Exosome-delivered miR-15a/16 target PD-L1 to inhibit immune escape in gastric cancer

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Abstract

Background: The programmed death-1 (PD-1)/PD-ligand 1 (PD-L1) pathway plays crucial role in various types of cancer. However, the underlying mechanism of PD-L1 expression dysregulation in GC has not been revealed. High expression of PD-L1 and low expression of miR-15a/16 often leads to poor prognosis of gastric cancer, therefore PD-L1 was regarded as a key target for the treatment of gastric cancer. This study aims to determine whether exosomes with miR-15a/16 could target PD-L1 and inhibit immune escape in gastric cancer (GC).

Methods: The protein expression levels of PD-L1 in tumor tissues and normal tissues of patients were evaluated by Western blot (WB) and immunohistochemistry (IHC), then confirmed that PD-L1 was a target of miR-15a/16 by luciferase reporter assay. HEK293T cells were transfected with miR-15a/16, exosomes were isolated by sequential differential centrifugation then cultured with gastric cancer cell lines, the protein and RNA levels of the PD-L1 were determined by western blotting and real-time qPCR. A mouse xenograft model was adopted to assess the association between exosome loaded miR-15a/16 and tumor growth in vivo. Flow cytometry was performed to analysis the effect of exo-miR-15a/16 on CD8^{+}T lymphocytes and INFγ^{+} of mice.

Results: We found that compared to adjacent noncancerous tissues, PD-L1 protein expression was significantly increased while miR-15a/16 expression level was significantly inhibited in tumor tissues. In addition, we found that miR-15a/16 mimics could downregulate PD-L1 expression, while miR-15a/16 inhibitors enhanced PD-L1 expression in human gastric cancer cell line SGC7901. Exo-miR-15a/16 could target PD-L1 and inhibit immune escape in gastric cancer.

Conclusions: Our study confirmed that miR-15a/16 in exosomes could suppress tumor growth and inhibit immune escape by targeting PD-L1 in vivo and in vitro, which might serve as a potential biomarker in monitoring the activity of gastric cancer.

Background

Gastric cancer is the fifth most common cancer around the globe and the third leading cause of cancer-related mortality, accounts for over 750,000 deaths per year[1].

Surgical resection, the main treatment for patients with gastric cancer currently, merely with a 5-year survival rate of approximately 20%-25%[2]. There for, chemotherapy and radiotherapy serve as main additional treatments associated with surgical resection. Nevertheless, the survival effect of them is not satisfactory, with a 5-year overall survival rate of only 10%-15%[3]. The median overall survival time for advanced gastric cancer patients with conventional chemotherapy is merely 9-11 months[4,5]. In recent years, targeted therapy and immunotherapy has attracted more and more attention and make non-negligible achievements.

In the past decade, immunotherapy made dramatic achievements in treatment of cancer, especially immune checkpoint inhibitors. They target molecules that regulate T cells which are soldiers of the immune system rather than targeting tumor cells. The goal of this therapy is to eliminate inhibition pathways which prevent effective anti-tumor T cell responses[6]. The programmed death-1 (PD-1)/PD-ligand 1 (PD-L1) pathway is of the most representative one, PD-L1 expressed on tumor cells binds to PD-1 on activated T cells of tumors and produces a signal that inhibits T cell activation, resulting in inhibition of T cell-induced immune responses and failure to destroy tumor cells[7,8]. Studies have demonstrated that treatment can enhance the host's anti-cancer immune response by inducing antibody-mediated blockage of the PD-1/PD-L1 pathway and then inhibiting the checkpoint[9]. PD-1/PD-L1 pathway was approved for the treatment of different tumors by FDA, including melanoma, renal cell carcinoma, non-small cell lung cancer (NSCLC), squamous cell carcinoma of the head and neck, Hodgkin's lymphoma and Merkel cell carcinoma[9-11]. The disease-free survival (DFS) and overall survival (OS) of PD-L1-positive patients were often poorer than those PD-L1-negative patients in a variety kinds of cancer[12] and studies have indicated a possible association between PD-L1 polymorphisms and the development of gastric cancer[9]. The upregulation of PD-L1 is found in a variety of gastrointestinal cancers, including pancreatic, colorectal and gastric cancer, and is associated with poor prognosis[13].

miRNAs are endogenous single-stranded non-coding RNAs of approximately 21- to 22-nt in length and regulate gene expression at the post-transcriptional level which play an important regulatory role by targeting mRNA[14,15]. The biogenesis of miRNAs is tightly controlled, and miRNAs dysregulation is associated with various biological processes involving tissue development, immune responses, metabolic diseases and tumorigenesis[14,16]. Current reports suggest that miR-105 secreted by metastatic breast cancer cells promotes cell migration by targeting the tight junction protein ZO-1[17]. Secreted miR-150 promotes migration of targeted endothelial cell[18]. The miR-15a/16 gene cluster is located at human chromosome region 13q14 and are dysregulated in a variety of malignancies including differentiation and maturation[19,20], in particular, they are abnormally expressed in hepatocellular carcinoma[21], Chronic lymphocytic leukemia[19], gastric cancer[22], prostate cancer[23] compared with healthy tissues.

