OncoTargets and Therapy

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ORIGINAL RESEARCH

RETRACTED ARTICLE: Upregulated miR-27a-3p Indicates a Poor Prognosis in Pancreatic Carcinoma Patients and Promotes the Angiogenesis and Migration by Epigenetic Silencing of GATA6 and Activating VEGFA/VEGFR2 Signaling Pathway

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Background: Abnormal miR-27a-3p expression has been frequently reported in several types of human cancer and contributes to tumor progression. However, the role and potential molecular mechanism of miR-27a-3p in the progression of pancreatic carcinoma have not been clarified.

Materials and methods: The expression of miR-27a-3p and GATA binding protein 6 (GATA6) in pancreatic carcinoma tissues and cell lines was evaluated by quantitative real-time PCR and Western blot analysis. The relationship between clinical pathologic features and miR-27a-3p expression was analyzed with Chi-square test. The regulatory mechanism of miR-27a-3p on GATA6 was confirmed by luciferase reporter assay and bioinformatics analysis. The effects of miR-27a-3p by targeting GATA6 on cell angiogenesis and migration were assessed by capillary tube formation and wound healing assays.

Results: miR-27a-3p expression was significantly upregulated in pancreatic carcinoma tissues and cell lines. Highly expressed miR-27a-3p was closely related to more lymph node metastasis, present peritoneal metastasis, and poor prognosis in patients with pancreatic carcinoma. MiR-27a-3p promoted migration and angiogenesis of pancreatic carcinoma cells by activating vascular endothelial growth factor A (VEGFA) and vascular endothelial growth factor receptor 2 (VEGFR2) expression. A significantly negative correlation between GATA6 mRNA and miR-27a-3 expression was found in pancreatic carcinoma samples. Modulation of miR-27a-3p could alter GATA6 expression in pancreatic carcinoma cells. GATA6 was identified as a functional target gene of miR-27a-3p, and GATA6 knockdown partially reversed the effects of miR-27a-3p siliencing on the migration and angiogenesis of pancreatic carcinoma cells by regulation of VEGFA/VEGFR2 pathway.

Conclusion: Upregulated miR-27a-3p indicates a poor prognosis in pancreatic carcinoma patients and promotes the angiogenesis and migration by epigenetic silencing of GATA6 and activating VEGFA/VEGFR2 signaling pathway, and indicating miR-27a-3p may be a promising therapeutic target for pancreatic carcinoma treatment.

Keywords: miR-27a-3p, pancreatic carcinoma, GATA6, migration, VEGFA/VEGFR2

Introduction

Pancreatic cancer is a highly lethal malignancy with a 5-year survival rate of less than 5% and is characterized by early metastasis, rapid invasion, and resistance to standard treatments.[1,2] Pancreatic ductal adenocarcinoma is the most common form, which

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accounts for more than 90% of all the pancreatic cancer cases.[3] Curative resection is the core of successful pancreatic carcinoma therapy, but only 15–20% of the patients are diagnosed during the early stages of cancer when surgical resection can be offered. A large proportion of patients are diagnosed with locally advanced or metastatic cancer at the time of presentation. Although an increasing number of therapies, including chemotherapy, radiotherapy, and molecular therapy, have been progressed in recent years, the overall 5-year survival rate of pancreatic carcinoma patients is still less than 7%.[4] During past decades, though several risk factors have been illustrated to associate with the tumorigenesis of pancreatic carcinoma, little progress has made on the molecular mechanisms underlying the progression of pancreatic carcinoma.[5–7] Therefore, identifying novel biomarkers involved in pancreatic carcinoma progression is necessary to provide early diagnosis and develop effective therapeutic options.

MicroRNAs (miRNAs, miRs) are a family of small noncoding RNA that inhibit gene expression by directly binding with the 3′-untranslated regions (UTRs) of messenger RNA (mRNA).[8] The dysregulation of miRNAs in carcinogenesis has been extensively examined in the past decade, which are closely associated with tumor initiation, metastasis, a relapse.[9,10] Changes in miRNA expression pattern have bee linked to profound effects on tumor cell phenotype in pancreatic carcinoma. For instance, miR-10a-5p gulate transcription factor AP-2 gamma (TFAP2 to mote gemcitabine resistance in pancreatic ca noma l iR-148a suppresses epithelial–mesenchymal sition and asion of pancreatic carcinoma cells by ta geting t family mber 10B (WNT10B).[12] MiR-337 rgets homeo B7 (HOXB7) to suppress pancreatic arcinoma cell proliferation and invasion.[13] In addition, iR-18 -5p, ETS proto-oncogene 1 (ETS1), and MET oto-on ene (c-M t) signaling pathway exacerbate a p or prog osis of p eatic carcinoma patients after radiati therap [14]

MiR-27a-3 s an isoform of mature miR-27a, is located at human romosome 19p13 and has been found to be frequently aberrant expressed and contribute to tumor progression in various types of cancer.[15] Using HiSeq 2000 sequencing from three independent cohorts (healthy control, benign pancreatic diseases, and pancreatic cancer), Wang et al identified that combination of serum CA19-9 and peripheral blood mononuclear cell's miR-27a-3p level can differentiate pancreatic cancer from benign pancreatic diseases.[16] Recently, Silvestris et al speculated that miR-27a-3p has an important angiogenic activity in pancreatic carcinoma.[17] However, the biological role and potential mechanism of miR-27a-3p in pancreatic carcinoma remain to be elucidated. In this study, we detected the expression patterns of miR-27-3p in pancreatic carcinoma tissues and cell lines, and determined the biological roles of miR-27a-3p on pancreatic carcinoma cell migration and angiogenesis in vitro. We further identified the underlying mechanism of miR-27a-3p on its target gene, GATA6, in the migration and angiogenesis of pancreatic carcinoma, which may shed light on their targeted applications in these cancer ther ies.

Materials and Metho

Clinical Samples

Twenty-eight pairs of esh pa reatic ca inoma tissues and their corresp ing umor ti es were collected between Janua 2006 to e 2 4 following radical surgical res ion biopsy fro Department of General Surgery, Jiangxi Prov ial People's Hospital Affiliated to Nan ang University (N nchang, China). None of the pan eatic carci ma patients received any preoperative and ostoperativ chemotherapy and/or radiotherapy. All specim had een confirmed diagnosis based on hema- lin-eosin and immunohistochemical staining by pathologists. The study was approved by the ethics committee of Jiangxi Provincial People's Hospital Affiliated to Nanchang University. For the use of these clinical materials for research purposes, consent forms were signed from all patients and conducted in accordance with the regulations of Declaration of Helsinki in 1964. The clinical data were collected from each patient, including age, gender, tumor size, tumor differentiation, clinical stage, location, lymph node metastasis, vascular invasion, and peritoneal metastasis. After excision, tissue specimens were immediately frozen in liquid nitrogen for subsequent analysis.

Cell Lines and Cell Culture

The normal human pancreatic ductal epithelial line (HPDE6-C7) and pancreatic carcinoma cell lines (AsPC-1 and Panc-1) were obtained from the Cell Bank of Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). These cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, NY, USA), supplemented with 15% fetal bovine serum (FBS; Gibco, NY, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma-Aldrich, MO, USA). All cells were maintained in a 5% CO_2-humidified atmosphere at 37°C.

RNA Oligonucleotides and Cell Transfection

The miR-27a-3p inhibitor and the inhibitor scrambled control, miR-27a-3p mimic and the mimic scrambled control, and GATA6 small interfering RNA (siRNA) and the siRNA control were purchased from GeneCopoeia Company (Guangzhou, China). Cell transfection was performed by using Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's protocols. AsPC-1 and Panc-1 cells were cultured in 12-well plates and transiently transfected with equal amounts (100 pmol) miR-27a-3p inhibitor and the inhibitor scrambled control, or miR-27a-3p mimic and the mimic scrambled control, and in some cases together with 2 μg of GATA6 siRNA and the siRNA control. At 48-hr posttransfection, the transfected cells were collected for quantitative real-time PCR analysis.

