Cancer associated fibroblasts (CAFs) derived exosomes with A promoted liver metastasis of pancreatic cancer by regulating C through transcription factor B

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Abstract

Cancer-associated fibroblasts (CAFs) comprise the majority of the tumor bulk of pancreatic ductal adenocarcinomas (PDACs). Despite the long-standing recognition of the prominence of CAFs in PDAC, the effect of extracellular vesicles derived from CAFs on the liver metastasis of PDACs cells is not well characterized. The aim of this study was to determine whether specific derived from CAFs might be involved in liver metastasis in PDACs. We found that CAFs are intrinsically related with liver metastasis and thus the potential role of CAFs in PDACs was highlighted. Further, CAFs significantly increase the release of extracellular vesicles wit A and it caused increased liver metastasis. Advanced experiments demonstrated that, C, in recipient epithelial cells and promote proliferation, was up-regulated by A in EVs from CAFs. Besides, treatment of an inhibitor of A as well as an inhibitor of exosome release, GW4869, significantly reduces the activation of B, thus decreased the activity of C. Collectively, these findings show the potential for exosome inhibitors as treatment options for overcoming PDAC liver metastasis.

Introduction

Pancreatic cancer is a frequent lethal cancers and reported as the leading cause of cancer death worldwide [1]. During the last several decades, great improvement of the surgical resection and chemo-radiotherapy was made in pancreatic cancer. However, the high mortality rate has not been improved significantly with poor survival of pancreatic cancer patients [2,3]. Therefore, it is urgent for the further clarify the underlying mechanisms and identification therapeutic targets in pancreatic cancer.

Long non-coding RNAs (lncRNAs), a class of ~200bp noncoding RNAs [4], are poorly conserved and are involved in a various biological processes including chromatin modification, epigenetic changes, transcription and post-transcriptional processing[5] . Accumulating studies revealed lncRNAs as an oncogene or tumor suppressor, playing crucial role in tumourigenesis[6]. Recent studies demonstrate lncRNAs as a competing endogenous RNA (ceRNA), involved tumor progression by interacting with miRNAs[7,8].

SPRY4-IT1 (LncRNA SPRY4 intronic transcript 1) was reported as an oncogene in various cancers, including squamous cell carcinoma, prostate cancer, colorectal cancer, melanoma, glioma and bladder cancer[9,10,11,12,13,14]. Khaitan et al. showed that the upregulated SPRY4-IT1 was involved in the apoptosis and invasion of melanoma [15]. SPRY4-IT1 also reported to promote tumor progression by targeting epithelial-mesenchymal transition in various cancers including esophageal squamous cell carcinoma, non-small-cell lung cancer and colorectal cancer [16,17,18] However, the expression and underlying molecular mechanism of SPRY4-IT1 in pancreatic cancer remains poorly understood.

In present study, we attempted to explore the potential role of SPRY4-IT1 in pancreatic cancer. We found that SPRY4-IT1 expression was upregulation in pancreatic cancer and associated with worse outcome for pancreatic cancer patients.Moreover, SPRY4-IT1 was involved the cell proliferation, migration and invasion by targeting miR-138-5p. Finally, EZH2 as a downstream gene and SPRY4-IT1-mediated miR-

138-5p/ EZH2 axis involved the EMT of pancreatic cancer. In conclusion, our results revealed the role of SPRY4-IT1/miR-138-5p/ EZH2 axis in pancreatic cancer, providing novel insights into underlining therapeutic target of pancreatic cancer.

Materials And Methods

Patients sample

46 pairs of primary pancreatic cancer and adjacent non-tumor tissues from patients were obtained from The First Affiliated Hospital of Dalian Medical University from 2017 to 2020. All specimens were frozen at 80℃ immediately after surgery. Patients recruited to this study did not receive any pre-operative treatments. The clinical characteristics of all the patients were summarized in Table 1.

