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ORIGINAL ARTICLE

MicroRNA-204-3p inhibits metastasis of pancreatic cancer *via* downregulating MGAT1

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Summary

Purpose: *We aimed to clarify the relationship between microRNA-204-3p level and clinical indicators in pancreatic cancer patients, and to provide theoretical references for target therapy.*

Methods: *Quantitative real-time polymerase chain reaction (qRT-PCR) was conducted to detect relative levels of microRNA-204-3p and MGAT1 in 60 paired pancreatic cancer tissues and adjacent normal ones. The relationship between microRNA-204-3p level and clinical indicators in pancreatic cancer patients was analyzed. MicroRNA-204-3p overexpression model was established in AsPC-1 and CFPAC-1 cells. Transwell and wound healing assay were carried out to illustrate the influence of microRNA-204-3p on the migratory potential in pancreatic cancer. Finally, luciferase assay and rescue experiments were performed to demonstrate the potential mechanism between microRNA-204-3p and MGAT1.*

Results: *MicroRNA-204-3p was lowly expressed in pancreatic cancer tissues. Low level of microRNA-204-3p predicted high rates of lymphatic metastasis and distant metastasis, as well as poor prognosis in pancreatic cancer patients. Overexpression of microRNA-204-3p inhibited pancreatic cancer cells to migrate in vitro. MicroRNA-204-3p could be targeted by MGAT1 through specific binding sites in the 3'UTR. A negative correlation between MGAT1 and microRNA-204-3p was identified in pancreatic cancer tissues. The interaction between MGAT1 and microRNA-204-3p was responsible for inhibiting metastasis of pancreatic cancer.*

Conclusions: *MicroRNA-204-3p is closely linked to lymphatic metastasis, distant metastasis and prognosis in pancreatic cancer patients. It inhibits the migratory ability in pancreatic cancer cells via negatively regulating MGAT1 level.*

Key words: *microRNA-204-3p, MGAT1, pancreatic cancer, metastasis*

Introduction

Pancreatic cancer is an extremely malignant tumor of the digestive system. Its 5-year survival is less than 5% and the median survival is shorter than 6 months [1-3]. Pancreatic ductal adenocarcinoma (PDAC) is the most common histological subtype of pancreatic cancer, accounting for 85-90% [4,5]. High mortality and poor prognosis in pancreatic cancer may be attributed to the deficiency of effective hallmarks. More seriously, pancreatic cancer is poorly responding to chemotherapy/radiotherapy, leading to a high tendency to tumor relapse [6,7]. Therapeutic efficacy for pancreatic cancer patients needs to be largely improved [8,9]. Early diagnosis and surgical resection can enhance the survival from 6% in stage IV pancreatic cancer patients to 50% in stage I [9]. Hallmarks for pancreatic cancer, diagnosis at the precancerous stage and screening for high-risk populations are beneficial to remarkably improve the prognosis in affected people [10,11]. In addition, clarifying mo-

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lecular variations and epigenetic changes in the development of pancreatic cancer will guide a new direction in clinical treatment [11-13].

MicroRNAs (miRs) are endogenous, single-strand, non-coding RNAs 19-24 nucleotides (nts) long. They are able to post-transcriptionally regulate about 30% protein-encoding genes by complementary base pairing [14-16]. One miR may correspond to multiple targets, and multiple miRs may also regulate the same target [17,18]. Abnormally expressed miRs are involved in multiple aspects during tumor development [19,20]. So far, several miRs have been identified to serve as potential hallmarks for pancreatic cancer [21,22]. However, the consistency of specific miRs in reported studies is poor, and their sensitivity and specificity need to be improved in clinical applications [14,17]. This study mainly focuses on discovering more advantageous miRs in pancreatic cancer development, and providing new entry points for the diagnosis, treatment and prognosis [22]. miR-204-3p is found downregulated in many types of tumors, and its level is linked to tumor staging. In this article we explored the role of miR-204-3p in influencing the migratory potential in pancreatic cancer and the involvement of its downstream target.

Methods

Patients and pancreatic cancer samples

A total of 60 paired pancreatic cancer tissues and adjacent normal ones were surgically collected. Tissue samples were pathologically confirmed by two experienced pathologists independently and stored at -80°C. This study got approval by Ethics Committee of West China Hospital and was conducted after signed informed consent was received from each subject.

