Identification of a *VHL* gene mutation in a Chinese family with Von Hippel-Lindau syndrome

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Received October 17, 2017; Accepted April 16, 2018

DOI: 10.3892/mmr.2018.8974

Abstract. Von Hippel-Lindau (VHL) syndrome is an autosomal dominant neoplastic disorder. The VHL tumor suppressor (*VHL*) gene has previously been identified to represent the causative gene of VHL. Previous studies have demonstrated that >506 different mutations in *VHL* are associated with VHL syndrome. The aim of the present study was to determine the *VHL* gene mutation present in a VHL syndrome pedigree and to investigate the pathogenesis of the mutant protein. Briefly, a family suffering from VHL syndrome in a Chinese Han population was recruited, and a missense mutation (c.345 C>A: p.H115Q) was revealed to be present within the *VHL* gene in the proband. Furthermore, Sanger sequencing revealed two carriers of the mutation within the family. The results of the present study also demonstrated a mutation in *VHL* associated with the VHL syndrome phenotype, which may be of future therapeutic benefit for the diagnosis of VHL syndrome. These results may also be relevant to further studies aiming to investigate the molecular pathogenesis of VHL syndrome.

Introduction

Von Hippel-Lindau (VHL) syndrome (Online Mendelian Inheritance in Man:193300) is an autosomal dominant neoplastic disorder. VHL syndrome is characterized by retinal and central nervous system haemangioblastomas, clear cell renal cell carcinoma (RCC), pheochromocytoma (PCC), pancreatic islet tumors and endolymphatic sac tumors (1). Furthermore, renal and pancreatic cysts as well as epididymal or broad ligament cystadenomas are commonly associated with these tumors. VHL syndrome can be classified into four groups based on phenotypic characteristics: Types 1, 2A, 2B and 2C. PCCs are not present in type 1; types 2A and 2B may exhibit PCCs and are distinguishable by the risk of additional accompanying RCCs; and type 2C presents with RCCs alone (2-4).

The prevalence of VHL Syndrome is 1 in every 36,000 live births (5). It has previously been established that mutations in the VHL tumor suppressor (*VHL*) gene are causative of VHL syndrome. Latif *et al* (6) cloned *VHL* in 1993 and Seizinger *et al* (7) located it to chromosome 3p25-p26. The penetrance of VHL syndrome in affected populations is estimated to be >90% by 60 years old (8).

VHL encodes a 4.7 kb mRNA, which produces two VHL tumor suppressor protein (pVHL) products: pVHL19 and pVHL30 (9). pVHL contains two functional domains: α-domain and a β-domain. The α-domain binds to elongation factors B and C (10). The β-domain functions as the pVHL recognition domain, and has an important role in cellular oxygen sensing via targeting of hypoxia-inducible factors (HIFs) for ubiquitination and proteasomal degradation; a mechanism that has been previously demonstrated to be involved in the pathogenesis of cancer (10). HIFs have an important function in the transcriptional response to changes in oxygen availability. When associated with an E3 ligase complex, pVHL regulates the stability of HIFs via its recognition domain. HIF-1α binding to pVHL is dependent on a 20-residue oxygen-dependent degradation (ODD) domain (11,12). Hydroxylation of a core proline residue (HIF-1α-P564) within the ODD domain is necessary for this binding (10-13).

Studies have demonstrated that mutant VHL proteins exhibit abnormal interactions with HIF-α proteins: The pVHL-R167Q mutant retains the ability to bind strongly to HIF-1α, whereas pVHL-W88S and pVHL-L158P exhibit marked decreases in HIF-1α binding activity (10,14). This illustrates the importance of wild-type pVHL in HIF-1α-induced carcinogenesis.

In the present study, Sanger sequencing was performed to investigate all exons of *VHL* in a Chinese family suffering from VHL syndrome, and the results revealed a heterozygous missense mutation responsible for a functional deficit in pVHL.

