An 11-bp duplication in the promoter region of the *VHL* gene in a patient with cerebellar hemangioblastoma and renal oncocyoma

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Abstract Central nervous system hemangioblastomas are benign vascular tumours that may present sporadically or as manifestation of the von Hippel-Lindau (VHL) disease. VHL Syndrome is a rare autosomal dominant disorder characterized, besides hemangioblastomas, by susceptibility to multifocal and bilateral renal cell carcinoma and cysts, retinal angiomas, pheochromocytoma, epididymis cystadenoma, pancreatic cysts and/or islet cell tumours. Germline mutations of *VHL* tumour suppressor gene cause the VHL disease, while somatic mutations have been associated with sporadic hemangioblastomas and clear-cell renal carcinomas. We identified an 11-bp duplication in the promoter region of the *VHL* gene in a VHL-affected individual. Functional analysis revealed that this variant affects the binding or the binding affinity of one or more transcription factors that regulate the transcription of *VHL* in vivo, reducing the endogenous levels of *VHL* mRNA. Moreover, consistent with the “two hits” model, microsatellite analysis of hemangioblastoma tissue from this patient revealed Allelic Imbalance for the chromosomal region near the *VHL* gene. We propose that these molecular events, through a loss of pVHL function, lead to the onset of the VHL-related tumours in that individual.

Keywords von Hippel Lindau · Hemangioblastomas · Allelic imbalance · qPCR · EMSA

Introduction

von Hippel-Lindau syndrome is a heritable autosomal dominant syndrome predisposing to central nervous system (CNS) and retinal hemangioblastomas, clear-cell renal carcinomas and pheochromocytomas (Choyke et al. 1995; Clifford and Maher 2001; Linehan et al. 1995; Maher and Kaelin 1997). Other lesions associated with VHL syndrome include multiple renal and pancreatic cysts, endolymphatic sac tumours of the inner ear, neuroendocrine tumours, oncocyomas, and epididymal and broad ligament cystadenomas (Choyke et al. 1997; Maher and Kaelin 1997; Manski et al. 1997). The disease affects approximately 1:36,000 live births with over 90% penetrance by 65 years of age (Maddock et al. 1996).

The *VHL* gene (NM_000551) located on chromosome 3p25-26 is widely expressed in both fetal and adult tissues (Latif et al. 1993; Richards et al. 1996) and is well conserved throughout evolution (Woodward et al. 2000). The *VHL* gene encodes two protein isoforms of 213 and 160 amino acid residues, respectively, with a predominantly cytoplasmic localization (Lee et al. 1996).

Most VHL index cases that are positive for VHL mutations have a positive family history of the disease. However, de novo mutations have been reported in some individuals without any VHL history in their parental lineage (Glenn et al. 1999).

Genotype–phenotype correlations have been well established on the types of mutation in the *VHL* gene and the specialized clinical features of the disease (Friedrich 2001; Gallou et al. 2004).

Here, we describe preliminary functional characterization of a novel 11-bp duplication located in a regulatory site of the *VHL* promoter region in a patient with cerebellar hemangioblastoma and renal oncocyoma.

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Materials and methods

Human samples and clinical data

The affected individual was a 40-year-old male who came to our attention because of symptomatic cerebella hemangioblastoma diagnosed by magnetic resonance imaging scan. There was no indication of a family history for this phenotype. Total removal of the lesion by surgery was achieved, with improvement of the neurological status. Paraffin-embedded tissues from the excised lesion were collected. The tumour sample was histopathologically diagnosed as hemangioblastoma according to the criteria of the World Health Organization (Kleihues and Cavenee 2000).

As the presence of a hemangioblastoma is the first clinical manifestation of the disease in approximately 40% of patients with VHL, our patient had undergone clinical and abdominal ultrasonography for other VHL-related tumours. This analysis showed the presence of a left kidney oncocytoma, a very rare clinical manifestation associated with the disease (Fiske et al. 2005; Teh et al. 1998).

Clinical available records reported the presence of renal cysts in the mother of the proband, whereas one of his siblings (36 years old) presented two right epididymal cysts. Finally, a second sibling (42 years old) does not show any evident clinical manifestation of the disease.

