Tissue Biomarkers in Predicting Response to Sunitinib Treatment of Metastatic Renal Cell Carcinoma

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Abstract. *Aim: To identify tissue biomarkers that are predictive of the therapeutic effect of sunitinib in treatment of metastatic clear cell renal cell carcinoma (mCRCC). Materials and Methods: Our study included 39 patients with mCRCC treated with sunitinib. Patients were stratified into two groups based on their response to sunitinib treatment: non-responders (progression), and responders (stable disease, regression). The effect of treatment was measured by comparing imaging studies before the initiation treatment with those performed at between 3rd and 7th months of treatment, depending on the patient. Histological samples of tumor tissue and healthy renal parenchyma, acquired during surgery of the primary tumor, were examined with immunohistochemistry to detect tissue targets involved in the signaling pathways of tumor growth and neoangiogenesis. We selected mammalian target of rapamycine, p53, vascular endothelial growth factor, hypoxia-inducible factor 1 and 2 and carbonic anhydrase IX. We compared the average levels of biomarker expression in both, tumor tissue, as well as in healthy renal parenchyma. Results were evaluated using the Student's t-test. Results: For responders, statistically significant differences in marker expression in tumor tissue versus healthy parenchyma were found for mTOR (4%/16.7%; p=0.01031), p53 (4%/12.7%; p=0.042019), VEGF (62.7%/45%; p=0.019836) and CAIX (45%/15.33%; p=0.001624). A further significant difference was found in the frequency of high expression (more than 60%) between tumor tissue and healthy parenchyma in VEGF (65%/35%; p=0.026487) and CAIX (42%/8%; p=0.003328). CAIX was expressed at high levels in the tumor tissue in both evaluated groups. Conclusion: A significantly higher expression of VEGF in CRCC in comparison to healthy parenchyma can predict a better response to sunitinib.*

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Key Words: Metastatic renal carcinoma, targeted-therapy, prediction, biomarkers, immunohistochemistry.

Renal cell carcinoma (RCC) represents 2-3% of all solid malignant tumors in the adult population of Western Europe. The Czech Republic has one of the highest incidences worldwide. During 2003-2007, the highest incidence worldwide was found in the Czech Republic. World population age-standardized rates (ASRs) were 22.1 per 100,000 in men and 9.9 per 100,000 in women. Among the regions of Czech Republic, the highest incidence was found in the Pilsen region, with ASR incidence 31,4/100,000 in men (1). Despite the increase in the detection of tumors at a low stage, about 20% of cases present with distant metastases at the time of diagnosis. Approximately half of all patients with localized disease are found to have progressed during the follow-up examination. Metastatic renal cell carcinoma (mRCC) is an incurable disease because of its chemoresistence and poor response to immunotherapy. These characteristics make RCC the most lethal urological malignancy.

In the systemic treatment of mRCC, targeted therapeutics such as tyrosine kinase inhibitors (TKI) are currently used as first choice drugs for favorable and intermediate risk mRCC according to Memorial Sloan-Kettering Cancer Center (MSKCC) criteria (2).

Sunitinib malate (Sutent®; Pfizer, USA) targets the receptors for vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and other tyrosine kinase receptors. Sunitinib achieves an objective response rate of up to 47% in mRCC, in comparison to the previously first-choice drug, interferon-α, which had up to a 13% objective response rate (3).

Despite these results, treatment with TKIs is considered palliative, achieving partial, temporary responses, and has practically unpredictable efficacy. Surgical removal of tumor masses is still the only curative option.

Indication of targeted-therapy is based upon the MSKCC criteria, which were created in the era of cytokine therapy (4). This clinical approach is currently insufficient and thus we seek new biomarkers for predicting the effect of targeted-agents.

Materials and Methods

Patients. Out of 119 patients in our Hospital treated in 2005-2012 with sunitinib for mRCC, 45 cases had available samples of both primary tumor tissue and healthy renal parenchyma. Of these 45, only 39 patients were included in the study. The other six patients were excluded due to non-clear cell histological tumor type (one case each of papillary RCC type 1, chromophobe RCC, and unclassified RCC), short course of sunitinib treatment (fewer than two cycles, one patient) or insufficient follow up data (two patients).

