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MiR-675 is frequently overexpressed in gastric cancer and enhances cell proliferation and invasion via targeting a potent anti-tumor gene PITX1

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ABSTRACT

Background: Gastric cancer (GC) is a common malignancy around the world. Irregular expression of microRNAs (miRNAs) contributes to the progression of malignancies. Our study illustrated that miR-675 facilitates GC proliferation and invasion via targeting paired-like homeodomain transcription factor 1 (PITX1) and promoting epithelial-mesenchymal transition (EMT) as well as Wnt/β-catenin signaling pathway.

Methods: We collected the RNA-seq data of GC and normal stomach tissues from TCGA database to analyze the expression of miR-675 and PITX1. Kaplan-Meier plotter on line tool was used to analyze the association between miR-675 or PITX1 expression and the overall survival of GC patients. The biological function of miR-675 in GC cells was evaluated via altering its expression using miR-675 agomiR or antamiR. Dual-luciferase reporter assay was applied for verifying whether miR-675 could direct bind to 3′UTR of PITX1. Rescue assays were applied for characterizing the effects of miR-675/PITX1 axis on GC growth and invasion. Western blot was performed to evaluate the protein expression levels of PITX1, EMT-related and Wnt signaling-related proteins.

Results: Our results showed that miR-675 is up-regulated and predictive of worse prognosis in GC patients. Overexpression of miR-675 in AGS cells notably promoted cell proliferation, migration and invasion, whilst down-regulation of miR-675 in SGC-7901 cells gained the opposite results. PITX1 is down-regulated in GC and identified as a direct target of miR-675. Overexpression of PITX1 in AGS cells reverses cell viability and invasion that enhanced by miR-675 up-regulation. Conversely, depletion of PITX1 in SGC-7901 cells rescues cell viability and invasion that inhibited by miR-675 down-regulation. Western bolt results revealed that miR-675 positively regulated EMT and Wnt/β-catenin signaling pathway in GC cells via targeting PITX1.

Conclusions: Our study emphasized the functional mechanism of miR-675 in GC and intimated that miR-675/PITX1 axis possibly affects proliferative and invasive properties of GC cells via regulating EMT and Wnt/β-catenin signaling pathway. Furthermore, miR-675 and PITX1 may be served as early diagnostic markers as well as therapeutic targets for GC.

1. Introduction

Gastric cancer (GC) is one of the most common malignant disease around the world, which possesses particularly high incidence in China and Japan [1,2]. Although substantial efforts have been made during the past years, the efficacy of clinical therapy is limited. Notably, the survival rate of GC patients relies on at which stage the disease was diagnosed [3]. Given this, a deeper understanding of the mechanism under GC progression could potentially improve GC diagnosis and therapies.

MicroRNAs (miRNAs) are small endogenous non-coding regulatory RNAs (17–25 nucleotides), which are currently demonstrated to take vital part in the progression of tumor [4,5]. MiRNAs play a negative regulation role in gene expression via binding to the 3′- untranslated region (UTR) of mRNAs, which leads to translation inhibition or mRNA degradation [6,7]. Increasing evidences illustrated the different expression of miRNA profiles in tumor tissues and a large amount of dysregulated miRNAs were implied to be promising biomarkers for diagnosis and even target for the treatment of cancer [2]. Many miRNAs that involve in the progression of GC have been identified in recent

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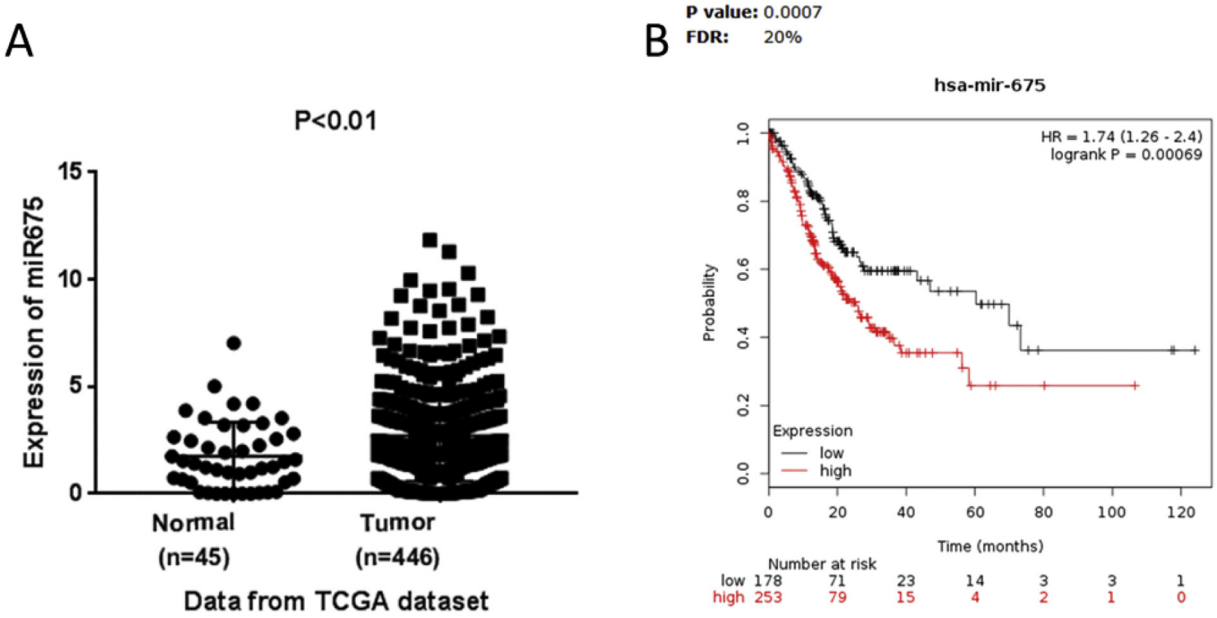

Fig. 1. MiR-675 was up-regulated in GC and associated with poor prognosis. (A) The expression of miR-675 in 446 GC tissues and 45 normal stomach tissues were analyzed based on the RNA-seq data obtained from TCGA database. (B) Kaplan–Meier curve for overall survival in the cohort of 431 GC patients was drawn utilizing the online Kaplan–Meier plotter tool. The patients were divided into low and high expression group based on auto selected best cutoff (cutoff value = 6).

years. For example, miR-21 is overexpressed in GC and contributes to GC progression via enhancing cell viability and suppressing apoptosis [8]. MiR-632 is also up-regulated in GC tissues and facilitates the development of GC by promoting angiogenesis in a TFF1-dependent manner [9]. Long Noncoding RNA H19-derived miR-675 promotes GC cell growth and invasion via suppressing runt domain transcription factor 1 (RUNX1) expression [10] or modulating FADD/Caspase 8/Caspase 3 signaling pathway [11]. However, the precise underlying mechanism of how miR-675 participates in GC development still needs further exploration, since that the regulation mechanism is intricate and one miRNA often regulates multiple mRNAs.

Paired-like homeodomain transcription factor 1 (PITX1) belongs to RIEG/PITX homeobox family, and is firstly recognized as a bicoid-related transcription factor that participates in pro-opiomelanocortin gene transcription [12]. Recently, PITX1 was characterized as an anti-tumor gene in several types of cancers, including GC. Its expression was reported to be modulated by miR-19a-3p, and lower expression of PITX1 predicts a worse prognosis of GC patients [13,14]. However, it still remains largely unclear about the detailed molecular mechanisms underlying how PITX1 involved in the development and progression of GC. In our present study, we demonstrated that PITX1 was a target of miR-675 as well. And we revealed that miR-675 stimulated the proliferation and migration of GC cells in vitro, which could be partially attributed to the inhibition of PITX1 expression.

2. Methods

2.1. Bioinformatics analysis

The RNA-seq data of GC tissue samples and normal stomach tissue samples were obtained from The Cancer Genome Atlas (TCGA, https://cancergenome.nih.gov/) and applied for analyzing the expression of miR-675 (in 446 GC tissues and 45 normal tissues) and PITX1 (in 375 GC tissues and 45 normal tissues). Kaplan-Meier plotter on line tool was applied for analyzing the correlation between miR-675 or PITX1 expression and the overall survival of GC patients.

