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Different angiogenic potential in low and high grade sporadic clear cell renal cell carcinoma is not related to alterations in the von Hippel–Lindau gene

Marcella M. Baldewijns [a,*], Iris J.H. van Vlodrop [a], Kim M. Smits [b], Peter B. Vermeulen [c], Gert G. Van den Eynden [c], Fiona Schot [a], Tania Roskams [d], Hein van Poppel [e], Manon van Engeland [a] and Adriaan P. de Bruïne [a]

[a] *Department of Pathology, GROW-School for Oncology and Developmental Biology, Maastricht University, Maastricht, The Netherlands*
[b] *Department of Epidemiology, GROW-School for Oncology and Developmental Biology, Maastricht University, Maastricht, The Netherlands*
[c] *Translational Cancer Research Group, Lab Pathology University of Antwerp/University Hospital Antwerp, Edegem; Oncology Center, General Hospital St. Augustinus, Wilrijk, Belgium*
[d] *Department of Pathology, University Hospital of Leuven, Leuven, Belgium*
[e] *Department of Urology, University Hospital of Leuven, Leuven, Belgium*

Abstract. *Background*: *von Hippel–Lindau (VHL)* inactivation is common in sporadic clear cell renal cell carcinomas (ccRCC). pVHL is part of the ubiquitin ligase complex that targets the alpha subunits of hypoxia-inducible transcription factor (HIF) for degradation under well-oxygenated conditions. In the absence of wild-type pVHL, as observed in VHL patients and most sporadic ccRCCs, constitutive upregulation of HIF results in transcriptional activation of angiogenesis-related genes, such as VEGF. Differences in angiogenic activity within the group of ccRCCs were reported and strong genotype-phenotype correlations were found in patients with VHL disease, raising a question about the importance of *VHL* inactivation status in angiogenic behaviour and tumour progression.

Methods: To address this question, we investigated the influence of *VHL* mutation (direct sequencing)/hypermethylation (methylation-specific PCR) on angiogenesis/tumour parameters (immunohistochemistry) in 150 patients with sporadic ccRCC.

Results: We found no significant association between *VHL* mutation or methylation and angiogenesis/tumour parameters.

Conclusion: These data indicate that tumour progression and angiogenesis are not directly influenced by *VHL* alterations and that additional genetic/epigenetic events should be considered to explain the diverse angiogenic and proliferative behaviour during tumour progression.

Keywords: VHL, angiogenesis, renal cell carcinoma

Abbreviations

CAIX	carbonic anhydrase IX
CcRCC	clear cell renal cell carcinoma
ECP%	endothelial cell proliferation fraction
HIF	hypoxia-inducible transcription factor
MVD	microvessel density
RCC	renal cell carcinoma
TCP%	tumour cell proliferation fraction
VEGF	vascular endothelial growth factor
VHL	von Hippel–Lindau

*Corresponding author: M.M. Baldewijns, Department of Pathology, AZ Maastricht, P. Debyelaan 25, NL 6229 HX Maastricht, The Netherlands. Tel.: +31 433876612; Fax: +31 433876613; E-mail: m.baldewijns@mumc.nl.

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1. Introduction

The most widely observed genetic aberration in clear cell RCC (ccRCC) is loss of chromosome 3p, harbouring the *von Hippel–Lindau* (*VHL*) tumour suppressor gene, which is located on chromosome 3p25 and plays an important role in hereditary and sporadic ccRCC [1]. 50–75% of sporadic ccRCCs show biallelic *VHL* inactivation [1–3]. The *VHL* gene exists of three exons and *VHL* mutations are heterogeneous distributed throughout the coding sequence, except that intragenic missense mutations are rarely seen within the first 50 codons [4]. In 5–20 % of reported cases the *VHL* gene is silenced by promoter hypermethylation [5–8]. The best characterized function of pVHL is its role as a component of the ubiquitin E3 ligase complex that targets hypoxia inducible factor HIF-α for ubiquitin-dependent proteasome degradation [9, 10]. The α-subunits of HIF are rapidly degraded by the proteasome under normal conditions, but are stabilized by hypoxia [11]. In hypoxic cells and in RCC, lacking functional pVHL, HIF-α degradation is suppressed, leading to enhanced transcription of target genes, including genes involved in cell proliferation, apoptosis, extracellular matrix formation, energy metabolism and pro-angiogenic genes such as vascular endothelial growth factor (VEGF) contributing to the activation of the angiogenic switch [1,12–15]. Therefore it can be hypothesized that a difference in angiogenesis/hypoxia can be expected in ccRCC patients with versus without functional *VHL*.

Previously, we observed significant differences in angiogenesis and hypoxia parameters between low grade (Fuhrman grade 1–2) and high-grade (Fuhrman grade 3–4) ccRCC [16].

