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miR-218 inhibits gastric tumorigenesis through regulating Bmi-1/Akt signaling pathway

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ABSTRACT

Background: Previous studies indicated that miR-218 was deregulated in gastric cancer patients and correlated with tumor invasion and prognosis. The aim of this study was to clarify the effect of miR-218 on the malignant behavior of gastric cancer and its role in regulating Bmi-1/Akt signaling pathway.
Materials and methods: We used miR-218 mimic to transfect gastric cancer cell lines AGS and SGC-7901, and the overexpression efficiency was validated using qRT-PCR assay. MTT assay and Transwell chamber system were performed to detect the effect of miR-218 on cell proliferation, invasion and migration on gastric cancer. Western blot and qRT-PCR assay was used to test the role of miR-218 in regulating Bmi-1/Akt signaling pathway.
Results: As shown in our research, ectopic expression of miR-218 in gastric cancer cells inhibits the proliferation, invasion and migration of gastric cancer cells. In addition, miR-218 re-expression inhibits the expression of Bmi-1 and its downstream target p-Akt^{473}, as well as MMPs and EMT process.
Conclusions: miR-218 inhibits the proliferation, invasion and migration of gastric cancer cells through modulating EMT process and the expression of MMPs via Bmi-1/Akt signaling pathway.

1. Introduction

Gastric cancer is the fifth most frequently diagnosed cancer and the third leading cause of death from cancer worldwide [1]. According to incomplete statistics in 2016, there were more than 26,370 new cases of gastric cancer patients and 10,730 people will eventually die of the disease in the United States [1–3]. What's more, the incidence of gastric cancer is much higher in Asian countries including China. Although endoscope diagnosis combined with multi-mode complex treatments, such as surgery, chemotherapy and radiotherapy have appeared constantly, a typical symptom of early gastric cancer isn't easy to find. Most of the gastric cancer has entered advanced stage after diagnosis, which has poor prognosis and low 5-year survival rate [4,5].

Heretofore, multiple factors including molecular, genetic, and epigenetic changes as well as environmental factors have been shown to be associated with the formation and progression of gastric cancer. MicroRNA (miRNA) is a kind of small endogenous non-coding RNAs, which inhibit the expression of the downstream target genes involved in regulating cell proliferation, invasion, migration, apoptosis and differentiation [6–8]. By targeting different genes such as IKK-β [9], Slits-Robo [10], nuclear factor-κB [11], Survivin [12], and Rictor-Akt [13], miR-218 has been proved to be one of the most important microRNAs in numerous cancers [14–17]. Previous researchers have found miR-218 acted as a novel potential biomarker in gastric cancer patients and strongly correlated with tumor stage, grade and metastasis, but the mechanisms remain unclear [18–23]. Oncogenic Bmi-1 (B-lymphoma Moloney murine leukemia virus insertion region-1) belongs to the Polycomb group (PcG) family [24,25]. Overexpressed Bmi-1 could stimulate malignant transformation, proliferation, invasion, distant metastasis and was associated with poor survival in various human cancers, including gastric cancer through numerous signaling pathways [26–28]. In this study, we found that the expression of miR-218 was significantly downregulated in gastric cancer patients in TCGA database, and decreased miR-218 also predict poor survival of cancer patients. In addition, the downregulated Bmi-1 was correlated with the expression of miR-218 in cancer tissues. Previous studies and bioinformatics analysis showed that miR-218 could bind to the 3′UTR region of Bmi-1 which is involved in the regulation of EMT process and the expression of MMPs in tumorigenesis [17]. Therefore, we hypothesize that miR-218 inhibits gastric tumorigenesis through regulating the Bmi-

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1/Akt signaling pathway.

2. Materials and methods

2.1. Cell culture and microRNA mimic cell transfection

Human gastric cancer cell lines AGS and SGC-7901 were used in this study. These cells were maintained in RPMI 1640 medium or Dulbecco's modified Eagle medium (Gibco, Grand Island, NY) with 10% fetal bovine serum (FBS; Hyclone, Logan, UT). Cells were transfected at 70% confluence using Lipofectamine 2000 (Invitrogen, Grand Island, NY), with a final microRNA mimic or negative control concentration of 50 nM. All experiments were performed in three replicates.

