ORIGINAL ARTICLE—ALIMENTARY TRACT

miR-874 Inhibits cell proliferation, migration and invasion through targeting aquaporin-3 in gastric cancer

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Abstract

Background Aquaporin-3 (AQP3) is a water transporting protein which plays an oncogenic role in several malignant tumors. However, its regulatory mechanism remains elusive to date. In this study, we investigated the microRNA-mediated gene repression mechanism involved in AQP3's role.

Methods The potential microRNAs targeting *AQP3* were searched via bioinformatic methods and identified by luciferase reporter assays, microRNA RT–PCR and western blotting. The expression patterns of miR-874 and AQP3 in human gastric cancer (GC) specimens and cell lines were determined by microRNA RT-PCR and western blotting. 5-ethynyl-2′-deoxyuridine, cell migration and invasion assays and tumorigenicity in vivo were adopted to observe the effects of miR-874 depletion or ectopic miR-874 expression on GC cell phenotypes. Cell apoptosis was evaluated by FACS and TUNEL in vitro and in vivo respectively.

Results miR-874 suppressed AQP3 expression by binding to the 3′UTR of *AQP3* mRNA in GC cells. miR-874 was significantly down-regulated and reversely correlated with AQP3 protein levels in clinical samples. Analysis of the clinicopathological significance showed that miR-874 and AQP3 were closely correlated with GC characteristics. Functional analyses indicated that ectopic miR-874 expression suppressed the growth, migration, invasion and tumorigenicity of GC cells, whereas miR-874 knockdown promoted these phenotypes. Down-regulation of Bcl-2, MT1-MMP, MMP-2 and MMP-9 and upregulation of caspase-3 activity and Bax were involved in miR-874 inducing cell apoptosis, and inhibiting migration and invasion.

Conclusions These results provide a mechanism by which AQP3 is upregulated, as well as highlight the importance of miR-874 in gastric cancer development and progression.

Keywords miR-874 · AQP3 · Gastric cancer

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Abbreviations

AQP3	Aquaporin-3
miRNA	microRNA
miR-874	microRNA-874
RT-PCR	Real-time polymerase chain reaction
GC	Gastric carcinoma
UTR	Untranslated region
mRNA	Messenger RNA

MT1-MMP   Membrane type-1 matrix metalloproteinase
MMP   Matrix metalloproteinase

Introduction

Gastric cancer (GC) is one of the most common lethal neoplasm worldwide, particularly in Eastern Asia, Eastern Europe and South America. Although GC morbidity and mortality have declined substantially in the recent decades, approximately 989,600 new cases and 738,000 deaths are estimated to have happened in 2008 [1]. In the past few years, with advances in surgical techniques, adjuvant therapy, radiochemotherapy, molecular targeted therapy and earlier detection of the disease, the prognosis of GC is improved [2]. However, GC patients' long-term outcomes remain dismal, especially for the advanced GC with a 5-year overall survival rate of 25 % or less [3, 4]. Currently the main virulence factors for its occurrence include chronic Helicobacter pylori infection, environmental exposures, dietary factors and genetic susceptibility. Recently, many microRNAs (miRNAs), oncogenes and tumor suppressor genes were confirmed to be closely associated with GC, but the depth molecular mechanisms underlying its carcinogenesis, progression and aggressiveness are still under investigation.

As known, AQPs (aquaporins) are a family of small, hydrophobic, transmembrane water-channel proteins related to the major intrinsic proteins. They function as membrane channels for the selective transport of water, glycerol and other small solutes across the cell membrane and are widely expressed in animals and plants. Thirteen members, AQP0 to AQP12, of this family have been identified to date [5]. As to the role of AQPs in human cancers, recent evidence has suggested that AQP3 is involved in the proliferation, migration and invasion of cancer cells such as human gastric adenocarcinoma, ovarian cancer, pancreatic cancer, esophageal and oral squamous cell carcinoma [6–9]. In our previous study, we showed that AQP3 exhibited differential expression between human gastric carcinomas and corresponding normal tissues, and AQP3 expression was associated with pathological differentiation and lymph node metastasis in GC patients [10]. These findings indicated that the *AQP3* gene might function as an oncogene, but the mechanism by which *AQP3* was regulated in GC had yet to be clearly defined.

Recently, miRNAs were identified as short (20–24 nt) non-coding RNAs which play important regulatory roles in gene expression by binding to partial homology of target gene mRNA 3′UTR region. Growing evidence indicates that miRNAs are involved in cancer development and progression, acting as tumor oncogenes or suppressors. To the best of our knowledge, limited information is obtainable concerning the expression and significance of miRNA-874 in GC thus far. In the present work, we demonstrated that the 3′UTR of *AQP3* contains a highly conserved miRNA-874 binding motif and its direct interaction with miR-874 downregulated endogenous AQP3 protein level. Based upon these data, we confirmed that *AQP3* is a target of miR-874, and further determined miRNA-874 expression pattern and its inhibition of GC cell growth, migration, invasion and tumorigenicity in vivo. The current work revealed that miRNA-874 is significantly downregulated in human gastric tumor specimens and affected cellular biological processes by targeting the transcriptional expression of *AQP3*. Ectopic expression of AQP3 was able to counteract the suppression of cell phenotypes caused by miR-874 overexpression. Our data is the first report showing the role of miRNA-874 in the regulation of AQP3 expression in GC cells. The results identifying a new tumor-suppressive miR-874-mediated GC pathway provide new insights into the pathogenesis of gastric oncogenesis and will aid the development of novel therapeutic strategies.

Materials and methods

Tissue specimens

Paired tumorous and adjacent non-tumorous human gastric tissues were obtained from 75 patients with GC who underwent surgical resection at the Department of General Surgery, First Affiliated Hospital, Nanjing Medical University, China. All patients were diagnosed pathologically according to the criteria of the American Joint Committee on Cancer. Histopathological diagnoses were performed by two professional pathologists independently. Written informed consents were obtained before specimen collection. Tissue samples were flash frozen immediately after resection and stored in liquid nitrogen until RNA and protein extraction. The acquisition of tissue specimens and study protocol were performed in strict accordance with the regulations of the Nanjing Medical University Institutional Review Board.

Cell lines and cell culture

The human GC cell lines AGS, BGC823, MKN28 (ATCC, Manassas, VA) and SGC7901 (CBTCCCAS, Shanghai, China) were cultured in RMPI-1640 medium supplemented with 10 % fetal bovine serum (FBS; Invitrogen, Grand Island, NY, USA) and antibiotics (1 % penicillin/streptomycin; Gibco). All cell lines were

grown in a humidified chamber supplemented with 5 % CO_2 at 37 °C.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from frozen tissues and cell lines using the Trizol Reagent (Invitrogen) according to the manufacturer's protocol, and was reverse transcribed into cDNA using Primescript RT Reagent (Takara). qRT-PCR was performed on a 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) using SYBR Premix Ex Taq Kit (Takara). The specific primers were as follows: AQP3, forward: 5′-TCAATGGCTTCTTTGACCAGTT CA-3′, reverse: 5′-CTTCACATGGGCCAGCTTCACA TT-3′; β-actin, forward, 5′-TCATGAAGTGTGACGTG GACAT-3′, reverse, 5′-CTCAGGAGGAGCAATGAT CTTG-3′. All procedures were performed in triplicate.

