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MicroRNA-582-5p suppressed gastric cancer cell proliferation via targeting AKT3

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Abstract. – OBJECTIVE: To dissect the functioning mode of miR-582-5p on gastric cancer cell growth and provide therapeutic targets for gastric cancer.

PATIENTS AND METHODS: Relative expression levels of miR-582-5p in human gastric cancer tissue samples and gastric cancer-derived cell lines were measured by using quantitative Real-time PCR. Cell proliferation and viability capacities were assessed by cell counting kit-8 (CCK8) assay and colony formation assay. Cell apoptosis and cell cycle distribution were identified by flow cytometry. Downstream target gene was confirmed by using luciferase and Western blotting assays.

RESULTS: MiR-582-5p was downregulated in gastric cancer tissues when compared with para-carcinoma tissues (n=42). Overexpressed miR-582-5p could attenuate cell proliferation and viability capacities, as well as promoted cell apoptosis and cell cycle arrest at G0/G1 phase. AKT3 was chosen as the target gene of miR-582-5p by bioinformatics analysis and luciferase reporter assay. Moreover, restoration of AKT3 could impair tumor suppression role of miR-582-5p on gastric cancer growth.

CONCLUSIONS: MiR-582-5p exerted tumor-suppressive effects on gastric cancer growth via targeting AKT3 *in vitro*, which provided an innovative and candidate target for diagnosis and treatment of gastric cancer.

Key Words:
microRNAs, Proliferation, AKT3, Gastric cancer.

Introduction

Gastric cancer (GC) is one of the most common malignant tumors in the world, which ranks fourth in malignancy and second in mortality of cancers[1]. The development and progression of GC are related to many factors and multiple steps, including changes in cancer genes and tumor suppressor genes[2]. Clinically, the early diagnosis of GC is quite difficult due to the lack of specific clinical symptoms[3]. Surgical resection is one of the effective methods for the treatment of GC, but the prognosis is still very poor in advanced GC[4]. There is a regional difference in the incidence of GC: the incidence of GC is significantly higher in the North-West and Eastern coastal areas of China than that in the Southern region[5]. GC mainly occurs in population aged over 50 years old, and the gender ratio (male/female) of incidence of GC is 2:1. GC is seriously threatening the human health[1]. Currently, prophylaxis and treatment of GC face various obstacles, namely the high incidence and mortality, and low early diagnosis rate, surgical radical rate and 5-year survival rate[6]. Surgical resection is the preferred in treatment of GC, and the 5-year survival rate reaches over 90% among patients in Stage I. However, the curative effect is not ideal for patients in advanced stage, and the 5-year survival rate in Stage II-III is only 11-40%. The active comprehensive therapy based on surgery has become the major direction of clinical research on GC[7]. In recent years, the basic, preventive and clinical studies on GC have been greatly improved and a comprehensive treatment has been accepted by the medical staff at all levels. The standardized lymph node dissection to the second station (D2), neoadjuvant therapy at preoperative staging, postoperative adjuvant chemotherapy, radiotherapy, palliative exploration of effective programs as well as immune gene therapy, are also explored[8]. microRNA (miRNA) is a kind of single-stranded, noncoding, small molecule RNA in length of 18-25 nucleotides (nt) in eukaryotes, of which the encoding genes exist in the intergenic regions or introns of the genome. They are involved in the post-transcriptional control with important effects on biological events such as cell development, proliferation, differentiation and tumorigenesis[9]. miRNA regulates nearly 1/3

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of gene expression in the genome. Studies have confirmed that miRNA is closely related to the pathological processes including cell development, metastasis and drug resistance of tumors; about 50% of the annotated miRNA is located in fragile sites, tumor genes or breaking regions of tumor suppressor genes associated with tumors in the genome, indicating that miRNA is critical to tumorigenesis, and the effect of miRNA is just similar to those of oncogenes and tumor suppressor genes[10]. In this study, we aimed to search for the miRNAs associated with GC, so as to elucidate its mechanism, which will potentially enrich the etiology and molecular pathology of GC. The study on GC-related miRNA can provide important theoretical supports for early diagnosis, prognosis evaluation and development of new anticancer drugs. The expression difference of miRNAs in GC tissues and para-carcinoma tissues was mainly investigated, and the role of miR-582-5p in the occurrence and development of GC was explored, including the cell proliferation, viability, apoptosis and cycle, etc. Furthermore, the relevant target gene was predicted using the bioinformatics, and its regulatory effect and mechanisms involved in the pathogenesis of GC were studied, to provide a theoretical basis for the in-depth exploration of pathogenesis and new treatment methods of GC.

