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Expression of nuclear FIH independently predicts overall survival of clear cell renal cell carcinoma patients

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ABSTRACT

Aim: The hypoxia inducible factor (HIF) pathway plays an important role in sporadic clear cell renal cell carcinoma (ccRCC) by stimulating processes of angiogenesis, cell proliferation, cell survival and metastases formation. Herein, we evaluate the significance of upstream proteins directly regulating the HIF pathway; the prolyl hydroxylases domain proteins (PHD)1, 2 and 3 and factor-inhibiting HIF (FIH), as prognostic markers for ccRCC.

Methods: Immunohistochemical marker expression was examined on a tissue microarray containing tumour tissue derived from 100 patients who underwent nephrectomy for ccRCC. Expression levels of HIF, FIH and PHD1, 2 and 3 were correlated with overall survival (OS) and clinicopathological prognostic factors.

Results: HIF-1α was positively correlated with HIF-2α ($p < 0.0001$), PHD1 ($p = 0.024$), PHD2 ($p < 0.0001$), PHD3 ($p = 0.004$), FIH ($p < 0.0001$) and VHL ($p = 0.031$). HIF-2α levels were significantly associated with FIH ($p < 0.0001$) and PHD2 ($p = 0.0155$). Mutations in the *VHL* gene, expression variations of HIF-1α, HIF-2α and PHD1, 2, 3 did not show a correlation to OS or clinicopathological prognostic factors. Tumour stage, grade, diameter, metastastic disease and intensity of nuclear FIH were significantly correlated to OS in univariable analysis ($p = 0.023$). Low nuclear FIH levels remained a strong independent prognostic factor in multivariable analysis ($p = 0.009$).

Conclusion: These results show that low nuclear expression of FIH is a strong independent prognostic factor for a poor overall survival in ccRCC.

1. Introduction

With a worldwide incidence of approximately 200,000 new cases and a mortality of 102,000 patients each year renal cell carcinoma (RCC) is one of the most lethal genitourinary malignancies.[1] Of all RCC patients approximately 30% will develop local or distant recurrences within 5 years after initial curative treatment.[2] Observational follow-up, succeeded by

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targeted therapy once metastases are present, remains the postoperative standard of care for these patients. Even with first line therapy, 5 year survival of metastasised RCC (mRCC) is less than 20%.[3] Improvements in identification of patients at risk of developing metastatic disease are therefore important.

Currently available prognostic models for non-metastasised disease, such as the University of California Integrated Staging System (UISS) and Stage, Size, Grade and Necrosis (SSIGN) score, are mainly based on clinical and pathologic variables.[4,5] The most important conventional features are tumour-node-metastasis (TNM) stage, Fuhrman grade[6] and Eastern Cooperative Oncology Group performance status (ECOG PS). Risk stratification with these features is possible, although the described accuracies vary.[7–9] In addition to clinical and pathologic prognostic parameters, tissue biomarkers can play a significant role in predicting prognosis. For example in breast cancer, tissue marker detection for gene expression profiles indicating prognosis and treatment response has become common practice.[10]

Since the understanding of molecular pathways underlying RCC has increased dramatically in recent years, the search for prognostic tissue biomarkers has been facilitated.

In clear cell RCC (ccRCC), the molecular hypoxia response pathway, in which hypoxia-inducible factors-α (HIF-1α and HIF-2α) play a central role, is crucial[11] (Fig. 1). HIF-α stimulates tumour growth and survival, angiogenesis, metastatic spread and glucose metabolism, amongst others. HIF-α levels are dependent of cellular oxygen levels and presence of the von Hippel–Lindau tumour suppressor protein (pVHL). In normoxia, the prolyl hydroxylase domain proteins (PHD1, PHD2 and PHD3) stimulate HIF-α degradation by enabling its recognition by pVHL by hydroxylation. Factor-inhibiting HIF (FIH) adds a further level of control by reducing the transcriptional activity of HIF-α.[12] Approximately 50–70% of sporadic ccRCC cases have *VHL* gene mutations.[13] The subsequent absence of functional pVHL causes an overexpression of HIF-α (both HIF-1α and HIF-2α) independent of oxygen concentrations. Expression of HIF-α and various downstream proteins of the HIF pathway in RCC has been studied for their prognostic value.[14,15] While of great importance for HIF-α regulation, proteins upstream in the HIF pathway have been less extensively investigated in RCC, despite recent reports on associations of levels of these proteins with aggressiveness of other tumour types.[16–18] The aim of this study was to determine whether the upstream factors of the HIF pathway PHD1,