2.2. RNA extraction and quantitative RT-PCR (qRT-PCR)

In brief, total RNA from tissues and cell lines were isolated by TRIzol reagent following manufacturer's instruction (Takara Inc., Dalian, P.R. China), and cDNA was prepared using PrimeScript RT reagent Kit (Takara Inc., Dalian, P.R. China). The specific primer sequences of miR-218 and U6 were GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTG CACTGGATACGACACATGGT and CGCTTCACGAATTTGCGTGTCAT. Quantitative RT-PCR (qRT-PCR) was carried out on a S1000™ Thermal Cycler real-time PCR system (Bio-Rad Laboratories, Inc., CA) using SYBR Premix Ex Taq™ (Takara Inc., Dalian, P.R. China). The mRNA expression of the indicated genes was normalized to 18S rRNA or U6 rRNA levels, and relative mRNA expression was calculated by using standard curves or according to $2^{-\Delta\Delta}$Ct method. Each sample was run in triplicate.

2.3. Western blotting

Cells were lysed in prechilled RIPA buffer containing protease inhibitors. Equal amounts of protein lysates were separated by SDS-PAGE and transferred onto PVDF membranes (Roche Diagnostics, Mannheim, Germany). The membranes were then incubated with specific primary antibodies at 4 °C overnight. Anti-Bmi-1 (Santa cruz), anti-phospho-AktSer473 (Bioworld Technology, co, Ltd), anti-total-Akt (Bioworld Technology, co, Ltd), and anti-Vimentin (Santa cruz), anti-E-cadherin (Santa cruz) were purchased from Santa cruz, Inc. Anti-GAPDH (Abmart) was purchased from Abmart. This was followed by incubation with species-specific HRP-conjugated secondary antibodies from ZSGB-BIO, and immunoblotting signals were visualized using the Western Bright ECL detection system (Advansta, CA).

2.4. Cell proliferation assays

Cell viability was evaluated every 2 days using MTT assay. Briefly, cells transfected with miR-218 mimic and miR-218 NC (1000/well) were seeded and cultured in 96-well plates. At the indicated times, 20 μl of 0.5 mg/ml MTT (Sigma, Saint Louis, MO) was added into the medium and the plates were further incubated for 4 h, followed by adding 150 μl of DMSO. The plates were then read on a microplate reader using a test wavelength of 570 nm and a reference wavelength of 670 nm. All MTT assays were done in triplicate.

2.5. Cell migration and invasion assays

Cell migration and invasion assays were assessed by transwell chambers (8.0 μm pore size; Millipore, MA). For cell invasion assay, chambers were pre-coated with 1:4 diluted Matrigel (Matrigel was diluted with cold RPMI 1640 medium or Dulbecco's modified Eagle medium; 15 μl/well; BD Bioscience, NJ). The indicated cells were starved overnight and then seeded in the upper chamber at a density of 2×10^4 cells/ml in 200 μl of medium containing 0.5% FBS (4000 cells each well). 1 ml of medium with 10% FBS (1 ml) was added to the lower chamber. After a 24 h incubation, non-migrating/ non-invading cells in the upper chamber were removed using a cotton swab, and migrating/ invading cells were then fixed in 100% methanol and stained with crystal violet solution (0.5% crystal violet in 2% ethanol). Photographs were taken randomly for five fields of each membrane. The number of migrating/invading cells was expressed as the average number of cells per microscopic field over five fields.

2.6. Statistical analysis

Wilcoxon-Mann-Whitney test was used for the comparison of two groups of continuous variables that were not normally distributed (expressed as medians and interquartile ranges). Independent *t* test was used for continuous variables that were normally distributed (expressed as means ± SD), and paired *t* test was used for paired samples. All in vivo experiments were performed in quintuplicate. All statistical analyses were performed using the SPSS statistical package (11.5, Chicago, IL). P values < 0.05 were considered significantly.

3. Results

3.1. Downregulated miR-218 in gastric cancers

To determine the role of miR-218 in gastric tumorigenesis, we first investigated the expression of miR-218 in a total of 476 stomach adenocarcinomas and 42 normal gastric tissues using TCGA data set from the Cancer Browser database. As expected, miR-218 expression in tumor tissues was significantly lower than that in normal controls ($P < 0.0001$; Fig. 1A). In addition, we also found that compared with matched non-cancerous gastric tissues, miR-218 expression was significantly downregulated in gastric cancer tissues ($P = 0.004$, n = 34; Fig. 1B). What's more, high expression of miR-218 in gastric cancer patients was most frequently found in patients with high tumor stage and patients without radiotherapy (Fig. 1C, 1D). To further examine the relationship of miR-218 with patient survival, we then categorized the cancer patients by using medium points of the expression of miR-218 in the cohort, Kaplan-Meier survival curves analysis was then used and we found the decreased miR-218 significantly correlated with the survival time of gastric cancer patients (Fig. 1E). In conclusion, the above date from the TCGA database indicated low expression of miR-218 is a predictor of poor survival for gastric cancer patients.