Patients and Methods

Patients

42 cases of GC tissue samples and corresponding para-carcinoma tissue samples were taken from patients receiving radical nephrectomy in the First Affiliated Hospital of Wenzhou Medical University from January 2016 to February 2017. The tissue samples were collected after the approval of Ethics Committee by the hospital. The patients signed the informed consent.

Methods

The tumor samples were cut from the gastric tissues within l0 min *in vitro*, while the para-carcinoma tissue was cut at more than 3 cm away from the distal tumor. All *in vitro* tissue samples were rinsed with RNase-free isotonic saline to remove the dirt or blood stain, placed into the cryopreserved tube, immediately frozen into liquid nitrogen and stored in the ultra-low temperature refrigerator (Haier, Qingdao, China) at -80°C for a long time used to extract RNA.

Cell Culture

The human GC cell lines MKN28, MKN-45, MGC803, SGC-7901 and normal human gastric epithelial cell line, GES-1, were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Cells were maintained in Roswell Park Memorial Institute-1640 (RPMI-1640) medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin. All the cells were incubated at 37°C in humidified atmosphere containing 5% CO_2.

Plasmid and Transfection

For upregulation of miR-582-5p in GC cells, miR-582-5p mimics and corresponding negative control (mimics-NC) were obtained from the RiboBio (Guangzhou, China). Transfections were performed using Lipofectamine 3000 Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols.

For overexpression of AKT3 for restoration, the AKT3 coding sequence was synthesized and inserted into vector pCDNA3.1 (Invitrogen, Carlsbad, CA, USA), and confirmed by sequencing. Empty pCDNA vector was used as control.

RNA Extraction and qRT-PCR

Total RNAs of cells were extracted by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). For miR-582-5p detection, Taqman assays were employed, and U6 was used as internal control. For mRNA analysis, total RNAs were reverse transcribed into cDNAs by using TaKaRa Reverse Transcription Kit (TaKaRa, Otsu, Shiga, Japan). Then, SYBR Green Premix Kit (Invitrogen, Carlsbad, CA, USA) was used to perform the qRT-PCR and GAPDH using as the control. All the relative expression RNA levels were calculated using the $2^{-\Delta\Delta CT}$ method.

Cell Counting kit-8 Assay

CCK8 (Dojindo, Kumamoto, Japan) assay was applied to investigate the cell proliferation. The cells were planted in a density of $1*10^3$ cells with 100 μL medium, and then were cultured for 24, 48, 72, 96 h after transfection. CCK8 reagent (10 μL per well) was added into the wells and absorbance of 450 nm was measured.

Colony Formation Assay

To further investigated cell growth of BC cells, cells were plated in 6-well plates at a density of 600 per well and maintained in normal

medium for 10 days. The colonies were fixed in 70% methanol for 20 min and then stained with 0.5% crystal violet for 10 min on ice, washing each well 3 times with phosphate buffered saline (PBS).

Cell Apoptosis Analysis

Flow cytometry was obtained to measure the cell apoptotic rate by using a fluorescein isothiocyanate (FITC) and propidium iodide (PI) kit (Vazyme, Nanjing, China). Cells were harvested after miR-582-5p mimics or inhibitors treatment and washed with pre-cooling PBS. Cells were resuspended in 1000 μL of binding buffer mixing 10 μL of FITC and PI, respectively. Then, cell apoptotic rate was measured by flow cytometry (BD Biosciences, San Jose, CA, USA), and the percentage of apoptotic cells was counted. Each measurement was repeated three times.

Cell Cycle Analysis

Transfected cells suspension were prepared and stained with PI using the BD Cycle test Plus DNA Reagent Kit (BD Biosciences, San Jose, CA, USA). The relative ratio of cells in G0/G1, S, or G2/M phase was analyzed by BD FACS Calibur flow cytometer (Brockport, NY, USA).

Luciferase Reporter Assay

The activity of luciferase was tested using the Dual-Luciferase reporter system (Promega, Madison, WI, USA). The AKT3 3'-UTR region containing the wild type or mutant miR-582-5p binding site was amplified and cloned into pGL3 luciferase vector (Promega, Madison, WI, USA). Treated cells were co-transfected with the established vector and miR-582-5p mimics or scrambled using lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). Then, the activity of luciferase was determined using luminometer (Promega, Madison, WI, USA) and measured as the fold-change to the basic pGL3 vector relatively.

