Restore of miR-541 resensitizes pancreatic cancer cells to gemcitabine-induced apoptosis through suppression of HAX-1

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Abstract

Background: MiR-541 acts as a tumor suppressor in some cancers. However, the role of miR-541 in regulating the chemosensitivity to cancer cells is still unclear. The aim of this study is to explore the effect of miR-541 on chemoresistance of pancreatic cancer (PCa) cells to gemcitabine-induced apoptosis.

Methods: Gemcitabine-resistant Panc-1 and Capan-2 PCa cell lines (Panc-1/R and Capan-2/R) were established through long term exposure to gemcitabine. Effect of miR-541 on changing the sensitivity of Panc-1/R and Capan-2/R to gemcitabine-induced cytotoxicity was evaluated by MTT assays. Regulation of miR-541 on HAX-1 was confirmed by bioinformatics, western blot analysis and luciferase reporter assays. Cell apoptosis and mitochondrial membrane potential (MMP) was measured by flow cytometry analysis.

Results: Comparison with Panc-1 and Capan-2, downregulation of miR-541 was observed in Panc-1/R and Capan-2/R cells. Overexpression of miR-541 was found to increase the cytotoxicity of gemcitabine to Panc-1/R and Capan-2/R cells. However, transfection with HAX-1 plasmid can abolish the effect of miR-541 on gemcitabine-induced cytotoxicity against Panc-1/R and Capan-2/R.

Conclusion: Downregulation of miR-541 is responsible for development of gemcitabine resistance in PCa. Overexpression of miR-541 may represent a potential strategy to reverse the chemoresistance of PCa.

Background

Pancreatic cancer (PCa) represents as the seventh leading cause of cancer death in the world [1]. Prognosis of PCa is very poor because PCa cells are highly invasive and highly metastatic. It has been reported that 60% of PCa patients have distant metastasis within the first 24 months after surgery [2]. To make matters worse, overall 5-year survival rate of PCa is less than 8% [3, 4]. Despite there have been great advances in medicine in the decades, chemotherapy is still an important approach to improve the overall survival of PCa patients [5].

Gemcitabine belongs to the pyrimidine antineoplastic drugs. Its main metabolites can incorporate into the DNA of cancer cells, followed by breaking the normal function of DNA and thus activating the apoptotic pathway of cancer cells [6, 7]. However, gemcitabine resistance is usually seen during the PCa treatment [8, 9]. Chemoresistance has been recognized as one of the leading causes of PCa-associated deaths [10]. At this point, it is vitally pressing to search the novel approach to intervene the formation of drug resistance.

MicroRNA (miRNA) is a class of short, endogenous and non-coding RNA molecules. MiRNAs negatively regulate 60% of coding genes by targeting the 3' untranslated regions (3'UTRs) of specific mRNAs [11–13]. Since miRNAs are involved in various cellular processes including proliferation, metabolism, survival

and apoptosis, dysregulation of miRNAs is an important factor for cancer pathogenesis in humans [14–16].

Recent studies have reported that deregulation of apoptosis-related proteins induces drug resistance of cancers [17, 18]. Among these proteins, hematopoietic cell-specific protein 1-associated protein X-1 (HAX-1) is usually overexpressed and is responsible for the drug resistance [19, 20]. In the present study, we showed the association between HAX-1 and resistance of gemcitabine in PCa.

Methods

Cell culture

Human PCa cell lines Capan-2 and Panc-1 were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured under the condition recommended by the vendor. Cells were cultured in 5% CO₂ incubator at 37 °C. For acquisition of gemcitabine-resistant PCa cell lines, Capan-2 and Panc-1 cells were gradually exposed to increasing concentrations of gemcitabine from 0.05 µM to 0.5 µM. The acquired gemcitabine-resistant Capan-2 and Panc-1 were named as Capan-2/R and Panc-1/R.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNAs of cell lines for PCR were extracted with TRIzol reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instruction. Subsequently, RNAs were reversely transcribed by One Step PrimeScript miRNA cDNA Synthesis Kit (Takara Bio, Inc., Otsu, Japan) to obtain the cDNAs of cell lines. Finally, the expression of miR-541 was measured by using SYBR Premix Ex Taq (TaKaRa) on the Applied Biosystems 7900 Sequence Detection System. The U6 snRNA was chosen as the endogenous control for the detection of miR-541. The

relative expression levels of each gene were calculated and normalized using the $2^{-\Delta\Delta C_t}$ method.

