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The long non-coding RNA H19-derived miR-675 modulates human gastric cancer cell proliferation by targeting tumor suppressor RUNX1

Ming Zhuang [a,b,1], Wen Gao [a,1], Jing Xu [a], Ping Wang [a], Yongqian Shu [a,*]

[a] Department of Oncology, The First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu, China
[b] Department of Oncology, The First People's Hospital of Lianyungang, Lianyungang, Jiangsu, China

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ABSTRACT

The lncRNA H19 has been recently shown to be upregulated and play important roles in gastric cancer tumorigenesis. However, the precise molecular mechanism of H19 and its mature product miR-675 in the carcinogenesis of gastric cancer remains unclear. In this study, we found that miR-675 was positively expressed with H19 and was a pivotal mediator in H19-induced gastric cancer cell growth promotion. Subsequently, the tumor suppressor Runt Domain Transcription Factor1 (RUNX1) was confirmed to be a direct target of miR-675 using a luciferase reporter assay and Western blotting analyses. A series of rescue assays indicated that RUNX1 mediated H19/miR-67-induced gastric cancer cell phenotypic changes. Moreover, the inverse relationship between the expression of RUNX1 and H19/miR-675 was also revealed in gastric cancer tissues and gastric cancer cell lines. Taken together, our study demonstrated that the novel pathway H19/miR-675/RUNX1 regulates gastric cancer development and may serve as a potential target for gastric cancer therapy.

1. Introduction

Gastric cancer is one of the most frequent causes of mortality worldwide, with an estimated 990,000 new cases and 738,000 deaths registered in 2008 [1,2]. Although the advanced setting and standard chemotherapy protocols have slightly reduced the 5-year mortality rate over the last few decades, there is still a pressing need to search for new prognostic biomarkers and therapeutic targets for this disease [3].

In recent years, it has become increasingly apparent that long non-coding RNAs (lncRNAs) are of crucial functional importance for disease occurrence. LncRNAs are a family of regulatory RNA molecules that are greater than 200 nucleotides in length. Different studies have demonstrated that lncRNAs are involved in the development of different types of cancer [4–6], for example, differential display code 3 (DD3PCA3) in prostate cancer, metastasis associated lung adenocarcinoma transcript 1 (MALAT-1) in non small cell lung cancer and HOX transcript antisense RNA (HOTAIR) in breast cancer and colorectal cancer [7–10]. There have also been reports demonstrating that the levels of H19 and colon cancer-associated transcript-1 (CCAT1) are markedly increased and play important roles in the molecular etiology of gastric cancer [11,12]. H19 is a paternally imprinted gene and is locate on chromosome 11p15.5, which does not encode for a protein but encodes for a 2.3 kb H19 noncoding RNA [13]. It is highly expressed in embryogenesis but is nearly completely downregulated in most tissues after birth [14]. Currently, several studies have shown that H19 is overexpressed in tumors and functions as an oncogene [15–17]. Yang et al. demonstrated that upregulated long non-coding RNA H19 contributed to the proliferation of gastric cancer cells and further verified that the association of H19 and p53 partially contributed to p53 inactivation in gastric cancer [11]. However, the precise underlying mechanism of H19 in gastric cancer tumorigenesis still needs to be further explored. The H19/miR-675 signaling axis has been shown to play important roles in colorectal cancer carcinogenesis [18]. Consequently, we speculated that the tumorigenesis process induced by H19 in gastric cancer may also be mediated via miR-675, similar to colorectal cancer. Thus, in the present study, we performed a series of rescue assays to confirm that H19 modulates human gastric cancer cell proliferation via miR-675. Furthermore, we identified a novel target of miR-675, RUNX1, a well-known tumor suppressor that mediates H19/miR-675-induced gastric cancer cell proliferation. Thus, we identified a new signaling pathway, H19/miR-675/RUNX1, which modulates gastric cell carcinogenesis and may serve as a potential diagnostic and therapeutic target for gastric cancer.

* Corresponding author. Address: Department of Oncology, The First Affiliated Hospital of Nanjing Medical University, 300 Guangzhou Road, Nanjing 210029, China. Fax: +86 025 83718836.
E-mail address: shuyongqian39000@163.com (Y. Shu).
[1] These authors contributed equally to this work.

