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Original Article

Regulation of MiR-224 on SMAD4 in pancreatic cancer and their association with pathological features

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Abstract: Objective: To investigate the expression of miR-224 and mothers against decapentaplegic homolog 4 (SMAD4) in pancreatic cancer (PC) and their association with pathological features of PC. Methods: Sixty-five cases of both tumor tissue and normal paracancerous tissue from pancreatic ductal adenocarcinoma (PDAC) patients who underwent surgery in our hospital from April 2016 to April 2019 were sampled. The expression of miR-224 and SMAD4 mRNA in PC tissues and paracancerous tissues were quantified by quantitative polymerase chain reaction (qPCR), and the expression of SMAD4 protein was quantified by Western blot. Meanwhile, the expression of miR-224 and SMAD4 mRNA in PC patients with different pathological features was analyzed. Human PC cell line (Aspc-1) was transfected. Before transfection, the cells were divided into miR-NC, miR-224 mimic, and miR-224 inhibitor groups. The proliferation, migration, and apoptosis of Aspc-1 cells were determined by cell counting kit-8 (CCK-8), Transwell, and flow cytometry, respectively. Dual-luciferase reporter (DLR) assay was employed to identify the targeting relation between miR-224 and SMAD4. The expression of SMAD4 mRNA and protein in the miR-224 mimic group and miR-224 inhibitor group were detected by the same method. Results: Compared with paracancerous tissues, the PC tissue showed remarkably higher expression of miR-224 and lower expression of SMAD4 mRNA and protein. Compared with the miR-NC group, the miR-224 mimics group showed elevated SMAD4 expression, accelerated cell growth, enhanced migration, as well as decreased apoptotic rate; while in the miR-224 inhibitor group, those results were reversed. In patients with moderately and poorly differentiated tumors, portal vein invasion, lymph node metastasis, and late pathological tumor-node-metastasis (pTNM) staging (stages III and IV); miR-224 expression was remarkably increased and SMAD4 mRNA and protein expression was remarkably decreased. Conclusion: MiR-224 regulates SMAD4 in a targeted manner, and SMAD4 expression decreases and miR-224 expression increases in PC tissues. The expression of miR-224 and SMAD4 are associated with pathological tumor features such as differentiation degree, portal vein invasion, lymph node metastasis, and pTNM staging.

Keywords: miR-224, SMAD4, pancreatic cancer, portal vein invasion

Introduction

Pancreatic cancer (PC) is a fatal malignant tumor found worldwide and will soon become the second primary cause of cancer-associated death. Pancreatic ductal adenocarcinoma (PDAC) is the most common PC with a 5-year survival rate of less than 7% [1-3]. The overall survival rate of most PDAC patients is low because they have been diagnosed in an advanced stage and have missed the optimal surgical timing. Due to the lack of early diagnosis methods, the efficacy of PDAC treatment is disappointing [4-6]. To develop an effective therapy for PDAC, it is necessary to understand the molecular mechanism of tumors and find molecular targets, such as mothers against decapentaplegic homolog 4 (SMAD4) [7].

SMAD4, a member of the SMAD family [8], functions as a tumor suppressor in various cancers and can be used as a prognostic indicator [9]. The frequent mutation or deletion of SMAD4 in PC indicates the importance of studying its rel-

Table 1. Sequence of related primers

Factor	Upstream primer	Downstream primer
miR-224	5'-AGTCTCTGGCTGACTACATCACAG-3'	5'-CTACTCACAAAACAGGAGTGGAATC-3'
SMAD4	5'-AGGATCAGTAGGTGGAATAG-3'	5'-TCTAAAGGTTGTGGGTCTGC-3'
U6	5'-ATTGGAACGATACAGAGAAGATT-3'	5'-GGAACGCTTCACGAATTTG-3'
β-actin	5'-CCGTTCCGAAAGTTGCCTTTT-3'	5'-ATCATCCATGGTGAGCTGGC-3'

