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Original Article

Immunochemical expression of fibroblast growth factor and its receptors in primary tumor cells of renal cell carcinoma

Maria Volkova[1], Ilya Tsimafeyeu[2,3], Anna Olshanskaya[1], Yulia Khochenkova[1], Eliso Solomko[1], Saida Ashuba[1], Vsevolod Matveev[1], Dmitry Khochenkov[1,4]

[1]N.N. Blokhin National Medical Research Center for Oncology, Moscow, Russian Federation; [2]Institute of Oncology, Hadassah Moscow, Moscow, Russian Federation; [3]Kidney Cancer Research Bureau, Moscow, Russian Federation; [4]Togliatti State University, Togliatti, Russian Federation

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Abstract: Background: There is the evidence of the role of the fibroblast growth factor and its receptors (FGF/FGFR) signaling pathway in tumorogenesis of renal cell carcinoma (RCC). We conducted a study aimed at evaluating of FGF2, FGFR1, and FGFR2 expression in primary tumor cells and assessing of these molecules expression levels effect on the characteristics of the tumor process and prognosis of patients with RCC. Methods: Expression of FGF2, FGFR1, and FGFR2 was investigated in 65 primary RCC specimens by immuhistochemical staining using the appropriate antibodies. Expression levels were evaluated by the semi-quantitative method. A search for correlations of expression levels of investigated growth factors and receptors with RCC features and patients outcomes was performed. Results: Cytoplasm and membrane expression of FGF2, FGFR1, and FGFR2 was found in the primary tumor cells of RCC patients. FGF2 staining was detected in 60.0% of cases (44.2 ± 5.4 HS). It was noted higher frequency and intensity of FGFR2 expression (66.2%; 46.6 ± 6.3 HS) comparing with FGFR1 (32.3%; 7.5 ± 2.2 HS). The correlation was revealed between the investigated markers overexpression and Fuhrman grade G3-4, as well as features of advanced RCC (paranephric fat tumor invasion, venous wall tumor invasion, adrenal and liver metastases). FGFR2 overexpression showed negative influence on cancer-specific survival in univariate analysis, however, lost its predictive value in multivariable analysis. Conclusion: Expression of FGF2 and its receptors was found on the surface and in the cytoplasm of RCC primary tumor cells and needs following investigations.

Keywords: Renal cell carcinoma, primary tumor, fibroblast growth factor, fibroblast growth factor receptor, expression, survival

Introduction

Deregulation of FGF/FGFR signaling in renal cell carcinoma (RCC) is now well understood [1, 2]. Fibroblast growth factors (FGFs) and fibroblast growth factor receptor 2 (FGFR2) regulate cellular proliferation, survival, migration and differentiation [3]. FGF also enhances the production of plasmin-plasminogen activator and matrix metalloproteinase in endothelial cells, which induces degradation of the intercellular matrix and promotes the formation of blood vessels [4]. The response of endothelial cells to FGF is regulated by integrins that promote cells adhesion, migration, proliferation, and morphogenesis [5]. FGF secreted by stromal fibroblasts induces proliferation of tumor cells via paracrine FGFR signaling [6]. In addition, fibroblasts in the tumor stroma can be activated by FGF secreted by endothelial and tumor cells [7]. Dissecting the FGF/FGFR pathway offers the hope of developing new therapeutic approaches that selectively target the FGF/FGF receptor axis in patients with genitourinary tumors with FGF/FGFR axis dysregulation [8]. Thus, there is the evidence base of the angiogenic, mitogenic and proliferative role of the FGF/FGFR signaling pathway in RCC.

However, there are only few studies describing the immunohistochemical expression of receptors on primary tumor cells. In addition, the

results of these studies are ambiguous about the correlation of these factors with prognosis. We conducted an investigation aimed at studying of FGF2, FGFR1, FGFR2 expression in primary tumor cells and assessing of these molecules expression levels effect on the characteristics of the tumor process and prognosis of patients with RCC.