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2. Materials and methods

2.1. Gastric cancer tissue and cell culture

Gastric cancer patient tissues were immediately preserved in RNA fixative (Bioteke, Beijing, China) after removal from the body and were stored at –80 °C until further use. Cancer tissue samples were obtained from The First Affiliated Hospital of Nanjing Medical University. This study was approved by the Ethics Committee of The First Affiliated Hospital of Nanjing Medical University. The median age of the patients was 65 years (48–79 years). All patients had adenocarcinoma at stages I–IV (8/I, 8/II, 4/III, 4/IV). The human gastric cancer cell lines used for analysis of the expression levels of H19, miR-675 and RUNX1 mRNA were selected as previously described by Yang et al. [11]. We selected AGS (p53 wild-type) and MGC803 (p53 mutant) for functional studies. All cell lines were cultured in RPMI1640 medium, supplemented with 10% fetal bovine serum (FBS) and 1% PS (100 U/ml penicillin, 100 μg/ml streptomycin) and maintained in a humidified incubator with 5% CO_2 at 37 °C.

2.2. RNA extraction and quantitative reverse transcription polymerase chain reaction

Total RNA containing miRNAs was extracted as previously described [18]. The method used to detect H19, miR-675 and RUNX1 messenger RNA (mRNA) was also based on a previous report [18]. For mRNA detection, the primers used in this study were as follows: H19 (forward: 5′-TACAACCACTGCACTACCTG-3′; reverse: 5′-TGGAATGCTTGAAGGCTGCT-3′); RUNX1 (forward: 5′-CCGAGAACCTCGAAGACATC-3′; reverse: 5′-GATGGTTGGATCTG CCTTGT-3′); and GAPDH as a loading control: F: 5′-ACCTGACCTGC CGTCTAGAA-3′, R: 5′-TCCACCACCCTGTTGCTGTA-3′[19].

2.3. MTT assay, colony formation assay

For the MTT assay, transfected cells were plated into 96-well plates at a density of 1000 cells/well. The transfected cells were incubated with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) for 4 h at 37 °C. The absorbance at 490 nm was measured. For the colony formation assay, cells were seeded into 6-well plates at a density of 500 cells/well after transfection. Approximately 10 days later, the number of colonies was counted, and images were obtained of the representative colonies. H19-RNAi was performed as previously described [11,14]. The siRNAs used in this study were mixtures of three siRNAs, and the sequences used were as follows: H19-siRNA1 (5′-UAAGUCAUUUGC ACUGGUUdTdT-3′), H19-siRNA2 (5′-GCAGGACAUGACAUGGUCC dTdT-3′), and H19-siRNA3 (5′-CCAACAUCAAAGACACCAUdTdT-3′).

2.4. miRNA target prediction

Targetscan, miRDB, and DIANA were used to predict the putative targets of miR-675.

2.5. Dual luciferase activity assay

The 3′-UTR or mutant 3′-UTR of human *RUNX1* cDNA containing the putative target site for miR-675 was chemically synthesized and inserted downstream of the luciferase gene in the pGL3-control vector (Promega, Madison, WI, USA). Hsa-miR-675 (MIMAT0004284): 2′-O-Me-UGGUGCGGAGAGGGCCCACAGUG and Anti-miR-675: 2′-O-Me--CACUGUGGGCCCUCUCCGCACCA were used for the luciferase assay. The MGC803 and AGS cells were plated at 1.2×10^5 cells/well in 24-well plates. Two hundred nanograms of pGL3-RUNX1-3′-UTR or pGL3-RUNX1-mut 3′-UTR (CACC to AGAA in seed sequence) plus 80 ng pRL-TK (Promega) were transfected in combination with 60 pmol of the miR-675 mimic, Anti-miR-675 mimic or controls using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. The luciferase activity was measured 24 h after transfection using the Dual Luciferase Reporter Assay System (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity for each transfected well.

2.6. Annexin V and PI staining

Evaluation of gastric cancer cell apoptosis was performed using FITC-conjugated Annexin V and PI (BD Pharmingen, San Jose, CA). The cells were washed twice in cold 1 × PBS and resuspended in Annexin V–binding buffer (BD Pharmingen, San Jose, CA) at a concentration of 3×10^6 per ml. This suspension (100 μl) was stained with 5 μl of Annexin V-FITC and 5 μl PI. These cells were gently vortexed and then incubated for 15 min at room temperature in the dark. After the addition of 400 μl of binding buffer to each tube, the cells were analyzed using flow cytometry.

2.7. Western blotting assays

MGC803 and AGS cells were harvested at 48 h after transfection and lysed in Triton X-100 lysis buffer containing 25 mM Tris–HCl (pH 7.5), 137 mM NaCl, 2.7 mM KCl, 1% Triton X-100 and protease inhibitor cocktail (Sigma, St. Louis, MO, USA) for 30 min at 4 °C. The primary antibodies included rabbit polyclonal anti-RUNX1 (DW71, Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:100 dilutions) and anti-GAPDH antibody (Abcam, San Francisco, USA, 1:1000 dilution). The secondary antibody included goat anti-rabbit IgG conjugated with HRP (horseradish Peroxidase) at a dilution of 1:1000. GAPDH was used as an internal control. Representative images from one of three independent experiments are shown. The band intensity was quantified using Image-J software.

