# miR-768-3p serves as a negative regulator in the progression of gastric cancer by targeting eIF4E

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**Abstract:** Gastric cancer (GC) remains one of the most common malignancy globally, with high incidence and mortality rates. The dysregulation of miR-768-3p has been observed in several types of tumors, and it is involved in the carcinogenesis of GC. This present study aimed to determine the regulatory mechanisms of miR-768-3p in GC progression. First of all, we found that miR-768-3p expression was downregulated in human GC tissues and cell line MGC803. Furthermore, Ectopic expression of miR-768-3p mimics interfering RNA resulted in a decrease in cell proliferation and G1/S transition, as well as promotion of apoptosis. Further investigation in the present study indicated that the expression of eIF4E protein and mRNA were decreased following upregulation of the expression of miR-768-3p. Moreover, eIF4E was demonstrated to be a target gene of miR-768-3p, according to the results of bioinformatics analysis and identified by dual luciferase assays. Collectively, these data suggest that miR-768-3p functions as a tumor suppressor in GC by targeting eIF4E. Therefore, miR-768-3p may be a novel biomarker and potential therapeutic target for GC.

**Key word:** miR-768-3p, gastric cancer, eIF4E

## Introduction

Although having a declining incidence and mortality in developed countries during the past two decades, GC exhibits its highest incidence rates in East Asia, especially in China. Nearly 950,000 new cases of gastric cancer were reported worldwide, leading to about 730,000 deaths annually, approximately 60% of GC incidence and mortality occur in China (1). In 2015, the morbidity of gastric cancer in China is 67.9/100,000, and the mortality rate is as high as 49.8/100,000, which was the second most common malignant tumor and the third leading cause of cancer-associated cases of mortality in whole country (2). The conventional treatments for advanced GC, including chemotherapy, radiotherapy and surgery, are associated with poor outcomes (3). Therefore, the combination therapy of conventional treatments and neoadjuvant therapy has become a new trend in the treatment of gastric cancer.

MicroRNAs (miRNAs), as a specific class of small non-coding RNAs, regulate the occurrence and development of gastric cancer via affecting cell proliferation, apoptosis, metastasis and susceptibility to therapy, and has become a new hot topic in GC research (4). Emerging evidence has reported that miR-768-3p functioned as oncogenes or tumor suppressors and was aberrantly expressed in various types of malignancy, including lung carcinomas (5), breast cancer (6), hematological malignancies (7), endometrial cancer (8), and thyroid cancer (9). Guo J *et al.* reported that the expression of miR-768-3p in GC tissues was lower compared with non-cancerous tissues, and we believed that this is first report about it associated with GC (10). In addition, microRNAs microarray analysis in GC showed that the expression of miR-768-3p was low in GC tissues (11). Therefore, we speculate that specific miR-768-3p may participate in the regulation of GC progression, and its potential role in tumorigenesis and cancer progression has not previously been elucidated.

In this study, we determined the abnormal expression of miR-768-3p in GC tissue

and cell lines by RT-qPCR. Furthermore, a series of functional experiments in vitro were carried out to detect the role of miR-768-3p in human gastric cell line MGC-803. Finally, the correlation between miR-768-3p and its potential target eIF4E was examined by using bioinformatics tools and dual-luciferase assay. Overall, these findings could have implications for providing a new potential therapeutic target for GC treatment.

## Materials and methods

### Human tissues

Human GC and paired adjacent non-cancerous tissues (n=33) were collected from patients during surgical resection at the Zigong Fourth People's Hospital (Zigong, China) from April 2015 to October 2018. Both tumor and non-cancerous tissues were confirmed histologically and the pathological type of cancer was adenocarcinoma. The complete clinicopathological characteristics of patients, including age, sex, tumor grade and TNM stage are described in Table I. Written informed consent was obtained in all cases, and all aspects of the present study were approved by the Ethics Committee of Zigong Fourth People's Hospital.

### Cell culture and transfection

The human gastric cell lines SGC-7901, AGS, HGC-27, MGC-803, MKN-7, MKN45, MKN74, BGC-823 and immortalized gastric epithelial cells GES-1 were purchased from the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI‑1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Life Technologies, Carlsbad, CA, USA), 50 U/ml penicillin and 50 μg/ml streptomycin (both from Gibco, Thermo Fisher Scientific, Inc. Waltham, MA, USA) at 37˚C in humidified 5% $CO_2$.

miR-768-3p mimics and corresponding negative control (miR-NC) were designed and synthesized by GenePharma (Shanghai, China). MGC-803 cells were plated into 6-well plates at a density of $5x10^5$ cells/well, and cultivated overnight at 37˚C. 24 hours after plating, miR-768-3p mimics (50 nM) and miR-NC (50 nM) were

transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Cells were collected at 48 h after transfection for RT-qPCR and western blot analysis. Each experiment was repeated at least three times.

