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ORIGINAL ARTICLE

Genotype–phenotype analysis of von Hippel–Lindau syndrome in fifteen Indian families

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Abstract The general prevalence of the familial multi-organ tumor disorder, von Hippel–Lindau syndrome (VHL), was estimated to be 1 in 25–40,000 in western studies two decades back. Few studies were done in Indian sub-continent, amidst a surge in clinical reports on VHL specific manifestations. The syndrome is correlated with mutations of the gene *VHL* (located in Chr 3p25.3). We aimed to conduct a prospective case series describing phenotypic and genotypic characteristics in Indian population. The VHL-specific clinical and radiological features were collected from patients and family members. Genotypic changes such as deletion/duplication or point mutation in the *VHL* locus were identified using sequencing and MLPA. Thirty-one subjects, from fifteen families with diagnosed VHL, were included in the study. Multicystic pancreas was found in 71 % (22/31), CNS hemangioblastoma in 68 % (21/31), renal cell carcinoma and retinal angiomas in 23 % (7/31) each, pheochromocytoma in 9.7 % (3/31) of the population and endolymphatic sac tumor in one subject. Four families (9 subjects) had full length deletion of *VHL*, three families (4 subjects) had a deletion of exon 3, eight families (18 subjects) had different exonic, splice-site and intronic point mutations and one subject had a de novo in-frame indel in exon 1. Multicystic pancreas and CNS hemangioblastomas were the most common manifestations in our population. The phenotypic expression patterns in terms of tumorigenesis, tissue tropism and penetrance in comparison to the genotypic features were found to be different from previous correlative studies.

Keywords Genotype–Phenotype correlation · von Hippel–Lindau syndrome · India · VHL · Mutation

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Introduction

More than a century ago, the vascular lesions of the retina identified by von Hippel [1] and associated cystic vascular tumors of the central nervous system (CNS) [2] were consolidated into one disorder with an autosomal dominant mode of inheritance, now known as von Hippel–Lindau syndrome (VHL, OMIM #193300) [3]. Since 1990, two genetic linkage analyses were done in twelve

British [4] and twenty-eight American pedigrees [5] of VHL families to map the gene locus between two closely flanking DNA markers in chromosome 3p. Further studies by positional cloning techniques on those flanking DNA markers helped to identify the causative gene *VHL* (NCBI Genbank gene ID: 608537), located in Chr 3p25.3 [6, 7]. According to the first major VHL registry from the United Kingdom with eighty-three individuals, cerebellar hemangioblastoma (Cere.HB) was the major manifestation in about 60 % of cases, followed by retinal angioma (RA) (34 %), renal cell carcinoma (RCC) (25 %) and an equal distribution of spinal hemangioblastoma (Sp.HB), pheochromocytoma (PCC) and multi-cystic pancreatic (MCP) lesions (14 %) [8]. Less frequent were hepatic lesions and endolymphatic sac tumors (ELST). Subsequently most of the clinical reports, retrospective clinical case studies, genotypic-phenotypic correlative studies done since then involved mostly western ethnic groups. The point prevalence of VHL was estimated to be 1 in 53,000 in southeastern UK in 1991 with an estimated birth incidence of 1 in 36,000 [9] and 1 in 85,000 in northwest UK in 1996 [8]. In genetic studies, new mutations accounted for only 1–3 % of cases [6, 7]. These prevalence studies have not been updated in recent times in other western populations.

Most of the existing literature pertaining to VHL in the Indian subcontinent consists of isolated clinical case reports on rare manifestations. An institutional retrospective study on CNS HB in northern India conducted between 1992 and 2003 identified six cases of VHL syndrome out of 69 patients with CNS HB (8.5 %) in 11 years [10]. A 10 year retrospective study done on 49 Southeast Indian patients with apparently sporadic HB, found clinical VHL syndrome in ten patients (20 %), with no relevant family history [11]. In case reports, such as that of bilateral multicentric RCC [12], sporadic pancreatic serous cystadenoma associated with neuroendocrine pancreatic carcinoma [13], multi-focal PCC [14], VHL was suspected, if not diagnosed, because of the specific cluster of manifestations occurred. A recent high throughput gene expression profiling study on three Asian Indian subjects with type-2 diabetes mellitus incidentally identified more than three-fold change in the co-expression of *VHL* compared to controls [15]. In 2012, Wu and coworkers analyzed 16 Chinese probands with clinically diagnosed VHL syndrome and identified 57 % of them (12/16) had de novo *VHL* mutations, without a family history [16]. Very recently, another study of nineteen Chinese probands with clinically confirmed VHL had novel mutations in nine families and six of them had no family history (~30 %) [17]. These studies show that there is improved awareness leading to increase in identification of VHL as well as *VHL* mutations during the last decade in Asian population.

