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MiR-99a suppresses cell migration and invasion by regulating IGF1R in gastric cancer

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Abstract. – OBJECTIVE: Gastric cancer is a common kind of gastrointestinal malignancies. Increasing evidence indicates dysregulation of microRNA-99a (miR-99a) in gastric cancer, and has been extensively investigated in terms of cancer formation, progression, diagnosis, therapy, and prognosis. The purpose of this study is to explore how miR-99a worked in gastric cancer on migration and invasion.

PATIENTS AND METHODS: The mRNA and protein levels of miR-99a and insulin-like growth factor 1 receptor (IGF1R) in gastric cancer were measured by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) and Western blot. Transwell assay was employed to analyze the migratory and invasive capacities. The Dual-Luciferase reporter assay was performed to confirm miR-99a mediated the expression of IGF1R by directly targeting its mRNA 3'-untranslated regions (3'-UTR) in gastric cancer cells.

RESULTS: MiR-99a was discovered to be significantly downregulated while IGF1R was upregulated in gastric cancer tissues and cell lines. The expression of miR-99a had a negative correlation with the IGF1R expression in gastric cancer tissues. Moreover, miR-99a was low expressed in gastric cancer cells HGC-27 and MGC-803 compared to the normal cell line. MiR-99a suppressed the migration and invasion through directly binding to the 3'-UTR of IGF1R mRNA in HGC-27 cells. In addition, IGF1R could reverse partial roles of miR-99a on migration and invasion in gastric cancer.

CONCLUSIONS: MiR-99a inhibited the migratory and invasive abilities by regulating the expression of IGF1R. MiR-99a was downregulated while IGF1R was upregulated in gastric cancer cell lines. The newly identified miR-99a/IGF1R axis provides novel insight into the pathogenesis of gastric cancer.

Key Words:
MiR-99a, IGF1R, Migration, Invasion, Gastric cancer.

Introduction

Gastric cancer (GC) is one of the most common gastrointestinal malignancies, and mortality ranks third in all the tumors[1,2]. Recently, due to changes in diet, increased work pressure, *Helicobacter pylori* infection and other reasons, gastric cancer was reported to have a tendency of younger. The general diagnosis of gastric cancer is at the advanced stage, at which stage the prognosis is poor and easy to relapse[3,4]. Moreover, there were little symptoms of the early stage; the current rate of gastric cancer early diagnosis is still low[5]. Thus, identifications of the new molecular biomarkers for early diagnosis of gastric cancer are necessary.

MicroRNAs (miRNAs) are small non-coding RNAs whose function is to repress gene expression by complementary pairing with target mRNA[6-8]. Altered expression of miRNAs affected the biological process in gastric cancer including miR-638, miR-152, miR-28 and miR-155[9-12]. MiR-99a, a common kind of microRNA, has been reported to be aberrantly expressed in several carcinomas including bladder cancer, non-small cell lung cancer, breast cancer and even in gastric cancer[13-16]. Feliciano et al[17] had expounded that miR-99a could repress lung cancer cell stemness. Moreover, in nasopharyngeal carcinoma, miR-99a inhibited cell invasion and metastasis[18]. Similar findings were found by Yu et al[19] that miR-99a suppressed the metastasis of non-small cell lung cancer cell. In addition, in gastric cancer, Zhang et al[16] have discovered that miR-99a regulated cisplatin resistance by directly targeting CAPNS1. However, almost no study explored the effect of miR-99a on the migration and invasion of nasopharyngeal carcinoma cells.

Insulin-like growth factor 1 receptor (IGF1R) is a transmembrane receptor of tyrosine kinase with α- and β-chains heterodimer that was acti-

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vated by its ligands IGF1 or IGF2[20]. IGF1R could influence tumor progression in many kinds of cancers including esophageal squamous cell carcinoma, hepatocellular carcinoma, colorectal cancer and prostate cancer[21-24]. Aleksic et al[24] have discovered that IGF1R was a recurrence post radiotherapy independent predictor. IGF1R impacted the survival and acquired cisplatin resistance in testicular germ cell tumor cells[25]. Moreover, in esophageal squamous cell carcinoma miR-99a has been reported to suppress proliferation, migration and invasion by inhibiting the IGF1R[22]. Considering these functions, we first propose that miR-99a regulated the expression of IGF1R to mediate the migration and invasion in gastric cancer.