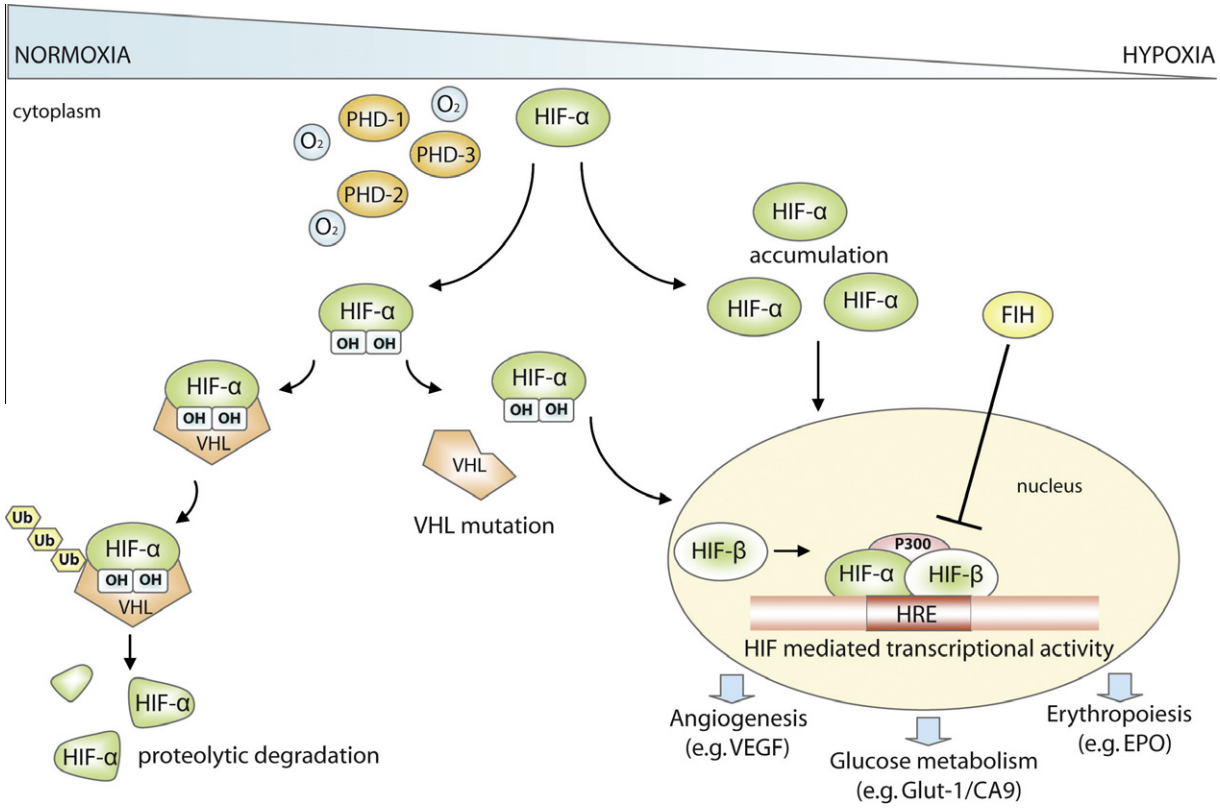

Fig. 1 – *The hypoxia pathway*. Under normoxic conditions the prolyl hydroxylase domain proteins (PHD1, PHD2 and PHD3) stimulate HIF-α degradation by enabling its recognition by the von Hippel–Lindau protein (pVHL) through hydroxylation. Factor-inhibiting HIF (FIH) adds a further level of control by reducing the transcriptional activity of HIF-α, until severe hypoxia occurs. In the presence of hypoxia or VHL mutations, HIF-α accumulates and mediates the transcription of factors stimulating tumour growth and survival, angiogenesis and glucose metabolism, amongst others. Ub: Ubiquitin, HRE: hypoxia response element.

