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Hypoxia inducible factors and hypoxia associated factor expression profiles and prognostic significance in renal cell carcinomas

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Abstract

Hypoxia Inducible Factor-1 alpha and Hypoxia Inducible Factor-2 alpha (HIF-1α and HIF-2α) are crucial in Renal Cell Carcinoma (RCC) formation. Hypoxia Associated Factor (HAF) prompts HIF-1α degradation regardless of oxygen levels, but no such link exists for HIF-2α. This study encompassed 239 cases, where tissue microarray (TMA) sections were exposed to HIF-1α, HIF-2α, and HAF antibodies. Staining intensity and tumor cell percentage determined scores for HIF-1α, HIF-2α, and HAF, with median histoscore establishing "high" or "low" cutoffs. Among the cases, 64.9% (155 cases) were negative for HIF-1α, 17.6% (42 cases) displayed low, and another 17.6% (42 cases) showed strong HIF-1α expression. HIF-1α expression correlated significantly with histological type and World Health Organization/International Society of Urological Pathology (WHO/ISUP) nuclear grade. Regarding HIF-2α, 15.5% (37 cases) were negative, 23.4% (56 cases) exhibited low, and 61.1% (146 cases) displayed high expression, with larger tumor size in the high HIF-2α group. Among 239 cases, 41.4% (99 cases) were negative for HAF, 36.8% (88 cases) showed low, and 21.8% (52 cases) displayed high HAF scores. HAF expression correlated with WHO/ISUP nuclear grade and metastasis presence. Notably, HIF-2α staining intensity directly correlated with increased HAF intensity (rho=0.146; p=0.024), while HIF-1α intensity decrease corresponded with heightened HAF intensity, showing a statistically significant negative correlation (rho=-0.180; p=0.005). In this rare examination of tumor tissue, a reverse connection between HIF-1α and HAF expression was uncovered, while a linear link emerged between HIF-2α and HAF expression. Overall, this study established that HIF-1α, HIF-2α, and HAF expression are associated with an unfavorable prognosis.

Keywords: HIFs, HAF, RCC, hypoxia, kidney

Introduction

Renal cell carcinoma (RCC) is a malignant tumor derived from the renal tubular epithelium. RCC is the most common urological malignant neoplasm and 9th most common cancer type in men and 14th in women, according to the World Health Organization (WHO) [1,2].

Hypoxia-inducible factors (HIFs) play critical roles in RCC tumorigenesis. HIFs consist of three subunits: HIF-1α, HIF-2α, and HIF-1β. HIF-1α and HIF-2α exhibit a high (48%) amino acid similarity and both can dimerize with HIF-1β [3]. Despite this high similarity rate, HIF-1α and HIF-2α are synthesized in different tissues and have distinct functions in cellular metabolism. HIF-2α is more effective in cellular proliferation and angiogenesis, while HIF-1α is the primary regulator of the glycolytic pathway [3,4]. Additionally, their roles in RCC

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carcinogenesis are contradictory. Several studies have shown that HIF-1α acts as a tumor suppressor in RCC carcinogenesis. On the other hand, limited data suggest that HIF-2α may have an oncogenic role in RCC tumorigenesis [4].

Regardless of oxygen levels, Hypoxia Associated Factor (HAF) causes HIF-1α to degrade. Despite this, no relation has been found between HAF and HIF-2α expression levels in the literature [5]. Also, HAF has been shown to play a role in the conversion of HIF-1α to HIF-2α in neuroblastoma and RCC cell cultures, but this effect could not be observed in tissue samples [5,6]. There are still some aspects of the effects of HIF-1α, HIF-2α, and HAF on tumorigenesis that need to be clarified.

In this study, our aim is to investigate the relationship in between HIF-1α, HIF-2α, and HAF expression levels and the relationship of these with the prognostic markers (age, histological type, surgical margin status, nuclear grade, tumor necrosis, etc.) in RCC tissue samples.

