

Original Paper

miR-106b Promotes Metastasis of Early Gastric Cancer by Targeting ALEX1 *in Vitro* and *in Vivo*

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Key Words

miR-106b • ALEX1 • EGC • Metastasis • JAK1/STAT3 signaling pathway • GLPG0643

Abstract

Background/Aims: Aberrant expression of miR-106b is a specific symptom of many solid carcinomas. Overexpression of miR-106b has been observed in gastric cancer. The effect of miR-106b on gastric cancer has been investigated in different cell culture models. However, the effect of miR-106b on metastasis of early gastric cancer (EGC) remains unknown. **Methods:** In the study, qRT-PCR, FISH, western blot, luciferase reporter assay, migration and invasion assays, flow cytometry and TUNEL staining were used to investigate the effect of miR-106b on metastasis of EGC. **Results:** To explore the function of miR-106b in EGC, we investigated the downstream signaling of miR-106b and found that ALEX1 was a direct target of miR-106b in gastric cancer cells. Up-regulation of ALEX1 effectively rescued the cell apoptosis induced by miR-106b inhibitor and promoted the expression levels of phosphorylation of JAK1 and STAT3. Moreover, overexpression of JAK1 reduced the cell apoptosis induced by miR-106b inhibitor and decreased the expression levels of the apoptotic proteins in gastric cancer cells. Furthermore, down-regulation of miR-106b promoted apoptosis of gastric cancer cells via inhibiting JAK1/STAT3 signaling pathway *in vitro* and *in vivo*. In addition, GLPG0643, a JAK1 inhibitor, enhanced the inhibitory effect of miR-106b inhibitor on gastric cancer growth *in vivo*. **Conclusion:** These findings provided a potential therapeutic manner for the treatment of metastasis of EGC in clinic.

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Introduction

Gastric cancer is one of the most common malignancies in the world. The incidence rate of gastric cancer has been gradually increasing in recent years [1, 2]. The diagnosis and treatment of early gastric cancer (EGC) are faced with huge challenges, which have caused widespread concern in the world [3-6]. EGC is defined as the invasion of stomach limited to the mucosal layer and submucosa and it has no direct correlation with lymph node metastasis [7]. Currently, the metastasis of EGC, the main cause that affects the survival of the patients, has no effective treatment manners [8-10]. Additionally, EGC is difficult to diagnose due to the non-specific symptoms. Although the application of endoscopic submucosal dissection can effectively improve the surgical outcome, the incidence rate of lymph node metastasis is still highly occurred, which achieves 5%-28% in patients with EGC [11, 12]. Thus, a deeper understanding of the molecular mechanism of the invasion and metastasis may lead to the better diagnosis and treatment of EGC.

miRNAs are a class of non-coding RNAs that act on posttranscriptional translation by targeting the target protein, which affect a wide range of biological functions such as cell proliferation, differentiation and apoptosis [13-15]. Different types of miRNAs may play different roles such as oncogenes or tumor suppressor genes in the tumorigenesis and development of gastric cancer [16-18]. Previous studies have shown that miR-106b promotes the occurrence of tumor metastasis in liver cancer and colorectal cancer [19, 20]. Moreover, the expression of miR-106b in serum can be used as a marker of high risk of EGC in a previous study [21]. Recent studies have shown that miR-106b has been involved in the regulation of invasion and migration of different tumor cell lines [22, 23]. However, the role of miR-106b in the invasion and metastasis of EGC and its detailed mechanism remain unclear. Arm protein lost in epithelial cancers, on chromosome X (ALEX) is a subgroup of armadillo family, which participates in a variety of cell processes such as cell proliferation, adhesion and apoptosis [24]. A recent study has revealed that overexpression of ALEX1 can inhibit the cell proliferation and induce the cell apoptosis, whereas depletion of ALEX1 leads to the increasing proliferation and the inhibitory apoptosis in breast cancer cells [25]. Hiroyoshi Iseki et al. found that overexpression of ALEX1 suppresses the colony formation in human colorectal carcinoma cells [24]. However, the relationship between ALEX1 and miR-106b and the probably mechanism in the metastasis of EGC are urgently needed to be solved. Here, we evaluate the function of miR-106b in EGC and investigate the mechanism of miR-106b mediated metastasis *in vitro* and *in vivo*. The findings will help reduce the incidence of EGC metastasis, and provide new ideas for the treatment of metastasis of EGC in clinic.

Materials and Methods

Patients and Specimens

The metastasis and non-metastasis tumor tissues were acquired from the patients with EGC. The patients with EGC underwent surgical resection at the Ruijin Hospital of Shanghai Jiaotong University. These patients did not have history of chemotherapy or radiotherapy before collecting specimens. The specimens were collected with the consent of the patients. The experiment was approved by the Ethics Committee of Shanghai Jiaotong University.

