Matrine inhibits ovarian cancer cell viability and promotes apoptosis by regulating the ERK/JNK signaling pathway via p38MAPK

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Abstract. Ovarian cancer displays the highest mortality rate among all types of gynecological cancer worldwide. The survival of patients with ovarian cancer remains poor due to poor responses to anticancer treatments. The present study aimed to investigate the therapeutic effects and potential mechanism underlying matrine in ovarian cancer tissues, ovarian cancer cells and a CAOV-3-derived tumor-bearing mouse model. MTT, migration, invasion, flow cytometry, immunofluorescence and immunohistochemistry assays were performed to assess the inhibitory effects of matrine on ovarian cancer. A xenograft ovarian cancer mouse model was established and treated with matrine or PBS. The results demonstrated that compared with the control group, matrine significantly induced ovarian cancer cell apoptosis by upregulating caspase-8 and Fas cell surface death receptor (Fas) expression levels, and downregulating Bcl-2 and Bcl-xl expression levels. Moreover, compared with the control group, matrine significantly inhibited ovarian cancer cell viability, migration and invasion by downregulating metastasis associated protein-1, fibronectin, angiotensin II type 2 receptor–interacting protein 3a and H high mobility group AT-hook 2 expression levels. Compared with the control group, matrine significantly increased p38MAPK, phosphorylated (p)ERK/ERK and pJNK/JNK expression levels in ovarian cancer cells. p38MAPK knockdown significantly downregulated p38MAPK, pERK/ERK and pJNK/JNK expression levels compared with the control group, which significantly promoted ovarian cancer cell viability, migration and invasion. In vivo experiments demonstrated that compared with the control group, matrine significantly suppressed tumor growth by markedly upregulating p38MAPK, ERK and JNK expression levels. The immunohistochemistry results demonstrated that caspase-8 and Fas expression levels were notably increased, whereas Bcl-2 and Bcl-xl expression levels were obviously decreased in matrine-treated tumors compared with PBS-treated tumors. In conclusion, the present study demonstrated that matrine inhibited ovarian cancer cell viability, migration and invasion, but induced cell apoptosis, suggesting that matrine may serve as a promising anticancer agent for the treatment of ovarian cancer.

Introduction

Ovarian cancer displays the highest mortality rate (47.4%) among all types of gynecological cancer worldwide (1). Despite the development of aggressive frontline treatments, including surgery and adjuvant chemotherapy, the 5-year survival rate of ovarian cancer is <25% for women diagnosed with stage III or IV disease worldwide (2). The majority of patients with ovarian cancer are diagnosed at an advanced stage, and the survival rate is largely dependent upon the stage of the cancer and the treatment strategies used (3). Clinical investigation has reported that metastasis is a primary contributor to the poor survival of patients with ovarian cancer (4). A systematic review and meta-analysis demonstrated that ovarian cancer often metastasizes throughout the peritoneal cavity, and even to the parenchyma of the liver or lung (5). Therefore, the survival of patients with ovarian cancer remains poor due to a poor responses to anticancer treatments (6).

Matrine (C₁₅H₂₄N₂O), an alkaloid extracted from the traditional Chinese herb Sophora alopecuroides L., has been reported to display potential therapeutic efficacy for epithelial cell-related human diseases (7,8). Previous studies have demonstrated that matrine displays a variety of pharmacological activities, including anticancer and anti-inflammatory activities, and potential therapeutic value in chronic liver and renal diseases, heart failure, diabetes mellitus and human malignancies (9,10). Moreover, it has been reported that matrine regulates numerous biological activities, including inflammation, apoptosis, fibrosis, oxidative stress and immune (11). It has also been demonstrated that matrine displays therapeutic benefits in the progression of cancer via inducing tumor cell
apoptosis in xenograft mouse models (12,13). Additionally, the antitumor activities of matrine and possible molecular targets for cancer prevention and treatment have been previously reviewed (14).

