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Predictive value of inflammatory regulators TGFb1 and CXCL8 in tumor tissue in colorectal cancer

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ABSTRACT

Background. Colorectal cancer is ranked third in terms of incidence and second in terms of mortality around the world. Molecular markers of chemoresistance allow to determine the prognosis of the disease and sensitivity of the tumor to drugs.

Aim. To assess the predictive value of expression of regulators of tumor-associated inflammation TGFb1 and CXCL8 in the tumor tissue in colorectal cancer.

Materials and methods. Patients were divided into 3 groups: group I included patients without relapse of the disease, group II encompassed patients with relapse of the disease (within 6–16 months after the end of chemotherapy), group III included patients with disease progression. Expression of TGFb1 and CXCL8 in the tumor tissue before treatment in patients with stage II–III colorectal cancer (n = 77) was determined using quantitative real-time polymerase chain reaction (PCR) on the Bio-Rad CFX-96 Touch Real-Time PCR Detection System (USA). Statistical data processing was performed using Statistica 13.0 software (StatSoft, USA).

Results. We found that in samples of poorly differentiated colorectal cancer, the level of TGFb and CXCL8 mRNA was significantly higher than in moderately and well differentiated tumors. We did not reveal any relationship of the level of TGFb1 and CXCL8 transcripts in tumor samples of patients with stage II–III colorectal cancer with age and the presence of mutations in the EGFR (Epidermal Growth Factor Receptor) signaling pathway (RAS, BRAF). We found a strong positive correlation between the levels of TGFb1 and CXCL8 transcripts for the entire sample of patients with colorectal cancer. We have found that the expression of *TGFb1* and *CXCL8* genes was significantly higher in the tumor tissue of patients with disease progression.

Conclusion. Overexpression of *TGFb1* and *CXCL8*, which are involved in the mechanism of tumor-associated inflammation, can be considered as a negative prognostic factor for the progression-free interval when using the FOLFOX / XELOX regimen for the treatment of colorectal cancer.

Keywords: colorectal cancer, CXCL8, TGFb1, EGFR, tumor progression

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Предиктивная значимость регуляторов воспаления TGFb1 и CXCL8 в опухолевой ткани при колоректальном раке

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РЕЗЮМЕ

Колоректальный рак (КРР) по заболеваемости в мире находится на 3-м месте и на 2-м – по смертности. Молекулярные маркеры химиорезистентности позволят определять прогноз заболевания и чувствительность опухоли к лекарственным препаратам.

Цель. Оценить предиктивную значимость экспрессии факторов TGFb1 и CXCL8 – регуляторов опухоль-ассоциированного воспаления в опухолевой ткани при КРР.

Материалы и методы. Пациенты были разделены на три группы: I – без рецидива, II – с рецидивом (в течение 6–16 мес после окончания химиотерапии), III – с прогрессированием заболевания. Экспрессию TGFb1 и CXCL8 в опухолевой ткани до начала лечения пациентов с КРР на II–III стадии ($n = 77$) определяли с использованием количественной полимеразной цепной реакции в реальном времени на амплификаторе CFX-96 BioRad (США). Статистическая обработка данных выполнена с использованием программного обеспечения Statistica 13.0 (StatSoft, США).

Результаты. В образцах низкодифференцированных опухолей при КРР уровень мРНК TGFb и CXCL8 был существенно выше, чем в опухолевых образцах с умеренной и высокой дифференцировкой. Зависимости уровня транскриптов TGFb1 и CXCL8 в образцах опухоли у пациентов на II–III стадии КРР от возраста и наличия мутаций EGFR (Epidermal Growth Factor Receptor) сигнального пути (RAS, BRAF) не выявлено. Установлена положительная сильная корреляционная связь между уровнями транскриптов TGFb1 и CXCL8 для всей выборки пациентов с КРР. Экспрессия генов *TGFb1* и *CXCL8* значимо выше в опухолевой ткани пациентов с прогрессированием заболевания.

