**miR-874 inhibits gastric cancer cell proliferation by targeting SPAG9**

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**Abstract**

**Background:** microRNAs (miRNAs) play essential roles in the development and progression of gastric cancer (GC). Although aberrant miR-874 expression has been reported in various human cancers, its role in GC remains obscure.

**Methods:** miR-874 expression was assessed by real-time quantitative polymerase chain reaction (RT-qPCR) in 62 matched GC and adjacent normal tissues, as well as in GC cell lines and immortalized human gastric epithelial cells. CCK8 assay, colony formation assay, and flow cytometry were used to assess the role of miR-874 in GC cell proliferation and apoptosis in vitro. Additionally, to determine the effects of miR-874 on GC cell proliferation and apoptosis in vivo, BALB/c nude mice were injected with GC cells transfected with a miR-874 mimic. The role of miR-874 in SPAG9 expression was assessed by luciferase assay, Western blotting, and RT-qPCR.

**Results:** miR-874 was downregulated in GC cell lines and tissues. miR-874 overexpression in GC cells led to inhibition of cell proliferation and induction of apoptosis. Moreover, SPAG9 was identified as a direct miR-874 target, the expression of which was suppressed by miR-874. SPAG9 overexpression markedly promoted GC cell proliferation.

**Conclusions:** miR-874 inhibited cell proliferation and induced apoptosis in GC cells. SPAG9 downregulation was crucial for the tumor-suppressive effects of miR-874. Hence, the miR-874/SPAG9 axis could serve as a novel therapeutic target in GC.

**Keywords:** Gastric cancer, miR-874, SPAG9, Proliferation, Apoptosis

**Background**

Gastric cancer (GC) is a common malignancy and important cause of mortality and morbidity, both in China and worldwide [1]. The 5-year survival rate of patients with GC after radical surgery ranges from 30 to 50%; the high malignancy and heterogeneity of GC, as well as its poor differentiation, are primary causes of poor prognosis [2]. Despite advances in surgical interventions, chemotherapy, targeted therapies, and immunotherapy, the overall prognosis of GC remains poor [3]. Therefore, elucidation of molecular mechanisms underlying GC cell proliferation, survival, and metastasis are imperative for developing novel therapeutic interventions GC. Additionally, identification of robust prognostic biomarkers and GC classification based on molecular profiles would enable personalized treatment of GC.

MicroRNAs (miRNAs) are 21–25-nucleotide, single-stranded, non-coding RNAs. miRNAs specifically bind to the 3′ untranslated region (3′-UTR) of target genes, which facilitates mRNA degradation or translation suppression. It has become evident that miRNAs play crucial roles in various biological processes, such that they regulate the expression of approximately 30% of all mRNAs expressed in a cell. Additionally, numerous miRNAs have been implicated in various human cancers, exerting either tumor suppressor or oncogenic functions [4]. miR-105 [5], miR-664a-3p [6], miR-451a [7], and miR-18b [8] have recently been identified.
as oncogenes in GC; moreover, miR-223-3p [9], miR-99b-3p [10], and miR-1297 [11] have been shown to suppress GC development and progression. miR-874 is a newly identified miRNA, which plays key roles in various malignancies, including nasopharyngeal carcinoma [12], non-small cell lung cancer [13], colorectal cancer [14], and hepatocellular carcinoma [15]. However, the role of miRNA-874 in GC remains unclear.

The oncogene sperm-associated antigen 9 (SPAG9) is a member of the cancer/testis antigen family; its expression is regulated by various miRNAs, including miR-524 [16] and miR-200a-3p [17]. In this study, we investigated the relevance of miR-874 in GC development and progression, by assessing the effects of miR-874 on GC cell proliferation, as well as the relationship between miR-874 and SPAG9.

Methods

Patients and ethics
Sixty two patients (35–72 years old) including 49 males and 13 females with surgical tumor specimens and adjacent non-tumor tissues were collected from Shandong Provincial Hospital Affiliated to Shandong First Medical University (Jinan, Shandong) after receiving written informed consent since July 2017 to May 2018. The histological diagnosis was made on sections stained with hematoxylin and eosin, according to the World Health Organization (WHO) classification guidelines. None of our study patients had received preoperative chemotherapy or radiotherapy. The study was approved by the Human Ethics Committee of Shandong Provincial Hospital Affiliated to Shandong First Medical University.

