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**Original Paper**

# Long Noncoding RNA EGFR-AS1 Promotes Cell Proliferation by Increasing EGFR mRNA Stability in Gastric Cancer

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**Key Words**
lncRNAs • Gastric cancer • EGFR-AS1 • EGFR • MRNA stability • PI3K

**Abstract**
***Background/Aims:*** LncRNA EGFR-AS1 is an antisense transcript of EGFR, which plays a key role in gastric cancer progression. This study was aimed to explore the effects of lncRNA EGFR-AS1 on GC and the underling mechanisms. ***Methods:*** The silencing of EGFR-AS1 expression was performed by using EGFR-AS1 shRNA lentivirus in MGC803 and SGC-7901 GC cell. The levels of lncRNA EGFR-AS1 and EGFR were detected by qPCR and western blot. Cell proliferation was assessed by CCK-8, EdU, and colony formation assays. The EGFR mRNA stability was explored by using RNA synthesis inhibitor α-amanitin. ***Results:*** In our study, EGFR-AS1 significantly up-regulated in GC tissues and correlated with tumor size. And the expression of EGFR-AS1 positively correlated with EGFR in tissues. Moreover, knock-down of EGFR-AS1 inhibited the proliferation of GC cells via suppressing EGFR-dependent PI3K/AKT pathway *in vitro* and *in vivo*. Mechanismly, depletion of EGFR-AS1 was found to decrease EGFR expression by reduction of EGFR mRNA stability. ***Conclusion:*** Our findings suggested that EGFR-AS1 might have an oncogenic effect on GC and serve as a potential target of GC.



## Introduction

Gastric cancer (GC) is one of the most common malignancies and the second leading cause of cancer-related death worldwide [1, 2]. Despite the diagnostic and treatment have

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developed quickly, the 5-year overall survival rate remains unsatisfactory [3]. Molecular targeted therapy could reduce side effects and provides a good new way for the treatment of GC [4-6]. Therefore, revealing the underlying molecular mechanisms that contribute to gastric carcinogenesis is essential for the development of novel effective therapies for GC patients.

Long noncoding RNAs (lncRNAs) is a class of functional RNAs that transcripts longer than 200 nucleotides and without protein coding potential [7, 8]. In recent years, thousands of lncRNAs have been discovered and findings have suggested that lncRNAs have vital roles in GC [9-15]. However, the functions and mechanisms of lncRNAs responsible for the development and progression of GC still need to be understood.

Epidermal growth factor receptor (EGFR) belongs to the family of receptor tyrosine kinases ErbB [16]. EGFR plays an important role in cell migration, proliferation, cell cycle regulation and other physiological processes [7]. EGFR is up-regulated in various cancers including gastric cancer [12, 13], and is recognized as oncogenic driver in tumorigenesis. LncRNA EGFR antisense RNA 1 (EGFR-AS1) is a 2.8-kb transcript that transcribes in the antisense strand of EGFR [17, 18]. Reports have shown that overexpression of EGFR-AS1 was sufficient to induce resistance to tyrosine kinase inhibitors and knockdown of EGFR-AS1 induces sustained tumor regression of squamous cell carcinoma [17]. EGFR-AS1 was relatively up-regulated in hepatocellular carcinoma (HCC) tissues and was determined to improve the ability of invasion and proliferation of HCC cells *in vitro* and *in vivo* via increasing the expression of EGFR [18]. However, the biological functions of EGFR-AS1 in GC have not been reported.

In the present study, we focused on the investigation of the function of EGFR-AS1 in GC. We analyzed the expression level of EGFR-AS1 in tumor and adjacent tissues and found that EGFR-AS1 was significantly upregulated in GC tumor tissues. We demonstrated that EGFR-AS1 up-regulates EGFR expression through enhancing EGFR mRNA stability, thus resulting in activating the EGFR-dependent pathway and promoting the proliferation of gastric carcinoma cells *in vitro* and *in vivo.*

## Materials and Methods

*Ethical conduct of research*

All the patients provided their written informed consent in accordance with the Declaration of Helsinki before enrollment in the study. The study was approved by the Institutional Review Board of the Nanjing Medical University (Nanjing, China).

*Patients and samples*

A total of 58 GC tissues and pair adjacent normal gastric tissues were obtained with informed consent from patients who underwent radical resection surgery at the First Affiliated Hospital of Nanjing Medical University. No patient had the history of exposure to either radiotherapy or chemotherapy before the surgery, and no other co-occurrence cancers was diagnosed. This study was performed with the approval of the Medical Ethics Committee of the Nanjing Medical University.

*Cell culture*

The human gastric epithelial cell line GES-1 and GC cell lines (SGC7901, BGC823, MGC803 and MKN-28) were purchased from the Cell Bank of the Chinese Academy of Science (Shanghai, China). The cells were grown in RPMI Medium 1640 (Gibco, CA, USA) supplemented with 10% fetal bovine serum (Gibco, CA, USA), 100 U/ml penicillin and 100 mg/ml streptomycin in a humidified incubator of 5% $CO_2$ and 95% air at 37 °C.

*RNA stability assay*

For the analysis of RNA stability, GC cells were treated with α-amanitin (50 μg/ml, Sigma-Aldrich, USA). Cells were collected at different time points (0, 6, 12, 18 and 24 h) and total RNA were extracted using Trizol

reagent (Invitrogen, USA). The level of mRNA was measured by qRT-PCR and normalized to 18S rRNA, the percentage of remaining RNA were relative to time 0.

*Plasmid construction*

The DNA segments of EGFR-AS1-shRNA (shRNA-1 and shRNA-2) and scramble negative controls (shNC) were synthesized by GENEWIZ (Suzhou, China). The annealed duplex DNA was ligated into the 5'Xho I and 3'Not I sites of the lentiviral pLL3.7-GFP vector. The EGFR-AS1 shRNA sequences are listed in Table 1.

