Germline mutations of the VHL gene in seven Chinese families with von Hippel-Lindau disease

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Abstract. Von Hippel-Lindau (VHL) disease is a hereditary tumor syndrome caused by mutations or deletions within the VHL tumor-suppressor gene, but VHL germline mutations in the Chinese have rarely been studied. To investigate the genetic profile of VHL mutations in the Chinese population, we evaluated the clinical characteristics of seven Chinese families suffering from VHL disease and determined the particular germline mutations in their VHL genes. Direct sequencing and real-time quantitative PCR was carried out. Disease-associated genetic abnormalities were identified in all of the seven families examined. Two novel intragenic germline mutations (645 G insertion and 417 G deletion) were identified and are reported for the first time. Partial VHL gene deletions in exon 1 were found in two of the seven families. Three clinically asymptomatic mutation carriers were also identified. The spectrum of VHL gene abnormalities in our group is distinct from that observed in North America, Europe and Japan. These mutations are also different from those previously identified in other Chinese VHL patients. Future meta-analysis will provide greater perspective on the Chinese VHL genetic profile. VHL gene screening can play a key role in identifying asymptomatic patients who are carriers of VHL-predisposing genetic abnormalities.

Introduction

Von Hippel-Lindau (VHL) disease (OMIM no. 19330) is a multisystem neoplastic disorder which manifests as a predisposition to tumor development, particularly of hemangioblastomas (HBs) in the central nervous system (CNS), retinal angiomas (RAs), renal clear cell carcinomas (RCCs) and cysts, pheochromocytomas, pancreatic cysts and islet tumors. The disease has an estimated incidence rate estimated between 1/36,000 and 1/52,000 live births (1-3) and a very high penetrance, as 97% of carriers of a VHL disease-causing mutation develop clinical symptoms by the age of 60 (3). VHL has been characterized as an autosomal dominant hereditary syndrome in accordance with the germline mutation which inactivates one of the VHL tumor suppressor gene alleles. The VHL gene resides on chromosome 3p25. In VHL patients, HBs are considered of the most frequent, characteristic, and clinically specific tumors due to this disease. Owing to their location and well-developed vascular network, CNS-HBs tend to bleed, rupture and cause a mass effect; thus, these types of tumors are regarded as a major cause of morbidity and mortality. Although progress has been made in the treatment of cerebella HBs, the potential for relapses and multifocal occurrences remains high. Another detriment to the prognosis of VHL patients is the fact that diagnosis of VHL disease is often missed for long periods of time, as additional lesions may be asymptomatic and the syndrome is often not immediately considered in patients with CNS-HBs. Early diagnosis, however, is the key to adequate management of VHL patients and identification of an at-risk kin.

More than 500 germline VHL mutations have been identified in populations from North and Central America, Japan and Europe. Unfortunately, there is a paucity of information available on VHL germline mutations in the Chinese. Only six manuscripts exist in the literature that describe germline mutations in Chinese VHL families. Two of those reported one VHL mutation in a particular Chinese family (4,5). Another manuscript by Zhang *et al* (6) described 26 germline mutations identified in 27 probands. Mao *et al* (7) reported VHL genetic findings in three other Chinese families, while Zhou *et al* (8) reported their findings of six distinctive germline mutations from ten Chinese families with VHL-associated CNS hemangioblastoma. Finally, Siu *et al* (9) found nine different mutations in nine other Chinese VHL families. The detection rate reported in China varies from 66-97% (6-9), and the so-called 'mutation hotspot' that has been identified in other ethnicities has yet to be defined in the Chinese. It is important to remember that China is geographically vast and is composed of many different districts that are characterized by distinct and long histories of genetic evolution. In order to eventually define the Chinese VHL hotspot more data on VHL-afflicted families of Chinese descent is necessary. Here, we performed a germline mutation analysis of the VHL gene

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Table I. Primers for amplification of VHL gene regions.

Primer set	Target	Sequences	Fragment size (bp)	Annealing temperature, ˚C
1	5'-UTR	Forward 5'-TAGTGGAAATACAGTAACGAGTTGG-3' Reverse 5'-TCTTCTTCAGGGCCGTACTCT-3'	326	62
2	VHL exon 1	Forward 5'-GGTGGTCTGGATCGCGGA-3' Reverse 5'-GGCTTCAGACCGTGCTATCG-3'	418	61
3	VHL exon 2	Forward 5'-GTGGCTCTTTAACAACCTTTGC-3' Reverse 5'-CCTGTACTTACCACAACAACCTTATC-3'	208	64
4	VHL exon 3	Forward 5'-AGTCTGTCACTGAGGATTTG-3' Reverse 5'-CTGAGATGAAACAGTGTAAG-3'	250	52

Table II. Primers for qPCR.

