

# Prognostic significance of *VHL*, *HIF1A*, *HIF2A*, *VEGFA* and *p53* expression in patients with clear-cell renal cell carcinoma treated with sunitinib as first-line treatment

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Received December 6, 2018; Accepted May 30, 2019

DOI: 10.3892/ijo.2019.4830

**Abstract.** Clear cell renal cell carcinoma (ccRCC) is the most common subtype of renal cell cancer, characterized by the highest mortality rate among other RCC subtypes due to the occurrence of metastasis and drug resistance following surgery. The Von Hippel-Lindau tumor suppressor (*VHL*)-hypoxia-inducible factor 1 subunit  $\alpha$  (*HIF1A*)/hypoxia-inducible factor 2 $\alpha$  (*HIF2A*)-vascular endothelial growth factor A (*VEGFA*) protein axis is involved in the development and progression of ccRCC, whereas sunitinib, a tyrosine kinase inhibitor, blocks the binding of *VEGFA* to its receptor. The aim of the present study was to examine the possible association of the gene expression of *VHL*, *HIF1A*, *HIF2A*, *VEGFA* and tumor protein P53 (*P53*) in cancer tissue with the outcome of ccRCC patients who were treated with sunitinib as first-line therapy following nephrectomy. A total of 36 ccRCC patients were enrolled, 11 of whom were administered sunitinib post-operatively. Tumor and control samples were collected, and mRNA and protein levels were assessed by reverse transcription-quantitative polymerase chain reaction and western blot analysis, respectively. High mRNA and protein expression levels of *HIF2A* and *VEGFA* were found to be associated with shorter overall survival (OS) and progression-free survival (PFS) rates, as well as with unfavorable risk factors of cancer recurrence and mortality. Resistance

to sunitinib was also observed; the OS and PFS rates were shorter (median OS and PFS: 12 and 6 months, respectively, vs. undetermined). Sunitinib resistance was associated with high *HIF2A* and *VEGFA* protein levels ( $b=0.57$  and  $b=0.69$  for OS and PFS, respectively;  $P<0.001$ ). Taken together, the findings of this study suggest that the protein levels of *HIF2A* and *VEGFA* in tumor tissue may serve as independent prognostic factors in ccRCC. ccRCC patients with increased intratumoral *HIF2A* and *VEGFA* protein levels, and unaltered *VHL* protein levels, are not likely to benefit from sunitinib treatment following nephrectomy; however, this hypothesis requires verification by large-scale replication studies.

## Introduction

Renal cell carcinoma (RCC) represents a serious health concern, with an estimated annual incidence of 69,330 new cases and 14,400 deaths in the USA in 2017 (1). Worldwide kidney cancer causes >100,000 deaths per year (2). Clear cell RCC (ccRCC) is the most common subtype of renal cancer (70-80%) (3), and is characterized by the highest mortality rate compared with other RCC subtypes (4). With a 50% risk of metastasis, the prognosis of patients with ccRCC is poor, with a 5-year survival rate of 40% (5), even with the administration of modern drugs (6). The development of ccRCC is associated with extrinsic factors, including smoking, obesity and hypertension. However, conditions such as hereditary von Hippel-Lindau (*VHL*) disease or sporadic somatic inactivation of the *VHL* gene have been found to have a very close genetic association with ccRCC (4). The *VHL* gene was mapped at chromosome 3p25.3 (7,8), and the protein encoded by this gene (pVHL) was subsequently shown to form a complex with elongin C, elongin B and cullin-2 (9), which possesses ubiquitin ligase E3 activity. Upon its formation, this complex specifically recognizes the  $\alpha$  subunits of two transcription factors, the hypoxia-inducible factors (HIFs) 1A and 2A, and directs them for degradation along the ubiquitin-proteasome pathway (8). Under normoxic conditions, oxygen-dependent hydroxylation of HIFs at specific proline residues by the enzyme prolyl hydroxylase (PHD)

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**Key words:** clear cell renal cell carcinoma, gene expression, hypoxia-inducible factor 1 subunit  $\alpha$ , hypoxia-inducible factor 2 $\alpha$ , metastatic disease, prognosis, reverse transcription-quantitative polymerase chain reaction, p53, sunitinib resistance, vascular endothelial growth factor A, Von Hippel-Lindau tumor suppressor, western blot analysis

triggers binding of pVHL, ubiquitination, and subsequent proteasomal degradation of either HIF1A or HIF2A (2). Apart from oxygen-dependent regulation, which requires pVHL for HIF ubiquitination, another pathway also leads to the degradation of the HIFs with the involvement of the P53 and mouse double minute 2 homolog (Mdm2) proteins (10). Under hypoxic conditions, P53 binds to HIF1A and triggers its removal through Mdm2-mediated ubiquitination and proteasomal degradation, controlled by the phosphatase and tensin homolog (PTEN)/phosphoinositide 3-kinase (PI3K)/AKT pathway (10-13). Consequently, under normal physiological conditions, the HIF1A and HIF2A proteins are degraded within minutes. However, during hypoxia, the hydroxylation of HIFs is inhibited, and the two proteins form a stable complex with the constitutively expressed HIF-1 $\beta$  subunit (ARNT protein); this complex is subsequently translocated to the cell nucleus. After binding with DNA at hypoxia response elements, the HIF complex activates the expression of a large variety of genes (14). Although HIF1A and HIF2A represent different proteins, and they partially overlap in the activation of target genes; for example, the gene expression of C-X-C chemokine receptor type 4 (*CXCR4*) and solute carrier family 2 member 1 (*SLC2A1*) has been shown to be activated by either HIF1A or HIF2A (15). One of the most well-characterized genes activated by HIFs is vascular endothelial growth factor A (*VEGFA*), which encodes heparin-binding protein. VEGFA robustly induces the proliferation and migration of vascular endothelial cells, and is essential for physiological and pathological angiogenesis (16). Currently, therapeutic approaches towards the treatment of advanced ccRCC comprise various VEGF pathway-targeted agents, including bevacizumab, sunitinib, sorafenib, pazopanib and axitinib (4). Sunitinib inhibits cellular signaling by blocking the membrane receptors of platelet-derived growth factor (PDGF), as well as VEGF, decreasing the rate of neoangiogenesis, which is one of the most important pathological mechanisms associated with RCC development (4). Although sunitinib was introduced as a first-line post-operative adjuvant therapy for metastatic ccRCC due to improvement of overall survival (OS) and progression-free survival (PFS) rates (17), it was reported that the outcome of ~15% of sunitinib-treated patients was grim, due to intrinsic drug resistance and cancer recurrence (6,18-20). The mechanisms of the resistance to tyrosine kinase inhibitors (TKIs) of the VEGF receptor (VEGFR) have yet to be fully elucidated, although one research group previously observed secondary resistance to angiogenesis inhibitors, reflected by cancer progression following a period of clinical improvement (21).

The deregulation of the *VHL* gene in ccRCC has been widely reported (5,22,23). Studies on the expression patterns of *HIF1A* (24-28), *HIF2A* (25-28), *VEGFA* (5,24,27-29) and *P53* (30,31) in ccRCC have revealed variations in terms of cancer progression and/or patient outcome. However, to the best of our knowledge, to date, no comprehensive study of all the above-mentioned genes in one cohort of ccRCC patients has been performed with the use of quantitative and semi-quantitative methods [i.e., reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis]. Therefore, the first aim of the present study was to identify the associations of the mRNA and protein levels of *VHL*,

*HIF1A*, *HIF2A*, *VEGFA* and *P53* with patient outcome. Furthermore, since the majority of patients receiving sunitinib as first-line post-operative treatment have a poor outcome, the possible associations between molecular signatures and clinicopathological data were further analyzed.

## Materials and methods

**Patients and samples.** Tissue samples were obtained from 36 patients with ccRCC who underwent radical nephrectomy at the Department of Urology, Medical University of Gdansk, Poland, between January, 2011 and September, 2013. The clinicopathological data of the patients are presented in Table I. A total of 11 patients received sunitinib following radical nephrectomy. The present study was approved by the Independent Bioethics Commission for Research of Medical University of Gdansk, and written consent was obtained prior to surgery from all patients.

**Sample acquisition.** Samples were obtained as previously described (13,32,33). In brief, dissected tissue samples of the primary ccRCC (n=36) and corresponding normal kidney (n=36) were collected in the operating theatre (by J.K.) and immediately placed in ~5 volumes of RNALater (Ambion Inc., now a brand of Thermo Fisher Scientific, Inc.). Tumor and normal kidney samples from 10 patients were placed in at least 10 volumes of 4% buffered formalin (pH 7.0-7.4; ChemPur). RNALater-stored samples were subsequently used for RNA and protein assessment; immunohistochemical (IHC) localization of proteins was accomplished using the formalin-stored tissues.

**Assessment of the mRNA expression of *VHL*, *HIF1A*, *HIF2A*, *VEGFA* and *P53*.** RNA isolation and cDNA synthesis were performed as previously described (13,32,33). Briefly, an ExtractMe RNA kit (Blirt) was used for RNA extraction from tissue samples. Total RNA samples (2  $\mu$ g) were reverse transcribed with the use of RevertAid Reverse Transcriptase (Fermentas; Thermo Fischer Scientific, Inc.). Details concerning the RT-qPCR methodology are provided in Table II. All reactions were run in duplicate; the measurement of glucuronidase beta (*GUSB*) gene expression was used for the normalization of qPCR results (33) with Livak and Schmittgen's  $2^{-\Delta\Delta C_q}$  method (34).

**Western blot analysis.** Western blot analysis was performed to compare the protein levels of HIF1A, HIF2A, VEGFA, VHL and P53 in paired tumor/unchanged kidney tissues of the 36 patients. Lysates were extracted according to the method previously described in our previous studies (13,32) with the use of a Mammalian Cell Extraction kit (BioVision). Since the rapid degradation of the HIF1A and HIF2A proteins occurs upon exposure to normal oxygen pressure (35), all further steps were performed immediately. Briefly, 10- $\mu$ g protein samples were loaded onto 10% polyacrylamide gels, resolved by 8% SDS-PAGE, and transferred onto polyvinylidene difluoride membranes using the Trans-Blot Turbo system (Bio-Rad Laboratories, Inc.). The membranes were blocked by incubation with 3% albumin fraction V in Tris-buffered saline (TBS) buffer at pH 7.4 (Sigma-Aldrich;

Table I. Patient characteristics.

