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ТРАНСКРИПТОМНЫЙ АНАЛИЗ КЛЕТОК МЕЛАНОМЫ, ПОЛУЧЕННЫХ ИЗ РАЗЛИЧНЫХ УЧАСТКОВ ПЕРВИЧНОЙ ОПУХОЛИ

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Аннотация

Введение. Внутриопухолевая гетерогенность представляет собой характерную черту большинства злокачественных новообразований, в том числе и меланомы кожи. Данное свойство является одним из препятствий для проведения эффективной таргетной терапии, поскольку у различных субклонов опухолевых клеток наблюдается вариабельная чувствительность к данным препаратам. С современных позиций терапия злокачественных новообразований требует персонифицированного подхода для каждого конкретного пациента. **Цель исследования** – оценка возможных различий между тканями меланомы, выделенными из различных участков первичной опухоли одного пациента на транскриптомном уровне. **Материал и методы.** В работе были использованы культуры клеток меланомы, полученные из центральной и периферической частей первичной опухоли двух пациентов. Исследование транскриптомов клеток проводили методом микрочипирования с последующим биоинформатическим анализом. **Результаты.** В клетках меланомы первого пациента, полученных из центрального и периферического участков одной опухоли, не было выявлено различий по транскриптомному профилю. У второго пациента имели место существенные различия (по 2953 транскриптам из 48226). В клетках, полученных из центрального участка опухоли, выявлено повышение мРНК генов, кодирующих белки, ассоциированные с иммунным ответом опухоли, транспортные белки ABC-семейства, сигнальные молекулы класса цитокинов. В культуре клеток, выделенной из периферического участка этой же опухоли, зарегистрировано увеличение уровня мРНК генов, кодирующих белки внеклеточного матрикса и воспалительного ответа. В целом различия между субклонами клеток второго пациента касались ряда сигнальных каскадов, играющих ведущую роль в онкогенезе (MAPK, PI3K-Akt-mTOR, VEGFA-VEGFR2 и др). **Заключение.** Проведенное исследование позволяет оценить возможные различия между клетками внутри опухоли на транскрипционном уровне с целью поиска новых подходов для персонифицированной терапии.

Ключевые слова: меланома, гетерогенность, транскриптом, микроокружение.

TRANSCRIPTOMIC ANALYSIS OF MELANOMA CELLS EXTRACTED FROM DIFFERENT SITES OF THE PRIMARY TUMOR

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Abstract

Introduction. Intratumor heterogeneity is a characteristic feature for most malignant tumors, including cutaneous melanoma. This property represents one of the main obstacles for effective targeted therapy, due to the different sensitivity to chemotherapeutic agents on various tumor cells subclones. Treatment of malignant tumors requires an individual approach to choose the most appropriate treatment regimen. **The purpose of the study** was to evaluate differences in melanoma tissue samples obtained from different parts of one patient's primary tumor at the transcriptomic level. **Material and Methods.** Melanoma cell cultures obtained from both central and peripheral parts of the primary tumor of two patients were used in the study. **Results.** Subclones from different parts of the first patient's tumor were similar, whereas the second patient demonstrated significant differences at the transcriptomic level (in 2953 transcripts out of 48226). In the cells of the central zone of the second patient's tumor, an increase in mRNA of the genes encoding proteins associated with tumor-specific immune response, as well as ABC-family transport proteins and cytokine signaling molecules, were noted. In the cells from the peripheral area of the same tumor, a more intensive transcription of genes encoding extracellular matrix and inflammatory response proteins was observed. Taken all round, the differences between the subclones of the second patient's cells were relevant to some signaling cascades playing a leading role in oncogenesis (MAPK, PI3K-Akt-mTOR, VEGFA-VEGFR2, etc.). **Conclusion**. The study allowed evaluation of differences between cancer cells within a tumor at the transcriptional level in order to search for further approaches to personalized melanoma therapy.

Keywords: melanoma, heterogeneity, transcriptome, microenvironment.

Introduction

Intratumor heterogeneity is a serious problem in terms of cancer treatment. With the development of a tumor cell genome, genetically heterogeneous subclones with different biological characteristics and variable sensitivity to chemotherapy appear in the primary tumor [1]. The development of malignant tumor and its growth is an active evolutionary process that results in a tumor consisting of cells with heterogeneous molecular characteristics [2]. This diversity entails the development of resistance to treatment in cancer patients [3].

