miRNA-148a-3p Regulates Immunosuppression in DNA Mismatch Repair–Deficient Colorectal Cancer by Targeting PD-L1

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Abstract

Immunotherapy against the interaction between programmed cell death ligand 1 (PD-L1) has emerged as a promising strategy for colorectal cancer with mismatch repair deficiency (dMMR) or microsatellite instability-high (MSI-H). The study aimed to identify miRNAs that posttranscriptionally control PD-L1 expression on tumor cells and also regulate immune evasion. A comprehensive miRNA screening using The Cancer Genome Atlas (TCGA) dataset (n = 260) combined with eight different miRNA target prediction programs resulted in the identification of a tumor suppressive miRNA, miR-148a-3p, as a potential negative regulator of PD-L1 expression, particularly in dMMR/MSI-H colorectal cancer. Using multiple cohorts of colorectal cancer, including TCGA data, a microarray dataset (n = 148), and formalin-fixed, paraffin-embedded samples (n = 395), we found that the expression of miR-148a-3p was decreased in dMMR/MSI-H tumors, correlating inversely with PD-L1 levels. We demonstrate that miR-148a-3p directly binds to the 3’-untranslated region of PD-L1, thereby reducing whole-cell and cell surface PD-L1 levels in HCT116 and SW837 cell lines. Overexpression of miR-148a-3p repressed IFNγ-induced PD-L1 expression on tumor cells and consequently diminished T-cell apoptosis in a coculture model of IL2-activated T cells and IFNγ-treated tumor cells. In conclusion, our data support a regulatory mechanism of PD-L1 expression on tumor cells and immune suppression via miR-148a-3p downregulation in colorectal cancer.

Implications: This study provides novel evidence that miR-148a-3p negatively regulates tumor cell PD-L1 expression and decreased levels of miR-148a-3p contributes to the immunosuppressive tumor microenvironment.

Introduction

Despite major advances in diagnosis and treatment, colorectal cancer remains the major cause of cancer-related death worldwide (1). Colorectal cancer is commonly grouped into two categories: approximately 15% of tumors with microsatellite instability-high (MSI-H), caused by defective function of the DNA mismatch repair (MMR) system, and the remaining 85% tumors that are microsatellite stable (MSS) exhibiting chromosomal instability (2, 3). Deficient MMR (dMMR) causes the accumulation of many insertion or deletion mutations at microsatellites spread along the genome and produces mutation-induced frameshift peptides (neoantigens), resulting in a high antigen-presenting ability (4, 5). Hence, dMMR cancers are highly immunogenic and thus exhibit a high density of tumor infiltrating lymphocytes (TIL) in the tumor microenvironment (4, 6). However, concomitant expression of multiple immune checkpoint molecules, including programmed cell death 1 (PD-1) and programmed cell death ligand 1 (PD-L1, also known as B7-H1 and CD274), was demonstrated selectively in dMMR colorectal cancer that may counteract the antitumoral immune responses, thereby dMMR cancer cells can evade immune eradication by TILs (6).

Immunotherapy with antibodies blocking the interaction between PD-1 and PD-L1 has emerged as a promising therapeutic strategy in various types of cancer. Although initial studies of immune checkpoint blockade against the PD-1/PD-L1 axis in colorectal cancer were not especially promising (7), recent clinical trials have revealed that the anti-PD-1 treatment with pembrolizumab or nivolumab resulted in considerable clinical benefits (8, 9). PD-L1 is expressed on the surface of immune cells and antigen-presenting cells, and is often upregulated in tumor cells. PD-1 expressed on TILs and its ligand PD-L1 interaction inhibits the effector phase of CD8 cytotoxic T-cell function through T-cell...
apoptosis and exhaustion (10–12). Transcriptional upregulation of PD-L1 in tumor cells can be induced in response to inflammatory cytokines, such as IFNγ secreted by TILs (12, 13), while it can also be intrinsically driven by oncogenic pathway activation (14, 15). Several posttranslational mechanisms to stabilize PD-L1 protein have recently been proposed (16–18). Although multiple pathways can contribute to the expression and function of PD-L1 involved in immune suppression, the detailed understanding of the upregulation of PD-L1 in tumor is limited.

miRNAs are a class of small (18–25 nucleotides), noncoding RNA molecules that posttranscriptionally regulate gene expression by binding to the 3′-untranslated region (3′-UTR) of protein-coding mRNAs with imperfect complementarity, leading to translational repression or cleavage of transcripts (19). Altered expression of miRNAs with oncocogenic or tumor suppressive functions have been extensively studied in various malignancies (19). Notably, recent studies have demonstrated that several miRNAs expressed in immune cells or cancer cells are crucially involved in cancer-related immune responses by targeting immunosuppressive or immunostimulating factors (20). Moreover, miRNAs may also regulate genes encoding immune checkpoint molecules, including PD-L1 (14).