Cell lines and reagents

Human pancreatic cancer cell lines (AsPC-1, PANC-1, MIA PaCa-2, CFPAC-1, BxPC-3) and the pancreatic ductal epithelial cell line (HPNE) were purchased from ATCC. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific, Waltham, MA, USA) in a 5% CO_2 incubator at 37°C. 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin were added in the culture medium. Cell passage was conducted when cells were grown to 80-90% confluence using trypsin.

Transfection

Transfection plasmids were constructed by GenePharma, Shanghai, China. Cells were cultured to 30-50% confluence in 6-well plates and transfected with plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). 48 h later, cells were collected for testing transfection efficacy and functional experiments.

Transwell migration assay

Transwell chambers (Millipore, Billerica, MA, USA) were inserted in each well of 24-well plates. 200 μL of suspension (5×10^5 cells/mL) was applied in the upper layer of the chamber with 700 μL of medium containing 20% FBS in the bottom. After 48-h incubation, migratory cells in the bottom were reacted with 15-min methanol, 20-min crystal violet and captured using a microscope. Migratory cells were counted in 10 random selected fields per sample.

Wound healing assay

Cells were inoculated in 6-well plates and grown to 90% confluence. After creation of an artificial wound in cell monolayer, medium with 1% FBS was replaced. 24 h later, wound closure was captured for calculating the percentage of wound healing.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Extracted RNAs by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) were purified by DNase I treatment, and reversely transcribed into cDNAs using Primescript RT Reagent (TaKaRa, Otsu, Japan). The obtained cDNAs underwent qRT-PCR using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Japan). Each sample was performed in triplicate. Relative level was calculated by $2^{-\Delta\Delta Ct}$. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were served as the internal references. MicroRNA-204-3p: forward: 5'-ACACTCCAGCTGGGGCTGGGAAGGCAAAGGG-3', reverse: 5'-CTCAACTGGTGTCGTGGA-3'; U6: forward: 5'-CTCGCTTCGGCAGCACA-3', reverse: 5'-AACGCTTCACGAATTTGCGT-3'; MGAT1: forward: 5'-GCGGCTCCTAGCCGAGAC-3', reverse: 5'-GTCTGGTGATGGACAGATCCC-3'; GAPDH: forward: 5'-TGACGCTGGGGCTGGCATTG-3', reverse: 5'-GCTCTTGCTGGGGCTGGTGG-3'.

Western blot

Cells or tissues were lysed on ice for isolating proteins. After detection of protein concentration by bicinchoninic acid (BCA) method (Beyotime, Shanghai, China), protein samples were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). They were subsequently loaded on polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Non-specific antigens were blocked in 5% skim milk for 2 h. Membranes were reacted with primary and secondary antibodies for indicated time. Band exposure and analyses were finally conducted.

Luciferase assay

Cells in good condition were co-transfected with negative control (NC) mimic/microRNA-204-3p mimic and pmirGLO-MGAT1-WT/pmirGLO-MGAT1-MUT/pmirGLO for 48 h. They were lysed and subjected to measurement of luciferase activity (Promega, Madison, WI, USA).

Statistics

GraphPad Prism 5 V5.01 (La Jolla, CA, USA) was used for data analysis. Data were expressed as mean

± standard deviation. Differences between groups were analyzed by t-test. Chi-square was conducted for analyzing the relationship between miR-204-3p level and clinical data of pancreatic cancer patients. Pearson's correlation test was applied for evaluating the relationship between two genes. Kaplan-Meier curves were applied for assessing the prognostic potential of miR-204-3p in pancreatic cancer. P<0.05 was considered statistically significant.

