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The putative tumor suppressor microRNA-497 modulates gastric cancer cell proliferation and invasion by repressing eIF4E

Weidong Li [a,1], Xuejun Jin [a,1], Xubin Deng [a,1], Gong Zhang [b], Bingqian Zhang [c], Lei Ma [a,*]

[a] Department of Medical Oncology, Affiliated Cancer Hospital of Guangzhou Medical University, Cancer Center of Guangzhou Medical University (CCGMU), Guangzhou, People's Republic of China
[b] Department of Radiotherapy, People's Hospital of Shanxi Province, Taiyuan, People's Republic of China
[c] Cancer Research Institution, Southern Medical University, Guangzhou, People's Republic of China

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ABSTRACT

Accumulating evidence has shown that microRNAs are involved in multiple processes in gastric cancer (GC) development and progression. Aberrant expression of miR-497 has been frequently reported in cancer studies; however, the role and mechanism of its function in GC remains unknown. Here, we reported that miR-497 was frequently downregulated in GC tissues and associated with aggressive clinicopathological features of GC patients. Further in vitro observations showed that the enforced expression of miR-497 inhibited cell proliferation by blocking the G1/S transition and decreased the invasion of GC cells, implying that miR-497 functions as a tumor suppressor in the progression of GC. In vivo study indicated that restoration of miR-497 inhibited tumor growth and metastasis. Luciferase assays revealed that miR-497 inhibited eIF4E expression by targeting the binding sites in the 3′-untranslated region of eIF4E mRNA. qRT-PCR and Western blot assays verified that miR-497 reduced eIF4E expression at both the mRNA and protein levels. A reverse correlation between miR-497 and eIF4E expression was noted in GC tissues. Taken together, our results identify a crucial tumor suppressive role of miR-497 in the progression of GC and suggest that miR-497 might be an anticancer therapeutic target for GC patients.

1. Introduction

MicroRNAs (miRNAs) are a class of endogenous non-coding RNAs of 19–25 nucleotides in length that suppress protein expression through base pairing with the 3′-untranslated region (3′-UTR) [1]. Mounting studies have documented a functional contribution of specific miRNAs in diverse biological processes [2,3]. In addition, miRNAs have been discovered to have a role in progression and metastasis of human cancers recently [4,5].

Gastric cancer (GC), one of the most common malignancies, ranks the second highest in the mortality rate worldwide [6]. The development of GC is a multistep process with accumulation of genetic and epigenetic changes. A growing body of evidence indicates that aberrant expression of miRNAs is involved in the progression of GC [7,8]. MiRNAs that deregulated in GC have been identified as modulators of cell growth, apoptosis, migration, and invasion [9,10]. A previous study indicated that miR-497 was down-regulated in GC [11]. However, the underlying mechanism of miR-497 in GC was unexplored.

Eukaryotic translation initiation factor 4E (eIF4E), a key player in translational control, has been found to contribute to tumor occurrence and development [12–14]. Over-expression of eIF4E results in the enhanced expression of various oncogenes, such as cyclin D1, VEGF, and MMP-9 [15]. Although eIF4E control of the translation of many oncogenes has been well documented, the upstream regulation of eIF4E itself is seldom reported.

In the present study, we found that low expression of miR-497 was associated with poor prognostic phenotype of GC. Furthermore, we identify that miR-497 can regulate the proliferation and invasion of GC cells by targeting directly the eIF4E gene.

2. Materials and methods

2.1. Cell lines and patient samples

Human GC cell lines and immortalized normal gastric mucosal epithelial cell line (GES-1) were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). All cell lines were maintained in a humid wet

* Corresponding author. Fax: +86 02062841833.
E-mail address: malei01@yeah.net (L. Ma).
[1] Equal contributors.

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atmosphere containing 5%CO_2 at 37 °C in RPMI-1640 medium supplemented with 10% newborn bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin. GC tissues were obtained from cancer center of Guangzhou Medical University. All GC specimens were obtained from Cancer Center of Guangzhou Medical University and had confirmed pathological diagnosis. This study was approved by the institute research ethics committee of the Cancer Center, Guangzhou Medical University.

