

The expression and function of miR-376a-3p/DLX axis in gastric cancer cells

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Abstract: Gastric cancer (GC) was referred to a malignant tumor of the digestive tract originating from the epithelium of gastric mucosa. Transcription factor DLX5 was verified as an oncogene in various types of tumors, while miR-376a-3p was speculated as a tumor suppressor. Based on the bioinformatics database, we hypothesized that miR-376a participated in the regulation of GC development by targeting DLX5. Compared with adjacent tissue, a significant increase of DLX5 expression was determined in GC tissues, but the expression level is significantly reduced in miR-376a. Similar expression signature of DLX5 and miR-376a was also determined between 4 GC cells (HGC, SGC, MGC, and AGS cell lines) and GES cell line. The level of DLX5 was notably reduced in HGC and MGC cell lines after miR-376a-3p overexpression, and increased after miR-376a-3p inhibition. Then, the inhibition role of miR-376a-3p on DLX5 was further proved by dual-luciferase reporter assay. Gain-of-function experiments showed that upregulation of miR-376a-3p in GC cells could inhibit the ability of epithelial-mesenchymal transition, proliferation, and invasion, and enhance the GC cell apoptosis level. However, these roles of miR-376a-3p could be abolished by DLX5 overexpression. This study confirmed that reduction of miR-376a-3p expression level in GC cells would lead to the increase in cell growth and invasion, indicating that upregulation of miR-376a-3p might have a potential therapeutic role on GC.

Introduction

Gastric cancer (GC) defines as a malignant tumor of the digestive tract originating from the epithelium of gastric mucosa, and is characterized by high incidence, insidious onset, and prone to metastasis (Seeneevassen *et al.*, 2021). Although the conversion surgery following chemotherapy has greatly improved the prognosis and survival, the recurrence and mortality of patients with advanced GC are still high (Biagioni *et al.*, 2019; Necula *et al.*, 2019). The progression of GC is caused by a series of cell behaviors involving proliferation, migration, and epithelial-mesenchymal transition of GC cells, which is affected by genetic and environmental factors, and multiple signal pathways (Ashrafizadeh *et al.*, 2020). The mechanism exploration of GC development could provide new therapeutic strategies for GC.

Distal-less homeobox 5 (DLX5) is a member of homeobox transcription factor gene family related to distal-less gene of *Drosophila* (Tan and Testa, 2021). In humans, DLX5 is convergently transcribed and share regulatory elements and

expression patterns with other DLX members (Depew *et al.*, 2002; Proudfoot *et al.*, 2016). It is reported that the proliferation of tumor cells was promoted by DLX5 in lymphomas and lung cancers through transcriptionally regulating MYC (Xu and Testa, 2009). A recent study revealed that DLX5 is essential for the development of squamous cell carcinomas by regulate thousands of cis-regulatory elements, and silencing of DLX5 could potently inhibit viability of squamous cell carcinomas (Huang *et al.*, 2021). However, it is still ill-defined how DLX5 regulates the pathogenesis of GC.

Increasing evidence has proved that microRNA (miRNA) is involved in multiple cellular processes by inhibiting the expression of target genes. Disordered expression of miRNA is also closely related to the progression of various diseases. miR-376a-3p, a member of miR-376 cluster, was identified as a potential predictor of pathological response of esophageal squamous cell carcinomas to neoadjuvant chemoradiotherapy (Wen *et al.*, 2016). A recent report manifested that restoration of miR-376a-3p expression could counteract the phenotype of bone-derived stromal cell giant cell tumor (Fellenberg *et al.*, 2016), indicating miR-376a-3p might be a tumor suppressor miRNA. It has been identified that overexpression of miR-376a-3p could alleviate the development of glioma

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(Chen *et al.*, 2020b). However, the role of miR-376a-3p in biological behavior of GC cells has not yet been discovered.

In this article, we discussed the expression profile of DLX5 and miR-376a-3p in GC cells and tissues, the mechanism by which miR-376a-3p regulated the expression of DLX5 in GC, and the inhibitory effect of miR-376a-3p on GC cell proliferation and invasion. We believed that our results would provide valuable therapeutic targets for GC treatment.

Materials and Methods

Clinical samples

GC tissue samples and adjacent normal tissue samples were gathered from GC patients that had undergone surgery at Department of General Surgery, the 6th Medical Center of PLA General Hospital from January 2020 to March 2021, and were cryopreserved in liquid nitrogen until further experiments. The study was approved by the Medical Ethics Committee in PLA General Hospital, and performed in accordance to the principles of the Declaration of Helsinki. Written informed consent was obtained from all subjects prior to enrollment.