Materials and Methods

Patient selection

In this study, the patients who were diagnosed with RCC in the partial/total nephrectomy specimens in the pathology department of Ankara Yildirim Beyazit University/Ankara Ataturk Training and Research Hospital between the years 2006-2014 were screened from the pathology report database of the department. Clinical and pathological characteristics, including age, gender, tumor size, World Health Organization/ International Society of Urological Pathology (WHO/ISUP) nuclear grade, coagulative tumor necrosis, microvascular invasion, renal sinus fat invasion, adrenal gland, ureter, renal vein invasion, distant metastasis, and overall survival were analyzed. Clinicopathological characteristics are summarized in Table 1.

Table 1. Clinical and histopathological characteristics of patients

Variables	
Sex	
Male	164
Female	75
Age (years)	60 (27-87)
Tumor size (cm)	
Min	1.5
Max	18
Nuclear grade	
1	24
2	88
3	81
4	46
5-year survey	
Localized disease	91.8%
Metastatic disease	12.1%
Tumor type	
Clear cell	146
Chromophobe	27
Papillary	48
Other	18
Metastases	
Yes	54
No	185
Sarcomatoid component	
Yes	12
No	227
Tumor necrosis	
Yes	93
No	146

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Tissue microarray and immunohistchemistry procedure

Tissue microarray (TMA) blocks using a precision mechanical system (Quick Ray, Manual Tissue Microarray, UNITMA, Korea) were constructed. From each patient with RCC, two tissue samples (diameter, 0.3 cm each) corresponding to the previously demarcated and most representative areas of respective hematoxylin–eosin-stained slides were removed. One of the core biopsies was sampled from the center and the other was sampled from the advancing edge of the tumor. Afterward, these samples were transferred to a recipient paraffin block at 3-mm intervals. Finally, TMA blocks were cut into 4-µm histological sections and used for immunohistochemistry. An immunohistochemical analysis for the expression of HIF-1α (H1alpha67; 1:50 dilution; Novus; USA), HIF-2α (ep190; 1:100 dilution; GeneTex, USA) and HAF (1:100 dilution, GeneTex; USA) were performed.

For HIF-2α and HAF antibodies; antigen recovery was performed by manually.

For each antibody, it was diluted in 90 ml distilled water by adding 10 ml of citrate buffer solution. The citrate solution was boiled in the heater for 60 minutes. It was kept for 20 minutes until it reached room temperature. The slides were passed 3 times with distilled water. To block the endogenous peroxide in tissues, 3% H_2O_2 application was performed in 37°C ethanol for 10 minutes. The slides were passed 3 times in distilled water. Slides were kept in a solution of phosphate-buffered saline (PBS) prepared as pH 7.4 for 10 minutes. Large Volume Ultra V Block was dropped and kept for 7 minutes. The solution was then removed from the slides. Antibodies on the slides were removed with distilled water and kept in PBS solution for 5 minutes. To verify antibody specificity, placenta tissue for HIF-2α and non-tumoral kidney tissue for HAF were used as positive controls.

For the HIF-1α antibody; Ventana BenchMark GX was used. After antigen retrieval treatment with citrate solution, HIF-1α antibody was manually applied and incubated overnight. Enrichment was done with Ventana Amplification Kit in the morning. The BMK Ultraview DAB paraffin kit was then used for staining. Also, counterstaining was performed as part of the automated staining protocol using hematoxylin. After staining, the slides were washed, dehydrated in graded alcohol and xylene, mounted, and coverslipped. To verify antibody specificity, breast carcinoma tissue was used as a positive control.

HIF-1α, HIF-2α and HAF antibodies were applied to the sections obtained from paraffin blocks of TMA prepared paraffin blocks and whole section slides. The presence of expression was evaluated in a light microscope. The presence of nuclear staining for the HIF-1α antibody, the presence of cytoplasmic staining for HIF-2α and HAF antibodies were considered to be positive (Figures 1-3).

