

Original Article

Gemcitabine enhances OSI-027 cytotoxicity by upregulation of miR-663a in pancreatic ductal adenocarcinoma cells

Bingfeng Huang^{1*}, Jianxin Wang^{1*}, Qi Chen^{1*}, Caidan Qu², Jian Zhang¹, Enjiang Chen¹, Yibo Zhang¹, Yi Wang¹, Lei Ni¹, Tingbo Liang¹

Departments of ¹Hepatobiliary and Pancreatic Surgery, ²Burn Surgery, The First Affiliated Hospital, School of Medicine, Zhejiang University, No. 79 Qingchun Road, Hangzhou 310003, China. *Equal contributors.

Received July 5, 2018; Accepted December 23, 2018; Epub January 15, 2019; Published January 30, 2019

Abstract: Pancreatic ductal adenocarcinoma (PDAC) is well-known to be the most deadly malignancy with the worst survival rate of all cancers. Gemcitabine-based chemotherapy is the most common treatment option for pancreatic ductal adenocarcinoma. However, it offers little therapeutic value in many cases due to the rapid development of chemoresistance. MicroRNAs (miRNAs) have been found to play pivotal roles in the chemotherapeutic resistance of PDAC. In the present study, we examined the molecular basis for the effective combination of OSI-027 and gemcitabine (GEM). Firstly, we identified a specific miRNA expression profile in PDAC cells after treatment with either of these drugs. We found that miR-663a was significantly upregulated after treatment with GEM and downregulated after OSI-027 treatment. With combination of the two drugs, miR-663a level was lower than the GEM group, but higher than the OSI-027 group. Real-time quantitative PCR confirmed these observations. To further establish the role of miR-663a in OSI-027 and GEM resistance in pancreatic cancer, we transfected PDAC cells with miR-663a mimic or miR-663a inhibitor. Cell viability and proliferation assays showed that miR-663a mimic enhanced drug sensitivity, while inhibitor promoted drug resistance. Moreover, we found that the combined effect of OSI-027 and GEM disappeared after inhibiting miR-663a. Our study clearly demonstrates that GEM upregulates miR-663a, thereby promoting the sensitivity of pancreatic cancer cells to OSI-027. Our study suggests that miR-663a expression may be a useful indicator of the potential for chemoresistance and provides a potential new therapeutic target to avert chemoresistance in PDAC.

Keywords: Pancreatic ductal adenocarcinoma (PDAC), chemoresistance, gemcitabine, OSI-027, microRNA

Introduction

Pancreatic ductal adenocarcinoma (PDAC), one of the most common lethal malignancies, is one of the main causes of cancer-related death in developed countries and has an extremely poor prognosis, with a 5-year survival rate of only 7% [1, 2]. Because its early symptoms are not obvious, patients with pancreatic ductal adenocarcinoma are often diagnosed at late stage, and miss the opportunity for curative surgery [3]. Hence, chemotherapy becomes the main treatment for patients with advanced stage PDAC [4]. Currently, GEM-based chemotherapy has been the first-line treatment for PDAC [5].

OSI-027 is a potent ATP-competitive mTORC1 and mTORC2 inhibitor that is currently in phase

II clinical trials. It has shown promise for treating a variety of tumor types, including leukemia [6], non-small cell lung cancer (NSCLC) [7], gallbladder carcinoma [8], pancreatic neuroendocrine tumors [9], and colorectal cancer [10]. Zhi et al. [11] demonstrated that combination OSI-027 and GEM was more effective than OSI-027 in the treatment of pancreatic ductal adenocarcinoma. However, the molecular mechanism(s) of how GEM enhances the therapeutic effect of OSI-027 remain unknown and require further investigation.

MicroRNAs (miRNAs), which are a class of small non-coding RNAs, have been demonstrated to serve as key gene regulators by binding to 3' untranslated regions (3'-UTRs) of their target mRNAs, thus inhibiting translation or promoting mRNA degradation [12]. They have been well-

established to play important roles in the regulation of basic cellular functions, including proliferation, apoptosis, differentiation, migration, and invasion, among others. Moreover, accumulating evidences implicate altered miRNA expression in the response of tumor cells to chemotherapy and the sensitivity of cancer cells to treatment. Downregulation of miR-21 [13] and miR-125a [14] increased GEM sensitivity in human pancreatic cancer cells. Chaudhary et al. demonstrated that ectopic expression of miR-205-3p in combination with GEM significantly reduced proliferation and tumor growth of pancreatic cancer cells in mouse models [15]. These results indicated that miRNAs could regulate GEM sensitivity in human pancreatic cancer cells. Therefore, in this study, we investigated the effects of miRNA in PDAC cells and the mechanism(s) by which GEM enhances the therapeutic effect of OSI-027.

Materials and methods

Cell lines and cell culture

Human PDAC cell lines (Panc-1, BxPC-3, T3-M4, MIApaca-2) were obtained from the Shanghai Institute for Biological Science (Shanghai, China) and were cultured in RPMI-1640 (Gibco) supplemented with 10% FBS (Gibco) and 1% penicillin/streptomycin (Sigma-Aldrich). Cells were maintained at 37°C in a humidified incubator with 5% CO₂ and were used within 3 months of resuscitation.

Reagents

OSI-027 and GEM were purchased from Selleck. Stock solutions were prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich), stored at -20°C, and diluted in fresh medium for each experiment. The final concentration of DMSO did not exceed 0.5% in any experiment.

