Влияние транскрипционных факторов, VEGF и протеиназ на прогрессирование рака почки

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Аннотация

Введение. Эффективность противоракового лечения зависит от биологических факторов опухоли. 
Цель исследования – определить активность протеасом и кальпаинов и выявить их связь с содержа-
нием VEGF, HIF-1α и NF-κB в опухолевых, неизмененных и метастатических тканях карциномы почек 
(RCC).

Материал и методы. В исследование были включены 93 пациента с почечно-клеточным раком. 
Содержание транскрипционных факторов и VEGF определяли методом ИФА. Количественный состав 
протеасом исследовали методом Вестерн-блоттинг. Активность протеасомы и кальпаина определяли 
с использованием специфического флуорогенного субстрата.

Результаты. Выявлена инактивация 
протеолиза у пациентов с раком почки. Прогрессирование заболевания было связано со значительным 
снижением уровня клеточного протеолиза и ростом содержания транскрипционных и ростовых факторов 
в тканях первичной опухоли. Активация протеолиза была обнаружена в метастатических тканях. Вы-
воды. В результате проведенного исследования показано, что факторы транскрипции NF-κB, HIF-1α, 
VEGF и внутриклеточные протеолитические системы участвуют в прогрессировании рака почки.

Ключевые слова: рак почки, метастазы, NF-κB, HIF-1α, VEGF.

IMPACT OF TRANSCRIPTION FACTORS, VEGF AND 
PROTEASES ON KIDNEY CANCER PROGRESSION

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Abstract

Introduction. The efficacy of anticancer treatment depends on biological factors of tumor. The aim of the 
study was to determine the activity of proteasomes and calpains and to reveal their association with VEGF, 
HIF-1α and NF-κB expressions in normal, primary and metastatic renal cell carcinoma (RCC) tissues.

Methods. Ninety-three patients with renal cell carcinoma were included into the study. The expression levels 
of transcription factor and VEGF were measured using ELISA kits. The levels of proteasome subunits were 
measured by Western Blotting. Proteasome and calpain activities were determined using specific fluorogenic
Introduction
Kidney cancer is among the cancers that have the highest growth rate and it is known as the most deadly urinary tract cancer [1]. In the past years, there has been an increased understanding of the tumor biology of renal cell carcinoma (RCC) [2–4]. Hypoxia-inducible factor (HIF-1) is a heterodimeric transcription factor consisting of an alpha and beta subunits. Hydroxylation of proline and asparagine residues in HIF-1α results in their binding to the von Hippel-Lindau protein (pVHL), and is followed by HIF-1α polyubiquitination and degradation in the proteasome [5, 6]. The HIV activation results in vascular endothelial growth factor (VEGF) production [3].

Our preliminary data showed the role of proteolytic regulation in kidney cancer progression [18]. The aim of our study was to determine the proteasome and calpain activities in normal, primary and metastatic human RCC tissues and to reveal their association with VEGF, HIF-1α and NF-κB expression levels.

Material and Methods
A total of 93 patients with RCC were treated at the Cancer Research Institute of Tomsk National Research Medical Center, Russian Academy of Sciences, Tomsk, Russian Federation (mean age 57.6 ± 2.2 years). Localized RCC (T1-3N0M0) was revealed in 50 patients. Forty-three patients had metastatic RCC (T2-4N0-1M1). All patients with localized RCC underwent surgery (partial nephrectomy or simple nephrectomy). Diagnosis verification and cancer stage estimation for patients with metastatic RCC included the biopsy analysis. The combined modality treatment included pre-operative pazopanib targeted therapy administered at a dose of 800 mg every day for 2 months. After completion of therapy, tumor response was evaluated according to the RECIST criteria, and radical nephrectomy was performed.

The study was approved by the Local Committee for Medical Ethics and all patients provided written informed consent. Specimens were reviewed by two pathologists separately. Normal, malignant and metastatic tissue samples taken at a distance of not less than 2 cm from the tumor border were used. The frozen samples were stored at -80°C.

Preparation of tissue homogenates. Tissue samples (100 mg) were homogenized and then resuspended in 300 μL of 50 mM Tris-HCl buffer (pH=7.5) containing 2 mM ATP, 5 mM MgCl₂, 1 mM dithiothreitol, 1 mM EDTA, and 100 mM NaCl. The homogenate was centrifuged at 10000×g for 60 minutes at 4°C.