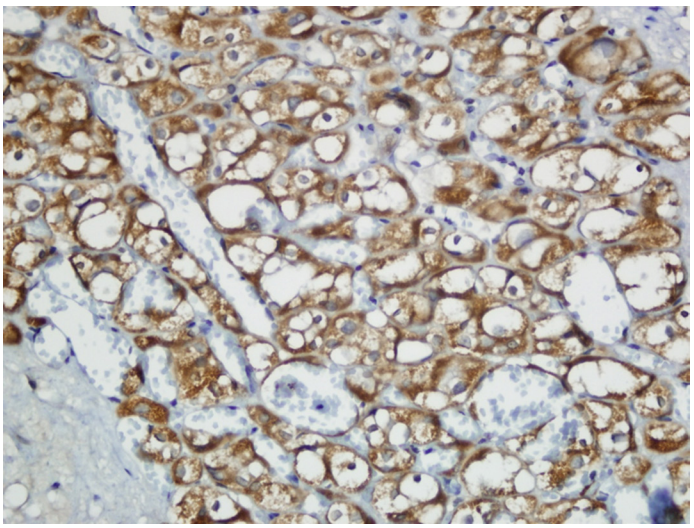

Figure 1. Chromophobe cell renal cell carcinoma, Score 3 HAF expression (x400)

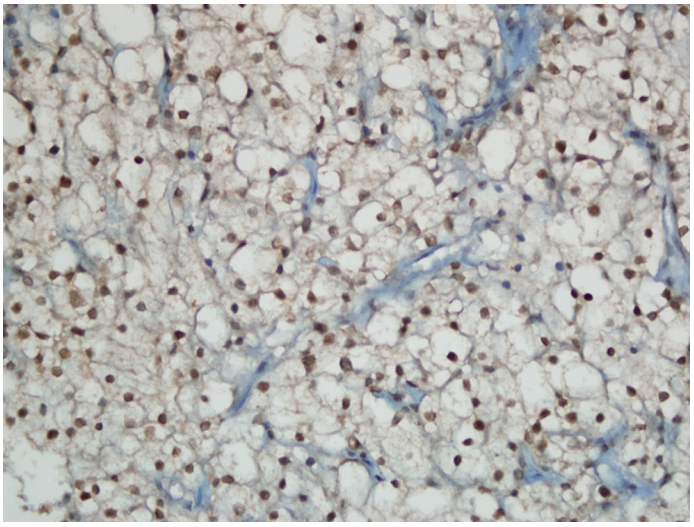

Figure 2. Clear cell renal cell carcinoma, Score 2 HIF-1α expression (x400)

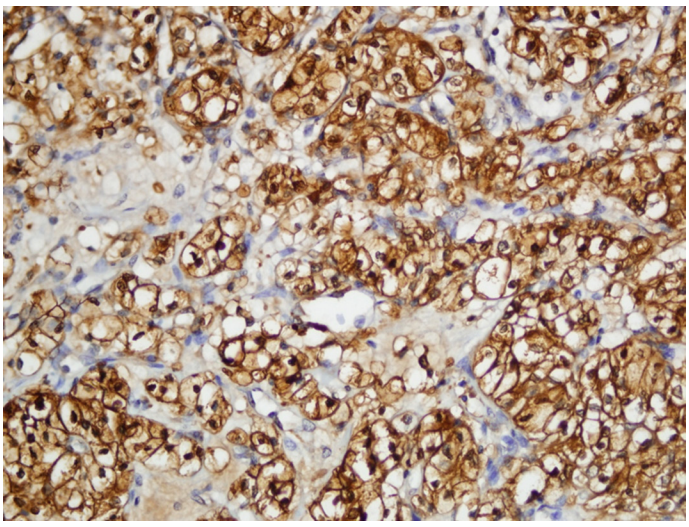

Figure 3. Clear cell renal cell carcinoma, Score 3 HIF-2α expression (x400)

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For HIF-1α, HIF-2α, and HAF, the staining was scored according to the intensity and percentage of tumor cells. The intensity was scored 0 (no staining), 1 (weak), 2 (moderate), and 3 (strong staining). The percentage staining was scored as 0 (no staining), 1 (1–10%), 2 (11–50%), 3 (51–90%), and 4 (91–100%). Also, the histoscore was calculated for analysis using the product of these two scores, giving a score from 0 to 12. The final histoscore used for the data analysis was the mean score from the two tissue cores from each RCC tumor block. Finally, the median histoscore was used to evaluate the cut-off scores for "high" or "low" staining for each protein. The score data for each 3 antibody expressions are shown in Table 2.

