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ORIGINAL RESEARCH

Identification of miR-200a-5p targeting the peptide transporter TAP1 and its association with the clinical outcome of melanoma patients

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ABSTRACT

Tumor escape is often associated with abnormalities in the surface expression of the human leukocyte antigen class I (HLA-I) antigens thereby limiting CD8$^+$ cytotoxic T cell responses. This impaired HLA-I surface expression can be mediated by deficient expression of components of the antigen processing and presentation machinery (APM) due to epigenetic, transcriptional and/or post-transcriptional processes. Since a discordant mRNA and protein expression pattern of APM components including the peptide transporter associated with antigen processing 1 (TAP1) has been frequently described in tumors of distinct origin, a post-transcriptional control of APM components caused by microRNAs (miR) was suggested. Using an *in silico* approach, miR-200a-5p has been identified as a candidate miR binding to the 3' untranslated region (UTR) of TAP1. Luciferase reporter assays demonstrated a specific binding of miR-200a-5p to the TAP1 3'-UTR. Furthermore, the miR-200a-5p expression is inversely correlated with the TAP1 protein expression in HEK293T cells and in a panel of melanoma cell lines as well as in primary melanoma lesions. High levels of miR-200a-5p expression were associated with a shorter overall survival of melanoma patients. Overexpression of miR-200a-5p reduced TAP1 levels, which was accompanied by a decreased HLA-I surface expression and an enhanced NK cell sensitivity of melanoma cells. These data show for the first time a miR-mediated control of the peptide transporter subunit TAP1 in melanoma thereby leading to a reduced HLA-I surface expression accompanied by an altered immune recognition and reduced patients' survival.

Abbreviations: Ab: antibody; ACTB: β-actin; APM: antigen processing and presentation machinery; ATCC: American tissue culture collection; β$_2$-m: β$_2$-microglobulin; BSA: bovine serum albumin; CTL: cytotoxic T lymphocyte; FCS: fetal calf serum; FFL: firefly luciferase; FFPE: formalin-fixed paraffin-embedded; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; HC: heavy chain; HLA: human leukocyte antigen; HLA-I: HLA class I; HRP: horseradish peroxidase; IFN: interferon; im-miR: immune modulatory miRNA; LMP: low molecular weight protein; luc: luciferase; MFI: mean fluorescence intensity; MHC: major histocompatibility complex; miR: microRNA; NC: negative control; NK: natural killer; NSCLC: non-small cell lung carcinoma; OS: overall survival; PBMC: peripheral blood mononuclear cells; RBP: RNA-binding proteins; RL: Renilla; RLU: relative light units; TAP: transporter associated with antigen processing; tpn: tapasin; UTR: untranslated region.

Introduction

The immune system operates as a significant barrier to tumor formation and progression[1] mediated by the innate and adaptive immune system, which significantly contributes to immune surveillance and thus to tumor eradication.[2,3] However, tumor cells often develop escape strategies including human leukocyte antigen class I (HLA-I) alterations. The physiologic function of HLA-I and components of the antigen processing and presentation machinery (APM) is required for T cell-mediated anti-tumor or anti-viral immunity. In contrast, tumor survival indicates a failure of the host immune surveillance, which is often associated with a dysfunction in the HLA-I antigen presentation.[4] This is mainly mediated by an impaired expression of HLA-I APM components, which is accompanied by disease progression, reduced patients' survival as well as a diminished recognition by CD8$^+$ cytotoxic

T lymphocytes (CTLs).[5,6] During the last years, a number of studies revealed that defects in the HLA-I APM component expression is mainly caused by deregulation rather than by structural alterations of these molecules[7-9] and is associated with a reduced HLA-I surface expression.[10] Although a transcriptional downregulation of HLA-I APM components frequently occurs in tumor cells, a post-transcriptional control of these molecules has been recently suggested due to altered expression of small non-coding microRNAs (miRs) or RNA-binding proteins (RBPs).[11]

MiRs have a length of approximately 20–23 nucleotides and play important roles as fine tuners in animals and plants by pairing to the mRNAs of protein-coding genes to direct their post-transcriptional repression in normal and cancer cells.[12] This is mediated by their binding to the 3' untranslated region (UTR) of the targeted mRNA thereby preventing its translation or inducing

its degradation.[13] Many miRs are abnormally expressed in hematologic and solid tumors[14] including melanoma. MiRs could act as oncogenes or tumor suppressor genes, which influence tumor formation, infiltration, metastasis formation and disease progression.[15] Recent evidence supports the deregulation of miRs controlling signal transduction cascades, such as e.g. PI3K/AKT or RAS/MAPK pathways, but also of other cellular processes including immune modulation.[16] Indeed, miRs could be involved in the escape from immune surveillance or altered immune responses by affecting the expression of different immune modulatory molecules in tumor and immune cells.[11] The characterization of miRs involved in immune evasion mechanisms might contribute to a better understanding of the development and progression of tumors.[17] These deregulated miRs might also improve patients' prognostic classification and provide new diagnostic and therapeutic tools.

Using distinct strategies, like e.g. *in silico* prediction, miR arrays, small RNA sequencing, RNA affinity approaches and luciferase (luc) reporter gene assays, we and others have identified a number of immune modulatory miRs (im-miRs).[11,18,19] These include members of the miR-148/-152 family, which have been shown to post-transcriptionally regulate the classical HLA-A, -B and -C molecules as well as the non-classical HLA-G antigen.[20-25] Despite their emerging role as fine tuners in the regulation of anti-tumoral immune responses, there exists limited information about miRs suppressing components of the HLA-I APM[20,26] in cancer cells.

So far, only a small number of im-miRs affecting APM components, and in particular the transporter associated with antigen processing 1 (TAP1), have been identified.[27-29] For example, the mimic-based overexpression of the tumor suppressive miR-9 in nasopharyngeal carcinoma exerts a broad effect on many well-known interferon (IFN)-induced genes of the HLA-I APM pathway. TAP1, β_2-microglobulin (β_2-m), the proteasome subunits PSMB8 and PSMB10, the classical HLA-B, HLA-C antigens as well as the non-classical HLA-F antigen were up-regulated at the mRNA level by miR-9.[29] Despite details about the underlying mechanisms of this deregulation remain unknown, it appears to occur indirectly. Furthermore, overexpression of the ER stress-induced miR-346 in Calu-3 and HeLa cells resulted in a reduced TAP1 mRNA expression, which correlated with decreased expression of major histocompatibility complex (MHC) class I surface antigens.[28]

Based on these results it was postulated that the post-transcriptional downregulation of HLA-I pathway in tumors might be controlled by the expression of APM component-specific miRs. This study identified and characterized miR-200a-5p as a regulator of TAP1 expression in human melanoma cells, which was accompanied by a decreased HLA-I surface expression and an increased NK cell response. Furthermore, miR-200a-5p expression was inversely correlated to TAP1 expression in melanoma lesions, which was associated with a worse patients' outcome.