Patients n=36	Subgroups	No.
Age (years)	≤62 <sup>a</sup>	19
Median, 60.58±11.9 years	62	17
Range, 33-82 years		
Sex	Female	17
	Male	19
Body mass index (BMI)	≤25 <sup>b</sup>	14
Median, 28.57±6.71	>25	22
Range, 19.82-45.52		
Creatinine (mg/dl)	≤1.21 <sup>b</sup>	32
Median, 1.08±1.04	>1.21	4
Range, 0.65-7.06		
Blood urea nitrogen (BUN) (mg/dl)	7-20 <sup>b</sup>	26
Median, 16.43±5.12	>20	10
Range, 7.4-28.2		
Estimated glomerular filtration rate (eGFR) (ml/min/1.73 m <sup>2</sup> )	≤60	8
	Range, 7.22-59.92	
	>60 <sup>b</sup>	28
Hematocrit (HCT) (%)	≤40.55	18
Median, 39.5±4.77	>40.55	18
Range, 28.6-46.9		
Hemoglobin (HGB) (g/dl)	≤11 <sup>b</sup>	5
Median, 13.05±1.82	>11	31
Range, 9-15.7		
Glucose (GLC) (mg/dl)	≤99 <sup>b</sup>	20
Median, 102.7±19.58	>99	16
Range, 77-167		
Sodium (Na <sup>+</sup> ) (mmol/l)	≤145 <sup>b</sup>	36
Median, 139.22±2.35	>145	0
Range, 133-144		
Potassium (K <sup>+</sup> ) (mmol/l)	≤3.5	1
Median, 4.33±0.46	>3.5	35
Range, 3.3-4.35		
Tumor location	Left kidney	17
	Right kidney	19
Tumor size (cm)	≤7 cm	17
	>7 cm	19
Fuhrman's histological grade	1	2
	2	16
	3	11
	4	7
TNM stage		
Non-metastatic, n=21	T1-2N0M0	21
Metastatic, n=15	T1-2N1M0	0
	T3N0-1M0	7
	T1-4N2M0	6
	T1-4N0-2M1	2
Sunitinib (anti-VEGFR)		
Yes		11
No		25

<sup>a</sup>Divided according to median value; <sup>b</sup>divided according to laboratory cut-off values.

Table II. Details of qPCR assays.

Gene name, GeneBank ID	Primer sequences	Amplicon size (bp)	qPCR efficiency	qPCR reaction conditions	qPCR reaction content
<i>VHL</i> , NM_000551.3	5'-CGGACAGCCTATTTTGCCAAT 5'-ATGTTTGCCCCCTAAACATCACA	400	96.4%	95°C, 3 min; 37x (95°C, 5 sec; 58°C, 10 sec; 72°C, 10 sec; 75°C, 10 sec - sample reading) Melting curve: 95°C, 15 sec; 60°C, 1 min; 60°C → 95°C reading every 0.3°C	5 µl SensiFast NoRox SYBR-Green (with SYBR-Green fluorophore) (BioLine, London, GB), 200 nM each primer, Σ 10 µl
<i>HIF1A</i> , NM_001243084.1	5'-ACCTGAAGAATTGGAAGAAATCAGA 5'-ATATCCAAATCACCGCATCCA	243	94.6%		
<i>HIF2A</i>	5'-CGTCCTGAGTGAGATTGAGAAAG 5'-GACTCCTCGAAGTTCTGATTCC	246	96.3%		
<i>VEGFA</i>	5'-GGGCTCATGGACGGGTGA 5'-ATCCATGAACCTTCACCACTTCG	328	92.7%		
<i>P53</i>	5'-ACGACGGTGACACGCTTCCCTG 5'-CGCTAGGATCTGACTGCGGCTC	84	99.1%		
<i>GUSB</i>	5'-ATGCAGGTGATGGAAGAAAGTGGTG 5'-AGAGTTGCTCACAAAGGTCACAGG	177	99.6%		
<i>VHL</i> , Von Hippel-Lindau; <i>HIF</i> , hypoxia-inducible factor; <i>VEGF</i> , vascular endothelial growth factor; <i>GUSB</i> , glucuronidase beta.					

now a brand of Merck KGaA) with 0.1% Tween-20 (TBST) for 1 h at room temperature, and were subsequently incubated overnight at 4°C with specific primary antibodies dissolved in 2% albumin/TBS at a dilution of 1:1,000. The following specific rabbit antibodies were used: Polyclonal anti-HIF1A (cat. no. LS-B674), polyclonal anti-HIF2A (EPAS1) (cat. no. LS-B4223), polyclonal anti-VEGFA (cat. no. LS-B10263), polyclonal anti-VHL (cat. no. LS-C99277) and polyclonal anti-P53 (cat. no. LS-B4558) (all purchased from LifeSpan BioSciences). After washing 3 times with TBST, the blots were incubated for 2 h at room temperature with horseradish peroxidase-conjugated anti-rabbit IgG antibodies (1:10,000; cat. no. A0545; Sigma-Aldrich; Merck KGaA). Anti-GAPDH peroxidase-conjugated IgM antibodies (1:50,000; cat. no. G9295; Sigma-Aldrich; Merck KGaA) were applied for 1 h at room temperature as the loading control. Following triple washing with TBST, immunoreactive bands were detected on medical X-ray films (Agfa HealthCare) using Clarity Western ECL Blotting substrate (Bio-Rad Laboratories, Inc.). Densitometric analysis of immunoreactive protein bands was performed with Quantity One software (Bio-Rad Laboratories, Inc.) and calculated as units = Intensity/mm<sup>2</sup>. After normalizing the levels against GAPDH for each sample, semi-quantitative results for the HIF1A, HIF2A, VEGFA, VHL or P53 proteins extracted from tumor samples were expressed according to the ratio: Mean no. of units<sub>Tumor</sub> / mean no. of units<sub>Control</sub>.

**Immunohistochemistry (IHC) for VHL, HIF1A, HIF2A, VEGFA and P53 proteins.** IHC staining was performed as previously described (32,33). Formalin-fixed paraffin-embedded tissue sections (6 µm) from the tumor and normal kidney tissues of 10 patients with ccRCC were deparaffinized and hydrated through xylenes and a graded alcohol series. Following antigen retrieval in hot (90°C) acidic citrate buffer (Epitope Retrieval Solution, pH 6.0; Leica Biosystems Ltd.), the samples were blocked for endogenous peroxidase activity by incubation with 3% hydrogen peroxide for 10 min, followed by incubation with 2.5% normal horse serum [ImmPRESS™ Anti-Rabbit Ig (peroxidase) Polymer Detection kit, Vector Laboratories Inc.; part of Maravai LifeSciences] to block the non-specific binding of immunoglobulin. IHC staining was performed using the same primary antibodies as those used for western blot analysis at a 1:100 dilution (with the exception of the anti-P53 antibody, which was used at a dilution of 1:50). Following a 2-h incubation with primary antibodies at room temperature, the slides were washed in PBS and incubated with an appropriate secondary antibody [ImmPRESS™ Anti-Rabbit Ig (peroxidase) Polymer Detection kit or ImmPRESS™ Anti-Mouse Ig (peroxidase) Polymer Detection kit; Vector Laboratories, Inc.) for 30 min. The slides were rinsed in PBS, and immunoreactive cells were visualized by the addition of 3,3'-diaminobenzidine solution (DAB Peroxidase Substrate kit, Vector Laboratories, Inc.) and counterstained with hematoxylin. The sections were subsequently dehydrated, mounted in DPX Mountant, viewed under a Nikon Eclipse E800 light microscope, and the acquisition of the microphotographs was performed using NIS software (Nikon). The specificity of IHC staining was determined by a negative control, which was prepared under conditions identical to those described above; however, the primary antibodies were replaced

with 2.5% normal horse serum [ImmPRESS™ Anti-Rabbit Ig (peroxidase) Polymer Detection kit; Vector Laboratories, Inc.].

**Statistical analysis.** Statistical analysis was performed using GraphPad Prism version 6.07 (GraphPad Software) and Statistica version 13 (Dell Inc.) software. The following statistical tests were used: Non-parametric Mann-Whitney U, Wilcoxon signed-rank and Fisher's 2x2 exact tests, Spearman's correlation, multivariate regression, Kaplan-Meier survival tests with log-rank (Mantel-Cox) test, and the Cox proportional hazard regression model. Survival associations were presented as hazard ratios (HRs) with their 95% confidence interval (CI) and P-values (36) using Cox and Kaplan-Meier estimations. The OS and PFS rates were calculated separately. In all analyses, a two-sided P<0.05 was considered to indicate a statistically significant value, with a 95% confidence interval.

## Results

**Characteristics of the patients.** The clinical, pathological and summary results of the laboratory assessments of the 36 patients with ccRCC enrolled in the present study are presented in Table I. Despite the malignancy, the majority of the patients were in a relatively good condition, as evidenced by the results of blood and urine laboratory tests and physical examinations; none of the patients was diagnosed with cachexia. The patients enrolled for sunitinib treatment had passed the ESMO guidelines (37).

Of the 36 patients with ccRCC aged 60.6±11.9 years [mean ± standard deviation (SD); Table I], 21 were diagnosed [according to the tumor-necrosis-metastasis (TNM) staging system] as stage I (T1-2N0M0), 7 as stage III (T1-2N1M0 or T3N0-1M0) and 8 as stage IV (T4N0-2M0 or T1-4N2M0 or T1-4N0-2M1), according to anatomic stage and prognostic groups based on the 2010 TNM 7th classification of RCC (38). At the time of surgery, 41.7% of the patients with ccRCC were diagnosed with local or distant metastases. Histological nuclear staging was based on the Fuhrman grading system (39), which revealed that tumor tissues from 2 patients were classified as grade 1, 16 were grade 2, 11 were grade 3, and 7 were classified as grade 4. None of the patients had undergone chemo- or radiotherapy prior to surgery. The mean follow-up period was 21 months (range, 3-48 months), and until the end of follow-up collection, 23 patients were alive (64%); all deaths were associated with ccRCC progression. The median OS rate was undefined. During follow-up, metastases occurred in 14 (39%) of the patients (data not shown), whereas the median PFS rate was undefined. Post-operative treatment included sunitinib (an anti-VEGFA agent), which was administered to 9 patients with clinically advanced ccRCC (T1-2N1M0, T3N0-1M0, T1-4N2M0 and T1-4N0-2M1), and to 2 patients with early ccRCC (T1-2N0M0), according to a generally accepted schedule (40) of 1 cycle: 50 mg/day for 4 weeks, followed by a 2-week interval. During follow-up, 9 and 10 sunitinib-treated patients succumbed to the disease or experienced cancer recurrence, respectively.

