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Prognostic value of the levels of CTLA-4 and its ligand B7.2 in patients with colorectal cancer

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ABSTRACT

Aim. To develop a computer program to determine the probability of colorectal cancer based on the assessment of the levels of CTLA-4 and its ligand B7.2.

Materials and methods. The study included 44 patients with colorectal cancer (CRC) and 25 patients with benign tumors of the colon. The control group consisted of 25 individuals who had been operated for colon injury. We determined the levels of CTLA-4 and B7.2 in the blood serum and in the supernatants of tumor tissue and lymph node homogenates using flow cytofluorometry.

Results. We found that the level of CTLA-4 in the blood serum increased by 2.77 times in CRC patients compared to the control group ($p < 0.001$). The concentration of CTLA-4 in the tumor tissue in patients with CRC was 2.34 times higher than in the control group ($p = 0.007$). The concentration of the B7.2 ligand in the blood serum of patients with CRC exceeded this parameter in the control group by 2.51 times ($p = 0.002$). The concentration of B7.2 in the tumor tissue of CRC patients was 1.68 times higher ($p = 0.004$) than in the control group. The analysis of the obtained data determined the parameters that have prognostic value in the structure of the diagnostic model. Using these parameters, we developed a computer program to determine the probability of CRC in the patient.

Conclusion. The data obtained demonstrate an increase in the levels of CTLA-4 and its ligand B7.2 in the serum and tumor tissue of patients with CRC.

Keywords: immune checkpoints, CTLA-4, B7.2, colorectal cancer

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Conformity with the principles of ethics. All patients signed an informed consent to participate in the study. The study was approved by the local Ethics Committee at Chita State Medical Academy (Protocol No. 98 of 27.11.2019).

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Прогностическое значение уровня белка CTLA-4 и его лиганда B7.2 у больных раком толстого кишечника

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РЕЗЮМЕ

Цель. Разработать программу для определения вероятности онкологической патологии толстого кишечника на основании оценки уровня белка CTLA-4 и его лиганда B7.2.

Материалы и методы. В исследование включены 44 пациента с колоректальным раком (КРР) и 25 больных с доброкачественными опухолями толстого кишечника. Контрольную группу составили 25 пациентов, оперированных в плановом порядке (пластика колостомы), сформированной ранее по поводу травмы толстой кишки. Концентрацию CTLA-4 и B7.2 определяли в сыворотке крови, а также в супернатантах гомогенатов ткани опухоли и лимфатических узлов с помощью метода проточной цитофлуометрии.

Результаты. Установлено, что у пациентов с раком толстой кишки уровень CTLA-4 в сыворотке крови увеличивается в 2,77 раза в сравнении с группой контроля ($p < 0,001$). Концентрация CTLA-4 в ткани новообразования у пациентов с КРР была выше аналогичного показателя группы контроля в 2,34 раза ($p = 0,007$). Концентрация лиганда B7.2 в сыворотке крови у пациентов с КРР превышала данный показатель в группе контроля в 2,51 раза ($p = 0,002$). Концентрация лиганда B7.2 в ткани опухоли у пациентов с КРР превышала таковую в группе контроля в 1,68 раза ($p = 0,004$). При анализе полученных данных определены параметры, которые имеют значимость в структуре диагностической модели. На основании этих параметров разработана компьютерная программа для определения вероятности наличия онкологической патологии толстого кишечника.

Заключение. Полученные данные демонстрируют увеличение уровня CTLA-4 и его лиганда B7.2 в сыворотке крови и ткани опухоли у пациентов с колоректальным раком.

Ключевые слова: иммунные контрольные точки, CTLA-4, B7.2, колоректальный рак

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

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INTRODUCTION

Colorectal cancer (CRC) is one of the late-diagnosed tumors. Moreover, it ranks third worldwide for mortality among malignant neoplasms [1, 2]. Its ability to escape the immune surveillance plays an essential role in the development and growth of the tumor. To escape the immune surveillance, tumor cells use certain molecular pathways, known as immune checkpoints (ICP) [3, 4]. The main function of ICP is to regulate immune processes and prevent the activated immune system from attacking cells indiscriminately [5]. These data made it possible to develop a new type of targeted immunotherapy for cancer based on blocking ICP [6].

Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4, CD152) is an ICP mainly expressed by T cells [7, 8]. B 7.2 (CD86) is CTLA-4 ligand. The interaction between CTLA-4 and this ligand is an important mechanism in the immunosuppressive regulation of T cell activity [9–11]. The mechanism of this regulation is triggered when CTLA-4 captures B 7.2

from the surface of an antigen-presenting cell (APC) or cancer cell and transfers them into the T lymphocyte via transendocytosis [12]. A number of studies devoted to the analysis of the effectiveness of monoclonal antibodies to CTLA-4 demonstrated objective positive responses in cases of breast cancer, melanoma, and kidney cancer [11]. However, studies confirming the effective use of monoclonal antibodies to CTLA-4 in patients with CRC are insufficient.

The aim of our research was to study the level of CTLA-4 and B7.2 in the blood serum and tumor tissue, as well as to assess the diagnostic value of these parameters in patients with CRC.

MATERIALS AND METHODS

A total of 44 patients with CRC were included in the study. The comparison group consisted of 25 patients with benign neoplasms of the colon who were treated at the Regional Oncology Dispensary in Chita from 2019 to 2020. The control group consisted of 25 patients admitted to the Regional Clinical Hospital for elective surgery (reconstruction of colostomy) due to prior colon injuries. All the patients were examined in accordance with the clinical guidelines approved by the Ministry of Healthcare of Russia [13]. In each case, a patient signed an informed consent. The study was approved by the Ethics Committee of Chita State Medical Academy of the Ministry of Healthcare of the Russian Federation and complied with the requirements of the Declaration of Helsinki of the World Medical Association (2013). Inclusion criteria were a patient's consent to participate in the study and a history of colon tumor. Exclusion criteria were HIV-positive status; autoimmune diseases; viral and bacterial infections; chemotherapy or radiation treatment before surgery.

A histologic examination of tumor tissue specimens showed that 39 CRC patients (88.6%) had moderately differentiated adenocarcinoma (G2). Well differentiated adenocarcinoma (G1) was diagnosed in three cases (6.8%). Two CRC patients (4.6%) had poorly differentiated adenocarcinoma (G3). Six patients had stage I of the disease, 24 patients were diagnosed with stage II. Stages III and IV were diagnosed in eight and six patients, respectively.

Blood sampling was carried out in the morning, 2 hours before the surgery. The day before sampling, patients received standard preoperative medication. Biopsies of the tumor tissue, lymph node tissue, and colon specimens in the control group weighing up to 1 gram were homogenized using the Ultra-Turrax T 10 basic homogenizer (IKA, Germany) in phosphate-buffered saline (pH 7.4). Then they were centrifuged at 5,000 rpm for 10 minutes, and a supernatant was selected. The concentration of CTLA-4 and B7.2 in the blood serum and tissue homogenate supernatant was determined by flow cytofluorometry on the CytoFlex LX analyzer (Beckman Coulter, USA) using the LEGENDplex™ HU multiplex immunoassay panel (Immune Checkpoint, USA) in accordance with the manufacturer's instructions.

In the statistical analysis, we followed the recommendations of the International Committee of Medical Journal Editors (ICMJE) and Statistical Analysis and Methods of Published Literature (SAMPL) Guidelines [14, 15]. Nominal data were described in absolute and relative values. The Pearson's chi-square test ($\chi 2$) was used to compare the nominal data of the study, which allowed to assess the significance of differences between the actual number of outcomes or qualitative characteristics of the sample falling into each category and the theoretical number that can be expected in the studied groups if the null hypothesis is valid [16].

