RESEARCH PAPER

Biological role and diagnostic utility of ribosomal protein L23a pseudogene 53 in cutaneous melanoma

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ABSTRACT

Background: Skin melanoma is one of the deadliest types of skin cancer and develops from melanocytes. The genetic aberrations in protein-coding genes are well characterized, but little is known about changes in non-coding RNAs (ncRNAs) such as pseudogenes. Ribosomal protein pseudogenes (RPPs) have been described as the largest group of pseudogenes which are dispersed in the human genome.

Materials and methids: We looked deeply at the role of one of them, ribosomal protein L23a pseudogene 53 (*RPL23AP53*), and its potential diagnostic use. The expression level of *RPL23AP53* was profiled in melanoma cell lines using real time quantitative reverse transcription polymerase chain reaction (qRT-PCR) and analyzed based on the Cancer Genome Atlas (TCGA) data depending on *BRAF* status and clinicopathological parameters. Cellular phenotype, which was associated with *RPL23AP53* levels, was described based on the REACTOME pathway browser, Gene Set Enrichment Analysis (GSEA) analysis as well as Immune and ESTIMATE Scores.

Results: We indicted *in vitro* changes in *RPL23AP53* level depending on a cell line, and based on *in silico* analysis of TCGA samples demonstrated significant differences in *RPL23AP53* expression between primary and metastatic melanoma, as well as correlation between *RPL23AP53* and overall survival. No differences depending on *BRAF* status were observed. *RPL23AP53* is associated with several signaling pathways and cellular processes.

Conclusions: This study showed that patients with higher expression of *RPL23AP53* displayed changed infiltration of lymphocytes, macrophages, and neutrophils compared to groups with lower expression of *RPL23AP53*. *RPL23AP53* pseudogene is differently expressed in melanoma compared with normal tissue and its expression is associated with cellular proliferation. Thus, it may be considered as an indicator of patients' survival and a marker for the immune profile assessment.

Key words: *RPL23AP53*; pseudogene; non-coding RNA; lncRNA; TCGA; BRAF mutation; biomarkers; immunology

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Introduction

Skin melanoma is one of the three main types of skin cancer along with basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) [1], and develops from melanocytes. Melanoma is often diagnosed in the advanced stage with metastasis to the small intestine, liver, lung, and brain tissues [2]. Its early diagnosis is still challenging. Molecular profiling could help to overcome this problem. Moreover, it allows for a personalized treatment [3]. The currently used therapeutic options could be divided into two main strategies: i) conventional treatment including surgical intervention and radio- or chemotherapy [4, 5], and ii) advanced therapy including several systemic and intratumoral approaches [6]. The treatment strategy depends on the clinical-pathological status and more often on molecular characteristics of patients [2].

Changes in the genome which are closely connected with ultraviolet radiation (UVA and UVB) emitted by the sun or artificial sources cause DNA damage by direct photodamage and reactive oxygen species (ROS) production and immune suppression which is the basis of the cancerogenesis [1]. The molecular mechanism of melanoma development is well known and mutations within *KIT*, *NRAS*, *BRAF*, *GNAQ*, and *GNA11* genes are commonly detected [3]. Also, changes in the expression of suppressor genes like *NF1*, *PTEN*, and *TP53* are indicated [3]. Moreover, the changes in *RAC1*, *PREX2*, *MiTF*, *CDK4*, and *CDKN2A* genes also play an important role in melanoma-specific signaling pathways and processes [1]. Based on the Cancer Genome Atlas (TCGA) data melanoma has been divided into four main molecular subgroups: mutant *BRAF*, mutant *RAS*, mutant *NF1*, and Triple-WT (wild-type) [7].

The initiation and development of melanoma are a consequence of changes in genes and pathways which finally keep cells in an activated proliferation state and block their terminal differentiation ability [8].

Except for known changes in protein-coding genes, epigenetic modification in melanoma seems to be one of the key events in disease development. Several different non-coding RNAs take part in this process, including lncRNAs. LncRNAs function as regulators of transcription in the nucleus, as scaf-folding proteins necessary for creating the transcriptional complexes and keeping their stability and activity, and function as competing endogenous RNAs interacting with miRNAs. That makes lncRNAs important elements of the regulation of cellular processes, including proliferation, cellular phenotype, metastasis ability, as well as response to pro- and anti-survival stimulus [9]. Similar to lncRNAs, but not the same, are pseudogenes. These transcripts interact with RNA, DNA, and protein molecules and are newly described elements of the epigenetic machinery. However, in contrast to the lncRNAs, their function and involvement in the cancerogenesis process are largely unknown, especially in the context of melanoma, which has been described by us previously [10]. The development of RNAseq techniques enables a deeper investigation of pseudogenes. One of the newly uncovered groups of pseudogenes are ribosomal protein pseudogenes (RPPs). It is estimated that about 2000 RPP transcripts can be found in the human transcriptome and it is thought to be the largest class of known pseudogenes [11, 12]. That constitutes a large number of transcripts in comparison to the 79 transcripts coding ribosomal proteins (RPs) in the human genome. Some of RPPs are not disturbed by stop codons or frameshifts and can be mistaken as functional RPs' genes. RPPs are distributed on a large scale in the whole genome and probably their location results from random insertions. Moreover, the number of pseudogenes is higher in the regions rich in GC-intermediate regions of the genome. Most of RP processed pseudogenes are located in the GC-poor regions [12]. RPP transcription depends on the tissue type and for some of them the level of RPPs is similar to the RP [11]. In contrast to the RPs, tissue-specific transcription of RPPs is most likely connected with biological processes [11], but it is not fully documented and understood. In contrast to pseudogenes, the RPs' parental genes are well characterized. Some evidence indicated that disturbance in RPs expression had an important influence on cancer cell behavior. Ribosome biogenesis is altered during neoplastic transformation and growing evidence shows that translation of RPs is upregulated which causes the nucleolar/ribosomal stress and influences cell cycle and proliferation, maintenance of genome integrity, and DNA repair, apoptosis, autophagy,

cell migration, invasion processes, and drug resistance [13, 14]. These proteins together with rRNAs are not only components of ribosomes for production of extra "cancer" proteins but also take part in the cellular regulation via eg. *uL18* and *uL5* adjusting the *TP53* homeostasis [13]. Moreover, the *5S RNP-MDM2* pathway is implicated in cellular stress caused by chemotherapeutic drugs, and nutrient starvation, including hypoxia and oxidative stress, in response to over-suppressor and oncogenic transcription and replicative stress [15]. However, the function of RPPs remains unknown.

This work is focused on the biological role and diagnostic utility of ribosomal protein L23a pseudogene 53 (*RPL23AP53*). We engaged human primary epidermal melanocytes, melanoma cell lines, and TCGA data of melanoma patients to study its function *in vitro* and *in silico*. There is no information about ribosomal protein L23a pseudogene 53 in cancer biology, but some evidence exists that L23a protein is upregulated in human hepatocellular carcinoma. [16] and prostate cancer [17]. It should be emphasized that ribosomal protein L23a has 107 pseudogenes, whose function is not known and should be described [18].

Materials and methods

Cell lines

The mutation status of the *BRAF* gene in the cell lines was obtained from the American Type Culture Collection (ATCC) or Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) cell culture databases and verified as described previously [19]. Cell lines used in the study were human adult and neonatal primary epidermal melanocytes: HEMa (ATCC® PCS-200-013) and HEMn (ATCC® PCS-200-012), respectively. Cells were cultured according to the ATCC (American Type Culture Collection, Manassas, Virginia, United States) protocol whereas human melanoma cell lines MEWO, SK-MEL28, WM115, WM266, WM9, and A549, were cultured as described previously [20].

qRT-PCR

Total RNA isolation and cDNA synthesis were performed as described previously [20]. Real time quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using 2x concentrated SYBR Green Master Mix (Roche)

with specific primers to detect: *RPL23AP53* (ENST00000521854) forward 5'-GAA GAT CCG CAT GTC ACT CA-3' and reverse 5'-TGG TCA GCG GAA ACT TGA TA-3' designed using The Universal ProbeLibrary Assay Design Center online tool from Roche. All primers were verified using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST). RT-PCR was performed on a LightCycler 480 (Roche) with the melting curve to discriminate between non-specific products. All real-time PCR data were analyzed by calculating the $2^{-\Delta CT}$, normalizing against the *GAPDH* expression amplified using forward 5'-CCA CTC CTC CAC CTT TGA CG-3' and reverse 5'-CCA CCA CCC TGT TGC TGT AG-3' primers [21].

Data sets

The TCGA data of selected transcripts was downloaded from XenaBrowser University of California, Santa Cruz, cohort: TCGA Melanoma (SKCM), cBioportal [22], the University of ALabama at Birmingham CANcer (UALCAN) [23], and ENCORI [24] databases. For validation of *RPL23AP53* expression levels, Gene Expression Omnibus (GEO) datasets were used: GSE8401 [25], GSE3189 [26], and GDS1989 [27]. All data from XenaBrowser and GEO is available online with unrestricted access which does not require any permission from the patient or institution.

