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MicroRNA-328 acts as an anti-oncogene by targeting ABCG2 in gastric carcinoma

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Abstract. – OBJECTIVE: To explore the regulatory mechanism of microRNA-328 expression level by targeting the protein ATP Binding Cassette Transporter G2 (ABCG2) in gastric cancer cells and seek for a biological marker of predicting gastric cancer.

PATIENTS AND METHODS: SGC-7901 and MKN-28 human gastric cancer cell lines were cultured. Meanwhile, paired gastric cancer pathological tissues and the corresponding adjacent normal tissues were collected. Western blot analysis was used to validate the protein expression of ABCG2. Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) analysis was used to detect the mRNA expression level of miR-328 and ABCG2. Cell counting kit-8 (CCK-8) and colony formation assay were performed to validate the proliferous ability of human gastric cancer cells. The transwell invasion and migration were operated to determine the migratory and invasive capacity. Dual-Luciferase reporter assay, qRT-PCR and Western blot were used to prove the target of miR-328.

RESULTS: Bioinformatics analysis made a prediction that ABCG2 was a direct functional target of miR-328. Position 619-625 of ABCG2 3'-UTR had a space structure that was complementary to miR-328 by bioinformatics analysis, and there was a significant reduction in the level of miR-328 in human gastric cancer cell lines and tissues. The expression of miR-328 down-regulated proliferation, invasion and migration of human gastric cancer cells *in vitro*, while silencing of miR-328 accelerated proliferation, invasion and migration of human gastric cancer cells *in vitro*. All results displayed ABCG2 was direct target protein of miR-328 owing the binding site and they presented a negative correlation.

CONCLUSIONS: ABCG2 is the target protein of miR-328. It presents a negative correlation of the expression level between miR-328 and ABCG2. Down-regulation of miR-328 inhibits the proliferation, invasion and migration of gastric cancer cell lines. MiR-328 could predict generation and development of gastric cancer as a biomarker.

Key Words:
MiR-328, ABCG2, Gastric cancer cells, Downregulation, Biomarker.

Introduction

Gastric cancer is a common malignant tumor of the digestive system[1]. It has the fourth highest cancer incidence on a global scale[2]. China possesses high occurring rate of gastric cancer[3]. Therefore, it has an important clinical value to explore the mechanism for invasion and metastasis of gastric cancer and to seek intervention target[4,5].

The function of miRNAs in the occurrence and development of malignancies has received extensive attention in recent years[6-8]. MiRNAs are endogenous, 20-25 nucleotides-long non-coding RNAs. MiRNAs can bind to the 3'-untranslated region (3'-UTR) of target mRNA sequences to modulate target protein expression at the post-transcriptional level[9,10]. Abnormal expression of miRNAs might function as either tumor activators or suppressors in the development of various tumors[11-13]. It has been confirmed that miRNAs play a significantly critical role in regulating a wide array of malignant phenotypes, such as proliferation, metastasis, differentiation and invasion of tumor cells[14]. Many studies[15-17] have focused on the role of miRNAs as therapeutic, prognostic, diagnostic, or response predictive molecular biomarkers in malignancies. MiR-328 is under-expressed in many cancers including gastric cancer stem cells[18-20] and can contribute to tumor resistance to chemotherapy. However, the role of miR-328 in the development of gastric cancer is not clear.

As a member of ATP-binding cassette membrane transporter protein superfamily, ATP Binding Cassette Transporter G2 (ABCG2) expressed omnipresently in breast cancer[21], brain tumor[22], lung cancer[23], nasopharyngeal carcinoma[24] and leukaemia[25]. ABCG2 has been gradually concerned with the resistance of tumor and linked to the patient's prognosis. It has been observed that ABCG2 has been used as a molecular marker

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for cancers[26]. Pan et al[27] have shown ABCG2 is the target spot for miRNA-328 in breast cancer, and gene amplification has been proved to be a vital mechanism for controlled ABCG2 expression in drug-resistant cancer cells[28,29]. They impact through base-pairing to complementary segments in the 3'-UTR of mRNA, leading to reversed regulation of the target gene. Underlying regulation of ABCG2 at its 3'-UTR is just beginning to be explored[30]. Whether ABCG2 in gastric cancer is the target of miR-328, the connection between ABCG2 and physiological activity of gastric cancer cells remains unknown. As a starting point, the aberrant expression of miR-328 on the regulation of ABCG2 is reviewed in gastric cancer. We have speculated that modulating ABCG2 expression by targeting miRNA-328 could represent a promising strategy for therapeutic manipulation to increase the efficacy of chemotherapeutic agents for gastric cancer[31], a highly lethal type of cancer.

Patients and Methods

Patients and Tissue Samples

100 patients diagnosed with cancers of the stomach by histopathology were convened at the Hospital of Weifang People's Hospital from October 2014 to March 2016. All patients aged between 26 and 78 (median= 52 years) have signed informed consent and the information was gathered by interview when hospitalized in the department or from the record. 100 pairs of surgical specimens were derived from the cancerous parts and pericarcinomatous normal tissues (five centimeters away from the cancerous parts, noncancerous tissues by pathologic examination). No chemotherapy or radiation was received before the operation. All tissue samples were made anonymous disposal according to the moral and legal provisions frozen in -70°C. This study was approved by the Ethics Committee of the Weifang People's Hospital.

Bioinformatics

The stem-loop structure of miR-328 was retrieved on miRBase. ABCG2 3'-UTR sequence was sought on GenBank (NM_004827). We strived to seek antisense matches of ABCG2 3'-UTR sequences against miR-328 by means of miRBase Targets, TargetScan and PITA.

