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The regulatory roles of miRNA and methylation on oncogene and tumor suppressor gene expression in pancreatic cancer cells

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ABSTRACT

Carcinogenesis is driven by an accumulation of mutations and genetic lesions, which leads to activation of oncogenes and inactivation of tumor suppressor genes. However, the molecular mechanisms by which the expression of these genes was regulated in pancreatic cancer remains unclear. In this study, we investigated the regulatory effects of microRNA and methylation on the expression of *k-ras*, *TP53* and *PTEN* genes in pancreatic cancer cells. The protein and miRNA levels were measured by Western blotting and Northern blotting, respectively. Xenograft pancreatic tumor models were established by inoculating BxPC-1, Capan-2, and Panc-1 tumor cells into athymic nu/nu mice. A disparate level of KRAS, p53, PTEN, Dnmts, and Dicer 1 proteins as well as let-7i, miR-22, miR-143, and miR-29b miRNA was observed in BxPC-1, Capan-2, and Panc-1 cells. Knockdown of *Dicer 1* expression in BxPC-3 and Panc-1 cells resulted in significant increases in KRAS, p53, PTEN, and Dnmts protein levels and significant decreases in miR-22, miR-143, let-7i, and miR-29b expression. Knockdown of *Dicer 1* expression in Capan-2 cells significantly increased p53 and PTEN expression, while significantly decreased miR-22 and miR-143 expression, but had no effects on PTEN, Dnmts, let-7i, and miR-29b expression. Knockdown of *Dicer 1* expression significantly inhibited xenograft BxPC-3 tumor growth, but promoted xenograft Panc-1 tumor growth. In contrast, knockdown of *Dicer 1* expression had no effect on xenograft Capan-2 tumor growth. Our study suggested that different pancreatic cancer cell lines exhibited obvious discrepancies in gene expression profiles, implying that different molecular mechanisms are involved in the carcinogenesis of pancreatic cancer subclasses. Our study highlighted the importance of personalized therapy.

1. Introduction

Pancreatic cancer is the fourth leading cause of cancer-related deaths in the United States and the eighth leading cause of cancer-related deaths worldwide [1]. Both the prevalence and incidence of pancreatic cancer are increasing worldwide. Despite recent progress in targeting therapy, the overall survival rate of pancreatic cancer patients is still less than 5% [2]. A report from the American Cancer Society revealed that approximately 43,100 patients were diagnosed with pancreatic cancer, and 36,800 patients died in the United States in 2010 [3]. A major reason for the high mortality rate is that pancreatic cancer is highly aggressive and is characterized by very early invasion and metastasis [1]. The lack of success in effective therapy may suggest that key molecular mechanisms believed to be involved in the pathogenesis of pancreatic cancer have not been uncovered.

In general, oncogenes and tumor suppressor genes control the tumor cell cycle, proliferation, differentiation, and apoptosis [4]. Mutation of oncogenes commonly causes dominant gain of function, whereas mutations in tumor suppressor genes usually result in loss of their inhibitory function [5]. Mutations in *k-ras* oncogene are very common in pancreatic cancer, occurring in about 95% of primary tumors [6]. Mutations in *k-ras* lead to the high expression of oncogenic RAS protein [7]. The p53 is a crucial tumor-suppressor and mutation in TP53 gene is observed in 50–75% of PDAC patients [8]. Abrogation of the p16 tumor-suppressive pathway is also thought to be a key mechanism involving in the development of many human tumors. Inactivation of p16 function occurs in up to 95% of sporadic pancreatic cancer cases [9]. Phosphatase and tensin homolog deleted on chromosome 10 (*PTEN*) is another key tumor suppressor which plays a crucial role in cell migration and spreading [10]. Although mutations of the *PTEN* gene have been widely observed in a variety of malignancies [11], PTEN mutation is rarely observed in pancreatic cancer [12]. The disparate genotypes suggest that different mechanisms may lead to the development of pancreatic cancer.