Western Blot Analysis

A protein assay (Bio-Rad, Beijing, China) was conducted for measuring the total protein concentration. The target proteins were replaced to the polyvinylidene difluoride (PVDF) membrane, which was then blocked in 5% dry milk at 37˚C for 1 h after fractionated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Next, the membrane was immunostained with antibodies (Cell Signaling Technology, CST, Danvers, MA, USA) overnight at 4˚C: 1:1000 rabbit anti-AKT3, and 1:5000 rabbit anti-GAPDH. Subsequently, 1:1000 goat anti-rabbit secondary antibody was used for cultivation. The bands were measured using ChemiDoc XRS imaging system (Hercules, CA, USA) and ImageJ software (Rawak Software, Inc., Hamburg, Germany).

Statistical Analysis

All the statistical analysis was handled by using SPSS 19.0 software (IBM, Armonk, NY, USA). All quantitative results were displayed as mean ± SD. One-way ANOVA test was used to compare between groups followed by Least Significant Difference (LSD). $p < 0.05$ indicated significant difference.

Results

MiR-582-5p Expression was Decreased in GC Tissues and Cell Lines

To examine the expression level of miR-582-5p in human GC, we examined miR-582-5p expression level in 42 GC tissue samples and adjacent normal tissue samples. As clearly shown in Figure 1A, miR-582-5p expression levels in GC group were significantly higher than that in adjacent normal group. Besides, we investigated expression of miR-582-5p in several GC cell lines and normal gastric epithelial cell line with qRT-RCR. It showed that, compared with GES-1 cell line, all these GC cell lines expressed a relatively lower level of miR-582-5p, in which MGC803 expressed the relatively lowest (Figure 1B). To identify the mode of action of miR-582-5p in GC tumorigenesis *in vitro*, MGC803 cell line was transfected with miR-582-5p mimics and mimics-NC for overexpression of miR-582-5p (Figure 1C).

Overexpression of miR-582-5p Inhibited GC Cell Growth in vitro

As shown in CCK8 assay, the viability of GC cells was significantly inhibited after transfected with miR-582-5p mimics compared with mimics-NC, in a time-dependent manner (Figure 2A).

Meanwhile, we carried out colony formation assay to further explore cell viability capacity. It showed that there were fewer formed colonies of MGC803 cells transfected with miR-582-5p mimics when compared with mimics-NC (Figure 2B). Collectively, all these results showed that miR-582-5p could inhibit GC proliferation and viability.

