Upregulation of microRNA-520a-3p inhibits the proliferation, migration and invasion via spindle and kinetochore associated 2 in gastric cancer

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Abstract. MicroRNAs (miR) serve important roles in the development and progression of tumors by targeting different genes. miR-520a-3p reported in lung and breast cancers as a tumor suppressor gene. However, the expression and functional significance of miR-520a-3p is not completely understood in gastric cancer (GC). In the present study, it was demonstrated that the expression levels of miR-520a-3p were significantly downregulated in GC tissues and cells using RT-qPCR. In addition, downregulated expression of miR-520a-3p was associated with the clinical stage of the tumor and invasion in patients with GC. Furthermore, overexpression of miR-520a-3p significantly inhibited cell proliferation, invasion and migration in SGC-7901 and MGC-803 GC cell lines using proliferation, wound healing and cell invasion assays. Spindle and kinetochore associated 2 (SKA2) was upregulated in GC cells using western blot analysis and a target gene of miR-520a-3p; miR-520a-3p mimics significantly reduced SKA2 expression. In addition, upregulation of SKA2 protein expression SKA2 reversed the miR-520a-3p-mediated inhibition of SGC-7901 cell proliferation, migration and invasion. In conclusion, miR-520a-3p functioned as a tumor suppressor gene by targeting SKA2 in GC cell lines, and may serve as a novel prognostic and potential therapeutic marker.

Introduction

Gastric cancer (GC) is a common cancer worldwide and remains the third leading cause of cancer-related death, following lung and liver cancer, accounting for 1.3 million cases of GC and 819,000 GC-associated cases of mortality in 2015 (1). The high incidence and mortality rates of GC have been demonstrated throughout the developed world, and GC adversely affects patients' health and quality of life (2). Although notable progress has been achieved in treating patients with GC based on advances in technology (3,4), the precise molecular mechanisms underlying GC development and progression remain unknown, and require further investigation.

MicroRNAs (miRNAs/miRs) can function as oncogenes or tumor suppressor genes, and regulate gene expression through translational repression or mRNA degradation by binding to the 3'-untranslated region (3'UTR) of their target mRNAs (5,6). Previous studies have identified that miRNAs exhibit aberrant expression and serve essential roles in GC (7-10). Therefore, further investigation into the expression and function of miRNAs may improve understanding of the molecular mechanisms responsible for the pathogenesis of GC.

Previous studies have demonstrated that the expression levels of miR-520a-3p are downregulated in non-small cell lung cancer cells. Decreased levels of miR-520a-3p expression were associated with poorer overall survival (11). Additionally, the upregulation of miR-520a-3p significantly suppressed the proliferation, cell cycle progression and metastatic activity by targeting mitogen-activated protein kinase kinase kinase2, HOXD8 or PI3K/AKT/mTOR (12,13). miR-520a-3p was also shown to inhibit proliferation and metastatic activity in breast cancer through cyclinD1, and in colorectal cancer via epidermal growth factor receptor (14,15). In osteosarcoma, dexmedetomidine has been reported to upregulate miR-520-3p, which directly targeted AKT1 to inhibit MG63 cell proliferation and migration and promote apoptosis. In addition, LINC01116 has been reported to target miR-520a-3p, which affected interleukin-6 receptor, thereby promoting proliferation and migration via the JAK-STAT signaling pathway (16,17). However, the expression profile and biological significance of miR-520a-3p in GC are unknown at present, to the best of our knowledge.

The present study examined the expression of miR-520a-3p in GC tissues and cells and demonstrated the functions of miR-520a-3p overexpression in GC cell proliferation, invasion and migration. Together, these data demonstrate that miR-520a-3p may be a novel therapeutic target for the treatment of GC.
Materials and methods

**Patients.** GC specimens and adjacent tissues were received from patients who were diagnosed with GC and underwent surgery at Ningbo No. 2 Hospital (Zhejiang, China) between August 2016 and May 2017. A total of 80 female patients, aged between 41-75 (55.3±11.4) years, and had not received local or systemic therapy prior to surgery at Ningbo No. 2 Hospital. The present study was approved by the Research Ethics Committee of Ningbo No. 2 Hospital, and all subjects provided the information written consent. The samples were graded according to the 8th edition of the AJCC staging classification system (18). The relationship between miR-520a-3p levels and the clinical characteristics of enrolled patients were analyzed.

