Long non-coding RNA MEG-3 suppresses gastric carcinoma cell growth, invasion and migration via EMT regulation

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Abstract. Gastric carcinoma is one of the most frequently diagnosed gastrointestinal tumors. Long non-coding RNAs (lncRNAs) are broadly defined as endogenous cellular non-coding RNA molecules. Studies have demonstrated that they may be associated with human cancer progression. In the present study, the role of lncRNA-maternally expressed gene 3 (MEG3) in the progression of gastric carcinoma cells was investigated in vitro and in vivo. It was demonstrated that lncRNA-MEG3 expression was downregulated in gastric carcinoma cells compared with normal gastric cells. lncRNA-MEG3 transfection increased E-cadherin expression and markedly inhibited gastric carcinoma cell growth, migration and invasion. Flow cytometric analysis revealed that lncRNA-MEG3 transfection promoted the apoptosis of gastric carcinoma cells. Western blot analysis demonstrated that lncRNA-MEG3 transfection inhibited the expression of anti-apoptotic proteins B cell lymphoma-2 (Bcl-2) and Bcl-2-like protein 2 and increased the expression of pro-apoptotic proteins caspase-3 and caspase-9 in gastric carcinoma cells. lncRNA-MEG3 transfection upregulated the expression of epithelial marker E-cadherin and inhibited the expression of mesenchymal markers vimentin and fibronectin in gastric carcinoma cells, which suggested that lncRNA-MEG3 inhibited epithelial-mesenchymal transition (EMT), which may subsequently inhibit progression in gastric carcinoma cells. The present study also revealed that lncRNA-MEG3 transfection suppressed tumor growth mainly by decreasing the expression of vascular endothelial growth factor and increasing the expression of Bcl-2 in vivo. In conclusion, these results indicated that lncRNA-MEG3 may regulate EMT-associated signaling pathways and has the potential as a therapeutic target in gastric carcinoma.

Introduction

Gastric cancer is one of the most common human gastrointestinal cancers and remains the second leading cause of cancer-associated mortality worldwide (1,2). Gastric cancer has the highest morbidity and mortality rate amongst the digestive system-derived cancers (3,4). Previous studies have demonstrated that apoptosis resistance of gastric cancer is inevitable as the cancer progresses (5,6). Tumor resistance to apoptosis is currently the greatest challenge in gastric cancer therapy as it often leads to tumor metastasis (7,8). Previous study has indicated that targeted therapies for advanced gastric cancer are effective (9). However, the treatment of patients with gastric cancer is particularly challenging for those with apoptotic resistance and tumor metastasis. Therefore, the discovery of more effective therapeutic targets in gastric cancer is essential.

Long non-coding RNAs (lncRNAs) are endogenous cellular non-coding RNA molecules >200 nucleotides in length that regulate gene expression and may function tumor suppressor in several cancers (14-16). However, the effect of lncRNA-MEG3 in gastric cancer is not yet fully understood.

In the present study, the role of lncRNA-MEG3 in gastric cancer cells in vitro and in vivo was investigated. It has been reported that epithelial-to-mesenchymal transition (EMT) could mediate gastric cancer progression (17,18). EMT is recognized as an important mechanism for cancer metastasis; it relies on epithelial cell morphology changes from epithelial cobblestone phenotype to elongated fibroblast phenotype (17). The process of EMT involves the disassembly of cell-cell junctions, actin cytoskeleton reorganization and enhancement of cell motility and invasion (18). Zn-finger E-box binding homeobox 1 (ZEB1) is expressed at the invasive front of carcinomas where it affects gene expression to induce EMT, which upregulates expression of vimentin and downregulates expression of E-cadherin, which is a key event for EMT and metastasis (19). Furthermore, inhibiting the EMT process can...
induce tumor cell apoptosis and autophagy in human gastric cancer (20), and ZEB1 has been suggested to be an important inducer of the EMT and a promoter of tumor metastasis (21). Therefore, the effects of IncRNA-MEG3 on EMT processes in gastric cancer cells were analyzed. The role of IncRNA-MEG3 on the apoptotic resistance of gastric cancer cells induced by the chemotherapeutic drug cisplatin was also investigated.