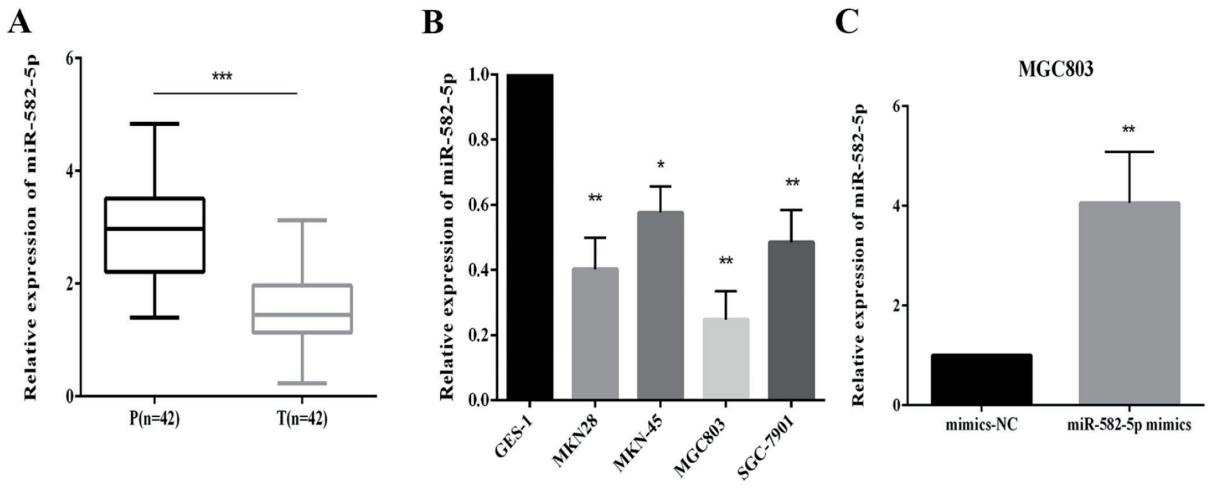

Figure 1. MiR-582-5p expression was decreased in GC tissues and cell lines. ***A,*** Analysis of miR-582-5p expression in paracarcinoma tissues (P) and tumor tissues (T). ***B,*** Analysis of miR-582-5p expression in several GC cell lines and normal cell line. ***C,*** Analysis of transfection efficiency in MGC803 cells transfected with miR-582-5p mimics and mimics-NC. Total RNA was detected by qRT-PCR and GAPDH was used as an internal control. Data are presented as the mean ± SD of three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Overexpression of miR-582-5p Promoted Cell Apoptosis and Induced cell Cycle Arrest at G0/G1 Phase

To better illustrate how miR-582-5p inhibited cell proliferation, we analyzed whether cell apoptosis and cell cycle were also affected by miR-582-5p. As shown in flow cytometric analysis, the apoptotic rate of GC cells transfected with miR-582-5p mimics increased remarkably compared with mimics-NC (Figure 2C). Moreover, the percentage of GC cells transfected with miR-582-5p mimics increased in G0/G1 phase while decreased in S-phase compared with mimics-NC (Figure 2D). These results indicated that miR-582-5p impaired GC cell proliferation capacity by promoting cell apoptosis and inducing cell cycle arrest at G0/G1 phase.

AKT3 is Directly Targeted by MiR-582-5p

According to microRNA target analysis of several database (TargetScan, miRanda, PicTar), we speculated AKT3 as a candidate target for miR-582-5p. The binding site of miR-582-5p with AKT3 3'-UTR was shown in Figure 3A. To further verify our assumption, we performed dual-luciferase assay. Using conducted wild type or mutant AKT3 3'-UTR vector, we found that luciferase activity significantly decreased in wild type group but no difference in mutant group (Figure 3B). In addition, we next measured the AKT3 mRNA and protein levels of established cell lines by qRT-PCR and Western blotting. Clearly, up-regulating miR-582-5p increased AKT3 expression in MGC803 cells (Figure 3C-D) compared to each control group. These findings suggested miR-582-5p could inhibit AKT3 expression.

Restoration of AKT3 Impaired Tumor Suppression Role of miR-582-5p

As mentioned before, overexpressed miR-582-5p could decrease the cell proliferation and might function via targeting AKT3.

Firstly, we measured the expression of AKT3 in GC tissues. The results showed that AKT3 was upregulated in GC tissues compared with the para-carcinoma tissues on the mRNA level (Figure 4A), and the expression of AKT3 was negatively correlated with the expression of miR-582-5p in GC tissues (Figure 4B).

Secondly, we explored whether AKT3 is responsible for the functional effects of miR-582-5p in GC tumorigenesis. We overexpressed AKT3 expression by transfected with pCDNA3.1-AKT3 in miR-582-5p-overexpressed MGC803 cells (Figure 4C). AKT3 restoration not only increased the proliferation of miR-582-5p-transfected cells (Figure 4D), but also attenuated cell apoptosis and cell cycle distribution at G0/G1 phase (Figure 4E-F). These results implied that miR-582-5p suppressed GC tumorigenesis by repressing AKT3 expression partially.

Discussion

GC is one of the most common malignant tumors in the world. It is the second leading cause of cancer death in males and the third cause in females. In 2008, it was estimated that there were 21,500 new cases of GC in the United States, with about 10,880 new cases of GC deaths[11]. In China, 360,000-400,000 new GC patients are found each year, and the mortality rate ranks second in leading causes of cancer death[12]. The incidence and mortality of GC are high at present. The early diagnosis rate and resection rate are low, and the proportion of patients with GC undergoing radical gastrectomy is lower than 60-70%[13]. The 5-year survival rate after various therapies is only about 40%[14]. Currently, there remains no comprehensive or in-depth understanding on the pathogenesis of GC. GC is also a multifactorial disease, in which genetic and environmental factors are considered to be involved in the occurrence and development of the disease[15]. A large, multicenter study revealed that many genes were involved in the incidence of GC, such as protein 53 (p53), p21, nuclear factor kappa B (NFκB), matrix metalloproteinases (MMPs), epidermal growth factor receptor (EGFR), E-cadherin, methylguanine methyltransferase (MGMT), cyclooxygenase 2 (COX2), phosphatase and tensin homolog (PTEN), adenomatous polyposis coli (APC), human epidermal growth factor receptor 2 (HER2), runt-related transcription factor 3 (RUNX3), ataxia telangiectasia mutated (ATM) gene and Krüppel-like factor 6 (KLF6)[16]. To find out the related genes of incidence and development of GC, and to understand the molecular genetic