*Lentivirus Production and Infection*

Recombinant lentivirus was generated from 293T cells by co-transfection of pdelta-8.91 and pVSVG together with pLL3.7-shNC and pLL3.7-shRNA1/2 using Lipofectamine 2000 (Invitrogen, CA, USA). Lentivirus particles were harvested at 48 and 72 hours after transfection, and filtered through a 0.45μm filter, followed by concentrating 100-fold via ultracentrifugation. The virus-containing pellet was re-suspended in DMEM, then aliquoted and stored at -80°C. Lentivirus particles were added to the GC cells in the presence of polybrene (Sigma, St. Louis, MO, USA). The supernatant was replaced with complete culture media after 24 hours. The interference efficiency of EGFR-AS1 in the infected cells was confirmed by qRT-PCR at 48 hours after infection.

*Cell proliferation assays*

The cells were seeded in 96-well plates and measured using Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). Cell proliferation was determined every 24 h for 4 days. The optical density was measured with a microplate reader (Bio-Rad, Hercules, CA, USA). For the colony formation assay, $1.5 \times 10^3$ cells were seeded in a six-well plate. After 14 days, colonies were fixed and stained with 0.05% crystal violate (Invitrogen, Carlsbad, CA, USA) before counting. EdU immunofluorescence staining were performed using an EdU kit (Roche, Mannheim, Germany). Briefly, cells were incubated with EdU (50μM) for 5 hours, fixed with 4% paraformaldehyde and followed with permeabilization using PBS containing 0.5% Triton X-100. Then the cells were incubated in Apollo staining solution, then repeated permeation and wash, and incubated in Hoechst 33342. All experiments were performed in triplicate.

*RNA extraction and quantitative RT-PCR (qRT-PCR)*

Total RNA was extracted from cells using a TRIzol reagent kit (Invitrogen, CA, USA). Subsequently, RNA was reverse transcribed to cDNA from 1μg of total RNA using Oligo(dT) and random 6 mers oligos for priming. The qRT-PCR was carried out using the SYBR Premix Ex Taq in the StepOne Plus system (Applied Biosystems, Foster City, CA). The expression level of each gene was normalized by 18S rRNA and reported as relative levels ($2^{-\Delta\Delta CT}$ method). The primers for qRT-PCR are listed in Table 1.

**Table 1.** Primers and RNA sequences used in this study

| oligonucleotide sequences | Sequence (5'to 3') |
|---|---|
| primers for Real-time PCR | |
| EGFR-AS1-qF | GGCCATCACGTAGGCTTCCT |
| EGFR-AS1-qR | TGCGTCTTCACCTGGAAGGG |
| EGFR-qF | CCAAGGCACGAGTAACAAGCT |
| EGFR-qR | GCACATAGGTAATTTCCAAA |
| 18s rRNA-qF | TTAATTCCGATAACGAACGAGA |
| 18s rRNA-qR | CGCTGAGCCAGTCAGTGTAG |
| U6-qF | CGCTTCGGCAGCACATATACTA |
| U6-qR | CGCTTCACGAATTTGCGTGTCA |
| GAPDH-qF | ACAACTTTGGTATCGTGGAAGG |
| GAPDH-qR | GCCATCACGCCACAGTTTC |
| Oligos for plasmid construction | |
| EGFR-AS1-sh1 | TCGAGCGCAGCATCTCCTGATTACCTATTCAAGAGATAGGTAATCAGGAGATGCTGTTTTTTTGC |
| EGFR-AS1-sh2 | TCGAGCGCAGGCACTGATTTGTGCACAATCAAGAGTTGTGCACAAATCAGTGCCTGTTTTTTTGC |
| EGFR-AS1-shNC | TCGAGCGGCTTCTAACACCGGAGGTCTTTCAAGAGAAGACCTCCGGTGTTAGAAGCTTTTTTTGC |

*Western blot analysis*

The proteins were extracted as previously described [19]. Equal amounts of protein (30 μg) were loaded into and separated by 10% or 12% SDS–PAGE and transferred onto nitrocellulose membranes (Millipore, MA, USA). Then incubated at room temperature in block buffer BSA (5%w/v in PBS) and followed by incubating with specific antibodies. Primary antibodies against GAPDH (Bioworld Technology), EGFR, p-EGFR, PI3K and p-PI3K (Cell Signaling Technology) were incubated overnight at 4°C The signal was detected with horseradish peroxidase-conjugated secondary antibodies and revealed using an ECL kit (Thermo Scientific). GAPDH protein was used as an internal control. Each experiment was repeated at least 3 times.

*Subcellular fractionation location*

RNA of nuclear and cytosolic fractions was separated using the PARIS Kit (Life Technologies, Carlsbad, CA, USA) as previously described [19]. The expression of U6, GAPDH and EGFR-AS1 were detected by qRT-PCR. The primers for qRT-PCR are listed in Table 1.

*Xenograft HCC mice model*

Approximately 1 × $10^6$ cells stably infected with EGFR-AS1 shRNA were harvested and injected subcutaneously into 4-week-old BALB/C nude mice. Tumor growth were measured every week from injection and calculated with the formula (length × width$^2$ × 0.5). After 5 weeks, mice were sacrificed, and the tumors were excised and measured. All animal experiments were conducted in accordance with the institutional standard guidelines of Nanjing Medical University and all experimental protocols were approved by the Use Committee for Animal Care of Jiangsu Province.

