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ORIGINAL ARTICLE

THE NUMBER OF CANCER STEM CELLS IN THE TUMOR TISSUE AND PERIFOCAL TISSUE OF NON-MUSCLE INVASIVE BLADDER CANCER

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ABSTRACT

Purpose of the study. Determine the content of cancer stem cells (CSCs) in the tumor tissue (TT) and perifocal tissues (PT) in muscle-non-invasive bladder cancer.

Materials and methods. We've examined fragments of TT and PT of 7 muscle-non-invasive bladder cancer (NMIBC) after surgical intervention – transurethral resection of the urinary bladder (TUR). In tissue samples that were used to obtain cell suspension of TT and PT using the BD Medimachine apparatus (BD, USA) was treated with monoclonal antibodies CD45-APC-Cy7, CD44-FITC, CD133-PE, CD24-PE (BD, USA) and were assessed on flow cytometer FacsCantoII (BD, USA). The percentage of cells with CSC phenotypic markers was determined in the analysis sample: $CD45\text{-}CD44^{+}CD24^{+}$, $CD45\text{-}CD44^{+}$, $CD45\text{-}CD24^{+}$, $CD45\text{-}CD133^{+}$, $CD45\text{-}CD44^{+}CD133^{+}$. The presence of significant differences in the groups was evaluated using the STATISTICA 13 software package and the differences between the samples were considered significant at $p < 0.05$. The percentage of cells of the corresponding phenotype was calculated relative to the total number of cells. The percentage of cells with the corresponding phenotype was calculated relative to the total number of cells.

Results. The relative numbers of cells with CSC phenotypic markers, such as CD24, CD44, were 77 % and 58 % higher in TT than in PT: 18.3 ± 3.5 vs. 4.3 ± 2.1, $p \leq 0.044$ and 15.5 ± 5.3 vs. 6.5 ± 0.8, $p \leq 0.043$, respectively. The number of $CD133^{+}$ cells was 83 % higher in PT compared to TT – 41.6 ± 12.1 vs. 22.7 ± 7.6, $p \leq 0.047$.

Conclusion. The study of CSCs is a promising direction for the study of oncogenesis and can be used to assess the nature of the further development of relapse and / or progression of the disease, as well as various therapeutic approaches that are aimed at eliminating with CSC phenotypic markers and blocking the pathways leading to the emergence and maintenance of this cell population in patients with NMIBC.

Keywords:
non-muscle-invasive bladder cancer, cancer stem cells, perifocal tissues

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ОРИГИНАЛЬНАЯ СТАТЬЯ

ОТНОСИТЕЛЬНОЕ СОДЕРЖАНИЕ ОПУХОЛЕВЫХ СТВОЛОВЫХ КЛЕТОК В ТКАНИ ОПУХОЛИ И ПЕРИТУМОРАЛЬНОЙ ЗОНЕ МЫШЕЧНО-НЕИНВАЗИВНОГО РАКА МОЧЕВОГО ПУЗЫРЯ

