Genetic and Epigenetic Analysis of von Hippel-Lindau (*VHL*) Gene Alterations and Relationship with Clinical Variables in Sporadic Renal Cancer

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Abstract

Genetic and epigenetic changes in the von Hippel-Lindau (VHL) tumor suppressor gene are common in sporadic conventional renal cell carcinoma (cRCC). Further insight into the clinical significance of these changes may lead to increased biological understanding and identification of subgroups of patients differing prognostically or who may benefit from specific targeted treatments. We have comprehensively examined the VHL status in tissue samples from 115 patients undergoing nephrectomy, including 96 with sporadic cRCC. In patients with cRCC, loss of heterozygosity was found in 78.4%, mutation in 71%, and promoter methylation in 20.4% of samples. Multiplex ligation–dependent probe amplification identified intragenic copy number changes in several samples including two which were otherwise thought to be VHL-noninvolved. Overall, evidence of biallelic inactivation was found in 74.2% of patients with cRCC. Many of the mutations were novel and approximately two-thirds were potentially truncating. Examination of these and other published findings confirmed mutation hotspots affecting codons 117 and 164, and revealed a common region of mutation in codons 60 to 78. Gender-specific differences in methylation and mutation were seen, although not quite achieving statistical significance (P = 0.068 and 0.11), and a possible association between methylation and polymorphism was identified. No significant differences were seen between VHL subgroups with regard to clinicopathologic features including stage, grade, tumor size, cancer-free and overall survival, with the exception of a significant association between loss of heterozygosity and grade, although a possible trend for survival differences based on mutation location was apparent. (Cancer Res 2006; 66(4): 2000-11)

Introduction

Almost 210,000 cases of renal cancer are diagnosed annually worldwide (GLOBOCAN 2002).[6] The most common subtype of renal parenchymal carcinoma is the conventional (clear cell) type (cRCC) accounting for ~75% of cases. The most frequent karyotypic change in cRCC is loss of 3p with several candidate genes implicated, in particular, the von Hippel-Lindau (*VHL*) tumor suppressor gene at 3p25. The *VHL* gene was identified in 1993 from familial studies of VHL disease which predisposes to tumors of the central nervous system, adrenal gland, kidney, and eye (1), and is now known to be involved in 50% to 75% of sporadic cRCC cases (2, 3). In the familial cases, germ line mutations are followed by mutation, methylation, or loss of the remaining wild-type VHL allele in the tumor, and in sporadic cases, the biallelic loss of function occurs through a combination of somatic allele loss, mutation, and/or methylation (2, 3).

The gene consists of three exons with two translation initiation sites resulting in two protein isoforms of 160 and 213 amino acids, pVHL19 and pVHL30, both seeming to have tumor suppressor activity. Consisting of a β-sheet domain and a smaller α-helical domain held together by two linkers and a polar interface, VHL normally functions as the substrate recognition subunit of a multiprotein E3 ubiquitin ligase complex involving elongins B and C, Cul-2, and Rbx1 with members of the hypoxia-inducible factor (HIF-α) family of transcription factors being principal targets for ubiquitination and subsequent proteasomal degradation. In hypoxic conditions or in the absence of functional VHL, HIF accumulates, up-regulating many genes involved in angiogenesis, cellular metabolism, and cell growth (reviewed in refs. 2, 3). However, VHL has also been implicated in a variety of other cellular processes including cell cycle regulation, extracellular matrix assembly, and cytoskeleton stability and there is evidence that some VHL activities are HIF-independent.

In familial disease, genotype-phenotype correlations exist (2, 3). Type 1 disease has a low risk of phaeochromocytoma and germ line mutations are often large deletions or truncating mutations. Type 2 has a high risk of phaeochromocytoma and is subdivided into high risk (2B), low risk (2A), or absence (2C) of cRCC, with germ line missense mutations more commonly seen. Defective regulation of HIF is found in all types except type 2C. The mechanism underlying the tissue-specific influences of the germ line mutations may reflect unknown tissue-specific functions of VHL driven by different regions of the protein. Recently, increased risk of renal involvement has been associated with germ line mutations leading to truncations (4) and specific missense mutations affecting protein structural integrity (4, 5).

In sporadic cRCC, studies have analyzed *VHL* mutation and methylation (6–34) but few have examined the clinical relevance of

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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[6] http://www-dep.iarc.fr/.

these alterations. However, such information may be of critical importance in terms of prognosis and for developing therapies targeting specific downstream pathways in patient subgroups (2). Several studies have found no differences in mutation frequency in relation to tumor grade, stage or tumor size (12, 24, 25, 34), or microvessel density and tumor cell proliferation (25), although an increased rate of mutation or methylation in pT3 tumors has been described (16). Poorer overall and progression-free survival have been reported in patients with "loss of function" (LOF) mutations (25, 32), although a much larger study found VHL mutation or methylation to be strongly associated with better prognosis in stage I to III patients but not stage IV patients (35).

The need for further large comprehensive clinical studies of sporadic RCC and *VHL* status is clear. We have analyzed renal tissue samples from 115 patients including 107 with sporadic RCC, with mutation analysis, loss of heterozygosity (LOH) analysis, promoter-specific methylation, and multiplex ligation–dependent probe amplification (MLPA) to detect intragenic deletions. Findings have been related to clinicopathologic features such as tumor grade, size, stage, as well as survival. The results have also been analyzed in the context of a comprehensive review of existing published[7] results to highlight potential areas of interest.

Materials and Methods

Patient samples. A total of 117 renal samples from 115 previously untreated patients undergoing nephrectomy (two bilateral) were analyzed following informed consent. The samples were selected from a total of 155 samples banked during October 1998 to November 2001, to include all the cRCC cases (n = 96; Table 1) and examples of other subgroups [RCC: seven papillary, one chromophobe, one collecting duct, two unclassified (mixed), and two familial VHL, three TCC renal pelvis, one metanephric nephroma, one oncocytoma, and one retention cyst]. For the cRCC cases, follow-up time ranged from 36 to 70 months with a median relapse-free survival of 40.3 months and cancer-specific survival of 40.4 months. Samples of tumor from each patient were snap-frozen and stored in liquid nitrogen. For genomic DNA, the buffy coats from venous blood samples (EDTA) were stored at −80°C. Frozen tissue sections (50 × 20 μm thickness) or the equivalent of 200 μL of frozen buffy coat were placed in microcentrifuge tubes and DNA extracted using QIAamp DNA Mini kit (Qiagen, Crawley, United Kingdom) and quantified using the PicoGreen dsDNA kit (Molecular Probes, Leiden, Netherlands).

Mutation detection using denaturing high-pressure liquid chromatography and DNA sequencing. Primers were designed using the Primer3 software (Whitehead Research Institute, Cambridge, MA) with two overlapping primer pairs (1a and 1b) used to amplify the promoter region and exon 1 with a further pair (1a/b) used for some sequencing, and one pair for each of exons 2 and 3 (Supplementary Table S1).

For denaturing high-pressure liquid chromatography (DHPLC) screening, PCR products (3-10 μL) from the renal tissue samples were denatured at 95°C for 5 minutes and allowed to cool to 65°C for the formation of homo- and heteroduplexes. DHPLC was carried out using a Transgenomic WAVE HPLC and DNasep column with (A) 0.1 mol/L triethylammonium acetate/0.1 mmol/L EDTA and (B) 0.1 mol/L triethylammonium acetate/0.1 mmol/L EDTA/25% v/v acetonitrile (Transgenomic, Elancourt, France). Analysis was carried out at a flow rate of 0.9 mL/min and gradient increase of buffer B of 2%/min for 4 minutes with start and end concentrations of buffer B being determined empirically for each fragment. Elution of DNA from the column was determined by absorbance at 260 nm. The optimum temperature for mutation detection for each fragment is ~1°C below T_m and was determined empirically for each fragment. Samples were analyzed both alone and with the addition of 50% wild-type reference DNA to ensure the detection of homozygous and heterozygous mutations.

