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Original Article

MicroRNA-27a-3p promotes the metastasis and epithelial-mesenchymal transition of gastric cancer via targeting RUNX1

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Abstract: Increasing evidence shows that microRNAs (miRNAs) are a novel class of gene regulators, and play a vital role in tumor development and progression. MicroRNA-27a (miR-27a) was previously reported dysregulated expression in human carcinoma, including gastric cancer (GC). However, till now, the mechanism that miR-27a functions as an oncogene is still not well known. As we all known, there are two isoforms of mature miR-27a, miR-27a-5p and miR-27a-3p. In this study, we figure out the link between miR-27a-3p and RUNX1 and the overexpression of miR-27a-3p markedly promote gastric cancer cell proliferation, invasion, and migration in vitro. We also describe that miR-27a-3p have effect on the EMT process. Furthermore, miR-27a-3p repressed RUNX1 expression by directly binding to 3-untranslated region (UTR) of RUNX1 in gastric cancer, and the inverse correlation was observed between the expressions of miR-27a-3p and RUNX1 mRNA in primary GC tissues. In turn, the restoration of RUNX1 led to suppressed proliferation, invasion, and migration of GC cells. In vivo, miR-27a-3p promotes tumor growth of GC. Taken together, these results suggested that the miR-27a-3p/RUNX1 axis promotes GC progression by directly downregulating RUNX1 expression and may be employed as a novel prognostic marker and therapeutic target in the management of gastric cancer.

Keywords: miR-27a-3p, gastric cancer, RUNX1, epithelial-mesenchymal transition (EMT), metastasis

Introduction

Gastric cancer (GC) is among the most common malignancies and the second leading cause of cancer-related death worldwide [1]. It is proposed that the incidence of the GC has significant region differences, particularly in Eastern Asia, Eastern Europe, and South America [2]. Particular clinical methods are currently underway to improve diagnosis and treatment for the GC patients, including advanced surgical resection, anti-Her-2 molecular-targeted therapies, radiation and chemotherapeutic therapy; nevertheless, the overall 5-year survival rates remains as poor as 10% to 40% [3]. Gastric cancer is a complex genetic disease accompanying various genetic and epigenetic alteration. However, the common molecular mechanism underlying the proliferation, migration and invasion of GC remain to be elucidated.

MicroRNAs (miRNAs) are a kind of diverse, short, non-coding RNA with 20-22 nucleotides in length that suppress gene expression by directly binding to the complementary sequences in 3'-untranslated region (UTR) of their target gene mRNA to induce mRNA degradation and suppression of translation [4, 5]. Rapidly growing evidence suggests that miRNAs were involved in diverse physiological and pathological processes including cell proliferation, differentiation, motility, apoptosis, angiogenesis and metastasis [6]. An increasing number of miRNAs were shown to be involved in metastasis and invasion of GC, including miR-21 [7], miR-25 [8], miR-29 [9, 10], miR-30b [11], miR-141 [12], and miR-223 [13]. Although compelling evidences indicated that miR-27a-3p is associated with gastric carcinogenesis, where miR-27a-3p as an oncogene and a promising diagnostic biomarker [14], the role of miR-27a-3p in GC metastasis and invasion remains to be demonstrated.

RUNX1 belongs to the RUNX family with the other two members, RUNX2 and RUNX3, each

having distinct tissue-specific express patterns and cell-context dependent functions through interactions with their common partner, core binding factor beta CBFβ [15]. As a transcription factor, RUNX1 is involved in multiple signaling pathways, including the RAS, ERK, TGF-β and WNT pathways, in biological process and cancer [16-19]. Early evidences have described that RUNX1 is deeply associated with leukemia [20] and breast cancer [21, 22]. However, the regulatory activity of RUNX1 is not confined to hematopoietic lineage and breast tissue. Accumulating evidence suggests significant contributions by RUNX1 as a tumor suppressor in development of gastric cancer [23].

In this present study, we examined the expression patterns of miR-27a-3p and RUNX1 in gastric cancer tissues and cell lines, and validated the negative relationship between miR-27a-3p and RUNX1. We found that RUNX1 is a direct target of miR-27a-3p, and RUNX1 overexpression could partially attenuate the effect of miR-27-3p in GC. Furthermore, our data showed that miR-27a-3p directly downregulated RUNX1 expression through binding to RUNX1-3'-UTR, and suggest an important regulatory role in the EMT process in GC.

Materials and methods

Patients and cancer specimens

Fresh frozen human gastric GC samples and their corresponding normal gastric tissue samples were obtained from the Tumor Bank Facility of Tianjin Medical University Cancer Institute and Hospital and National Foundation of Cancer Research (TBF of TMUCIH & NFCR). The types of all the tumors were confirmed by the pathologic analysis. The use of all the human materials were in accordance with the ethical guidelines of the Declaration of Helsinki and were approved by the Institutional Review Board.

