Long non-coding RNA GATA6-AS inhibits gastric cancer cell proliferation by downregulating microRNA-25-3p

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Abstract. The abnormal growth of endothelial cells is involved in several types of diseases, including gastric cancer. The long non-coding RNA GATA6-AS is a key regulator of endothelial cell growth and may therefore also play an important role in gastric cancer. In the present study it was found that GATA6-AS was downregulated in tumor tissues compared with adjacent normal tissues. Moreover, plasma levels of GATA6-AS were linearly associated with GATA6-AS expression levels in tumor tissues and not in normal tissues. MicroRNA (miR)-25-3p was upregulated in tumor tissues compared with adjacent normal tissues and was inversely associated with GATA6-AS in tumor tissues only. The overexpression of miR-25-3p in gastric cancer cells resulted in no significant changes in the expression levels of GATA6-AS, whereas overexpression of GATA6-AS led to significantly downregulated miR-25-3p levels. Furthermore, overexpression of GATA6-AS inhibited cancer cell proliferation, with no effect on migration and invasion. The overexpression of miR-25-3p resulted in increased proliferation of cancer cells and attenuated the effects of GATA6-AS overexpression. Thus, it is postulated that GATA6-AS inhibits proliferation of gastric cancer cells by downregulating miR-25-3p.

Introduction

Although the incidence rate of gastric cancer ranks fourth among all malignancies worldwide in 2011, it is the second major cause of death among patients with cancer due to its aggressive nature (1). In 2011, gastric cancer caused ~700,000 deaths and affected >900,000 new cases worldwide (2). The development of gastric cancer at early stages lack classic symptoms, and the majority of patients are diagnosed at advanced stages (3). Patients with early stage gastric cancer benefit from surgical resections, however more than half of these patients develop recurrent cancer and <30% live longer than 5 years (4,5). Therefore, in-depth investigations on the molecular mechanism of gastric cancer are required to improve therapy and prognosis.

Long non-coding (lnc)RNAs (>200 nt) are RNA transcripts with limited protein-coding capacity (6). These RNAs were initially classified as non-functional background transcripts of the transcriptome however, an increasing number of studies have shown that they are associated with diverse cellular processes and the pathogenesis of human diseases (7,8). Differential expression of lncRNAs is frequently observed during the development of cancer (9), and their regulation may contribute to cancer therapy by affecting the expression of downstream oncogenes and/or tumor suppressors (10). Therefore, characterizing the function of lncRNAs in cancer is of utmost importance. The lncRNA GATA6-AS is a key regulator of endothelial cell growth, and abnormal growth of endothelial cells participates in several types of diseases, including gastric cancer (11). The present study therefore investigated the role of GATA6-AS in gastric cancer.

Materials and methods

Patients. Yue Bei People's Hospital admitted 109 patients with gastric cancer during the time period between January 2015 and March 2018. Of these patients, 60 (32 males and 28 females) with ages ranging from 41-68 years (mean ± standard deviation, 53.4±6.0 years) were recruited to the present study according to strict inclusion and exclusion criteria. Inclusion criteria: Patients i) who were diagnosed for the first time; ii) with no history of previous malignancies; and iii) who had received no therapy before admission. Exclusion criteria: Patients i) with other gastric diseases, such as gastric ulcer; ii) who had other malignancies; and iii) who had received treatment within 3 months before admission. All patients provided written informed consent prior to participation. The Ethics Committee of Yue Bei People's Hospital approved the present study.

Specimens and cell lines. Gastric cancer tissues and non-cancer tissues were collected from each patient through

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gastric biopsy. Blood (5 ml) was collected in an EDTA tube (cat. no. 367861; BD Biosciences) from each patient before any treatment. The blood was centrifuged at room temperature for 20 min at 1,400 x g to isolate the plasma. All tissues and plasma samples were stored in liquid nitrogen before use.

In order to perform *in vitro* experiments, gastric cancer cell lines SNU-1 and AGS were purchased from the American Type Culture Collection (ATCC). The cells were cultured in RPMI-1640 medium (ATCC) containing 10% fetal bovine serum (Sangon Biotech Co.) and penicillin-streptomycin (100 U/ml) at 37˚C with 5% CO_2.