**Cell culture.** All GC cell lines (SGC-7901, BGC-823, and MGC-803) and a normal gastric epithelial cell line (GES-1) used in the present study were obtained from the Institute of Biochemistry and Cell Biology (Shanghai, China) and maintained in DMEM (HyClone; GE Healthcare Life Sciences) medium (SGC-7901, BGC-823) or RPMI 1640 (HyClone; GE Healthcare Life Sciences) medium (MGC-803, GES-1) containing 10% FBS (Shanghai ExCellBiology, Inc.) at 37°C, in a humidified incubator with 5% CO₂ (Memmert GmbH).

**Cell transfection.** Cells (SGC-7901 and MGC803) were transfected with miR-520a-3p mimics (100 pmol; forward, 5'-AAA GUGCUUUCCUUUGGACUGU-3' and reverse, 5'-AGU CCAAUGGAGACACUUUU-3') or miR-520a-3p negative control (NC; 100 pmol; forward, 5'-UUCUCCGAACGU GUCAGCUTT-3' and reverse, 5'-ACGUAGCAACUCUGCG GAAAT-3'; both from Shanghai GenePharma Co. Ltd.), or spindle and kinetochore associated 2 (SKA2) cDNA plasmid (pLenti-SKA2-Puro) or NC (pLenti-C-Myc-DDK-P2A-Puro) (4 µg; both from OriGene Technologies, Inc.) using Lipofectamine™ 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. Cells were incubated with the transfection reagent for 24 h, following which RNA and protein were extracted. No treatment or transfection was performed in the MOCK group.

The SKA2 cDNA plasmid (without the 3’UTR) and miR-520a-3p mimics were co-transfected into SGC-7901 cells, which subsequently underwent western blot analyses, cell proliferation assays, wound healing assay and cell invasion assays.

**Western blotting.** Total cell protein was extracted by 1XSDS (Sigma-Aldrich; Merck KGaA) and quantified by a bicinchoninic acid assay (Beyotime Institute of Biotechnology). Cellular proteins (40-50 µg) were loaded onto a 12% polyacrylamide gel and resolved by SDS-PAGE. After separation, the proteins were transferred onto polyvinylidene difluoride membranes (EMD Millipore) for immunoblotting. Membranes were blocked with 5% BSA (Beijing Solarbio Science & Technology Co., Ltd.) for 2 h at room temperature, the membranes were incubated with primary antibodies overnight at 4°C. The primary antibodies used were Ki-67 (1:1,500; Abcam; cat no. ab15580), matrix metalloproteinase (MMP)-2 (1:1,500; Abcam; cat no. ab7033), MMP-9 (1:1,500; Abcam; cat no. ab137651), SKA2 (1:1,500; Abcam; cat no. ab91551) and GAPDH (1:2,000; Cell Signaling Technology, Inc.; cat. no. 5174). The membranes were subsequently incubated for 1 h at room temperature with goat anti-rabbit IgG-horseradish peroxidase (HRP) or goat anti-mouse IgG-HRP (1:5,000; Wuhan Boster Biological Technology, Ltd.; cat. nos. BA1054 and BA1050, respectively). The protein signals on the membrane were detected using an ECL reagent (Absin Biotechnology Co., Ltd.) and visualized using a chemiluminescence imaging system (LI-COR Biosciences).

