Cancer Management and Research

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ORIGINAL RESEARCH

Long noncoding RNA SNHG16 silencing inhibits the aggressiveness of gastric cancer via upregulation of microRNA-628-3p and consequent decrease of NRP1

This article was published in the following Dove Press journal:
Cancer Management and Research

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Background: MicroRNA-628-3p (miR-628-3p) has been reported to play important roles in the progression of multiple human cancers. Nonetheless, whether the expression profile of miR-628 is altered in gastric cancer remains unclear and whether its aberrant expression plays a crucial part in the aggressiveness of gastric cancer is yet to be determined. Therefore, in this study, we systematically investigated the involvement of miR-628 in gastric cancer progression.

Materials and methods: MiR-628 expression in gastric cancer tissues and cell lines were determined via reverse transcription-quantitative polymerase chain reaction (RT-qPCR). A CCK-8 assay, flow cytometric analysis, Transwell assays, and a xenograft model experiment were performed to evaluate the influence of miR-628 overexpression on gastric cancer cells. Notably, mechanisms underlying the tumor-suppressive activity of miR-628 in gastric cancer cells were explored by bioinformatics analysis, a luciferase reporter assay, RT-qPCR, and Western blotting.

Results: MiR-628 expression was low in gastric cancer tissue samples and cell lines. The low expression of miR-628 was closely associated with the lymph node metastasis, invasive depth and TNM stage among patients with gastric cancer. Further clinical analysis indicated that patients with gastric cancer underexpressing miR-628 had a worse prognosis than did the patients with high miR-628 expression in the tumor. Overexpressed miR-628 restrained proliferation, migration, and invasion; induced apoptosis; and impaired tumor growth of gastric cancer cells. In addition, neuropilin 1 (NRP1) mRNA was validated as the direct target of miR-628 in gastric cancer. Long noncoding RNA small nucleolar RNA host gene 16 (SNHG16) was demonstrated to sponge miR-628 in gastric cancer. Moreover, miR-628 knockdown abrogated the influence of SNHG16 silencing on gastric cancer cells.

Conclusion: Our findings elucidate how the SNHG16–miR-628–NRP1 pathway serves as a regulatory network playing crucial roles in gastric cancer progression, suggesting that this pathway may be a novel target of anticancer therapy.

Keywords: gastric cancer, microRNA-628-3p, long noncoding RNA, neuropilin 1, small nucleolar RNA host gene 16

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Introduction

Gastric cancer is the fifth most prevalent type of malignant tumor and the third leading cause of cancer-associated deaths globally.[1] The morbidity of gastric cancer and resulting deaths decreased in the past decade owing to notable progress in the

diagnostic and therapeutic methods.[2] Unfortunately, there are still many thousands of patients with gastric cancer who have received the diagnosis at late stages, thus, resulting in unsatisfactory clinical outcomes.[3] The recurrence and metastasis after surgical management are the major causes of the poor prognosis of patients with gastric cancer.[4] Various factors, such as heredity, Helicobacter pylori infection, dietary habits, smoking, and drinking, are known to be implicated in gastric carcinogenesis and progression;[5,6] however, the mechanisms are still incompletely understood, and this situation is another barrier to successful therapy for gastric cancer. Therefore, studying the molecular alterations that occur during the initiation and progression of gastric cancer may facilitate the identification of promising therapeutic strategies and should improve the prognosis.

MicroRNAs (miRNAs) are a family of evolutionarily conserved and noncoding short RNAs with a length of ~23 nucleotides.[7] MiRNAs can recognize and directly bind to complementary sequences in the 3′-untranslated regions (3′-UTRs) of their target mRNAs, thereby causing translation inhibition and/or mRNA degradation.[8] Thus far, over 1800 miRNAs have been confirmed in the human genome, and their aberrant expression has been observed in nearly all human cancer types.[9] Some studies have indicated that a number of miRNAs are dysregulated in gastric cancer, and the dysregulation of miRNAs is involved in gastric cancer progression by affecting numerous tumor behaviors.[10–12] These findings have collectively proved the regulatory importance of miRNAs in the progression of gastric cancer, suggesting that miRNAs may be effective therapeutic targets in gastric cancer.

Long noncoding RNAs (lncRNAs) are a novel group of noncoding RNAs of >200 nucleotides.[13] Thousands of lncRNA genes have been identified in the human genome, many of which perform regulatory functions in the aggressive behaviors of cancer cells during tumorigenesis including tumor progression.[14] LncRNAs are reported to be regulators of gene expression through a variety of mechanisms, including genomic interactions, protein amounts, miRNA competition, and chromatin modifications.[15,16] Various lncRNAs are abnormally expressed in gastric cancer and regulate multiple pathological processes, including cell proliferation, cell cycle, apoptosis, metastasis, and angiogenesis.[17–19] Hence, lncRNAs might be potential molecular targets for the diagnosis and treatment of gastric cancer.

In recent years, miR-628-3p (miR-628) was reported to substantially participate in the progression of several types of human cancer, including colorectal cancer,[20] acute myeloid leukemia,[21] pancreatic cancer,[22] and non-small-cell lung cancer.[23] Nevertheless, whether the expression profile of miR-628 is altered in gastric cancer remains unclear and whether its aberrant expression is important for the aggressiveness of gastric cancer is yet to be studied. Therefore, the aim of our current study was to evaluate miR-628 expression in gastric cancer and explore its specific roles in the regulation of the malignant characteristics of gastric cancer.

Materials and methods

Patient samples

In total, 54 pairs of gastric cancer tissue samples and matched adjacent normal tissue samples were collected at Suihua First Hospital in Heilongjiang Province. None of the patients had received preoperative radiotherapy, chemotherapy, or other anticancer interventions. All tissue specimens were obtained after surgical resection, immediately frozen in liquid nitrogen, and then stored at −80 °C. All the participants agreed to take part in this study and provided written informed consent prior to the surgical operation. The study protocol was approved by the Ethics Committee of Suihua First Hospital in Heilongjiang Province and was carried out in compliance with the ethical standards of the Declaration of Helsinki.

Cell culture

Human gastric cancer cell lines (BGC-823, SGC-7901, MKN-45, and AGS) and immortalized human gastric epithelial cells (GES-1) were bought from the American Type Culture Collection (Manassas, VA, USA). All the above cells were maintained at 37 °C in a humidified 95% air and 5% CO_2 atmosphere and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% of fetal bovine serum (FBS),100 U/mL penicillin, and 100 μg/mL streptomycin (All from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Oligonucleotides, small interfering RNAs (siRNAs), plasmids, and cell transfection

MiR-628 agomir (agomir-628), negative control (NC) agomir (agomir-NC), miR-628 antagomir (antagomir-628), and NC antagomir (antagomir-NC) were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). The siRNA that was used to silence SNHG16 (si-SNHG16) and negative control siRNA (si-NC) were generated by

Guangzhou RiboBio Co., Ltd. (Guangzhou, China). The plasmid expressing NRP1 (pcDNA3.1-NRP1) was constructed by GenScript Biotech Corp. (Nanjing, China). Cells were seeded in 6-well plates 24 h before transfection. The above-mentioned oligonucleotides and plasmid were transfected into cells by means of Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.).

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated from tissue samples or cells using the TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and was reverse-transcribed into complementary DNA (cDNA) with the miScript Reverse Transcription Kit (Qiagen GmbH, Hilden, Germany). MiR-628 expression was detected via qPCR with the miScript SYBR Green PCR kit (Qiagen GmbH, Hilden, Germany) and normalized to U6 small nuclear RNA. To quantify NRP1 and SNHG16 expression, the synthesis of cDNA was conducted using the PrimeScript RT Reagent Kit, followed by qPCR with SYBR® Premix Ex Taq™ (both from Takara Biotechnology Co., Ltd., Dalian, China). The expression levels of NRP1 and SNHG16 were normalized to GAPDH. The $2^{-\Delta\Delta Cq}$ method was employed for quantification.

Cell Counting Kit-8 (CCK-8) assay

Transfected cells were seeded in 24-well plates at a density of 2×10^3 cells/well. After 0–72 h of incubation, the CCK-8 assay was carried out every 24 h to determine cell proliferation. Briefly, 10 µL of the CCK-8 solution (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was added into each well, and the transfected cells were incubated at 37 °C for additional 2 h. The absorbance was measured at a 450 nm wavelength on a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Detection of cell apoptosis by flow-cytometric analysis

Transfected cells were treated with trypsin (Gibco; Thermo Fisher Scientific), harvested by centrifugation, and subjected to cell apoptosis detection by means of the Annexin V Fluorescein Isothiocyanate (FITC) Apoptosis Detection Kit (Biolegend, San Diego, CA, USA). In particular, transfected cells were resuspended in 100 µL of 1× binding buffer, double-stained with 5 µL of the Annexin V solution and 5 µL of the propidium iodide solution, and incubated at room temperature in the dark for 15 min. Finally, the apoptotic rate (early stage + late stage) was determined on a flow cytometer (FACScan; BD Biosciences, Bedford, MA, USA).

