RESEARCH ARTICLE

Effect of Targeted Therapy With Pazopanib on Expression Levels of Transcription, Growth Factors and Components of AKT/m-TOR Signaling Pathway in Patients with Renal Cell Carcinoma

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Abstract

Background: The effect of the targeted therapy on cancer molecular markers remains currently unknown. The aim of the study was to investigate the expression and content of transcription, growth factors and components of the AKT/m-TOR signaling pathway in kidney cancer patients before and after targeted therapy with pazopanib. **Methods:** A total of 157 patients with renal cell carcinoma were enrolled into the study. The level of mRNA expression was investigated by real-time PCR, and the contents of transcription and growth factors, as well as the levels of AKT/m-TOR signaling pathway components were determined by ELISA and Western blotting. **Results:** Targeted therapy with pazopanib resulted in a 3.1-fold decrease in HIF-2α expression that was accompanied by a reduction in the levels of NF-κB p65 and p50, HIF-1α and CAIX. The levels of GSK-3ß and AKT mRNA were increased; however, the levels of corresponding proteins remained low. The targeted therapy with pazopanib did not influence the level of PTEN phosphatase. A 1.9-fold increase in the level of p70 S6 (S371) was observed after therapy. **Conclusion:** The efficacy of tyrosine kinase inhibitors is associated with the changes in the angiogenic factors. Molecular characteristics of cancer could determine markers of disease progression as well as potential targets for anticancer therapies

Keywords: kidney cancer- metastasis- pazopanib

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Introduction

Stable genetic alterations in VHL gene (von Hippel-Lindau) followed by HIF (hypoxia inducible factor) and vascular endothelial growth factor (VEGF) overexpression are known to contribute to the development of renal cell carcinoma (Badewiijns et al., 2010). It should be noted that the pVHL mutations do not affect the cancer prognosis and disease outcome (Choueiri et al., 2008). There is also no association between the pVHL status and expression of tumor markers (Badewiijns et al., 2010; Spirina et al., 2008; Yumazov et al., 2016). In addition, there is evidence that cancer progression is associated with the decreased HIF-1α gene expression in tumor tissue, accompanied by high levels of HIF-2α (Sakamoto et al., 2009; Sourbier et al., 2012) and carbonic anhydrase IX (CAIX) mRNA (Driessen et al., 2006). The transcription factor κB (NF-κB) is one of the important intracellular agents, regulating the processes of proliferation and apoptosis in renal cancer (Fan et al., 2008).

Signal transduction involves binding to tyrosine kinase receptor VEGFR2 and results in AKT-mTOR pathway stimulation (Badalian et al., 2007; Szajewski et al., 2015). The main AKT substrates are variety of proteins involved in cell growth, proliferation and apoptosis (c-RAF (serine/threonine-protein kinase), GSK-3-beta (glycogen synthase kinase-3-beta)) (Guo et al., 2015; Spirina et al., 2015). The pathway is antagonized by various factors including PTEN (Gao et al., 2005; Schultz et al., 2011). It is known that hypoxia-inducible transcription factor (HIF-1) can stimulate activity of m-TOR (Hudson et al., 2002).

The AKT/mTOR signaling pathway activation has been shown in different tumors (Pópulo et al., 2012). There have been contradictory results about the association of investigated molecular markers with the kidney cancer growth and progression of kidney cancer. Almatore et al., (2005) reported that the activation of AKT/m-TOR signaling cascade was detected in only 40% of kidney tumor cells. Recently there has been evidence of overexpression of AKT signaling pathway with an increased content of its components in tissues of clear cell renal carcinoma (RCC) (Li et al., 2013; Akbani et al., 2014; Spirina et al., 2015).

Pazopanib, a targeted tyrosine kinase inhibitor, is

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widely used for treating patients with advanced renal cell carcinoma. However, there have been no published reports indicating the effects of tyrosine kinase inhibitors on molecular markers in renal cell carcinoma. The aim of the study was to investigate the expression and content of transcription, growth factors and components of the AKT/m-TOR signaling pathway in kidney cancer patients before and after targeted therapy with pazopanib.

