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miR-135a promotes tumor invasion by targeting BACH1 and GATA3 in gastric cancer

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Abstract:	AIM The aim of this study was to investigate the effect of miR-135a/BACH1/GATA3 signaling on the proliferation, apoptosis, invasion, and migration of gastric cancer cells. METHODS Sixty specimens of cancer tissues and sixty specimens of corresponding adjacent tissues in gastric cancer patients were collected. qPCR and immunohistochemistry were used to detect the expression levels of miR-135a, BACH1 and GATA3 in cancer tissues and adjacent tissues. miR-135a inhibitor, miR-141 mimic, BACH1 inhibitor and GATA3 inhibitor carriers were established. Cell proliferation was detected by CCK8, the invasion ability of cells in vitro was evaluated by MTT, and the cell apoptosis of each group was detected by flow cytometry. RESULTS The results of RT–PCR showed that the expression levels of miR-135a in gastric cancer tissues were significantly higher than those in adjacent tissues, while the expression levels of BACH1 and GATA3 in gastric tissues were significantly lower than those in adjacent tissues ($P<0.05$). Interfering with miR-135a increased both BACH1 and GATA3 expression levels; overexpression of miR-135a significantly reduced BACH1 and GATA3 expression levels. Interference with BACH1 and GATA3 significantly enhanced the proliferative capacity of gastric cancer cells. Overexpression of miR-135a significantly increased apoptosis, whereas interference with BACH1 and GATA3 increased apoptosis. CONCLUSION miR-135a participates in the development of gastric cancer by regulating gastric cancer cell proliferation and apoptosis by targeting BACH1 and GATA3.

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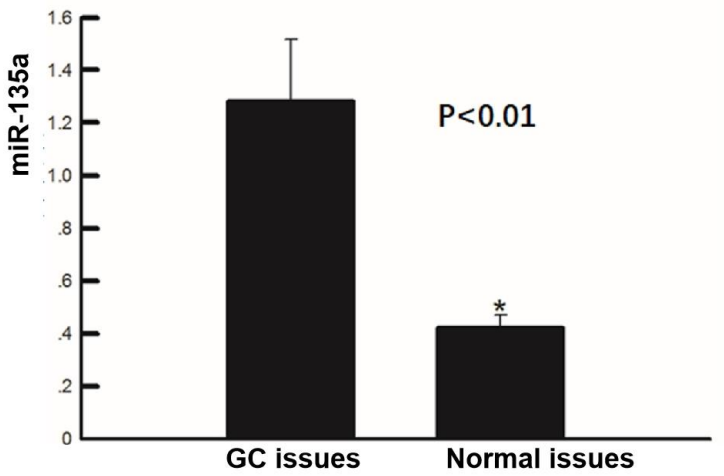
Authors’ contributions

Zhu YF were responsible for the overall study concept. Zhang YC, Jiang ZY and Tao GQ analyzed and interpreted the data, and Zhang Y was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

Fig. 1

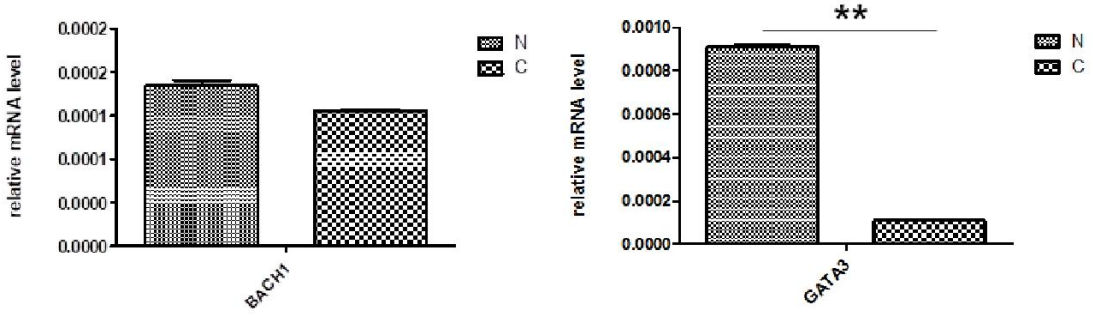
Expression of miR-135a in GC tissues



The miR-135a level was significantly different between the GC tissues and matched normal tissues (P<0.01).