Though VHL is a rare disease, frequent requirement of multiple surgeries for CNS HB and RCC as well as the disabling complication of retinal detachment and vision loss from undetected RA make living with VHL very challenging for affected individuals. Our objective was to conduct a prospective case series describing phenotypic and genotypic characteristics of VHL in Indian population.

Patients and methods

Patients

We set up a screening program at our institution (Amrita Institute of Medical Sciences) for early detection and surveillance of VHL related tumors, in which clinico-radiological screening and surveillance is possible. The subjects included in this study were on long term surveillance and management for VHL through our institution's multidisciplinary phakomatosis clinic (Amrita Center for Phakomatoses) over a period of eight years from 2006 to 2014. The study was approved by the Institutional Ethics Committee. After obtaining informed consent for genotype-phenotype analysis, a peripheral blood sample was taken and pedigree analysis completed. The clinical screening included (1) dilated retinal exam by our retinal specialists, (2) CNS imaging included gadolinium contrast enhanced MRI of the brain and entire spinal cord, (3) abdominal ultrasonography, (4) 24 h urine catecholamine study. Patients diagnosed with symptomatic CNS HB underwent surgical excision in nineteen subjects and stereotactic radiosurgery in one. Whereas subjects with asymptomatic CNS HB were maintained on periodic clinical and imaging follow-up. Laser photocoagulation was advised for retinal angiomas detected on screening. Renal cell carcinoma and pheochromocytoma were managed surgically according to our uro-oncology department protocols. After genetic counseling, first degree relatives consenting for the study were included for genetic screening. These individuals were then included in the study and subjected to the same clinical screening protocols only when VHL mutations were detected.

Molecular genetic analysis

From the collected blood samples, peripheral blood monocytes were separated by centrifugation (5000×*g* for 2 min) after RBC lysis (0.01 M Tris–HCl pH 7.6, 320 mM sucrose, 5 mM $MgCl_2$, 1 % Triton X-100), DNA was isolated using Chloroform-isopropanol extraction as described previously with minor modifications [18], and was analyzed using Nanodrop-1000® spectrophotometer (Thermo scientific Inc., PA USA).

Point mutation analysis

Using previously described specific primers for the three exons of *VHL* (Genbank Refseq ID: NM_000551.3), 100 ng of genomic DNA was PCR amplified and sequenced [19] using Bigdye direct cycle sequencing kit v3.1 (Life Technologies., CA USA) and capillary electrophoresis (ABI 3130XL, Life Technologies Inc., CA USA). Chromatograms thus obtained from samples were compared against that of normal controls using Codoncode® Aligner software (Codoncode Corporation, MA, USA), which automatically determine the point mutations. We verified and compared all the germline mutations with in genomic DNA with publically available and curated mutation databases such as Human Gene Mutation Database (HGMD) [20] (http://www.hgmd.cf.ac.uk/ac/index.php).

Deletion/duplication analysis

Heterozygous deletions of the *VHL* locus were identified using multiplex ligation dependent probe amplification (MLPA) procedure [21, 22]. VHL specific MLPA reaction kit (SALSA MLPA P016-C2 VHL obtained from MRC Holland, Netherlands) containing a cocktail oligonucleotide probe-mix was used. Probe-mix contains nine probes targeted to *VHL*, six probes targeted to four different genes adjacent to *VHL* (*FANCD2*, *BRK1/HSPC300*, *IRAK2*, and *GHRL*), two reference probes for Chr 3p region and twelve probes for other chromosomal regions. All reactions were performed according to protocols described by the manufacturer. Final hybridized products were separated using ABI 3130XL capillary electrophoresis (Life technologies, CA USA) and the fluorescent intensity electropherogram evaluated using Coffalyser® automated software.