As the number of participants in the study groups was less than 50, we used the Shapiro – Wilk test to assess the normality of the distribution of quantitative variables. Taking into account that the distribution of characteristics in all groups was different from normal, the data obtained were presented in as the median and the interquartile range Me [Q_1; Q_3]. The Kruskal – Wallis H test was performed to compare three independent groups in terms of one quantitative characteristic. If there were statistically significant differences, a pairwise comparison was performed using the Mann – Whitney U test with the Bonferroni correction [17]. The Spearman's rank correlation coefficient was used to measure correlations between the studied parameters. The strength of the relationship between the studied parameters was determined by the Chaddock scale [18].

The diagnostic model was constructed by binary logistic regression. To determine the value of the model, we applied the ROC analysis, which made it possible to assess the sensitivity, specificity, and accuracy of the model. Statistical processing of the research results was carried out using the IBM SPSS Statistics Version 25.0 software package (International Business Machines Corporation, USA).

RESULTS

We found that the level of CTLA-4 in the blood serum in CRC patients increased by 2.77 times compared to the control group ($U = 119.0$, $p < 0.001$). There were

no significant differences in the level of CTLA-4 in the blood serum of CRC patients and those with benign tumors of the colon (Table 1). The data obtained indicate that an increase in the concentration of CTLA-4 in the blood serum may be a marker of a colon tumor, but it does not allow to determine its nature.

Table 1

CTLA-4 level in patients with colon tumor, pg / ml, *Me* [Q_1; Q_3]

Parameter	Group			Test statistics, df = 2
	Control group, *n* = 25	Benign tumor, *n* = 25	Colorectal cancer, *n* = 44	
Blood serum	4.88 [4.38; 6.22]	10.48 [10.30; 14.50]	13.50 [13.07; 20.80]	$H = 34.26$, $p < 0.001$
Tumor tissue	6.06 [6.03; 8.40]	9.42 [8.84; 11.37]	14.17 [13.72; 27.28]	$H = 8.82$, $p = 0.012$

Note: *H* – the Kruskal–Wallis *H* test, *p* – the achieved level of significance (here and in Table 2).

Similar changes were observed in the study of the CTLA-4 concentration in the tumor tissue. The level of CTLA-4 in the tumor tissue was 2.34 times higher in CRC patients compared to the control group ($U = 334.0$; $p = 0.007$). This parameter in CRC patients was 1.5 times higher than in patients with benign colon tumors ($U = 371.0$; $p = 0.02$) (Table 1). The results obtained allowed to note that an increase in the CTLA-4 concentration was associated with the nature of the tumor. However, these statistically significant data are of no practical interest, since a histologic examination of the obtained material can determine the nature of a neoplasm.

The concentration of the B7.2 ligand in the blood serum of CRC patients was 2.51 times higher than in the control group ($U = 302.5$; $p = 0.002$). We also found that the levels of B7.2 in the blood serum of patients with colon cancer and patients with benign colon tumors had no statistically significant differences (Table 2).

Table 2

B7.2 level in patients with colon neoplasms, pg / ml, *Me* [Q_1; Q_3]

Parameter	Group			Test statistics, df = 2
	Control group, *n* = 25	Benign tumor, *n* = 25	Colorectal cancer, *n* = 44	
Blood serum	33.00 [30.08; 40.11]	79.00 [78.68; 97.46]	82.93 [76.70; 113.26]	$H = 23.08$, $p < 0.001$
Tumor tissue	37.09 [34.11; 44.34]	40.40 [43.36; 48.90]	62.31 [61.74; 79.93]	$H = 9.96$, $p = 0.007$

The data obtained demonstrate an increase in the concentration of B7.2 in patients with colon tumors. However, it is impossible to differentiate between a benign and a malignant tumor by analyzing the concentration of this biological marker.

A similar increase in the parameters was noted in the tumor tissue. The concentration of the B7.2 ligand in the tissue in CRC patients was 1.68 times higher than in the control group ($U = 319.0$; $p = 0.004$). The level of B7.2 in CRC patients was 1.54 times higher than in patients with benign colon tumor ($U = 387.0$; $p = 0.04$) (Table 2). The concentration of the B7.2 ligand was increased in the tumor tissue, as was the CTLA-4 level in the tissue. The data obtained allow to differentiate between a benign and a malignant neoplasm in the colon.