*Statistical analysis*

Statistical analyses were performed using SPSS 20.0 and GraphPad Prism 7. Expression of EGFR-AS1 in samples between GC tissues and adjacent gastric tissues was compared by a Wilcoxon signed-rank test. Correlations between the EGFR-AS1 expression levels and pathological features were analyzed with the chi square ($\chi 2$) test. The Student t-test or one-way ANOVA was used to compare the results and expressed as mean ± s.d. between any two preselected groups. Pearson's correlation coefficient was calculated using Prism7 software (GraphPad). A $P$ value < 0.05 was considered statistically significant.

## Results

*The antisense transcribed lncRNA EGFR-AS1 upregulated in GC tissues and cell lines*

Firstly, we tested EGFR-AS1 expression in GC tissues and pair-matched adjacent gastric tissues from 58 GC patients by quantitative RT-PCR (qRT-PCR). As shown in Fig. 1A, EGFR-AS1 expression was significantly increased in GC tissues compared with adjacent gastric tissues

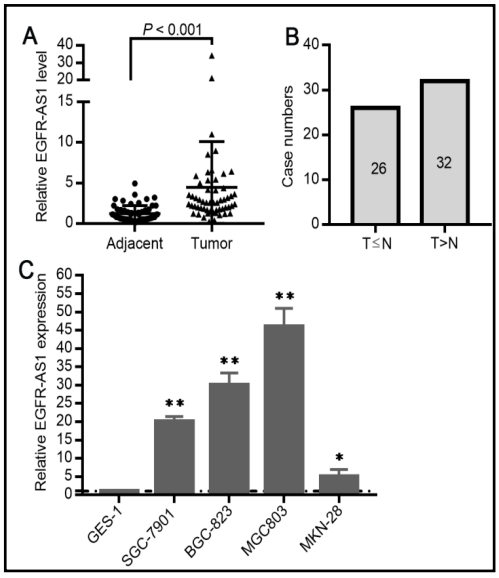


**Fig. 1.** EGFR-AS1 is highly expressed in GC specimens and cell lines. (A) The expression of EGFR-AS1 in 58 paired GC and adjacent gastric tissues were detected by qRT-PCR, the 18S rRNA served as internal control. Significant differences were analyzed according to the Wilcoxon signed-rank test (P<0.05 are considered as significant). (B) The expression of EGFR-AS1 was at higher level in 55% tumor tissues as compared to adjacent gastric tissues (32/58). T ≤ N: The expression of EGFR-AS1 in GC tissues was less than or equal to that in paired adjacent gastric tissues. T > N: The expression of EGFR-AS1 in GC tissues was more than that in paired adjacent gastric tissues. (C) The levels of EGFR-AS1 were detected in GES-1 and four human GC cell lines by qRT-PCR. The values indicate the mean ± s.d. for three separate experiments (*P<0.05, **P<0.01, ANOVA).

(Wilcoxon signed-rank test, $P < 0.001$). Compared to corresponding adjacent gastric tissues, the expression level of EGFR-AS1 was upregulated in 55% tumor tissues (32/58) (Fig. 1B).

To investigate the correlation between EGFR-AS1 expression and patient clinical features, patients of GC were divided into EGFR-AS1 high and low expression groups depending on whether EGFR-AS1 expression was upregulated compared with the corresponding adjacent gastric tissues.) (Table 2). Here we found that higher EGFR-AS1 expression levels (67.6%) were significantly correlated with larger tumor size ($P = 0.023$). However, there was no significant correlation between EGFR-AS1 expression and other clinicopathological features.

We further examined the expression level of EGFR-AS1 in normal gastric epithelium cell line GES1 and GC cell lines (SGC7901, BGC823, MGC803 and MKN-28). EGFR-AS1 expression in GC cell lines were significantly higher than in normal gastric epithelium cell line GES1 (Fig. 1C). All of the results above indicated a potential vital oncogenic role of EGFR-AS1 in GC.

*Knockdown of EGFR-AS1 suppressed GC cell proliferation in vitro*

As EGFR-AS1 was significantly upregulated in GC and associated with tumor size of patients, we next explored whether EGFR-AS1 plays a vital role in the cell proliferation of GC. MGC803 and SGC-7901 cell lines were selected for further study. We silenced the expression of EGFR-AS1 by using EGFR-AS1 shRNA lentivirus (two specific shRNAs target EGFR-AS1), and the interference efficiency was evaluated by qRT-PCR (Fig. 2A). Then, shRNA-2 was selected for following experiments due to its higher interference efficiency. CCK8 assay showed that cells displayed a markedly reduced proliferation rate with knockdown of EGFR-AS1 in both of MGC803 and SGC-7901 cells (Fig. 2B). EdU staining assay demonstrated that decreased EGFR-AS1 contributed to less EdU-positive cells than control (Fig. 2C). To further confirm our observation, colony-formation assay was performed. Findings showed that silenced EGFR-AS1 expression significantly inhibited the clonogenicity of the MGC803 and SGC-7901 cells (Fig. 2D). These results suggest that EGFR-AS1 plays an important role in regulating GC cell proliferation.

