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ORIGINAL ARTICLE

Mutational status of VHL gene and its clinical importance in renal clear cell carcinoma

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Abstract The most common subtype of renal cell carcinoma is the clear cell type (ccRCC), accounting for 75 % of cases. Inactivation of *VHL* gene is thought to be an early event in ccRCC carcinogenesis. Our intention was to assess whether *VHL* mutational status might provide useful predictive or prognostic information in patients with ccRCC. *VHL* messenger RNA (mRNA) expression was analyzed by in situ hybridization and its protein by immunohistochemistry on a tissue microarray containing samples from 148 cases. This was validated by qRT-PCR on 62 cases, for which RNA was available. The mutation status was assessed in 91 cases by Sanger sequencing. *VHL* was found mutated in 57 % of cases, with missense mutations in 26 %, nonsense in 5 %, splice site in 13 %, deletions in 39 %, indels in 8 %, duplications in 8 %, and insertions in 2 % of the cases. The prevalence of mutations by exon was the following: exon 1, 47 %; exon 2, 27 %; and exon 3, 13 %. *VHL* protein was expressed in a high number of cases (80 %), but significant correlations were not found between protein expression, clinical data, and survival. Importantly, of the 91 samples evaluated by sequencing, 45 were mutated, and 87 % of those were strongly positive. We found 32 novel mutations in the *VHL* gene in ccRCC. The presence of mutations was not concordant with mRNA or protein expression. Nonsense mutations of the *VHL* gene appear to be related with poorer prognosis and survival.

Keywords Renal carcinoma · Clear cell · Prognosis · Mutational status · VHL

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Abbreviations

ccRCC	Clear cel renal carcinoma
Cul2	Cullin 2
FFPE	Formalin-fixed paraffin-embedded
HGMD	The Human Gene Mutation Database
HIF1α	Hypoxia-inducible factor 1-alpha
IHC	Immunohistochemistry
ISH	In situ hybridization
Rbx-1	Ring-box protein 1
rcc	Renal cell carcinoma
RT-PCR	Reverse transcription polymerase chain reaction
SAME	Department of Medical Records and Statistics
TNM	Classification of malignant Tumors
VHL	Von Hippel–Lindau

Introduction

The most common subtype of renal cell carcinoma (RCC) is the clear cell type (ccRCC), accounting for 75 % of all RCC cases [1]. The most frequent somatic mutation in ccRCC is loss of 3p, in particular the von Hippel–Lindau (VHL) tumor suppressor gene at 3p25, in combination with point mutations, genomic loss, or hypermethylation of the second *VHL* allele. Inactivating mutations of *VHL* are the main cause of sporadic ccRCC and familial von Hippel–Lindau disease-associated ccRCC. However, mutations of the *VHL* gene are also associated with an increased frequency of tumors in other organs such as the central nervous system, retina, or adrenal gland [2, 3].

The product of the *VHL* gene, pVHL, is a protein with multiple functions that has been implicated in a variety of cellular processes, including cell cycle regulation, extracellular matrix assembly, cytoskeleton stability, maintenance of the primary cilium, control of microtubule dynamics, regulation of neuronal apoptosis, and transcriptional regulation [4, 5]. These processes are much less thoroughly characterized than those involving the hypoxia-inducible factor 1-alpha (HIF1α) [6]. pVHL acts as a substrate recognition component of an E3 ubiquitin ligase complex [4, 6, 7] that polyubiquitylates the catalytic α-subunit of HIF, which results in constitutive activation of hypoxic responses [8, 9]. These are caused by deregulation of HIF-α subunits and are crucial for transcriptional activation of hypoxia-inducible genes that encode angiogenic and growth factors [10–12]. A previous study has demonstrated that specific *VHL* mutations are associated with clinical characteristics in a large majority of ccRCC cases [13], indicating that *VHL* may represent a sensitive biomarker of ccRCC. Conceivably, particular *VHL* defects result in loss of different *VHL* functions, in turn responsible for specific clinical phenotypes [14]. While inactivation of the *VHL* tumor suppressor gene clearly has an important role in the development of ccRCC, the question is whether *VHL* mutational status—the presence or absence of a mutation and when present its location, type, and effects on the resulting protein structure—could provide useful predictive or prognostic information in patients with ccRCC.

Currently, prediction of patient survival is based on traditional, clinical, and pathological parameters, including tumor size and Fuhrman nuclear grade. Since inactivation of the *VHL* gene is thought to be an early event in ccRCC initiation, we evaluated the presence of *VHL* somatic mutations in a large set of ccRCC samples and correlated the observed mutation patterns with VHL protein expression by immunohistochemistry (IHC), level of messenger RNA (mRNA) expression, and clinical data.

