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RESEARCH ARTICLE

Management of Gene Variants of Unknown Significance: Analysis Method and Risk Assessment of the *VHL* Mutation p.P81S (c.241C>T)

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Abstract: ***Background:*** Evaluation of the pathogenicity of a gene variant of unknown significance (VUS) is crucial for molecular diagnosis and genetic counseling, but can be challenging. This is especially so in phenotypically variable diseases, such as von Hippel-Lindau disease (vHL). vHL is caused by germline mutations in the *VHL* gene, which predispose to the development of multiple tumors such as central nervous system hemangioblastomas and renal cell carcinoma (RCC).

Objective: We propose a method for the evaluation of VUS pathogenicity through our experience with the *VHL* missense mutation c.241C>T (p.P81S).

Method: 1) Clinical evaluation of known variant carriers: We evaluated a family of five *VHL* p.P81S carriers, as well as the clinical characteristics of all the p.P81S carriers reported in the literature; 2) Evaluation of tumor tissue via genetic analysis, histology, and immunohistochemistry (IHC); 3) Assessment of the variant's impact on protein structure and function, using multiple databases, *in silico* algorithms, and reports of functional studies.

Results: Only one family member had clinical signs of vHL with early-onset RCC. IHC analysis showed no VHL protein expressed in the tumor, consistent with biallelic *VHL* inactivation. The majority of *in silico* algorithms reported p.P81S as possibly pathogenic in relation to vHL or RCC, but there were discrepancies. Functional studies suggest that p.P81S impairs the VHL protein's function.

Conclusion: The *VHL* p.P81S mutation is most likely a low-penetrant pathogenic variant predisposing to RCC development. We suggest the above-mentioned method for VUS evaluation with use of different methods, especially a variety of *in silico* methods and tumor tissue analysis.

Keywords: Genetic screening, Missense mutation, Renal cell carcinoma, *VHL* gene, Von Hippel-Lindau disease, Variant of unknown significance.

1. INTRODUCTION

Unclassified gene variants, also referred to as variants of unknown significance (VUS), are gene mutations that have an unknown or unclear effect on protein function [1]. VUSs are mostly amino acid substitutions and intronic variants or small in-frame-insertions/deletions. How they impact on gene function is uncertain, and consequently, evaluation of their clinical significance is challenging [1]. Depending on the gene(s) tested, the frequency of VUS occurrence after genetic tests is variable. Up to almost half of the gene variants reported in the *BRCA1* and *BRCA2* genes are classified as VUSs [2]. An unclear interpretation generates confusion, creates anxiety for the VUSs carriers and their families, and difficulties for the clinicians who are responsible for clinical management. Multiple approaches are used to classify VUSs, while a standard universal method is not yet available [3]. It is important to adopt a reliable and valid method of classification, as a major problem is that many variants are not classified in time to help clinical management [4]. We aim to set up a general method, which can be used for the evaluation of the pathogenicity of VUS, through our experience with a missense mutation in the *VHL* tumor suppressor gene (OMIM: 608537). Our strategy is based on several approaches for assessing VUSs and thereby evaluating a mutation from different perspectives.

Pathogenic germline mutations in the *VHL* gene are associated with von Hippel-Lindau disease (vHL) (OMIM no.:193300), which is an autosomal dominantly inherited familial cancer syndrome. Predisposed individuals are at risk of developing a variety of malignant and benign neoplasms such as retinal, cerebellar, and spinal hemangioblastomas

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(HB), renal cell carcinoma (RCC), pheochromocytoma, and pancreatic tumors [5]. vHL-related tumors are associated with loss of heterozygosity (LOH), somatic mutations, or other inactivation of the wild-type allele in accordance with Knudson's two-hit model [6]. Germline mutations have been detected throughout most of the *VHL* gene, and the spectrum includes missense mutations, microdeletions, insertions, splice site, nonsense mutations, and large deletions [7]. Somatic *VHL* mutations and allele loss have also been detected in sporadic tumors, especially in sporadic RCC and sporadic CNS HB [7]. The VHL protein (pVHL) is involved in numerous cellular pathways, the best described is the regulation of hypoxia-inducible factors (HIFs). Along with the Elongin B and Elongin C proteins, pVHL is part of an ubiquitin ligase complex, which marks HIFs for proteosomal degradation under normal oxygen conditions [5, 7]. Under hypoxia, or when pVHL function is impaired, HIFs are stabilized and activate the transcription of genes primarily involved in angiogenesis [5, 7]. The severity of the disease varies markedly among patients, both in relation to the age at manifestation development but also which organs are affected [5]. Nevertheless, all carriers of pathogenic *VHL* germline mutations are advised to follow comprehensive surveillance guidelines to detect new lesions at early stages and to facilitate timely treatment [5, 8]. We present a family with five *VHL* missense mutation c.241C>T (p.P81S)(Reference sequence: NM_000551.3) carriers, of whom only one presented with early-onset RCC. The single manifestation in the proband and the absence of clinical signs in the four other mutation carriers questions the pathogenicity of p.P81S and complicates the choice of a surveillance plan for the family. We investigated the pathogenicity of this VUS using a step-by-step method, in order to understand its possible involvement in the development of RCC or even a vHL phenotype.

2. MATERIALS AND METHODS

We investigated the *VHL* mutation p.P81S using the following tools: 1) Clinical evaluation and 2)*VHL* germline mutation analysis of family members, 3) Genetic tumor analysis and 4) histology and immunohistochemistry (IHC) analysis of the proband's RCC, 5) literature search for reported *VHL* p.P81S mutation carriers, 6) database and *in silico* analysis of the mutation, and evaluation of reported functional studies of the variant.