Materials and methods

Cell lines and cell culture conditions

The human embryonic kidney cell line HEK293T was obtained from the American Tissue Culture Collection (ATCC), the human melanoma cell lines FM3 (ESTDAB-007) and FM81 (ESTDAB-026) from the European Searchable Tumor Cell Line and Data Bank (ESTDAB project; www.ebi.ac.uk/ipd/estdab)[30,31] and BUF1379 from Soldano Ferrone (Department of Surgery, Massachusetts General Hospital, Harvard Medical School, Boston, USA). The HEK293T cells were cultured in Dulbecco's Modified Eagles Medium (DMEM, Invitrogen, California, USA), while the melanoma cells were maintained in Roswell Park Memorial Institute 1640 medium (RPMI 1640, Invitrogen) supplemented with 10% (v/v) fetal calf serum (FCS) (PAN, Aidenbach, Germany), 2 mM L-glutamine (Lonza, Basel, Switzerland) and 1% penicillin/streptomycin (v/v, Sigma-Aldrich, Missouri, USA) at 37°C in 5% (v/v) CO_2 humidified air. For IFN-γ treatment, the cell lines were cultured in the presence of recombinant 200 U/mL IFN-γ (PAN) for 24 h or 48 h, respectively.

Human melanoma tissues

Tissue samples from cutaneous malignant melanoma (n = 26) were collected between 2008 and 2016 in the Department of Dermatology of the University Hospital of Zurich, Zurich, Switzerland[32] and the University Hospital in Salzburg, Austria. The study was performed according to the declaration of Helsinki and approved by the ethical committees of the University Hospital in Zurich (KEK-ZH-No. 647 and 800) as well as of the University Hospital in Salzburg (E-Nr. 2142). The clinical data from the melanoma patients as well as the PD-L1 expression and immune cell infiltration of the tumor lesions are available.

Cloning of the miR expression vector

The miR-200a-5p expression vector was generated by cloning of miR-200a-5p into the multiple cloning site of the pmRm-cherry vector (Clontech, Mountain View, CA, USA) as previously described.[23] The used oligonucleotides are listed in the Supplementary Table S1.

Isolation of cellular RNA, miR and qPCR analysis

Total cellular RNA and miR from tissue cultures were isolated using the NucleoSpin RNA kit (Macherey Nagel, Schkeuditz, Germany) or the TRIzol reagent (Invitrogen) according to the manufacturer's instructions, respectively. For RNA isolation from paraffin-embedded tissue sections, total RNA was extracted using the NucleoSpin Tissue kit (Macherey Nagel) according to the manufacturers' protocol. The RNA was treated with DNaseI (New England Biolabs (NEB), Ipswich, MA, USA) for 30 min at 37 °C, inactivated with 50 mM EDTA for 10 min at 75°C and then served as template for cDNA synthesis.

Reverse transcribed quantitative PCR (RT-qPCR) was performed as previously described.[31,33] Briefly, RNA was reversed transcribed into cDNA using RevertAid[TM] H Minus First Strand cDNA synthesis kit (Thermo Scientific, Waltham, Massachusetts, USA). For miR specific cDNA synthesis, miR specific stem loop primers where used[33,34] (Supplementary Table S1) whereas for the reverse transcription reaction of mRNA, oligo dT primers (Thermo Scientific) were used. For

RT-qPCR reaction, the 2x SYBR Green qPCR Master Mix (Absource, Munich, Germany) was employed with target-specific primers (Supplementary Table S1). The reverse transcription reactions were carried out in a 96-well labcycler (Sensoquest, Göttingen, Germany) and the qPCR reactions in a BIO-RAD 96-well iCycler (BIO-RAD Laboratories, Inc., Hercules, CA, USA) for quantifying the expression of miRs and mRNAs. For qPCR, relative changes of RNA abundance were determined by the ΔCT method using the house-keeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin (ACTB) or delta-aminolevulinate synthase 1 (ALAS1) for normalization, whereas the relative miR expression levels were normalized to corresponding expression levels of the non-coding RNA RNU6A. The absolute copy numbers of miRs in the plasmid transfectants were determined by an external miR-specific TOPO-TA plasmid standards (Invitrogen Cat. no. K4510–22) generated by cloning the respective stem-loop PCR product of the miR of interest into this plasmid.[23] The reactions were performed at least in triplicates of biological replicates.

Protein extraction and Western blot analysis

For Western blot analysis, 50 µg protein/lane was separated in 10% SDS-PAGE gels, transferred onto nitrocellulose membranes (Schleicher & Schuell, Munich, Germany) and stained with Ponceau S as previously described.[35] Immune detection was performed with the following APM-specific primary antibodies (Ab): anti-TAP1 (ab13516, Abcam, Cambridge, United Kingdom), anti-TPN (ab13518, Abcam) and anti-TAP2, anti-MHC class I heavy chain (HC), anti-LMP2 and anti-LMP10, kindly provided by Soldano Ferrone. Staining with anti-GAPDH (Cell Signaling Technology) served as loading control. The membranes were then stained with suitable horseradish peroxidase (HRP)–conjugated secondary antibodies (DAKO, Hamburg, Germany or Cell Signaling Technology, Danvers, USA), before the signal was visualized with the Pierce Western Blot Signal Enhancer substrate (Thermo Scientific) and recorded with a LAS3000 camera system (Fuji LAS3000, Fuji GmbH, Düsseldorf) using the Image Reader LAS3000 software. The immunostaining signals were subsequently analyzed using the ImageJ software (NIH, Bethesda, MD). Relative protein expression levels are provided as arbitrary units by setting the peak values of the corresponding GAPDH signals to 1.

Luciferase reporter assay

The TAP1 3′-UTR was cloned in the pmiR-Glo Dual-Luciferase miR target expression vector (Promega, Madison, Washington, USA) with the restriction enzymes NheI and SalI (Thermo Scientific). For the deletion of the binding side of miR-200a-5p on the TAP1 3′-UTR, specific primers were designed according to the NeBaseChanger software (https://nebasechanger.neb.com/, NEB) and the Q5® Site-Directed Mutagenesis kit (NEB) was employed according to manufacturer's instructions. On day zero, 1×10^4 HEK293T cells were seeded into 96-well plates. After 12–16 h, the cells were co-transfected with 30 nM miR mimics and 5 ng TAP1 3′-UTR pmiR-Glo vector using Lipofectamin 2000 (Invitrogen). The

cells were washed with phosphate-buffered saline (PBS) 48 h post-transfection and lysed in lysis buffer (Promega). The firefly and renilla luciferase (luc) activities were determined using the DualGlo reagent (Promega) with the GloMax 96-Microplate luminometer (Promega) kindly provided by G. Posern (Institute of Biophysical Chemistry, Martin Luther University Halle-Wittenberg, Halle (Saale), Germany) with the Dual-Luciferase® Reporter Assay System (Promega) according to manufacturer's instructions. Firefly luciferase (FFL) activities were internally normalized by Renilla (RL) activities yielding relative light units (RLU). An empty vector only containing the multiple cloning site served as negative control. RLU ratios were normalized to control populations. All experiments were performed at least three times in triplicates.

Transfection of miRs

To determine the impact of miRs on the expression and function of APM components, 3×10^5 cells/well were seeded in 6-well plates and transiently transfected after 12–16 h with 30 nM mimics (miR-200a-5p or the mimic negative control (NC; Qiagen, Hilden, Germany) using 9 µL Lipofectamine RNAiMAX (Invitrogen) or with 2 µg/mL plasmids (miR expression or empty vector) using Effectene (Qiagen), according to the manufacturer's instructions. The cells were harvested 48 h post-transfection for subsequent RNA, Western blot and flow cytometric analyses.