Cell culture

Human gastric cancer cell AGS was acquired from Chinese Academy of Sciences and cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA). The cells were maintained at 37°C in an atmosphere of 5% CO₂.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen). Reverse transcription was performed and cDNA was prepared for amplification. The expression levels of miR-106b were quantified using a miRNA-specific TaqMan miRNA Assay Kit (Applied Biosystems). The reaction was performed with incubation at 95°C for 5 min, 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s (36 cycles), and at 72°C for 10 min using the primers (F: 5'-AGCCGTCAAGAGCAATAACGAA-3', R: 5'-GTGCAGGG TCCGAGGT-3'). U6 was used as a reference gene. Analysis of expression levels of miR-106b was performed in triplicate and calculated using the $2^{-\Delta\Delta ct}$ method.

Fluorescence in situ hybridization (FISH)

Tissue was prepared and performed ISH using the miR-106b probe (5'-ATCTGCACTGTCTCAGCACTTTA-3' and the microRNA ISH Kit (Exiqon, Vedbaek, Denmark). The signals of IF were examined with a fluorescence microscope (Olympus).

Transwell migration and invasion assay

Cell suspension was added into the upper cup of transwell chambers (Corning Incorporated, NY, USA). The lower chamber was supplied with 10% FBS-supplemented media. After 16 h, non-migrating cells inside upper cup were removed by cotton swab. Cells migrating through the membrane to bottom surface of the cup were fixed in methanol, stained with 0.5% crystal violet, and subjected to microscopic analysis. Numbers of migrating cells were counted in ten randomly selected fields. Invasion assay was conducted using 24-well transwell chambers coated with 3 mg/ml matrigel (Corning Incorporated, NY). The invading cells were analyzed after 24 h.

Luciferase Reporter assays

The promoter of ALEX1 was cloned in pGL 3.0 luciferase reporter plasmids. Cells were cotransfected with pRL-CMV renilla luciferase reporter and the pGL 3.0 luciferase reporter plasmid containing the promoters of ALEX1. The luciferase activities were measured by a dual luciferase reporter assay system (Promega).

Lentivirus vector construction, transfection and infection

HEK-293T cells were fostered in culture dish. The lentivirus vectors containing ALEX1 (PLVX-ALEX1) and JAK1 (PLVX-JAK1) were transfected into HEK-293T. Viral lysates were harvested and purified. After AGS cells were incubated in plate for 24 h. PLVX vectors were added into the medium and infected the cells.

Western blot

For western blots, samples were separated on SDS-PAGE gels and then transferred to PVDF membranes (Millipore). After blocking with milk, membranes were processed following the ECL Western blotting protocol (GE). Bcl-2, Bax, Bim, ZEB1, SNAI2, TWIST1, E-cadherin, pJAK1, pSTAT3 and GAPDH antibodies were acquired from Cell Signaling Technology (Beverly, MA, USA). ALEX1 antibody was obtained from Abcam (Cambridge, MA).

Flow cytometry

Cell suspension was prepared and added 10 μ L of Annexin V, incubated in dark for 15 min, then added 5 μ L of 7AAD, and analyzed using the flow cytometer within 5 minutes. The results were expressed as percentage of apoptotic cells.

Animals

BALB/c nude mice were used in the experiments at the age of 4 weeks. The mice were provided from the Animal Center of the Second Military Medical University. All animal experiments were conducted in strict accordance with the guidelines for the care and use of experimental animals and were approved by the Animal Care Committee of Ruijin Hospital, Shanghai Jiaotong University. Mice bearing AGS tumor xenografts were randomly divided into five groups: Control, NC, GLPG0634, miR-106b inhibitor, miR-106b inhibitor + GLPG0634. Volumes of tumor were measured every week.

TUNEL staining

Tissues were embedded and incubated with proteinase K (40 µg/mL) for 1 hour at 37°C, then treated with 2% H₂O₂ in distilled water for 30 min at 37°C. After enzymatic reaction, sections were washed with PBS, incubated with anti-digoxigenin peroxidase conjugate for 30 min at 37°C in a humidified chamber. Sections were stained with diaminobenzine and counterstained with hematoxylin, and observed under a light microscope.

Statistical analysis

The data were expressed as the means ± standard deviations of three independent experiments and data were considered statistically significant when values reached P<0.05.