Since strong genotype–phenotype correlations were found in patients with VHL disease, whereby the tumour phenotype was influenced by the nature of *VHL* mutations [17], the question emerges whether *VHL* alterations can explain the different angiogenic phenotypes and different biological behaviour in sporadic ccRCC. Here we examine the influence of *VHL* alterations (mutation/promoter hypermethylation) on angiogenesis and hypoxia parameters in ccRCC and correlate these results with clinical outcome. To the best of our knowledge, this is the first study investigating the association between *VHL* alterations and active angiogenesis, reflected by endothelial cell proliferation measurement.

2. Material and methods

2.1. Patients and tissue samples

Tumour samples of 150 patients with sporadic ccRCC, treated with radical or partial nephrectomy without adjuvant treatment, have been collected retrospectively. The samples were derived from the archives of the Departments of Histopathology of the University Hospitals of Leuven and Maastricht. The original histological slides, stained with hematoxylin and eosin, were reviewed to confirm nuclear Fuhrman grading. All patients were evaluated post-operatively at regular intervals by means of physical examination, chest ray, abdominal computed tomography or ultrasound. When indicated, a bone scan was performed. The median follow-up time was 64 months (range 1–153 months) and mean follow-up of 9 years. The clinicopathological data of the study population are summarized in Table 1.

2.2. VHL mutation analysis

Tumour tissue was selected on the basis of haematoxylin and eosin stained sections to ensure a minimum of 80% tumour cells in the samples. Genomic DNA was extracted from 20 µm slides from each case. After

Table 1
Clinical and histopathological characteristics

	Clinicopathological data ($N = 150$)
Gender	
Male/Female	99/51 (66/34%)
Age[a] (years)	66.6 (± 12.1 s.d.)
Mean tumour diameter (cm)	6.36 (± 3.45 s.d.)
Fuhrman grade	
1	15 (10%)
2	72 (48%)
3	46 (30.7%)
4	17 (11.3%)
Tumour stage[a]	
I	82 (54.7%)
II	24 (16%)
III	42 (28%)
IV	2 (1.3%)
Lymph node status[a]	
1	4
2	11

[a]T and N status were assigned according to the TNM classification of the International Union Against cancer 2002.

dewaxing of the tumour slides in xylene and rehydration through sequential changes of alcohol, DNA was isolated and purified with Puregene® DNA purification kit (BIOzym, Landgraaf, The Netherlands). PCR primers used for amplification are available upon request. DNA was amplified using a (multiplex) semi-nested PCR approach. The first (multiplex) PCR was performed using 200 ng of the extracted DNA and subjected to 35 cycles of PCR: 30 s at 94°C, 30 s at 66°C and 30 s at 72°C. The second PCR with 4 µl diluted (1:100) PCR product was subjected to 35 cycles of PCR: 30 s at 94°C, 30 s at T_m and 30 s at 72°C. Exon amplification was performed in 25 µl (1 × PCR buffer, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 0.2 µl Primer (each), 1 unit Platinum® Taq DNA polymerase (Invitrogen™, Breda, The Netherlands) and template DNA). The products were analyzed by agarose gel electrophoresis and visualized by Gelstar® (Cambrex, New Jersey, USA).

For direct sequence analysis, the PCR products were purified using multiscreen HTS® 96 wells plate (Millipore, Billerica, USA) and collected in 50 µl sterile water. For the sequence reaction, 2 µl purified DNA, BigDye vl.1, sequence buffer and primers (3.2 pmol) were used. The PCR was subjected to 25 cycles of PCR: 10 s at 96°C, 5 s at 50°C and 4 min at 60°C. The sequence products were precipitated with ethanol in a 96 well plate and dissolved in 20 µl sterile water and measured with the ABI 3710 Genetic Analyzer. Mutations were identified by Mutation Surveyor DNA Variant Analysis Software version 3.0 (Soft Genetics LLC, USA) and checked manually. *VHL* mutation analysis succeeded in 127 of 150 tumour samples.

2.3. VHL promoter CpG island methylation analysis

DNA methylation in the CpG island of the *VHL* gene promoter was determined by chemical modification of genomic DNA with sodium bisulfite and subsequent methylation-specific PCR (MSP) as described in detail elsewhere [18,19]. Briefly, 0.5 µg of DNA was modified by sodium bisulfite using the EZ DNA methylation kit (Zymo Research) according to the manufacturer's instruction. To facilitate MSP analysis on DNA retrieved from formalin-fixed, paraffin embedded tissue, DNA was first amplified with flanking PCR primers that amplify bisulfite-modified DNA but do not preferentially amplify methylated or unmethylated DNA. The resulting fragment was used as a template for the VHL MSP reaction. Primer sequences and PCR conditions are available upon request.

