Reconsideration of biallelic inactivation of the VHL tumour suppressor gene in hemangioblastomas of the central nervous system

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Abstract

Objectives—Cerebellar haemangioblastoma occurs sporadically or as a component tumour of autosomal dominant von Hippel-Lindau disease. Biallelic inactivation of the VHL tumour suppressor gene, which is located on chromosome 3p, has been shown to be involved in the pathogenesis of both tumour entities. Mechanisms of VHL inactivation are intragenic mutations, mitotic recombination events, and hypermethylation of the promoter region. The systematic and complete examination of these genetic and epigenetic phenomena in large series of von Hippel-Lindau disease related and sporadic hemangioblastomas has, thus far, not been performed.

Methods—In the largest series to date, 29 von Hippel-Lindau disease associated and 13 sporadic hemangioblastomas were investigated for all suggested inactivating mechanisms of the VHL gene using single strand conformational polymorphism (SSCP), loss of heterozygosity (LOH), and methylation analyses. Additionally, corresponding blood samples of all patients were screened for VHL germline mutations by SSCP and Southern blotting.

Results—Germline mutations were identified in 94% of patients with von Hippel-Lindau disease and their tumours and 62% of these hemangioblastomas showed LOH of chromosome 3p. Of the 13 sporadic tumours, 23% showed a single somatic mutation of the VHL gene that was not present in the germline. 3p LOH was identified in 50% of informative sporadic tumours. No von Hippel-Lindau disease related or sporadic tumour demonstrated VHL promoter hypermethylation.

Conclusions—For most von Hippel-Lindau disease related haemangioblastomas, the inactivation or loss of both alleles of the VHL gene, as predicted by the Knudson two hit theory, is required. However, in a subset of tumours including most sporadic haemangioblastomas, the genetic pathways involved in tumorigenesis have yet to be defined and may represent alterations of a different pathway or pathways.

Keywords: hemangioblastoma; von Hippel-Lindau disease; tumour suppressor gene; DNA methylation

Haemangioblastomas are benign and usually cystic tumours which are predominantly found in the cerebellum of adults. They occur as a sporadic entity or as part of von Hippel-Lindau disease, an autosomal dominant disorder which is characterised by CNS haemangioblastomas and, in addition, by retinal angiomas, renal cell carcinomas, and pheochromocytomas.1

The VHL susceptibility tumour suppressor gene maps to 3p25–26 and was identified in 1993.2 The three exons contain a coding sequence of 852 nucleotides. Germline mutations within the VHL gene have been identified in up to 100% of affected families.3 Suggested functions of the VHL gene are (1) down regulation of hypoxia inducible mRNAs; (2) proper assembly of the extracellular fibronectin matrix; (3) regulation of exit from the cell cycle4 and (4) regulation of expression of carbonic anhydrases 9 and 12.5 The VHL gene seems to be a classic tumour suppressor gene with biallelic inactivation by two genetic alterations. In von Hippel-Lindau disease, the first hit is an inherited germline mutation which is present in one allele in each cell of the body. It is often a “small” intragenic mutation. The second hit is a somatic DNA alteration which is acquired during the patient’s lifetime and is present only in the tumour tissue. Known somatic inactivating mechanisms include recombination events resulting in a loss of heterozygosity (LOH) of the VHL gene, and to a lesser extent intragenic point mutations. Recently, hypermethylation of normally unmethylated sites of the promoter that are rich in 5’-CG-3’ dinucleotides, so called CpG islands, has been suggested as an epigenetic mechanism of tumour suppressor gene inactivation.6

Genomewide changes in methylation pattern are known to occur in all forms of neoplasia. Although non-island CpGs become hypomethylated, certain CpG islands become densely hypermethylated. Furthermore, in normal tissues, extensive methylation of promoter region CpG islands is associated with transcriptional silencing. This is well known for imprinted alleles and genes on the inactive X chromosome.7 The role of tumour suppressor gene hypermethylation, however, is still unclear in many tumours.

Previous genetic studies on CNS haemangioblastomas either did not analyse both von Hippel-Lindau disease related and sporadic tumours or did not investigate all known mechanisms of gene inactivation. These studies have yielded a broad range of different
Reconsidering molecular pathogenesis of CNS haemangioblastoma

Health Organisation classification.18

MoMCl are were performed. All tumours were classi-

frozen in liquid nitrogen and were stored at

3, which may be a consequence of the

investigation of only small series.

To get a more complete and reliable picture of

VHL gene function in haemangioblastoma

pathogenesis, we sought to examine and

compare an extensive series of 29 von Hippel-

Lindau disease associated and 13 sporadic

hemangioblastomas for all suggested mecha-
nisms of VHL inactivation.

