Published in final edited form as:

Cancer Lett. 2017 August 28; 402: 1–8. doi:10.1016/j.canlet.2017.05.007.

Chemosensitization and Inhibition of Pancreatic Cancer Stem Cell Proliferation by Overexpression of microRNA-205

Amit Kumar Chaudhary, Goutam Mondal, Virender Kumar, Krishna Kattel, and Ram I. Mahato*

Department of Pharmaceutical Sciences, University of Nebraska Medical Center, Omaha, NE 68198, USA

Abstract

Treatment of pancreatic cancer with gemcitabine (GEM) is limited due to its rapid plasma metabolism and development of chemoresistance. MicroRNA (miRNA) regulates cancer stem cell (CSC) maintenance and induces chemoresistance in cancer cells. In this study, we observed differential downregulation of miR-205 (miR-205-5p) in human pancreatic cancer tissues and cells. Compared to GEM-sensitive MIA PaCa-2 cells, miR-205 was highly downregulated in GEM-resistant MIA PaCa-2R cells. Lentivirus-mediated overexpression of miR-205 inhibits MIA PaCa-2R cell proliferation after GEM-treatment. Further investigation confirmed that miR-205 alone significantly reduces the proliferation of CSCs and tumor growth in mouse models. However, miR-205 in combination with GEM was more efficient in reducing the proliferation of CSCs and 3D spheroids. Moreover, miR-205 overexpressing MIA PaCa-2R cells induced orthotopic tumor growth was significantly inhibited after intravenous administration of GEM-conjugated methoxy poly(ethylene glycol)-block-poly(2-methyl-2-carboxyl-propylene carbonate)-graft-gemcitabine-graft-dodecanol (mPEG-b-PCC-g-GEM-g-DC) (mPEG-b-PCC-g-GEM-g-DC) polymeric micelles. Also, a reduction in CSCs, EMT and chemoresistance markers was observed in miR-205 overexpressing MIA PaCa-2R cells. Immunohistochemical analysis of orthotopic tumors showed a decrease in drug resistance protein caveolin-1 and cell proliferation marker Ki-67 in combination treatment. Overall, our findings suggest that miR-205 resensitizes GEM-resistant pancreatic cancer cells to GEM and acts as a tumor suppressor miRNA.

Graphical abstract

* Address for correspondence: Dr. Ram I. Mahato, Department of Pharmaceutical Sciences, University of Nebraska Medical Center (UNMC), 986025 Nebraska Medical Center, Omaha, NE 68198-6025, Tel: (402) 559-5422; Fax: (402) 559-9543, ram.mahato@unmc.edu.

Conflict of Interest: The authors declare no potential conflicts of interest.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

Pancreatic cancer; Chemoresistance; Drug delivery; Gemcitabine; MicroRNA; Tumor suppressor

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is aggressive cancer to treat, with the limited success in the therapeutic management of patients. As per American Cancer Society, PDAC accounts for 3% of all cancers in the United States and about 7% of cancer-related deaths in 2016. Mortality rates for PDAC have increased by 0.4% per year since 2000 with estimated 41,780 deaths in 2016. Due to its late-stage clinical prognosis, 85% of patients have metastatic disease making surgical and therapeutic interventions ineffective [1,2]. Gemcitabine (GEM) is a first-line FDA-approved drug used alone or in combination to improve the lifespan of PDAC patients. Although the mechanisms by which GEM induces cell death are well known, their effectiveness is incomplete due to the acquisition of drug resistance after prolonged treatment and desmoplastic microenvironment [3]. Several therapeutic interventions have been developed for GEM-resistant PDAC. For instance, zidovudine sensitizes GEM-resistant pancreatic cancer cells through Akt/GSK3 β -Snail pathway [4]. Similar effects have also been shown by eribulin mesylate and the combination of cotylenin A and phenethyl isothiocyanate in killing GEM-resistant pancreatic cancer cells [5,6]. Although the combination of GEM with nab-paclitaxel [7] and the chemotherapy regimen FOLFIRINOX has shown the overall survival of metastatic PDAC patients; a significant rate of grade 3/4 toxicity was observed [8].

Pancreatic cancer stem cell (CSC) displays enhanced chemoresistance and metastatic activity, which in turn is regulated by miRNAs. Aberrantly expressed miRNAs contribute to many biological processes including tumor initiation and progression, apoptosis, and maintenance of CSCs and EMT phenotype [9-11]. Studies including ours have shown the importance of miRNAs and CSCs in cancer progression and metastasis, making their significance as biomarkers and potential targets for reversing drug resistance [12,13]. Although miR-15a [14], miR-21 [15], miR-34 [16], miR-181b [17], miR-200b/c and let-7 family [18,19] have shown their association with GEM-resistant PDAC, only few have been examined for their roles in drug resistance.

The development of miRNA-based therapeutics in combination with small molecule provides an unprecedented opportunity to counteract chemoresistance. Therefore, efficient delivery of miRNAs to the target organ and cells could improve treatment outcome in a broad range of human cancers. However, *in vivo* delivery of miRNAs remains a challenge due to their poor biostability, rapid excretion, immunogenicity, poor cellular uptake, endosomal escape and improper intracellular release. Our earlier study has shown miR-205 (miR-205-5p) to promote CSC proliferation, resulting in chemosensitization of GEM-resistant pancreatic cancer cells after its transient expression [12]. Therefore, in this study, instead of transiently overexpressing miR-205, we used lentivirus particles to overexpress this miRNA in GEM-resistant pancreatic cancer cells to determine its long-term effect alone or in combination with GEM.

2. Materials and Methods

2.1 Human pancreatic cancer tissue samples and cells

Human frozen specimens from Rapid Autopsy Program (RAP) were obtained from surgical resections following well-known protocols and the informed consent waiver provided by the Institutional Review Board, University of Nebraska Medical Center (UNMC). Tissues were classified based on the diagnosis made by a pathologist. All cells were purchased from ATCC. GEM-resistant MIA PaCa-2R cells were maintained in DMEM containing GEM supplemented with 1% penicillin/streptomycin and 10% FBS throughout the study. HPDE cells were maintained under the same culture condition in Keratinocyte-SFM supplemented with keratinocytes supplements.