2.2. RNA isolation and quantitative real-time PCR

Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, California, USA). For qPCR, RNA was reverse transcribed to cDNA from 1 mg of total RNA using a Reverse Transcription Kit (Takara). Real-time PCR analyses were conducted with Power SYBR Green (Takara). Results were normalised to the expression of U6 or GAPDH. The primers used for miR-497 were previously described [16]. The primers for U6 5′-CTCGCTTCGGCAGCACA-3′ (forward) and 5′-AACGCTTCACGAATTTGCGT-3′ (reverse). The primers for eIF4E were used as previously described [17]. The primers for GAPDH were 5′-AACGTGTCAGTGGTGGACCTG-3′ (forward) and 5′-AGTGGGTGTCGCTGTTGAAGT-3′ (reverse). The relative expression of each gene was calculated and normalized using the $2^{-\Delta\Delta Ct}$ method relative to U6 snRNA or GAPDH.

2.3. Plasmid, microRNA mimic and inhibitor

Full-length eIF4E cDNA entirely lacking the 3′-UTR was subcloned into the eukaryotic expression vector pcDNA3.1(+) (Invitrogen). The eIF4E 3′UTR target site for miR-497 was amplified by PCR cloned into the XbaI site of pGL3 control (Promega, Madison, USA). This vector was sequenced and named WT eIF4E 3′UTR. Site-directed mutagenesis of the miR-497 target-site in the eIF4E 3′UTR was carried out using the Quick-change mutagenesis kit (Strata-gene, Heidelberg, Germany) and named Mut eIF4E 3′UTR.The miR-497 mimics and miR-control were chemically synthesized by Shanghai GenePharma Company (Shanghai, China). Oligonucleotide transfection was performed with Lipofectamine 2000 reagent (Invitrogen).

2.4. Luciferase reporter assay

For the reporter assays, Wt or Mut 3′UTR vector and the control vector pRL-CMV ((cytomegalovirus) coding for Renilla luciferase, Promega) were cotransfected. Luciferase activity was measured 36 h after transfection using the Dual-Luciferase Reporter Assay System (Promega).

2.5. MTT, cell cycle and invasion assays

The MTT assay was carried out as described previously [18]. For cell cycle assay, cell pellets harvested by centrifugation were washed for twice with ice-cold PBS and fixed with ice-cold 70% ethanol for 48 h at 4 °C. The fixed cells were rehydrated in PBS and subjected to PI/RNase staining followed by fluorescence-activated cell sorter scan (FACS) analysis. The cell invasion assay was carried out as a previous study [19].

2.6. Western blot and in situ hybridization analysis

Protein samples were resolved by 10% SDS–PAGE and then transferred to PVDF membranes. The membranes were blocked and probed with antibodies against eIF4E, cyclin D1, MMP-9 and GAPDH, followed by probed with the secondary antibodies accordingly. Band detection via enzyme-linked chemiluminescence was performed according to the manufacturer's protocol (ECL; Pierce Biotechnology Inc., Rockford, USA).

In situ hybridization was performed by using antisense locked nucleic acid (LNA) modified oligonucleotide probes according to the protocol of In Situ Hybridization kit (Boster, Wuhan, China). Briefly, Sections were deparaffinized and deproteinated, and then prehybridized for 1 h in hybridization liquid in a humidified chamber (50% formamide, 5× SSC). Then the sections were incubated with 20 μl LNA-miR-497 hybridization solution at 42 00BC for 16 h, followed by washing with phosphate-buffered saline (PBS) 3 times. Then the sections were labeled with FITC for 1 h at room temperature in the dark. Nuclei were counterstained with a DAPI karyotyping kit (GenMed, Boston, MA, USA).

2.7. Lentivirus production and infection

The pLV-has-miR-497 plasmid and the negative control pLV-miRNA-vector were purchased from Biosettia Inc. (Biosettia, San Diego, USA). Viral packaging and infection were performed according to standard protocols as recommended by the manufacturer. The packaged lentiviruses were named LV-miR-497. The empty lentiviral vector LV-ctrl was used as a control.

2.8. In vivo growth and metastasis assay

For the in vivo tumor growth studies, 1×10^6 SGC-7901 cells, stably expressing miR-497, or the control vector, were injected subcutaneously in the upper back of BALB/C-nu/nu athymic nude mice. After 25 days, tumor samples were carefully removed and weighed. The experimental metastasis studies were conducted as previously described [20]. All animal procedures were performed in accordance with institutional guidelines.

