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ORIGINAL ARTICLE

Correlation of VEGF and EGFR in peripheral blood with clinical stage and pathological grade of renal cell carcinoma and analysis of prognosis

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Summary

Purpose: *To detect the expression of VEGF and EGFR in peripheral blood and cancer tissues of patients with renal cell carcinoma (RCC), and to explore the correlations with clinical stage, pathological grade and prognosis of disease.*

Methods: *A total of 64 patients with RCC who were diagnosed and treated from June 2016 to August 2017 in our hospital were enrolled. Patients were divided into different clinical stages and pathological grades, and ELISA and immunohistochemistry were used to detect the expression of VEGF and EGFR in peripheral blood. Peripheral blood was also taken from 24 healthy individuals to serve as control group. Real-time fluorescence quantitative PCR (qRT-PCR) was used to detect the expression of VEGF and EGFR in RCC tissues and paracancer tissues. All patients were followed up after discharge to record their survival.*

Results: *Significant differences in the expression levels of VEGF and EGFR were found between stage III and IV ($p<0.05$), but not between stage I and II. Expressions level of VEGF and EGFR in serum of well-differentiated, moderately-differentiated, and poorly-differentiated RCC were all higher than those in the healthy control group, and significant differences were found between different pathological grades ($p<0.05$). Patients with higher expression levels of VEGF and EGFR showed shorter survival compared to patients with lower expression levels ($p<0.05$).*

Conclusion: *VEGF and EGFR in peripheral blood can be used as one of the effective indicators of prognosis of RCC. Our study provided reference for clinical treatment and prediction of prognosis of RCC.*

Key words: *EGFR, prognosis, renal cell carcinoma, VEGF*

Introduction

RCC, as the most common type of primary renal tumor in adults, accounts for about 3% of malignant solid tumors, and more than 100,000 people die of this disease each year worldwide [1-3]. The early diagnosis rate of RCC is low, and most patients are diagnosed at advanced stages [4]. About 30% of patients with RCC at the time of first diagnosis have metastases, and the 5-year survival rate after metastasis is less than 20% [5]. It is generally believed that tumor growth is closely related to angiogenesis in tumor tissues, and VEGF as a vascular endothelial growth factor is a key factor regulating tumor angiogenesis [6-9]. EGFR as a natural transmembrane receptor is overexpressed in a variety of malignancies [10,11]. In this study, ELISA and qRT-PCR were used to detect the expression of VEGF and EGFR in peripheral blood and tumor tissues of paients with RCC, respective-

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ly. Correlations between expression of VEGF and EGFR and the TNM clinical stages and pathological grades were explored to provide reference for clinical treatment and prediction of prognosis of this disease.

Methods

Subjects

A total of 64 patients of our hospital with RCC were enrolled from June 2016 to August 2017 and included 41 males and 23 females, with age range from 42 to 75 years with a median of 60 years. Thirty-nine patients had cancer of the left kidney and 25 of the right kidney. All patients were diagnosed by clinical and postoperative pathological examinations. Peripheral blood was collected 2 hrs before surgery. TNM clinical staging: 35 cases with stage I, 18 with stage II, 8 with stage III, and 3 cases with stage IV. All patients were diagnosed with clear cell RCC, of which 41 patients had well differentiated, 15 moderately differentiated, and 8 poorly differentiated disease. Peripheral blood was also extracted from 24 healthy people to serve as control group.

Reagents

RNA Extraction Kit was purchased from Invitrogen (Waltham, MA, USA); Fluorescent Quantitative PCR Kit was purchased from Wuhan Biofavor Biotech Services Co., Ltd. (Wuhan, China); VEGF ELISA Kit was purchased from Wuhan Huamei Bioengineering Co., Ltd. (Wuhan, China); EGFR ELISA Kit was purchased from Beijing Longke Fangzhou Biological Engineering Technology Co., Ltd., (Beijing, China); rabbit anti-human VEGF and EGFR primary antibody was purchased from Beijing Biolebo Technology Co., Ltd., (Beijing, China); rat anti-rabbit secondary antibody was purchased from Beijing Huatai Biotechnology Co., Ltd (Beijing, China).

Peripheral blood sample collection

Fasting peripheral blood (66ml) was collected from patients before and after operation and placed in EDTA-K2 tubes. Peripheral blood mononuclear cells were isolated by density gradient centrifugation at 2000rpm for 20min within 4 hrs after collection.

