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ARID1A promotes chemosensitivity to gemcitabine in pancreatic cancer through epigenetic silencing of RRM2

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Received September 10, 2021, accepted November 5, 2021

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Pharmazie 77: 224-229 (2022) *doi: 10.1691/ph.2022.1881*

Pancreatic cancer is one of the most common malignancies with very poor prognosis due to its broad resistance to chemotherapy. ARID1A, a subunit of SWI/SNF complex, is involved in pancreatic carcinogenesis through epigenetic silencing of oncogenes. In this study, we aimed to explore whether ARID1A was implicated in the gemcitabine resistance in pancreatic cancer patients via regulating RRM2. We examined the effect of ARID1A depletion on the gemcitabine sensitivity in pancreatic cancer cells and explored the role of RRM2 in ARID1A-mediated pancreatic cancer cells chemosensitivity to gemcitabine. We found that Knockout of ARID1A led to gemcitabine resistance in pancreatic cancer cells, effect of which could be reversed by RRM2, a gemcitabine resistance related gene. ARID1A decreased the transcription of RRM2, and directly bound to the promoter of RRM2. Moreover, expression of RRM2 was negatively correlated with ARID1A in pancreatic cancer tissues. Thus, ARID1A-mediated RRM2 epigenetic suppression is crucial for enhancement of pancreatic cancer chemosensitivity to gemcitabine, and ARID1A could be used as a biomarker to guide the gemcitabine chemotherapy of pancreatic cancer.

1. Introduction

Pancreatic cancer is one of the leading causes of cancer-related mortality with a five-year survival rate of less than 6% (Bray et al. 2018). The majority of patients were diagnosed with unresectable or metastatic pancreatic cancer due to its nonspecific symptoms and the lack of reliable screening methods for early detection (Neuzillet et al. 2015). At present, gemcitabine-based chemotherapy remains the first-line treatment for patients with advanced stage of pancreatic cancer (Zhang et al. 2018). Nonetheless, the inherent or acquired resistance to gemcitabine is a major cause of treatment failure in pancreatic cancer (de Sousa et al. 2014). Therefore, a better understanding of the resistance mechanism of pancreatic cancer against gemcitabine is of paramount importance. It has been shown that the inherent or acquired resistance to gemcitabine occurs mainly through alteration of gemcitabine metabolism, most notably via upregulation of the small subunit of ribonucleotide reductase, RRM2 (Zhou et al. 2013). Ribonucleotide reductase (RR), consisting of M1 and M2 subunits, is a rate-limiting enzyme that catalyzes the conversion of ribonucleotides to deoxyribonucleotides, which is essential for DNA replication and repair. A body of evidence indicates that RR plays a positive role in tumor growth, metastasis, and drug resistance, including the resistance to gemcitabine (Vena et al. 2015; Xia et al. 2017). Clinical studies have shown that overexpression of either RRM1 or RRM2 in pancreatic cancer was associated with poor response to gemcitabine (Fujita et al. 2010; Nakano et al. 2007). In addition, *in vitro* studies have shown that upregulation of RRM2 increased drug resistance and invasive potential, whereas RRM2 suppression reversed drug resistance and decreased proliferation in cultured pancreatic cancer cells (Duxbury et al. 2004). Accumulating data has shown that the dysregulation of RRM2 in chemoresistant cells was caused by gene amplification, transcriptional activation, and perhaps other unidentified mechanisms (Zhou et al. 2001).
ARID1A is a subunit of the SWI/SNF complex, which is involved in remodeling chromatin through mobilizing nucleosomes in an ATP-dependent manner. ARID1A has been generally considered as a tumor suppressor gene because widespread loss-of-function mutations were found in multiple cancer types and the down-regulation of ARID1A was related to poor prognosis of several types of cancer (Huang et al. 2018; Sun et al. 2017; Mathur et al. 2017). Recently, ARID1A has been demonstrated to be involved in pancreatic carcinogenesis, whose genomic deletions, mutations, and rearrangements have been frequently detected in pancreatic cancer patients (Roy et al. 2018; Jäkel et al. 2017; Witkiewicz et al. 2015), and a recent study has shown that ARID1A restrains pancreatic neoplasia formation in a mouse model (Kimura et al. 2018). In addition, previous studies have reported that ARID1A loss contributes to multiple drug resistance of ovarian cancer through transcriptional activation of MRP2 and that ARID1A mutation is associated with a poor prognosis in biliary tract cancer patients treated with gemcitabine (Ahn et al. 2016; Luo et al. 2018). However, the association between ARID1A expression and the response of pancreatic cancer cells to gemcitabine is elusive and needs to be further investigated.
In the present study, we explored the ARID1A expression in pancreatic cancer and analyzed the association between ARID1A level and gemcitabine resistance. Moreover, we investigated the potential role of ARID1A in regulating gemcitabine sensitivity and found that ARID1A enhances pancreatic cancer cells response to gemcitabine by suppressing the transcription of RRM2.