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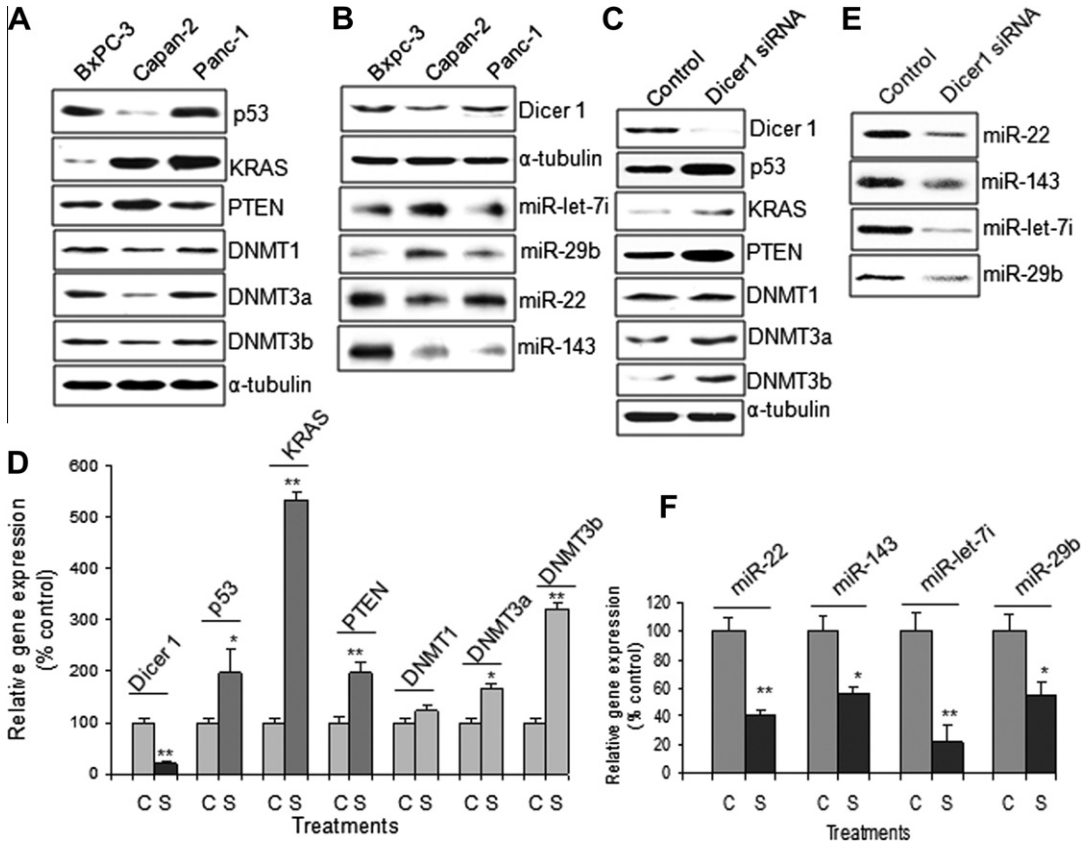

Fig. 1. Genes expression in BxPC-3, Capan-2, and Panc-1 without Dicer 1 silence and in BxPC-3 cells under Dicer 1 silencing. (A) Western blot of protein expression. (B) Northern blot of miRNA expression. (C) Western blot of gene expression in BxPC-3 cells under Dicer 1 silencing. (D) Percent densitometric values of genes in (C) normalized to that in control group. (E) Northern blot of miRNA expression in BxPC-3 cells under Dicer 1 silencing. (F) Percent densitometric values of miRNAs in (E) normalized to that in control group. $^{*}p < 0.01$, $^{**}p < 0.001$. B, BxPC-3; C, Capan-2; P, Panc-1. $N = 5$.

Recently, the identification of microRNAs (miRNA) which target oncogenes and tumor suppressor gene provides new insights on their role in carcinogenesis, progression, metastasis, and prognosis of tumors. The regulatory role of miRNA is complex. For example, a single miRNA can regulate the expression of hundreds of genes while a single gene can be regulated by multiple miRNAs [13]. miRNAs can act as tumor-suppressor genes as well as oncogenes and are widely reported to be abnormally expressed in many types of cancers [14]. However, the functional role of most miRNAs remains unexplored, and it is unclear how these miRNAs were regulated. In the process of miRNA genesis, Dicer is a key enzyme that controls miRNA production. Dicer cuts pre-miRNA into mature double-stranded miRNA fragments of approximately 15–30 nucleotides in length [15]. Therefore, the protein level or activity of Dicer determines miRNA levels. Given the roles played by Dicer, it has been hypothesized that changes in the expression or activity of this enzyme may be responsible for the global down-regulation of miRNA expression in tumors [16]. However, the mechanism by which the expression of oncogenes and tumor suppressor genes was regulated by miRNAs in different subclasses of pancreatic cancer has not been elucidated.