Materials and Methods

Material and methods of investigation

A total of 157 patients with RCC were enrolled in the study. The retrospective study included patients with histopathologically verified RCC, admitted to and nephrectomised at the Cancer Research Institute, Tomsk National Research Center, Russian Academy of Medical Sciences, Tomsk, Russian Federation. The patients underwent a physical examination, chest radiography, and computer tomography (CT) of the abdomen. When vena cava tumour thrombus invasion was suspected, cavography or magnetic resonance imaging (MRI) was performed. Patients with skeletal-associated pain or elevated serum alkaline phosphatase were assessed with bone scintigraphy. The patients were followed-up according to a program including regular clinical and radiological examinations. The median age of the patients was 57 years. The treatment for kidney cancer depends on the size of the cancer and whether it has spread to other parts of the body.

Localized RCC (T1-3N0M0) was diagnosed in 90 patients and metastatic RCC (T2-4N0-1M1) in 63 patients. All patients with localized RCC underwent surgery (partial nephrectomy or simple nephrectomy) and then followed-up according to a program including regular clinical and radiological examinations. Patients with metastatic RCC received 2 cycles of preoperative targeted therapy with pazopanib at a dose of 800 mg daily, for 2 months. Tumor response to targeted therapy was evaluated according to RECIST criteria. All patients underwent radical nephrectomy. Diagnosis was verified on the basis of biopsy results.

The study was approved by the Local Committee for Medical Ethics and all patients provided written informed consent. Tumor tissue samples and histologically normal tissue samples adjacent to tumors were used for investigation. Specimens were reviewed separately by two independent pathologists.

RNA extraction

The postoperative tumor samples were incubated in RNAlater solution (Ambion, USA) for 24-hours at + 4 °C and then stored at -80 ºC. Total RNA was extracted using RNeasy Mini Kit (Qiagen).

RT-qPCR. PCR was conducted in 25 µl reaction volumes containing 12.5 µl BioMaster HS-qPCR SYBR Blue (2X) ("Biolabmix" Russia) and 300 nanoM of each primers. CAIX: F 5′-GTTGCTGTCTCGCTTGGAA-3′, R 5′-CAGGGTGTCAGAGAGGGTGT-3′; HIF-1′: F 5′- CAAGAACCTACTGCTAATGCCA-3′, R 5′- TTTGGTGAGGCTGTCCGA-3′; EPAS1: F 5′- TGGAGTATGAAGAGCAAGCCT-3′, R 5′-GGGAACCTGCTCTTGCTGT-3′; NFKB1: F 5′-CGTGTAAACCAAAGCCCTAAA-3′, R 5′-AACCAAGAAAGGAAGCCAAGT-3′; RELA: F 5′-GGAGCACAGATACCACCAAGA-3′, R 5′-GGGTTGTTGTTGGTCTGGAT-3′; VEGFA: F 5′-AGGGCAGAATCATCACGAA-3′, R 5′-TCTTGCTCTATCTTTCTTTGGTCT-3′; KDR: F 5′-AACACAGCAGGAATCAGTCA-3′, R 5′-GTGGTGTCTGTGTCATCGGA-3′; 4E-BP1: F 5′- CAGCCCTTTCTCCCTCACT -3′, R 5′- TTCCCAAGCACATCAACCT -3′; AKT1: F 5′- CGAGGACGCCAAGGAGA -3′, R 5′-GTCATCTTGGTCAGGTGGTGT -3′; C-RAF: F 5′- TGGTGTGTCCTGCTCCCT -3′, R 5′-ACTGCCTGCTACCTTACTTCCT -3′; GSK3b: F 5′- AGACAAGGACGGCAGCAA -3′, R 5′-TGGAGTAGAAGAAATAACGCAAT -3′; 70S kinase alpha: F 5′- CAGCACAGCAAATCCTCAGA -3′, R 5′- ACACATCTCCCTCTCCACCTT -3′; m-TOR: F 5′- CCAAAGGCAACAAGCGAT-3′, R 5′- TTCACCAAACCGTCTCCAA -3′; PDK1: F 5′- TCACCAGGACAGCCAATACA -3′, R 5′-CTCCTCGGTCACTCATCTTCA -3′; GAPDH: F 5′- GGAAGTCAGGTGGAGCGA-3′, R 5′-GCAACAATATCCACTTTACCAGA-3′. A pre-incubation at 95°C for 10 min was to activate the Hot Start DNA polymerase and denature DNA, and was followed by 45 amplification cycles of 95°C denaturation at 95 0 for 10 sec, 60°C annealing at 60 0 for 20 sec (iCycler iQ™, BioRad).

