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Research Article

Immunohistochemical diagnosis of tumor-associated macrophages in patients with muscle-invasive bladder cancer after radical cystectomy

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ABSTRACT

BACKGROUND: Bladder cancer is a serious problem of oncourology. The gold standard of treatment for muscle-invasive bladder cancer is radical cystectomy with previous neoadjuvant chemotherapy. Unfortunately, the effectiveness of radical treatments is severely limited in the long term. For this reason, research in the field of predicting survival can significantly improve long-term oncological results. The determination of the levels of macrophages associated with the tumor appears promising.

AIM: to determine the effect of the expression levels of macrophages associated with a tumor on survival rates in patients diagnosed with muscle-invasive bladder cancer after radical cystectomy.

MATERIALS AND METHODS: the study was conducted on the basis of the Clinic of the Bashkir State Medical University in the period from 01.05.2021 to 01.07.2023. The study involved 66 patients with an established diagnosis of muscle-invasive bladder cancer. After surgical treatment, histological and immunohistochemical studies were performed to determine CD68 and CD163 levels. After 24 months, a survival analysis was performed to determine the levels of general, tumor-specific and relapse-free survival and the construction of Kaplan–Mayer graphs.

RESULTS: According to the results of the analysis, there was a significant decrease in survival in groups with high CD68 and CD163 expression rates ($p < 0.05$). In the study groups, there was a significantly significant correlation between high levels of CD68 and CD163 ($p < 0.05$).

CONCLUSIONS: CD68 and CD163 can act as independent markers of predicted survival in patients with muscle-invasive bladder cancer after radical cystectomy.

Keywords: bladder cancer; radical cystectomy; macrophages; immunohistochemical diagnostics; CD68; CD163.

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Научная статья

Иммуногистохимическая диагностика макрофагов, ассоциированных с опухолью, у пациентов с мышечно-инвазивным раком мочевого пузыря после радикальной цистэктомии

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АННОТАЦИЯ

Актуальность. Серьезная проблема онкоурологии — рак мочевого пузыря. Золотым стандартом лечения мышечно-инвазивного рака мочевого пузыря выступает радикальная цистэктомия с предшествующей неоадъювантной химиотерапией. К сожалению, эффективность радикальных методов лечения резко ограничена в долгосрочной перспективе. По этой причине исследования в области прогнозирования выживаемости могут существенно улучшить отдаленные онкологические результаты. Большой перспективой обладает определение уровней макрофагов, ассоциированных с опухолью.

Цель — определение влияния уровней экспрессии макрофагов, ассоциированных с опухолью, на показатели выживаемости у пациентов с диагнозом мышечно-инвазивный рак мочевого пузыря после радикальной цистэктомии.

Материалы и методы. Исследование было проведено на базе Клиники ФГБОУ ВО «Башкирский государственный медицинский университет» в период с 01.05.2021 по 01.07.2023. В исследовании принимали участие 66 пациентов с установленным диагнозом «мышечно-инвазивный рак мочевого пузыря». После оперативного лечения осуществлялось гистологическое и иммуногистохимическое исследование с определением уровней CD68 и CD163. По истечении 24 мес. проводился анализ выживаемости с определением уровней общей, опухолеспецифической и безрецидивной выживаемости с построением графиков Каплана – Майера.

Результаты. По результатам анализа наблюдается достоверное снижение выживаемости в группах с высокими показателями экспрессии CD68 и CD163 ($p < 0,05$). В исследуемых группах наблюдается достоверно значимая корреляция между высокими уровнями CD68 и CD163 ($p < 0,05$).

Заключение. CD68 и CD163 могут выступать в качестве независимых маркеров прогнозируемой выживаемости у пациентов с мышечно-инвазивным раком мочевого пузыря после радикальной цистэктомии.

Ключевые слова: рак мочевого пузыря; радикальная цистэктомия; макрофаги; иммуногистохимическая диагностика; CD68; CD163.

