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Von Hippel–Lindau (VHL) disease and VHL-associated tumors in Indian subjects: VHL gene testing in a resource constraint setting

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Abstract

Background: Von Hippel–Lindau (VHL) syndrome is a familial cancer syndrome caused by mutations in VHL gene. It is characterized by the formation of benign and malignant tumors like retinal angioma, cerebellar hemangioblastoma, spinal hemangioblastoma, renal cell carcinoma, pheochromocytoma, pancreatic and renal cysts, and endolymphatic sac tumors. Germline mutations in VHL gene have also been reported in isolated VHL-associated tumors. VHL gene is a small gene with 3 coding exons and can be easily tested even in a resource constraint setting.

Objective: To describe clinical presentation and estimate the diagnostic yield of in VHL and VHL-associated tumors.

Methods: This is a descriptive study in a hospital setting. Here, we describe the clinical and molecular data of 69 patients with suspected VHL or having VHL-associated tumors. Sanger sequencing of coding sequences and conserved splice sites of VHL gene were done in all patients. Multiplex ligation-dependent probe amplification (MLPA) of VHL gene to detect large deletions/duplications was performed for 18 patients with no pathogenic sequence variations.

Results: Among tumor types at presentation, pheochromocytoma was seen in 49% (34/69), hemangioblastoma was seen in 30% (21/69), and renal cell carcinoma was seen in 7% (5/69). Rest had other tumors like paraganglioma, endolymphatic sac papillary tumors, cerebellar astrocytoma and pancreatic cyst. Seven patients (10%) had more than one tumor at the time of diagnosis. Pathogenic variations in VHL gene were identified in 31probands by Sanger sequencing; 18 were missense, 2 nonsense and 2 small indels. A heterozygous deletion of exon 3 was detected by MLPA in one patient among 18 patients for whom MLPA was done. Overall, the molecular yield was 46% cases (32/69). Family history was present in 7 mutation positive cases (22%). Overall, 11 families (16%) opted for pre-symptomatic mutation testing in the family.

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Conclusions: Mutation testing is indicated in VHL and VHL-associated tumors. The testing facility is easy and can be adopted easily in developing countries like India. The yield is good, and with fairly high incidence of familial cases, molecular testing can help in pre-symptomatic testing and surveillance.

Keywords: Von Hippel–Lindau syndrome, Molecular sequencing data, Copy number variation

Introduction

Von Hippel–Lindau (VHL) syndrome (OMIM#193,300) is an autosomal dominant disorder caused by mutation in VHL gene and is mainly characterized by the formation of benign and malignant tumors. VHL is a tumor suppressor gene which regulates the transcription factors HIF1 and HIF2. Its inactivation leads to over expression of target genes implicated in angiogenesis, cell growth and proliferation [1].

The tumors seen in VHL are retinal hemangiomas, cerebellar hemangioblastoma, spinal hemangioblastoma, renal cell carcinoma, pheochromocytoma, pancreatic and renal cysts, and endolymphatic sac tumors. Clinical diagnostic criteria have been proposed. For simplex cases, in the absence of family history, at least two characteristic lesions are required to make the diagnosis while in patients with positive family history; one characteristic lesion is enough to make the diagnosis of VHL [2, 3]. Appearance of only one feature has been reported in 50% of the patients. The VHL disease has been characterized as type I (without pheochromocytoma) and type II (with pheochromocytoma) and certain genotype–phenotype correlations have been described [4, 5]. Among all cases with a mutation in VHL gene, about 90% cases are due to point mutations which include missense, nonsense, small insertion and deletions and splice site variants. Large deletions/duplications account for rest 10% cases.

VHL is a small gene with 3 coding exons with 642 nucleotides coding for 213 amino acids [6]. In this study, we compiled the clinical and molecular data of 69 Indian patients with suspected VHL and also include pre-symptomatic testing of some families. We further attempted to analyze the spectrum of mutations in VHL gene.

Subjects and methods

Study design

A descriptive study in a tertiary hospital setting.

Subjects

Sample size: 69 consecutive subjects who have met the inclusion criteria for the study period of 5 years.

Inclusion criteria: Patients with suspected VHL (with one or more tumors known to be associated with VHL).

Exclusion criteria: Patients with other familial cancers.

