Germline *VHL* gene mutations in three Serbian families with von Hippel-Lindau disease

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Von Hippel-Lindau (VHL) disease is an autosomal dominantly inherited cancer predisposition syndrome due to germline mutations in the VHL tumor suppressor gene which is associated with virtually complete penetrance. The VHL syndrome has a highly variable phenotypic expressivity including retinal and CNS haemangioblastomas, pheochromocytomas, renal clear cell carcinomas, and multifocal cysts. In order to establish *VHL* gene testing, we analyzed three families affected by VHL disease, using SSCP mutation screening and DNA sequencing. Among 18 family members with and without clinical manifestations, eight cases with germline *VHL* mutations were detected. In family A, a c.490G>T/p.Gly93Cys substitution was found. In family B, with pheochromocytoma only phenotype, we detected a previously not described c.463G>A/p.Val84Met replacement. Within this family, a prenatal diagnosis was also performed. Affected members of the third family with a VHL type 1 disease carried a c.475T>C/p.Trp88Arg exchange. All these mutations were located in exon 1 of the *VHL* tumor suppressor gene. Alterations in this hydrophobic region of the core β domain of the VHL protein are known to have a variety of phenotypic consequences. We observed also intrafamiliar variation in time of onset and severity of the disease.

Key words: VHL gene, germline mutations, VHL syndrome*, prenatal diagnosis*

Von Hippel-Lindau (VHL) disease is associated with the development of multifocal, highly vascularized tumors in mesenchymal and neural crest-derived tissues of several organ systems. Clinically most important are tumors of the central nervous system (haemangioblastoma – HB CNS), eye (retinal haemangioblastoma – RB), kidney (renal clear cell carcinoma – RCC), adrenal medulla (pheochromocytoma – PHE), inner ear (endolymphatic sac tumor), and endocrine pancreas (islet cell tumor) [1, 2].

In most VHL patients, autosomal dominantly inherited germline mutations can be identified in the *VHL* gene locus [3]. Exceptions to the rule appear to be epigenetic gene silencing [4] and genetic mosaicism [5]. Among the characterized gene alterations are point mutations in about 60% of the cases, partial deletions in approximately 30%, and deletions of the entire *VHL* gene in about 10% [6–8].

From VHL families, more than 250 distinct mutations have been identified which are distributed randomly throughout the *VHL* gene. There is also a significant number of recurrent mutants [9]. Databases of *VHL* gene mutations (www.vhl.org/research/beroud.htm, http://www.umd.be:2020) help to establish genotype–phenotype correlations that allow classification into distinct VHL disease subtypes. Most striking is the fact that VHL families with a high risk of PHE (type 2 VHL disease) almost invariably harbour a *VHL* missense mutation. Type 2 VHL families are usually also characterized by either a low (type 2A) or a high risk (type 2B) of RCC. Type 2C families, in contrast, have an increased risk of PHE without the other classical clinical features of VHL disease [1–3].

Nonsense mutations and large deletions, that lead to complete loss of *VHL* gene function, or mutations that are predicted to alter the functional conformation of the VHL protein (pVHL), in contrast, are associated with a markedly reduced risk of type 2 VHL disease [10, 11]. Recent investigations also demonstrated mutations in two gene regions coding for

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amino acids 74-90 and 130-136 to be associated with a high risk of developing RCC or type 1 VHL disease (8).

These findings led to the tempting hypothesis that PHE pathogenesis in the setting of VHL disease reflects a specific pVHL "gain of function" such that PHE development requires partial, but not complete loss of protein function [3, 10–12]. pVHL is engaged in the inhibition of angiogenesis and apoptosis, cell cycle exit, fibronectin matrix assembly, and proteolysis. It appears that the primary consequence of pVHL inactivation is to uncouple the hypoxia-inducible gene expression from changes in oxygen availability. Several lines of evidence demonstrate that the pVHL β-domain binds directly to hypoxia-inducible factor-α (HIF-α) subunits and catalyzes the polyubiquitination, hence destruction, of these subunits. In cells depleted of pVHL function, HIF-α accumulated, leading to overexpression of growth factors such as transforming growth factor-α (TGF-α), platelet-derived growth factor-β chain (PDGF-β), vascular endothelial growth factor (VEGF), and erythropoietin [3, 10, 11].