2.2. miRNA profiling in human pancreatic tissue

Total RNAs from human unaffected pancreatic tissue and pancreatic cancer lesions were isolation using spin technology (Qiagen, MD). The miScript II RT Kit was used for cDNA synthesis for checking miRNA expression. SYBR Green-based pathway-focused miScript miRNA PCR Array was performed to detect miRNAs (catalog number 102ZF, Qiagen, MD) using Roche Light Cycler 480® (Roche, Indianapolis) [12]. Unaffected pancreatic tissues were considered as controls.

2.3. Measurement of miR-205 in pancreatic cancer cells and other target genes

For quantification of miR-205 (miR-205-5p, 5' - UCCUUCAUUCCACCGGAGUCUG-3') in MIA PaCa-2, HPAF-II, BXPC-3, HPDE and MIA PaCa-2R cells, the miScript primer assays (Qiagen, MD) was used per the manufacturer's instructions. U6 was used as internal controls. For mRNA quantification, quantitative real-time RT-PCR (qRT-PCR) was performed to amplify cDNA using SYBR Green dye universal master mix per the manufacturer's instructions using GAPDH as internal controls [12].

2.4. Generation of lentivirus-mediated overexpression of miR-205 containing MIA PaCa-2R cells

2×10⁵ MIA PaCa-2R cells/well in 6-well plate were grown overnight and transduced by adding 8 µg/mL of polybrene and 10 µl of lenti-miR205 and lenti-miRScramble lentiviral particles. After 72 h of incubation, GFP-positive MIA PaCa-2R+lenti-hsamiR205 and MIA

PaCa-2R+lenti-hsamiRScramble cells were isolated using an FACSCalibur flow cytometer. Expression of miR-205 was analyzed by qRT-PCR as mentioned above at 24, 48 and 72 h.

2.5. Effect of miR-205 in subcutaneous tumor bearing mice

All *in vivo* experiments were performed per the NIH animal use guidelines and protocol approved by the Institutional Animal Care and Use Committee, UNMC. Tumor flank was established in 6–8-week-old male NSG mice (n=4) by subcutaneous injection of 3×10^6 MIA PaCa-2R+lenti-hsamiR205 and MIA PaCa-2R+lenti-hsamiRScramble cells as before [20]. After 55 days, tumors were excised and images were taken.

2.6. Cell viability, cell migration and invasion assay

5×10^3 MIA PaCa-2R+lenti-hsamiR205 and MIA PaCa-2R+lenti-hsamiRScramble cells/well were seeded in 96-well plate in triplicate. MTT assay was carried out to determine the cell viability after 72 h post-treatment with GEM (50-1000 nM) [20]. For migration assay, 5×10^4 MIA PaCa-2R+lenti-hsamiR205 and MIA PaCa-2R+lenti-hsamiRScramble cells were cultured in the upper chambers of Transwell plates (8.0 μ m transparent PET Membrane) (Corning Inc.) in 2.5 ml DMEM supplemented with 1% FBS. The lower chamber was filled with 3 ml DMEM supplemented with 10% FBS. For invasion study, overnight grown 125×10^2 MIA PaCa-2R+lenti-hsamiR205 and MIA PaCa-2R+lenti-hsamiRScramble cells were cultured in upper chambers of Transwell plates using Matrigel in 2.5 ml DMEM supplemented with 1% FBS. The lower chamber was filled with 3 ml DMEM supplemented with 10% FBS. After 72 h incubation, cells were treated with 500 nM of GEM and further incubated for 72 h. Migration and invasion of cells were observed under a fluorescent microscope after staining with crystal violet. Also, crystal violet stained cells were dissolved in methanol and quantified at 540 nm using a UV–Vis spectrophotometer (Thermo Fisher Scientific) [21].

2.7. Cell cycle and apoptosis analysis

MIA PaCa-2R+lenti-hsamiR205 and MIA PaCa-2R+lenti-hsamiRScramble cells were treated with 500 nM GEM after 72 h of culture. After 72 h of post-treatment, floating and attached cells were harvested, washed with cold PBS and fixed in 1 mL of 70% ethanol overnight at -20 °C. The next day, cells were washed and resuspended in 500 μ l FxCycle PI/RNase staining solution (Molecular Probes) and analyzed by an FACSCalibur flow cytometer. For apoptosis analysis, harvested cells were resuspended in 500 μ l of 1 $\times$ Annexin V binding buffer, 5 μ l of Annexin V-Cy5, 5 μ l of PI (BioVision Inc.) and analyzed by an FACSCalibur flow cytometer.

2.8. Aldefluor assay

After 72 h of post-seeding of MIA PaCa-2R+lenti-hsamiR205 and MIA PaCa-2R+lenti-hsamiRScramble cells, a set of cells were analyzed for ALDH⁺ without GEM-treatment using AldeRed ALDH detection assay per the manufacturer's instructions (Merck Millipore) [22]. Post 72 h of 500 nM GEM-treatment, another set of cells were analyzed for ALDH⁺ with GEM-treatment using an FACSCalibur flow cytometer. GFP-positive and DEAB-treated cells were used to set up a gated region.

2.9. Western blot analysis

Post 72 h of culture, MIA PaCa-2R+lenti-hsamiR205 and MIA PaCa-2R+lenti-hsamiRScramble cells were lysed using RIPA buffer and protein expression was analyzed as before [49] by incubating with primary antibodies for CD44 (3578S) (Cell Signaling Technology, Inc.), ALDH1A1 (sc-166362), ZEB1 (sc-25388) and Actin (sc-1616) (Santa Cruz Biotechnology, Inc.). Post-incubation with primary antibody for 16 h at 4 °C, HRP-conjugated secondary antibodies (sc-2020, sc-2030 and sc-2031) (Santa Cruz Biotechnology, Inc.) were applied for 1 h in phosphate buffered saline with Tween[®] 20 (PBST) at RT. Target proteins were detected by ImmunoCruz Western blotting luminol reagent (Santa Cruz Biotechnology, Inc.).

2.10. 3D hanging drop spheroid culture

25×10² MIA PaCa-2R+lenti-hsamiR205 and MIA PaCa-2R+lenti-hsamiRScramble cells/well were seeded in Perfecta3D[®] 96-Well Hanging Drop Plates (3D Biomatrix, Inc.) in 40 µl DMEM medium in triplicate. After the formation of 3D spheroids, they were treated with 500 nM of GEM. Following treatment, images were taken at 3rd, 7th and 9th day to check the cytotoxic effect.

