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# MiRNA-146b-5p inhibits the malignant progression of gastric cancer by targeting TRAF6

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**Abstract. – OBJECTIVE: MicroRNAs (miRNAs) are 22 nucleotides long that are extensively expressed in eukaryotes. They are vital regulators in pathological processes. This study aims to illustrate the role of miRNA-146b-5p in the development of gastric cancer (GC).**

**PATIENTS AND METHODS: MiRNA-146b-5p levels in 62 GC species and matched paracancerous ones were detected. Influences of miRNA-146b-5p level on clinical parameters of GC patients were assessed. Phenotype changes of AGS and SGC-7901 cells overexpressing miRNA-146b-5p were evaluated by Cell Counting Kit-8 (CCK-8) and transwell assay, respectively. Luciferase assay and rescue experiments conducted to uncover the mechanism of 146b-5p in regulating the development of**

**RESULTS: MiRNA-146b-5p was downregu in GC species than paracancerous ones. Lo level of miRNA-146b-5p was ob rved in patients combined lymphatic m and dis tant metastasis than those out stases. *In vitro* overexpression of A-146b attenuated proliferative and mi y pot cells. TRAF6 was the rg m A-1 which was respons for t elopment of GC regulated by A-146b-5p**

**CONCLUSION NA-146b-5p is negatively correl to phatic meta asis and distant me sis ra GC. It suppresses the malig development GC by targeting TRAF6.**

*Key ds:*
M 14 p, TRAF Gastric cancer (GC), Malignant ment.

## Introduction

Gastric cancer (GC) is a popular digestive system tumor. Its incidence has been sharply reduced in loped countries. In the United States, the incidence of GC reduced about 20% in the past two decades. However, Asia is the area with high morbidity and mortality of GC. GC remains to be the second leading fatal dis n coun [1-5]. It is estimated that e are 00 mi newly onsets of GC. more than illi eople die of this tum China, abou GC patients are initial agne s advanced GC. Seriously, more than 50% GC p develop postoperative recu with a media vival of shorter than 10 nths[5,6]. Low detective ate of early-stage GC, n nsitive chem herapy and radiotherapy, as as deficienc effective biomarkers, all results in the poor gnosis of GC[7,8]. It is urgent to clar nesis and etiology of GC, thus develop or biomarkers of GC[9-11].

MicroRNAs (miRNAs) are non-coding RNAs ed in eukaryotes[12,13]. They exert post-tran al regulation on target gene silencing through degrading or inhibiting translation of mRNAs after recognizing their 3'UTRs[14,15]. MiRNAs are featured by high conservation and tissue specificity, which are involved in cell behavior regulations[16,17]. By targeting oncogenes or tumor-suppressor genes, miRNAs are able to influence tumor development[18]. Previous studies[19,20] have shown the potentials of miRNAs as diagnostic and therapeutic targets of GC.

In this paper, a total of 62 GC species and paired paracancerous ones were collected. The role of miRNA-146b-5p/TRAF6 axis in mediating the malignant progression of GC was explored.

## Patients and Methods

### Patients and Samples

62 paired GC species and paracancerous ones (5 cm away from tumor edge) were collected and stored at -80°C. None of enrolled subjects were preoperatively treated with anti-tumor therapy. Their clinical data were recorded. Patients and their families in this study have been fully informed. This study was approved by Ethics Committee of the Second Hospital of Shandong University.

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### Cell Culture

GC cell lines (AGS, BGC-823, SGC-7901, MKN28, and MKN45) and epithelial cells of gastric mucosa (GES-1) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 Ul/mL penicillin, and 100 µg/mL streptomycin in a 5% $CO_2$ incubator at 37°C. Cell passage was conducted in 1×typsin+EDTA (ethylenediaminetetraacetic acid) at 80-90% confluence.

### Transfection

Cells were grown at 50-70% confluence in 6-well plates and transfected with plasmids constructed by GenePharma (Shanghai, China), using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Transfected cells for 48 h were harvested for functional experiments.