Material and methods

Patients

To be eligible, patients with localized or locally advanced RCC were required to have no any systemic or radiation therapy before surgical treatment. All participants should be aged ≥18 years at the time of inclusion. Patients' data were depersonalized and anonymous. The exclusion criteria were the unsuitability of the histological material for the immunohistochemical study, the patient's participation in another study, and the presence of distant metastases at the time of diagnosis.

The study was conducted according to the Declaration of Helsinki. The study was approved by the medical ethics review board of N.N. Blokhin Russian Cancer Research Center, and signed informed consent was obtained from all patients.

Immunohistochemistry

Prospectively collected surgical samples of the tumor tissue were used for the examination. A routine histological examination was performed in all cases. Expression of FGF2, FGFR1, and FGFR2 was studied in the RCC tissue by immunohistochemistry with anti-FGFR1 (M2-F12, Santa Cruz, 1:200), anti-FGFR2 (C-8, Santa Cruz, 1:200) and anti-FGF-2 (G-2, Santa Cruz, 1:200) antibodies and REAL™ EnVision™ Detection System, Peroxidase/DAB+ Rabbit/Mouse (Agilent). Expression of FGF2, FGFFR1, and FGFR2 was studied in the RCC tissue by immunohistochemistry using appropriate Abcam/Santa Cruz Biotech antibodies from the Invitrogen immunohistochemical staining kit. Expression levels were evaluated using a semi-quantitative method by characterizing of the staining intensity (0, 1+, 2+ and 3+) and counting the relative number of stained cells which was expressed as a percentage (0-100%). The immunohistochemical expression level (H-score; HS) was calculated by multiplying the percentage of stained cells by their staining intensity [9].

Statistical analysis

Statistical analysis was performed with commercially available software (IBM SPSS Statistics Base v21.0 (SPSS, Inc., Chicago, IL, USA). The significance of differences between the quantitative factors was calculated with t-test for normally distributed values or with non-parametric Mann-Whitney U test. To compare the qualitative parameters, the Fisher's exact test and 2 were used taking into account nonparametric data and the Poisson distribution. Differences were recognized as significant at $P<0.05$. To assess the relationship between factors, the Pearson correlation coefficient (r) was calculated and its significance was evaluated. To evaluate predictive efficacy of analyzed factors, ROC curves were constructed, and threshold values were selected according to the coordinates of the ROC-curves. Overall survival (OS) was defined as the time from the date of surgery until the last known date alive. Cancer-specific survival (CSS) was defined as the time from the date of surgery until the last known date alive or death from RCC. Recurrence-free survival (RFS) was defined as the time from the date of radical surgery to the date of radiologically confirmed relapse or death from RCC. Progression-free survival (PFS) was defined as the time from the date of cytoreductive surgery to the date of radiologically confirmed progression of the disease or death from RCC. CSS, RFS, and PFS were analyzed with the Kaplan-Meier method, the Mantel-Haenszel log-rank test, and Cox regression model.

Results

Patient characteristics

The study included 65 patients with RCC pT1a-T4N0/+M0/+ (**Table 1**). The median age of the patients was 59.0 (33-79) years, a male-to female ratio was 1.9:1. All patients were diagnosed with RCC. Most patients (59; 90.8%) had unilateral, and 6 (9.2%) had bilateral kidney tumors. The median diameter of a renal parenchyma tumor was 10 (2.5-26) cm. Tumor venous thrombosis was noted in 50 (76.9%) cases. Forty-five (69.2%) patients had distant

