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REPORTS OF PRACTICAL ONCOLOGY AND RADIOTHERAPY

ISSN: 1507-1367

e-ISSN: 2083-4640

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DOI: 10.5603/RPOR.a2023.0030

Article type: Research paper

Published online: 2023-05-31

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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 methods: 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 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

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 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 scaffolding 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 ATCC or 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, USA) protocol whereas human melanoma cell lines MEWO, SKMEL28, 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]. 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 NCBI Basic Local Alignment Search Tool (BLAST). Real-time 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 ($R \geq 0.3$) and negative ($R \leq -0.3$) Pearson correlations for *RPL23AP53* transcript with genes were downloaded from cBioPortal [22] and analyzed using REACTOME pathway analysis ($p < 0.05$ and $FDR < 0.05$; FDR — false discovery rate) 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 $FDR \leq 0.25$ 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, USA). 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. $3.680e-007 \pm 3.055e-007$; $p = 0.0028$) (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).

Table 1. The expression levels of *RPL23AP53* depending on clinicopathological parameters in the TCGA melanoma patients; $p < 0.05$ considered as significant

| Parameter | Group | Cases | <i>RPL23AP53</i> | |
|-------------|-----------|-------|-------------------------|---------|
| | | | Mean \pm SEM | p-value |
| Gender | Male | 290 | 0.3804 \pm 0.05149 | 0.2386 |
| | Female | 178 | 0.3172 \pm 0.06585 | |
| Age | ≤ 58 | 236 | 0.344 \pm 0.05770 | 0.6921 |
| | > 58 | 227 | 0.3663 \pm 0.05737 | |
| Ulceration | Yes | 167 | 0.3449 \pm 0.07375 | 0.3751 |
| | No | 147 | 0.3088 \pm 0.06253 | |
| Clark level | I | 6 | -0.8354 \pm 0.8310 | 0.2577 |
| | II | 18 | 0.4998 \pm 0.1706 | |
| | III | 78 | 0.4666 \pm 0.06916 | |
| | IV | 167 | 0.3722 \pm 0.06869 | |
| | V | 53 | 0.2564 \pm 0.1158 | |
| Breslow | < 1.0 | 51 | 0.3575 \pm | 0.8240 |

| | | | | |
|----------------------------------|-----------------------------|-----|---------------------|--------|
| depth | | | 0.08995 | |
| | 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 | |
| Neoplas m disease stage | 0 | 6 | -0.8765 ± 0.8283 | 0.1866 |
| | I-II | 218 | 0.3859 ± 0.05387 | |
| | III-IV | 194 | 0.3584 ± | |

| | | | | |
|--|--|--|---------|--|
| | | | 0.06433 | |
|--|--|--|---------|--|

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).

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

Next, positive (> 0.3) and negative (< -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 GSEA analysis of patients with low and high expression of *RPL23AP53* was performed. In the patients group with lower *RPL23AP53* expression, changes 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 Fig. 4A. Based on the GSEA results signaling pathways connected with melanoma, such as *MTOR*, *EGFR* and *MEK*, were chosen 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 ESTIMATE 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 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 (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 features 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 nondividing 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 level 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 transcripts 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.

Acknowledgments

We thank the Greater Poland Cancer Center for supporting our work and providing a fully equipped laboratory to perform the necessary analyses.

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.

Funding

This work was supported by the Greater Poland Cancer Center grant no.: 1/2023(263), 13/01/2023/PGN/WCO/001; Alicja Braska received “The best in nature 2.0. The integrated program of the Poznań University of Life Sciences” program scholarship at the time of writing this manuscript from the University of Life Sciences in Poznań; Joanna Kozłowska-Masłoń received a PhD program scholarship at the time of writing this manuscript from the Adam Mickiewicz University in Poznań; Kacper Guglas received a scholarship at the time of writing this manuscript from the European Union POWER PhD program and from Medical University of Warsaw.

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.

