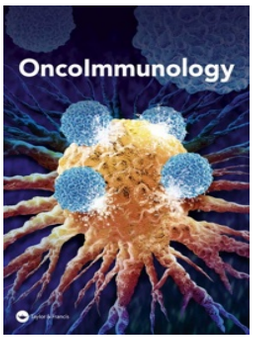

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Identification of RNA-binding protein hnRNP C targeting the 3'UTR of the TAP-associated glycoprotein tapasin in melanoma

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ABSTRACT

Deregulation or loss of the human leukocyte antigen class I (HLA-I) molecules on tumor cells leading to inhibition of $CD8^+$ T cell recognition is an important tumor immune escape strategy, which could be caused by a posttranscriptional control of molecules in the HLA-I pathway mediated by RNA-binding proteins (RBPs). So far, there exists only limited information about the interaction of RBPs with HLA-I-associated molecules, but own work demonstrated a binding of the heterogeneous ribonucleoprotein C (hnRNP C) to the 3' untranslated region (UTR) of the TAP-associated glycoprotein tapasin (tpn). In this study, *in silico* analysis of pan-cancer TCGA datasets revealed that hnRNP C is higher expressed in tumor specimens compared to corresponding normal tissues, which is negatively correlated to tpn expression, T cell infiltration and the overall survival of tumor patients. Functional analysis demonstrated an upregulation of tpn expression upon siRNA-mediated downregulation of hnRNP C, which is accompanied by an increased HLA-I surface expression. Thus, hnRNP C has been identified to target tpn and its inhibition could improve the HLA-I surface expression on melanoma cells suggesting its use as a possible biomarker for T-cell-based tumor immunotherapies.

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Antigen processing and presentation; HNRNPC; melanoma; RNA-binding protein; tapasin; tumor immune evasion

Introduction

During the last two decades, tumor immunotherapy has rapidly developed, but still has a limited efficacy in solid tumors including melanoma.[1] Although immune checkpoint inhibitors (ICPi) represent an important pillar of the current immunotherapeutic options and combination therapies, which have dramatically changed the outcome of melanoma patients, long-term success of this treatment is only reported for <20% of patients.[2–4] Therefore, novel immunotherapeutic targets and better insights into the mechanisms associated with melanoma development and immune evasion are urgently required.

A few tumor immunotherapies, including cancer vaccines, ICP blockade and chimeric antigen receptor (CAR) T cells, are currently FDA approved or implemented in clinical studies, and the different tumor immunotherapeutic strategies have some disadvantages and not every patient will respond to this treatment option.[5] The characterization of the underlying mechanisms of tumor immune escape is the cornerstone for the selection of the appropriate immunotherapeutic approach. In addition to the expression of inhibitory ICP, the loss or downregulation of molecules of the human leukocyte antigen class I (HLA-I) antigen processing and presentation pathway is a major immune evasion process, since their deficiencies block the presentation of tumor-associated antigens to $CD8^+$ cytotoxic T lymphocytes (CTL).[6] A number of studies demonstrated an impaired expression of different components of the HLA-I antigen processing and presentation machinery (APM) in multiple tumor types at distinct tumor stages.[7,8] However, the underlying mechanisms of the absent and/or reduced function of HLA-I APM components are not uniform and significantly vary including the changes at the genetic, transcriptional, posttranscriptional, posttranslational and epigenetic levels.[9,10] In this context, the posttranscriptional regulation of the expression of HLA-I APM components mediated by microRNAs (miRNAs) and RNA-binding proteins (RBPs) has recently rekindled interest.[11–13]

RBPs are proteins binding to the RNA sequences including in cells thereby participating in the formation of ribonucleoprotein (RNP) complexes.[14,15] Due to their wide range of regulatory functions, RBPs play an important role not only in physiological process but also in the development of many diseases.[16,17] There exists evidence that RBPs also have crucial functions in tumors based on their deregulation in different tumor specimens compared to normal tissues.[18] RBPs have been shown to modulate tumor metastasis and epithelial mesenchymal transition (EMT) progression. For example, PCBP1 regulates breast cancer invasiveness,[19] heterogeneous ribonucleoprotein A1 ((hnRNP) A1 affects hepatocellular carcinoma migration[20] and MEX3A promotes angiogenesis in colorectal cancer.[21] Nevertheless, there exists only limited information about the interaction of RBPs with HLA-I-associated molecules. So far, only MEX3B and Syncrip have been identified to target HLA-A.[13,22] In addition, hnRNP R has been shown as a positive regulator of both classical HLA-I and the non-classical HLA-G antigens,

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thereby modulating the NK cell cytotoxicity.[23] Other HLA-I APM components, such as tapasin (tpn), have not yet been investigated regarding their association with RBPs. Tpn is an important component of the HLA-I APM that coordinates with the transporter associated with antigen processing (TAP) proteins the peptide loading onto HLA-I molecules, which is a key determinant for successful immune responses.[24] New studies reveal a selector function of the tpn-ERp57 complex by regulating the peptide kinetic stability, peptide abundance and peptide proofreading,[25,26] suggesting an important role of tpn for peptide loading and editing processes on HLA-I molecules. Own previous mass spectrometric data revealed that the heterogeneous nuclear ribonucleoprotein C1/C2 (hnRNP C), an RBP with heterogeneous nuclear RNA (hnRNA) and associated with pre-mRNAs in the nucleus thereby influencing the pre-mRNA processing,[27] targets the 3' untranslated region (UTR) of tpn. Based on its wide range of functions, in particular the modulation of growth properties, recent studies have linked the expression of this RBP to the development of many tumors,[28–30] including lung cancer,[31] gastric cancer[32] and hepatocellular carcinoma.[33] In melanoma, hnRNP C increases the tumor cell migration and invasion via the interaction with several proteins.[34–36] However, the role of hnRNP C on the expression and function of immune modulatory molecules remains to be explored.

In this study, we identified that hnRNP C can bind to tpn, leading to an inhibition of its expression, while knockdown of hnRNP C can upregulate tpn expression, thereby enhancing HLA-I surface expression and $CD8^+$ T cell recognition. These experimental data were confirmed by bioinformatics analyses of the Cancer Genome Atlas (TCGA) and might have important implications for the implementation of T cell-based immunotherapies.