*EGFR was positively correlated with the level of EGFR-AS1 in GC tissues*

EGFR-AS1 is transcribed in the opposite strand of EGFR. The genomic location region of EGFR-AS1 and EGFR is shown in Fig. 3A. The RNA-seq projects of PRJNA280600 and PRJEB4337 which are provided by NCBI database (Fig. 3B and 3C) and the GTEX RNA-seq

**Table 2.** Correlation between EGFR-AS1 expression and clinicopathological characteristics of gastric cancer patients (n = 58) **#**EGFR-AS1 levels in tumor tissues were categorized as low or high depending on whether EGFR-AS1 expression was upregulated compared with the corresponding adjacent gastric tissues. TNM, Tumor node metastasis; *, P < 0.05

| | EGFR-AS1 expression# | | | |
|---|---|---|---|---|
| Characteristics | Low（n = 26） | High（n = 32） | Chi-square | P value |
| Age | | | 0.120 | 0.655 |
| ≤ 60 | 19 | 25 | | |
| > 60 | 7 | 7 | | |
| Gender | | | 0.104 | 0.747 |
| Male | 16 | 21 | | |
| Female | 10 | 11 | | |
| Differentiation grade | | | 0.586 | 0.444 |
| Well and Moderate | 14 | 14 | | |
| Poorly | 12 | 18 | | |
| Tumor size | | | 5.17 | 0.023* |
| < 5cm | 15 | 9 | | |
| > 5cm | 11 | 23 | | |
| TNM stages | | | 0.330 | 0.567 |
| I-II | 16 | 22 | | |
| III-IV | 10 | 10 | | |
| Metastasis | | | 0.22 | 0.639 |
| Yes | 17 | 19 | | |
| No | 9 | 13 | | |

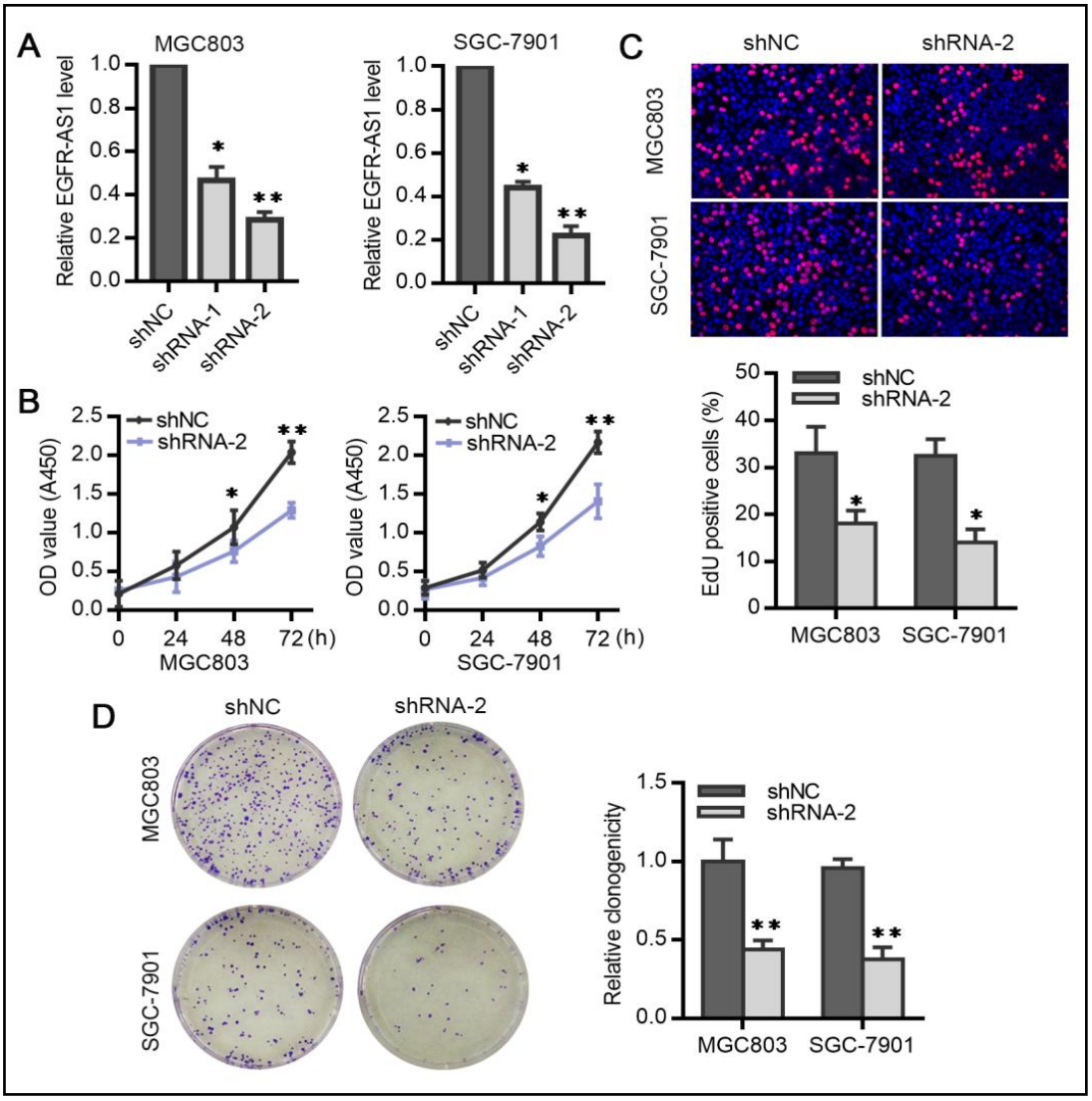


**Fig. 2.** EGFR-AS1 affect gastric carcinoma cell growth.(A) Expression level of EGFR-AS1 in MGC803 and SGC-7901 cells after infected with shNC and shEGFR-AS1-expressing lentivirus (represented by shNC or shRNA1/2). The values indicate the mean ± s.d. for three separate experiments (*P<0.05, **P<0.01, ANOVA). (B) CCK-8 assay was detected in different time points including 0, 24, 48 and 72 h showed a decreased level of EGFR-AS1 inhibited the growth in MGC803 and SGC-7901 cell lines. The values indicate the mean ± s.d. for three separate experiments (*P<0.05, **P<0.01, ANOVA). (C) EdU staining analysis of cell proliferation. The blue color represents the nuclei, and the red color indicates EdU-positive nuclei (upper). The graphs show the percentage of EdU-positive nuclei (lower). The values indicate the mean ± s.d. for three separate experiments (*P<0.05, Student t-test). (D) Colony-forming assays were used to determine the colony ability of shRNA-2-infected MGC803 and SGC-7901 cells. The colonies were counted and are depicted in a bar chart (right panel). The values indicate the mean ± s.d. for three separate experiments (**P<0.01, Student t-test).

projects provided by UCSC database (Fig. 3D) show a similar expression pattern between EGFR and EGFR-AS1, especially with a very high level in placenta compared to other normal tissues (Fig. 3B and 3C). The database analysis suggests that EGFR-AS1 may have positive correlation with EGFR. Thus, we evaluated the correlation between EGFR-AS1 and EGFR. The mRNA levels of EGFR in 58 paired tumors and adjacent nontumor tissues were quantified by qRT-PCR, and we found a significantly increased level of EGFR in tumor group (Fig. 4A).