Target	Sequences
VHL exon 1	
Forward	5'-CCCAGGTCATCTTCTGCAATC-3'
Reverse	5'-GCCGTCGAAGTTGAGCCATA-3'
VHL exon 2	
Forward	5'-CCTTTGCTTGTCCCGATAGG-3'
Reverse	5'-TGAGAGATGGCACAAATAATTCAGTT-3'
VHL exon 3	
Forward	5'-TGCCCTTCCAGTGTATACTCTGAA-3'
Reverse	5'-GACGATGTCCAGTCTCCTGTAATTC-3'
GAPDH	
Forward	5'-AACAGCGACACCCATCCTC-3'
Reverse	5'-CATACCAGGAAATGAGCTTGACAA-3'

in east-Chinese families with VHL disease by using direct sequencing and real-time quantitative PCR.

Materials and methods

Patients. A diagnosis of VHL disease was made on the basis of conventional criteria (10). Patients with CNS-HBs were admitted to the Department of Neurosurgery at the First Affiliated Hospital of Soochow University. Between 1996 and 2010, 12 of those patients came from seven families. We approached each of these families to request permission to investigate the members' health situation; ultimately, 13 non-symptomatic (and presumably healthy, non-VHL) relatives volunteered for genetic analysis. The clinical findings of all patients and healthy kin are summarized in Table III.

Genetic screening for VHL gene mutation. Peripheral blood samples were obtained for VHL mutation analysis. Genomic DNA was extracted by means of the Wizard Genomic DNA Purification kit (Promega, Inc., Madison, WI, USA). Eight sets of primers were designed to cover all three exons and the 5'- untranslated region (UTR) of the VHL gene (Table I); primers were manufactured by Sbsgene (Shanghai, China). Genomic DNA (100-200 ng) was amplified by PCR in 25 μl reaction mixtures containing 2.5 μl 10X EX Taq buffer, 2 μl $MgCl_2$ (25 mmol/l), 0.5 μl deoxynucleoside triphosphate (10 mmol/l), 0.3 μl EX Taq DNA polymerase (5 U/μl) and 2 μl of each primer (10 mmol/l). PCR amplification conditions were as follows: 94˚C for 3 min; 40 cycles of 94˚C for 30 sec, 60˚C for 30 sec and 72˚C for 30 sec; and, a final extension of 72˚C for 2 min. After PCR, 1 μl aliquot of the amplification product was verified by agarose gel electrophoresis. The remaining product volume was purified with Promega's Magic PCR Purification kit and directly sequenced by an automatic sequencer (ABI 3730 Genetic Analyzer; Applied Biosystems, Foster City, CA, USA). The mutations detected were confirmed by two-way DNA strand sequencing, and compared with a previously affirmed VHL cDNA sequence (11). Then, the mutations were compared with the Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/index.php).

In the cases in which direct sequencing failed to show any VHL gene mutation, DNA samples were analyzed for germline deletion of the VHL gene. To detect partial or complete VHL gene deletions, qPCR was performed. SYBR-Green (Applied Biosystems) was used as a DNA-binding dye and detected on a Roche LightCycler 480 (Roche Co., Shanghai, China). Oligonucleotide primers for the three VHL exons, a 5'-UTR fragment, and the reference gene GAPDH were created according to the protocol described by Casarin *et al* (12) (Table II).

qPCR reactions were carried out with 25 ng of genomic DNA template, 1X SYBR-Green PCR Master mix and 300 nM each of the appropriate forward and reverse primers, in a final volume of 15 μl. The following conditions were used: initial denaturation for 10 min at 94˚C and 40 cycles of 15 sec at 94˚C and 1 min at 60˚C. Fluorescence detection occurred at the end of the 60˚C annealing/extension step, and the dissociation curve analysis was performed between 60-95˚C.

Every qPCR reaction plate included four controls (three known positives and one negative, without template) corresponding to each of the fragments to be analyzed. In every qPCR run, each sample and its controls were amplified in triplicate, in order to minimize the variability of the results. Furthermore, each qPCR assay was performed twice to confirm the data obtained and validate the method. A dissociation curve analysis was performed at the end of each PCR run in order to exclude the presence of non-specific amplification products.

Table III. Clinical manifestations of VHL in Chinese families.