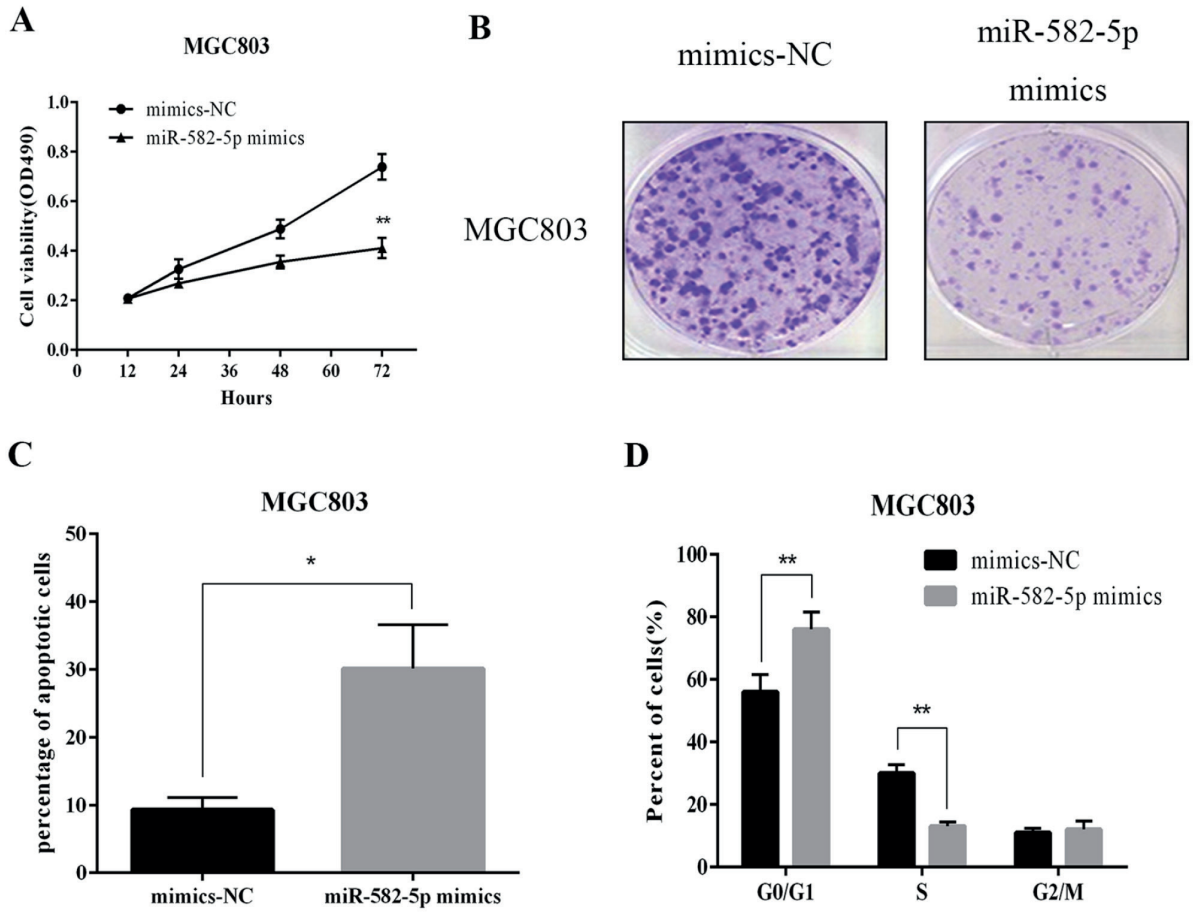

Figure 2. Overexpression of miR-582-5p inhibited GC cell growth *in vitro*. ***A,*** CCK8 assay was performed to determine the viability of transfected MGC803 cells. ***B,*** Colony formation assay was performed to determine the proliferation of transfected MGC803 cells. ***C,*** Flow cytometric analysis was performed to detect the apoptotic rates of transfected MGC803 cells. ***D,*** Flow cytometric analysis was performed to detect cell cycle progression of transfected MGC803 cells. $^{*}p < 0.05$; $^{**}p < 0.01$.

