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# miR-494 inhibits invasion and proliferation of gastric cancer by targeting IGF-1R

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**Abstract. – OBJECTIVE: The aim of this study was to investigate the target gene of miR-494 and its roles in tumor growth of gastric cancer (GC).**

**PATIENTS AND METHODS: Expression of miR-494 was evaluated by quantitative real-time polymerase chain reaction (qRT-PCR) in gastric cancer tissues and cell lines. Then, luciferase reporter assay was used to elucidate whether insulin-like growth factor 1 receptor (IGF1R) is a target gene of miR-494. Finally, the roles and mechanism of miR-494 in the regulation of tumor invasion were further investigated.**

**RESULTS: Relative miR-494 level was found to be significantly lower in patients with GC than healthy controls ($p < 0.01$). Over-expression of miR-494 could inhibit gastric cancer cell proliferation, migration, and invasion *in vitro*. Furthermore, we demonstrated that miR-494 binds to the 3'-untranslated region (UTR) of IGF1R and inhibits the expression of the IGF1R protein.**

**CONCLUSIONS: Our data showed that miR-494 acted as a tumor suppressor in GC.**

*Key Words:*
miR-494, insulin-like growth factor 1 receptor, gastric cancer.

## Introduction

Gastric cancer (GC) is the most frequent cause of cancer-related death, with an incidence of approximately 934,000 cases per year in East Asia[1]. Despite recent advances in diagnostic method, surgical technique and new chemotherapy regimens, the long-term survival rate for GC is still quite low[2,3]. So many researchers devote themselves to find pathogenesis and more effective tumor therapy.

MicroRNAs (miRNAs) are small non-coding RNAs that target at a majority of protein-coding transcripts and act as guiding molecules in RNA silencing[4,5]. Notably, it is reported that miRNA families play an essential role in a couple of oncogeneses, including survival, growth, apoptosis, migration, and metastasis[6]. Accumulating reports have revealed that dysregulation of miRNA expression occurs in various types of human cancers. For example, Li et al[7] showed that miR-23b suppresses ovarian cancer progression by targeting runt-related transcription factor-2. Zhong et al[8] showed that microRNA-30b/c inhibits non-small cell lung cancer cell proliferation by targeting Rab18. Cheng et al[9] found that microRNA-218 inhibits bladder cancer cell proliferation, migration, and invasion by targeting BMI-1. Ma et al[10] showed that mir-138 suppresses cell proliferation by targeting bag-1 in gallbladder carcinoma. These findings suggested that miRNAs may be involved in cancer progression.

In the present study, we found that miR-494 is downregulated in GC tissues and cell lines. Over-expression of miR-494 inhibits GC cell growth, migration, invasion. Furthermore, IGF1R were the direct targets of miR-494. These results suggested miR-494 might be a valuable target for GC treatment

## Patients and Methods

### Patients and Tissue Samples

In this work, 25 pairs of GC tissues and adjacent non-tumor tissues were obtained from patients with GC resection at Department of Digestive Diseases, Shandong Provincial Hospital Affiliated to Shandong University. None of the patients had undergone preoperative treatment, such as radiation therapy or chemotherapy. The present study was approved by the Ethics Com-



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mittee of Shandong Provincial Hospital Affiliated to Shandong University. Written informed consent was obtained from all the patients.

### Cell Culture and Cell Transfection

BGC823 and SGC7901 gastric cancer cell lines were from Shanghai Cancer Institute (Shanghai, China). Normal human gastric epithelial cells GES-1 were obtained from the Cell Bank of Xiangya Central Laboratory, Central South University (Changsha, Hunan, China). Those above cells were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) medium (Invitrogen, Carlsbad, CA, USA) supplemented with 15% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA) in a humidified chamber with 5% $CO_2$ at 37 °C.

miR-494 mimics was purchased from Shanghai GenePharma Co., Ltd. (Shanghai, People's Republic of China), and siRNA targeting IGF1R mRNA was purchased from Sigma-Aldrich Co. (St Louis, MO, USA). The cells were transfected with NC and mimics using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Transfection efficiency was monitored by qRT-PCR.

### Cell Proliferation Assay

For MTT assay, cells were seeded into a 96-well plate. Next, 20 l of MTT was added to each well and incubated for 4 h at 37°C in 5% of $CO_2$. Dimethylsulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) was added to dissolve the formazan crystals and the absorbance was measured at 490 nm. The data were presented as optical density (OD) value. The proliferative rate of H460 was then evaluated using a Cell-Light™ EdU Cell Proliferation Detection kit (RiboBio, Pudong, Shanghai, China) following the manufacturer's instructions.