Cell Culture

Human gastric cancer cell lines SGC-7901 (poorly differentiated, metastatic), MKN-28 (well differentiated) and normal gastric epithelial cell lines GES-1 were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). All cell lines were fostered in medium of Roswell Park Memorial Institute-1640 (RPMI-1640) (HyClone, South Logan, UT, USA) added with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA), 100 units penicillin per milliliter, 100 μg/mL streptomycin per milliliter in an atmosphere of 5% CO_2 at 37°C until 90% coverage.

Cell Transfection

The synthesized miR-328 mimic, the negative control (miR-NC), miR-328 antagomir (miR-328-aI) and miR antagomir negative control (miR-NCI) (Applied Biosystems, Foster City, CA, USA) were applied to transfect into cells with the aid of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The negative control was used to validate the specificity of transfection. SGC-7901 and MKN-28 were plated onto 6-well plates (Seebio Biotech, Shanghai, China) until 80% confluence approximately before transient transfection and incubated 48 h for further research.

Western Blot Analysis

Western blot analysis was carried out to verify the protein levels of ABCG2 in two gastric cancer cell lines (SGC-7901 and MKN-28) after transfection. The harvested cells were dissociated in radioimmunoprecipitation assay (RIPA) cell lysis solution (Invitrogen, Carlsbad, CA, USA) after being washed by Phosphate-Buffered Saline (PBS). The whole-protein samples from cell lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis system including 12% separation gel and 5% stacking gel (Sinopharm, Shanghai, China), electrotransferred to nitrocellulose membranes (PALL, New York, NY, USA). Then, the menbranes were blocked overnight in Tris-Buffered Saline including 0.1% (v/v) Tween 20 (Sinopharm, Shanghai, China) and 5% (w/v) skimmed milk powder at 4°C. After incubating with primary antibodies against ABCG2 (diluted 1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), β-Actin (diluted 1:300, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h and anti-mouse IgG secondary antibodies marked horseradish peroxidase (diluted 1:5000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1.5 h, the visual analysis was arrived at a conclusion using the enhanced che-

miluminescence detection system (ECL; Pierce, Waltham, MA, USA). β-Actin was used as an internal control for the standardization of ABCG2. Independent Western blot analysis was carried out in triplicate.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) Analysis

MRNA expression levels of miR-328 and ABCG2 from 100 pairs of cancerous parts and corresponding pericarcinomatous normal tissues were testified by qRT-PCR analysis as described. Total RNA was isolated from tissue samples by means of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). To obtain the concentration, the absorbance ratios of extracted RNA samples were measured at 260 nm/280 nm employing the NanoDrop ND-1000spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RT reaction was performed using SYBR® Green analysis through Prime Script™ RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). U6 was served as an internal reference to normalize the expression level of miR-328. RT system includes 5× Prime Script Buffer 2 μL, Prime Script RT Enzyme Mix I 0.5 μL, Oligo dT Primer 0.5 μL, Random 6 mers 0.5 μL, Total RNA 1 μL, RNase Free dH_2O up to 10 μL. The reaction was conducted in the tube for 15 min at 37°C, 5 sec at 85°C and then held at 4°C. The Real Time-PCR volume that contained SYBR® Premix Ex Taq II 10 μL, Forward Primer 0.8 μL, Reverse Primer 0.8 μL, cDNA 2 μL, ROX Reference Dye 0.4 μL, dH_2O 6 μL was operated using SYBR® Premix Ex Taq™ II (TaKaRa,Otsu, Shiga, Japan) on Applied Biosystems 7500 (Thermo Fisher Scientific, Waltham, MA, USA) for 30 sec at 95°C, and 3 sec at 95°C, 30 sec at 60°C for 40 cycles. The relative target miRNA or ABCG2 mRNA expression values were evaluated by the $2^{-\Delta\Delta CT}$ method. All samples were conducted in triplicate. The same experiment was done on the three kinds of cell lines to further detect the expression of miR-328. Primers were as follows: miR-328 forward, 5'-GCTGGCCCTCTCTGCCC-3', reverse, 5'-CGTCAGATGTCCGAGTAGAGG-3', U6 forward, 5'-CTCGCTTCGGCAGCACA-3', reverse, 5'-AACGCTTCACGAATTTGCGT-3', ABCG2 forward, 5'- CAGGTGGAGGCAAATCTTCGT-3', reverse, 5'-ACACACCACGGATAAACTGA-3', and GAPDH forward, 5'-GGAGCGAGATCCCTCCAAAAT-3', reverse, 5'- GGCTGTTGTCATACTTCTCATGG-3'.

Cell Counting Kit-8 (CCK-8) Assay

Cell proliferation *in vitro* for human gastric cancer cell lines SGC-7901 and MKN-28 transfected with miR-328 mimic and miR-NC was handled with the aid of Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). Incubation for 24 h, 48 h, 72 h was performed after transfection in 96-well plates (Seebio Biotech, Shanghai, China) at a density of 10^4-10^5 cells per well at 37°C and then was added 10 μL CCK-8 solution to each well. Absorbance ratio at 450 nm was measured employing the NanoDrop ND-1000 μL ultraviolet spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) after incubating the plates for 4 hours in the incubator. All cell proliferation assays *in vitro* were conducted in triplicate.

Colony Formation Assay

In brief, SGC-7901 and MKN-28 cells transfected with miR-328 mimic and miR-NC respectively were seeded in 60 mm diameter plates (Seebio Biotech, Shanghai, China) at a density of 1×10^3 cells per well loaded with 5 mL RPMI-1640 medium and 10% FBS for 17 days. Colonies were cleaned with PBS, fixed with methanol (Sinopharm, Shanghai, China), stained with Giemsa (Sinopharm, Shanghai, China) and counted under the CX-31 (Olympus, Tokyo, Japan).

