ГИПЕРСЕГМЕНТАЦИЯ ЯДЕР НЕЙТРОФИЛОВ КРОВИ У БОЛЬНЫХ С ЛОКАЛИЗОВАННЫМ И РАСПРОСТРАНЕННЫМ РАКОМ ГОРТАНИ И ГОРТАНОГЛОТКИ

Кологривова Е.Н.[1], Плешко Р.И.[1], Черемисина О.В.[2], Болдышевская М.А.[2]

[1] ФГБОУ ВО «Сибирский государственный медицинский университет» Министерства здравоохранения РФ, г. Томск, Россия

[2] Научно-исследовательский институт онкологии ФГБНУ «Томский национальный исследовательский медицинский центр Российской академии наук», г. Томск, Россия

Резюме. Нейтрофильные гранулоциты обладают широким спектром функциональной активности. В последние годы обсуждается функциональная значимость нейтрофилов в развитии и течении злокачественных новообразований. Показано, что при опухолевом росте нейтрофильные гранулоциты могут играть двоякую роль, проявляя про- или противоопухолевую активность. Результаты клинических и экспериментальных исследований свидетельствуют о возможности перепрограммирования нейтрофилов под влиянием факторов микроокружения. Цель исследования – оценка структурных и функциональных особенностей нейтрофильных гранулоцитов у больных с разной степенью распространенности рака гортани и гортаноглотки. Проведено обследование 41 пациента (мужчины в возрасте от 35 до 67 лет) с впервые выявленным раком гортани и гортаноглотки. После окончательной верификации диагноза пациенты были разделены на подгруппы в соответствии с классификацией TNM: первую подгруппу с локализованным опухолевым процессом ($T_{1-3}N_0M_0$) составили 14 пациентов, вторая подгруппа состояла из 27 пациентов с распространенным опухолевым процессом ($T_3N_{1-2}M_0$). В периферической крови оценивали относительное и абсолютное количество нейтрофилов, определяли нейтрофильно-лимфоцитарное соотношение (NLR). Подсчитывали относительное содержание в крови нейтрофилов с разной степенью сегментированности ядер, цитохимически определяли активность кислородозависмых и кислородонезависмых механизмов микробицидности (миелопероксидазы, катионных белков, щелочной фосфатазы и степень активации нейтрофилов в НСТ-тесте). Концентрацию интерлейкина-8 определяли с помощью иммуноферментного анализа. Выявлено, что у больных раком гортани и гортаноглотки на фоне повышенной в сравнении со здоровыми концентрации интерлейкина-8 возрастает численность циркулирующих в крови нейтрофиль-

Адрес для переписки:

Кологривова Елена Николаевна
ФГБОУ ВО «Сибирский государственный медицинский университет» Министерства здравоохранения РФ
634050, Россия, г. Томск, Московский тракт, 2.
Тел.: 8 (913) 876-80-69.
E-mail: enkologrivova@mail.ru

Address for correspondence:

Elena N. Kologrivova
Siberian State Medical University
2 Moskovsky trakt
Tomsk
634050 Russian Federation
Phone: +7 (913) 876-80-69.
E-mail: enkologrivova@mail.ru

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ных гранулоцитов (p = 0,045) и NLR (p = 0,033). В популяции нейтрофилов по мере распространения опухолевого процесса увеличивается доля клеток, обладающих гиперсегментированными ядрами (p < 0,001) и повышенным цитотоксическим потенциалом. Выявлена прямая корреляционная связь средней силы (r = 0,42, p = 0,03) между индексом Т, отражающим объем опухоли, и относительным содержанием гиперсегментированных нейтрофилов. На основании результатов настоящего исследования можно утверждать, что такой простой и доступный для анализа лабораторный параметр, как степень сегментации ядер нейтрофильных гранулоцитов, может быть использован в качестве одного из критериев, позволяющих оценивать и прогнозировать особенности течения опухолевого процесса.