Table 2. Score data of HIF-1α, HIF-2α, and HAF expression

Antibody	Negative (0–1) n (%)	Low (2–3) n (%)	High (4–12) n (%)
HIF-2α	37 (15.5)	56 (23.4)	146 (61.1)
HAF	99 (41.4)	88 (36.8)	52 (21.8)
HIF-1α	155 (64.9)	42 (17.6)	42 (17.6)

Statistical analysis

Using the Shapiro-Wilk test, the normal distribution of continuous variables was graphically evaluated. The median (minimum; maximum) was given for the descriptive statistics and mean±standard deviation values were given as additional information. Number (n) and percentage (%) were given to show the distribution in categorical variables.

The distribution of HIF-1α, HIF-2α, and HAF scores were determined by Pearson's chi-square test. Kruskal-Wallis test was used to compare the dimension variables in the groups.

The relationship between HIF-2α, HAF, and HIF-1α scores was analyzed with the Spearman rho correlation coefficient. The correlation coefficient between 0.00 and 0.19 is interpreted as "low or no relation", in the range of 0.20 to 0.39 is "low relation", in the range of 0.40-0.69 is "moderate relation", in the range of 0.70-0.89 is "strong (high) relationship" and in the range of 0.90-1.0 is "very strong relation ".

For statistical analysis and calculations, IBM SPSS Statistics 21.0 (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.) was used. The level of statistical significance was accepted as $p < 0.05$.

Survival analyses of the cases were also evaluated by the Kaplan-Meier method.

Results

A total number of 239 cases included in this study cohort. A hundred and sixty-four (68.6%) patients were male and 75 (31.3%) were female, and the median age was 60 (27-87) years. A hundred and forty-six (61%) patients were clear cell RCC, 27 (11.3%) were chromophobe RCC, 48 (20.1%) were papillary RCC, and 18 (7.5%) were other subtypes. Twenty-four tumor were classified as ISUP grade 1, 88 were ISUP grade 2, 81 were ISUP grade 3, and 46 were ISUP grade 4. Tumor characteristics and demographic data were summarized in Table 1.

Tumor size used by TNM system for RCC staging. In this study, the smallest tumor size was 1.5 cm and the largest tumor size was 18 cm.

The longest follow-up period of the patients was 113 months, and the survival time ranged between 1-113 months. Overall survival rate was high (82%), as most of the cases in the study were in the early stage and limited to the kidney.

Twelve (5%) cases showed sarcomatoid differentiation. Of the 12 cases with sarcomatoid differentiation, 8 were clear cell RCC, one was papillary RCC, one was chromophobe RCC, and 2 were other subtypes. In this study 22.6% of the cases were metastatic. The metastatic sites were lung (10.4%) and lymph nodes (7.9%).

HIF-1α immunohistochemistry

Of the 239 cases included in the study, 155 cases were negative (64.9%), 42 cases (17.6%) were showed low expression, and 42 cases (17.6%) were showed strong HIF-1α expression.

There was a strong correlation between HIF-1α expression score and histologic subtypes. HIF-1α expression was higher in clear cell RCC than other types. 73.8% (n=31) of 42 cases with low expression and 83.3% (n=35) of 42 cases with high expression were clear cell RCC ($p<0.001$).

Also, HIF-1α expression was lower in cases with WHO/ISUP grade 3 and 4 than the others. Of the 46 WHO/ISUP grade 4 cases, 35 of them showed HIF-1α negative, 7 were low expressed, and 4 were strong HIF-1α expressed ($p=0.011$). Of the 81 ISUP grade 3 cases, 61 of them showed HIF-1α negative, 8 were low expressed, and 12 were strong expressed ($p=0.011$).

No association between HIF-1α expression and other clinicopathological parameters were found.

HIF-2α immunohistochemistry

Of the 239 cases included in the study, 37 cases were negative (15.5%), 56 cases (23.4%) were showed low expression, and 146 cases (61.1%) were showed strong HIF-2α expression.

