieke taak in een cel kunnen verrichten.

Bij de gezonde mens zorgen de twee kopieën van het VHL-gen ervoor dat een cel zich niet ongeremd kan vermenigvuldigen. Men noemt het VHL-gen daarom een tumorsuppressorgen. Een kind dat een gemuteerd VHL-gen erft van een ouder zal dit gemuteerde gen in alle cellen hebben. We noemen dit een kiembaanmutatie. Het feit dat iemand drager is van een kiembaanmutatie wil niet zeggen dat alle cellen zich ongeremd zullen gaan delen. Omdat genen in paren voorkomen, is er ook nog een gezond gen aanwezig dat wel normaal functioneert. Dit gen is geërfd van de niet aangedane ouder. Iemand met een geërfd, gemuteerd VHL-gen loopt echter de kans dat toevallig ook het andere gezonde gen wordt beschadigd. Het gevolg is dat de rem op celdeling, in bijvoorbeeld een niercel, wegvalt. Uit deze niercel kan vervolgens een niercelcarcinoom ontstaan. Het is bij deze ziekte overigens nog niet duidelijk waarom sommige organen juist wel en andere niet aangedaan zijn.

Kinderen van een VHL-patiënt, zowel jongens als meisjes, hebben 50% kans om het gemuteerde gen te erven. De aangedane ouder heeft immers ook nog een niet-gemuteerd exemplaar van het VHL-gen. Hierdoor hebben kinderen ook 50% kans dat zij de aanleg voor de ziekte niet krijgen. Een VHL-patiënt is dus een persoon met een erfelijke aanleg voor de ziekte, omdat hij of zij in alle lichaamscellen een kiembaanmutatie in het VHL-gen draagt. Omdat binnen één VHL-familie vrijwel iedere drager van een VHL-kiembaanmutatie één of meer tumoren krijgt, spreekt men van een ziekte met een dominant overervingspatroon.

Doelstelling van dit onderzoek

Het belangrijkste doel van het in dit proefschrift beschreven onderzoek is het identificeren van VHL-patiënten en families door middel van DNA-onderzoek. Vervolgens zijn 'voorspellend' genetisch onderzoek en erfelijkheidsadvisering mogelijk. Aanvragers van een DNA-test kunnen worden verlost van de onzekerheid omtrent hun risico voor het krijgen van deze ziekte. Familieleden die géén drager zijn, kunnen uit levenslange controle worden ontslagen. Dragers van een VHL-kiembaanmutatie kunnen, reeds vanaf jonge leeftijd, regelmatig gecontroleerd worden op uitingen van de ziekte. De verwachting is dat hierdoor tijdig operatief kan worden ingegrepen en dat zowel de levensverwachting als de kwaliteit van leven verbeteren.

Omdat het periodiek klinisch onderzoek (kostbaar en tijdrovend) tot alleen die personen die drager zijn van een VHL-kiembaanmutatie beperkt kan worden, is de verwachting dat de kosten-effectiviteitsratio van onderzoek naar VHL-tumoren verbetert.

Patiënten en methoden

Nieuwe families met een erfelijke aanleg voor deze ziekte kunnen worden gevonden door patiënten met typische VHL-tumoren met een belaste familiegeschiedenis, te onderzoeken op mutaties in het VHL-gen. VHL-kiembaanmutaties kunnen ook voorkomen bij sporadische patiënten met één of meer typische VHL-tumoren. Omdat een kiembaanmutatie in alle lichaamscellen aanwezig is, wordt DNA gebruikt dat geïsoleerd is uit bloedmonsters. DNA-onderzoek voor VHL wordt verricht bij de Divisie Medische Genetica van het Universitair Medisch Centrum Utrecht en het Klinisch Genetisch Centrum van het Academisch Ziekenhuis Rotterdam. Klinische gegevens van geteste personen en hun familieleden werden verzameld nadat men een toestemmingsformulier hiervoor had ondertekend.

