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IFN-γ induces the upregulation of RFXAP via inhibition of miR-212-3p in pancreatic cancer cells: A novel mechanism for IFN-γ response

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Abstract. Previous studies have demonstrated that pancreatic cancer-derived microRNA (miR)-212-3p can inhibit the expression of regulatory factor X-associated protein (RFXAP), an important transcription factor for major histocompatibility complex (MHC) class II, and thereby lead to downregulation of MHC class II in dendritic cells. It has also been established that interferon (IFN)-γ can increase the expression of MHC class II in immune cells. It was therefore hypothesized that IFN-γ can inhibit miR-212-3p expression in pancreatic cancer, leading to the upregulation of RFXAP and MHC class II expression. This may represent a novel molecular mechanism underlying the use of IFN-γ in immunotherapy. Data from the present study revealed that miR-212-3p was inhibited by IFN-γ in a dose and time-dependent manner in the pancreatic ductal adenocarcinoma cell line PANC-1. RFXAP and MHC class II expression were increased following IFN-γ stimulation. A luciferase assay was performed to validate RFXAP as a target gene of miR-212-3p. The expression levels of RFXAP and MHC class II were decreased by miR-212-3p mimics and increased by miR-212-3p inhibitors. In PANC-1 cells transfected with miR-212-3p mimics, IFN-γ stimulation could not increase the RFXAP and MHC class II. The results from the present study suggest that IFN-γ increases RFXAP and MHC class II expression by inhibiting miR-212-3p. To the best of our knowledge, this is the first report of this novel molecular mechanism underlying the effects of IFN-γ on pancreatic cancer, which may aid with the development of immunotherapies for patients with pancreatic cancer.

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Abbreviations: RFXAP, regulatory factor X-associated protein; MHC, major histocompatibility complex; IFN, interferon; DCs, dendritic cells; PDAC, pancreatic ductal adenocarcinoma

Key words: pancreatic cancer, RFXAP, MHC class II, interferon-γ, miR-212-3p

Introduction

Pancreatic cancer is one of the most fatal tumors, with a 5-year survival rate of 3-6% (1). An estimated 48,960 new cases of pancreatic cancer and 40,560 mortalities from pancreatic cancer occurred in the United States of America in 2015, making this cancer type the fourth leading cause of cancer-associated mortality (2). Surgery is the only reported cure for pancreatic cancer; however, the resectability rate is only 18% due to its high rate of vascular invasion and metastasis (3). Radiotherapy and chemotherapy may extend survival time to some extent, but their overall effectiveness remains limited (4).

Major histocompatibility complex (MHC) class II expression has previously been demonstrated in a proportion of patients with pancreatic cancer, and has been shown to be correlated with better prognosis (5). Class II MHC transactivator-induced expression of MHC class II can inhibit pancreatic cancer metastasis and improve prognosis (6); however, the specific molecular mechanisms underlying MHC class II expression in pancreatic cancer remain unclear. Previous studies have demonstrated that regulatory factor X-associated protein (RFXAP) is a key transcription factor for the synthesis of MHC class II molecules in dendritic cells (DCs) (7). Exosomal microRNA (miRNA/miR)-212-3p secreted by pancreatic cancer cells can inhibit RFXAP expression in DCs, and thus suppress MHC class II expression and promote the immune escape of pancreatic tumors (8). This may explain why MHC class II expression is downregulated in pancreatic cancer, which leads to poor outcomes. Interferon (IFN)-γ has been demonstrated to induce MHC class II expression in immune cells, including DCs (9); however, whether IFN-γ can stimulate pancreatic cancer cells to express RFXAP and MHC class II molecules has not been reported, to the best of our knowledge. Therefore, we hypothesized that IFN-γ may inhibit the expression of miR-212-3p in pancreatic cancer and thereby increase the expression of RFXAP and

MHC class II molecules. To the best of our knowledge, this is a novel mechanism for the effect of IFN-γ on pancreatic cancer, and is the first observed correlation between RFXAP deficiency and tumor progression. The data from the present study may aid with the development of immunological and biological treatments for patients with pancreatic cancer, and improve their prognosis.