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РЕЗЮМЕ

Цель исследования. Определить содержание опухолевых клеток с фенотипом стволовых (ОСК) в ткани опухоли и перитуморальной зоне при мышечно-неинвазивном раке мочевого пузыря (МНИРМП).
Материалы и методы. Исследованы фрагменты опухолевой ткани (ОП) и ткани перитуморальной зоны (ПЗ) 7 пациентов с впервые выявленным мышечно-неинвазивным раком мочевого пузыря (МНИРМП) после проведения оперативного вмешательства в объеме трансуретральной резекции мочевого пузыря (ТУР). В образцах тканей, которые использовались для получения клеточной суспензии ОП и ПЗ с помощью аппарата BD Medimachine (BD, USA), с использованием моноклональных антител CD45-APC-Cy7, CD44-FITC, CD133-PE, CD24-PE (BD, USA), осуществляли определение фенотипических характеристик клеток на проточном цитометре FacsCantoII (BD, USA). В анализируемых образцах определяли процентное содержание клеток с фенотипом стволовых: $CD45\text{-}CD44^{+}CD24^{+}$, $CD45\text{-}CD44^{+}$, $CD45\text{-}CD24^{+}$, $CD45\text{-}CD133^{+}$, $CD45\text{-}CD44^{+}CD133^{+}$. Наличие достоверности различий в группах оценивали при помощи программного пакета Statistica 13, различия между выборками считали достоверными при $p < 0,05$. Расчёт процентного содержание клеток соответствующего фенотипа производился относительно общего числа клеток.
Результаты. Относительное содержание клеток, имеющих фенотипические маркеры ОСК такие как CD24, CD44, в ОП были на 77 % и 58 % больше, чем в ПЗ, соответственно 18,3 ± 3,5 против 4,3 ± 2,1, $p \leq 0,044$ 15,5 ± 5,3 против 6,5 ± 0,8, $p \leq 0,043$. Количество $CD133^{+}$ – клеток оказалось больше на 83 % в ПЗ по сравнению с ОП – 41,6 ± 12,1 против 22,7 ± 7,6, $p \leq 0,047$.
Заключение. Изучение опухолевых стволовых клеток в настоящее время является перспективным направлением для изучения развития злокачественного процесса и может быть использовано для предикции и оценки характера дальнейшего развития рецидива и/или прогрессирования заболевания, а также, в дальнейшем, для применения различных подходов терапии, которые будут направлены на устранение клеток с фенотипом стволовых и блокирования путей, которые приводят к возникновению и поддержанию данной популяции клеток у больных с МНИРМП.

Ключевые слова:
мышечно-неинвазивный рак мочевого пузыря, опухолевые стволовые клетки, перитуморальная зона

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Bladder cancer (BC) is one of the main problems in the structure of the general oncourological morbidity accounts for 4.6 %, inferior to malignant kidney formations [1; 2]. Without appropriate and timely assistance, this malignant neoplasm (MN) can lead to severe disability and a significant deterioration in the quality of life of patients. About 400 thousand new cases of the disease are registered annually in the world [3]. BC is on the 7th place in the structure of cancer incidence in men and 17th place in women in the world [2]. In the structure of the general (both sexes) oncological morbidity in Russia, BC occupies the 13th place (2.7 %), in men this pathology occupies the 9th place (4.6 %), and the 16th in women, thereby forming a fairly significant group of malignant neoplasms of the genitourinary system, accounting for 25.1 % of all MN. The average age of men who became ill in Russia is 66.7 years, women – 68.8 years [4]. In 2020 1,594 people with newly diagnosed BC were registered in the Southern Federal District, and 389 people were registered in the Rostov Region [5].

Muscle-noninvasive BC (NMIBC) at the stages of Ta, T1, q carcinoma (CIS) according to the TNM classification accounts for about 70 % of cases [2], muscle-invasive BC (MIBC), as well as metastatic forms – about 30 % [6]. The metastatic form is characterized by a rather aggressive course and high mortality. The 5-year survival rate for patients with metastatic BC is less than 6 % [7].

Currently, treatment methods and prognosis of the further course of BC are based on the classification of TNM and for NMIBC on prognosis groups, taking into account a number of factors. Despite this, the long-term results of treatment of patients belonging to the same classification groups and receiving identical treatment may vary significantly. In this regard, in order to fully predict the course of BC, it is necessary not only to determine the histological structure of the tumor, the degree of its differentiation, but also to take into account the influence of individual factors that determine the clinical behavior and biological aggressiveness of the tumor [8].