Cell culture and treatment

HGC, SGC, MGC, and AGS cell lines and gastric mucosal cells (GES) were got from National Collection of Authenticated Cell Culture (Shanghai, China). After resuscitation, cells were passaged and grown in M199 medium containing 10% FBS (Gibco, Grand Island, USA), 100 units/mL penicillin antibiotic, and 100 µg/mL streptomycin antibiotic (Thermo Fisher Scientific, Waltham, USA).

Cells transfection

miR-376a-3p mimic, miR-376a-3p inhibitor control were ordered from GENEWIZ (Jiangsu, China). Co-transfection of indicated constructs into cells by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Cells were collected and detected after 48 h transfection.

RNA and protein isolation

Total RNA was extracted from tissues or cells with RNAiso Plus reagent (Takara, Dalian, China). The extracted RNA samples were examined on a Nanodrop spectrophotometer (Thermo Fisher Scientific) for quality and quantity evaluation. The protein samples were separated from cells or tissues by using RIPA buffer plus PMSF (Beyotime, Shanghai, China). The concentration of protein samples was determined by bicinchoninic acid assay.

Dual-luciferase reporter assay

The wild-type or mutant 3' UTR of DLX5 was synthesized and subcloned into luciferase reporter plasmid (WT-DLX5 or MUT-DLX5). The WT-DLX5 (or MUT-DLX5) plasmid, renilla plasmid, and miR-376a-3p mimic (or negative control) were co-transfected into HEK293T cells by using Lipofectamine 3000 (Thermo Fisher Scientific). Cells were harvested at 48 h after transfection. Luciferase activities were detected with dual luciferase reporter assay kit (Beyotime, Shanghai, China). The experiment was repeated for 3 times.

Quantitative real-time PCR (qRT-PCR)

Equal amounts of RNA (200 ng) were used as template to synthesize cDNA with PrimeScript RT reagent Kit (Takara). qRT-PCR assay was carried out in a Real Time System III (Takara) using SYBR Green. ACTB was set as reference gene for mRNA detection, while U6 was set for miRNA detection. The data were evaluated based on $2^{-\Delta\Delta C_t}$ method.

Western blot assay

Equal amounts (30 µg) of protein samples were electrophoresed by SDS-Polyacrylamide gel electrophoresis (10%). After transferring, the PVDF membrane was blocked by BSA solution (1%) at room temperature for 2 h. Then, the diluted primary antibodies (1:1000 dilution for anti-DLX5, Abcam, Cambridge, UK; 1:2000 dilution for anti-ACTB, Proteintech, Wuhan, China) were added, respectively, and incubated with the membrane at 4°C overnight. After washing by TBS-T buffer for 5 times, the corresponding secondary antibody was added for 1-h incubation at room temperature. The immune band was developed with ECL chemiluminescence substrate (Boster, Wuhan, China) on ChemiDoc MP system (Bio-Rad, Hercules, USA). The reference gene ACTB was set as loading control.

Cell Counting Kit-8 (CCK-8) assay

CCK-8 assay was used to detected GC cell viability. Briefly, the indicated number of GC cells in each well were reseeded in 96-well plates and maintained in complete medium overnight. At the following day, cells were transfected with indicated plasmids. 10 µL of CCK-8 solution (Beyotime, Shanghai, China) was mixed with cells in each well under 37°C incubator with 5% CO₂ for 2 h. Microplate spectrophotometer (Biotec, Basel, Switzerland) was subsequently applied to test the absorbance of 450 nm per well. The derived data represented cell viability.

Trans-well invasion assay

Invasion assay was performed with invasion chambers (Millipore, Eschborn, Germany) plus Matrigel (BD Biosciences, San Jose, USA). Briefly, the chamber inserts were coated by Matrigel (20 mg/mL), and dried overnight. The upper chambers placed serum-free M199 medium containing GC cells (5×10^3), while the lower compartments placed M199 medium plus 20% FBS. Crystal violet (0.1%) was used to stain the migrated GC cells after 48 h. Counting of the invaded cells were conducted under 5 random fields by ImageJ software.

Flow cytometry assay for apoptosis

Flow cytometry assay was used to examine the apoptosis of GC cells with an Annexin V-FITC kit (Beyotime). Annexin V-FITC and propidium iodide-stained GC cells were measured within 30 min by FACS Canto II flow cytometer (BD Biosciences).

Statistical analysis

Difference analysis of statistical data was made by SPSS version 22.0. The difference was analyzed by independent sample *t*-test for 2 groups, and analyzed by one-way ANOVA for multiple groups. A statistically *P*-value of less than 0.05 was indicated to be significant.