Total RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). In order to detect the expression levels of GATA6-AS, TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract total RNA from tissue specimens (0.1 g), plasma (0.2 ml) and cultured cells ($1x10^5$ cells). cDNA was synthesized using the High Capacity cDNA Reverse Transcription kit (Bio-Rad Laboratories, Inc.). The qPCR reactions were prepared using Applied Biosystems Power SYBR™ Green PCR Master mix (GE Healthcare Life Sciences) and the endogenous control 18S ribosomal (r)RNA.

In order to detect the expression of miR-25-3p, total miRNA was extracted from the aforementioned tissues and cell lines using the mirVana miRNA Isolation kit (Thermo Fisher Scientific, Inc.). TaqMan MicroRNA Reverse Transcription kit (Thermo Fisher Scientific, Inc.) was used to perform reverse transcription, and qPCR reactions were prepared using Applied Biosystems TaqMan™ MicroRNA assay (Thermo Fisher Scientific, Inc.) with U6 as the endogenous control.

The thermocycling conditions were: 95˚C for 2 min, followed by 40 cycles of 95˚C for 20 sec and 56˚C for 40 sec. The primer sequences were the following: GATA6-AS forward, 5'-GGA CTCGGAATGCCTTGCTT-3'; GATA6-AS reverse, 5'-CCA CAACCCATTTCCGTCAG-3'; 18S rRNA forward, 5'-GCT TAATTTGACTCAACACGGGA-3'; 18S rRNA reverse, 5'-AGCTATCAATCTGTCAATCCTGTC-3'; and miR-25-3p forward, 5'-ATTGCACTTGTCTCGGTCTG-3'. The miR-25-3p reverse and U6 primers were included in the kit. Three biological replicates were used for each PCR reaction. All data normalizations were performed based on the $2^{-\Delta\Delta Cq}$ method (12).

Transient transfection. Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to achieve transient transfections, using $3x10^5$ cells in 2 ml cell culture medium in a 6-well plate. GATA6-AS (accession no. NR_102763.1)-expressing vectors were constructed using pcDNA3.1 vector (Sangon Biotech Co.). MISSION® microRNA mimic hsa-miR-25-3p (CAUUGCACUUGU CUCGGUCUGA) and miRNA negative control (NC; GGU UCGUACGUACACUGUUCA) were purchased from Sigma-Aldrich; Merck KGaA. For the transient transfection, 10 nM vectors and 40 nM miRNAs were used. Two control groups, including control (cells without transfection) and NC (cells transfected with miRNA NC or empty vectors) were included in this study. Cells were harvested 24 h after transfection for subsequent experiments.

In vitro cell proliferation assay. AGS and SNU-1 cells were harvested at 24 h after transfection to perform the Cell Counting Kit-8 (CCK-8) assay (Sigma-Aldrich; Merck KGaA). Single-cell suspensions were prepared and the cell concentration was adjusted to $4x10^4$ cells/ml. Each well of a 96-well plate was filled with 0.1 ml cell suspension and incubated at 37˚C in 5% CO_2. Cells were incubated with CCK-8 solution (10 μl) for 4 h prior to the measurement of optical density at 450 nm. Cells were measured every 24 h until 96 h.

Basic Local Alignment Search to predict the interaction between miR-25-3p and GATA6-AS. The potential interaction between miR-25-3p and GATA6-AS was predicted using the Basic Local Alignment Search (https://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastDocs&DOC_TYPE=Download) with miR-25-3p as query sequence and GATA6-AS as target sequence. All other parameters were default.

Statistical analysis. Each experiment included three biological replicates. Statistical analysis was performed using the GraphPad Prism 6 software (GraphPad Software, Inc.). Paired Student's t-test was used for comparisons between gastric cancer and non-cancer tissues. Comparisons among different cell treatment groups were performed by ANOVA (one-way) and Tukey's post hoc test. Linear regression was used for the regression analyses. $P<0.05$ was considered to indicate a statistically significant difference.

