

RESEARCH ARTICLE

MiR-21 Upregulation Induced by Promoter Zone Histone Acetylation is Associated with Chemoresistance to Gemcitabine and Enhanced Malignancy of Pancreatic Cancer Cells

Wei-Feng Song^{1,2}, Lei Wang^{1,2}, Wei-Yi Huang¹, Xun Cai¹, Jiu-Jie Cui¹, Li-Wei Wang^{1,2*}

Abstract

Background and Aims: MicroRNA-21 (miR-21) is reported to be overexpressed and to contribute to proliferation, apoptosis and gemcitabine resistance in pancreatic ductal adenocarcinomas (PDACs). The aims of this study were to explore regulation of miR-21 expression by epigenetic change and its impact on chemoresistance and malignant properties of pancreatic cancer. **Materials and methods:** We retrospectively collected 41 cases of advanced pancreatic cancer patients who were sensitive or resistant to gemcitabine and assessed levels of serum circulating miR-21 for correlation with cytotoxic activity. Histone acetylation in the miR-21 promoter was also studied in gemcitabine-sensitive and gemcitabine-resistant PDAC cells. Gemcitabine-resistant HPAC and PANC-1 cells were transfected with pre-miR-21 precursors (pre-miR-21) and antisense oligonucleotides (anti-miR-21), and were treated with TSA. Finally, invasion and metastasis assays were performed and alteration in miR-21, PTEN, AKT and pAKT level was evaluated in these cells. **Results:** Serum miR-21 levels were increased in gemcitabine-resistant PDAC patients compared with gemcitabine-sensitive subjects. The miR-21 levels were increased in 6 PDAC cells treated with gemcitabine significantly, associated with 50% inhibitory concentrations (IC₅₀s). Histone acetylation levels at miR-21 promoter were increased in PDAC cells after treatment with gemcitabine. Enhanced invasion and metastasis, increased miR-21 expression, decreased PTEN, elevated pAKT level were demonstrated in gemcitabine-resistant HPAC and PANC-1 cells. Pre-miR-21 transfection or TSA treatment further increased invasion and metastasis ability, decreased PTEN, and elevated pAKT levels in these two lines. In contrast, anti-miR-21 transfection could reverse invasion and metastasis, and PTEN and pAKT expressions induced by gemcitabine. **Conclusions:** MiR-21 upregulation induced by histone acetylation in the promoter zone is associated with chemoresistance to gemcitabine and enhanced malignant potential in pancreatic cancer cells.

Keywords: Serum miR-21 - advanced pancreatic cancer - chemosensitivity - histone acetylation

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer-related death, with only 3% of patients alive 5 years after diagnosis (Siegel et al., 2012). Surgical resection is the only curative treatment option, but more than 80% of patients are diagnosed at advanced stages (Li et al., 2004). Despite advances in clinical management, the high mortality rate of pancreatic cancer, which is almost equal to its incidence, remains largely unchanged (Siegel et al., 2012). The main reasons for this poor prognosis include early metastatic spread, high local recurrence rate, and multifactorial resistance to treatments (Li et al., 2004).

Gemcitabine has been the first line of treatment for locally advanced and metastatic PDAC (Burriss et

al., 1997). However, the objective response rate of gemcitabine is lower than 10%, and only a minority of patients attain disease control, with <15% of patients progression-free at 6 months from diagnosis (Burriss et al., 1997; Li et al., 2004).

The mechanisms regulating PDAC cell growth and resistance to chemotherapy are poorly understood. In recent years, considerable interest has been placed in understanding the role of microRNAs in cancer. MicroRNAs (miRNAs) are small noncoding RNAs ranging in size from 17 to 25 nucleotides, which reduce gene expression after transcription by recognizing complementary target sites in the 3'-untranslated regions or coding sequence (CDS) of target mRNAs (Bartel, 2004; Lim et al., 2005). miRNAs play an important role in cell differentiation, apoptosis, tumor development, invasion,

¹Department of Medical Oncology, Shanghai First People's Hospital, Shanghai Jiao Tong University, ²Shanghai Key Laboratory of Pancreatic Diseases Research, Shanghai, China *For correspondence: liweiwangdr@163.com

Table 1. The Clinical Factors of Gemcitabine-sensitive and Gemcitabine-resistant Advanced PDCA Patients (n=41)

	No. of patients	Advanced PDCA patients		P-value
		Gemcitabine -sensitive n=21	Gemcitabine -resistant n=20	
Age (years)				
Mean±SD	66	59.6±10.8	60.0±10.6	0.828*
Gender				
Male	26	12	14	0.393
Female	15	9	6	
Location				
Head	17	10	7	0.412
Body, tail	24	11	13	
Stage				
III	16	9	7	0.606
IV	25	12	13	
CA19-9				
<500 IU/ml	19	10	9	0.867
≥500 IU/ml	22	11	11	

and metastasis (Negrini et al., 2007). With profound understanding of the miRNA target genes and the cellular behaviors influenced by them, miRNA may become exciting targets for cancer therapy (Cho, 2010). Growing evidence suggests that aberrant miRNA expression contributes to the development and progression of PDAC (Bloomston et al., 2007; Szafranska et al., 2007; Mardin & Mees, 2009).