Results

miR-204-3p was lowly expressed in pancreatic cancer samples

We collected 60 paired pancreatic cancer tissues and adjacent normal ones. QRT-PCR data showed that miR-204-3p was downregulated in pancreatic cancer tissues and cell lines (Figure

Table 1. Association of miR-204-3p expression with clinicopathologic characteristics of pancreatic cancer

Parameters	*Number of cases*	*miR-204-3p expression*		*p value*
		High (n=35)	*Low (n=35)*	
Age (years)				0.999
<60	24	14	10	
≥60	36	21	15	
Gender				0.651
Male	22	12	10	
Female	38	23	15	
T stage				0.057
T1-T2	36	22	14	
T3-T4	24	13	11	
Lymph node metastasis				0.015
No	35	25	10	
Yes	25	10	15	
Distance metastasis				0.028
No	34	24	10	
Yes	26	11	15	

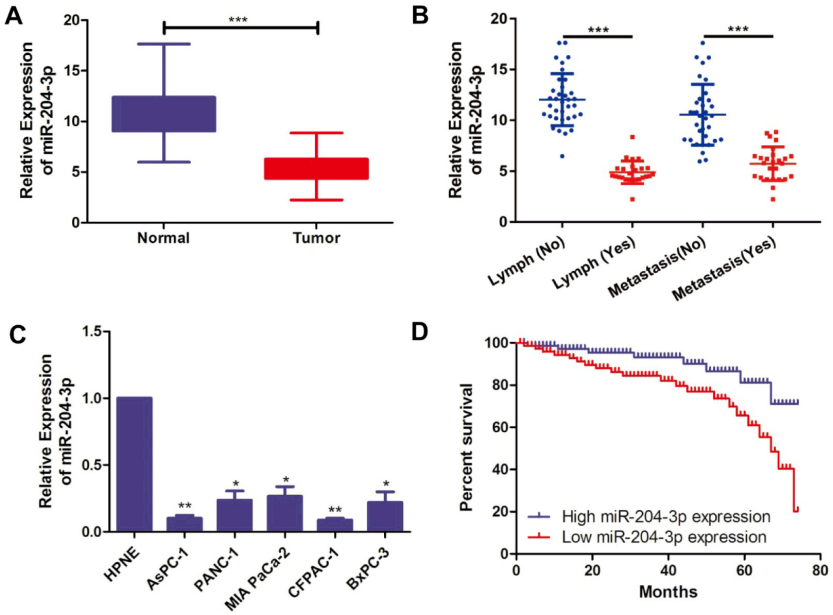

Figure 1. MiR-204-3p was lowly expressed in pancreatic cancer samples. **A:** miR-204-3p level in pancreatic cancer tissues and adjacent normal ones. **B:** miR-204-3p level in pancreatic cancer patients either with lymphatic metastasis, distant metastasis or not. **C:** miR-204-3p level in pancreatic cancer cell lines. **D:** Kaplan-Meier curves depicted based on the overall survival in pancreatic cancer patients with high or low expression of miR-204-3p. Data were expressed as mean±SD. *p<0.05, **p<0.01, ***p<0.001.

1A,1C), suggesting its potential anti-cancer effect. According to the miR-204-3p level in pancreatic cancer tissues, recruited patients were assigned into high and low miR-204-3p expression groups. Chi-square analysis showed that miR-204-3p level was negatively related to rates of lymphatic metastasis and distant metastasis, while it was unrelated to age, gender and tumor staging in pancreatic cancer patients (Table 1). As expected, lower abundance of miR-204-3p was detected in pancreatic cancer patients with lymphatic metastasis or distant metastasis than those without metastases (Figure 1B). Kaplan-Meier curves revealed poor prognosis in pancreatic cancer patients expressing low level of miR-204-3p (Figure 1D). It is suggested that miR-204-3p could be a novel hallmark for predicting the prognosis in pancreatic cancer.

Overexpression of miR-204-3p inhibited the migratory potential in pancreatic cancer

To analyze the influence of miR-204-3p on the cellular functions of pancreatic cancer, miR-204-3p overexpression model was established in AsPC-1 and CFPAC-1 cells (Figure 2A). Migratory potential changes were examined by transwell and wound healing assay which showed that overexpression of miR-204-3p markedly decreased the migratory cell number and wound closure percentage in pancreatic cancer cells (Figures 2B, 2C), indicating the inhibitory effect of miR-204-3p on pancreatic cancer migration.

miR-204-3p was bound to MGAT1

Bioinformatics analysis depicted the predicted consequential pairing in the 3'UTR of miR-204-3p