Patients and Methods

Patients and Clinical Samples

A collection of 55 patients were selected from patents that sought treatment at the Heze Municipal Hospital from 2014 to 2016, and pairs of gastric cancer and corresponding paracancerous tissue samples were obtained. The patents had not accepted any treatment before surgery and the tissues were frozen in liquid nitrogen and saved in -80°C freezer after surgical excision. All the specimens of this study got informed consent from patients and were approved by the Ethical Committee of Heze Municipal Hospital.

Cell Lines and Culture Condition

We purchased a normal gastric epithelium cell line GES-1 and two gastric cancer cell lines (HGC-27 and MGC-803) from the American Type Culture Collection (ATCC; Manassas, VA, USA). All the cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) at 37°C in a humidified atmosphere of 5% CO_2.

RNA Isolation and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) assay was performed using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) or the miRcute and Separation of miRNAs Kit (Tiangen, Beijing, China) to identify the expression of total mRNAs or miRNAs from gastric cancer tissues and cells. At first, we employed the PrimeScript™ II 1st Strand complementary deoxyribose nucleic acid (cDNA) Synthesis Kit (TaKaRa, Dalian, China) to reverse transcribe and synthesize the first cDNA chain. Then, we applied the SYBR Prime Script miRNA RT-PCR Kit and the SYBR Premix Kit (both purchased from TaKaRa, Dalian, China) to quantify the expression of miR-99a and IGF1R, whose internal reference was U6 or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) respectively. We calculated the relative expression of mRNA and miRNA based on the $2^{-\Delta\Delta CT}$ method. Primer sequences used in this study were as follows: miR-99a, F: 5'-GTCAGGACCTCCTTACTC-3', R: 5'-GCTAGGGATGCGACAGGA-3'; IGF1R, F: 5'-GCCGGGTGTAAGCATCCTCGTG-3', R: 5'-ATGTGCGTCGTGGAGTCCCG-3'; ; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Protein Extraction and Western Blotting

The radioimmunoprecipitation assay (RIPA) Lysis Buffer, containing phenylmethylsulfonyl fluoride (PMSF; both from Beyotime, Shanghai, China) was utilized to lysed the cells to extract the total protein on ice. We determined the protein concentration used the Bicinchoninic Acid (BCA) Reagent Kit (Solarbio, Beijing, China) and measured the absorbance used a microplate reader. Separated the proteins with equal quality we utilized sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred the protein blots onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) and incubated anti-IGF1R rabbit polyclonal antibody (1:500; GeneTax, San Antonio, TX, USA) at 4°C overnight after blocking in 5% nonfat dried milk for 1 h. Then, the blots were incubated with the anti-rabbit antibody (1:3000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 hours at room temperature. Finally, the blots signal was determined on the Bio-Rad Gel Doc XR instrument (Bio-Rad, Hercules, CA, USA).

Transwell Assay

We performed cell invasive and migratory abilities using transwell chambers with a member pore size of 8 μm (Costar, Beijing, China), which the filter coated with or without Matrigel (Clontech, Mountain View, CA, USA). Inserted transwell chambers in a 24-well plate, 200 μL of cell

suspension were seeded into the top of the chamber, in which the medium was free of FBS; then 500 μL of medium (containing 10% FBS) was added into the lower chamber acted as an inducer. After incubating at 37°C for 48 h, the cells still on the top side of the chamber were removed by a cotton swab. The migrated or invaded cells were fixed and stained with methanol and 0.1% crystal violet respectively, the membranes were separated and photographed under a microscope.