Table 1. Baseline demographic and clinical patient characteristics

Characteristics	Study population, N=65
Age (years), median (range)	59.0 (33-79)
Gender, N (%)	
Male	43 (66.2)
Female	22 (33.8)
Histology, N (%)	
Clear-cell RCC	59 (90.8)
Non-clear cell RCC	6 (9.2)
RCC with sarcomatoid features	0
Fuhrman grade, N (%)	
G1-2	29 (44.6)
G3-4	36 (65.4)
Size of the primary tumor, diameter, median (range), cm	10 (2.5-26)
The primary tumor side, N (%)	
Unilateral	59 (90.8)
Bilateral	6 (9.2)
TNM stage, N (%)	
pT1-T2	12 (18.5)
pT2-T3	53 (81.5)
pT4	0
N0	53 (81.5)
N1	12 (18.5)
M0	20 (30.8)
M1	45 (69.2)
Tumor venous thrombus, N (%)	50 (76.9)
Tumor invasion of paranephric fat, N (%)	29 (44.6)
Metastatic sites, N (%)	
1	22 (33.8)
≥2	23 (35.4)
Sites of metastases, N (%)	
adrenal gland	28 (43.1)
lungs	22 (33.8)
bones	5 (7.7)
liver	2 (3.1)
Surgery, N (%)	
Nephrectomy, retroperitoneal lymphadenectomy	65 (100)
Thrombectomy	50 (76.9)
Metastasectomy, N (%)	28 (43.1)
adrenalectomy	24 (36.9)
contralateral partial nephrectomy	1 (1.5)
bone metastasectomy	1 (1.5)
pulmonary resection	1 (1.5)
liver resection	1 (1.5)
Complete removal of all tumor sites, N (%)	40 (61.5)
Cytoreductive nephrectomy, N (%)	25 (39.5)
Systemic therapy following cytoreductive nephrectomy, N (%)*	22 (88.0)
immunotherapy	3 (5)
targeted therapy	19 (29)

*from 25 patients undergone cytoreductive nephrectomy.

Table 2. FGF2, FGFR1, FGFR2 expression in primary tumor cells in RCC patients

Growth factor and it's receptors (n 65)	Specimens with expression, n (%)	Expression level, median ± σ, HS
FGF2	39 (60.0)	44.2 ± 5.4
FGFR1	21 (32.3)	7.5 ± 2.2
FGFR2	43 (66.2)	46.6 ± 6.3

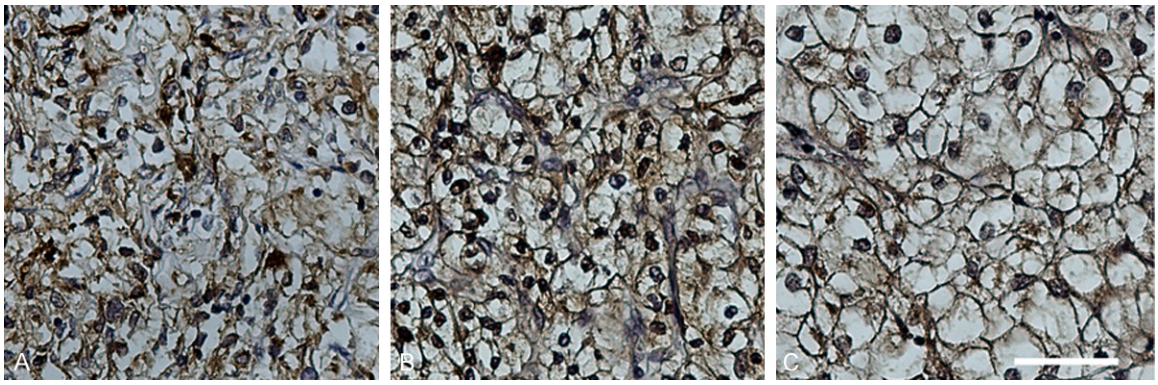

Figure 1. Expression of FGF (A), FGFR1 (B) and FGFR2 (C) in the primary tumor cells (scalebar, 50 μm).

metastases at the time of surgery. Solitary and multiple metastatic lesions were diagnosed in 22 (33.8%) and 23 (35.4%) cases, respectively. More than one metastatic site was found in 11 (16.9%) patients. RCC metastases were localized in the adrenal gland, lungs, bones, liver in 28 (43.1%), 22 (33.8%), 5 (7.7%), and in 2 (3.1%) patients, respectively.