Cell culture

Human GC cell lines of HGC-27, MGC-803 and BGC823 were purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM/high glucose (HGC-27, BGC823 or HGC-27) medium (HyClone) supplemented with 10% FBS penicillin (100 U/mL) and streptomycin (100 ug/mL) in a humidified environment with 5% CO_2 at 37°C, respectively.

RNA extraction and quantitative RT-PCR

Total RNA from frozen tissues or cells were extracted by TRIzol (Invitrogen) according to the instructions of manufacturer. Isolated total RNA was using a reverse transcription kit (Cat. No. RR037a; Takara, Tokyo, Japan) to obtain the miRNAs or mRNAs SYBR® Premix Ex Taq™ (Cat. No. RR420A, Takara) were employed for quantitative RT-PCR of miRNA and mRNA on an ABI 7500 PCR system (Applied Biosystems). The primers of used in this study are listed as follows: RUNX1-forward: 5'-CTGCCCATCGCTTTCAAG--GT-3'; RUNX1-reverse: 5'-GCCGAGTAGTTTTC ATCATTGCC-3'; GAPDH-forward: 5'-AGAAGGCTGGGGCTCATTTG-3'; GAPDH-reverse: 5'-AGGGGCCATCCACAGTCTTC-3'. Primers for U6 and miR27a-3p were purchased from GeneCopoeia (RiboBio). U6 RNA and GAPDH were used as miRNA and mRNA internal control respectively. Each sample was run in triplicate. The relative expression was calculated using the relative quantification equation (RQ) = $2^{-\Delta\Delta Ct}$.

Western blot assay

Total protein was extracted from cultured cells by lysing in RIPA buffer, and the lysates were analyzed using the standard Western blotting analyses. GAPDH proteins serving as a loading control. Antibodies used: RUNX1 from Abcam; Fibronectin, Vimentin from Sigma; E-cadherin, α-catenin, γ-catenin, N-cadherin from BD Bioscience. The detection was achieved by incubation with HRP-conjugated secondary Abs (Santa Cruz Biotechnology). Bound proteins were visualized with ECL Plus Western Blot Detection Reagents (GE Healthcare).

Plasmid construction and transfection

The full length cDNA of RUNX1 was inserted into the empty pCMV-Tag2B vector in the restriction digestion sites EcoRI and XhoI. For the construction of human RUNX1-3'UTR, the potential miR-27a-3p-binding sequences containing wild-type or mutant seed region were synthesized and cloned into the SpeI and HindIII restriction enzyme digestion sites of pMIR-REPORT luciferase vector (Applied Biosystems). The following primers were used to generate specific fragments: RUNX1-forward:5'-

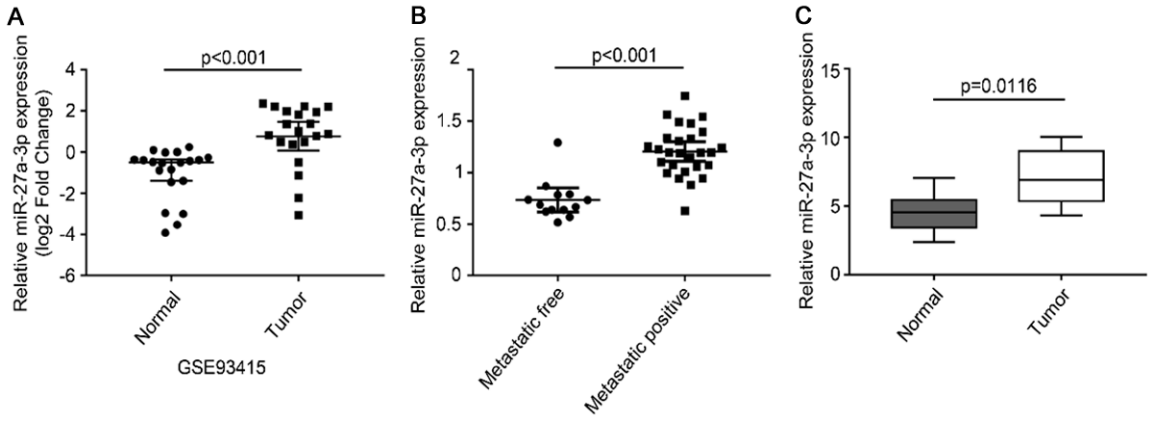

Figure 1. miR-27a-3p was increased in GC tissues and correlated with lymph node metastatic capacity in GC tissues. A. Expression of miR-27a-3p was upregulated in GC tissues compared to normal gastric tissues based on the data downloaded from the GEO database (GSE93415). B. qPCR data of miR-27a-3p in primary gastric cancer (with or without lymph node metastasis). C. Measurement of miR-27a-3p expression in 21 GC specimens and adjacent normal gastric tissues.

CGGAATTCATGGCTTCAGACAGCATA--TT-3', RUNX1-reverse: 5'-CCCTCGAGTCAGTAGGGCCTCCACACGG-3',UTR-wildtype-forward: 5'-GGACTAGTCAGGATCTCGCTGTAGGT-3', UTR-wildtype-reverse: 5'-CCAAGCTTATGGTCAAAGCAAGAAAGAA-3'. The mutant fragment were also constructed as a negative control (mut1: ACUGUGAA to AAGGGGCA, mut2: CUGUGAA to CGAUACA). Lipofectamine 2000 reagent (Invitrogen) was used for the transfection of plasmids, miRNA mimics and miRNA inhibitor.