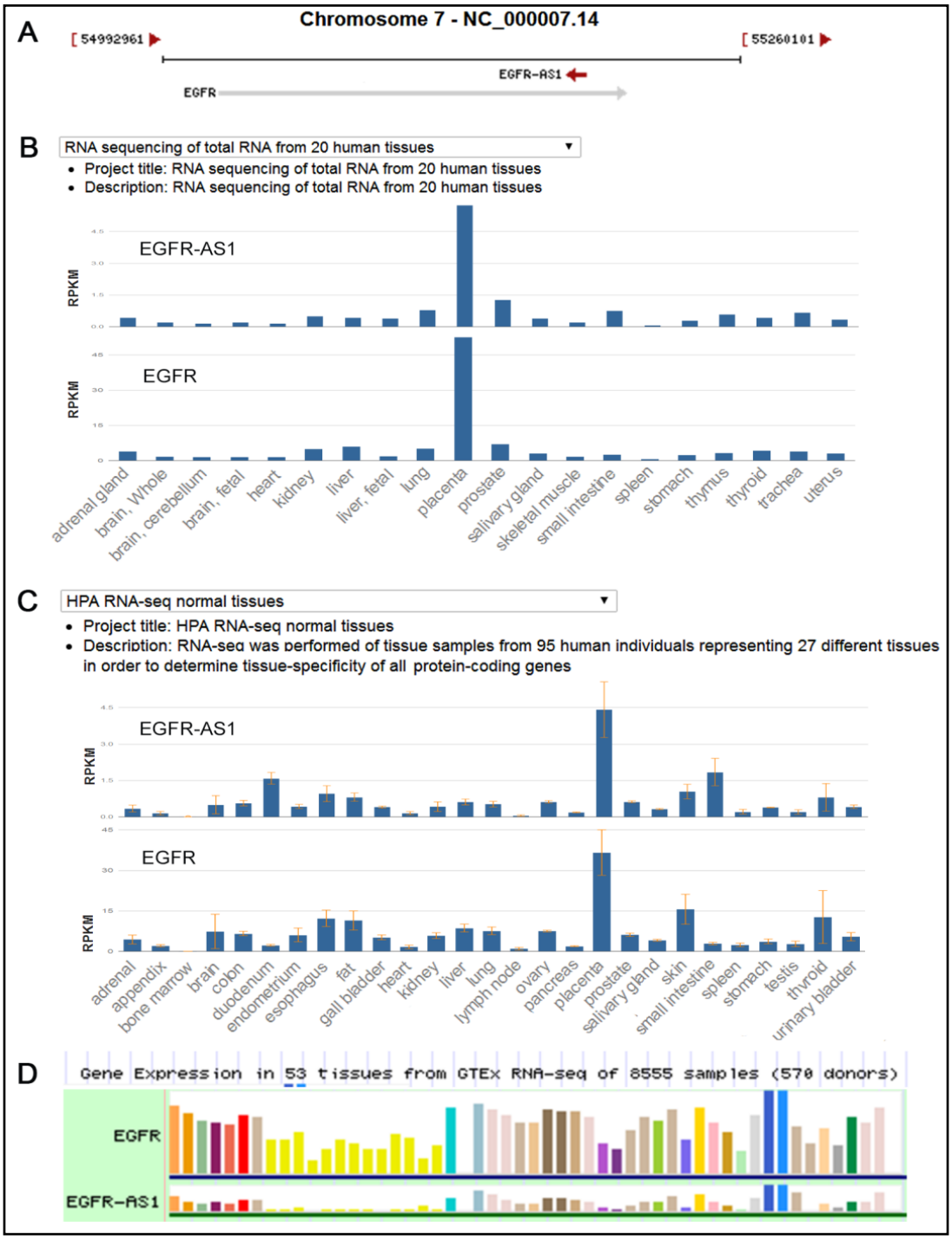


**Fig. 3.** Positive correlation between the level of EGFR and EGFR-AS1 in GC tissues.(A) Showing the genomic region of EGFR and EGFR-AS1 in NCBI database; arrows show the direction of transcription. Human EGFR-AS1 is transcribed on the opposite strand. (B) The relative expression level of EGFR and EGFR-AS1 by RNA sequencing of total RNA from 20 human tissues in BioProject: PRJNA280600, and provided by NCBI database. (C) The relative expression level of EGFR and EGFR-AS1 by HPA RNA-seq of 27 different normal tissue samples from 95 human individuals in BioProject: PRJEB4337, and provided by NCBI database. (D) The relative expression level of EGFR and EGFR-AS1 in 53 tissues from GTEX RNA-seq of 8555 samples of 570 donors, and provided by UCSC database.