Consent and ethical approval

Informed consent was obtained from all subjects and guardians (in case of minors) before data collection and testing. Ethical approval was obtained from Institutional Ethics Committee of the Institute where data collection and molecular testing were done. Ethical committee approval number: IEC 2016-110-EMP-92.

Acquisition of clinical data

After obtaining informed consent, all relevant clinical information was collected. It included age at first diagnosis, types of the tumors, other associated features. A detailed family history was taken, all at-risk relatives identified, and their disease status determined. All subjects and their available family members were provided genetic counselling, which included at least three sittings: one pre-test, one post-test and one extended family screening and surveillance plan.

Molecular test

EDTA blood (3 ml) of the subjects and their at-risk relatives (where available) were collected as a part of clinical testing. Genomic DNA from the blood samples was extracted using commercial kit (QIAamp DNA Blood extraction

Table 1 Primers for VHL (NM_000551)

Sl.No	Exons	Forward primers	Reverse primers	Band sizes
1	Exon 1	GCGAAGACTACGGAGGTCG	GATGTGTCCTGCCTCAAGGG	565
2	Exon 2	ACAACCTTTGCTTGTCCCGA	GGCAAAAATTGAGAACTGGGCT	239
3	Exon 3	TACTGAGACCCTAGTCTGCC	ACTAAGGAAGGAACCAGTCC	318

kit). Sanger sequencing of all three coding exons of *VHL* gene (NM_000551) was done. The entire coding regions and exon–intron boundaries of *VHL* were amplified by polymerase chain reaction (PCR) method with 3 primer sets (Table 1). Purified PCR products were sequenced bi-directionally using ABI 310 capillary sequencer (Applied Biosystems, San Diego, USA). All variations were looked in HGMD (www.hgmd.cf.ac.uk), clinvar (https://www.ncbi.nlm.nih.gov/clinvar/), specific VHL mutation database, PubMed (https://www.ncbi.nlm.nih.gov/pubmed/) as well as population databases like 1000 G (http://www.internationalgenome.org/), EXaC (http://exac.broadinstitute.org/) to see if they were already reported mutations or polymorphisms. In-silico analysis for pathogenicity of mutations were checked using 3 prediction tools namely Mutation Taster (www.mutationtaster.org), SIFT (sift.jcvi.org) and Polyphen-2 (genetics.bwh.harvard.edu/pph2). Annotations for sequence variations were rechecked and online prediction was done using *MobiDetails* (https://mobidetails.iurc.montp.inserm.fr/MD/). Pathogenicity for each sequence variation was ascertained based on the criteria laid down by the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG) [7]. Other at-risk family members were checked for the sequence variations found in the affected patient. Some patients (where blood sample was available) with wild type VHL were subsequently reanalyzed for genomic rearrangements using multiplex ligation-dependent probe amplification (MLPA) assay. MLPA was carried out as per manufacturer's instructions (SALSA MLPA P016 VHL probemix, MRC-Holland, Amsterdam, The Netherlands).

Protein modelling for the Novel variant

The superfamily and domains of deduced VHL protein were validated by superfamily version 1.75 and SMART tools analysis, respectively [8, 9]. Secondary structure prediction for both wild and mutant VHL proteins was performed by PDBsum analysis at European Molecular Biology Laboratory (EMBL) server. Homology models were constructed on SWISSMODEL workspace (http://swissmodel.expasy.org/workspace) [10], based on template, VHL subunit A of *Homo sapiens* (PDB id: 4WQO) [11]. The quality of generated protein models were evaluated by QMEAN score (http://swissmodel.expasy.org/qmean/cgi/index.cgi) and PROCHECK tools [12]. Further, constructed models were visualized in University of California, San Francisco (UCSF) Chimera [13].