The surgical procedures were conducted between 2002 and 2012. In all these cases, the histological material of both the primary tumor and healthy renal parenchyma were acquired from radical nephrectomy and partial nephrectomy specimens immediately after surgery, and were stored as tissue blocks embedded in paraffin in the archives of the Department of Pathology of the University Hospital in Pilsen.

Of the study group, 14 patients (36%) were pre-treated with immunochemotherapy for at least 1 month; in four patients (10%), sunitinib was the second-line treatment after sorafenib. Patient´s characteristics are summarized in Table I. Sunitinib was self-administered orally at a daily dose of 50 mg, 37.5 mg or 25 mg, in accordance with the individual's tolerance. Each treatment cycle consisted of 4 weeks on treatment followed by 2 weeks of discontinuation, according to the recommended schedule. Treatment and follow-up data are summarized in Table II.

The effect of treatment was evaluated according to the findings on imaging studies [*i.e.* computed tomography (CT) of chest and abdomen, chest X-ray, the whole body 18F-Fludeoxyglucose Positron Emission Tomography / Computed Tomography (18F-FDG PET/CT) conducted after 2-5 cycles of the treatment, for an average of 4.3 months (range=2.6-6.8 months). These findings were compared to studies performed before the initiation of the treatment using Response Evaluation Criteria in Solid Tumours (RECIST) version 1.1 (5). The average interval between these studies was a median of 5.4 months (range=2.7-9.1 months).

In 11 patients, progression of disease was observed. Such patients were considered as non-responsive, and grouped accordingly. Patients who also had newly-formed metastases were grouped under the non-responsive group. In nine patients, the condition was evaluated as stable disease. In 19 of the patients, the condition was evaluated as regressive. Patients with regression and those with stable disease where defined together as the responsive group. Complete remission was not observed. Patients were then stratified into groups according to progression-free survival (PFS). PFS was defined as the time from the initiation of sunitinib until the time of the first signs of disease progression were observed. Eighteen out of 39 patients were in the group with PFS less than 1 year (n=18) and 21/39 were in the group with PFS exceeding 1 year (n=21).

Histological analysis and immunohistochemistry. During the histological analysis, the tissue samples were fixed in 4% buffered formalin, embedded routinely in paraffin, and 5-μm sections were cut and stained with hematoxylin and eosin. For each case, sections containing approximately equal volumes of tumoral and nontumoral tissue were chosen using light microscopy. Immunohistochemical assays were performed using the Ventana Benchmark XT automated stainer (Ventana Medical System, Inc., Tucson, AZ, USA). The following primary antibodies were used: Mammalian Target of Rapamycine (mTOR) (polyclonal, 1:50; Cell Signaling Technology, Danvers, MA, USA), p53 (DO-7, 1: 50; monoclonal, Dako, Glostrup, Denmark), Vascular endothelial Growth Factor (VEGF) (Y103, monoclonal, 1:50; ABCAM, Cambridge, UK), Hypoxia-inducible Factor 1 (HIF1) (ESEE 122, monoclonal, 1:300; ABCAM, Cambridge, UK), Hypoxia-inducible Factor 2 (HIF2) (ep190b, monoclonal, 1:30; ABCAM), Carbonic anhydrase IX (CAIX; rhCA9, monoclonal, 1:100; RD Systems, Abingdon, UK). Appropriate positive and negative controls were employed for all immunohistochemical assays. The pattern of staining was scored separately for tumoral and nontumoral tissue components: negative, if no staining was observed; scattered positive, if single or few cells were clearly positive (up to 5%); focally positive, if large groups of cells were clearly positive (40-60%), and diffusely positive, if the majority (>50%) of tumor cells were positive. Positivity ranging between 5% and 40% did not occur. The intensity of positive staining ranged from 1+ to 3+ and was evaluated for every amount of reacting tissue. In samples with diffuse positivity, the marks 1+ to 3+ correspond to percentage positive expression, where 3+ was considered as intensity equal to positive control, therefore 100%. Marks 1+ and 2+ match the intensity of staining of 33% and 66% respectively. In focal reactivity of tissue, a level of staining more than 2+ was considered as overall positivity 1+.