Transfection

Mature human miR-541 mimics (5'-UGGUGGGCACAGAAUCUGGACU-3') and negative control oligonucleotides (NCO, 5'-GGGACACACUGGUGUAAUGGC-3') were purchased from GenePharma Co. Ltd. (Shanghai, China). HAX-1 small interfering RNA (siRNA) was purchased from Santa Cruz Biotechnology (sc-43365) (Santa Cruz, CA, USA). Open reading frame of HAX-1 gene was inserted into the pcDNA3.1 (Invitrogen, Carlsbad, CA, USA) to construct a recombinant eukaryotic expression plasmid of HAX-1. For transfection, RNA oligonucleotides (50 pmol/ml) or plasmid (2 µg/ml) was coated with Lipofectamine 2000 (Invitrogen) and then mixed in serum-free medium. Subsequently, the serum-free medium was added to the cells. After incubation for 6 hours, the serum-free medium was removed and fresh RPMI-1640 medium containing 10% fetal bovine serum was added for 24 hours.

Cell viability assay

Cells were seeded into 96-well plates overnight. Next, the adherent PCa cells were treated with different concentrations of gemcitabine followed by incubation for 48 h. Subsequently, 20 µl of 5 mg/ml MTT reagent (Sigma-Aldrich, St.Louis, MO, USA) was added into the culture medium for more 4 h incubation. Next, the supernatant were removed followed by addition with dimethyl sulfoxide. OD values were then measured at 570 nm wavelength on an ELISA microplate reader (Sunrise Microplate Reader, TECAN, Switzerland). 50% inhibiting concentration (IC50) of gemcitabine to PCa cells was measured according to the cell viability curve.

Luciferase reporter assay

A fragment of HAX-1 3' UTR containing predicted miR-541 binding site was cloned into the pGL3 Luciferase Reporter Vectors (Promega, Madison, WI, USA). The recombinant reporter was named as pGL3-wt HAX-1. The mutant HAX-1 reporter was created by mutating the seed region of the miR-541 binding site (UGCCCACCA) by using the site-directed mutagenesis kit (Takara). The mutant reporter was named as pGL3-mt HAX-1. For luciferase reporter assay, cells were plated into 48-well plates overnight. Next, PCa cells were co-transfected with RNA oligonucleotides (miR-541 or NCO, 50 pmol/ml), recombinant pGL3 reporter (2 µg/ml) and Renilla luciferase pRL-TK vector (100 ng/ml, Promega). After 48 h incubation, luciferase activities were measured by using the Dual-Luciferase Reporter assay system (Promega) according to the manufacturer's instruction.

Western blot assay

To test the expression level of HAX-1, total proteins were extracted by using RIPA buffer (Cell Signaling Technologies, Danvers, MA, USA). To test the level of cytochrome c and apoptosis-inducing factor (AIF) in mitochondria and cytosol respectively, Mitochondria/Cytosol Fraction Kit (BioVision, Milpitas, CA, USA) was used according to the manufacturer's instruction. Next, 50 µg of the obtained samples were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by transference to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) through electroblotting. The membranes were then probed by anti-human HAX-1 (Santa Cruz Biotechnology), anti-human GAPDH (Cell Signaling Technologies), anti-cytochrome c (Cell Signaling Technologies) and anti-AIF (Cell Signaling Technologies). Subsequently, the membranes were washed and incubated with horseradish peroxidase-conjugated antibodies. Protein bands were detected by using an enhanced chemiluminescence detection kit (Pierce, Rockford, IL, USA).

Flow cytometry

cells were harvested through centrifugation at 2,000 r.p.m for 5 min. Subsequently, cells were washed with PBS for twice. For evaluation of mitochondrial membrane potential (MMP), cells were stained with 5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethyl imidacarbo cyanine iodide (JC-1, Molecular Probes; Waltham, MA, USA) as an indicator. Cells emitting red fluorescence were considered as cells with high MMP. For measurement of cell apoptosis, Annexin V-FITC Apoptosis Detection Kit (Sigma-Aldrich) was used according to their manufacturer's instruction. The Annexin V-positive cells were considered as the apoptotic cells.

Statistical analysis

All data are represented as the mean $\pm$ standard deviation (SD) and carried out by at least three independent experiments. For comparison analysis, two-tailed Student's t-tests were used to estimate the statistical differences between two groups. One-way analysis of variance (ANOVA) and Bonferroni's post hoc test were used to determine the differences between three or more groups. Statistical analysis was performed by using SPSS 16.0 software. Values of $P < 0.05$ were considered significant.