Results

Negative expression correlation of miR-376a-3p and DLX5 in GC

To investigate the correlation between miR-376a-3p expression and DLX5 level in GC, the relative expression levels of miR-376a-3p and DLX5 was determined in multiple GC cell lines. Analysis of qRT-PCR assay (Fig. 1A) confirmed that the mRNA level of DLX5 in GC cell lines (HGC, SGC, MGC, and AGS) was obviously higher than that in gastric mucosal cells (GES). The elevated protein expression of DLX5 in the 4 GC cell lines (Fig. 1B) was then approved in western blot detection. In contrast, the mRNA level of miR-376a-3p in the 4 GC groups (HGC, SGC, MGC, and AGS) was obviously lower than that in the GES group (Fig. 1C). The negative expression correlation between miR-376a-3p and DLX5 was more obvious in HGC and MGC cell lines. Then, the levels of miR-376a-3p and DLX5 were determined in tissues from normal and GC groups (n = 20). Compared with the normal group (Fig. 1F), the expression levels of DLX5 mRNA and protein were notably increased in tumor group (Figs. 1D and 1E), accompanied by the decreased expression level of miR-376a-3p in tumor group.

The inhibitory effect of miR-376a-3p in DLX5 expression

To clarify whether DLX5 was a direct target of miR-376a-3p in GC, the gain-of-function experiment was firstly performed in HGC and MGC cell lines. Analysis of qRT-PCR assay confirmed the elevated expression level of miR-376a-3p in HGC and MGC cell lines after transfection of miR-376a-3p mimic (Fig. 2A). Analysis of western blot assay demonstrated that the expression level of DLX5 protein was significantly decreased in HGC and MGC cell lines overexpressing miR-376a-3p (Fig. 2B). Then, loss-of-function experiment confirmed that the protein expression of DLX5 were significantly increased in HGC and MGC cell lines with miR-376a-3p inhibition (Figs. 2C and 2D).

Subsequently, one miR-376a-3p binding site with high affinity was identified in the 3' untranslated region (3'UTR) of DLX5 mRNA through bioinformatics algorithm analysis. Results of dual-luciferase reporter assay (Fig. 2E) confirmed that the was remarkably reduced after transfection of miR-376a-3p mimic in wild-type DLX5 (WT-DLX5) group. However, the luciferase activity did not notably change in the mutant (MUT-DLX5) groups with or without miR-376a-3p overexpression.

The effect of miR-376a-3p/DLX5 in EMT and proliferation of GC cells

Epithelial to mesenchymal transition (EMT) is well-known as an important process during the development of various tumors. Result of western blot assay (Fig. 3A) showed that the expression of mesenchymal markers (claudin-1, vimentin, and N-cadherin) was down-regulated in HGC and MGC cell lines transfected with miR-376a-3p mimic, while the level of epithelial marker (E-cadherin) was up-regulated. However, overexpression of DLX5 could abolish the inhibitory role of miR-376a-3p in GC cell EMT, demonstrated by the decreased expression of mesenchymal markers and the increased expression of E-cadherin. Then, CCK-8 assay was used to confirm the effect of miR-376a-3p/DLX5 in GC cell growth. Both in HGC and MGC cell lines, the growth vitality of GC cells was significantly reduced in miR-376a-3p overexpression group, but was obviously alleviated in miR-376a-3p/DLX5 co-overexpression group (Figs. 3B and 3C).

The inhibitory effect of miR-376a-3p/DLX5 in GC cell invasion

Trans-well invasion assay was implemented to examine the roles of miR-376a-3p/DLX5 in GC cell invasion. Compared with control group in HGC cell line, the number of transmembrane cells was significantly reduced in miR-376a-3p overexpression group. The anti-invasive effect of miR-376a-3p in GC cells could be partially offset by overexpression of DLX5. The number of transmembrane cells in miR-376a-3p/DLX5