Cell culture and and transfection

The human pancreatic cancer cell lines (PANC-1, SW1990, BxPC-3 and AsPC-1) and human pancreatic duct epithelial cell lines (HPDE6-C7) line were purchased from the Cell Bank Chinese Academy of Sciences (Shanghai, China). The cells were cultured in DMEM (Gibco BRL, Grand Island, NY, USA), with 10% fetal bovine serum (FBS, Gibic o BRL, Grand Island, NY, USA) and 100 ug/ml penicillin/streptomycin at 37°C with 5% CO_2.

The SPRY4-IT1 plasmid and SPRY4-IT1 siRNA were used to overexpress or knockdown SPRY4-IT1 in pancreatic cancer cells. At 24 h post transfection, the expression of SPRY4-IT1 was detected by qPCR.

RNA extraction and Quantitative real-time PCR (qRT-PCR)

Total RNA was collected from pancreatic cancer tissues or cells using Trizol (Invitrogen, CA, USA). Next, we used the Taqman™ microRNA reverse transcription kit to synthesis cDNA from RNA. Finally, qPCR analysis was used to detect miRNA expression using TaqMan™ MicroRNA Assay kit (Applied Biosystems, USA). The relative expression was present as fold changes by the comparative Ct ($^{\Delta\Delta}$Ct) method.

MTT assay

The cell viability was detected by MTT assay using the MTT Reagent (Roche Applied Science) at 6, 24, 48 and 72 h.

Invasion and migration assay

The cell invasion capability was detected using transwell invasion assay. The ell migration capability was detected using wound healing assay as previous work[19].

Western blot assay

Total protein was obtain from pancreatic cancer tissue or cells using RIPA and then separated by 10% SDS gels. The anti-EZH2 (E7031, sigma) or anti-GAPDH (G9545, sigma) antibodies was used to detected EZH2 and GAPDH expression.

Luciferase Reporter Assays

The human EZH2 3'-UTR fragment was subcloned into the pGL3 plasmid (Promega). And the putative binding sites for miR-138-5p was mutated in pGL3- EZH2-mut vector. Luciferase activity was detected using the Dual-Luciferase Reporter Assay System (Promega).

Tumor xenograft model and tumorigenicity assay

AsPC-1 cells stably transfected with sh-SPRY4-IT1 or sh-control vector were subcutaneously injected into 4-week-old female nude mice with $2*10^6$ cells. Tumor volume and the tumor growth curves were calculated. The mice were sacrificed after 5 weeks.

Statistical analysis

The Data analyzed using SPSS 17.0 (SPSS, Chicago, USA) and represented as mean ± standard deviation (SD). Statistical significance was assessed by T-test for two-group comparison. $P<0.05$ was considered as statistically significant.

Results

SPRY4-IT1 expression in pancreatic cancer tissue and cell lines

SPRY4-IT1 has been observed as oncogenic regulator, involving in cancer progression in several cancers. However, the expression and role of SPRY4-IT1 in pancreatic cancer remains unknown. Herein, we firstly detected SPRY4-IT1 expression in pancreatic cancer tissues and adjacent normal tissues by qRT-PCR. We found that SPRY4-IT1 expression was significantly upregulated in pancreatic cancer tissues compared with corresponding adjacent non-tumor tissues (Figure 1A). Moreover, SPRY4-IT1 expression in four human pancreatic cancer cell lines also upregulated compared with HPDE6- C7 cells (Figure 1B). Collectively, these results demonstrate that SPRY4-IT1 expression was involved in pancreatic cancer pathogenesis. Furthermore, we analyzed the relationship between SPRY4-IT1 expression and clinical characteristics in 46 pancreatic cancer patients (Table 1). Noticeably, SPRY4-IT1 expression was significantly associated with TNM stage and depth of invasion. Finally, we found that the lower expression of SPRY4-IT1 shown longer survival in patients with pancreatic cancer (Figure. 1C).

