microRNA-23a promotes cell growth and metastasis in gastric cancer via targeting SPRY2-mediated ERK signaling

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Abstract. microRNAs (miRs) serve important roles in various human cancer types. Recently, miR-23a has been indicated as an oncogene in gastric cancer, but the underlying mechanism remains unclear. In the present study, reverse transcription-quantitative polymerase chain reaction and western blot analysis was used to explore the effects of miR-23a in gastric cancer. Additionally, cell proliferation, migration and invasion were examined using an MTT assay, wound healing assay and Transwell assay, respectively. Furthermore, a luciferase reporter gene assay was used to confirm the target association. It was determined that miR-23a was significantly upregulated in gastric cancer tissues and cell lines compared with adjacent tissues, and a normal gastric epithelial cell line. Furthermore, its upregulation was significantly associated with cancer progression and poor prognosis of patients. Knockdown of miR-23a caused a notable reduction in the proliferation, migration and invasion of gastric cancer AGS cells. Sprouty homolog 2 (SPRY2) was then predicted to be target gene of miR-23a. A luciferase reporter gene assay data demonstrated that miR-23a has the ability to directly bind to the 3'-untranslational region of SPRY2 mRNA. Further investigation demonstrated that SPRY2 was significantly downregulated in gastric cancer tissues and cell lines, and the protein expression of SPRY2 was negatively regulated by miR-23a in AGS cells. Furthermore, knockdown of SPRY2 reduced the suppressive effects of miR-23a inhibition in AGS cell proliferation, migration and invasion. In addition, the activity of extracellular signal-regulated kinase (ERK) signaling was also inhibited by the miR-23a/SPRY2 knockdown in AGS cells. The present study indicated that miR-23a serves a promoting role in gastric cancer via targeting SPRY2 and downstream ERK signaling.

Introduction

Gastric cancer is one of the most common human cancer types (1,2). Due to the high rate of recurrence and metastasis, patients with gastric cancer have demonstrated a poor 5-year survival time (1,2). Although there have been great improvements in cancer diagnosis and treatment in recent years, gastric cancer still remains among the top five for cancer-associated mortality globally (1-3). Revealing the molecular mechanism underlying gastric cancer development and progression may assist in developing more effective therapeutic strategies against gastric cancer.

miRNAs (miRs) are a type of small non-coding RNAs containing 22-25 nucleotides, and act as key regulators for gene expression via directly binding the mRNA 3'-untranslated region (UTR) of their target genes, which leads to translation inhibition or mRNA degradation (4,5). Through these functions, miRs participate in the regulation of various biological processes, including cell development and differentiation, angiogenesis, cell survival, proliferation, cell cycle progression, migration, invasion and tumorigenesis (6,7). In recent years, numerous miR-targeting oncogenes and tumor suppressors have been identified in numerous human cancer types (8-10). Through inhibiting the expression of these oncogenes and/or tumor suppressors, these miRs serve suppressive or promoting roles in tumor growth, and metastasis (11,12).

miR-23a has been demonstrated to serve different roles in various human cancer types (13,14). It functions as a tumor suppressor in osteosarcoma, and the ectopic expression of miR-23a led to reduced proliferation, migration and invasion of osteosarcoma cells (14). Additionally, miR-23a inhibits epithelial-mesenchymal transition (EMT) in endometrial endometrioid adenocarcinoma by targeting mothers against decapentaplegic homolog 3 (13). On the contrary, miR-23a was identified as an oncogene in gastric cancer (15,16), promoting the growth of gastric adenocarcinoma cell line MGC803 and downregulating the interleukin-6 receptor (15). miR-23a inhibits PPP2R5E expression, which facilitates the growth of gastric cancer cells (16); however, the exact regulatory mechanism underlying miR-23a in gastric cancer remains unclear.

Sprouty homolog 2 (SPRY2) is a member of the sprouty family and inhibits the activity of receptor tyrosine kinase signaling, which is required for the growth factor-stimulated
translocation of the protein to membrane ruffles (17,18). Previous studies have indicated that SPRY2 serves a suppressive role in different human cancer types (19-21); however, the regulatory mechanism underlying SPRY2 expression in gastric cancer remains unknown.