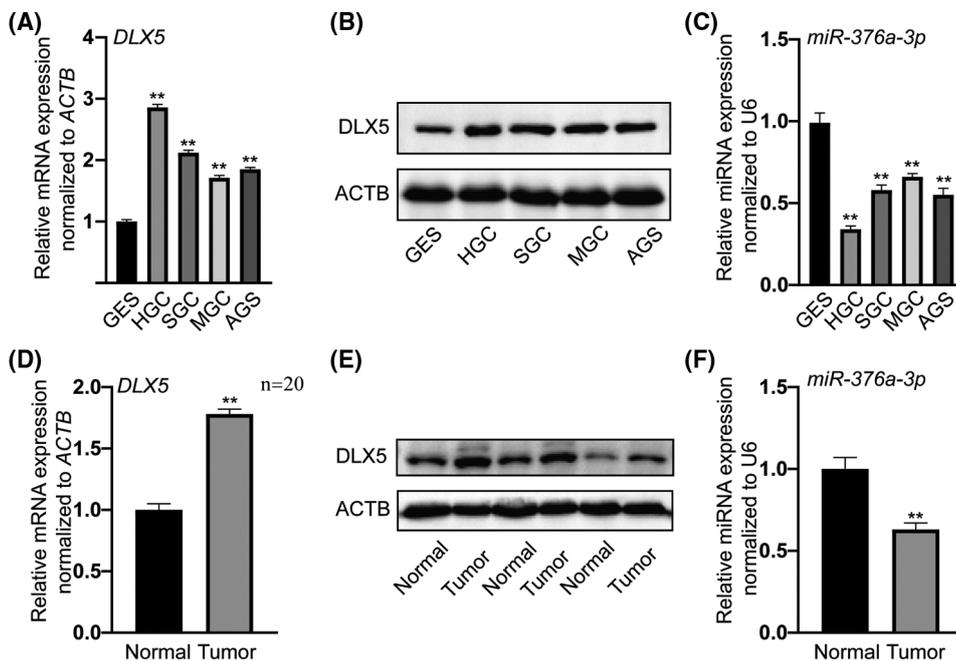


FIGURE 1. Negative expression correlation of miR-376a-3p and DLX5 in GC, all the cells were seeded in a 12-well plate and harvested after 2-day cultivation. A, qRT-PCR analysis of DLX5 between 4 GC cell lines (HGC, SGC, MGC, and AGS) and GES cell line. $**P < 0.01$. B, Western blot results of DLX5 protein between GC cell lines and GES cell line. ACTB was the loading control. C, qRT-PCR detection of miR-376a-3p between GC and GES cell lines. $**P < 0.01$. D, qRT-PCR analysis of DLX5 in tissues from normal and tumor groups. $**P < 0.01$. E, Western blot results of DLX5 protein in tissues from normal and tumor groups. ACTB was the loading control. F, qRT-PCR analysis of miR-376a-3p in tissues from normal and tumor groups. $**P < 0.01$.