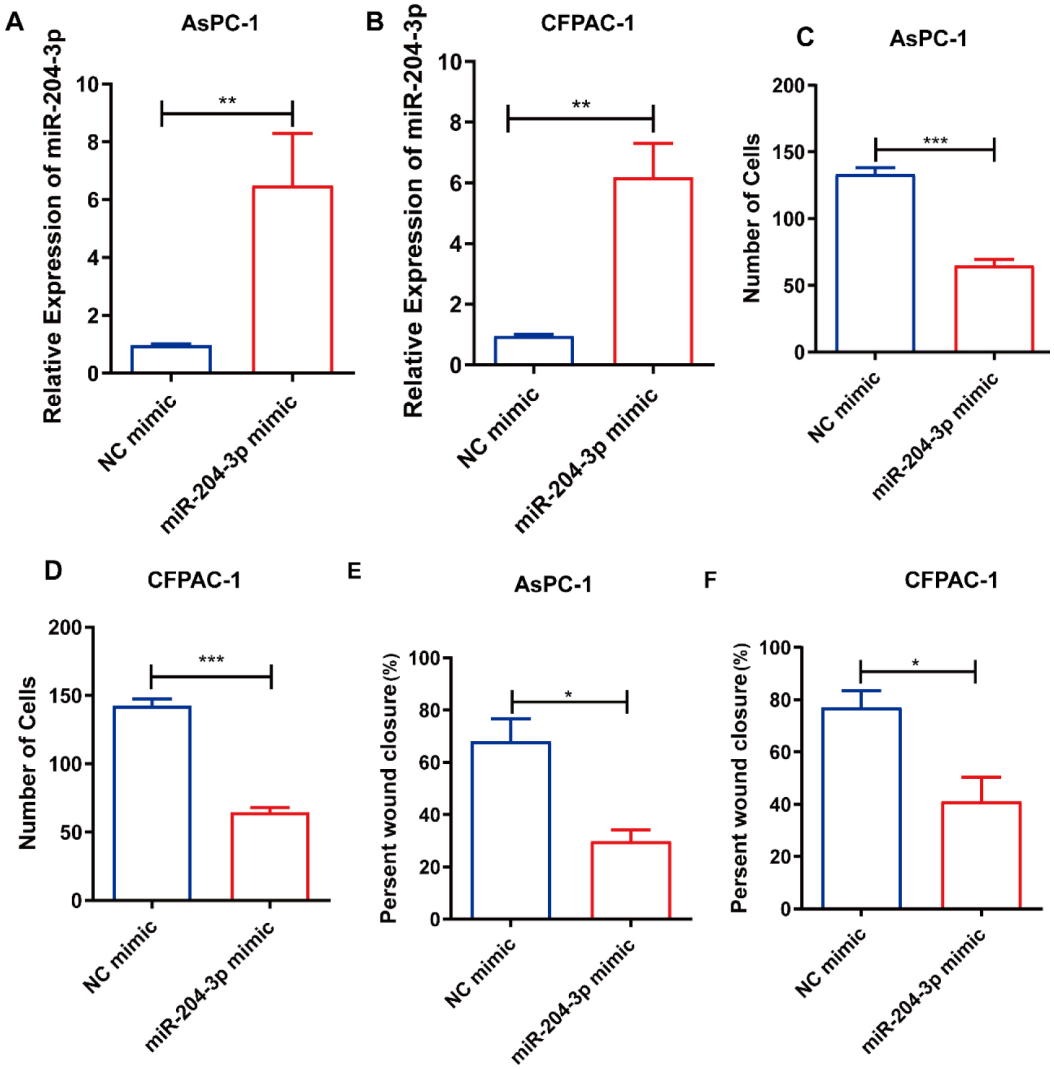

Figure 2. Overexpression of miR-204-3p inhibited migratory potential in pancreatic cancer. **A:** Transfection efficacy of miR-204-3p mimic in AsPC-1 and CFPAC-1 cells. **B:** Migration in AsPC-1 and CFPAC-1 cells transfected with NC mimic or miR-204-3p mimic (Magnification: 40×). **B [DOUBLE "B"?]:** Wound closure percentage in AsPC-1 and CFPAC-1 cells transfected with NC mimic or miR-204-3p mimic (magnification: 40×). Data were expressed as mean±SD. *p<0.05.