Methods and materials

Cell culture and transfection

The human metastatic melanoma cell lines Buf1379 and Buf1402 were kindly provided by Soldano Ferrone (Department of Surgery, Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA). Both melanoma cell lines were cultured in Roswell Park Memorial Institute 1640 medium (RPMI1640, Invitrogen, Carlsbad, CA, USA) at 37°C in 5% CO_2 humidified air supplemented with 10% fetal calf serum (FCS) (PAN, Aidenbach, Germany), 3% L-glutamine (Lonza, Basel, Switzerland) and 3% pen/strep (Sigma-Aldrich, Missouri, USA).

For transfection, 2.5×10^5 melanoma cells/well were seeded into six-well plates. After 16–20 h, cells were transfected with siRNA (si-hnRNP C, 2 ng/ml, EHU133931, Sigma-Aldrich, St. Louis, MO, USA) or the negative control (NC, EHUEGFP, Sigma-Aldrich) using lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. Furthermore, untreated parental melanoma cells served as additional control. The cells were collected 48 h after transfection or approx. 64 h after seeding for subsequent RNA, protein and flow cytometric analyses.

RNA isolation and real-time quantitative reverse-transcription PCR (RT-qPCR)

Total RNA was isolated using the TRIzol reagent (Invitrogen) following the manufacturer's instruction and then subjected to cDNA synthesis (Thermo Scientific, Rockford, IL, USA). For RT-qPCR, the SYBR qPCR master mix (Vazyme, Nanjing, PRC) was used with the primers listed in Table S1, and the data were normalized to the housekeeping genes including β-actin, delta-amino levulinate synthase (ALSA1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and mRNA levels were normalized to parental cells.

Protein extraction and Western blot analysis

Total cellular protein was isolated with a lysis buffer (Thermo Scientific) containing protease and phosphatase inhibitors (Thermo Scientific) followed by the quantification of proteins with the Pierce BCA Protein Assay kit (Thermo Scientific). About 25 µg protein/sample was separated in Bolt™ 4 to 12% mini protein gels (Invitrogen) and transferred onto membranes through Blot2 Transfer Stacks (Invitrogen). Subsequently, the membranes were blocked with milk for 1 h at room temperature and incubated with the following primary antibodies (Abs) overnight at 4°C: anti-HNRNPC (1:1000, PA5-24221 Thermo Scientific), anti-tpn (1:1000, ab13518, Abcam, Cambridge, UK), anti-β-actin (1:1000, ab6276, Abcam) and anti-HLA-I heavy chain (HC) (1:750; HC-10), which was kindly provided by Prof. Dr Soldano Ferrone (Harvard University, Boston, USA). This was followed by staining of the membranes with a horseradish peroxidase-conjugated goat anti-mouse/rabbit Ab (Cell Signaling Technology, Inc., Danvers, USA) as a secondary Ab for 1 h at room temperature. A LAS-3000 imaging system (Fujifilm, Tokyo, Japan) was employed to image the chemiluminescent blots. The signal intensity was determined by densitometric analysis using ImageJ (NIH, Bethesda, Maryland, USA) and normalized to the β-actin staining.

Flow cytometry

For determination of the HLA-I surface expression by flow cytometry, 1×10^5–1×10^6 cells were incubated with an anti-HLA-ABC (Beckman, Brea, California, USA) or anti-HLA-BC (Invitrogen) mAb, respectively, for 15 min at room temperature, then washed with PBS and measured on a Navios 3L10C (Beckman Coulter GmbH, Krefeld, Germany) or a LSRFortessa (BD Biosciences, Heidelberg, Germany) flow cytometer. The data were evaluated with the Kaluza (Beckman Coulter) or FACS Diva (BD Biosciences) analysis software and calculated as mean specific fluorescence intensity (MFI).

Gene set enrichment analysis (GSEA)

To explore the hnRNP C-mediated regulation of gene expression, the RNA-seq dataset from 67 metastatic melanoma cases from the TCGA-SKCM dataset was analyzed using the GSEA software (UC SanDiego, Broad Institute).[37] The molecular

signature database (MSigDB) was used to determine which gene sets correlated with the hnRNP C expression.

Bioinformatics and statistical analysis

The probability of melanoma patients' overall survival (OS) in dependence of hnRNP C expression was determined by the Kaplan–Meier estimation employing 459 cases from the "SKCM Cancer" and "Bhardwaj" and 44 cases of a metastatic melanoma patient's dataset from R2 database (https://hgserver1.amc.nl/cgi-bin/r2/main.cgi). In addition, using 67 samples of distantly metastasized melanoma from the TCGA-SKCM dataset, the link between immune infiltration and the expression levels of HnRNP C^{high} and hnRNP C^{low} was determined by CIBERSORT. For the correlation of the hnRNP C expression, in pan-cancer with tpn as well as MHC-I expression, GAPIA, ENCORI and the GSEA web tools were employed. The data were presented as mean ± standard deviation (SD) and analyzed by GraphPad Prism (GraphPad Software, LLC, San Diego, USA), ImageJ software and Microsoft Office (Microsoft Corporation, Redmond, WA, USA). Paired and/or unpaired t-tests were performed. $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***) were statistically significant.

Results

Clinical relevance of hnRNP C expression regarding the survival of pan-cancer patients

Since hnRNP C overexpression was reported in several cancer types including melanoma, the prognostic relevance of hnRNP C expression in melanoma patients was investigated using the datasets "R2: Tumor Melanoma – Bhardwaj – 44 – MAS5.0 – u133p2" and "R2: Tumor Melanoma – TCGA – 470 – rsem – tcgars" from the R2 genomic analysis web tool. In both datasets, lower hnRNP C mRNA transcript levels were correlated with a higher OS probability (Figure 1a and b), suggesting an association of lower hnRNP C expression with a better OS of melanoma patients. These data were in line with a majority of cancer types also demonstrating a correlation of increased patients' survival with reduced hnRNP C expression (Figure 1c and Figure S1). In contrast, the hnRNP

