

## Mutations in the von Hippel-Lindau Tumour Suppressor Gene in Central Nervous System Hemangioblastomas

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### Abstract

Central nervous system hemangioblastomas (cHAB) are rare tumours which most commonly arise in the cerebellum. Most tumours are sporadic, but as many as one third of cHABs occur in the course of the hereditary disorder – von Hippel-Lindau disease (VHL). In order to diagnose new VHL families in Poland we performed sequencing of the entire VHL gene in archival material (paraffin embedded hemangioblastoma tissues) in a large series of 203 unselected patients with cHAB. VHL gene mutations were detected in 70 (41%) of 171 tumour samples from which DNA of relatively good quality was isolated. We were able to obtain blood samples from 19 of mutation positive cases. Eight (42%) of these harboured germline mutations in persons from distinct undiagnosed VHL families.

## Introduction

Central nervous system hemangioblastomas are rare tumours which most commonly arise in the cerebellum. Most tumours are sporadic, but as many as one third occur in the course of the hereditary disorder – von Hippel-Lindau disease (VHL) [1]. VHL disease is a rare autosomal dominant disorder characterized by a predisposition to hemangioblastomas of the central nervous system (cHAB) and retina, renal cell carcinomas and pheochromocytomas. The predisposition is caused by germline mutations in the VHL tumour suppressor gene on chromosome 3p25-26 [2]. Germline mutations in the VHL gene are present in almost all VHL families [3, 4]. From a clinical perspective it is very important to distinguish between sporadic and VHL-associated hemangioblastomas because of the risk of early onset and multiple organ tumours in VHL families. Early diagnosis of VHL disease allows for the proper management of not only hemangioblastomas but of other VHL-related lesions as well. Clinical screening according to a carefully planned surveillance schedule results in an improved prognosis for VHL subjects [5].

Given the occurrence of VHL disease with a frequency of 1:36 000 live births, it is estimated that there are as many as 1000 VHL patients in Poland. However, to date, the Polish VHL Registry includes only about 100 VHL patients from 34 Polish families [4]. Thus, in order to diagnose new VHL families in Poland, we performed sequencing of the entire VHL gene in all available archival material (paraffin embedded cHAB tissues) from a series of 203 unselected patients with cHAB diagnosed in Poland primarily in the period between 1999 and 2003.

## Material and methods

Tissues embedded in paraffin blocks were collected from 203 cHAB patients operated in different Medical Centres in Poland. Most patients were operated between 1999 and 2003, but some of them were diagnosed prior to 1999 (Poznań, Kraków). Paraffin blocks were obtained from 7 neuropathology departments (Szczecin, Poznań, Lublin, Warszawa, Katowice, Wrocław, Częstochowa). Cases were unselected for age, clinical presentation or family history.

DNA isolation was performed as described previously [6]. In brief, formalin fixed, paraffin embedded tissues were sectioned into slides, then deparaffinized in two changes of xylene. Sections were hydrated through a series of graded alcohols. Tissues were placed in 1.5 ml eppendorf tubes and digested with proteinase K. After digestion, proteinase was heat

inactivated. After purification in Microcon-100 tubes (Amicon), a solution containing DNA was diluted in 50  $\mu$ l dH<sub>2</sub>O.

The entire coding sequence of the VHL gene was amplified in nested PCR using 6 pairs of primers (available on request). In brief, 3 exons of the VHL gene were initially amplified in 25 cycles each of 95°C 30s, 60°C 30s, 72°C 30s with external primers. Then, reamplified with internal primers in 25 PCR cycles using the same conditions. Negative controls were used to avoid false results. After purification, PCR products were sequenced with the internal primers, using fluorescently labelled dideoxy chain terminators from an ABI Prism kit (Applied Biosystems) in an ABI 377 automated sequencer.

## Results

Amplification of the coding sequence of the VHL gene was successful in 171 of 203 cHAB samples. Automated sequencing showed VHL gene mutations in 70 (41%) of 171 tumours (Table 1). Single VHL changes were identified in 67 tumours. In the remaining 3 tumours two different VHL mutations were identified.

Altogether 52 different VHL changes were identified. Forty eight mutations were observed in single hemangioblastoma cases. The following four mutations were recurrent: 233A>G was detected in 4 cases, 239G>A present in 2 cases, 266T>A observed in 2 cases, and IVS3-1G>T identified in 17 cases. All changes except one silent variant (Ala50Ala) resulted in alteration of VHL protein sequence.

Intragenic VHL mutations were non-randomly distributed, all changes (except one silent variant) were localized downstream codon 53. Of the 171 tumours, 36 (21%) had mutations within exon 1, 9 (5%) within exon 2, and 5 (3%) within exon 3. In addition to these mutations in the coding sequence we identified one of four splice site mutations in 20 (28%) tumours (IVS1 + 1G>A, IVS2 + 2T>C, IVS2 + 2T>G in single cases and IVS3-1G>T in 17 tumours).

We were able to obtain blood samples from 19 of the 70 mutation positive cases. Eight (42%) of these harboured germline mutations and eleven (58%) had somatic mutations present only in their tumours, but not in peripheral blood leukocytes.

## Discussion

We identified mutations of the VHL gene in 70 (41%) of unselected hemangioblastoma tumours. Our results are in line with previous smaller studies showing that VHL gene mutation is a critical event in the pathogenesis of both familial and sporadic hemangioblastoma [7-13].

