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MicroRNA-215 functions as a tumor suppressor and directly targets ZEB2 in human pancreatic cancer

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ABSTRACT. It has been shown that microRNA-215 (miR-215) is downregulated in several human malignancies, and this correlates with tumor progression. However, its expression and function in pancreatic cancer is still unclear. The aim of this study was to explore the effects of miR-215 on pancreatic cancer formation and progression. Using quantitative RT-PCR, we detected miR-215 expression in pancreatic cancer cell lines and primary tumor tissues. The association of miR-215 expression with clinicopathological factors and prognosis was also analyzed. We then observed the effects of miR-215 on the biological behavior of pancreatic cancer cells. Lastly, the potential regulatory function of miR-215 on ZEB2 expression was investigated. miR-215 expression levels were significantly downregulated in pancreatic cancer samples and cell lines. Decreased miR-215 expression was significantly associated with large tumor size, advanced TNM stage, lymph node metastasis, vessel invasion, and lower overall survival. Multivariate regression analysis corroborated that downregulation of miR-215 was an independent unfavorable prognostic factor. Overexpression of miR-215 inhibited pancreatic cancer cell

proliferation, invasion, and migration; promoted cell apoptosis *in vitro*; and suppressed tumorigenicity *in vivo*. Further, ZEB2 was confirmed as a direct target of miR-215 by using a luciferase reporter assay. These findings indicate that miR-215 may act as a tumor suppressor in pancreatic cancer cells, and could serve as a novel therapeutic target for miR-based therapy.

Key words: Pancreatic cancer; MicroRNA-215; Proliferation; Prognosis; Cancer invasion

INTRODUCTION

Pancreatic cancer is the fourth major cause of cancer-related deaths in the world and has the lowest survival rate of any solid tumor cancer (Wong et al., 2014; Xue et al., 2014). Despite advances in clinical and experimental oncology, the overall 5-year survival rate of patients with pancreatic cancer is approximately 3-5% (Jemal et al., 2010), primarily because of late diagnosis, rapid disease progression, and resistance to chemotherapy and radiotherapy. Previous studies have demonstrated diverse genetic alterations in pancreatic cancer (Ch et al., 20 , but the molecular mechanisms underlying its formation and progression are highly complex. Further identification of new candidate molecules that take part in these proces s is important for improving the diagnosis, prevention, and treatment of this disease.

MicroRNAs (miRs) are a class of short (~22 nucleotides in length), endogenous, single-stranded, non-protein-coding RNAs that bind directly to the 3'-untranslated regions (3'-UTRs) of target messenger RNAs (mRNAs), leading to mRNA degradation or translational suppression (Bartel 2009). It is now well known that miRs are involved in many different biological processes, including cell growth, apoptosis, development, differentiation and endocrine homeostasis (Bartel 2004). Accumulating research also suggests that miRs play essential roles in the biology of human cancers, which may provide a new and promising treatment target for cancer (Heneghan et al., 2010). Dysregulation of miR expression has frequently been reported to be closely associated with tumor initiation, promotion, and progression. miR-215 is upregulated in cervical cancer (Liang et al., 2014), hepatocellular carcinoma (Liu et al., 2014), gastric cancer (Deng et al., 2014), and prostate cancer (Walter et al., 2013), and it acts as a potential oncogene in these tumors. On the contrary, the expression of miR-215 was found to be significantly decreased in esophageal adenocarcinoma (Wijnhoven et al., 2010), colon cancer (Karaayvaz et al., 2011), and renal cell carcinoma (RCC) (White et al., 2011), where it acts as a candidate tumor suppressor. However, the correlation between miR-215 dysregulation and clinicopathological characteristics of pancreatic cancer has not yet been evaluated, and the biological roles of miR-215 and its direct functional targets in pancreatic cancer remain poorly understood.

Epithelial-to-mesenchymal transition (EMT) has been recognized as an important physiological process that is associated with cancer progression and metastasis. Zinc finger E-box-binding homeobox 2 (ZEB2), an important member of the ZEB family, induces EMT through repression of E-cadherin and promotes tumor development (Comijn et al., 2001). High ZEB2 expression was found in diverse types of cancers including pancreatic cancer (Imamichi et al., 2007; Usova et al., 2013; Lee et al., 2014; You et al., 2014). Intriguingly, several miRs, such as miR-132 (Zheng et al., 2014), miR-144 (Guan et al., 2014), and miR-200c (Lu et al., 2014), participate in the regulation of the activity of ZEB2 in different tissues, but the potential regulatory effect of miR-215 on ZEB2 expression in pancreatic cancer has not been confirmed.

In this study, we examined miR-215 expression in pancreatic cancer tissues and cell lines using real-time PCR. The association of miR-215 levels with clinicopathological features and disease prognosis was also analyzed. Furthermore, we investigated the effects of miR-215 on the biological behavior of pancreatic cancer cells. Finally, ZEB2 was identified as a direct target of miR-215 using a luciferase reporter assay.