In this study, we investigated the expressions of KRAS, p53, PTEN, DNA methyltransferases (DNMTs) protein and let-7i, miR-22, miR-29b, and miR-143 microRNAs in BxPC-3, Capan-2, and Panc-1 pancreatic cancer cells. We further evaluated the regulatory role of Dicer 1 on the expression of these genes and miRNAs in three cell lines by knock down of Dicer 1 gene expression.

2. Materials and methods

2.1. Cell culture

BxPC-3 is a human pancreatic cancer cell line expressing wild-type KRAS, wild-type PTEN, and mutated p53 proteins. Capan-2 is a human pancreatic cancer cell line expressing mutated KRAS, mutated p53, and functional PTEN proteins. PANC-1 is a human pancreatic cancer cell line expressing functional PTEN, mutated KRAS, and mutated p53 proteins. These three cell lines were obtained from the American Type Culture Collection (ATCC) and grown in RPMI 1640 medium supplemented with L-glutamine, antibiotics, and 10% fetal bovine serum (Gibco Laboratories, USA). Cells were cultured at 37 °C in 5% CO_2.

2.2. Animals

Sixty female athymic nu/nu mice (7 weeks old) were provided by the Animal Center of Central South University. The animals were housed socially (four mice per cage) in a room under standard lighting conditions and temperature. Water and food were provided *ad libitum*. All animal experiments were conducted with an animal protocol approved by Central South University and performed in accordance with the animal care guidelines of the Chinese Council.

2.3. Northern blot analysis

Total RNA was extracted from control or treated cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The micoRNA was

further isolated with PureLink™ miRNA isolation Kit (Invitrogen). About 2 μg of denatured small RNA was loaded onto a 12.5% urea–polyacrylamide gel and then run in 0.5× TBE buffer. RNA was then transferred to a nylon membrane in 0.5× TBE buffer. Prehybridization and hybridization were performed with the ExpressHyb buffer (Invitrogen) at 68 °C. The membrane was rinsed following the manufacturer's instructions (Invitrogen) and developed for signal detection. To detect miRNAs, the antisense oligonucleotides of miR-22, miR-29b, miR-143, and let-7i were ^{32}P-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase. The ^{32}P-end labeled probe was used directly for hybridization. The ZR small-RNA™ ladder (ZYMO Research, Orange, CA, USA) was used to determine miRNA band size.

2.4. Western blot analysis

The treated or untreated BxPC-3, Capan-2, and Panc-1 cells were harvested in RIPA buffer containing 1% protease inhibitor cocktail (Sigma–Aldrich, St. Louis, MO, USA). Protein concentrations were measured using BCA Protein Assay Kit (Beyotime, Shanghai, China). About 20 μg of total protein was loaded onto a 10% SDS–PAGE gel and transferred to PVDF membranes. After blocking with 5% non-fat milk for 2 h, membranes were then incubated with primary antibody overnight at 4 °C. Membranes were washed with PBS and incubated with HRP-labeled secondary antibody (1:5000 dilution) for 2 h at room temperature the next day. Immunoreactive proteins were detected using an enhanced chemiluminescence reagent (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. To control for loading efficiency, the blots were stripped and re-probed with α-tubulin antibody (1:2000 dilution, Sigma–Aldrich, St. Louis, MO, USA). The antibodies for PTEN, p53, Dnmt1, Dnmt3a, Dnmt3b, Dicer 1, KRAS and the peroxidase-labeled secondary antibody were purchased from Cell Signaling Technology (Beverley, MA, USA).

2.5. Knock down of Dicer 1 gene expression

The target siRNA sequence of Dicer 1 gene (AAGGCTTACCTTCTCCAGGCT) was designed as previously described [17] and synthesized by Integrated DNA Technologies, Inc. (Coralville, IO, USA). BxPC-3, Capan-2, and Panc-1 cells were passaged 24 h before transfection. Cells in 10-cm dishes were transfected with a scrambled oligonucleotide (oligo) (AATTCTCCGAACGTGTCACGT) that does not specifically target any known gene, or Dicer 1 siRNA duplex using Lipfectamine 2000 following the user manual (Invitrogen, Grand Island, NY). Forty-eight hours later, the cells were harvested for total RNA isolation or homogenization for Western blot assay.

