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CLINICAL STUDY

Clinical presentation and mutation analysis of VHL disease in a large Chinese family

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Abstract Von Hippel-Lindau (VHL) disease is an autosomal dominantly inherited neoplastic disorder characterized by marked phenotypic variability and age-dependent penetrance. This disease is caused by germline mutations in the VHL tumor suppressor gene. Systematic physical examinations, imaging assessments and molecular genetic tests for the VHL gene were performed in a large Chinese VHL family. The examined Chinese VHL family, which has 25 members from four generations, including 7 diagnosed VHL patients and 2 asymptomatic mutation carriers. The average ages of first onset for generations I, II, and III were 37, 30 and 16, respectively. The male:female ratio among VHL patients was 6:1. Molecular genetic investigations detected the c.433C>T [p.Q145X] nonsense mutation in the VHL gene. Molecular modeling of the VHL-EloninC- EloninB-HIF-1α complex predicted that the p.Q145X mutation markedly alters the L7 loop structure of the β-domain of the VHL protein (pVHL), destabilizes the VHL-HIF-1αcomplex, and induces the truncation of pVHL. We speculate that the p.Q145X nonsense mutation leads to relatively obvious familial aggregation. This mutation causes the type I phenotype of VHL disease and is associated with a high risk of retinal and central nervous system (CNS) hemangioblastomas (HGBs) and visceral cysts, but a low risk of renal cell carcinoma. Moreover, within a VHL family, the average ages of first onset became younger in successive generations, and the CNS HGBs are more likely to occur in male patients.

Keywords Von Hippel-Lindau disease ·
Hemangioblastoma · c.433C>T mutation · p.Q145X

Abbreviations
VHL	Von Hippel-Lindau
TSG	Tumor suppressor gene
pVHL	VHL protein
HGB	Hemangioblastoma
RCC	Renal cell carcinoma
HIF	Hypoxia-inducible factor
MRI	Magnetic resonance imaging
H&E	Hematoxylin and eosin,
PCR	Polymerase chain reaction

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Introduction

Von Hippel-Lindau (VHL) disease is an autosomal dominantly inherited neoplastic disorder, characterized by marked phenotypic variability and age-dependent penetrance. The incidence of VHL disease is estimated to be approximately 1/36,000 live births [1, 2]. The penetrance of this disease is greater than 90 % by the age of 65 [3], and the leading causes of death are central nervous system (CNS) hemangioblastoma (HGB) and renal carcinoma [2, 4].

The VHL tumor suppressor gene (TSG), which is located on the short arm of chromosome 3(3p25.3), consists of three exons. Due to alternative translation initiation

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at position 54, this gene encodes two VHL proteins (pVHLs): $pVHL_{30}$ (30 kDa in length, 213 amino acids) and $pVHL_{19}$ (19 kDa in length, 160 amino acids), which lacks the first 53 amino acids. Both protein isoforms have tumor suppressor effects [5, 6]. pVHL has two structural domains: an $\sim$100 residue amino-terminal domain rich in β-sheets (the β-domain) and a smaller carboxyterminal α-helical domain (the α-domain). The β-domain binds the α regulatory subunits of the transcription factors hypoxia-inducible factor-1 (HIF-1) and hypoxia-inducible factor-2 (HIF-2). The α-domain binds the Elongin B and Elongin C (Elongin BC) complex, then interacts with Cullin-2 and transfers to the E3 ubiquitin ligase protein complex, which binds (via two hydroxylated prolines) to the HIF-1α and HIF-2α regulatory subunits and targets these subunits for ubiquitylation and proteolytic degradation [7, 8]. Under hypoxia conditions involving no pVHL activity, HIF-1 and HIF-2 become stabilized and activate the hypoxic gene response, which consists of a large repertoire of target genes (e.g., VEGF, PDGF β, TGF α, CyclinD1, etc.) involved in diverse processes, such as angiogenesis, proliferation, apoptosis and metabolism.

According to Knudson's two-hit model [9], VHL disease is caused by germline and somatic mutations (two hits). In this case, the first hit is a germline mutation (a rearrangements, large deletion or point mutation) that inactivates one copy of the VHL gene in all cells [10]. The second hit involves a somatic mutation or deletion of the wild-type allele or the hypermethylation of this allele's promoter. In this study, we examine a large Chinese VHL family with 25 members across four generations that includes 7 VHL patients and 2 mutation carriers. Correlated genetic findings, clinical phenotypes and their internal connections are discussed.

