Clinical and Genetic Investigation of a Multi-generational Chinese Family Afflicted with Von Hippel-Lindau Disease

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Abstract

Background: Von Hippel-Lindau (VHL) disease is a hereditary tumor disorder caused by mutations or deletions of the VHL gene. Few studies have documented the clinical phenotype and genetic basis of the occurrence of VHL disease in China. This study aimed to present clinical and genetic analyses of VHL within a five-generation VHL family from Northwestern China, and summarize the VHL mutations and clinical characteristics of Chinese families with VHL according to previous studies.

Methods: An epidemiological investigation of family members was done to collect the general information. A retrospective study of clinical VHL cases was launched to collect the relative clinical data. Genetic linkage and haplotype analysis were used to make sure the linkage of VHL to disease in this family. The VHL gene screening was performed by directly analyzing DNA sequence output. At last, we summarized the VHL gene mutation in China by the literature review.

Results: A five-generation North-western Chinese family afflicted with VHL disease was traced in this research. The family consisted of 38 living family members, of whom nine were affected. The individuals afflicted with VHL exhibited multi-organ tumors that included pheochromocytomas (8), central nervous system hemangioblastomas (3), pancreatic endocrine tumors (2), pancreatic cysts (3), renal cysts (4), and paragangliomas (2). A linkage analysis resulted in a high maximal LOD score of 8.26 (theta = 0.0) for the marker D3S1263, which is in the same chromosome region as VHL. Sequence analysis resulted in the identification of a functional C>T transition mutation (c. 499 C>T, p.R167W) located in exon 3 of the 167th codon of VHL. All affected individuals shared this mutation, whereas the unaffected family members and an additional 100 unrelated healthy individuals did not. To date, 49 mutations have been associated with this disease in Chinese populations. The most frequent VHL mutations in China are p.S65 W, p.N78 S, p.R161Q and p.R167 W.

Conclusions: The results supported the notion that the genomic sequence that corresponds to the 167th residue of VHL is a mutational hotspot. Further research is needed to clarify the molecular role of VHL in the development of organ-specific tumors.

Key words: Cancer; Linkage Analysis; Mutation; Von Hippel-Lindau Disease

Introduction

Von Hippel-Lindau (VHL) syndrome is a rare, autosomal inherited disorder with an estimated incidence of 1/52,000–1/36,000 and a penetrance of more than 97%.1 It is thought to disrupt tumor-suppression through hypoxia-inducible factor (HIF)-1α-mediated effects that result in the degradation of HIF. Through transcriptional regulation, HIF can increase the glucose uptake and the expression of genes (i.e. vascular endothelial growth factor, platelet-derived growth factor, erythropoietin and transforming growth factor-β) that have been associated with the regulation of pathogenic angiogenesis.6-9 Recently, Neal et al.10 focused on roles of VHL-regulated microRNAs in tumorigenesis, and found that the expression of miR-210 is increased in renal cancers and might contribute to the VHL-associated RCC.
VHL is involved in a complex functional network that is important for tumor suppression.\textsuperscript{[11]} Faulty genes within the network can act synergistically to induce tumors and the network can result in a cascade effect that affects tumor suppression by activating and inhibiting downstream genes. Burnichon \textit{et al}\textsuperscript{[12]} found that the succinate dehydrogenase x family and \textit{VHL} act synergistically in the development of the pheochromocytoma. Similarly, Gimelli \textit{et al}\textsuperscript{[13]} found that haploinsufficiency of ring finger protein 139 (RNF139) might facilitate the development of clear-cell RCC in association with \textit{VHL} mutations.

Clinically, \textit{VHL} disease can be classified into four subtypes based on the presence of pheochromocytoma and/or RCC. Type I \textit{VHL} is not associated with pheochromocytoma, and type II is associated with both hemangioblastoma and pheochromocytoma, with either a relatively low incidence (Type IIA) or a high incidence (Type IIB) of RCC and pancreatic tumors. In contrast, \textit{VHL} IIC is characterized by a pheochromocytoma-only phenotype.\textsuperscript{[14,15]}

Currently, based on the analysis of more than 300 families afflicted with \textit{VHL} disease, more than 823 distinct mutations have been detected and registered in the Universal \textit{VHL}-Mutation Database.\textsuperscript{[16]} To date, a limited number of studies of \textit{VHL} disease have been conducted in China. In the present study, we describe a five-generation \textit{VHL} family from Northwestern China and present clinical and genetic analyses of \textit{VHL} within this family. Moreover, we summarize the \textit{VHL} mutations and clinical characteristics of Chinese families with \textit{VHL} according to previous studies.