Om te bereiken dat zoveel mogelijk potentiële VHL-patiënten voor DNA-onderzoek werden aangemeld, werden de volgende stappen ondernomen:

1) het aanschrijven van de universiteitsklinieken voor Oogheelkunde (patiënten met retinale hemangioblastomen);
2) het plaatsen van een oproep in het tijdschrift van de Nederlandse vereniging voor Neurologie (patiënten met hemangioblastomen in het centraal zenuwstelsel);
3) het oprichten van een Landelijke VHL-werkgroep bestaande uit ongeveer 80 medisch specialisten met belangstelling voor erfelijke tumoren (klinisch genetici, internisten, oogartsen, neurologen, neurochirurgen, chirurgen, urologen, en kinderartsen);
4) het benaderen van de acht Klinisch Genetisch Centra (bekende VHL-families, maar nog niet aangemeld voor DNA-onderzoek);
5) het verspreiden van informatie-materiaal binnen de Belangenvereniging VHL en op het Internet (zodat familieleden ook zelf initiatief konden nemen voor DNA-onderzoek).

Samenvatting van de doelstellingen

1) Het opsporen van VHL-families en het identificeren van de kiembaanmutatie;
2) Het diagnosticeren van presymptomatische (voordat er klachten optreden) familieleden met een VHL-kiembaanmutatie;
3) Het identificeren van VHL-kiembaanmutaties bij sporadische patiënten met een typische VHL-tumor, maar zonder een VHL-familiegeschiedenis;
4) Het verbeteren van DNA-analysetechnieken om genetische afwijkingen aan te tonen in families waarin tot nog toe géén VHL-kiembaanmutaies zijn gevonden;
5) Het verzamelen van genetische en klinische gegevens om mogelijke relaties aan te tonen tussen specifieke VHL-kiembaanmutaties en de expressie van het ziektebeeld (genotype-fenotype correlaties);
6) Het opstellen van landelijke richtlijnen voor diagnostiek en periodiek klinisch onderzoek.

Hoofdstuk 2, klinisch onderzoek

In dit hoofdstuk komen voornamelijk klinische aspecten van de ziekte aan de orde. Het hoofdstuk beschrijft radiologische technieken voor het in beeld brengen van organen in de bovenbuik (2.1) en richtlijnen voor diagnostiek en periodiek klinisch onderzoek (2.4). Twee organen worden in het bijzonder besproken, de nieren (2.2) en de ogen (2.3).

Radiologische afbeelding van nieren, bijnieren en alvleesklier

Diverse beeldvormende technieken, zoals MRI (Magnetic Resonance Imaging of magnetische kernspinresonantie), CT (Computer Tomografie-scan) en echografie, spelen een belangrijke rol in het periodiek onderzoek van VHL-patiënten. Met deze technieken kunnen VHL-tumoren vroegtijdig opgespoord worden en nauwlettend in de gaten worden gehouden. Vanwege mogelijk stralingsgevaar verdienen MRI en echo de voorkeur boven CT bij het radiologisch onderzoek van organen van VHL-patiënten.

Niercelcarcinomen bij VHL-patiënten

Bij VHL-patiënten komen vaak multipele tumoren in beide nieren voor. Er moet dan een afweging gemaakt worden tussen een afwachtend beleid met periodieke controles, niersparende chirurgie of verwijdering van één of beide nieren. Deze beslissing hangt af van de grootte, de groei en het gedrag van de tumor. Indien het technisch mogelijk is, wordt niersparende chirurgie verricht. Niersparende chirurgie heeft als belangrijk voordeel dat de nierfunctie meestal behouden kan blijven. Bij VHL-patiënten met een sterk progressief ziektebeeld rest soms geen andere oplossing dan de verwijdering van beide nieren. Deze patiënten worden dialyse afhankelijk en/of komen in aanmerking voor niertransplantatie.