VHL gene testing procedures have several important bearings in molecular medicine. Firstly, characterization of the mutation provides information about the molecular basis of disease in a certain family and thereby accurate predictive testing. Secondly, genotype-phenotype correlations may have implications for subsequent screening and prognosis [8, 13, 14]. Genetic testing is also expected to expand the existing mutation databases and make them more informative and applicable. For these reasons, we studied three Serbian families affected by VHL disease. In order to obtain a very early diagnosis, we also performed a prenatal analysis in cells from the amniotic fluid of a female married to a *VHL* mutation carrier.

Patients and methods

Subjects. The study was approved by the appropriate institutional review board and was conducted in accordance with the Declaration of Helsinki. Informed consent was obtained from all patients and their relatives. Eighteen members from three families were analyzed. Patients were hospitalized either in the Military Medical Academy in Belgrade or in the Institute for Endocrinology, Diabetes and Metabolic Disorders at the Clinical Center of Serbia in Belgrade. They came to our attention because of their medical and familial histories. Figure 1 shows the pedigrees of the families studied.

DNA analysis. Genomic DNA was extracted from peripheral blood leukocytes or archived tumor tissue by standard phenol/chloroform procedures [15]. In case of the pregnant woman, amniotic fluid (20 mL) was obtained by amniocentesis and centrifuged at 1700 g for 10 minutes at room temperature. The cell pellet was washed two times with 0.9% NaCl solution and then resuspended in 400 μL TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0) containing 2% SDS and 0.2 mg/mL proteinase K (Qiagen, Hilden, Germany). The cells were incubated at 56°C for 1 hour and overnight at 37°C. Then genomic DNA was isolated by standard phenol/chloroform procedures [15].

PCR primers were designed for the three coding exons as well as for the promoter region of the *VHL* gene. Amplicon 1 included nucleotides 590 to 880, amplicon 2 included nucleotides 780 to 1111, amplicon 3 included nucleotides 5311 to 5551, and amplicon 4 included nucleotides 8611 to 8886 with respect to the *VHL* gene sequence in the GenBank accession number AF010238. The primers used for the amplification of the *VHL* promoter were 5'-GTTACAACAGCCTACGGTG-3' and 5'-CCGTCTTCAGGGCCG-3', covering the region of nucleotides 512 to 800 [16]. Briefly, for all amplifications 100–200 ng of genomic DNA and 1 U of Taq polymerase (Roche, Germany) were used per PCR reaction. Amplification conditions were 35 cycles of 45 seconds at 94°C, 45 sec at 61°C or 63°C, and 45 sec at 72°C, followed by a 5 min final extension step at 72°C. Family members were screened by single-stranded conformational polymorphism (SSCP) analysis. SSCP profiles were resolved on 8% polyacrylamide gels (8 V/cm, +4°C, 4 h) and stained with silver nitrate. Amplicons of interest were bidirectionally sequenced by the ABI PRISM™ BigDye™ Terminator v3.1 Ready Reaction Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and by Automated DNA sequencer-ALFexpressII with the Thermo Sequenase, Cy5day Terminator Sequencing kit (Amersham Pharmacia Biotech, Freiburg, Germany) in accordance with the manufacturer's instructions.

Results

We analyzed the *VHL* gene in three families from the Serbian population. Selection of the families was based on the clinical data of the probands and their family histories. *VHL* gene amplicons of all patients were screened by SSCP analysis, and those with variant bands were subjected to sequencing and confirmatory re-sequencing. In order to eliminate possible experimental artifacts, confirmatory analyses always included a second, independently prepared DNA sample. Once the SSCP profile was linked to a specific mutation, it provided a reliable tool for mutation screening within a certain family, as carriers of identical germline mutations had identical SSCP profiles.