Results

Our study included 69 unrelated probands (45 males and 24 females) who presented with one or more tumors classically associated with VHL syndrome (Table 2). The average age at diagnosis was 27.2 years (range = 10–68 years). Among tumor types at presentation, pheochromocytoma was found in 49% (34/69), hemangioblastoma was found in 30% (21/69), renal cell carcinoma was found in 7% (5/69), and paraganglioma was found in 4% (3/69). Pancreatic cysts and cerebellar astrocytoma were present in 2 patients each and endolymphatic sac tumor in a single patient. Diagnostic criteria for VHL were met by 12 patients in whom pathogenic sequence variation was detected in 11 patients (yield 92%). Fifty seven probands did not meet diagnostic criteria and pathogenic sequence variation was detected in 21 of them (yield 37%). Seven patients had more than one tumor type at presentation. Of 21 different pathogenic sequence variations in VHL (identified in 31 probands) by Sanger sequencing; 18 were missense, 2 nonsense and 2 small indels. The overall diagnostic yield of Sanger sequencing was 44.9%. A positive family history was present in 7 cases in all of whom mutation was detected. Out of 38 patients in whom Sanger sequencing failed to detect any pathogenic sequence, MLPA to detect large deletions/duplications could be performed for 18 patients. A heterozygous deletion of exon 3 of VHL gene was detected in one patient from this subset. Pre-symptomatic testing of at-risk relatives was requested for 19 individuals from 11 families, of whom 9 pre-symptomatic individuals were found to harbor a pathogenic variation.

Only one novel variant was identified in this study, c.597delG (p.Glu199fsTer3). It was classified a VUS as per ACMG guidelines. The population frequency in various databases and predictions have been depicted in Table 3. Secondary structure and homology modeling of mutated VHL with this variant consisted of 2 sheets, 7 strands, 2 beta hairpins, 10 beta turns and 4 helices (Fig. 1). The Ramachandran Plot statistics of generated homology model of VHL-M showed that, 87.2% residues were in most favored regions, 12.8% residues in additionally allowed regions. The estimated absolute quality QMEAN4 was − 0.01, which was greater than the acceptable value − 0.50. Moreover, the main-chain G-factor and overall PROCHECK score of mutated VHL protein were found to be 0.37 and 0.08, respectively.

Discussion

VHL is a well-described familial cancer syndrome with well-established clinical characteristics and a database of mutations (http://www.umd.be/VHL/). However, there is limited data from the Indian population.

The average age at presentation in our cohort was 27.2 years which is comparable to previously reported data [5]. Pheochromocytomas constituted the most common tumor seen in 49% (34/69) subjects. This contrasts previous reports including the Indian study by Vikkath et al. 2015 where pancreatic cysts and hemangioblastomas were

Table 2 Clinical features and genotype of patients with variants detected in VHL gene (NM_000551)

Case no	Sex	Age at diagnosis in years	Presenting tumor	Other tumors (s)/ feature(s)	Variation identified	ACMG classification	Criteria	Family history	Clinical diagnostic criteria met[#]	Pre-symptomatic testing of family members
01	M	15	Cerebellar hemangioblastoma	No	c.337C > T p.Arg113Ter	Pathogenic	PVS1, PM2, PP3, PP5	+	Yes	Father- same variation identified
02	M	46	Cerebellar hemangioblastoma	No	c.556G > A p.Glu186Lys	Likely pathogenic	PS1, PM1, PM2, PP3 (Reported in clinvar)	–	No	No
03	M	37	Renal cell carcinoma (bilateral)	No	c.194C > T p.Ser65Leu	Likely pathogenic	PM1, PM2, PP3, PP5	+	Yes	Son-no variation identified
04	M	13	Pheochromocytoma (B/L)	No	c.556G > A p.Glu186Lys	Likely pathogenic	PS1, PM1, PM2, PP3 (Reported in clinvar)	–	No	No
05	M	27	Cerebellar hemangioblastoma	Pheochromocytoma (B/L)	c.500G > A p.Arg167Gly	Likely pathogenic	PM1, PM2, PM5, PP3	+	Yes	No
06	M	30	Renalangiomyolipoma, pancreatic and epididymal cyst	–	c.337C > T p.Arg113 Ter	Pathogenic	PVS1, PM2, PP3, PP5	+	Yes	Brother-same variation identified
07	M	31	Renal cell carcinoma (B/L)	Pheochromocytoma (B/L)	c.597delG p.Glu199fsTer3[##]	VUS	PVS1, PM2	–	Yes	Daughter-no variation identified Son-same variation identified
08	F	27	Cerebellar hemangioblastoma	No	c.556G > A p.Glu186Lys	Likely pathogenic	PS1, PM1, PM2, PP3 (Reported in clinvar)	–	No	No
09	M	11	Pheochromocytoma(B/L)	No	c.382C > T p.Leu128Phe	VUS; upgraded to Likely pathogenic	PM1, PM2, PP3, PS4	–	No	Brother-same variation identified Father-no variation identified
10	M	36	Cerebellar hemangioblastoma	No	c.233A > G p.Asn78Ser	Likely pathogenic (Pathogenic in Clinvar)	PM1, PM2, PP3, PP5	–	No	Sister-no variation identified
11	F	14	Endolymphatic sac papillary tumor	No	c.563 T > C p.Leu188Pro	Likely pathogenic	PM1, PM2, PP3, PP5	–	No	No
12	M	28	Pancreatic cyst	No	c.277G > C p.Gly93Arg	Likely pathogenic	PM1, PM2, PM5, PP3	+	Yes	No
13	M	16	Cerebellar hemangioblastoma	Pheochromocytoma (B/L)	c.277G > C p.Gly93Arg	Likely pathogenic	PM1, PM2, PM5, PP3	–	Yes	No
14	M	13	Pheochromocytoma (B/L)	No	c.500G > A p.Arg167Gln	Likely pathogenic	PM1, PM2, PM5, PP3	–	No	No