Material and methods

Study population

We selected 148 patients with clinically diagnosed sporadic ccRCC from the A. C. Camargo Cancer Center registry. All

Table 1 Demographics and clinicopathological data of the 148 patients with conventional (clear cell) renal cell carcinoma

Characteristic	*N* (%)
Sex	
Female	59 (39.9)
Male	89 (60.1)
Age (median)	56 (range)
Grade (Fuhrman)	
1	23 (15.5)
2	80 (54.1)
3	30 (20.3)
4	13 (8.8)
Stage tumor (T)	
T1a	31 (20.9)
T1b	37 (25.0)
T2[a]	16 (10.8)
T2b	11 (7.4)
T3[a]	36 (24.3)
T3b	1 (0.7)
T4	11 (7.4)
Node (N)	
X	60 (40.5)
0	72 (48.6)
1	4 (2.7)
2	7 (4.7)
Metastasis (M)	
X	1 (0.7)
0	129 (87.2
1	18 (12.2)
Stage	
I	79 (53.4)
II	15 (10.1)
III	32 (21.6)
IV	18 (12.2)

X, the presence of distant metastasis/lymphnodes cannot be assessed

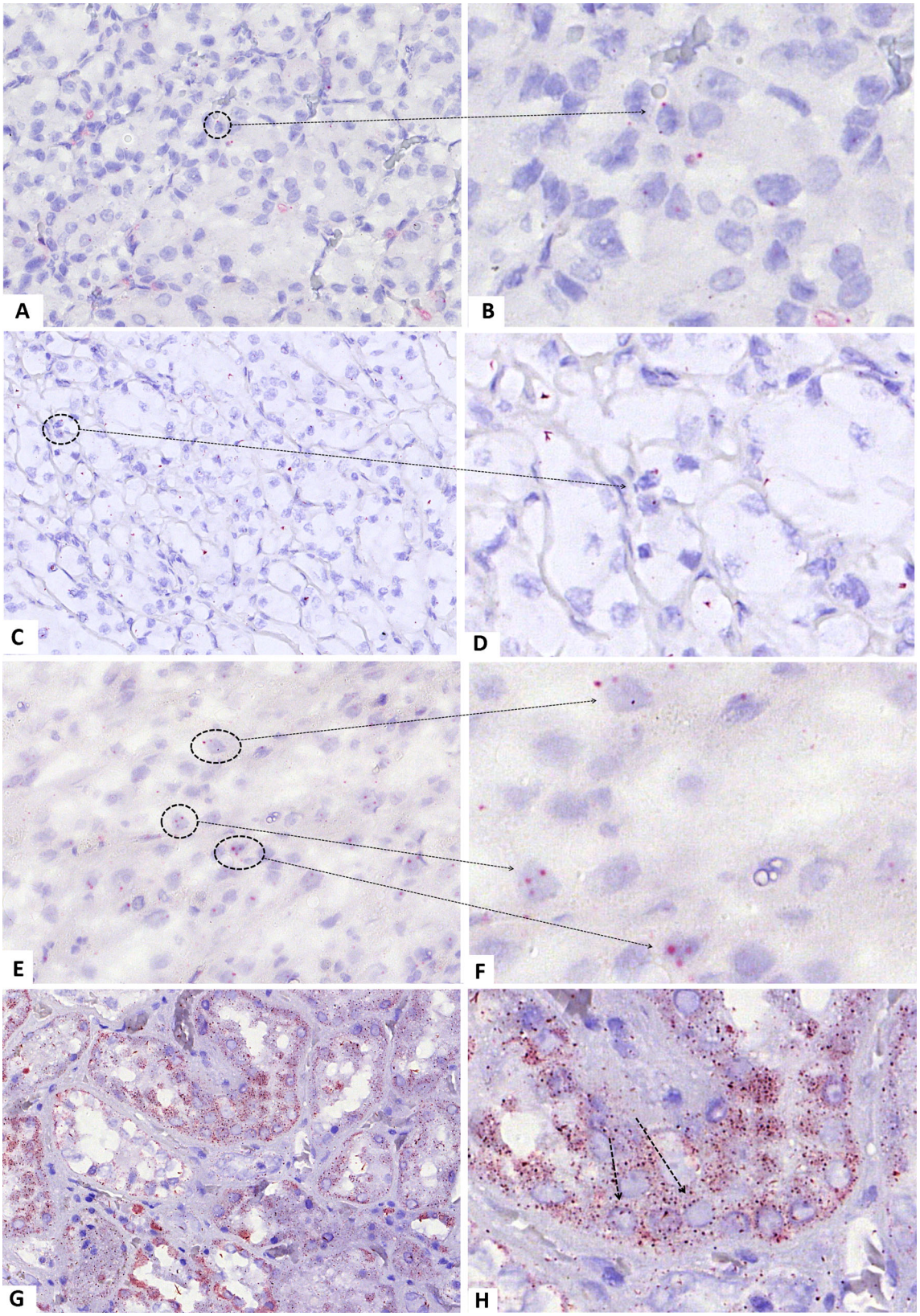

Fig. 1 RNA-ISH analysis of *VHL* gene expression in clear cell renal carcinoma. **a**–**f** paired images of different tumors showing dots and their cellular localization indicated by *arrows* (magnification×40 and×100, respectively). **g**, **h** Images of the same normal renal tissue at×40 and×100, respectively, with dots indicated by *arrows*