3.2. miR-218 overexpression inhibits gastric cancer cell growth

Frequent downregulated of miR-218 in primary gastric cancers but not in control subjects suggests that miR-218 may play a tumor-suppressor role in gastric tumorigenesis. To elucidate the role of miR-218 in gastric carcinogenesis, we tested the growth-suppressive effect by upregulating miR-218 expression in gastric cancer cell lines AGS and SGC-7901 using microRNA mimics. Over-expression of miR-218 in AGS and SGC-7901 cells was confirmed by qRT-PCR assay (Fig. 2A). As shown in Fig. 2B, the up-regulated miR-218 significantly inhibited cell proliferation as compared with the negative control both in AGS and in SGC-7901 cells.

3.3. miR-218 overexpression inhibits gastric cancer cell migration and invasion

Given that metastasis is the main cause of cancer-related death and miR-218 has been demonstrated to be involved in cancer metastasis, we thus attempted to investigate the effect of overexpressed miR-218 on the migration and invasion abilities of gastric cancer cells in this study. As shown in Fig. 3A, there were a significantly lower number of migrated cells in miR-218 mimics transfected cells than in negative control ones both in AGS and SGC-7901 cells. Moreover, the invasion assay showed that miR-218 mimics significantly decreased the ability of cells

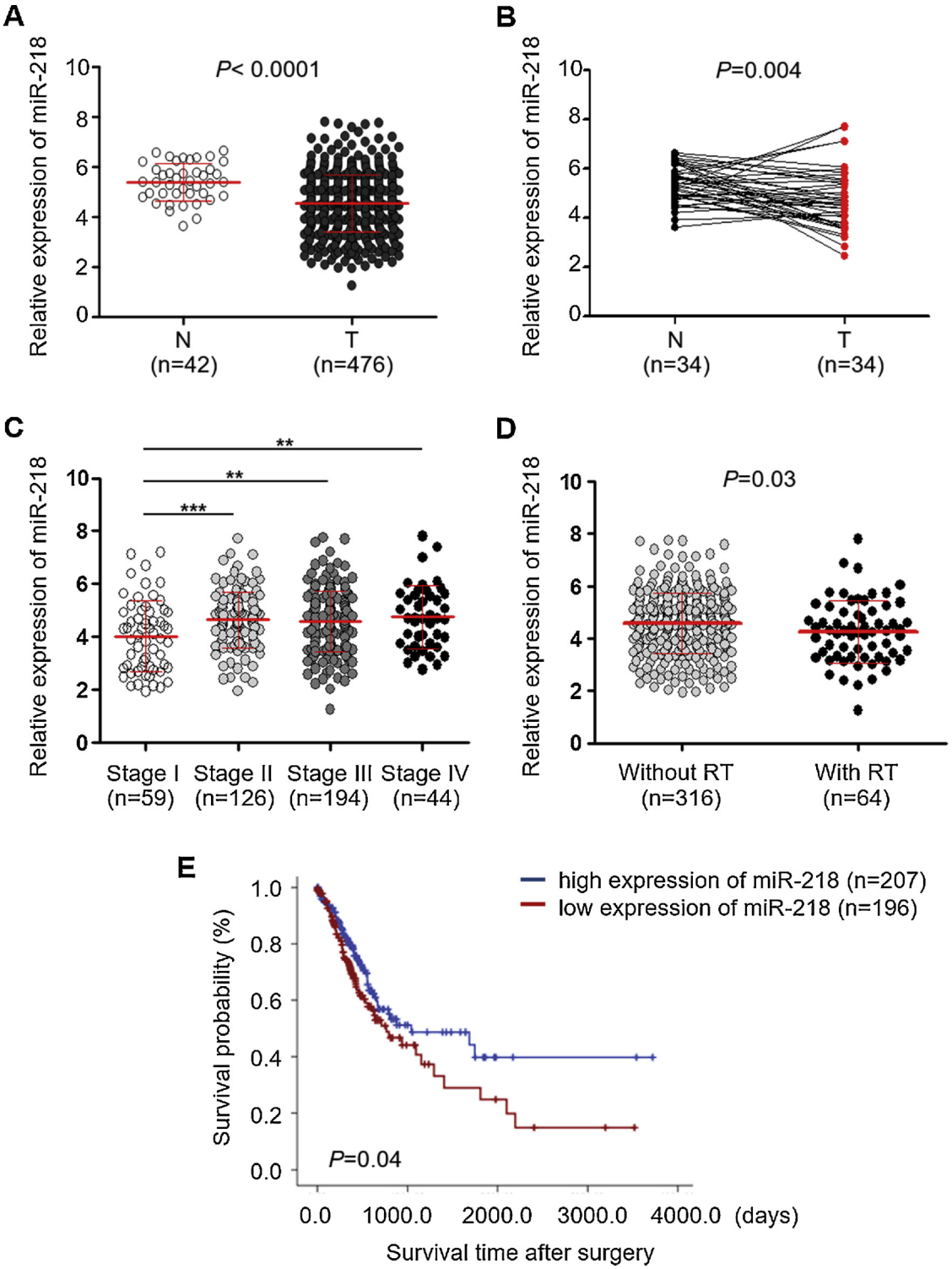

Fig. 1. Downregulation of miR-218 in gastric cancer. The expression of miR-218 was significantly downregulated either in gastric cancers (T; n = 476) compared to the normal tissues (N; n = 42) (A) or in primary gastric cancers (T; n = 34) compared to their matched non-cancerous normal tissues (N; n = 34) (B) in TCGA dataset. High expression of miR-218 was also found in patients with high-grade tumor (C) and patients without radiotherapy (D). (E) Kaplan-Meier analysis was performed according to the expression of miR-218 in TCGA dataset. Kaplan-Meier survival curves indicated decreased miR-218 shortens the survival time of these patients. RT, Radiotherapy; N, normal; T, tumor; $^{**}P < 0.01$; $^{***}P < 0.001$.

