АНГИОГЕННЫЙ ПОТЕНЦИАЛ ЦИРКУЛИРУЮЩИХ НЕЙТРОФИЛОВ ПЕРИФЕРИЧЕСКОЙ КРОВИ ПРИ РАКЕ ПОЧКИ

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Резюме. В настоящее время актуальным является изучение роли нейтрофилов при раке почки. Их роль в канцерогенезе неоднозначна. Являясь одним из наиболее распространенных лейкоцитов крови, нейтрофилы играют важную роль в прогрессировании рака посредством множества механизмов, включая стимулирование ангиогенеза, иммуносупрессии и метастазирования рака. Нейтрофилы синтезируют и высвобождают проангиогенные факторы, которые способны прямо или косвенно стимулировать рост и миграцию эндотелиальных клеток, что, в свою очередь, вызывает образование новых кровеносных сосудов из ранее существовавших. Продукция нейтрофилами различных факторов, в том числе и проангиогенных, опосредована экспрессией генов данных молекул. Функциональная гетерогенность характеризуется различиями в паттернах экспрессии генов нейтрофилов. Целью данного исследования была оценка ангиогенного потенциала циркулирующих нейтрофилов при раке почки. Объектом исследования явились нейтрофилы крови пациентов с верифицированным раком почки светлоклеточного типа на I стадии (T1N0M0G1, n = 28, медиана возраста 60), II стадии (T2N0M0G2, n = 15, медиана возраста 61) и III стадии (T3N0M0G2, n = 15, медиана возраста 63) до хирургического лечения. Группу контроля составляли условно здоровые доноры (n = 15, медиана возраста 54). Методом иммуноферментного анализа оценивались уровни IL-8 и VEGF-A в сыворотке крови. Экспрессия генов CXCL8 и VEGF-A в циркулирующих нейтрофилах была определена методом количественной ПЦР с обратной транскрипцией. В результате проведенного нами исследования выявлено повышение уровня IL-8 и VEGF-A в сыворотке крови пациентов с раком почки во всех исследуемых группах по сравнению с группой контроля. Мы наблюдали прямую корреляционную связь между уровнем IL-8 и VEGF-A в сыворотке у пациентов с раком почки (r = 0,429; p = 0,016), которая подтверждает взаимосвязь данных ангиогенных факторов. Было установлено значимое повышение экспрессии гена CXCL8 циркулирующими нейтрофилами у пациентов на II (2,91, $Q_{0,25}$-$Q_{0,75}$: (1,296-4,99), p = 0,02) и III (1,93, $Q_{0,25}$-$Q_{0,75}$: (0,755-11,36, p = 0,014) стадии рака почки по сравнению с контрольной группой (1,50, $Q_{0,25}$-$Q_{0,75}$: (0,80-4,05)), однако экспрессия гена VEGF-A циркулирующими нейтрофилами не отличалась от аналогичных показателей в группе контроля. На основании полу-

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ченных результатов можно предположить, что циркулирующие в крови нейтрофилы при раке почки осуществляют свой ангиогенный потенциал через продукцию IL-8.

Ключевые слова: нейтрофилы, ангиогенез, VEGF-A, IL-8, фенотип нейтрофилов, рак почки