Clinical-pathological data analysis

The expression levels of transcripts were analyzed depending on the clinicopathological parameters, such as: sample type (primary *vs.* metastasis), cancer type (cutaneous melanoma *vs.* other), cancer localization (extremities *vs.* trunk *vs.* regional lymph node *vs.* head and neck *vs.* distant metastasis *vs.* cutaneous or subcutaneous tissue *vs.* other), gender (women *vs.* men), age (< 58 *vs.* > 58), ulceration (absent *vs.* present), Clark level (I *vs.* II *vs.* III–IV *vs.* V), Breslow depth (< 1 *vs.* 1–2 *vs.* 2.1–4 *vs.* > 4), mitotic rate (0–2 *vs.* 2–3 *vs.* > 4), cancer stage (0 *vs.* I + II *vs.* III + IV), M-stage (M0 *vs.* M1), T-stage (T0 *vs.* T1 + T2 *vs.* T3 + T4), similarly as described previously [28]. Next, from a group of 472 patients, high- and low-expressing groups of RPL23AP53 were obtained using the quartile expression as a cutoff: low (< 25) and high (> 75). The expression level was analyzed in the group of all patients, as the median

of *RPL23AP53* and separately depending on *BRAF* status, with mutation (MUT) and wild type (WT). Progression-free interval (PFI) and overall survival (OS) were assessed in these subgroups, similarly as described previously [28].

Phenotype analysis

Positive (R ≥ 0.3) and negative (R ≤ −0.3) Pearson correlations for *RPL23AP53* transcript with genes were downloaded from cBioPortal [22] and analyzed using REACTOME pathway analysis [p < 0.05 and false discovery rate (FDR) < 0.05] for distinguishing specific pathways and cellular processes. Functional enrichment analysis and prediction of gene function were performed using Gene Set Enrichment Analysis (GSEA) software version 3.0 (http://www.gsea-msigdb.org/gsea/index.jsp). Melanoma patients were divided into two groups with high and low expression of selected transcripts by the mean of the expression level. The input file contained expression data for 20'530 genes and 565 patients. Gene set permutations with a parameter set as 1000 were used for the analysis. Pathways (hallmark gene sets (H) and collection from MSigDB) with nominal p-value ≤ 0.05 and FDR ≤ 0.25 were considered significant, similarly as described previously [29].

Immune cell infiltration analysis

Immune and ESTIMATE Scores (Estimation of STromal and Immune cells in MAlignant Tumor tissues using Expression data) were downloaded from https://bioinformatics.mdanderson.org/estimate/disease.html and used to assess the infiltration of immune cells into tumor tissue and to infer tumor purity, as described previously [30]. Subpopulations of specific immune cells were estimated based on data presented by Thorsson et al. [31].

Statistical analysis

All statistical analyses were performed using GraphPad Prism9 (GraphPad, San Diego, CA, United States). T-test, Mann-Whitney U test, or one-way ANOVA test were used depending on data normality estimated using the Shapiro-Wilk normality test. The RFS and OS analyses were performed using the log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests, respectively. All t-tests and ANOVA tests were performed as two-tailed and considered significant at $p < 0.05$, similarly as described previously [29].

Results

Expression of RPL23AP53 is changed in cell lines and melanoma patients

The expression level of ribosomal protein L23a pseudogene 53 was tested in normal melanocytes, melanoma cell lines, and the TCGA melanoma patients. No significant changes between melanoma and melanocytes were observed (0.05573 ± 0.01591 *vs.* 120.0 ± 76.82; $p = 0.1314$) for *RPL23AP53*. The specified melanoma cell lines displayed different *RPL23AP53* levels ($p = 0.0032$), which was higher in cells with *BRAF* V600E than *BRAF* V600D mutation (240.1 ± 146.6 *vs.* $3.680\text{e-}007 \pm 3.055\text{e-}007$; $p = 0.0028$) (Fig. 1A).

Next, the *RPL23AP53* pseudogene was analyzed using the TCGA data. It displayed significant differences in expression between primary and metastatic samples (0.229 *vs.* 0.599; $p = 2.43 \times 10^{-6}$ and 1.94 *vs.* 2.51; $p = 0.0316$, respectively), and downregulation of *RPL23AP53* in N0 compared to N3 samples (2.403 *vs.* 1.755; $p = 0.0250$), as well as N1 *vs.* N3 (2.611 *vs.* 1.755; $p = 0.0314$). There were no significant differences in *RPL23AP53* expression between the samples depending on the *BRAF* mutation status. However, when the correlation between the expression of *RPL23AP53* and *BRAF* mRNA in *BRAF* WT, *BRAF* MUT, and *BRAF* V600E patient subgroups was analyzed, significant correlations between the expression of these transcripts in a group of *BRAF* WT ($R = 0.2560$, $p = 0.0020$), *BRAF* MUT ($R = 0.3215$, $p < 0.0001$) and *BRAF* V600E ($R = 0.3889$, $p < 0.0001$) were observed (Fig. 1B).

The expression of RPL23AP53 is independent of clinicopathological parameters

Next, the expression levels of *RPL23AP53* regarding clinicopathological parameters were analyzed using the TCGA database. No significant differences ($p > 0.05$) in the expression levels of *RPL23AP53* and gender, age, ulceration, Clark level, Breslow depth, mitotic rate, M-, T-stages, cancer type nor neoplasm disease stage were noticed (Tab. 1).
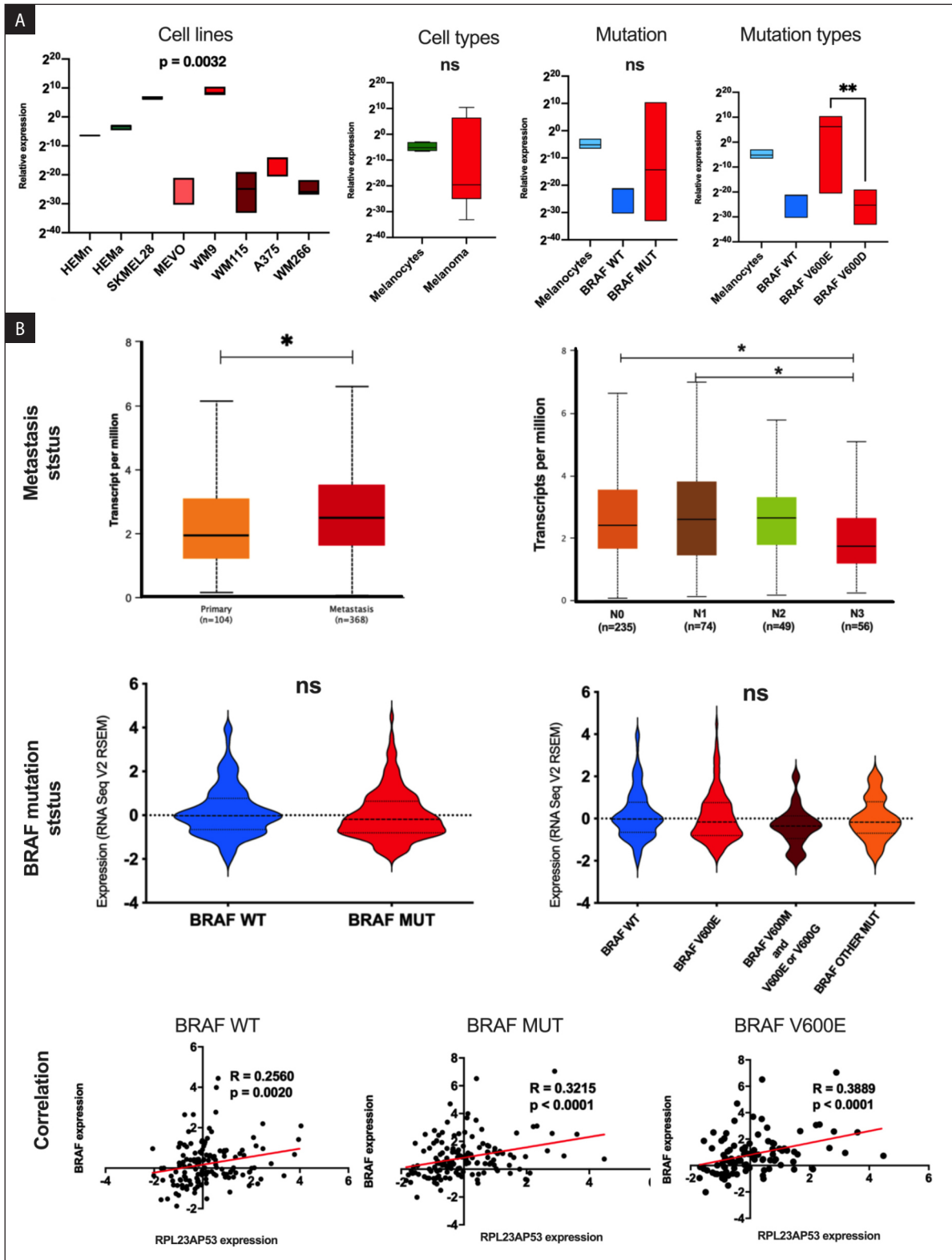
Figure 1. The expression level of *RPL23AP53* pseudogene in: melanocytes and melanoma cell lines: *BRAF* WT (MEVO), *BRAF* V600E (SKMEL28, WM9, and A549), and *BRAF* V600D (WM115 and WM266) (**A**), and melanoma patients from the Cancer Genome Atlas (TCGA) database depending on metastasis status and regional nodal metastases status [graphs from the University of ALabama at Birmingham CANcer (UALCAN) database, modified], *BRAF* mutation status and *BRAF* expression level. *BRAF* wild type (WT), *BRAF* mutated (MUT) and *BRAF* V600E (c. 1799T>A) subgroups (**B**); T-test, one way ANOVA with Kruskal-Wallis test and Spearman correlation; solid lines in the boxplots and dashed lines in violin plots represent mean of expression; p < 0.05 considered as significant, ns - no significant, * p ≤ 0.05, ** p ≤ 0.01