Patients and methods

Patients

The proband (III-9) was a 14-year-old girl who presented with a 6-month history of back pain and chest tightness as well as 2 weeks of constipation-associated weakness in her left lower extremity. Magnetic resonance imaging (MRI) of the brain and spine revealed 3 enhancing lesions in the thoracic cord at T6-T8, T9 and T12 (Fig. 1f). Abdominal ultrasonography revealed multiple cysts in the pancreas and left adrenal gland (Fig. 1h). Because the patient had a family history of headaches, her family members were examined for VHL. The pedigree of her family is presented in Fig. 2a and the clinical features of the proband and her family members are indicated in Table 1. This study was approved by the ethic committees at local hospitals. All the living family members gave written informed consent for this genetic study.

Histology and immunostaining

Brain tumors were surgically removed from patients and postoperatively assessed for pathological confirmation. Sections of these tumors were fixed in formalin, processed, embedded in paraffin and stained with hematoxylin and eosin (H&E) for morphological evaluation.

DNA sequence analysis

DNA sequence analysis of the VHL gene was performed for the proband (III-9) and for her parents (II-11, II-12), sister (III-10), uncle (II-5), aunts (II-6 and II-7) and cousin (III-4). Written informed consent was obtained from all tested family members (clinically diagnosed VHL patients and asymptomatic relatives). Venous blood was obtained, and genomic DNA was isolated from peripheral blood leukocytes. The VHL gene was amplified by polymerase chain reaction (PCR). The following primers were used for both PCR and sequencing: 5′-TGGAAATACAGTAACGAGTTGG-3′ and 5′-GGCTTCAGACCGTGCTAT-3′, the forward and reverse primers for exon 1, respectively; 5′-TGGGATTACAGGTGTGGG-3′ and 5′-GAAGTCAGCAACAAATAAGC-3′, the forward and reverse primers for exon 2, respectively; and 5′-GGTAGTTGTTGGCAAAGC-3′ and 5′-TCAAAGAGGAGAAAGACTTG-3′, the forward and reverse primers for exon 2, respectively (Table 2).

The PCR amplification protocol included 35 cycles of 94 °C for 60 s, 60 °C for 60 s, and 72 °C for 60 s. Sanger DNA sequencing was performed. Products were sequenced using the ABI 3700 DNA Genetic Analyzer, and then assessed using DNAMAN.

Modeling the three-dimensional structure of pVHL

The coordinates of the HIF-1α-pVHL-EloninB-EloninC complex [11] were obtained from the RCSB protein data bank (PDB ID:1LM8). The corresponding three-dimensional image was generated using PyMOL Viewer [12].

