VEGF Expression and Response to Sunitinib in Patients with Metastatic Clear Cell Renal Cell Carcinoma

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Abstract. *Aim: To verify whether vascular endothelial growth factor (VEGF) is associated with distant metastasis free survival (DMFS) and Overall Survival (OS) of patients with renal cell carcinoma (RCC) treated with sunitinib. Patients and Methods: We have studied 41 patients with metastatic RCC treated with radical nephrectomy, between 2008 and 2010, and sunitinib. Pathological features were compared with the Memorial Sloan-Kettering Cancer Center (MSKCC) score, DMFS, and with OS, and PFS after first-line therapy. Results: Tumor stage and grade, VEGF expression and H-score correlated with MSKCC score, DMFS, and with OS; VEGF expression correlated with stage and OS. Patients with higher H-score and higher VEGF expression had a significantly shorter survival; OS after first-line sunitinib therapy and PFS correlated with MSKCC score and DMFS but not with VEGF expression and H score. Conclusion: Our data suggest the potential use of tumor cell VEGF expression as a prognostic marker for DMFS and OS, but VEGF does not appear promising as a marker of response to therapy.*

Renal cell carcinoma (RCC) represents 2-3% of all tumors (1), 80-90% of which are of the clear cell type. Although most patients with early-stage RCC can be cured surgically, approximately 33% present with disease are in an advanced state at diagnosis, and for them, treatment is not curative (2); approximately 50% of patients who undergo potentially curative surgical resection for less advanced disease can be expected to develop distant metastases during follow-up (3-6).

A high incidence of metastatic spread is regarded as one of the most unique characteristics of RCC (6-8). RCC can be highly resistant to conventional cytotoxic chemotherapy, hence immunotherapy was applied for patients with metastatic RCC as first-line therapy. However, only limited efficacy was achieved by this treatment, with an objective response rate of <20%; therefore metastatic RCC generally has a poor prognosis, with a median overall survival of about one year (9, 10).

Recent advances in the understanding of the molecular biology of clear cell RCC have established the importance of inactivated von Hippel-Lindau (*VHL*) gene and its role in the pathogenesis of sporadic clear cell RCC. *VHL* gene alterations lead to overexpression of vascular endothelial growth factor (VEGF) and other growth factors, resulting in endothelial cell migration, tumor growth, and tumor angiogenesis (11-14). Consequently, several systemic therapeutic agents targeting VEGF and the mammalian target of rapamycin (mTOR) pathways as first- and second-line treatments for metastatic disease were developed, with impressive improvements in progression-free survival (PFS) and overall survival (OS) (6, 15, 16). Sunitinib is one such a small-molecule inhibitor of multiple tyrosine kinases, including VEGF and its receptors. Although these molecularly-targeted agents dominate the therapeutic landscape of advanced RCC, treatment decisions are made

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exclusively on the basis of clinical criteria, given the absence of clinical and molecular predictive and prognostic-validated biomarkers. These are potentially important for therapy selection, patient counseling, and clinical trial stratification.

The aim of the present study was to evaluate the correlation between VEGF expression on endothelial and tumor RCC cells and the outcome in patients with metastatic RCC who received sunitinib as first-line therapy.

Patients and Methods

The procedure for this research project conforms with the provisions of the Declaration of Helsinki.

We considered patients with histologically-confirmed diagnosis of clear cell RCC (17) treated with radical nephrectomy at the Institute of Urology of the Polytechnic University of the Marches Region, A.O. Ospedali Riuniti – Ancona - Italy, between 2008 and 2010. Metastases were present in all patients, whether at diagnosis or early during follow-up. At metastatic recurrence, all patients received first-line anti-VEGF therapy with sunitinib at the dose of 50 mg daily for four weeks followed by two weeks' rest.

The features considered when evaluating patients were patient age, tumor grade, and tumor cell and endothelial cell VEGF expression detected by immunohistochemistry on the histological specimens.

Our data were compared with the Memorial Sloan-Kettering Cancer Center (MSKCC) score (18), a nomogram predicting for likelihood that RCC will recur at five years after surgery, following a new diagnosis, and with time from surgery to distant metastasis, also referred to as distant metastasis-free survival (DMFS).

