

## Improved Identification of von Hippel-Lindau Gene Alterations in Clear Cell Renal Tumors

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**Abstract Purpose:** To provide a comprehensive, thorough analysis of somatic mutation and promoter hypermethylation of the *von Hippel-Lindau* (*VHL*) gene in the cancer genome, unique to clear cell renal cancer (ccRCC). Identify relationships between the prevalence of *VHL* gene alterations and alteration subtypes with patient and tumor characteristics.

**Experimental Design:** As part of a large kidney cancer case-control study conducted in Central Europe, we analyzed *VHL* mutations and promoter methylation in 205 well-characterized, histologically confirmed patient tumor biopsies using a combination of sensitive, high-throughput methods (endonuclease scanning and Sanger sequencing) and analysis of 11 CpG sites in the *VHL* promoter.

**Results:** We identified mutations in 82.4% of cases, the highest *VHL* gene mutation prevalence reported to date. Analysis of 11 *VHL* promoter CpG sites revealed that 8.3% of tumors were hypermethylated and all were mutation negative. In total, 91% of ccRCCs exhibited alteration of the gene through genetic or epigenetic mechanisms. Analysis of patient and tumor characteristics revealed that certain mutation subtypes were significantly associated with Fuhrman nuclear grade, metastasis, node positivity, and self-reported family history of RCC.

**Conclusion:** Detection of *VHL* gene alterations using these accurate, sensitive, and practical methods provides evidence that the vast majority of histologically confirmed ccRCC tumors possess genetic or epigenetic alteration of the *VHL* gene and support the hypothesis that *VHL* alteration is an early event in ccRCC carcinogenesis. These findings also indicate that *VHL* molecular subtypes can provide a sensitive marker of tumor heterogeneity among histologically similar ccRCC cases for etiologic, prognostic, and translational studies.

Considerable progress has been made in understanding the genetic basis of kidney cancer (1, 2). The susceptibility genes associated with several forms of inherited renal cell cancer (RCC) have been identified by rigorous analysis of families

using genetic linkage analysis and positional cloning (3–7). The most common subtype of RCC is the conventional clear cell type (ccRCC), which accounts for ~75% of cases. In both familial and sporadic ccRCC, allelic inactivation of the *von*

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*Hippel-Lindau* (*VHL*) gene has been shown to occur through mutation, methylation, and/or chromosomal loss in the majority of ccRCCs analyzed (8–13).

Molecular studies examining tumor DNA from sporadic cases of ccRCC have provided strong evidence that *VHL* alteration is a common, early event in the carcinogenic process (12). In addition, specific types of *VHL* mutations may be associated with etiologic factors, disease progression, and prognosis (1). However, several studies have examined *VHL* alterations in multiple patient populations and have reported significant differences in the prevalence of mutations observed, ranging from 50% to 71% (8–10, 12, 13). Differences in mutation prevalence could be due to several factors, including (a) the patient population examined, (b) tumor histopathology, (c) the ratio of tumor to normal DNA in a sample, or (d) the method of mutation detection used. For example, inclusion of non-ccRCC tumors in a molecular study would be expected to decrease the *VHL* mutation prevalence observed, as *VHL* mutations are rare in other types of RCC (14).

Accurate, sensitive, and practical high-throughput mutation detection methods must be used to analyze large numbers of well-characterized samples to correlate the prevalence, type, and location of *VHL* mutations with etiologic or prognostic risk factors. Previous studies have relied on scanning techniques, such as denaturing high-performance liquid chromatography or single-strand conformational polymorphism, followed by annotation of variants using Sanger sequencing. Neither of these scanning techniques is amenable to a level of throughput that matches data generation by fluorescent Sanger sequencing on a capillary electrophoresis platform.

Recently, a new scanning technique has been developed that offers key advantages over previous approaches, especially when used in combination with Sanger sequencing. Endonuclease scanning uses enzymes purified from celery (*Cel I* and *II*) to detect mutations in amplified PCR products (15–17). The endonucleases cleave heteroduplexed DNA at mismatch sites and digests are separated by their size. Cleavage products and their sizes do not only indicate the presence of a variant but also provide information of its location, allowing quick and easy identification of mutations when PCR products are sequenced. The primary objective of the current study was to apply this new mutation detection method to analyze *VHL* gene mutations in a set of tumor samples that were collected as part of a large international molecular epidemiologic study of kidney cancer etiology and survival, selected to represent a distribution by histopathologic variables and known RCC risk factors. The second goal was to provide a comprehensive genetic and epigenetic analysis to elucidate the relationship between somatic mutations and promoter hypermethylation of the *VHL* gene in the cancer genome unique to ccRCC. This goal would be accomplished by using only DNA extracted from frozen tissue sections that were each histologically confirmed clear cell cases (ccRCC) by a National Cancer Institute expert in renal tumor pathology (M.M.), by exhaustively searching for and confirming all mutations using a combination of analytic methods that could be practical, sensitive, but suitable for analysis of a large number of cases, and, lastly, by using Sanger sequencing to evaluate 11 CpG sites in the *VHL* promoter in place of methylation-specific PCR, which can result in both false-positive and false-negative results.

## Materials and Methods

**Tumor DNAs.** A subset of patient tumor samples ( $n = 205$ ) from cases enrolled in a large case-control study of kidney cancer conducted in central and eastern Europe was selected to include a distribution of cases by tumor stage, grade, and known risk factors such as sex, body mass index (BMI), hypertension, and smoking. We obtained informed consent from potential participants in accordance with the National Cancer Institute, IARC, and local Institutional Review Boards. Tumor DNA extraction was done following pathologic review, and manual macrodissection to remove nontumor tissue. Sample areas that seemed to contain at least 70% tumor cells were used for DNA extraction. For each sample, 5 mm<sup>3</sup> of tissue were sectioned and digested with 0.4 µg proteinase K per µL of digestion buffer (500 mmol/L KCl, 100 mmol/L Tris-HCl, 15 mmol/L MgCl<sub>2</sub>, 0.5% Tween 20) at 50°C overnight. A standard protocol<sup>16</sup> was used to extract DNA from the digested samples. DNA was quantitated using a ND-1000 spectrophotometer (Nano-Drop).

