Original Article

Von Hippel-Lindau "Black Forest" mutation inherited in a large Chinese family

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Background: The Von Hippel-Lindau (*VHL*) *p.Tyr98His* (*Y98H*) mutation is designated as the "Black Forest" founder mutation and has been previously reported in Western countries. This study reports the first recorded Chinese VHL family with the "Black Forest" mutation in Asia.

Methods: Paired whole-exome sequencing (WES), Sanger sequencing and immunohistochemistry (IHC) were performed on samples from a large Chinese family to confirm the causative mutation and mutation carriers in the family. Clinical manifestations of the family were summarized and compared with those reported from other patients with the *VHL Y98H* mutation.

Results: The Chinese pheochromocytoma (PCC) family was identified as a VHL type 2 family with a *Y98H* mutation. There were 4 VHL patients and 11 currently healthy individuals with the mutation. Copy number analysis and SDHB IHC were performed to exclude interference from other pathogenic genes of PCC or paraganglioma (PGL).

Conclusions: We report the first recorded instance of a Chinese VHL type 2 family with the "Black Forest" mutation by using WES and Sanger sequencing, which widens the currently recorded presence of the "Black Forest" mutation to China and potentially elsewhere in Asia and indicates that the "Black Forest" mutation does not uniquely evolve in occidental countries. A personalized surveillance approach, which may be more appropriate for affected families, has been recommended to improve quality of life.

Keywords: Von Hippel-Lindau disease (VHL disease); whole-exome sequencing (WES); pheochromocytoma (PCC); Black Forest mutation

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Introduction

Von Hippel-Lindau (VHL) disease, an autosomal dominant inherited neoplastic syndrome, presents with various clinical phenotypes and age-dependent penetrance. Many kinds of tumors are related to VHL, such as central nervous system (CNS) haemangioblastomas, retinal angiomas (RAs), clear-cell renal cell carcinomas (ccRCCs), pheochromocytomas (PCCs), pancreatic neuroendocrine tumors, pancreatic cysts, and endolymphatic sac tumors (1-3). Meanwhile, paragangliomas (PGLs), and epididymal and broad ligament cystadenomas may also be possible manifestations of VHL disease (4-6). The incidence of VHL disease is approximately 1/36,000 (7). VHL has over 90% penetrance by the age of 65, and the onset of symptoms can even occur during childhood (8).

VHL disease has traditionally been classified into 2 types based on its clinical manifestations (1,9): type 1 (reduced risk of PCCs) and type 2 (with PCCs). Type 2 is further subdivided in to type 2A (low risk of ccRCCs), type 2B (high risk of ccRCCs), and type 2C (only with PCCs, without ccRCCs or haemangioblastomas) (9,10). Patients were diagnosed as having VHL disease according to their clinical characteristics before the genetic testing was complemented in clinics. However, there were several patients who had a clinical diagnosis of VHL disease but lacked VHL mutations, indicating that genetic testing is the gold standard rather than exclusively relying on diagnosis based on clinical features (11). As a result, genetic testing can be used to verify clinical diagnoses in affected VHL patients and rule out VHL disease in individuals with VHL-related tumors without family history (12). After the mutation of VHL has been confirmed in the proband of the family, genetic testing can also be utilized for diagnosis or pre-symptomatic screening of other family members. In addition, genetic testing has shown great potential for use in prenatal screening (12,13).

The *VHL p.Tyr98His* (*Y98H*) mutation was designated as the "Black Forest" founder mutation (14), which was previously identified in two VHL type 2A families that lived in Pennsylvania (USA), but was later shown to have originated and spread in the isolated mountain valleys of the Black Forest in Germany (14). Additionally, *VHL Y98H* was also detected in Sweden and some other non-Asian countries (4,6,15,16).

In Xiangya Hospital, South Central China, we carried out paired whole-exome sequencing (WES) and Sanger sequencing on a Chinese PCC family, which was eventually identified as being a VHL type 2 family with the *VHL Y98H* mutation. As far as we know, this is the first VHL family with the *VHL Y98H* mutation that has been reported in Asia. Furthermore, we summarized and analyzed its clinical manifestations and compared it with *VHL Y98N* mutation.

Methods

Subjects and samples

A large PCC family with four generations was followed by the Department of Urology, Xiangya Hospital, Central South University in China. This study was approved and supervised by the Ethics Committee of Xiangya Hospital (No. 201403307), and informed written consents were signed by each participant. Approximately 2 mL of peripheral venous blood was collected from each family member in tubes that contained anticoagulant (EDTA-K_2). DNA samples from peripheral venous blood of all the members were extracted with the Dzup kit (Dzup, Sangon Biotech Co. Ltd, Shanghai, China). The DNA samples were assessed for quantity and quality before sequencing.

