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The Profound Impact of von Hippel-Lindau Gene Mutations in Renal Cell Cancers: A Study of the Kashmiri Population

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ABSTRACT

Introduction: The primary aim of this study was to evaluate the incidence of von Hippel-Lindau (VHL) gene mutations among a group of Kashmiri patients diagnosed with renal cell tumors. Correlation of these mutations was explored with clinical pathological status of the illness.

Methods: PCR-SCCP and DNA sequencing evaluated the DNA samples of both the tumor and adjacent normal tissue for the occurrence of VHL gene mutations. In addition, blood samples were used from all the cases to rule out any germ-line mutation.

Results: Mutations of the VHL gene identified in renal-cell cancer (RCC) patients were 52.5% (21 of 40), including 9 missense, 10 frame shift, and 2 non-sense mutations. Of the mutations, 52.38% were detected in exon 1, 38.1% in exon 2, and 9.52% in exon 3. Nineteen out of 23 (82.6%) cases of the clear-cell type and 2 out of 2 (100%) of angiomyolipomas of RCC were positive for VHL gene mutation. No correlation was found between tumor grade and/or stage and the presence of VHL mutation.

Conclusions: In conclusion, sporadic RCC shows mutations in the VHL gene, which mainly appear in the clear-cell subtype in our patients. Thus alteration in the VHL gene has been implicated in the pathogenesis of renal-cell sporadic cancer of the patients in our population.

INTRODUCTION

Renal-cell carcinoma (RCC) is the most common malignant kidney tumor in adults. It accounts for 2 to 3% of all human malignancies [1]. Among cancers of the urinary system, RCC is associated with the worst clinical outcome [2]. The incidence of RCC is increasing, and it is estimated that RCC accounts worldwide for 95 000 cancer-related deaths per year [3]. Overall, 8.9 new cases are diagnosed per 100 000 per year, with a male-to-female predominance of 3:2 [4]. In this region, urinary tract cancers comprised 9.5% of all cancers in which renal-cell carcinoma comprised about 1.5% of all cancers. The age-standardized rate (ASR) incidence in the case of RCC is 0.4 cases/100 000/year [5].

The development of RCC from normal renal epithelium may also involve alterations in genes that control cell division. These include genes that participate directly in controlling the cell cycle, such as the retinoblastoma (Rb) gene, the Tp53 tumor-suppressor gene, and the RAS gene family [6,7]. RCC is a morphologically and genetically heterogeneous tumor that includes, among several rare entities, 4 major subtypes, namely clear-cell, papillary, chromophobe, and collecting duct (Bellini duct) carcinomas [8-10]. The clear-cell subtype accounts for 70 to 80% of all RCCs [10]. Clear-cell RCC is thought to arise from the proximal tubule and presents as both a hereditary and a sporadic form. Hereditary clear-cell RCC occurs in patients with von Hippel-Lindau (VHL) disease because of germ-line mutations in the VHL tumor suppressor gene located on the short arm of chromosome 3 [11]. Clear-cell carcinomas make up 75 to 85 percent of tumors and are characterized by a deletion of one or both copies of chromosome 3p [12]. Biallelic von

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Table 1. A sequence of primers used for amplification of exon 1, 2, and 3 of the VHL gene.

Primer	Primer Sequence*	AT (° C)	Product Size (bp)
MA2A-F MA2A-R	f 5'- GGCCCGTGCTGCGCTCGGTGAACT -3' r 5'CCCAGCTGGGTCGGGCCTAAGCGCCGGGCCCGT-3'	55	141
TK-F TK-R	f 5' GGCCCGTGCTGCGCTCGGTGAACT 3' r 5' CGGCCCGTGCCAGGCGGCAGCGTTGGAT 3'	57	190
VHL-3 F VHL-3 R	f 5' CCGAGGAGGAGATGGAGGCC 3' r 5' GACCGTGCTATCGTCCCTGC 3'	62	250
VHL-4 F VHL-4 R	f 5' CGGTGTGGCTCTTTAACAACC 3' r 5' CAAGTGGTCTATCCTGTACTT 3'	55	230
VHL- 5F VHL-5R	f 5' TTCCTTGTACTGAGACCCTAGT 3' r 5' TACCATCAAAAGCTGAGATGAAACAGTGTAAGT 3'	55	280

AT: annealing temperature

Hippel-Lindau (VHL) gene defects, a rate-limiting event in the carcinogenesis, occur in approximately 75% of sporadic clear-cell RCC. The disease is caused by mutations of the VHL gene on the short arm of the third chromosome (3p26 to p25).