Exosomes are vesicles secreted by mammalian cell types with a diameter of 40-100nm[24]. Exosomes could contain transmembrane proteins, noncoding RNAs and DNA derived from donor cell and serve as a delivery of intercellular communication[25,26]. With advantages of non-toxic and higher biocompatibility than liposomes, exosome recently serve as a new carrier to deliver a variety of substances for treatment[27]. Recent studies have shown that they can act as biomarkers for diseases or as deliveries for drugs[28,29]. In addition, studies have shown that exosomes could deliver miRNAs and play an important role of therapy in cancer. Exosomes deliver MTA1 to regulate hypoxia and estrogen signaling in breast cancer cells[30]. Exosomes from tumor microenvironment contribute to cancer cell metabolism[31]. Exosome transmitted miR-128-3p to increase chemosensitivity in colorectal cancer. Studies have shown that exosomes could deliver a serious of biological substance including miRNAs to regulate tumor growth.

Materials And Methods

Human Tissue

GC tissues and paired noncancerous tissues were obtained from patients undergoing surgery in Tianjin Medical University Cancer Institute and Hospital (Tianjin, China). Both tumor tissues and noncancerous tissues were histologically confirmed. The pathological type of cancer tissues was confirmed as adenocarcinoma. Written consent was provided by all of the patients, and all aspects were approved by the Ethics Committee of Tianjin Medical University Cancer Institute and Hospital. Tissues were immediately frozen in liquid nitrogen after surgery and stored at -80°C.

Tumor Models

Our experimental procedures were approved by Institutional Animal Care and Research Advisory Committee of Tianjin Medical University. Female mice (BALB/c, 4 weeks) were purchased from Model Animal Center of Nanjing University. barrier facilities on a high efficiency particulate air (HEPA) filter rack. Feeding in the middle. $3×10^6$ MFC cells were injected to the subcutaneous region of the left groin area of each mouse, 5 mice in each group. On the 14th day, a tumor model was established subcutaneously in nude mice (n = 15), when the tumor grows to a diameter of about 8 mm, 20ug of different exosomes (suspended in 40ul PBS) or PBS are injected through the tail vein every 2 days, and 5 mg/kg DDP is injected intraperitoneally every 4 days (Figure 6A). The long and short diameters of tumors were recorded every other day and then calculated tumor volume.

Cell Culture

SGC7901(human gastric adenocarcinoma cell line) and HEK293T (human embryonic kidney epithelial cell line) were acquired from the Chinese Academy of Sciences Cell Bank in Shanghai, China. The mycoplasma contamination was excluded. Cells were incubated in a humidified incubator (95% air and 5% CO2 at 37°C). DMEM (GIBCO) containing 10% fetal bovine serum (GIBCO) and 1% penicillin/streptomycin (Solarbio, China). Six pairs of gastric cancer tissues and adjacent normal tissues were collected from patients at the Tumor Research Institute and Hospital of Tianjin Medical University (Tianjin, China). Specimens of this study were approved by the Ethics Committee of Tianjin Medical University Cancer Institute and Hospital and the informed consent of patients was provided.

Isolation of Exosomes from Cell Culture Medium

After transfection of HEK293T cells for 48h, the cell culture medium was centrifuged at 3,000g for 20 minutes to remove cell debris, the supernatant was then centrifuged at 10,000g for 20 minutes to remove larger vesicles. Finally, centrifuged the supernatant at 110,000g for 80 minutes, discarded supernatant and added 100ul PBS in it, gently pipetting several times and filtered by a 0.2mm filter to obtain the exosome suspension. All steps were performed at 4 °C.

Transmission Electron Microscopy

Exosomes were immersed in a droplet of 2.5% glutaraldehyde and fixed at 4°C for more than 12hrs. Samples were washed in PBS for 3 times and fixed in 1% osmium tetroxide for 1 hour (RT), embedded in 10% gelatin and fixed in glutaraldehyde at 4°C and then cut into small pieces (<1 mm^3). Dehydrated specimens with increasing concentrations of alcohol (30%, 50%, 70%, 90%, 95%, and 100%; ×3), replace the pure alcohol with propylene oxide and then infiltrate the samples in increasing concentrations (25%, 50%, 75%, and 100%) of Quetol-812 epoxy resin mixed with propylene oxide. Then, specimens were embedded in pure Quetol-812 epoxy resin. Ultrathin cyttings (100 nm) were cut with a Leica UC6 ultramicrotome, followed by stained with uranyl acetate (10min) and lead citrate (5 min) at RT before observation via FEI Tecnai T20 transmission electron microscope (120 kV).

Cell Transfection

SGC7901 was seeded in 6-well plates, HEK293T was in 10cm dishes, transfected with Lipofectamine 2000 (Invitrogen) and Opti-MEM (GIBCO) according to manufacturer's instructions. For miRNA upregulation and downregulation, a 100-pmol dose of miR-15a/16 mimics, inhibitors, and NC was transfected, then cell culture medium was replaced with complete medium 4-6hrs after transfection.