Family	Patient	Gender	History	VHL phenotype	Diagnosis	Age at diagnosis (years)
A	Proband	M	No	I	CNSHB	48
					PC	48
	Son	M		Asymptomatic		
B	Proband	M	Yes	I	CNSHB	29
	Cousin	M		I	CNSHB	35 (multiple)
	Cousin	M		Asymptomatic		
	Cousin	M		Asymptomatic		
	Daughter	F		Asymptomatic		
	Nephew	M		Asymptomatic		
	Niece	F		Asymptomatic		
C	Proband	F	Yes	IIA	CNSHB	29 (multiple)
	Sister	F		IIA	CNSHB	34 (multiple)
					APMO	35
	Nephew	M		IIA	CNSHB	19
					PH	14
	Daughter	F		Asymptomatic		
D	Proband	F	Yes	I	CNSHB	34 (multiple)
	Son	M		Asymptomatic		
E	Proband	m	Yes	I	CNSHB	21 (multiple)
	Brother	M		I	CNSHB	29 (multiple)
	Sister	F		Asymptomatic		
	Nephew	M		Asymptomatic		
	Niece	F		Asymptomatic		
	Aunt	f		Asymptomatic		
F	Proband	M	Yes	I	CNSHB	23 (multiple)
					PC	25
	Father	M		I	CNSHB	53 (multiple)
					PC	53
G	Proband	M	Yes	I	CNSHB	33 (multiple)
					RA	27
					PCC	29
	Son			Asymptomatic		

CNSHB, hemangioblastoma of the central nervous system; RA, retinal angioma; RCC, renal cell carcinoma; PH, pheochromocytoma; PC, pancreatic cyst; APMO, adnexal papillary cystadenoma of probable mesonephric origin.

Results

Clinical material. VHL manifestations of the 7 families with VHL disease examined in our study are shown in Table III. Twelve VHL patients were initially diagnosed based on clinical characteristics. All presented with CNS hemangioblastomas; 3 of the patients were determined to have isolated CNS-HBs. Nine patients presented with multifocal CNS-HBs; 7 of these patients had tumors located in both the cerebellum and spinal tissues, while only 2 patients had tumors located in the cerebellum and brain stem.

The ages of the patients at initial diagnosis ranged from 16-56 years (mean, 33 years). The gender distribution was 25% female (n=3) and 75% male (n=9). Over the course of the study period, 14 hemangioblastoma specimens were resected from a total of 12 operations. All of the patients were followed-up after the operation, for at least 6 months. Two patients relapsed, one at six years post-resection and the other at 13 years (mean, 9.5 years).

VHL gene mutation. Genetic abnormalities known to be associated with VHL were identified in all seven families (Table IV). The PCR products were detected by agarose gel electrophoresis, and the electrophoresis strip matched the theory size. Genetic analysis was performed on 12 VHL patients and 13 non-symptomatic kin volunteers. Four intragenic mutations were detected by direct sequencing. Two types of VHL abnormalities were identified in exon 2. The first mutation was

Table IV. Germline mutation found in affected families.

Family	Nucleotide change	Effect on coding sequence	Location within the VHL gene	VHL phenotype	Family history
A	Exon 1 deletion		Exon 1	I	No
B	645G insertion[a]	Frameshift	Exon 2	I	Yes
C	553+5G>C	Splice	Intron 1	IIA	Yes
D	646C>T	Glu145stop	Exon 2	I	Yes
E	Exon 1 deletion		Exon 1	I	Yes
F	646C>T	Glu145stop	Exon 2	I	Yes
G	417G deletion[a]	Frameshift	Exon 1	I	Yes

[a]Novel mutations.

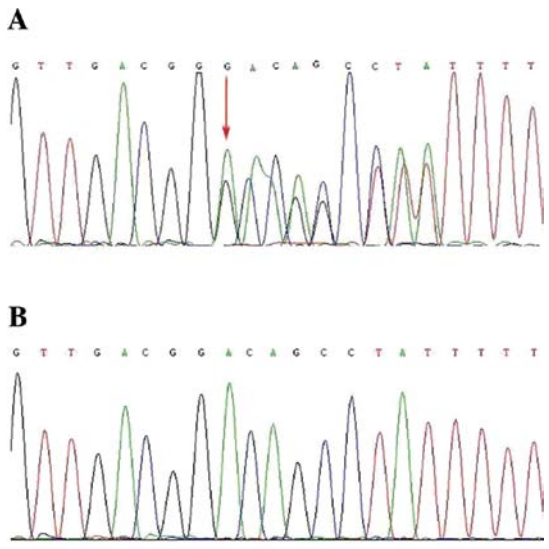

Figure 1. A guanine insertion at position 645 in stemma B. This mutation is expected to result in a frameshift. (A) Mutation in VHL patient; (B) normal VHL sequence in healthy kin.