SPRY4-IT1 induces cell proliferation, migration, and invasion in pancreatic cancer

To further demonstrate the potential biological function of SPRY4-IT1 in pancreatic cancer development, we used siRNA to knockdown SPRY4-IT1 expression in AsPC-1 and BxPC-3 cells. As shown in Figure 2A, siRNA evidently decreased SPRY4-IT1 expression level in both two cells. MTT results revealed that the

silenced SPRY4-IT1 evidently suppressed the proliferation ability BxPC-3 and AsPC-1 cells (Figure 2B). Moreover, we found that silenced SPRY4-IT1 also evidently repressed the migration and invasion ability in both BxPC-3 and AsPC-1 cells (Figure 2C and D). These data indicated that SPRY4-IT1 as oncogene, plays important role on tumor progression of pancreatic cancer. Next, we used a xenograft nude mice model to reveal the effects of SPRY4-IT1 on pancreatic cancer growth. AsPC-1 cells transfected with silenced SPRY4-IT1 or shControl were subcutaneously injected into mice. As shown in Figure 2E, tumor growth was evidently inhibited by sh-SPRY4-IT1. Tumor grew significantly more slowly in mice with silenced SPRY4-IT1. Four weeks later, the mean tumor volume was evidently downregulated by sh-SPRY4-IT1. These results disclose that SPRY4-IT1 improved pancreatic cancer progression in vivo and vitro.

miR-138-5p expression was downregulated in pancreatic cancer tissues and negatively associated with SPRY4-IT1 expression

Recently, LncRNA was reported as a regulator of miRNA and interfere with miRNA pathways at posttranscriptional regulation. Firstly, we used the bioinformatics database (FINDTAR3, http://bio.sz.tsinghua.edu.cn/) to predict the target miRNA of SPRY4-IT1 and found that miR-138-5p has two putative binding sites with SPRY4-IT1 (Figure 3A). Previous studies showed that downregulated miR-138-5p was involved in the regulation of cell growth and autophagy in pancreatic cancer [20,21]. Consistent with previous studies, miR-138-5p was downregulated in pancreatic cancer tissues compared with corresponding adjacent non-tumor tissues (Figure 3B). Moreover, we found a significant upregulation of miR-138-5p in the silenced SPRY4-IT1 group compared with control group (Figure 3B). Then we found an inverse correlation between SPRY4-IT1 and miR-138-5p in pancreatic cancer(Figure 3C). Next, we investigated effects of SPRY4-IT1 on miR-138-5p expression levels. As shown in Figure 3D, the miR-138-5p expression level was evidently upregulated in SPRY4-IT1 siRNA group but evidently downregulated in SPRY4-IT1 vector compared with control cells. Finally, we used mimics to upregulate miR-138-5p expression in pancreatic cancer. Interestingly, miR-138-5p restoration abrogated the effects of SPRY4-IT1 on pancreatic cancer cells with reduced cell proliferation, migration and invasion process (Figure 3E, F and G). Taken together, these data demonstrated that SPRY4 -IT1 involves pancreatic cancer progression via regulating miR-138-5p expression.

SPRY4-IT1 regulates EZH2 expression via miR-138-5p

EZH2 function as a oncogene in various cancers [22,23,24]. Recent studies showed that LncRNA SPRY4-IT1 was related with EZH2 expression in several tumors progression[18,25,26] and EZH2 is highly expressed in pancreatic carcinoma. In this study, EZH2 was predicted as a direct target of miR-138-5p (Figure 4A). We used a luciferase reporter assay to detect the target role of miR-138-5p on the 3'-UTR of EZH2. As shown in Figure 4B, luciferase activity of wt EZH2 was significantly reduced by miR-138-5p mimics, but miR-138-5p did not affect the luciferase-reporter activity of mutated EZH2. Furthermore, miR-138-5p evidently repressed EZH2 expression in two pancreatic cancer cells (Figure 4C). Next, we investigated the effects of SPRY4-IT1 on the expression levels of EZH2 in pancreatic cancer. As shown in

Figure 4D, silenced SPRY4-IT1, evidently downregulated the expression of EZH2 mRNA and protein, while SPRY4-IT1 vector was significantly induced the expression of EZH2 mRNA and protein pancreatic cancer cells. In conclusion, these results indicated that SPRY4-IT1 promotes progression of pancreatic cancer via miR-138-5p/EZH2 axis.