Wij toonden aan dat niercelcarcinomen relatief langzaam groeien (0,3 cm per jaar) en omgeven worden door een bindweefselachtig pseudokapsel. In vijf van 17 bestudeerde tumoren werd ingroei van de tumor in het kapsel gezien en bij drie van deze vijf tumoren werd een doorbraak van de tumor door het pseudokapsel heen waargenomen. Het aantal tumoren (4,6 per nier) was relatief laag. Bij drie patiënten werden één of beide nieren verwijderd en microscopisch bestudeerd. Bij twee van de drie patiënten werd in één nier, in de directe omgeving van het niercelcarcinoom, slechts één enkel satelliet tumortje waargenomen. Bij vijf patiënten werden zes niercelcarcinomen (met een grootte van 1,8 tot 5,5 cm) met niersparende chirurgie verwijderd. Regelmatige controle van deze patiënten na de operatie, met een gemiddelde controle-duur van 30 maanden, toonde aan dat hun nierfunctie goed behouden bleef. Patiënten met kapsel in- of doorgroei vertoonden geen ongunstiger verloop van de ziekte. Wij concludeerden dat, in de onderzochte VHL-patiënten, niercelcarcinomen langzaam groeien, relatief goedaardig zijn en een stevig kapsel bezitten. Hierdoor zijn VHL-patiënten met niercelcarcinomen goede kandidaten voor niersparende chirurgie.

Hemangioblastomen in de ogen

Wij beschrijven het natuurlijk beloop van oogheelkundige afwijkingen bij 20 patiënten uit zes VHL-families. Er worden vijf stadia in de ontwikkeling van een hemangioblastoom onderscheiden en geïllustreerd door foto's (fluoresceïne angiografie). Retinale

hemangioblastomen worden in het algemeen behandeld met laserbestraling, of cryo(vries)therapie. Tevens wordt er een casus (familie C) beschreven die de waarde van DNA-onderzoek illustreert voor het opsporen van VHL-patiënten met een negatieve familiegeschiedenis. Bij een jonge patiënt die alleen haemangioblastomen in de ogen had, werd een VHL-kiembaanmutatie gevonden. Omdat VHL zich niet zelden voor het eerst in de ogen manifesteert, bevelen wij aan om periodiek oogheelkundig onderzoek van personen met een hoog risico voor VHL zo jong mogelijk te laten beginnen. De verwachting is dat door vroege opsporing (indien nodig gevolgd door behandeling) het aantal VHL-patiënten met verminderd gezichtsvermogen beperkt kan worden.

Richtlijnen voor diagnostiek en periodiek klinisch onderzoek

De Landelijke VHL werkgroep geeft richtlijnen om het beleid van vroege opsporing en behandeling van VHL-patiënten in Nederland te ondersteunen.

Voor het stellen van de diagnose VHL bij een patiënt zijn zowel klinische verschijnselen als familiegeschiedenis belangrijk. Typische met VHL geassocieerde tumoren zijn hemangioblastomen, feochromocytomen, niercelcarcinomen, binnenoortumoren en multipele pancreascysten. Multipele pancreascysten zijn VHL-specifiek omdat zij zeldzaam zijn in de normale populatie. Cysten in nieren of geslachtsorganen daarentegen, komen relatief vaak voor in de populatie en zijn minder specifiek voor VHL. De klinische diagnose VHL wordt gesteld bij een patiënt met een typische VHL-tumor in combinatie met een *positieve* familie-anamnese. Bij een patiënt met een *negatieve* familie-anamnese zijn twee of meer hemangioblastomen, of een hemangioblastoom in combinatie met een andere typische VHL-tumor vereist voor het stellen van de diagnose VHL.

De klinische diagnose kan worden bevestigd door het identificeren van een VHL-kiembaanmutatie. DNA-onderzoek van het VHL-gen toont een kiembaanmutatie aan bij vrijwel alle klassieke VHL-families (grote families met verschillende tumoren) en klassieke VHL-patiënten (individuen met verschillende tumoren). Voor DNA-onderzoek komen zowel patiënten met een VHL-tumor en een positieve familiegeschiedenis als patiënten met een typische VHL-tumor en een negatieve familiegeschiedenis in aanmerking. Tevens is (verdenking op) VHL een indicatie voor erfelijkheidsadvisering.