Table 1. Summary of the clinical characteristics of the 96 patients with sporadic conventional RCC

Variables	Number of patients
Sex	39 female/57 male
Age	range, 38-86; median, 63 (y)
Grade	
1	6
2	32
3	34
4	24
Tumor	
1a	19
1b	20
2	7
3a	23
3b	25
3c	0
4	2
Node	
X	8
0	79
1	7
2	2
Metastasis	
0	74
1	22
Stage	
I	38
II	5
III	31 (4 at least stage III)
IV	22

NOTE: Pathologic diagnosis is according to Fuhrman's grading system and UICC tumor-node-metastasis staging system. For the six tumors in which accurate assessment of tumor stage was not possible, the minimum T value is indicated.

DNA sequencing of tissue samples found to be positive by DHPLC, and corresponding buffy coat genomic DNA samples was carried out following treatment of the PCR products (5 μL) for 30 minutes at 37°C with shrimp alkaline phosphatase (2 μL of 1 unit/μL; USB-GE Healthcare-Biosciences, Amersham, United Kingdom), and exonuclease I (1 μL of 10 units/μL; USB), with subsequent inactivation at 80°C for 15 minutes. Sequencing was carried out in 10 μL reactions using 3 μL of the purified PCR products, 1.6 pmol of the appropriate forward or reverse primer and the BigDye (v1.1) Terminator kit (Applied Biosystems, Warrington, United Kingdom) according to the manufacturer's protocol. Reactions were carried out for 25 cycles using a GeneAmp 9700 Thermal and sequencing was carried out by fluorescence capillary electrophoresis using an ABI PRISM 3100 Genetic Analyzer with denaturing POP-6 polymer/Tris-TAPS-EDTA buffer (Applied Biosystems).

LOH analysis of 3p. Six highly polymorphic microsatellite markers flanking the *VHL* gene (Fig. 1) were selected.[8] Initially *D3S1317* and *D3S1597* were used for all samples and those found to be noninformative or equivocal were then also analyzed using further markers as necessary. The marker, primer, and PCR conditions are provided in Supplementary Table S2. Fluorescent PCR products were electrophoretically separated and

[7] A. Harris, some unpublished data.

[8] http://www.gdb.org/ and http://www.ensembl.org.

detected using an ABI PRISM 3100 Genetic Analyzer with PCR products sized by comparison with Genescan-500 ROX size standards. Data was analyzed using Genescan Analysis software version 3.1. LOH was assessed by determining the ratio of the peak heights or areas of the tumor alleles to the normal alleles to calculate the allelic imbalance ratios (AIR), i.e., AIR = (T1 / T2) / (N1 / N2), where T1 and T2 denote the two tumor alleles and N1 and N2 denote the two normal alleles. Samples scored as negative for LOH showed an AIR for all informative markers to be >0.8 with most in each case being >0.9, similar scores to those found for a normal kidney run as a control and the benign oncocytoma and retention cysts samples. Samples with AIR < 0.8 for the closest informative marker to *VHL* or markers flanking *VHL* were scored as positive for LOH. The majority of these had an AIR < 0.6; a small subgroup clearly showed allelic imbalance and scored 0.6 to 0.8, possibly reflecting normal tissue contamination or tumor aneuploidy.

Promoter methylation analysis. The methylation status of the *VHL* promoter was examined by sodium bisulfite modification and methylation-specific PCR (36). Briefly, 0.5 to 1 μg of genomic DNA was denatured in 0.3 mol/L NaOH for 15 minutes at 37°C and then unmethylated cytosine residues were sulfonated by incubation in 3.12 mol/L sodium bisulfite (pH 5.0; Sigma, Poole, United Kingdom) and 5 mmol/L hydroquinone (Sigma) in a thermocycler (Hybaid, Hemel Hempstead, United Kingdom) for 30 seconds at 95°C and then for 15 minutes at 50°C for 20 cycles. The sulfonated DNA was recovered using the Wizard DNA clean-up system (Promega, Southampton, United Kingdom) and the conversion reaction was completed by desulfonating in 0.3 mol/L NaOH for 10 minutes at room temperature. The DNA was ethanol precipitated and resuspended in water. Methylation-specific PCR was done using specific primers designed to amplify methylated and unmethylated *VHL* promoter sequences (Supplementary Table S3).

Multiplex ligation–dependent probe amplification. MLPA analysis to detect copy number changes in the *VHL* gene was carried out using the SALSA P016B *VHL* probe kit (MRC-Holland, Amsterdam, Netherlands). The kit contains eight probes to the *VHL* gene (four in exon 1 and two in each of exons 2 and 3), an additional five probes to three other genes on 3p and two control probes to regions telomeric and centromeric from *VHL*, and 14 probes to regions of other chromosomes with the amplification products differing by 9 bp (Fig. 1). Briefly, 50 ng DNA was denatured at 98°C for 5 minutes, the MLPA probe cocktail was added to a total volume of 8 μL and allowed to hybridize for 16 hours at 60°C. Following addition of Ligase-65 and ligation at 54°C for 15 minutes, the ligase was inactivated at 98°C for 5 minutes. PCR primers, deoxynucleotide triphosphates, and polymerase mix were then added and PCR was carried out for 33 cycles of (95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 60 seconds). Products were then analyzed using an ABI PRISM 3100 Genetic Analyzer with ROX-500-labeled internal size standard. Data was generated using Genescan software and relative probe signals were calculated by dividing each measured peak height by the sum of all peak heights in that sample and then normalizing the result to that obtained on control DNA samples. The possibility of copy number changes for control probes in aneuploid tumors, differences in reaction efficiency between samples, and the presence of relatively small but variable amounts of normal cells in the tumor samples complicated the analysis, and accordingly, each sample was compared with multiple controls and those reproducibly giving the best fit were used for analysis.

Statistical analysis. SAS version 8.2 (SAS Institute, Inc., Cary, NC) was used. Summary statistics were calculated using contingency table analysis and Fisher's exact test for categorical exploratory measures, or using a *t* test or ANOVA in which the exploratory measure was continuous. Relapse-free survival was calculated in patients with localized disease at presentation (M0) and was defined as the time from surgery until relapse or cancer-related death, with cancer-specific survival calculated for all patients from nephrectomy to cancer-related death, and analyzed using Kaplan Meier curves and log-rank test. A two-sided 5% level of significance was used.

Results

The results are summarized in Table 2. Of the 97 sporadic cRCC samples (96 patients, including 1 bilateral case), 76 (78.4%) showed LOH with almost all of these showing a likely loss of the whole of the region of 3p examined. LOH was also clearly seen in both familial VHL cases, three of eight papillary RCC cases, one of three TCC kidney cases, and the metanephric nephroma sample.