Under the influence of carcinogens in the epithelium of the bladder, the probability of changing the functional state of a heterogeneous cell population increases, the mechanisms of cell cycle control are disrupted, various mutations occur, which leads to changes in the processes of cell proliferation and differentiation. Studies on transgenic mice have shown that epithelial stem cells with HRAS or FGFR3 mutations can transform into tumor stem cells of bladder cancer that develop in NMIBC (local mutations in 12, 13 or 61 codons of the oncogene HRAS1 [9], activating local mutation in 7 and 10 exons of the fibroblast growth factor receptor gene 3 [10], PIK-3CA missense mutations [11]), whereas stem cells with mutations of the p53/Rb/PTEN gene transform into tumor stem cells of the urothelium, which cause NMIBC (deletion of chromosome 9p21, etc.) [9; 12]. The characteristic features of NMIBC are activating mutations and overexpression of proto-oncogenes (FGFR3, HRAS, etc.), in most cases, which are acquired gene abnormalities [9].

Currently, the main method of diagnosis of NMIBC is histological analysis of the material obtained after transurethral resection of the bladder, with which it is possible to determine the depth of invasion and the degree of differentiation of the tumor [13].

Recently, the question of early prediction and prevention of BC development has become acute, on which the course of the disease and it's outcome depend, as well as the possibility of timely qualified care.

Table 1. Bladder cancer biomarkers' characteristics in terms of urine study

Marker	Sensitivity, %	Specificity, %
UroVysion	71	66
BTA assessment test (Bladder Tumor Antigen)	64 for G_1, 92 for G_3	90
Cytokeratin level measurement 19 (CYFRA 21-1)	55.7 for G_1, 91.9 for G_3	85.5
NMP22	55.7	85.7
ImmunoCyt/uCyt+ essay	79.3 for G_1, 92.1 for G_3	80 for G_1, 92 for G_3

The diagnostic spectrum of BC biomarkers is diverse, but the accuracy of the techniques and their prognostic value aren't high enough and have limited use in the clinic, as can be seen from the Table 1, which presents some markers for the diagnosis of BC and their main characteristics, according to various studies published at the moment [14].

Recently, the role of tumor stem cells (CSC) in the diagnosis and evaluation of the effectiveness of cancer treatment has been actively studied. CSC (CSC–Cancer Stem Cells) is a specific tumor cell, which is characterized by the ability to asymmetric division, self–renewal *in vivo*, causes the growth of a tumor identical to the original one. A distinctive feature of CSS is their increased resistance to antitumor effects. It is known that antitumor drugs are aimed at eliminating most of the tumor masses sensitive to the antitumor agent, however, it has been proven that the nucleus of cells in the form of CSC remains in the body, which, in turn, may have the ability to restore, proliferate and progress the disease [15]. In this regard, the identification of CSC is an important aspect in assessing the effectiveness of the methods used to treat cancer pathology.

Despite the experimental and theoretical data accumulated to date, many biological properties of CSC, their involvement in the pathological process and their influence on the processes of recurrence and progression remain poorly understood.

The purpose of the study: was to determine the content of tumor cells with the stem cell phenotype in tumor tissue and peritumoral area in noninvasive muscle BC.

MATERIALS AND METHODS

Fragments of tumor tissue (TT) and tissue of the peritumoral zone (PZ) of 7 patients with newly diagnosed noninvasive muscle bladder cancer (NMIBC), all patients have given written consent to the transfer of biological material and the processing of personal data. Histological structure – papillary urothelial carcinoma of low malignancy (low-grade). In 2 people, the tumor is localized along the back wall of the bladder, in 5 people, the tumor is localized on the side walls of the bladder. 5 patients had 1–2 tumors in the bladder, in 2 patients the tumor had a multifocal character. The 1st patient has a history of MN of a different localization (prostate cancer), 1 patient has a history of chronic viral hepatitis C and HIV of art. III, 1 patient is a convalescent of COVID-19 pneumonia.

