miR-221 regulates proliferation and apoptosis of ovarian cancer cells by targeting BMF

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Abstract. To observe the expression of microRNA-221 (miR-221) in ovarian cancer tissues and its effect and associated mechanism on proliferation and apoptosis in the ovarian cancer SKOV3 cell line. The expression of miR-221 and B-cell lymphoma 2 modifying factor (BMF) mRNA in ovarian cancer and para-carcinoma tissues was detected by reverse transcription-quantitative polymerase chain reaction, the expression of BMF was detected by western blot. MicroRNA.org online predicted that BMF was the possible target gene of miR-221, and the regulatory association was validated by a dual-luciferase reporter gene assay. SKOV3 cells were divided into 8 transfection groups: Anti-miR-negative control (NC); anti-miR-221; phosphorylated internal ribosome entry site 2 (pIRES2)-blank, pIRES2-BMF, small interfering (si)-NC, si-BMF, anti-miR-221+si-BMF and anti-miR-221+pIRES2-BMF groups. Cell proliferation was detected by EdU staining flow cytometry. The effect of transfection on cell apoptosis was detected by Annexin V/PI double staining, and the activity of caspase-3 was detected by spectrophotometry. The effect of anti-miR-221 or pIRES2-BMF transfection on SKOV3 cell proliferation was detected by MTT assay and flow cytometry, and the effect on apoptosis was detected by the Annexin V/PI double staining. Compared with para-cancer tissues, the miR-221 expression was significantly upregulated ($P<0.001$), the BMF mRNA expression was significantly downregulated ($P<0.001$), and the expression of BMF proteins was significantly downregulated in the ovarian cancer tissues. Dual-luciferase reporter gene assay confirmed a targeted regulatory association between miR-221 and BMF. The anti-miR-221 or pIRES2-BMF transfection significantly upregulated BMF expression in SKOV3 cells, significantly decreased cell proliferation and significantly increased cell apoptosis. The overexpression of BMF may enhance the proapoptotic and proliferation-inhibition effect of anti-miR-221 on SKOV3 cells. The transfection of si-BMF significantly promoted cell proliferation, reduced cell apoptosis and attenuated the proapoptotic and proliferation-inhibition effect of anti-miR-221 on cells. The expression of miR-221 was significantly upregulated and the expression of BMF was significantly down-regulated in ovarian cancer tissues. The overexpression of miR-221 antagonized the apoptosis of ovarian cancer SKOV3 cell and promoted the cell proliferation by targeted inhibition of the expression of BMF, which may serve a role in the pathogenesis of ovarian cancer.

Introduction

Ovarian cancer is the fifth most common cause of cancer-associated mortality in females worldwide, and 90% of cases of ovarian cancer are epithelial-derived malignancies (1). Although ovarian cancer has an improved response to chemotherapy, the prognosis of patients with ovarian cancer is not ideal clinically, with very low survival rate (2,3). Studies have indicated that clinical pathological parameters, including age, tumor stage, patient's body function and residual tumor volume, are independent predictors of the prognosis of ovarian cancer (1-3); however, for patients with similar status and treatments, their survival rates were not the same (4). Therefore, it is urgent to identify the factors relating to the occurrence and development of ovarian cancer to provide a scientific basis for the development of effective treatment strategies of ovarian cancer and enhance the survival rate of patients with ovarian cancer.

B-cell lymphoma 2 (Bcl-2) protein family includes a number of members, including anti-apoptosis factors Bcl2-like (Bcl-2L), Bcl-extra-large (xL) and Mcl1, and the pro-apoptosis factor containing the structure of BH3-only (5,6), and includes Bcl2-2L 11, p53 upregulated modulator of apoptosis (PUMA), BH3 interacting domain death agonist, Bcl-2 associated agonist of cell death, Bcl-2 and Bcl-2 modifying factor (BMF) (7,8). Similar to PUMA, BMF serves a role in initiating cell apoptosis by binding to the anti-apoptosis factors B-cell lymphoma (Bcl)2, Bcl-xL and Bcl-w proteins (9). During the occurrence of a variety of tumor types including lymphoma (10), colorectal cancer (11) and breast cancer (12), BMF serves a role of tumor suppressor gene.