RNA Isolation and Quantitative Real-Time PCR Analysis

Total RNA was isolated by using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The RNA samples (1 μg each) were then reverse-transcribed into cDNA using the Omniscript reverse transcription kit (Qiagen, Germany). Quantitative real-time PCR reaction was performed by using the Quantitect SyBr green PCR system (Qiagen). RNAU6B snRNA (U6) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as miRNA and mRNA internal control, respectively. The specific forward primer of miR-27a-3p was 5′-ACGGTTCGTGGTTCACAGTGGCTAAGTTCCG-3′. The specific forward primer of U6 was 5′-ACGCAAATTCGTGAAGCGTT-3′. Reverse primers for miR-27a-3p and U6 were provided by Qiagen. The primers for GATA6 and GAPDH were as follows: GATA6: 5′-TTCCTACGCTTCGGCATCG-3′ (sense), 5′-TGGTCGAGGTCAGTGAACAGC-3′ (antisense); GAPDH: 5′-GAGTCAACGGATTTGGTCGT-3′ (sense), 5′-TTGATTTTGGAGGGATCTCG-3′ (antisense). The relative expression of GATA6 mRNA and miR-27a-3p was calculated by using $2^{-\Delta\Delta Ct}$ method.

Wound Healing Assay

The impact of miR-27a-3p on the migration of pancreatic carcinoma cells was determined by wound healing assay. Briefly, AsPC-1 and Panc-1 cells were seeded to 6-well plates at a confluence of 60% and incubated overnight. At 24 hrs after transfection, an artificial wound was created onto the monolayer using a sterile 100 μL tip. After scratching, the floating cells were washed with phosphate buffer saline (PBS) for three times and the medium was replaced with fresh serum-free medium. Images of cell migration were captured at 0- and 12-hr time-points under a Zeiss inverted light microscope (LSM710, Zeiss, Germany) at × 100 or 200 magnification.

Capillary Tube Formation Assay

In vitro capillary tube formation assay was performed to evaluate the effect of miR-27a-3p on angiogenesis. In brief, 200 μL of Matrigel (BD Biosciences Pharmingen, CA, USA) was added to each well of 24-well plates and allowed to polymerize at 37°C for 30 mins. Before the capillary tube formation assay, to serum-starve human umbilical vein endothelial cells (HUVECs; Cyagen Biosciences, Guangzhou, China), the cells were incubated in MCDB 131 medium (Gibco, NY, USA) containing 2% microvascular growth supplement (Thermo Fisher Scientific, MA, USA) for 8 h at 37°C. Then, HUVECs (2×10^4 cells/well) were grown in tumor cell-derived media (CM) in a coated plate at 37°C. After 6 hrs, the cells were photographed under a Zeiss inverted light microscope to assess the formation of capillary-like structures. The number of branches represented the degree of in vitro angiogenesis.

Luciferase Reporter Assay

The GATA6 3′-UTR-wild type (WT) vector was constructed by inserting the amplified 3′-UTR of human GATA6 into pmirGLO luciferase reporter plasmid. Subsequently, the binding sequences interacting with the miR-27a-3p "seed" (UGACACU) were mutated from ACUGUGA to A-—A, and the mutant (MUT) GATA6 3′-UTR was also inserted into pmirGLO luciferase reporter plasmid to construct the GATA6 3′-UTR-mutant (MUT) vector. Then, GATA6 3′-UTR-WT or MUT vector (1 μg) and equal amounts (50 pmol) of the miR-27a-3p inhibitor, inhibitor scrambled control, miR-27a-3p mimic and mimic scrambled control were cotransfected into AsPC-1 and Panc-1 cells using Lipofectamine 2000. At 48-hr posttransfection, the cells were collected and analyzed for luciferase activity by using a Dual-Luciferase Reporter Assay system (Promega, WI, USA) under a Modulus Luminometer (Turner Biosystems, Sunnyvale, USA).

Western Blotting Analysis

Protein was quantified using the BCA™ protein assay kit (Pierce, IL, USA). Protein (25 μg each) was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then blotted onto PVDF membranes (Millipore, CA,

USA). The membranes were blocked with 5% non-fat milk for 2 hrs at 37°C and incubated with anti-GATA6 antibody (1:1000; #5851; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-VEGFA antibody (1:500; #sc-7269; Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-VEGFR2 antibody (1:500; #9698; Cell Signaling Technology), and anti-GAPDH antibody (1:2000; #sc-47724; Santa Cruz Biotechnology, Inc.) for overnight at 4°C. After washing with Tris-Buffered Saline and Tween 20 (TBST) 3 times, the membranes were incubated with an anti-rabbit or mouse secondary antibody (1:2500; #sc-2357 and #sc-2005; Santa Cruz Biotechnology, Inc.) for 1 hr at 37°C. Finally, a 40:1 peroxide:luminol solution (Pierce) was added to the membranes and incubated for 5 mins at 37°C. The signals were captured and the band intensity was quantified using Bio-Rad Chemidoc XRS Gel Imaging System (Bio-Rad, CA, USA).

Statistical Analysis

The SPSS 17.0 statistical software package (SPSS, Chicago, IL, USA) was applied for statistical analysis. The association between miR-27a-3p expression and clinicopathological features was evaluated using χ^2 test. Survival data were analyzed using Kaplan–Meier method and the log-rank test. One-way ANOVA with a Bonferroni correction or Student's *t*-test was used to assess the differences between groups. All data were represented as the mean of an average of three independent experiments. Significant differences were indicated as $P<0.05$.

Results

MiR-27a-3p Expression Is Upregulated in Pancreatic Carcinoma Tissues and Cell Lines

To examine the expression patterns of miR-27a-3p in pancreatic carcinoma samples, quantitative real-time PCR analysis was performed on 28 paired pancreatic carcinoma tissues and their corresponding non-tumor tissues. Our results showed that miR-27a-3p had significantly increased expression in pancreatic carcinoma tissues as compared to the corresponding non-tumor tissues (Figure 1A, $P<0.05$). In most cases, miR-27a-3p expression in tumor tissues was obviously higher than that in non-tumor tissues (Figure 1B, $P<0.05$). In addition, consistent with the results from pancreatic carcinoma samples, the expression levels of miR-27a-3p were also found to be markedly upregulated in the two pancreatic carcinoma cell lines (AsPC-1 and Panc-1) compared with that of the normal human pancreatic ductal epithelial line (HPDE6-C7) (Figure 1C, $P<0.05$). Collectively, these data indicated that miR-27-3p is frequently overexpressed in pancreatic carcinoma tissues and cell lines.

Upregulated miR-27a-3p Is Correlated with Tumor Metastasis and Poor Prognosis in Patients with Pancreatic Carcinoma

We further examined the association between miR-27a-3p expression and clinicopathological features in pancreatic carcinoma patients using chi-square test. The average expression level of miR-27a-3p in pancreatic carcinoma tissues was used as a cut-off value (0.49): of the 28 samples of pancreatic carcinoma, 12 samples (≥0.49, 42.86%) and 16 samples (<0.49, 57.14%) were classified into high and low miR-27a-3p expression groups, respectively. As shown in Table 1, high miR-27a-3p expression was significantly associated with more lymph node metastasis ($P<0.05$) and present peritoneal metastasis ($P<0.05$). There was no statistically significant association between miR-27a-3p expression and the other clinicopathological parameters, including age, gender, tumor size, tumor differentiation, location, vascular invasion, and clinical stage. In addition, the prognostic value of miR-27a-3p expression was evaluated by Kaplan–Meier method and log-rank test, we found that highly expressed miR-27a-3p was closely related to poor prognosis in patients with pancreatic carcinoma (Figure 1D, $P<0.05$). To conclude, these results provided some hint of potential metastatic role and poor prognosis for miR-27a-3p in pancreatic carcinoma.