Despite growing evidence that upregulated PD-L1 in tumor cells plays a key role in immune evasion particularly in dMMR colorectal cancer, posttranscriptional mechanisms controlling PD-L1 expression and consequent T-cell dysfunction in colorectal cancer are not fully understood. Here we hypothesize that miRNAs are involved in the immunosuppressive microenvironment in dMMR colorectal cancer via suppression of PD-L1 expression. Using comprehensive miRNA screening combined with sequence-based target prediction algorithms, we identified a tumor suppressive miRNA, miR-148a-3p, as a potential regulator of PD-L1 in dMMR colorectal cancer. We demonstrate that miR-148a-3p plays an important role in modulating PD-L1 expression that can functionally affect T-cell apoptosis.

Materials and Methods
TCGA Data analysis
The cancer genome atlas (TCGA) level 3 data for colon adenocarcinoma (COAD), including CD274 (PD-L1) expression (Illumina RNA-Seq), miRNA expression profiles (Illumina HiSeq), and MMR status based on MSI testing (MSI-H, MSI-Low, and MSS), were obtained through the TCGA website (https://cancergenome.nih.gov/; ref. 2) and cBioPortal for Cancer Genomics (http://www.cbioportal.org/) in March 2016 (23). Association of miRNA expression with PD-L1 expression or with MMR status was analyzed by BRB-ArrayTools (http://brb.nci.nih.gov/BRB-ArrayTools.html) using correlation analysis or class comparison analysis.

Microarray data analysis
We utilized microarray gene expression and miRNA expression profiles from 148 patients with MSS colorectal cancer (24). These datasets are publicly available from the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo), deposited as GSE19380 on the basis of Affymetrix Human Genome U133 Plus 2.0 array, and GSE19981 based on Agilent-070156 Human miRNA array. The normalized expression values were obtained from each dataset and were not processed further. If a gene or miRNA is represented by multiple probe sets, the expression values of multiple probes were averaged.

miRNA Target prediction
To screen potential miRNAs that potentially bind to the 3′-UTR of CD274, we employed eight target prediction programs, including miRMap (http://mirmap.czbiohub.org/), RNA22 (https://cm.jefferson.edu/ma22/), PITA (https://genie.weizmann.ac.il/pubs/ pit07/mir07_data.html), miRanda (www.microrna.org), Targetscan (www.targetscan.org), microT-CDS (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=micrOT_CDS/), miRWalk (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/), and miRDB (http://www.mirdb.org/mirDB/).

Patient samples
We obtained formalin-fixed, paraffin-embedded (FFPE) tissue samples from 395 consecutive patients with primary colorectal cancer, who had undergone surgical resection without preoperative chemotherapy or radiotherapy between 1990 and 2013 in Fukushima Medical University (FMU) Hospital (Fukushima, Japan). All 395 samples were used for IHC and 72 of them were used for qRT-PCR. The study was conducted in accordance with the Declaration of Helsinki and was approved by the Institutional Review Board of Fukushima Medical University (Fukushima, Japan).

IHC
For PD-L1 staining, rabbit mAb against PD-L1 [catalog no. 13684, PD-L1 (E1L3N) XP, Cell Signaling Technology] was used (18, 25–29). Four-micron-thick sections were deparaffinized, rehydrated, and endogenous peroxidases were blocked with 0.3% hydrogen peroxide in methanol. Antigens were retrieved by autoclaving for 10 minutes in Tris-EDTA buffer solution (120°C, pH 9.0). The primary antibody was incubated in 1:400 dilution of 10 mmol/L PBS containing Tween 20 (Sigma-Aldrich) at 4°C overnight, and subsequently detected by a horseradish peroxidase (HRP)-coupled anti-rabbit polymer (Envisionþ-System-HRP, Dako). Sections were then incubated with DAB (Dako), before counterstaining with hematoxylin. Negative controls were synthesized by replacing primary antibody with PBS. Several carcinoma tissues from lung, esophagus, breast, and stomach were used as positive controls. Tumor specimens were considered PD-L1–positive when more than 5% of tumor cells showed membranous staining of any intensity with or without cytoplasmic staining (25, 27, 28).

IHC for MMR proteins including MLH1, MSH2, MSH6, and PMS2 was performed using Dako EnVisionþ System with mouse or rabbit mAbs against MLH1 (clone ES05, 1:50; Dako), MSH2 (clone FE11, 1:50; Dako), MSH6 (clone EP49, 1:200; Dako), and PMS2 (clone EP51, 1:50; Dako), as described previously (30). Loss of a MMR protein was defined as the absence of nuclear staining of tumor cells in the presence of positive nuclear staining in internal controls.

