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Original Article

Co-regulation of miR-143, miR-218 and miR-338-3pin inhibits gastric cancer migration and invasion by targeting *collagen type I*

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Abstract: Gastric cancer remains to be the fourth malignant and has the second mortality worldwide. This study was aimed to investigate the co-regulation roles of three miRNAs in the metastasis and invasion of gastric cancer and to illustrate its potential mechanism. A total of 30 gastric cancer patients and human gastric cancer AGS cell line were enrolled in this study. Expression of targets in the tumor tissue and cells were detected using the RT-PCR and western blotting analysis. Correlation between miRNA expression and target gene expression was analyzed using the Pierson correlation. Effects of miRNAs on cell migration and invasion were analyzed using Transwell assay. The results showed that the three miRNAs including miR-143, miR-218 and miR-338-3p were all down-regulated while collagen type I (COL1A1) was up-regulated in the tumor tissue than in the adjacent normal tissue. Expression of three miRNAs was negatively correlated COL1A1 expression. COL1A1 expression was negatively regulated by the three miRNAs, and this correlation was more apparent between the co-regulation of the three miRNAs and COL1A1. The migrated and invaded cells were both decreased by the up-regulated miRNAs, whereas this effect was more apparent by the co-regulation of the three miRNAs. Taken together, our study revealed that co-regulation of miR-143, miR-218 and miR-338-3p played curial roles in suppressing the metastasis and invasion of gastric cancer by targeting COL1A1.

Keywords: Gastric cancer, miR-143, miR-218 and miR-338-3p, collagen type I (COL1A1), migration, invasion

Introduction

Gastric cancer remains to be the fourth most common malignant and has the second most frequent cause of cancer-related deaths worldwide [1]. To data, treatment methods for gastric cancer including surgery, chemotherapy, and radiation therapy have play certain roles in curing gastric cancer, but the results still remain unsatisfactory due to its easy metastasis and hard diagnose [2, 3]. Hence, to explore several new treatment approaches will be benefit for the clinical cure of gastric cancer.

microRNAs are some endogenous, highly conserved non-coding RNAs 20- to 22-nt in length that function in various of biological processes at the transcriptional or post-transcriptional level by targeting the 3'-UTR of genes [4]. In recent years, the therapeutic important roles of miRNAs in the development, progression and metastasis of gastric cancer have been widely studied. For example, Guo et al reported that miR-338-3p functioned as a suppressor in the progression of gastric cancer by targeting P-ERX2α [5]. Similar studies such as the important roles of miR-218 in gastric cancer detection and progression [6, 7], and miR-143 in regulating the cell growth and apoptosis in gastric cancer development [8, 9] have also revealed the pivotal roles of miRNAs in the biology of gastric cancer. Besides, increasing evidence has revealed the crucial roles of miRNAs in the cancer metastasis [10]. miR-29 functioned as an anti-metastatic factor in gastric cancer by regulating ITGB1 [11], and miR-218 inhibited the cell invasion and migration of gastric cancer by targeting Robo1 receptor [6]. From another point of view, collagen, members of the matrix metalloproteinase that can degrade the basement membrane and extracellular matrix, has been widely known to play

Table 1. Primers used for targets amplification in this study

Target	Primer	Sequence (5'-3')
GAPDH	Sense	GGGTGGAGCCAAACGGGTC
	Antisense	GGAGTTGCTGTTGAAGTCGCA
COL1A1	Sense	TCCTGGTCCTGCTGGCAAAGAA
	Antisense	CACGCTGTCCAGCAATACCTTGA
U6	Sense	CGCTTCACGAATTTGCGTGTCAT
	Antisense	AACGCTTCACGAATTTGCGT

crucial roles in the development of tumors including tumor invasion and metastasis [12, 13]. Collagen type I (COL1A1) enhanced the metastasis and invasion of ovarian epithelial cancer through PTEN signal [14], as well as the important roles of collagen type IV in gastric cancer [15]. In particular, Naito et al reported that miR-143 regulated collagen type III in stromal fibroblasts of scirrhous type gastric cancer [16]. However, few have been searched for the important roles of co-regulation of miR-143, miR-218, and miR-338-3p in the metastasis and invasion of gastric cancer, as well as its mechanism.