clinical data for this retrospective study were obtained from the Department of Medical Records and Statistics (SAME). This study was approved by the Ethics Committee of this institution.

Tumor characteristics

All tumor samples were from primary tumors treated with intended curative surgery in the Department of Urology Division of Pelvic Surgery, between 2002 and 2011. Frozen tissue and formalin-fixed paraffin-embedded (FFPE) samples were selected from A. C. Camargo Cancer Center Biobank. FFPE samples were microarrayed for subsequent pVHL immunohistochemical analysis and RNA in situ hybridization. Frozen tissue was retrieved to extract DNA for capillary sequencing and RNA for gene expression analysis. Clinicopathological and demographical data, including age at diagnosis, gender, tumor staging, grading, and presence of metastasis, are summarized in Table 1.

Pathological analysis

FFPE sections were stained with hematoxylin and eosin for light microscopy. Macroscopic and histological parameters analyzed included the following: tumor size, tumor necrosis, and nuclear Fuhrman grade, according to Fuhrman et al. [15] and Rioux-Leclercq et al. [16] and tumor stage according to the TNM AJCC/UICC 2009 classification [17].

Immunohistochemistry

IHC was performed using a Ventana Benchmark XT (Ventana Medical Systems Inc., Tucson, AZ) and a monoclonal anti-*VHL* antibody, (diluted 1:1,000) obtained from LifeSpan BioSciences (Seattle, WA, USA). Normal kidney was used as positive control and primary antibody omission as negative control. A semiquantitative H score was obtained by visual assessment of the staining intensity and the percentage of positively stained cells [18, 19].

RNA in situ hybridization

We assessed mRNA expression in individual cells using RNAscop (Advanced Cell Diagnostics, Inc., Hayward, CA, USA), a novel RNA ISH technology with a unique probe design strategy that allows simultaneous signal amplification and background suppression to achieve single-molecule visualization while preserving tissue morphology (Fig. 1). Assays using archival FFPE specimens were performed in parallel with positive and negative controls in order to ensure interpretable results. An endogenous housekeeping gene was used as positive control to assess both tissue mRNA integrity and assay procedure. Negative control was used to assess background signals. The staining results were evaluated examining tissue sections under a standard bright field microscope at× 20–40 magnification. Each dot was counted in each cell until 100 cells were counted. The sum of all dots found in this area was then used for further analysis. Subsequently, all the results from all cases were used to obtain a median of expression that was used as cutoff point to segregate low-expression cases (<15 dots/100 cells) and high expression (>15 dots/100 cells).

VHL mutation analysis

Genomic DNA from tumor samples was extracted using Precellys® Tissue homogenizer. Primers were designed to amplify the entire coding sequence and exon–intron boundaries of the *VHL* gene. Primer sequences were exon 1F-GAAGACTA CGGAGGTCGA; exon 1R-GATGTGTCCTGCCTCAAG; exon 2F GTCTTGATCTCCTGACCTCATG; exon 2R CATA ACGTACAAATACATCACTTCC; exon 3F CATCAGTAGT ACAGGTAGTTG; exon 3R CACAATGCCTAGTGAAGT CAG. PCR reactions were performed using the GoTaq Green Master Mix (Promega, Madison, WI, USA) according to the manufacturer's instructions. All PCR products were treated with ExoSAP-IT (USB Corporation, Cleveland, OH, USA) and sequenced in both directions using the ABI 3130xl Genetic

Table 2 Subtypes of VHL gene mutations observed among 91 histologically confirmed ccRCCs

VHL status			Genomic change			Type			Locartion		
	n	%		*n*	%		*n*	%		*n*	%
Mutation	52	57.1	Frameshift	30	48.4	Insertions	1	1.6	Exon 1	29	46.7
No mutation	39	42.9	Inframe	5	8.0	Deletions	24	38.8	Intron 1	4	6.5
			Missense	16	25.9	Duplications	5	8.0	Exon 2	17	27.4
			Nonsense	3	4.8	Substitutions	19	30.7	Intron 2	4	6.5
			Splicing	8	12.9	Indel	5	8.0	Exon 3	8	12.9
						Splice site	8	12.9			
			*Total of 62 events								

* It was observed 52 patients with mutations. However, some of these patients had more than one event, totaling 62 events.