Determination of MMR status
Tumors demonstrating MSI-H or loss of at least one MMR protein were collectively designated as dMMR, and tumors with
non-MSI-H or intact MMR protein expression as proficient MMR (pMMR; ref. 30).

Cell culture

The human colorectal cancer cell lines, including SW837 and HCT116, were obtained from JCRB Cell Bank (Osaka, Japan) and RIKEN Cell bank (Ibaraki, Japan), respectively, and were used within 6 months of culture after they were received. Both cell lines were grown in RPMI1640 (Thermo Fisher Scientific) containing 10% FBS and penicillin/streptomycin (100 IU/mL; Thermo Fisher Scientific). Cells were cultured at 37°C and 5% CO₂ in tissue culture incubator.

Transfection

During the exponential growth phase, cells were transiently transfected with 30 nmol/L of mirVana miR-148a-3p mimic (ID: MC10263), mirVana mir-148a-3p inhibitor (ID:MH10263), mirVana miRNA mimic negative control#1, or mirVana miRNA inhibitor negative control#1, using Lipofectamine RNAiMAX Reagent (Thermo Fisher Scientific) according to the manufacturer’s protocol. Following 48 hours of incubation, cells were used for each experiment.

Luciferase reporter assay

Complementary 38-bp DNA oligonucleotides containing the putative miR-148a-3p–binding site in the 3’-UTR of human CD274 (antisense: 5’-tcctagtGTCCTATTTGCTCTTGGG Caagcttg; sense: 5’-caagcttcGCCCCAGCAGCTGATAAGGAAACactaggag-3’) were synthesized (PD-L1 wild-type), and complementary 38-bp DNA oligonucleotides containing mutant miR-148a-3p–binding site in the 3’-UTR (CCACTG to GCAGAC) of CD274 (antisense: 5’-tcctagtGTCCTATTTGCTCTTGGG Caagcttg; sense: 5’-caagcttcGCCCCAGCAGCTGATAAGGAAACactaggag-3’) were also synthesized as mutant controls (PD-L1 mut). HindIII and SpeI restriction sites were inserted at both ends of the oligonucleotides. The sense and antisense strands were annealed by adding 1,000 ng of each oligonucleotide to 50 μL of 1× NEBuffer 4 (NEB) at 90°C for 10 minutes and then at 37°C for 1 hour, and then digested with HindIII and SpeI. The annealed oligonucleotides were ligated into the pMIR-REPORT Luciferase Vector (Ambion). A total of 300 ng of each reporters were cotransfected with 30 nmol/L of mir-148a-3p mimic or negative control into HCT116 cells in 24-well plates. pRL-TK (Promega Corporation) was used as an internal control. Forty-eight hours after transfection, dual-luciferase activity was assessed with the Dual-Luciferase Reporter Assay System (Promega). Luciferase activity was normalized to internal control according to the manufacturer’s specification.

IFNγ Treatment

Twenty-four hours before treatment, tumor cells were transiently transfected with 30 nmol/L of mirVana miR-148a-3p mimic or negative control. Then, cells were treated with 10 ng/mL of IFNγ (Recombinant Human IFNγ; R&D Systems) (31). Cells were harvested 48 hours after treatment initiation and used for each experiment.

RNA Extraction

Total RNA from cultured cells was isolated using TRIzol Reagent (Thermo Fisher Scientific) according to the manufacturer’s instruction. For isolation of total RNA from tissue samples, 72 FFPE tumor specimens were obtained, as described above, including eight dMMR/PD-L1–positive, 16 pMMR/PD-L1–positive, and 48 pMMR/PD-L1–negative patients. Unstained FFPE blocks were marked for carcinoma area and each marked area was macro-dissected and selectively sliced into 5–10-μm sections for RNA isolation. RecoverAll Total Nucleic Acid Isolation Kit (Thermo Fisher Scientific) was used following the manufacturer’s protocol. Isolated total RNA was quantified by Nanodrop.

qRT-PCR

For the expression of PD-L1, 1 μg of total RNA was reverse transcribed to cDNA using the SuperScript III First-Strand Synthesis System (Thermo Fisher Scientific) and qRT-PCR was conducted using TaqMan assays with TaqMan Gene Expression Master Mix (Thermo Fisher Scientific) on the 7500 real time PCR system in triplicate. Relative expression levels were determined with SDS software by the 2-ΔΔCt method as described by the manufacturer, with ACTB used as the calibrator gene. The expression levels of mature miRNAs were analyzed using TaqMan microRNA reverse transcription kit according to the manufacturer’s instruction. Briefly, 40 ng of total RNA was reverse transcribed using miRNA specific RT primers and PCR was performed using TaqMan microRNA assays with the 7500 real time PCR system in triplicate. We used small-nuclear RNAs, including RNU66 (for colorectal cancer cell lines) or RNU48 (for patient’s samples), as endogenous normalization controls. Relative expression levels were determined by the 2-ΔΔCt method. All TaqMan probes were purchased from Thermo Fisher Scientific; CD274 (HS0125391_m1), ACTB (Hs99999903_m1), hsa-miR148a-3p (ID: 000470), RNU66 (ID: 001002), and RNU48 (ID: 001006).