All reagents were kept at room temperature for 30 min. Fifty μL of serum sample were put into well of 96-well plate, followed by addition of 50 μL of conjugated antibody. The solution was gently shaken, followed by incubation at 37°C for 30 min. The above reaction solution was removed, followed by washing with phosphate buffered saline (PBS). After that, 50 μL of color development reagents A and B were added, followed by incubation at 37°C for 10 min. Finally, 50 μL of stop solution were added and absorbance was measured at 450 nμ. Standard curve was plotted to calculate the levels of VEGF and EGFR.

Detection of VEGF and EGFR expression by qRT-PCR

Reverse transcription reaction conditions: 37°C for 15min, 85°C for 5s and 4°C for preservation. Reverse transcription reaction system (10 μL): 5× Prime ScriprTM Buffer; Prime ScriprTM RT Enzyme Mix I; Oligo Dt Primer (50 μM) ×1; Random 6 mers (100 μM) ×1; Total RNA; Rnase Free dH_2O. PCR reaction system (50 μL): RPremix Ex Taq TM II (2×); PCR Forward Primer (10 μM); PCR Reverse Primer (10 μM); ROX Reference Dye (50×); DNA template; Rnase Free dH_2O.

Primers were synthesized by Nanjing Shengxing Biotechnology Co., Ltd (Nanjing, China). Primer sequences are listed in Table 1.

PCR reaction conditions: 94°C for 3 min, followed by 40 cycles of 94°C for 20 s, 58°C for 20 s, and 72°C for 30 s. The relative expression levels of VEGF and EGFR mRNA were normalized to endogenous control.

Table 1. Primer sequences

VEGF	Forward primer	5'-TCGGGCCTCCGAAACCATGA -3'
	Reverse primer	5'-CCTGGAGAGAGATCTGGTTC -3'
EGFR	Forward primer	5'-ACCCCAGCAGTTTCTGCAA-3'
	Reverse primer	5'-AGCCACCTCCTGGATGGTC-3'
GAPDH	Forward primer	5'-TGGGTGTGAACCACGAGAA-3'
	Reverse primer	5'-GGCATGGACTGTGGTCATGA-3'

Immunohistochemistry

RCC tissues and paracancer tissues were fixed with formalin and embedded in paraffin. Tissue sections were dewaxed, followed by washing with PBS and blocking with 10% fetal bovine serum (FBS) for 10 min at room temperature. Tissue sections were then incubated with primary antibodies (VEGF monoclonal antibody and EGFR monoclonal antibody, 1:50 dilution) at 4°C overnight. After washing with PBS, secondary antibody (1:50 dilution) was added, followed by incubation at room temperature for 30 min. After incubation with protein-peroxidase solution at room temperature for 30 min, slides were washed, followed by DAB color development. Finally, tissue sections were sealed.

Analysis of the results

All sections were observed under an optical microscope with 100x magnification. Five visual fields were randomly selected, and light yellow to brown color indicated positive staining. The proportion of stained tumor cells was calculated. Staining results were assessed by the proportion of positive cells and the degree of staining. Scoring criteria: 0 points (percentage of positive cells 0 to 5%), 1 point (6 to 30%), 2 points (31 to 60%), 3 points (61 to 100%). Staining intensity score: 0 points (no coloring); 1 point (light yellow); 2 points (brown); 3 points (tan). Two scores were multiplied to obtain the final score: 0 to 3 were divided into low expression group, and 4 to 9 were divided into high expression group.

Statistics

SPSS19.0 statistical software was used to analyze all data. Quantitative data were expressed as mean ±

standard deviation. Numerical data was expressed as percentage (%). One way ANOVA was used for inter-group comparisons and Kaplan-Meier method was used for survival analysis with comparisons performed with log-rank test . α=0.05 was used as the test standard. A p value <0.05 was considered to be statistically significant.

Results

Serum levels of VEGF and EGFR in patients with different clinical stages

As shown in Table 2, with the gradual increase of TNM staging, the serum levels of VEGF and EGFR gradually decreased. Compared with stage I, significant decrease was found in stages III and IV (p<0.05), but not in stage II.

Serum levels of VEGF and EGFR in patients with different pathological grades

As shown in Table 3, with the gradual increase in the grade of tissue differentiation, serum levels of VEGF and EGFR gradually increased. Compared with the control group, significantly higher expression levels of VEGF and EGFR were found in patients with high, moderate, and poor differentiation (p<0.05). Significant differences were also found among patients with high, moderate and poor differentiation.

Expression of VEGF and EGFR in RCC tissues and paraneoplastic tissues

As shown in Table 4, the mean expression level of VEGF in RCC tissues was 8.73±1.05, which was significantly lower than that in the control and paraneoplastic tissues (14.03±0.94, 13.96±1.42, respectively; p<0.05). The mean expression level of EGFR in RCC tissues was 5.02±0.35, which was significantly lower than that of the control and paraneoplastic tissues (9.46±0.52, 9.17±0.44, respectively; p<0.05).