2 and 3 and FIH have independent prognostic value as tissue biomarker for ccRCC.

2. Material and methods

2.1. Patients

This retrospective study included patients who underwent nephrectomy for ccRCC between 1994 and 2006 at the University Medical Center Utrecht (UMCU). The study was carried out in accordance with the ethical guidelines of our institution concerning informed consent about the use of patient's materials after surgical procedures. Patients with von Hippel–Lindau's disease, tuberous sclerosis, Wilms' tumour or RCC subtypes other than ccRCC were excluded. Hereafter, a random ccRCC population of 100 patients was included of which the clinicopathologic characteristics are summarised in Table 1. Patient clinical, pathologic and survival data were obtained by reviewing hospital records and by information from the general practitioner, and included age, sex, 2002 tumour-node-metastasis (TNM) classification, Fuhrman grade, ECOG performance data, primary tumour size, presence of tumour necrosis and SSIGN score. Tumour stage and grade were determined by one pathologist (TGNJ). Patients were evaluated from time of diagnosis to 10 year follow-up. OS was defined as the time from nephrectomy till the date of death or last clinical follow-up.

Table 1 – Patient characteristics.

Features	Median (inter-quartile range)	N (%)
Number of patients		100
Age (years)	64 (54–73)	
Gender		
Female		32 (32)
Male		68 (68)
Overall survival (months)	55 (22–93)	
Tumor classification		
pT1a		18 (18)
pT1b		14 (14)
pT2		12 (12)
pT3a		17 (17)
pT3b/c		35 (35)
pT4		3 (3)
Fuhrman nuclear grade		
Total		88 (88)
1		12 (12)
2		45 (45)
3		28 (28)
4		3 (3)
Tumor size	7.5 (4.5–10)	
<5 cm		26 (26)
⩾5 cm		74 (74)
Lymph node involvement		
pNx + pN0		90 (90)
pN1 + pN2		10 (10)
Metastases		
Synchronous		17 (17)
Metachronous		18 (18)
Tumor necrosis		
Yes		10 (10)
No		90 (90)
SSIGN score		
Total		88 (88)
0–1		19 (22)
2		13 (15)
3		3 (3)
4		14 (16)
5		13 (15)
6		6 (7)
7		7 (8)
8		5 (6)
9		3 (3)
⩾10		5 (6)
VHL mutation		
Sequencing completed		37
Yes		16 (43)
Missense		4 (11)
Deletion		11 (30)
Indel		1 (3)
No		21 (57)

2.2. Tissue microarray construction

Formalin-fixed paraffin-embedded (FFPE) renal tumour material was obtained from the Biobank of the UMCU after approval of the UMCU Institutional Review Board in accordance with Dutch medical ethical guidelines. A tissue microarray (TMA) was constructed by taking 3 cores (1 mm diameter) from each of the 100 cancer specimens and 3 cores from benign renal parenchyma surrounding the tumour and arranging them in a new composite paraffin block using an arrayer (Beecher instruments, Sun Prairy, United States of America (USA)).

2.3. Immunohistochemistry

TMA sections (5 μm) were deparaffinised and rehydrated. Immunohistochemistry procedures included antigen retrieval using citrate buffer (pH 6) (FIH, VHL, PHD1 and PHD3) or ethylenediaminetetraacetic acid buffer (pH 9) (PHD2, HIF-1α and HIF-2α). The primary antibodies and their respective dilutions were as follows: VHL (Clone Ig32, BD Biosciences, Temse, Belgium, 1:100), PHD1 (Abcam, Cambridge, United Kingdom (UK), 1:100), PHD2 (Abcam, Cambridge, UK, 1:100), PHD3 (Novus Biologicals, Cambridge, UK, 1:400), HIF-1α (BD Biosciences, Temse, Belgium, 1:50), HIF-2α (Abcam, Cambridge, UK, 1:500) and FIH (Novus Biologicals, Cambridge, UK, 1:100). PowerVision (Immunologic, Duiven, The Netherlands) was used as secondary antibody. All reactions were visualised using diaminobenzidine/H_2O_2. Finally, array sections were counterstained with haematoxylin.

