Assessment of AKT/mTOR Signaling Pathway Components in Kidney Cancers and Their Connection with Metastases

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L.V. Spirina, MD, DSc, Associate Professor, Leading Researcher, Laboratory of Tumor Biochemistry1; Professor, Department of Biochemistry and Molecular Biology with a Course of Clinical Laboratory Diagnostics2; E.A. Usynin, MD, PhD, Senior Researcher, Department of General Oncology1; Associate Professor, Department of Oncology3; Z.A. Yurmazov, Physician, Department of General Oncology1; I.V. Kondakova, MD, DSc, Professor, Head of the Laboratory of Tumor Biochemistry1; E.M. Slonimskaya, MD, DSc, Professor, Department of Oncology3

1Cancer Research Institute, Tomsk National Research Medical Center of the Russian Academy of Sciences, 5 Kooperativny St., Tomsk, 634009, Russia; 2Siberian State Medical University, 2 Moskovsky trakt, Tomsk, 634050, Russia; 3Saint Petersburg State University, 7–9 Universitetskaya naberezhnaya, Saint Petersburg, 199034, Russia

Introduction

Phosphatidylinositol-3-kinase AKT/mTOR signaling pathway (PI3K/AKT/mTOR) is activated under the influence of numerous growth factors and regulates the process of cell division and its apoptosis. Its components participate in the molecular mechanisms of kidney cancer development and progression and are used for targeted therapy of the disease [1, 2]. They are presented by phosphoinositide-3-kinase (PI3K), AKT and mTOR kinases but taking into account the importance of the two latter ones the cascade is often called AKT/mTOR. AKT protein kinase is activated by PDK1 (pyruvate dehydrogenase kinase) and mTORC2 enzymatic complex which in addition to mTOR includes also mLST8 (mammalian lethal with Sec13 protein 8) known as GβL (G-protein β-subunit-like protein), rictor (rapamycin-insensitive companion of mTOR), mSin1 (mammalian stress-activated protein kinase (SAPK)-interacting protein 1), protor (protein observed with rictor) [3].

Numerous proteins involved in the processes of growth, cell proliferation, and apoptosis are the substrates of the AKT kinase. c-RAF (serine/threonine-protein kinase) and a glycogen metabolism enzyme GSK-3beta (glycogen synthase kinase-3beta) are considered as key ones [4, 5]. The AKT also plays a substantial role in activation of a rapamycin-sensitive complex mTORC1 consisting of mTOR, raptor (regulatory-associated protein of TOR), mLST8, and PRAS40 (proline-rich PKB/AKT substrate 40 kDa) [6].

Protein kinase mTOR, as described above, exists in a cell as a subunit of the intracellular multimolecular signaling complexes mTORC1 and mTORC2 [7]. The most important substrates of mTOR in the mTORC1 complex are p70 S6 kinase (ribosomal protein S6 kinase) and 4E-BP1 (initiating factor 4E binding protein 1) [8–10] which serve as the key regulators of mRNA translation and stimulate protein synthesis.

AKT signaling pathway activity is controlled with PTEN phosphatase [11]. It catalyzes the removal of
the phosphate group in 3D position of the inositol ring from phosphatidylinositol-3-phosphates inhibiting the translation of the signal via PI3K/AKT/mTOR signaling pathway. Phosphatase PHLP (PH domain and leucine-rich repeat protein phosphatase) which catalyzes dephosphorylation at Ser473 in the AKT molecule refers also to the protein substrates PTEN. Increase of this enzyme activity results in activation of apoptosis and deceleration of tumor cell proliferation which allows it to be referred to the important oncosuppressor proteins [12]. Transcription factor HIF-1 is also known to stimulate the activity of protein kinase mTOR [5].

The activation of this signaling pathway has been shown in various types of tumor diseases [13]. However, data concerning kidney tumors are rather contradictory. Thus, Almatore et al. [14] believe that activation of AKT/mTOR signaling pathway is detected only in 40% of tumor cells in kidney cancer. There are facts demonstrating that a marked hyperexpression of the AKT signaling pathway with the increase of its component content is observed in the tissue of a clear cell tumor [15–17]. They may have various values in the cancerous tissue of the kidney depending on the size of the primary tumor and presence of hematogenic metastases of the disease [18, 19]. The level of expression of the examined metabolites and their association with the content of the protein components in the tissue of the kidney affected with clear cell cancer have not been actually studied.