Table 1. The expression levels of *RPL23AP53* depending on clinicopathological parameters in The Cancer Genome Atlas (TCGA) melanoma patients; p < 0.05 considered as significant

Parameter	Group	Cases	RPL23AP53	
			Mean ± SEM	p-value
Gender	Male	290	0.3804 ± 0.05149	0.2386
	Female	178	0.3172 ± 0.06585	
Age	≤ 58	236	0.344 ± 0.05770	0.6921
	> 58	227	0.3663 ± 0.05737	
Ulceration	Yes	167	0.3449 ± 0.07375	0.3751
	No	147	0.3088 ± 0.06253	
Clark level	I	6	-0.8354 ± 0.8310	0.2577
	II	18	0.4998 ± 0.1706	
	III	78	0.4666 ± 0.06916	
	IV	167	0.3722 ± 0.06869	
	V	53	0.2564 ± 0.1158	
Breslow depth	< 1.0	51	0.3575 ± 0.08995	0.8240
	1.0–2.0	88	0.406 ± 0.08832	
	2.1–4.0	78	0.4712 ± 0.07299	
	> 4.0	143	0.3458 ± 0.08105	
Mitotic rate	<1	3	0.44 ± 0.6814	0.8363
	1–4	65	0.369 ± 0.08878	
	> 4	88	0.3793 ± 0.09413	
M Stage	M0	418	0.3572 ± 0.04292	0.8753
	M1	24	0.3756 ± 0.1354	
T Stage	T0	23	0.3326 ± 0.1736	0.5480
	T1–T2	121	0.3088 ± 0.07192	
	T3–T4	244	0.383 ± 0.05653	
Cancer Type	Cutaneous melanoma	69	0.3008 ± 0.1088	0.3570
	Desmoplastic melanoma	3	0.826 ± 0.3703	
	Melanoma	28	0.1691 ± 0.1249	
	Acral melanoma	2	0.627 ± 0.4657	
	Lentigo maligna melanoma	1	1.186 ± 0.0	
Neoplasm disease stage	0	6	−0.8765 ± 0.8283	0.1866
	I–II	218	0.3859 ± 0.05387	
	III–IV	194	0.3584 ± 0.06433	

SEM — standard error of the mean

A higher expression of RPL23AP53 is associated with better survival of melanoma patients

Next, melanoma patients were divided into groups with high and low *RPL23AP53* expression, and progression-free survival (PFI) as well as overall survival (OS) were analyzed. Patients with higher *RPL23AP53* expression showed slightly longer PFI (p = 0.1211 and p = 0.3061, respectively), while significantly longer OS (p = 0.0121 and p = 0.0088, respectively) (Fig. 2A). When patients were divided into *BRAF* MUT, no significant differences were observed in the case of PFI (p = 0.0818 and p = 0.0729) but patients with higher levels of *RPL23AP53* displayed longer survival time (OS) (p = 0.0313 and p = 0.0268) (Fig. 2B). No associations (p > 0.05) between PFI nor OS and expression levels of *RPL23AP53* were observed in the group of patients with wild type *BRAF* gene (Fig. 2C).
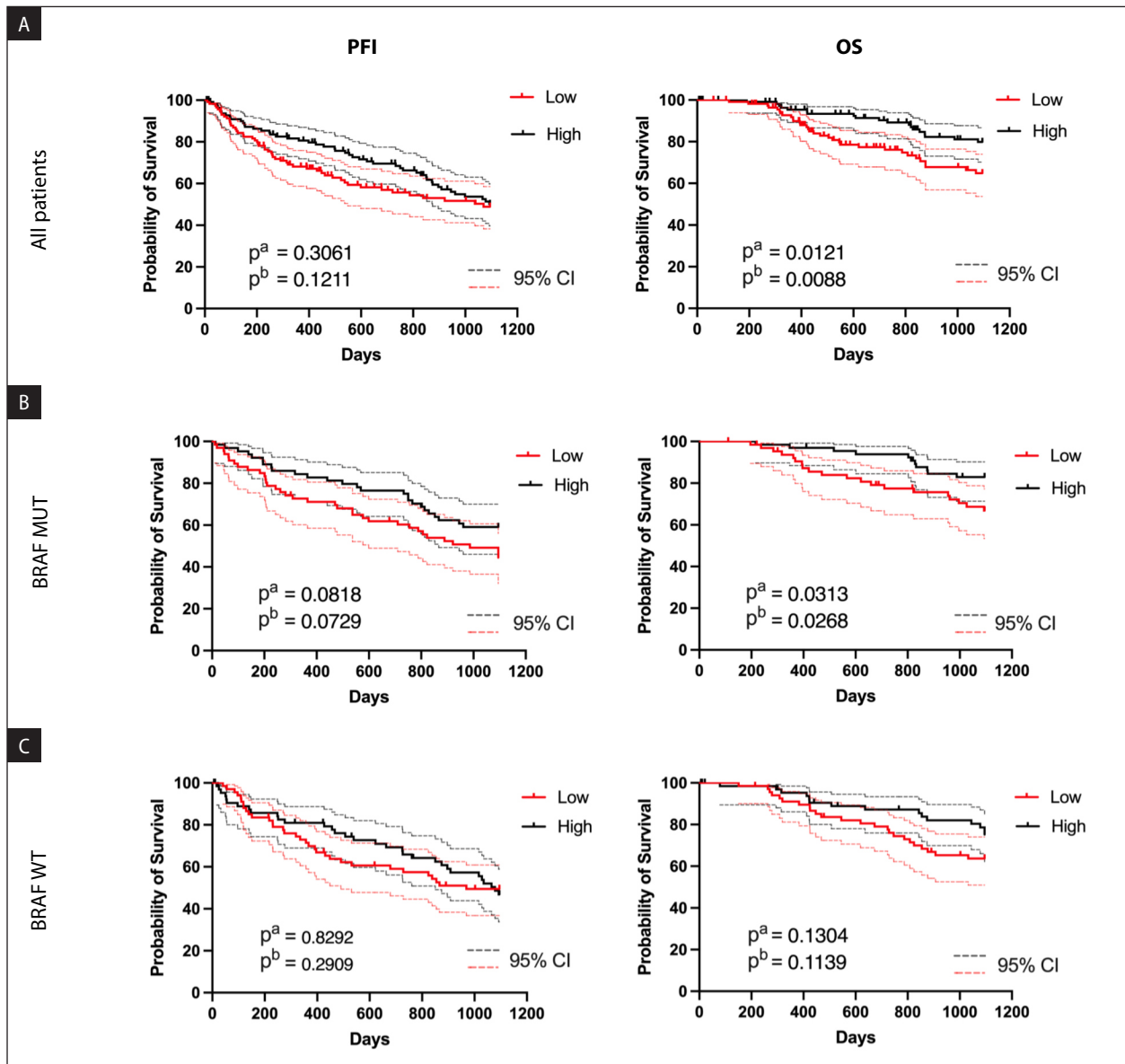
Figure 2. Progression-free interval (PFI) and overall survival (OS) of melanoma patients depending on the *RPL23AP53* expression levels in: group of all patients (**A**), patients with *BRAF* mutation (**B**) and patients with wild type of *BRAF* gene (**C**); results presented for 3 years of observation; low (red solid lines) and high (black solid lines) subgroups represent patients with 1st and 4th quartile of gene expression level for A panel and median expression level for panels B and C; p^a — Log-rank (Mantel-Cox) test; p^b — Gehan-Breslow-Wilcoxon test; 95% CI — 95% of confidence interval marked as dashed red and black lines; $p < 0.05$ considered as significant

A higher level of RPL23AP53 is associated with changes in essential signaling pathways

Next, positive ($R \geq 0.3$) and negative ($R \leq -0.3$) Pearson correlations of *RPL23AP53* transcript with genes involved in selected cellular processes were analyzed using the REACTOME tool. *RPL23AP53* was 5701 positively correlated with genes found in processes such as cell cycle and mitosis, RNA transcription, and metabolism, ($p < 0.05$). Only 68 genes were negatively correlated with *RPL23AP53* and associated with electron transport, translation, cellular responses to stress, and viral processes ($p < 0.05$). All results are presented in Figure 3.