Transfection

The miR-99a mimic and pcDNA3.1-IGF1R were employed to overexpress miR-99a or IGF1R, while the miR-99a inhibitor was utilized to knockdown miR-99a. The miR-99a mimic and inhibitor, as well as pcDNA3.1-IGF1R, were purchased from GenePharma (Shanghai, China). HGC-27 cells were seeded into a 6-well plate with 80% density, and the transfection was performed using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA). In addition, the cells were harvested after transfected 48 h.

Plasmid Construction and Luciferase Reporter Assay

TargetScan (http://www.targetscan.org/vert_71/) was applied to predict target genes of miR-99a, and IGF1R was determined to be a direct target gene of miR-99a. At first, the miR-99a mimic or the mimic NC was inserted into the pmirGlo vector. Then, the 3'-untranslated regions (3'-UTR) sequence of IGF1R mRNA containing the binding site was cloned and inserted into the pmirGlo Luciferase vector (WT). Meanwhile, the sequences in 3'-UTR of IGF1R mRNA were mutated utilized the QuickChange Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) and inserted into the pmirGlo vector (MUT).

Cells with confluence were 80% in 6-well plates, transfected the miR-99a mimic or NC and WT or MUT using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The Luciferase activity was measured after transfected 48 h, using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Statistical Analysis

All data are presented as the mean ± standard deviation (SD). The analysis was performed using Statistical Product and Service Solutions (SPSS) 16.0 (SPSS Inc., Chicago, IL, USA). We applied unpaired *t*-test to perform the statistical comparisons between the two groups, while the comparison between three groups was carried out using One-way analysis of variance (ANOVA) test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ was considered statistically significant.

Results

The Correlation of the Expression of MiR-99a and IGF1R in Gastric Cancer Tissues

The miR-99a level in 55 pairs of gastric cancer tissues and corresponding paracancerous tissues were measured using RT-PCR, and we found it was lowly expressed in cancer tissues than that in the corresponding paracancerous tissues ($p<0.001$; Figure 1A). The expression of IGF1R was also determined and we found that the mRNA level was higher in gastric cancer tissues than corresponding paracancerous tissues ($p<0.001$; Figure 1B). Thus, the expression of miR-99a and IGF1R showed that the expression of miR-99a had a negative correlation with IGF1R expression in gastric cancer tissues ($p<0.001$, $r=-0.5349$; Figure 1C). In addition, miR-99a was verified to be downregulated in gastric cancer cell lines HGC-27 ($p=0.0014$) and MGC-803 ($p=0.0006$) compared to the normal gastric epithelium cell line GES-1 (Figure 1D).

MiR-99a Inhibits the Migration and Invasion of Gastric Cancer Cells

To determine the role of miR-99a in gastric cancer, we evaluated the numbers of migration and invasion, which represented the migratory and invasive abilities. At first, we transfected the miR-99a mimic or NC mimic into HGC-27 cells, and showed the mRNA level of miR-99a was increased ($p=0.0009$), while the mRNA level of miR-99a was decreased ($p=0.0102$) when HGC-27 cells were transfected the miR-99a inhibitor (Figure 2A). Next, we validated that the migratory ($p=0.0026$) and invasive ($p=0.0020$) numbers were reduced when transfected the miR-99a mimic in HGC-27 cells. On the contrary, the miR-99a inhibitor increased the migratory ($p=0.0022$) and invasive ($p=0.0010$) numbers in HGC-27 cells (Figure 2B), which indicate that miR-99a inhibited the migration and invasion of gastric cancer cells.

