miR-519 regulates the proliferation of breast cancer cells via targeting human antigen R

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Abstract. Breast cancer is one of the most prevalent types of cancer among women that leads to millions of deaths worldwide every year. The mechanisms of breast cancer pathogenesis remain unclear. It has been reported that aberrant expression of miR-519, is associated with breast cancer development; however, the effects of miR-519 on breast cancer cell proliferation remain unknown. Therefore, the present study aimed to determine whether miR-519 could regulate breast cancer cell proliferation. A total of 20 pairs of primary breast cancer and adjacent normal tissues were collected from patients with breast cancer. miR-519 expression level was determined by reverse transcription-quantitative polymerase chain reaction. Furthermore, miR-519 mimics or inhibitors were transfected into breast cancer MCF-7 cells in order to up- or downregulate miR-519 expression. Subsequently, human antigen R (HUR), BCL-2 and BAX protein levels were analyzed by western blotting. MCF-7 cell proliferation was assessed using MTT and colony formation assays. A luciferase assay was performed to verify whether miR-519 could directly bind to HUR mRNA. The results demonstrated that miR-519 expression level was lower in primary breast cancer tissues compared with adjacent normal tissues. Furthermore, miR-519 overexpression and downregulation inhibited and stimulated MCF-7 cell proliferation, respectively. In addition, the results from luciferase assay demonstrated that HUR was a target of miR-519. HUR overexpression could reverse the effect of miR-519 mimics on MCF-7 cell proliferation, whereas HUR silencing could rescue the effect of miR-519 inhibitors on MCF-7 cell proliferation. These findings suggested that miR-519 may regulate MCF-7 cell proliferation by targeting HUR.

Introduction

Breast cancer is one of the most common types of cancer in women that is responsible for high number of cancer-associated mortality cases worldwide (1). Each year, ~1.7 million new patients are diagnosed with breast cancer, and >0.5 million women die from breast cancer worldwide (2). Early diagnosis and treatment could reduce the mortality rate of patients with breast cancer. However, numerous patients are diagnosed at an advanced stage, which is the major reason for frequent mortality (3). The main treatments for breast cancer include chemotherapy, surgery, targeted therapy, hormone replacement therapy and radiation therapy (4). There are numerous causes of breast cancer, including lifestyle (obesity, alcohol, Western-style diet) (5), depression and anxiety disorder (6) and gene mutations, including breast cancer (BRCA) gene mutation (7); however, the mechanisms underlying breast cancer pathogenesis remain unclear.

MicroRNAs (miRs) represent a class of 21-23 nucleotides long small non-coding RNAs that can act as onecogens (8) or tumor suppressors (9) in various types of cancer, including gastrointestinal (10), liver (11), pancreatic (12), ovarian (13), lung (14) and breast cancer (15). miRs can bind directly to the 3'-untranslated region (UTR) of target genes to negatively regulate protein expression (16). It has been reported that miRs can regulate the proliferation, differentiation, migration, metastasis and apoptosis of cancer cells. In addition, numerous miRs are associated with breast cancer pathogenesis. For example, miR-454-3p can promote breast cancer metastasis by regulating Wnt/β-catenin signaling pathway (15). Furthermore, miR-29a contributes to breast cancer cell epithelial-mesenchymal transition, migration and invasion by targeting suppressor of variegation 4-20 homolog 2 (17). In addition, miR-449b-5p suppresses the proliferation and invasion of breast cancer cells by regulating the cell cycle-related and expression elevated protein in tumor-mediated Wnt/β-catenin signaling (18).

The miR-519 family is located on human chromosome 9 and includes miR-519a-3p, miR-519b-3p, miR-519c-3p, miR-519a-5p and miR-519b-5p. miR-519a-3p, miR-519b-3p and miR-519c-3p have similar sequences and share the same seed sequence (19). It has been reported that the miR-519 family is associated with cancer development. For example, miR-519
suppresses nasopharyngeal carcinoma cell proliferation by targeting the oncogene upregulated gene 4/upregulator of cell proliferation (20). However, the role of miR-519 in the development of breast cancer remains unknown. The present study demonstrated that miR-519 was downregulated in primary breast cancer tissues. The findings from this study suggested that miR-519 may regulate breast cancer cell proliferation by targeting human antigen R (HUR).