**METHODS**

**Patients**

The Chinese family study included five-generations comprising of 38 living family members, of whom nine were affected [Figure 1]. All living members were examined clinically. The clinical and radiographic images were published with the consent of the patient. The study was approved by the research Ethics Committees of Xi’an Jiaotong University School of Medicine. Written informed consents were obtained from all participants.

**Epidemiological investigation of the family afflicted with Von Hippel-Lindau**

Family members were interviewed to collect socio-demographic information that included age, gender, locality, education, income, and occupation. Blood pressure and ultrasound data were collected during the initial screening, and additional examinations were performed on the affected subjects at Shaanxi Sengong Hospital. The medical charts of the \textit{VHL}-patients were reviewed, and relevant social and clinical data (e.g. biochemical tests, images, pathologic reports, etc.) were collected.

**Genetic linkage and haplotype analyses**

Peripheral blood leukocytes were collected from 33 of the 38 living family members, and human genomic DNA was extracted using the DNA Isolation Kit for Mammalian Blood (Tiangen Biotech Co., Ltd., Beijing, China). We genotyped three polymorphic microsatellite markers that flanked \textit{VHL} on chromosome 3p25 (D3S3691, D3S1597 and D3S1263). The microsatellite markers were amplified by PCR using fluorescently labeled primers. The products were analyzed with an Applied Biosystems 3730 Genetic

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**Figure 1:** Haplotypes of the Chinese family affected by Von Hippel-Lindau (\textit{VHL}). The open and filled blackened symbols indicated unaffected and affected individuals, respectively. The squares and circles symbolized the males and females, respectively. The proband, IV-3, was indicated by an arrow. The haplotyping was conducted using three polymorphic microsatellite markers. The marker order was determined from the Marshfield map and the UCSC Human Genome database (February 2009). The dark boxes symbolized haplotypes that cosegregated with the affected individuals and suggested linkage of \textit{VHL} to disease in this family. The boxes with slash symbolized the dead family members.
Analysis (Applied Biosystem, Foster City, CA, USA). LOD scores were calculated using the MLINK program of the LINKAGE package 5.1 (Perkin Elmer, Waltham, USA). The parameters used for linkage analysis were autosomal dominant inheritance, complete penetrance, a mutation rate of 0, equal male-female recombination rates, equal allele frequency, and the disease allele frequency of 1 in 10,000.[17]

**Von Hippel-Lindau sequence analysis**

Mutation screening was performed by directly analyzing the DNA sequence output. The exons of VHL were amplified with primers flanking the exon-intron boundaries [Table 1]. The PCR thermal cycling program was as follows: One cycle of 2 minutes for denaturation at 95°C, 35 cycles for 30 seconds at 95°C, 35 seconds at 57°C, 45 seconds at 72°C, and one 7 minutes extension step at 72°C. The PCR products were sequenced on an Applied Biosystems 3730 Genetic Analyzer.[17,18]

**Summary of the mutations in the Chinese families with Von Hippel-Lindau disease**

We searched for studies in PubMed (http://www.ncbi.nlm.nih.gov/sites/entrez?db=pubmed) using the following terms: “VHL,” “Mutation” and “Chinese.” All of the analyzed data have previously been published.[3]

**RESULTS**

**Clinical analysis**

The proband in the family was a 42-year-old female [Figure 1: IV-3] who was first admitted to The First Affiliated Hospital of Xi’an Jiaotong University with a 2-week history of right upper abdomen pain in August 2011. An abdominal radiograph revealed neoplasms in the pancreas, bilateral adrenal gland, and spleen, bilateral renal cysts, cholecystolithiasis and pancreas atrophy with calcification [Figure 2]. Based on the radiographic findings and the endocrinological results, a surgical treatment was performed. Ultimately, the histopathologic examination confirmed the diffuse PETs and the right pheochromocytoma.