RNA extraction, reverse transcription PCR, and quantitative real-time PCR

Total RNA was extracted from cells with Trizol (Invitrogen, CA, USA) and reverse transcribed using the Prime Script reagent RT Kit (Takara Biotechnology, Dalian, China). PCR (SYBR Green dyestuff, TaKaRa Biotechnology) was performed on an ABI Prism 7900HT Real-Time System (Applied Biosystems, Inc). RT-PCR was performed with the following cycling conditions: 95°C for 30 s, 40 cycles of 95°C for 5 s, 60°C for 30 s. The expression levels of miRNAs in

each group were calculated by relative quantification ($2^{-\Delta\Delta Ct}$ or $2^{-\Delta Ct}$) [16].

Transfection

Asynchronously growing cells were seeded at 2×10^5 cells/well of a six-well plate. The miR-663a mimic, miR-663a inhibitor, and negative control were all purchased from RiboBio (Guangzhou, China), and were transfected into PDAC cell lines using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Cell viability and proliferation assays

To evaluate relative cell viability, PDAC cells (5,000 per well) were seeded into 96-well microplates and incubated overnight. The culture medium was then replaced with complete media containing diverse concentrations of GEM (2, 4, 6, and 8 $\mu\text{mol/L}$) or OSI-027 (20, 40, 60, and 80 $\mu\text{mol/L}$) for 48 hours, then the cell counting kit-8 assay (CCK-8; KeyGEN) was conducted following the manufacturer's instructions. Cell viability was expressed relative to untreated control cells. To evaluate cell proliferation, cells were treated with OSI-027 (IC50 concentrations), GEM (IC50 concentrations), or OSI-027 plus GEM. Then cells were assayed using the Click-iT 5-ethynyl-20-deoxyuridine (EdU) Imaging Kit (Invitrogen) following the manufacturer's instructions and counterstained with Hoechst 33342. The percentage of proliferating cells in five random fields of view per slide was determined under an inverted fluorescence microscope (Olympus) and expressed relative to untreated control cells.

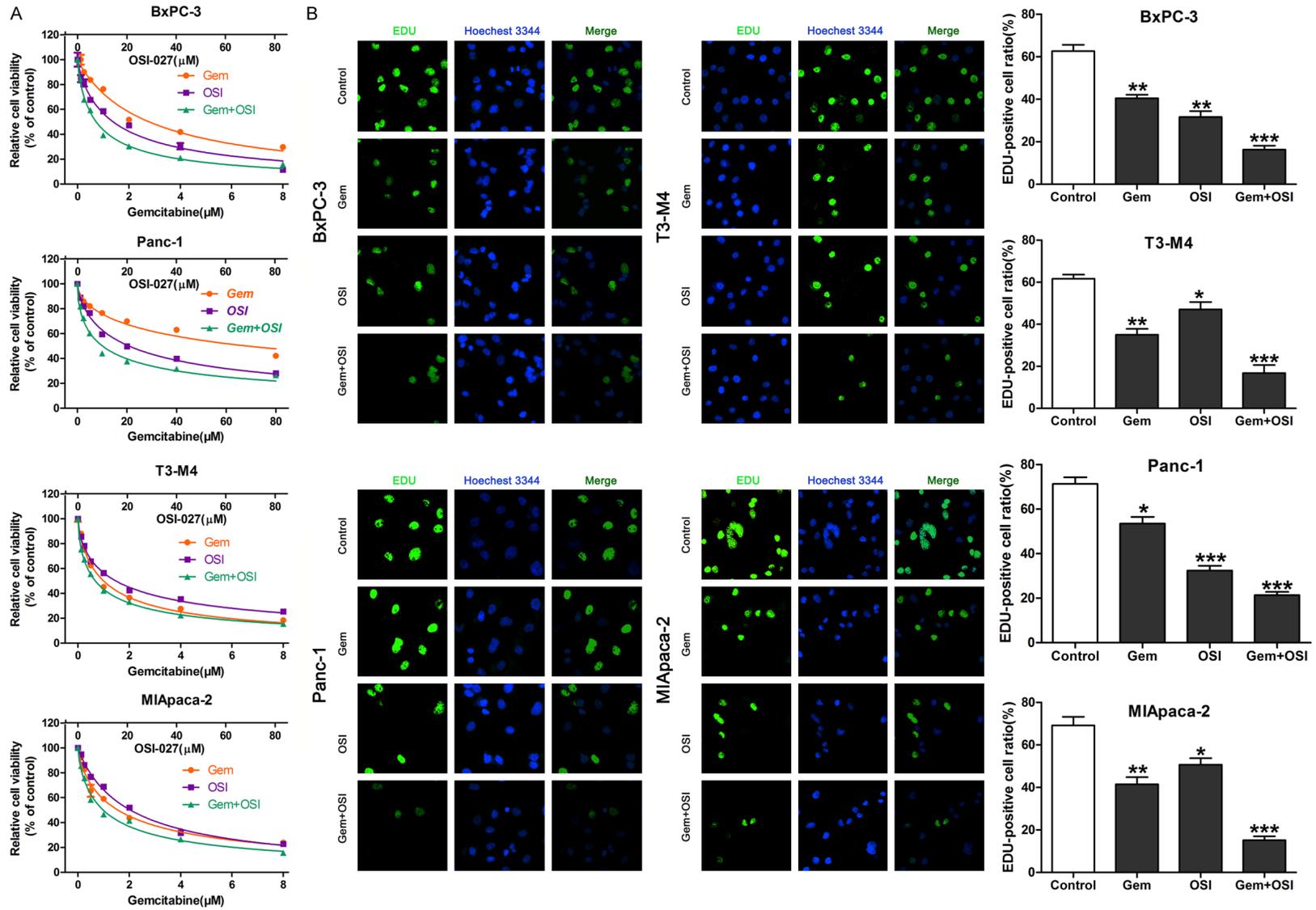
Apoptosis

PDAC cells or transfected cells were treated with OSI-027 (IC50 concentrations), GEM (IC50 concentrations), or OSI-027 plus GEM for 48 hours, stained using the Annexin V-PE/7AAD apoptosis kit (BD Biosciences) according to the manufacturer's protocol, and analyzed using a BD FACS Caliber flow cytometer using BD Cell Quest software.