2.5. Effect of miR-205 in subcutaneous tumor bearing mice

The orthotopic pancreatic cancer mouse model was generated using MIA PaCa-2R+lenti-hsamiR205 and MIA PaCa-2R+lenti-hsamiRScramble cells as described earlier [23]. At 40 days after post implantation, animals were randomly divided into four groups (n=3). Group 1 (MIA PaCa-2R+lenti-hsamiRScramble) and 2 (MIA PaCa-2R+lenti-hsamiR205) received normal saline, group 3 (MIA PaCa-2R+lenti-hsamiRScramble) and 4 (MIA PaCa-2R+lenti-hsamiR205) received GEM-conjugated polymeric micelles. GEM-conjugated micelles were prepared using methoxy poly(ethylene glycol)-block-poly(2-methyl-2-carboxyl-propylene carbonate)-graft-gemcitabine-graft-dodecanol (mPEG-b-PCC-g-GEM-g-DC) as reported earlier [24] and administered intravenously thrice a week for two weeks at an equivalent dose of 40 mg/kg. The tumors were excised, size was measured, and images were taken at the end of the study.

2.12. Immunohistochemical and immunofluorescence staining

To stain K-Ras, tumor sections (4 µm) were fixed in precooled acetone for 30 min and washed with PBS thrice. Permeabilization was done by washing the slides twice for 5 min in TBS + 0.025% Triton X- 100. After blocking in 10% goat serum with 1% BSA in TBS for 2h at RT, sections were incubated with K-Ras antibody (sc-30) (1:50) (Santa Cruz Biotechnology, Inc.) overnight at 4 °C. Next day, slides were washed with TBS + 0.025% Triton X-100 and further incubated with anti-mouse IgG H&L (DyLight[®] 550) (ab96872) for mice tumors and anti-mouse IgG H&L (Alexa Fluor[®] 488) (ab150113) for human tumors (Abeam, Cambridge, MA). Mouse tumor sections were stained with Ki-67 and caveolin-1 antibodies to determine cell proliferation and drug resistance. Positively stained color (brown in each case) was selected for quantification of the relative intensity.

2.13. Statistical analysis

All experiments were repeated three times. Student's unpaired t-test was used to compare the mean values of individual groups. A p-value <0.05 was considered as statistically significant.

3. Results

3.1. miR-205 suppressed GEM-resistant pancreatic cancer

miRNA profiling from primary pancreatic cancer lesions showed differential expression of various miRNAs, where miR-205 was significantly downregulated in cancer lesions compared to unaffected pancreatic tissue from matched patients (Fig. 1A). However, it was highly downregulated in a patient with the highest metastatic site (10 sites) and less in the least metastatic sites (2 sites). Similar expression pattern of miR-205 was also evident in GEM-sensitive MIA PaCa-2, HPAF-II and BXPC-3 pancreatic cancer cells compared to HPDE normal cells. However, its expression was highly downregulated in GEM-resistant MIA PaCa-2R cells (Fig. 1B). Also, upregulation of K-Ras was observed in the clinical samples compared to normal pancreatic tissue (Fig. 1C).

To evaluate the role of miR-205 in GEM-resistant pancreatic cancer cells, we overexpressed miR-205 in MIA PaCa-2R cells and used for *in vivo* studies (Fig. 2A). As shown in Figure. 2B, a significant reduction in subcutaneous tumor growth was observed in MIA PaCa-2R +lenti-hsamiR205 cells bearing mice compared to MIA PaCa-2R+lenti-hsamiRScramble cells bearing mice. Since miR-205 expression was increased over time, we treated cells with GEM after 72 h for MTT assay. As predicted, a significant inhibition of MIA PaCa-2R +lenti-hsamiR205 cell proliferation was observed after GEM-treatment in a dose-dependent manner (Fig. 2C). Further, we observed a significant reduction in TUBB3, RRM1 and ZEB1 transcripts by 3.37, 3.84 and 1.34 –folds, respectively in MIA PaCa-2R+lenti-hsamiR205 cells at the transcriptional level (Fig. 2D). Also, ZEB1 expression was decreased in MIA PaCa-2R+lenti-hsamiR205 cells at translation level compared to MIA PaCa-2R+lenti-hsamiRScramble cells (Fig. 2E).

3.2. Regulation of migratory and invasive properties

To determine the effect of miR-205 and GEM in combination on migratory and invasive properties of MIA PaCa-2R cells, we performed Transwell chamber assays. Overexpression of miR-205 followed by GEM-treatment significantly decreased the degree of migratory MIA PaCa-2R+lenti-hsamiR205 cells by 1.87 –fold compared to MIA PaCa-2R+lenti-hsamiRScramble cells after GEM-treatment (Fig. 3A&B). Similarly, the level of invading MIA PaCa-2R+lenti-hsamiR205 cells was also decreased significantly by 1.36 –fold compared to MIA PaCa-2R+lenti-hsamiRScramble cells after GEM-treatment (Fig. 3C&D).

3.3. Effect of miR-205 and GEM combination on 3D spheroids

3D spheroids are a fast and standardized method for drug screening. Therefore, we generated 3D spheroids of MIA PaCa-2R+lenti-hsamiR205 and MIA PaCa-2R+lenti-hsamiRScramble cells followed by GEM treatment. As a result, a reduction in spheroid size

was observed compared to non-treated and GEM-treated scrambled miRNA containing cells (Fig. 3E).

3.4. Regulation of cell cycle, apoptosis and pancreatic CSCs

To further evaluate the combination effects of miR-205 and GEM on cell cycle and apoptosis, MIA PaCa-2R+lenti-hsamiR205 and MIA PaCa-2R+lenti-hsamiRScramble cells were grown with and without GEM. Compared to MIA PaCa-2R+lenti-hsamiRScramble cells, miR-205 significantly increased the percentage of MIA PaCa-2R cells in G0/G1 phase from $43.52 \pm 0.06\%$ to $52.62 \pm 0.002\%$, which was further increased to $78.54 \pm 0.01\%$ after GEM-treatment (Fig. 4A). Also, a significant increase in the percentage of apoptotic cells from $47.2 \pm 3.97\%$ in MIA PaCa-2R+lenti-hsamiRScramble cells to $53.6 \pm 3.47\%$ in MIA PaCa-2R+lenti-hsamiR205 cells was observed (Fig. 4B).

