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. Therefore, it is crucial to develop effective targeted therapy to perform successful intervention. 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 and play a crucial role in tumors development by regulating tumor suppressor genes and transcription factors. For example, Yu et al. found that miR-6852 could inhibit the GC cells proliferation and invasion through forkhead box J1. Xu et al. demonstrated that miR-543 promotes GC cells migration and invasion by down-regulating speckle-type POZ protein. Previous results have demonstrated that miR-124-3p acts as a potential marker and suppresses tumor growth in gastric cancer. Interestingly, some researchers found that miR-124-5p expression in patients with positive lymphatic metastasis of the primary gastric tumor was down-regulated. 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.
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 collected. 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% CO2. 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 psiCEHK-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 × 10^3 cells / well on a 24-well plate under the incubation condition of at 37 °C, 5% CO2 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% CO2 in an incubator. We observed cells and took pictures after 24 h.

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 GenBank, and produced in Shanghai Sangon Biotech Co., Ltd. The following primer sequences were used: miR-124-5p (5’-CGTGTTGACGCAGACCTTGAT-3’), U6 (forward primer: 5’-GCTTCGGCAGCACATATACTAAAAT-3’, reverse primer: CGCTTCAGAAATTTCGCTGTCA-3’, MIEN1 (forward primer: 5’-CAGTGTCTGTTGGAGCACACTATACATATAAT-3’, reverse primer: 5’-GACGGCTGTTGGTGATCTTT-3’, GAPDH (forward primer: 5’-gagcgagatccctccaa-3’, reverse primer: 5’-GACTGGTCATGTCCTCA-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 ≤ 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...
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).
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. 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%. 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. Migration and invasion enhancer 1 (MIEN1), neighboring the HER2/neu locus, was highly expressed in prostate cancer phenotypes with different stages and grades. It has been considered as a novel biomarker of breast cancer, prostate cancer, and especially a key factor in the progression of GC. Emerging literature suggest MIEN1 as a new tumor-specific target protein as
it facilitates cancer progression that plays key role in distinct processes of migration/invasion of cancer cells. And it’s reported that miR-136 promotes proliferation and metastasis of gastric cancer by upregulating MIEN1 expression. It was previously shown that MIEN1 exerts a critical influence on the progress of GC. 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. It is close to HER2/neu site on chromosome 17, so it frequently amplifies by HER2 amplicon (in 79% of breast carcinoma). 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. 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. We previously evidenced that cells that overexpress MIEN1 harbor higher metastatic capability,
which doesn’t indicate the faster initiation or onset of tumors.\textsuperscript{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.\textsuperscript{27} Through targeting SMC4, low miRNA-124-5p expression is concerned with unfavorable prognosis of colorectal carcinoma.\textsuperscript{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...
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. Many miRNAs play biological functions by targeting MIEN1, including miRNA-26b, miRNA-940. In this study, luciferase report experiment proved that MIEN1 is the direct functional target of miR-124-5p in GC.
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,