to pass through the Matrigel-coated membrane (Fig. 3B). These results suggest that miR-218 overexpression significantly inhibits the migration and invasive potential of gastric cancer cells. To determine whether the effect of miR-218 overexpression on cell metastasis was associated with matrix metalloproteinase (MMPs), qRT-PCR was used to assess the expression of MMP-2,-7 -9 and -14 genes, 4 representative MMPs involved in cancer cell invasion. As shown in Fig. 4A, miR-218 mimics significantly inhibited the expression of these 4 genes in these cells, suggesting that the decrease in the metastasis-associated phenotypes may be link to the inhibition of MMP-2, -7, -9 and -14 genes. To further clarify the mechanism of miR-218 contributing to cell migration and invasion, we investigated the effect of miR-218 on the process of

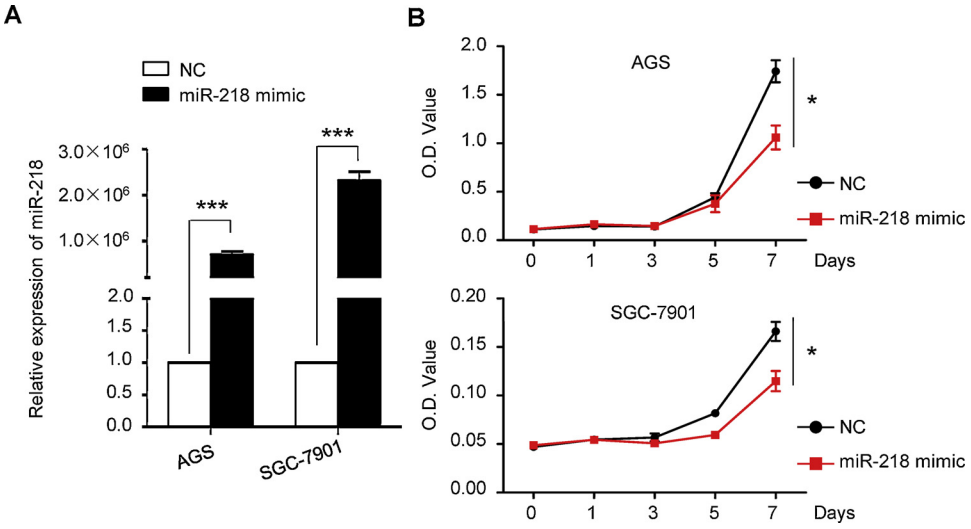

Fig. 2. miR-218 mimic overexpression inhibits cell growth in gastric cancer cells. (A) The expression of miR-218 mimic was confirmed using qRT-PCR assay. *U6*rRNA was used as a normalized control for qRT-PCR assay. (B) miR-218 mimic significantly inhibited cell proliferation in gastric cancer cells AGS and SGC-7901. * $P < 0.05$; ***$P < 0.001$.

epithelial-mesenchymal transition (EMT), which is one of the critical steps during tumor metastasis and plays a central role in the invasion and metastasis of various cancers, including gastric cancer. As shown in Fig. 4A and B, qRT-PCR and western blot indicated that miR-218 overexpression in AGS and SGC-7901 cells increased E-cadherin expression, and decreased the expression of Vimentin as well as the transcription suppressors Twist, Snail-1 and -2. Collectively, these findings suggest that inhibition of the EMT process by miR-218 mimics may contribute to suppression of gastric cancer cell migration and invasion.