Results

Families

Over the eight year period, we collected clinical details of 248 subjects from sixteen families. Subjects with age ranging from 5 to 66 years were screened. From these, 48 subjects with either clinically confirmed VHL or their first degree relative were selected for further clinical and genetic screening. Amongst these, 30 subjects were confirmed to have both clinical features and mutations in the *VHL* locus and one with only genotypic change. The complete set of data of patients is given in Table 1. Thirteen families were from Kerala state, one each from Karnataka and Orissa states. One subject of a family from Eastern European Country Belarus was excluded from the study. Pleiotropic changes were seen with in families in terms of type of occurrence of tumors, age of onset etc. Genetic heterogeneity was seen in two subjects in one family (Sl No. 10 and 14 of family AD). Five subjects from five different families (~16 %) had no family history suggestive of VHL.

Phenotype genotype analysis

The average age of onset of the first symptom was 36.3 ± 12.5 years. Further details of the age of presenting manifestations are given in Fig. 1 (box–whisker plot). The earliest age of onset was 20 years, presented with RCC and PCC and latest was 66 years, presented with MCP and CNS HB. The earliest onset of CNS HB was 23 years and that of MCP was 25 years. RA and PCC presented at an earlier mean age of onset (29.4 ± 11 and 31.3 ± 13.3 years) and RCC at later age (41.2 ± 10.7 years) when compared to other tumors (see Fig. 1; Table 2). One of the rare manifestations, ELST, occurred in a 40 year old male. Multiple occurrences of one phenotypic manifestation is described in Table 2—CNS HB being the most common. Tables 3 and 4 and Fig. 2 depict the summary of germline mutations in subjects.

MCP was the most common manifestation seen in our population. In most of the subjects, MCP was an incidental feature in screening abdominal ultrasonography. MCP was symptomatic only in three cases—presented with features such as common bile duct obstruction/pancreatitis, in females between 34 and 50 years. About 71 % (22/31) of the subjects who had *VHL* genotypic changes had MCP. MCP was invariably expressed in all the families with full length deletion of the entire gene (FLD). All the subjects had a definite family history with an autosomal dominant type of inheritance. Three out of four patients with exon 3 deletions had MCP. Subjects having point mutations in exon 1 had MCP in 10 out of 14 (71 %). The c.282–302 indel (p.95–101) which is in the β-domain region was spared of MCP as similar to the c293 A>G. The donor splice-site mutation at c.340 + 1 G>A (p.G114D) in particular was the most common mutation noted in association with MCP, though this may represent a sampling bias. The two other subjects, who did not present with MCP are from the same family (with c353 T>C). These subjects presented with other tumors at a younger than average age of onset of MCP.

CNS HB was found in 68 % of subjects (21/31) with *VHL* mutations. Out of these cases, there were eight large deletions (three families), three cases of exon 3 deletion (three families), one indel in exon 1 and nine point mutations spanning the entire locus. Except two de novo probands with mutations c.282–302 indel and c.293A>G, all of them had autosomal dominant type of inheritance.