Fig. 2

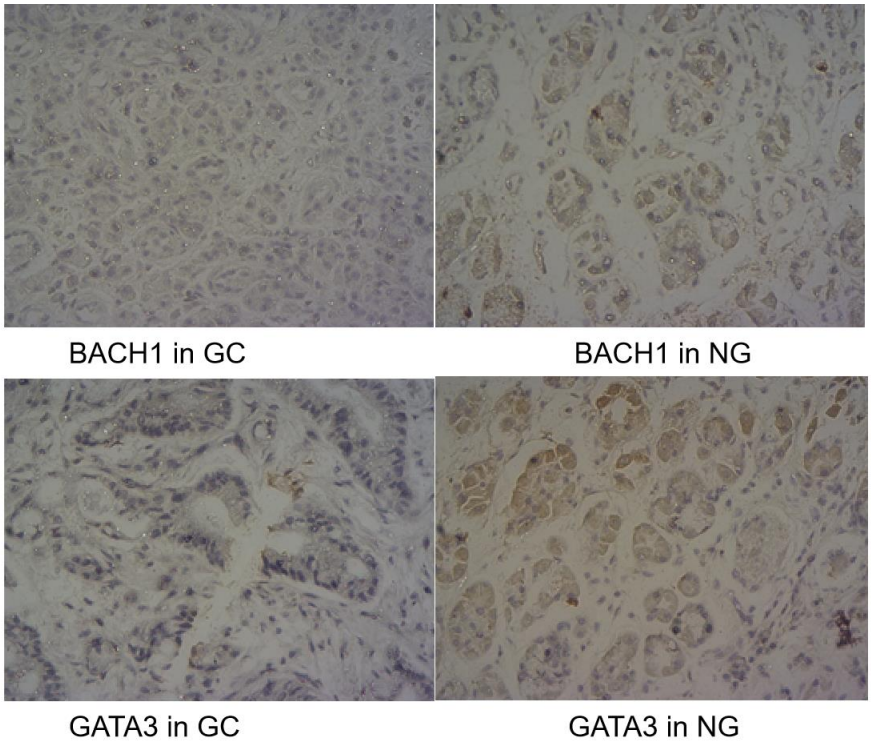
Expression of BACH1 and GATA3 in GC tissues



The expression of BACH1 was decreased in GC tissues (0.0001±0.00002 vs. 0.000923±0.00004, $P<0.05$). The expression of GATA3 was significantly decreased in GC tissues (0.00091±0.00002 vs. 0.00012±0.00004, $P<0.01$)

Fig 3

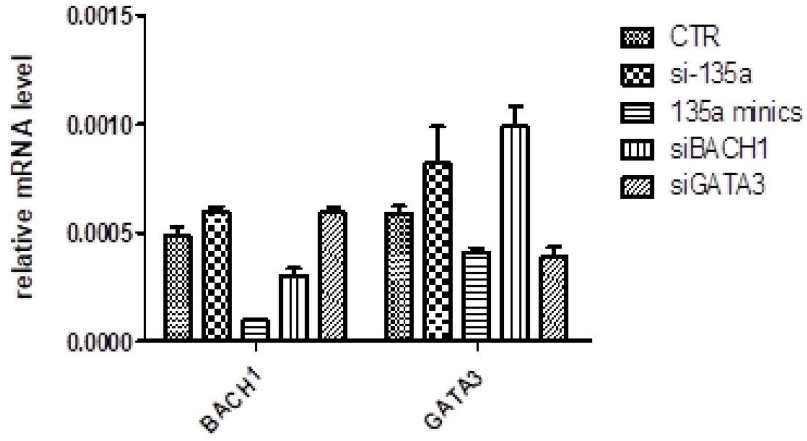
BACH1 and GATA3 expression evaluated by immunohistochemistry



The expression of BACH1 in GC tissues was much lower. The expression of GATA3 in GC tissues was much lower.

Fig 4

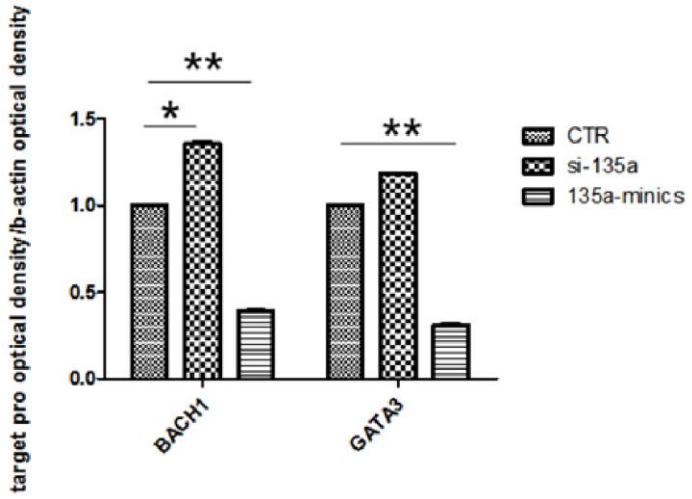
miR-135a, BACH1 and GATA3 expression evaluated by qPCR



