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MiR-124-5p Inhibits the Progression of Gastric Cancer by Targeting MIEN1

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Abstract

Objective: To observe the effect of miR-124-5p on progression of gastric cancer (GC) and explore the targeting mechanism. **Methods:** After collecting the specimens, we used real-time fluorescence quantitative PCR to detect the miR-124-5p level of GC tissue and corresponding adjacent tissue. Then MTT test and scratch wound-healing assay were hired to evaluate the influence of miR-124-5p in GC cell (SGC-803 and SGC7901) migration and proliferation ability. The binding of miR-124-5p to migration and invasion enhancer 1 (MIEN1) was detected through dual luciferase reporter gene experiment and western blot was utilized to assay the protein level of MIEN1. **Results:** Compared with adjacent tissues, miR-124-5p level in GC tissues was lower significantly. MiR-124-5p mimic inhibited the metastasis and proliferation ability of SGC7901 cells and miR-124-5p inhibitor promoted the migration and proliferation ability of SGC803 cells. In addition, miR-124-5p targeted MIEN1 and negatively modulated the MIEN1 expression in SGC-803 and SGC7901 cells. Silencing MIEN1 negatively regulated the metastasis and proliferation ability of SGC7901 cells. **Conclusion:** MiR-124-5p inhibited the GC cell proliferation and metastasis phenotypes through MIEN1, which probably becomes a novel molecular target for clinical GC treatment.

Keywords

miR-124-5p, gastric cancer, MIEN1, cell migration and invasion

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Introduction

As the fourth leading cause of mortality, gastric cancer (GC) usually appear at more advanced stages with more aggressive histology patterns and worse prognosis.[1,2] Therefore, it is crucial to develop effective targeted therapy to perform successful intervention.[3] Invasion and metastasis are important processes that mediate tumor initiation and development, better understand of which provide information for finding treatment target of GC. So the underlying molecular mechanisms about tumor invasion and metastasis need to be fully elucidated.

It reported that miRNAs are important regulative factor of gene expression[4-6] and play a crucial role in tumors development by regulating tumor suppressor genes and transcription factors.[7-12] For example, Yu *et al.* found that miR-6852 could inhibit the GC cells proliferation and invasion through forkhead box J1.[13] Xu *et al*. demonstrated that miR-543 promotes GC cells migration and invasion by down-regulating speckle-type POZ protein.[14] Previous results have demonstrated that miR-124-3p acts as a potential marker and suppresses tumor growth in gastric cancer.[15,16] Interestingly, some researchers found that miR-124-5p expression in patients with positive lymphatic metastasis of the primary gastric tumor was down-regulated.[17] But the specific relationship between miR-124-5p and GC need to be further clarified.

In this study, we observed the miR-21-5p level in GC cancer and investigated the effect of miR-21-5p on GC cells metastasis and proliferation. We also explored the interaction of MIEN1 and miR-21-5p.

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Methods and Materials

Specimens and Participants

From January 2017 to February 2019, the GC tissue and corresponding adjacent tissue specimens (3 cm away from the tumor) derived from 50 patients who were pathologically diagnosed with GC in our hospital were colledted. After surgery, samples from GC tissues and tissues adjacent to the cancer were kept in liquid nitrogen immediately. All participants had signed written informed consent. This study was authorized by Ethics Committee of Affiliated Hospital of Qingdao University.

Cell Culture

In this study, the gastric cancer cell lines SGC-7901 and SGC803 were obtained from the American Type Culture Collection (Manassas, VA, USA). All of the cells came from American type culture collections and been cultivated in RPMI 1640 medium (Gibco). Cells were replenished 10% FBS treated with thermal inactivation (Gibco) under the conditions of 37°C, humidified incubator, 5% CO_2. Mimic or inhibitor came from Shanghai Gene Pharmaceutical Co., Ltd. Cells have been transfected on Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) Inhibitor NC). Transfection reagent has been added separately to the cells as a mimic. In negative control which was detected by RT qPCR) after 48 h.

Double luciferase Reporter Assay

Luciferase reporter assay was performed to verify if MIEN1-was a direct target of miR-124-5p. Construction of psiCEHCK-2 dual luciferase vector (Promega Corporation, Fitchburg, WI, USA) was named as wild type of MIEN1 or the mutant type of MIEN1. The constructs and miR-124-5p mimics or miR-mimics (Genechem Co., Ltd, Shanghai, China) were transiently co-transfected with the luciferase reporter plasmid in HEK-293 T cells. Transfected cells were collected after 48 hours of incubation at 37°C. Luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega, Fitchburg, WI, USA) in accordance with the manufacturer's instructions.

Viability Assay

Cell growth and viability were measured with CCK-8 reagent (Beyotime, Shanghai, China). The cells were inoculated in 96-well plates at a density of 1×10^3 cells per well for 7 days. Add 10 L ccK-8 to the well every 24 hours for 1 h. The absorbance at 450 nm was then measured using the Epoch (Bio-Tek, VT, USA).Finally. The growth curve is drawn according to absorbance. Make 3 parallel holes and repeat the measurement in triplicate.

