

# The expression of RUNX3 gene in renal cell cancer and its clinical relevance with serum vascular endothelial growth factor

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## ABSTRACT

**Background:** Latest advances indicate that RUNX3 is a candidate tumor suppressor in several types of human cancers, including renal cell cancer. However, its definitive role is not yet established. Vascular endothelial growth factor (VEGF) has been widely studied as a surrogate marker of angiogenic activity and prognostic marker in renal cancer for monitoring treatment response and detection of early relapse. The aim of the study was to examine the clinical significance of RUNX3 expression and serum VEGF in series of renal cancer patients using quantitative real-time polymerase chain reaction and standard enzyme-linked immunosorbent assay kit and find its correlation with renal cancer stage, grade, and histopathology.

**Materials and Methods:** We reviewed our prospectively collected renal cancer database of 47 patients. All patients were evaluated preoperatively and staged and underwent partial or radical nephrectomy as per the feasibility criteria. RUNX 3 expression in tumor tissue and adjoining parenchyma was sampled in all patients, and serum levels of VEGF were measured in pre-and post-operative period on day 7 and day 30 after surgery. 10 age- and sex-matched healthy volunteers served as control group. **Results:** We observed that RUNX3 gene expression was significantly lower in tumor tissue than in normal renal parenchyma of a renal cancer patient. The serum VEGF levels were significantly increased in patients with renal cell carcinoma (RCC) compared to normal healthy volunteers and showed decreasing trend after the surgery. Loss of RUNX3 gene expression and higher VEGF levels strongly correlated with high-grade tumors; however, it was not related to tumor size and histopathology. There was no correlation of RUNX 3 with VEGF levels in RCC patients. **Conclusion:** The results of this study showed that renal cancer patients had increased VEGF levels which were effectively alleviated by curative resection. Lower expression of RUNX3 in renal cancer suggests its tumor suppressive role and new insights into targeted therapies linking RUNX3 gene may have some diagnostic and therapeutic implications in RCC patients. We did not find any correlation between RUNX3 gene and serum VEGF.

**Key words:** Renal cell cancer, RUNX3, Vascular endothelial growth factor

## Introduction

Renal cell carcinoma (RCC) is the most common renal cancer and accounts for 2-3% of malignant tumors in adults.<sup>[1]</sup> The incidence of RCC has increased in the last decade. Surgery is the only effective curative treatment for localized RCC. The clinical outcome of RCC patients is challenging and not always possible with prognostic factors such as staging and grading, which are primarily used when assessing cancer prognosis. Metastasis is a complex process wherein cancer cells migrate out of primary tumors and invade into neighboring tissue, intravasate into the blood or the lymphatic circulation and target specific organs to initiate cancerous outgrowth.<sup>[2]</sup> Metastatic RCC accounts for 20% of cases even after surgery and portends poor prognosis due to its resistance to chemotherapy, radiotherapy and hormonal therapy. However, recent targeted molecular therapies have been effective and showed promising response by offering an improved progression-free survival.<sup>[3]</sup> The interplay of tumor-suppressor genes and oncogenes form

a crucial part of cancer initiation and progression. Runt-related (RUNX) family genes are mainly regulators of gene expression in cell proliferation and differentiation. RUNX family members, RUNX1, RUNX2 and RUNX3, encode DNA-binding subunits that bind a common b subunit, core-binding factor subunit beta, to generate heterodimeric transcription regulators.<sup>[4]</sup> RUNX 3, in particular, is shown to play a tumor suppressor role in several cancers and its expression levels are down-regulated in breast cancer, colorectal cancer, glioma, and melanoma.<sup>[5,6]</sup> In analysis of gastric cancer patients with peritoneal metastasis, evidence shows that RUNX3 expression decreased significantly in the metastatic tissue, compared to normal gastric mucosa or primary main tumors.<sup>[7]</sup> Importantly, the decrease in RUNX3 protein expression is significantly associated with decreased survival of gastric cancer and melanoma patients.<sup>[8,9]</sup> The angiogenic factors are involved in neovascularization of RCC.<sup>[10]</sup> Being a highly vascular tumor, it is of interest to measure the serum vascular endothelial growth factor (VEGF) levels in RCC and compare with healthy individuals and further study

the correlation of serum VEGF and RUNX3 gene expression in tumor tissue in RCC patients.