Results

GATA6-AS levels are downregulated in gastric cancer tissue and are linearly associated with plasma levels. RT-qPCR was performed to analyze the differential expression of GATA6-AS in cancer and non-cancer tissues of patients with gastric cancer. It was observed that the expression levels of GATA6-AS were significantly lower in gastric cancer tissues compared with non-cancer tissues ($P<0.05$; Fig. 1A). Plasma levels of GATA6-AS were also measured by RT-qPCR. Linear regression analysis was used to test the association between the expression levels of GATA6-AS in tissues and the plasma levels. It was observed that GATA6-AS plasma levels were linearly associated with the levels in tumor tissues ($P<0.0001$; Fig. 1B), and also in normal tissues ($P<0.0001$; Fig. 1C).

miR-25-3p is upregulated in gastric cancer tissues and inversely associated with GATA6-AS levels. RT-qPCR was performed to analyze the differential expression of miR-25-3p in cancer and non-cancer tissues of patients with gastric cancer. It was observed that the expression levels of miR-25-3p were significantly higher in gastric cancer tissues compared with non-cancer tissues ($P<0.05$; Fig. 2A). Linear regression analysis showed that miR-25-3p was inversely associated with GATA6-AS in gastric cancer tissues only ($P<0.0001$; Fig. 2B); no association was observed with the levels in the adjacent non-cancer tissues ($P>0.05$; Fig. 2C).

Overexpression of GATA6-AS in gastric cancer cells leads to downregulation of miR-25-3p. Gastric cancer SNU-1 and AGS cells were transfected with GATA6-AS-expressing vector and miR-25-3p mimic to further investigate the association between GATA6-AS and miR-25-3p. Compared with the two

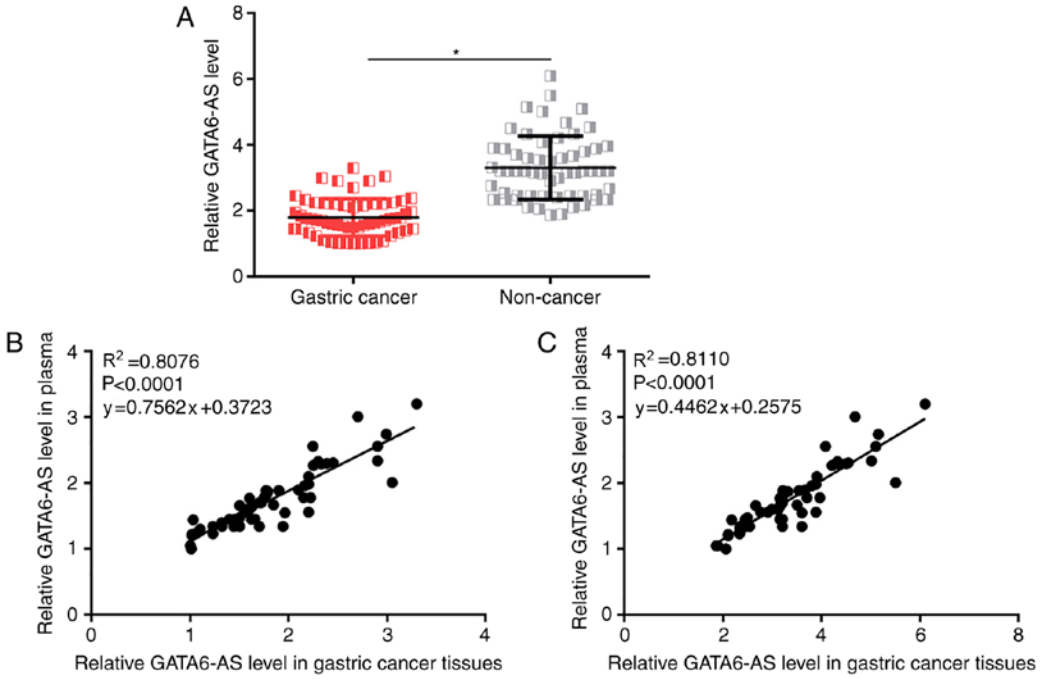

Figure 1. GATA6-AS is downregulated in gastric cancer tissue and is associated with the plasma levels. (A) Reverse transcription-quantitative PCR results show downregulation of GATA6-AS in gastric cancer tissues compared with adjacent non-cancer tissues. Linear regression analysis demonstrates that GATA6-AS plasma levels are linearly associated with the levels in (B) tumor and (C) normal tissues. *P<0.05.