The expression of miR-21 has been shown to be involved in the progression of several types of cancers (Kwak et al., 2011; Yang et al., 2011), including PDAC (Bloomston et al., 2007; Szafranska et al., 2007; Nagao et al., 2012), and the up-regulation of miR-21 expression has been shown to correlate with a lower cancer survival rate (Dillhoff et al., 2008; Jamieson et al., 2012). Recent studies also demonstrated that miR-21 modulates the chemosensitivity of cancer cells primarily by targeting PTEN or PDCD4 (Moriyama et al., 2009; Ali et al., 2010; Giovannetti et al., 2010; Tomimaru et al., 2010). In addition, cancer tissue miR-21 has been shown to be a biomarker for chemoresistance and clinical outcome following adjuvant therapy in resectable pancreatic cancer (Hwang et al., 2010). Further study showed that the serum level of miR-21 can be used as a predictor of chemosensitivity in advanced PDAC (Wang et al., 2013a). We also found that gemcitabine-resistant advanced PDAC patients had higher serum miR-21 level compared with gemcitabine-sensitive subjects. So we explore the effects of miR-21 expression on gemcitabine sensitivity, invasion and metastasis in PDAC cells.

Previous studies showed that (1) miR-21 regulated expression of PTEN and phosphorylation of its downstream kinase Akt (Meng et al., 2006; Meng et al., 2007; Li et al., 2009) and (2) the reduction of phospho-Akt (pAkt) correlated with enhanced apoptosis and antitumor activity induced by gemcitabine in vitro and in vivo, suggesting that Akt pathway plays a significant role in mediating drug resistance in PDAC cells (Ng et al., 2001). Therefore, we investigate the PTEN expression and

Akt phosphorylation status after pre-miR-21, anti-miR-21 transfection and gemcitabine treatment.

Currently the mechanisms that regulates miR-21 expression are not elucidated. Cancer epigenetics are characterized by DNA methylation and chromatin modification which are mediated by DNA methyltransferases (DNMTs), histone acetyltransferase, histone deacetylases (HDACs), and others (Yoo & Jones, 2006). HDACs selectively affect gene transcription through removing acetyl groups from histones and subsequently leading to chromatin condensation. There are 18 HDACs isoforms at known and divided into four subgroups dependent on structure homology. Among these, the ubiquitous expressed class I HDACs including HDAC1-3 and eight are the most characterized proteins. HDACs inhibitors represent a promising new class of compounds for the treatment of cancer (Miyanaga et al., 2008). The anti-tumor effects of these drugs are due to the hyperacetylation of histones, which finally lead to activation of tumor suppressor genes (Lane & Chabner, 2009). Treatment of inhibitors trichostatin A (TSA) reduced viability and induced apoptosis in NSCLC (Choi, 2005). HDACs inhibitors depsipeptide (FK228) and TSA decreased expression and altered localization of Aurora kinases and survivin, resulting in mitotic catastrophe and cell death in lung cancer cells (Zhang et al., 2008a). So we investigate the correlation of histone acetylation at miR-21 promoter zone with chemoresistance to gemcitabine, invasion and metastasis in PDAC cancer cells.

Materials and Methods

Patient characteristics and clinical features

A total of 41 patients with pathologically confirmed locally advanced or metastatic pancreatic adenocarcinoma were retrospectively recruited between January 2010 and December 2012 from Shanghai First People's Hospital. All of the patients received gemcitabine as first-line palliative chemotherapy. Response evaluations were done every six weeks according to RECIST (Response Evaluation Criteria In Solid Tumors) Version 1.1. Among them, 21 patients were gemcitabine-sensitive (Complete Regression for 4 patients and Partial Regression for 17 patients) and 20 patients were gemcitabine-resistance (progression at first response evaluation). Patient characteristics and clinical features were balanced between two groups (Table 1). Each participant donated 5 ml of venous blood before initiating chemotherapy. The blood was collected in tubes with an EDTA anticoagulant and was separated into the serum and cellular fractions within 2 hour after the sample collection. The serum was then stored immediately at -80 °C until analysis. This study was approved by the Ethics Committee of Shanghai First People's Hospital, and written informed consent was obtained from each participant, in accordance with the institutional guidelines of our hospital.

Cell lines

Six PDAC cell lines were obtained from Shanghai Institute for life science, Chinese Academy of Sciences. Cells were grown in 5% CO₂ saturated humidity, at 37°C

and cultured in DMEM (Gibco, USA) supplemented with penicillin/streptomycin, 2 mmol/L glutamine and 10% FBS. The cell growth-inhibitory effect of 72-hour gemcitabine exposure was studied as described previously (Giovannetti et al., 2006). Gemcitabine-resistant cells were acquired after 72-hour gemcitabine exposure at IC₅₀ (HPAC and PANC-1 cells).

Cytotoxicity studies

Cell growth inhibition was determined from three separate experiments using both MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and SRB (sulforhodamine-B) assays and was expressed as the percentage of control (vehicle-treated cells) absorbance, corrected for absorbance before drug addition. The 50% inhibitory concentration (IC₅₀) of cell growth for each cell line was determined by non-linear least squares curve fitting of the dose-response curves (GraphPad PRISM, Intuitive Software for Science).

Reverse transcription and quantitative PCR

Reverse transcription and quantitative PCR analysis of miR-21. RNA (10–100 ng) was reverse transcribed and the resulting cDNA was amplified using the specific Taqman MicroRNA assays (Applied Biosystems) for miR-21 and RNU43 (assay ID, 000397 and 001095, respectively). The quantification of DNA was done by real-time PCR, using SYBR Premix Ex Taq™ (Takara) and ROTOR-GENE RG-3000A (Corbett Research). Ct values from each sample were obtained using the operating software of RG-3000A. Relative quantification of amplified template was performed as described by Chakrabarti et al. (2002). Each PCR reaction, run in triplicate for each sample, was repeated at least twice independently. Quantification of relative expression [reported as arbitrary units (a.u.)] was performed using the Δ Ct method. Δ Ct was obtained by normalizing to corresponding control.

miR-21 transfection.