A number of clinical reports have indicated that the ERK and JNK signaling pathways are associated with ovarian cancer cell viability, migration and invasion (15,16). The JNK signal pathway contributes to cisplatin resistance of ovarian cancer cells and is a potential target to overcome ovarian cancer cell resistance to apoptosis (17). Another study reported that targeting the PI3K/mTOR and RAS/ERK signaling pathways can inhibit ovarian cancer cell viability, migration and invasion (18). Moreover, the p38MAPK signaling pathway is associated with cisplatin resistance in human ovarian cancer cells (19). Therefore, it was hypothesized that matrine might regulate ovarian cancer cell viability, migration, invasion and apoptosis via the p38MAPK/ERK/JNK signaling pathway.

A previous study has reported that angiotensin II type 2 receptor-interacting protein 3a (ATIP3a) downregulation was associated with enhanced salivary adenoid cystic carcinoma cell migration and invasion (20). Molina et al (21) demonstrated that ATIP3a limits cancer cell migration and metastatic progression. In addition, Malek et al (22) reported that high mobility group AT-hook 2 (HMG2) might serve as a potential target for the treatment of patients with ovarian cancer. Several previous studies have indicated that metastasis associated protein-1 (MTA-1) is associated with human cancer cell metastasis (23-25). Furthermore, Kenny et al (26) demonstrated that fibronectin (FN) overexpression could increase ovarian cancer metastasis, suggesting that FN expression serves crucial roles in ovarian cancer metastasis (27). Therefore, the present study assessed the effects of matrine on ATIP3a, HMG2, MTA-1 and FN expression in ovarian carcinoma cells.

The present study aimed to assess the role of matrine in the progression of ovarian carcinoma by investigating the inhibitory effects of matrine on ovarian cancer cell viability both in vitro and in vivo. In addition, the present study analyzed the potential mechanism underlying matrine in ovarian cancer cells. Following treatment with matrine, the ERK/JNK signaling pathway was analyzed and tumor growth in xenograft mice was also assessed.

Materials and methods

Cell culture. The CAOV-3 cell line was purchased from American Type Culture Collection. Cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) at 37°C with 5% CO₂. CAOV-3 cells were treated with matrine (0.20 mg/ml; cat. no. M5319; Sigma-Aldrich; Merck KGaA) or PBS at 37°C for 12 h.

Cell migration and invasion assays. CAOV-3 cells (1×10⁶) in 500 μl serum-free DMEM medium were seeded into the upper chamber (6-well inserts; pore size, 8 μm; BD Biosciences). For Transwell assays, cells were seeded into the upper chamber with 500 μl DMEM, whereas DMEM supplemented with 10% FBS was plated into the lower chamber at 37°C. To assess invasion, Matrigel-coated Invasion Chambers (BD Biosciences) were used according to the manufacturer's protocol. Following incubation for 24 h at 37°C with 5% CO₂, cells were fixed with 4% paraformaldehyde for 10 min at room temperature and stained with 0.5% crystal violet (Sigma-Aldrich; Merck KGaA) for 15 min at room temperature. Stained cells were visualized using a light BZ-X700 microscope (Keyence Corporation; magnification, x10).

p38MAPK knockdown. Small interfering (si)RNA targeted against p38 (si-p38) and siRNA negative control (NC; scrambled control) were synthesized by Guangzhou RiboBio Co., Ltd. The sequences of the siRNAs were as follow: si-p38 forward, 5'-AGUGCGGUAUAGGACCUAUACCUA-3' and reverse, 5'-AAUUGGUGGCAGGCUCUU-3'; and control forward, 5'-CUCGUCUCAUUGCATGAGT-3' and reverse, 5'-AAAAUUCCCGUGUGAGCCAGUUU-3'. siRNA concentrations were determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc.). Subsequently, CAOV-3 cells (1×10⁶) were transfected with 100 pmol si-p38 or NC using RNAi MAX (Thermo Fisher Scientific, Inc.) at 37°C according to the manufacturer's protocol. At 72 h post-transfection, cells were used for subsequent experiments.

Cell viability assay. The effect of matrine on CAOV-3 ovarian cancer cell viability was analyzed by performing MTT assays. Briefly, CAOV-3 cells (1×10⁶ cells/well) were seeded into 96-well plates. Following incubation for 24 h at 37°C, 20 μl MTT solution was added to each well for 30 min at 37°C. Subsequently, the culture medium was removed and 500 μl DMSO was used to dissolve the MTT formazan crystals. An ELISA plate reader (Bio-Rad Laboratories, Inc.) was used to determine optical density at a wavelength of 540 nm.