Materials and methods

Cell culture. The human pancreatic ductal adenocarcinoma (PDAC) cell lines, PANC-1, CFPAC-1, SW1990, BxPC-3 and MIAPaCa-2 were obtained from the Chinese Academy of Sciences (Shanghai, China). The cell lines were cultured in 25-cm^2 cell culture flask at 37˚C in a humidified atmosphere of 5% CO_2 with RPMI-1640 medium (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The cell lines were harvested following treatment with IFN-γ (1,000 IU/ml; Peprotech, Inc., Rocky Hill, NJ, USA) or equal amounts of PBS for 24 h at 37˚C in a humidified atmosphere of 5% CO_2. Human embryonic kidney 293T cells obtained from the Chinese Academy of Sciences (Shanghai, China) were maintained in Dulbecco's modified Eagle's medium (Corning Incorporated, Corning, New York, USA) supplemented with 10% fetal bovine serum under the aforementioned conditions.

Cell transfection. Cells were washed with PBS and switched to FBS-free growth medium for 24-48 h prior to transfection. Cells were transiently transfected with human miR-212-3p mimics or inhibitors, or negative controls (NC) (cat. nos. miR10000269-1-5, miR20000269-1-5, miR01201-1-5 and miR02201-1-5; Guangzhou Ribo Bio Co., Ltd., Guangzhou, China). Briefly, $2x10^5$ cells were seeded in 6-well plates. miR-212-3p mimics (final concentration, 50 nM) or inhibitors (final concentration, 100 nM) were transfected into the cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. At 48 h after transfection, the cells of each group were harvested for polymerase chain reaction (PCR) and western blot analyses.

Luciferase assay. 293T cells were co-transfected with pLMP vectors (Invitrogen; Thermo Fisher Scientific, Inc.) containing the wild-type RFXAP3'-untranslated region (CUGAAA ACUGUU) or mutant sequences (AAUGACGUAUCU), and miRNA mimics or inhibitors. The potential binding sequences of miR-212-3p on the RFXAP 3'UTR were mutated using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, United States). Cells were harvested and subjected to lysis at 48 h following transfection. *Renilla* luciferase activity was used for normalization, and firefly luciferase activity was detected with a Dual-Luciferase Reporter Assay kit (Promega Corporation Madison, WI, USA), according to the manufacturer's protocol.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Total RNA from the cells was isolated and purified using an RNeasy Mini kit (Qiagen, Inc., Valencia, CA, USA), according to the manufacturer's protocol. The concentration, purity, and amount of total RNA were quantified using a Nano Drop spectrophotometer (ND-1000 version 3.5.2 software; Thermo Fisher Scientific, Inc., Wilmington, DE, USA). Reverse transcriptase reactions contained RNA samples, 50 nM stem-loop RT primer (miRQ0000269-1-2, Ribo Bio Co., Ltd., Guangzhou, China), 1x RT buffer (Applied Biosystems; Thermo Fisher Scientific, Inc.), 0.25 mM each of dNTPs, 3.33 U/ml MultiScribe reverse transcriptase (Applied Biosystems; Thermo Fisher Scientific, Inc.), and 0.25 U/ml RNase inhibitor (P/N:N8080119; Applied Biosystems; Thermo Fisher Scientific, Inc.). The reverse transcriptase reactions conditions were as follows: 16˚C for 30 min, 42˚C for 30 min, and 85˚C for 5 min. qPCR was performed on an Applied Biosystems® 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, Inc.), including 2 μl of RT product, 10 μl of Power SYBR Green PCR Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.), 4 μl of RNase-free water, and 2 μl forward and reverse primers. The qPCR thermocycling conditions were as follows: 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. The relative expression ratio of miR-212-3p and *RFXAP* was presented as the fold change, which was normalized to an endogenous reference (U6 RNA) using the $2^{-\Delta\Delta Cq}$ method (10). The primers were purchased from Guangzhou Ribo Bio Co., Ltd., and the sequences were as follows: miR-212-3p forward, 5'-GGT AACAGTCTCCAGTCA-3', and reverse, 5'-GCAATTGCA CTGGATACG-3'; *RFXAP* forward, 5'-CAGTAGAATTCG GCCAAGCAGGTGCTAAAAG-3', and reverse, 5'-CAGAGG ATCCATGTAGATGTTCTTGGTAAG-3'. Experiments were performed in triplicate.