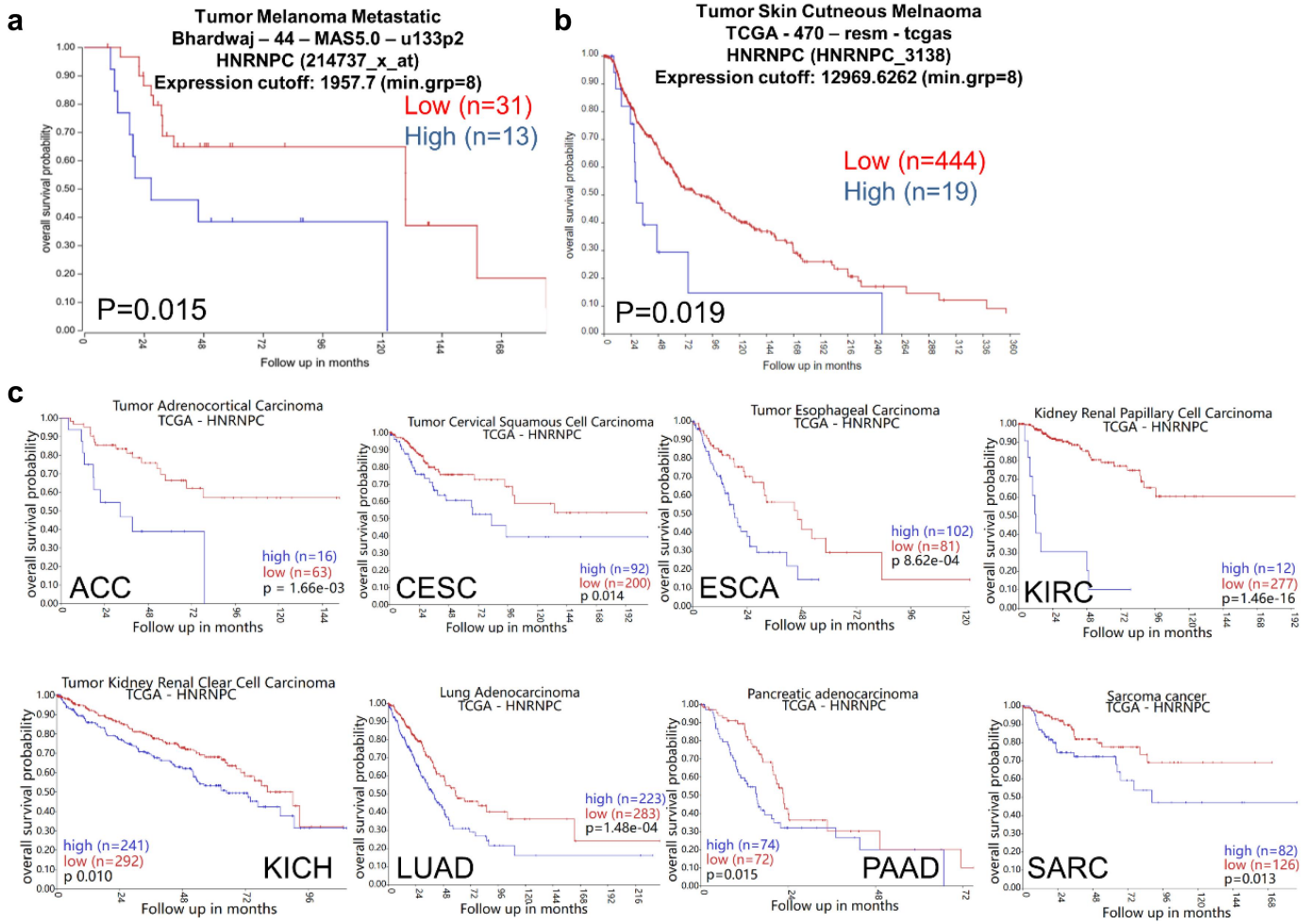

Figure 1. Correlation of increased patients' overall survival with reduced hnRNP C expression in different cancer types including melanoma. The OS probability of melanoma patients with Kaplan–Meier estimation over time depending on hnRNP C expression including (A) 44 cases metastasis melanoma patients and (B) 459 cases from "SKCM Cancer" dataset, both analyzed from R2 web database (https://hgserver1.amc.nl/cgi-bin/r2/main.cgi) is shown. (C) Correlation of the expression of hnRNP C with the OS in several cancer via TCGA dataset using R2 web tool is demonstrated and presented as overall survival probability (ACC: adrenocortical carcinoma, CESC: cervical squamous cell carcinoma and endocervical adenocarcinoma, ESCA: esophageal carcinoma, KIRC: kidney renal clear cell carcinoma, KIRP: kidney renal papillary cell carcinoma, LUAD: lung adenocarcinoma, PAAD: pancreatic adenocarcinoma, SARC: sarcoma).

C expression levels were not linked to the patients' survival in gastric and ovarian cancers (Figure S2), while the expression of hnRNPC positively correlated with the patients' survival in lung and breast carcinoma (Figure S3). Comparison of the expression of hnRNP C in different tumor types to that of corresponding normal tissues using the TCGA dataset analyzed with the GAPIA web tool demonstrated higher hnRNP C transcript levels in most cancer types than in the corresponding non-neoplastic tissues suggesting that hnRNP C expression has a possible tumor-promoting effect (Figure 2). Similar results were also obtained *in vitro* with higher hnRNP C expression in Buf1379 and Buf1402 melanoma cell lines than in melanocytes (Figure S4). In contrast, for acute myeloid leukemia (LAML), subsequent correlation analyses suggest that hnRNP C has no tumor-promoting effect in this disease.

Correlation between hnRNP C, tpn and HLA-I expression in pan-cancer than adjacent normal tissue

Since hnRNP C has been shown to bind to the 3'UTR of tpn, the link between hnRNP C mRNA levels and tpn expression was analyzed in the TCGA dataset using the ENCORI web tool. A negative correlation of both genes was found in 13 different cancer types including melanoma with a high correlation coefficient (*R* value) (Figure 3), suggesting that the binding of hnRNP C to the 3'UTR of tpn inhibits its expression. In contrast, there exists no correlation between hnRNP C and tpn in some tumors, such as breast cancer (Figure S5A), while, e.g., in pancreatic cancer, a positive correlation was detected (Figure S5B). To further verify the relationship between hnRNP C and MHC class I APM components, GSEA was performed using 67 metastatic melanoma cases from the TCGA dataset. This bioinformatics analysis in combination with the Molecular Signatures Database demonstrated a negative correlation of hnRNP C mRNA levels and the MHC class I complex "GOBP-PEPTIDE-ANTIGEN-ASSEMBLY-WITH-MHC-PROTEIN-COMPLEX" (Figure S6, Table S2), thereby strengthening the evidence of an inverse correlation of hnRNP C and tpn.