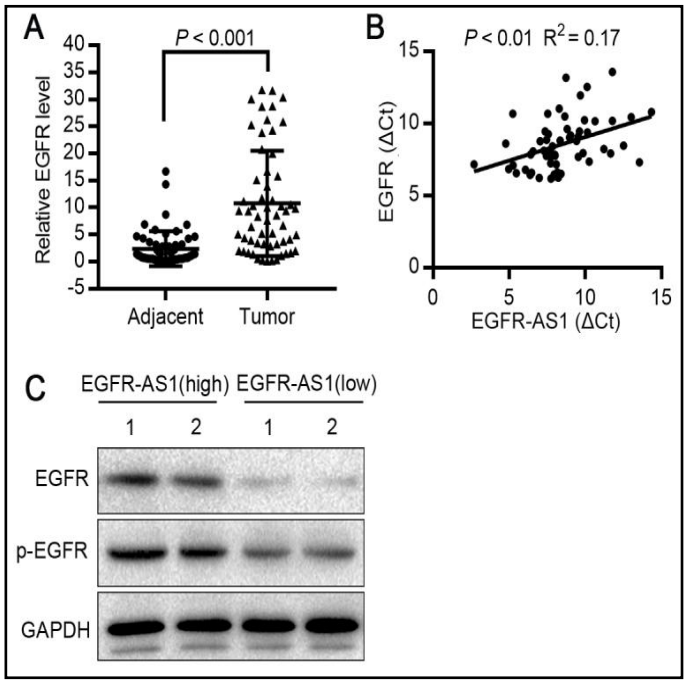


**Fig. 4.** positive correlation between the level of EGFR and EGFR-AS1 in GC tissues.(A) qRT-PCR analysis of EGFR expression in 58 paired GC and adjacent gastric tissues, the 18S rRNA served as internal control. Significant differences were analyzed according to the Wilcoxon signed-rank test (P<0.05 are considered as significant, Student t-test). (B) The correlation between EGFR mRNA level and EGFR-AS1 level was be analyzed in tissues. The ∆Ct values (normalized to 18S rRNA) were subjected to Pearson correlation analysis. (C) Patients with GC were divided into EGFR-AS1 high/low-expression groups based on whether EGFR-AS1 expression was upregulated compared with the corresponding adjacent gastric tissues. The expression of EGFR protein was compared in the two groups by western blot assay. GAPDH served as the internal control.

A significantly positive correlation was also observed between the expression of EGFR and EGFR-AS1 ($R^2$ = 0.17, $P$ < 0.01, Pearson correlation analysis, Fig. 4B). Furthermore, the protein level of EGFR was also higher in EGFR-high samples compared to EGFR-low samples of the GC tissues (Fig. 4C).

*Knockdown of EGFR-AS1 inhibited EGFR expression via reducing EGFR mRNA stability*

We observed above that the expression of EGFR was positively correlated with EGFR-AS1. Thus, we investigated whether EGFR-AS1 could influence the expression of EGFR. The knockdown efficiency of EGFR-AS1 in MGC803 and SGC-7901 cells were detected (Fig. 5A). The mRNA and protein expression of EGFR were dramatically decreased in EGFR-AS1 knockdown cells (Fig. 5B and 5C). EGFR-dependent PI3K/AKT pathway plays a critical role in cell proliferation [20-22]. Consequently, we aim to investigate whether EGFR-AS1 promoted GC cell proliferation through activation of EGFR-dependent PI3K/AKT pathway. Results showed that EGFR-AS1 knockdown resulted in inhibition of the phosphorylation of EGFR and PI3K (p-EGFR and p-PI3K) in MGC803 cells treated with shRNA. (Fig. 5C).

We further identified the underlying mechanism for the upregulated expression of EGFR by EGFR-AS1. Because the EGFR-AS1 is transcribed on the opposite strand of EGFR, we analyzed the RNA sequence base pairing between EGFR-AS1 and EGFR mRNA. As shown in Fig. 5D, a long base-pairing with a length of 194bp has been predicted by NCBI BLAST (http://blast.ncbi.nlm.nih.gov/). It has been reported that a long base-pairing (104bp) of BACE1-AS and BACE1 mRNA promoted the mRNA stability of BACE1. Therefore, we tested the effect of EGFR-AS1 on EGFR mRNA stability. Cells were treated with RNA synthesis inhibitor α-amanitin or DMSO (negative control), then the level of EGFR mRNA was measured by qRT-PCR and the percentage of remaining mRNA were relative to time 0. Results showed a decreased stability of EGFR mRNA in EGFR-AS1 silenced MGC803 cells, however the knockdown of EGFR-AS1 did not altered the GAPDH (negative control) mRNA stability (Fig. 5E). Collectively, our data demonstrated that knockdown of EGFR-AS1 decreased EGFR expression by reducing EGFR mRNA stability. Finally, we also showed that EGFR-AS1 localized both in the cytosol and nucleus in MGC803 cells (Fig. 5F), indicating a wide range of regulatory mechanism of EGFR-AS1 for target gene.