Scratch Wound-Healing Assay

SGC7901 and SGC803 cells in logarithmic growth phase were inoculated at the density of 2.5×10^5 cells / well on a 24-well plate under the incubation condition of at 37° C, 5% CO_2 for 24 hours. Then the culture and rinse were discarded with PBS for 2 times. Every plate was streak 3 to 4 lines with a 200μl pipette tip evenly and forcefully. After rinsing each well with PBS for 3 times, 1 mL of serum-free culture solution was added to each well and the cells were incubated under the condition of 37 °C, 5% CO_2 in an incubator. We observed cells and took pictures after 24 h.l

Transwell Invasion

Transwell chamber (Corning, NY, USA) was coated with Matrigel (200 mg/ml) and been incubated overnight. All non-invasive cells were removed after 24 h. Fixed the Matrigel membrane and paraformaldehyde, then stained by crystal violet solution. The experiment was repeated and measured for 3 times. Phase contrast microscopy (Olympus, Tokyo, Japan) was used to count the invading cells.

qRT-PCR

Primers was designed with Primers 5.0 software in the light of the human miR-124-5p and MIEN1 mRNA sequences in GeneBank, and produced in Shanghai Sangon Biotech Co., Ltd. The following primer sequences were used: miR-124-5p (5'-CGTGTTCACAGCGGACCTTGAT-3'), U6 (forward primer: 5'-GCTTCGGCAGCACATATACTAAAAT-3', reverse primer: CGCTTCACGAATTTGCGTGTCAT-3'), MIEN1 (forward primer: 5'-CAGTGCTGTGGAGCAGT-3', reverse primer: 5'-GACGGCTGTTGGTGATCTTT-3'), GAPDH (forward primer: 5'-gagcgagatccctccaa-3', reverse primer: 5'-actgtggtca tgagtccttc-3'). Total RNA was isolated by Trizol reagent. The purity and concentration of RNA were detected by ultraviolet spectrophotometry. The first strand of cDNA was synthesized by reverse transcription (Vazyme Biotech Co., Ltd., Nanjing, China). GAPDH was the MIEN1 mRNA internal reference gene and U6 as the miR-124-5p internal reference gene on ABI7300 fluorescence quantitative PCR instrument. The relative expression level of target gene RQ = 2-ΔΔCT.

Statistical Analysis

The consequences were expressed as mean ± SEM of independent assays. One-way ANOVA was used for multiple comparisons, then paired comparison with post-group test was carried out as necessary. $P \leq 0.05$ was considered to harbor statistical difference.

Results

miR-124-5p Expression in GC

The level of miR-124-5p in GC tissue was significantly lower than that in adjacent tissues (Figure 1A) and level of miR-124-5p in GC tissue increased with stage of GC (Figure 1B). Furthermore, level of miR-124-5p in human GC SGC-803 and SGC7901 cells is significantly lower than in human gastric