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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, * $p \leq 0.05$, ** $p \leq 0.01$

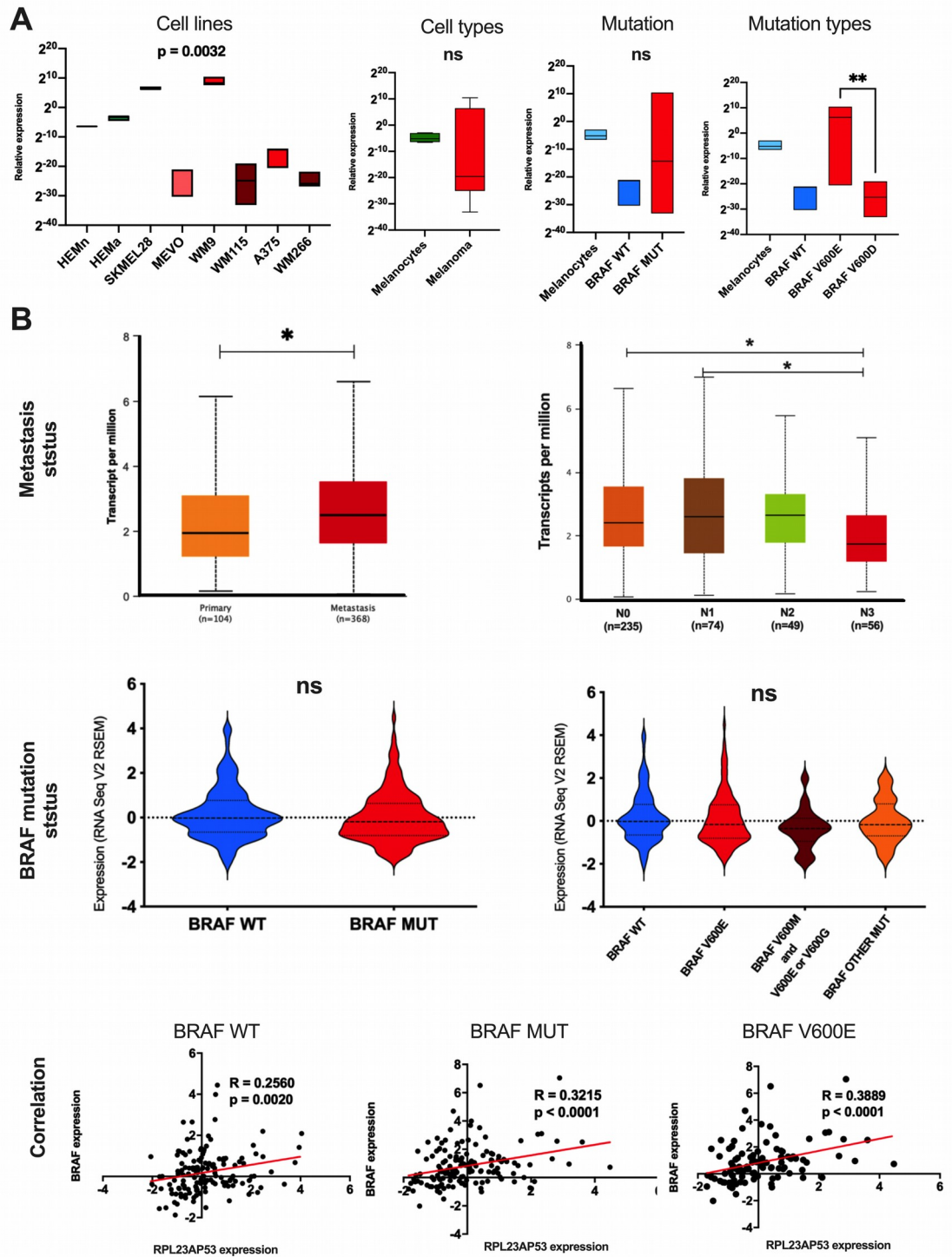


Figure 2. Progression-free interval (PFI) and overall survival (OS) of melanoma patients depending on the *RPL23AP53* expression levels in: group of all patients (A), patients with *BRAF* mutation (B) and patients with wild type of *BRAF* gene (C); results presented for 3 years of observation; low (red 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