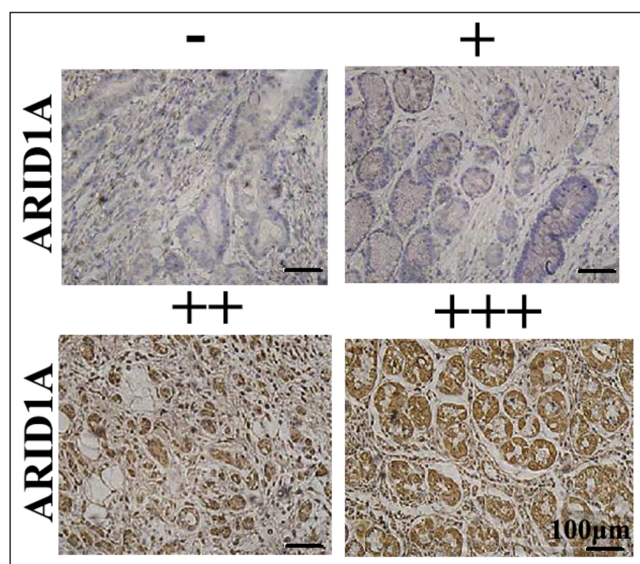
2. Investigations and results

2.1. *ARID1A level is related to gemcitabine sensitivity in pancreatic cancer*

To investigate the association between ARID1A expression and gemcitabine resistance in pancreatic cancer patients, we performed immunohistochemical analysis on 95 human pancreatic cancer specimens. General clinical information for patients is shown in Table1. Representative immunostaining for differential expres-

Table 1: General information of pancreatic cancer patients (n=95)

Category	n(%)
Gender	
Male	49 (51.6)
Female	46(48.4)
Age (years)	
Average	63.2±11.4
Range	36-84
Gemcitabine treatment	
Yes	56 (58.9)
No	39 (41.1)
Pathology stages	
	8 (8.4)
	72 (75.8)
III	15 (15.8)
Grade	
High/moderate	50(52.6)
Low	45(47.4)
Vascular invasion	
No	78(82.1)
Yes	17(17.9)
Lymph node status	
Negative	39(41.1)
Positive	56(58.9)



SUPPLEMENTARY Fig.1: Representative images of ARID1A expression in paraffin-embedded tissues from 95 patients with pancreatic cancer [-, +, ++, +++].

sions of ARID1A in pancreatic cancer samples were shown in Fig. S1. The expressions of ARID1A in most pancreatic cancer patients were low with negative/weak ARID1A staining, whereas high ARID1A expressions were only found in 37 patients with middle/strong staining. In patients with high ARID1A expressions, those treated with gemcitabine had a markedly longer survival time than those without gemcitabine treatment (shown in Fig.1A). In contrast, among patients with low ARID1A expression, the survival time in those treated with gemcitabine was similar to those without gemcitabine treatment (shown in Fig.1B). In patients who received gemcitabine treatment, those with low ARID1A expression had a significantly shorter overall survival time than those with high ARID1A expression, but among patients not treated with gemcitabine, there was no significant difference in survival time between the two groups (shown in Fig.1C and 1D).