Cell culture and transfection
Human gastric mucosal epithelial cells (GES-1), human GC cell lines (MKN-74, BGC-823, MGC-803, MKN-45) in this study were purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China) and used in our experiments. Cells were cultured into T25 flasks in RPMI-1640 medium supplemented with 10% FBS, and grown in a humidified chamber supplemented with at 37 °C with 5% CO_2. The miR-874 mimic and inhibitor, SPAG9 siRNA (si-SPAG9) and SPAG9 were obtained from Santa Cruz. Transfection was achieved using the LipofectamineTM 3000 kit (Invitrogen) according to the manufacturer’s instructions.

Real-time quantitative polymerase chain reaction (RT-qPCR)
Total RNA was extracted from tissues or cultured cells with TRIzol reagent (Thermo Fisher Scientific) according to the protocol [18]. RT-qPCR was performed using SYBR Green PCR master mix (Applied Biosystems) in a total volume of 20 μl on 7900HT Fast Real-Time PCR System (Applied Biosystems) as follows: 95 °C for 30 s, 40 cycles of 95 °C for 5 s, and 60 °C for 30 s. A dissociation step was performed to generate a melting curve to confirm the specificity of the amplification. The U6 small nuclear RNA and GAPDH mRNA were used to normalize the expression for miR-874 and SPAG9 mRNA, respectively. The following primers were as follows: SPAG9 forward primer: 5′-CAA GGC GGA TCT AAA GCT ACC -3′, reverse primer: 5′-TTG GCC CAT CTC TAA CCT TCA-3′; GAPDH forward primer: 5′- CTG GCC TAC ACT GAG CAC C-3′, reverse primer: 5′- AAG TGG TCG TTG AGG GCA ATG-3′, L6 small nuclear RNA forward primer: 5′-CTC GTG TCG GCA GCA CA-3′, reverse primer: 5′-AAC GCT TCA CGA ATT TGC GT-3′; miR-874 forward primer: 5′-CAC GCA CCA GGG TAA GAG AG-3′, reverse primer: 5′-CCA GCC AGT CCG TCC CT-3′.

Luciferase activity assay
According to TargetScan (http://www.targetscan.org) and MiRanda (http://www.microrna.org/microrna/home.do) databases, the wild-type SPAG9 3′UTR (SPAG9-Wt) or the mutant SPAG9 3′UTR (SPAG9-Mut) was constructed into the pGL3 luciferase reporter vector. The above luciferase reporter plasmid was co-transfected with miR-874 mimic or NC mimic into BGC-823 cells, and the pRL-TK luciferase reporter vector was used as negative control. Luciferase assay was performed the firefly luciferase 48 h post-transfection and measured using the Dual-Luciferase® Reporter Assay System (E1910, Promega Corporation, Madison, WI, USA).

Cell proliferation assay
CCK8 assay in this study was performed to determine the cell proliferation. In brief, cells were added into 96-well plates and cultured for another 48 h. Then 20 μl 5 mg ml·1 CCK-8 solution was added for another 4-h culture. With the supernatant removal, 150 μl dimethyl sulfoxide was added into each well of the plate in a shaking table at low speed at room temperature for 10 min. The optical density (OD) at 450 nm was measured.

Colony formation assay
The capacity of cell proliferation was further detected using the colony formation assays. In brief, after cultured for 14 days, transfected BGC-823 cells (~ 3 × 10^5/6-well plate) were fixed with formaldehyde (4%) and then stained with 0.5% crystal violet solution. Lastly, a light microscope was used to count the number of colonies (> 50 cells).

Cell apoptosis analysis
BGC-823 cells were collected, washed twice with cold 1 x PBS. Then the cells were binding buffer to a concentration of 1–5 × 10^6/ml. Next, 100 μl of cell suspension was added into a 5 ml tube followed by adding 5 μl of Annexin V/FITC and 5 μl of Propidium Iodide (PI) into
the tube. After being incubated for 15 min in the dark, 400 μl of 1 × Annexin V binding buffer were added into the tube. The cells were analyzed by flow cytometry (BD, USA).

**Western blot analysis**

The total protein content was isolated from collected cell samples using radioimmuno-precipitation assay (RIPA) buffer (Thermo Fisher Scientific, Waltham, MA) on ice for 30 min and centrifuged at 10000 g for 30 min. Subsequently, the protein concentration in each sample was measured by utilizing a BCA assay (Thermo Fisher Scientific, Waltham, MA), the pallets were discarded and supernatants were mixed with the loading buffer for electrophoresis. Proteins were separated by electrophoresis on 10% SDS-polyacrylamide gels, before being transferred to polyvinylidene difluoride membranes (MerckMillipore, Billerica, MA, USA). Blots were blocked with a 5% skim milk solution and incubated overnight with an antibody against SPAG9 (1:1000; Abcam, UK) or GAPDH (1:5000, Santa Cruz Biotechnology, USA). Membranes were then exposed to the corresponding horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature. Bio-Rad Chemidoc XRS chemiluminescence imaging system was performed to detect the bands, and the luminescence images were acquired for quantitative analysis.