Table 1 The phenotypic and genotypic changes in the population

Family ID	Sl no.	Age of onset	Gender	F/H	MCP	CNS HB	RA	RCC	PCC	ELST	Gene change	Predicted pVHL protein change	Domain/other genes involved
S3	1	35	F	+	+	+	−	−	−	−	c.214 T>C	p.S72P	β
JK	2*	5	F	+	−	−	−	−	−	−	c.233 A>G	p.N78S	β
JK	3	40	M	+	+	+	+	+	−	+	c.233 A>G	p.N78S	β
A	4	25	M	+	+	+	−	−	−	−	c.280 G>A	p.E94K	β
S2	5	23	F	−	−	+	+	−	−	−	c.282–302 indel	p. 95–101	β
E	6	57	M	−	−	+	−	−	−	−	c.293 A>G	p.Y98C	β
AD	7	27	M	+	+	−	−	−	−	−	c.340 + 1 G>A	p.G114D	β
AD	8	34	F	+	+	−	−	−	−	−	c.340 + 1 G>A	p.G114D	β
AD	9	38	F	+	+	−	−	−	−	−	c.340 + 1 G>A	p.G114D	β
AD	10	17	M	+	−	+	+	−	−	−	c.340 + 1 G>A, (340 + 5 G>C)	p.G114D	β
AD	11	40	M	+	+	+	−	+	−	−	c.340 + 1 G>A	p.G114D	β
AD	12	50	F	+	+	+	−	−	−	−	c.340 + 1 G>A	p.G114D	β
AD	13	50	M	+	+	−	−	+	−	−	c.340 + 1 G>A	p.G114D	β
AD	14	50	F	+	+	+	−	+	−	−	c.340 + 1 G>A, (340 + 5 G>C)	p.G114D	β
NM	15	28	M	+	−	−	−	+	+	−	c.353 T>C	p.L118P	β
NM	16	33	M	+	−	+	−	−	−	−	c.353 T>C	p.L118P	β
S4	17	20	F	−	−	−	+	−	+	−	c.464 T>G (463 + 43 A>G)	p.V155G	α–β interface
D	18	46	M	−	−	−	+	−	+	−	c.499 C>T	p.R167W	α
MM	19	33	F	+	+	+	−	−	−	−	Exon 3 del	–	α, distal β[b]
MM	20	35	M	+	+	−	−	+	−	−	Exon 3 del	–	α, distal β
NS	21	35	M	+	−	+	+	+	−	−	Exon 3 del	–	α, distal β
P	22	25	F	+	+	+	+	−	−	−	Exon 3 del	–	α, distal β
KS	23	27	M	+	+	+	−	−	−	−	*VHL* FLD[a]	–	*BRK1, FANCD2*
KS	24	29	F	+	+	+	−	−	−	−	*VHL* FLD	–	*BRK1, FANCD2*
KS	25	36	M	+	+	+	−	−	−	−	*VHL* FLD	–	*BRK1, FANCD2*
KS	26	51	F	+	+	+	−	−	−	−	*VHL* FLD	–	*BRK1, FANCD2*
KS	27	66	M	+	+	+	−	−	−	−	*VHL* FLD	–	*BRK1, FANCD2*
PM	28	31	F	+	+	+	−	−	−	−	*VHL* FLD	–	*BRK1, FANCD2*
PM	29	35	F	+	+	−	−	−	−	−	*VHL* FLD	–	*BRK1, FANCD2*
PM	30	49	M	+	+	+	−	−	−	−	*VHL* FLD	–	*BRK1, FANCD2*
S1	31	34	M	+	+	+	−	−	−	−	*VHL* FLD	–	*BRK1, FANCD2*

The whole set phenotypic and genotypic features of 31 subjects who had confirmed VHL in our population is listed. The subjects and the respective families were listed according to the location of mutation in the *VHL* gene and the type of mutation in the order point mutation, partial or full length deletion (FLD). The various manifestations are indicted by their presence as "+" and absence as "−" in the middle part of the table. The last two columns show the suggested change in the VHL protein, the location of change in the tertiary structure of the protein (α or β domain) in point mutations and partial deletions or involvement of any other genes (*BRK1*/*FANCD2*) in FLD. Asymptomatic subjects who were diagnosed during the genotypic screening were also included in the study and marked in the table with an asterisk (*) sign

[a] FLD-full length deletion

[b] A 23-residue distal β domain in the sequence comprising H4 helix. See the location of this in the primary sequence depicted in Fig. 2. The mutations are much common in the β-domain than the α-domain in our population. See the detailed analysis of the VHL phenotype and genotype in the Results and Discussion sections

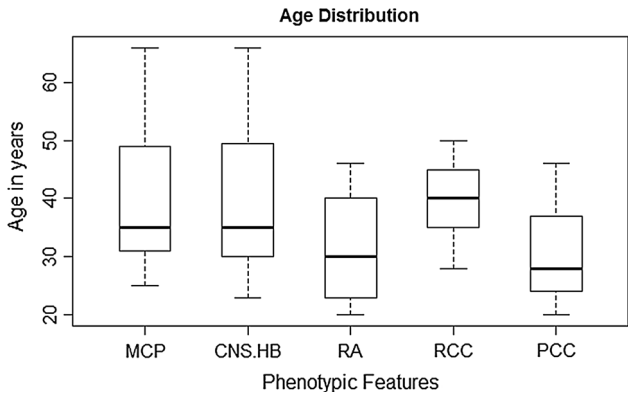

Fig. 1 Distribution of age of onset/detection of the first symptom in VHL patients are represented in a *box–whisker plot*. The *box* represents the inter-quartile range, the *thick horizontal line* inside the *box* is the median age of onset, and *dotted line error bar* is the range of age of onset. The median age of onset of MCP and CNS.HB is 35 years and both have similar range of age of occurrence. Similarly in RA and PCC presented at an earlier median age (30 and 28 years respectively) with similar range of age distribution. Median age of presentation of RCC is 40 years, relatively later than other tumors