2.6. Tumor growth study

Approximately 5 × 10^6 BxPC-3, Capan-2, and Panc-1 tumor cells were subcutaneously injected into the right hind limbs of mice. After the tumors have grown to 7–8 mm in diameter, mice were randomly divided into 2 groups: control and silenced group. Each group contains 10 animals. Mice in the control group were given polyethylenimine/scrambled oligo injection and mice in the silenced groups were injected with polyethylenimine/Dicer 1 siRNA duplex as previously described [18]. Each injection contained

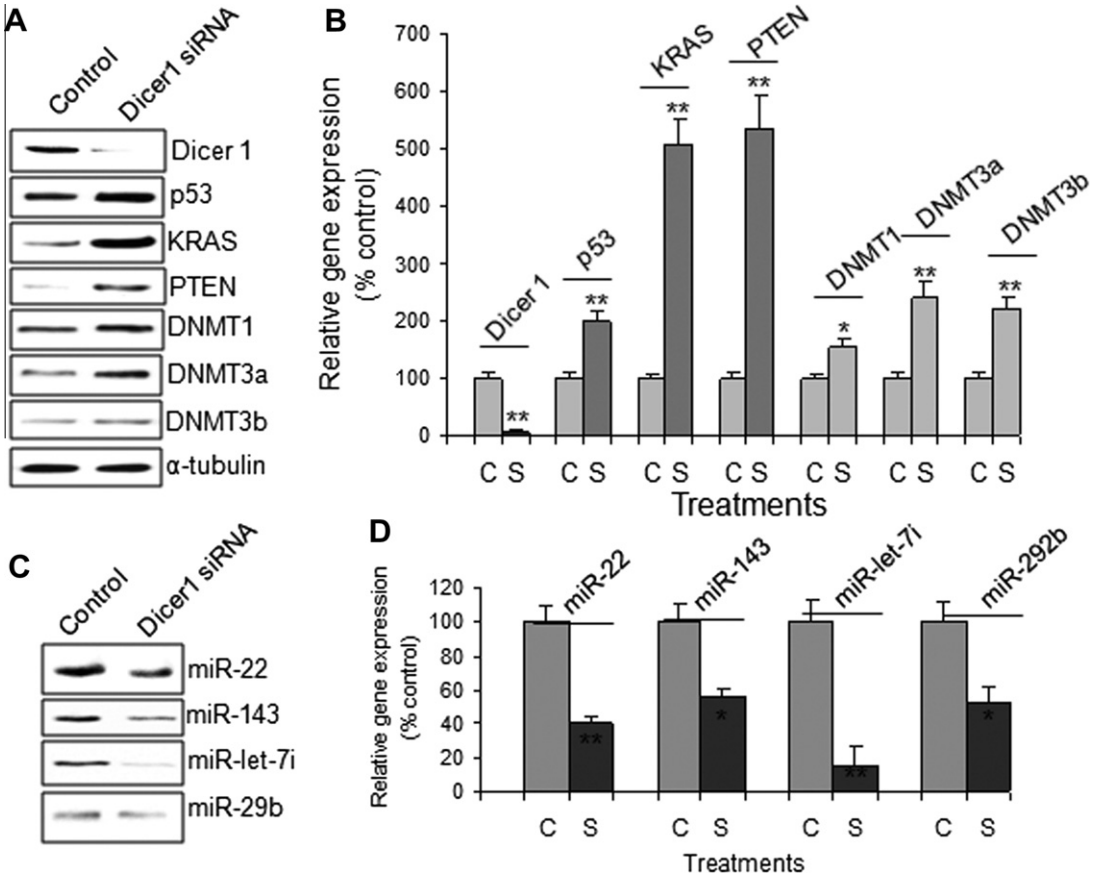

Fig. 2. Gene expression in Panc-1 cells under Dicer 1 silencing. (A) Western blot of gene expression. (B) Percent densitometric values of genes in (A). (C) Northern blot of miRNA expression. (D) Percent densitometric values of miRNAs in (C). Densitometric values were normalized to that in control group. $^*p < 0.01$, $^{**}p < 0.001$. C, control; S, Dicer 1 silencing. $N = 5$.

30 μg of scrambled oligo or Dicer 1 siRNA. Animals were injected once a week for 4 weeks. Mice with xenograft BxPC-3 and Panc-1 tumors were euthanized 30 days after the first injection of siRNA and tumors were excised and weighed. Mice with xenograft Capan-2 tumors were sacrificed 45 days after the first injection. Tumor specimens were snap frozen and kept at −80 °C.

2.7. Statistical analysis

Data were analyzed using Statistical Package for the Social Sciences, version 13.0 (Chicago, IL). Differences between groups were detected with two tailed student *t*-test. All data are presented as mean ± standard error of the mean. A $p < 0.05$ was considered statistically significant.