### Migration and Invasion Assays

The migration and invasion assays were performed using transwell chambers. For the migration assays, $2 \times 10^4$ transfected cells suspended in serum-free DMEM were added into the upper chamber of 8- m transwells (BD Biosciences, Franklin Lakes, NJ, USA). For the invasion assays, the 24-well transwell chambers (Promega, Madison, WI, USA) were used for the cell invasion assay in accordance with the manufacturer's protocol. The cells were incubated at 37°C in 5% of $CO_2$ for 12 h and 14 h for the migration and invasion assays, respectively. Then, the membrane inserts were removed and non-invading cells were removed from the upper surface of the membrane. Cells that had moved to the bottom of the chamber were fixed with 100% methanol for 30 min and stained with 0.1% crystal violet for 30 min. Assays were independently conducted three times.

### Quantitative Real-time PCR

Total RNA was isolated using TrIzol (Invitrogen, Carlsbad, CA, USA). Total RNA was reverse-transcribed using a RevertAid™ First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's instructions. TaqMan miRNA assays (Applied Biosystems, Foster City, CA, USA) were used to detect the expression level of mature miR-494 according to the provided protocol, and the small nuclear RNA U6 was selected as an internal control. The results were represented as fold changes, which were calculated by the $2^{-\Delta CT}$ method.

### Western Blot Analysis

Whole cell extracts were prepared with a cell lysis reagent (Sigma-Aldrich, St. Louis, MO, USA) according to the manual. Treated cells were washed twice with PBS and proteins were extracted with SDS lysis buffer (Beyotime, Haiding, Beijing, China), and separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. Subsequently, the membrane was incubated with primary antibodies against AEG-1 (1:1000) and β-actin (1:2000; all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 4 h at 37°C. The next day, the membranes were washed with PBS and then incubated with secondary antibody (Santa Cruz, Pudong, Shanghai, China) at room temperature for 2 h. Finally, protein bands were detected by enhanced chemiluminescence (Santa Cruz, Pudong, Shanghai, China) according to the manufacturer's protocol. Three independent experiments were performed.

### Luciferase Reporter Assay

WT or MUT Rab11a 3'-UTR reporter constructs and the control pRL-TK vector coding for Renilla luciferase were co-transfected with miR-494 mimics into BGC823 and SGC7901 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Cells were transfected with the pMIR-REPORT vectors containing the 3 UTR variants and miR-203 mimics for 24 h. Lu-

ciferase values were determined using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

## Results

### miR-494 is Down-Regulated in GC Tissues and Cells

To analyze the expression of miR-494 in GC, q-PCR using Taqman probes was conducted to measure the levels of miR-494. As shown in Figure 1A, the expression levels of miR-494 were distinctly reduced in GC tissues compared to adjacent non-tumor tissues ($p < 0.01$). Furthermore, miR-494 expression was markedly downregulated in all GC cell lines compared to that in Normal human gastric epithelial cells GES-1 ($p < 0.01$), as shown in Figure 1B.

### Effects of miR-494 on Cell Proliferation, Invasion and Migration in GC Cells

To investigate the potential effect of miR-494 on the progression of GC, we transfected GC cell line BGC823 cells with either miR-494 mimics (miR-494) or negative control miRNA mimics (miR-NC). miR-494 expression in BGC823 cells was determined using qRT-PCR (Figure 2A). MTT assay showed that the growth rate of BGC823 cells with miR-494 overexpression was significantly lower than the normal control (Figure 2B). The role of miR-494 on cell migration and invasion, two key determinants of malignant progression and metastasis, was assessed in human GC cells. Our results showed that miR-494 overexpression significantly reduced the migration and invasion of BGC823 cells ($p < 0.05$) (Figure 2C and Figure 2D). Taken together, these results suggested that miR-494 might participate in BGC823 proliferation and metastasis.