The results of the molecular genetic analyses are shown in Figure 1 and Table I. In family A, the pedigree indicated a type 2 VHL syndrome, with only HB medullae spinalis in patient AI1, HB CNS and PHE in patient AII5, and PHE only in patients AII2 and AIII3. Members AII2, AII6, AIII3, AIII4, AIII5, AIII6, AIII7, AIII8, and AIII9 were screened by SSCP analyses. Subjects AII2 and AIII3 had discriminatory SSCP profiles for exon 1 amplicons, and sequencing revealed a c.490G>T/p.Gly93Cys substitution. Although both individuals carried the identical germline mutation in heterozygous form, their course of disease was different. The mother (AII2) was diagnosed with PHE at the age of 33. After three years, cholecystectomy was performed due to acute cholelithiasis. Her son (AIII3) suffered by severe

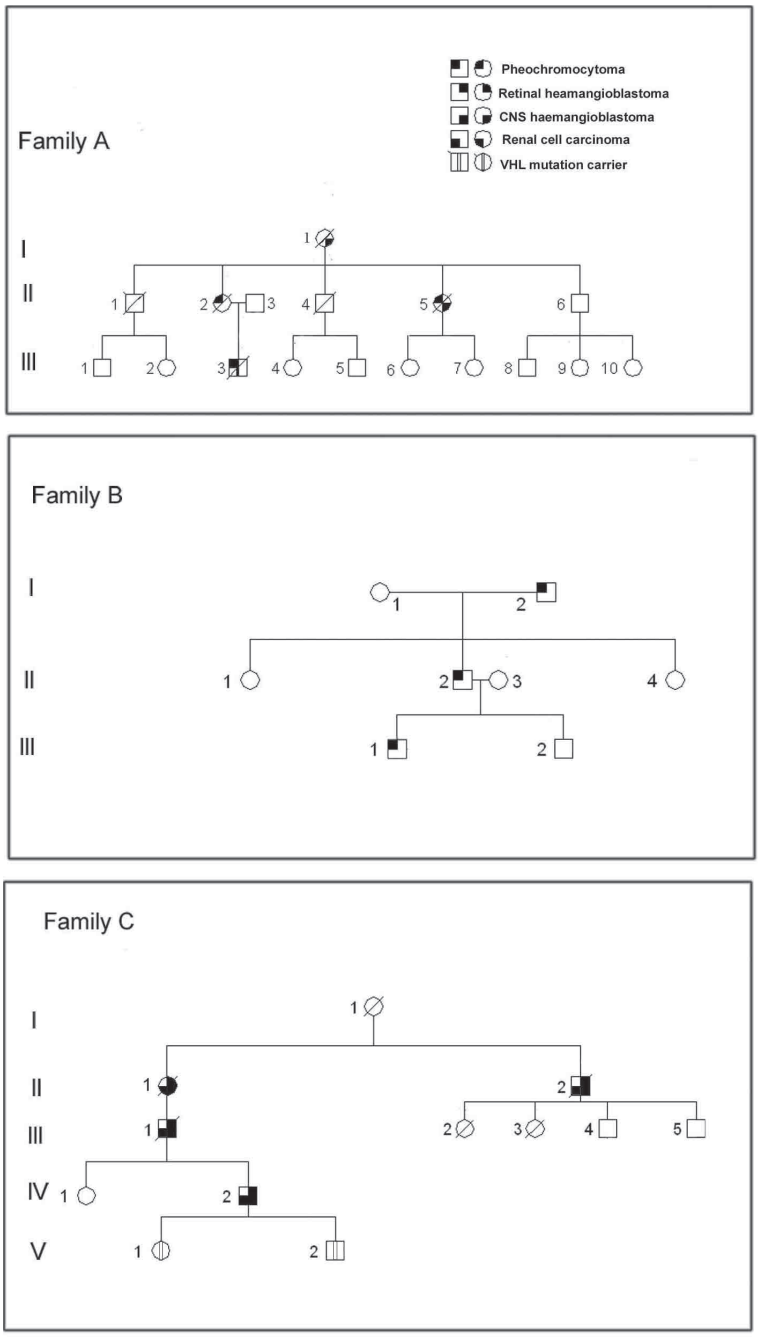

Figure 1. Pedigree of the families studied.
Legend: *VHL* gene analyses were performed in members II2, II6, III3, III4, III5, III6, III7, III8, and III9 of family A, members II2, II3, III1, and III2 of family B, and members III5, IV1, IV2, V1, and V2 of family C. All six individuals with VHL symptoms (affected members) carried germline *VHL* gene mutations, while two other heterozygous carriers (CV1 and CV2) are asymptomatic at present.