Paired WES

Paired WES was first performed on genomic DNA from peripheral venous blood of the proband's mother and younger brother at the Beijing Genome Institute in Shenzhen, China. The appropriately qualified genomic DNA samples were randomly fragmented by the Covaris Acoustic System, and the library fragments were mainly distributed between 200 and 300 bp. Adapters were then ligated to both ends of the resulting fragments. The adapter-ligated templates were purified by using Agencourt AMPure SPRI beads, and the fragments with insert size of approximately 176 bp were excised. Extracted DNA was amplified by ligation-mediated PCR (LM-PCR), purified, and hybridized to the Nimblegen SeqCap EZ Library v3.0 (Roche/NimbleGen, Madison, WI) for enrichment, and the magnitude of enrichment was estimated for the products. Each qualified library was then loaded on the Hiseq2500 platform (Illumina, San Diego, CA, USA) and sequenced. Finally, the raw image files were processed by Illumina base-calling Software v1.7, and paired-end readings were generated and stored in FASTQ format (raw data).

Bioinformatics analysis

Clean data were produced by filtering the raw data. The Burrows-Wheeler Aligner was used to compare

the clean data of each sample to the human reference genome (GRCh37/HG19) (17,18). Local realignment around insertion/deletions (*InDels*) and base quality score recalibration was performed using the Genome Analysis Toolkit (GATK) (v3.3.0) (19,20). All genomic variations, including single nucleotide polymorphisms (*SNPs*) and *InDels*, were detected by the state-of-the-art GATK (v3.3.0) haplotype caller module. Moreover, exome copy number variants (CNVs) were identified by ExomeCNV. Annotation of the genetic variants was performed using the Human Gene Mutation Database (HGMD), dbSNP, ExAC, ClinVar and 1000 Genomes Project databases.

Sanger sequencing

When the causative mutation was confirmed, Sanger sequencing was further performed on all the other family members. The target gene was amplified via polymerase chain reaction (PCR) with the following primers: F-5'-GTACGGCCCTGAAGAAGACG-3'; R-5'-GTCACCCTGGATGTGTCCTG-3'. PCR was performed in a total volume of 20 μL, including 0.3 μL DNA (15 ng), 0.3 μL forward primer (20 μmol/L), 0.3 μL reverse primer (20 μmol/L), 10 μL PrimeSTAR (Takara Biotechnology Co, Ltd., Japan) and 9.1 μL deionized water. The PCR programme was as follows: 98 ℃ for 5 min; 98 ℃ for 10 s, 58 ℃ for 5 s and 72 ℃ for 1 min for a total of 40 cycles; 72 ℃ for 5 min. After that, the PCR products were purified and sent to Biosune, Shanghai, China for Sanger sequencing.

Immunohistochemistry (IHC)

The antibodies against SDHB, mouse monoclonal clone 21A11 (NB600-1366; Novus Biologicals, Littleton, CO, USA; 1:50), were applied on the four patients' formalin fixed paraffin-embedded (FFPE) tissues. At first, the sections were deparaffinised, rehydrated, and heated in a microwave oven in Tris-EDTA buffer (10 mM Tris, 1mM EDTA) at 100 ℃ for 15 min. Later, after rinsing in tap water, they were incubated in 3% H_2O_2 in PBS for 20 min. After that, the primary SDHB antibodies were diluted, and slides were incubated with 100 μL per slide at 37 ℃ for 1 hour, followed by rinsing in 0.5% Tris-Tween buffer (pH =8.0). The sections were then incubated for 45 minutes with peroxidase-labeled polymers coupled with goat anti-mouse immunoglobulin (EnVision Dual Link System-HRP Kit; Dako Cytomation, Carpinteria, CA, USA). Finally, diaminobenzene tetrahydrochloride was applied twice (5 min each time) and the glass slides were rinsed with distilled water.

Results

Presentation of the extended family

The proband was a 34-year-old female with bilateral adrenal masses detected by ultrasonography. The patient did not have headaches, palpitations, predilection to sweating, nor other complaints. Physical examination was normal for her, including blood pressure. Family history of diseases was initially denied. Plasma cortisol, renin, angiotensin and aldosterone were all within normal values, except for urinary vanilmandelic acid (VMA), which was 114.2 μmol/day (normal range: 10–30 μmol/day). Computed tomography of the adrenal glands showed: (I) bilateral adrenal masses, both suggestive of PCCs; (II) a hepatic haemangioma in the left lobe (*Figure 1A*). Therefore, PCCs were highly suspected. The patient was given Prazosin regularly for 2 weeks, and laparoscopic bilateral partial adrenalectomy (LBPA) was finally performed. Later, histopathological examination confirmed that she had bilateral PCCs.

In addition, the proband's younger brother, a 33-year-old male, was also found to have a right adrenal mass by an imaging examination 7 months after his sister's LBPA. He was asymptomatic, and the results of physical examination, including blood pressure, were unremarkable. Urinary VMA was 55.0 μmol/day. Computed tomography of the adrenal glands indicated (*Figure 1B*) that a right PCC was strongly suspected in light of his sister's condition. Therefore, laparoscopic right partial adrenalectomy (LRPA) was performed after treatment with Prazosin for one month. The diagnosis was confirmed by histopathological examination.