The previously described single nucleotide polymorphisms (A/G; National Center for Biotechnology Information dbSNP rs779805) at nucleotide 520 located 195 bases upstream of the first ATG and at nucleotide 183, codon 61 C/G (13), were detected in 61 of 115 (53.0%) individuals and in 1 patient, respectively. Novel SNPs were found at nucleotide 788, codon 25 (C/T) in two patients and at

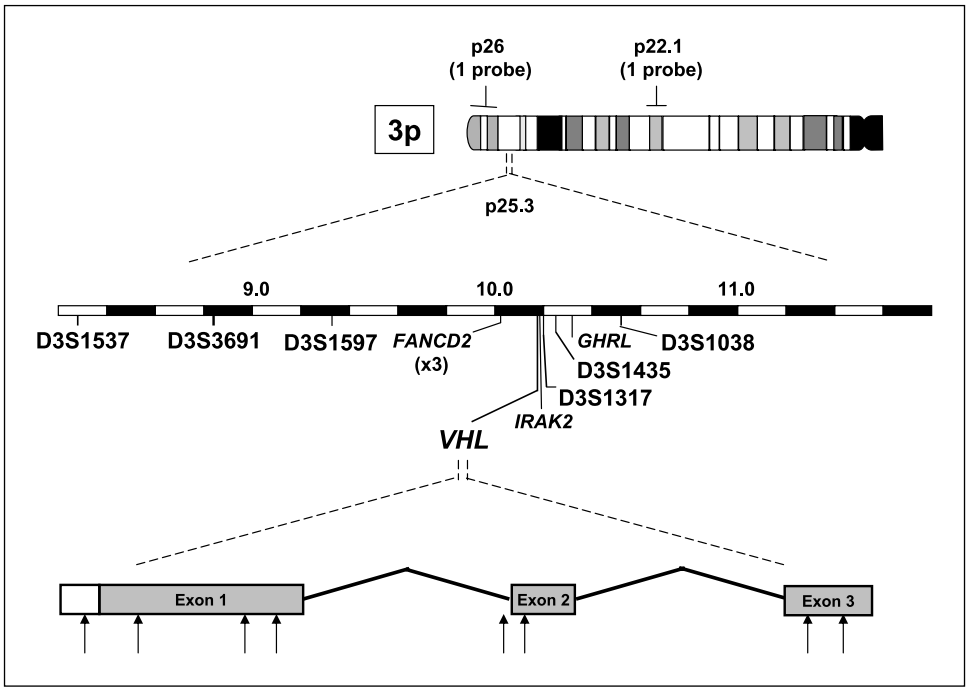

Figure 1. Schematic illustration of the location of the *VHL* gene on chromosome 3p and the locations of the microsatellite markers used for the LOH analysis and the MLPA probes. Control MLPA probes ~10 Mb telomeric and 26 Mb centromeric of the *VHL* gene are shown with an expanded section of p25.3 showing the five probes located in genes flanking VHL. Within the *VHL* gene, four probes lie within exon 1 or the promoter, one in intron 1 immediately before exon 2, and one within exon 2, and an additional two within exon 3 (*arrows*).

Table 2. Summary of the genetic changes seen in the *VHL* gene in patients with sporadic conventional (clear cell) RCC (n = 96) or familial VHL (n = 2)

ID	Histology	Grade and pTNM	LOH?	Methylation?	MLPA?	Mutation?					
						Genomic change	mRNA change	Exon	Codon and predicted amino acid change	Mutation type	Previously found?
1	RCC Conv	G3 T3a,N0,M1	+	+	+	1046_1055 del10	332_340 +1 del10	1	splicing effect (111)	Spl/FS	N*
4	RCC Conv	G1 T1b,N0,M0	−	−	−	—	—	—	—	—	—
5	RCC Conv	G3 T3b,N0,M1	+	−	nd	5401 G>T	350 G>T	2	W117L	MS	N*
6	RCC Conv	G3 T3a,N0,M0	+	+	nd	—	—	—	—	—	—
7	RCC Conv	G2 T3a,N0,M0	−	−	−	964_967 del4	250_253 del4	1	V84fsX157	FS	N
8	RCC Conv	G3 T3b,N0,M0	+	+	nd	—	—	—	—	—	—
9	RCC Conv	G2 T3b,N0,M1	+	−	nd	948 T>A	234 T>A	1	N78K	MS	N*
10	RCC Conv	G3 T3b,N0,M0	+	+	nd	5394 C>G	343 C>G	2	splicing effect (H115D)	Spl/MS	Y
14	RCC Conv	G2 T1b,N0,M0	+	−	−	—	—	—	—	—	—
17	RCC Conv	G2 T1a,N0,M0	+	−	nd	5394 C>A	343 C>A	2	splicing effect (H115N)	Spl/MS	N*
18	RCC Conv	G2 T3a,N0,M0	+	−	nd	994_995 insCG	280_281 insCG	1	E94fsX159	FS	N*
19	RCC Conv	G3 T3a,N0,M0	+	−	nd	917 C>A	203 C>A	1	S68X	N	Y
20	RCC Conv	G2 T1a,N0,M0	+	+	−	1015_1016 ins10	100_101 ins10	1	L101fsX166	FS	N*
22	RCC Conv + sc	G3 T3a,N0,M0	+	−	nd	923_928 del6	209_214 del6	1	E70IFD	IFD	N*
24	RCC Conv	G2 T1a,N0,M0	+	−	+	—	—	—	—	—	—
26	RCC Conv	G3 T3a,N0,M1	+	+	nd	—	—	—	—	—	—
28	RCC Conv	G2 T3b,N0,M0	+	−	nd	8676_8677 insT	473_474 insT	3	L158fsX173	FS	Y
29	RCC Conv	G2 T1b,N0,M0	+	−	nd	899_900 del9 insGC	185_193 del9 insGC	1	V62fsX64	FS	N*
30	RCC Conv	G3 T3b,N0,M1	+	nd	nd	5432 del1	381 del1	2	G127fsX158	FS	Y
31	RCC Conv	G4 T3a,N0,M1	−	−	−	941_942 insAA	227_228 insAA	1	L76fsX159	FS	N*
33	RCC Conv	G3 T3b,NX,M1	+	−	+	—	—	—	—	—	—
34	RCC Conv	G3 T3b,N0,M0	+	−	nd	DHPLC not yet confirmed by sequencing		1			
35	RCC Conv	G3 T1a,N0,M0	+	−	nd	908 C>A	194 C>A	1	S65X	N	Y
36	RCC Conv	G2 T1a,N0,M0	+	−	−	510 C>G	—	5′ UTR	—	5′ UTR	N
37	RCC Conv	G2 T1a,N0,M0	+	−	−	957_961 del5	243_247 del5	1	P81fsX129	FS	N*
38	RCC Conv	G2 T1b,N0,M0	+	+	nd	971 C>T	257 C>T	1	P86L	MS	Y
39	RCC Conv + sc	G4 T3a,N2,M1	+	−	−	8694 A>C	491 A>C	3	Q164P	MS	N*
41	RCC Conv	G1 T1b,N0,M0	+	−	+	—	—	—	—	—	—
42	RCC Conv	G3 T3b,NX,M1	+	+	nd	894_898 del5	180_184 del5	1	R60fsX129	FS	N*
43	RCC Conv	G2 T1a,N0,M0	+	−	nd	927 del1	213 del1	1	P71fsX158	FS	N*
47	RCC Conv	G4 T3a,N0,M1	−	nd	−	—	—	—	—	—	—
49	RCC Conv	G2 T1b,N0,M0	+	+	+	5516 T>C	IVS2+2 T>C	IVS 2+2	Splicing effect	Spl	Y
51	RCC Conv	G3 T3b,N0,M0	+	+	−	880 del1	166 del1	1	A56fsX66	FS	N*
52	RCC Conv	G3 T3b,N0,M0	+	−	−	—	—	—	—	—	—
53	RCC Conv	G3 T3b,N0,M1	+	+	nd	5421 del A	370 del A	2	T124fsX158	FS	N*
54	RCC Conv	G2 T1a,N0,M0	+	+	nd	5477 del T	426 del T	2	V142fsX158	FS	N*
55	RCC Conv	G4 T3b,NX,M1	+	−	nd	915_920 del6 insA	201_206 del6 insA	1	N67fsX129	FS	N
58	RCC Conv + sc	G4 T3b,N1,M0	−	−	−	—	—	—	—	—	—
62	RCC Conv	G4 T2,N0,M0	+	−	nd	8694 A>C	491 A>C	3	Q164P	MS	N*
64	RCC Conv	G2 T1a,NX,M1	+	−	nd	—	—	—	—	—	—
65	RCC Conv	G1 T1a,N0,M0	+	−	nd	5490_5491 insA	439_440 insA	2	I147fsX173	FS	N*
69	RCC Conv	G4 T3a,N1,M0	+	−	nd	5392 G>C	341 G>C	2	splicing effect (G114D)	Spl/MS	N*
70	RCC Conv	G2 T3a,N0,M0	+	+	−	1016 T>C	302 T>C	1	L101P	MS	N*
73	RCC Conv	G2 T2,N0,M0	−	−	−	—	—	—	—	—	—
74	RCC Conv + sc	G4 T3b,N1,M1	−	−	−	—	—	—	—	—	—
75	RCC Conv	G2 T1b,N0,M0	+	−	nd	943_951 del9	229_237 del9	1	C77_R79 del	IFD	N*
77	RCC Conv	G3 T4,N0,M1	+	−	nd	8743_8747 del5 insT	540_544 del5 insT	3	I180fsX200	FS	N*
78	RCC Conv	G4 T3b,N0,M0	+	−	nd	8689 C>A	486 C>A	3	C162X	N	N*