2.4. Scoring methods

Protein expression was analysed by a pathologist (PJvD) blinded to other data. Cytoplasmic staining (PHD1, 2, 3, VHL), percentage of positively stained nuclei (PHD1, 2, 3, FIH, HIF-1α, HIF-2α) and nuclear intensity (FIH) were assessed in a semiquantitative fashion. For cytoplasmic and nuclear staining, tumours were scored as 0, no intensity; 1, weak; 2; moderate; 3, strong intensity. Percentages of positively stained nuclei were scored as 0, <15%; 1, 15–75%; 2, >75%. The mean score of three cores of a tumour was used for statistical analysis.

Table 2 – PCR primers VHL gene.

	Sequence
PCR primers	
Exon 1A forward	5′ GGT-GGT-CTG-GAT-CGC-GGA-GGG-A 3′
Exon 1A reverse	5′ CGC-GAG-TTC-ACC-GAG-CGC-AGC-A 3′
Exon 1B forward	5′ AAC-TGG-GCG-CCG-AGG-AGG-AGA-T 3′
Exon 1B reverse	5′ GGG-CTT-CAG-ACC-GTG-CTA-TCG 3′
Exon 2 forward	5′ TTC-ACC-ACG-TTA-GCC-AGG-AC 3′
Exon 2 reverse	5′ GGT-CTA-TCC-TGT-ACT-TAC-CA 3′
Exon 3 forward	5′ AGC-CTC-TTG-TTC-GTT-CCT 3′
Exon 3 reverse	5′ GGA-ACC-AGT-CCT-GTA-TCT 3′
Sequence primers	
Exon 1A forward	5′ GAT-CGC-GGA-GGG-AAT-GCC 3′
Exon 1A reverse	5′ CCG-CCC-GGC-CTC-CAT-CTC 3′
Exon 1B forward	5′ GCC-GAG-GAG-GAG-ATG-GAG 3′
Exon 1B reverse	5′ TTC-AGA-CCG-TGC-TAT-CGT 3′
Exon 2 forward	5′ CAG-GAC-GGT-CTT-GAT-CTC 3′
Exon 2 reverse	5′ TTA-CCA-CAA-CAA-CCT-TAT-CT 3′
Exon 3 forward	5′ TGT-TCG-TTC-CTT-GTA-CTG 3′
Exon 3 reverse	5′ ACT-CAT-CAG-TAC-CAT-CAA-AA 3′

2.5. DNA extraction and amplification

Tumour tissue was separated from normal kidney on paraffin tissue sections by scratching, guided by H&E staining of adjacent paraffin sections, lysed in Tris/HCl buffer, pH 8.0 with Tween-40 mg/ml proteinase K (2 mg/ml) at 56 °C o/n. The lysate was boiled for 10′ and subsequently cooled down on ice. After 2′ centrifugation at 14,680 rpm, the DNA concentration of the supernatant was measured using the Isogen Nanodrop Spectrophotometer ND-1000. The DNA was subsequently stored at –20 °C. Amplification of patient tumour DNA was carried out in 25 μl reactions using 1 μg of tumour DNA. Primers used for amplification are listed in Table 2; exon 1 was amplified in two parts.

2.6. DNA purification and VHL gene sequencing

Residual primers and single-stranded DNA were removed from PCR products using exonuclease I and Shrimp Alkaline Phosphatase at 37 °C for 30 min followed by 20′ termination at 80 °C. Sequencing was carried using the Big Dye system and the primers were listed in Table 2. Sequencing runs were performed with the Gene-amp PCR system 9600 (Applied Biosystems). Sequence reaction products were purified using Sephadex columns prior to running them on a 3130×/genetic analyser (Applied Biosystems, Foster City, USA). Results were analysed using Mutation Surveyor software (SoftGenetics, LLC, State College, PA, USA v3.24).

