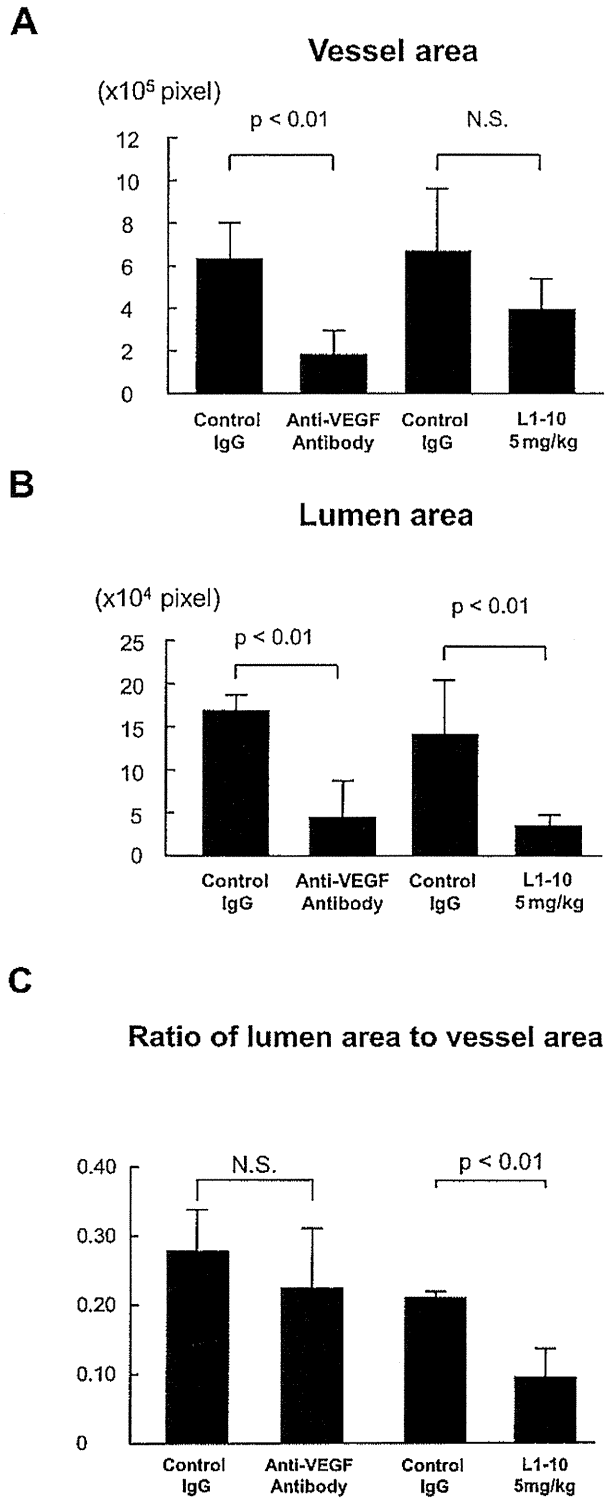

Figure 5. Image analysis for lumen formation. The outer contour of vessel or lumen was traced using the image analysis software, and the (A) vessel area, (B) lumen area, and (C) the ratio of lumen to vessel was estimated. The number of mice; anti-VEGF antibody (n=5) and the control group (n=3), L1-10 (n=5) and the control group (n=3).

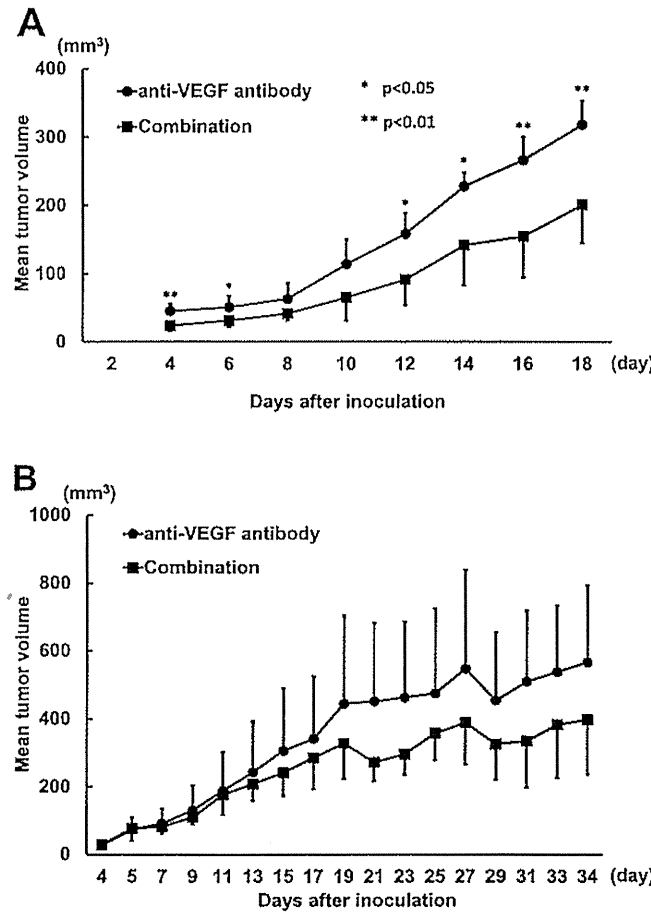

Figure 6. Growth curve of combination treatment. L1-10, anti-VEGF antibody, control IgG were administered along with routes, intervals and doses summarized in Table I. Combination treatment with early administration: injection of L1-10 and anti-VEGF antibody were started immediately after inoculation. Treatment continued to day 18. Mice were sacrificed at day 20. Combination treatment inhibited tumour growth. The decrease in tumour volume was statistically significant except for days 8 ($p=0.0895$) and 10 ($p=0.0558$) by t-test. * and ** represent p-value <0.05 and <0.01, respectively. Combination treatment with late administration: injection of L1-10 and anti-VEGF antibody were started 5 days after inoculation. Treatment was continued to day 31. Mice were sacrificed at day 34. No significant differences were observed between VEGF treatment and combination treatment in any time-point.

behaviour of HUVECs or levels of the proteins (Fig. 3). By *in vivo* system we found that Ang2 inhibition with treatment of L1-10 dose dependently decreased tumour growth. Furthermore, we found that L1-10 led to extension of the tumour-associated vessels whilst it suppressed formation of a sound lumen. Ratio of lumen area to vessel area was significantly decreased by L1-10 treatment compared to that of VEGF. The double staining of both endothelial cells and pericytes revealed that the endothelial cells were tightly covered with abundant pericytes in the tumour-associated vessels of L1-10-treated mice, when compared to control groups (Fig. 4B). Therefore, the difference in effects endowed by L1-10 could be the existence of both endothelial cells and pericytes *in vivo*, but not *in vitro* in which pericytes are lacking. Our data suggest that Angl/Ang2 balance plays an essential role in *in vivo* vascular differentiation. Thus, it is assumed that Angl/Ang2 balance may be shifted to Angl dominance by L1-10, which should facilitate recruitment of pericytes along the endothelial cells, considering established model on angiogenesis by Angs-Tie2 signaling (1,27). Indeed, several recent studies provided evidence that Ang2 inhibition causes an increase in pericyte-coverage over the endothelial cells (9,10,22).

We postulate that lack of pericytes in the *in vitro* system unables the demonstration of the relevance of Ang2 in vascular differentiation.

Several agents against Ang2, Ab536 (7) or anti-Ang2 monoclonal antibody 3.19.3 (8), or peptide antibody fusions including 2xCon4, L1-7, L1-10, CovX-Bodies (7,9,10,13,22,23) generally suppressed *in vivo* tumour growth implanted in nude mice. These studies provided histological features of the tumours such as deceased vessel density, increased apoptosis and pericyte coverage. We first report here that vascular endothelial cells were extended, but lacked lumen formation by L1-10 mediated Ang2 inhibition. One may suppose that such characterized phenotype is rather specific to the KM12SM cells. However, we consider that our finding is not unique to just the cell type because Chae *et al* reported that forced expression of Ang2 caused enlargement of vascular lumen in xenografts of U87 glioma cells (24), in which coverage of the endothelial cells with pericytes was rather diminished by overexpression of Ang2. Therefore, it is likely that *in vivo* vascular differentiation is highly dependent on endothelial cell-pericyte interaction through Ang2 mediated mechanism.

We found that combination of the anti-VEGF antibody and L1-10 enhanced tumour inhibitory effects as compared to anti-VEGF alone in nude mice. This is consistent with a recent report by Hashizume *et al* (22). Since histological analysis revealed that anti-VEGF or anti-Ang2 treatment caused a distinct inhibitory effect on formation of tumour-associated vessels, it is probable that the suppressive effects on KM12SM tumour could be through a tumour angiogenesis-mediated mechanism. VEGF inhibition is already clinically feasible when applied with chemotherapy including 5-FU/leucovorin, oxaliplatin and irinotecan. One of its mechanisms is thought to be normalization of tumour-associated vessels which facilitate the entry of chemo-agents to the tumour cells (28). With a view of pericyte-endothelial cell interaction, Ang2 inhibition would contribute to intense coverage of vascular endothelial cells with pericytes and this is in the line with anti-VEGF for vascular normalization in tumour tissues. In conclusion, we propose that Ang2 is essential to *in vivo* vascular differentiation.

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MicroRNA-29a induces resistance to gemcitabine through the Wnt/β-catenin signaling pathway in pancreatic cancer cells

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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 Bioresources (JCRB, Tokyo, Japan). BxPC-3 and Panc-1 cell lines were obtained from the American Type

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Key words: pancreatic cancer, gemcitabine, microRNA, miR-29a, Wnt/β-catenin signaling pathway

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% CO_2-95% 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'-GGTGTTTGAGG AGATCTGGG-3'; *AXIN2* reverse primer, 5'-TGCTCACAGC CAAGACAGTT-3'; *CCND1* forward primer, 5'-AAGGCCTG AACCTGAGGAG-3'; *CCND1* reverse primer, 5'-CTTGACTC CAGCAGGGCTT-3'; *MYC* forward primer, 5'-AAGAGGAC TTGTTGCGGAAA-3'; *MYC* reverse primer, 5'-CTCAGCCA AGGTTGTGAGGT-3'; *TACSTD1* forward primer, 5'-TCCAG AAAGAAGAGAATGGCA-3'; *TACSTD1* reverse primer, 5'-AAAGATGTCTTCGTCCCACG-3'; *TCF3* forward primer, 5'-ATCTGTGTCCCATGTCCCAG-3'; *TCF3* reverse primer, 5'-CCAGGGTAGGAGACTTGCAG-3'; and *PBGD* forward primer, 5'-TGTCTGGTAACGGCAATGCGGCTGCAAC-3'; *PBGD* reverse primer, 5'-TCAATGTTGCCACCACACTGT CCGTCT-3'.