Luciferase reporter plasmids and assays

MGC-803 cells were seeded in 24-well plates at a density of 5×10^4 cells per well and transiently transfected with mutant or wild-type luciferase reporter plasmids and Renilla luciferase control vector (pRL-TK), and then the cells were transfected with miR-NC or miR-27a-3p mimics (purchased from RiboBio) at a final concentration of 50 nmol/L. after transfection for 48 hours, luciferase activity was measured using a dual-luciferase reporter system (Promega) via GloMaxLuminometer. The firefly luciferase activity was normalized to the Renilla luciferase activity.

Growth curve analysis

At the beginning, each well of a 24-well pallet were seeding 1×10^4 BGC823 cells in triplicate, and transfect with microRNA mimics. The cells were maintained in culture media for 96 hours and counted the number every 12 hours. To plot the growth curve, the seeding cells were trypsinized and manually countedbyusing Neubauer chamber under the microscope. The growth curve was then plotted using the cell counting data.

Cell invasion assay

Transwell chamber filters (Millipore) were coated with Matrigel. After transfection with miR-NC, miR-27a-3p mimics, empty vector, and RUNX1 vector respectively, BGC823 cells were suspended in serum-free DMEM media at a concentration of 1×10^5/mL, and then the count of 50,000 cells (500 μL) per well were seeded into the upper chamber of the transwell. Following, the chamber was transferred to a well including 500 μL of DMEM media containing 10% fetal bovine serum. After incubation for 24 hours, the media in the top well were discarded and the cells attached on the top of the membrane were removed by wiping with cotton swabs. The membranes were then stained with crystal violet and the remaining cells were counted. Four high powered fields were counted for each membrane.

Cell proliferation assay

To evaluated the role of miR-27a-3p in cell proliferation, a MTT assay was performed following the manufacturer's instructions. The cells transfected with anti-miR-27a-3p and anti-miR-NC (5000 cells/well, 6 repeated wells) were seeded into 96-well plates and cultured for 24