Next, the Gene Set Enrichment Analysis (GSEA) analysis of patients with low and high expression of *RPL23AP53* was performed. In the patients group with lower *RPL23AP53* expression, changes
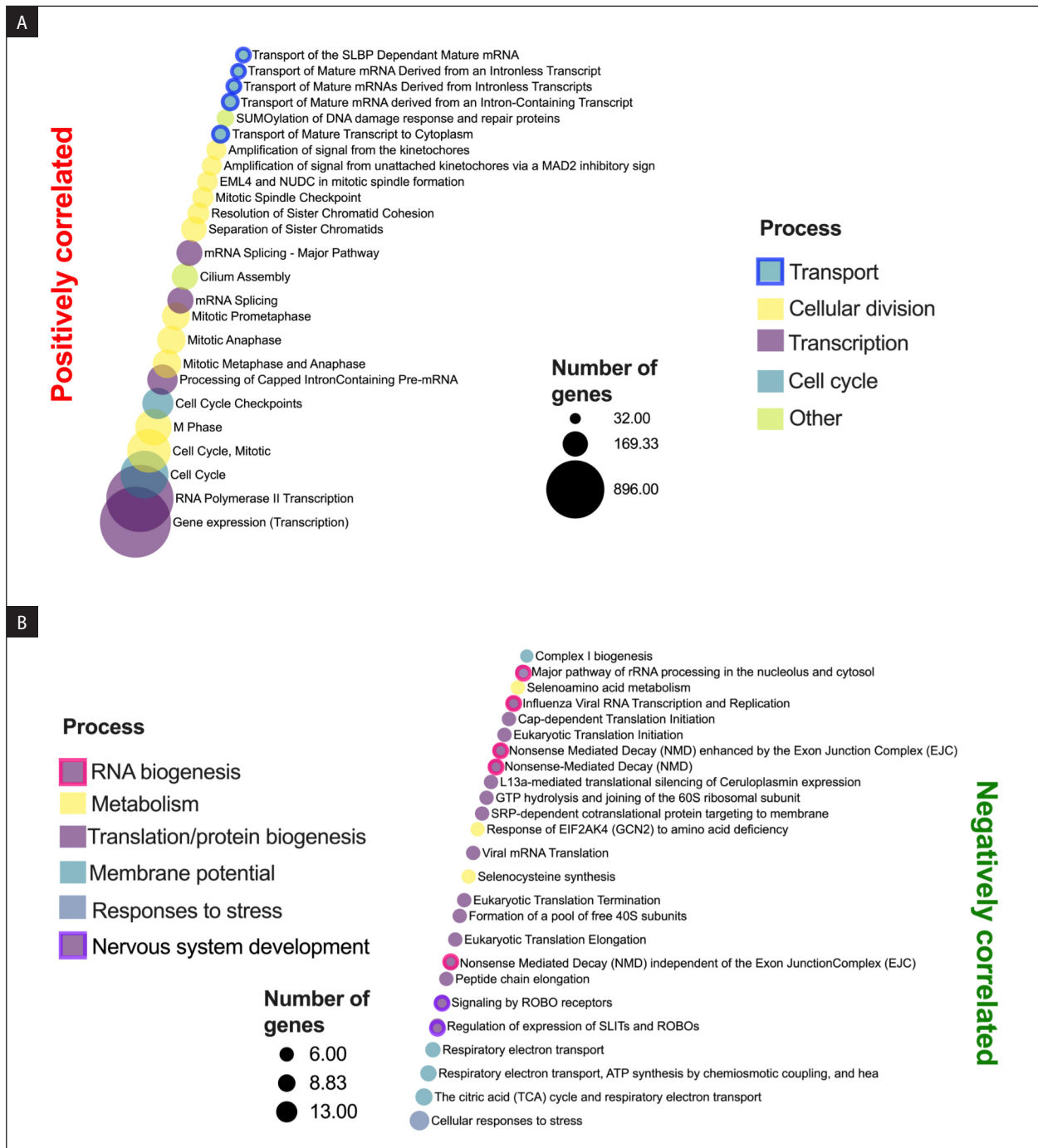
Figure 3. Genes correlated with *RPL23AP53*: positive (> 0.3) (**A**) and negative (< −0.3) (**B**) Pearson correlations of *RPL23AP53* gene with genes and analyzed using REACTOME pathway analysis for distinguishing cellular pathways and processes. Only results with p < 0.05 and false discovery rate (FDR) < 0.05 were shown

were found in RB_P130_DN.V1_DN, EIF4E_DN, TBK1.DF_DN and TBK1.DF_UP, PIGF_UP.V1_UP, ERBB2_UP.V1_DN, MEK_UP.V1_DN (genes down-regulated in cells positive for *ESR1* and stably overexpressing constitutively active MAP2K1), VEGF_A_UP.V1_DN, JAK2_DN.V1_DN, EGFR_UP.V1_DN (genes down-regulated in cells positive for ESR1 and express ligand-activatable EGFR), RAF_UP.V1_DN, and MTOR_UP.N4.V1_DN. All data are presented in Figure 4A. Based on the GSEA results signaling pathways connected with melanoma, such as *MTOR, EGFR* and *MEK,* were chosen
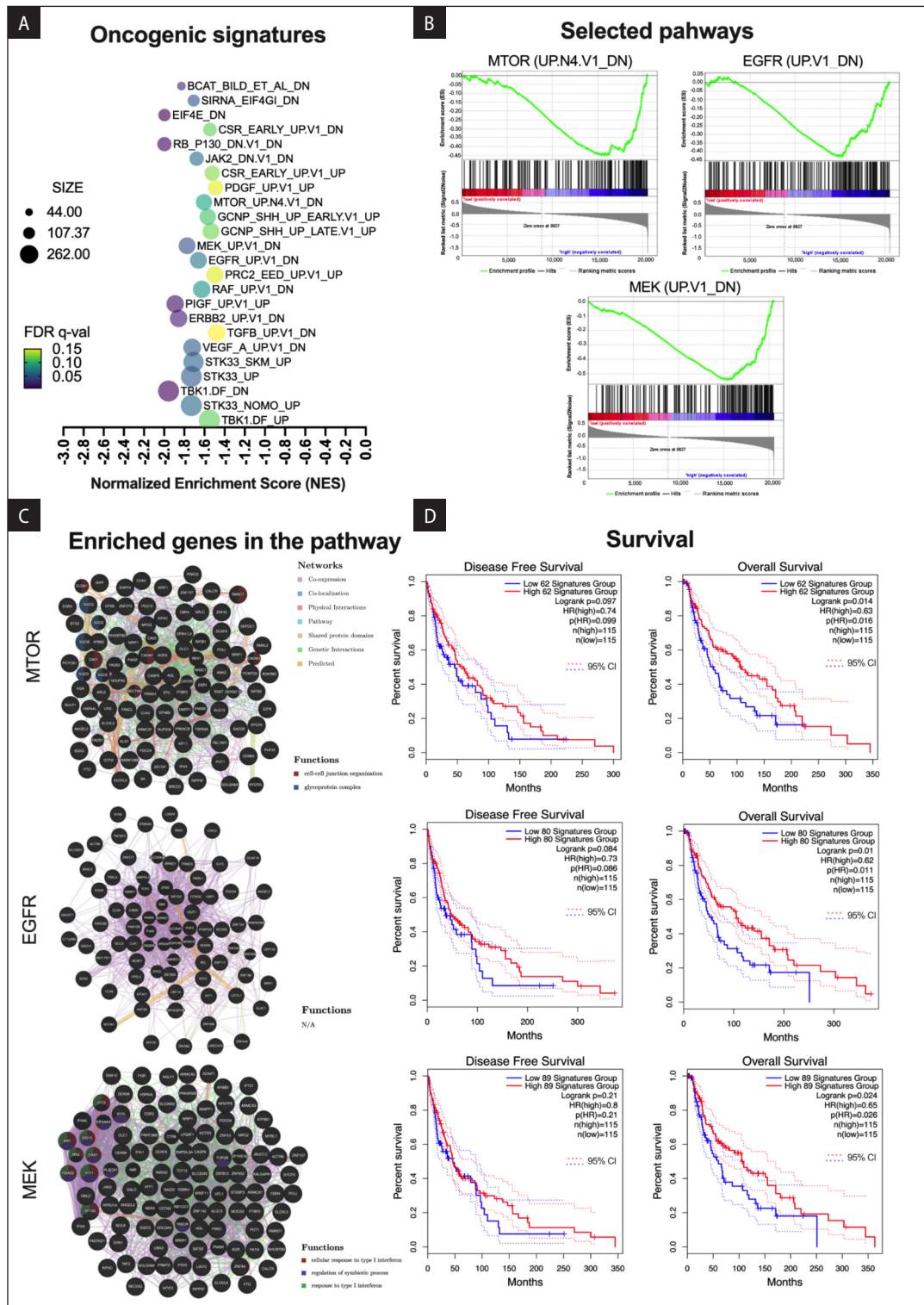
Figure 4. The phenotype associated with low or high expression levels of *RPL23AP53* in the Cancer Genome Atlas (TCGA) melanoma patients based on Gene Set Enrichment Analysis (GSEA) results. **A.** Changed molecular pathways in patients with lower expression of *RPL23AP53*; **B.** selected for melanoma *MTOR*, *MEK*, and *EGFR*; **C.** Involvement in cellular processes based on the GENEmania analysis tool; **D.** Association of genes from *MTOR*, *MEK* and *EGFR* pathways with disease-free survival and overall survival based on Gene Expression Profiling Interactive Analysis 2 (GEPIA2) database analysis; red and blue solid lines represent the group of patients with high and with low expression level of selected genes from the pathway, respectively; 95% CI — 95% of confidence interval marked as dashed red and blue lines; NES — normalized enrichment score; p-val — nominal p-value), FDR q-value — false discovery rate); only results set with p ≤ 0.05 and FDR ≤ 0.25 are shown; p < 0.05 considered as statistically significant