Flow cytometry. CAOV-3 cell apoptosis was analyzed using an Annexin V-FITC and PI apoptosis detection kit (Becton-Dickinson and Company). CAOV-3 cells (1×10⁶) were stained with FITC-Annexin V and PI for 1 h at 4°C in the dark. Cell apoptosis (early and late apoptosis) was measured using a FACSCalibur flow cytometer (Becton-Dickinson and Company) and analyzed using CellQuest Pro software (version 4.0.2; Becton-Dickinson and Company).

Animal study. A total of 20 male nude mice (age, 6-8 weeks; weight, 25-30 g) were purchased from the Animal Experimental Center of Shandong University. Mice were housed at 25±1°C with 12-h light/dark cycles, 50±5% humidity, and free access to food and water. CAOV-3 cells (2x10⁶) suspended in 0.5 ml PBS were subcutaneously injected into the right flank of each nude mouse. During the experimental period (day 0-30), gross tumor volume was measured using vernier calipers every 3 days. CAOV-3-derived tumor-bearing mice were randomly divided into two groups (n=10 per group): i) oral treatment with matrine (200 mg/kg/day) once a day; or ii) oral treatment with PBS (200 mg/kg/day) once a day. The dosage of matrine was determined according to a previous study (28). On day 30, three mice from each group were sacrificed and tumor weight was measured to assess the inhibitory effects of matrine. The remaining rats (n=7 per group) were used to evaluate 120-day survival. When the tumor diameter reached 18 mm, mice were anesthetized with 1% pentobarbital (40 mg/kg) and sacrificed by decapitation. Animals were monitored for signs of distress.
Western blotting. CAOV-3 cells (1x10^7) were homogenized using RIPA buffer (Sigma-Aldrich; Merck KGaA). Protein concentrations were quantified using the BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). Proteins (30 µg) were separated via 15% SDS-PAGE and transferred to PVDF membranes. Following blocking with 5% BSA (Sigma-Aldrich; Merck KGaA) at 4°C overnight, the membranes were incubated at 4°C overnight with primary rabbit anti-mouse primary antibodies targeted against: Caspase-9 (cat. no. ab52298; 1:1,000; Abcam), Bcl-2 (cat. no. ab182858; 1:1,000; Abcam), Bcl-xl (cat. no. ab32370; 1:1,000; Abcam), phosphorylated (p)ERK (cat. no. ab76299; 1:1,200; Abcam), ERK (cat. no. ab196883; 1:1,000; Abcam), JNK (cat. no. ab31419; 1:1,000; Abcam), pJNK (cat. no. ab32385; 1:1,000; Abcam), caspase-8 (cat. no. ab25991; 1:1,200; Abcam), cytochrome c (Cyto c; cat. no. ab133504; 1:1,000; Abcam), Fas cell surface death receptor (Fas; cat. no. ab82419; 1:1,000; Abcam), p38MAPK (cat. no. ab170099; 1:1,000; Abcam), ATIP3a (cat. no. ab127159; 1:1,000; Abcam), HMGA2 (cat. no. ab52039; 1:1,000; Abcam), MTA-1 (cat. no. ab71153; 1:1,000; Abcam), FN (cat. no. ab24213; 1:1,000; Abcam) and β-actin (cat. no. ab8226; 1:2,000; Abcam). Following washing with PBST (0.01% Tween-20), the membranes were incubated with a HRP-conjugated goat anti-rabbit IgG secondary antibody against β-actin as the loading control.