2.2. Cell culture

Human gastric normal epithelial mucosa cell line GES-1 and GC cell lines (MGC-803, SGC-7901 and AGS) were bought from the Cell Bank, Shanghai Institutes for Biological Sciences (Shanghai, China) and cultivated in RPMI 1640 medium supplemented with FBS (10%) and penicillin G/streptomycin (1%) under a standard condition (5% CO_2, 95% air, 37 °C).

2.3. Transfection

MiR-675 agomiR, miR-675 antamiR and their negative control (NC) were synthesized by GenaPharma (Shanghai, China). MiR-675 agomiR was used to up-regulate miR-675 expression in AGS cells. MiR-675 antamiR was used to down-regulate miR-675 in SGC-7901 cells. pcDNA3.1- PITX1 plasmid was constructed and used to overexpress PITX1 in AGS cells. Si- PITX1 (5′-GCTCTCCTCTCAATCCATGTTCTC-3′) was used to silence PITX1 in SGC-7901 cells and si-con (5′-GCAATTC TCCGAACGGTCACGT-3′) was used as a negative control. Lipofectamine 2000 (Invitrogen) was utilized to conduct cell transfection. MiR-675 agomiR alone or both miR-675 agomiR and pcDNA3.1- PITX1 were transfected into AGS cells. MiR-675 antamiR alone or both miR-675 antamiR and si- PITX1 were transfected into SGC-7901 cells. In addition, to explore the effect of miR-675 on normal epithelial mucosa cells, miR-675 agomiR and miR-675 antamiR were transfected into GES-1 cells, respectively. Cells treated with transfection reagent were considered as control. Transfection efficiency was evaluated 24 h after transfection.

2.4. RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from GC cells using TRIzol® reagent (Invitrogen, USA). The isolated RNA was then reverse transcribed using PrimeScript RT Reagent Kit (Takara, Japan) or MiScript Reverse Transcription kit (Qiagen), as appropriate. qPCR was then performed on ABI7500 Real-Time PCR System (Applied Biosystems, USA) to evaluate the expression of miR-675 and PITX1 using SYBR Premix Ex Taq II (TaKaRa, Japan) or MiScript SYBR-Green PCR kit (Qiagen). The relative expression level of miR-675 and PITX1 was computed utilizing

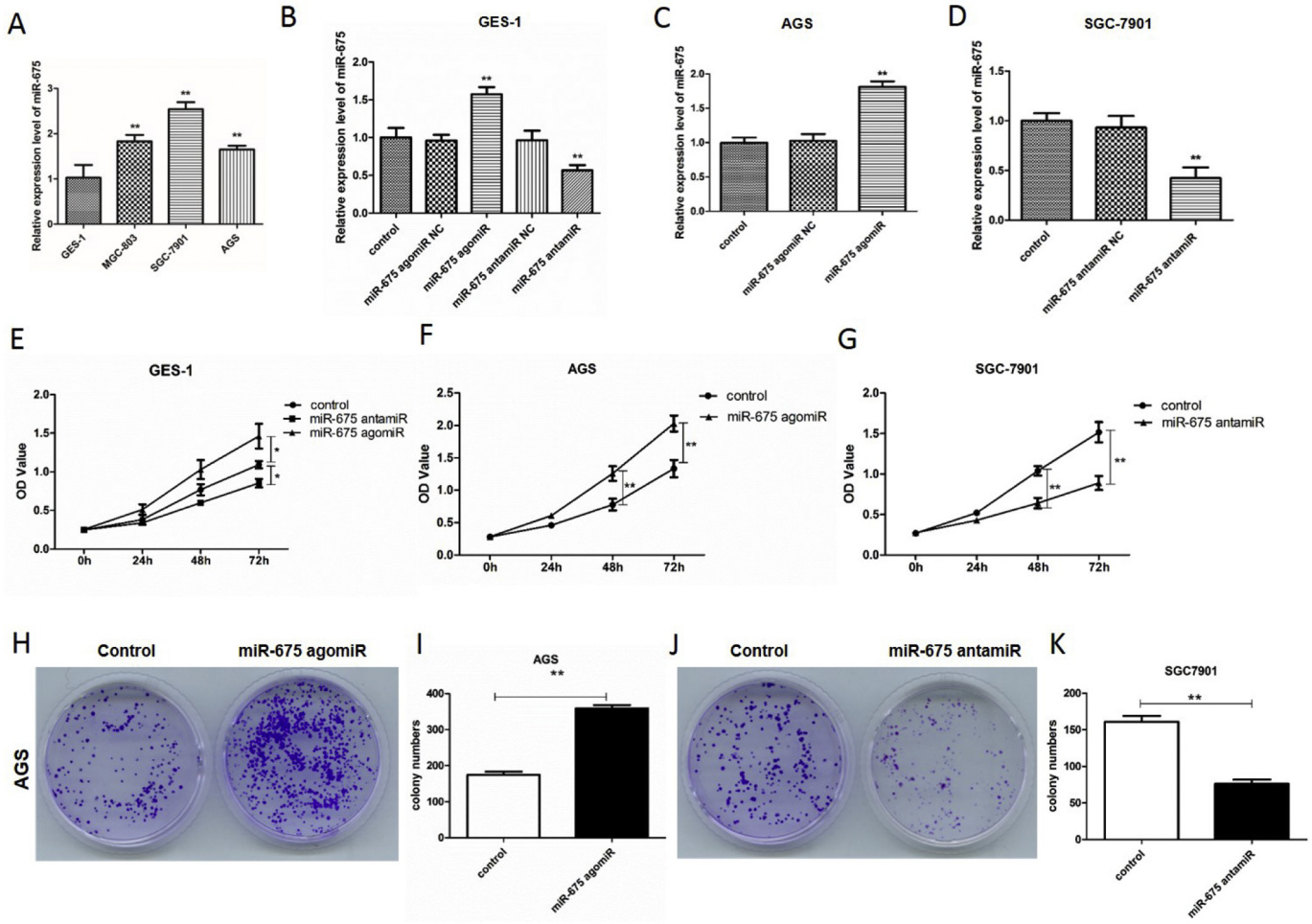

Fig. 2. MiR-675 promoted GC cell proliferation in vitro. (A) The expression of miR-675 in GC cell lines and normal stomach cell line was evaluated using qRT-PCR. (B)The expression level of miR-675 in GES-1 cells was increased by miR-675 agomiR and decreased by miR-675 antamiR. (C) The expression level of miR-675 was increased in AGS cells after transfected with miR-675 agomiR. (D) The expression level of miR-675 was decreased in SGC-7901 cells after transfected with miR-675 antamiR. (E–G) The proliferation of GES-1, AGS and SGC-7901 cells were determined by CCK8 assay. (H–I) The representative images of the colony formation assay results for AGS cells and the numbers of colonies. (J–K) The representative images of the colony formation assay results for SGC-7901 cells and the numbers of colonies. N = 6, **p < 0.01 vs. control group.

a $2^{-\Delta\Delta Ct}$ method. GAPDH and U6 were utilized as internal references. The primers used were listed as follows:

miR-675 F: 5′-TGGTGCGGAGAGGGC-3′,
miR-675 R: 5′-GAACATGTCTGCGTATCTC-3′;
U6 F: 5′-CGCAAGGATGACACGCAAAT-3′,
U6 R: 5′-ATTTGCGTGTCATCCTTGCG-3′;
PITX1F: 5′-GTACGCACTTCACAAGCCAGCA-3′,
PITX1R: 5′-GCTCGGTGAGGTTGGTCCACA-3′;
GAPDHF: 5′-GTCTCCTCTGACTTCAACAGCG-3′,
GAPDHR: 5′-ACCACCCTGTTGCTGTAGCCAA-3′.

2.5. Cell counting kit-8 (CCK8) assay

Single cell suspension was prepared after 24 h transfection, and seeded into 96-well plates (1000 cells/well). CCK- 8 solution (10 μl/well, Dojindo, Kumamoto, Japan) was added to the plates after being cultivated for 0, 1, 2, 3, 4 days. After 1.5 h incubation at 37 °C, a microplate reader (Bio-Rad, CA, USA) was utilized to detect the optical density (OD) at 450 nm. Each group includes 3 parallel wells and all experiments were conducted for 6 independent times.