Upregulation of tpn expression, but not of other HLA-I APM components by knockdown of hnRNP C

To gain functional insights into the hnRNP C-mediated tpn regulation, hnRNP C was silenced in the two metastatic melanoma cell lines Buf1379 and Buf1402. Successful knockdown of hnRNP C (Figure 4a) significantly increased the tpn mRNA expression levels (Figure 4b). To investigate whether hnRNP C also influenced other HLA-I APM molecules, the mRNA expression levels of TAP1, TAP2, β_2-microglobulin (β_2m) and HLA-I HC were determined by q-PCR in hnRNP C^{low} (hnRNP C knockdown) vs. hnRNP C^{high} (NC, negative controls) cells. As shown in Figure 4c to 4i, the mRNA levels of TAP1, TAP2, and β_2m of the hnRNP C^{high} and hnRNP C^{low} transfectants were comparable, while the HLA-B and HLA-C mRNA expression levels were slightly altered upon siRNA-mediated silencing of hnRNP C and HLA-A expression was only affected in hnRNP C^{low} Buf1402 cells. Since interferon-γ (IFN-γ) is master regulator and most potent inducer of major HLA-I APM components,[38] the effect of hnRNP C silencing on IFN signaling components was also investigated. As shown in Figure S7,

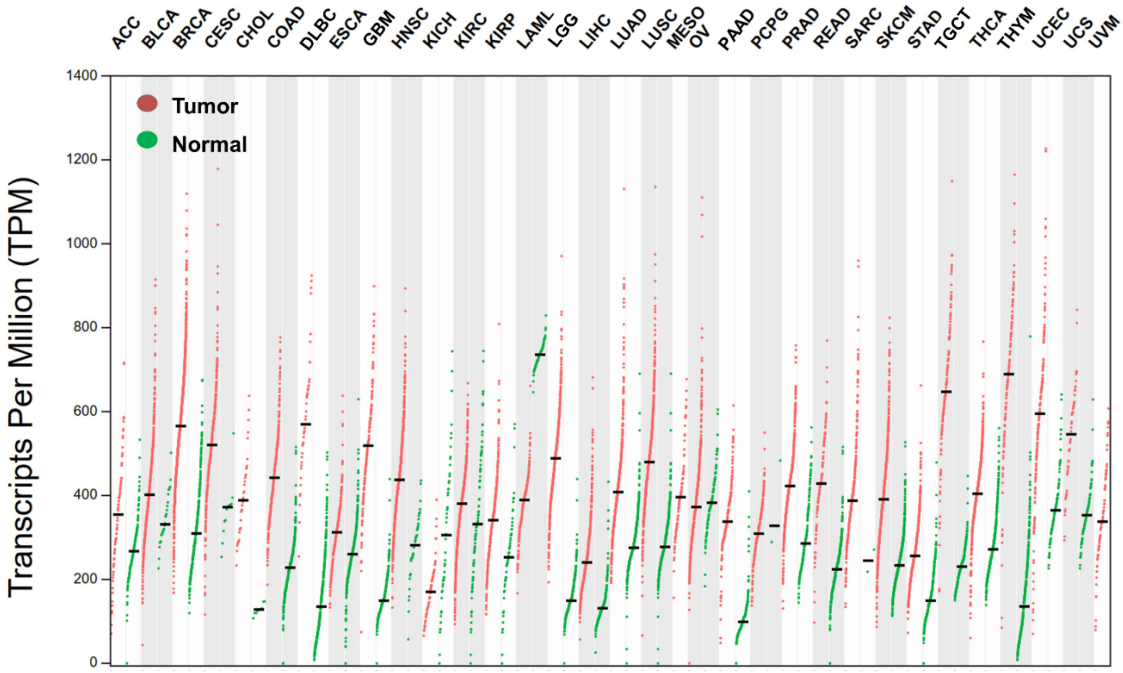

Figure 2. Higher hnRNP C expression levels in tumor samples compared to paired tumor adjacent samples in pan-cancer. The expression of hnRNP C mRNA levels in tumor tissues and paired normal tissues was compared in 31 TCGA cancer datasets including melanoma via the GAPIA web tool (Each dots represent expression of samples. Log Scale: using log2(TPM +1) transformed expression data for plotting). (BLCA: bladder urothelial carcinoma, BRCA: breast invasive carcinoma, CHOL: cholangiocarcinoma, COAD: colon adenocarcinoma, DLBC: lymphoid neoplasm diffuse large B cell lymphoma, GBM: glioblastoma multiforme, HNSC: head and neck squamous cell carcinoma, KICH: kidney chromophobe, LAML: acute myeloid leukemia, LGG: brain lower grade glioma, LIHC: liver hepatocellular carcinoma, LUSC: lung squamous cell carcinoma, OV: ovarian serous cystadenocarcinoma, PCPG: pheochromocytoma and paraganglioma, PRAD: prostate adenocarcinoma, READ: rectum adenocarcinoma, SKCM: skin cutaneous melanoma, STAD: stomach adenocarcinoma, TGCT: testicular germ cell tumors, THCA: thyroid carcinoma, THYM: thymoma, UCEC: uterine corpus endometrial carcinoma, UCS: uterine carcinosarcoma).