ANGIOGENIC POTENTIAL OF CIRCULATING PERIPHERAL BLOOD NEUTROPHILS IN KIDNEY CANCER

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Abstract. The role of neutrophils in kidney cancer is currently being studied. Their role in carcinogenesis is ambiguous. As one of the most abundant blood leukocytes, neutrophils play an important role in cancer progression through multiple mechanisms, including promotion of angiogenesis, immunosuppression, and cancer metastasis. Neutrophils synthesize and release pro-angiogenic factors that are able to directly or indirectly stimulate the growth and migration of endothelial cells, which in turn causes the formation of new blood vessels from pre-existing ones. The production of various factors by neutrophils, including proangiogenic ones, is mediated by the expression of the genes of these molecules. Functional heterogeneity is characterized by differences in neutrophil gene expression patterns. The aim of this study was to evaluate the angiogenic potential of circulating neutrophils in kidney cancer. The object of the study were blood neutrophils of patients with verified clear cell kidney cancer at stage I (T1N0M0G1, n = 28, median age 60), stage II (T2N0M0G2, n = 15, median age 61) and stage III (T3N0M0G2, n = 15, median age 63) before surgery. The control group consisted of apparently healthy donors (n = 15, median age 54). Serum levels of IL-8 and VEGF-A were assessed by enzyme immunoassay. Expression of the CXCL8 and VEGF-A genes in circulating neutrophils was determined by reverse transcription quantitative PCR. As a result of our study, an increase in the level of IL-8 and VEGF-A in the blood serum of patients with kidney cancer in all studied groups compared with the control group was revealed. We observed a direct correlation between serum levels of IL-8 and VEGF-A in patients with kidney cancer (r = 0.429; p = 0.016), which confirms the relationship of these angiogenic factors. A significant increase in CXCL8 gene expression by circulating neutrophils was found in patients on II (2.91, $Q_{0.25}$-$Q_{0.75}$: (1.296-4.99), p = 0.02) and III (1.93, $Q_{0.25}$-$Q_{0.75}$: (0.755-11.36, p = 0.014) stages of kidney cancer compared with the control group (1.50, $Q_{0.25}$-$Q_{0.75}$: (0.80-4.05)). However, VEGF-A gene expression by circulating neutrophils did not differ from those in the control group. Blood neutrophils in kidney cancer exercise their angiogenic potential through the production of IL-8.

Keywords: neutrophils, angiogenesis, VEGF-A, IL-8, neutrophil phenotype, kidney cancer

Introduction

The most common type of kidney cancer (KC) is clear cell (about 70% of cases). KC is considered to be an immunogenic tumor but is known to mediate immune dysfunction to a large extent by causing the entry of immunoinhibitory cells, such as regulatory T cells and myeloid suppressor cells, into the tumor microenvironment [4]. The microenvironment of a tumor can contribute to its development from initiation to metastasis [7]. Neutrophils (Nph) are the most common population of granulocytes in human blood and, as a rule, make up a significant proportion of tumor-infiltrating immune cells in KC [3, 13]. In response to chemokine signals, neutrophils quickly migrate from the bloodstream to the focus of inflammation [11]. These tumor-associated Nph (TANs) are the main effector cells of the tumor microenvironment [9]. Nph can support tumor growth through various mechanisms, including suppression of T cell activation, stimulation of genetic instability, tumor cell proliferation, angiogenesis, and metastasis [8].

Nph are known to play an important role in stimulating tumor angiogenesis through the production of pro-angiogenic factors, including MMP-9, Bv8, vascular endothelial growth factor A (VEGF-A), and chemokines [3]. The proangiogenic activity of Nph is crucial in the early stages of tumor progression, since Nph-produced MMP-9 triggers angiogenesis, facilitating the mobilization of VEGF-A and subsequent binding to VEGFR2 [3].

In addition to its pro-inflammatory function, IL-8 enhances the proliferation, survival, and migration of endothelial cells, thereby activating and maintaining the development of angiogenesis [2]. IL-8 and its receptors are widely expressed by both tumor cells and a variety of non-malignant cells present in the tumor microenvironment, including tumor-associated macrophages, Nph, and endothelial cells [2, 6]. It was found that elevated serum levels of IL-8 in KC were associated with the prevalence of the process and worse overall survival [12]. The production of various factors by neutrophils, including proangiogenic ones, is mediated by the expression of the genes of these molecules. The functional heterogeneity of neutrophils is characterized by differences in the patterns of neutrophil gene expression [10].

The aim of the study was to assess the angiogenic potential of circulating Nph in KC.

Materials and methods

The object of the study was blood Nph of patients with verified KC, clear cell type I stage (T1N0M0G1, n = 28, median age 60), II stage (T2N0M0G2, n = 15, median age 61) and III stage (T3N0M0G2, n = 15, median age 63) before surgical treatment. The control group consisted of apparently healthy donors (n = 15, median age 54). Informed consent to participate in the study was obtained from all patients. The study was approved by the Ethics Committee of the Institute of Medicine, Ecology and Physical Culture of Ulyanovsk State University (protocol No. 1 dated January 15, 2020).