Table 1. VHL gene mutations in hemangioblastoma of the central nervous system

Case	Mutation	Consequence	Character				
				28	266T>A	Leu89His	Uv
<b>exon1</b>				29	266T>A	Leu89His	Somatic mutation
1	150C>T	Ala50Ala	Uv	30	266T>C 194C>T	Leu89Pro Ser65Leu	Somatic mutation Germline mutation
2	163G>A	Glu55Lys	Uv	31	266T>G	Leu89Arg	Uv
3	170G>C	Gly57Ala	Uv	32	268A>T	Asn90Tyr	Uv
4	173G>A	Arg58Gln	Uv	33	275A>G	Asp92Gln	Uv
5	174del149	In frame	Somatic mutation	34	334 T>C 254del54	Tyr112His In frame	Somatic mutations
6	184G>A	Val62Met	Uv	35	338G>A	Arg113Gln	Uv
7	193T>C	Ser65Pro	Germline mutation	36	340G>C	Gly114Arg	Uv
8	194C>G	Ser65Trp	Uv	37	IVS1+1G>A	Splice	Germline mutation
9	196G>A	Val66Met	Uv	<b>exon2</b>			
10	206insG	Frameshift	Somatic mutation	38	353T>C	leu118pro	Uv
11	218A>G 302T>C	Gln73Arg Leu101Pro	Uv	39	357delC	Frameshift	Somatic mutation
12	220G>A	Val74Ile	Uv	40	363delT	Frameshift	Uv
13	227T>A	Phe76Tyr	Uv	41	379G>A	Gly127Arg	Uv
14	227T>C	Phe76Ser	Uv	42	382C>T	Leu128Phe	Uv
15	232A>G	Asn78Asp	Somatic mutation	43	403T>A	Leu135Ile	Uv
16	233A>G	Asn78Ser	Germline mutation	44	437delC	Frameshift	Uv
17	233A>G	Asn78Ser	Uv	45	463G>T	Val155Leu	Uv
18	233A>G	Asn78Ser	Uv	46	463G>A	Val155Met	Germline mutation
19	233A>G	Asn78Ser	Uv	47	IVS2+2T>C	Splice	Uv
20	239G>A	Ser80Asn	Uv	48	IVS2+2T>G	Splice	Uv
21	239G>A	Ser80Asn	Uv	<b>exon3</b>			
22	240T>G	Ser80Arg	Uv	49	474 delG	Frameshift	Uv
23	250 G>A	Val84Met	Uv	50	477-478insCA	Frameshift	Germline mutation
24	254T>C	Leu85Pro	Uv	51	481C>T	Arg161X	Germline mutation
25	257 C>T	Pro86Leu	Uv	52	486C>G	Cys162Trp	Uv
26	259delTAT	In frame	Uv	53	499C>T	Arg167Trp	Germline mutation
27	263G>A	Trp88X	Somatic mutation	54-70	IVS3-1G>T*	Splice	Somatic mutation?

Germline mutation – mutation detected in DNA from tumour and from peripheral blood leukocytes

Somatic mutation – mutation present in tumour but not in peripheral blood leukocytes

Uv – unverified variant (detected in tumour – blood sample not tested)

\* – mutation present in tumour tissue, but not detected in DNA isolated from blood leukocytes of four available cases – probably somatic mutation

Previous investigations demonstrated that the VHL gene acts as a classic tumour suppressor gene for sporadic and VHL-related hemangioblastomas (and also renal cell carcinoma, pheochromocytoma and pancreatic tumours), as inactivation of both VHL alleles either by point mutations, loss of the entire allele or VHL promoter methylation has been shown in these tumours [7-13]. Although we did not investigate such somatic events as loss of heterozygosity or promoter methylation, in one case (no. 34) we identified two different point mutations and demonstrated that the two somatic events affected both VHL alleles. This is an interesting and rare example of VHL gene inactivation by two somatic point mutations in hemangioblastoma. Also, tumours no. 11 and 30 harboured two different small intragenic mutations, however we did not verify whether the mutations affected both VHL alleles.

In the present study we detected 52 different VHL changes. We observed different types of VHL mutations (missense, nonsense, in-frame and frameshift deletions and insertions, and splice site alterations), localized in all exons of the VHL gene and downstream codon 53. DNA alterations (including missense variants) localized 3' of codon 53 (highly conserved region of the VHL gene) are predicted to be true mutations [15], thus all VHL changes (except one silent variant) reported in this series are believed to be pathogenic.

In the current series, most mutations were observed in single cases. Only the IVS3-1G>T splice site mutation was relatively common (identified in 17 cases). This variant was present in tumour tissue, but it was not detected in DNA isolated from blood leukocytes of four patients. Thus, it seems that the IVS3-1G>T mutation may be a somatic event relatively common in hemangioblastoma. However, in the remaining cases, the germline character of the IVS3-1G>T mutation cannot be excluded.

The incidence of VHL disease among cHAB patients is between 5% and 30% [16, 17]. More recent reports describe an incidence of about 40% [18, 19] and suggest that VHL disease was underdiagnosed prior to the introduction of modern imaging techniques and molecular analysis of the VHL gene. In regards to the 41% prevalence of small intragenic mutations in our group of 171 cHAB patients, and that approximately 42% of these mutations are germline (of the 19 mutation positive hemangioblastoma tumours from whom blood samples were available, eight (42%) harboured germline mutations), we calculate that VHL disease caused by intragenic mutations occurs in about 17% of unselected hemangioblastoma patients in Poland. Given that the sensitivity of direct sequencing is about 60% in the diagnosis of VHL disease in the Polish population [4],

we estimate that VHL disease is present in about 25% of unselected patients with cHAB in Poland.

We believe that the most efficient way of diagnosis of VHL is to provide genetic counselling to all VHL suspected patients at the time of diagnosis of VHL-associated lesions. In this study we showed that the analysis of archival material from paraffin sections is relatively efficient in searching for new VHL cases, when DNA from peripheral blood is not available. Using this approach, we diagnosed eight novel VHL families in Poland and offered appropriate surveillance and treatment of disease to these families.

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