The effect of miR-21 on chemoresistance, invasion and metastasis was evaluated by transfecting the PDAC cells with pre-miR-21 precursors (pre-miR-21) or antisense oligonucleotides (anti-miR-21) purchased from Ambion (assay ID, PM10206 and AM10206, respectively) at 30 nmol/L final concentration. Cells were plated at 200,000 per well in 3 mL RPMI 1640 with 10% fetal bovine serum (FBS) and 1% antibiotics. After 24 hours, cells were exposed to 9 μ L Oligofectamine (Invitrogen) in serum-free medium and mixed for 10 minutes, followed by addition of 3 μ L miR-21 precursor/inhibitor. Cells were also incubated with miRNA-negative controls and FAM-labeled pre-miR/anti-miR (Ambion). After 24 hours, the medium was replaced with RPMI 1640 with 10% FBS, without antibiotics. To evaluate the effects on cell growth, cells were allowed to grow for additional 48 or 72 hours in drug-free medium or treated with gemcitabine, as described previously (Giovannetti et al., 2006; Funel et al., 2008).

Invasion assay

Invasion by PDAC cell lines were assayed using

12-well cell culture chambers containing inserts with 8 μ m pores coated with matrigel (Corning, USA). The cells were added to the upper chamber at a density of 4×10^4 cells/insert. After 24 h of incubation, cells on the upper surface were wiped off with a cotton swab. Cells that had invaded the lower surface were fixed with 70% ethanol, stained with 0.01% Hoechst. Invasiveness was quantitated by selecting ten different views (100 times) and calculating the number of invading cells.

Migration assay

Migration assays were performed using two-chamber-Transwell (Corning, USA). The lower surface of a polycarbonate filter with 8 μ m pores was coated with 1 μ g/ml bovine collagen IV. Cells were trypsinized and suspended in a serum-free medium containing 1% BSA at a concentration of 4×10^4 cells/insert. The cells were placed in the upper chamber and free DMEM was placed in the lower chamber. After 12 hr at 37 °C, the cells in the upper chamber were wiped off with a cotton swab. The cells on the lower surface of the filter were fixed with 70% ethanol, stained with 0.01% Hoechst, migration was quantitated by selecting ten different views (100 times) and calculating the number of migrated cells.

Western blotting

Cells were lysed in RIPA buffer (150 mM NaCl, 100 mM Tris-HCl, 1% Tween-20, 1% sodium deoxycholate and 0.1% SDS) with 0.5 mM EDTA, 1 mM PMSF, 10 μ g/ml aprotinin and 1 μ g/ml pepstatin. Proteins were resolved in SDS-PAGE and transferred to PVDF membranes. In Western blotting analysis, the blots were incubated with IRDye 700CW or 800CW-conjugated secondary antibody, the infrared fluorescence image was obtained using Odyssey infrared imaging system (Li-Cor Bioscience), and the optical density of the bands were quantified using the Image-Pro Plus 5.1 (Media Cybernetics), and the optical density of each Ach3, Ach4, AKT, P-AKT, or PTEN bands were normalized to the optical densities of the corresponding H3, H4, or β -actin bands respectively. Data were obtained from at least three independent experiments.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed using the Upstate Biotechnology ChIP kit and following a modified protocol from the manufacturer. Briefly, cell lysates were sheared by sonication in 1% SDS lysis buffer to generate chromatin fragments with an average length of 200-1000 bp. The chromatin was then immunoprecipitated overnight at 4 °C with antibodies specific to Ach3, Ach4, or an equivalent amount of control IgG. Protein-DNA-antibody complexes were precipitated with protein A-agarose beads for 2 h at 4 °C. Input or DNA in the complex was subjected to quantitative PCR.

Sense Primer 'TTCTTGCCGTTCTGTAAGT; Anti-sense Primer 'GAGATGTCACGATGGTAGG

Statistical analysis

All statistical analyses were performed using SPSS 10.0. Data were expressed as mean \pm SD. The statistical

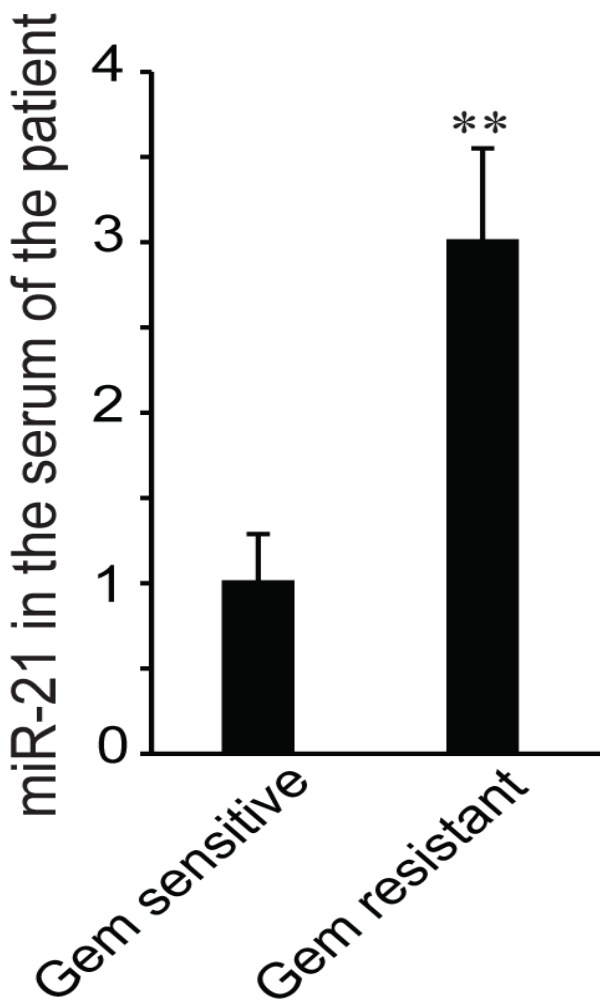


Figure 1. Serum miR-21 Levels Before Initiating Chemotherapy Increased Significantly in Gemcitabine-resistant Advanced PDAC Patients Compared with Gemcitabine-sensitive Patients. Columns, mean from three independent experiments; bars, SD (* $P < 0.001$, Mann-Whitney)

correlation of data between groups was analyzed by ANOVA, Student's t test, Mann-Whitney U test or Pearson χ^2 test, where $P < 0.05$ were considered significant.