Discussion

Accumulating studies suggested that lncRNAs are involved in tumor pathogenesis and progression, and may yield novel therapeutic concepts for the treatment of these tumors including pancreatic cancer[27,28,29]. Here, our studies showed that SPRY4-IT1 expression evidently upregulated in pancreatic cancer tissues and cells, moreover, the high expressed SPRY4-IT1 is closely associated with TNM stage and depth of invasion, and shorter overall survival. These data indicated SPRY4-IT1 as a novel biomarker of pancreatic cancer, indicating a therapeutic target for pancreatic cancer patients.

Accumulating studies indicates that lncRNAs SPRY4-IT1 dysregulated in severial tumors and involved tumor progression. Zhou et al. showed that SPRY4-IT1 promotes the progression of hepatocellular carcinoma [26]. Khaitan et al. showed that lncRNA SPRY4-IT1 regulates apoptosis and invasion of melanoma [15]. Zhang and Xie et al. have reported that SPRY4-IT1 was involved in the metastasis of esophageal squamous cell carcinoma and gastric cancer via inducing epithelial-mesenchymal transition [17,30]. SPRY4-IT1 was also reported to affect cell proliferation in bladder cancer and esophageal squamous cell carcinoma [13,31]. In present study, we found that silenced SPRY4-IT1 significantly suppressed the growth, migration and invasion of pancreatic cancer cells, indicating that SPRY4-IT1 involves pancreatic cancer progression.

Recently, studies have reported that lncRNAs modulated miRNA expression in tumor progression. For instance, Mei et al. showed that LncRNA GAS5 regulates the tumorigenesis of non-small cell lung cancer by inhibiting miR-23a [32]. Zhao et al. identified LncRNA Taurine-Upregulated Gene 1 as an oncogene by Inhibiting MicroRNA-9 in breast cancer[33]. Previous studies showed that miR-138-5p regulated cell growth and autophagy in pancreatic cancer [20,21]. Consistently, we show that miR-138-5p expression was regulated by SPRY4-IT1 and negatively associated with SPRY4-IT1 expression in pancreatic cancer. EZH2 function as oncogene in various cancers [22,23,24]. Recent studies showed that LncRNA SPRY4-IT1 was related with EZH2 expression in severial tumors progression [18,25,26] and EZH2 is highly expressed in pancreatic carcinoma. Here, these data showed that EZH2 is a direct target of miR-138-5p and SPRY4-IT1 function as oncogene through miR-138-5p/EZH2 axis in pancreatic cancer.

In conclusion, the present study showed that PRY4-IT1 was upregulated in pancreatic cancer. PRY4-IT1 involved pancreatic cancer progression through miR-138-5p/EZH2 axis, indicating that PRY4-IT1 may be used as a biomarker pancreatic cancer patients and sheds novel diagnostics and therapeutics for the pancreatic cancer.

Declarations

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JD and GT conducted the experiments. ZKL and HFL participated in the sequence alignment. ZN and CM participated in the design of the study and performed the statistical analysis. JD conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

The present study was approved by the Institutional Animal Care and Use Committee of The First Affiliated Hospital of Dalian Medical University.

Consent for publication

Conflict of interest

Guang Tan，Zhikun Lin，Haifeng Luo，Zhen Ning，Chi Ma，Jian Du declare that they have no conflict of interest.

Abbreviations

Long non-coding RNAs (lncRNAs); SPRY4-IT1 (LncRNA SPRY4 intronic transcript 1)

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Tables

Table 1

The relationship between SPRY4-IT1 expression and clinicopathological factors in 46 PC patients.

Clinicopathologic parameters	All cases	SPRY4-IT1 expression		P-value
		High	Low	
Age		36	10	
<60	28	22	6	0.514
≥60	18	14	4	
Gender				
Male	22	17	5	0.431
Female	24	19	5	
Tumor size(cm)				
≤3	27	21	6	0.674
>3	19	15	4	
TNM stage				
Stage I + II	29	25	4	0.0052*
Stage III+IV	17	11	6	
Lymph node Metastasis				