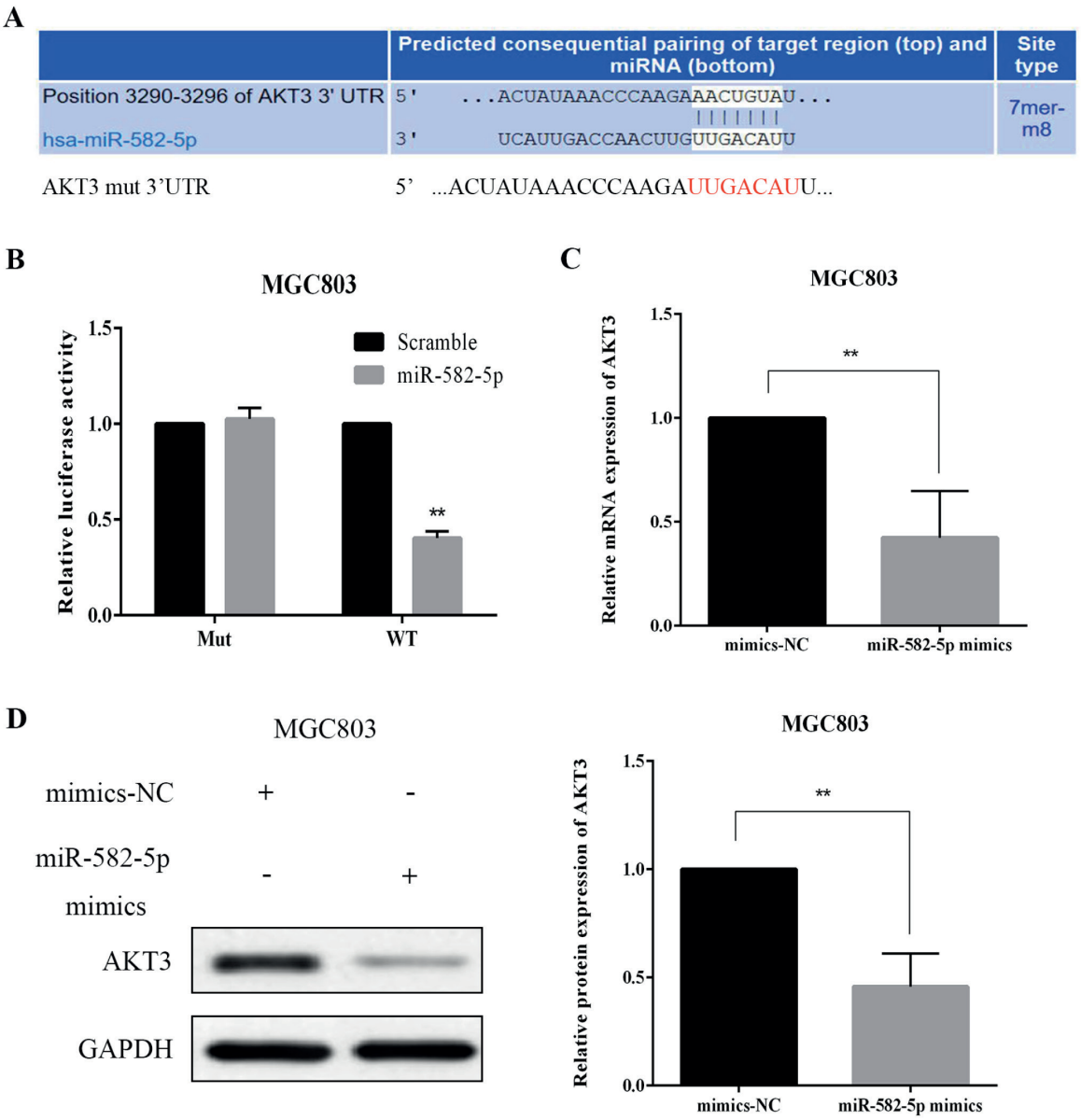

Figure 3. AKT3 is the target gene of miR-582-5p. ***A,*** AKT3 was selected as the potential downstream of miR-582-5p *via* using bioinformatics analysis. ***B,*** Luciferase activities of MGC803 cells transfected with the wild type or the mutated AKT3 3′UTR together with miR-582-5p mimics or mimics-NC. ***C,*** Analysis of AKT3 mRNA expression level of MGC803 cells transfected with miR-582-5p mimics or mimics-NC. ***D,*** Analysis of AKT3 protein expression level of MGC803 cells transfected with miR-582-5p mimics or mimics-NC. Data are presented as the mean ± SD of three independent experiments. $^{**}p < 0.01$.

