DOI: 10.21294/1814-4861-2018-17-4-67-74
УДК: 616.61-006.6:612.015.1:577.2

Для цитирования: *Спирина Л.В., Кондакова И.В., Усынин Е.А., Слонимская Е.М., Юрмазов З.А.* Влияние транскрипционных факторов, VEGF и протеиназ на прогрессирование рака почки. Сибирский онкологический журнал. 2018; 17 (4): 67–74. – doi: 10.21294/1814-4861-2018-17-4-67-74.
For citation: *Spirina L.V., Kondakova I.V., Usynin E.A., Slonimskaya E.M., Yurmazov Z.A.* Impact of transcription factors, VEGF and proteases on kidney cancer progression. Siberian Journal of Oncology. 2018; 17 (4): 67–74. – doi: 10.21294/1814-4861-2018-17-4-67-74.

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 содержанием 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 флюорогенного 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. **Выводы.** 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

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substrates. **Results.** We revealed inactivation of proteolysis in patients with kidney cancer. Disease advance was associated with a significant depression of cellular proteolysis and increase in transcription and growth factor levels in primary kidney cancer tissues. The proteolysis activation was found in metastatic tissues. **Conclusions.** Our results suggest that NF-κB, HIF-1α and VEGF transcription factors and intracellular proteolytic systems are involved in kidney cancer progression.

Keywords: renal cell carcinoma, metastasis, NF-κB, HIF-1α, VEGF.

Introduction

Kidney cancer is among the cancers that have the highest growth rate and it is known as the most deadly urinary tract caner [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].

NF-κB is the key transcription factor that controls the transcription of 150 target genes [7]. The processing of p105 and p100 (NF-κB precursors) is mediated by the proteasome pathway [8]. The active form of the NF-κB transcription factor is p65/p50 heterodimers [9–12]. It is known that NF-κB can regulate the HIF-1α level [13].

Proteolysis is one of the mechanisms for regulation of NF-κB and HIF-1α transcription factors, and the proteasome and calpain systems are the most possible factors to be involved. Proteasome is the multicatalytic complex that consists of the catalytic core (20S) with one or two regulatory particles attached to it [14]. Both the 20S and 26S proteasomes may be divided into two large groups of immune and constitutive forms that consist of either constitutive (β1β2β3β4β5β6β7) or immune (LMP2, LMP7, MECL-1) subunits, respectively [15–19]. Calpains belong to the calcium-dependent proteases [20]. The calpain activity is implicated in several fundamental physiological processes, including cytoskeletal remodelling, cellular signalling, apoptosis, and cell survival [21–25].

A decrease in HIF-1α degradation in the presence of proteasome inhibitors or under hypoxia leads to a significant increase in both VEGF and its mRNA expression in tumor cells [26, 27]. Moreover, calpains were shown to be involved in HIF-1α destruction [5]. Activation of NF-κB is effected by proteasomes. The key moment of NF-κB activation belongs to breaking the ties between the transcription factor and repressor protein known as I-κB [8, 11]. Currently, even greater significance is attached to the investigation on the participation of calpains in I-κB destruction [28]. The additional mechanism in NF-κB regulation serves the NF-κB protein forming from precursors, which are mediated by proteasomes through the modification of p105 protein [29].

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 $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, and 100 mM NaCl. The homogenate was centrifuged at 10000×g for 60 minutes at 4°C.

Proteasome fractiation. 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-

threitol, and 30 μM Suc-LLVY-AMC. The 26S proteasome activity solution additionally contained 5 mM $MgCl_2$ and 1 mM ATP. The reaction was carried out for 20 minutes at 37°C and then was stopped by the addition of 1 % sodium dodecyl sulfate. We used the proteasome inhibitor MG-132 to estimate the influence of other proteases.

Calpains activity assay. The calpains activity was performed 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 activity solution contained 100 мМ Tris-HCl (pH=7.3), 145 мМ NaCl and 30 μM Suc-LLVY-AMC. Incubations were performed at room temperature for 30 minutes in absence or presence of 10 mM $CaCl_2$ 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 buffer (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-/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 according to the method described in the manual accompanying the unit. Before incubation with antibodies the PVDF membrane was incubated in 10 mM Tris-HC1 buffer (pH 7.5), containing 150 mM NaC1 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 anti-human LMP2, PA28β (Santa Cruz, USA) at 20 ºC for 1 h, followed by three consecutive washes in the 10 mM Tris-HC1 buffer (pH 7.5), containing 150 mM NaCI. PVDF samples were incubated in Amersham ECL western blotting detection analysis system according to the method described in the manual accompanying the unit and then were exposed to ECL-films (Amersham, USA). The results were standardized using the beta-actin expression in a sample and were expressed in percentages to the proteasome subunits content in non-transformed tissues. The expression of proteasome subunit in normal non-altered tissue was indicated as 100 %.