Thereafter, following the start of anti-VEGF therapy, all considered parameters were correlated with patients overall survival (OS), that is, patient survival from the diagnosis, first-line OS (that is, patient survival from the start of the first-line treatment), and progression-free survival (PFS).

Immunohistochemistry. Immunohistochemistry was performed on conventional 6-μm-thick histological paraffin-embedded tissue sections on poly-L-lysine-coated glass slides. After heat-drying, sections were de-paraffinized in xylene and sequentially rehydratated in gradients of ethanol. To better unmask antigenic sites, sections were treated with Target Unmasking Fluid solution (Histo-line Laboratories, Milano, Italy) at 90˚C for 10 min and incubated overnight at 4˚C with a monoclonal antibody against VEGF-165 (diluted 1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The reaction was revealed using the streptoavidin-biotin-peroxidase technique (Envision peroxidase kit; Dako-cytomation, Carpinteria, CA; USA). After incubation with 3,3 diaminobenzidine (0.05 diaminobenzidine in 0.05 M Tris buffer, pH 7.6, and 0.01% hydrogen peroxide), sections were counterstained with Mayer's hematoxylin and coverslipped with Permount (Histo-Line Laboratories, Milano, Italy). Positive controls were paraffin-embedded sections from gastric carcinomas, previously shown to react with the primary antibodies. For negative controls, the primary antibody was replaced with non-immune serum.

The percentage of VEGF-stained tumoral cells (RCC VEGF) was semi-quantitatively assessed independently by two different operators on 10 fields at ×400 magnification, and differences in interpretation were resolved by consensus. They evaluated the staining according to a 4 point arbitrary scale of 0 to 4 for the percentage of positive cells, that is: 0, 0%, 1, 1-25%, 2, 26-50%, 3 >50%. In the statistical evaluation, VEGF scores were grouped together by 0-1 and 2-3. VEGF staining intensity was also graded using the following scale: 0, negative; 1, weak; 2, intermediate; 3, strong (Figure 1 A and B). The percentage and intensity of VEGF staining were also considered together as the H-score (19), applying the following formula: H score=(% of cells stained at intensity category 1×1) + (% of cells stained at intensity category 2×2) + (% of cells stained at intensity category 3×3). An H-score between 0 and 300 was thus obtained, where 300 was equal to 100% of tumour cells stained strongly (3+). In Kaplan Meier analysis, the H score was divided as low <100 and high >100.

VEGF expression intensity was also evaluated in endothelial cells (endothelial VEGF) of the vessels branching within the tumoral cells, according to a 3-point arbitrary scale, that is: 0, negative; 1, weak staining intensity; 2, intermediate to strong staining intensity.

Table I. *Patients' demographic and disease characteristics.*

Median age, years (range)	64 (47-69)
Gender	
Male	32/41
Female	9/41
Pathological stage	
T2 G2	2
G3	3
T3 G2	11
G3	23
T4 G3	2
Distant metastasis	
At diagnosis	14/41
During follow-up	27/41
Median DMFS, months	45.34
Most common site of metastasis, no (%)	
Lung	27 (66)
Lymph nodes	10 (24)
Bone	8 (20)
Liver	9 (22)
Kidney	4 (10)
Brain	2 (5)
Other	11 (27)
ECOG performance status	
0	19/41
1	22/41
MSKCC risk category	
Favorable	12/41
Intermediate	21/41
Poor	8/41
Median OS from diagnosis, months (range)	55.40 (4.08-128.52)
Median first-line OS (range)	28.87 (2.30-54.18)
Median first line PFS (range)	7.05 (1.51-39.02)

DMFS: Distant metastasis-free survival; ECOG: Eastern Cooperative Oncology Group; MSKCC: Memorial Sloan–Kettering Cancer Center; OS: overall survival; PFS: progression-free survival.