2.6. Colony formation assay

Single cell suspension was prepared and approximately 400 cells were seeded into dishes for 2 weeks cultivation under standard conditions. Colonies were washed by PBS buffer, fixed by 4% paraformaldehyde and stained by 0.1% crystal violet staining solution. The number of the colonies was manually counted.

2.7. Transwell assay

Transwell chambers (BD Biosciences, USA) coated with or without matrigel (BD Bioscience) were applied for evaluating the invasive or migratory ability of GC cells, accordingly. Cell suspension was prepared using serum-free medium and seeded into the upper chamber (10,000 cells for invasion assay and 5, 000 cells for migration assay). The lower chamber was added with 600 μl complete medium. The chamber were then taken into an incubator and incubated for 24 h at 37 °C. Cells remained on the upper chamber were erased, while cells in the lower membranes were fixed by 4% paraformaldehyde and stained by 0.1% crystal violet staining solution, successively. Then cells were pictured under a light microscope (Olympus, Tokyo, Japan) and counted in 5 independent and random fields.

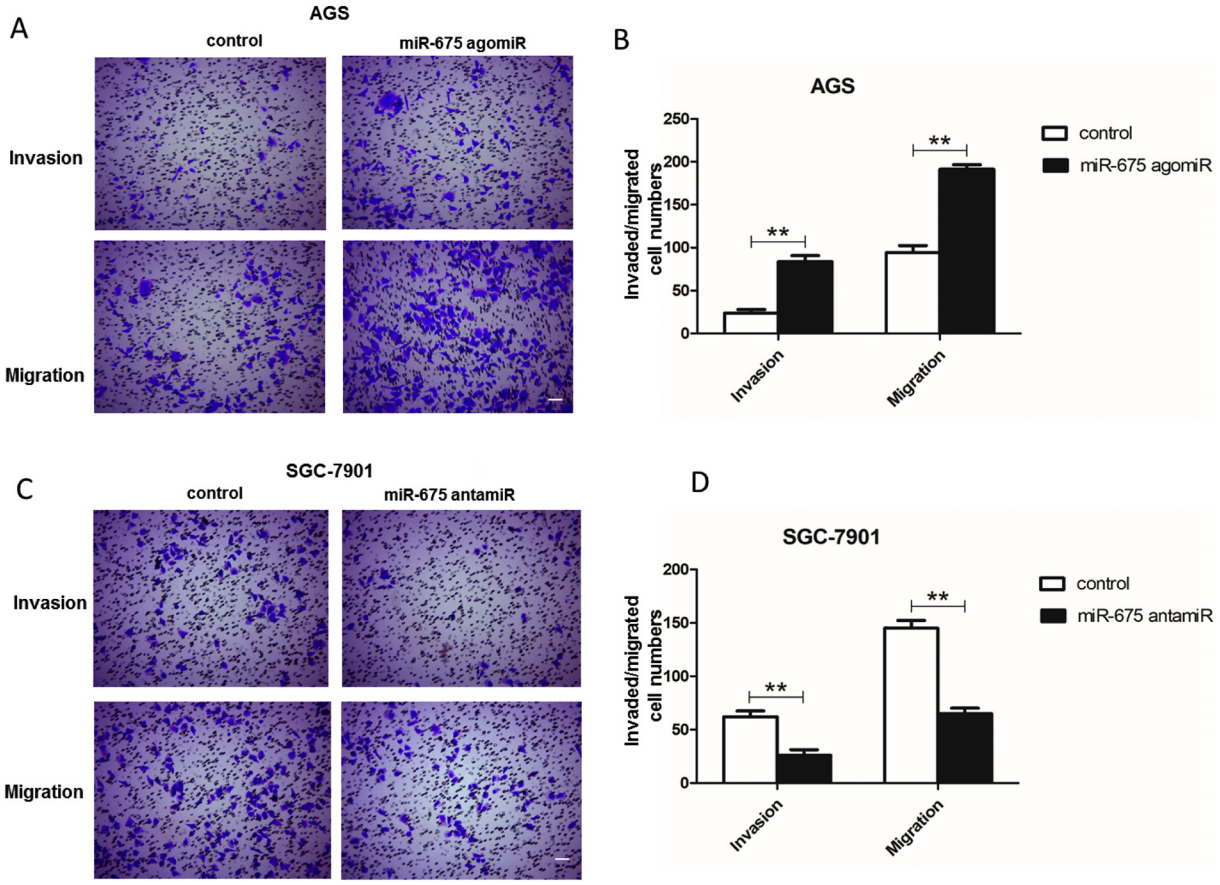

Fig. 3. MiR-675 facilitated GC cell migration and invasion in vitro. (A) The invasion and migration ability of AGS cells was evaluated by transwell assays. (B) The number of invaded/migrated AGS cells. (C) The invasion and migration ability of SGC-7901 cells was evaluated by transwell assays. (D) The number of invaded/migrated SGC-7901 cells. N = 6, **p < 0.01 vs. control group. Bars = 100 μm.

2.8. Luciferase reporter assay

Fragment from PITX1 that harboring the forecasted binding sites or the mutant binding sites between PITX1 and miR-675 were sub-cloned into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA) to construct the pmirGLO-PITX1-WT and pmirGLO-PITX1-MUT vectors. Then pmirGLO-PITX1-WT and pmirGLO-PITX1-MUT were co-transfected with miR-675 mimic or miR-675 mimic NC into cells. After 48 h transfection, the dual luciferase reporter assay system (Promega, Madison, WI, USA) was applied for evaluating luciferase activity of pmirGLO- PITX1-WT and pmirGLO- PITX1-MUT.

2.9. Western blot assay

Cells were collected after 48 h transfection and lysed in RIPA lysis buffer containing protease inhibitors. Protein of each group was separated on a 12% SDS-PAGE gel followed by being transferred onto polyvinylidene fluoride membranes (Merck Millipore, Germany). After that, membranes were incubated with 5% non-fat milk for blockage. Hereafter the membranes were probed with the primary and secondary antibodies, successively. Signals were detected using an enhanced chemiluminescence (ECL) plus detection kit (Thermo Fisher Scientifc, Inc.) according to the instructions.

Nuclei and cytosol proteins of AGS and SGC7–901 cells were extracted using a Nuclear and Cytoplasmic Protein Extraction Kit (KeyGEN Biotech, Jiangsu, China) in accordance with the instructions provided by the manufacturer. GAPDH and Histone H3 were used as internal reference of the cytoplasmic and nuclear protein fraction, respectively.

2.10. Statistical analysis

The experimental data was analyzed by SPSS22.0 statistical analysis software (SPSS Inc., Chicago, IL, USA). Mean comparisons between multiple samples were performed using one-way analysis of variance (ANOVA) followed by Tukey or Dunnett post hoc test. All data were exhibited as Mean ± SD. The value of $p < 0.05$ was regarded as statistically significant.

3. Results

3.1. MiR-675 was elevated in GC tissues and associated with poor prognosis

We investigated the expression of miR-675 in both GC tissues and normal stomach tissues based on the RNA-seq data downloaded from TCGA database. The results exhibited that the expression of miR-675 was notably up-regulated in GC tissues (n = 446) than that in the normal stomach samples (n = 45, Fig. 1A, p = 5.56E-06). The enhanced expression of miR-675 indicated that it possibly participates in the initiation and metastasis of GC. Then we evaluated the effect of miR-675 expression on the overall survival of GC patients using Kaplan–Meier plotter online tool. We found that high level of miR-675 was remarkably correlated with shorter overall survival time (Fig. 1B, p = 0.00069).