### Cell Proliferation Assay

Cells were inoculated in a 96-well plate with $2\times10^3$ cells per well. At the appointed time points, absorbance value at 490 nm of each sample was recorded using the Cell Counting Kit-8 (CC-8) kit (Dojindo Molecular Technologies, Kumamoto, Japan) for plotting the viability curves.

### Transwell Assay

200 µL of suspension (1.0×10⁵) was applied in the upper side of the Transwell chamber (Millipore, Billerica, MA, USA) inserted in a 24-well plate with 700 µL medium containing 10% FBS in the bottom. After incubation, the cells in the bottom were fixed with methanol for 15 min, dyed with crystal violet for 30 min, and counted using microscope. Migratory cell number was counted in 5 randomly selected fields per sample (magnification 40×).

### Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Extracted RNAs by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) were purified by DNase treatment, subsequently transcribed into complementary deoxyribose nucleic acids (cDNAs) using PrimeScript RT Reagent (TaKaRa, Otsu, Shiga, Japan). Obtained cDNAs underwent qRT-PCR using SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). β-actin and U6 were used as the internal references. Each sample was performed in triplicate, and the relative level was calculated by $2^{-\Delta\Delta Ct}$. miRNA-146b-5p: forward: 5'-TGACCCATCCTGGGCCTCAA-3', reverse: 5'-CCAGTGGGCAAGATGTGGGCC-3'; U6: forward: 5'-CGCTTCGGCAGCACATATAC-3', reverse: 5'-TTCACGAATTTGCGTGTCAT-3'; TRAF6: forward: 5'-TGCTTGATGGCATTACGACA-3', reverse: 5'-CATTTGGACATTTCA GAG-3'; β-actin: forward: 5'-CCTG CCCAG CACAAT-3', reverse: 5'-TGCC GGTGTC-CCTTTG-3'.

### Western Blot

Cells were lysed for extracting cellular protein and electrophoresed. Protein samples were loaded on polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Subsequently, non-specific antigens were blocked with 5% skim milk for 2 h. Membranes were reacted with primary and secondary antibodies for indicated time. Band exposure and analyses were finally conducted.

### Luciferase Assay

Cells inoculated in a 24-well plate were co-transfected with pmirGLO-TRAF6-WT/pmirGLO-TRAF6-MUT/pmirGLO and NC/miRNA-146b-5p mimics, respectively. 48 hours later, the cells were lysed for determining relative Luciferase activity (Promega, Madison, WI, USA).

### Statistical Analysis

GraphPad Prism 5 V6.01 (La Jolla, CA, USA) was used for data analyses. Data were expressed as mean ± standard deviation (SD). The differences between the two groups were analyzed by the *t*-test. Chi-square analysis was used to assess the relationship between miRNA-146b-5p level and clinical parameters of GC patients. $p<0.05$ was considered as statistically significant.

## Results

### Downregulated MiRNA-146b-5p in GC

Compared with paracancerous species, miRNA-146b-5p was downregulated in GC tissues (Figure 1A). Similarly, miRNA-146b-5p was lowly expressed in GC cell lines than that of GES-1 cell line (Figure 1B).

### MiRNA-146b-5p Level was Linked to Lymphatic Metastasis and Distant Metastasis of GC

Based on median level of miRNA-146b-5p in enrolled GC patients, they were assigned into