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microRNA (miRNA) is a class of endogenous non-coding RNAs ~22 nucleotides in size (12). miRNA can regulate the translation of its target gene mRNA or degrade mRNA by regulating the post-transcriptional level, which is involved in the regulation of numerous biological functions, including cell proliferation, migration, invasion and apoptosis (13-15). miR-221 is a miRNA that is closely associated with tumorigenesis and serves a tumor-promoting role in a variety of tumor types, including breast (16), pancreatic (17), prostate (18,19) and non-small cell lung cancer (20), and miR-221 serves a role as a tumor suppressor gene (21). A number of studies have demonstrated that the expression of miR-221 in tumor tissue or peripheral blood of patients with ovarian cancer was significantly increased, indicating that miR-221 may serve a role of a cancer-promoting gene in the occurrence of ovarian cancer (21,22). MicroRNA.org online (22) predicted that BMF was the possible target gene of miR-221. In the present study, the expression levels of miR-221 and BMF in ovarian cancer tissues were detected by reverse transcription-quantitative polymerase chain reaction (RT-qPCR), and transfected the siRNA of anti-miR-221 or BMF into the ovarian cancer cell line SKOV3 to observe the effect of miR-221 on SKOV3 cell proliferation and apoptosis, and validate the association between miR-221 and target gene BMF and the regulatory effect on the proliferation and apoptosis of SKOV3 cells, to identify a novel treatment target for the clinical treatment of ovarian cancer.

Materials and methods

Clinical data. A total of 30 patients with ovarian cancer who received their treatment in The Department of Obstetrics and Gynecology in The First Affiliated Hospital of Fujian Medical University from February 2013 to December 2014 were collected. None of the patients underwent chemotherapy and radiotherapy prior to surgery. The patients were aged from 33-72 years, with a mean age of 51.3±21.4 years. The tumor specimens and normal para-carcinoma tissue specimens were collected by surgery. Tumor tissue specimens were pathologically confirmed as ovarian cancer, whilst the normal tissues were confirmed as having no cancerous tissue invasion by two pathologists from the First Affiliated Hospital of Fujian Medical University. Specimens were preserved in liquid nitrogen within 10 min following acquisition. All specimens were collected with the patient's informed consent and approved by the Ethics Committee of the First Affiliated Hospital of Fujian Medical University.

Cells and main reagents. Ovarian cancer cell line SKOV3 cells were purchased from the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences in Beijing, China. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Horseradish peroxidase-labeled goat anti-mouse IgG secondary antibody (catalog no. SA00001) was purchased from OriGene Technologies, Inc. (Beijing, China). Annexin V/PI staining kits and caspase-3 enzyme activity test kits were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). TRIzol® and transfection reagent Lipofectamine® 2000 were purchased from Invitrogen (Thermo Fisher Scientific, Inc.). Mimic NC, miR-221 mimic, anti-miR-NC and anti-miR-221 were purchased from Guangzhou Ribobio Co., Ltd. (Guangzhou, China). Luciferase activity detection kits were purchased from Promega Corporation (Madison, WI, USA). Mouse anti-human β-actin (catalog no. MS123A1) and BMF antibodies (catalog no. A21422), and si-NC (catalog no. A24185) and si-BMF (catalog no. A36521) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The cell proliferation detection kit (Click-iT EdU Alexa Fluor 488 Flow Cytometry Assay Kit) was purchased from Molecular Probes (Thermo Fisher Scientific, Inc.). Overexpression plasmid phosphorylated internal ribosome entry site 2 (pIRES2) was purchased from Beijing BioVector (Beijing, China; http://www.biovector.net/).