Immunohistochemistry assay

Paraffin-embedded tissue samples (8 pairs, total 16 samples) of gastric cancer tissues and paired adjacent noncancerous tissues were sliced and stained with a 1:50 dilution of anti-PD-L1 monoclonal antibody (Santa Cruz Biotechnology). Positive staining was identified using the DAB system (Zhongshan Jinqiao, China). Five regions were randomly selected for each sample.

Protein Extraction and Western Blot

The expression of PD-L1was assessed by Western blot and normalized to β-actin. Cells and tissues were lysed in RIPA buffer with freshly added Protease inhibitor cocktail. The pyrolysis product was separated on SDS-PAGE Gel and then transferred onto polyvinylidene fluoride Membrane (Millpore). Immunoblots were blocked with 2% BSA for 0.5h at RT, then incubated with anti-PD-L1 (1:150, Santa Cruz Biotechnology) and anti-GAPDH (1:3000, Abcam) antibodies at 4 °C for more than 12hrs. After incubation with corresponded secondary antibody, membranes were visualized by enhanced chemiluminescence system Kit (Millipore, USA).

RNA Isolation and qRT-PCR

Total RNA from cultured cells and tissues was extracted with TRIzol reagent (Invitrogen). cDNA was obtained via avian myelobastosis virus (AMV) reverse transcriptase (TaKaRa) and the reaction process was as follows: 30 min at 16°C, 30 min at 42°C and 5 min at 85°C. Real-time qPCR was then initiated at 95°C for 5min; the cDNA was denatured at 95°C for 15s and then extended at 60°C for 1 min, which was performed for 40 cycles. All the reactions assayed in triplicate. The cycle threshold (Ct) data was

calculated according to the formula of $\triangle$Ct = Ct gene _ Ct control. The relative levels of genes were normalized using the equation $2^{-\triangle Ct}$. The PD-L1 and GAPDH primers were designed as follows:

5'- GCTCCAAAGGACTTGTACGTG -3' (PD-L1, sense),

5'- TGATCTGAAGGGCAGCATTTC -3' (PD-L1, anti-sense),

5'- AGGTCGGTGTGAACGGATTTG -3' (GAPDH, sense),

5'- TGTAGACCATGTAGTTGAGGT -3' (GAPDH, anti-sense).

Flow Cytometry

Spleen T lymphocytes were isolated from wild-type BALB/c mice and then resuspended in PBS. In brief, fluorescein isothiocyanate(FITC)-conjugated monoclonal antibodies against mouse INFγ^{+} and phycoerythrin(PE)-conjugated monoclonal antibodies against mouse CD8^{+} (eBioscience, Thermofisher Scientific, San Diego, CA, USA) were incubated with isolated cells at 4 °C for 20 min. Cells were washed and purified to 99% by flow cytometry (Fig. 6A).

Statistical Analyses

All experiments were repeated in parallel at least three times and datas were expressed as mean ± SE. P < 0.05 was considered statistically significant by Student's t test: * p < 0.05; ** p < 0.01; and *** p < 0.001.

Discussion

Currently, surgery is considered to be the only radical treatment for gastric cancer. However, most patients were diagnosed at an advanced stage and missed the best surgical opportunity. The 5-year survival rate of advanced gastric cancer is no more than 10%[32,33]. Therefore, further exploration of early diagnosis and treatment are urgently needed to improve the survival of patients with gastric cancer.

Exosomes are small particles which could act as an information mediator in many biological processes. Because of its high biocompatibility and delivery efficacy, exosome was used as a specific pathway of intercellular communication and attracted more and more attention in recent years. miRNAs loaded in exosomes can be transferred to receptor cells for regulation of gene expression and play a crucial role in establishing the tumor microenvironment to promote tumorigenesis, tumor progression, migration and invasion[34].

PD-L1 is broadly expressed in tissues and can be further induced upon exposure to IFN-γ.[35] PD-L1 expression is associated with poor prognosis in many cancers, including lung, stomach, colon, breast, cervix, ovary, renal cell, bladder and liver, as well as in glioma and melanoma[36], such tumors may evade the host immune system by weaken of tumor-specific T-cell responses through PD-1/PD-L1 pathway[37]. According to the prediction, PD-L1 protein expression was positively correlated with the prognosis of GC

and the expression level of PD-L1 was negatively correlated with the expression level of miR-15a/16. From our bioinformatics analysis, we found that miR-15a/16 could target PD-L1 mRNA with a high probability of conservation in SGC7901. Reports indicate that miR-15a/16 is a critical tumor suppressor and plays a crucial role in the regulation of tumor proliferation, invasiveness, metastasis and angiogenesis[19,38,39]. Regarding the role of miR-15a/16 in tumor suppression, previous studies have shown that miR-15a/16 inhibit tumorigenesis in chronic lymphocytic leukemia[40]. miR-15a/16 inhibit tumor progression by targeting MYCN in neuroblastoma[41]. miR-15a regulate BCL2 and SOX2 and exhibit tumor suppressor properties in colorectal carcinomas[42]. In our study, we identified PD-L1 as a target of miR-15a/16 in mediating tumor suppression. We found that exosomes carrying miR-15a/16 can be transfer into SGC7901 and inhibit tumor growth by targeting PD-L1.