2.9. Statistical analysis

Statistical analysis was performed using a SPSS software package (SPSS Standard version 16.0, SPSS Inc). Data were expressed as the mean ± SD from at least three independent experiments. For survival analysis, we analyzed all patients with GC by Kaplan–Meier analysis. A log rank test was used to compare different survival curves. P values <0.05 were considered significant.

3. Results

3.1. Lower levels of miR-497 are frequently detected in GC tissues and associates with poorer overall survival of GC patients

Real-time PCR was performed to examine the expression levels of miR-497 in 36 pairs of GC and adjacent non-neoplastic tissues. As shown in Fig. 1 A, the expression levels of miR-497 were significantly decreased in GC tissues compared to adjacent non-neoplastic tissues ($P < 0.05$). The in situ hybridization assay also showed that miR-497 was down-regulated in GC tissues when compared with the non-neoplastic tissues (Fig. 1B).We further found that the expression level of miR-497 was lower in GC cell lines compared with immortalized normal gastric mucosal epithelial cell line GES-1 (Fig. 1C, $P < 0.05$). The SGC-7901 cell line, which had the lowest expression of miR-497, was chosen for further study.

The levels of miR-497 in a large cohort of 97 GC tissues (including the 36 samples used before) were examined to further investigate the clinicopathological and prognostic significance of miR-497 levels in patients with GC. The median value of all 97 GC samples was chosen as the cut-off point for separating tumors with low-level expression of miR-497 from high-level expression miR-497 tumors. Therefore, 49/97 (50.5%) GC cases had low-level

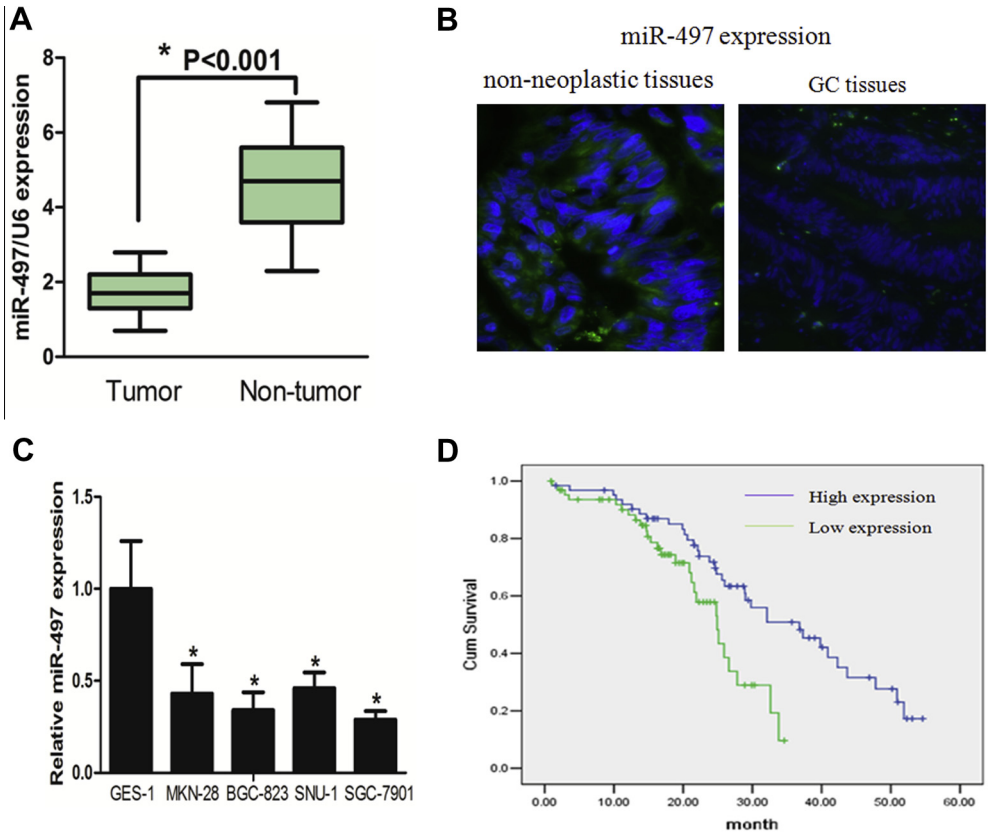

Fig. 1. Low expression of miR-497 was frequently found in GC tissues and associated with poorer overall survival. (A) The expression levels of miR-497 were significantly decreased in GC tissues compared to adjacent non-neoplastic tissues. (B) The in situ hybridization assay indicated that miR-497 was down-regulated in GC tissues. (C) The expression level of miR-497 was lower in GC cell lines compared with immortalized normal gastric mucosal epithelial cell line GES-1. (D) The Kaplane–Meier analysis revealed that low-level expression of miR-497 was associated with shorter overall survival of patients with GC.