3.4. *miR-218 negative regulating PI3K/Akt signaling via Bmi-1*

To gain insights into molecular mechanisms underlying tumor suppressor role of miR-218 in gastric cancer, bioinformatics analysis was then used to investigate the target genes of miR-218. Consistent with previous studies, we found that miR-218 can bind to the 3′UTR region of Bmi-1. Thus, we speculate that miR-218 may play a tumor suppressor role in gastric cancer through targeting Bmi-1. As shown in Fig. 5A, Bmi-1 expression in tumor tissues was significantly higher than that in normal controls ($P < 0.001$; Fig. 5A). In addition, we also found that compared with matched non-cancerous gastric tissues, Bmi-l expression was significantly upregulated in gastric cancer tissues ($P = 0.001$; Fig. 5B). Moreover, correlation analysis indicated that Bmi-1 expression was significantly negatively correlated with the expression of miR-218 (Fig. 5C). It has been reported that PI3K/Akt signaling pathway is a direct downstream target of Bmi-1, which is involved in the regulation of EMT process and the expression of MMPs in tumorigenesis. However, previous studies also indicated that Bmi-1 activated the PI3K/Akt pathway through inhibiting the expression of PTEN. Therefore, we hypothesize that miR-218 inhibits gastric tumorigenesis through regulating the Bmi-1/Akt signaling pathway. As shown in Fig. 5D, miR-218 mimics could significantly inhibit Bmil expression as well as the phosphorylated Akt both in AGS and SGC-7901 cell lines. Taken together, our data implied that miR-218 inhibits gastric cancer cell growth and invasiveness through negatively regulating the PI3K/Akt signaling pathways (Fig. 6).

4. Discussion

GC is a complex and molecularly heterogeneous disease involving deregulation of canonical oncogenic pathways [29,30], such as p53 [31,32], wnt/β-catenin [33], nuclear factor (NF)-κB [34] and PI3K/Akt pathways [35]. Moreover, like other human cancers, gastric tumorigenesis can also be profoundly influenced by epigenetic abnormalities, such as aberrant gene methylation, histone modification and microRNAs [36,37]. miR-218 was demonstrated to be an important tumor suppressor in a number types of cancer. In hepatocellular carcinoma, miR-218 has been demonstrated to be downregulated, and associated with increased tumor size [38]; Moreover, miR-218 inhibits human lung adenocarcinoma cell migration and invasion via the suppression of Ecop and Robo1 expression, suggesting miR-218 as a potential therapeutic target [39]; Similarly, overexpression of miR-218 significantly inhibited cell proliferation, arrested the cell cycle in the G0/G1 phase and induced apoptosis by targeting Bmi-1, thus the miR-218/Bmi-1 signaling axis may be a potential novel diagnostic marker and therapeutic target for the treatment of APL [40]. In this study, we found that miR-218 was frequently downregulated in gastric cancer tissues as compared with matched normal gastric tissues in TCGA database, and decreased expression of miR-218 predict a poor survival in these patients, suggesting that miR-218 may be a potential tumor suppressor gene in gastric cancer.

Considering that the downregulated miR-218 may contribute to gastric tumorigenesis, tumor suppressor effect of miR-218 was validated in gastric cancer cells. miR-218 overexpression in the downregulated gastric cancer cell lines showed significant growth-inhibitory effect by suppression of cell proliferation as well as induction of cell apoptosis, further confirming its potential tumor suppressor function. Notably, miR-218 overexpression strongly inhibited gastric cancer cell migration and invasion. Western blot indicated that miR-218 over-expression in AGS and SGC-7901 cells increased E-cadherin expression, and decreased Vimentin expression suggesting that miR-218 overexpression inhibited the EMT process in gastric cancer cells. In addition, given that matrix metalloproteinase (MMPs) play a key role in tumor metastasis [41], the effect of miR-218 on expression of *MMP-2,-7 -9 and -14* was tested in gastric cancer cells. As expected, our data showed that miR-218 knockdown significantly induced the expression of these genes, implicating that the decreased metastasis-associated phenotypes may be mediated by suppressing the expression of *MMPs* genes.

To better understand the tumor suppressor role of miR-218 in gastric cancer, PicTar (http://pictar.mdc-berlin.de/), TargetScan (http://www.targetscan.org/) and DIANA (http://diana.cslab.ece.ntua.gr/microT/) was used to investigate target genes of miR-218. As with previous studies, bioinformatics analysis indicated that miR-218 can bind to the 3′UTR region of Bmi-1. What's more, by using TCGA database, we also found the expression of Bmi-1 was significantly elevated in cancer patients. Moreover, compared with matched non-cancerous gastric tissues, Bmi-l was also upregulated in gastric cancer tissues. Next, correlation analysis showed that Bmi-1 expression was significantly negatively correlated with the expression of miR-218.