After diagnosis of two PCC patients in this family, we considered the possibility of hereditary pheochromocytoma (HPCC). To test this hypothesis, the patients' other family members were enrolled and screened by adrenal ultrasonography. As expected, more PCC patients were found in this family. At first, their mother, a 61-year-old female who had been treated for hypertension but lacked any other relevant PCC symptoms, was found to have a large right adrenal mass. After preoperative computed tomography of adrenal glands (*Figure 1C*) and Prazosin treatment for 1 month, she also had LRPA, and a right cystic tumour was found during the operation (*Figure 1D*).

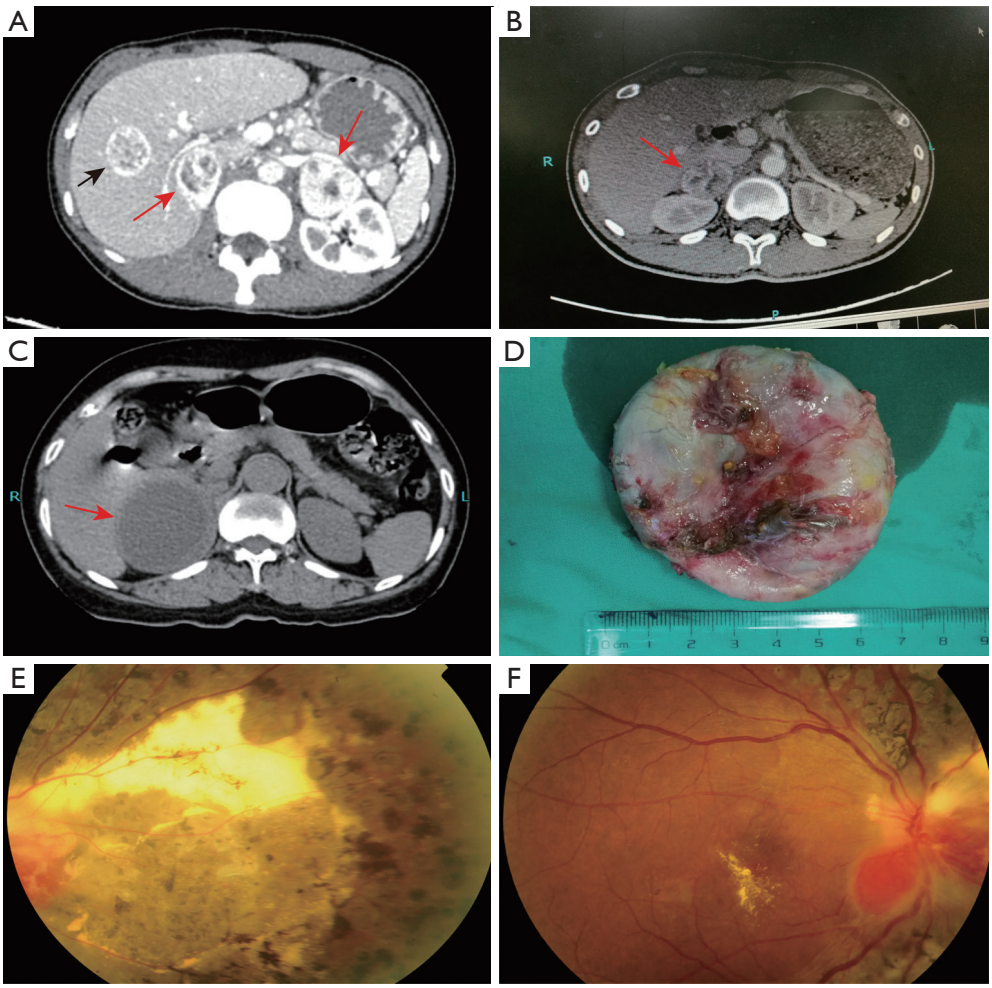

Figure 1 Image findings of PCC patients. (A) Bilateral adrenal masses (red arrows) and a hepatic haemangioma (black arrow); (B) right adrenal mass (red arrow); (C) cystic lesion of the right adrenal gland (red arrow); (D) Cystic tumour (right adrenal mass) identified in the proband's mother; (E) large laser spots and scattered haemorrhage; (F) upper and lower vascular arch pigmentation, anterior papillary haemorrhage [(E) & (F) referring to fundus examination of II-1, and images revealing performance after laser treatment]. PCC, pheochromocytomas.

The right PCC was identified by the histopathological examination after LRPA. Later, it was discovered that she had RA approximately 10 years ago, and a recent fundus examination supported this finding (*Figure 1E,F*).

In addition, the proband's cousin, a 31-year-old male, who had LRPA 10 years ago for right PCC, was found to have a left adrenal mass during the imaging examination. However, he had neither clinical symptoms nor any other physical findings associated with PCC in the examinations. In addition, his urinary VMA level within 24 hours was normal. Finally, he underwent a laparoscopic left partial adrenalectomy, and left PCC was confirmed by histopathology.