Transwell Invasion and Migration Assay In Vitro

The invasion and migration capacity of SGC-7901 and MKN-28 cells transfected with miR-328 mimic and miR-NC was detected with transwell chambers (8 μm pore diameter; Corning, Corning, NY, USA). SGC-7901 and MKN-28 cells were starved in serum-free medium for 24 h after transfection. Cells were resuspended and put into the upper chamber of the plate at a density of 1×10^5 cells with 80 μL of Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) and then was added 100 μL of serum-free RPMI-1640 medium. RPMI-1640 mixed with 10% FBS was placed into the lower chambers. The upper cells were wiped using swabs after 24 h of incubation. Cells passed through the polycarbonate membrane were disposed of 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) and crystal violet (Sigma-Aldrich, St. Louis, MO, USA) respectively. Under the CX-31 (Olympus, Tokyo, Japan), we counted from five fields. Transwell was operated in triplicate. *In vitro* migration assay, there was no need for Matrigel.

Luciferase Reporter Assay

All of the transfection reactions were operated using Lipofectamine 2000 as described previously. The binding-site of miR-328 gene promoter illatived by PROMO was sub-cloned into the upstream of PGL3-Basic (Promega, Madison, WI, USA) after PCR amplification. The total RNA was extracted from the SGC-7901/MKN-28 and amplified the 3'-UTR of ABCG2, then connected to the PmirGLO (Sangon Biotech, Shanghai, China). SGC-7901 and MKN-28 cells were co-transfected with 3'-UTR-Luciferase reporter plasmid constructs and miR-328 mimic, miR-328-aI (RiboBio, Guangzhou, China), miR-NC, miR-NCI, phRL-TK plasmid (Promega, Madison, WI, USA) internal reference, incubated at 37°C in 96-well plates (Seebio Biotech, Shanghai, China). Firefly Luciferase and Renilla Luciferase substrate joined after lysis and then Luciferase activity was analyzed through the DualGlo™ Luciferase Assay System (Promega, Madison, WI, USA). All Luciferase assays were conducted in triplicate.

Statistical Analysis

Statistical computations were performed using the software of IBM Statistical Product and Service Solutions (SPSS) Statistics version 21.0 (Armonk, NY, USA). All analysis results were shown as mean ± standard deviation. The significance of difference was analyzed by the Dunnett-*t* test for two groups or one-way ANOVA for multiple groups followed by Post-Hoc Test (Least Significant Difference). The correlation coefficient between miR-328 and ABCG2 expression level was measured by Spearman's analysis. The results were considered statistically significant when $p<0.05$.

Results

ABCG2 3'-UTR is a Potential Binding Site of MiR-328 Based on Bioinformatics Analysis

To explore the functional regulation of miR-328 for ABCG2 and structural relationships, various miRNA online databases possessing unique algorithms were used to look for the miRNA recognition element (MRE) sequence, which is 3'-UTR of the target gene, such as TargetScan, miRBase Targets, PITA. The stem-loop structure is displayed (Figure 1A) with the aid of miRBase. The complementary binding site located in region 619-625 of ABCG2 3'-UTR has come under observation (Figure 1B) by TargetScan and PITA, whereas this site was not identified by miRBase Targets. We speculated that this predicted discrepancy may be due to the difference in methods and databases used by the individual procedure.

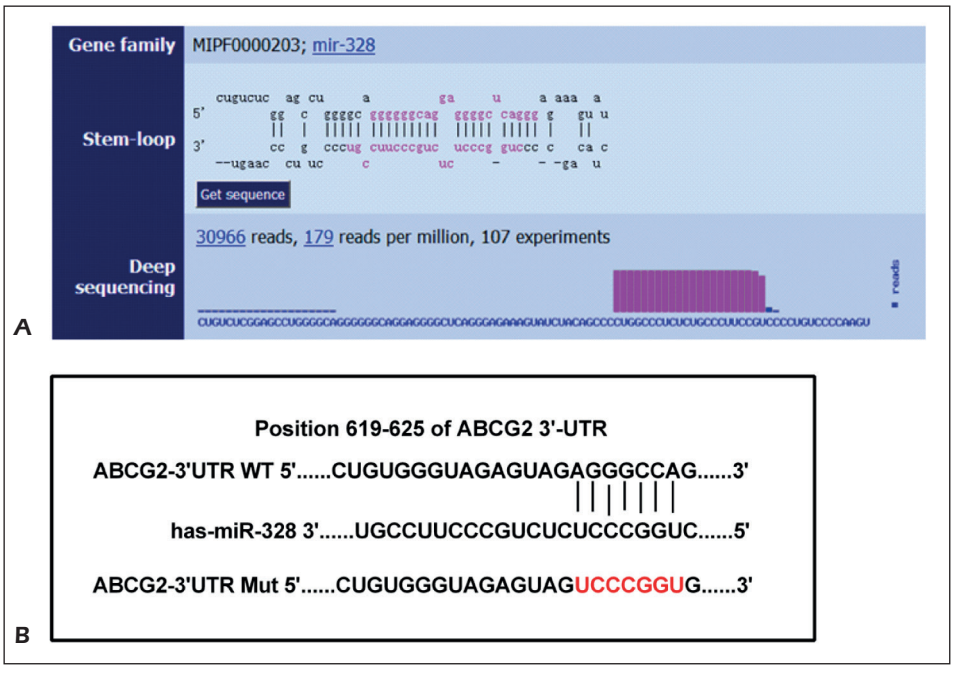

Figure 1. ABCG2 3'-UTR is identified as the potential binding site of miR-328. **A**, The stem-loop structure of 3'-UTR is displayed with the aid of miRBase. **B**, The complementary binding site located in region 619-625 of ABCG2 3'-UTR has come under observation by TargetScan and PITA.