Western blot analysis. Cells were lysed with RIPA buffer (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) containing a protease inhibitor cocktail (Thermo Fisher Scientific, Inc.). The lysates were cleared by centrifugation at $1.2x10^4$ x g at 4˚C for 15 min, and the concentrations of proteins were measured using a BCA protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). The proteins were denatured in 2X SDS buffer at 95˚C and 10 μl of proteins were loaded per well to a 10% SDS-PAGE gel, then transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% skim milk powder for 1 h at room temperature and probed with the following antibodies overnight at 4˚C: RFXAP (dilution, 1:200; cat. no. ab172281; Abcam, Cambridge, UK), or MHC class II (dilution, 1:500; cat. no. ab17101; Abcam), or β-actin (dilution, 1:1,000; cat. no. ab8226; Abcam). The samples were incubated with secondary goat anti-mouse antibodies conjugated to horseradish peroxidase (dilution, 1:5,000; ab6789; Abcam.) for 1 h at room temperature. The blots were visualized by GE Healthcare enhanced chemiluminescence kit (GE Healthcare, Chicago, IL, USA) using Kodak X-OMATLS film (Eastman Kodak, Rochester, NY, USA). Each sample was measured three times.

Statistical analysis. All data are presented as mean ± standard deviation and were analyzed using a Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference. All data were analyzed using SPSS software (version 19.0;