The diverse molecular portrait of the cells that form one tumor can be a result of a set of changes at the genetic and epigenetic levels [4]. Among such changes, special attention should be given to differences in transcriptome profile of the cell. This is due to the fact that the transcription is a dynamic process that projects functional cell characteristics and predetermines different aspects of cells' biological activity, forming an individual molecular landscape of the development of cancer cells' subclones [5].

The aim of this research is a comparative analysis of transcriptomic profiles of melanoma cells derived from different parts of one patient's primary tumor.

Material and Methods

In the present study we used surgical specimens of two patients treated at the General Oncosurgery Department of A.I. Kryzhanovsky Krasnoyarsk Region Clinical Oncology Center, Krasnoyarsk, Russia.

The study was approved by the ethical Committee of Professor V.F. Voino-Yasenetsky Krasnoyarsk State Medical University (record No. 73/2016 dated 16.12.2016) and the ethical Committee of A.I. Kryzhanovsky Krasnoyarsk Region Clinical Oncology Center (record No. 8 dated 14.062.2017). Patients' clinical characteristics are shown in Table 1.

Immediately after surgical excision, a tumor fragment of at least 8 mm^3 and weighing at least 300 mg was immersed in a tube containing a culture medium RPMI-1640 (Gibco, Life Technologies, Paisley, UK) with the addition of 20% fetal bovine serum (Gibco, Life Technologies, Paisley, UK) and antibiotic-antimicotic complex: 100 U/ml penicillin G, 100 µg/ml streptomycin, 0.025 µg/ml amphotericin B (HyClone laboratories, USA) and was transported in ice. Two pieces were then aseptically separated from the tumor tissue: one from the central part of the tumor and one from the peripheral part of the tumor. If necessary, the obtained fragments were mechanically cleaned of necrotic tissue, washed in a sterile balanced Hanks salt solution (HBSS) (Gibco®, Life Technologies, Paisley, UK) and crushed by crossed scalpels.

The obtained tumor fragments were disaggregated by incubation in 0.25% trypsin solution with EDTA (Gibco, Life Technologies, Paisley, UK) in an amount of 1 ml of trypsin for every 100 mg of tissue at +37°C for 15-20 minutes. At the end of incubation, the suspension was centrifuged on a centrifuge CLMN-P10-01-Elecon ("Liston", Zhukov, Russia) at a speed

of 1000 rpm for 5 minutes. The obtained cellular precipitate was resuspended in 5 ml of RPMI-1640 nutrient medium with L-glutamine (Gibco®, Life Technologies, Paisley, UK), 10% fetal bovine serum (Gibco®, Life Technologies, Paisley, UK) and a mixture of antibiotic-antimicotic complex (HyClone laboratories, USA) and placed in a culture tube, in which further cultivation was carried out in CO_2-incubator (Sanyo, Osaka, Japan) with 5% of carbon dioxide, at +37°C. The first change of the nutrient medium was carried out 48 hours after the beginning of cultivation after the visual cells' adhesion to the adhesive surface of the tube. The first cell passage was carried out 4 days later and subsequent cell passages were carried out every 3 days of incubation.

To study the transcriptome of melanoma cells isolated from the central and peripheral parts of the tumor, the cells were transplanted into a 24-well plate at a concentration of 2×10^5 cells/ml. The culture medium was changed 24 hours after the cells' adhesion to the adhesive surface, with a subsequent culturing during 72 hours. At the end cells were removed using 0.25% trypsin with EDTA, washed in 0.01M phosphate-buffered saline and then the total RNA was isolated. RecoverAll™ Total Nucleic Acid Isolation kit (Ambion, Lithuania) was used for isolation according to the manufacturer's protocol. The concentration of purified total RNA was determined by Qubit® 2.0 fluorimeter (Singapore) using Qubit™ RNA HS Assay Kit (Ref. Q32852, Invitrogen™, Eugene, Oregon, USA).

The transcriptome of melanoma cells isolated from different parts of the tumor was studied using the GeneAtlas® system (Affymetrix, USA). For this purpose, 10 ng of purified total RNA were pretreated with the sample preparation kit titled GeneChip™ WT Pico Kit (Applied Biosystems™, Santa Clara, California, USA) in accordance with the manufacturer's protocol. Then, molecules were hybridized to GeneChip™ Human Gene 2.1 ST Array Strip microchips (Applied Biosystems™, Santa Clara, California, USA) with the reagents from GeneAtlas Hybridization, Wash, and Stain Kit for WT Array Strips (Applied Biosystems™, Santa Clara, California, USA). After 20 hours of hybridization microchips were washed and stained with solutions for washing and staining from the same set in the Fluidic station of the GeneAtlas® system. The detection of the microarray results was made in the Imaging station of this system.