(*Continued on the following page*)

Table 2. Summary of the genetic changes seen in the *VHL* gene in patients with sporadic conventional (clear cell) RCC (n = 96) or familial VHL (n = 2) (Cont'd)

ID	Histology	Grade and pTNM	LOH?	Methylation?	MLPA?	Mutation?					
						Genomic change	mRNA change	Exon	Codon and predicted amino acid change	Mutation type	Previously found?
79	RCC Conv	G3 T2,N0,M1	−	−	nd	—	—	—	—	—	—
80	RCC Conv	G4 T1b,N0,M0	−	+	−	—	—	—	—	—	—
81	RCC Conv	G2 T3a,N0,M0	−	−	−	DHPLC not yet confirmed by sequencing		1			
82	RCC Conv	G3 T1a,N0,M0	+	−	nd	5459 del T	408 del T	2	F136fsX158	FS	N*
83	RCC Conv	G2 T3b,N0,M0	+	−	−	8706 del1	503 del1	3	S168fsX169	FS	N*
86	RCC Conv	G1 T1b,N0,M0	+	+	−	1055 G>T	IVS1+1 G>T	IVS1+1	Splicing effect	Spl	N*
87	RCC Conv	G3 T1b,N0,M0	+	−	nd	5451 G>T	400 G>T	2	E134X	N	Y
88	RCC Conv	G4 T2,N0,M0	−	−	−	—	—	—	—	—	—
96	RCC Conv	G4 T1b,N0,M0	+	+	nd	980 T>G	266 T>G	1	L89R	MS	Y
97	RCC Conv	G3 T3b,NX,M1	+	−	nd	976 T>A	262 T>A	1	W88R	MS	Y
98	RCC Conv	G3 T3b,N2,M1	+	−	nd	8696 G>A	493 G>A	3	V165I	MS	N*
						8712 T>A	509 T>A	3	V170D	MS	Y
101	RCC Conv	G2 T1a,N0,M0	+	−	nd	8735 del1	532 del1	3	L178fsX201	FS	N*
107	RCC Conv	G2 T1b,N0,M0	+	−	nd	897 del1	183 del1	1	P61fsX66	FS	Y
108	RCC Conv	G4 T1a,N0,M0	−	−	nd	—	—	—	—	—	—
112	RCC Conv	G3 T3a,N0,M0	−	−	nd	—	—	—	—	—	—
114	RCC Conv	G1 T1a,N0,M0	+	−	nd	954 T>A	240 T>A	1	S80R	MS	Y
115	RCC Conv + sc	G4 T3a,N1,M1	L+	−	nd	5401 G>T	350 G>A	2	W117X	N	Y
			R+	−	nd	5401 G>T	350 G>A	2	W117X	N	Y
116	RCC Conv	G3 T2,N0,M0	+	−	nd	5424 del1	373 del1	2	H125fsX158	FS	N*
117	RCC Conv	G4 T3a,N0,M0	+	−	nd	8667_8677 del11	464_474 del11	3	splicing effect (155)	Spl/FS	N*
118	RCC Conv	G2 T3b,N0,M0	+	−	−	5512 C>T	461 C>T	2	splicing effect (P154L)	Spl/MS	Y
120	RCC Conv	G4 T3b,N1,M0	−	−	nd	1029_1044 del16	315_330 del16	1	T105fsX153	FS	N
122	RCC Conv	G4 T3b,N0,M0	+	−	nd	5400 del1	349 del1	2	W117fsX158	FS	N*
123	RCC Conv	G2 T1b,N0,M0	+	−	nd	5503 T>A	452 T>A	2	I151N	MS	N*
124	RCC Conv	G3 T3a,N0,M1	+	−	nd	946 A>G	232 A>G	1	N78D	MS	N*
125	RCC Conv + sc	G4 T3a,N0,M0	+	−	nd	931 del1	217 del1	1	Q73fsX158	FS	N*
128	RCC Conv	G4 T3a,N0,M0	−	−	+	—	—	—	—	—	—
129	RCC Conv	G3 T1a,N0,M0	+	−	nd	5411_5412 ins A	359_360 insA	2	D121fsX131	FS	N*
130	RCC Conv	G2 T3a,N0,M0	+	−	−	911 del1	197 del1	1	V66fsX158	FS	N*
132	RCC Conv	G3 T1b,N0,M0	−	nd	+	—	—	—	—	—	—
133	RCC Conv	G3 T3a,N0,M0	+	−	nd	937_938 ins A	223_224 insA	1	I75fsX131	FS	N
134	RCC Conv	G3 T1a,N0,M0	+	−	−	8684 C>T	481 C>T	3	R161X	N	Y
135	RCC Conv	G3 T2,N0,M0	+	−	nd	—	—	—	—	—	—
136	RCC Conv	G2 T1b,N0,M0	+	−	+	5391 G>A	IVS1-1 G>A	IVS1-1	splicing effect	Spl	Y
137	RCC Conv	G3 T3b,N0,M0	+	+	nd	DHPLC not yet confirmed by sequencing		1			
138	RCC Conv	G2 T1a,N0,M0	+	−	nd	922 G>T	208 G>T	1	E70X	N	Y
139	RCC Conv	G1 T1a,N0,M0	−	+	−	8666 G>A	IVS2-1 G>A	IVS2-1	splicing effect	Spl	Y
140	RCC Conv + sc	G4 T4,N0,M1	+	−	−	—	—	—	—	—	—
141	RCC Conv	G2 T1a,N0,M0	+	−	nd	1031_1037 del7	317_323 del7	1	G106fsX156	FS	N*
142	RCC Conv	G3 T3a,N0,M0	+	−	−	5457 T>G	406 T>G	2	F136V	MS	N*
143	RCC Conv + sc	G4 T3b,N1,M0	−	−	−	DHPLC not yet confirmed by sequencing		2			
144	RCC Conv	G3 T1b,N0,M0	+	−	−	—	—	—	—	—	—
146	RCC Conv	G3 T2,NX,M1	+	−	+	5495_5496 insT	444_445 insT	2	A149fsX173	FS	N*
147	RCC Conv	G4 T3b,NX,M0	+	+	−	—	—	—	—	—	—
148	RCC Conv	G2 T1b,N0,M0	+	−	+	5441_5442 insT	390_391 insT	2	N131fsX131	FS	N*
150	RCC Conv	G3 T3a,NX,M0	−	−	−	—	—	—	—	—	—