Western blot analysis. Cells grown to semiconfluence 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 Trasylol, 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, Calmbacher, 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.

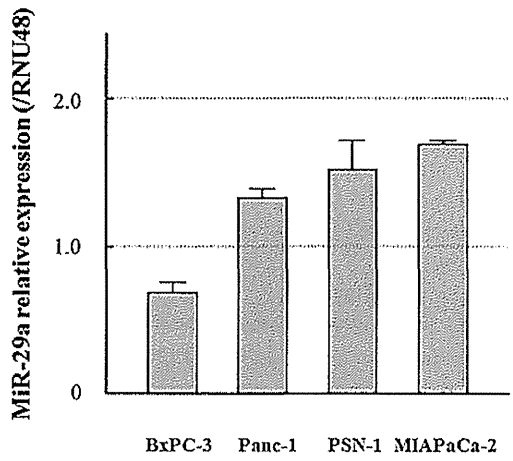

Figure 1. The expression level of miR-29a in four human pancreatic carcinoma cell lines including MIAPaCa-2, PSN-1, BxPC-3 and Panc-1. The miR-29a expression was normalized by the average expression of BxPC-3 with the lowest expression of miR-29a in the pancreatic cancer cell lines. Data are mean ± SD.

Statistical analysis. Data were expressed as mean ± SD. Clinicopathological parameters were compared using the χ^2 test and continuous variables were compared using the Student's t-test. A $p<0.05$ denoted the presence of a statistically significant difference. Statistical analysis was performed using the StatView software (SAS Institute Inc., Cary, NC, USA).

Results

Transfection of anti-miR-29a reduces resistance to GEM. All the four cell lines used in the present study expressed miR-29a though the level varied among the cells (Fig. 1). The relative expression of miR-29a was significantly lower in the transfected cells by qRT-PCR (Fig. 2A). To evaluate the effect of miR-29a on the response to GEM, we transfected anti-miR-29a in MIAPaCa-2 and PSN-1 cells, which showed higher miR-29a expression levels than the other cell lines. The MTT assay showed that cells transfected with anti-miR-29a were significantly less resistant to GEM compared to control cells (Fig. 2B). Next, we evaluated the extent of apoptosis of these cells at 24 h after treatment with GEM (MIAPaCa-2; 40 ng/ml, PSN-1; 2 ng/ml) by the Annexin V assay. The percentages of early and late apoptotic cells were significantly higher in the two cancer cell lines transfected with anti-miR-29a than in the control cells (Fig. 2C).

We also examined the influence of miR-29a on the cell cycle in MIAPaCa-2 and PSN-1. The distribution of cells in G_0/G_1 phase, S phase and G_2/M phase was similar between the miR-29a-suppressed cells and the control cells in the absence of GEM (Fig. 2D). However, 24 h after GEM treatment (MIAPaCa-2; 20 ng/ml, PSN-1; 1 ng/ml), the percentage of cells at the S phase among the miR-29a-suppressed cells was higher than the control cells (Fig. 2D).

MiR-29a activates Wnt/β-catenin signaling pathway. We examined next the expression levels of these molecules in MIAPaCa-2 and PSN-1. Western blot analysis showed significantly higher protein expression levels of these molecules in the anti-miR-29a-transfected cells compared with the control cells (Fig. 3A). Furthermore, the luciferase reporter assay showed that TCF/LEF transcriptional activity, representing the activity of the Wnt/β-catenin signaling pathway, was significantly lower in the miR-29a-suppressed cells than in the control cells (Fig. 3B). We also examined the expression of five Wnt/β-catenin signaling targeted genes (*AXIN2, CCND1, MYC, 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). In

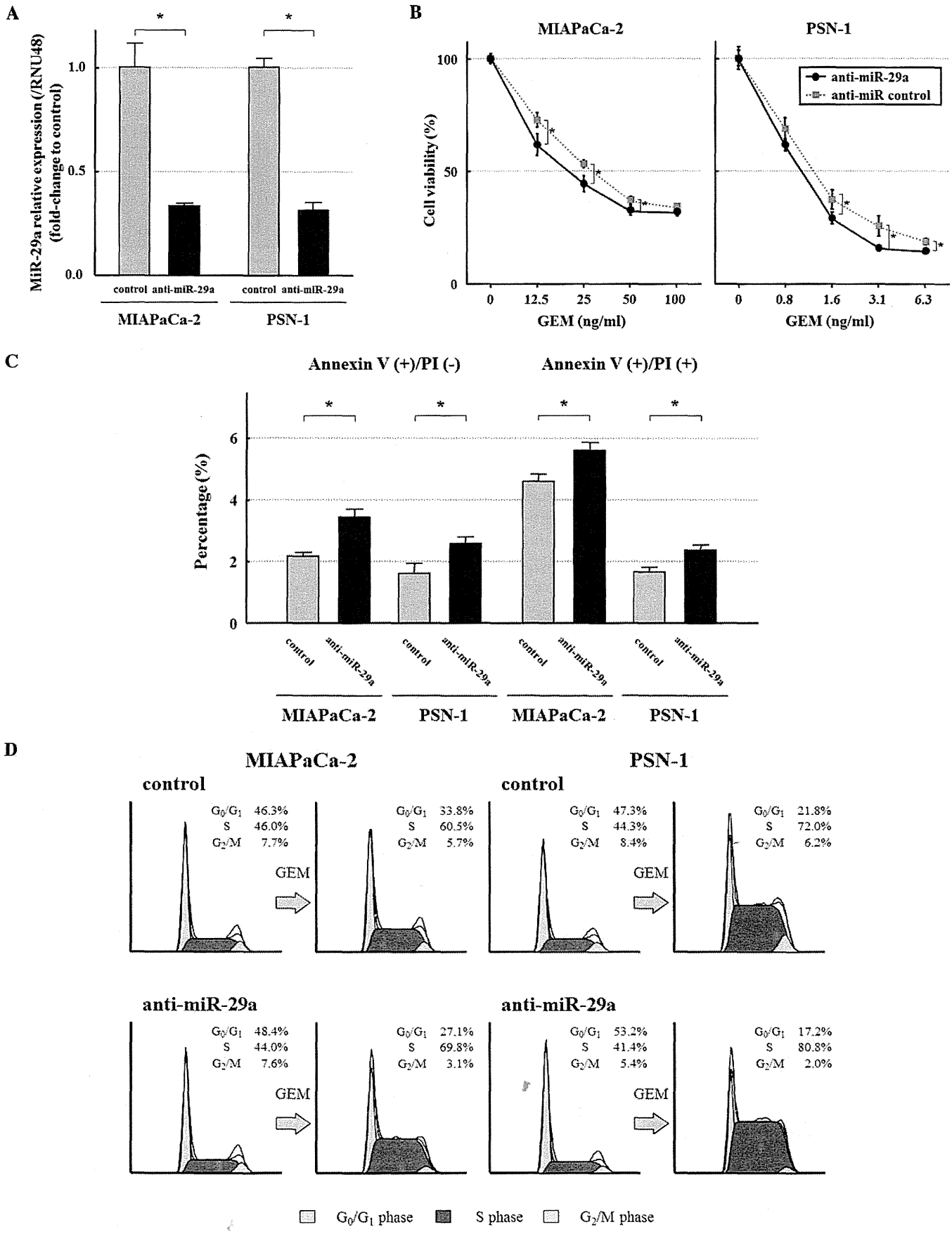

Figure 2. Transfection of anti-miR-29a into MIAPaCa-2 and PSN-1. (A) qRT-PCR showed significant suppression of miR-29a in the transfected cells compared to the control cells (*p<0.05). (B) MTT assay showed that the growth-inhibitory effects of GEM in the miR-29a-suppressed cells was significantly stronger 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.