3′UTR luciferase construct and assay

The 3′UTR of AQP3 mRNA containing the wild-type or mutated miR-874/miR-194 binding sequences were synthesized by Shenggong (Shanghai, China). The sequence was cloned into the FseI and XbaI restriction sites of the pGL3 luciferase control reporter vector (Promega, USA) to generate the AQP3 3′UTR reporter. Total 5×10^5 AGS cells were seeded in 24-well plates. Cells were cotransfected with 0.12 μg of either pGL3-WT-AQP3 or pGL3-MUT-AQP3 3′UTR reporter plasmid together with 40 nM of miRNA mimics or negative control (miR-874 or miR-194) oligoribonucleotides using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. Meanwhile, 0.01 μg of Renilla luciferase expression plasmid was also transfected into the above cells as a reference control. Firefly and Renilla luciferase activities were measured by Dual-Luciferase reporter assay (Promega, E1910, WI, USA) at 36 h after transfection according to the manufacturer's instruction. The relative luciferase activity was calculated as firefly fluorescence/Renilla fluorescence.

miRNA RT-PCR

Total RNA was extracted as above. Target-specific reverse transcription and Taqman microRNA assay were performed using the Hairpin-it™ miRNAs qPCR Quantitation Kit (Genepharma, ShangHai, China) according to the manufacturer's instructions. The reactions were performed on the 7500 Real-Time PCR System. The snRNA U6 was selected as an endogenous reference to calculate the relative expression levels of miR-874 and miR-194 in every sample using the $2^{-\Delta\Delta Ct}$ method. All experiments were done independently in triplicate.

Vector constructs, lentivirus production and cell transfection

We modified the commercial LV3-has-miR-874-pre-microRNA vector (pre-miR-874) and LV3-has-miR-874-sponge inhibitor vector (miR-874 inhibitor) lentiviral constructs (Genepharma, ShangHai, China) to overexpress or knockdown miR-874 in GC cells. LV3 empty lentiviral construct (miR-NC) served as a negative control. The constructed vectors were verified by DNA sequencing. When AGS and SGC7901 cells grew to 50 % confluence, cells were infected with lentiviral vectors miR-NC, pre-miR-874 or miR-874 inhibitor, respectively, at an appropriate multiplicity of infection. The above four groups of cells SGC7901-NC, SGC7901-miR-874 inhibitor, AGS-NC and AGS-pre-miR-874 were selected with 3.5 μg/ml puromycin (Sigma, Aldrich) for 5 days to build stable cell lines. Thereafter, the cells were analyzed using Hairpin-it™ miRNAs qPCR Quantitation Kit for miR-874 expression. Stably infected cells were selected for further experiments. Lentiviral vectors encoding AQP3 and shR-NAs targeting AQP3 were produced as described previously [11].

Western blot assay

Total protein from frozen tissues and cell lines were extracted using a lysis buffer [50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 % Triton X-100, 0.1 % SDS, 1 mM EDTA, protease inhibitors (1 mM phenylmethanesulfonyl fluoride, and a cocktail of protease inhibitors)]. The protein extracts were loaded, size-fractionated by SDS–polyacrylamide gel electrophoresis and transferred to PVDF membranes (Bio-Rad Laboratories). After blocking, the membranes were incubated with specific first antibodies in dilution buffer at 4 °C overnight. The blotted membranes were incubated with HRP-conjugated anti-mouse or anti-rabbit IgG (1:2000) at room temperature for 2 h. Then the targeting protein expression level was detected using an enhanced chemiluminescence (ECL) detection system following the manufacturer's instructions. GAPDH was used as an internal control. Mouse antibodies to AQP3, Bcl-2, and Bax (Santa Cruz Biotechnology), and rabbit antibodies to membrane type-1 matrix metalloproteinase (MT1-MMP), matrix metalloproteinase-2 (MMP-2) and MMP-9 (cell signaling technology) were used.

5-ethynyl-2′-deoxyuridine (EdU) assay and colony formation assay

Cell proliferation was measured by EdU assay kit (Ribobio, China). Briefly, SGC7901-NC, SGC7901-874 inhibitor, AGS-NC and AGS-pre-miR-874 stable cells were seeded

into 96-well plates at a density of 5×10^3 cells per well and cultured for 24 h. The cells were then treated with 50 μM of EdU for an additional 2 h at 37 °C. The cells were then fixed in 4 % formaldehyde for 15 min and permeabilized with 0.5 % Triton X-100 at room temperature for 20 min. After washing with PBS, the cells were treated with 100 μl of 1 × ApolloR reaction cocktail for 30 min. Subsequently, the cellular nuclei were stained with 100 μl of Hoechest 33342 (5 μg/ml) for 20 min and visualized under a Nikon microscope (Nikon, Japan). Data were analyzed using a mean number of three fields for each sample. For colony formation, four groups of GC stable cells (200 per well, 12-well plates) were cultured in RMPI-1640 medium for 3 weeks. These colonies were photographed and analyzed statistically.

Cell apoptosis analysis

Flow cytometry was used to analyze cell apoptosis. GC cells stably transfected with miR-874 inhibitor, pre-miR-874 or empty vector were harvested, washed and resuspended in ice-cold PBS. Staining of apoptotic cells was achieved by incubating the cells with propidium iodide (10 μg/ml; Sigma) and Annexin V-FITC (50 μg/ml, BD) in the dark for 15 min at room temperature. Data were acquired on a FACScan flowcytometer (Becton–Dickinson, Franklin Lakes, NJ, USA).

Caspase-3 activity assay

To measure caspase-3 activity, GC cells (1×10^6 cells) were treated with miR-874 inhibitor, miR-874 precursor or empty vector. The caspase-3 activity was determined with a Caspase-Glo® 3/7 Assay kit (Promega, USA) according to the manufacturer's protocol.

Cell migration and invasion assays

Cell migration and invasion assays were performed using a chamber of 6.5 mm in diameter (8 μm pore size, Corning). SGC7901-NC, SGC7901-miR-874 inhibitor, AGS-NC and AGS-pre-miR-874 stable cells (1×10^5 cells which were resuspended in 100 μl serum-free medium) were added to the upper chamber separately. Similarly, approximately 2×10^5 cells resuspended in 100 μl serum-free medium were seeded independently in the top chamber coated with 1 mg/ml Matrigel for invasion assays. Subsequently, 0.5 ml of 10 % FBS-RMPI-1640 was added to the lower chamber. Cells were incubated at 37 °C for 24 h, and then cells in the upper chamber were removed using cotton swabs, respectively. Cells migrating to or invading the bottom of the membrane were stained with 0.1 % crystal violet for 30 min at 37 °C, followed by washing with PBS.

Four random fields of each membrane were photographed and counted for statistical analysis.

Tumorigenicity in vivo

A total of sixteen nude mice (BALB/c nude mice, Vitalriver, Nanjing, China; 4 weeks old) were randomly divided into four groups. SGC7901-NC, SGC7901-miR-874 inhibitor, AGS-NC and AGS-pre-miR-874 stably transfected cells were inoculated bilaterally and subcutaneously into the flanks of nude mice. Bidimensional tumor measurements were taken with vernier calipers every 4 days, and the mice were euthanized after 3 weeks. The volume of the implanted tumor was calculated using the formula: volume = (width^2 × length)/2.

Immunohistochemistry (IHC) for subcutaneous grafts

All specimens were fixed in 4 % formalin and embedded in paraffin. MaxVision^{TM} techniques (Maixin Bio, China) were used for IHC according to the manufacturer's instruction. After blocking endogenous peroxides and proteins, the 4 μm of slices were incubated with diluted primary antibody against AQP3 or Ki-67 (Maixin Bio, China) overnight at 4 °C. After washing with PBS, the slices were incubated with HRP-Polymer-conjugated secondary antibody at 37 °C for 1 h. Then the slices were stained with the 3,3-diaminobenzidine solution for 3 min and counterstained with hematoxylin. Tumor slices were examined in a blinded manner. Three fields were selected for examination as the percentage of positive tumors and cell-staining intensity.

TUNEL assay

The implanted tumors were fixed in 4 % formalin and embedded in paraffin. The 4 μm slices were obtained before HRP-conjugated dUTP staining. A TUNEL apoptosis detection kit (Keygen biotech, KGA7051, China) was used to detect cell apoptosis in the implanted tumors according to the manufacturer's instruction. All slices were assessed under the microscope. The apoptotic cells and total number of cells in five random fields (magnification, ×100) were photographed and counted for each group. The apoptotic index of the cancer cells = apoptotic cells/total cells × 100 %.