evant mechanism independent of PDAC. Loss of functional SMAD in PDAC interferes with the transforming growth factor Beta (TGF-β)/SMAD signaling pathway, resulting in the reduction of gene inhibition in tumors [10, 11]. MicroRNA targeting has attracted much attention recently, it regulates mRNA by binding to the 3'-untranslated region (3'-UTR) of target mRNA and further regulates gene expression, and its abnormal expression contributes to tumorigenesis [12, 13]. It is reported that miR-224 is up-regulated in PDAC and accelerates cell proliferation and migration, suggesting its close association with the deterioration of PC [14]. Besides, miR-224 regulates the expression of SMAD4 in liver cancer [15]. However, the regulatory relationship between miR-224 and SMAD4 in PC, the association between the two, and the pathological features remain largely unexplored. Therefore, this study was designed to investigate these factors.

Data and methods

Data

Sixty-five cases each of tumor tissue and normal paracancerous tissue from PDAC patients who underwent surgery in our hospital from April 2016 to April 2019 were sampled.

The study acquired approval from the Ethics Committee of Suizhou Hospital, Hubei University of Medicine. Patients and their families were informed in advance and signed an informed consent.

Inclusive criteria: patients diagnosed with PDAC in our hospital; patients with no other diseases that had an impact on this study; patients with complete clinical data. Exclusive criteria: patients receiving chemotherapy prior; patients suffering from diseases that had an impact on this study; patients with severe hepatic and renal insufficiency.

Main reagents, instruments, and detection methods

Main reagents and instruments: Human PC cell line (Aspc-1) and Dulbecco's Modified Eagle Medium (DMEM) (Hunan Fenghui Biotechnology Co., Ltd.); 10% fetal bovine serum (FBS) (Thermo-Fisher Technology (China) Co., Ltd); cell cycle detection kit (Shanghai ML Biotechnology Co., Ltd.); cell apoptosis detection kit and transfection reagent Lipofectamine TM3000 (Sigma-Aldrich (Shanghai) Trading Co., Ltd.); Trizol (Shanghai Yuanye Biotechnology Co., Ltd.); primer sequences of miR-224, SMAD4, and internal references (**Table 1**), and synthesis of transfection plasmids (Shanghai Sangon Bioengineering Co., Ltd.); ultraviolet spectrophotometer (Beijing Jiayuan Industrial Technology Co., Ltd.); CoulterCytoFLEX flow cytometer (ABC, USA); ABI 7500 real-time PCR instrument (Beijing Long Jump Biotechnology Development Co., Ltd.); Transwell (Shanghai SBO Biomedical Technology Co., Ltd.); microplate reader (Beijing Image Trading Co., Ltd.).

Expression of miR-224 and SMAD4 mRNA: The expression of miR-224 and SMAD4 mRNA in PC tissues and transfected cells was detected by qPCR. Extraction of total RNA: 50 mg of tissues were put into a RNase-free microfuge tube (1.5 mL), mixed with 0.5 ml Trizol, then ground into a homogenate by a homogenizer, afterwards, 0.5 ml of Trizol was added and let to rest. The process lasted for about 0.5 h. Then, 200 μL chloroform was added to every 1 ml of Trizol. After rapid shaking and mixing for 30 s, the mixture was placed on ice for 5 min, then centrifuged at 1500×g at 4°C for 10 min. The supernatant (400-600 μL) was transferred to a new centrifuge tube with a pipette, then mixed with isopropyl alcohol and trizol (500 μL/1 mL). The tube was covered, and the liquid was mixed evenly, let stand for 10 min, and centrifuged (1500×g) at 4°C for 10 min. The supernatant was discarded, isopropyl alcohol was removed