**RT-qPCR.** The expression levels of miR-520a-3p and SKA2 were determined using RT-qPCR. Total RNA was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The RNA was reverse transcribed to cDNA using a TaqMan reverse transcription kit (Thermo Fisher Scientific, Inc.) with the following RT protocol; 15 min at 37°C and 5 sec at 85°C. cDNA was amplified using SYBR-Green PCR Master mix (Roche Diagnostics) on a LightCycler 480 system. The thermocycling conditions were as follows: 95°C for 10 min; and followed by 45 cycles, 95°C for 10 sec and 60°C for 60 sec. Fold changes were calculated by relative quantification (2^−ΔΔCt) using endogenous U6 and GAPDH as references for miR-520a-3p and SKA2 expression, respectively (19). The primers used for miR-520a-3p were: Forward, 5’-ACACTCCACGTGGGA AAGTGCTTCCC-3' and reverse, 5’-CTCAAATCTGTTGCT GGA-3'. For U6, the primers used were: Forward, 5’-CTC GCTTCGGACGCACA-3’ and reverse, 5’-AACGCTTCACGA ATTGGCGT-3’. For SKA2, the primers used were: Forward, 5’-CTGAAACTATGCTAATGGGGAG-3’ and reverse, 5’-TTCCAAACATCTGTGACACTCAAAG-3’. For GAPDH, the primers used were: Forward, 5’-AAGCTCTGCGGTGAC TAAC-3’ and reverse, 5’-GCATCACCAGGGAGAGAAT-3’.

**Cell proliferation assay.** miR-520a-3p mimics, SKA2 cDNA and negative control-transfected GC cells (SGC-7901 and MGC-803) were seeded in 96-well plates (1x10^4 cells/well). A total of 20 µl Cell Titer 96® Aqueous One Solution (Promega Corporation) was added to each well to determine cell viability at 4, 24, 48, 72 and 96 h after seeding. Cells were incubated at 37°C, in a humidified incubator with 5% CO₂ for 3 h. Subsequently the absorbance of each well was measured at 490 nm on a spectrophotometer (Beckman Coulter, Inc.).

**Wound healing assay.** Cells were cultured in 6-well plates (1x10^5 cells/well) for 24 h. Once the cellular density had reached ~100%, a vertical line was scraped in the culture using a 1-ml pipette tip. The cells were rinsed with PBS and incubated with fresh serum-free medium at 37°C. The widths of the gap at 0 and 24 h were compared to assess the distance of migration using fluorescence microscopy.

**Cell invasion assays.** Cell invasion assays were performed using a Matrigel invasion chamber (24-well plates; 8-µm pore size; Corning Inc.) according to the manufacturer’s protocol. A total of 5x10^4 cells were seeded in the upper chambers of the wells in 100 µl FBS-free medium, and the lower chambers contained DMEM supplemented with 20% FBS. The cells were incubated for 24 h at 37°C, after which the cells on the
filter surface were stained with 0.1% crystal violet for 30 min at room temperature and images were captured with an inverted fluorescence microscope (Nikon Corporation), representative images were captured at x100 magnification. The absorbance was measured at 590 nm.

**Bioinformatics.** Potential miR-520a-3p targets were predicted and analyzed using the following two publicly available databases: TargetScanv7.2 (http://www.targetscan.org), and miRanda (http://www.microrna.org/microrna/getGeneForm.do).

**Luciferase assay.** The predicted miR-520a-3p binding sites on the 3'UTR of wild-type (WT) SKA2 (MirTarget-SKA2-3U-WT), together with a corresponding mutant (mut) miR-520a-3p binding sites on the 3'UTR of SAK2 (MirTarget-SKA2-3U-Mut), were synthesized and transfected into the pGL3 vector (Promega Corporation). SGC-7901 cells were seeded in a 24-well plates (5x10^4 cells/well), the WT or Mut 3'UTR vectors, and miR-520a-3p mimics or NC were co-transfected using Lipofectamine™ 2000. The relative luciferase activity was measured after 48 h using a Dual-Luciferase Reporter assay system according to the manufacturer's protocols (Promega Corporation). Luciferase activity was normalized to Renilla luciferase activity.

**Statistical analysis.** All experiments were repeated three times and data are presented as the mean ± standard deviation. One-way ANOVA and Fisher's least significant difference tests were used to calculate P-values between multiple groups. Data in Table I were analyzed using χ^2 tests of four-fold table. Statistical analyses were performed using SPSS 15.0 software (SPSS, Inc.). P<0.05 was considered to indicate a statistically significant difference.