Immunohistology. CAOV-3-derived tumor tissues were collected from experimental mice (n=3 per group) in each group on day 30. Antigen retrieval was performed using eBio-science™ IHC Antigen Retrieval Solution (cat. no. 00-4955-58; Invitrogen; Thermo Fisher Scientific, Inc.). Tissues were fixed with 10% formalin overnight at room temperature, embedded in paraffin, deparaffinized and rehydrated with ethanol and 0.05% TBST (0.01% Tween-20). Tissues were cut into 4-µm sections, then incubated with 3% hydrogen peroxide for 15 min at room temperature. Subsequently, the sections were blocked with 5% BSA for 2 h at room temperature, washed with PBS, and then incubated overnight at 4°C with rabbit anti-mouse primary antibodies targeted against: Caspase-8 (cat. no. ab25901; 1:1,500; Abcam), Fas (cat. no. ab24533; 1:1,500; Abcam), p38MAPK (cat. no. ab170099; 1:1,500; Abcam), ERK (cat. no. ab196883; 1:1,000; Abcam), JNK (cat. no. ab31419; 1:1,500; Abcam), Bcl-2 (cat. no. ab182858; 1:1,500; Abcam), Bcl-xl (cat. no. ab32370; 1:1,500; Abcam). Following washing with PBS, the sections were incubated with a goat anti-rabbit secondary antibody (cat. no. ab50077; 1:5,000; Abcam) for 1 h at 37°C. Following washing with PBS, DAB was applied for visualization of the proteins. Images were captured using a fluorescence BZ-9000 microscope (Keyence Corporation).

Immunofluorescence. For immunofluorescence, CAOV-3 cells were fixed with 4% paraformaldehyde for 30 min at room temperature. Cells were washed with PBS, blocked with 5% BSA for 2 h at room temperature and washed with PBS. Cells were then stained with DAPI and incubated with rabbit anti-mouse DR2 (cat. no. 8049; 1:2,000; Cell Signaling Technology, Inc.), DR5 (cat. no. 8074; 1:2,000; Cell Signaling Technology, Inc.), AKT (cat. no. 9272; 1:2,000; Cell Signaling Technology, Inc.) and NF-κB (cat. no. 8242; 1:2,000; Cell Signaling Technology, Inc.) primary antibodies. Subsequently, cells were incubated with a HRP-conjugated secondary antibody (cat. no. 7074; 1:2,000; Cell Signaling Technology, Inc.) for 2 h at room temperature. Cells were washed with PBS and counterstained with 5% DAPI for 30 min at room temperature. Stained cells were visualized using a BZ-9000 fluorescence microscope (Keyence Corporation).

TUNEL assay. TUNEL assays were conducted using the ApoTag® Peroxidase In Situ Apoptosis Detection Kit (Sigma-Aldrich; Merck KGaA) according to the manufacturer's protocol. Briefly, tissue sections were fixed with 5% paraformaldehyde overnight at 4°C, washed with PBS and then incubated with proteinase K (15 µg/ml) for 15 min at 37°C, washed with PBS and then incubated with 3% H2O2 for 5 min at 37°C. Subsequently, tissue sections were incubated with TdT buffer for 1 h at 37°C. Following washing with PBS, tissue sections were incubated with an anti-HRP-conjugated antibody for 1 h at 37°C. Signal detection was performed using DAB. Tissues were washed with PBS and counterstained with 5% DAPI for 30 min at room temperature. Data are presented as the fold change in TUNEL-positive nuclei compared with the control. Following mounting with Antifade mounting medium (cat. no. P0126; Beyotime Institute of Biotechnology), apoptotic cells were imaged using a BZ-9000 fluorescence microscope (Keyence Corporation) under five randomly selected fields of views.

Statistical analysis. Data are presented as the mean ± SEM of three independent experiments. Statistical analyses were performed using SPSS software (version 19.0; IBM Corp). Comparisons between two groups were analyzed using the unpaired Student's t-test. Comparisons among multiple groups were analyzed using one-way ANOVA followed by Tukey's post hoc test. Survival was assessed using Kaplan-Meier plots, which were compared using the log-rank test. P<0.05 was considered to indicate a statistically significant difference.