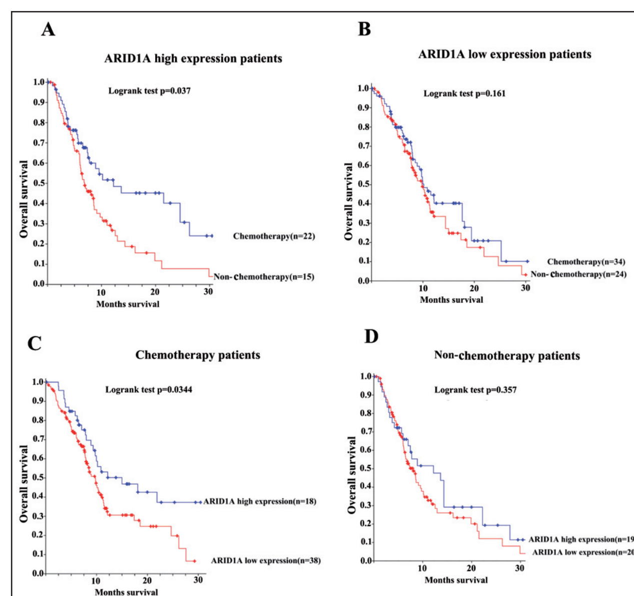


Fig. 1: ARID1A level is correlated with pancreatic cancer response to gemcitabine. (A) Gemcitabine improves the survival of patients with high expression of ARID1A. (B) Gemcitabine does not affect survival time of patients with low expression of AIRD1A. (C) expression of ARID1A affects the survival time of patients treated with gemcitabine. (D) Survival analysis of patients with high or low expression of ARID1A, who did not receive gemcitabine treatment. * P<0.05, ** P<0.01 compared with control group.

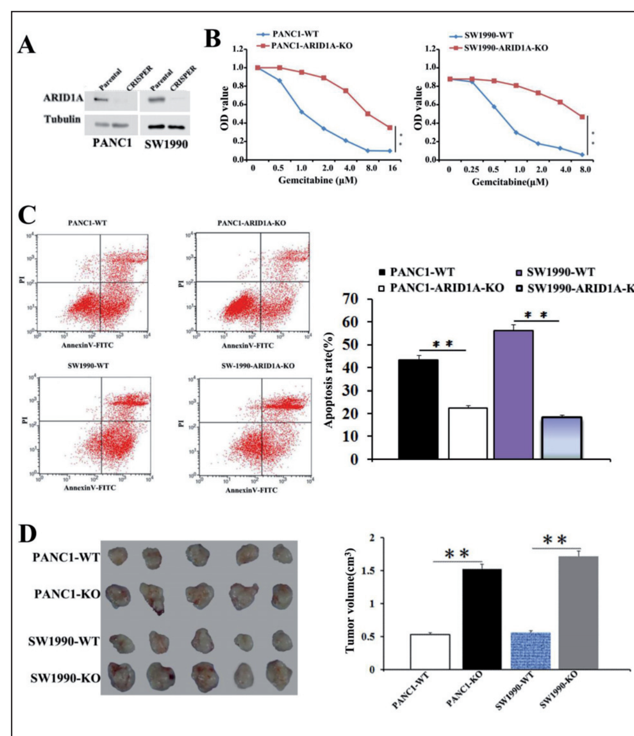


Fig. 2: ARID1A knockout increased the chemoresistance in pancreatic cancer cells to gemcitabine *in vitro* and *in vivo*. (A) Western blotting analysis of ARID1A expression in PANC1-WT/KO and SW1990-WT/KO cells. (B) MTT assay to determine the viability of indicated pancreatic cancer cells treated with various concentrations of gemcitabine for 48h. (C) Flow cytometric analysis to determine the apoptosis rate of indicated pancreatic cancer cells treated with 2μM gemcitabine for 24h. (D) Tumor size and volumes were shown after 28 days of gemcitabine treatment.

2.2. *Depletion of ARID1A enhances the chemoresistance in pancreatic cancer cells to gemcitabine in vitro and in vivo*

To explore whether ARID1A is involved in the response of pancreatic cancer cells to gemcitabine, we established ARID1A knockout human pancreatic cancer cell lines PANC1-KO and SW1990-KO using the CRISP/Cas9 technology, both of which cell lines lack any detectable ARID1A protein expression by western blot (shown in Fig.2A). Firstly, we performed a cell viability assay to explore the effects of ARID1A depletion on the chemosensitivity of pancreatic cancer cells to gemcitabine. We found that ARID1A knockout significantly reduced the chemosensitivity to gemcitabine both in PANC-1 and SW1990 cells at 48h (shown in Fig.2B). Secondly, we investigated gemcitabine-induced apoptosis. The flow cytometric results showed that the apoptosis induced by gemcitabine was significantly decreased in ARID1A-KO cells (shown in Fig.2C).