2.1. Clinical Evaluation of the Family

The described family was identified as part of a clinical diagnostic visit at the Department of Clinical Genetics, Odense University Hospital. The family underwent genetic counseling and the proband initially underwent diagnostic mutation analysis on DNA obtained from peripheral blood of the *VHL, FLCN, FH, MET, BRCA1, BRCA2, HNF1A,* and *HNF1B* genes (direct sequencing of exons and exon-intron boundaries or Next-generation sequencing (*BRAC1* and *BRAC2*), and Multiplex Ligation-dependent Probe Amplification (MLPA)). Chromosomal analysis for chromosomal rearrangements involving chromosome 3p was also performed. No mutations or chromosomal imbalances were identified in genes other than the *VHL* gene. Expression of pMLH1, pMSH2, pMSH6, and pPMS2 was initially assessed and found to be normal in the proband's tumor by immunohistochemistry (IHC). When the *VHL* p.P81S mutation was identified, the proband's living first-degree relatives, as well as his maternal grandmother, underwent *VHL* mutation analysis, as described in the next section. The proband's RCC tumor was also further analyzed, as described below. Family and clinical history concerning all family members was obtained by interview, and confirmed through the evaluation of medical records.

2.2. *VHL* Germline Mutation Analysis

Genomic DNA was purified from whole blood by using the PureGene Blood Core Kit C (Qiagen, Hilden, Germany, cat. no. 158389) according to the manufacturer's instructions. The *VHL* mutation analysis was performed using direct Sanger sequencing of exons and exon-intron boundaries (Supplementary Table **1**). PCR was carried out in a 15 µl reaction mixture containing 1.5 µl 10x Key Buffer, 0.3µl dNTPs (10µM), 1 µl forward and reverse primer (10µM), 0.1 µl Taq DNA polymerase, and 1µl DNA sample template (50 ng/µl). The thermal cycling setting for the primer sets include a 5 min initial denaturation at 95°C followed by 40 amplification cycles (20 sec denaturation at 95°C, 20 sec annealing at 55-65°C depending on the primer sets, and 30 sec extension at 72°, finished with a final extension at 72°C for 5 min). PCR products were bi-directionally sequenced with an ABI PRISM 3130XL Genetic Analyzer (Applied Biosystems, California, USA) using the BigDye Terminator Cycle Sequencing-Kit version 1.1 (Applied Biosystems, California, USA) according to the manufactures' manual. The sequence was visualized and evaluated using ChromasPro, version 1.7.6 (Technelysium Pty Ltd, South Brisbane, Australia).

Moreover, genomic DNA was examined for intragenic deletions by MLPA analysis using the MLPA-*VHL* test kit (P016-C2, MRC-Holland, Amsterdam, Netherlands), and carried out according to the manufacturer's protocol. Products were analyzed using an ABI3130 XL Genetic Analyzer (Applied Biosystems, California, USA) with LIZ500-labeled internal size standard. Data was evaluated using Coffalyser (version 140101.0000, MRC-Holland, Amsterdam, Netherlands).

2.3. Genetic Tumor Analysis

Tissue samples from the proband's RCC had been formalin fixed and paraffin-embedded after the tumor was surgical removed. Sections for DNA purification, histology, and IHC analysis were cut consecutively from the same tissue block, and therefore the individual sections represent neighboring sections of the same tissue sample.

Tumor DNA was extracted by first subjecting four 10 µm thick tissue sections to 1 mL of Ultra Clear^{TM} treatment in order to dissolve the paraffin. The paraffin was removed after an hour of incubation at 37 °C and 100% methanol treatment that separated the paraffin from the tissue. To digest the tissue, we subsequently added 10,5 µl Proteinase K (20 mg/ml) mixed with 5 µ BSA (bovine serum albumin, 10 mg/ml) and 500 µl extraction buffer (10 mM Tris-HCl (PH 8,3), 50 mM KCl, 1,5 mM $MgCl_2$, 0,35% TWEEN 20, 0,45% TRITONx100). We incubated the sample at 54°C for

two days, and thereafter stopped the enzyme activity by incubating at 100,5°C for 10 minutes. Precipitation of the DNA was done by adding sodium acetate (NaAc) and 96% Ethanol and using high-speed centrifugation.

2.3.1. Loss of Heterozygosity (LOH) Analysis

The DNA samples were analyzed for LOH by PCR using the microsatellite markers D3S1597, D3S3601, and D3S3691, which flank the *VHL* gene (Supplementary Figure **1**). Each PCR sample contained 5 µl of template DNA (10ng/µl), 0.12 µl or 0.30 µl of each primer (10 µM), 0.24 µl of dNTPs (10 µM), 1.5 µL of 10x Key Buffer, 0.24µl fluorescence primer #1799, and 0.24 µl of Taq DNA polymerase in a total volume of 15 µl. Labeled amplified DNA was analyzed on a polyacrylamide gel. Afterwards, PCR products were mixed with 10 µl formamide and 0.5 µl GeneScan™ 600LIZ® DNA internal size ladder. The samples were denatured for 5 min at 95°C and analyzed using an ABI3130 XL Genetic Analyzer (Applied Biosystems, California, USA).

To determine whether there was LOH in the tumor DNA, a ratio of allele peak height was calculated between the signals of each of the two alleles of the tumor DNA and the blood DNA: (Height of tumor DNA allele one/Height of tumor DNA allele two)/(Height of blood DNA allele one/Height of blood DNA allele two). LOH was assigned when more than 30% in the signal reduction of one allele was observed in the tumor sample compared to the corresponding blood sample, in accordance with other reported LOH thresholds [9-11].

2.3.2. Sanger Sequencing of Tumor DNA

We looked for somatic point mutations in the *VHL* gene with direct sequencing of the three *VHL* exons and exon-intron boundaries, using a total of nine PCR primer sets (Supplementary Table **1**). PCR was carried out in a 15 µl reaction mixture containing 1.5 µl 10x Key Buffer, 0.3 µl dNTPs (10µM), 1 µl forward and reverse primer (10µM), 0.1 µl Taq DNA polymerase, and 10 µl DNA sample template (10 ng/µl). The thermal cycling setting for the primer sets include a 1 min initial denaturation at 96°C followed by 40 amplification cycles (30 sec denaturation at 96°C, 1 min annealing at 62-64°C depending on the primer sets, and 1 min extension at 72°, finished with a final extension at 72°C for 5 min).PCR products were directly sequenced in both orientations on an ABI PRISM 3130XL Genetic Analyzer (Applied Biosystems, California, USA) using the BigDye Terminator Cycle Sequencing-Kit version 1.1 (Applied Biosystems, California, USA) according to the manufacturers' manuals. Data was generated and evaluated using Chromas Pro version 1.7.6. (Technelysium, South Brisbane, Australia).