arterial hypertension (36.0/24.0 kPa) since the age of 12 and was operated because of left PHE at the age of 24. A malignant pheochromocytoma invading the left kidney together with metastatic deposits in the right basal lung was diagnosed.

In patients BII2 and BIII1 from family B, bidirectional sequencing of the *VHL* gene revealed a c.463G>A mutation affecting the first nucleotide of codon 84 and resulting in the replacement of valine (GTG) by methionine (ATG). In patient BIII1, this alteration was associated with PHE at the age of one year. Three years later, the disease had progressed into a bilateral PHE. In patient BII2, the first symptoms of PHE appeared at the age of 40 (type 2C VHL syndrome). A sample of amniotic fluid was taken from individual BII3 during the 16th week of pregnancy for prenatal testing, and the same point mutation as in her husband BII2 was detected.

In persons CII2, CIV2, CV1 and CV2 of the C kindred, genetic analyses revealed a germline c.475T>C/p.Trp88Arg exchange. Figure 1 shows the pedigree of this family, which is indicative of a type 1 VHL syndrome. In proband CIV2, RB was diagnosed at the age of 12. Multiple pancreatic and kidney cysts were present at the age of 29 and left kidney cancer was found at the age of 35. Individuals CV1 and CV2 are carriers of this genetic defect, but are at present asymptomatic. They are 15 and 12 years old, respectively, and their parents avoid medical surveillance. Subject CII2 had the first symptoms of RB at the age of 60 and deceased due to RCC in both kidneys aged 63 (Table I).

Discussion

We describe germline missense mutations in exon 1 of the *VHL* gene in three families with VHL syndrome, affecting codons 84, 88, and 93. The c.463G>A/p.Val84Met substitution present in family B has not been detected previously. Finding a novel germline mutation in VHL families is not uncommon, because *VHL* gene defects are very heterogeneous. Based on one of the *VHL* mutation databases (http://www.umd.be:2020), only missense mutations affect 69 or about 32% of the 213 codons. Aside from the mutational diversity, some clustering of mutations exists. In the β domain, there is stretch with a high mutation rate ranging from amino acid 67 to 117 (especially delPhe76, Asn78Ser/His/Thr, which are mostly associated with VHL type 1, and Pro86Leu). Hot spots also occur in the α domain, between amino acids 157-170, especially codons Arg161Term, Cys162Tyr/Pro/Trp, Arg167Gln/Trp (the most frequent in PHE), and Leu178Pro [3,6,7,11].

Although all three amino acid substitutions are located in the same hydrophobic region of the core β domain of pVHL, phenotypic manifestations are quite different in the three families. In families A and B, substitutions p.Gly93Cys and

p.Val84Met were associated with PHE development. This is in accordance with the literature. Substitutions p.Gly93Cys/Ser/Asp/Arg have been described to be associated with PHE and/or HB CNS, while p.Val84Leu/Pro were associated with PHE only [6, 7, 18–20]. It is generally accepted that the majority of mutations associated with PHE are missense mutations positioned in the α domain of pVHL which preserve the binding function and ubiquitination of Hif protein. Although positioned in the region mostly associated with the appearance of RCC, alterations in codons 93 and 84 most likely lead to a gain-of-function which is the main characteristic of PHE, the β domain of pVHL partially preserving its role in the degradation and inhibition of transcription of Hif 1α [3, 8, 10, 11].