Methods

PATIENTS AND TUMOUR SAMPLES

A total of 42 CNS haemangioblastoma samples were obtained from 31 patients, who were consecutively treated between 1993 and 1997 at the Department of Neurosurgery of the Freiburg University Medical Centre. The series comprised 29 von Hippel-Lindau disease associated tumours obtained from 18 patients with the disease and 13 sporadic tumours, which were obtained from 13 patients with no clinical signs of a tumour syndrome. Eighty three per cent of disease associated and 62% of sporadic tumours were located in the cerebel-
lum. The diagnosis of von Hippel-Lindau dis-

ease was established by the criteria of Neu-

mann et al.17 The tumours were from 11 male

and 20 female patients. The age at operation

ranged from 16 to 71 years with an average of

38 years. The tumour samples were snap

frozen in liquid nitrogen and were stored at

−80°C until the molecular genetic investiga-
tions were performed. All tumours were classi-
fied histopathologically according to the World

Health Organisation classification.18

For germ line analysis blood samples were

available from all patients. Our study was

approved by the ethics committee of the

University of Freiburg and our patients gave

informed consent.

MOLECULAR GENETIC ANALYSES

Genomic and tumour DNA was isolated by

standard methods.16 Southern blotting was

performed to detect large deletions in the VHL

gene. Genomic DNA (7 µg) was digested with

excess Eco RI (Boehringer Mannheim). The

fragments were separated in a 0.6% agarose gel

with 1×TBE buffer and transferred to a

positively charged nylon membrane (Boe-

hringer Mannheim) by capillary blot. Frag-

ments were visualised with the DIG high prime

labelling and detection starter kit I (Boehringer

Mannheim) according to the supplier. The

probes for hybridisation of the Southern blot

were made by two sets of primers, one in the

very beginning of exon 1 and another set in the

3′ untranslated region of exon 3.

Single strand conformation polymorphism

(SSCP) analysis was used to find point

mutations, small deletions, or insertions. Four

sets of primers were needed to cover three

exons. Mixtures contained 100 ng genomic

DNA, 0.2 mM dNTP, 0.5 pmol/µl of each

primer, MgCl2, and 0.1 µ/l Taq DNA

polymerase (Gibco BRL). Polymerase chain

reaction (PCR) conditions and sets of primers

have been previously described.20 Polymerase

chain reaction amplified fragments (10 µl) were
denatured by adding 15 µl denaturing solution

(containing 95% formamide, 10 mM NaOH, 0.05% xylene cyanol, 0.05% bromophenol
blue) and heating to 96°C for 3 minutes before

chilling on ice. Denatured fragments were

separated on a polyacrylamide gel (MDE17

Gel Solution, FMC Bioproducts Europe) with

0.5×MDE and 0.6×TBE buffer according to

the manufacturer. After separation at 200 V for

16 hours the fragments were stained with silver

water, and reamplified for sequencing, which

was performed with nested fluorescence la-

belled primers using the dideoxy method and a

semiautomatic sequencer (Alf, Pharmacia). All

mutations were confirmed by sequencing. Thus

we newly detected or reconfirmed germ-

line mutations as previously described in a

larger series of our patients.21

Altogether, three polymorphic markers on

chromosome 3p were used for the LOH analy-
sis of the VHL gene including one dinucleotide

repeat polymorphism (D3S1038), which is

located close to the VHL locus at 3p25–26,22

and two restriction fragment length polymor-

phism (RFLP) markers, which are located
directly within the VHL gene: One Hae III

RFLP marker is located at the nucleotide 19 of

the VHL gene,23 the other one is a PCR gener-

ated Acc I RFLP marker, which is located at

nucleotide 1149 in the 3′ untranslated region of

VHL.24

For investigation of VHL promoter hyper-
methylation, we performed a PCR based tech-
nique using the methylation sensitive enzyme

Eco RI, which has two recognition sites within

cpG island of the VHL promoter: Genomic

DNA (1 µg) was digested with 10 U Ehe I (MBI

Fermentas) and buffer Y/Tango17 in a total

volume of 150 µl at 37°C for 16 hours. Eco I has

the recognition sequence 5′-GGCGGC-3′

within exon 1 and will not cleave if the internal

cytosine is methylated. To ensure complete
digestion, a further 5 U Eco I was added after
digestion and the sample was incubated for

another hour at 37°C. Before the PCR was per-
fomed, the sample was purified with Gene-

CLean II DNA purification Kit (Bio 101)

according to the supplier’s instructions. The
digested DNA was redissolved in 20 µl Tris-