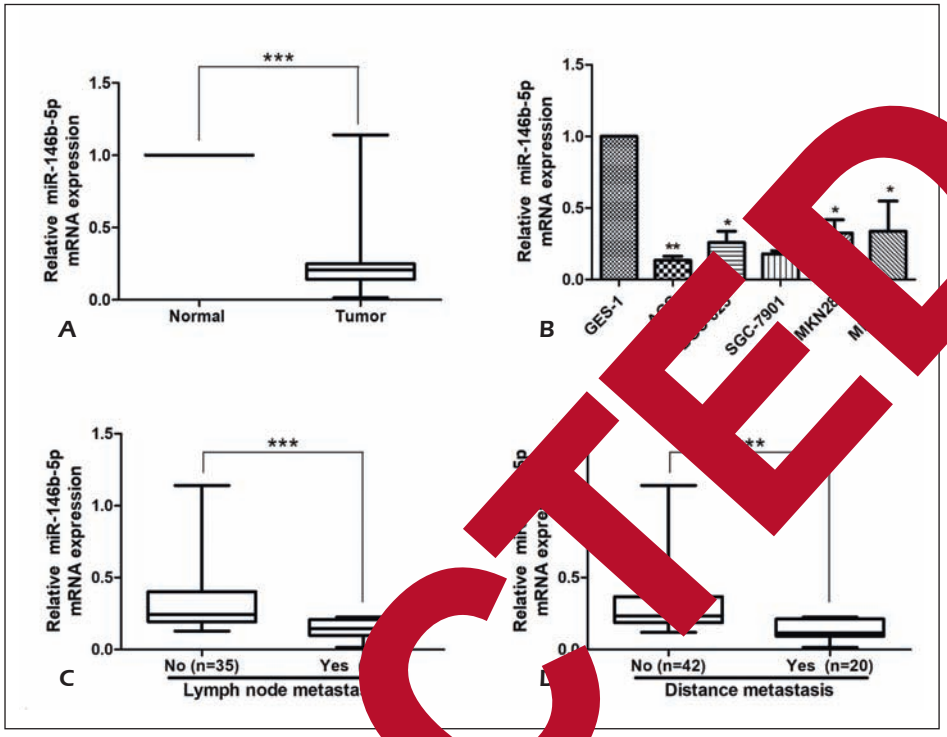


**Figure 1.** Downregulated miRNA-146b-5p in GC. **A**, MiRNA-146b-5p levels in GC species and paracancerous ones. **B**, MiRNA-146b-5p levels in GC cell lines. **C**, MiRNA-146b-5p levels in GC patients either with lymph node metastasis or not. **D**, MiRNA-146b-5p levels in GC patients either with distant metastasis or not. Data were expressed as mean±SD. *$p$<0.05, **$p$<0.01, ***$p$<0.001.

high or low-level groups. Chi-square an... showed that miRNA-146b-5p level was li... rates of lymphatic metastasis and distant ... tasis, while it was not correlated to age, sex, ... tumor grade of GC patients (Table I). Moreo... lower level of miRNA-146b-5p ... served ... GC patients combined lymph ... asis and ... distant metastasis than tho... without ... tastases (Figure 1C, 1D). MiRNA ... 5p m... biomarker of GC.

### *Overe... on of MiRNA-146b-5p Suppressed Proliferative and Migratory ...tials of GC*

... fection efficacy of miRNA-146b-5p mimics was first verified in AGS and SGC-7901 cells (Figure 2A). Viability of GC cells was markedly reduced after overexpression of miRNA-146b-5p (Figure 2B). Besides, reduced migratory cell number was seen in GC cells overexpressing miRNA-146b-5p (Figure 2C).

**Table I.** LncRNA ... AS1 expressio... linical features of patients with ovarian cancer.

| Parameters | ...mber of ...ses | miR-146b-5p expression | | *p*-value |
|---|---|---|---|---|
| | | High (%) | Low (%) | |
| *Ag... rs)* | | | | 0.200 |
| | 27 | 11 | 16 | |
| ≥... | 35 | 20 | 15 | |
| *...nder* | | | | 0.529 |
| ...le | 39 | 18 | 21 | |
| ...ale | 22 | 12 | 10 | |
| *T... size (cm)* | | | | 0.596 |
| | 22 | 10 | 12 | |
| ≥3 | 40 | 21 | 19 | |
| *...ymph node metastasis* | | | | 0.021 |
| ...lo | 35 | 22 | 13 | |
| | 27 | 9 | 18 | |
| *Distance metastasis* | | | | 0.030 |
| No | 42 | 25 | 17 | |
| Yes | 20 | 6 | 14 | |

