Abstract. Although we studied previously the mechanisms of resistance of pancreatic cancer cells to gemcitabine (GEM), prediction of the response to GEM remains unsatisfactory. The aim of this study was to investigate the relationship between miR-29a expression and the response to GEM in pancreatic cancer cells. Changes in the growth-inhibitory effect of pancreatic cancer cells (MIAPaCa-2, PSN-1, BxPC-3 and Panc-1) to GEM were examined after overexpression or suppression of miR-29a. We also examined the effect of miR-29a on the Wnt/β-catenin signaling pathway and investigated whether the altered growth-inhibitory effect by miR-29a suppression was weakened after the addition of Wnt3a, a Wnt/β-catenin signaling activator. MIAPaCa-2 and PSN-1 cells transfected with anti-miR-29a showed significantly lower resistance to GEM. In the anti-miR-29a-transfected cells, GEM induced significantly larger numbers of apoptotic cells and S phase accumulation compared to control cells, demonstrated by Annexin V assay and flow cytometric analysis of the cell cycle, respectively. The transfected cells showed overexpression of putative target molecules including Dkk1, Kremen2 and sFRP2 and lower activation of the Wnt/β-catenin signaling pathway. The addition of Wnt3a weakened the augmented growth-inhibitory effect of anti-miR-29a transfection. Our findings suggest that miR-29a expression correlates significantly with the growth-inhibitory effect of GEM and that activation of the Wnt/β-catenin signaling pathway mediated the miR-29a-induced resistance to GEM in pancreatic cancer cell lines.

Introduction

Pancreatic cancer is one of the most common malignancies worldwide (1). The prognosis of patients with pancreatic cancer remains poor even after curative resection and more than half of patients develop tumor recurrence at distant or local sites, with an estimated 5-year survival rate of only 20% (2-4). Chemotherapy plays an important role in the treatment of pancreatic cancer. Gemcitabine (GEM), a cell cycle specific inhibitor of DNA synthesis and a ribonucleotide reductase, has become the gold standard chemotherapeutic agent for pancreatic cancer (5,6). However, the response rate to GEM is <20%, indicating that the outcome remains unsatisfactory (5). We reported that the expression of ribonucleotide reductase M1 subunit (RRM1) was significantly associated with the response to GEM in pancreatic cancer cell lines and clinical specimens (7,8). However, the clinical response to GEM based on the expression of RRM1 cannot be predicted satisfactorily. Therefore, it is necessary to find novel biological markers that can accurately predict the clinical response to GEM.

Recently, microRNA (miRNA) has emerged as a critical class of negative regulators of gene expression through modulation of the post-transcriptional activity of its multiple target mRNAs by repression of translation or direct cleavage (9,10). MiRNAs control a wide array of biological processes, including cell proliferation, differentiation and apoptosis. Aberrant expression of miRNAs is widely reported in human cancers with both up- and downregulation detected in cancer cells compared with their normal counterparts (11,12). Employing gene manipulation protocols, the present study was designed to identify the miRNA linked to the response of pancreatic cancer cells to GEM through the modulation of Wnt/β-catenin signaling pathway. The results showed a significant relationship between miR-29a and response to GEM in pancreatic cancer cells. Additional experiments using Wnt3a, a Wnt/β-catenin signaling activator, demonstrated that the miR-29a-induced resistance to GEM correlated significantly with the activation of Wnt/β-catenin signaling in pancreatic cancer cell lines.

Materials and methods

Pancreatic cancer cell line and clinical samples. Four human pancreatic carcinoma cell lines (MIAPaCa-2, PSN-1, BxPC-3 and Panc-1) were used in the present study (8). MIAPaCa-2 and PSN-1 cell lines were obtained from the Japanese Collection of Research Bioreresources (JCRB, Tokyo, Japan). BxPC-3 and Panc-1 cell lines were obtained from the American Type
Culture Collection (ATCC, Rockville, MD, USA). These cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in a humidified incubator under 5% CO₂-95% air.