De werkgroep heeft een protocol voor periodiek klinisch onderzoek opgesteld. Hiervoor komen de volgende personen in aanmerking: dragers van een VHL-kiembaanmutatie; familieleden uit VHL-families waarin geen kiembaanmutatie werd gevonden; familieleden uit VHL-families waarin de kiembaanmutatie bekend is, die geen DNA-diagnostiek wensen; patiënten bij wie de diagnose VHL wordt overwogen maar geen mutatie werd gevonden.

Hoofstuk 3, genetisch onderzoek

In dit hoofstuk worden de genetische aspecten van VHL belicht. Klassieke VHL-patiënten en families met VHL-kiembaanmutaties worden beschreven (3.1 en 3.2) en tevens komen sporadische patiënten met een typische VHL-tumor aan de orde (3.3 en 3.4). VHL-kiembaanmutaties worden namelijk ook gevonden bij een patiënt met één of meer typische VHL-tumoren zonder een belaste familiegeschiedenis. Dit kan optreden wanneer: 1) de familiegeschiedenis niet voldoende is (of kan worden) uitgevraagd; 2) één van de ouders drager is van een VHL-kiembaanmutatie maar geen uiting heeft van de ziekte (non-penetrantie); 3) een mutatie in het VHL-gen is opgetreden in de zaadcel of eicel van respectievelijk de vader of moeder (*de novo* mutatie). Deze personen worden aangeduid als (schijnbaar) sporadische VHL-patiënten.

VHL-kiembaanmutaties in Nederland

Tot nu toe werden 32 kiembaanmutaties in het VHL-gen aangetoond: 25 in VHL-families en zeven in sporadische VHL-patiënten. Bovendien vonden wij VHL-kiembaanmutaties in twee sporadische patiënten die niet aan de huidige VHL-criteria voldeden. Acht van de 34 gevonden mutaties werden volgens de literatuur nog niet eerder aangetoond bij VHL-patiënten. Een analyse van correlaties tussen de diverse genotypes (kiembaanmutaties) en het fenotype (uiting van de ziekte) bevestigde de bevindingen uit de literatuur. Zo hebben families met feochromocytomen vaak een bepaald type missense mutatie (waarbij slechts één aminozuur is veranderd in het VHL-eiwit) terwijl families met andere soorten kiembaanmutaties in het algemeen juist geen feochromocytomen hebben.

Bij de negen sporadische patiënten met één of meer typische VHL-tumoren kon in vier gevallen worden bevestigd dat een *de novo* mutatie betrof, doordat de mutatie niet bij één van beide ouders aanwezig was. Bij één patiënt werd de kiembaanmutatie juist wel bij één van beide ouders gevonden. Bij de vier overige patiënten kon geen bewijs voor een *de novo* mutatie geleverd worden omdat de ouders niet beschikbaar waren voor DNA-onderzoek. Bij drie van deze vier patiënten bleef echter de familiegeschiedenis, mede door het ontbreken van VHL-gerelateerde tumoren ook bij naaste familieleden op oudere leeftijd, suggestief voor een *de novo* mutatie. Deze resultaten doen vermoeden dat *de novo* mutaties tussen de 12% (4/34) en 21% (7/34) van het totale aantal gevonden VHL-kiembaanmutaties in Nederland vormen. Dit onderzoek beschrijft tevens vier families met VHL-kiembaanmutaties en een verlaagde of zelfs geheel afwezige expressie van het ziektebeeld. Deze twee bevindingen onderstrepen het belang om ook bij schijnbaar sporadisch patiënten met één of meer typische VHL-tumoren een DNA-test te verrichten.