### Results

miR-520a-3p expression levels are downregulated in GC and are associated with clinical stage and tumor invasion. miR-520a-3p expression levels in 80 GC samples and adjacent tissue samples were measured by RT-qPCR, and the levels were normalized to U6. The results demonstrated that miR-520a-3p expression levels were significantly decreased in GC tissues compared with the adjacent normal tissues (P<0.01; Fig. 1A). Furthermore, the expression levels of miR-520a-3p in three human GC cell lines (SGC-7901, BGC-823 and MGC-803) were evaluated and compared with the normal gastric epithelial cell line (GES-1). miR-520a-3p expression was significantly upregulated in the GES-1 compared with the other three cell lines (Fig. 1B).

To investigate the clinical significance of miR-520a-3p in patients with GC, miR-520a-3p expression in a cohort of 80 specimens was analyzed (Table I). The miR-520a-3p levels were significantly associated with clinical stage (P<0.05) and tumor invasion (P<0.001) in GC tissues; however, they were not associated with patient age, tumor size, histological grade or pathological type (P>0.05).

**Table I. Association between miR-520a-3p expression and clinicopathological factors in 80 primary gastric cancer tissues.**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patients, n</th>
<th>miR-520a-3p expression level</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤50</td>
<td>38</td>
<td>Low, n (%) 28 (73.7)</td>
<td>0.990</td>
</tr>
<tr>
<td>&gt;50</td>
<td>42</td>
<td>High, n (%) 10 (26.3)</td>
<td></td>
</tr>
<tr>
<td>Tumor diameter, cm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤3</td>
<td>24</td>
<td>Low, n (%) 21 (87.5)</td>
<td>0.067</td>
</tr>
<tr>
<td>&gt;3</td>
<td>56</td>
<td>High, n (%) 3 (12.5)</td>
<td></td>
</tr>
<tr>
<td>Tumor TNM staging</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I+II</td>
<td>43</td>
<td>Low, n (%) 27 (62.8)</td>
<td>0.016a</td>
</tr>
<tr>
<td>III+IV</td>
<td>37</td>
<td>High, n (%) 16 (37.2)</td>
<td></td>
</tr>
<tr>
<td>Tumor histological overall NHS grade</td>
<td></td>
<td></td>
<td>0.305</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>Low, n (%) 4 (57.1)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>41</td>
<td>High, n (%) 3 (42.9)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>32</td>
<td>Low, n (%) 33 (80.5)</td>
<td></td>
</tr>
<tr>
<td>Invasion</td>
<td></td>
<td></td>
<td>0.001b</td>
</tr>
<tr>
<td>T1+T2</td>
<td>27</td>
<td>Low, n (%) 14 (51.9)</td>
<td></td>
</tr>
<tr>
<td>T3+T4</td>
<td>53</td>
<td>High, n (%) 13 (48.1)</td>
<td></td>
</tr>
<tr>
<td>Pathological type</td>
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</tr>
<tr>
<td>Adenocarcinoma</td>
<td>69</td>
<td>Low, n (%) 52 (75.4)</td>
<td></td>
</tr>
<tr>
<td>Non-adenocarcinoma</td>
<td>11</td>
<td>High, n (%) 4 (36.4)</td>
<td></td>
</tr>
</tbody>
</table>

*miR, microRNA; TNM, Tumor-Node-Metastasis; NHS, National Health Service.*
miR-520a-3p suppresses the proliferation of GC cells. To investigate the functional effects of miR-520a-3p expression in GC, SGC-7901 and MGC-803 cells were transfected with MOCK, miR-NC or miR-520a-3p mimics. Treatment with miR-520a-3p mimics significantly increased the expression levels of miR-520a-3p (P<0.001), compared with the MOCK group (Fig. 2A). Following the upregulation of miR-520a-3p expression, the effect on cell proliferation was examined using cell proliferation assay. Compared with the control cells, cells transfected with miR-520a-3p mimics demonstrated significantly reduced levels of proliferation (Fig. 2B). Recent studies have demonstrated that Ki-67 is closely associated with cell proliferation (20,21). Investigating the levels of Ki-67 showed that miR-520a-3p mimics significantly decreased Ki-67 expression in GC cells (P<0.01; Fig. 2C).

miR-520a-3p decreases the migration and invasion of GC cells. To examine how miR-520a-3p affects the migration and invasion of GC cells, the effects on cell migration and invasion were investigated in vitro using scratch wound healing and Transwell assays, respectively. The data demonstrated that transfection of cells with miR-520a-3p mimics significantly inhibited GC cell migration and invasion (Fig. 3A and B) compared with MOCK and NC groups.