Western blotting

To extract total protein, cells were lysed in RIPA Lysis Buffer (Thermo Fisher Scientific) supplemented with Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific). The concentration of the protein lysates was measured and then the lysates were boiled in Tris-Glycine SDS Sample Buffer (Thermo Fisher Scientific). Equal amount of protein was separated by 4%–20% Tris-Glycine Gel (Thermo Fisher Scientific) and then transferred onto polyvinylidene difluoride membrane using the iBlot2 Dry Blotting System (Thermo Fisher Scientific). The membrane was blocked with 5% nonfat dried skimmed milk powder (Cell Signaling Technology) and incubated with HRP-conjugated rabbit anti-PD-L1 antibody [catalog no. 51296, PD-L1 (E1L3N) XP, 1:1000; Cell Signaling Technology; refs 17, 18, 29] or primary mouse anti-β-actin (catalog no. SC-69789, 1:2,000; Santa Cruz Biotechnology). Then the membrane was incubated with goat anti-mouse or anti-rabbit HRP secondary antibody (Santa Cruz Biotechnology) and protein signals were developed with the SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) using LAS4000 Imager (GE Healthcare; ref. 29).

Flow cytometry and detection of apoptosis

For the analysis of cell surface PD-L1 expression, cultured cells were harvested and resuspended in ice-cold PBS with 1% FBS, and then cell suspensions were incubated with PE-conjugated anti-human CD274 (PD-L1, B7-H1) mAb (M11H1; 12-5983, 1:40; eBioscience; refs. 17, 29, 31) for 1 hour on ice in the dark. For detection of apoptotic cells, we used Annexin V-PE/7-Amino-Actinomycin D (7-AAD) Apoptosis Detection Kit (BD Bioscience) according to the manufacturer’s specification. Annexin V and
7-AAD staining was measured by FACS Canto II (Becton Dickinson Bioscience) and data were analyzed with FlowJo Software (TOMY Digital Biology).

**Cell proliferation and colony formation assays**

To evaluate cell proliferation, WST-cell proliferation assay using Cell Counting Kit-8 (CCK-8, Dojindo) was performed according to the manufacturer’s specification. Briefly, HCT116 cells transfected with miR-148a-3p mimics or negative controls were harvested and plated at 2,000 cells in 100 μL media per well in 96-well plates. After 24, 48, 72, and 96 hours of incubation in complete medium, the cells were treated with 10 μL of the CCK-8 reagent and incubated at 37°C for 2 hours, and then the absorbance at 450 nm was measured by a microplate reader.

For the colony formation assay, transfected cells were plated at 500 cells in 6-well plates and cultured for 10 days. At the end of 10 days, the cells were fixed with fixation solution (87.5% methanol, 12.5% acetic acid) for 5 minutes and stained with 0.5% crystal violet. Colonies containing more than 50 cells were counted.

**Coculture experiments**

Peripheral blood mononuclear cells (PBMC) were purified from the fresh blood of healthy donors by using Lymphoprep (Stemcell Technologies). PBMCs were stimulated with 200 IU/mL of human IL2 (Sigma-Aldrich) in AIM-V Medium (Thermo Fisher Scientific) for more than 7 days, in which PD-1 expression on T cells can be forced (29, 32). IL2-stimulated cells were cocultured with tumor cells treated with IFNγ and miR-148a-3p mimic at a ratio in 24-well plates. After 48 hours incubation, the population of apoptotic CD3+ cells and T cells were analyzed with Annexin V-PE/7-AAD apoptosis detection kit using flow cytometry (29).

**Statistical analysis**

Fisher exact test or χ² test was used to assess associations between categorical variables. Comparison between gene or miRNA values across groups was assessed using Mann–Whitney U test, or Kruskal–Wallis test with Dunn post hoc test, as appropriate. All statistical analyses were conducted using GraphPad Prism v6.0 (GraphPad Software). All P-values were two-tailed, and P less than 0.05 were considered statistically significant.