Materials and methods

Cell lines and cell culture. Gastric cancer cell lines HGC-27 (catalog no. BNCC338546) and BGC-823 (catalog no. BNCC337689), as well as the normal gastric cell lineGES-1 (catalog no. BNCC337970), were all purchased from BeNa Culture Collection (Beijing, China; http://www.bnc.org.cn). All cells were cultured in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal calf serum (Invitrogen; Thermo Fisher Scientific, Inc.) at 37°C in a humidified chamber with 5% CO₂.

IncRNA-MEG3 transfection. HGC-27 cells (1x10⁶ cells/well) were seeded on 6-well plates and were incubated at 37°C for a 24 h prior to transfection. Cells were subsequently transfected at 37°C for 30 min with 100 μM pcDNA3.1-MEG3 overexpression vector (Thermo Fisher Scientific, Inc.) or pcDNA3.1 empty vector control using Lipofectamine™ 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer’s instructions. At 48 h post-transfection, efficiency was validated by reverse transcription-quantitative polymerase chain reaction (RT-qPCR).

ZEB1 transfection. The human ZEB gene (GenBank: BC107781.1) was synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China) and amplified by polymerase chain reaction (PCR). Primers were synthesized by Invitrogen (Thermo Fisher Scientific, Inc.) and had the following sequences: ZEB, forward, 5'-GGGGACAAGGCAGGAGT CAT-3' and reverse, 5'-TTTTGAGAAACATTGGAATAA-3'; β-actin, forward, 5'-CCGAGTCAAAGGTATGGTGC-3' and reverse, 5'-AGGCTCTTCCATGGTCTGA-3'. The thermocycling conditions were as follows: Initial denaturation 94°C for 30 sec; followed by 25 cycles of 94°C for 30 sec, 59°C for 30 sec, 72°C for 2 min and a final elongation step at 72°C for 10 min. HGC-27 cells (1x10⁵ cells/well in a 6-well plate) were transfected at 37°C for 30 min, following construction of a ZEB1 pcDNA3.1 overexpression plasmid (pZEB; 100 nM; Invitrogen; Thermo Fisher Scientific, Inc.) and Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer’s instructions was used as the transfection reagent. The pcDNA3.1 empty vector was used as a transfection negative control group. HGC-27 cells that were stably infected with pZEB were selected using 2 µg/µl puromycin (Invitrogen; Thermo Fisher Scientific, Inc.) 48 h post-infection and subsequently transfected with IncRNA-MEG3, as aforementioned.

RT-qPCR. Total RNA was extracted from tumor cells (1x10⁵ cells/well) by using RNAEasy Mini kit (Qiagen Sciences, Inc., Gaithersburg, MD, USA). cDNA was synthesized at 42°C for 50 min using High Capacity cDNA Reverse Transcription kit (cat. no. 4368814; Invitrogen; Thermo Fisher Scientific, Inc.) according to manufacturer’s instrument. IncRNA-MEG3 expression was detected by qPCR using an ABI7300 Thermocycler (Applied Biosystems; Thermo Fisher Scientific, Inc.), with β-actin as an endogenous control (Life Technologies; Thermo Fisher Scientific, Inc.) (22). Primers were synthesized by Invitrogen (Thermo Fisher Scientific, Inc.) and the sequences were as follows: IncRNA-MEG3, forward, 5'-CATGAGGATCCCAGCATGCT-3' and reverse, 5'-CATGGTCATCCTAGT3-3'; β-actin, 5'-CGGAGTCACAGGATTTGGCT-3' and reverse, 5'-AGCTTCTCAAGTGTCGTA-3'. Initial denaturation was performed at 94°C for 2 min, which was followed by 45 cycles at 95°C for 30 sec, 57.2°C for 30 sec and 72°C for 10 min. The reaction mixture (20 µl) contained 50 ng of genomic DNA, 200 nM dNTPs, 2.5 units TaqDNA polymerase (Takara Biotechnology Co., Ltd., Dalian, China), 200 µM primer sequences and SYBR-Green qPCR Master Mix (5 µl; Invitrogen; Thermo Fisher Scientific, Inc.). Relative mRNA expression was calculated using 2^ΔΔCq (23); relative gene expression levels were normalized to β-actin.