Statistics

All experiments were performed in triplicate. Quantitative values were expressed as the mean \pm standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism 6 software (GraphPad Software,

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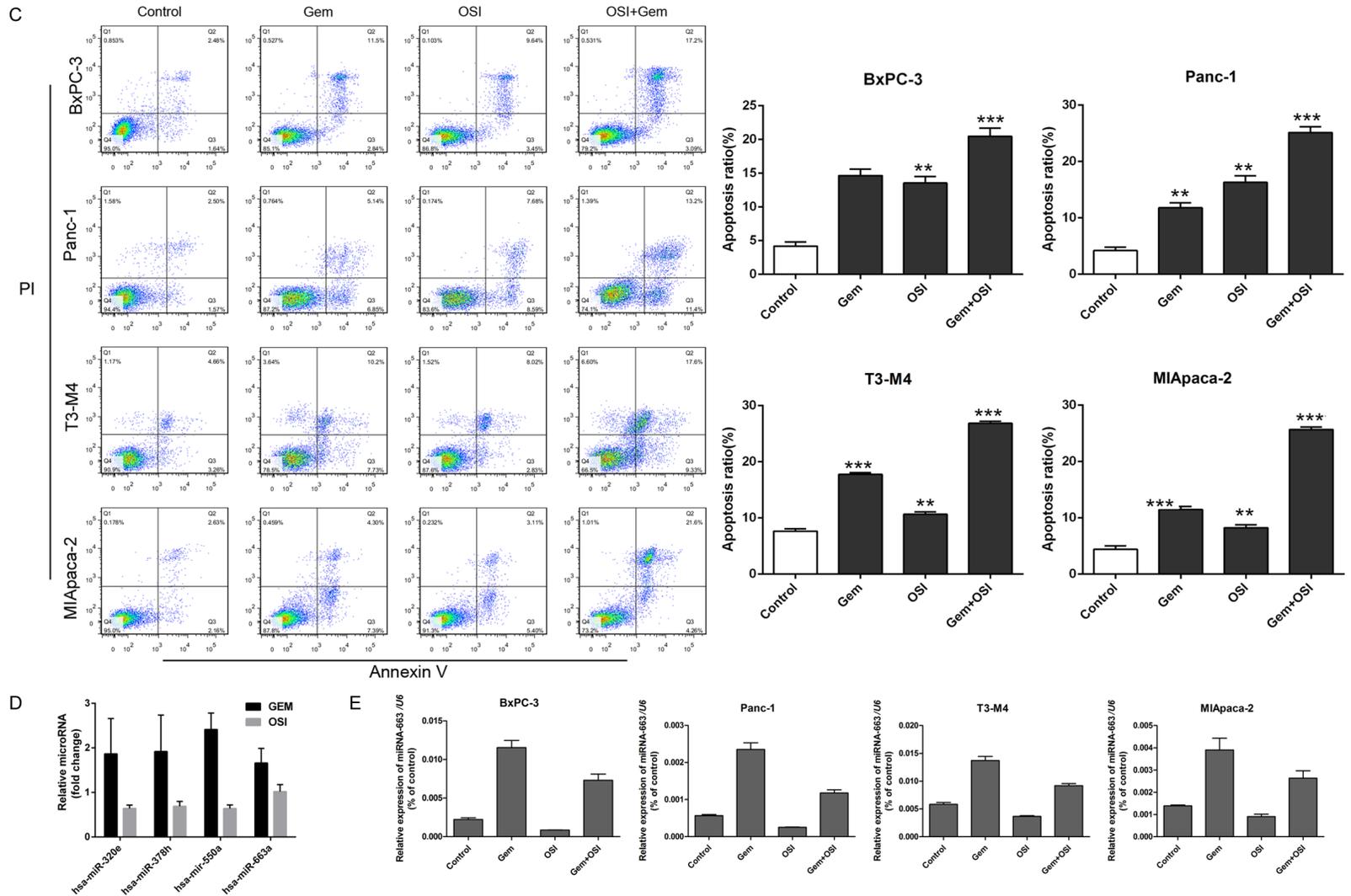


Figure 1. Effect of OSI-027 and GEM on four PDAC cell lines (Panc-1, BxPC-3, T3-M4, and MIApaca-2). A. Cell viability was measured in PDAC cells treated with GEM, OSI-027, or a combination of both for 48 h by CCK-8 assay. B. Relative EdU-positive cell ratios for PDAC cells treated with GEM, OSI-027, or a combination of both for 48 h, quantified by EdU staining; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. C. Quantification of apoptosis in PDAC cells treated with GEM, OSI-027, or a combination of both for 48 h, determined by flow-cytometric analysis. ** $P < 0.01$ and *** $P < 0.001$. D. Differentially expressed microRNAs after treatment with OSI-027 and GEM examined by qRT-PCR. E. The expression of miR-663 in PDAC cells treated with GEM, OSI-027, or a combination of both, examined by qRT-PCR analysis.