Results

Serum miR-21 levels increase in gemcitabine-sensitive compared to gemcitabine-resistant PDAC patients

Serum miRNA-21 expression level was examined by qRT-PCR in the 21 patients resistant to gemcitabine and 20 patients sensitive to gemcitabine. The serum miRNA-21 level in gemcitabine-resistant patients was significantly higher than in gemcitabine-sensitive patients ($p < 0.001$) (Figure 1).

miR-21 expression and gemcitabine cytotoxicity in PDAC cells

MiR-21 was expressed in all six PDAC cells and significantly increased after gemcitabine treatment. The expression of miR-21 was detectable in all PDAC cell lines. However, this expression differed among cells, ranging from 4.6 a.u. in PL-45 to 0.1 a.u. in BxPc3 cells (Figure 2A). To evaluate whether gemcitabine affects miR-

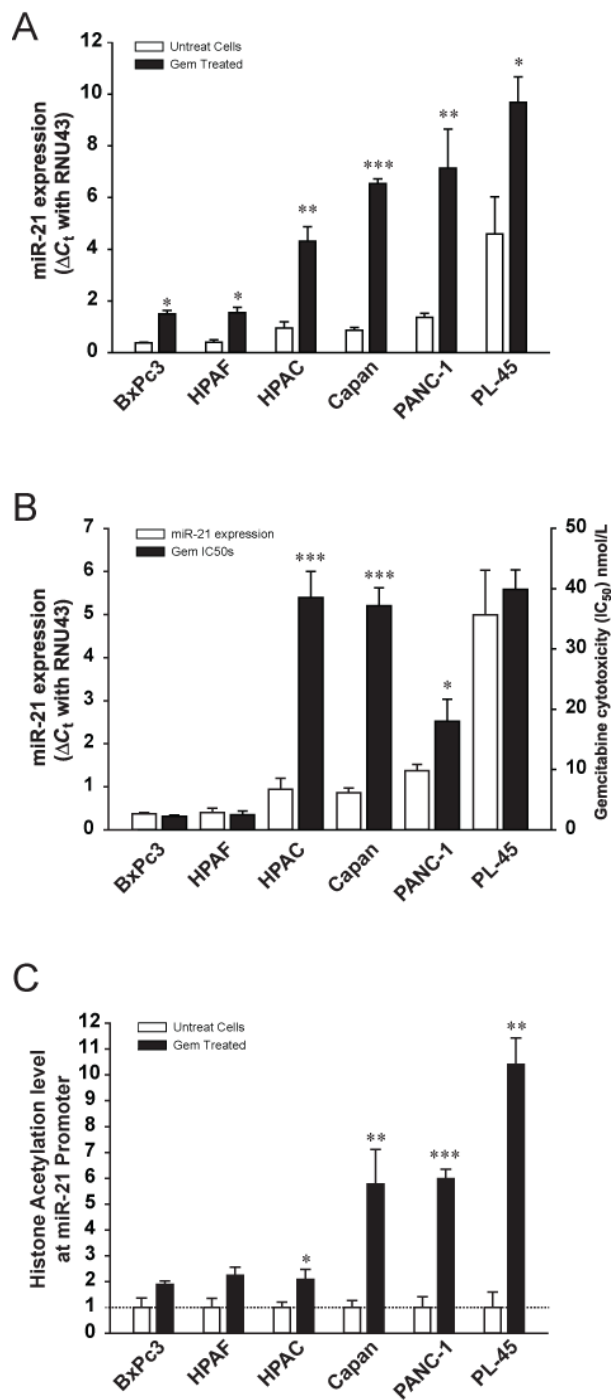


Figure 2. A, significant modulation of miR-21 expression in PDAC cells treated with gemcitabine at IC₅₀s. **B,** expression values of miR-21 in PDAC cell lines (white columns) and gemcitabine IC₅₀s (black columns). **C,** significant elevation of histone acetylation level at miR-21 promoter in PDAC cells treated with gemcitabine at IC₅₀. Columns, mean from three independent experiments; bars, SD (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Mann-Whitney)

21 expression in vitro, we measured the levels of miR-21 in 6 PDAC cells after 72-hour exposure to gemcitabine at IC₅₀. The gemcitabine treatment resulted in a significant increase of miR-21 expression, ranging from 2.1- to 19.1-fold, in comparison with basal expression (Figure 2A).