2.7. Statistical analysis

TMA marker expression was correlated to clinicopathological markers using Spearman rank correlation for non-parametric measures. Marker expression was correlated to overall survival (OS) using Kaplan–Meier survival curves followed by log rank analysis to estimate differences in levels of the analysed variables. Marker independence for prediction of survival was determined by univariable– followed by multivariable Cox regression analysis.

3. Results

3.1. Patients

Comprehensive clinicopathological features of 100 ccRCC patients are depicted in Table 1. Median OS was 55 months (IQR 22–93). At the time of final analysis, 25 patients had died because of their disease and 41 patients were still alive, having a median survival time of 82 months (IQR 53–100). Seventeen patients presented metastases at the time of nephrectomy, 18 patients developed metastases within follow-up. Of patients with metastasized disease 7 were treated with immunotherapy, 2 with a combination of immunotherapy and metastasectomy and 4 with metastasectomy. Thirteen patients received adjuvant radiotherapy for their bone metastases.

3.2. Sequencing of the VHL gene

Quality of the DNA derived from formalin-fixed paraffin-embedded tissue was sufficient for sequencing of the *VHL* gene in 37 patients (Table 1). In total, 16 mutations (43%) were identified of which 1 was located in an intron (Table 2). Mutations were equally distributed along the gene. Most mutations were single or double-basepair deletions (11×) or point muta-

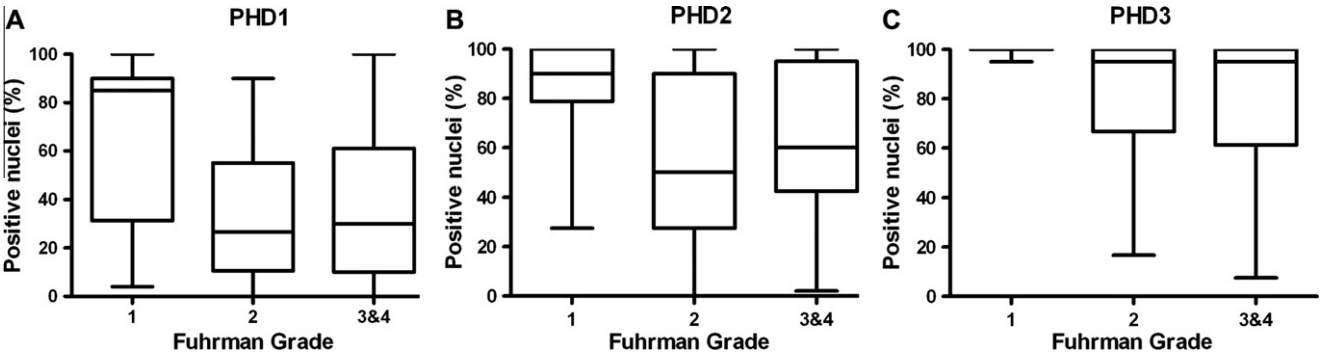

Fig. 2 – *PHD1, 2 and 3 expression levels by Fuhrman grade*. The percentage of PHD1 (A), PHD2 (B) and PHD3 (C) positive nuclei is highest in lesions with Fuhrman grade 1. Fuhrman grade 1: 12 patients, Fuhrman grade 2: 45 patients, Fuhrman grade 3&4: 31 patients.

tions (4×), one insertion–deletion was detected. Twelve mutations resulted in frameshifts. The presence or absence of a mutation in the *VHL* gene did not correlate with clinicopathological parameters, survival of the patients or expression of any of the markers under investigation in this study.

3.3. Expression of HIF

Most tumours showed nuclear presence of HIF-1α (94%) and HIF-2α (82%). Median percentages of positive nuclei within a tumour were 27.5% (IQR 6.7–62.5%) for HIF-1α and 2.7% (IQR 0.5–13.5%) for HIF-2α. Expression of HIF-1α and HIF-2α was not associated with any of the clinicopathological parameters considered in this study. HIF-1α was positively correlated with HIF-2α ($p < 0.0001$), PHD1 ($p = 0.024$), PHD2 ($p < 0.0001$), PHD3 ($p = 0.004$), FIH ($p < 0.0001$) and VHL ($p = 0.031$) (Spearman rank correlation). HIF-2α levels were significantly associated with FIH ($p < 0.0001$) and PHD2 ($p = 0.0155$).