Interactions between cell-surface proteins and the extracellular matrix (ECM) serve a critical role in tumor migration and invasion (22). As MMPs, particularly MMP-2 and MMP-9, digest ECM components, they are closely associated with the metastatic capabilities of cancerous cells (23). Transfection of miR-520a-3p mimics led to significantly decreased protein expression levels of MMP-2 and MMP-9 in GC cells compared with the MOCK and NC groups.

miR-520a-3p directly targets SKA2 in the SGC-7901GC cell line. To investigate the molecular mechanism underlying the effects of miR-520a-3p on GC cellular functions, SKA2 mRNA was identified as a potential miR-520a-3p target using bioinformatics (Fig. 4A). Subsequently, the WT or Mut target region sequence of the SKA2 3'UTR were cloned and inserted into a luciferase reporter vector (Fig. 4A). The luciferase reporter assays revealed that miR-520a-3p significantly decreased the luciferase activity in the reporter gene containing the WT 3'UTR (P<0.01); however, no difference was observed with the Mut 3'UTR (P>0.05; Fig. 4B). To demonstrate whether miR-520a-3p affected SKA2 expression in GC, expression of SKA2 in SGC-7901 cells transfected with miR-520a-3p mimics was measured. The data demonstrated that transfection with miR-520a-3p mimics significantly decreased SKA2 mRNA and protein expression levels, compared with the MOCK group (P<0.01; Fig. 4C and D). Furthermore, the protein expression of SKA2 in SGC-7901, BGC-823, and MGC-803 was significantly in all 3 GC cell lines compared with GES-1 (P<0.001; Fig. 4E).

Overexpression of SKA2 reverses the miR-520a-3p-mediated inhibition of SGC-7901 cell proliferation, migration and invasion. To determine whether SKA2 overexpression reversed the inhibitory effects of miR-520a-3p mimics on SGC-7901 cells, SKA2 cDNA without the 3'UTR and miR-520a-3p mimics were co-transfected into SGC-7901 cells (P<0.01; Fig. 5A). Western blotting and cell viability assays showed that the decrease in SKA2 expression and cell viability following miR-520-3p overexpression was reversed when SKA2 was co-transfected with miR-520a-3p mimics (Fig. 5B and C). Migration and invasion assays demonstrated that SKA2 co-transfection significantly reversed the miR-520a-3p-mediated decrease in cell migration and invasion in SGC-7901 cells (Fig. 5D and E).

Discussion

miR-520a-3p serves an tumor suppressive role in certain types of cancer (11-17). However, its functions and expression in GC have not been studied, to the best of our knowledge.

The results demonstrated that miR-520a-3p was downregulated both in human GC tissues and GC cell lines compared with adjacent tissues and a normal gastric cell line. In addition, the expression of miR-520a-3p was associated with clinical stage and tumor invasion in patients with GC.
experimentation revealed that the upregulation of miR-520a-3p inhibited the proliferation, migration and invasion of GC cells (SGC-7901 and MGC-803), potentially by directly inhibiting SKA2. Furthermore, miR-520a-3p significantly inhibited the level of SKA2, and overexpression of SKA2 rescued the miR-520a-3p-mediated inhibition of SGC-7901 cell proliferation, migration and invasion. Therefore, the present study further confirmed the several biological functions attributed to miR-520a-3p, including the reduction in GC cell viability, and inhibition of cell migration and invasion following transfection with miR-520a-3p mimics, as well as the molecular mechanism underlying the miR-520a-3p-mediated inhibitory effect (11-17). SKA2 is located on chromosome 17 of the human genome and is a conserved protein involved in the kinetochore complex (24). SKA2, together with its cofactors SKA1 and SKA3, constitute the SKA complex, which maintains the metaphase plate and/or spindle checkpoint silencing (25,26). Recently, SKA2 was also identified to exert a function in tumorigenesis. Aberrant expression patterns of SKA2 have been reported in lung cancer (27-29), breast cancer (30-32), osteosarcoma (33,34) and kidney cancer (35). Nevertheless, the potential roles of SKA2 in GC are unknown. The results,
Figure 3. Overexpression of miR-520a-3p inhibits the migratory and invasive capabilities of GC cells. (A) Migratory distance and (B) number of invasive cells in the miR-520a-3p mimics group were significantly decreased compared with the MOCK and NC groups in the wound healing and Transwell invasion assays. *P<0.05 vs. MOCK and NC. (C) Protein expression levels of MMP-2 and MMP-9 were significantly lower in the miR-520a-3p mimics group compared with the MOCK and NC groups. **P<0.01 vs. MOCK and NC. Magnification, x100. Scale bar, 100 µm. miR, microRNA; GC, gastric cancer; MOCK, mock transfected cells; NC, negative control; MMP, matrix metalloproteinase.