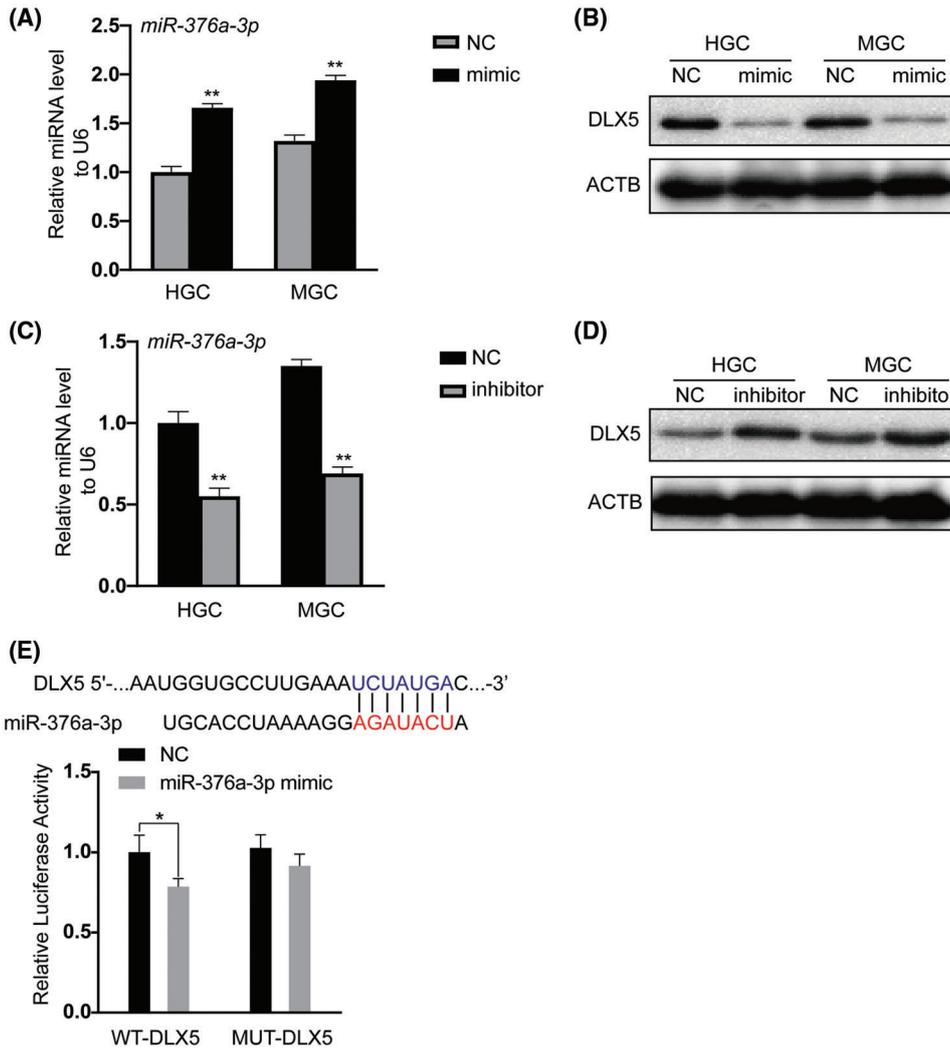


FIGURE 2. The inhibitory role of *miR-376a-3p* in DLX5 expression. A, qRT-PCR analysis of *miR-376a-3p* in HGC and MGC cell lines after mimic transfection. ***P* < 0.01. B, Analysis of DLX5 protein expression in HGC and MGC cell lines after transfection of mimic through western blot detection. ACTB was the loading control. C, qRT-PCR results of *miR-376a-3p* in HGC and MGC cell lines after transfection of inhibitor. ***P* < 0.01. D, Expression of DLX5 protein in HGC and MGC cell lines after transfection of inhibitor. ACTB was the loading control. E, Dual luciferase verification of the direct action between *miR-376a-3p* and DLX5. **P* < 0.05.

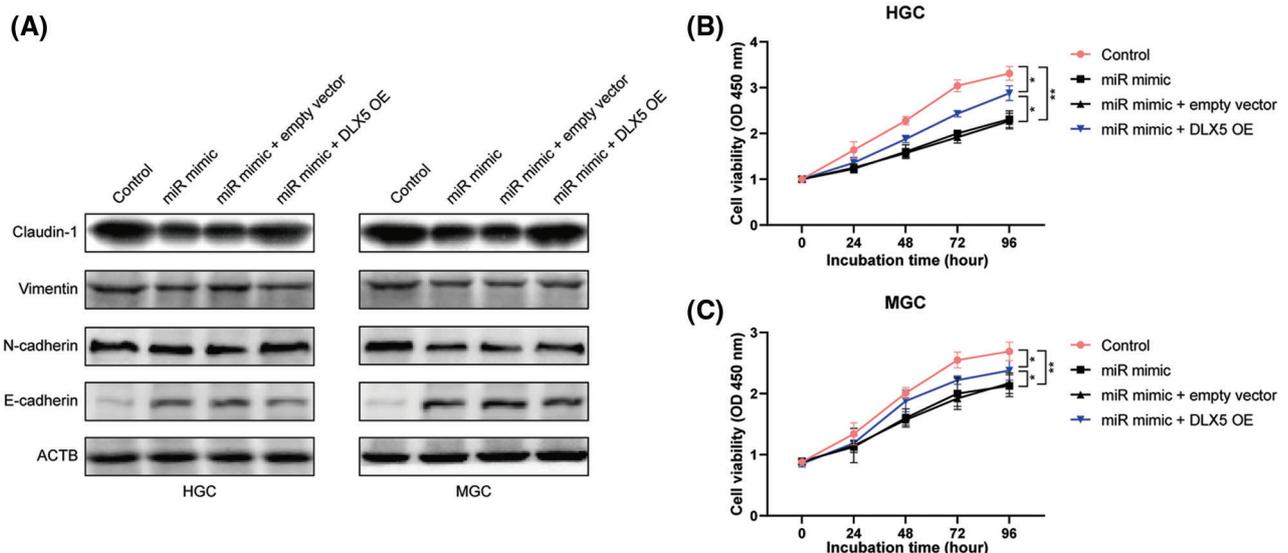


FIGURE 3. The effect of *miR-376a-3p*/DLX5 in EMT and proliferation of GC cells. A, Western blot results of EMT markers in HGC and MGC cell lines after overexpression of *miR-376a-3p* and/or DLX5. B–C, CCK-8 results of HGC and MGC cell lines after overexpression of *miR-376a-3p* and/or DLX5.

co-overexpression group was significantly more than that in *miR-376a-3p* overexpression group (Figs. 4A and 4B). Although the invasion ability of MGC cell line was weaker

than that of HGC cell line, the roles of *miR-376a-3p*/DLX5 in invasion of GC cells could be also tested and verified in MGC cell line (Figs. 4A and 4B).