## Materials and Methods

### Study population

A total of 55 patients with renal tumors based on imaging studies underwent surgery between July 2013 and December 2014. Four patients with histopathology as benign tumor and four patients who did not complete the study protocol were excluded from the study. The control group comprised 10 age- and sex-matched healthy volunteers. Clinical characteristics included patients age, sex, complete blood count, renal, and liver function test and all patients underwent contrast computed tomography for staging purpose. Radical or partial nephrectomy was done based on feasibility and negative margin status. Tumors were graded according to the Fuhrman nuclear grading system and staged according to the American Joint Committee on Cancer TNM system.

### Sample collection

#### Tissue

Tumor tissue and adjoining normal renal parenchyma samples were collected from patients who underwent nephrectomy. All specimens were snap-frozen with liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until nucleic acid extraction. Histopathologically confirmed cases of RCC were included and benign tumors were excluded.

#### Serum

A total of 4 ml blood was collected in plain vials by venipuncture and allowed to clot at room temperature for 30 min. Serum was separated from the blood and stored at  $-80^{\circ}\text{C}$  till further analysis. Blood samples in the study group were collected 7 and 30 days postsurgery in follow-up period. Blood samples from 10 healthy adults served as baseline control.

### Laboratory measurement

#### Tissue

Tumor and adjoining tissue were separately homogenized in 0.1 M phosphate buffered saline and centrifuged at 10,000 rpm. Total RNA from adjoining normal parenchyma and tumor tissue was isolated as by using TRIzol reagent (Invitrogen) as per standard protocol. The quantity and quality of RNA was measured by reading the samples at 260 nm and 280 nm (ratio at 260/280  $>1.8$ ) using ultraviolet spectrophotometer (Model-DU 640, Beckman Coulter, USA) as described by Sambrook and Russell (2001). From 1  $\mu\text{g}$  of RNA, cDNA was synthesized by using iScript<sup>TM</sup>cDNA synthesis kit (BIORAD) according to manufacturer's protocol. Relative quantification of RUNX3 gene expression was done by real time polymerase chain reaction (PCR) (Roche Diagnostics, Basel Switzerland) using SYBR<sup>®</sup> Green I chemistry. The expression of the gene was normalized to the housekeeping gene  $\beta$ -actin.

#### Serum

Plasma was separated from whole blood by centrifugation at 2000 rpm for 10 min. VEGF in the serum was estimated

by Ray Bio<sup>®</sup> Human VEGF enzyme-linked immunosorbent assay (ELISA) Kit as per manufacturer's instructions (RAY BIOTECH).

## Results

During the period between July 2013 and December 2014, 55 patients underwent surgery for renal tumors. Four patients with histopathological diagnosis of benign tumor and four patients who did not complete the study protocol were excluded. Their demographic and surgery details are given in Table 1. The mean age of the patients was 50.51 years (range 30–74 years). Final histopathology was clear cell cancer in 42, papillary variant in two patients and chromophobe variant in three patients. On Fuhrman grading, 14 had Grade 1, 21 had Grade 2 and 12 had Grade 3 disease.

### RUNX3 expression is decreased in human RCC

First, we determined the quantitative RUNX3 expression in RCC patients. DNA was extracted from tumor tissue and adjoining normal parenchyma and quantified with real-time PCR. A significantly lower expression of RUNX3 was observed in the carcinoma tissues. These results showed that RUNX3 was commonly expressed in normal human renal cells but decreased in renal cancer cells. The reference value of RUNX3 gene in normal renal parenchyma was taken as 1 and majority of patients with RCC showed almost three-fold decrease in RUNX3 gene expression [Figure 1].