**RNA isolation and RT-qPCR**

Total RNA was isolated from the cultured cells and tissues using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. RT reactions were performed to synthesize cDNA by using a TaqMan miRNA RT kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). For detection of miR-768-3p expression, RT-qPCR was performed by using SYBR Premix Ex Taq kit (Takara, Dalian, China) in ABI7500 (Applied Biosystems). U6 were used as internal controls and was analyzed using the 2-ΔΔct method. The experiments were repeated three times.

**Western blotting**

Protein was extracted from cells and tissues using RIPA buffer (Sigma-Aldrich) containing a freshly added protease inhibitor cocktail. Then, proteins concentration were resolved through 10% SDS-polyacrylamide gel electrophoresis, transferred to PVDF membranes (Bio-Rad, Berkeley, CA, USA), after blocking in 5% non-fat milk to block the membranes for 2 h at room temperature, probed with antibodies against eIF4E (dilution, 1:1,000)、bcl-2 (dilution, 1:2,000)、caspase3 (dilution, 1:2,000) or β-actin (dilution, 1:5,000, Abcam, Cambridge, MA,USA) and then with secondary antibodies (dilution, 1:5,000 , Abcam). The protein bands were analyzed by the Bio-Rad Gel imaging system.

**Cell proliferation assays**

MGC-803 cells were seeded in 96-well plates at a density of $5x10^3$ cells/well in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) and allowed to attach overnight. The cells were then transfected with miR-768-3p mimic or miR-NC used as controls. After transfection for 24, 48 or 72 h, 20 μl MTT (5 mg/ml; Beyotime, Shanghai,

China) was added to each well, followed by incubation at 37˚C for an additional 4 h. Absorbance was recorded at 570 nm with a 96−well plate reader following the addition of dimethyl sulfoxide (DMSO).

**Cell cycle assay**

Cells were harvested by trypsinization 48 h after transfection, washed three times with ice‑cold PBS, and fixed with 70% ethanol overnight at 4˚C. The fixed cells were washed in PBS and subjected to propidium iodide (PI)/RNase A staining followed by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA). The percentage of cells in each phase of the cell cycle was estimated using Modfit LT software, (version, 3.0; BD Biosciences).

**Apoptosis assay**

MGC-803 cells were seeded in 6‑well plates at a density of $10^5$ cells/well. Following 24 h post‑transfection, cells were stained with Annexin V‑fluorescein isothiocyanate and PI (Bestbio, Shanghai, China), according to the manufacturer's instructions. Apoptosis rates were analyzed by BD FACSCalibur™ system (BD Biosciences).

**Luciferase reporter assay**

HEK-293T cells were used for the luciferase reporter assay. Cells were cultured in a 24-well plate and co-transfected with the pGL3-3'-UTR (500 ng) of eIF4E or mutated 3'-UTR and miR-768-3p expressing vector or negative control vector. The 3'-UTR and 3'-UTR mutation were amplified using the primers as Fig. 5C. The 3'-UTR mutation was generated using the QuikChange II XL Site‑Directed Mutagenesis kit (Stratagene, Santa Clara, CA, USA). At 48 h after transfection, the cells were collected and analyzed using the Dual Luciferase Assay System (Promega, Fitchburg, WI, USA) according to the manufacturer's instructions. The relative firefly luciferase activities were normalized to *Renilla* luciferase activities. The experiments were repeated at least three times.

**Statistical analysis**

Statistical analysis was performed with SPSS software, version 16 (SPSS, Inc.,

Chicago, IL, USA). Data were presented as means ± SD. The difference between two groups was analyzed using ANOVA and Chi-squared test. Differences were considered significant for $P<0.05$.

**Table I.** Clinicopathological characteristics and miR-768-3p expression in 33 patients with GC.

| Clinicopathological characteristics | Cases (n=33) | miR-768-3p expression | | $\chi 2$ | P-value |
|---|---|---|---|---|---|
| | | Low (%) (n=20) | High (%) (n=13) | | |
| Sex | | | | 1.669 | 0.289 |
| Male | 17 | 11(64.7) | 6(35.3) | | |
| Female | 16 | 9(56.3) | 7(43.7) | | |
| Age (years) | | | | 1.408 | 0.361 |
| ≤60 | 15 | 10(66.7) | 5(33.3) | | |
| >60 | 18 | 10(55.6) | 8(44.4) | | |
| Hp infection | | | | 2.179 | 0.157 |
| Yes | 21 | 12(57.1) | 9(42.9) | | |
| No | 12 | 8(66.7) | 4(33.3) | | |
| Tumor size (mm) | | | | 3.105 | 0.089 |
| ≤5.0 | 20 | 13(65.0) | 7(35.0) | | |
| >5.0 | 13 | 7(53.8) | 6(46.2) | | |
| TNM stage | | | | 6.793 | 0.019[a] |
| I-II | 19 | 9(47.4) | 10(52.6) | | |
| III-IV | 14 | 11(78.6) | 3(21.4) | | |
| Local invasion | | | | 0.100 | 0.895 |
| T1-T2 | 22 | 12(54.5) | 8(45.5) | | |
| T3-T4 | 11 | 6(54.5) | 5(45.5) | | |
| Lymph-node metastasis | | | | 8.672 | 0.006[a] |
| 0-2 | 23 | 11(47.8) | 12(52.2) | | |
| >2 | 10 | 9(90.0) | 1(10.0) | | |

miR, microRNA; Hp, Helicobacter pylori; TNM, tumor-node-metastasis. [a]$P<0.05$.