All PCRs were performed with controls for unmethylated alleles (DNA from normal lymphocytes), methylated alleles (normal lymphocyte DNA treated *in vitro* with SssI methyltransferase (New England Biolabs)), and a control without DNA. Ten µl of each MSP reaction were directly loaded onto 2% agarose gels, stained with Gelstar (Cambrex), and visualized under UV illumination. MSP reactions succeeded in 134 of 150 tumour samples. To assess reproducibility, MSP reactions have been performed in duplicate starting from DNA amplification with flanking PCR primers. The reproducibility was 96%.

2.4. Assessment of angiogenesis and hypoxia parameters

Serial sections of 4 micron were made from the paraffin embedded kidney tumours. Methods for quantification of microvessel density (MVD), endothelial cell proliferation, tumour cell proliferation and assessment of HIF-1α, carbonic anhydrase IX (CAIX), VEGF expression are described more in detail elsewhere [16].

Briefly, by means of a CD34/Ki67 double-stain procedure [20] MVD, the fraction of proliferating endothelial cells and tumour cells were evaluated. One section was analyzed per tumour. For assessment of the vascular density within a tumour section, 1 hotspot (most vascularised microscopic field) was selected and 4 areas were chosen randomly. Vessel counts were done at 200× magnification using an optical grid. The presence of a vascular lumen was not necessary to identify a microvessel. MVD was expressed as vessels per mm^2. Since, the mean vessel density of the tumour gives an estimate of the net result of phases of angiogenesis and angioregression a tumour went through, we also assessed the fraction of proliferating endothelial cells, which is a better measure for active angiogenesis. A total number of approximately 500 intratumoural endothelial cells and 500 tumour cells were evaluated on consecutive fields at a 400× magnification and the fractions of proliferating endothelial cells (ECP%) and tumour cells (TCP%) were assessed. ECP% and TCP% were calculated according to the following formulas:

ECP% = (the number of endothelial cells with Ki67-stained nuclei/total number of endothelial cells evaluated) × 100;

TCP% = (the number of tumour cells with Ki67-stained nuclei/total number of tumour cells evaluated) × 100.

For CAIX immunohistochemical staining [20], a score of 0–3 for intensity of staining within the tumour was given (0: no staining, 1: weak staining, 2: moderate staining, 3: strong staining). The percentage of immunostained tumour cells was estimated. The product (intensity score $\times$ the percentage of immunoreactive tumour cells) yielded a final score of 0–300.

The HIF-1α immunohistochemical staining was carried out as described elsewhere [20]. For HIF-1α expression analysis, only tumour cells with completely and darkly stained nuclei were scored as positive. The fraction of HIF-1α-positive tumour cells was estimated.

Furthermore a VEGF immunohistochemical staining was performed [16] and VEGF expression was semi-quantitatively assessed according to a four point grading scale: 0 absence of tumour staining, 1 + membrane staining tumour cells, 2 + strong membrane staining and cytoplasmic staining of $<50\%$ of the tumour cells and 3 + strong cytoplasmic staining in $>50\%$ of all tumour cells. As VEGF is know to be a secreted protein, attention was also given to stromal expression. However, as ccRCC typically contains very sparse stroma, mainly existing of microvasculature in which the endothelial cells express VEGF, we did not include this in our final analysis, as the endothelial cell population is already represented in the MVD assessment by CD34 staining.

The assessments of all the higher mentioned immunohistochemical stainings were made by two independent observers (inter-observer variability $< 5\%$).

2.5. *Data analysis*

For statistical analysis, ccRCCs were first categorized according to presence or absence of loss of function mutations (defined as truncating mutations, predicted to alter the open reading frame, including nonsense mutations and frameshift mutations) on the one hand and presence or absence of *VHL* promoter methylation on the other hand. Secondly ccRCCs were subdivided into a group with no mutations or silent mutations, a second group with loss of function mutations and a third group with mutations of unknown biological significance (missense mutations without abrogating the *VHL* start codon, in-frame mutations). Thirdly, ccRCCs were categorized according to presence or absence of *VHL* changes, by which *VHL* change was defined as a loss of function mutation and/or *VHL* promoter methylation.

Since angiogenesis and hypoxia parameters were not normally distributed, non-parametric statistics were used to compare continuous variables (e.g. vessel density, endothelial or tumour cell proliferation, percentage of HIF-1α immunoreactive tumour cells, CAIX score, VEGF score) between tumours with different *VHL* mutation or methylation status. To compare two groups Mann–Whitney and more than two groups Kruskall–Wallis tests were used, respectively. For the association between non-continuous variables (e.g. Fuhrman grade, tumour stage) and *VHL* mutation or methylation status, a chi-square test was used. The above mentioned statistical analyses were performed using the SPSS 12.0. software package.