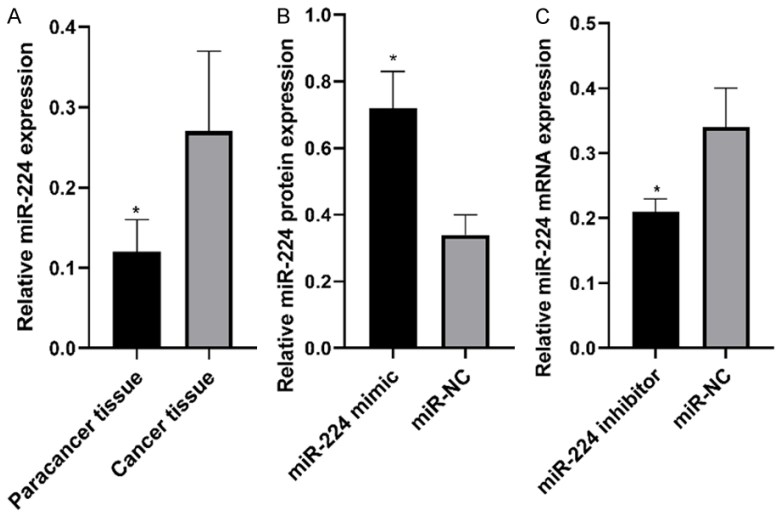

Figure 1. Expression of miR-224. A. Expression of miR-224 in paracancerous tissues and tumor tissues: the relative expression of miR-224 in tumor tissues is remarkably higher than that in paracancerous tissues (P<0.05). Note: * P<0.05 *vs* paracancerous tissues. B. Expression of miR-224 in the miR-224 mimic group after transfection: the relative expression of miR-224 in the miR-224 mimic group is remarkably higher than that in the miR-NC group (P<0.05). C. Expression of miR-224 in the miR-224 inhibitor group after transfection: the relative expression of miR-224 in the miR-224 inhibitor group is remarkably lower than that in the miR-NC group (P<0.05). Note: * P<0.05 *vs* miR-NC group.

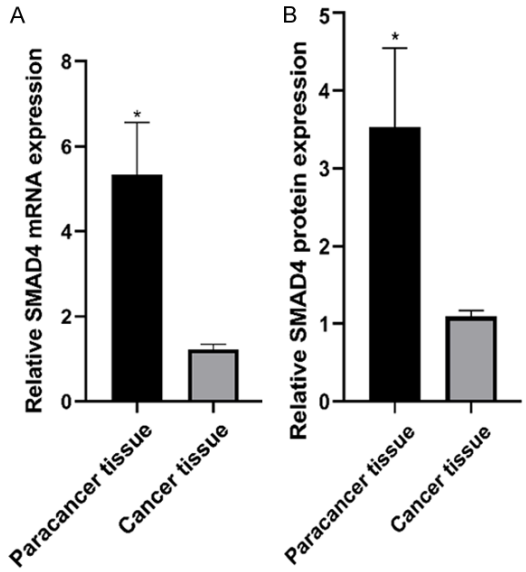

Figure 2. Expression of SMAD4 mRNA in paracancerous tissues and tumor tissues. A. Relative expression of SMAD4 mRNA in paracancerous tissues and tumor tissues: the relative expression of SMAD4 mRNA in tumor tissues is remarkably lower than that in paracancerous tissues (P<0.05). B. Relative expression of SMAD4 protein in tumor tissues and paracancerous tissues: the relative expression of SMAD4 protein in tumor tissues is remarkably lower than that in paracancerous tissues (P<0.05). Note: * P<0.05 *vs* miR-NC group.

and 1 mL of 75% ethanol was added, and mixed thoroughly. The mixture was centrifuged (1500×g) at 4°C for 10 min. The RNA obtained was washed. The supernatant was discarded, and precipitated RNA was air-dried for 5-10 min, and fully dissolved with 20 μL of diethyl pyrocarbonate (DE-PC) treated water. Then qPCR was carried out using an ABI 7500 real-time PCR instrument. Reaction conditions: 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s, then 60-95°C. The results were compared with those of an internal reference.

Expression of SMAD4 protein: After grinding with liquid nitrogen, the tissues were mixed with radio immunoprecipitation assay (RIPA) lysis buffer, homogenated, and centrifuged at low temperature and high speed. Supernatant was subpackaged and stored in a freezer at -80°C. The expression of SMAD4 protein was measured by Western blot, and the SMAD4/GAPDH ratio represented the relative expression.