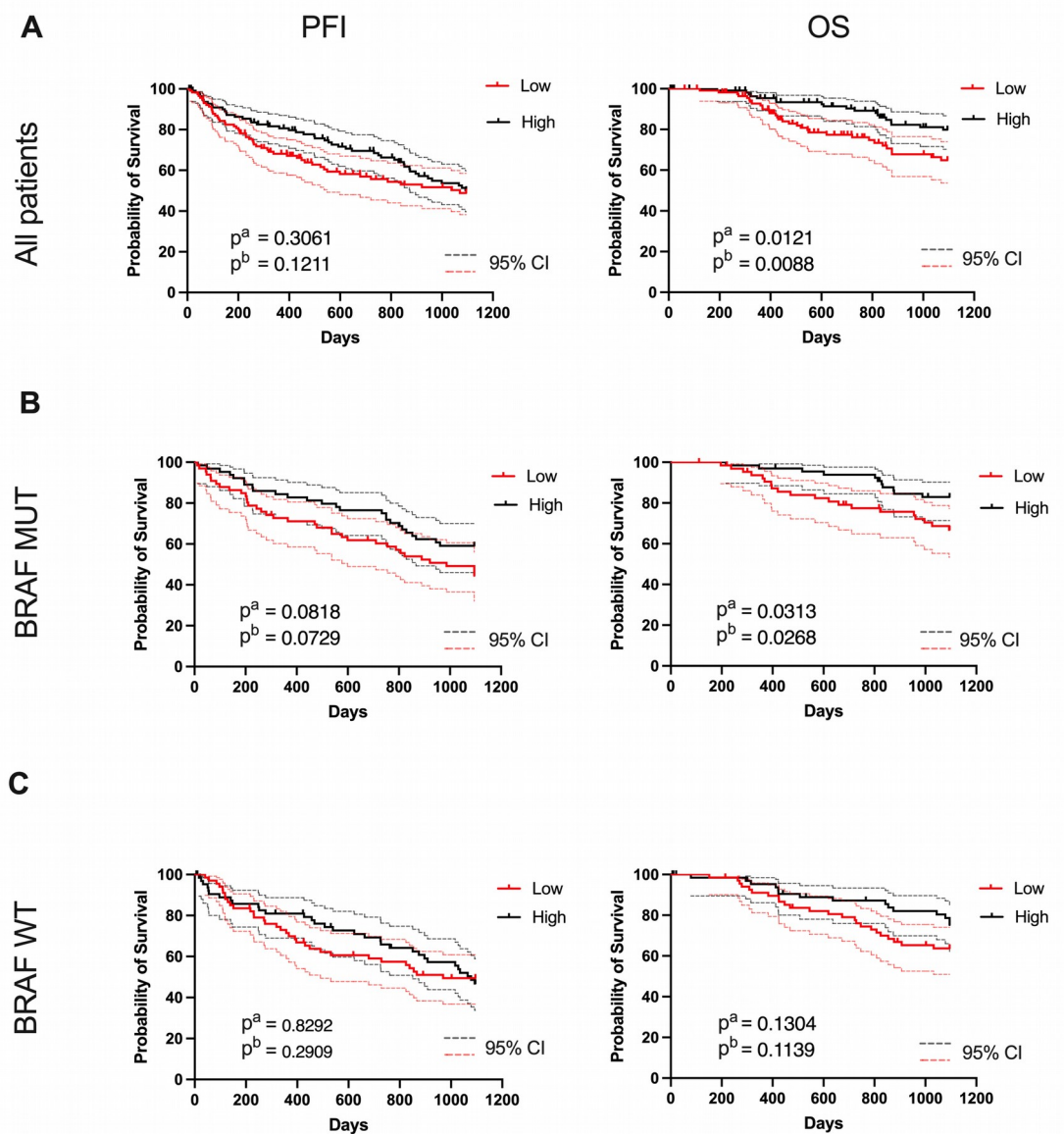


Figure 3. Genes correlated with *RPL23AP53*: positive (> 0.3) (A) and negative (< -0.3) (B) Pearson correlations of *RPL23AP53* gene with genes and analyzed using REACTOME pathway analysis for distinguishing cellular pathways and processes. Only results with $p < 0.05$ and false discovery rate (FDR) < 0.05 were shown