Interfering with miR-135a increased BACH1 and GATA3 expression, whereas miR-135a overexpression decreased BACH1 and GATA3 expression; interfering with BACH1 decreased BACH1 gene expression, and GATA3 decreased BACH1 gene expression

Fig 5

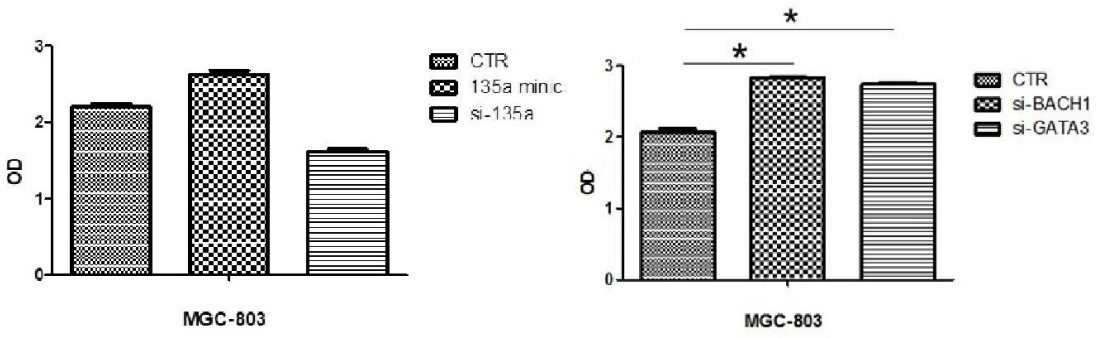
BACH1 and GATA3 expression evaluated by Western blot



Interfering with miR-135a increased both BACH1 and GATA3 expression, while BACH1 expression levels were significantly elevated. The expression levels of BACH1 and GATA3 were significantly reduced in the miR-135a mimic group (*P<0.05, **P<0.01)

Fig.6

Detection of Cell Proliferation by MTT



Overexpression of miR-135a enhanced the proliferative capacity of MGC-803 cells, which was not significantly different from the control cells.

Interfering with BACH1 significantly enhanced the proliferative capacity of MGC-803 cells, and the proliferative capacity of MGC-803 cells was significantly enhanced after interfering with GATA3 (*P<0.05)