Table 2 (continued)

Case no	Sex	Age at diagnosis in years	Presenting tumor	Other tumors (s)/ feature(s)	Variation identified	ACMG classification	Criteria	Family history	Clinical diagnostic criteria met[#]	Pre-symptomatic testing of family members
15	M	22	Pheochromocytoma (right)	No	c.467A > G p.Tyr156Cys	Likely pathogenic	PM1, PM2, PP3, PP5	−	No	No
16	F	25	Pheochromocytoma (B/L)	No	c.466 T > C p.Tyr156His	VUS, upgraded to Likely pathogenic	PM1, PM2, PP3, PP4	−	No	Son and daughter-same variation identified
17	F	27	Cerebellar hemangioblastoma	No	c.257C > T p.Pro86Leu	Likely pathogenic	PM1, PM2, PP3, PP5	−	No	No
18	M	16	Pheochromocytoma (B/L)	No	c.467A > G p.Tyr156Cys	Likely pathogenic	PM1, PM2, PP3, PP5	−	No	Father-same variation identified Mother and sister-no variation identified
19	F	12	Pheochromocytoma (B/L)	No	c.482G > A p.Arg161Gln	Likely pathogenic	PM1, PM2, PP3, PP5	+	Yes	Brother and sister-same variation identified
20	F	12	Pheochromocytoma (Right)	No	c.239G > A p.Ser80Asn	Likely pathogenic	PM1, PM2, PM5, PP3	+	Yes	No
21	M	14	Pheochromocytoma (B/L)	No	c.340G > A p.Gly114Ser	Likely pathogenic	PM1, PM2, PP3, PP5	−	No	No
22	M	12	Pheochromocytoma (B/L)	No	c.256C > T p.Pro86Ser	Likely pathogenic	PM1, PM2, PP3, PP5	−	No	No
23	F	17	Pheochromocytoma (B/L)	No	c.382C > T p.Leu128Phe	VUS; upgraded to Likely pathogenic	PM1, PM2, PP3, PS4	−	No	No
24	M	13	Intramedullary hemangioblastoma	No	c.217insC (c.217dup) p.Gln73ProfsTer 59	Pathogenic (Reported in HGMD)	PVS1, PS1, PM1, PM2	−	No	No
25	M	34	Pheochromocytoma (B/L)	No	c.583C > T p.Gln195 Ter	Pathogenic (Reported in Clinvar)	PVS1, PM2, PP5	−	No	No
26	M	17	Pheochromocytoma (B/L)	No	c.499C > T p.Arg167Trp	Pathogenic	PS3, PM1, PM2, PP3, PP5	−	No	Father, mother and sister-no variation identified
27	M	23	Pheochromocytoma (B/L)	No	c.499C > T p.Arg167Trp	Pathogenic	PS3, PM1, PM2, PP3, PP5	−	No	No
28	F	46	Pheochromocytoma (B/L)	No	c.335A > G p.Asn112Cys	Likely pathogenic	PM1, PM2, PP3, PP5	−	No	Mother-no variation identified

Table 2 (continued)

