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## Expression pattern of immune- and cancer-associated genes in peripheral blood of mice bearing melanoma cells

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**Aim.** To identify putative non-invasive expression markers, based on relative expression (RE) of cancer- and immune-associated genes, in peripheral blood of mice, bearing melanoma cells. **Methods.** RE of 56 cancer- and immune-associated genes was assessed by quantitative PCR in peripheral blood of C57BL/6j mice inoculated with B16 mouse melanoma cells and in control animals. **Results.** Eleven genes showed significant differences in the RE levels in mice, bearing melanoma: six genes (*Ccl5*, *Il1b*, *Mif*, *Rnase1*, *S100a1* and *Tgfb1*) were expressed at higher levels, and five genes (*Erb2*, *Ifnb1*, *Il6*, *Pdcd1* and *Prom1*) were downregulated in comparison with the control animals. We have demonstrated a stable immunosuppressed state of mice inoculated with melanoma cells as evidenced by decreased RE levels of *Ifnb1* and *Pdcd1* and increased RE levels of *Lbp*, *Tlr3*, *Tlr8*, *Gstp1*, *Prom1*, *Oas1a*, *Oas3* and *Il1b*. **Conclusions.** Assessment of expression of cancer- and immune-associated genes in peripheral blood during growth of malignant cells in experimental animals may result in discovery of effective noninvasive expression markers for the prognosis of the cancer outcome and chemotherapy efficiency.

**Keywords:** melanoma, relative gene expression, immune-associated genes, putative expression markers

## Introduction

Melanoma represents high malignant neoplasms. It occurs in a variety of tumor groups that differ in clinical and histological characteristics, profiles of metastasis, ethnic distribution, a causative role of UV radiation and mutational profile for each patient [1]. For now, such modern approaches to cure mela-

noma as immunotherapy [2] and combined methods [3] are widely used; however, none of them provides 100 % efficiency of treatment. Melanoma, like many solid tumors, is a quite heterogeneous disease with certain molecular features of individual tumors, hence, it is important, to define the markers for progn-

sis, describing a status of immune system and specific characteristics of each tumor [4, 5, 6]. The least invasive methods to analyze markers are preferential. The analysis of liquid biopsies (blood, urine, saliva, *etc.*) is the most non-invasive for this purpose.

To characterize the growth of tumor cells in experimental animals, 56 cancer-associated genes and several immune-associated genes were chosen, namely, the genes encoding cytokines (*Ifna2, Ifnb1, Ifne, Ifnk, Ifng, Il6, Il1b, Il12a, Tnf*), chemokines (*Ccl3, Ccl4, Ccl5, Cxcl9, Cxcl10, Cxcl11*), interferon-stimulated genes (*Oas1a, Oas2, Oas3, Mx1, Tlr3, Tlr7, Tlr8, Rela*), immune therapy target genes (*Pdcd1, Cd274*), markers of cancerous cells (*Prom1, Nfkb1, Gstp1, Tgfb1*) and genes involved in inflammation (*Nlrp3, Arg2, Mif, S100A1, Xdh et al.*)[7].

Many of these genes are expressed differently in various cell types, that is why it is quite difficult to correlate their RE levels with the tumor progression.

We hypothesized a possibility to find the correlation between the RE levels of above-mentioned genes and the growth of melanoma cells in mice, using the peripheral blood of experimental animals to monitor the expression. We wanted to detect the specific changes in the RE levels and propose the novel non-invasive expression markers for the cancer diagnosis and prognosis.

## Materials and Methods

**Cell line.** The mouse melanoma B16 cell line was obtained from the Bank of Cell Lines (R.E.Kavetsky IEPOR, NAS of Ukraine). The cells were cultured in DMEM (Sigma) medium with the addition of 10 % FBS (Sigma),

100 units/ml penicillin and 100 µg/ml streptomycin at 37°C in a CO<sub>2</sub> incubator. The cells were detached by an EDTA/trypsin solution and rinsed in a phosphate buffered saline (PBS). Cells were counted, and suspension of 2x10<sup>5</sup> cells was injected into each mouse.