Vijf families met een deletie van het VHL-gen

Een specifiek type mutatie is een gedeeltelijke of volledige afwezigheid van het VHL-gen. Dit type mutatie noemt men een deletie. Bij vier families was er sprake van een gedeeltelijke deletie van het VHL-gen en bij één familie was het gehele VHL-gen afwezig in de kiembaan. Deze deleties werden aangetoond met behulp van de zogeheten ‘kwantitatieve Southern blot’ techniek. De deletie van het gehele VHL-gen werd bevestigd met een andere techniek: ‘Fluorescence in situ hybridisation’, kortweg FISH

genoemd. Met dit onderzoek toonden wij aan dat kwantitatieve Southern blot analyse een betrouwbare methode is om deleties in het VHL-gen op te sporen. Deze techniek moet daarom geïmplementeerd worden in de reguliere DNA-diagnostiek bij VHL.

In dit onderzoek probeerden wij ook correlaties tussen genotype en fenotype aan te tonen. We bevestigden de bevinding dat patiënten met een geërfde deletie in het VHL-gen in het algemeen een laag risico op het ontwikkelen van feochromocytomen hebben. Daarnaast viel het ons op dat bij deze vijf families en vooral bij de familie met een deletie van het gehele VHL-gen, relatief veel hemangioblastomen in het centraal zenuwstelsel voorkomen.

Sporadische patiënten met hemangioblastomen in het centraal zenuwstelsel

Wij onderzochten hoeveel sporadische patiënten met louter hemangioblastomen in het centraal zenuwstelsel drager van een VHL-kiembaanmutatie zijn. De onderzochte populatie bestond uit 25 Nederlandse en 63 Engelse patiënten. Bij drie (3,6%) van de 84 patiënten met een solitair hemangioblastoom werd een kiembaanmutatie in het VHL-gen aangetroffen. Aangezien alle mutaties werden aangetroffen bij patiënten jonger dan 50 jaar concludeerden wij dat vooral in deze leeftijdscategorie DNA-onderzoek voor een mutatie in het VHL-gen verricht moet worden.

Vier patiënten hadden veelvoudige hemangioblastomen en hierbij werd bij twee patiënten (50%) een VHL-kiembaanmutatie aangetroffen. Het feit dat we geen VHL-kiembaanmutatie vonden bij de twee patiënten met multipele hemangioblastomen kan een aanwijzing zijn voor: 1) de aanwezigheid van nog-niet-ontdekte genen die van invloed zijn op de ontwikkeling van hemangioblastomen; 2) het feit dat deze patiënten niet in alle lichaamscellen een VHL-mutatie dragen (een ‘mozaïek’ patroon); 3) de toevallige aanwezigheid van verschillende hemangioblastomen bij één patiënt.

Patiënten en families met feochromocytomen

Wij onderzochten 24 patiënten met louter feochromocytomen op een VHL-kiembaanmutatie. Bij 14 patiënten was er sprake van een solitaire tumor, zeven patiënten hadden veelvoudige tumoren en bij drie patiënten betrof het een familiaire vorm van feochromocytomen. Bij geen van deze 24 patiënten werd een kiembaanmutatie in het VHL-gen aangetroffen. Zelfs bij de patiënten met een sterke verdenking op de aanwezigheid van een geërfde mutatie (zoals een jonge leeftijd, veelvoudige tumoren of familiaire feochromocytomen) kon geen VHL-kiembaan-mutatie worden aangetoond. Aangezien DNA-onderzoek van het VHL-gen in bijna alle VHL-families een kiembaanmutatie aantoont, sluit de afwezigheid van een mutatie de aanwezigheid van het ziektebeeld praktisch uit. Daarom lijkt het niet aanbevelens-waardig om patiënten mét feochromocytomen maar zonder VHL-kiembaanmutatie te onderzoeken op het eventuele voorkomen van andere typische VHL-tumoren.

Hoofdstuk 4, dicussie

Dit hoofdstuk beschrijft de voornaamste bevindingen en de belangrijkste implicaties en conclusies van het onderzoek.