for further investigation (Fig. 4B). In the *MTOR* pathway, 62 genes were observed as enriched and involved in cell-cell junction organization (6 genes) and glycoprotein complex (5 genes) as well as other processes. For 80 genes from the *EGFR* pathway, no association with any processes was indicated. In the case of the *MEK* pathway, 89 genes were associated with the regulation of a cellular response to type I interferon (8 genes), regulation of symbiotic process (10 genes), response to type I interferon (8 genes), and response to the viral infection (Fig. 4C). Next, the patients' disease free (DFS) and overall survival (OS) depending on the expression levels of genes indicated in GSEA analysis and connected with melanoma were analyzed. Significantly longer overall survival times of patients with higher levels of *MTOR* (p = 0.014), *EGFR* (p = 0.01), and *MEK* (p = 0.024) genes were observed. No differences (p < 0.05) were indicated in the disease-free survival (Fig. 4D).

RPL23AP53 expression is correlated with the immune profile of melanoma patients

Significant differences between higher and lower levels of *RPL23AP53* and the immune and ESTI-MATE scores (p = 0.0483 and p = 0.0030, respectively) were found and no differences in the stromal cells and levels of *RPL23AP53* (p > 0.05) were observed (Fig. 5A). Patients with higher expression of *RPL23AP53* displayed a lower fraction of lymphocytes (p = 0.0108) and higher fraction of macrophages (p = 0.0146), and the population of neutrophils was raised (p = 0.0158) (Fig. 5B). In the case of higher expression of *RPL23AP53*, lower expression of CD8 T cells and higher CD4 memory resting (p = 0.0367 and p = 0.0009, respectively) as well as lower expression of regulatory Tregs (p < 0.0001), and significantly higher infiltration of macrophages M2 (p = 0.0004) were observed; no differences in B cell lymphocytes (p > 0.05) in
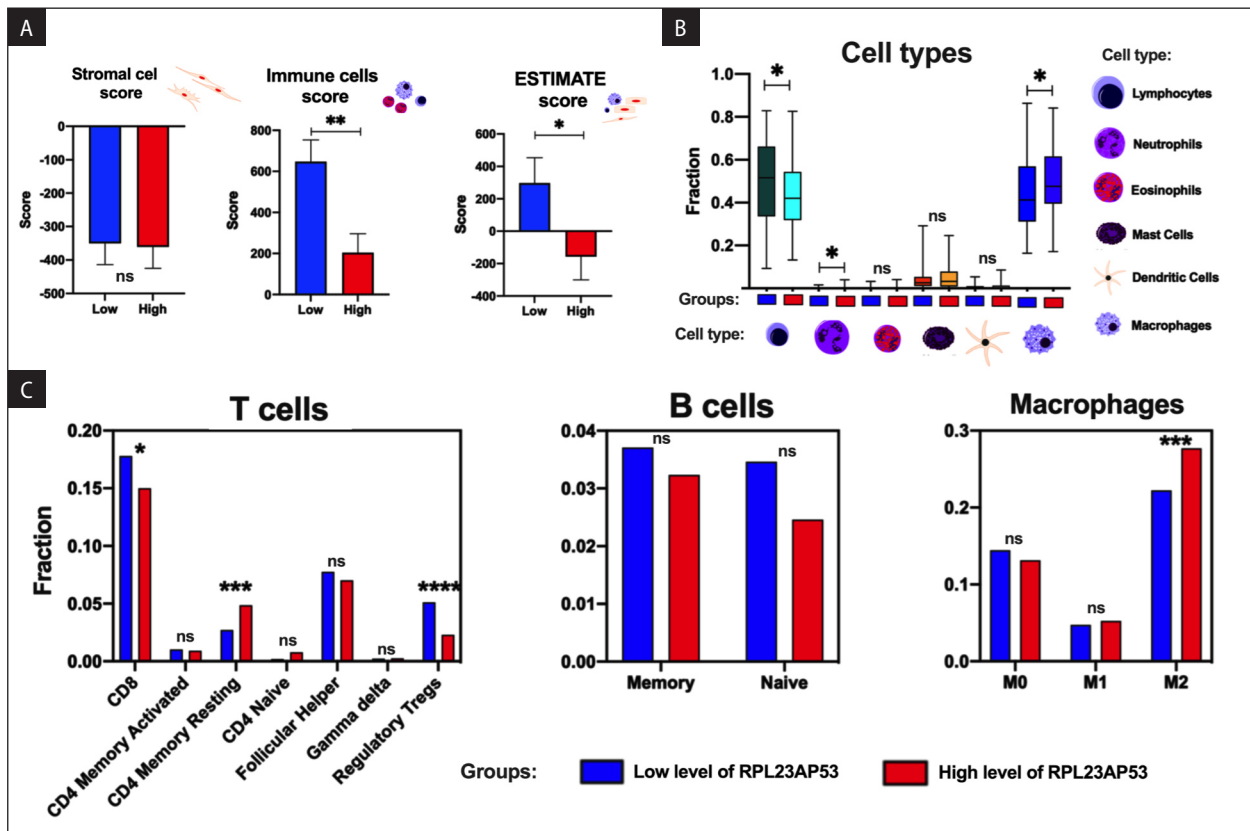
Figure 5. Association of the immunological profile of the Cancer Genome Atlas (TCGA) melanoma patients with the expression levels of *RPL23AP53*: **A.** ESTIMATE, immune cells, and stromal cells scores; **B.** differences of lymphocytes, neutrophils, eosinophils, mast cells, dendritic cells, and macrophages; **C.** The fraction of specific subpopulation of T cells, B cells, and macrophages depending on the low and high expression levels of *RPL23AP53*; T-test or Mann Whitney test, p < 0.05 considered as significant; ns — non significant; *p < 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001

patients with higher levels of *RPL23AP53* were indicated (Fig. 5C).