**In vivo tumor growth model**

All mouse experiments were approved by the Ethics Committee of the Shandong Provincial Hospital Affiliated to Shandong First Medical University. BALB/c nude mice (female, 4–6 weeks) were randomly divided into NC group, miR-874 mimic group and miR-874 inhibitor group (n = 6 per group). The cells of each group were collected and made into single-cell suspension with a concentration of 5 × 10^6 cells/mL. The mice were sacrificed by cervical dislocation at the end of 4 weeks, and all the solid tumors were stained with TUNEL staining to observe the apoptosis of the tumor.

**Statistical analysis**

All data were presented as mean ± standard deviation (SD) and analyzed using a professional SPSS software 20.0 (SPSS Inc., Chicago, UL, USA). Differences between two groups were analyzed using student’s t-test and among three or more groups were analyzed by one-way ANOVA. P < 0.05 was considered statistically significant.

**Results**

miR-874 is downregulated in GC

miR-874 expression levels in 62 matched GC and tumor-adjacent normal tissues were measured with RT-qPCR. Notably, miR-874 was expressed at substantially lower levels in GC tissues, compared to paired normal tissues (Fig. 1a). Similarly, miR-874 was expressed at considerably lower levels in GC cell lines MKN-74, BGC-823, MGC-803, and MKN-45, compared to GES-1 cells (Fig. 1b); BGC-823 cells exhibited the lowest miR-874 expression.
miR-874 inhibits proliferation and promotes apoptosis in vitro in GC cells

Various GC cell lines were transfected with a miR-874 mimic, and miR-874 overexpression was confirmed by RT-qPCR (Fig. 2a). Subsequently, the proliferation rate of GC cells was measured using the CCK8 method and colony formation assays. Both assays revealed that miR-874 overexpression dramatically suppressed cell proliferation in all three GC cell lines (Fig. 2b, c). The role of miR-874 in cell apoptosis was also evaluated by flow cytometry; the cell apoptosis was measured in cells containing miR-874 mimics or inhibitor via flow cytometer (d). * P < 0.05; ** P < 0.01. GC, gastric cancer; RT-qPCR, reverse transcription-quantitative PCR.
miR-874 mimic significantly increased GC cell apoptosis, compared with the control group (Fig. 2d).

**miR-874 inhibits proliferation and promotes apoptosis in vivo in GC cells**

To further confirm the tumor-suppressive effect of miR-874, BALB/c GC xenograft models were employed. Notably, miR-874-overexpressing tumors were significantly smaller at 4 weeks after cancer cell implantation, compared to control tumors (Fig. 3). Conversely, miR-874 inhibition accelerated tumor growth in our GC mouse model. Furthermore, TUNEL assay analysis revealed enhanced cancer cell apoptosis in miR-874-overexpressing tumors.

**miR-874 directly targets SPAG9 in GC cells**

Using miRNA seed sequence targeting prediction analysis, a potential miR-874 binding site was identified in the 3'UTR of SPAG9 (Fig. 4a). Subsequent use of a dual-luciferase reporter assay system showed that luciferase activity was significantly reduced in pGL3-PIK3CA-SPAG9-expressing cells after transfection with the miR-874 mimic, compared with pGL3-PIK3CA-NC and pGL3-PIK3CA-SPAG9-Mut cells (P < 0.01); this finding confirmed that miR-874 binds to the 3'UTR of SPAG9, thereby suppressing its expression (Fig. 4b). RT-qPCR and Western blotting were also performed to confirm the effects of miR-874 on SPAG9 expression at the mRNA and protein levels, respectively. Notably, transfection with the miR-874 mimic significantly reduced SPAG9 protein levels (P < 0.01). Conversely, miR-874 inhibition increased SPAG9 protein levels (P < 0.001) (Fig. 4c). Consistent with these findings, RT-qPCR analysis showed that the mRNA levels of SPAG9 were significantly reduced after transfection with the miR-874 mimic (Fig. 4d). Overall, these results suggest that miR-874 directly binds SPAG9, thus suppressing its expression.