Since miR-205 plays a major role in regulating many biological processes, we observed upregulation of stemness markers OCT3/4 and NANOG in MIA PaCa-2 cells by 18.38 and 2.8 –folds compared to HPDE cells. They were further increased by 9.38 and 13.67 –folds in MIA PaCa-2R compared to MIA PaCa-2 cells (Figure. 5A). In contrast, they significantly decreased by 3.70 and 1.20 –folds after miR-205 overexpression in MIA PaCa-2R compared to MIA PaCa-2R+lenti-hsamiRScramble cells (Fig. 5B). Similarly, following Aldeflour staining, the population of ALDH⁺ cells was decreased by 1.9 –fold in MIA PaCa-2R+lenti-hsamiR205 compared to MIA PaCa-2R+lenti-hsamiRScramble cells (Fig. 5C). However, the population further decreased by 2.9 –fold in MIA PaCa-2R+lenti-hsamiR205 compared to MIA PaCa-2R+lenti-hsamiRScramble cells when treated with GEM (Fig. 5D). To further confirm the reduction in CSC population by miR-205 is through the inhibition of CSC-specific markers, we checked the expression of ALDH1 and CD44 by Western blot. A significant decrease in their levels was evident in MIA PaCa-2R+lenti-hsamiR205 compared to MIA PaCa-2R+lenti-hsamiRScramble cells (Fig. 5E).

3.5. miR-205 sensitizes GEM-resistant orthotopic tumor

We further explored the combination effect of miR-205 and GEM in orthotopic pancreatic tumor-bearing NSG mice. Following intravenous administration of mPEG-b-PCC-g-GEM-g-DC, a significant reduction in tumor growth was observed in MIA PaCa-2R+lenti-hsamiR205 cells generated tumor bearing mice ($77.83 \pm 21 \text{ mm}^3$) compared to MIA PaCa-2R+lenti-hsamiRScramble cells generated tumor bearing mice ($172.85 \pm 17 \text{ mm}^3$). Additionally, a significant reduction in tumor size was also seen in MIA PaCa-2R+lenti-hsamiR205 cells generated tumor bearing mice ($298.46 \pm 54 \text{ mm}^3$) compared to MIA PaCa-2R+lenti-hsamiRScramble cells generated tumor bearing mice ($464.94 \pm 94 \text{ mm}^3$) without GEM-treatment (Fig. 6A&B). Unlike K-Ras upregulation in pancreatic cancer tissue (Fig. 1B), we observed its downregulation in MIA PaCa-2R+lenti-hsamiR205 cells generated orthotopic tumors compared to MIA PaCa-2R+lenti-hsamiRScramble cells when these micelles were treated with GEM-conjugated polymeric micelles (Fig. 7). Similarly, immunohistochemical analysis of orthotopic pancreatic tumor specimens overexpressing miR-205 obtained from GEM-treated animals showed a reduction in cell proliferation (Ki-67) and drug resistance (caveolin-1) proteins compared to the non-treated and scrambled miRNA containing groups (Fig. 7).

4. Discussion

In this study, investigated the expression of miRNAs in clinically defined pancreatic cancer lesions. Like others, we also observed downregulation of miR-205 in cancer lesions where it was highly downregulated in a patient with highest metastasis sites and low with least metastasis sites (Fig. 1A) [12]. Since most cancer-related mortality is metastasis-dependent, our observation suggests metastasis in PDAC might relate to the level of miR-205 expression. Although GEM is the first-line chemotherapeutic for PDAC, its effectiveness is limited due to the acquisition of drug resistance [3]. Therefore, we explored the expression of miR-205 in GEM-sensitive and GEM-resistant pancreatic cancer cells. As evident from our clinical samples and previous studies, similar expression pattern was observed in all cells [12,25,26]. However, it was highly downregulated in MIA PaCa-2R compared to MIA PaCa-2 cells signifying its correlation with GEM-resistance (Fig. 1B).

Several miRNAs based therapeutics have been developed for GEM-resistant PDAC. For instance, the combination of miR-223 inhibitor/genistein [27] and overexpression of miR-210/miR-101 [28,29] inhibited tumor growth by targeting EMT phenotype and multidrug resistance proteins ABCC5/RRM1, respectively. Therefore, we generated miR-205 overexpressing MIA PaCa-2R cells using lentiviral particles to study the long-term impact brought after its restoration (Fig. 2A). Interestingly, a reduction in subcutaneous tumor growth was observed after miR-205 overexpression compared to scrambled miRNA (Fig. 2B). Also, the proliferation of MIA PaCa-2R+lenti-hsamiR205 cells was significantly reduced after GEM-treatment in a dose-dependent manner (Fig. 2C). These findings suggest miR-205 inhibits the growth of GEM-resistant pancreatic cancer and helps in its sensitization to GEM.

It is known that overexpression of microtubule protein TUBB3 and RRM1 regulate chemoresistance in cancers including PDAC [30,31]. Therefore, we observed a significant reduction in TUBB3 and RRM1 transcripts in MIA PaCa-2R+lenti-hsamiR205 compared to MIA PaCa-2R+lenti-hsamiRScramble cells (Fig. 2D). This observation correlates with the previous finding where inhibition of TUBB3 reduces the growth of PDAC and metastasis, making them an attractive target for cancer therapy [32]. It has been shown that cells lose their epithelial features resulting in migration, invasion and increased tumorigenic potential during EMT process. EMT-type cells including CSCs have also been reported to play important roles in chemoresistance and metastasis in several malignancies. As miR-205 regulates the EMT process by inhibiting the expression of ZEB1 and ZEB2 [26,33], we observed a significant reduction in ZEB1 expression in miR-205 overexpressing cells compared to the cells expressing scrambled miRNA (Fig. 2D&E) [26,34,35]. We also observed a significant decrease in the degree of migratory and invasive properties of highly invasive and metastatic MIA PaCa-2R cells when miR-205 was overexpressed followed by GEM-treatment (Fig. 3A-D). This evidence supported that the combination of miR-205 and GEM reduces the tumorigenic potential of pancreatic cancer affecting EMT phenotypes.