Заключение. Гиперэкспрессия *TGFb1* и *CXCL8*, участвующих в механизме опухоль-ассоциированного воспаления, может рассматриваться как негативный фактор прогноза времени без прогрессирования при использовании схемы FOLFOX/XELOX лечения колоректального рака.

Ключевые слова: колоректальный рак, TGFb1, CXCL8, EGFR, опухолевая прогрессия

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INTRODUCTION

Colorectal cancer (CRC) is a malignant tumor that develops in the colonic and rectal mucosa. CRC is ranked third in terms of incidence and second in terms of mortality around the world [1]. Molecular markers of chemoresistance can be used for early diagnosis of CRC, assessment of patient prognosis, and prediction of tumor sensitivity to chemotherapy. In ordinary cells, transforming growth factor beta 1 (TGFb1) stimulates production of collagen and fibronectin, reducing secretion of enzymes that are responsible for degrading the extracellular matrix [2]. At different stages of malignant transformation in colonic epithelial cells, TGFb1 acts both as a suppressor and a promoter of tumor growth [3]. TGFb1 is involved in inhibition of cell proliferation, induces apoptosis and angiogenesis, and has immunosuppressive effects [4–7]. Previous studies have shown a relationship between a high level of TGFb1 in the blood serum in patients with CRC and a poor disease prognosis [8]. TGFb1 is also involved in the epithelial – mesenchymal transition (EMT) [9–11].

It has been shown that CRC cells can produce interleukin (IL)-8 (IL-8 / CXCL8), which mediates neutrophil chemotaxis [12]. Activated neutrophils secrete CXCL8, which can interrupt the apoptotic effect of Bcl-2, prolong the presence of neutrophils in the tumor stroma, and block the anti-inflammatory effect of factors [13, 14]. CXCL8 is also involved in tumor vascularization [15].

Due to conflicting literature data on the role of inflammatory mediators in carcinogenesis, the aim of the study was to assess the predictive value of TGFb1 and CXCL8 expression in the tumor tissue in CRC.

MATERIALS AND METHODS

A retrospective study was carried out at Ulyanovsk Regional Clinical Oncology Center and Research Medical and Biological Center of Ulyanovsk State University from 2014 to 2020. The study protocol was approved by the Ethics Committee at the Institute of Medicine, Ecology, and Physical Education of Ulyanovsk State University (Protocol No. 9 of 15.09.2014).

Detailed characteristics of patients are given in Table 1.

Table 1

Characteristics of patients with colorectal cancer included in the study, *n* = 77

Parameter	Number of patients
Gender:	
– male;	42
– female	35
Age, years:	
– 25–44	9
– 45–59	40
– 60–75	28
Stage of the disease:	
– II;	16
– III;	37
– IV	24
Assessment of regional lymph node metastasis (N):	
–N0;	34
–N1;	44
–N2	16
Degree of tumor differentiation:	
– poorly differentiated;	7
– moderately differentiated;	44
– well differentiated	26
Tumor location (side):	
– left-sided	59
right-sided	18
The presence of mutations in the EGFR signaling pathway:	
– nRAS;	5
– kRAS;	21
– BRAF;	5
– undefined	12
Family history of the disease:	
– yes;	12
– no;	49
– undefined	16
Polychemotherapy according to the FOLFOX / XELOX regimen:	
– adjuvant;	53
– palliative	24
Assessment of prevalence of stage II–III primary tumor:	
– T_2	3
– T_3	34
– T_{4a}	9
– T_{4b}	7
Presence of negative prognostic factors (stage II–III tumors):	
– yes	23
– no	30

Treatment efficacy was evaluated every 2 months (after 4 courses of FOLFOX / 2 courses of XELOX), as well as after completion of all chemotherapy courses. The examination plan included: complete blood count and blood biochemistry, urinalysis, assessment of serum carcinoembryonic antigen (CEA) and carbohydrate antigen (CA 19-9) levels, chest radiography in two projections, abdominal, pelvic, and retroperitoneal ultrasound, and endoscopic methods (fiberoptic colonoscopy, if indicated). In case of doubtful results of standard examination methods, contrast-enhanced multislice computed tomography (MSCT) or magnetic resonance imaging (MRI)

of the chest, abdomen, and pelvis was performed. At the end of treatment, patients were followed up by an oncologist, with periodic health checkups in accordance with standard WHO guidelines.