Ключевые слова: рак гортани, рак гортаноглотки, объем опухоли, нейтрофилы, гиперсегментация ядер, цитотоксичность нейтрофилов, IL-8

HYPERSEGMENTATION OF NEUTROPHIL NUCLEI IN PERIPHERAL BLOOD OF PATIENTS WITH LOCALIZED AND ADVANCED CANCER OF THE LARYNX AND LARYNGOPHARYNX

Kologrivova E.N.[a], Pleshko R.I.[a], Cheremisina O.V.[b], Boldyshevskaya M.A.[b]

[a] *Siberian State Medical University, Tomsk, Russian Federation*
[b] *Research Institute of Oncology, Tomsk National Research Medical Center, Russian Academy of Sciences, Tomsk, Russian Federation*

Abstract. Neutrophilic granulocytes have a wide spectrum of functional activity. In recent years, the functional significance of neutrophils in the development and course of malignant neoplasms has been discussed. It has been shown that neutrophilic granulocytes can play pro- or antitumor activity. The aim of the study was to assess the structural and functional features of neutrophils in patients with varying degrees of prevalence of cancer of the larynx and laryngopharynx. Forty-one patients (aged 35-67) with newly diagnosed cancer of the larynx and laryngopharynx were examined and divided into subgroups according to the TNM classification: the first subgroup (14 patients) with a localized tumor process consisted; and the second subgroup (27 patients) with a widespread tumor process. The relative and absolute number of neutrophils was assessed, and the neutrophil-lymphocyte ratio (NLR) was determined. The content of neutrophils with varying degrees of nuclear segmentation in the blood was calculated, the activity of myeloperoxidase, cationic proteins, alkaline phosphatase, and the degree of neutrophil activation in the NBT test was determined cytochemically. Concentration of interleukin-8 was determined using ELISA. In patients with cancer of the larynx and laryngopharynx the number of neutrophils (p = 0.045) and NLR (p = 0.033), as well as serum concentration of interleukin 8 (p = 0.011), increased compared to healthy individuals. The proportion of cells with hypersegmented nuclei in the neutrophil population (p < 0.001) and cytotoxic potential increased with the spread of tumor process. A direct correlation (r = 0.42, p = 0.03) was found between the T index, which reflects the volume of the tumor, and the content of hypersegmented neutrophils. It can be argued that such a simple and accessible laboratory parameter as the degree of segmentation of the nuclei of neutrophilic granulocytes can be used as one of the criteria to assess and predict the course of the tumor process.

Keywords: cancer of the larynx, cancer of the laryngopharynx, tumor volume, neutrophil, hypersegmentation of nuclei, cytotoxicity of neutrophils, IL-8

Introduction

Neutrophilic granulocytes (NGs), also called polymorphonuclear or segmented leukocytes, are the dominant cell population among circulating leukocytes. Their most important function is considered to be antimicrobial protection implemented in the innate immune system through phagocytosis, secretory degranulation, and the formation of neutrophil extracellular "traps" (NETs) [1].

In recent years, information has appeared that NGs, along with the phagocytic ability, have a wide variety of functional activities. It has been shown that neutrophils are able to produce cytokines, modulate the activity

of cells in the microenvironment, and thus actively participate in the pathogenesis of various diseases, including cancer [1, 8]. Relatively recently, it has become clear that neutrophilic granulocytes can play a dual role during tumor growth, exhibiting either pro- or antitumor activity [1]. The subpopulation of neutrophils with antitumor properties is commonly referred to as N1, with protumor properties as N2 [1, 4, 7]. The results of clinical and experimental studies demonstrate the ability of neutrophils to transform under the influence of tumor microenvironment factors [4, 7]. Currently, close attention of researchers is directed to the search for morphological, phenotypic, and molecular genetic markers of pro- and antitumor NGs phenotypes and to assess the possibility of using these cells as diagnostic and therapeutic targets [2, 4, 6].

The aim of this study was to evaluate the structural and functional features of neutrophilic granulocytes circulating in the blood in patients with varying degrees of prevalence of cancer of the larynx and laryngopharynx.