All patients underwent nephrectomy with retroperitoneal lymphadenectomy. In 50 (76.9%) cases tumor thrombectomy was also performed. Distant metastases were removed in 28 (43.1%) cases. Adrenalectomy, contralateral partial nephrectomy, pulmonary resection, bone metastasectomy were performed in 24 (36.9%), 1 (1.5%), 1 (1.5%), 1 (1.5%) patients, respectively. Complete removal of all RCC tumor sites was done in 40 (61.5%) cases; 25 (39.5%) patients underwent cytoreductive surgery.

Pathologic validation showed RCC in all samples of the removed primary tumor, and clear-cell RCC subtype was detected in the overwhelming majority of cases (59; 90.8%). Non-clear cell RCC was confirmed in 6 (9.2%) specimens. Fuhrman grade G1-2 occurred in 29 (44.6%) and G3-4 in 36 (65.4%) patients. Categories pT1-T2 and pT3-T4 were diagnosed in 12 (18.5%) and 53 (81.5%) cases, respectively. Paranephric fat tumor invasion was detected in 29 (44.6%) samples. Thrombotic masses had a structure similar to a kidney tumor in all thrombectomy specimens and venous wall tumor invasion was noted in 4 (6.2%) cases. Lymph node metastases were diagnosed in 12 (18.5%) cases, while more than one lymph node was affected in 8 (12.3%) patients. Histological examination confirmed that all removed tumors of other locations had the structure of RCC and were metastases of the primary tumor located in the removed kidney.

Patients who undergone complete removal of all metastatic sites were under close follow-up. Systemic therapy was administered in 22 (88.0%) of 25 patients following cytoreductive surgery (cytokines-3 patients with pulmonary metastases, antiangiogenic targeted therapy-19 patients).

Expression of FGF/FGFR and its predictive value

Cytoplasm and membrane expression of FGF2, FGFR1, and FGFR2 was found in the primary tumor cells of RCC patients (**Table 2**). FGF2 staining was detected in 60.0% of cases (44.2 ± 5.4 HS). It was noted higher frequency and intensity of FGFR2 expression (66.2%; 46.6 ± 6.3 HS) comparing with FGFR1 (32.3%; 7.5 ± 2.2 HS). **Figure 1** demonstrates expression of FGF2, FGFR1, and FGFR2.

The analysis of potential relationship of tumor characteristics (number of affected kidneys,

Table 3. Correlation of FGF2, FGFR1, and FGFR2 expression levels on primary tumor cells with the primary tumor characteristics in RCC patients

Characteristics	Pirson correlation, r/Significance, p		
	FGF2	FGFR1	FGFR2
Fuhrman grade	0.248*/0.046	0.261/0.230	0.161/0.200
Paranephric fat tumor invasion	0.354**/0.004	0.245*/0.049	0.112/0.215
Venous wall tumor invasion	-0.029/0.822	0.313*/0.012	0.177/0.101
Adrenal gland metastases	0.171/0.262	0.385**/0.009	-0.122/0.425
Liver metastases	0.358*/0.016	-0.134/0.381	0.404**/0.006

**Correlation is significant at 0.01 (two-tailed). *Correlation is significant at 0.05 (two-tailed).

the primary tumor size, histological variant, Fuhrman grade, pT category, paranephric fat invasion, tumor venous thrombosis, venous wall tumor invasion, pN and M categories, number and sites of metastases) with expression levels of FGF2, as well as receptors of tyrosine kinases FGFR1, and FGFR2 was performed (**Table 3**). Significant correlation of FGF2, FGFR1, and FGFR2 overexpression with unfavorable morphological features and advanced tumor was noted. Fuhrman grade directly correlated with the FGF2 expression, paranephric fat invasion correlated with FGF2 and FGFR2 expression, venous wall tumor invasion correlated with FGFR2 expression, adrenal gland metastases correlated with FGFR-1 expression, and liver metastases were associated with FGFR2 expression. No other significant relationships were found.