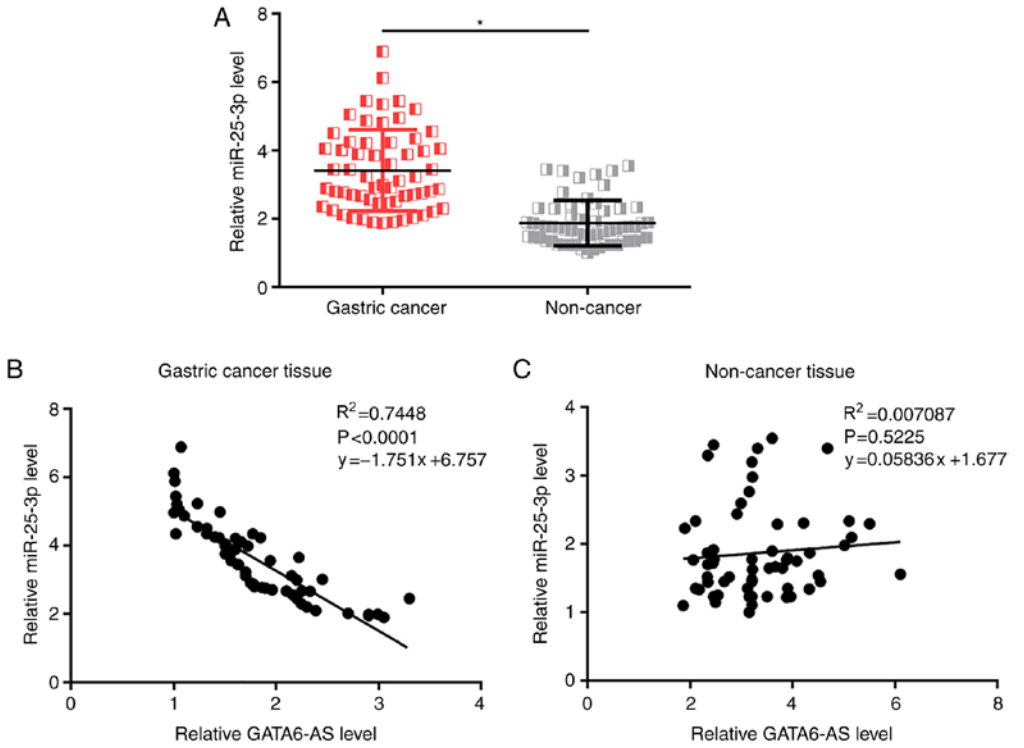

Figure 2. miR-25-3p is upregulated in gastric cancer tissues and is inversely associated with GATA6-AS levels. (A) Reverse transcription-quantitative PCR results show significant downregulation of miR-25-3p in gastric cancer tissues compared with non-cancer tissues. Linear regression analysis of miR-25-3p vs. GATA6-AS levels demonstrates (B) an inverse linear association in gastric cancer tissues, and (C) no association in adjacent non-cancer tissues. *P<0.05. miR, microRNA.

control groups (NC and control), GATA6-AS and miR-25-3p were overexpressed in both cell lines 24 h after transfection (all P<0.05; Fig. 3A). In addition, overexpression of miR-25-3p in gastric cancer cells resulted in no significant changes in the expression levels of GATA6-AS (Fig. 3B), whereas overexpression of GATA6-AS led to significantly downregulated miR-25-3p expression (both P<0.05; Fig. 3C).

Overexpression of GATA6-AS inhibits proliferation of gastric cancer cells via miR-25-3p. GATA6-AS overexpression led to