Table 2. Comparison of mean serum levels of VEGF and EGFR among patients with different clinical stages

Stages	*Case*	*VEGF (ng/L)*	*EGFR (pg/ml)*
Control	24	502.35±93.64	254.22±39.26
I	35	923.58±173.56	573.34±69.49
II	18	854.42±154.63	558.47±77.47
III	8	798.39±159.47*#	492.26±48.21*#
IV	3	775.38±148.42*#	473.93±69.36*#

*compared with stage I, p<0.05; #compared with stage II, p<0.05

Table 3. Mean serum levels of VEGF and EGFR in patients with different pathological grades

Grades	*Cases*	*VEGF (ng/L)*	*EGFR (pg/ml)*
Control	24	502.35±93.64	254.22±39.26
High	41	953.74±162.43*	595.37±83.47*
Moderate	15	865.72±159.93*#	524.66±79.25*#
Low	8	784.62±146.29*#‡	473.53±39.56*#‡

*compared with control, p<0.05; #compared with high differentiation group, p<0.05; ‡compared with moderate differentiation group, p<0.05

Table 4. Mean expression of VEGF and EGFR in renal cell carcinoma tissues and paraneoplastic tissues

	Cases	*VEGF*	*EGFR*
Control	24	14.03±0.94	9.46±0.52
Paraneoplastic tissues	64	13.96±1.42	9.17±0.44
Renal cell carcinoma tissues	64	8.73±1.05*	5.02±0.35#

*compared with paraneoplastic tissues, p<0.05; #compared with control tissues, p<0.05

Table 5. Mean expression of VEGF and EGFR in renal cell carcinoma and adjacent tissues and the correlations with clinical stages

Stage	*Cases*	*VEGF*		*EGFR*	
		Cancer tissues	*Adjacent tissues*	*Cancer tissues*	*Adjacent tissues*
I	35	9.47±0.88	13.27±0.79	5.35±0.41	9.01±0.48
II	18	9.16±0.79	12.94±0.98	5.17±0.43	8.95±0.45
III	8	7.73±0.68*	11.46±0.83	4.16±0.37*	8.79±0.38
IV	3	6.99±0.65*#	8.77±0.74†	3.86±0.31*#	7.27±0.33†

*compared with stage I and II, p<0.05; #compared with stage III, p<0.05; †compared with stage I-III, p<0.05

Table 6. Mean expression levels of VEGF and EGFR in renal cell carcinoma tissues and adjacent tissues

Expression level	*Cases*	*VEGF*		*EGFR*	
		Cancer tissues	*Adjacent tissues*	*Cancer tissues*	*Adjacent tissues*
High	41	9.53±0.92#	13.85±0.92	5.72±0.48#	9.85±0.53
Moderate	15	8.25±0.88*#	12.04±0.85*	4.84±0.41*#	8.27±0.48*
Low	8	7.06±0.75*†#	10.93±0.77*†	3.95±0.37*†#	7.44±0.41*†

*compared with high differentiation group, p<0.05; †compared with moderate differentiation group, p<0.05; #compared with adjacent tissues, p<0.05

Expression of VEGF and EGFR expression in RCC and adjacent normal tissues and the correlations with clinical stages

As shown in Table 5, the levels of VEGF and EGFR in RCC tissues and adjacent normal tissues gradually decreased with the increase of clinical stages. Significant differences in the expression of VEGF and EGFR in cancer tissues were found between patients with different clinical stages except between stage I and II (p<0.05). There was no significant difference in the expression of VEGF and EGFR in adjacent tissues among patients with clinical stages I, II and III, while significantly decreased expression levels of VEGF and EGFR in adjacent normal tissues were found in patients with clinical stage IV (p<0.05).

Expression of VEGF and EGFR expression in RCC and adjacent normal tissues and the correlations with pathological grades

As shown in Table 6, the expression levels of VEGF and EGFR in RCC tissues and adjacent normal tissues gradually increased with the increase of pathological grades. Significant differences in the expression levels of VEGF and EGFR in RCC tissues and adjacent normal tissues were found among patients with different pathological grades (p<0.05).

Prognostic analysis

As shown in Figures 1 and 2 the survival of patients with high expression levels of VEGF and EGFR was significantly shorter than that of patients with low expression levels of VEGF and EGFR (p=0.002 and p=0.005, respectively).