H. pylori culture and co-culture with gastric cells

H. pylori bacteria were cultured and co-cultured with gastric cells as previously described [12]. Briefly, *H. pylori* (strain ATCC 26695) were grown on Columbia agar plates (bioMe′rieux, Marcy l'Etoile, France) with 100 U/ml *H.*

pylori selective supplement (Oxoid, Basingstoke, United Kingdom) at 37 °C in an anaerobic chamber (BBL Campy Pouch System, Becton–Dickinson Microbiology Systems). After 48–72 h, *H. pylori* bacteria were harvested and resuspended in antibiotic-free culture medium with 2 % fetal calf serum. The *H. pylori* bacterial densities were adjusted and the bacteria were then incubated with GC cells at a cell-to-bacterium ratio of 1:100 for 24 h in the medium according to a previous report [12].

Modified Giemsa staining of *H. pylori* in mucous tissues of human GC

The gastric mucous tissues of GC patients were fixed in 4 % formaldehyde and embedded in paraffin. Histological slices were stained with modified Giemsa, as previously described [13].

Statistical analysis

The data were expressed as mean ± standard deviation (SD). Clinicopathological findings were compared using unpaired *t*-tests or Pearson χ^2 tests. Analysis of variance (ANOVA) was used to compare the control and the treated groups. *P* values <0.05 were considered as statistically significant.

Results

AQP3-3′UTR is putatively targeted by miR-874

Our previous observation that AQP3 is highly upregulated in GC tissues led us to explore the mechanism of AQP3 overexpression [10]. To investigate whether the expression of AQP3 is regulated by miRNAs, TargetScan (http://www.targetscan.org/), PicTar (http://pictar.bio.nyu.edu) and miRanda (http://www.sanger.ac.uk) were used in combination to predict miRNAs which might target *AQP3*. As shown in Fig. 1a, bioinformatics analysis revealed the top two possible miRNAs, miR-874 containing a putative 8-mer-binding motif and miR-194 possessing a 7-mer-binding site in the 3′UTR of *AQP3* transcript.

miR-874 directly interacts with a putative binding site of *AQP3*-3′UTR

The luciferase reporter assay was employed to validate whether *AQP3* was a direct target of miR-874 or miR-194. Wild-type and mutant *AQP3* 3′UTRs containing the site-directed mutations in the putative target sites of miR-874 or miR-194, were cloned into reporter plasmids respectively (Fig. 1a). The fluorescent activity of the reporter gene was

significantly declined in the group which was cotransfected with pGL3-WT-AQP3 3′UTR and pre-miR-874 compared with the control (Fig. 1b). In addition, there was no significant reduction of the fluorescent intensity in the group that was cotransfected with pGL3-MUT-AQP3 3′UTR and pre-miR-874, further demonstrating that miR-874 induced the decrease of AQP3 expression by binding to complementary nucleotide sequences in the 3′UTR of *AQP3* mRNAs. Of note, there was no difference in the fluorescent intensity of AQP3 among the miR-194 groups under different treatments (Fig. 1c).

miR-874 suppresses AQP3 protein expression through translational repression

To investigate the impact of miR-874 on AQP3 expression in GC cell lines, we performed both miR-874 overexpression and knockdown experiments. Figure 1d shows miR-874 expression after lentiviral transfection. Notably, miR-874 silencing in SGC7901 cells which lack endogenous AQP3 expression resulted in the upregulation of the AQP3 protein compared with the negative control (Fig. 1e, f). Conversely, the protein level of AQP3 was significantly reduced in AGS cells with inherent high expression level of AQP3, after transfection with pre-miR-874 (Fig. 1e, f). Nevertheless, no significant changes in the level of *AQP3* mRNA were observed after miR-874 inhibitor or pre-miR-874 were transfected into GC cells compared with the control group (Fig. 1g). These results indicated that miR-874 suppressed AQP3 protein expression through translational repression.

miR-874 is frequently downregulated in GC

To evaluate the expression pattern of miR-874 in GC tissues, we employed miRNA RT-PCR in 75 pairs of GC and non-GC samples. The results indicated that miR-874 was significantly downregulated in GCs compared with the non-cancerous tissues (Fig. 2a, $P < 0.001$). Among the 75 pairs of clinical samples, 54/75 (72.0 %) showed miR-874 down-regulation (T/N < 1.0), and 21/75 (28.0 %) were upregulated (T/N > 1.0). The protein expression levels of AQP3 in 75 paired GCs and non-neoplastic tissues were measured by western blotting. Sixty-four of 75 (85.3 %) were significantly elevated in GCs compared with matched non-GC tissues, which was in line with the previous study (Fig. 2c, and Table 1, $P < 0.001$) [10]. The AQP3 protein expression of six random matched GC samples was shown in Fig. 2b. Furthermore, we assessed the correlation between miR-874 or AQP3 expression levels and clinicopathological features. As shown in Table 1, miR-874 expression level was lower in samples with moderately and poorly histological type, lymph node metastasis N1–N3
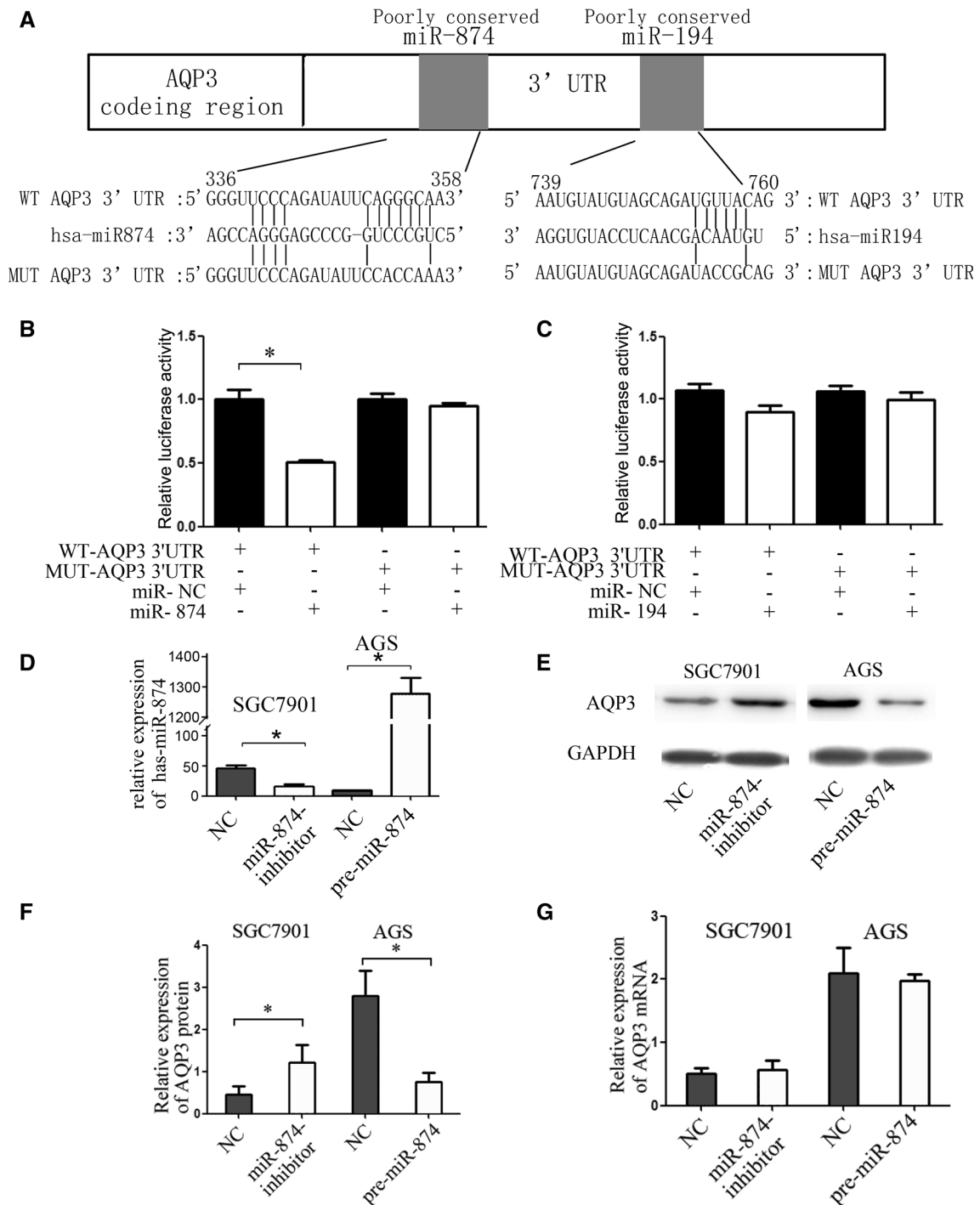
Fig. 1 Identification of AQP3 as a potential target of miR-874. **a** The AQP3 3′UTR regions containing the wild-type or mutant binding site and sequence complementarity between miRNAs (miR-194 and miR-874) and the *AQP3* 3′UTR (WT and MUT) are shown. **b, c** Relative AQP3 luciferase activity was analyzed after the wild-type or mutant 3′UTR reporter plasmids were cotransfected with miR-NC, miR-874 or miR-194 in AGS cells. The *histogram* represents the mean of the normalized AQP3 luciferase intensity from three independent experiments (mean ± SD). *$P < 0.05$. **d–g** The specific miR-874 inhibitor, pre-miR-874 and empty lentiviral construct vector were transfected into SGC7901 or AGS cells. Western blot assay, miRNA RT-PCR and qRT-PCR were used to analyze the expression levels of AQP3 and miR-874 in SGC7901 and AGS cells transfected with miR-NC, miR-874 inhibitor or pre-miR-874, *$P < 0.05$