Case no	Sex	Age at diagnosis in years	Presenting tumor	Other tumors (s)/ feature(s)	Variation identified	ACMG classification	Criteria	Family history	Clinical diagnostic criteria met[#]	Pre-symptomatic testing of family members
29	M	15	Cerebellar hemangioblastoma	Pheochromocytoma (B/L)	c.482G > A p.Arg161Gln	Likely pathogenic	PM1, PM2, PP3, PP5	–	Yes	No
30	M	10	Pheochromocytoma (B/L)	No	c.499C > T p.Arg167Trp	Pathogenic	PS3, PM1, PM2, PP3, PP5	–	No	No
31	F	29	Cerebellar hemangioblastoma	Pheochromocytoma (B/L)	c.500G > A p.Arg167Gln	Likely pathogenic	PM1, PM2, PM5, PP3	–	Yes	No
32	M	46	B/L Renal cell carcinoma	–	Heterozygous deletion of exon 3 (MLPA)			–	No	No

B/L Bilateral; *MLPA* Multiplex ligation-dependent probe amplification

[#] Diagnostic criteria- For simplex cases, in the absence of family history, at least two characteristic lesions are required to make the diagnosis while in patients with positive family history, one characteristic lesion is enough to make the diagnosis of VHL (Lancer et al. 2003; Maher et al. 2011)

[##] Novel variant

Table 3 Population frequencies and predictions for the novel variant c.597delG (p.Glu199AspfsTer3)

Population databases	**Values**	**Descriptions**
gnomAD exome:	No match in gnomAD exome	v2.0.1 Exomes global MAF
gnomAD genome:	No match in gnomAD genome	v2.0.1 Genomes global MAF
gnomAD v3:	No match in gnomADv3	v3 Genomes global MAF
dbSNP rsid:	No match in dbSNP v154	Identifier for NCBI dbSNP
Clinvar:	No match in Clinvar v20220702	Clinvar interpretation
LOVD Matches:	No match in LOVD public instances	LOVD match in public instances
Features	**Values**	**Descriptions**
Overall predictions		
CADD raw:	No match in CADD v1.6	[-6.41;35.5] The higher the less likely to be observed
CADD phred:	No match in CADD v1.6	Phred-like scaling of raw score
Eigen raw:	None	[-3.33;6.84] The higher the less likely to be observed
Eigen phred:	None	Phred-like scaling of raw score
MPA score:	10	Raw score [0;10], 10: high impact
MPA impact:	Frameshift	Impact type

the most common presentations [14]. Overall diagnostic yield was 46.3% in our cohort which included VHL and isolated tumors associated with VHL. Out of 12 patients, who met the diagnostic criteria at the time of presentation, pathogenic variations in VHL gene were detected in 11 patients (92% yield). However, pathogenic variations were also identified in 21 patients who did not meet diagnostic criteria at the time of presentation (yield 37%). This stresses the limitations of relying on the criteria especially in young patients where the full spectrum of the disorder has not manifested. Out of 37 patients with isolated PPGLs (Pheochromocytomas and Paragangliomas), pathogenic variation in VHL gene was found in 16 patients (43%). PPGLs are caused by a germline pathogenic mutation in one of the 27 susceptibility genes which include RET, NF1, MET, KIF1B, MAX, etc. besides VHL [15]. A mutation in one of these remaining genes may be causative. In an Indian study by Khadilkar et al. on PPGLs VHL gene was among the 5 genes they studied. In their study, VHL accounted for 33.3% and 13.18% PPGLs cases in pediatric and adult patients, respectively [16]. In a similar Indian study by Lomte et al. on patients with PPGLs, missense mutation was found in 81% and the rest were small indels or large deletions. However, their study was a retrospective study of mutation positive cases only and did not take in to account the yield of each test unlike our study [17].

In our cohort, diagnostic yields for CNS hemangioblastoma and renal cell carcinoma were 45% (9/21) and 60% (3/5), respectively. Of particular interest was a 14-year-old girl (Case 11) who had only endolymphatic sac tumor at presentation. So, VHL germline mutation testing should be offered even to those who do not fulfill the diagnostic criteria and have seemingly sporadic tumors.