Results

Matrine inhibits ovarian cancer cell viability, migration and invasion by downregulating metastasis-associated genes. Matrine significantly inhibited CAOV-3 cancer cell viability compared with the control group (Fig. 1A). Similarly, CAOV-3 cell migration and invasion were significantly inhibited by matrine compared with the control group (Fig. 1B and C). The western blotting results demonstrated that ATIP3a, HMGA2, MTA-1 and FN protein expression levels were significantly downregulated by matrine in CAOV-3 cells compared with the control group (Fig. 1D and E). The results suggested that matrine inhibited ovarian cancer cell viability, migration and invasion by downregulating metastasis-associated genes.
Matrine promotes ovarian cancer cell apoptosis via the extrinsic apoptotic signaling pathway. Following treatment with matrine for 48 h, CAOV-3 cell apoptosis was significantly increased compared with the control group (Fig. 2A). Caspase-8 and Fas protein expression levels were significantly increased, whereas Bcl-2 and Bcl-xl protein expression levels were significantly decreased in matrine-treated CAOV-3 cells compared with the control group (Fig. 2B and C). Moreover, caspase-9 and Cyto c expression levels were significantly increased in matrine-treated ovarian cancer cells compared with the control group (Fig. 2D). The immunofluorescence assay results demonstrated that DR2, DR5, AKT and NF-κB expression levels were significantly
upregulated by matrine in CAOV-3 cells compared with the control group (Fig. 2E and F). The aforementioned results demonstrated that matrine regulated ovarian cancer cell apoptosis via the extrinsic apoptotic signaling pathway.
Figure 3. Continued.
Matrine suppresses ovarian cancer cell viability, migration and invasion by upregulating the p38-mediated ERK/JNK signaling pathway. Compared with the control group, matrine significantly upregulated p38MAPK, pERK/ERK and pJNK/JNK expression levels in CAOV-3 cells (Fig. 3A). p38MAPK knockdown significantly decreased p38MAPK expression levels in CAOV-3 cells compared with the control group (Fig. 3B). p38MAPK knockdown also significantly decreased pERK/ERK and pJNK/JNK expression levels in CAOV-3 cells compared with the control group (Fig. 3C). However, p38MAPK knockdown did not markedly alter the expression levels of ERK and JNK in CAOV-3 cells compared with the control group. At 24 h post-transfection, p38MAPK knockdown significantly promoted ovarian cancer cell viability, migration and invasion compared with the control group (Fig. 3D and E). Matrine-induced CAOV-3 cell apoptosis was inhibited by p38 knockdown, as demonstrated by the rate of apoptosis not being significantly altered between the control and si-p38 + matrine groups (Fig. 3F). The western blotting results demonstrated that p38 knockdown significantly increased ATIP3a and HMGA2 expression levels compared with the si-NC group (Fig. 3G). However, ATIP3a and HMGA2 expression levels were not significantly different between the si-NC and si-p38 + matrine groups. The immunofluorescence results indicated that p38MAPK knockdown significantly decreased the expression levels of DR2 and DR5 in CAOV-3 cells compared with the control group (Fig. 3H). Collectively, the results demonstrated that matrine suppressed ovarian cancer cell migration and invasion by upregulating the p38MAPK-mediated ERK/JNK signaling pathway.

Matrine inhibits tumor growth and prolongs survival rate in CAOV-3 tumor-bearing mice. Matrine treatment significantly decreased tumor weight compared with the control group (Fig. 4A). The immunohistochemistry results demonstrated that p38MAPK, ERK and JNK expression levels were markedly upregulated by matrine treatment compared with the control group (Fig. 4B). The TUNEL assay results indicated that apoptotic bodies were significantly increased in matrine-treated mice compared with the control group (Fig. 4C). The immunohistochemistry results demonstrated that matrine notably increased caspase-8 and Fas expression levels, and obviously decreased Bcl-2 and Bcl-xl expression levels in tumor tissues compared with the control group (Fig. 4D and E). Long-term observation demonstrated that matrine treatment significantly prolonged the survival rate of CAOV-3-derived tumor-bearing mice compared with the control group (Fig. 4F). The aforementioned results indicated that matrine inhibited tumor growth and prolonged the survival rate of CAOV-3-derived tumor-bearing mice during the 120-day observation period.
Figure 4. *In vivo* effects of matrine treatment on ovarian cancer growth and the survival of ovarian carcinoma-bearing mice. (A) Matrine inhibits tumor growth in CAOV-3-derived tumor-bearing mice compared with the control group. (B) p38MAPK, ERK and JNK expression levels in CAOV-3-derived tumors. (C) Apoptotic bodies in CAOV-3-derived tumors were detected by performing TUNEL assays. Expression levels of (D) Caspase-8, Fas, (E) Bcl-2 and Bcl-xl in CAOV-3-derived tumors. (F) Survival of CAOV-3-derived tumor-bearing mice in 120-day observation. **P<0.01. Fas, Fas cell surface death receptor.
Discussion

The present study investigated the effects of matrine on ovarian cancer cells and the p38MAPK-mediated ERK/JNK signaling pathway. Compared with the control group, matrine significantly inhibited ovarian cancer cell viability, migration and invasion, and induced apoptosis by upregulating the p38MAPK-mediated ERK/JNK signaling pathway. In vivo experiments further verified the hypothesis, suggesting the anticancer potential of matrine for the treatment of ovarian cancer.