mechanism of GC so as to provide the theoretical basis for the prevention, diagnosis and treatment of GC are research hotspots at present. miRNA is a type of noncoding, single-stranded, small molecule RNA with a length of about 22 nucleotides, which arranges on chromosomes in the non-random and clustering forms and combines with the 3' UTR of a specific mRNA, so that the miRNA can control the mRNA stability and efficiency of further translation. In 2002, through a large number of basic experiments, Calin et al[17] found that in the chronic lymphocytic leukemia, when about 30 base pairs deletion occurred at 1st zone 4th belt on the long arm of chromosome, the expressions of miR-15 and miR-16 were downregulated or even absent when transcription was performed at the downstream region. Based on this, researchers began an intensive study on the relationship

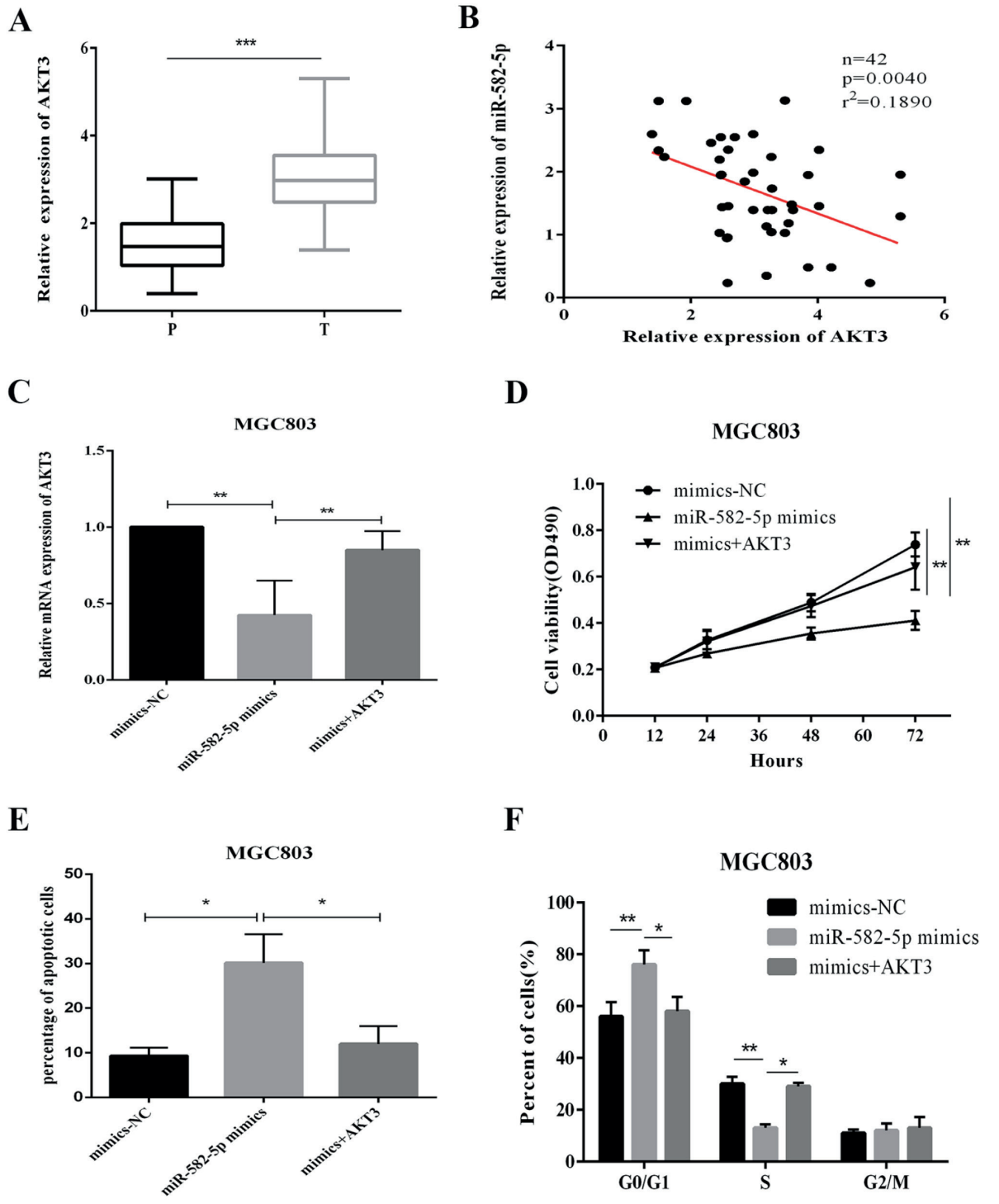

Figure 4. Restoration of AKT3 impaired tumor suppression role by miR-30c. ***A,*** Analysis of AKT3 expression level in GC tissues (T) and matched paracarcinoma tissues (N), n = 42. ***B,*** Correlation between miR-582-5p and AKT3 expression in GC tissues (n = 42). ***C,*** Analysis of transfection efficiency in MGC803 cells transfected with miR-582-5p negative control (NC), mimics and/or pCDNA3.1-AKT3. ***D,*** Overexpressed AKT3 rescued suppressed cell proliferation by miR-582-5p. ***E,*** Overexpressed AKT3 attenuated cell apoptosis. F, Overexpressed AKT3 attenuated cell cycle distribution at G0/G1 phase. Data are presented as the mean ± SD of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

between gene expression and miRNA. In recent years, the research on GC and miRNA has been developed and unceasingly deepened, mainly focused on the effects of miRNAs in the occurrence and metastasis of GC. Gene chip, PCR, Western blot and bioinformatics, have also been widely used in research on the detection of miRNA expression and prediction of target genes. The emergence of miRNA has revolutionized the traditional idea that ribonucleic acid (RNA) is only a medium between deoxyribonucleic acid (DNA) and protein, and more and more basic researches have confirmed that miRNA is closely related to the biological characteristics of tumor cells, such as unlimited clonal proliferation, anti-apoptosis and easy metastasis. Recent studies have shown that as an important member of miRNA, the significant role of miR-582-5p in development of tumor has drawn people's attention. A large number of researches have proved that miR-582-5p is involved in many biological processes such as growth, invasion and migration of tumor cells. In bladder cancer, miR-582-5p can inhibit proliferation, invasion and migration of tumor, indicating that miR-582-5p may exert a significant role in the