Depending on the response to FOLFOX / XELOX chemotherapy regimens, the patients were divided into 3 groups: group I included patients without relapse of the disease (more than 3 years after the end of chemotherapy), group II encompassed patients with relapse of the disease (within 6–16 months after the end of chemotherapy), group III included patients with disease progression during chemotherapy.

A molecular genetic study of formalin-fixed paraffin-embedded (FFPE) tumor samples by polymerase chain reaction (PCR) was performed in the following way. Histological sections of tumors containing at least 80% of cancer cells were used as biomaterial for the study. Sections of tissue blocks obtained from resection margins of the same tumors were taken as a conditional norm. DNA / RNA was isolated from FFPE blocks from 10–15 μm-thick tumor sections (with a total area of at least 2 cm^2) using SileksMagNA magnetic particles (Kit KIRFFPE0100, Sileks LLC, Moscow, Russia).

Using the QuantumDNA-211 kit, the concentration of DNA isolated from paraffin-embedded colorectal tissue and suitable for amplification was determined, and the presence of PCR inhibitors in the sample was identified. In 92% of cases, the samples did not contain PCR inhibitors and had a concentration of DNA fragments suitable for PCR. Furthermore, using the Insider NRAS-3 and Insider KRAS-2 Mutation Detection Kits (StepOne Plus, Evrogen Lab, Moscow, Russia), the presence of RAS mutations was determined. To analyze mutations in the *BRAF* (*V600E*) gene in tumor DNA samples, the kit manufactured by Syntol (Moscow, Russia) was used. For the transcript analysis, a reverse transcription PCR was performed immediately after the isolation. Then quantitative real-time PCR was performed in triplets on the Bio-Rad CFX-96 Touch Real-Time PCR Detection System (USA) using the DNA intercalating dye SYBR. Primer sequences synthesized at Evrogen Lab are given in Table 2. The *GAPDH* gene was used as a housekeeping gene. Normalized expression of target genes with respect to the housekeeping gene was calculated using the Bio-Rad CFX Manager Software [16].

Statistical data processing was performed using Statistica 13.0 (StatSoft, USA). Non-normally distributed variables were compared using the nonparametric Mann – Whitney test and the Pearson's correlation coefficient. To analyze regression for overall and relapse-free survival, the Cox regression model and the Kaplan – Meier analysis were used. The data were presented as the median and the interquartile range *Me* (Q_1–Q_3).

Table 2

Primer sequences in the studied genes [17]

Studied gene	Sequence	Primer annealing temperature, °C
TGFb1	F5'-CGA CTC GCC AGA GTG GTT AT -3' R 5'- AGT GAA CCC GTT GAT GTC CA-3'	59
CXCL8	F5'- CTC CAA ACC TTT CCA CCC C -3' R5'-GAT TCT TGG ATA CCA CAG AGA ATG - 3'	60
GAPDH	F5'-GCA CCG TCA AGG CTG AGA AC - 3' R5' -TGG TGA AGA CGC CAG TGG A - 3'	59

RESULTS

Following our studies, we found that the level of TGFb1 mRNA in the tumor in patients with stage II–III CRC did not depend on patient's age and the presence of mutations in the EGFR signaling pathway (RAS, BRAF). Pronounced differences in the levels of TGFb1 mRNA in CRC were detected in poorly differentiated tumors (Table 3).

A strong positive correlation was found between the levels of TGFb1 and CXCL8 transcripts in all CRC samples (r = 0.730; Rho = 0.852; p = 0.00001) (Fig. 1).