Table I. *Characteristics of patients*.

Parameter	Case number
Total patients	39 (100%)
Gender	
Male	27 (69%)
Female	12 (31%)
pT	
1	7
2	6
3a	14
3b	11
4	1
Fuhrman grading	
1	13
2	21
3	2
4	3
Metastasis at the time of diagnosis	
N+, M+	11 (28%)
TNM stage	
I	7
II	5
III	15
IV	12
Age at surgery (years)	mean 56.7 (39.6-71.1)
Age at initiation of therapy (years)	mean 59.1 (41.0-78.3)

Number of patiens, gender, TNM classification and stage of disease at time of surgery, age at time of surgery and the initiation of therap, respectively, are shown.

Table II. *Treatment data*.

		Number	Percentage of all
Patients pre-treated with immunochemotherapy		14	36%
		Mean	Range
Time between surgery and diagnosis of metastases (months)		18.2	0.0-74.3
Time between diagnosis of metastases and initiation of treatment (months)		10.2	0.2-49.2
Duration of sunitinib treatment (months)	Total	15.8	3.4-57.4
	Group of responders	19.4	3.9-57.4
	Group of non-responders	4.9	3.4-8.5

Results

After stratification of patients according to the effect of treatment, the concordance between longer PFS, as well as OS, and objective response to treatment was verified.

Average PFS reached 21.4 months (range=8.5-56.8 months) in responders, and 5.4 months (range=3.4-8.5 months) in non-responders (p=0.0003218). OS in responders was 35.1 months (range=4.6-83.4 months) *vs*. 17.0 months (range=5.5-30.3 months) in non-responders (p=0.00187421).

In the group of responders to treatment, statistically significant results for mTOR, p53, VEGF and CAIX were found when comparing the levels of expression of individual markers between tumor tissue and healthy parenchyma (n=26, 19 cases of regression, seven of stable disease). mTOR and p53 were observed to have had significantly lower expression in tumoral *versus* healthy tissue. The average positivity of mTOR was 4% in tumor tissue, whereas in healthy renal parenchyma the positivity was 16.7% (p=0.01031). Results of p53 expression were similarly lower in tumor tissue *versus* healthy tissue. (4% *versus* 12.7%; p=0.042019). There were also significant differences found in the expression of VEGF (in tumor 62.7%, in healthy parenchyma 45%; p=0.019836) and CAIX (45% *versus* 15.33%; p=0.001624). In contrast to mTOR and p53, VEGF and CAIX had higher expression in the tumor *versus* healthy parenchyma. Differences in HIF1 and HIF2 were not statistically significant.

In the group of non-responders (n=11), statistically significant differences were found in p53 and VEGF. For p53, the marker expression was lower in tumor tissue (3% *versus* 21.3%; p=0.02824). For VEGF, the expression was higher in the tumoral tissue (36.3% *versus* 12%; p=0.011921). When comparing expression of various markers between responders and non-responders, there were no statistically significant differences found (neither in tumoral tissue nor in the healthy tissue).

In the group with an objective response, using the frequency analysis of high expression *i.e*. the relative values of 2+ and 3+ on the scale of expression (in over 60% of cells), significant differences in VEGF expression were found between tumor tissue and healthy parenchyma (65% *versus* 35%; p=0.026487) and CAIX (42% *versus* 8%, p=0.003328). CAIX was highly expressed in tumoral tissue in both responders and non-responders.