Flow cytometry

The antibodies (Abs) employed for flow cytometry were the PE-Cyanine7-labeled-anti-HLA-ABC (BioLegend, California, US), the APC-labeled-anti-HLA-BC (BioLegend), the unconjugated anti-HLA-A2 (kindly provided by Soldano Ferrone) and the PE-labeled-secondary goat anti-mouse Ab (Jackson ImmunoResearch, Cambridgeshire, UK). Flow cytometry was performed as previously described.[36] Briefly, $1–5 \times 10^5$ cells were incubated with the appropriate amounts of antibodies at 4 °C in darkness for 30 min. The stained cells were further stained with propidium iodide (Biolegend), measured on a BD FACS LSRFortessa (Becton Dickinson (BD), New Jersey, USA) and subsequently analyzed with the FACS Diva analysis software (BD). The data are expressed as mean specific fluorescence intensities (MFI).

CD107a degranulation assay

Tumor cell susceptibility to NK cells was evaluated by a CD107a degranulation assay.[37,38] Briefly, total peripheral blood mononuclear cells (PBMCs) were stimulated for 18 h with IL-12 (Immunotools), IL-15 (Immunotools) and IL-18 (Biovision) and then co-incubated at a 1:1 ratio with target cells at 37°C. After one hour of incubation, the APC-anti-CD107a Ab (Biolegend) was added and after additionally 3 h the effector cells were stained with anti-CD3, anti-CD16 (both from Biolegend) and anti-CD56 Ab (Thermo Scientific), before cells were analyzed by flow cytometry. The experiments were performed with PBMCs from at least three different donors. The data are expressed as percentage of CD107a positive NK

cells after subtraction of the spontaneous degranulation obtained by incubating the effector cells in the absence of any target. Daudi cells served as controls to evaluate NK cell functionality (data not shown).

Immunohistochemical staining of the paraffin-embedded tissue sections of melanoma patients

Formalin-fixed paraffin-embedded (FFPE) tumor samples were processed and analyzed in collaboration with the Department of Dermatology of the University Hospitals in Zurich (Switzerland) and Salzburg (Austria). 4 μm thickness sections were cut from each FFPE tissue block. After deparaffinization in xylene and rehydration in decreasing concentrations of ethanol, tumor sections were boiled for epitope retrieval in pre-heated buffers in a pressure cooker for 40 min.

Thereafter, sections were cooled for 20 min and rinsed with deionized water. If necessary, endogenous peroxidases were blocked with 3% H_2O_2 solution for 10 min. Further staining steps were performed using the Dako Autostainer Plus platform (Agilent Technologies Inc., Santa Clara, USA) and have been described elsewhere in detail.[32]

Immunohistochemical stains were evaluated by three independent dermatopathologists. A consensus-based score was derived for every single evaluation. Cell count was estimated by averaging at least ten high powered fields (HPF, 400x magnification) representative of the entire tumor. Expression of TAP1 in tumor cells was graded into four categories regarding the frequency (0%, 1–10%, 11–30%, >30%). For the present study, TAP1^{high} (>30 %) and TAP1^{low} (0–10 %) lesions were processed.

Bioinformatics – survival analysis

In silico analysis was performed using the R2 web tool (http://r2.amc.nl) in order to predict the association of the expression of miR-200a-5p, TAP1 and HLA-I molecules with the survival of metastatic melanoma patients. The statistical differences in the gene expression values between the patients' groups with 'high' and 'low' mRNA expression levels were evaluated by ANOVA tests implemented in the R2 web tool. The p-values were corrected for multiple testing according to the false discovery rate. All cutoff expression levels and their resulting groups were correlated with the patients' survival and used for the generation of the Kaplan-Meier curves, which allowed to discriminate patients into 'good' and 'bad' prognosis cohorts. Kaplan Meier analysis was performed to estimate the disease specific survival probability and distant metastasis free survival probability according to mRNA expression status using this tool.

For this reason, the "R2: Tumor Melanoma – Jöhnsson – 214 – custom – ilmnht12v4"[39] dataset was chosen and 214 unique metastatic melanoma patients were included for the analysis. Total RNA was extracted from fresh-frozen melanoma tumors and genome-wide expression profiling was performed using Illlumina Human HT-12V4.0 BeadChip arrays by standard methods and after removal of replicate samples, 214 unique melanoma samples were analyzed. For determination of high and low expression of TAP1, HLA-A and miR-200a, the cutoff modus "median" divided the patients into two groups containing the same number of patients. The raw p-value significance was calculated for every graph with the web database. Correlation of miR-200a expression with TAP1 and HLA-A was determined with the same dataset. Pearson's correlation was calculated with the transform 2log setting. The 2log expression ratio was compared and a linear regression was calculated. The expression pattern of TAP1 and HLA-I molecules were compared to that of miR-200a and correlated to the clinical parameters. A p-value < 0.05 was considered as significant.

Statistical analysis

Microsoft Excel 2010 (Microsoft Corporation), SPSS version 15.0 and GraphPad Prism version 8 were used for analysis and a p-value < 0.05 was considered statistically significant using the t test.