The survival analyses were performed by use of the statistical package STATA 10.0.

Cause-specific overall survival (OS) was defined as the time from nephrectomy until renal cancer-related death or until the end of follow-up. Metastatic-free survival (MFS) was defined as the time from nephrectomy until the occurrence of a metastasis or until the end of follow-up. Kaplan–Meier curves and log-rank tests were used to estimate the overall influence of *VHL* loss of function mutations, *VHL* promoter hypermethylation and *VHL* changes on OS and MFS. Hazard ratios (HRs) and corresponding 95%-confidence intervals (CI) were assessed by use of Cox proportional hazard models. Factors were considered possible confounders if they were known prognostic factors for renal cancer and influenced the crude HR. Confounders that were eventually included in the model were sex, age at diagnosis, cancer stage, tumour size (cm), Fuhrman grading, endothelial cell proliferation, tumour cell proliferation, HIF-1α, VEGF score and vessel density. CAIX score was evaluated as a possible confounder, however, was observed to have no influence, therefore CAIX score was not included in the model. As the number of stage IV cancers was small ($n = 2$), we decided to exclude these cases from the analyses that were adjusted for cancer stage. The proportional hazard assumption was tested using the Schoenfeld residuals.

In all statistical analyses all quoted p-values are two sided, and p-value $\leqslant 0.05$ was considered statistically significant.

3. Results

3.1. *VHL mutation and promoter CpG island methylation is mutual exclusive in ccRCC*

Eighty-seven of 127 ccRCCs (68%) showed *VHL* mutations. In the 87 *VHL* mutant ccRCCs, 108 muta-

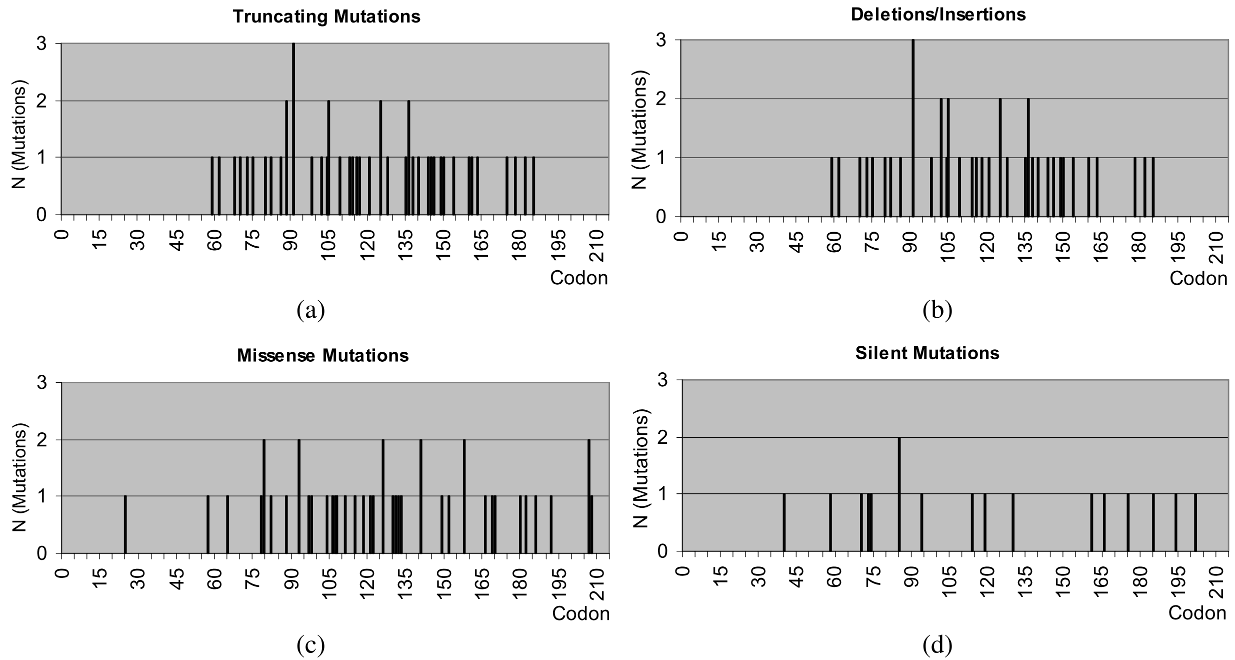

Fig. 1. Type of mutation plotted against the codon number. Codon 1–114 encodes exon 1, codon 114–155 encodes exon 2 and codon 155–213 encodes exon 3.

tions were observed. Seventeen ccRCC cases had two mutations and two ccRCC cases showed three mutations. Figure 1 shows the type of mutation plotted against the codon number. Except for one single nucleotide polymorphism at codon 25 and silent mutation at codon 40, no further mutations were seen within the first 57 codons.