The majority of the investigators adhere to the opinion that a ratio of the component expression level and the content of the appropriate proteins in oncogenesis is of great importance determining the biological character of tumor growth and prognosis of the disease [20].

The aim of our investigation was to study the expression of the AKT signaling pathway components, mTOR substrates, and contents of their protein products in the cancerous kidney tissue depending on the tumor process extension.

Materials and Methods

34 patients with clear cell cancer of the kidney T1–3N0–1M0 (the mean age 57.6±2.2 years) were included in the study. The patients were distributed in the following way depending on the size of the primary tumor: in 10 patients the neoplasm corresponded to T1, in 14 to T2, and in 10 to T3. A localized form of the disease (T1–3N0M0) was revealed in 24 patients, a disseminated one in 10. The range of diagnostic and treatment procedures for patients with kidney cancer complied with the recommended algorithms on the diagnosis and treatment of malignant neoplasms endorsed by the Ministry of Health of the Russian Federation.

The study was approved by the Ethical Committee of the Cancer Research Institute, Tomsk National Research Medical Center of the Russian Academy of Sciences. All procedures involving patients were carried out in compliance with the Declaration of Helsinki on human rights (2013). Written informed consent was obtained from all patients.

Samples of the tumor and histologically unchanged tissue being at a distance of not less than 1 cm from the border of the tumors served as the material for the study. To perform Western blotting, the harvested tissue samples were frozen and stored at −80°C. To isolate mRNA and determine a relative expression level, the tissue samples were placed into the RNAlater solution (Ambion, USA) and after the 24-hour incubation at +4°C were stored at −80°C.

Determining the expression level of the AKT/mTOR signaling pathway components

RNA isolation. RNA was isolated using RNeasy Mini Kit containing DNAsé I (Qiagen, Germany). To determine the quantity of the isolated RNA, a NanoDrop-2000 spectrophotometer (Thermo Scientific, USA) was used to assess its concentration and isolation purity. The DNA concentration was 80–250 ng/μl. The purity of the isolated DNA was estimated by counting the ratio of absorbance at the wavelengths of 260, 280, and 230 nm. The A260/A280 ratio was from 1.95 to 2.05; that of A260/A230 from 1.90 to 2.31. RNA integrity was analyzed by means of capillary electrophoresis using a TapeStation system (Agilent Technologies, USA) and R6K ScreenTape Kit (Agilent Technologies). RIN (RNA integrity number), an index developed for finding errors in the evaluation of DNA quality, was equal to 5.6–7.8.

Quantitative real-time PCR with reverse transcription. The level of component expression was assessed with the help of quantitative real-time reverse transcription PCR (RT-qPCR) using SYBR Green and iCycler amplificator (Bio-Rad Laboratories, USA). In order to obtain cDNA on the RNA template the reaction of reverse transcription was performed using Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific) with the random hexanucleotide primers according to the instructions supplied with the kit. The PCR was set up in three replicas in the volume of 25 μl containing 12.5 μl Maxima SYBR Green qPCR Master Mix (2X) (Thermo Scientific), 300 nmol of forward and reverse primers, and 50 ng of cDNA.

Two-step amplification program included: 1 cycle — 94°C, 10 min — predenaturation; 40 cycles: step 1 — 94°C, 10 s and step 2 — 60°C, 20 s at 60°C. Primers were selected using Vector NTI Advance 11.5 program and NCBI database (http://www.ncbi.nlm.nih.gov/nuccore) (Table 1).

The housekeeping gene of the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) enzyme was used as a reference gene and the expression level of each target gene was normalized relative to GAPDH expression.

Determining the content of AKT/mTOR signaling pathway components

Homogenates production. The frozen tissue (100 mg) was homogenized in liquid nitrogen,
resuspended in 300 μl 50 mM tris-HCl buffer (pH 7.5) containing 2 mM ATP, 5 mM magnesium chloride, 1 mM dithiothreitol, 1 mM EDTA, and 100 mM sodium chloride. The homogenate was centrifuged for 60 min at 10,000 g and 4°C.

Electrophoresis. Electrophoresis was performed according to Laemmli in 13% polyacrylamide gel.