Figure 4. SKA2 is a direct target gene of miR-520a-3p in GC cells. (A) Sequence alignment of miR-520a-3p with 3'UTR of SKA2 predicted by TargetScan and the Mut 3'UTR miR-520a-3p binding sequence. (B) Luciferase activity of SGC-7901 cells was examined via a luciferase reporter assay. **P<0.01 vs. mutant. miR-520a-3p decreased SKA2 expression at both the mRNA (C) and protein (D) levels compared to MOCK in SGC-7901 cells. **P<0.01, ***P<0.001 vs. MOCK group. (E) SKA2 expression was analyzed in three GC cell lines (SGC-7901, BGC-823 and MGC-803) and a normal gastric epithelial cell line (GES-1). ***P<0.001 vs. GES-1. miR, microRNA; GC, gastric cancer; MOCK, mock transfected cells; NC, negative control; WT, wild-type; SKA2, spindle kinetochore-associated protein 2; UTR, untranslated region.
to the best of our knowledge, are the first to demonstrate that SKA2 was significantly upregulated in gastric cell lines. As a potential target gene of miR-520a-3p predicted by miRanda and TargetScan software, the present study analyzed the relationship between SKA2 and miR-520a-3p in SGC-7901 cells. The results demonstrated that miR-520a-3p mimics significantly attenuated the mRNA and protein expression of SKA2. Furthermore, downregulation of SKA2 by miR-520a-3p mimics was reversed by SKA2 cDNA plasmid. These results suggested that miR-520a-3p directly repressed the expression of SKA2 in GC by binding to its 3'UTR.

A previous study reported that miR-520d-3p inhibited GC cell growth and metastatic activity by targeting EphA2 (36). miR-520d-3p and miR-520a-3p are members of the same microRNA family and share a large amount of homology. As they have different sequences and binding sites of target genes, they play a role in tumorigenesis through different regulatory mechanisms. In the present study, the potential value of miRNA-520a-3p and SKA2 as therapeutic targets in cancer diagnosis and targeted therapy was demonstrated.

In summary, miR-520a-3p was downregulated in GC tissues and cell lines. miR-520a-3p also serves a role as a tumor suppressor gene in GC cell lines, decreasing cell proliferation, invasion and migration by targeting SKA2. The novel interaction between miR-520a-3p and SKA2 may contribute to the development of improved targeted therapies for patients with GC. However, due to the complex signaling pathways regulated by microRNAs and the limited sample size, further investigation into the function and regulatory mechanisms of miR-520a-3p in vivo is required. In particular, the expression levels of miR-520a-3p and SKA2 should be determined in a range of cancer types, both in vitro and in vivo, to determine whether the mechanisms underlying miR-520a-3p-mediated oncogenic activity in GC cell lines are replicated elsewhere.

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Availability of data and materials

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

Authors' contributions

HS and FR collected and analyzed the data, and wrote and revised the manuscript. XF conceived and designed the experiments. HJ and YC conducted the experiments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by The Research Ethics Committee of Ningbo No. 2 Hospital (Zhejiang, China), and all patients provided written informed consent.
Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