MiR-27a-3p Promotes the Migration of Pancreatic Carcinoma Cells in vitro

In order to evaluate the potential role of miR-27a-3p on the metastasis of pancreatic carcinoma, we introduced miR-27a-3p inhibitor or inhibitor scrambled control into AsPC-1 and Panc-1 cells, meanwhile transfecting cells with miR-27a-3p mimic or mimic scrambled control. The data of quantitative real-time PCR assay confirmed that transfected miR-27a-3p inhibitor significantly decreased the expression levels of miR-27a-3p in AsPC-1 and Panc-1 cells (Figure 2A, $P<0.05$), whereas pancreatic carcinoma cells treated with miR-27a-3p mimic had significant higher miR-27a-3p levels than that cells transduced with mimic scrambled control (Figure 2B, $P<0.05$). Subsequently, wound-healing assay was conducted in pancreatic carcinoma cells, and data showed that overexpression of miR-27a-3p significantly increased AsPC-1 and

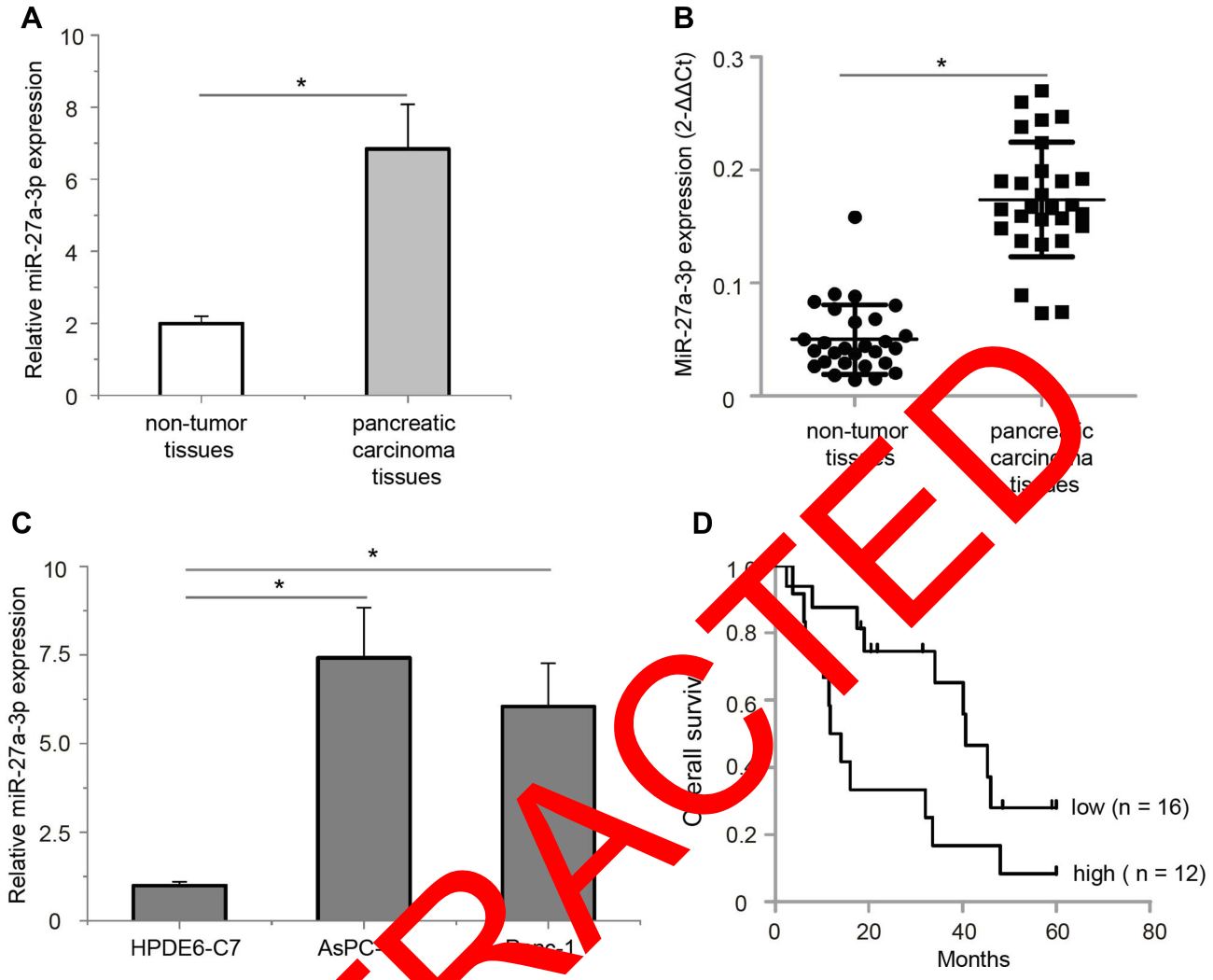

Figure 1 Expression levels of miR-27a-3p in pancreatic carcinoma samples and cell lines. (**A**) The average expression level of miR-27a-3p in pancreatic carcinoma tissues was over 3.48-fold higher than non-tumor tissues. (**B**) MiR-27a-3p expression was calculated by the $2^{-\Delta\Delta Ct}$ method in 28 pancreatic carcinoma samples; in most cases, miR-27a-3p levels in pancreatic carcinoma tissues were obviously higher than those in non-tumor tissues. (**C**) The expression levels of miR-27a-3p were remarkably higher in the AsPC-1 and Panc-1 cell lines than in the HPDE6-C7 cell line. (**D**) Pancreatic carcinoma patients with high miR-27a-3p expression (n = 12) exhibited significantly poorer overall survival rate than those patients with low miR-27a-3p expression (n = 16) as defined by log-rank test. *P<0.05.

Panc-1 cells migration in vitro (Figure 2C, P<0.05). Nevertheless, the migrated ability of pancreatic carcinoma cells was significantly blocked by miR-27a-3p inhibitor (Figure 2C, P<0.05). In short, these results demonstrated that miR-27a-3p promotes the migratory capability of pancreatic carcinoma cells.

MiR-27a-3p Induces HUVECs Angiogenesis in vitro

Angiogenesis is a hallmark of cancer, and numerous studies have demonstrated the important role of angiogenesis in facilitating tumor metastasis.[18] Therefore, we attempted to explore whether miR-27a-3p had a promotive effect on angiogenesis. HUVECs were used as the source of endothelial cells to perform in vitro capillary tube formation assay. Firstly, we transfected miR-27a-3p mimic or mimic scrambled control and miR-27a-3p inhibitor or inhibitor scrambled control into HUVECs to examine changes in tube formation. As shown in Figure 3A, miR-27a-3p overexpression significantly increased tube formation capability compared with the mimic scrambled control group (P<0.05), while miR-27a-3p inhibitor reduced tube formation (Figure 3B, P<0.05). Since the VEGFA/VEGFR2 pathway plays a key role in angiogenesis both physiologically and pathologically, we next detected the expression levels of VEGFA and VEGFR2 in AsPC-1 and Panc-1 cells after transfection of miR-27a-3p mimic and inhibitor. The data showed that overexpression of miR-27a-3p upregulated the protein expression levels of VEGFA and

Table 1 miR-27a-3p Expression and Its Association with Clinicopathological Features of Pancreatic Carcinoma Patients

Characteristics	Case (No.)	Expression of miR-27a-3p		χ^2	*P*-Value
		High	Low		
Age (years)					
<50	7	3	4	<0.001	1.000
≥50	21	9	12		
Gender					
Male	15	7	8	0.191	0.662
Female	13	5	8		
Location					
Head	10	6	4	1.867	0.243
Body/tail	18	6	12		
Tumor size (cm)					
<4	19	10	9	2.306	0.223
≥4	9	2	7		
Tumor differentiation					
High/medium	11	7	4	3.194	0.121
Low	17	5	12		
Vascular invasion					
Absent	23	9	14	0.730	0.624
Present	5	3	2		
Peritoneal metastasis					
No	21	6	15	7.000	0.023*
Yes	7	6	1		
Lymph node metastasis					
Absent	10	1	9	6.[illegible]7	0.[illegible]*
Present	18	11	7		
Clinical stage					
I/II	12	3		2.734	0.136
III/IV	16	9	7		

Note: *P<0.05.