Results

HAX-1 is overexpressed in gemcitabine-resistant PCa cells

To study the gemcitabine resistance in PCa, we established the gemcitabine-resistant Capan-2 and Panc-1 cell lines (Capan-2/R and Panc-1/R) through gradual exposure of routine Capan-2 and Panc-1 cell lines to increasing concentrations of gemcitabine. Results of MTT assays confirmed the resistance of gemcitabine to Capan-2/R and Panc-1/R (Fig. 1a). We showed that IC₅₀ of gemcitabine to the established Capan-2/R and Panc-1/R cells was obviously lower than their parental Capan-2/R and Panc-1/R cells (Fig. 1b). Next, we compared the expression of HAX-1 between gemcitabine-resistant PCa cells and their parental PCa cell lines. We found that expression of HAX-1 in Capan-2/R and Panc-1/R cells was obviously overexpressed at both the mRNA level (Fig. 1c) and protein level (Fig. 1d). These results suggested that overexpression of HAX-1 may be associated with gemcitabine resistance of PCa.

Knockdown of HAX-1 reduced the resistance of gemcitabine to Capan-2/R and Panc-1/R

To investigate whether overexpression of HAX-1 was associated with the gemcitabine resistance of PCa, we transfected the Capan-2/R and Panc-1/R cells with HAX-1 siRNA. Effect of HAX-1 siRNA on Capan-2/R and Panc-1/R was shown in Fig. 2a. We then found that knockdown of HAX-1 increased the sensitivity of gemcitabine to Capan-2/R and Panc-1/R (Fig. 2b). Under the treatment of HAX-1 siRNA, IC₅₀ of gemcitabine to Panc-1/R cells decreased by 74.9% and IC₅₀ of gemcitabine to Capan-2/R cells decreased by 78.2% (Fig. 2c). On the other hand, we transfected the Capan-2 and Panc-1 cells with HAX-1 plasmid to test the effect of HAX-1 on the formation of gemcitabine resistance. Effect of HAX-1 plasmid on Capan-2 and Panc-1 was shown in Fig. 2d. We then found that overexpression of HAX-1 significantly increased the IC₅₀ of gemcitabine to Panc-1 and Capan-2/R (Fig. 2e). These results indicated that overexpression of HAX-1 was an important factor to induce the gemcitabine resistance of PCa. Knockdown of HAX-1 can reduce the resistance of gemcitabine to Capan-2/R and Panc-1/R.

MiR-541 targets HAX-1 in PCa

As 60% of coding genes were regulated by miRNAs [11–13], we searched the upstream regulator of HAX-1 through the public databases of TargetScan, miRanda and PicTar. All of the three miRNA databases

showed that HAX-1 mRNA 3' UTR contained sequence which was complementarily paired with miR-541 (Fig. 3A). Furthermore, by contrast to the overexpression of HAX-1 in Capan-2/R and Panc-1/R cells (Fig. 1c and 1d), expression of miR-541 was significantly decreased in these cells (Fig. 3b). These results suggested that HAX-1 may be the target of miR-541 in PCa. To prove the miR-541/HAX-1 axis, we tested the expression of HAX-1 in Capan-2/R and Panc-1/R cells after they were transfected with miR-541 mimics. We then found that recovery of miR-541 expression reduced the expression of HAX-1 in Capan-2/R and Panc-1/R cells (Fig. 3c). Furthermore, results of luciferase assay showed that miR-541 significantly decreased the luciferase activities of the pGL3-wt HAX-1 reporter, but not the pGL3-mt HAX-1 reporter or pGL3-control reporter (Fig. 3d). All of these results indicated that HAX-1 was the target of miR-541, and overexpression of HAX-1 was induced by absence of miR-541 in Capan-2/R and Panc-1/R cells.

Increase of miR-541 reduced the resistance of gemcitabine to Capan-2/R and Panc-1/R through suppression of HAX-1

Our preceding results indicated that overexpression of HAX-1 was induced by absence of miR-541 in Capan-2/R and Panc-1/R cells. We thus investigated whether increase of miR-541 can reduce the gemcitabine resistance of Capan-2/R and Panc-1/R cells. As shown in Fig. 4a, transfection with miR-541 mimics increased the sensitivity of Panc-1/R cells to gemcitabine. Under the treatment of miR-541 mimics, IC₅₀ of gemcitabine to Panc-1/R cells decreased by 80.9%. As shown in Fig. 4b, transfection with miR-541 mimics increased the sensitivity of Capan-2/R cells to gemcitabine. Under the treatment of miR-541 mimics, IC₅₀ of gemcitabine to Capan-2/R cells decreased by 83.2%. On the other hand, under the co-treatment of gemcitabine and miR-541, cell viability of HAX-1 plasmid-transfected Capan-2/R and Panc-1/R cells was significantly higher than the control plasmid-transfected Capan-2/R and Panc-1/R cells (Fig. 4c). These results indicated that increase of miR-541 reduced the resistance of gemcitabine to Capan-2/R and Panc-1/R through suppression of HAX-1.