Figures

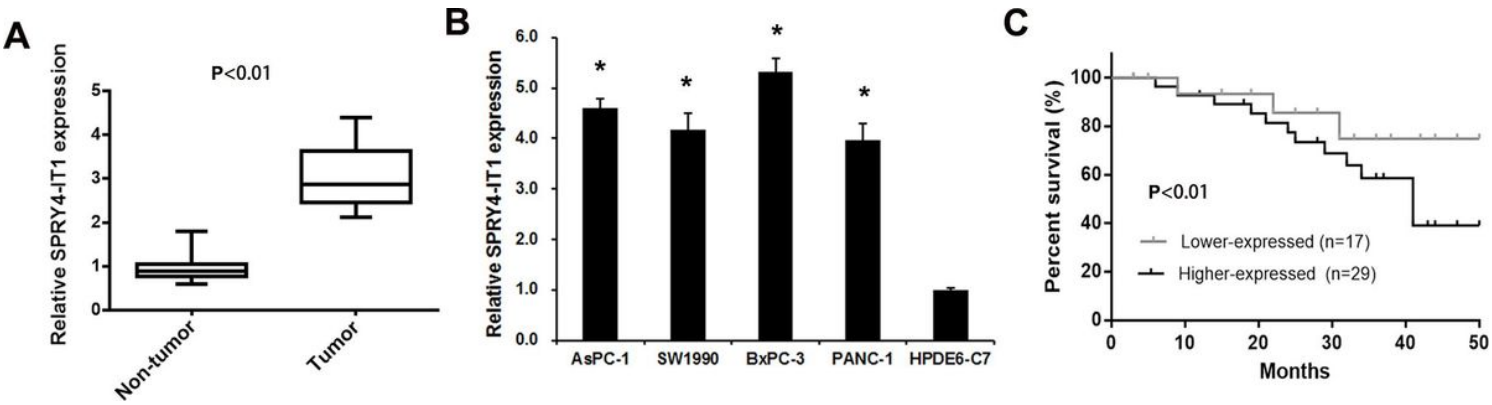

Figure 1

The expression levels of SPRY4-IT1 pancreatic cancer tissue and cell lines. (A) Expression level of SPRY4-IT1 in bladder cancer tissue and adjacent non-tumor tissues. (B) Expression level of SPRY4-IT1 in human pancreatic cancer cell lines (AsPC-1, SW1990, BxPC-3 and PANC-1) and human pancreatic duct epithelial cell lines (HPDE6-C7) line. * P< 0.05 compared with SV-HUC-1 cells. C, Kaplan-Meier curves for overall survival in patients with pancreatic cancer according to SPRY4-IT1 expression