In the current study, we aimed to investigate the co-regulation correlation of the three miRNAs including miR-143, miR-218, and miR-338-3p in the metastasis and invasion of gastric cancer and to reveal its mechanism of action. Comprehensive experimental studies were used to detect the expressions of the three miRNAs and COL1A1 in the gastric cancer patients, and to analyze the correlation between miRNA expression and COL1A1 expression in gastric cancer cells. This study may provide theoretical basis for the therapeutic target application of the three miRNAs in the treatment of gastric cancer.

Materials and methods

Patient samples and cell culture

A total of 30 patients who were diagnosed as gastric cancer in the General Hospital of the People's Liberation Army between November, 2014 and December, 2015 were enrolled in this study. The informed consent and approval of patients were obtained from the General Hospital of the People's Liberation Army. This study was approved by the Ethics Committee of General Hospital of the People's Liberation Army. Gastric cancer diagnose was pathologically confirmed, and cancer tissues and their adjacent normal tissue were obtained from clinically ongoing surgical specimens with informed consent [17]. Tissues were snap frozen with liquid nitrogen, and stored at -80°C till RNA extraction.

Human gastric cancer AGS cell line was cultured in the RPMI 1640 medium (Invitrogen, USA) containing 10% fetal bovine serum (FBS; Sigma, USA) at 37°C under a humidified atmosphere containing 5% CO_2.

Cell transfection

AGS cells were plated onto 60-mm dishes. After 24 h of incubation, cells were transfected with the siRNA (si-COL1A1, miR-143/miR-218/miR-338-3p inhibitor or mimic; purchased from Sangon Biotech, Shanghai, China) with Lipofectamine® 2000 Transfection Reagent [18]. Cells transfected without any vectors are considered as the control. After 48 h of incubation, cells were prepared for further analysis.

RNA preparation and real-time PCR

Total RNA extraction from the cells or tissues was performed using the TRIzol Reagent (Invitrogen) according to the manufacture's protocol. The concentration and purity of the isolated RNA were detected using SMA 400 UV-VIS (Merinton, Shanghai, China). Purified RNA at density of 0.5 μg/μL with nuclease-free water was used for cDNA synthesis with the PrimerScript 1st Strand cDNA Synthesis Kit (Invitrogen, USA). Expressions of targets were detected in an Eppendorf Mastercycler (Brinkman Instruments, Westbury, NY) using the SYBR ExScript RT-qPCR Kit (Takara, China). The total reaction system of 20 μL volume was as follows: 1 μL cDNA from the above PCR, 10 μL SYBR Premix EX Taq, 1 μL each of the primers (10 μM), and 7 μL ddH_2O. The PCR program was as follows: denaturation at 50°C for 2 min; 95°C for 10 min; followed by 45 cycles of 95°C for 10 s, and 60°C for 1 min. Melting curve analysis of amplification products was performed at the end of each PCR to confirm that only one product was amplified and detected. Phosphoglyceraldehyde dehydrogenase (GAPDH) or U6 was chosen as the internal control. Primers for miRNAs amplification were purchased from Taqman probe and the other prim-

ers used for targets amplification were shown in **Table 1**.

Western blotting

After 48 h of incubation, cells were lapped with RIPA assay (radioimmunoprecipitation, Sangon Biotech) lysate containing PMSF (phenylmethanesufonyl fluoride, Sigma), and then were centrifuged at 12,000 rpm for 10 min at 4°C. Supertanant was collected for the measurement of protein concentrations using BCA protein assay kit (Pierce, Rochford, IL). For Western blotting [19], 50 μg protein per cell lysate was subjected to a 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transferred onto a Polyvinylidencefluoride (PVDF) membrane (Mippore). Then the PVDF membranes were blocked in Tris-Buffered Saline Tween (TBST) containing 5% non-fat milk at room temperature for 1 h. The membranes were incubated with rabbit anti-human antibodies (type I collagen, 1:100 dilution, Invitrogen) and overnight at 4°C. Then membrane was incubated with horseradish-peroxidase labeled goat anti-rat secondary antibody (1:1000 dilution) at room temperature for 1 h. Finally, the PVDF membranes were washed 3 times with 1× TBST buffer for 10 min each. The signals were detected after incubation with a chromogenic substrate using the enhanced chemiluminescence (ECL) method. Additionally, GAPDH served as the internal control.