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BACKGROUND

Malignant neoplasms of the urinary system are among the most common among cancers worldwide, with bladder cancer (BC) representing a serious problem. Recent data reveal that BC causes approximately 213,000 deaths annually worldwide. Moreover, the incidence tends to increase annually; accordingly, BC ranks 10th currently [1]. This situation is due to not only an increase in the population's cancer alertness and quality of diagnostic procedures but also to BC being a polyetiological disease affecting patients of different ages. BC is classified into nonmuscle-invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC), and the criterion for diagnosing the malignant process is the absence or presence of invasion into the muscle layer of the bladder. This classification is actively used by practicing oncologists and urologists because of the fundamental differences in treatment regimens depending on the BC type. The clinical presentations of these forms also vary significantly. In contrast to NMIBC, MIBC is characterized by rapid progression and aggressive course with the development of regional and distant metastases. In BC, MIBC is diagnosed in approximately 25% of cases, with a 5-year survival rate of approximately 50% [2].

The gold standard treatment for high-risk MIBC and NMIBC is radical cystectomy with pelvic lymphadenectomy and prior neoadjuvant platinum-based chemotherapy. For male patients, the scope of the surgical intervention includes the excision of the bladder, paravesical tissue, prostate gland, and seminal vesicles in a single block. For female patients, the scope of the surgical intervention consists of supralevator pelvic evisceration. Despite the radical principle of surgical treatment and compliance with the principles of oncological safety, neoadjuvant chemotherapy is recommended [3]. For some patients, if the preoperative treatment was ineffective, adjuvant chemotherapy was recommended [4]. Unfortunately, the long-term effectiveness of radical treatment methods is sharply limited, and according to world literature, the survival rate has not significantly increased over the past 30 years [5]. Thus, research into predicting the survival value in MIBC may improve significantly long-term oncological outcomes. For this purpose, the use of biological tumor markers, which affect directly the treatment outcomes, is justified. The stratification of patients into groups according to the levels of biological predictors will allow a personalized approach to the treatment of MIBC.

Over the past decades, vast knowledge has been gained in the molecular biology of malignant processes. Moreover, the study of the microenvironment of tumors of various localizations is of particular interest in this field. Tumor-associated macrophages (TAMs) are among the most significant in this cytological structure. TAMs are widely represented in the microenvironment of solid tumors and can function depending on the signals they receive [6–8]. In this regard, TAMs can have two different functional states, namely, the M1 and M2 phenotypes [9]. According to current data, when the neoplastic process is initiated, macrophages surrounding the pathological focus have the M1 phenotype. Moreover, they are activated by the influence of interferon-gamma, interleukin-12, and interleukin-23 [9] and are programmed to eliminate tumor cells and induce a Th-1 immune response. By acquiring a proinflammatory and antitumor phenotype, M1 macrophages are involved in local immune reactions in response to the neoplastic process [10]. As a malignant tumor progresses, M1 macrophages transform into M2 macrophages, which have an alternative activation pathway because of oncogenic signals [11]. Such macrophages have anti-inflammatory and protumor activity, induce a Th-2 immune response, and have an immunosuppressive effect. Thus, M2 macrophages contribute to tumor evasion from immune surveillance and ensure further propagation of the neoplastic process [12]. The activation of multiple signaling pathways in the tumor microenvironment also affects TAMs, resulting in a constant transformation of M1 phenotype to M2. Thus, M2 macrophages are the most common representatives of TAMs in the tumor microenvironment. The change in phenotypes characterizes TAMs as a rapidly changing component of the tumor microenvironment, which can be used to assess and predict the malignant process [13]. TAMs constantly secrete various cytokines and chemokines (programmed death-ligand 1 [PD-L1], arginase 1, interleukin-10, and transforming growth factor-β) and express specific receptors (CD68 and CD163) on their surface [14–18]. Determining the levels of surface antigens (CD68 and CD163) using immunohistochemical analysis is the most reliable method for diagnosing TAMs [19, 20].

This study aimed to investigate the relationship between TAM levels in patients diagnosed with MIBC who underwent radical cystectomy with pelvic lymphadenectomy and postoperative survival rates.