All patients underwent transurethral resection of the bladder (TUR), in which material was taken: a fragment of the tumor (up to 1.5 cm in size), a fragment of the perifocal zone (retreating from the tumor by at least 0.8 cm, but not more than 1.5 cm). The obtained tissue fragments immediately after sampling and delivery to the laboratory were used to obtain a cell suspension using BD Medimachine (BD, USA). The cell suspension was treated with a panel of monoclonal antibodies: $CD45^-$APC-Cy7, CD44-FITC, CD133-PE, CD24-PE in accordance with the manufacturer's instructions (BD, USA). The phenotypic characteristics of the cell suspension in order to identify cells with the USC phenotype were evaluated on a FacsCantoII flow cytometer (BD, USA). In the analyzed samples, the percentage of cells with the USC phenotype was determined: $CD45^-CD44^+CD24^+$, $CD45^-CD44^+$, $CD45^-CD24^+$, $CD45^-CD133^+$, $CD45^-CD44^+CD133^+$. The percentage of cells of the corresponding phenotype relative to the total number of cells was calculated.

Patients after the treatment in the volume of the TOUR are under dynamic observation, continue to receive adequate treatment in accordance with the clinical recommendations of the AOR in the volume of intravesical chemotherapy, followed by a control study and, if necessary (the presence of relapse or progression of the disease), a decision on further diagnosis and treatment tactics.

Statistical processing was performed using the STATISTICA 13 package (StatSoft Inc., USA). The nature of the distribution of the obtained data was evaluated using the Shapiro-Wilk criterion. Since the obtained data had a normal distribution, the results were presented in the form of the arithmetic mean and the standard error of the arithmetic mean (M ± s). To compare the average values of quantitative indicators in groups, in the case of a normal distribution law, the parametric Student criterion was used, in another case, the nonparametric Mann-Whitney criterion. The differences were considered significant at $p < 0.05$.

RESEARCH RESULTS

The conducted research revealed a number of features of the relative content and distribution of tumor cells with the stem phenotype. It should be noted that the decisive role in the development of

cancer is played by the environment of the tumor, i.e. those interactions formed between the tumor cell and different types of surrounding cells in the peritumoral zone, changes in which can contribute to further invasion of the process. The number of $CD45^-$cells was analyzed, the pool of which is highly likely to include tumor cells with a stem phenotype. The number of CD45 cells in TT and PZ did not differ significantly, amounting to 61.3 ± 5.8 and 71.8 ± 12.6. The relative content of cells with phenotypic CSC markers such as CD24, CD44 in TT were 77 % and 58 % higher than in PZ, respectively, 18.3 ± 3.5 vs. 4.3 ± 2.1, $p \le 0.044$, 15.5 ± 5.3 vs. 6.5 ± 0.8, $p \le 0.043$. The number of $CD133^+$ cells was 83 % higher in PZ compared to TT – 41.6 ± 12.1 vs. 22.7 ± 7.6, $p \le 0.047$. In tumors of the BC content of the cells with the phenotype $CD44^+CD24^+$ and $CD44^+CD133^+$ exceeded the values in PZ 80 % and 63 %, respectively, of 10.3 ± 4,9 vs 2,1 ± 0,4, $p \le 0.039$ inch, 9,0 ± 4,5 versus 3.3 ± 0,9, $p < 0,046$.

So, cells with the CSC phenotype ($CD45^-CD44^+CD24^+$, $CD45^-CD44^+$, $CD45^-CD44^+CD24^+$ and $CD45^-CD44^+CD133^+$) predominate in the tumor tissue. The peritumoral zone was dominated by cells with the $CD45^-CD133^+$ phenotype (Fig. 1).

DISCUSSION

For the first time, CSCs were isolated by D. Bonnet and Y. E. Dick (1997) in acute myeloid leukemia $CD34^+/CD38^-$, and later in various solid tumors [16].