and stage III–IV. AQP3 protein level was closely associated with histological type and lymphatic invasion. Notably, by analysis of their expression levels in GC tissues, we found the level of miR-874 expression was inversely related to AQP3 protein expression (Fig. 2d, $P < 0.001$, $r = -0.511$). Taken together, these collective data

Fig. 2 Expression patterns of miR-874 and AQP3 in GC specimens and cell lines. **a** The expression levels of miR-874 in 75 paired GCs and non-GCs were detected by miRNA RT–PCR assay and normalized to RNU6B. The miR-874 expression levels in GC tumor tissues and matched non-tumorous tissues were measured, analyzed and shown by a *scatterplot*. *$P < 0.001$. **b** The protein levels of AQP3 in six paired GC tissues were detected by western blotting. GAPDH was used as the loading reference. **c** The expression levels of AQP3 protein in 75 paired GCs and non-GCs were measured by western blotting. AQP3 expression was normalized to GAPDH. **d** The correlation between miR-874 and AQP3 was analyzed and the results showed that miR-874 expression was inversely related to AQP3 protein expression ($P < 0.001$, $r = -0.511$). **e–g** The expression levels of AQP3 and miR-874 in cell lines were detected by western blot assay and miRNA RT-PCR
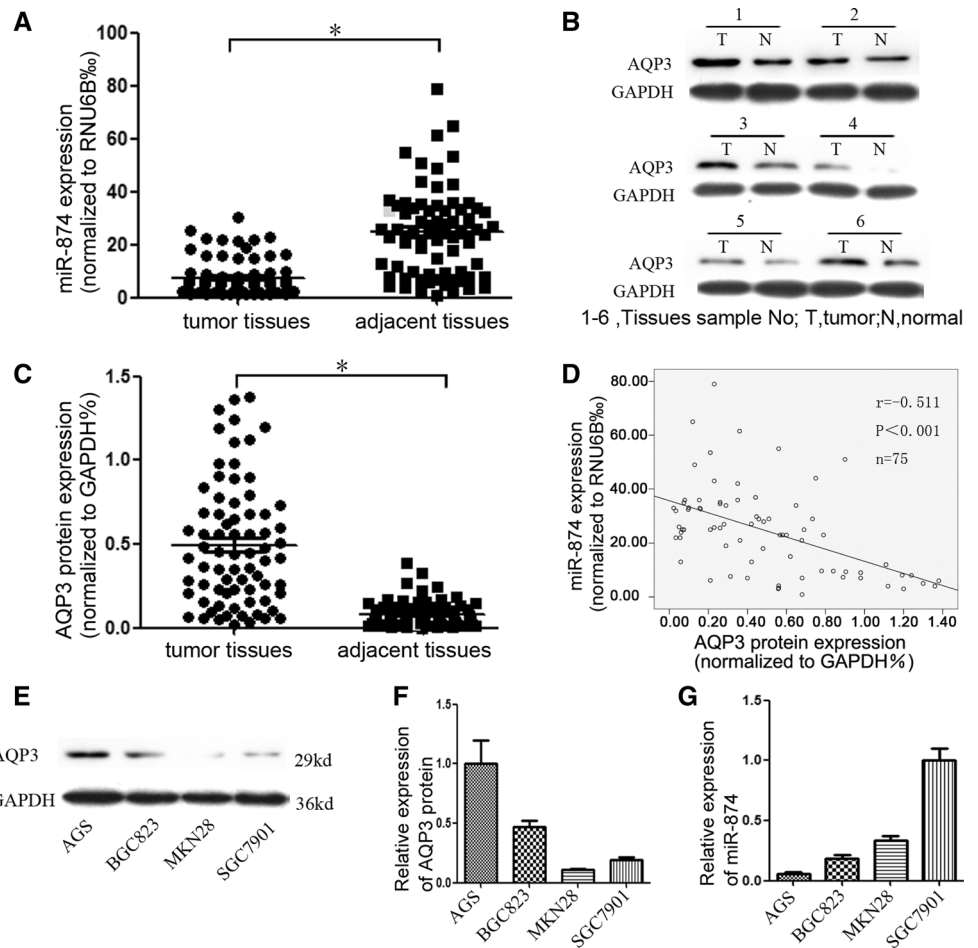
provided evidence that miR-874 downregulation may play an important role in the pathogenesis of GC by regulating AQP3 expression.

Additionally, the AQP3 protein level was higher in AGS and BGC823 cells, but lower in MKN28 and SGC7901 cells, which was inversely correlated with AQP3 protein expression (Fig. 2e–g).

miR-874 inhibits cellular proliferation and colony formation

To confirm the hypothesis that miR-874 functions as a candidate suppressor in GC, we also investigated the influence of miR-874 expression on cellular growth and colony formation. Here, we used the EdU incorporation assay, which is proven to be a more sensitive and specific method [14, 15], to assess the effects of miR-874 on cell proliferation. As shown in Fig. 3a, the number of SGC7901 cells incorporating EdU in the miR-874 inhibitor-treated group significantly increased relative to the control group in SGC7901 cells ($P < 0.05$). By contrast, AGS cells transfected with the miR-874 precursor showed a significant decrease of cell proliferation compared with the

negative control group (Fig. 3b, $P < 0.05$). Furthermore, to evaluate the long-term effects of miR-874 on cell proliferation, the colony formation assay was employed. The results implied that miR-874 downregulation significantly enhanced the colony formation ability of SGC7901 cells as compared with the negative control (Fig. 3c, d, $P < 0.05$). Conversely, fewer colonies were formed in AGS cells transfected with pre-miR-874 compared with the empty vector control (Fig. 3c, d, $P < 0.05$), suggesting that miR-874 negatively regulated cell proliferation of GC cells in vitro.

To address the mechanism of miR-874-modulated cell growth, annexin V-FITC/PI double staining assay by flow cytometry was employed. The proportion of apoptotic SGC7901 cells transfected with miR-874 inhibitor was significantly lower than the negative control group (Fig. 3e, f, $P < 0.05$). Moreover, upregulation of miR-874 by transfection with miR-874 precursor promoted AGS cell apoptosis.