### Comparison of RUNX3 expression with clinicopathological parameters

The clinicopathologic features of the 47 RCC patients are summarized in Table 1. There were 23 men and 24 women. The mean body mass index (BMI) was 23.98 kg/m<sup>2</sup>. The

**Table 1: The demographic details of patients**

Characteristics	No.
Total number of patients	47
Age	50.51
Sex	23/24
BMI	23.98
Surgical approach	
Open	20
Lap	27
Surgery performed	
Radical	31
Partial	16
Histopathology	
Clear	42
Papillary	3
Chromophobe	2
Fuhrman grade	
Grade 1	14
Grade 2	21
Grade 3	12

BMI: Body mass index

RUNX3 expression showed an increasing trend with grade of the disease, though it was not statistically significant [Figure 2]. Four patients with renal tumor tissue had values higher as compared to normal parenchyma, all of them were high-grade tumors according to Furman's grading system. The RUNX3 gene expression was not related to tumor size and histopathology.

**Serum VEGF assessment in RCC patients**

The serum VEGF levels in RCC patients were compared with 10 healthy volunteers. The mean baseline serum value of VEGF in RCC patients was significantly higher (mean  $\pm$  standard deviation [SD] -  $1177.93 \pm 1019.18$ ) as compared to serum levels of VEGF in healthy volunteers using Student's *t*-test ( $570.24 \pm 584.21$ ) ( $P < 0.04$ ). The serum VEGF levels showed decreasing trend and returned to near baseline at day 30 after surgery ( $592.07 \pm 576.09$ ) [Figure 3].

**Comparison of serum VEGF with clinicopathological parameters**

Patients with high-grade tumors had high serum levels of VEGF compared with low-grade disease and it was statistically significant using Student's *t*-test ( $P < 0.05$ ) [Figure 4]. The majority of the patients in our study were diagnosed as clear cell cancer and had higher serum levels of VEGF compared to other histopathological variants. Serum VEGF levels were not correlated with size of the tumor (data not shown).

**Correlation of RUNX3 gene expression with VEGF levels**

There was one to one correlation between RUNX3 and serum VEGF in all patients and correlation efficient value was 0.58. RUNX3 gene expression was decreased in RCC patients, and serum VEGF was higher in this group of patients [Figure 5].

**Discussion**

RUNX family members are major regulators of gene expression in developmental pathways and are critically involved in cellular proliferation and differentiation. Its role in tumorigenesis has been studied extensively in recent years. RUNX family genes were originally cloned as AML2 and localized to chromosome 1p36.1 which is the most frequently affected region in various type of cancers.<sup>[11]</sup> RUNX proteins have essential functions in both cell proliferation and differentiation in humans. They can act both as protooncogenes and tumor suppressor genes. Li *et al.* investigated the expression and localization of RUNX3 gene in pancreatic tissues and reported that RUNX3 expression was low to absent in normal pancreatic tissues and pancreatic cancer tissues expressed higher RUNX3 gene expression.<sup>[12]</sup> This is in contrast to studies related to gastric cancer in which RUNX3 expression was lost in 45–60% of gastric cancer cells,<sup>[13]</sup> but present in normal gastric epithelial cells. A meta-analysis study by Wang *et al* suggested that RUNX3 methylation was associated with an increased risk and progression of esophageal

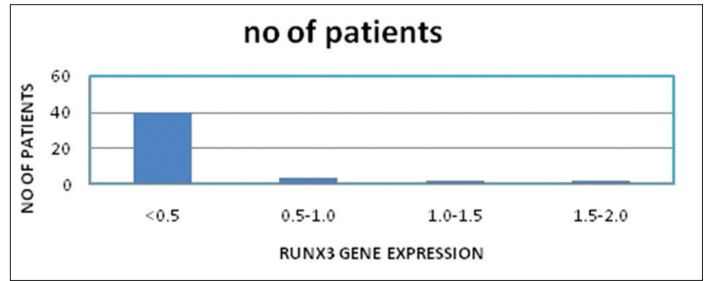


Figure 1: The RUNX3 gene expression in tumor tissue of renal cell carcinoma patients. Reference value taken as 1 for RUNX3 expression in normal parenchyma