Cell migration and invasion assay

Cell migration and invasion assay were evaluated using Transwell migration chambers (8 μm pore size; Corning, USA). For migration assay, cells (5×104 cells/well) were seeded in the upper portion of a chamber with serum-free RPMI 1640 medium after transfection. Medium containing 10% FBS with or without mimic or inhibitor or control, were served as s chemoattractant in the lower chamber. After 24 h of incubation at 37°C, cells were calculated. For invasion assay, the Transwell membranes were coated with the diluted Matrigel (Invitrogen) and then air-dried at 4°C.

Immunohistochemistry

Sections at 5 μm were floated onto positively charged slides (SuperFrost Plus, Menzel-Glaser, Germany) for 20 min, and then were representative formalin-fixed and paraffin-embedded. Sections were dewaxed in xylene and rehydrated by a graded series of ethanol (100% 10 min; 95% 5 min; 80% 5 min; and 70% 5 min). Heat-induced epitope retrieval was carried out by immersing the slides in citrate buffer (pH6) and microwaving at 600 W for 20 min. Endogenous peroxidase activity was quenched by incubating the sections in 3% H_2O_2 at 37°C for 10 min, and then were washed with PBS buffer for 3 times with 5 min each. Non-specific binding sites were then blocked by pre-incubating with 20% normal horse serum (Sigma) and 1% bovine serum albumin (BSA) in PBS/0.3% Triton X-100 for 20 min at room temperature. Sections were incubated with polyclonal rabbit anti-COL1A1 antibody (1:50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. After washing with 0.25% BSA and 0.05% Tween-20 in PBS, sections were incubated with biotinylated secondary antibody (Dako Ltd) (1:500 dilution) for 1 h at room temperature. Then sections were washed with 0.05% Tween-20, followed by incubated with horse radish peroxidase-conjugated Streptavidin biotin complex (HRP-StrepABC) (Vectastain Universal Quick Kit PK-7800, Vector Laboratories, Burlingame, CA, USA) for 30 min. All sections were again washed with PBS, and immunoreactivity was visualized in the presence of 0.05% 3, 3'-diaminobenzidine (DAB). Nuclei were then lightly counterstained with Harris Haematoxylin, before rehydrating and mounting with DPX.

Statistical analysis

Total experiments were conducted independently 3 times. All data are expressed as the mean ± SD. Statistically analysis was performed using Graph prism 5.0 software. Statistical difference for data was calculated using the one-way analysis of variance (ANOVA) with post-hoc test. $P<0.05$ was considered as statistically significant.

Results

Expression of COL1A1 and miRNAs in gastric cancer tissue

The relative mRNA level for collagen I in gastric cancer tissue was significantly increased compared to that in the adjacent normal tissue