The median follow-up for all patients was 19.9 (1-133) months. RCC progression occurred in 17 (42.5%) of 40 cases following radical surgery. All 17 patients developed distant metastases. Two patients with solitary liver lesions underwent complete removal of RCC metastases. Fifteen patients received targeted antiangiogenic therapy. The best response was stabilization.

Forty-two of 65 (64.6%) patients are alive (24 (36.9%) without evidence of the disease; 18 (27.7%) with metastases). Twenty-three (35.4%) patients died from RCC progression (22; 33.8%) or surgical complications (1; 1.5%). Median OS and CSS survival of all 65 patients was 43.8 and 52.1 months, respectively. Median DFS of 40 patients following radical surgery was 79.2 months, median PFS of 25 patients after cytoreductive surgery was 7.4 months. In univariate analysis negative prognostic factors of CSS were unilateral kidney tumor, Fuhrman grade 3-4, pT>T2, tumor venous thrombosis, multiple metastases, and cytoreductive surgery (P<0.05 for all).

Predictive value of FGF2, FGFR1, and FGFR2 expression levels for RCC progression following radical or cytoreductive surgery, and death from kidney cancer was assessed. FGFR2 overexpression was revealed to be of negative predictive value for CSS. According to the ROC-curve border value of FGFR2 for CSS was 80 HS, and it`s expression ≥80 HS was associated with decrease of CSS from 52.1 to 15.7 months (P=0.014) (**Figure 2**). Prognostic value of FGFR2≥80 HS for CSS was lost in multivariable analysis (P>0.05). No other relationships between FGF2, FGFR1, FGFR2 expression levels with RCC prognosis were found (P>0.05 for all).

Discussion

The FGF/FGFR signaling plays the important role in embryonic development, maintenance of adult organ systems, tissue regeneration, wound healing, and hematopoiesis. The FGF family consists of 23 signaling polypeptides that act as potent mitogens stimulating the growth of fibroblasts, endothelial, and cancer cells. FGF2, also known as basic FGF (bFGF), is the most studied member of the growth factor family [10, 11]. Five FGFRs have been identified, four of which (FGFR-1-4) are transmembrane receptor tyrosine kinases. FGFR activation provides FGF signaling. Endothelial cells predominantly express FGFR1 and, to a lesser extent, FGFR2. FGF/FGFR is one of the signaling pathways that depend on the expression of a hypoxia-induced factor, which is often overexpressed in clear-cell RCC. Data on the expression and, especially, the prognostic value of FGF/FGFR in RCC patients are scarce and contradictory [12, 13].

We investigated FGF/FGFR expression at prospectively collected surgical samples of primary RCC tumors. Immunohistochemical study with the semi-quantitative assessment of protein production by tumor cells, evaluating both number of stained cells and degree of staining

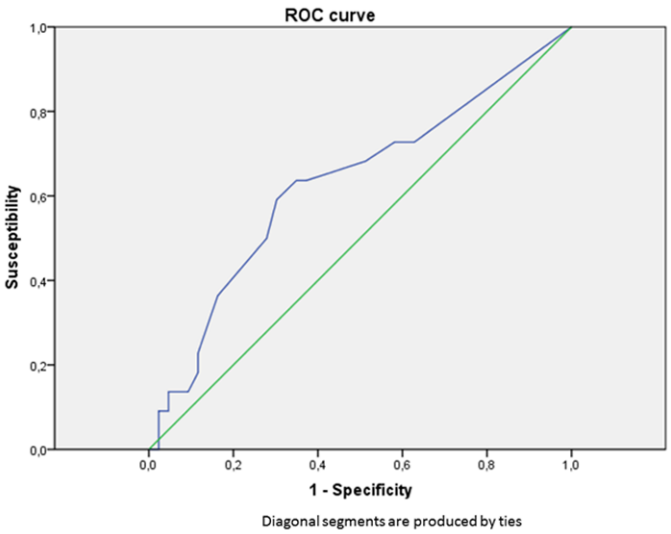

Figure 2. Value of FGFR2 expression level for prediction of death from RCC by ROC-curve.