Analyzer (Applied Biosystems). The sequences were compared with the *VHL* reference [refseq NM_000551] using the CLCBio Genomics Workbench software. All *VHL* alterations were searched in the HGMD (www.hgmd.org) and UMD (www.umd.be:2020) databases. Unreported mutations that generated a premature stop codon (nonsense and frameshift) were classified as pathogenic. Missense alterations were characterized using two in silico protein prediction algorithms: Sift (http://sift.jcvi.org/) and Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/) in order to predict the pathogenicity of the variants.

Real-time PCR (RT-PCR)

Total RNA was extracted from surgically removed tumors and ten adjacent noncancerous frozen tissue samples using RNeasy Mini Kit (QIAGEN, Austin, TX). First-strand cDNA was treated with TURBO DNA-free™ Kit (LIFE

Table 3 Univariate analisys for OS, CSS, and DFS in ccRCC patients

Variables	Overall survival		Cancer-specific survival		Disease-free survival	
	Mean survival		Mean survival		Mean survival	
Clinical stage	56.4	<0.001	58.7	<0.001	55.3	<0.001
(III/IV vs I/II)	37.2		37.2		37.3	
Tumor size	55.8	<0.001	57.6	<0.001	55.3	<0.001
(>7,0 cm vs <7,0 cm)	38.8		39.4		37.8	
Fuhrman	54.7	<0.001	56.2	<0.001	51.1	0.264
grade (III/IV vs I/II)	36.6		37.5		46.7	
Necrosis	56.4	<0.001	57.8	<0.001	51.5	0.433
	41.8		42.9		48	
Metastasis	53.8	<0.001	55.4	<0.001		-
	18.1		18.1			
Lymphatic vascular invasion	52.3	<0.001	53.8	<0.001	51.8	0.007
	25.3		25.3		30.4	
Microscopic vascular invasion	52.2	0.002	54	<0.001	52.5	0.001
	39.0		39		39.6	
Lymph nodes	52	<0.001	53.5	<0.001	51.1	0.085
	23.5		23.5		30.4	
Renal sinus fat invasion	52.7	0.001	54.4	<0.001	54	<0.001
	38.3		38.3		34.3	
Incidental Tumor	57.2	<0.001	57.2	0.001	52.2	0.223
	43.3		45.5		48.1	
Renal vein invasion	51.4	0.007	53	<0.001	51.5	0.011
	38.1		38.1		39.1	
Inferior cava vein invasion	50.1	0.015	51.5	0.005	50.4	0.619
	25		25		41.6	
pVHL	50.4	0.392	51.6	0.319	51	0.184
(Strong vs weak)	48.7		50.2		50.3	
ISH	51.4	0.609	85.1	0.652	49.9	0.855
	46.6		124.0		40.7	
Status VHL	53.6	0.911	53.6	0.911	53.2	0.405
(mutaded vs no mutated)	52.8		52.8		50.6	
Location (codon)	50.9	0.870	50.9	0.870	49	0.714
	51.7		51.7		55	
Loss of function	51.3	0.758	57.3	0.758	48.5	0.761
	53		53.8		51.8	
qRT-PCR	49.1	0.622	85.3	0.493	45.6	0.598
	49.8		61.8		50.8	

TECHNOLOGIES; Carlsbad, CA) to remove contaminating DNA from RNA preparations. Reverse transcription was performed using an 18-oligo(dT) primer and Superscript III reverse transcriptase Invitrogen, (Carlsbad, CA) using total RNA for 2 h at 42 °C. Quantitative RT-PCR was performed using Applied Biosystems 7500HT Fast Real-Time PCR System (Applied Biosystems). A set of primers and TaqMan probes for *VHL* were purchased from Applied Biosystems [Hs00184451_m1]. As positive control, we used a pool of normal kidney tissue. Sample omission was used as negative control. β-Actin and 18S housekeeping genes were used to normalize the reactions. The values of cycle threshold in the corresponding reference group were considered as normal. This relative comparative quantification method was applied to determine gene expression levels.

Statistical analysis

Analyses were performed using SPSS for Windows, version 17.0 (SPSS®, Chicago, IL). To assess the association between *VHL* expression and other variables, Pearson chi-square test was performed. Fisher's exact test was applied to cases in which the expected frequency was less than 5. The level of significance was 5 %. Comparisons between the degree of expression of *VHL* in non-neoplastic renal parenchyma and ccRCC tissue were made by Kruskal–Wallis test and Mann–Whitney test. Disease-specific survival was defined as the interval between primary surgery and death from disease. DSS and overall survival were examined using Kaplan–Meier curves. To compare the estimated curves for each category within a given variable, the log-rank test was used. The level of significance was 5 %. Cox proportional hazard model was used to determine which variables influenced survival. Variables that significantly impacted survival in univariate analysis were included in multivariate analyses. The confidence interval was 5 %.

