Original Article

Analysis of expression pattern of proteins associated with AKT/mTOR signaling pathway in kidney cancer development

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Abstract

Introduction: The purpose of this study was to analyze the expression status, reciprocal interplay, and prognostic significance of AKT1 and hypoxia-inducible factor 1 alpha (HIF-1α) in AKT/mechanistic target of the rapamycin pathway and to enable them to be studied as possible therapeutic targets.

Materials and Methods: This prospective study included 25 patients with clear cell renal cell carcinoma (ccRCC) operated between December 2019 and January 2022. Tumor and adjacent normal tissue samples were subjected to immunohistochemical analysis, RNA extraction, cDNA synthesis, and quantitative real-time polymerase chain reaction for AKT and HIF-1α. The fold changes were then calculated by ΔΔCt method.

Results: The included 25 ccRCC patients had 1.5-fold greater HIF-1 mRNA expression and 0.9-fold higher AKT1 gene expression in the ccRCC tissues compared to the corresponding healthy control. High, moderate, and low expression of HIF-1α was seen in 15, 6, and 1 of 25 samples, respectively. High, moderate, and low expression of p-AKT1 was seen in 18, 2, and 3 of 25 samples, respectively.

Conclusion: Our study data predicted higher gene expression as well as protein expression of HIF-1α and AKT. The proteins HIF-1α and AKT are localized in the nucleus of the RCC tumor samples compared to normal. Overexpression of these proteins might play significant roles in tumor development and differentiation as reported by others previously. This study can help clarify the biological role of HIF-1α and AKT in RCC to develop new strategies for this malignancy.

Keywords: AKT, hypoxia-inducible factor 1 alpha, mechanistic target of the rapamycin, renal cell carcinoma

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INTRODUCTION

Renal cell carcinoma (RCC) contributes to 2%–3% of all adult malignant neoplasms and is one of the most fatal urologic malignancies.[1] Men are at greater risk (1.9:1) than females.[1] Tobacco smoking, obesity, hereditary factors, hypertension and related medication, and chronic renal failure are some of the common risk factors associated with RCC.[2]

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RCC originates from the tubular structures of the kidney and is classified into four major histological cell types. Clear cell RCC (ccRCC) is the most common type, accounting for about 75%–80% of all cases of RCC. Around 4% of RCCs are hereditary.[3] Nearly, all familial cases of ccRCC are due to inherited mutation in the Von Hippel–Lindau (VHL) tumor suppressor gene. In at least two-thirds of sporadic

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cases of ccRCC, the VHL gene is inactivated either by point mutation, deletion, or promoter hypermethylation.[4-6]

Cancer is unvaryingly accompanied by changes in signal transduction. Alterations in proto-oncogenes and tumor suppressor genes lead to dysregulated signal transduction that underlies the abnormal growth and proliferation of cancer cells. In some cases, tumor-associated mutations specifically target genes coding for critical signaling proteins. Signaling proteins that play an important role in cancer-associated signaling networks can serve as therapeutic targets even though their function is not specifically altered as part of the disease etiology.[7,8]

AKT/protein kinase B is a part of the serine/threonine protein kinase family. It has been implicated in the pathogenesis and progression of many human malignant tumors, such as prostate, breast, lung, ovary, and thyroid cancers, by regulating some key steps that control the balance of cell survival and apoptosis.[9,10] Akt activates and phosphorylates the mechanistic target of rapamycin (mTOR); consequently, activated mTOR regulates S6K1 activation and phosphorylation.[11]

MATERIALS AND METHODS

The study was conducted in a tertiary care hospital from December 2019 to January 2022. The present study protocol was reviewed and approved by the institutional ethical committee. Informed consent was obtained by all subjects when they were enrolled in the study.

This prospective, observational study recruited 25 patients who underwent surgery for suspected renal malignancy. Tumor biopsy and adjacent normal tissue were collected. The tissue samples were divided into three parts: one part was stored at −80°C until protein isolation, another part was stored in 10% formalin for paraffin-embedded tissue blocks, and the remaining part was kept in TRIzol reagent for RNA isolation

Immunohistochemical expression

The protein expression of AKT and VHL product HIF-1α was done by immunohistochemical analysis of 25 primary tumor samples according to the standard protocol.[12] In brief, 10% formalin-fixed tissues were used to make paraffin-embedded blocks. Deparaffinized tissue sections (3 μm) were incubated for 15 min in a methanol solution containing 3% H_2O_2 to block endogenous peroxidase activity. Following antigen retrieval with a 10 mM citrate buffer, the tissue sections were incubated overnight at 4°C with a polyclonal rabbit antibody (1:100) specific for AKT1 and HIF-1α (Abcam, Cambridge, UK). After rinsing in citrate buffer, a biotinylated goat anti-mouse antibody (1:500) labeled with streptavidin and chromogen as substrate was added (UltraVision Detection System Anti-Polyvalent, HRP/AEC, LabVision Corporation, Fremont, CA, USA). The evaluation of expression involved site and degree of reactivity. Degree of reactivity included evaluation for maximal staining intensity using a 0–3 scale (0, negative; 1, weak; 2, moderate; 3, strong). To obtain representative images, slides were scanned by the Leica 1000 DM ergonomic system.