Together with the loss of the homologous chromosome 3p allele (3p LOH), VHL mutations are rate-limiting events in the carcinogenesis of clear-cell RCC [13,14]. Mutations have been observed in the entire gene and usually lead to a truncated inactive protein [15]. The VHL gene is involved in cell cycle regulation, the regulation of hypoxia inducible genes, and proper fibronectin assembly in the extracellular matrix [16,18]. In approximately 19% of sporadic clear-cell RCC, methylation of the VHL gene promoter appeared to be involved [16,18]. In approximately 10 to 20% of sporadic clear-cell RCC no alteration in the VHL alleles was detected, indicating that other genes are involved in clear-cell RCC carcinogenesis, possibly affecting the same signaling pathway as VHL.

Owing to the fact that there is no data on genetic alterations on RCC available in our population or given the backdrop of a significant presence of RCC patients, the highest among males, it is the first initiative to study the gene alterations in RCC patients of Kashmir Valley by DNA sequencing.

METHODS AND MATERIALS

Specimen

A total of 40 histologically confirmed, previously untreated renal-cell cancer patients attending the Department of Urology of the Sher-I-Kashmir Institute of Medical Sciences (SKIMS), Srinagar were included in this study. Blood samples were taken from all cases to rule out the possibility of germ-line mutation in the VHL gene to confirm the sporadic nature of mutation in our RCC cases.

Information on tumor grade and stage was obtained for all patients. The diagnostic slides were reviewed by a panel of 2 expert pathologists to confirm the diagnosis and insure uniformity of the classification criteria. We reviewed all cases and classified them according to the 1987 version of the TNM classification. Written pre-informed consent was obtained from all cases and controls. The demographic and clinic-pathological characteristics of each patient were recorded in a questionnaire. This study was approved by the ethical committee of the SKIMS. $\chi 2$ was applied to calculate the *P* value of VHL gene mutation with clinico-pathological characteristics. A P value < 0.05 was considered significant.

DNA Extraction

In this study, single-strand conformation polymorphism and DNA sequencing were used to analyze the regions of VHL genes harboring the point mutations in a series of 40 renal carcinoma samples. Tumor samples (both tumor and adjacent normal tissue) collected after radical nephrectomy of renal-cell tumors were immediately snap-frozen and stored at -70° C. DNA from tissues, as well as blood, was extracted using a DNA easy tissue kit (Qiagen GmbH; Hilden, Germany) according to the enclosed protocol.

Polymerase Chain Reaction

Exons 1, 2, and 3 of the VHL gene containing hotspot codons were amplified using previously described specific primers (Table 1). PCR amplification was carried out in a 50 µL volume container with 25 ng of genomic DNA; a 1X PCR buffer containing 1.5 mM

Table 2. Clinicoepidemiological variables of patients with a renal tumor used for mutational analysis

Variable	Parameter	Cases N = 40, %
sex[a]	males females	25 (62.5%) 15 (37.5%)
age	≤ 50 > 50	31 (77.5%) 9 (22.5%)
dwellingb	rural urban	27 (67.5%) 13 (32.5%)
smoking status[c]	smokers nonsmokers	22 (55%) 18 (45%)
differentiation guide	I II III IV	13 (32.5%) 22 (55%) 4 (10%) 1 (2.5%)
histological type[d*] (TNM)	clear-cell pappillary oncocytoma angiomyolipoma leomyoma	23 (57.5%) 10 (25%) 3 (7.5%) 2 (5%) 2 (5%)
sitee	upper pole middle lower	14 (35%) 17 (42.5%) 9 (22.5%)
lymph node status	no yes	36 (90%) 4 (10%)
stage	stage I stage II stage III stage IV	21 (52.5%) 5 (12.5%) 13 (32.5%) 1 (2.5%)

[a]age/sex: M = male, F = female; [b]rural/urban: R = rural, U = urban; [e]smoking status: S = smokers, NS = nonsmokers

MgCl2; 100 µM each of dATP, dGTP, dTTP, dCTP; 1.5 U of Taq DNA polymerase (Biotools; Madrid, Spain); and 1 µM of forward and reverse primers (Genescript; Piscataway, NJ, USA). The PCR and thermal conditions are given in Table 1. The PCR products were run on 2% agarose gel and analyzed under an ultraviolet illuminator. The single-strand conformation polymorphism (SSCP) analysis of the amplicons of exon 7, 10, and 15 was performed on 6% non-denaturing polyacrylamide gel (PAGE) utilizing nonradioactive silver staining [17]. The purified PCR amplicons of the tumor samples showing mobility shift on SSCP analysis, and randomly chosen normal samples were used for direct DNA sequencing using the automated DNA sequencer ABI PRISM® 310 Genetic Analyzer (Applied Biosystems, Life Technologies; Carlsbad, CA, USA).