2.4. Histology and Immunohistochemical Analysis

Paraffin embedded sections were deparaffinized and rehydrated in graded alcohol. For general histology, one section was stained with hematoxylin and eosin (H&E) by standard methods, and the neighboring section was used for IHC analysis. For IHC, a 5 µm tissue section was deparaffinized in xylol and rehydrated sequentially in ethanol. Deparaffinized tissue sections were quenched with 3% H_2O_2 for 15 min, and then washed with phosphate-buffered saline (PBS) and blocked for 30 min with 10% goat serum in PBS and 0.5% Triton-X. Tissue sections were incubated at 4°C overnight with the antibody VHL FL-181; sc-5575 (Santa Cruz, Biotechnology Inc.; 1:200 dilution). pVHL expression was determined using Dako Envision+ System-HRP Kit (Agilent Technologies, Glostrup, Denmark) as per the manufacturer's instructions.

2.5. Literature Search for Reported VHL p.P81S Mutation Carriers

We identified reports of patients with a germline *VHL* mutation in the scientific literature, through a search of the PubMed database. We searched for publications in English, using combinations of the search words: "VHL", "variants", "P81S/Pro81Ser", "germline mutation", "c.241C>T". All identified abstracts were reviewed and the main text of each article was searched for: "P81S"/"Pro81Ser", "VHL". We selected articles reporting patients with the *VHL* p.P81S variant and identified additional reports through a review of the papers referenced in these articles.

2.6. Databases, *In Silico* Analysis, and Reports of Functional Studies

2.6.1. Databases

Human Genome Mutation Database (HGMD® Professional) [12] collects known gene variants causing human inherited disease along with disease-associated/functional polymorphisms reported in peer-reviewed literature. HGMD collects data through a combination of manual and computerized search procedures. The Single Nucleotide Polymorphism database (dbSNP) [13] is a public archive for genetic variation within and across different species, hosted by the National Center for Biotechnology Information **(**NCBI) in collaboration with the National Human Genome Research Institute (NHGRI). It accepts submissions from academic research laboratories, as well as from private research companies. ClinVar database [14] is a public archive of human variants and associated phenotypes, hosted by the NCBI. ClinVar collects information derived from clinical testing, research and extraction from literature through semi-automated data streams: Online Mendelian Inheritance in Man (OMIM) [15], GeneReviews [16], dbSNP [13], and the NIH Genetic Testing Registry (GTR) [17], but it also accepts direct submissions from both organizations and individuals. Universal Protein Resource (Uniprot) [18] is a comprehensive catalog of protein information (protein sequences and functional annotation). UniProt provides both manual curation and automatic annotation as it interprets and integrates information from the scientific literature as well as large data sets [18]. Exome Sequencing Project (ESP) collects human genetic variants in coding regions through the application of Exome-Next-generation sequencing in different, well-phenotyped populations [19]. The project is a collaboration between several institutions, and so far more than 200,000 individuals from large United States populations are included.

2.6.2. Computational Prediction (In silico) Methods

SIFT (Sort Intolerant from Tolerant) [20] evaluates evolutionary conservation at the position a missense substitution is observed. Given a protein sequence, it chooses related

proteins and aligns these proteins with the query (multiple sequence alignments). SIFT calculates the probability that an amino acid at a given position will be tolerated, relative to the most frequent amino acid at that position. A cutoff value is used for the probability (range: from 0 to 1) of classifying the missense mutation as "tolerated" (<0.05) or "deleterious" (≥0.05) [20]. SIFT calculates the median conservation value, which measures the diversity of the sequences in the alignment, ranging from 4.32 (log2 (20)) (high degree of conservation, only one amino acid observed) to zero (all 20 amino acids observed at a specific position). Values >3.25 should be interpreted with caution, due to the risk of a false positive error (if sequence alignments are closely related, SIFT will predict most substitutions as deleterious, even if they are neutral). Align-GVGD [21] also considers evolutionary conservation, combining an alignment with amino acid physicchemical features to calculate the range of variation at each position in the alignment (GV) and the distance of missense substitution from the edge of that range of variation (GD). The two scores are combined to provide a classifier which gives a series of grades, ranging from "C0" (unlikely to be deleterious) to "C65" (most likely deleterious) [21]. MutationTaster [22] evaluates the disease-causing potential of variants through analysis and by integrating information from different databases, such as the dbSNP database [13], the 1000 Genomes Project [23], HGMD [12], and ClinVar [14]. The analysis assesses evolutionary conservation, splice-site changes, loss of protein features, changes that might affect gene expression [22]. Variants are categorized as either 1) disease causing – *i.e.* probably deleterious, 2) disease causing automatic – *i.e*. known to be deleterious, 3) polymorphism – *i.e*. probably harmless, or 4) polymorphism automatic – *i.e.* known to be harmless. The employed Bayes classifier calculates the probability of the prediction, i.e. a value close to one indicates a high reliability of the prediction. Polyphen-2 (Polymorphism Phenotyping version 2) classifier [24] is based on a decision tree and integrates evolutionary conservation principles and the physiochemical properties of the two residues, with protein structure based parameters. The used parameters encompass sequence annotations downloaded from the UniProt database and sequence features calculated by PolyPhen2 as well as other programs. Prediction outcome can be either 1) probably damaging – *i.e*. predicted to be damaging with a high confidence, 2) possibly damaging – i.e. predicted as damaging but with a low confidence, or 3) benign –*i.e.* predicted as benign with a high confidence, along with a numerical score ranging from 0.0 (benign) to 1.0 (damaging). In this study we chose to use the HumVar model for assessment of the damaging effect, as this model is recommended for use in a clinical setting [25]. Project HOPE (Have (y)Our Protein Explained) server [26] collects structural protein information from numerous sources, including calculations on the 3D protein structure sequence annotations in UniProt database, and sequence-based predictions from DAS-servers (Distributed Annotation System) [27]. It uses the submitted sequence as a query for BLAST (Basic Local Alignment Search Tool) searches in the UniProt database and the Protein Data Bank (PDB)[28]. The collected information and known properties of the wild-type and mutated amino acid (such as size, charge and hydrophobicity) are used to predict the variant's effect on the protein structure and function. Symphony [29] is an optimized binary classification system that integrates predictions from different *in silico* systems, in order to predict the functional effects of missense *VHL* variants and to classify clear cell RCC risk. Each computational method uses a different methodology to independently evaluate the effect of missense mutations on protein stability and protein interactions with other ligands. The output predictions obtained through a regression model tree, are used to train and test a binary classifier, which outputs the predicted risk of clear cell RCC in a binary classification scheme (high or low).