### Flot1 was a Target Gene of miR-494 in GC Cells

To identify the microRNA that targets IGF1R, we queried several microRNA target databases, such as TargetScan, PicTar and miRanda, and predicted that miR-494 could bind directly to the 30-UTR of IGF1R (Figure 3A). Then, we performed Western blot and luciferase reporter assays to detect the expression of IGF1R responses to the changes of miR-494 expression in BGC823 cell lines. The luciferase activity of IGF1R -3'UTR-wt construct was significantly decreased upon the over-expression of miR-494 in BGC823 cells, whereas its mutant counterpart was not (Figure 3B). Western blot analyses showed that over-expression of miR-494 significantly decreased the expression of IGF1R in BGC823 cells (Figure 3C). Taken together, these data revealed that IGF1R is a direct target of miR-494 at least in GC.

### Effect of IGF1R on GC Cells Proliferation, Migration and Invasion

To further determine if IGF1R affected BGC823 cell proliferation, migration and inva-

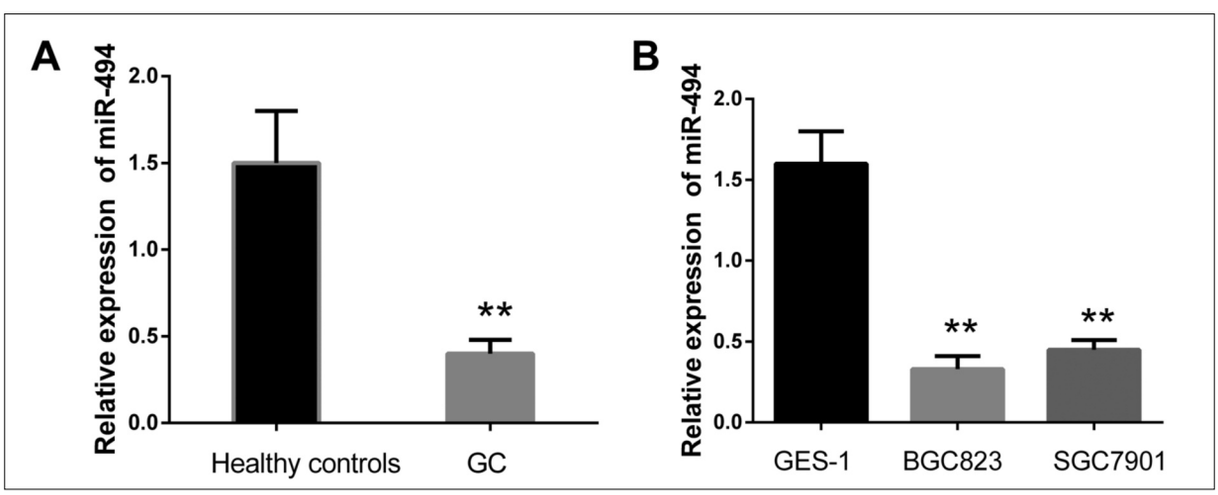


**Figure 1.** Downregulation of miR 494 in GC tissues and cell lines. ***A,*** qPCR was performed to determine the miR 494 expression levels in 25 paired human GC and adjacent non-tumor tissues. *$p < 0.05$; **$p < 0.01$. ***B,*** The Normal human gastric epithelial cells GES-1and gastric cancer cell lines BGC823, SGC7901, were analyzed by qPCR to measure miR 494 expression levels. *$p < 0.05$; **$p < 0.01$, compared to normal.