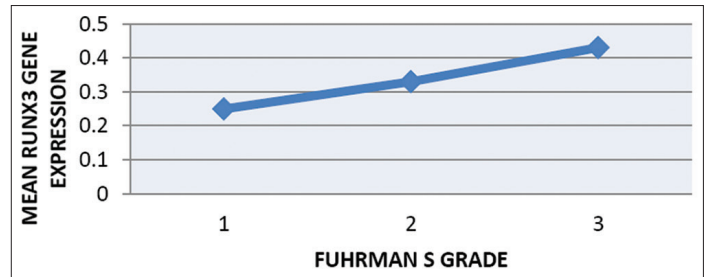


Figure 2: RUNX3 expression with respect to renal cell carcinoma Fuhrmann grading system

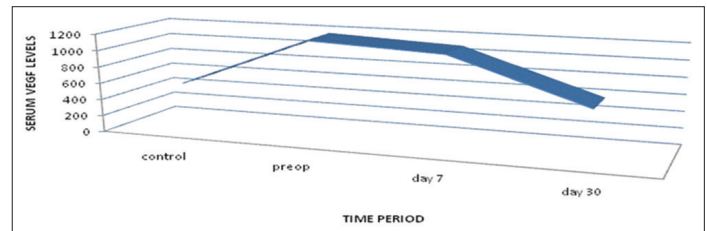


Figure 3: Serum vascular endothelial growth factor levels in renal cell carcinoma patients in pre- and post-operative period

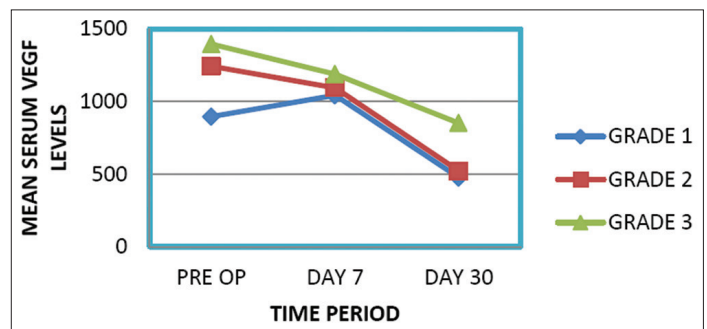


Figure 4: Serum vascular endothelial growth factor in pre- and post-operative period with respect to renal cell carcinoma Fuhrmann grading system

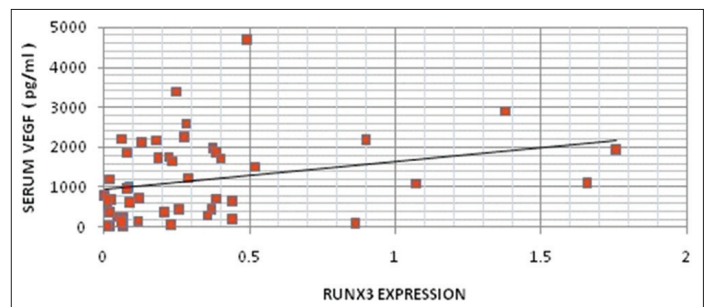


Figure 5: One to one correlation of RUNX3 and vascular endothelial growth factor comparison in 47 patients of renal cell cancer

cancer. The prevalence of lymph node involvement, tumor size, and histological grade increased in RUNX3 negative patients and was associated with worse survival in esophageal cancer patients.<sup>[14]</sup> Our study, however, did not show any significant correlation with tumor size, grade, and histopathology.

Studies have shown that *in vitro* expression of RUNX3 in cancer cells reduces cell migration, invasion and angiogenesis abilities, which was consistent with the function of RUNX3 *in vivo* in many cancers such as prostate and renal cancers.<sup>[15,16]</sup> In this context, new insights into targeted therapies linking RUNX3 gene may have some diagnostic and therapeutic implications in RCC patients.