RESULTS

Table 2 contains the clinicoepidemiological characteristics of the patients with RCC. The number of cases in the age group of ≥ 50 (N = 31; 77.5%) exceeded < 50 years (N = 9; 22.5%). Histological breakup of the RCC cases were stage I, 21 (52.5%); stage II, 5 (12.5%); stage III, 13 (32.5%); and stage IV, 1 (2.5%). All the cases after surgical resection were subjected for histopathological examination (HPE), and all of the samples resected were histologically confirmed as renal-cell carcinomas of different types, as depicted in Table 2.

Overall mutations in exon 1, 2, and 3 of VHL identified in this study aggregated to 52.5 % (21/40) (Table 3). In all, there were 8 missense mutations (6 transitions and 2 transversions), 6 were C > T transitions, and 2 G > T transversions. We detected 10 frame shifts in which 4 were insertion mutations of AGGT > AGAGT (insertion A) in exon 1 and six insertion mutations of GTTG > GTATG (insertion A) in exon 2. We found 3 nonsense mutations of GGA > TGA resulting in Gly > stop codon in exon 2 (Table 3).

Among 21 mutations detected in our study, 20% were seen in exon 1, 20% in exon 2, and 5% in exon 3 (Table 4). Representative partial electropherogram of few mutations are given in Figure 1, Figure 2, Figure 3, and Figure 4. Among 21 mutations found in this study, 5 (38%) were found in grade I and 12 (54.5%) in grade II, whereas 4 (75%) and 1 (100%) were detected in grade III and grade IV, respectively (Table 3).

Figure 1. Partial reverse sequence of exon 1 showing mutation (above) in codon 246 CCC > CAC and a normal sequence (below).

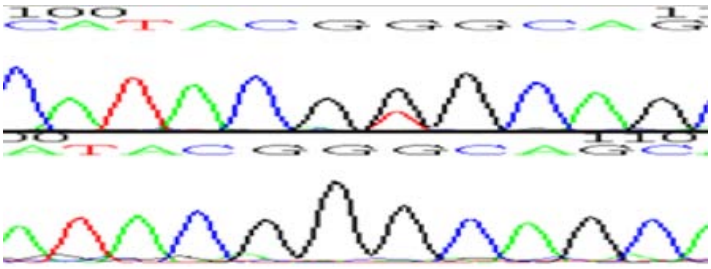
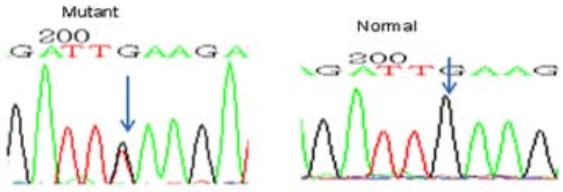


Table 3. Clinicoepidemiological characteristics and mutational status of patients with a renal tumor

Characteristics	Cases (N = 40)		Mutant		Wild		*P*
	N	N%	N	N%	N	N%	
	40	100%	21	52.5%	19	47.5%	0.327
sex							
male	25	62.5%	14	56%	11	44%	0.567
female	15	37.5%	7	46.7%	8	53.5%	
smoking status							
smoker	18	45%	10	55.6%	8	44.4%	
nonsmoker	22	55%	11	50%	11	50%	0.726
histopathological grade							
grade I	13	32.5%	5	38.5%	8	61.5%	0.427
grade II	22	55%	12	54.5%	10	45.5%	
grade III	4	10%	3	75%	1	25%	
grade4	1	2.5%	1	100%	0	0%	
histopathological type							
clear cell							
pappillary	23	57.5%	19	82.6%	4	17.4%	0.000
oncocytoma	10	25%	0	0%	10	100%	
angiomyolipoma	3	7.5%	0	0%	3	100%	
leomyoma	2	5%	2	100%	0	0%	
	2	5%	0	0%	2	100%	
stages							
stage I	21	52.5%	11	52.4%	10	47.6%	
stage II	5	12.5%	1	20%	4	80%	0.327
stage III	13	32.5%	8	61.5%	5	38.5%	
stage IV	1	2.5%	1	100%	0	0%	

This difference showed a non-significant correlation with VHL mutation patterns ($P > 0.05$). Mutations in 11 of 21 (52.4%) were detected in stage I tumors and 1 of 5 (20.0%) in stage II, and 8 out of 13 (61.5%) were detected in stage III while 1 mutation was found in stage IV (100%; $P > 0.5$). Nineteen of 21 (82.6%) mutations were found in clear-cell RCC while as 2 of 2 (100%) were found in angiomyolipoma. This difference in mutation frequency between different histological tumor types was seen as statistically significant in clear-cell RCC ($P < 0.05$).