In the future, the level of miR-15a/16 in exosomes might be a biomarker for monitoring tumorigenesis of gastric cancer. Patients with higher miR-15a/16 expression might have better prognosis.

This finding indicates that suppressing the expression of these exosomes with overexpressed miR-15a/16 might be a novel therapeutic strategy in GC. In brief, our study provides a potential biomarker and a novel target for tumor growth.

Results

High Level of PD-L1/PD-1 Expression Is Positively Correlated with the Poor Prognosis of GC

The effect of PD-L1/PD-1 on the prognosis of GC was analyzed by the open access database Kaplan-Meier plotter (http://kmplot.com/analysis/index.php?p=service&cancer=gastric). The survival curves of GC patients associated with PD-L1/PD-1 expression are shown in Figure 1A and 1B. During the follow-up period, the survival rate of the PD-L1/PD-1 high expression group was always lower than that of the low expression group.

PD-L1 Protein Expression Is Upregulated in GC Tissues Compared to Normal Adjacent Tissues

As predicted, higher expression of PD-L1 is an adverse prognostic factor.

Then, we hypothesized that the upregulation of PD-L1 expression might be a factor that promote tumorigenesis. We analyzed the level of PD-L1 expression in gastric cancer tissues and adjacent noncancerous tissues of 6 GC patients. As detected by immunohistochemistry in Figure 1C, among those patients whom we can detect PD-L1 expression, the expression level of PD-L1 protein was dramatically upregulated in the GC tissues compared with that of normal adjacent tissues, then we verified it by western blot and got consistent results (Figure 1D▯E). The positive ratio of PD-L1 in tissues of GC patients (Figure 1F).

Identification of PD-L1 as a Direct Target of miR-15a/16

Based on our predictive bioinformatics analysis, we found that miR-15a/16 can directly target the 3'UTR of PD-L1 mRNA in a highly conserved pattern among species (Figure 2A).

miR-15a/16 Is downregulated in GC Tissues

To detect the level of miR-15a/16 expression, we performed RT-PCR to detect the number of copies of miR-15a/16 in plasma and found that copies of miR-15a/16 was significantly decreased in GC tissues compared with that in normal subjects and we found that the higher the tumor stages, the more significant the differences are (Figure 2B). In tissues of the 6 patients, we found that miR-15a/16 expression was significantly decreased in GC tissues (Figure 2C) compared with that in noncancerous tissues.

Further Validation of PD-L1 as a Direct Target of miR-15a/16

With bioinformatics tools mentioned above, we preliminarily confirmed that miR-15a/16 can directly target the 3'UTR of PD-L1 mRNA (Figure 3A). The luciferase assay showed that the relative luciferase activity was clearly inhibited when miR-15a/16 mimics were cotransfected with the luciferase reporters and was noticeably increased when it was miR-15a/16 inhibitors. However, the interaction was lost when the plasmid sequence was mutant (Figure 3B). Additionally, we detected the interrelationship between miR-15a/16 and PD-L1 in SGC7901 directly. In the mimics group, two groups of cells were transfected with miR-15a/16 mimics respectively and a group of miR-15a and miR-16 cotransfected and a NC group transfected with NC mimics. The inhibition group was transfected similarly. As shown in Figures 3C, the expression of PD-L1 protein (detected by western blotting) in each group was changed accordingly. Overexpression of miR-15a/16 led to inhibition in PD-L1 expression and the inhibition was the strongest when miR-15a/16 transfected simultaneously. While the inhibition of miR-15a/16 enhanced the expression of PD-L1 and the expression was strongest when the two was transfected at the same time (Figure 3C、D). In addition, as expected, there were no obvious changes in c-MYB mRNA expression among SGC7901 with different transfections (Figure 3E).

Exosome-delivered miR-15a/16 could sharply reduce PD-L1 expression in gastric cancer cells

Exosomes were extracted from HEK293-T cell medium and photographed by transmission electron microscopy (Figure 4A) and the relative concentration of exosome was in Figure 4B. Western blots were performed to certify that the expression of the marker proteins of exosomes were higher than exosomes from donor cells (Figure 4C). To obtain exosome with miR-15a/16, we transfected HEK293-T cells with miR-15a/16 mimics and incubated for 48hrs, then the exosomes were loaded with miR-15a/16. (Figure 4D). Subsequently, the expression level of PD-L1 was remarkably downregulated in exosomes derived from the medium of miR-15a/16-mimics-transfected cells compared with those from control exosomes and the inhibition is most pronounced in the cotransfected group (Figure 4E、F). However, the mRNA level of PD-L1 was relatively stable in SGC7901 cocultured with control exosomes or miR-15a/16 exosomes (Figure 4G).

Inhibition of exo-miR-15a/16 on tumor growth in vivo.