**Experimental animals.** Adult female mice of the C57BL/6j line were used. Suspension of the B16 mouse melanoma cell was subcutaneously introduced into the right posterior paw. Mice, not bearing melanoma cells, served as the control animals. Five mice were studied in each group. On the day 19<sup>th</sup> following the injection of melanoma cells, the peripheral blood was collected for the analysis. All manipulations with animals were conducted in accordance with the rules of handling of experimental animals, approved by the Bioethic Committee of IMBG of NAS of Ukraine and the rules, described in “European Convention for the Protection of Vertebrate Animals used for experimental and other scientific purposes” (Strasburg, 1986).

**Total RNA isolation and cDNA synthesis.** 100 µl of whole blood were thoroughly mixed with 300 µl of Trizol (Sigma). Total RNA was isolated, using a Direct-zol RNA MiniPrep total RNA kit (Zymo Research), according to the manufacturer’s protocol. DNaseI treatment was performed on columns. The quality and concentration of total RNA samples were analyzed on spectrophotometer (NanoDrop Technologies Inc. USA) and agarose gel electrophoresis. cDNA synthesis was performed, using Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific, USA), according to the manufacturer’s protocol.

**Quantitative PCR (qPCR).** The RE levels of the investigated genes were assessed as

described earlier [8, 9], using 5x HOT FIREPolEvaGreen qPCR Mix Plus (Solis BioDyne, Estonia) on Bio-Rad CFX96 Real-Time PCR Detection System (USA) and the following program: denaturing - 95°C for 12 min and then 40 cycles (95°C-15 s, 60°C – 20 s , 72°C - 20 s). Primers were selected, using an algorithm <https://www.ncbi.nlm.nih.gov/tools/primer-blast/> and the databases <https://primerdepot.nci.nih.gov/> and <https://www.origene.com> (see [10]). The reference *TBP* gene was used to normalize RE calculations by  $2^{-\Delta\text{dCt}}$  and  $2^{-\Delta\Delta\text{dCt}}$  methods, as described earlier [9].

*Statistical analysis.* A STATISTICA10 software was used to perform the statistical analysis. The Kruskal-Wallis and Fischer exact tests with correction on multiple comparisons, according to the Benjamini - Hochberg procedure with FDR = 0.2 [11] were used to calculate differences between groups.

## Results and Discussion

After the RE levels of 56 cancer- and immune-associated genes were assessed in peripheral blood of experimental animals, we could divide the genes in three groups, (Figure 1 A, B). Thus, the genes such as *Ccl5*, *Il1b*, *Mif*, *Rnasel*, *S100a1* and *Tgfb1* were highly expressed, whereas the genes *ErbB2*, *Ifnb1*, *Il6*, *Pdcd1* and *Prom1* were low expressed genes. The RE levels differ between the highly and low expressed genes more than 100-fold.

13 genes were differently expressed, when the mice bearing melanoma cells were compared with the control animals, as was calculated by a statistical analysis. 11 genes were upregulated ( $p < 0.05$ ) (Table 1A) and 2 genes were downregulated ( $p < 0.05$ ) (Table 1B).