VEGFR2 (Figure 3C, P<0.05). While miR-27a-3p knockdown downregulated VEGFA and VEGFR2 expression (Figure 3D, P<0.05). Together, these results demonstrated that miR-27a-3p facilitates the angiogenesis of pancreatic carcinoma cells by activating VEGFA/VEGFR2 signaling pathway.

GATA6 Is the Target Gene of miR-27a-3p

To elucidate the potential mechanism of miR-27a-3p in pancreatic carcinoma metastasis, we predicted that the "seed" sequence (UGACACU) of miR-27a-3p could completely bind the ACUGUGA of GATA6 3′-UTR sequences using TargetScan and PicTar computer-aided algorithms (Figure 4A). Then, we analyzed the correlation between GATA6 mRNA levels and miR-27a-3p expression in 28 pancreatic carcinoma samples. As shown in Figure 4B, a significantly negative correlation between GATA6 mRNA levels and miR-27a-3 expression was found in pancreatic carcinoma samples (P<0.01). Further study showed that transfection of AsPC-1 and Panc-1 cells with miR-27a-3p mimic significantly decreased the mRNA and protein expression levels of GATA6 compared with the mimic scrambled control treated cells (Figure 4C and E, P<0.05), while transfection with miR-27a-3p inhibitor prominently increased the levels of GATA6 expression in AsPC-1 and Panc-1 cells (Figure 4D and F, P<0.05).

To identify whether miR-27a-3p could directly target GATA6 expression, a luciferase reporter assay was conducted. Luciferase reporter plasmids containing the GATA6 3′-UTR-WT (Figure 5A) or MUT (Figure 5B) sequence was constructed to verify the binding site between GATA6 and miR-27a-3p. When AsPC-1 and Panc-1 cells were co-transfected with GATA6 3′-UTR-WT or MUT vector, miR-27a-3p mimic or mimic scrambled control, and miR-27a-3p inhibitor or inhibitor scrambled control, the data showed that miR-27a-3p mimic significantly downregulated relative luciferase activity of GATA6 3′-UTR-WT vector in pancreatic carcinoma cells compared with the mimic scrambled control (Figure 5C, P<0.05), but not GATA6 3′-UTR-MUT vector. In contrast, transfected with miR-27a-3p inhibitor in pancreatic carcinoma cells obviously increased relative luciferase activity of GATA6 3′-UTR-WT vector compared with the inhibitor scrambled control (Figure 5D, P<0.05), but not GATA6 3′-UTR-MUT vector. These observations indicated that GATA6 is the target gene of miR-27a-3p, and miR-27a-3p directly targets GATA6 to downregulate endogenous GATA6 expression in pancreatic carcinoma cells.

MiR-27a-3p/GATA6/VEGFA/VEGFR2 Signaling Pathway Is Responsible for the Migration and Angiogenesis of Pancreatic Carcinoma Cells

To test the hypothesis that miR-27a-3p/GATA6/VEGFA/VEGFR2 signaling pathway was involved in the metastasis and angiogenesis of pancreatic carcinoma cells, GATA6 siRNA or the siRNA control was transfected into pancreatic carcinoma cells to knockdown GATA6 expression. The results revealed that AsPC-1 and Panc-1 cells transfected with GATA6 siRNA exhibited lower

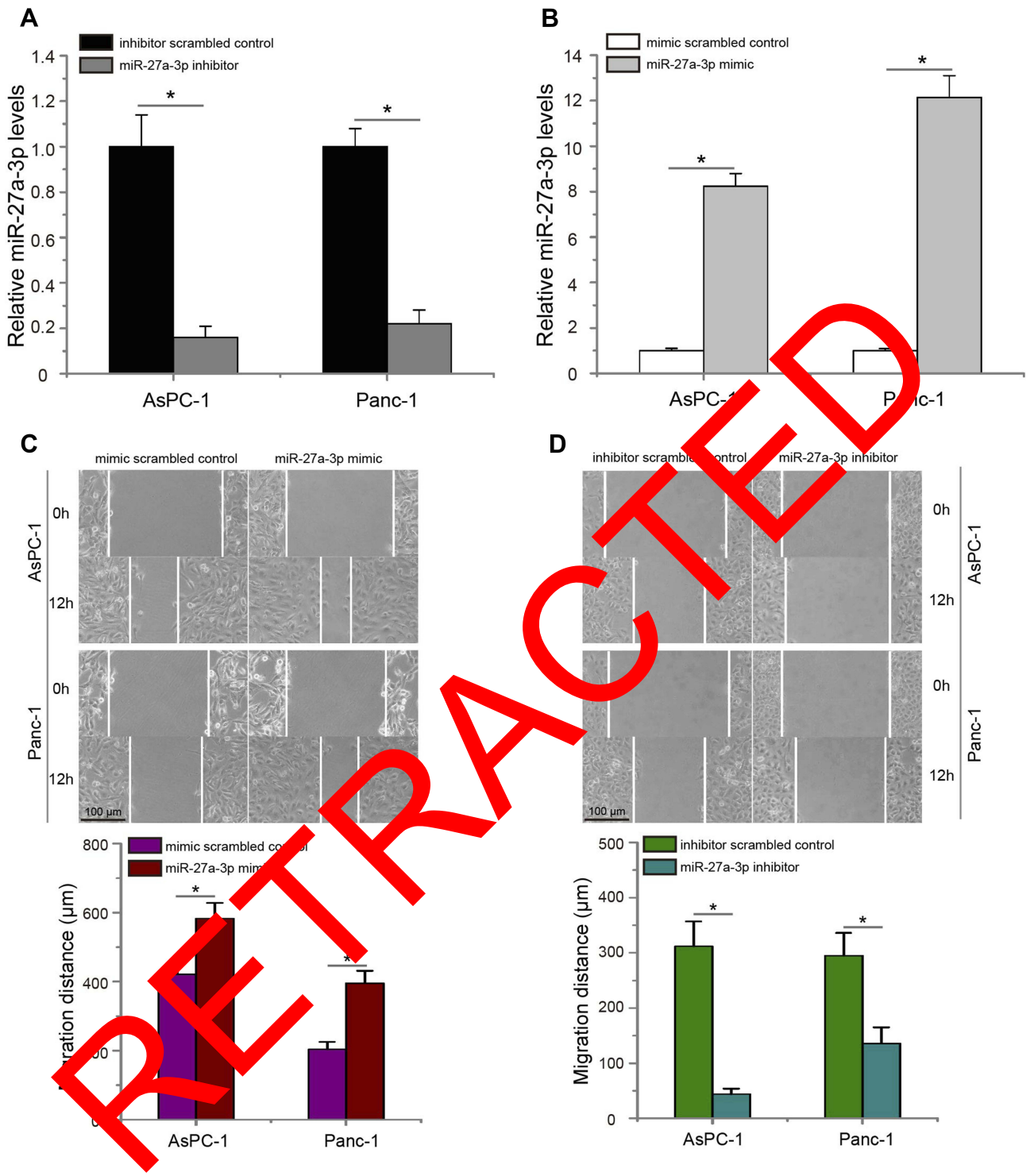

Figure 2 MiR-27a-3p promotes the metastasis of pancreatic carcinoma cells in vitro. (**A**) Expression changes of miR-27a-3p in AsPC-1 and Panc-1 cells after transfection of miR-27a-3p inhibitor or inhibitor scrambled control. (**B**) Pancreatic carcinoma cells treated with miR-27a-3p mimic had significant higher miR-27a-3p expression than that cells transfected with mimic scrambled control. (**C**) MiR-27a-3p mimic significantly increased AsPC-1 and Panc-1 cells migration compared with the mimic scrambled control group. (**D**) MiR-27a-3p inhibitor significantly suppressed AsPC-1 and Panc-1 cells migration. *P<0.05.