3.4. Expression of prolyl hydroxylases

PHD1, 2 and 3 were localised to the nuclei of RCC cells. PHD1 was detected in on average 39% of all nuclei, PHD2 in 63% and PHD3 in 84%. Although no association between the levels of PHD proteins and OS of patients was identified, a significant correlation (PHD1 $p = 0.024$, PHD2 $p = 0.0067$, PHD3 $p = 0.0012$, Kruskal–Wallis) between Fuhrman grade and expression of all three PHD proteins was established (Fig. 2). The percentage of nuclei staining positive for PHD1, 2 and 3 was high in tumours with Fuhrman grade 1.

3.5. Expression of FIH is associated with overall survival

In normal kidney tissue, FIH was primarily localised to the cytoplasm. In ccRCC cells, however, FIH staining was predominantly nuclear. The percentage of RCC nuclei with positive FIH staining ranged widely from 1% to 100%. In addition to the percentage of positive nuclei, the intensity of FIH staining varied between patients (Fig. 3). Therefore, both the percentage of positive cells and the intensity of FIH staining (score 1, 2 or 3) were recorded. A low (<15%) percentage of FIH-positive nuclei and a low intensity of FIH staining (score 1) were both significantly correlated with a shorter OS of patients ($p = 0.001$ and $p = 0.003$ log rank) (Fig. 4). FIH was positively associated with tumour diameter ($p = 0.016$, Spearman rank) but not with any of the other clinicopathological parameters including stage and grade.

We evaluated the following variables for prognostic value: tumour stage, tumour grade, tumour diameter, presence of metastatic disease, lymph node involvement, presence of necrosis and expression of FIH (Table 3). Of these, stage, grade, diameter, metastatic disease and intensity of FIH showed a statistically significant ($p = 0.023$) correlation with OS in univariable analysis. Inclusion of these factors in a multivariable backwards conditional Cox regression analysis resulted in a model with metastatic disease, stage and FIH intensity as independent predictors for OS ($p = 0.009$) (Table 3).

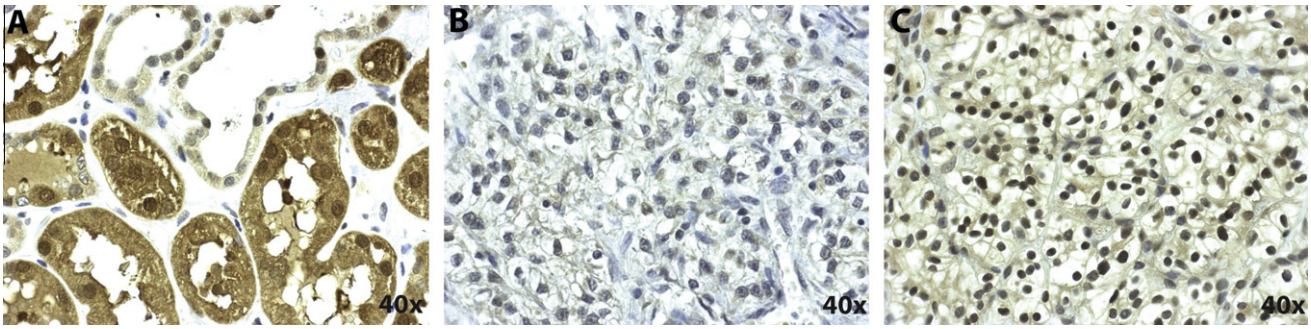

Fig. 3 – *FIH immunohistochemistry*. (A) Expression of FIH in normal kidney, FIH is predominantly present in the cytoplasm of tubuli. (B) Low expression of FIH in ccRCC. (C) High nuclear expression of FIH in ccRCC.