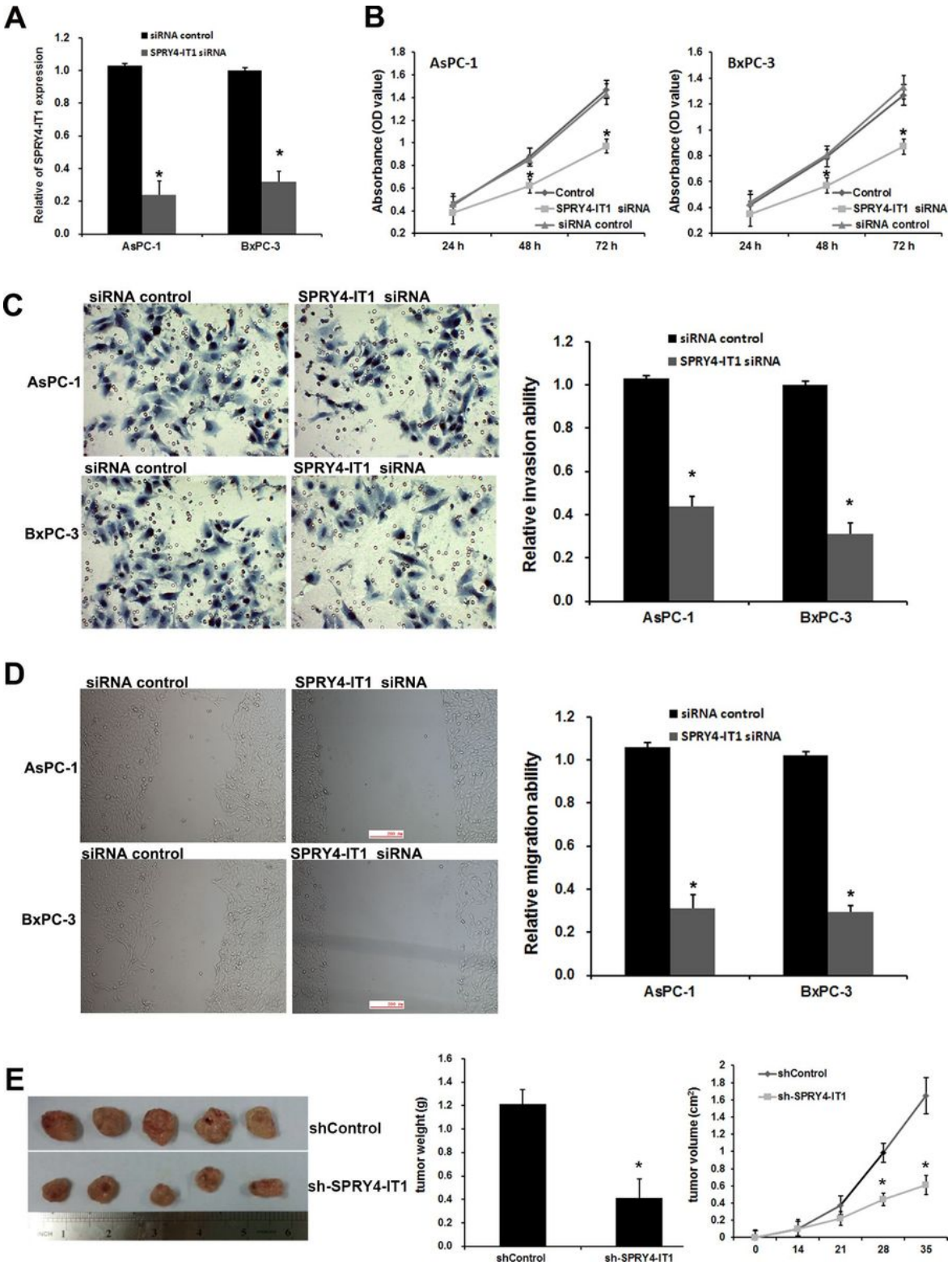

Figure 2

Knockdown of SPRY4-IT1 inhibits cell proliferation, migration, and invasion, and promotes apoptosis of bladder cancer cells in vitro. A, Silencing of SPRY4-IT1 with siRNAs in AsPC-1 and BxPC-3 cell lines. B, MTT assay revealed the effects of SPRY4-IT1 siRNAs on pancreatic cancer cell proliferation. C, Transwell invasion assay was used here to detected the effects of SPRY4-IT1 siRNAs on pancreatic cancer cell invasion capability. D, The wound healing assay was used here to detected the effects of SPRY4-IT1

siRNAs on pancreatic cancer cell migration capability. E, sh-SPRY4-IT1 inhibited tumorigenicity in vivo. Tumor volume and weight were calculated and all data are shown as mean ± SEM. *P < 0.05, ***P < 0.001.