3.2. MiR-675 accelerated GC cell proliferation and invasion

Then we quantified the expression of miR-675 in normal stomach and GC cell lines using qRT-PCR analysis. We identified that miR-675

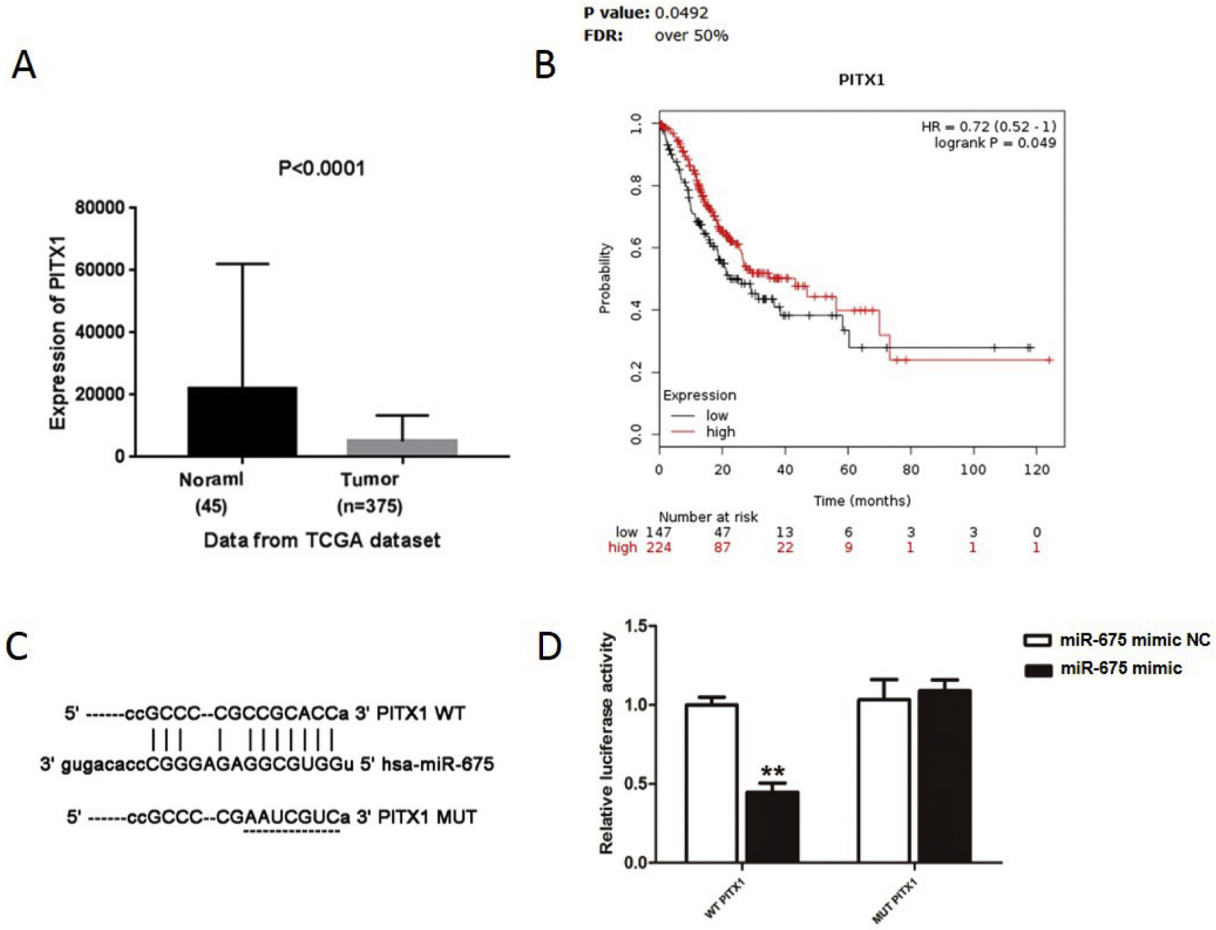

Fig. 4. PITX1 was a target of miR-675. (A) The expression of PITX1 in 375 GC tissues and 45 normal stomach tissues were analyzed based on the RNA-seq data obtained from TCGA database. (B) Kaplan–Meier curve for overall survival in the cohort of 371 GC patients was drawn using the online Kaplan–Meier plotter tool. (C) The predicted binding sites between miR-675 and PITX1. (D) Dual-luciferase reporter assay was applied for verifying whether miR-675 could direct bind to the 3′UTR of PITX1 via the predicted sites. WT PITX1: pmirGLO-PITX1-WT; MUT PITX1: pmirGLO-PITX1- MUT. N = 6, **p < 0.01.

expression is enhanced in GC cell lines (MGC-803, SGC-7901 and AGS) compared with that in GES-1 cell line (Fig. 2A, $p < 0.01$). This observation further verified our results obtained from the TCGA data. Since AGS possess the lowest while SGC-7901 possess the highest miR-675 expression among the 3 GC cell lines, we selected AGS cells to perform miR-675 agomiR experiments while SGC-7901 cells to conduct miR-675 antamiR experiments. Then miR-675 agomiR and antamiR were respectively transfected into AGS and SGC-7901 cells to achieve miR-675 up-regulation or down-regulation. In addition, GES-1 cells were transfected with miR-675 agomiR or antamiR to explore the effect of miR-675 on normal gastric cells. The results showed that miR-675 expression was successfully increased or decreased by miR-675 agomiR or antamiR in GES-1 cells (Fig. 2B, $p < 0.01$). In AGS cells transfected with miR-675 agomiR, the level of miR-675 significantly increased to about 1.8 folds of that in control group (Fig. 2C, $p < 0.01$). While, the level of miR-675 in SGC-7901 cells transfected with miR-675 antamiR decreased to < 0.5 fold of that in control group (Fig. 2D, $p < 0.01$).

Then we assessed the effects of miR-675 on GES-1 and GC cell viability using CCK8 assay. The results showed that down-regulation of miR-675 inhibited the proliferation whilst up-regulation of miR-675 promoted the proliferation of GES-1 cells (Fig. 2E, $p < 0.05$). Consistent with the tendency observed in GES-1 cells, overexpression of miR-675 notably accelerates the proliferation of AGS cells (Fig. 2F, $p < 0.01$), while reduction of miR-675 significantly hinders the viability of SGC-7901 cells (Fig. 2G, $p < 0.01$). Significant difference was observed at 48 h and 72 h (Fig. 2F–G). We can easily observed from Fig. 2E–G that altering the expression of miR-675 has less influence on GES-1 cell proliferation than on GC cells. This difference may be caused by the different nature between normal and tumor cells. Hence, in the following section, experiments were only performed in AGS and SGC-7901 cells. Colony formation assays were conducted to further verify the effect of miR-675 on GC cell proliferation and the outcomes were consistent with the results of CCK8 assay. We can easily observed that enhanced miR-675 expression in AGS cells significantly increased the number of clones formed compared with the control (Fig. 2H and I, $p < 0.01$). On the other side, decrease the level of miR-675 greatly reduced the colony-forming ability of SGC-7901 cells (Fig. 2J and K, $p < 0.01$). These outcomes hinted that miR-675 appears to be a proliferation facilitator in GC.

The outcomes of transwell assays exhibited that overexpression of miR-675 apparently increased the numbers of migrated and invaded AGS cells compared with the corresponding control (Fig. 3A and B, $p < 0.01$). Conversely, when the level of miR-675 in SGC-7901 cells was decreased by miR-675 antamiR, the numbers of migrated and invaded SGC-7901 cells were decreased compared with the corresponding control (Fig. 3C and D, $p < 0.01$). These data illustrated that miR-675 promoted the motility of GC cells in vitro.