2.6.3. Reports of Functional Studies

As part of the above-mentioned search for p.P81S mutation carriers, we identified two reports of functional studies of the P81S variant.

2.7. Approvals and Patient Consents

The examined carriers of the *VHL* p.P81S mutation all gave their informed written consent to participate in the study, which was approved by the Danish Regional Committees of Research Ethics (H-2-2010-012) and the Danish Data Protection Agency (2009-41-3994).

3. RESULTS

3.1. Clinical Evaluation and VHL Germline Mutation Analysis of the Family

Among the five confirmed carriers of the p.P81S mutation, only the proband was diagnosed with clear cell RCC at the age of 29, and was treated with partial nephrectomy. Genetic testing revealed the missense mutation p.P81S along with a silent mutation p.P2P (c.6C>G) in the *VHL* gene. A genetic work-up of the immediate family identified the proband's mother (52 years), his two brothers (32 and 36 years), and his maternal grandmother (71 years) to also be p.P81S mutation carriers (Fig. **1**).

The proband, his mother, and his grandmother all underwent the full diagnostic vHL program, according to Danish vHL guidelines [8]: Magnetic Resonance Imaging (MRI) of the CNS, Computed Tomography (CT)/Ultra Sound (UL) of the abdomen, ophthalmological examination, measurement of plasma-metanephrines, plasma-Chromgranin A, and audiometry. The proband's two brothers both had UL of the abdomen. Besides the proband's RCC, none of the five carriers had any other signs of vHL-related manifestations. The proband's extended family has not been tested for the mutation, and no other family members have had any subjective signs of vHL manifestations. Even though the mutation was found to originate from the mother's side of the family, it should be noted that the proband's paternal uncle died from metastatic RCC at 46 years of age, and the proband's father died from urosepsis in relation to bladder cancer in situ at the age of 56. Both men had been smokers. No tissue samples could be obtained from either of them for genetic testing.

3.2. Genetic Analysis of the Proband's RCC Tumor

We screened the proband's RCC for somatic *VHL* mutations. There was no evidence of LOH at the sites of the D3S1597 (allelic decrease of 19%) or the D3S3601 primer (allelic decrease of 25%). The LOH analysis for D3S3691

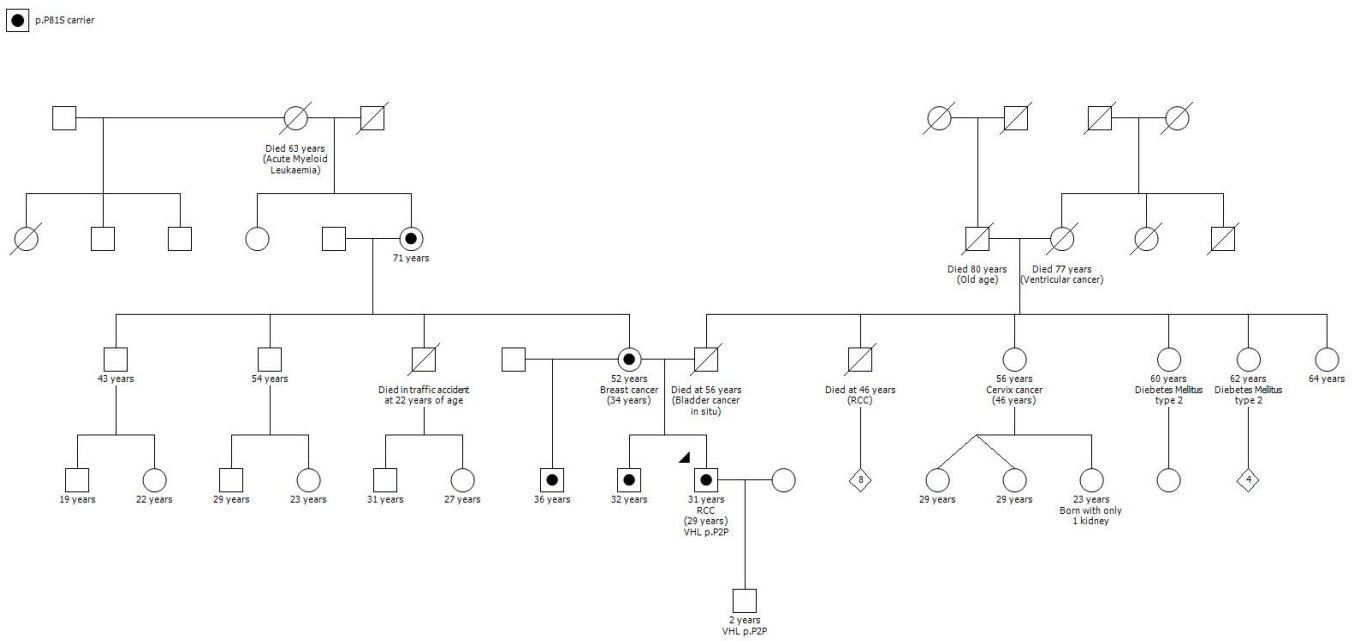

Fig. (1). Pedigree of the presented family.