A total of 38 living members (19 males and 19 females) of the family were identified, nine of these members (two males and seven females) were affected. The ages of the living family members ranged from 16 to 61 years (mean age: 32.20 ± 4.75 years). The onset ages of the living patients ranged from 20 to 40 years (mean age: 29.11 ± 5.74 years). In the nine affected subjects, there were three cases of HBs, two cases of PETs, three cases of pancreatic cysts, four cases of renal cysts, eight cases of pheochromocytomas (including four hemi- and four bilateral cases), and two cases of paragangliomas. Patient III-4 had a combination of HBs and pheochromocytoma, IV-7 exhibited HBs and pheochromocytoma; IV-10 exhibited HBs and renal cysts; IV-3 and IV-5 exhibited combined pancreatic lesions (PETs and cysts), renal cysts, pheochromocytomas and paragangliomas, III-8, and IV-11, IV-15 and IV-20 suffered only from pheochromocytomas. Blood pressures above 140/90 were observed in 13 members, of whom six were also diagnosed with pheochromocytomas. The relevant imaging, social, and clinical data of the proband and the affected relatives were shown in Figure 2, Tables 2 and 3.

**Linkage and haplotype analyses**

We performed linkage analysis using three markers that spanned VHL. A maximal LOD score of 8.26 was obtained for marker D3S1263 at θ = 0.00, which suggested linkage [Table 4]. Haplotype analysis revealed that haplotype 3-1-3 cosegregated with the disease in this family.

| Table 1: Primers used for amplification and sequencing of VHL |
|-------------------|-----------------|-----------------|-----------------|
| Exon | Physical Location* | Primers’ sequence | Annealing Temperature (°C) |
|       | Forward | Reverse |       |
| 1     | 10,183,426 | 5’-CGCGAAGACTACGGAGGT | 5’-GGATGTGTCTCTGCTCAAG | 57 |
| 2     | 10,188,045 | 5’-AGACGAGTGTTTTCAACGTTAG | 5’-CAAGTGGTTTTGAGACACCAT | 57 |
| 3     | 10,191,389 | 5’-GATGGTTTGGCAAAGCCTCTT | 5’-GTTTGCCCCTAAACATCACAAT | 57 |

*UCSC browser, February 2009;http://genome.ucsc.edu/cgi-bin/hgGateway.
which indicated that the disease locus was linked to the chromosome region harboring \textit{VHL}, and hence, indicated \textit{VHL} as a candidate gene [Figure 1].

\textbf{Mutation analysis}

A functional C>T transition mutation located within exon 3 of \textit{VHL} (c. 499 C>T, p.R167W) was identified by mutation screening. This alteration was observed only in the affected members of the family and was not detected in any of the unaffected family members [Figure 3]. Moreover, this nucleotide substitution was not found in 100 healthy control individuals (200 alleles), which indicated that this alteration was not a polymorphic variant of \textit{VHL}.

\textbf{Summary of the studies of Von Hippel-Lindau mutations in afflicted Chinese families}

Currently, nine studies including the present study have been conducted on 77 Chinese families and have described 49 mutations of \textit{VHL} \([19-27]\). The mutations were summarized according to the position, nucleotide change, effect on coding sequence, and associated \textit{VHL} phenotype(s) [Table 5]. Independent of phenotype, the most frequent mutations were