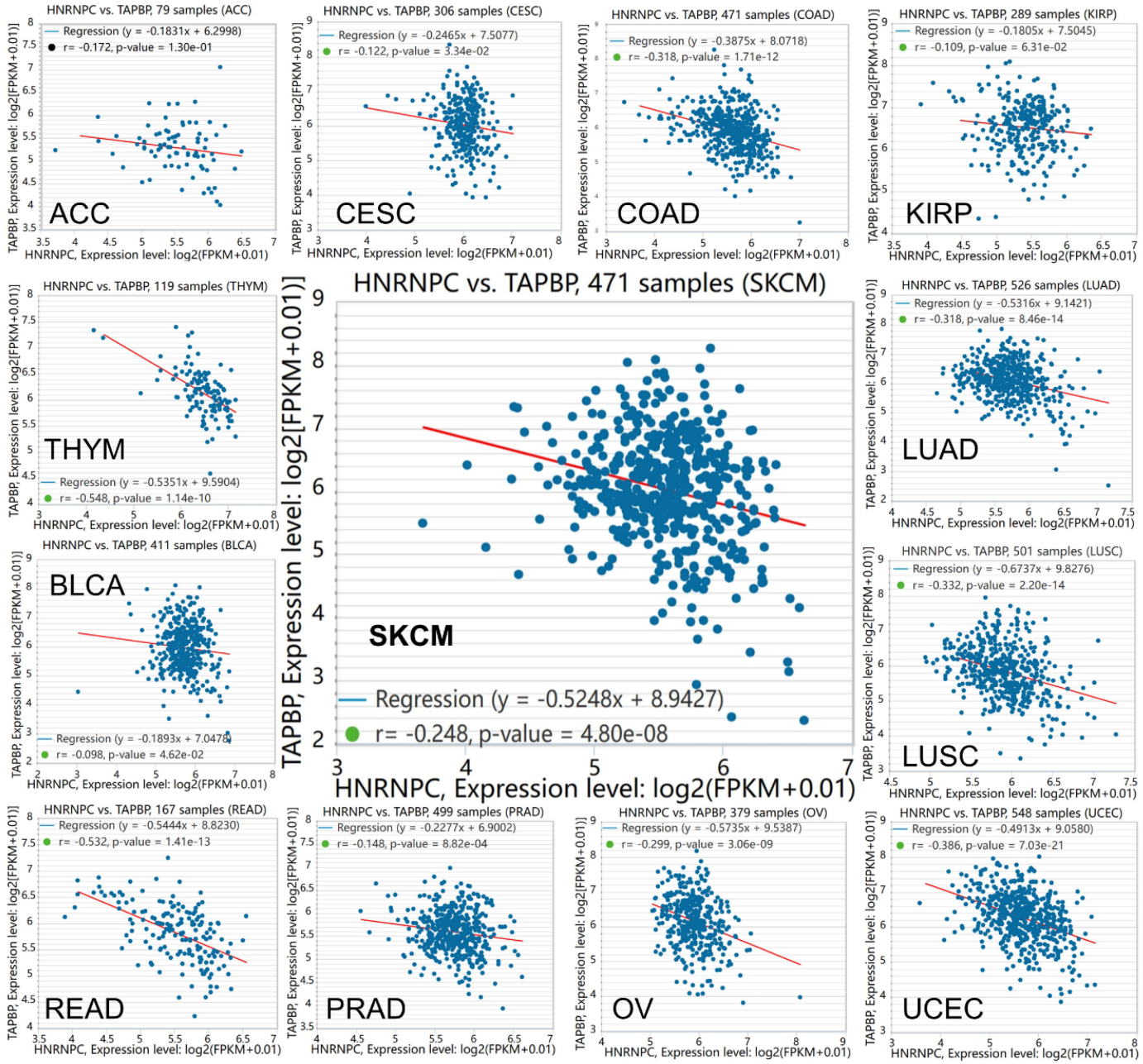

Figure 3. Inverse correlation of hnRNP C and tpn expression in different cancer types. The link of mRNA expression between hnRNP C and tpn was determined in different cancer types using the TCGA data set via ENCORI web tool (https://rnasysu.com/encori/index.php). Each dot represents mRNA expression levels of a tumor sample. The expression values of these two genes from RNA-seq data were scaled with log2 (FPKM +0.01) and using linear regression and spearman correlation (ACC: adrenocortical carcinoma, CESC: cervical squamous cell carcinoma and endocervical adenocarcinoma, COAD: colon adenocarcinoma, KIRP: kidney renal papillary cell carcinoma, LUAD: lung adenocarcinoma, LUCS: lung squamous cell carcinoma, UCEC: uterine corpus endometrial carcinoma, OV: ovarian serous cystadenocarcinoma, READ: rectum adenocarcinoma, PRAD: prostate adenocarcinoma, THYM: thymoma, BLCA: bladder urothelial carcinoma, SKCM: skin cutaneous melanoma).

an upregulation of JAK1, STAT2 and STAT1 was detected after hnRNP C knockdown in Buf1379 and Buf1402 cells. Next to mRNA levels, the tpn protein was 2-fold upregulated after hnRNP C knockdown (Figure 5a,b) in both melanoma cells compared to the NC group. Although the non-targeting siRNA also slightly influenced the hnRNP C protein expression in both cell lines, tpn protein expression was significantly altered in hnRNP C^{low} Buf1402 and Buf1379, which is accompanied by a slight upregulation of the HLA-I HC as determined by staining of the Western blot with the anti-HC10 mAb (Figure 5c,d).

Inverse correlation of hnRNP C and activation of HLA-I antigens and tumor immune cell infiltration

Silencing hnRNP C can upregulate the expression of tpn mRNA and protein level, which is also inversely correlated with HLA-I pathway-associated molecules, as determined by flow cytometry. After hnRNP C knockdown, HLA-ABC and HLA-BC are upregulated on the cell surface of Buf1379 cells (Figure 6a–d). While only HLA-BC was slightly upregulated in Buf1402 cells (Figure 6c), which correlated with the Western blot results of the HLA-I and for HLA-A, in contrast, hnRNP