Expression levels of RPL23AP53 differ depending on the type of melanoma samples

Next, the expression levels of *RPL23AP53* in patients' samples and cell lines were validated using available data from GEO. First, based on the data from a study made by Talantov et al. (data set GSE3189) it was observed that the expression level of *RPL23AP53* is significantly lower in benign nevi and malignant melanomas in comparison to normal samples (73.51 ± 9.971 *vs.* 77.45 ± 7.209 *vs.* 164.1 ± 15.21, p = 0.0019 and p = 0.0007, respectively). Surprisingly, no differences were observed between nevus and melanoma (p > 0.9999) (Fig. 6A). Using expression data from cell lines
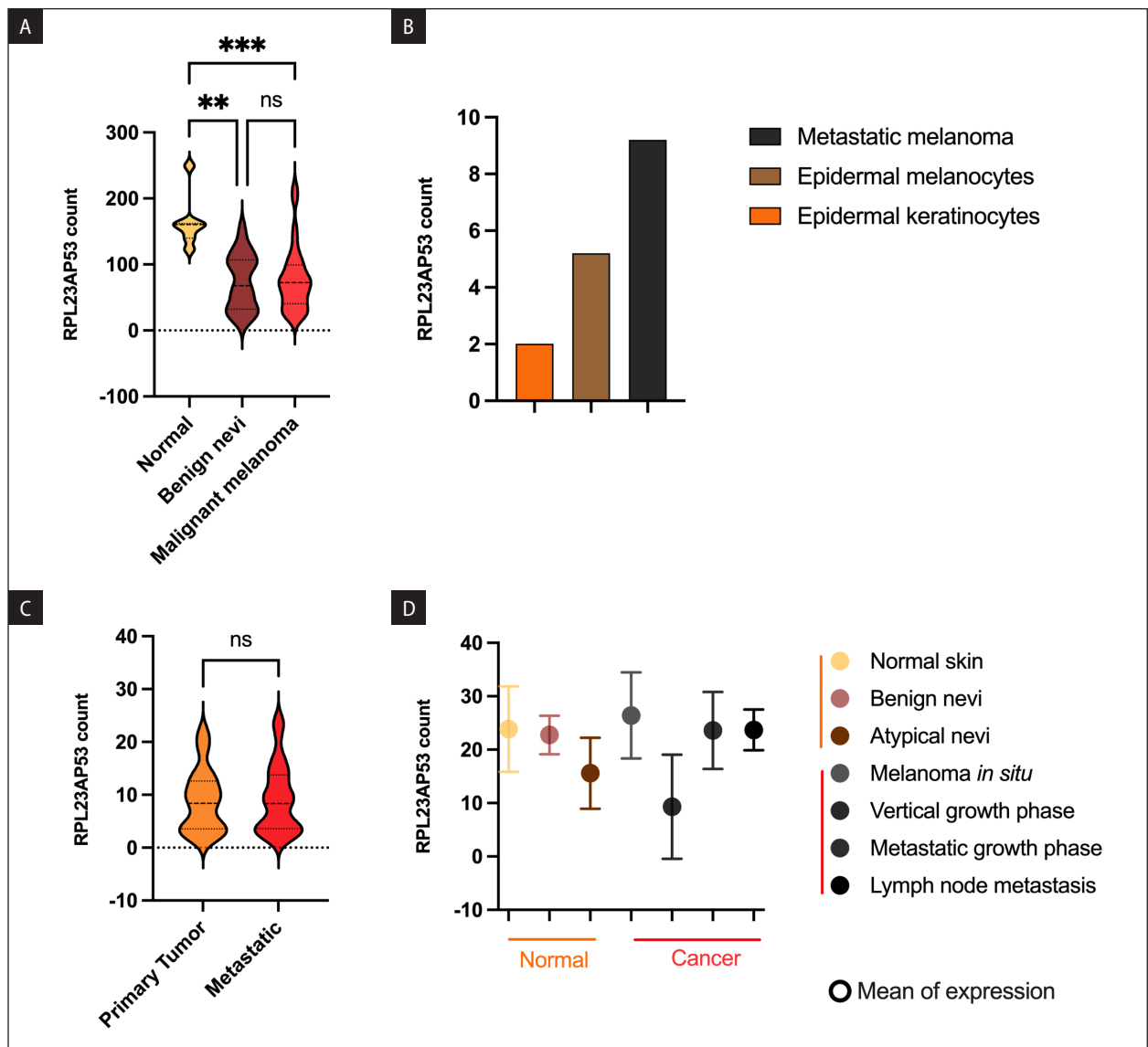
Figure 6. Validation of *RPL23AP53* expression levels using data from Gene Expression Omnibus (GEO). **A.** Differences in expression in normal, benign nevi, and malignant melanoma samples based on GSE3189, One way Anova with Dunn's multiple comparisons test; **B.** Expression in epidermal keratinocytes and melanocytes compared to metastatic melanoma cell lines (GSE3189), only one replicates included in data set per sample type; **C.** Expression levels based on primary tumor and metastatic samples from GSE8401, Mann Whitney test; **D.** Comparison of normal (normal skin, benign nevi, atypical nevi) and cancer (melanoma *in situ*, vertical and metastatic growth phases, lymph node metastasis) samples in GDS1989 data set, dots represent mean of value, only two replicates available per sample type; dashed lines in violin plots represent mean of expression; p < 0.05 considered as significant; ns — non significant, **p ≤ 0.01, ***p ≤ 0.001

(GDS1989), *RPL23AP53* expression was higher in a metastatic melanoma cell line (value = 9.2) than in epidermal melanocytes (value = 2) and keratinocytes (value = 5.2), but those data lack replicates for statistical analysis (Fig. 6B). However, based on the other data set (GSE8401) no differences were indicated between primary and metastatic melanomas (8.952 ± 1.066 *vs.* 9.282 ± 0.8906, p = 0.8405) (Fig. 6C). Smith et al. presented also data from different normal and cancer samples, where expression levels of *RPL23AP53* depended on the various histological types of samples. Estimated samples included normal skin (23.85 ± 5.65), benign nevi (22.75 ± 2.55), atypical nevi (15.60 ± 4.70) and melanoma *in situ* (26.40 ± 5.70), vertical (9.30 ± 6.90), and metastatic (23.60 ± 5.10) growth phases, lymph node metastasis (23.70 ± 2.21) (Fig. 6D).

Discussion

It is the first report, where the biological role and diagnostic utility of ribosomal protein L23a pseudogene 53 (*RPL23AP53*) were studied based on human primary epidermal melanocytes and melanoma cell lines as well as melanoma patients using the TCGA data. *RPL23AP53* pseudogene is dysregulated in specified melanoma cell lines and in melanoma patients' metastatic tissue. However, its expression seems to depend on the cell line or tissue features, and can be either upregulated or downregulated. In melanoma samples lower expression of this pseudogene correlates with more advanced stages and lymph node metastases. The *BRAF* mutation status is known to be an important marker in melanoma, and the gene is mutated in approximately 50% of melanoma patients. We investigated the association of *RPL23AP53* with *BRAF* mutation and observed increased expression of *RPL23AP53* in *BRAF* V600E mutated cell lines but not in the TCGA patients. However, the expression levels of *RPL23AP53* positively correlated with *BRAF* expression in the group of patients without mutation in *BRAF* gene, but especially in the group with mutated forms of *BRAF* gene. However, patients with higher levels of *RPL23AP53* displayed better survival rates than those with lower expression of this pseudogene. One of the reasons is the probable connection of *RPL23AP53* with proliferating cancers. It should be noted that one of the futures of cancer stem cells is a slow proliferation ratio and high self-renewing capacity [32]. Moreover, the cells with features of cancer stem cells are resistant to radiotherapy and chemotherapy, and they are most likely responsible for metastasis [33, 34]. Fattore et al. discovered the molecular pathways characteristic of slow-cycling melanoma stem cells. They identified 25 genes and divided them into four groups: i) kinases and metabolic genes, ii) melanoma-associated proteins, iii) Hippo pathway, and iv) slow cycling/CSCs factors [35]. In our work we indicated that melanoma patients with higher levels of *RPL23AP53* are correlated with transcription, cell cycle, RNA maturation and mitosis. Moreover, based on the GSEA analysis, we observed that patients with lower levels of *RPL23AP53* displayed upregulation of *MTOR*, *EGFR* and *MEK* pathways. It is known that the *MTOR* signaling is crucial for melanoma pathogenesis and, at the same time, is a modulator of cancer stem cells [36]. The Epidermal Growth Factor Receptor (*EGFR*) has been described as an essential receptor tyrosine kinase-regulator of cancer stem cells and modulation of this pathway could be used for radiotherapy due to involvement of *EGFR* pathway in DNA repair process [37]. The *MEK* pathway is frequently activated in melanomas and many different inhibitors of this pathway are used; however, it is characterized by *de novo* and acquired resistance which leads to cancer stem cells division and cancer progression [38].

Smith et al. based on gene expression profiling of different stages of melanoma development indicated that genes involved in mitotic cell cycle regulation and cell proliferation were the two main groups of genes engaged in melanoma progression [27]. They observed that nevus constituted of non-dividing cells and *in situ* melanomas are characterized by comparatively low mitotic/proliferation ratios. In contrast, cells in the vertical and metastatic growth phases aggressively divide [27]. However, it should be emphasized that changes in some genes characterized for metastatic melanomas can also be observed in nevus, which reflects the underlying complexity of transcriptome changes during cancer progression [27]. Hust et al. observed that vertically growing melanomas are highly tumorigenic and metastatic, and cells are expansive with their anchorage-independent growth ability [39]. Interestingly, we observed that the expression lev-

el of *RPL23AP53* is the lowest in vertical growth phase melanomas compared to pre-malignant and advanced melanoma stages. We suppose, based on the results presented here, that the expression level of *RPL23AP53*, and probably other ribosomal protein pseudogenes, is negatively correlated with cancer stem cells content in melanoma. However, this hypothesis needs to be experimentally verified.

It should be noted that *RPL23AP53* pseudogene and its role, especially in cancer, has not been described previously. Balasubramanian et al. reported a large-scale comparative analysis of ribosomal protein pseudogenes in four mammalian genomes and observed that all of them contain large numbers of ribosomal protein pseudogenes assessed between 1'400 to 2'800 transcripts. Moreover, most of the pseudogenes originated from independent lineage-specific retro-transposition activities taking place in the genomes [34]. It has been indicated that in humans, ribosomal protein pseudogenes are constitutively but differentially expressed in almost all tissues in contrast to genes encoding ribosomal proteins. These transcripts could participate in biological processes in a tissue-specific way [11]. Some studies indicated the disturbance of ribosomal protein pseudogenes in cancer, such as *RPSAP52* in pituitary adenomas, which regulates *p21Waf1/CIP* expression by interacting with the RNA binding protein HuR [40]. Wang et al., based on the TCGA data and patients' samples, indicated that *RPSAP52* is upregulated in glioblastoma and its high expression predicted poor survival. *In vitro* model showed that *RPSAP52* upregulates *TGF-β1* and causes higher cancer stemness [41]. Ribosomal protein L23A (*rpL23A*), also known as melanoma differentiation-associated gene (*mda-20*), was reduced in human cells including melanoma cells treated with human leukocyte (*IFN-α*) and immune (*IFN-γ*) interferons, but not by growth inhibition resulting from serum starvation. It has been shown that the expression of *rpL23A* was necessary for cell growth [42]. Kondo et al. observed that *S8*, *L12*, *L23a*, *L27*, and *L30* ribosomal protein mRNAs are upregulated in hepatocellular carcinoma compared to adjacent normal tissues and are important for cancer development [16]. In addition, the expression level of *rpL23A* was upregulated in cancer compared to normal and hyperplasia cells [17].