Inc., La Jolla, CA, USA). Two groups were compared using Student's *t*-test, and multiple group comparisons were conducted using one-way analysis of variance. $P < 0.05$ was considered statistically significant.

Results

Effect of OSI-027 and GEM on PDAC cell lines

In agreement with a previous study [11], the CCK-8 assay demonstrated that the combination of OSI-027 and GEM was more cytotoxic than either compound alone in all PDAC cell lines tested (**Figure 1A**). Moreover, we treated PDAC cells with IC50 concentrations of OSI-027, GEM, or a combination of both. EdU incorporation showed that the combination of OSI-027 and GEM also significantly inhibited PDAC proliferation (**Figure 1B**). Apoptosis analysis revealed that OSI-027 could not induce cell apoptosis; however, when combined with GEM, it significantly enhanced apoptosis induced by GEM (**Figure 1C**). These results indicate that OSI-027 combined with GEM can effectively inhibit the activity and proliferation of pancreatic cancer cells and promote apoptosis.

Altered miR-663 expression in PDAC cell lines promotes sensitivity to GEM

Many reports show that miRNAs participate in drug sensitivity in a number of cancers, including breast cancer and PDAC [17-19]. Considering the important role of miRNAs, we used microarray to detect changes in miRNA expression profile after treatment with OSI-027 and GEM, respectively. Among the differentially expressed miRNAs, we found that miR-663a changed after OSI-027 and GEM treatment (**Figure 1D**). qRT-PCR was used to evaluate relative miRNA-663a expression levels in treated cells (**Figure 1E**, $P < 0.05$). After OSI-027 treatment, miR-663a expression was downregulated, while in the presence of GEM it was upregulated. With combination of the two drugs, miR-663a was lower than with GEM treatment alone, but higher than with OSI treatment alone. These results showed that miR-663a might be involved in chemoresistance.

To understand the functional role of miR-663a in GEM resistance in pancreatic cancer cells, PDAC cells were transfected with miR-663a mimics or miR-663a inhibitor, respectively. The

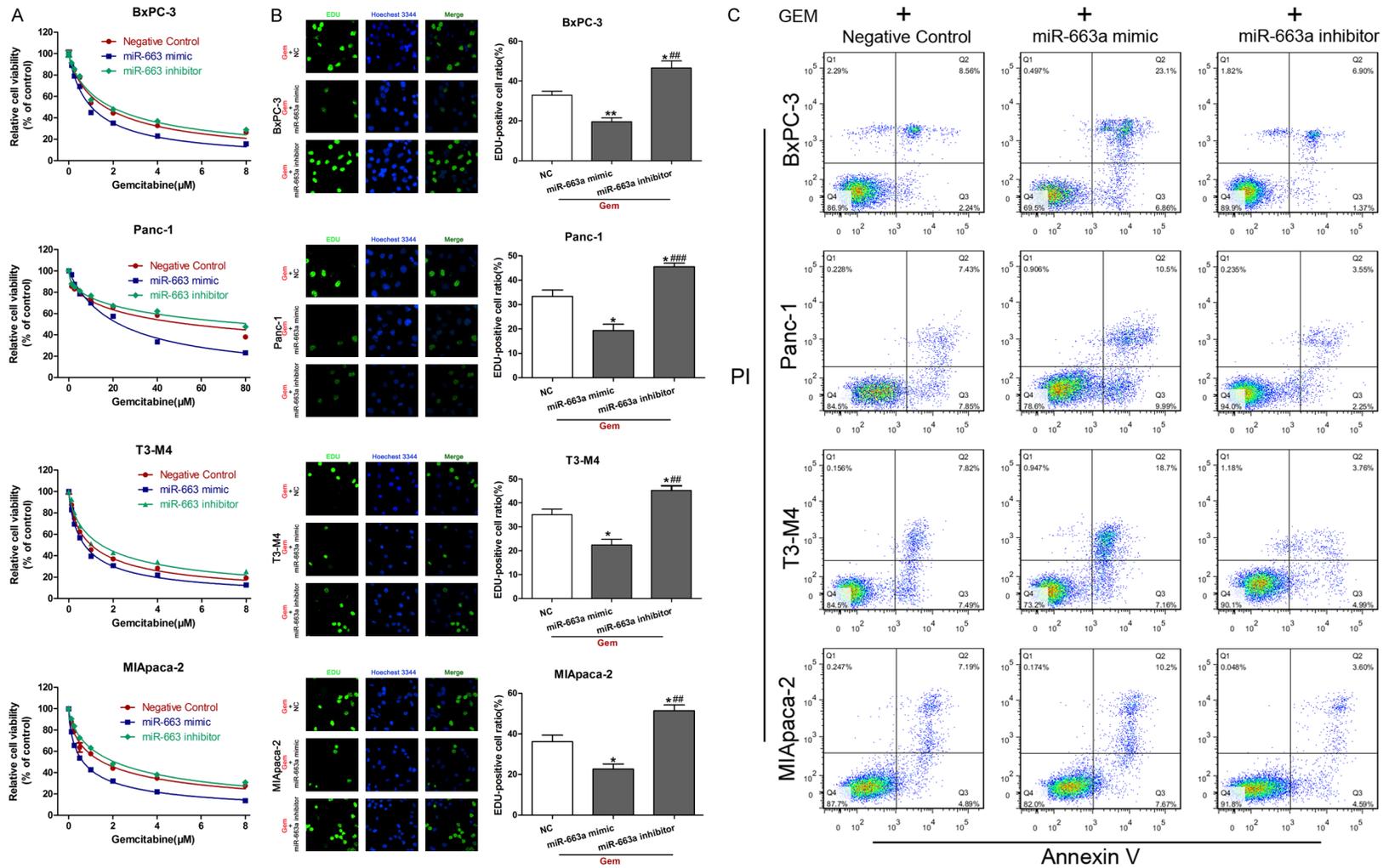
transfected cells were treated with GEM for EdU and apoptosis assays. Cell survival assay was employed to detect chemoresistance after treatment with GEM. Results showed that after treatment with GEM, miR-663a mimic significantly reduced cell viability in PDAC cells, while miR-663a inhibitor increased cell viability (**Figure 2A**, $P < 0.05$). We further investigated the functional significance of miR-663a on cell proliferation and apoptosis in PDAC cells. First, EdU analysis showed that the PDAC cells treated with miR-663a mimic had lower levels of proliferation than the negative control, while cells treated with miR-663a inhibitor had higher levels of proliferation than the negative control (**Figure 2B**, $P < 0.05$). Second, Annexin V/PI staining showed that miR-663a mimic significantly increased apoptotic cell rate and miR-663a inhibitor reduced apoptotic rate (**Figure 2C**, $P < 0.05$). Finally, we measured the levels of miR-663a expression in PDAC cells transfected with the miR-663a negative control, inhibitor, and mimics. The efficiency of transfection was assayed by qRT-PCR analysis. As expected, the expression of miR-663a was remarkably elevated after transfection with miR-663a mimic and declined with miR-663a inhibitor (**Figure 2D**). These data suggested that miR-663a played a role in GEM resistance.