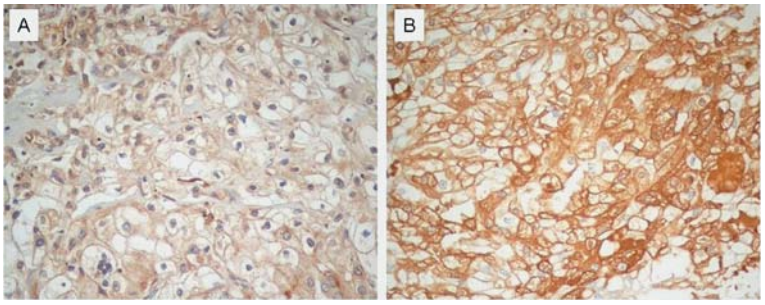

Figure 1. *Clear cell RCC showing weak cytoplasm and cell membrane intensity (A) and strong intensity (B) staining for vascular endothelial growth factor (VEGF). ×200 magnification.*

Statistical analysis. Statistical analysis was performed using the Kolmogorov-Smirnov normality test for all considered parameters. Student's *t*-test was used to compare the staining intensity and the percentage of the stained cells at each intensity. Correlations between continuous variables were analyzed using Spearman's rank correlation test. The Fischer and chi-squares tests were used to compare nominal data. Kaplan Meier curves with the log-rank test were designed to compare survival parameters (20). The influence of each parameters on survival was assessed using Cox proportional hazard models. Statistical analyses were performed using the SPSS 16 package (SPSS Inc., Chicago, IL, USA). Significance was set at p less than 0.05.

Results

Forty-one patients (mean age=64 years, range=47-79 years) were included in this analysis; in all the patients, metastases were present, whether at-diagnosis (14 patients) or early in the follow-up (27 patients, after a mean time of 45.34 months). Thirty-two patients were males and nine females; pathological stage was T3 (TNM 2009) or more in the majority of patients. The mean follow-up period was 47.49 months.

The demographic and clinical characteristics of patients are outlined in Table I. According to the MSKCC score, 12 patients had favorable criteria, 21 intermediate, and eight poor.

There was no statistically significant difference in the results obtained when considering the 14 patients with metastases at diagnosis and the 27 patients who developed metastases early in the follow-up, so all data were considered together.

In Figures 1 and 2, clear cell RCC intensity staining for VEGF is shown. Tumor stage was significantly associated with tumor VEGF expression (p=0.025); tumor stage and Fuhrman grade were correlated with MSKCC score (p=0.002 and p=0.039, respectively) and with DMFS (p=0.004 and 0.015 respectively). The prognostic value of VEGF expression for MSKCC criteria and DMFS was evaluated by comparing the patients with high score (3-4) and low score (0-2) VEGF expression and patients with high (>100) and low (<100) H-score: endothelial VEGF was significantly correlated with MSKCC criteria (p=0.34, data not shown) and DMFS (p=0.031, Figure 3) and H-score was also correlated with MSKCC score (p=0.035, data not shown) and DMFS (p=0.042, Figure 4). The median DMFS was 45.34, 6.94 and 1.08 months in patients with scores of 0, 1 and 2 endothelial VEGF expression, respectively.

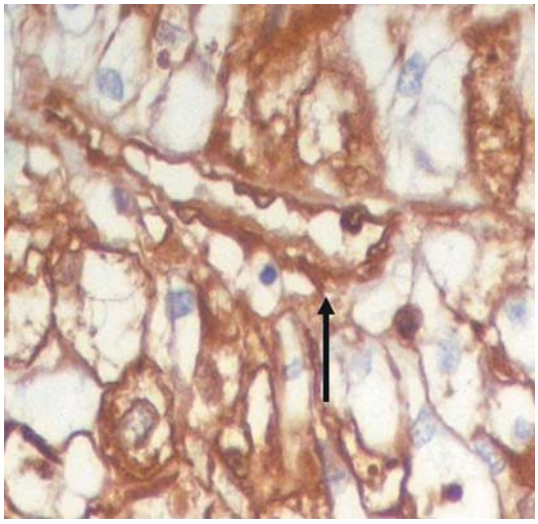

Figure 2. *Clear cell RCC: Strong intensity staining of vascular endothelial growth factor (VEGF) in the endothelial cells on the blood vessel within the neoplasm (see arrow). ×400 magnification.*

The median OS after radical nephrectomy was 55.40 (95% confidence interval, 4.08-128.52) months, and that from the occurrence of metastasis and from the start of first-line sunitinib was 28.87 (2.30-54.18) months, with a PFS of 7.05 (1.51-39.02) months.