Cell culture and transfection: Aspc-1 cells were routinely subcultured in a cell incubator at 37°C and 5% CO_2 with high glucose DMEM containing 10% FBS. Cells were transferred to 96-well plates before transfection and then grouped into a miR-NC group, miR-224 mimic group, and miR-224 inhibitor group. Afterwards, the cells were transfected with Lipofectamine TM3000 kit, then the expression of miR-224 in Aspc-1 cells of each group was determined.

Cell proliferation detection: Transfected Aspc-1 cells were inoculated into 96-well plates. Each sample was tested in 3 parallel wells and cultured for 24 h, 48 h, and 72 h, separately. Afterwards, 20 μL of cell proliferation colorimetric reagent (MTS Cell Proliferation Colorimetric Assay Kit, also known as Cell Counting Kit-8) was added to each well 2 hours before the end of the culture, and placed in a 37°C, 5%

Table 2. Association between pathological features of PC and miR-224 expression

Pathological parameters	Cases (n)	MiR-224 expression	t	p
Tumor diameter (cm)			1.59	0.116
<3	26	0.23±0.08		
≥3	39	0.27±0.11		
Tumors			1.53	0.130
Single	52	0.19±0.04		
Multiple	13	0.21±0.05		
Degree of differentiation			17.43	<0.001
Moderately and poorly differentiated	41	0.29±0.03		
Highly differentiated	24	0.17±0.02		
Portal vein invasion			5.24	<0.001
Yes	32	0.22±0.09		
No	33	0.13±0.04		
Lymph node metastasis			2.47	0.042
Yes	12	0.21±0.10		
No	53	0.15±0.07		
pTNM staging			3.72	<0.001
Stages I and II	30	0.16±0.08		
Stages II and IV	35	0.25±0.11		

CO_2 incubator. Two hours later, the optical density (OD) value was read with a fully-automatic microplate reader at 490 nm wavelength to evaluate cell proliferation.

Cell migration detection: Migration assay as conducted with Transwell. First, matrigel (10 μg/μL) was melted at 4°C, diluted to 0.25 μg/μL in DMEM, and put into an ice box for standby. Then, 100 μL was added to each well of the 24-well transwell, and cultured in an incubator with 37°C and 5% CO_2 for 1 h. After the matrigel solidified, the remaining non-solidified liquid was dried with filter paper. Cells were inoculated, and 100 μg DMEM containing 10% FBS was added to the apical chamber, 600 μL DMEM containing 20% FBS was added to the basolateral chamber. The cells were cultured with 37°C and 5% CO_2 for one day, then taken out and counted.

Apoptosis detection: Apoptosis of cells transfected for 48 h and stained with Annexin V and propidium iodide (PI) in a 6-well plate and were measured by CoulterCytoFLEX flow cytometer (ABC, USA). The test was repeated 3 times.

Targeting relationship between miR-224 and SMAD4: Aspc-1 cells seeded in 96-well plates were transfected with miR-224 mimic, miR-224 inhibitor, SMAD4 mutant (Mut), and SMAD4 wild type (Wt) using a Lipofectamine TM3000 kit. The activities of firefly and renilla luciferases were evaluated using dual-luciferase reporter (DLR) assay (Promega).

Statistical analysis

Data processing was performed with SPSS 19.0 (Asia Analytics Formerly SPSS China). Measurement data presented as mean ± standard deviation ($\bar{x}$ ± sd) were analyzed by *t*-test, such as expression of miR-224, SMAD4 mRNA and protein in tissues, cell proliferation and apoptosis. Additionally, miR-224 and SMAD4 mRNA in patients with different pathological features was also analyzed by *t*-test. MiR-224 and SMAD4 mRNA and protein in transfected cells were tested by One-way analysis of variance (ANOVA). Post-hoc analysis was performed with the Least Significant Diffirence (LSD). Values of $P<0.05$ indicated statistically significant differences.