In the present study, the aim was to explore the molecular mechanism underlying miR-23a gastric cancer growth and metastasis.

Materials and methods

Tissue collection. The present study was approved by the Ethical Committee of The Third Xiangya Hospital (Changsha, China). A total of 78 gastric cancer tissues, as well as the adjacent non-tumor tissues were obtained from The Third Xiangya Hospital between January 2010 and March 2011. These patients with gastric cancer included 45 male and 33 female aged between 43-83 years, with a mean of 67.4 years. Written informed consent was obtained from all patients. All tissue samples were confirmed using histopathological evaluation and stored at -80°C until further use. The clinical information for patients with gastric cancer is summarized in Table I.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted using the TRIzol® reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA), which was then converted to cDNA using a Tagman® miRNA Reverse Transcription kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The expression of miR was determined using a Hairpin-it miRNAs qPCR Quantitation kit (Shanghai GenePharma Co., Ltd., Shanghai, China). U6 was used as the internal reference. The expression of mRNA was examined using a SYBR® Green qPCR Assay kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. GAPDH was used as the internal reference for miRNA. The thermocycling conditions were initial denaturation at 95°C for 10 min, then 40 cycles at 95°C for 15 sec and annealing/elongation at 60°C for 15 sec. The relative expression was analyzed by the 2-ΔΔCq method (22). The primers for miR-23a and U6, which was used as the internal reference for microRNAs, were purchased from Fulgene (Guangzhou, China; cat nos. HmiRQP0345 and HmiRQP9001, respectively). The primer sequences were as follows: miR-23a forward, 5'-CCTACTCTGCTGCTTCAAGAGACAT-3' and reverse, 5'-GGGGCTCGTGAGAGAAAGTTTGCCTC-3'; U6 forward, 5'-ACACCTTTGATGTTGAAAGG-3' and reverse, 5'-GGTACGCACGCAAGTTTGC-3'. GAPDH forward, 5'-GAGACCAGATCCCTCCCAAAAT-3' and reverse, 5'-GGCTTGTTCATACCTTCTCATGG-3'.

Cell culture and transfection. Human gastric mucosa epithelial line GES-1, and gastric cancer lines HGC-27, BGC-823, SGC-7901 and AGS were obtained from the Cell Bank of Central South University (Changsha, China). The cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS; both from Thermo Fisher Scientific, Inc.) at 37°C in a humidified incubator containing 5% CO2. Cell transfection was conducted using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. AGS cells were transfected with 100 nM of scrambled miR mimic (miR-NC; Guangzhou Fulengen Co., Ltd., Guangzhou, China), miR-23a mimic (miR-23a; Guangzhou Fulengen Co., Ltd.), miR-23a inhibitor (Fulangen), negative control inhibitor (NC inhibitor; Guangzhou Fulengen Co., Ltd.), co-transfected with miR-23a inhibitor and non-specific siRNA (miR-23a in+siNC; OriGene Technologies, Inc., Beijing, China) or co-transfected with miR-23a inhibitor and SPRY2-specific siRNA (miR-23a in+siSPRY2; OriGene Technologies, Inc.). Following transfection for 48 h, the following experiments were performed.

Western blot analysis. Cells were lysed in RIPA buffer (Thermo Fisher Scientific, Inc.), and the protein was extracted and quantified using a BCA Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.). Proteins (50 µg) were separated with 10% SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane (Thermo Fisher Scientific, Inc.). The membrane was incubated with TBS with Tween 20 containing 5% non-fat milk at room temperature for 3 h, and then with rabbit anti-mouse SPRY2 (dilution, 1:50; cat no. ab58670), total-ERK (dilution, 1:100; cat no. ab180570), phospho-ERK (dilution, 1:100; cat no. ab29272; Abcam) at room temperature for 1 h. Chemiluminescence detection was performed using a Pierce™ Fast Western Blot kit (Pierce; Thermo Fisher Scientific, Inc.). The relative protein expression was analyzed using Image-Pro Plus software version 6.0 (Media Cybernetics, Inc., Rockville, MD, USA), represented as the density ratio vs. GAPDH.