MATERIAL AND METHODS

Patients and clinical specimens

Paired pancreatic primary cancer tumors and adjacent normal pancreatic tissues were obtained postoperatively from 97 patients at the Department of Oncology, Dongfang Hospital of Beijing, University of Chinese Medicine (Beijing, P. R. China) between March 2007 and February 2011. These tissues were flash-frozen in liquid nitrogen immediately after resection and stored at -80°C until use. None of the patients received neoadjuvant chemo- or radio-therapy before surgery. Patient characteristics are shown in Table 1. Follow-up information was available for all patients. Overall survival (OS) was defined as the time from the day of operation to death or, for living patients, the date of last follow-up. This study was approved by the Research Ethics Committee of the Hospital of Beijing and written informed consent was obtained from each patient.

Table 1. Association between miR-215 expression and various clinicopathological features in patients with pancreatic cancer.

Clinicopathological features	No. of cases	MiR-215 expression		P
		Low [(N, (%)]	High [(N, (%)]	
Age (years)				
<60	55	31 (56.4%)	24 (43.6%)	0.222
≥60	42	18 (42.9%)	24 (57.1%)	
Gender				
Female	56	27 (48.2%)	29 (51.8%)	0.682
Male	41	22 (53.7%)	19 (46.3%)	
Tumor size (cm)				
<2.0	38	13 (34.2%)	25 (65.8%)	0.013
≥2.0	59	36 (61.0%)	23 (39.0%)	
Differentiation				
Well-moderate	63	29 (46.0%)	34 (54.0%)	0.289
Poor	34	20 (58.8%)	14 (41.2%)	
Lymph node metastasis				
Positive	61	40 (65.6%)	21 (34.4%)	
Negative	36	9 (25.0%)	27 (75.0%)	<0.001
Vessel invasion				
Positive	58	36 (62.1%)	22 (37.9%)	
Negative	39	13 (33.3%)	26 (66.7%)	0.007
TNM stage				
IA + IB	23	7 (30.4%)	16 (69.6%)	0.033
IIA + IIB	74	42 (56.8%)	32 (43.2%)	

TNM stage: tumor-node-metastases stage.

miR transfection of cell lines

Human pancreatic cancer cell lines PANC-1, AsPC-1, BxPC-3 and SW1990, and the normal pancreatic epithelial cell line H6C7 were obtained from American Type Culture Collection (Manassas, VA, USA). H6C7 cells were cultured in keratinocyte serum-free medium (Invitrogen,

CA, USA). Pancreatic cancer cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mM streptomycin. All cells were incubated at 37°C in a humidified atmosphere with 5% CO_2.

For miR transfection, the cells were seeded into a 24-well plate and incubated overnight, then transfected the following day with mature miR-215 mimics, miR-215 inhibitors (anti-miR-215), or negative control (miR-NC or anti-miR-NC) (GenePharma, Shanghai, China) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer protocol.

RNA extraction and quantitative real-time PCR

Total RNA was isolated using TRIzol® reagent (Invitrogen) according to manufacturer instructions. The reverse transcription reaction was carried out with 100 ng of total RNA using the looped primers. Real-time PCR was performed using the standard Taqman miR assay protocol on the ABI7500 real-time PCR detection system (Applied Biosystems, USA) with cycling conditions of 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s and 74°C for 5 s. U6 small nuclear RNA was used as an internal control. The threshold cycle (Ct) was defined as the fractional cycle number at which the fluorescence passed the fixed threshold. Each sample was measured in triplicate and the relative amount of miR-215 to U6 was calculated using the equation $2^{-\Delta Ct}$, where $\Delta Ct = (Ct^{miR\text{-}215} - Ct^{U6})$.

MTT assay

After transfection, pancreatic cancer cells were harvested, seeded into 96-well culture plates at a density of 200 cells in 200 µL/well, and incubated at 37°C. At different time points (24, 48, 72 or 96 h), 100 µL of MTT solution (0.5 mg/mL; Sigma, USA) was added to each well, and the plates were incubated for another 4 h. Next, the MTT solution was removed and 150 µL dimethyl sulfoxide (DMSO) was added to each well to stop the reaction. The plates were gently shaken on a swing bed for 10 min and spectrometric absorbance at 490 nm was measured using a microplate reader. This experiment was performed in triplicate for each sample.

Detection of apoptosis by flow cytometry

Apoptosis was detected by flow cytometric analysis. Briefly, the cells were washed and resuspended at a concentration of 1 x 10^6 cells/mL. Then, the cells were stained with Annexin V and propidium iodide (PI) using the Annexin V apoptosis detection kit. After incubation at room temperature in the dark for 15 min, cell apoptosis was analyzed on a FACSCalibur (Becton, Dickinson and Company, San Jose, CA, USA).

Transwell invasion assay

The invasion assay was performed using 24-well transwell chambers (8 µm; Corning). After transfection, tumor cells were resuspended in serum-free DMEM medium and 2 x 10^5 cells were seeded into the upper chambers covered with 1 mg/mL matrigel. DMEM containing 10% FBS was added to the bottom chambers. Following a 24-h incubation, cells on the upper surface of the membrane were physically detached, fixed with 95% ethanol, stained with 0.1% crystal violet, and counted under a light microscope.