(*Continued on the following page*)

Table 2. Summary of the genetic changes seen in the *VHL* gene in patients with sporadic conventional (clear cell) RCC (n = 96) or familial VHL (n = 2) (Cont'd)

ID	Histology	Grade and pTNM	LOH?	Methylation?	MLPA?	Mutation?					
						Genomic change	mRNA change	Exon	Codon and predicted amino acid change	Mutation type	Previously found?
151	RCC Conv + sc	G4 T1b,N0,M0	−	−	−	8676 T>A	473 T>A	3	L158Q	MS	Y
153	RCC Conv	G2 T1b,N0,M0	−	−	nd	—	—	—	—	—	—
155	RCC Conv	G2 T1b,N0,M0	+	−	nd	8678_8683 del6 insT	475_480 del6 insT	3	K159fsX171	FS	N*
21	RCC familial VHL	G1 T1a,N0,M0	+	nd	−	942_943 insC	228_229 insC	1	C77fsX131	FS	N*
149	RCC familial VHL	G2 T2,N0,M0	+	−	+	943_944 insC	228_229 insC	1	C77fsX131	FS	N*

NOTE: Mutation nomenclature is in accordance with recent recommendations (47). All nucleotide numbering is in accord with the GenBank sequence AF010238 for genomic VHL and L15409 for mRNA with A of the first initiator ATG being 1. Codon numbers are in accordance with L15409. Previous description of the mutation in HGMD or Necker VHL mutations database was ascertained using the web sites http://www.hgmd.org and http://www.umd.be:2020 (N* indicates that the precise mutation wasn't seen but that a mutation of the same type involving the same codon or intronic nucleotide was reported) and potential effects of mutations on splicing were checked using http://www.fruitfly.org/seq_tools/splice.html. Conv, conventional; sc, plus sarcomatoid change; nt, nucleotides; del, deletion; ins, insertion; MS, missense; FS, frameshift; N, nonsense; Spl, splice error; S, silent error; IFD, in-frame deletion; nd, not determined.

nucleotide 5557 (A/G) in the intron 43 bp 3′ to the end of exon 2 in 8 of 115 (7.0%) individuals, confirmed as polymorphisms by comparison with constitutional DNA. *VHL* mutations were only seen in the sporadic and familial cRCC cases. Unequivocal mutation status was determined in 93 sporadic cRCC tumors, 66 (71%) of which had mutations with 65 of these being single mutations. An additional four tumors positive for DHPLC were not confirmed by sequencing. The distribution of mutations is shown in Fig. 2*A* and *B*, illustrating the absence of mutations in the first half of exon 1, 50% of the exonic mutations in the second half of exon 1, 31% in exon 2, and only 19% in exon 3.

Frameshifts accounted for 48.5% of mutations with 30.9% being missense, 11.8% nonsense, 5.9% intronic, and 2.9% in-frame deletions. Ten mutations potentially affect exon splicing. Insertions or deletions were more common in exon 1 and nucleotide substitutions were more frequent in exon 3. Approximately two-thirds of the mutations would be predicted to result in truncated or absent proteins due either to frameshift or substitutions resulting in stop codons. Many of these mutations are novel, with no previous descriptions either in the literature or VHL databases. Codons affected in >1 tumor (78, 115, 164, and 117) and mutations affecting splicing were also among those previously reported in sporadic cRCC and featured as major or moderate "hotspots" (Fig. 2*A-D*). Other than these, particular clusters of frameshift/nonsense mutations were apparent in codons 60 to 62, 65 to 68, and 70 to 76, which also match the pattern of mutations seen in previous studies (n = 886) when collated (Fig. 2*A-D*).

MLPA analysis was carried out on 41 sporadic cRCC cases, 11 of which had intragenic deletions/duplications (Fig. 3; Table 2). Deletion of all or part of 3p in a diploid background was found in five cases (samples 24, 41, 49, 146, and 148). In sample 149, the detected mutation falls on the ligation point of one of the MLPA probes resulting in clear underrepresentation. Sample 128 showed relative 3p loss by MPLA without corresponding LOH, which may be due to the masking effects of normal DNA or may represent two copies of 3p in a triploid cellular background. Sample 33 is subtriploid and showed probable loss of two identical regions in 3p, suggesting that chromosome duplication followed the deletion event. In contrast, sample 1 contained three copies of 3p with two nonidentical deletions, so that in this case, at least one deletion event followed duplication. Sample 136 with three copies of parts of 3p, two copies of other parts, and for some probes, just one copy, may be explained by a deletion and nonreciprocal translocation with a breakpoint within exon 1 of VHL. Sample 132 shows a >3-fold duplication of a small region of exon 1 which can't be explained by simple translocations and at least two breakpoints are likely to be involved. Importantly, the changes detected by MLPA in samples 128 and 132 were found in samples which otherwise would have been assessed as having no disruption of the *VHL* gene.

Promoter methylation was shown in 19 of 93 (20.4%) sporadic cRCC samples examined and in one of three TCC kidney, four of eight papillary RCC, and one of two unclassified RCC samples. LOH or mutation were present in all but one of the methylated cRCC cases. Of the 75 cRCC tumors with LOH, 61 also had a confirmed mutation of which 11 were also positive for methylation. Therefore, of the 93 samples for which sufficient information was available, 69 (74.2%) showed evidence of biallelic inactivation of *VHL*. A total of 10 samples (11.5%) of the 87 for whom LOH, methylation analysis, and confirmed mutation status was available showed no evidence of any alteration of the *VHL* gene, although not all of these were examined by MLPA to rule out intragenic deletions.

No significant differences were seen between the presence/absence of methylation, LOH, mutation, or potential biallelic inactivation when examined with regard to age, grade, stage (pTNM individual categories or groupings), maximum tumor diameter, or the presence of microvascular invasion. Possible associations between methylation and gender (15 of 19 methylated samples occurred in male patients, i.e., 27.3% of male patients compared with 10.5% of female patients), mutation and gender (31 of 39 female patients had a mutation compared with 33 of 53 males), and methylation and the A>G SNP at nucleotide 520 were seen (14 of 19 methylated cases being associated with the SNP), although just

failing to reach statistical significance (P = 0.067, P = 0.108, and P = 0.119, respectively). LOH and grade were significantly related (P = 0.008) with only 13 of 24 grade 4 tumors showing LOH compared with 30 of 34 grade 3, 28 of 32 grade 2, and 4 of 6 grade 1. Of the 13 grade 4 tumors with LOH, 11 also had a mutation whereas mutations were seen in only 5 of the 11 grade 4 tumors with no LOH.