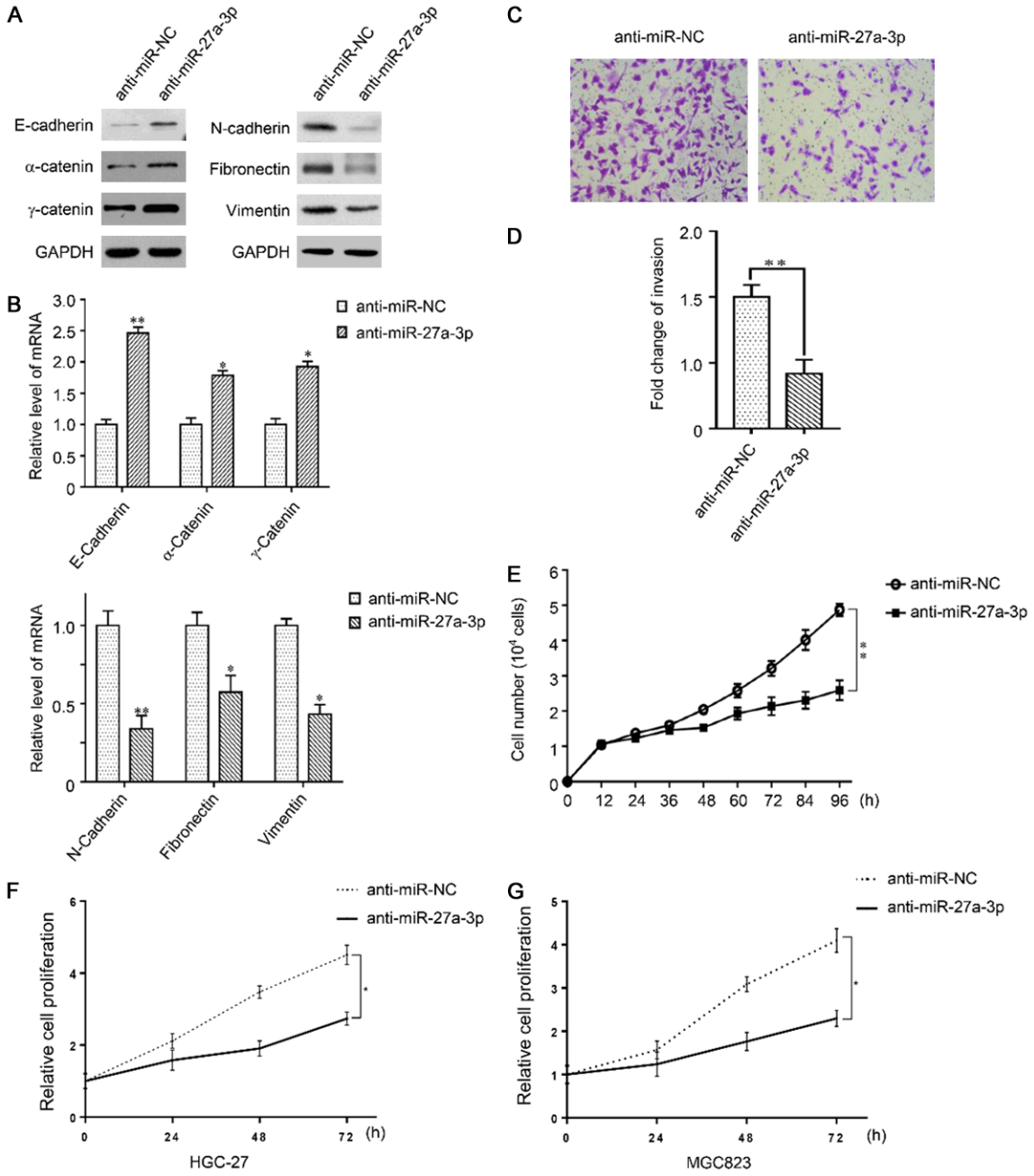

Figure 2. Decreasing the expression of miR-27a-3p suppressed invasion, proliferation and EMT process in BGC823 cells. (A and B) Western blot assays and qPCR showed the increase of epithelial markers and the decrease of mesenchymal markers after transfected with anti-miR-27a-3p compared to control. *P<0.05, **P<0.01. (C and D) miR-27a-3p is associated with the invasion of GC cells. Transwell invasion assays were showed (C) and statistically analyzed showed in (D). *P<0.05 and **P<0.01 (two-tailed unpaired t-test). (E) anti-miR-27a-3p inhibits cellular growth. BGC823 cells transfected with anti-miR-NC and anti-miR-27a-3p were subjected on growth analysis. Cell proliferation in HGC-27 (G) and MGC823 (F) cells transfected with anti-miR-27a-3p or anti-miR-NC was assessed by MTT assay at 24 h, 48 h and 72 h. Each bar represents the mean ± s.d. for triplicate measurements. *P<0.05.

h, 48 h and 72 h. Subsequently, 20 uL of a 5 mg/ml MTT solution (Sigma) was added to each well and plate was incubated for 4 hour. Thereafter the supernatant was aspirated and 200 uL of DMSO was added to dissolve formazan crystals for 10 min. The absorbance was determined spectrophotometrically at a wave length of 490 nm using an ELISA reader (Mole-

cular Devices, Sunnyvale CA, USA). All experiments were repeated three times.

Statistical analysis

Statistical analysis utilized two-tailed Student t-test. The relationship between the expressions of miR-27a-3p and RUNX1 mRNA was analyzed by Pearson's correlation. A *p*-value of less than 0.05 was considered statistically significant. Results are presented as means ± SD form three independent experiments.

Results

MiR-27a-3p is overexpressed in GC tissues and correlated with metastatic capacity in GC tissues

Since previous studies have indicated that miR-27a-3p play a critical role in the GC initiation and progression and functions as an oncogene [14], we carefully investigated the role of miR-27a-3p in gastric cancer progression. We downloaded miRNA expression profiles of gastric cancer tissues and normal gastric tissues form GEO database (GSE93415). The analyses showed that miR-27a-3p level was significantly upregulated in gastric cancer tissues than that of in normal tissues (**Figure 1A**). Then the patients and cancer specimens were stratified, based on clinical progression, the expression of miR-27a-3p was markedly increased in the GC tissues of patients with lymph node metastasis (**Figure 1B**). This finding reminding us that miR-27a-3p may also has effect on GC metastasis process. Consistent with this finding, real-time PCR analyses showed that miR-27a-3p in clinical specimens was overexpressed in gastric cancer tissues compared to levels in matched adjacent normal gastric tissues (**Figure 1C**). Together, these results demonstrated that miR-27a-3p was overexpressed in human GC was associated with enhanced tumor progression and metastasis in patients with GC.

Decrease miR-27a-3p level suppress the epithelial-mesenchymal transition (EMT) process, invasion and proliferation of gastric cancer cells

Increasing evidences shows that miRNA-27a is a biomarker for predicting chemosensitivity and prognosis in metastatic gastric cancer [24], but the mechanism is not clear till now. To further probe the function of miR-27a in the regulation of EMT process, we performed transfections of anti-miR and anti-miR-27a-3p (miR-27a-3p is the major isoform of mature miR-27a) in BGC823 cells. The Western blot analysis showed that down-regulation of miR-27a-3p significantly promoted the protein expression of epithelial markers (E-cadherin, α-catenin and γ-catenin) and decreased the protein expression of mesenchymal markers (N-cadherin, fibronectin and vimentin) compared to the control (**Figure 2A**). Then the qPCR is performed to detect the mRNA level change of EMT-related genes, including E-cadherin, α-catenin, γ-catenin, N-cadherin, fibronectin and vimentin, and the results were in consistent with their protein levels (**Figure 2B**).