Table 1
Correlation between the clinicopathologic characteristics and expression of miR-497 in GC.

Variables	miR-497			
	All case (n = 97)	Low expression (n = 49)	High expression (n = 48)	P value*
Age (years)				
⩽60	54	29	25	0.482
>60	43	20	23	
Gender				
Male	61	32	29	0.618
Female	36	17	19	
Differentiation				
High, middle	34	15	19	0.355
Low	63	34	29	
Location				
Distal third	40	19	21	0.619
Middle third, proximal third	57	30	27	
Local invasion				
T1, T2	33	11	22	0.015*
T3, T4	64	38	26	
Lymph node metastasis				
No	39	13	26	0.006*
Yes	58	36	22	
TNM stage				
I, II	43	19	24	0.266
III, IV	54	30	24	

* $P < 0.05$.

expression of miR-497, while 48/97 (49.5%) GC cases had high-level expression of miR-497. The miR-497 low-expression group was significantly associated with lymph node metastasis rate ($P = 0.006$) and local invasion ($P = 0.015$). However, the miR-497 expression levels did not show significant relationship with age, gender, tumor differentiation, tumor location and TNM stage (Table 1). The Kaplane–Meier analysis revealed that low-level expression of miR-497 was associated with shorter overall survival of patients with GC (Fig. 1D, $P < 0.05$).

3.2. eIF4E is a direct target of miR-497 in GC cells

Using the algorithms for target gene prediction, including miRanda and TargetScan, eIF4E was identified as one of the potential targets of miR-497. To verified that eIF4E was a direct target of miR-497, eIF4E wild-type (WT) or mutant 3′-UTR (Fig. 2A) was subcloned into a luciferase reporter vector and co-transfected with miR-497 mimics or negative control into SGC-7901 cells. The results showed that miR-497 significantly inhibited the luciferase activity of the eIF4E WT 3′-UTR but not that of the mutant in SGC-7901 cells (Fig. 2B). To directly assessed the effect of miR-497 on eIF4E expression, we transfected miR-497 into SGC-7901 cells and found that overexpression of miR-497 reduced the eIF4E mRNA and protein levels (Fig. 2C). Taken together, these results indicated that eIF4E was a direct target of miR-497 in GC cells.

3.3. Ectopic miR-497 expression alters GC cells proliferation and invasion by down regulation of eIF4E

Given that miR-497 might act as a tumor suppressor, we asked whether restoration of miR-497 could affect proliferation and invasion in GC cells. We found that the proliferation of cells transfected with miR-497 mimics was decreased compared with that of cells