**Expression of the VHL, HIF1A, HIF2A, VEGFA and P53 genes at the mRNA level.** The mRNA levels of the selected genes were assessed by RT-qPCR in the tumor tissues and



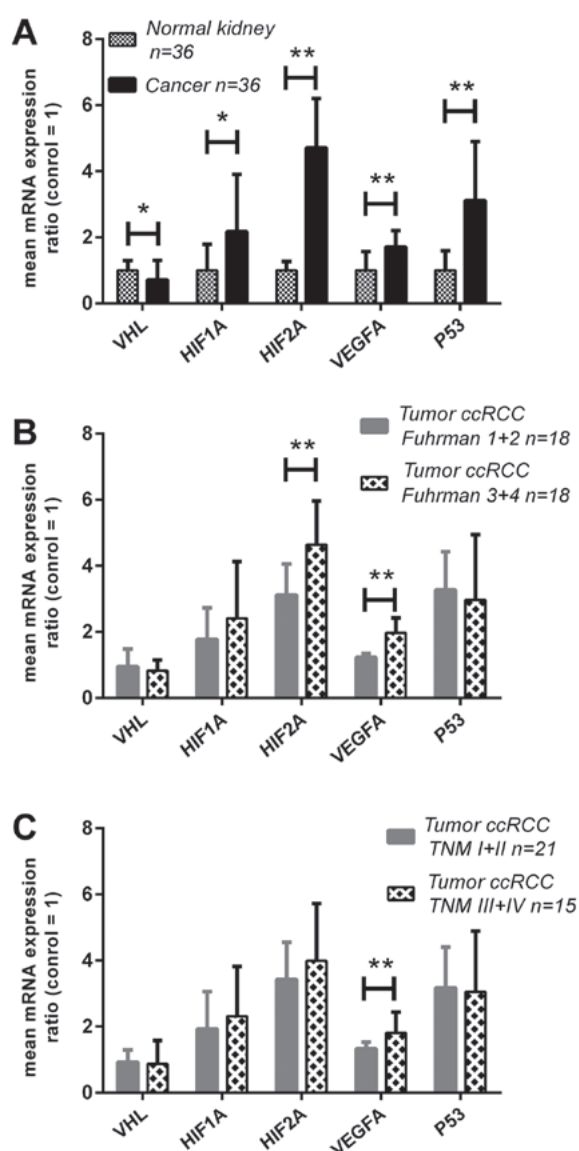


Figure 1. Expression analysis of the *VHL*, *HIF1A*, *HIF2A*, *VEGFA* and *P53* genes in ccRCC at the mRNA level. Gene expression was assessed as described in the Materials and methods. (A) Comparison between tumor and normal kidney samples; (B) gene expression in tumor samples related to Fuhrman grade; and (C) gene expression in tumor samples related to TNM stage. Bars and whiskers represent mean  $\pm$  standard deviation normalized to control kidney samples. \* $P<0.05$ , \*\* $P<0.01$ . (Wilcoxon signed-rank test for A, Mann-Whitney U test for B and C). ccRCC, clear cell renal cell carcinoma; VHL, Von Hippel-Lindau; HIF, hypoxia-inducible factor; VEGF, vascular endothelial growth factor; qPCR, quantitative polymerase chain reaction.

corresponding normal kidney samples of 36 patients with ccRCC. Different ratios of expression for all genes between the cancerous tissues and microscopically unaltered renal tissues were observed; the *VHL* mRNA ratio was 30% lower in the cancer tissues ( $P<0.05$ ; Fig. 1A), and its decreased levels were observed in 20/36 (56%) of the ccRCC patients (Table III). The other analyzed genes exhibited statistically significantly higher mRNA levels in the ccRCC compared with the normal kidney tissue: *HIF1A* mRNA was upregulated ~2-fold ( $P<0.05$ ; Fig. 1A), and its level was increased in 56% of the cancer samples (Table III); by comparison, *HIF2A* mRNA was upregulated ~5-fold ( $P<0.01$ ; Fig. 1A), and its higher expression was observed in 58% (Table III) of the patients. The *VEGFA*

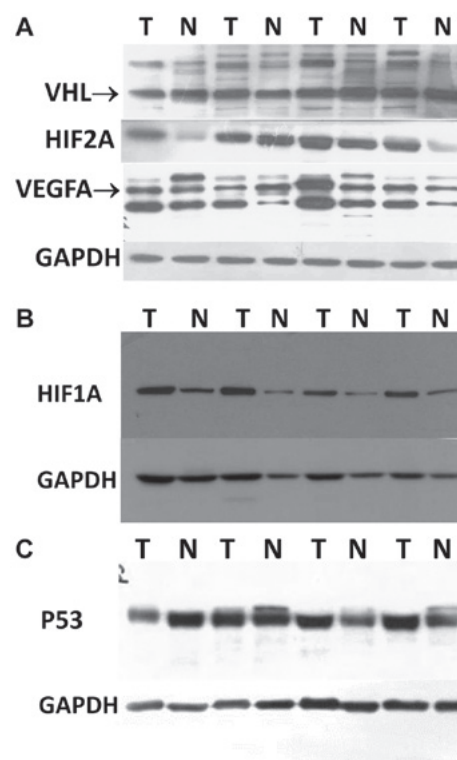


Figure 2. Western blot analysis images of *VHL*, *HIF1A*, *HIF2A*, *VEGFA* and *P53* proteins in ccRCC. Protein assessment was performed as described in the Materials and methods. (A) Merged image of *VHL*, *HIF2A*, *VEGFA* and *GAPDH* proteins as they were analyzed in the same western blot membrane. (B and C) Western blot analysis images of *HIF1A* and *P53* proteins with corresponding *GAPDH* bands, respectively. ccRCC, clear cell renal cell carcinoma; VHL, Von Hippel-Lindau; HIF, hypoxia-inducible factor; VEGF, vascular endothelial growth factor.

and *P53* mRNA levels were increased by 2- and 3-fold, respectively ( $P<0.01$ ; Fig. 1A), and increased mRNA expression rates of these genes were detected in 78 and 70% of the ccRCC patients, respectively (Table III). When the molecular assessment at the mRNA level was compared with the clinicopathological data, it was observed that the increased expression of the *HIF2A* and *VEGFA* genes was associated with a higher Fuhrman's grade (3 and 4); additionally, the mRNA levels increased by ~1.5- and 1.6-fold (Fig. 1B) between grades 3+4 and 1+2, respectively ( $P<0.01$ ). The mean expression rate of *VEGFA* was also associated with clinically advanced ccRCC, since its mRNA level was ~1.4-fold higher in the metastatic tumor samples compared with early-stage tumors ( $P<0.01$ ; Fig. 1C). When the expression levels for each gene were divided according to their median levels in normal samples (13,41) (Table III), increased expression rates of *HIF2A* (42%) and *VEGFA* (47%) were observed in samples with higher Fuhrman's grades, whereas higher *HIF2A* (33%) and *P53* (39%) mRNA levels were observed in clinically advanced ccRCCs (Table III). No other associations were observed between the laboratory or clinicopathological data of the patients and the expression of the studied genes at the mRNA level.

*Expression of the VHL, HIF1A, HIF2A, VEGFA and P53 genes at the protein level.* Semi-quantitative assessment of the protein expression levels was performed in paired tumor and normal

Table III. Association between *VHL*, *HIF1A*, *HIF2A*, *VEGFA* and *P53* mRNA levels and clinical data.

Patients n=36	Subgroups	<i>VHL</i>			<i>HIF1A</i>			<i>HIF2A</i>			<i>VEGFA</i>			<i>P53</i>		
		↓	↑	P-value <sup>a</sup>	↓	↑	P-value <sup>a</sup>	↓	↑	P-value <sup>a</sup>	↓	↑	P-value <sup>a</sup>	↓	↑	P-value <sup>a</sup>
Age (years)	≤6, n=19	10 (28)	9 (25)	0.74	8 (22)	11 (31)	1.0	9 (25)	10 (28)	0.52	5 (14)	14 (39)	0.69	8 (22)	11 (31)	0.15
Median, 60.58±11.9	>62, n=17	10 (28)	7 (19)		8 (22)	9 (25)		6 (17)	11 (30)		3 (8)	14 (39)		3 (8)	14 (39)	
Range, 33-82,																
Sex	Female, n=17	11 (31)	6 (16)	0.23	5 (14)	12 (33)	0.1	9 (25)	8 (22)	0.31	6 (17)	11 (31)	0.11	7 (19)	10 (28)	0.28
	Male, n=19	9 (25)	10 (28)		11 (31)	8 (22)		6 (17)	13 (36)		2 (5)	17 (47)		4 (11)	15 (42)	
Tumor size (cm)	≤7 cm, n=17	7 (19)	10 (28)	0.18	9 (25)	8 (22)	0.5	7 (19)	10 (28)	1.00	3 (8)	14 (39)	0.69	5 (14)	12 (33)	1.00
	>7 cm, n=19	13 (36)	6 (16)		7 (19)	12 (33)		8 (22)	11 (31)		5 (14)	14 (39)		6 (17)	13 (36)	
Fuhrman's histological grade,	1+2, n=18	10 (28)	8 (22)	1.00	5 (14)	13 (36)	0.09	12 (33)	6 (17)	<b>0.006</b>	7 (19)	11 (31)	<b>0.04</b>	8 (22)	10 (28)	0.15
	3+4, n=18	10 (28)	8 (22)		11 (31)	7 (19)		3 (8)	15 (42)		1 (3)	17 (47)		3 (8)	15 (42)	
TNM stage	Non-metastatic, n=21	12 (33)	9 (25)	0.9	7 (19)	14 (39)	0.17	12 (33)	9 (25)	<b>0.04</b>	7 (19)	14 (39)	0.10	10 (28)	11 (31)	<b>0.011</b>
	Metastatic, n=15	8 (22)	7 (19)		9 (25)	6 (17)		3 (8)	12 (33)		1 (3)	14 (39)		1 (3)	14 (39)	
Sunitinib treatment	Yes, n=11	6 (17)	5 (14)	1.00	7 (19)	4 (11)	0.16	2 (6)	9 (25)	0.07	1 (3)	10 (28)	0.38	4 (11)	7 (19)	0.7
	No, n=25	14 (39)	11 (31)		9 (25)	16 (44)		13 (36)	12 (33)		7 (19)	18 (50)		7 (19)	18 (50)	

<sup>a</sup>P-values were calculated by Fisher's 2x2 test. Downward arrows indicate decreased levels and upward arrows indicate increased levels. Values in bold indicate statistical significance (P<0.05). VHL, Von Hippel-Lindau; HIF, hypoxia-inducible factor; VEGF, vascular endothelial growth factor.