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Key words: Von Hippel-Lindau syndrome, Von Hippel-Lindau gene, mutation, gene, genetics

Materials and methods

Subject recruitment and clinical examination. A family with VHL syndrome from a Han Chinese population was recruited for the present study in Hunan Cancer Hospital (Changsha, China) between May 2014 and June 2014. There were originally 23 patients in this pedigree; however, 6 patients succumbed to mortality prior to recruitment. In total, 17 subjects [including 8 males and 9 females; aged 4-65 years old; one patient suffering from VHL syndrome (proband)] were enrolled and underwent detailed clinical examinations, including the Romberg sign test, in which the standing patient is asked to his/her close eyes with their heels together and if the patient loses balance, a positive result is recorded (15). In addition, finger-nose tests were performed, which requires the patient to alternately touch his/her nose and the examiner's finger as quickly as possible. If the patient fails to touch his/her nose accurately, a positive result is recorded (15). All members of the family were examined by two experienced surgeons. Diagnoses of VHL syndrome were determined via investigation of medical histories and performance of physical examinations, including a complete neurological examination and brain magnetic resonance imaging (MRI). In addition, 200 unrelated volunteers (aged 35-65 years old; 116 males and 84 females) that did not have VHL syndrome or present any associated phenotypes were recruited as controls in the Health Management Center, Xiangya Hospital of Central South University (Changsha, China) between September 2014 and October 2014. The present study was approved by the Institutional Review Board of the Xiangya Hospital of Central South University, and all subjects enrolled in the present study provided written informed consent prior to examination.

Immunostaining. Biopsy tissues isolated from the intracranial cyst of the proband were obtained via surgical excision, fixed in 4% paraformaldehyde at room temperature for one week and then embedded in paraffin. Histopathological analysis of paraffin-embedded biopsy tissue sections (6 μm) from the proband was performed using a Hematoxylin and Eosin Staining Kit (cat. no. M020; GEFAN Biotechnology, Co., Ltd., Shanghai, China) (16). Sections were blocked with 5% horse serum (cat. no. HQ90041; Shanghai Bangyi Biotechnology Co., Ltd., Shanghai, China) at room temperature for 30 min and subsequently incubated at 4˚C overnight with antibodies against glial fibrillary acidic protein (GFAP; 1:200; cat. no. KIT-0031), epithelial membrane antigen (EMA; 1:80; cat. no. KIT-0011), S-100 protein, E3 SUMO-protein ligase nse (NSE; 1:100; cat. no. MAB-0584), hematopoietic progenitor cell antigen CD34 (CD34; 1:50; cat. no. KIT-0004), Factor VIII-related Antigen (F8; 1:100; cat. no. RAB-0070) and proliferation marker protein Ki-67 (Ki-67; 1:100; cat. no. MX006) (16). All primary antibodies were purchased from Fuzhou Maixin Biotech Co., Ltd. (Fuzhou, China). Following this, sections were incubated with horseradish peroxidase-conjugated anti-mouse/rabbit secondary antibodies (working dilution was provided by the kit; cat. no. KIT-5010; Fuzhou Maixin Biotech Co., Ltd.) at room temperature for 2 h. Images of the sections were captured using Photographs were taken using a light microscope Olympus BX51 (Olympus Corporation, Tokyo, Japan; magnification x400) and a Nikon N6006 camera (Nikon Corporation, Tokyo, Japan). The number of positive cells was calculated as the total area of positive staining divided by the area of an individual positive cell using Image Pro Plus software (version 6.0; Media Cybernetics, Inc., Rockville, MD, USA). The percentage of positive cells was calculated as the number of positive cells divided by the total number of cells.