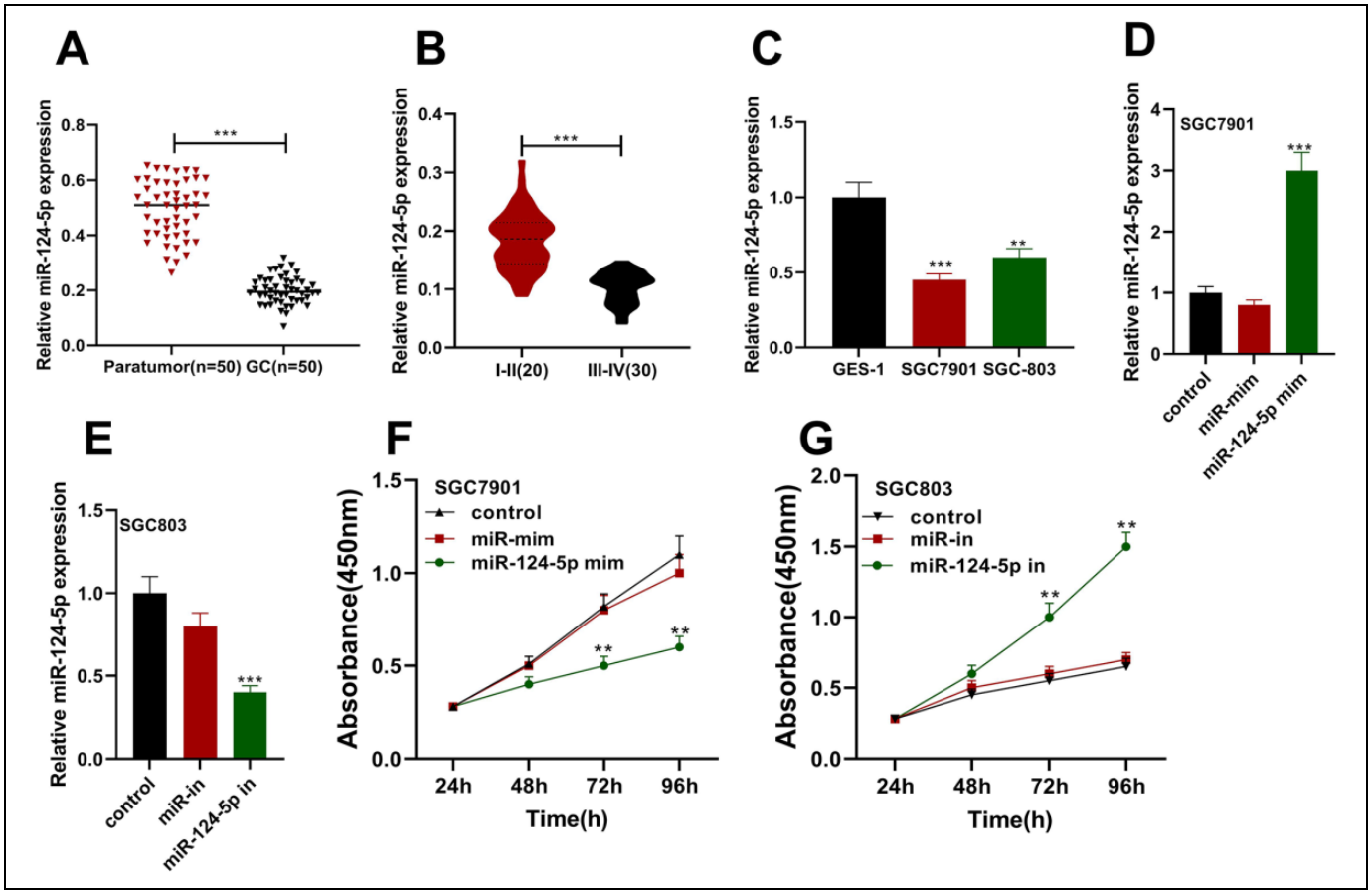

Figure 1. MiR-124-5p is down-regulated in GC tissues and cells and inhibits GC cell proliferation. QRT-PCR to assay miR-124-5p expression in GC tissues and adjacent tissues (A), GC patients with different stages (B), human gastric epithelial cell lines GES-1 and GC cell lines SGC7901 and SGC-803 (C), cells after transfecting miR-124-5p mimics (D), cells after transfecting miR-124-5p inhibitor (E). After transfecting miR-124-5p mimics (F) and inhibitors (G), MTT was used to examine GC cell proliferation. **P < 0.01, ***P < 0.001 (in contrast to miR-mim or miR-in group).

mucosa epithelial cells GES-1(Figure 1C). The miR-124-5p in SGC7901 cells significantly increased when miR-124-5p mimic administrated (Figure 1D), while miR-124-5p in SGC-803 cells significantly reduced when miR-124-5p inhibitor administrated (Figure 1E). MiR-124-5p mimic transfection inhibited the growth of SGC7901 cells (Figure 1F), and miR-124-5p inhibitor promoted the proliferation of SGC803 cells (Figure 1G).

MiR-124-5p Overexpression Inhibits Invasion and Migration Ability of GC Cells

The wound gap of SGC7901 cells in miR-124-5p mimic group was larger than in control groups (Figure 2A), which means that the invasion ability of SGC7901 cells had been significantly inhibited by miR-124-5p overexpression. After administration of miR-124-5p mimic, the number of invaded cell decreased (Figure 2B), which means that miR-124-5p mimic decreased the GC cells proliferation ability. Therefore, over miR-124-5p expression suppressed not only invasion but also migration ability of GC cell lines *in vitro*.

Effect of Low Expression of miR-124-5p on GC Cells

The wound gap of SGC-803 cells transfected with miR-124-5p mimic was significantly less than control cells (Figure 3A), whereas the low level of MIR-124-5p promoted the invasion of SGC-803 cells (Figure 3B), implying that miR-124-5p is capable of inhibiting the malignant phenotype of GC cells.

MiR-124-5p Directly Binds to MIEN1's 3'-UTR

The results found that miR-124-5p remarkably lessened the luciferase activity of HEK-293 T cells transfected with pmirGLO plasmid carrying wt-MIEN1, but not the HEK-293 T cells transfected with (Mut)3'-UTR (mut-MIEN1) (Figure 4B). In contrast to normal gastric epithelial cell line GES-1, MIEN1 mRNA level markedly increased (Figure 4C). In addition, MIEN1 was highly expressed in GC tissues (Figure 4D). The relationship between miR-124-5p and MIEN1 in GC specimens was also studied. MiR-124-5p levels were negatively correlated with MIEN1 expression as shown in Figure 4E. In addition, after transfected by miR-124-5p mimics, the MIEN1 expression was significantly down-regulated (Figure 4F). The