MiR-541 facilitates the mitochondrial apoptosis induced by gemcitabine in Capan-2/R and Panc-1/R cells

Our preceding results had indicated that miR-541 reduced the gemcitabine resistance of PCa. We next explored the pathway of cell death in the gemcitabine and miR-541-co-treated Capan-2/R and Panc-1/R cells. First, the mitochondrial membrane potential (MMP) was tested. Despite miR-541 single treatment can not affect the MMP of Capan-2/R and Panc-1/R, miR-541 enhanced the collapse of MMP induced by gemcitabine (Fig. 5a). Next, We separated the mitochondria fraction from the cytosol of Capan-2/R and Panc-1/R cells. Comparing to the single gemcitabine-treated Capan-2/R and Panc-1/R cells, the gemcitabine and miR-541-co-treated Capan-2/R and Panc-1/R cells released more amount of cytochrome c and apoptosis-inducing factor (AIF) (Fig. 5b). Cytochrome c and AIF are potent apoptosis inducers [21, 22]. As the results of the release of these apoptosis inducers, caspases and apoptosis were triggered (Fig. 5c and 5d). These results indicated that treatment with miR-541 can facilitate the mitochondrial apoptosis induced by gemcitabine in Capan-2/R and Panc-1/R cells.

Discussion

Development of drug resistance is a major obstacle during the chemotherapy with gemcitabine. Gemcitabine is a pyrimidine antineoplastic drug that induces apoptosis pathway of cancer cells [23, 24]. However, cancer cells usually develop resistance of apoptosis through deregulation of apoptosis-related proteins [25, 26]. Among these proteins, hematopoietic cell-specific protein 1-associated protein X-1 (HAX-1) is reported to act as a key anti-apoptotic protein. HAX-1 is mainly localized at mitochondria outer membrane. HAX-1 on mitochondria inhibits collapse of mitochondria and loss of mitochondrial membrane potential (MMP) [27–30]. Therefore, overexpression of HAX-1 reduces the damage of mitochondria and inhibits the cell apoptosis induced by drugs and environmental factors [31]. Studies have reported that overexpression of HAX-1 is usually observed in multiple human cancers. Furthermore, overexpression of HAX-1 has been reported to induce resistance to many chemotherapeutic drugs [32, 33]. HAX-1 has become an important target in the chemotherapy.

The aim of this study is to explore the association between gemcitabine resistance and HAX-1 in PCa. We thus established the models of gemcitabine-resistant PCa cell lines. We discovered that expression level of HAX-1 in the gemcitabine-resistant PCa cells was significantly higher than that in the routine PCa cells. It indicated that overexpression of HAX-1 was an important change when the PCa cells developed the gemcitabine resistance. Furthermore, we observed that transfection with HAX-1 siRNA resensitized the gemcitabine-resistant PCa cells to gemcitabine treatment. It indicated that overexpression of HAX-1 was an important factor that induced resistance of gemcitabine in PCa.

MicroRNA (miRNA) is a class of endogenous RNA molecules that negatively regulate 60% of coding genes [11–13]. In this study, we observed that expression level of miR-541 was significantly decreased in gemcitabine-resistant PCa cells. Moreover, we proved that overexpression of HAX-1 in gemcitabine-resistant PCa cells was induced by absence of miR-541 expression. MiR-541 acts as a tumor suppressor in some cancers including lung cancer and pancreatic cancer. Overexpression of miR-541 has been reported to reverse cancer progression through suppression proliferation and invasion of cancer cells [34–36]. We discovered that recovery of miR-541 expression in gemcitabine-resistant PCa cells resensitized them to gemcitabine treatment. Furthermore, we proved that the effect of miR-541 was dependent on the suppression of HAX-1. It indicated that miR-541/HAX-1 axis was an important target in the therapy of gemcitabine.

In our mechanism research, we demonstrated that the apoptotic signaling induced by gemcitabine can be expanded by miR-541 treatment. Overexpression of miR-541 inhibited the expression of HAX-1 and thus facilitate the collapse of mitochondria induced by gemcitabine. As a result, cytochrome c and AIF which are potent apoptosis inducers [21, 22] were released into the cytosol. These apoptosis inducers triggered caspases and the following apoptosis of gemcitabine-resistant PCa cells (Fig. 6).