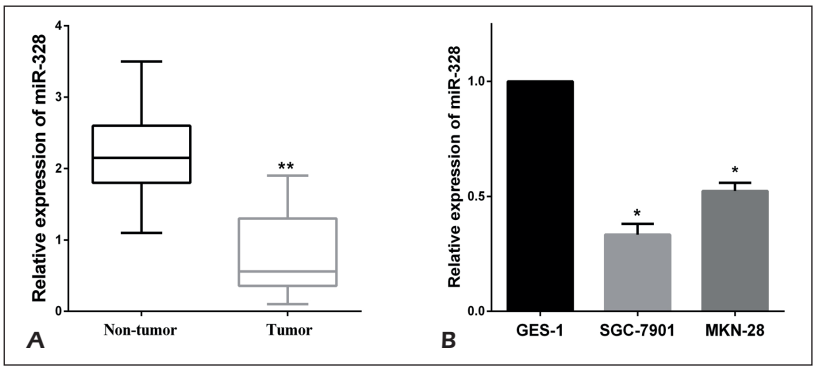

Figure 2. Relative expression level of miR-328 is detected in human gastric cancerous tissues and cell lines by qRT-PCR. **A**, miR328 expression profile in gastric cancer tissues compared with normal adjacent tissues. **B**, Relative expression of miR-328 in different gastric cancerous cell lines *in vitro*. $^{**}p<0.01$, $^{*}p<0.05$.

MiR-328 Expression Level is Significantly Downregulated in Human Gastric Cancerous Tissues and Cell Lines

To validate miR-328 expression on 100 cases of collected cancerous and corresponding adjacent normal tissues, as well as the correlation between expression level and clinical outcome, we have used qRT-PCR analysis in the study. MiR-328 expression level in gastric cancerous tissues was significantly decreased compared with that in the corresponding normal tissues (Figure 2A, $p<0.05$). Moreover, the correlation between miR-328 expression and physiological or pathological factors was analyzed, respectively. We found that miR-328 expression of gastric cancerous tissue specimens from patients with metastasis was markedly lower than those with non-metastasis ($p=0.0159$) (Table I). There was no significant difference between miR-328 and age, gender, local invasion, except tumor size, tumor node metastasis (TNM) stage, lymph node metastasis, respectively. To further verify the role of miR-328 on tumorigenesis, its mRNA expression level was examined in SGC-7901, MKN-28 and GES-1, respectively. Compared with GES-1, miR-328 expression level was remarkably reduced in SGC-7901 and MKN-28 cell lines (Figure 2B). All results indicated that the down-regulation of miR-328 in gastric cancer tissues and cell lines acts as a potentially important role in the occurrence and progression of human gastric cancer.

Table I. Clinicopathological characteristics and miR-328 expression in 100 patients with gastric cancer.

Clinicopathologic features	Cases (n=100)	miR-328 expression		*p*-value
		High (%)	Low (%)	
Gender				
Male	62	32 (51.6)	30 (48.4)	0.423
Female	38	13 (34.2)	25 (65.8)	
Age (years)				
≤52	70	40 (57.1)	30 (42.9)	0.078
>52	30	12 (0.4)	18 (0.6)	
Tumor size (mm)				
≤5.0	31	15 (48.4)	16 (51.6)	0.035*
>5.0	69	39 (56.5)	30 (43.5)	
TNM stage				
I-II	44	16 (36.4)	28 (63.6)	0.039*
III-IV	56	30 (53.6)	26 (46.4)	
Local invasion				
T1-T2	45	30 (66.7)	15 (33.3)	0.082
T3-T4	55	20 (36.3)	35 (63.6)	
Lymph-node metastasis				
0-2	60	40 (66.7)	20 (33.3)	0.019*
>2	40	15 (37.5)	25 (62.5)	

*χ^2-test; TNM, tumor-node-metastasis.

Expression of MiR-328 Downregulates Proliferation, Invasion, Migration of Human Gastric Cancer Cells

To better probe the function of miR-328 over-expression on cell proliferation, CCK-8 and colony formation assay were performed. We transfected miR-328 mimics to overexpress miR-328. Enforced successfully over-expression of miR-328 was identified by qRT-PCR (Figure 3A). In addition, the results of CCK-8 and colony formation assay revealed that enforced up-regulation of miR-328 performed inhibition on the proliferation rate of SGC-7901 and MKN-28 cells compared with miR-NC (Figure 3B-3C, $p<0.05$). Given that metastasis of gastric cancer could be significantly correlated with miR-328, the abil-