Altered miR-663 expression in PDAC cell lines modifies sensitivity to OSI-027

To further examine the role of miR-663a in OSI-027 chemosensitivity, PDAC cells were transfected with miR-663a mimics or miR-663a inhibitor, respectively. The transfected cells were treated with OSI-027 for EdU and apoptosis assays. CCK8 assay showed that miR-663 mimic combined with OSI-027 led to a remarkable decline in cell viability compared with cells treated only with OSI-027. Accordingly, cell viability in cells treated with OSI-027 combined with miR-663 inhibitor was significantly higher than cells treated with OSI-027 alone (**Figure 3A**, $P < 0.05$).

We further investigated the functional significance of miR-663a on cell proliferation and apoptosis in PDAC cells. First, EdU analysis showed that PDAC cells in the presence of the miR-663a mimic had lower levels of proliferation than the negative control, while cells with miR-663a inhibitor had higher levels of proliferation than the negative control (**Figure 3B**, P

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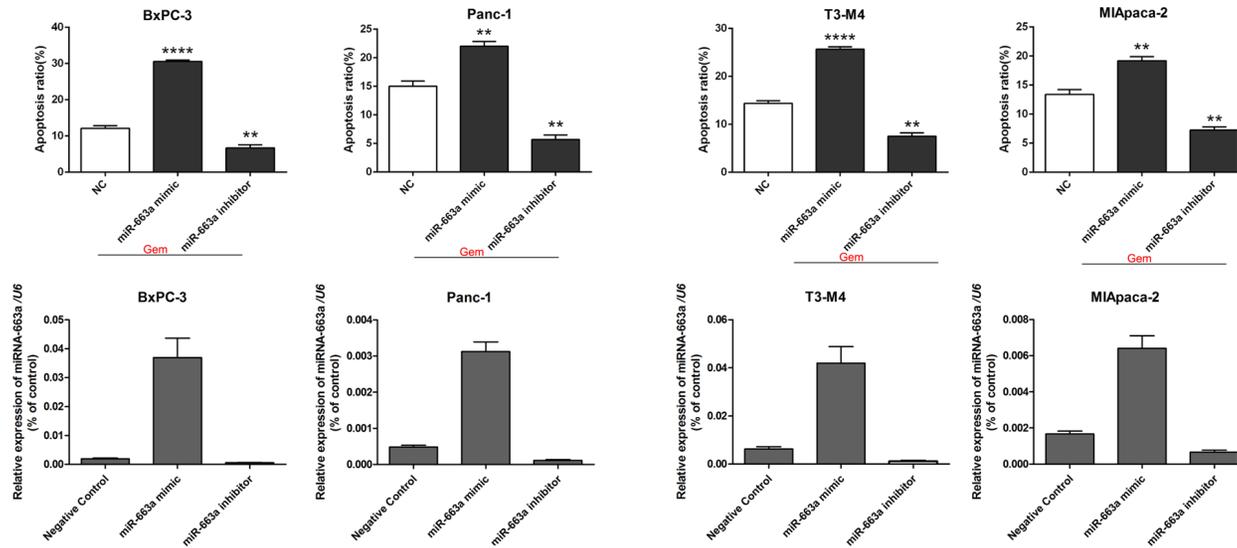
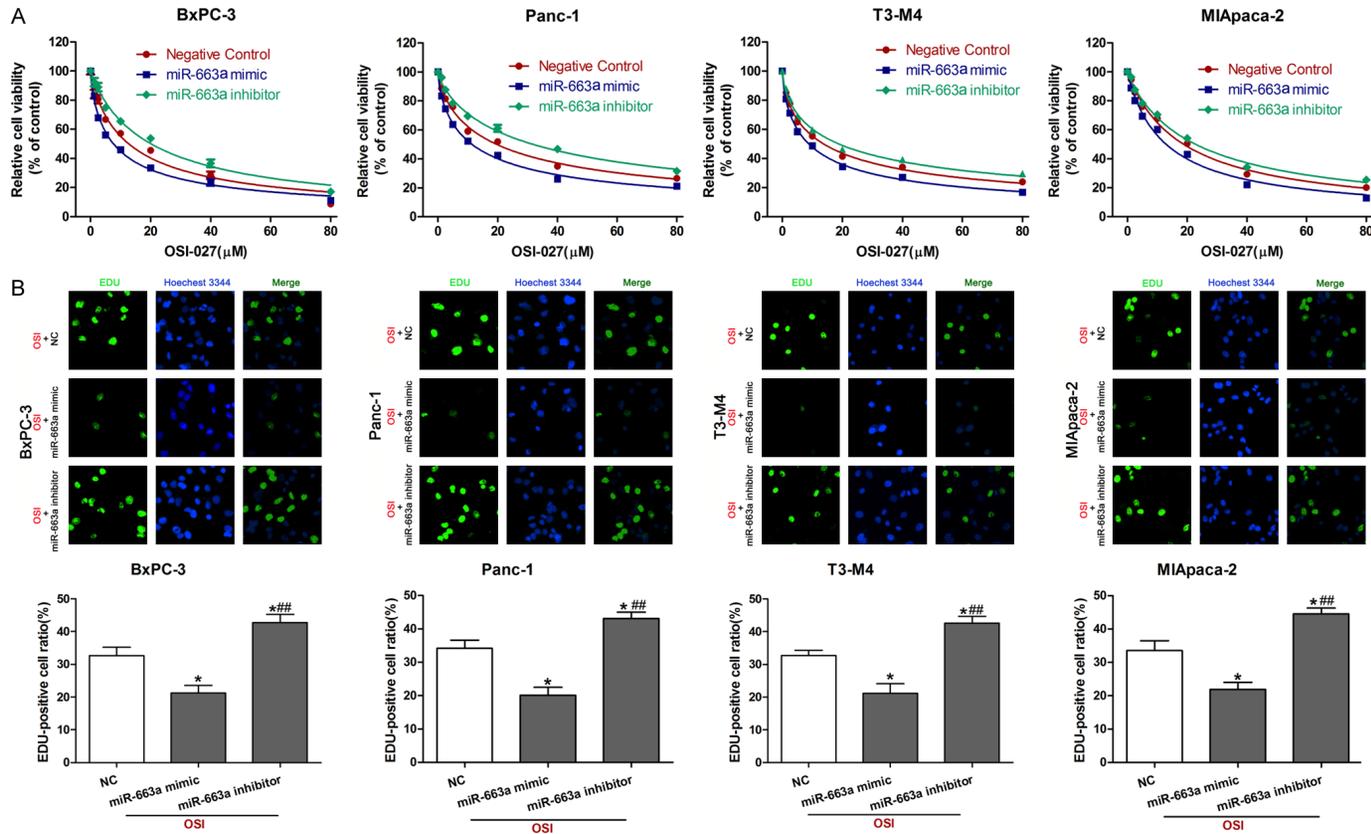


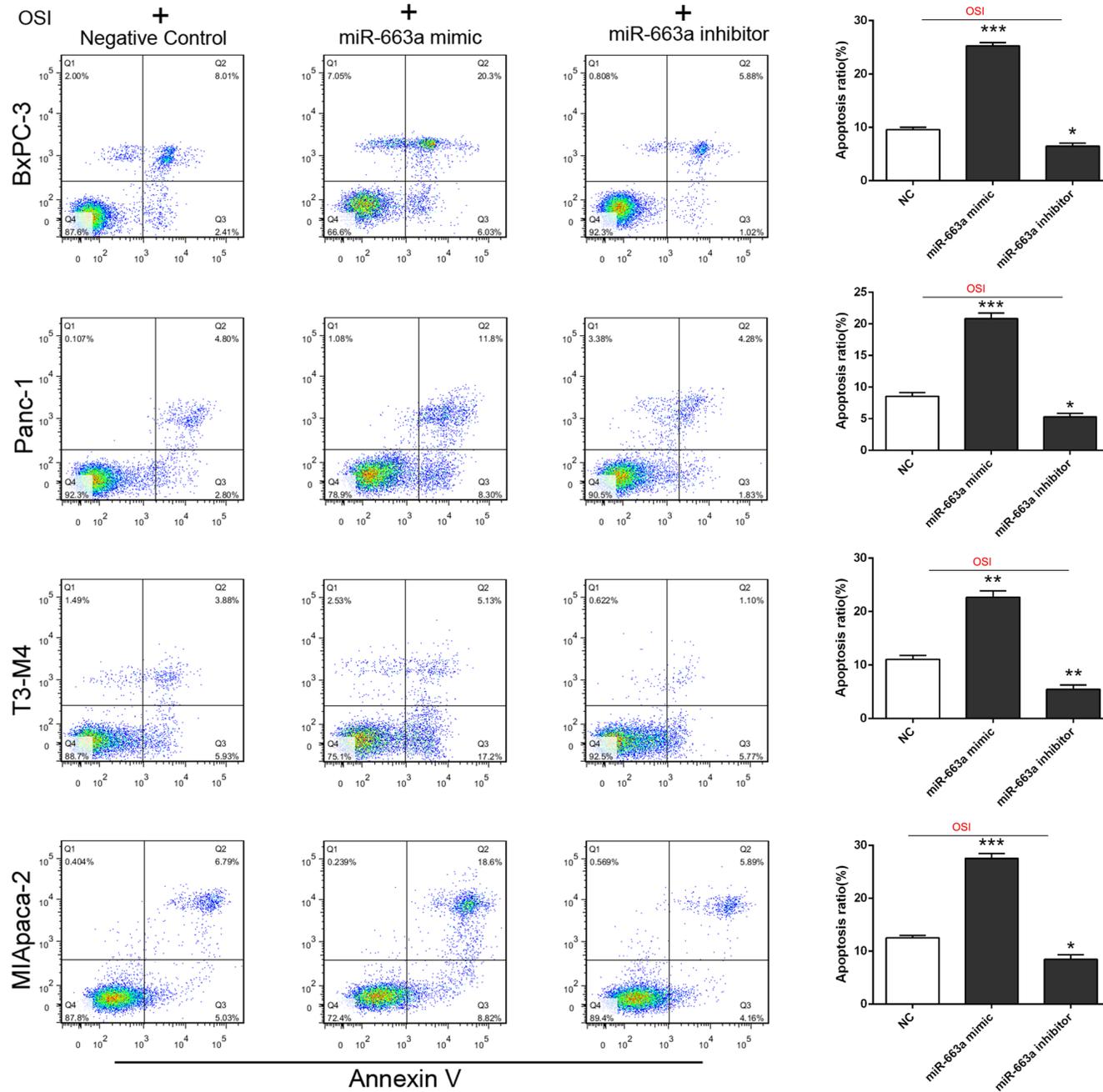
Figure 2. Altered miR-663 expression in PDAC cell lines promotes sensitivity to GEM. A. CCK-8 was used to analyze cell viability in PDAC cells transfected with miR-663 mimic, miR-663 inhibitor, or negative control. B. Relative EdU-positive cell ratios in PDAC cells transfected with miR-663 mimic, miR-663 inhibitor, or negative control after GEM treatment (IC50) for 48 h, quantified by EdU staining; *P < 0.05, **P < 0.01 vs. NC, ###P < 0.01, ####P < 0.001 vs. miR-663 mimic. C. Quantification of Annexin V/PI positive apoptotic cells in PDAC cells transfected with miR-663 mimic, miR-663 inhibitor, or negative control after GEM treatment (IC50), determined by flow cytometry. **P < 0.01 and ***P < 0.001. D. The expression of miR-663 in PDAC cells transfected with miR-663 mimic, miR-663 inhibitor, or negative control, examined by qRT-PCR analysis.