Fig.7

Apoptotic rate (%) of each group

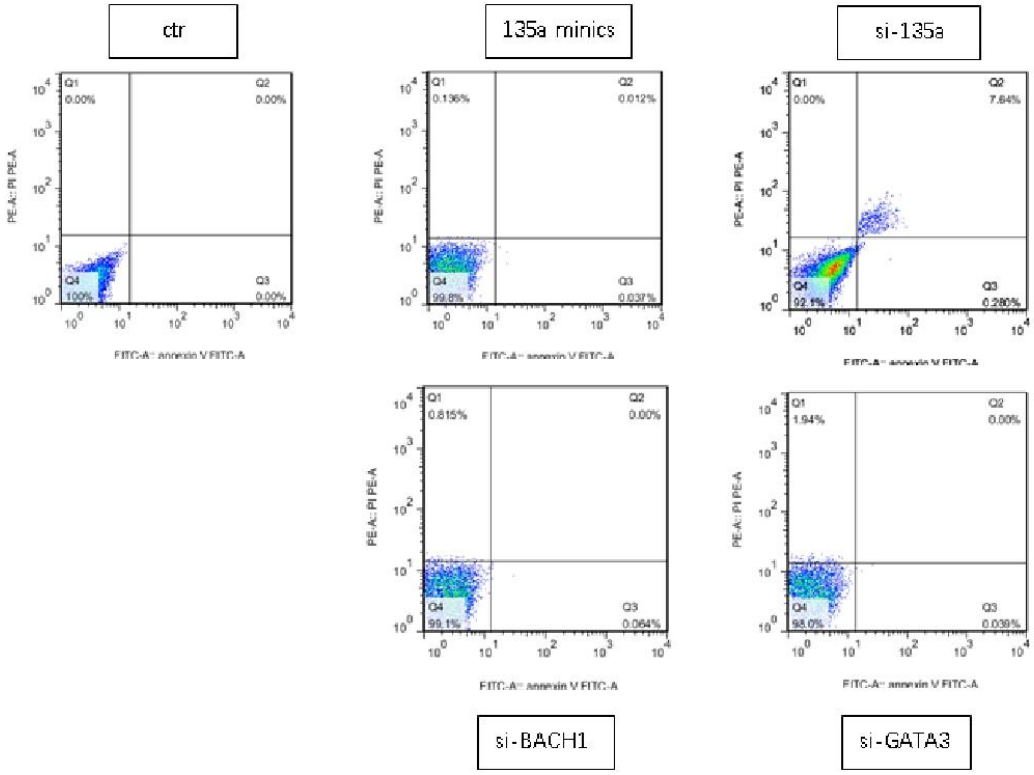
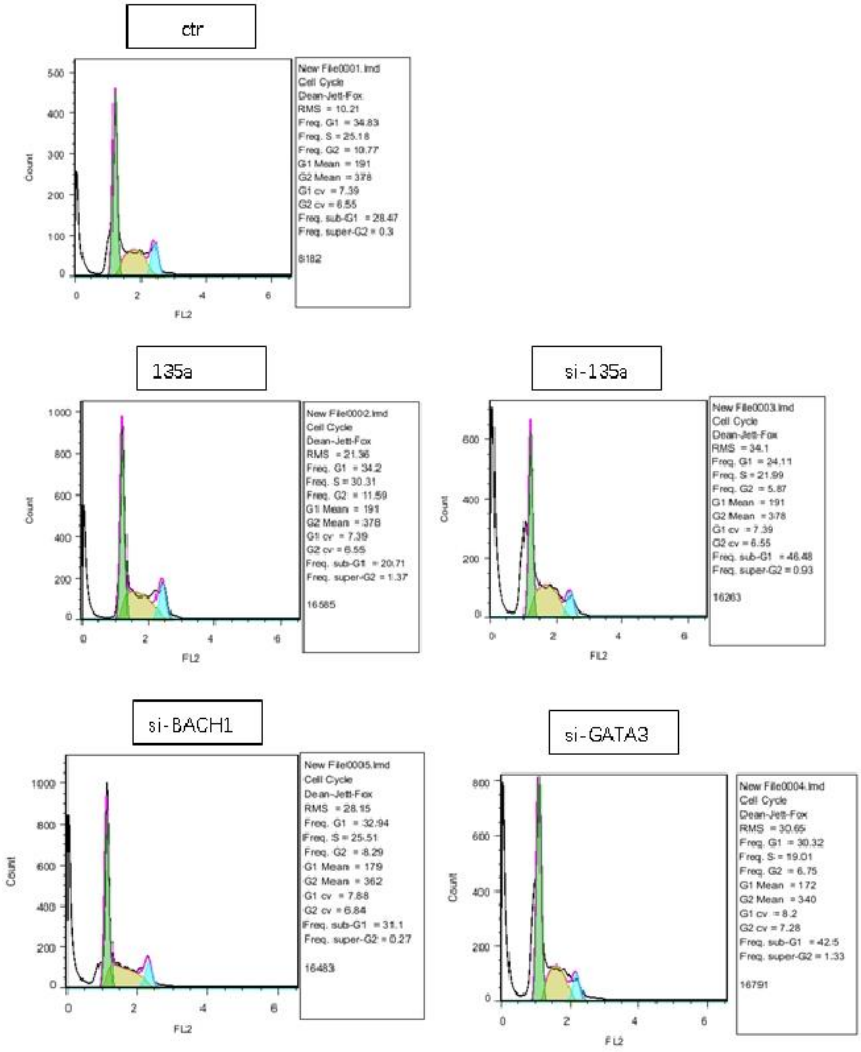


Fig.7 Apoptotic rate (%) of each group

The apoptosis rate of the miR-135a mimic group showed little change, while it significantly increased to 7.64% in the miR-135a inhibitor group. There was no significant difference in apoptosis rates between the BACH1 inhibitor group, GATA3 inhibitor group and control group

Fig.8

Comparison of cell cycle of MGC-803 cells in each group after transfection.



The cell cycle was slightly affected in the miR-135a mimic group, while apoptosis was increased after interference with miR-135a, BACH1, and GATA3

miR-135a promotes tumor invasion by targeting BACH1 and GATA3 in gastric cancer

Abstract

AIM

The aim of this study was to investigate the effect of miR-135a/BACH1/GATA3 signaling on the proliferation, apoptosis, invasion, and migration of gastric cancer cells.