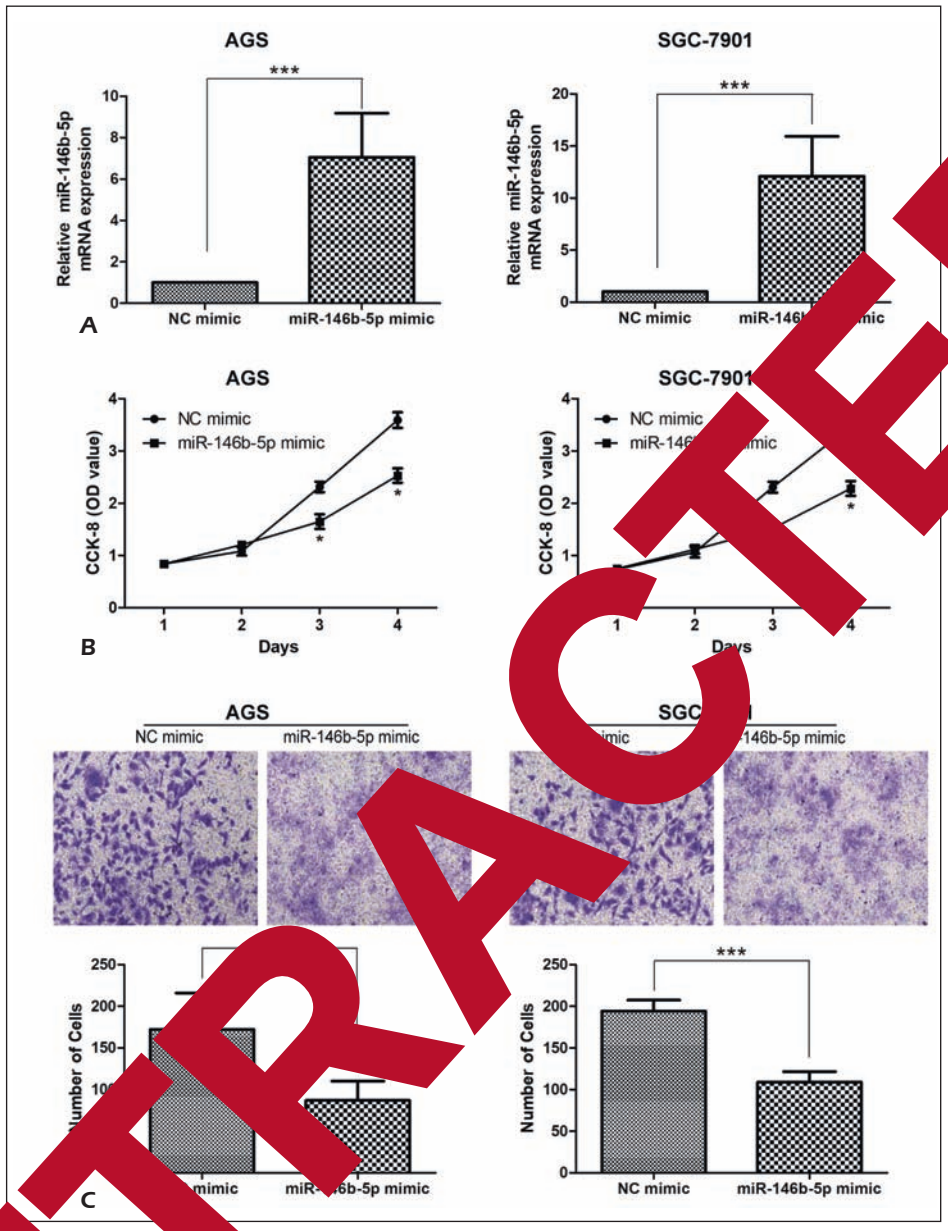


**Figure 7** ...overexpression of miR...6b-5p suppressed proliferative and migratory potentials of GC. **A**, Transfection efficacy o...NA-146...5p mimics. **B**, ...liability in AGS and SGC-7901 cells transfected with NC mimics and miRNA-146b-5p mim..., Migr...in AGS and SGC-7901 cells transfected with NC mimics and miRNA-146b-5p mimics (magnification 40×)...ver...ressed a...ean±SD. *$p$<0.05, ***p<0.001.