A dose-dependent inhibition of cell growth was observed after gemcitabine treatment in all PDAC cells (Figure 2B), with IC₅₀s ranging from 4.5 ± 0.7 nmol/L (BxPc3) to 40.1 ± 4.2 nmol/L (PL-45). The Spearman

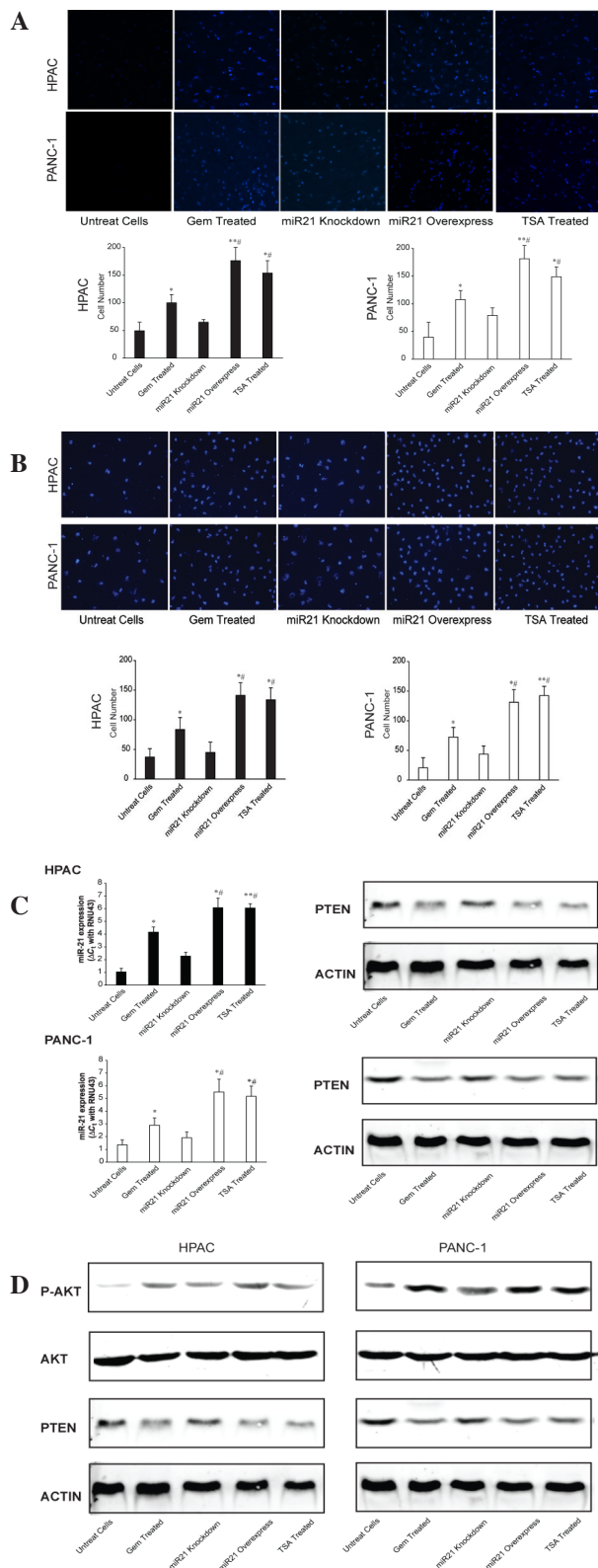


Figure 3. A, HPAC and PANC-1 Cells invasion assays. Representative photographs and quantification are shown. B, HPAC and PANC-1 Cells migration assays. Representative photographs and quantification are shown. C, miR-21 expression and PTEN protein level in HPAC and PANC-1 cells. D, Western blot analysis for PTEN, AKT and p-AKT protein in HPAC and PANC-1 cells. Cells untreated or treated with gemcitabine (chemoresistance to gemcitabine), cells (gemcitabine treated) transfected with pre-miR-21 or anti-miR-21, cells (gemcitabine treated) treated with TSA. Columns, mean from three independent experiments; bars, SD (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Mann-Whitney)

test showed significant correlation ($P = 0.04$) between the miR-21 expression and gemcitabine IC_{50} s.

Histone acetylation level at miR-21 promoter increased in PDAC cells after treatment with gemcitabine

To evaluate whether gemcitabine affects histone acetylation level at miR-21 promoter in vitro, we measured the levels of histone acetylation level in 6 PDAC cells after 72-hour exposure to gemcitabine at IC_{50} . This treatment resulted in a significant increase of histone acetylation level, ranging from 1.9- to 10.1-fold, in comparison with basal level (Figure 2C).

Chemoresistance to gemcitabine in PDAC cells correlates within vasion, metastasis and miR-21/PTEN/Akt expression

We assayed whether gemcitabine treatment can change the capacity of PDAC cells for invasion, metastasis. PDAC cells were suitable for the invasion and metastasis assay, because they showed good invasion to the Matrigel membranes. The cell invasion and metastasis assay showed that gemcitabine-resistant PDAC cells (HPAC and PANC-1 after treatment with gemcitabine) had higher invasion and metastasis rate compared with the control cells ($P < 0.05$, Figure 3A, B). Gemcitabine-resistant HPAC and PANC-1 cells had increased miR-21 expression ($P < 0.05$), reduced PTEN level and increased pAkt/Akt ratio (Figure 3C, D).

miR-21 affects invasion, metastasis and PTEN and Akt expression

We assayed whether miR-21 could change the capacity of PDAC cells for invasion and metastasis. The cell invasion and metastasis assay showed that anti-miR-21 inhibitor transfection can reverse increased invasion and metastasis capability of gemcitabine-resistant HPAC and PANC-1 cells compared with the control cells ($P < 0.05$, Figure 3A, B). In contrast, pre-miR-21 transfection in gemcitabine-resistant PDAC cells resulted in further increased cell invasion and metastasis in transwell assays ($P < 0.05$, Figure 3A, B). Gemcitabine-resistant HPAC and PANC-1 cells transfected with Pre-miR-21 had further reduced PTEN expression and increased pAkt/Akt ratio compared with gemcitabine-resistant cells (Figure 3A, B), whereas anti-miR-21 inhibitor transfection could reverse low PTEN expression and high pAkt/Akt ratio induced by gemcitabine resistance (Figure 3C, D).