Nph was isolated from leukocyte suspension on a double density gradient of sterile ficoll-verografin solutions. The isolated Nph were washed from the gradient three times with sterile saline sodium chloride solution and adjusted to a concentration of 5×10^6 cells/ml. The purity of the Nph fraction was 92–94%. The viability of Nph in the test with 0.5% trypan blue was 95%. From the general blood test, the following were calculated: the absolute number of leukocytes in patients with KC, median 6.36 ($Q_{0.25}$-$Q_{0.75}$: 5.00-7.18), neutrophils, median 3.072 ($Q_{0.25}$-$Q_{0.75}$: 0-4.14). RNA was isolated from the peripheral blood Nph fraction using SileksMagNA magnetic particles (LLC Sileks, Moscow, Russia) at a KingFisher automated nucleic acid isolation station. After isolation, the reverse transcription reaction was set up. Expression of CXCL8 and VEGF-A genes was determined by quantitative PCR with reverse transcription using primers (CJSC Evrogen, Moscow, Russia). The level of IL-8 and VEGF-A in serum was determined using ELISA (CJSC Vector-Best-Volga, Russia). Sets of quantitative indicators, the distribution of which differed from normal, were described using the values of the median (Me) and the lower and upper quartiles ($Q_{0.25}$-$Q_{0.50}$). The statistical significance of differences was assessed using the Mann–Whitney U test. In order to study the relationship between quantitative variables, the calculation of the Spearman correlation coefficient and the linear regression model were used. Statistical processing was performed using Statistical 13.

Results and discussion

As a result of our study, an increase in the level of IL-8 in the blood serum of patients with KC in all the studied groups compared with the control group was revealed (Table 1). High concentrations of IL-8 have also been found by Wu L. et al. (2021) in serum and tissue samples from patients with various types of cancer and have been shown to correlate with tumor progression and worse overall survival. High levels of IL-8 in the blood promote the migration of Nph into the tumor, and blocking the signaling of this chemokine, according to Schimek V. et al. (2022), suppresses this process. Accordingly, an increase in the level of this chemokine in patients with KC can lead to an increase in Nph chemotaxis into the tumor.

We also found an increase in the serum level of VEGF-A at all stages of KC relative to the control group (Table 1), which is consistent with previous studies [5]. In addition, the studies of Apte R.S. et al. (2019) found that a high serum level of VEGF-A correlates with invasiveness, vascular density, me-

TABLE 1. SERUM LEVELS OF IL-8 AND VEGF-A AT VARIOUS STAGES OF KIDNEY CANCER, Me ($Q_{0.25}$-$Q_{0.75}$)

Group / Indicator	Control n = 22	Stage I n = 28	Stage II n = 15	Stage III n = 15
IL-8, pg/mL	11.04 (10.30-14.99)	94.26 (36.07-387.50) p = 0.00001	409.08 (29.23-422.15) p = 0. 00023	96.37 (61.83-275.22) p = 0.0001
VEGF-A, pg/mL	136.34 (91.01-168.95)	372.99 (282.99-545.82) p = 0.0001	337.52 (329.33-692.18) p = 0.00047	227.34 (227.34-399.49) p = 0.0017

Note. Statistical significance p was calculated relative to the control group; the values were considered statistically significant at $p \le 0.05$.

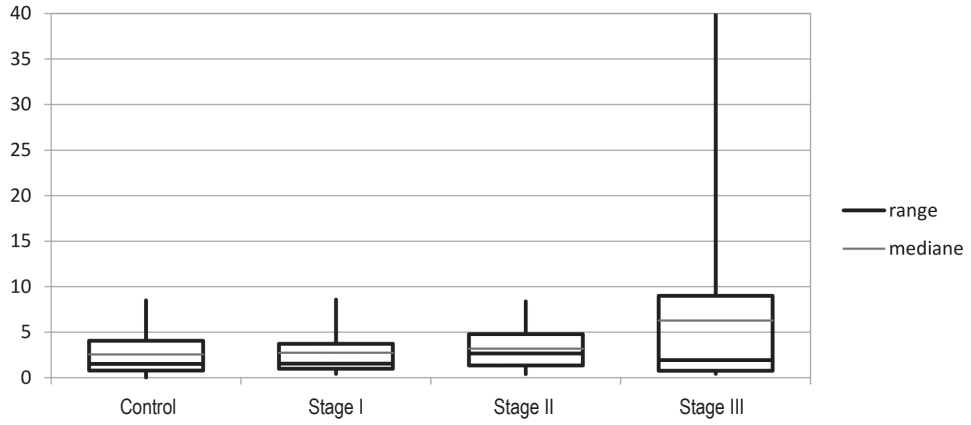

Figure 1. CXCL8 gene expression in circulating neutrophils

tastasis, and relapses in KC and can serve as a biomarker for the development of angiogenesis.