**Results**

**Identification of miR-148a-3p as a putative regulator of PD-L1**

To explore the miRNA-mediated regulatory mechanisms of PD-L1 in colorectal cancer, we conducted an integrative analysis utilizing the TCGA-COAD dataset of comprehensive mRNA/miRNA-sequencing combined with MMR status (n = 260; ref. 2). This approach was based on the principle that specific miRNAs with altered expression should be related to different tumor phenotypes (MMR status) and be correlated inversely with the expression of their functional target genes (PD-L1). In the TCGA dataset, PD-L1 (encoded by CD274 gene) mRNA levels were significantly upregulated in dMMR tumors compared with pMMR tumors, as depicted in Fig. 1A and reported in recent studies (33, 34). Of 47 miRNA probes showing significant negative correlation with PD-L1 expression (P < 0.0001; Supplementary Table S1), 19 mature miRNAs were found to be significantly decreased in dMMR tumors (P < 0.05), as demonstrated in Fig. 1A. We then tested those 19 miRNAs using a panel of eight different sequence-based miRNA target prediction algorithms, leading to the identification of miR-148a-3p being common to seven of eight searches (Fig. 1B). Those algorithms consistently demonstrated a putative miR-148a-3p–binding site at position 133–138 (GCAACUG) in the 3’-UTR of PD-L1 mRNA (Fig. 1C). We also analyzed miR-200b and miR-429, because the miR-200 family miRNAs have recently been shown to regulate PD-L1 expression by directly targeting its 3’-UTR (21). We found that miR-200b and miR-429 were each predicted to target PD-L1 by five of eight programs, and that their expression levels were inversely correlated with PD-L1, irrespective of MMR status (Fig. 1A and B), confirming the validity of our computational screening.

**Association between miR-148a-3p, PD-L1, and MMR status**

According to the recent TCGA analysis by Ock and colleagues (33), we also attempted to divide TCGA tumors into PD-L1High or PD-L1Low subgroups based on the median CD274 mRNA expression, showing that PD-L1High tumors had significantly lower levels of miR-148a-3p, than those of PD-L1Low (P < 0.0001; Fig. 2A). As compared with tumors with pMMR/ PD-L1Low, miR-148a-3p expression was significantly decreased in pMMR/PD-L1High tumors (P < 0.05) as well as in dMMR/PD-L1High tumors (P < 0.01), and it appears that miR-148a-3p down-regulation was found predominantly in dMMR/PD-L1High tumors (Fig. 2A). Furthermore, the inverse relationship between PD-L1 and miR-148a-3p expression was confirmed in an additional mRNA/miRNA microarray dataset of pMMR colorectal cancer by Low and colleagues (24), in which miR-148a-3p was significantly decreased in PD-L1High tumors (P = 0.0073; Fig. 2B). To further address the association between the expression of miR-148a-3p, PD-L1, and MMR status, we next evaluated PD-L1 protein expression in 395 samples of resected colorectal cancer specimens by IHC in the FMU cohort (Fig. 2C–F). As demonstrated in Fig. 2C and D, membranous PD-L1 staining in tumor cells with or without cytoplasmic staining was examined. We found that 23 of 395 tumors (5.8%) were considered positive for tumor cell PD-L1 expression. As shown in Supplementary Table S2, the expression of PD-L1 showed no significant association with clinicopathologic parameters, such as age, gender, tumor location, histological type, or TNM staging. In contrast, tumors with positive PD-L1 staining were significantly enriched in dMMR (P < 0.0001; Fig. 2E; Supplementary Table S2), which is consistent with previous IHC studies on colorectal cancer (25, 26, 28). We also measured the expression levels of miR-148a-3p by qRT-PCR using 72 tumor RNA samples isolated from FFPE tissues. We found a trend toward decreased miR148a-3p expression in PD-L1 positive and/or dMMR tumors, although this did not reach statistical significance (Fig. 2F).

**miR-148a-3p as a tumor suppressor in colorectal cancer**

Recent reports have suggested that miR-148a-3p can work as a tumor suppressive miRNA that is frequently downregulated in colorectal cancer and other malignancies (35–37). To confirm the tumor suppressive function of miR-148a-3p in vitro, we used exogenous overexpression by transfecting miR-148a-3p mimic using a colorectal cancer cell line, HCT116 (Fig. 3A). Indeed, forced miR-148a-3p expression exhibited significant growth suppressive effects on HCT116 cells analyzed by cell proliferation (Fig. 3B) and colony formation assays (Fig. 3C).
Tumor cell PD-L1 was directly regulated by miR-148a-3p

