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Original article

Involvement of microRNA-181b in the gemcitabine resistance of pancreatic cancer cells

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ABSTRACT

Background/objectives: MicroRNAs (miRs) have been shown to regulate the sensitivity to several chemotherapeutic agents in various types of cancers. MiR-181b is one of such regulators, yet its importance in pancreatic cancer is not determined so far. The aim of this study was to investigate the relationship between microRNA (miR)-181b expression and gemcitabine resistance in pancreatic cancer cells.

Methods: The effects of overexpression or knockdown of miR-181b on four pancreatic cancer cell lines exposed to gemcitabine were examined. The induction of apoptosis and the changes of the cylindromatosis (CYLD) protein were examined. Furthermore, the effect of small interference RNA for CYLD (si*CYLD*) on cell viability and the relationship between CYLD and nuclear factor kappa B (NF-κB) were investigated.

Results: The expression of miR-181b was higher in BxPC3, Panc1 and PSN1 cells compared with MiaPaCa2 cells. Pre-miR-181b transfection into MiaPaCa2 cells increased their gemcitabine resistance, whereas anti-miR-181b transfection into the other pancreatic cancer cell lines reduced their resistance to gemcitabine and led to the induction of apoptosis. The protein levels of CYLD were increased by anti-miR-181b in Panc1 and PSN1 cells. Inhibition of CYLD increased the NF-κB activity and gemcitabine resistance in Panc1 and PSN1 cells.

Conclusions: The present study demonstrated that miR-181b was associated with the resistance of pancreatic cancer cells to gemcitabine, and verified that miR-181b enhances the activity of NF-κB by inhibiting *CYLD*, leading to the resistance to gemcitabine. Our results suggest that miR-181b is a potential target for decreasing gemcitabine resistance.

1. Introduction

Pancreatic cancer is the 4th leading cause of cancer-related death in the United States, and has the poorest prognosis among digestive cancers, with a relative 5-year survival rate of only 6% [1]. Although the only curative treatment for pancreatic cancer is a surgical resection, the resectable cases are limited, and 80% of the resected cases will experience a relapse within 1–2 years [2]. Consequently, the 5-year survival rate is only ~20% even after "curative" resection [3]. Surgery alone is thus considered to be insufficient to improve the prognosis, and multimodal treatments, including chemotherapy, radiotherapy and immunotherapy, have been pursued. Nevertheless, the response rates to these treatments are poor, ranging from 20 to 30% [4–7]. Clarification of the mechanisms of resistance to these treatments, and the establishment of an effective system or method for predicting sensitivity are therefore important.

Recently, research on microRNA (miR) has proceeded at an accelerated rate. MiRs are small RNA molecules of 21–22 base pairs, which work as modulators of gene expression in the post-transcriptional phase [8]. MiRs have drawn the attention of oncologists because their aberrant expression correlates with carcinogenesis and/or the progression of cancers. Moreover, several studies have indicated that miRs regulate the sensitivity of various types of cancers to several chemotherapeutic agents [8–14]. We have recently reported that miR-21 and miR-146a play an

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important role in determining the sensitivity of HCC cells to IFN-α, and that the expression level of these miRs significantly correlated with the clinical efficacy of IFN-based chemotherapy and the overall survival in patients with HCC [12,13].

Among several candidate miRs regulating chemoresistance, miR-181b has been shown to be one of the important determinants of the prognosis and/or chemosensitivity in several kinds of cancers [15–20]. MiR-181b was first reported to be downregulated in glioblastoma in 2005 [21]. Thereafter, it has shown that the target genes of miR-181b include *cylindromatosis* (*CYLD*), B-cell lymphoma 2 (*BCL2*), tissue inhibitor of metalloproteinases-3 (*TIMP3*), Homeobox protein Hox-A11 (*HOXA11*), and T Cell Leukemia/Lymphoma 1 (*TCL1*) [17,22,23]. Among these genes, *CYLD* has been shown to be involved in NF-κB signaling, and is closely involved in regulating the apoptosis of cancer cells [24–31]. With regard to pancreatic cancer, it has been reported that several miRs have an association with the resistance to gemcitabine, a key drug used for the treatment of pancreatic cancers [9–11,32–37]. However, the relationship between pancreatic cancer and the expression of miR-181b has not yet been elucidated.

In the present study, we focused on miR-181b and examined the relationship between miR-181b and gemcitabine resistance in pancreatic cancer cells, and further verified that miR-181b enhances the activity of NF-κB by inhibiting *CYLD*, thus leading to the resistance to gemcitabine.

2. Materials and methods

2.1. Cell lines and culture conditions

The BxPC3, MiaPaCa2, Panc1, and PSN1 pancreatic cancer cell lines were obtained from the Japan Cancer Research Resources Bank (Tokyo, Japan) and cultured in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum and antibiotics (PenStrep 15140, Invitrogen, Carlsbad, CA).