MTT assay. AGS cells in 100 µl DMEM containing 0.5 g/l MTT (Thermo Fisher Scientific, Inc.) were seeded in a 96-well plate at a density of 1x104 cells/well. Following incubation at 37°C for 0, 24, 48 or 72 h, 50 µl DMSO (Thermo Fisher Scientific, Inc.) was added. Following incubation at 37°C for 10 min, the wavelength of 570 nm for each sample was measured using the Tecan Infinite® M200 plate reader (Tecan Group, Ltd., Mannedorf, Switzerland).

Wound healing assay. A wound healing assay was conducted to determine the cell migratory capacity. AGS cells were cultured to full confluence, and wounds of ~1 mm width were created with a plastic scribe. Then, cells were washed once using PBS. Prior to migration, AGS cells were photographed under an inverted microscope (magnification, x40; Olympus Corporation, Tokyo, Japan) as the negative control. Following being cultured at 37°C with 5% CO2 for 48 h, AGS cells were observed under an inverted microscope (magnification, x40; Olympus Corporation). This experiment was repeated for 3 times.

Transwell assay. A Cell Invasion Assay kit (Chemicon International, Inc., Temecula, CA, USA), with an adhesion matrix supplied in the kit, was used to perform the Transwell assay, according to the manufacturer's guidelines. An AGS cell suspension was prepared in 300 µl DMEM containing
Table I. Association between miR-23a expression and clinicopathological characteristics of patients with gastric cancer.

<table>
<thead>
<tr>
<th>Variables</th>
<th>miR-23a expression</th>
<th></th>
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<th>P-value</th>
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<tbody>
<tr>
<td></td>
<td>High (n=41)</td>
<td>Low (n=37)</td>
<td></td>
<td></td>
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<td>Age, years</td>
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<tr>
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<td></td>
<td></td>
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<tr>
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<td>15</td>
<td>18</td>
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<tr>
<td>T stage</td>
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<td></td>
<td></td>
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<tr>
<td>T1-2</td>
<td>14</td>
<td>18</td>
<td></td>
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</tr>
<tr>
<td>T3-4</td>
<td>27</td>
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<td></td>
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<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Present</td>
<td>29</td>
<td>12</td>
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<tr>
<td>Absent</td>
<td>12</td>
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<tr>
<td>Distant metastasis</td>
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<tr>
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<td>0.006*</td>
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*P<0.001; T, tumor; miR, microRNA.

50,000 AGS cells and was seeded in the upper chamber. Then, 500 µl DMEM with 10% FBS was added into the lower chamber. Following incubation at 37°C for 24 h, the non-invasive AGS cells on the upper face of the membrane were scraped, while the invading AGS cells on the lower face of the membrane were stained with 0.5% crystal violet at room temperature for 20 min and counted under an inverted microscope (magnification, x200).

Bioinformatics analysis. The putative target genes of miR-23a were predicted using Targetscan software (www.targetscan.org), in accordance with the manufacturer's protocol.

Luciferase reporter gene assay. The mutant type (MT) 3'-UTR of SPRY2A was constructed using a Quik-Change Site-Directed Mutagenesis kit (Agilent Technologies, Inc., Santa Clara, CA, USA), according to the manufacturer's protocol. The wild type (WT) or MT 3'-UTR of SPRY2 were then inserted into the p51-CHECK2 vector (Promega Corporation, Madison, WI, USA). For the luciferase reporter gene assay, AGS cells were transfected using Lipofectamine® 2000 (Invitrogen, Thermo Fisher Scientific, Inc.) with 100 nM of WT SPRY2-3'-UTR or MT SPRY2-3'-UTR plasmid, with or without 100 nM of miR-23a mimics, respectively. The luciferase activity was then measured at 48 h following transfection using the Dual-Luciferase Reporter assay system (Promega Corporation) on the Lmax Multiwell luminometer (Molecular Devices, LLC, Sunnyvale, CA, USA). Renilla luciferase activity was normalized to firefly luciferase activity.