We then investigated the roles of miR-27a-3p in cellular behavior of gastric cancer cells in vitro using transwell invasion assays. As shown in **Figure 2C**, down expression of miR-27a-3p led to a decrease in the invasive potential of BGC823 cells. Taken together, these results support a critical role for the miR-27a-3p in the regulation of EMT and invasion.

To further clarify the function of miR-27a-3p in gastric cancer growth, we seeded 1×10^4 BGC823 cells per well and transfected anti-miR-NC mimics and anti-miR-27a-3p mimics, respectively. To evaluated the effects, we counted the cell number per 12 hours, in total 96 hours, and found that silencing miR-27a-3p expression dramatically reduced the growth of BGC823 cells (**Figure 2D**). MTT assay was used to examine the effect of miR-27a-3p knockdown on GC cell proliferation and the results indicated that miR-27a-3p knockdown dramatically inhibited cell viability at 24 h, 48 h and 72 h compared with that of anti-miR-NC cell groups *in vitro* in both GC cell lines HGC-27 (**Figure 2G**) and BGC-823 (**Figure 2F**). Collectively, these experiments indicate that miR-27a-3p promotes the proliferation, invasion and EMT transition of GC cells.

RUNX1 is a direct functional target of miR-27a-3p in gastric cancer cells

The function of miRNAs in tumor initiation and progression is dependent on targeting their key target genes, so it is crucially important to identify the targets of miR-27a-3p. We used publicly available algorithms TargetScan to predict the targets of miR-27a-3p, and identified RUNX1

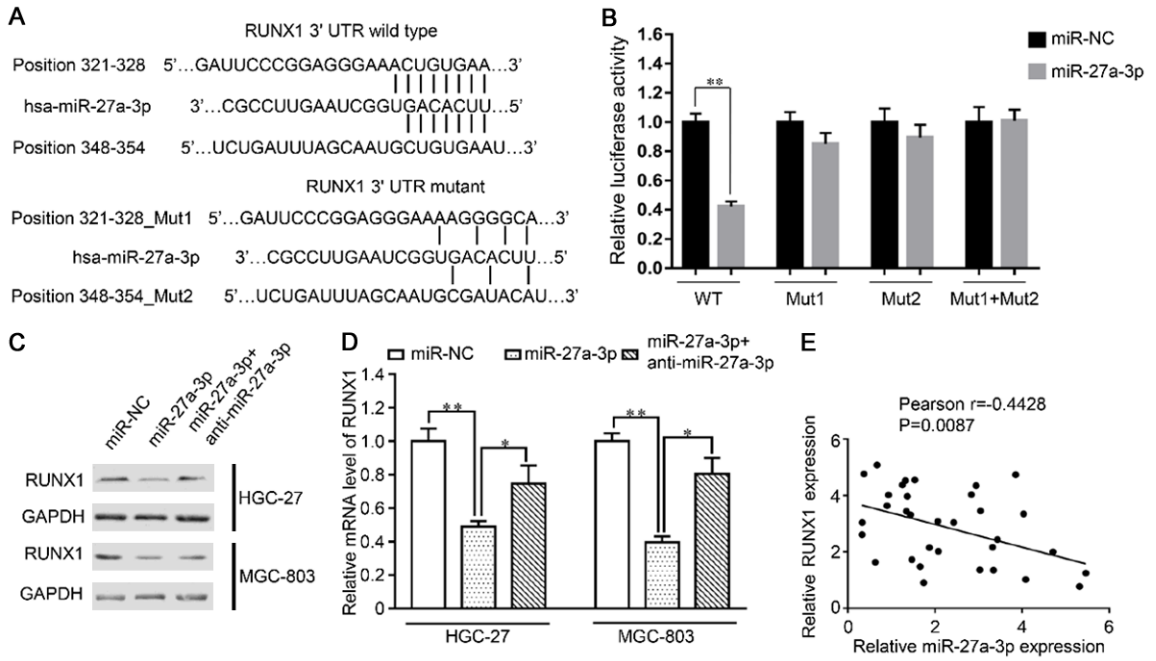

Figure 3. RUNX1 was a direct target of miR-27a-3p. (A) Bioinformatic analysis suggested that the 3'UTR of RUNX1 contained two putative miR-27a-3p binding sites. Site-directed mutagenesis of the miR-27a-3p binding sites were performed. (B) Luciferase activity assays of luciferase vectors containing wild-type or mutant RUNX1-3'-UTR were performed after transfection with miR-NC or miR-27a-3p. Western blot and qPCR assays show the expression of RUNX1 protein (C) and RUNX1 mRNA (D) in HGC-27 and SGC-7901 cells after transfection with miR-NC or miR-27a-3p. For qPCR assays repeated in duplicates, GAPDH served as an internal control for RUNX1. *P<0.05, **P<0.01. (E) Correlation analysis between miR-27a-3p and RUNX1 mRNA level in GC tissues.