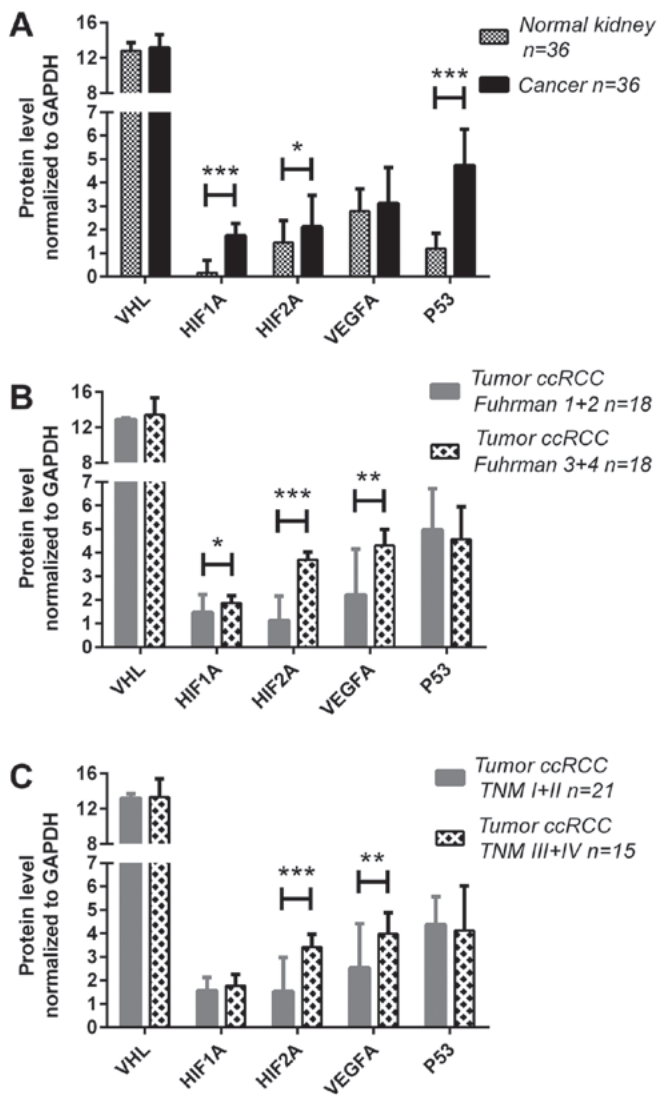


Figure 3. Expression analysis of the *VHL*, *HIF1A*, *HIF2A*, *VEGFA* and *P53* genes in ccRCC at the protein level. Gene expression at protein level was assessed as described in the Materials and methods. (A) Comparison between tumor and normal kidney samples; (B) gene expression at protein level in tumor samples related to Fuhrman grade; and (C) gene expression at protein level in tumor samples related to TNM stage. Bars and whiskers represent mean  $\pm$  standard deviation normalized to GAPDH level in each sample. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . (Wilcoxon signed-rank test for A, Mann-Whitney U test for B and C). ccRCC, clear cell renal cell carcinoma; VHL, Von Hippel-Lindau; HIF, hypoxia-inducible factor; VEGF, vascular endothelial growth factor; qPCR, quantitative polymerase chain reaction.

kidney samples of the 36 patients with ccRCC by western blot analysis and the exemplary images are shown in Fig. 2. The expression levels of the analyzed proteins were increased in the tumor samples: The HIF1A, HIF2A and P53 levels were  $\sim 10$ - ( $P < 0.001$ ), 1.5- ( $P < 0.05$ ) and 4.8-fold ( $P < 0.001$ ) higher in the cancer tissues, respectively, compared with those in the normal kidney samples (Fig. 3A). No differences were observed in the pVHL and VEGFA protein levels between the cancerous and normal tissues (Fig. 3A). However, the expression levels of other proteins were increased in the tumor samples: When the expression levels were divided by their median values in normal tissues, higher levels of the HIF1A, HIF2A and P53 proteins in cancer tissues were detected in 63, 64 and 75% of the patients, respectively (Table IV). The complete absence

of the HIF1A protein was found in 10 normal and 3 cancer samples, whereas the absence of the HIF2A protein band was found in 4 normal samples and 1 tumor sample (data not shown). Such a result was not observed for the other proteins examined. When the western blot analysis results were compared with the clinicopathological data, no significant association between sex, age, tumor progression or tumor size and VHL or P53 protein levels was observed (Figs. 3B and C; Table IV). On the other hand, the protein ratios of HIF1A, HIF2A and VEGFA were markedly increased in the tumors of patients with higher Fuhrman's grades; the HIF1A levels were increased  $\sim 1.3$ -fold ( $P < 0.05$ ) in 22% of the samples (Fig. 3B and Table IV), whereas those of HIF2A were 3.3-fold higher ( $P < 0.001$ ) in 44% of the samples (Fig. 3B and Table IV). The VEGFA protein level was increased  $\sim 2$ -fold (44% of the samples) in samples with advanced Fuhrman's grades in comparison with samples with Fuhrman's 1+2 grades ( $P < 0.01$ ; Fig. 3B and Table IV). In addition, the expression levels of the HIF2A and VEGFA proteins were associated with metastatic ccRCC: The HIF2A protein level was  $\sim 2$ -fold higher ( $P < 0.001$ , Fig. 3C) in advanced tumor samples (36% of the samples, Table IV), whereas that of VEGFA protein was upregulated  $\sim 1.6$ -fold ( $P < 0.01$ ; Fig. 3C) in metastatic cases (39% of the samples; Table IV). A higher expression of HIF2A protein was also noted in larger tumors, with 17 of the 19 cases with tumors  $> 7$  cm exhibiting an increased HIF2A level (Table IV).

*Association between mRNA and protein expression levels of the analyzed genes.* The Spearman's correlation test was used to examine the possible associations between VHL-HIF1A/HIF2A-VEGFA expression at the mRNA and protein level, as well as P53 expression, in ccRCC samples. The results are presented in Table V. First, medium-to-strong positive correlations were observed between the mRNA and protein levels of each gene [from Spearman's rank correlation coefficient ( $r_s$ )=0.57 for P53 mRNA-protein to  $r_s$ =0.74 for HIF1A mRNA-protein;  $P < 0.05$ ; Table V]. Subsequently, it was observed that the expression of P53 did not correlate with the levels of any other analyzed genes. Notably, a weak negative correlation was observed between the levels of the VHL protein and the HIF1A and HIF2A proteins in ccRCC tissue ( $r_s$ =-0.13 and -0.19, respectively;  $P < 0.05$ ; Table V). There was also a medium-to-strong positive correlation between either *HIF1A* or *HIF2A* and *VEGFA* (at both the mRNA and protein level); the associations were stronger for *HIF1A* mRNA-*VEGFA* mRNA and *HIF2A* mRNA-*VEGFA* mRNA ( $r_s$ =0.71 and 0.73, respectively;  $P < 0.05$ ) compared with HIF1A protein-*VEGFA* protein or HIF2A protein-*VEGFA* protein ( $r_s$ =0.58 and 0.69, respectively; Table V). A weak positive correlation was also observed between the levels of *HIF1A* and *HIF2A* mRNA ( $r_s$ =0.42;  $P < 0.05$ ).

*Tissue localization of proteins.* Immunohistochemical staining for VHL, HIF1A, HIF2A, VEGFA and P53 was performed on paired tumor and normal kidney samples of 10 patients with ccRCC (3 patients with non-metastatic and 7 patients with metastatic ccRCC, of whom 3 were treated with sunitinib). As shown in Fig. 4A, in the unaffected part of the kidney, pVHL immunoreactivity was strong in the cytoplasm and nuclei of the epithelial cells of the proximal and distal



Table IV. Association between VHL, HIF1A, HIF2A, VEGFA and P53 protein levels and clinical data.

Patients n=36	Subgroups	pVHL			HIF1A			HIF2A			VEGFA			P53		
		↓	↑	P-value <sup>a</sup>	↓	↑	P-value <sup>a</sup>	↓	↑	P-value <sup>a</sup>	↓	↑	P-value <sup>a</sup>	↓	↑	P-value <sup>a</sup>
Age (years)	≤62, n=19	13 (36)	6 (17)	0.73	5 (14)	14 (39)	0.72	5 (14)	14 (39)	0.29	8 (22)	11 (31)	0.15	6 (17)	13 (36)	0.45
Median,	>62, n=17	10 (28)	7 (19)		6 (17)	9 (31)		8 (22)	9 (25)		3 (8)	14 (39)		3 (8)	14 (39)	
60.58±11.9																
Range, 33-82																
Sex	Female, n=17	13 (36)	4 (11)	0.17	4 (11)	13 (36)	0.48	9 (25)	8 (22)	0.08	8 (22)	9 (25)	0.07	8 (22)	9 (25)	0.07
	Male, n=19	10 (28)	9 (25)		7 (19)	12 (33)		4 (11)	15 (42)		3 (8)	16 (44)		3 (8)	16 (44)	
Tumor	≤7 cm, n=17	10 (28)	7 (19)	0.73	4 (11)	13 (36)	0.48	11 (31)	6 (17)	<b>0.001</b>	6 (17)	11 (31)	0.72	5 (14)	12 (33)	0.71
size (cm)	>7 cm, n=19	13 (36)	6 (16)		7 (19)	12 (33)		2 (5)	17 (47)		5 (14)	14 (39)		4 (11)	15 (42)	
Fuhrman's	1+2, n=18	10 (28)	8 (22)	1.00	1 (3)	17 (47)	<b>0.003</b>	12 (33)	7 (19)	<b>0.005</b>	9 (25)	9 (25)	<b>0.02</b>	7 (19)	11 (31)	0.12
histological	3+4, n=18	10 (28)	8 (22)		10 (28)	8 (22)		2 (6)	16 (44)		2 (6)	16 (44)		2 (6)	16 (44)	
grade																
TNM stage	Non-metastatic, n=21	15 (42)	6 (17)	0.31	6 (17)	15 (42)	1.00	11 (31)	10 (28)	<b>0.03</b>	10 (28)	11 (31)	<b>0.011</b>	8 (22)	13 (36)	0.05
	Metastatic, n=15	8 (22)	7 (19)		5 (14)	10 (28)		2 (5)	13 (36)		1 (3)	14 (39)		1 (3)	14 (39)	
Sunitinib	Yes, n=11	7 (19)	4 (11)	1.00	6 (20)	5 (17)	0.23	1 (3)	10 (31)	<b>0.01</b>	0 (0)	11 (31)	<b>0.015</b>	4 (11)	7 (19)	0.4
treatment	No, n=25	16 (44)	9 (25)		5 (17)	14 (47)		12 (38)	9 (28)		11 (31)	14 (38)		5 (14)	20 (56)	

<sup>a</sup>P-values were calculated by Fisher's 2x2 test. Downward arrows indicate decreased levels and upward arrows indicate increased levels. Values in bold indicate statistical significance (P<0.05). VHL, Von Hippel-Lindau; HIF, hypoxia-inducible factor; VEGF, vascular endothelial growth factor.

Table V. Summary results of Spearman's correlation tests between molecular data of *VHL*, *HIF1A*, *HIF2A*, *VEGFA* and *P53* gene expression.

Molecular data/rs <sup>a</sup>	<i>VHL</i> mRNA	<i>VHL</i> protein	<i>HIF1A</i> mRNA	<i>HIF1A</i> protein	<i>HIF2A</i> mRNA	<i>HIF2A</i> protein	<i>VEGFA</i> mRNA	<i>VEGFA</i> protein	<i>P53</i> mRNA	<i>P53</i> protein
<i>VHL</i> mRNA		0.63	NS	NS	NS	NS	NS	NS	NS	NS
<i>VHL</i> protein	0.63		NS	-0.13	NS	-0.19	NS	NS	NS	NS
<i>HIF1A</i> mRNA	NS	NS		0.74	0.42	NS	0.71	0.49	NS	NS
<i>HIF1A</i> protein	NS	-0.13	0.74		NS	0.69	0.60	0.58	NS	NS
<i>HIF2A</i> mRNA	NS	NS	0.42	NS		0.68	0.73	0.64	NS	NS
<i>HIF2A</i> protein	NS	-0.19	NS	0.69	0.68		0.72	0.69	NS	NS
<i>VEGFA</i> mRNA	NS	NS	0.71	0.60	0.73	0.72		0.73	NS	NS
<i>VEGFA</i> protein	NS	NS	0.49	0.58	0.64	0.69	0.73		NS	NS
<i>P53</i> mRNA	NS	NS	NS	NS	NS	NS	NS	NS		0.57
<i>P53</i> protein	NS	NS	NS	NS	NS	NS	NS	NS	0.57	

<sup>a</sup>rs, correlation slope; results shown if  $P < 0.05$ . VHL, Von Hippel-Lindau; HIF, hypoxia-inducible factor; VEGF, vascular endothelial growth factor.