Given that PD-L1 was predicted to be a potential target of miR-148a-3p and that miR-148-3p was negatively correlated with PD-L1 expression, we next evaluated whether miR-148a-3p directly interacts with the 3'UTR of PD-L1 mRNA. Luciferase reporter assays were performed using reporter plasmids containing either wild-type PD-L1 3'UTR or mutant PD-L1 3'UTR. As shown in Fig. 4A, transfection of wild-type PD-L1 3'UTR luciferase reporter construct together with miR-148a-3p mimic into HCT116 cells significantly inhibited the luciferase reporter activity compared with that of negative control, while this effect was reversed when the predicted 3'UTR-binding site was mutated. This result demonstrated that PD-L1 is a direct target of miR-148a-3p. We then assessed the effect of miR-148a on the expression of PD-L1 using two colorectal cancer cell lines, including a dMMR cell line HCT116 and a pMMR cell line SW837 (Fig. 4B). In both cell lines, transfection of miR-148a-3p mimic reduced PD-L1 mRNA levels measured by qRT-PCR, by approximately 59% in HCT116 cells, and 38% in SW837 cells (Fig. 4C). Moreover, overexpressed miR-148a-3p also resulted in a decrease of PD-L1 protein.
expression by Western blot analysis of whole-cell lysate (Fig. 4D), and in cell surface levels by flow cytometry (Fig. 4E and F). On the other hand, although transfection of miR-148a-3p inhibitor could decrease miR-148a-3p levels evaluated by qRT-PCR in both cell lines (Supplementary Fig. S1A), no significant change of PD-L1 expression was observed in mRNA or protein levels by qRT-PCR, Western blotting, and flow cytometry (Supplementary Fig. S1B–S1E).

miR-148a-3p reduced IFNγ-induced PD-L1 expression

It is well accepted that cell surface PD-L1 expression in tumor cells are mainly regulated by the cytokine IFNγ secreted by immune cells in the tumor microenvironment (10–13). To confirm the effect of IFNγ on PD-L1 expression, HCT116 and SW837 cells were treated with IFNγ at different doses and different exposure intervals, and then PD-L1 expression on tumor cells was examined by flow cytometry. As shown in Fig. 5A and B, the stimulation effect of IFNγ on cell surface PD-L1 expression in HCT116 and SW837 was demonstrated in a dose-dependent manner, and IFNγ treatment for 48 hours at 10 ng/mL was used for further experiments. Western blotting analysis further confirmed the upregulation of PD-L1 protein expression in whole-cell lysate under 10 ng/mL of IFNγ treatment in HCT116 cells (Fig. 5C). We next sought to determine the effect of miR-148a-3p on IFNγ-induced PD-L1 expression. We transfected HCT116 cells with miR-148a-3p mimic or negative control for 24 hours before IFNγ treatment. As demonstrated in Fig. 5C, the expression of PD-L1 protein stimulated by IFNγ treatment was diminished in
Tumor suppressive functions of miR-148a-3p in HCT116 cells. HCT116 cells were transfected with miR148a-3p mimic or negative control, and used for WST-cell proliferation and colony formation assays. A, Transfection of miR-148a-3p mimic increased the expression levels of miR-148a-3p analyzed by qRT-PCR. Forced expression of miR-148a-3p in HCT116 cells resulted in decreased cell proliferation at different time points (B), and reduced colony formation (C), compared with negative control. Data represent the mean ± SD from three independent experiments (*, P < 0.05; **, P < 0.01).