We observed a direct correlation between the level of IL-8 and VEGF-A in serum in patients with KC ($r = 0.429$; $p = 0.016$), which confirms the relationship of these angiogenic factors. It is known that IL-8 promotes a proinflammatory state, stimulates angiogenesis and is a powerful chemoattractant for Nph [7, 14], and also activates pro-angiogenic pathways, which is confirmed by previous studies by Zhou N.A.N. et al. (2016).

The phenotype of neutrophils is characterized by gene expression in them [10]. We found a significant increase in CXCL8 gene expression by circulating neutrophils in patients on II (2.91, $Q_{0.25}$-$Q_{0.75}$: (1.296-4.99), $p = 0.02$) and III (1.93, $Q_{0.25}$-$Q_{0.75}$: (0.755- 11.36, $p = 0.014$) of the KC stage compared with the control group (1.50, $Q_{0.25}$-$Q_{0.75}$: (0.80-4.05)) (Figure 1), which may indicate an increase in the ability to produce IL-8 Nph in the studied groups.

From previous studies by Apte R.S. et al. (2019) it is known that Nph synthesize VEGF-A, which is stored in them and released during inflammatory angiogenesis. VEGF-A is one of the factors that determine protumor (N2) neutrophil polarization [7]. In studies by Amorim C. et al. (2022) also demonstrated that a high level of VEGF-A released by neutrophils is characteristic of the N2 phenotype. As a result of our analysis, the expression of the VEGF-A gene by circulating Nph in KC did not differ from similar indicators in the control group (Figure 2). Expression of the VEGF-A gene (Me = 1.47, $Q_{0.25}$-$Q_{0.75}$: (0.645-3.96)) by circulating Nph in patients with KC was significantly lower than the expression of the CXCL8 gene (Me = 2.34, $Q_{0.25}$-$Q_{0.75}$: (0.904-8.32), $p = 0.0023$). Analysis of the linear regression model of the VEGF-A gene expression Nph and the serum

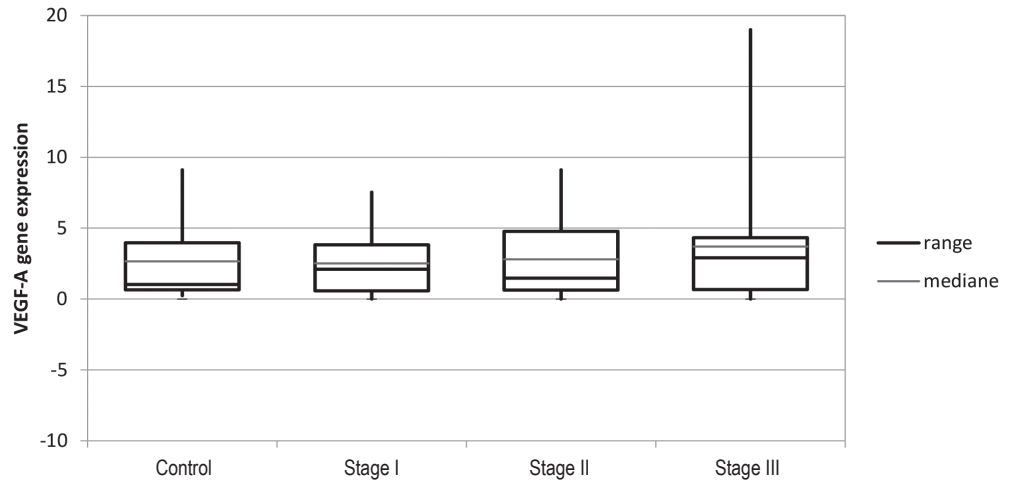

Figure 2. VEGF-A gene expression in circulating neutrophils

level of IL-8 in the group of patients at stage II showed an inverse relationship between these indicators ($R^2 = 0.8$, $p = 0.0054$).

Conclusion

Thus, our results suggest that Nph circulating in the blood during KC exercise their angiogenic potential through the production of IL-8, and are not the main producers of serum VEGF-A. An elevated serum level of IL-8 probably contributes to the formation of a protumor phenotype of circulating Nph. It can be assumed that an increase in the level of this chemokine in the future will lead to a change in the expression of proangiogenic factor genes in neutrophils and their protumor polarization in the process of recruitment to the tumor.

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