Results

Point mutations

VHL gene was mutated in 57 % (52 of 91) of the cases. Double mutations were found in nine cases (17 %). A total of 19 tumors (31 %) had substitutions, of which 16 (26 %) were characterized

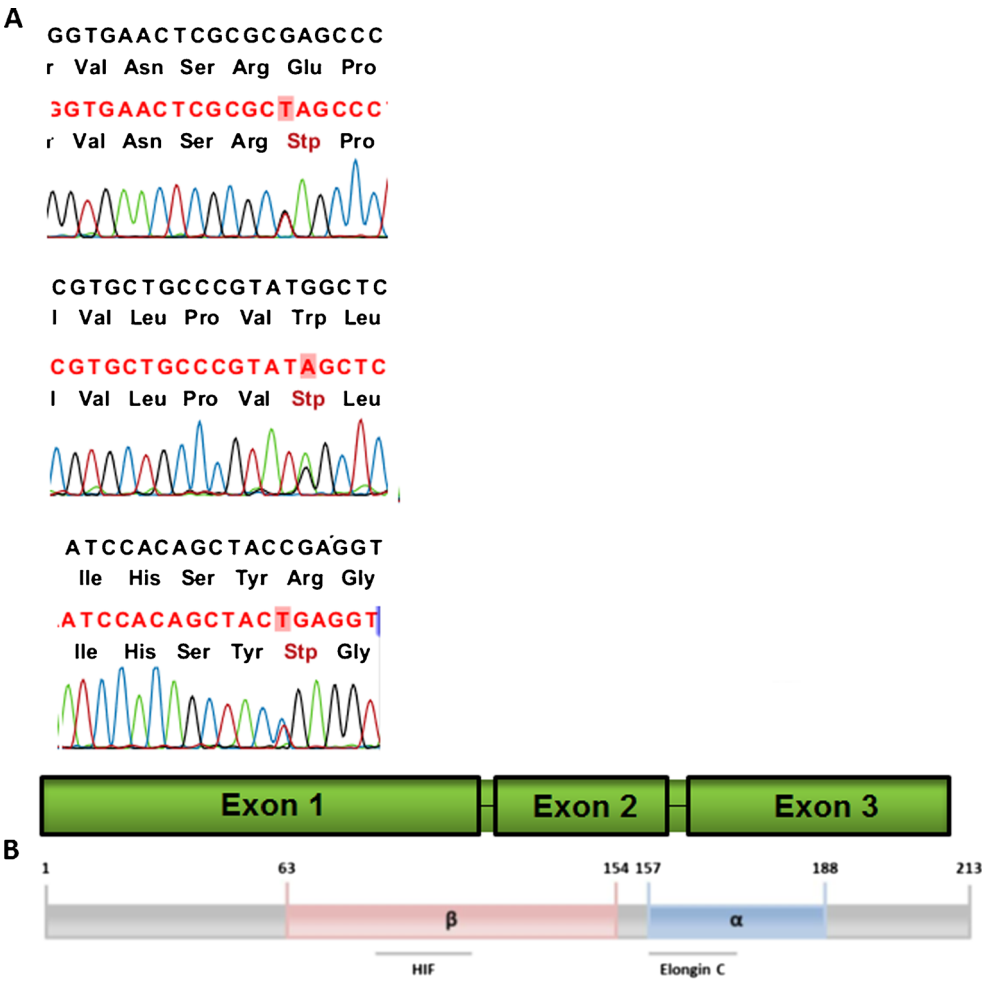

Fig. 2 **a** Electropherogram generated by analysis of three cases of a nonsense mutation located at exon 1. Alteration described in **a** c.208G>T, **b** c.263G>A, and **c** c.337C>T. **b** Schematic illustration of VHL domains

