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ОРИГИНАЛЬНОЕ ИССЛЕДОВАНИЕ
ORIGINAL RESEARCH

Killing potential of circulating neutrophils in renal tumors

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Abstract. Relevance. Currently, the study of the role of neutrophils in the development of renal cancer is of considerable interest. The study of the immunopathogenesis of renal cancer is determined by the need to use combined treatment with immunotherapy. It is known that neutrophils have both pro- and antitumor properties, which are associated with the level of surface receptors CD11b, CD16, CD63, CD66b and the killing activity of neutrophils. *The aim* of the study was to assess the killing potential of circulating neutrophils in renal tumors. *Materials and Methods.* The object of the study was circulating neutrophils of patients with verified renal cancer ($n = 74$), patients with renal benign neoplasms ($n = 18$) and conditionally healthy donors ($n = 22$). The study of the phenotype of the isolated neutrophils was carried out by flow cytometry. Neutrophil extracellular traps were counted using the method by I.I. Dolgushin. *Results and Discussion.* Analysis of the percentage of neutrophil extracellular traps showed an increase in their number in the groups of patients with renal cancer, both stages I–II and III–IV, relative to the control group and the group of patients with renal benign neoplasms. An increase in the neutrophil trap index was found in the groups of patients with renal cancer stages I–II and III–IV relative to the control group and the group of patients with renal benign neoplasms. When assessing the phagocytic activity and the phagocytic activity index, a significant increase in these indicators was found in the groups of patients with renal cancer relative to the control group and the group of patients with renal benign neoplasms. A correlation was found between the percentage of neutrophil extracellular traps ($r = 0.438$, $p = 0.001$), the phagocytic activity ($r = 0.431$, $p = 0.001$) and the phagocytic activity index ($r = 0.507$, $p = 0.001$) of neutrophils and the stage of renal cancer. A significant increase in the percentage of neutrophils expressing CD66b receptors was found both at the initial and widespread stages of renal cancer relative to the group with renal benign neoplasms and the control group. Multivariate Cox regression revealed an increase in the risk of renal cancer with an increase in CD66b expression, the neutrophil extracellular traps index, the phagocytic activity and the phagocytic activity index of circulating neutrophils ($R^2 = 0.728$, $\chi^2 = 58.1$, $p = 0.001$). For differential diagnostics between renal benign neoplasms and renal cancer, the percentage of CD66b+ neutrophils, the neutrophil extracellular traps index, the phagocytic activity of neutrophils and the phagocytic activity index of neutrophils demonstrated statistical

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significance together. The area under the curve (AUC) of the model was 0.983, and could be diagnosed with a probability of 94.3% (Spec. = 0.889, Sens. = 0.962). **Conclusion.** Thus, an increase in CD66b+ neutrophils and activation of extracellular trap release indicate an increase in the killing activity of neutrophils in renal cancer. Simultaneous determination of the amount of CD66b+ neutrophils, the index of neutrophil extracellular traps, phagocytic activity and the index of phagocytic activity can be used for differential diagnosis between the renal benign neoplasms and renal cancer.

Keywords: neutrophils, renal cancer, CD11b, CD16, CD66b, CD63, neutrophil extracellular traps

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Introduction

Clear cell renal cell carcinoma (RC) is the most common type of renal cancer (about 70% of cases). The leading role in its treatment belongs to the surgical method. However, already at the time of diagnosis, a significant proportion of patients are diagnosed with disseminated RC, which determines the need for combined treatment with immunotherapy [1]. Currently, the number of studies aimed at studying the role of neutrophils (Nph) in RC is growing [2, 3]. The ability of Nph to exhibit both pro- and antitumor properties has been shown [4].

The level of expression of surface receptors determines the functional activity of Nph. Thus, CD11b, CD16, CD63 and CD66b allow identifying mature

and activated Nph [5]. Thus, CD11b mediates chemotaxis of Nph to the site of inflammation, adhesion, phagocytosis, respiratory burst and degranulation [6]. CD16 (FcγRIII) is a marker of Nph capable of cytotoxic action, degranulation, oxygen burst and proliferation [6]. CD66b receptors are expressed by mature, activated and degranulating Nph [7]. CD11b+CD16+ is a subpopulation of mature Nph capable of phagocytosis and degranulation [8]. The receptors of maturity, activation, degranulation and cytotoxicity CD16, CD63, CD66b determine the phenotype of neutrophils, which have a high capacity to form extracellular neutrophil traps (NETs) [9].

