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.
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 μg/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). 2×10⁶ 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 μl of whole mice blood was thoroughly mixed with 300 μl 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^ΔΔCt and 2^ΔCt 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 B16 cells.
of lymphocytes and tumor-associated macrophages (Cd3, Cd4, Cd8, Cd68, Cd163, Nos2, Ccl17, Ccl22, Ctda4, 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-ΔΔCt 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  | Cox-2   | 22.237       | P < 0.0001 |
| 2  | Ccl22   | 5.486        | P < 0.0001 |
| 3  | Cd68    | 3.165        | P < 0.0001 |
| 4  | Fap     | 2.417        | P < 0.001  |
| 5  | Cxcl14  | 2.260        | P < 0.001  |
| 6  | Ccl17   | 2.854        | P < 0.05   |
| 7  | Acta2   | 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  | Cxcl12  | 0.235        | P < 0.001 |
| 2  | Cd4     | 0.486        | P < 0.050 |
| 3  | Cd8     | 0.518        | P = 0.055 |
| 4  | Cd163   | 0.540        | P = 0.057 |
| 5  | Cd3     | 0.629        | P = 0.059 |
| 6  | Nos2    | 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 microenvironment 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 endothyme 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 progression, 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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вити експресію ПАФ- та ПАМ-асоційованих генів у периферійній крові хворих на рак для моніторингу перебігу захворювання. Метою роботи було оцінити відносну експресію (ВЕ) пухлино- асоційованих генів у периферійній крові миші з експериментальною меланомою. Кількісна ПЛР була використана для встановлення рівнів ВЕ 15 генів у крові інтактних C57BL/6j миші та миші із введенням В16 клітинами меланоми. Тести Краскела-Уолліса та точний тест Фішера з поправками на множинні порівняння за процедурою Бенжамін–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 миші із введенням клітинами меланоми порівняно з контрольними тваринами. Так, рівні експресії маркерів ПАФ Acta2, Cxcl14, Fap та ПАМ маркери Cd68, Ccl2 та Ccl17 були істотно підвищеніми, тоді як ВЕ Cd4, C3 та Cd8 були значно зниженими. Ці дані з підвищеною експресією імуносупресивного маркера Cox-2 свідчать про стійкий імуносупресивний стан експериментальних тварин із введеннями клітинами меланоми. Виявлено найбільше посилення ВЕ Cox-2 (більше 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 периферійній крові миші з експериментальними меланомами можуть бути потенційними маркерами для неінвазивного детектування прогресії пухлин.

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

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