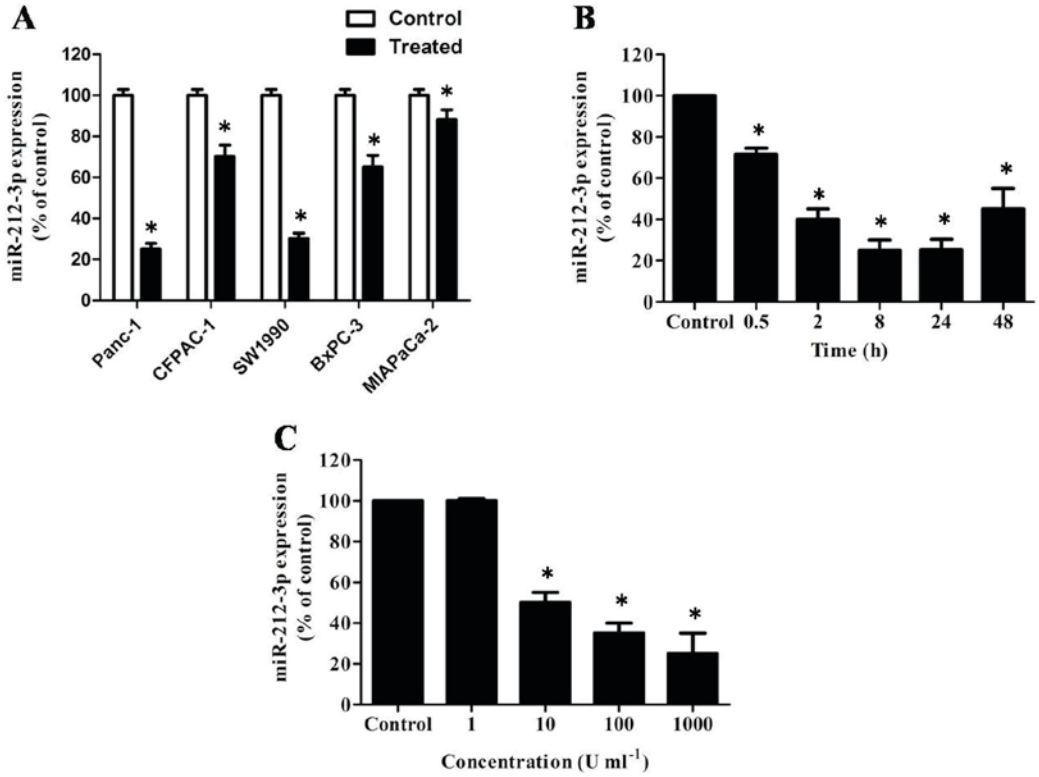

Figure 1. miR-212-3p is downregulated by IFN-γ in PDAC cell lines. (A) RT-qPCR analysis of relative miR-212-3p expression in IFN-γ- or PBS-treated PDAC cell lines, including PANC-1, CFPAC-1, SW1990, BxPC-3 and MIAPaCa-2. miR-212-3p was markedly reduced in IFN-γ-stimulated PDAC cells. Transcript levels were normalized to U6 expression. (B) Time-course of miR-212-3p induction by IFN-γ. PANC-1 cells were stimulated with 1,000 U/ml IFN-γ for the indicated times and miR-212-3p expression was quantified by RT-qPCR. miR-212-3p expression decreased over time during the first 24 h after treatment. (C) Dose-response analysis of miR-212-3p induction by IFN-γ. Decreased expression of miR-212-3p was observed between 1 and 1,000 U/ml IFN-γ. PANC-1 cells were stimulated with the indicated doses of IFN-γ for 24 h and miR-212-3p expression was quantified by RT-qPCR. miR, microRNA; IFN-γ, interferon-γ; PDAC, pancreatic ductal adenocarcinoma; RT-qPCR, reverse transcription-quantitative polymerase chain reaction. *P<0.05 vs. PBS stimulated PDAC cells.

IBM Corp., Armonk, NY, USA) and GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA, USA).

Results

miR-212-3p is down regulated by IFN-γ in PDAC cell lines. RT-qPCR analysis demonstrated that the expression of miR-212-3p was markedly reduced in IFN-γ-stimulated PDAC cells compared with the control, particularly in PANC-1 cells (Fig. 1A, P<0.05). A time-course analysis of miR-212-3p expression in PANC-1 cells following IFN-γ treatment revealed that miR-212-3p expression decreased over time during the first 24 h after treatment (Fig. 1B, P<0.05). In addition, the decrease in miR-212-3p expression was dose-dependent between 1 and 1,000 U/ml IFN-γ (Fig. 1C, P<0.05).

RFXAP is a direct target of miR-212-3p in PANC-1 cells. A dual-luciferase activity assay demonstrated that miR-212-3p mimics significantly suppressed the luciferase activity of reporter vectors containing the wild-type *RFXAP* 3'-UTR but not in the mutant (P<0.05; Fig. 2A). To assess whether miR-212-3p has a functional role in the downregulation of *RFXAP*, the expression level of miR-212-3p was manipulated in in PANC-1 cells. RT-qPCR analysis demonstrated that overexpression of miR-212-3p significantly inhibited *RFXAP* and MHC class II mRNA expression in PANC-1 cells, while inhibition of miR-212-3p exhibited opposite effects (P<0.05; Fig. 2B). Western blot analysis demonstrated that overexpression of miR-212-3-p resulted in a marked decrease in RFXAP protein levels, whereas inhibition of miR-212-3p resulted in a marked increase in RFXAP protein levels (Fig. 2C).