2.8. Statistical analysis

All data are shown as the mean ± SD, and the experiments were repeated three times. Two-tailed Student' *t*-test was performed, and $P < 0.05$ was considered statistically significant.

3. Results

3.1. miR-675 upregulation in gastric cancer is correlated with H19 expression

We examined the expression of H19 and miR-675 in gastric cancer cells and gastric cancer tissues using quantitative real-time PCR. These results were consistent with findings obtained from a report by Yang et al. (Fig. 1A). Moreover, the expression levels of H19 and miR-675 in five gastric cancer cell lines were correlated with each other ($r = 0.9910$, $P = 0.0315$, data not shown). We next assessed the correlation of miR-675 and H19 expression in 24 human gastric cancer tissues. As shown in Fig. 1B, the expression of H19 and miR-675 in gastric cancer tissues was correlated with each other ($r = 0.8214$, $P < 0.01$). These results were consistent with the findings of Cai et al. and Tsang et al. [13,18], in which lncRNA H19 RNA was found to be the primary precursor of miR-675, but also indicated that miR-675 may mediate H19-induced gastric cancer progression.

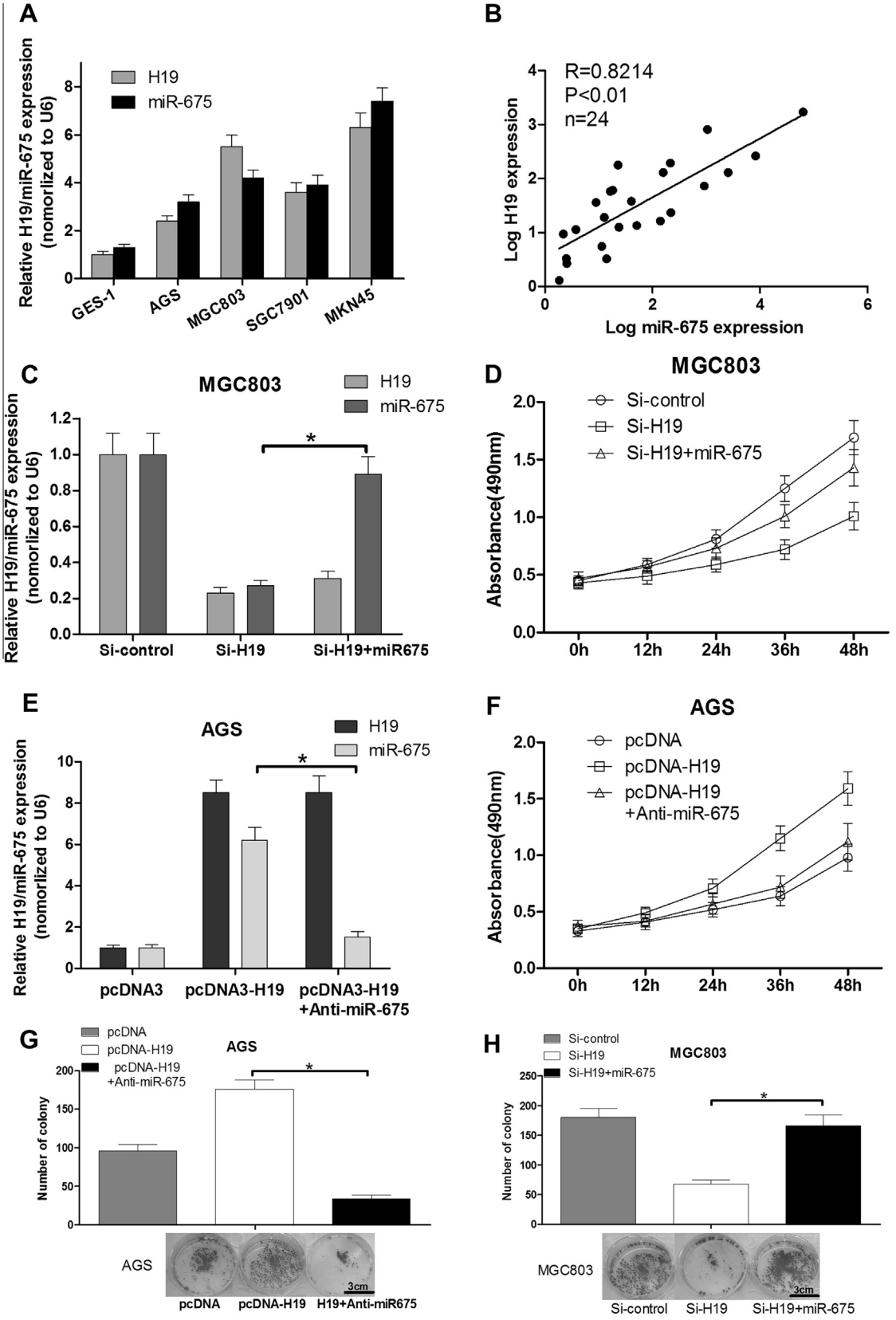

Fig. 1. Role of H19-derived miR-675 in gastric cancer cell proliferation. (A) Expression of H19 and miR-675 in human gastric cancer cell lines, which were normalized to U6 with respect to GES-1. (B) Positive correlation