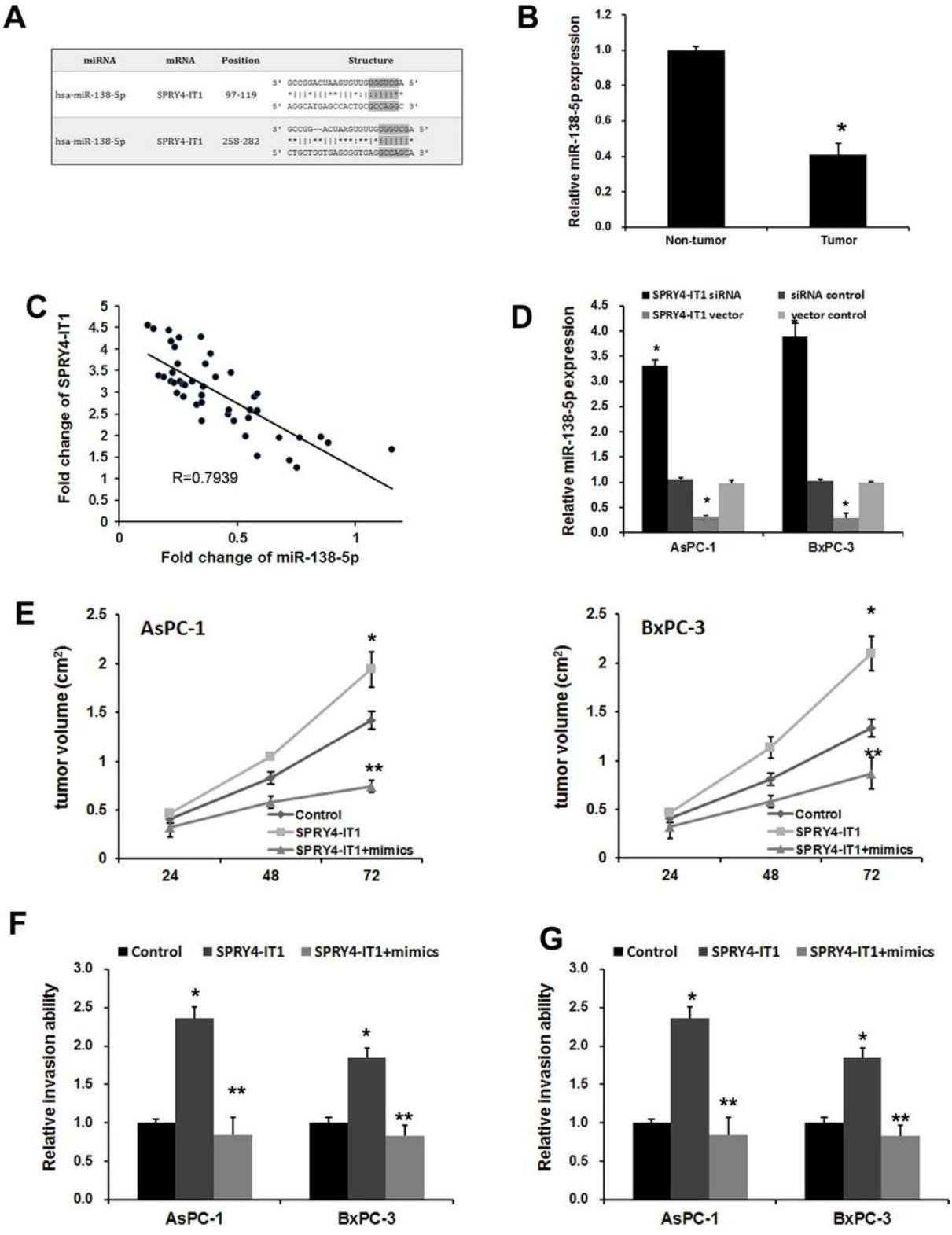

Figure 3

miR-138-5p as a downstream of SPRY4-IT1 was downregulated in pancreatic cancer. A, Putative binding sites of SPRY4-IT1 with miR-138-5p. B, miR-138-5p expression levels in pancreatic cancer. C, The correlateship between SPRY4-IT1 and miR-138-5p in pancreatic cancer. D, SPRY4-IT1 regulated miR-138-5p expression in pancreatic cancer cells. E,MTT assay revealed the effects of SPRY4-IT1 and miR-138-5p mimics on pancreatic cancer cell proliferation. F, Transwell invasion assay was used here to detected the effects of SPRY4-IT1 and miR-138-5p mimics on pancreatic cancer cell invasion capability. G, The wound healing assay was used here to detected the effects of SPRY4-IT1 and miR-138-5p mimics on pancreatic cancer cell migration capability All assays have been performed three times independently and data are presented as mean ± SEM. *p< 0.01 vs control group, **p < 0.01 vs SPRY4-IT1 group.