Proteasome fractionation. All procedures were carried out at 4°C. Proteins from tissue homogenates were fractionated with stepwise concentrations of ammonium sulfate [30]. The fractions were assayed for the proteasome activity.

Proteasome activity assay. Chymotrypsin-like activity of the total proteasome, 26S and 20S pools was measured in cancer, metastatic and non-transformed tissue homogenates, and in the proteasome fractions, using the fluorogenic substrate N-Succinyl-Leu-Leu-Val-Tyr-7-Amido-4-Methylcoumarin (Suc-LLVY-AMC) in a Hitachi-850 (Japan) fluorimeter at an excitation wavelength of 380 nm and an emission of 440 nm [31]. The 20S proteasome activity solution contained 20 mM Tris-HCl (pH=7.5), 1 mM dithio-
in absence or presence of 10 mM CaCl2 and N-Acetyl-
were performed at room temperature for 30 minutes
мМ NaCl and 30 μM Suc-LLVY-AMC. Incubations
ity solution contained 100 мМ Tris-HCl (pH=7.3), 145
MgCl2
some activity solution additionally contained 5 mM
threitol, and 30 μM Suc-LLVY-AMC. The 26S protea-
Calpains activity assay. The calpains activity
service in tissue homogenates using the fluorogenic
substrate N-Succinyl-Leu-Leu-Val-Tyr-7-Amido-4-
Methylcoumarin (Suc-LLVY-AMC) in a Hitachi-850
(Japan) fluorimeter at an excitation wavelength of 380
nm and an emission of 440 nm [32]. The calpains activ-
ity solution contained 100 мМ Tris-HCl (pH=7.3), 145
mM NaCl and 30 μM Suc-LLVY-AMC. Incubations
were performed at room temperature for 30 minutes
in absence or presence of 10 mM CaCl2, and N-Acetyl-
L-leucyl-L-leucyl-L-norleucinal (calpain inhibitor I).

Electrophoresis. SDS-PAGE was used, according
to the method of Laemmli [33]. The samples were
incubated for 5 to 10 min in 62.5 mM Tris-HCl buf-
er (pH 6.8), containing 2.0 % (w/v) SDS, 5.0 % (v/v)
mercaptoethanol, 10 % (v/v) glycerol, and 0.0012 %
Bromophenol blue.

Western Blot Analysis. After SDS-PAGE, the gels
were allowed to equilibrate for 10 min in 25 mM
Tris and 192 mM glycine in 20 % (v/v) methanol.
The protein was transferred to 0.2-μm 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 according to the
method described in the manual accompanying the
unit. Before incubation with antibodies the PVDF
membrane was incubated in 10 mM Tris-HCl buf-
er (pH 7.5), containing 150 mM NaCl and 0.1 %
(v/v) tween-20 for 2 hours. Then it was incubated in a
1:2500 dilution of monoclonal mouse anti-human
α1α2α3α5α6α7, LMP7(Santa Cruz, USA), Rpt6
(Enzo Life Science, USA) and of polyclonal rabbit
α1α2α3α5α6α7, LMP7(Santa Cruz, USA), Rpt6
was 30% (Figure 1). The decreased 20S proteasome
activity depression was accompanied by a low calpain
activity. The calpain activity was 1.4 times lower in
cancer tissues than in non-transformed tissues.
Proteasome and calpain activities were decreased
in kidney cancer tissues. The total proteasome and 26S
and 20S proteasome activities were found to be lower
in RCC tissues than in non-transformed tissues.
The proteasome activity is known to be associated with the
content of proteasome subunits [16, 34]. The expression
of the proteasome subunits α1α2α3α5α6α7 was
higher in cancer tissues than in non-transformed tissues
(agreed standard) (p<0.05). However, the expression
levels of LMP2 immune proteasome subunit
and PA28β proteasome activator subunit were decreased
in cancer tissues. The difference in their levels be-
tween cancer tissues and normal surrounding tissues
was 30% (Figure 1). The decreased 20S proteasome
activity was likely dependent on a low level of PA28β
subunit of the proteolytic complexes. A nonparametric
correlation was revealed between the LMP2 level and
proteasome activity (r=0.26; p=0.04). Proteasome
activity depression was accompanied by a low calpain
activity. The calpain activity was 1.4 times lower in
RCC tissues than in normal tissues. It should be noted
that proteasomes and calpains play a significant role in
cancer development, tumor progression and metastatic
spread due to their impact on transcription and growth
factor levels. Thus, changes in the protease activity in
kidney cancer tissues could be followed by the altered
expression of transcription and growth factors.