**PCR.** Amplification of patient tumor DNAs was carried out in 50 µL reactions using 10 to 15 ng of tumor DNA and 1.25 units of either HotMaster Taq DNA Polymerase (Eppendorf) or HotStart Taq DNA polymerase (Denville Scientific) with their respective 1× reaction buffers, and 0.2 mmol/L of each deoxynucleotide triphosphate and 0.2 µmol/L each of forward and reverse primer. Thermal cycling was accomplished using MJ Research (Bio-Rad) Dyad and Tetrad DNA Engines and a program of 95°C for 2 min, 10 cycles of touchdown PCR, and then 30 cycles of 95°C for 30 s, 58°C for 30 s, and 68°C for 30 s, followed by a final 5-min extension at 68°C. PCR products were heteroduplexed using a program of 95°C for 5 min, cooling to 85°C at -2.0°C/s, cooling to 25°C at -0.2°C/s, and then incubation at 4°C in a thermocycler. PCR products (5 µL) were analyzed by 2% agarose gel electrophoresis in 1× Tris-acetate-EDTA and visualized with ethidium bromide.

**Endonuclease scanning.** Heteroduplexed PCR samples were combined with 15 units of Surveyor Nuclease W and 1 µL of Surveyor Enhancer W (Transgenomic) and incubated at 42°C for 20 min. Digestions were terminated by addition of 2 µL stop solution [0.5 mol/L EDTA (pH 8.0)] and analyzed on a WAVE HPLC instrument equipped with a High Sensitivity Detection system and a DNASep HT column (Transgenomic). Run variables were a 12 µL injection volume using the double-strand sizing multiple fragment application at a 50°C oven temperature. WAVE HPLC gradient variables are described in Supplementary Materials and Methods. Detection was 260 nm for UV and 495 nm excitation/537 nm emission for fluorescence. A 100-bp DNA ladder (New England Biolabs) was run as a size marker. Instrument control, data acquisition, and data analysis were done using WAVE Navigator software. Positive and negative controls were included with each plate of PCR products to monitor endonuclease cleavage efficiency.

**VHL gene sequencing.** Excess PCR primers were removed from 10 µL of PCR product using the AMPure PCR Purification system (Agencourt Bioscience Corp.). Purified product was eluted in 30 µL of deionized water. Reaction chemistry using BigDye version 3.1 (Applied Biosystems) and cycle sequencing were adapted from the manufacturer's recommendations. Cycle sequencing products were purified using CleanSEQ reagents (Agencourt Bioscience). Purified sequencing products were eluted in 40 µL of 0.01 µmol/L EDTA and 30 µL was run on an ABI 3100 Genetic Analyzer. Sequence chromatograms were analyzed by several methods as described in Supplementary Materials and Methods.

**VHL promoter methylation.** Standard methods were used for bisulfite modification of 100 to 500 ng of tumor DNA (Zymo Research Laboratories). Primers (Supplementary Materials and Methods) were designed to amplify both methylated and unmethylated alleles across

<sup>16</sup> [waldman.ucsf.edu/Protocols/index.html](http://waldman.ucsf.edu/Protocols/index.html)

**Table 1.** Distribution of patient and tumor characteristics among cases

	<i>n</i>	%
Total cases	205	100
Center		
Romania	36	18
Poland	21	10
Russia	19	9
Czech Republic*	129	63
Sex		
Male	122	60
Female	83	40
Smoking status		
Never	89	45
Ever	110	55
BMI †		
<25	54	26
25-35	147	72
>35	4	2
Self-reported hypertension		
No	108	53
Yes	97	47
Tumor stage		
T	42	20
T2	92	45
T3/T4	54	26
Missing	17	8
Metastasis		
Mx	37	18
M0	148	72
M1	9	4
Missing	11	5
Fuhrman nuclear grade		
I	2	1
II	154	75
III	39	19
IV	3	1
Missing	7	3

\*Czech centers include Brno, Olomouc, Prague, and Ceske Budejovice.

† Body Mass Index (BMI) at time of interview.

11 CpG dinucleotides of the *VHL* promoter. PCR was done on 2  $\mu$ L treated DNA in a MJ Research PTC200 thermal cycler: 5 min of denaturation at 95°C, 10 cycles of touchdown to a 50°C annealing temperature, and then 35 cycles of 30 s at 95°C, 30 s at 50°C, and 60 s at 72°C, followed by a final 5-min extension at 72°C. Nested PCR was done on 1  $\mu$ L of a 1:10 dilution of first-round product using cycling

conditions as described above. PCR products (5  $\mu$ L) were visualized in 2.0% agarose and bidirectionally sequenced. Cytosine positions in CpGs were inspected for thymine or cytosine signal in chromatograms as follows: T only, not methylated; both cytosine and thymine, partially methylated; C only, fully methylated. Tumor samples containing at least four methylated CpGs analyzed (>36%) were considered methylated. All analyses were run in duplicate, blinded to *VHL* mutation status, and with positive (CpGenome Universal Methylated DNA, Chemicon/Millipore) and negative (K562 Human Genomic DNA, Promega) controls.

**Subcloning.** Cloning of PCR products used topoisomerase-activated vector (18) and a TOPO cloning kit (Invitrogen). Amplified inserts were prepared by colony PCR (19) and sequenced as described above before quantifying the number of clones containing mutant alleles.