WES and Sanger sequencing results

To identify the inherited germline mutation in this family, the genomes of II-1 and III-3 (*Figure 2*) were sequenced by paired WES. Filtering steps were performed as follows. First, we removed genomic variants that didn't belong to PCC/PGL susceptibility genes, including *RET*, *VHL*, *NF1*, *SDHA*, *SDHB*, *SDHC*, *SDHD*, *SDHAF2*, *TMEM127*, *MAX*, *FH*, *EGLN1*, *MEN1*, *GDNF*, *GNAS*, *CDKN2A*, *P53*, *BAP1*, *BRCA1*, *BRCA2*, *ATRX*, *KMT2D*, *RAS*, *EPAS1*, *MDH2*, *IDH and KIF1B* (18). After this filtering step, there were 136 and 28 SNP and InDel, respectively, variants

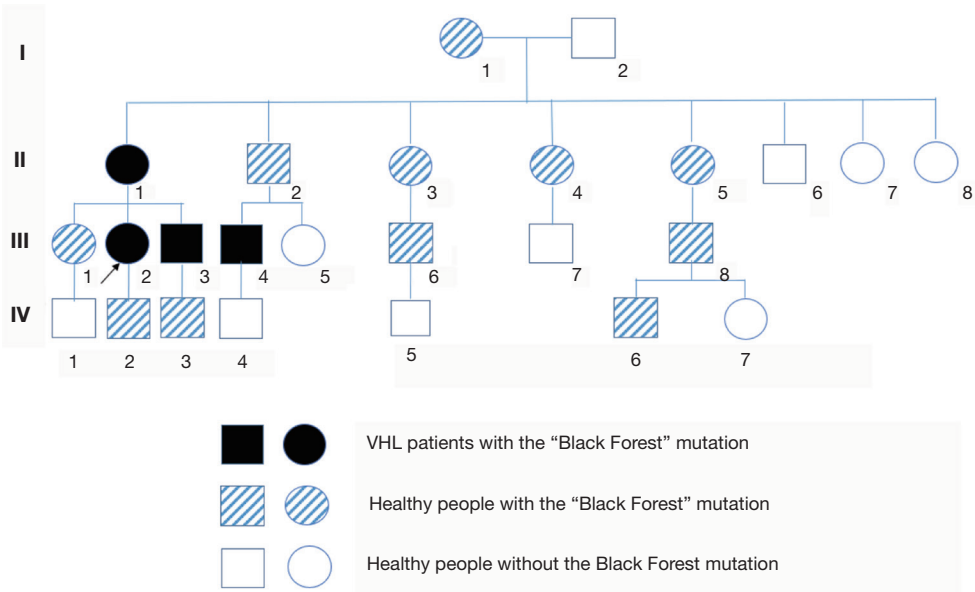

Figure 2 Pedigree of the VHL family. III-2 was the first detected PCC patient and is indicated by the arrow. VHL, Von Hippel-Lindau; PCC, pheochromocytomas.

Table 1 Filtering steps of paired WES

Patients	Total number of genomic variants		Number of variants in susceptibility genes for PCC/PGL		Number of variants in coding sequences and splice region[a]		Number of variants after removing variants with MAF >0.1%[b]		Identical mutant genes in II-1 and III-3
	SNP	InDel	SNP	InDel	SNP	InDel	SNP	InDel	
II-1	98,989	16,657	136	28	12	1	2	0	2
III-3	100,717	16,985	134	32	12	1	2	0	

[a], removing 5'-prime-UTR, 3'-prime-UTR, intronic, downstream gene and synonymous variants (*Table S1*); [b], removing variants with MAF (minor allele frequency) >0.1% in 1,000 genomes and ESP 6500 databases, but retaining the variants without MAF data in 1,000 genomes and ESP 6500 databases. WES, whole-exome sequencing; PCC, pheochromocytomas; PGL, paraganglioma.

identified for II-1; the corresponding figures for III-3 were 134 and 32 (*Table 1*). Then, 5'-prime-UTR, 3'-prime-UTR, intronic and synonymous variants were excluded, remaining 12 SNP and 1 InDel variants for both II-1 and III-3 (*Table S1*). Next, we used a stringent filtering algorithm, with which we removed variants with minor allele frequency (MAF) >0.1% in 1,000 genomes and ESP6500 databases. Finally, there were 2 genomic variants left, one in *VHL* and the other in *KMT2D*, in which were found in both of II-1 and III-5. The *VHL NM_000551.3*: *c.292 T > C* mutation was demonstrated as a pathologic mutation, whereas the *KMT2D NM_003482.3*: *c.12911 C > T* mutation was found in healthy individuals in the ExAC database (http://exac.broadinstitute.org/variant/12-49425577-G-A), and was not suggested to be a pathologic germline mutation of PCC.

To further exclude interference from other pathogenic genes of PCC/PGL, we performed copy number analysis and discovered that there were no copy number variants identified in genes related to PCC/PGL. Moreover, SDHB IHC was positive in the four patients of this family, ruling out the possibility of *SDHB* mutation again (*Figure 3*).