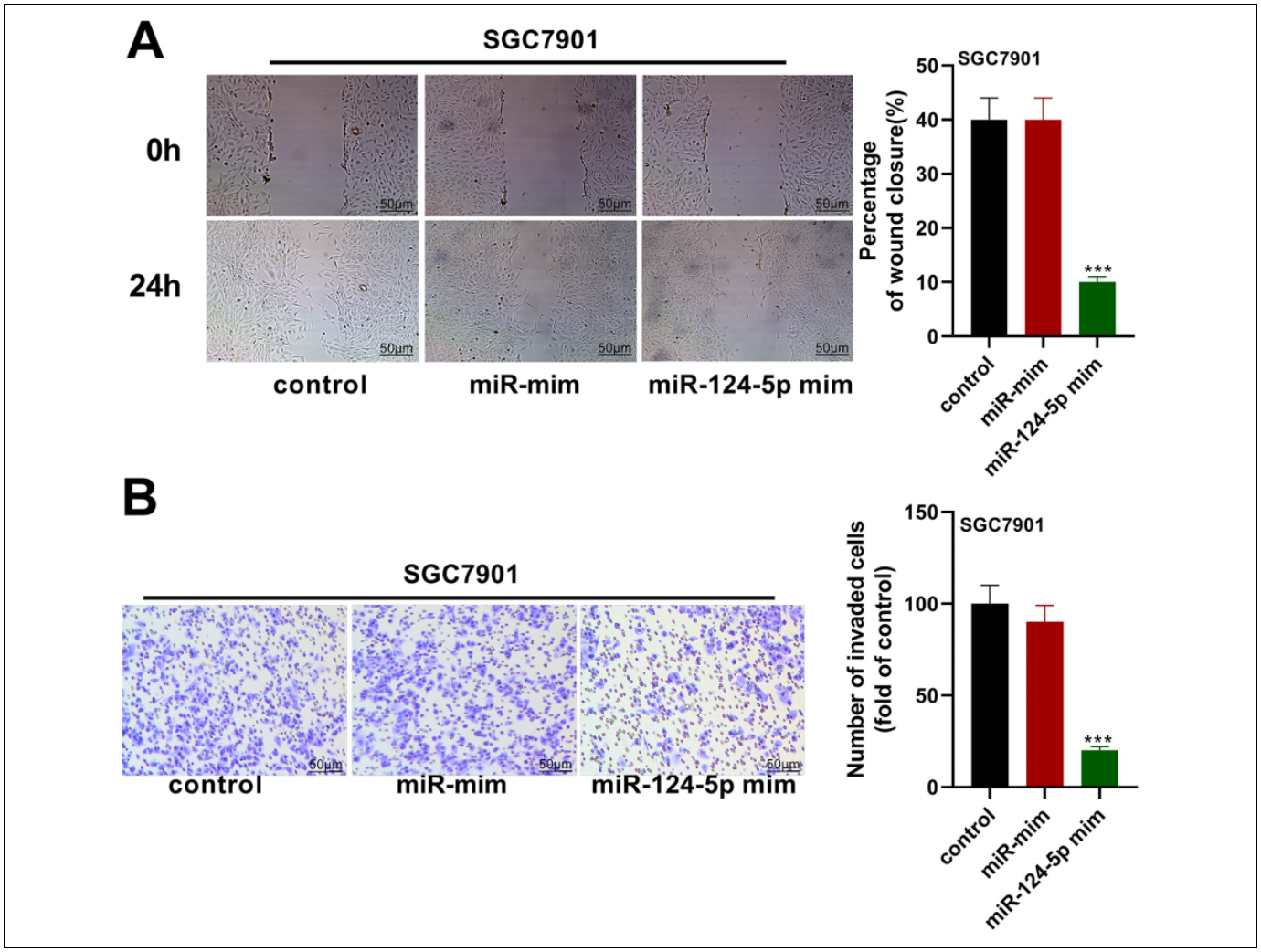

Figure 2. High-expressed miR-124-5p restrains invasion and migration of SGC7901 cells. After transfection with miR-124-5p mimics, the migration (A) and invasion (B) of SGC7901 cells was detected adopting the scratch wound-healing assay and transwell experiment, separately. ***$P < 0.001$ (in contrast to miR-mim group).

protein level of MIEN1 in GC cells transfected with miR-124-5p mimic was lower compared to cells transfected with miR-NC (Figure 4G).

MIEN1 Overexpression Reversed the Effect of miR-124-5p on GC Cells

Previous study showed that MIEN1 is involved in the progress of GC.[18] In this study, the MIEN1 mRNA and protein level in GC cells transfected with sh-MIEN1 were significantly reduced (Figure 5A and B). MIEN1 level was down-regulated, and GC cell proliferation was significantly inhibited (Figure 5C). At the same time, the down-regulation of MIEN1 significantly reduced the SGC7901 cells' migration and invasion ability (Figure 5D and E). Co-transfecting SGC7901 cells with miR-124-5p mimic and pLV-MIEN1 can resume MIEN1 expression in it (Figure 6A and B). The overexpression of MIEN1 promoted the malignant phenotype of GC cells which was inhibited by miR-124-5p (Figure 6C-E).

Discussion

GC is one of the most ubiquitous carcinomas across the globe. Currently, the treatment method has a survival rate of only 20%, resulting in severe death worldwide. Most patients are confirmed as metastatic or advanced GC at the first diagnosis, and 5-year survival of patients who suffer from advanced GC is not more than 15%.[19] The emergence of new therapeutic targets for GC is crucial to the early diagnosis and improvement of survival rate.