The fold changes were calculated by $\Delta\Delta C_t$ method (the total $\Delta\Delta C_t$ = fold of cancerous/normal tissue gene level), using normal tissue. A ratio of specific mRNA/GADPH (GADPH as a respective control) amplification was then calculated.

VEGF, VEGFR2, CAIX, HIF-1α, HIF-2, NF-κB p50 и NF-κB p65 determination. HIF-1α and NF-κB (p50 and p65) expressions were measured using Caymanchem ELISA kits (USA) in Anthos 2020 ELISA-microplate reader (Biochrom, UK). Nuclear extracts were prepared and purified according to manufacturer's instructions. CAIX, HIF-2α determination was performed using Cusabio Elisa kits (China).

Determination of expression levels of AKT/m-TOR signaling pathway components

Electrophoresis

SDS-PAGE (Laemmli) was used.

Western Blot Analysis

The protein was transferred to 0.2-/xm pore-sized PVDF membrane (GE Healthcare, UK), either at 150 mA or 100 V for 1 h by using a Bio-Rad Mini Trans-Blot electrophoresis cell. The membrane was incubated in a 1:2500 dilution of monoclonal mouse anti-human phospho-PTEN (Ser380), AKT (pan), phospho -AKT (T308), phospho-GSK-3-beta (Ser9), phospho-PDK1 (Ser241), phospho-c-Raf (Ser259), m-TOR, phospho-mTOR (Ser2448), phospho-p70 S6 (Ser371), phospho-4E-BP1 (Thr37/46) (Cell Signaling, USA) at 4 ºC overnight.

PVDF samples were incubated in Amersham ECL western blotting detection analysis system (Amersham, USA). The results were standardized using the beta-actin expression in a sample and were expressed in percentages to the protein content in non-transformed tissues. The level of protein in normal non-altered tissue was indicated as 100%.

Statistical analysis

Statistical analysis was performed using SPSS 19.0 software. The gene expression was presented as mean ± error of the mean. Data were expressed as median and ranges. Either a Student's t-test or Mann-Whitney test was used for comparing differences in mean values. Correlation analysis of data was carried out using a Spearman Rank Correlation test.

Results

Expression and content of transcription and growth factors in patients with kidney cancer are linked to the metastasis development. The level of HIF-2α mRNA was 3 times higher in patients with metastatic RCC than in patients with localized RCC (Figure 1). The expression level of mRNA of HIF-1-dependent proteins was increased in patients with metastatic RCC. In patients with metastatic RCC, a 8.5-, 19.8- and 13.4-fold increase was observed in the VEGF, CAIX and VEGFR2 expressions, respectively.

The study showed that the level of HIF-1α transcription factor was 1.5 times higher in patients with disseminated RCC compared to that observed in patients with localized RCC (Figure 2). The levels of VEGF and VEGFR2 receptor were respectively 2.3 and 1.78 times higher in metastatic compared to non-metastatic cancer tissues. On the contrary, the CAIX level was 1.5 times lower in patients with metastatic disease compared to those who had localized cancer.