of H19 and miR-675 expression in gastric cancer tissues, $r = 0.8214$, $P < 0.01$, $n = 24$. (C, E) The miR-675 expression in cells after transfection with miR-675 or anti-miR-675 was validated using quantitative reverse transcription-PCR. Relative H19/miR-675 expression in cells was compared with those of the control. (D, F) H19 knockdown- or overexpressed-cells were transfected with miR-675 or anti-miR-675 for 24 h, allowed to grow for 48 h and were subsequently evaluated using the MTT assay. In addition, the absorbance at 490 nm was measured at 0, 12, 24, 36 and 48 h. (G and H) H19 knockdown- or overexpressed-cells were transfected with miR-675 or anti-miR-675 for 24 h and then plated in 6-cm dishes for 10 days.*$P < 0.05$. Scale bars = 3 cm.

3.2. Overexpression of miR-675 restores siRNA-H19-induced inhibition, while knockdown of miR-675 rescues pcDNA-H19-induced promotion of gastric cancer cell proliferation

To elucidate whether miR-675 plays pivotal roles in H19-induced gastric cancer cell proliferation, we transfected AGS and MGC803 cells with H19 siRNA and miR-675 mimics and examined their proliferation using MTT and colony formation assays. As shown in Fig. 1C, miR-675 mimics could significantly restore inhibition of miR-675 expression via si-H19 in MGC803 cells. In addition, miR-675 mimics also restored the proliferation inhibition of MGC803 cells by si-H19. The same effect was observed in the col-