Results

Effect of miR-106b on the metastasis of EGC

The expression of miR-106b was explored in the tissues of patients with EGC metastasis by Q-PCR and FISH. The result of Q-PCR revealed that the expression of miR-106b in the metastasis tissues showed nearly 2-fold increase compared with non-metastasis tissues (Fig. 1a). Moreover, FISH result showed that the expression of miR-106b was slightly increased in the metastasis tissues compared with non-metastasis tissues (Fig. 1b). To investigate the effect of miR-106b on the metastasis of EGC, we up-regulated and down-regulated the expression of miR-106b by the mimics or inhibitor of miR-106b in gastric cancer cells. The result showed the expression of miR-106b was significantly decreased following treatment with the inhibitor of miR-106b (Fig. 1i), and significantly increased after treatment with miR-106b mimics (Fig. 1c). The migration and invasion results revealed that miR-106b mimics treatment significantly increased the migrated (Fig. 1d and e) and invaded (Fig. 1f and g) cells when compared with the control. Meanwhile, miR-106b mimics also promoted the expression levels of EMT-related proteins, including SNAI2, ZEB1, TWIST1 and E-cadherin (Fig. 1h). On the other hand, we detected the effect of miR-106b inhibitor on the apoptosis of gastric cancer cells. The result showed that the apoptosis proportion was remarkably increased after treatment with miR-106b inhibitor compared with the control (Fig. 1j). In addition, miR-106b knockdown obviously enhanced the expression levels of apoptotic proteins such as Bax and Bim, and reduced Bcl-2 expression levels (Fig. 1k), which suggest miR-106b inhibitor could promote the apoptosis of gastric cancer cells.

miR-106b targeted ALEX1 in gastric cancer cells

ALEX1, a member of the armadillo family, is reported to inhibit cancer progress and metastasis. To study the effect of ALEX1 on miR-106b regulating the function of gastric cancer cells, we detected ALEX1 expression levels following treatment with or without miR-106b and up-regulated ALEX1 by infection of PLVX-ALEX1 (Fig. 2a and b). The result showed that miR-106b inhibitor obviously enhanced the expression levels of ALEX1 and miR-106b mimics reduced ALEX1 expression levels in AGS cells (Fig. 2a). Moreover, in order to determine the relationship between ALEX1 and miR-106b in gastric cancer cells, the luciferase reporter plasmids containing the wt or mutant 3'-UTR segments of ALEX1 were constructed. The result demonstrated that the luciferase activities were remarkably promoted by miR-106b inhibitor combined with wt reporter plasmid treatment, while they were no difference in the treatment of miR-106b inhibitor combined with mutant reporter plasmid compared with the control. On the other hand, miR-106b mimics combined with wt reporter plasmid treatment significantly decreased the luciferase activities compared with the control. However, miR-106b mimics combined with mutant reporter plasmid treatment showed no change of the luciferase activities (Fig. 2c). Interestingly, we also found the apoptotic cells induced by miR-106b inhibitor were significantly decreased after cells were infected with PLVX-ALEX1 (Fig. 2d). In accordance with the result of apoptosis, the expression levels of apoptotic proteins were obviously reduced after combined treatment with miR-106b inhibitor and PLVX-ALEX1