EDTA-buffer. Two microlitres were used for

the following 15 µl multiplex PCR. One set of

primers flanked the Eco I sites within exon 1

(F:5′-GAG GCA GGC GTC GAA GAG TAC

GCC ACT GAG-3′ R: 5′-CAA AAG CTG

AGA TGA AAC AGT GTA-3′) to amplify exon

3. As a second control, the same

Eco I did not cleave—that is, in the

presence of VHL gene methylation. As a control

for failure of the PCR, a second pair of primers

was used (F: 5′-CTG AGA CCC TAG TCT

GCC ACT GAG-3′ R: 5′-CAA AAG CTG

AGA TGA AAC AGT GTA-3′) to amplify exon

3, which has no Eco I restriction site. The

reaction volumes contained 2 µl primers, 0.2 mM

dNTPs, and 0.5 U Taq polymerase (GIBCO

BRL). Fragments were amplified for 27 cycles of

30 seconds at 95°C, 40 seconds at 62°C, and 30

seconds at 72°C. As a second control, the same

procedure was carried out for each DNA sample
without enzyme to ensure correct amplification of exon 1.

Results
All CNS haemangioblastomas were investigated for alterations of the VHL gene by SSCP, LOH, and methylation analyses. Additionally, corresponding blood samples of all von Hippel-Lindau disease and sporadic patients were screened for VHL gene mutations by SSCP and Southern blot analysis. The results are listed in table 1.

Analysis of the blood samples by SSCP disclosed abnormal electrophoretic patterns in the germline of 14 out of 18 patients with von Hippel-Lindau disease. Subsequent sequencing showed that 10 patients had missense mutations, two had in-frame insertions, and one had a nonsense mutation. In one patient (%), no mutation could be detected by sequencing, although the SSCP electrophoretic pattern was abnormal. The results of sequencing are summarised in table 1. Southern blot analysis of the germline DNA disclosed deletions of 2 kb within the VHL gene in four patients with von Hippel-Lindau disease, who had no SSCP aberrations. Thus, a germline mutation was identified in 17 of 18 (94%) patients with the disease.

All disease related tumours showed the same mutation as the corresponding blood sample and no disease related tumour showed a somatic mutation in addition to the germline mutation. Three of the 13 sporadic tumours had a single somatic mutation including one nonsense and two frameshift mutations (table 1). The nonsense mutation (R161X) in the sporadic tumour 28 has been described before as a germline mutation. The two other mutations have not been described so far.

The results of the LOH analysis are summarised in figure 1. Allelic losses on chromosome 3p were found in 13 of 21 informative von Hippel-Lindau disease tumours (62%) and in

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**Table 1 Mutations of the VHL gene in VHL related and sporadic haemangioblastomas of the CNS. 42 tumours from 31 patients were investigated**