Transwell assay

The migratory and invasive abilities were assessed using noncoated or Matrigel-coated Transwell chambers (BD Biosciences), respectively. Transfected cells were collected after 48 h incubation, centrifuged, and resuspended in FBS-free DMEM. The cell concentration was adjusted to 1×10^5 cells/mL. A total of 200 µL of the cell suspension was added into the upper chambers, while the bottom chambers were covered with 500 µL of DMEM supplemented with 10% of FBS. The chambers were next kept at 37 °C and 5% CO_2 for 24 h, following by cell fixation with 4% paraformaldehyde, staining with 0.5% crystal violet, and washing with phosphate-buffered saline (PBS; Gibco; Thermo Fisher Scientific, Inc.). After that, the images of the migratory and invading cells were captured under an inverted microscope (×200 magnification; IX41SF; Olympus Corporation, Tokyo, Japan). The numbers of migratory and invading cells in five random visual fields per group were determined, and the mean and standard deviation (SD) were calculated to describe the migratory and invasive abilities.

Xenograft model experiment

Agomir-628–transfected or agomir-NC–transfected cells in the logarithmic growth phase were collected and injected subcutaneously into female 4–6-week-old BALB/c nude mice (n=4 for each group; the Laboratory Animal Center of Yangzhou University; Yangzhou, China). Two weeks after the injection, the tumor volume was measured using the formula: volume (mm3) = $0.5\times\text{length}\times\text{width}^2$. All the nude mice were then euthanized by dislocation of cervical vertebrae at 4 weeks after the inoculation for excision of the tumor xenografts. The tumor xenografts were stored for the isolation of total RNA and protein. The study protocol was approved by the Animal Care and Use Committee of Suihua First Hospital in Heilongjiang Province and was performed in compliance with the Animal Protection Law of the People's Republic of China-2009 for experimental animals.

Bioinformatics analysis

Bioinformatics tools, namely, miRDB (http://mirdb.org/) and TargetScan (http://www.targetscan.org), were utilized to search for the putative target of miR-628. DIANA tools -LncBase Experimental v2 (http://carolina.imis.athena-inno

vation.gr/diana_tools/web/index.php?r=lncbasev2%2findex-experimental) was applied to analyze the binding site for miR-628 in SNHG16.

Luciferase reporter assay

The 3′-UTR of NRP1, which contains the predicted wild-type (wt) miR-628–binding site, and the mutant (mut) NRP1 3′-UTR, were amplified by Shanghai GenePharma Co., Ltd. The synthesized DNA fragments were cloned into the pmirGLO vector (Promega Corporation, Madison, WI, USA) to generate the wt-NRP1 and mut-NRP1 reporter plasmids. The wt-SNHG16 and mut-SNHG16 reporter plasmids were chemically produced in the same way. For the reporter assay, cells were seeded in 24-well plates, followed by cotransfection with agomir-628 or agomir-NC and either the wt or mut reporter plasmid. Following a 48 h transfection period, the luciferase activity was determined using a dual-luciferase reporter assay system (Promega Corporation). The Renilla luciferase activity was assayed for normalization.

Protein extraction and Western blot analysis

RIPA lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) was utilized to isolate total protein from tissue samples, cells, or tumor xenografts. The isolated total protein was quantified with the Bradford Kit (Beyotime Institute of Biotechnology). Protein samples containing equal amounts of protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis in a 10% gel and transferred onto polyvinylidene difluoride membranes. Blocking with 5% nonfat milk was performed at room temperature for 2 h to prevent nonspecific binding of antibodies. The membranes were next probed with primary antibodies against NRP1 (cat. # ab81321; 1:1000 dilution; Abcam) or GAPDH (ab181603; 1:1000 dilution; Abcam) followed by incubation with a goat anti-rabbit IgG horseradish peroxidase-conjugated antibody (ab6721; 1:5000 dilution; Abcam) (secondary antibody) at room temperature for 1 h and visualization with an enhanced chemiluminescence detection system (GE Healthcare, Chicago, IL, USA).

Statistical analysis

All data are presented as mean ± SD and were subjected to analysis in SPSS 13.0 software (IBM Corporation, Armonk, NY, USA). Pearson's χ^2 test was conducted to investigate the association between miR-628 expression and clinical parameters among the patients with gastric cancer. The expression correlation between miR-628 and NRP1 in gastric cancer tissue samples was evaluated by Spearman's correlation analysis, which was also carried out to test the expression correlation between miR-628 and SNHG16. The Kaplan–Meier method was employed to build a survival curve, and the differences among groups were assessed by the logrank test. A comparison between two groups was performed with Student's *t*-test, whereas one-way analysis of variance along with Tukey's post-hoc test was performed to assess differences among multiple groups. Data with $P<0.05$ were considered statistically significant.

Results

Downregulation of miR-628 is associated with an unfavorable prognosis among patients with gastric cancer

To detect the expression profile of miR-628 in gastric cancer, we measured miR-628 expression in 54 pairs of gastric cancer tissue samples and matched adjacent normal tissue samples through RT-qPCR. The results showed that expression levels of miR-628 were lower in gastric cancer tissue samples relative to adjacent normal tissues (Figure 1A, $P<0.05$). In addition, miR-628 turned out to be underexpressed in the four tested gastric cancer cell lines (BGC-823, SGC-7901, MKN-45, and AGS) in comparison with the immortalized human gastric epithelial cells (GES-1; Figure 1B, $P<0.05$).

To uncover the clinical relevance and prognostic significance of miR-628 in gastric cancer, we subdivided all the 54 patients with gastric cancer into two groups: low-miR-628-expression group and high-miR-628-expression group, according to the median value of miR-628 among the gastric cancer tissue samples. Low miR-628 expression was significantly associated with lymph node metastasis ($P=0.013$), invasive depth ($P=0.001$) and TNM stage ($P=0.002$) among the patients with gastric cancer (Table 1). By contrast, no obvious correlation with age, gender, tumor size or histological grade was detected (all $P>0.05$). Moreover, patients with gastric cancer harboring low miR-628 expression had a lower probability of better overall survival than did patients in the high-miR-628-expression group (Figure 1C, $P=0.0264$). These results suggested that miR-628 may play a critical part in the aggressiveness of gastric cancer.

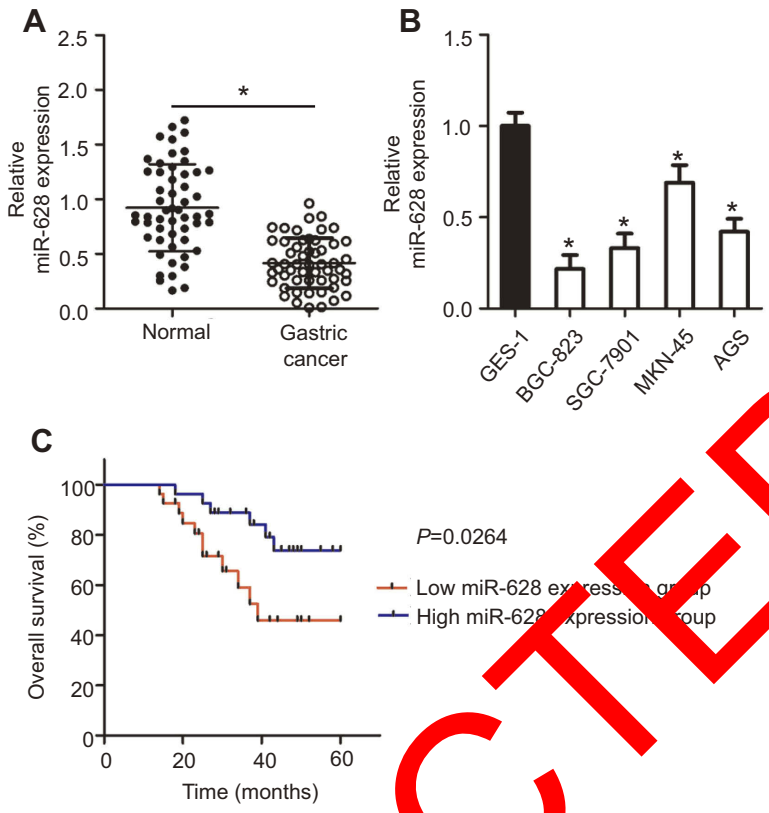

Figure 1 The expression of miR-628 in gastric cancer and its correlation with patients' survival. (**A**) MiR-628 expression in 54 pairs of gastric cancer tissue samples and matched adjacent normal tissues was analyzed using RT-qPCR. All tissue samples were collected from First Hospital in Heilongjiang Province. *P<0.05 vs normal tissues. (**B**) MiR-628 levels in four gastric cancer cell lines (BGC-823, SGC-7901, MKN-45, and AGS) and immortalized human gastric epithelial cells (GES-1) were measured by RT-qPCR. *P<0.05 vs GES-1. (C) The correlation between miR-628 expression and overall survival among patients with gastric cancer. P=0.0264.