Western blotting. After electrophoresis, polypeptides were transferred to the PVDF membrane (Immobilon; Millipore, USA). Immunodetection was carried out with antibodies to phospho-PTEN (Ser380), AKT (pan), phospho-AKT (T308), phospho-GSK-3beta (Ser9), phospho-PDK1 (Ser241), phospho-c-RAF (Ser259), mTOR, phospho-mTOR (Ser2448), phospho-p70 S6 kinase (Ser371), phospho-4E-BP1 (Thr37/46) (Cell Signaling, USA). The membrane was treated at the ECL chemiluminescence detection system (GE Healthcare, Great Britain). Band intensity was assessed using a computer program ImageJ. Standartization was performed relative to β-actin. The results were expressed in the percentage of the index values for the unchanged tissue.

Statistics. The results were statistically processed using Statistica 8.0 software package. Significance of differences between the groups was determined by means of nonparametric Mann–Whitney test for independent samples, the results were presented as M (median) with interquartile range (25th and 75th percentiles). Gene expression results were presented as M±m. The differences were considered significant at p<0.05. A nonparametric median test and Kruskal–Wallis ANOVA test were used to compare two or more independent samples. The presence of relationship between the studied indices was examined using correlation analysis, Spearman’s rank correlation coefficient (r) was calculated to assess the strength of relationship between the variables.

Results and Discussion

The investigation performed showed no significant differences in the expression of the components PTEN, AKT, GSK-3beta, PDK1, c-RAF depending on the size of the primary tumor in patients with kidney cancer (Table 2). Similar phenomenon was also registered for the mRNA of mTOR protein kinase. At the same time, the fact of the 4.2-fold increase of 70 S6 kinase expression was detected in patients with the tumor size corresponding to T2N0M0 relative to the tumor T1N0M0 and a marked 8.3-fold increase of the mRNA level of the 4E-BP1 transcription inhibitor in patients with the tumor size corresponding to T3N0M0−1 compared to the tumor T1N0M0. However, median test and Kruskal–Wallis ANOVA test did not confirm statistical significance of these differences.

The study of the expression of these indices in patients with the presence and absence of hematogenic metastasis signs revealed growth of the mRNA level of the AKT kinase and PDK1 protein kinase responsible for its phosphorylation by 7.4 and 1.87 times, respectively. There also has been noted a 2.2-fold reduction in

### Table 1

| Components | Amplicon (bp) | Sequence |
|------------|--------------|----------|
| 4E-BP1     | 244          | F 5′-CAGCCTTTTCTCCCTGTACCT-3′  
R 5′-TTCCCCAGCATCTCACT-3′ |
| AKT1       | 181          | F 5′-CAGAGGGCCAGCGAAGGAG-3′  
R 5′-GTCACTTGGTCAAGTTGTGTT-3′ |
| c-RAF      | 152          | F 5′-TGTTGTGTCCTGCTCCTGC-3′  
R 5′-ACTGCTGTCACCTTACTCCT-3′ |
| GSK-3beta  | 267          | F 5′-AGACAGAGCGGGACGCAA-3′  
R 5′-TGAGGATAGAAGAATAAGCATTG-3′ |
| 70S kinase | 244          | F 5′-CAGCACAGCAAATCTCCTCAG-3′  
R 5′-ACACATCTCCCTCTCCACCT-3′ |
| mTOR       | 160          | F 5′-CCAGGAGCAAGCAGCAT-3′  
R 5′-TTCCCAAAACCGTCCCAA-3′ |
| PDK1       | 187          | F 5′-TCACAGGAGCAGCCAAATAC-3′  
R 5′-CTCCTCTGGTCACTCATCTCA-3′ |
| GAPDH      | 138          | F 5′-GGAGATCAGGTGGAGAGCA-3′  
R 5′-GCAACATATCCACTTCTTACCAGA-3′ |

Note: NM is number of RNA sequence in NCBI Nucleotide Database (http://www.ncbi.nlm.nih.gov/nuccore); F is forward primer; R is reverse primer.
expression of the gene-regulating synthesis of glycogen GSK-3beta in the tumor tissue in metastatic cancer of the kidney in comparison with the localized form of the disease (Table 3). The described changes were accompanied by the decrease of PTEN expression: the level of mRNA phosphatase was significantly lower in patients having distal metastases relative to the similar index in the localized process.