Interestingly, the only two hotspot mutations in our population, c464T>G (p.VHL^{V155G}) and c.499C > T (p.VHL^{R167W}) of age 20 and 46 years-old respectively, did not present with CNS HB. Notably, different subjects from one family with same donor splice site mutations after exon 1 had presented with and without CNS HB. Similar pleiotropic changes were seen in the case of other families as well (see Tables 3, 4 for details) suggesting a variable expressivity for CNS HB in our population.

RA was seen in seven cases (22.6 %). Three of them had missense mutations, one had the short indel, one splice-site mutation and two had exon 3 deletions from separate families. Of note, RA was found to be more associated with partial deletion/missense mutations of *VHL* than full length deletion (Fisher's exact test $p < 0.1$), when grouped as large deletions versus non-large deletions (i.e. point mutations and smaller deletions spanning not more than one exon). RCC was seen in seven cases (22.6 %), two of them had missense mutations (in exon 1 and 2), three subjects had a splice-site mutation, two exon 3 deletions from four separate families. PCC was seen in three cases (9.7 %) who had mutations, c.353 T>C, c.464 T>G (463 + 43 A>G) and c.499 C>T. One case of ELST had c.233A>G (pN78S). Notably, all subjects who had large deletions of entire *VHL* locus had a deletion of two adjacent genes, namely, SCAR/WAVE actin nucleating complex subunit (*BRK1* or *HSPC300*) and Fanconi anemia complementation group D2 (*FANCD2*). Eight out of nine subjects in this group presented with CNS HB and

Table 2 Mean age of initial diagnosis of each manifestation of the VHL

Manifestation	Mean age	Range	No. of subjects (%)	No. of subjects in which manifestations occurred more than once	Total no of diagnosis made
MCP	38.6 ± 11.3	25–66	22 (71 %)	NA	23
CNS HB	37.5 ± 12.4	17–66	21 (68 %)	9	29
RA	29.4 ± 11	17–46	7 (22.6 %)	0	6
RCC	41.2 ± 10.7	28–60	7 (22.6 %)	3	10
PCC	31.3 ± 13.3	20–46	3 (9.7 %)	0	3
ELST	40	NA	1 (3.2 %)	0	1

This table shows occurrence of different VHL manifestations in our population with mean and range of age of onset, relative frequency of each manifestation and recurrence of more than once during the study period. The MCP had the highest frequency of occurrence, following by CNS HB. RA and RCC had same occurrence. Recurrence was seen with only CNS HB and PCC. More details are given in the Results and Discussion sections

Table 3 Genotypic changes and phenotypic manifestations in 30 VHL patients

Genotypic changes	No. of subjects affected	No. of families affected	Phenotypic manifestations					
			MCP	HB	RA	RCC	PCC	ELST
Full length deletion	9	3	9	8	–	–	–	–
Exon deletion	4	3	3	3	2	2	–	–
Short deletion (exon 1)	1	1	–	1	1	–	–	–
Point mutation	17	8	10	8	4	5	3	1

This table shows the correlation of different manifestations in our population associated with different gentotypic changes

Table 4 Phenotypic characterizations of specific point mutations

Type of point mutation	Changes in the translated protein	Exon No.	No. of subjects affected/ No. of families	Phenotypic characteristics
c.214 T>C	S72P	Exon 1	1/1	MCP, CNS HB
c.233 A>G	N78S	Exon 1	2/1	MCP, CNS HB, RA, RCC, ELST
c.280 G>A	E94K	Exon 1	1/1	MCP, CNS HB
c.293 A>G	Y98C	Exon 1	1/1	CNS HB
c.340 + 1 G>A	G114D	Exon 1	8/1	MCP, CNS HB,RA, RCC
c.353 T>C	L118P	Exon 2	2/1	CNS HB, RA, RCC, PCC
c.464 T>G	V155G*	Exon 3	1/1	RA, PCC
c.499 C>T	R167W*	Exon 3	1/1	RA, PCC