Quality control (QC) of microarray was performed by the software system automatically due to the introduction of control RNA molecules into the study at the stages of sample preparation and hybridization. The fluorescence data analysis with its transformation into relative mRNA expression levels was carried out using the Expression Console ("Affymetrix", USA). Transcriptome Analysis Console 3.0 ("Affymetrix", USA) was used for statistical processing of microarray data. The non-parametric t-test ANOVA was used for data comparing. The statistical significance in transcriptomic profile differences was evaluated after an adjustment to the false discovery rate (FDR) criterion proposed by Benjamin, Hochberg for multiple comparisons [6]. The differences were statistically significant at $p_{FDR}<0.05$.

To assess the biological significance of individual genes, the GO (Gene ontology) project databases were used (https://www.ebi.ac.uk/QuickGO/, last access 06.11.2017), the analysis of genes' ontology of differentially expressed genes was carried out using Panther 13.1 database. Analysis of the clusters' biological role based on the microarray results was performed in the database DAVID 6.8 (https://david.ncifcrf.gov/home.jsp, last access 10.11.2017).

Results

The analysis of transcriptomic profiles of melanoma cells obtained from different parts of the first patient's tumor (Krsgmu-HS-Mel-CD-T-230317-Centr and Krsgmu-HS-Mel-CD-T-230317-K) revealed no differences between them. However, different results were shown in melanoma cells of the second patient – Krsgmu-HS-Mel-RI-T-040417-Centr and Krsgmu-HS-Mel-RI-T-040417-K. Profiles of these cultures

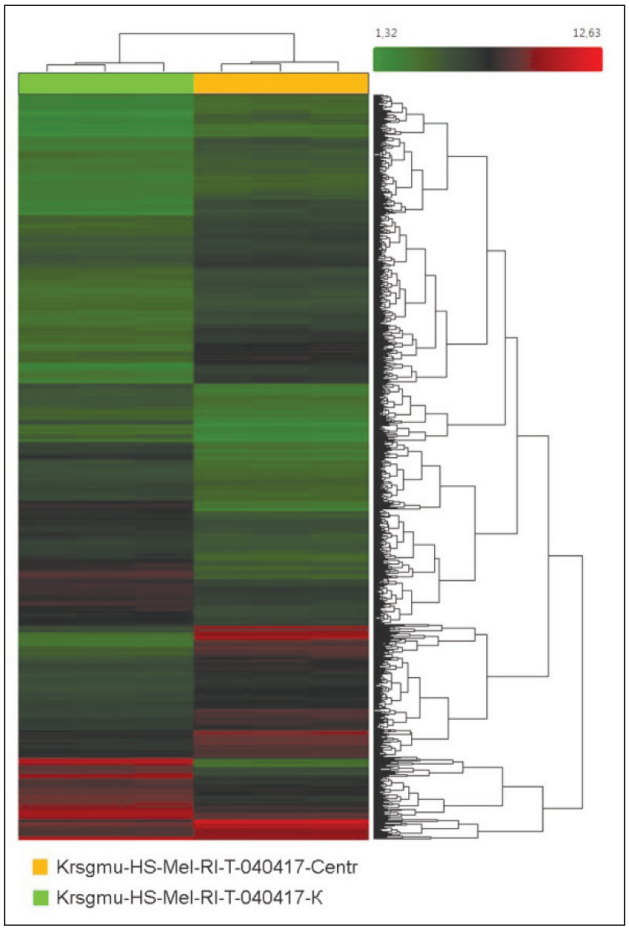

Figure 1. Hierarchical clustering of genes with different levels of transcription in melanoma cells isolated from the central part (Krsgmu-HS-Mel-RI-T-040417-Centr) and peripheral part (Krsgmu-HS-Mel-RI-T-040417-K) of the same tumor

differed in the level of 2953 transcripts. Out of them, the level of 1735 transcripts was two or more times higher, while the level of 1218 transcripts was two or more times lower in the cells of the central part of the tumor compared to the cells of the peripheral part of the tumor (Figure 1).