3.3. MiR-675 negatively regulated PITX1 expression through binding to the 3′UTR of PITX1

To uncover how miR-675 promotes GC cell proliferation and invasion, TargetScan (http://www.targetscan.org/vert_71/) online tool was applied for forecasting the targets of miR-675. Based on the forecast,

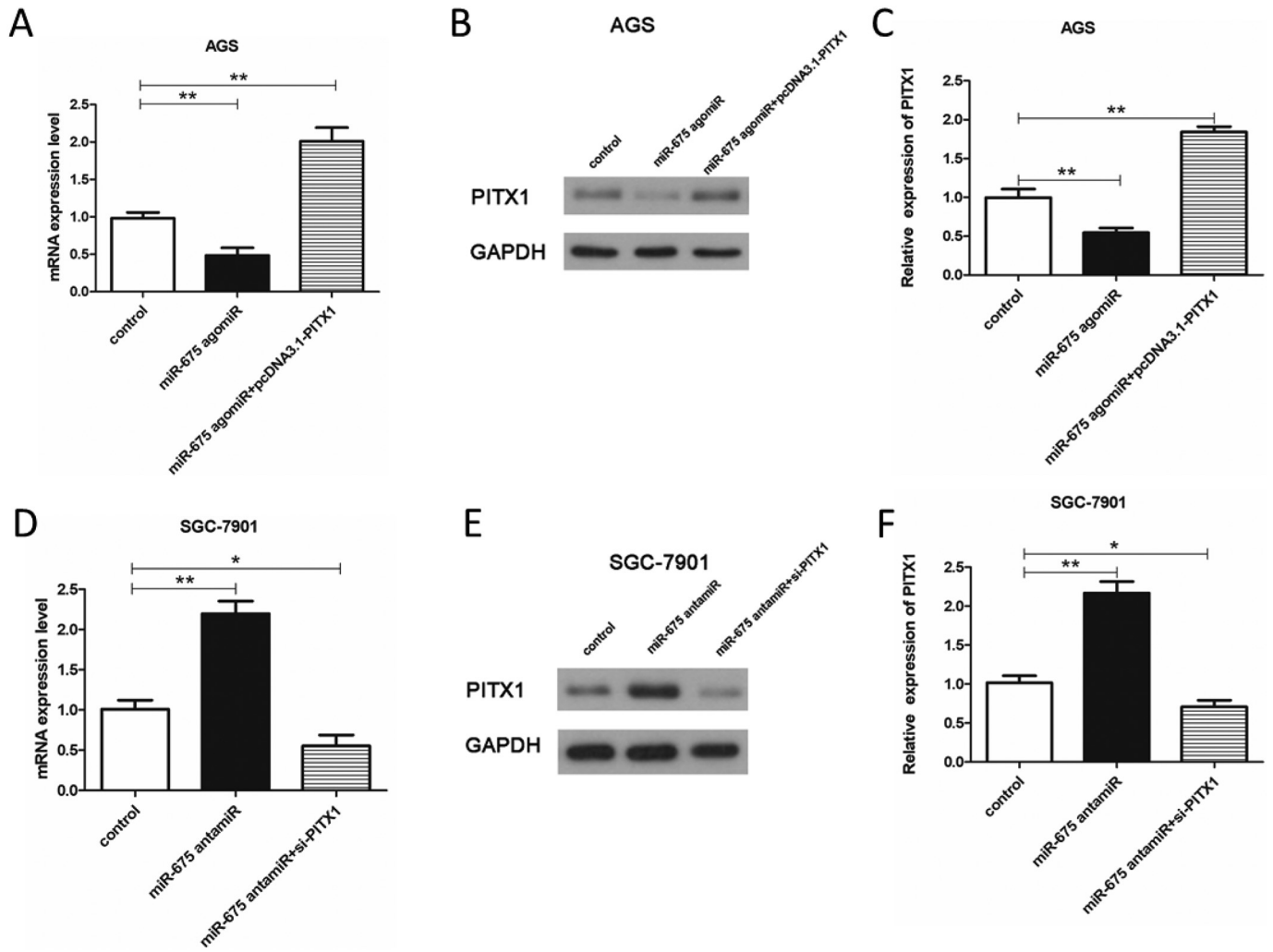

Fig. 5. PITX1 was negatively regulated by miR-675. (A) The mRNA expression of PITX1 in AGS cells. (B)The protein expression of PITX1 in AGS cells. (C) Quantification of the protein expression level of PITX1 in AGS cells. (D) The mRNA expression of PITX1 in SGC-7901 cells. (E) The protein expression of PITX1 in SGC-7901 cells. (F) Quantification of the protein expression level of PITX1 in SGC-7901 cells. N = 6, *p < 0.05, **p < 0.01.

bioinformatics and statistic analysis of RNA-seq expression data and clinic prognosis data from TCGA datasets coupled with literature search results aiming at it functioning as a noteworthy transcription factor participates in the regulation of genes that involve in cell differentiation, proliferation etc., PITX1 was selected for next experiments. Analysis of the RNA-seq data of 375 GC samples and 45 normal samples obtained from TCGA database, we discovered that the level of PITX1 was greatly down-regulated in GC compared with that in normal samples (Fig. 4A, $p < 0.0001$). Kaplan–Meier survival analysis clarified that high expression of PITX1 was associated with good prognosis of patients with GC (Fig. 4B, $p = 0.049$). The forecasted binding sites between miR-675 and PITX1 were shown in Fig. 4C. Luciferase reporter assay was then carried out to prove our prediction and revealed that the luciferase activity of pmirGLO-PITX1-WT was significantly decreased in miR-675 mimic transfected cells ($p < 0.01$), while the luciferase activity of pmirGLO-PITX1-MUT was almost not changed (Fig. 4D). These outcomes suggested that miR-675 could bind to the predicted binding sites in PITX1 mRNA and PITX1 is a direct target of miR-675. The mRNA and protein expression of PITX1 in response to miR-675 up-regulation or down-regulation were monitored by qRT-PCR and Western blot, respectively. Up-regulation of miR-675 in AGS cells leads to a significant decrease of PITX1 expression at both RNA and protein levels (Fig. 5A–C, $p < 0.01$), and down-regulation of miR-675 in SGC-7901 cells caused an increase in PITX1 expression (Fig. 5D–F, $p < 0.01$). These outcomes indicated that the expression level of PITX1 is negatively regulated by miR-675 in GC.

3.4. PITX1 reverses GC cell proliferation and invasion mediated by miR-675 overexpression

In order to verify that whether miR-675 involved in the regulation of GC proliferation and invasion via targeting PITX1, si-PITX1 was used to silence PITX1 and pcDNA3.1- PITX1 was used to overexpress PITX1 (Fig. 5). Based on the CCK8 and colony formation assays, we found that the elevated cell proliferation ability mediated by miR-675 up-regulation was decreased after overexpression of PITX1 in AGS cells (Fig. 6A, C and D). Transwell assays illustrated that increased migration and invasion ability of AGS cells, which was mediated by miR-675 overexpression, was canceled by overexpressing PITX1 (Fig. 7A and B). In contrast, the reduced proliferation ability of SGC-7901 cells caused by miR-675 down-regulation was recovered by depleting PITX1 (Fig. 6B, E and F). Similarly, the decreased migration and invasion ability of SGC-7901 cells induced by miR-675 down-regulation was also rescued by depleting PITX1 (Fig. 7C and D). Thus, we surmised that miR-675 modulates GC cell viability and motility through targeting PITX1.