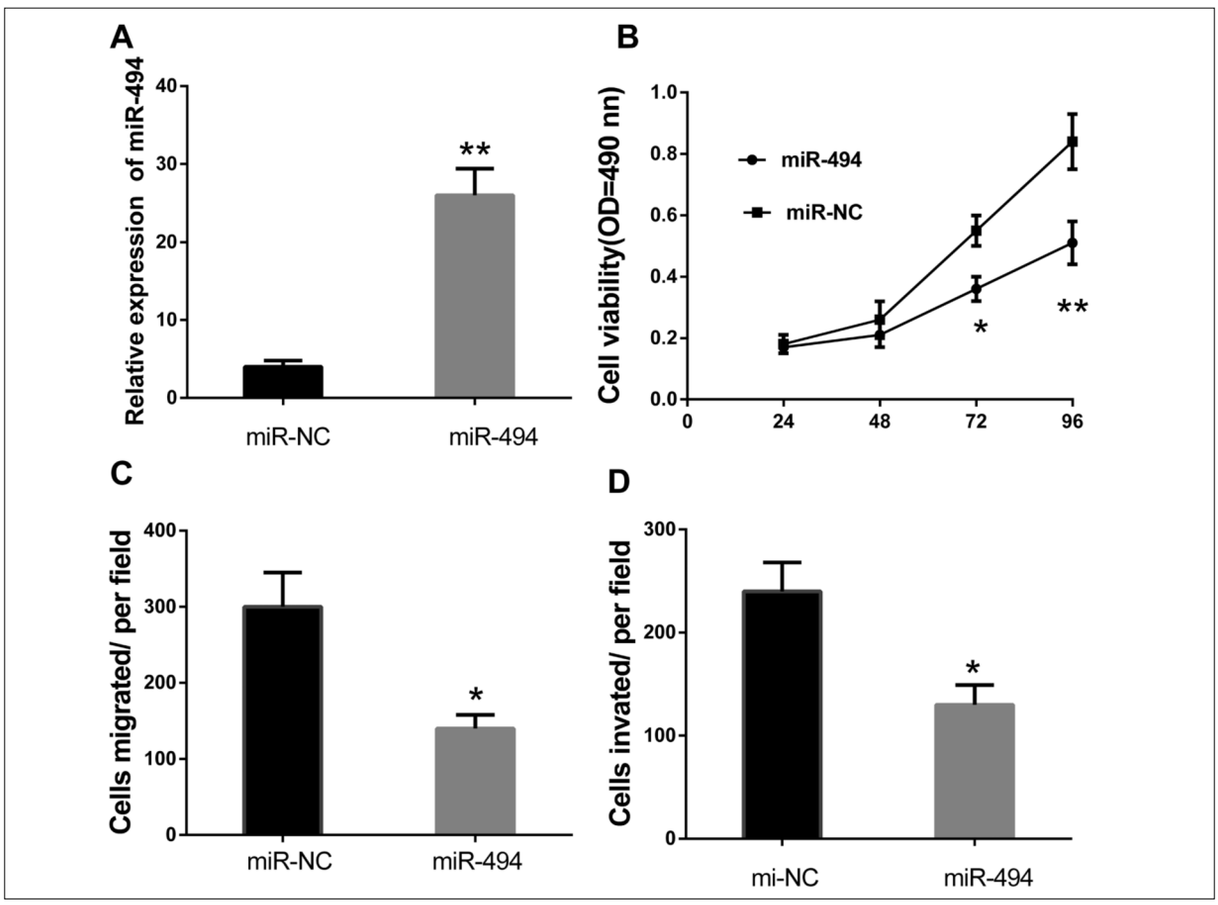


**Figure 2.** miR-494 inhibited GC cell proliferation, migration and invasion. ***A,*** Relative 494 expression in BGC823 cells when transfected with miR-494 mimics (miR-494) or its negative control (miR-NC) detected by qRT-PCR. ***B,*** Proliferation of transfected BGC823 cells evaluated by MTT assay. ***C,-D,*** Migration and invasion abilities were determined in BGC823 cells transfected with miR-494 mimics or negative control (NC). *$p < 0.05$, **$p < 0.01$ compared with NC.

sion, the efficiency of IGF1R siRNA was confirmed by qRT-PCR (Figure 4A). Our results showed down-regulation of IGF1R efficiently inhibited cell proliferation of GC ($p < 0.05$ Figure 4B), the cell migration ($p < 0.05$; Figure 4C) and the cell invasion ($p < 0.05$; Figure 4D). These data confirmed that miR-494 promoted GC cells proliferation by repressing endogenous IGF1R expression and that IGF1R played important role in miR-494-mediated GC cell proliferation.

## Discussion

Accumulating evidence reveals the crucial role of miRNAs in GC progression. For example, Xiong et al[11] found that Down-Regulated miRNA-214 induced a cell cycle G1 Arrest in gastric cancer cells by up-regulating the PTEN protein. Wang et al[12] showed that MiR-30a increased cisplatin sensitivity of gastric cancer cells through suppressing epithelial-to-mesenchymal transition (EMT). Shen et al[13] found that Expression level of microRNA-195 in the serum of patients with gastric cancer was related to the clinicopathological staging of cancer. These findings demonstrated that miRNAs might play multiple roles inGC progression.