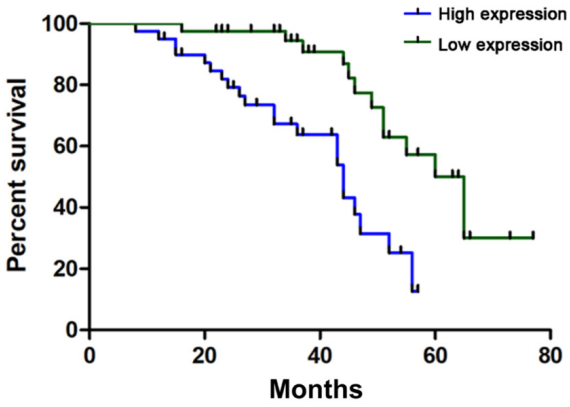

Figure 1. Kaplan-Meier survival of patients with high and low expression level of VEGF (log rank, p=0.002).

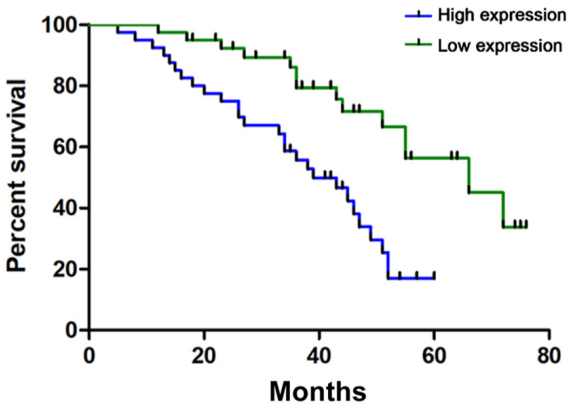

Figure 2. Kaplan-Meier survival of patients with high and low expression level of EGFR (log-rank, p=0.005).

Discussion

VEGF can induce increased calcium function, promote vascular permeability, endothelial cell migration, and ultimately lead to angiogenesis [12,13]. EGFR is one of the members of the HER receptor family and can influence cell proliferation and differentiation by binding to its own ligand [14]. Studies on mouse Wilms tumor model showed a significant correlation between the expression of VEGF and the course of disease [15]. Moreover, it has been confirmed that EGFR is upregulated in many malignant tumors, such as breast cancer [16], bladder cancer [17], ovarian cancer [18], upper gastrointestinal cancer [19], and pancreatic cancer [20]. However, application of VEGF and EGFR is the diagnosis of patients with RCC has not been reported.

In this study, ELISA and qRT-PCR were used to detect the expression of VEGF and EGFR in peripheral blood and tumor tissues. The results showed that with the gradual increase in the grade of differentiation, serum levels of VEGF and EGFR gradually increased. The expression levels of VEGF and EGFR in serum were low in the control group, but their expression levels were relatively high in the high, moderate, and low differentiation groups and were significantly increased with increased grade of differentiation ($p<0.05$). The mean expression level of VEGF in RCC tissues was 8.73±1.05, which was significantly lower compared with the control and paraneoplastic tissues (14.03±0.94, 13.96±1.42, respectively; $p<0.05$). The mean expression level of EGFR in RCC tissues was 5.02±0.35, which was significantly lower than that of the control and paraneoplastic tissues (9.46±0.52, 9.17±0.44, respectively; $p<0.05$). The expression levels of VEGF and EGFR in RCC tissues and adjacent tissues gradually decreased with the increase of clinical stages ($p<0.05$). With the gradual increase in the grade of differentiation, the expression levels of VEGF and EGFR in RCC tissues and adjacent tissues gradually increased ($p<0.05$). The survival of patients with low expression levels of VEGF and EGFR was better than those of patients with high expression levels, indicating a promising prognostic value of VEGF and EGFR for patients with RCC.

Conclusions

In summary, the expression levels of VEGF and EGFR in peripheral venous blood and cancer tissues may serve as promising prognostic markers for RCC. We believe that our study provided useful information for the treatment and prediction of prognosis of this disease.

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Conflict of interests

The authors declare no conflict of interests.