**Statistical analysis.** *VHL* mutation and promoter methylation were considered as dichotomous variables per case (no/yes). Tumor and subject characteristics, such as clinical stage, grade, node stage (N0, N1, N2), BMI (<25, 25-35, 35+), pack-years of smoking (0, 1-20, 20+), and smoking status (never, former, current), were considered as categorical variables. Other variables, such as metastasis (M0, M1), self-reported hypertension (no/yes), family history of cancer (no/yes-kidney and no/yes-any), sex, and age at diagnosis (<50,  $\geq$ 50 y), were considered as dichotomous variables. Prevalences of *VHL* alterations were calculated by dividing the number of cases with an alteration by the total number of cases analyzed. Cases with missing information were excluded from analyses.  $\chi^2$  tests were applied to contingency table (2  $\times$  2) analysis to test for differences between the number of cases with or without an alteration in each group. Ordered logistic regression was used to analyze associations between categorical variables and cases with particular *VHL* alterations. All analyses were conducted using STATA 9.0 (Stata Corp.) and all statistical tests were two sided.

## Results

**Pilot study.** DNA extracted from 22 RCC patient tumors with previously characterized mutations in the *VHL* gene was used to compare the specificity and sensitivity of endonuclease scanning relative to denaturing high-performance liquid chromatography. All mutations were detected using both techniques. To assess the sensitivity of endonuclease scanning, serial dilutions of PCR products from six tumor DNA samples with known mutations were combined with normal amplicons and analyzed. Both techniques successfully detected mutations in DNA dilutions that contained 3% to 5% mutant/normal DNA (data not shown).

**Patient and tumor characteristics.** Cases were selected to include a distribution by sex, age at diagnosis, histopathologic variables, such as tumor stage and grade, and other kidney

**Table 2.** Subtypes of *VHL* gene mutations observed among 205 histologically confirmed ccRCCs

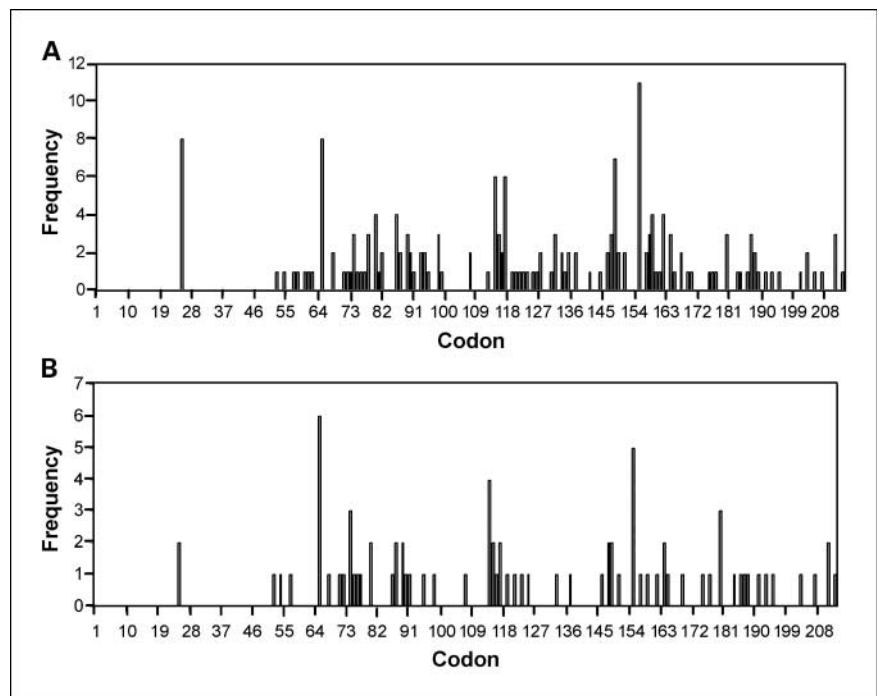
No. cases ( <i>n</i> = 205)			No. mutations ( <i>n</i> = 176)											
<i>VHL</i> status	<i>n</i>	%	Type	<i>n</i>	%	Location	<i>n</i>	%	RSI bin*	<i>n</i>	%	Database	<i>n</i>	%
Mutated	169	82	Deletion	59	33.5	Exon 1	62	35	Low	80	45	Yes	124	70
1 Mutation	162	79	Insertion	42	23.9	Intron 1	4	2	Medium	92	52	No	52	30
2 Mutations	7	3	Missense	42	23.9	Exon 2	50	28	High	4	2			
No mutation	36	18	Nonsense	18	10.2	Intron 2	11	6						
P25L †	8	4	Splicing ‡	15	9	Exon 3	49	28						
Silent †	7	3												

\*RSI of mutant allele compared with wild-type.

† P25L and silent mutations are shown for reference and were not classified as functional mutations in future calculations.

‡ Splicing mutations include intronic nucleotide changes within three bases of the intron-exon boundary.

Fig. 1. Distribution of *VHL* mutations by codon. A, all mutations. B, mutations with a low RSI value (5-30%).



cancer risk factors that were prevalent in this population, such as hypertension, high BMI, tobacco smoking, and family history of cancer. Table 1 provides a frequency distribution of patient and tumor characteristics for cases that were included in this study. Most cases (63%) were from the Czech Republic. Sex, smoking habits, BMI category, and prevalence of hypertension were similar in those in the entire study population.