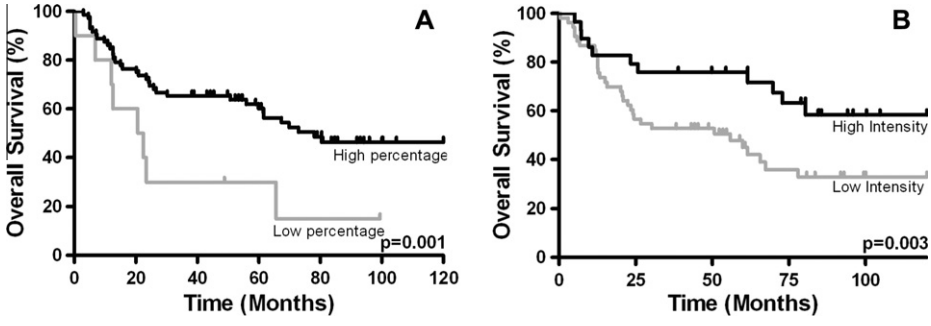

Fig. 4 – *Overall survival*. Kaplan–Meier curves showing the overall survival of ccRCC patients with low versus high percentages of nuclear FIH (A) and low versus high intensity of nuclear FIH staining (B).

Table 3 – Uni- and multivariable analysis.

Univariable analysis Variable	HR (95% confidence interval (CI))	Significance
Tumor classification		
T1	1.00	
T2	1.28 (0.46–3.55)	0.631
T3	3.13 (1.51–6.48)	0.002
T4	5.11 (0.64–40.7)	0.123
Fuhrman nuclear grade		
1	1.00	
2	2.25 (0.77–6.59)	0.138
3	2.45 (0.79–7.55)	0.120
4	12.01 (2.11–68.42)	0.005
Tumor diameter	1.08 (1.01–1.15)	0.026
Distant metastases	4.09 (2.14–7.83)	0.000
Lymph node involvement	1.17 (0.64–2.15)	0.616
Necrosis	1.14 (0.47–2.73)	0.772
FIH (% positive nuclei)	0.99 (0.98–1.02)	0.141
FIH (intensity)	0.45 (0.23–0.90)	0.023
Multivariable Cox regression analysis Variable	**HR**	**Significance**
Tumor classification		
T1	1	
T2	0.43 (0.05–3.58)	0.437
T3	2.08 (0.82–5.26)	0.121
T4	7.60 (1.82–31.64)	0.005
Distant metastases	4.45	0.000
FIH (intensity)	0.40	0.009

4. Discussion

In this study nuclear FIH expression in the primary tumour was shown to have a significant independent prognostic value for ccRCC patients. FIH inhibits HIF-α in an oxygen-dependent manner. However, FIH remains active unless severe hypoxia occurs.[19] This suggests that FIH may have an important function as one of the final checks on HIF-α transcriptional activity. Although HIF is not the only target for hydroxylation by FIH, it is the most intensively studied and best characterised to date. The presence of FIH has been investigated in various normal and neoplastic human tissues, in which the intensity and subcellular localisation are very heterogeneous. Although in normal human tissues FIH is predominantly cytoplasmic, nuclear expression of FIH can be relatively strong in certain neoplasms.[20] Moreover, FIH has provided variable prognostic values in several tumour types.[16,21] In pancreatic endocrine tumours (PETs) cytoplasmic FIH levels were significantly higher in more malignant PETs, but were not associated with survival. Nuclear FIH did not correlate with any histopathologic variables in this study.[16] In invasive breast cancer, both cytoplasmic FIH expression and absence of nuclear FIH were independent prognostic factors for a shorter disease-free survival.[21] Our study showed for the first time in ccRCC patients that low expression of nuclear FIH is a significant independent predictor for worse OS. In RCC, FIH can be detected both in the nucleus and cytoplasm, and the specific subcellular localisation varies between different RCC patients.[20] This was similar in our study (Fig. 3). The mechanism and function of nuclear FIH in RCC, and the reason why low nuclear FIH levels have such strong prognostic value are currently unknown. The absence of nuclear FIH in more aggressive phenotypes could be explained by increasing gene mutations within these tumours, including FIH gene mutations. This is, however, unlikely since FIH gene mutations have not been found in RCC.[22] It is therefore possible that FIH is actively retained in the cytoplasm or exported out of the nucleus in tumours associated with worse prognosis.