3. Results

3.1. Gene and miRNA expression patterns in pancreatic cancer cell lines

As shown in Fig. 1A and B, BxPC-3 cells exhibited lower KRAS, let-7i, and miR-29b levels. Capan-2 showed lower p53, Dnmt3a, Dicer 1, and miR-143 levels. Panc-1 cells exhibited lowered PTEN, let-7i, miR-29b, and miR-143 levels. These results suggested highly disparate genotypes, which may be representative of pancreatic cancer subclasses.

3.2. Silencing of Dicer 1 expression in BxPC-3 cells

Silencing of Dicer 1 gene was established by transfecting Dicer 1 siRNA duplex into BxPC-3 cells for 48 h. Western blot analysis showed that Dicer 1 siRNA achieved around 80% inhibition in Dicer 1 protein expression (Fig. 1C). At the same time, the protein levels of p53, KRAS, PTEN, Dnmt3a, and Dnmt3b were upregulated (Fig. 1C and D). Northern blot analysis showed that knockdown of Dicer 1 expression significantly downregulated the expression of miR-22, miR-143, let-7i, and miR-29b (Fig. 1E and F), but had no effect on the expression of Dnmt1 (Fig. 1C and D). miR-29b expression negatively correlated with Dnmts expression, miR-143 expression negatively correlated with KRAS expression, and miR-22 negatively correlated with p53 and PTEN expression. Dnmt expressions are positively associated with KRAS, p53, and PTEN expression implying that the expression of these genes was not regulated by methylation machinery.

3.3. Silencing Dicer 1 expression in Panc-1 cells

Western blot analysis showed about 90% inhibition in Dicer 1 protein level after siRNA transfection. Knockdown of Dicer 1 expression in Panc-1 cells significantly increased p53, KRAS, PTEN, Dnmt1, Dnmt3a, and Dnmt3b protein levels (Fig. 2A and B). Northern blot analysis showed that knockdown of *Dicer 1*