Materials and methods

On the basis of the endoscopic department of the Research Institute of Oncology of the Tomsk Research Medical Center of the Russian Academy of Sciences (Tomsk), 41 patients with newly diagnosed cancer of the larynx and hypopharynx (men aged 35 to 67 years) were examined before starting antitumor therapy. After the final verification of the diagnosis, all patients with cancer of the larynx and laryngopharynx were divided into subgroups in accordance with the TNM classification, in which the T (tumor) criterion characterizes the size of the primary tumor, the N (nodus) criterion describes the state of regional lymph nodes, and the M (metastasis) criterion indicates the presence or absence of distant metastases. The first subgroup with a localized tumor process (T1-3N0M0) included 14 patients, the second subgroup consisted of 27 patients with a widespread tumor process (T3N1-2M0). As a control group, 55 apparently healthy individuals (men aged 39 to 69 years) were examined, for whom the inclusion criteria were: the absence of malignant and benign neoplasms in history, the absence of chronic diseases of the oropharynx and nasopharynx, the absence of acute respiratory viral infections within 3 weeks before the examination, sanitized oral cavity (absence of carious teeth and bleeding gums). All examined persons underwent the procedure of signing the informed consent to participate in the scientific and practical research.

The total number of leukocytes was determined in the blood and the leukocyte formula was calculated using a Carl Zeiss Axioskop 40 FL microscope in the standard mode and the ratio of the relative number of neutrophils and lymphocytes (Neutrophil-Lymphocyte Ratio – NLR) was determined. The percentage of active neutrophils and the intensity of activation of oxygen-dependent mechanisms of cytotoxicity were assessed using the NBT-test in the form of an average cytochemical coefficient (ACC) according to the L.S. Kaplow [3]. Cytochemically, the intracellular expression of neutrophil marker enzymes (myeloperoxidase, alkaline phosphatase) and cationic proteins involved in the implementation of the cytotoxic function was determined [3]. In addition, when analyzing 100 neutrophilic granulocytes, groups of cells with varying degrees of nuclear segmentation were identified: 1) cells with nuclei containing 2-3 segments; 2) neutrophils containing 4 segments; 3) neutrophils containing 5 or more segments (hypersegmented nuclei). To analyze the structural features, the average segmentation coefficient (ASC) was introduced, which reflects the distribution of neutrophils with different nuclear morphology. The average segmentation coefficient was determined by the formula:

$$ASC = (1 \cdot a + 2 \cdot b + 3 \cdot c)/N,$$

where a is the relative number of neutrophils with a nucleus consisting of 2-3 segments; b is the relative number of neutrophils with a nucleus consisting of 4 segments; c is the relative number of hypersegmented neutrophils with a nucleus of 5 or more segments; N is the number of counted cells.

In blood plasma, ELISA was used to determine the concentration of interleukin (IL)-8, one of the main chemokines for NG. To determine the concentration of IL-8, a set of reagents Interleukin-8-ELISA-BEST (CJSC Vector-Best, Russia) was used.

For statistical analysis of the obtained data, the software package "SPSS Statistics 17.0" was used. The results were processed using the Mann-Whitney U-test and presented as a median and quartiles – Me ($Q_{0.25}$-$Q_{0.75}$). Differences between groups were considered significant at a significance level not exceeding 0.05. Spearman's paired correlation analysis was used to identify the relationships between the parameters.

Results and discussion

Statistically significant differences in the group of patients with cancer of the larynx and laryngopharynx from the group of healthy ones were manifested in the shift of the leukocyte formula towards the predominance of neutrophilic cells: 59.00 (54.00-66.00) % – in patients and 55.00 (51.00-61.00) % in healthy people ($p = 0.045$). Changes in the hemogram were also reflected in the increase in NLR: in the group of patients, this indicator was 2.12 (1.66-3.00), while in healthy people it was 1.75 (1.31-2.13), $p = 0.033$. In the last decade, many publications have appeared on the change in NLR in cancers of various localizations, and our data are consistent with the results of other

authors, who recorded an increase in this indicator in tumors of various localizations [13].

It should be noted that an increase in the relative and absolute number of neutrophils in the blood was noted against the background of an increase in the concentration of IL-8, the content of which was 17.36 (15.14-41.45) pg/mL in patients with cancer of the larynx and laryngopharynx compared with 10.75 (6.86-32.95) pg/mL in healthy people ($p = 0.011$). Information about the increased concentration of IL8 in the blood serum of cancer patients has been presented in recent years in a number of publications, while the position on the possibility of using an elevated level of this cytokine as a negative prognostic criterion in various malignant neoplasms is very well substantiated [6].

Analysis of the structural features of neutrophils, conducted in this study, revealed in patients with cancer of the larynx and laryngopharynx an increased number of NGs circulating in the blood with four, five or more segments of the nuclei (Table 1).