VEGF, HIF-1α and NF-κB (p65 and p50) determination. The pellets left after preparing tissue homogenates were resuspended in 50 μL of 50 mM Tris-HCl buffer (pH=7.5) containing 2 mM ATP, 5 mM $MgCl_2$, 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) in Anthos 2020 ELISA-microplate reader (Biochrom, UK). Nuclear extracts were prepared and purified according to manufacturer's instructions.

Statistical analysis. Statistical analysis was performed using SPSS 19.0 software. Data were expressed as median (interquartile ranges). To evaluate the difference, either the Student t test or the Mann-Whitney test was applied. Correlation analysis on data was carried out with Spearman Rank Correlation test. Kruskal-Wallis ANOVA and median Test were used for comparing two or more independent groups.

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-kB [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 mg 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 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 between 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

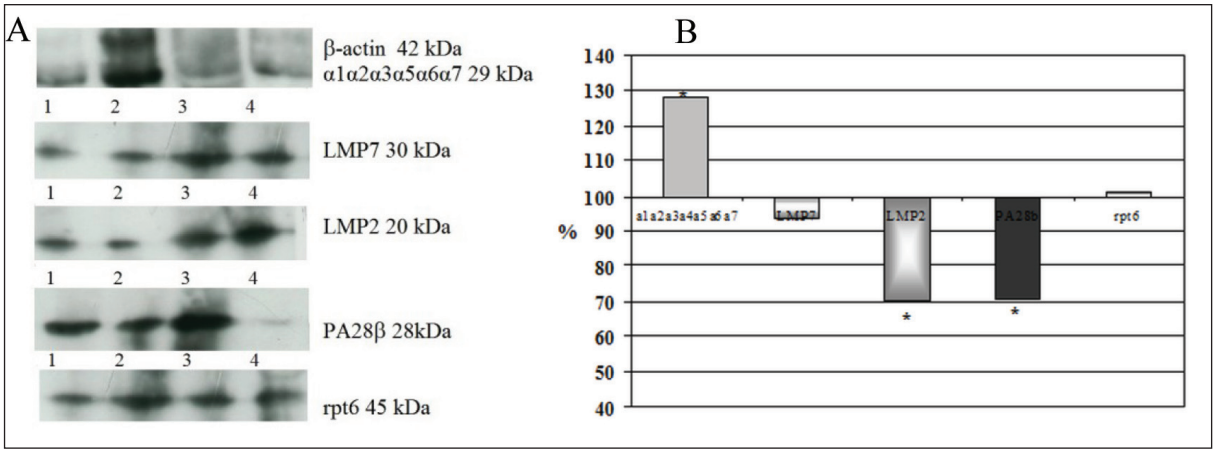

Figure 1. Proteasome subunit content in RCC.
A - Western Blotting analysis shows the expression of proteasome subunits in cancer and non-transformed tissues:
2, 3 – Western Blots of cancer tissues; 1, 4 - Western Blots of non-transformed tissues.
B – The expression of proteasome subunit in normal non-altered tissue is indicated as 100%.
* - in comparison with normal tissue, p<0.05; the results are standardized using the β-actin expression in a sample and are expressed in percentages to the proteasome subunits content in non-transformed tissues

and growth factors in primary cancer tissues. 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 in metastatic than in primary RCC tissues. No difference in calpain activity was found between metastatic tis-

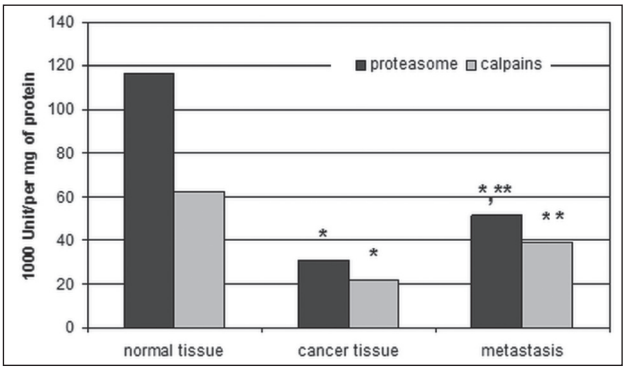

Figure 2. Total proteasome activity, 26S and 20S proteasome activity and calpain activity in kidney cancers. The results represent the Me (Q1-Q3); * in comparison with normal tissue, p<0.05. ** in comparison with cancer tissues. Proteasome depression is accompanied with fall of calpains activity. It is detected the growth in total proteasome and calpain activity in metastasis tissues compared to primary cancer tissues

Table 1

NF-κBp65, NF-κBp50, HIF-1α and VEGF expressions in normal and kidney cancer tissues

Samples	NF-κBp65 expression, RLU /per mg of protein in well	NF-κBp50 expression, RLU /per mg of protein in well	HIF-1α expression, RLU /per mg of protein in well	VEGF expression, pg/mg of protein
Non-transformed normal tissue, n=16	4,88 (4,66–5,21)	4,16 (3,76–4,66)	0,98 (0,62–1,18)	10,28 (8,46–14,3)
Kidney cancer tissue, n=69	7,0 (4,7–15,4)*	7,0 (4,7–14,7)*	4,88 (2,56–8,6)*	69,3 (27,5–139,75)*

Note: the results represent the Me (Q1-Q3); * in comparison with normal tissue, p<0,05.