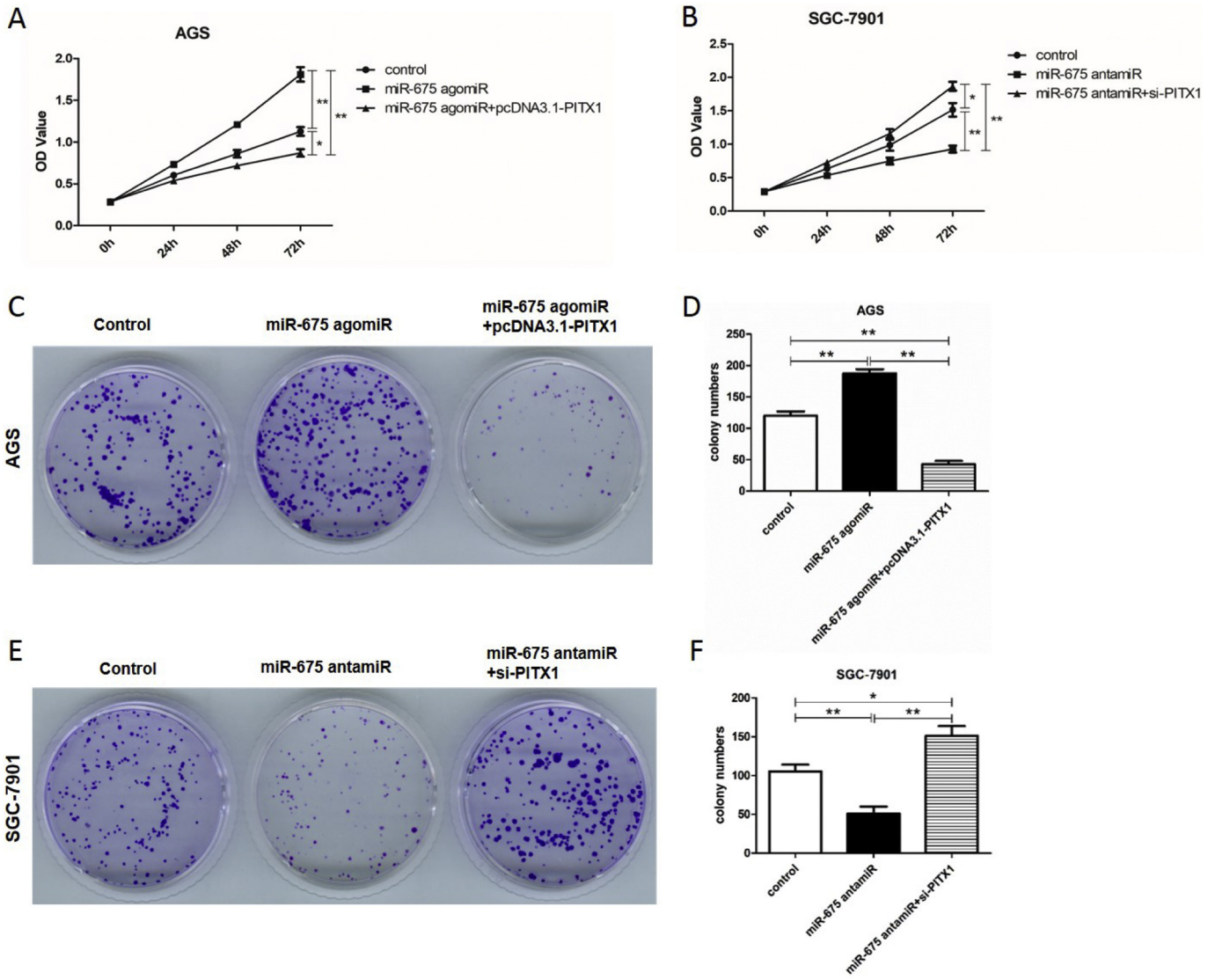

Fig. 6. PITX1 reversed GC cell proliferation mediated by miR-675. (A) Overexpression of PITX1 reversed proliferation ability of AGS cells that promoted by miR-675 up-regulation. (B) Knockdown of PITX1 rescued the proliferation ability of SGC-7901cells that suppressed by miR-675 down-regulation. (C–D) Overexpression of PITX1 reversed colony formation ability of AGS cells that promoted by miR-675 up-regulation. (E–F) Knockdown of PITX1 rescued the colony formation ability of SGC-7901cells that suppressed by miR-675 down-regulation. N = 6, *p < 0.05, **p < 0.01.

3.5. The effect of miR-675/PITX1 axis on epithelial-mesenchymal transition (EMT)

The effect of miR-675/PITX1 axis on the expression of EMT-related markers was determined by Western blot. The outcomes revealed that the level of E-cadherin was declined, while the levels of N-cadherin, Vimentin and MMP9 were enhanced in AGS cells transfected with miR-675 agomiR compared with the control. When co-transfected with miR-675 agomiR and pcDNA3.1-PITX1, the expression of E-cadherin was enhanced, while the levels of N-cadherin, Vimentin and MMP9 were decreased in AGS cells, compared with the miR-675 agomiR as well as the control groups (Fig. 8A–E).

On the contrary, the expression of E-cadherin was up-regulated, while the levels of N-cadherin, Vimentin and MMP9 were decreased in SGC-7901 cells transfected with miR-675 antamiR compared with the control. And when down-regulation of miR-675 and PITX1 together in SGC-7901 cells, the expression of E-cadherin was reduced, while the expression of N-cadherin, Vimentin and MMP9 were increased compared with the miR-675 antamiR as well as the control groups (Fig. 8A, F–I). Collectively, these data intimated that the miR-675 promoted EMT of GC cells via suppressing PITX1 expression.

3.6. The effects of miR-675/PITX1 axis on the Wnt/β-catenin signaling pathway

Hyperactivation of Wnt/β-catenin signaling is related to the aggressive progression of GC [15,16]. A high activity of β-catenin evaluated the migration and invasion of GC cells [17]. Thus the effect of miR-675/PITX1 expression on Wnt/β-catenin signaling pathway was determined by Western blot. We found that upregulation of miR-675 in AGS cells significantly increased the nuclear accumulation of β-catenin and enhanced the expression of Cyclin D1 and c-Myc. However, overexpression of PITX1 canceled the effect of miR-675 up-regulation on the expression of β-catenin, Cyclin D1 and c-Myc (Fig. 9A–B). On the contrary, down-regulation of miR-675 in SGC-7901 cells markedly reduced the expression of nucleus β-catenin and decreased the expression of Cyclin D1 and c-Myc. Knockdown of PITX1 restored the level of nucleus β-catenin, and revived the protein expression of Cyclin D1 and c-Myc that decreased by miR-675 down-regulation (Fig. 9C–D). These outcomes verified that miR-675 activated Wnt/β-catenin signaling