<table>
<thead>
<tr>
<th>Tumour No</th>
<th>Tumour location</th>
<th>Germline mutation</th>
<th>Somatic mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Nucleotide / AA</td>
<td>LOH / Methylation</td>
</tr>
<tr>
<td>VHL related tumours (n=29 investigated):</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1a Cerebellum</td>
<td>446 A/G</td>
<td>N78S Missense</td>
<td>LOH Negative</td>
</tr>
<tr>
<td>1b Cerebellum</td>
<td>446 A/G</td>
<td>N78S Missense</td>
<td>LOH Negative</td>
</tr>
<tr>
<td>1c Cerebellum</td>
<td>446 A/G</td>
<td>N78S Missense</td>
<td>LOH Negative</td>
</tr>
<tr>
<td>2 Cerebellum</td>
<td>746 T/A</td>
<td>L178P Missense</td>
<td>LOH Negative</td>
</tr>
<tr>
<td>3a Cerebellum</td>
<td>699 C/G</td>
<td>C162W Missense</td>
<td>Negative Negative</td>
</tr>
<tr>
<td>3b Cerebellum</td>
<td>699 C/G</td>
<td>C162W Missense</td>
<td>LOH Negative</td>
</tr>
<tr>
<td>4 Cerebellum</td>
<td>479 T/C</td>
<td>L89P Missense</td>
<td>NI Negative</td>
</tr>
<tr>
<td>5a Cerebellum</td>
<td>761 C/A</td>
<td>S183X Nonsense</td>
<td>NI Negative</td>
</tr>
<tr>
<td>5b Cerebellum</td>
<td>761 C/A</td>
<td>S183X Nonsense</td>
<td>NI Negative</td>
</tr>
<tr>
<td>6 Cerebellum</td>
<td>2 kb del</td>
<td>Deletion</td>
<td>LOH Negative</td>
</tr>
<tr>
<td>7a Cerebellum</td>
<td>2 kb del</td>
<td>Deletion</td>
<td>LOH Negative</td>
</tr>
<tr>
<td>7b Spine T8/9</td>
<td>2 kb del</td>
<td>Deletion</td>
<td>LOH Negative</td>
</tr>
<tr>
<td>8a Cerebellum</td>
<td>443 ins TCT</td>
<td>77insL</td>
<td>In frame insertion</td>
</tr>
<tr>
<td>8b Cerebellum</td>
<td>443 ins TCT</td>
<td>77insL</td>
<td>In frame insertion</td>
</tr>
<tr>
<td>8c Cerebellum</td>
<td>443 ins TCT</td>
<td>77insL</td>
<td>In frame insertion</td>
</tr>
<tr>
<td>8d Cerebellum</td>
<td>443 ins TCT</td>
<td>77insL</td>
<td>In frame insertion</td>
</tr>
<tr>
<td>9 Spine C4/5</td>
<td>Not detected</td>
<td>L89P Missense</td>
<td>NI Negative</td>
</tr>
<tr>
<td>10 Cerebellum</td>
<td>479 T/C</td>
<td>L89P Missense</td>
<td>NI Negative</td>
</tr>
<tr>
<td>11 Cerebellum</td>
<td>505 T/C</td>
<td>Y98H Missense</td>
<td>NI Negative</td>
</tr>
<tr>
<td>12 Vermis</td>
<td>665 T/C</td>
<td>I151T Missense</td>
<td>LOH Negative</td>
</tr>
<tr>
<td>13 Cerebellum</td>
<td>505 T/C</td>
<td>Y98H Missense</td>
<td>Negative Negative</td>
</tr>
<tr>
<td>14a Cerebellum</td>
<td>Partial deletion*</td>
<td>106insR</td>
<td>In frame insertion</td>
</tr>
<tr>
<td>14b Cerebellum</td>
<td>Partial deletion*</td>
<td>Deletion</td>
<td>NI Negative</td>
</tr>
<tr>
<td>15 Cerebellum</td>
<td>529 ins GCC</td>
<td>106insR</td>
<td>In frame insertion</td>
</tr>
<tr>
<td>16 Cerebellum</td>
<td>2 kb del</td>
<td>Deletion</td>
<td>LOH Negative</td>
</tr>
<tr>
<td>17b Spine C8</td>
<td>505 T/C</td>
<td>Y98H Missense</td>
<td>NI Negative</td>
</tr>
<tr>
<td>18a Cerebellum</td>
<td>475 T/A</td>
<td>W88R Missense</td>
<td>LOH Negative</td>
</tr>
<tr>
<td>18b Cerebellum</td>
<td>475 T/A</td>
<td>W88R Missense</td>
<td>LOH Negative</td>
</tr>
<tr>
<td>Sporadic tumours (n=13 investigated):</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19 Cerebellum</td>
<td>Not detected</td>
<td>Not detected</td>
<td>LOH Negative</td>
</tr>
<tr>
<td>20 Cerebellum</td>
<td>Not detected</td>
<td>Not detected</td>
<td>NI Negative</td>
</tr>
<tr>
<td>21 Med. obl</td>
<td>Not detected</td>
<td>Not detected</td>
<td>NI Negative</td>
</tr>
<tr>
<td>22 Cerebellum</td>
<td>Not detected</td>
<td>Not detected</td>
<td>LOH Negative</td>
</tr>
<tr>
<td>23 Med. obl</td>
<td>Not detected</td>
<td>Not detected</td>
<td>NI Negative</td>
</tr>
<tr>
<td>24 Cerebellum</td>
<td>Not detected</td>
<td>Not detected</td>
<td>NI Negative</td>
</tr>
<tr>
<td>25 Cerebellum</td>
<td>Not detected</td>
<td>Not detected</td>
<td>LOH Negative</td>
</tr>
<tr>
<td>26 Med obl</td>
<td>Not detected</td>
<td>Not detected</td>
<td>NI Negative</td>
</tr>
<tr>
<td>27 Cerebellum</td>
<td>Not detected</td>
<td>694 C/T R161X</td>
<td>Negative Negative</td>
</tr>
<tr>
<td>28 Spine, C1/2</td>
<td>Not detected</td>
<td>681 ins 11bp frameshift</td>
<td>LOH Negative</td>
</tr>
<tr>
<td>29 Cerebellum</td>
<td>Not detected</td>
<td>Not detected</td>
<td>NI Negative</td>
</tr>
<tr>
<td>30 Spine, T11</td>
<td>Not detected</td>
<td>Not detected</td>
<td>LOH Negative</td>
</tr>
</tbody>
</table>

LOH=Loss of heterozygosity; NI=Not informative for LOH analysis; Med obl=medulla oblongata.