It is known that NETs play an important role in the occurrence and development of pathological processes

[10,11]. While the positive effects of NETs in the fight against pathogens have already been largely described, their negative role has also become known, including in carcinogenesis. NETs can have potential antitumor effects due to the direct destruction of cancer cells or activation of the immune system, releasing a number of cytotoxic substances that destroy tumor cells, block tumor growth and the formation of metastases [12, 13]. However, many studies indicate that NETs, ensuring the spread of tumor cells to organs and tissues, are capable of exerting a protumorigenic effect and play an important role in the progression and metastasis of tumors, including in RC [14]. The phenotype of Nph is associated with the tumor process and stage, and can determine the growth and progression of RC [9]. However, this question has not been fully studied.

The aim of this work was to evaluate the killing potential of circulating Nph in renal tumors.

Materials and methods

The object of the study were circulating Nph of patients with verified RC (Table 1), conditionally healthy donors (control group) and patients with renal benign neoplasms (RBN) (oncocytoma, angioliipoma, renal cyst) (Table 1). Nph were isolated from leukocyte suspension on a double density gradient of Ficoll-Verografin solutions. The isolated Nph were brought 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%. The phenotype of the isolated Nph was studied by flow cytometry (BioSino, China) using monoclonal antibodies (Sony Biotechnology, USA) labeled with FITC (fluorescein isothiocyanate), PE (phycoerythrin), PC5 (phycoerythrin-cyanin 5): CD11b, CD16, CD63, CD66b, CD95. NETs were counted according to the method of Dolgushin I.I. et al. (2010). The number traps (NT,%) of neutrophil was determined — the number of neutrophil traps containing yeast activator cells from 100 counted network-like structures and the neutrophil trap index (TI, c.u.) — the number of yeast activators in 100 counted traps per 1 structure, phagocytic activity (PA,%) and phagocytic index (PI, c.u.).

Statistical analysis

The sets of quantitative indicators, the distribution of which differed from the normal one, were described using the median (Me) values and the lower and upper quartiles ($Q_1 - Q_3$). The statistical significance of the differences was assessed using the Mann-Whitney U-test. To study the relationship between quantitative variables, the Spearman correlation coefficient was calculated. To assess the association of the studied indicators and the degree of differentiation, sensitivity and specificity were calculated, as well as ROC analysis. Statistical processing was performed using Statistica v. 13 and Jamovi 2.3.28 software. Differences were considered statistically significant at $p < 0.05$.

Table 1

Clinical characteristics of patients included in the study		
Group	Clinical characteristics	Meaning
Renal cancer, n = 74	Age – median ($Q_1 - Q_3$), years	66 (58–70)
	Absolute leukocyte count $\times 10^9/l$	7.02 (5.80–8.31)
	Absolute neutrophil count $\times 10^9/l$	3.46 (2.70–4.90)
	NLR ($Q_1 - Q_3$)	1.33 (1.01–2.19)
	Sex	
	male	36
	female	38
	Histotype:	
	Clear cell carcinoma	74
	Stage of the disease:	
	I–II	40
	III–IV	34

Ending tabl. 1

Group	Clinical characteristics	Meaning
Renal benign neoplasms n = 18	Age – median ($Q_1 - Q_3$), years	68 (56–75)
	Absolute leukocyte count $\times 10^9/l$	4.60 (4.30–5.65)
	Absolute neutrophil count $\times 10^9/l$	2.58 (2.10–2.68)
	NLR ($Q_1 - Q_3$)	1.19 (1.18–1.68)
	Sex	
	male	0
	female	18
Control group n = 22	Age – median ($Q_1 - Q_3$), years	54(52–66)
	Absolute leukocyte count $\times 10^9/l$	6.10 (5.28–6.88)
	Absolute neutrophil count $\times 10^9/l$	3.30 (2.82–3.97)
	NLR ($Q_1 - Q_3$)	1.73 (1.23–1.88)
	Sex	
	male	8
	female	14

Note: NLR – neutrophil-lymphocyte ratio.