RCC is known for its unpredictable clinical behavior. Considerable efforts have been made to find markers conclusively associated with risk for progressive disease. Neovascularization, an essential event for the growth of solid tumors, is regulated by a number of angiogenic factors. The more vascular tumors are associated with higher risk of metastasis and a less favorable prognosis. The VEGF is thought to play a major role in tumor angiogenesis. Increased expression and prognostic relevance of VEGF have been described in various epithelial and mesenchymal neoplasms. VEGF mRNA is found overexpressed in tumor cells, and secreted VEGF has been localized to the supplying vascular endothelium of tumor cells.<sup>[17]</sup> The microenvironment with gradients of critical nutrients, metabolites, and hypoxia are believed to facilitate VEGF mRNA expression in tumor cells.<sup>[18]</sup> VEGF is perhaps the most studied in cancer progression. VEGF is an important target of HIF and a potent mediator of angiogenesis. VEGF increases vascular permeability; induces endothelial cell (EC) proliferation, survival, migration, and differentiation; and promotes the degradation of the extracellular matrix around sprouting ECs by inducing the expression of proteases. This results in decreased junctional integrity and enhanced vascular permeability. In our present study, we measured serum VEGF using ELISA kit in all RCC patients and healthy volunteers. We observed a significantly higher level of VEGF in RCC patients as compared to healthy volunteers. The higher serum VEGF levels may be due to the fact that VEGF is liberated into the blood circulation by tumor cells secreting various cytokines which induce the expression of VEGF.

Intercurrent disease, medication or platelets count were not taken into account in our study. No difference in serum levels of VEGF was found in relation to gender and all age groups. The serum level of VEGF was assessed in the post-operative period and it was found that serum levels significantly decreased 4 weeks after surgery. There was no significant change in serum levels of VEGF on day 7 after the surgery. This suggests that high levels of VEGF required in physiologic process of wound healing after the surgery and gradual decrease to baseline after healing process. Jan *et al.* studied serum levels of VEGF in patients with RCC and found that serum

levels were significantly higher in RCC patients compared to controls and serum levels of VEGF correlated with clinical stage, histopathological grade of the disease and was associated with adverse survival.<sup>[19]</sup> In our study, patients with high-grade disease had high serum levels of VEGF compared with low-grade disease and it was statistically significant ( $P < 0.05$ ). The majority of the patients in our study were diagnosed as clear cell cancer and had higher serum levels of VEGF compared to other histopathological variants. However, we did not find any correlation between serum VEGF and other clinicopathological variables including age, gender, tumor size, and BMI.

In the previous studies, it has been shown that *in vitro* expression of RUNX3 gene suppresses migration, invasion, and angiogenesis in RCC.<sup>[16]</sup> It reduces the capacity of RCC cells supernatant to stimulate proliferation and tube formation of human ECs compared with those of control cells; Peng *et al.* demonstrated that RUNX3 down-regulated VEGF expression via transcriptional repression in human gastric cancer.<sup>[13]</sup> However, we did not find any correlation between RUNX3 gene and serum VEGF levels.

## Conclusion

The results of our study suggest tumor suppressive role of RUNX3 gene in RCC patients. However, it was not correlated with tumor size, grade, and histopathology of the disease. The serum VEGF in RCC patients was markedly higher suggesting it highly vascular tumor. The serum VEGF levels correlated with tumor size and grade in RCC patients and serum levels lowered to baseline at day 30 after surgery. The RUNX3 gene assessed quantitatively was not correlated with serum levels of VEGF. New insights into targeted therapies linking RUNX3 gene may present intriguing possibilities with diagnostic and therapeutic implications in RCC patients.

## Limitations

This study has potential limitations. First, the number of patients included in the study was small. Large-scale studies are warranted to establish utility of RUNX3 gene in RCC. Second, follow-up of patients was done only till 30 days after surgery. Longer follow-up and number of patients presenting with recurrence and death due to advanced disease could help identify the prognostic utility of RUNX3 gene and VEGF. Third, studies to address the molecular mechanism of RUNX3 are needed to better understand the biology and links to VEGF.

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