| Variables | Living members \((n = 38), n(\%)\) |
|-----------|---------------------------------|
| Gender    |                                 |
| Male      | 19 (50)                         |
| Female    | 19 (50)                         |
| Age (years) |                                 |
| 16-20     | 3 (7.89)                        |
| 21-30     | 4 (10.53)                       |
| 31-40     | 7 (18.42)                       |
| 41-50     | 18 (47.37)                      |
| ≥50       | 6 (15.78)                       |
| \textit{VHL} patients |                                 |
| Patients  | 9 (23.68)                       |
| Healthy   | 29 (76.32)                      |
| Income/month (CNY) |                                 |
| <3000     | 15 (39.47)                      |
| ≥3000     | 23 (60.53)                      |
| Occupation |                                 |
| Farmer    | 22 (57.89)                      |
| Worker    | 11 (28.95)                      |
| Student   | 2 (5.26)                        |
| Teacher   | 3 (7.90)                        |
| Education |                                 |
| Illiterate | 1 (2.63)                        |
| Primary   | 7 (18.42)                       |
| Secondary | 17 (44.74)                      |
| High school certificate | 9 (23.68)                   |
| Bachelor  | 4 (10.53)                       |
| Marital status |                                 |
| Married   | 25 (65.79)                      |
| Unmarried | 13 (34.21)                      |
| Blood pressure (highest) |                                 |
| ≥140/90 mmHg | 13 (34.21)                  |
| <140/90 mmHg | 25 (65.79)                   |

\textit{VHL}: Von Hippel-Lindau, CNY: ChinaYuan.

\textbf{Discussion}

\textit{VHL} disease is an autosomal dominant familial neoplasm syndrome caused by mutations in \textit{VHL} that can lead to the development of tumors and cysts in multiple organs. In our study, we identified a functional C>T transition mutation (c. 499 C>T, p.R167 W) in the \textit{VHL} gene in a North-western Chinese family suffering from \textit{VHL} disease by linkage and sequencing analyses. A high maximal-LOD-score of 8.26 (θ = 0.0) for the marker D3S1263 was obtained through the linkage analysis. The p.R167W mutation resulted in the substitution of arginine at position 167 with a tryptophan residue that co-segregated only in affected individuals. This mutation was only present in the affected \textit{VHL} patients and was completely absent from the other healthy family members and an additional 100 healthy controls. The results of this research firmly indicated that the p.R167W mutation of \textit{VHL} caused the disease in this family. According to the diagnostic and classification criterion, one patient was classified as Type I, four as Type IIA, and four as Type IIC. To our knowledge, there are very few studies of PETs in the world, and this is the first such report in China.

Mutations in \textit{VHL} that result in amino acid changes between codons 157–189 tend to be associated with increases in the
knowledge, the first reported the association of p.R167W mutation in Type II VHL, which is critical to the normal functioning of VHL. Indeed, replacement of residues by tryptophan in other polypeptides has been associated with various diseases.[31,33] The reason for these differences might be related to genetic heterogeneity between the two different ethnic populations. In China, there were reports of associations of p.Rl67W with each subtype of VHL.[23,24] To the best of our knowledge, the first reported the association of p.R167W with Type II VHL in a family of Chinese origin.

The p.R167W mutation resulted in a change from arginine to tryptophan at position 167. The isoelectric point of tryptophan is 10.76, while that of arginine is 5.89 isoelectric. Moreover, the benzene ring structure of tryptophan can affect the secondary and three-dimensional structure of proteins, and indeed, replacement of residues by tryptophan in other polypeptides has been associated with various diseases.[31,33] Indeed the p.VHL is the component of the VHL-Elongin BC-CUL2 (VCB) complex that acts as an ubiquitin-ligase E3 and directs proteasome-dependent degradation of HIF-1α. Interestingly, the regional residues (p. 157–166) are involved in the interaction with the Elongin BC complex. Thus, the p.Rl67W mutation might affect the VCB complex by impeding binding to the Elongin BC complex.[28,34]

Our summary of the numbers of VHL mutations that have been reported in various Chinese families revealed that these numbers are substantially lower than the numbers of mutations that have been reported in populations of European origin. The most frequent mutations in China are p.S65W, p.N78S, p.R161Q and p.R167W, and the most frequent mutations in Western countries are p.F76del, p.N78S, p.R161Stop, p.R167Q, p.R167W and p.L178P. The most common VHL phenotype was Type II (40 mutations), and 11 mutations were associated with Type I (40:11). Zbar et al.[16] also showed that 208 mutations were associated with Type I, and 66 mutations were associated with Type II (208:66). The reason for these differences might be related to genetic