Genomic DNA preparation and molecular analysis. Genomic DNA was isolated from peripheral blood lymphocytes of all subjects using the standard phenol-chloroform method (17). VHL was amplified via polymerase chain reaction (PCR). Three pairs of primers were designed to cover all of the exons and splicing junctions of VHL (Table I). The thermocycling conditions of PCR used were as follows: Initial Denaturation at 95˚C for 30 sec; followed by 30 cycles of denaturation at 95˚C for 30 sec, annealing at 58˚C for 30 sec and elongation 72˚C for 30 sec; and a final extension at 75˚C for 5 min. PCR amplification was performed using an ABI 2720 PCR system (Thermo Fisher Scientific, Inc., Waltham, MA, USA) using a 10 μl mixture containing premixed DNA polymerase (5 μl; Takara Biotechnology Co., Ltd., Dalian, China), primers (2 μl), genomic DNA (1 μl) and double distilled H_2O (2 μl). PCR products were detected using 6% polyacrylamide gel electrophoresis with silver staining. Sanger sequencing was performed using an ABI 3100/3130 Genetic Analyzer automated sequencer (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The sequencing results were analyzed using SeqMan 3.0 (DNASTAR, Inc., Madison, WI, USA), and compared with the reference sequences listed in the National Center for Biotechnology Information database (https://www.ncbi.nlm.nih.gov/). Mutation naming followed the nomenclature recommended by the Human Genomic Variation Society (http://www.hgvs.org/).

RNA preparation and RNA stability investigation. Total RNA was isolated from lymphocytes obtained from the patient using a GeneJET RNA Purification Kit (Fermentas; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. First strand complementary (c)DNA was synthesized from total RNA at 42˚C for 60 min and then at 70˚C for 5 min using a RevertAid First Strand cDNA Synthesis Kit (Fermentas; Thermo Fisher Scientific, Inc.), according to the manufcaturer's instructions. Primer sequences for semi-quantitative (sq)PCR were designed using Primer 3 (version 4.1.0; http://primer3.ut.ee/) and were as follows: pVHL forward, 5'-CTCCCAGGT CATCTTCTGCA-3' and reverse, 5'-CTTGACTAGGCTCCG GACAA-3'. sqPCR procedures were performed according to the following protocol: Initial denaturation at 95˚C for 30 sec; followed by 10 cycles at 95˚C for 30 sec, 62˚C for 30 sec and 72˚C for 30 sec; a further 23 cycles at 95˚C for 30 sec, 59˚C for 30 sec and 72˚C for 30 sec; and a final extension at 75˚C for 5 min. Following this, 5% agarose gel electrophoresis was performed to verify the PCR products. Proteins were visualized using ethidium bromide. Normality of distributions was determined using the Shapiro-Wilk test. Statistical significance was assessed with an unpaired two-tailed Student's t-test, using GraphPad Prism 6.0 (Graphpad Software, Inc.,

Table I. Primers used for amplification of the Von Hippel-Lindau disease tumor suppressor gene.

Primer	Primer sequences
VHL-1F	AGCGCGTTCCATCCTCTAC
VHL-1R	AAGATTGGATAACGTGCCTGA
VHL-2F	GGAGAAAATAGGTGCCCTGAC
VHL-2R	AAGATTGGATAACGTGCCTGA
VHL-3F	GCAAAGCCTCTTGTTCGTTC
VHL-3R	GCCACCACCTTCTCCTGATA

F, forward; R, reverse; VHL, Von Hippel-Lindau disease tumor suppressor.

La Jolla, CA, USA). Data are presented as mean ± standard deviation.

Bioinformatics analysis. Lasergene MegAlign 9.0 (DNAStar, Inc.) was used to investigate whether the identified mutation of pVHL was conserved between different species. MutationTaster (http://mutationtaster.org), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/index.shtml) and SIFT (http://sift.jcvi.org) software, all available online, were selected to investigate the effect of the mutated amino acid on the protein's structure and function. SWISS-MODEL (https://swissmodel.expasy.org) and PyMol software (version 1.5.0.3; DeLano Scientific, San Carlos, CA, USA) were used to predict and visualize three-dimensional protein models.