Statistical analysis. The data are expressed as the mean ± standard deviation following at least three independent experiments. Statistical analysis of differences was performed via one-way analysis of variance and Turkey's post-hoc test for multiple groups or Student's t-test for two groups, using the SPSS version 20.0 software package (IBM Corp., Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference. A χ2 test was performed for Table I, with a log-rank test conducted on the Kaplan-Meier survival curve.

Results

Upregulation of miR-23a is associated with gastric cancer progression and poor prognosis of patients. In the present study, RT-qPCR was conducted first to examine the expression levels of miR-23a in gastric cancer. The data indicated that miR-23a was significantly upregulated in gastric cancer tissues compared with adjacent non-tumor tissues (Fig. 1A). Further investigation revealed that the increased expression of miR-23a was significantly associated with node metastasis and advanced clinical stage (Table I). The results indicated that miR-23a may contribute to gastric cancer growth and metastasis. Furthermore, the patients with gastric cancer with high miR-23a expression demonstrated significantly reduced survival time, compared with those with low miR-23a levels (Fig. 1B); therefore, miR-23a may be used as a potential predictor for the prognosis of patients with gastric cancer.

Knockdown of miR-23a decreases AGS cell proliferation, migration and invasion. The miR-23a levels in gastric cancer cell lines, including HGC-27, BGC-823, SGC-7901 and AGS cells, were further examined. The data indicated that miR-23a was also significantly upregulated in gastric cancer cell lines compared with normal gastric epithelial GES-1 cells (Fig. 2A). AGS cells were used to conduct in vitro experiments. As miR-23a was demonstrated to be significantly upregulated in gastric cancer, AGS cells were transfected with a miR-23a inhibitor to knockdown its expression. Following transfection, the miR-23a levels were significantly reduced in the miR-23a inhibitor group compared with the NC inhibitor group (Fig. 2B). The MTT assay data further demonstrated that knockdown of miR-23a caused a significant decrease in AGS cell proliferation after 48 h (Fig. 2C). The results of a wound healing assay and Transwell assay demonstrated that inhibition of miR-23a significantly suppressed AGS cell migration and invasion (Fig. 2D and E). According to this data, it was indicated that miR-23a serves a promoter role in gastric cancer growth and metastasis, and may be used as a potential therapeutic target.

SPRY2, which is downregulated in gastric cancer, is a target of miR-23a in AGS cells. Bioinformatics analysis was conducted to predict the target of miR-23a, which indicated SPRY2 to be a potential target (Fig. 3A). To further confirm this target association, the luciferase reporter gene plasmid containing the WT or MT of SPRY2-3'-UTR (Fig. 3B) was constructed. Following this, a luciferase reporter assay in AGS cells was performed.
Figure 1. (A) Reverse transcription-quantitative polymerase chain reaction was conducted to examine the miR-23a levels in gastric cancer tissues and adjacent tissues (P<0.0001). (B) Patients with gastric cancer with high miR-23a expression demonstrated reduced survival time, when compared with those with low miR-23a levels (P=0.029). miR, microRNA.

Figure 2. (A) RT-qPCR was conducted to examine the miR-23a levels in gastric cancer cell lines, compared with normal gastric mucosa epithelial GES-1 cells. **P<0.01 vs. GES-1. Following this, AGS cells were transfected with miR-23a inhibitor or NC inhibitor. (B) RT-qPCR was conducted to examine the miR-23a levels. (C) MTT assay, (D) wound healing assay and (E) Transwell assay were conducted to examine cell proliferation, migration and invasion, respectively. **P<0.01 vs. NC inhibitor. The magnifications for the wound healing and Transwell assay images were x40 and x200, respectively. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; NC, negative control; miR, microRNA; OD, optical density.
The luciferase activity was significantly reduced following co-transfection with the miR-23a mimics and WT 3'-UTR of SPRY2 plasmid, but unaltered in cells co-transfected with miR-23a mimics and MT SPRY2-3'-UTR plasmid (Fig. 3C). This data demonstrated that miR-23a directly binds to the 3'-UTR of SPRY2 mRNA in gastric cancer AGS cells.