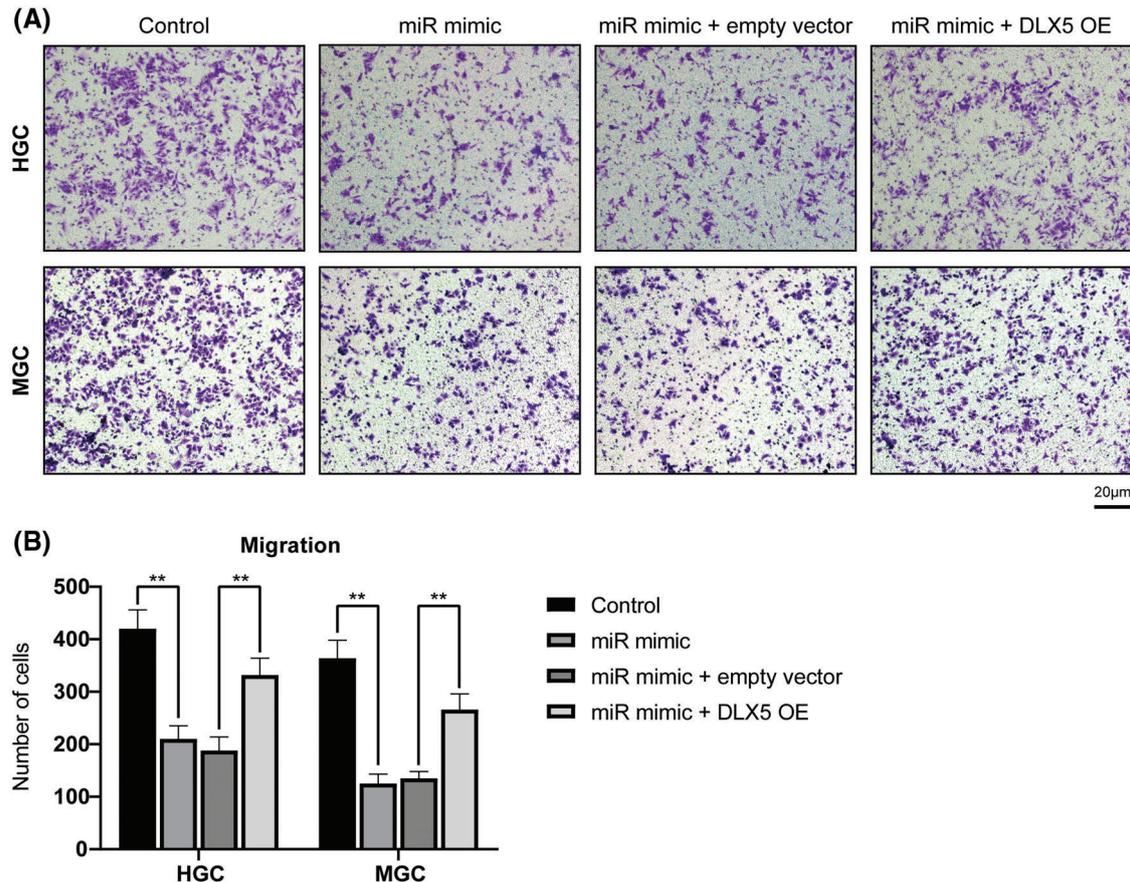


FIGURE 4. The functions of miR-376a-3p/DLX5 in GC cell invasion. A, Trans-well invasion results of HGC and MGC cell lines after overexpression of miR-376a and/or DLX5. Scar bar: 20 um. B, Counting results of invaded cells in GC cells after overexpression of miR-376a and/or DLX5. Upregulation of DLX5 could abolish the anti-invasive role of miR-376a-3p on GC cells. $**P < 0.01$.

The promotion effect of miR-376a-3p/DLX5 in GC cell apoptosis ability

The roles of miR-376a-3p/DLX5 in apoptosis level of GC cells was investigated through flow cytometry detection. The results confirmed the promotion effect of miR-376a-3p overexpression in GC cell apoptosis. The apoptotic rates of GC cells was significantly elevated after miR-376a-3p overexpression both in HGC cell line and MGC cell lines. The pro-apoptotic effect of miR-376a-3p could be partially abolished by DLX5 overexpression. The apoptosis rate of GC cells in the miR-376a-3p/DLX5 co-overexpression group was notably less than that in the miR-376a-3p-sole-overexpression group (Figs. 5A and 5B).

Discussion

In the present study, the expression levels of miR-376a-3p and DLX5 were determined in GC tissues and cell lines. DLX5 was proved to be a novel target of miR-376a-3p in GC. Increased levels of miR-376a-3p could reduce the ability to proliferate and invade of GC cells by targeting DLX5. The apoptosis level of GC cells could be increased after miR-376a-3p overexpression. The present result suggested that upregulation of miR-376a-3p in GC patients might have a potential therapeutic role.