Current age or age at death is given under each individual symbol. Cancer diagnoses with the age at diagnosis given in parentheses, and/or cause of death, are shown below.

was uninformative. Direct sequencing revealed no somatic mutations in the *VHL* gene, but detected the patient's germline mutations.

3.3. Histology and Immunohistochemical Analysis

Histological analysis of renal tissue revealed clear cell carcinoma, numerous cysts, and areas with apparently normal cortex (Fig. **2A**). Immunostaining for pVHL showed a cytoplasmic reaction in the distal tubules and the proximal tubules, whereas the glomeruli lacked staining (Fig. **2B**). In areas with clear cell carcinoma and cyst formation, staining for pVHL was absent (Fig. **2C** and **D**).

3.4. Literature Search for Reported VHL p.P81S Mutation Carriers

We identified sixty-seven articles in our search, and identified twenty-three *VHL* p.P81S mutation carriers in nine articles [30-38] (Table **1**). When our family was included, seventeen out of twenty-eight (61%) of the mutation carriers had a vHL phenotype. However, of these, two did not completely fulfill the clinical diagnostic criteria for vHL [8]; one 44 year-old woman was reported to have only one cerebellar HB [34], and one patient had only a RCC at the age of 29 (present study).

3.5. Databases, *In silico* Analysis and Reports of Functional Studies

Predictions for the impact of the p.P81S substitution in the *VHL* gene, obtained by *in silico* analysis, showed a discrepancy. The variant was predicted as tolerated by SIFT, Mutation Taster and Polyphen, but in contrast, was predicted to affect the protein function by HOPE and Symphony. Align GVGD placed it in an intermediate area of genetic risk. (Table **2**) shows the results from computational methods and databases.

We identified two reports of functional studies of the p.P81S variant. These studies employed both *in vivo* and *in vitro* methods, and concluded that p.P81S affects the normal function of pVHL.

4. DISCUSSION

We evaluate the role of the *VHL* mutation p.P81S in relation to vHL and RCC development, and propose a method for analysis of such a VUS. We examined a family with five p.P81S carriers over three generations, where only the proband had early-onset clear cell RCC. We evaluated the family clinically and genetically, performed genetic analysis of the proband's tumor, assessed the phenotypes of all other p.P81S mutation carriers reported in the literature, and used databases, *in silico* analysis methods, as well as reports of functional studies to determine pathogenicity. (See Fig. **3**) for an overview of the evaluation method.

The p.P81S variant does not seem to be a polymorphism, as the allele frequency was 0% in three different healthy control populations (in total 2,424 alleles) [34, 36, 38, 39].

In our family, the proband was diagnosed with clear cell RCC at the young age of 29, consistent with an inherited susceptibility. The lack of pVHL expression in the proband's tumor tissue found by IHC analysis is consistent with a vHL-associated tumor, as it points toward inactivation of both *VHL* alleles in accordance with Knudson's two hit model [6]. The first hit would be the p.P81S germline mutation, while the wild-type allele could be inactivated by other mechanisms than those analyzed here, such as intragenic deletions or hypermethylation of the promotor region [40]. However, biallelic *VHL* inactivation is also reported in up to 74% of