**Drugs and reagents.** GEM was purchased from Eli Lilly and Co. (Indianapolis, IN, USA). Polyclonal rabbit anti-human DKK1 antibody (Cell Signaling Technology, Beverly, MA, USA), polyclonal rabbit anti-human sFRP2 antibody (Abcam Inc., Cambridge, MA, USA), polyclonal mouse anti-human Kremen2 antibody (Abcam Inc.) and polyclonal rabbit anti-human β-actin (Sigma-Aldrich Co., St. Louis, MO, USA) were used for western blot analysis. Recombinant human Wnt3a (R&D Systems, Minneapolis, MN, USA) was used as a Wnt/β-catenin signaling activator. In this study, Wnt3a was used at 50 ng/ml based on the protocol described in a previous study (13).

**Transfection.** Antisense miR-29a inhibitor (anti-miR-29a), miR-29a precursor (pre-miR-29a) and their negative control oligonucleotides were obtained from Ambion Inc. (Austin, TX, USA). These were used to transfect pancreatic cancer cells by using siPORT NeoFx (Ambion Inc.) according to the instructions provided by the manufacturer. The transected cells were resuspended and cultured in regular culture medium for 24-72 h before analysis.

**RNA extraction.** Total RNA and miRNA fractions were isolated from tissue samples and cell lines by TRIZol agent (Invitrogen, Carlsbad, CA, USA) and the quality of the RNA was assessed with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) at 260 and 280 nm (A260/280).

**Real-time quantitative reverse transcription-polymerase chain reaction for miRNA expression.** Reverse transcription reaction and real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) were performed using TaqMan human miRNA assay kit (Applied Biosystems, Foster City, CA, USA) according to the instructions supplied by the manufacturer. The expression of the target miRNA was normalized relative to that of the internal control; RNU48. Data were analyzed according to the comparative Ct method (14).

**Real-time qRT-PCR for mRNA expression.** RT reaction was performed with SuperScript II (Invitrogen) based on the protocol provided by the manufacturer, followed by qRT-PCR. The expression of the target gene was normalized relative to the expression of porphobilinogen deaminase (PBGD), which was used as an internal control. The designed PCR primers were as follows: AXIN2 forward primer, 5'-GTTGTGGAGAGTTCCAC-3'; AXIN2 reverse primer, 5'-CTCGCCGCTCTGTCGGCTAC-3'; CCND1 forward primer, 5'-AACGT-GGCGCTTCAAC-3'; CCND1 reverse primer, 5'-CTGGATCCGCCGCGAC-3'; MYC forward primer, 5'-AAGACGACGACGGCAGC-3'; MYC reverse primer, 5'-CACTGTCGAGCGGCTAC-3'; TACSTD1 forward primer, 5'-CAGGATCGTCCGTCTGCTACTCCC-3'; TACSTD1 reverse primer, 5'-ATCGGATGAGAAGGACG-3'; TCF3 reverse primer, 5'-CCAGGCTAGAGATCTGAG-3'; and PBGD forward primer, 5'-TGTCTGTGTAAGGCATCCTGA-3'; PBGD reverse primer, 5'-TCAATTGTGACCACACTGTCGCTT-3'.

**Western blot analysis.** Cells grown to semifusion were lysed in RIPA buffer [25 mM Tris (pH 7.5), 50 mM NaCl, 0.5% sodium deoxycholate, 2% Nonidet P-40, 0.2% sodium dodecyl sulfate, 1 mM phenylmethylsulphonyl fluoride and 500 KIE/ml Tryosyl, proteinase inhibitor (Bayer, Leverkusen, Germany)]. Western blot analysis was carried out as described previously (15,16).

**Growth-inhibitory assay.** Inhibition of cell growth in the presence of chemotherapeutic agents was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma-Aldrich Co.) assay as described previously (15,17). Briefly, the cells were incubated for 72 h under various concentrations of GEM. After re-incubation for 4 h in MTT solution, acid-isopropanol was added to dissolve the resultant formazan crystals. The absorbance of the plate was measured in a microplate reader at a wavelength of 570 nm with a 650-nm reference and the results were expressed as the percentage of absorbance relative to untreated controls.

**Annexin V assay.** The binding of Annexin V was used as a sensitive method for measuring apoptosis, as described previously (15). Twenty-four hours after treatment, cells were stained with Annexin V-FITC and propidium iodide (PI) (BioVision Research Products, Mountain View, CA, USA) and analyzed on a FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA). For the assessment of apoptosis, Annexin V-positive and PI-negative cells and Annexin V-positive and PI-positive cells were considered as early apoptotic cells and late apoptotic cells, respectively.