## Result

### Low expression of miR-768-3p in gastric cancer tissues and cell lines.

To investigate the function of miR-768-3p in the pathogenesis of GC, we performed RT‑qPCR in 33 pairs of GC tissues and adjacent non-cancerous tissues. As demonstrated in Fig. 1A, miR-768-3p expression was markedly decreased in GC

tissues compared with the adjacent non-cancerous tissues (**$P<0.01$, Fig. 1A). Based on the median expression level of miR-768-3p (=0.55), GC patients were categorized into two groups on the basis of sex, age, tumor size, TNM stage, local invasion, lymph‐node metastasis, respectively, and the detailed grouping is shown in Table I. These data shown that decreased expression of miR-768-3p was significantly associated with lymph node metastasis and advanced tumor‐nodes‐metastasis (TNM) stage(*$P<0.05$), there was no significant correlations were observed between the miR-768-3p expression level and age, sex, Hp infection, location or tumor size. These findings suggested that the downregulation of miR-768-3p may be involved in gastric cancer development.

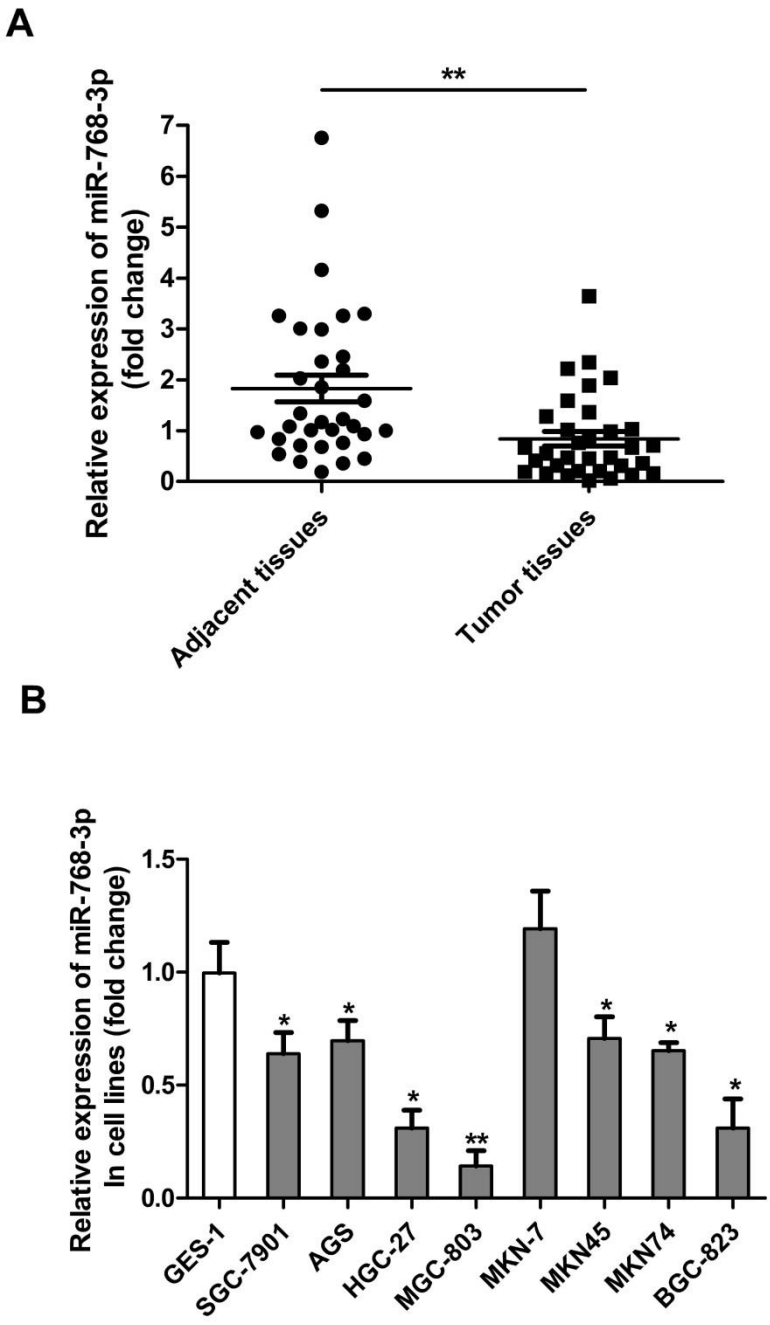


Figure 1. miR-768-3p is downregulated in gastric cancer. (A) The expression of miR-768-3p was significantly downregulated in cancer tissues compared with adjacent non-cancerous as control. U6 RNA served as an internal control. (B) Relative expression of miR-768-3p in MGC-803 and other GC cells compared with the normal gastric epithelial cell (GES-1). *$P<0.05$, **$P<0.01$.