Table 3

| Components | Localized kidney cancer (n=24) | Disseminated kidney cancer (n=10) |
|------------|------------------------------|----------------------------------|
| **Components of AKT/mTOR signaling pathway** | | |
| PTEN       | 22.4±5.40                    | 8.34±6.70*                      |
| AKT        | 9.8±4.76                     | 72.97±40.12*                    |
| GSK-3beta  | 27.6±5.71                    | 12.4±3.8*                       |
| PDK1       | 16.1±3.19                    | 30.2±17.3*                      |
| c-RAF      | 24.0±8.33                    | 41.13±10.83                     |
| **Protein kinase mTOR and its substrates** | | |
| mTOR       | 20.6±5.91                    | 54.8±23.09                      |
| p70 S6 kinase | 31.9±12.82                | 32.6±17.22                      |
| 4E-BP1     | 112.5±5.40                   | 33.2±11.05*                     |

* Statistically significant differences of values in comparison with localized kidney cancer (p<0.05).

Activation of AKT/mTOR signaling pathway in the kidney tumor associated with the von Hippel–Lindau gene defect is known to be accompanied by the decrease of GSK-3beta kinase activity concurrently with downregulation of E3 ubiquitin-ligase (von Hippel–Lindau protein) [21]. There are also data that the expression level of this index is associated with recurrence-free survival of patients with breast cancer [22, 23]. In this relation, it becomes clear that it is the reduced expression level of mRNA of GSK-3beta kinase that is connected with kidney cancer progression when the metastatic form of the disease is developing.

Table 4

| Components | Stage of the disease |
|------------|----------------------|
|            | T1,N2,M0 (n=10)     | T2,N2,M1 (n=14) | T3,N2,M0 (n=10) |
| **Components of AKT/mTOR signaling pathway** | | | |
| Phospho-PTEN | 104.8 [67.9; 165.3] | 110.3 [67.4; 194.4] | 83.3 [55.3; 105.0] |
| AKT (pan)    | 170.0 [98.6; 142.9] | 168.4 [113.7; 224.1] | 284.5 [79.6; 447.8] |
| Phospho-AKT (T308) | 125.3 [73.2; 150.4] | 134.1 [83.1; 210.9] | 127.8 [101.0; 114.0] |
| Phospho-AKT (S473) | 120.3 [73.2; 150.4] | 139.1 [93.1; 200.9] | 137.8 [106.0; 124.0] |
| Phospho-GSK-3beta | 186.8 [116.7; 267.5] | 151.5 [100.1; 227.0] | 107.5 [78.5; 107.3] |
| Phospho-PDK1 | 177.7 [116.7; 167.6] | 150.9 [54.2; 170.2] | 95.6 [78.0; 119.2] |
| Phospho-c-RAF | 126.3 [78.0; 162.0] | 164.0 [18.1; 234.5] | 286.9 [247.0; 311.1]* |
| **Protein kinase mTOR and its substrates** | | | |
| mTOR       | 179.9 [68.8; 193.0] | 211.0 [154.5; 260.4] | 124.2 [50.7; 194.1] |
| Phospho-mTOR (Ser2448) | 257.1 [106.4; 318.9] | 123.0 [50.0; 164.7] | 108.8 [72.6; 181.7]* |
| Phospho-p70 S6 kinase | 106.8 [66.7; 95.0] | 101.1 [67.5; 117.7] | 102.6 [31.2; 195.5] |
| Phospho-4E-BP1 | 107.1 [63.1; 136.6] | 141.1 [71.8; 151.8] | 266.7 [105.5; 489.9] |

* Statistically significant differences of values in comparison with the group of patients with stage T1,N2,M0 (p<0.05).
and phospho-AKT (S473) content in the tumor tissue (Table 5).

Of special attention is the dynamics of changes in the expression level of the AKT and its protein product which were obtained during our study. Data presented in Figure 1 characterize expression and content of the AKT protein kinases, phospho-AKT (T308) and phospho-AKT (S473) in patients with localized and disseminated cancer of the kidney. As the disease progressed, the AKT expression has been found to grow and the content of the protein kinase to decrease in patients with hematogenic metastases. These changes were followed by the expression increase of the protein kinase PDK1 involved in the activation of the examined AKT. The level of mRNA PDK1 during metastases development may be supposed to be an additional proof of the role of the AKT/mTOR signaling pathway in the progression of kidney cancer.