MiR-494 has been proven as tumor-suppressive miRNA in several tumors. For example, Liu et al[14] found that miR-494 promotes cell proliferation, migration and invasion, and increased sorafenib resistance in hepatocellular carcinoma by targeting PTEN. Song et al[15] found that miR-494 suppressed the progression of breast cancer in vitro by targeting CXCR4 through the Wnt/β-catenin signaling pathway. Notably, He et al[16] found that miR-494 acted as an anti-oncogene in gastric carcinoma. However, the detailed role of miR-494 in GC carcinogenesis is still unclear.

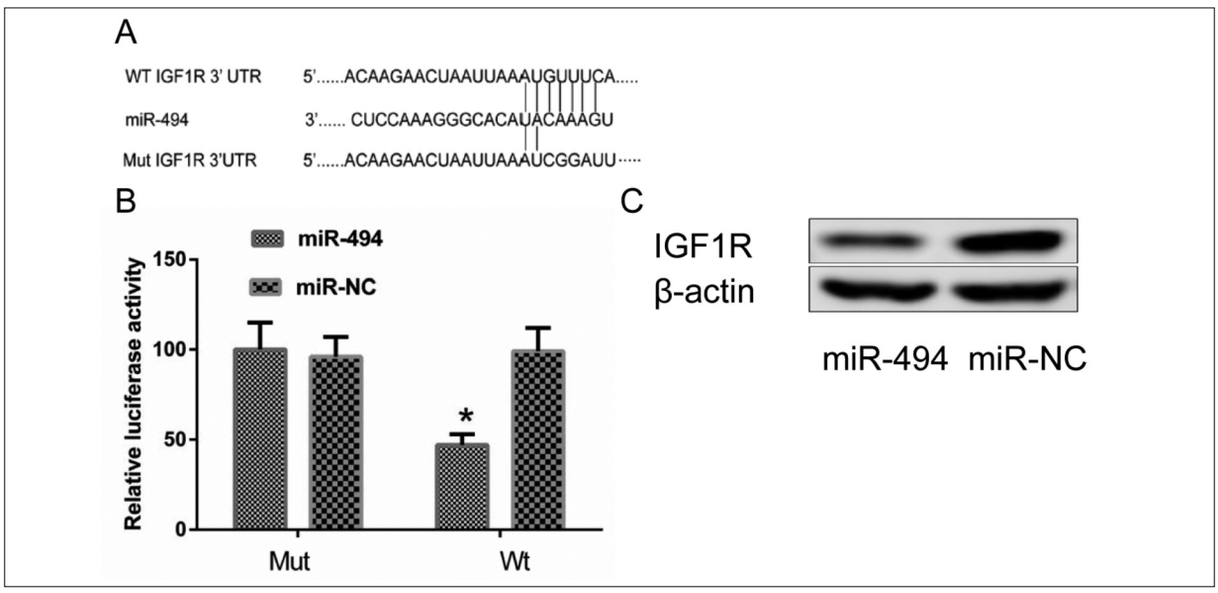


**Figure 3.** miR-494 negatively regulates IGF1R by binding to the IGF1R 3′UTR. ***A,*** Schematic representation of the miR-494 sequences, putative miR-494 targeting site in the 3′UTR of IGF1R, and the generated mutant IGF1R 3′UTR. ***B,*** Luciferase reporter assay showed the inhibitory effect of miR-494 on IGF1R 3′UTR luciferase activity in BGC823 cells. ***C,*** Expression of IGF1R was detected by Western blot in A549 cells transfected with miR-494 mimics or miR-NC. β-actin was used as a control. Data are presented as means ± SD from three independent experiments. *$p < 0.05$.