Detection of gene expression by RT-qPCR. A total of 1 ml TRIzol was added to every 20 mg of tissue or every $3x10^6$ cells. Following full lysis, chloroform was added for extraction. Following separation, the RNA supernatant was transferred to a new Eppendorf (EP) Tube®, precipitated with isopropanol and washed with 70% ethanol, and then dissolved in diethyl pyrocarbonate water to extract RNA. The RNA was reverse transcribed into cDNA using the PrimeScript™ RT reagent Kit (Perfect Real Time, Takara, Japan; URL: http://www.takara-bio.com/), then using cDNA as a template, qPCR was performed for detection of gene expression. The reverse transcription reaction system included 0.5 μl oligdT Primer (50 μM), 0.5 μl Random 6-mers (100 μM), 0.5 μl PrimeScript RT Enzyme Mix, 1.0 μg RNA, 2 μl 5X PrimeScript Buffer, then RNase-free water was added to a total volume of 10.0 μl. The reverse transcription reaction condition: 37˚C for 15 min; and 85˚C for 5 sec. The RT-qPCR reaction system included SYBR Fast qPCR Mix 10.0 μl, 0.8 μl forward Primer (10 μM), 0.8 μl Reverse Primer (10 μM), 2.0 μl cDNA and 6.4 μl RNase Free dH_2O. The qPCR reaction conditions: Initial denaturation 10 min at 95˚C; denaturation 10 sec at 95˚C; annealing 20 sec at 60˚C; and extension 15 sec at 72˚C; 40 cycles, and finally the gene expression was detected in ABI ViiA 7 real-time qPCR instrument. The $2^{-\Delta\Delta Cq}$ method was used to compare gene expression between clinical samples (23).

The primer sequence used were: miR-221P forward, 5'-GTTGGTGGGAGCTACATTGTCTGC-3', and reverse, 5'-GTGTCGTGGACTCGGCAATTC-3'; U6P forward, 5'-ATTGGAACGATACAGAGAAGATT-3', and reverse, 5'-GGAACGCTTCACGAATTTG-3'; BMFP forward, 5'-TTTATGGCAATGCTGGCTATCG-3', and reverse, 5'-GCAATCTGTACCTCTGCTTGATG-3'; and β-actin forward, 5'-GAACCCTAAGGCCAAC-3', and reverse, 5'-TGTCACGCACGATTTCC-3'.

Construction of BMF overexpression plasmids. The fragments of the coding region of the BMF gene were amplified and the size of target fragments was identified by gel electrophoresis (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). SDS PAGE gel (9%) was prepared as follows: 50 g of protein sample was loaded for 3 h and then placed into the electric transducers, in which the buffer was added to the transmembrane for 1.5 h and the target protein was transferred onto nitrocellulose membranes. PCR product was recovered from gel, and following dual-enzyme digestion they were ligated into pIRES2 plasmids, then following transformation the positive strains were selected, and strains were amplified and cultured

to extract the recombinant plasmid containing the target fragments. Sequencing was performed to confirm that the target fragment of BMF gene was inserted correctly, and termed as pIRES2-BMF; in addition, the blank vector pIRES2-blank was used as control.

SKOV3 cell culture and transfection. SKOV3 cells were cultured in DMEM containing 10% FBS and 1% streptomycin, and cultured in a cell incubator at 37˚C and an atmosphere containing 5% CO_2. Medium was replaced once every two days. Cells were subcultured according to the ratio of 1:4; cells in good growth condition at the logarithmic period were taken to carry out experiments. One day prior to transfection, cells were inoculated in a 10 cm culture dish, to ensure that the cell density could be ≤50-60% on the day of transfection. A total of 2 μl Lipofectamine 2000, 30 nmol anti-miR-221p, 20 nmol pIRES2-BMF, and 20 nmol si-BMF were diluted into 50 ml Opti-MEM (Invitrogen; Thermo Fisher Scientific, Inc.) and added to the cells. Following incubation at room temperature for 5 min, the diluted Lipofectamine 2000 and all transfectants were gently mixed well and placed at room temperature for 20 min, then according to difference of the grouping, the transfected mixture was added to cells free of serum and double antibody culture medium (Invitrogen; Thermo Fisher Scientific, Inc.), incubated in 37˚C incubator for 6 h, and then the medium was changed to fresh complete DMEM medium containing serum (Invitrogen; Thermo Fisher Scientific, Inc.) and double antibody, to continue to culture 48 h, and cells were collected for detection. The transfected cells were divided into four groups: Anti-miR-NC, anti-miR-221, pIRES2-blank and pIRES2-BMF.