**Materials and methods**

**Clinical breast cancer tissues.** A total of 20 pairs of breast cancer tissues and adjacent normal breast tissues were collected from patients with breast cancer at the Department of General Surgery of The Fourth Hospital of Hebei Medical University between January 2017 and October 2018. All patients (age range, 36-84 years; average age, 58 years) underwent primary surgical treatment without any preoperative chemotherapy or radiotherapy. Adjacent normal breast tissues were obtained from the margins of tumors (≥5 cm from the tumor). The tissues were flash-frozen in liquid nitrogen and stored at -80°C. All clinicopathological information was recorded. Each patient had read and signed informed consent for tissue donation. This study was approved by the Ethical Review Committee of Hebei Medical Hospital.

**Breast cancer cell culture.** The human breast cancer cell line MCF-7 was purchased from the American Type Culture Collection. MCF-7 cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS; both HyClone; GE Healthcare Life Sciences), 80 U/ml penicillin (Gibco; Thermo Fisher Scientific, Inc.) and 100 µg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.) and placed at 37°C in a 95% humidified atmosphere containing 5% CO₂.

**Cell transfection.** The miR-519 family contains miR-519b-3p, miR-519c-3p and miR-519a. The miR oligos for miR-519 mimic, miR mimic negative control, miR-519 inhibitor and miR inhibitor negative control were provided by Shanghai GenePharma Co., Ltd. The sequences of the miRs were as follows: miRNA mimics negative control forward, 5'-UUCUCCGAACGU GUCACGUU-3' and reverse, 5'-ACGUGACACGUUCCGG AGAAUU-3'; miR-519a mimic forward 5'-AAAGUGCAU CCUUUAAGAGU-3' and reverse, 5'-ACACUCUAAAG GAUCGACU-3'; miR-519b mimic forward 5'-AAAGUG CAUCCUUUAAGAGU-3' and reverse, 5'-AAGCUCUAA AAGGAUGCACU-3'; miR-519c mimic forward, 5'-AAA GUGCAUCCUUUAAGAGAUGU-3' and reverse, 5'-AUGC UCUCUAAAGGAUGACGAU-3'; miR inhibitor negative control forward 5'-UUCUCCGAACGUUCCGGAGAUU-3' and reverse, 5'-ACGUGACACGUUCCGGAGAAUU-3'; miR-519a inhibitor, 5'-UCACACUCUAAAGGAUGACAU-3'; miR-519b inhibitor, 5'-UCUCUAAAGGAUGACAU-3'; and miR-519c inhibitor, 5'-UCAUCCUUAAGGAUGACAU-3'. One day prior to transfection, MCF-7 cells were seeded in 6-well plates at the density of 3.5x10⁵ cells/well in 1.6 ml RPMI-1640 medium containing 10% FBS. Of miRNA oligos were individually diluted into diethylyl pyrocarbonate (DEPC)-treated H₂O to the concentration of 20 nM and mixed together in a 1:1:1 ratio (v/v). A volume of 4 µl miR-519 mimics or inhibitors (containing mimics or inhibitors for miR-519a, miR-519b and miR-519c) were diluted in serum-free RPMI-1640 medium (100 µl). Then, HiperFect transfection reagent (2 µl) was diluted in serum-free RPMI-1640 medium (100 µl). Then, the two reagents were mixed together and added to each well. The cells were harvested 48 h following transfection for subsequent experiments.