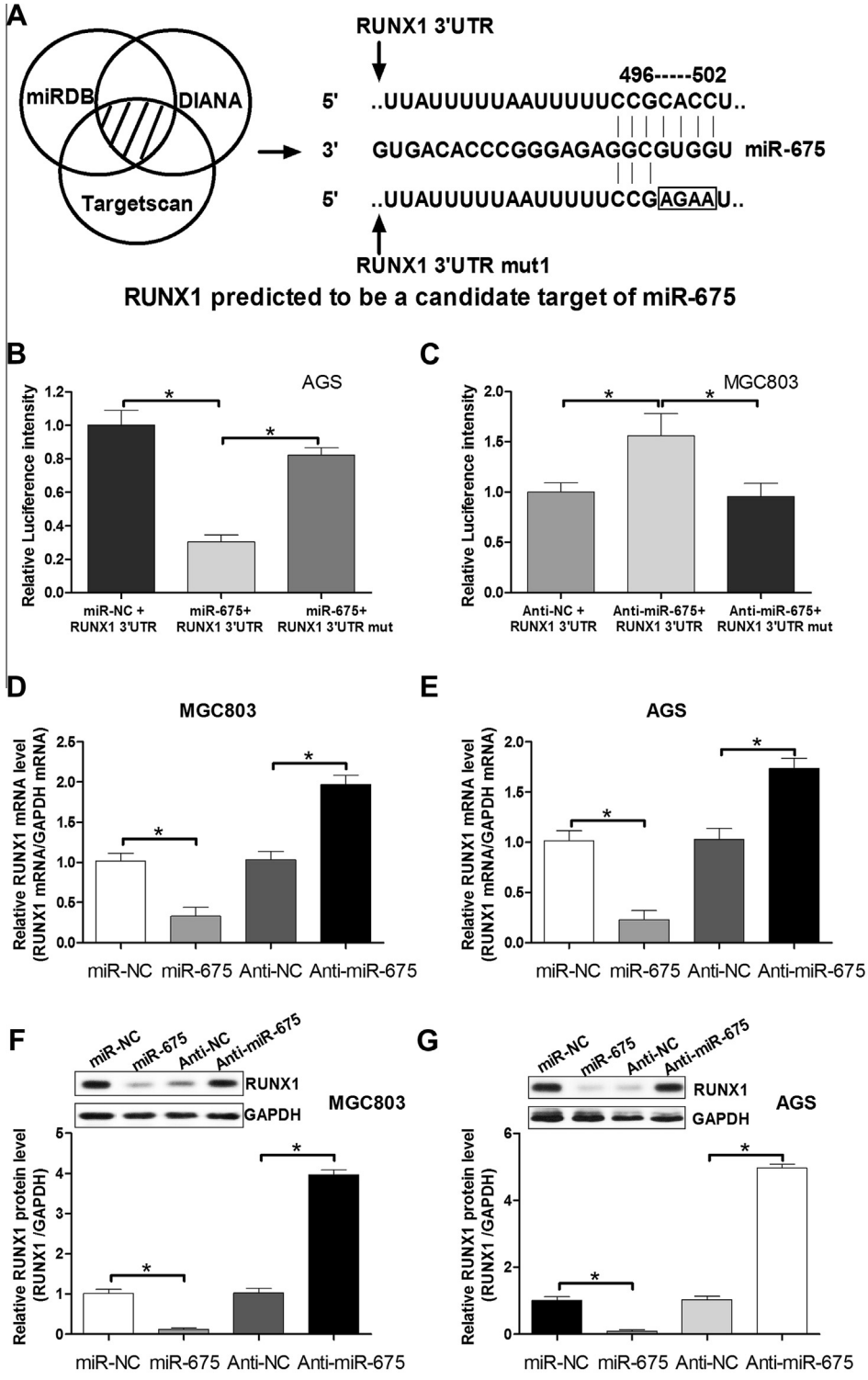

Fig. 2. miR-675 directly targets RUNX1. (A) RUNX1 is potentially targeted by miR-675 using bioinformatic analyses. Algorithms between miR-675 and the 3′UTR of RUNX1 and also the mutant RUNX1 3′UTR. (B and C) The luciferase reporter assay was performed to detect the effect of miR-675 or anti-miR675 on the luciferase intensity controlled by the 3′UTR of RUNX1. $^{*}P < 0.05$. (D and E) qRT-PCR was performed to detect the effects of miR-675 on RUNX1 mRNA expression. (F and G) Western blotting analyses were performed to detect the effect of miR-675 on the protein levels of RUNX1. $^{*}P < 0.05$.

ony formation assay (Fig. 1H). Furthermore, we used pcDNA-H19 to increase the H19 expression level in H19-low expressed AGS cells. Next, we investigated whether anti-miR-675 could also rescue the promotion of pcDNA-H19-induced gastric cancer cell proliferation. As shown in Fig. 1E–G, when the miR-675 expression level was reduced using anti-miR-675, transfection with pcDNA-H19 resulted in an impairment in the proliferation and colony formation in AGS cells. These results suggested that H19 regulates the gastric cancer cell proliferation phenotype via miR-675.

3.3. MiR-675 downregulates RUNX1 expression at the mRNA and protein level in gastric cancer cells

A greater specificity in miRNA prediction can be attained using the consensus of multiple algorithms, and thus, genes predicted to be a target of miR-675 by at least three algorithms (Targetscan, miRDB and DIANA) were selected for further study (Fig. 2A). As a result, an important tumor suppressor gene RUNX1 was identified, and the sequence alignments for miR-675 and the 3′-UTR of RUNX1 mRNA are shown in Fig. 2A. To confirm whether the 3′UTR of RUNX1 was a functional target of miR-675 in gastric cancer, we established a luciferase reporter system. The 3′-UTR of RUNX1 mRNA flanking the entire putative target sequence was subcloned into the firefly luciferase reporter vector pGL3. In addition, we also constructed a mutated 3′UTR vector that contains four mutant nucleotides in the seed sequence (Fig. 2A, CACC–AGAA). When miR-675 was overexpressed in AGS cells, the luciferase expression level was significantly lower compared to the control group. However, the luciferase intensity with the mutated 3′UTR was not affected by miR-675 (Fig. 2B). When blocking the expression of miR-675 with anti-miR-675 in MGC803 cells, the luciferase intensity was increased in RUNX1-3′UTR wild-type cells (Fig. 2C). These results suggested that RUNX1 was a direct target of miR-675 in gastric cancer cells.