VHL mutation status in terms of either location or type of mutation (truncation, missense or none) was not significantly associated with clinical variables. This was also the case for mutations grouped into "loss of function" versus "wild-type" (defined as truncating mutations versus missense/silent/no mutation corresponding to earlier studies; refs. 25, 32). However, if the mutations most likely to result in truncations, i.e., the frameshifts, nonsense, and splice mutations (n = 48) were grouped by location of clusters with three groupings in the β domain (1, codons 60-83; 2, 84-122; and 3, 123-156) and the remaining group in the α domain (4, codons 157-213), 6 of 7 of the group 4 samples had microvascular invasion compared with 4 of 13 for group 3, 5 of 13 for group 2, and 5 of 15 for group 1, approaching statistical significance (P = 0.0990).

Conventional prognostic factors such as stage and grade showed a significant relationship with cancer-specific survival (Fig. 4*A* and *B*) but none of the VHL variables examined showed a significant association with cancer-specific survival (Fig. 4*C-F*). The same was true for relapse-free survival (data not shown). Similarly, there was no significant relationship between mutation or methylation and survival in stage I to IV or I to III patients (Fig. 4*G-H*).

Discussion

From our comprehensive analysis of *VHL* alterations, potential biallelic inactivation was found in almost three-quarters of the sporadic cRCC patients. Intragenic deletions or rearrangements are often missed in mutation screening, and using Southern blotting, a recent study found no *VHL* gene rearrangements in 189 cases of sporadic cRCC (26). Our study describes for the first time the use of MLPA for the analysis of intragenic *VHL* gene deletions, detecting the involvement of VHL in several cases which otherwise seemed uninvolved, as well as detecting gene duplications. MLPA results

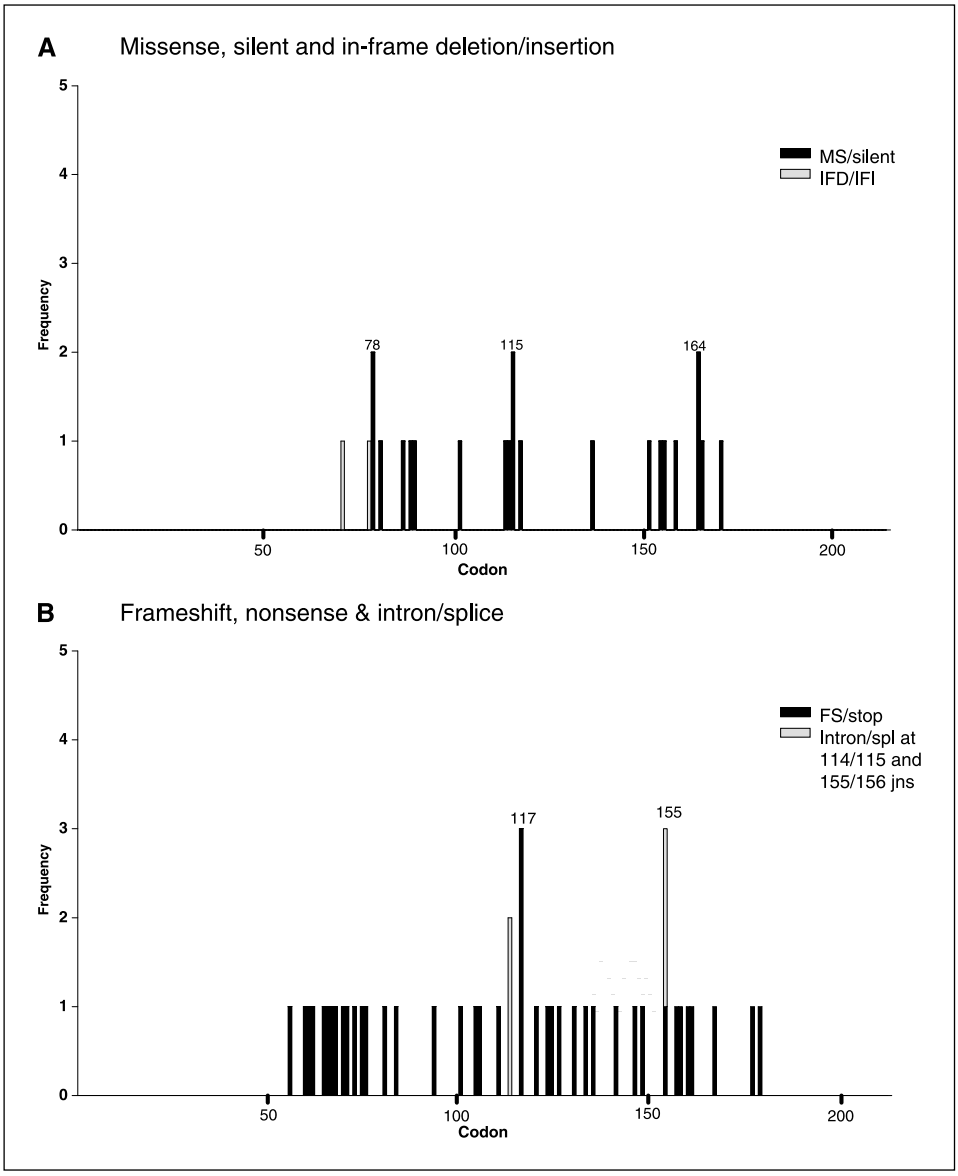

Figure 2. VHL mutation spectrum in sporadic cRCC related to the different regions of the gene and protein with potential binding sites shown. *A* and *B*, mutations found in our study (n = 67).

overlap with those of LOH analysis but this depends on the region of deletion and LOH caused by somatic recombination would not result in copy number change.

Studies examining VHL methylation in sporadic cRCC report rates of 5% to 15% (16, 26, 32, 37–39), although not all promoter-specific, with our finding of 20.4% extending this range. The trend towards increased methylation rates in male patients, also seen in a previous study (39), might be linked to factors such as age, alcohol consumption, and smoking but may have clinical relevance as has been described for estrogen receptor-α promoter hypermethylation in patients with lung cancer (40). The gender-specific trends in mutation and methylation, and the possible association between methylation and the presence of the SNP in the 5′ region of the gene (nucleotide 520) requires further confirmation but may indicate different influences on different *VHL* inactivation mechanisms.

Many of the mutations are novel. Our finding of 71% of sporadic cRCC having *VHL* mutations is higher than the 33% to 69% previously reported (6–32, 34), probably reflecting the techniques used. Examination of our data and published data reveals clusters in sporadic cRCC cases (e.g., codons 65-90), which are only apparent when large numbers of cases are examined. The absence of events at codon 81 also illustrates the specificity of the association between trichloroethylene exposure and such mutations (41). A single mutation involving a thymidine repeat region in exon 2 affecting codons 147 to 148 was found in our study, although previously