and MGAT1. Subsequently, decreased luciferase activity in AsPC-1 and CFPAC-1 cells co-transfected with miR-204-3p mimic and pmirGLO-MGAT1-WT confirmed the binding between miR-204-3p and MGAT1 (Figure 3A). Overexpression of miR-204-3p markedly downregulated protein and mRNA levels of MGAT1 in pancreatic cancer cells (Figure 3B). Showing a negative correlation with miR-204-3p level in pancreatic cancer tissues, MGAT1 was highly expressed in pancreatic cancer samples (Figures 3C, 3D, 3F). In addition, Kaplan-Meier curves demonstrated that highly expressed MGAT1 was unfavorable to the prognosis in pancreatic cancer patients (Figure 3E).

The interaction between MGAT1 and miR-204-3p was responsible for metastasis of pancreatic cancer

W e thereafter explored the influence of MGAT1 on the migratory ability in pancreatic cancer cells. First, higher level of MGAT1 was observed in pancreatic cancer cells co-overexpressing MGAT1 and miR-204-3p than those overexpressing miR-204-3p (Figure 4A). Interestingly, co-overexpression of MGAT1 and miR-204-3p could markedly reverse the suppressed migratory ability in pancreatic cancer cells overexpressing miR-204-3p (Figures 4B, 4C).

Discussion

Pancreatic cancer is a very critical disease characterized by rapid progression, poor prognosis and frequent metastasis. It is the number eight tumor killer and 5% of pancreatic cancer patients can live for 5 years [1-5]. So far, the molecular mechanisms underlying metastasis of pancreatic cancer have remained largely unknown. We believed that molecular target therapy is able to greatly improve the survival and prognosis in pancreatic cancer patients [10-13].

MiRs are produced by hairpin-structural pre-miRs with 70 nt long following a series of restriction endonuclease reaction in cells. They are widely distributed in animals, plants and microorganisms [14-16]. Typically, the pairing between miRs and target mRNAs in the 3'UTR leads to mRNA degradation or its translation suppression, ultimately regulating biological behaviors [16-19]. To seek for effective means that aim for a reverse or cure, differentially expressed miRs in pancreatic cancer profiling are analyzed. At last, the most available candidate miR-204-3p is selected [21,22]. Our findings showed that miR-204-3p was downregulated in pancreatic cancer tissues and cell lines. Low level of miR-204-3p was linked to high metastasis rate