Concomitantly, Expression of miR-768-3p was assessed by RT‑qPCR in several GC cell lines, including SGC-7901, AGS, HGC-27, MGC-803, MKN-7, MKN45,

MKN74, BGC-823, and immortalized gastric epithelial cells GES-1 as control. miR-768-3p expression levels were significantly reduced in a majority of GC cell lines compared with the level in gastric epithelial cells GES-1 (*$P<0.05$). The results demonstrated the expression of miR-768-3p was markedly decreased in MGC803 cells compared with other cell lines (**$P<0.01$, Fig. 1B). As a result, the MGC803 cell line was selected for subsequent experiments. These results revealed that miR-768-3p may serve an antitumor role in gastric cancer carcinogenesis.

**Effect of overexpressed miR-768-3p on proliferation, cell cycle, and apoptosis of MGC-803 cells.**

In order to evaluate the tumor‑suppressing potential of miR-768-3p in GC cells, miR-768-3p mimics or miR‑NC were transiently transfected into MGC-803 cells, and the transfection efficiency was determined by RT-qPCR. As indicated in Fig. 2A, transfection of cells with miR-768-3p mimic revealed a significant increase of miR-768-3p expression compared to that in cells transfected with miR‑NC (***$P<0.001$, Fig. 2A). For cell proliferation detection, MTT assay was used to investigate the cell viability and found that miR-768-3p mimics induced a significant suppression of cell proliferation compared with the respective controls (*$P<0.05$, Fig. 2B). Flow cytometry analysis was used to detect the cycle of MGC803 cells and observed that miR-768-3p mimics inhibited G1/S transition in GC cells , G0/G1 phase of which was increased compared with the miR‑NC group (*$P<0.05$, Fig. 2C).