The oxygen-dependent cytotoxicity of NGs was assessed by the results of a spontaneous NBT-test, reflecting the activity of NADPH oxidase, and by the activity of myeloperoxidase (Table 2). The relative number of NBT-positive neutrophils and the average cytochemical coefficient (ACC) in this test in patients with cancer of the larynx and laryngopharynx were increased in comparison with the control group, mainly due to cells with an average and high degree of activation (Table 2). Myeloperoxidase activity was also increased in NGs in cancer patients (Table 2). Similar features were revealed when assessing the state of oxygen-independent mechanisms of neutrophil cytotoxicity: cytochemical coefficients characterizing the content of cationic proteins and alkaline phosphatase in patients with laryngeal cancer exceeded the corresponding values in the healthy group (Table 2). The ability to implement cytotoxic reactions is regarded ambiguously when considering the pro- and antitumor effects of NGs. On the one hand, this cytotoxicity can be realized in relation to tumor target cells, on the other hand, it can contribute to disruption of the structural organization of the extracellular matrix, abnormal angiogenesis, and metastasis [2].

Analyzing the relationship between the parameters characterizing the morphofunctional status of NGs with the clinical characteristics of the tumor process, determined using the TNM classification, we found that in the subgroup of patients with advanced tumor process (T3N1-2M0), the number of hypersegmented cells (5 or more segments) exceeded the corresponding indicator patients with localized cancer of the larynx and laryngopharynx (T1-3N0M0): (10.0 (7.5-14.5)) % and (4.0 (2.0-8.5)) %, respectively ($p = 0,04$). When conducting a paired correlation analysis, a direct relationship of medium strength ($r = 0.42$, $p = 0.03$) was revealed between the T index, which reflects the volume of the tumor, and the relative content of hypersegmented neutrophils.

Currently, the prevailing opinion is that a segmented nucleus is necessary for neutrophils for accelerated migration into tissues, while the mechanisms and functional significance of the hypersegmentation phenomenon are not fully understood [5]. It is generally accepted that hypersegmented forms of NGs (more than 5 segments) appear in the blood in pathological conditions [5]. Nuclear hypersegmentation is one of the earliest, most sensitive and specific signs of megaloblastic anemia, and is also observed in myelodysplastic syndrome, chronic infections, and conditions associated with a decrease in the concentration of granulocyte colony-stimulating factor (G-CSF) [5]. It is well known that NGs are the first immunocompetent cells migrating from blood vessels to the area of tissue damage or infectious inflammation. It has been proven that, due to their pronounced ability to extravasation and interstitial migration, NGs, along with the implementation of

TABLE 1. DISTRIBUTION OF NEUTROPHILS ACCORDING TO THE DEGREE OF NUCLEAR SEGMENTATION IN PATIENTS WITH CANCER OF THE LARYNX AND LARYNGOPHARYNX, Me ($Q_{0.25}$-$Q_{0.75}$)

Indicator	Study group		p
	Cancer of the larynx and laryngopharynx (n = 41)	Healthy volunteers (n = 55)	
Neutrophils with a moderate degree of segmentation (2-3 segments), %	48 (36-60)	76 (70-82)	p < 0.001
Neutrophils with a high degree of segmentation (4 segments), %	39 (31-50)	20 (18-29)	p < 0.001
Hypersegmented neutrophils (5 and > segments), %	11 (7-17)	1 (0-2)	p < 0.001
Average segmentation coefficient (ASC)	1.66 (1.47-1.79)	1.24 (1.18-1.33)	p < 0.001

Note. n, the number of examined individuals, p, the level of significance of differences.