Results

Heterogeneous expression of HLA class I components in melanoma cell lines

A large panel of melanoma cell lines (n = 42) has been previously analyzed for the mRNA and/or protein expression of TAP1 and HLA-I heavy chain (HC) and/or HLA-I surface expression. Based on their "APM score" and/or on their HLA-I surface expression in comparison to melanocyte samples, the melanoma cell lines were categorized into three groups named high, intermediate and low expressors (Figure 1). The HLA-I APM score represents the sum of TAP1, TAP2, tapasin (TPN), β_2-m and HLA-ABC mRNA or protein expression levels normalized to housekeeper genes/ proteins. A low, intermediate and high APM score was detected at the mRNA level in 40.38%, 33.33% and 26.19% of the melanoma cell lines when compared to the melanocyte samples (Figure 1A), respectively, while a low, intermediate and high APM score was found in 38.11%, 21.43% and 40.48% of the melanoma cell lines at the protein level relative to melanocytes (Figure 1B), respectively. The mRNA levels of the HLA-I APM components were quantified using reverse transcribed quantitative PCR (RT-qPCR) and compared with the protein expression data obtained by Western blot analysis for the different melanoma cell lines. When compared to melanocytes, lower, intermediate and higher HLA-I surface expression was found in 45.24%, 26.19% and 28.57% of melanoma cell lines, respectively (Figure 1C). This was accompanied by a heterogeneous, but often statistically significant reduced mRNA and/or protein expression of APM components and HLA-I HC in melanoma cell lines. The impaired APM component expression was mainly due to deregulation of these molecules, since it could be upregulated by IFN-γ (Supplementary Figure S1). Based on their profiles, three melanoma cell lines BUF1379, FM3 and FM81 belonging to the high, intermediate and low HLA-I APM expressor categories, respectively, were selected for further analyses.
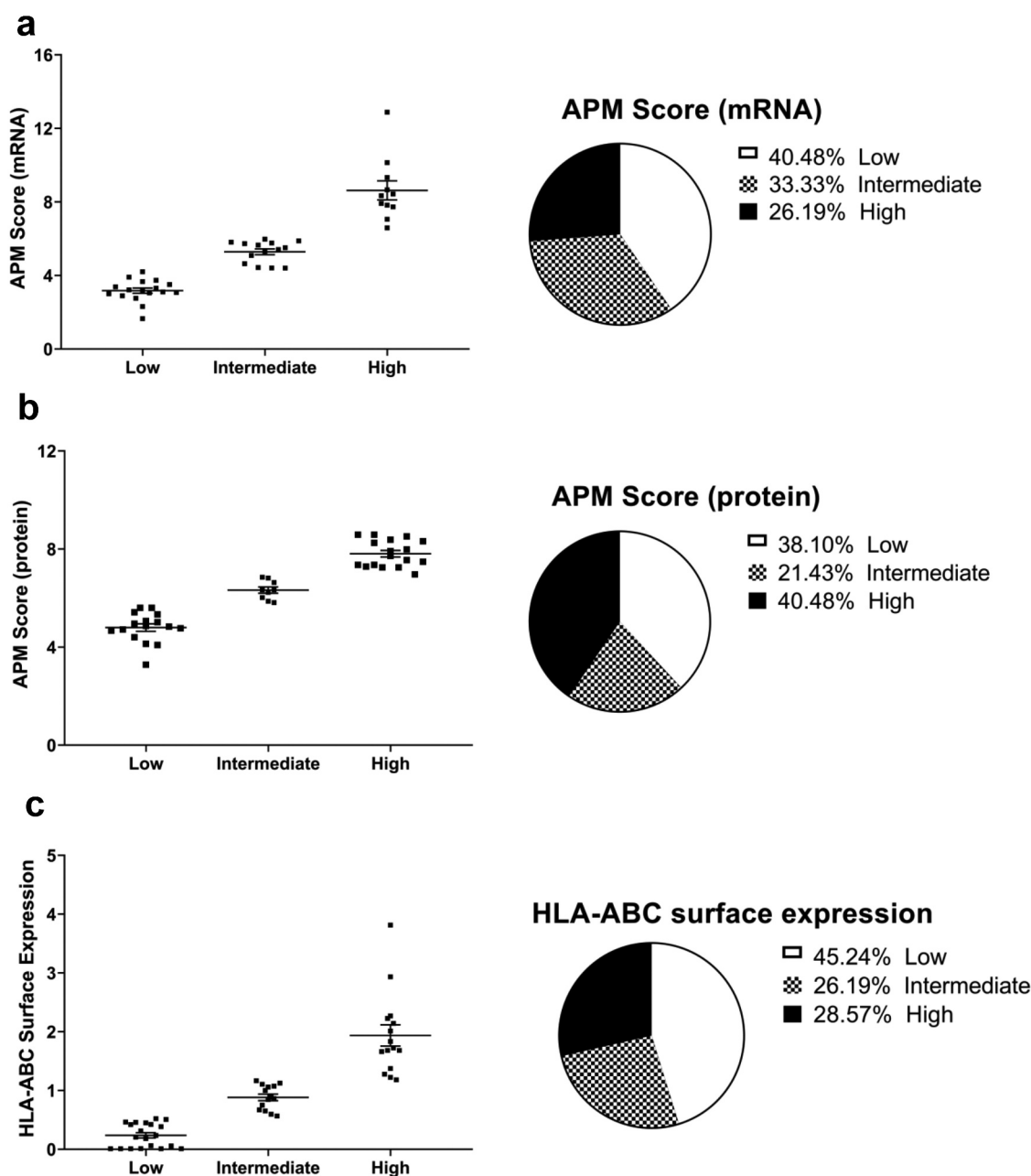
Figure 1. Characterization of the expression of HLA-I APM components in melanoma cell lines. Melanoma cell lines (n = 42) have been characterized for the expression of the different HLA-I APM components TAP1, TAP2, TPN, β_2-m and HLA-ABC at the mRNA (A) and protein level (B) by RT-qPCR and Western blot analysis, respectively. An APM score was calculated by adding the expression levels of the 5 APM components upon normalization to their expression levels in melanocytes. Surface expression of HLA-ABC was determined by flow cytometry (C). Shown are the values for each individual cell line (*left*) as well as a pie chart providing the percentage of high, intermediate and low expressors (*right*).

Clinical relevance of TAP1 and HLA-I molecules with survival of metastatic melanoma patients

Independent studies reported an association of a decreased expression of TAP1 and HLA-I molecules (HLA-A, HLA-B and HLA-C) with poor prognosis in several cancer types including melanoma. This was reevaluated using the dataset "R2: Tumor Melanoma – Jöhnsson – 214 – custom – ilmnht12v4"[39] available at the R2 genomics analysis and visualization platform (http://r2.amc.nl) in order to determine the prognostic relevance of TAP1 and HLA–I molecules (HLA-A, HLA-B and HLA-C) expression patterns in 214 melanoma patients.

As shown in Figure 2, higher TAP1 and HLA-A mRNA transcript levels were correlated with an increased disease specific survival probability and higher distant metastasis free survival probability (Figure 2A,B) linking higher TAP1 and HLA-I levels with a better survival of metastatic melanoma patients. In particular the TAP1 expression was significantly correlated with the HLA-A mRNA level (Figure 2C).

Direct interaction of miR-200a-5p with TAP1 3′-UTR

Since a discordant TAP1 mRNA and protein level was observed in some melanoma cell lines analyzed, a post-transcriptional
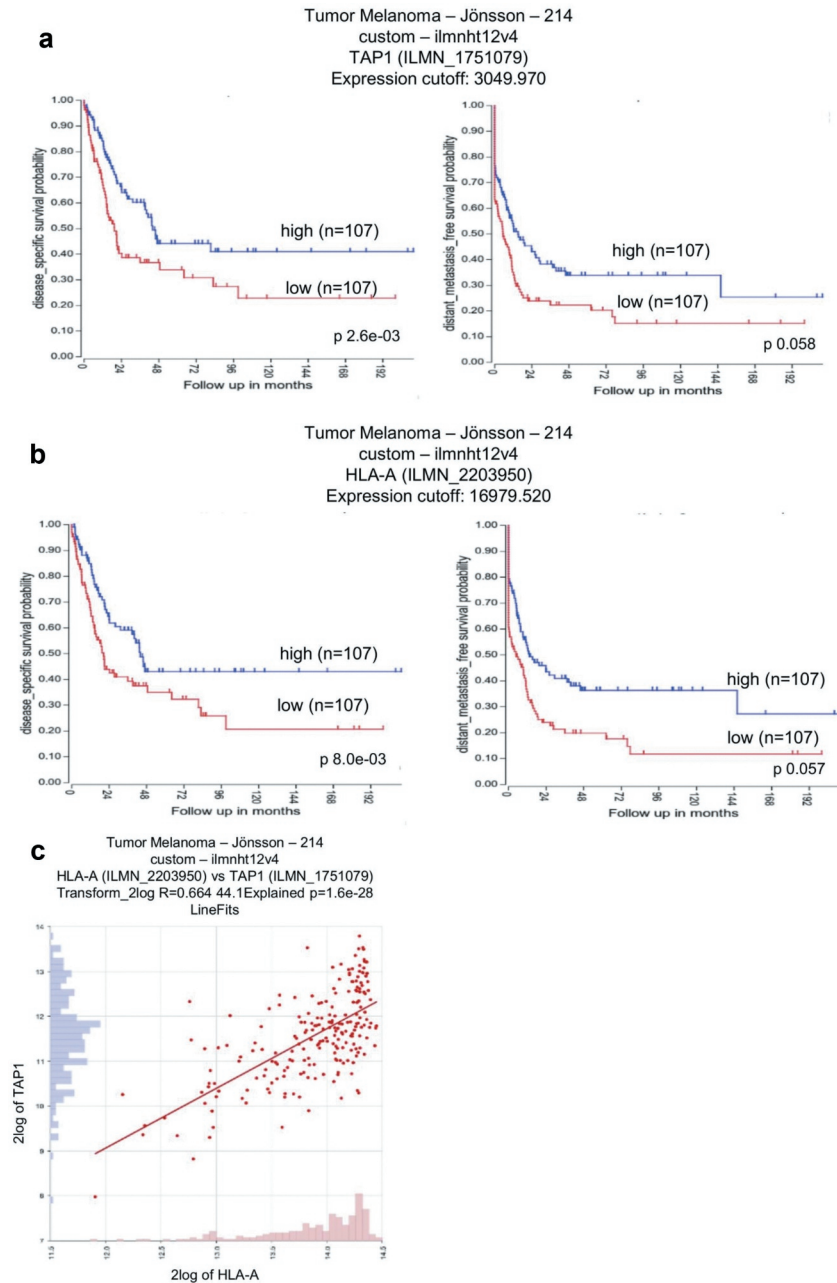
Figure 2. Correlation of TAP1 and HLA-A expression with the survival probability in metastatic melanoma patients. Kaplan Meier estimation curves for survival probability of individual metastatic melanoma patients based on the expression of TAP1 (A) and HLA-A (B) were generated by using the "R2: Tumor Melanoma – Joehnsson – 214 – custom – ilmnht12v4" dataset. Shown are the curves both for disease specific (*left*) and distant metastasis free survival probability (*right*) for the two genes. The raw *p*-values were based on log-rank tests and calculated for every graph with the web database (http://r2.amc.nl). (C) Correlation of TAP1 and HLA-A mRNA expression in the metastatic melanoma patients using the same dataset.