*Result with kind permission of Dr Stolle, Department of Medical Genetics, University of Pennsylvania. Some of the listed germline mutations were detected in collaboration with Dr Brauch and coworkers. Patients 4 and 10 had the same mutation 479 T/C and were related. Furthermore the patients with the mutation 505 T/C (patients 11, 13, and 17) were related. All other patients were Not related.
The methylation analysis did not show hypermethylation of the VHL gene promoter region in any of the investigated tumours. This was indicated by failure of amplification of the promoter fragment in all tumour and blood samples that were digested with EcoRI. All undigested samples amplified correctly and the exon 3 control fragment also amplified in each case.

**Discussion**

Previous reports on mutation analysis of the VHL gene in von Hippel-Lindau disease related and sporadic CNS haemangioblastomas have yielded a broad range of different results. This phenomenon might be the consequence of only a few cases in the investigated series and the incomplete analysis of VHL gene inactivating mechanisms. Here we have systematically and rigorously investigated the VHL gene for all known inactivating mechanisms in the largest study to date.

Reported frequencies of LOH on chromosome 3p in disease related haemangioblastomas are 14% (1/7), 27% (3/11), 66% (2/3), and 100% (4/4) with an average of 40%. Our investigations showed 3p LOH in 13 of 21 informative von Hippel-Lindau disease tumours (62%), indicating that a classic two hit inactivation of the VHL gene is a common mechanism in disease associated haemangioblastomas (fig 2). In our sporadic tumours we detected LOH of the telomeric 3p region in 50% of cases. These results are in good agreement with two previous studies, which have found allelic loss on 3p in 1/2 and 10/19 sporadic CNS haemangioblastomas.

Previous investigations on hypermethylation of the VHL gene in CNS haemangioblastomas have yielded different results and were based on small series. Prowse et al found hypermethylation in four of eight investigated von Hippel-Lindau disease related CNS haemangioblastomas. By contrast, Tse et al investigated eight (three disease associated and five sporadic) haemangioblastomas and did not detect hypermethylation of the VHL gene in any of the tumours. In agreement with the second study we could not find any hypermethylation of the VHL gene promoter region. Both previous groups used methods that were similar to ours, which is digestion with a methylation sensitive restriction enzyme and subsequent PCR with flanking primers. However, the particular enzymes used were different (SmaI and NotI in our study).

The different results can eventually be explained by different levels of sensitivity. Quantitative analyses of methylation might help to clarify this controversial issue.

With our SSCP method, which has been shown to have a sensitivity of 86% in detecting VHL germline mutations we have found only three somatic VHL gene mutations, all of which occurred in sporadic haemangioblastomas. Only one of these tumours showed biallelic VHL gene inactivation due to an additional LOH of the VHL gene region at 3p. The relatively low frequencies of somatic VHL gene mutations in sporadic haemangioblastomas and
biallelic VHL gene inactivation agree with three previous studies, in which somatic mutations have been reported with an average of 18% (7/38). In previous studies, no sporadic tumour has been shown to have biallelic VHL gene inactivation, as these studies did not investigate all inactivating mechanisms, or the tumours retained heterozygosity. By contrast, our investigation of all known VHL gene inactivating mechanisms is now highly suggestive that biallelic VHL gene inactivation is not a common mechanism in the tumorigenesis of sporadic CNS haemangioblastoma. This is very interesting, as until now, the “classic” Knudson two hit mechanism was viewed as dogma for the VHL gene in both von Hippel-Lindau disease associated and sporadic haemangioblastoma. Furthermore, the fact that five of 10 sporadic tumours showed 3p LOH, but four of them lacked structural VHL gene alterations implies that the VHL gene plays a minor role in the sporadic tumours and that there are other genes on chromosome 3p with involvement in haemangioblastoma tumorigenesis. This has already been suggested for clear cell renal cancer, where mutations in tumour suppressor genes at 3p14-p21 seem to have a primary role in tumorigenesis in tumours with 3p LOH but without VHL gene inactivation.

In summary, our data suggest that the genetic pathways involved in pathogenesis in von Hippel-Lindau disease haemangioblastoma versus sporadic haemangioblastoma are distinct. Further investigations on chromosome 3p in CNS haemangioblastomas might clarify this hypothesis further.

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