**Cell cycle analysis.** Cell cycle analysis was performed based on flow cytometric analysis, as described previously (16,17). Briefly, PI and RNase (Sigma-Aldrich Co.) were added and data were acquired on the FACSCalibur (BD Biosciences). The cell cycle was analyzed using ModFIT software (BD Biosciences).

**Luciferase reporter assay.** To evaluate the activity of the Wnt/β-catenin signaling pathway, TCF/LEF transcriptional activity was examined. For the examination, the reporter assay kit (SA Biosciences, Frederick, MD, USA) was used according to the instructions provided by the manufacturer. In brief, cells were transiently transfected with the transcription factor-responsive reporter or negative control by the Lipofectamine 2000 reagent (Invitrogen). After the transfection, the cells were transfected with anti-miR-29a or its negative control oligonucleotide. After 48 h, luciferase activity was measured with the Dual-Luciferase Assay System (Promega, Madison, WI, USA) using luminometer, Lumat LB9507 (Berthold Technologies, Camburgacher, Germany). The Firefly luciferase activity, indicating TCF-dependent transcription, was normalized to the Renilla luciferase activity as an internal control to obtain the relative luciferase activity.
and AXIN2, CCND1, TACSTD1 and TCF3) in anti-miR-29a-transfected MIAPaCa-2 and PSN-1 cell lines by qRT-PCR. Suppression of miR-29a significantly reduced the mRNA expression of the targeted genes (Fig. 3C). Taken together, the results suggest that miR-29a activates the Wnt/β-catenin signaling pathway through the suppression of Dkk1, Kremen2 and sFRP2.

**Transfection of pre-miR-29a induces resistance to GEM.** To further assess the effects of miR-29a, pre-miR-29a was transfected into BxPC-3 and Panc-1, which expressed lower levels of miR-29a than the other cell lines (Fig. 1). Transfection of cells with pre-miR-29a increased miR-29a level compared to the control cells (Fig. 4A). The miR-29a-overexpressing cells were significantly more resistant to GEM than the control cells, as evident by the MTT assay (Fig. 4B).

**MiR-29a-induced resistance is mediated by Wnt/β-catenin signaling activation.** Finally, we analyzed the mechanism responsible for the miR-29a-induced resistance to GEM. We focused on the Wnt/β-catenin signaling pathway, based on the results reported by Kapinas et al (13). The addition of Wnt3a to the cultures of MIAPaCa-2 and PSN-1 resulted in the activation of Wnt/β-catenin signal in the cell lines (Fig. 5A). Furthermore, the MTT assay showed the Wnt3a-treated cells were more resistant to GEM (MIAPaCa-2; 25 ng/ml, PSN-1; 1.6 ng/ml, Fig. 5B). In addition, both the inactivated Wnt/β-catenin signal and the augmented growth-inhibitory effect by the afore-mentioned anti-miR-29a transfection were weakened after the addition of Wnt3a (Fig. 5). These findings suggest that activation of the Wnt/β-catenin signaling mediates, at least in part, the miR-29a-induced resistance to GEM.

**Discussion**

The present study demonstrated that the expression of miR-29a correlated significantly with the growth-inhibitory effect of GEM and that the Wnt/β-catenin signal mediates the miR-29a-induced resistance to GEM in pancreatic cancer cell lines. Kapinas et al reported previously that miR-29a activates the Wnt/β-catenin signal through direct regulation of the negative regulators of the signal, Dkk1, Kremen2 and sFRP2 (13). Other studies indicated that activation of the Wnt/β-catenin-signaling, which is observed in 65% of pancreatic cancer cases, also plays an important role in the proliferation and differentiation of stem cells and that some chemotherapeutic drugs often induce tumor cell death, but not cancer stem cells (18-22). Moreover, the Wnt/β-catenin signal was reported to correlate significantly with chemoresistance (16,23-25). Thus, the results of the present study are in agreement with the above previous reports.