To analyze the effect of ARID1A on pancreatic cancer response to gemcitabine *in vivo*, we inoculated wild type pancreatic cancer cells (PANC1-WT/SW1990-WT) or ARID1A knockout pancreatic cancer cells (PANC1-KO/SW1990-KO) into nude mice, respectively, and treated mice with gemcitabine when tumors reached a volume of ~50mm^3. As shown in Fig.2D, the tumors from ARID1A knockout pancreatic cancer cells displayed a significantly larger mass than those from the corresponding wild type pancreatic cancer cells. Moreover, we performed IHC assays on the xenograft tumor for proliferation index and we found that ki67 expression level was significantly higher in tumors from ARID1A knockout pancreatic cancer cells than in control cell–derived tumors (shown in Fig.3). Taken together, these results indicated that knockout ARID1A can promote the resistance in pancreatic cancer cells to gemcitabine *in vitro* and *in vivo*.

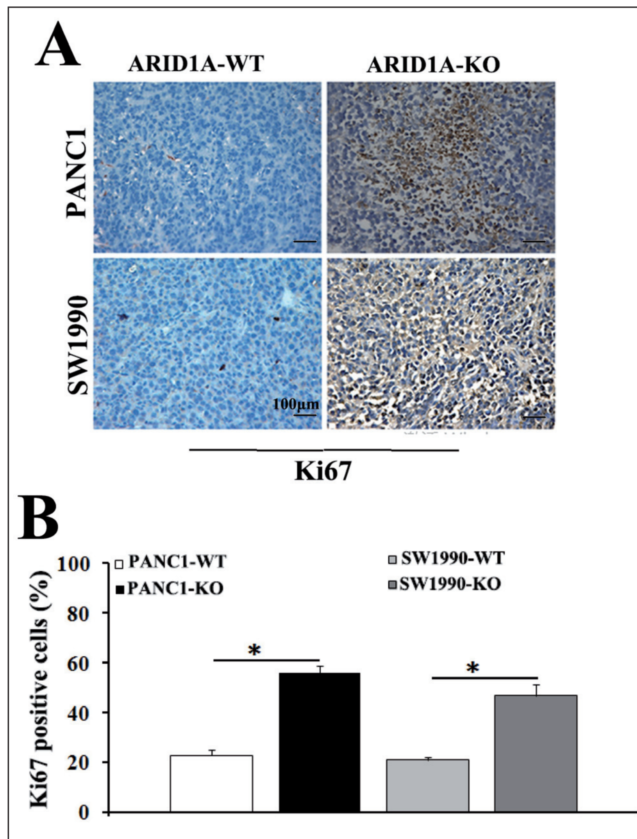


Fig. 3: Representative images of IHC staining of Ki-67 in the tumor tissue from wild type pancreatic cancer cells or ARID1A knockout pancreatic cancer cells(A), and the figure showing the quantitative analysis(B). * P<0.05

2.3. *RRM2 is a novel target of ARID1A*

As we found above that ARID1A enhanced gemcitabine sensitivity in pancreatic cancer and previous reports have shown that ARID1A suppresses carcinogenesis mainly through epigenetic silencing of oncogenes, we speculated that ARID1A may increase gemcitabine sensitivity in pancreatic cancer through inhibition of gemcitabine resistance associated genes. To identify which gemcitabine resistance-associated genes were targeted by ARID1A, we compared the expression levels of hENT1, CDA, 5′-NT, RRM1, RRM2, TS, ERCC1, MRPs and FASN in wild-type PANC1 and SW1990 cells with those expressed in corresponding ARID1A knockout cells. We found that only RRM2 expression was markedly upregulated both in ARID1A knockout PANC-1 and SW1990 cells compared with their parental cells (shown in Fig.4A and 4B). We further found that RRM2 was markedly downregulated in ARID1A knockout pancreatic cancer cells when restoration of ARID1A (shown in Fig.5D), indicating that RRM2 might be a potential downstream gene of ARID1A in pancreatic cancer cells. To validate whether RRM2 is directly regulated by ARID1A, we investigated whether ARID1A directly binds to RRM2 promoter via chromatin immunoprecipitation. In line with a previous study which indicated that there is an ARID1A peak enriched on the promoter of RRM2(Raab et al. 2015), we found that ARID1A was enriched on RRM2 promoter region (shown in Fig.4C), suggesting that RRM2 is a novel target directly regulated by ARID1A.