Eventually, Sanger sequencing revealed that in addition to the proband and his mother, 13 other family members had carried the *VHL Y98H* mutation, of which 2 members were PCC and 11 members remained healthy at the end of this study (*Figure 2*).

Genotype-phenotype associations

In the present family, 15 members (54%) from 4 generations

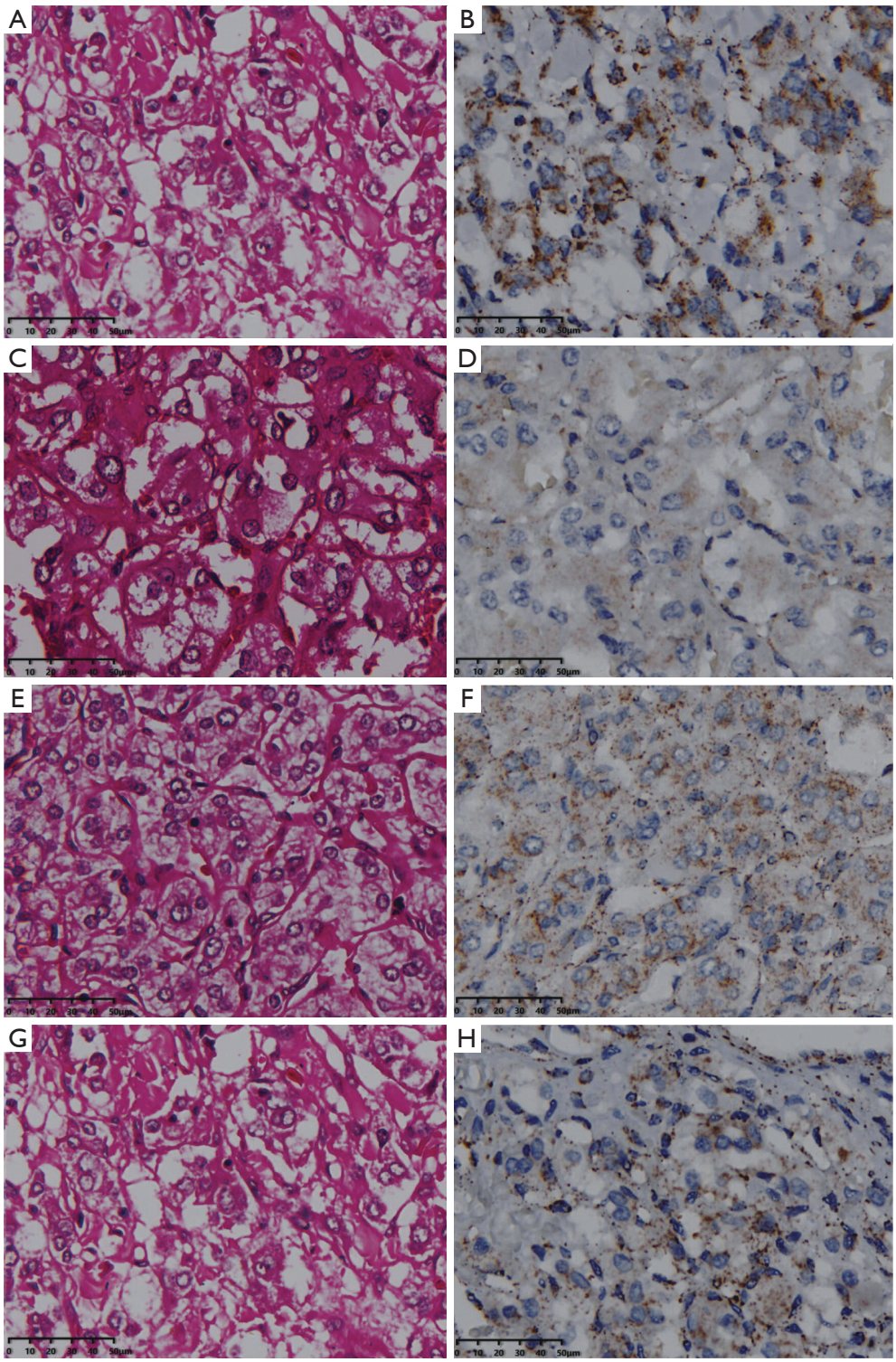

Figure 3 HE staining and SDHB IHC of the patients (A, C, E and G are HE staining images from II-1, III-2, III-3 and III-4; B, D, F and H are SDHB IHC images from II-1, III-2, III-3 and III-4, respectively).