METHODS

Sixty specimens of cancer tissues and sixty specimens of corresponding adjacent tissues in gastric cancer patients were collected. qPCR and immunohistochemistry were used to detect the expression levels of miR-135a, BACH1 and GATA3 in cancer tissues and adjacent tissues. miR-135a inhibitor, miR-141 mimic, BACH1 inhibitor and GATA3 inhibitor carriers were established. Cell proliferation was detected by CCK8, the invasion ability of cells in vitro was evaluated by MTT, and the cell apoptosis of each group was detected by flow cytometry.

RESULTS

The results of RT–PCR showed that the expression levels of miR-135a in gastric cancer tissues were significantly higher than those in adjacent tissues, while the expression levels of BACH1 and GATA3 in gastric tissues were significantly lower than those in adjacent tissues ($P<0.05$). Interfering with miR-135a increased both BACH1 and GATA3 expression levels; overexpression of miR-135a significantly reduced BACH1 and GATA3 expression levels. Interference with BACH1 and GATA3 significantly enhanced the proliferative capacity of gastric cancer cells. Overexpression of miR-135a significantly increased apoptosis, whereas interference with BACH1 and GATA3 increased apoptosis.

CONCLUSION

miR-135a participates in the development of gastric cancer by regulating gastric cancer cell proliferation and apoptosis by targeting BACH1 and GATA3.

Key words: MiR-135a, BACH1, GATA3, Gastric Cancer

INTRODUCTION

Gastric cancer (GC) has become one of the leading causes of death worldwide, but early diagnosis is difficult because of the lack of specific symptoms. The incidence of GC ranks fourth among malignant tumors, and the case fatality rate ranks third. However, it remains difficult to diagnose at an early stage, which mainly limits the availability of therapy. Most GC patients are diagnosed at an advanced stage with metastasis to lymph nodes and distant organs, which leads to extremely poor clinical outcomes.

MicroRNAs (miRNAs) are a class of endogenous noncoding single-stranded RNAs that can act as either oncogenes or tumor suppressors. MiR-135a is a member of the miR-135 family and is dysregulated in various cancers. In recent studies, miR-135a has been shown to play important but contradictory roles in cancer progression. For example, the expression of miR-135a increases in hepatocellular carcinoma, colorectal cancer and human bladder cancer, which is implicated in their development. In contrast, some studies show that miR-135a decreases and plays a suppressive role during the development of malignant glioma, such as epithelial ovarian cancer and renal cell carcinoma.[1-3] Therefore, miR-135a may be a promising molecule in cancer diagnosis and treatment.

As one of its potential target genes, BTB and CNC homology 1(BACH1) plays a vital role in the adjustment of oxidative stress and is ascribed as a repressor of its main target hemoxygenas-1 (HO-1). BACH1 promotes proliferation and differentiation in a variety of tissues and cell types. However, there are few reports on the relationship between miR-135a and BACH1.[4] GATA binding protein 3(GATA3), another target gene of miR-135a, is expressed in many epithelial and mesenchymal tumors. A small number of studies have suggested that GATA3 expression in GC is intrinsically related to the clinical stage and pathological grade of GC, but the mechanism of action has not been reported.[5] Based on the clinical samples of 60 gastric cancers, the effects of miR-135a/BACH1/GATA3 signaling on gastric cancer cell proliferation, apoptosis, invasion, and migration were examined by qPCR and immunohistochemistry. The findings of this study provide new insight into the

pathogenesis of GC, and miR-135a/BACH1/GATA3 signaling promises to be a potential tumor biomarker and therapeutic target for GC.

MATERIALS AND METHODS

1.1 Clinical tissue collection

Sixty surgically resected gastric cancer patients with tumor and corresponding normal tissue samples were identified from the Affiliated Wuxi People's Hospital of Nanjing Medical University between March 2019 and October 2019. All patients received radical gastrectomy without preoperative radiotherapy or chemotherapy. All fresh specimens were stored in liquid nitrogen immediately at −80 °C until use.

The study was approved by the Ethics Committee of the Affiliated Wuxi People's Hospital of Nanjing Medical University.