Expression and content of AKT m-TOR signaling

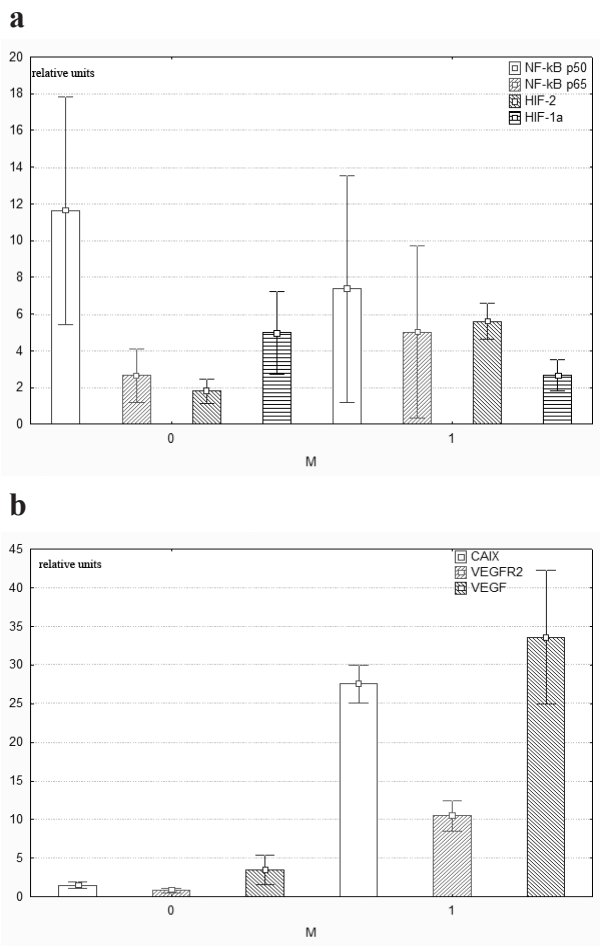

Figure 1. Expression of NF-kB p50, NF-kB p65, HIF-2, HIF-1α (a), VEGF, рецептора VEGFR2 и CAIX (b) in cancer tissues, M – is a stage of cancer: 0 – non-metastatic cancer, 1 – metastatic cancer.In patients with metastatic RCC, a 8.5-, 19.8- and 13.4-fold increase was observed in the VEGF, CAIX and VEGFR2 expressions, respectively. The study showed that the level of HIF-1α transcription factor was 1.5 times higher in patients with disseminated RCC compared to that observed in patients with localized RCC.

Table 1. Relative Expression and Content of PTEN, AKT, GSK-3ß, PDK1, c-RAF, m-TOR, 70 S6 Kinase and 4E-BP1 in Cancer Tissues of Kidney

Parameter, Relative units	n	Localized cancer	Metastatic cancer	Parameter, %	Localized cancer	Metastatic cancer
AKT/m-TOR signaling pathway components expression				AKT/m-TOR signaling pathway components content		
PTEN	33	22.45±9.4	8.34±6.70*	phospho -PTEN	99.7 (70.4-139.2)	55.3 (55.2-57.0)*
AKT	33	9.84±4.76	72.97±40,12*	AKT (pan)	139.95 (110.68-229.4)	160.35 (112.3-175.4)
				phospho -AKT (308)	105.1 (76.1-156.58)	73.1 (55.9-84.7)*
				phospho -AKT (473)	118.6 (93.2-164.1)	85.22 (53.44-106.7)*
GSK-3-beta	33	27.65±9.71	12.4±3.8*	phospho -GSK-3-b	156.1 (106.85-250.5)	147.5 (97.4-196.9)
PDK1	33	16.13±3.19	30.2±17.3*	phospho -PDK1	132.0 (102.35-168.0)	119.2 (111.7-132.6)
c-RAF	33	24.01±8.33	41.13±10.83	phospho -c-Raf	157.1 (102.9-240.75)	146.35 (75.8-164.9)
m-TOR and its substrates expression				m-TOR and its substrates expression content		
m-TOR	33	20.68±9.91	54.83±23.09	m-TOR	158.6 (90.35-218.0)	129.4 (78.8-138.4)
70 S6 kinase	33	31.98±12.82	32.6±17.22	phospho -mTOR (Ser2448)	128.1 (93.0-205.6)	108.1 (81.7-160.4)
				phospho-p70 S6 (S371)	93.6 (67.1-117.8)	101.9 (39.0-173.7)
				phospho-p70 S6 kinase (T389)	131.0 (78.1-188.7)	128.0 (105.4-136.8)
4E-BP1	33	12.15±5.4	33.32±11.05*	phospho-4E-BP1	109.7 (83.4-155.5)	101.2 (93.8-108.6)

*- Level of significance compared to the localized kidney cancer patients; $p<0.05$

Table 2. Expression and Content of Transcription Factors NF-κB p65, NF-κB p50, HIF-1α, HIF-2α in Disseminated Cancer Tissues of Kidney before (Untreated Group) and after Pazopanib Targeted Therapy (Pazopanib Treated Group)