Current advancement of 3D spheroids has offered improved correlating results than traditional 2D culture systems. 3D cultures are more resistant to chemotherapy and radiotherapy than 2D cultures which accurately mimic the features of solid tumor

microenvironment. These unique features highlight the potential of 3D spheroids and offer a simple and highly reproducible model for *in vitro* drug screening. Therefore, we checked the effects of miR-205 in combination with GEM in 3D spheroids of MIA PaCa-2R cells. As evident from our 2D culture (Fig. 2C), we observed a significant reduction in spheroid size in GEM-treated MIA PaCa-2R+lenti-hsamiR205 compared to MIA PaCa-2R+lenti-hsamiRScramble cells (Fig. 3E). Based on the role of miR-205 in inhibiting the proliferation of pancreatic cancer, we next performed cell cycle analysis. We observed a significant increase in the percentage of MIA PaCa-2R cells at G0/G1 phase as reported earlier after miR-205 overexpression [36,37], which further increases after GEM-treatment (Fig. 4A). Also, the population of apoptotic cells significantly increased in MIA PaCa-2R+lenti-hsamiR205 cells compared to MIA PaCa-2R+lenti-hsamiRScramble cells (Fig. 4B). These phenomena further suggest that miR-205 alone was sufficient to induce cell cycle arrest and apoptosis in GEM-resistant cells, thus inhibit tumor growth in an animal model (Fig. 2B) [37,38].

Most tumors including PDAC are heterogeneous in nature with a small population of CSCs providing enhanced chemoresistance and EMT properties which have been attributed to miRNAs in solid tumors including PDAC [12]. miRNAs including miR-199a, miR-590, miR-145 and miR-200c inhibit CSCs in cancers including PDAC by targeting CSC-specific markers [39-42]. Stemness factors OCT3/4 and NANOG associate themselves with induced pluripotent stem cells, suggesting their correlation with CSC proliferation. Our observation showed a significant increase in OCT3/4 and NANOG in MIA PaCa-2R cells compared to MIA PaCa-2 cells providing greater tumorigenic potential [43] (Fig. 5A). However, their expression decreased significantly after miR-205 overexpression in MIA PaCa-2R cells (Fig. 5B). Since ALDH activity is the hallmark of pancreatic CSCs [12], we observed a significant decrease in the population of ALDH⁺ cells after miR-205 overexpression which further decreases after GEM-treatment (Fig. 5C&D). Additionally, a decrease in ALDH1 and CD44 expression was also evident in MIA PaCa-2R+lenti-hsamiR205 cells compared to scrambled miRNA (Fig. 5E). These findings suggest that miR-205 decreases the proliferation of ALDH⁺ cells, which further decreases when combined with GEM. This observation also provided evidence that the population of ALDH⁺ cells increases over time (Fig. 5C&D).

In addition to the induction of chemoresistance [3], GEM effectiveness is also restricted due to its rapid plasma metabolism requiring high dose resulting in systemic toxicity [23]. However, the use of appropriate nano-formulations has shown to overcome these bottlenecks. For instance, in our earlier studies, we have demonstrated mPEG-b-PCC-g-GEM-g-DC polymer improves the stability, loading and accumulation of GEM in the pancreatic tumor [23,24]. However, unlike intratumoral administration of polymeric micelles containing miR-205 mimic and GEM in subcutaneous tumor model which lack original tumor microenvironment and clinically infeasible [20], in this study, we administered mPEG-b-PCC-g-GEM-g-DC micelles intravenously in orthotopic pancreatic tumor-bearing mice. As a result, a significant inhibition of orthotopic tumor growth was observed after miR-205 overexpression compared to scrambled miRNA (Fig. 6). Also, a significant reduction in tumor growth was also noted with miR-205 only which was consistent with a subcutaneous model (Fig. 2B&6).

EMT plays a major role in cancer metastasis and progression through Ras signaling. As K-Ras mutation denoted one of the initial changes in PDAC [44,45] and upregulated in the clinical sample (Fig. 1C), we observed a reduction in K-Ras signal in MIA PaCa-2R+lenti-hsamiR205 generated orthotopic pancreatic tumors after GEM-treatment (Fig. 7). Caveolin-1, an essential constituent of caveolae is upregulated and positively associated with ABC transporters, aerobic glycolysis activity and autophagy. It mediates MDR in colon cancer, adriamycin-resistant in breast cancer, taxol/GEM-resistant in lung cancer and GEM-resistant in pancreatic cancer cells [46-48]. It mediates MDR in colon cancer, adriamycin-resistant in breast cancer, taxol/GEM-resistant in lung cancer and GEM-resistant in pancreatic cancer cells. Since caveolin-1 has been found to be highly expressed in CSCs affecting its chemosensitivity [48], we checked its expression in orthotopic pancreatic tumors. As a result, we observed a decrease in caveolin-1 expression in tumors after miR-205 and GEM-treatment, an agreement with Chatterjee et al. study [46]. Which was further supported by the reduction in cell proliferation marker Ki-67 (Fig. 7). These findings further suggest that miR-205 sensitizes GEM-resistant pancreatic cancer by inhibiting drug resistance protein and act as tumor suppressor.

In conclusion, our study highlights tumor-suppressive function of miR-205 in GEM-resistant PDAC. When overexpressed, miR-205 resensitizes GEM-resistant pancreatic cancer cells to GEM by reducing the proliferation of ALDH⁺ cells, inhibition of EMT phenotype and chemoresistance markers. Therefore, this study provides a platform for combination therapy of miR-205 and GEM as a viable strategy to treat advanced pancreatic cancer. However; further study is needed to correlate the relationship between miR-205, chemoresistance and CSCs at the molecular level.

Acknowledgments

This work was supported by the National Institutes of Health (1R01EB017853, P50CA127297), Fred and Pamela Buffett Cancer Center and the Faculty Start-up fund to RIM.