Following this, the expression levels of SPRY2 in gastric cancer tissues and cell lines were investigated. As depicted in Fig. 3D, the mRNA levels of SPRY2 were significantly reduced in gastric cancer tissues compared with normal adjacent tissues. Similarly, the mRNA and protein levels of SPRY2 were downregulated in gastric cancer cell lines (Fig. 3E and F).

The effects of miR-23a on the protein expression of SPRY2 in AGS cells were studied. The data indicated that knockdown of miR-23a enhanced the mRNA and protein expression of SPRY2 in AGS cells (Fig. 4A and B). To further confirm this data, AGS cells were transfected with the miR-23a mimic to increase its expression. Following transfection, the miR-23a levels were upregulated in the miR-23a group compared with the miR-NC group (Fig. 4C). The upregulation of miR-23a reduced the mRNA and protein levels of SPRY2 (Fig. 4D and E); therefore, miR-23a negatively regulates the expression of SPRY2 in AGS cells.

**SPRY2 is involved in the miR-23a-mediated AGS cells.** Based on the aforementioned data, it was speculated that SPRY2 may be involved in the miR-23a-mediated AGS cells. To clarify this speculation, AGS cells were co-transfected with the miR-23a inhibitor and SPRY2-specific siRNA plasmid, or co-transfected with the miR-23a inhibitor and non-specific siRNA in the control group. Following transfection, the mRNA and protein levels of SPRY2 were significantly downregulated...
in the miR-23a in+siSPRY2 group compared with the miR-23a in+siNC group (Fig. 5A and B). Furthermore, it was demonstrated that the proliferation, migration and invasion of AGS cells were significantly upregulated in the miR-23a inhibitor+SPRY2 siRNA group compared with the miR-23a inhibitor+NC siRNA group (Fig. 5C-E). The results indicated that the suppressive effect of miR-23a knockdown on AGS cells may occur via the upregulation of SPRY2.

**Activity of ERK signaling is mediated by miR-23a and SPRY2 in AGS cells.** SPRY2 has been reported to have inhibitory effects on the activity of ERK signaling, which is essential for cancer cell growth and metastasis (23). The activity of ERK signaling was thus studied. As depicted in Fig. 6A, knockdown of miR-23a reduced the phosphorylation levels of the ERK protein; however, the phosphorylation levels of the ERK protein were increased in the miR-23a in+siSPRY2 group compared with the miR-23a in+siNC group (Fig. 6B); therefore, it is suggested that miR-23a promotes the activity of ERK signaling via targeting SPRY2.

**Discussion**

The regulatory mechanism underlying miR-23a in gastric cancer remains unclear. In the present study, it was demonstrated that miR-23a was significantly upregulated in gastric
cancer tissues and cell lines, and its upregulation was significantly associated with cancer progression and poor prognosis of patients. Knockdown of miR-23a significantly decreased the proliferation, migration and invasion of AGS cells. SPRY2, which, in the present study, is significantly downregulated in gastric cancer tissues and cell lines, was identified as a target gene of miR-23a, and its expression was negatively regulated by miR-23a at the post-transcriptional levels in AGS cells. siRNA-induced SPRY2 inhibition reduced the suppressive effects of miR-23a downregulation in AGS cells. In addition, the activity of ERK signaling was inhibited by the miR-23a/SPRY2 knockdown in AGS cells.

It has been demonstrated that numerous miRs are deregulated and serve important roles in gastric cancer (24,25). For example, miR-218 inhibits proliferation, migration and EMT of gastric cancer cells via targeting WAS protein family member 3 (9). miR-181b inhibits the glycolysis in gastric cancer cells via inhibiting the expression of hexokinase 2 (26). The present study demonstrated that the expression of miR-23a was significantly increased in gastric cancer tissues and cell lines, compared with adjacent non-tumor tissues or normal gastric epithelial cells, respectively. The results of the current study were consistent with previous studies (15,27,28). Following this, it was determined that the increased expression of miR-23a was significantly associated with gastric cancer progression and the poor prognosis of patients. Similarly, Ma et al (29) demonstrated that the co-expression of miR-23a and miR-23b in gastric cancer tissues was significantly associated with advanced stage, lymph node metastasis and invasion, and it was an independent predictor for unfavorable prognosis.