mRNA expression study

Total RNA was isolated from paired tissue samples (renal tumor and adjacent nonmalignant renal tissue from patients) by using the TRIzol reagent according to Maiti *et al*., 2015.[12] After that, real-time quantitative polymerase chain reaction (qRT-PCR) analysis was performed to analyze the expression label of VHL and AKT with an ABI Prism 7500 using Power SYBR Green PCR Master Mix (Applied Biosystems, USA). Beta 2 microglobulin was used as a control.

Statistical analysis

For multivariate analysis, the Cox regression analysis was used. $P < 0.05$ was considered statistically significant. Statistical analysis employed the statistics software package SPSS 24.0.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Table 1 shows the demographic and clinicopathologic characteristics of the study participants.

Quantitative polymerase chain reaction analysis

We examined the difference in gene expression levels of HIF-1α and AKT1 between tumor and adjacent normal tissues. We found a significant 1.5-fold higher mRNA expression of HIF-1α and 0.9-fold higher expression of AKT1 gene in the ccRCC tissues compared to the respective healthy control, respectively ($P < 0.05$ compared to normal).

Expression of hypoxia-inducible factor 1 alpha

The RCC tumor samples showed strong immunoreactivity to HIF-1α at the level of the nucleus, whereas control samples showed either none or weak, diffuse immunoreactivity. The integrity of morphology, the degree of vascularization, and the differentiation of cellular components are deformed in the case of RCC in comparison to normal cells. HIF-1α staining intensities were generally increased in RCC compared with normal tissues. Among the samples, high expression of HIF-1α was seen in 60% (15/25) of the

samples. Moderate expression was observed in 24% (6/25) of the samples and 4% (1/25) has low expression. Compared to normal tissues, significant increase in the expression of HIF-1α protein was seen in stage I + II tumor cells (27.85%, $P = 0.004$) and stage III + IV tumor cells (57.14%, $P = 0.009$). It is previously reported that overexpression of HIF-1α had significantly worse overall survival (177) [Figure 1]. Table 2 explains the percentage of patients and the scoring of expression.

Expression of AKT

Tumor-positive tissue had a higher frequency of activated Akt (p-Akt) than nearby normal tissues [Figure 1]. Furthermore, increased p-Akt was more often located in the nucleus than in the cytoplasm. Among the samples, high expression of p-AKT was seen in 72% (18/25) of the samples. Moderate expression was observed in 8% (2/25) of the samples and 12% (3/25) has low expression. Compared to normal tissues, a significant increase in the expression of AKT protein was seen in stage I + II tumor cells (18.85%, $P = 0.005$) and stage III + IV tumor cells (43.14%, $P < 0.0003$). Table 3 explains the percentage of patients and scoring of expression.

DISCUSSION

Multiple signaling pathways are coupled to the development of RCC. Among them, PI3K/AKT/mTOR is one of the universal signaling pathway characteristics of most cells, the central elements of which are the enzymes PI3K, AKT, and mTOR kinase. All of these occurrences set off unchecked mechanisms that aid in the development of cancer. Analysis of expression pattern of proteins associated with AKT/mTOR signaling pathway showed that (a) components of the mTOR pathway are constitutively activated in all cases (100%) of RCC; (b) higher expression of p-Akt1, an upstream effector, and the mTOR pathway component, HIF-1α are significantly upregulated and overexpressed in both gene and protein level expression

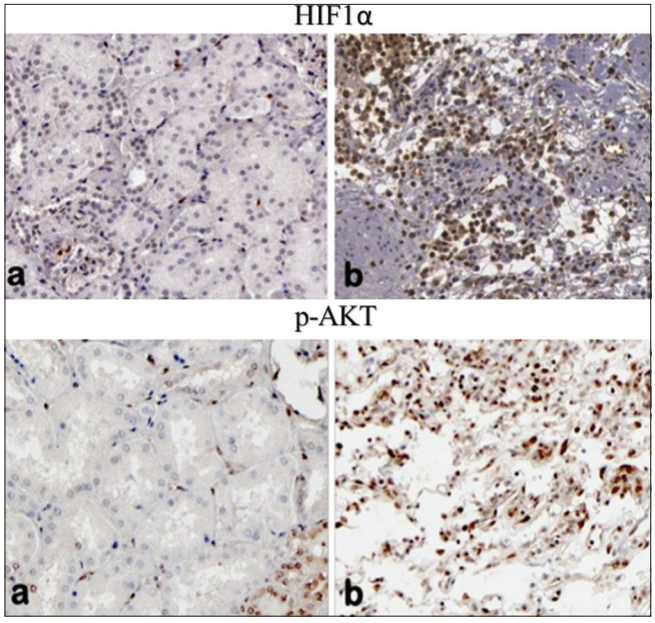

Figure 1: Representative expression image of HIF-1α and p-AKT by immunohistochemistry in renal tumor tissues (40). (a) Normal tissue (b) Renal tumor tissue, HIF-1α: Hypoxia-inducible factor 1 alpha