Changes of the PTEN phosphatase at the mRNA levels and the appropriate protein are shown in Figure 2 where decrease of the component expression level was followed by the reduction of the phosphatase content. Kidney cancer progression is known to be associated with functional inferiority of PTEN that leads to a more intensive activation of the AKT/mTOR signaling pathway [2, 6]. This may be proved by the fact, revealed and verified by us, that the AKT expression grew with the development of the disseminated process. The content reduction of the activated AKT forms, phospho-AKT (T308) and phospho-AKT (S473), was also registered. It allows us to suppose that high activity of this signaling cascade under the conditions of PTEN phosphatase inferiority is implemented due to a small amount of the enzyme and results in the decrease of its phosphorylated forms.

The detected multidirectional expression changes of the AKT kinase and its protein products agree with our previously obtained data in relation to the transcription and growth factors (HIF-1, HIF-2, VEGF, CAIX) [15, 18] which are also confirmed by other authors [25]. It is interesting to note

Table 5
Content of AKT/mTOR signaling pathway in patients with localized and disseminated kidney cancer (%) (Me [Q1; Q3])

| Components                  | Localized kidney cancer (n=24) | Disseminated kidney cancer (n=10) |
|-----------------------------|-------------------------------|----------------------------------|
| **Components of AKT/mTOR signaling pathway** |                               |                                  |
| Phospho-PTEN                | 99.7 [70.4; 139.2]             | 55.3 [55.2; 57.0]*               |
| AKT (pan)                   | 139.9 [110.7; 229.4]           | 160.4 [112.3; 175.4]             |
| Phospho-AKT (T308)          | 105.1 [76.1; 156.6]            | 73.1 [55.9; 84.7]*               |
| Phospho-AKT (S473)          | 118.6 [93.2; 164.1]            | 85.2 [53.4; 106.7]*              |
| Phospho-GSK-3beta           | 156.1 [106.8; 250.5]           | 147.5 [97.4; 196.9]              |
| Phospho-PDK1                | 132.0 [102.4; 168.0]           | 119.2 [111.7; 132.6]             |
| Phospho-c-RAF               | 157.1 [102.9; 240.8]           | 146.4 [75.8; 164.9]              |
| **Protein kinase mTOR and its substrates** |                           |                                  |
| mTOR                        | 158.6 [90.4; 218.0]            | 129.4 [78.8; 138.4]              |
| Phospho-mTOR (Ser2448)      | 128.1 [93.0; 205.6]            | 108.1 [81.7; 160.4]              |
| Phospho-p70 S6 kinase       | 93.6 [67.1; 117.8]             | 101.9 [39.0; 173.7]              |
| Phospho-4E-BP1              | 131.0 [78.1; 188.7]            | 128.0 [105.4; 136.8]             |

* Statistically significant values in comparison with the localized kidney cancer.

Figure 1. Expression and content of AKT protein kinase in the tissue of the kidney tumor:
M₀ — in patients with localized kidney cancer; M₁ — with disseminated kidney cancer; (a) AKT expression, results are presented in arbitrary units (Y-axis); (b) content of phospho-AKT (T308) and phospho-AKT (S473), results are presented in percentage of the content values in the non-transformed tissue (Y-axis)
that the dynamics of changes was similar only for the oncosuppressor protein PTEN both at the level of mRNA and protein though it was not validated statistically by the correlation analysis.

Changes in the process of mRNA synthesis and degradation, dysregulation of translation processes, and activation of posttranslational modification of proteins may underlie these discrepancies [26].

Conclusion

The study performed has found association between the expression of the AKT/mTOR signaling pathway components, the content of their protein products, and progression of the disease. Changes of the mRNA level and the content of the appropriate protein product have been shown for the AKT, PTEN phosphatase, 4E-BP1, and GSK-3beta. The findings of the conducted study expand the notion of oncogenesis in cancer of the kidney and serve also as a fundamental base for determining the risk connected with the generalization of the tumor process.

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Conflicts of interest. The authors have no conflicts of interest to declare.

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