miR-628 inhibits gastric cancer cell proliferation, migration, and invasion in vitro and promotes apoptosis

BGC-823 and SGC-7901 cell lines manifested relatively low miR-628 expression as compared with MKN-45 and AGS cells; therefore, the first two cell lines were chosen as the model to explore the detailed functions of miR-628 in the oncogenicity of gastric cancer. Agomir-628 was transfected into BGC-823 and SGC-7901 cells to increase endogenous miR-628 expression (Figure 2A, P<0.05). Data from the CCK-8 assays showed that transfection with agomir-628 greatly reduced the proliferative ability of BGC-823 and SGC-7901 cells (Figure 2B, P<0.05). In line with this finding, upregulation of miR-628 notably increased the apoptosis of BGC-823 and SGC-7901 cells, as revealed by flow-cytometric analysis (Figure 2C, P<0.05). After that, Transwell assays were carried out to test the effects of miR-628 upregulation on the migration and invasiveness of gastric cancer cells. The migratory (Figure 2D, P<0.05) and invasive (Figure 2E, P<0.05) abilities of BGC-823 and SGC-7901 cells were decreased by miR-628 upregulation. The above results indicated that miR-628 may function as a tumor-suppressive modulator in gastric cancer.

NRP1 mRNA is directly targeted by miR-628 in gastric cancer

To gain an in-depth understanding of the mechanisms behind the activity of miR-628 in gastric cancer, the putative targets of miR-628 were predicted via bioinformatics analysis. NRP1 was chosen for further verification because this gene has two major miR-628 binding sites in the 3′-UTR of its mRNA (Figure 3A) and substantially participates in gastric carcinogenesis.[24–26] To test our assumption, the wt-NRP1 (1 and 2) and mut-NRP1 (1 and 2) reporter plasmids were constructed based on the predicted binding site and were cotransfected with agomir-628 or agomir-NC into BGC-823 and SGC-7901 cells. The transfection with agomir-628 efficiently impaired the luciferase activity of the plasmid containing the wild-type NRP1-binding site (both 1 and 2; P<0.05). Conversely, no obvious alterations in the

Table 1 The association between miR-628 expression and clinicopathological features in patients with gastric cancer

Features	miR-628 expression		*P*
	Low	High	
Age (years)			0.785
<60	13	11	
≥60	14	16	
Gender			0.387
Male	16	20	
Female	11	7	
Tumor size (cm)			0.327
<5	19	23	
≥5	8	4	
Histological grade			0.577
Well-intermediately differentiation	15	18	
Poor differentiation	12	9	
Lymph node metastasis			0.013[a]
No	10	20	
Yes	17	7	
Invasive depth			0.001[a]
T1+T2	6	19	
T3+T4	21	8	
TNM stage			0.002[a]
I-II	9	21	
III-IV	18	6	

Note: [a]*P*<0.05.

luciferase activity were seen in the BGC-823 and SGC-7901 cells cotransfected with agomir-628 and the mut-NRP1 reporter plasmid (both 1 and 2; Figure 3B). To investigate whether NRP1 is scientifically and clinically relevant to the expression of miR-628, the expression profile of NRP1 was determined in the 54 pairs of gastric cancer tissue samples and matched adjacent normal tissues. The mRNA level of NRP1 was higher in the gastric cancer tissue samples than in the adjacent normal tissues (Figure 3C, *P*<0.05). Additionally, Spearman's correlation analysis of the 54 gastric cancer tissue samples confirmed that the expression of NRP1 inversely correlated with miR-628 expression (Figure 3D; R^2=0.4138, *P*<0.0001). Furthermore, the mRNA (Figure 3E, *P*<0.05) and protein (Figure 3F, *P*<0.05) levels of NRP1 obviously diminished after overexpression of miR-628 in BGC-823 and SGC-7901 cells. In summary, NRP1 is a direct target of miR-628 in gastric cancer.

Tumor-suppressive activities of miR-628 in gastric cancer cells are NRP1 dependent

MiR-628 inhibited the growth and metastasis of gastric cancer cells in vitro, and NRP1 mRNA was validated as a direct target of miR-628 in the experiments above. Hence, we assumed that the tumor-suppressive roles of miR-628 in gastric cancer are dependent on NRP1. We restored NRP1 expression in the agomir-628–transfected BGC-823 and SGC-7901 cells by cotransfecting the plasmid expressing NRP1 pcDNA3.1-NRP1 (Figure 4A, *P*<0.05). Then, the results of CCK-8 and flow cytometric assays revealed that ectopic miR-628 expression decreased the proliferation (Figure 4B, *P*<0.05) and increased the apoptosis rate (Figure 4C, *P*<0.05) of BGC-823 and SGC-7901 cells; these effects were attenuated by cotransfection with pcDNA3.1-NRP1. Furthermore, restoration of NRP1 expression weakened the miR-628–mediated inhibitory actions on the migration (Figure 4D, *P*<0.05) and invasiveness (Figure 4E, *P*<0.05) of BGC-823 and SGC-7901 cells. Taken together, the above observations suggested that NRP1 is the functional target of miR-628 and that NRP1 down-regulation is essential for the tumor-suppressive activities of miR-628 in gastric cancer.

SNHG16 functions as a sponge for miR-628 in gastric cancer

A plethora of studies indicate that lncRNAs can act as competing endogenous RNAs (ceRNAs) to sponge miRNAs. Therefore, we next attempted to test whether miR-628 can be sponged by a certain lncRNA in gastric cancer. Bioinformatics analysis was carried out and identified two potential miR-628–binding sites in an lncRNA called SNHG16 (Figure 5A). The luciferase reporter assay was then conducted to confirm the prediction, and the results showed that restoration of miR-628 expression greatly decreased the luciferase activities of wt-SNHG16 (both 1 and 2; Figure 5B, *P*<0.05) but not mut-SNHG16 (both 1 and 2) in BGC-823 and SGC-7901 cells.

To further examine the interaction between miR-628 and SNHG16 in gastric cancer, we quantitated SNHG16 expression in the 54 pairs of gastric cancer tissue samples and the matched adjacent normal tissue samples. In line with other studies,[27,28] the expression of SNHG16 turned out to be higher in the gastric cancer tissue samples than in the adjacent normal tissues (Figure 5C, *P*<0.05).