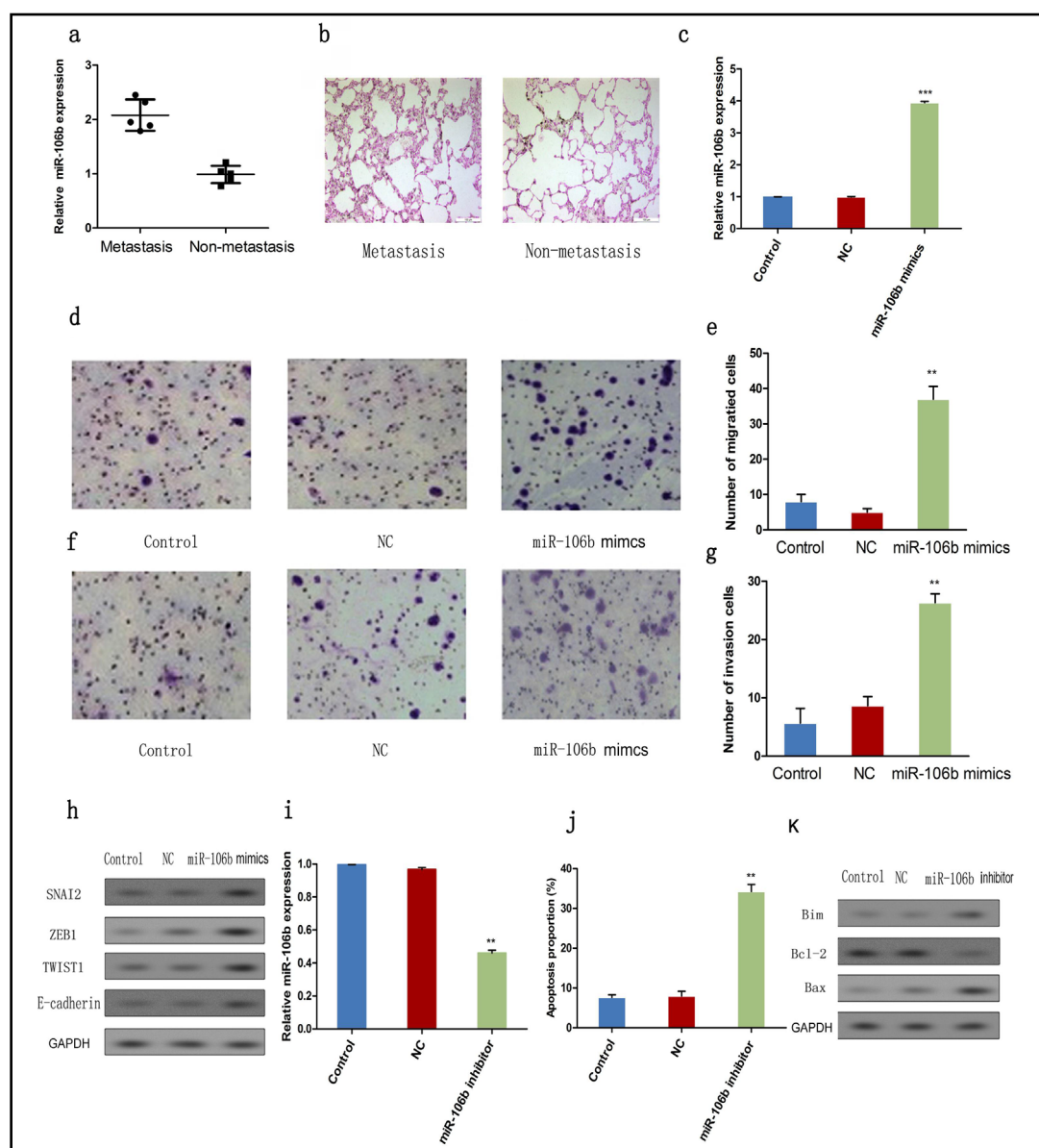


Fig. 1. Effect of miR-106b on migration and invasion of gastric cancer cells. (a, b) miR-106b expression was detected in the tissues of EGC by Q-PCR and FISH. (c) Expression of miR-106b was verified following treatment with miR-106b mimics. (d, e) Migration ability of gastric cancer cells was detected following treatment with miR-106b mimics. (f, g) Invasion ability of gastric cancer cells was detected following treatment with miR-106b mimics. (h) Detection of EMT related proteins SNAI2, ZEB1, TWIST1, E-cadherin after cells treated with miR-106b inhibitor. (i) Expression of miR-106b was verified following treatment with miR-106b inhibitor. (j) AGS cells were treated with miR-106b inhibitor, then stained with annexin V-FITC/7-AAD and analyzed by flow cytometry. (k) Apoptotic protein expression levels of Bim, Bcl-2, Bax were detected following treatment with miR-106b inhibitor. **P<0.01 versus control. ***P<0.001 versus control.

when compared with miR-106b inhibitor treatment alone (Fig. 2g and 2h). In addition, the EMT related protein expression levels were decreased following combined treatment with miR-106b inhibitor and PLVX-ALEX1 when compared with miR-106b mimics treatment alone (Fig. 2e and 2f).