3.4. Ectopic expression of RUNX1 counteracts the effects of miR-675, while knockdown of RUNX1 reverses the effects of anti-miR-675 in MGC803 and AGS gastric cancer cell growth

To confirm that the phenotype of miR-675 in gastric cancer cells is the result of the repression of RUNX1, a rescue experiment was performed. First, we confirmed the expression of pcDNA3/RUNX1 (RUNX1) or siRNA/RUNX1 (si-RUNX1) in AGS or MGC803 cells, respectively, using Western blotting analyses (upper-left in Fig. 3A and B). Next, AGS cells were transfected with miR-675 and RUNX1. Over-expression of RUNX1 counteracts the cell growth promotion caused by miR-675, as observed in the MTT assay (Fig. 3A) and colony formation assay (Fig. 3C). In addition, MGC803 gastric cancer cells were transfected with anti-miR-675 plus siRUNX1. Similarly, knockdown of RUNX1 reverses the cell growth retardation caused by anti-miR-675, as observed in Fig. 3B and D. These results indicated that RUNX1 is an important mediator of cell growth regulation via miR-675 in gastric cancer.

3.5. RUNX1 mRNA is inversely expressed with miR-675 and H19 in gastric cancer

We demonstrated that H19 regulates the gastric cancer cell proliferation phenotype via miR-675 and that miR-675 can down-regulate the expression of RUNX1 to regulate gastric cancer cell growth. To determine whether this regulatory mechanism exists in gastric cancer tissues, we examined these three types of transcripts in 24 human gastric cancer tissues. The data were analyzed using the $2^{-(\Delta CT)}$ method, and the means are represented as Log(X). These results indicated that RUNX1 mRNA was inversely expressed with miR-675 and H19 in gastric cancer tissues (Fig. 4B and C). This concordant inverse correlation between RUNX1 and miR-675 or H19 was also observed in five types of gastric cancer cell lines (Figs. 4A and 1A).

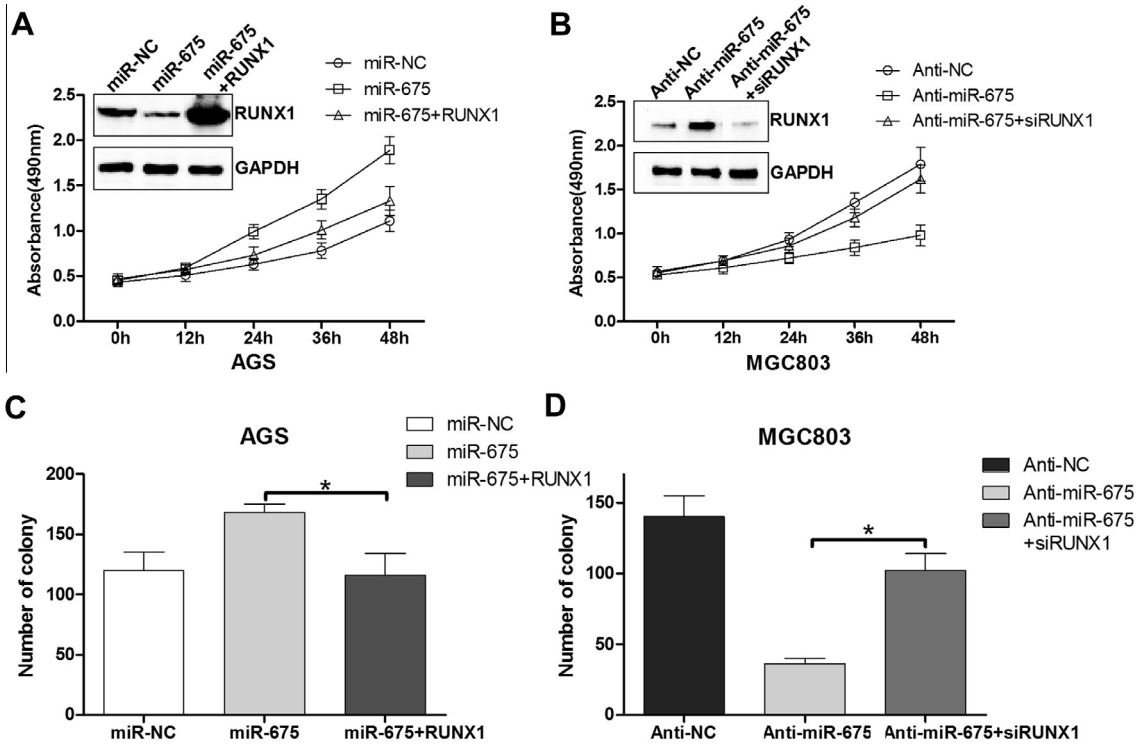

Fig. 3. The effect of miR-675/RUNX1 on cell proliferation and clonogenicity in gastric cancer cells. (A, C) AGS cells were transfected with miR-675 with or without RUNX1 for 24 h and subsequently analyzed using the MTT assay and colony formation assay. (B, D) MGC803 cells were transfected with anti-miR-675 with or without siRNA RUNX1 (siRUNX1) for 24 h and subsequently analyzed using the MTT assay and colony formation assay.