**Analysis of *VHL* mutations.** Detailed annotation of all mutations and single nucleotide polymorphisms can be found in Supplementary Table S1. Representative results from analysis of endonuclease digests are shown in Supplementary Figs. S1 and S2A-F. Endonuclease cleavage product sizes were determined by overlay of a digest profile with a sizing ladder using Navigator software. This allowed rapid location of variants in sequence chromatograms. We observed 100% concordance

between mutations identified using endonuclease and those detected in forward and reverse sequencing chromatograms. Overall, *VHL* was mutated in 82.4% (169 of 205) of cases (Table 2). Double mutations were found in 3.4% (7 of 205) of cases. The P25L variant that has been previously described (10, 20) was present in eight patients (4%). As in previous studies, this variant was not considered a mutation; however, six of these cases (75%) also possessed another *VHL* alteration. A total of 3% ( $n = 7$ ) of tumors exhibited coding single nucleotide polymorphisms and 2% ( $n = 4$ ) possessed an IVS 2+43 intronic single nucleotide polymorphism. A total of 176 *VHL* mutations were identified, including deletions (34%,  $n = 59$ ), insertions (24%,  $n = 42$ ), missense (24%,  $n = 42$ ), nonsense (10%,  $n = 18$ ), and splice junction alterations (9%,  $n = 15$ ). Ten of 15 splice junction mutations

**Table 3.** Comparison of mutant allele frequencies estimated by subcloning and RSI

Tumor ID	Exon	Sequencing of tumor DNA*	Sequencing of clones	Mutant allele frequency		
				RSI (%) <sup>†</sup>	Cloning <sup>‡</sup>	(%)
4_1	3	763 deletion	763 delCTCTACG	20	18/56	32
4_6	2	613 G>T	613 G>T	40	17/48	35
4_8	2	652 A>G	652 A>G	50	21/38	55
4_12	1	469 C>T	469 C>T	40	9/31	29
4_16	2	581 deletion	581 deletion	30	21/58	36
4_31	3	IVS2-1 G>A	IVS2-1 G>A	25	3/40	8
4_47	3	752 deletion	752 delTCGTCA	30	18/54	33
4_53	2	574 insertion	574 delGA	10	20/57	35
4_65	2	570 deletion	570 delCAGAGAT	20	24/55	44
4_70	2	665 T>C	665 T>C	30	21/50	42

\*Insertions and deletions were not characterized in initial tumor DNA sequences.

<sup>†</sup>RSI of mutant allele compared with total fluorescence.

<sup>‡</sup>Number of positive transformants.



occurred in the first base of an intron and all were observed within three bases of an exon. The prevalence of mutations by exon was as follows: exon 1, 37% ( $n = 65$ ); exon 2, 34% ( $n = 59$ ); and exon 3, 30% ( $n = 52$ ). We found a high (6%,  $n = 11$ ) prevalence of alterations that seem to affect exon 2/3 splicing and may truncate the protein downstream of codon 155. Figure 1A shows that alterations of the *VHL* gene were distributed from codon 52 (a deletion) to 213 (an insertion), excluding the P25L variant (10, 20). Subtypes of mutations were distributed as previously reported (8–10, 13).

**Confirmation of *VHL* mutant status.** Analysis of 20 mutation-positive cases from half of the *VHL* mutant cases ( $n = 20$ ) and a subset that was wild-type ( $n = 19$ ) was repeated and scored in a blinded manner. All but one mutation was confirmed (95% agreement, 38 of 39), and this false positive was excluded from subsequent calculations. The second half of cases were reamplified (268 of 273 amplicons) using a high-fidelity polymerase (different from the first-round analyses) and *VHL* mutation or wild-type status was confirmed in all cases. Five samples failed to amplify and were not repeated due to limited DNA quantities.

**Relative signal intensity.** Examination of mutant and wild-type peak heights averaged from forward and reverse sequencing chromatograms revealed that the fluorescence signal of mutant nucleotides in DNA amplified from many tumors was dramatically less than those from wild-type nucleotide peaks. Figure 1B presents the distribution of mutations with a low

relative signal intensity (RSI). To calculate RSI of mutant peak heights, we averaged visual estimates of peak heights from forward and reverse sequencing traces, divided by total signal (mutant + wild-type peaks) present at a single nucleotide position. These RSI estimates were grouped into high (>60%), medium (31-60%), and low (5-30%) bins to estimate the proportion of mutations that might be difficult to analyze using sequencing software. For example, mutations in the low RSI bin exhibited <30% signal compared with the total fluorescence, whereas >70% of signal was observed from wild-type nucleotides. As shown in Table 2, 46% of variants exhibited a low RSI value. These samples represent cases whose mutant signal could be easily masked by wild-type sequence, especially in the presence of high background. Automated detection of low RSI mutations was unreliable by commercially available sequencing software.

**Confirmation of allele frequencies by subcloning.** To determine whether visual estimates of RSI reflected the proportion of mutant allele in the original tumor DNA or whether PCR and sequencing were introducing a bias toward wild-type, PCR products from 10 tumors containing mutations with RSI values between 10% and 50% were amplified and subcloned (Table 3). Mutant and wild-type alleles were counted in an average of 48 subclones per tumor, each of which was sequenced on both strands. Generally, mutant allele frequency determined by subcloning agreed to within  $\pm 12\%$  of the visual RSI estimate (7 of 10 cases). Five tumors with deletions each

Mutation Status	Tumor ID	CpG 1	CpG 2	CpG 3	CpG 4	CpG 5	CpG 6	CpG 7	CpG 8	CpG 9	CpG 10	CpG 11	Methylated CpGs
		Methylation Status											
NVD	4-11												0/11
NVD	4-13												6/11
NVD	4-15												10/11
NVD	4-19												6/11
NVD	4-21												5/11
NVD	4-22												5/11
NVD	4-28												0/11
NVD	4-32												9/11
NVD	4-37												0/11
NVD	4-42												8/11
NVD	4-45												0/11
NVD	4-52												6/8
NVD	4-73												0/11
NVD	4-80												1/11
NVD	4-83												8/11
NVD	4-86												9/11
deletion_ex 3	4-1												0/11
S65stop_ex 1	4-2												0/11
deletion_ex 3	4-3												0/11
P25L+IVS 1-1	4-10												0/11
deletion_ex 2	4-23												0/11
S80N_ex 1	4-24												0/11
deletion_ex 3	4-26												0/11
deletion_ex 3	4-29												1/11
insertion_ex 2	4-34												0/11
S65L_ex 1	4-66												0/11
no data	Positive Cntrl												11/11
NVD	Negative Cntrl												0/11