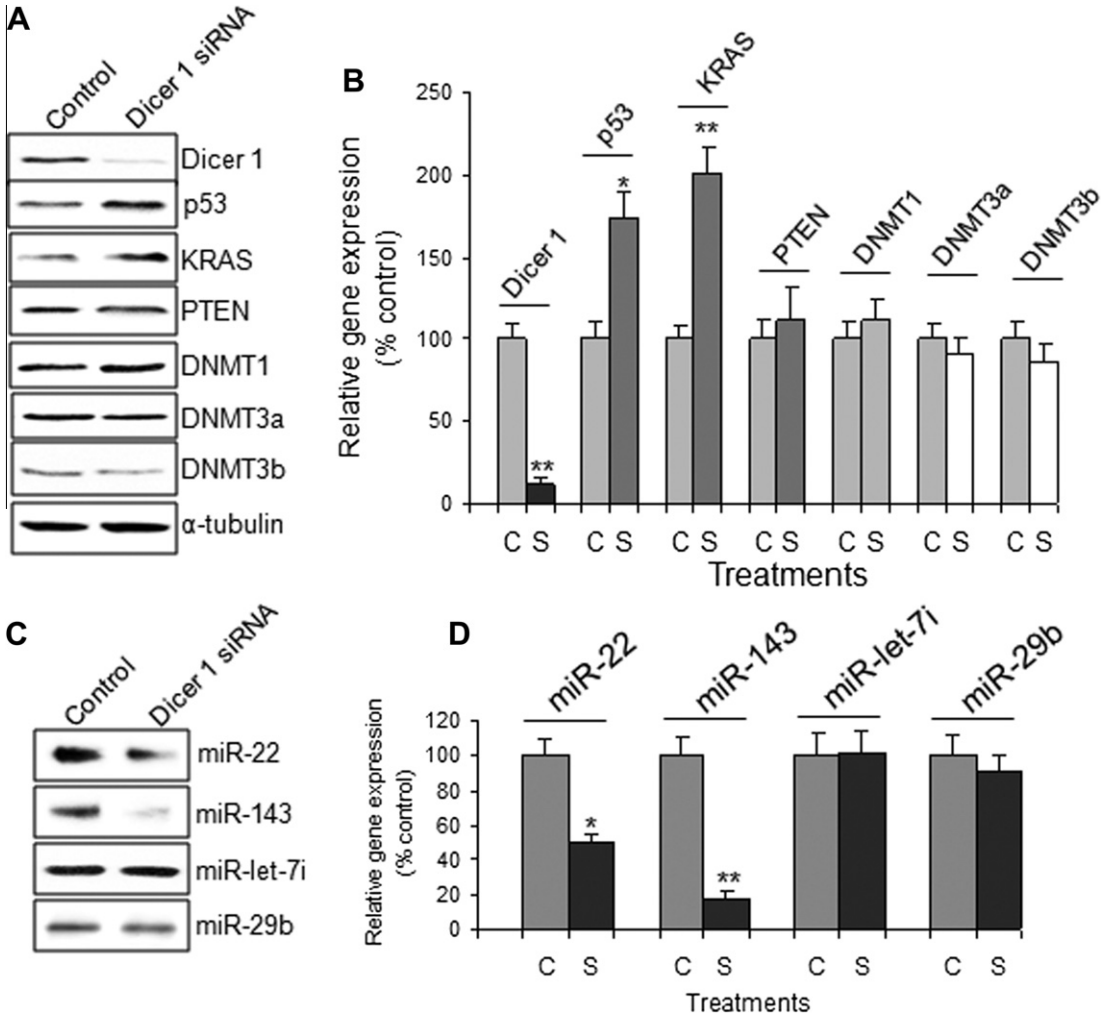

Fig. 3. Gene expression in Capan-2 cells under Dicer 1 silencing. (A) Western blot of gene expression. (B) Percent densitometric values of genes in (A). (C) Northern blot of miRNA expression. (D) Percent densitometric values of miRNAs in (C). Densitometric values were normalized to that in control group. $^{*}p < 0.01$, $^{**}p < 0.001$. C, control; S, Dicer 1 silencing. $N = 5$.

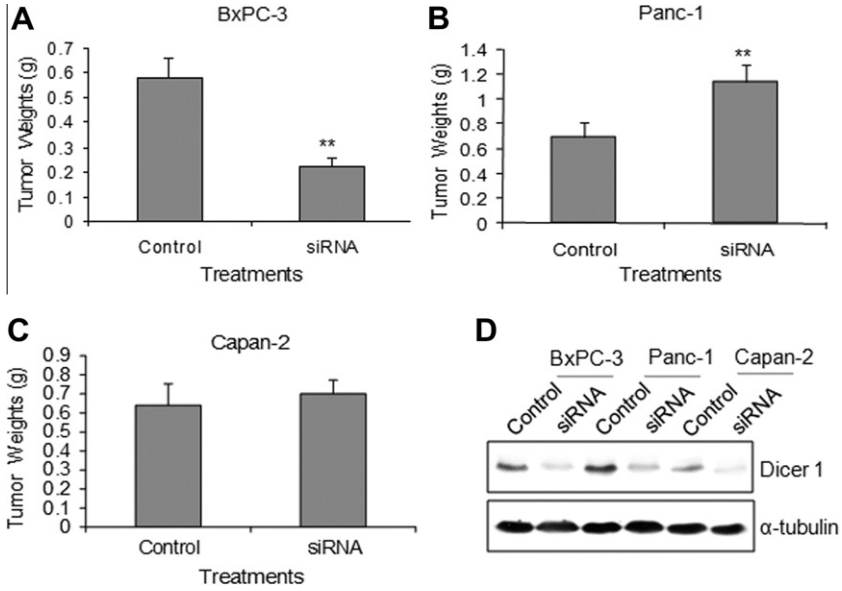

Fig. 4. The effect of Dicer 1 silencing on tumor growth. (A) Tumor growth in xenograft BxPC-3 tumor model. (B) Tumor growth in xenograft Panc-1 tumor model. (C) Tumor growth in xenograft Capan-2 tumor model. (D) Western blot of Dicer 1 protein expression in tumor tissues injected with crample oligo (Control) and Dicer 1 siRNA (siRNA). $^{**}p < 0.01$ *vs.* control. $N = 10$.

expression significantly decreased miR-22, miR-143, let-7i, and miR-29b expression in Panc-1 cells (Fig. 2C and D). These findings suggested that gene expression in BxPC-3 and Panc-1 cells were regulated by a similar mechanism.