It should be strongly emphasized that knowledge related to lncRNA and, especially, pseudogene tran-

scripts in melanoma is very limited. Guo et al. identified seven pseudogenes, *SRP9P1*, *RP4-800G7.1*, *CH17-264B6.3*, *C1DP1*, *MTND4P12*, *LDHAP3*, and *RP11-359E7.3* as upregulated, based on the TCGA data [43]. Moreover, there are no studies on pseudogene transcript alteration depending on the BRAF gene status in melanoma. Only few studies have focused on lncRNAs and BRAF gene, and they examined BRAF-activated non-coding RNA (*BANCR*) and *RMEL 1, 2*, and *3* [44, 45].

Finally, we checked whether the expression level of *RPL23AP53* pseudogene is connected with the infiltration of immune cells in patients' samples. Our results indicated that patients with higher levels of *RPL23AP53* pseudogenes present distinct immunological profiles. Our results also indicated that patients with higher levels of *RPL23AP53* had a lower fraction of CD8+ T cells and higher levels of neutrophils. Yang et al. based on the TCGA data calculated the immune and stromal scores using the ESTIMATE algorithm and the abundance of six infiltrating immune cells using the TIMER algorithm in melanoma patients. They observed that the prognosis of the patients with higher numbers of CD8+ T cells and neutrophils was better [46]. Better patients' survival for patients with higher expression of *RPL23AP53* could be associated with a higher level of T cell CD4 memory resting and a higher level of neutrophils. However, we also observed that patients with higher levels of *RPL23AP53* displayed lower levels of Treg T cells. It was observed that Tregs are frequently infiltrated into melanoma and the CD8+ T and Treg cells ratio is the predictive indicator for patient survival. Moreover, depletion of Tregs seems to be a promising strategy in melanoma treatment [47]. We also observed a change in M2 macrophages levels which were up-regulated in the group of patients with higher levels of *RPL23AP53*. It is known that two types of tumor associated macrophages (TAMs) can be distinguished: M1 responsible for activation of the adaptive immune system and M2 which are described as pro-tumor [48]. As in the case of Tregs, estimation of M2 macrophages can be used for future therapeutic strategies [48].

Conclusion

This is the first study demonstrating the association of *RPL23AP53* pseudogene with melanoma,

better survival rates and favorable immune profiles. However, the exact role of ribosomal pseudogenes is not understood. They could potentially regulate the expression levels of selected miRNAs and act as molecular sponge [49]. More studies need to be conducted to indicate the biological role of both ribosomal protein L23A and *RPL23AP53* pseudogene.

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Authors' contributions

Authors' individual contributions: conceptualization — T.K.; methodology — T.K.; investigation — T.K., K.G., P.P., J.K.M., A.B.; data curation — T.K., K.G., P.P., J.K.M.; writing — original draft preparation: T.K., P.P.; writing — review and editing: T.K., U.K., A.T.; visualization — T.K.; supervision — A.T.; funding acquisition — A.T., K.G. All authors read and approved the final manuscript.

Conflict of interests

The authors declare that there is no conflict of interest regarding the publication of this paper. The use of the data does not violate the rights of any person or any institution.

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Availability of data and materials

The datasets used during the current study are available online from the XenaBrowser University of California, Santa Cruz, cohort: TCGA Melanoma (SKCM), cBioportal, UALCAN, ENCORI, and GEO databases, or from the corresponding author on reasonable request.

Ethics approval

All data is available online, access is unrestricted and does not require the patient's consent or other permissions. The use of the data does not violate the rights of any person or any institution.

Consent for publication

All authors have read and approved the final manuscript.

References

1. Liu-Smith F, Jia J, Zheng Y. UV-Induced Molecular Signaling Differences in Melanoma and Non-melanoma Skin Cancer. Adv Exp Med Biol. 2017; 996: 27–40, doi: 10.1007/978-3-319-56017-5_3, indexed in Pubmed: 29124688.

2. Naik PP. Cutaneous Malignant Melanoma: A Review of Early Diagnosis and Management. World J Oncol. 2021; 12(1): 7–19, doi: 10.14740/wjon1349, indexed in Pubmed: 33738001.

3. Scatena C, Murtas D, Tomei S. Cutaneous Melanoma Classification: The Importance of High-Throughput Genomic Technologies. Front Oncol. 2021; 11: 635488, doi: 10.3389/fonc.2021.635488, indexed in Pubmed: 34123788.

4. Davis LE, Shalin SC, Tackett AJ. Current state of melanoma diagnosis and treatment. Cancer Biol Ther. 2019; 20(11): 1366–1379, doi: 10.1080/15384047.2019.1640032, indexed in Pubmed: 31366280.

5. Dabestani PJ, Dawson AJ, Neumeister MW, et al. Radiation Therapy for Local Cutaneous Melanoma. Clin Plast Surg. 2021; 48(4): 643–649, doi: 10.1016/j.cps.2021.05.008, indexed in Pubmed: 34503724.

6. Kasakovski D, Skrygan M, Gambichler T, et al. Advances in Targeting Cutaneous Melanoma. Cancers (Basel). 2021; 13(9), doi: 10.3390/cancers13092090, indexed in Pubmed: 33925915.

7. Cancer Genome Atlas Network. Genomic Classification of Cutaneous Melanoma. Cell. 2015; 161(7): 1681–1696, doi: 10.1016/j.cell.2015.05.044, indexed in Pubmed: 26091043.

8. Darp R, Ceol C. Making a melanoma: Molecular and cellular changes underlying melanoma initiation. Pigment Cell Melanoma Res. 2021; 34(2): 280–287, doi: 10.1111/pcmr.12950, indexed in Pubmed: 33283422.

9. Wozniak M, Czyz M. The Functional Role of Long Non-Coding RNAs in Melanoma. Cancers (Basel). 2021; 13(19), doi: 10.3390/cancers13194848, indexed in Pubmed: 34638331.

10. Stasiak M, Kolenda T, Kozłowska-Masłoń J, et al. The World of Pseudogenes: New Diagnostic and Therapeutic Targets in Cancers or Still Mystery Molecules? Life (Basel). 2021; 11(12), doi: 10.3390/life11121354, indexed in Pubmed: 34947885.

11. Tonner P, Srinivasasainagendra V, Zhang S, et al. Detecting transcription of ribosomal protein pseudogenes in

diverse human tissues from RNA-seq data. BMC Genomics. 2012; 13: 412, doi: 10.1186/1471-2164-13-412, indexed in Pubmed: 22908858.

12. Zhang Z, Harrison P, Gerstein M. Identification and analysis of over 2000 ribosomal protein pseudogenes in the human genome. Genome Res. 2002; 12(10): 1466–1482, doi: 10.1101/gr.331902, indexed in Pubmed: 12368239.

13. Pecoraro A, Pagano M, Russo G, et al. Ribosome Biogenesis and Cancer: Overview on Ribosomal Proteins. Int J Mol Sci. 2021; 22(11), doi: 10.3390/ijms22115496, indexed in Pubmed: 34071057.

14. Penzo M, Montanaro L, Treré D, et al. The Ribosome Biogenesis-Cancer Connection. Cells. 2019; 8(1), doi: 10.3390/cells8010055, indexed in Pubmed: 30650663.

15. Nishimura K, Kumazawa T, Kuroda T, et al. Perturbation of ribosome biogenesis drives cells into senescence through 5S RNP-mediated p53 activation. Cell Rep. 2015; 10(8): 1310–1323, doi: 10.1016/j.celrep.2015.01.055, indexed in Pubmed: 25732822.

16. Kondoh N, Shuda M, Tanaka K, et al. Enhanced expression of S8, L12, L23a, L27 and L30 ribosomal protein mRNAs in human hepatocellular carcinoma. Anticancer Res. 2001; 21(4A): 2429–2433, indexed in Pubmed: 11724303.

17. Vaarala M, Porvari K, Kyllönen A, et al. Several genes encoding ribosomal proteins are over-expressed in prostate-cancer cell lines: Confirmation of L7a and L37 over-expression in prostate-cancer tissue samples. Int J Cancer. 1998; 78(1): 27–32, doi: 10.1002/(sici)1097-0215(19980925)78:1<27::aid-ijc6>3.0.co;2-z, indexed in Pubmed: 9724090.