Scratch migration assay

The scratch migration assay was performed to observe the influence of miR-215 on pancreatic cancer cell migration. When cells transfected with miR-215 mimics, miR-215 inhibitors or NC were grown to confluency, and a scratch in the cell monolayer was made with a cell scratch spatula. After the cells were incubated under standard conditions for 24 h, pictures of the scratches and subsequent growth were taken using a digital camera system coupled with a microscope.

Luciferase reporter assays

The pGL3-reporter luciferase vector was used for the construction of the pGL3-ZEB2 and pGL3-ZEB2-mut vectors. The pGL3-ZEB2-mut vector was designed with ZEB2 having the miR-215 target site mutated using the Stratagene Quik-Change site-directed mutagenesis kit (Stratagene, Germany). For the luciferase reporter assay, cells were cultured in 24-well plates and transfected with the reporter plasmids and miR-215 mimics using Lipofectamine 2000. 24 h after transfection, the luciferase activity was measured using the dual luciferase reporter assay system (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity for each sample.

Western blot analysis

Protein lysates were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. After blocking, the membranes were incubated with purified rabbit anti-ZEB2 antisera at 4°C overnight. The next day, the membranes were washed with PBS and then incubated with peroxidase-conjugated goat anti-rabbit IgG. Immunodetection was conducted with chemiluminescence (ECL) reagents (Pierce) and exposed on an X-ray film. β-actin was used as an internal reference for relative quantification.

Tumorigenicity *in vivo*

Tumor formation was studied by establishing a xenograft model in mice. Commercial lentiviral vectors containing miR-215 (LV-miR-215) (GeneChem Co. Ltd., Shanghai, China) were used to infect pancreatic cancer cells according to the manufacturer's instructions. An empty lentiviral construct served as a negative control (LV-NC) and the stably transfected cells were selected using puromycin (1.5 µg/mL). Female BALB/c athymic nude mice (3-4 weeks old) were purchased from Model Animal Research Center of Nanjing University. 1 x 10^6 tumor cells (100 µL cell suspension) stably overexpressing miR-215 or NC were inoculated subcutaneously. Bidimensional tumor measurements were taken with Vernier calipers every 4 days, and tumor volume (mm^3) was calculated using the formula: volume = (length x $width^2$)/2. The mice were sacrificed and the tumors were weighed 3 weeks after inoculation.

Statistical analysis

All statistical analyses were carried out using the SPSS 16.0 software package (SPSS, Chicago, IL, USA) and all data are reported as means ± standard deviation. Differences between groups were analyzed using the Student's *t*-test or the chi-square test. Relationships between miR-215 expression and ZEB2 protein levels were explored by Pearson correlation analysis. Survival

curves were constructed with the Kaplan-Meier method and compared by log-rank tests. To evaluate independent prognostic factors associated with survival, a multivariate Cox proportional hazard regression analysis was used. P values <0.05 were considered to be statistically significant.

RESULTS

Decreased expression of miR-215 in pancreatic cancer and its correlation with ZEB2 levels

The expression level of miR-215 in pancreatic cancer tissues and cell lines was detected by qRT-PCR and normalized to U6 small nuclear RNA. Figure 1A shows that the expression levels of miR-215 were significantly lower in pancreatic cancer specimens (mean ± SD: 9.4 ± 1.9) than in the corresponding adjacent non-cancerous tissues (mean ± SD: 19.9 ± 4.3; P < 0.001). The expression of miR-215 in four pancreatic cancer cell lines was also clearly downregulated (Figure 1B). Since PANC-1 cells exhibited the lowest miR-215 expression while SW1990 cells expressed relatively high levels of miR-215 among the four pancreatic cancer cell lines tested, two cell lines were selected for testing of mature miR-215 mimics or miR-215 inhibitors and further studies.

ZEB2 protein levels were detected using western blot analysis in clinical specimens and cell lines. The results show that ZEB2 protein levels in tumor samples were higher than in the adjacent normal tissues (P < 0.001; Figure 1C). ZEB2 levels in pancreatic cancer cells were also higher than in normal pancreatic epithelial cells (Figure 1D). In addition, we obtained a clear inverse correlation (R = -0.4328, P = 0.0003) between ZEB2 levels and miR-215 expression in pancreatic cancer specimens (Figure 1E).

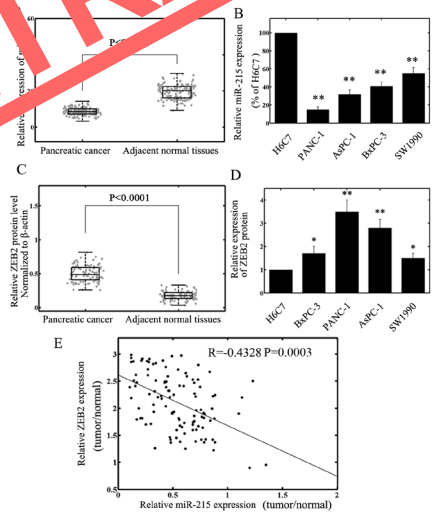

Figure 1. Expression of miR-215 and ZEB2 in pancreatic cancer tissues and cell lines. **A.** miR-215 expression levels (means ± SD) were calculated by qRT-PCR and normalized to U6 small nuclear RNA. miR-215 expression was significantly lower in pancreatic cancer tissues than in the corresponding non-cancerous tissues. **B.** miR-215 expression was downregulated in pancreatic cancer cell lines PANC-1, AsPC-1, BxPC-3, and SW1990 compared to the human normal pancreatic epithelial cell line H6C7. **P < 0.01. **C.** Relative ZEB2 protein levels (means ± SD) in pancreatic cancer and corresponding non-cancerous tissues, as measured by western blot analysis and normalized to β-actin. **D.** ZEB2 protein levels in pancreatic cancer cells were higher than in H6C7 cells. **E.** The inverse correlation of ZEB2 protein levels with miR-215 expression was examined by Pearson correlation analysis. *P < 0.05, **P < 0.01.