To explore whether the above results are also clinically relevant, we analyzed the relation of ARID1A and RRM2 in pancreatic cancer samples *via* immunohistochemical staining. In line with results from *in vitro* cultured cells, the expression levels of RRM2 were negatively associated with that of ARID1A expression in pancreatic cancer tissues (shown in Figs.4D and 4E).

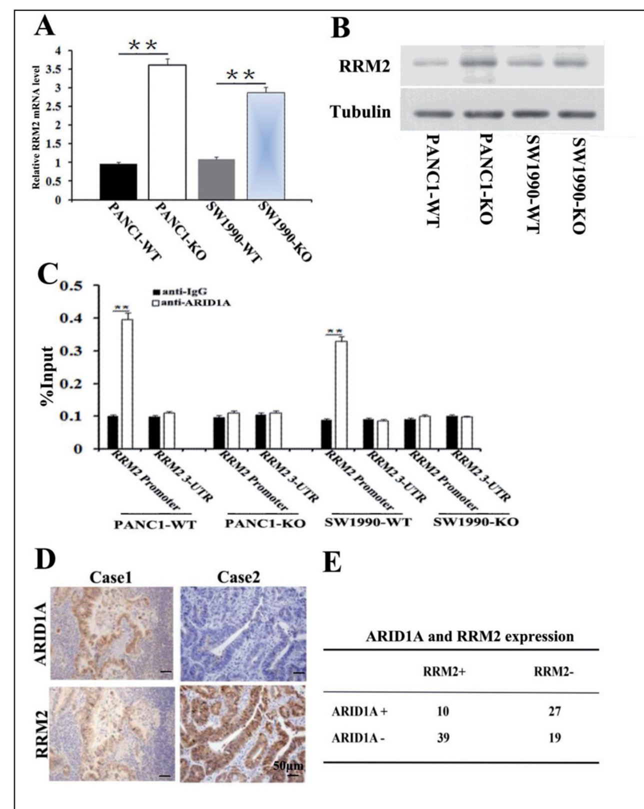


Fig. 4: RRM2 is a direct target of ARID1A. (A) RRM2 mRNA was determined by qRT-PCR in wild type PANC1/SW1990 cells and in ARID1A knockout PANC1/SW1990 cells. (B) the protein level of RRM2 in wild type PANC1/SW1990 cells and in ARID1A knockout PANC1/SW1990 cells. (C) The interaction between ARID1A and RRM2 promoter was examined by ChIP. (D) Level of ARID1A and RRM2 in pancreatic cancer tissues was examined by Immunohistochemistry. (E) The association between ARID1A and RRM2 was examined through Pearson'χ^2-test, p<0.01.

2.4. Enhancement of gemcitabine resistance in ARID1A-knockout pancreatic cancer is dependent on upregulating RRM2

To further explore whether RRM2 was responsible for the ARID1A-attenuated gemcitabine resistance in pancreatic cancer cells, we first knocked down RRM2 by its specific siRNA in ARID1A knockout PANC1 and SW1990 cells, and found that downregulation of RRM2 significantly reversed the gemcitabine resistance (shown in Figs. 5A, 5B and 5C). Then, ARID1A knockout pancreatic cells were overexpressed ARID1A alone or overexpressed ARID1A and RRM2 simultaneously. Overexpression of ARID1A significantly reduced the expression of RRM2 and increased the gemcitabine chemosensitivity in pancreatic cancer cells. More important, the reversion of gemcitabine resistance in ARID1A KO pancreatic cancer cells by restoring ARID1A expression was impaired by upregulating RRM2 with RRM2 cDNA transfection (shown in Figs. 5D, 5E and 5F). Thus, RRM2 mediates the effects of ARID1A on the chemosensitivity in pancreatic cancer cells to gemcitabine.