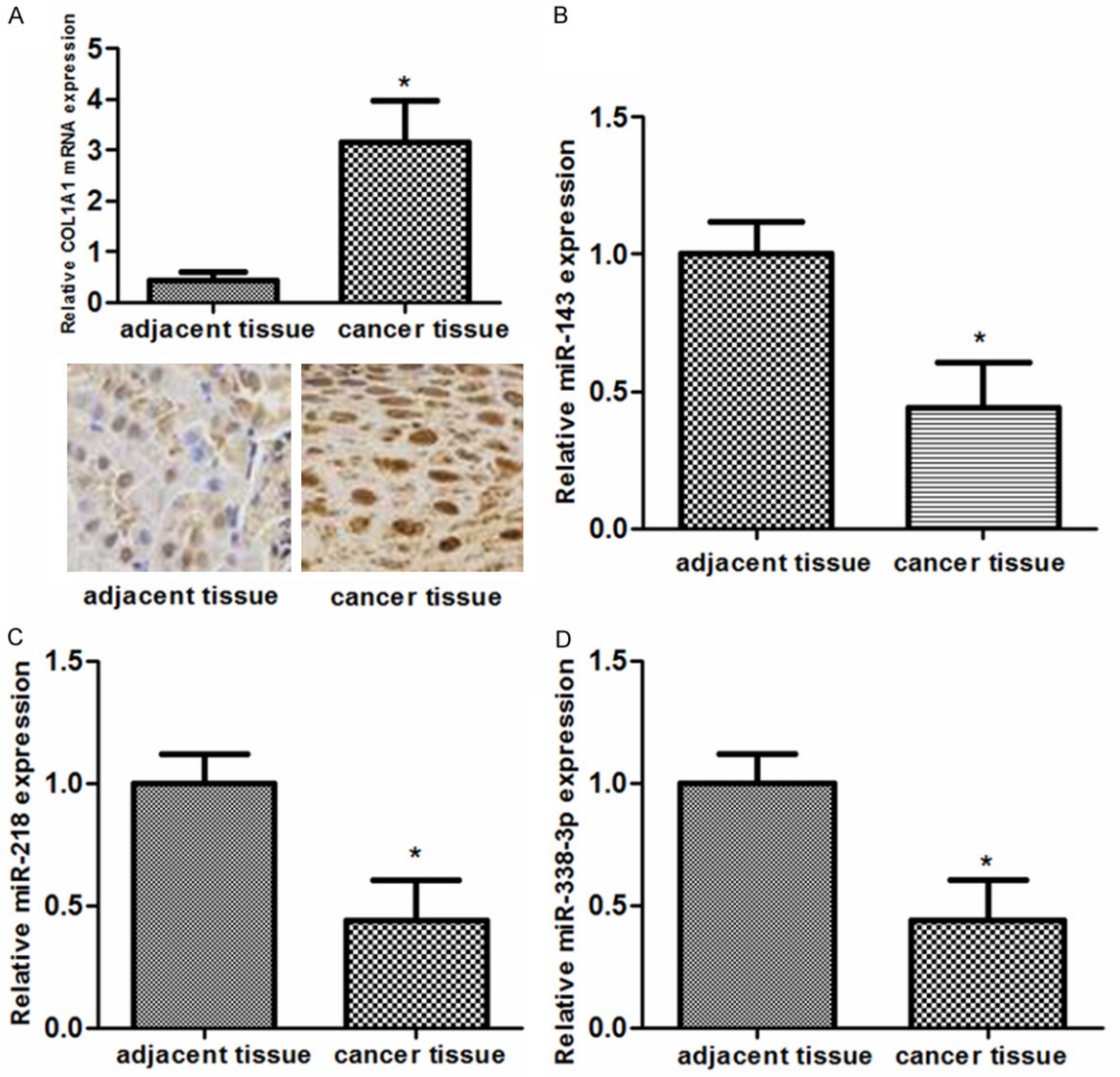

Figure 1. Expression of collagen type I (COL1A1) and miRNAs in gastric cancer tissue (n=30). (A) The relative mRNA and protein level for collagen I in gastric cancer tissue was higher than that in its adjacent tissue; (B-D) RT-PCR analysis revealed that the relative mRNA level for miR-143 (B), miR-218 (C) and miR-338-3p (D) in gastric cancer tissue was lower than that in the adjacent tissue. *: P<0.05 compared to the adjacent tissue.

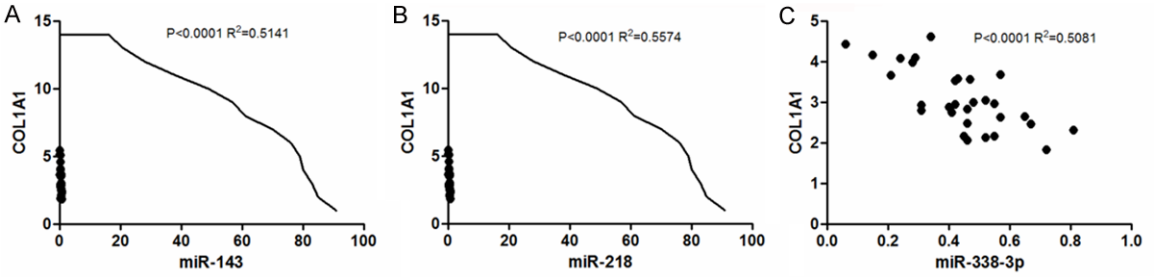

Figure 2. Correlation analysis of the miRNA expression and the COL1A1 expression. A: miR-143 was negatively correlated to COL1A1 expression (P<0.0001, R^2=0.5141); B: miR-218 was negatively correlated to COL1A1 expression (P<0.0001, R^2=0.5574); C: miR-338-3P was negatively correlated to COL1A1 expression (P<0.0001, R^2=0.5031).