Most of the 22 pathogenic variations identified in this study are clustered in exons 1 and 3 of VHL (NM_000551, Fig. 2). Of these only one is novel. The mother of proband with this novel variant was deceased at 55 years of age with unknown cause and father at 75 years of age. In extended family history recently, proband's niece is found to have pheochromocytoma; however, sample was not available for molecular testing. This variant is predicted to be deleterious by in-silico prediction tools and protein homology modeling. The variants c.499C > T, c.500G > A and c.556G > A were each present in three unrelated families.

To facilitate genotype–phenotype correlations, VHL disease was categorized into Type 1 and Type 2, according to the absence or presence of pheochromocytoma. As previously reported, we also found that missense substitutions were associated with a higher risk of pheochromocytoma while truncating mutations and large genomic rearrangements were associated with renal tumors [18, 19]. Patients with the same mutation presented with different tumors. This was evident even in the same family. Patient 12, 28-year-old male, presented with pancreatic cyst, while his father and paternal uncles presented with CNS hemangioblastomas.

Pathogenic mutations were identified in all the 7 familial cases. Nine at-risk relatives were found to harbor the pathogenic variant found in the respective probands. Thus, we were able to counsel these patients and also put them on appropriate surveillance. Early detection and management of tumors have been shown to improve the outcome. Anxiety of mutation-negative at-risk relatives was allayed, and they were prevented from undergoing unnecessary surveillance.

Though, a multigene panel by next-generation sequencing (NGS), to look for VHL and VHL-associated tumors is

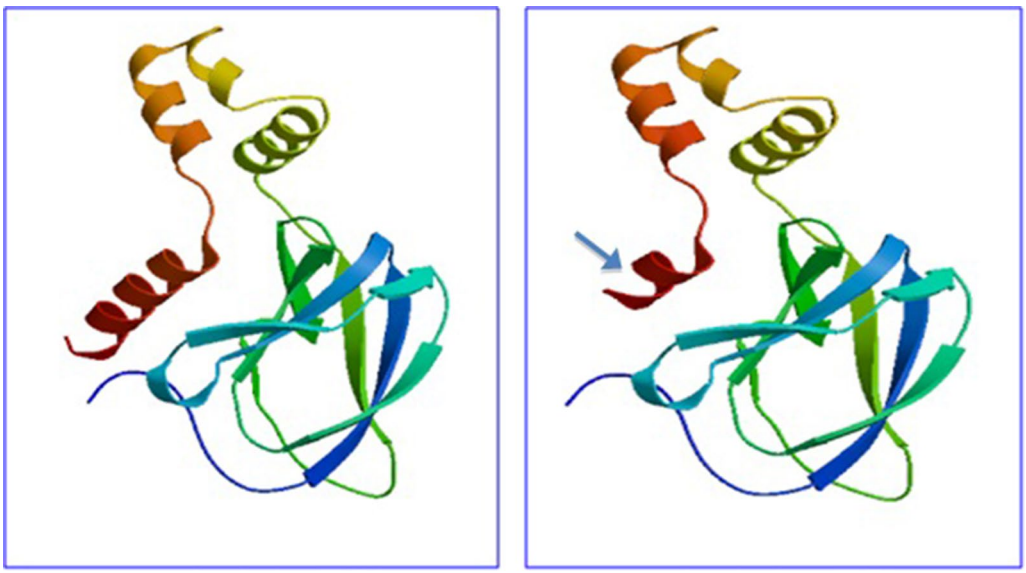

Fig. 1 Schematic view of the VHL-Wild type (left panel) and VHL-Mutated (right panel) homology model where VHL-Mutated shows truncated α helix (indicated by arrow)