MTT assay. The IncRNA-MEG3-transfected HGC-27 cells (1x10⁵) were seeded into 96-well plates for 48 h at 37°C in triplicate for each condition. Following incubation, 20 µl MTT (5 mg/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in PBS solution was added to each well and the plate was incubated for 4 h. The medium was subsequently removed and 100 µl of dimethyl sulfoxide was added into the wells to dissolve the purple formazan crystals. Cell proliferation was determined by optical density using a microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA) at wavelength of 490 nm.

Cell growth assay. The IncRNA-MEG3-transfected HGC-27 (1x10⁵) were seeded in six-well plates and cultured in RPMI-1640 medium at 37°C for 14 days. Medium was subsequently removed and the cells were fixed with 100% methanol for 20 min at 37°C and stained with 0.1% (w/v) crystal violet for 5 min at 37°C (Sigma-Aldrich; Merck KGaA). Cell colonies (>500 cells) were counted in at least three fields under an Olympus BX51 light microscope (Olympus Corporation, Tokyo, Japan) and counted with Image-Pro Plus 5.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Apoptosis assay. The transfected HGC-27 cells (1x10⁵) were seeded into 6-well plates for 12 h at 37°C in a humidified incubator with 5% CO₂. Cells were subsequently incubated with cisplatin (5.0 mg/ml) for 24 h at 37°C. Cells were removed, collected and washed with PBS three times. Subsequently, cells were stained with fluorescein isothiocyanate (FITC)-conjugated Annexin V and propidium iodide using the Annexin V-FITC Apoptosis Detection kit (BD Biosciences, San Jose, CA, USA), according to manufacturer’s instructions. A flow cytometer (BD Biosciences) was used to analyze the percentage of apoptotic HGC-27 cells.

Western blot analysis. HGC-27, BGC-823 GES-1 and IncRNA-MEG3 transfected cells were homogenized in radioimmunoprecipitation assay lysis buffer containing protease inhibitor (both from Sigma-Aldrich; Merck KGaA) and...
centrifuged at 8,000 x g for 10 min at 4°C. A BCA protein assay kit (Thermo Fisher Scientific, Inc.) was used to measure protein concentration. A total of 30 µg protein was separated by 12% SDS-PAGE, as described previously (24). Proteins were transferred onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA), blocked in 5% skimmed milk for 1 h at 37°C and were subsequently incubated with the following primary antibodies for 12 h at 4°C: Anti-B cell lymphoma-2 (Bcl-2; 1:1,000; cat. no. ab32124; Abcam, Cambridge, UK), Bcl-2-like protein 2 (Bcl-w; 1:1,000; cat. ab38629; Abcam), Bcl-associated X protein (Bax; 1:1,000; cat. no.ab182733; Abcam), Bcl-2-associated agonist of cell death (Bad; 1:1,000; cat. no. ab90435; Abcam), E-cadherin (1:1,000; cat. no. ab1416; Abcam), vimentin (1:1,000; cat. no. ab92547; Abcam), fibronectin (1:1,000; cat. no. ab2413; Abcam) and β-actin (1:1,000; cat. no. ab8227; Abcam). Membranes were then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG monoclonal secondary antibody (1:2,000; Abcam) or rabbit anti-mouse IgG monoclonal secondary antibody (1:2,000; cat. no. 6728; Abcam) for 2 h at 37°C. Protein were visualized using the SuperSignal West Pico Chemiluminescent Substrate Trial kit (Pierce; Thermo Fisher Scientific, Inc.). Images were obtained using the ChemiDoc XRS system with Quantity One software (version 4.0; Bio-Rad Laboratories, Inc., Hercules, CA, USA), and protein expression was analyzed using BandScan 5.0 software (Glyko, Inc., Novato, CA, USA).