(n=30; P<0.05). Immunohistochemistry analysis showed that collagen I protein in the gastric tissue was more than that in the adjacent tissue (**Figure 1A**). Besides, RT-PCR analysis

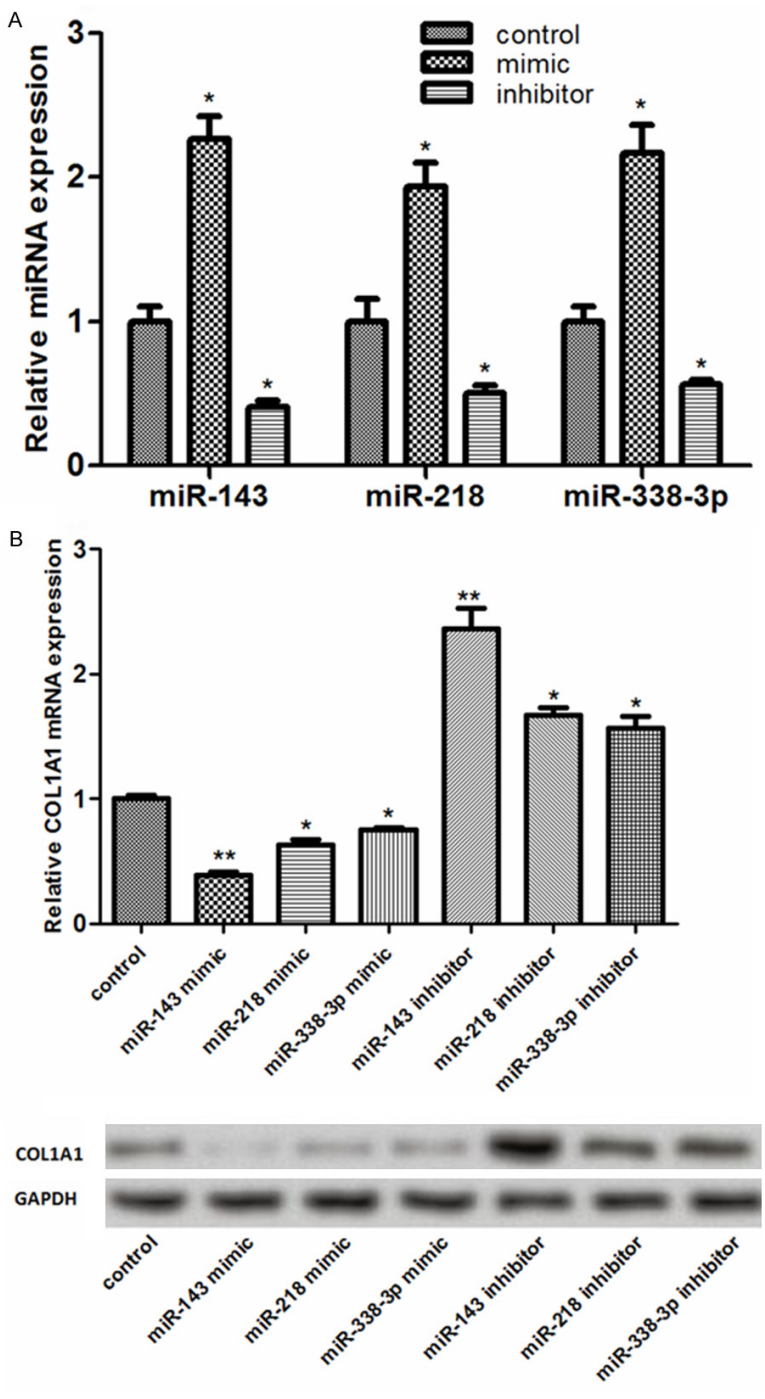

Figure 3. Expression of miRNAs and COL1A1 in the gastric cancer AGS cell line. A: Relative expression of all the three miRNAs was significantly increased by the mimic transfection compare to the control; B: mRNA and protein expression of COL1A1 was significantly suppressed by any one of the three miRNAs mimic transfection, but was increased by their inhibitor. *: P<0.05 and **: P<0.01 compared to the control cells.