Forty-three of 108 (39.8%) *VHL* mutations were missense mutations, 45/108 (41.7%) were truncating mutations (of which 38 frameshift and 7 nonsense mutations), 17/108 (15.7%) were silent mutations and 2 (1.9%) were in-frame deletions. Forty-seven of 108 (43.5%) mutations affected exon 1, 36/108 (33.3%) mutations were located within exon 2 and 25/108 (23.1%) mutations were detected within exon 3. Deletions were more common in exon 1 ($n = 47$) than exon 2 ($n = 36$) and exon 3 ($n = 25$).

In 11 of 134 (8%) ccRCCs methylation of the *VHL* promoter CpG island was present. In 38 of 134 ccRCCs (28%) mutations nor methylation of the VHL promoter CpG island was found. Both the frequencies of *VHL* mutations and *VHL* promoter methylation, as well as types of mutations are in accordance with ranges described in literature [1–3,5–7,21].

Table 2 shows that *VHL* mutation and *VHL* promoter methylation are almost mutually exclusive.

Table 2a

The presence of *VHL* loss of function mutations in relation to *VHL* promoter methylation

VHL mutation	VHL promoter methylation		
	Absent	Present	Total
0	38	8	46
1	44	1	45
Total	82	9	91

Notes: 0 = no mutation or silent mutation, 1 = loss of function mutation (frameshift, nonsense). Pearson $\chi^2 = 5.8732$; Pr = 0.015.

Table 2b

The presence of *VHL* mutations in relation to *VHL* promoter methylation

VHL mutation	VHL promoter methylation		
	Absent	Present	Total
0	38	8	46
1	44	1	45
2	34	2	36
Total	116	11	127

Notes: 0 = no mutation or silent mutation, 1 = loss of function mutation, 2 = mutation of unknown biological significance (missense mutations without abrogating the *VHL* start codon, in-frame mutations). Pearson $\chi^2 = 7.2287$; Pr = 0.027.

3.2. Immunohistochemical results

As reported previously [16], high-grade ccRCC showed a significant higher ECP% ($p = 0.049$), higher TCP% ($p = 0.009$), higher VEGF protein expression ($p < 0.001$), lower MVD ($p < 0.001$) and lower HIF-1α protein expression ($p = 0.002$) than low grade ccRCC. No significant differences were seen in CAIX expression between both groups. Furthermore, a strong positive correlation was found between ECP% and TCP% ($\rho = 0.82, p < 0.001$).

3.3. Alterations in the VHL gene do not influence tumour or angiogenesis/hypoxia parameters

No significant differences were observed between the presence/absence of *VHL* promoter methylation and/or mutations when examined with regard to Fuhrman grade, tumour stage, TCP%, angiogenesis and hypoxia related parameters (MVD, ECP%, VEGF protein expression, HIF-1α protein expression) (Table 3 and 4). The type of *VHL* mutation was not significantly associated with angiogenesis/hypoxia and clinical variables (Table 4). Only a significant correlation was present between CAIX expression and *VHL* change (Table 4).

3.4. Alterations in the VHL gene do not influence clinical outcome

The median OS was 64 months (range 1–153) and the median MFS was 56 months (range 0–153). Median OS in the patients with *VHL* loss of function mutations was 63 months, median OS in patients with *VHL* wildtype was 61.4 months. Median MFS in the patients with *VHL* loss of function mutations was 54 months, median MFS in patients with *VHL* wildtype was 56 months.

Kaplan–Meier curves showed no statistically significant difference in OS for *VHL* loss of function mutations (log-rank $p = 0.552$), *VHL* mutations (including loss of function mutations and mutations of unknown biological significance, log-rank $p = 0.489$) or *VHL* changes (log-rank $p = 0.424$) (Fig. 2(a), (c), (e)). For MFS no statistically significant differences were observed for *VHL* loss of function mutations (log-rank $p = 0.661$), *VHL* mutations (including loss of function mutations and mutations of unknown biological significance, log-rank $p = 0.758$) or *VHL* changes (log-rank $p = 0.400$) either (Fig. 2(b), (d), (f)).