Caspase-3, a cytoplasmic aspartate-specific cysteine protease, is proven to play a pivotal role in the apoptotic signaling transduction pathway. Figure 3g shows that the caspase-3 activity was significantly suppressed in

Table 1 Expression of miRNA-874 and AQP3 protein in human gastric cancer according to clinicopathological features of patients

Clinicopathological variables	miR-874 expression		P value	AQP3 protein expression		P value
	T > N ($n = 21$)	T < N ($n = 54$)		T > N ($n = 64$)	T < N ($n = 11$)	
Age (year)						
<50	9	25		27	7	
≥50	12	29	0.788	37	4	0.321
Gender						
Male	13	31		36	8	
Female	8	23	0.722	28	3	0.488
Tumor size (cm)						
<3	8	22		25	5	
≥3	13	32	0.834	39	6	0.947
Histological type						
Well	10	9		12	7	
Moderately and poorly	11	45	0.006*	52	4	0.005*
Depth						
Localized in subserosa	8	12		18	2	
Beyond subserosa	13	42	0.163	46	9	0.749
Lymph node metastasis						
$n0$	12	15		20	7	
$n1$–$n3$	9	39	0.017*	44	4	0.084
Lymphatic invasion						
Absent	13	21		33	1	
Present	8	33	0.072	31	10	0.022*
Stage						
I, II	12	16		22	6	
III, IV	9	38	0.027*	42	5	0.347

* $P < 0.05$ Statistically significant difference

SGC7901 cells treated with miR-874 inhibitor compared with the control. By contrast, miR-874 overexpression increased the caspase-3 activity in AGS cells. These results indicated that miR-874 could efficiently activate caspase-3 in GC cells. Furthermore, the expression levels of Bcl2 and Bax protein in miR-874 knockdown SGC7901 cells and miR-874-overexpressing AGS cells were assessed by western blotting. As shown in Fig. 3h–j, miR-874 depletion resulted in a decrease of Bax protein, whereas increased Bcl-2 protein expression level was observed in SGC7901 cells. Correspondingly, miR-874 overexpression induced an upregulation of the Bax protein accompanied by suppression of Bcl-2 protein expression. These data suggested that miR-874 affects the progression of cell apoptosis through the caspase-3, Bax and Bcl2 related signal pathway.

miR-874 is involved in the negative regulation of GC cell migration and invasion

The above clinical significance analysis of miR-874 expression suggested that downregulation of miR-874 may be associated with cellular metastasis and perhaps even invasion in GC (Table 1). Here, we used cell migration and Matrigel invasion assays to investigate the effects of miR-874 on cell migration and invasion ability. Our data revealed that cell migration ability was enhanced by miR-874 knockdown in SGC7901 cells, while it was suppressed by miR-874 overexpression in AGS cells (Fig. 4a, b, $P < 0.05$). Additionally, compared with the control cells, miR-874 silencing in SGC7901 cells also dramatically boosted cell invasion whereas miR-874 upregulation inhibited AGS cell invasion, as assessed by Matrigel invasion assay (Fig. 4a, b, $P < 0.05$).

Since MT1-MMP, MMP-2 and MMP-9 play a key role in tumor cell metastasis and invasion, we further examined the effect of miR-874 expression alteration on their expression levels. As shown in Fig. 4c, d, the expressions of MT1-MMP, MMP-2 and MMP-9 were markedly increased in SGC7901 cells after treatment with miR-874 inhibitor. By contrast, miR-874 overexpression induced the down-regulation of MT1-MMP, MMP-2 and MMP-9 protein in AGS cells (Fig. 4c, e). These findings suggested that downregulation of miR-874 expression contributed to GC cellular migration and invasion.
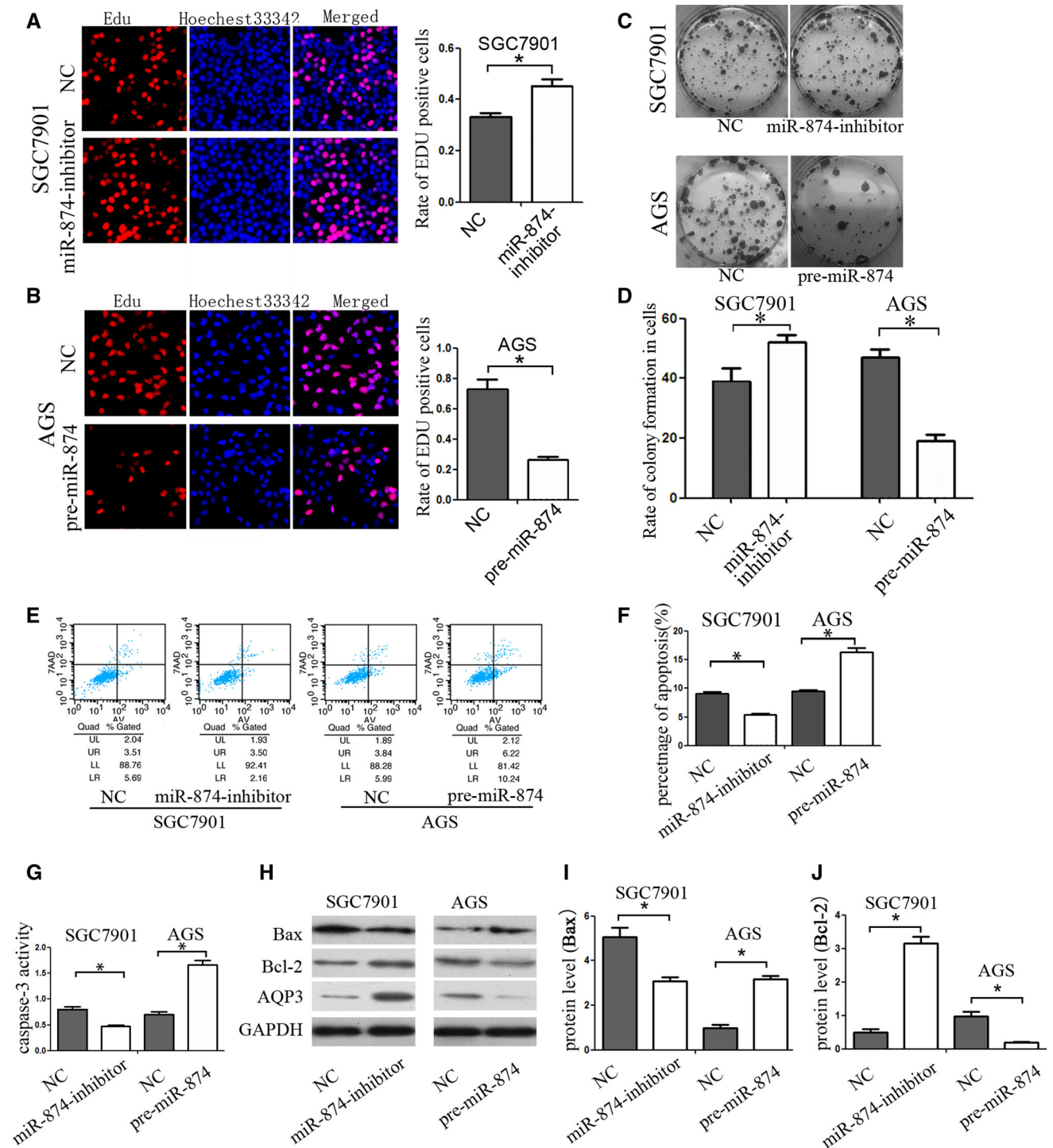
Fig. 3 miR-874 suppresses cell proliferation and promotes cell apoptosis. **a** Representative profiles of Edu cell growth in SGC7901 cells after transfection with miR-874 inhibitor compared to the negative control. **b** The intensity-distribution plots of Edu-incorporated positive cells when miR-874 was overexpressed in AGS cells were shown. The cells transfected with empty vector were used as negative control. **c, d** Effects of miR-874 on the colony formation of GC cells. The number of colonies was calculated and analyzed. **e, f** FACS analysis of the effect of miR-874 expression alteration on cell apoptosis. The empty vector was used as control. **g** The caspase-3 activities of gastric cancer cells after treatment with miR-874 inhibitor, miR-874 precursor or the empty vector were evaluated by Caspase-3 activity assay. **h–j** Expression levels of Bax, Bcl-2 and AQP3 protein were detected by western blotting. GAPDH was used as loading control. All proteins were normalized to the expression of GAPDH. The bars on the histograms are the standard deviation of three independent experiments (mean ± SD), *$P < 0.05$
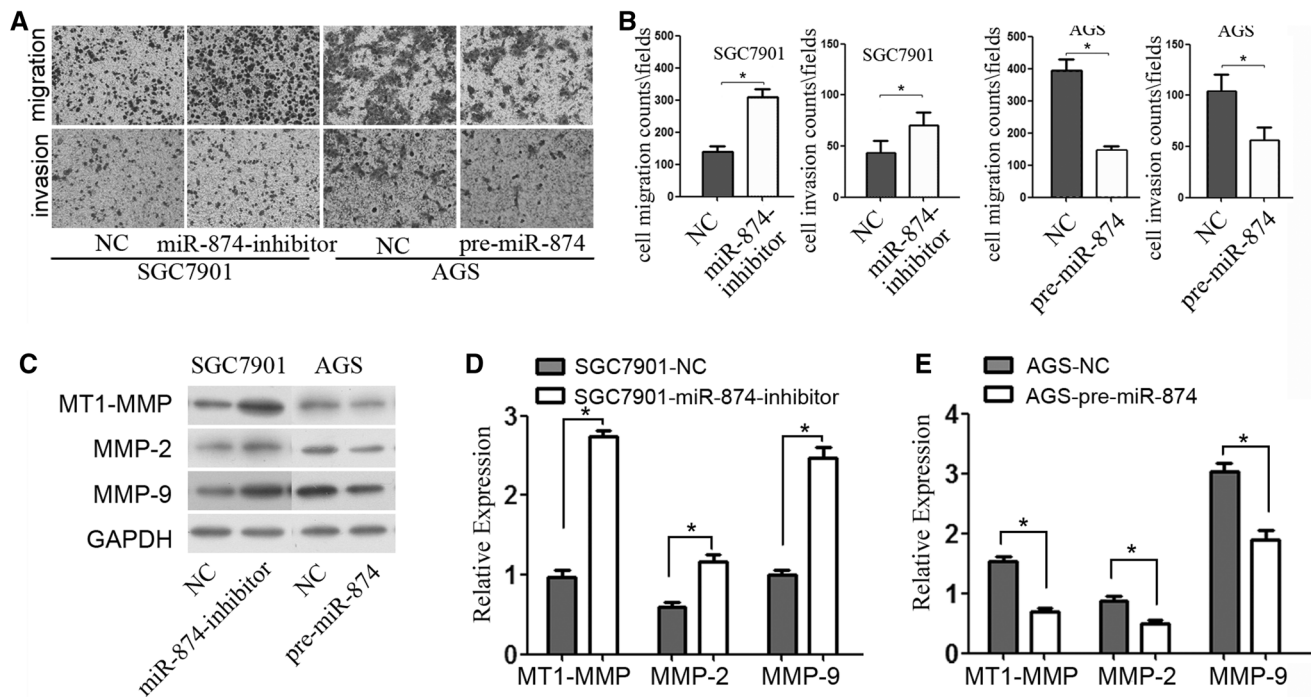
Fig. 4 miR-874 negatively regulates GC cell migration and invasion. **a**, **b** Transwell migration and Matrigel invasion assays of GC cells were performed after transfection with miR-874 inhibitor, pre-miR-874 or empty vector. Compared with the control cells, miR-874 silencing increased the migration and invasion ability of SGC7901 cells. Ectopic expression of miR-874 dramatically reduced the number of AGS cells that migrated to and invaded the lower surface of membrane than the control cells. **c–e** Western blot results showed a significant upregulation in the expression of MT1-MMP, MMP-2 and MMP-9 protein in miR-874-silenced cells compared with the control (*left lanes*). Ectopic expression of miR-874 in AGS cells suppressed MT1-MMP, MMP-2 and MMP-9 expression (*right lanes*). The experiment was performed in triplicate. *$P < 0.05$