showed that the relative mRNA levels for three miRNAs including miR-143, miR-218, and miR-338-3p in gastric cancer tissue (n=30) were all significantly down-regulated compared to that in adjacent tissue (P<0.05; **Figure 1B**, **1C**).

miR-143, miR-218 and miR-338-3p are negatively correlated to COL1A1 expression

Pierson correlation analysis was used to analyze the correlation between miRNA expression and COL1A1 expression [20], and the results revealed that COL1A1 can be targeted by miRNAs including miR-143, miR-218 and miR-338-3p. The correlation between COL1A1 expression and the 3 kinds of miRNAs were analyzed using Pierson's correlation analysis, and the results showed that the three kinds of miRNAs were all negatively correlated to COL1A1 expression (P<0.0001, R^2= 0.5141 for miR-143; P< 0.0001, R^2=0.5574 for miR-218; P<0.0001, R^2=0.5081 for miR-338-3p; **Figure 2**).

miR-143, miR-218 and miR-338-3p negatively regulated COL1A1 expression

To further assess whether COL1A1 expression was regulated by the three miRNAs, the gene-mediated silencing was used to evaluate the COL1A1 expression under difference conditions in AGS cells (**Figure 3**). The relative mRNA levels for miR-143, miR-218 and miR-338-3p were all highly expressed by the mimic RNA transfection, but were all down-regulated by the inhibitor transfection in AGS cells compared to the control cell (P<0.05; **Figure 3A**). Besides, the COL1A1 expression was significantly decreased in AGS cells that transfected with the mimic miRNA, but its expression