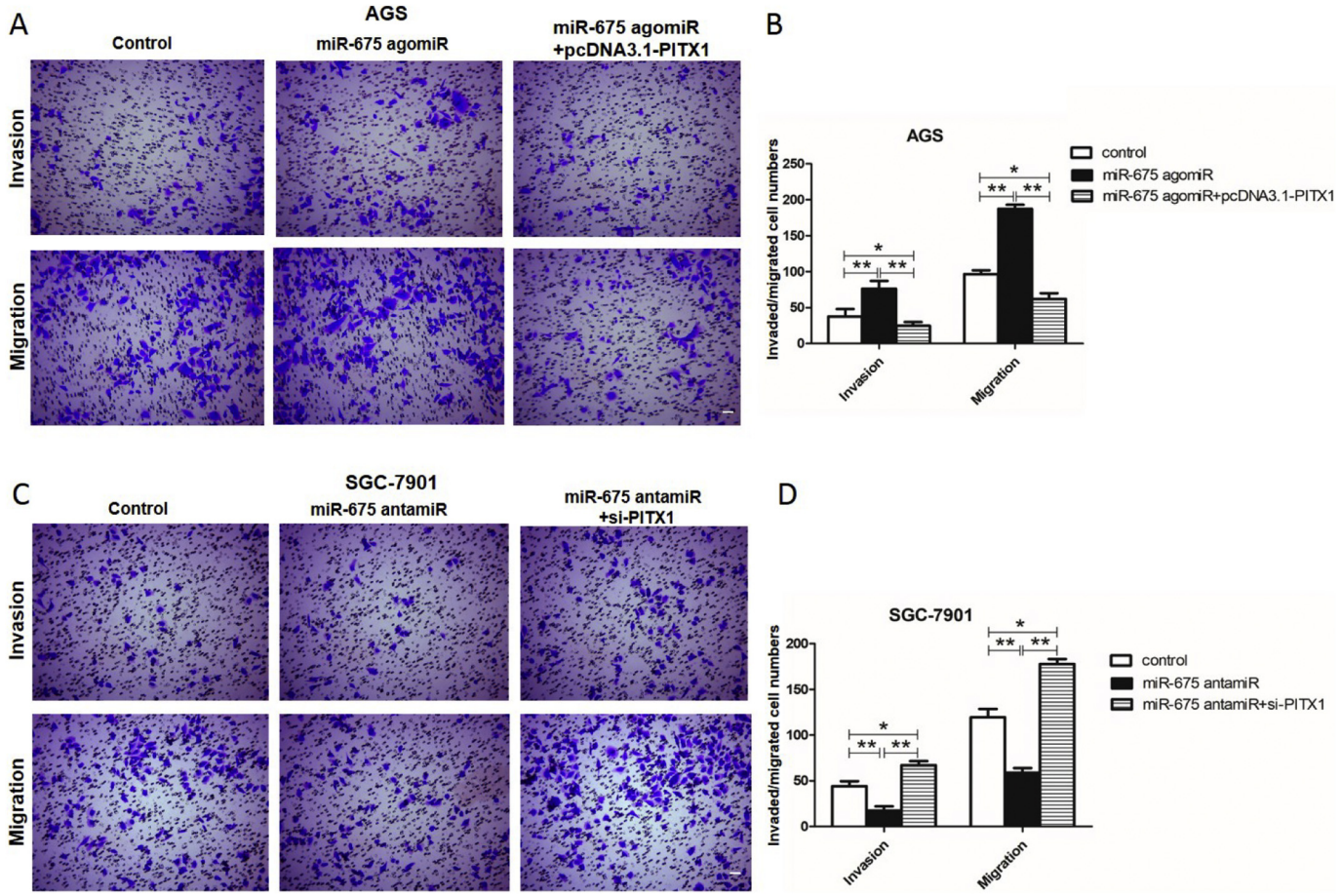

Fig. 7. PITX1 reversed GC cell invasion and migration mediated by miR-675. (A) Overexpression of PITX1 reversed invasion and migration abilities of AGS cells that promoted by miR-675 up-regulation. (B) The numbers of invaded and migrated AGS cells. (C) Knockdown of PITX1 rescued the invasion and migrated abilities of SGC-7901 cells that suppressed by miR-675 down-regulation. (D) The numbers of invaded and migrated SGC-7901 cells. N = 6, *p < 0.05, **p < 0.01. Bars = 100 μm.

pathway via repressing PITX1. Collectively, all the above data prompted us to conjecture that miR-675 accelerated GC cell viability, migration and invasion via promoting EMT and activating Wnt/β-catenin signaling by targeting PITX1.

4. Discussion

As vital modulators of gene expression, miRNAs are involved in many basic cellular processes, such as proliferation, invasion and/or metastasis, and death [18,19]. Abnormal expression of miRNAs has been recognized as a common feature of malignancies [18]. Moreover, miRNAs can be served as potential diagnostic markers for “early and less malignant” GC tumors, as well as be used in endoscopic treatment [20]. In our present study, we choose miR-675 as the study object since we revealed that its expression level was dramatically up-regulated in both GC tissues and cell lines, and its expression was negatively associated with the survival of GC patients. This result is consistent with the previous outcomes in GC obtained by other researchers [21,22]. In some other tumors, such as glioma [23], colorectal [24] and hepatocellular [25] tumors, the expression of miR-675 was also found to be enhanced. However, in prostate cancer [26], non-small cell lung cancer [27] and adrenal cortical carcinoma [28], the level of miR-675 was lower than that in their corresponding normal tissues. These contradictory phenomena entailed that miR-675 may play different roles in different tumors. From in vitro assays, we identified that up-regulation of miR-675 in AGS cells enabled them exhibited greater proliferation and invasive abilities; whereas, silencing of miR-675 in SGC-7901 cells inhibited the growth and motility of this cell line. The above data intimated that miR-675 acts as a tumor- forwarder in the progression of GC.

Accumulating evidences revealed that the effect of miRNAs on carcinogenesis is achieved by regulating their downstream target genes [19,29]. Studies have uncovered that > 30% of human genes are modulated by miRNAs and one miRNA often regulates hundreds of RNA [30]. Hence, we next predicted the candidate targets using the TargetScan online tool. Among all the candidates, we found that PITX1 was down-regulated in GC and its expression was positively associated with the survival of GC patients, hence we further explored the relationship between miR-675 and PITX1 in GC cells. Our results verified that miR-675 could direct bind to the 3′UTR of PITX1 and then negatively regulate its expression. Rescue assays revealed that PITX1 reverses GC cell proliferation and invasion mediated by miR-675, which hinted that miR-675 involve in GC cell growth and motility via hindering PITX1 expression. Recently, PITX1 was found to act as a suppressor in TERT transcription via binding to TERT promoter, which finally modulated telomerase activity (important for cellular immortalization and cancer development) [31]. In addition, there were evidences showed that PITX1 inhibited tumorigenicity via suppressing RAS pathway through RASAL1 [32]. To date, the inhibitory role of PITX1 in the development of several malignancies (for instance, lung cancer, colorectal carcinoma and GC etc.) has been reported [13,32,33]. Fengchang Qiao et al. reported that PITX1 was down-regulated by miR-19a-3p in GC and down-regulation of PITX1 enhanced cell malignancy via affecting PDCD5 [13]. In our study, we clarified the targeting relationship between miR-

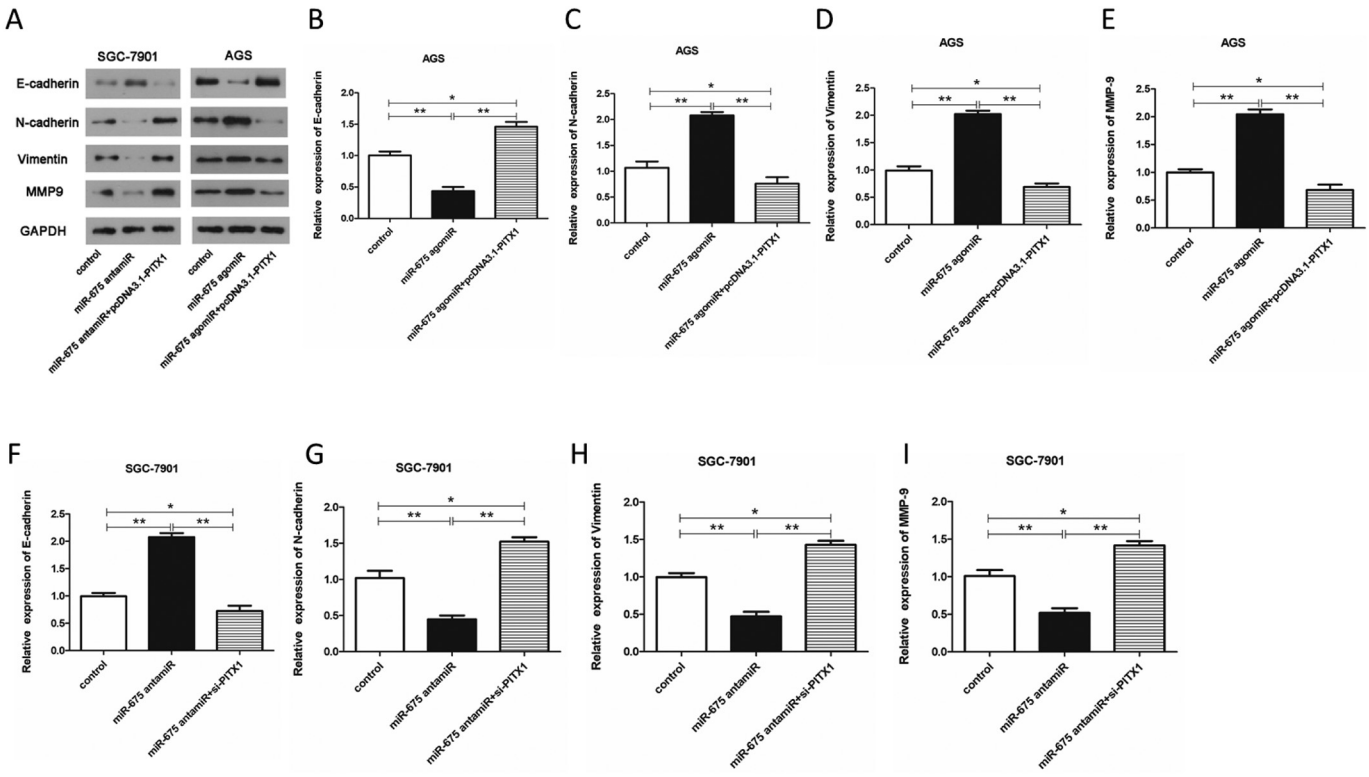

Fig. 8. MiR-675/PITX1 axis affected EMT of GC cells. (A) The expressions of EMT-related proteins were detected by western blot. (B–E) The relative expression of E-cadherin, N-cadherin, Vimentin and MMP9 in AGS cells transfected with miR-675 agomiR or miR-675 agomiR and pcDNA3.1- PITX1. (F–I) The relative expression of E-cadherin, N-cadherin, Vimentin and MMP9 in SGC-7901 cells transfected with miR-675 antamiR or miR-675 antamiR and si-PITX1. N = 6, *$p < 0.05$, **$p < 0.01$.