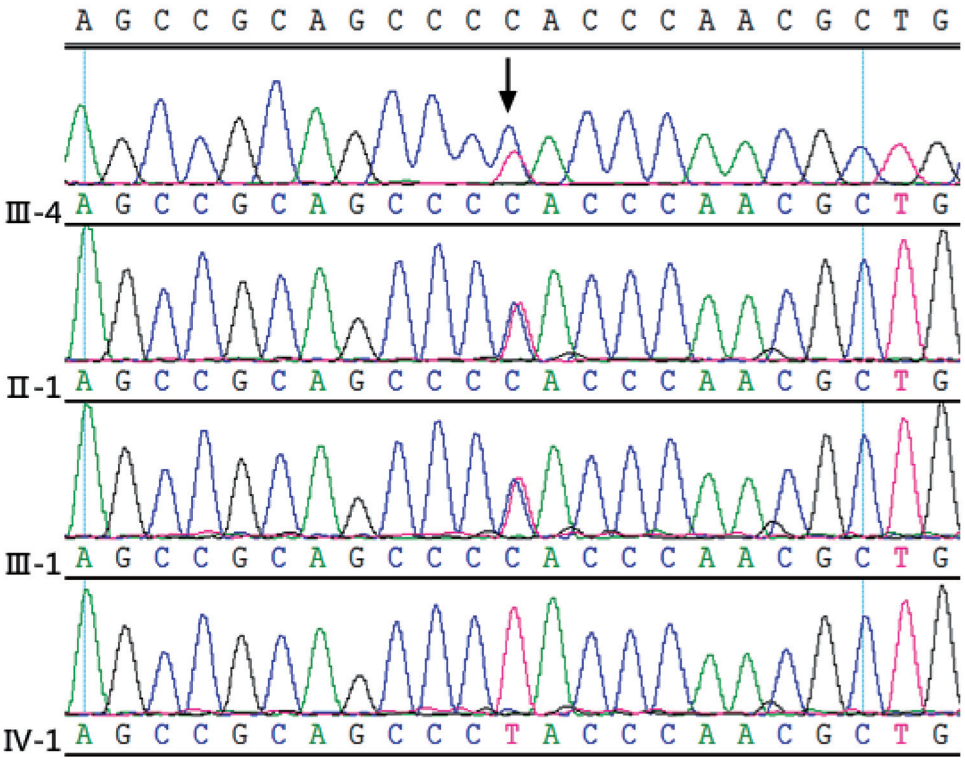

Figure 4 Base sequence of *VHL Y98H* mutation (black arrow) (from III-4, II-1 and III-1, the individuals with the *VHL Y98H* mutation) and corresponding normal sequence (from IV-1, the individual without the *VHL Y98H* mutation) by Sanger sequencing. VHL, Von Hippel-Lindau.

were identified to have the *VHL Y98H* mutation, but only 4 members were diagnosed with PCC (*Figures 2*,*4*). Most of the mutation-positive individuals have had VHL-related check-ups subsequently, and clinical presentations of the mutation-positive individuals are described (*Table 2*).

CNS haemangioblastomas are cardinal features of VHL disease and occur in 60–80% of VHL patients (21,22). Therefore, the medical history of each mutation carrier was obtained again. Initially, II-1 recalled that she had sudden blindness in her right eye approximately 10 years ago, and a RA, which has an identical histopathological appearance to a CNS haemangioblastomas, was eventually diagnosed; however, her other examination results were negative.

Additionally, III-2 was the first diagnosed PCC patient. The lesion in her liver was shown to be a haemangioma by ultrasound-guided percutaneous biopsy. Several rounds of radiofrequency ablation were performed on the benign lesion. Related check-ups were also performed on III-2, III-3 and III-4, and CNS magnetic resonance, fundus examination and abdominal ultrasonography results were negative except for a hepatic hemangioma that was also found on III-4 (*Table 2*).

The oldest member with *VHL Y98H* mutation, I-1, was 80 years old. She refused to do any check-ups because she felt she was healthy and could not accept any potentially bad results from the check-ups. The II-2, II-3, II-4, II-5, III-1, III-6 and III-8 individuals were healthy adults with *VHL Y98H* mutations. They felt healthy, and several examinations were conducted that showed no presence of VHL-related symptoms.

IV-2, IV-3 and IV-6 individuals were 10, 12 and 5 years old, respectively. All of them were healthy individuals with *VHL Y98H* mutations because they were too young to form any VHL-related tumors.

Overall, among all the family members with *VHL Y98H* mutation, II-1 was a VHL type 2A patient; III-2, III-3 and III-4 were VHL type 2C patients; and the other eleven individuals were asymptomatic.