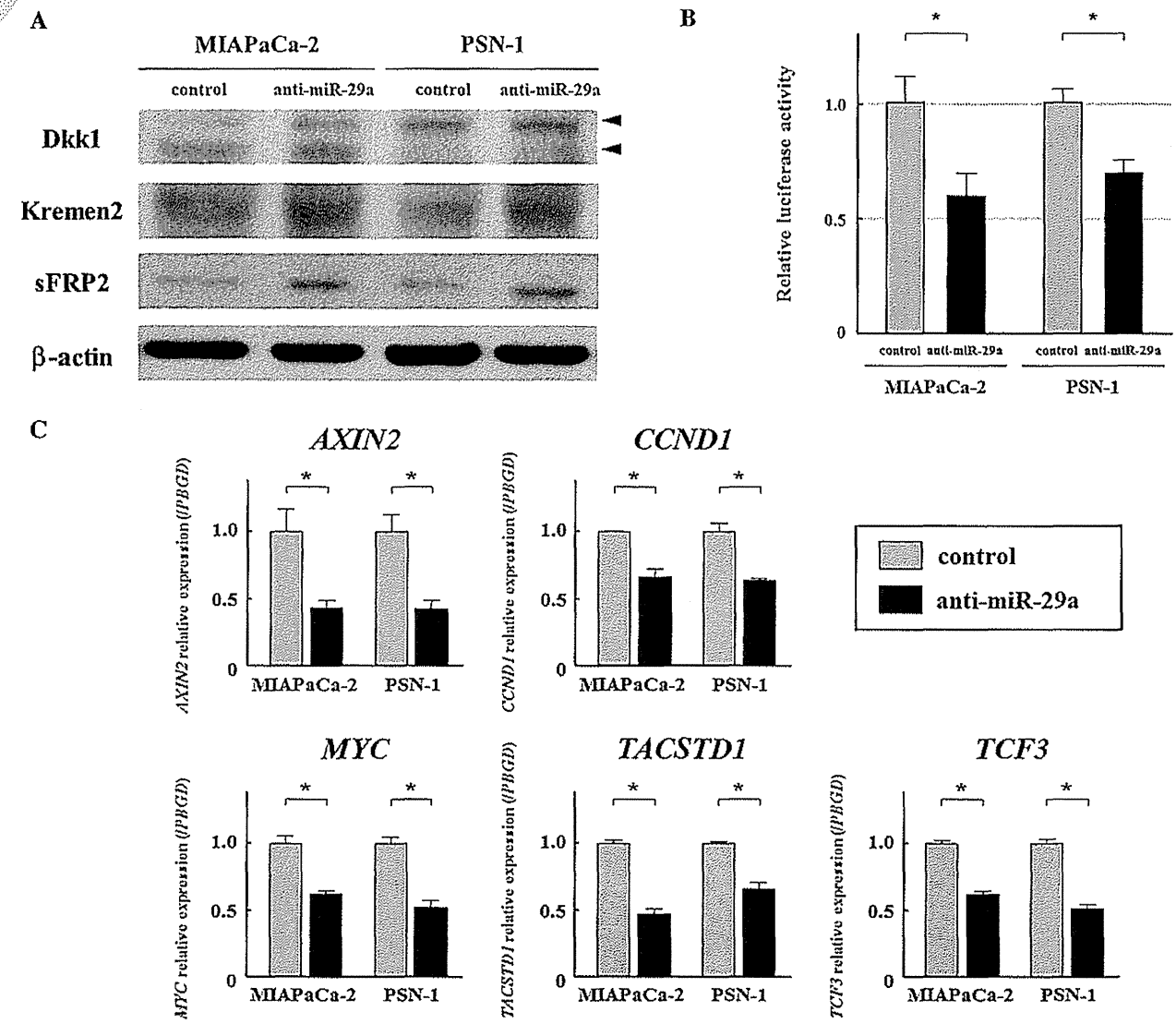

Figure 3. Evaluation of the influence of the Wnt/β-catenin signaling pathway in MIAPaCa-2 and PSN-1. (A) Western blot analysis demonstrated significant overexpression of Dkk1, Kremen2 and sFRP2 proteins in the anti-miR-29a-transfected cells compared to the control cells. (B) Luciferase reporter assay 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.

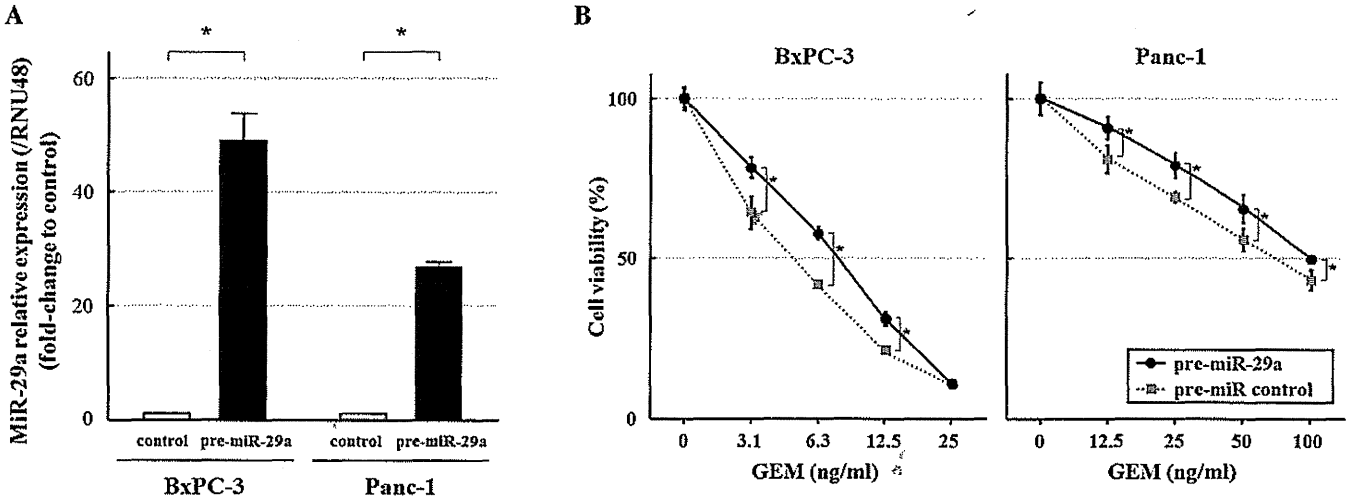

Figure 4. Effects of transfection of pre-miR-29a into BxPC-3 and Panc-1. (A) Overexpression of miR-29a in the transfected cells was confirmed by qRT-PCR (*p<0.05). (B) MTT assay showed that the growth-inhibitory effects of GEM in the miR-29a-overexpressed cells was significantly weaker than in the control cells (*p<0.05). Data are mean ± SD.

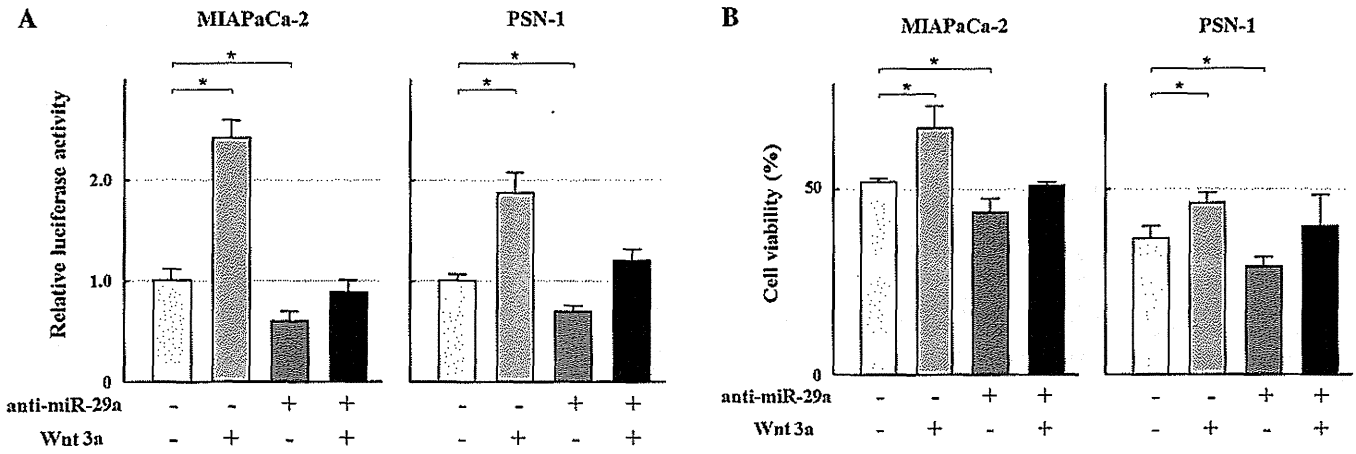

Figure 5. Changes in Wnt/β-catenin signaling activity and growth-inhibitory effects of GEM after anti-miR-29a transfection and/or Wnt3a addition. MTT assay indicated a weaker growth-inhibitory effect for GEM (MIAPaCa-2; 25 ng/ml, PSN-1; 1.6 ng/ml) following the addition of Wnt3a at 50 ng/ml and that the strengthened growth-inhibitory effect by anti-miR-29a transfection was also weakened after the addition of Wnt3a (*$p<0.05$). 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 Dikkopf-1 (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 hepatocellular carcinoma (HCC) (13), while others indicated that such expression is associated with response to GEM 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.

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Subcuticular sutures versus staples for skin closure after open gastrointestinal surgery: a phase 3, multicentre, open-label, randomised controlled trial

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Summary

Background Staples have been widely used for skin closure after open gastrointestinal surgery. The potential advantages of subcuticular sutures compared with staples have not been assessed. We assessed the differences in the frequency of wound complications, including superficial incisional surgical site infection and hypertrophic scar formation, depending on whether subcuticular sutures or staples are used.

Methods We did a multicentre, open-label, randomised controlled trial at 24 institutions between June 1, 2009, and Feb 28, 2012. Eligible patients aged 20 years or older, with adequate organ function and undergoing elective open upper or lower gastrointestinal surgery, were randomly assigned preoperatively to either staples or subcuticular sutures for skin closure. Randomisation was done via a computer-generated permuted-block sequence, and was stratified by institution, sex, and type of surgery (ie, upper or lower gastrointestinal surgery). Our primary endpoint was the incidence of wound complications within 30 days of surgery. Analysis was done by intention to treat. This study is registered with UMIN-CTR, UMIN000002480.

Findings 1080 patients were enrolled and randomly assigned in a one to one ratio: 562 to subcuticular sutures and 518 to staples. 1072 were eligible for the primary endpoint and 1058 for the secondary endpoint. Of the 558 patients who received subcuticular sutures, 382 underwent upper gastrointestinal surgery and 176 underwent lower gastrointestinal surgery. Wound complications occurred in 47 of 558 patients (8·4%, 95% CI 6·3–11·0). Of the 514 who received staples, 413 underwent upper gastrointestinal surgery and 101 underwent lower gastrointestinal surgery. Wound complications occurred in 59 of 514 (11·5%, 95% CI 8·9–14·6). Overall, the rate of wound complications did not differ significantly between the subcuticular sutures and staples groups (odds ratio 0·709, 95% CI 0·474–1·062; p=0·12).

Interpretation The efficacy of subcuticular sutures was not validated as an improvement over a standard procedure for skin closure to reduce the incidence of wound complications after open gastrointestinal surgery.