Association between miR-215 expression and clinicopathological features and prognosis in patients with pancreatic cancer

Using the median miR-215 expression in all 97 pancreatic cancer patients as a cutoff, the patients were divided into 2 groups: high miR-215 expression and low miR-215 expression. We found that low miR-215 expression was significantly associated with large tumor size, advanced TNM stage, lymph node metastasis, and vessel invasion, but not with patients' age, gender, or tumor differentiation (Table 1). Furthermore, patients with low levels of miR-215 expression had a significantly shorter OS than those with high levels of miR-215 expression (Figure 2). Meanwhile, multivariate survival analysis showed that TNM stage, lymph node metastasis, vessel invasion, and miR-215 expression were independent prognostic markers for OS of pancreatic cancer patients (Table 2).

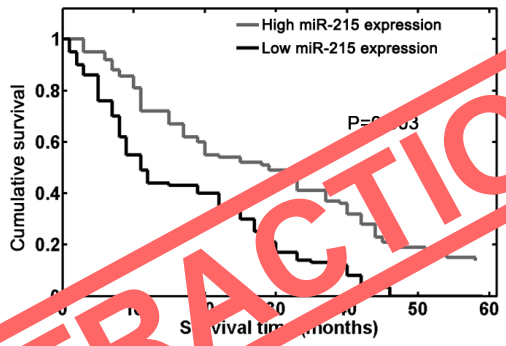

Figure 2. Overall survival curves of two groups of patients with pancreatic cancer defined by low and high expression of miR-215. Low miR-215 expression levels were significantly associated with poor outcome (P = 0.003, log-rank test).

Table 2. Univariate and multivariate analysis of overall survival in patients with pancreatic cancer.

Variables	Univariate log-rank test (P)	Cox multivariable analysis (P)	Relative risk (RR)
Age at diagnosis (ye			
<60 *vs* ≥60	0.42	-	-
Gender			
Female *vs* male	0.65	-	-
Tumor size (cm)			
<2.0 *vs* ≥2.0	0.74	-	-
Differentiation			
Well-moderate *vs* poor	0.09	-	-
Lymph node metastasis			
Negative *vs* positive	0.005	0.02	5.4
Vessel invasion			
Negative *vs* positive	0.008	0.03	4.6
TNM stage			
(IA-IB) *vs* (IIA-IIB)	<0.001	0.006	9.5
miR-215 expression			
High *vs* low	0.003	0.011	6.8

TNM stage: tumor-node-metastases stage.

Effects of miR-215 on the biological behavior of pancreatic cancer cells

In order to selectively overexpress or downregulate miR-215, mature miR-215 mimics or miR-215 inhibitors were transfected into PANC-1 or SW1990 cells. qRT-PCR analysis confirmed

increased miR-215 expression after transfection with miR-215 mimics and decreased miR-215 expression following transfection with miR-215 inhibitors (Figure 3A). The MTT assay showed that cell proliferation was significantly impaired in PANC-1 cells transfected with miR-215 mimics, while proliferation of SW1990 cells was increased after transfection with miR-215 inhibitors compared with negative controls (Figure 3B).

Flow cytometry was employed to determine the effect of miR-215 on cell apoptosis. The proportion of apoptotic PANC-1 cells transfected with miR-215 mimics was significantly higher than the negative control group. Moreover, downregulation of miR-215 reduced SW1990 cell apoptosis (Figure 3C).

A transwell invasion assay was performed to investigate whether miR-215 had a direct influence on cell invasion. As shown in Figure 3D, the upregulation of miR-215 impeded the invasion of PANC-1 cells, when compared with negative control. Conversely, transfection of SW1990 cells with miR-215 inhibitors promoted cell invasion ability. Scratch migration assay also confirmed the inhibitory effect of miR-215 on pancreatic cancer cell migration (Figure 3E).