OS was significantly correlated with tumor stage (p=0.001), Furhman grade (p=0.033) and MSKCC criteria (p<0.001). The prognostic value of tumor cell VEGF expression for OS in our patients was evaluated by comparing patients as described above. The OS after radical nephrectomy was significantly correlated with VEGF expression in tumor cells (p=0.0145)

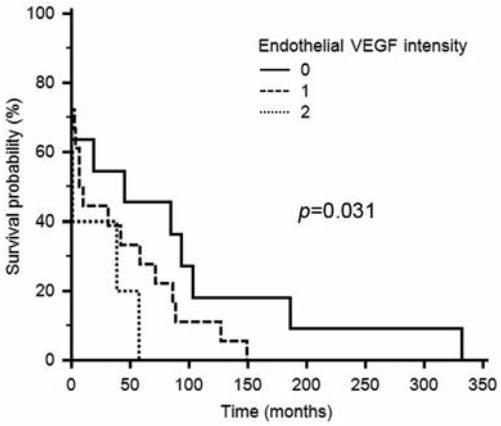

Figure 3. *Kaplan-Meier curves showing distant metastasis-free survival (DMFS), stratified by endothelial vascular endothelial growth factor (VEGF) intensity. DMFS was negatively-correlated with endothelial VEGF (p=0.031).*

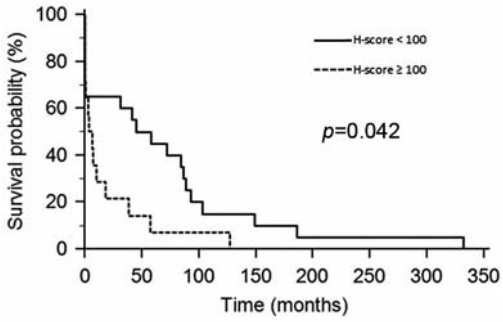

Figure 4. *Kaplan-Meier curves showing distant metastasis-free survival (DMFS), stratified by H-score for tumor cells. The prognostic value of vascular endothelial growth factor (VEGF) expression for DMFS was evaluated by comparing the patients with high (>100) and low (<100) H-score: DMFS was negatively correlated with VEGF H-score (p=0.042).*

(Figure 5) and H-score (p=0.004); a statistically significant correlation was also found between OS and endothelial VEGF (p=0.016), (median OS was 229.12, 71.44 and 64.73 months respectively in patients with 0, 1 and 2 intensity scores).

In multivariate analysis, first-line PFS in patients treated with sunitinib was significantly affected by MSKCC group (p=0.049) and DMFS (p=0.038).

First-line OS and PFS after sunitinib therapy were significantly associated with tumor stage (p=0.016 and p=0.015 respectively); no statistically significant correlation was found between PFS after first-line sunitinib treatment and tumor VEGF intensity (p=0.319), endothelial VEGF expression (p=0.421) or H-score (p=0.497).

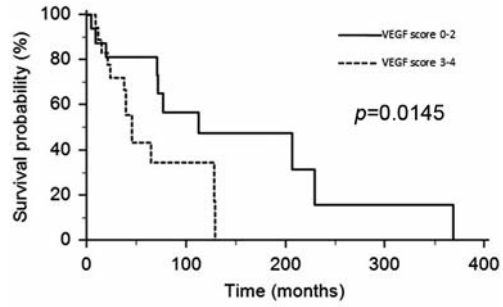

Figure 5. *Kaplan-Meier curves showing overall survival (OS), stratified by vascular endothelial growth factor (VEGF) expression on renal cell carcinoma (RCC) tumor cells. The prognostic value of RCC VEGF expression for OS in our patients was evaluated by comparing patients with high score (3-4) and low score (0-2) VEGF expression. OS from radical nephrectomy was significantly negatively correlated with VEGF expression in RCC cells (p=0.0145).*

Discussion

Angiogenesis in RCC is the result of genetic and epigenetic events and is supported by a complex functional interaction between endothelial cells, pericytes, extracellular matrix (ECM) and stroma cells in the tumor microenvironment. VEGF is a potent promoter of tumor angiogenesis (21, 22).