Table 1: Demographic and clinicopathologic characteristics of study participants

Characteristics	Number of cases
Age at diagnosis (years), mean	
Male	53
Female	54
Gender	
Male	15
Female	10
Stage at diagnosis	
I	6
II	12
III	4
IV	3
Location	
Right	9
Left	16
Smoking status	
Smoking	20
Nonsmoking	5
Drinking status	
Alcoholic	3
Nonalcoholic	22
Hypertension	
Yes	17
No	8
Obesity	
Yes	18
No	7

Table 2: Comparative analysis between Stage I+II and III+IV tumors for hypoxia-inducible factor 1 alpha

IHC scoring	Stage	
	I+II	III+IV
n=25	18	7
0	1	2
1	0	1
2	5	1
3	12	3

IHC scoring: 0: Negative, 1: Weak, 2: Moderate, 3: Strong.
IHC: Immunohistochemistry

Table 3: Comparative analysis between Stage I+II and III+IV tumors for AKT1

IHC scoring	Stage	
	I+II	III+IV
n=25	18	7
0	1	1
1	1	2
2	1	1
3	10	3

IHC scoring: 0: Negative, 1: Weak, 2: Moderate, 3: Strong.
IHC: Immunohistochemistry

in RCC tumor tissues compared to the normal kidney; and (c) correlative expression is evident of activated phosphorylation sites and signal transduction from p-Akt to HIF-1α. In support of our observations regarding a constitutively activated mTOR pathway in human RCC are various studies. These include (a) the Chuang *et al*.'s study[13] of the overexpression of glutathione s-transferase (GST)-α in the majority of primary and metastatic ccRCC, given that GST-α can be increased through PI3K/Akt/mTOR/p70S6K signaling;[14] (b) the efficacy of rapamycin as an inhibitor of human renal cancer pulmonary metastasis in a xenograft model;[15] and (c) the study by Thomas *et al*.[16] demonstrating that kidney cancer cells become more sensitive to the mTOR inhibitor CCI-779 when the VHL tumor suppressor gene VHL is lost and that VHL-deficient tumors exhibit increased uptake of the PET tracer fluorodeoxyglucose in an mTOR-dependent manner. HIF-1a plays a central role in RCC tumorigenesis by acting as a transcription factor for several proteins that are important in tumoral adaptation to a tissue microenvironment that is low in oxygen. Higher HIF-1α expression in the cytoplasm might indicate that HIF-1α has been translocated to the cytoplasm, does not transcribe DNA, and therefore leads to less aggressive tumors and better prognosis. Because high HIF-1α expression was associated with poor survival, targeting HIF-1α may be a promising therapeutic approach.[17] HIF-1α regulates angiogenesis, tumor growth, progression, metastatic spread, and glucose metabolism by acting as a transcription factor for crucial proteins, such as vascular endothelial growth factor, platelet-derived growth factor, epidermal growth factor receptor, insulin-like growth factor, glucose transporter-1), chemokine receptors, and carbonic anhydrase IX and XII. In addition, HIF-1α plays an important role in regulating the cell cycle and apoptosis.[18] We identified a significant higher expression of HIF-1α among tumor samples compared to normal. We observed a correlation between the gene and protein expression of HIF-1α and AKT genes and that both were found to be upregulated and overexpressed, respectively.

Activated Akt (p-Akt) was found more frequently in tumor positive than in uninvolved tissues. The activated serine/threonine kinase Akt (p-Akt) controls proteins involved in apoptosis and cell proliferation.[19] Overexpression of p-Akt, however, may contribute to the development and progression of various malignancies and consequently have a negative impact on prognosis.[19] It was previously shown that Akt, once activated, translocates from the cytoplasm to the nucleus. The role of this nuclear translocation, the specific nuclear target structures, and, above all, the consecutively initiated regulatory processes, which may increase tumor proliferation rate or dedifferentiation or a combination of both, have not been fully clarified so far. In conclusion, the present study found significantly increased nuclear p-AKT expression in RCC. Enhanced AKT kinase activity and subsequent downstream signal transduction are now accepted to play an important role in human malignancy.

CONCLUSION

Our study data predicted a higher gene expression as well as protein expression of HIF-1α and AKT. The proteins HIF-1α and AKT are localized in the nucleus in RCC tumor samples compared to normal. Overexpression of these proteins might play significant roles in tumor development and differentiation as reported by others previously. This study can help clarify the biological role of HIF-1α and AKT in RCC to develop new strategies for this malignancy.

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Conflicts of interest
There are no conflicts of interest.

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