MFC was injected to set up tumor models in BALb/C mice subcutaneously. When tumors grew to about 8mm in diameter, 20 mg of different exosomes (suspended in 40ml PBS) or PBS were injected through the caudal vein every 2 days, (Figure 5A). The length and short diameter of tumors were recorded every other day (Figure 5C). Compared with the control group, the tumor volume sharply decreased in treatment groups, especially the group of exo-miR-15a/16 (Figure 5B). After 2 weeks, tumors were removed and weighed (Figure 5D). Extracted RNA from each tumor and detected the level of mRNA by qRT-PCR in each group of tumors. The results showed that the level of PD-L1 miRNA in the experiment groups was significantly lower than that in control group (Figure 5H), while there was no change in mRNA level of PD-L1 (Figure 5G). Then verified by western blot, the expression level of PD-L1 was also sharply decreased in Exo-15a/16 groups (Figure 5E▯F). These results demonstrate that Exo-miR-15a/16 could downregulate the expression of PD-L1 and inhibit

Exo-miR-15a/16 target PD-L1 to inhibit immune escape in gastric cancer.

fluorescein isothiocyanate(FITC)-conjugated monoclonal antibodies against mouse INFγ and phycoerythrin(PE)-conjugated monoclonal antibodies against mouse CD8 (eBioscience, Thermofisher Scientific, San Diego, CA, USA) were incubated with isolated cells at 4°C for 20min. The number of CD8$^+$T cells and INFγ$^+$ T cells were significantly higher in exo-miR-15a/16 groups than that in control group (Figure 6A▯B). And the survival rate of mice in groups of exo-miR-15a/16 is higher than mice in control group (Figure 6C).

Declarations

Ethics declarations

Ethics approval and consent to participate

The current research was ratified by the Ethics Committee of Tianjin Tumor Hospital.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no Competing interest.

Funding

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors. /All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. /All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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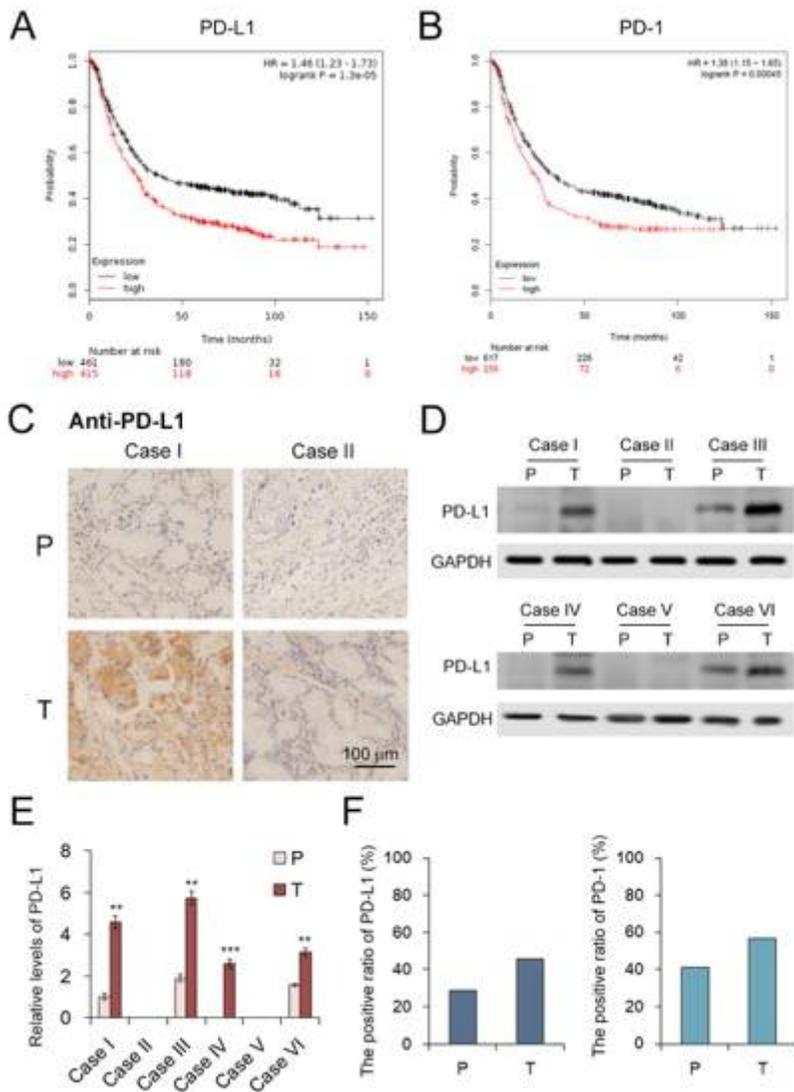
Figures