Results and discussion

During the study, we did not find any significant change in the absolute amount of Nph in the blood of patients in the study groups. According to studies [15, 16], NETs are associated with tumor cell proliferation

and affect tumor development. Analysis of the percentage of NETs showed an increase in their number in groups of patients with RC, both I–II and III–IV stages of RC, relative to the control group and the group of patients with RBN (Table 2).

Table 2

Indicators of neutrophil extracellular traps and phagocytic activity of circulating neutrophils in the study groups, Me ($Q_1 - Q_3$)

Indicators	Control group (n = 22)	Renal benign neoplasms (n = 18)	Renal cancer stage I–II (n = 50)	Renal cancer stage III–IV (n = 34)
Number traps, %	4.00 (3.00–4.75)	3.00 (3.00–4.00)	5 (4.00–7.00)*	6.00 (5.00–8.00)*
p	-	-	p1 = 0.001, p2 = 0.001	p1 = 0.001, p2 = 0.001
Index trap, y. e.	1.33(1.00–1.90)	1.55 (1.45–1.60)	1.69 (1.48–1.85)*	1.63 (1.50–2.00)*
p	-	-	p1 = 0.007, p2 = 0.022	p1 = 0.012, p2 = 0.024
Phagocytic activity, %	13.00 (10.30–12.30)	7.00 (5.00–11.00)*	10.00 (8.75–12.30)*	14.00 (10.00–17.00)*
p	-	p1 = 0.001	p2 = 0.006	p1 = 0.189, p2 = 0.001, p3 = 0.001
Phagocytic activity index, y. e.	0.24 (0.19–0.29)	0.17 (0.07–0.39)	0.26 (0.20–0.39)*	0.32 (0.29–0.36)*
p	-	-	p2 = 0.026	p1 = 0.004, p2 = 0.024, p3 = 0.001

Note: p1 – reliability of differences in indicators from the values of the control group; p2 – reliability of differences in indicators from the values in the group with benign neoplasms; p3 – reliability of differences in indicators from the values of the previous stage of renal cancer; reliability of differences according to the Mann-Whitney criterion: * ($p < 0.05$).

We also found an increase in IT in the groups of patients with stages I–II and III–IV RC relative to the control group and the group of patients with RBN. When assessing PA and PI, a significant increase in these parameters was found in the groups of patients with RC relative to the control group and the group of patients with RBN. In addition, PA and PI of circulating Nph changed depending on the prevalence of RC, so a significant increase in these parameters was noted in the group of patients at stages III–IV RC relative to the group with stages I–II RC. According to previous studies [17], at the initial stages of lung cancer, NETs had cytotoxic properties in relation to cancer cells, and at later stages they demonstrated a pro-tumor effect. There is evidence that in breast cancer, Nph more actively form NETs in the presence of a tumor with G3 differentiation than G1 [18]. The formation of NETs is one of the mechanisms of “evasion” or shielding of cancer cells from the antitumor mechanisms of the immune system [19].

Our data indicate an increase in the ability of circulating Nph to form NETs, as well as an increase in their killing activity in groups with RP relative to patients with RBN and the control group. The correlation we found between the percentage of NETs ($r = 0.438$, $p = 0.001$), PA ($r = 0.431$, $p = 0.001$) and IP ($r = 0.507$, $p = 0.001$) of Nph with the stage of RC may indicate an increase in their cytotoxic properties depending on the prevalence of RC. We assessed the expression of surface receptors that determine activation, degranulation, phagocytic activity and maturity of Nph. A significant increase in the number of Nph expressing CD66b receptors was found both at the initial and widespread stages of RC (Table 3). An increase in the number of CD66b+ Nph may indicate an increase in activated, mature, degranulating circulating Nph in patients in the study groups. There is evidence of an increase in the number and ability to form IL-1 in CD66b+ Nph in other types of cancer [20], which is consistent with the results of our study in RC.