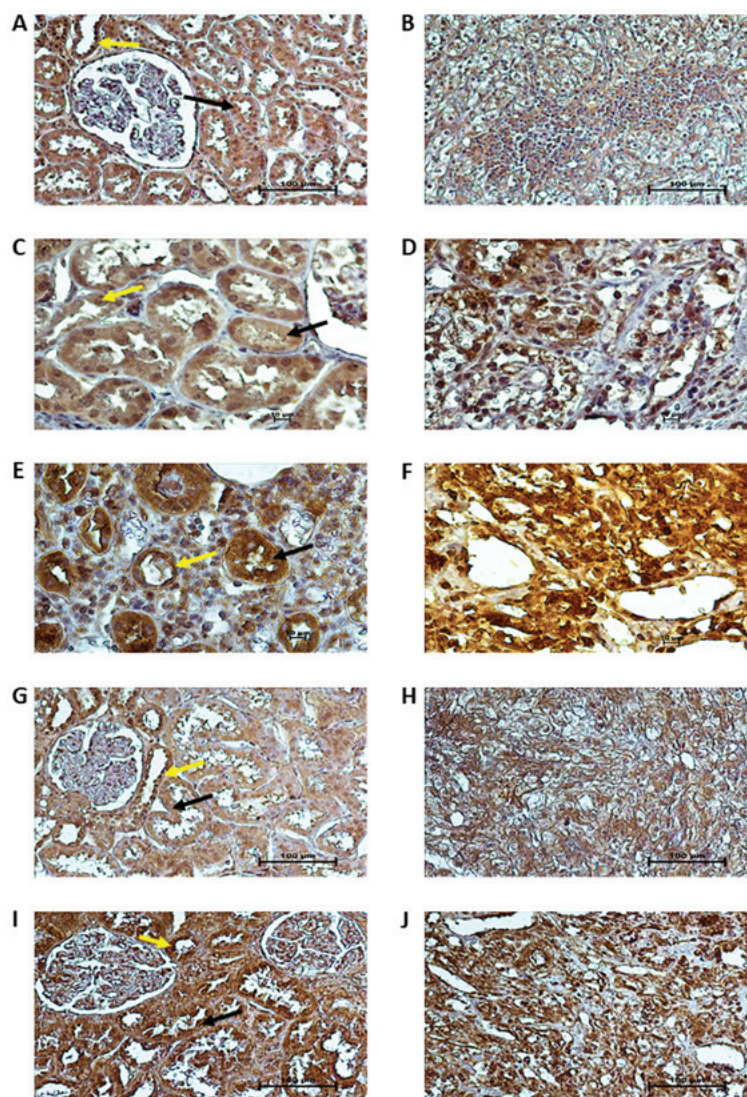


Figure 4. Localization of VHL, HIF1A, HIF2A, VEGFA and P53 proteins in ccRCC and normal kidney. Immunoreactivity for (A and B) VHL, (C and D) HIF1A, (E and F) HIF2A, (G and H) VEGFA and (I and J) P53 proteins in normal kidney (A, C, E, G and I) and TNM stage 3 and Fuhrman grade 3 ccRCC sections (B, D, F, H and J) was demonstrated by immunohistochemical staining, as described in the Materials and methods. Scale bars, 10  $\mu$ m (C-F) and 100  $\mu$ m (A, B and G-J). Black and yellow arrows indicate proximal and distal tubules of unchanged kidney morphological structure, respectively (A, C, E, G and I). ccRCC, clear cell renal cell carcinoma; VHL, Von Hippel-Lindau; HIF, hypoxia-inducible factor; VEGF, vascular endothelial growth factor.

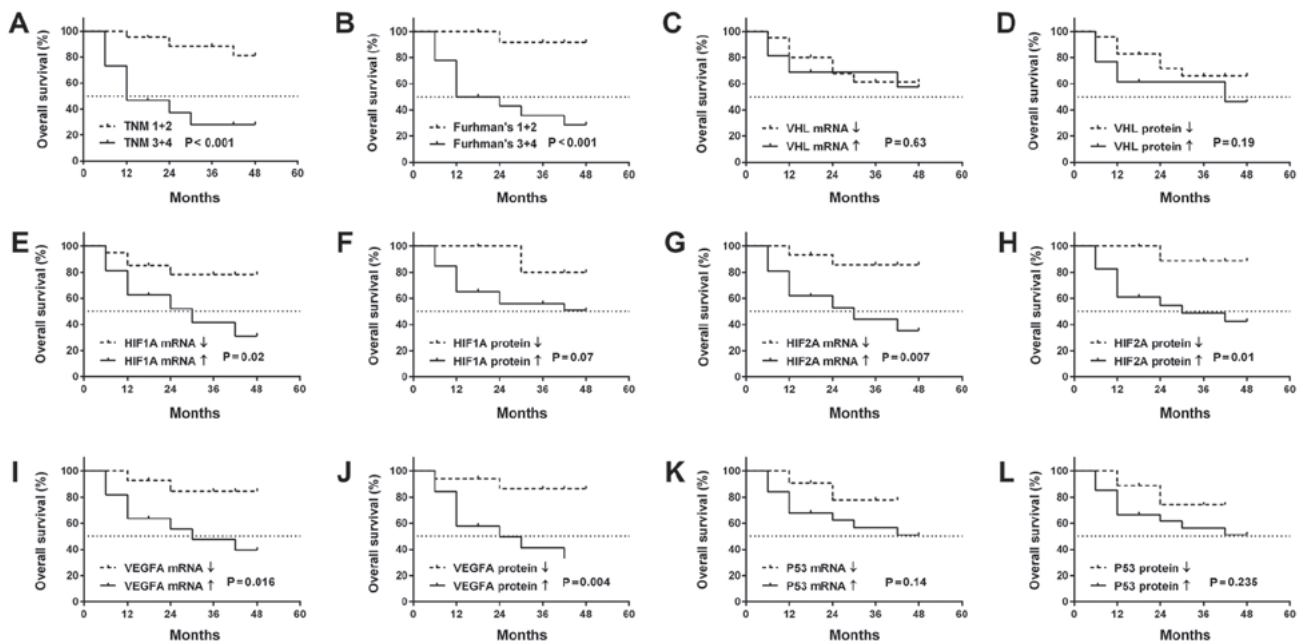


Figure 5. Kaplan-Meier's overall survival analysis for ccRCC patients related to clinicopathological and molecular data. (A) TNM classification. (B) Fuhrman grade. (C and D) *VHL* mRNA and protein expression, respectively. (E and F) *HIF1A* mRNA and protein levels, respectively. (G and H) *HIF2A* mRNA and protein expression, respectively. (I and J) *VEGFA* mRNA and protein levels, respectively. (K and L) *P53* mRNA mRNA and protein levels, respectively. Cut-off values between increased and decreased expression rates were arbitrarily classified using median expression values of each gene/protein in control samples. Log-rank (Mantel-Cox) test was applied. ccRCC, clear cell renal cell carcinoma; *VHL*, Von Hippel-Lindau; *HIF*, hypoxia-inducible factor; *VEGF*, vascular endothelial growth factor.

tubules (PT - black arrows and DT - yellow arrows in figures, respectively), whereas weaker pVHL immunoreactivity was noted in the cytoplasm and nuclei of tumor cells (Fig. 4B). *HIF1A* and *HIF2A* immunoreactivity was predominantly present in the nuclei of cancer cells (Fig. 4D and F), with a particularly strong expression of *HIF2A* (Fig. 4F). In normal kidney tissue, *HIF1A* protein expression was observed in the cytoplasm and nuclei of PT and DT epithelial cells (black and yellow arrows, respectively, Fig. 4C), whereas *HIF2A* protein expression was mainly localized in the cytoplasm of PT and DT cells (black and yellow arrows, respectively, Fig. 4E). The immunoreactivity of *VEGFA* was moderate in the cytoplasm of PT and DT cells (black and yellow arrows, respectively, Fig. 4G), whereas in the tumor specimens, *VEGFA* protein expression was strongly and homogeneously distributed in the cytoplasm of cancer cells (Fig. 4H). Finally, we observed very strong immunoreactivity of the *P53* protein in the cytoplasm and nuclei of both tumor (Fig. 4J) and normal kidney cells (stroma, black arrows for PT and yellow arrows for DT; Fig. 4I).

**Associations of clinicopathological and molecular data with patient outcome.** We found that higher Fuhrman's grade and TNM stage were associated with OS (Fig. 5A and B). No association was observed between the OS of patients and other clinicopathological parameters (data not shown). It was observed that patients with an increased expression of *HIF1A* (mRNA), *HIF2A* (mRNA and protein) and *VEGFA* (mRNA and protein) in ccRCC exhibited a shorter OS (Fig. 5E and G-J). There was no observed association between OS and *VHL* or *P53* gene expression, neither at the mRNA nor the protein level (Fig. 5C, D, K and L).

Similarly to OS, PFS was associated with a higher Fuhrman's grade and TNM stage (Fig. 6A and B), while no other clinicopathological values were related to PFS (data not shown). Patients with increased levels of *HIF1A* (mRNA and protein), *HIF2A* (mRNA and protein), as well as *VEGFA* (mRNA and protein) were characterized by a shorter PFS (Fig. 6E-J). There was no connection between levels of mRNA or protein of the *VHL* and *P53* genes and the occurrence of cancer progression (Fig. 6C, D, K and L).

Cox proportional hazards test with multivariable regression indicated that an increased *HIF2A* expression at the mRNA and protein level, as well as increased *VEGFA* protein levels, were independent prognostic factors of a worse outcome (Table VI). Moreover, increased levels of the *HIF2A* and *VEGFA* proteins were independent prognostic factors of earlier cancer progression (Table VII). The administration of sunitinib was not introduced to the Cox hazards test, since the treatment was applied after the acquisition of the biological material.

**Gene expression in the tissues of patients treated with sunitinib.** The molecular data of the *VHL*, *HIF1A*, *HIF2A*, *VEGFA* and *P53* genes at the mRNA and protein level in ccRCC tissues were analyzed according to adjuvant sunitinib treatment. As shown in Fig. 7A, the *HIF2A* and *VEGFA* mRNA levels were ~1.3- and 1.5-fold higher in the tissues of sunitinib-treated patients compared with those in patients not receiving adjuvant treatment ( $P < 0.05$ ). However, when the samples were divided according to the median values of the gene expression in the controls, no statistically significant differences were observed (Table III, bottom section). Unlike the results observed at the mRNA level, parallel statistical associations were observed at

Table VI. Univariable and multivariable Cox regression analysis of the overall survival rate of ccRCC patients.