**Fig. 2.** Analysis of *VHL* promoter methylation across 11 CpGs among mutation-negative ( $n = 16$ ) and mutation-positive ( $n = 10$ ) ccRCC DNAs. Methylation status of each CpG is indicated as follows: yellow, unmethylated; blue, partial methylation; red, fully methylated. Hatched box, CpG was uninformative. Bottom, positive (methylated) and negative (unmethylated) controls included in each analysis. Left, *VHL* mutation status. Far right column, fully and partially methylated CpG sites. Cases that had at least four methylated CpG sites in the *VHL* promoter were considered methylation positive.

possessed a RSI value that was lower than the mutant allele frequency determined from subcloning. This may indicate that either visual estimation of RSI for deletions was inaccurate or, alternatively, that deletions actually show lower fluorescent signal compared with that of wild-type alleles in sequence chromatograms.

***VHL promoter methylation.*** Another mechanism by which genes can be inactivated is through promoter hypermethylation. Most studies have used methylation-specific PCR, which relies on methylation of a few CpGs to determine the methylation status of the promoter (8, 10, 13, 21). As with methylation-specific PCR, we analyzed bisulfite-treated DNA, but primers were designed to equally amplify both methylated and unmethylated alleles. To provide a comprehensive analysis, after bisulfite treatment, we used Sanger sequencing to evaluate cytosine positions in CpGs. Cytosine (methylated) to thymine (unmethylated) ratios in 11 sequential CpGs of the *VHL* promoter. Mutation-negative (94%, 34 of 36) and mutation-positive (12%, 21 of 169) tumors were selected for analysis. Seventeen (8.3%, 17 of 205) cases possessed at least four methylated CpGs and were considered hypermethylated and potentially silenced. All methylated cases were negative for *VHL* mutation (Fig. 2). In summary, 91% (186 of 205) of cases showed potential *VHL* inactivation through mutation (82.4%,  $n = 169$ ) or hypermethylation (8.3%,  $n = 17$ ).

***VHL status by patient and tumor characteristics.*** *VHL* alterations were subsequently stratified by tumor histopathologic and patient characteristics as summarized in Table 4. The overall prevalence of mutated cases was not associated with any of the tumor or patient characteristics examined; however, the prevalence of certain subtypes of mutations was. For example, tumors with distant metastases (M1) had significantly more double mutations than those without metastases (M0; 22.2% versus 2.7%;  $P = 0.003$ ). Nonsense mutations were associated with Fuhrman nuclear grade ( $P = 0.03$ ) and lymph node positivity ( $P = 0.05$ ) and were more prevalent among M1 than M0 cases ( $P = 0.01$ ). The location of alterations also seemed to differ between groups. For example, exon 3 mutations were more prevalent in M1 than M0 cases (70.0% versus 25.2%;  $P = 0.01$ ) and among cases with a family history of kidney cancer (66.7% versus 25.2%;  $P = 0.007$ ). Of note, all six cases with a positive family history of kidney cancer possessed tumor DNA harboring a *VHL* mutation and four of six mutations (66.7%) were located in exon 3. Lastly, the prevalence of *VHL* alterations located in exon 3 increased from 8.3% among subjects with low BMI (<25) to 25.5% among those with high BMI (>35;  $P_{\text{trend}} = 0.07$ ).

## Discussion

In this study, DNA from 205 histologically confirmed, macrodissected ccRCC tumors was analyzed for *VHL* gene alterations using a novel combination of endonuclease scanning and fluorescent Sanger sequencing. When applied in parallel, mutations were detected in 82.4% (169 of 205) of ccRCC cases. This is the highest prevalence of *VHL* mutations thus far reported and the largest number of histologically confirmed ccRCC tumors analyzed in a single study. Detailed analysis of the *VHL* promoter identified an additional 8.3% of tumors that were hypermethylated and potentially silenced. Notably, in this study, methylation and mutation were

mutually exclusive. Together, a total of 91% of cases exhibited genetic and epigenetic alteration of the *VHL* gene. Analysis of patient and tumor characteristics revealed that the overall prevalence of mutations was not associated with clinical variables normally associated with disease progression, but that particular mutation subtypes were associated with Fuhrman nuclear grade, metastasis, node positivity, and a family history of kidney cancer. Sensitive and accurate methods of mutation detection such as those described here will be an advantage to future studies by minimizing misclassification of cases by *VHL* mutation/promoter methylation status and reducing the risk of biasing associations toward the null.

Some of the largest studies (between 93 and 205 RCC cases) that had previously examined *VHL* mutation and methylation have reported fewer *VHL* gene mutations [between 42% (13) and 71% (10)]. These studies used single-strand conformational polymorphism and denaturing high-performance liquid chromatography (8–10, 13). As observed previously (10), we document a high prevalence of alterations at codons 65, 114, 147, and 155, although only 6% of mutations occurred in codons 147/148 (13). Mutations were distributed evenly across exons, whereas other studies reported higher frequencies in exons 1 (8, 10) and 2 (13). Lastly, 3.4% (7 of 205) of tumors in this study displayed two *VHL* alterations, Banks et al. (10) reported a single case (1%, 1 of 93), whereas most other studies reported none (8, 13). Hypermethylation of the *VHL* promoter was observed exclusively in mutation-negative tumors and the prevalence observed is within the range previously reported (5% and 17%, respectively; refs. 8, 13). In contrast, one earlier study reported that 21% of tumors were hypermethylated and half of these (11 of 19, 58%) possessed *VHL* mutations (10). This discrepancy may result from different approaches used to assess promoter methylation status or from differences between the histologic RCC subtypes examined. In this study, cases were exhaustively reviewed by one expert to ensure reliability that all cases included in this study were histologically confirmed ccRCC.