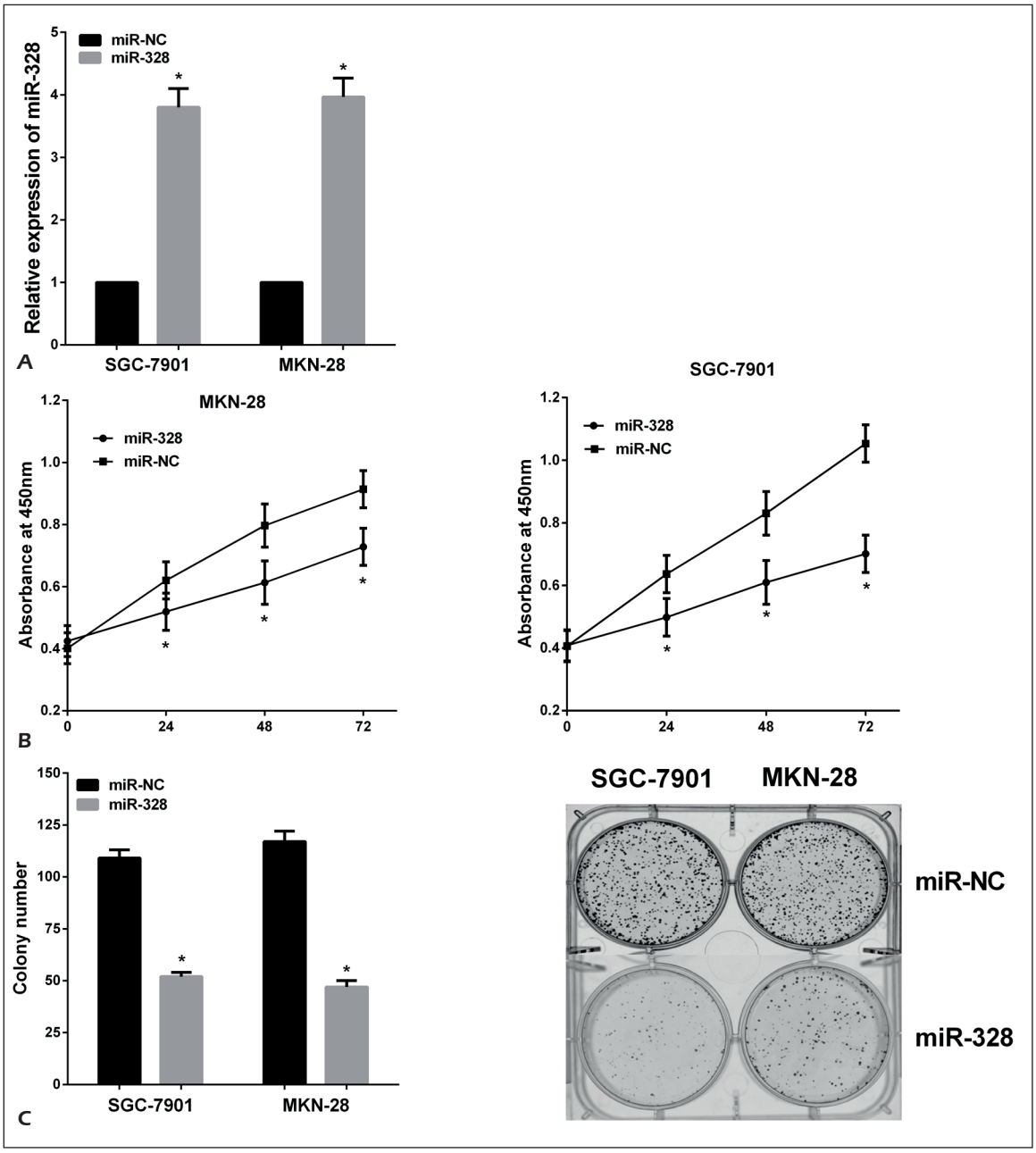

Figure 3. Enforced expression of miR-328 inhibits cell proliferation of human gastric cancer cell lines in vitro. **A**, Enforced successfully over-expression of miR-328 was identified by qRT-PCR. Proliferation capacity of SGC-7901 and MKN-28 cells following transfections was confirmed using CCK-8 kit **B**, and colony formation assay (magnification: 40×) **C**. *$p<0.05$.

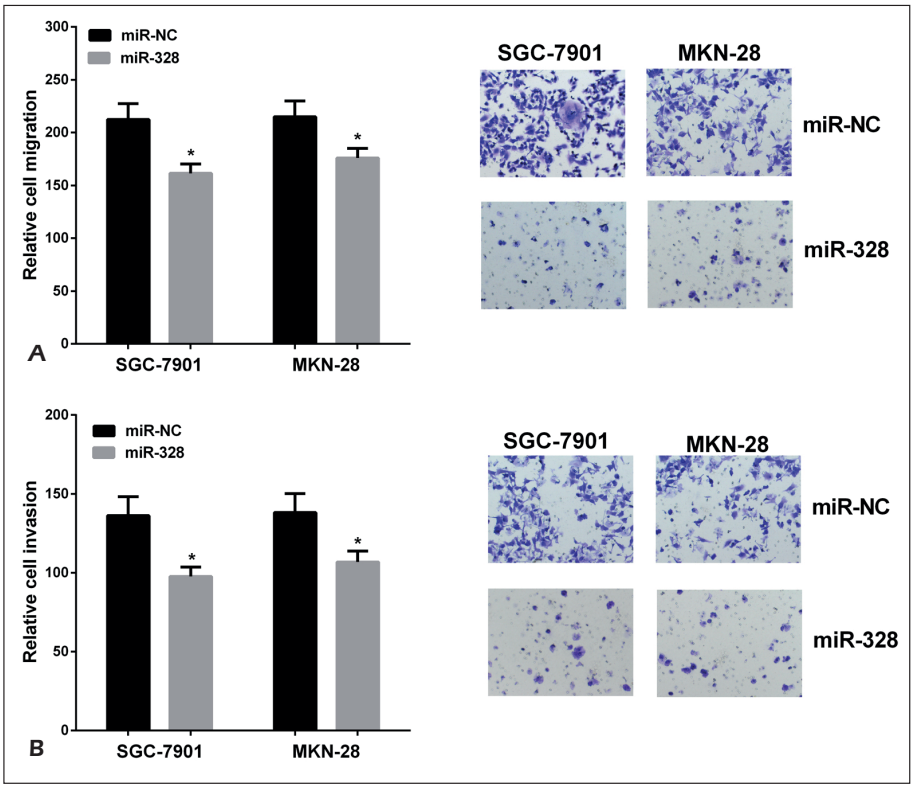

Figure 4. Enforced expression of miR-328 inhibits cell migration and invasion of human gastric cancer cell lines in vitro. **A**, Effect of miR-328 over-expression on migratory and invasive ability was measured using transwell migration and transwell invasion (magnification: 40×) **B**, After transfection of miR-328 mimic or miR-NC in SGC-7901 and MKN-28 cells (magnification: 40×). *p<0.05.

ity of invasion and metastasis in gastric cancer cells was determined through transwell tests. As expected, miR-328 significantly suppressed the invasion and migration of SGC-7901 and MKN-28 cells (Figure 4A-4B, p<0.05).