Results from the age and sex adjusted Cox proportional hazard analyses did not show a statistically significant influence of *VHL* mutation (HR 1.18, 95%-CI 0.57–2.44), *VHL* promoter methylation (HR 0.89, 95%-CI 0.27–2.95) or *VHL* changes (HR 1.37, 95%-CI 0.64–2.96) on OS or MFS (*VHL* mutation HR 1.16, 95%-CI 0.59–2.25; *VHL* promoter methylation HR 1.00, 95%-CI 0.36–2.78; and *VHL* changes HR 1.33, 95%-CI 0.67–2.64). In the multivariate analyses, neither *VHL* mutations nor *VHL* promoter methylation had a statistically significant influence on OS (*VHL* mutation: HR 0.39, 95% CI 0.14–1.11; *VHL* promoter methylation: HR 0.44, 95%-CI 0.06–3.11; *VHL* changes: HR 0.42, 95%-CI 0.14–1.23) or on MFS (*VHL* mutation: HR 0.88, 95% CI 0.35–2.21; *VHL* promoter methylation: HR 1.29, 95%-CI 0.33–5.06; *VHL* changes: HR 1.21, 95%-CI 0.46–3.19).

Clinicopathologic parameters did have a significant influence on OS and MFS in the multivariate analyses. Mortality and metastasis risk was increased for patients with a higher cancer stage (OS stage II cancer HR 3.48, 95%-CI 1.14–10.63; OS stage III cancer HR 6.39, 95%-CI 2.49–16.39; MFS stage II cancer HR 2.84, 95%-CI 1.11–7.28; MFS stage III cancer HR 4.12, 95%-CI 1.83–9.24) and patients with higher tumour cell proliferation (increment of 1%: OS HR 1.10, 95%-CI 1.03–1.17; MFS HR 1.05, 95%-CI 0.99–1.12).

4. Discussion

The aim of our study was to investigate whether *VHL* mutation and methylation status can explain differences in angiogenic potential between low and high grade ccRCCs.

In agreement with most other studies [5,22–25] we found no significant relationship between *VHL* mutation (presence or type) or *VHL* promoter methylation and tumour parameters (grade, stage, tumour cell proliferation). In our previous study [16], we observed that high grade ccRCCs were characterized by a higher angiogenic activity than low grade ccRCCs. This difference cannot be explained by presence/absence of alterations in the *VHL* gene. No significant association could be found between *VHL* alterations and angiogenesis (ECP%, MVD,VEGF) or HIF-1α expression. Only a significant association was observed between presence of *VHL* change (VHL promoter methylation and/or *VHL* loss of function mutation) and CAIX expression. However with a p-value of 0.047, it is not a strong significant correlation and it can be questioned