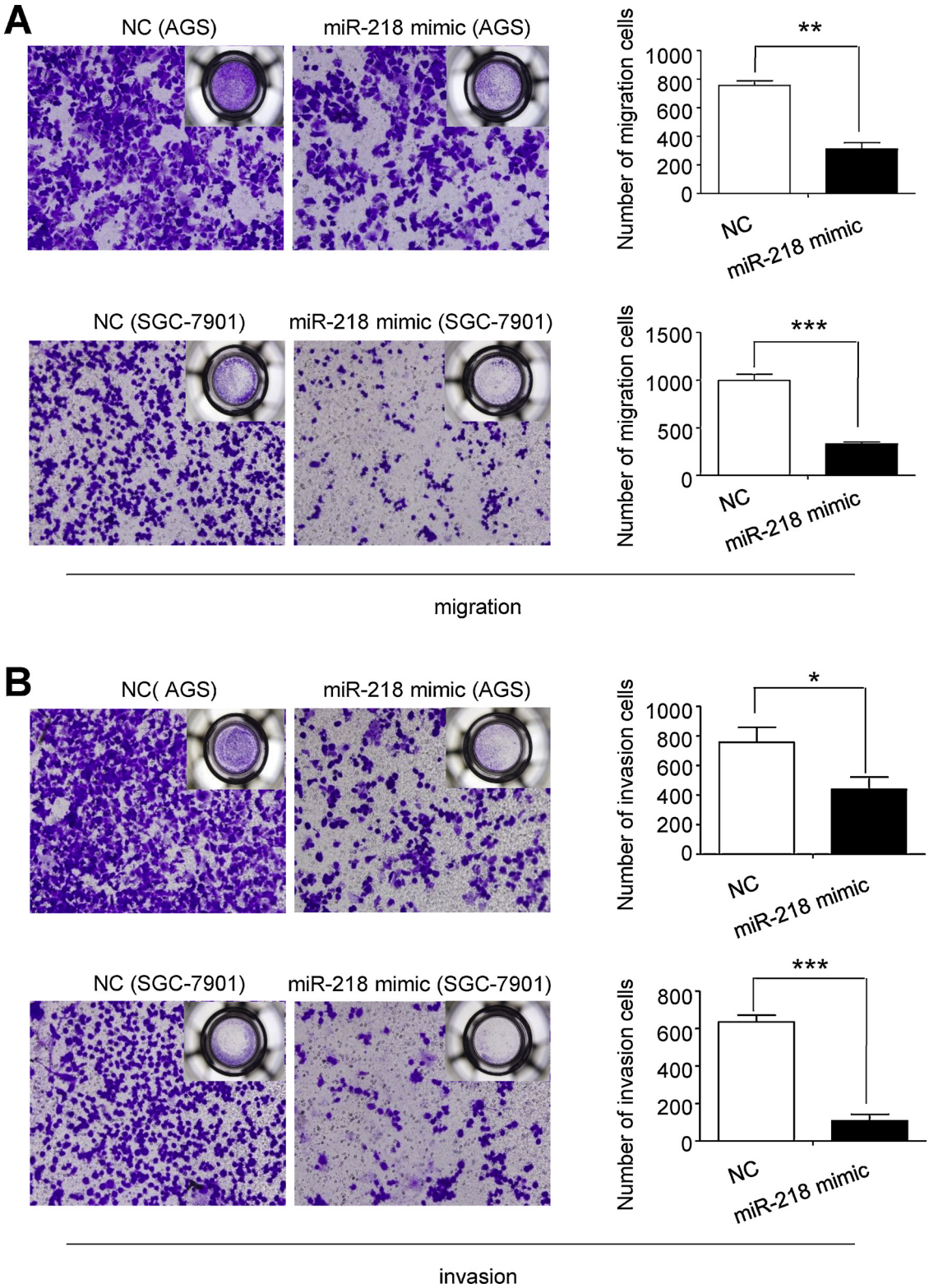

Fig. 3. miR-218 mimic inhibits gastric cancer cell migration and invasion. The representative images of migrated (A) or invaded (B) cells. Histograms, corresponding to show means ± S.E. of cell numbers from three independent assays. Magnification for each set: × 200. Scale bars, 50 μm. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Previous studies found PTEN is a direct downstream target of Bmi-1, which is involved in PI3K/Akt signaling pathway in tumorigenesis of gastric cancer [42,43]. Therefore, we hypothesize that miR-218 inhibits gastric tumorigenesis through regulating the Bmi-1/Akt signaling pathway. As expected, miR-218 overexpression significantly inhibits Bmi-l expression as well as the AKT S473 phosphorylation in the above two gastric cancer cell lines. Taken together, our data implied that miR-218 inhibits gastric cancer cell growth and invasion through negatively regulating the PI3K/Akt signaling pathways.