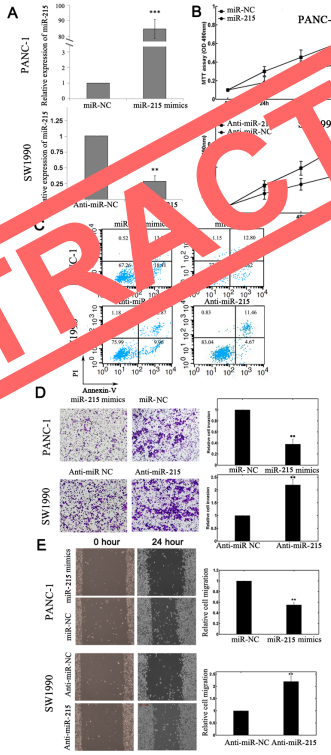

Figure 3. Effects of miR-215 mimics or inhibitors on biological behavior of PANC-1 and SW1990 cells. **A.** qRT-PCR analysis confirmed increased miR-215 expression in PANC-1 cells transfected with miR-215 mimics, and decreased miR-215 expression in SW1990 cells transfected with miR-215 inhibitors. U6 RNA was used as an internal control. **P < 0.01, ***P < 0.001. **B.** MTT assay shows that miR-215 reduced cell proliferation *in vitro*. Data are reported as means ± SD of the experiments performed in triplicate. *P < 0.05, **P < 0.01. **C.** Cell apoptosis was detected by flow cytometric analysis after transfection with miR-215 mimics, miR-215 inhibitors (anti-miR-215), or negative controls (miR-NC or anti-miR-NC). PI: propidium iodide. **D.** Transwell invasion assay shows that upregulation of miR-215 impeded the invasion of PANC-1 cells, while transfection of SW1990 cells with miR-215 inhibitors promoted cell invasion. **P < 0.01. **E.** Scratch migration assay confirmed the inhibitory effect of miR-215 on pancreatic cancer cell migration. **P < 0.01.

ZEB2 is a target gene of miR-215

Using the bioinformatics software TargetScan (http://www.targetscan.org) for target gene prediction, ZEB2 was identified as one of the potential targets of miR-215. The predicted binding of miR-215 with ZEB2 3'UTR is illustrated in Figure 4A. To further confirm that ZEB2 is a direct target of miR-215 in pancreatic cancer, we first transfected PANC-1 cells with miR-215 mimics and found that this resulted in reduced ZEB2 protein levels (Figure 4B). Then, we used constructs (pGL3-ZEB2 and pGL3-ZEB2-mut plasmids) to perform a reporter assay, which revealed that transfection with miR-215 mimics triggered a marked decrease of luciferase activity in pGL3-ZEB2-transfected PANC-1 cells, without a change in luciferase activity of pGL3-ZEB2-mut-transfected cells (Figure 4C). These data indicate that ZEB2 is a direct target of miR-215 in pancreatic cancer.

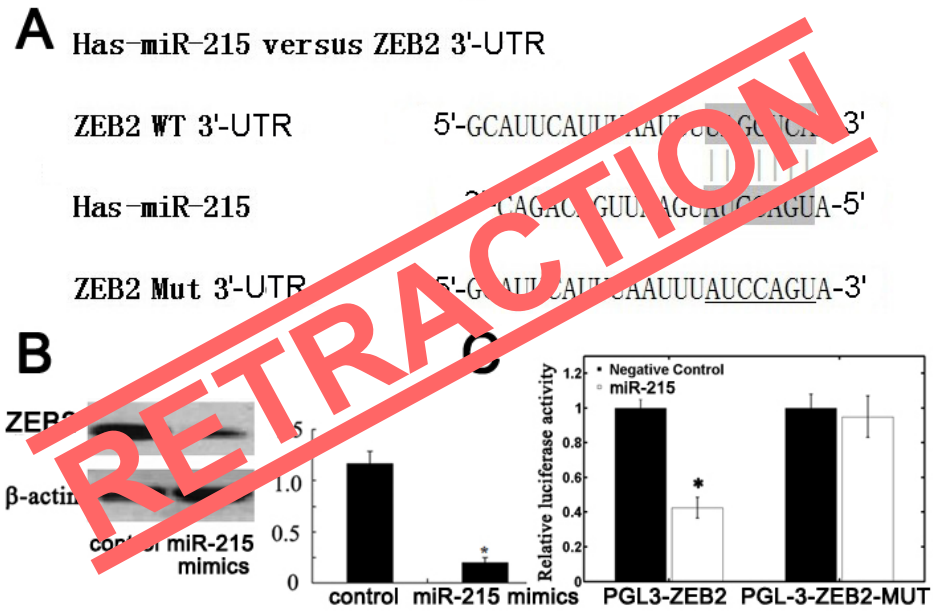

Figure 4. ZEB2 is a direct target of miR-215. **A.** MiR-215 binding sites in the ZEB2 3'-UTR. ZEB2-mut indicates the ZEB2 3'-UTR with mutations in miR-215 binding sites. **B.** Western blot analysis showed that transfection of miR-215 decreased ZEB2 protein expression. *P < 0.05. **C.** Relative luciferase activity comparing the pGL3-ZEB2 and pGL3-ZEB2-MUT vectors in A549 cells. Firefly luciferase activity was normalized to Renilla luciferase activity. *P < 0.05.