Results

A general physical examination of the proband (III-9) revealed a weakened superficial abdominal reflex. A detailed neurological examination at the time of admission revealed an alert, intelligent, and cooperative teenage girl. Her muscle strength was grade IV in the left lower extremity but normal in the remaining limbs. MRI of the brain and spine revealed 3 enhancing lesions in the thoracic
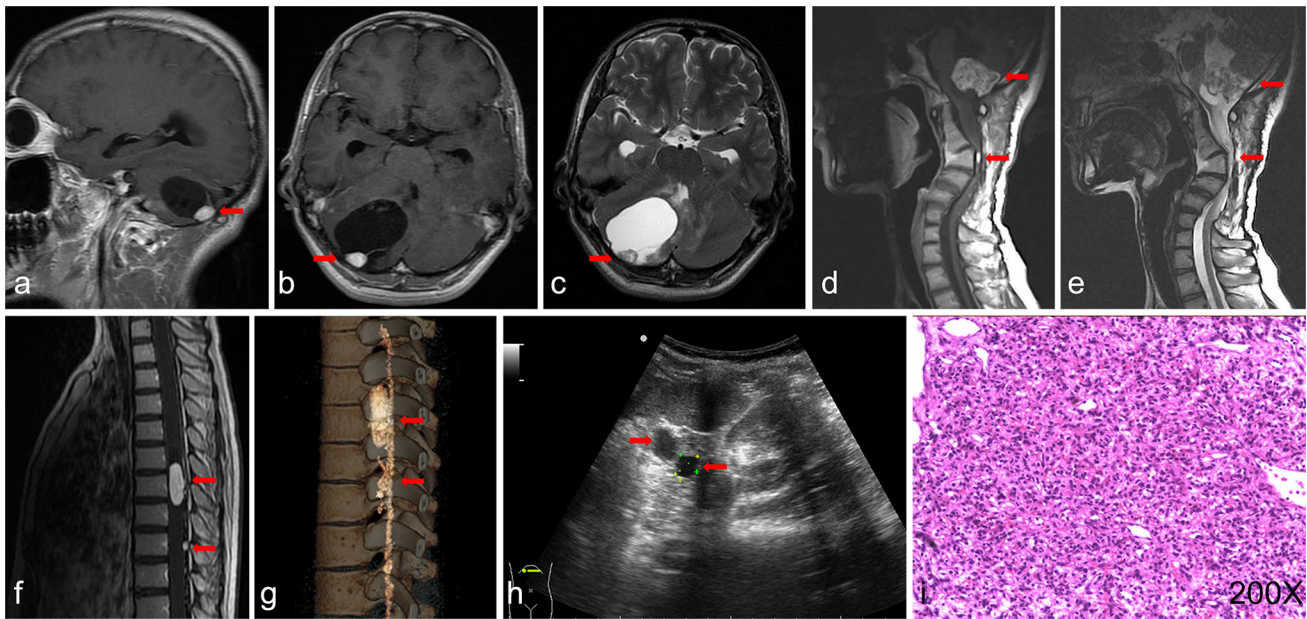
Fig. 1 Imaging and Pathological findings. **a–c** A right cerebellar hemangioblastoma with a large peritumoral cyst (sagittal and axial T1-weighted contrast-enhanced MRI and axial T2-weighted MRI). **d, e** Multiple hemangioblastomas in the cerebellar vermis and at the C-3 level (sagittal T1-weighted contrast-enhanced MRI and sagittal T2-weighted MRI). **f, g** Spinal hemangioblastomas (sagittal T1-weighted contrast-enhanced MRI and a multi-slice helical CT three-dimensional reconstruction). **h** Multiple pancreatic cysts (abdominal ultrasonograph). **i** Tumor sections from the proband were stained with H&E (original magnification: ×200)

cord at T6-T8, T9 and T12 (Fig. 1f). An abdominal ultrasonography revealed multiple cysts in the pancreas and left adrenal gland (Fig. 1h).

The T6-T8 and T9 tumors were resected. A diagnosis of HGB was comfirmed by the pathological evaluation. H&E staining revealed the characteristic HGB phenotype, which included a mixture of diffuse small spindle cells and clear cytoplasmic lipid-laden stromal cells within an extensive capillary network (Fig. 1i). The patient's clinical signs and symptoms had resolved by a 6-month follow-up examination.

The heterozygous nonsense mutation in exon 2 of the VHL gene (c.433C>T [p.Q145X]) was detected in 5 individuals, including 3 diagnosed patients (II-11, III-4, and III-9) and 2 asymptomatic mutation carriers (II-5, and III-10) (Fig. 2b). This mutation has previously been reported and is classified as pathogenic [13].

The pedigree and clinical features of the proband's family members were evaluated (Fig. 2a; Table 1); 7 of these individuals were diagnosed as VHL patients, and 2 of these individuals were classified as mutation carriers (II-5 and III-10). The ages of onset for VHL patients ranged from 12 to 37 years. The average ages of first onset for generations I, II, and III were 37, 30 and 16, respectively. The male : female ratio among VHL patients was 6:1 (Table 1).

Discussion

VHL disease, a progressive multisystem neoplastic disorder, is characterized by a tendency to develop retina and CNS HGBs, renal cell carcinoma (RCC), pheochromocytomas, visceral cysts, and other lesions [14]. Approximately 80 % of VHL patients have a multigenerational family history of the disease, and the remaining cases of VHL disease may result from a de novo mutation. In the previous situation, only a single simple manifestation (a CNS HGB or visceral lesion) is needed in order to diagnose this disease. However, 2 manifestations, including a CNS or retinal HGB, are required for a diagnosis of VHL disease in the absence of a confirmed family history. With the advent of genetic testing, the clinical diagnostic criteria for this disease have been improved [15].