1.2 Real-time quantitative PCR (qPCR)

Total RNA was extracted from frozen tissues using TRIzol, and then cDNA of miRNA was synthesized with Reverse Transcriptase M-MLV (RNase H-) (TaKaRa, Tokyo, Japan) by using specific primers (Ribobio, Guangzhou, China) according to the manufacturer’s instructions. Gene cDNA was synthesized with M-MLV Reverse Transcriptase (Promega Corporation, Fitchburg, WI, USA) according to the manufacturer’s instructions. The reaction mixture used for qPCR was Toyobo PCR Master Mix (Toyobo, Osaka, Japan). The relative expression levels of each miRNA or gene were calculated and normalized using the $2^{-\Delta\Delta Ct}$ method relative to U6 or GAPDH.

1.3 Immunohistochemistry

Immunohistochemical study was performed using the EnVision method (Dako, Glostrup, Denmark) on 2-mm formalin-fixed, paraffin-embedded sections. The staining intensity was scored semiquantitatively as described by 2 independent observers without knowledge of the clinical status of the samples. All images were captured using a digital camera mounted on a light microscope (Axioscrop, Zeiss, Gottingen, Germany).

1.4 Cell culture and transfection

The human GC MGC-803 cell line was purchased from the Shanghai Institutes for Biological Sciences (Shanghai, People's Republic of China) and routinely cultured in Roswell Park Memorial Institute 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin. All cells were maintained in a humidified incubator containing 5% carbon dioxide at 37 °C.

miR-135a mimics, miR-135a inhibitor, siBACH1, siGATA3 and miRNA negative controls were purchased from GenePharma Company (Shanghai, China). Transfections were performed by using Lipofectamine 2000 reagent (Thermo Fisher Scientific) according to the manufacturer's instructions.

The cells were seeded into six-well plates, incubated for 24 hours, and then transfected with miR-135a mimics, inhibitors, siBACH1, and siGATA3 by using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's suggestions. At 48 hours posttransfection, cells were harvested and used in subsequent experiments.

Real-time quantitative PCR (qPCR)

Total RNA was extracted from GC cells. The exact procedure was the same as before.

Western blot analysis

Total protein was extracted from cells by using RIPA buffer (Beyotime) supplemented with phenylmethylsulfonyl fluoride (Beyotime). A BCA kit (Beyotime) was used to detect the concentration of protein in the supernatant. Protein expression was assessed by immunoblot analysis of 40 μg of cell lysate in the presence of antibodies against BACH1 and GATA3 (Cell Signaling Technology, Danvers, MA, USA). The relative protein abundance of total protein was determined by normalization to the endogenous control protein GAPDH.

Cell proliferation assay

Cell proliferation was monitored by the colorimetric water-soluble tetrazolium salt assay using a cell counting kit-8 (Dojindo, Kumamoto, Japan) following the manufacturer's instructions. Cells (2×103 cells per well) were seeded in 96-well plates, and cell proliferation was documented every 24 hours for a period of 4 days.

The number of viable cells was assessed by determining the absorbance at 450 nm using a monochromator microplate reader (Safire II, Tecan, Switzerland).

Cell apoptosis in each group detected by flow cytometry.

Cells were collected by centrifugation (2000 rpm for 5 min) and washed twice (2000 rpm for 5 min) with PBS to collect 1 to 5×10^5 cells. Then, 500 μL of PBS-suspended cells was added. Five microliters of Annexin V-FITC was added, and 5 μL of propidium iodide was added. The cells were placed at room temperature and protected from light for 5 to 15 min. Observation and detection were performed by flow cytometry within 1 hour.

Cells were washed twice with PBS at 1000 rpm for 5 min before removal of the fixative; 300 cells were filtered, centrifuged at 1000 rpm for 5 min, and the PBS was discarded. Cells were resuspended in 0.5 mL RNase A (10 mg dissolved in 10 ml PBS, filtered out). Cells were resuspended in 0.5 mL 0.1 mg/mL PI staining solution for 30 min at 4 °C before flow cytometry for 1 h, and cell cycle distribution was analyzed by flow cytometry.

1.5 Statistical analyses

Data were analyzed using SPSS 19.0 software (SPSS Inc., Chicago, Illinois). The comparison between the two groups was performed with the $\chi 2$ test; the measurement data are expressed as the mean ± SD, and the comparison between groups was performed by t test or F test; $P<0.05$ was considered to indicate a statistically significant difference.

RESULTS

Expression levels of miR-135a, BACH1 and GATA3 in GC tissues and adjacent tissues.

The miR-135a level was significantly different between the GC tissues and matched normal tissues ($P<0.01$). The expression of miRNA-135a was significantly increased in GC tissues (Fig. 1).