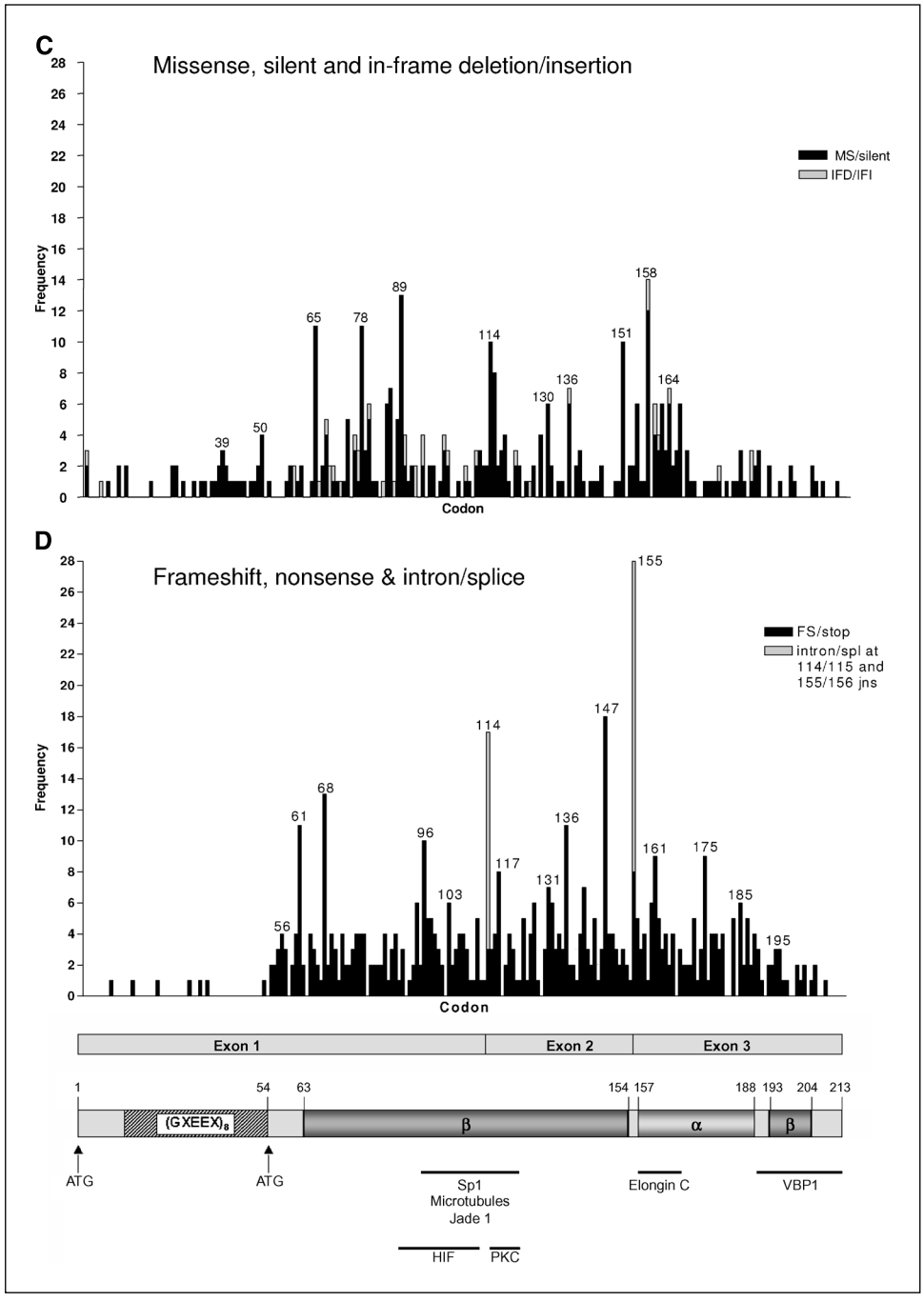

Figure 2. *Continued. C* and *D,* composite analysis of these and results from other studies (n = 884). The studies from which the data has been extracted (refs. 6–34) have all been checked to ensure that data published in multiple reports are not duplicated with some publications omitted for this reason (1, 35, 48–50). Gene and protein locations have been renumbered where necessary according to GenBank sequence: AF010238 for genomic *VHL* and L15409 for mRNA, with A of the first initiator ATG being 1 and codon numbers in accordance with L15409. Only two studies (6, 17) include cell lines, said to accord with tissue where examined. Data presented is only that from conventional (clear cell) RCC cases with the exception of one study (7) in which samples were included although not specifically indicated as being clear cell. Results from patients with known exposure to trichloroethylene (41) or patients with end-stage renal disease following dialysis have been excluded, similar to the 15 nucleotide deletions reported in six cases (19), which, on the basis of initial evidence, may be polymorphic.

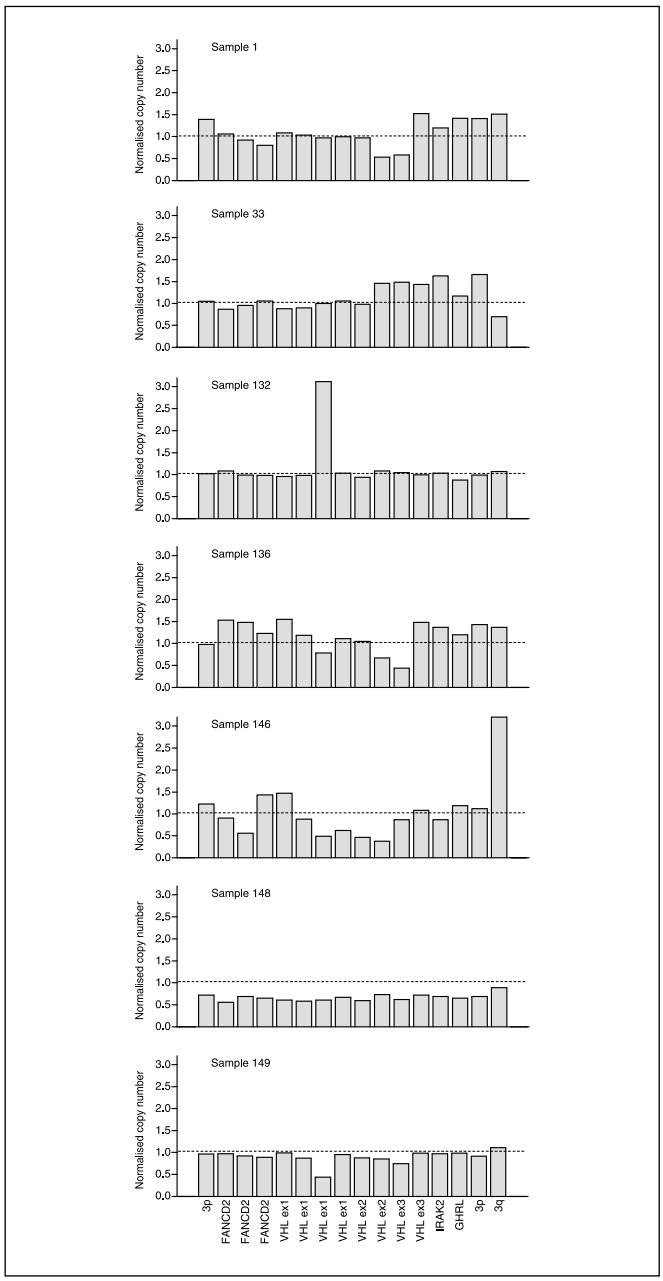

Figure 3. MLPA analysis showing histograms of relative probe intensities for seven samples, with probes ordered by position on chromosome 3.

reported in 9 of 77 cRCC cases (16), but the composite analysis shows an additional 7 cases confirming the hotspot, with environmental or geographic influences possibly affecting frequency.

We found no mutations affecting the first 53 amino acids of the VHL protein, a region potentially involved in interacting with fibronectin and recently reported to mediate tumor suppression via phosphorylation-dependent effects (42). When the composite data is reviewed, 50 mutations affect codons 1 to 54, although predominantly in studies using paraffin-embedded tissue (44 of 50) with three studies accounting for 41 of the cases (19, 25, 34). Formalin-fixed paraffin-embedded material and lower amounts of template are associated with increasing artifacts in PCR analyses (43, 44). However, biological and geographic variation may be involved as not all *VHL* studies using paraffin-embedded tissue found mutations in this region, the frequency of mutations overall in these studies is not anomalously high (although higher frequencies of multiple mutations were often seen) and many results were reproducible.