Once mutations were identified using endonuclease scanning and sequencing, the RSI value was estimated to determine the proportion that would be difficult or impossible to detect using automated sequence analysis. To avoid misclassification of low RSI *VHL* mutants, PCR product from a subset of mutant cases was subcloned to confirm their identity. Mutation status was confirmed in 10 of 10 cases and in 7 of 10 tumors; the mutant allele frequency calculated from the percentage of mutation-positive transformants agreed with the RSI visual estimate.

To reduce risk of false-negative results, before DNA extraction, all frozen tumor biopsies were macrodissected to remove normal tissue based on a pathology review of one H&E-stained slide to obtain at least 70% tumor tissue per sample. For this reason, the observed high percentage of variants (46%) with low RSI values was unexpected. Tumors in general contain various amounts of normal tissue and cells (22, 23). Kidney tumors in particular can be highly vascularized (24). Tumor infiltration by normal tissue or blood cells, such as lymphocytes, can reduce the proportion of tumor to normal DNA in a sample. Linehan et al. (25) examined seven RCC tumors that were free of any visible normal tissue. After enzymatic dispersion of RCC tissue, they observed that only 20% to 50% were tumor cells (mean,  $26 \pm 15\%$ ). Similarly, Belldegrun et al. (26) examined 37 tumors and found that 6% to 75% of

**Table 4.** Von Hippel–Lindau (VHL) alteration subtypes associated with patient descriptive and clinical characteristics

<b>Total</b>	<b>All cases* (%)</b>	<b>Normal cases (%)</b>	<b>Methylated (%)</b>	<b>1 Mutation (%)</b>	<b>Total mutated (%)</b>	<b>&gt;1 Mutation (%)</b>	<b>Deletion (%)</b>
	<b>205 (100)</b>	<b>18 (16.8)</b>	<b>17 (8.3)</b>	<b>162 (79.8)</b>	<b>169 (83.2)</b>	<b>7 (3.4)</b>	<b>59 (28.8)</b>
<b>Tumor characteristics</b>							
<b>Stage</b>							
T	42 (20.5)	4 (9.5)	3 (7.1)	34 (81.0)	35 (83.3)	1 (2.4)	12 (28.6)
T2	92 (44.9)	8 (19.0)	9 (9.8)	73 (79.3)	76 (82.6)	3 (3.3)	28 (30.4)
T3	64 (31.2)	8 (19.0)	5 (7.8)	48 (75.0)	51 (79.7)	3 (4.7)	14 (21.9)
T4	7 (3.4)	0 (0.0)	0 (0.0)	7 (100.0)	7 (100.0)	0 (0.0)	5 (71.4)
<i>P</i>			0.79		0.84	0.70	0.75
<b>Furhman nuclear grade</b>							
I	2 (1.0)	1 (50.0)	1 (50.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
II	154 (77.8)	13 (8.4)	12 (7.8)	125 (81.2)	130 (84.4)	5 (3.2)	48 (31.2)
III	39 (19.7)	4 (10.3)	3 (7.7)	31 (79.5)	32 (82.1)	1 (2.6)	10 (25.6)
IV	3 (1.5)	1 (33.3)	0 (0.0)	2 (66.7)	2 (66.7)	0 (0.0)	1 (33.3)
<i>P</i>			0.47		0.72	0.80	0.79
<b>Node</b>							
N0	153 (89.5)	16 (10.5)	12 (7.8)	122 (79.7)	126 (82.4)	4 (2.6)	46 (30.1)
N1	12 (7.0)	0 (0.0)	1 (8.3)	9 (75.0)	11 (91.7)	2 (16.7)	5 (41.7)
N2	6 (3.5)	1 (16.7)	1 (16.7)	4 (66.7)	4 (66.7)	0 (0.0)	2 (33.3)
<i>P</i>			0.45		0.63	0.30	0.56
<b>Metastasis</b>							
Mx	37 (19.1)	3 (8.1)	4 (10.8)	29 (78.4)	30 (81.1)	1 (2.7)	10 (27.0)
M0	148 (76.3)	16 (10.8)	12 (8.1)	117 (79.1)	121 (81.8)	4 (2.7)	44 (29.7)
M1	9 (4.6)	0 (0.0)	0 (0.0)	7 (77.8)	9 (100.0)	2 (22.2)	3 (33.3)
<i>P</i> †			0.45		0.14	0.003	0.82
<b>Patient characteristics</b>							
<b>Age group (y)</b>							
<50	36 (17.6)	4 (11.1)	2 (5.6)	27 (75.0)	30 (83.3)	3 (8.3)	14 (38.9)
>50	169 (82.4)	16 (9.5)	15 (8.9)	135 (79.9)	139 (82.2)	4 (2.4)	45 (26.6)
<i>P</i>			0.58		0.67	0.07	0.14
<b>Sex</b>							
Male	122 (59.5)	11 (9.0)	9 (7.4)	101 (82.8)	102 (83.6)	1 (0.8)	31 (25.4)
Female	83 (40.5)	9 (10.8)	8 (9.6)	61 (73.5)	67 (80.7)	6 (7.2)	28 (33.7)
<i>P</i>			0.78		0.87	0.01	0.20
<b>Hypertensive</b>							
Yes	97 (47.3)	8 (8.2)	11 (11.3)	75 (77.3)	79 (81.4)	4 (4.1)	25 (25.8)
No	108 (52.7)	10 (15.1)	6 (5.6)	87 (80.6)	90 (83.3)	3 (2.8)	34 (31.5)
<i>P</i>			0.21		0.51	0.61	0.37
<b>BMI</b>							
<25	54 (26.3)	6 (11.1)	5 (2.5)	42 (77.8)	43 (79.6)	1 (1.9)	2 (3.7)
25_35	101 (49.3)	9 (8.9)	8 (3.9)	82 (81.2)	84 (83.2)	2 (2.0)	34 (33.7)
35+	50 (24.4)	5 (10.0)	4 (2.0)	38 (76.0)	42 (84.0)	4 (8.0)	13 (26.0)
<i>P</i>			0.54		0.69	0.11	0.64
<b>Tobacco smoking</b>							
Never	89 (43.6)	7 (7.9)	8 (9.0)	71 (79.8)	75 (84.3)	4 (4.5)	30 (33.7)
Former	45 (22.1)	3 (6.7)	4 (8.9)	36 (80.0)	38 (84.4)	2 (4.4)	12 (26.7)
Current	70 (34.3)	10 (14.3)	5 (7.1)	54 (77.1)	55 (78.6)	1 (1.4)	17 (24.3)
<i>P</i>			0.88		0.37	0.32	0.19
<b>Pack-years</b>							
0	89 (43.6)	7 (7.9)	8 (9.0)	71 (79.8)	75 (84.3)	4 (4.5)	30 (33.7)
1_20	48 (23.5)	6 (12.5)	6 (12.5)	33 (68.8)	36 (75.0)	3 (6.3)	11 (22.9)
>20	67 (32.8)	7 (10.4)	3 (4.5)	57 (85.1)	57 (85.1)	0 (0.0)	18 (26.9)
<i>P</i>			0.50		0.95	0.17	0.32
<b>Family history of kidney cancer †§</b>							
No	138 (67.3)	16 (11.6)	13 (9.4)	105 (76.1)	110 (79.7)	5 (3.6)	40 (29.0)
Yes	6 (2.9)	0 (0.0)	0 (0.0)	6 (100.0)	6 (100.0)	0 (0.0)	2 (33.3)
<i>P</i>			0.45		0.24	NA	0.82
<b>Family history of cancer †§</b>							
Yes_any	61 (29.8)	4 (6.6)	4 (6.6)	51 (83.6)	53 (86.9)	2 (3.3)	17 (27.9)
<i>P</i>			0.50		0.20	0.80	0.93