GATA6 protein expression than cells treated with the siRNA control (Figure 6A, P<0.05). Interestingly, GATA6 siRNA could decrease the protein expression of GATA6 in AsPC-1 and Panc-1 cells treated with miR-27a-3p inhibitor (Figure 6B, P<0.05). Of note, miR-27a-3p inhibitor-treated AsPC-1 and Panc-1 cells transfected with the GATA6 siRNA exhibited significantly higher cell migration (Figure 6C, P<0.05) and tube formation

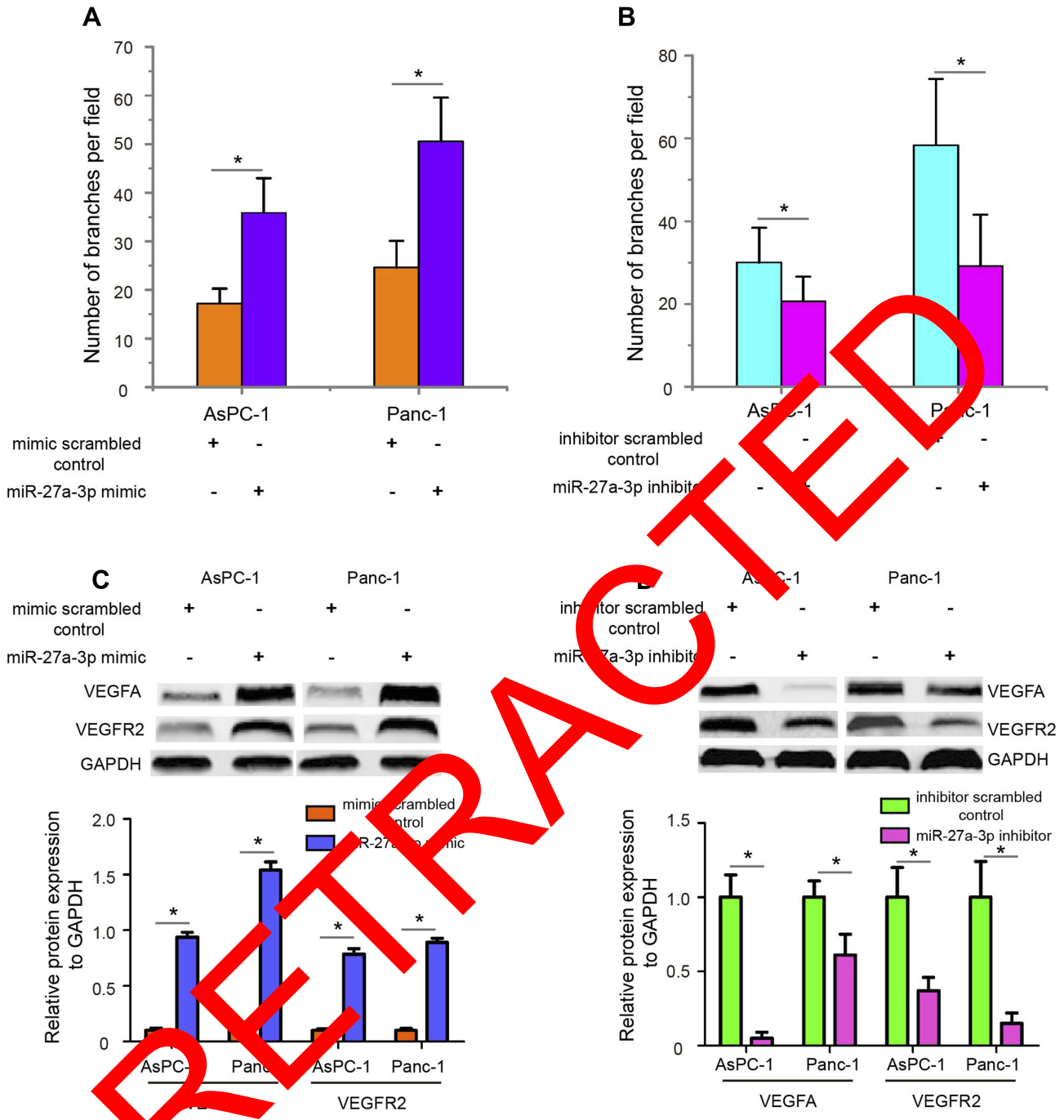

Figure 3 MiR-27a-3p enhances the angiogenesis of pancreatic carcinoma cells in vitro. (**A**) The tube formation capacities of HEVECs were observed after transfected miR-27a-3p mimic or mimic scrambled control. (**B**) MiR-27a-3p knockdown significantly decreased tube formation capability compared with inhibitor scrambled control group. (**C**) The protein expression of VEGFA and VEGFR2 in AsPC-1 and Panc-1 cells was detected by Western blotting analysis after overexpression of miR-27a-3p. GAPDH was used as an internal control. (**D**) MiR-27a-3p knockdown downregulated the VEGFA and VEGFR2 protein expression in pancreatic carcinoma cells. *P<0.05.

capability (Figure 6D, P<0.05) than cells co-transfected with the miR-27a-3p inhibitor and siRNA control. Moreover, the protein expression levels of VEGFA and VEGFR2 in miR-27a-3p inhibitor-treated AsPC-1 and Panc-1 cells were partially reversed upon knockdown of GATA6 (Figure 6E, P<0.05). This model of miR-27a-3p/GATA6/VEGFA/VEGFR2 signaling pathway in pancreatic carcinoma is thus summarized in Figure 7. Thus, our study suggested that upregulated miR-27a-3p promotes cell angiogenesis and migration of pancreatic carcinoma via epigenetic silencing of GATA6 and activating VEGFA/VEGFR2 signaling pathway.