Sample preparation and mutational analysis

After written informed consent, genomic DNA was extracted from peripheral blood leukocyte (PBL) and from surgically excised paraffin-embedded cerebella hemangioblastoma using standard phenol-chloroform procedure and QIAamp DNA Mini Kit (Qiagen), respectively.

The entire *VHL* coding sequence and promoter region were PCR amplified using the set of primers listed in Table 1. PCR products were purified (GFX^{TM} PCR DNA and Gel Band Purification Kit [GE Healthcare]) and sequenced.

The PCR promoter region fragment was subcloned using TOPO TA cloning vector (Invitrogen), amplified directly from crude lysates of single bacterial colonies, and examined by *MspI* restriction enzyme digestion and scored as either wild type or mutant depending on fragment sizes. Normal allele produces six fragments of 118, 72, 65, 30, 5 and 4-bp in size, while allele with duplication produces a 129-bp fragment instead of the 118-bp fragment. The presence of the *VHL* promoter variant was ascertained in a collection of 250 healthy blood donors without family history of VHL disease or related symptoms. The control samples were recruited from Southern Italy and analyzed by *MspI* restriction digestion.

VHL gene deletions analysis

Analysis of genomic deletions of the *VHL* gene was performed in Long PCR by using a set of primers' pairs described in (Cybulski et al. 1999). Briefly, large fragments were amplified in a final volume of 25 µl using 2 U of Expand Long Template Enzyme (Roche), 20 pm of each primer, 500 µM dNTPs and 400 ng of template DNA.

LOH analysis

PBL and tumour DNA samples were analyzed using fluorescently-labelled polymorphic microsatellite markers (Table 1). Independent PCR amplifications were performed in duplicate as described in Kuroki et al. (2003) and PCR products were detected by capillary electrophoresis. The results were analyzed by the Prism Genescan software and then exported by Prism Genotyper Analysis software (Livak and Schmittgen 2001) for semi-automatic allelic imbalance estimation. Semi-quantitative expression (Q^{LOH}) for the degree of imbalance was calculated as described in Skotheim et al. (2001) (see also Fig. 2).

Electrophoretic mobility shift assay (EMSA)

EMSAs were performed using the Gel Shift Assay System Kit (Promega). Double-stranded DNA probes containing either the wild type (wt) or the 11-bp variant (see below) were end-labelled with γ 32P (ATP) using T4 polynucleotide kinase (Promega), incubated with HeLa nuclear extract (Promega) and separated on a 4% nondenaturing acrylamide gel. Competition assays were carried out using unlabeled probes with the indicated fold molar excess. Band intensities were analyzed and quantified with autoradiography and PhosphorImager (Molecular Image FX, Biorad), respectively.

The nucleotide sequences used are as follows: 5'-AGCGCGCACGCAGCTCCGCCCCGCGTCCGACCCGCGGAT-3' for the wild type and 5'-AGCGCGCACGCAGCTCCGCCCCGCGTCCGACCCGCGGAT-3' for the 11-bp variant, respectively.

Reporter luciferase assay

The -175 to -35 sequence of wild type and 11-bp variant of *VHL* promoter was cloned into pGL3 basic vector (Promega). HeLa cells were transfected with 800 ng of pGL3 basic, pGL3 basic wt *VHL* and pGL3 basic variant *VHL* constructs using Fugene6 (Roche) according to the manufacturer's instructions. 10 ng of pRL-SV40 (Promega) was added to measure transfection efficiency. At 24 h post-transfection, cells were harvested and luciferase activity was measured by Dual Luciferase Reporter Assay System