miR-874 suppresses the tumorigenicity in vivo

To assess the effect of miR-874 on tumorigenicity in vivo, we constructed a recombinant lentiviral vector producing the miR-874 inhibitor that was used to transfect into SGC7901 cells. The transfected cells were injected into a flank of nude mice to form ectopic tumors. The cells transfected with negative lentiviral vector which was used as negative control were inoculated into the opposite flank of the same mice. Notably, miR-874 silencing significantly promoted tumorigenicity in vivo, where the tumor size was dramatically increased compared with that of the controls (Fig. 5a, $P < 0.05$). Furthermore, western blotting and immunohistochemistry results of the implanted tumors in mice certified that the AQP3 protein was significantly overexpressed in the miR-874 inhibitor transfection group compared with the controls (Fig. 5b–e, $P < 0.05$). Growth curves of the implanted tumors revealed that the tumors of the SGC7901-miR-874 inhibitor group were significantly larger than the control group after 16 days (Fig. 5f). Meanwhile, we also constructed a recombinant lentiviral vector producing the miR-874 precursor. We observed the suppressive effects on tumorigenicity of AGS cells in vivo as compared with the controls in the miR-874 precursor transfection group (Fig. 5a, g, $P < 0.05$). To further confirm that miR-874 could inhibit cell growth, the distribution of Ki67 was determined in the implanted tumors. We observed that the group of miR-874 silencing samples possessed a higher level of Ki67 positive cells compared to the negative control group (Fig. 5h, i, $P < 0.05$). By contrast, a significant downregulation of the Ki67 rate was observed in the ectopic tumors. TUNEL results also showed the number of TUNEL positive cells in the tissues of the miR-874 silencing group was significantly decreased relative to the negative control (Fig. 5h, j, $P < 0.05$). However, the miR-874-overexpressing tumors had a significantly higher apoptosis index than the control group. This revealed that miR-874 could be considered as a candidate therapeutic target against human GC.

AQP3 is involved in the regulation of cell proliferation, migration and invasion by miR-874