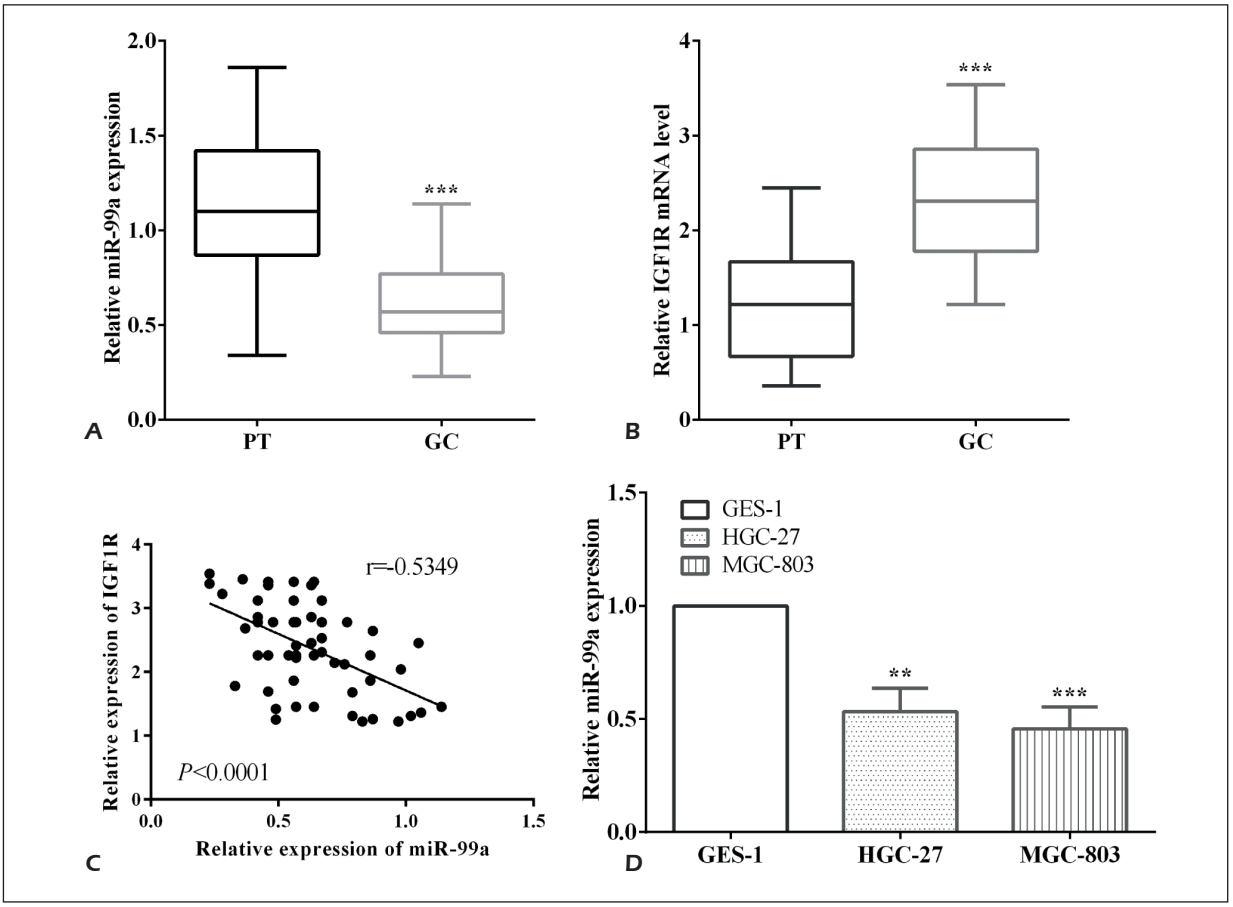

Figure 1. The correlation between the expression of miR-99a and IGF1R in gastric cancer tissues. ***A,*** The relative mRNA level of miR-99a in 55 pairs of gastric cancer and corresponding paracancerous tissues were measured using RT-PCR. ***B,*** The mRNA level of IGF1R was determined in gastric cancer tissues and corresponding paracancerous tissues by RT-PCR. ***C,*** The correlation between the expression of miR-99a and IGF1R in gastric cancer tissues. ***D,*** The relative expression of miR-99a in gastric cancer cell lines HGC-27 and MGC-803 and normal gastric epithelium cell line GES-1.