PD-L1 expression regulated by miR-148a-3p. A, Luciferase reporter activity was analyzed 48 hours after cotransfection of miR-148a-3p mimic or negative control and wild-type (wt) PD-L1 3′-UTR luciferase reporter construct or mutant (mut) construct into HCT116 cells. The relative firefly luciferase activity was normalized to Renilla luciferase, and data represent the mean ± SD from three independent experiments. **, P < 0.01. B, Transfection of miR-148a-3p mimic or negative control into HCT116 and SW837 cell lines. The expression levels of miR-148a-3p were analyzed by qRT-PCR. **, P < 0.01. HCT116 and SW837 cells were transfected with miR-148a-3p mimic or negative control. After 48 hours of incubation, cells were lysed and analyzed for the expression of PD-L1 in mRNA levels and in whole-cell protein levels by qRT-PCR (C) and Western blotting (D), respectively. **, P < 0.01. PD-L1 expression on cancer cell surface was analyzed by flow cytometry in HCT116 and SW837 cells 48 hours after transfection of miR-148a-3p mimic or negative control (E). Representative histograms of PD-L1 are shown (F), and data are expressed as the mean ± SD from three independent experiments (**, P < 0.01).
Figure 5.
miR-148a-3p reduced IFNγ-induced PD-L1 in tumor cells and diminished T-cell apoptosis. A, Dose-dependent escalation of cell surface PD-L1 levels in HCT116 cells following IFNγ stimulation. HCT116 cells were exposed to various doses of IFNγ (0, 0.5, 1.0, 10, and 50 ng/mL) for 24, 48, or 72 hours followed by flow cytometry for PD-L1. B, HCT116 and SW837 cells were exposed to 10 ng/mL of IFNγ for 24, 48, or 72 hours. C, HCT116 cells were treated with 0 (control) or 10 ng/mL of IFNγ for 48 hours, or HCT116 cells were transfected with negative control (NC) or miR-148a-3p mimic for 24 hours and then treated with 10 ng/mL of IFNγ for 48 hours. PD-L1 protein expression in whole-cell lysate was analyzed by Western blotting. HCT116 and SW837 cells were transfected with miR-148a-3p mimic or negative control for 24 hours and then exposed to 10 ng/mL of IFNγ for 48 hours, and cell surface PD-L1 expression was analyzed by flow cytometry. Representative flow cytometric histograms of PD-L1 are shown (D), and data are expressed as the mean ± SD from three independent experiments (E). **, P < 0.01. HCT116 cells transfected with miR-148a-3p or negative control were exposed to IFNγ for 48 hours, and then cocultured with IL2-activated T cells for 48 hours followed by flow cytometry. CD3+ cells were gated and analyzed for the proportion of apoptotic T cells determined by Annexin V/7-AAD staining. The proportion of apoptotic T cells was considered early apoptotic (Annexin V+, 7-AAD−) and late apoptotic/dead (Annexin V+, 7-AAD+) cells. Representative flow cytometric plots of apoptotic T cells are shown (F). The proportion of CD3-gated apoptotic cells for T-cells alone culture, or T cells cocultured with HCT116 cells transfected with miR-148a-3p mimic or negative control, are shown (G), and data represent the mean ± SD from three independent experiments (**, P < 0.01).
miR-148a-3p overexpression in tumor cells diminished T-cell apoptosis

Because miR-148a-3p could decrease PD-L1 expression on tumor cell surface, we speculated that miR-148a-3p may not only function as tumor suppressor, but also modulate immune response by inhibiting PD-L1 expressed on tumor cells. As the PD-1/PD-L1 signaling pathway is known to inhibit T-cell antitumor immune responses and lead T cells to apoptosis (10), we investigated whether decreased PD-L1 expression on tumor cell by miR-148a-3p functionally reduce T-cell apoptosis using a coculture model of cancer cells and T cells. We harvested IL-2-activated T cells expressing PD-1 (29, 32), and they were then cocultured with cancer cells transfected with miR-148a-3p mimic or negative control under treatment of IFNγ. After 48 hours of incubation, apoptotic T cells were measured by Annexin V/7-AAD staining in CD3-gated population. As shown in Fig. 5F–G, we found a significant decrease particularly in the percentage of late apoptotic or dead T cells (Annexin V+/7-AAD+ +) when T cells were cocultured with HCT116 cells overexpressing miR-148a-3p (18.3% ± 0.3%) in comparison with that of negative control (25.4% ± 2.5%). A similar trend was observed in the analysis of T cells cocultured with SW837 cells overexpressing miR-148a-3p (Supplementary Fig. S2A and S2B).

Discussion

The regulation of PD-L1 in cancer has been extensively investigated. The expression of PD-L1 gene is induced primarily by transcriptional mechanisms through several distinct pathways (12, 14, 15), while posttranslational regulation of PD-L1 protein has been demonstrated, in which PD-L1 protein stability can be modulated by glycosylation and ubiquitination (16–18).

Aside from these mechanisms, recent studies focused on posttranscriptional regulation of PD-L1 transcript targeted by miRNAs, including miR-200 and miR-34 in NSCLC (21, 22), miR-424 (322) in ovarian cancer (38), and miR-152 in gastric cancer (39). Although one study reported that miR-138a-5p may target PD-L1 in colorectal cancer (40), miRNA-mediated PD-L1 expression and function in colorectal cancer remains largely unclear. This study investigated potential miRNAs that were negatively correlated with PD-L1 expression and downregulated in MSI-H tumors using comprehensive miRNA-sequence data followed by computational target prediction algorithms. This led us to the identification of tumor suppressive miRNA, miR-148a-3p as a negative regulator of PD-L1 in colorectal cancer for the first time with our knowledge. Using colorectal cancer cell lines, we found that overexpression of miR-148a-3p reversed IFNγ-induced PD-L1 expression in tumor cell surface that functionally correlated with diminished T-cell apoptosis in the tumor microenvironment. We thus propose a novel mechanism by which tumor immune evasion is regulated at least in part by miR-148a-3p/PD-L1 axis in colorectal cancer.