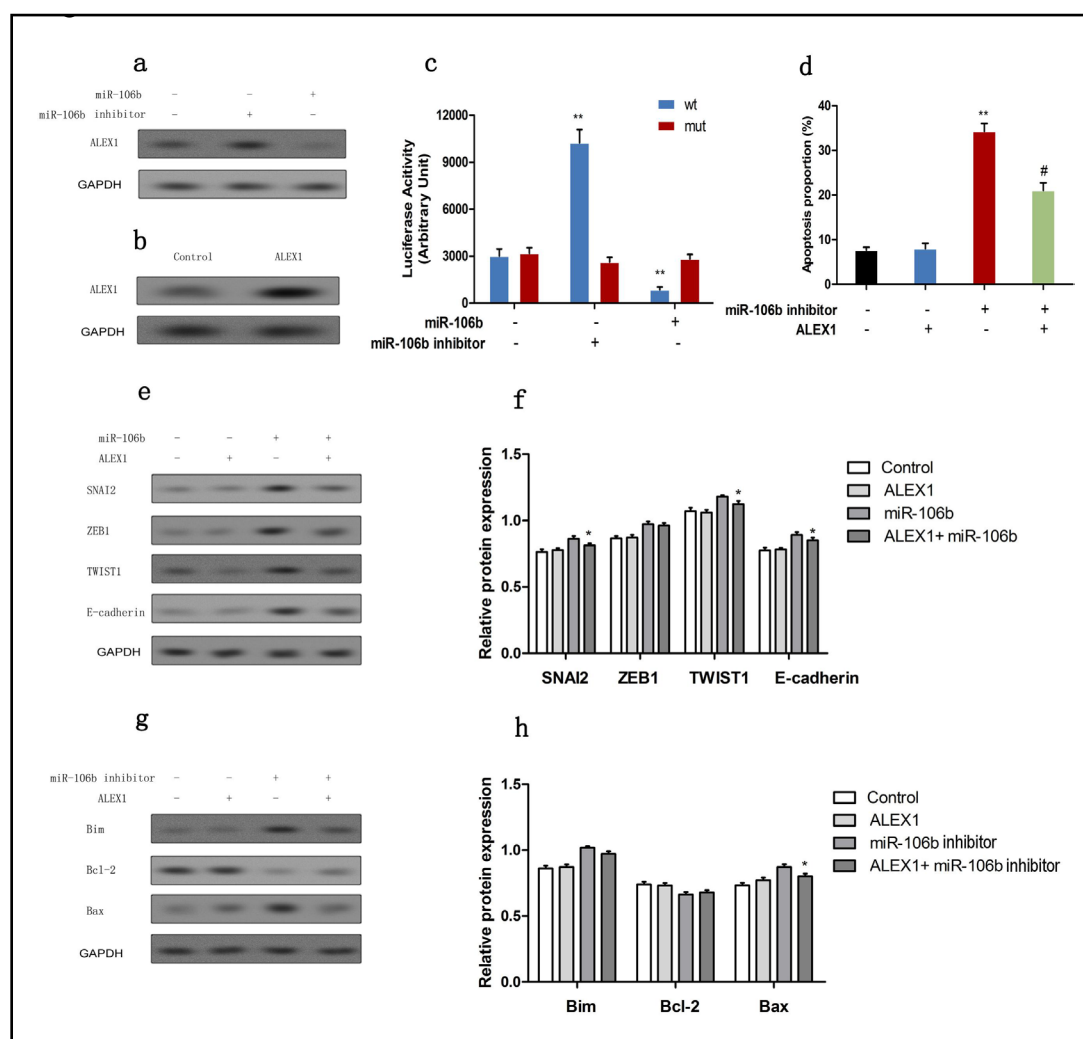


Fig. 2. miR-106b targeted ALEX1 in gastric cancer cells. (a) miR-106b mimics and miR-106b inhibitor were used to treat gastric cancer cells, and the expression of ALEX1 was detected. (b) ALEX1 expression level was detected following PLVX-ALEX1 treatment. (c) Luciferase activity of ALEX1 was detected by luciferase reporter assay following treatment with miR-106b mimics or miR-106b inhibitor. **P<0.01. (d) Cell apoptosis was detected following treatment with miR-106b inhibitor and/or PLVX-ALEX1. **P<0.01 versus control. # P<0.05 versus miR-106b inhibitor treatment. (e, f) Detection of EMT related proteins SNAI2, ZEB1, TWIST1, E-cadherin after cells treated with miR-106b mimics and/or PLVX-ALEX1. *P<0.05 versus miR-106b mimics treatment. (g, h) Detection of apoptotic proteins after cells treated with miR-106b inhibitor and/or PLVX-ALEX1. *P<0.05 versus miR-106b inhibitor treatment.

Down-regulation of miR-106b promoted apoptosis of gastric cancer cells through JAK1/STAT3 signaling pathway

To further investigate the molecular mechanism of miR-106b mediated apoptosis of gastric cancer cells, JAK/STAT signaling pathway related proteins were studied after treatment with miR-106b inhibitor. The results revealed that miR-106b inhibitor treatment led to a decrease of pJAK1 and pSTAT3 expression levels compared with the control (Fig. 3a). In order to study the effect of ALEX1 on miR-106b inhibitor regulating JAK/STAT signaling pathway, the gastric cancer cells were infected with PLVX-ALEX1 following treatment with miR-106b inhibitor. As expected, PLVX-ALEX1 infection treatment reversed the expression levels of pJAK1 and pSTAT3 inhibited by miR-106b inhibitor (Fig. 3b). The relative expression levels of pJAK1 and pSTAT3 were significantly increased by PLVX-ALEX1 combined with miR-