Results

Clinical evaluations. A four-generation family with diagnosed VHL syndrome was recruited for the present study. The family consists of 23 individuals, including 11 males and 12 females (Fig. 1). The proband was a 28-year-old male, who presented with an episodic headache for 10 years. Both the finger-nose test and the Romberg's sign were recorded as positive in the physical examination. A cyst and a nodule were revealed via brain MRI (Fig. 2). The cyst was demonstrated to be a hemangioblastoma tumor following surgical excision followed by histopathological analysis of the biopsy tissues. H&E staining revealed that the lesion contained a large number of thin-walled capillaries (Fig. 3). The sole neoplastic element, known as the 'stromal cell', inhabited the interstices between ramifying vascular arcades. Stromal cells stained for S-100 and NSE exhibited dense-core intracytoplasmic granules, however did not demonstrate expression of GFAP or EMA (Fig. 3), which has previously been detected immunohistochemically in most nonneoplastic epithelia (18). Stromal cells did not exhibit positive staining for endothelial markers such as CD34, whereas the thin-walled capillaries were positive; stromal cells occasionally exhibited the expression of vascular marker F8 (Fig. 3). The expression of Ki-67, which is a proliferation marker (19), was <1% (Range of normal value 1-2%; Fig. 3). Therefore, the proband was diagnosed with VHL syndrome at the Hunan Cancer Hospital (Changsha, China). A further five affected members of the family had previously died from this syndrome.

Mutation analysis. A heterozygous mutation c.345C>A (p.H115Q) in exon 2 of the *VHL* gene was revealed to be present in the proband III:2 (Fig. 4A). In addition, two healthy individuals (III:4 and III:8) in the pedigree were demonstrated to carry the mutation, as revealed by Sanger sequencing (Fig. 1). The mutation was not present in the 200 healthy patients.

Bioinformatics analysis. Alignment of VHL amino acid sequences in numerous species revealed that the histidine at the 115th amino acid site is highly conserved (Fig. 4B). The functional prediction software packages Polyphen-2, SIFT and MutationTaster; demonstrated that the variant is likely to be deleterious (data not shown). The structures of pVHL and p.H115Q, as predicted using SWISS-MODEL, were markedly different from one another: The wild type pVHL exhibited an imidazole ring structure (Fig. 4Ca), and the p.H115Q mutant exhibited a non-imidazole ring structure (Fig. 4Cb). Protein surface analysis revealed marked changes between the wild type pVHL and the p.H115Q mutant pVHL. In wild type pVHL, the area highlighted by the red arrow is non-polar charged and forms a continuous hydrophobic cleft, which facilitates its interaction with HIF-α (Fig. 4D). However, the H115Q mutant introduces a negative charge in this area (right panel), subsequently disrupting the hydrophobic environment required for binding with HIF-α (Fig. 4D).

Levels of VHL mRNA in the proband and healthy controls. cDNA was generated by reverse transcribing RNA extracted from peripheral blood samples obtained from patients in the pedigree and the healthy controls. No significant difference in the expression of pVHL was demonstrated between the mutated and wild type cDNA, as demonstrated by RT-sqPCR analysis (Fig. 5).

Discussion

VHL is located on chromosome 3p25-p26 and encodes a single 4.7 kb mRNA. To date, >506 mutations in the *VHL* gene associated with the development of VHL syndrome have been reported in the Human Gene Mutation Database (http://www.hgmd.org), the majority of which are deletion and missense mutations. The *VHL* gene and pVHL are widely expressed in tissues, thus tissue-specific expression cannot explain the complex tumor pattern in VHL syndrome (2).

VHL proteins exhibit numerous functions. A previous study demonstrated that the functions of pVHL include regulating the expression of HIFs, mediating the invasion and metastasis of tumor cells via the regulation of microtubules, and regulating the proliferation and apoptosis of cells (20). pVHL contains two domains, the α-domain and the β-domain (20). The α-domain is responsible for binding to elongation factor C, which contains three α helices located at amino acid residues 155-192; the β-domain is the substrate recognition region for pVHL, and is located at amino acid residues 63-154 (20). The α-domain binds to elongation factors B and C, and Cullin 2, and has an important role in the maintenance of the spatial conformation stability of pVHL (20). The β domain binds to the subunit of HIF and participates in the degradation of the