was a putative target of miR-27a-3p. RUNX1 is regarded as a tumor suppressor gene and involved in cell division, invasion, growth and differentiation of several cancers cells. According to the prediction, miR-27a-3p has two different putative binding sites within the 3'UTR of RUNX1 (**Figure 3A**). To identify whether RUNX1 is a target of miR-27a-3p, we constructed vectors containing the wild-type 3'-UTR, and the mutant of seed-region complementary sites in 3'-UTR (including Mut1, Mut2 and Mut (1+2)) of RUNX1 mRNA, which was individually fused directly downstream of the firefly luciferase gene (**Figure 3A**). For the luciferase assays, the wild-type or mutant vector was co-transfected into MGC-803 cells with miR-NC or miR-27a-3p. As shown in **Figure 3B**, miR-27a-3p significantly reduced the relative luciferase activity of the wild-type 3'-UTR of RUNX1 (P<0.01) compared with miR-NC control transfected cells. We also observed that the luciferase activities of the Mut1 and Mut2 were not significantly changed (**Figure 3B**).

To further confirm that RUNX1 is a functional target of miR-27a-3p in GC, we examined changes of endogenous RUNX1 protein level in HGC-27 and MGC-803 cells after transfection of miR-NC, miR-27a-3p mimic and anti-miR-27a-3p mimic. As shown in **Figure 3C**, endogenous RUNX1 expression was significantly decreased by transfection of miR-27a-3p mimic, but the reduction of protein level was restored by anti-miR-27a-3p. Uncropped blots for **Figure 3C** were showed in Supplementary Data. The mRNA changes of RUNX1 after the transfection of miR-NC, miR-27a-3p and anti-miR-27a-3p were in agreement with the protein variation (**Figure 3D**). Additionally, RUNX1 mRNA level was inversely correlated with miR-27a-3p expression levels (**Figure 3E**). Taken together, these results suggest that miR-27a-3p can downregulated RUNX1 expression in GC cells through direct binding to the 3'-UTR of RUNX1.

Mir-27a-3p promoted GC cell growth, invasion and EMT via targeting of RUNX1

To further investigate whether the effect of miR-27a-3p on promoting proliferation, invasion, and EMT transition of GC cells was via targeting RUNX1, we performed the experiments that the human BGC823 cells were transfected