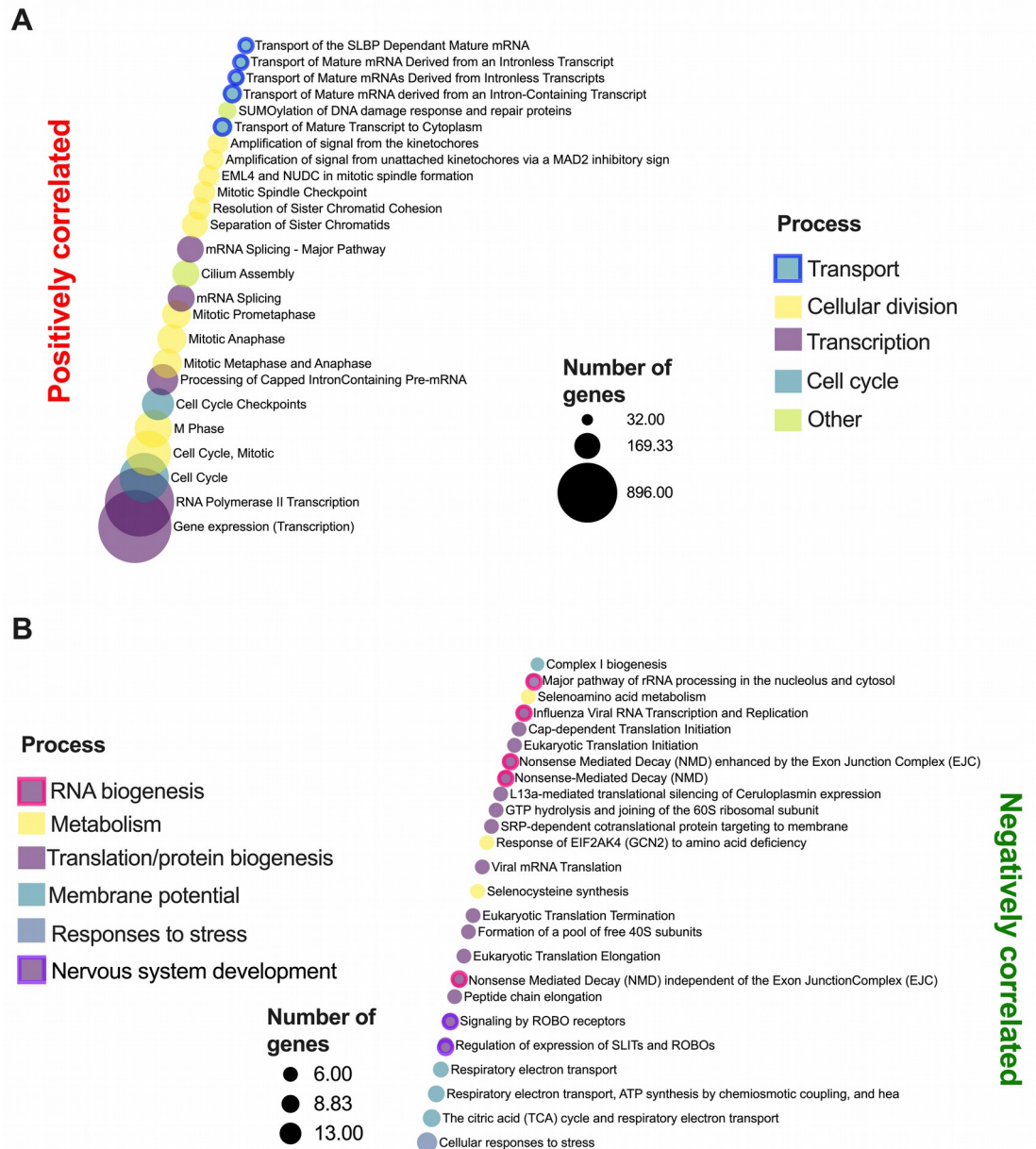


Figure 4. The phenotype associated with low or high expression levels of *RPL23AP53* in the Cancer Genome Atlas (TCGA) melanoma patients based on Gene Set Enrichment Analysis (GSEA) results. **A.** Changed molecular pathways in patients with lower expression of *RPL23AP53*; **B.** selected for melanoma