Figure 2. Partial forward sequence of exon 3 showing mutation (left) in codon 614 TGA > TTA and a normal sequence (right).



DISCUSSION

In the present study, the mutational spectrum of the VHL gene (exon 1, 2, and 3) were studied in 40 cases of RCC. The VHL

Table 4. Characteristics of patients with RCC showing VHL gene mutations.

ID	Age	Sex	SS	G	St	L.N.	HPE	Cdn.	NC	Base Change	AA	Mut
KT3	65	M	S	2	III	yes	CC	246	C > T	CCC > CTC	Pro > Leu	M
KT5	45	M	NS		III	No	CC	246	C > T	CCC > CTC	Pro > Leu	M
KT10	45	M	NS	3	III	yes	CC	614	G>T	TGA > TTA	SC > Leu	M
KT1	50	M	S	1	I	No	CC		Ins T	AGGT > AGAGT		FS
KT19	60	F	S	2	I	No	CC		Ins T	AGGT > AGAGT		FS
KT25	60	M	S	1	I	No	AM	424	G>T	GA > TGA	Gly > SC	NS
KT33	80	M	S	2	II	No	AM	424	G > T	GGA > TGA	Gly > SC	NS
KT6	55	F	N.S	2	III	No	CC	422	Insertion A	GTTG > GTATG		FS
KT28	50	M	S	1	I	No	CC	422	Insertion A	GTTG > GTATG		FS
KT39	60	F	NS	2	I	No	CC	422	Insertion A	GTTG > GTATG		FS
Kt29	75	M	S	1	III	No	CC	246	C > T	CCC > CTC	Pro > Leu	MS
KT8	55	F	NS	2	I	No	CC	246	C > T	CCC > CTC	Pro > Leu	MS
KT17	50	M	S	2	I	No	CC	246	C > T	CCC > CTC	Pro > Leu	MS
KT26	45	M	NS	1	I	No	CC	614	G > T	TGA > TTA	SC > Leu	MS
KT11	42	F	S	2	III	No	CC		Ins T	AGGT > AGAGT		FS
KT23	65	M	NS	4	I	No	CC		Ins T	AGGT > AGAGT		FS
KT40	55	M	NS	2	I	No	CC	422	Insertion A	GTTG > GTATG		FS
KT20	45	M	S	2	III	No	CC	422	Insertion A	GTTG > GTATG		FS
KT37	50	M	NS	3	I	No	CC	422	Insertion A	GTTG > GTATG		FS
KT17	60	F	NS	3	III	No	CC	246	C > T	CCC > CTC	Pro > Leu	MS
KT13	70	F	NS	2	IV	yes	CC	246	C > T	CCC > CTC	Pro > Leu	MS

SS: Smoking status; G: Grade; St: Stage; Ex: exon; Cdn: Codon; AA: Amino acid change; NC: Nucleic acid change; Mut: mutation; M: Missense; FS: Frame shift

gene is a tumor suppressor gene predisposed to both sporadic clear-cell (conventional) RCC and VHL disease. The frequency of mutations in this series aggregated to 52.5%. No germ-line mutations were detected in the VHL gene in extracted DNA from peripheral blood of the studied patients. The most common mutations found in our study were frame shifts, missense, and nonsense mutations from all the 3 exons of the VHL gene. Individuals with VHL disease carry 1 wild type VHL allele and 1 inactivated VHL allele. In other words, VHL patients are VHL heterozygotes. Tumor or cyst development in VHL disease is linked to somatic inactivation or the loss of the remaining wild type VHL allele. Approximately 20 to 37% of VHL patients have large or partial germ-line deletions, 30 to 38% have missense mutations, and 23 to 27% have nonsense

Figure 3. Partial reverse sequence of exon 1 showing insertion (left) resulting in AGGT > AGAGT and a normal partial sequence (right).