Results

Expression of miR-224

The relative expression of miR-224 in tumor tissues was remarkably higher than that in paracancerous tissues (0.27±0.10 *vs* 0.12±0.04)

Table 3. Association between pathological features of PC and SMAD4 mRNA and protein expression

Pathological parameters	Cases (n)	SMAD4 mRNA expression	t	p	SMAD4 protein expression	t	p
Tumor diameter (cm)			0.85	0.397		0.32	0.748
<3	26	1.22±0.12			0.60±0.11		
≥3	39	1.19±0.15			0.59±0.13		
Tumors			0.77	0.446		1.15	0.255
Single	52	1.20±0.12			0.61±0.08		
Multiple	13	1.17±0.15			0.58±0.10		
Degree of differentiation			8.98	<0.001		10.58	<0.001
Moderately and poorly differentiated	41	1.12±0.05			0.52±0.04		
Highly differentiated	24	1.41±0.19			0.79±0.15		
Portal vein invasion			11.85	<0.001		8.51	<0.001
Yes	32	1.05±0.08			0.54±0.07		
No	33	1.41±0.19			0.83±0.18		
Lymph node metastasis			6.28	<0.001		4.39	<0.001
Yes	12	1.21±0.06			0.51±0.02		
No	53	1.56±0.19			0.74±0.18		
pTNM staging			10.74	<0.001		11.62	<0.001
Stages I and II	30	1.72±0.17			0.72±0.11		
Stages II and IV	35	1.32±0.13			0.50±0.02		

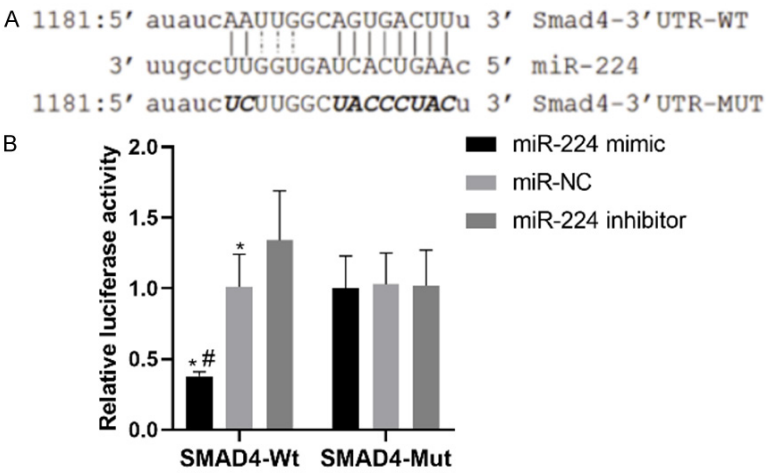

Figure 3. Targeting relationship between miR-224 and SMAD4. A. SMAD4 shares binding loci with miR-224. B. Luciferase activity of SMAD4-Wt in the miR-NC group and miR-224 mimic group is lower than that in the miR-224 inhibitor group, and the miR-224 mimic group is lower than the miR-NC group, but the luciferase activity of SMAD4-Mut is not affected, indicating that the luciferase activity is significantly reduced when SMAD4 bound to miR-224 (P<0.05). Note: *P<0.05 *vs* miR-224 inhibitor group, #P<0.05 *vs* miR-NC group.

(P<0.05). The expression of miR-224 in the miR-224 mimic group, miR-224 inhibitor group, and miR-NC group were (0.72±0.11), (0.21±0.02), (0.34±0.06), respectively. Therefore, the relative expression of miR-224 in the miR-NC group was remarkably lower than the miR-224 mimic group, and remarkably higher than the miR-224 inhibitor group (P<0.05). See **Figure 1** for details.

Expression of SMAD4 in tumor tissues and paracancerous tissues

The relative expression of SMAD4 mRNA in paracancerous tissues and tumor tissues were (5.34±1.22) and (1.23±0.11), respectively, and that of SMAD4 protein was (3.53±1.02), (1.09±0.08), respectively. The relative expression of SMAD4 mRNA and protein in tumor tissues were remarkably lower than those in paracancerous tissues (P<0.05). See **Figure 2** for details.