In general, a VHL family includes several generations of VHL patients. Typically, complete and detailed information regarding the history of such families cannot be easily obtained. In the present case, the proband's grandfather (I-1) was diagnosed with a CNS HGB, and he was the only individual in his generation who was known to have VHL-associated lesions. He underwent a craniotomy and tumor resection at a local hospital, and pathological examination confirmed that his lesion was a HGB. In subsequent generations, VHL-associated tumors were found in several
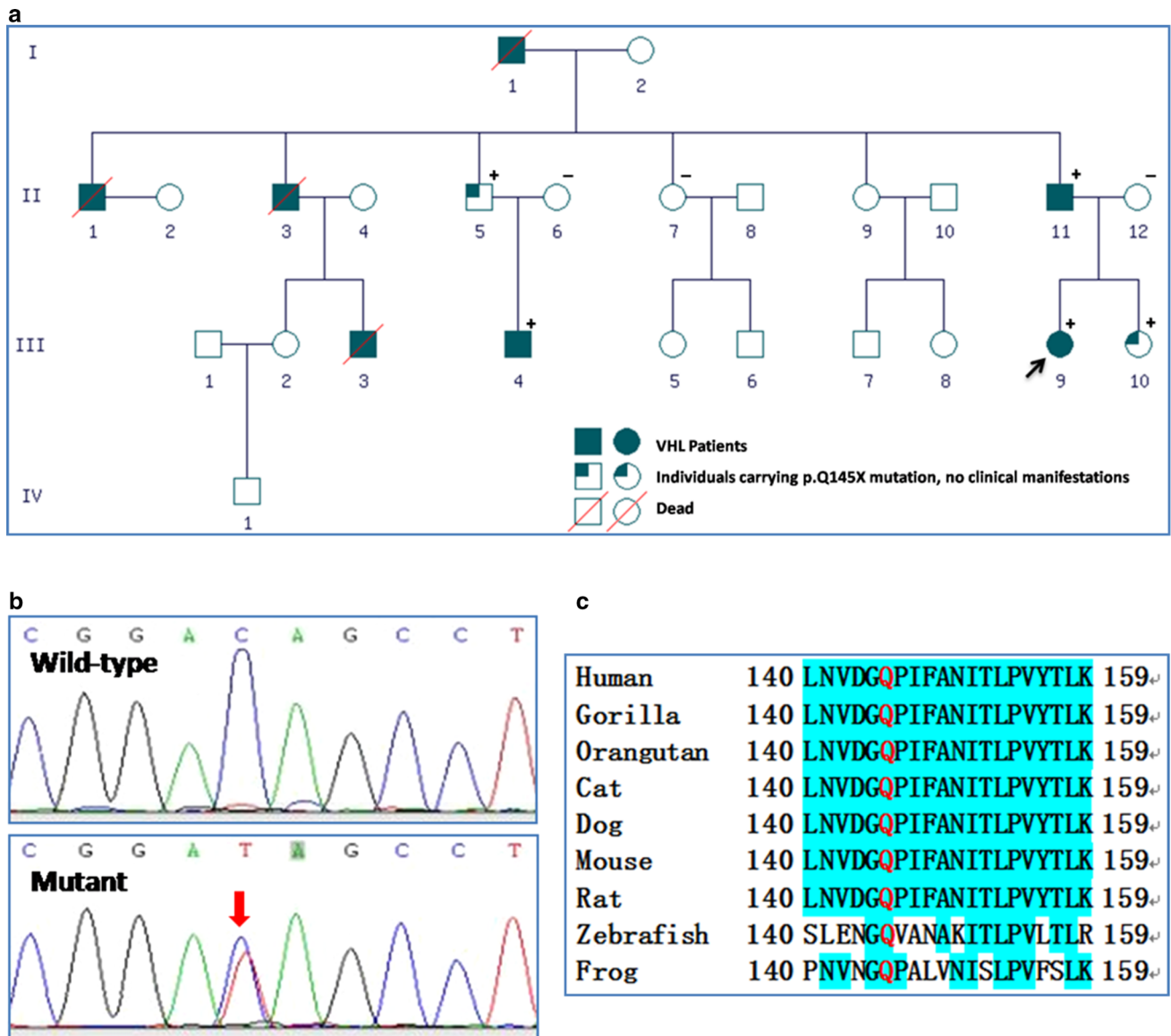
Fig. 2 Detection of the c.433C>T VHL gene mutation in the examined family. **a** The pedigree of the VHL family. **b** DNA sequence traces of a representative wild-type allele (*upper panel*) and a representative heterozygous c.433C>T mutation (*lower panel*) from family members. Relevant examinations were conducted, and DNA sequence analyses were performed. Therefore, a relatively intact VHL family pedigree was determined. The average ages of first onset became younger in successive generations. The male : female ratio for individuals with CNS HGBs indicates that the burden of a CNS HGB in VHL disease may be associated with male patients [16].