IFN-γ increases RFXAP and MHC class II expression by inhibiting miR-212-3p in PANC-1 cells. Western blot analysis revealed that RFXAP was markedly up regulated following treatment with IFN-γ, which was concomitant with an upregulation in MHC class II expression (Fig. 3A). However, following transfection withmiR-212-3pmimics, IFN-γ stimulation did not increase the expression of RFXAP or MHC class II (Fig. 3B). These results suggest that IFN-γ increases RFXAP and MHC class II expression by inhibiting the expression of miR-212-3p in PANC-1 cells.

Discussion

As an immune adjuvant, IFN-γ has demonstrated some efficacy in treating pancreatic cancer (11,12). It can enhance the immunogenicity of tumor vaccines and promote the immune response of antigen-specific helper T-cells (13). When used in combination with chemotherapeutic drugs, including gemcitabine, it can effectively prolong survival time for patients with pancreatic cancer (14). Thus far, few studies have explored the molecular mechanisms by which

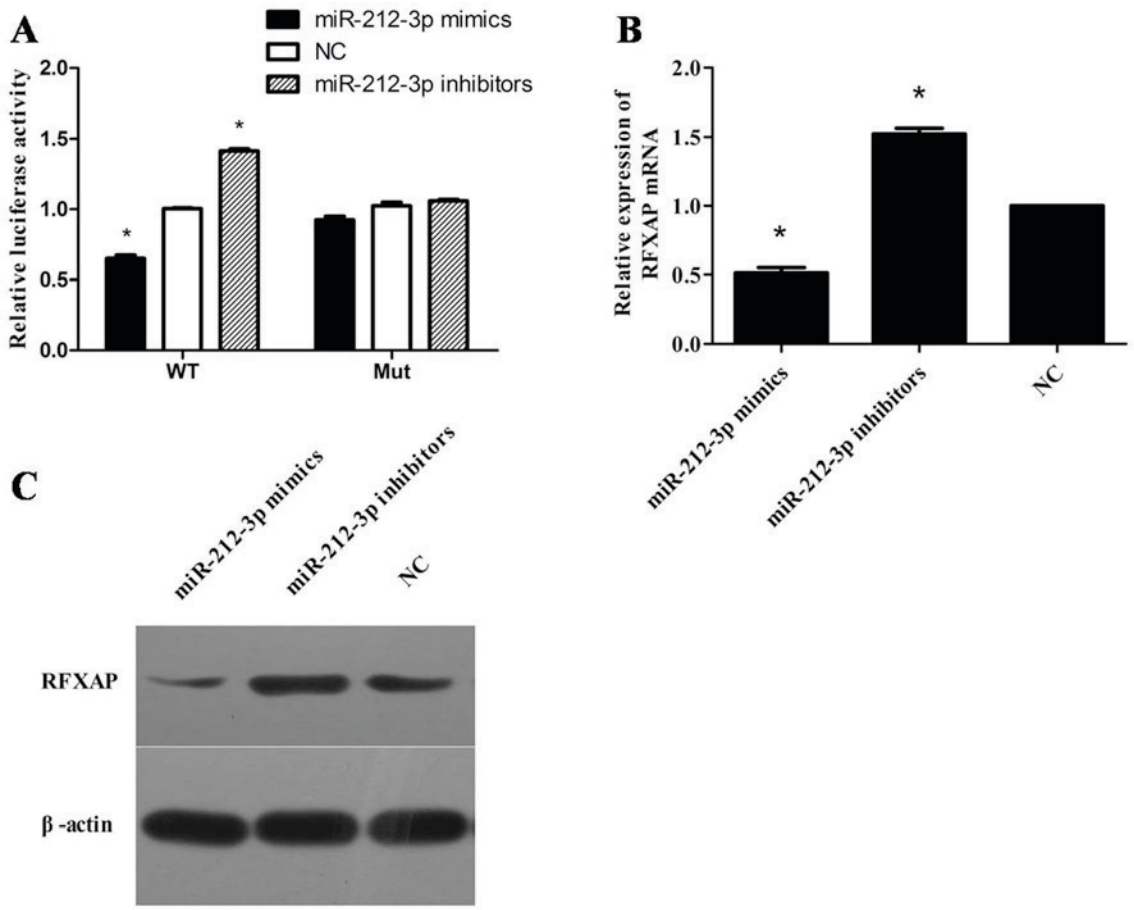

Figure 2. RFXAP is a direct target of miR-212-3p in PANC-1 cells. (A) Luciferase activity was determined in 293T cells 48 h following co-transfection with reporter vectors containing a WT or Mut RFXAP 3'untranslated region, and miR-212-3p mimics, miR-212-3p inhibitors or NC. Luciferase activity is expressed relative to the NC. (B) RFXAP mRNA was detected by reverse transcription-quantitative polymerase chain reaction in PANC-1 cells transfected with miR-212-3p mimics, inhibitor or NC. Levels were normalized to