Cell proliferation activity detected by MTT assay. The SKOV3 cells transfected with anti-miR-NC, anti-miR-221, pIRES2-blank and pIRES2-BMF were inoculated into 96-well culture plates. Following incubation for 24, 48 and 72 h at 37˚C following transfection, 10 μl MTT solution (Qiagen GmbH, Hilden, Germany) was added to each well for 4 h at 37˚C, the supernatant was removed, and 150 μl dimethyl sulfoxide solution was added, following vibration for 10 min at low speed, the absorbance A450 at the wavelength of 450 nm was measured.

Detection of cell proliferation by EdU staining flow cytometry. Cells in all transfection groups were re-suspended using the complete medium (Qiagen GmbH). The kit used was the Click-iT® EdU flow cytometry cell proliferation assay (Qiagen GmbH, Hilden, Germany). Following incubation for 120 min with 10 μM EdU at 37˚C, cells were cultured for 48 h at 37˚C. Following digestion with trypsin, cells were centrifuged at 1,000 x g for 5 min at 37˚C and washed once with 1% bovine serum albumin (BSA) in PBS. The cells were fixed with 100 μl Click-iT fixative for 15 min at room temperature, centrifuged at 1,000 x g for 5 min at 37˚C and washed once with 1% BSA in PBS. A total of 100 μl permeabilization and washing reagent (0.2%) was added for permeabilization 15 min at room temperature, then 500 μl of reaction solution containing PBS (496 ml), $CuSO_4$ (1 mM), Alexa Fluor 488 (10 μM), buffer additive (4 ml, component F; Qiagen GmbH, Hilden, Germany) was added to incubate for 30 min in the darkness at room temperature. Following this, 3 ml permeabilization and wash reagent was added and the mixture was centrifuged at 1,000 x g for 5 min at 37˚C and washed with PBS once. The cells were then re-suspended with 500 μl permeabilization and wash reagent, and cell proliferation was detected by the Beckman Coulter FC 500 MCL/MPL flow cytometer.

Detection of apoptosis. Cells in all transfection groups were digested and harvested by trypsin, centrifuged for 5 min at 1,000 x g 37˚C and washed once using 1% BSA PBS. Cells were re-suspended in 100 μl binding buffer (part of the apoptosis detection kit), then cells were incubated with a cell apoptosis detection kit [10 μl (15 M) Annexin V dye (FITC) and 5 μl (15 M) PI dye; Beyotime Institute of Biotechnology, Haimen, China] in the dark for 15 min at 37˚C. Cell apoptosis was detected with a Beckman Coulter FC 500 MCL/MPL flow cytometer and analyzed using FCS Express 3.0 software (DeNovo software, Glendale, CA, USA).

Detection of Caspase-3 activity. The pNA standard was prepared according to the protocols of the Caspase-3 activity assay kit, and A405 was measured and a standard curve was plotted. Cells in all treatment groups were digested by lysis solution on ice (100 μl lysate for $5x10^6$ cells), then the supernatant was transferred to a new pre-cooled EP Tube for standby. The buffer, test article, Ac-DEVD-pNA (Qiagen GmbH, Hilden, Germany) were added to the 96-well plate successively, and incubated at 37˚C for 120 min. When an evident color change was determined, the absorbance value A405 was measured immediately using a microplate reader. The absorbance value A405 reflected the activity of Caspase-3 of the test article.