Patients were subsequently stratified into groups according to PFS. The median PFS of the whole group was 12.7 months (range=3.4-56.8 months). The median OS was 29.9 months (range=4.6-83.4 months). At the time of analysis, 15 patients (38.5%) were still alive. Three out of the 15 continued on sunitinib treatment. We analyzed the expression of biomarkers in groups with PFS shorter than 1 year (n=18; median PFS=4.9 months, range=3.4-9.8 months) and the group with PFS exceeding 1 year (n=21; median PFS=26.7 months, range=12.2-56.8 months). In both groups, a higher expression of CAIX was apparent in the tumoral tissue compared to healthy renal tissue. In the group with PFS<1 year, average expression was 33.33% in the tumoral tissue compared to 9.8% in the healthy parenchyma (p=0.00169647). In patients with PFS≥1 year, average expression was 50.0% in tumor compared to 18.33% in healthy tissue (p=0.00187404). Opposite results were shown for p53: statistically significantly lower expression was found in tumor compared to healthy parenchyma (PFS <1 year: 5.88% *versus* 15.69%; p=0.04237081, PFS≥1 year: 1.67% *versus*13.33%; p=0.0035556). Statistically significant results were achieved for mTOR in the group with PFS≥1 year (1.67% *versus* 13.33%; p=0.00547403). In the group with PFS<1 year, a mere statistical trend (5.88% *versus* 17.56%; p=0.09153799) was observed. An even higher difference was observed using division into groups with PFS<1/2 year and PFS≥1/2 year (4% *versus* 20%; p=0.00294457). In accordance with the results for the groups of responders and non-responders, a statistically significant difference between the expression of VEGF in the tumoral tissue compared to healthy parenchyma in patients with PFS≥1 year (60% *versus* 45%; p=0.04599199) was observed. In the group with PFS <1 year, this difference was not statistically significant (62.75% *versus* 52.94%; p=0.12992678).

Discussion

TKIs of VEGF and PDGF receptors are now the first line of treatment for metastatic clear cell RCC. Sunitinib, sorafenib, pazopanib and axitinib are currently available for clinical use. These molecules inhibit cellular signaling by targeting multiple receptor tyrosine kinases. After passing through the cell membrane, they block the function of growth factor receptor tyrosine kinases (PDGF and VEGF), stem cell growth factor receptor receptor (KIT/CD117), Fms-like tyrosine kinase-3, and others. The result is the interruption the signaling pathways for tumor neovascularization. Sorafenib additionally blocks RAF kinase, part of the RAS/RAF/MEK/ERK (Rous-adenosarcoma protein/RAS-associated factor/mitogen-activated protein ERK kinase 1/extracellular signal-related kinase) cascade essential for cell proliferation. Other agents used in mRCC include monoclonal antibodies against VEGF (of which the only currently used drug is bevacizumab), and mTOR inhibitors temsirolimus and everolimus.

Biomarkers of tumoral tissue. Nowadays, the immunohisto-chemical and genetic examination of tumor samples is considered an integral part of pathological examination. Determination of tissue biomarkers is one of the ways in which it is possible to detect the activity of particular intracellular pathways. This study was based on the examination of tissue acquired from primary tumor nephrectomy specimens; histological samples of metastases are rarely available for such analysis. Possible problems complicating further application of the study findings may be intratumor heterogeneity (6). This feature of RCC is currently repeatedly reported in the literature. The accuracy of the examination of tissue markers, from this point of view, is therefore limited by the number of examined samples. This may be the major limitation of similar studies which are based on examination of only a single tumor sample. Whether this feature affects tumor response to targeted treatment is a matter for further studies.

HIFα. HIFs are transcription factors regulating more than 200 genes involved in the major pathways of tumorigenesis. HIFα regulates genes influencing neoangiogenesis in conditions of hypoxia. The proteins encoded by these genes include VEGF and PDGF. Better response to sunitinib treatment (partial and complete regression) was observed in patients with high expression of HIF1α and HIF2α in the tumor tissue, *e.g.* an objective response was observed in 76% of patients with high expression of HIF2α and only in 13% with undetectable HIF2α (7). Lindgren *et al.* in 2005 described the high expression of HIF1α as being an independent positive predictive factor for clear cell RCC (8), but a subsequent study by the same authors on larger cohort did not confirm these findings (9). No statistically significant difference in HIF1α or HIF2α among the tested groups was found in our study. HIFα still remains controversial as a predictive marker in mRCC.