References

1. Willett CG, Czito BG, Bendell JC, Ryan DP. Locally advanced pancreatic cancer. *J Clin Oncol*. 2005; 23:4538–4544. [PubMed: 16002845]
2. Sener SF, Fremgen A, Menck HR, Winchester DP. Pancreatic cancer: A report of treatment and survival trends for 100,313 patients diagnosed from 1985-1995, using the national cancer database. *J Am Coll Surg*. 1999; 189:1–7. [PubMed: 10401733]
3. Bailey JM, Swanson BJ, Hamada T, Eggers JP, Singh PK, Caffery T, Ouellette MM, Hollingsworth MA. Sonic hedgehog promotes desmoplasia in pancreatic cancer. *Clin Cancer Res*. 2008; 14:5995–6004. [PubMed: 18829478]
4. Namba T, Kodama R, Moritomo S, Hoshino T, Mizushima T. Zidovudine, an antiviral drug, resensitizes gemcitabine-resistant pancreatic cancer cells to gemcitabine by inhibition of the akt-GSK3beta-snail pathway. *Cell Death Dis*. 2015; 6:e1795. [PubMed: 26111057]
5. Kasukabe T, Honma Y, Okabe-Kado J, Higuchi Y, Kato N, Kumakura S. Combined treatment with cotylenin A and phenethyl isothiocyanate induces strong antitumor activity mainly through the induction of ferroptotic cell death in human pancreatic cancer cells. *Oncol Rep*. 2016; 36:968–976. [PubMed: 27375275]
6. Takezaki Y, Namikawa T, Koyama T, Munekage E, Munekage M, Maeda H, Kitagawa H, Hanazaki K. Antitumor effects of eribulin mesylate in gemcitabine-resistant pancreatic cancer cell lines. *Anticancer Res*. 2016; 36:6077–6082. [PubMed: 27793935]

7. Von Hoff DD, Ervin T, Arena FP, et al. Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. *N Engl J Med*. 2013; 369:1691–1703. [PubMed: 24131140]
8. Conroy T, Desseigne F, Ychou M, et al. FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. *N Engl J Med*. 2011; 364:1817–1825. [PubMed: 21561347]
9. Kent OA, Mendell JT. A small piece in the cancer puzzle: microRNAs as tumor suppressors and oncogenes. *Oncogene*. 2006; 25:6188–6196. [PubMed: 17028598]
10. Zhang W, Dahlberg JE, Tam W. MicroRNAs in tumorigenesis: A primer. *Am J Pathol*. 2007; 171:728–738. [PubMed: 17724137]
11. Wen D, Danquah M, Chaudhary AK, Mahato RI. Small molecules targeting microRNA for cancer therapy: Promises and obstacles. *J Control Release*. 2015; 219:237–247. [PubMed: 26256260]
12. Singh S, Chitkara D, Kumar V, Behrman SW, Mahato RI. miRNA profiling in pancreatic cancer and restoration of chemosensitivity. *Cancer Lett*. 2013; 334:211–220. [PubMed: 23073476]
13. Singh S, Chitkara D, Mehrazin R, Behrman SW, Wake RW, Mahato RI. Chemoresistance in prostate cancer cells is regulated by miRNAs and hedgehog pathway. *PLoS One*. 2012; 7:e40021. [PubMed: 22768203]
14. Zhang XJ, Ye H, Zeng CW, He B, Zhang H, Chen YQ. Dysregulation of miR-15a and miR-214 in human pancreatic cancer. *J Hematol Oncol*. 2010; 3:46–8722. 3–46. [PubMed: 21106054]
15. Giovannetti E, Funel N, Peters GJ, et al. MicroRNA-21 in pancreatic cancer: Correlation with clinical outcome and pharmacologic aspects underlying its role in the modulation of gemcitabine activity. *Cancer Res*. 2010; 70:4528–4538. [PubMed: 20460539]
16. Ji Q, Hao X, Zhang M, et al. MicroRNA miR-34 inhibits human pancreatic cancer tumor-initiating cells. *PLoS One*. 2009; 4:e6816. [PubMed: 19714243]
17. Takiuchi D, Eguchi H, Nagano H, et al. Involvement of microRNA-181b in the gemcitabine resistance of pancreatic cancer cells. *Pancreatol*. 2013; 13:517–523. [PubMed: 24075517]
18. Li Y, VandenBoom TG 2nd, Kong D, Wang Z, Ali S, Philip PA, Sarkar FH. Up-regulation of miR-200 and let-7 by natural agents leads to the reversal of epithelial-to-mesenchymal transition in gemcitabine-resistant pancreatic cancer cells. *Cancer Res*. 2009; 69:6704–6712. [PubMed: 19654291]
19. Ali S, Ahmad A, Banerjee S, Padhye S, Dominiak K, Schaffert JM, Wang Z, Philip PA, Sarkar FH. Gemcitabine sensitivity can be induced in pancreatic cancer cells through modulation of miR-200 and miR-21 expression by curcumin or its analogue CDF. *Cancer Res*. 2010; 70:3606–3617. [PubMed: 20388782]
20. Mittal A, Chitkara D, Behrman SW, Mahato RI. Efficacy of gemcitabine conjugated and miRNA-205 complexed micelles for treatment of advanced pancreatic cancer. *Biomaterials*. 2014; 35:7077–7087. [PubMed: 24836307]
21. Hingorani P, Zhang W, Gorlick R, Kolb EA. Inhibition of src phosphorylation alters metastatic potential of osteosarcoma in vitro but not in vivo. *Clin Cancer Res*. 2009; 15:3416–3422. [PubMed: 19447875]
22. Minn I, Wang H, Mease RC, Byun Y, Yang X, Wang J, Leach SD, Pomper MG. A red-shifted fluorescent substrate for aldehyde dehydrogenase. *Nat Commun*. 2014; 5:3662. [PubMed: 24759454]
23. Kattel K, Mondal G, Lin F, Kumar V, Mahato RI. Biodistribution of self-assembling polymer-gemcitabine conjugate after systemic administration into orthotopic pancreatic tumor bearing mice. *Mol Pharm*. 2016
24. Chitkara D, Mittal A, Behrman SW, Kumar N, Mahato RI. Self-assembling, amphiphilic polymer-gemcitabine conjugate shows enhanced antitumor efficacy against human pancreatic adenocarcinoma. *Bioconjug Chem*. 2013; 24:1161–1173. [PubMed: 23758084]
25. Zhang Y, Li M, Wang H, Fisher WE, Lin PH, Yao Q, Chen C. Profiling of 95 microRNAs in pancreatic cancer cell lines and surgical specimens by real-time PCR analysis. *World J Surg*. 2009; 33:698–709. [PubMed: 19030927]
26. Gregory PA, Bert AG, Paterson EL, Barry SC, Tsykin A, Farshid G, Vadas MA, Khew-Goodall Y, Goodall GJ. The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. *Nat Cell Biol*. 2008; 10:593–601. [PubMed: 18376396]