### ...A-14...rgeted TRAF6

...ential bi...g sequences in 3'UTR of miR-N...5p and TRAF6 were predicted (Figure ...d Luciferase activity in GC cells ...-transfected with miRNA-146b-5p mimics and ...GLO-TRAF6-WT further showed the bind-in...tween miRNA-146b-5p and TRAF6. Protein and mRNA levels of TRAF6 were downregulated in AGS and SGC-7901 cells overexpressing miRNA-146b-5p (Figure 3A). In addition, TRAF6 was highly expressed in GC species and cell lines (Figure 3B, 3C).

### *Overexpression of TRAF6 Abolished Inhibitory Effect of overexpressed MiRNA-146b-5p on Malignant Phenotypes of GC*

Rescue experiments were designed for clarifying the involvement of TRAF6 in GC development regulated by miRNA-146b-5p. First of all, the over-

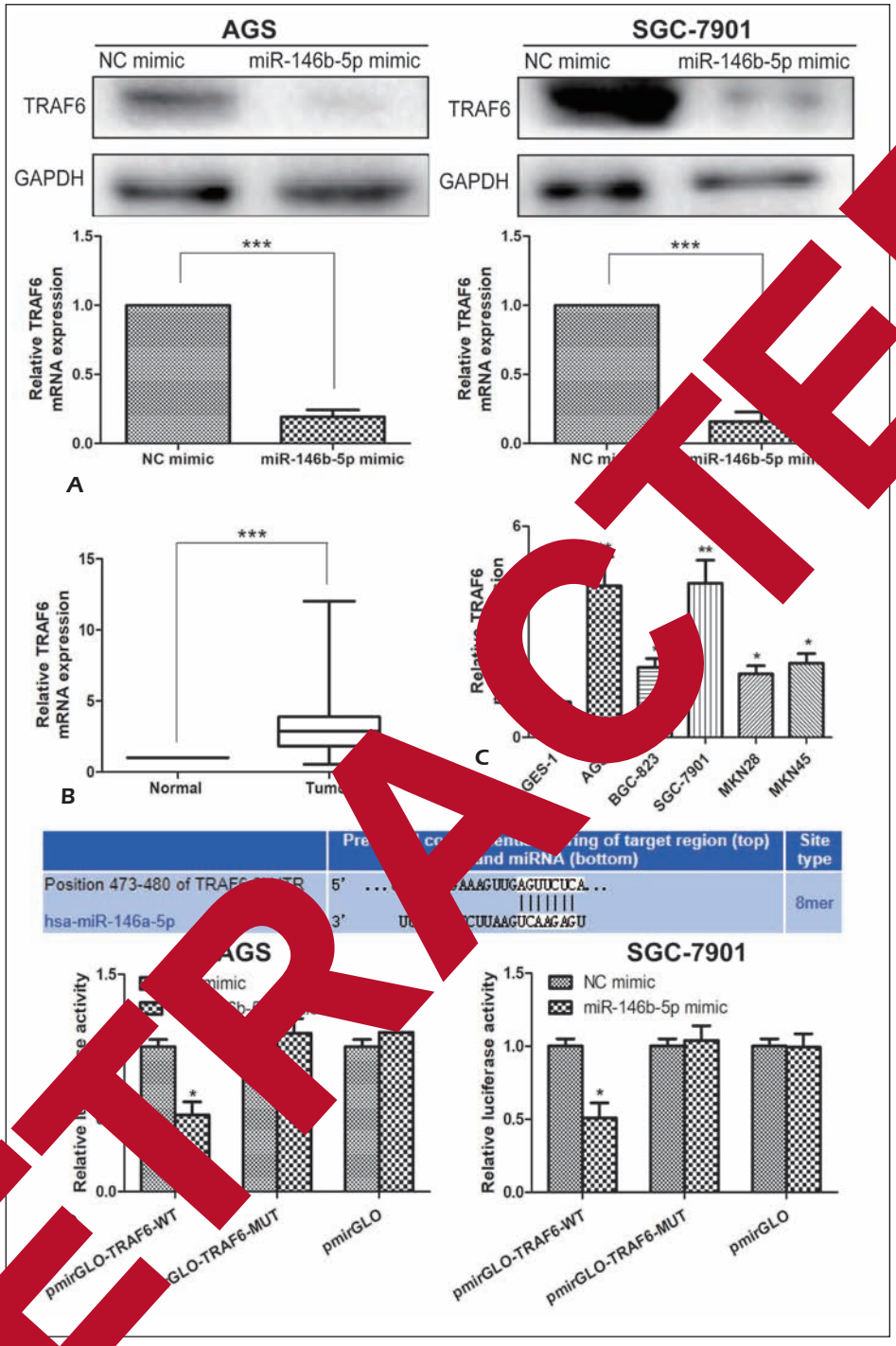




**Fig. 3.** MiRNA-146b-5p targeted TRAF6, **A**, Protein and mRNA levels of TRAF6 in AGS and SGC-7901 cells transfected with NC mimics and miRNA-146b-5p mimics. **B**, TRAF6 levels in GC species and paracancerous ones. **C**, TRAF6 levels in GC cells. Potential binding sequences in 3'UTR of miRNA-146b-5p and TRAF6. Luciferase activity in AGS and SGC-7901 cells co-transfected with pmirGLO-TRAF6-WT/pmirGLO-TRAF6-MUT/pmirGLO and NC/miRNA-146b-5p mimics. Data were expressed as mean±SD. *$p$<0.05, **$p$<0.01, ***$p$<0.001.

expression of TRAF6 was able to downregulate miRNA-146b-5p in GC cells overexpressing miRNA-146b-5p (Figure 4A) and upregulate TRAF6 as well (Figure 4B). Notably, the overexpression of TRAF6 reversed the reduced migratory potential in GC cells overexpressing miRNA-146b-5p (Figure 4C). Hence, TRAF6 was responsible for malignant development of GC regulated by miRNA-146b-5p.