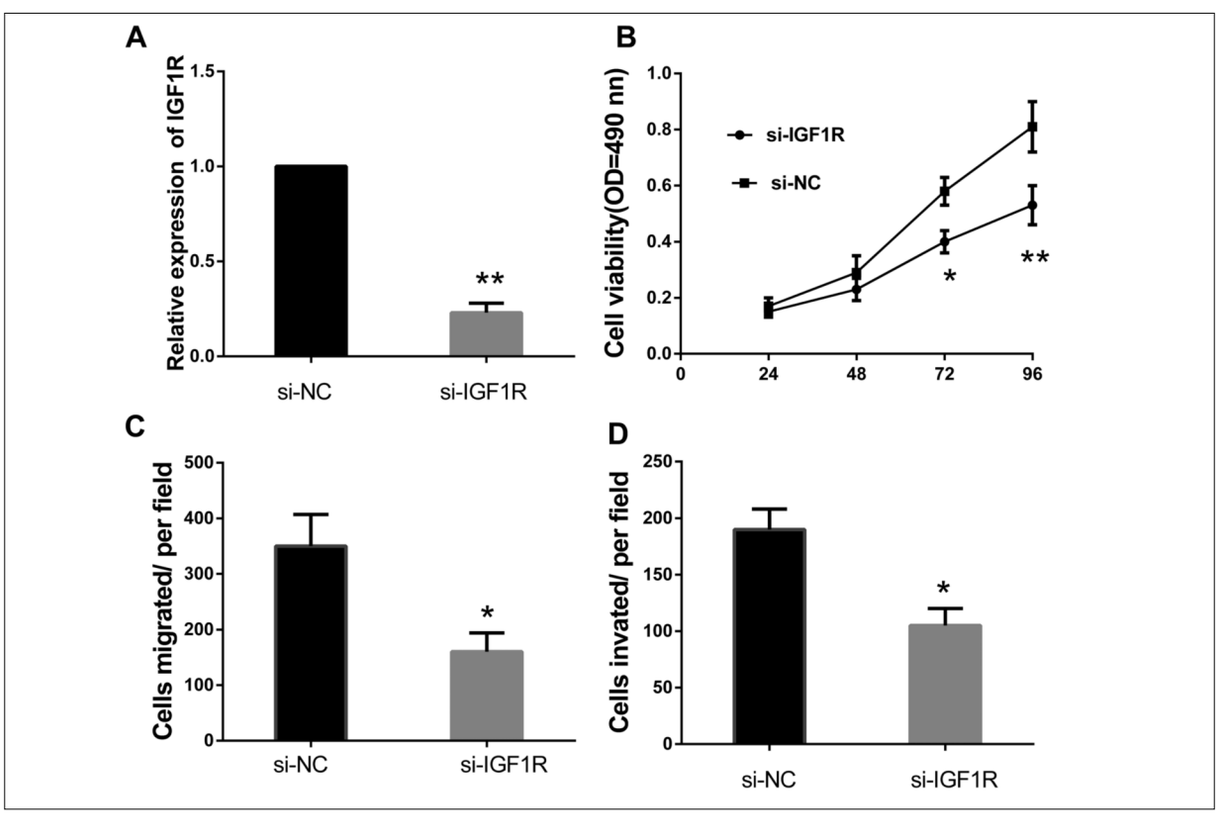


**Figure 4.** Decreased expression of IGF1R showed similar effect with miR-494 over-expression. ***A,*** Knockdown of IGF1R by siRNA (si-IGF1R) was confirmed by qRT-PCR in BGC823 cells. ***B,*** The vitality of BGC823 cells transfected with si-IGF1R or si-NC was detected using the MTT assay. ***C,-D,***) Transwell assays were used to detect migration ***(C)*** and invasion ***(D)*** of BGC823 cells transfected with si-IGF1R or si-NC. Data are presented as means ± SD from three independent experiments. *$p < 0.05$.

IGF-1R is a member of the insulin receptor (IR) family, which is well known for its role in the resistance of NSCLC cells to EGFR-TKIs[17]. IGF-1R functions as a key oncogene in the development and maintenance of cancer[18]. IGF1R has been found to be regulated by a variety of miRNAs in many cancers. Yuan Y et al[19] reported that miR-140 inhibited tumor growth and metastasis of NSCLC by targeting IGF1R. Li et al[20] found that miR-494 suppressed tumor growth of epithelial ovarian carcinoma by targeting IGF1R. Wang et al[21] found that miR-195 inhibited the growth and metastasis of NSCLC cells by targeting IGF1R. Those results informed that IGF-1R might play an important role in suppressing tumor cells. However, how IGF-1R was regulated in GC has not been studied.

In the present work, our results showed that miR-494 expression was decreased in GC tissue and cell lines. Furthermore, up-regulated expression of miR-494 could inhibit cell proliferation, migration and invasion. Luciferase reporter assay confirmed IGF1R as a new target of miR-494. Our data suggested that the miR-494 plays a central role in GC development and may serve as a novel target for the treatment of GC.

## Conclusions

The present results elucidate a potential mechanism underlying the tumor-suppressor role of miR-494, and indicate that miR-494 could be a useful marker and potential therapeutic target in GC.

### Acknowledgements

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### Conflict of Interest

The Authors declare that there are no conflicts of interest.

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