HIF-2α expression was found to be correlated with tumor size, adrenal gland metastasis, and renal sinus infiltration status.

The median tumor size was found to be greater in highly expressed HIF-2α positive cases (median 5.5cm).

No metastases were found in HIF-2α negative cases, and all 2 adrenal metastatic cases highly expressed HIF-2α. Not only negative cases were metastasis free, but also HIF-2α low expressed cases were metastasis free, too.

Thirty-nine (16.3%) of 239 cases demonstrated renal sinus infiltration. Thirty-four (87.2%) of 39 cases were HIF-2α positive ($p=0.704$). Despite that, these relationships were not to be found

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statistically significant.

No association was found between HIF-2α expressions and other clinicopathological parameters (p>0.05).

HAF immunohistochemistry

Of the 239 cases included in the study, 99 cases were negative (41.4%), 88 cases (36.8%) were in low HAF score group, and 52 cases (21.8%) were high HAF score group.

There was a strong correlation between HAF expression score and histologic subtypes. Clear cell RCCs (51.4%) had a lower HAF score than chromophobe RCCs (66.6%) (p=0.002).

HAF was positive in 76% of RCCs with WHO/ISUP nuclear grade 4 ($\chi 2$=18.213; p=0.006). Of the 46 WHO/ISUP grade 4 cases, 18 were in high HAF score group, 17 were in low HAF score group, and 11 were HAF negative.

Furthermore, although statistically insignificant, rate of HAF positivity was higher in metastatic RCC cases than non-metastatic ones. Of the 54 metastatic RCC cases, 37 were HAF positive ($\chi 2$=4,697; p=0.096).

No association was found between HAF expression score and other clinicopathological parameters (p>0.05).

Correlation in between HIF-1 α, HIF-2 α, and HAF

In this study, as HIF-2α staining intensity increased, an increase in HAF intensity was noticed, and a positive relationship was found between HIF-2α and HAF scores (rho=0.146; p=0.024). On the other hand, while HIF-1α intensity decreased, HAF intensity increased. Statistically significant negative correlation was found between these two findings (rho=-0.180; p=0.005). There was no correlation between HIF-1α and HIF-2α expression (p=0.414).

In this study, the survival time of the cases and the expression scores of HIF-1α, HAF, and HIF-2α were examined by Kaplan-Meier method, and no relationship was found between them(p>0.05).

Discussion

HIF-1α expression

Studies have reported that the presence of HIF-1α expression is seen in patients with clear cell RCC rather than other types [6-9]. In accordance with the literature, in our study HIF-1α expression score was higher in clear cell RCCs than other types (p<0.001).

According to the previous studies, HIF-1α expression was reported to be less in tumors with higher nuclear grade [8,10,11]. Concordant with the previous results, HIF-1α expression scores were lower in cases with WHO/ISUP grade 3 and 4 than others in this study.

In a study conducted by Motzer et al HIF-1α antibody was administered immunohistochemically in 149 cases, and a relationship was found between expression scores and progression-free survival [12]. Vice versa, in another meta-analysis study, it was reported that the overall survival of patients with HIF-1α expression was shorter than those without expression [11]. In this study, no relationship was found between HIF-1α expression and overall survival. But the number of patients and median follow-up time may not be enough to document this relationship.

HIF-2α expression

In the literature, it was stated that HIF-1α and HIF-2α have different roles in the pathogenesis of clear cell RCC [13]. HIF-2α has a more tumorigenic effect in the development of RCC and is associated with advanced stage, and chemo/radio-resistance [13]. On the other hand, high HIF-2 expression was found to be correlated with better prognosis and lower tumor grade in some studies [14]. In this study, the median tumor size was found to be larger in HIF-2α high score group than negative group (respectively 5.5 cm and 4.5 cm). In line with other studies, this finding suggests that HIF-2α expression may be associated with advanced stage.

Prior studies demonstrated that metastasis and invasive behavior were related to metalloproteinase activity of HIF-2α [15,16]. In our study, even though it was not statistically significant, the presence of renal sinus infiltration and adrenal gland metastasis were found to be related with HIF-2α expression. As well, it can also be used to guide the clinic in terms of the presence of HIF-2α expression in needle biopsies and early stage cases, the patient's invasion capacity and the frequency of screening for the existence of metastasis.