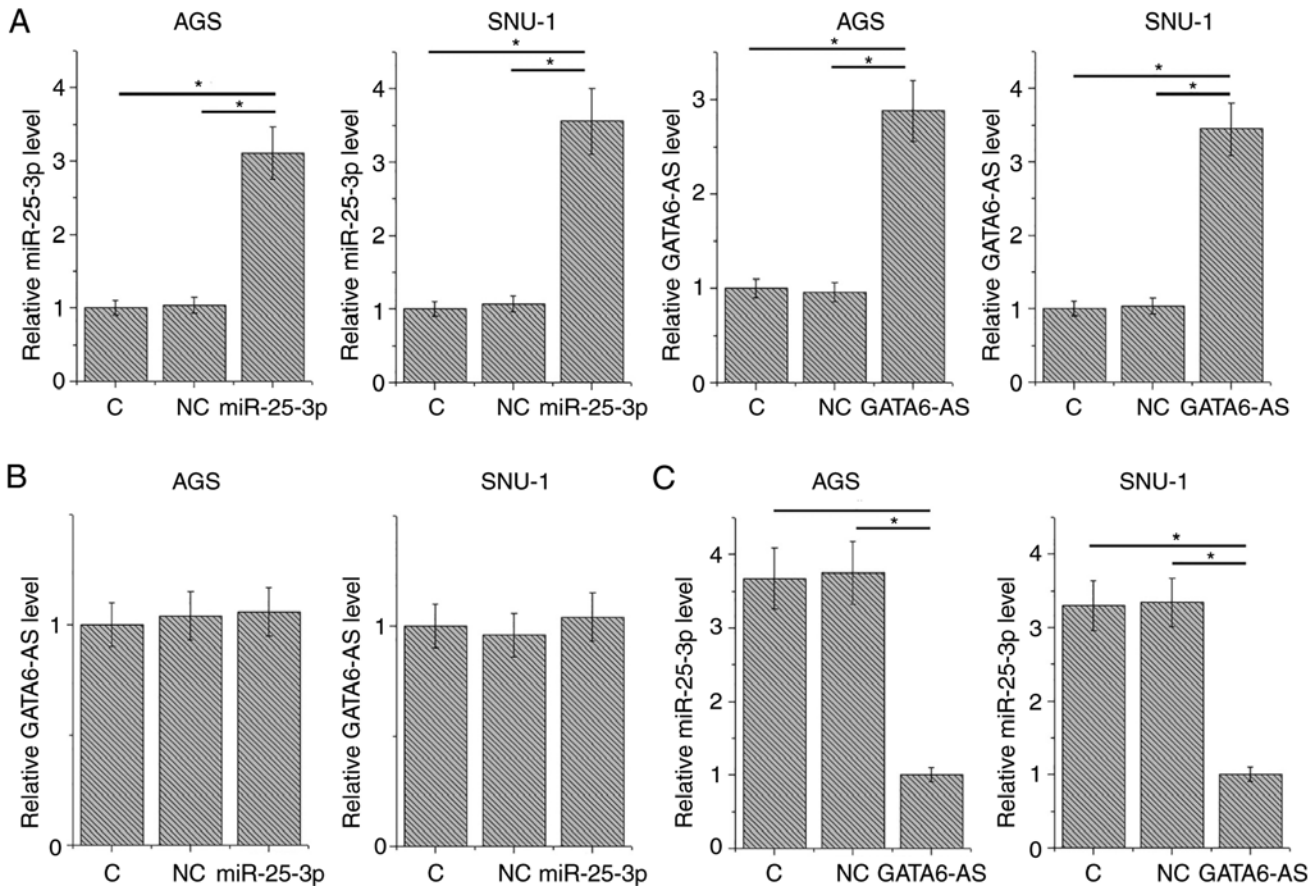

Figure 3. GATA6-AS overexpression causes downregulation of miR-25-3p in gastric cancer cells. (A) Compared with NC and C groups, GATA6-AS and miR-25-3p are overexpressed in gastric cancer AGS and SNU-1 cells 24 h after transfection with an overexpression plasmid and a miR mimic, respectively. (B) miR-25-3p overexpression in gastric cancer cells results in no significant changes in the expression levels of GATA6-AS. (C) GATA6-AS overexpression leads to significantly downregulated miR-25-3p expression. *P<0.05. miR, microRNA; C, control; NC, negative control.