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Introduction

Wound complications are among the most common issues reported after surgery, and are often very problematic for patients in terms of cosmetic appearance, decreased quality of life, prolonged hospital stays, and increased health-care costs.[1,2] Several publications have addressed ways to reduce the risk of wound complications associated with surgery,[3–6] such as intraoperative administration of antimicrobial prophylaxis,[4,5] skin preparation, barrier retractional wound protection,[7] use of absorbable sutures during intraperitoneal procedures,[8,9] and pulsatile lavage irrigation of wounds before closure.[10,11] Triclosan-coated sutures significantly reduced the rate of surgical site infections compared with conventional uncoated sutures in various types of surgery.[12]

Because of the increase in the number of patients with preoperative comorbidities that are risk factors for wound complications, such as malnutrition,[13] diabetes mellitus,[14] and obesity,[15] new, innovative approaches will be necessary to decrease the risk of wound complications after surgery. Subcuticular suturing for skin closure is an attractive alternative for skin approximation in most types of surgery. It is often used in plastic surgery because of the low incidence of wound complications and good cosmetic appearance.[16–18] Compared with staples, several clinical trials have shown that subcuticular sutures are associated with a significantly lower incidence of wound complications and better cosmetic results after orthopaedic surgery,[19] cardiovascular surgery,[20,21] and caesarean section.[22,23]

In 242 patients undergoing coronary artery bypass graft surgery, Johnson and colleagues[24] prospectively closed half of each sternal and saphenous vein harvest wound with staples and half with intradermal sutures. The incidence of wound infection was similar with both methods, but significantly fewer wound complications were noted with subcuticular sutures than with staples. Additionally, patients who expressed a preference preferred sutures to staples. Basha and investigators[25] randomly assigned 435 patients undergoing caesarean delivery to stainless steel staples or subcuticular 4-0 monocryl sutures. They

reported that staple closure was associated with a four-times increased risk of wound separation (adjusted odds ratio [OR] 4·66, 95% CI 2·07–10·52; p<0·001) and poor patient satisfaction.

These trials had been done for class 1 surgical procedures—ie, clean surgery. However, the benefit of subcuticular sutures in gastrointestinal surgery, a class 2 (clean-contaminated) surgery that is associated with a high incidence of wound complications,[15,26,27] has not been fully examined.[28] Staples are the most commonly used technique for skin closure during gastrointestinal surgery because of convenience and speed. Because no consensus has been reached about how to apply findings from class 1 surgery to class 2 surgery, an optimum method of skin for gastrointestinal surgery remains to be established.

We investigated differences in prevention of wound complications between subcuticular sutures and staples after elective upper and lower gastrointestinal open surgery.

Methods

Study design and participants

We did a large-scale, multicentre, open-label, phase 3 randomised controlled trial at 24 institutions in Japan from June 1, 2009, to Feb 28, 2012. The study was organised by the Clinical Study Group of Osaka University on Risk Management (OSGO-RM), which is composed of hospitals affiliated from the Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University.

Eligible patients were undergoing elective upper or lower gastrointestinal surgery, aged 20 years or older, and had adequate organ function. Patients undergoing abdominoperineal resection for rectal cancer were also eligible, but we only assessed abdominal wounds for outcomes. We excluded patients needing emergency or laparoscopic surgery, with a history of laparotomy with a midline incision, or with long-term corticosteroid use; active infection such as peritonitis, pneumonia, or urinary tract infection; massive ascites; coagulopathy or other disorders that would preclude study participation; uncontrolled or insulin-treated diabetes; mental illness, poor general condition; severe cardiopulmonary disease; or who were deemed by surgeons to be inappropriate for participation in a randomised trial. The institutional review board of each hospital approved the protocol. All patients provided written informed consent before randomisation. We did not collect data on the number of patients approached and assessed for eligibility.

Randomisation and masking

Patients were recruited by the investigators and treatment allocation was made preoperatively after confirming eligibity.

Enrolment was done through a web-based system established for this trial and randomisation by a computer-generated permuted-block sequence. The size of the blocks used for randomisation was four. Patients were randomly assigned (1:1) to either subcuticular sutures or staples for skin closure and balanced according to institution, sex, and type of surgery (ie, upper or lower gastrointestinal open surgery). Investigator surgeons were informed of the treatment allocation via the internet and did the procedures. Patients and investigators were not masked to group assignment. The data centre, based at the Multicenter Clinical Study Group at Osaka University was responsible for treatment allocation, central monitoring, and statistical analyses under the supervision of the statistician in charge.

Procedures

In the subcuticular suture group, surgeons used interrupted subcuticular sutures with 3-0 or 4-0 monofilament absorbable suture (polydioxanone; PDS-II Ethicon, Tokyo, Japan). The interval of the sucuticular sutures was 15–25 mm and the length of the bite of sutures was 15–25 mm from the edge of the skin. Under this condition, the skin could be closed tightly. Use of sterile strips or skin glue for epidermal approximation in addition to subcuticular sutures was an institutional choice. In the staples group, metallic skin staples, which were the choice of individual institutions, 10–15 mm apart were used. Approximation of the fat layer was not allowed in the either group. Before the trial, investigators from participating institutions were instructed on how to do subcuticular sutures during the trial. A video in which a plastic surgeon used the subcuticular suturing technique (adopted as the standard) was provided to each participating institution. The standard procedure was also demonstrated at each investigator meeting. Investigators and physicians in training met yearly to examine how subcuticular sutures were done.

All participating institutions were asked to follow the guidelines about prevention of surgical site infections issued by the US Centers for Disease Control and Prevention (CDC).[29] Surgical gloves and instruments were changed before wound closure. Absorbable monofilament sutures were used for approximation of the fascia, and the subcutaneous space was irrigated with saline without added antibiotics. Intra-abdominal drain placement through a separate incision away from the operative incision was permitted but drainage of the wound was not allowed. Skin preparation techniques, prophylactic antibiotic administration, the volume of saline used for intra-abdominal irrigation, dressing methods, and timing of postoperative staple removal, perioperative care, and wound management were according to each participating institution's respective standards.

Our primary outcome was incidence of wound complications within 30 days of surgery. The secondary outcome was the incidence of hypertrophic scar formation 6 months after surgery. Wound complications were defined as the presence of at least one of several signs or symptoms necessitating treatment: wound disruption,

stitch abscess, abscess caused by metal allergy, seroma or haematoma, or superficial incisional surgical site infections. Superficial incisional surgical site infections are defined by the CDC[29] as infections occurring within 30 days of surgery that implicate only the skin or subcutaneous tissue of the incision. Diagnosis of superficial incisional surgical site infection must satisfy one or more of several criteria: purulent drainage (with or without laboratory confirmation) from the superficial incision, organisms isolated from an aseptically obtained culture of fluid or tissue from the superficial incision, or at least one of the signs or symptoms of infection (pain or tenderness, localised swelling, redness or heat, and superficial incision deliberately opened by the surgeon, unless the incision is culture-negative). Infection control personnel monitored and detected surgical site infections during patients' hospital stays. Changes noted in the wound were not defined as wound complications if they did not necessitate treatment. When superficial incisional surgical site infections and other wound complications coexisted in the same patient, we defined the complication as superficial incisional surgical site infections. We defined hypertrophic scar as a widened or elevated unsightly scar with erythema or pigmentation.

Responsible surgeons checked for the presence or absence of wound complications every day during the hospital stay and at every outpatient visit until 30 days after surgery. They were also responsible for checking for the presence or absence of hypertrophic scar formation at 6 months after surgery, and measured the width and length of detected hypertrophic scars. Before starting the trial, the principal investigator showed typical cases of various wound complications and hypertrophic scars, and consensus about all types of wound complications was reached by the investigators.

Statistical analysis

We planned a sample size of 530 patients per treatment group when we designed the trial. Such a sample size would provide power of 80% with a two-sided significance level of 0·05 to detect superiority in the reduction of the frequency of wound complications. Wound complications were anticipated in 11% of patients in the staples group and 6% in the subcuticular sutures group, allowing for a loss to follow-up of roughly 10%. The projected accrual period was 2 years and no interim analyses were planned.

We did the analysis on a modified intention-to-treat basis. We expressed continuous numerical data as medians and IQRs or means and SDs, when appropriate, and distribution of dichotomous data in percentages with 95% CIs. We used Fisher's exact test to compare binary variables and the Mann-Whitney *U* test to compare continuous variables. All p values of less than 0·05 were deemed significant.

The primary outcome was analysed with Fisher's exact test, and we used the Mantel-Haenszel test to adjust for the type of surgery, a potential confounding factor, which was not prespecified in the protocol. We used Fisher's exact test to analyse the secondary outcome and to calculate and compare outcomes as a post-hoc analysis on the basis of type of surgery.

We analysed thickness of subcutaneous fat (objectively classified by the surgeon as either thin, normal, or thick), American Society of Anesthesiologists (ASA) physical status classification,[30] operative time, intraoperative blood loss volume, duration of prophylactic antibiotics, presence of drainage tube and duration of drainage, and use of postoperative anticoagulant therapy as variables. Subgroups were analysed with logistic regression to assess for statistical interactions between treatments in various subgroups. Because of the exploratory nature of subgroup comparisons, we report test results without multiplicity adjustments for type I error. This study is registered with UMIN-CTR, UMIN000002480. UMIN-CTR is one of the network members of the Japan Primary Registries Network, which meets WHO registry criteria.