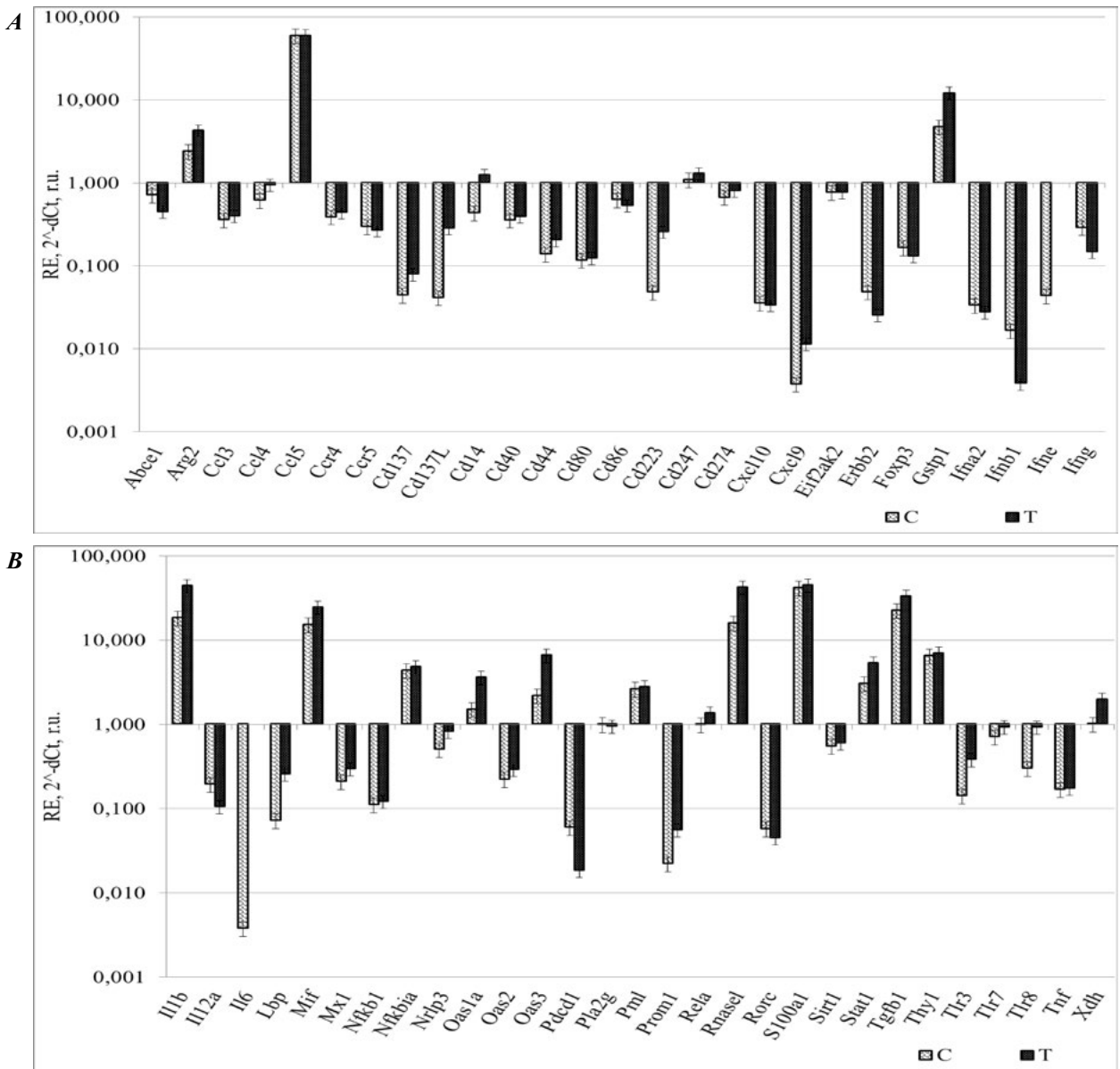
Difference in the RE levels was considered significant, when the change was 2-fold or more. Several genes showed the tendency in changes, for example, *Xdh* ( $p = 0.055$ ) and *Arg2* ( $p = 0.058$ ) were upregulated, and three genes, namely *Ifng*, *ErbB2* ( $p = 0.055$ ) and *Il12a* ( $p = 0.057$ ) were downregulated. We believe that in larger groups the changes in the RE levels could be significant.

The highest upregulation, above 3-fold, was found for *Lbp*, *Tlr8*, *Cxcl9*, *Oas3* ( $p < 0.001$ ) in the mice, bearing melanoma cells.

The protein, encoded by *Lbp*, is involved in the TLR signaling pathway and in the acute phase of the immunological response to gram-negative bacterial infections and also is a marker of fibrin neutropenia and a poor state in cancer patients [12, 13]. *Tlr8* and *Tlr3*, receptors of the TLR family, were upregulated in the mice bearing melanoma cells. It was shown, that an increased expression of *TLR8* leads to a faster proliferation of tumor cells and also promotes chemoresistance of the pancreatic tumors. NF- $\kappa$ B and COX2 are upregulated with the *TLR8* increase [14]. Moreover, it is known that *TLR3* can stimulate invasiveness of cancerous cells [15].

Noteworthy, the expression levels and a role of *Cxcl9* in the development of various tumor types is controversial [16]. Even so, *Cxcl9* is a promising target for the creation of new approaches to treat cancer [17].