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Figure 3. Altered miR-663 expression in PDAC cell lines modifies sensitivity to OSI-027. A. CCK-8 was used to detect cell viability in PDAC cells transfected with miR-663 mimic, miR-663 inhibitor, or negative control. B. Relative Edu-positive cell ratios for PDAC cells transfected with miR-663 mimic, miR-663 inhibitor, or negative control after OSI-027 treatment (IC50) for 48 h, were quantified by Edu staining: *P < 0.05 vs. NC, ##P < 0.01 vs. miR-663 mimic. C. Quantification of Annexin V/PI positive apoptotic cells in PDAC cells transfected with miR-663 mimic, miR-663 inhibitor, or negative control after OSI-027 treatment (IC50), examined by flow cytometry. *P < 0.05, **P < 0.01 and ***P < 0.001.

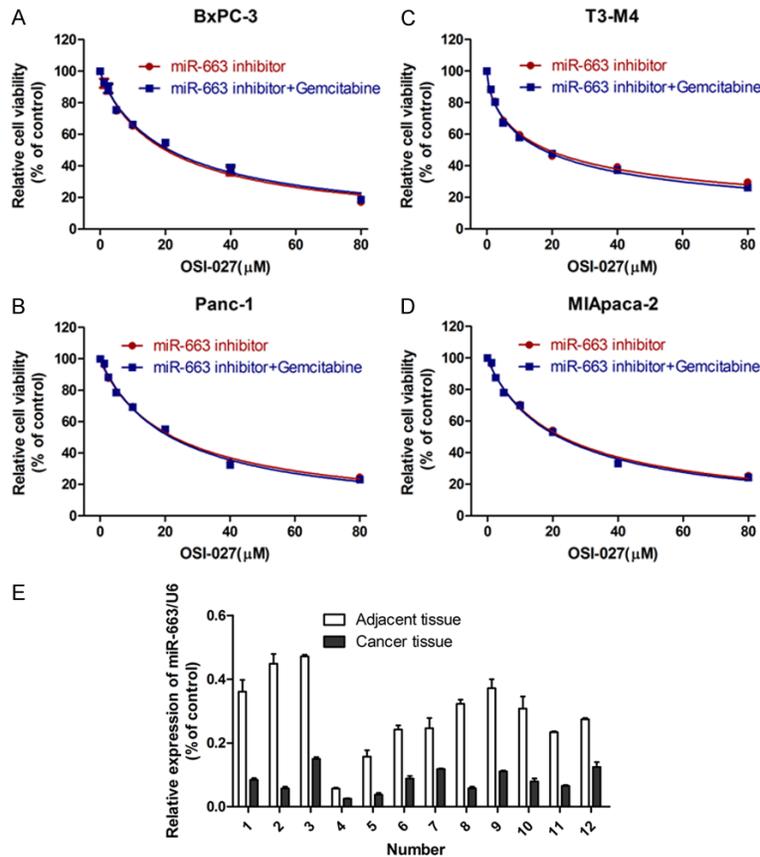


Figure 4. GEM desensitizes PDAC cells to OSI-027 treatment. A-D. Comparison of cell viability in PDAC cells with miR-663 inhibitor alone or miR-663 inhibitor combined with GEM. There was no significant difference in IC50 value. E. The level of miR-663 between adjacent tissue and cancer tissue was determined by qRT-PCR.