TSA affects invasion, metastasis and miR-21/PTEN/Akt expression

We assayed whether TSA treatment can change the capacity of PDAC cells for invasion and metastasis. The cell invasion and metastasis assay showed that gemcitabine-resistant HPAC and PANC-1 cells treated with TSA had higher invasion and metastasis rate compared with the gemcitabine-resistant cells ($P < 0.05$, Figure 3A, B). At the same time gemcitabine-resistant HPAC and PANC-1 cells treated with TSA had higher miR-21 expression, lower PTEN level and higher pAkt/Akt ratio compared with the gemcitabine-resistant control cells (Figure 3C, D).

Discussion

The current studies showed that a number of miRNAs were differentially expressed in cancer and normal tissues. microRNA-21 has been suggested to function as an oncogene because it is overexpressed in many types of malignancy such as lung cancer (Liu et al., 2013), ovarian cancer (Iorio et al., 2007), colon cancer (Slaby et al., 2007), gastric cancer (Chan et al., 2008) and esophageal squamous cell carcinoma (Hiyoshi et al., 2009). Particularly, it had a high expression in the more aggressive PDAC cell and tissue samples (Giovannetti et al., 2010). However, most of these reports focused on studying the miRNA expression in cancerous tissues and safely obtaining sufficient quantities of tumor tissue is difficult for PDAC. Therefore, noninvasive methods for detection are warranted. Recent studies have shown that miRNAs are stably detectable in plasma or serum, and a strong correlation exists between tumor-derived circulating miRNAs and miRNAs directly within tumor cells (Chen et al., 2008; Mitchell et al., 2008). The serum level of miR-21 could serve as a surrogate for the expression level of miR-21 in the tumor tissue and be used as a predictor of the chemosensitivity of advanced PDAC treated with gemcitabine (Wang et al., 2013a). In our study, serum miR-21 levels were significantly higher in gemcitabine-resistant PDAC patients compared with gemcitabine-sensitive subjects. The miR-21 expression also significantly increased in 6 PDAC cells treated with gemcitabine. The miR-21 expression was associated with gemcitabine resistance. We therefore further explored the biological function of microRNA-21 in PDAC cells in vitro. Our current findings showed that microRNA-21 could significantly increase Chemoresistance, invasion and metastasis capacity in PDAC cells.

Various studies have reported that miR-21 plays an important role not only in tumor growth but also in the invasion and metastasis by targeting multiple tumor suppressor genes including PTEN, PDCD4, BCL-2, TPM1, and RECK (Zhu et al., 2007; Asangani et al., 2008; Gabriely et al., 2008; Zhang et al., 2008b; Zhu et al., 2008; Nagao et al., 2012; Zhang et al., 2012). PTEN (phosphatase and tensin homolog deleted on chromosome 10) (Li et al., 1997) is one of the most frequently inactivated tumor suppressors in different tumor types. PTEN negatively regulates intracellular levels of PI-3K/Akt, most likely via direct dephosphorylation, suggesting that it exerts its role as a tumor suppressor by negatively regulating the PI-3K/Akt signaling pathway (Leslie & Downes, 2002). This pathway is known to play a key role in numerous cellular functions including proliferation, adhesion, angiogenesis, migration, invasion, metabolism, survival and chemoresistance (Bader et al., 2005). PTEN was reported as a direct target of miR-21 that was involved in miR-21-mediated effects on tumor biology: cell growth, migration, and invasion in human hepatocellular carcinoma (Meng et al., 2007), and gemcitabine-induced apoptosis in human cholangiocarcinoma (Meng et al., 2006). PTEN has been shown to inhibit tumor cell growth and invasion by blocking the PI3K/AKT pathway (Vogt et al., 2009). Several mechanisms such as genetic mutation, promoter

methylation, and post-transcriptional modification, may contribute to PTEN inactivation. We demonstrated that miR-21 inhibitor increases chemosensitivity, and restrains invasion and metastasis in PDAC cells. So we focused our attention on PTEN. Based on these various aspects of the microRNA-21 function, we hypothesized that miR-21 played an important role in the progression of PDAC and regulated PTEN expression. Indeed, our current study revealed that gemcitabine-resistant PDAC cells transfected with miR-21 inhibitor could reverse reduction of PTEN protein level, pAKT/AKT ratio increase induced by chemoresistance. Thus, we postulated that PTEN was also an important target of miR-21 in PDAC. Therefore, restraint on PDAC cell chemoresistance, invasion and metastasis by miR-21 inhibitor may be partially mediated via up-regulation of PTEN. Indeed, other mechanisms and targets of miR-21 besides PTEN may play roles in mediating PDAC cell chemoresistance, invasion and metastasis. Nevertheless, these other mechanisms still need to be elucidated.

Expression of miRNAs in human cancer was considered to be regulated at multiple levels. miR-21 transcription was inhibited by estradiol and was stimulated by activation protein 1 (AP-1) and signal transducer and activator of transcription 3 (STAT3) in cancer (Krichevsky & Gabriely, 2009; Wickramasinghe et al., 2009). In addition, TGF- β increased miR-21 expression at the post-transcriptional level (Wickramasinghe et al., 2009). Therefore, deregulation of miR-21 expression in PDAC may occur at the transcriptional or post-transcriptional level. Epigenetics is a rapidly expanding field that focuses on stable changes in gene expression that are not accompanied by any changes in the DNA sequence, and that are mediated primarily by DNA methylation, histone modifications and small non-coding RNA molecules (Boumber & Issa, 2011). Many miRNAs are epigenetically regulated by either histone modifications or by combined DNA methylation and histone modification (Wang et al., 2013b). Histone acetylation regulates miR-375 in esophageal squamous cell carcinoma (Isozaki et al., 2012). We demonstrated that miR-21 expression correlated with acetylation level at miR-21 promoter. MiR-21 level was significantly elevated after TSA treatment. At the same time the invasion and metastasis capability of PDAC cells also markedly increased after TSA treatment.