In the literature, a meta-analysis study reported the presence of HIF-2α expression has been shown to be associated with shorter cancer-specific survival [7]. Nevertheless, in another study, it was reported that HIF-2α expression was not associated with survival [11]. In our study, the presence of HIF-2α expression has not been found associated with overall survival. On the other hand, this result may have been seen due to the limited number of cases.

HAF expression

HAF is a multifunctional DNA ubiquitin ligase that has been shown to have an effect on angiogenesis. HAF overexpression was demonstrated in tumor cells [17]. HAF plays a role in HIF-1α regulation under normoxia, hypoxia or growth hormone stimulation [18]. Besides, unlike HIF-2α, HAF has an effect on HIF-1α regulation regardless of the Von-Hippel Lindau (VHL) state [19].

In studies performed on cell lines, it was found that HIF-1α and HIF-2α had a reciprocal relationship, and when one isoform was increased, expression of the other isoform was decreased [17]. In the literature, studies on this subject have been done mostly in cell lines, and there are few studies applied immunohistochemically in the clear cell RCC subtype [14,19]. Furthermore, to the best of our knowledge, there is no study showing the relationship between HAF expression and RCC subtypes. In this study, a

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significant relationship was found between HAF expression and histological type (p=0.002). HAF expression was found in 51.3% of clear cell RCC subtypes, 64% of chromophobe RCC subtypes and 72.9% of papillary RCC subtypes.

In this study, HAF expression was higher in patient with high WHO/ISUP nuclear grade. In contrast, no specific study of the relationship between HAF expression levels and WHO/ISUP nuclear grade has been reported in the literature so far.

In the literature, a study examining mRNA levels and cell behavior in cell lines and animal models were found that HAF overexpression levels were associated with invasive course and metastasis [19]. Supporting this finding, in this study, HAF expression was higher in metastatic RCC cases than others, although this finding was statistically insignificant.

In in-vivo studies was found while HAF expression levels was increased, HIF-1α mRNA expression levels was decreased regardless of VHL status and oxygen level [14,15]. At the same time, HAF has no effect on HIF-2 degradation. However, there are a limited number of studies on tumor tissues. In this study, which is one of the few studies conducted on tumor tissue, while HAF expression increased, HIF-1α expression decreased but HIF-2α expression increased. A statistically significant correlation was found between them (respectively p=0.005 and p=0.024). But there is no correlation found between HIF-1α expression and HIF-2α expression.

Conclusions

In our study, in accordance with the literature, a significant relationship was found between HIF-1α expression levels and histological type, as well as WHO/ISUP nuclear grade. A larger tumor size was detected in the group showing HIF-2α expression compared to the negative group, and these results indicate a relationship between HIF-2α and poor prognosis.

In this study, a relationship was found between HAF expression levels and WHO/ISUP nuclear grade, as well as the presence of metastasis. Consistent with the literature, these findings indicate that HAF expression is associated with a poor prognosis.

Although studies have reported on the relationship between HAF and prognosis so far, to the best of our knowledge, there has not been a study on HAF and survival in RCCs. In our study, no relationship was found between HAF and overall survival. However, these results may need to be confirmed by more homogeneous and larger working groups.

In this study, which is one of the few studies examining tumor tissue, a reverse relationship was found between HIF-1α and HAF expression, while a linear relationship was found between HIF-2α and HAF.

To sum up, in this study, HIF-1α, HIF-2α, and HAF expression were found to be associated with poor prognostic markers, consistent with the literature. These markers can be used in needle biopsy samples to assess the patients' prognosis, monitor metastasis or invasion development, and guide clinical follow-up for cases with subtyping. Additionally, this study aimed to contribute to the literature on renal cell carcinogenesis and the relationship between HIFs and HAF.

Conflict of Interests
The authors declare that there is no conflict of interest in the study.

Financial Disclosure
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Ethical Approval
Ethics committee approval was obtained for the study with the decision of Clinical Research Ethics Committee (date10.06.2015 and numbered 126).

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