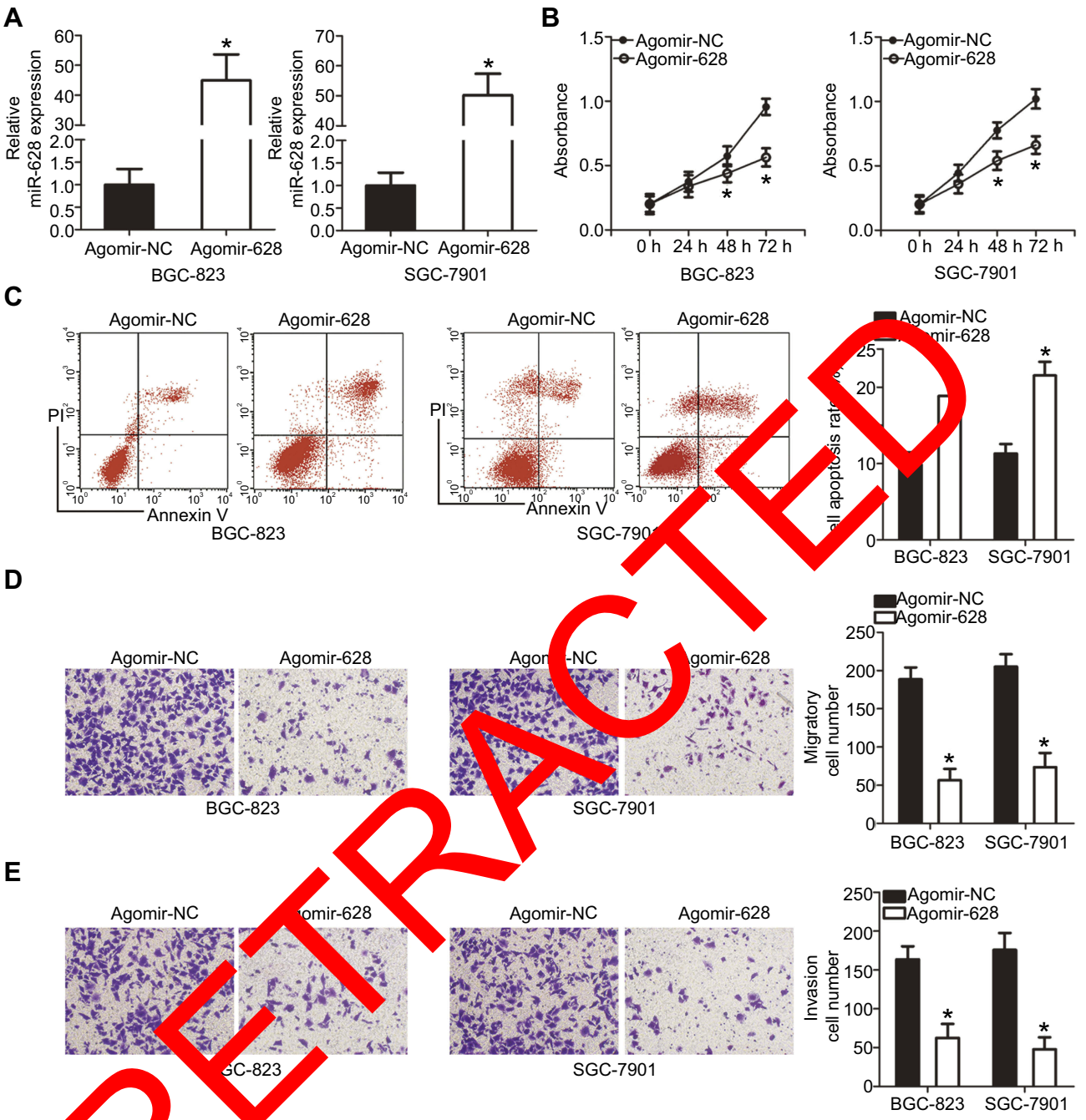

Figure 2 M[illegible]628 [illegible]overexpress[illegible]uppresses the proliferation, induces apoptosis, and decreases the migration and invasiveness of BGC-823 and SGC-7901 cells. (**A**) RT-qPCR was con[illegible]ed to measure miR-628 expression in BGC-823 and SGC-7901 cells after transfected with agomir-628 or agomir-NC. *P<0.05 vs agomir-NC. (**B**) The proliferative abilit[illegible] BGC-823 and SGC-7901 cells treated with agomir-628 or agomir-NC was examined using the CCK-8 assay. *P<0.05 vs agomir-NC. (**C**) Flow-cytometric analysis w[illegible]arried out to determine the influence of agomir-628 transfection on the apoptosis of BGC-823 and SGC-7901 cells. *P<0.05 vs agomir-NC. (**D, E**) The migration and invasiveness of miR-628–overexpressing BGC-823 and SGC-7901 cells were assessed in Transwell assays. The migration and invasion abilities were quantified as cell numbers (× 200 magnification). *P<0.05 vs agomir-NC.

Moreover, SNHG16 expression was inversely related with miR-628 expression among the gastric cancer tissue samples, as revealed by Spearman's correlation analysis (Figure 5D; R^2=0.4296, P<0.0001). Lastly, si-SNHG16 silenced SNHG16 expression (Figure 5E, P<0.05), increased miR-628 expression (Figure 5F, P<0.05), and reduced NRP1 protein expression (Figure 5G, P<0.05) in BGC-823 and SGC-7901 cells. Collectively, these findings confirmed that SNHG16 functions as a molecular sponge for miR-628 in gastric cancer.

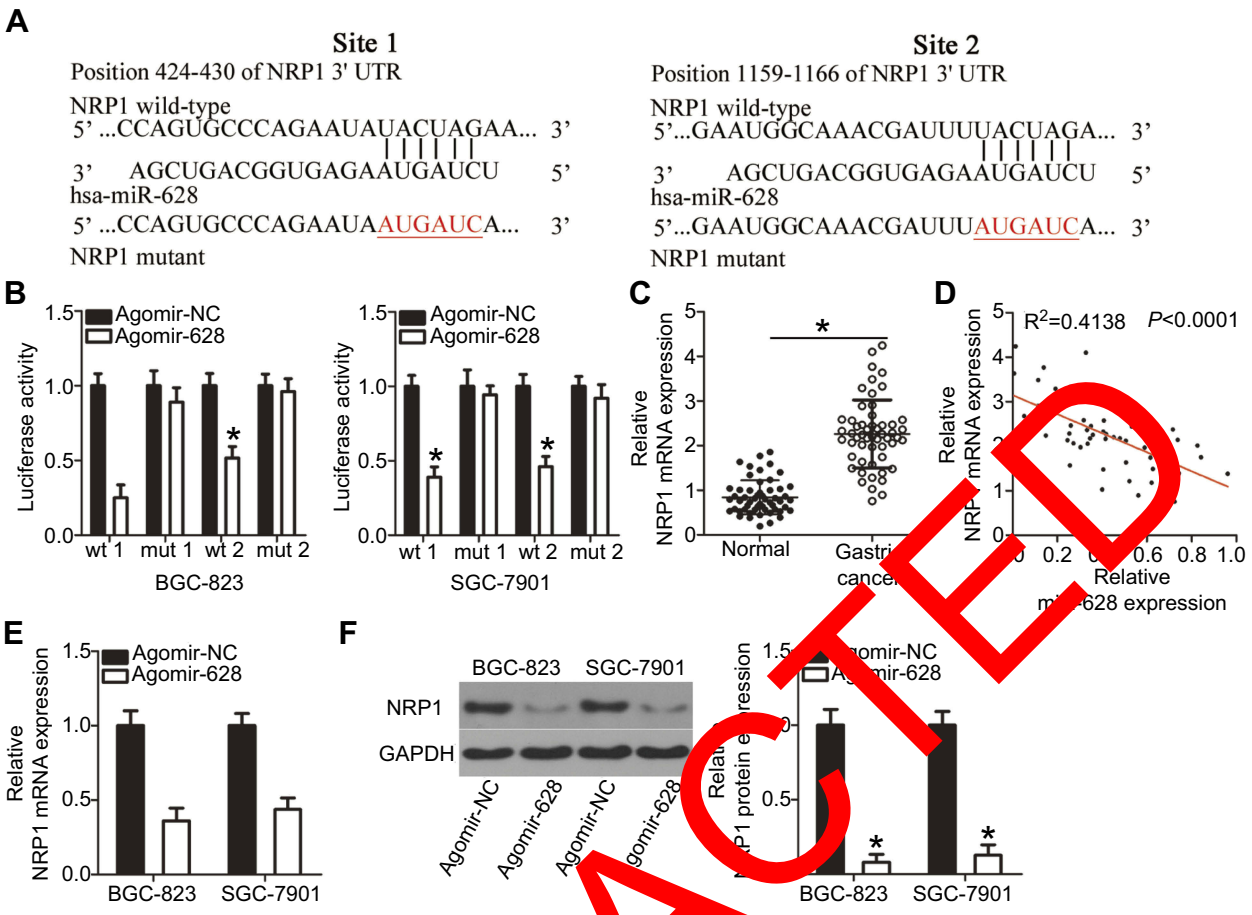

Figure 3 Validation of NRP1 mRNA as a direct target of miR-628 in gastric cancer. (**A**) The putative miR-628 target sequences in the 3′-UTR of NRP1 revealed by bioinformatics software. The 3′-UTR regions of NRP1 containing the wt or mut binding sites as well as miR-628 sequences are presented. (**B**) BGC-823 and SGC-7901 cells were cotransfected with agomir-628 or agomir-NC and wt-NRP1 or mut-NRP1. Following transfection, a luciferase reporter assay was performed to assess the interaction between miR-628 and NRP1 mRNA in gastric cancer. *P<0.05 vs agomir-NC. (**C**) RT-qPCR was carried out to measure the expression levels of NRP1 mRNA in 54 pairs of gastric cancer tissue samples and matched adjacent normal tissue samples. *P<0.05 vs normal tissue samples. (**D**) A negative expression correlation between miR-628 and NRP1 in gastric cancer tissue samples was confirmed via Spearman correlation analysis. R^2=0.4138, P<0.0001. (**E, F**) NRP1 mRNA and protein expression levels in BGC-823 and SGC-7901 cells with restored miR-628 expression were detected through RT-qPCR and Western blotting. *P<0.05 vs agomir-NC.