occurrence and development of human tumors[18]. Importantly, the detection of tissue in colon cancer revealed that the expression of miR-582-5p was inhibited, suggesting that miR-582-5p plays an important role in the cancer[19]. This study aimed to elucidate the relationship between miR-582-5p and the occurrence and development of GC. In our present study, we first identified that the expression of miR-582-5p in 42 GC tissue samples was significantly downregulated than adjacent normal tissues. Furthermore, we conducted miR-582-5p-overexpressed cell lines and employed several cell function experiments, which demonstrated that the upregulation of miR-582-5p inhibited cell proliferation in GC. As a conclusion, we considered that miR-582-5p functioned as a suppress-factor in GC.

MiRNAs could bind to the 3'-UTR region of target genes and influence their expression to change cellular processes. We further searched potential target gene of miR-582-5p in some databases and found AKT3 as a direct target mRNA. The v-akt murine thymoma viral oncogene homolog 3 (AKT3) is a member of the serine/threonine protein kinase AKT subfamily, and activated AKT plays an important role in mediating cell growth and proliferation, cell motility and invasion, apoptosis and resistance to chemotherapy and radiotherapy[20,21]. The activation of AKT is closely related to the occurrence and development of tumor, which can be simulated by multiple growth factors, hormones, cytokines, inactivation of phosphatase and tensin homolog deleted on chromosome ten (PTEN) and activation of Ras[22]. In recent years, the significant progress has been made in the study on the activation of AKT and occurrence and development of tumors. AKT can promote cell growth and proliferation, inhibit apoptosis, advance cell invasion and metastasis, and promote angiogenesis. However, the underlying upstream mechanism of AKT3 in GC has not been well identified and reported yet. In our present study, we initially revealed that AKT3 was directly targeted by miR-582-5p, and AKT3 expression was negatively correlated with miR-582-5p in GC tissues and cell lines. Moreover, restoration of AKT3 could rescue tumor suppression role by downregulated miR-582-5p on GC cell growth. Evidence indicated that miR-582-5p might be the upstream of AKT3 involved in GC tumorigenesis.

Conclusions

We showed that miR-582-5p was markedly downregulated in GC. Increased miR-21 expression could suppress cell proliferation, viability and promote cell apoptosis and G0/G1 arrest abilities via targeting AKT3. These findings indicated miR-582-5p could be a potential target for prognostic prediction and therapeutic strategies.

Conflict of interest

The authors declare no conflicts of interest.

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