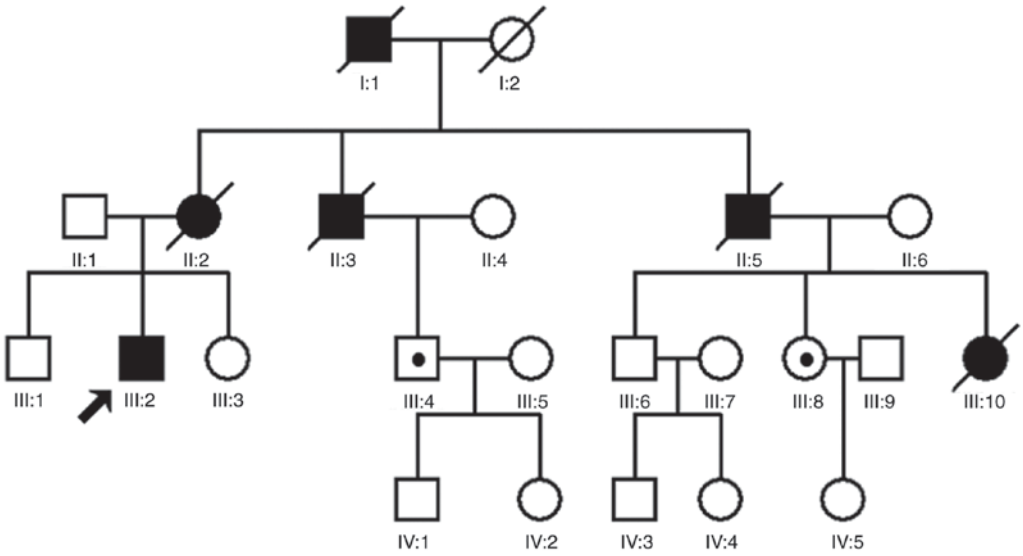

Figure 1. The pedigree of the family with Von Hippel-Lindau syndrome. Squares indicate males; circles represent females. Shading indicates affected individuals. A slash through the symbol indicates that the individual is deceased. The arrow indicates the proband. Dots indicate carriers of the variation.

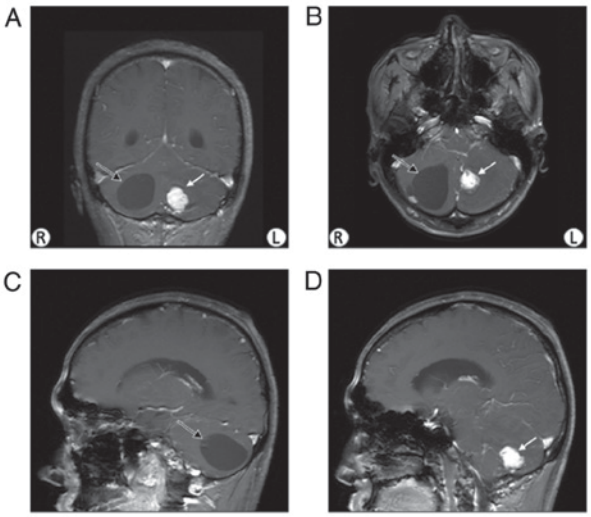

Figure 2. Magnetic resonance imaging of the brain of the proband. (A and B) Through coronal and axial scanning, a cyst was detected deep inside the right bilateral cerebellum, in addition to a nodule in the left side. (C and D) The sold cyst and the nodule were also detected in sagittal scanning. The white and black arrows indicate the solid cyst and the nodule, respectively.

HIF subunit under aerobic conditions (21). According to previous studies, the most prevalent mutations present in VHL syndrome are R167, R161, V155, Y98, G114, F76, P86, P81, L158 and C162 (20,22,23). The majority of the mutation sites are located within the HIF-1α and elongation factor C domains (24).

The stability of HIF-1α binding to pVHL may affect the degradation of the HIF protein, which may lead to tumorigenesis. Previous studies have demonstrated that hydroxylation of the P564 residue of HIF-1α has an important role in its binding to pVHL, and pVHL-S111 and pVHL-H115 are important regulators of this mechanism (10,12,13).

In the present study, a heterozygous c.345C>A (p.H115Q) mutation in *VHL* was successfully revealed via Sanger sequencing in the affected proband and gene carriers within the pedigree; however, the mutation was revealed to not be present within the non-affected members of the pedigree and the 200 healthy control patients. The RT-sqPCR results revealed that the expression levels of the mutant *VHL* were not significantly different compared with the control group and the patients with VHL.