Parameters	Univariable analysis		Multivariable analysis	
	P-value	HR (95% CI)	P-value	HR (95% CI)
Sex	0.38	1.63 (0.53-5.01)		
Female vs. male				
Age	0.91	0.93 (0.31-2.78)		
>62 vs. ≤62 (years)				
Tumor size	0.48	1.49 (0.49-4.41)		
>7 vs. ≤7 (cm)				
Tumor grade	<b>0.003</b>	<b>6.96 (1.89-25.68)</b>	0.11	3.04 (0.77-11.92)
T3+4 vs. T1+2)				
Histological grade				
F3+4 vs. F1+2	<b>0.008</b>	<b>16.25 (2.11-125.53)</b>	<b>0.038</b>	9.64 (1.12-82.32)
VHL mRNA levels	0.65	0.77 (0.26-2.32)		
↓ vs. ↑				
VHL protein levels	0.21	4.57 (1.35-15.37)		
↓ vs. ↑				
HIF1A mRNA levels	<b>0.032</b>	<b>0.27 (0.08-0.89)</b>	0.09	0.99 (0.21-4.55)
↑ vs. ↓				
HIF1A protein levels	0.089	5.85 (0.76-45.06)		
↑ vs. ↓				
HIF2A mRNA levels	<b>0.021</b>	<b>5.98 (1.31-27.44)</b>	<b>0.03</b>	<b>3.21 (0.52-19.72)</b>
↑ (>17.363) vs. ↓ (≤17.363)				
HIF2A protein levels	<b>0.031</b>	<b>8.51 (1.11-65.62)</b>	<b>0.04</b>	<b>3.07 (0.22-42.19)</b>
↑ vs. ↓				
VEGFA mRNA levels	<b>0.037</b>	<b>5.02 (1.11-22.94)</b>	0.09	1.09 (0.13-9.24)
↑ vs. ↓				
VEGFA protein levels	<b>0.014</b>	<b>6.58 (1.44-30.03)</b>	<b>0.019</b>	<b>2.34 (0.34-16.06)</b>
↑ vs. ↓				
P53 mRNA levels	0.18	2.78 (0.61-12.57)		
↑ vs. ↓				
P53 protein levels	0.33	2.11 (0.46-9.53)		
↑ vs. ↓				

Values in bold indicate statistical significance (P<0.05). Downward arrows indicate decreased levels and upward arrows indicate increased levels. Values in bold indicate statistical significance (P<0.05). VHL, Von Hippel-Lindau; HIF, hypoxia-inducible factor; VEGF, vascular endothelial growth factor.

the protein level; the HIF2A protein level was ~2-fold higher in 10 of the 11 sunitinib-treated patients (P<0.05), whereas the VEGFA protein level was ~2.5-fold higher in the cancer tissues of sunitinib-treated patients compared with patients not receiving adjuvant treatment (Table IV, bottom section; Fig. 7B).

*Effect of sunitinib treatment on patient outcome.* As shown in Fig. 8A, post-operative treatment with sunitinib had no positive effect on the survival of patients with ccRCC, with a median survival of sunitinib-treated patients of 12 months. On the contrary, 80% of the patients with ccRCC who did not receive sunitinib had a positive outcome (Fig. 8A, solid

line). Comparable results were obtained for cancer recurrence: The median PFS for sunitinib-treated patients was 12 months, whereas the cancer-related deterioration of the health status was not observed in >60% patients not receiving sunitinib treatment (Fig. 8B). The molecular data presented in Fig. 7 and Tables III and IV revealed the possible association between patient outcome, sunitinib treatment and the cancer tissue levels of the VEGFA and HIF2A proteins. In the sunitinib-treated ccRCC patients who had high HIF2A and VEGFA protein levels in their tumor tissues, the median OS was not altered (Fig. 8B); however, the PFS decreased to 3 months (Fig. 8D). The multivariable regression revealed moderate-to-strong associations between sunitinib treatment, increased levels of



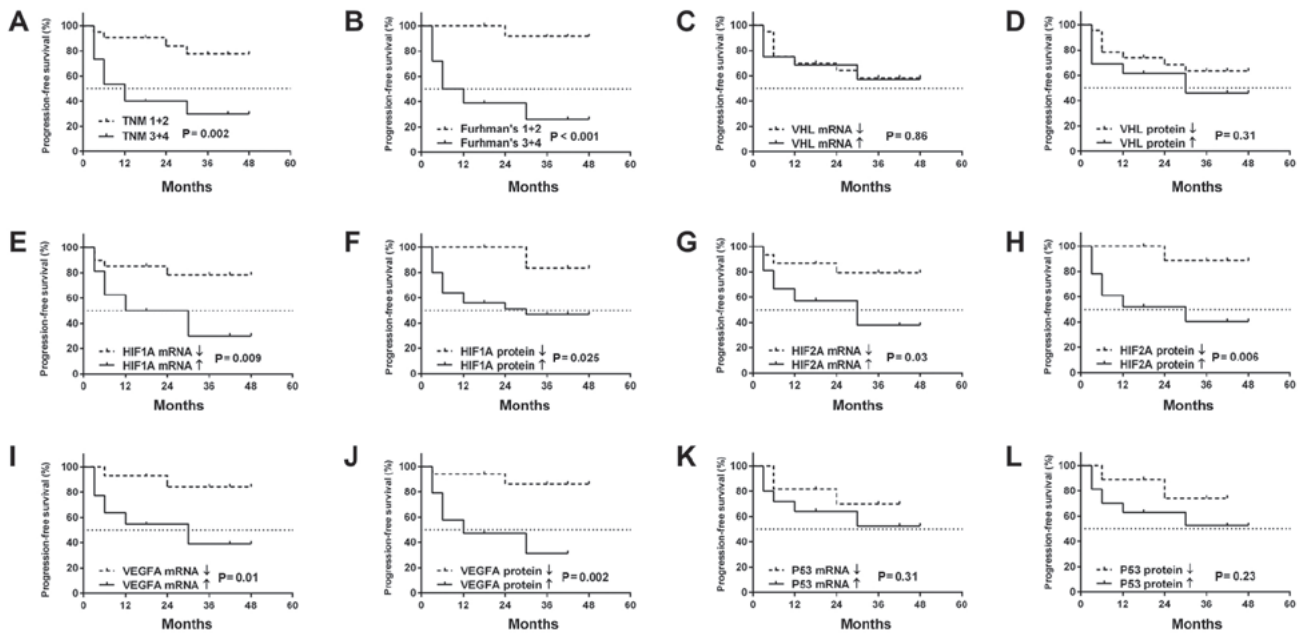


Figure 6. Kaplan-Meier's progression-free survival analysis of ccRCC patients related to clinicopathological and molecular data. (A) TNM classification. (B) Fuhrman grade. (C and D) *VHL* mRNA and protein expression, respectively. (E and F) *HIF1A* mRNA and protein levels, respectively. (G and H) *HIF2A* mRNA and protein expression, respectively. (I and J) *VEGFA* mRNA and protein levels, respectively. (K and L) *P53* mRNA and protein levels, respectively. Cut-off values between increased and decreased expression rates were arbitrarily classified using median expression values of each gene/protein in control samples. Log-rank (Mantel-Cox) test was applied. ccRCC, clear cell renal cell carcinoma; VHL, Von Hippel-Lindau; HIF, hypoxia-inducible factor; VEGF, vascular endothelial growth factor.

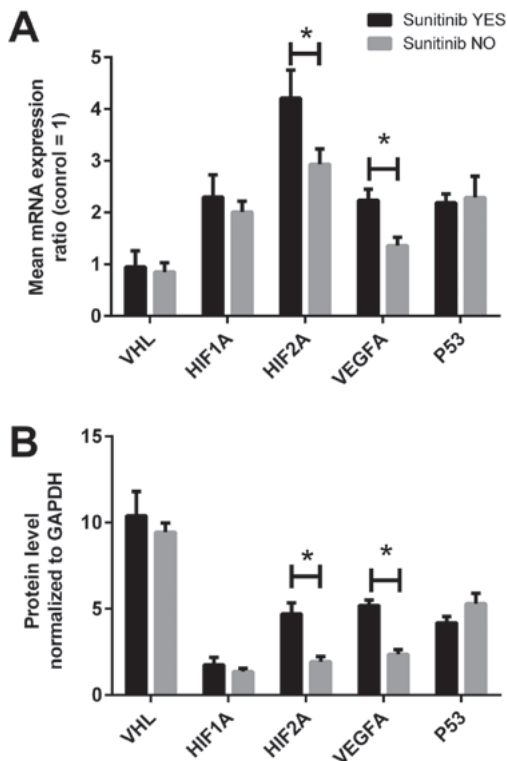


Figure 7. Expression profiles of *VHL*, *HIF1A*, *HIF2A*, *VEGFA* and *P53* genes in tumor samples of ccRCC patients divided by sunitinib administration. Comparison between patients treated postoperatively with sunitinib (n=11) vs. those receiving no adjuvant therapy (n=25). (A) mRNA levels of genes assessed by qPCR, results normalized to the  $\beta$ -glucuronidase gene followed by normalization to control samples (not shown); (B) protein ratios assessed by western blotting, results normalized to GAPDH protein. Bars and whiskers represent mean  $\pm$  standard deviation. \* $P<0.05$  (Mann-Whitney U test). ccRCC, clear cell renal cell carcinoma; HIF, hypoxia-inducible factor; VEGF, vascular endothelial growth factor.

both *VEGFA* and *HIF2A* proteins, and either death ( $b=0.57$ ,  $P<0.001$ , Fig. 9A) or disease progression ( $b=0.69$ ;  $P<0.001$ , Fig. 9B).

## Discussion

RCC is the 6th and 10th most common malignancy in males and females, respectively, in the USA, according to the estimated number of new cases in 2017 (1). More than 300,000 patients are diagnosed annually, of whom ~143,000 succumb to the disease each year, thus rendering RCC the 16th most common cause of cancer-related mortality worldwide (1,22). ccRCC is the most common histological subtype of RCC. At the molecular level, deregulation of the expression of the pVHL-HIFs-*VEGFA* pathway proteins is frequently observed in ccRCC (42,43).

The aim of the present study was to simultaneously assess the status of the VHL-HIF1/2A-*VEGFA* pathway in a group of ccRCC patients from one clinical center. Similar to our previous studies on ccRCC (13,33,41) qPCR was selected to access gene expression at the mRNA level. At the protein level, a semi-quantitative technique (western blot analysis) was applied for all studied ccRCC samples and IHC staining for the material available from 10 patients. The statistical compatibility of the western blot analysis and IHC methods has been noted by authors who analyzed human epidermal growth factor receptor 2/neu (44) or periostin (45) proteins in human breast cancer tissues (44) or cells (45), claudin-2 and -5 proteins in gastric carcinoma (46), or for the validation of antibody clones for immunochemistry in non-small-cell lung cancer (47).