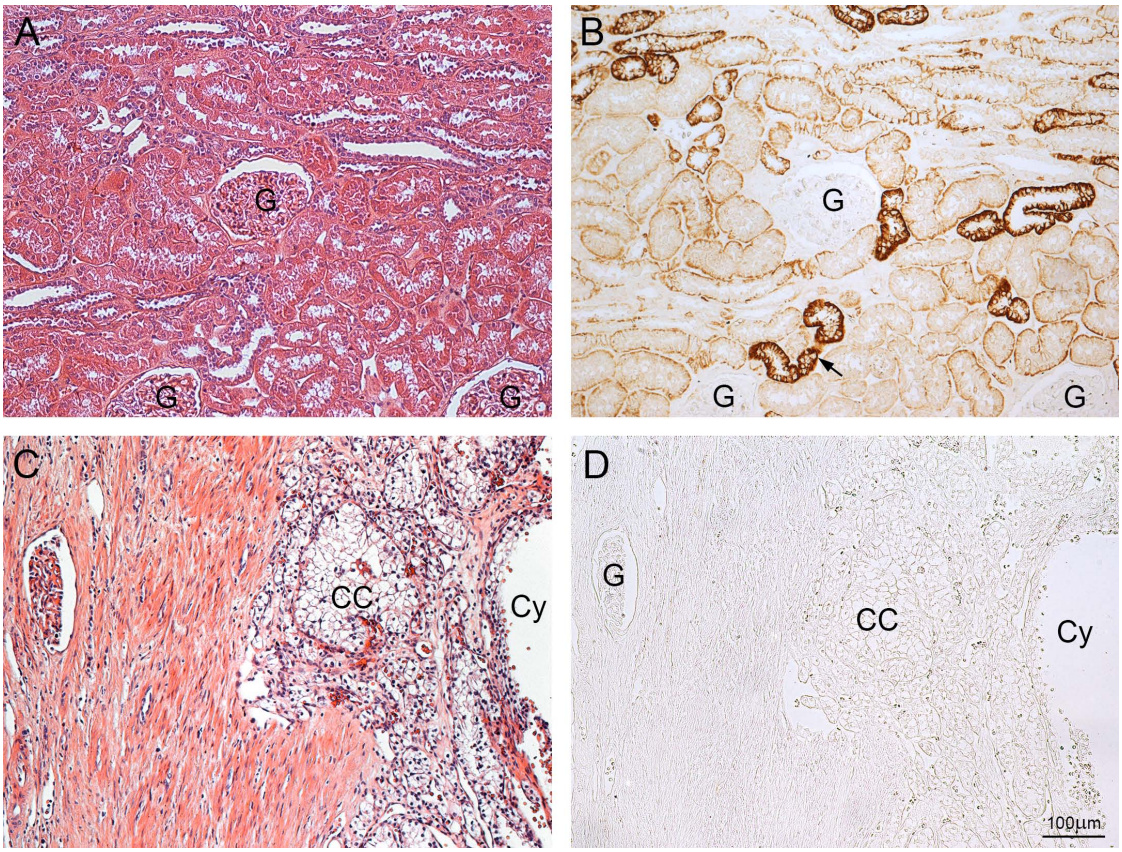

Fig. (2). A-D: Histology and immunohistochemistry on renal tumor tissue with apparently normal cortex. A: H&E stained cortex with glomeruli (G). **B**: Neighbor section; strong cytoplasmic staining for pVHL in distal tubules (arrow) and weaker staining in proximal tubules; glomeruli were unstained. **C**: H&E stained tumor tissue with clear cell carcinoma (CC), cyst (Cy), glomerulus, and connective tissue. **D**: Neighbor section was without immunoreaction for pVHL. Bar: 100µm.

sporadic RCCs [41-43]. We cannot exclude the possibility that the proband's RCC is sporadic or due to yet unknown hereditary factors. In this context, the p.P81S variant could be coincidental, and not causative of the proband's phenotype. The p.P81S mutation originated from the proband's maternal family who had no history of vHL or RCC. It is noteworthy that the proband's paternal uncle died from metastasizing RCC, and his father died at an early age (Fig. **1**). Unfortunately, we could not obtain information about the genetic statuses of the father or uncle to evaluate whether a genetic RCC predisposition originated from the paternal family. The initial screening of other known RCC-associated genes limits the likelihood that other differential diagnoses, such as Birt-Hogg-Dube syndrome, Hereditary Leiomyomatosis and Renal Cell Cancer, or Hereditary Papillary Renal Carcinoma should be the cause of a hereditary RCC-phenotype in this family.

Overall, including our family, twenty-eight carriers of the p.P81S mutation are reported in the literature. Ten of these carriers from two different families were found to have another concurrent *VHL* mutation, a large deletion and p.L188V mutation, respectively [36, 38]. Both concurrent mutations were previously assessed to be pathogenic [36, 38], and are in all likelihood responsible for the families' vHL phenotypes. In the majority of the families with only the p.P81S mutation, most carriers have no vHL-related manifestations [31, 34, 36, 38]. Only about a third (5 of 18 individuals) fulfilled the clinical diagnostic criteria for vHL [8], and no families had more than one family member with a vHL phenotype (Table **1**). In one family, a 35-year old patient had a retinal HB and islet cell tumor of the pancreas, and his father was reported with a pheochromocytoma, but the father's genotype was unknown [33, 34]. In our family, as well as one other family, the two probands did not fulfill the clinical diagnostic criteria for vHL, but presented with only a single vHL-associated manifestation (Table **1**).

The majority of the *in silico* algorithms employed reported p.P81S to possibly be pathogenic. HOPE and Symphony considered the mutation to affect the protein function and to entail a high risk for developing RCC, while Align GVGD assigned it an intermediate genetic risk (Table **2**). Many of the *in silico* methods base their pathogenicity predictions on a molecular evolutionary approach, as there is correlation between highly conserved residues and the intolerance of mutations which are likely to cause disease [44]. Some also consider the mutation's position in the context of the protein structure, such as MutationTaster, Polyphen-2, Project HOPE, and Symphony. Among the *in silico* tools, we consider Align GVGD and Symphony to be more reliable, based on the accuracy of the parameters and algorithms used.

RCC development is correlated with *VHL* mutations that affect HIF regulation [45]. This might not be the result of the

Table 1. Overview of all reported carriers of the *VHL* p.P81S mutation in the literature.

Individuals with the vHL phenotype* out of all reported p.P81S mutation carriers (%)	vHL-related manifestations found in the affected individuals with the p.P81S mutation	Ages of the reported p.P81S mutation carriers	Comment	References
10 of 12 (83%)	1. family: 1 pt. with CNS HB + pancreatic cysts (p.P81S + *de novo VHL* deletion) and his vHL affected son (without p.P81S, but instead the *VHL* deletion). 2. family** (9 cases with a common ancestor, all also had a *VHL* p.L188V mutation on the same allele): Pt. 1: a single pheo, Pt. 2+3+4: multiple pheos, Pt. 5: retinal HB + spinal HB + renal cysts, Pt. 6+7: retinal HB + spinal HB, Pt. 8 + 9: spinal HB + Pheo	1. family: Affected proband 50 years + healthy father (with p.P81S, unknown age) 2. family: 13 carriers over three generations,10 clinically evaluated: 9 affected (unknown ages) + 1 clinically unaffected (age 36)	In this study only patients with double *VHL* mutations + pheos were included. p.P81S was found in two separate families, but in both concomitant with other *VHL* mutations assessed to be the causes of the disease.	a
3 of 3 (100%)	First pt.: cerebellar and spinal HBs + RCC + renal cysts + pancreatic cysts + epididydimal cysts Second pt.: retinal HB + pancreatic islet cell tumor (+ father with pheo, but unknown genotype) Third pt.: multiple CNS HBs	First pt.: Unknown Second pt.: 35 years Third pt.: 34 years	p.P81S found in three families fulfilling vHL diagnostic criteria, specifics found and given in [34] First pt.: isolated case Second pt.: father with pheo but unknown mutation status Third pt.: unknown family status	b
1 of 1 (100%)	Multiple CNS HBs + RCC	Unknown	No information about the pt.'s age or family members.	c
1 of 2 (50%)	CNS HB+ RCC + pancreatic cysts	Unknown	The pt.'s father was also a p.P81S mutation carrier; died unaffected at age 89 years	d
1 of 5 (20%)	1 Cerebellar HB***	17,77,43,44, 64 years	All 5 subjects from the same family. A 44 old woman was the proband in this family with a single cerebellar HB.	e
1 of 5 (20%)	1 patient with ccRCC***	29, 32, 36, 52, 71 years	All 5 subjects from the same family. A 29-year-old man was the proband in this family, with a single RCC.	Our study
In total: 17 of 28 (61%)				

Pt. = Patient, CNS= Central Nervous System, HB = Hemangioblastoma, ccRCC = Clear cell Renal Cell Carcinoma, Pheo= pheochromocytoma

* Diagnostic clinical criteria for vHL: 2 manifestations or 1 manifestation AND affected 1st degree relative with vHL [8].