Role of the funding source

The sponsor had no roles in the study design; data collection, analysis, or interpretation; or writing of the Article. The corresponding author had full access to all

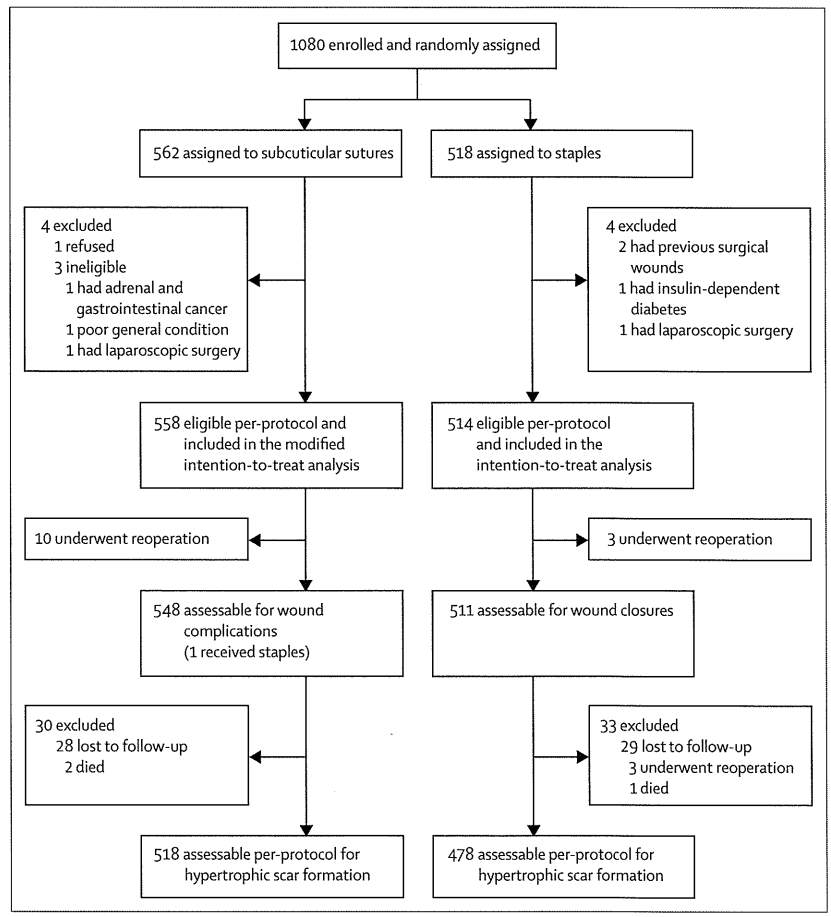

Figure 1: **Trial profile**

	Subcuticular sutures (n=562)	Staples (n=518)
Age (years)	68 (61–75)	68 (61–74)
Sex		
Male	388 (69·0%)	365 (70·5%)
Female	174 (31·0%)	153 (29·5%)
Surgery		
Upper gastrointestinal	385 (68·5%)	417 (80·5%)
Lower gastrointestinal	177 (31·5%)	101 (19·5%)
Thickness of subcutaneous fat*		
Thick	143 (25·6%)	109 (21·2%)
Standard	328 (58·8%)	317 (61·6%)
Thin	87 (15·6%)	89 (17·3%)
ASA physical status classification*		
1	201 (36·0%)	201 (39·0%)
2	313 (56·1%)	273 (53·0%)
3	44 (7·9%)	41 (8·0%)
Operative time (min)*	228 (180–270)	218 (175–264)
Blood loss (mL)	230 (100–430)	244 (120–450)
Wound protection†		
Surgical drape	535 (95·9%)	497 (96·3%)
Gauze	10 (1·8%)	6 (1·2%)
None	13 (2·3%)	13 (2·5%)
Duration of antibiotic prophylaxis (days)*		
1	379 (67·9%)	373 (72·4%)
2	33 (5·9%)	22 (4·3%)
3	100 (17·9%)	86 (16·7%)
≥4	46 (8·2%)	34 (6·6%)
Duration of drain insertion (days)*		
0 (ie, no drain)	118 (21·1%)	108 (21·0%)
1–3	50 (9·0%)	34 (6·6%)
≥4	390 (69·9%)	373 (72·4%)
Duration of hospital stay after surgery (days)	14 (11–21)	15 (12–21)
Anticoagulation therapy‡		
Yes	130 (23·3%)	96 (18·6%)
No	429 (76·7%)	420 (81·4%)

Data are n (%) or median (IQR). ASA=American Society of Anesthesiologists.[30] *Data missing for four patients in the subcuticular sutures group and three patients in the staples group. †Data missing for four patients in the subcuticular sutures group and two patients in the staples group. ‡Data missing for three patients in the subcuticular sutures group and two patients in the staples group.

Table 1: **Baseline demographic and clinical characteristics**

the data and was responsible for the decision to submit for publication.

Results

Figure 1 shows the trial profile. 1080 patients from 24 institutions were enrolled and randomly assigned—562 to subcuticular sutures and 518 to staples. Assessment of case report forms showed that four patients in each group were ineligible for inclusion, and thus the modified intention-to-treat population comprised 558 patients in the subcuticular sutures group and 514 in the staples group

	Subcuticular sutures (n=385)	Staples (n=417)
Diseases		
Gastric cancer	375 (97·4%)	403 (96·6%)
Gastric submucosal tumour	6 (1·6%)	9 (2·2%)
Other	4 (1·0%)	5 (1·2%)
Procedures		
Total gastrectomy	149 (38·7%)	143 (34·3%)
Distal gastrectomy	186 (48·3%)	219 (52·5%)
Proximal gastrectomy	19 (4·9%)	16 (3·8%)
Exploratory laparotomy	4 (1·0%)	4 (1·0%)
Other	27 (7·0%)	35 (8·4%)

Data are n (%).

Table 2: **Types of diseases and surgical procedures in patients undergoing upper gastrointestinal surgery**

	Subcuticular sutures (n=177)	Staples (n=101)
Diseases		
Colon cancer	98 (55·4%)	51 (50·5%)
Rectal cancer	71 (40·1%)	48 (47·5%)
Anal cancer	2 (1·1%)	1 (1·0%)
Other	6 (3·4%)	1 (1·0%)
Procedures		
Right hemicolectomy	41 (23·2%)	28 (27·7%)
Left hemicolectomy	44 (24·9%)	8 (7·9%)
Low anterior resection	61 (34·5%)	38 (37·6%)
Abdominoperineal resection	11 (6·2%)	10 (9·9%)
Partial resection of colon	9 (5·1%)	10 (9·9%)
Other	11 (6·2%)	7 (6·9%)

Data are n (%).

Table 3: **Types of diseases and surgical procedures in patients undergoing lower gastrointestinal surgery**

(figure 1). Ten patients in the subcuticular sutures group and three in the staples group needed reoperation within 30 days, which met the exclusion criterion, a history of laparotomy, and thus were not assessed for wound complications.

Distribution of most demographic and clinical characteristics of enrolled patients was balanced between groups except type of surgery (table 1). Tables 2 and 3 show details of the diseases and surgical procedures in the two groups. 417 patients who underwent upper gastrointestinal surgery were allocated to the staples group and 385 to the subcuticular sutures group, and 177 patients who underwent lower gastrointestinal surgery were allocated to the subcuticular sutures group and 101 to the staples group.

In the subcuticular sutures group, wound complications occurred in 47 of 558 (8·4%, 95% CI 6·3–11·0) patients, including 36 (6·4%, 4·6–8·8) patients with superficial incisional surgical site infections. In the staples group, wound complications occurred in 59 of 514 patients

	All patients				Upper gastrointestinal surgery				Lower gastrointestinal surgery			
	Subcuticular suture (n=558)	Staples (n=514)	Odds ratio (95% CI)	p	Subcuticular sutures (n=382)	Staples (n=413)	Odds ratio (95% CI)	p	Subcuticular sutures (n=176)	Staples (n=101)	Odds ratio (95% CI)	p
Primary outcome												
Wound complication rate*	47 (8·4%)	59 (11·5%)	0·709 (0·474–1·062)	0·12	29 (7·6%)	39 (9·4%)	0·788 (0·459–1·339)	0·38	18 (10·2%)	20 (19·8%)	0·463 (0·217–0·978)	0·0301
Component outcomes												
Surgical site infection (superficial incisional)	36 (6·4%)	36 (7·0%)	0·928 (0·558–1·543)	0·81	23 (6·0%)	20 (4·8%)	1·259 (0·649–2·461)	0·53	13 (7·4%)	16 (15·8%)	0·425 (0·179–0·992)	0·0399
Non-surgical-site infection	11 (2·0%)	23 (4·5%)	0·435 (0·189–0·940)	0·0238	6 (1·6%)	19 (4·6%)	0·331 (0·107–0·875)	0·0149	5 (2·8%)	4 (4·0%)	0·710 (0·149–3·666)	0·73
Wound separation	3 (0·5%)	8 (1·6%)	0·346 (0·059–1·453)	0·13	1 (0·3%)	6 (1·5%)	0·178 (0·004–1·480)	0·13	2 (1·1%)	2 (2·0%)	0·570 (0·041–7·979)	0·62
Seroma	5 (0·9%)	12 (2·3%)	0·383 (0·105–1·179)	0·09	3 (0·8%)	11 (2·7%)	0·290 (0·052–1·108)	0·06	2 (1·1%)	1 (1·0%)	1·149 (0·059–68·457)	1·00
Haematoma	1 (0·2%)	2 (0·4%)	0·466 (0·008–8·969)	0·61	0 (0·0%)	1 (0·2%)	..	..	1 (0·6%)	1 (1·0%)	0·573 (0·007–45·300)	1·00
Other	2 (0·4%)	1 (0·2%)	1·867 (0·097–110·358)	1·00	2 (0·5%)	1 (0·2%)	2·166 (0·112–128·141)	0·61	0 (0·0%)	0 (0·0%)	..	..

Significance was calculated with Fisher's exact test. *Adjusted odds ratio 0·658 (95% CI 0·438–0·988; p=0·0438 [calculated with Mantel-Haenszel test]).

Table 4: **Primary outcome and its components in modified intention-to-treat population**

(11·5%, 8·9–14·6), including 36 (7·0%, 5·0–9·6) with superficial incisional surgical site infections (table 4). As a primary outcome, the number of wound complications did not differ significantly between the two groups (OR 0·709, 95% CI 0·474–1·062; p=0·12). Since we identified confounding with the stratified factor, type of surgery, adjustment was done to show a significant difference (0·658, 0·438–0·988; p=0·0438), although this was not prespecified.

Post-hoc exploratory analyses showed that wound complications excepting surgical site infections occurred significantly less often in the subcuticular suture group than in the staples group overall (OR 0·435, 95% CI 0·189–0·940; p=0·0238) and in patients who underwent upper gastrointestinal surgery (0·331, 0·107–0·875; p=0·0149). In patients who underwent lower gastrointestinal surgery, significantly fewer wound complications (0·463, 0·217–0·978; p=0·0301) and superficial incisional surgical site infections (0·425, 0·179–0·992; p=0·0399) were noted in the subcuticular sutures than in the staples group (table 4).

Table 5 summarises secondary outcomes. Significantly fewer hypertrophic scars formed in the subcuticular sutures group than in the staples group overall (OR 0·726, 0·528–0·998; p=0·0429) and specifically in patients who underwent upper gastrointestinal surgery (0·672, 0·465–0·965; 0·0282).