There is a common consensus that the majority of ccRCCs are initiated by the somatic inactivation of the VHL tumor suppressor gene (48,49). pVHL is a multifunctional factor

Table VII. Univariable and multivariable Cox regression analysis of the progression-free survival rate of ccRCC patients.

Parameters	Univariable analysis		Multivariable analysis	
	P-value	HR (95% CI)	P-value	HR (95% CI)
Sex	0.29	1.81 (0.61-5.4)		
Female vs. male				
Age	0.71	0.82 (0.28-2.35)		
>62 vs. ≤62 (years)				
Tumor size	0.76	1.17 (0.41-3.35)		
>7 vs. ≤7 (cm)				
Tumor grade	<b>0.007</b>	<b>5.01 (1.54-16.18)</b>		
T3+4 vs. T1+2				
Histological grade				
F3+4 vs. F1+2	<b>0.004</b>	<b>18.66 (2.42-143.35)</b>	<b>0.028</b>	6.64 (1.12-16.32)
VHL <i>mRNA</i> levels	0.86	0.91 (0.31-2.65)		
↓ vs. ↑				
VHL protein levels	0.34	0.59 (0.21-1.72)		
↓ vs. ↑				
HIF1A <i>mRNA</i> levels	<b>0.02</b>	<b>0.25 (0.08-0.81)</b>	0.09	0.96 (0.21-4.55)
↑ vs. ↓				
HIF1A protein levels	0.06	6.83 (0.98-52.27)		
↑ vs. ↓				
HIF2A <i>mRNA</i> levels	0.05	3.62 (0.99-13.22)		
↑ (>17.363) vs. ↓ (≤ 17.363)				
HIF2A protein levels	<b>0.029</b>	<b>9.57 (1.25-73.44)</b>	<b>0.031</b>	<b>3.75 (0.28-49.27)</b>
↑ vs. ↓				
VEGFA <i>mRNA</i> levels	<b>0.028</b>	<b>5.37 (1.19-24.26)</b>	0.07	2.31 (0.33-15.69)
↑ vs. ↓				
VEGFA protein levels	<b>0.009</b>	<b>7.32 (1.62-32.97)</b>	<b>0.04</b>	<b>2.34 (0.31-17.27)</b>
↑ vs. ↓				
P53 <i>mRNA</i> levels	0.34	1.86 (0.51-6.68)		
↑ vs. ↓				
P53 protein levels	0.26	2.34 (0.53-10.48)		
↑ vs. ↓				

Values in bold indicate statistical significance ( $P < 0.05$ ). Downward arrows indicate decreased levels and upward arrows indicate increased levels. Values in bold indicate statistical significance ( $P < 0.05$ ). VHL, Von Hippel-Lindau; HIF, hypoxia-inducible factor; VEGF, vascular endothelial growth factor.

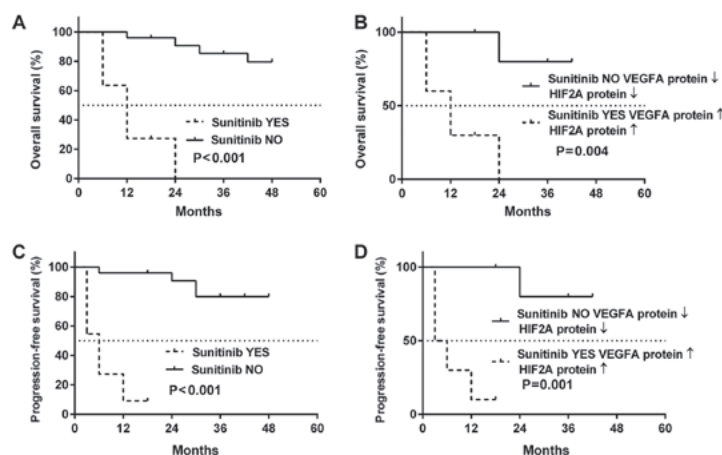


Figure 8. Overall and progression-free survival analyses of ccRCC patients with regard to post-operative treatment with sunitinib and selected molecular markers. (A and C) Survival and cancer recurrence plots of 11 patients who received postoperative treatment with sunitinib. The outcome analysis of (B) overall and (D) progression-free survival was extended by taking the protein levels of both HIF2A and VEGFA into consideration, as detailed in Results. Log-rank (Mantel-Cox) test was applied. ccRCC, clear cell renal cell carcinoma; HIF, hypoxia-inducible factor; VEGF, vascular endothelial growth factor.

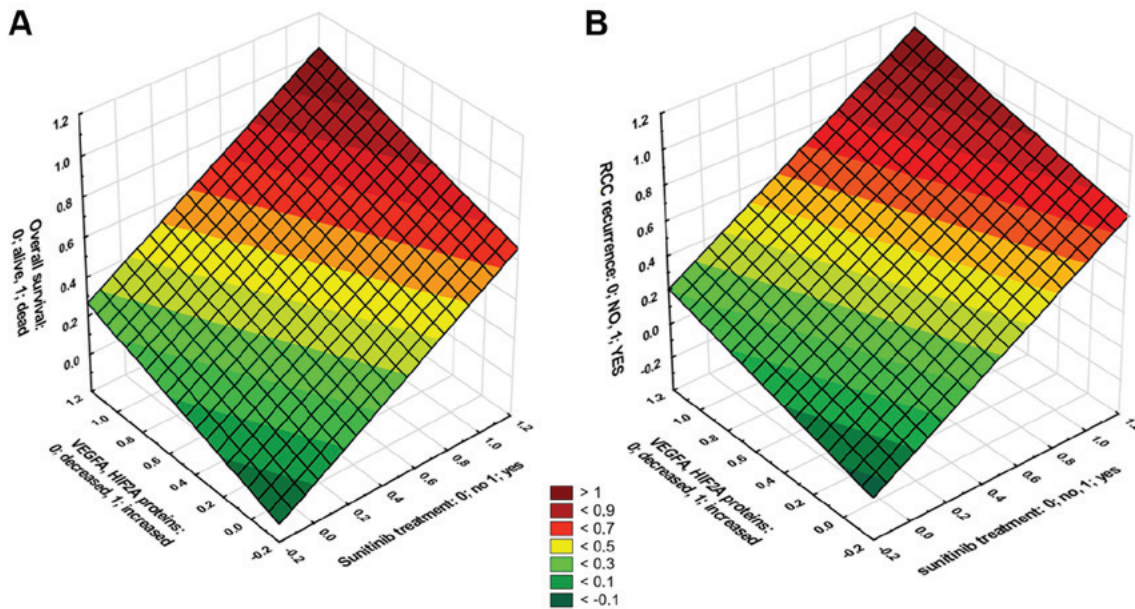


Figure 9. Association between VEGFA and HIF2A protein expression, patient outcome and sunitinib treatment. Graphic presentation of multivariate regression analysis of the possible association between overexpression of the VEGFA and HIF2A proteins in tumor samples (independent variable), (A) overall or (B) progression-free survival and postoperative treatment with sunitinib. The variables were counted in non-parametric (0, 1) order, according to events (sunitinib usage or outcome) or VEGFA and HIF2A expression rates (high or low). Yellow-to-red areas represent increasingly significant associations between variables. HIF, hypoxia-inducible factor; VEGF, vascular endothelial growth factor.

that acts as an adaptor protein recruiting different effector proteins to different cellular targets, thereby regulating various cellular processes (22). It has been demonstrated that pVHL may trigger various processes, such as glucose uptake and metabolism (50), angiogenesis (50,51), the suppression of epithelial-to-mesenchymal transition (52), cell proliferation, survival or apoptosis (50,51), or activation of the P53 pathway (22,53). Since all the mentioned processes may be involved in the mechanisms underlying carcinogenesis, the expression of the *VHL* gene in ccRCC has been extensively studied. Although the contribution of the mutated *VHL* gene to ccRCC initiated in the course of Von Hippel-Lindau disease has been described (22,54), further studies of this gene in sporadic ccRCC are warranted. We have previously identified molecular prognostic markers of ccRCC that were involved in the Hippo pathway (13,41,55). Although in the present study, a decreased *VHL* mRNA level was observed in tumor samples, this was not found to be associated with the clinicopathological data of the patients or with their outcome or sunitinib treatment. Similar to our results, Xiao-Fen *et al* reported the underexpression of *VHL* mRNA in tumor samples from 75 RCC patients (the number of ccRCC cases was not specified) in comparison to normal kidney tissues (23). However, in contrast to our findings, Xiao-Fen *et al* observed that this decreased expression was associated with cancer TNM progression and tumor size, although the lack of data on the histological subtypes of RCC precludes a direct comparison between their results (23) and ours. Högner *et al* (56), using qPCR, observed a strong underexpression of *VHL* mRNA in 80.6% of the studied 69 ccRCC patients; however, they investigated the associations between clinicopathological and molecular data. On the contrary to the listed studies, deep-genome and mRNA studies on 48 ccRCC cases conducted by Girgis *et al* did not reveal any changes in *VHL* mRNA levels, despite the

observed hypermethylation of the *VHL* genomic locus (57). Other studies on *VHL* gene expression were mainly based on DNA analysis focusing on the hypermethylation status of the *VHL* gene promoter locus or mutational analysis of the *VHL* exons (5,22,23,57-59). Since previous investigations of the tumor tissues from patients with ccRCC treated with sunitinib focused on *VHL* mutations (60-62), to the best of our knowledge, the present study is the first to investigate the expression of *VHL* at both the mRNA and protein level in patients with ccRCC treated with first-line sunitinib. Finally, our findings of weaker immunoreactivity of pVHL demonstrated by IHC staining are only mentioned in a few studies (63,64) in which the authors reported either markedly decreased pVHL immunoreactivity in early ccRCC (63), or a reduction trend of pVHL expression in association with ccRCC progression (64).

The most well-known function of the VHL protein is the regulation of cellular response to oxygen depletion via maintenance of the cellular lifespan of HIF transcription factors. There is a rapid proteasomal degradation of HIFs during normoxia; however, no association between pVHL and HIFs has been established during tumor-associated hypoxia (65). In this study, although high levels of *HIF1A* and *HIF2A* expression were observed at both the mRNA and protein level in ccRCC samples, there was no observed association between *HIF1A/2A* and *VHL* protein levels. Therefore, it may be hypothesized that the control of pVHL upon HIF degradation was incomplete in the analyzed ccRCC samples. This is supported by Nyham *et al* (66), who observed the overexpression of *HIF1A* and *HIF2A* at the protein level, as assessed by western blot analysis in 88 and 100% of 17 ccRCC patients, respectively, regardless of the genetic mutations of *VHL* that were detected in 43.5% of the tumors (66); therefore, they proposed that control of the degradation of HIFs by an intact pVHL is not effective during



ccRCC progression (66). The results of this study, demonstrating highly increased levels of HIF proteins in samples with intact levels of pVHL, may support this hypothesis, although our focus was not VHL genetic alterations.