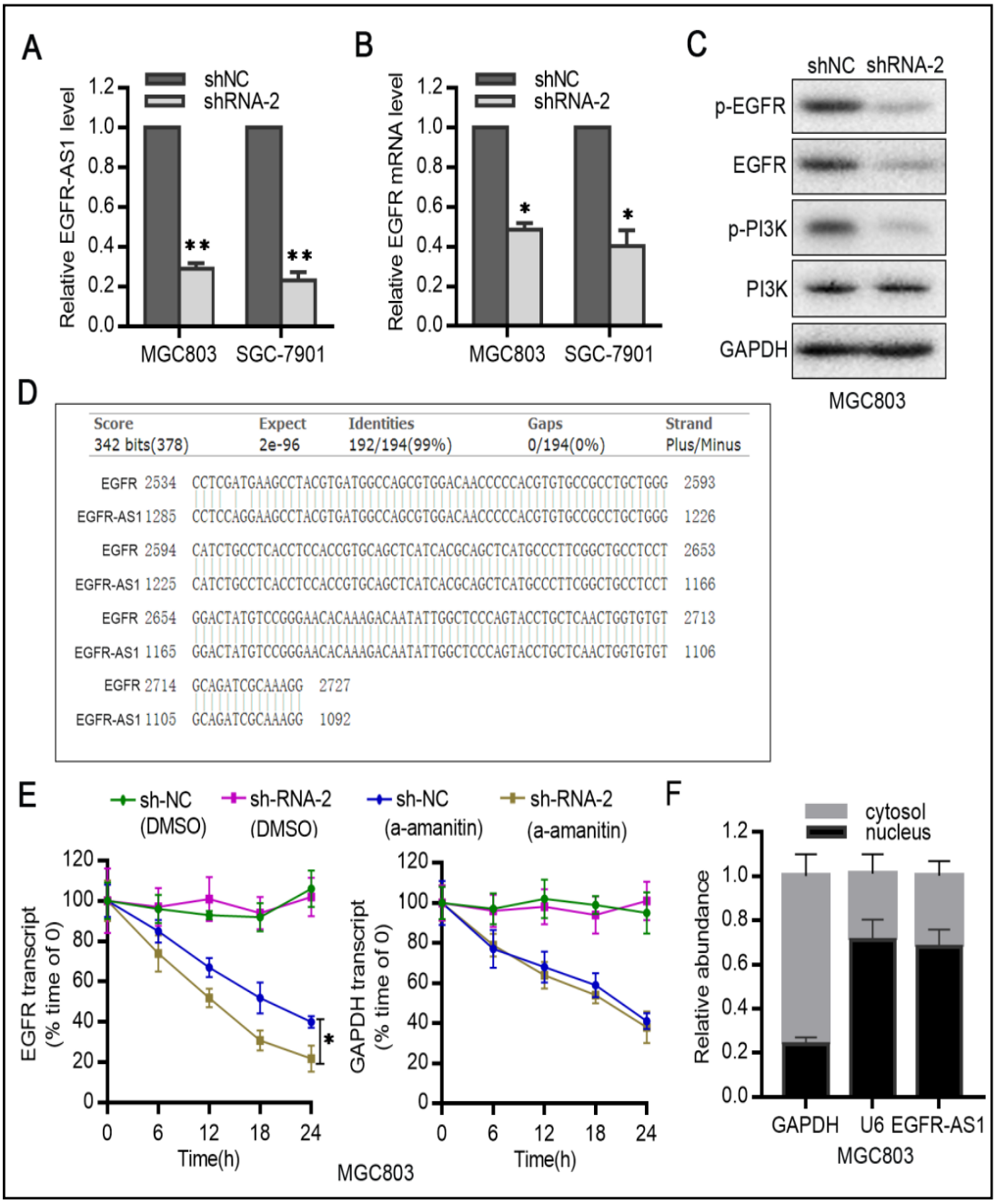


**Fig. 5.** knockdown of EGFR-AS1 decrease EGFR expression by reducing EGFR mRNA stability. (A) EGFR-AS1 expression levels after infection of EGFR-AS1 shRNA or control in MGC803 and SGC-7901 cells. The values indicate the mean ± s.d. for three separate experiments (*P<0.05, **P<0.01, Student t-test). (B) The relative levels of EGFR mRNA were examined by qRT-PCR, knockdown of EGFR-AS1 decrease EGFR mRNA expression. (C) Western blot assay showing the protein level of EGFR, p-EGFR, PI3K and p-PI3K upon silencing of EGFR-AS1 in MGC803. GAPDH served as the internal control. (D) The sequence presented here were the overlap region of EGFR-AS1 with EGFR mRNA (194 bp). (E) The mRNA stability of EGFR and GAPDH were tested by qRT-PCR every 6 h relative to time 0 after using α-amanitin (50 μM) (RNA synthesis inhibitor) or dimethylsulfoxide (DMSO, negative control) in MGC803 cells, and normalized to 18S rRNA. The values indicate the mean ± s.d. for three separate experiments (*P<0.05, ANOVA). (F) GAPDH, U6 and EGFR-AS1 RNA expression were detected in separated cytoplasm and nuclear RNA in MGC803 cells by qRT-PCR. GAPDH was used as the control for cytoplasmic expression and U6 for nuclear expression.