We determined the concentration of CTLA-4 and B7.2 in the tissue of regional lymph nodes in CRC patients. The level of CTLA-4 in the lymph node tissue was 132.22 [117.36; 174.40] pg / ml; the concentration of B7.2 was 537.35 [466.76; 650.84] pg / ml.

The analysis of the data obtained indicated that the level of CTLA-4 in the blood serum had a moderate correlation with the level of CTLA-4 in the tissue ($\rho = 0.37$; $p < 0.01$). No statistically significant correlations were found for B7.2 ligand. The level of B7.2 in the blood serum did not correlate with its level in the tumor tissue ($\rho = 0.008$; $p = 0.94$). At the same time, a pronounced correlation was revealed between the levels of CTLA-4 and B7.2 in the blood serum ($\rho = 0.57$; $p < 0.01$). At the same time, there was a weak direct correlation between the described membrane molecules in the tissue ($\rho = 0.28$; $p = 0.004$), which confirmed the role of B7.2 as a ligand for CTLA-4, and not as a separate biomarker.

When analyzing the data, we identified parameters that may be essential in the diagnostic model for determining the probability of cancer (Table 3) and obtained the following equation:

Table 3

Value of CTLA-4 and B7.2 in the structure of the diagnostic model

Parameter	B	Root mean square error	Wald test	Degree of freedom, df	Significance, p	Exp (B)	95% CI for Exp B
CTLA-4 in the blood serum	0.42	0.132	9.98	1	0.002	1.52	1.17–1.96
B7.2 in the blood serum	0.03	0.013	3.78	1	0.05	1.03	1.01–1.05
Constant	–3.25	0.968	11.28	1	0.001	0.04	–

Note: CI – confidence interval.

$$C = \frac{1}{1+e^{3,25-0,42*CTLA4ser-0,03*B7.2ser}},$$

where CTLA-4ser is the level of the CTLA-4 protein in the blood serum; B7.2ser is the level of B7.2 in the blood serum; 3.25 is the constant of the logistic regression level; 0.42 and 0.03 are non-standardized B coefficients, e-exponent ~ 2.72. When the coefficient C is ≥ 0.59, the development of a tumor in the colon is diagnosed. In the control group, this parameter (C) was 0.40 [0.36; 0.50], it was 0.86 in the patients with cancer [0.82; 0.87]. The controls were found to have C ≥ 0.59 in 20% of cases (5 / 25), CRC patients had C ≥ 0.59 in 94.2% (65 / 69) of cases (Sensitivity of this conclusion is 0.94, specificity and accuracy are 0.80 and 0.90, respectively (AUC = 0.88 [95% CI 0.79–0.97], $p < 0.001$) (Figure).