The induction of ovarian cancer cell apoptosis via eliciting molecular stimulation or accumulation of intracellular metabolic disturbance status has been systematically reviewed and analyzed in epithelial ovarian cancer cells (29). Apoptotic resistance of ovarian cancer cells induced by various antitumor drugs exhibits a great challenge in neoplastic therapy and has attracted research interest worldwide (30). In addition, caspase-8 upregulation induces apoptosis, reducing the survival of ovarian cancer cells (31). Furthermore, activation of the JNK signaling pathway is associated with the apoptotic signaling pathway induced by chemotherapy via upregulating BRCA1, Fas and Fas ligand expression levels in ovarian cancer cells (32,33). The present study demonstrated that p38MAPK was involved in matrine-induced ovarian cancer cell apoptosis via upregulating Fas and caspase-8 expression levels. The results of the present study provided a possible mechanism underlying matrine-induced ovarian cancer cell apoptosis.

Metastasis is the primary reason leading to higher mortality of patients with ovarian cancer (34). MTA-1 regulates ovarian cancer cell invasion via the EMT signaling pathway, which could be considered as a master regulator in tumorigenesis (35). DN is overexpressed in ovarian tumors, and contributes to local migration and long-distance metastasis of tumor cells (36). Additionally, previous reports have indicated that high HMGA2 and ATIP3a expression levels negatively affect the prognosis of patients with ovarian cancer (37,38). The present study demonstrated that matrine significantly suppressed ovarian cancer cell viability, migration and invasion by downregulating MTA-1, FN, ATIP3a and HMGA2 expression levels compared with the control group. However, the therapeutic effects of matrine on other ovarian cancer cell lines and other cancer cells require further investigation.

It has been reported that amplification of ERK/JNK signaling leads to a reduction in ovarian cancer cell chemosensitivity, which increases the median survival of patients with ovarian cancer (39). Activation of the JAK/STAT, MAPK/ERK and PI3K/Akt signaling pathways contributed to chemotherapy-induced ovarian cancer cell apoptosis, which is associated with favorable patient outcomes (40,41). In the present study, matrine significantly downregulated Bcl-2 and Bcl-x1 expression levels, but significantly upregulated p38MAPK, pERK/ERK, pJNK/JNK, caspase-8 and Fas expression levels compared with the control group. Moreover, compared with the control group, matrine significantly inhibited CAOV-3-derived xenograft growth in nude mice by inducing apoptosis in vivo. Therefore, the present study aimed to investigate the relationship between the ERK/JNK signaling pathway and the anticancer mechanism underlying matrine. The results demonstrated that compared with the control group, matrine significantly increased p38MAPK expression and further upregulated the ERK/JNK signaling pathway, which led to increased ovarian cancer cell apoptosis and suppressed cell migration and invasion. Compared with the control group, matrine significantly improved the survival probability of CAOV-3-derived tumor-bearing mice. Moreover, the results indicated that matrine inhibited CAOV-3 cell viability by regulating the ERK/JNK signaling pathway via p38MAPK. However, the associations between matrine and other signaling pathways, including NF-κB, PI3K/Akt/mTOR and EGFR signaling pathways, should be investigated in future studies.

In conclusion, the present study demonstrated that matrine not only increased ovarian cancer cell apoptosis, but also inhibited ovarian cancer cell viability, migration and invasion, which contributed to tumor growth inhibition and improved long-term survival rates of CAOV-3-derived tumor-bearing mice. Collectively, the results of the present study suggested that matrine suppressed ovarian cancer cell viability, migration and invasion via the p38MAPK-mediated ERK/JNK signaling pathway.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article or available from the corresponding author on reasonable request.

Authors' contributions

LX performed the experiments. JJ designed the study. LX and JJ confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Hongqi Hospital Affiliated to Mudanjiang Medical University (approval no. 20160112CA1).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