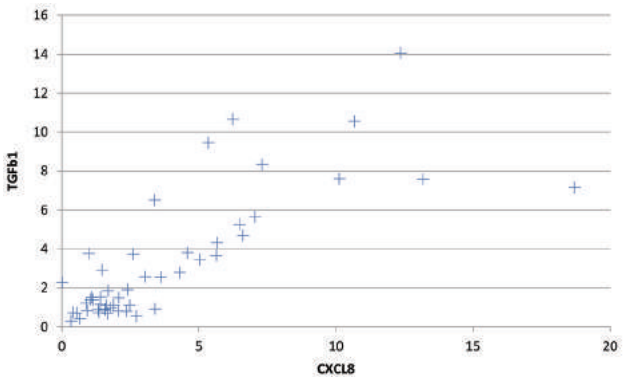

Fig. 1. Scatterplot (Pearson's correlation coefficient) of TGFb1 and CXCL8 mRNA values in the tumor in CRC patients

Table 3

Transcript level in *TGFb1* and *CXCL8* genes in FFPE colorectal cancer samples, *Me* (Q_1–Q_3)

Parameter	Normalized expression of *TGFb1* in colorectal cancer samples	Normalized expression of *CXCL8* in colorectal cancer samples
Patient's age: – over 55 years; – under 55 years	 1.845 (0.910–3.906) 2.702 (0.895–4.145) $p = 0.690$	 1.968 (1.127–5.114) 2.210 (1.549–4.997) $p = 0.560$
CRC stage: – II – III	 2.558 (1.427–7.167) 1.490 (0.867–3.769) $p = 0.114$	 2.446 (1.469–5.348) 2.212 (1.320–5.657) $p = 0.819$
Degree of tumor differentiation: – poorly differentiated; – moderately differentiated; – well differentiated	 7.168 (4.120–12.553) 2.568 (1.856–6.345) 1.427 (0.809–2.628) $p_1 = 0.035$, $p_2 = 0.023$	 8.770 (1.127–15.114) 2.262 (1.454–6.872) 1.408 (0.849–2.997) $p_1 = 0.004$, $p_2 = 0.012$
The presence of mutations in the EGFR signaling pathway (RAS, BRAF): – yes; – no	 1.630 (0.840–3.843) 2.578 (1.12–4.411) $p = 0.371$	 1.597 (1.107–3.224) 2.822 (1.647–5.294) $p = 0.246$

Note: the nonparametric Mann – Whitney test was used; the differences between two independent groups were assessed; the differences were statistically significant at $p \leq 0.05$.

It was found that the expression of TGFb1 in the tumor differed significantly in groups of patients with CRC, depending on the tumor response to standard chemotherapy. In the group of patients with disease progression during chemotherapy, the levels of TGFb1 mRNA were higher than in the group of CRC patients with relapse of the disease (within 6–16 months after the end of chemotherapy – group II) and the group of patients without relapse of the disease (for more than 2 years) – group I ($p_1 = 0.009$; $p_2 = 0.0007$). A similar trend was observed when the level of CXCL8 mRNA was analyzed (Fig. 2). Overexpression of CXCL8 in CRC was observed in the group of patients with disease progression (group III) ($p_1 = 0.0008$; $p_2 = 0.001$).

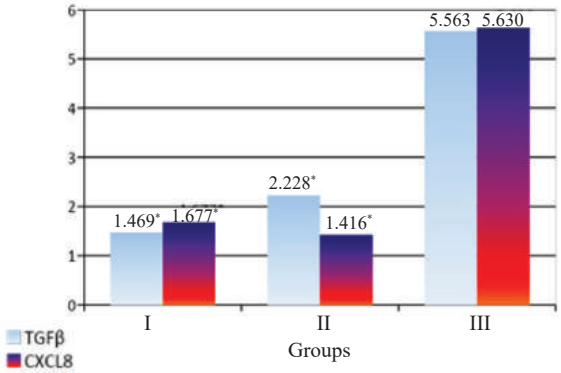

Fig. 2. TGFb1 and CXCL8 transcript levels in tumors of CRC patients depending on the tumor response to chemotherapy: * data are significantly different from those in group III ($p \leq 0.05$)

The Cox regression analysis showed that the progression-free survival depended on the expression of *TGFb1* in the primary tumor ($\chi 2 = 8.158$; $p = 0.0043$). The Kaplan – Meier analysis of relapse-free survival (PFS) in CRC patients also showed the effect of *TGFb1* expression on PFS. In the group of patients with tumor *TGFb1* expression of more than 2 (group I), the follow-up median was 11.3 months versus 62.9 months (group 0) (log-rank-test, $p = 0.041$) (Fig. 3).