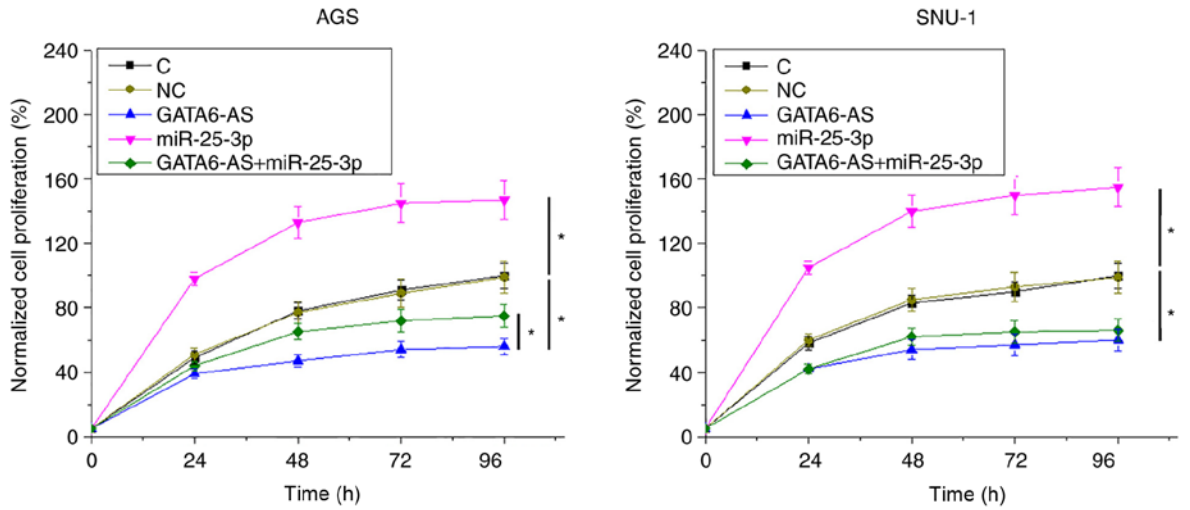

Figure 4. GATA6-AS overexpression causes inhibition of proliferation via miR-25-3p regulation GATA6-AS overexpression leads to inhibited proliferation of gastric cancer SNU-1 and AGS cells. In addition, miR-25-3p overexpression results in increased proliferation of the cancer cells and attenuates the effects of GATA6-AS overexpression. *P<0.05. miR, microRNA; C, control; NC, negative control, negative control of empty vector transfection only shown here because the two NCs were almost perfectly overlapped.

the inhibition of proliferation in both SNU-1 and AGS cells (both P<0.05). In addition, miR-25-3p overexpression resulted in increased proliferation of cancer cells and attenuated the effects of GATA6-AS overexpression (all P<0.05) (Fig. 4).

Discussion

GATA6-AS is a key regulator of endothelial cell growth and the abnormal growth of endothelial cells has been associated with

the pathogenesis of several types of diseases, including gastric cancer (11). The present study investigated the involvement of GATA6-AS in patients with gastric cancer and revealed that the overexpression of GATA6-AS inhibited proliferation of gastric cancer cells, by downregulating miR-25-3p, which is known to promote gastric cancer (13).

miR-25-3p is an oncogenic miRNA with functionalities characterized in several types of cancer (14,15). miR-25-3p regulates cancer cell behaviors, such as apoptosis and proliferation, through different tumor suppression or oncogenic pathways, such as via TNF-related apoptosis inducing ligand death receptor-4 (14) and large tumor suppressor homolog 2 (15). During the development of gastric cancer, miR-25-3p is reported to be overexpressed in tumor tissues and associated with cancer cell proliferation, migration and invasion (16). Consistent with previous studies, miR-25-3p was overexpressed in gastric cancer tissues and its overexpression led to the inhibition of proliferation in gastric cancer cells, further confirming the oncogenic role of miR-25-3p in gastric cancer.

The present study demonstrated that GATA6-AS was downregulated in gastric cancer tissues, indicating its involvement in this disease. Spatial and temporal expression analyses revealed that lncRNAs are expressed in certain types of cells or at certain developmental stages of disease progression to regulate gene expression (17,18). The existence of circulating lncRNAs in the blood has also been reported, indicating their potential roles as systemic regulators of gene expression (19). The present study detected the expression of GATA6-AS in patients with gastric cancer and found that the plasma levels were linearly associated with the expression levels in tumor and normal tissues. Therefore, GATA6-AS may systemically regulate downstream gene expression.

The present study also demonstrated that GATA6-AS downregulated miR-25-3p to inhibit the proliferation of gastric cancer cells. To investigate this further, the Basic Local Alignment Search tool was used, with miR-25-3p as the query sequence and GATA6-AS as the target. However, no promising target site of miR-25-3p was found on GATA6-AS, suggesting the presence of an indirect interaction. Thus, further studies are required to elucidate the interaction between GATA6-AS and miR-25-3p.

In conclusion, GATA6-AS is downregulated in gastric cancer, and overexpression of GATA6-AS may downregulate miR-25-3p to inhibit gastric cancer cell proliferation.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

DL designed the experiments. TW, JL, TZ, XZ, DZ and ZH conducted the experiments. DL and TW analyzed the data. DL wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All patients provided written informed consent prior to participation. The protocol of the present study was approved by the Ethics Committee of Yue Bei People's Hospital, Shaoguan, China.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have competing interests.

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