Discussion

In this study, the *VHL* "Black Forest" mutation that was

Table 2 Clinical features of family members with *VHL Y98H* mutation

Patient	Age	HP	PCC	CNS hemangioma	Retinal angioma	ccRCC	Pancreatic neuroendocrine tumor	Pancreatic and renal cysts	Other lesions
I-1	80	No	N/A	N/A	N/A	N/A	N/A	N/A	N/A
II-1	61	Persistent	Right	No	Right	No	No	No	N/A
II-2	53	No	No	No	No	No	No	No	N/A
II-3	53	No	No	No	No	No	No	No	N/A
II-4	57	No	No	No	No	No	No	No	N/A
II-5	55	No	No	No	No	No	No	No	N/A
III-1	41	No	No	No	No	No	No	No	N/A
III-2	34	No	Bilateral	No	No	No	No	No	Hepatic hemangioma
III-3	33	No	Right	No	No	No	No	No	N/A
III-4	31	No	Bilateral	No	No	No	No	No	Hepatic hemangioma
III-6	28	No	No	No	No	No	No	No	N/A
III-8	26	No	No	No	No	No	No	No	N/A
IV-2	10	No	No	No	No	No	No	No	N/A
IV-3	12	No	No	No	No	No	No	No	N/A
IV-6	5	No	No	No	No	No	No	No	N/A

HP, hypertension; PCC, pheochromocytoma; CNS, central nervous system; ccRCC, clear-cell renal cell carcinoma; N/A, not available.

believed to have evolved in the white population was identified in a large Chinese family for the first time. Furthermore, clinical features and genotype-phenotype associations of patients with *VHL Y98H* mutation were summarized and analysed.

VHL disease is an autosomal dominant inherited neoplastic syndrome caused by inactivation of the *VHL* tumor suppressor gene mapped to chromosome 3p25.3 (2,23). The clinical diagnosis of VHL disease is traditionally described as follows: (I) at least two haemangioblastomas in the CNS or retina; (II) one haemangioblastoma in the CNS or retina plus at least one VHL-related visceral tumor (excluding epididymal and renal cysts); (III) any one of above items with a family history of VHL (24). Apart from the diseases mentioned above, other VHL-related visceral tumours include endolymphatic sac tumours occurring from head and neck, pancreatic cysts, serous cystadenomas, pancreatic neuroendocrine tumours (NETs), renal cysts, ccRCCs, epididymal cysts, papillary cystadenomas of the epididymis and broad ligament cystadenomas, indicating the variability and complexity of VHL disease (25). Therefore, when diagnosing VHL disease, a systematic examination should be done to avoid a missed diagnosis of VHL-related visceral tumors. In addition, the occurrence of multiple diseases is more conducive to the diagnosis.

Different mutations cause different types of VHL disease. Patients with truncating mutations, exon deletions, or missense mutations that disrupt the integrity of the VHL protein structure probably present with type 1 VHL disease, but individuals with type 2 VHL disease would carry the surface missense mutations (mutation site at the surface of the VHL protein) (24-26). *VHL Y98H* is a surface missense mutation, whose carriers have been widely regarded as type 2A. The *VHL* Y98H mutation was also called the "Black Forest" founder mutation (14). In the past, this mutation was reported only in the Occident (*Table 3*). However, the present study is the first report of a large Chinese family with *VHL Y98H* mutations, proving that there is no geographical limitation for this mutation. In fact, approximately 20% of patients have VHL disease as the result of a *de novo* mutation, which means *de novo* mutation at codon 98 can occur independently of the race

Table 3 Black Forest mutation cases obtained from PubMed

Series	Cases	Nation	Age*	RA	CNS	PCC	ccRCC	OIs
Brauch *et al.*, 1995 (14)	61	Germany	N/A	31	12	43	4	0
Chen *et al.*, 1995 (16)	55	America	N/A	26	5	30	0	0
Boedeker *et al.*, 2009 (4)	2	NM* (not Asia)	20.5 [7–34]	0	0	2	0	2
Klingler *et al.*, 2013 (15)	2	Germany	N/A	1	1	2	0	2
Gaal *et al.*, 2009 (5)	1	Germany	7	0	0	0	0	1
Crona *et al.*, 2015 (27)	1	Sweden	48	N/A	N/A	N/A	N/A	N/A
Glasker *et al.*, 1999 (28)	21	Germany	N/A	N/A	N/A	N/A	N/A	N/A
Dahia *et al.*, 2014 (29)	2	America	N/A	N/A	N/A	N/A	N/A	N/A
Crossey *et al.*, 1994 (6)	1	UK	N/A	N/A	N/A	N/A	N/A	N/A

Age*, mean age at first diagnosis; RA, retinal angioma; CNS, central nervous system haemangioblastoma; PCC, pheochromocytoma; ccRCC, clear-cell renal cell carcinoma; OIs, other lesions; N/A, not available; NM*, not mentioned (from one of the following countries: Germany, France, Spain or the Netherlands).

or of the geographic location. Therefore, the occurrence of this mutation is likely to be random. Furthermore, there are no distinct differences in clinical manifestations for the present patients compared with former cases (*Table 3*). However, there are still a small number of patients with *Y98H* mutations having ccRCCs, indicating that the genotype-phenotype mismatch of *VHL Y98H* mutations is not impossible. In addition, the similar mutation in the same codon, *VHL Y98N* mutation, is associated with type 2B (high risk of ccRCC). Knauth *et al.* found that the *Y98H mutation* results in higher binding affinities for key cellular substrates when compared to the *Y98N* mutation, indicating the internal mechanism of the difference between the two types of manifestations (30,31).