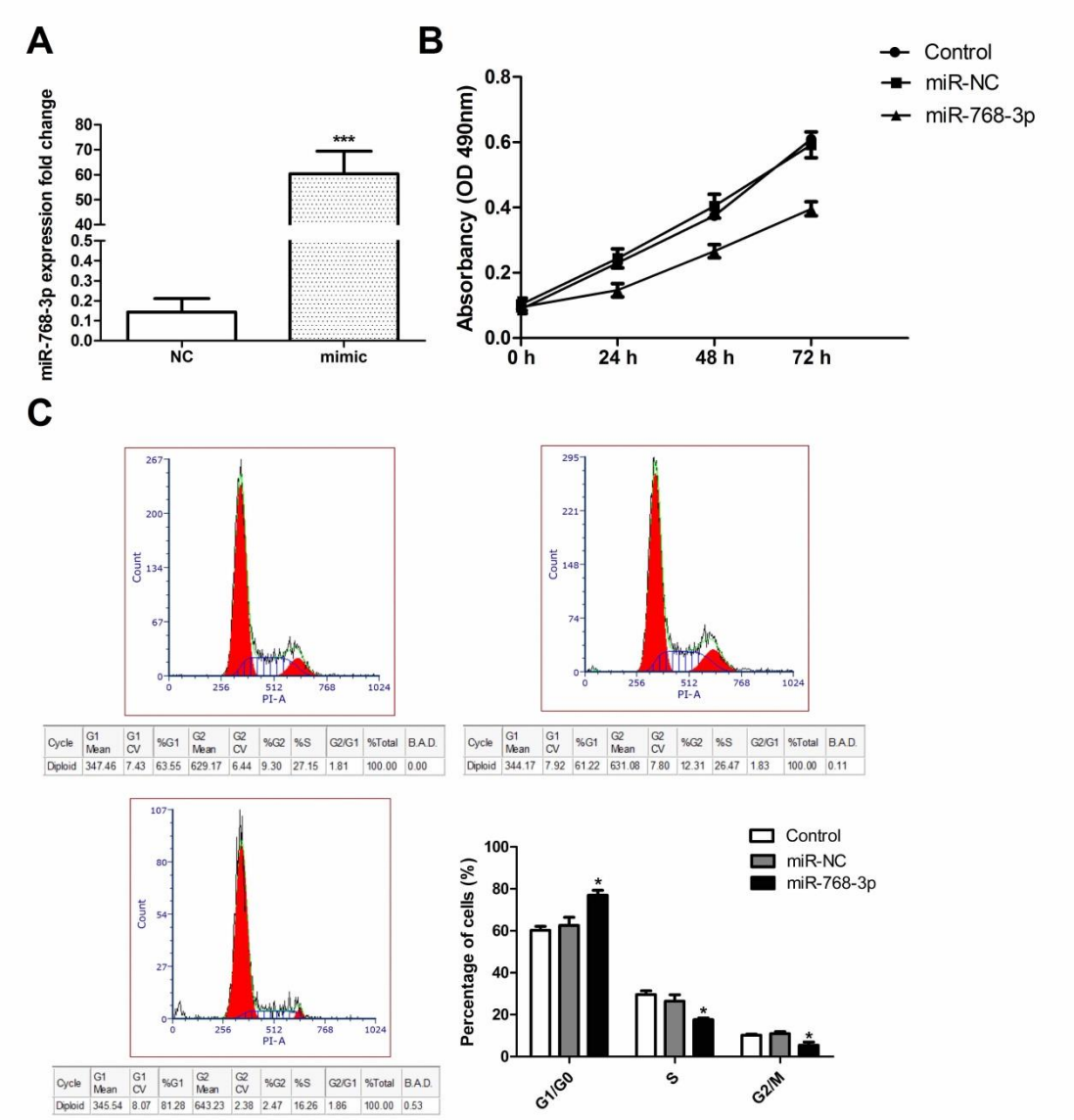


Figure 2. miR-768-3p suppressed cell proliferation and cell cycle in MGC803. (A) miR-768-3p was overexpressed by transfected miR‑133a mimic. (B) miR-768-3p overexpression inhibits gastric cell proliferation. (C) miR-768-3p overexpression inhibits G1/S transition in MGC803 cells. ****P<0.001*, **P<0.05*. miR, microRNA; NC, negative control.

Furthermore, we assessed the effect of miR-768-3p on the apoptosis of GC cells by flow cytometry. The results of the FITC apoptosis detection revealed that the apoptosis rates were increased in the miR-768-3p mimics groups compared with the miR‑NC group (**P<0.05*, Fig. 3).

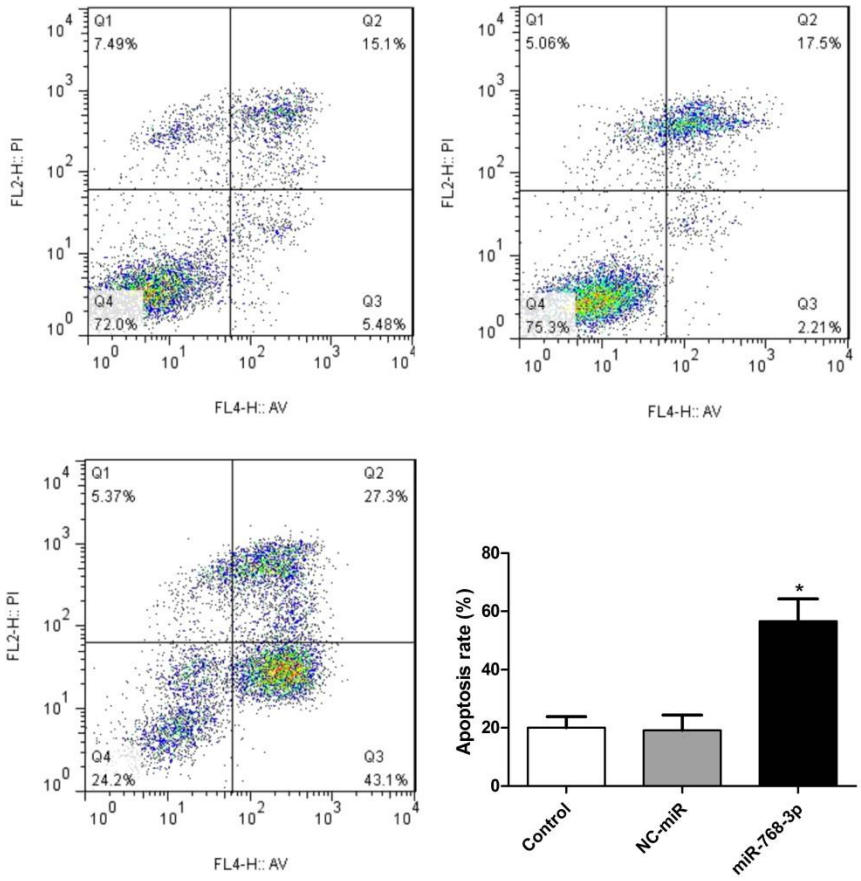


Figure 3. miR-768-3p suppressed cell apoptosis rate of difference groups in MGC803 cell.

*: $P < 0.05$, compared with NC group

**Effect of overexpression levels of miR-768-3p on the expression of eIF4E, bcl-2, and caspase3 protein in MGC-803 cells**

In order to detect whether miR-768-3p can inhibit eIF4E, bcl-2, and caspase3 protein expression, we transfected MGC803 cells with miR-768-3p mimics and miR-NC. Then western blotting was performed to detect the proteins. The results showed that eIF4E, bcl-2, and caspase3 protein were downregulated in MGC803 cells transfected with miR-768-3p mimics, compared to that in cells transfected with miR-NC (*$P<0.05$, Fig. 4).