18. GeneCards. The human gene database. https://www.genecards.org/cgi-bin/carddisp.pl?gene=RPL23A (7 March 2022).

19. Mackiewicz-Wysocka M, Czerwińska P, Filas V, et al. Oncogenic BRAF mutations and p16 expression in melanocytic nevi and melanoma in the Polish population. Postepy Dermatol Alergol. 2017; 34(5): 490–498, doi: 10.5114/ada.2017.71119, indexed in Pubmed: 29507566.

20. Czerwinska P, Jaworska AM, Wlodarczyk NA, et al. Melanoma Stem Cell-Like Phenotype and Significant Suppression of Immune Response within a Tumor Are Regulated by TRIM28 Protein. Cancers (Basel). 2020; 12(10), doi: 10.3390/cancers12102998, indexed in Pubmed: 33076560.

21. Paszkowska A, Kolenda T, Guglas K, et al. C10orf55, CASC2, and SFTA1P lncRNAs Are Potential Biomarkers to Assess Radiation Therapy Response in Head and Neck Cancers. J Pers Med. 2022; 12(10), doi: 10.3390/jpm12101696, indexed in Pubmed: 36294833.

22. Gao J, Aksoy BA, Dogrusoz U, et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Sci Signal. 2013; 6(269): pl1, doi: 10.1126/scisignal.2004088, indexed in Pubmed: 23550210.

23. Chandrashekar DS, Bashel B, Balasubramanya SA, et al. UALCAN: A Portal for Facilitating Tumor Subgroup Gene Expression and Survival Analyses. Neoplasia. 2017; 19(8): 649–658, doi: 10.1016/j.neo.2017.05.002, indexed in Pubmed: 28732212.

24. Li JH, Liu S, Zhou H, et al. starBase v2.0: decoding miRNA-ceRNA, miRNA-ncRNA and protein-RNA interaction networks from large-scale CLIP-Seq data. Nucleic Acids Res. 2014; 42(Database issue): D92–D97, doi: 10.1093/nar/gkt1248, indexed in Pubmed: 24297251.

25. Xu L, Shen SS, Hoshida Y, et al. Gene expression changes in an animal melanoma model correlate with aggressiveness of human melanoma metastases. Mol Cancer Res. 2008; 6(5): 760–769, doi: 10.1158/1541-7786.MCR-07-0344, indexed in Pubmed: 18505921.

26. Talantov D, Mazumder A, Yu JX, et al. Novel genes associated with malignant melanoma but not benign melanocytic lesions. Clin Cancer Res. 2005; 11(20): 7234–7242, doi: 10.1158/1078-0432.CCR-05-0683, indexed in Pubmed: 16243793.

27. Smith AP, Hoek K, Becker D. Whole-genome expression profiling of the melanoma progression pathway reveals marked molecular differences between nevi/melanoma in situ and advanced-stage melanomas. Cancer Biol Ther. 2005; 4(9): 1018–1029, doi: 10.4161/cbt.4.9.2165, indexed in Pubmed: 16251803.

28. Kazimierczak U, Kolenda T, Kowalczyk D, et al. BNIP3L Is a New Autophagy Related Prognostic Biomarker for Melanoma Patients Treated With AGI-101H. Anticancer Res. 2020; 40(7): 3723–3732, doi: 10.21873/anticanres.14361, indexed in Pubmed: 32620611.

29. Tomaszewska W, Kozłowska-Masłoń J, Baranowski D, et al. Influences HNSCC Development and Progression through Regulation of the Epithelial-to-Mesenchymal Transition Process and Could Be Used as a Potential Biomarker. Biomedicines. 2021; 9(12), doi: 10.3390/biomedicines9121894, indexed in Pubmed: 34944712.

30. Koteluk O, Bielicka A, Lemańska Ż, et al. The Landscape of Transmembrane Protein Family Members in Head and Neck Cancers: Their Biological Role and Diagnostic Utility. Cancers (Basel). 2021; 13(19), doi: 10.3390/cancers13194737, indexed in Pubmed: 34638224.

31. Thorsson V, Gibbs DL, Brown SD, et al. Cancer Genome Atlas Research Network. The Immune Landscape of Cancer. Immunity. 2018; 48(4): 812–830.e14, doi: 10.1016/j.immuni.2018.03.023, indexed in Pubmed: 29628290.

32. Zimmerer RM, Korn P, Demougin P, et al. Functional features of cancer stem cells in melanoma cell lines. Cancer Cell Int. 2013; 13(1): 78, doi: 10.1186/1475-2867-13-78, indexed in Pubmed: 23915418.

33. Kolenda T, Przybyła W, Kapałczyńska M, et al. Tumor microenvironment - Unknown niche with powerful therapeutic potential. Rep Pract Oncol Radiother. 2018; 23(3): 143–153, doi: 10.1016/j.rpor.2018.01.004, indexed in Pubmed: 29760589.

34. Czerwińska P, Mazurek S, Wiznerowicz M. Application of induced pluripotency in cancer studies. Rep Pract Oncol Radiother. 2018; 23(3): 207–214, doi: 10.1016/j.rpor.2018.04.005, indexed in Pubmed: 29760595.

35. Fattore L, Mancini R, Ciliberto G. Cancer Stem Cells and the Slow Cycling Phenotype: How to Cut the Gordian Knot Driving Resistance to Therapy in Melanoma. Cancers (Basel). 2020; 12(11), doi: 10.3390/cancers12113368, indexed in Pubmed: 33202944.

36. Yang L, Shi P, Zhao G, et al. Targeting cancer stem cell pathways for cancer therapy. Signal Transduct Target Ther. 2020; 5(1): 8, doi: 10.1038/s41392-020-0110-5, indexed in Pubmed: 32296030.

37. Talukdar S, Emdad L, Das SK, et al. EGFR: An essential receptor tyrosine kinase-regulator of cancer stem cells. Adv Cancer Res. 2020; 147: 161–188, doi: 10.1016/bs.acr.2020.04.003, indexed in Pubmed: 32593400.

38. Wang AX, Qi XY. Targeting RAS/RAF/MEK/ERK signaling in metastatic melanoma. IUBMB Life. 2013; 65(9): 748–758, doi: 10.1002/iub.1193, indexed in Pubmed: 23893853.

39. Hsu MY, Meier F, Herlyn M. Melanoma development and progression: a conspiracy between tumor and host. Differentiation. 2002; 70(9-10): 522–536, doi: 10.1046/j.1432-0436.2002.700906.x, indexed in Pubmed: 12492494.

40. D'Angelo D, Arra C, Fusco A. RPSAP52 lncRNA Inhibits p21Waf1/CIP Expression by Interacting With the RNA Binding Protein HuR. Oncol Res. 2020; 28(2): 191–201, doi: 10.3727/096504019X15761465603129, indexed in Pubmed: 31831098.

41. Wang S, Guo X, Lv W, et al. LncRNA RPSAP52 Upregulates TGF-β1 to Increase Cancer Cell Stemness and Predict Postoperative Survival in Glioblastoma. Cancer Manag Res. 2020; 12: 2541–2547, doi: 10.2147/CMAR.S227496, indexed in Pubmed: 32368136.

42. Jiang H, Lin JJ, Tao J, et al. Suppression of human ribosomal protein L23A expression during cell growth inhibition by interferon-beta. Oncogene. 1997; 14(4): 473–480, doi: 10.1038/sj.onc.1200858, indexed in Pubmed: 9053844.

43. Guo Y, Wei Q, Tan L, et al. Inhibition of AURKB, regulated by pseudogene MTND4P12, confers synthetic lethality to PARP inhibition in skin cutaneous melanoma. Am J Cancer Res. 2020; 10(10): 3458–3474, indexed in Pubmed: 33163283.

44. Hussen BM, Azimi T, Abak A, et al. Role of lncRNA BANCR in Human Cancers: An Updated Review. Front Cell Dev Biol. 2021; 9: 689992, doi: 10.3389/fcell.2021.689992, indexed in Pubmed: 34409032.

45. Sousa JF, Torrieri R, Silva RR, et al. Novel primate-specific genes, RMEL 1, 2 and 3, with highly restricted expression in melanoma, assessed by new data mining tool. PLoS One. 2010; 5(10): e13510, doi: 10.1371/journal.pone.0013510, indexed in Pubmed: 20975957.

46. Yang S, Liu T, Nan H, et al. Comprehensive analysis of prognostic immune-related genes in the tumor microenvironment of cutaneous melanoma. J Cell Physiol. 2020; 235(2): 1025–1035, doi: 10.1002/jcp.29018, indexed in Pubmed: 31240705.

47. Huang L, Guo Y, Liu S, et al. Targeting regulatory T cells for immunotherapy in melanoma. Mol Biomed. 2021; 2(1): 11, doi: 10.1186/s43556-021-00038-z, indexed in Pubmed: 34806028.

48. Kolenda T, Guglas K, Kopczyńska M, et al. Quantification of long non-coding RNAs using qRT-PCR: comparison of different cDNA synthesis methods and RNA stability. Arch Med Sci. 2021; 17(4): 1006–1015, doi: 10.5114/aoms.2019.82639, indexed in Pubmed: 34336028.