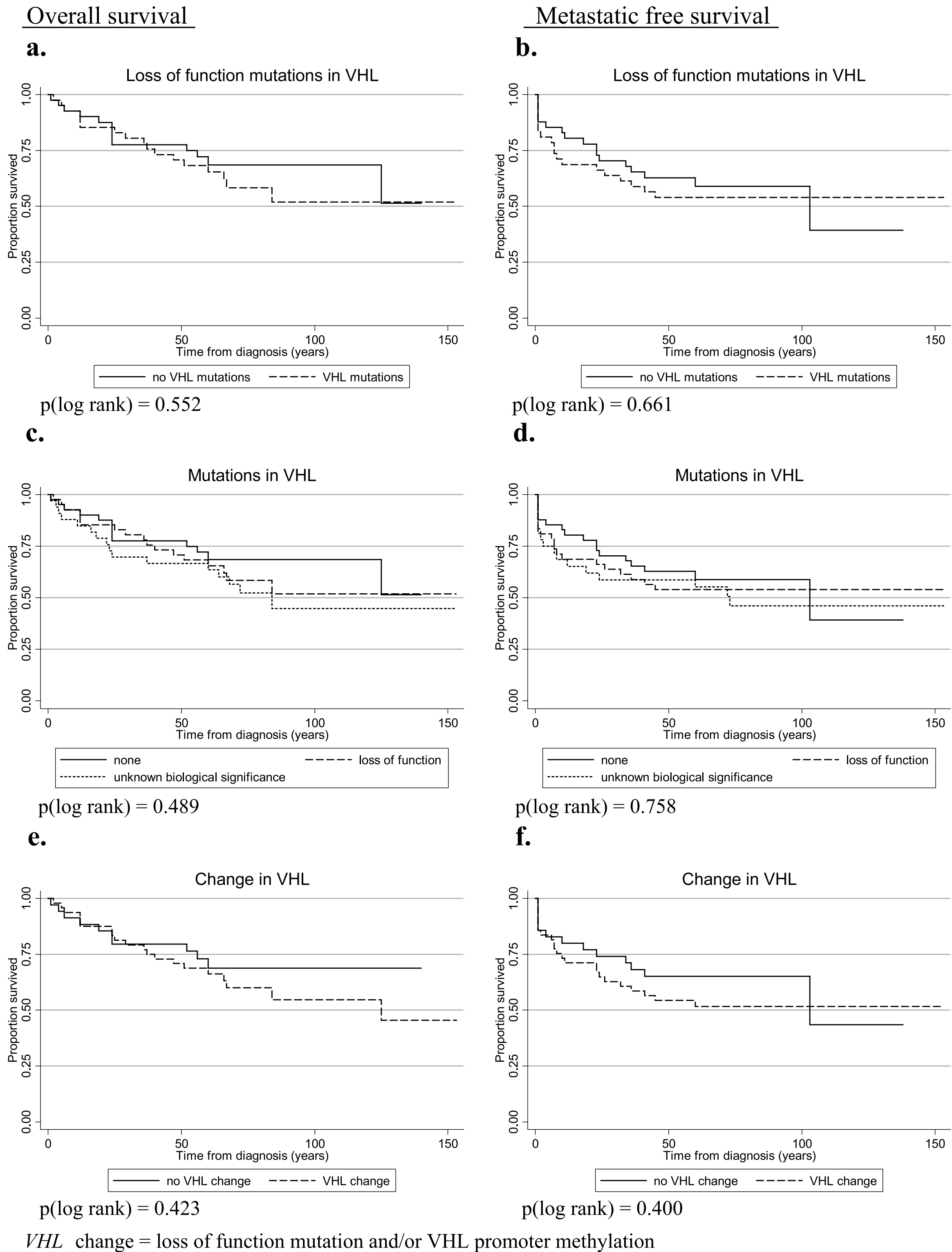

Fig. 2. Kaplan–Meier survival analyses.

Table 3
Tumour parameters stratified by *VHL* mutation/methylation status

	N	No mutation	Silent *VHL* mutation	Loss of function *VHL* mutation	*VHL* mutation of unknown biological significance	*VHL* promoter methylation absent	*VHL* promoter methylation present
Grade							
1	12	7 (58%)	0 (0%)	2 (16%)	3 (25%)	11 (92%)	1 (8%)
2	64	16 (25%)	5 (8%)	23 (35%)	18 (28%)	58 (91%)	6 (9%)
3	43	15 (35%)	10 (23%)	15 (35%)	10 (23%)	40 (93%)	3 (7%)
4	15	5 (33%)	2 (13%)	5 (33%)	5 (33%)	14 (93%)	1 (7%)
Stage							
1	73	26 (36%)	7 (9%)	22 (30%)	18 (25%)	64 (88%)	9 (12%)
2	23	8 (35%)	1 (4%)	7 (30%)	7 (30%)	22 (96%)	1 (4%)
3	37	8 (22%)	9 (24%)	16 (43%)	11 (30%)	36 (97%)	1 (3%)
4	1	1 (100%)	0 (0%)	0 (0%)	0 (0%)	1 (100%)	0 (0%)

Table 4
Relationship between *VHL* alterations and angiogenesis/tumour parameters

	VHL promoter methylation[a] ($n = 134$) $0 = 123$; $1 = 11$ p-value	*VHL* mutation[b] ($n = 91$) $0 = 46$; $1 = 45$ p-value	*VHL* mutation[c] ($n = 127$) $0 = 46$; $1 = 45$; $2 = 36$ p-value	*VHL* change[d] ($n = 93$) $0 = 38$; $1 = 55$ p-value
Angiogenesis parameters				
MVD	0.277	0.499	0.798	0.937
ECP%	0.300	0.817	0.464	0.248
HIF-1α	0.789	0.151	0.255	0.150
CAIX	0.909	0.087	0.150	0.047
VEGF	0.650	0.267	0.503	0.147
Tumour parameters				
TCP%	0.113	0.817	0.867	0.409
Fuhrman grade (1–4)	0.686	0.762	0.944	0.953
Tumour stage (1–4)	0.108	0.212	0.424	0.437

Notes: MVD: microvessel density; ECP%: endothelial cell proliferation fraction; TCP%: tumour cell proliferation fraction.

[a] *VHL* promoter methylation: 0 = unmethylated; 1 = methylated.

[b] *VHL* mutation: 0 = no or silent mutation; 1 = loss of function mutation.

[c] *VHL* mutation: 0 = no or silent mutation; 1 = loss of function mutation; 2 = mutation of unknown significance.

[d] *VHL* change: 0 = no methylation and/or loss of function mutation; 1 = *VHL* methylation and/or loss of function mutation.

whether it has any biological significance, since no significant association is found between CAIX expression and *VHL* promoter methylation or CAIX expression and *VHL* mutation status separately. CAIX plays an important role in allowing cancer cells to buffer their intracellular pH in hypoxic and/or in extensive glycolysis conditions [26,27], but no effect of CAIX on active angiogenesis has been shown in literature. An earlier study showed that CAIX expression is not solely regulated by HIF-1α accumulation (through hypoxia and/or defective *VHL*), resulting in binding to hypoxia response element (HRE), but also by factors binding to the juxtaposed SP1/SP3 site PR1 of the CAIX promoter, whereby induction of CAIX may be the result of a cooperation between SP1/SP3 and HIF-1α transcription factors [28].