TABLE 2. INDICATORS CHARACTERIZING THE CYTOTOXIC POTENTIAL OF NEUTROPHILS IN PATIENTS WITH CANCER OF THE LARYNX AND LARYNGOPHARYNX, Me ($Q_{0.25}$-$Q_{0.75}$)

Indicator	Study group		p
	Cancer of the larynx and laryngopharynx (n = 41)	Healthy volunteers (n = 55)	
NBT-positive neutrophils, %	20 (10-30)	10 (10-12)	0.042
ACC in the NBT-test	0.28 (0.13-0.57)	0.12 (0.10-0.16)	0.003
Myeloperoxidase activity, ACC	1.92 (1.74-2.12)	1.58 (1.39-1.80)	0.010
Cationic proteins, ACC	1.61 (1.44-1.68)	1.34 (1.22-1.51)	0.039
Alkaline phosphatase, ACC	0.66 (0.61-0.79)	0.33 (0.25-0.40)	< 0.001

Note. n, the number of examined patients; p, the level of significance of differences; ACC, the average cytochemical coefficient.

phagocytosis, are actively involved in tissue remodeling during wound healing, angiogenesis, tumor formation, and metastasis [9]. Moreover, neutrophils very quickly have to switch between different modes of migration. The segmented nucleus allows cells to change their shape and easily overcome intercellular contacts as part of tissue cell layers. However, other cells with non-segmented nuclei, such as lymphocytes and monocytes (mononuclear cells), are also able to migrate into tissues [11]. It is believed that the severity of segmentation of the NGs nuclei characterizes the degree of their maturity, and the hypersegmented nucleus, which has increased flexibility, indicates the readiness of the cell to complete the life cycle [5]. Interestingly, "old" neutrophils migrate faster to the sites of inflammation [9]. To date, there is no unequivocal answer about the functional significance of hypersegmented neutrophils present in the tumor growth zone. Experimental studies have shown that nuclear hypersegmentation, which is a morphological sign of more mature neutrophils, is characteristic of cells with antitumor properties, i.e. for N1-neutrophils [4]. At the same time, there is evidence that the presence of a large number of hypersegmented neutrophils in tumor tissue is associated with an unfavorable outcome of oncological diseases [6].

There is still no unequivocal answer to the question: are the morphofunctional features of cells formed during differentiation in the bone marrow, or are mature neutrophils, exposed to any factors in the systemic circulation, capable of plasticity? Experimental studies have demonstrated the possibility of *in vitro* induction of a hypersegmented neutrophil phenotype, corresponding in its functional characteristics to the antitumor subpopulation N1 [10, 12].

The results of this study indicate that the number of cells with hypersegmented nuclei circulating in the blood increases as the tumor process spreads, and simultaneously with an increase in the degree of nuclear segmentation, the cytotoxic potential of NGs increases. Questions about the functional significance and biological feasibility of morphological modifications of the nuclear apparatus of neutrophils circulating in the blood during malignant growth remain open. Based on the results of this study, it can be argued that such a simple and accessible for analysis laboratory parameter as the degree of segmentation of NCs nuclei can be used as one of the criteria to assess and predict the course of the tumor process.

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Авторы:

Кологривова Е.Н. *— д.м.н., профессор, профессор кафедры иммунологии и аллергологии ФГБОУ ВО «Сибирский государственный медицинский университет» Министерства здравоохранения РФ, г. Томск, Россия*

Плешко Р.И. *— д.м.н., профессор, профессор кафедры морфологии и общей патологии ФГБОУ ВО «Сибирский государственный медицинский университет» Министерства здравоохранения РФ, г. Томск, Россия*

Черемисина О.В. *— д.м.н., заведующая эндоскопическим отделением, Научно-исследовательский институт онкологии ФГБНУ «Томский национальный исследовательский медицинский центр Российской академии наук», г. Томск, Россия*

Болдышевская М.А. *— врач клинической лабораторной диагностики патолого-анатомического отделения, Научно-исследовательский институт онкологии ФГБНУ «Томский национальный исследовательский медицинский центр Российской академии наук», г. Томск, Россия*

Authors:

Kologrivova E.N., *PhD, MD (Medicine), Professor, Department of Immunology and Allergology, Siberian State Medical University, Tomsk, Russian Federation*

Pleshko R.I., *PhD, MD (Medicine), Professor, Department of Morphology and General Pathology, Siberian State Medical University, Tomsk, Russian Federation*

Cheremisina O.V., *PhD, MD (Medicine), Head, Endoscopic Department, Research Institute of Oncology, Tomsk National Research Medical Center, Russian Academy of Sciences, Tomsk, Russian Federation*

Boldyshevskaya M.A., *Doctor of Clinical Laboratory Diagnostics, Pathological and Anatomical Department, Research Institute of Oncology, Tomsk National Research Medical Center, Russian Academy of Sciences, Tomsk, Russian Federation*

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