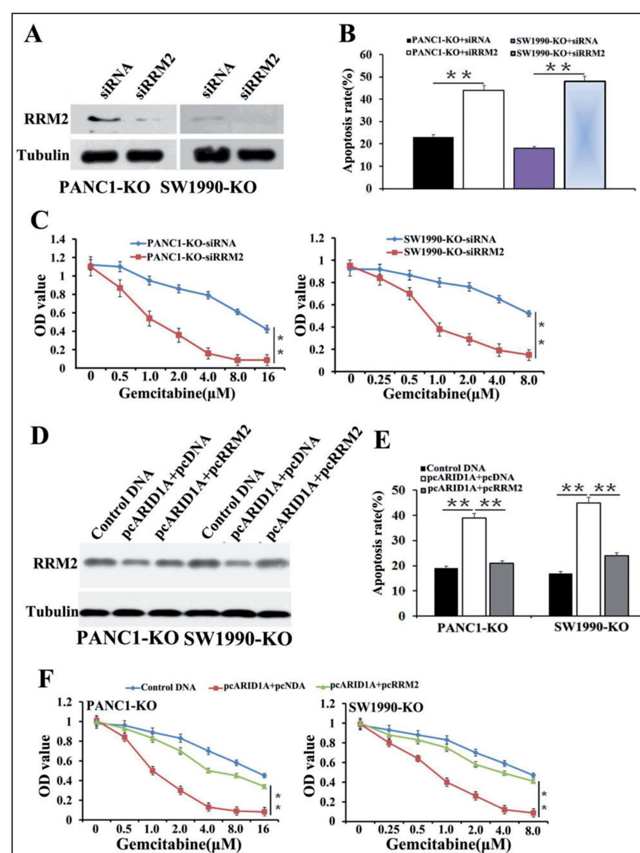


Fig. 5: RRM2 is involved in ARID1A mediated chemosensitivity of pancreatic cancer cells to gemcitabine. (A) RRM2 was determined by Western blotting in PANC1-KO and SW1990-KO cells transfected with RRM2 specific siRNAs and control siRNAs, respectively. (B) Apoptosis rate was determined by flow cytometry to test the effect of downregulating RRM2 on PANC1-KO and SW1990-KO cells response to 200nM gemcitabine treatment for 24h. (C) Cellular viability was determined by MTT assay to test the effect of downregulating RRM2 on PANC1-KO and SW1990-KO cells response to various concentrations of gemcitabine for 48h. (D) Western blotting analysis of ARID1A and RRM2 expression in PANC1-KO and SW1990-KO cells , which were transfected with ARID1A expression vector alone or cotransfected with ARID1A and RRM2 expression vector. (E) PANC1-KO and SW1990-KO cells, which were transfected with ARID1A expression vector alone or cotransfected with ARID1A and RRM2 expression vector, were treated with 2μM gemcitabine for 24h. Flow cytometry analysis of the cell apoptosis rate. (F) PANC1-KO and SW1990-KO cells, which were transfected with ARID1A expression vector alone or cotransfected with ARID1A and RRM2 expression vector, were treated with various concentrations of gemcitabine for 48h. MTT assay analysis of cellular viability.