Parameter, Relative units	n	Untreated group	Pazopanib treated group	Parameter	Untreated group	Pazopanib treated group
Transcription factors expression				Transcription factors content		
NF-kB p65	33	3.1±1.5	0.98±0.66	NF-kB p65, Units/mg protein in well	9.8 (6.6-15.3)	4.6 (3.9-9.0)*
NF-kB p50	33	31.1±19.3	1.16±0.85	NF-kB p50, Units/mg protein in well	7.0 (5.12-20.62)	4.47 (3.34-6.33)*
HIF-1a	33	3.0±1.22	0.48±0.27	HIF-1a, Units/mg protein in well	6.2 (3.1-7.1)	1.0 (0.34-2.0)*
HIF-2a	33	17,8±10,9	0,37±0,23*	HIF-2a, pg/mg of protein	332,0 (246,0-551,9)	352,8 (300,0-1007,2)
VEGF, VEGFR2 and CAIX expression				VEGF, VEGFR2 and CAIX content		
VEGF	33	4.9±1.9	0.3±0.2	VEGF, pg/mg of protein	16.6 (10.8-52.5)	9.2 (8.52-12.4)
VEGFR2	33	9.1±6.9	1.2±0.7	VEGFR2, pg/mg of protein	43.3 (23.5-62.5)	29.8 (19.1-41.6)
CAIX	33	11.5±6.13	3.7±3.7	CAIX, pg/mg of protein	246.9 (111.2-523.6	20.5 (14.8-81.5)*

*- Level of significance compared to the patients untreated with pazopanib; $p<0.05$