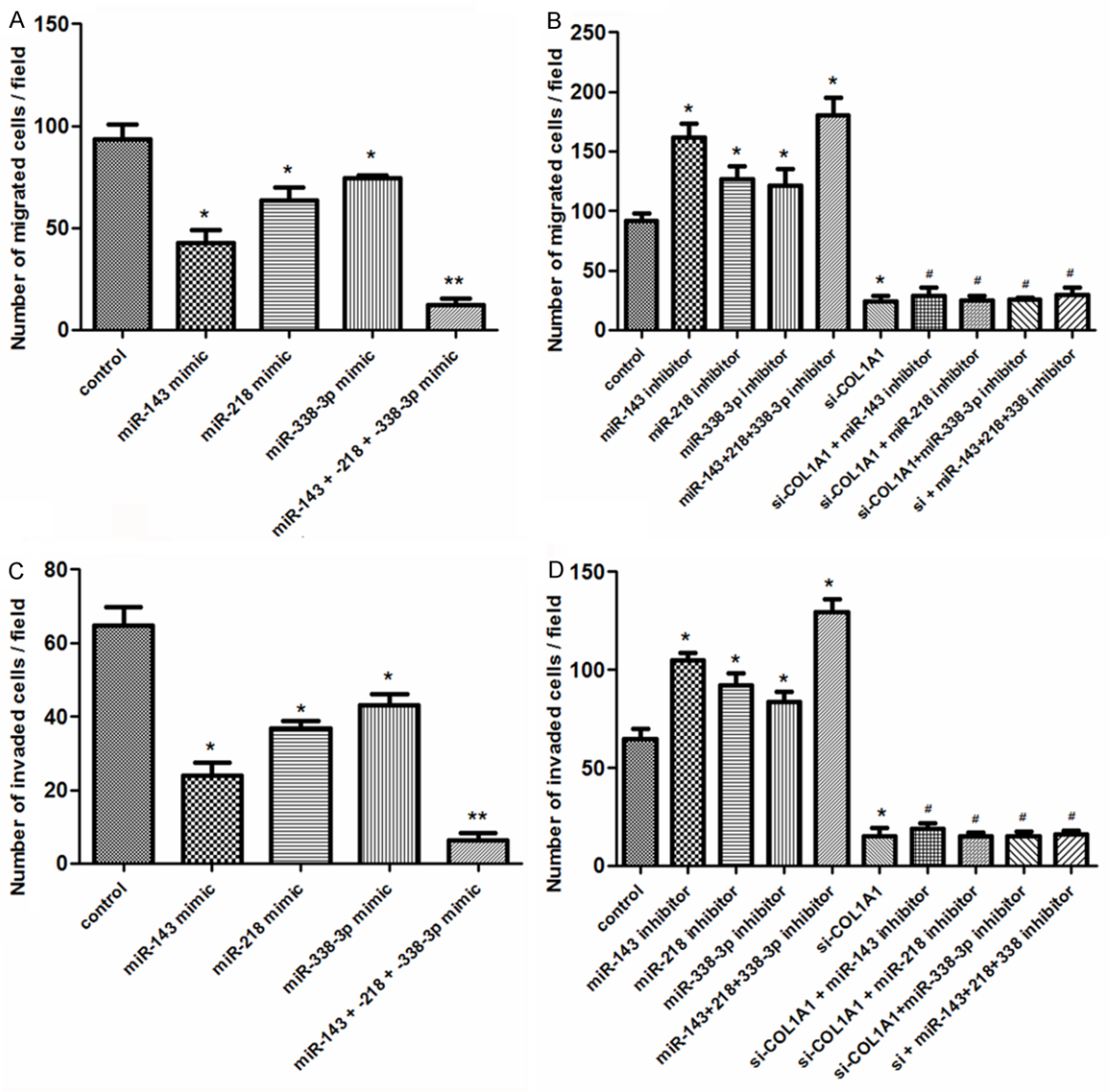

Figure 4. Influence of miRNA expression on tumor cell metastasis and invasion. A: Overexpression of the three miRNAs including miR-143, miR-218, and miR-338-3p significantly suppressed the migrated cells; while miR-143 performed an apparent suppress effect among the three miRNAs. Whereas, co-regulation of the three miRNAs could drastically suppress the migrated cells; B: Cell migration ability was increased by the miRNA inhibitor transfection, however, silencing COL1A1 could significantly decrease the number of migrated cells. When cells were transfected with the silencing COL1A1 and miRNA inhibitor, number of migrated cells was decreased, which was similar to that in cells transfected with silencing COL1A1 and the three miRNAs; C: Overexpression of the three miRNAs including miR-143, miR-218, and miR-338-3p significantly suppressed the invaded cells, while miR-143 performed an apparent suppress effect among the three miRNAs. Whereas, co-regulation of the three miRNAs could drastically suppress the invaded cells; D: Cell invasion ability was increased by the miRNA inhibitor transfection, however, silencing COL1A1 could significantly decrease the number of invaded cells. When cells were transfected with the silencing COL1A1 and miRNA inhibitor, the number of invaded cells was decreased, which was similar to that in cells transfected with silencing COL1A1 and the three miRNAs. *: $P<0.05$ and **: $P<0.01$ compared to cells without transfection. #: $P<0.05$ compared to cells transfected with each according kind of miRNA.

was up-regulated in the miRNA inhibitor transfected AGS cells ($P<0.05$; **Figure 3B**), suggesting that the three miRNAs may negatively regulate the COL1A1 expression in AGS cells, while miR-143 performed a more regulatory effect on AGS cell migration and invasion.

miRNA panel with AGS cell migration and metastasis

We further analyzed the influence of miRNA expression on gastric AGS cell migration and invasion. The results showed that the migrated

and invaded cells were both significantly decreased in various degree by the overexpressed miRNAs including miR-143, miR-218 and miR-338-3p ($P<0.05$). Interestingly, the number of migrated or invaded cells in AGS cells transfected with the 3 miRNAs was declined more apparent than the control, as well as the cell-transfected with one of the three miRNAs ($P<0.01$; **Figure 4A** and **4C**). However, the influences of the three miRNA inhibitors or the co-transfected miRNA inhibitors on AGS cell migration and invasion were opposite to that transfected with the mimic (**Figure 4B** and **4D**). Consequently, we further transfected the si-COL1A1 and si-miRNA into AGS cells to observe the cell migration and invasion abilities. The results showed that when cells were transfected with si-COL1A1 alone, the migrated and invaded cells were significantly declined compared to the control ($P<0.05$). In addition, the migrated and invaded cells were both significantly decreased by the co-transfection of si-COL1A1 and miRNA inhibitor in AGS cells (**Figure 4B** and **4D**). Also, co-transfection of the three kinds of miRNAs induced the more apparent suppress effects on AGS cell migration and invasion. These results suggested that the overexpressed miR-143/miR-218/miR-338-3p could suppress AGS migration and invasion by down-regulating COL1A1.