Out of several molecular-targeted agents, sunitinib is regarded as one of the most powerful drugs against RCC (23). In pre-clinical RCC models, sunitinib was shown to have inhibitory effects on tumor cell proliferation, as well as on angiogenesis through the inactivation of multiple receptor tyrosine kinases, including VEGF receptors 1-3, and platelet-derived growth factor receptors (PDGFR-A and PDGFR-B) (24). In a clinical setting, Motzer *et al*. reported the excellent antitumor activity of sunitinib against RCC, showing significantly favorable prognostic outcomes (25). However, the acquisition of a phenotype resistant to this agent is of major clinical concern: the vast majority of patients with metastatic RCC with an initial positive response to sunitinib eventually develop progressive disease (26, 27).

In the present study, we evaluated the correlation between VEGF expression in tumoral and endothelial tumor cells and the outcome of patients affected by metastatic RCC. Tumor extension, Fuhrman grade, tumor and endothelial cell VEGF expression, and H-score were separately analyzed and correlated with DMFS and patient survival.

Tumor stage and grade, VEGF expression and H-score correlated with MSKCC score and DMFS, and also with OS; VEGF expression was associated with tumor stage and OS. Patients with a higher H-score and higher VEGF expression had a significantly shorter survival compared to those having lower expression.

We also observed that first-line OS after sunitinib therapy, and PFS correlate with MSKCC score and DMFS but not with VEGF expression and H-score.

These results confirm that RCC tumor cells, producing high levels of VEGF, display a great ability to grow and spread and suggest that angiogenic activity might be stronger in tumors with a higher ability to invade, as previously observed (28). Therefore VEGF expression has a good prognostic value for DMFS and for OS in RCC; on the contrary, our findings show that tumor and endothelial VEGF expression did not correlate with the outcome of patients treated with first-line sunitinib for metastatic RCC, therefore suggesting that these parameters are not important predictors of response to therapy.

As far as we are aware, no predictive tissue biomarker of response to sunitinib has been identified for metastatic RCC.

Some authors have pointed-out controversy on VEGF expression in primary tumor tissues as prediction of outcome, and intratumoral levels of VEGF have not been shown to predict survival outcome of anti-VEGF therapy (29, 30). Porta *et al*. found that serum levels of VEGF were significant predictors of PFS in patients with renal carcinoma treated with sunitinib (31); and Bernard *et al*. showed that the levels of VEGF soluble isoforms (VEGF121 and VEGF165) were associated with the response to sunitinib in patients affected by metastatic RCC (32).

Some studies have shown the involvement of signal transduction pathways in the acquisition of resistance to a wide variety of molecular-targeted agents (32, 33). VEGF pathway genes are highly polymorphic, with multiple common single nucleotide polymorphisms in regulatory regions able to affect function or expression of proteins and to alter risk and prognosis of various diseases that are tightly regulated by angiogenesis (33). This genetic variability has been studied as a potential predictive biomarker of outcome in patients treated with anti-VEGF therapy for colorectal, breast, and ovarian cancer (34-37), and may explain, in part, the acquisition of a phenotype resistant to sunitinib in RCC cells. Combined treatment with sunitinib and potential agents targeting activated regulatory regions could be regarded as one promising approach for overcoming sunitinib resistance in patients with RCC.

In conclusion, our data suggest the potential use of tumor cell VEGF expression as a prognostic marker for DMFS and OS in patients with metastatic clear cell RCC after radical nephrectomy. Considering the limitations of tissue VEGF evaluation, VEGF does not appear promising as a marker of response to therapy. Further studies are required to incorporate tumor VEGF expression into clinical prognostic models, also in consideration of VEGF pathway polymorphism that can affect response to therapy.

Conflicts of Interest

None declared.

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