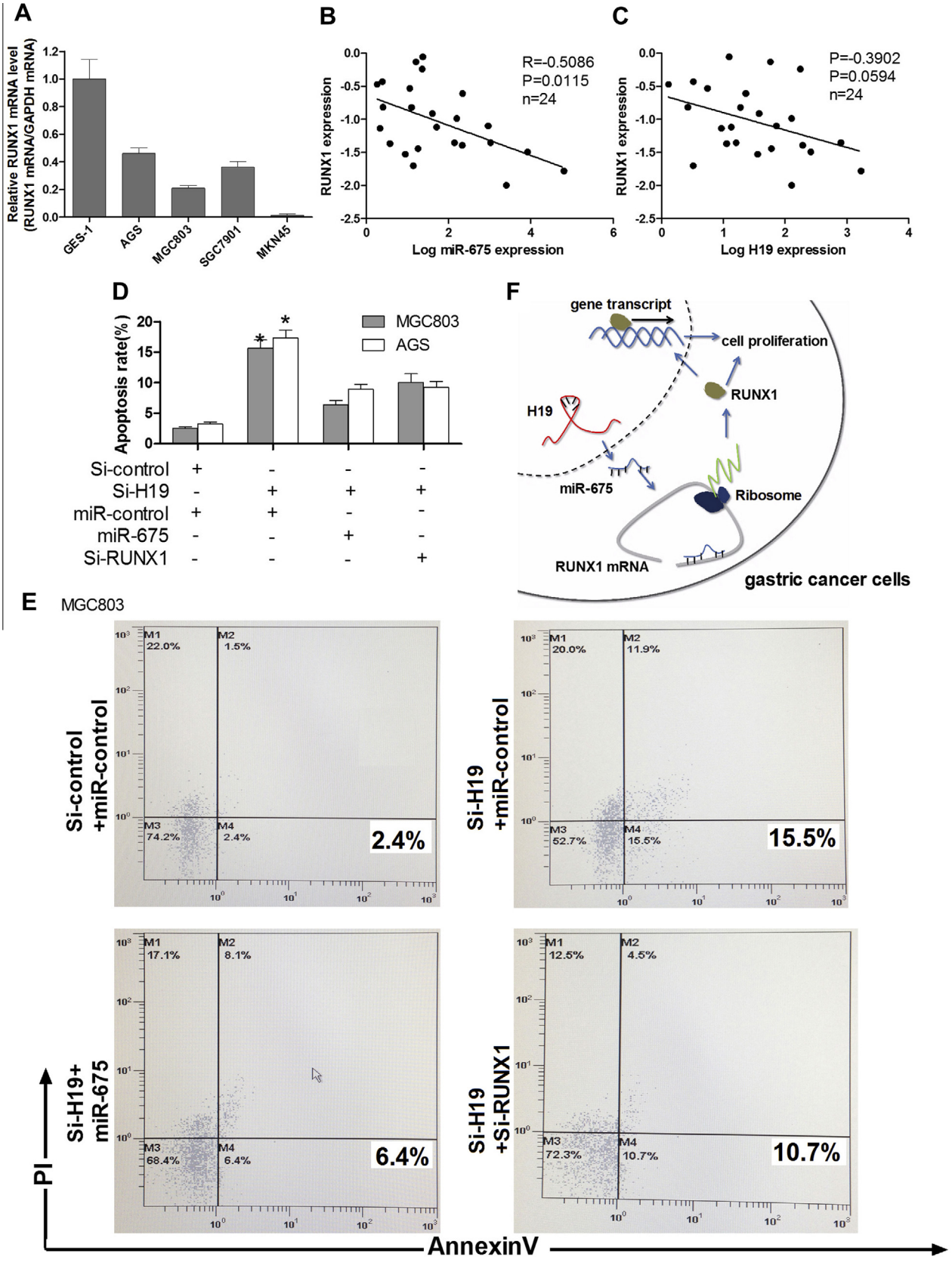

Fig. 4. H19/miR-675/RUNX axis in gastric cancer. (A) Relative expression of RUNX1 in human gastric cancer cell lines, which was normalized to GAPDH with respect to GES-1. (B and C) RUNX1 mRNA expression was inversely correlated with H19 and miR-675 in gastric cancer tissues as detected using quantitative reverse transcription-PCR, Mean ± SD, $n = 24$. (D, F) H19-deficient AGS or MGC803 cells were transfected with miR-675 or siRUNX1 and then analyzed using flow cytometry. Representative images are shown (MGC803 cells), and the data are presented as the mean ± S.D. from three independent experiments. $^{*}P < 0.05$. (E) Schematic representation of the hypothetical molecular mechanism of H19/miR-675/RUNX1 regulation on the growth of gastric cancer cells.