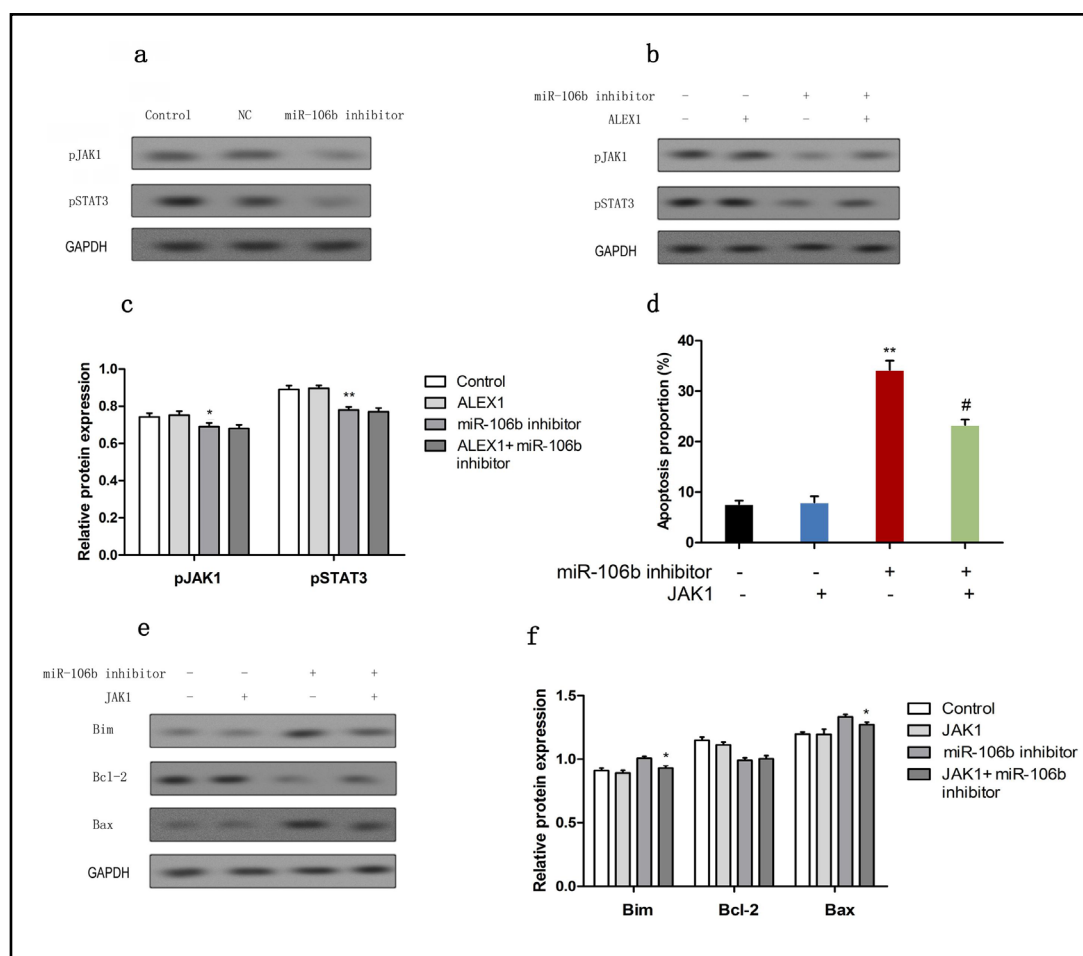


Fig. 3. miR-106b inhibitor regulated JAK1/STAT3 Signaling Pathway. (a) Detection of JAK/STAT pathway related protein levels after cells treated with or without miR-106b inhibitor. (b, c) The expression levels of pJAK1 and pSTAT3 were detected following treatment with with miR-106b inhibitor and/or PLVX-ALEX1. * $P < 0.05$ versus control, ** $P < 0.01$ versus control. (d) Cell apoptosis was detected following treatment with miR-106b inhibitor and/or PLVX-JAK1. ** $P < 0.01$ versus control. # $P < 0.05$ versus miR-106b inhibitor treatment. (e, f) Detection of apoptotic proteins after cells treated with miR-106b inhibitor and/or PLVX-JAK1. * $P < 0.05$ versus miR-106b inhibitor treatment.

106b inhibitor treatment when compared with miR-106b inhibitor treatment alone (Fig. 3c). To further determine the effect of JAK/STAT signaling pathway on miR-106b inhibitor induced apoptosis, we constructed the PLVX-JAK1 vectors and infected the gastric cancer cells. The result showed that PLVX-JAK1 combined with miR-106b inhibitor treatment remarkably decreased the apoptotic cells when compared with miR-106b inhibitor treatment alone (Fig. 3d). Consistently, the apoptotic protein expression levels of Bax and Bim were obviously reduced, and the Bcl-2 expression levels were increased after PLVX-ALEX1 combined with miR-106b inhibitor treatment (Fig. 3e and f).