Cell migration and invasion assay. The IncRNA-MEG3-transfected HGC-27 cells were cultured in Matrigel-coated and -uncoated Transwell inserts (8 µm pore size; Merck KGaA) for the invasion and migration assays, respectively. HGC-27 cells (1x10^6 cells/well) with 150 µl serum free DMEM (Invitrogen; Thermo Fisher Scientific, Inc.) was placed into the upper chamber and DMEM with 5% fetal calf serum (Invitrogen; Thermo Fisher Scientific, Inc.) was placed in the lower chamber. Following 24 h incubation at 37°C, HGC-27 cells in the upper chamber were removed with a cotton swab and those in the lower chamber were fixed in 4% paraformaldehyde for 15 min at 37°C, stained with 0.1% crystal violet dye (Sigma-Aldrich; Merck KGaA) for 20 min at 37°C and counted at three randomly selected views using an Olympus BX51 light microscope (Olympus Corporation, Tokyo, Japan).

Animal study. The present study was approved by the Ethics Committee of Xi’an Jiao Tong University. A total of 40, 6-8-week-old female C57BL/6 mice (30-35 g) were used (Shanghai SLAC Laboratory Animals Co., Ltd., Shanghai, China). All mice were housed in a temperature-controlled environment (23±2°C), 50±5% humidity, normal atmosphere with a 12-h light/dark cycle and provided free access to food and water. The IncRNA-MEG3- or IncRNA-vector control transfected HGC-27 cells (1x10^6) were inoculated one time on a single side of the posterior flank (n=10/group). The tumor volumes were calculated according to the following formula: Length x width^2 x 0.52. On day 28, mice were euthanized with diethyl ether and 1.5% pentobarbital sodium (100 mg/kg) tail vein injection, and the tumors were isolated. The largest tumor size was ~2,000 mm^3. Mice were sacrificed when tumor diameter reached 16 mm.

Immunohistochemical (IHC) analysis. Gastric tumor xenografts were fixed using 10% formaldehyde for 2 h at room temperature, embedded in paraffin and sectioned (4 µm). The sections were deparaffinized in xylene for 15 min at room temperature, rehydrated through graded ethanol concentrations and endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 10 min at room temperature, as previously described (25). Tumor sections were incubated with specific primary antibodies against vascular endothelial growth factor (VEGF; 1:1,000; cat. no. ab69479; Abcam) and Bcl-2 (1:1,000; cat. no. ab32124; Abcam) for 12 h at 4°C. Tumor tissues were then incubated with HRP-conjugated rabbit anti-mouse IgG monoclonal secondary antibody (1:10,000; cat. no. 6278; Abcam). A Ventana Benchmark Automated Staining system (Ventana Medical Systems, Inc., Tucson, AZ, USA) was used to analyze protein expression in tumor tissues. The staining results were semi-quantified by the percentage of positively stained cells as examined using an Olympus BX51 light microscope (magnification, x400; Olympus Corporation).

Statistical analysis. Data was expressed as the mean ± standard deviation, and each experiment was performed at least three times. All data were analyzed by SPSS 19.0 software (IBM Corp., Armonk, NY, USA) and GraphPad Prism 5.0 (GraphPad Software, Inc. La Jolla, CA, USA) using Student’s t-tests or one-way analysis of variance followed by Tukey’s multiple comparison post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

IncRNA-MEG3 expression levels are decreased in gastric cancer cell lines. To identify the role of IncRNA-MEG3 in gastric cancer, the expression of IncRNA-MEG3 in gastric cancer cells was detected by RT-qPCR. IncRNA-MEG3 expression levels were significantly lower in gastric cancer cell lines HGC-27 and BGC-823 compared with expression in the GE1 normal gastric cell line (Fig. 1A). E-cadherin expression levels were significantly decreased in gastric cancer cell lines HGC-27 and BGC-823 compared to the normal gastric cell line GE1 (Fig. 1B and C), which suggested that IncRNA-MEG3 may be associated with gastric cancer progression.

IncRNA-MEG3 transfection upregulates E-cadherin expression in gastric cancer cells. To investigate the role of IncRNA-MEG3 on E-cadherin expression, E-cadherin gene and protein expression in HGC-27 cells was detected following IncRNA-MEG3 transfection. IncRNA-MEG3 expression was significantly increased in HGC-27 cells following overexpression vector transfection, compared with cells transfected with empty vector (Fig. 2A). Further, E-cadherin expression levels were significantly increased in HGC-27 gastric cancer cells following transfection of IncRNA-MEG3 (Fig. 2B), which indicated that IncRNA-MEG3 transfection may upregulate E-cadherin expression in gastric cancer cells.