Increased miR-215 expression suppresses xenograft tumor formation

To further evaluate the effects of miR-215 on tumor growth *in vivo*, we engineered PANC-1 cells to stably overexpress miR-215 using lentivirus infection. These cells were injected subcutaneously into nude mice to form ectopic tumors. The cells transfected with negative lentiviral vector LV-NC were also inoculated. As shown in Figures 5A-C, the tumors formed by miR-215-overexpressing PANC-1 cells were smaller and had lower tumor weights than LV-NC infected control tumors. qRT-PCR analysis of the tumor tissues confirmed elevated miR-215 in miR-215-overexpressing tumors (Figure 5D)

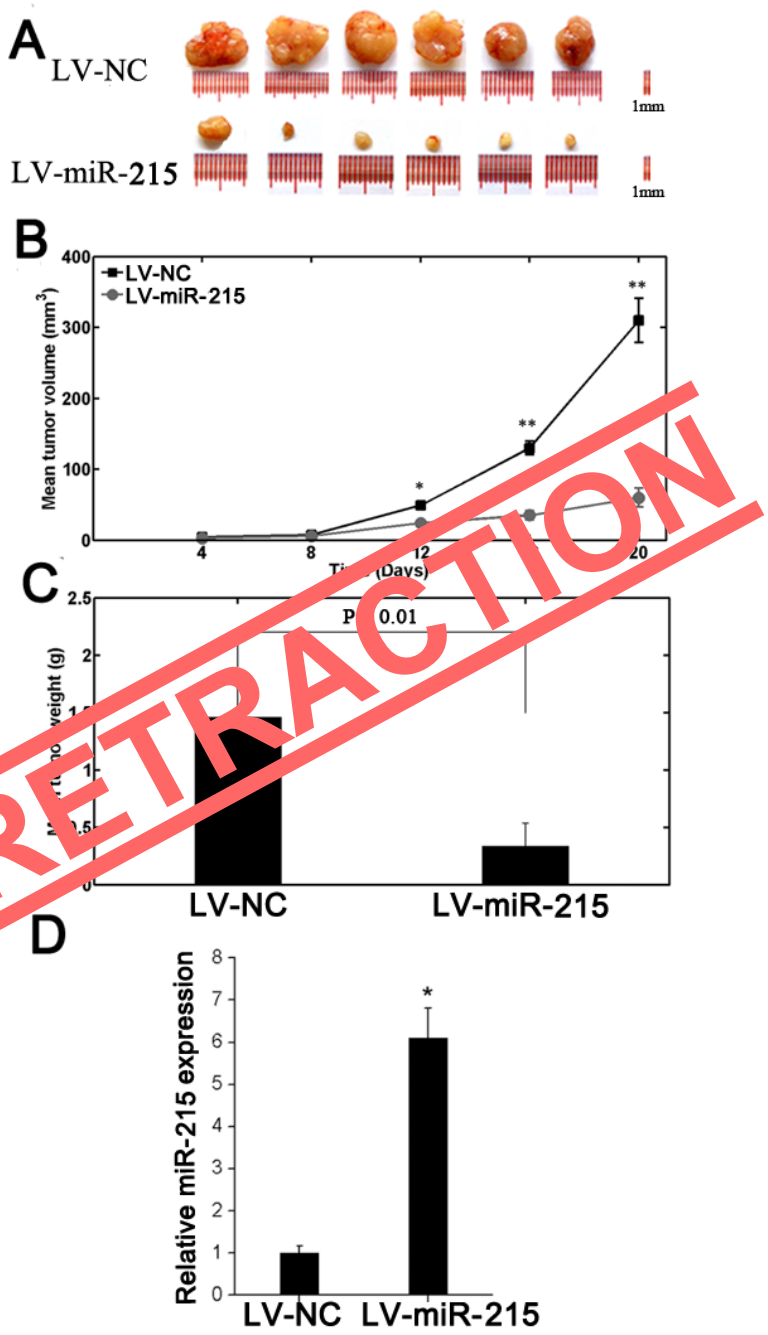

Figure 5. Upregulation of miR-215 resulted in inhibition of xenograft tumor growth *in vivo*. **A.** and **B.** Tumors formed by miR-215-overexpressing (LV-miR-215) PANC-1 cells were significantly smaller than the control group (LV-NC). *P < 0.05, **P < 0.01. **C.** Tumors were weighed 3 weeks after inoculation. The average tumor weight is indicated as means ± SD. **D.** qRT-PCR analysis of the tumor tissues confirmed elevated miR-215 in miR-215-overexpressing tumors. *P < 0.05.

DISCUSSION

Dysregulation of miRs has been shown to be involved in tumorigenesis and progression in various types of tumors; however, elucidation of their potential roles in pancreatic cancer remains poorly understood. In this study, we found that miR-215 was downregulated in pancreatic cancer cell lines and primary tumor samples. Decreased miR-215 expression significantly correlated with aggressive clinicopathological features and poor survival. Moreover, overexpression of miR-215 significantly inhibited cell proliferation, invasion and migration; promoted cell apoptosis *in vitro*; and suppressed tumorigenicity *in vivo*. Finally, ZEB2 was identified as a direct target of miR-215. To our knowledge, this is the first study to analyze the clinical significance and biological function of miR-215 in pancreatic cancer.