Among the interferon (INF alpha) targeted genes, *Oas3*, *Oas1a* and *Rnasel* were upregulated in the group of mice bearing melanoma cells. Functioning of OAS1 and OAS3 in the immune pathways and response to RNA viruses are closely related to RNASEL [18]. It is known that downregulation of these genes



**Fig. 1.** Pattern of RE levels in the control mice (C) and mice, bearing melanoma cells (T).

is associated with the poor prognosis for cancer patients and also with the resistance to chemotherapy [19].

It could be that the mice still have the immune system reserves to fight the tumor growth at this stage after the melanoma cells were

inoculated. However, several symptoms of the immunosuppressive state were detected in experimental animals, as was evident by the expression data.

Next, we observed a group of genes that showed moderate upregulation in the mice,

**Table 1. Gene RE levels, that were upregulated RE (A) and downregulated (B), when mice, bearing melanoma cells and the control animals were compared.**

<b>A</b>	№	Genes	RE, increase	p-value*	<b>B</b>	№	Genes	RE, decrease	p-value*
	1	<b>Lbp</b>	3.56	p<0.001		1	<b>Ifnb1</b>	0.23	p<0.001
	2	<b>Tlr8</b>	3.10			2	<b>Pdcd1</b>	0.31	
	3	<b>Cxcl9</b>	3.04			3	<i>Ifng</i>	0.51	p=0.055
	4	<b>Oas3</b>	3.01			4	<i>ErbB2</i>	0.52	
	5	<b>Cd14</b>	2.89	p<0.050		5	<i>Il12a</i>	0.54	p=0.057
	6	<b>Tlr3</b>	2.68						
	7	<b>Rnasel</b>	2.67						
	8	<b>Gstp1</b>	2.55						
	9	<b>Prom1</b>	2.51						
	10	<b>Il1b</b>	2.42						
	11	<b>Oas1a</b>	2.40						
	12	<i>Xdh</i>	1.96	p=0.055					
	13	<i>Arg2</i>	1.77	p=0.058					

Note:\* - Fisher exact test with FDR=0.2; genes, showing the significant differences in RE levels, are indicated in bold.

bearing melanoma cells, i.e. RE changes were 2-3 fold. The Cd14 upregulation could be due to the infiltration of monocytes into tumor, and could serve as a marker for cancer progression and metastasizing [20, 21].

It has been reported already that *GSTP1*, encoding Glutathione S-transferase PI, is over-expressed in many types of cancers [22], for example, is associated with the K-ras mutation in colorectal cancer [23] and with downregulation of miR-133a in head and neck squamous cell carcinoma [22]. An increased *GSTP1* expression is also indicated in an enhanced detoxification activity, protecting cancer cells against cytotoxic and cytostatic drugs [24, 25]. However, there are no data on the *Gstp1* expression in the blood of cancer patients. Noteworthy, an increased *GSTP1* expression enhances oncogenicity of breast cancer by regulating glycolytic and lipid metabolism as well as the energy and oncogenic signaling pathways, resulting in the activation of glyce-

raldehyde-3-phosphate dehydrogenase [26]. Moreover, the genetic or pharmacological inactivation of *GSTP1* worsens the survival of tumor cells, due to abnormalities in the underlying signaling pathways.

*PROM1* (*CD133*) is a marker of cancer stem cells, for lung and colorectal tumors [27, 28]. High levels are correlated with the poor prognosis in colorectal cancer [29]. Importantly, the increased RE levels of *Prom1* were detected in the mice, bearing melanoma cells. This could be an evidence of the presence of circulating tumor cells in the mouse bloodstream as well as of an elevated expression of *Cd14* and *Gstp1*, described above.

Additionally, the *Il1b* expression increased in the mice, bearing melanoma cells. This interleukin can be expressed by tumor cells and different stromal elements, such as myeloid-derived suppressor cells and tumor-associated macrophages [30]. IL1B enhances metastasizing [31].

Importantly, the experimental animals, bearing tumor cells, showed lower RE levels of *Ifnb1*, *Pdcd1* and *Ifng*. This indicates the immunosuppressive state of the mice [32, 33].

Summarizing, several studied genes showed significant differences in the RE levels in the mice, bearing melanoma cells, compared with the control animals. The detected alterations refer to the genes, associated with the immune and cancer cells and could serve as putative noninvasive biomarkers of tumor growth. These genes are *Lbp*, *Tlr3*, *Tlr8*, *Prom1* and *Cd14*. Additionally, the *Oas3*, *Rnase1*, *Gstp1* and *Cxcl9* genes could be the markers of sensitivity to chemotherapy.