Figure 5. AGS cells were co-transfected with miR-23a inhibitor and SPRY2-specific siRNA, miR-23a in+siSPRY2, or co-transfected with miR-23a inhibitor and non-specific siRNA, miR-23a in+siNC. Following this, (A) reverse transcription-quantitative polymerase chain reaction and (B) western blot analysis were used to examine the mRNA and protein expression of SPRY2. (C) An MTT, (D) wound healing and (E) Transwell assay were conducted to examine cell proliferation, migration and invasion, respectively. **P<0.01 vs. miR-23a in+siNC. The magnification for wound healing assay image was x40, for Transwell assay was x200. si, small interfering; miR, microRNA; NC, negative control; SPRY, sprouty homolog 2; in, inhibitor.
overall survival time. Furthermore, it was demonstrated that knockdown of miR-23a could be able to reduce the proliferation, migration and invasion of AGS cells in the current study. Additionally, Zhu et al. (30) demonstrated that inhibition of miR-23a was able to inhibit the proliferation and invasion of gastric cancer MGC803 cells; however, the underlying molecular regulatory mechanism of miR-23a in gastric cancer migration and invasion remains unknown.

It has been well established that SPRY2 is an important tumor suppressor, and is regulated by a number of miRs (19-21). For instance, SPRY2 is able to inhibit the phosphorylation of ERK downstream of receptor tyrosine kinase signaling, which is important for tumor growth, metastasis and drug-resistance in human cancer types (20,21). Additionally, miR-27b promotes cell invasion of glioma U251 cells via targeting SPRY2 (19). miR-21 upregulates the ERK-mitogen-activated protein kinase signaling pathway via targeting SPRY2 during human mesenchymal stem cell differentiation (31). In the present study, it was demonstrated that miR-23a was able to directly target SPRY2, which was involved in the miR-23a-mediated proliferation, migration and invasion of AGS cells. The downstream ERK signaling in AGS cells was further studied. The results indicated that the knockdown of miR-23a significantly inhibited the activity of ERK signaling in AGS cells, which was reversed via SPRY2 downregulation. This data indicated that miR-23a was able to activate the ERK signaling via the inhibition of SPRY2, and thus promote the malignant phenotypes of AGS cells. Similarly, a previous study also indicated that miR-27a was able to promote the migration and invasion of gastric cancer cells via inhibiting the expression of SPRY2, and thus activating ERK signaling (32).

To the best of our knowledge, this is the first study to report that miR-23a promoted the proliferation, migration and invasion of gastric cancer cells via targeting the tumor suppressor SPRY2, and thus upregulating the activity of ERK signaling. Therefore, the results of the present study suggest that miR-23a may be a potential therapeutic target for gastric cancer. In addition, further investigations are required to explore the regulatory effects of miR-23a/SPRY2 in gastric cancer cell in vivo.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Author’s contributions

YL collected clinical tissues, did the clinical experiments, wrote the manuscript and submitted it. HC designed the present study and revised this manuscript. PS, TC, LC, JY and BJ performed the in vitro experiments.

Ethics approval and consent to participate

The present study was approved by the Ethical Committee of The Third Xiangya Hospital. Written informed consent was obtained from all patients involved in the present study.

Consent for publication

Written informed consent was obtained from all participants for publication of the present study.

Figure 6. (A) Western blot analysis was used to examine the levels of the p-ERK and t-ERK protein levels in AGS cells transfected with miR-23a inhibitor or negative control (NC) inhibitor. **P<0.01 vs. NC inhibitor. (B) Western blot analysis was used to examine levels of the p-ERK protein and t-ERK protein levels in AGS cells co-transfected with miR-23a inhibitor and SPRY2-specific siRNA (miR-23a in+siSPRY2), or co-transfected with miR-23a inhibitor and non-specific siRNA (miR-23a in+siNC). **P<0.01 vs. miR-23a in+siNC. ERK, extracellular signal-regulated kinases; NC, negative control; miR, microRNA; si, small interfering; SPRY2, sprouty homolog 2; p, phospho; t, total; in, inhibitor.
Competing interests

The authors declare that they have no competing interests.

References


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