In BC, USCS were first described in 2009 by K. S. Chan et al., their greater content was found in MIBC than in NMIBS [17]. Markers of CSC in BC are a number of phenotypic determinants CD44, CD133, CD47, CD49, 67LR (67-kD laminin receptor), as well as a characteristic set of cytokeratins (keratin 14, 5 and others) [15]. The use of CD133 for the detection of CSC in MN of the bladder is not often noted, its study continues in terms of informativeness in this pathology.

Based on the sequencing of 59 cells from three bladder cancer samples (including BC stem cells, non-BC stem cells, epithelial bladder stem cells, epithelial non-bladder stem cells) Yangetal. the origin of BC tumor cells from epithelial stem cells of the bladder or epithelial non-stem cells of the bladder has been suggested [18]. Probably, urothelial stem cells are located in the basal cell layer and are able to repair damage to the bladder. Based on the conducted studies of the experimental mod-

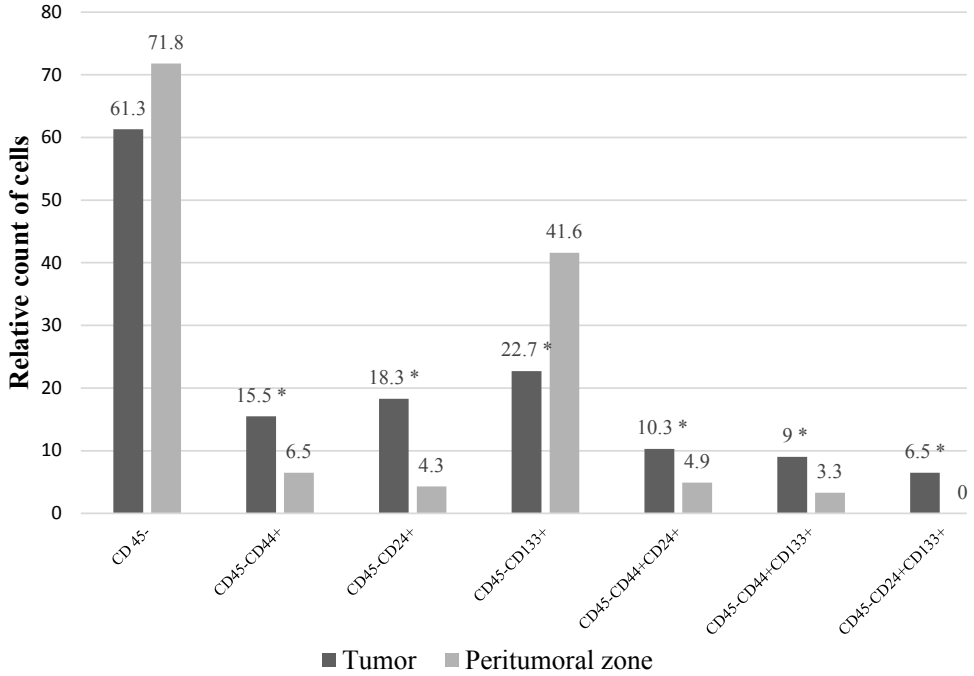

Fig. 1. Relative count of CSC, TT and PZ.

el, the origin of MIBC from urothelial stem cells of the basal cell layer was confirmed. BC tumor stem cells are $CD44^+CK5^+CK20^-$, have phenotypic markers characteristic of basal cells [19]. $CD44^+$ cells were detected in the basal layer of normal urothelium and urothelial carcinoma, in addition, the cells of the intermediate layer also express CD44. It has been shown that due to a mutation in the FGFR3 gene, intermediate layer cells transform into malignant papillary carcinoma of low malignancy and bladder hyperplasia [20].

Experiments to track clones on a mouse tumor model, to which cells isolated *in vivo* were injected intradermally, demonstrated that papillary tumor cells mainly originate from the intermediate layer. In the study of more than 300 samples of patients with transitional cell carcinoma of the bladder, 40 % contained $CD44^+$ cells. Histological analysis showed that the xenografts of the tumor retained a histology similar to that of the patient's original tumor. Cells with the $CD44^+$ phenotype have a high oncogenicity (200 times higher than CD44-tumor cells of BC), and the ability to self-renew. The frequent and significant expression of CD44 in normal tissues and tumors contradicts the idea of the relative rarity of USC, and therefore there is a need to combine CD44 with other markers, for example, CD133 or CD24 to detect CSC [14].