**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.
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 Conflcting 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 Fifth 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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References
1. Hartgrink HH, Jansen EP, van Grieken NC, van de Velde CJ. Gastric cancer. *Lancet*. 2009;374(9688):477-490.
2. Shi Y, Zhou Y. The role of surgery in the treatment of gastric cancer. *J Surg Oncol*. 2010;101(8):687-692.
3. Durães C, Almeida GM, Seruca R, Oliveira C, Carneiro F. Biomarkers for gastric cancer: prognostic, predictive or targets of therapy. *Virchows Arch*. 2014;464(3):367-378.
4. Mohr AM, Mott JL. Overview of microRNA biology. *Semin Liver Dis*. 2015;35(1):3-11.
5. Fabian MR, Sonenberg N, Filipowicz W. Regulation of mRNA translation and stability by microRNAs. *Annu Rev Biochem*. 2010;79:351-379.
6. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*. 2004;116(2):281-297.
7. Ambros V. The functions of animal microRNAs. *Nature*. 2004;431(7006):305-355.
8. Xie M, Ma L, Xu T, et al. Potential regulatory roles of microRNAs and long noncoding RNAs in anticancer therapies. *Mol Ther Nucleic Acids*. 2018;13:233-243.
9. Kwak PB, Iwasaki S, Tomari Y. The microRNA pathway and cancer. *Cancer Sci*. 2010;101(11):2309-2315.
10. Farazi TA, Spitzer JI, Morozov P, Tuschl T. MiRNAs in human cancer. *J Pathol*. 2011;223(2):102-112.
11. Qu H, Xu W, Huang Y, Yang S. Circulating miRNAs: promising biomarkers of human cancer. *Asian Pac J Cancer Prev*. 2011;12(5):1117-1125.
12. Gentilin E, Degli UE, Zatelli MC. Strategies to use microRNAs as therapeutic targets. *Best Pract Res Clin Endocrinol Metab*. 2016;30(5):629-639.
13. Yu H, Zhang J, Wen Q, et al. MicroRNA-6852 suppresses cell proliferation and invasion via targeting forkhead box J1 in gastric cancer. *Exp Ther Med*. 2018;16(4):3249-3255.
14. Xu J, Wang F, Wang X, He Z, Zhu X. MiRNA-543 promotes cell migration and invasion by targeting SPOP in gastric cancer. *Onco Targets Ther*. 2018;11:5075-5082.
15. Liu F, Hu H, Zhao J, et al. MiR-124-3p acts as a potential marker and suppresses tumor growth in gastric cancer. *Biomed Rep*. 2018;9(2):147-155. doi:10.3892/br.2018.1113
16. Wu Q, Zhong H, Jiao L, et al. MiR-124-3p inhibits the migration and invasion of Gastric cancer by targeting ITGB3. *Pathology, Res Pract*. 2020;216(1):152762.
17. Yang B, Jing C, Wang J, et al. Identification of microRNAs associated with lymphangiogenesis in human gastric cancer. *Clin Transl Oncol*. 2014;16(4):374-379.
18. Evans EE, Henn AD, Jonason A, et al. C35 (C17orf37) is a novel tumor biomarker abundantly expressed in breast cancer. *Mol Cancer Ther*. 2006;5(11):2919-2930.
19. Uprak TK, Attaallah W, Çeliker ÇA, Ayrançı G, Yeğen C. CHER-2 incidence in gastric cancer, its association with prognosis and clinicopathological parameters. *Ulus Cerrahi Derg*. 2015;31(4):207-213.
20. Pan Y, Wu A, Xu F, Chen C, Jiang L, Jin R. Lentivirus-mediated overexpression of miR-124 suppresses growth and invasion by targeting JAG1 and EZH2 in gastric cancer. *Oncol Lett*. 2018;15(5):7450-7458. doi:10.3892/ol.2018.18194. Epub 2018 Mar 7. PMID: 29731896; PMCID: PMC5921033.
21. Dasgupta S, Wasson LM, Rauniar N, Prokai L, Borejdo J, Vishwanatha JK. Novel gene C17orf37 in 17q12 amplicon promotes migration and invasion of prostate cancer cells. *Oncogene*. 2009;28(32):2860-2872.
22. Dasgupta S, Cushman I, Kpetemey M, Casey PJ, Vishwanatha JK. Prenylated C17ORF37 induces filopodia formation to promote cell migration and metastasis. *J Biol Chem*. 2011;289(9):25935-25946.
23. Kushwaha PP, Gupta S, Singh AK, Kumar S. Emerging role of prenylated C17orf37 in 17q12 amplicon promotes migration and invasion enhancer 1 (mien1) in cancer progression and metastasis. *Front Oncol*. 2019;9:868.
24. Yu X, Xiao W, Song H, Jin Y, Xu J, Liu X. *CircRNA_100876* sponges miR-136 to promote proliferation and metastasis of gastric cancer by upregulating MIEN1 expression. *Gene*. 2020;748:144678.
25. Staaf J, Jonsson G, Ringner M, et al. High-resolution genomic and expression analyses of copy number alterations in HER2-amplified breast cancer. *Breast Cancer Res Treat*. 2010;12(3):R25.
26. Pogue-Geile KL, Kim C, Jeong JH, et al. Predicting degree of benefit from Adjuvant Trastuzumab in NSABP trial B-31. *J Natl Cancer Inst*. 2013;105(23):1782-1788.
27. Katz E, Dubois-Marshall S, et al. A gene on the HER2 amplicon, C35, is an oncogene in breast cancer whose actions are prevented by inhibition of Syk. *Br J Cancer*. 2010;103(3):401-410.
28. Chen Q, Lu G, Cai Y, et al. MiR-124-5p inhibits the growth of high-grade gliomas through posttranscriptional regulation of LAMB1. *Neuro Oncol*. 2014;16(5):637-651.
cancer via targeting of SMC4. Cancer Med. 2014;3(6):1544-1552.

30. Rajendiran S, Kpetemey M, Maji S, et al. MIEN1 promotes oral cancer progression and implicates poor overall survival. Cancer Biol Ther. 2015;16(6):876-885.

31. Kpetemey M, Dasgupta S, Rajendiran S, et al. MIEN1, a novel interactor of Annexin A2, promotes tumor cell migration by enhancing AnxA2 cell surface expression. Mol Cancer. 2015;14:156.

32. Li D, Wei Y, Wang D, Gao H, Liu K. MicroRNA-26b suppresses the metastasis of non-small cell lung cancer by targeting MIEN1 via NF-kappaB/MMP-9/VEGF pathways. Biochem Biophys Res Commun. 2016;472(3):465-470.

33. Rajendiran S, Parwani AV, Hare RJ, Dasgupta S, Roby RK, Vishwanatha JK. MicroRNA-940 suppresses prostate cancer migration and invasion by regulating MIEN1. Mol Cancer. 2014;13:250.