Downregulation of SNHG16 inhibits the proliferation, migration and invasiveness and induces apoptosis of gastric cancer cells

To explore the roles of SNHG16 in the biological characteristics of gastric cancer, si-SNHG16 was used to silence endogenous SNHG16 expression in BGC-823 and SGC-7901 cells and then a series of functional assays were conducted. The influence of SNHG16 downregulation on gastric cancer cell proliferation and apoptosis was investigated in the CCK-8 assay and flow-cytometric experiment. The proliferative capacity (Figure 6A, P<0.05) of the BGC-823 and SGC-7901 cells transfected with si-SNHG16 diminished, whereas the apoptosis rate (Figure 6B, P<0.05) increased. We also performed the Transwell assay to determine the actions of the SNHG16 knockdown on the migration and invasiveness of gastric cancer cells. BGC-823 and SGC-7901 cells transfected with si-SNHG16 had weaker migratory (Figure 6C, P<0.05) and invasive (Figure 6D, P<0.05) abilities. The results revealed that SNHG16 may have an oncogenic influence on the aggressive phenotypes of gastric cancer.

SNHG16 exerts its effects in gastric cancer via the miR-628–NRP1 axis

Because the above results indicated that SNHG16 plays oncogenic roles in gastric cancer progression and could regulate NRP1 expression by sponging miR-628, we next conducted rescue experiments to determine whether silencing of SNHG16 expression inhibits the growth and metastasis of gastric cancer cells in vitro by releasing

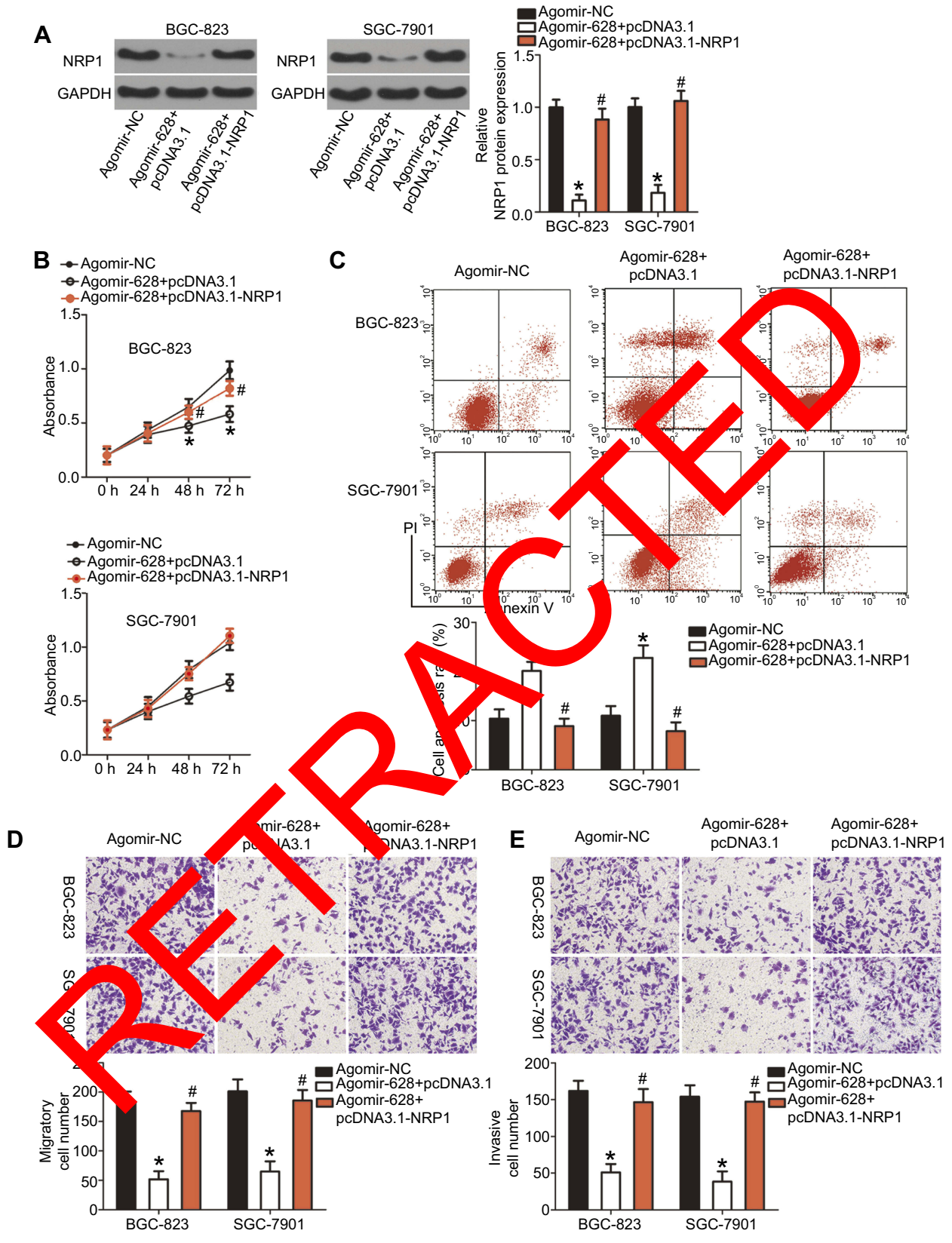

Figure 4 Restoring NRP1 expression neutralizes the influence of miR-628 overexpression on gastric cancer cells. (**A**) Western blot analysis of NRP1 protein expression in BGC-823 and SGC-7901 cells after cotransfection with agomir-628 and pcDNA3.1-NRP1 or pcDNA3.1. **P*<0.05 vs group agomir-NC. #*P*<0.05 vs group agomir-628+pcDNA3.1. (**B, C**) The proliferation and apoptosis of BGC-823 and SGC-7901 cells with restored NRP1 expression were quantified by the CCK-8 assay and flow-cytometric analysis. **P*<0.05 vs group agomir-NC. #*P*<0.05 vs group agomir-628+pcDNA3.1. (**D, E**) The migratory and invasive abilities of BGC-823 and SGC-7901 cells treated with the above-mentioned constructs were evaluated in Transwell assays. **P*<0.05 vs group agomir-NC (× 200 magnification). #*P*<0.05 vs group agomir-628+pcDNA3.1.