MATERIALS AND METHODS

The study was conducted at the Clinic of the Bashkir State Medical University from 05/01/2021 to 07/01/2023. All study participants were informed and signed a voluntary consent. The study was approved by the local independent ethical committee of the Bashkir State Medical University of the Ministry of Health of Russia dated June 15, 2021, No. 4595–07. The study enrolled 66 patients diagnosed with MIBC based on cystoscopy/transurethral resection biopsy. Data on the presence of distant metastases were not obtained. The experimental group included 42 (63.6%) men and 24 (36.4%) women,

and the average patient age was 68.3 ± 2.4 years. The stages of the oncological process and tumor grade are presented in Table 1.

Cisplatin-based neoadjuvant chemotherapy was used in 50 (75.8%) patients. Radical cystectomy with pelvic lymphadenectomy was indicated for all patients. The border of the lymph node dissection was the aortic bifurcation, and in the presence of severe lymph node damage, dissection was performed to the level of the origin of the inferior mesenteric artery. The surgical material was subsequently sent for histological examination to determine the stage of the malignant neoplasm in accordance with the postsurgical tumor, lymph node, metastase (pTNM) classification and the grade according to the World Health Organization classification (1973). The material was fixed in a 1% neutral formalin solution and embedded in paraffin. Sections of 5 μm thick were prepared on a Leica microtome (Germany) and stained with hematoxylin and eosin. Subsequently, a morphological analysis was performed to determine the stage and degree of tumor differentiation. Immunohistochemical analysis was performed using a Bond-maX apparatus (Australia). For this purpose, serial paraffin sections of 5 μm thick were used with the application of the streptavidin–biotin method. Commercial monoclonal antibodies CD68 and CD163, which are macrophage markers, were used for the study. After the reaction with monoclonal antibodies, microslide images were digitized using a Leica Aperio scanner (Germany). The Digital Pathology software package was used to evaluate expression. The intensity of the expression was assessed semiquantitatively on a point scale, ranging from 0 to 3, taking into account the severity of staining, where 0 implied no reaction; 1, weak reaction; 2, moderate reaction; and 3, strong reaction.

Twenty-four months after the surgery, a survival analysis was performed to determine the overall, tumor-specific, and relapse-free survival rates and construct Kaplan–Meier plots. Statistica 10.0 software (StatSoft Inc., USA) was used for the statistical analysis. The experimental groups were compared using the Mann–Whitney *U*-test and Student's *t*-test. The log-rank test was used to compare survival curves. The level of significance when processing statistical data was considered critical at $p < 0.05$.

RESULTS

The surgical material from all patients was subjected to a pathomorphological review. The differentiation of the stages and degrees of the tumor process is presented in Table 2.

After an immunohistochemical study, the expression level of membrane antibodies was determined. Subsequently, the patients were distributed into two groups according to the staining intensity for CD68 and CD163, where group 1 showed the absence or a low level of expression, whereas group 2 had moderate or a high level of expression. The data obtained are presented in Table 3. Patients with high CD163 expression levels also had high CD68 expression levels ($p < 0.05$); however, two patients with a high CD68 expression level also had low CD163.

Twenty-four months after radical surgeries, the overall, tumor-specific, and relapse-free survival rates were analyzed by plotting Kaplan–Meier curves in the study groups. Survival results are presented in Figs. 1–6.

The analysis results revealed a significant decrease in the survival rates in groups with high expression levels of CD68 and CD163 ($p < 0.05$). Two patients, who had high CD68 expression levels and low CD163 expression levels, did not have relapses and were withdrawn from follow-up because of death. In the studied groups, a significant correlation was found between high CD68 and CD163 expression levels ($p < 0.05$). Thus, CD68 and CD163 may be independent markers of predicted survival in patients with MIBC who underwent radical cystectomy. The findings demonstrate the value of using tumor-associated

Table 1. Characteristics of bladder tumors in patients before radical cystectomy ($n = 66$)

Таблица 1. Характеристика опухолей мочевого пузыря у пациентов до радикальной цистэктомии ($n = 66$)

Disease stage	Number of patients, *n*
Clinical stage	
cT2	36 (54.5%)
cT3	18 (27.3%)
cT4	12 (18.2%)
Grade (biopsy)	
G1	8 (12.1%)
G2	32 (48.5%)
G3	26 (39.4%)