Silencing of MiR-328 Accelerate Proliferation, Invasion, Migration of Human Gastric Cancer Cells

To further determine the role of endogenous miR-328 silencing on tumor occurrence and progression, we also transfected SGC-7901 and MKN-28 cells with miR-328-aI and miR-NCI. Enforced successfully inhibition of miR-328 expression was confirmed by qRT-PCR (Figure 5A). CCK-8 and colony formation assay implied that SGC-7901 and MKN-28 cells transfected with miR-328-aI performed higher viability of proliferation compared with miR-NCI (Figure 5B-5C, p<0.05). The transwell test also demonstrated that the inhibition of miR-328 increased cells migration and invasion (Figure 6A-6B, p<0.05). All those results indicated that the knockdown of miR-328 could promote human gastric cancer cells growth, migration and invasion.

ABCG2 Is a Direct Functional Target of MiR-328 in Human Gastric Cancer Cells

To probe in further detail whether the potential mechanism of miR-328 on tumorigenesis was mediated by ABCG2, we carried out qRT-PCR and Western-blot assay to discuss the assumed negative correlation between the abnormal expression of miR-328 and mRNA, the protein level of ABCG2 subsequent to bioinformatics analysis. The results illustrated that up-expression of miR-328 suppressed, while knockdown of miR-328 accelerated the expression of ABCG2 at both mRNA and protein levels (Figure 7A-7B, p<0.05). Those results proved that ABCG2 may be a direct target of miR-328. To verify miR-328 recognition element (MRE) site, as well as the functionary connection between miR-328 and ABCG2 once again, we performed Luciferase reporter assay on SGC-7901 and MKN-28 cells cotransfected with 3'-UTR-Luciferase reporter plasmid constructs and miR-328 mimic, miR-328-aI, miR-NC, miR-NCI, phRL-TK plasmid, respectively. Our results revealed that up-expression of miR-328 significantly weakened the Luciferase activity index of the reporter plasmids, while knockdown of

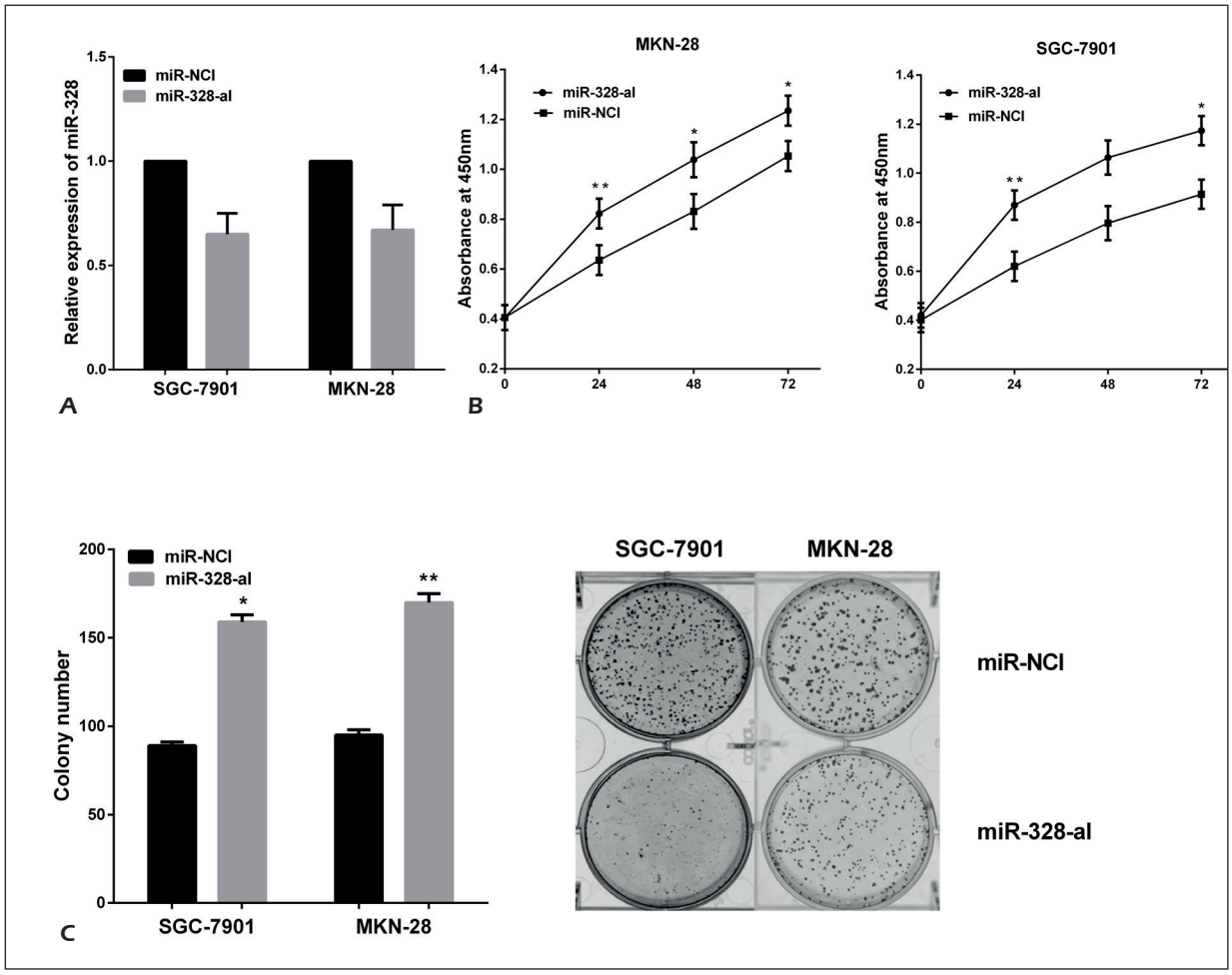

Figure 5. Enforced silencing of miR-328 promotes cell proliferation of human gastric cancer cell lines *in vitro*. **A**, Enforced successfully inhibition of miR-328 was identified by qRT-PCR. Proliferation capacity of SGC-7901 and MKN-28 cells following transfections was confirmed using CCK-8 kit **B**, and colony formation assay (magnification: 10×) **C**. *$p<0.05$.

miR-328 enhanced the Luciferase activity index (Figure 7C, $p<0.05$). All those results suggested that ABCG2 is direct target protein of miR-328 owing the binding site and they present a negative correlation.