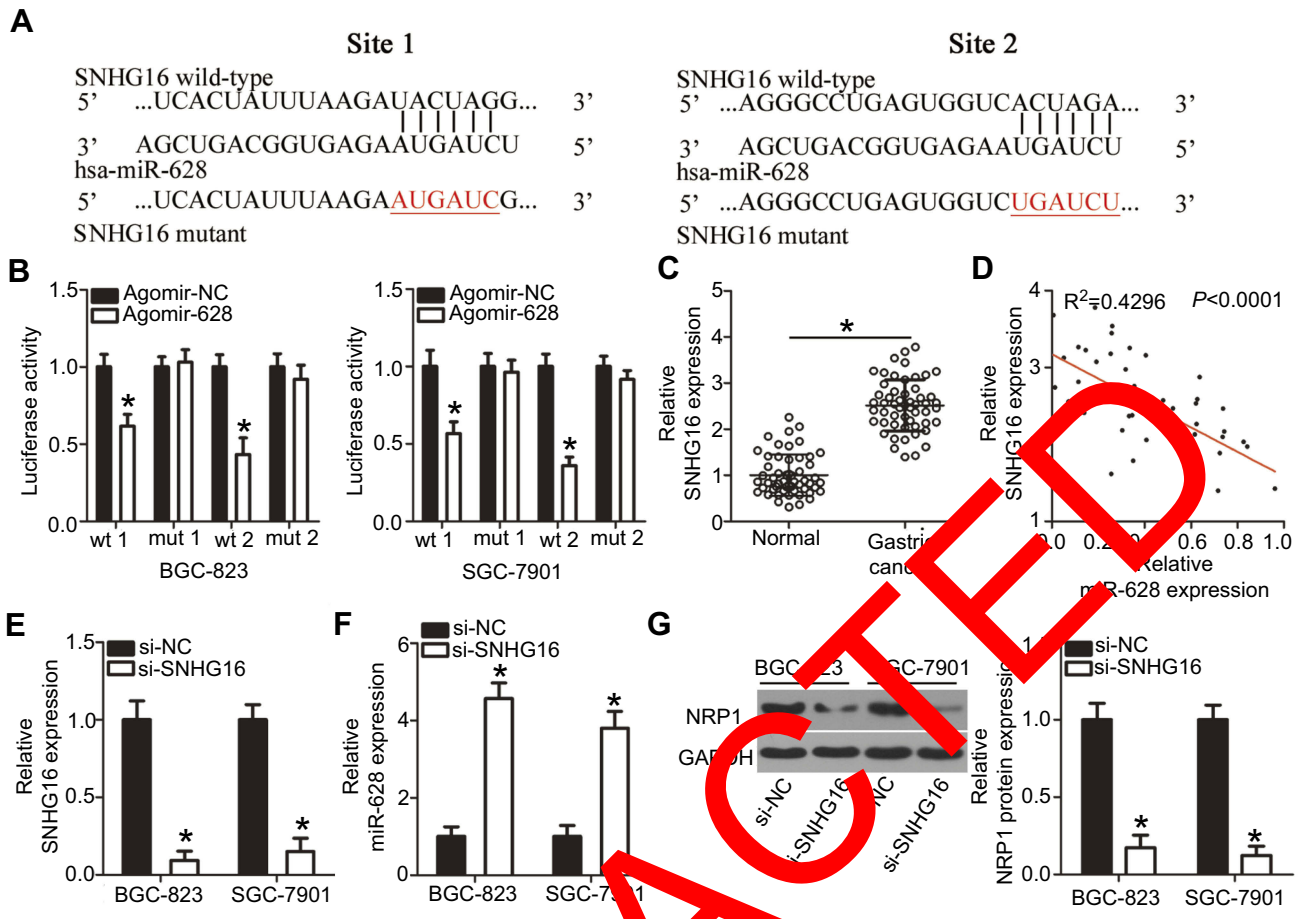

Figure 5 SNHG16 functions as a ceRNA and regulates NRP1 expression in gastric cancer by competitively binding miR-628. (**A**) The predicted two binding sites for miR-628 on SNHG16 as predicted by bioinformatics software. (**B**) Antagomir-628 or agomir-NC was cotransfected with wt-SNHG16 or mut-SNHG16 into BGC-823 and SGC-7901 cells, and sequentially the luciferase activity was quantified. *P<0.05 vs agomir-NC. (**C**) Expression of SNHG16 in 54 pairs of gastric cancer tissue samples and matched adjacent normal tissue samples was examined via RT-qPCR. *P<0.05 vs normal tissues. (**D**) Spearman's correlation analysis uncovered an inverse association between miR-628 and SNHG16 in gastric cancer tissue samples. R^2=0.4296, P<0.0001. (**E**) The expression levels of SNHG16 in BGC-823 and SGC-7901 cells when they were treated with si-SNHG16 or si-NC were determined by RT-qPCR. *P<0.05 vs si-NC. (**F, G**) RT-qPCR and Western blot analysis were performed to assess miR-628 and NRP1 protein expression, respectively, in SNHG16-depleted BGC-823 and SGC-7901 cells. *P<0.05 vs si-NC.

sponged miR-628 and decreasing NRP1 expression. Hence, antagomir-628, which was used to knock down miR-628 expression (Figure 7A, P<0.05), was cotransfected with si-SNHG16 into BGC-823 and SGC-7901 cells, and the miR-628 amount and NRP1 protein levels were detected via RT-qPCR and Western blotting. After the transfection, the increased level of miR-628 (Figure 7B, P<0.05) and decreased level of the NRP1 protein (Figure 7C, P<0.05) in SNHG16 knockdown BGC-823 and SGC-7901 cells were almost reversed by cotransfection with antagomir-628. Furthermore, cotransfection with antagomir-628 abrogated si-SNHG16–mediated effects on the proliferation (Figure 7D, P<0.05), apoptosis (Figure 7E, P<0.05), migration (Figure 8A, P<0.05), and invasiveness (Figure 8B, P<0.05) of BGC-823 and SGC-7901 cells. These findings suggested that SNHG16 performs its biological activities in gastric cancer cells at least in part via the miR-628–NRP1 axis.

miR-628 suppresses the growth of gastric cancer in vivo

The xenograft model experiment was conducted to test whether miR-628 can hinder tumor growth of gastric cancer cells in vivo. Agomir-628–transfected BGC-823 cells were injected subcutaneously into nude mice, and cells treated with agomir-NC served as the control. Consistently with the in vitro results, the agomir-628 group showed an obvious decrease in tumor volume compared with that in the agomir-NC group (Figure 9A and B, P<0.05). Meanwhile, measurements of the tumor xenografts revealed that miR-876 overexpression markedly reduced tumor weight (Figure 9C, P<0.05).

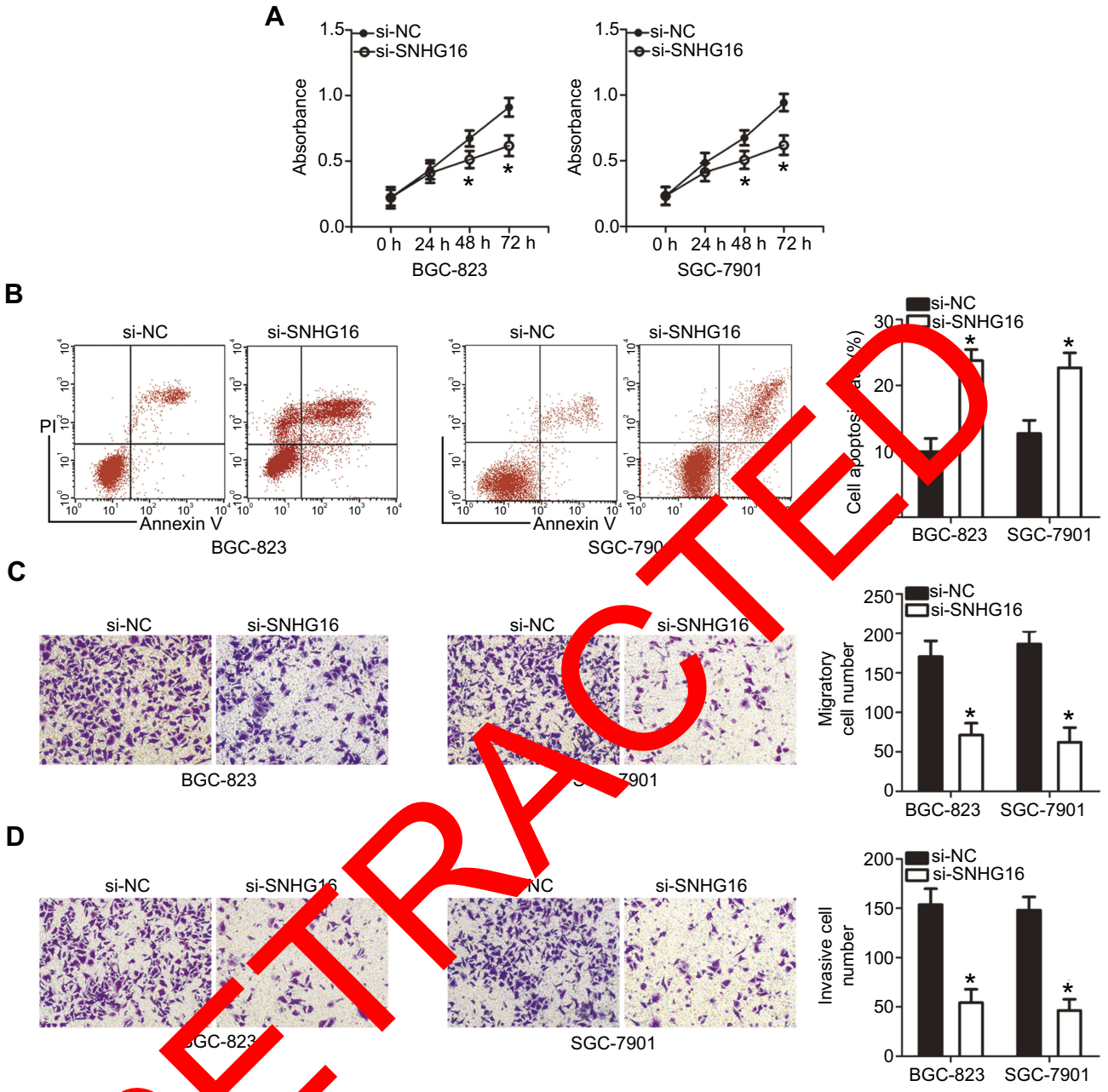

Figure 6 The knockdown of SNHG16 inhibits the proliferation, migration and invasion as well as promotes apoptosis of BGC-823 and SGC-7901 cells. (**A, B**) The CCK-8 assay and flow-cytometry analysis were carried out to evaluate the proliferation and apoptosis of BGC-823 and SGC-7901 cells after si-SNHG16 or si-NC transfection. *P<0.05 vs si-NC. (**C**) The influence of si-SNHG16-induced SNHG16 silencing on the migration and invasiveness of BGC-823 and SGC-7901 cells was tested in Transwell assays (× 200 magnification). *P<0.05 vs si-NC.