3.4. Silencing Dicer 1 expression in Capan-2 cells

To knockdown Dicer 1 expression, Dicer 1 siRNA duplex was transfected into Capan-2 cells for 48 h. Western blot analysis showed that transformation of Dicer 1 siRNA duplex resulted in 87% inhibition in Dicer 1 protein level (Fig. 3A and B). Knockdown of *Dicer 1* expression in Capan-2 cells significantly increased p53 and KRAS protein levels, but had no effect on PTEN, Dnmt1, Dnmt3a, and Dnmt3b protein levels (Fig. 3A and B). Northern blot analysis showed that knock-down of *Dicer 1* expression significantly decreased the expression of miR-22 and miR-143, but had no effect on let-7i and miR-29b expression in Capan-2 cells (Fig. 3C and D). These results suggested that: (1) knock-down of *Dicer 1* expression had no effect on Dnmt expression or on methylation; (2) let-7i and miR-29b were regulated by multiple mechanisms including Dicer 1.

3.5. The effect of inhibiting Dicer 1 expression on tumor growth in xenograft pancreatic tumor models

After tumor mass reached 7–8 mm in diameter, mice were given direct polyethylenimine/Dicer 1 siRNA injection into the tumor mass once per week for 4 weeks. BxPC-3 and Panc-1 tumors were excised 30 days after first siRNA injection. Capan-2 tumors were excised 45 days after first siRNA injection. Wet tissue weight was determined for excised tumors. Injection of Dicer 1 siRNA significantly decreased the wet weight of BxPC-3 tumors (Fig. 4A), but significantly increased the wet weight of Panc-1 tumors (Fig. 4B). Interestingly, injection of Dicer 1 siRNA had no effect on Capan-2 tumor growth (Fig. 4C). The silencing of Dicer 1 expression was verified in tumor tissues after siRNA delivery (Fig. 4D).

4. Discussion

Pancreatic cancer is an aggressive cancer with a high mortality rate. Despite recent progress in targeting therapy of cancer, effective treatment has not yet been established in pancreatic cancer. In this study, we explored the regulatory role of microRNA and methylation on the expression of the *k-ras* oncogene as well as *TP53* and *PTEN* tumor suppressor genes in three pancreatic cancer cell lines. Different gene expression profiles were observed between cell lines. Methylation was found to regulate gene expression in Capan-2 cells, but not in BxPC-3 or Panc-1 cells. High expression of Dicer 1 is one of the main mechanisms responsible for high expression of miR-22, let-7i, miR-143, and miR-29b in BxPC-3 and Panc-1 cells. In contrast, the expression of let-7i and miR-29b in Capan-2 cells was regulated by different mechanisms including Dicer 1. Silencing of *Dicer 1* gene expression produced different effects on tumor growth in xenograft BxPC-3, Capan-2, and Panc-1 tumor models. The current study implies that different mechanisms are involved in the tumor growth of pancreatic cancer subclasses.

The accumulation of inherited and acquired genetic defects results in the carcinogenesis and progression of cancers [19]. For instance, mutations in *k-ras* inhibit the GTPase activity of RAS, leading to an oncogenic RAS protein. Mutated RAS protein induces multiple signaling pathways involved in tumor cell growth [7]. Mutations in tumor-suppressor genes have been demonstrated to cause functional loss of their proteins, which are thought to involve in the development and prognosis of pancreatic cancer [20]. BxPC-3 cell line was derived from an adenocarcinoma locating at the body of pancreas and genotype analysis revealed a mutation in *TP53* gene, wild-type *k-ras*, and *p16* deletion. Capan-2 cell line was established from an infiltrated pancreatic cancer. This cell line has wild-type *TP53 gene*, mutated *k-ras*, and *p16* deletion. Panc-1 was cultured from an adenocarcinoma locating at the head of pancreas with invasion to the duodenal wall. A previous study demonstrated that *k-ras* and *TP53* were mutated, while *p16* was deleted in Panc-1 cells [7]. In contrast, *PTEN* gene is functional in all of these cell lines. In this study, we found that miRNAs exert similarly regulatory roles on KRAS, p53 and PTEN expressions in BxPC-3 and Panc-1 cells, but obviously different role in Capan-2 cells. Dicer is a key enzyme that controls miRNA processing. In this study, silencing of *Dicer 1* expression revealed that Dicer 1 expression is required for the growth of BxPC-3 cells, but not Capan-2 cells. In contrast, Dicer 1 is a tumor suppressive gene in Panc-1 cells.