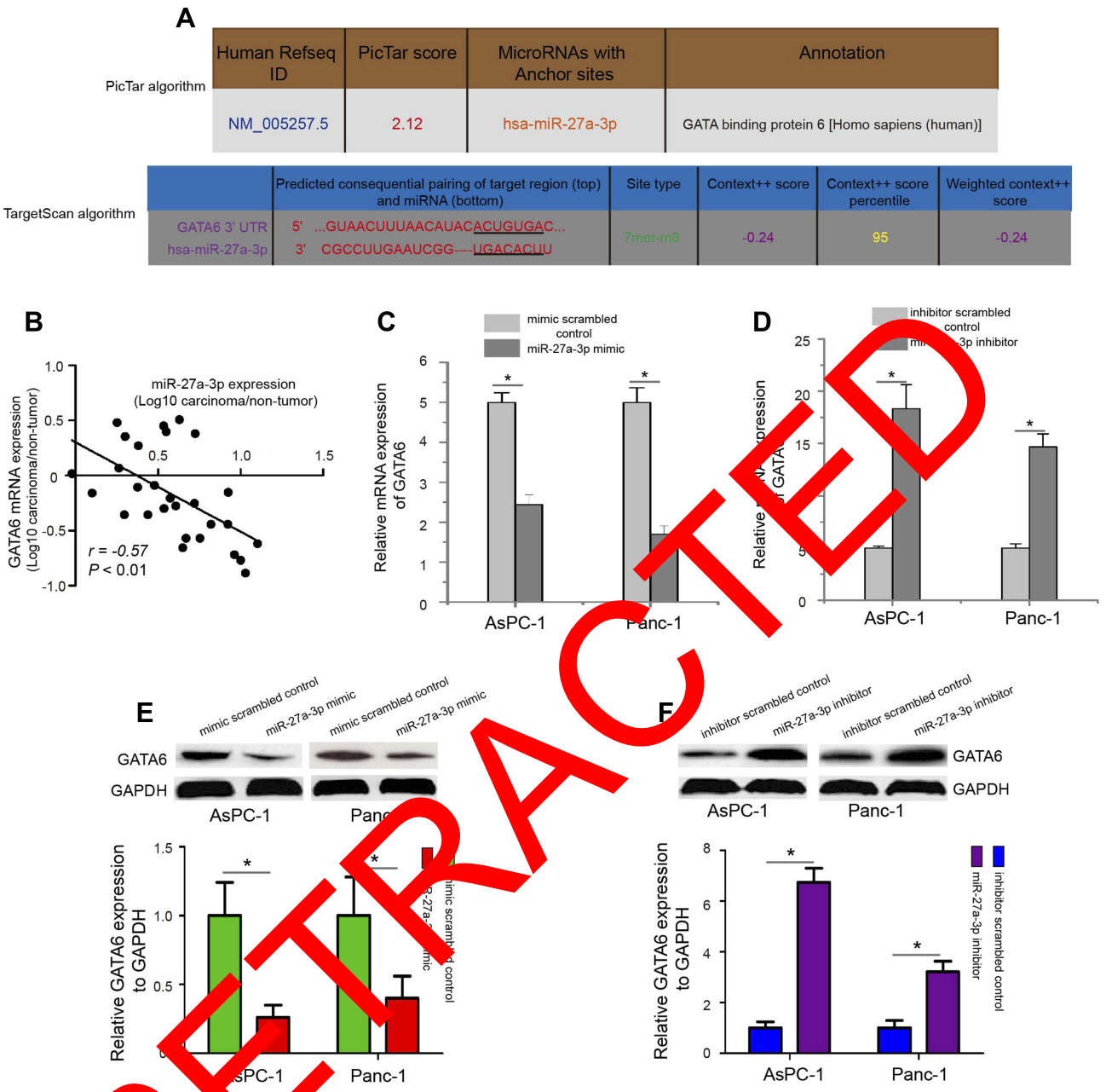

Figure 4 Mo[illegible]ation of [illegible]R-27a-3p a[illegible] GATA6 expression in pancreatic carcinoma cells. (**A**) MiR-27a-3p could completely bind with the ACUGUGA of GATA6 3′-UTR sequence[illegible]ng TargetS[illegible] and PicTar computer-aided algorithms. (**B**) The expression relationship of GATA6 mRNA and miR-27a-3p in pancreatic carcinoma samples by Spearman c[illegible]elatio[illegible] a[illegible] mRNA and protein expression levels of GATA6 were evaluated in AsPC-1 and Panc-1 cells after miR-27a-3p overexpression. (**D** and **F**) MiR-27a-3p in[illegible]-treated pancreatic carcinoma cells significantly increased GATA6 mRNA and protein expression. *P<0.05.

Discussion

Increasing number of studies has demonstrated that aberrant expression of miRNAs contributes to tumor initiation and progression in pancreatic carcinoma.[19] Some of miRNAs are associated with the metastatic abilities of pancreatic carcinoma cells. For example, Fu et al reported that downregulated miR-98-5p promotes pancreatic carcinoma cell proliferation and metastasis by downregulation of MAP4K4 and inhibition of the downstream MAPK/ERK signaling.[20] Xiong et al showed that deregulated expression of miR-107 inhibits metastasis of pancreatic carcinoma cells through inhibition PI3K/Akt signaling pathway.[21] Yu et al demonstrated that miR-448 suppresses metastasis of pancreatic carcinoma cells through targeting JAK1/STAT3 signaling pathway.[22] Fan et al suggested that miR-454 acts as a suppressor

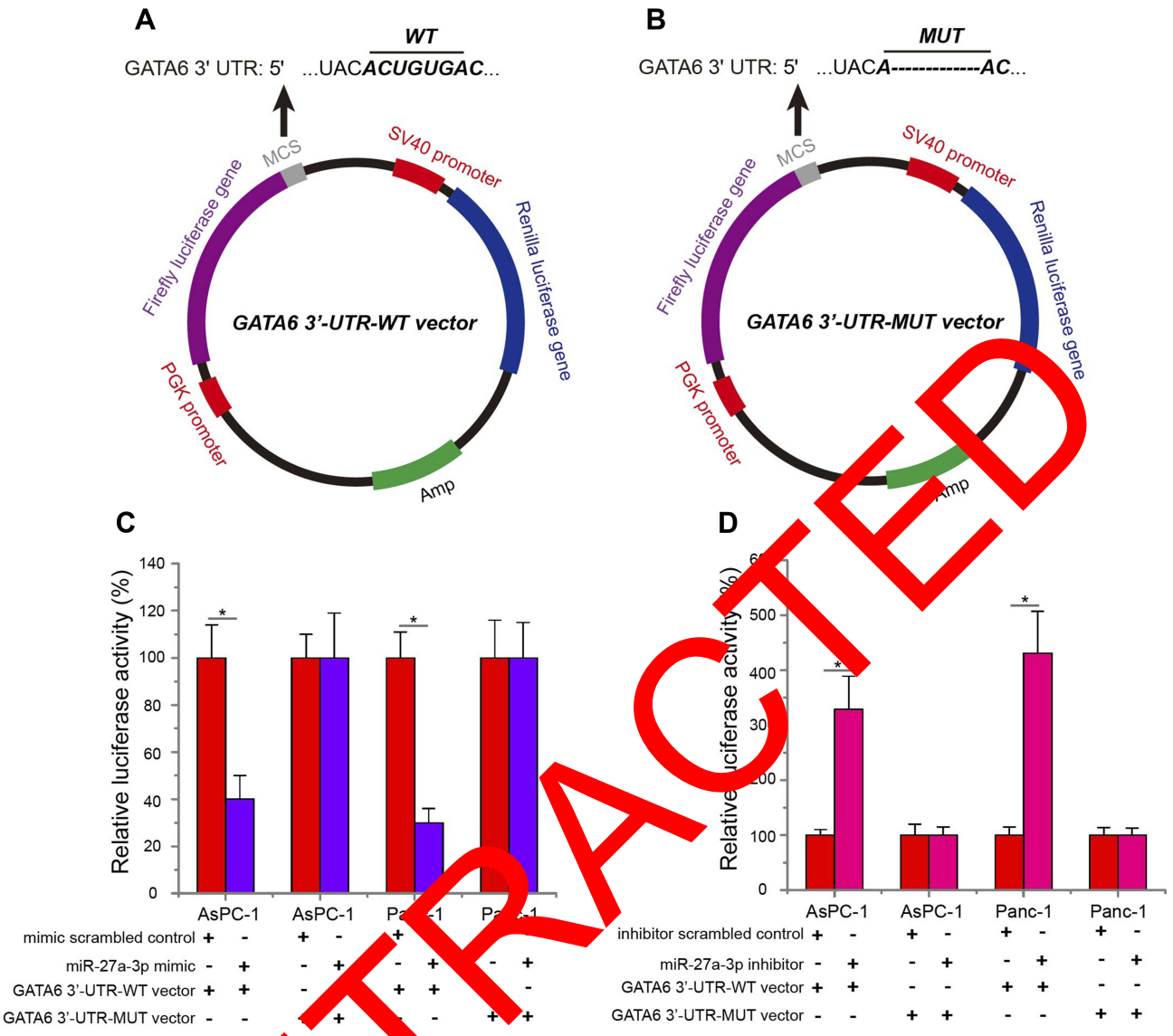

Figure 5 GATA6 was the direct target gene of miR-27a-3p. pmirGLO luciferase reporter plasmid containing the GATA6 3′-UTR-WT (**A**) or MUT (**B**) sequence was constructed to verify the binding site between GATA6 and miR-27a-3p. (**C**) In pancreatic carcinoma cells, miR-27a-3p overexpression suppressed the relative luciferase activity of GATA6 3′-UTR-WT vector. miR-27a-3p mimic-treated pancreatic carcinoma cells have no influence on the relative luciferase activity of GATA6 3′-UTR-MUT vector. (**D**) In pancreatic carcinoma cells, miR-27a-3p inhibition augmented the relative luciferase activity of GATA6 3′-UTR-WT vector. miR-27a-3p inhibitor-transfected pancreatic carcinoma cells have no influence on the relative luciferase activity of GATA6 3′-UTR-MUT vector. *P<0.05.

in tumor proliferation, angiogenesis, and metastasis in pancreatic carcinoma cells by targeting LDL-receptor-related protein 6.[23] However, the biological function and potential mechanism of miR-27a-3p in pancreatic carcinoma are rarely reported.

