

EXPERIMENTAL WORKS

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PATTERN OF EXPRESSION OF IMMUNE- AND STROMA-ASSOCIATED GENES IN BLOOD OF MICE WITH EXPERIMENTAL B16 MELANOMA

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*The interaction between malignant and stromal cells represents a major cross-talk pathway upon carcinogenesis. Cellular elements of the reactive tumor stroma are a heterogeneous population which are represented specifically by cancer-associated fibroblasts (CAFs) and tumor-associated macrophages (TAM). It is not known whether expression of CAF- and TAM-associated genes could be detected in the peripheral blood of cancer patients to monitor a course of disease. The aim of the study was to assess the relative expression (RE) of cancer-related genes in peripheral blood of mice with experimental melanoma. Quantitative PCR was used to determine RE of 15 genes in the blood of C57BL/6j control mice and mice with injected B16 melanoma cells. The Kruskal-Wallis and the Fischer exact tests with correction on multiple comparisons, according to the Benjamini-Hochberg procedure with FDR = 0.2 were used for statistical analysis. Analysis of 15 immune and stromal markers RE showed differentiated expression of several CAF and TAM markers in mice with experimental melanoma in comparison with the control animals. Thus, CAF markers *Acta2*, *Cxcl14*, *Fap* and TAM markers *Cd68*, *Ccl22* and *Ccl17* were significantly upregulated, while *Cd4*, *Cd3* were downregulated. This, together with increased expression of *Cox-2* suggested a stable immunosuppressive state of mice with experimental melanomas. The results of the study showed that potential markers of cancer-associated fibroblasts and tumor-associated macrophages in peripheral blood of mice with experimental melanoma could be used for non-invasive detection of melanoma cell progression.*

Key words: melanoma, relative gene expression, cancer-associated genes, cancer-associated fibroblasts, tumor-associated macrophages.

The interaction between cancerous and stromal cells represent a major cross-talk pathway upon carcinogenesis, influencing tumor growth and metastasizing [1, 2]. Cellular elements of the reactive tumor stroma are a heterogeneous population which are represented by fibroblasts, cancer-associated fibroblasts (CAFs), macrophages, tumor-associated macrophages (TAM), pericytes, endothelial cells, mesenchymal stem cells, dendritic cells and also other cells of the immune system, such as T-lymphocytes (Th1, Th2, Th17, T_{reg}), B-cells,

eosinophils, basophils, neutrophils, NK-cells, etc. [3-6]. The major focus in the studies on reactive tumor stroma is aimed at the identification of the CAFs and analysis of their features, due to the fact, that they interact directly with tumor cells, regulating tumor growth and spread, being the main “sculptor” of the extracellular matrix [7-9]. Unlike normal fibroblasts, CAFs show distinct properties, including increased proliferation rate, enhanced collagen production, secretion of growth factors, stimulation of tumor cell proliferation upon co-culturing [10].

Moreover, CAFs differ from normal fibroblasts by expression of a number of specific markers, including Smooth muscle α -actin (*ACTA2*), Vimentin (*VIM*), Fibroblast specific protein (*FSP*), Fibroblast activation protein (*FAP*), Thrombosporin-1 (*TSP-1*), Tenascin with (*TNC*), Platelet-derived growth factor- α and β (*PDGFRA* and *PDGFRB*) receptors and others [11-13]. In addition, they express Matrix metalloproteinases-1 and -3 (*MMP1*, *MMP3*), produce collagen and release cytokines (*IL-6*, *IL-8*) and chemokines (*CXCL12*, *CXCL8*, *CXCL14*, *CCL5*, *CCL2*, *CCL7*), which leads to the activation of tumor growth and metastasizing [6, 8, 14, 15]. Recent studies showed a regulatory function of CAF in recruiting of monocytes into a tumor development zone, polarization of M2-like macrophages, providing an immune-suppressive role in particular, through the PD-1 axis [16]. Thus, TAMs is yet another important cellular element of the tumor stroma; they are markers of poor prognosis [17, 18]. TAMs change their metabolism and interact with CAFs; they take part in the reconstruction of the extracellular matrix, which allows both, the local spread of tumor cells and metastasizing (dissemination) [19, 20].

We have to mention that all these findings describe characteristics of solid tumors. It is not known whether expression of CAF- and TAM-associated genes could be detected in the peripheral blood or other biological fluids of cancer patients, to monitor and/or predict a course of the disease as a non-invasive assessment. Therefore, we selected a set of CAF- TAM- and immune-markers to monitor their expression in the blood of animals with experimental melanomas, to identify putative non-invasive markers of tumor growth.