The expression of BACH1 was decreased in GC tissues (0.0001±0.00002 vs. 0.000923±0.00004, P<0.05) (Fig. 2).

Compared with the normal tissue group, a significant decrease in GATA3 in CC tissues was also observed (0.00091±0.00002 vs. 0.00012±0.00004, P<0.01) (Fig. 2).

BACH1 and GATA3 expression evaluated by immunohistochemistry

Compared with that in the normal group, the expression of BACH1 in GC tissues was much lower. In addition, a significant decrease in GATA3 in GC tissues was also observed (Fig. 3).

Relative expression levels of miR-135a, BACH1 and GATA3 in each group after transfection

Interfering with miR-135a increased BACH1 and GATA3 expression, whereas miR-135a overexpression decreased BACH1 and GATA3 expression; interfering with BACH1 decreased BACH1 gene expression, and GATA3 decreased BACH1 gene expression (Fig. 4).

Interfering with miR-135a increased both BACH1 and GATA3 expression, while BACH1 expression levels were significantly elevated. The expression levels of BACH1 and GATA3 were significantly reduced in the miR-135a mimic group (*P<0.05, **P<0.01) (Fig. 5).

Detection of cell proliferation by MTT

Overexpression of miR-135a enhanced the proliferative capacity of MGC-803 cells, but this finding was not significantly different from the control cells. Interference with miR-135a decreased the proliferative capacity of MGC-803 cells, but again, this finding was not significantly different from the control cells. Interfering with BACH1 significantly enhanced the proliferative capacity of MGC-803 cells, and the proliferative capacity of MGC-803 cells was significantly enhanced after interfering with GATA3 (*P<0.05) (Fig. 6).

Comparison of apoptosis ability of MGC-803 cells in each group after transfection

The apoptosis rate of the miR-135a mimic group showed little change, while it significantly increased to 7.64% in the miR-135a inhibitor group. There was no

significant difference in apoptosis rates between the BACH1 inhibitor group, GATA3 inhibitor group and control group (Fig. 7).

Comparison of the cell cycle of MGC-803 cells in each group after transfection.

The cell cycle was slightly affected in the miR-135a mimic group, while apoptosis was increased after interference with miR-135a, BACH1, and GATA3 (Fig. 8).

DISCUSSION

According to the World Health Organization's 2018 Global Cancer Statistics, the number of new cases of GC worldwide is 10.4 million annually, ranking fifth (5.7%) in terms of the incidence of malignancies and third (8.2%) in terms of deaths (780,000). China is a large country with a high incidence of GC, ranking second in terms of both incidence and mortality, with a high incidence of advanced carcinoma of more than 80% and a low 5-year survival rate (35.1%), which is lower than that of South Korea and Japan. Nevertheless, GC remains difficult to diagnose at an early stage, which mainly limits the availability of therapy. Most GC patients are diagnosed at an advanced stage with metastasis to lymph nodes and distant organs, which leads to extremely poor clinical outcomes and prognoses and a median overall survival of less than one year. With a 1% increase in the clinical early diagnosis rate, approximately 7.6 million deaths are averted worldwide, and approximately 160,000 newborns are born in China. Early diagnosis and early treatment are important for gastric cancer control. Therefore, understanding the genetic and epigenetic alterations underlying GC development and progression is still needed.

MicroRNAs (miRNAs) are small noncoding RNAs that affect posttranscriptional regulation by binding to the 3'-untranslated region of target messenger RNAs. As a member of the miR-135 family, miR-135a is a critical miRNA that can produce an identical and active sequence by being encoded by 2 genes localized on different chromosomes. Current reports have shown that the effects of miRNA-135a on cancer progression are contradictory.[1,2] Upregulation of miR-135a has been reported in colorectal cancer, breast cancer, ependymoma, melanoma, bladder cancer and hepatocellular carcinoma, indicating that miR-135a may serve as a tumor promoter in

these cancers.[6,7]