U6 RNA and expressed relative to the NC. (C) Protein levels of RFXAP were examined by western blot analysis following transfection with miR-212-3p mimics, inhibitors or NC. β-actin was used as a loading control. *P<0.05 vs. NC. RFXAP, regulatory factor X-associated protein; WT, wild-type; Mut, mutant; NC, negative control; miR, microRNA.

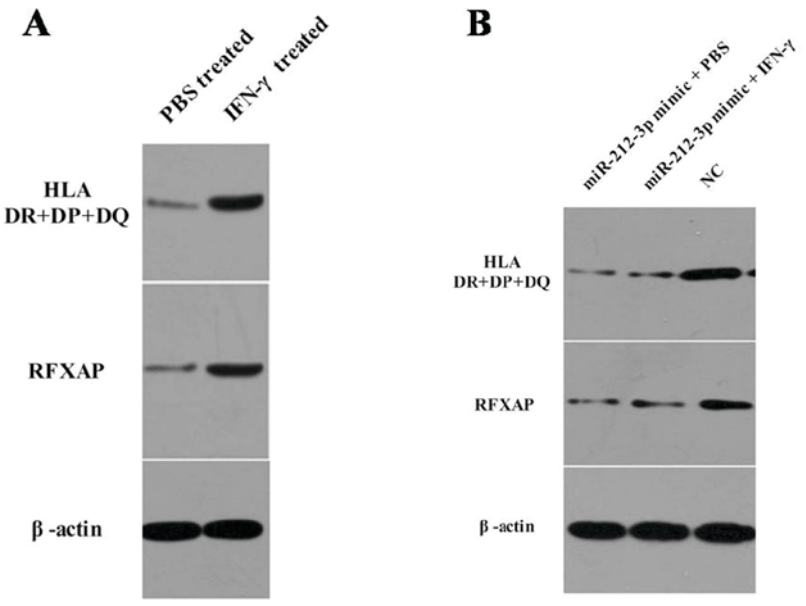

Figure 3. IFN-γ increases RFXAP and MHC class II expression by inhibition of miR-212-3p. Western blot analysis of MHC class II (HLADR+DP+DQ) and RFXAP in IFN-γ- or PBS-treated PANC-1 cells. (A) In untransfected PANC-1 cells, RFXAP and MHC class II were markedly upregulated following IFN-γ stimulation compared with the PBS treatment. (B) In miR-212-3p mimic-transfected cells, IFN-γ stimulation did not markedly increase the levels of RFXAP or MHC class II compared with the PBS treatment. β-actin was used as a loading control. IFN-γ, interferon-γ; MHC, major histocompatibility complex; RFXAP, regulatory factor X-associated protein; miR, microRNA; HLA, human leukocyte antigen; NC, negative control.

IFN-γ suppresses the proliferation and metastasis of pancreatic cancer cells. Detjen *et al* (15) demonstrated that IFN-γ suppressed the proliferation of pancreatic cancer cells through the caspase-1-mediated apoptosis pathway. In the present study, IFN-γ upregulated RFXAP and MHC class II expression in the PDAC cell line PANC-1. This novel mechanism provides experimental evidence for the use of IFN-γ in adjuvant treatment for pancreatic cancer, which may improve patient prognosis.