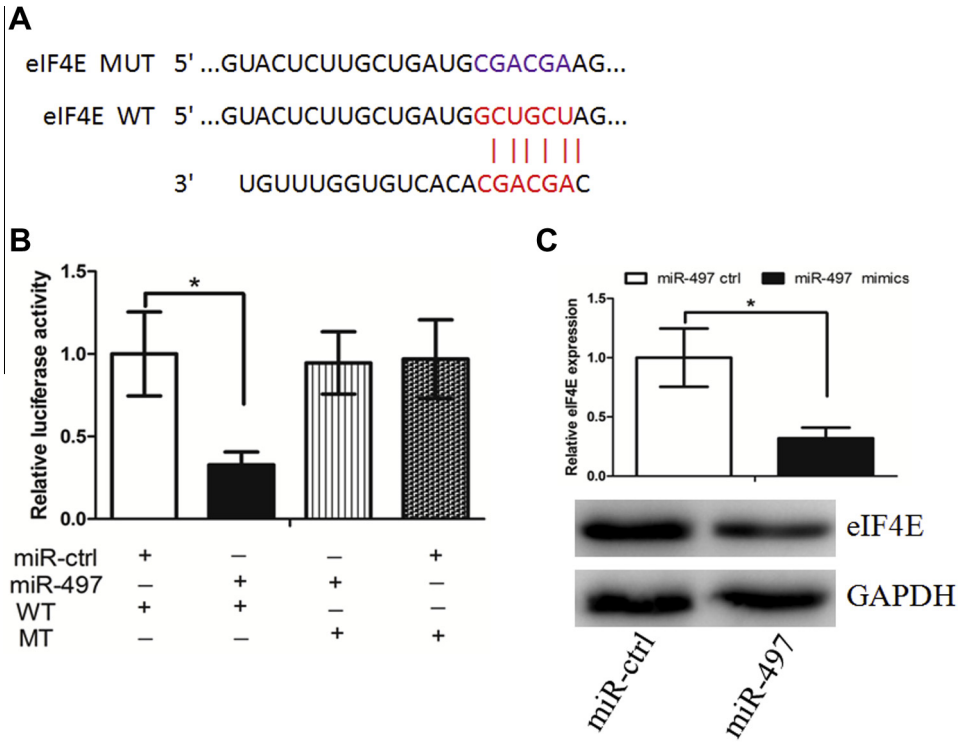

Fig. 2. eIF4E is a direct target of miR-497 in GC cells. (A) The wild type and mutant complementary sequences of the eIF4E mRNA 3′-UTR are shown with the miR-497 sequence. (B) Luciferase assay in SGC-7901 cells co-transfected with miR-497 and a luciferase reporter containing the eIF4E 3′-UTR (WT) or a mutant (Mut). (C) miR-497 transfection affects eIF4E mRNA and protein levels.