Table 2. Characteristics of bladder tumors in patients after pathomorphological examination (*n* = 66)
Таблица 2. Характеристика опухолей мочевого пузыря у пациентов после патоморфологического исследования (*n* = 66)

Parameter	Number of patients, *n*
Stage of bladder cancer according to histology results	
pT1	8 (12.1%)
pT2	30 (45.5%)
pT3	16 (24.2%)
pT4	12 (18.2%)
Grade (surgical material)	
pG1	12 (18.2%)
pG2	28 (42.4%)
pG3	26 (39.4%)
Regional lymph nodes	
pN0	22 (33.3%)
pN+/pNx	44 (66.7%)

Table 3. Expression levels of membrane antibodies CD68 and CD163
Таблица 3. Уровень экспрессии мембранных антител CD68 и CD163

Parameter	Group 1	Group 2	*p*-value
CD68			
Number of patients, *n*	30 (42.4%)	36 (57.6%)	<0.05
Expression intensity	0.7 ± 0.3	2.4 ± 0.6	<0.05
Neoadjuvant chemotherapy, *n*	16 (32%)	34 (68%)	<0.05
CD163			
Number of patients, *n*	32 (45.5%)	34 (54.5%)	<0.05
Expression intensity	0.6 ± 0.4	2.3 ± 0.7	<0.05
Neoadjuvant chemotherapy, *n*	18 (36%)	32 (64%)	<0.05

macrophage markers to estimate postoperative survivability. Moreover, survival rates may be affected by concomitant diseases diagnosed before surgery, as well as adjuvant chemotherapy performed in the postoperative period. In this study, no patients received adjuvant chemotherapy. Conversely, 50 (75.8%) patients received neoadjuvant chemotherapy, which also significantly affected the survival rates. Moreover, no statistically significant differences were noted in the distribution of patients receiving neoadjuvant chemotherapy between the groups ($p < 0.05$); therefore, the influence of this factor on the analysis results was not significant. The study results revealed a predominance of TAMs in patients with worse survivability, which may be due to the increased potential of M2 macrophages and increased protumor activity.

DISCUSSION

Despite significant advances in recent years in the surgical treatment of MIBC, the prognosis for patients who underwent radical cystectomy remains virtually unchanged [21]. The stratification of patients by risk groups is necessary to determine subsequent treatment and diagnostic measures. The prospect of using biological predictors also involves predicting responses to neoadjuvant chemotherapy and determining indications for adjuvant treatment [22]. The estimation of the predicted survival rate may allow for a more personalized approach to the choice of subsequent treatment methods, which will improve long-term oncological outcomes.

Sun et al. [23] analyzed the levels of infiltration, TAM polarization, and their effect on the prognosis

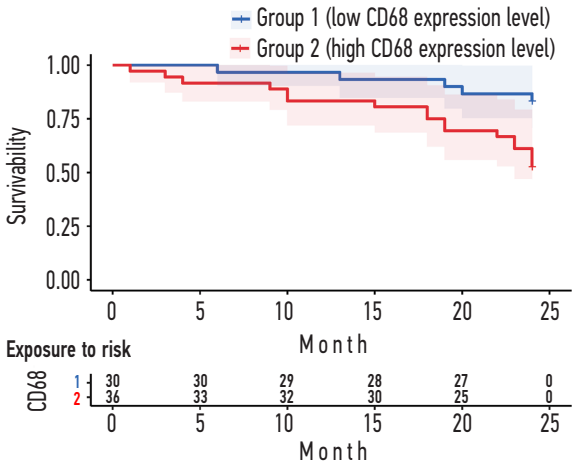

Fig. 1. Overall survival and CD68 expression levels, $p = 0.0096$
Рис. 1. Общая выживаемость и уровни экспрессии CD68, $p = 0{,}0096$