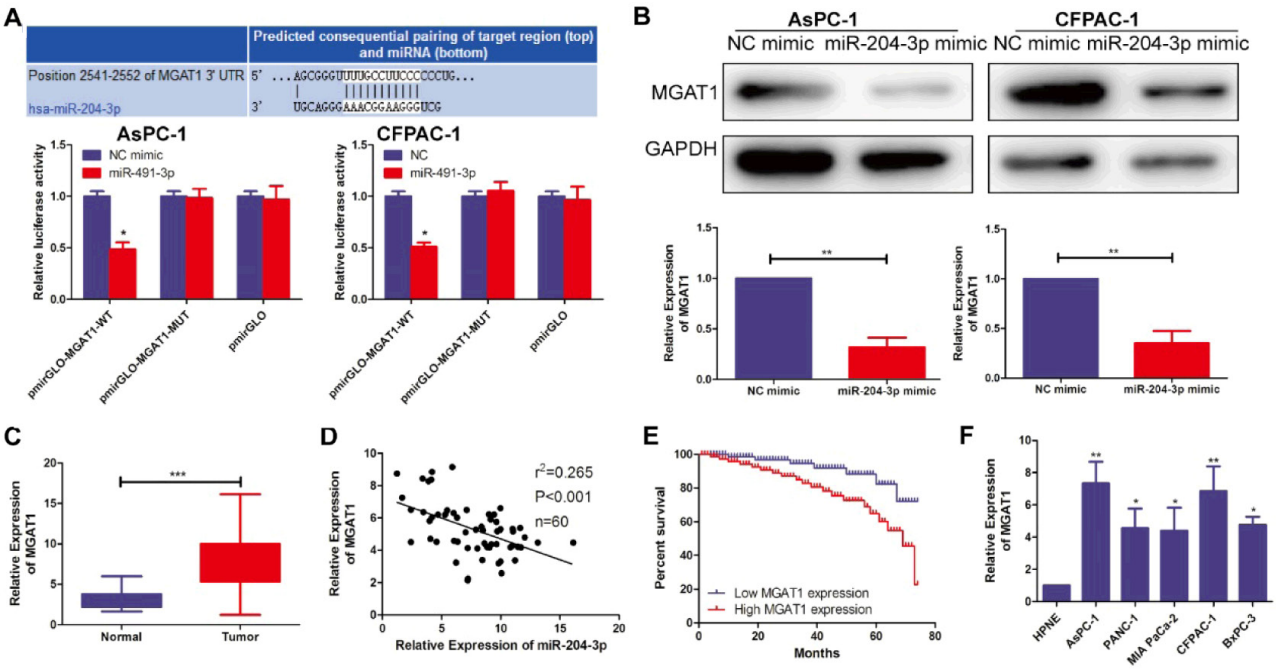

Figure 3. miR-204-3p was bound to MGAT1. **A:** Luciferase activity in AsPC-1 and CFPAC-1 cells co-transfected with NC mimic/miR-204-3p mimic and pmirGLO-MGAT1-WT/pmirGLO-MGAT1-MUT/pmirGLO. **B:** MGAT1 level in AsPC-1 and CFPAC-1 cells transfected with NC mimic or miR-204-3p mimic. **C:** MGAT1 level in pancreatic cancer tissues and adjacent normal ones. **D:** A negative correlation between levels of miR-204-3p and MGAT1 in pancreatic cancer tissues. **E:** Kaplan-Meier curves depicted based on the overall survival in pancreatic cancer patients with high or low expression of MGAT1. **F:** MGAT1 level in pancreatic cancer cell lines. Data were expressed as mean±SD. *p<0.05, **p<0.01, ***p<0.001.