the examined family. **c** Multiple sequence alignment for pVHL, with the Gln145 residue indicated in red. The glutamine amino acid in this position is highly conserved across different species

DNA sequence analysis is an irreplaceable supplement to clinical diagnostic criteria, particularly with respect to facilitating the early diagnosis of VHL disease. Affected individuals with small asymptomatic VHL-associated tumors but no complications or metastases, and mutation carriers without tumors can now be detected via genetic recognition of the responsible mutation; thus, patients' survival rates and quality of life may improve. In this study, 4 diagnosed patients (I-1, II-1, II-3, and III-3) died before genetic testing was conducted, whereas testing revealed that 3 diagnosed patients (II-11, III-4, and III-9) and 2 asymptomatic mutation carriers (II-5, III-10) possessed the heterozygous p.Q145X nonsense mutation in exon 2 of the VHL gene (Fig. 2a).

The clear familial aggregation exhibited in the examed pedigree is closely related to the nonsense mutation c.433C>T [p.Q145X]. The fact that residue 145 in pVHL is

Table 1 Clinical features of patients with VHL disease

	I-1	II-1	II-3	II-11	III-3	III-4	III-9
Age at onset (years)	37	27	31	32	12	23	14
Mean age at onset for each generation (years)	37		30			16	
Age of death (years)	38	28	43	–	22	–	–
Sex	M	M	M	M	M	M	F
VHL gene sequence analysis	–	–	–	+	–	+	+
CNS hemangioblastomas	+	+	+	+	+	–	+
Pancreatic lesions	–	–	–	+	–	+	+
Renal cysts	–	–	–	+	–	–	–
Adrenal cysts	–	–	–	–	–	–	+

M male, *F* female

Table 2 Primers used for sequencing the VHL gene and their T_m values

Gene name	Sequence of primers	T_m (°C)	Amplicon (bp)
VHL exon 1	F:5′-TGGAAATACAGTAACGAGTTGG-3′	55.00	636
	R:5′-GGCTTCAGACCGTGCTAT-3′	55.00	
VHL exon 2	F:5′-TGGGATTACAGGTGTGGG-3′	53.00	570
	R:5′-GAAGTCAGCAACAAATAAGC-3′	53.00	
VHL exon 3	F:5′-GGTAGTTGTTGGCAAAGC-3′	52.00	731
	R:5′-TCAAAGAGGAGAAAGACTTG-3′	52.00	

evolutionarily conserved (Fig. 2c) suggests that this amino acid is important for maintaining the protein's structure and function. This codon is located within the β-domain, which is known to interact with HIF-1α. In addition, a recent computational study suggests that the canonical configuration of the wild-type β-domain is vital to the efficient function of the HIF-1α-pVHL-ElonginB-ElonginC complex and that the mutation of any of residues in the H-bond network at the binding site disrupts HIF binding [17]. Research has demonstrated that the focus for the dynamic organization of pVHL–HIF-1α recognition is the Gln145 residue within the L7 loop of the β-domain; in the tumorigenic mutant of pVHL, nearly all of the correlated dynamic motions are abolished [18].

Two different mutations of residue 145 have previously been reported: the c.435G>T (p.Gln145His) missense mutation, which was reportedly associated with sporadic RCC [18], and the c.433C>T [p.Q145X] nonsense mutation. Peppa et al. first found this germline nonsense mutation in a pedigree involving 2 VHL patients from 2 generations (the proband and her father). Both patients presented with type 1 VHL and exhibited a high risk of retinal and CNS HGBs and pancreatic cysts but a low risk of RCC. In accordance with this finding, individuals in the Chinese family examined in this study all presented a high frequency of CNS HGBs and visceral cysts but a low frequency of RCC or pheochromocytoma, among other manifestations. Moreover, our kindred, which consisted of 4 generations, involved more patients and a broader age range than the kindred examined by Peppa et al. All of the patients examined in this study were diagnosed with type 1 VHL, and none of these patients presented with RCC. This result further demonstrated that the c.433C>T [p.Q145X] nonsense mutation is associated with type 1 VHL and that the low risk of RCC among patients with this mutation is not caused by age-dependent penetrance. This phenomenon, which is inconsistent with Morgan's statement that nonsense mutations in the VHL gene are associated with a higher age-related risk for RCC [19], is a distinctive feature of the mutation in question. Therefore, we conclude that the p.Q145X nonsense mutation is likely to be associated with retinal and CNS HGBs and visceral cysts, but unlikely to be associated with RCC. Notably, although the c.435G>T (p.Gln145His) missense mutation and the c.433C>T [p.Q145X] nonsense mutation both involve residue 145, these two mutations exhibit opposing tendencies with respect to the incidence of RCC. This phenomenon may help to improve our understanding of the molecular mechanisms of tumorigenesis in VHL disease.