Table 1 Primer sequences for VHL gene amplification and LOH analysis

Exon	Primer name	Primer sequences (5' → 3')
Promoter	5'UTR-prom_fw	TAGCCTCGCCTCCGTTACAA
	5'UTR-prom_rv	TTCTTCAGGGCCGTA CTCTTCGA
1	VHL-ex1a_fw	GTCTGGATCGCGGAGGGAAT
	VHL-ex1a_rv	GGACTGCGATTGCAGAAGAT
	VHL-ex1b_fw	TGCGCTCGGTGA ACTCGCGCGA
	VHL-ex1b_rv	TTCAGACCGTGCTATCGTCC
2	VHL-ex2_fw	CACCGGTGTGGCTCTTTAAC
	VHL-ex2_rv	TGGGCTTAATTTTCAAGTGG
3	VHL-ex3_fw	GCAAAGCCTCTTGTTCTGTTT
	VHL-ex3_rv	CCATCAAAAGCTGAGATGAAA
Flanking	D3S1335_F	AAGGCACTGAGGAGATGAGA
	D3S1335_R	TAGGAAAATTAGACGGAAGCAA
	D3S1317_F	TACAAGTTCAGTGGAGAACC
	D3S1317_R	CCTCCAGGCCATACACAGTCA
	D3S1038_F	TCCAGTAAGAGGCTTCCTAG
	D3S1038_R	AAAGGGGTTCAGGAAACCTG
	D3S3950_F	CATCCTCCTTTTTATTTC AAGAGG
	D3S3950_R	ACAGAGTGGCAAGCTTTGGA
	D3S1304_F	TCGCTCTTTGATAGGC
	D3S1304_R	ATTTCATTTGTAATTTACTAGCAG
	D3S1263_F	CTGTTGACCCATTGATACCC
	D3S1263_R	TAAAATCACAGCAGGGGTTC
	D1S2836_F	TTTAACCAAGGNGGTGAAAG
	D1S2836_R	CTGGAATGAAATCCTCCC
	D2S125_F	GCAACAGAGTGAGACCCTGA
	D2S125_R	TTCTGAGAACCAGATTGTGATTG
LOH control marker	EEF1A1_for	AGCAAAAATGACCCACCAATG
	EEF1A1_rev	GGCCTGGATGGTTCAGGATA
	GAPDH_for	GAAGGTGAAGGTCGGAGTC
	GAPDH_rev	GAAGATGGTGATGGGATTTC
	HPRT1_for	TGACACTGGCAAAACAATGCA
	HPRT1_rev	GGTCCTTTTCACCAGCAAGCT
	VHL_for	GGCCGCCGCATCCA
	VHL_rev	CATCGTGTGTCCCTGCATCTC

following the manufacturer's protocol (Promega). The results are expressed as the relative luciferase activity (RLA) of the appropriate control. The experiments were carried out twice in triplicate.

Quantitative real time PCR (qPCR)

Total RNA from PBL of the affected individual and ten healthy independent controls previously screened for sequence alterations in *VHL* promoter region, were prepared using RNeasy Mini Kit (Qiagen). Reverse transcription (RT) was performed using Superscript II (Invitrogen) primed with poly d(T) according to the manufacturer's instructions. Real-time quantitative PCRs were carried out in four replicates using Platinum SYBR Green qPCR

SuperMix-UDG (Invitrogen) and run on ABI 7900 sequence detection system with the defaults amplification conditions. The geometric mean of Ct values of GAPDH (NM_002046), EEF1A1 (NM_001402) and HPRT1 (NM_000194) genes were used to normalize input for each sample analyzed, while ten control cDNAs were used to define normal relative expression levels. The calculations were made using the comparative CT method as reported (User Bulletin #2, Applied Biosystems; Livak and Schmittgen 2001; Vandesompele et al. 2002).

Binding sites prediction and statistical methods

To predict and compare binding sites in alternative alleles we used Alibaba2 (<http://www.gene-regulation.com/>) and

TESS interface (<http://www.cbil.upenn.edu/teess/>), respectively.

The *t* test for independent samples was used as statistical method.

Results and discussion

Germline inactivation of the *VHL* gene causes the von Hippel-Lindau cancer-predisposing syndrome, and somatic mutations of this gene have been associated to sporadic hemangioblastoma and clear-cell renal carcinoma. The entire coding sequence and the promoter region of the *VHL* gene in peripheral blood and cerebellar hemangioblastoma was analyzed in an individual that presented a symptomatic cerebella hemangioblastoma and a left kidney oncocyoma.