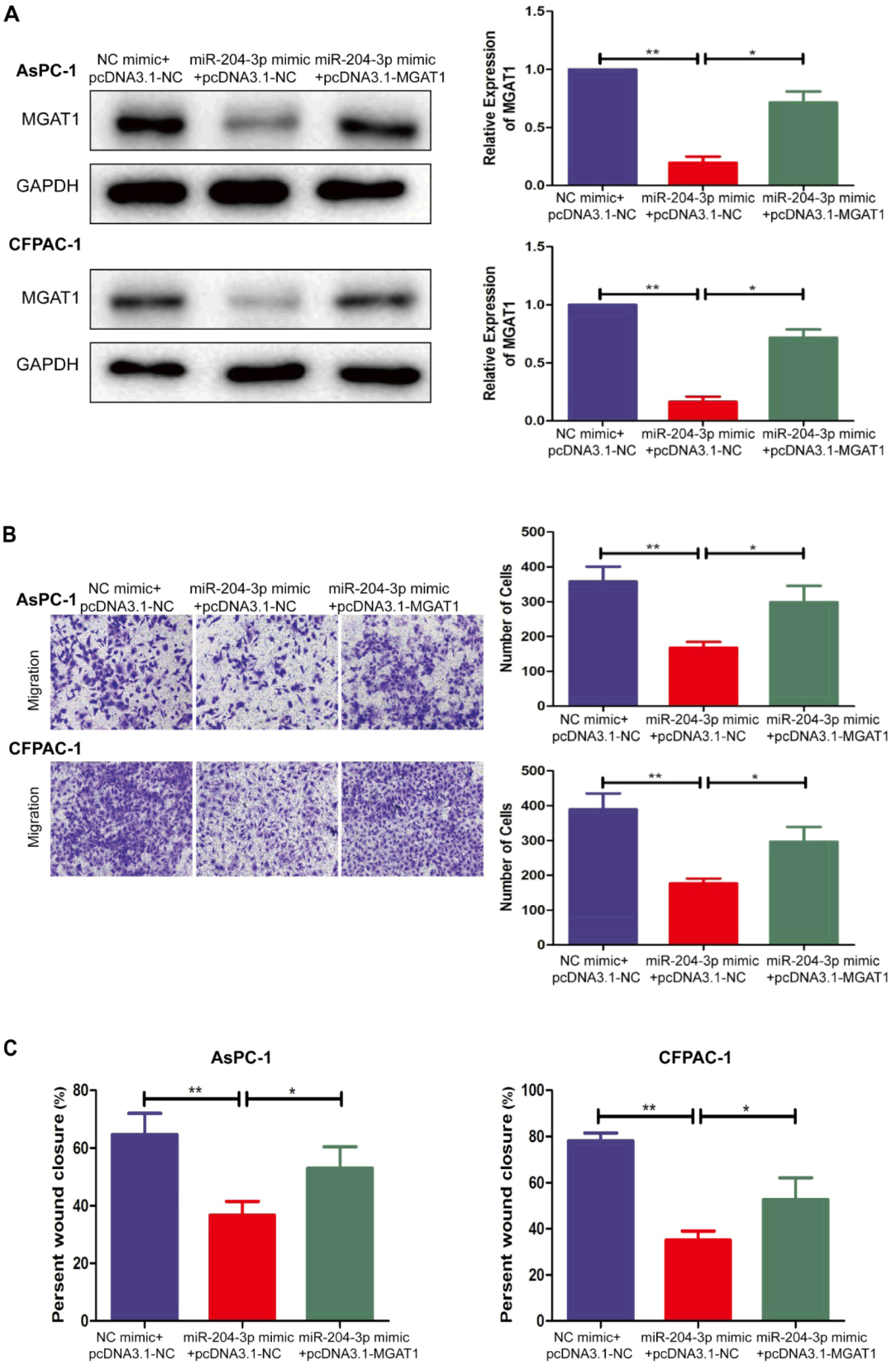

Figure 4. The interaction between MGAT1 and miR-204-3p was responsible for metastasis of pancreatic cancer. **A:** MGAT1 level in AsPC-1 and CFPAC-1 cells co-transfected with NC mimic+pcDNA3.1-NC, miR-204-3p mimic+pcDNA3.1-NC or miR-204-3p mimic+pcDNA3.1-MGAT1. **B:** Migration in AsPC-1 and CFPAC-1 cells co-transfected with NC mimic+pcDNA3.1-NC, miR-204-3p mimic+pcDNA3.1-NC or miR-204-3p mimic+pcDNA3.1-MGAT1 (Magnification: 40×). **C:** Wound healing percentage in AsPC-1 and CFPAC-1 cells co-transfected with NC mimic+pcDNA3.1-NC, miR-204-3p mimic+pcDNA3.1-NC or miR-204-3p mimic+pcDNA3.1-MGAT1 (Magnification: 40×). Data were expressed as mean±SD. *p<0.05, **p<0.01.

and poor prognosis in pancreatic cancer. Hence, we believed that miR-204-3p exerted an anti-cancer role in the development of pancreatic cancer. Subsequently, functional experiments yielded a conclusion that miR-204-3p was capable of inhibiting migratory potential in pancreatic cancer cells.

With the development of bioinformatics and molecular biology, biological functions of a growing number of miRs have been identified [17,18]. The interaction between a miR and its targets, which can be predicted by the software, determines their impacts on life activities [18,23]. The current methods on predicting miR targets mainly follow the five basic principles: (1) Complementary base paring between a miR and the target gene; (2) Thermal stability between miR and mRNA duplexes; (3) Highly conserved miR target sites among different species; (4) No complex secondary structure at the miR target site; (5) Stronger binding capacity in the miR 5'terminal than that of 3'terminal [23,24].

In this paper, we found out predicted sequences in 3'UTR miRNA-204-3p that bound MGAT1. Furthermore, luciferase assay confirmed the direct binding between miR-204-3p and MGAT1. MGAT1 was downregulated in pancreatic cancer cells overexpressing miR-204-3p. Notably, overexpression of MGAT1 could reverse the inhibitory effect of miR-204-3p on pancreatic cancer migration. Therefore, it was verified that the inhibitory effect of miR-204-3p on the malignant progression of pancreatic cancer cells was caused by negatively regulating MGAT1 level.

Conclusions

MiR-204-3p is closely linked to lymphatic metastasis, distant metastasis and prognosis in pancreatic cancer patients. It inhibits migratory ability in pancreatic cancer cells *via* negatively regulating MGAT1 level.

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Conflict of interests

The authors declare no conflict of interests.

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