IncRNA-MEG3 inhibits gastric cancer cell growth, migration and invasion. To investigate the role of IncRNA-MEG3 in gastric cancer progression, the growth, proliferation,
Figure 1. IncRNA-MEG3 and E-cadherin expression is downregulated in gastric cancer cell lines. (A) IncRNA-MEG3 expression levels in gastric cancer cell lines HGC-27 and BGC-823 compared to the normal gastric cell line GES-1. (B and C) E-cadherin (B) protein and (C) mRNA expression levels in gastric cancer cell lines HGC-27 and BGC-823 compared to the normal gastric cell line GES-1. Data are presented as the mean ± standard deviation; each experiment was performed in triplicate; **P<0.01. IncRNA, long non-coding RNA; MEG3, maternally expressed gene 3.

Figure 2. IncRNA-MEG3 transfection upregulates E-cadherin expression in gastric cancer cells. (A) IncRNA-MEG3 transfection increases the expression of IncRNA-MEG3 mRNA. (B) E-cadherin protein expression in transfected HGC-27 cells. Data are presented as the mean ± standard deviation; each experiment was performed in triplicate; **P<0.01. IncRNA, long non-coding RNA; MEG3, maternally expressed gene 3.
invasion and migration of HGC-27 cells were measured. The results revealed that lncRNA-MEG3 transfection decreased HGC-27 cell growth and proliferation compared to the control group (Fig. 3A and B, respectively). Results from the migration and invasion assays demonstrated that lncRNA-MEG3 transfection significantly inhibited the migratory and invasive ability of HGC-27 cells compared with the control group (Fig. 3C and D, respectively). These results indicated that lncRNA-MEG3 transfection may inhibit gastric cancer cell growth, proliferation, migration and invasion.

**lncRNA-MEG3 promotes apoptosis of gastric cancer cells.**

The role of lncRNA-MEG3 in apoptosis of gastric cancer cells was subsequently investigated. Flow cytometry analysis revealed that lncRNA-MEG3 transfection further promoted the apoptotic rates (early + late stage apoptosis) of gastric cancer compared with the control group (Fig. 4A). Western blot analysis demonstrated that lncRNA-MEG3 transfection downregulated the expression of anti-apoptotic proteins Bcl-2 and Bcl-w (Fig. 4B) and upregulated the expression of pro-apoptotic proteins caspase-3 and caspase-9 in gastric cancer cells (Fig. 4C). These results suggested that lncRNA-MEG3 may promote the apoptosis of gastric cancer cells.

**lncRNA-MEG3 inhibits gastric cancer cells growth via EMT regulation.**

EMT is involved in the progression of cancer metastasis (26). To understand the molecular mechanism by which lncRNA-MEG3 suppressed gastric cancer growth, EMT marker expression was detected in HGC-27 cells following lncRNA-MEG3 transfection. LncRNA-MEG3 transfection
significantly increased epithelial marker E-cadherin expression and significantly inhibited mesenchymal marker vimentin and fibronectin expression in HGC-27 cells (Fig. 5A). Successful establishment of stable ZEB overexpression cells was verified by RT-PCR (Fig. 5B). In addition, co-transfection of lncRNA-MeG3 into the ZEB overexpressing cells upregulated epithelial marker E-cadherin expression levels and down-regulated mesenchymal markers Vimentin and Fibronectin expression levels and inhibited gastric cancer cell proliferation compared with ZEB-only transfection group (Fig. 5C and D). These results indicate that lncRNA-MeG3 may inhibit gastric cancer cell growth via regulation of EMT.