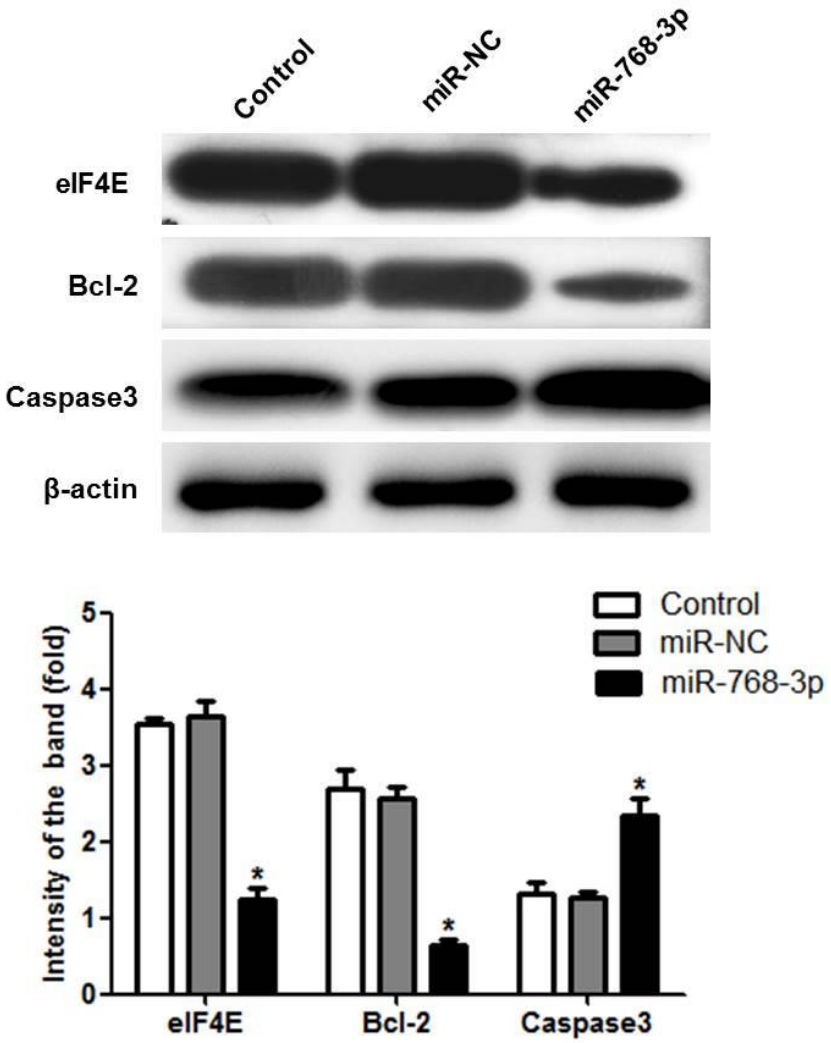


Figure 4. miR-768-3p affected the relative proteins expressions of difference group in MGC803 cell.*: $P < 0.05$, compared with NC group

**miR-768-3p downregulates eIF4E expression by directly targeting its 3'-UTR.**

Given that eIF4E is a potent cancer promoter in GC (12) and miR-768-3p consistently suppressed the expression of eIF4E protein and mRNA in GC cells (Fig. 5A,B), we focused on the cooperation between eIF4E and miR-768-3p. To verify whether miR-768-3p could bind to 3'-UTR of eIF4E, luciferase report assays were performed in HEK293T cells. Our results showed that the luciferase activity of the wild-type (WT) 3'-UTR of DNMT1 was significantly decreased in cells co-transfected with miR-768-3p compared with that in miR-NC control, while the

luciferase activity of the mutated type (MT) 3'-UTR of eIF4E was not obviously altered in cells co-transfected with miR-768-3p compared with control (**$P<0.01$, Fig. 5C, D). Additionally, we detected whether the expression of eIF4E is regulated by miR-768-3p in mRNA level. In MGC-803 cells, the overexpression of miR-768-3p reduced the expression of eIF4E by RT-qPCR (**$P<0.01$, Fig. 5A).