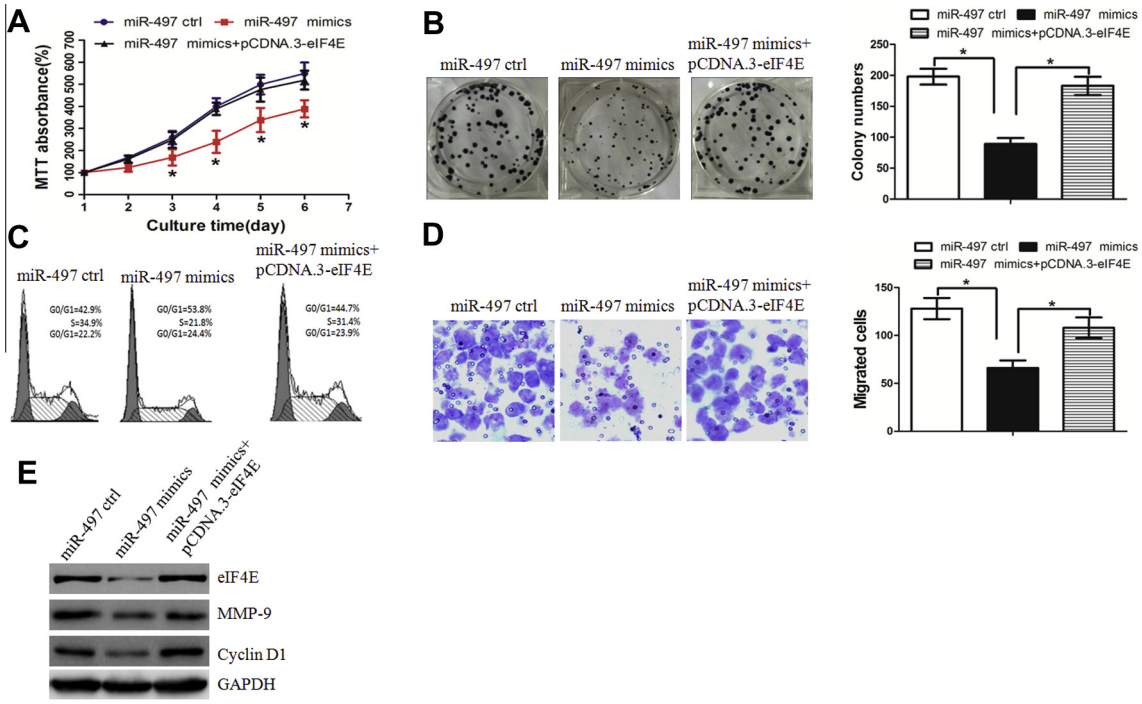

Fig. 3. Ectopic miR-497 expression alters GC cells proliferation and invasion by down regulation of eIF4E. (A) MiR-497 inhibited cell proliferation, while over-expression of eIF4E counteracted this effect. (B) MiR-497 decreased the colony formation ability of SGC-7901 cells, while ectopic expression of eIF4E abrogated the effect. (C) Restoration of eIF4E in miR-497-treated cells abrogated the miR-497-induced G1 cell cycle arrest. (D) The Boyden assay showed that miR-497 inhibited GC cell invasion, while this effect was abrogated by restoration of eIF4E. (E) Western blot assays showed that ectopic expression of eIF4E abrogated the miR-497-downregulated eIF4E, MMP-9 and cyclin D1.

transfected with miR-ctrl, according to the results of the MTT assay (Fig. 3A). A colony formation assay was performed to further demonstrate the anti-proliferative effect of miR-497 on GC cells. As shown in Fig. 3B, the colony numbers of SGC-7901 cells transfected with miR-497 mimics were significantly lower than those transfected with miR-ctrl. To further examined whether the effect of

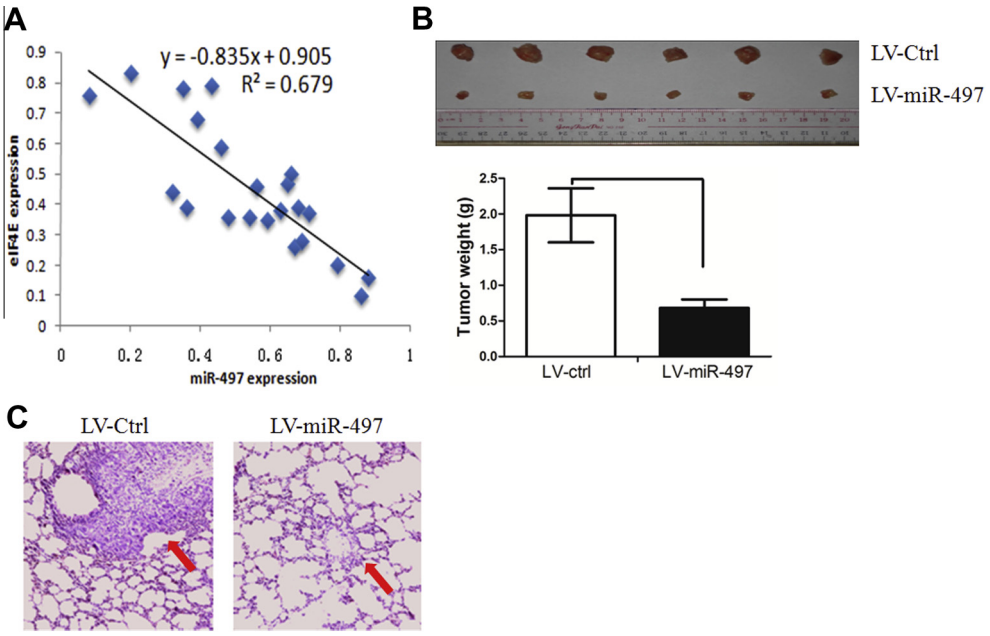

Fig. 4. The expression of miR-497 was inversely correlated with eIF4E protein level in GC tissues and miR-497 inhibited tumor growth and invasion in vivo. (A) The expression of miR-497 was inversely correlated with eIF4E protein level in GC tissues ($P < 0.05$). (B) Restoration of miR-497 decreased tumor growth in vivo. (C) SGC-7901 cells stably transfected with miR-497 established smaller lung metastatic colonies than mock group.

miR-497 on the proliferation of SGC-7901 cells reflected a cell-cycle arrest, cell-cycle progression was analyzed by flow cytometric analysis. The results revealed that over-expression of miR-497 increased the percentage of cells in the G1/G0 phase and decreased the percentage of S-phase cells (Fig. 3C). Based on these data, we proposed that miR-497 inhibited proliferation by decreasing the S-phase fraction of GC cells. We then investigated the effect of ectopic expression of miR-497 on cell invasion. The Boyden assay showed that miR-497-transfected SGC-7901 cells exhibited significantly decreased invasiveness when compared with the negative control group (Fig. 3D). We further showed that restoration of miR-497 affected the down-stream targets of eIF4E (Fig. 3E). Interestingly, with the restoration of eIF4E in mimic- transfected cells, the role of miR-497 overexpression in SGC-7901 cells was eliminated (Fig. 3A–E).