MTOR, *MEK*, and *EGFR*; **C.** Involvement in cellular processes based on the GENmania 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 $p \leq 0.05$ and $FDR \leq 0.25$ are shown; $p < 0.05$ considered as statistically significant

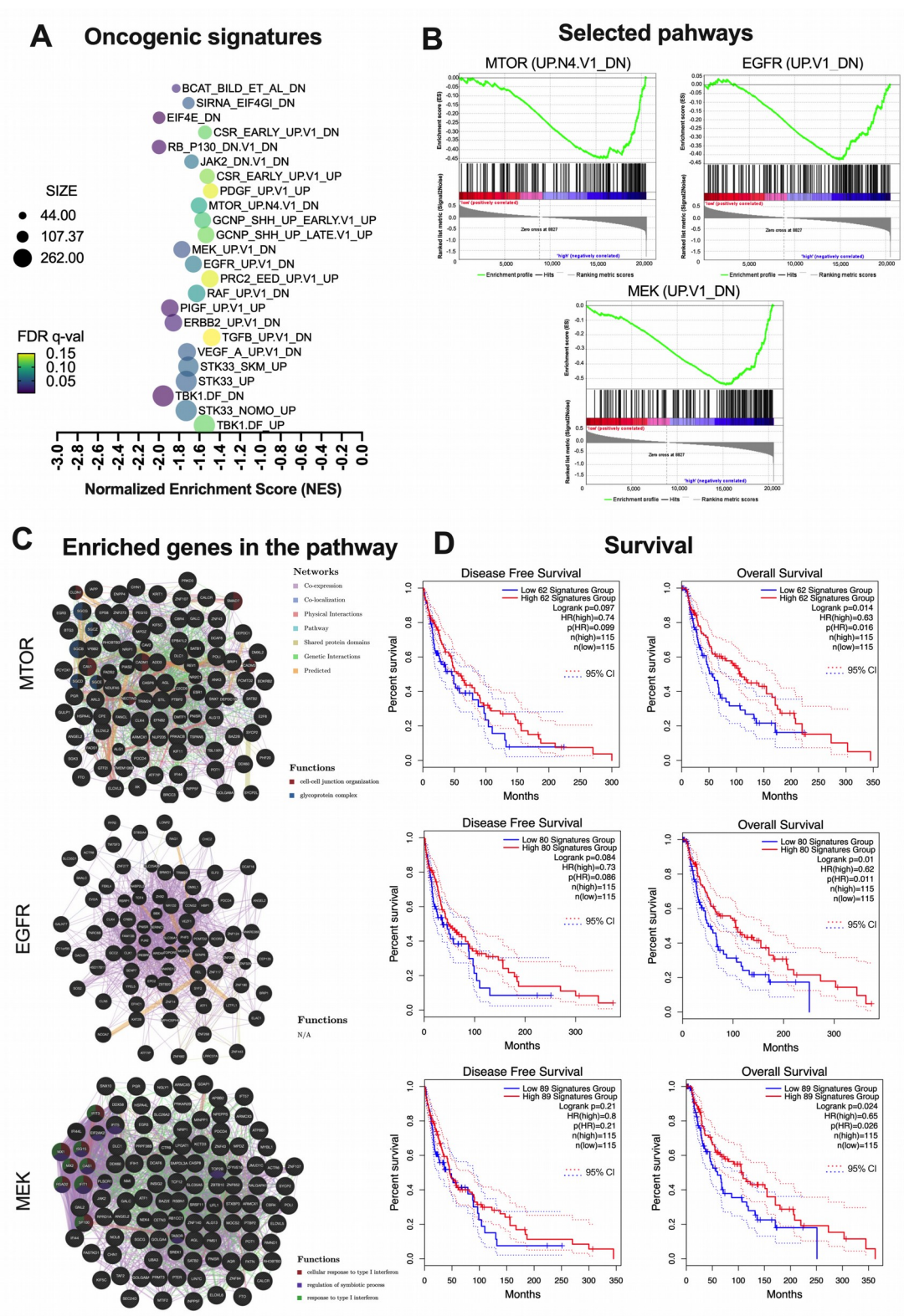


Figure 5. Association of the immunological profile of the Cancer Genome Atlas (TCGA) melanoma patients with the expression levels of *RPL23AP53*: **A.** ESTIMATE, immune cells, and stromal cells scores; **B.** differences of lymphocytes, neutrophils, eosinophils, mast cells, dendritic cells, and