A heterozygous (ca. -44_-54dup11) 11-bp duplication was identified in the *VHL* promoter region 65 bp upstream from the start codon (hereafter referred to as the 11-bp variant) in both patient tissues examined (Fig. 1a). No other nucleotide change was detected and Long PCR to detect genomic deletions was negative confirming that the 11-bp duplication was the only variant found in the patient.

The same variant was absent in 250 healthy unrelated blood donors analyzed by MspI restriction digestion, as described in [Materials and methods](#), confirming its absence in the general population (data not shown).

Genetic analysis of other available family members showed that the variant is present in both the siblings of propositus. The mother was negative for the 11-bp duplication, while the DNA of the father was not available. The father died of lung cancer without any evident signs of VHL disease, and thus we were unable to draw the familial inheritance of this variant although a paternal transmission might be highly likely. Interestingly, one of the sibling positive for the 11-bp variant shows no evident VHL-related clinical manifestation after instrumental VHL disease protocol screening (Catapano et al. 2005). This aspect could be explained by the low penetrance of this variant, both with respect to late disease onset and predisposition to benign tumours. In fact, there is a large variability in the age onset of this disorder, and environmental factors and genetic modifiers influence the phenotypic expression of the disease (Glenn et al. 1991; Stolle et al. 1998; Webster et al. 1998).

The 11-bp variant is located in a regulatory region that affects *VHL* promoter activity (Kuzmin et al. 1995; Zatyka et al. 2002). Analysis of this region using different bioinformatics algorithms revealed several multiple predicted binding sites for transcription factors (TFs) in agreement with previous reports (Zatyka et al. 2002). Thus, the duplication of this region could result in altered transcriptional regulation. In order to investigate this possibility,

electrophoretic mobility shift assays (EMSAs) were carried out. HeLa nuclear extracts were incubated with end-labelled double-stranded oligonucleotides containing the 11-bp variant and increasing amounts of either unlabeled wild type or 11-bp variant probes as competitors. As depicted in Fig. 1b, the unlabeled wild-type competitor bound the HeLa nuclear extract with greater affinity than the unlabeled 11-bp variant competitor as demonstrated by the statistically significant reduced intensity of the radiolabelled HeLa-DNA complex at equivalent competitor concentrations [Fig. 1b, compare lane 3 with 7 (*P* value < 0.01) and lane 4 with 8 (*P* value < 0.01), respectively]. Similar differences were obtained when the radiolabelled wild type sequence was used as probe instead of the variant sequence (data not shown).

Biocomputational analysis revealed that Sp1 was one of the several transcriptional factors putatively binding to the duplicated region. Thus, in order to investigate whether the observed EMSA patterns were due to differential Sp1 binding efficiency, EMSA competition experiments were performed using labelled wild type and 11-bp variant probes with increasing amounts of unlabelled Sp1 canonical sequence. Interestingly, we did not find any significant difference in Sp1 mediated competition (data not shown) suggesting that the Sp1 sites are probably not responsible for the different binding affinity observed in HeLa in our experimental conditions, supporting the hypothesis that the binding of other proteins could be involved.

The location within the promoter suggests that the 11-bp variant can influence the *VHL* promoter activity. To ascertain this, we performed Luciferase Reporter Assay using wild-type and mutated VHL promoter sequences in HeLa cells. As reported in Fig. 1c, the 11-bp variant construct produced a significant (*P* < 0.01) decrease of luciferase activity compared with the wt construct, suggesting that the duplication influences *VHL* promoter activity.

In addition, to get insight on the possible physiological consequences of this novel promoter variant in the context of full-length promoter, quantitative real-time PCR was carried out to determine the mRNA expression level of *VHL* in PBL cDNAs from the propositus and ten healthy controls. As reported in Fig. 1d, the affected individual showed a significantly lower mRNA expression level of *VHL* compared to the average of ten healthy independent controls (*P* value 0.0009). It is worth underlining that the value of the healthy control with the lowest mRNA expression level is significant higher than that of the propositus (*P* < 0.04).

Assessing the *VHL* mRNA expression level in the relatives it would be of great interest to define whether this mutation is functional. However, the impossibility of obtaining RNA samples from these individuals precluded this analysis.