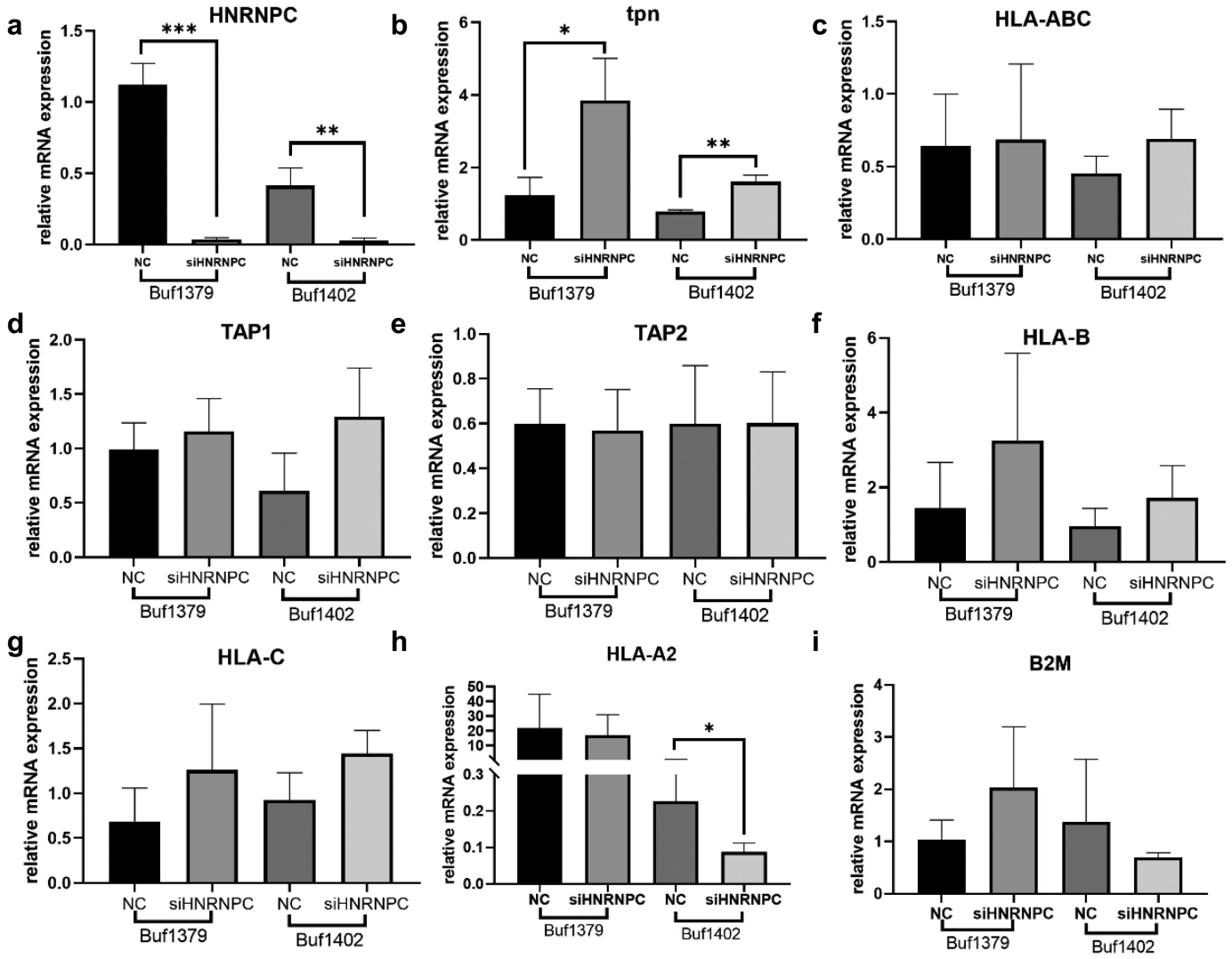

Figure 4. Upregulation of tpn mRNA levels by knockdown of hnRNP C. RT-qPCR was performed to determine the mRNA expression levels of hnRNP C and tpn as well as other MHC-I-associated molecules in two metastatic melanoma cell lines 48 h after transfection with si-hnRNP C or with a siRNA negative control (NC). The qPCR data were normalized to parental cells without any treatments and presented as mean of relative expression levels ± SD from three independent biological replicates.

C silencing did not affect HLA-A2 surface expression in both melanoma cell lines (Figure 6e,f). Several studies revealed that proteasomal subunits[39] contribute to the development of $CD8^+$ T cells and HLA-I-mediated activation of $CD8^+$ T cells accompanied by IFN-γ production,[40] suggesting that hnRNP C inhibits HLA-I pathway components in melanoma and consequently the $CD8^+$ T cell activation. In addition, in breast and lung cancer, the loss of HLA-I and associated molecules significantly correlated with a reduced number of tumor infiltrating T lymphocytes,[41] while high HLA-I expression levels were associated with an increased immune cell infiltration.[42] Since the HLA-I pathway inhibition by hnRNP C overexpression might affect the immune cell infiltration in melanoma cells, the correlation between hnRNP C expression and immune cell infiltration was investigated using 67 metastatic melanoma cases from the TCGA-SKCM dataset, which were divided into hnRNP C^{high} and hnRNP C^{low} expressors. It is well established that $CD8^+$ T cells recognize antigens presented by HLA-I,[43] and HLA-I expression affects $CD8^+$ T cell activation and is associated with the level of T cell infiltration. As shown in Figure 6g, the frequency of $CD8^+$ T cell infiltration was inversely correlated with the hnRNP C^{high} group, suggesting that hnRNP C overexpression might impair the $CD8^+$ T cell-mediated tumor recognition due to downregulation of the HLA-I pathway.

Discussion

Our study focused on the role of hnRNP C in the regulation of tpn mRNA, a component of the HLA class I pathway. *In silico* analysis as well as experimental approaches showed that hnRNP C binds to the tpn 3' UTR and down-regulation of hnRNP C significantly increases the expression of tpn thereby improving the expression of HLA-I on the surface of melanoma cells. HnRNP C, a member of the subfamily of ubiquitously expressed hnRNPs, was reported to be highly expressed in most tumor entities, but the underlying molecular mechanisms of hnRNP C function in tumors have not yet been well