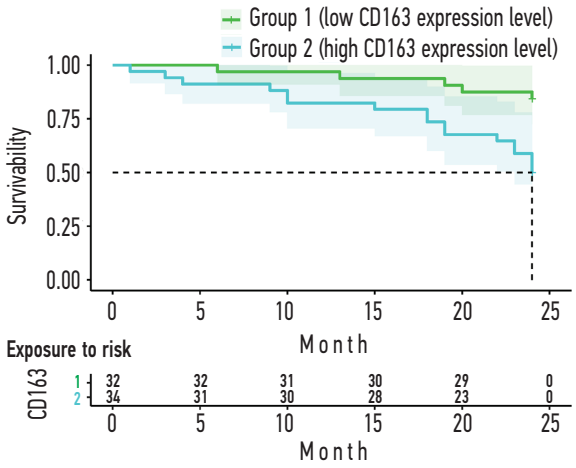

Fig. 2. Overall survival and CD163 expression levels, $p = 0.0033$
Рис. 2. Общая выживаемость и уровни экспрессии CD163, $p = 0{,}0033$

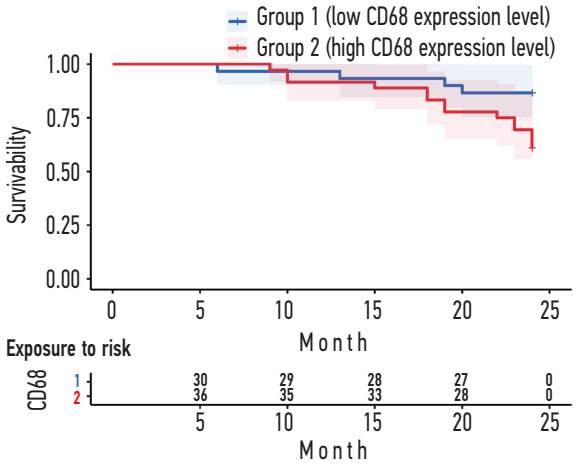

Fig. 3. Tumor-specific survival and CD68 expression levels, $p = 0.028$
Рис. 3. Опухолеспецифическая выживаемость и уровни экспрессии CD68, $p = 0{,}028$

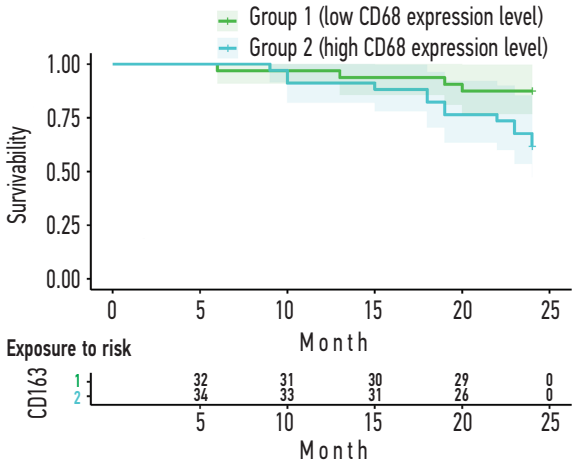

Fig. 4. Tumor-specific survival and CD163 expression levels, $p = 0{,}022$
Рис. 4. Опухолеспецифическая выживаемость и уровни экспрессии CD163, $p = 0{,}022$

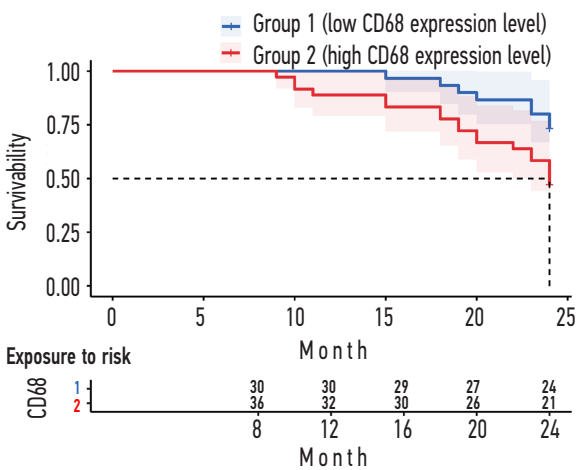

Fig. 5. Disease free survival and CD68 expression levels, $p = 0.025$
Рис. 5. Безрецидивная выживаемость и уровни экспрессии CD68, $p = 0{,}025$