Development of metastasis leads to changes in
the protease activity and expression of transcription

Results
High expression levels of NF-κB, HIF-1α and VEGF
were found in RCC tissues. The NF-κBp50 expression
was 1.5 times higher in cancer tissues than in normal
tissues (Table 1). Our results estimated the prevalence
of active forms of NF-κB transcription factor in tumors.
The NF-κBp65 and NF-κBp50 expression levels were
5.5 (2.8-8.7) and 6.3 (3.3-10.0) RLU per mg protein in a
well, respectively. The NF-κB p65 to NF-κBp50
ratio of less than 1.0 is known to be a sign of nonactive
dimmers of NF-κB [12]. The NF-κB p65/p50 ratio of
1.1 in cancer tissues indicated NF-κB activation. In
cancer tissues, the HIF-1α and VEGF expression levels
were 4.2 and 12.45 times higher than those observed
in non-transformed tissues (4.2 (2.2-7.9)) RLU per
gmg protein and 128.0 (81.8-168.2) pg /per mg protein,
respectively. Thus, the high levels of transcription and
growth factors were revealed in RCC tissues.

Proteasome and calpain activities were decreased
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Development of metastasis leads to changes in
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КЛИНИЧЕСКИЕ ИССЛЕДОВАНИЯ

VEGF, HIF-1α and NF-κB (p65 and p50) determina-
nation. The pellets left after preparing tissue homoge-
nates were resuspended in 50 μL of 50 mM Tris-HCl
buffer (pH=7.5) containing 2 mM ATP, 5 mM MgCl2,
1 mM dithiothreitol, 1 mM EDTA, and 100 mM NaCl
and then centrifugated at 14000×g for 10 minutes at
4 °C. HIF-1α and NF-κB (p50 and p65) expression
were measured with Caymanchem ELISA kits (USA)
Kidney cancer progression and metastasis are followed by significant changes in proteolysis. Table 2 shows the activity of proteasomes and calpains in metastatic and non-metastatic tissues. Patients with T2-4N0-1M1 (metastatic cancer) had a 1.3-fold decrease in the total proteasome activity and a 1.3-fold increase in the calpain activity compared to T1-3N0-1M0 patients (non-metastatic tumors). Calpains are known to be responsible for limited protein proteolysis in cells. In the absence of proteasomes, they could damage and modify proteins and peptides [19]. I-κB, the endogenous inhibitor of NF-κB and HIF-1α transcription factors, is a substrate of proteases, and its incomplete proteolysis could lead to the accumulation of angiogenic factors in cancer cells and metastasis development [5, 19].

The development of metastatic spread was found to be accompanied by increased levels of transcription factors and VEGF expression (Table 3). The production of NF-κBp65 was 3.2 times higher in tumor tissues than in normal non-transformed tissues. So, this fact was accompanied by a 5.0-fold increase in the NF-κBp65 to NF-κBp50 ratio. The HIF-1α and VEGF expression levels were respectively 9- and 2.8 times higher in metastatic RCC tissues than in non-metastatic tissues. Thus, the progressive depression of protease activity in RCC tissues was associated with high expression levels of transcription factors and VEGF. Their accumulation in tumors led to cancer progression and metastasis.