Recent studies showed that the expression level of miR-124 was significantly decreased in cancer tissues and had a tumor suppressor role in various types of cancer.[20] Migration and invasion enhancer 1 (MIEN1), neighboring the HER2/neu locus, was highly expressed in prostate cancer phenotypes with different stages and grades.[21] It has been considered as a novel biomarker of breast cancer,[19] prostate cancer[21,18] and especially a key factor in the progression of GC.[22] Emerging literature suggest MIEN1 as a new tumor-specific target protein as

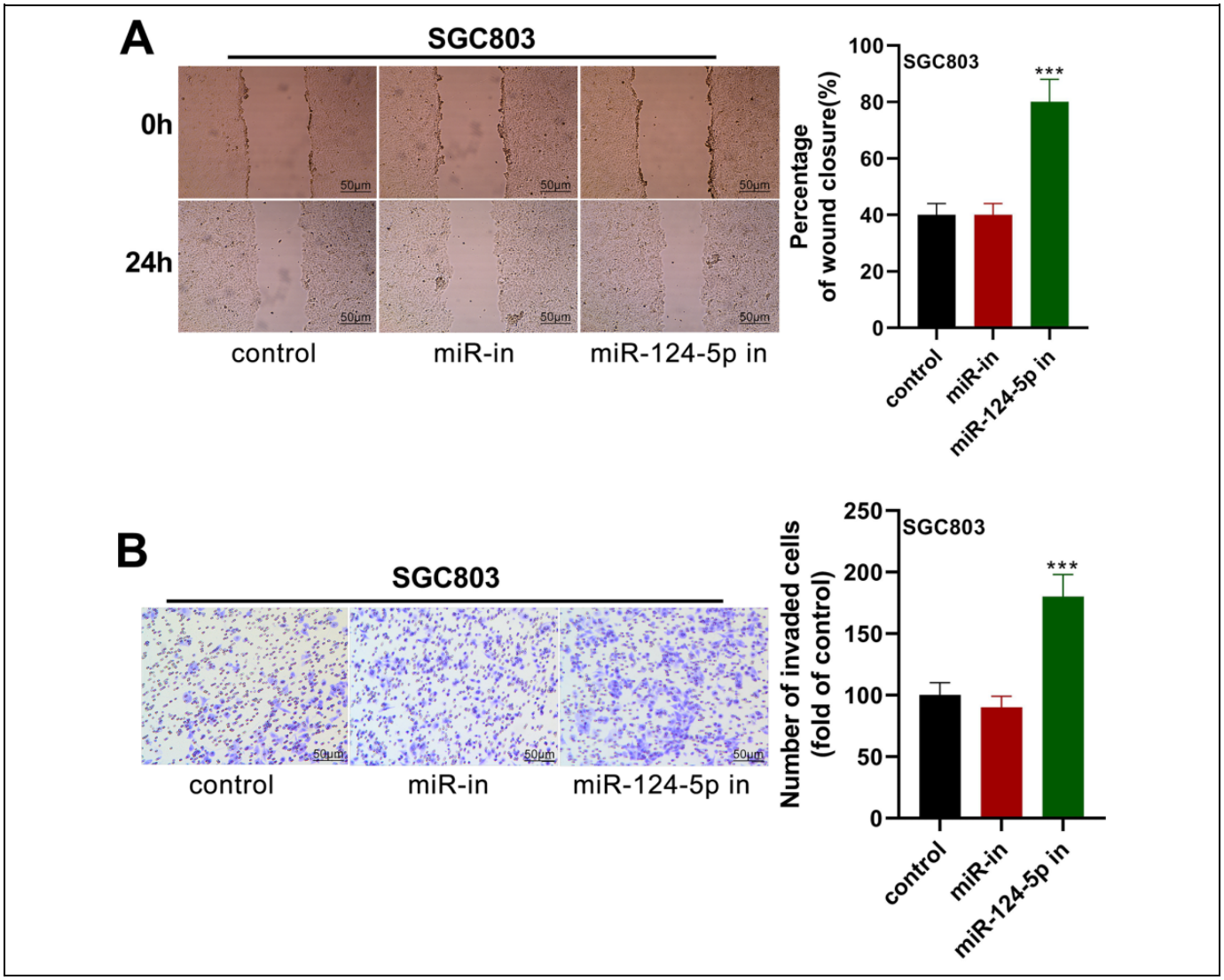

Figure 3. Low-expressed miR-124-5p facilitates the invasion and migration of SGC803 cells. After transfection with miR-124-5p inhibitor, the migration (A) and invasion (B) of SGC803 cells was detected adopting the scratch wound-healing assay and transwell experiment, separately. ***P < 0.001 (in contrast to miR-mim group).

it facilitates cancer progression that plays key role in distinct processes of migration/invasion of cancer cells.[23] And it's reported that miR-136 promotes proliferation and metastasis of gastric cancer by upregulating MIEN1 expression.[24] It was previously shown that MIEN1 exerts a critical influence on the progress of GC.[22] This is consistent with the inhibitory impact of MINE1 on the malignant phenotype of GC cells in this study. We also evidenced that MINE1 has undergone post-transcriptional modulation of miR-124-5p. Additionally, miR-124-5p reduces not only migration but also invasion capability of GC cells. We believe that miR-124-5p is a new target for GC, which may restrain the progress of GC by inhibiting MIEN1 signal.

In this study, we discovered a new miRNA, miR-124-5p, which can regulate MIEN1 in GC. Our research shows that miR-124-5p may negatively correlated with tumor progression, and the absence of miR-124-5p lead to increased expression of MIEN1, so as to accelerating the progress of clinical GC. Ectopic expression of miR-124-5p not only reduces the expression of MIEN1, but also bring down the ability of cell migration and invasion. It is demonstrated that miR-124-5p may be expected to be a new therapeutic agent for GC.