27. Ma J, Zeng F, Ma C, Pang H, Fang B, Lian C, Yin B, Zhang X, Wang Z, Xia J. Synergistic reversal effect of epithelial-to-mesenchymal transition by miR-223 inhibitor and genistein in gemcitabine-resistant pancreatic cancer cells. *Am J Cancer Res.* 2016; 6:1384–1395. [PubMed: 27429851]
28. Amponsah PS, Fan P, Bauer N, Zhao Z, Gladkich J, Fellenberg J, Herr I. microRNA-210 overexpression inhibits tumor growth and potentially reverses gemcitabine resistance in pancreatic cancer. *Cancer Lett.* 2017; 388:107–117. [PubMed: 27940128]
29. Fan P, Liu L, Yin Y, et al. MicroRNA-101-3p reverses gemcitabine resistance by inhibition of ribonucleotide reductase M1 in pancreatic cancer. *Cancer Lett.* 2016; 373:130–137. [PubMed: 26828016]
30. Akita H, Zheng Z, Takeda Y, et al. Significance of RRM1 and ERCC1 expression in resectable pancreatic adenocarcinoma. *Oncogene.* 2009; 28:2903–2909. [PubMed: 19543324]
31. Nakahira S, Nakamori S, Tsujie M, et al. Involvement of ribonucleotide reductase M1 subunit overexpression in gemcitabine resistance of human pancreatic cancer. *Int J Cancer.* 2007; 120:1355–1363. [PubMed: 17131328]
32. McCarroll JA, Sharbeen G, Liu J, et al. betaIII-tubulin: A novel mediator of chemoresistance and metastases in pancreatic cancer. *Oncotarget.* 2015; 6:2235–2249. [PubMed: 25544769]
33. Korpai M, Lee ES, Hu G, Kang Y. The miR-200 family inhibits epithelial-mesenchymal transition and cancer cell migration by direct targeting of E-cadherin transcriptional repressors ZEB1 and ZEB2. *J Biol Chem.* 2008; 283:14910–14914. [PubMed: 18411277]
34. Cao LY, Yang J, Fu XG, Lin YH, Lin F, Huang BY. The MicroRNA miR-205 inhibits epithelial-mesenchymal transition in HK-2 cells by down-regulating ZEB1 and ZEB2 expressions. *Nan Fang Yi Ke Da Xue Xue Bao.* 2016; 36:1700–1705. [PubMed: 27998868]
35. Zhao W, Wang H, Han X, et al. DeltaNp63alpha attenuates tumor aggressiveness by suppressing miR-205/ZEB1-mediated epithelial-mesenchymal transition in cervical squamous cell carcinoma. *Tumour Biol.* 2016; 37:10621–10632. [PubMed: 26864590]
36. Salajegheh A, Vosgha H, Md Rahman A, Amin M, Smith RA, Lam AK. Modulatory role of miR-205 in angiogenesis and progression of thyroid cancer. *J Mol Endocrinol.* 2015; 55:183–196. [PubMed: 26342107]
37. Yang G, Zhang P, Lv A, Liu Y, Wang G. MiR-205 functions as a tumor suppressor via targeting TGF-alpha in osteosarcoma. *Exp Mol Pathol.* 2016; 100:160–166. [PubMed: 26708425]
38. Tian L, Zhang J, Ge J, Xiao H, Lu J, Fu S, Liu M, Sun Y. MicroRNA-205 suppresses proliferation and promotes apoptosis in laryngeal squamous cell carcinoma. *Med Oncol.* 2014; 31:785-013–0785-3. Epub 2013 Dec 3. [PubMed: 24297308]
39. Cheng W, Liu T, Wan X, Gao Y, Wang H. MicroRNA-199a targets CD44 to suppress the tumorigenicity and multidrug resistance of ovarian cancer-initiating cells. *Febs J.* 2012; 279:2047–2059. [PubMed: 22498306]
40. Ma C, Huang T, Ding YC, Yu W, Wang Q, Meng B, Luo SX. MicroRNA-200c overexpression inhibits chemoresistance, invasion and colony formation of human pancreatic cancer stem cells. *Int J Clin Exp Pathol.* 2015; 8:6533–6539. [PubMed: 26261532]
41. Zhou L, Zhao LC, Jiang N, Wang XL, Zhou XN, Luo XL, Ren J. MicroRNA miR-590-5p inhibits breast cancer cell stemness and metastasis by targeting SOX2. *Eur Rev Med Pharmacol Sci.* 2017; 21:87–94. [PubMed: 28121351]
42. Zhou X, Yue Y, Wang R, Gong B, Duan Z. MicroRNA-145 inhibits tumorigenesis and invasion of cervical cancer stem cells. *Int J Oncol.* 2017; 50:853–862. [PubMed: 28112371]
43. Matsuoka J, Yashiro M, Sakurai K, Kubo N, Tanaka H, Muguruma K, Sawada T, Ohira M, Hirakawa K. Role of the stemness factors sox2, oct3/4, and nanog in gastric carcinoma. *J Surg Res.* 2012; 174:130–135. [PubMed: 21227461]
44. Kumar V, Mondal G, Slavik P, Rachagani S, Batra SK, Mahato RI. Codelivery of small molecule hedgehog inhibitor and miRNA for treating pancreatic cancer. *Mol Pharm.* 2015; 12:1289–1298. [PubMed: 25679326]
45. Zhong X, Zheng L, Shen J, et al. Suppression of MicroRNA 200 family expression by oncogenic KRAS activation promotes cell survival and epithelial-mesenchymal transition in KRAS-driven cancer. *Mol Cell Biol.* 2016; 36:2742–2754. [PubMed: 27550813]

46. Chatterjee M, Ben-Josef E, Thomas DG, et al. Caveolin-1 is associated with tumor progression and confers a multi-modality resistance phenotype in pancreatic cancer. *Sci Rep.* 2015; 5:10867. [PubMed: 26065715]
47. Shatz M, Liscovitch M. Caveolin-1 and cancer multidrug resistance: Coordinate regulation of pro-survival proteins? *Leuk Res.* 2004; 28:907–908. [PubMed: 15234566]
48. Wang Z, Wang N, Liu P, et al. Caveolin-1, a stress-related oncotarget, in drug resistance. *Oncotarget.* 2015; 6:37135–37150. [PubMed: 26431273]