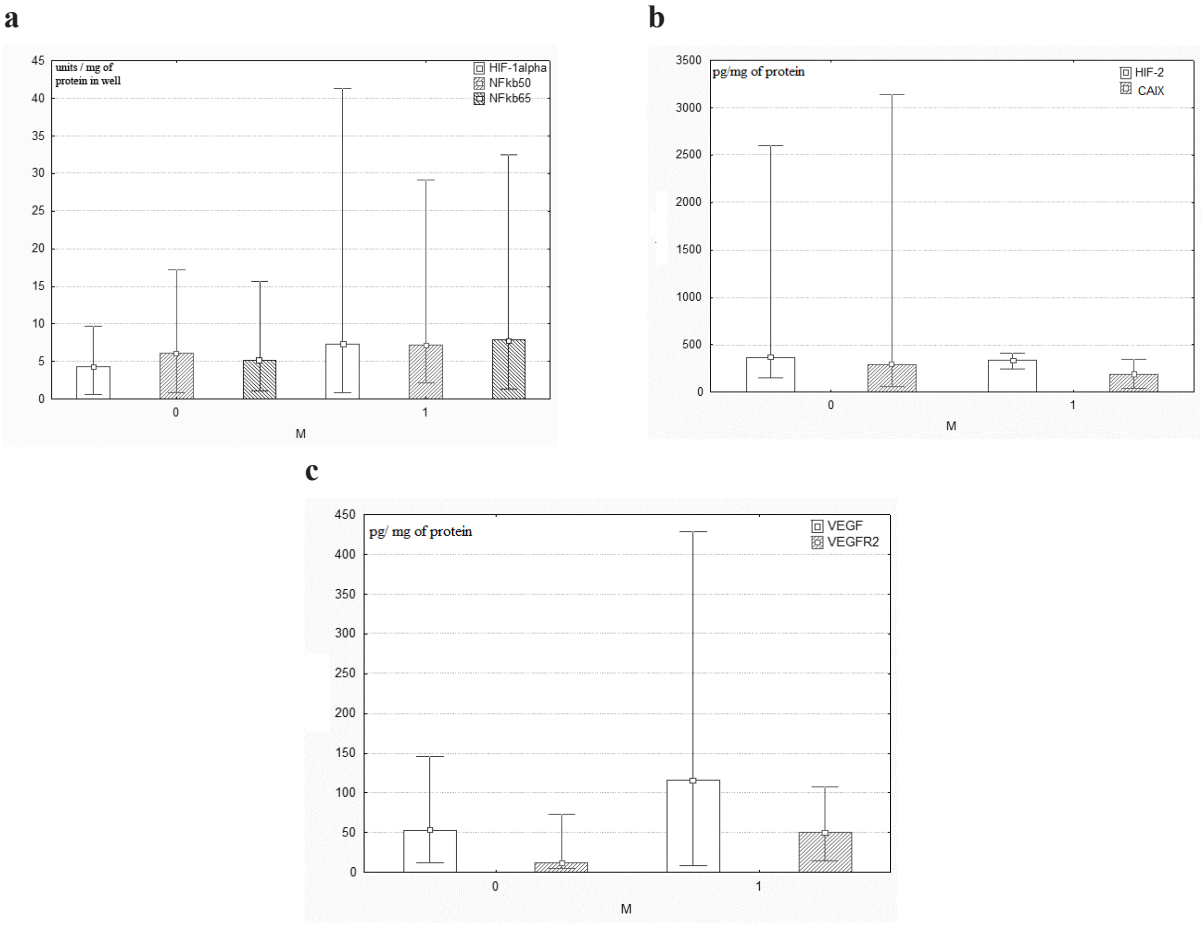

Figure 2. Levels of NF-kB p50, NF-kB p65, HIF-1α (a); HIF-2, CAIX b); VEGF. VEGFR2 (c) in Cancer Tissues, M – is a Stage of Cancer: 0 – non-metastatic cancer, 1 – metastatic cancer. The levels of VEGF and VEGFR2 receptor were respectively 2.3 and 1.78 times higher in metastatic compared to non-metastatic cancer tissues. On the contrary, the CAIX level was 1.5 times lower in patients with metastatic disease compared to those who had localized cancer.

pathway components in kidney cancer patients. A 7.4- and 1.87- fold increase in AKT mRNA and PDK1 levels, respectively, was revealed in patients with hematogenous metastasis (Table 1). In addition, a 2.2-fold decrease in GSK-3ß expression was observed in tissue of metastatic RCC compared to that of localized RCC. The PTEN mRNA level was significantly lower in patients with distant metastases compared to patients with localized cancer.

The 4E-BP1 expression was also associated with hematogenous metastases, being 2.7 times higher in patients with metastatic renal cancer than in localized cancer. The AKT/m-TOR signaling pathway components were determined in cancer patients. The activation of this

Table 3. Relative Expression and Content of PTEN, AKT, GSK-3ß, PDK1, c-RAF, m-TOR, 70 S6 Kinase and 4E-BP1 in Disseminated Cancer Tissues of Kidney before (Untreated Group) and after Pazopanib Targeted Therapy (Pazopanib Treated Group)

Parameter, Relative units	n	Untreated group	Pazopanib treated group	Parameter, %	Untreated group	Pazopanib treated group
AKT/m-TOR signaling pathway components expression				AKT/m-TOR signaling pathway components content		
PTEN	33	1.0±0.5	2.0±0.5	phospho -PTEN	96.8 (64.9-112.45)	56.14 (55.2-57.0)*
AKT	33	34.7±23.7	105.0±41.6*	AKT (pan)	129.7 (103.8-223.75)	212.7 (175.4-250.1)
				phospho -AKT (308)	105.1 (73.1-156.6)	70.3 (55.9-84.7)*
				phospho -AKT (473)	101.95 (72.5-137.7)	118.9 (105.2-132.6)
GSK-3-beta	33	22.7±8.1	46.5±24.6*	phospho -GSK-3-b	144.8 (101.7-232.5)	199.8 (147.5-252.1)
PDK1	33	21.1±10.1	74.5±34.5	phospho -PDK1	129.5 (86.9-162.4)	144.3 (132.6-156.0)
c-RAF	33	35.2±9.9	52.6±36.6	phospho -c-Raf	145.9 (93.6-240.7)	155.6 (93.6-240.7)
m-TOR and its substrates expression				m-TOR and its substrates expression content		
m-TOR	33	33.8±15.5	25.1±10.2	m-TOR	157.3 (75.35-218.0)	133.9 (129.4-138.4)
70 S6 kinase	33	32.1±14.2	13.1±12.7	phospho -mTOR (Ser2448)	116.7 (79.5-173.5)	179.0 (160.4-197.7)
				phospho-p70 S6 (S371)	90.35 (44.7-117.8)	175.2 (101.9-248.5)*
				phospho-p70 S6 kinase (T389)	109.7 (83.4-155.5)	101.2 (93.8-108.6)
4E-BP1	33	18.4±7.9	49.8±23.7	phospho-4E-BP1	109.7 (83.4-155.5)	101.2 (93.8-108.6)