Abbreviation: NA, not available.

\*Subgroups that do not add to 205 are due to missing clinical or risk factor information.

† *P* value for comparison of M0 versus M1 only.

‡ Both groups are compared with cases without family history of cancer.

§ First-degree relative with cancer or kidney cancer.

**Table 4.** Von Hippel-Lindau (VHL) alteration subtypes associated with patient descriptive and clinical characteristics (Cont'd)

<b>Insertion (%)</b>	<b>Missense (%)</b>	<b>Splice (%)</b>	<b>Nonsense (%)</b>	<b>Exon 1<sup>†</sup> (%)</b>	<b>Exon 2<sup>†</sup> (%)</b>	<b>Exon 3<sup>†</sup> (%)</b>
<b>42 (20.5)</b>	<b>40 (19.5)</b>	<b>15 (7.3)</b>	<b>18 (8.8)</b>	<b>62 (30.2)</b>	<b>50 (24.4)</b>	<b>49 (23.9)</b>
12 (28.6)	7 (16.7)	1 (2.4)	3 (7.1)	12 (34.3)	16 (45.7)	7 (20.0)
16 (17.4)	22 (23.9)	6 (6.5)	7 (7.6)	27 (37.0)	16 (21.9)	30 (41.1)
13 (20.3)	11 (17.2)	7 (10.9)	8 (12.5)	22 (46.8)	14 (29.8)	11 (23.4)
1 (14.3)	0 (0.0)	1 (14.3)	0 (0.0)	1 (16.7)	4 (66.7)	1 (16.7)
0.34	0.50	0.07	0.57	0.93	0.76	0.51
0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
35 (22.7)	31 (20.1)	10 (6.5)	10 (6.5)	46 (36.8)	39 (31.2)	40 (32.0)
5 (12.8)	8 (20.5)	4 (10.3)	6 (15.4)	14 (48.3)	7 (24.1)	8 (27.6)
0 (0.0)	0 (0.0)	0 (0.0)	1 (33.3)	0 (0.0)	1 (50.0)	1 (50.0)
0.17	0.86	0.57	0.03	0.65	0.70	0.70
31 (20.3)	31 (20.3)	9 (5.9)	12 (7.8)	52 (43.0)	35 (28.9)	34 (28.1)
2 (16.7)	1 (8.3)	1 (8.3)	4 (33.3)	2 (16.7)	4 (33.3)	6 (50.0)
0 (0.0)	0 (0.0)	1 (16.7)	1 (16.7)	2 (66.7)	1 (33.3)	0 (0.0)
0.27	0.17	0.31	0.05	0.50	0.90	0.67
8 (21.6)	8 (21.6)	2 (5.4)	2 (5.4)	7 (24.1)	13 (44.8)	9 (31.0)
29 (19.6)	28 (18.9)	10 (6.8)	13 (8.8)	52 (45.2)	34 (29.6)	29 (25.2)
2 (22.2)	2 (22.2)	1 (11.1)	3 (33.3)	1 (10.0)	2 (20.0)	7 (70.0)
0.84	0.81	0.62	0.01	0.14	0.94	0.01
7 (19.4)	7 (19.4)	4 (11.1)	1 (2.8)	10 (34.5)	11 (37.9)	8 (27.6)
35 (20.7)	33 (19.5)	11 (6.5)	17 (10.1)	52 (39.4)	39 (29.5)	41 (31.1)
0.86	0.99	0.34	0.16	0.83	0.27	0.96
26 (21.3)	23 (18.9)	11 (9.0)	11 (9.0)	33 (35.9)	32 (34.8)	27 (29.3)
16 (19.3)	17 (20.5)	4 (4.8)	7 (8.4)	29 (42.0)	18 (26.1)	22 (31.9)
0.72	0.77	0.26	0.89	0.25	0.60	0.67
22 (22.7)	18 (18.6)	6 (6.2)	11 (11.3)	29 (37.7)	22 (28.6)	26 (33.8)
20 (18.5)	22 (20.4)	9 (8.3)	7 (6.5)	33 (39.3)	28 (33.3)	23 (27.4)
0.46	0.74	0.56	0.22	0.73	0.64	0.40
7 (13.0)	15 (27.8)	6 (11.1)	4 (7.4)	19 (50.0)	15 (39.5)	4 (10.5)
22 (21.8)	14 (13.9)	7 (6.9)	9 (8.9)	27 (34.2)	19 (24.1)	33 (41.8)
13 (26.0)	11 (22.0)	2 (4.0)	5 (10.0)	16 (36.4)	16 (36.4)	12 (27.3)
0.10	0.43	0.17	0.64	0.51	0.87	0.07
21 (23.6)	15 (16.9)	4 (4.5)	8 (9.0)	27 (36.0)	22 (29.3)	26 (34.7)
8 (17.8)	12 (26.7)	3 (6.7)	4 (8.9)	17 (45.9)	10 (27.0)	10 (27.0)
13 (18.6)	12 (17.1)	8 (11.4)	6 (8.6)	17 (35.4)	18 (37.5)	13 (27.1)
0.42	0.90	0.11	0.93	0.56	0.91	0.21
21 (23.6)	15 (16.9)	4 (4.5)	8 (9.0)	27 (36.0)	22 (29.3)	26 (34.7)
5 (10.4)	14 (29.2)	3 (6.3)	5 (10.4)	14 (38.9)	10 (27.8)	12 (33.3)
16 (23.9)	10 (14.9)	8 (11.9)	5 (7.5)	20 (40.8)	18 (36.7)	11 (22.4)
0.93	0.87	0.09	0.76	0.92	0.80	0.13
26 (18.8)	30 (21.7)	8 (5.8)	9 (6.5)	45 (42.1)	35 (32.7)	27 (25.2)
1 (16.7)	2 (33.3)	0 (0.0)	1 (16.7)	1 (16.7)	1 (16.7)	4 (66.7)
0.89	0.51	NA	0.34	0.42	0.64	0.007
15 (24.6)	8 (13.1)	7 (11.5)	8 (13.1)	16 (33.3)	14 (29.2)	18 (37.5)
0.40	0.25	0.23	0.10	0.31	0.68	0.11