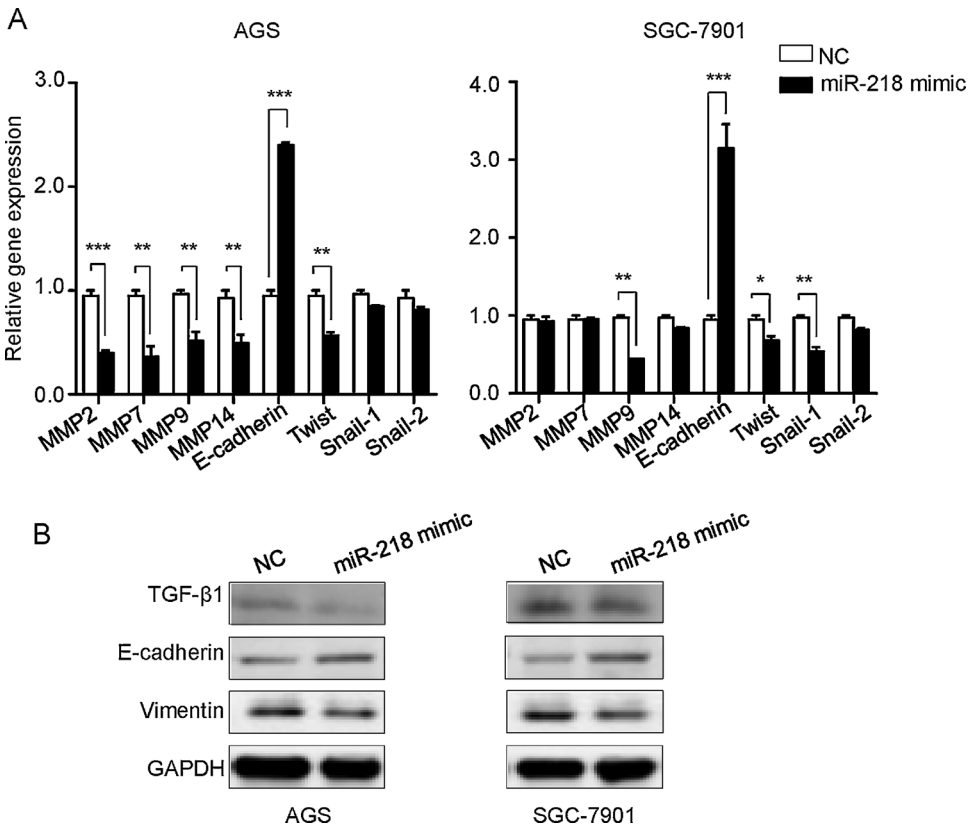

Fig. 4. miR-218 mimic inhibits EMT process in gastric cancer cells. (A) qRT-PCR was performed to test the effect of miR-218 mimic on the expression of metastasis-related genes MMP2, 7, 9, 14 and E-cadherin as well as its transcription suppressors Twist, Snail-1 and -2 in gastric cancer cells. Expression levels of these genes were normalized with *18S*rRNA levels. Data were presented as mean ± S.E. (B) The expression of TGF-β1, E-cadherin and Vimentin was determined in the indicated cells by western blot analysis. GAPDH was used as loading control. $^{**}P < 0.01$; $^{***}P < 0.001$.