3. Discussion

Mutation and/or loss of ARID1A have been involved in pancreatic cancer development and progression, and are associated with poorer prognosis in pancreatic cancer. One of the main reasons for the poor prognosis of pancreatic cancer is resistance to the mainline chemotherapeutic drug, gemcitabine. Ahn et al. (2016) found that mutation of ARID1A is related to the poor prognosis in biliary tract cancer patients treated with gemcitabine, suggesting that ARID1A may be involved in regulating the chemosensitivity of tumor cells to gemcitabine. In this study, we explored the relationship between ARID1A level and chemotherapy sensitivity of pancreatic cancer to gemcitabine. Our survival analysis for the first time showed that pancreatic cancer patients with high ARID1A expression were more sensitive to gemcitabine chemotherapy than those with low ARID1A expression, suggesting that ARID1A might be involved in pancreatic cancer sensitivity to gemcitabine. To further explore the effect of ARID1A on gemcitabine response, we established ARID1A knockout pancreatic cancer cell lines and found that loss of ARID1A in pancreatic cancer cells could decrease their chemosensitivity to gemcitabine *in vitro* and *in vivo*. Gemcitabine-based chemotherapy is a main therapeutic approach for pancreatic cancer, which has improved the survival of pancreatic cancer patients, however, the improvement in prognosis of pancreatic cancer patients remains poor, due to majority of pancreatic cancer patients eventually developing resistance to gemcitabine. It has been shown that the response of pancreatic cancer to gemcitabine can be affected by the expression level of genes involved in gemcitabine metabolism (Amrutkar et al. 2017; de Sousa et al. 2014). hENT1, hCNT1, and hCNT3 are involved in gemcitabine taken up into cells, whose expression levels are related to gemcitabine sensitivity and better patient prognosis, and dCK is involved in the activation of gemcitabine in the cells. Thus, these genes positively contribute to gemcitabine activity and enhance the chemosensitivity of pancreatic cancer cells to gemcitabine, whereas upregulation of RRM1, RRM2, CDA, and 5′-NT contributes to gemcitabine resistance. In this study, we found that the expression of a gemcitabine resistance related gene, RRM2, increased in ARID1A knockout pancreatic cancer cells and decreased in these cells after restoring ARID1A expression. Immunohistochemical analysis further indicated that the expression of ARID1A was negatively associated with RRM2 in pancreatic cancer tissues. Moreover, our ChIP analysis for ARID1A has shown that ARID1A indeed bound to RRM2 promoter region, indicating that RRM2 is a potential target directly inhibited by ARID1A in pancreatic cancer. Our biological function studies indicated that the chemosensitivity of pancreatic cancer cells to gemcitabine enhanced by ARID1A was dependent on suppressing the expression of RRM2, which is consistent with a previous study indicating that downregulation of RRM2 increased chemosensitivity to gemcitabine in pancreatic cancer cells (Duxbury et al. 2004).

In summary, ARID1A functions as a gemcitabine-sensitive factor and loss of ARID1A promotes gemcitabine resistance in pancreatic cancer by upregulation of RRM2. Therefore, ARID1A may be used as a biomarker to guide gemcitabine against pancreatic cancer.

4. Experimental

4.1. Clinical specimens and cell lines

95 Pancreatic ductal adenocarcinoma samples were collected from surgical specimens in Wuhan Tumor Hospital, China, during January 2014 to December 2016. This study was approved by the Medical Ethics Review Committee of Wuhan Tumor Hospital on human subject research (No.WHTH2018-1035) and conducted according to the ethical guidelines of the Declaration of Helsinki. All patients involved in the study have signed the informed consent. A portion of pancreatic cancer patients (56/95) received gemcitabine-based monotherapy after resection for pancreatic ductal adenocarcinoma. The remaining portion of the pancreatic cancer patients (39/95) did not undergo any chemotherapy or radiotherapy after surgery due to frailty (N = 18) or to non-medical reasons (N = 21). All cases were followed up from the date of surgery to January 2020.

Human PANC1 and SW1990 pancreatic cancer cell lines were purchases from the American Type Culture Collection (Manassas, VA, USA). These cells were cultured at 37°C with 5% CO_2 according to the provider's instructions.

4.2. Establishment of ARID1A knockout pancreatic cells

ARID1A gene was knocked out in PANC-1 and SW1990 cells with CRISPR/Cas9 system as described previously (Liu et al. 2019). Briefly, a human ARID1A gRNA sequence (5′-CGGACCTGAAGAACTCGAAC-3′) or a control gRNA sequence (5′-ACGGAGGCTAAGCGTCGCAA-3′) was subcloned into the pGEM-T Easy vector (Promega, Madison, WI, U.S.A.). Then, pancreatic cancer cells were cotransfected with vector containing ARID1A gRNA or control gRNA, plasmid carrying the human codon-optimized Cas9, and the enhanced GFP (eGFP) plasmid. After 24 hours, FACS was used to isolated GFP-positive cells which were then subjected to cloning by limiting dilution. 10–14 days later, Clonal populations with the loss of ARID1A expression were screened through immunoblot.