severity of tumors. The mutation presented in this study occurred at codon position 167, which is critical to the normal functioning of VHL.[28,29] Notably, the p.R167W mutation was found in four VHL phenotypes. Therefore, further examination of this codon might provide key insight into the function of VHL. Previous research conducted by Neumann et al.[10] reported that germline mutations of codon 167 convey a high risk for the development of pheochromocytoma. Moreover, the codon 167 mutation is correlated with the subtypes that are characterized by pheochromocytoma with (p.R167Q and p.Rlc67W) or without renal carcinoma (p.Y98H and p.Y112H). However, in the family we studied, the p.Rlc67W mutation was connected to the pheochromocytoma without renal carcinoma phenotype. This discrepancy might be due to genetic heterogeneity between the two different ethnic populations. In China, there were reports of associations of p.Rlc67W with each subtype of VHL.[23,24] To the best of our knowledge, the first reported the association of p.Rlc67W with Type II VHL in a family of Chinese origin.

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### Table 3: The social and clinical characteristics of the nine living patients

| Items                          | III - 4 | III - 8 | IV - 3 | IV - 5 | IV - 7 | IV - 10 | IV - 11 | IV - 15 | IV - 20 |
|-------------------------------|---------|---------|--------|--------|--------|---------|---------|---------|---------|
| Age/OSA/OA (years)            | 56/38/38| 45/26/30| 41/31/41| 37/20/24| 34/34/34| 28/23/28| 24/24/24| 42/35/35| 32/29/29|
| Gender                        | Male    | Female  | Female  | Female  | Female  | Female  | Male    | Female  | Female  |
| Education                     | Primary | Secondary| Secondary| Secondary| Secondary| Secondary| Bachelor| Bachelor| Secondary|
| Occupation                    | Farmer  | Worker  | Farmer  | Worker  | Teacher | Worker  | Worker  | High school certificate | Worker |
| Marital status                | Married | Married | Married | Married | Married | Married | Unmarried| Married | Married |
| Highest blood pressure (mmHg)| 160/110 | 180/100 | 200/110 | 170/100 | 110/80 | 120/80 | 110/80 | 130/90 | 140/80 |

- Cerebellar: +; Pancreatic lesions: –; Renal lesions: –; Pheochromocytoma: –; Bilateral: +; Hemi: –. Previous research conducted by Neumann et al.[10] reported that germline mutations of codon 167 convey a high risk for the development of pheochromocytoma. Moreover, the codon 167 mutation is correlated with the subtypes that are characterized by pheochromocytoma with (p.R167Q and p.Rlc67W) or without renal carcinoma (p.Y98H and p.Y112H). However, in the family we studied, the p.Rlc67W mutation was connected to the pheochromocytoma without renal carcinoma phenotype. This discrepancy might be due to genetic heterogeneity between the two different ethnic populations. In China, there were reports of associations of p.Rlc67W with each subtype of VHL.[23,24] To the best of our knowledge, the first reported the association of p.Rlc67W with Type II VHL in a family of Chinese origin.

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### Table 4: Two-point LOD scores obtained from linkage analysis between VHL and chromosome 3p25.3 in the pedigree

| Markers | Physical location | 0.0 | 0.01 | 0.05 | 0.1 | 0.2 | 0.3 | 0.4 | Zmax | θmax |
|---------|------------------|-----|------|------|-----|-----|-----|-----|------|------|
| D3S3691 | 8,840,396        | 4.45| 4.38 | 4.10 | 3.71| 2.84| 1.86| 0.80| 4.45 | 0.0  |
| D3S1597 | 9,365,414        | 5.09| 4.99 | 4.58 | 4.05| 2.93| 1.74| 0.57| 5.09 | 0.0  |
| D3S1263 | 11,517,247       | 8.26| 8.12 | 7.58 | 6.86| 5.32| 3.60| 1.71| 8.26 | 0.0  |

*UCSC browser, February 2009; http://genome.ucsc.edu/cgi-bin/hgGateway.
In summary, the present study identified a functional C>T transition mutation (c. 499 C>T, p.R167W) located within exon 3 of VHL that is likely the cause of VHL disease in this family. VHL mutations suggest that the function of VHL might be similar to other check-point proteins in that when mutations are present, uncontrolled cell proliferation (or more precisely, neoplasias) arise in various tissues. Further research is needed to clarify the molecular mechanisms of VHL’s role in organ-specific tumors.

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