MiR-27a-3p is a member of the miR-23a~27-24a cluster. Recent studies have demonstrated that miR-27a-3p is frequently upregulated in several metastatic tumors, including osteosarcoma, colorectal cancer, nasopharyngeal carcinoma, and gastric cancer, and contributes to tumor progression and metastasis.[15,24–26] To confirm the feature of miR-27a-3p expression with pancreatic carcinoma, we examined miR-27a-3p expression in 28 paired pancreatic carcinoma samples with real-time PCR analysis. The results showed that miR-27a-3p levels were significantly upregulated in pancreatic carcinoma tissues and cell lines compared with non-tumor tissues and normal human pancreatic ductal epithelial lines, respectively, and high miR-27a-3p expression was associated with more lymph node metastasis, present peritoneal metastasis, and poor prognosis. The data were in line with previous works of literature showing an upregulation of miR-27a-3p in several metastatic

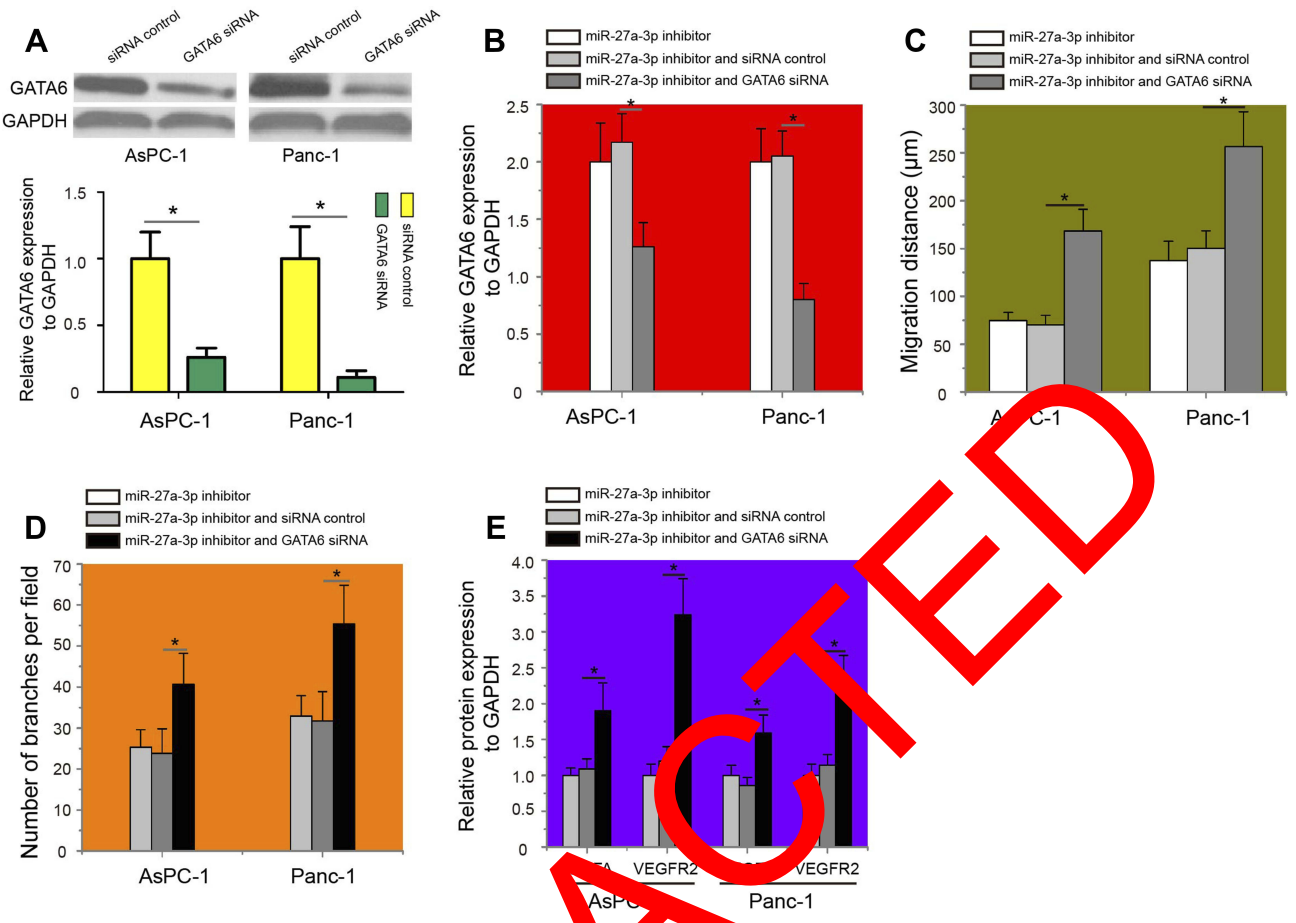

Figure 6 GATA6 knockdown partially reversed the effects of miR-27a-3p on the metastasis and angiogenesis of pancreatic carcinoma cells by regulation of VEGFA/VEGFR2 pathway. (**A**) Transfection of GATA6 siRNA could decrease the GATA6 protein expression in AsPC-1 and Panc-1 cells. (**B**) Western blotting analysis of GATA6 expression in miR-27a-3p inhibitor-treated pancreatic carcinoma cells after transfected with either GATA6 siRNA or the siRNA control. (**C**) GATA6 inhibition reversed the migration-suppressive effects of miR-27a-3p inhibitor on pancreatic carcinoma cells. (**D**) miR-27a-3p inhibitor-treated AsPC-1 and Panc-1 cells transfected with the GATA6 siRNA exhibited significantly higher tube formation capacity. (**E**) Western blotting was performed to determine the protein expression levels of VEGFA in miR-27a-3p inhibitor-treated pancreatic carcinoma cells after transfected with GATA6 siRNA. The protein expression levels of VEGFR2 in miR-27a-3p inhibitor-treated AsPC-1 and Panc-1 cells were partially reversed upon knockdown of GATA6. *P<0.05.

tumors,[24–26] not only confirming the overexpression pattern of miR-27a-3p but also suggesting an important metastatic function and prognosis of miR-27a-3p in the progression of pancreatic carcinoma.

As for the role of miR-27a-3p in metastatic tumor, overexpression of miR-27a-3p promoted gastric cancer cell migration in vitro and in vivo.[15] MiR-27a-3p inhibition suppresses malignant phenotypes of osteosarcoma cells.[24] Consistent with these reports,[15,24] our results showed that overexpression of miR-27a-3p significantly promoted the migration of pancreatic carcinoma cells and induced HUVECs angiogenesis in vitro. Consistently, miR-27a-3p knockdown was found to reduce the migration and angiogenesis in vitro. Angiogenesis is a fundamental process for tumor progression, and tumor cells can produce a number of growth factors to modulate angiogenesis. Evidence has shown that VEGFA/VEGFR2 pathway plays a critical role in regulating angiogenesis.[27] VEGFA induces biological effects in a paracrine/autocrine manner by binding to its receptors, especially VEGFR2.[28] In the current study, we also found that miR-27a-3p promoted the angiogenesis of pancreatic carcinoma cells by activating VEGFA/VEGFR2 signaling pathway, and blockade of miR-27a-3p reduced angiogenesis via suppressing VEGFA/VEGFR2 expression. The data provided substantial pieces of evidence that miR-27a-3p promotes the migration and angiogenesis of pancreatic carcinoma cells.