It is recommended that all *VHL* mutation carriers should be offered surveillance in which regular follow-up visits were adopted to check each organ involved in the VHL disease. To be specific, (I) ophthalmoscopy is recommended to be done yearly from infancy; (II) plasma or urinary catecholamines and metanephrines should be done (yearly and when blood pressure is raised) from 2 years of age; (III) MRI of craniospinal axis is recommended to be done yearly from 11 years of age; (IV) ultrasound of abdomen is advised to be done yearly from 8 years of age; and (V) CT of abdomen should be done yearly from 18 years of age or earlier if it is clinically indicated (13). As it is widely documented that many mutations are related to its clinical types, surveillance could also be done according to the genotype of VHL patients, suggesting that genetic consulting is imperative for VHL patients. As a result, personalized surveillance has been recommended to the family members with *VHL Y98H*, especially for the younger ones (IV-2, IV-3 and IV-6).

In the present study, WES and Sanger sequencing were utilized to diagnose VHL disease, though they are not often used to diagnose VHL disease in China. We were initially unable to confirm presence of a pathogenic gene of the PCC family. Therefore, WES was performed because it was more efficient than Sanger sequencing to screen all susceptible genes. Moreover, WES of two or more familial patients could exclude non-shared mutations to narrow the scope of candidate genes. In addition, combination of WES and Sanger sequencing offers the opportunity to detect pathogenic mutations in familial and sporadic PCC cases, especially for clinically atypical or asymptomatic individuals. Meanwhile, with the achievements of genotype-phenotype studies, precision medicine of PCC and VHL was estimated to have a bright future, such as treating tumours in a timely fashion.

In conclusion, we report the first recorded Chinese VHL type 2 family with the "Black Forest" mutation using WES and Sanger sequencing, thus widening the currently recorded presence of the "Black Forest" mutation to China and potentially elsewhere in Asia and indicating that the "Black Forest" mutation does not uniquely evolve in occident countries. In addition, a personalized surveillance approach, which may be more appropriate for affected families, has been recommended to improve quality of life.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was approved and supervised by the Ethics Committee of Xiangya Hospital (No. 201403307), and informed written consents were signed by each participant.

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Supplementary

Table S1 Variants after first two steps of filtration

Consequence	Gene	Impact	Mutation frequency in 1000G_ALL	Chr	Chromosomal position	Reference	Variant	Mutation frequency in ESP6500_ALL
SNP (II-1)								
missense_variant	*MEN1*	Moderate	0.834465	chr11	64572018	T	C	0.91
missense_variant	*BRCA1*	Moderate	0.355831	chr17	41223094	T	C	0.3
missense_variant	*BRCA1*	Moderate	0.352636	chr17	41244000	T	C	0.3
missense_variant	*BRCA1*	Moderate	0.335663	chr17	41244435	T	C	0.28
missense_variant	*BRCA1*	Moderate	0.54393	chr17	41244936	G	A	0.49
missense_variant	*BRCA2*	Moderate	0.975839	chr13	32929387	T	C	0.98
missense_variant	*ATRX*	Moderate	0.552318	chrX	76937963	G	C	0.51
missense_variant	*KMT2D*	Moderate	–	chr12	49425577	G	A	–
missense_variant	*VHL*	Moderate	–	chr3	10183823	T	C	–
splice_region_variant	*SDHA*	Low	0.0105831	chr5	256451	C	T	–
missense_variant	*SDHD*	Moderate	0.945487	chr11	111963860	A	G	–
missense_variant	*MDH2*	Moderate	0.522165	chr7	75677504	C	T	0.39
SNP (III-3)								
missense_variant	*MEN1*	Moderate	0.834465	chr11	64572018	T	C	0.91
missense_variant	*BRCA2*	Moderate	0.975839	chr13	32929387	T	C	0.98
missense_variant	*BRCA1*	Moderate	0.355831	chr17	41223094	T	C	0.3
missense_variant	*BRCA1*	Moderate	0.352636	chr17	41244000	T	C	0.3
missense_variant	*BRCA1*	Moderate	0.335663	chr17	41244435	T	C	0.28
missense_variant	*BRCA1*	Moderate	0.54393	chr17	41244936	G	A	0.49
missense_variant	*KMT2D*	Moderate	–	chr12	49425577	G	A	–
missense_variant	*ATRX*	Moderate	0.552318	chrX	76937963	G	C	0.51
missense_variant	*VHL*	Moderate	–	chr3	10183823	T	C	–
missense_variant	*SDHA*	Moderate	0.003195	chr5	224487	T	C	–
missense_variant	*SDHD*	Moderate	0.945487	chr11	111963860	A	G	–
missense_variant	*MDH2*	Moderate	0.522165	chr7	75677504	C	T	0.39
InDel (II-1)								
splice_region_variant	*EPAS1*	Low	–	chr2	46583283	T	TCT	0.58
InDel (III-3)								
splice_region_variant	*EPAS1*	Low	–	chr2	46583283	T	TCT	0.58