Taken together, these results suggest that miR-497 could functionally reduce cell proliferative and invasive potential by regulating eIF4E expression.

3.4. The expression of miR-497 is inversely correlated with the expression of eIF4E protein in GC tissues

We further measured the mRNA levels of eIF4E in 25 GC specimens and correlated them with miR-497 expression in the same specimens. As shown in Fig. 4A, when eIF4E mRNA levels were plotted against miR-497 expression, a significant inverse correlation was observed (2-tailed Spearman's correlation, $r = -0.835$; $P < 0.01$).

3.5. Restoration of miR-497 inhibited tumor growth and invasion in vivo

We next investigated the efficacy of miR-497 against tumor growth and invasion in vivo. Stable transfection of miR-497 into SGC-7901 cells resulted in decreased growth and tumor weight of subcutaneous xenograft tumors in nude mice, when compared with those stably transfected with empty vector (Fig. 4B). In the experimental metastasis studies, SGC-7901 cells stably transfected with miR-497 established smaller lung metastatic colonies than mock group (Fig. 4C). These results suggested that miR-497 could inhibit the growth and metastasis of GC cells in vivo.

4. Discussion

Despite the advancements in treatment options, improvements in GC patient survival have been limited owing to lack of early detection. Biomarkers to improve GC diagnosis, prognosis and prediction of treatment response therefore represent opportunities to improve patient outcome. In recent years, investigation of epigenetic biomarkers such as miRNA expression, have implicated that these alterations may be enticing translational biomarker candidates in GC. In the present study, we sought to provide evidence that down-regulation of miR-497 promoted proliferation and invasion of GC cells in vitro and in vivo.

In this study, expression of miR-497 in GC specimens was significantly lower than that in corresponding adjacent tissues. Decreased expression of miR-497 is associated with a more aggressive tumor phenotype in GC patients. In addition, GC patients with low expression of miR-497 had a poorer overall survival than those with high expression of miR-497.

EIF4E has been identified as an oncogene in many malignant diseases, including GC. Aberrant eIF4E expression has been linked to development, progression, and prognosis of multiple tumors by regulation of various cell signaling pathways. Silencing of eIF4E gave rise to slowed proliferation and arrested cell cycle inG0/G1 phase in cancer cells of larynx, stomach, and breast [21–23]. In addition, inhibition of eIF4E could decrease the invasion of cancer cells [24,25]. These findings indicated that targeting the eIF4E expression may provide a potential molecular target for the cancer therapy.

In the present study, the luciferase assay using a reporter containing the wild type miR-497 binding sequence at the 3′-UTR of eIF4E mRNA indicated that the luciferase activity could be significantly reduced by over-expression of miR-497. Restoration of miR-497 could down-regulate the expression of eIF4E mRNA and protein levels in SGC-7901 cells. Furthermore, miR-497 levels were

found to be inversely correlated with mRNA expression of eIF4E in GC tissues. By restoring miR-497 expression in GC cells, we found that miR-497 indeed suppressed cell proliferation through inducing G1 cell cycle arrest. Additionally, miR-497 inhibited SGC-7901 cell invasion. Interestingly, restoration of eIF4E can eliminate these effects of miR-497 overexpression. Furthermore, ectopic expression of miR-497 suppressed the downstream effectors of eIF4E. Taken together, these data suggested that miR-497 impacted on GC cells partially by inactivation of eIF4E.

In summary, our data provided evidence that decreased expression of miR-497 was associated with a more aggressive tumor phenotype and poorer overall survival in GC patients. In addition, we identified eIF4E as a direct target of miR-497 and restoration of miR-497 suppressed proliferation and invasion partially by inactivation of eIF4E in SGC-7901 cells. Restoration of miR-497 inhibited proliferation and invasion both in vitro and in vivo. As the limit on the number of GC samples and cell types, more elaborate studies will be necessary for further exploration of the potential role of miR-497 in development of GC. However, our findings on miR-497 are encouraging and suggest that this miRNA could be a potential target for the treatment of GC in the future.

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