**IncRNA-MEG3 inhibits gastric tumor growth in vivo.** To determine whether endogenous expression of IncRNA-MEG3 was able to affect gastric tumor growth in vivo, HGC-27 cells stably transfected with IncRNA-MEG3 or empty vectors were subcutaneously injected into female mice. Transfection with IncRNA-MEG3 markedly inhibited tumor growth compared to empty vector group mice following a 28-day inoculation (Fig. 6A). Immunohistochemical analysis revealed that IncRNA-MEG3 transfection suppressed tumor growth in vivo, predominantly by decreasing VEGF and Bcl-2 expression (Fig. 6B). These results suggested that IncRNA-MEG3 expression may inhibit gastric tumor growth in vivo.

**Discussion**

Emerging evidence has demonstrated that IncRNAs are involved in various biological processes, including cell differentiation, growth, apoptosis and cancer metastasis (27-29). In recent years, several IncRNAs have been demonstrated to be associated with gastric tumor growth and metastasis (30,31). Additionally, IncRNA-MEG3 may act as an inhibitor to regulate gastric cancer progression (32,33). In the present study, the
The role of lncRNA-MeG3 in gastric cancer cells was analyzed. The results demonstrated that lncRNA-MeG3 transfection promoted the apoptosis and markedly inhibited the growth and metastasis of gastric cancer cells. lncRNA-MeG3 may also inhibit gastric cancer cell growth through the regulation of EMT.

Numerous studies have revealed the emerging significance of lncRNAs in various human tumors. lncRNAs serve crucial roles in tumor invasion and metastasis through the regulation of tumor suppressor and oncogenic pathways. The link between lncRNAs and tumors is gaining increasing attention in oncology. A previous study reported that lncRNA-MeG3 upregulation and downregulation of antisense non-coding RNA at the INK4 locus may be a clinically relevant strategy in the treatment of gallbladder cancer (34). The present study demonstrated that lncRNA-MeG3 transfection...
significantly inhibited gastric cancer cell growth, migration and invasion.

Tumor resistance to apoptosis in human cancer has a crucial role in metastasis. A previous study indicated that lncRNA-MEG3 upregulation induced renal cell carcinoma cell apoptosis by activating the mitochondrial pathway (35). In the present study, lncRNA-MEG3 promoted the apoptosis of gastric cancer cells. lncRNA-MEG3 can also inhibit the proliferation of cervical carcinoma cells through the induction of cell cycle arrest and apoptosis (36). It may also inhibit non-small cell lung cancer cell proliferation and promote apoptosis through the regulation of p53 expression (37). The present study demonstrated that lncRNA-MEG3 transfection increased the expression pro-apoptotic proteins caspase-3 and caspase-9 in gastric carcinoma cells. Additionally, lncRNA-MEG3 transfection inhibited the expression anti-apoptotic proteins Bc1-2 and Bc1-w. Thus, the results indicated that lncRNA-MEG3 transfection reduced the apoptotic resistance of gastric carcinoma cells.

Several reports have demonstrated that EMT has an essential role in the regulation of gastric carcinoma cell growth, proliferation and apoptosis (38,39). A previous report indicated that EMT in gastric cancer cells can be induced through the activation of the interleukin-6/signal transducer and activator of transcription 3 signaling pathway (40). In the current study, lncRNA-MEG3 transfection upregulated the expression of the epithelial marker E-cadherin and downregulated the expression of mesenchymal markers vimentin and fibronectin in gastric cancer cells. EMT induction prevented the lncRNA-MEG3 transfection-inhibited growth of gastric cancer cells. The role of lncRNA-MEG3 in suppressing gastric carcinoma cell proliferation, migration and invasion was primarily investigated; however, the present study did not analyze EMT-associated signaling pathways.

In conclusion, the potential involvement of lncRNA-MEG3 in gastric cancer was explored. The results suggest that lncRNA-MEG3 may inhibit the growth of gastric cancer cells through the regulation of EMT. However, further study is required to identify other potential mechanisms of lncRNA-MEG3 involvement in gastric cancer progression.

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Availability of data and materials

The datasets generated and analyzed during the current study are not publicly available due to further research being performed, but are available from the corresponding author on reasonable request.

Authors' contributions

JJ and SZ designed the study. JJ and SZ performed the experiments. SZ analyzed the data.

Ethics approval and consent to participate

The protocol was approved by The Ethics Committee of Xi'an Jiao Tong University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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