We did a post-hoc subset analysis to identify potential interactions between wound complications and background factors (figure 2). Significant risk reduction for wound complications was noted with subcuticular sutures compared with staples in male patients (*vs* female patients), lower gastrointestinal surgery (*vs* upper gastrointestinal surgery), cases with operative time of 220 min or greater (*vs* those with operative times <220 min), and patients receiving postoperative anticoagulant therapy (*vs* those not receiving such therapy). We did not identify any important treatment-related adverse events for stapling or subcuticular sutures.

	n	Hypertrophic scar formation	Odds ratio (95% CI)	p
All patients			0·726 (0·528–0·998)	0·0429
Subcuticular sutures	558	93 (16·7%)		
Staples	514	111 (21·6%)		
Upper gastrointestinal surgery			0·672 (0·465–0·965)	0·0282
Subcuticular sutures	382	66 (17·3%)		
Staples	413	98 (23·7%)		
Lower gastrointestinal surgery			1·226 (0·576–2·729)	0·72
Subcuticular sutures	176	27 (15·3%)		
Staples	101	13 (12·9%)		

Data for hypertrophic scar formation are n (%). Significance was calculated with Fisher's exact test.

Table 5: **Secondary outcomes in the modified intention-to-treat population**

Discussion

Subcuticular sutures for skin closure have been advocated instead of staples in clean (class 1) surgery, including cardiovascular surgery,[24] orthopaedic surgery,[19] and caesarean delivery,[25] on the basis of the results of randomised studies. Whether these results can be applied to class 2 surgery, as represented by gastrointestinal surgery, is of concern. Classification of the types of surgery is described in panel 1. Our results show that subcuticular sutures did not significantly reduce the frequency of

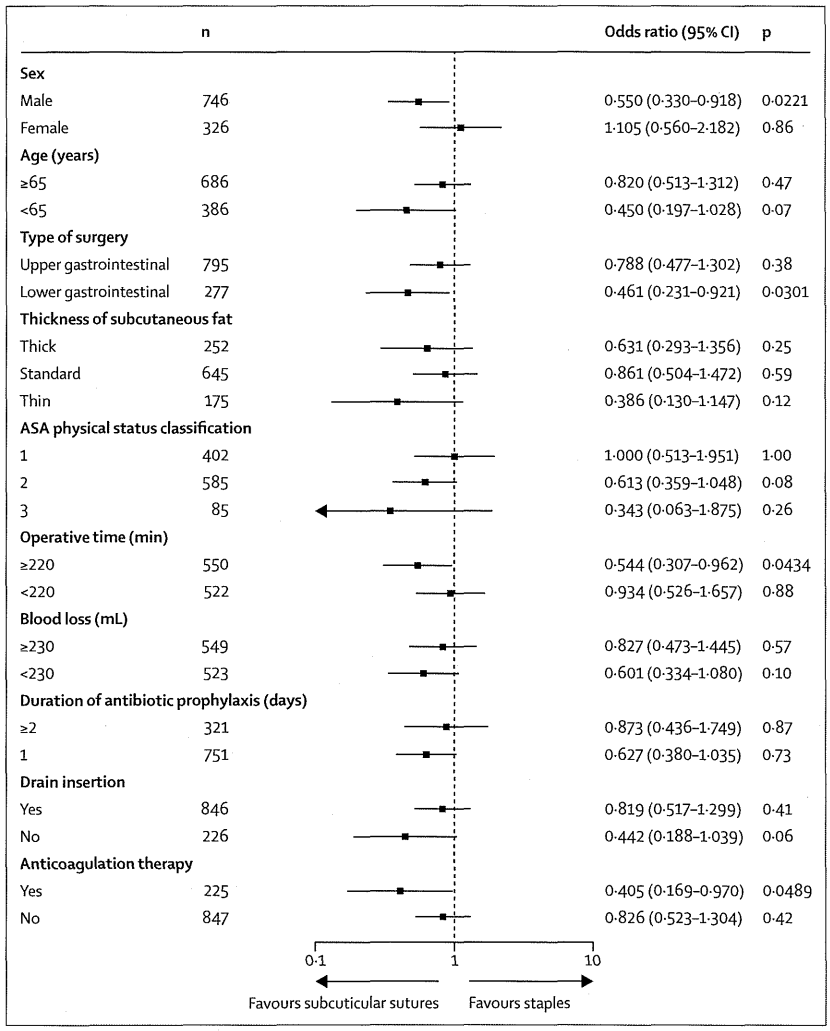

Figure 2: **Subset analysis of wound complications in the modified intention-to-treat population**
Significance was calculated with Fisher's exact test. ASA=American Society of Anesthesiologists.

wound complications as a primary outcome and therefore subcuticular sutures are not validated as a new standard procedure for skin closure after gastrointestinal surgery (panel 2). As a secondary outcome, we noted fewer hypertrophic scars formed when subcuticular sutures were used than when staples were used.

Our sample size calculation was done on the assumption that the incidence of wound complications was 7·5% with upper gastrointestinal surgery and 15% with lower gastrointestinal surgery when staples were used and the expected number of patients receiving the respective surgery was equal (1:1), which gave the incidence of wound complications as 11%. We postulated that a 5% reduction of the incidence of wound complications by subcuticular sutures was necessary to be a new standard procedure for skin closure. There are several reasons why we did not obtain the results we expected. We showed that the incidences of wound complications were 8·4% in the subcuticular sutures group and 11·5% in the staples group (p=0·12) in all patients, 7·6% and 9·4% (p=0·38) in upper gastrointestinal surgery, and 10·2% and 19·8% (p=0·0301) in lower gastrointestinal surgery (table 4). Subcuticular sutures were more effective in lower gastrointestinal surgery, whereas enrolment of patients receiving open lower gastrointestinal surgery was substantially lower than that of patients receiving open upper gastrointestinal surgery (278 *vs* 802) because laparoscopic surgery has become more prevalent in lower gastrointestinal surgery. Although we included type of surgery as one of our stratification variables, more patients who underwent lower gastrointestinal surgery received subcuticular sutures than staples (177 *vs* 101) and more patients who underwent upper gastrointestinal surgery received staples than subcuticular sutures (417 *vs* 385) as a result of the unexpected unbalanced allocation (tables 2, 3), which might be caused by participation of many institutions and the presence of three stratification factors. These factors attenuated the postulated effect of subcuticular sutures and the analysis of the primary outcome did not reach significance. When adjusting for the type of surgery, subcuticular sutures seemed to confer a benefit, although this result is not conclusive. Thus, preferential use of subcuticular sutures might be supported in some circumstances. Although we did not analyse outcomes of individual institutions, there was possibility of heterogeneity with regard to the effect of subcuticular sutures caused by as many as 24 institutions.

Before this trial, few data for potential differences in the rate of wound complications and hypertrophic scar formation between upper and lower gastrointestinal surgery were available. That the incidence of superficial incisional surgical site infections was higher with lower gastrointestinal surgery than with upper gastrointestinal surgery had been previously reported,[15] which was the reason why we used type of surgery as a stratification factor. We showed that the incidence of total wound complications and superficial incisional surgical site infections was significantly higher in lower than in upper gastrointestinal surgery, whereas the incidence of

***Panel 1:* Classification of types of surgery (class 1 and 2)[29]**

Class 1 (clean)
An uninfected operative wound in which no inflammation is encountered and the respiratory, alimentary, genital, or uninfected urinary tract is not entered. For example, skin procedures (ie, biopsies), simple orthopaedic surgery, vascular surgery, and elective caesarean section.

Class 2 (clean-contaminated)
An operative wound in which the respiratory, alimentary, genital, or urinary tracts are entered under controlled conditions and without unusual contamination. For example, gastrointestinal surgery, thoracic procedures, gynaecological procedures, and emergency caesarean section.

hypertrophic scar formation was higher in upper than in lower gastrointestinal surgery. Subcuticular sutures reduced the incidence of wound complications compared with staples in lower gastrointestinal surgery and the formation of hypertrophic scars in upper gastrointestinal surgery, possibly because of the higher number of events of those types in these types of surgery, respectively.

Subset analysis showed that subcuticular sutures resulted in significantly fewer wound complications in some subgroups, such as lower gastrointestinal surgery, longer operative time, and postoperative anticoagulant therapy, and the frequency of wound complications in almost all subsets of patients was lower in the subcuticular sutures group than in the staples group.

It is reasonable to employ subcuticular sutures in other types of gastrointestinal surgery, especially hepatobiliary or pancreatic surgery, which exert extensive surgical stress and are associated with large volumes of blood loss, long operative times, and a high incidence of surgical site infections.[41,42] We did not include hepatobiliary or pancreatic surgery in this trial because they contain a wide variety of surgical procedures and different levels of surgical site infection rates. The results of our subset analysis imply that subcuticular sutures could be applied to other types of gastrointestinal surgery and might reduce wound complications.[43]

We persuaded investigators to follow the US national surgical infection prevention guidelines, which recommend that antibiotic prophylaxis should be discontinued within 24 h of surgery.[4] As a result, 67·9% in the subcuticular sutures group and 72·4% in the staples group received prophylaxis with antibiotics for 1 day in this trial. Compared with the result of a national cohort study in the USA,[44] reporting that about 60% of patients who had major surgery were still receiving antimicrobial prophylaxis at 24 h after surgery, our results were acceptable. We did not find an imbalance between the groups.

Our study had several limitations. First, the absence of masking could have biased the detection of wound complications. However, assessment of surgical site infections was done by infection control personnel at the participating institutions who did not have roles in trial design or conduct. Detection of other wound complications was based on whether some treatment (dressing or surgical intervention) for wound management was documented in the medical record, which could minimise bias. However, it was possible that the open nature of our trial might have affected the findings. The Japanese insurance system and common clinical practice permitted examination of patients by responsible surgeons at outpatient clinics 1 month and 6 months after surgery, which allowed for accurate assessment of the wound even though allocation was not masked.

Second, it has been reported that subcuticular sutures for skin closure have advantages compared with staples with regard to cosmetic considerations,[16–18] patient satisfaction,[24,25] and wound handling.[24,25] Nevertheless, we did not assess patients' satisfaction, patients' preference, or potential overall effects on the health-care system, and we did not use a validated scale to assess scars. We did not directly compare costs either, but the price of one stapling device and that of two packs of PDS-II sutures were roughly the same and median operative time was 10 min longer in the subcuticular sutures group (table 1).