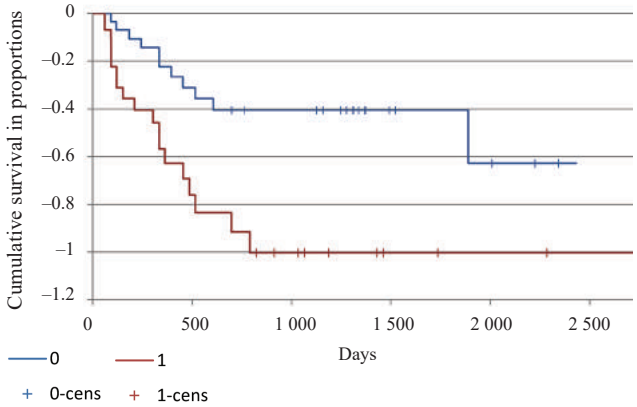

Fig. 3. Progression-free survival curve in CRC patients depending on the expression of TGFb1 in the tumor

DISCUSSION

Ambiguous functioning of TGFb1 in malignant transformation and tumor progression may be explained by the fact that, besides the two main pathways in

which TGFb is involved [18], the cytokine contributes to a number of signaling cascades, which are linked through activation of TGFb-EGFR proteins [19, 20]. During CRC progression, mutation-associated inactivation of the TGFb1 signaling pathway occurs. TGFb1 is believed to inhibit tumor growth in the intestine due to inactivation of TGF beta receptors (TGFb-R1 and R2) or intracellular SMADs (SMAD 2 / 3 / 4) [21]. Cells that lack signals from TGFb1 increase production of proinflammatory cytokines and thereby cause transformation of colonic epithelium [22, 23].

Our data on the increase in TGFb1 mRNA expression in group III with a decrease in tumor differentiation confirm the results of studies by A. Calon et al. (2012) on more frequent cancer relapses, advanced cancer stage at diagnosis, and reduced survival of patients with colon cancer [24]. The loss of the ability to suppress tumor growth (group III), which accompanies TGFb1 overexpression, determines cell selection for survival in CRC. In turn, secretion of chemokines in the tumor activates immune infiltration in the tissue and promotes migration of cancer cells to the vessels, accelerating angiogenesis. The observed coexpression of *TGFb1* and *CXCL8* genes in the CRC samples may indicate a relationship between the factors involved in the control over proliferation (TGFb1) and proinflammatory microenvironment, in particular CXCL8, during progression of CRC [24]. A shorter relapse-free interval during chemotherapy in patients with overexpression of *TGFb1* and *CXCL8* can be explained by the fact that TGFb1 protects cancer cells from apoptosis by activating the ErK signaling pathway [25].

Therefore, we found significant differences in the levels of TGFb1 and CXCL8 expression in the tumor tissue of CRC patients depending on the tumor response to chemotherapy, tumor differentiation, and the duration of the progression-free interval during FOLFOX / XELOX chemotherapy.

CONCLUSION

Overexpression of *TGFb1* and *CXCL8*, which are involved in activating the mechanisms of tumor-associated inflammation, can be considered as a negative prognostic factor for progression-free survival using the FOLFOX / XELOX treatment regimen for CRC.

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Bogomolova I.A. – selection of the clinical site for the analysis. Antoneeva I.I. – conception and design. Myagdieva I.R., Abakumova T.V. – analysis and interpretation of the data. Dolgova D.R. – justification of the manuscript and critical revision of the manuscript for important intellectual content. Peskov A.B. – selection of the methods for the statistical analysis. Gening T.P – final approval of the manuscript for publication.

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