Association between pathological features and miR-224 expression in PC tissues

MiR-224 expression in PC tissue was associated with tumor differentiation, portal vein invasion, lymph node metastasis, and pTNM staging. MiR-224 expression was remarkably increased in patients with moderately and poorly dif-

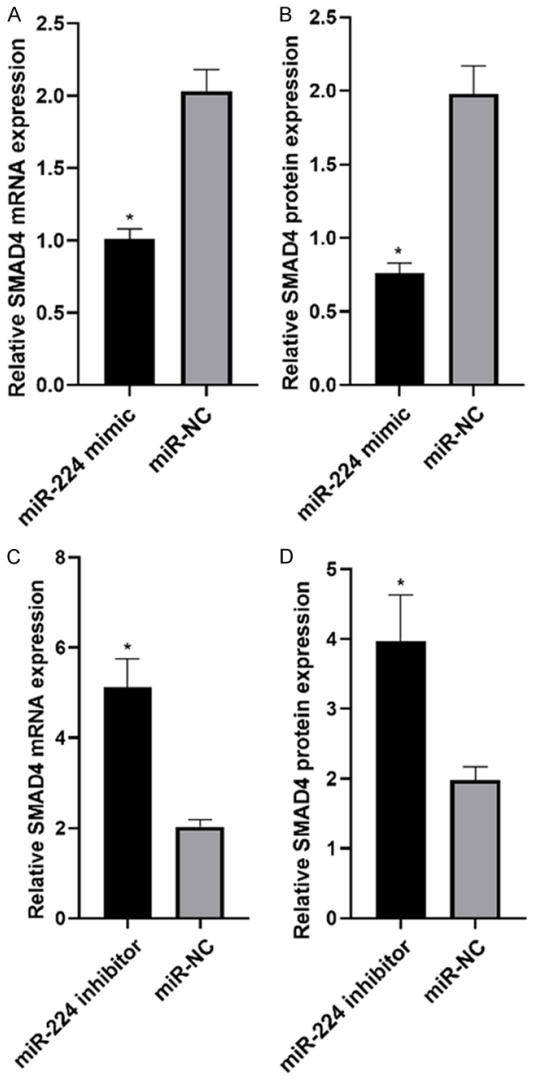

Figure 4. Inhibitory effect of miR-224 on SMAD4. A. Expression of SMAD4 mRNA in the miR-224 mimics group: expression of SMAD4 mRNA in the miR-224 mimics group is remarkably lower than that in miR-NC group (P<0.05). B. Expression of SMAD4 protein in the miR-224 mimics group: expression of SMAD4 protein in the miR-224 mimics group is remarkably lower than that in the miR-NC group (P<0.05). C. Expression of SMAD4 mRNA in the miR-224 inhibitor group: expression of smad4 mRNA in the miR-224 inhibitor group is remarkably higher than that in the miR-NC group (P<0.05). D. Expression of SMAD4 protein in the miR-224 inhibitor group: expression of SMAD4 protein in the miR-224 inhibitor group is remarkably higher than that in the miR-NC group (P<0.05). Note: * P<0.05 *vs* miR-NC group.

ferentiated tumors, portal vein invasion, lymph node metastasis, and late pTNM staging (stages III and IV) (P<0.05). See **Table 2** for details.

Association between pathological features and expression of SMAD4 mRNA and protein in PC tissue

The expression of SMAD4 mRNA and protein in PC tissue was associated with tumor differentiation, portal vein invasion, lymph node metastasis, and pTNM staging. The expression was remarkably reduced in patients with moderately and poorly differentiated tumor, portal vein invasion, lymph node metastasis, and late pTNM staging (stages III and IV) (P<0.05). See **Table 3** for details.

Targeting relationship between miR-224 and SMAD4

After reading the literature [16], we found there were binding loci between SMAD4 and miR-224. DLR assay revealed that miR-224 mimics reduced and miR-224 inhibitor enhanced the luciferase activity of SMAD4-Wt, but that of SMAD4-Mut was not affected, indicating that the luciferase activity was remarkably reduced when SMAD4 bound to miR-224 (P<0.05). See **Figure 3** for details.

miR-224 exerts an inhibitory effect on SMAD4

Expression of SMAD4 mRNA in the miR-NC group (2.03±0.15) was remarkably higher that that in the miR-224 mimics group (1.01±0.07) (P<0.05), and remarkably lower than that in the miR-224 inhibitor group (5.12±0.63) (P<0.05). Expression of SMAD4 protein in the miR-NC group (1.98±0.19) was remarkably higher that in the miR-224 mimics group (0.76±0.07) (P<0.05), and remarkably lower than that in the miR-224 inhibitor group (3.97±0.66) (P<0.05). See **Figure 4** for details.