Bioinformatics analysis using SIFT, PolyPhen-2 and MutationTaster revealed that the c.345C>A (p.H115Q) mutation is deleterious and thus suggested that it may be pathogenic. SWISS-MODEL software was used to investigate the three-dimensional structure of the protein and to compare wild-type VHL with the p.H115Q mutant, and it was revealed that the substitution of an alkaline histidine with a neutral glutamine changes the structure of pVHL from an imidazole ring to a non-imidazole ring. Furthermore, the mutant H115Q introduces a negative charge in this area, which subsequently disrupts the viral hydrophobic environment. This may explain why the interaction between HIF-α and VHL decreases dramatically following the H115Q mutation (14,25). The bioinformatics results in the present study suggested that the mutant protein may have also affected the binding ability of pVHL with other interacting proteins, thus affecting the biological function of the protein.

A previous study demonstrated that two steps are required for the initiation of carcinogenesis following gene mutation: The first step being the inheritance of a mutation via germinal cells, and the second step being a mutation arising in somatic cells (24,26). Thus, it was speculated that the development of VHL syndrome may follow this hypothesis. Furthermore, it can be suggested that the mutations in the somatic cells of two carriers (III:4 and III:8) in the pedigree investigated in the present study have not yet been activated, and thus may not produce an associated phenotype.

In addition, studies of *VHL* knockout mice have revealed an important role of pVHL in developmental biology. Homozygous $VHL^{-/-}$ mice die at 10.5-12.5 days post-birth due to placental angiogenesis dysfunction (27). $VHL^{+/-}$ heterozygous

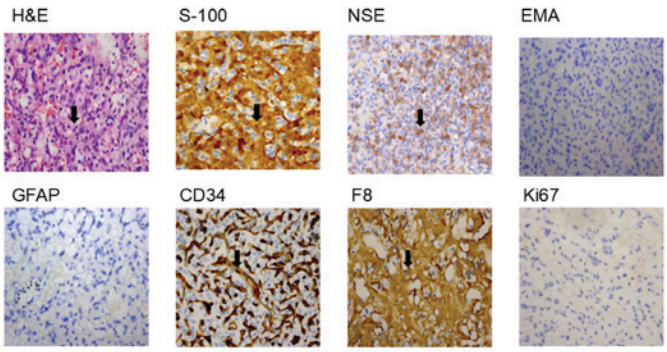

Figure 3. Histopathology of the biopsy tissues from the patient with VHL. Pathological analysis results from the cerebellum cyst tissue sections, examined via staining. Numerous small blood vessels, and a number of stroma cells, fat-like cells and fibroblasts were observed (magnification, x400). Immunohistochemical analysis of the tissue biopsy samples from the patient with VHL. HE, S-100, NSE and F8 were revealed to be present in cells; and EMA, GFAP, CD34 and Ki67 were revealed to be absent in cells (magnification, x400). Black arrows indicate histopathological changes. VHL, Von Hippel-Lindau; HE, hemagglutinin-esterase; S-100, protein S-100; NSE, E3 SUMO-protein ligase nse; F8, coagulation factor VIII; EMA, epithelial membrane antigen; GFAP, glial fibrillary acidic protein; CD34, hematopoietic progenitor cell antigen CD34.