** We considered the patients with the concurrent mutations p.P81S and p.L188V, reported by [36] and [38],to belong to the same family, based on a personal correspondence with the two respective corresponding authors.

***The affected probands in these families do not strictly fulfill the clinical diagnostic criteria for vHL.

a:[36], [38], b: [32], [33], c: [30], [35], d:[31], [36], e:[34], [37]

absence of pVHL, but rather the incapacity of the mutated protein to interact with other proteins, such as HIF and Elongin C, that is responsible for its role in RCC development [45]. The p.P81S mutation is located within the β-sheet domain of pVHL (residues 63-154). Even though Elongin C binding has been assigned mainly to the pVHL α-helical domain, P81 and R82 have been shown to be located at the site of α-pVHL, β-pVHL, and Elongin C interactions [46]. Residue p.P81S might affect the orientation of the neighboring residue R82, whose side chain is central in a hydrogen

Table 2. Results from *in silico* analysis and databases.

Method	Description	Results	Reference
Databases			
HGMD*	Known gene lesions responsible for human inherited diseases and functional polymorphisms	Pathogenic. Phenotype: von Hippel-Lindau syndrome	a
dbSNP*	Identified genetic variations	Validated dbSNP entry, Clinical significance: Other	b
ClinVar*	Correlation between human genetic variations and phenotypes	Pathogenic, von Hippel-Lindau syndrome. Uncertain significance, Neoplastic Syndromes, Hereditary	c
Uniprot*	Protein sequence and functional information	Possibly pathogenic	d
ESP*	Genetic variants in coding regions (large-scale population study)	Eur. Am.: T=0.06% Afr.Am.: T=0.00%	e
***In silico* methods**			
SIFT*	Evolution sequence information	Tolerated, score: 0.08 Median: 3.47	f
Align GVGD*	Evolution sequence information and biophysical characteristics	C15 (GV:37.56-GD:49.72)	g
Mutation Taster*	Protein features, DNA and protein sequence, genotype frequencies, conservation analysis, splice site, polyadenylation signal	Polymorphism Probability value: 0.902	h
PolyPhen2	Protein features and evolutionary considerations	*HumDiv prediction model:* Possibly damaging, score: 0.945. Sensitivity: 0.80; Specificity: 0.95 *HumVar prediction model****:* Benign, score 0.322. Sensitivity: 0.86; Specificity: 0.77	i
HOPE	Amino acids biophysical features and protein 3D-structure	*Contacts:* multimer contacts involving the residue affected. *Structure*: the special conformation given by proline (very rigid) can be disturbed. *Variants*: "DISEASE". *Conservation:* possibly damaging. *Domains:* "von Hippel-Lindau disease tumor suppressor, beta domain". *Amino acids properties*: mutant residue smaller than wild-type (possible loss of external interactions). Wild-type residue more hydrophobic than mutant (loss of hydrophobic interactions with other molecules on the surface of the protein).	j
Symphony	Protein stability and protein interaction with other ligands	*Predicted risk of ccRCC:* YES *Molecule interface:* Elongin C	k

HGMD: Human Genome Mutation Database, dbSNP: the Single Nucleotide Polymorphism database, ClinVar: ClinVar database, Uniprot: Universal Protein Resource, ESP: Exome Sequencing Project, SIFT: Sort Intolerant from Tolerant, Polyphen-2, Polymorphism Phenotyping 2, HOPE: Have (y)Our Protein Explained, ccRCC: Clear cell renal cell carcinomas, Eu.Am: European American, Afr.Am.: African American.

*Approached through Alamut Visual version 2.6 (Interactive Biosoftware, Rouen, France)

**Model used in the presented results

a:[12], b:[13], c:[14], d:[18], e:[19], f:[20], g:[21], h:[22], i;[24], j:[26], k:[29].

bond network stabilizing both the α/β domain interface of pVHL and the pVHL/Elongin C interface [47]. The VHL-Elongin C interface is almost completely hydrophobic, with only a few hydrogen bonds on the periphery involving the residue R82. The replacement of proline with serine at residue 81 may disturb the native pVHL structure because of the loss of hydrophobic interactions, the loss of a special rigid conformation associated with proline, and the creation of hydrogen bonds with neighboring polar amino acids [36]. A functional study investigated different mutant pVHL, both alone and in combinations [47]. Alone, p.P81S did not cause significant structural perturbation of the protein, but it had cumulative severity when combined with p.L188V. These results suggest that p.P81S may modify known germline high-penetrance mutations [36, 48]. The position within the β sheet is compatible with the impairment of the protein

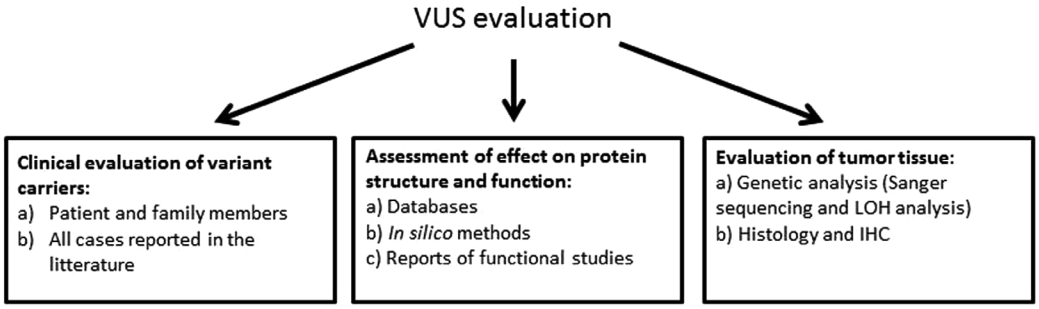

Fig. (3). Overview of the evaluation of VUS pathogenicity. VUS: Variant of unknown significance, LOH: Loss of Heterozygosity, IHC: Immunohistochemistry.