**miR-874 regulates the progression of GC by modifying SPAG9 expression**

BGC-823 cells were transfected with SPAG9, miR-874, or a combination of these (Fig. 5a). Importantly,
SPAG9 overexpression significantly promoted cell proliferation (Fig. 5b, c), whereas it inhibited cell apoptosis (Fig. 5d). Transfection with the miR-874 mimic reversed the effects of SPAG9 overexpression on GC cell proliferation and apoptosis.

**Discussion**

GC incidence and mortality remain high [19]. With recent advances in the fields of molecular and cell biology, the understanding of molecular mechanisms underlying cancer has advanced considerably. Additionally, many genes have been shown to regulate cancer cell proliferation [20]. Notably, gene therapy has emerged as a promising therapeutic approach for cancer, as well as for other human diseases. Several miRNAs have been shown to regulate cell differentiation, proliferation, and survival, through binding interactions with complementary target mRNAs [21]. miR-874 has recently been identified as a tumor-suppressor and is often downregulated in certain types of cancer, including GC [22]. In this study, we confirmed that miR-874 expression was reduced in GC tissues and cells. We also demonstrated that miR-874 overexpression suppressed GC cell proliferation and promoted apoptosis.

Furthermore, we investigated the mechanism underlying the tumor-suppressive effects of miR-874 in GC. Aberrant SPAG9 expression has been reported in several malignancies, including renal, breast, thyroid, and cervical cancer. However, the relevance of SPAG9 in human GC remains elusive. In the present study, we identified SPAG9 as a miR-874 target. We also demonstrated that SPAG9 overexpression enhanced cell proliferation and inhibited cell apoptosis in GC cells. Consistent with these findings, a recent study showed that SPAG9 overexpression promoted proliferation in human prostate cancer cells [23]. Furthermore, SPAG9 has been shown to regulate HEF1 expression, promoting epithelial to mesenchymal transition in urothelial carcinoma in a Rac1 pathway-dependent manner [24]. In prostate cancer, SPAG9 promotes cell survival, angiogenesis, and tumor metastasis by activating the MAPK signaling pathway [25].

**Conclusions**

In conclusion, we demonstrated that miR-874 inhibited cell proliferation and induced apoptosis in GC
cells. We also identified the SPAG9 oncogene as a target of miR-874 and showed that SPAG9 downregulation was crucial for the tumor-suppressive effects of miR-874. Therefore, the miR-874/SPAG9 axis could serve as a novel therapeutic approach for GC.

Supplementary information
Supplementary information accompanies this paper at https://doi.org/10.1186/s12885-020-06994-z.

Additional file 1. Original data of western blot (SPAG9 and GAPDH) in Fig. 4, the cropping of the blot by figure processing software was clearly mentioned with red rectangle.

Fig. 5 miR-874 regulated the progression of GC through affecting SPAG9 expression. The mRNA expression of SPAG9 was measured in cells containing SPAG9 plasmid with or without miR-874 (a). The cell proliferation in cells containing SPAG9 plasmid with or without miR-874 via CCK8 (b) and colony formation assay (c). The cell apoptosis was measured in cells containing SPAG9 plasmid with or without miR-874 via flow cytometer (d). *P < 0.05, **P < 0.01. GC, gastric cancer

Acknowledgements
Not applicable.

Authors' contributions
Q.H.S., S.K. and C.N.Z. conceived the study; S.K., Z.X.Y., Z.L., S.B.T., H.C.W., F.X.Z. and L.P.L. performed experiments; Q.H.S., C.N.Z. and S.K. contributed patients' samples; S.K. and C.N.Z. wrote the manuscript. All authors have read and approved the manuscript.

Funding
Not applicable.

Availability of data and materials
All data generated or analysed during this study are included in this published article and its supplementary information files.

Ethics approval and consent to participate
The present study was approved by the Ethics Committee of Shandong Provincial Hospital Affiliated to Shandong First Medical University. Written informed consent was obtained from all enrolled subjects.

Consent for publication
Not applicable.

Abbreviations
miRNAs: microRNAs; GC: Gastric cancer; SPAG9: Sperm associated antigen 9; CT: Cancer testis; RIPA: Radioimmuno-precipitation assay; ANOVA: Analysis of variance; 3’UTR: 3’-Untranslated region
Competing interests
There are no conflicts of interest to declare.

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Received: 13 February 2020 Accepted: 22 May 2020
Published online: 05 June 2020

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