An earlier report investigated the effect of *VHL* mutation status on vessel density and also showed that

these two features are not associated with one another [22]. Additionally, in accordance with other reports [29,30], no significant correlation was found between presence/absence or type of *VHL* alterations and clinical outcome. However, contrasting results about the prognostic relevance of *VHL* alterations are present in literature [31,32]. Differences between studies may be due to low samples sizes or small effect sizes [33–35], length of follow-up and postoperative treatment.

Our results suggest that regulation of angiogenesis and tumour progression of ccRCC is apparently not directly influenced by *VHL* alterations. The equal frequencies of *VHL* alterations between tumour stages 1, 2 and 3 supports the belief that *VHL* gene inactivation is an early event in ccRCC tumourigenesis [36,37]. Subsequent to this inactivation, additional genetic and epigenetic events might cause the diverse angiogenic and proliferative behavior during tumour progression. To that end, the strong association between tumour cell proliferation rate and endothelial cell proliferation rate, observed in our study, implies that tumour angiogenesis and tumour cell proliferation in ccRCCs may be driven by similar mechanisms.

The HIF pathway, involved in renal angiogenesis and carcinogenesis, is complex. Tumour behavior may be dictated by additional genetic defects in the hypoxia-HIF-VHL upstream, non-VHL oncogenic pathways on HIF activation (P13 kinase pathway [38, 39]/MAPK kinase pathway [40,41]), and downstream pathways (VEGF and other target genes). For instance, VEGF by itself acts through multiple cell surface receptors and signaling pathways to stimulate endothelial cell proliferation, survival, and migration [42]. By inducing other growth factor expression, VEGF stimulates a cascade of angiogenic activity [42].

Studies of HIF target genes have defined examples with putative antitumourigenic properties [43]. Also contrasting properties of HIF-1α and HIF-2α were observed in VHL-defective RCC cells, whereby, HIF-2α yielded more protumourigenic effects than HIF-1α [44], suggesting that it is the overall balance of target gene activation that determines the outcome of HIF activation.

Besides the HIF transcriptional cascade, often considered as the regulating system of tumour angiogenesis, accumulating evidence indicates that HIF-independent pathways also can control angiogenesis [45]. These mechanisms still have to be elucidated in sporadic ccRCC.

In our study, the lack of a direct association between *VHL* inactivation status and HIF-1α expression can be explained by an earlier report of Jiang et al. This study demonstrated the expression of a different number of hypoxia-inducible genes between RCC 786-0 VHL^+ and 786-0 VHL^- cell lines. Four genes were induced in both cell lines. These findings suggest *VHL*-dependent and *VHL*-independent mechanisms in RCC cells in response to hypoxic stress [46]. Other studies reveal that, even in the absence of *VHL* mutations, the frequency of HIF-1α protein expression still varies from 14% to 75% [14,47,48]. In this case, loss of HIF-1α protein was not only associated with truncated HIF-1α mRNA transcripts, but also with transcriptional silencing [49]. In these HIF-1α defective cell lines, the knockdown of the HIF-2α gene demonstrated that HIF-2α regulated the VEGF production, irrespective of the *VHL* gene mutation status [49].

Finally, 27% of our patient group showed no *VHL* mutations or *VHL* promoter methylation. In the literature, in approximately 25–50% of sporadic ccRCC no alterations in the *VHL* alleles were detected [25,50, 51].These findings indicate that *VHL* alterations may be central to the development of the vast majority of ccRCC, but not the only tumourigenic pathway. Additional inactivation of 3p12-p21 tumour suppressor genes appears to be necessary in the development of ccRCC irrespective of *VHL* gene inactivation [52, 53]. Furthermore, though 3p is the most frequently lost chromosome arm in ccRCC, other regions of genetic loss on other chromosomes have been identified in ccRCC [54–56]. The presence of multiple genetic losses in ccRCC is associated with a poor prognosis [53,55].

In conclusion, our data suggest that *VHL* alterations, though one of the key events in both hereditary and sporadic ccRCCs, may not independently influence angiogenesis, tumour proliferation or prognosis. The variable nature of ccRCC is most likely strongly determined by the complex interplay of additional downstream modifications, among which the role of epigenetic alteration of gene expression is becoming more and more acknowledged. Elucidation of these modifications will be necessary for successful targeted therapy.

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