Table 3

Changes in the expression of surface receptors of circulating neutrophils in the study groups, Me (Q₁–Q₃)

Indicators		Control group (n = 22)	Renal benign neoplasms (n = 18)	Renal cancer stage I–II (n = 50)	Renal cancer stage III–IV (n = 34)
CD11b+	%	89.10 (82.00–90.90)	93.30 (85.00–93.90)	87.40 (72.64–95.20)	94.40 (73.30–97.90)
	10 ³ /л	2.94 (2.70–3.00)	2.4 (2.19–2.50)	3.02 (2.51–3.30)	3.27 (2.53–3.38)
CD16+	%	86.10 (82.00–89.70)	83.40 (82.00–89.50)	89.00 (72.20–95.00)	88.30 (61.20–94.40)
	10 ³ /л	2.84 (2.71–2.96)	2.15 (2.11–2.42)	3.08 (2.49–3.31)	3.06 (2.12–2.27)
CD66b+	%	66.50 (57.80–77.20)	65.60 (60.10–67.60)	75.70 (62.80–86.90)	70.30 (59.50–79.30)
	10 ³ /л	2.26 (2.18–2.76)	2.24 (2.14–2.51)	2.62 (2.17–3.01)*	2.43 (2.06–2.74)*
p		-	-	p2 = 0.001	p1 = 0.012, p2 = 0.001
CD63+	%	82.80 (78.10–95.60)	78.30 (67.00–98.00)	86.10 (68.30–96.00)	81.30 (35.10–92.00)
	10 ³ /л	2.73 (2.58–3.15)	2.02 (1.72–2.53)	2.98 (2.36–3.32)	2.81 (1.21–3.18)
CD11b*CD16+	%	86.30 (77.70–89.40)	81.70 (80.30–89.50)	82.00 (64.40–90.60)	86.50 (61.10–93.50)
	10 ³ /л	2.84 (2.56–2.95)	2.10 (2.07–2.31)	2.84 (2.23–3.13)	2.99 (2.11–3.24)

Note: p1 – reliability of differences in indicators from the values of the control group; p2 – reliability of differences in indicators from the values in the group with benign neoplasms; reliability of differences according to the Mann-Whitney criterion: * ($p < 0.05$).

Changes in the expression values of CD11b, CD16, CD63 receptors of circulating Nph in the study groups were statistically insignificant. In multivariate Cox regression, an increase in the risk of developing RC was

revealed with an increase in the expression of CD66b, TI, PA and the PI of circulating Nph ($OR^2 = 0.728$, $\chi^2 = 58.1$, $p = 0.001$). In the univariate logistic regression analysis for the differential diagnosis between renal

RBN and RC, the following factors showed statistical significance: the percentage of CD66b+ Nph (OR 0.344 95% CI 0.128–0.559, $p=0.002$), the TI (OR 9.173 95% CI 1.474–16.872, $p=0.020$), PA of Nph (OR 1.555 95% CI 0.437–2.672, $p=0.006$) and Plof Nph (OR 42.940 95% CI 12.259–73.622, $p=0.006$). The area under the curve (AUC) of the model was 0.983, and RC could be diagnosed with a probability of 94.3% (Spec. = 0.889, Sens. = 0.962) (Figure).

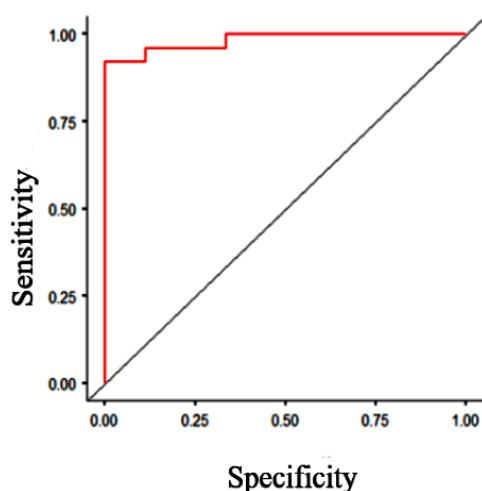


Fig. ROC curve for the regression model of differential diagnosis of renal cancer and renal benign neoplasms taking into account the expression indicators of the CD66b receptor, the neutrophil extracellular traps index, phagocytic activity and the phagocytic activity index

We found a direct correlation between the change in the expression of the CD66b receptor and CD16 ($r=0.501$, $p=0.001$), as well as the CD66b receptor and CD63 ($r=0.688$, $p=0.001$) in groups of patients with stages I–II and stages III–IV RC. This may indicate an increase in the killing activity of neutrophils in the studied groups, since these receptors are markers of maturity, PA, the ability to degranulate and form NETs [21].