Human MHC class II molecules mainly comprise human leukocyte antigen-DP, -DQ, and -DR antigens, which are primarily expressed in antigen-presenting cells, including DCs and monocytes/macrophages (16). The regulatory factor X (RFX) complex, which contains regulatory factor X-associated ankyrin-containing protein, RFXAP and regulatory factor X 5, is a key initiating component that regulates MHC class II transcription (17). RFXAP is the core transcription factor of the RFX complex, and its normal expression and functioning are essential for the transcription of MHC class II molecules (18). *RFXAP* mutation or deletion may result in bare lymphocyte syndrome (18). As tumor cells typically lack the specific mechanism to directly activate cluster of differentiation (CD) 8^+ T-cells via MHC class I molecules, the MHC class II molecule-activated $CD4^+$ T-cells serve a key role in anti-tumor immunity (19,20). Certain tumor cells, including pancreatic cancer cells, can also express MHC class II molecules, which is associated with relatively good prognosis, suggesting that activation of MHC class II expression could inhibit pancreatic cancer metastasis and improve prognosis (21). The present study confirmed that IFN-γ upregulates MHC class II expression in pancreatic cancer cells, and thus provided evidence for use of IFN-γ as an immunological and biological therapy for pancreatic cancer.

A previous study demonstrated miR-212-3p involved in the generation of pancreatic cancer-derived exosomes and could suppress RFXAP protein expression in DCs and thus elicit DC immune tolerance (8). Other studies have also demonstrated the key function of miR-212-3p in pancreatic cancer metastasis; Park *et al* (22) demonstrated that miR-212 expression increased in pancreatic cancer cells and promoted their proliferation by inhibiting Retinoblastoma 1 gene expression in a targeted manner. In addition, Ma *et al* (23) demonstrated that miR-212 promoted tumor cell proliferation and infiltration by the targeted inhibition ofPatch-1 gene expression. Data from the present study confirmed that IFN-γ could inhibit miR-212-3p expression in pancreatic cancer cells, and thus upregulate the expression of RFXAP and MHC class II. Following transfection with miR-212-3p mimics, IFN-γ could not stimulate expression of RFXAP and MHC class II, which implies that IFN-γ stimulates MHC class II expression by suppressing miR-212-3p and thereby upregulating RFXAP expression. To the best of our knowledge, this function of IFN-γ had not been reported previously. IFN-γ treatment in patients may inhibit miR-212-3p expression, promote RFXAP and MHC class II expression, and thus lead to immune effects on pancreatic cancer cells and improve the prognosis of patients.

Surgery is currently the only curative treatment for patients with pancreatic cancer. Together with post-operative chemotherapy, it may achieve a 5-year survival rate of 15-40%. However, only 10-15% of patients with pancreatic cancer are candidates for radical resection as ~85% of patients with pancreatic cancer initially present with local major vessel invasion or distant metastasis (24). Although chemotherapy, radiotherapy and interventional treatment have extended the survival time of patients with pancreatic cancer, the overall effectiveness of these approaches remains limited (25). Immunotherapies, biological therapies and adjuvant therapies may extend survival times for pancreatic cancer. The present study revealed that IFN-γ treatment of tumor cells can promote *RFXAP* expression. RFXAP is not only a key transcription factor for MHC class II expression but may also promote the expression of other tumor suppressor genes. IFN-γ may serve an important role in tumor immunotherapy by promoting *RFXAP* expression and therefore the targeting of downstream genes. However, as genes associated with *RFXAP* transcription have not been identified, further studies are required to investigate the roles of RFXAP in tumorigenesis.

In conclusion, IFN-γ may inhibit miR-212-3p expression in pancreatic cancer, leading to upregulation of RFXAP and MHC class II. This may reflect a novel molecular mechanism underlying the effects of IFN-γ on pancreatic cancer.

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