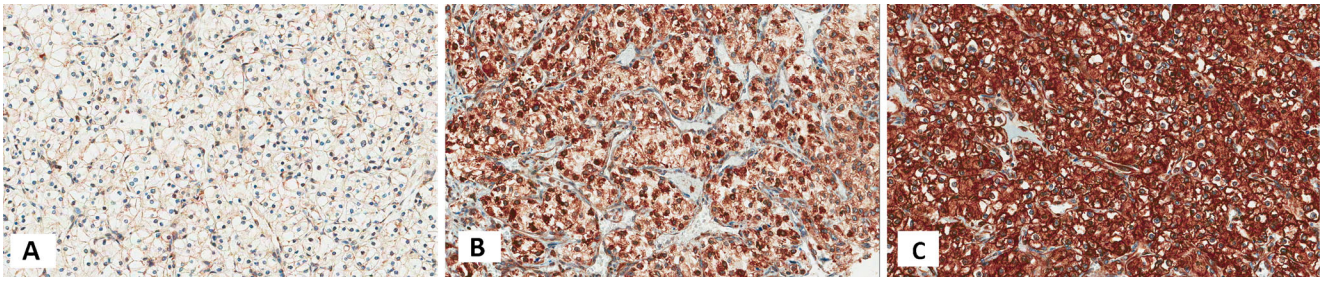

Fig. 3 IHC results of *VHL* protein expression in renal clear cell carcinoma. The *panel* shows the intensity of staining: **a** weak *VHL* staining, score 1; **b** moderate staining, score 2; **c** strong staining, score 3 (×20 magnification)

as missense mutation. Nonsense mutations were found in three cases (5 %). Splice site mutations were found in eight cases (13 %). The other 35 *VHL* mutations comprised deletions of 24 cases (39 %), small indels of 5 cases (8 %), small duplications of 5 cases (8 %), and small insertions of 1 case (2 %). The prevalence of mutations by exon was the following: exon 1, 28 mutations (47 %); exon 2, 16 mutations (27 %); and exon 3, 8 mutations (13 %). The results are summarized in Table 2.

All *VHL* alterations were searched in the HGMD and UMD database. The majority of the mutations (32 cases—52 %) described in this study was considered new according to these databases (Supplementary Table 1). Computational prediction analysis of 16 missense variants indicated all to be pathogenic in at least one of the algorithms. However, in nine cases (56 %), these mutations would likely be tolerated, while seven mutations (44 %) were shown to potentially affect VHL protein function (Supplementary Table 2).

Clinicopathological features versus mutation status

Associations between *VHL* mutational status and clinicopathological variables including sex, tumor grade, TNM stage, microvascular invasion, Furhman grade, and necrosis were examined. All these variables were significantly associated with cancer-specific, disease-free, and overall survival ($p<0.05$, Table 3 and Supplementary Table 3).

Point mutations versus clinicopathological features

The three cases with a nonsense mutation located at exon 1 (Fig. 2a) were strikingly different from all others. These cases presented poor prognosis and short survival ($p=0.007$). On the other hand, there was no significant association between the presence or absence of mutation, "wild-type" (silent or no mutation) versus mutations grouped into "loss of function" (truncating mutations) and cancer-specific or overall survival (Table 3).

The mutations were distributed throughout different exons, and they were grouped by different domains (α and β). The β-domain, localized between codons 63 and 154 (among exon 1 and exon 2), harbored 79 % ($n=48$), and the α-region, 13 % ($n=7$) of the mutations, all of them between codons 155 and 213 (Fig. 2b). No mutations were found between codons 1 and 63. The location of the mutations at the protein level was not significantly associated with cancer-specific, disease-free, or overall survival (Table 3).

Of the 52 mutated cases, 45 (87 %) showed immunoreactivity for VHL of which 7 cases (13 %) showed moderate or weak staining. Considering all samples included in the TMA, 6 (4 %) were classified as weak staining, 24 (17 %) as moderate staining, and 112 cases (79 %) as strong staining (Fig. 3) (Table 4). VHL immunostaining was not significantly associated with prognosis, cancer-specific, or overall survival (Table 4). Furthermore, we found no association between immunostaining and mutation status. IHC expression was higher in males than that in females ($p=0.029$).

Expression of mRNA analyzed by ISH evaluation showed a significant difference between tumor and nontumor tissue, the latter showing more dots than the former ($p<0.001$, Fig. 4). Expression of mRNA was corroborated by IHC results, as all cases with more than 15 dots by ISH were strongly positive by IHC ($p=0.008$). No statistical difference was observed between *VHL* expression in tumor samples and cancer-specific, disease-free, or overall survival (Table 3).