Discussion

Increasing evidence has revealed the pivotal roles of miRNAs in the progression and metastasis of gastric cancer, such as miR-29 and miR-143 [9, 11], but the mechanisms remain complicate [21]. In this study, we detected the expression of three miRNAs including miR-143, miR-218, and miR-338-3p and the expression of COL1A1 in gastric cancer tissue based on the reviewed literatures, and analyzed the correlation between the miRNA and COL1A1 expression. In agreement with previous results [5, 7, 9], our data showed that expressions for miR-143, miR-218, and miR-338-3p were all down-regulated in the gastric cancer tissue, as well as COL1A1 was up-regulated in the tumor tissue [22], suggesting the important roles for these factors in the biology of gastric cancer. Accordingly, we further analyzed the correlation between the three miRNAs and the gene of COL1A1. The results showed that any one of the three miRNAs was negatively correlated to the expression of COL1A1, indicating the correlation of COL1A1 and the three miRNAs in regulating gastric cancer.

Furthermore, we investigated the significant roles of miRNAs and COL1A1 in the biology of gastric cancer metastasis using the gene-mediated silencing. Our study showed that the three miRNAs including miR-143, miR-218, and miR-338-3p were all up-regulated by the miRNA mimic transfection, whereas COL1A1 was down-regulated by the miRNA mimic transfection, indicating that any one of the three miRNAs could suppress the COL1A1 expression. Role of COL1A1 in the metastasis and invasion for gastric cancer has not been fully discussed. However, Honma et al demonstrated that the tumor growth and invasion of malignant human glioma cell can be suppressed by COL1A1 [23]. Similar evidence was also mentioned in the skin metastasis of dermatofibrosarcoma protuberans [24]. Also, Maolin et al proved that COL1A1 polymorphism was associated with the risk of osteosarcoma susceptibility [25]. Additionally, the migrated and invaded cells were significantly decreased by the silenced COL1A1, indicating the significant roles of COL1A1 in promoting the metastasis and invasion of gastric cancer. On the other hand, previous studies have proved that miR-143, miR-218, and miR-338-3p were involved in the metastasis of several kinds of tumors, such as miR-143 inhibited the metastasis of pancreatic cancer and colorectal cancer [26, 27], miR-218 in the metastasis of prostate cancer [28], and miR-338-3p in liver cancer [29]. Nonetheless, the influence of co-regulation of the three miRNAs in cancer metastasis remains in complicate, especially in the metastasis of gastric cancer. However, studies have revealed the suppress roles of the three miRNAs including miR-143, miR-218, and miR-338-3p in gastric cancer by different mechanisms [6, 30, 31]. In this study, the migrated and invaded gastric cancer cells were suppressed by the overexpression of any one of the three miRNAs. Interestingly, this inhibit role was more apparent by the co-regulation of the three miRNAs, implying that co-regulation of miRNAs including miR-143, miR-218, and miR-338-3p may be negatively correlated to the cell metastasis and invasion by suppressing COL1A1.

To sum up, our study suggests that miR-143, miR-218, and miR-338-3p are all down-regulated in gastric cancer, and the co-regulation of the three miRNAs may negatively regulate the metastasis and invasion of gastric cancer by targeting the COL1A1. This study may provide theoretical basis for illustrating the crucial roles of miR-143, miR-218, and miR-338-3p in affecting the metastasis and invasion of gastric cancer and for elucidating the mechanism of co-regulation action in the biology of gastric cancer. Further experimental studies are still needed to explore the roles of the miRNA panel in the diagnosis of gastric cancer.

Disclosure of conflict of interest

None.

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