MiR-99a Directly Targeted to IGF1R and Inhibited its Expression

TargetScan was employed to predict the potential target gene of miR-99a, and IGF1R was found to be a target gene of miR-99a. As predicted, the binding site of miR-99a was located at 5602–5609 on IGF1R 3'-UTR, which was 5'-…UUAAGUCCAGUAGAUUACGGGUA…-3' (WT) and inserted into the pmirGlo vector. Subsequently, the binding sequences were mutated from 5'-UACGGGU-3' to 5'-AUGCCCA-3' and then inserted into the vector, which was designated as MUT in HGC-27 cells (Figure 3A). Then, the Luciferase reporter assay was performed to verify that IGF1R was a target of miR-99a by directly binding to its 3'-UTR of mRNA. As expected, the Luciferase activity was reduced (p=0.0030) caused by WT, whereas MUT showed no change (p=0.7598) in HGC-27 cells (Figure 3B). To further determine that miR-99a mediated the expression of IGF1R, the expression of IGF1R was evaluated using RT-qPCR. With treatment by the miR-99a mimic, the mRNA level of IGF1R was repressed (p=0.0067), while increased (p=0.0046) after being transfected with the miR-99a inhibitor (Figure 3C).

IGF1R could Reverse Partial Function of MiR-99a on the Migration and Invasion

To further explore the mechanism of miR-99a on the migration and invasion by directly binding to the 3'-UTR of IGF1R mRNA, the miR-99a mimic and IGF1R were co-expressed into HGC-27 cells. The mRNA and protein levels of IGF1R were reduced (p=0.0009) when transfected with the miR-99a mimic and it was reversed when re-transfected with pcDNA3.1-IGF1R overexpressed plasmid (p=0.0272), as demonstrated in Figure 4A. The number of migration was enhanced (p=0.0166) when co-transfected with the miR-99a mimic