We demonstrated that ectopic expression of miR-874 suppressed the proliferation, migration and invasion of GC cells and inhibited AQP3 protein expression. By contrast, miR-874 knockdown promoted these phenotypes and enhanced AQP3 protein expression (Figs. 3, 4). To further illustrate
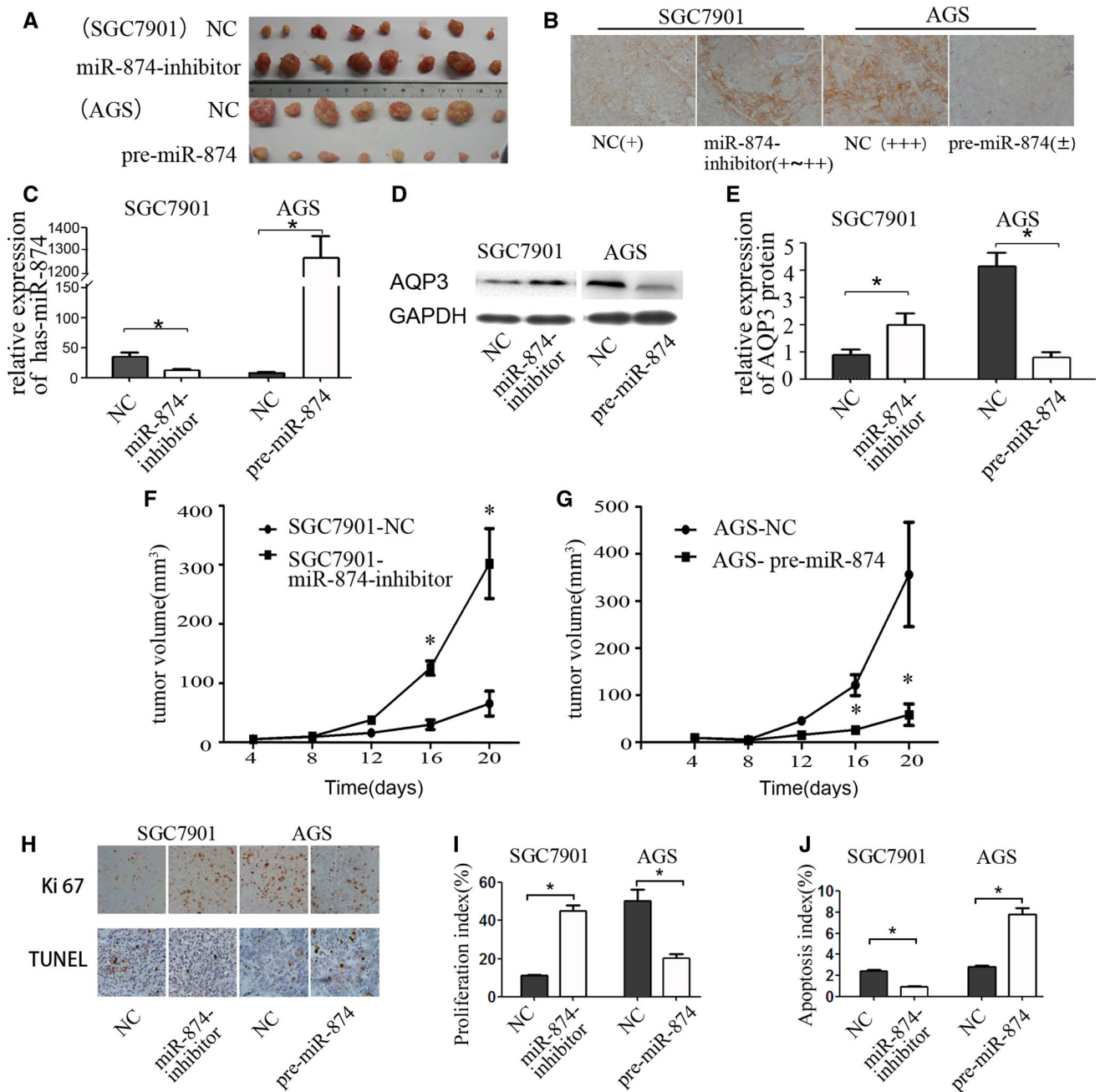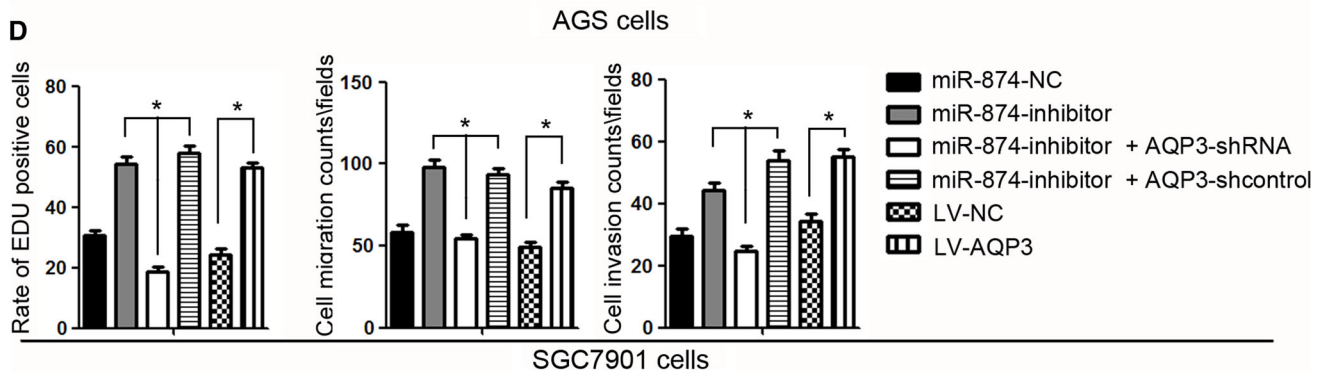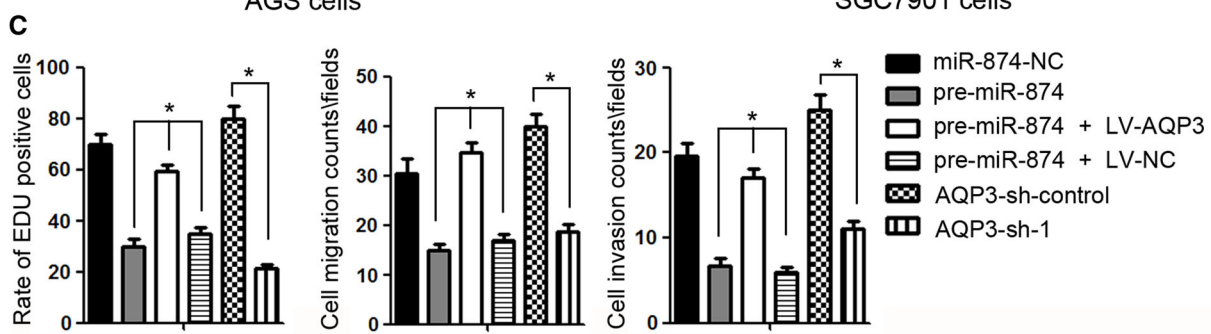
Fig. 5 miR-874 inhibits xenograft tumor growth of gastric cancer cells. **a** SGC7901 cells transfected with miR-874 inhibitor showed significantly enhanced tumor formation in the left flank of nude mice ($n = 8$). Cells infected with miR-874-NC were used as a negative control and were injected into the opposite flank of the same mice. The suppression effects were observed in AGS cells transfected with pre-miR-874. **b–e** The expression levels of miR-874 and AQP3 protein in the implanted tumors that were treated with different lentiviral vector of miR-874 were evaluated by miRNA RT–PCR, western blot and immunohistochemistry assays ($*P < 0.05$). **f, g** Tumor volume significantly increased after miR-874 inhibitor administration ($*P < 0.05$, $n = 8$), where miR-874-NC was used as control. Tumor volume remarkably decreased after pre-miR-874 administration compared to the control group ($*P < 0.05$, $n = 8$). Tumor volume was calculated as $V = (a \times b^2)/2$ where **a** and **b** are the long and short axes of the implanted tumors respectively. **h–j** The effect of miR-874 expression on cell proliferation and apoptosis in the above implanted tumors was determined by immunohistochemical assay against Ki-67 and TUNEL assay. The index of the cancer cells = positively stained cells/total cells $\times$ 100 %. $*P < 0.05$

miR-874 that affects cell growth, migration and invasion by regulating AQP3, we identified that knockdown of endogenous AQP3 inhibited the proliferation, migration and invasion of AGS cells (Supplemental Fig. S1, S2). Enhanced expression of AQP3 promoted these cell phenotypes in SGC7901 cells. Intriguingly, the inhibitory effect of *AQP3*

A

a. miR-874-NC
b. pre-miR-874
c. pre-miR-874 + LV-AQP3
d. pre-miR-874 + LV-NC
e. AQP3-sh-control
f. AQP3-sh-1

AGS cells

B

a. miR-874-NC
b. miR-874-inhibitor
c. miR-874-inhibitor + AQP3-shRNA
d. miR-874-inhibitor + AQP3-shcontrol
e. LV-NC
f. LV-AQP3

SGC7901 cells

C

AGS cells

- miR-874-NC
- pre-miR-874
- pre-miR-874 + LV-AQP3
- pre-miR-874 + LV-NC
- AQP3-sh-control
- AQP3-sh-1

D

SGC7901 cells

- miR-874-NC
- miR-874-inhibitor
- miR-874-inhibitor + AQP3-shRNA
- miR-874-inhibitor + AQP3-shcontrol
- LV-NC
- LV-AQP3

silencing on cell phenotypes was consistent with the effect of miR-874 overexpression (Fig. 6a, c). Subsequently, we investigated whether AQP3 counteracted the suppression of cell phenotypes caused by miR-874 overexpression in GC cells. The vector LV-AQP3, which contains only the *AQP3* coding sequence, was constructed for *AQP3* expression