Discussion

Currently, the incidence of gastric cancer is the fourth highest in the world, and it has the second highest mortality rate[32]. Surgery is still considered to be the first choice for the treatment of gastric cancer[33,34]. Although there is a significant improvement in the survival time of patients after surgery owing to new-born adjuvant therapy, much remains to be clarified and there are no good target gene therapy drugs[35]. Therefore, it is important to explore the targeting carcinogenic critical gene drugs in gastric cancer.

In recent years, many studies[36,37] have shown that mutation or abnormal expression of miRNAs was closely related to various human malignant tumors, suggesting that miRNAs might play a role as tumor suppressors or promoters. With the deepening of the research, the statement that miRNA can regulate a variety of tumorigenic processes, such as cell proliferation, differentiation, apoptosis, cell cycle, migration and invasion has gradually been recognized[38-41]. Thus, studying miRNAs expression and their functions on the incidence, development of tumors could help to evaluate the early diagnosis, treatment and prognosis.

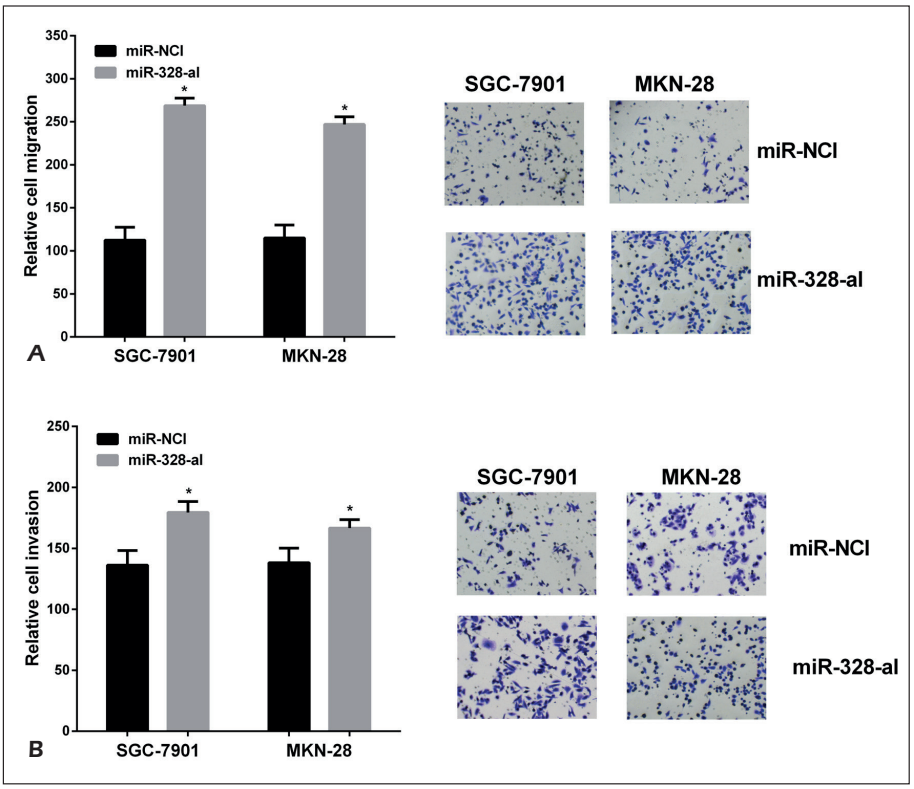

Figure 6. Enforced silencing of miR-328 promotes cell migration and invasion of human gastric cancer cell lines in vitro. Effect of miR-328 under-expression on migratory and invasive ability was measured using transwell migration **A**, and transwell invasion (magnification: 40×) **B**, after transfection of miR-328-aI or miR-NCI in SGC-7901 and MKN-28 cells (magnification: 40×). *$p<0.05$.

MicroRNAs are endogenous, non-coding small RNAs of 20-25 nt, which are widely present in eukaryotic cells and are more conservative in evolution[42]. The functionary mechanism is to cause post transcriptional translation blocked or degradation of the target gene mRNA by complete or incomplete base-pairing with the 3'-UTR of target gene mRNA[43]. MiR-328 derives from chromosome 16 q22.1[44]. Similar to other miRNAs, miR-328 also exerts its biological functions by regulating the expression of its target genes. The validated target genes include γ-H2AX (non-small cell lung cancer)[45], TGFβRIII (heart)[46], PIM-1 (pulmonary artery)[47], TCF7L2 (cervical cancer)[48], hERG (breast cancer)[49], TGFβ (malignant melanoma)[50], CD44 (renal tubules and gastrointestinal secretion)[51], SFRP1 (glioma)[52], ABCG2 (colorectal cancer)[53], and so on. We can see the target of miR-328 is also different in different tissues. But so far, the study of miR-328 and its target gene in gastric cancer is unknown. We first used bioinformatics methods to predict the target genes of miR-328.

We explored possible complementary candidates of miR-328 and the corresponding recognition element (MRE) site applying multiple algorithms, Targetscan, PITA, miRBase. According to previous studies, ABCG2 could be the most matched candidate because of the lowest free binding energy and interaction energy from RNAcofold. So ABCG2 was selected as the subject of research among lots and lots of MRE sites.