The increased levels of *HIF1A* and *HIF2A* at both the mRNA and protein level in ccRCC samples observed in the present study confirm previous findings (24,26-28,67). For example, Turner *et al* investigated a group of 34 ccRCC patients and observed overexpression of HIFs at the mRNA and protein level; the *HIF1A* mRNA level was ~2-fold and the *HIF2A* mRNA was 5-fold higher in tumor samples compared with normal kidneys (28). Using western blot analysis, the authors revealed the upregulation of HIF1A and HIF2A protein levels in 77 and 79% of the tumor cases, respectively (28). The level of HIF proteins were positively associated with the increased number of blood vessels in tumor tissue, suggestive of neoangiogenesis (28). Similar to our findings, a positive correlation between HIFs and *VEGFA* expression at both the mRNA and protein level was also reported (28).

The finding of a shorter OS in ccRCC patients exhibiting increased levels of HIF1A, HIF2A and VEGFA in the present study was consistent with the results reported by Ebru *et al* (27). Using IHC in a group of 72 ccRCC patients, they observed a strong association between shorter OS and high expression of HIF1A, HIF2 and VEGFA, as well as Ki-67 protein and microvessel density (27). Another study has also reported a high immunoreactivity of HIF1A, HIF2 and VEGFA in the nuclei or/and cytoplasm of ccRCC cells (68).

High levels of HIF2A, but not HIF1A, were found to be associated with the risk of death and cancer recurrence, independent of sunitinib treatment. The stronger impact of HIF2A, rather than HIF1A, on ccRCC progression has also been previously reported (65,69-75). Maroto *et al* observed a similar pattern of shorter PFS in 10 sunitinib-treated patients, but only for both HIF2A- and c-Myc-positive cases (at the protein level) (76). Philips and Atkins also reported that HIF2A is more relevant in ccRCC development and progression compared with HIF1A, assuming that HIF2A is the dominant oncogene in RCC (75). Shen *et al* further suggested the oncogenic role of HIF2A and the tumor-suppressive role of HIF1A in the development of pVHL-defective RCCs (69). The dual role of HIF1A was also reported by Lindgren *et al* (70); favorable prognosis of 92 RCC patients (including 66 ccRCC cases) was observed in association with increased HIF1A protein levels, as determined by western blot analysis (70). Furthermore, studies on pre-neoplastic kidney lesions of patients with VHL disease also support the key role of HIF2A in the transformation of dysplastic cells, as the HIF2A expression was increased while that of HIF1A was decreased, as assessed by IHC (71,72). The association between high levels of HIF2A and ccRCC progression has also been observed in cell lines representing various stages of RCC progression (73,74) or in mouse xenograft models (77). In a clinical study, Kamai *et al* analyzed tumor samples obtained from 129 patients with ccRCC, and found that increased expression of the HIF2A protein (measured by western blot analysis) was associated with worse clinical status, local and distant metastasis, and shorter OS (78). The possibility of using HIF2A inhibitors to block ccRCC progression and recurrence was recently suggested (75,79,80). Therefore, novel

HIF2A antagonists were developed, such as PT2399 (80) and PT2385 (81), which block the PAS-B domain of the HIF2A subunit. Furthermore, new-generation dual mammalian target of rapamycin inhibitors (GDC-0980 and BKM120), which block both TORC1 and TORC2 activity, including HIF2A expression, have been introduced to phase I trials of advanced RCC (75,82). Whereas these data confirm the importance of HIF2A inhibition in modern ccRCC chemotherapy, the results of the present study strongly suggest that the measurement of HIF2A protein levels may serve as independent prognostic marker in ccRCC, also in sunitinib-treated patients (76). However, it must be noted that Beuselinck *et al* (62) observed high levels of *HIF2A* mRNA in sunitinib-sensitive ccRCCs. The differences in methodology may explain the opposite observations, since Beuselinck *et al* used  $\beta$ -actin as a reference gene (62), which may have affected the results (33), whereas we used  $\beta$ -glucuronidase, which was carefully normalized for ccRCC samples in our previous study (33).

It has been widely confirmed that HIF1A, as well as HIF2A, trigger transcription of the *VEGFA* gene to VEGFA, and its receptors (VEGFRs) play pivotal roles in vasculogenesis and angiogenesis under physiological conditions, as well as in cancer, including ccRCC (10,24,50,54,65,74,80,83). The present study also revealed that the expression of *VEGFA*, either at the mRNA or protein level, was strongly associated with ccRCC progression, and that patients with higher VEGFA expression exhibited a poorer outcome and earlier recurrence of cancer. Additionally, Cox analysis revealed that ccRCC patients with high levels of VEGFA mRNA in tumor tissues had an increased risk of cancer progression, while an increased risk of death was associated with high levels of the VEGFA protein in tumor samples. Other studies have also reported the important effect of VEGFA expression on the progression of ccRCC (27,83-85), while Wang *et al* also revealed that high levels of *VEGFA* mRNA may serve as a prognostic marker in ccRCC (83).

The IHC detection of VEGFA has been widely used for the assessment of its expression in ccRCC tissues (5,27,83-85). Our findings demonstrated an increased cytoplasmic presence of VEGFA in cancer cells, consistent with previous reports (5,27,83-85). For example, Veselaj *et al* (84) and Dagher *et al* (5) observed that increased VEGFA immunoreactivity in tumor samples from ccRCC patients was associated with cancer progression and disease-free survival (5,84), as well as with an increased risk of death (84).

Finally, in this study, we investigated whether the *P53* gene expression pattern was associated with either ccRCC progression or the expression of VHL-HIFs-VEGFA axis components, since such an approach has not yet been reported, at least to the best of our knowledge. Although higher *P53* mRNA and protein levels were detected in tumor samples, there was no obvious association between *P53* expression and clinicopathological or molecular data of the other analyzed genes and proteins. Our finding of the strong immunoreactivity of the *P53* protein in tumor cells is consistent with other reports on *P53* in ccRCC (27). Shi *et al* observed a higher expression of *P53* at the mRNA and protein level, as assessed by qPCR, western blot analysis and IHC, in tumor samples, but without any association with clinical variables (86). Ebru *et al* did not identify a connection



between P53 protein expression, as determined by IHC, and the outcome of 62 ccRCC patients (27). Furthermore, the authors did not observe any associations between P53 and HIF1A, HIF2A or VEGFA protein immunoreactivity in the same samples (27). As regards first-line sunitinib treatment, Zhu *et al* observed that high levels of P53, as determined by IHC, were associated with favorable OS; however, they did not include sunitinib-resistant cases in their study (87). Based on the results of the present and previous studies, it may be suggested that *P53* gene expression at the mRNA or protein level is not associated with ccRCC progression. Moreover, the present study is, to the best of our knowledge, the first to analyze P53 expression in sunitinib-resistant ccRCC.

Sunitinib is selective inhibitor of multiple receptor tyrosine kinases, including VEGFRs (1-3), PDGFRs and c-kit, and it was approved by FDA in 2006 (2009 in Poland) for the treatment of metastatic RCC (17). It has been demonstrated that patients treated with sunitinib had longer median PFS compared with those treated with interferon (11.0 vs. 5.1 months, respectively) (17). However, later studies did not yield such optimistic values, since most of the advanced RCCs treated with sunitinib developed intrinsic drug resistance (18-20). The results of this study demonstrated that patients who were treated with sunitinib post-operatively had significantly shorter OS and PFS compared with ccRCC patients who did not receive such treatment. Since there was no difference in the pre-operative clinical status between sunitinib-treated and non-treated patient groups, it may be hypothesized that the poor outcome may be due to the drug treatment. In addition, Busch *et al* analyzed the outcome of 35 metastatic RCC cases (29 ccRCCs) who received first-line sunitinib treatment, and reported a poor prognosis due to intrinsic drug resistance (88). Lim *et al* observed a shorter OS and PFS in 33 out of 134 metastatic ccRCC patients treated with first-line sunitinib (21). A recent meta-analysis of adjuvant therapy in metastatic RCC did not reveal an increase of OS or DFS in association with sunitinib treatment; however, such therapy was associated with severe adverse events (20). Duran *et al* stated that, eventually, all patients with advanced RCC will become resistant to first-line TKIs, suggesting that second-line treatment should be introduced (43). The molecular mechanism underlying intrinsic resistance to first-line sunitinib treatment remains elusive (21); however, molecular studies on sunitinib resistance in RCC are currently in progress. Giuliano *et al* observed overexpression of the ABCB1 gene, which participates in the accumulation of the drug in autolysosomes of 786-O and RCC10 cell lines, favoring cellular efflux of sunitinib (89). Butz *et al* observed downregulation of miR-1 and miR-663a targeting FRAS1 and MDGA1 gene expression in a sunitinib-resistant ccRCC xenograft model (90). The results of the present study, showing higher HIF2A and VEGFA mRNA and protein expression, with no underexpression of pVHL, in sunitinib-treated ccRCC patients compared with those receiving no adjuvant treatment, must be verified by further studies, since only Beuselinck *et al* observed the opposite pattern of high HIF2A and VEGFA levels in sunitinib-sensitive tumor samples (62). Notably, recent results on sunitinib-resistant RCC cell lines (786-0, Caki-1, Caki-2 and SN12K1) reported by Kamli *et al*

partially support our findings, as Caki-2 and SN12K1 cells exhibited overexpression of VEGFA (91).

In conclusion, based on the observation that sunitinib-treated ccRCC patients with high levels of VEGFA and HIF2A protein expression and unchanged levels of pVHL in tumor samples are characterized by higher risk of death and cancer recurrence, we recommend that cRCC patients with this molecular profile are not administered sunitinib as first-line treatment. However, since only 11 of the 36 analyzed ccRCC patients were treated with sunitinib, this conclusion is merely a hypothesis, and large-scale replication studies in independent subject panels are required to validate the results. Moreover, the assessment of VHL, VEGFA and HIF2A protein levels in ccRCC tissues in the future may prove to be helpful in selecting an effective drug treatment.

### Acknowledgements

Not applicable.

### Funding

The present study was funded by the ST-12 and ST-02-0117/07 internal funds of the Medical University of Gdańsk, Poland.

### Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

### Authors' contributions

PMW performed the statistical analyses and drafted the manuscript. PMW and JK conceived and designed the study. JK collected tissue samples and patient data and revised the manuscript. AKC, AW, MS, AR and AL performed molecular analyses and statistical tests. ZK substantially contributed in the interpretation of the results, as well as revised the manuscript and provided funds, and MM collected tissue samples and provided funds. All the authors have read and approved the final version of this manuscript.

### Ethics approval and consent to participate

The study was approved by the Independent Bioethics Commission at the Medical University of Gdańsk (permission no. NKEBN/4/2011) and written consent was obtained prior to surgery from all patients. All experimental procedures were performed according to the regulations and internal biosafety and bioethics guidelines.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests to disclose.

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