In conclusion, the efficacy of subcuticular sutures was not validated as an improvement over a standard procedure for skin closure after gastrointestinal surgery.

***Panel 2*: Research in context**

Systematic review

We searched Medline and the Cochrane Database of Systematic Reviews with the terms "subcuticular suture, cutaneous closure, or dermal closure", "staple or staple closure", and "randomised controlled trial or phase 3 trial". We identified 11 randomised trials: four for caesarean delivery,[25,31–33] three for cardiovascular surgery,[24,34,35] two for orthopaedic surgery,[19,36] one for gynaecological surgery,[41] and one for laparotomy.[28] All these surgical procedures are class 1 (clean) surgery except for laparotomy, for which the details of the specific surgical procedures were not specified in the report. Six trials recommended subcuticular sutures[19,24,25,28,32,34] and four[31,35–37] showed equivalent results for sutures and staples. Only one trial recommended staples.[33] Most were small-scale trials (n=48–435). The number of patients in the trials with equivalent results ranged from 77 to 187. Three[23,38,39] of the four meta-analyses about caesarean delivery recommended subcuticular sutures; the other showed similar outcomes with sutures and staples.[40] A meta-analysis[21] of cardiovascular surgery recommended subcuticular sutures to reduce the number of wound complications. We identified no randomised trials in gastrointestinal surgery.

Interpretation

To our knowledge, our trial is the first done in gastrointestinal surgery (a class 2 surgery). Although the results of most randomised trials done in class 1 surgery support the use of subcuticular sutures to reduce wound complications and improve cosmetic outcomes, the benefits of subcuticular sutures in clean-contaminated surgeries remain unclear. This trial failed to prove subcuticular sutures were a new standard procedure for skin closure after gastrointestinal surgery; however, the formation of hypertrophic scars was significantly reduced with subcuticular sutures compared with staples.

Contributors

TT and KY drafted the paper. TT designed the protocol. YD and MM supervised the design of the trial and assisted with doing the trial. SK and TS obtained and analysed the data. TT, KU, and TI were the main investigators. All other authors participated in study conduct and recruitment of patients.

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Conflicts of interest

We declare that we have no conflicts of interest.

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FULL PAPER

BJC

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Keywords: microRNA; miR-320c; pancreatic cancer; gemcitabine resistance; SMARCC1

miR-320c regulates gemcitabine-resistance in pancreatic cancer via SMARCC1

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Background: Gemcitabine-based chemotherapy is the standard treatment for pancreatic cancer. However, the issue of resistance remains unresolved. The aim of this study was to identify microRNAs (miRNAs) that govern the resistance to gemcitabine in pancreatic cancer.

Methods: miRNA microarray analysis using gemcitabine-resistant clones of MiaPaCa2 (MiaPaCa2-RGs), PSN1 (PSN1-RGs), and their parental cells (MiaPaCa2-P, PSN1-P) was conducted. Changes in the anti-cancer effects of gemcitabine were studied after gain/loss-of-function analysis of the candidate miRNA. Further assessment of the putative target gene was performed *in vitro* and in 66 pancreatic cancer clinical samples.

Results: miR-320c expression was significantly higher in MiaPaCa2-RGs and PSN1-RGs than in their parental cells. miR-320c induced resistance to gemcitabine in MiaPaCa2. Further experiments showed that miR-320c-related resistance to gemcitabine was mediated through SMARCC1, a core subunit of the switch/sucrose nonfermentable (SWI/SNF) chromatin remodeling complex. In addition, clinical examination revealed that only SMARCC1-positive patients benefited from gemcitabine therapy with regard to survival after recurrence ($P = 0.0463$).

Conclusion: The results indicate that miR-320c regulates the resistance of pancreatic cancer cells to gemcitabine through SMARCC1, suggesting that miR-320c/SMARCC1 could be suitable for prediction of the clinical response and potential therapeutic target in pancreatic cancer patients on gemcitabine-based therapy.

Pancreatic cancer is one of the leading causes of tumour-related mortalities. The prognosis of patients after complete resection is poor, and >50% of patients develop tumour recurrence at distant or locoregional sites, with an estimated 5-year survival of only 20% (Li *et al*, 2004). Treatment modalities for pancreatic cancer include surgery, chemotherapy, radiation therapy, and combination therapy. Gemcitabine-based chemotherapy has formed the core of the multimodal therapy and improved the prognosis of patients with pancreatic cancer (Oettle *et al*, 2007), but its effect is modest because of high drug resistance. The selection of patients who derive a true benefit from gemcitabine could be an important stepping stone toward improvement of outcome of pancreatic cancer.

Several molecular mechanisms in drug resistance in pancreatic cancer have been elucidated. We have previously described that RRM1, the gene that encodes the regulatory subunit of ribonucleotide reductase, is strongly associated with gemcitabine resistance in pancreatic cancer (Nakahira *et al*, 2007; Akita *et al*, 2009). However, the development of drug resistance appears to be a multifactorial process, so our understanding is still fragmentary.

Recently, several studies have indicated that microRNAs (miRNAs) regulate this drug resistance (Tomimaru *et al*, 2010; Tomokuni *et al*, 2011). miRNAs are endogenous, single-stranded, non-coding RNAs and modulators of gene expression in the post-transcriptional phase, composed of 18–25 nucleotides. Currently, 1600 human miRNAs have been identified (miRBase 19, http://www.mirbase.org/). miRNAs are predicted to control the activity of approximately 30% of all protein-coding genes in mammals, and each miRNA can regulate up to 100 different messenger RNAs. Currently, the most promising miRNAs in association with

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pancreatic cancer drug resistance against gemcitabine are miR-15a (Zhang *et al*, 2010), miR-21 (Park *et al*, 2009; Ali *et al*, 2010; Giovannetti *et al*, 2010; Hwang *et al*, 2010), miR-34 (Ji *et al*, 2009), miR-200b and miR-200c (Li *et al*, 2009; Ali *et al*, 2010), miR-214 (Zhang *et al*, 2010), miR-221 (Park *et al*, 2009), and members of the let7 family (Li *et al*, 2009). However, only selected miRNAs have been investigated for their role in drug resistance in pancreatic cancer.

In this study, we developed gemcitabine-resistant cell clones from human pancreatic cancer cell lines and performed comprehensive expression profiling of miRNAs. The results indicate that miR-320c confers resistance to gemcitabine in pancreatic cancer cells through SMARCC1.

MATERIALS AND METHODS

Cell lines. The human pancreatic cell lines MiaPaCa2 and PSN1 were obtained from the Japan Cancer Research Resources Bank (Tokyo, Japan). These cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U ml^{-1} penicillin, and 100 mg ml^{-1} streptomycin at 37 °C in a humidified incubator with 5% CO_2 in air.

Establishment of gemcitabine-resistant cell clones. Gemcitabine-resistant cells were generated by exposure to gradually increasing concentrations of the drug for 2 months as described previously (Goan *et al*, 1999; Davidson *et al*, 2004). Parental MiaPaCa2 cells (MiaPaCa2-P) and parental PSN1 cells (PSN1-P) were exposed to gemcitabine at an initial concentration of 1 ng ml^{-1}. When cells adapted to the drug, the gemcitabine concentration was increased. The final concentrations were 20 ng ml^{-1} gemcitabine for MiaPaCa2 and 10 ng ml^{-1} gemcitabine for PSN1. Through this process, we successfully established gemcitabine-resistant cells (Nakahira *et al*, 2007). Limiting the dilution of the established cells allowed the production of MiaPaCa2 cell clones and PSN1 cell clones that were resistant to gemcitabine. Four clones (MiaPaCa2-RGs: MiaPaCa2-RG1, MiaPaCa2-RG2, MiaPaCa2-RG3, and MiaPaCa2-RG4) and three clones (PSN1-RGs: PSN1-RG1, PSN1-RG2, and PSN1-RG3) from each line were used in these experiments.

Drugs and reagents. Gemcitabine was kindly supplied by Eli Lilly Pharmaceuticals (Indianapolis, IN, USA). In reference to previous studies, polyclonal rabbit anti-human SMARCC1 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for western blot analysis (DelBove *et al*, 2011) and monoclonal mouse anti-human SMARCC1 antibody (Santa Cruz Biotechnology Inc.) for immunohistochemistry (Andersen *et al*, 2009) were used as primary antibodies. Monoclonal rabbit anti-human actin for western blot analysis was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Transfection. The precursor oligonucleotide of hsa-miR-320c (pre-miR-320c), antisense oligonucleotide inhibitor of hsa-miR-320c (anti-miR-320c), SMARCC1 small interfering (si) RNA oligonucleotide (siSMARCC1), and their scrambled oligonucleotides were obtained from Ambion Inc. (Austin, TX, USA). Pre/anti-miR-320c and siSMARCC1 were transfected using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) according to the protocol provided by the manufacturer. Each scrambled oligonucleotide was transfected in the same way as a matched negative control.

RNA extraction. Total RNA, including the small RNA fraction, was isolated from cell lines with Trizol reagent (Invitrogen) as previously described (Yang *et al*, 2009). The quality of the RNA was assessed with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) at 260 and 280 nm ($A_{260/280}$) wavelengths.

Real-time quantitative reverse-transcription-PCR for miRNA expression. The reverse transcription (RT) reaction was performed with the TaqMan MicroRNA RT Kit (Applied Biosystems, Foster City, CA, USA), and real-time quantitative (q) PCR was performed with TaqMan MicroRNA Assays (Applied Biosystems) using the ABI7900HT system (Applied Biosystems). The expression of the target miRNA was normalised relative to that of the endogenous control, RNU48. Data were analysed according to the comparative Ct method (Schmittgen *et al*, 2004).