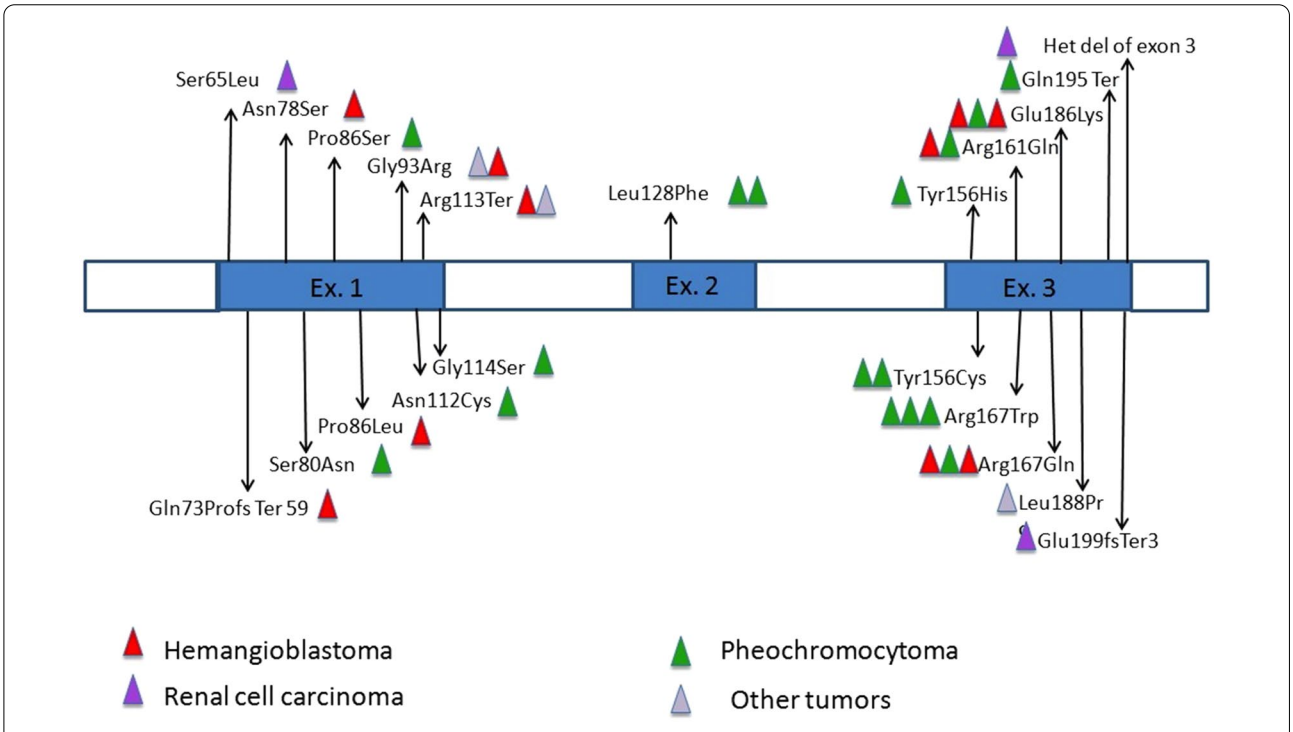

Fig. 2 Distribution of Mutations across VHL gene along with the tumors at presentation (NM_000551)

being employed exceedingly, Sanger sequencing of VHL gene should be the first step, considering the small size of the gene and high yield of the test, especially in resource constraint settings.

Conclusions

Due to the high sensitivity and specificity of VHL mutation testing, it should be offered not only to patients fulfilling the diagnostic criteria but also in those with sporadic tumors. Variant detection helps in genetic counselling and pre-symptomatic diagnosis by testing possible carriers in the family, thus improving outcome.

Abbreviations

VHL: Von Hippel–Lindau; MLPA: Multiplex ligation-dependent probe amplification; EDTA: Ethylenediamine tetraacetic acid; PCR: Polymerase chain reaction; ACMG: American college of medical genetics and genomics and the association for molecular pathology criteria for variant classification; PPGLs: Pheochromocytomas and paragangliomas; NGS: Next-generation sequencing; B/L: Bilateral.

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Author contributions

DA and MA contributed to data curation and writing original draft; PH, SP, SP, SD, and YP contributed to methodology**,** formal analysis and investigation; DP, GN, KM, GR, BR, MS, BD, CS, AA, and PS contributed to resources and editing; MK contributed to conceptualization, writing—reviewing and editing, and supervision. Each author believes that the manuscript represents honest work and consented for publication. All authors read and approved the final manuscript.

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Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

Ethical approval was obtained from Institutional Ethics Committee of SGPGIMS, Lucknow, India, the Institute where data collection and molecular testing were done. Ethical committee approval number: IEC 2016-110-EMP-92. Informed consent was obtained from all subjects and guardians (in case of minors) before data collection and testing.

Consent for publication

All subjects and guardians (in case of minors) consented for publication of data. Identity was kept anonymous.

Competing interests

All authors declare that they have no competing interests.

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