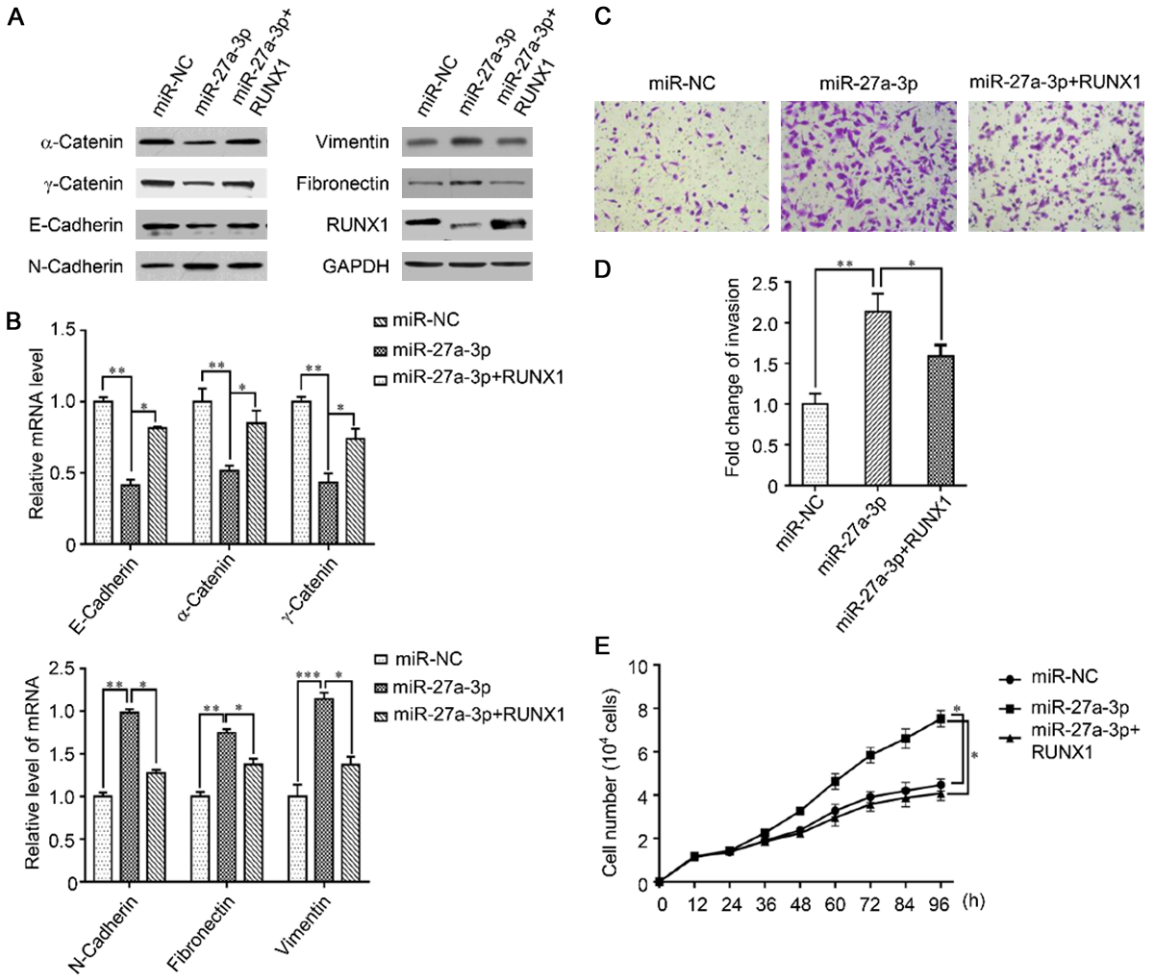

Figure 4. miR-27a-3p promotes invasion, growth and EMT process via targeting RUNX1 in BGC823 cells. A and B. Western blot assays and qPCR showed the decrease of epithelial markers and the increase of mesenchymal markers after transfected with miR-27a-3p compared to control, while co-transfection with RUNX1 weaken the EMT process induced by miR-27a-3p. *P<0.05, **P<0.01, ***P<0.001. C and D. Overexpression of miR-27a-3p significantly promoted cell invasion, while co-transfection with RUNX1 significantly suppressed the cell invasion in miR-27a-3p transfected cells, as determined in a transwell assay. E. miR-27a-3p promoted GC cell growth by targeting RUNX1. Growth analysis showed that overexpression of RUNX1 can suppress the growth ability induced by miR-27a-3p. Each bar represents the mean ± s.d. for triplicate measurements. *P<0.05.

with miR-NC, miR-27a-3p mimic or co-transfected with miR-27a-3p and RUNX1 vector. The results showed endogenous epithelial proteins (E-cadherin, α-catenin and γ-catenin) expression level were dramatically reduced and mesenchymal proteins (N-cadherin, fibronectin and vimentin) expression were upregulated by miR-27a-3p. However, co-transfection with miR-27a-3p and RUNX1 overexpressing plasmid significantly weaken the promotion of EMT process by miR-27a-3p **Figure 4A**). In consistent with the protein level, the mRNAs expression level of epithelial markers were decreased and mesenchymal markers in BGC823 cells after the transfection of miR-27a-3p mimic were also rising up. The overexpression of RUNX1 still shows out the ability to inhibit the EMT transition induced by miR-27a-3p in mRNA level (**Figure 4B**).

To further determine whether overexpression of miR-27-3p affect cell invasion via the direct targeting of RUNX1, we performed transwell invasion assays with BGC823 cells. In the transwell invasion assays, we found that overexpression of miR-27a-3p significantly enhanced the ability of cells to invade through the membrane (**Figure 4C** and **4D**), whereas RUNX1 co-transfection significantly decreased the likelihood of cell invasion ability.

The significant promotion of proliferation by miR-27a-3p expression let us to further explore the possible biological significance of miR-27a-3p in tumor growth. Then, the capacity for cancer cells growth was evaluated in BGC823 cells that were transfected with miR-NC, miR-27a-3p or co-transfection with miR-27a-3p and RUNX1 plasmid. The results showed that miR-27a-3p-transfected cells displayed stronger ability for growth compared to control, whereas the ability was also suppressed by the restoration of RUNX1 (**Figure 4E**). Together, miR-27a-3p promotes the invasion, proliferation and EMT transition potential of GC cells by targeting RUNX1.

Discussion

miRNAs have been accepted as key regulators of tumorigenesis, as they can regulate many biological processes, such as cell proliferation, transformation, differentiation, metastasis and survival [25]. Although a number of molecular drivers of gastric cancer have been described over the years, only very recently, miRNAs have emerged as key players in the pathogenesis of this disease [26, 27]. Previous evidences have described dysregulate expression of miR-27a in GC [28, 29], and in this study, we found that miR-27a-3p has a closely relationship with GC and determined it was significantly upregulated in tumor tissues of GC patients compared with matched adjacent normal tissues. We also observed that the higher expression of miR-27a-3p in diffuse-type gastric carcinoma was correlated with a more aggressive phenotype of GC in patients. Loss-of-function studies further revealed that miR-27a-3p inhibition repressed the proliferation, invasion, and migration of GC cells in vitro.

Having characterized the role of miR-27a-3p in GC, we next attempted to identify the potential target genes mediating its function. As we all known that a single miRNA can regulate a large number of target genes, consequently eliciting various biological response [30]. According to the bioinformatics analysis, we predicted RUNX1 as a putative target gene of miR-27a-3p with two binding sites. The dual luciferase reporter assays were performed and confirmed that RUNX1 was a direct target gene of miR-27a-3p and both of the two binding sites had direct interaction with the miR-27a-3p. In agreement with reporter assays, transfection of miR-27a-3p mimic significantly repressed the expression of RUNX1 in GC cells. Furthermore, our observation for an inverse correlation between miR-27a-3p expression and RUNX1 expression in GC cells fills this important void in literature for the missing experimental evidence for the function of miR-27a-3p and RUNX1 in gastric pathogenesis.