◀ **Fig. 6** The roles of miR-874 and AQP3 in the regulation of gastric cancer cell proliferation, migration and invasion. **a** miR-874 upregulation or *AQP3*-shRNA suppressed AGS cell growth, migration and invasion. The cells transfected with the empty vector or constructed vectors were subjected to the EdU assay, cell migration and invasion assays. The rescue experiments for miR-874 overexpression were performed by ectopic expression of AQP3 without its 3′-UTR in AGS cells. **b** A similar rescue effect wherein the promotion of cell phenotypes by miR-874 silencing was counteracted by downregulation of AQP3 was also observed in SGC7901 cells. **c**, **d** The data came from at least three independent experiments. A representative data is displayed as mean ± SD. *$P < 0.05$

without miR-874 targeting. AGS cells were cotransfected with miR-874 precursor and either LV-AQP3 or LV empty vector. The data clearly confirmed that ectopic expression of AQP3 effectively reversed the suppression of cell growth, migration and invasion caused by miR-874 overexpression (Fig. 6a, c, $P < 0.05$). In the SGC7901 cells, we also observed a similar rescue effect in which the promotion of cell phenotypes by miR-874 silencing was counteracted by downregulation of AQP3 (Fig. 6b, d, $P < 0.05$). Taken together, these results are consistent with our hypothesis that miR-874 affects GC cell proliferation, migration and invasion by regulating AQP3 expression.

miR-874 is not involved in *H. pylori*-regulated AQP3 expression in human GC

Helicobacter pylori infection is reported to be the strongest known risk factor for gastric carcinogenesis [16, 17]. In this study, we also explored the role of AQP3 and miR-874 in *H. pylori* carcinogenesis in vitro. AGS or SGC7901 cells were cultured with *H. pylori*. The expression level of miR-874 in GC cells treated with *H. pylori* was evaluated by miRNA RT-PCR. The results showed that *H. pylori* infection did not affect miR-874 expression both in AGS and SGC7901 cell lines (Supplemental Fig. S3a). However immunoblotting data suggested that *H. pylori* infection increases the protein level of AQP3 in AGS and SGC7901 cells, which is consistent with Wang's report (Supplemental Fig. S3b) [12]. These results indicated that *H. pylori* infection could affect AQP3 expression but did not regulate miR-874 expression of GC cells.

Moreover, we also examined *H. pylori* infection status and analyzed its correlation with AQP3 and miR-874 expression in GC tissues. The results suggested that *H. pylori* infection is not statistically correlated with age, tumor size, histological type or other clinicopathological characteristics (Supplemental Table 1, $P > 0.05$), but it was significantly associated with AQP3 reactivity both in GC and normal tissues (Supplemental Fig. S3e, f, $P < 0.05$). By contrast, miR-874 expression was not associated with *H. pylori* infection in GC and corresponding non-cancerous tissues ($P > 0.05$; Supplemental

Fig. S3c, d). The data further suggested that AQP3, but not miR-874, is involved in *H. pylori* infection-related gastric cancer.

Discussion

Aberrant expression of miRNAs, abnormal activation of oncogenes and inactivation of tumor suppressor genes play prominent roles in the initiation and development of cancer [18]. Herein, we demonstrated that miR-874 could regulate cell growth, migration and invasion by targeting oncogene *AQP3* in GC. In our previous study, we used bioinformatics software to predict the candidate miRNAs targeting *AQP3*. Indeed, we focused our attention on miR-874, a novel miRNA of 22 nucleotides. miR-874 is located at 5q31.2 and is conserved in most of mammals [19]. Bioinformatics analysis revealed that 3′UTR of *AQP3* contains an 8-mer target site for miR-874. To confirm the direct regulation of *AQP3* by miR-874, we used luciferase reporter assays using a reporter with the 3′UTR of *AQP3* and observed reduced luciferase activity after miR-874 overexpression. In this study, our data also demonstrated that miR-874 could repress AQP3 protein expression in GC cells while the mRNA expression level of AQP3 did not change. Furthermore, compelling evidences proved that miR-874 expression was inversely correlated with AQP3 protein level in GC specimens and cell lines.

miRNAs are a class of small non-coding RNAs that are involved in posttranscriptional regulation of gene expression. They affect both the stability and translation of mRNAs by binding to partly complementary sequence motifs in the 3′UTR of target mRNAs. Recently, large numbers of miRNAs aberrant expression, biological functions and related carcinogenesis pathways in various cancers suggests that numerous miRNAs exert tumor suppressive or oncogenic roles by regulating their target genes. Thus, finding novel microRNAs and potential target genes is a research hotspot.

Currently the expression pattern and functions of miR-874 in cancer are inadequately reported. Until recently, miR-874 was only observed to be frequently downregulated in maxillary sinus squamous cell carcinoma [20]. However, Nadine Ratert [21] reported that there were no significant differences between non-cancerous and cancerous specimens in urothelial carcinoma. miR-874 may also act as a tumor suppressor and affect cell biological behavior by regulating PPP1CA directly in maxillary sinus squamous cell carcinoma [20]. Here, we revealed that miR-874 was highly and widely downregulated in 72 % of GC tissues compared with their nontumorous counterparts. Importantly, an additional characteristic of miR-874 expression was observed in GC. Low miR-874 expression

was closely associated with GC clinicopathological features, such as histological type, lymph node metastasis and TNM stage.

Moreover, miR-874 depletion promoted GC cell proliferation, colony formation in vitro and tumorigenicity in vivo. By contrast, enhanced expression of miR-874 suppressed cell growth, colony formation and tumor formation of GC cells. Further study revealed that miR-874 could promote cell apoptosis by upregulating expression of Bax, activating caspase-3 and inhibiting Bcl-2 expression. Additionally, we verified that miR-874 is involved in the negative regulation of GC cell migration and invasion by mediating MT1-MMP, MMP-2 and MMP-9 expression in vitro. These results strongly suggested that miR-874 boosts the development and progression of GC. More intriguingly, ectopic expression of AQP3 effectively reversed the suppression of cell proliferation, migration and invasion caused by miR-874 overexpression, suggesting that the inhibitory effect of miR-874 upregulation on cell phenotypes may be dependent upon AQP3.

The AQP family members are key regulators of a variety of human diseases, such as diarrhea, motion sickness, brain edema and cancer [22–24]. AQP3, as a member of the AQP family, is reported to be closely associated with tumors. AQP3 overexpression has been observed in many types of cancers, including glioma [25], lung adenocarcinoma [26], renal tumor [27] and colorectal cancer [28]. In our previous studies, we reported that higher AQP3 expression could be detected in GC tissues than that in normal mucosa, and AQP3 expression was associated with pathological differentiation and lymph node metastasis in GC patients [10]. We also demonstrated that AQP3 promoted GC cell proliferation and migration, suggesting increased risk of cancer invasion and metastasis is associated with AQP3 overexpression [6]. However, the mechanism by which AQP3 is upregulated in human gastric cancer had not been clearly defined. Our previous studies confirmed that hepatocyte growth factor receptor (c-Met) upregulated AQP3 expression in human gastric carcinoma cells via the ERK signaling pathway [29]. Ji et al. [7, 30] found that EGF could induce ovarian cancer and skin fibroblast cell migration by upregulation of AQP3 expression. Notably, Sepramaniam et al. [31, 32] demonstrated that miRNA-320a and miRNA-130a could function as important modulators of AQP1 and AQP4 in cerebral ischemia and infarct. However, the regulation of AQPs by miRNAs through 3′UTR in cancer remains unreported to date. Our study is the first report to show that miR-874 inhibits proliferation, migration and invasion in GC cells through a possible mechanism of negative regulation of AQP3 expression. Recent evidence suggested that AQP3 is involved in Helicobacter pylori-related gastric tumors [12]. Here, the role of miR-874 and AQP3 in *H. pylori*-induced

carcinogenesis was investigated. We found that AQP3, but not miR-874, expression was affected by *H. pylori* infection in GC cells and was closely related with *H. pylori* infection in gastric tumor tissues and normal tissues. The signaling mechanism of *H. pylori*-induced AQP3 upregulation requires further investigation.

In summary, our study demonstrated that miR-874 has a suppressive role in cell growth, migration and invasion of GC cells. More importantly, the mechanism of miR-874-mediated inhibition of GC cell biological behavior is related to the direct regulation of its target gene *AQP3*. The feature of miR-874 as a tumor suppressor indicates it to be a novel therapeutic target for cancer therapy. It will be valuable to explore whether miR-874 could act as a therapeutic agent for GC patients.

Conflict of interest The authors declare no conflict of interest.

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