2.2. Transfection

The cells were cultured to 80% confluence and transfected with 5 nmol/L of an anti/pre-miR-181b antisense oligonucleotide, 10 μmol/L of *CYLD* siRNA oligonucleotide (si*CYLD*), or a negative control oligonucleotide (Applied Biosystems, Foster City, CA), using the siPORT NeoFX Transfection Agent (Ambion Inc., Austin, TX) in accordance with the manufacturer's protocol. After transfection, cells were cultured for 24 h, and then the medium was replaced with DMEM containing 10% fetal bovine serum and antibiotics.

2.3. RNA extraction

Total RNA was isolated from cells using the TRIzol agent (Invitrogen), according to the protocol provided by the manufacturer. Briefly, each cell line was treated with 1.0 ml of TRIzol and 0.2 ml of chloroform. After incubation at room temperature for 3 min, the cells were centrifuged at 12,000 *g* for 15 min at 4 °C, then the supernatant was removed, and the pellet was washed with 75% ethanol and centrifuged at 7,500 *g* for 5 min at 4 °C. The RNA pellet was dried and dissolved in RNase-free-water. The quality and the concentration of the RNA were quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

2.4. Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) to determine the microRNA expression

Reverse transcription (RT) reactions and real-time qRT-PCR were performed using a TaqMan human miRNA assay kit (Applied Biosystems) according to the instructions supplied by the manufacturer. Briefly, the cDNA was synthesized from 10 ng of total RNA using the TaqMan human miRNA assay kit and specific stem-loop reverse transcription primers (Applied Biosystems) according to the manufacturer's protocol. The reverse transcription conditions were as follows: 16 °C for 30 min followed by 40 °C for 30 min and 85 °C for 5 min. Real-time PCR reaction was done using TaqMan Universal PCR Master Mix, No AmpErase UNG, and TaqMan miRNA specific PCR-primers (Applied Biosystems). A 20 μl aliquot of the reaction product was incubated in a 96-well optical plate at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, and then at 60 °C for 1 min, using an ABI PRISM 7900HT instrument (Applied Biosystems). The expression of the target miR in cell lines was normalized to the expression of RNU48, which was used as an internal control. The data were analyzed according to the comparative Ct method ($2^{-\Delta CT}$).

2.5. MTT (3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, yellow tetrazole) assay

The MTT assay was used to determine the IC50 (the concentration that inhibits the viability in 50% of the cells) value for gemcitabine. Cells were seeded into 96-well plates each at 5000/100 μl, and were cultured under the usual culture conditions. After 24 h, the cells were exposed to gemcitabine for 72 h, then 10 μl of MTT solution was added to each well. The plates were incubated for 3 h at 37 °C and the culture solution was replaced with 100 μl of 0.04 N HCl–isopropanol. After 24 h, the absorbance of individual wells was read at a 550 nm test wavelength and a 655 nm reference wavelength, using a microplate reader (Bio-Rad Laboratories, Hercules, CA). The IC50 value for gemcitabine was calculated from the dose–response curve.

2.6. Annexin V assay

The binding of annexin V was used as a sensitive method for measuring apoptosis. At 24 h after transfection of the anti-miR-181b into the PSN1 cells, the cells were exposed to gemcitabine for 24 h at 25 ng/ml, then stained with Annexin V-APC (BD Biosciences, Franklin Lakes, NJ, USA) and PE-propidium iodide (PI) (Bio Vision Research Products, Mountain View, CA, USA) according to manufacturer's protocol. The analysis was performed using a FACSAria™ II Cell Sorter (BD Biosciences). Approximately 50,000 cells were tested for each sample. Annexin V-positive and PE-

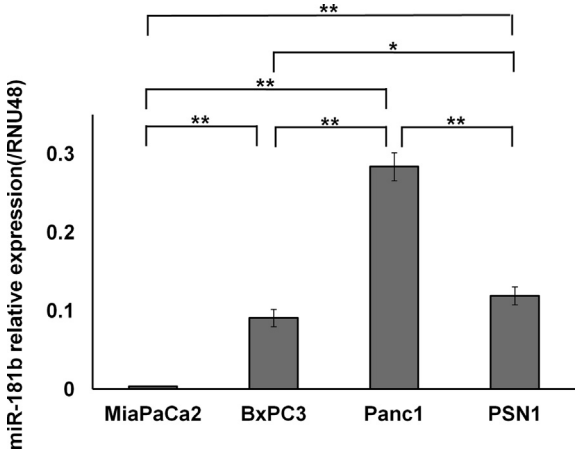

Fig. 1. miR-181b expression in pancreatic cancer cell lines. The expression of miR-181b was determined using quantitative real-time PCR (*$p < 0.05$, **$p < 0.01$).