In contrast, significant downregulation of miR-135a has been found in prostate cancer, ovarian cancer, pancreatic cancer, renal cell carcinoma, gall bladder cancer and osteosarcoma, leading to its role as a tumor suppressor.[8,9] Even within the same cancer type, the expression of miR-135a in GC can differ dramatically.[10] Zhang et al. reported that miR-135a has an important role in the suppression of GC and presents a novel mechanism of miRNA-mediated KIFC1 expression in cancer cells.[11] Xie et al. indicated that miR-135a plays a suppressive role in GC cell migration by targeting TRAF5 and the downstream NF-κB pathway.[12] However, Yan et al. found that miR-135a was upregulated in GC, which promoted GC pathogenesis and appeared to suppress E2F1 expression and Sp1/DAPK2 pathway signaling.[13] In our study, the expression levels of miR-135a were significantly increased in GC tissues and MGC-803 cells, which was in accordance with Yan's study. Furthermore, interfering with miR-135a significantly increased the apoptosis of MGC-803 cells. These results supported the hypothesis that miRNA-135a was involved in GC progression and might function as an oncogenic factor. Compared with Zhang and Xie's research, the dramatic difference in sample volumes or patient states (with or without chemotherapy before surgery) might contribute to the discrepancy; hence, the underlying mechanisms remain to be further explored.

BACH1, a member of the basic leucine zipper transcription factor family, is a critical participant in oxidative stress. BACH1 is known to regulate multiple physiological processes, including heme homeostasis, the oxidative stress response, senescence, the cell cycle, and mitosis. BACH1 mainly contains two functional domains: the N-terminal BTB/POZ domain, which is mainly involved in the formation of protein dimers, and the C-terminal leucine zipper bZIP domain, which can form heterodimers with small Maf proteins in the nucleus, bind to target genes in the nucleus, and inhibit antioxidant response element (ARE)-mediated target gene expression.[14,15] Downregulation of BACH1 expression in endothelial cells promotes endothelial cell HO-1 mRNA and protein expression and increases VEGF transcriptional activity and mRNA and protein expression.[16,17] Studies have

demonstrated that inhibition of BACH1 expression increases the expression of HO-1 and VEGF in endothelial cells and promotes migration and lumen formation in human microvascular endothelial cells. BACH1 promotes the development of bone metastases in breast cancer by upregulating the expression of the key transfer genes CXCR4 and MMP1[18-20]. Although BACH1 is one of the miR-135a predicted target genes, there are few reports about the relationship between miRNA-135a and BACH1. This study shows that BACH1 expression was reduced in GC tissues. Interference with miR-135a expression significantly promoted BACH1 expression, while BACH1 expression was significantly reduced after overexpression of miR-135a. Interference with BACH1 significantly enhanced MGC-803 proliferation. Therefore, miR-135a may be involved in the development of GC through the negative regulation of BACH1.

GATA3, a zinc-finger transcription factor belonging to the GATA family, is expressed in many epithelial and mesenchymal tumors. GATA3 promotes proliferation and differentiation in a variety of tissues and cell types. The Zn-finger construct GATA3 transcription factor not only plays a role in the growth regulation and differentiation of multiple malignant tumor cells but also is closely related to malignant tumor grade and prognosis.[21-24] GATA3 expression has been linked to a favorable prognosis in breast cancers, neuroblastomas, endometrial carcinomas, colorectal cancer, urothelial carcinomas, soft tissue sarcomas and head and neck squamous cell carcinoma.[25-28] A small number of studies have suggested that GATA3 expression in GC is intrinsically related to the clinical stage and pathological grade of GC, but the mechanism of action has not been reported. Although GATA3 is one of the predicted targets for miR-135a, there have been few reports on miR-135a and GATA3 expression and their relationship in cancer. In the present study, GATA3 expression was significantly reduced in GC tissues, and in vitro experiments showed an inverse correlation between miR-135a and GATA3 expression, with disruption of miR-135a significantly promoting GATA3 expression, whereas overexpression of miR-135a significantly reduced GATA3 expression, and the proliferative capacity of MGC-803 cells was significantly enhanced after disruption of miR-135a. Thus,

miR-135a may be involved in the development of GC through negative regulation of GATA3.

Conclusion

In summary, miR-135a is highly expressed in GC tissues, while the expression levels of BACH1 and GATA3 are reduced in GC tissues. Overexpression of miR-135a and silencing of BACH1 and GATA3 increased the proliferation of GC MGC-803 cells. Silent expression of miR-135a promoted apoptosis of MGC-803 cells. It is concluded that miR-135a, BACH1 and GATA3 are involved in the biological process of GC and could be used as diagnostic markers and therapeutic targets for GC.

Abbreviations

GC: gastric cancer; BACH1: BTB and CNC homology 1; GATA3: GATA binding protein 3; qPCR: Real-time quantitative Polymerase Chain Reaction; LNM: lymph node metastasis; Nrf2: nuclear factor (erythroid-derived 2)-like 2.

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