Table 2

Total proteasome activity, 26S and 20S proteasome activities and calpain activity in metastatic and non-metastatic RCC

Samples	Proteasome activity, ·103 Unit/per mg of protein			Calpains activity, ·103 Unit/per mg of protein
	Total proteasome activity	26S proteasome activity	20S proteasome activity	
Non-metastatic RCC tissue, n=63	36.1 (20.0–102.0)	12.5 (7.3–21.3)	28.0 (16.7–65.7)	33.5 (16.8–33.5)
Metastatic RCC tissue, n=30	27.0 (5.5–43.1)*	10.2 (3.3–23.3)	30.1 (20.0–49.4)	44.7 (18.6–54.3)*

Note: the results represent Me (Q1-Q3); * – in comparison to non-metastatic RCC, p<0,05.

Table 3

NF-κBp65, NF-κBp50, HIF-1α and VEGF expressions in metastatic and non-metastatic RCC

Samples	NF-κBp65 expression, RLU / per mg of protein in well	NF-κBp50 expression, RLU / per mg of protein in well	Coefficient NF-κBp65/ p50	HIF-1α expression, RLU /per mg of protein in well	VEGF expression, pg/mg of protein
Non-metastatic RCC, n=45	5.0 (2.0–7.9)	6.4 (3.3–10.0)	0.6 (0.3–2.0)	4.2 (2.2–7.8)	71.5 (38.3–139.7)
Metastatic RCC, n=30	11.3 (6.8–15.7)*	6.2 (4.4–9.7)	3.0 (1.6–3.0)*	7.9 (6.2–8.5)*	205.4 (131.7–261.5)*

Note: the results represent the Me (Q1–Q3); * in comparison with non-metastatic RCC tissue, p<0,05.

sues and normal kidney tissues. However, the calpain activity was 1.75 times higher in metastatic RCC tissues than in primary kidney cancer tissues. The results of non-parametric ANOVA analysis (Kruskal-Wallis test and Median test) showed significant changes in the total proteasome (p=0.0001; p=0.0001) and calpain (p=0.03; p=0.06) activities in primary non-metastatic and metastatic tissues. Despite the fact that the contribution of the cellular protease system to tumorigenesis remains unclear, we decided to evaluate the NF-κB, HIF-1α and VEGF expression levels in primary and metastatic kidney cancer tissues. The recent study have documented that the increase of proteasome and calpains activity is required for sufficient carcinogenesis [20]. Thus, the protease activation in metastatic tissues is accompanied with increased NF-κBp50, HIF-1α and VEGF expression levels. Metastatic cancer cells are known to have the altered biological features. In our study we found the correlation between the aggressive behavior of cancer cells and proteolysis activity.

Correlations between the proteasome activity, transcription factors and VEGF expression were found in RCC tissues. To further examine the potential importance of proteases in RCC metastasis development, we analyzed the correlations between them and transcription and growth factor levels. Positive relationships between the activities of all proteasome pools in tumors were found. We observed statistically significant correlations between the 20S and 26S proteasome activities (r=0.60; p<0.05), total proteasome and 20S activities (r=0.80; p<0.05), and between the total and 26S proteasome activities (r=0.78; p<0.05). A positive correlation was found between the total proteasome activity, 26S proteasome activity and calpain activity (r1=0.56, p=0.00001; r2=0.57, p=0.00002; r3=0.63, p=0.00001, respectively). Goldberg A.L. [8] reported that the HIF-1α expression level was influenced by NF-kB transcription factors. The analysis of our data showed the relationships between NF-κB p65 and HIF-1α expression levels (r=0.9; p=0.0001) and between VEGF and HIF-1α expression levels (r=0.8; p=0.001). The effect of NF-κB on angiogenic growth factor was likely to be mediated through HIF-1 expression changes. The association between HIF-1α and VEGF expression was also detected in cancer tissues (r=0.97; p=0.0001).

Discussion

The proteolytic regulation of transcription and growth factors is of great importance in kidney cancer progression. In RCC, HIF-1α level was associated with low 26S proteasome activity (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 miltiple crosstalks between the expression 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 m RNA 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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Received 28.05.18
Accepted 27.06.18

Funding
This study required no funding.

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

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Финансирование
Авторы данной статьи подтвердили отсутствие финансовой поддержки.
Конфликт интересов
Авторы объявляют, что у них нет конфликта интересов.

Поступила 28.05.18
Принята в печать 27.06.18