Compared with its overexpression in cancer, MIEN1 has the lowest expression in several normal tissues.[18] It is close to HER2/neu site on chromosome 17, so it frequently amplifies by HER2 amplicon (in 79% of breast carcinoma).[24] A recent study using 8 genes including MIEN1 showed that even in HER2-negative breast carcinoma, trastuzumab therapy had moderate response, confirming that MIEN1 is vital in the response to new assisted therapy.[25] Studies manifested that the overall survival rate of breast carcinoma patients is lower when MIEN1 has high-expression, whereas low-expression implicates a greater prognosis.[26] We previously evidenced that cells that overexpress MIEN1 harbor higher metastatic capability,

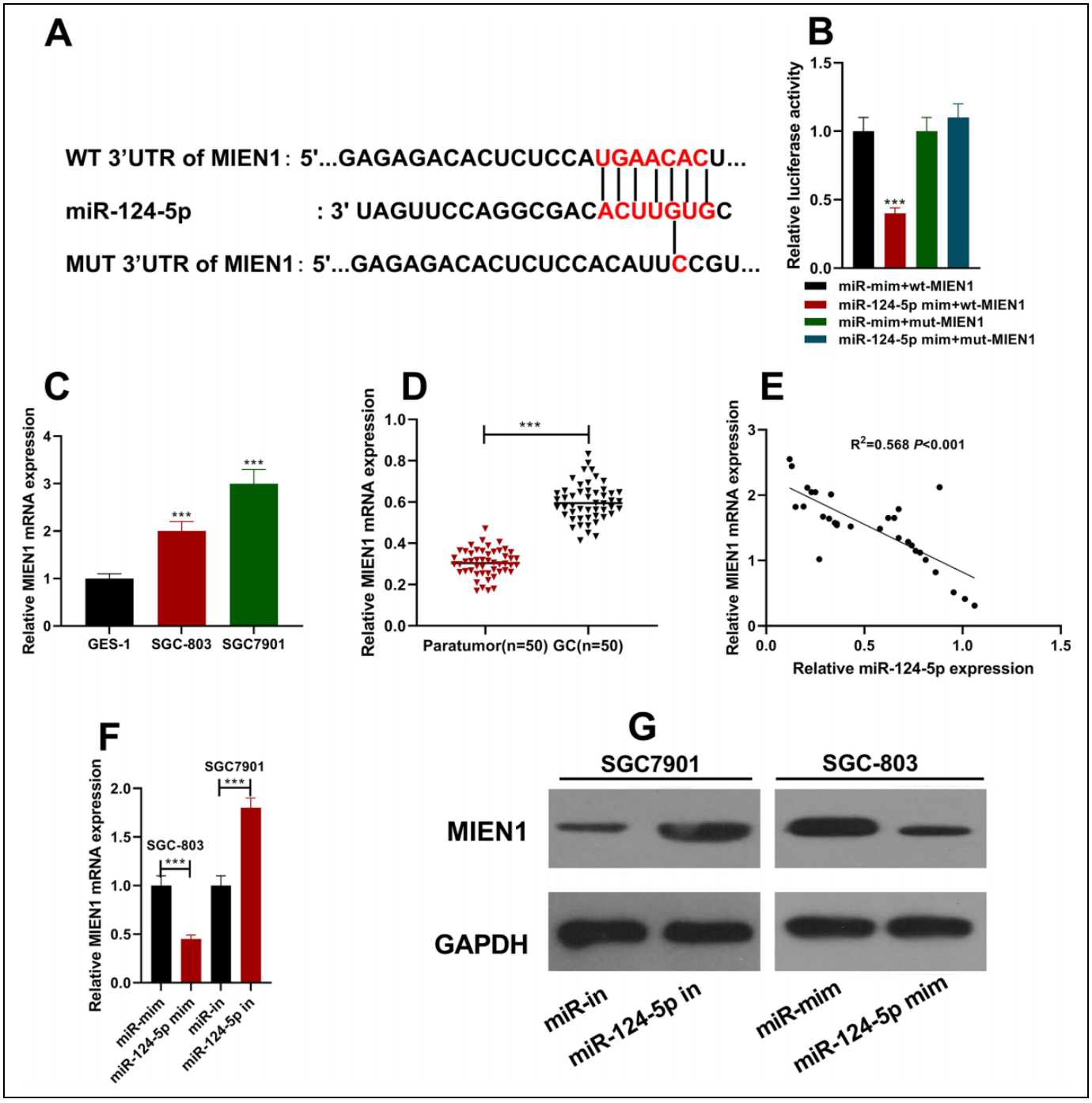

Figure 4. miR-124-5p is negatively correlated with MIEN1. A: Bioinformatics database showed that miR-124-5p has a binding site with MIEN1. B: miR-124-5p has a binding relationship with MIEN1. C: MIEN1 mRNA levels in human gastric epithelial cells and GC cells. D: MIEN1 mRNA expression in GC tissues and tissues adjacent to cancer. E: Relationship between miR-124-5p and MIEN1 in GC tissues. F-G: MIEN1 mRNA and protein expression in GC cells after transfection of miR-124-5p mimics and inhibitors. ***P < 0.001.

which doesn't indicate the faster initiation or onset of tumors.[18] Now, we also found the high MINE1 expression in GC cells and its role in promoting migration and invasion.