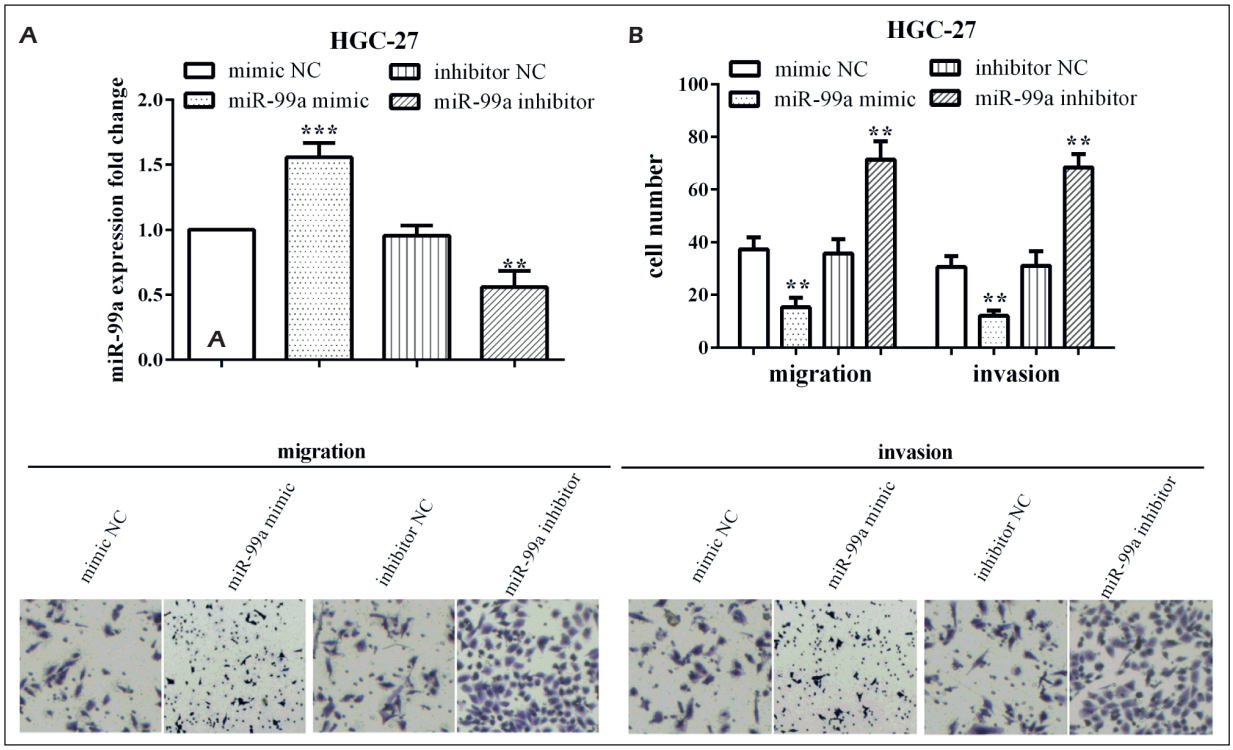

Figure 2. MiR-99a inhibits the migration and invasion of gastric cancer cells. ***A,*** miR-99a mRNA level was measured after being transfected with the miR-99a mimic or the miR-99a inhibitor in HGC-27 cells. ***B,*** The migratory and invasive abilities were validated when transfected the miR-99a mimic or the miR-99a inhibitor in HGC-27 cells (magnification: 40×).

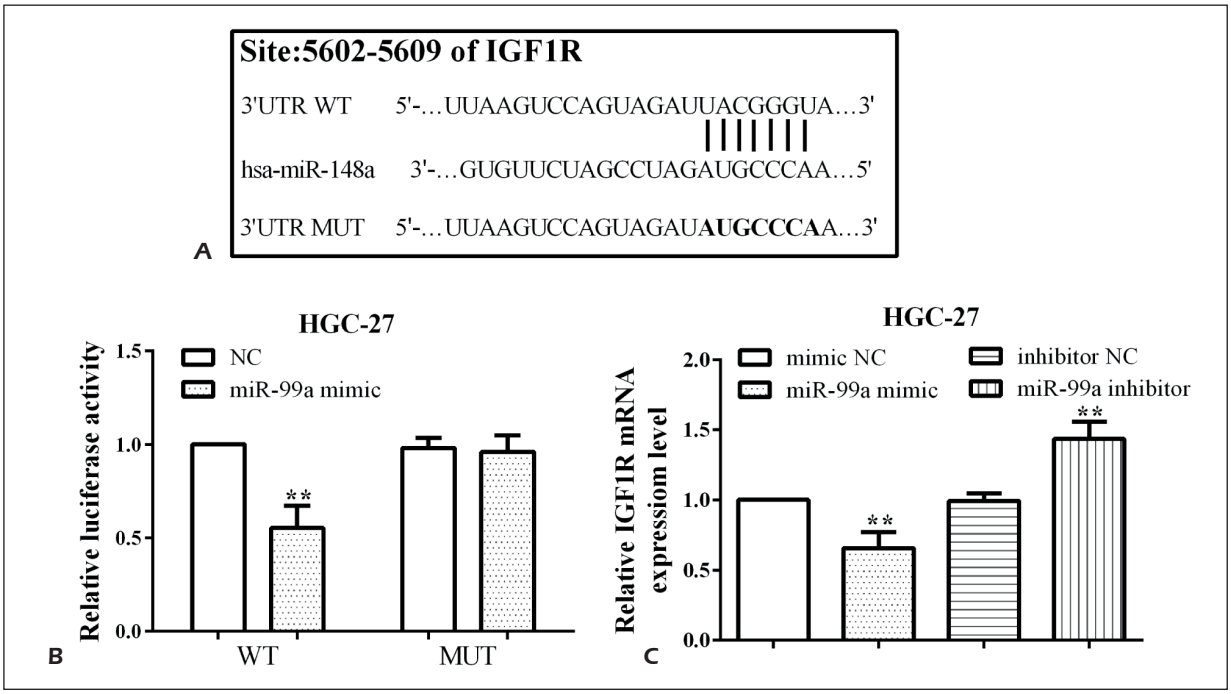

Figure 3. MiR-99a directly targeted to the 3'-UTR of IGF1R to inhibit its expression. ***A,*** The complementary sequences of miR-99a and IGF1R were consulted by TargetScan, and the mutant sequences were revealed. ***B,*** The comparison of the Luciferase activity was calculated when co-transfected with the miR-99a mimic and WT or MUT. ***C,*** The changes in IGF1R mRNA with treatment by the miR-99a mimic or miR-99a inhibitor.