*- Level of significance compared to the patients untreated with pazopanib; p<0.05;

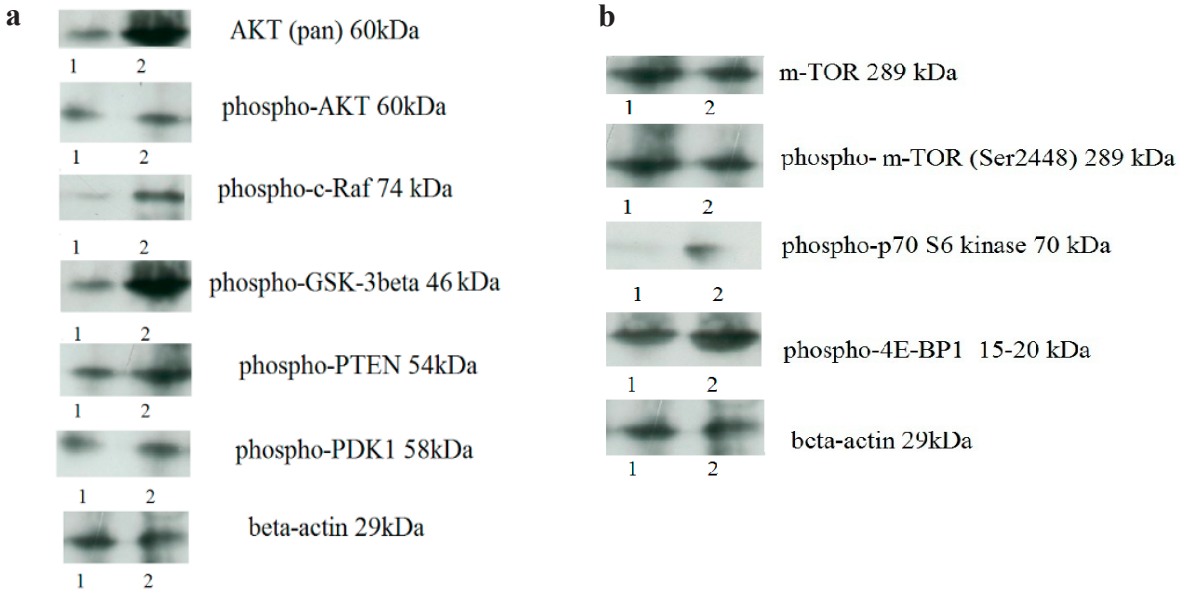

Figure 3. Immunoblots of AKT/m-TOR Components in Kidney Cancer Tissues. a – AKT, phospho-PDK1, phospho-c-Raf, phospho-GSK-3beta and phospho-PTEN level in non transformed (1) and cancer (2) tissues; b – m-TOR, phospho-m-TOR, 70S 6 kinase and 4E-BP1 levels in non transformed (1) and cancer (2) tissues. Note: The results were standardized using the beta-actin expression in a sample and were expressed in percentages to the protein content in non-transformed tissues. The level of protein in normal non-altered tissue was indicated as 100%. It is found the activation of AKT/m-TOR signaling pathway in kidney cancers by increase of AKT, its phosphorylated form, proteinkinase m-TOR, glycogen regulator GSK-3-ß and transcription inhibitor 4E-BP1.

pathway was found in kidney cancers by increase of AKT, its phosphorylated form, proteinkinase m-TOR, glycogen regulator GSK-3-ß and transcription inhibitor 4E-BP1 (Figure 3). It was noted that the development of distant metastasis was accompanied by a significant decrease of the PTEN and phospho-AKT content in 1.8 and1.4 fold, respectively, in the tumor tissues compared to the non-metastatic ones.

Effect of targeted therapy with pazopanib on expression and content of transcription, growth factors and components of the AKT / m-TOR signaling pathway.

Targeted agents are currently administered for patients with metastatic kidney cancer. In a study involving 63 patients with metastatic renal cancer, therapy with

pazopanib resulted in partial tumor regression in 26.9% of cases, disease stable in 61.5%, and progression in 11.5% of cases. Expression levels of growth and transcription factors tended to decrease after therapy with pazopanib (Table 2). The HIF-2α expression was found to be 48 times lower after therapy than before therapy.