Conclusion

Thus, an increase in CD66b+ Nph and activation of extracellular traps release indicate an increase in the killing activity of Nph in RC. Simultaneous deter-

mination of the amount of CD66b+ Nph, TI, PA and the PI can be used for differential diagnosis between RBN and RC.

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Киллинговый потенциал циркулирующих нейтрофилов при опухолях почки

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Аннотация. Актуальность. В настоящее время значительный интерес представляет изучение роли нейтрофилов в развитии рака почки. Исследование иммунопатогенеза рака почки определяется необходимостью использования комбинированного лечения с применением иммунотерапии. Известна способность нейтрофилов проявлять как про- так

и противоопухолевые свойства, которые связаны с уровнем поверхностных рецепторов CD11b, CD16, CD63, CD66b и киллинговой активностью нейтрофилов. **Цель** — оценить киллинговый потенциал циркулирующих нейтрофилов при опухолях почки. **Материалы и методы.** Объектом исследования явились циркулирующие нейтрофилы пациентов с верифицированным раком почки ($n = 74$), пациентов с доброкачественными новообразованиями почки ($n = 18$) и условно здоровых доноров ($n = 22$). Исследование фенотипа выделенных нейтрофилов проводили методом проточной цитометрии. Подсчет внеклеточных нейтрофильных ловушек проводили по методу Долгушина И.И. **Результаты и обсуждение.** Анализ процента внеклеточных нейтрофильных ловушек показал увеличение их количества в группах пациентов с раком почки, как I–II, так и III–IV стадий относительно группы контроля и группы пациентов с доброкачественными новообразованиями почки. Было обнаружено повышение индекса нейтрофильных ловушек в группах пациентов с раком почки I–II и III–IV стадий относительно контрольной группы и группы пациентов с доброкачественными новообразованиями почки. При оценке фагоцитарной активности и индекса фагоцитарной активности было обнаружено значимое увеличение данных показателей в группах пациентов с раком почки относительно контрольной группы и группы пациентов с доброкачественными новообразованиями почки. Обнаружена корреляционная связь между процентом внеклеточных нейтрофильных ловушек ($r = 0,438$, $p = 0,001$), фагоцитарной активностью ($r = 0,431$, $p = 0,001$) и индексом фагоцитарной активности ($r = 0,507$, $p = 0,001$) нейтрофилов со стадией рака почки. Установлено значимое повышение процента нейтрофилов, экспрессирующих рецепторы CD66b как на начальных, так и на распространенных стадиях рака почки относительно группы с доброкачественными новообразованиями почки и группы контроля. В мультивариантной регрессии Кокса выявлено возрастание риска возникновения рака почки при повышении экспрессии CD66b, индекса внеклеточных нейтрофильных ловушек, фагоцитарной активности и индекса фагоцитарной активности циркулирующих нейтрофилов ($R^2 = 0,728$, $\chi^2 = 58,1$, $p = 0,001$). Для дифференциальной диагностики между доброкачественными новообразованиями почки и раком почки статистическую значимость демонстрировали совместно процент CD66b+ нейтрофилов, индекс внеклеточных нейтрофильных ловушек, фагоцитарной активности нейтрофилов и индекса фагоцитарной активности. Площадь под кривой (AUC) модели составила 0,983, и рак почки мог быть диагностирован с вероятностью 94,3% (Spec. = 0,889, Sens. = 0,962). **Выводы.** Таким образом, увеличение CD66b+ нейтрофилов и активация высвобождения внеклеточных ловушек свидетельствует о повышении киллинговой активности нейтрофилов при раке почки. Одновременное определение количества CD66b+ нейтрофилов, индекса внеклеточных нейтрофильных ловушек, фагоцитарной активности и индекса фагоцитарной активности может использоваться для дифференциальной диагностики между доброкачественными новообразованиями почки и рака почки.

Ключевые слова: нейтрофилы, рак почки, CD11b, CD16, CD66b, CD63, внеклеточные нейтрофильные ловушки

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