regulation of TAP1 by miRs was suggested. *In silico* analysis using the microrna.org[40] (http://www.microrna.org/microrna/home.do) and RNAhybrid[41] (https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid) algorithms predicted miR-200a-5p as a candidate miR targeting the TAP1 3′-UTR (Figure 3A). The binding affinity of complementary structures between the putative miRs and the target mRNA demonstrated a high free binding energy of −26.3 kcal/mol for miR-200a-5p and TAP1 3′-UTR (Figure 3B) indicating a high probability of interaction.

Furthermore, the direct interaction between miR-200a-5p and the TAP1 3′-UTR was validated by the dual luc reporter assay. After the transient transfection of HEK293T cells with the pMir-Glo vector containing the TAP1 3′-UTR

in the presence of miR-200a-5p mimics, the luc activity significantly decreased upon miR-200a-5p overexpression in comparison to the miR mimic negative control (NC) (Figure 3C). As expected, the deletion of the miR-200a-5p binding site did neither alter the luc activity with miR-200a-5p nor with the NC (Figure 3C,D).

Differential expression of miR-200a-5p in melanoma cell lines

The different melanoma cell lines were analyzed by RT-qPCR for the expression of miR-200a-5p in correlation to TAP1. MiR-200a-5p was heterogeneously expressed in these cells
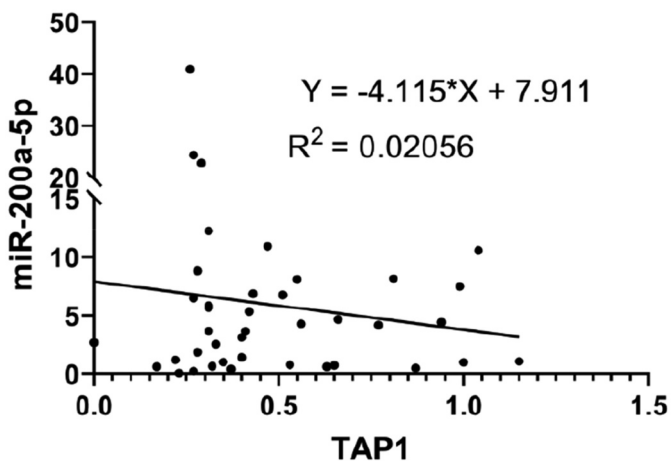
Figure 3. Identification of miR-200a-5p interaction with the 3′-UTR of TAP1. (A) Graphic representation of the TAP1 3′-UTR (NM_000593.5) with the *in silico* predicted binding site for miR-200a-5p (red box). (B) Sequence alignment, secondary structure and free energy (mfe = −26.3 kcal/mol) for predicting the interaction of TAP1 3′-UTR (red) and miR-200a-5p (green) were obtained using the free online data base RNAhybrid (https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid). (C) The dual luciferase reporter assay was performed with HEK293T cells as described in Materials and Methods. Briefly, HEK293T cells were transfected with the miR-200a-5p or miR mimic negative control (NC) together with the plasmid encoding for the Firefly luciferase (FFL) cloned downstream the TAP1 3′-UTR in its wild type form (TAP1 3′-UTR wt) or upon deletion of the predicted binding site for miR-200a-5p (TAP1 3′-UTR del). FFL activities were internally normalized to Renilla luciferase activities yielding relative light units (RLU). Shown are the mean ± SE from 3 to 6 independent experiments upon normalization to the miR mimic NC. * $p < .05$ in un-paired t-test. (D) Alignment of the TAP1 3′-UTR wt and the TAP1 3′-UTR del upon plasmid sequencing. The gray box highlights the deletion of the binding site of miR-200a-5p.

(Figure 4). A threshold of < 20 cycles difference between the expression of miR-200a-5p and the housekeeper gene RNAU6 was considered as "detectable" miR-200a-5p expression, while melanoma cell lines with > 20 cycles difference between the expression of miR-200a-5p and RNAU6 were defined as undetectable miR-200a-5p expression. In 32/42 melanoma cell lines (76.19%) including BUF1379, FM3 and FM81 cells, miR-200a-5p expression was detectable. In 12 melanoma cell lines (28.57%), low TAP1 protein expression was accompanied by miR-200a-5p higher expression levels compared to melanocyte samples.

Downregulation of TAP1 expression by miR-200a-5p

As an initial proof of principle, HEK293T cells were transiently transfected with miR-200a-5p recombinant plasmid and the mock control vector, respectively. MiR-200a-5p

overexpression in HEK293T cells (Figure 5A) results in a more than 40% downregulation of TAP1 mRNA (Figure 5B) and protein expression levels (Figure 5C) when compared to the mock control vector transfectants.

To further determine the effect of miR-200a-5p on TAP1 expression in melanoma cell lines, miR-200a-5p mimics and a miR mimic NC were transiently transfected into BUF1379, FM3 and FM81 cells. Overexpression of miR-200a-5p was obtained in all three melanoma cell lines, while cells transfected with the NC showed a relative expression pattern when compared to parental cells (Figure 6A). For Western blot analysis, melanoma cells only transfected with the transfection reagent named "Control" served as an additional control to the NC. Overexpression of miR-200a-5p in BUF1379 and FM3 cells caused a 40% downregulation of TAP1 mRNA and protein levels compared to that of the NC or control

Figure 4. Basal expression of miR-200a-5p and TAP1 in different melanoma cell lines. The different melanoma cell lines were analyzed by RT-qPCR for the expression of miR-200a-5p and TAP1. Shown are the expression levels upon normalization to melanocytes as well as the linear regression equation and its R^2.

samples (Figure 6B,G,H). Furthermore, miR-200a-5p overexpression only caused a downregulation of TAP1, but not of other APM components, such as TAP2, TPN, LMP2 and HLA-I HC (Figure 6C–G). This was accompanied by a decreased HLA-I surface expression particularly of HLA-BC in the miR-200a-5p transfectants (Figure 6I–kK). As expected, the mRNA and protein expression levels of

control transfectants were comparable to that of parental samples.