Material and Methods

Cell line. B16 mouse melanoma cell line was obtained from the Bank of Cell Lines (R. E. Kavetsky IEPOR, NAS of Ukraine). Cells were cultured in a DMEM (Sigma) medium with the addition of 10% FBS (Sigma), 100 units/ml penicillin and 100 μ g/ml streptomycin at 37 °C in a CO₂ incubator. Cells were collected after treatment with EDTA/trypsin solution and rinsed in Phosphate-buffered saline (PBS). 2 \times 10⁵ of B16 cells were injected into one mouse.

Experimental animals. The cell suspension was injected subcutaneously to the right posterior paw of the adult female mice of a C57BL/6j line. Intact C57BL/6j females served as a control group. Both the control group and mice with melanomas

consisted of five animals. The blood was collected for analysis on the day 19th after melanoma cells were injected. All experiments were carried out, according to European Convention for the Protection of Vertebrate Animals used for experimental and other scientific purposes (Strasbourg, 1986) and followed the rules of handling of experimental animals, approved by Bioethics Committee of IMBG NAS of Ukraine.

Total RNA isolation and cDNA synthesis. 100 μ l of whole mice blood was thoroughly mixed with 300 μ l Trizol (Sigma). A total RNA was isolated by Direct-zol RNA MiniPrep total RNA kit (Zymo Research), according to the manufacturer's protocol. The quality and the concentration of the total RNA was determined by the mean of a spectrophotometer (NanoDrop Technologies Inc., USA). cDNA synthesis was performed, using the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, USA), according to the manufacturer's protocol.

Quantitative PCR (qPCR). Relative gene expression (RE) levels were determined, using a 5xHOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne, Estonia) and a Bio-Rad CFX96 Real-Time PCR Detection System (USA) with the following program: denaturation at 95 °C for 12 min, and then 40 cycles (95 °C – 15 s, 60 °C – 20 s, 72 °C – 20 s) as described earlier [21, 22]. Primers were selected, using the software at <https://www.ncbi.nlm.nih.gov/tools/primer-blast/> and <https://primerdepot.nci.nih.gov/> and the site <https://www.origene.com>. The reference *TBP* gene was used to normalize RE levels, using the 2^{- Δ Ct} and 2^{- $\Delta\Delta$ Ct} methods, as described earlier [22].

Statistical analysis. A statistical analysis was performed using a STATISTICA10 software for the Kruskal-Wallis and the Fischer exact tests with correction on multiple comparisons according to the Benjamini-Hochberg procedure with FDR = 0.2 [23].

Results and Discussion

Experimental tumors, induced after injection of B16 melanoma cells, were detected by palpation. The first tumors were observed at the days 12th-13th. By the day 19, tumors increased to a volume of 4.0 cm³ and reached the maximal allowed size (8.5 cm³) in 28 days. We have chosen the day 19th to perform our experiments.

We assessed the RE levels of 15 immune-associated genes and stromal markers in mouse blood. Namely, there were markers of various populations

of lymphocytes and tumor-associated macrophages (*Cd3*, *Cd4*, *Cd8*, *Cd68*, *Cd163*, *Nos2*, *Ccl17*, *Ccl22*, *Ctla4*, *Cox-2*), and also genes characteristic for tumor-associated fibroblasts (*Acta2*, *Fap*, *Cxcl12*, *Cxcl14*, *Hif1a*). The levels of the above-mentioned genes RE in mice with experimental melanomas (T) and in control mice (C) were compared using the $2^{-\Delta Ct}$ method (Fig.).

As is seen from Figure, all studied genes showed moderate and low expression levels.

The statistical analysis of RE levels in mice with experimental melanomas and the control animals allowed to identify 6 genes that were significantly up-regulated (Table 1) and 2 genes that were downregulated (Table 2) in animals.

More than two-fold increase/decrease of RE levels was considered as the significant changes. The *Cox-2* (*Ptgs1*) gene, encoding cyclooxygenase 2, showed the highest RE level in peripheral blood of animals with experimental melanomas. It catalyzes the first step of prostaglandin synthesis and is associated with inflammatory diseases and cancer [24]. *Cox-2* is expressed in many types of human tumors and its elevated expression is associated with poor prognosis [25, 26].