**Small interfering (si)RNA targeting HUR**

Small interfering (si)RNA targeting HUR (cat. no. sc-35619) was obtained from Santa Cruz Biotechnology, Inc. and is a pool of three HUR-specific siRNAs. The sequences of siRNAs were as follows: Si-HUR-1 forward, 5'-GGCGAGGUUGAUAUCGCAA AUU-3' and reverse, 5'-UUUGCAGAUCAUCUGC CUU-3'; si-HUR-2 forward, 5'-GACCAUGCAAAACUA UGAAUU-3' and reverse, 5'-UCAUAGUUGUCAUAGGU CUU-3'; si-HUR-3 forward, 5'-GCUGUGAUCUCAU CUAUU-3' and reverse, 5'-UAGAUGAAGAGUGACACG CUU-3' siRNA negative control forward, 5'-UUUCUCGAA CGUGUCAGCUU-3' and reverse, 5'-ACUGUGACAGU CGAGAATT-3'. One day prior to transfection, MCF-7 cells were seeded in 6-well plates at the density of 3x10⁵ cells/well in 1.6 ml RPMI-1640 medium containing 10% FBS. siRNA oligos were individually diluted into diethyl pyrocarbonate (DEPC)-treated H₂O to the concentration of 20 nM and mixed together in a 1:1:1 ratio (v/v). A volume of 4 µl siRNAs (containing three siRNAs) were diluted in serum-free RPMI-1640 medium (100 µl). Then, HiperFect transfection reagent (2 µl) was diluted in serum-free RPMI-1640 medium (100 µl). The two reagents were mixed together and added to each well. The cells were harvested 48 h following transfection for subsequent experiments.

**Adenovirus vector transfection.** Recombinant adenoviruses expressing HUR (AD-HUR) and control adenovirus vector containing GFP (AD-CON) were purchased from Shanghai GeneChem Co., Ltd. One day prior to transfection, MCF-7 cells were seeded in 6-well plates at the density of 3.5x10⁵ cells/well in 2 ml RPMI-1640 medium containing 10% FBS. The adenovirus transfection was performed at a multiplicity of infection) of 30. At 48 h following infection, the cells were harvested.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** After 48 h of miR oligo transfection, total RNA was extracted from MCF-7 cells using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.). RNA (1 µg) was reverse transcribed into cDNA. RNA (1 µg), miR-specific stem-and-loop RT-primers (1 µl), dNTP (2 µl; 10 mM) and DEPC-treated H₂O (6 µl) were mixed together and incubated at 70°C for 10 min, then cooled on ice. Recombinant RNase inhibitor (0.5 µl; 40 U/µl), M-MuLV reverse transcriptase (0.5 µl; 200 U/µl; New England Biolabs, Inc.), 2 µl 10X reverse transcriptase buffers and 6 µl DEPC-treated H₂O were added. The RT reactions were performed as follows: 42°C for 60 min, 95°C for 5 min, and 4°C forever. The sequences of the RT primers were as follows: miR-519a RT, 5'-GTGAGTTATTCGCTGAGGTGC GAGGATTTGCAGCCTC-3'; miR-519b RT, 5'-GTGAGTTATTCGCTGAGGTGC GAGGATTTGCAGCCTC-3'; miR-519c RT, 5'-GTGAGTTATTCGCTGAGGTGC GAGGATTTGCAGCCTC-3'.
membranes (EMD Millipore). Membranes were blocked with a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Inc.). Total protein was extracted by using 100 µl of RIPA Buffer per well (Cell Signaling Technology, Inc.). The cell lysate was centrifuged at 10,000 x g for 10 min at 4˚C. Supernatant fraction was collected, and protein concentration was determined using a protein assay kit (Qiagen China Co., Ltd.) for 48 h. The luciferase activity was measured by using a dual-luciferase reporter assay system (Promega Corporation). Luciferase activity was normalized to Renilla luciferase activity. A total of six samples were measured for each group. The experiment was repeated three times.

**MTT assay.** MCF-7 cell proliferation was determined using MTT assay (Sigma-Aldrich; Merck KGaA). MCF-7 cells were cultured in 96-well plate at the density of 5,000 cells per well in 100 µl RPMI-1640 medium containing 10% FBS. After 24, 48 and 72 h, 20 µl MTT (10 mg/ml) was added into each well for 4 h. Supernatant was discarded and 150 µl DMSO was added to dissolve purple formazan crystals. Absorbance was read at 490 nm on a microplate reader (Bio-Rad Laboratories, Inc.).