The transcript levels of genes encoding 42 proteins were more than 25 times higher, whereas transcripts of genes encoding 43 proteins were more than 25 times lower in cells from the central tumor part than in cells from the peripheral part. Proteins with increased mRNA levels in cells obtained from the central tumor parts belonged to the proteins responsible for various intracellular processes, including intercellular adhesion, transport of ions and metabolites. The most significant changes were detected in mRNA levels encoding proteins ABCC2, SNAP25, CCL2, OAS1, MAGEA3, MAGEB2, MAGEA6, DNER, TPTE, HLA-DRB5, DNER. Proteins, in which the mRNA levels were 25 times lower in cells from the central tumor part than in cells from the peripheral tumor part, included intercellular adhesion molecules, extracellular matrix proteins, growth factors, signal transduction proteins, apoptotic proteins (NEXN, PPP1R14A, POSTN, chipboard, B3GALT2, SEMA7A, FGF7, RIMS1, FBLN5, SCUBE3, PCDH18, COL1A2, TNFRSF10D, ADAMTS5, BGN).

Among the transcripts of genes that have a moderate increase in the level of cells obtained from the central part of the tumor compared with the periphery of the tumor (more than 5 times but less than 25 times), 423 transcripts were identified, with a 5-25 fold decrease in the level of 275 transcripts.

Proteins, which had increased mRNA levels in cells from the central part of the tumor compared to its periphery, were represented by the proteins of the Wnt-signaling cascade, proteins for intercellular contacts and adhesion, cadherins, regulators of the p53 signaling pathway, molecules of the EGF signaling pathways, and various cytokines. A similar trend was observed for the proteins with mRNA levels, which were lower in the culture of cells obtained from the central tumor sites compared to its periphery. The most common were the transcripts of genes of intercellular adhesion molecules, cadherin: CDH8, PCDHB3, PCDHB2, EN1, TGFBR1, ACTA2, TCF7L2, EDN1, PLCB4, SFRP4, and genes encoding proteins involved in angiogenesis: PRKD1, CRYAB, TCF7L2, PDGFRB, tissue factor F3.

Compared to the peripheral part of the tumor, genes' transcripts with a moderate decrease (a 5-25-fold) in mRNA levels in the central part represented the largest group since it contained 1249 altered transcripts of isolated genes, and 896 transcripts had a 2-5-fold decrease in mRNA levels. Among the altered gene transcripts, there were mRNAs of proteins involved in angiogenesis (the corresponding proteins are components of the integrin and endothelin signaling pathways.

There were identified 950 genes' transcripts with slightly elevated levels (more than 2 times, but less than 5 times) in cells obtained from the central tumor part compared to its periphery, and a 2-5-fold decrease was observed in the levels of 750 genes' transcripts. Proteins, the mRNA levels of which were elevated in the cells from the central parts of the tumor compared to its periphery, were represented by proteins-participants of the inflammatory response: PIK3CB, PLA2G4A, CXCL8, COL14A1, IL1B, NFATC2, PRKX, PLCG2, CCL5, RELB, RGS17, NFKBIA, ARRB1, GNA1, PDK1, as well as T-lymphocytes' activators: HLA-DQA1, MAP3K1, NFATC2, LCP2. Proteins of intercellular adhesion, cadherins and integrins dominated among proteins with a 2-5-fold decrease in the mRNA levels.

The results obtained showed that the altered genes' transcripts were the components of 642 signaling pathways. Among these signaling cascades, we identified signaling pathways, which included 10 or more proteins, the mRNA expression level of which was changed according to our findings. The transcriptomic profile revealed that mRNAs of genes with the increased transcription level in cells obtained from the central part of the tumor compared to cells obtained from the peripheral part of the tumor were involved into 40 signaling cascades**,** and mRNAs of genes with the decreased transcription level were involved only into 19 signaling pathways (Table 2).

Discussion

Transcriptomic profiling data analysis showed an increase in mRNAs of proteins, such as: melanoma-associated antigen-3 and melanoma-associated antigen B2. A 93.81-fold increase in the mRNA level of these proteins was observed in the center of the tumor. According to the literature review results, these genes are considered to be target genes recognized by cytotoxic T-cells under antigen-specific immunotherapy in skin melanoma patients [7].