Ethische, psychische en maatschappelijke aspecten

Wanneer de diagnose VHL gesteld is (of overwogen wordt), is verwijzing naar een klinisch genetisch centrum van belang voor bespreking van de erfelijke aspecten. Het stellen van de diagnose VHL heeft namelijk niet alleen gevolgen voor de patiënt maar ook voor zijn of haar familieleden. Bij het overwegen van erfelijkheidsonderzoek kan echter bij arts en patiënt een conflict van belangen ontstaan tussen enerzijds het respecteren van de privacy van de familieleden en anderzijds de verantwoordelijkheid voor de gezondheid van de familieleden. Met een voorlichtingsbrochure (verspreid via de patiënt en/of geïnformeerde familieleden) kunnen familieleden van informatie over VHL worden voorzien en gewezen worden op de mogelijkheid zelf erfelijkheids-advisering te vragen. Op deze manier kunnen zij zelf kiezen of zij (of hun nageslacht) voor onderzoek in aanmerking willen komen.

Presymptomatisch DNA-onderzoek vormt een aanzienlijke psychische belasting. De psychische last, ontstaan uit de angst om ziek te zullen worden en de onzekerheid over dragerschap van de erfelijke aanleg, kan groter zijn dan de belasting voortvloeiend uit de wetenschap dat er een grote kans bestaat op het ontwikkelen van de ziekte. Derhalve is deskundige begeleiding van de patiënt, volgens de daarvoor door de klinisch genetische centra landelijk gehanteerde richtlijnen, tijdens de procedure van erfelijkheidsonderzoek gewenst.

Een erfelijke ziekte kan van invloed zijn indien men een nieuwe verzekering wil afsluiten of van verzekering wil veranderen. Kennis omtrent de aanleg voor een erfelijke ziekte stelt (ziektekosten)verzekeraars in staat om deze personen een verhoogde premie te berekenen of zelfs een verzekering te weigeren. In deze situatie zou men kunnen spreken van genetische discriminatie. Gelukkig bestaat er tussen overheid en verzekeraars sinds 1990 een afspraak die betrekking heeft op het afsluiten van verzekeringen in relatie tot erfelijkheidsonderzoek. Deze afspraak beschermt de patiënt tegen misbruik van informatie betreffende de aanleg voor een erfelijke ziekte door verzekeraars. Voorts is in 1997 de Wet op Medische keuringen aanvaard: werkgevers mogen geen erfelijkheidsonderzoeken laten verrichten of gegevens daarover opvragen.

VHL-patiënten kunnen zich voor voorlichting en advies over de hierboven genoemde aspecten wenden tot de Belangenvereniging Von Hippel-Lindau. De Belangenvereniging kan tevens een rol spelen om een vertraging in de diagnosticering van VHL te voorkomen. Door middel van brochures, een VHL-krant en een website op het Internet worden personen met een verhoogd risico op deze ziekte alsmede artsen, voorzien van informatie over de klinische en genetische aspecten van deze ziekte. Het blijkt dat niet alle artsen die VHL-patiënten of patiënten met typische VHL-tumoren behandelen, op de hoogte zijn van de klinische en genetische aspecten van deze complexe ziekte. Een samenwerking van zowel artsen die VHL-patiënten periodiek onderzoeken en/of behandelen als klinieken, is daarom van groot belang. De Landelijke VHL werkgroep heeft een protocol opgesteld voor periodiek klinisch onderzoek dat nu in veel ziekenhuizen in Nederland op één dag wordt uitgevoerd.

Conclusies

Door middel van DNA-diagnostiek en een inventarisatie van VHL-families die nog niet voor DNA-onderzoek zijn aangemeld, zijn er momenteel bijna 50 VHL-families in Nederland bekend. Wij constateerden dat *de novo* mutaties tussen de 12% en 21% van het totaal aantal VHL-kiembaanmutaties vertegenwoordigen. Wij vonden ook VHL-kiembaanmutaties bij patiënten die niet aan de huidige diagnostische VHL-criteria voldoen. Deze bevindingen onderstrepen het belang om bij sporadische patiënten met één (of meer) typische VHL-tumoren een DNA-test te verrichten om de diagnose VHL aan te tonen of uit te sluiten, omdat: 1) dragers van een VHL-kiembaanmutatie en hun familieleden een verhoogd risico hebben op het ontwikkelen van tumoren; 2) DNA-analyse van het VHL-gen relatief eenvoudig uitvoerbaar is en in bijna alle klassieke VHL-families en patiënten een kiembaanmutatie aantoont.