Table 4 IHC staining in a set of 148 FFPE samples and comparison with the pattern of *VHL* mutations (52 samples)

N (%)	H score 0–100	H score 101–200	H score 201–300
148 total cases	6 (4.2 %)	24 (16.9 %)	112 (78.9 %)
52 mutated case	2 (3.8 %)	5 (9.6 %)	45 (86.6 %)

H score obtained by summing the percentages of cell staining at each intensity: score 0–100 represents a weak staining, score 101–200 a moderate staining, and score 201–300 a strong staining

Quantitative real time PCR

Real-time quantitative PCR (qRT-PCR) was used to verify the expression of *VHL* gene. Non-neoplastic tissue from renal parenchyma was used for comparison with tumor samples. No statistically significant difference was observed between

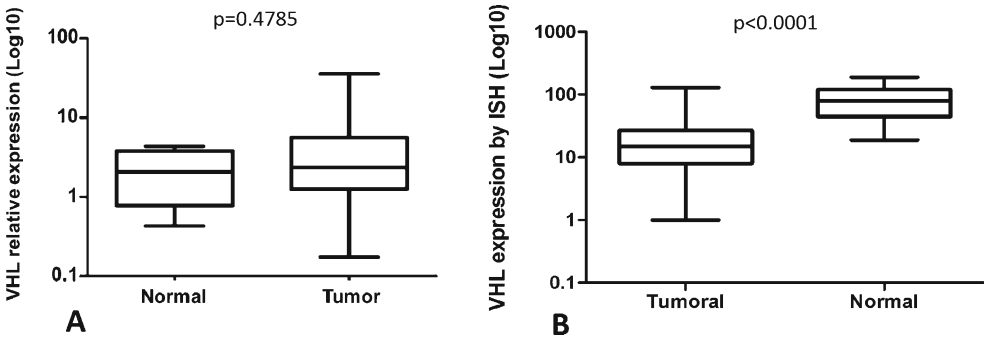

Fig. 4 Comparison of *VHL* expression between tumor and non-neoplastic samples. **a** relative *VHL* expression by qRT-PCR and **b** *VHL* expression determined by ISH

VHL expression in tumor samples and that in normal kidney samples (p=0.4785) (Fig. 4).

Discussion

Inactivation of *VHL* gene is considered as one of the initiating molecular events in ccRCC. The gene appears to play a critical role in the tumorigenesis of ccRCC. pVHL binds to elongin C, elongin B, Cul2, and Rbx-1 proteins, and this complex targets various proteins, including HIF1α for ubiquitin-dependent proteolysis [20, 21]. Consequently, pVHL controls hypoxia-inducible genes, including among others the genes encoding the vascular endothelial growth factors. Somatic mutation of the *VHL* gene is among the most frequent genetic alterations observed in ccRCC [22]. Therefore, the purposes of this study were to describe *VHL* gene mutations and evaluate their consequences in terms of mRNA and protein expression as well as their clinical significance in a set of 148 ccRCC.

Mutations that result in loss of protein function have been associated with poor prognosis when compared with missense mutations or wild type [10, 23, 24]. Nickerson et al. [13] found a relationship between the prevalence of particular subtypes of mutations, such as nonsense mutations (18 of 176 cases) and tumor grade, nodal status, and metastasis (p=0.01). We found the three cases with a nonsense mutation strikingly different from all others as they presented poorer prognosis and shorter survival (p=0.007). However, this result has to be cautiously interpreted in view of the very limited number of cases.

Young et al. [14] analyzed 177 samples of sporadic conventional RCC for somatic allele loss, mutation, and promoter methylation, in association with clinical and pathological variables. They found an association between mutation and gender, 84.6 % of women harboring mutations compared with 68.2 % in men (p=0.0189) [19]. We found a significant association (p=0.029) between IHC expression and gender with more frequent IHC staining in males (66.1 %) than in females (Supplementary Table 4). Banks et al. [25] also performed genetic and epigenetic analysis of *VHL* in 96 tissue samples of sporadic ccRCC and did not observe an association between different subtypes of mutations, clinical data, and survival rates. Our results corroborate these findings as mutation status or location of mutations did not correlate with overall or disease-free survival.

We found a high frequency of strong *VHL* protein expression by IHC (78.9 %). Crisp and strong cytoplasmic staining is considered a good indicator of specificity [26]. We found no