Differences in the transcriptomic profile were revealed in ABC transporter proteins family. These proteins are considered to be associated with the formation of a multiple drug resistance. The ABCC2 protein mRNA level was 29.9 times higher in the center of the tumor. It is known that an increase in the expression activity of this protein on the tumor cells membrane can result in more intensive elimination of therapeutic agents from the cell, causing a decrease in the intracellular drug concentration, which is necessary for the efficient tumor growth inhibition [8]. This fact can explain why different tumor subclones yield a different therapeutic response to the anti-tumor agent.

A 98.14-fold increase in CCL2 chemokine mRNA expression in the center of the tumor indicates that there are different types of immunological reactions in central and peripheral parts of the tumor. This protein is known to be actively expressed by tumor-

Table 1

Characteristics of the studied samples

No	Patients' clinical data	Code number of the cell cultures, obtained from the tumor fragment	Tumor fragment localization
1	Patient K., a 30-year-old female, superficial spreading melanoma of the right lower leg	Krsgmu-HS-Mel-CD-T-230317-Centr	Center
		Krsgmu-HS-Mel-CD-T-230317-K	Peripheral part
2	Patient B., a 64-year-old male, superficial spreading melanoma of the interscapular area	Krsgmu-HS-Mel-RI-T-040417-Centr	Center
		Krsgmu-HS-Mel-RI-T-040417-K	Peripheral part

Table 2

Signaling pathways, involved in the regulation of differentially altered genes, according to the transcriptome analysis in the cell culture, obtained from different parts of the tumor

Signaling pathway *	Number of transcripts increased in the central tumor part compared to the peripheral one	Signaling pathway *	Number of transcripts decreased in the central tumor part compared to the peripheral one
Nuclear Receptors Meta-Pathway	33	miR-targeted genes in lymphocytes	37
miR-targeted genes in lymphocytes	27	miR-targeted genes in muscle cell	36
Retinoblastoma (RB) in Cancer	26	Focal Adhesion-PI3K-Akt-mTOR-signaling pathway	27
Cell Cycle	19	Focal Adhesion	21
Circadian rythm related genes	19	Mesodermal Commitment Pathway	20
PI3K-Akt Signaling Pathway	17	MAPK Signaling Pathway	19
MAPK Signaling Pathway	17	Wnt Signaling Pathway	14
Ectoderm Differentiation	17	ESC Pluripotency Pathways	14
Endoderm Differentiation	17	Vitamin D Receptor Pathway	13
Mesodermal Commitment Pathway	15	Regulation of Actin Cytoskeleton	13
VEGFA-VEGFR2 Signaling Pathway	15	miR-targeted genes in leukocytes	10
Chemokine Signaling Pathway	15	EGFR1 Signaling Pathway	10

* This table shows a total of 15 signaling pathways for both increased and decreased transcripts with the largest number of mRNAs involved into the signaling cascade.

associated macrophages, as well as by tumor cells themselves. In addition, the biological effect of this protein correlates with its concentration in the tumor tissue: at high concentrations of CCL2, a stimulation of classically activated M1 macrophages takes place, and a cytostatic effect in tumor cells occurs, whereas at low concentrations of CCL2, an accumulation of already alternatively activated M2 macrophages in the tissue takes place, which produces an inverse effect and induces an enhanced proliferation activity of the tumor cells [9].

The proteins whose mRNA levels were more than 25 times lower in the center than in the peripheral part of the tumor were mainly proteins-components of extracellular matrix. This fact can be due to a higher level of stromal components in the peripheral part of the tumor than in its central part. The change of the expression profile corresponds to data obtained from colorectal cancer research that reported the predominance of proteins forming components of extracellular matrix: collagens, tissue inhibitors of matrix metalloproteins (COL6A3, COL1A2, POSTN, TIMP2 and others) at the peripheral part of the tumor [10].

Among both increased and decreased gene transcripts in the central part of the tumor in comparison with its periphery, there were mRNAs, coding proteins that were components of the Wnt signaling pathway. The components of the Wnt signaling pathway are known to take part at all stages of skin melanoma development. For example, the transcription of a well-known c-myc oncogene whose enhanced expression is characteristic for various neoplasms, including melanoma of the skin, is induced by means of the β-catenin signaling cascade [11], the gene expression of negative regulators of a canonical Wnt signaling pathway being often suppressed. For example, the production of Dkk-1, 2 and 3 that inhibit the β-catenin signaling cascade by means of binding with co-receptor LRP5/6 is significantly reduced or absent in melanoma cells [12].