On the other hand, the Wnt/β-catenin signaling pathway, which plays important roles in the development of various malignancies, cell proliferation and differentiation, has been also reported to correlate with chemoresistance (23-25).
Figure 2. Transfection of anti-miR-29a into MIAPaCa-2 and PSN-1. (A) qRT-PCR showed significant suppression of miR-29a in the transfected cells compared to the control cells (p<0.05). (B) MTT assay showed that the growth-inhibitory effects of GEM in the miR-29a-suppressed cells was significantly stronger than in the control cells (p<0.05). (C) Annexin V assay indicated that the percentages of early apoptotic cells and late apoptotic cells induced by GEM (MIAPaCa-2; 40 ng/ml, PSN-1; 2 ng/ml) in the miR-29a-suppressed cells were significantly higher than in the control cells (p<0.05). (D) Flow cytometric analysis of the cell cycle indicated that GEM treatment (MIAPaCa-2; 20 ng/ml, PSN-1; 1 ng/ml) resulted in accumulation of cells in the S phase among the miR-29a-suppressed cells compared to the control cells. Data are mean ± SD.
Figure 3. Evaluation of the influence of the Wnt/β-catenin signaling pathway in MIAPaCa-2 and PSN-1. (A) Western blot analysis demonstrated significant overexpression of Dkk1, Kremen2 and sFRP2 proteins in the anti-miR-29a-transfected cells compared to the control cells. (B) Luciferase reporter assay showed that TCF/LEF transcriptional activity was significantly lower in the miR-29a-suppressed cells than in the control cells (*p<0.05). (C) qRT-PCR demonstrated significantly lower expressions levels of five Wnt/β-catenin signaling targeted genes in the miR-29a-suppressed cells than in the control cells (*p<0.05). Data are mean ± SD.

Figure 4. Effects of transfection of pre-miR-29a into BxPC-3 and Panc-1. (A) Overexpression of miR-29a in the transfected cells was confirmed by qRT-PCR (*p<0.05). (B) MTT assay showed that the growth-inhibitory effects of GEM in the miR-29a-overexpressed cells was significantly weaker than in the control cells (*p<0.05). Data are mean ± SD.
fact, we reported previously the activation of the Wnt/β-catenin signaling pathway in HCC with poor response to interferon and 5-fluorouracil therapy (16). Recently, Kapinas et al (13) reported that miR-29a activates the Wnt/β-catenin signal by directly regulating Dkk1 (Dkk1), Kremen2 and secreted frizzled related protein 2 (sFRP2), which are negative regulators of the signal transduction, suggesting that miR-29a induces chemoresistance to chemotherapeutic agents through the activation of the Wnt/β-catenin signaling pathway.

We reported previously that RRM1 expression correlates significantly with the response to GEM (7,8). Therefore, in the present study, we also investigated the effects of anti-miR-29a transfection on RRM1 expression. The result showed no significant change in RRM1 expression after anti-miR-29a transfection (data not shown). Several investigators also reported that the expression of miRNAs correlates significantly with chemoresistance in several types of cancers (15,26,27). For example, we reported that the expression of miR-21 is associated with resistance to interferon and 5-fluorouracil in pancreatic cancer and cholangiocarcinoma cells (15,26,27).

Furthermore, since miRNA is associated with the response to GEM, miR-21 has been reported in some studies of pancreatic and other cancers (15,26,27). Therefore, in the present study, the effects of transfection of anti-miR-29a on miR-21 expression were also examined and the results showed no significant changes in miR-21 expression (data not shown). These results suggest that the chemoresistance induced by miR-29a is different from that related to RRM1 and miR-21.

To date, evidence suggests that miR-29a acts as an oncomiRNA as well as an anti-oncomiRNA (28-31). Xiong et al (28) reported that miR-29a promotes apoptosis and represses tumorigenicity in HCC cells, while the present study showed contradictory results. The reason for this contradiction remains unresolved, but it is speculated that miR-29a can act as either an oncomiRNA or an anti-oncomiRNA, depending on the tumor circumstances, suggesting that the exact role of miR-29a in cancer is still unclear and needs to be fully investigated in the future.

Several studies have reported that miR-29a is detected in the sera of patients with ovarian and colorectal cancers, suggesting its potential use as a biomarker for cancer detection (32,33). Confirmation of the present findings in larger population multicenter studies may allow the measurement of plasma levels of miR-29a to predict the clinical response to GEM in patients with pancreatic cancer.

In conclusion, the present study demonstrated a significant association between miR-29a expression and the response to GEM in pancreatic cancer cell lines by genetic manipulation experiments. The results showed that the miR-29a-induced resistance to GEM is mediated by activation of the Wnt/β-catenin signaling pathway. These findings suggest that miR-29a could be potentially used as a marker for the prediction of the clinical response to GEM and serves as a potential target for therapy against pancreatic cancer.

References