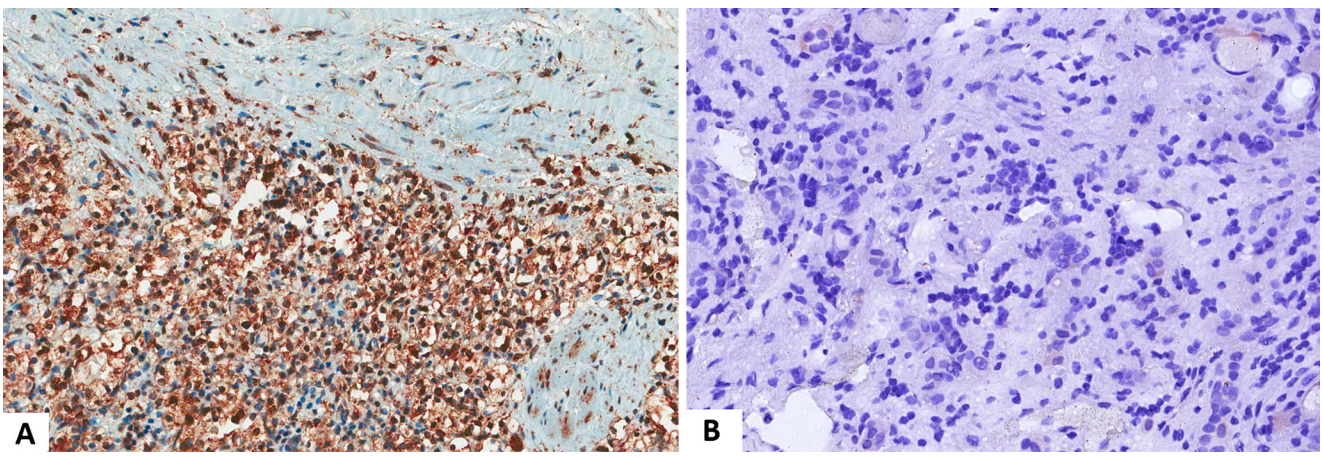

Fig. 5 Staining pattern of *VHL* mRNA and protein. **a** Specific cytoplasmic immunohistochemical staining of *VHL* protein by IHC (×20 magnification), **b** area of inflammation around tumor showing no ISH dots (×40 magnification)

immunoreactivity in stromal or inflammatory cells (Fig. 5a) and yet; protein expression did not correlate with clinical data or survival (p=0.392). Other authors [27] found an association between absence of pVHL expression and lower *N* stages (p=0.02), *M* stages (p=0.01), Fuhrman grades (p=0.001), and more favorable outcome (p=0.02). Among the 91 samples with *VHL* gene-sequencing results, we found 52 mutated, 45 of which (87 %) strongly positive by IHC. However, we found no difference in gene expression by qRT-PCR between mutated and nonmutated cases (p=0.5516).

We found no differences in gene expression or IHC staining between tumor samples and normal kidney. Results of qRT-PCR did not correlate with H score values. Methodological considerations should be introduced here. Positive *VHL* immunostaining might not necessarily indicate wild-type *VHL* status as anti-VHL antibodies may be able to bind epitopes in altered proteins with aberrant conformation and aberrant function. Furthermore, qRT-PCR might not distinguish between altered mRNAs or an effect of alternative splicing. Overall, IHC evaluation of VHL expression does not provide better understanding of the integrity of the protein or of its function. The ISH results did correlate well with those of IHC, both methods validating each other mutually. The staining pattern by ISH is illustrated in Fig. 5b.

One of the main issues in the molecular diagnosis of *VHL* gene is the effect of missense variants on protein function. Several computational approaches have been used to predict potentially deleterious mutations that affect protein function, such as Polyphen and Sift [28, 29]. Leonard et al. [30] suggested that missense mutations of the *VHL* gene, located in regions predicted to affect protein folding and coding for interactive protein domains, interfere with protein–protein interactions. However, this hypothesis was not validated using functional studies. The majority of the missense mutations we found were predicted to be pathogenic by Polyphen but not by Sift. Functional analysis will have to be performed to clarify this issue.

The suppressor function of *VHL* through its negative regulation of HIF is well established. According to Forman et al. [31], pVHL mutations might alter a protein residue crucial for protein–protein interactions or destabilize a protein domain, altering such interaction and leading to incorrect protein function. Therefore, it might not be the absence of pVHL, but rather the incapacity of the mutated protein to interact with other proteins that is responsible for its role in ccRCC development. Mutations occur in two different domains of *VHL*: the β-sheet domain that recognizes and binds with HIF and the α-helical domain that interacts with elongin B and elongin C. We found no significant associations between these mutation categories and clinicopathological variables.

Differences between previously reported somatic *VHL* mutations and our data might be caused by numerous factors, including the amount of non-neoplastic DNA in tumor samples, the method of mutation detection used, the different mutational stage, the cancer cells may be in, and also differences between populations. Gerlinger et al. [32] investigated phenotypic consequences of intratumoral genetic heterogeneity in ccRCC and observed that in terms of the *VHL* mutation status, ccRCC are not heterogeneous, which goes along with its purported role as a driver of ccRCC.

In summary, we report 32 novel mutations in the *VHL* gene that were not previously published, which enriches the database on this gene. The clinical significance of these mutations remains obscure, as the results of the analysis of gene mutation and mRNA and protein expression are not concordant. Nonsense mutations of the *VHL* gene seem to be important, as they appear to be related with poor prognosis and shorter survival. As the nonsense mutations were found in exon 1, we suggest that investigation of this gene region might contribute important prognostic information to clinical practice, in allowing the selection of patients for less conservative surgery or closer clinical follow-up.

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