The transcriptome profile revealed that, mRNAs of genes with the elevated transcription level in cells obtained from the tumor center were involved in 40 signaling cascades, whereas mRNAs of genes with decreased transcription level were involved in only 19 signaling pathways. According to the bioinformatics analysis data, mRNA profile changes in melanoma

cells obtained from both the central and peripheral parts of the tumor affected genes-components of signaling pathways traditionally associated with cancerogenesis (MAPK, PI3K-Akt-mTOR, VEGFA-VEGFR2, Focal adhesion, Wnt signaling pathway). Proteins of the MAPK signaling cascade are one of the key targets in skin melanoma therapy although many patients suffering from metastatic melanoma showed acquired resistance to MAPK inhibitors.

One of the major mechanisms of MAPK-inhibitor resistance formation is the realization of the intratumor heterogeneity phenomenon. High plasticity of melanoma cells provokes both a clonal evolution of genetic resistance (for example due to a mutation in genes that code components of the MAPK or PI3K/AKT/PTEN signaling cascades) and an appearance of cel phenotypes that are functionally and metabolically resistant to various therapeutic agents [13]. Thus, melanoma cells, like 'a moving target', drift among different metabolic influences, cell cycles and states of cell differentiation, this projecting a high dynamical potential for adaptation to exogenous stress factors including medicaments.

Among the altered transcripts, there were mRNAs coding proteins responsible for the interaction between mRNAs and their genes-targets in different cells: lymphocytes, myocytes, and epithelial cells. This is consistent with the fact that mRNAs can act as paracrine and autocrine regulators of a biological behavior of the tumor micro-environment [14].

The changes in the mRNA profile in melanoma cells originated both from the center and the periphery of the tumor showed dysregulation of VEGFA-VEGFR2 signaling pathway, which took part in melanoma pathogenesis; the up-regulation of this signaling pathway proteins maintained a proliferation activity of melanoma cells [15].

The expression of mRNAs, coding THBS1 and SPARC proteins, was significantly lower in cells obtained from the center of the tumor than in cells obtained from the peripheral part of the tumor. These genes are components of autophagy signaling pathway, and according to previous reports, they can take part in a chemoresistance formation associated with autophagy [16].

At the same time, for the cells obtained from the tumor central part, some unique signaling pathways were determined (Cell Cycle, Circadian rhythm related genes) which were not determined for the cells obtained from the tumor peripheral part. The change of an expression profile of Circadian rhythm genes is known to be an early occurrence in case of skin melanoma and is connected with the involvement of tumor microenvironment into this process [17].

A special role is given to melatonin that prevents DNA potential damage and can provide an endogenous enzymatic system to protect from oxidative stress by means of growth factor regulators and through activating some antioxidant enzymes, such as superoxide dismutase and catalase. Besides, melatonin in pharmacological concentrations suppresses proliferation of the melanoma cells [18].

For the tumor periphery cells, there were determined signaling cascades that were not characteristic for the cells from central part of the tumor: miR-targeted genes in leukocytes, Vitamin D Receptor Pathway, Regulation of Actin Cytoskeleton. Apart from calcium metabolism regulation, vitamin D3 active forms are also known to have an anticarcinogenic effect, whereas alterations in vitamin D signaling, including both D3 activation and inactivation, as well as expression and activity of the corresponding receptors, influence the melanoma progression and the outcome of the disease [19].

Conclusion

Cells obtained from the central part of the tumor demonstrated the increased mRNA levels of genes indicating a high sensitivity of cells to immunotherapy; in particular, these cells were characterized by enhanced expression of CT-antigens, as well as MAGE-A3 and MAGE B2 genes. These genes were considered to be gene-targets detected by cytotoxic T-cells in antigen-specific immunotherapy of melanoma patients.

Melanoma cells derived from the peripheral part of the same tumor, had increased level of mRNA genes coding an extracellular matrix and inflammation-related proteins. According to bioinformatic analysis, the changes in the mRNA profile in the melanoma cells from the tumor periphery were observed in genes-components of the signaling pathways that took part in the antioxidant response. This fact suggested that the peripheral part of the tumor was more sensitive to antioxidant therapy.

Thus, the study results showed a difference between melanoma cells obtained from various parts of the primary tumor at the transcriptomic level. This fact should be taken into consideration when administering anticancer therapy.

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Conflict of interest

The authors declare that they have no conflict of interest.

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