675 and PITX1 in GC and revealed their effects on GC cell replication and motility for the first time, which enriched our understanding of GC pathology.

Epithelial-mesenchymal transition (EMT) is known as a vital sponsor of epithelial-derived tumors invasiveness, which is classical characterized by reduction of cell-cell junctions and the re-establishment of the cytoskeleton [34]. Hence, we explored the effect of miR-675/PITX1 on EMT by measuring the expression of EMT markers using Western bolt. E-cadherin is one of the most commonly used markers for epithelial trait, and N-cadherin and vimentin are common markers for the mesenchyma [35]. Matrix metalloproteinase 9 (MMP-9), a member of MMPs family, has been identified in large quantities in tumors and supposed to associated with tumor invasiveness [36]. In addition, previous reports revealed that MMP-9 mediated the degradation of E-cadherin via cleaving E-cadherin ectodomain near plasma membrane into sE-cad [37]. Our results illustrated that overexpression of miR-675 improved the expression of N-cadherin, Vimentin and MMP9, while decreased the expression of E-cadherin. Overexpression of PITX1 reversed the effect on EMT markers caused by miR-675 up-regulation. These phenomena suggested that miR-675/PITX1 axis possibly affected GC invasiveness via regulating EMT.

The Wnt signaling is known as an evolutionarily conserved pathway which is implicated in many events during embryonic development as well as tissue homeostasis, for instance cell proliferation, differentiation etc. [38]. Aberrant Wnt/β-catenin signaling has been illustrated to be involved in tumor progression in many types of cancers including GC [16,39]. In GC, it has been suggested that Wnt/β-catenin signaling promoted the development of tumor through facilitating the proliferation and invasion of GC cells [40]. What's more, it was identified as one of the main signaling pathways that implicated in EMT and then took a vital part in tumor metastasis [41]. Blocking Wnt/β-catenin signaling exhibited anti-metastatic effect on GC cells [42]. As we observed from the above results that miR-675/PITX1 axis could regulate the proliferation, invasion and EMT of GC cells, we wondered that whether these regulation was mediated via Wnt/β-catenin signaling. Our data showed that up-regulation/down-regulation of miR-675 notably promoted/suppressed the Wnt/β-catenin signaling pathway in GC cells, meanwhile, PITX1 overexpression/depletion canceled up-regulation/down-regulation of miR-675 caused effect on Wnt/β-catenin signaling pathway. The promoting effect of miR-675 on Wnt/β-catenin signaling pathway observed in our study was consistent with Viviana Costa et al' research, in which they illustrated that miR-675-5p plays a positive role in activating Wnt/β-catenin signaling pathway during hMSC osteoblastic differentiation [43]. Moreover, overexpression of PITX1 presented an inhibitory effect on the Wnt/β-catenin signaling pathway in senile osteoporosis [44], which was also in line with our data. Collectively, these outcomes hinted that miR-675 promoted the activation of Wnt/β-catenin signaling pathway possibly via targeting PITX1 and provided evidence for our speculation that miR-675/PITX1 axis affect GC cell proliferation and metastasis via modulating Wnt/β-catenin signaling pathway. However, the detailed mechanism of how miR-675/PITX1 linked with Wnt/β-catenin signaling needs our further exploration.

Taken together, the major findings in our study were summarized as follows: a) miR-675 was up-regulated in GC and predictive of poor prognosis. b) miR-675 facilitated GC cell proliferation and invasion via targeting PITX1. c) miR-675/PITX1 axis modulated GC cell proliferation and invasion via regulating EMT and Wnt/β-catenin signaling pathway. The miR-675/PITX1 axis that identified in our study might be necessary in modulating GC progression and possess the potential to serve as early diagnostic markers as well as therapeutic targets for GC.

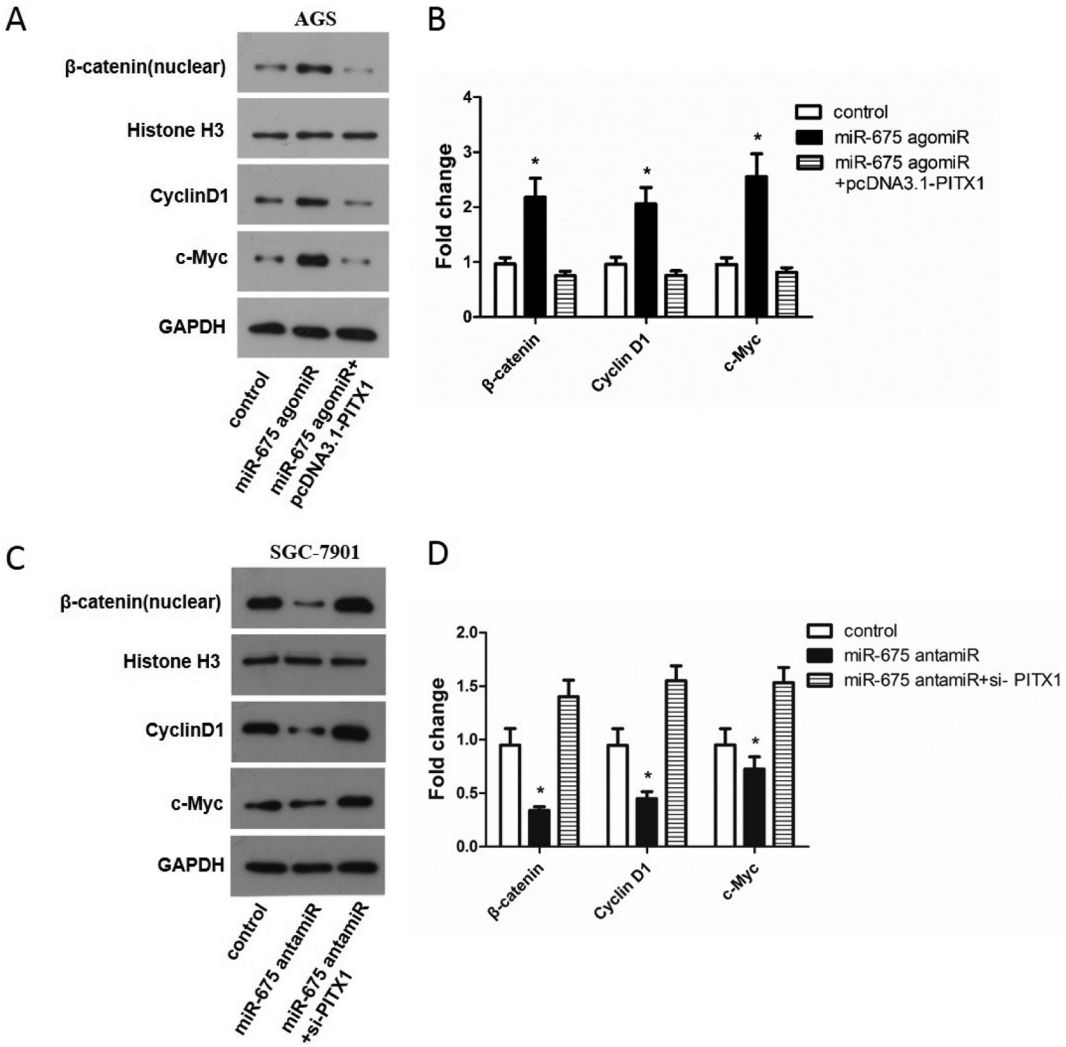

Fig. 9. The effect of miR-675/PITX1 axis on Wnt signaling pathway was determined by Western blot. (A–B) The expression of Wnt signaling pathway-related proteins in AGS cells transfected with miR-675 agomiR or miR-675 agomiR and pcDNA3.1- PITX1. (C–D) The expression of Wnt signaling pathway-related proteins in SGC-7901 cells transfected with miR-675 antamiR or miR-675 antamiR and si-PITX1. *$p < 0.05$ vs. control group.

Declaration of Competing Interest

None.

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