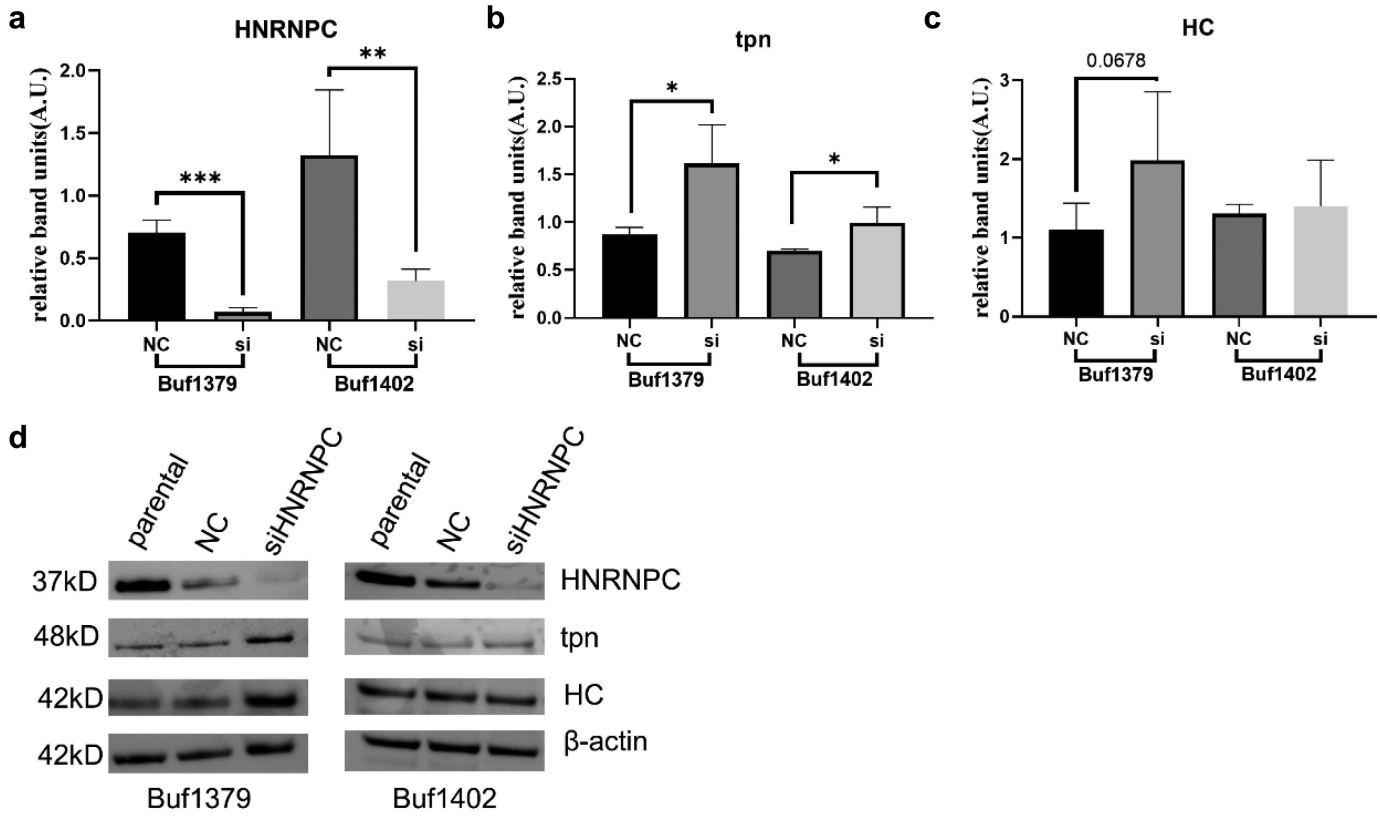

Figure 5. Upregulation of tpn protein levels by knockdown of hnRNP C. (A-D) The protein expression of hnRNP C, tpn and HLA-I HC was determined 48 h after transfection of Buf1379 and Buf1402 cells with si-hnRNP C or NC using Western blot analysis as described in Methods and Materials. The relative band intensities (A.U., arbitrary units) were compared to parental melanoma cells and normalized to staining with an anti-β-actin Ab (mean ± SD, $n = 3$ biological replicates). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

defined.[44] So far, high levels of hnRNP C expression in tumors strongly correlated with the tumor phenotype, like increased proliferation and migration,[31,45] but its effect on immunomodulatory molecules has not yet been addressed. Own studies and published data demonstrated that the loss or downregulation of HLA-I molecules in tumors is one major tumor immune escape mechanism.[46–48] This abnormality can be caused by gene mutations or loss of heterogeneity of, e.g., HLA-I HC and β_2m.[49] In addition, epigenetic, transcriptional and/or post-transcriptional regulation can control the HLA-I APM component expression.[10,50] These different causes gave rise to the same result: impaired HLA class I surface expression and T cell-mediated immune evasion. So far, some information is available about the impact of miRNAs on the HLA-I molecules,[11,47] while the effects of RBPs on these molecules are with few exceptions mainly unexplored.

During the last years, hnRNP C has been reported to regulate the growth properties of tumors,[44,51] which was associated with a worse survival of patients.[33,52] These data are in accordance with recent studies indicating that lower hnRNP C expression levels were associated with a better survival for patients with different cancer types. In melanoma patients, two TCGA datasets revealed that hnRNP C expression is associated with the patients' outcome. In addition, hnRNP C transcript levels were higher in most cancer types when compared to paired normal tissues, which confirmed earlier studies[53,54] suggesting that hnRNP C might be an accelerator in these tumors. Furthermore, our study demonstrated a strong negative correlation of hnRNP C with tpn in many cancer types, suggesting a functional association of both molecules. This is in line with the fact that hnRNP C can bind to the tpn 3'UTR.[55] Subsequently, additional *in silico* analyses were performed, which confirmed our results. HnRNP C is negatively linked to the expression of HLA-I, which might be due to the binding of hnRNP C to the tpn 3' UTR.

In eukaryotes, genes are also subjected to processing, translocation and stabilization prior to translation. The transcriptional process and post-transcriptional co-regulation are strictly controlled by RBPs, which bind to the 3'UTR of respective mRNAs,[56] thereby mainly leading to a reduced mRNA expression accompanied by a diminished protein expression.[57,58] Consistent with these findings, hnRNP C impaired tpn expression at the mRNA and protein levels, which was reverted by siRNA-mediated hnRNP C knockdown. In addition, other molecules of this pathway, such as HLA-B and HLA-C, and/or the HLA-I HC mRNA levels were also upregulated upon hnRNP C knockdown. One possible explanation might be that hnRNP C also binds to the HLA-I 3' UTR accompanied by a reduced HLA-I surface expression, which could be reverted after hnRNP C knockdown. Abnormal expression of hnRNP C in many malignant tumors negatively correlated with patients' prognosis,[28–33] indicating its value as prognostic marker and therapeutic target. Interestingly, Wu and coauthors have summarized the implementation of small-