Real-time qRT-PCR for messenger RNA expression. Complementary DNA was synthesised from 8.0 μg total RNA using the SuperScript first-strand synthesis system (Invitrogen), according to the instructions supplied by the manufacturer. Real-time quantitative PCR was performed using designed oligonucleotide primers and the LightCycler 480 Real-Time PCR system (Roche Diagnostics, Mannheim, Germany). For detection of the amplification products, the LightCycler-DNA master SYBR green I (Roche Diagnostics) was used as described previously (Yamamoto *et al*, 2004), and the amount of target gene expression was calculated. The expression of the target gene was normalised relative to the expression of GAPDH, which was used as an endogenous control. The designed PCR primers were as follows: GAPDH forward primer 5′-GTCGGAGTCAACGGATTTGGT-3′ and GAPDH reverse primer 5′-GCCATGGGTGGAATCATATTGG-3′; and SMARCC1 forward primer 5′-TCATGCGGATGCTCCTACCA-3′ and SMARCC1 reverse primer 5′-AAACCTCCGCCATCCCT GTT-3′.

MiRNA microarray experiments. The purified RNAs obtained from MiaPaCa2-P, MiaPaCa2-RGs (MiaPaCa2-RG1, MiaPaCa2-RG2, MiaPaCa2-RG3, and MiaPaCa2-RG4), PSN1-P, and PSN1-RGs (PSN1-RG1, PSN1-RG2, and PSN1-RG3) were used as samples and assessed as being of high quality by the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and NanoDrop (NanoDrop Technologies). Next, 500 ng of extracted total RNA was labeled with Hy5 using the miRCURY LNA Array microRNA Power Labeling kit (Exiqon, Vedbaek, Denmark). The labeled RNAs were hybridised onto 3D-Gene Human miRNA Oligo chips containing 1011 anti-sense probes printed in duplicate spots (Toray, Kamakura, Japan). The annotation and oligonucleotide sequences of the probes conformed to the miRBase miRNA database (http://microrna.sanger.ac.uk/sequences/). After stringent washes, the fluorescent signals were scanned with the ScanArray Express Scanner (Perkin Elmer, Waltham, MA, USA) and analysed using GenePix Pro version 5.0 (Molecular Devices, Sunnyvale, CA, USA). The raw data for each spot were normalised by substitution with the mean intensity of the background signal determined by the signal intensities of all blank spots with 95% confidence intervals. Measurements of both duplicate spots with signal intensities >2 s.ds. of the background signal intensity were considered to be valid. The relative expression level of a given miRNA was calculated by comparing the signal intensities of the averaged valid spots with their mean value throughout the microarray experiments after normalisation by their median values adjusted equivalently.

Construction of reporter plasmids and evaluation of luciferase reporter activity. To construct a luciferase reporter plasmid, a SMARCC1-3′UTR fragment containing the miR-320c target site was subcloned into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA) located 3′ to the firefly luciferase gene. Nucleotide sequences of the constructed plasmids were confirmed by DNA sequencing analysis. For luciferase reporter assays, MiaPaCa2-P were seeded in 96-well plates and then cotransfected with the pmirGLO-SMARCC1-3′UTR construct and either pre-miR-320c or scrambled oligonucleotide for negative control (Ambion) using Lipofectamine 2000

(Invitrogen). Assays were conducted 48 h after transfection using Dual-Glo Luciferase Assay System (Promega). Firefly luciferase signals were normalised to renilla luciferase signals. All transfection experiments were conducted in triplicate.

Western blot analysis. Western blot analysis was performed as described previously (Yamamoto *et al*, 2003). Briefly, total protein was extracted from cells grown to semiconfluence in radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Inc., Rockford, IL, USA). Aliquots of total protein (12 μg) were electrophoresed on sodium dodecyl sulfate polyacrylamide, 10% Tris-HCl gels (Bio-Rad Laboratories Inc., Hercules, CA, USA). The separated proteins were transferred to polyvinylidene difluoride membranes (Millipore Co., Billerica, MA, USA) and incubated with primary antibodies for 1 h.

Growth inhibitory assay. The growth inhibitory assay was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich Co.) assay as described previously (Eguchi *et al*, 2000). In brief, cells were incubated for 72 h under several concentrations of gemcitabine. After reincubation for 4 h with MTT solution, an acid–isopropanol mixture was added to dissolve the resulting formazan crystals. The absorbance of the plate was measured in a microplate reader at a wavelength of 550 nm with a 650-nm reference, and the results were expressed as a percentage of absorbance relative to that of untreated controls.

Patients and specimens. The study subjects were 66 patients with pancreatic cancer, recruited as described previously (Akita *et al*, 2009). Between January 1992 and March 2008, 166 patients underwent surgery for pancreatic cancer at Osaka University Hospital, Osaka, Japan. We excluded 100 patients for the following reasons: tumours were not resectable in 26 patients because of liver metastases or peritoneal carcinomatosis; surgery resulted in R1 (residual microscopic cancer) or R2 (residual macroscopic cancer) resections in 21 patients; chemotherapy or chemoradiotherapy was provided preoperatively to 37 patients; lack of neutral-buffered formalin-fixed and paraffin-embedded tumour blocks or/and clinical follow-up information for study purposes in 14 cases; and radiotherapy or immunotherapy was provided postoperatively to 2 patients. Because the natural history of variant pancreatic neoplasms differs from that of the usual pancreatic ductal adenocarcinoma, patients with intraductal papillary mucinous neoplasms, mucinous cystic adenocarcinomas, and medullary adenocarcinomas were excluded from this study. Supplementary Table S1 summarises the characteristics of the 66 patients who were enrolled. They included 31 men and 35 women with a mean age of 65.3 ± 7.6 years (± s.d.). All patients had R0 (no residual cancer) resections by pancreaticoduodenectomy in 53 patients, distal pancreatectomy in 11 patients, and other resections in 2 patients. The histopathological grading showed well, moderately, and poorly differentiated adenocarcinoma in 26, 30, and 10 patients, respectively. The UICC-TNM classification was 2, 1, and 63 patients with pT1, pT2, and pT3; 28, 32, and 6 patients with pN0, pN1, and pM1lym; and 1, 1, 26, 32, and 6 patients with stage IA, IB, IIA, IIB, and IV, respectively. None of the patients had received neoadjuvant therapy preoperatively. All 66 patients were followed until disease recurrence and/or death. The median follow-up period was 17.0 months (3.5–147.7), the 5-year survival rate was 25.0%, and recurrence of disease was observed in 51 patients. Treatment with gemcitabine was carried out in 26 patients; 3 patients received it as adjuvant chemotherapy, and 23 patients received it after disease recurrence. Radiation therapy was not carried out during all the follow-up period.

Immunohistochemical staining. Immunohistochemical staining for SMARCC1 in 66 pancreatic cancer samples was performed using the method described previously (Kondo *et al*, 1999). Briefly, formalin-fixed, paraffin-embedded 4-μm-thick sections were deparaffinised in xylene, then treated with an antigen-retrieval procedure, and incubated in methanol containing 0.3% hydrogen peroxide to block endogenous peroxidase. After incubation with normal protein block serum, the sections were incubated overnight at 4 °C with an anti-SMARCC1 antibody as the primary antibody. Thereafter, the sections were detected with avidin–biotin complex reagents (Vector Laboratory Inc., Burlingame, CA, USA) and diaminobenzidine. All sections were counterstained with haematoxylin. The positivity for SMARCC1 staining was defined as detectable nuclear staining of $>10\%$ of cancer cells.

Statistical analysis. Data are expressed as means ± s.d. Clinicopathological parameters were compared using the χ^2 test, and continuous variables were compared using the Student's *t*-test. Survival curves were computed using the Kaplan–Meier method, and differences between survival curves were compared using the log-rank test. A P value <0.05 denoted the presence of a statistically significant difference. Statistical analysis was performed using JMP software version 8.0.2 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Characteristics of established gemcitabine-resistant cells. The morphology of MiaPaCa2-RGs resembled that of MiaPaCa2-P. Although MiaPaCa2-RGs showed similar growth curves compared with MiaPaCa2-P in the absence of gemcitabine (data not shown), MiaPaCa2-RGs were significantly resistant to gemcitabine compared with MiaPaCa2-P, which MTT assays confirmed (Figure 1A). On the other hand, the morphology of PSN1-RGs was slightly spindle-like in comparison with that of PSN1-P, and the growth rate of PSN1-RGs was slightly slower than that of PSN1-P in the absence of gemcitabine (data not shown). MTT assays showed significant resistance to gemcitabine in PSN1-RGs compared with PSN1-P (Figure 1B).

miR-320c expression was significantly higher in gemcitabine-resistant clones than in parental cells. To identify the candidate miRNAs related to resistance to gemcitabine, miRNA microarray analysis was performed using a MiaPaCa2 combination (MiaPaCa2-P and MiaPaCa2-RGs) and PSN1 combination (PSN1-P and PSN1-RGs). The analysis showed that, among the 1011 miRNAs, the miRNA expression levels of 20 (2.0%) in MiaPaCa2-RGs (Supplementary Table S2) and 74 (7.3%) in PSN1-RGs (Supplementary Table S3), respectively, were altered by >1.5 average fold relative to parental, keeping adequate expression quantities and excluding miRNA*s. Furthermore, eight miRNAs were identified in common both in MiaPaCa2-RGs and PSN1-RGs (Figure 1C). These miRNAs are listed with average fold change and P values of MiaPaCa2-RGs relative to MiaPaCa2-P and PSN1-RGs relative to PSN1-P in Table 1. Among them, miR-320c showed the highest alteration (1.97 average fold change; 2.20-fold increase in MiaPaCa2-RGs, and 1.73-fold increase in PSN1-RGs) and was statistically significant ($P=0.0033$ in MiaPaCa2-RGs, $P=0.0366$ in PSN1-RGs). The results of real-time qRT-PCR for miR-320c confirmed the upregulation in MiaPaCa2-RGs (Figure 1D). Therefore, miR-320c was selected for further analysis.

Gain-of-function and loss-of-function of miR-320c alters the resistance of MiaPaCa2 cells to gemcitabine. To evaluate the effect of miR-320c on the response to gemcitabine in MiaPaCa2 cells, pre-miR-320c was first transfected into MiaPaCa2-P. Real-time qRT-PCR showed that transfection of pre-miR-320c markedly increased the miR-320c expression level for over 72 h (Figure 2A). The MTT assay demonstrated that transfection of pre-miR-320c into MiaPaCa2-P induced resistance to gemcitabine treatment