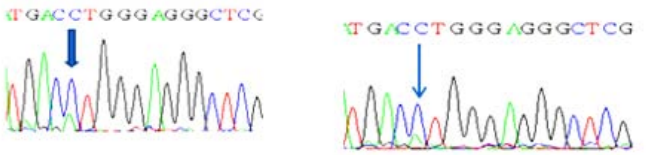
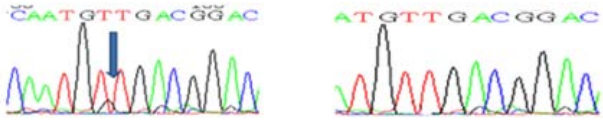


Figure 4. Partial reverse sequence of exon 2 showing insertion of A in codon 422 (left) resulting in GTTG > GTAGT and a normal partial sequence (right).



or frame-shift mutations [15,19]. In general, VHL mutations are extremely heterogeneous and are distributed throughout the coding sequence, except that intragenic missense mutations are rarely seen within the first 50 codons. With 12 in total, more than 150 different germ-line VHL mutations linked to VHL disease have been reported [20]. VHL seems to be mutated somatically in approximately 50%, and hypermethylated in another 10 to 20% of sporadic clear-cell renal carcinomas. However, mutation of the VHL gene is rarely detected in other histologic subtypes of RCC. This shows that mutational frequency detected in RCC in our study is in accordance with most of the studies that have been done to date (our study: 52.5%, vs Gnara et al. [20]: 57%, vs Gallou et al. [21]: 56%, vs Kondou et al. [22]: 51%). Some of the studies were not in conformity with our report in terms of the frequency of the mutations seen in the VHL gene (our study: 52.5%, vs Foster et al. [23]: 42%, vs Whaley et al. [24]: 33%, vs Brauch et al. [25]: 42%, vs Igarash et al. [26]: 66%). This difference in the observed proportion of mutations in the VHL gene may be attributed to the use of different techniques; e.g., SSCP vs direct sequencing, to contamination with normal tissue components, or to the type of material analyzed (fresh or archival paraffin material). In keeping with the Knudson 2-hit model, biallelic VHL inactivation (as a result of mutation or hypermethylation) is common in both sporadic hemangioblastomas and sporadic clear-cell renal carcinomas [27]. VHL seems to be mutated somatically in approximately 50%, and hypermethylated in another 10 to 20% of sporadic clear-cell renal carcinomas. However, mutation of the VHL gene is rarely detected in other histologic subtypes of RCC. The percentage of frame-shift mutations was approximately 50% in most studies [28,29], comparable to the 48% observed in the current study. The percentage of in-frame deletions/insertions was higher in our study (12%) as compared to the percentage in other studies (< 5%) [28]. These frame-shift mutations may cause the altered 11 function of VHL protein, which leads to the abnormal tumor suppressor activity of the VHL gene. The present study found VHL mutations in 19 of the 23 cases (82.6%) of clear cell renal carcinoma, and presentation of a higher number of mutations in this tumor type was found to be statistically significant ($P < 0.05$). In this study we found 42.8% (9 of 21) mutations were of missense in nature compared to the distribution of mutation data present in the universal VHL. With mutation data based on 747 mutations [30], the relative amount of point mutations is much higher (64%) compared to the percentage of point mutations we observed (42.8%). Our observations of missense mutations are in accordance with Kjeld et al. [30]. The percentage of nonsense mutations is somewhat similar as thouse found in the Universal VHL Mutation Database (11% vs 9.5%). The missense mutations in sporadic cases of RCC that have already lost 1 allele may impair VHL protein function, resulting in the loss of tumor suppressor function of the VHL gene. The distribution of histological subtypes was comparable to the distribution described before. Surprisingly, our study also found VHL mutations in one of the non-clear-cell RCC samples that were collected. In angiomyolipoma, we found 2 mutations out of 2 samples (100%). These results are in contrast with the current opinion that VHL mutations are exclusively restricted to clear-cell RCC. In this study however, we did not find any mutation in any other non-clear-cell renal cancer. Our results and results from others [25,32] showed no association with nuclear grades. We did not observe a difference in stages between mutated and wild type tumors, as was confirmed by 2 other studies [32]. In a small number of tumors analyzed, which is also a limitation in this study, our results showed that VHL gene mutations were observed irrespective of pathological grade or stage. This finding suggests that VHL gene mutation may be an early event in the development of renal-cell carcinoma. Furthermore, our study could not find any association with age, tumor location, tumor size, or any other clinicopathological characteristics.

CONCLUSION

We conclude that sporadic RCC shows mutations in the VHL gene, which mainly appear in the clear-cell subtype in our patients. Thus alteration in the VHL gene has been implicated in the pathogenesis of renal-cell sporadic cancer of the patients in our population. Such alterations result in severe disturbances in the protein, likely disturbing its tumor suppressing function, and is then implicated in the development of RCC because the protein functioning was not evaluated in this study.

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