Correlation of the miR-200a-5p-mediated downregulation of TAP1 with enhanced NK cell recognition

To assess the functional relevance of miR-200a-5p-induced suppression of TAP1 and consequently of HLA-I surface expression, NK cell-mediated recognition was determined using the CD107a degranulation assay. Due to a comparable effect of miR-200a-5p on BUF1379 and FM3 cells, analysis was performed with FM3 cells, since they express a Bw4 allele and thus have one additional "restriction" element for inhibition of NK cells. Higher levels of CD107a positive NK cells were found in response to FM3 cells overexpressing miR-200a-5p than to NC transfectants (Figure 7). This might be due to the miR-200a-5p-mediated silencing of TAP1 expression followed by a reduced HLA-I surface expression.

Correlation between miR-200a-5p expression with TAP1 and immune cell infiltration

To determine the *in vivo* relevance of the *in vitro* data, miR-200a-5p levels were evaluated by RT-qPCR analysis in 26 FFPE sections of human primary melanoma that have been scored as TAP1 low (n = 10) and TAP1 high (n = 16) based on immunohistochemical
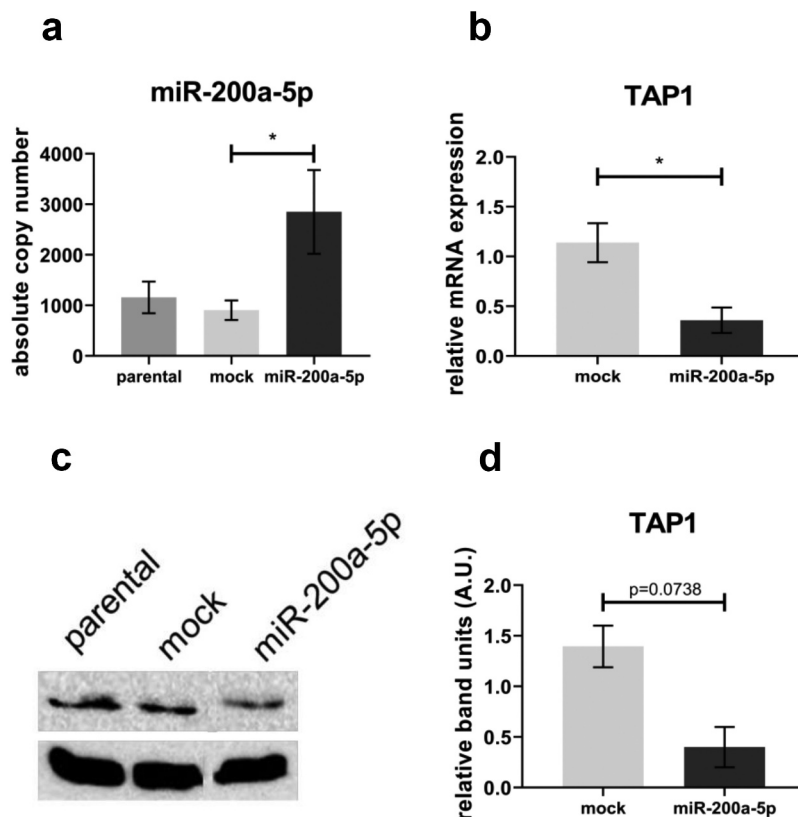

Figure 5. Effect of miR-200a-5p overexpression on TAP1 expression in HEK293T cells. HEK293T cells were either left untreated (parental) or transiently transfected with 2 μg/mL empty pmRm-cherry vector (mock) or with miR-200a-5p plasmid (miR-200a-5p). After 48 h miR-200a-5p overexpression in absolute copy number (A) as well as the mRNA expression levels of TAP1 were determined by RT-qPCR (B). Protein expression was evaluated by Western blot (C) and for quantification of the results, the relative band density (A.U., arbitrary units) of transfectants was calculated to the respective parental cells and normalized to GAPDH expression (D). Shown are the normalized mean ± SE from a minimum of three different biological replicates, *p < .05 in un-paired t-test.
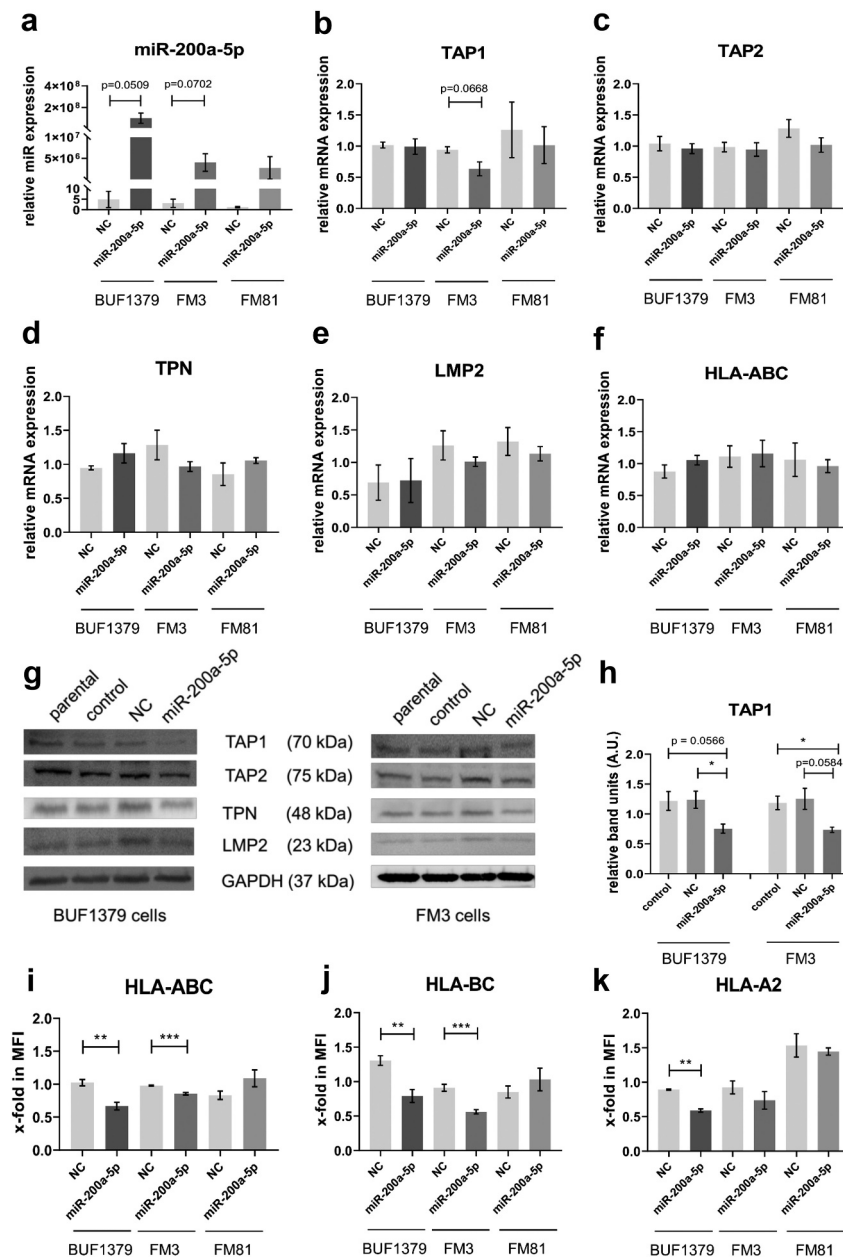
Figure 6. Effect of miR-200a-5p overexpression on the expression of HLA-I APM components in melanoma cell lines. BUF1379, FM3 and FM81 melanoma cells were left untreated (parental) or transiently transfected with RNAimax alone (control) or together with 30 nM miR mimic negative control (NC) or miR-200a-5p mimic (miR-200a-5p). After 48 h miR-200a-5p overexpression (A) as well as the mRNA expression levels of the indicated APM components were determined by RT-qPCR (B – F). Protein expression was evaluated by Western blot (G – H) and flow cytometry (I – K). For quantification of Western blot results, the relative band density (A.U., arbitrary units) of transfectants was calculated to the respective parental melanoma cells and normalized to GAPDH expression. Shown are the normalized mean ± SE from a minimum of 3 different biological replicates and one representative Western blot, *p < .05, **p < .01, ***p < .001 in un-paired t-test.