Conclusion

overexpression of HAX-1 is responsible for development of gemcitabine resistance in PCa. HAX-1 can be suppressed by miR-541 which can promote the mitochondrial apoptosis induced by gemcitabine. Therefore, miR-541/HAX-1 axis may represent a promising target in the treatment of PCa.

Abbreviations

qRT-PCR, quantitative Real-Time Polymerase Chain Reaction; HAX-1, hematopoietic cell-specific protein 1-associated protein X-1; Panc-1/R, gemcitabine-resistant Panc-1; Capan-2/R, gemcitabine-resistant Capan-2; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; IC50, 50% inhibiting concentration; PCa, pancreatic cancer; mRNA, message RNA; siRNA, small interfere RNA; MMP, mitochondrial membrane potential; AIF, apoptosis inducing factor.

Declarations

Acknowledgments

Not applicable.

Authors' contributions

HL, XMG, JZ, XDZ, JX, FLL, ZQY, XXW and MZL conducted the experiments and wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

HAX-1 was overexpressed in Panc-1/R and Capan-2/R. a MTT assays were performed to detect the sensitivity of Panc-1/R and Capan-2/R cells to different concentrations (0~80 μM) of gemcitabine. b Comparison of IC50 between gemcitabine-resistant PCa cell lines and their parental PCa cell lines. c QRT-PCR analysis was performed to test the differences of HAX-1 expression between gemcitabine-resistant

PCa cell lines and their parental PCa cell lines at the mRNA level. d Western blot analysis was performed to test the differences of HAX-1 expression between gemcitabine-resistant PCa cell lines and their parental PCa cell lines at the protein level. *P<0.05.

Figure 2

HAX-1 partially determined the gemcitabine resistance of PCa. a Effect of HAX-1 siRNA on decreasing the expression of HAX-1 in Capan-2/R and Panc-1/R cells. b HAX-1 siRNA enhanced the cytotoxicity of gemcitabine to Capan-2/R and Panc-1/R cells. c HAX-1 siRNA decreased the IC50 of gemcitabine to Capan-2/R and Panc-1/R cells. d Effect of HAX-1 plasmid on increasing the expression of HAX-1 in Capan-2 and Panc-1 cells. e HAX-1 plasmid increased the IC50 of gemcitabine to Capan-2 and Panc-1 cells. *P<0.05.

Figure 3

HAX-1 is the target of miR-541 in PCa. a TargetScan, miRanda and PicTar miRNA databases showed the putative miR-541 binding site on 3' UTR of HAX-1 mRNA. b QRT-PCR analysis was performed to test the differences of miR-541 expression between gemcitabine-resistant PCa cell lines and their parental PCa cell lines. c Effect of miR-541 on decreasing the HAX-1 expression in Capan-2/R and Panc-1/R cells. d MiR-541 decreased the luciferase activities of the pGL3 reporters containing wild type HAX-1 3' UTR. *P<0.05.

Figure 4

MiR-541 increased the sensitivity of gemcitabine to Capan-2/R and Panc-1/R through suppression of HAX-1. a MiR-541 decreased the IC50 of gemcitabine to Panc-1/R. b MiR-541 decreased the IC50 of gemcitabine to Capan-2/R. c Treatment with HAX-1 plasmid inhibited the effect miR-541 on the cytotoxicity of gemcitabine to Capan-2/R and Panc-1/R. * $P < 0.05$. # $P < 0.05$ vs. gemcitabine+miR-541 group. & $P < 0.05$ vs. gemcitabine+NCO group.

Figure 5

MiR-541 enhanced the mitochondrial apoptosis of Capan-2/R and Panc-1/R cells which were treated with gemcitabine. a MiR-541 enhanced the collapse of MMP in Capan-2/R and Panc-1/R cells which were treated with gemcitabine. b MiR-541 enhanced the release of cytochrome c and AIF in Capan-2/R and Panc-1/R cells which were treated with gemcitabine. c MiR-541 enhanced the cleavage of caspase-9 and caspase-3 in Capan-2/R and Panc-1/R cells which were treated with gemcitabine. d MiR-541 enhanced the gemcitabine-induced apoptosis of Capan-2/R and Panc-1/R cells. * $P < 0.05$ vs. NCO group. # $P < 0.05$ vs. gemcitabine+NCO group.

Figure 6

Schema of the predicted mechanisms by which miR-541 partially reversed the gemcitabine resistance of PCa. Overexpression of miR-541 inhibited the expression of HAX-1 and thus facilitate the collapse of mitochondria induced by gemcitabine. As a result, cytochrome c and AIF which are potent apoptosis inducers were released into the cytosol. These apoptosis inducers triggered caspases and the following apoptosis of gemcitabine-resistant PCa cells.

Supplementary Files

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