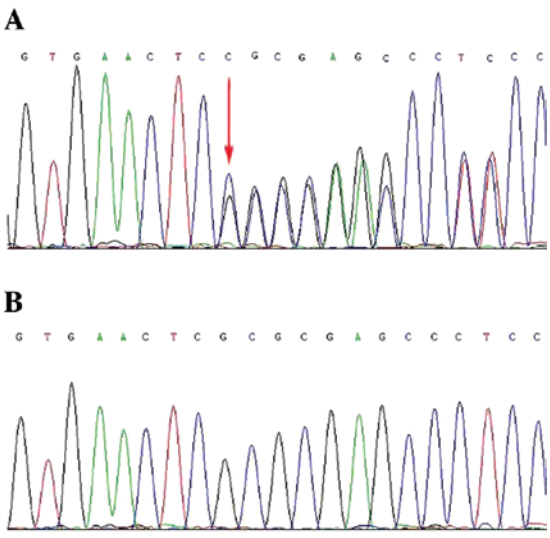

Figure 2. A guanine deletion at position 417 in stemma G. This mutation is expected to result in a frameshift. (A) Mutation in VHL patient; (B) normal VHL sequence in healthy kin.

identified in stemma B, where a guanine inserted at position 645 caused a frameshift (Fig. 1). This mutation is novel and is reported here for the first time. The second mutation, a cytosine replacement by thymine at position 646 (Glu145stop), occurred in stemmas D and F, and caused a CAG codon to become a termination UAG. The third mutation, a frameshift-causing guanine deletion at position 417, was found in stemma G and is also novel (Fig. 2). The fourth mutation was identified as a splice site mutation, 553+5G>C. Partial VHL gene deletions in exon 1 were found in two (stemmas A and E) of the seven families carrying VHL mutations (Figs. 3 and 4). In addition, our analysis revealed that three clinically asymptomatic volunteer kin were mutation carriers.

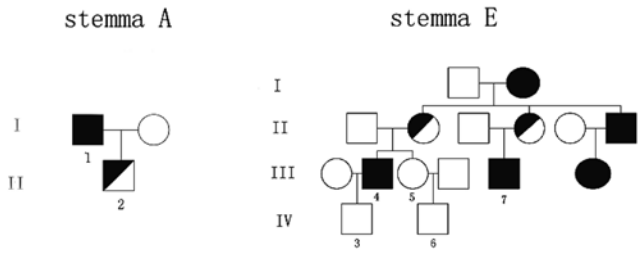

Figure 3. Pedigree analysis of VHL mutations detected in two Chinese families. ■, Denotes male VHL patients; ●, denotes female VHL patients; semi-black denotes non-symptomatic VHL gene mutation carriers.

Discussion

To date, more than 500 distinct mutations have been detected and registered in the Universal VHL-Mutation Database (http://www.umd.be). Each represents a unique family strain which manifests as tumor development, albeit in various tissues.

In familial hemangioblastomas patients, the VHL gene mutation detection rate has been reported to reach between 82-100% (13-16). Germline VHL mutations have been classified among three groups: large deletions, which account for ~40% of all mutations; intragenic missense mutations (~30%); and, protein truncating mutations, including nonsense, frameshift insertions and deletions, and splice site mutations (~30%) (17).

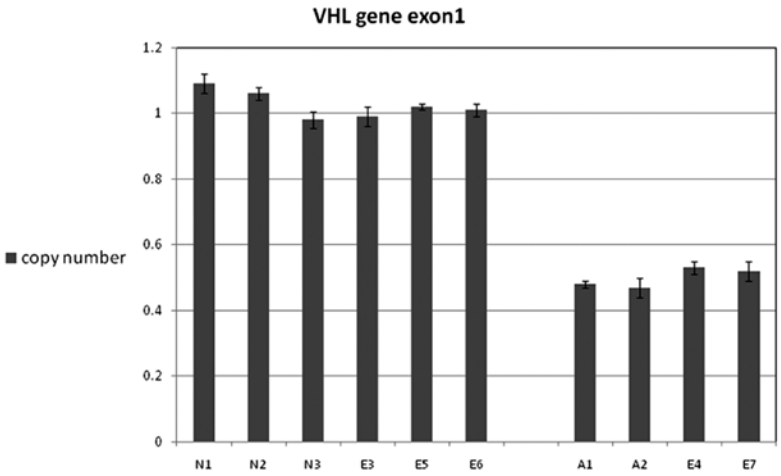

Figure 4. Quantification of the deletions in exon 1 of the VHL gene detected in two VHL families (stemmas A and E) and three healthy volunteers (N1, N2, N3). Healthy individuals and non-carriers (E3, E5 and E6) showed a ratio close to 1.0 for the GAPDH vs. VHL exon 1 transcript levels, as measured by qPCR. In contrast, all carriers of the exon 1 deletion (A1, A2, E4 and E7) showed a clear reduction in this ratio, which was calculated to be close to 0.5.