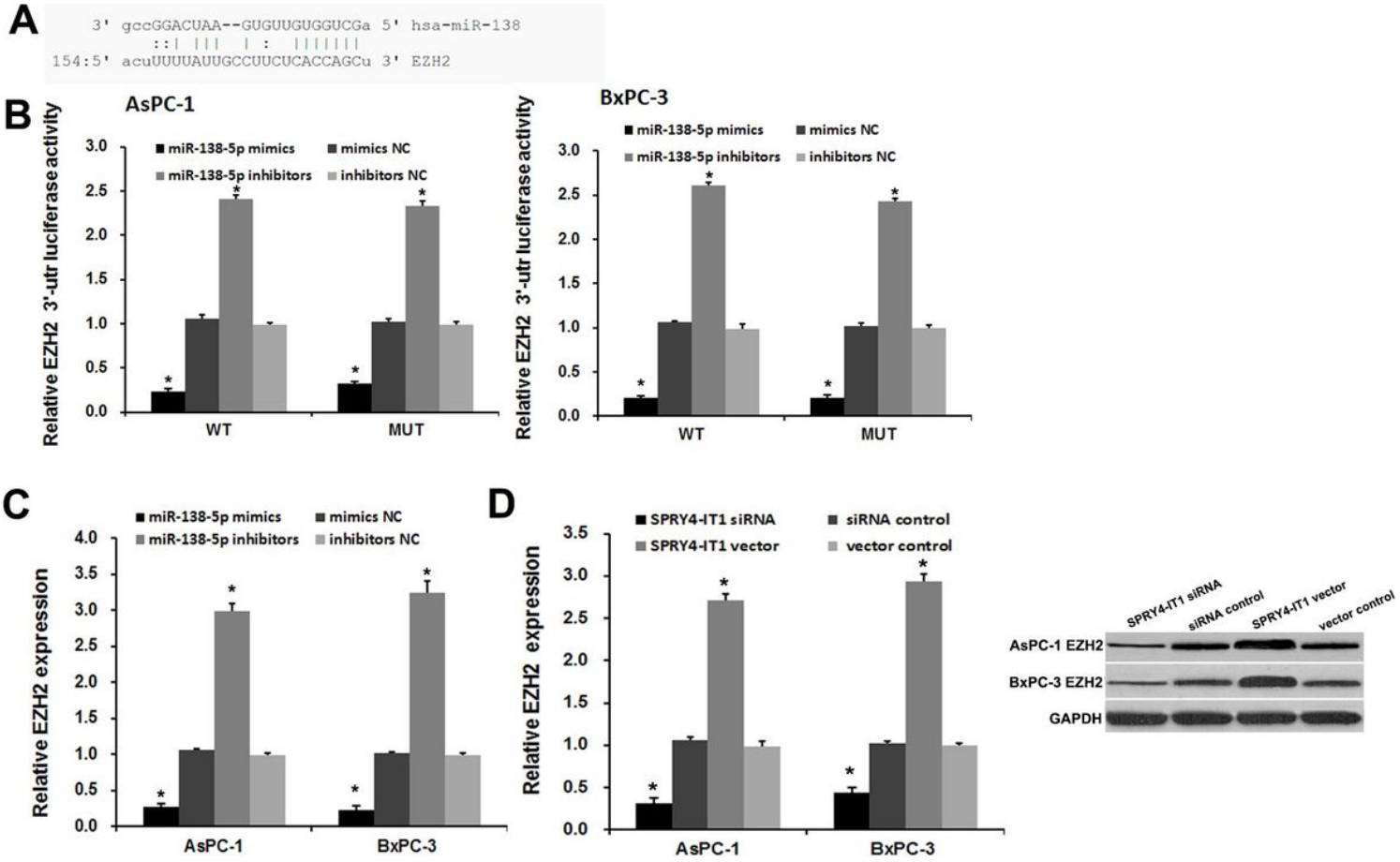

Figure 4

The effect of SPRY4-IT1 on miR-138-5p/EZH2 axis. A, B, SPRY4-IT1,regulated the expression of EZH2 in pancreatic cancer cells. C, luciferase-reporter assay the target role between miR-138-5p and EZH2. D, miR-138-5p repressed EZH2 expression in pancreatic cancer cells.