Effects of knock-out and over-expression of miR-224 on cells

(1) Growth of Aspc-1 cells after miR-224 knockdown and over-expression. There was no significant difference in cell growth among the miR-224 mimic group, miR-224 inhibitor group and miR-NC group at the 24th hour (P>0.05), but the growth in the miR-224 mimic group was accelerated compared with the miR-NC group from 48 h to 72 h, and that in the miR-224 inhibitor group was significantly slowed down (P<0.05). Cell growth was significantly different between 24 h and 72 h in the same group (P<0.05). See **Table 4** for details. (2) Migration

Table 4. Growth of Aspc-1 Cells in different time periods (n=65)

Time	MiR-224 mimic group	MiR-224 inhibitor group	MiR-NC group	F	p
24 h	1.42±0.13	1.44±0.14	1.44±0.12	0.51	0.601
48 h	3.67±0.67*	2.05±0.37*	2.98±0.54*	146.90	<0.001
72 h	6.55±1.63*,#	3.32±0.54*,#	4.26±0.70*,#	156.50	<0.001

Note: *P<0.05 *vs* 24 h, #P<0.05 *vs* 48 h.

Table 5. Migration of Aspc-1 cells (n=65)

Group	MiR-224 mimic group	Mir-224 inhibitor group	MiR-NC group	F	P
Migrated cell number (n)	152.78±14.30	78.56±7.25	137.95±11.57	35.25	<0.001

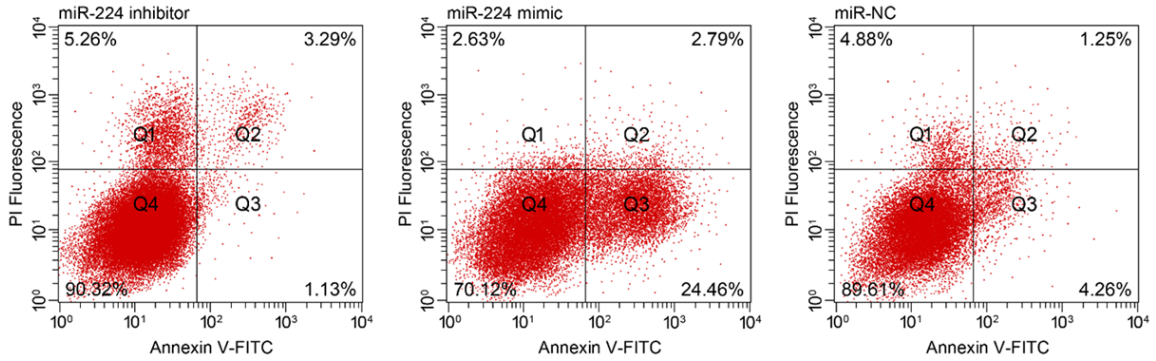

Figure 5. Apoptotic rate of Aspc-1 cells. Flow cytometry shows that the apoptosis rate in the miR-224 inhibitor group was higher than that in the miR-NC group, while that in the miR-224mimic group was lower than that in the miR-NC group (P<0.05).

Table 6. Apoptosis of Aspc-1 Cells (n=65)

Group	miR-224 mimic group	miR-224 inhibitor group	miR-NC group	F	P
Apoptotic rate (%)	4.42±0.74	27.25±2.34	5.51±0.74	71.42	<0.001

of Aspc-1 cells after miR-224 knock-down and over-expression. The number of migrated cells in the miR-NC group (139.78±11.98) was remarkably lower than that in the miR-224 mimics group (152.78±14.30), and remarkably higher than that in the miR-224 inhibitor group (78.56±7.25) (P<0.05). See **Table 5** and **Figure 5** for details. (3) Apoptosis of Aspc-1 cells after miR-224 knock-down and over-expression. The apoptotic rates in the miR-224 mimic group, miR-224 inhibitor group, and miR-NC group were (4.42±0.74)%, (27.25±2.34)%, (5.51±0.74)%, respectively. The apoptotic rate in the miR-224 mimic group was lower than that in the miR-NC group (P<0.05). See **Table 6** for details.