and pcDNA3.1-IGF1R, whose partial function was reversed by re-transfected pcDNA3.1-IGF1R (p=0.0011). The results of the invasion were similar to migration, it was also increased (p=0.0256) when co-transfected with the miR-99a mimic and pcDNA3.1-IGF1R compared to only transfected the miR-99a mimic (Figure 4B).

Discussion

Gastric cancer is a common kind of gastrointestinal malignancies. Usually diagnosed at the advanced stage, it has a poor prognosis and is prone to relapse[1,2]. In addition, there were almost no significant symptoms in the early stage, and the current early diagnosis rate of gastric cancer is still low[5]. Thus, it is necessary to identify new molecular biomarkers for early diagnosis of gastric cancer. MiRNAs are small non-coding RNAs, whose function is to repress gene expression through complementary pairing with target mRNA[6-8]. Increasing evidence suggested that miR-99a could act as a tumor suppressor and inhibited tumorigenesis in prostate cancer and anaplastic thyroid cancer[26,27]. In nasopharyngeal carcinoma, miR-99a inhibited the invasion and metastasis by directly binding to HOXA1[18]. Moreover, miR-99a inhibited the proliferation, migration and invasion of esophageal squamous cells[21]. Similar findings were observed by Chen et al[28] that miR-99a suppressed the proliferation, migration and invasion by regulating IGF1R in breast cancer cells. Our findings were consistent with all the findings: miR-99a was downregulated in gastric cancer tissue samples and cells. In addition, we first proposed that miR-99a suppresses the migration and invasion in gastric cancer.