Increasing evidence has linked miRNA expression profile with the development of diseases, and some miRNAs have been proved as predictive markers of tumor staging or

prognosis (Rajasegaran *et al.*, 2021). The abnormal miRNA expression levels are tightly correlated to the pathogenesis, chemotherapy sensitivity, and prognosis of GC (Ouyang *et al.*, 2021). Recently, a 4-miRNA expression signature (miR-200b, miR-103a, miR-199, and miR-152) was identified as a predictor for GC chemoresistance, which outperformed those clinicopathological factors (Cheng *et al.*, 2021). A novel signature composed of 8 genome instability-related miRNAs was also constructed to stratify GC patients into distinct risk groups with different survival outcomes and immunotherapy efficacy (Liu *et al.*, 2021). In this study, the reduced expression of miR-376a-3p was identified both in GC cell lines and tissues, indicating that miR-376a-3p might be a potential supplement to the current miRNA expression signature for GC diagnosis, treatment, or prognosis.

The etiological mechanism leading to GC has not been fully clarified. Recent reports have confirmed that a range of miRNAs are participated in regulation of GC development (Ouyang *et al.*, 2021). Recently, miR-25, widely expressed in multiple tumors, was identified as a regulator of the growth and apoptosis of GC cells (Yang *et al.*, 2021). Defect of miR-497 could contribute to tumorigenesis and progression of GC, and overexpression of miR-497 might be a promising strategy in preventing the development of GC (Zhang *et al.*, 2021a). Furthermore, miR-199b-5p and miR-486-5p were also proved to function in the repression of GC tumorigenesis and progression (Chen *et al.*, 2021; Dai *et al.*, 2021).