After that, the expression levels of miR-876 and NRP1 protein in the tumor xenografts were determined. The results meant that in the agomir-628 group, the expression of NRP1 protein (Figure 9D, P<0.05) was lower, whereas miR-628 (Figure 9E, P<0.05) was overexpressed. These results suggested that miR-628 overexpression inhibited gastric cancer tumor growth in vivo by decreasing NRP1 expression.

Discussion

In recent decades, dysregulation of miRNAs has been reported to be involved in gastric cancer initiation and progression, and it has become clear that miRNAs may serve as oncogenic or tumor-suppressive factors.[29–31] Hence, exploring the specific functions of cancer-associated miRNAs in gastric cancer should be useful for identifying promising targets for the diagnosis and

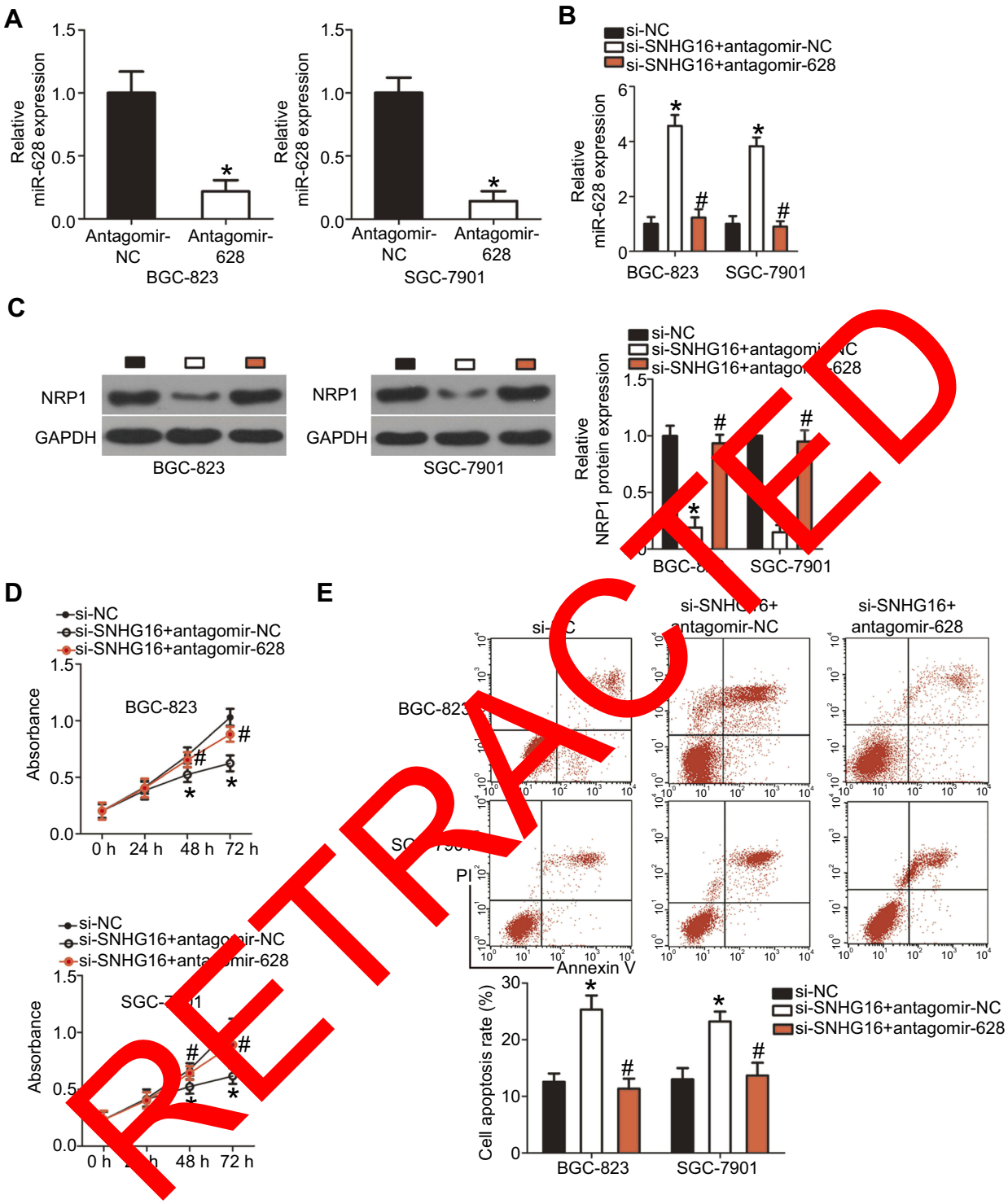

Figure 7 The miR-628 knockdown abrogates the effects of SNHG16 silencing on the proliferation and apoptosis of BGC-823 and SGC-7901 cells. (**A**) The transfection efficiency of antagomir-628 in BGC-823 and SGC-7901 cells was examined via RT-qPCR. **P*<0.05 vs antagomir-NC. (**B**) RT-qPCR analysis was conducted to measure miR-628 expression in BGC-823 and SGC-7901 cells pretransfected with si-SNHG16 and transfected with antagomir-628 or antagomir-NC. **P*<0.05 vs the si-NC group. #*P*<0.05 vs the si-SNHG16+antagomir-NC group. (**C**) Total protein was extracted from the aforementioned cells and then subjected to Western blotting for the quantification of NRP1 protein expression. **P*<0.05 vs group si-NC. #*P*<0.05 vs group si-SNHG16+antagomir-NC. (**D, E**) The proliferation and apoptosis of BGC-823 and SGC-7901 cells treated as described above were assessed respectively by the CCK-8 assay and flow-cytometric analysis. **P*<0.05 vs the si-NC group. #*P*<0.05 vs the si-SNHG16+antagomir-NC group.

treatment of gastric cancer. To the best of our knowledge, this study is the first to systematically investigate the involvement of miR-628 in gastric cancer. The expression status and prognostic value of miR-628 in