This tabe represents phenotypic characterizations of individual point mutations. The phenotypic manifestations given in Table 4 comprise the extent of manifestations in type of mutation in the whole population, not necessarily present one subject

* The hotspot mutations, the common mutation types previously identified in other ethnic populations. More details of the phenotype–genotype analysis and correlation are given in the Results and Discussion sections

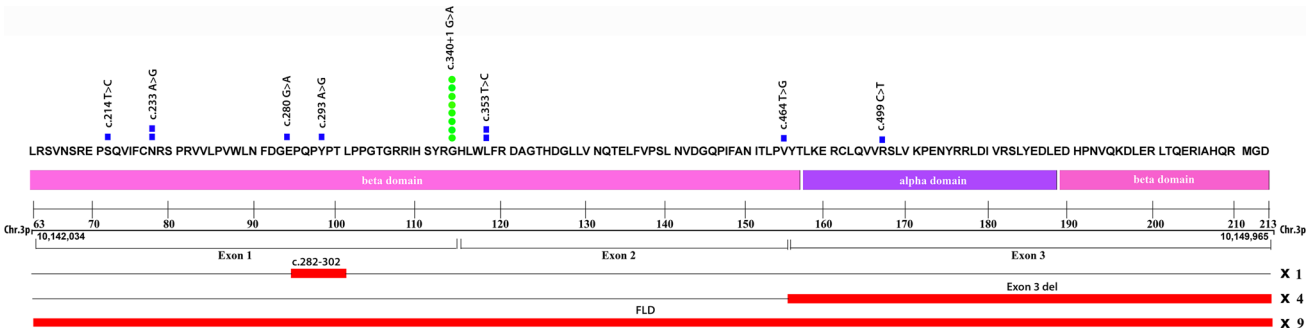

Fig. 2 The profile of *VHL* showing the regions of mutations identified in our population. The cDNA sequence-the second line from top marked as Chr 3p, divided into three exons 1, 2 and 3. The line above depicts the protein sequence, with its single letter amino acid sequence aligned above. Each *dot* (*square* and *rounded*) represent mutation in one individual. *Square dots* represent non-synonymous point mutations, *round dots* may be larger deletions/ truncations. The *bar* above the cDNA *line*, represents the different domains of pVHL. The long deletions were all depicted in the *last three lines* below, with the *thicker regions* showing the deleted region of the gene. The germline mutations are denoted above the *dots* and *bars*. The *numbers on the right hand side* are the frequencies of those large deletions from our population. The figure shows that the predominant point mutations are seen in β-domain (15/16). Note that exon 3 comprises entire α-domain and distal 23 residue of β-domain (H4 helix), which are lost in four subjects with exon 3 deletions. One α-domain mutation was on c.499 C>T (pVHL^{R167W}), which affects stability of the entire protein (see Discussion section for more details)

MCP. None of them developed RA, RCC or PCC. One subject in this group, a 35 years old lady, had an isolated MCP detected during the screening program.

Discussion

An understanding of the genotypic and phenotypic profile of an inherited disease is essential both in clinical management and to develop a scientific understanding of its pathogenesis. *VHL* gene mutation screening is presently helpful in confirming the diagnosis in asymptomatic first degree relatives, thereby allowing earlier detection and improved life-span through long-term disease surveillance. The few genotypic-phenotypic correlations described to date [23–26], provide the clinician with tools for predicting and prognosticating the disease course in individual patients. Gene mutation detection has also now found a role in prenatal screening [27]. Correlation between specific genotypic change, altered protein expression pattern and phenotypic profile hopefully provide insight into the pathogenesis of the individual manifestations of the syndrome. Hence it is important to first characterize the genotypic and phenotypic profiles of the illness separately for different ethnic groups.

In order to understand the occurrence of CNS HB by different point mutations of *VHL* in our study, we analyzed