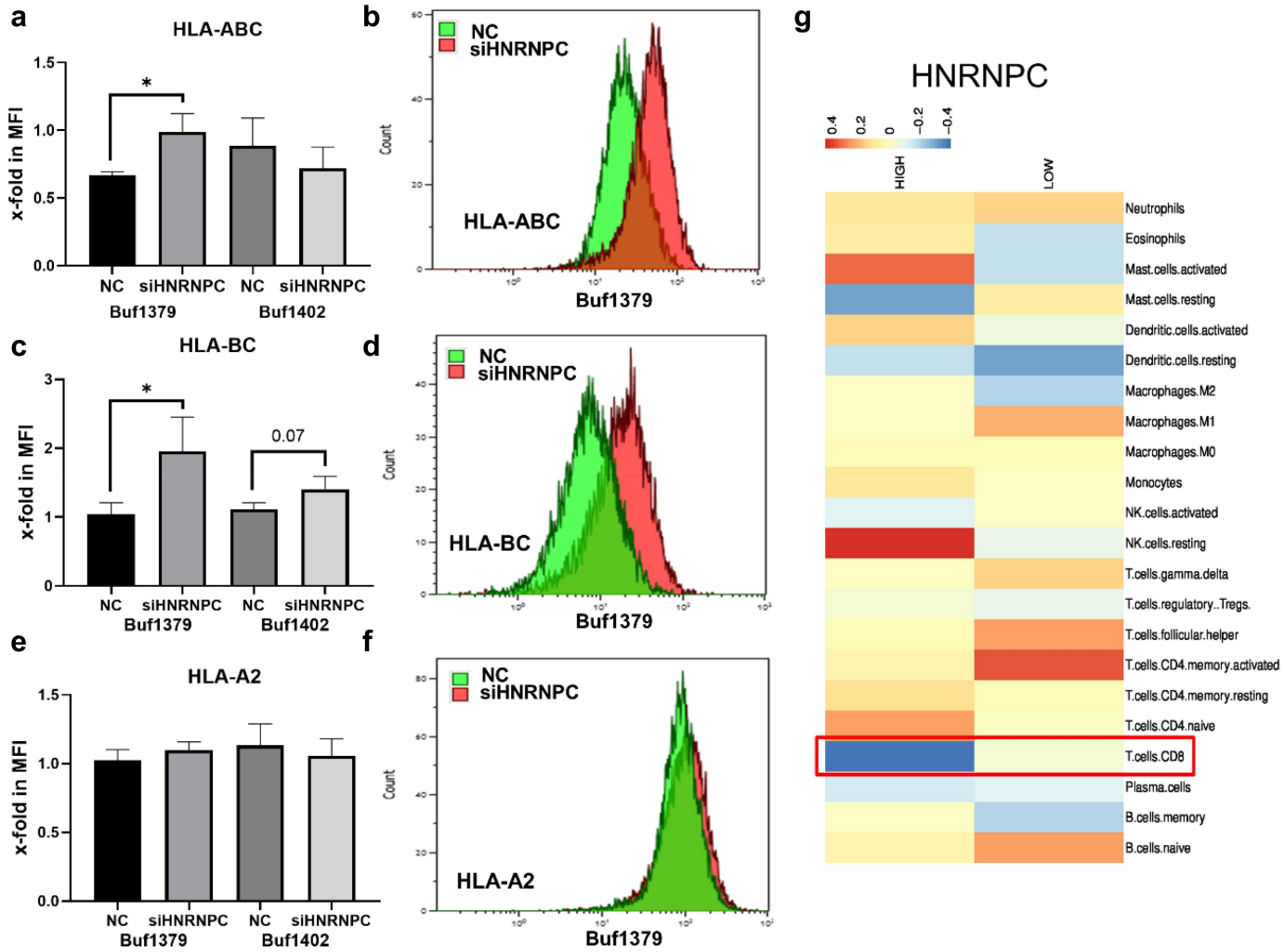

Figure 6. Effect of siRNA-mediated downregulation of hnRNP C on HLA-I cell surface expression and immune cell infiltration. (A-B) Flow cytometry was performed as described in Methods and Materials to determine the HLA-I surface expression of melanoma cells upon transfection with si-hnRNP C and NC. Melanoma cells were stained with antibodies directed against HLA-ABC and HLA-BC. The data were presented as x-fold change in the mean fluorescence intensity (MFI) to parental cells (mean ± SD, $n = 3$ biological replicates). (C) The 67 metastatic melanoma cases divided into hnRNP C^{high} and hnRNP C^{low} expressors were correlated with the immune cell infiltration via CIBERSORT in TCGA-SKCM dataset. Data are presented in a heat map. *$p < 0.05$.

molecule inhibitors targeting RBPs, which makes hnRNP C a possible therapeutic target, but altering its expression or inhibiting its function using small-molecule inhibitors,[59] thereby improving HLA-I expression. Thus, hnRNP C not only has the potential as an important molecular biomarker for disease outcome as well as a possible therapeutic target but also as a small inhibitory molecule, which might be used as a single treatment or in combination with immunotherapies to improve the patients' outcome.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Author contributions

Conceptualization: Y.W., B.S. Methodology Y.W., B.S. Investigation: Y.W., B.S. Visualization: Y.W. Funding acquisition: B.S. Writing: Y.W., B.S.

Data availability statement

All the data are available in the manuscript or the supplementary materials.

Abbreviations

Ab	Antibody
ALSA1	Delta-amino levulinate synthase
APM	Antigen processing machinery
CAR	Chimeric antigen receptor
CTL	Cytotoxic T lymphocyte
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GSEA	Gene set enrichment analysis
HC	Heavy chain
HLA	Human leukocyte antigen
hnRNA	Heterogeneous nuclear RNA
hnRNP C	Heterogeneous nuclear ribonucleoproteins C1/C2
hnRNP	Heterogeneous ribonucleoprotein family
ICP	Immune checkpoint
ICPi	Immune checkpoint inhibitor
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
miRNA	microRNA
OS	Overall survival
RBP	RNA-binding protein
RNP	Ribonucleoprotein
SD	Standard deviation
TAP	Transporter associated with antigen processing
TCGA	The Cancer Genome Atlas
tpn	Tapasin
UTR	Untranslated region

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