Op dit moment is het niet mogelijk om een betrouwbare schatting te maken van de prevalentie van VHL in Nederland. Wij komen in dit proefschrift tot een voorlopige schatting dat de ziekte bij één op de 64.000 mensen vóórkomt. De werkelijke prevalentie zal waarschijnlijk hoger zijn omdat er nog steeds nieuwe VHL-families geïdentificeerd worden en nog niet alle familieleden van bekende dragers van een VHL-kiembaanmutatie onderzocht zijn.

Het is moeilijk gebleken om strikte (klinisch diagnostische) criteria voor VHL op te stellen. Ondanks het feit dat bij alle klassieke VHL-patiënten en families een kiembaanmutatie wordt gevonden, blijkt anderzijds geen simpele relatie te zijn tussen een kiembaanmutatie in het VHL-gen en de uiting van tumoren (genotype/fenotype-correlatie). Bij niet-verwante dragers van een identieke kiembaanmutatie kunnen VHL-tumoren zich verschillend manifesteren. Maar ook binnen één familie ontstaan tumoren op verschillende leeftijden en ontwikkelen familieleden verschillende soorten tumoren. De expressie van het ziektebeeld kan afhankelijk zijn van omgevingsfactoren (leefstijl, roken en voeding) en van andere genen dan het VHL-gen. Bij sommige kiembaanmutaties komt het ziektebeeld verminderd of zelfs geheel niet tot expressie.

Bij sporadische patiënten met één type VHL-tumor wordt in een laag percentage (0-5%) een VHL-kiembaanmutatie gevonden. Dit succes-percentage neemt toe wanneer de tumor op jonge leeftijd voorkomt of dat één type VHL-tumor multipel, dubbelzijdig of familiair voorkomt. Indien er bij laatstgenoemde patiënten en families geen VHL-kiembaanmutatie wordt geïdentificeerd, is het van belang om te onderzoeken of andere genen een rol spelen in de ontwikkeling van de betreffende VHL-gerelateerde tumor.

Een belangrijke conclusie van het onderzoek is dat, uitgaande van een verbeterde prognose door periodiek onderzoek en behandeling, het opsporen van VHL-patiënten door middel van DNA-diagnostiek zinvol is. Bij het nut van vroege opsporing van VHL-patiënten dienen echter een paar kanttekeningen geplaatst te worden:
1) er is nog onvoldoende bewijs voor een verbeterde kwaliteit van leven en een langere levensduur; 2) een betrouwbare kosten-effectiviteits-analyse ontbreekt;
3) de psychologische gevolgen zijn nog onvoldoende bestudeerd.
Deze drie punten kunnen een basis vormen voor nader klinisch onderzoek. Uitgebreider genetisch onderzoek blijft aangewezen voor families en patiënten met typische VHL-tumoren maar bij wie geen VHL-kiembaanmutatie is aangetoond.

Woorden van dank

Vijf jaar geleden maakte ik, als dienstplichtig arts met veel vrije tijd, via de neurologen Hanlo en Krouwer en de internist Lips voor het eerst kennis met het onderwerp van dit proefschrift. Op dat moment wist ik niet zo heel veel meer over deze zeldzame erfelijke vorm van kanker te vertellen dan dat het op de laatste bladzijde van het leerboek neurologie beschreven stond. Tijdens mijn dienstplicht werd er subsidie voor een onderzoeksproject aangevraagd en kon ik mij storten op een geheel te herziene versie van de patiënten-informatiefolder. Nooit had ik kunnen bevroeden dat ik door deze relatief zeldzame aandoening met zovele klinische en genetische collegae zou gaan samenwerken. Dit maakt het onmogelijk om iedereen te noemen, laat staan persoonlijk te bedanken.