Previous findings demonstrated that miR-124-5p targets tumor suppressor factors or certain oncogenes in cancer progress. For example, miR-124-5p inhibits glioma growth through post-transcriptional modulation of LAMB1.[27] Through targeting SMC4, low miRNA-124-5p expression is concerned with unfavorable prognosis of colorectal carcinoma.[28] Nonetheless, the impact of miR-124-5p on GC metastasis has not been thoroughly elucidated, and its mechanism is still unclear. In our research, we have proved that the over miR-124-5p regulation

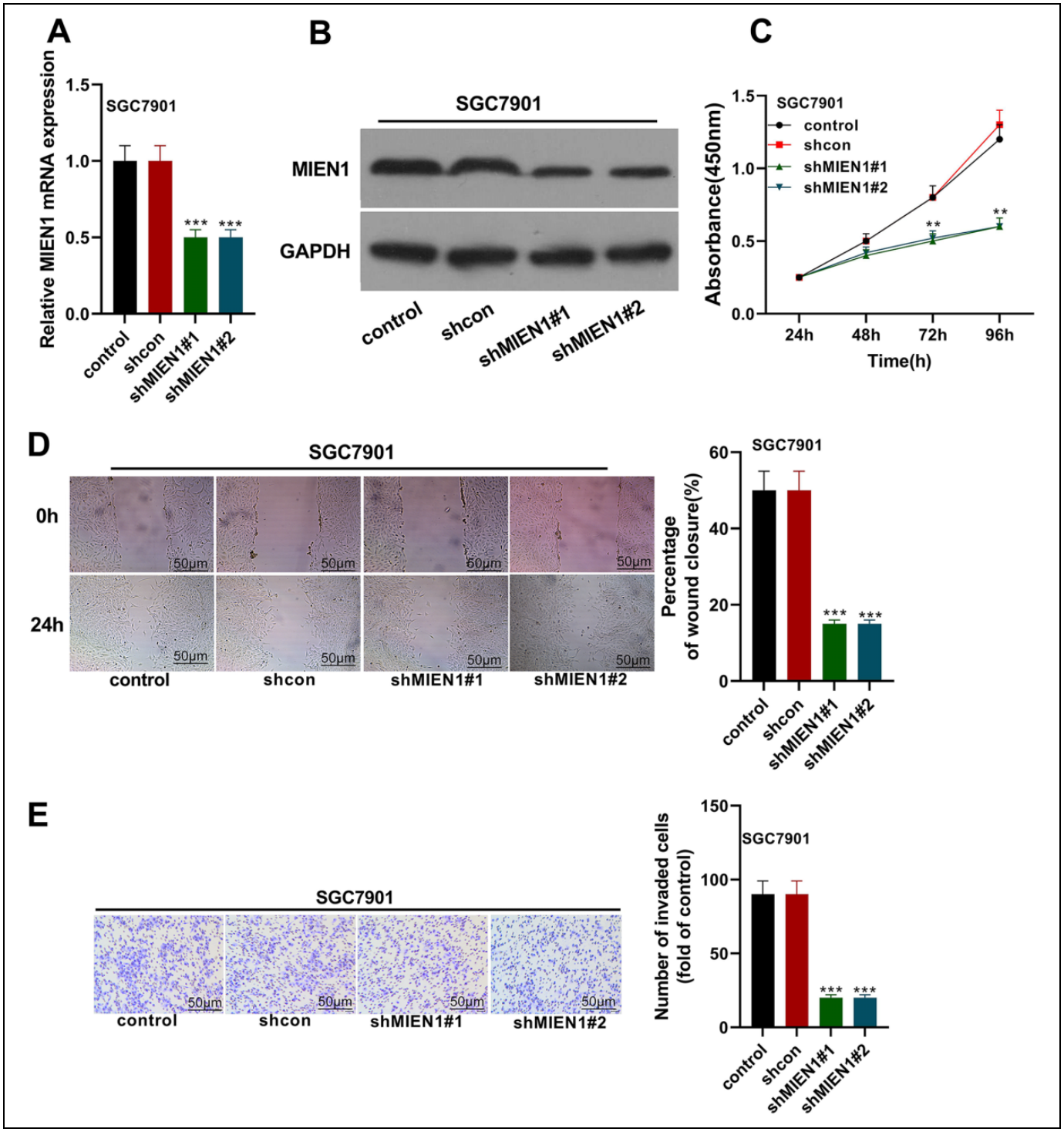

Figure 5. Low-expressed MIEN1 inhibits the proliferation, migration and invasion of GC cells. After low MIEN1 expression in SGC7901 cells, qRT-PCR was applying to assay the MIEN1 mRNA expression (A); Western blot to assay the MIEN1 protein expression (B); MTT to assay the proliferation (C); scratch wound-healing assay to detect the migration (D) as well as invasion (E). **P < 0.01, ***P < 0.001. (in contrast to shcon group).

restrains the proliferation, invasion as well as migration of GC, while the low miR-124-5p expression has opposite effect.

MIEN1 is located in the 17q12 region of human chromosome and is dysregulated in various cancer tissues.[29-31] Many miRNAs play biological functions by targeting MIEN1, including miRNA-26b,[32] miRNA-940.[33] In this study, luciferase report experiment proved that MIEN1 is the direct functional target of miR-124-5p in GC.