**Colony formation assay.** One day following transfection of miRNA mimics or inhibitors, ~300 MCF-7 cells were seeded in each well of 6-well plate and cultured at 37˚C for 2 weeks. The medium was removed once a week and replaced with fresh medium containing the miRNAs mimics or inhibitors transfection mixture. After 14 days, cells were washed three times with PBS and fixed with 4% polymerized formaldehyde (Beijing Solarbio Science & Technology Co., Ltd.) for 20 min at room temperature. Cells were stained with 2.5% crystal violet staining solution (Beijing Solarbio Science & Technology Co., Ltd.) for 30 min at room temperature. Cells were washed with PBS three times and air-dried. The colonies that contained >50 cells were counted with the naked eye in each well. The relative colony number was calculated as the ratio of cells transfected with miR-519 mimics (519 m) to cells transfected with negative control mimics (NCm), or cells transfected with miR-519 inhibitors (519i) to cells transfected with negative control inhibitors (NCi). All experiments were carried out in triplicate each time and repeat three times.

**Statistical analysis.** SPSS 13.0 statistical software package (SPSS, Inc.) was used to perform statistical analysis. All data are presented as the means ± standard error of the mean. The non-parametric Mann-Whitney U test was used to compare two groups. One-way analysis of variance followed by Turkey's post hoc test was used to compare three or more groups. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**miR-519 is downregulated in breast cancer tissues.** The sequences of miR-519a-3p, miR-519b-3p and miR-519c-3p were highly conserved (Fig. 1A). miR-519 expression level was analyzed in 20 breast cancer tissues and adjacent normal tissues. miR-519 may therefore serve a role in breast cancer development.

**miR-519 regulates breast cancer cell proliferation.** miR-519a-3p, miR-519b-3p and miR-519c-3p shared similar sequences, and their expression levels were decreased in breast cancer tissues. The mimics of miR-519a-3p, miR-519b-3p and miR-519c-3p were then mixed equally and transfected into MCF-7 cells for 48 h. The results from RT-qPCR demonstrated that miR-519a-3p, miR-519b-3p and miR-519c-3p expression levels were...
significantly increased in MCF-7 cells following transfection with miR-519 mimics compared with MCF-7 cells transfected with negative control mimics (Fig. 2A). The results from MTT and colony formation assays demonstrated that MCF-7 cell proliferation was inhibited following transfection with miR-519 mimic (Fig. 2B and C). To verify the effect of miR-519 on MCF-7 cell proliferation, the inhibitors of miR-519a-3p, miR-519b-3p and miR-519c-3p were mixed equally and transfected into MCF-7 cells for 48 h. The results from RT-qPCR demonstrated that miR-519a-3p, miR-519b-3p and miR-519c-3p expression levels were significantly lower in MCF-7 cells transfected with miR-519 inhibitors compared with MCF-7 cells transfected with negative control inhibitors (Fig. 2D). The results from MTT and colony formation assays demonstrated that MCF-7 cell proliferation was increased following transfection with miR-519 inhibitors (Fig. 2E and F). These results suggested that miR-519 could regulate MCF-7 cell proliferation.

miR-519 directly targets HUR. It has been reported that miRs execute their biological functions by directly regulating downstream target genes. Furthermore, it was predicted that HUR mRNA possesses two binding sites (19). One was at 886-980 nt in the coding region of HUR and the other was at 4332-4337 nt in the 3'UTR of HUR (Fig. 3A). A luciferase activity assay was used to determine whether miR-519 could bind directly to HUR mRNA. The results demonstrated that miR-519 mimic transfection significantly decreased the luciferase activity of cells transfected with a pmiRGLO vector containing the binding sites (pmiRGLO-3'utr) (Fig. 3B, left panel) compared with cells transfected with pmiRGLO-3'UTR only. Furthermore, luciferase activity was significantly inhibited in MCF-7 cells co-transfected with pmiRGLO-3'utr and miR-519 mimics (Fig. 3B, middle panel) compared with MCF-7 cells transfected with pmiRGLO control vector and miR-519 mimic. Transfection with miR-519 inhibitors had no effect on luciferase activity (Fig. 3B, right panel). The results from western blotting demonstrated that miR-519 overexpression significantly decreased HUR and BCL-2 protein levels and significantly increased BAX protein level (Fig. 3C). Conversely, HUR and BCL-2 protein levels were significantly increased and BAX protein level was significantly decreased in MCF-7 cells transfected with miR-519 inhibitors (Fig. 3D). HUR may therefore be a direct target gene of miR-519.