Discussion

Mutation and deletion of SMAD4 induces mutations of relevant signaling pathway factors in various cancers, leading to loss of inhibition on cell growth and deterioration of disease [17-19]. Our study was designed to investigate the expression of SMAD4 and miR-224 in PC tissues, their relationship in PC, as well as the association between SMAD4 and pathological features of PC.

First, we found that the expression of miR-224 in PC tissues was remarkably higher than that in paracancerous tissues. MiR-224 is highly expressed in various cancers including PC [20-22], which is similar to our finding. The expression of SMAD4 decreased in cancer due to the inhibitory effect of its related pathways on cell growth [23]. In our study, the expression of SMAD4 in PC tissues was remarkably lower than that in paracancerous tissues. Therefore we preliminarily deduced that miR-224 was negatively correlated with SMAD4 in PC. Ac-

cording to Yao G, SMAD4 is regulated by miR-224 in liver cancer [24]. We transfected miR-224 into human PC cells (Aspc-1), and found that miR-224 mimics reduced and miR-224 inhibitor increased the luciferase activity of SMAD4-Wt. Compared with the miR-NC group, the miR-224 mimics group showed elevated SMAD4 expression, accelerated cell growth, enhanced migration, as well as decreased apoptotic rate; while in the miR-224 inhibitor group, those results were reversed. This indicates that miR-224 plays a regulatory role on SMAD4 in PC, which is similar to that in liver cancer.

The study also tested the expression of SMAD4 and miR-224 in PC tissues of patients with different pathological features. It turned out that the expression of miR-224 was remarkably increased, while the expression of SMAD4 mRNA and protein was remarkably decreased in patients with moderately and poorly differentiated tumors, portal vein invasion, lymph node metastasis, and late pTNM staging (stages III and IV). Loss or inactivation of SMAD4 portends reduced cell adhesion, enhanced motility and decreased differentiation [25]. Moreover, miR-224 can accelerate cell proliferation and enhance invasiveness in cancer. Meanwhile, SMAD4 is one of the targets of miR-224 in several cancers, including PC [26, 27]. Therefore, SMAD4 is able to promote the differentiation of Aspc-1 cells. When the expression of SMAD4 was down-regulated due to the targeted inhibition of miR-224, cell differentiation decreased, so the expression of SMAD4 mRNA and protein in moderately and poorly differentiated tumors decreased remarkably, while that of miR-224 increased remarkably. It was precisely because of the targeted inhibition relationship between miR-224 and SMAD4 that the decrease of SMAD4 led to the enhancement of cell mobility and the increase of migrated cell number. Therefore, miR-224 was elevated and SMAD4 was suppressed in tumors of patients with portal vein invasion, lymph node metastasis, and advanced PC. The expression of SMAD4 was reported to be decreased in advanced cancers previously, while the level of miR-224 was significantly increased [28, 29], similar to the results of our study. This indicates that regulation of miR-224 on SMAD4 is associated with differentiation degree, portal vein invasion, lymph node metastasis, and pTNM staging.

However, there were still limitations in our study. We fail to test pathway signaling proteins related to SMAD4, which leads to the lack of clarity on the specific role of miR-224 in the regulation of SMAD4 in PC. We will determine these proteins to elaborate the effects of miR-224 and SMAD4 on PC.

To sum up, miR-224 regulates SMAD4 in a targeted manner, and SMAD4 expression decreases and miR-224 expression increases in PC tissues. The expression of miR-224 and SMAD4 are associated with tumor pathological features such as differentiation degree, portal vein invasion, lymph node metastasis, and pTNM staging. These findings may provide references for the development of molecular detection and targeted therapy for patients with those pathological features.

Disclosure of conflict of interest

None.

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