In family C, a T→C mutation at nucleotide position 475 resulted in the replacement of tryptophane by arginine at amino acid position 88. Na et al. [21] examined the influence of this pVHL substitution on the expression of Hif 1α and consequently on the expression of VEGF in RCC tissue obtained after surgery. It was demonstrated the p.Trp88Arg leads to an elevated expression of Hif 1α that exceeds 24 times the expression of VEGF. This *in vitro* result suggests that the p.Trp88Arg mutation perturbs the β sheet integrity and induces a loss-of-function, the primary prerequisite for RCC development [11, 21, 22].

However, the same substitution as well as p.Trp88Ser/Cys/Thr were detected in individuals diagnosed with only HB CNS [6, 22–24]. In case of point mutations, interfamilial differences in some manifestations of the VHL syndrome suggest the possibility that other genetic alterations (mutations and/or polymorphisms) affect the VHL phenotype [3, 20, 25]. Early in life, VHL type 1 is the most frequent manifestation (including HB CNS and especially RB), while RCC appears much later, indicating tissue-specific effects of distinct *VHL* mutations in addition [3].

In family B, there is prominent difference in the time of onset of the first VHL symptoms. In patient BIII1, PHE appeared very early so that surgery was necessary at the age of two, while the first symptoms in his father and grandfather appeared only in advanced age, beyond 40 (Table I). Inter and intrafamiliar differences of this kind are common in the VHL syndrome. Inactivation of both alleles either by a point mutation and/or by a deletion is a major feature of tumor suppressor genes such as the *VHL* gene and one of the prerequisites of malignant transformation. In patients with a germline point mutation of the *VHL* coding sequence, the second hit is usually a somatic deletion of the wild-type *VHL* allele [3, 10]. However, PHE may also appear without loss of heterozygosity in early childhood. It is generally accepted for this type of tumor that the alteration of a single allele is sufficient for the development of a tissue-specific neoplasia [26]. In addition, a gene dosage effect may be a major determinant of the tumor development in chromaffin cells. PHE could also emerge by yet another mechanism without a second hit – a pathway characterized by the loss of ability to bind fibronectin, leading to PHE in spite of the retained ability to down-regulate Hif [3, 10].

Table I. Genotype and phenotypic characteristics of family members with mutations in the *VHL* gene

Family	Type of VHL syndrome	Mutations	No	Age at diagnosis
A	2C	c.490G>T/p.Gly93Cys	AII2	33
			AIII3	12
B	2C	c.463G>A/p.Val84Met	BII2	40
			BIII1	2
C	1	c.475T>C/p.Trp88Arg	CIII5	60
			CIV2	12
			CV1	–
			CV2	–

Legend: Patient numbers are from Figure 1. Nucleotides are numbered according to GeneBank database accession no. L15409. Codons have been numbered with the first methionine as codon 1 (17).

In case of the B family, we were approached by husband BII2 (*VHL* mutation carrier) and by his wife BII3 (*VHL* mutation not affected) who expected a baby, to perform a prenatal mutation analysis in the fetus. The c.463G>A mutation detected in cells of the amniotic fluid had thus to originate from the zygote. To our knowledge, this is the first report of a prenatal diagnosis of *VHL*, aside from preimplantation gene testing [27]. Prenatal and preimplantation gene testing is generally recommended in families with heritable cancer predisposition syndromes such as MEN2, VHL syndrome, Li-Fraumeni syndrome, retinoblastoma, etc. [28, 29]. In our experience, the molecular genetic approach was very helpful in accurately detecting the *VHL* mutation *in utero* and subsequently for the appropriate genetic counseling and delicate decision-making process related to the termination of the pregnancy.

Accumulated and systematized data about germline *VHL* mutations demonstrate that specific trends can be observed regarding genotype-phenotype correlations in spite of evident intra and interfamilial differences. Correlation between a specific *VHL* mutation and the clinical presentation of VHL disease could be a major improvement in the clinical management of affected patients, who are not curable at present. Nevertheless, any progress in predicting disease course will improve control and follow-up of the patient, thus hopefully also reducing the consequences.

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