HUR protein level is upregulated in breast cancer tissues. HUR protein level was analyzed in 20 pairs of breast cancer and adjacent normal tissues. The results demonstrated that HUR and BCL-2 protein levels were significantly higher, whereas BAX protein level was significantly lower in breast cancer tissues compared with adjacent normal tissues. (Fig. 4A). Next, HUR-specific siRNA (si-HUR) was transfected into MCF-7 cells. As presented in Fig. 4B, HUR protein level was decreased to ~50% following si-HUR transfection. Furthermore, BCL-2 protein level was significantly lower, and the BAX protein level was significantly higher in MCF-7 cells transfected with si-HUR compared with cells transfected with negative control siRNA (NC). HUR may therefore serve a role in breast cancer development.

miR-519 regulates MCF-7 cell proliferation via targeting HUR. To verify whether miR-519 could regulate MCF-7 cell proliferation by negatively regulating HUR, HUR protein expression was up- or downregulated in MCF-7 cells. An adenovirus vector expressing HUR was constructed and transfected into MCF-7 cells. The results demonstrated that HUR overexpression could reverse the effects of miR-519 mimic on BAX and BCL-2 protein levels (Fig. 5A) and MCF-7 cell proliferation (Fig. 5B and C). In addition, HUR-specific siRNA was designed and transfected into MCF-7 cells to silence HUR protein expression. The results demonstrated that HUR downregulation could
Figure 2. miR-519 regulates MCF-7 cell proliferation. (A) Relative mRNA levels of miR-519a-3p, miR-519b-3p and miR-519c-3p in MCF-7 cells transfected with miR-519 mimics (miR-519m group) and negative control mimics (NC group). (B) Colony formation in MCF-7 cells transfected with miR-519 mimics. (C) MCF-7 cell proliferation determined by MTT in MCF-7 cells transfected with negative control mimics (NC group) or miR-519 mimics (miR-519m group). (D) Relative mRNA levels of miR-519a-3p, miR-519b-3p and miR-519c-3p in MCF-7 cells transfected with miR-519 inhibitors (miR-519i group) and negative control inhibitors (NCi group). (E) Colony formation in MCF-7 cells transfected with miR-519 inhibitors. (F) MCF-7 cell proliferation determined by MTT in MCF-7 cells transfected with negative control inhibitors (NCi group) or miR-519 inhibitors (miR-519i group). *P<0.05, **P<0.01 vs. control group. n=5. 519i, miR-519 inhibitors; 519m, miR-519 mimics; NC, negative control; NCi, negative control inhibitors; OD, optical density.
Figure 3. miR-519 directly targets HUR. (A) A bioinformatics database was used to predict the binding sites of miR-519 on HUR mRNA. One was at 886-980 nt in the coding region and the other was at 4332-4357 nt in the 3’-UTR. (B) Luciferase activity assay was performed in MCF-7 transfected with negative control mimics (NC group), miR-519 mimics (miR-519m group), negative control inhibitors (NCi group) or miR-519 inhibitors (miR-519i group). Western blotting was used to measure HUR, BAX and BCL-2 protein levels in MCF-7 cells transfected with (C) miR-519 mimics or (D) miR-519 inhibitors. *P<0.05, **P<0.01 vs. control group. n=5. 519i, miR‑519 inhibitors; 519m, miR‑519 mimics; HUR, human antigen R; NC, negative control; NCi, negative control inhibitors; nt, nucleotide.
rescue the effects of miR-519 inhibitors on BAX and BCL-2 protein levels (Fig. 5D). The results from colony formation and MTT assays indicated that HUR silencing could reverse the effects of miR-519 on MCF-7 cell proliferation. Taken together, these results suggested that miR-519 may regulate MCF-7 cell proliferation by targeting HUR.

**Discussion**

Numerous factors can lead to breast cancer, including lifestyle (obesity, inactivity, alcohol) (22) and BRCA gene mutation (23). Aberrant expression of miRs, including miR-148a-3p (24), miR-124-3p (25) and miR-301a-3p (26), can also contribute to the pathogenesis of breast cancer. The present study focused on the effects of miR-519 on breast cancer cell proliferation. The results suggested that miR-519 may serve a crucial role in breast cancer cell proliferation.