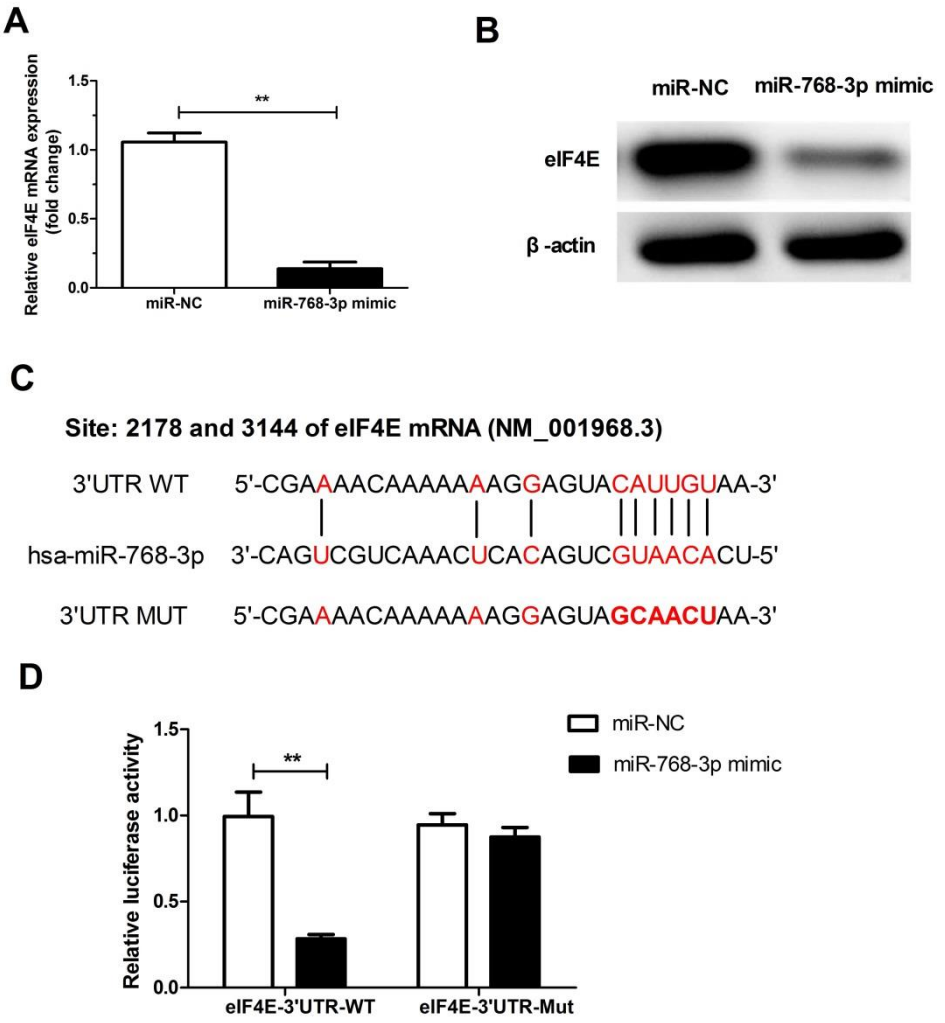


Figure 5. miR-768-3p downregulates eIF4E expression by directly targeting its 3'-UTR. (A) The mRNA expression level and (B) the protein expression level of eIF4E. (C) The putative and corresponding mutant binding site of miR-768-3p in the eIF4E 3'-UTR. (D) Analysis of luciferase activity. **: $P<0.01$.

## Discussion

Despite improvements in diagnosis and treatment, the outcomes of patients with GC remain poor (13). Thus, a better understanding of gastric carcinogenesis and the identification of novel molecular targets to improve the diagnosis and therapy of GC are warranted.

eIF4E (Eukaryotic initiation factor 4E), as an mRNA 5' cap-binding protein, is known to have the potential to mediate translation of almost every mRNA. Increasing evidence supports that eIF4E overexpressed in a variety of solid tumors and tumor cell lines, and has been found to have anti-tumor activity *in vitro* and *in vivo* (14-16). Overexpression of eIF4E in GC has been demonstrated to correlate with oncogenesis, and their silencing may effectively inhibit cancer growth, cell apoptosis, metastasis (17,18). Therefore, Targeting eIF4E appears to be an attractive anticancer strategy for treatment of gastric cancer.

The identity of miR-768-3p, which is located within the sequence of the small nucleolar RNA HBII-239, as an microRNA has been argued and even its entry has been removed from miRBASE (19). Whereas further studies have found that miR-768-3p was associated with Argonaute2 (Ago2), a core component of miRNA-mediated silencing complex, to the same extent as let-7a, a well-established mature miRNA that directly binds Ago2 and mediates messenger RNA repression in cells, suggesting that miR-768-3p possesses the characteristics of a functional miRNA *in vivo* (20). Consequently, miR-768-3p is processed into a mature miRNA through a noncanonical miRNA biogenesis pathway as proposed by hypothesis that miRNAs could be generated by small nucleolar RNA (21).

Previous studies have indicated that microRNA act as tumor suppressor modulates gastric cancer cell proliferation and invasion by repressing eIF4E (22). In this report, we present evidence that miR-768-3p has an important role in inhibiting eIF4E expression and mRNA translation, and in regulating GC cell proliferation, cell cycle and apoptosis. Further research shows that miR-768-3p could bind to 3'-UTR of eIF4E by luciferase report assays, which is consistent with that of miR-768-3p targeting by eIF4E in melanoma (23). These findings indicate that miR-768-3p

function as tumor suppressor in GC by targeting eIF4E.