Down-regulation of miR-106b inhibited gastric cancer *in vivo*

To testify the effect of miR-106b knockdown on gastric cancer *in vivo*, we constructed the mouse model of gastric cancer using AGS gastric cancer cells. The tumor volume was observed in different treatments for indicated time. The result revealed miR-106b inhibitor significantly reduced the tumor volume of gastric cancer; and this effect was further promoted by the treatment of GLPG0643 (Fig. 4a). Moreover, down-regulation of miR-106b enhanced the protein expression levels of ALEX1 *in vivo* (Fig. 4b). Meanwhile, down-regulation of miR-

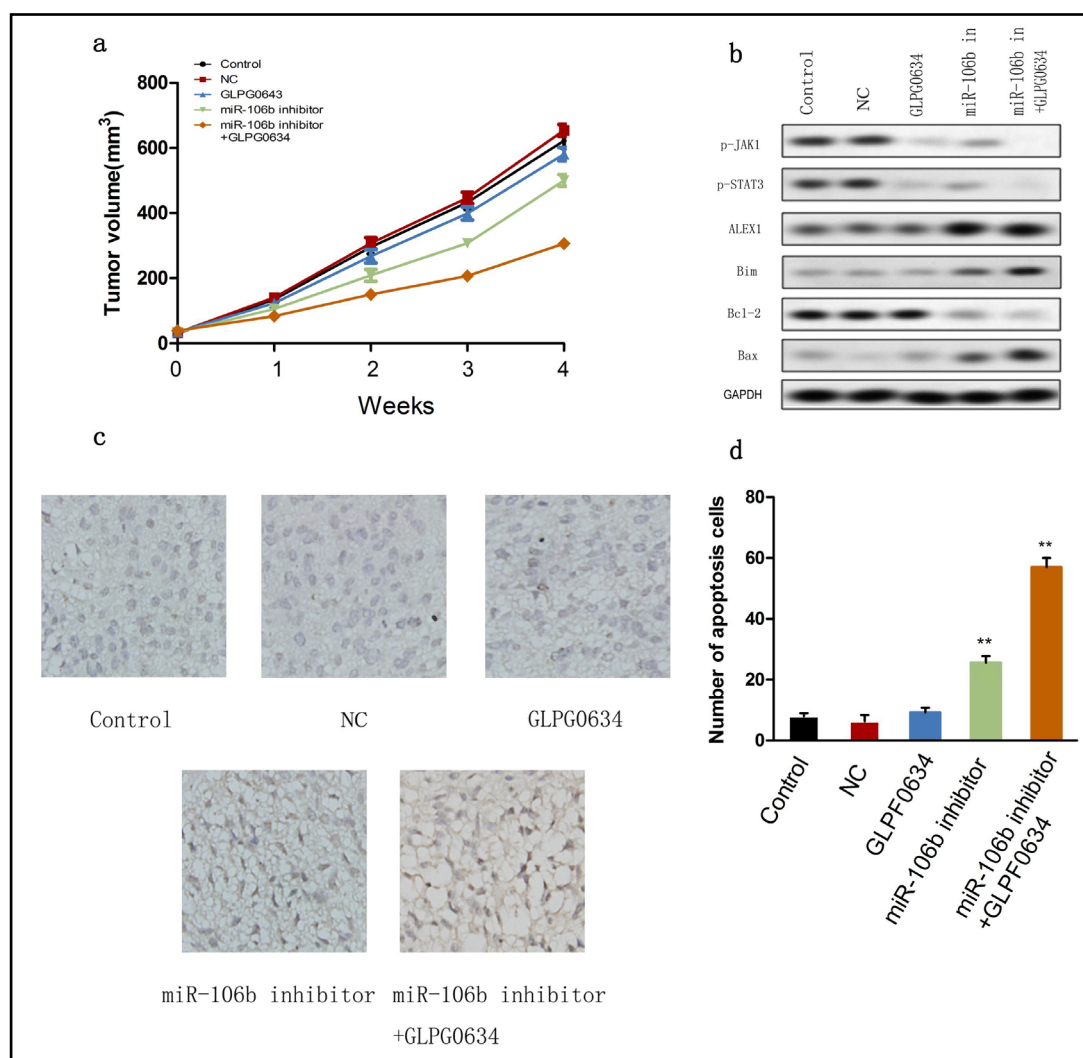


Fig. 4. miR-106b inhibited gastric cancer *in vivo*. (a) Tumor volume was measured with different treatments for indicated time points. (b) The protein expression levels of pJAK1, pSTAT3, ALEX1, Bim, Bcl-2 and Bax were detected following with different treatments. (c, d) Apoptosis was detected using TUNEL staining for the tumor tissue treated with different treatments. ** $P < 0.01$ VS Control.