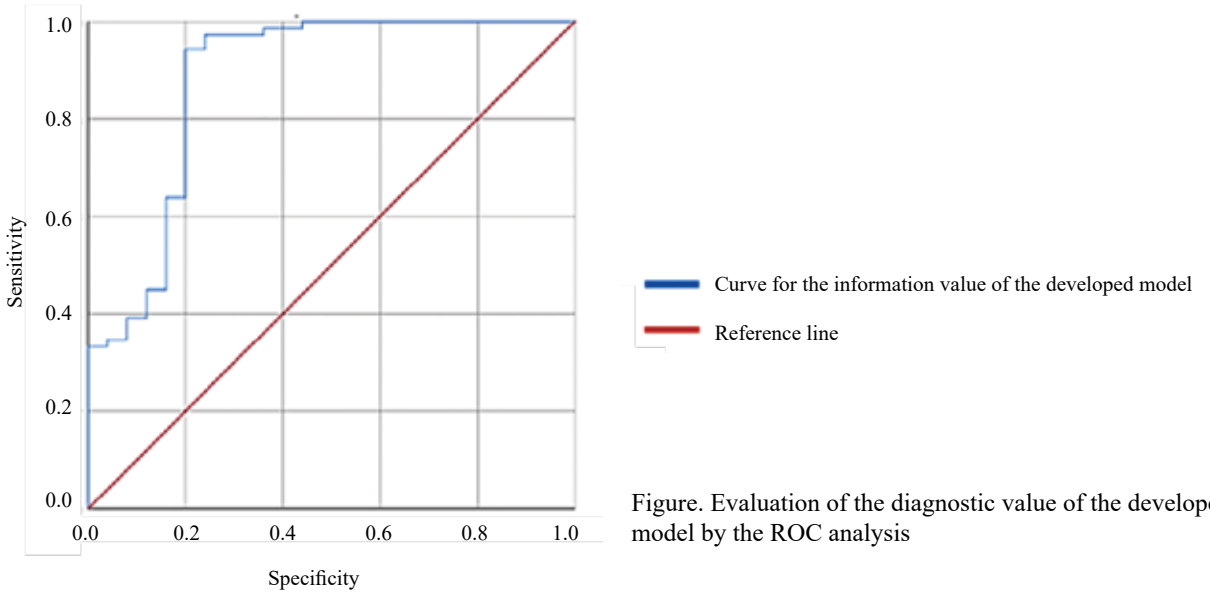

Figure. Evaluation of the diagnostic value of the developed model by the ROC analysis

The described method can be used in both outpatient departments and surgical hospitals to predict the presence of cancer in the colon. We developed a program for the Windows-based Object Pascal development environment (Borland Delphi) to simplify the method when used in clinical practice. A set of actions is created in a special mode of the user window, in which the user gets access to entering data on the level of CTLA-4 (pg / ml) and its B7.2 ligand in the blood serum (pg / ml) in patients with complaints of functional intestinal disorders. The program is applicable and provides an opportunity to determine the probability of cancer in the colon, which makes it possible to identify a risk group and optimize the strategy for their examination and treatment [19].

DISCUSSION

We found that the levels of CTLA-4 and B7.2 increased in the blood serum of CRC patients. There were no significant differences between the concentrations of CTLA-4 and B7.2 in the blood serum of CRC patients and patients with benign intestinal neoplasms. The data obtained demonstrate that an increase in the level of CTLA-4 and B7.2 indicates the presence of a colon tumor, but it is impossible to determine whether it is malignant or benign.

Similar changes in their concentration were observed in the study of these markers in the tumor tissue. The levels of CTLA-4 and B7.2 in the tumor tissue in CRC patients were higher than in the control

group. In addition, the concentration of these markers in CRC patients was increased in comparison with patients with a benign colon tumor.

We also determined cut-off values for CTLA-4 and B7.2 markers in the blood serum, which are significant in the structure of the diagnostic model. The developed computer program makes it possible to suspect a colon tumor, which will allow to form risk groups and optimize the examination strategy.

Similar data on high CTLA-4 expression in the tumor tissue were obtained in patients with breast cancer and cholangiocarcinoma [20, 21]. This fact indicates that the presence of CTLA-4 in the tumor microenvironment is one of the markers of immune suppression development, which contributes to the growth and spread of tumor cells. We believe that the CTLA-4 protein is an important link in the pathogenesis of cancer cells escaping from the immune surveillance. X.J. Guo (2021) discussed the role of the CTLA-4 protein in the activation of regulatory T cells (Treg), which are the strongest inhibitors of the immune response [21]. Therefore, the role of CTLA-4 in the activation of regulatory T cells in malignant neoplasms of various localizations requires further comprehensive studies.

CONCLUSION

The data obtained demonstrate an increase in the levels of CTLA-4 and B7.2 in the blood serum and tumor tissue in CRC patients, as well as their direct correlation with each other. Thus, we suppose that these proteins are essential in the pathogenesis of cancer cells escaping from immune surveillance in colon cancer. Based on the established patterns, we developed a computer program to determine the probability of colon cancer in the patient.

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