Identification of intragenic mutations in the VHL gene has largely been carried out by PCR-based direct sequencing methods. However, gross deletions are likely to be missed by direct sequencing. Quantitative Southern blot analysis or universal primer quantitative fluorescent multiplex PCR have been employed for complete or partial deletions and more complex rearrangements of the VHL gene (6,15). Casarin *et al* (12) reported that qPCR was a rapid and efficient technique to analyze the VHL gene, as it combines high sensitivity and specificity, and requires very small amounts of nucleic acid template. qPCR, therefore, gained popularity as an alternative method for the detection of hemizygosity at the VHL locus. In particular, Ciotti *et al* (18) combined direct sequencing with the qCR method to perform successful laboratory diagnoses of Italian patients based on the VHL gene. In our study presented herein, mutation analyses were performed in Chinese patients as described by Ciotti *et al* (18).

A total of six disease-causing genetic alterations were detected in seven families, and included one insertion mutation (645 G insertion), one nonsense mutation (646C>T), one deletion mutation (417 G deletion), one splice mutation (553+5G>C) and two large deletions of the VHL gene. Two unrelated stemma, D and F were found to have the same nonsense mutation (646C>T) in exon 2 that had been previously reported by Gläsker *et al* (14). The splice mutation (553+5G>C) had also been previously detected in a papillary type 2 RCC patient with a family history of VHL disease (19). According to the Universal VHL-Mutation Database, two intragenic germline mutations (417 G deletion and 645 G insertion) were also previously reported. There were no missense mutations identified in our study.

Genotype-phenotype correlations in the VHL syndrome have been established in some studies. Deletion and insertion mutations, nonsense mutations, and splice site mutations have been associated with a type I family (20). Our results are in agreement; each of the six families we found harboring deletions, frameshift, and nonsense mutations were type I families. An exception existed in our study, however; stemma C with a splice site mutation represented a type IIA family. Interestingly, it was also reported that 92% of VHL families with pheochromocytoma had a missense mutation (20).

In another large international study, three regions of frequent germline mutation in the VHL gene were identified in North America, Europe and Japan: between codons 75-82, between codons 157-189 and at the splice site between exons 2 and 3 (20). Moreover, ninety of the 137 intragenic mutations were located in the defined hotspot for these ethnicities. In contrast, the three largest VHL Chinese patient studies found the percentage of mutations occurring in this same hotspot were notably lower [22% (8), 27% (6) and 77% (9)]. In our group, no mutations were identified in the hotspot. The common germline VHL mutations in patients from North America, Europe and Japan were: delPhe76, Asn78Ser, Arg161stop, Arg167Gln, Arg167Trp and Leu178Pro (20). These mutations occurred in four or more VHL families, and the occurrence rate of common mutations is estimated to be nearly 10% in the Western population (20,21). Although we did not identify any common mutations in our study, these mutations have been reported by others studying Chinese patients (6,8,9). China is geographically vast, and as such it is expected to present different mutation spectrums in different areas. Indeed the small number of reports in the literature focusing on Chinese VHL patients and families (4-9) present a strikingly distinct spectrum of mutation among the Chinese population. Moreover, the investigators for each of these different manuscripts are specialists in many different fields, including urology, pathology, neurosurgery, and endocrinology. We believe that 'selection bias', such as inclusion of study cohorts consisting of people predominantly from one area or individuals representing only a restricted subset of tumor types, may account for the apparent genetic discrepancies that characterize the Chinese VHL patients to date.

By gathering statistical information on larger pools of patients in the future, we hope to gain greater perspective on VHL, which will eventually aid in identifying and developing effective treatment. Furthermore, the relationships between particular VHL mutations and their associated tumor types remain to be fully understood; large sample testing will also facilitate this type of study.

Molecular genetic analysis has enabled the early diagnosis of VHL disease in patients who do not otherwise satisfy the conventional clinical criteria for diagnosis, and has proven to be an effective means by which to identify VHL gene mutation carriers. In our study, three asymptomatic carriers were discovered among the non-symptomatic (healthy) volunteer kin. As the usual age of symptomatic presentation correlates with the second and third decades of life, it is possible that these individuals (of ages 14, 16 and 22) will eventually develop VHL-related tumors. Thus, we recommend close follow-up in hopes of achieving early treatment.

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