Changes in NF-κB, HIF-1α and VEGF expression levels in primary cancer and metastatic tissues. NF-κBp50 expression in metastatic and primary RCC tissues was higher than in normal non-transformed tissues. The NF-κBp65 level was low both in metastatic and normal kidney parenchyma. And this molecular factor is reduced in 2.0 fold in comparison to RCC tissue. Interestingly, the HIF-1α content was 1.7 times higher in metastatic than in normal tissues. The VEGF expression was higher in metastatic and primary RCC tissues than in normal kidney tissues. We used the Kruskal Wallis (nonparametric ANOVA) Test and Median Test to analyze variances of multiple groups. We found significant differences in HIF-1 (p=0.0001; p=0.0001), NF-kBp50 (p=0.0002; p=0.0004), NF-kBp65 (p=0.02; p=0.01) and VEGF (p=0.0002; p=0.004) expression levels between normal, primary and metastatic RCC tissues.

Proteasome and calpain activities in primary and metastatic RCC tissues. Figure 2 shows the protease activity in distant metastatic tissues. Proteasome activity was 2.2 times lower in metastatic RCC tissues than in normal kidney tissues and was 1.5 times lower than in primary RCC tissues. No difference in calpain activity was found between metastatic tis-

Figure 1. Proteasome subunit content in RCC. A - Western Blotting analysis shows the expression of proteasome subunits in cancer and non-transformed tissues; 2, 3 – Western Blots of cancer tissues; 1, 4 - Western Blots of non-transformed tissues. B – The expression of proteasome subunit in normal non-altered tissue is indicated as 100%.

Figure 2. Total proteasome activity, 26S and 20S proteasome activity and calpain activity in kidney cancers. The results represent the Me (Q1-Q3); * in comparison with normal tissue, p<0.05. ** in comparison with cancer tissues. Proteasome depression is accompanied with fall of calpains activity. It is detected the growth in total proteasome and calpain activity in metastasis tissues compared to primary cancer tissues.
Влияние активности протеаз на экспрессию NF-κB, HIF-1α и VEGF в нормальных и почечноклеточных опухолях.

**Таблица 1**

<table>
<thead>
<tr>
<th>Препараты</th>
<th>NF-κBp65, RLU/мг белка</th>
<th>NF-κBp50, RLU/мг белка</th>
<th>HIF-1α, RLU/мг белка</th>
<th>VEGF, пг/мг белка</th>
</tr>
</thead>
<tbody>
<tr>
<td>Нормальные ткани, n=16</td>
<td>4,88 (4,66–5,21)</td>
<td>4,16 (3,76–4,66)</td>
<td>0,98 (0,62–1,18)</td>
<td>10,28 (8,46–14,3)</td>
</tr>
<tr>
<td>Рак почек, n=69</td>
<td>7,0 (4,7–15,4)*</td>
<td>7,0 (4,7–14,7)*</td>
<td>4,88 (2,56–8,6)*</td>
<td>69,3 (27,5–139,75)*</td>
</tr>
</tbody>
</table>

Примечание: результаты представлены медианой (Q1-Q3); * в сравнении с ненормальной тканью, p<0,05.

**Таблица 2**

<table>
<thead>
<tr>
<th>Препараты</th>
<th>Общая активность протеаз, -103 Уе/мг белка</th>
<th>Активность 20S протеаз, -103 Уе/мг белка</th>
<th>Активность 26S протеаз, -103 Уе/мг белка</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ненарастителенное опухолевое, n=63</td>
<td>36.1 (20.0–102.0)</td>
<td>12.5 (7.3–21.3)</td>
<td>28.0 (16.7–65.7)</td>
</tr>
<tr>
<td>Нарастителенное опухолевое, n=30</td>
<td>27.0 (5.5–43.1)*</td>
<td>10.2 (3.3–23.3)</td>
<td>30.1 (20.0–49.4)</td>
</tr>
</tbody>
</table>

Примечание: результаты представлены медианой (Q1-Q3); * в сравнении с ненарастителенном опухолевым, p<0,05.