Although *VHL* gene mutations are considered as an early and important event in the development of ccRCC, studies that investigated *VHL* alterations and survival have provided contradictory results.[13,23–26] Our data suggests that VHL status is not correlated to common clinical prognostic parameters or survival of ccRCC patients.

Both HIF-1α and HIF-2α, which are central proteins of the HIF pathway, failed to show a correlation to survival and the most important clinicopathological parameters in our study. In ccRCC, both HIF-1α and HIF-2α are important regulators of angiogenesis and cell proliferation.[27,28] HIF-1α, but not HIF-2α, has prognostic value in colorectal cancer, lymph node negative breast carcinoma and ovarian carcinoma.[29–31] In ccRCC HIF-1α did correlate with good prognosis in a study using Western blot, however, immunohistochemical staining of the same population could not confirm this finding.[32,33] Another study reported prognostic value for HIF-1α, but only in metastatic RCC patients.[34] HIF-2α has been related to worse survival in several cancer types.[35–37] In ccRCC, evidence has accumulated revealing the importance of HIF-2α in tumourigenesis.[28,38,39] However, although HIF-2α has shown to be in-

versely correlated to TNM and nuclear grade in RCC patients, we and others have shown no evidence for a correlation of HIF-2α expression with survival.[40] HIF-1α and HIF-2α levels did correlate positively to expression of PHD1, 2 and 3 and FIH. Under hypoxic conditions both HIF-1α and HIF-2α upregulate PHD protein expression. This may serve as a negative feedback to decrease HIF-α activity.[41,42] Since hypoxia is common in RCC and many other solid tumours,[43] a correlation between molecules participating in the hypoxia response pathway is not surprising and indicates the intricate interplay between these proteins. Alternatively, FIH may exert its effects on tumour biology in a HIF-independent fashion. In addition to the interaction of FIH with the hypoxia pathway, it was recently discovered that in the presence of oxygen HIF competes with many ankyrin-repeat domain-containing proteins (ARDs) for hydroxylation by FIH. Many ARDs have a proposed function in cell proliferation and differentiation. Due to this competition hypoxia or a decreased presence of FIH may lead to accumulation of non-hydroxylated ARDs. Although the exact mechanisms have not yet been elucidated, downstream effects of this accumulation may lead to increased tumour aggressiveness.[44,45]

PHD proteins have been studied in several other tumour types as potential prognostic markers.[16,17] In these studies high nuclear PHD levels were associated with tumour aggressiveness and worse survival. For ccRCC, as far as we know no information was present for PHD expression in relation to patient survival. We show that there is no correlation between PHD1, 2, 3 and RCC patient survival. Surprisingly, in this study high nuclear PHD levels were significantly correlated to a low Fuhrman grade, indicating that in contrast to other tumour types, increased nuclear PHD levels are primarily present in less aggressive (grade 1) ccRCC.

Herein we show that FIH is a very promising prognostic marker for ccRCC. However, for its definitive clinical value a prospective clinical trial will be needed. Like most described prognostic markers in literature this study was performed on tissue obtained in the time that immunotherapy was first line therapy for RCC. In the current era of targeted therapy correlation of these prognostic tissue markers to survival is therefore not completely certain, especially when markers have a function in tumour specific immunology. Since only a minority of our patient population underwent immunotherapy and the here described markers are not known to have a function in the immune system, it is highly unlikely that the prognostic significance will be altered under current treatment standards. Furthermore, prospective clinical trials are needed to determine the additional clinical significance and application of FIH to the currently available prognostic models for non-metastasized disease.

In conclusion, this study shows that nuclear FIH is an important indicator of prognosis in ccRCC patients, with low nuclear FIH expression predicting a poor OS. Unlike previous studies focussing on HIF and its downstream targets, the results presented herein highlight the importance of upstream regulatory proteins of the HIF pathway as independent prognostic factors for ccRCC. We anticipate that integration of FIH in established prognostic models could result in a more accurate prediction of survival.

Conflict of interest statement

The authors declare no conflict of interest.

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