Figure 1

The Expression level of PD-L1 in GC (A、B) The survival curve of GC patients associated with PD-L1、PD-1 expression. (p < 0.001). (C) Immunohistochemistry of human gastric cancer tissues and paired normal tissues (n=6, total 12 samples). (D) Western blotting analysis of PD-L1 expression in GC cancer tissues and the corresponding normal tissues (n=6, total 12 samples). (E) Gray analysis of (D). (F) The positive ratio of PD-L1. *p < 0.05、**p < 0.01, and ***p < 0.001. All error bars stand for SE.
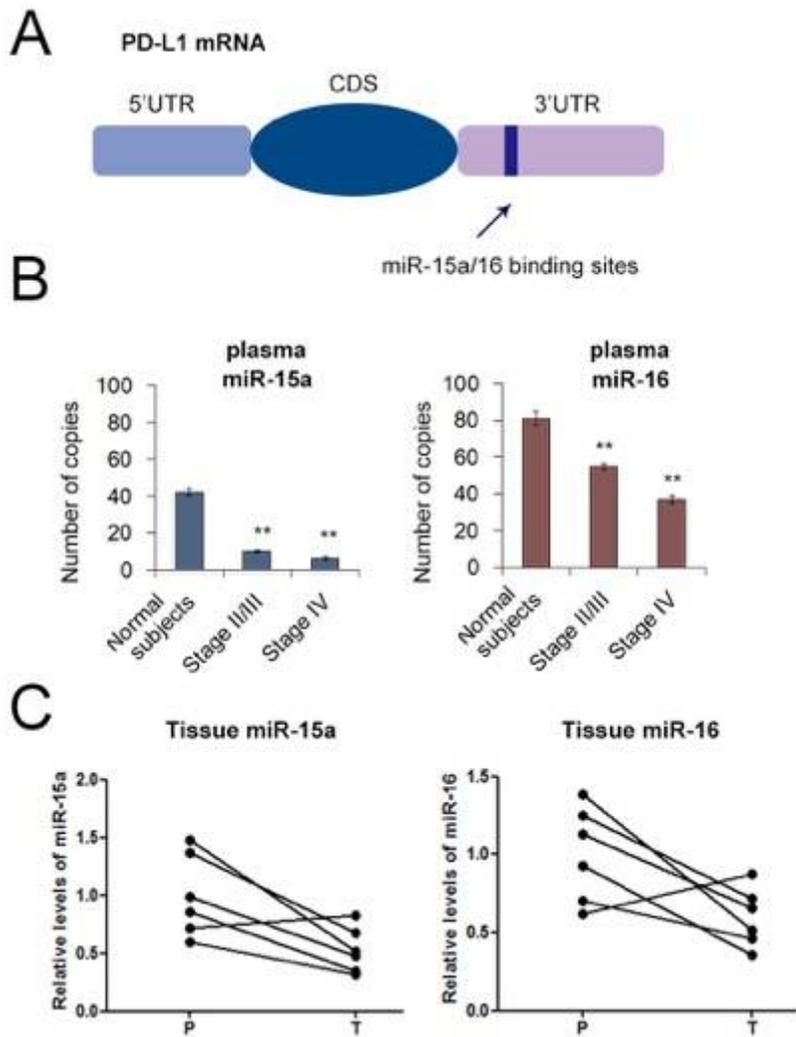
Figure 2

The Expression level of miR-15a/16 in GC (A) Predicted binding sites of miR-15a/16 within the 3'UTR of PD-L1 mRNA. (B) qRT-PCR analysis of miR-15a/16 levels in plasma of GC patients. (C) qRT-PCR analysis of miR-15a/16 levels in tissues of GC patients. **p < 0.01.
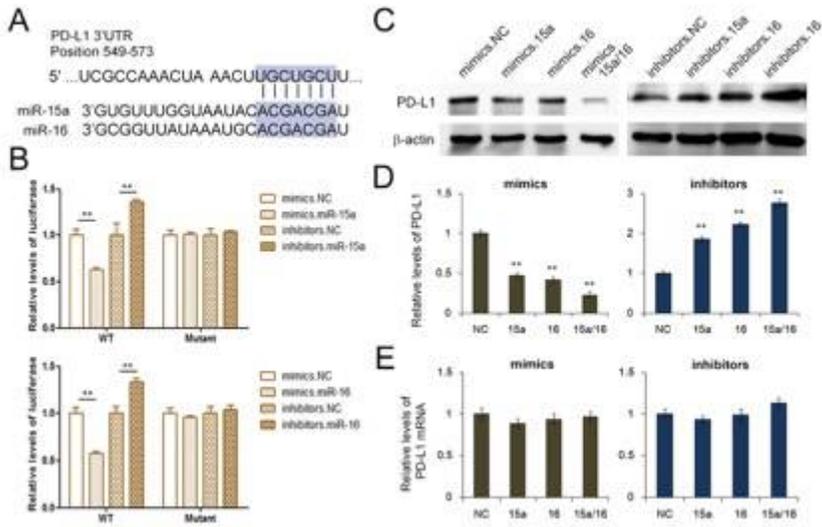
Figure 3