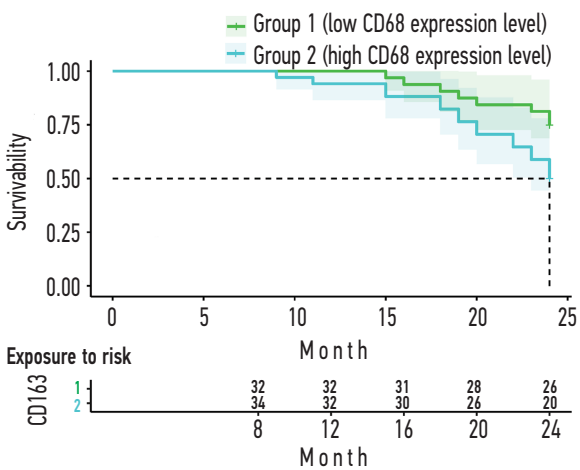

Fig. 6. Disease free survival and CD163 expression levels, $p = 0.037$
Рис. 6. Безрецидивная выживаемость и уровни экспрессии CD163, $p = 0{,}037$

and expected response to adjuvant chemotherapy and PD-L1 inhibitor therapy in MIBC. They revealed that patients with elevated M1 macrophages had a better response to immunotherapy, whereas infiltration by M2 macrophages contributed potentially to immunotherapy resistance. Moreover, they noted that tumors with significant TAM infiltration and an M2-like configuration were characterized by immunosuppression, with an abundance of oncogenic immune cells and immunosuppressive cytokines. In another large study, Taubert et al. [24] examined the prognostic effect of TAM levels with responses to adjuvant chemotherapy and predicted survivability in patients with MIBC who underwent radical cystectomy. In that study, monoclonal antibodies CD68, CD163, and CCL2 were used to diagnose TAM. The authors noted the high prognostic significance of CD68 for stratifying patients according to the level of response to chemotherapy, taking into account pT and pN stages. Moreover, high TAM levels correlated with worse survival rates but better responses to adjuvant chemotherapy. Koll et al. [2] analyzed the relationship between TAM levels and tumor-infiltrating immune cells with survival rates and responses to therapy in 101 patients who underwent surgery between 2010 and 2020. The study used multiplex immunohistochemistry and multispectral imaging. Antibodies CD68 and CD163 were used to verify TAMs. They noted a high correlation between CD68 and CD163 levels, although some cells expressed CD163 in the absence of CD68. Increased TAM infiltration and high expression levels of CD68 and CD163 were associated with worse survivability in the study groups. The researchers noted the promise of using TAMs as biological predictors and stated the need for a more accurate characterization of TAM and M1/M2 polarization. Regarding the routine use of immunohistochemical diagnostics of TAMs by determining the expression levels of CD68 and CD163 using digital analysis, the authors believed that this method is highly reliable [2].

The results correspond to the global experience in this field. The present study also has some limitations. The experimental group did not include patients receiving courses of adjuvant chemotherapy. For this reason, the possible prognostic effect of TAM levels on therapeutic response was not assessed. The distribution of pTNM stages and the presence of concomitant diseases in the experimental groups were also not considered, which could affect the TAM levels and survivability after surgical treatment.

CONCLUSION

This study demonstrates worse survival rates in patients with MIBC who underwent radical cystectomy and had high expression levels of CD68 and CD163. A significant correlation was noted between these TAM markers. More detailed characterization of TAMs enables the identification of subsets of macrophages that have prognostic significance and may be potential targets in the treatment of BC.

ADDITIONAL INFORMATION

Authors' contribution. Thereby, all authors made a substantial contribution to the conception of the study, acquisition, analysis, interpretation of data for the work, drafting and revising the article, final approval of the version to be published and agree to be accountable for all aspects of the study. Personal contribution of each author: V.N. Pavlov — research design development, manuscript editing; M.F. Urmantsev — collection of material, analysis of the data obtained; M.R. Bakeev — collecting material, analyzing the data obtained, writing the text of the manuscript.

Competing interests. The authors declare that they have no competing interests.

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Ethics approval. The present study protocol was approved by the local Ethics Committee of the Bashkir State Medical University (reference number: 4595-07 15 June 2021).

Consent for publication. Written consent was obtained from the patients for publication of relevant medical information within the manuscript.

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