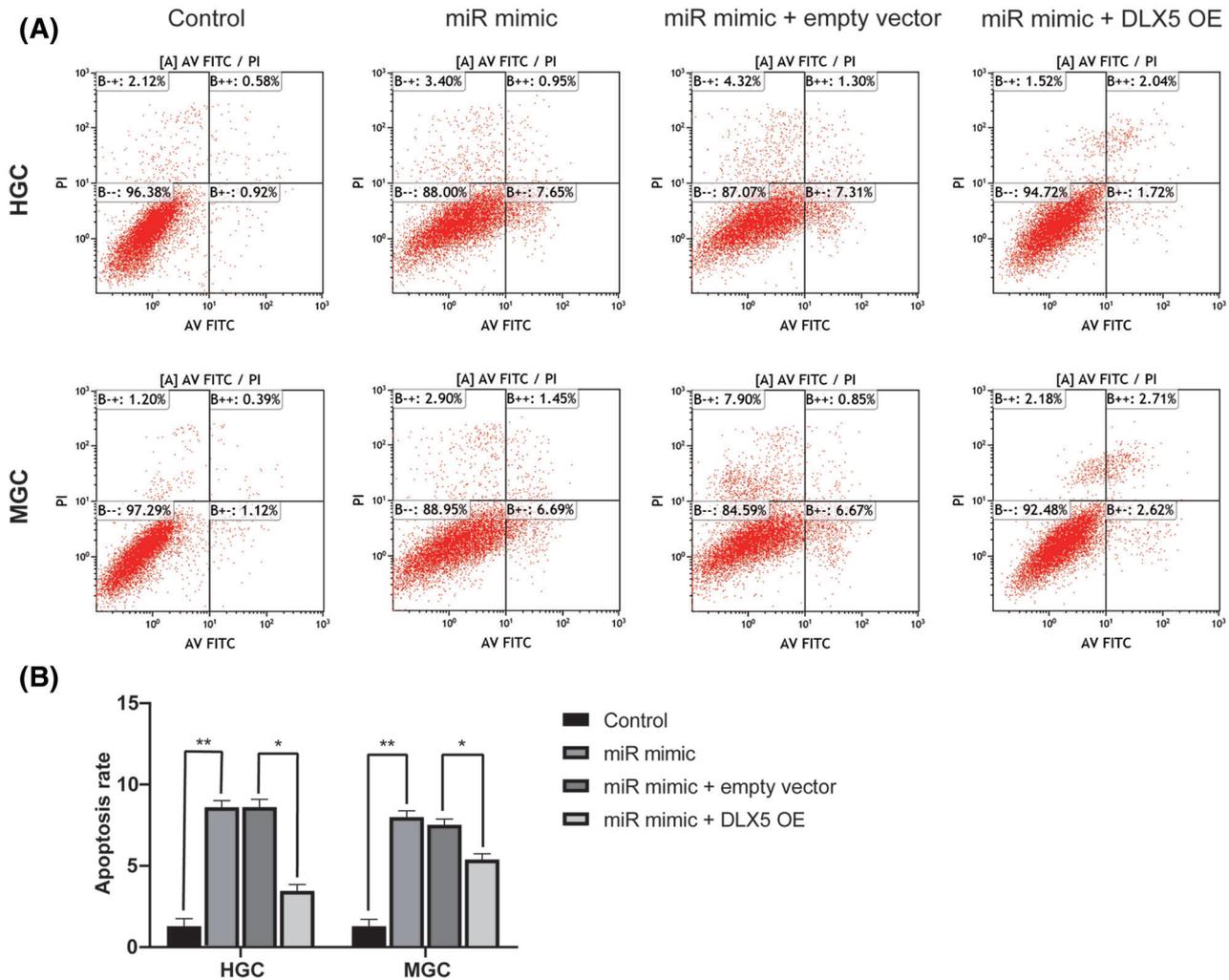


FIGURE 5. The roles of miR-376a-3p/DLX5 in GC cell apoptosis. A, Flow cytometry results of HGC and MGC cell apoptosis after overexpression of miR-376a and/or DLX5. B, Analysis results of the apoptotic proportion in HGC and MGC cell after overexpression of miR-376a and/or DLX5. * $P < 0.05$, ** $P < 0.01$.

Chen *et al.* (2020a) found that miR-217 had a low level expression in gastric tumor tissues of 40 patients with GC, and a lower expression in the gastric tumor tissues of the patients with GC metastasis. Moreover, miR-217 markedly suppressed the metastasis and invasion of gastric cancer cell line *in vitro*. Furthermore, miR-217 inhibited the expression of PTPN14 by directly targeting its 3'UTR. Moreover, the down-regulation of PTPN14 reduced the metastasis and invasion, whereas up-regulation of PTPN14 led to the enhanced metastases and invasion of GC cells. Our results revealed the regulatory role of miR-376a-3p in inhibiting GC cell growth and apoptosis, indicating that overexpression of miR-376a-3p might be a feasible strategy for GC treatment.

As a member of DLX gene family, transcription factor DLX5 has been verified as an oncogene in a variety of tumors, including squamous cell carcinoma or osteosarcoma (Huang *et al.*, 2021; Zhang *et al.*, 2021b). Transcriptional activation of DLX5 could regulate thousands of enhancers and promoters, which converge on activating cancer-promoting pathways (Tan and Testa, 2021). MiRNAs targeting DLX5 have also investigated in various pathological processes. miR-141 and miR-200a were found to modulate pre-osteoblast differentiation through the translational

repression of DLX5 (Itoh *et al.*, 2009). miR-214 was also found to inhibit myogenic differentiation of mesenchymal stem cells via targeting DLX5 (Qadir *et al.*, 2014). However, the function and mechanism of miRNA-DLX5 on tumorigenesis and progression have not been completely investigated. The present study found that miR-376a-3p could reduce the expression level of DLX5 in GC cells, and inhibit the ability of EMT, proliferation, and invasion. The results have broadened the regulatory network of DLX5 in GC. Additionally, the abnormally expressed DLX2 has been reported in various human hematological malignancies and solid tumors, including acute lymphoblastic leukemia, acute myeloid leukemia, etc. (Morini *et al.*, 2010; Sunwoo *et al.*, 2008; Yilmaz *et al.*, 2011). A recent study showed that DLX2 was expressed in transforming growth factor β (TGF- β) and it plays a key role in transforming its tumor suppressive function into tumor promoting function (Sunwoo *et al.*, 2008). In addition, abnormal TGF- β Expression is involved in tumor progression, metastasis, angiogenesis and poor survival of GC. Tang *et al.* (2013) demonstrated that increased expression of DLX2 may correlate with the advanced stage of gastric adenocarcinoma, and it may contribute to tumor development.

Overall, our findings identified the role of miR-376a-3p as a suppressor of GC, which was mediated through DLX5 expression inhibiting. This study highlighted the post-transcriptional regulation of miR376a-3p on DLX5 expression in GC cells, and provided novel molecular mechanisms on GC development.

Availability of Data and Materials: All the data could be accessed via reasonable request to the corresponding author.

Author Contribution: Conceptualization, supervision, project administration, writing–review & editing: Lin Chen; formal analysis, investigation, writing–original draft, validation: Yan Zhang; supervision, project administration, funding acquisition: Chao-jun Zhang; methodology, software: Zhen Cao; methodology, resources: Zhan-wei Zhao. All authors reviewed the results and approved the final version of the manuscript.

Ethics Approval: The study was approved by the Ethics Committee of Chinese PLA General Hospital (No. 2020054; date: December 30, 2019).

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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