VEGF. VEGF is a dimeric glycoprotein promoting tumor angiogenesis. Production of VEGF is elevated in renal carcinoma with altered Von Hippel-Lindau gene (10), thus mainly in clear cell RCC, but it was also found in papillary RCC, where high expression correlated with worse survival (11). Overexpression of VEGF in tumor was found to be associated with higher stage and grade, tumor necrosis (12), a more aggressive tumor phenotype and significantly shorter survival (9).

The possible use of tissue VEGF as a predictor of the effect of systemic treatment is suggested in this study, but confirmation on a larger cohort is needed.

CAIX. CAIX is an HIF1α-regulated transmembrane protein regulating intracellular acidosis, depending on the level of anaerobic metabolism in tumor tissue. In many solid metastatic tumors, a high expression of CAIX indicates an aggressive phenotype and poor prognosis (13, 14). On the contrary, however, in RCC this feature was associated with a favorable prognosis. Due to *VHL* gene mutations in most RCCs, there is a strong relationship between an expression of VEGF and CAIX. A low level of CAIX expression, especially when associated with VEGF overexpression and the absence of *VHL* gene mutation, indicates an aggressive phenotype and poor prognosis of RCC (15-17). A role of CAIX in prediction of the effect of TKI in mRCC remains unclear. The different effect of two types of TKIs, sunitinib and sorafenib, in association with CAIX expression was described by Choueiri *et al*. The average reduction in tumor mass in a sunitinib-treated cohort was 17% in the group with high expression *versus* 25% in the group with low expression. In patients treated with sorafenib, the difference between these groups was more significant, 13% regression *versus* 9% progression (18).

Another study describes an association of low CAIX level and poor survival of patients with mRCC and a lower response to TKI treatment (15).

mTOR. Signaling pathway of mTOR regulates cell growth, survival and angiogenesis. Autocrine stimulation of tumor cells mediated by VEGF and PDGF binding to receptors on the tumor cell surface activates the PI3K/AKT/mTOR (phosphatidil-inositol-3-kinase/Akt-protein kinase-B/mTOR) signaling pathway. Subsequent products have antiapoptotic effect, promoting cell growth and angiogenesis (19, 20). Dysregulation of the PI3K/AKT/mTOR signaling pathway is a hallmark of clear cell-RCC and papillary RCC (21, 22). Positive correlation between high expression of

phosphorylated mTOR and better objective response, as well as PFS, was described in patients treated with everolimus (23). Altered signaling pathway of mTOR also interferes with sunitinib resistance of mRCC. Particularly, lack of the tumor-suppressor phosphatase and tensin homolog coincides with sunitinib resistance in RCC *in vitro* (24). There is still a lack of data about influence of mTOR pathway and the response to sunitinib in clinical studies. Significant findings in the comparison of the levels of mTOR expression in tumor and in healthy parenchyma were observed in patients with PFS exceeding 6 months (4% *versus* 20%; 0.00294457) in our study.

The possibility of predicting the response of mRCC to targeted-therapy could improve treatment strategies and thus save time and money. RCC is a heterogeneous group, not only with variability in the histopathological type but also with genetic and histological variability, even within a single tumor.

There is still a need for further studies in order to find a suitable predictive marker for mRCC treatment. Accurate diagnosis of the tumor is currently not possible without genetic testing. In the future, histological, immunohistochemical and genetic mapping of tumoral tissue may lead the way to personalized medicine.

Conclusion

We found significant results in the expression of p53 and CAIX between healthy tissue and tumor in all tested groups. The production of p53 was found to be suppressed in tumoral tissue in all of the tested groups whereas CAIX was typically more highly expressed in tumor compared to normal tissue in all groups. These features seem to be the typical signs of the tissue of RCC.

Significantly higher expression of VEGF in tumoral tissue of clear cell-RCC compared to healthy parenchyma can predict a better response to sunitinib. On the other hand, the high expression of VEGF in healthy renal parenchyma can predict worse response to treatment.

Acknowledgements

Supported by IGA NT 12010-5, MH CZ - DRO (Faculty Hospital in Pilsen - FNPl, 00669806) and by the Charles University Research Fund (project number P36).

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Received June 3, 2015
Revised July 23, 2015
Accepted July 24, 2015