MiR-215, a p53-induced miR, has been reported to play important roles in cancer progression. Recent studies confirmed miR-215 downregulation in esophageal adenocarcinoma (Wijnhoven et al., 2010), colon cancer (Karaayvaz et al., 2011), and RCC (White et al., 2011). Decreased miR-215 levels in colorectal cancer were associated with increased tumor sizes and shorter disease-free survival after radical surgery (Chiang et al., 2012; [illegible] et al., 2013). Ectopic expression of miR-215 inhibited cell proliferation and triggered cell cycle arrest at G2 phase in HCT 116 colon cancer cells (Song et al., 2010), and decreased cellular migration and invasion in a RCC cell line model (White et al., 2011). In contrast to the antitumor properties described above, miR-215 also acts as an oncogene in several cancers. In cervical cancer, miR-215 expression was significantly higher in the cancerous tissues of patients with lymph node metastasis, advanced FIGO stage, and poor survival (Liang et al., 2014). In gastric cancer, high miR-215 expression showed significant correlation with tumor invasion and TNM stage (Deng et al., 2014). Functionally, miR-215 was able to promote the proliferation of hepatoma cells and gastric cancer cells (Deng et al., 2014; Liu et al., 2014). Anti-miR-215 remarkably inhibited the tumor growth of hepatoma cells in nude mice (Liu et al., 2014). Taken together, these findings indicate that the role of miR-215 in human malignancies may be multifaceted, depending on the tissue involved.

It is clear that miRs execute their oncogenic or tumor suppressor functions by regulating the expression of target genes (Liu et al., 2014). With regard to miR-215, several targets have been confirmed in previous studies including protein tyrosine phosphatase receptor type T (PTPRT) (Liu et al., 2014), thymidylate synthase (TS) (Song et al., 2010), dihydrofolate reductase (DHFR) (Song et al., 2010), retinoblastoma tumor suppressor gene 1 (RB1) (Deng et al., 2014), activated leukocyte cell adhesion molecule (ALCAM) (Jin et al., 2011), and activin receptor type 2B (ACVR2B) (Senanayake et al., 2012). ZEB2, as a tumor-promoting gene, has been found to be upregulated in different tumor types, and identified as a target gene of a number of miRs. The important role of ZEB2 has been strongly underlined in numerous studies, due to its function in inducing EMT and facilitating the metastasis of cancer cells. Usova et al. (2013) corroborated the contribution of ZEB2 in pancreatic cancer progression. White et al. (2011) revealed that miR-215 could directly target ZEB2 in RCC. Using a luciferase reporter assay, our study demonstrated that ZEB2 was a direct target of miR-215 in pancreatic cancer. However, there is no 'one-to-one' connection between miRs and target mRNAs and a typical miR can have more than 100 targets (Brennecke et al., 2005). Conversely, several miRs can converge on a single transcript target (Krek et al., 2005). ZEB2 is not the only miR-215 target dysregulated in pancreatic cancer; other functional targets of miR-215, such as RB1 and TS, also modulate pancreatic cancer pathogenesis (van der Zee et al., 2012; Hagen et al., 2014). Therefore, the potential regulatory circuitry afforded by miR-215 is enormous and accurate mechanisms are needed to describe how miR-215 influences pancreatic cancer progression.

In summary, our study reveals that the expression level of miR-215 is significantly decreased in pancreatic cancer and associated with tumor development. Therefore, low miR-215 expression may imply poor prognosis. The anti-tumor effects of miR-215 were also observed in functional analysis and ZEB2 was confirmed as a direct target of miR-215. These findings suggest that miR-215 acts as a tumor suppressor in pancreatic cancer, and could be a potential candidate for miR-based therapy against this disease.

Conflicts of interest

The authors declare no conflict of interest.

REFERENCES

Bartel DP (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116: 281-297.

Bartel DP (2009). MicroRNAs: target recognition and regulatory functions. *Cell* 136: 215-233.

Brennecke J, Stark A, Russell RB and Cohen SM (2005). Principles of microRNA-target recognition. *PLoS Biol.* 3: e85.

Chiang Y, Song Y, Wang Z, Liu Z, et al. (2012). microRNA-192, -194 and -215 are frequently down-regulated in colorectal cancer. *Exp. Ther. Med.* 3: 560-566.

Cho IR, Park JC, Park CH, Jo JH, et al. (2014). Pre-treatment neutrophil to lymphocyte ratio is a prognostic marker to predict chemotherapeutic response and survival outcomes in metastatic advanced gastric cancer. *Gastric Cancer* 17: 703-710.

Comijn J, Berx G, Vermassen P, Verschueren K, et al. (2001). The two-handed E box binding zinc finger protein SIP1 downregulates E-cadherin and induces invasion. *Mol. Cell* 7: 1267-1278.

Deng Y, Huang Z, Xu Y, Jin J, et al. (2014). MiR-215 modulates gastric cancer cell proliferation by targeting RB1. *Cancer Lett.* 342: 27-35.

Guan H, Liang W, Xie Z, Li H, et al. (2014). Down-regulation of miR-144 promotes thyroid cancer cell invasion by targeting ZEB1 and ZEB2. *Endocrine* 46: 566-574.

Hagen J, Muniz VP, Falls KC, Reed SM, et al. (2014). RAI/GL6A promotes G1-S phase progression and pancreatic neuroendocrine tumor cell proliferation in a p21-dependent manner. *Cancer Res.* 74: 6661-6670.