Prof. Pearson, dear Peter, since you always have to do your best to speak Dutch, I feel that I should now make an effort to address you in English. When I first met you, the former Department of Human Genetics fitted easily into a small coffee corner in Stratenum. Since 1995, your department has expanded to such an extent that it would now be simply impossible to drink coffee with all our colleagues in the one room, let alone do any work. I consider it my privilege to be the first post-graduate to be awarded a Ph.D. during your second career as a professor in the Netherlands. I am very grateful for all the times that you were prepared to discuss my research with me, and that you were prepared to bat for me when it really mattered.

Prof. Lips, beste Kees, nu dit proefschrift tot een goed einde is gekomen, trek ik eindelijk de stoute schoenen aan om u, de VHL-goeroe, bij de voornaam te noemen. U bent de initiator van het onderzoeksproject en het is voor mij bijzonder leerzaam geweest om van u de veelzijdige klinische aspecten van het ziektebeeld te leren kennen. U weet als geen ander een multidisciplinair samenwerkingsverband te creëren waarin klinische problemen door bijdragen uit verschillende invalshoeken opgelost kunnen worden. Goede herinneringen bewaar ik aan het congres dat wij bezocht en in Honolulu. Alhier had ik het voorrecht om, reeds vroeg in het onderzoek, met u de andere VHL-grootheden van de wereld te ontmoeten. Reikhalzend kijk ik uit naar de volgende gelegenheid waarop wij met een bloemenkrans een Luau mogen bijwonen.

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om mijn vragen omtrent het promoveren zelf voor te leggen. Ten tweede ben ik veel dank verschuldigd aan Prof. Niermeijer voor zijn waardevolle suggesties bij het schrijven van dit proefschrift.

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Lieve Kathalijne, wederom bewees jij je talenten als illustratrice. Helaas schrijf ik waarschijnlijk maar één keer in mijn leven een proefschrift, maar ik hoop dat op nog vele andere boeken een echte Hes de omslag mag sieren.

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Curriculum vitae

De schrijver van dit proefschrift werd geboren op 25 februari 1968 te Dordrecht. Na het eindexamen gymnasium ß aan het Stedelijk Gymnasium te Utrecht, begon hij in 1986 met de studie geneeskunde aan de Universiteit Utrecht, alwaar hij het artsexamen behaalde in 1995. Tijdens zijn studie werkte hij in vaatfunctie-laboratorium van het Universitair Medisch Centrum Utrecht en voor het farmaceutisch bedrijf Eli Lilly. Buitenlandse stages en co-schappen werden gevolgd in Jaipur (India), Harare (Zimbabwe) en Aberdeen (Schotland).De dienstplicht vervulde hij als eerste luitenant-arts bij de cavalarie in de Bernhardkazerne te Amersfoort. Tijdens deze periode begon hij onder begeleiding van Prof. dr. C.J.M. Lips met het opzetten van de studie die uiteindelijk tot dit proefschrift heeft geleid. Van 1996 tot 1999 was hij tevens werkzaam als forensisch geneeskundige bij de GG&GD West-Utrecht. De auteur zal in maart 2000 met de opleiding tot internist beginnen in het Ziekenhuis-centrum Apeldoorn, lokatie Lukas.

The author was born on 25th February 1968 in Dordrecht. After successfully completing his grammar school education at the *Stedelijk Gymnasium* in Utrecht in 1986, he started studying medicine at Utrecht University, where he graduated in 1995. During his studies he worked as a technician at the Laboratory of Blood Vessel Function in the University Medical Centre Utrecht, and as an information assistant in Eli Lilly, the pharmaceutical company. He also undertook practical work in Jaipur, Harare, and Aberdeen. Whilst fulfilling his compulsory national service in 1995 in the cavalry at the Bernhard Military Barracks, Amersfoort, he started the work that led to this thesis. Between 1996 and 1999 he also worked as a forensic doctor for the municipal health authority. In March 2000 the author will begin as a resident at the Department of Internal Medicine, Apeldoorn Hospital Centre.