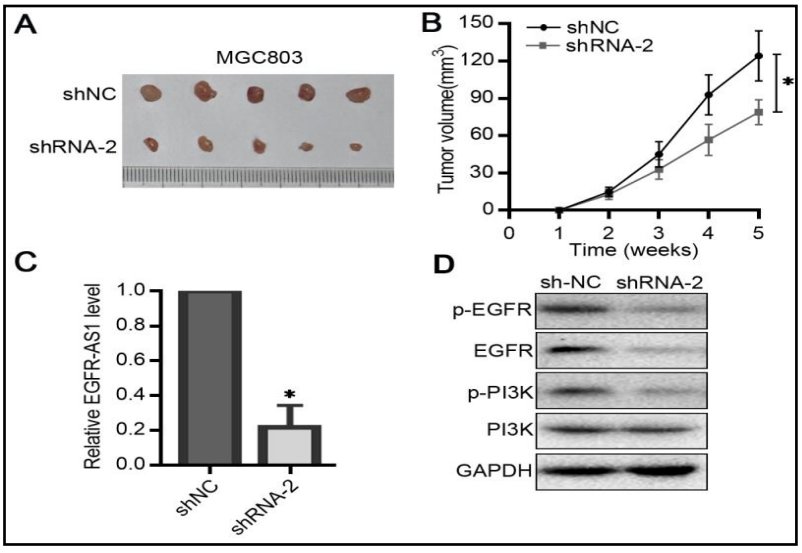


**Fig. 6.** Inhibition of tumor growth by knockdown of EGFR-AS1 in vivo.(A) Photograph of actual tumor after removal from mice at 5 weeks after injection of MGC803 cells infected with EGFR-AS1 shNC and shRNA-2. (B) The volume of the subcutaneous tumors were measured after injection every week. The tumor growth curve shows that knockdown of EGFR-AS1 significantly inhibited tumor growth in the mice. Data are the mean ± s.d., Asterisk indicates a significant change (*P<0.05, **P<0.01). (C) EGFR-AS1 expression levels were detected by qRT-PCR in tumors harvested form mice. 18S rRNA was used as the control. The values indicate the mean ± s.d. (*P<0.05, Student t-test). (D) The protein level of EGFR, p-EGFR, PI3K and p-PI3K were detected by western blot in tumors harvested form mice. GAPDH served as the internal control.

*EGFR-AS1 knockdown inhibited tumorigenesis of GC cells in vivo*

We examined whether EGFR-AS1 influenced the growth of GC cells in nude mice *in vivo*. EGFR-AS1 silenced MGC803 cells (shRNA-2) or control cells (shNC) were subcutaneously injected into BALB/C nude mice, and tumor size was monitored every week. The results demonstrated that cells with EGFR-AS1 silenced MGC803 cells developed significantly smaller tumors than control cells (Fig. 6A), and as presented in Fig. 6B, knockdown of EGFR-AS1 significantly suppressed the tumor growth curve compared with negative control group. Moreover, inhibition of EGFR-AS1, p-EGFR and p-PI3K was also observed in tumors of mice with EGFR-AS1 silenced MGC803 cells injection (Fig. 6C and 6D).

## Discussion

LncRNAs have attracted great interest recent years due to its ability involved in multiple cellular processes, including proliferation, apoptosis, cell migration and invasion [23, 24]. LncRNAs display complicated regulatory function by a wide range of regulatory mechanism through transcriptional regulation, epigenetic modulation through chromatin modification and post-transcriptional regulation [25-30]. However, the involvement of lncRNAs in human GC development and progression are still need to be illustrated.

LncRNA EGFR-AS1 originates from the opposite strand of EGFR [17, 18]. EGFR-AS1 has been reported relatively high expressed and up-regulated the expression of EGFR in HCC tissue, thus promoting HCC development by improving the ability of invasion and proliferation of HCC cells *in vitro* and *in vivo* [18]. EGFR-AS1 knockdown was sufficient to induce sustained tumor regression of squamous cell carcinoma *in vivo* by mediating EGFR addiction and activation of the EGFR pathway [17]. Thus, we attempted to investigate the role and molecular mechanism of EGFR-AS1 in gastric cancer.

We report here, for the first time, that EGFR-AS1 was expressed to a high degree in GC samples compared with adjacent gastric tissues. We observed that high expression level of EGFR-AS1 in GC patients was significantly correlated with tumor size, but no significant correlation between EGFR-AS1 expression and other clinicopathological features were

found. Depletion of EGFR-AS1 expression inhibited GC cell proliferation *in vitro* and *in vivo*. These data suggest that EGFR-AS1 serves as an oncogenic lncRNA in gastric cancer. The expression of EGFR in clinical samples was found to be significantly higher in GC tissues than in adjacent non-tumor tissues, which is consistent with other reports [31]. Also, a significantly positive correlation was observed between the expression of EGFR and EGFR-AS1. Additionally, decreased level of EGFR was found upon the knockdown of EGFR-AS1 expression. We also detected the activation of EGFR-dependent PI3K/AKT pathway, which played an important role in cell proliferation [20-22]. Our findings suggest that EGFR-AS1 induction of cell proliferation through activation of EGFR-dependent PI3K/AKT pathway *in vitro* and *in vivo*.

We further to identify the underlying molecular mechanism of EGFR-AS1 upregulated EGFR expression. LncRNAs have been reported to promote mRNA decay and repress the translation of mRNA by short partial base-pairing with specific target mRNAs [32, 33]. On the contrary, longer base-pairing (104bp) of BACE1-AS (BACE1 antisense lncRNA) and BACE1 mRNA promoted the mRNA stability of BACE1 by protection of BACE1 mRNA protected from degradation [34]. A long base-pairing with a length of 194bp has been predicted between EGFR-AS1 and EGFR mRNA. Therefore, we investigated whether EGFR-AS1 promote the expression of EGFR through affecting mRNA stability. Results demonstrated that silence of EGFR-AS1 decreased EGFR mRNA stability. Collectively, our data demonstrate that knockdown of EGFR-AS1 decreases EGFR expression by reducing mRNA stability. What are the mechanisms about the upregulation of EGFR-AS1 in GC samples? The gene region with H3K4me1 and/or H3K27Ac modification of histone proteins is suggestive of enhancer and, to a lesser extent, other regulatory activity, which were observed on the EGFR-AS1 in 7 tumor cell lines by ChIP-seq results provided by UCSC database [35, 36]. Whether upregulation of EGFR-AS1 in GC due to the modification status of histone H3, and other mechanisms regarding EGFR-AS1 regulate EGFR expression are still need to be investigated in the future.

## Conclusion

In conclusion, EGFR-AS1 expression were significantly increased in GC tissues and cell lines. Additionally, knockdown of EGFR-AS1 inhibited cell proliferation *in vivo* and *in vitro* through suppression of EGFR expression by reducing EGFR mRNA stability. Our findings suggest that high expression of EGFR-AS1 may have an oncogenic effect in GC, and may serve as potential targets for GC therapy in the future.

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## Disclosure Statement

The authors declare no conflicts of interest.

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