Abbreviations

ABC

ATP binding cassette transporters

ALDH

Aldehyde dehydrogenase

CSC

Cancer stem cell

DEAB

Diethylaminobenzaldehyde

EMT

Epithelial-mesenchymal transition

GEM

Gemcitabine

GFP

Green fluorescent protein

HPDE

Human pancreatic ductal epithelial

HRP

Horseradish peroxidase

mPEG-b-PCC-g-GEM-g-DC

Methoxy poly(ethylene glycol)-block-poly(2-methyl-2-carboxyl-propylene carbonate-graft-gemcitabine-graft-dodecanol

miRNA

microRNA

MDR

Multidrug resistance

OCT

Optimum cutting temperature

PDAC

Pancreatic ductal adenocarcinoma

PI

Propidium iodide

RRM1

Ribonucleotide reductase catalytic subunit M1

TUBB3

Tubulin beta 3 class III

ZEB1

Zinc finger E-box-binding homeobox 1

2D

Two-dimensional

3D

Three-dimensional

Highlights

- Differential downregulation of miR-205 (miR-205-5p) in pancreatic cancer tissues and cells.
- Compared to gemcitabine (GEM)-sensitive cells, miR-205 is highly downregulated in GEM-resistant pancreatic cancer cells.
- miR-205 regulates pancreatic cancer stem cells, which in turn result in chemoresistance.
- Overexpression of miR-205 in GEM-resistant pancreatic cancer cells (CSCs) reduce the proliferation of pancreatic cancer stem cells, inhibits tumor growth by resensitizing the cells to GEM.

Fig. 1. miR-205 is downregulated in pancreatic cancer

(A) Hierarchical clustering of differentially expressed miRNAs in human primary pancreatic cancer lesions by human cancer pathway finder miRNA PCR array. Red denotes high expression levels, whereas green denotes low expression levels. Sample 6, 8, 9 are pancreatic cancer lesions and samples 1, 3, 4 are unaffected pancreatic tissues. (B) miR-205 expression levels in HPDE, MIA PaCa-2, HPAF-II, BXPC-3 and MIA PaCa-2R cells. (C) Immunofluorescence analysis of K-Ras from pancreatic cancer lesion and unaffected pancreatic tissue (10 $\times$ and 40 $\times$). Data are presented as the mean $\pm$ SD of three independent tests. * $p < 0.05$. Scale bar, 200 μ m.

Fig. 2. Overexpression of miR-205 inhibits the growth of GEM-resistant pancreatic cancer *in vivo* and sensitizes it to GEM

(A) mir-205 expression level in MIA PaCa-2R cells containing lenti-hsamiRScramble and lenti-hsamiR205 at 24, 48 and 72 h post-incubation. (B) *In vivo* efficacy of miR-205 in subcutaneous tumor-bearing mice with representative tumor size of various miR-205 overexpressing MIA PaCa-2R cells generated tumor groups compared to scrambled miRNA groups (n=4). (C) MTT assay of MIA PaCa-2R cells containing lenti-hsamiR205 and lenti-hsamiRScramble at 72 h time point. (D) The expression level of biomarkers related to chemoresistance (TUBB3, RRM1) and EMT (ZEB1) in MIA PaCa-2R cells containing lenti-hsamiRScramble and lenti-hsamiR205 at 72 h time point. (E) Western blot analysis of ZEB1 and Actin (control) from MIA PaCa-2R+lenti-hsamiR205 and MIA PaCa-2R+lenti-hsamiRScramble cells post 72 h of culture. Data are presented as the mean $\pm$ SD of three independent tests. $*p<0.05$.

Fig. 3. The combination of miR-205 and GEM inhibits migration, invasion and of 3D spheroids of GEM-resistant pancreatic cancer cells

(A, B) Transwell membrane assay for cell migration. Data are presented as the mean $\pm$ SD of three independent tests. $*p < 0.02$. (C, D) Transwell membrane assay for cell invasion. Data are presented as the mean $\pm$ SD of three independent tests. $*p < 0.04$. Scale bar, 100 μ m. (E) Fluorescent microscope images of 3D spheroids for cell cytotoxicity assay of MIA PaCa-2R+lenti-hsamiR205 compared to MIA PaCa-2R+lenti-hsamiRScramble cells. Scale bar, 200 μ m.

Fig. 4. The combination of miR-205 and GEM has an effect on cell cycle and induction of apoptosis on GEM-resistant pancreatic cancer cells

(A) Analysis of cell cycle phase distribution using flow cytometry of MIA PaCa-2R+lenti-hsamiR205 and MIA PaCa-2R+lenti-hsamiRScramble cells before and after GEM-treatment. (B) Analysis of the percentage of apoptotic MIA PaCa-2R+lenti-hsamiR205 compared to MIA PaCa-2R+lenti-hsamiRScramble cells. NT: non-treated with GEM. The data are the mean $\pm$ S.D. of three independent experiments. $*p<0.02$.

Fig. 5. The combination of miR-205 and GEM inhibits CSC population of GEM-resistant pancreatic cancer cells

The expression of stemness markers OCT3/4 and NANOG in HPDE, MIA PaCa-2 and MIA PaCa-2R cells before (A) and after transduction (B) with lenti-hsamiR205 and lenti-hsamiRScramble. Aldefluor staining of MIA PaCa-2R+lenti-hsamiR205 and MIA PaCa-2R+lenti-hsamiRScramble cells before (C) and after treatment with GEM (D). (E) The expression of pancreatic CSCs markers CD44 and ALDH1 in MIA PaCa-2R+lenti-hsamiR205 and MIA PaCa-2R+lenti-hsamiRScramble cells (NT: non-treated with GEM).

Fig. 6. Overexpression of miR-205 chemosensitizes GEM-resistant pancreatic cancer in orthotopic tumor model to GEM

(A) *In vivo* efficacy of mPEG-b-PCC-g-GEM-g-DC in orthotopic tumor-bearing mice generated using MIA PaCa-2R+lenti-hsamiR205 and MIA PaCa-2R+lenti-hsamiRScramble cells. (B) Tumor volume. Data expressed as the mean $\pm$ SE (n=3). * $p < 0.05$.

Fig. 7. Effect of miR-205 and its combination with GEM on K-ras, Caveolin-1 and Ki-67
Immunofluorescence staining of cryosections for K-Ras from orthotopic tumor-bearing mice. Scale bar, 50 μm (40×). Immunohistochemical analysis for drug resistance protein caveolin-1 and cell proliferation marker Ki-67 in orthotopic tumor specimens. (NT: non-treated with GEM and T: treated with GEM). Scale bar, 200 μm (40×). Arrow indicate the stained cells.