cells were tumor cells (mean,  $39 \pm 3\%$ ). It is plausible that the low amount of mutant allele estimated by RSI in approximately half of the tumors examined here resulted from a low ratio of tumor to normal tissue that was not removed by macro-

dissection. Lymphocyte infiltration, vascular architecture, and surrounding normal tissue seem to be a significantly greater component of kidney tumors compared with other types of tumor tissues. Contamination of tumor tissue with normal



tissue in patient samples may also explain discrepancies in the reported *VHL* mutation frequency in ccRCC. The presence of significant amounts of normal cells in tumor tissue strongly argues for application of highly sensitive and accurate mutation detection methods such as those presented here to ensure that all mutations are detected. This conclusion is based on the observation that the *VHL* mutation frequency we detected with the methods described surpasses the renal cancer *VHL* somatic mutation frequency detected in all previous studies (14).

After we completed this comprehensive analysis of somatic mutations and methylation in the cancer genome of ccRCC, we correlated the genetic findings with patient clinical and descriptive characteristics. Analysis of mutation prevalence among patient and tumor subgroups revealed that total mutation prevalence was not associated with any of the variables examined. This finding is similar to other studies and supports the hypothesis that *VHL* gene inactivation is likely an early event in the ccRCC carcinogenic process (8–10, 27–29). Unlike some previous studies, we did not observe a higher prevalence of *VHL* mutation/hypermethylation among high-stage tumors (13) nor did we observe differences in the prevalence of hypermethylation or double mutations by sex (10). We did find that late-stage metastatic lesions had more double mutations, nonsense mutations, and mutations located in exon 3 than M0 or Mx cases. We also observed that the prevalence of nonsense mutations was significantly associated

with grade and node positivity and that cases with a family history of kidney cancer had significantly more mutations located in exon 3. Survival studies will be necessary to determine whether these molecular subtypes have any prognostic relevance.

A goal of personalized medicine is to identify tumors with particular alterations that can be used to predict a clinical response to treatment in the patient. A recent study showed that patients with metastatic RCC that possessed *VHL* methylation or truncating mutations had prolonged time to tumor progression during treatment with antiangiogenic therapy (21). We show that segregation of tumors by *VHL* alteration subtype could be achieved in 91% of ccRCC cases. These findings indicate that *VHL* molecular subtypes could provide a sensitive biomarker of ccRCC tumor heterogeneity among histologically similar cases for etiologic, prognostic, and translational studies (1, 2, 21, 30, 31).

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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# Clinical Cancer Research

## Improved Identification of von Hippel-Lindau Gene Alterations in Clear Cell Renal Tumors

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