Table 1. A list of genes, showing increased relative expression in mice with experimental melanomas compared with the control animals

No	Genes	RE, increase	P-value
1	<i>Cox-2</i>	22.237	$P < 0.0001$
2	<i>Ccl22</i>	5.486	$P < 0.0001$
3	<i>Cd68</i>	3.165	$P < 0.0001$
4	<i>Fap</i>	2.417	$P < 0.001$
5	<i>Cxcl14</i>	2.260	$P < 0.001$
6	<i>Ccl17</i>	2.854	$P < 0.05$
7	<i>Acta2</i>	1.925	$P = 0.055$

Table 2. A list of genes, showing decreased relative expression in mice with experimental melanomas compared with the control animals

No	Genes	RE, decrease	P-value
1	<i>Cxcl12</i>	0.235	$P < 0.001$
2	<i>Cd4</i>	0.486	$P < 0.050$
3	<i>Cd8</i>	0.518	$P = 0.055$
4	<i>Cd163</i>	0.540	$P = 0.057$
5	<i>Cd3</i>	0.629	$P = 0.059$
6	<i>Nos2</i>	0.638	$P = 0.059$

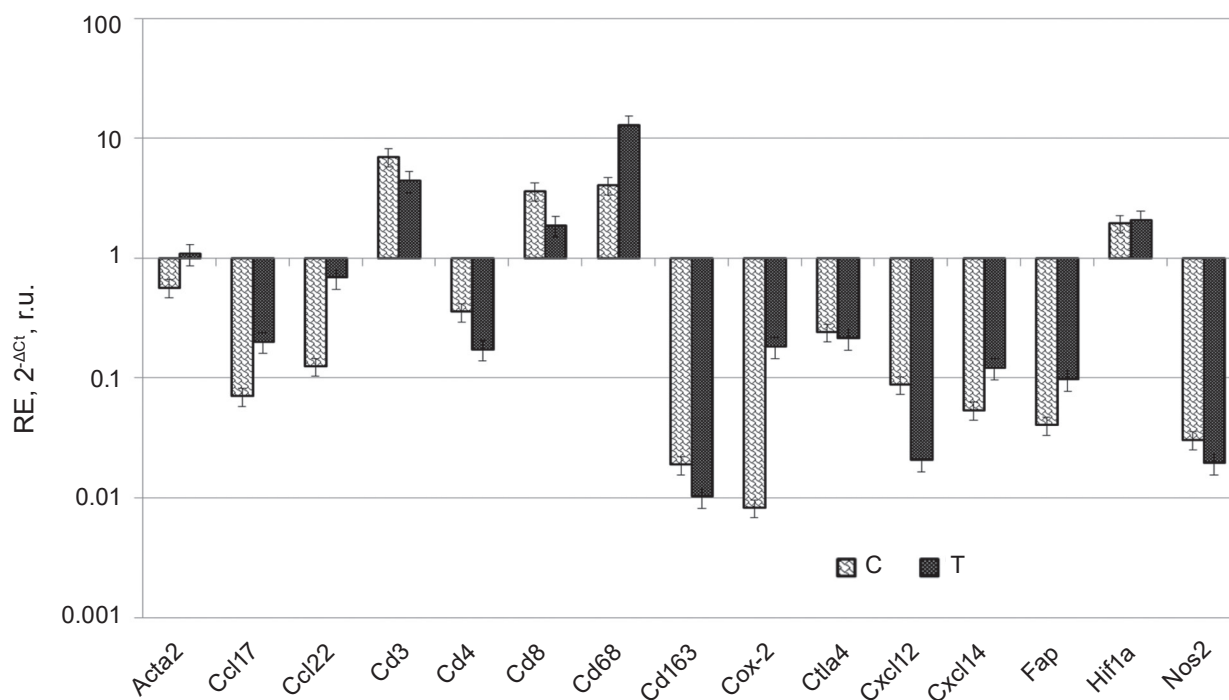


Fig. RE levels of cancer-associated genes in the blood of the control animals (C) and in mice with experimental melanoma (T)

Cox2 contributes to angiogenesis, tissue invasion and resistance to apoptosis [27, 28]. In addition, COX2 promotes tumor evasion from immune reaction and cancer resistance to immunotherapy [29]. Increased COX2/PGE2/E-prostaglandin receptor signaling may suppress dendritic cells, natural killer (NK) and T cells, responsible for immunosuppression upon tumor progression [30]. *COX2* gene is expressed in both cancerous cells and tumor-associated fibroblasts and macrophages [31, 32]. Our earlier study on prostate cancer showed a positive correlation between RE of *COX2*, CAF and lymphocytes markers, and correlated negatively with prostate cancer and epithelial cell markers [33]. Noteworthy, *Cox-2* relative expression was the highest increase in (more than 20 fold, Table 1, 2) indicating the necessity of COX-2 inhibitors using for melanoma treatment in addition to well-known immunotherapy methods.

Noteworthy, *Ccl22*, *Cd68* and *Ccl17* were significantly upregulated as well. The RE of *Ccl22* gene, encoding immunosuppressive chemokine was increased more than 5 fold. Usually, it is expressed by tumor-associated macrophages [34] and promotes migration of cancerous cells through the CCL22-CCR4 axis [35, 36]. Another marker of tumor-associated macrophages, CD68, showed more than 3-folds increase in mice with experimental melanomas. It is known that CD68 contributes to the establishment of immunosuppressive micro-environment upon tumor progression [37]. The third TAM marker, *Ccl17*, is closely connected with *Ccl22* in the CCL2-CCR2 pathway, which is involved in the regulation of tumor metastasizing [35].