3.6. Both miR-675 and siRNA-RUNX1 can restore siRNA-H19-induced gastric cancer cell apoptosis

To establish the H19/miR-675/RUNX1 signaling pathway in the regulation of gastric cancer cell proliferation, we further analyzed the roles of three molecules in the apoptotic process of gastric cancer cells using flow cytometry. We performed a rescue assay using transfected MGC803 cells with miR-675 and siRUNX1 to restore the cell apoptosis induced by siH19. These results showed that when miR-675 or siRUNX1 was transfected, the cell apoptosis rate was recovered to 6.4% and 10.7%, respectively, compared to H19-deficient MGC803 cells. Moreover, a similar result was obtained in AGS cells (Fig. 4D). These findings indicated that both miR-675 and siRNA-RUNX1 can restore siRNA-H19-induced gastric cancer cell apoptosis.

4. Discussion

The carcinogenesis of gastric cancer is complex and consists of multiple processing steps, involving numerous genetic and epigenetic alterations. Profiles of cDNA arrays have demonstrated hundreds of genes that are up- or downregulated in gastric cancer [20]. Among these genes, several genes have been shown to be involved in gastric development, such as NF-kB, HER-2 and PAK4, among others [21,22]. Moreover, the profiles of miRNA arrays have shown that numerous miRNAs are differentially expressed in gastric cancer tissues compared with non-tumor tissues [2,23] and play prominent roles in tumorigenesis and the metastasis of gastric cancer, such as miR-106a, miR-202-3p and miR-363, among others [24–26]. Recently, lncRNA expression profiles in gastric cancer have revealed the potential role of lncRNAs in gastric cancer occurrence and development [27], such as the SUMO1 pseudogene 3 (SUMO1P3), BM742401, MEG3 and H19 [11,28–30]. However, these studies focused on the mechanism of a single species in tumor development, namely protein coding genes, microRNAs and long non-coding RNAs. In our study, we established a potential mechanism of the H19/miR-675/RUNX1 signaling axis in gastric development, which links protein encoding genes, microRNAs and long non-coding RNAs. This approach may provide new clues for future therapeutic treatments of gastric cancer.

Mature miRNAs have been shown to be critical regulators in cancer-related processes, such as let-7 and miR-15 [31,32]. Currently, differential expression of miR-675 in benign and malignant adrenocortical tumors has been demonstrated [33]. Hernandez et al. have demonstrated that miR-675 plays important roles in hepatocellular carcinoma [34]. However, the role of miR-675 and its regulatory network in gastric cancer is still largely unknown, despite the recent identification of the association of H19 with gastric cancer. Thus, we focused our investigation on the biological effects of miR-675 in gastric cancer cell lines. It is essential to explore its target genes for a working mechanism of miR-675 in gastric cancer. Many targets of miR-675 have been proposed in different models of disease, such as Twist1 and Rb in AFP-secreting hepatocellular carcinoma and Igf1r in both embryonic and extra-embryonic cell lineages [34,35]. In this study, we identified a novel target of miR-675, RUNX1, in gastric cancer, which highlights the miR-675 regulatory network in cancer development.

Runx1 is an important tumor suppressor in cancers. Although the involvement of miR-mediated post-transcriptional regulation was investigated, such as miR-27a [36], H19/miR-675 regulation of RUNX1 was also demonstrated. In the present study, we demonstrated that ectopic expression of RUNX1 rescued the effects caused by miR-675 in gastric cancer cells. Furthermore, our findings support the hypothesis that H19/miR-675 functions in gastric cancer by regulating the expression of RUNX1 (Fig. 4E). To the best of our knowledge, this is the first report to provide evidence of H19/miR-675/RUNX1 function in gastric cancer.

Taken together, the major findings of this study can be summarized as follows: (i) H19 regulates the gastric cancer cell proliferation phenotype via miR-675. (ii) H19-derived miR-675 modulates cell proliferation of gastric cancer cells by targeting the tumor suppressor RUNX1. (iii) The H19/miR-675/RUNX1 signaling axis plays an important role in the tumorigenesis of gastric cancer and may also serve as potential prognosis markers and potential targets for therapy.

Acknowledgments

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