106b significantly decreased the pJAK1 and pSTAT3 protein expression levels compared with the control, which was consistent with the results *in vitro*, and the effect of miR-106b knockdown on the pJAK1 and pSTAT3 protein expression was enhanced by the treatment of GLPG0643 (Fig. 4b). To further determine the role of miR-106b knockdown in gastric cancer, the apoptosis of tumor tissues was detected using a TUNEL assay. The result revealed that the TUNEL positive cells were remarkably increased by down-regulation of miR-106b when compared with the control, and further promoted by GLPG0643 treatment (Fig. 4c and d). In addition, the apoptotic protein expression levels of Bax and Bim were obviously increased, and the Bcl-2 expression levels were decreased following down-regulation of miR-106b, which further strengthened by GLPG0643 treatment (Fig. 4b).

Discussion

miR-106b is reported to overexpression in various types of cancers and plays an important role in the regulation of cell invasion and migration *in vitro* and *in vivo* [26-29]. However, the expression of miR-106b and its possible functions in metastasis of cancer cells are not clear in EGC. In this study, the expression levels of miR-106b in metastasis tissues of EGC were increased when compared with non-metastasis tissues. ALEX1, a novel tumor suppressor gene, suppresses metastasis of gastric cancer, which involved in PAR-1/Rho GTPase signaling pathway [30]. The tentative inhibition of miR-106b in metastasis of EGC and the possible mechanism are urgent to be solved.

Previous study demonstrated that miR-106b was up-regulated in cancer associated fibroblasts (CAFs) from patients with gastric cancer, and CAFs with down-regulated miR-106b could significantly inhibit gastric cancer cell migration and invasion by targeting PTEN [31]. In this study, we revealed the up-regulation of miR-106b in the metastasis tissues from patients with EGC. *In vitro* results showed that the migration and invasion ability of gastric cancer cells were significantly increased following treatment with miR-106b mimics. On the other hand, compared with the control, miR-106b inhibitor treatment showed a high level of apoptosis proportion, as well as the expression of apoptotic proteins in gastric cancer cells. Furthermore, the expression levels of ALEX were promoted by the treatment of miR-106b inhibitor, and reduced by the treatment of miR-106b mimics. The luciferase reporter result demonstrated that ALEX1 was a direct target of miR-106b in gastric cancer cells. Overexpression of ALEX1 rescued the cell apoptosis and apoptotic proteins expression levels induced by miR-106b inhibitor, which was in accordance with the result of previous study [25]. Moreover, we noted that phosphorylation of JAK1 and phosphorylation of STAT3 were suppressed by miR-106b inhibitor treatment, which partially reversed following up-regulation of ALEX1. JAK1/STAT3 signaling pathway was reported to serve as a target for gastric cancer treatment [32]. A recent study showed that inhibiting STAT3 phosphorylation involved in the suppression of inflammation and proliferation in gastric cancer cells [33]. Interestingly, we found overexpression of JAK1 reduced apoptosis and apoptotic proteins expression levels in gastric cancer cells. Moreover, GLPG0643 treatment enhanced the inhibitory effect of miR-106b inhibitor on gastric cancer growth *in vivo*. The TUNEL positive cells were also significantly increased by the combined treatment of GLPG0643 and miR-106b inhibitor when compared with the control. In addition, the apoptotic protein expression levels of Bax, Bim were further strengthened by GLPG0643 combined with miR-106b inhibitor treatment. These results suggested that JAK1 might be a tentative target that enhanced the effect of miR-106b inhibitor on the suppression of gastric cancer growth.

Conclusion

Taken together, miR-106b was a potent factor of promoting metastasis of EGC. Moreover, miR-106b inhibitor exhibited anti-cancer characteristics both *in vitro* and *in vivo*. The identification of ALEX1 expression correlating to miR-106b highlighted the significance of ALEX1 in miR-106b inhibitor suppressed gastric cancer, suggesting ALEX1 was a direct target of miR-106b. In addition, down-regulation of miR-106b promoted apoptosis of gastric cancer cells through inhibiting JAK/STAT signaling pathway. These findings could be translational into clinical trials and would shed light on the potential and valid strategy in the treatment of EGC.

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Disclosure Statement

The authors declare that they have no competing interests.

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