Recent studies showed that p53 regulates Dicer expression through transcriptional miRs, such as let-7. The promoters of

miRNA genes in let-7 family contain p53 reaction elements (REs). Thus, p53 can enhance let-7 expression and subsequently down-regulate Dicer expression [21]. Dicer's promoter also contains several p53-REs, indicating that Dicer could be a transcriptional target of p53 [21]. A previous study demonstrated that knockdown of Dicer expression can upregulate p53 level [22]. We therefore proposed that there is a regulation loop between p53, Dicer, and let-7. In BxPC-3 and Panc-1 cells, knockdown of *Dicer 1* expression increased p53 level, but decreased let-7i expression. This is because p53 was mutated and dysfunctional in these two cell lines. The regulatory role of p53 on let-7i was further verified in Capan-2 cells. Capan-2 cells have functional p53. Knockdown of *Dicer 1* expression increased p53 expression, which subsequently upregulated let-7i expression. But this effect was counteracted by the decrease in Dicer level. We also demonstrated that miR-29b level is dependent on p53 activity.

Previous studies demonstrated that miR-22 inhibits the expression of both *TP53* and *PTEN* genes, p53 in turn suppresses miR-22 expression [23]. miR-143 has been revealed to regulate KRAS expression in pancreatic cancer cells [24]. In this study, knockdown of Dicer 1 expression decreased miR-22 and miR-143 expression, but increased KRAS, PTEN and p53 protein level in BxPC-3 and Panc-1 cells. These observation confirmed previous findings that miR-22 regulates p53 and PTEN expression, while miR-143 regulates KRAS expression. However, in Capan-2 cells, p53 is functional. miR-22 and miR-143 expression negatively correlated with KRAS and p53, but not PTEN protein levels. This suggested that PTEN expression was mainly regulated by another mechanism, such as methylation.

A recent study suggested the involvement of miRNA in controlling DNA methylation by inhibiting Dnmts expression [25]. For example, miR-29b was reported to induce global DNA hypomethylation and tumor suppressor gene re-expression in acute myeloid leukemia by directly targeting Dnmt3a, Dnmt3b, and indirectly targeting Dnmt1 [26]. In BxPC-3 and Panc-1 cells, decreased miR-29b expression correlated with the upregulation of Dnmts expression. In contrast, silencing of *Dicer 1* gene in Capan-2 cells did not affect miR-29b and Dnmts expression. This suggests that miR-29b regulates methylation in all three pancreatic cancer cell lines. In BxPC-3 and Panc-1 cells, Dnmts expression was increased, but PTEN expression was also increased. This suggests that in these two cell lines, methylation is not the main mechanism that regulates PTEN expression. In contrast, miRNA is a dominant mechanism to control PTEN expression. However, decreased miR-22 expression did not increase PTEN expression in Capan-2 cells. This suggests that miR-29b-regulated methylation may play an important role in PTEN expression, which counteracted the role of miR-22. It has been demonstrated that KRAS mutations were found in about 95% of pancreatic cancers, while p53 mutation was found in about 75% of patients. This suggests that methylation is not a key cause for KRAS and p53 abnormalities in pancreatic cancer.

The current study also revealed that knockdown of *Dicer 1* expression induces global down-regulation of miRNA expression in tumors, which produced different outcomes in tumor growth. Knockdown of *Dicer 1* expression upregulated KRAS, p53 and PTEN expression, and inhibited xenograft BxPC-3 tumor growth. However, in BxPC-3 cells, *TP53* gene is mutated while *k-ras* is wild-type. Therefore, upregulation of PTEN expression plays a key role in BxPC-3 tumor growth delay. Knockdown of *Dicer 1* expression upregulated KRAS, p53 and PTEN expression in Panc-1 cells, but promoted xenograft Panc-1 tumor growth. In Panc-1 cells, *k-ras* and *TP53* were mutated. Although PTEN expression was upregulated, the mutated KRAS gains oncogenic function. Thus, KRAS mutation is the main power to drive Panc-1 tumor growth. Knockdown of *Dicer 1* expression upregulated p53 and KRAS expression in Capan-2 cells, but exhibited no effect on xenograft Capan-2 tumor growth. Capan-2 cells express wild-type TP53, wild-type PTEN and mutated KRAS. Therefore, the tumor suppressive role of p53 was counteracted by the oncogenic role of mutated KRAS. This suggests that whether global down-regulation of miRNA expression affects tumor growth is tumor cell genotype dependent.

In conclusion, the regulatory roles of miRNA and methylation on oncogenes or tumor suppressor genes are tumor cell genotype dependent. Different molecular mechanisms are involved in the carcinogenesis and progression of pancreatic cancer. Our study highlighted the importance of personalized therapy.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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