staining. MiR-200a-5p expression was found statistically significant inversely related to the TAP1 score (Figure 8A) thereby supporting our *in vitro* data. The same melanoma samples were also analyzed regarding their immune cell infiltration, in particular of CD8^{+}, CD4^{+}, Foxp3^{+} and CD163^{+} immune cells, using IHC. The TAP1 expression scores were directly correlated to the frequency of CD8^{+} (Figure 8B), CD163^{+} (Figure 8C), CD4^{+} and FoxP3^{+} (data not shown) immune cells, since TAP1^{low}/TAP1^{high} scored melanoma patients showed low/high scores of immune cell subpopulations, respectively. Furthermore, a direct link between the low TAP1 score with CD8^{+} T cell infiltration, but high miR-200a-5p expression and vice versa exists (Figure 8D).

Association of miR-200a-5p expression with clinical parameters

In order to correlate the expression of miR-200a-5p with the disease specific survival probability of metastatic melanoma patients and the expression of TAP1 and HLA-I molecules (HLA-A, HLA-B and HLA-C), the same melanoma dataset[39] of the R2 web tool was used. Higher expression of miR-200a was significantly associated with worse disease specific survival probability of patients with metastatic melanoma (Figure 8E), but not with the distant metastasis free survival probability (Supplementary Figure S2). Furthermore, the miR-200a expression was inversely correlated to the expression of TAP1 and of all
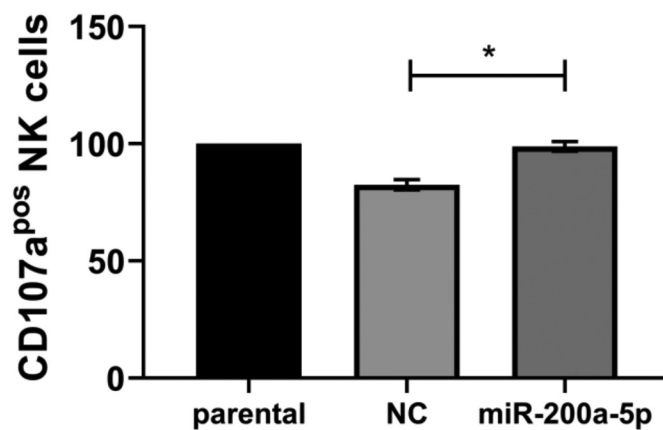
Figure 7. Increased recognition of miR-200a-5p overexpressing FM3 melanoma cells by NK cells. NK cells from healthy donors were co-incubated with FM3 cells, either left untreated (parental), transfected with the negative control (NC) or with miR-200a-5p mimics. After 4 h the percentage of CD107a expressing NK cells was determined by flow cytometry. Shown are the normalized mean ± SE from 3 independent experiments. *p < .05 in paired t-test.

HLA-I molecules as representatively shown for HLA-A (Figure 8F,G).

Discussion

Our study suggests that TAP1 expression in melanoma cells can be controlled by miR-200a-5p leading to a downregulation of HLA-I surface expression, which is linked to a reduced survival of melanoma patients. The miR-200 family has become a hot topic and includes 5 members (miR-200a, miR-200b, miR-200c, miR-141 and miR-429) located on two different genomic clusters: the first cluster consists of miR-200a, miR-200b and miR-429 on chromosome 1, the second of miR-200c and miR-141 on chromosome 12.[42,43] Moreover, miR-200a-5p overexpression has recently been described in non-small cell lung cancer (NSCLC),[44] ovarian cancer,[45] breast cancer[46] and papillary thyroid carcinoma, but so far not linked to HLA-I and APM component expression.[47]

Alterations in the surface expression and/or function of the HLA-I antigens are frequently found in human tumors and equip neoplastic cells with mechanisms to escape immune surveillance.[5] Furthermore, tumors with acquired resistance to checkpoint inhibitor therapy or adoptive T cell transfer can acquire genetic deficiencies or downregulation of APM components resulting in the loss or reduced expression of either constitutive or HLA-I surface antigens.[48] The abnormalities are significantly associated with a higher tumor staging, grading, disease progression and a reduced survival of patients as well as a failure to CD8^{+} T cell-based immunotherapies. The aberrant expression of HLA-I molecules can be caused by structural alterations or deregulations of genes encoding the classical HLA-I antigens and/or APM components. Inactivating mutations in the human HLA-I HC and β_2-m genes have been mainly described in melanoma and colorectal carcinoma with a low frequency,[49] while mutations in other APM components, like TAP1, TAP2, TPN and the proteasomal subunits occur more rarely in melanoma, lung and cervical cancer.[5] The deregulation of HLA-I APM components could also occur at the epigenetic, transcriptional or post-transcriptional level[50] providing tumors

cells with different mechanisms to inactivate immune responses, which result in tumor growth and evasion from host immune surveillance. In some malignancies, both "soft" (reversible) and "hard" (irreversible) downregulation of HLA-I may limit effective T cell immune responses.[51,52] Interestingly, reduced HLA-I expression is very common in melanoma prior to immune checkpoint therapy and associated with primary resistance to ipilimumab, but not to nivolumab.[51] In metastatic melanoma patients, regression of metastasis after immunotherapy is associated with the expression of HLA-I APM components and IFN-regulated rejections genes.[53] Furthermore, the expression of HLA-I molecules and APM components is directly correlated with an improved overall and progression-free survival of melanoma patients in several TCGA datasets.[39,54,55]

A reduced mRNA and/or protein expression of various APM components was identified in a large series of melanoma cell lines and lesions, which could be correlated with an increased tumor grading[17,56] implying a deregulated APM component and HLA-I molecule expression. During the last years, some miRs have been reported to target APM components and HLA-I molecules suggesting an important role for post-transcriptional control in the antigen processing and presentation process.[17] So far, only miR-346 has been identified to directly regulate TAP1 during ER stress,[28] suggesting that miRs might be key players and fine tuners of the post-transcriptional regulation of TAP1.[57,58] Their deregulation in melanoma was found by analysis of genomic characteristics of these tumors, which were significantly enriched in miR genes.[59] Several miRs profiles of melanoma cell lines or tissues revealed altered expression patterns compared to melanocytes or nevi, respectively.[60] Functional *in vitro* and/or *in vivo* testing of some deregulated miRs suggested potential contributions of specific miRs to the molecular complexity of this malignancy.[61]