Therapy with pazopanib resulted in a 2.1-, 1.6- and 6.2- fold reduction in NF-κB p65, NF-κB p50 and HIF-1α levels, respectively. The level of CAIX was 12 times lower after therapy than before therapy.

We revealed a 3-fold increase in AKT expression in cancer tissues after treatment (Table 3). Signaling pathway activation was also accompanied by a 2-fold increase in GSK-3beta mRNA level compared to that observed before treatment.

The AKT and phosphorylated AKT (T308) expressions were 1.5 times higher after treatment than before treatment. In addition, a 1.9-fold increase in the level of phospho-p70 S6 (S371) kinase was also observed. The use of targeted therapy with tyrosine kinase inhibitors led to a1.7-fold reduction in the level of phospho-PTEN

Discussion

Carbonic anhydrase IX (CAIX) is known to be a HIF-1α- dependent protein (Badewiijns et al., 2010). Changes in mRNA and protein levels of HIF-1α and CAIX in metastasis development were shown to be multidirectional. These results were similar to data shown by Linden et al., (2008). The authors found that in patients with clear-cell renal cell carcinoma, dissemination of tumor cells was associated with low HIF-1α expression and high content of the corresponding protein. We also revealed the HIF-1α mRNA suppression in metastatic cancer, accompanied by a high content of the HIF-1α protein.

Moreover, the increased CAIX mRNA level led to decrease in the content of its protein. The CAIX is involved in the regulation of various carcinogenic processes (Sourbier et al., 2012). These changes are likely can affect the growth and proliferation of tumor cells.

The development of haematogenous metastases from RCC is accompanied by the increased HIF-2α expression with the high level of corresponding protein. It is known that the activation of HIF-2 leads to a modification of proliferative potential of cancer cells (Driessen et al., 2006; Linden et al., 2008). This phenomenon is likely to be caused by specific peculiarities of tumor cells. We suppose that the metastatic phenotype activation and cancer progression are the main stages of cancerogenesis and they are closely related to the HIF-2α expression.

This marker is likely to be a predictor of unfavorable outcome. Therefore, 4E-BP1 mRNA could be an indicator of tumor aggressiveness, that was consistent with other recent studies (Biswas et al., 2010; Gerlinger et al., 2012; Darwish et al., 2013).

These data allow us to conclude that the metastasis activation is followed by a stimulation of AKT/m-TOR signaling pathway with PTEN deficiency. So, they can be the potential targets for pazopanib targeted therapies.

Effect of targeted cancer therapy on molecular markers is currently associated with its efficacy. We studied the relationship between the level of transcription/growth factors and their expression in kidney cancer. The changes in the levels of molecular markers and their expression were revealed. Our data indicated that the effect of pazopanib was associated with decreased expression and content of transcription factors responsible for tumor angiogenesis. We showed the expression and content of molecular markers in renal tumor untreated and treated with pazopanib. The progression of kidney cancer occurred due to a high levels of HIF-2α mRNA and its protein. Targeted therapy with pazopanib resulted in a decrease in the level of HIF-2α mRNA, thus indicating the effectiveness of this treatment. This positive effect was also confirmed by the analysis of NF-κB p65, NF-κB p50 and HIF-1α transcription factors. Reduction in these markers led to decreased production of transcription factors including CAIX.

The altered expression of AKT/m-TOR signaling pathway components also belongs to the molecular characteristics of RCC metastasis development. Although these facts are mailny theoretical, they may be useful in evaluating potential tumor markers for predicting disease outcome. It was detected an increase of GSK-3ß and AKT mRNA levels at low rates of corresponding proteins in cancer tissues after targeted therapy. The targeted therapy did not influence the level of PTEN, which remained low after therapy.

In conclusion, molecular events responsible for the development of cancer were shown to be the potential markers for targeted therapy. The use of tyrosine kinase inhibitors resulted in decreased expression of angiogenic factors. The level of p70 S6 (S371) was increased, while the level of AKT and GSK-3ß was decreased. The impact of this treatment on the PTEN content was not identified. Further studies are necessary to evaluate the impact of the targeted therapy on molecular markers, thus allowing the efficacy of therapy to be increased.

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