It is known that increased expression in CD44 tumor cells causes metastasis, self-maintenance of these cellular elements, and also contributes to the formation of drug resistance against the background of resistance to apoptosis. A number of studies have revealed a correlation between the presence of CD44 and the degree of prevalence of BC. The presence of $CD44^+$ showed a lower survival rate and incomplete response to previous therapy (chemo and/or radiotherapy), thus, a change in the expression of CD44, which is an adhesive protein and promotes cell migration, can act as one of the mechanisms causing the process of recurrence and progression of BC [21].

Summarizing the data obtained from our work, we found a greater number of $CD44^+$ cells in the tumor tissue, which is consistent with the literature data and may indicate an unfavorable course of the disease, as well as the possibility of using this marker as a marker for predicting disease recurrence after complex treatment.

CD133 (AC133, prominin 1) is a glycoprotein that was first discovered by H. Yu et al. in 1997 as a cell surface protein expressed on $CD34^+$ hematopoietic progenitor cells [22]. Transplantation of tumor stem cells expressing CD133 to mice with immunodeficiency generated histologically similar tumor tissue with self-renewal [23]. In a study by Huang P. and co-author in 2013. It was demonstrated that the $CD133^+$ subpopulation of human bladder cancer cells was characterized by activation of pluripotent stem cell markers – Oct-4 and Sox-2, while demonstrating more aggressive proliferation compared to the CD133-subpopulation. The $CD133^+$ subpopulation also tended to form colonies, which indicates a strong clonogenic ability, i.e. they have phenotypic features associated with CSC [24]. The presence of CD133 on the surface of tumor cells causes the preservation of their stem properties, as well as the launch of the formation of differentiated malignant cells [25].

In our work, CSC with the $CD133^+$ phenotype were found in greater numbers in the peritumoral zone. Based on this, it can be assumed that this marker functions as a modulator of the effects of a wide range of cytokines, affects the activity of various membrane receptors, and an increase in this marker can lead to structural and functional changes in cells with an increase in the probability of their tumor transformation.

Previously published studies have proven the important role of CD24 in the development of oncogenesis and the progression of various types of malignant neoplasms, including renal cell carcinoma (RCC), nasopharyngeal cancer, hepatocellular carcinoma (HCC), ovarian cancer, non-small cell lung cancer (NSCLC), breast cancer and others. This mucin-like cell membrane protein is expressed in many types of tumor tissue. In breast cancer, a correlation was noted between the overexpression of CD24, the prevalence and progression of the disease [26]. CD24 expression was slightly correlated with lymphovascular invasion of the BC tumor, whereas CD133 was associated with distant metastases and aggressiveness of the tumor process. Tumor cells with the phenotype of stem $CD133^+CD24^+$ are characteristic of more aggressive forms, low differentiated (high grade) bladder carcinomas of high malignancy [25].

CONCLUSION

Thus, based on a small sample size, it's possible to assume some phenotypic and quantitative features of

CSC in tumor tissue and peritumoral zone in NMIBC.

The study of CSC is a promising direction for the study of oncogenesis, and with further study, there is a high probability of using these markers to assess the nature of the development of relapse and/or progression of the disease, as well as for new different therapy approaches aimed at eliminating cells with the CSC phenotype by affecting surface markers and corresponding signaling pathways that lead to the emergence and maintenance of this cell population. Despite all the available scientific work related to the search for new effective methods of diagnosis, the study of CSC and their impact on the process of occurrence, metastasis in BC – have not been studied enough, and therefore it is planned to further study these cells with the stem phenotype.

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