The miR-200 family is known for its role in cancer metastasis by inhibiting epithelial-mesenchymal transition and differentially regulating morphological plasticity and mode of melanoma cell invasion.[62] The expression of three family members, miR-200a, miR-200b and miR-429, is upregulated in three epithelial cancers (breast, ovarian and melanoma) as determined by high-resolution
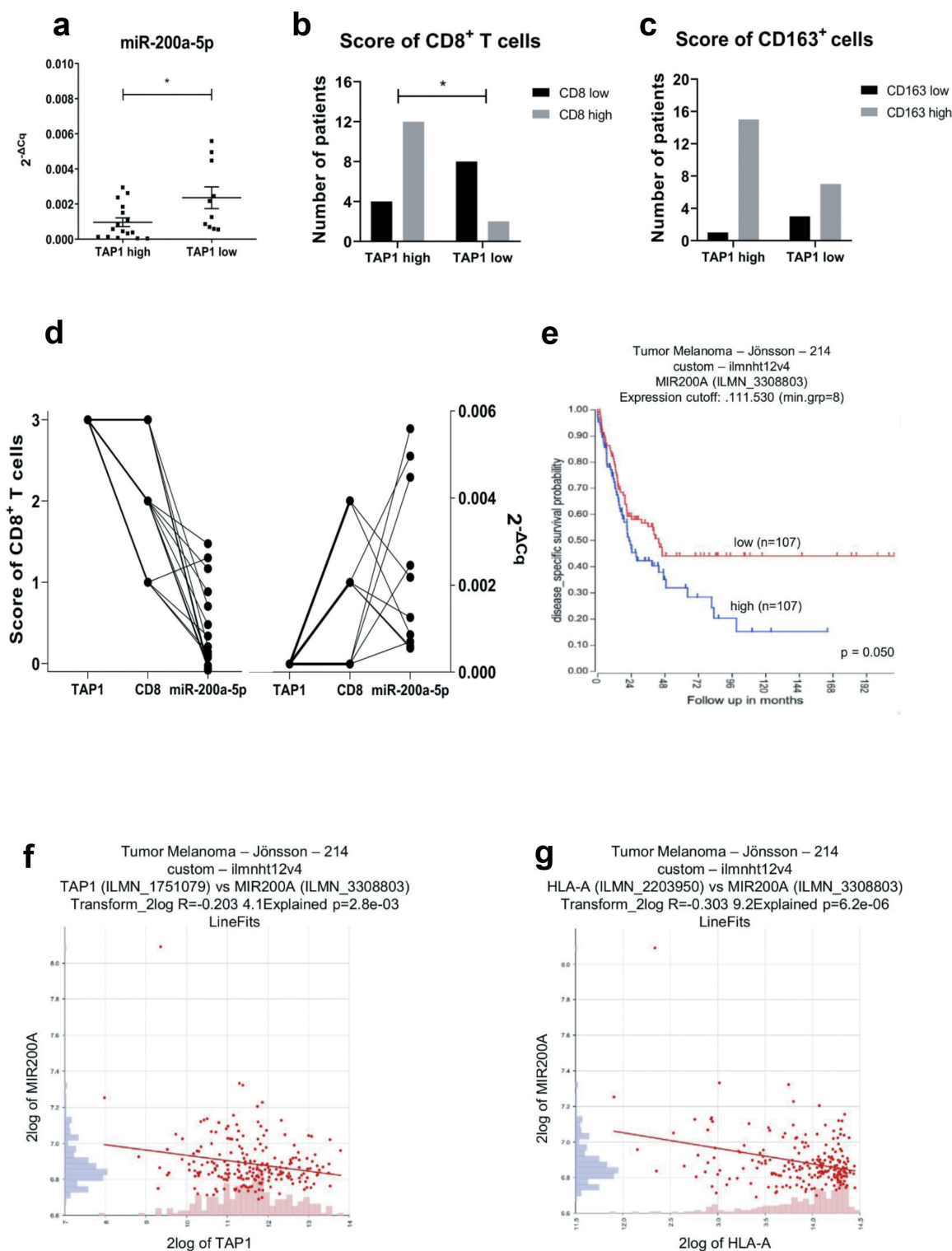
Figure 8. Inverse correlation of miR-200a-5p expression with TAP1 levels and survival probability in melanoma patients. (A-D) Paraffin-embedded tissue sections from 26 primary melanoma patients were analyzed for miR-200a-5p expression levels by RT-qPCR and scored for TAP1, CD8 and CD163 expression as high or low by immunohistochemical staining. Comparison between TAP1 high (n = 16) and TAP1 low (n = 10) melanoma lesions is shown for miR-200a expression (A) and CD8^{+} (B) and CD163^{+} cell infiltration (C). An overview of TAP1, CD8 (left y-axis) and miR-200a-5p expression (right y-axis) for each individual patient among the TAP1 high (*left*) and TAP1 low (*right*) group is provided (D). (E-G) The "R2: Tumor Melanoma – Jöhnsson – 214 – custom – ilmnht12v4" dataset was employed to evaluate the correlation between miR-200a expression and patients' disease specific survival probability by Kaplan Meier estimation curve (E) as well as with TAP1 (F) and HLA-A expression (G) The raw *p*-values were based on log-rank tests and calculated for every graph with the web database. (A: *p < .05 in paired t-test, B: *p < .05 in Fisher's exact test)

array based comparative genomic hybridization.[59] In particular, the expression of miR-200a-5p is significantly increased in several cancer types[44,45,47] and heart diseases.[63,64]

In our study, miR-200a-5p has been shown to regulate TAP1 expression in HEK293T and melanoma cells. MiR-200a-5p was initially found by *in silico* prediction to bind

to the TAP1 3′-UTR. This binding was further confirmed by the dual luc reporter assay. Moreover, a specific effect of miR-200a-5p overexpression on TAP1 protein expression, but not at the TAP1 mRNA level, was found in two melanoma cell lines. MiRs direct the RISC complex to downregulate target expression by mRNA cleavage and/or translational repression.[13,65-67] Although often typically relatively mild,[68,69] the most relevant readout of the miR regulatory effects are at the protein level.[65] Upon overexpression of miR-200a-5p either with mimics or an overexpression vector, a specific downregulation of TAP1 protein level was observed resulting in a downregulation of HLA-ABC surface expression. Since in particular HLA-BC expression was affected by this miR, possible HLA allele specific functional consequences of TAP1 downregulation could be suggested. Interestingly, this might depend at the HLA-I surface expression levels of melanoma cells, since miR-200a-5p affects HLA-I expression of BUF1379 and FM3 cells, but not of the HLA-I low expressing cell line FM81. Furthermore, the miR-200a-5p-mediated reduced HLA-I expression was accompanied by an increased number of CD107a positive NK cells upon co-culture with miR-200a-5p expressing melanoma cells. As expected, no effect of miR-200a-5p overexpression was detected on other APM components suggesting a TAP1-specific effect of miR-200a-5p.

MiR-200a-5p levels were also found inversely related with TAP1 levels of paraffin-embedded tissue sections of melanoma patients. High levels of miR-200a-5p were found in melanoma lesions with low TAP1 expression levels supporting our hypothesis of a post-transcriptional regulation of TAP1 via miR-200a-5p. Interestingly, the immune cell infiltrate, such as CD8^{+} cells determined by immunohistochemistry analysis, was also inversely correlated with the levels of miR-200a-5p further supporting its role as an immune modulatory miR.

In conclusion, we described for the first time a possible immune modulatory role of miR-200a-5p, since this miR targets the TAP1 3′-UTR accompanied by downregulation of TAP1 protein expression associated with a reduced HLA-I surface expression in melanoma cell lines and an increased NK cell recognition. It is noteworthy that miR-200a-5p only moderately affects TAP1 expression suggesting that other miRs controlling TAP1 expression might exist. Furthermore, the clinical relevance of miR-200a-5p should be analyzed in trials, in which treatment options are determined based on HLA-I expression and APM components expression. MiR-200a-5p might serve as a novel target for the treatment of melanoma or for selection/prediction of melanoma patients undergoing the most effective immunotherapy.

Acknowledgments

We would like to thank Maria Heise for excellent secretarial work.

Declaration of interest statement

The authors declare that they have no conflicts of interest.

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