4.3. Immunohistochemistry (IHC)

Immunohistochemical staining was performed as previously described (Yano et al. 2019; Wang et al. 2014) with antibodies against ARID1A (CST, USA) and RRM2 (Abcam, UK). Expression of ARID1A and RRM2 were evaluated as described previously (Sun et al. 2019).

Cell viability assay: 5×10^3 pancreatic cancer cells were seeded onto 96-well plates. 24 hours later, the cells were incubated with the various reagents for 48h. A 10% 5mg/ml solution of MTT agent (Sigma-Aldrich) was added for 2 hours. The medium was then removed and the cells were dissolved in DMSO (Sigma-Aldrich). Relative cytotoxicity was determined by measuring the absorbance at 570 nm using a luminometer (Molecular Devices, USA). The experiments were repeated three times.

4.4. Apoptosis assay

Cells with various treatments were analyzed by FACSCalibur flow cytometer following stained with fluorescein isothiocyanate-conjugated annexin V and propidium iodide (BD Bioscience) according to the manufacturer’s protocol. The experiments were repeated three times.

4.5. RNA isolation and quantitative RT-PCR

Total RNA was isolated from cells using Trizol reagent (Invitrogen). cDNA was synthesized with 1μg of total RNA by miScript II RT Kit (QIAGEN). The Real-time PCR was performed with a PCR kit (SYBR Premix Ex Taq II Kit ,TaKaRa Biotechnology). Primer sequences were as follows: RRM2, 5’-TTACATAAAAGATCCCAAAGAAAGG-3’ and 5’-AGCCTCTTTGTCCCCAATC-3’; and β-actin, 5’-GATCATTGCTCCTCCT-GAGC-3’ and 5’-ACTCCTGCTTGCTGATCCAC-3’. The relative expression levels of mRNAs were normalized to those for the control using the $2^{-\Delta\Delta CT}$method.

4.6. Chromatin immunoprecipitation (ChIP)

ChIP was conducted by a ChIP-IT Expression kit (Qiagen) according to the manufacture’ instruction. Immunoprecipitations were carried out with an anti-ARID1A (Santa Cruz) antibody. Precipitated DNA was analyzed by quantitative PCR with two pairs of primers for position of the RRM2 promoter region. The experiments were repeated three times.

4.7. Western blotting

Total protein was isolated by lysis buffer (Beyotime). Equal amounts of total protein from various treated cells were separated by SDS-PAGE and transferred onto PVDF membranes (Pierce, Biotechnology, USA). Membranes were immunoblotted by specific primary antibodies followed by secondary antibodies. The protein bands were visualized using ECL detection reagents (Thermo Fisher Scientific, USA).

4.8. Animal experiments

All animal experiments were approved by the Animal Care and Use Committee of Wuhan Tumor Hospital (ACU20180871) and performed in accordance with the care and use of laboratory animals and the Equator network guidelines in caring for experimental animal, and we made efforts to decrease the number of animals used and minimize their suffering according to Guide for the Care and Use of Laboratory Animals (8th edition. Washington (DC): National Academies Press (US), 2011.). Pancreatic cancer cells (5×10^6 cells per mouse) were injected subcutaneously into the right flank of 6-week-old athymic BALB/c mice (n = 5 per group, Slac Laboratory Animal Co. Ltd, Shanghai, China). The tumor size was monitored twice a week with calipers, and the tumor volume was calculated using the formula: Volume (mm^3) = length × width^2 × 0.5. When the tumors were ~50mm^3 in volume, gemcitabine of 75 mg/kg was administered by intraperitoneal injection every other day (Yu et al. 2018). The tumor-bearing mice were euthanized on the twenty-eighth day after gemcitabine treatment, and the primary tumor was excised, formalin-fixed, and paraffin embedded.

4.9. Statistical analysis

Statistical analysis was performed using SPSS (version 19) and GraphPad PRISM 6 software. Statistical methods included Pearson chi-square test, two-tailed Student’s t-test, and Kaplan-Meier survival analysis. All statistical tests included a two-way analysis of variance. Statistical significance was assumed when $P < 0.05$.

Acknowledgments: We appreciate all the contributors during our study.

Conflict of interest: None declared.

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