Area under the curve

Area	Std. error[a]	Asymptomatic sig.[b]	Asymptomatic 95% Confidence Interval	
			Lower board	Upper board
0.628	0.076	0.092	0.479	0.778

The test result variable: higher FGFR2 has at least one of tie between the positive actual state ground and the negative actual state group. Statistics may be biased.

(H-score), was chosen as a method for expression estimation. This technique is well reproducible, has proven itself well in early works [8] and is now widely used by other researchers [13, 15].

Expectedly, expression of FGF2, as well as its receptors FGFR1 and FGFR2, was detected on the surface and in the cytoplasm of primary tumor cells in patients with RCC pT1a-T4N0/+M0/+. We revealed FGF2 staining in 60% of RCC samples (44.2 HS) and noted greater frequency and intensity of FGFR2 expression (66.2%; 46.6 HS) compared to FGFR1 (32.3%; 7.5 HS). Horstmann M. et al. registered FGF2 staining in ≥5% of RCC cells (n=259) in 37.7% of specimens from the marginal zone and in 28.2% of samples from the central zone of the primary RCC tumor [16]. Tsimafeyeu I. et al. showed pronounced overexpression of FGFR1 in RCC cells (n=100). FGFR1 expression was revealed in 98% of primary tumor samples, FGFR2 staining was recorded much less frequently, in 4% of cases [17]. In a small series (n=36) of Iacovelli R. et al., the expression of FGFR1 and FGFR2 was unexpectedly low: staining of ≥5% of tumor cells occurred only in 16% and 30% of cases, respectively [18]. It is not possible to compare the results of different studies because of different methods of proteins expression assessment used, but it is obvious that RCC cells are characterized by relatively high content of FGF/FGFR.

The effect of FGF/FGFR on the prognosis of RCC patients has hardly been studied. However, this signaling axis activation seems to be associated with survival worsening. In our series, univariate analysis demonstrated that FGFR2 overexpression ≥80 HS had negative influence on CSS (P=0.014). However, prognostic value of FGFR2 was lost in multivariable analysis. Other authors also have noted negative impact of increased FGF/FGFR expression on the prognosis of RCC patients. Horstmann M. showed that FGF2 overexpression at the tumor growth margin was an independent risk factor for OS, along with Fuhrman grade and N+ category [16]. Iacovelli R. revealed the correlation of low FGFR2 expression with PFS increase in RCC patients receiving targeted antiangiogenic therapy [18]. Ho T. et al. reported, that overexpression of FGFR1 and FGFR2 was associated with lower PFS in metastatic RCC patients who received sorafenib as the third line treatment (n=40) [19].

Considering the importance of FGF receptors in the pathogenesis of RCC, it is advisable to examine the development of inhibitors [20-23]

and monoclonal antibodies [24] that block these receptors. In the case of suppression of the activity of the receptor by an inhibitor, its pathogenetic role will be proven.

In conclusion, expression of FGF2 and its receptors was found on the surface and in the cytoplasm of RCC primary tumor cells. The correlation was revealed between the investigated markers overexpression and Fuhrman grade G3-4, as well as features of advanced RCC (paranephric fat tumor invasion, venous wall tumor invasion, adrenal and liver metastases). FGFR2 overexpression ≥80 HS showed negative influence on CSS in univariate analysis, however, lost its predictive value in multivariate analysis.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Ilya Tsimafeyeu, Institute of Oncology, Hadassah Moscow, Bolshoi Boulevard, Building 46, Stroenie 1, Moscow 115478, Russia. E-mail: pirogovkatim@mail.ru

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