The effect of the c.435G>T (p.Gln145His) missense mutation on pVHL structure was assessed using molecular modeling. The Gln145 side chain participates in the stabilization of the turn in which it is located. The breaking of the loop-stabilizing H-bond interactions between Gln145 and Asn141, Gln145 and Val142, in combination with the replacement of the Gln145 residue with histidine markedly destabilizes this turn (Fig. 3). Thus, this missense mutation can cause partial or global misfolding. The most

remarkable effect of this structural reorganization on protein interactions is the high probability of the dissociation of the VHL and HIF-1α proteins. Despite the deformation of the turn involving residue 145, the disruption of the VHL-Elongin C interaction was not observed during molecular dynamics simulations.

The nonsense mutation c.433C>T, which results in the replacement of glutamine (CAG) with a stop codon (TAG) at codon 145 (p.Q145X), produces a truncated pVHL that lacks the final 68 amino acids. Thus, when this mutation occurs, the β domain's ability to bind and ubiquitinate HIF-1α is reduced, and the downstream α-domain (residues 155–192) cannot be synthesized; as a result, the truncated pVHL fails to bind to Elongin C, Elongin B and Cullin-2. Therefore, the function of pVHL is abolished. This inactivation leads to the stabilization of HIF-α and the subsequent transcriptional activation of HIF-induced target genes (hypoxia-inducible genes, including VEGF, PDGF β, TGF α and EPO) [20]. The overexpression of these genes may play a key role in the process of tumorigenesis, resulting in higher morbidity for VHL-associated tumors and relatively obvious familial aggregation. Based on the data from the current study, it may be speculated that the p.Q145X nonsense mutation leads to clear familial aggregation of VHL disease; based on this reasoning, every member of a VHL family with the p.Q145X nonsense mu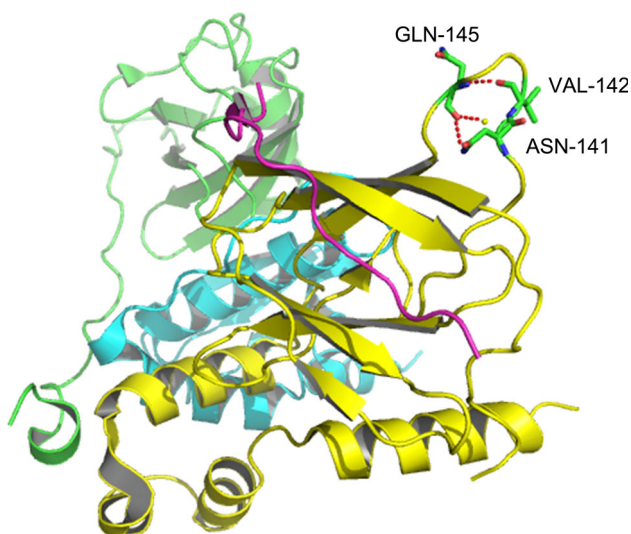tation should be systematically and comprehensively examined, and regular follow-up should be strictly conducted to ensure that VHL complications are recognized at a curable stage.

Conclusion

The p.Q145X nonsense mutation of the VHL gene in a large Chinese family was found to be associated with type 1 VHL disease, which presented with a high risk of retinal and CNS HGBs and visceral cysts but a low risk of RCC. In addition, this mutation may result in relatively obvious familial aggregation. Therefore, once this mutation has been detected, careful and strict surveillance of every member of the proband's family is requires.

Compliance with ethical standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

Fig. 3 Ribbon and ball-and-stick rendering of a representative snapshot from molecular simulations conducted to model the HIF-1α-pVHL-ElonginB-ElonginC complex The ribbon models of VHL, Elongin C, and Elongin B protein fragments are *yellow*, *blue* and *green*, respectively, whereas the fragment of the HIF-1α protein is presented in *pink*. Coloring by atomtype (with C, N, O, and H atoms in *green*, *blue*, *red* and *grey*, respectively) was used for the ball-and-stick models of residues. The loop-stabilizing H-bond interactions between Gln145 and Asn141, Gln145 and Val142 are depicted in *red*

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