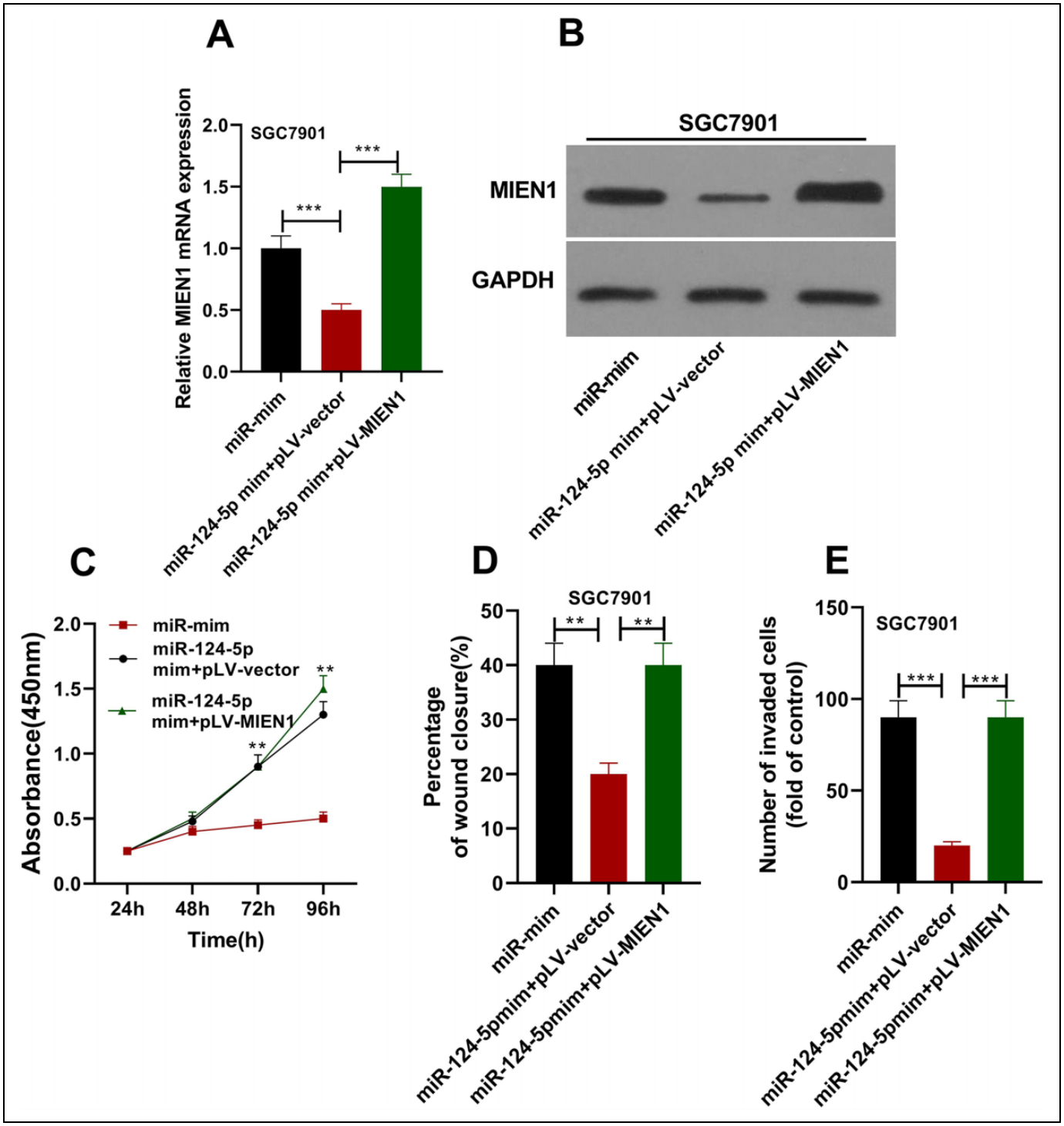

Figure 6. miR-124-5p influences the progress of GC by modulating MIEN1. After co-transfection of miR-124-5p and MIEN1 overexpression plasmids in SGC7901 cells, qRT-PCR was employed to monitor MIEN1 mRNA expression (A); western blot to assay MIEN1 protein expression (B); MTT to assay proliferation (C); scratch wound-healing assay to test migration (D); transwell test to assay the invasion (E). **P < 0.01, ***P < 0.001.

However, there are some deficiencies in this article. Firstly, in order to verify the conclusion of this study, animal experiments such as nude mice tumorigenesis are necessary in the following studies. Secondly, in the mechanism research, other downstream targets of miR-124-5p need to be further screened and verified, which will further clarify the downstream mechanism of miR-124-5p; Finally, in order to explore the value of miR-124-5p as a prognostic marker, more patients,

together with corresponding follow-up information, should be included to analyze the overall survival time and relapse-free survival time. In addition, due to the limitation of sample size, it is necessary to carry out further research in a larger research queue. However, in this study, we found that miR-124-5p is capable of inhibiting the progression of GC by targeting MIEN1, which promisingly provides a new molecular target for treating GC.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article

Ethics Statement

The experiment was often approved by the Ethics Committee of the The Fivth Medical Center of PLA General Hospital(NO.2063), and all patients participating in this study provided written informed consent in accordance with the "Helsinki Declaration."

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