This study first detected the miR-328 expression in paired pathological tissues and the corresponding adjacent normal tissues from 100 cases of patients diagnosed with gastric cancer. Meanwhile, we analyzed the relationship between the prognosis of patients and miR-328 expression, the correlation between miR-328 and patients gender, age, tumor size, TNM stage, local invasion, lymph-node metastasis. MiR-328 expression levels in gastric cancer pathological tissues were significantly decreased compared with corresponding normal tissues. However, there was no significant difference between miR-328 and age, gender, local invasion, except tumor size, TNM stage, lymph node metastasis. In addition, miR-328 level was markedly reduced in SGC-7901 and MKN-28 relative to GES-1.

In cell proliferation experiments, enforced up-regulation of miR-328 performed inhibition on the proliferation rate of SGC-7901 and MKN-28 cells compared with the control group. Similarly, miR-328 markedly suppressed invasion and

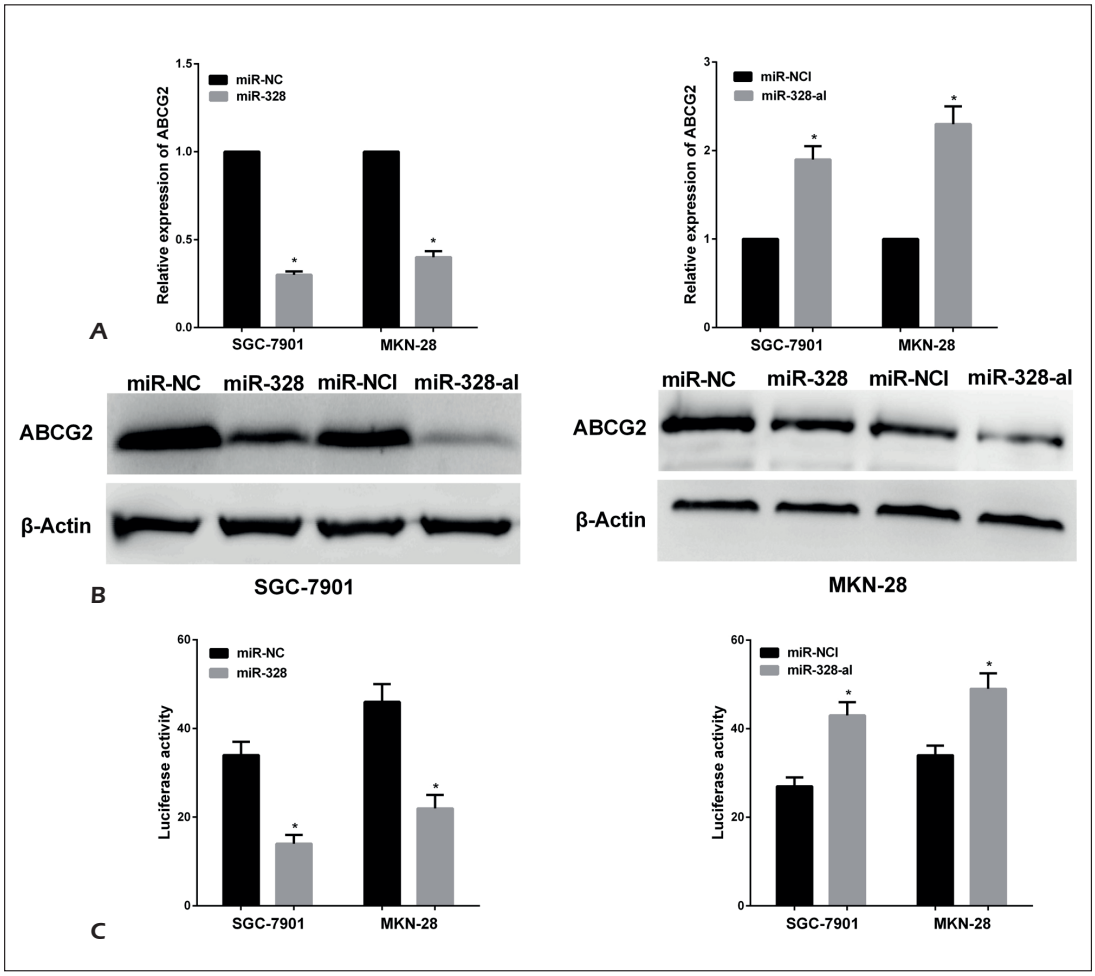

Figure 7. ABCG2 is a direct functional target of miR-328 in human gastric cancer cells. ABCG2 mRNA **A**, protein **B**, expression levels and Luciferase activity **C**, were measured by qRT-PCR, Western blot and Luciferase reporter assays in SGC-7901 and MKN-28 cells after miR-328 mimic or miR-328-aI transfection. *$p<0.05$.

migration of SGC-7901 and MKN-28 in transwell test. When miR-328 was knocked down, this inhibitory action is removed. The results of qRT-PCR and Western blot assay illustrated that up-expression of miR-328 suppressed, while silencing of miR-328 accelerated the expression of ABCG2 at both mRNA and protein levels. All those results confirmed our hypothesis. Additionally, Dual-Luciferase reporter assay revealed that up-expression of miR-328 significantly weakened, while knockdown of miR-328 enhanced the Luciferase activity index of the reporter plasmids. Those results suggested that the potential mechanism of miR-328 on tumorigenesis was mediated by ABCG2.

Conclusions

We first revealed that ABCG2 3'-UTR is a potential binding site of miR-328 in gastric cancer. The expression of miR-328 can regulate the proliferation, invasion, migration of human gastric cancer cells SGC-7901 and MKN-28. ABCG2 is a direct functional target of miR-328 in human gastric cancer cells. This newly identified miR-328 may provide further insight into the progression and offers a promising therapeutic target for gastric cancer.

Conflict of Interests

The authors declared no conflict of interest.

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