binding of HIF [36]. Fibronectin assembly might also be impaired by p.P81S mutation, due to its role in target capture [36]. Furthermore, p.P81S disrupts the binding of pVHL and heterochromatin protein 1 (HP1) in the conserved HP1-binding motif located in the β-sheet of pVHL [49]. A further indication that the position is important in pVHL functionality is that finding a mutation of the neighboring position, c.242C>T (p.P81L), presumably plays a causative role in sporadic paraganglioma development [50] (Table **2**). Both *in vivo* and *in vitro* models based on embryonic stem cells from *Vhl*$^{-/-}$ mice have shown that *VHL* p.P81S can initiate a cellular response that provides a tumor growth advantage through metabolic diversification, apoptosis suppression, and the alteration of DNA damage response [51]. Furthermore, p.P81S has been shown to impair the ability of pVHL to interact with Elongin C [51].

Another interesting aspect is the recent finding of an association between exposure to the industrial solvent trichloroethylene (TCE) and somatic *VHL* p.P81S mutations in sporadic RCC [7, 39]. A remarkably high frequency of somatic p.P81S mutations are observed in RCC tumor tissue from patients exposed to TCE (in 13 out of 44 patients, i.e. 39%) [39]. In addition, somatic p.P81S mutations are more frequently found in patients with high and medium levels of TCE exposure, but not in those with a low exposure level [39]. The hotspot mutation p.P81S was not observed in sporadic tumors from unexposed individuals (n=128 RCC patients) [39, 52]. Interestingly, in RCCs from TCE exposed patients, the p.P81S mutation was also found in apparently normal kidney parenchyma adjacent to the RCC (4 patients), indicating that the mutation may represent the "first-hit" in a somatic two-hit RCC tumorgenesis involving *VHL* brought on by TCE exposure [39]. The mechanism of TCE effect on the *VHL* gene is not fully known; it is hypothesized that p.P81S may be pro-tumorigenic, but without direct transforming activity [36, 39].

Our findings of the lack of pVHL expression in the tumor tissue, along with the proband's young age at diagnosis, indicate an association between *VHL* p.P81S and the vHL phenotype. However, due to the low frequency of vHL manifestations among reported p.P81S carriers, we cannot fully exclude that the proband's tumor is a sporadic RCC. When considering that the majority of the *in silico* methods predicted a possible pathogenic effect, the critical position of the nucleotide in relation to pVHL function, and the variant's association with TCE-related RCC development, p.P81S is in all likelihood a low-penetrant pathogenic variant for RCC development. We recommend annual ultrasound of the kidneys as surveillance for the presented family, instead of the full vHL surveillance program [8]. If a mutation carrier should later develop clinical signs of vHL, we advise the family to be re-evaluated.

CONCLUSION

We propose a method for the evaluation of the clinical impact of a VUS: 1) Clinical evaluation of all variant carriers (including those reported in the literature); 2) Evaluation of tumor tissue using genetic analysis, histology, and IHC; 3) Assessment of the variant's effect on protein structure and function through the use of multiple genetic databases, *in silico* algorithms, and results from any functional studies reported. It is noteworthy that we found discrepancies between the different *in silico* algorithms, which underlines the possible problem with applying just one method. Use of several different methods provides a better prediction that takes different aspects into account. Clinical uncertainty of such a variant might persist, despite of use of sensitive and specific methods such as those described above. Therefore, we strongly encourage the development of specific guidelines regarding how clinicians should manage VUS.

LIST OF ABBREVIATIONS

BSA	=	Bovine serum albumin
BLAST	=	Basic Local Alignment Search Tool
CNS	=	Central nervous system
DAS	=	Distributed Annotation System
dbSNP	=	Single Nucleotide Polymorphism database
ESP	=	Exome Sequencing Project
HGMD	=	Human Mutation Database
GTR	=	Genetic Testing Registry
HB	=	Hemangioblastoma
H&E	=	Hematoxylin and eosin
HIF	=	Hypoxia-inducible factors
HOPE	=	Have (y)Our Protein Explained
HP1	=	Heterochromatin protein 1
IHC	=	Immunohistochemistry

LOH = Loss of heterozygosity

MLPA = Multiplex Ligation-dependent Probe Amplification

NaAc = Sodium acetate

NCBI = National Center for Biotechnology Information

NHGRI = National Human Genome Research Institute

OMIM = Online Mendelian Inheritance in Man

Polyphen2 = Polymorphism Phenotyping version 2

PDB = Protein Data Bank

pVHL = VHL protein

RCC = Renal cell carcinoma

SIFT = Sort Intolerant From Tolerant

TCE = Trichloroethylene

UniProt = Universal Protein Resource

vHL = von Hippel-Lindau disease

VHL = von Hippel-Lindau tumor suppressor, E3 ubiquitin protein ligase gene

VUS = Variant of Unknown Significance

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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MLMB had the idea for the study and initiated it. LNK, MLB, and MLMB obtained the clinical data, blood and tissue samples, and counseled the family. MLMB and MLB coordinated and supervised the study. DA and MLMB analyzed the scientific literature. DA studied the mutation, using databases and *in silico* algorithms. SNH and HM carried out IHC and the genetic analysis of the RCC tumor. DA wrote the first draft of the manuscript. All of the authors edited the draft and approved the final manuscript.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article, and includes: Supplementary Table **1**: Primers used for Sanger Sequencing and LOH analysis. Supplementary Fig. **1**: Illustration of the chromosomal location of the three microsatellite markers used for the LOH analysis in relation to the *VHL* gene.

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