Many of these mutations potentially result in truncated or alternatively spliced proteins if translated and stable, and a recent familial study found predominantly germ line mutations leading to truncations or large rearrangement to be associated with susceptibility to renal lesions or RCC (4), although mainly deletions rather than frameshifts. The pattern of missense mutations found in sporadic cRCC reviewed here differs markedly from the 10 most common mutations in familial VHL (45), with even the most common mutation seen in type 2B disease (R167W) occurring relatively infrequently. Missense mutations may exert effects on specific protein-protein interactions (Fig. 2) such as the common familial mutations at residues interacting with HIF-1α or elongin B, whereas mutations in the hydrophobic core or buried polar residues may affect structural integrity. A recent familial study found missense mutation cluster regions associated with risk of renal lesions to affect codons 74 to 90 and 130 to 136, with the former, but not the latter, conferring genetic susceptibility to the development of RCC (4) and affecting the sporadic cases reviewed here. Familial missense mutations affecting codons 65, 128, and 167 predicted to result in loss of stability have also been associated with cRCC (5). Potentially, mutations may effectively give rise to dominant negatives, as illustrated by the VHL homologue, VHL-like protein, which contains a β domain only and therefore binds HIF and essentially protects it (46).

Few studies have examined the clinical significance of VHL alterations. The significantly lower rate of LOH in grade 4 tumors, most of which also had no detectable mutation or methylation, may in part reflect higher levels of contaminating normal DNA from lymphocytic infiltrates or there may be a subgroup of non-VHL involved tumors with a tendency to higher grade. We found mutations only in cRCC, and in common with most other studies, we found no significant association between *VHL* mutation or methylation and clinical variables (12, 24–26 34). In particular, we did not confirm the increased frequency of *VHL* mutation/hypermethylation in pT3 (non–organ-confined) tumors (16) but we did confirm the presence of mutations in even the smallest of tumors (26) which together with our approximately equal frequencies of alterations between pT1 and 2 versus pT3 groups, supports the hypothesis that the *VHL* gene is involved as an early event in RCC tumorigenesis.

Poorer survival has been associated with loss of function events (frameshift or nonsense mutations) compared with wild-type, missense mutations, and mutations of unknown biological consequence (25, 32) although numbers of cases in the LOF group were very small. We found no prognostic effect of *VHL* mutation but a trend (although not quite statistically significant) was seen for increased frequency of microvascular invasion in tumors where the presumed deletion would occur essentially from the elongin binding region onwards, possibly indicating a role for either HIF-regulated factors or other VHL substrates in this aspect of tumorigenesis. The largest previous study also found no relationship between clinicopathologic factors and VHL mutation/LOH in cRCC (26, 35). In marked contrast, however, is that 0 of 11 cRCC cases with methylation had an intragenic mutation whereas we found 14 of 19 methylated cases to have a confirmed mutation,

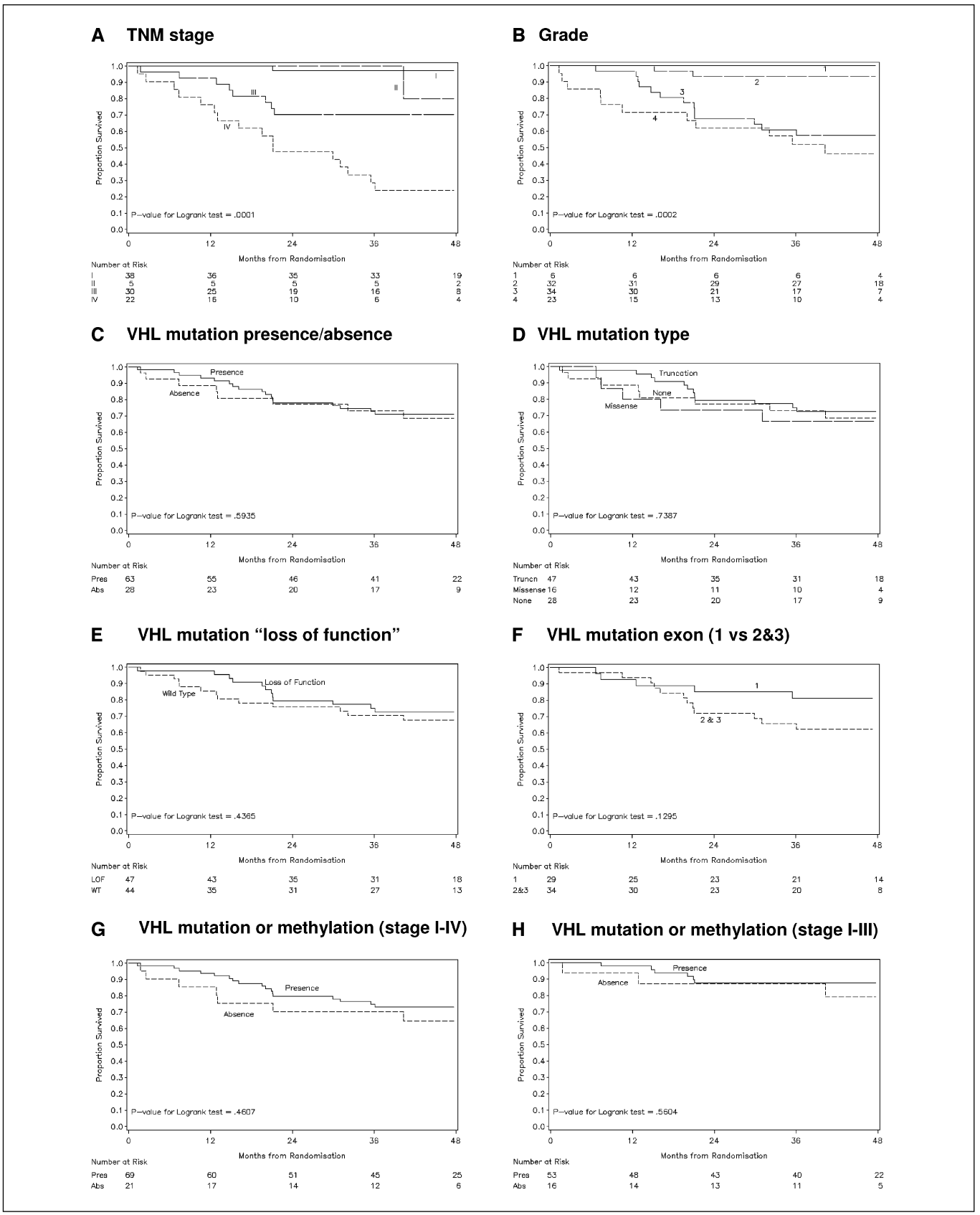

Figure 4. Overall survival curves for cRCC patients based on stage, grade, and VHL alterations as indicated. The numbers at the bottom of each figure include the censored data.

although their mutation and methylation frequencies overall were much lower (51% and 5.4%, respectively). However, a significantly better cancer-free and overall survival was seen in patients with stage I to III cancer with *VHL* alteration (mutation or methylation) but wasn't seen in stage IV disease (35). Our data fails to confirm this although when stage IV patients were included a similar trend was seen. Differences between studies might reflect samples sizes, length of follow-up, and postoperative treatment.

The involvement of VHL in cRCC is complex and warrants further large studies to clarify the potential clinical implications. This may aid prognosis, define subgroups of patients for specific targeted therapies, and patients where other genetic pathways may be involved. Given the uncertainty of the effects of mutations or methylation and whether single mutation events alone could result in haploinsufficiency, functional insight should be sought from linking such results with analysis of the resultant form(s), level and subcellular location of VHL protein and downstream pathways.

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