Collectively, the results demonstrated that abnormal declined miR-768-3p in both carcinoma tissues in GC cases and GC cell lines, were associated with early clinic pathological factors of GC. Our results suggested that Ectopic expression of miR-768-3p inhibited cell proliferation, and G1/S transition, as well as promoted cell apoptosis. In addition, just as we expected that the relationship between miR-768-3p and eIF4E expression was negatively correlated, eIF4E was a direct target of miR-768-3p in GC cells. These findings may contribute to the further elucidation of the molecular mechanisms underlying GC progression and provide candidate targets for the prevention and treatment of GC.

**Reference:**

1. Jemal A, Bray F, Center MM, et al: Global cancer statistics. CA Cancer J Clin 61:69-90, 2011
2. Chen W, Zheng R, Baade PD, et al: Cancer statistics in China, 2015. CA Cancer J Clin 66:115-132, 2016
3. Tsunehiro T, Yoshiro S, Yuko K: Gastric cancer: current status of diagnosis and treatment. Cancers (Basel) 5:48-63, 2013
4. Petrocca F, Visone R, Onelli MR, et al: E2F1-regulated microRNAs impair TGFbeta-dependent cell-cycle arrest and apoptosis in gastric cancer. Cancer Cell 13:272-86, 2008
5. Xie Z, Chen W, Chen Y, et al: miR-768-3p is involved in the proliferation, invasion and migration of non-small cell lung carcinomas. Int J Oncol 51:1574-1582, 2017
6. Dharanija M, Manuela Z, Markus W, et al: Circulating miRNAs as surrogate markers for circulating tumor cells and prognostic markers in metastatic breast cancer. Clin Cancer Res 18:5972-5982, 2015
7. Dzikiewicz-Krawczyk A: MicroRNA polymorphisms as markers of risk, prognosis and treatment response in hematological malignancies. Crit Rev Oncol Hematol 93:1-17, 2015
8. Zhou J, Song T, Gong S, et al: microRNA regulation of the expression of the estrogen receptor in endometrial cancer. Mol Med Report 3:387-392, 2008
9. Vriens MR, Weng J, Suh I, et al: MicroRNA expression profiling is a potential diagnostic tool for thyroid cancer. Cancer 118:3426-3432, 2012
10. Guo J, Miao Y, xiao B, et al: Differential expression of microRNA species in human gastric cancer versus non-tumorous tissues. J Gastroenterol Hepatol 24:652-657, 2010
11. Guo JJ, Xiao BX, Chen SC, et al: Non-coding RNA and Gastric Cancer. J Clin & Pathol Res 35:52-53, 2015
12. Ge Y, Wu Q, Ma G, et al: Hypermethylation of EIF4E promoter is associated with early onset of gastric cancer. Carcinogenesis 39:66, 2018

13. Greenlee RT, Murray T, Bolden S, et al: Cancer statistics, 2000. CA Cancer J Clin 50:7, 2000
14. Hans-Guido W, Elisa DS, Fridman JS, et al: Survival signalling by Akt and eIF4E in oncogenesis and cancer therapy. Nature 428:332-7, 2004
15. Mamane Y, Petroulakis E, Rong L, et al: eIF4E-from translation to transformation. Oncogene 23:3172-3179, 2004
16. Defatta RJ, Nathan CA, Benedetti A, De: Antisense RNA to eIF4E suppresses oncogenic properties of a head and neck squamous cell carcinoma cell line. Laryngoscope 110:928-933, 2010
17. Wu S, Yang L, Wu D, et al: AEG-1 induces gastric cancer metastasis by upregulation of eIF4E expression. J Cell Mol Med 21:3481, 2017
18. Liang S, Guo R, Zhang Z, et al: Upregulation of the eIF4E signaling pathway contributes to the progression of gastric cancer, and targeting eIF4E by perifosine inhibits cell growth. Oncol Rep 29:2422-2430, 2013
19. Valleron W, Ysebaert L, Berquet L, et al: Small nucleolar RNA expression profiling identifies potential prognostic markers in peripheral T-cell lymphoma. Blood 120:3997-4005, 2012
20. Arroyo JD, Chevillet JR, Kroh EM, et al: Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. PNAS 108:5003-5008, 2011
21. Miyoshi K, Miyoshi T, Siomi H: Many ways to generate microRNA-like small RNAs: non-canonical pathways for microRNA production. Mol Genet Genomics 284:95-103, 2010
22. Li W, Jin X, Deng X, et al: The putative tumor suppressor microRNA-497 modulates gastric cancer cell proliferation and invasion by repressing eIF4E. Biochem Biophys Res Commun 449:235-240, 2014
23. Jiang CC, Croft A, Tseng HY, et al: Repression of microRNA-768-3p by MEK/ERK signalling contributes to enhanced mRNA translation in human melanoma. Oncogene 33:2577-88, 2014