Fig. 1 Effect of the VHL promoter variant on transcriptional activity. **a** Representative sequence electropherogram of direct sequencing of PCR product illustrating the sequence of VHL promoter region in the wild type (*wt*) and mutated alleles. At the bottom, a cloned product of mutated allele was also reported. The 11-bp duplicated sequence is marked by the *bar* drawn below the sequence. **b** Different binding ability of wild type and variant sequence for HeLa nuclear extract. Representative electrophoretic mobility shift assay is depicted in which HeLa cell nuclear extract was incubated with radiolabelled 11-bp variant sequence. Competition experiments were done in the presence of the indicated fold molar excess of unlabelled wt or variant

sequences. The experiments were performed three times with similar results. **c** Decrease of relative luciferase activity (*RLA*) of 11-bp variant construct compared with wt construct. The luciferase activity values have been normalized to the level of activity obtained with pGL3 basic vector. **d** Decrease of endogenous *VHL* mRNA expression level in the propositus. Average relative mRNA expression level from affected and controls as measured by real-time quantitative PCR. The average of expression levels from ten healthy independent controls is reported. All values have been normalized to the level of GAPDH, *EEF1A1* and *HRPT1*

In carriers of *VHL* gene germline mutations, the development of the disease begins following the loss or inactivation of the remaining wild type allele according to the Knudson two-hit model (Knudson 1971; Linehan et al. 2001). Several possible mechanisms have been reported to lead to inactivation of the second allele, such as cytogenetic abnormalities (Lemeta et al. 2002), loss of heterozygosity (LOH) (Crossey et al. 1994; Tse et al. 1997) and

small intragenic mutations or hypermethylation (Glasker et al. 2001; Prowse et al. 1997).

To assess whether LOH was the mechanism leading to the inactivation of second *VHL* allele, LOH analysis was performed on several cerebellar hemangioblastoma sections using the set of microsatellites listed in Table 1. Different tumour-rich fractions of hemangioblastoma tissue of the affected individual showed slightly variable values

Fig. 2 Allelic imbalance (AI) in haemangioblastoma tissue. The peak heights are measured in relative fluorescence units. Two microsatellites located on chromosome 1 and 2 were used as controls. **a** AI of D3S1335 microsatellite marker. $Q^{LOH} = [2815/1739]/[2435/827] = 0.55$. **b** AI of D3S1038 microsatellite marker. $Q^{LOH} = [1494/956]/[1653/584] = 0.55$

of Allelic Imbalance for the D3S1335 and D3S1038 microsatellites located near to the *VHL* gene (see Fig. 2 for a representative example). Allelic imbalance describes the detection of a skewed intensity ratio between two alleles at a locus. It may reflect the complete loss of one allele that is masked by the presence of normal cells, by tumour heterogeneity or non-clonal loss. Consistently, it has been shown that the hemangioblastomas-associated vascular component is composed of reactive, nonneoplastic cells (Lee et al. 1998; Vortmeyer et al. 1997). Thus, our inability to detect complete LOH was likely due to the presence of normal tissue in the paraffin-embedded cerebella hemangioblastoma sections available.

The clinical signs of the patient reported here, namely singular CNS hemangioblastoma and renal oncocytoma, do not fit the classical definition of VHL diagnostic symptoms. Here, we showed that the analysis of the promoter region was crucial for the molecular characterization of this patient otherwise negative for *VHL* mutation screening of the coding region. Therefore, extending the molecular analysis to include the regulatory regions of the gene may be helpful for mutational screening of patients having a “VHL-like” disorder.

In summary, the identification and functional characterization of an 11-bp variant in the promoter of the *VHL* gene were reported in an individual affected by cerebellar

hemangioblastoma and renal oncocytoma. The experimental data reported in this work support the hypothesis that this variant and Allelic Imbalance are the two events that lead to a functional alteration of *VHL* expression causing the phenotype manifestation.

While we were submitting this work, Martella et al (2006) reported the identification of the same heterozygous variant in three individuals with VHL related lesions and 1/100 control individuals. Conversely to our results, the authors concluded that this variant is a neutral polymorphism based on their observation of equivalent expression levels of both alleles measured by PCR-RFLP.

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