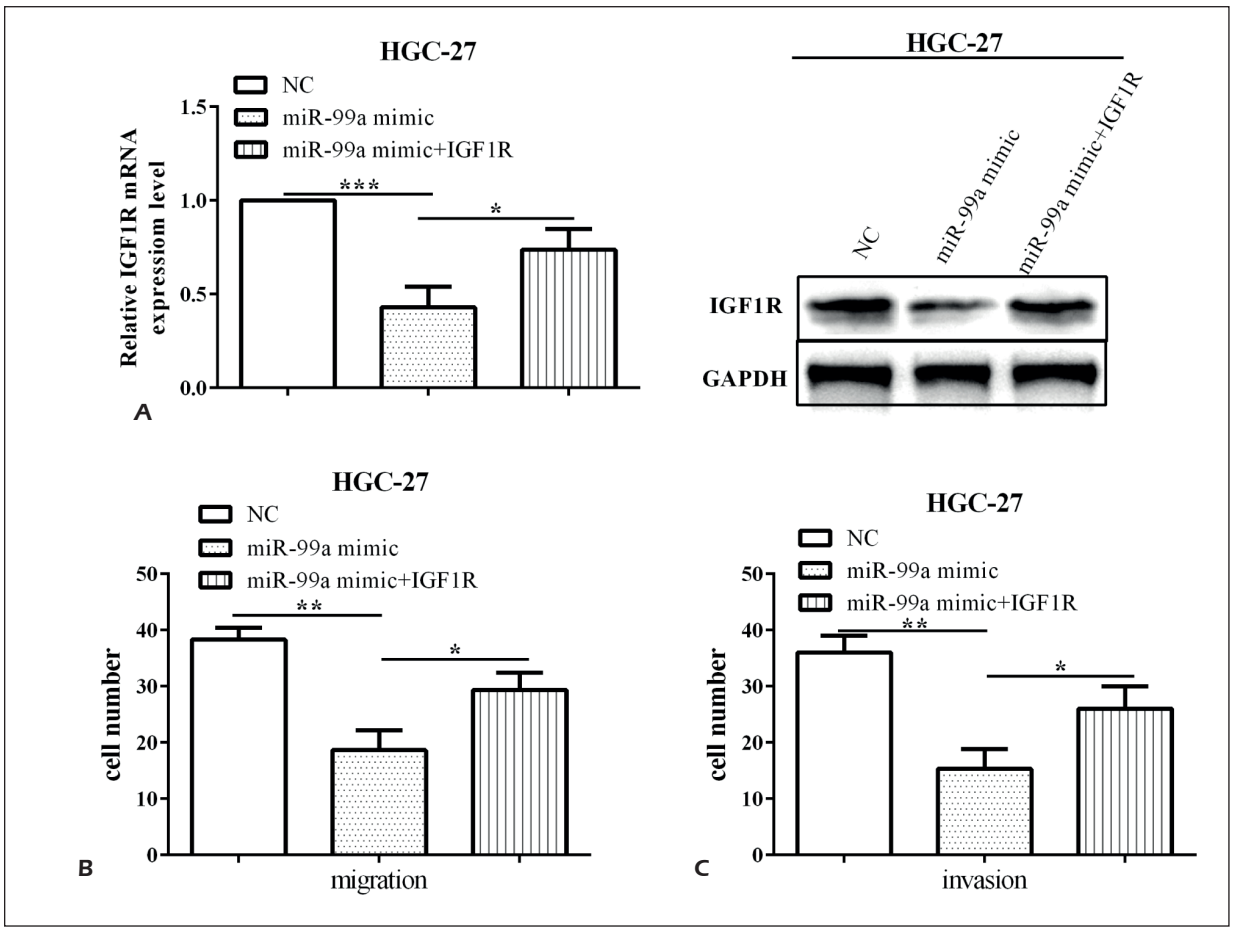

Figure 4. IGF1R could reverse partial functions of miR-99a on the migration and invasion. ***A,*** The mRNA and protein levels of IGF1R were verified when co-transfected with the miR-99a mimic and IGF1R compared with only transfected with the miR-99a mimic. ***B,*** The migratory number was enhanced when co-transfected with the miR-99a mimic and IGF1R, whose partial function was reversed by re-transfected IGF1R. ***C,*** The invasive ability was determined when co-transfected with the miR-99a mimic and IGF1R versus only transfected the miR-99a mimic.

IGF1R is a tyrosine kinase transmembrane receptor that could be activated by its ligands IGF1 or IGF2[20]. Aleksic T et al[24] have discovered that IGF1R functioned as a recurrence post radiotherapy independent predictor. Furthermore, IGF1R impacted the survival and acquired cisplatin resistance in testicular germ cell tumor cells[25]. Considering these results, we strongly believe that IGF1R played important roles in gastric cancer. We determined IGF1R was upregulated in gastric cancer tissues and the expression of IGF1R had a negative correlation with the expression of miR-99a in gastric cancer tissues. Moreover, miR-99a suppresses the proliferation, migration and invasion by inhibiting the IGF1R in esophageal squamous cell carcinoma and breast cancer[21,28]. In this study, we first verified that IGF1R was a direct target of miR-99a and that it could reverse the partial function of miR-99a in gastric cancer cells. We first proposed that miR-99a mediated the migration and invasion by directly binding to the 3'-UTR of IGF1R mRNA in gastric cancer cells.

Conclusions

MiR-99a was low expressed and IGF1R was overexpressed in cancer tissues than that in the corresponding paracancerous tissues. MiR-99a suppressed the migratory and invasive abilities by mediating IGF1R by directly targeting the 3'-UTR of its mRNA in gastric cancer. In addition, the expression of miR-99a has an inverse correlation with the IGF1R expression in gastric cancer tissue samples. IGF1R was a direct target gene of miR-99a, and the expression of IGF1R was mediated by exogenous altering the expression of miR-99a. IGF1R could reverse partial function of miR-99a on the migration and invasion. This novelty of miR-99a may offer a promising target for the treatment of gastric cancer.

Conflict of Interests

The authors declare that they have no conflict of interest.

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