the point mutations affecting each of the residues of the VHL protein against its molecular structure and function. The VHL protein (pVHL) is a 213 residue (see Fig. 2) structure containing two tightly coupled domains, a proximal (N-terminal) β-domain containing seven pleated β-sheets (containing residues from 63 to 155) followed by a middle thirty residue α-domain containing three helices (H1, H2, H3) (159–189) and a distal 23 residue β-domain which corresponds to an extra helix (H4) [28]. The pathogenesis of CNS HB was originally ascribed to the associative role of pVHL as a subunit in the Ubiquitin E3 ligase complex in the degradation of HIF-1α during normoxia and the lack of HIF degradation thereof in VHL [7, 28–33]. Ubiquitin E3 ligase complex contains pVHL, hetero-dimeric Elongin-C/B and Cullin family member CUL2 and ring finger protein Rbx1 to form a stable VEC complex [28, 29]. The critical residues of pVHL for the binding of HIF-1α were identified to be Trp^{88}, Tyr^{98}, Ser^{111}, His^{115} and Trp^{117}, all in the β-domain [34]. Out of these, Ser^{111} and His^{115} and Trp^{117} were considered to be critical for the binding of a twenty-residue *oxygen degradation domain* (ODD) of HIF-1α containing a critical residue Hydroxyproline (Hyp^{564}) [33, 34]. Other important sites of the pVHL are Val^{155} and Arg^{167}. These two residues are in the region of α-β interdomain interface and H1α helix respectively and were suggested to be critical for pVHL-ElonginC binding and stable assembly of VEC complex [31]. The frequency of VHL tumor tissue derived missense mutations in the residues Tyr^{98}, Val^{155} and Arg^{167} are much higher than any other regions and these are called hotspot mutations.

Four families, comprising eleven subjects, in our population had either point mutations or short deletions in region where HIF-1α interacts. Five subjects out of eleven in this group did not develop CNS HB. It is Interesting to note that, four out of seven subjects of a family having G114D mutation, who must have lost the His^{115} and Trp^{117} residues also did not develop CNS HB. All of them are above 27 years and even one subject was 50 years of age. Similarly, subjects who had point mutations in hotspot regions, such as in exon 3 region i.e. c.464 T>G (463 + 43 A>G) translating to $pVHL^{V155G}$ and c.499 C>T ($pVHL^{R167W}$), did not develop CNS HB or MCP during the study period. The proband with exon 2 missense transversion (c464T>G) with an additional deep intronic point mutation (463 + 43 A>G) translating to $pVHL^{V155G}$ is an unreported missense mutation. Both the cases presented with RA and bilateral PCC and not presented with CNS HB. One case with CNS HB and RA having a de novo short indel (c.282–302) must have lost the critical Tyr^{98} residue. The deletion of seven residues in this case could possibly have affected the stability of the VEC protein complex, as well as affected the microtubule stabilizing function [35]. From our data, occurrence of CNS HB in certain subjects in our population appears to have a different phenotypic-genotypic association compared to previous studies. The point mutations from our population suggests that HIF-1α binding residues might not have a decisive role for the development of HB. This has been implicated in a recent bioinformatics based computational analysis of the pVHL protein [36, 37].

Compared to majority of other studies, we found a higher prevalence of MCP over other manifestations, irrespective of genotype. The mean age of onset of both MCP and CNS HB was about the same (39.5 years, ranging 25–66 years), because most of the cases with MCP was incidentally identified when many subjects came to the clinic to evaluate for CNS HB. Those who did not have MCP in our population were all <50 years of age. Moreover, the frequency of MCP in our population is similar to a few of the earlier studies in larger VHL population specifically done for pancreatic cystic lesions [38, 39]. The current study shows that, perhaps, almost all the cases of CNS HB might have a sub-clinical MCP. In our Indian population, we found this to be a useful screening technique, in that first degree relatives of a proband demonstrating MCP on abdomen ultrasonography most likely have VHL germline mutations. There are several genes implicated for the pathogenesis of pancreatic cysts, including those involved in ciliogenesis and ciliary functions of pancreatic epithelial cells [39]. However, loss/mutations of *VHL* locus (Chr 3p) alone can cause pancreatic cysts in human as identified in recent whole-exome screening studies [40]. Three cases in our population who had point mutations in Exon 1 (c.214T>C, c.233 A>G and c.280 G>A translates to $p.VHL^{S72P}$, $p.VHL^{N78S}$ and $p.VHL^{E94K}$) developed CNS HB and MCP. In the case of N78S, additionally, RA, RCC and ELST were also manifested, but not PCC. Though Ser^{72} and Asn^{78} are far from the proper HIF-1α binding region, they are located in a different region of pVHL which has a role in the dynamic stabilization of HIF-1α for its effective degradation [32]. The $p.VHL^{E94K}$ is a novel mutation which was added very recently into the HGMD, while we were preparing this data. This is a de novo mutation and the 25 year old proband presented with RA with multiple CNS HB and no other manifestations. The point mutation with reversal of net charge (Glu > Lys) in this residue might have modified the molecular structure and interaction leading to occurrence of CNS HB and MCP. Interestingly, MCP was generally present more among point mutations of exon 1 region (ten subjects/three pedigrees) and absent in that of exon 2 and 3 of the *VHL*. This might suggest that the specific residues of pVHL for its interaction with other proteins such as microtubules and intracellular and extra-cellular matrix proteins for the pathogenesis of cystic pancreas might be located in the