Epithelial-mesenchymal transition (EMT) is involving the morphogenesis during embryonic development and is also critical for the conversion of early stage tumors into invasive malignancies [31]. Multiple miRNAs have been reported to strictly affect the EMT process [32]. However, the role of miR-27a-3p in the EMT process is not very well described. We transfected the anti-miR-27a-3p mimic to downregulate the miR-27a-3p in GC cells and found out the expression of epithelial markers were decreased on the contrary with the expression levels of mesenchymal markers. Our results suggested that miR-27a-3p function as an enhancer of EMT process and might be a therapeutic target for prevention or treatment of GC and other EMT-associated cancers.

RUNX1 is an essential transcription factor for definitive hematopoiesis, and has identified as one of the most frequently mutated genes in breast cancer patients. As a member of the RUNX family, RUNX1 is playing as an important EMT suppressor in breast cancer cells [33]. As a direct target of miR-27a-3p, we doubted that miR-27a-3p promotes EMT process via repressing the expression of RUNX1. To confirm our assumption, we co-transfected miR-27a-3p mimic with RUNX1 expression plasmid in GC cells and observed that RUNX1 with the ability to suppress EMT process induced by miR-27a-3p.

In conclusion, we found out the regulatory mechanism that miR-27a-3p-induced loss of RUNX1 enhanced the proliferation, invasion, and migration of GC cells and the miR-27a-3p/RUNX1 axis played a significant role in the EMT process. These findings indicated that miR-27a-3p may be a biomarker for the prognosis of GC patients and downregulation of it may provide a promising strategy for treating GC.

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Disclosure of conflict of interest

None.

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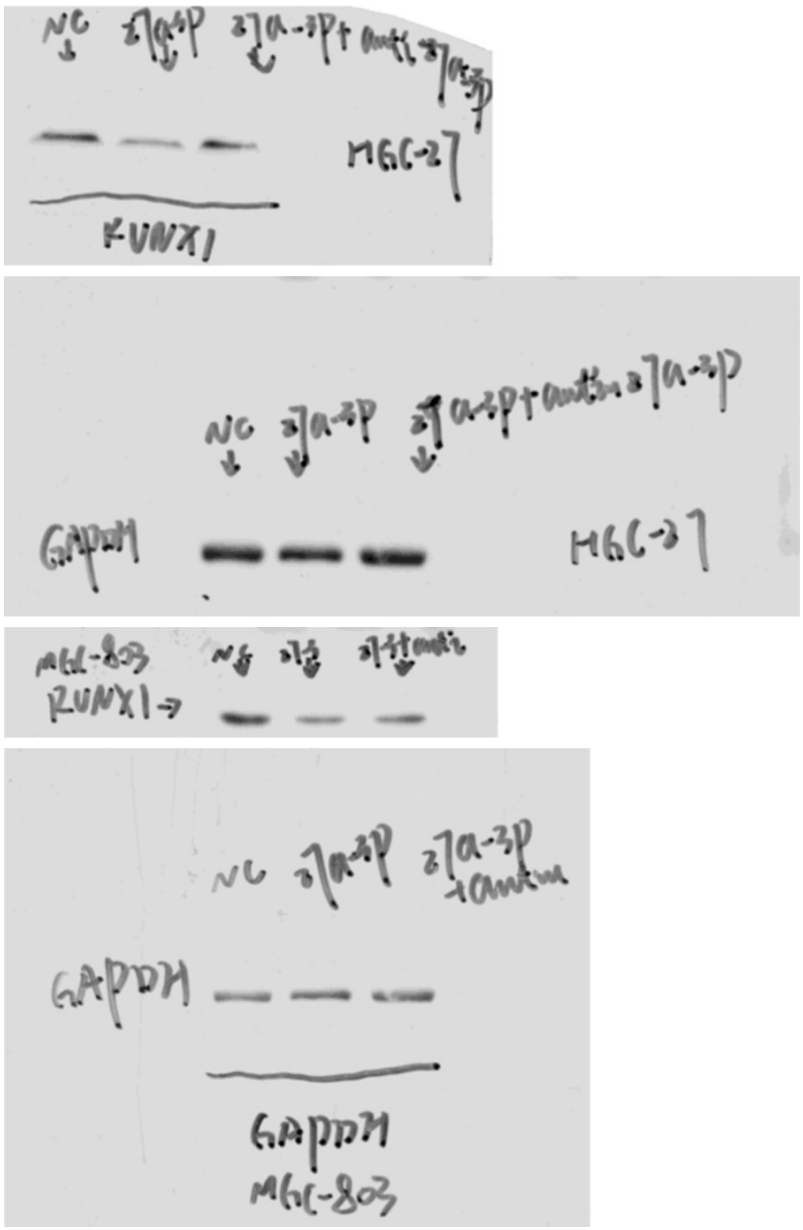

Supplementary Data. The full membrane of WB picture for **Figure 3C**.