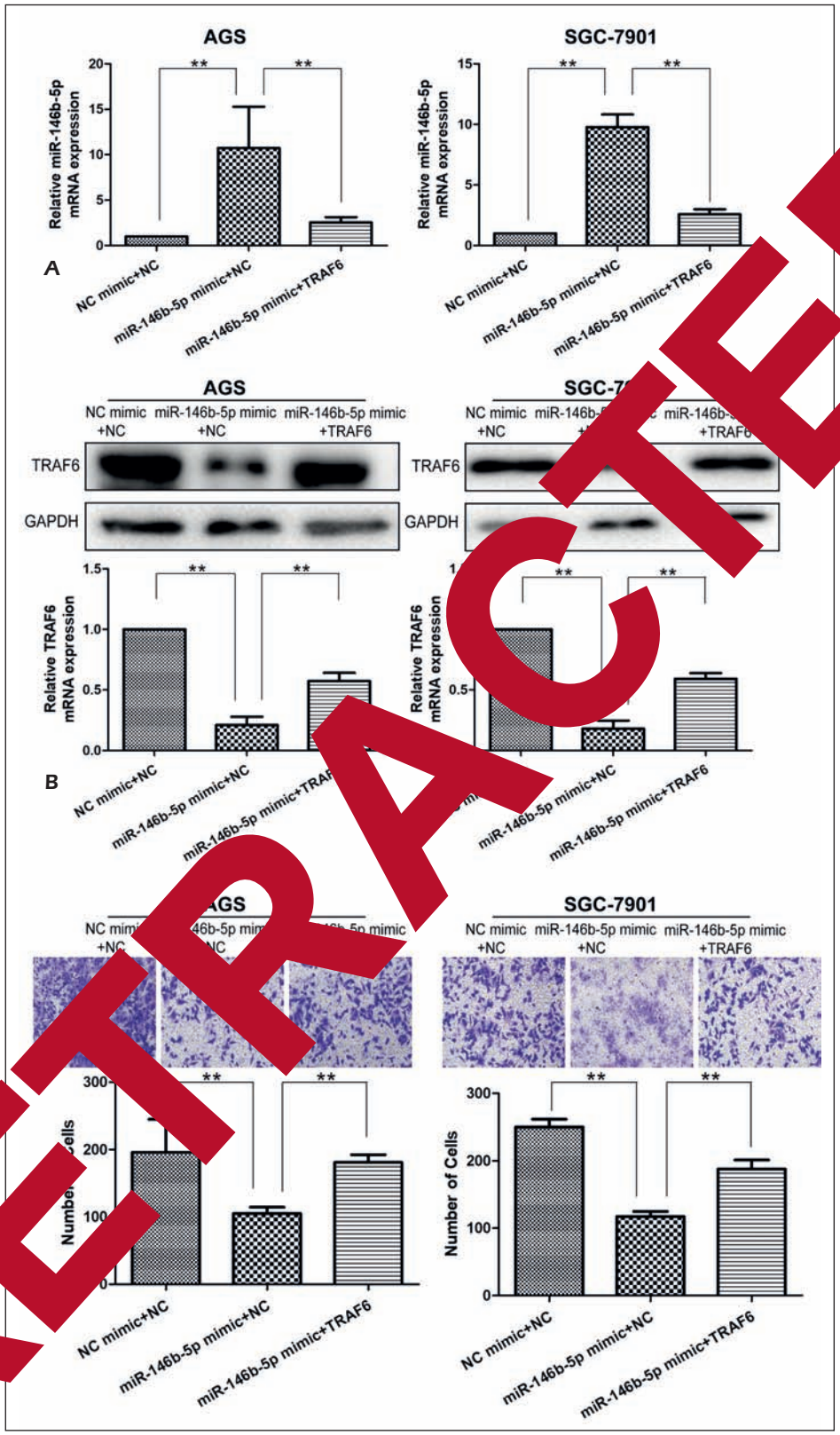




**Figure 4.** Overexpression of TRAF6 abolished inhibitory effect of overexpressed miRNA-146b-5p on malignant phenotypes of GC. AGS and SGC-7901 cells were transfected with NC mimics+NC, miRNA-146b-5p mimics+NC or miRNA-146b-5p mimics+pcDNA-TRAF6, respectively. **A**, The mRNA level of miRNA-146b-5p. **B**, Protein and mRNA levels of TRAF6. **C**, Migration (magnification 40×). Data were expressed as mean±SD. $^{**}p<0.01$.

## Discussion

Despite great advances have been made in the treatment and improved prognosis of GC, its mortality in China remains high[4,5]. Postoperative recurrence and metastasis are the main causes of GC-induced death. Therefore, it is of great significance to elucidate the mechanism underlying metastasis and recurrence of GC[6-8]. The occurrence and development of GC are complicated and regulated by epigenetics and genetics, involving multiple factors and genes[7-9]. In the past, abnormally expressed oncogenes and tumor-suppressor genes were believed to be the key links in the pathogenesis of GC[9,10].

MiRNAs are highly conserved non-coding RNAs[12,13]. They extensively participate in GC development[17]. Differences in pathological subtypes and differentiation levels of GC may be attributed to different pathways in which miRNAs are involved[17,18]. It is reported that miRNA-146b-5p dysregulation is closely linked to malignant development of tumor cells[21,22]. The