exon 1 region. Further studies focused on the residues of exon 1 might help to understand the molecular pathogenesis of pVHL in pancreatic lesions.

Whole gene deletions in our populations are all associated with mutations of adjacent genes *FANCD2* and *BRK1* (*HSPC300*). These adjacent genes were found to be involved in another recent study conducted in Brazilian population [41]. The chromosomal region containing *VHL* locus was found to have high density of transposons (*Alu* elements) which are susceptible for recombination, causing germline deletion of genes in this region [42]. An important feature of all the subjects who had whole gene deletion is the conspicuous absence of RA and RCC. The lower prevalence of RA in VHL subjects who had whole gene deletions has been recently identified in a larger study conducted by National Eye Institute, Bethesda USA [23, 24]. The loss of *HSPC300* in VHL had been associated with lower occurrence of RCC was described previously [22, 24]. But any evidence on the protective effect of *HSPC300* co-deletion in the occurrence of RA in VHL is not available from previous studies. In RCC, the protein HSPC300 is involved in actin polymerization and along with a group of proteins (WAVE, Arp 2/3 complexes), function as effectors of Rho GTPases, specifically Cdc42 and Rac [43] which are subsequently involved in the regulation of actin dynamics for cell motility, morphology as well as cytokinesis [44]. Perhaps, the observed protective role of HSPC300 in RA might be similar to that played in RCC. Further studies in RA tumor tissues might be required to understand this mechanism.

FANCD2 is a major DNA repair protein on the Fanconi Anemia (FA) pathway to resolve interstrand cross-linking of DNA during the S-phase of cell cycle [45]. The interstrand cross links, which prevent separation of DNA helix for elongation of replication fork, trigger ubiquitylation of a protein complex containing protein FANCD2 and a similar one called FANCL as intermediates [46]. The effects of FA pathway in the normal development of the cells and tumorigenesis is an emerging field. Few studies are currently available regarding its role in the pathogenesis of VHL. FA pathway, owing to its central role in DNA repair and cell cycle, might also contribute to the pathogenesis of VHL. With the phenotypic features of MCP and CNS HB seen in these subjects, it may be implicated that perhaps the loss of ciliogenesis and ciliary functions described in the development of pancreatic cyst [39] might have a predominant role in the pathogenesis of CNS HB as well via the alteration in actin cytoskeletal dynamics, in both intracellular and extra-cellular matrix. Moreover, the derangement of actin dynamics and loss of cytokinesis (with the loss of *HSPC300*) might be protective for the renal tissue and not for pancreatic tissue suggest that the micro environment in these specific tissues might have significant role in triggering the formation of cystic lesions. This is also supported by other evidences suggesting that pVHL has a major role in fibronectin assembly and stabilization in the extracellular matrix, and VHL specific tumorigenesis can be regulated at the level of matrix assembly [47, 48]. Further studies on the involvement of these gene products in extra-cellular matrix functions in specific tissues separately might be pertinent in this context.

In conclusion, we describe a higher occurrence of MCP in our population irrespective of *VHL* genotypic characteristics, absence of occurrence of HB even at the loss of critical residues of pVHL–HIF interaction, involvement of other genes critical for cell cycle and tissue development which also influences certain phenotypes such as expression of RA, RCC or PCC, which are in coherence with only a few of previous studies. From these observations it appears that the phenotypic tumor manifestations in VHL seems to be determined by involvement of more than one gene in the pathophysiology, perhaps even suggesting that VHL is a complex genetic disorder, certainly warranting for a more detailed examination including high-throughput whole genome screening studies.

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Conflict of interest The authors declare that there is no conflict of interest.

Ethical standard 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. This article does not contain any studies with animals performed by any of the authors.

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

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