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## Conclusions

Assessment of the expression of cancer- and immune-associated genes in the peripheral blood of the experimental animals upon the growth of malignant cells *in vivo* may result in the discovery of the effective noninvasive expression markers for the prognosis of the outcome of the cancer disease and effectiveness of the chemotherapy.

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#### **Патерни експресії імунно та пухлино-асоційованих генів у периферійній крові мишей з експериментальними меланомами**

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**Мета.** Визначити потенційні неінвазивні експресійні маркери на основі патернів відносної експресії (ВЕ) пухлино- та імунно-асоційованих генів, що вивчалися у периферійній крові мишей з введеними клітинами меланоми. **Методи.** Рівні ВЕ пухлино- та імунно-асоційованих генів були досліджені кількісною ПЛР у периферійній крові C57BL/6j мишей з введеними B16 клітинами меланоми та контрольних тварин. **Результати.** Для 11-и генів виявлено значущі відмінності рівнів ВЕ у мишей з введеними клітинами меланоми, з них 6 генів (*Ccl5*, *Il1b*, *Mif*, *Rnasel*, *S100a1* та *Tgfb1*) мали підвищені рівні ВЕ та 5 генів (*ErbB2*, *Ifnb1*, *Il6*, *Pdcd1* й *Prom1*) – знижені рівні ВЕ у порівнянні з контрольними тваринами. Ми показали стабільний імуносупресивний стан мишей з введеними клітинами меланоми, про що свідчать знижені рівні ВЕ *Ifnb1*, *Pdcd1* та підвищені рівні ВЕ *Lbp*, *Tlr3*, *Tlr8*, *Gstp1*, *Prom1*, *Oas1a*, *Oas3* та *Il1b*. **Висновки.** Оцінка експресії пухлино- та імунно-асоційованих генів у периферійній крові експериментальних тварин при прогресії у них злоякісних клітин може призвести до виявлення

ефективних неінвазивних експресійних маркерів для прогнозу онкологічних захворювань та ефективності хіміотерапії.

**Ключові слова:** меланома, відносна експресія генів, імунно-асоційовані гени, потенційні експресійні маркери.

#### **Паттерны экспрессии иммуно и опухоль-ассоциированных генов в периферической крови мышей с экспериментальными меланомами**

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**Цель.** Определить потенциальные неинвазивные экспрессионные маркеры на основе паттернов относительной экспрессии (ОЭ) опухоль- и иммуно-ассоциированных генов, которые исследовались в периферической крови мышей с введенными клетками меланоми. **Методы.** Уровни ОЭ опухоль- и иммуно-ассоциированных генов были исследованы количественной ПЦР в периферической крови of C57BL / 6j мышей с введенными B16 клетками меланоми и контрольных животных. **Результаты.** Для 11-и генов выявлены значимые отличия уровней ОЭ у мышей с введенными клетками меланоми, из них 6 генов (*Ccl5*, *Il1b*, *Mif*, *Rnasel*, *S100a1* и *Tgfb1*) имели повышенные уровни ОЭ, а 5 генов (*ErbB2*, *Ifnb1*, *Il6*, *Pdcd1* и *Prom1*) – пониженные уровни ОЭ по сравнению с контрольными животными. Мы показали стабильное иммуносупрессивное состояние мышей с введенными клетками меланоми, о чем свидетельствуют сниженные уровни ОЭ *Ifnb1*, *Pdcd1* и повышенные уровни ОЭ *Lbp*, *Tlr3*, *Tlr8*, *Gstp1*, *Prom1*, *Oas1a*, *Oas3* и *Il1b*. **Выводы.** Оценка экспрессии опухоль- и иммуно-ассоциированных генов в периферической крови экспериментальных животных при прогрессии у них злокачественных клеток может привести к выявлению эффективных неинвазивных экспрессионных маркеров для прогноза результата онкологических заболеваний и эффективности химиотерапии.

**Ключевые слова:** меланома, относительная экспрессия генов, иммуно-ассоциированные гены, потенциальные экспрессионные маркеры

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