< 0.05). Second, Annexin V/PI staining showed that miR-663a mimic significantly increased apoptotic cell rate and miR-663a inhibitor reduced apoptotic rate (Figure 3C, $P < 0.05$).

GEM desensitized PDAC cells to OSI-27 treatment

The above results showed that miR-663a was a tumor suppressor gene, that is, miR-663a mimics enhanced drug sensitivity, while inhibitor promoted drug resistance. Therefore, we hypothesized that the mechanism underlying the synergistic benefit of GEM in combination with OSI-027 may be that GEM upregulates

miR-663a and promotes sensitivity of pancreatic ductal cancer cells to OSI. In order to test the hypothesis that GEM enhances the cytokine effect of OSI-027 by upregulating miR-663a, we transfected PDAC cells with miR-663a inhibitor. The cell viability of PDAC cells showed that there was no significant increase in the calculated IC50 value with or without GEM for OSI-027 treatment (Figure 4A-D). Furthermore, we analyzed the level of miR-663 between cancer tissue and adjacent tissue, the results found that adjacent tissue had the higher level of miR-663 than cancer tissue. These data show that the combined beneficial effect of OSI-027 and GEM disappeared after suppression of miR-663a with inhibitor; specifically, GEM-associated enhancement of the effect of OSI-027 must depend on miR-663a. These data demonstrated that GEM enhanced the cytokine effect of OSI-027 by upregulating miR-663a.

Discussion

Pancreatic cancer is a highly malignant digestive system tumor, and chemotherapy is one of its main therapies. As the main chemotherapy drug for pancreatic cancer, the role of GEM in improving prognosis for patients with pancreatic cancer is not definitive due to the presence of chemoresistance. Therefore, a better understanding and quicker ability to identify mechanisms of GEM chemoresistance may provide new treatments and better drug selection strategies for improving the prognosis of this lethal disease.

MiRNAs are a class of non-coding RNA with a length of about 22 nucleotides that play important roles in regulating gene expression. With growing knowledge of the relationship between miRNAs and cancer, it also has been shown that the sensitivity of cancer cells to anticancer drugs is affected by miRNAs [20], such as miRNA-34 [21], miRNA-206 [22], miRNA-485 [23], and let-7c [24]. To date, several miRNAs and their corresponding targeted genes have been found to be associated with chemotherapy resistance [25]. Based on these findings, the identification of resistance-relevant miRNAs may be a potential therapeutic strategy to reverse chemoresistance.

MiR-663a, which is located at human chromosome 20q11.1, was reported to be differentially expressed in diverse cancers. This miRNA is increasingly thought to act as a tumor suppressor, with a decreased level found in human gastric cancer cells [26] and glioblastoma [27]. MiR-663 is also reported to be upregulated in lung cancer [28], breast cancer [29], and nasopharyngeal carcinoma cells [30], and was determined to function as an oncogene. However, the role of miR-663 in PDAC, especially its correlation with chemosensitivity, has been unclear.

The present study identified that miR-663a expression was altered after treatment with both OSI-027 and GEM. MiR-663a was upregulated with GEM treatment alone and downregulated with OSI-027 treatment alone. With combination of the two drugs, miR-663a was lower than with GEM alone, but higher than with OSI alone. qRT-PCR analysis confirmed this result. Next, we confirmed that miR-663a was involved in the drug resistance of the cell lines used in this study. We used CCK8, EdU, and apoptosis to evaluate this and found that miR-663a mimics enhanced drug sensitivity, while inhibition promoted drug resistance. Finally, after the inhibitor interfered, there was no difference in OSI-027 sensitivity after treatment with or without GEM. Altogether, these data support the conclusion that GEM can enhance the killing effect of OSI on cells by raising levels of miR-663a.

This study is not without several limitations. For instance, we performed microarray analysis after treatment with GEM and/or OSI-027 and selected miR-663a as a candidate miRNA due

to its upregulation with GEM treatment and downregulation with OSI-027 treatment. We demonstrated that miR-663a was involved in the phenotype of chemotherapy resistance; however, we did not elucidate the relationship between miR-663a and MDR1.

In summary, our study demonstrated that miR-663a is a tumor suppressor in pancreatic cancer, whose overexpression can sensitize pancreatic cancer cells to GEM or OSI-027. Meanwhile, the combined effect of OSI-027 and GEM disappeared with inhibition of miR-663a. miR-663a may therefore potentially be used as a new biological marker of chemotherapy and its effectors potentially new targets to overcome chemoresistance.

Acknowledgements

This work was financially supported by the National High Technology Research and Development Program of China (SS2015AA0204-05 and SS2014AA020533), the Zhejiang Provincial Natural Science Foundation of China (LQ13H160006), the Key Innovative Team for the Diagnosis and Treatment of Pancreatic Cancer of Zhejiang Province (2013TD06), the Key Program of the National Natural Science Foundation of China (81530079), and the National Natural Science Foundation of China (81300341, 81302071 and 81401954).

Disclosure of conflict of interest

None.

Address correspondence to: Tingbo Liang, Department of Hepatobiliary and Pancreatic Surgery, The First Affiliated Hospital, School of Medicine, Zhejiang University, No. 79 Qingchun Road, Hangzhou 310003, China. Tel: +86 571-87315005; Fax: +86 571-87315005; E-mail: liangtingbo@zju.edu.cn

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