**Таблица 3**

<table>
<thead>
<tr>
<th>Препараты</th>
<th>NF-κBp65, RLU/мг белка</th>
<th>NF-κBp50, RLU/мг белка</th>
<th>Коэффициент NF-κBp65/50</th>
<th>HIF-1α, RLU/мг белка</th>
<th>VEGF, пг/мг белка</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ненарастителенное опухолевое, n=45</td>
<td>5,0 (2.0–7.9)</td>
<td>6,4 (3.3–10.0)</td>
<td>0,6 (0,3–2,0)</td>
<td>4,2 (2.2–7.8)</td>
<td>71,5 (38,3–139,7)</td>
</tr>
<tr>
<td>Нарастителенное опухолевое, n=30</td>
<td>11,3 (6.8–15,7)*</td>
<td>6,2 (4,4–9.7)</td>
<td>3,0 (1,6–3,0)*</td>
<td>7,9 (6,2–8,5)*</td>
<td>205,4 (131,7–261,5)*</td>
</tr>
</tbody>
</table>

Примечание: результаты представлены медианой (Q1–Q3); * в сравнении с ненарастителенном опухолевым, p<0,05.

**Обсуждение**

Баланс между активностью протеаз и экспрессией антигенных факторов важен для развития метастазов. Набор корреляций между транскрипционными факторами и VEGF в нормальных и раковых тканях почек был обнаружен. Положительные корреляции между активностью всех протеазных пул в опухоли и HIF-1α экспрессией были обнаружены. Положительная корреляция была обнаружена между активностью всей протеазной и 26S протеазной активностью (r=0.56; p=0.0001) и между HIF-1α и VEGF экспрессией (r=0.8; p=0.001). Эффект NF-κB на ангиогенез фактор роста был вероятно опосредован через HIF-1 экспрессию.

Различия между экспрессией HIF-1α и VEGF в раковых тканях почек были также обнаружены в опухолевых тканях (r=0.97; p=0.0001).

**Обсуждение**

Протеолитический регулятор транскрипции и роста факторов является важным влиянием в развитии рака в почках. В РК, HIF-1α уровень был связан с низкой 26S протеазной активностью (r=-0,36; p=0,04).
The loss of von Hippel-Lindau gene was followed by HIF-1α overexpression through blockage of its proteasome degradation [26, 27]. The intensive angiogenesis is the result of this process. The NF-κBp50 level was correlated with 20S proteasome activity. The post-translational modification of p105 was performed by proteasome [28]. The nonparametric nonlinear regression analysis was carried out to study the impact of proteases on transcription factor expression. It was found that the induced 20S proteasome activity (Beta=0.93; p<0.05) and depressed 26S proteasome activity (Beta=-0.58; p<0.05) led to the increased NF-κBp50 level in cancer cells.

There are multiple crosstalks between the expressions of transcription and growth factors in RCC. Proteolysis is considered the most significant. The 26S proteasome plays the main role in HIF-1α degradation, and 20S proteasome participates in NF-κBp50 production.

Low proteasome and calpain activities are specific to RCC. The increase in the protease activity is followed by RCC metastasis development. The blockage of HIF-1α degradation and the increased level of VEGF result in non-effective proteolysis. In RCC culture, P. van Uden [13] revealed the influence of NF-κB on HIF-1α transcription. Our findings highlight the potential contribution of intracellular proteases to kidney cancer metastasis. The increased NF-κBp65 expression is likely can influence on HIF-1 production in metastatic RCC and can be the main factor of cancer progression. There are reports on the association between the NF-κB activity and VEGF mRNA expression in breast cancer cells [35]. Our results are consistent with other recent studies, which indicate that the VEGF level is positively correlated with NF-κB p65 expression in cancer tissues.

Conclusion

Our results showed the relationship between the proteasome/calpain activity and transcription factor expression in primary and metastatic RCC. The hypothetic schema of proteolytic regulation of transcription factors and VEGF expression in RCC was obtained. The depression of cellular proteolytic systems and increase in the levels of transcription and growth factors were found in primary cancer tissues, resulting in metastasis development. High levels of NF-κB, HIF-1α and VEGF were found in primary metastatic tumors. A significant difference in cancer cells was found between primary and metastatic tissues. In distant metastatic tissues, proteolysis activation was accompanied by high levels of transcription and growth factors. The altered biological features were shown in metastatic tissues. The increased protease activity in metastatic tissues was followed by high NF-κBp50, HIF-1α and VEGF levels. Thus, proteasomes and calpains play a significant role in metastasis development. A decreased protease activity is necessary for the aggressive behavior of cancer cells and kidney cancer dissemination. Induced proteolysis in metastatic tissues is required for RCC progression.

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Conflict of interest
The authors declare that they have no conflict of interest.

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