Heneghan HM, Miller N, Kerin MJ (2010). miRNAs as biomarkers and therapeutic targets in cancer. *Curr. Opin. Pharmacol.* 10: 543-550.

Imamichi Y, Konig A, Gress T and Menke A (2007). Collagen type I-induced Smad-interacting protein 1 expression downregulates E-cadherin in pancreatic cancer. *Oncogene* 26: 2381-2385.

Jemal A, Siegel R, Xu J and Ward E (2010). Cancer statistics, 2010. *CA Cancer J. Clin.* 60: 277-300.

Jin Z, Selaru FM, Cheng Y, Kan T, et al. (2011). MicroRNA-192 and -215 are upregulated in human gastric cancer *in vivo* and suppress ALCAM expression *in vitro*. *Oncogene* 30: 1577-1585.

Karaayvaz M, Pal T, Song B, Zhang C et al. (2011). Prognostic significance of miR-215 in colon cancer. *Clin. Colorectal Cancer* 10: 340-347.

Krek A, Grun D, Poy MN, Wolf R, et al. (2005). Combinatorial microRNA target predictions. *Nat. Genet.* 37: 495-500.

Lee H, Jun SY, Lee YS, Lee HJ, et al. (2014). Expression of miRNAs and ZEB1 and ZEB2 correlates with histopathological grade in papillary urothelial tumors of the urinary bladder. *Virchows Arch.* 464: 213-220.

Li S, Gao J, Gu J, Yuan J, et al. (2013). MicroRNA-215 inhibits relapse of colorectal cancer patients following radical surgery. *Med. Oncol.* 30: 549.

Liang H, Li Y, Luo RY and Shen FJ (2014). MicroRNA-215 is a potential prognostic marker for cervical cancer. *J. Huazhong Univ. Sci. Technolog. Med. Sci.* 34: 207-212.

Liu F, You X, Chi X, Wang T, et al. (2014). Hepatitis B virus X protein mutant HBxΔ127 promotes proliferation of hepatoma cells through up-regulating miR-215 targeting PTPRT. *Biochem. Biophys. Res. Commun.* 444: 128-134.

Liu GF, Tang D, Li P, Wang S et al. (2014). S-1-based combination therapy S-1 monotherapy in advanced gastric cancer: A meta-analysis. *World J. Gastroenterol.* 20: 310-318.

Lu YM, Shang C, Ou YL, Yin D et al. (2014). miR-200c modulates ovarian cancer cell metastasis potential by targeting zinc finger E-box-binding homeobox 2 (ZEB2) expression. *Med. Oncol.* 31: 134.

Senanayake U, Das S, Vesely P, Alzoughbi W, et al. (2012). miR-192, miR-194, miR-215, miR-200c and miR-141 are downregulated and their common target ACVR2B is strongly expressed in renal childhood neoplasms. *Carcinogenesis* 33: 1014-1021.

Song B, Wang Y, Titmus MA, Botchkina G, et al. (2010). Molecular mechanism of chemoresistance by miR-215 in osteosarcoma and colon cancer cells. *Mol. Cancer* 9: 96.

Usova EV, Kopantseva MR, Kostina MB, Van'kovich AN, et al. (2013). Expression of the ZEB2 gene in pancreatic stromal cells in pancreatic ductal adenocarcinoma, pancreatitis, and normal state. *Dokl. Biol. Sci.* 448: 61-64.

van der Zee JA, van Eijck CH, Hop WC, van Dekken H, et al. (2012). Expression and prognostic significance of thymidylate synthase (TS) in pancreatic head and periampullary cancer. *Eur. J. Surg. Oncol.* 38: 1058-1064.

Walter BA, Valera VA, Pinto PA and Merino MJ (2013). Comprehensive microRNA Profiling of Prostate Cancer. *J. Cancer* 4: 350-357.

White NM, Khella HW, Grigull J, Adzovic S, et al. (2011). miRNA profiling in metastatic renal cell carcinoma reveals a tumour-suppressor effect for miR-215. *Br. J. Cancer* 105: 1741-1749.

Wijnhoven BP, Hussey DJ, Watson DI, Tsykin A, et al. (2010). MicroRNA profiling of Barrett's oesophagus and oesophageal adenocarcinoma. *Br. J. Surg.* 97: 853-861.

Wong J, Rahman S, Saeed N, Lin HY, et al. (2014). Effect of Body Mass Index in Patients Undergoing Resection for Gastric Cancer: A Single Center US Experience. *J. Gastrointest. Surg.* 18: 505-511.

Xue H, Lu Y, Xue Z, Lin B, et al. (2014). The effect of CYP1A1 and CYP1A2 polymorphisms on gastric cancer risk among different ethnicities: a systematic review and meta-analysis. *Tumour Biol.* 35: 4741-4756.

You J, Li Y, Fang N, Liu B, et al. (2014). MiR-132 suppresses the migration and invasion of lung cancer cells via targeting the EMT regulator ZEB2. *PLoS One* 9: e91827.

Zheng YB, Luo HP, Shi Q, Hao ZN, et al. (2014). miR-132 inhibits colorectal cancer invasion and metastasis via directly targeting ZEB2. *World J. Gastroenterol.* 20: 6515-6522.