Consistent with our finding, tumor suppressive functions of miR-148a-3p have been demonstrated in many cancer types (35, 37), including colorectal cancer (36, 41). Indeed, miR-148a-3p has been reported to be downregulated in tumors, compared with their normal counterpart, which often correlated with advanced stage, where miR-148a-3p silencing was caused by DNA hypermethylation of its promoter (35, 37, 42). Decreased levels of miR-148a-3p were associated with poor clinical outcomes in a variety of cancers (43–45). Likewise, three independent studies of colorectal cancer reproducibly showed that low expression of miR-148a-3p was significantly associated with poor disease-free or cancer-specific survival in stage II and III patients (36, 41, 46). Therefore, it is clear that miR-148a-3p downregulation due to epigenetic mechanisms, can contribute to colorectal cancer progression and may serve as a prognostic biomarker for patients with stage II and III colorectal cancer. However, the involvement of miR-148a-3p in the tumor immune system has been so far unknown. Here we provide novel evidence that miR-148a-3p plays an important role in the immunosuppressive tumor microenvironment by directly targeting PD-L1, particularly in dMMR tumors. Collectively, methylated and downregulated miR-148a-3p in tumor cells is likely to contribute not only to the tumor progression but also to the increase of tumor cell surface PD-L1 expression that can consequently help tumor cells escape from adaptive immunity.

Patients with positive PD-L1 expression in tumor cells by IHC have exhibited trends toward increased rate of responses to anti-PD-1/PD-L1 therapies across various clinical trials (47, 48). PD-L1–negative expression, however, does not imply a lack of response and those patients can still achieve clinical benefit with PD-1/PD-L1 blockade. As predictive values of PD-L1 IHC alone are insufficient for patient selection, studies are being actively conducted to develop more effective, predictive biomarkers for the PD-1/PD-L1 immunotherapy. Recently, patients with dMMR/MSI-H colorectal cancer have emerged as a distinct biomaker-defined population who could benefit from PD-1 blockade (8, 9). Despite frequent PD-L1 expression in dMMR/MSI-H colorectal cancer, not all of these patients responded to the anti-PD-1 antibody nivolumab with approximately 31% of objective response rate and 69% of disease control rate, where PD-L1 IHC was not predictive of response (9). Conversely, a small subset of pMMR/MSS colorectal cancer with or without PD-L1 expression may still benefit from immunotherapy, although it is a more difficult challenge. In view of that, combination of multiple approaches to capture the immune status of colorectal cancer is considered more effective as predictive biomarkers for the response to anti-PD-1/PD-L1 therapies (48). Thus, multiple strategies, such as MMR/MSI testing, PD-L1 IHC, TIL density, neoantigens load, and immune gene signatures, combined with miR-148a-3p measurement described here may provide optimal characterization of the immune tumor microenvironment for precision cancer immunotherapy in colorectal cancer.

miRNA-based therapeutics are currently being evaluated in phase I and phase II clinical trials as new approaches for the treatment of malignancies and other diseases (49). In the treatment of cancer, therapeutic delivery of the tumor suppressive miRNA, miR-34, in which a miR-34 mimic encapsulated in lipid nanoparticles (MRX34), is the most advanced therapeutics tested in a phase I trial (ClinicalTrials.gov: NCT01829971; ref. 49). Intriguingly, as miR-34 can directly target PD-L1 in NSCLC cell lines, the in vivo delivery of miR-34 via MRX34-repressed tumor PD-L1 expression in a mouse model of NSCLC. MRX34 treatment also promoted CD8+ TILs and reduced the number of exhausted CD8+PD1+ T cells in the tumor microenvironment, indicating
that miR-334 had direct effects on immune evasion (22). Because miR-148a-3p can work as a tumor suppressor and also restore T-cell function by inhibiting PD-L1, further studies are needed to investigate the possibility of miR-148a-3p replacement strategy as immunotherapy for colorectal cancer.

One limitation of this study is the lack of in vivo assays, although our in vitro data are convincing. The role of miR-148a-3p in regulating PD-L1 expression would be more important in the in vivo setting, where various cellular types under diverse immune conditions are interactively involved in the immunosuppressive tumor microenvironment. Therefore, we suggest that in vivo functional experiments using a syngeneic mouse model would be interesting future directions.

In conclusion, this study demonstrated for the first time that downregulated miR-148a-3p expression was found particularly in dMMR colorectal cancer, correlating with higher levels of PD-L1 expression. Our findings suggest that miR-148a-3p suppresses IFNγ-induced PD-L1 expressed on tumor cell surface and consequently restores T-cell viability in the tumor microenvironment. Our findings provide novel evidence that miR-148a-3p regulates immune evasion via PD-L1 and may guide development of novel cancer biomarkers, as well as therapeutic interventions for colorectal cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References
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miRNA-148a-3p Regulates Immunosuppression in DNA Mismatch Repair–Deficient Colorectal Cancer by Targeting PD-L1

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