PD-L1 is a direct target of miR-15a/16. (A) Schematic description of the base pairing interaction between miR-15a/16 and PD-L1 mRNA. (B) Direct recognition of PD-L1 by miR-15a/16. SGC7901 cells were co-transfected with firefly luciferase reporters with either WT or mutant PD-L1 3'-UTR with miR-15a/16 mimics and inhibitors. The interaction between miR-15a/16 and the target was evident. (C) Western blot analysis of PD-L1 expression in SGC7901 treated with miR-15a/16 mimics and inhibitors and the corresponding quantitative analysis (D). (E) qRT-PCR analysis of PD-L1 mRNA levels in SGC7901 treated with miR-15a/16 mimics and inhibitors. **$p < 0.01$.
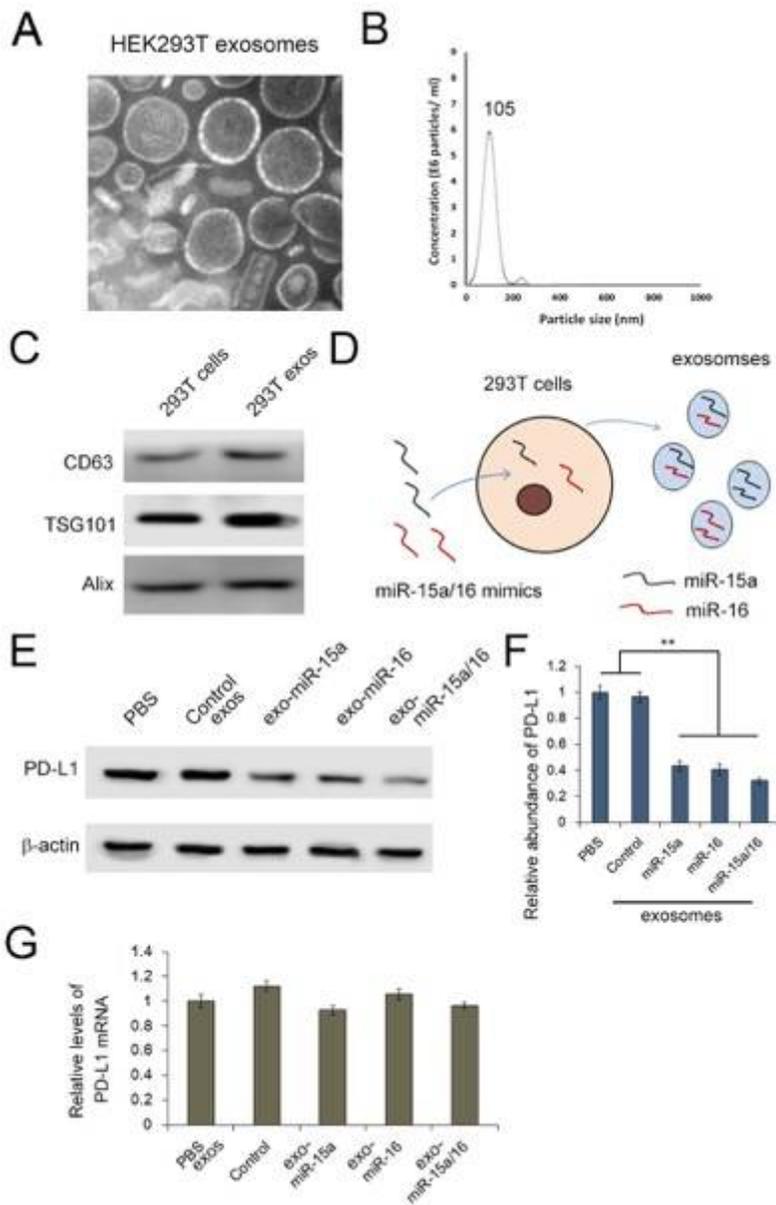
Figure 4

Exo-miR-15a/16 fuses into cells and regulates the expression of PD-L1. (A) Transmission electron microscope image of exosomes from HEK293T cell medium. (B)Diameter of exosomes. (C) Marker proteins of exosomes were determined via western blotting (D) Extraction of exosomes from HEK293-T cells. (E) Expression levels of the PD-L1 in SGC7901 cells were detected by western blotting. (F) Gray analysis of (E). (G) Expression levels of PD-L1 was tested by qRT-PCR. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. All error bars stand for SE.
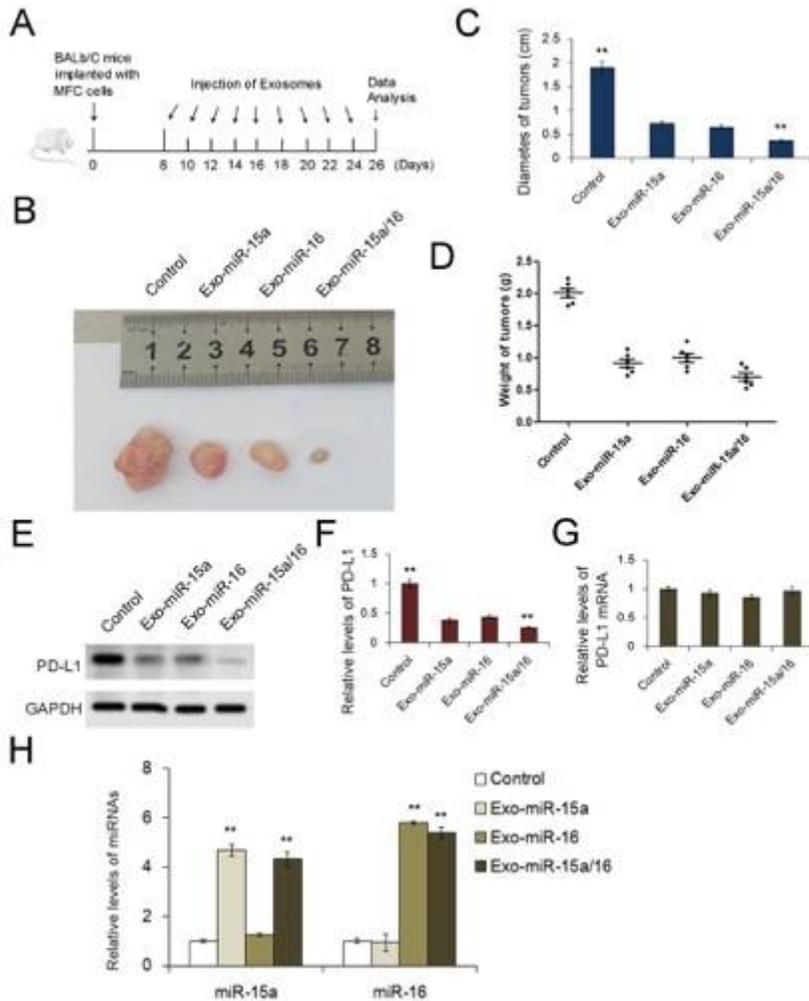
Figure 5