References

1. Ferlay J, Soerjomataram I, Dikshit R et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 2015;136:E359-86.
2. Voss MH, Hsieh JJ, Motzer RJ. Novel approaches targeting the vascular endothelial growth factor axis in renal cell carcinoma. Cancer J 2013;19:299-306.
3. Chen C, Chi H, Min l, Junhua Z. Downregulation of guanine nucleotide-binding protein beta 1 (GNB1) is associated with worsened prognosis of clear cell renal cell carcinoma and is related to VEGF signaling pathway. JBUON 2017;22:1441-6.
4. Dias F, Teixeira AL, Santos JI et al. Renal cell carcinoma development and miRNAs: a possible link to the EGFR pathway. Pharmacogenomics 2013;14:1793-1803.
5. Barata PC, Ornstein MC, Garcia JA. The Evolving Treatment Landscape of Advanced Renal Cell Carcinoma in Patients Progressing after VEGF Inhibition. J Kidney Cancer VHL 2017;4:10-8.
6. Sorich MJ, Kichenadasse G, Rowland A, Woodman RJ, Mangoni AA. Angiotensin system inhibitors and survival in patients with metastatic renal cell carcinoma treated with VEGF-targeted therapy: A pooled secondary analysis of clinical trials. Int J Cancer 2016;138:2293-9.
7. Matušan-Ilijaš K, Damante G, Fabbro D et al. EGFR expression is linked to osteopontin and NF-κB signaling in clear cell renal cell carcinoma. Clin Transl Oncol 2013;5:65-71.
8. Vano YA, Tartour E, Fournier LS, Beuselinck B, Mejean A, Oudard S. Prognostic factors in patients with advanced renal cell carcinoma treated with VEGF-targeted agents. Expert Rev Anticancer Ther 2014;14:523-42.
9. Funakoshi T, Lee CH, Hsieh JJ. A systematic review of predictive and prognostic biomarkers for VEGF-targeted therapy in renal cell carcinoma. Cancer Treat Rev 2014;40:533-47.
10. Harshman LC, Xie W, Bjarnason GA et al. Conditional survival of patients with metastatic renal-cell carcinoma treated with VEGF-targeted therapy: a population-based study. Lancet Oncol 2012;13:927-35.
11. Pal SK, Li SM, Wu X et al. Stool Bacteriomic Profiling in Patients with Metastatic Renal Cell Carcinoma Receiving Vascular Endothelial Growth Factor-Tyrosine Kinase Inhibitors. Clin Cancer Res 2015;21:5286-93.
12. Bayrak O, Sen H, Bulut E et al. Evaluation of EGFR, KRAS and BRAF gene mutations in renal cell carcinoma. J Kidney Cancer VHL 2014;1:40-5.
13. Lai S, Molfino A, Seminara P et al. Vascular Endothelial Growth Factor Inhibitor Therapy And Cardiovascular And Renal Damage In Renal Cell Carcinoma. Curr Vasc Pharmacol 2017 Jun 20. doi: 10.2174/1570161115666170621073715. [Epub ahead of print].

14. Song W, Dang Q, Xu D et al. Kaempferol induces cell cycle arrest and apoptosis in renal cell carcinoma through EGFR/p38 signaling. Oncol Rep 2014;31:1350-6.
15. He D, Li L, Zhu G et al. ASC-J9 suppresses renal cell carcinoma progression by targeting an androgen receptor-dependent HIF2α/VEGF signaling pathway. Cancer Res 2014;74:4420-30.
16. Feng ZH, Fang Y, Zhao LY et al. RIN1 promotes renal cell carcinoma malignancy by activating EGFR signaling through Rab25. Cancer Sci 2017;108:1620-7.
17. Kang SK, Volodarskiy A, Ohmann EL, Balar AV, Bangalore S. Efficacy and Safety of Selective Vascular Endothelial Growth Factor Receptor Inhibitors Compared with Sorafenib for Metastatic Renal Cell Carcinoma: a Meta-analysis of Randomised Controlled Trials. Clin Oncol (R Coll Radiol) 2016;28:334-41.
18. Chen F, Deng J, Liu X, Li W, Zheng J. Corrigendum: HCRP-1 regulates cell migration and invasion via EGFR-ERK mediated up-regulation of MMP-2 with prognostic significance in human renal cell carcinoma. Sci Rep 2016;6:19412.
19. Salinas-Sánchez AS, Serrano-Oviedo L, Nam-Cha SY, Roche-Losada O, Sánchez-Prieto R, Giménez-Bachs JM. Prognostic Value of the VHL, HIF-1α, and VEGF Signaling Pathway and Associated MAPK (ERK1/2 and ERK5) Pathways in Clear-Cell Renal Cell Carcinoma. A Long-Term Study. Clin Genitourin Cancer 2017 pii: S1558-7673(17)30153-2. doi: 10.1016/j.clgc.2017.05.016. [Epub ahead of print].
20. Liang L, Li L, Zeng J et al. Inhibitory effect of silibinin on EGFR signal-induced renal cell carcinoma progression via suppression of the EGFR/MMP-9 signaling pathway. Oncol Rep 2012;28:999-1005.