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; * $p < 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$

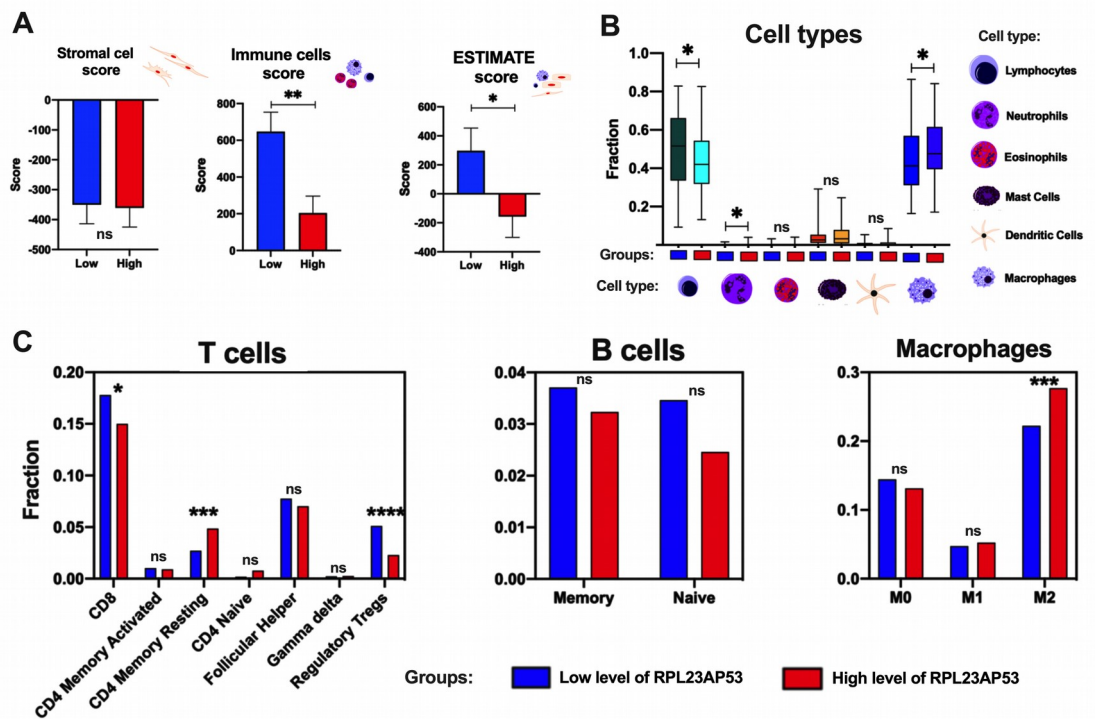


Figure 6. Validation of *RPL23AP53* expression levels using data from Gene Expression Omnibus (GEO). **A.** Differences in expression in normal, benign nevi, and malignant melanoma samples based on GSE3189, One way Anova with Dunn’s multiple comparisons test; **B.** Expression in epidermal keratinocytes 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, $**p \leq 0.01$, $***p \leq 0.001$