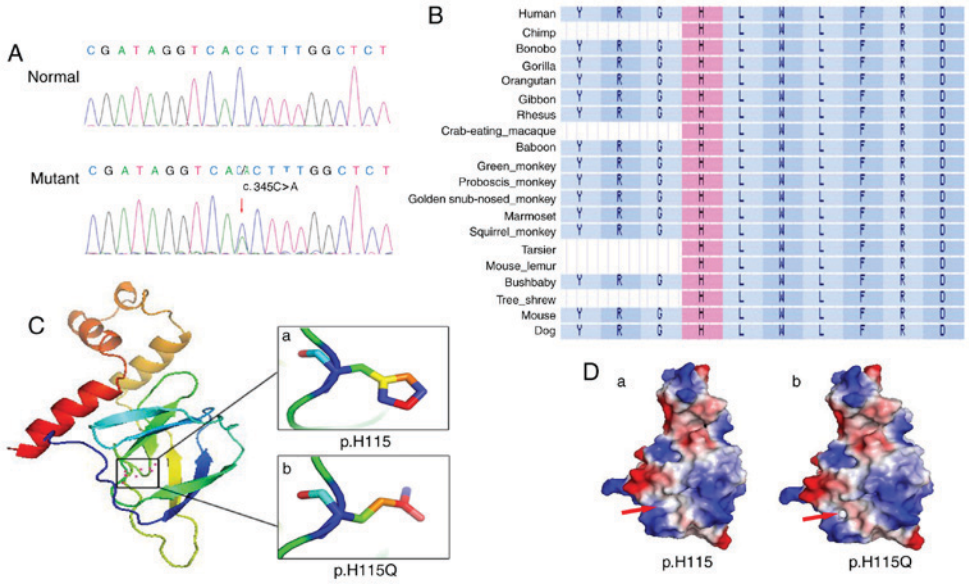

Figure 4. The VHL p.H115Q variant was analyzed using Sanger sequencing and bioinformatics analysis. (A) Mutation analysis of the *VHL* gene. The sequence chromatogram (red arrow) demonstrates a heterozygous C>A substitution mutation. (B) Evolutionary conservation of the p.H115Q residue. The *H* highlighted in pink represents the 115th amino acid position. (C and D) Three-dimensional protein structure, as predicted by modeling software. (Ca) An imidazole ring structure is present in the wild-type VHL protein, whereas a (Cb) non-imidazole ring structure is present in the mutant protein. (Da and Db) Significant changes to the surface of the mutant protein were observed, as indicated by the red arrow. VHL, Von Hippel-Lindau.

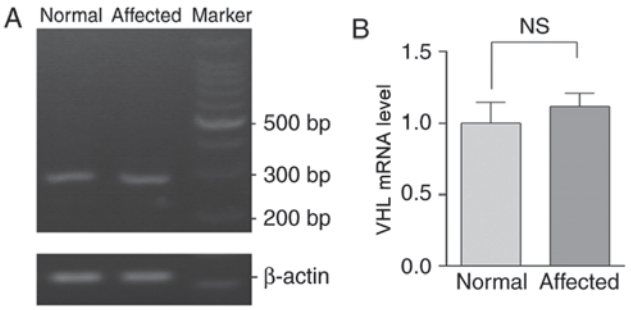

Figure 5. Mutations do not affect RNA stability. (A) PCR products were obtained via 5% agarose gel electrophoresis. (B) No statistically significant differences were demonstrated between the expression level of the mutant *VHL* gene in the patient with VHL, and the healthy controls. β-actin was selected as the loading control (n=3). NS, no significant difference; RT-PCR, reverse transcription-polymerase chain reaction; VHL, Von Hippel-Lindau; bp, base pairs.

mice develop vascular tumors of the liver (28). Further studies have confirmed that *VHL*-heterozygous mice tend to develop tumors with blood vessel hyperplasia phenotypes, and that pVHL is involved in regulating the expression of angiogenesis-associated genes, which are important for tumorigenesis (21,28).

In conclusion, a heterozygous p.H115Q mutation in the *VHL* gene was screened in a Han Chinese family with VHL syndrome. Despite this mutation having previously been revealed to cause VHL syndrome (28,29), it also has a great significance for validating the spectrum of gene mutations involved in the development of VHL syndrome.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Natural Science Foundation of Hunan Province, China (grant. no. 2017JJ3507), and by the Postdoctoral Science Foundation of Xiangya Hospital, Central South University, China.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZH recruited the family with VHL syndrome and diagnosed the proband with Von Hippel-Lindau syndrome. ZD collected peripheral blood lymphocytes from all individuals belonging to the family with Von Hippel-Lindau syndrome. LX and AL extracted DNA from peripheral blood lymphocytes, designed the primers and performed the Sanger sequencing. ZH and BL screened for the mutation site and performed the bioinformatics analysis. BL and ZH drafted the manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Review Board of the Xiangya Hospital of Central South University (Changsha, China), and all subjects enrolled in the present study provided written informed consent prior to examination.

Consent for publication

Written informed consent was provided.

Competing interests

The authors declare that they have no competing interests.

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