Exosome-miR-15a/16 inhibit the expression of PD-L1 and tumor growth in vivo (A) The flow chart depicting the in vivo experimental design. (B) The morphology of xenografted tumor tissues (n = 5). (C–D) Quantitative analysis of the diameters of tumors (C) and the weight of tumors (D). (E–G) Expression levels of PD-L1 in tumors by western blot (E) and qRT-PCR analysis(G). (F) Gray analysis of (E). **p < 0.01; ***p < 0.001. All error bars stand for SE.
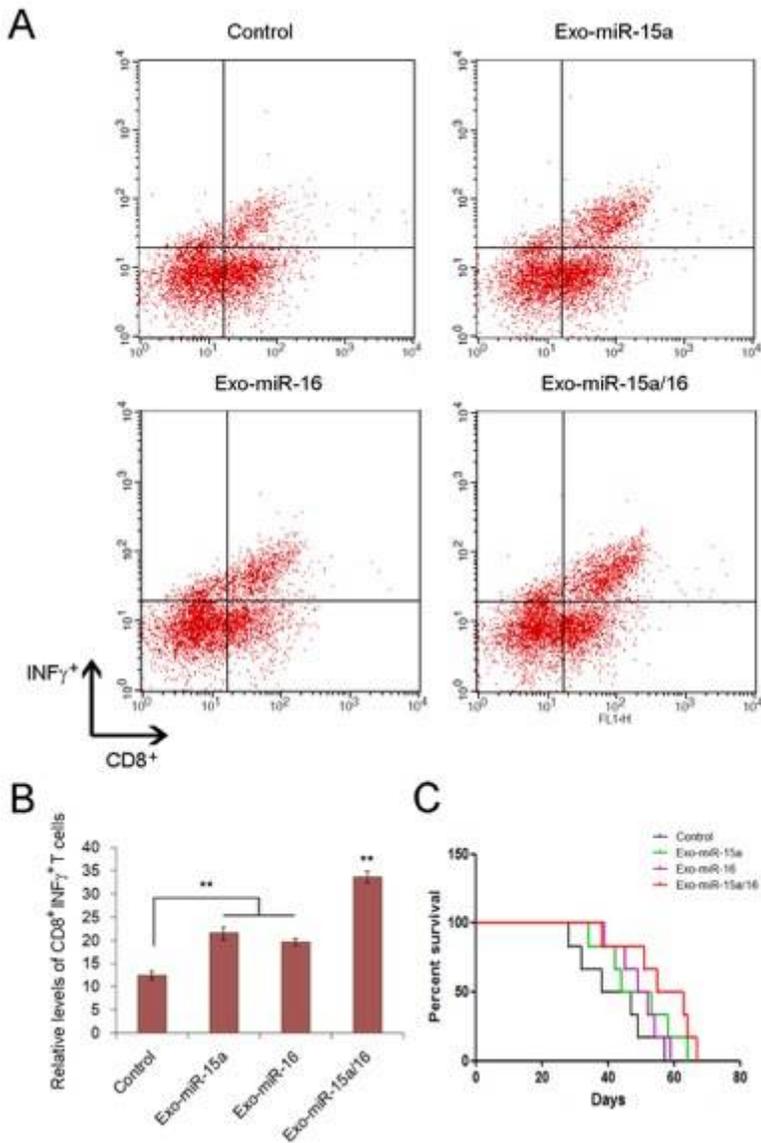
Figure 6

Exo-miR-15a/16 could enhance the number of CD8+ INFγ + T cells and inhibit immune escape
Representative flow cytometric analysis of immune checkpoint protein expression on CD8+ T
lymphocytes and INFγ + of mice in exo-miR-15a/16 therapy and control groups(A、B). The survival curve
of mice in exo-miR-15a/16 and control group(C).