strong invasive and metastatic potentials of GC result in the poor prognosis of affected patients. In this paper, our findings uncovered that miRNA-146b-5p was downregulated in GC species than paracan ones. GC patients combined lymphatic meta distant metastasis expressed a lower level of mi A-146b-5p than those without metastases. *In vitro* expression of miRNA-146b-5p attenuated prolifera and migratory potentials of GC cel

MiRNAs negatively regul gene ressions through complementary b pairing Mature miRNAs specifically re ize ta NA and thus result in inh tio NA tra e-tion[15]. Differentiall pressed NAs in different types of tu may be po biomarkers[16,17]. Our re d that TRA was the direct target b ding m -146b-5p. TRAF6 is a membe he adaptor y that couples the TNF re or family to the si ng pathway[23,24]. It is orted t the activation of the downstr ignal including NF-κB pathway, is involve F6-ind tumorigenesis[25]. Here, AF6 e tar miRNA-146b-5p, which spons e development of GC regulate y miR 46b-5p. Collectively, miRNA-14 ppressed the malignant development of by g TRAF6.

## Conclusions

MiRNA-146b-5p level is negatively correlated to lymphatic metastasis and distant metastasis rates of GC. It suppresses the malignant development of GC by targeting TRAF6.


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## Conflict of Interests

The authors declare that they h o conflict of

## Ref


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