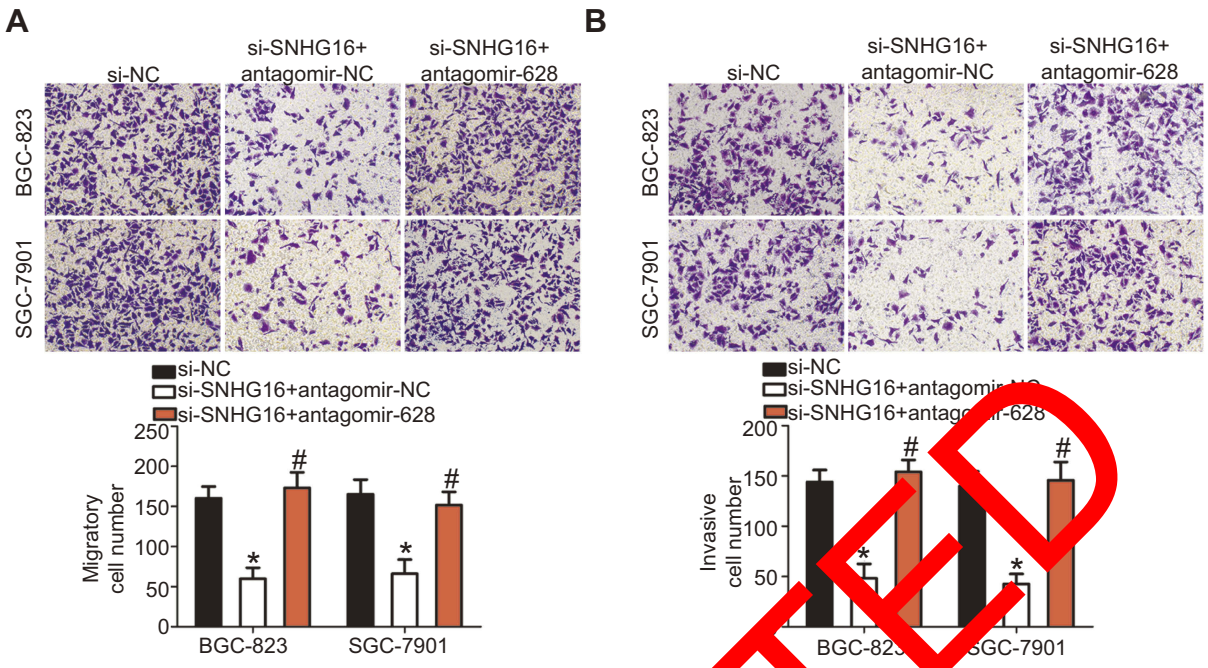

Figure 8 The miR-628 knockdown abolishes the influence of SNHG16 silencing on the migration and invasion of BGC-823 and SGC-7901 cells. (**A, B**) Transwell assays were employed to determine the migration and invasion of BGC-823 and SGC-7901 cells after cotransfected with si-SNHG16 and antagomir-628 or antagomir-NC. **P*<0.05 vs the si-NC group. #*P*<0.05 vs the si-SNHG16+antagomir-NC group (× 200 magnification).

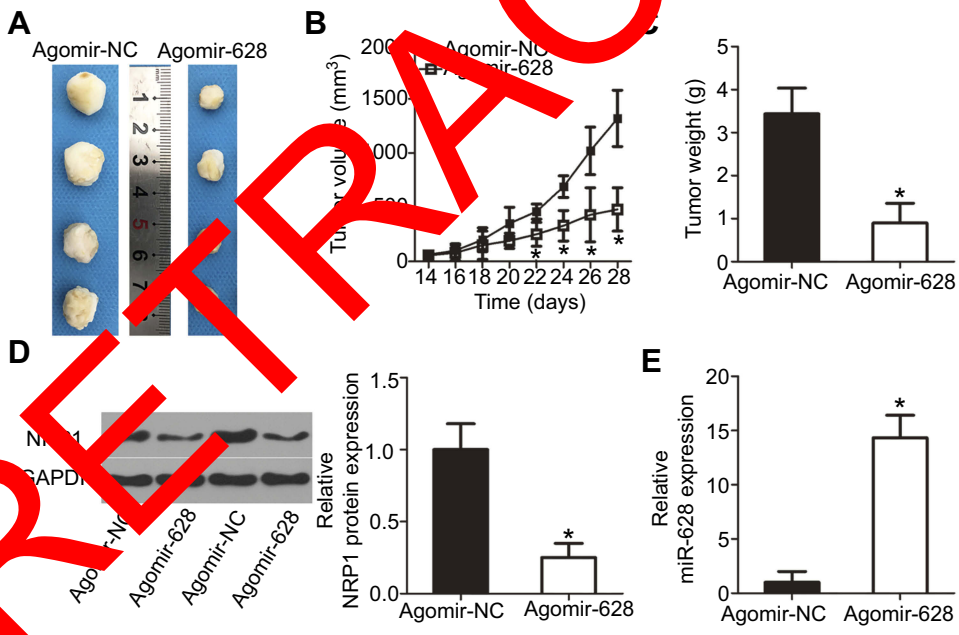

Figure 9 MiR-628 decreases tumor growth in vivo by decreasing NRP1 expression. BGC-823 cells transfected with agomir-628 or agomir-NC were harvested and then injected subcutaneously into female 4- to 6-week-old BALB/c nude mice. (**A**) A representative image of the tumor xenografts obtained from the agomir-628 and agomir-NC groups. (**B, C**) The tumor growth and tumor weight were obviously lower in the agomir-628 group than in the agomir-NC group. **P*<0.05 vs agomir-NC. (**D**) Western blotting was performed to detect NRP1 protein expression in the tumor xenografts obtained from the agomir-628 and agomir-NC groups. **P*<0.05 vs agomir-NC. (**E**) The expression of miR-628 in the tumor xenografts was determined by RT-qPCR. **P*<0.05 vs agomir-NC.

gastric cancer were explored in detail. In particular, we examined the detailed actions of miR-628 on the malignant characteristics of gastric cancer cells and unraveled the mechanisms of its action.

MiR-628 is downregulated in colorectal cancer,[20] acute myeloid leukemia,[21] and pancreatic cancer.[22] On the contrary, the expression of miR-628 is high in non-small-cell lung cancer.[23] These conflicting observations piqued our

interest in determining the expression profile of miR-628 in gastric cancer. Herein, we demonstrated aberrant downregulation of miR-628 in gastric cancer tissues and cell lines. Decreased miR-628 expression was found to be closely related to lymph node metastasis, invasive depth and TNM stage among our patients with gastric cancer. Patients with gastric cancer that underexpressed miR-628 had a worse prognosis than did the patients with high miR-628 expression. These results suggest that miR-628 might be an effective predictor of the clinical outcomes of patients with gastric cancer. However, in this study, we did not assess the correlation betwee nmiR-628 and disease-free survival rate among patients with GC. It was a limitation of our study, and we will resolve it in the near further.

MiR-628 plays tumor-suppressive roles by regulating the progression of multiple human cancer types. For instance, miR-628 overexpression suppresses acute myeloid leukemia cell proliferation, induces cell cycle arrest and promotes cell apoptosis in vitro, and decreases tumor growth in vivo.[21] Resumption of miR-628 expression restricts epithelial–mesenchymal transition and metastasis in breast cancer.[32] On the contrary, miR-628 performs oncogenic activities in non-small-cell lung cancer by promoting cell proliferation, motility, adhesion and decreasing apoptosis.[23] Nevertheless, the functions of miR-628 in the malignancy of gastric cancer remain poorly understood. In this study, we showed that restoration of miR-628 expression suppressed gastric cancer cell proliferation, migration, and invasion as well as increased apoptosis. Additionally, ectopic miR-628 expression inhibited tumor growth in vivo. These findings suggest miR-628 may be a target for the anticancer therapy of patients with gastric cancer.

MiRNAs function by repressing the expression of their target protein and can be changed by certain lncRNAs. In this study, we demonstrated that NRP1 mRNA is the direct target of miR-628 in gastric cancer. In addition, SNHG16 was found to act as a ceRNA to sponge miR-628, thereby regulating the expression of NRP1. NRP1, being a member of the neuropilin family, is a type I transmembrane glycoprotein expressed on the cell surface.[33] NRP1 is upregulated in gastric cancer, and its overexpression is closely associated with a diffuse subtype, poor differentiation grade, tumor size, tumor stage, lymph node metastasis, and TNM stage.[24,25] Patients with gastric cancer overexpressing NRP1 show shorter overall survival and median survival period than do the patients with low NRP1 expression in the tumor.[24] NRP1 exerts a tumorigenic effect on the malignant phenotype of gastric cancer and is implicated in the regulation of cell proliferation, apoptosis, migration, invasion, epithelial–mesenchymal transition, and chemotherapy responses.[24–26] Here, we report that miR-628 directly downregulates NRP1, thereby restraining the aggressive behaviors of gastric cancer.

SNHG16 is overexpressed in gastric cancer, and its high expression obviously correlates with invasion depth, lymph node metastasis, TNM stage, and histological differentiation.[27] Functionally, silencing of SNHG16 reduces cell proliferation, colony formation, and metastasis; induces cell cycle arrest; increases apoptosis in vitro; and decreases tumor growth in vivo.[27,28] The oncogenic activities of SNHG16 in gastric cancer are mediated by sponging of miR-135a and stimulation of the JAK2–STAT3 pathway.[28] In the present study, we demonstrated a new mechanism underlying the tumor-promoting action of SNHG16 in gastric cancer. SNHG16, which contains miR-628–binding sites, can act as a ceRNA to reduce the effective miR-628 amount, thereby upregulating NRP1. Consequently, targeting the SNHG16–miR-628–NRP1 pathway might be an innovative modality for managing gastric cancer.

Conclusion

In summary, we revealed that miR-628 has a tumor-suppressive influence on the progression of gastric cancer. In addition, NRP1 mRNA was identified as a direct target of miR-628 in gastric cancer, and miR-628 was found to be sponged by SNHG16. Our results may be applicable to the treatment of patients with gastric cancer and could improve their prognosis.

Disclosure

The authors report no conflicts of interest in this work.

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