In conclusion, miR-21 is over expressed in PDAC cells treated with gemcitabine, and miR-21 modulates Chemoresistance, cell invasion and metastasis in vitro. These effects are likely due to direct suppression of PTEN by miR-21. MiR-21 upregulation may be induced by histone acetylation at promoter zone. Our data suggest that miR-21 and HDAC are possible to become potential therapeutic targets for PDAC.

References

- Ali S, Ahmad A, Banerjee S, et al (2010). 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*, **70**, 3606-17.
- Asangani IA, Rasheed SA, Nikolova DA, et al (2008).

- MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pcdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer. *Oncogene*, **27**, 2128-36.
- Bader AG, Kang S, Zhao L, Vogt PK (2005). Oncogenic PI3K deregulates transcription and translation. *Nature reviews. Cancer*, **5**, 921-9.
- Bartel DP (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*, **116**, 281-97.
- Bloomston M, Frankel WL, Petrocca F, et al (2007). MicroRNA expression patterns to differentiate pancreatic adenocarcinoma from normal pancreas and chronic pancreatitis. *JAMA:J Am Med Assoc*, **297**, 1901-8.
- Boumber Y, Issa JP (2011). Epigenetics in cancer: what's the future? *Oncology (Williston Park, N.Y.)*, **25**, 220-6, 228.
- Burris HA, 3rd Moore MJ, Andersen J, et al (1997). Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *J Clin Oncol*, **15**, 2403-13.
- Chakrabarti SK, James JC, Mirmira RG (2002). Quantitative assessment of gene targeting in vitro and in vivo by the pancreatic transcription factor, Pdx1. Importance of chromatin structure in directing promoter binding. *J Biol Chem*, **277**, 13286-93.
- Chan SH, Wu CW, Li AF, Chi CW, Lin WC (2008). miR-21 microRNA expression in human gastric carcinomas and its clinical association. *Anticancer Res*, **28**, 907-11.
- Chen X, Ba Y, Ma L, et al (2008). Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res*, **18**, 997-1006.
- Cho WC (2010). MicroRNAs in cancer - from research to therapy. *BBA*, **1805**, 209-17.
- Choi YH (2005). Induction of apoptosis by trichostatin A, a histone deacetylase inhibitor, is associated with inhibition of cyclooxygenase-2 activity in human non-small cell lung cancer cells. *Int J Oncol*, **27**, 473-9.
- Dillhoff M, Liu J, Frankel W, Croce C, Bloomston M (2008). MicroRNA-21 is overexpressed in pancreatic cancer and a potential predictor of survival. *J Gastrointest Surg*, **12**, 2171-6.
- Funel N, Giovannetti E, Del Chiaro M, et al (2008). Laser microdissection and primary cell cultures improve pharmacogenetic analysis in pancreatic adenocarcinoma. *Lab Invest*, **88**, 773-84.
- Gabriely G, Wurdinger T, Kesari S, et al (2008). MicroRNA 21 promotes glioma invasion by targeting matrix metalloproteinase regulators. *Mol Cell Biol*, **28**, 5369-80.
- Giovannetti E, Del Tacca M, Mey V, et al (2006). Transcription analysis of human equilibrative nucleoside transporter-1 predicts survival in pancreas cancer patients treated with gemcitabine. *Cancer Res*, **66**, 3928-35.
- Giovannetti E, Funel N, Peters GJ, et al (2010). MicroRNA-21 in pancreatic cancer: correlation with clinical outcome and pharmacologic aspects underlying its role in the modulation of gemcitabine activity. *Cancer Res*, **70**, 4528-38.
- Hiyoshi Y, Kamohara H, Karashima R, et al (2009). MicroRNA-21 regulates the proliferation and invasion in esophageal squamous cell carcinoma. *Clin Cancer Res*, **15**, 1915-22.
- Hwang JH, Voortman J, Giovannetti E, et al (2010). Identification of microRNA-21 as a biomarker for chemoresistance and clinical outcome following adjuvant therapy in resectable pancreatic cancer. *PLoS one*, **5**, e10630.
- Iorio MV, Visone R, Di Leva G, et al (2007). MicroRNA signatures in human ovarian cancer. *Cancer Res*, **67**, 8699-707.
- Isozaki Y, Hoshino I, Nohata N, et al (2012). Identification of novel molecular targets regulated by tumor suppressive miR-375 induced by histone acetylation in esophageal squamous cell carcinoma. *Int J Oncol*, **41**, 985-94.
- Jamieson NB, Morran DC, Morton JP, et al (2012). MicroRNA molecular profiles associated with diagnosis, clinicopathologic criteria, and overall survival in patients with resectable pancreatic ductal adenocarcinoma. *Clin Cancer Res*, **18**, 534-45.
- Krichevsky AM, Gabriely G (2009). miR-21: a small multi-faceted RNA. *J Cell Mol Med*, **13**, 39-53.
- Kwak HJ, Kim YJ, Chun KR, et al (2011). Downregulation of Spry2 by miR-21 triggers malignancy in human gliomas. *Oncogene*, **30**, 2433-42.
- Lane AA, Chabner BA (2009). Histone deacetylase inhibitors in cancer therapy. *J Clin Oncol*, **27**, 5459-68.
- Leslie NR, Downes CP (2002). PTEN: The down side of PI 3-kinase signalling. *Cell Signal*, **14**, 285-95.
- Li D, Xie K, Wolff R, Abbruzzese JL (2004). Pancreatic cancer. *Lancet*, **363**, 1049-57.
- Li J, Huang H, Sun L, et al (2009). MiR-21 indicates poor prognosis in tongue squamous cell carcinomas as an apoptosis inhibitor. *Clin Cancer Res*, **15**, 3998-4008.
- Li L, Ernsting BR, Wishart MJ, Lohse DL, Dixon JE (1997). A family of putative tumor suppressors is structurally and functionally conserved in humans and yeast. *J Biol Chem*, **272**, 29403-6.
- Lim LP, Lau NC, Garrett-Engle P, et al (2005). Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature*, **433**, 769-73.
- Liu ZL, Wang H, Liu J, Wang ZX (2013). MicroRNA-21 (miR-21). expression promotes growth, metastasis, and chemoradioresistance in non-small cell lung cancer cells by targeting PTEN. *Mol Cell Biochem*, **372**, 35-45.
- Mardin WA, Mees ST (2009). MicroRNAs: novel diagnostic and therapeutic tools for pancreatic ductal adenocarcinoma? *Ann Surg Oncol*, **16**, 3183-9.
- Meng F, Henson R, Lang M, et al (2006). Involvement of human micro-RNA in growth and response to chemotherapy in human cholangiocarcinoma cell lines. *Gastroenterology*, **130**, 2113-29.
- Meng F, Henson R, Wehbe-Janek H, et al (2007). MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer. *Gastroenterology*, **133**, 647-58.
- Mitchell PS, Parkin RK, Kroh EM, et al (2008). Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci USA*, **105**, 10513-8.
- Miyayama A, Gemma A, Noro R, et al (2008). Antitumor activity of histone deacetylase inhibitors in non-small cell lung cancer cells: development of a molecular predictive model. *Mol Cancer Ther*, **7**, 1923-30.
- Moriyama T, Ohuchida K, Mizumoto K, et al (2009). MicroRNA-21 modulates biological functions of pancreatic cancer cells including their proliferation, invasion, and chemoresistance. *Mol Cancer Ther*, **8**, 1067-74.
- Nagao Y, Hisaoka M, Matsuyama A, et al (2012). Association of microRNA-21 expression with its targets, PDCD4 and TIMP3, in pancreatic ductal adenocarcinoma. *Modern Pathol*, **25**, 112-21.
- Negrini M, Ferracin M, Sabbioni S, Croce CM (2007). MicroRNAs in human cancer: from research to therapy. *J Cell Sci*, **120**, 1833-40.
- Ng SS, Tsao MS, Nicklee T, Hedley DW (2001). Wortmannin inhibits pkb/akt phosphorylation and promotes gemcitabine antitumor activity in orthotopic human pancreatic cancer xenografts in immunodeficient mice. *Clin Cancer Res*, **7**, 3269-75.
- Siegel R, Naishadham D, Jemal A (2012). Cancer statistics, 2012.