As miRNAs execute their biological functions by regulating the expression of their target genes, identifying the target genes of miR-27a-3p is important to illustrate the potential mechanism of miR-27a-3p in the metastasis of pancreatic carcinoma cells. Several genes have been

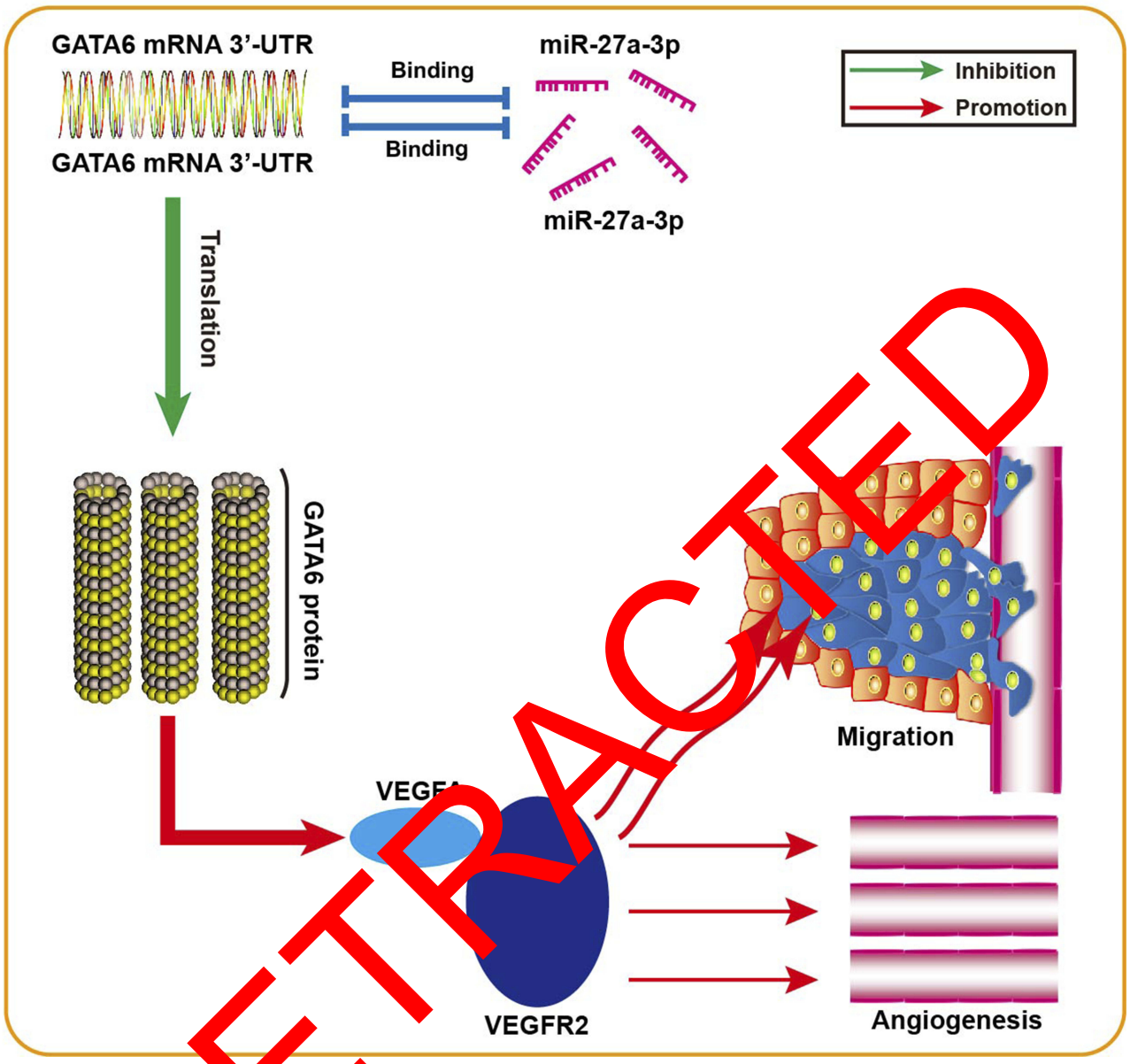

Figure 7 Schematic of [illegible]-27a-3p/GATA6/VEGFA/VEGFR2 signaling pathway in pancreatic carcinoma.

reported as potential targets of miR-27a-3p including MAX interactor 1, dimerization protein (MXI1), F-box and WD repeat domain-containing 7 (FBXW7), cadherin 5 (CDH5), and B-cell translocation gene 2 (BTG2).[15,29–31] GATA6 is a member of an evolutionarily conserved family of zinc finger transcription factors that bind to the (A/T)GATA(A/G) consensus sequence to activate or repress gene expression, which is found to be aberrantly expressed in several types of cancers and functions as an oncogene or tumor suppressor according to the tumor origin.[32] An oncogenic role was proposed for GATA6 in pancreatic carcinoma based on the occurrence of GATA6 gains/amplifications in a small proportion of tumors, whereas a tumor-suppressive role of GATA6 has been postulated in pancreatic carcinoma mouse model.[33,34] Recently, Martinelli et al provided mechanistic insight into the tumor-suppressive function of GATA6 in pancreatic carcinoma.[35] GATA6 is a regulator of canonical epithelial differentiation, and loss of GATA6 expression is both prognostic and predictive of response to adjuvant therapy. Importantly, our current analysis in pancreatic carcinoma samples also found that GATA6 mRNA levels inversely

correlated with miR-27a-3p expression. These results were consistent with Martinelli's report.[35]

Furthermore, bioinformatics analysis revealed that GATA6 would be theoretically a potential target of miR-27a-3p and GATA6 mRNA 3′-UTR has a putative miR-27a-3p binding site. As miRNAs usually negatively regulate gene expression by binding to 3′-UTR of target mRNAs, we speculated that miR-27a-3p could be capable of inhibiting GATA6 expression via the binding site in the mRNA 3′-UTR. Based on a luciferase reporter assay, we confirmed that miR-27a-3p directly binds to the 3′-UTR region of GATA6 mRNA in pancreatic carcinoma cells. In addition, we detected the endogenous expression of GATA6 after alteration of miR-27a-3p levels in AsPC-1 and Panc-1 cells. As expected, following knockdown of miR-27a-3p, the mRNA and protein expression levels of GATA6 expression were significantly increased, whereas transfection with miR-27a-3p mimic obviously decreased the levels of GATA6 expression.

The above data indicated that miR-27a-3p directly targeted GATA6 to downregulate endogenous GATA6 expression in pancreatic carcinoma cells. These studies encouraged us to hypothesize that miR-27a-3p might exert its metastatic role by downregulation of GATA6. In support of this hypothesis, reduced GATA6 expression by GATA6 siRNA was noted in miR-27a-3p inhibitor-treated pancreatic carcinoma cells. Rescue experiments provided pieces of evidence that miR-27a-3p inhibitor-transfected AsPC-1 and Panc-1 cells transfected with the GATA6 siRNA exhibited significantly higher cell migration and tube formation capability. Moreover, GATA6 knockdown reversed the protein expression levels of VEGFA and VEGFR2 in AsPC-1 and Panc-1 cells by miR-27a-3p inhibitor. Herein, our results implied that miR-27a-3p/GATA6/VEGFA/VEGFR2 signaling pathway is responsible for the migration and angiogenesis of pancreatic carcinoma cells. However, it should be mentioned that miR-27a-3p can also downregulate target genes other than GATA6. Therefore, whether there are other signaling pathways of miR-27a-3p-mediated metastatic role in pancreatic carcinoma needs further study.

In conclusion, our findings demonstrated that miR-27a-3p is upregulated in pancreatic carcinoma, and miR-27a-3p promoted the migration and angiogenesis of pancreatic carcinoma cells. GATA6 as miR-27a-3p targeting gene played a critical role in the activation of VEGFA/VEGFR2 signaling pathway during metastatic progression of pancreatic carcinoma. These observations highlight a rationale to investigate the therapeutic benefits of miR-27a-3p in the treatment of pancreatic carcinoma.

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Disclosure

The authors declare that they have no competing interests.

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