We have revealed earlier the increased RE of other TAM genes *Cd14*, *Tlr8* and *Il1b* in mice with experimental melanomas [38]. This could enhance metastatic spread and immunosuppression [39, 40]. Increased RE of *Tlr8* suggests that the special endotype of TAM rising, namely M2c [41].

The other group of genes showing increased RE in mice with injected melanoma cells consists of CAF markers, namely *Cxcl14*, *Fap* and *Acta2*. It is quite unexpected results, as there are no much data on CAF expression in animal with melanomas. The circulating cancer cells were found in the blood under condition of tumor progression and metastasizing [42, 43], however, the presence of tumor-associated fibroblasts and macrophages in the blood was demonstrated only in several studies [44, 45]. According to the literature data, this phenomenon can be observed at the later stages of tumor progres-

sion, i.e. in advanced cancers. As a rule, circulating CAFs express *FAP* and *ACTA2* simultaneously [44]. In prostate cancer tissue *CXCL14* showed the highest RE levels in CAFs, especially at the late stages and upon metastasizing [46]. This suggests that CAFs play a vital role in the dissemination and metastasizing of melanoma cells to other organs and systems, which is not a typical and well-known fact [44, 45]. It is known that CAFs are a highly abundant and heterogeneous cell population of mesenchymal lineage, which could disseminate to other organs to prepare specific cell niche for metastasizing tumor cells [47, 48].

Expression of *Cd3*, *Cd4*, *Cd8* is associated with different T cell phenotypes, especially, tumor-infiltrating lymphocytes. Decreased expression of these markers in blood indicates reduced immune response [49].

Our data showed that tumor growth was accompanied by CAF and TAM detection in peripheral blood of mice with experimental melanoma. TAM and CAF markers as putative markers of tumor growth and metastasizing could be used for non-invasive detection of melanoma cells.

Conflict of interest. Authors have completed the Unified Conflicts of Interest form at http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

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ПАТЕРНИ ЕКСПРЕСІЇ ІМУНО- ТА СТРОМАЛЬНО-АСОЦІЙОВАНИХ ГЕНІВ У КРОВІ МИШЕЙ З ЕКСПЕРИМЕНТАЛЬНОЮ МЕЛАНОМОЮ В16

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Клітинні елементи пухлинної строми є гетерогенною популяцією, представленою, зокрема, пухлиноасоційованими фібробластиками (ПАФ) та пухлиноасоційованими макрофагами (ПАМ). Залишається невідомим, чи можна вия-

вити експресію ПАФ- та ПАМ-асоційованих генів у периферійній крові хворих на рак для моніторингу перебігу захворювання. Метою роботи було оцінити відносну експресію (ВЕ) пухлиноасоційованих генів у периферійній крові мишей з експериментальною меланою. Кількісна ПЛР була використана для встановлення рівнів ВЕ 15 генів у крові інтактних C57BL/6j мишей та мишей із введеними В16 клітинами меланоми. Тести Краскала-Уолліса та точний тест Фішера з поправками на множинні порівняння за процедурою Бенжаміні-Хохбера з FDR = 0,2 були використані для статистичного аналізу.

Аналіз ВЕ 15 генів виявив диференційну експресію маркерів пухлиноасоційованих фібробластів (ПАФ) та макрофагів (ПАМ) у групі мишей із введеними клітинами меланоми порівняно з контрольними тваринами. Так, рівні експресії маркерів ПАФ *Acta2*, *Cxcl14*, *Fap* та ПАМ маркери *Cd68*, *Ccl22* та *Ccl17* були істотно підвищеними, тоді як ВЕ *Cd4*, *Cd3* та *Cd8* були значно зниженими. Ці дані разом з підвищеною експресією імуносупресивного маркера *Cox-2* свідчив про стійкий імуносупресивний стан експериментальних тварин із введеними клітинами меланоми. Виявлено найбільше посилення ВЕ *Cox-2* (більш ніж у 20 разів), що демонструє необхідність використання COX-2 інгібіторів для лікування меланоми на додаток до відомих імунотерапевтичних методів. Одержані дані вказують, що ПАФ та ПАМ у периферичній крові мишей з експериментальними меланомами можуть бути потенційними маркерами для неінвазивного детектування прогресії пухлин.

Ключові слова: меланома, відносна експресія генів, пухлиноасоційовані гени, пухлиноасоційовані фібробласти, пухлиноасоційовані макрофаги.

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