CA Cancer J Clin, **62**, 10-29.

- Slaby O, Svoboda M, Fabian P, et al (2007). Altered expression of miR-21, miR-31, miR-143 and miR-145 is related to clinicopathologic features of colorectal cancer. *Oncology*, **72**, 397-402.
- Szafrańska AE, Davison TS, John J, et al (2007). MicroRNA expression alterations are linked to tumorigenesis and non-neoplastic processes in pancreatic ductal adenocarcinoma. *Oncogene*, **26**, 4442-52.
- Tomimaru Y, Eguchi H, Nagano H, et al (2010). MicroRNA-21 induces resistance to the anti-tumour effect of interferon-alpha/5-fluorouracil in hepatocellular carcinoma cells. *Br J Cancer*, **103**, 1617-26.
- Vogt PK, Gymnopoulos M, Hart JR (2009). PI 3-kinase and cancer: changing accents. *Curr Opin Genet Dev*, **19**, 12-7.
- Wang P, Zhuang L, Zhang J, et al (2013a). The serum miR-21 level serves as a predictor for the chemosensitivity of advanced pancreatic cancer, and miR-21 expression confers chemoresistance by targeting FasL. *Mol Oncol*, **7**, 334-45.
- Wang Z, Yao H, Lin S, et al (2013b). Transcriptional and epigenetic regulation of human microRNAs. *Cancer Lett*, **331**, 1-10.
- Wickramasinghe NS, Manavalan TT, Dougherty SM, et al (2009). Estradiol downregulates miR-21 expression and increases miR-21 target gene expression in MCF-7 breast cancer cells. *Nucleic Acids Res*, **37**, 2584-95.
- Yang CH, Yue J, Pfeffer SR, Handorf CR, Pfeffer LM (2011). MicroRNA miR-21 regulates the metastatic behavior of B16 melanoma cells. *J Biol Chem*, **286**, 39172-8.
- Yoo CB, Jones PA (2006). Epigenetic therapy of cancer: past, present and future. *Nature Rev Drug Discov*, **5**, 37-50.
- Zhang BG, Li JF, Yu BQ, et al (2012). microRNA-21 promotes tumor proliferation and invasion in gastric cancer by targeting PTEN. *Oncology Rep*, **27**, 1019-26.
- Zhang XH, Rao M, Loprieto JA, et al (2008a). Aurora A, Aurora B and survivin are novel targets of transcriptional regulation by histone deacetylase inhibitors in non-small cell lung cancer. *Cancer Biol Ther*, **7**, 1388-97.
- Zhang Z, Li Z, Gao C, et al (2008b). miR-21 plays a pivotal role in gastric cancer pathogenesis and progression. *Lab Invest; a journal of technical methods and pathology*, **88**, 1358-66.
- Zhu S, Si ML, Wu H, Mo YY (2007). MicroRNA-21 targets the tumor suppressor gene tropomyosin 1 (TPM1). *J Biol Chem*, **282**, 14328-36.
- Zhu S, Wu H, Wu F, et al (2008). MicroRNA-21 targets tumor suppressor genes in invasion and metastasis. *Cell Res*, **18**, 350-9.