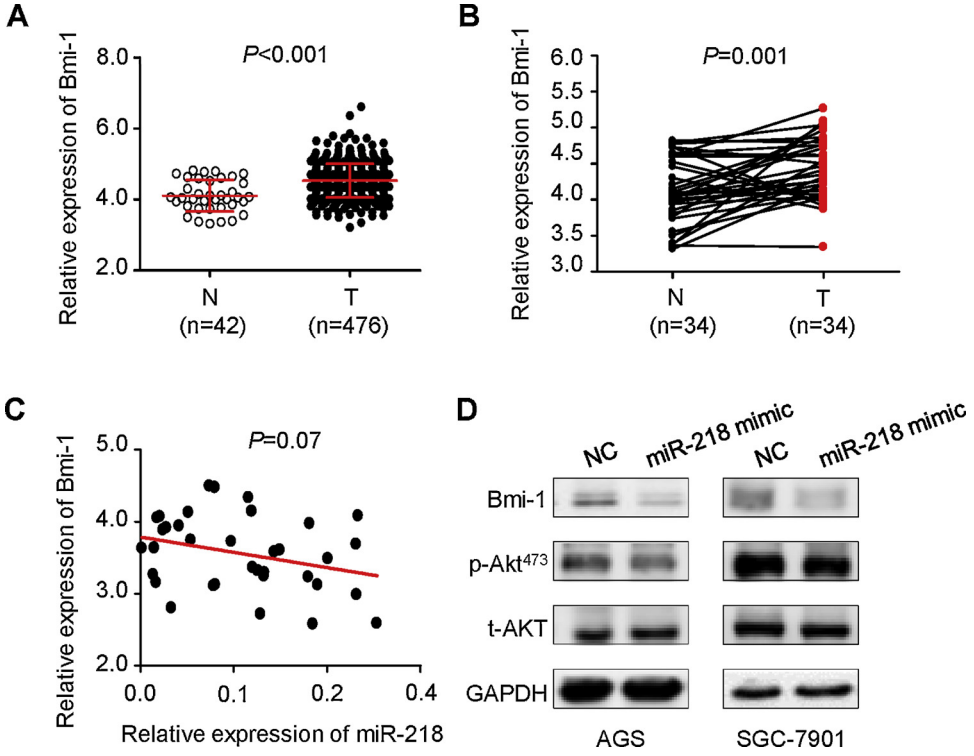

Fig. 5. Downregulated Bmi-1 by miR-218 mimic in gastric cancer. Bmi-1 is highly expressed either in gastric cancers (T; n = 476) compared with normal gastric tissues (N; n = 42) (A) or in primary gastric cancers (T; n = 34) compared with their matched non-cancerous gastric tissues (N; n = 34) (B) in TCGA dataset. (C) Linear regression analysis was performed to assess the correlations between Bmi-1 with miR-218 in gastric cancers in TCGA data set. (D) The effect of miR-218 mimic overexpression on the PI3K/Akt cascades by western blot analysis. GAPDH was used as loading control.

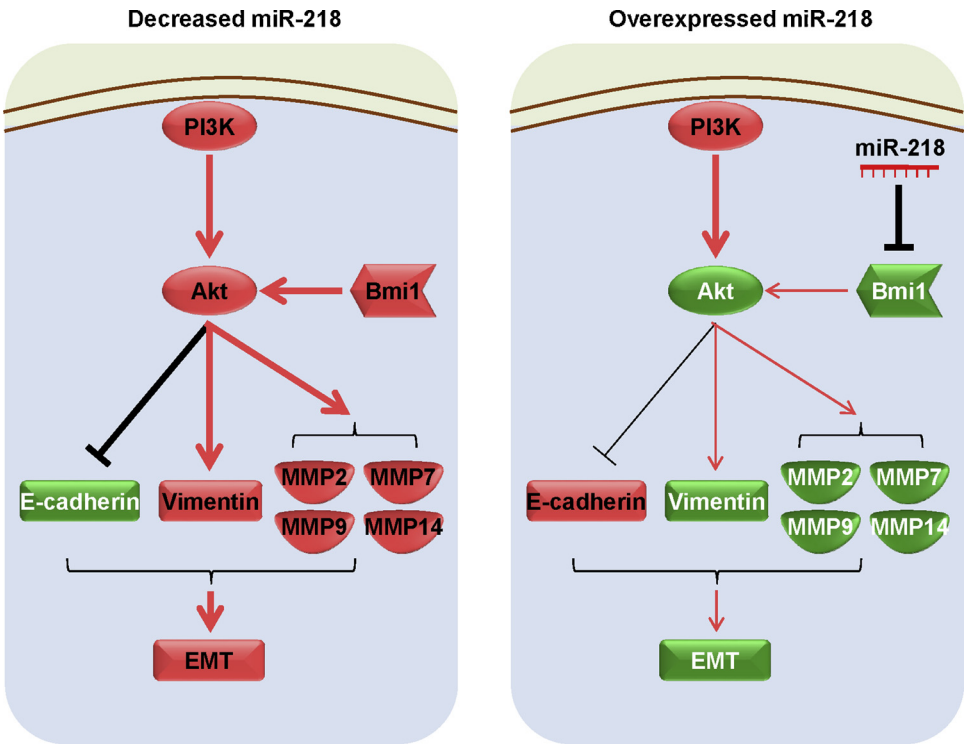

Fig. 6. Schematic model of molecular mechanisms underlying tumor suppressor role of miR-218 in gastric cancer. Downregulated miR-218 in gastric cancer cell induces a high level of Bmi-1, causing Akt phosphorylation and sequentially activates PI3K/Akt pathway. Activated PI3K/Akt pathway promotes EMT progression by increasing the expression of Vimentin, MMPs and decreasing the expression of E-cadherin. Taken together, downregulation of miR-218 promotes cell growth and invasion through activating Bmi-1/Akt signaling pathways, ultimately contributing to tumorigenesis of gastric cancer.

Declaration of interest

The authors declare that they have no competing interests.

Acknowledgments

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