The miR-519 family includes miR-519a-3p, miR-519b-3p, miR-519b-3p, miR-519c-3p, miR-519a-5p and miR-519b-5p. miR-519 robustly inhibits HeLa cell proliferation and induces HeLa cell senescence (27). miR-519 suppresses nasopharyngeal carcinoma cell proliferation (20). The present results demonstrated that the relative expression levels of miR-519a-3p, miR-519b-3p and miR-519c-3p were lower in primary breast cancer tissues compared with in adjacent normal tissues. The present study demonstrated that miR-519

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### Table

| miR      | Relative expression level |
|----------|---------------------------|
| miR-519a-3p | Lower                    |
| miR-519b-3p | Lower                    |
| miR-519c-3p | Lower                    |

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Figure 4. HUR protein level is upregulated in breast cancer tissues. (A) HUR protein level was analyzed in 20 pairs of breast cancer and adjacent normal tissues. B) HUR, BCL-2 and BAX protein levels were measured in MCF-7 cells transfected with HUR-specific siRNAs (si-HUR) and negative control siRNA (NC).
overexpression could inhibit MCF-7 cell proliferation, whereas miR-519 downregulation could promote MCF-7 cell proliferation. These results were consistent with those from a previous study (19,20).

miRs exert their biological function by regulating their target genes. It has been reported that miR-519 can regulate two prominent subsets of genes. One subset of target genes encodes proteins involved in DNA maintenance, including deoxyuridine 5'-triphosphate nucleotidohydrolase, exonuclease 1, replication protein A2 and DNA polymerase epsilon 4, accessory subunit. The other subset of target genes encodes proteins that control intracellular calcium levels, including ATPase secretory pathway Ca2+ transporting 1ATP2C1 and ORAI calcium release-activated calcium modulator 1 (27). The present study demonstrated that HUR was a target of miR-519. The results from luciferase assay confirmed that miR-519 could directly bind to HUR mRNA and negatively regulate HUR protein expression. HUR is a RNA-binding protein that can bind and stabilize AU rich element-mRNAs (28). HUR is abundantly expressed in cancer cells and associated-malignant phenotypes (29). HUR has been implicated in numerous cellular events, including...
proliferation, senescence, differentiation, apoptosis, and stress and immune responses. In turn, HUR can promote certain processes, including cancer pathogenesis and inflammation (30). HUR can exert its effects in two ways. Firstly, HUR acts as a positive effector for gene expression by binding and stabilizing their mRNA, for example cyclooxygenase 2, p21 and cyclin D1 (31-33). Secondly, HUR can decrease the translation efficiency of mRNA, including of TNF-α and p27 (34,35). The results from the present study demonstrated that HUR protein level was increased in primary breast cancer tissues compared with adjacent normal tissues, and that this effect was accompanied by increased BCL-2 expression and decreased BAX expression. Furthermore, the present study demonstrated that HUR could promote BCL-2 expression and inhibit BAX expression. HUR overexpression could rescue the effects of miR-519 mimics on MCF-7 cell proliferation and on BCL-2 and BAX expression, whereas HUR silencing could reverse the effects of miR-519 inhibitors on MCF-7 cell proliferation and on BCL-2 and BAX expression. Previous studies suggested that HUR could be a positive regulator of BCL-2 by promoting BCL-2 mRNA stability and translation efficiency (36,37). BCL-2 can promote the survival and proliferation of cells and therefore participate in the pathogenesis of cancer and tumor growth (38). BAX is a pro-apoptotic effector that can be inhibited by BCL-2 (39). Both proteins belong to the BCL-2 family, although they have opposite effects on cell apoptosis. BCL-2 overexpression can inhibit BAX insertion into the mitochondrial outer membrane but spontaneously increase BAX localization to the mitochondria (40). Taken together, the findings from the present study suggested that miR-519 may regulate breast cancer cell proliferation by targeting HUR, which may be considered as a potential therapeutic target in breast cancer.

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Availability of data and materials

The dataset used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LR, YL and QZ designed the experiments. LR, LF and BT performed the experiments. AZ and HY analysed the data. LR and YL wrote and revised the manuscript. All authors approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethical Review Committee of Hebei Medical University (Shijiazhuang, China). Written informed consent was obtained from each patient.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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