Dual-luciferase reporter gene assay. MicroRNA.org online prediction indicated that the sites 3673-3690 on the 3'-untranslated region (UTR) of BMF mRNA were possible binding sites for miR-221. The DNA fragments containing the sites and containing the site mutant were synthesized *in vitro*, then cloned to dual luciferase gene reporter vector pMIR (Thermo Fisher Scientific, Inc.). The pMIR vector and miR-221 mimic (mimic NC, anti-miR-NC and anti-miR-221) were co-transfected into SKOV3 cells using Lipofectamine® 2000 transfection reagent (Thermo Fisher Scientific, Inc.). Following 48 h of culture at 37˚C, the luciferase activity was detected by dual luciferase assay kit. The red firefly luciferase signal was utilized as a normalization control.

Detection of protein expression by western blot analysis. Tissues or cells were lysed by radioimmunoprecipitation assay lysate (Thermo Fisher Scientific, Inc.). Supernatant was removed and the protein concentration was determined with a bicinchoninic acid assay reagent (Pierce; Thermo Fisher Scientific, Inc.). A total of 40 μg solution was loaded onto SDS-PAGE for electrophoresis separation (10% separation gel and 5% spacer gel), transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with PBS with Tween 20 (PBST, Sigma-Aldrich; Merck KGaA) containing 5% BSA for 1 h at 37˚C, followed by incubation with primary antibody overnight at 4˚C (the dilution ratios of BMF and β-actin were 1:2,000 and 1:4,000, respectively). The next day, the membranes were washed three times using 0.1% tris-buffered saline and Tween 20 (TBST), 5 min each

time, then HRP-labeled secondary antibody (dilution ratio of 1:10,000) was added and incubated for 1 h at room temperature, then washed by 0.1% TBST x3. The bands of PVDF membrane were developed by Supersignal West Femto HRP sensitive chemiluminescent substrate (Sigma-Aldrich; Merck KGaA), using β-actin as the internal reference substance.

Statistical analysis. Statistical analysis was performed by SPSS 18.0 (SPSS, Inc., Chicago, IL, USA). Measurement data are expressed as mean ± standard deviation, the measurement data between the two groups were compared using Student's t-test; the measurement data between the multiple groups were compared first using one-way analysis of variance, then using the Bonferroni method for comparison. The expression levels of miR-221 and BMF mRNA in tissues were compared by Mann-Whitney U test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Abnormal change in the expression levels of miR-221 and BMF in ovarian cancer tissues. The results of RT-qPCR demonstrated that, compared with the para-cancer tissues, the expression of miR-221 in tumor tissues was significantly increased (Mann-Whitney U=48, $P<0.001$; Fig. 1A), and the expression of BMF mRNA was significantly decreased in patients with ovarian cancer (Mann-Whitney U=162, $P<0.001$; Fig. 1B). Western blot analysis indicated that the expression of BMF proteins in ovarian cancer tissue was significantly lower than that in para-cancer tissues ($P<0.001$). The representative test results were depicted in Fig. 1C.

Targeted regulatory effect between miR-221 and BMF. The microRNA.org online prediction indicated that BMF may be the target gene for miR-221. The predicted BMF 3'-UTR target site of miR-221 was depicted in Fig. 2A. The results of the luciferase reporter assay demonstrated that the co-transfection of the wild-type pMIR-BMF-3'-UTR-wt vector and miR-221 mimic significantly decreased the activity of luciferase ($P<0.05$), and the co-transfection of wild-type pMIR-BMF-3'-UTR-wt vector and anti-miR-221 significantly increased the activity of luciferase ($P<0.05$); however, the co-transfection of mutant pMIR-BMF-3'-UTR-mut vector and miR-221 mimic or anti-miR-221 did not significantly change the activity of luciferase, indicating a targeted regulatory association between miR-221 and BMF mRNA (Fig. 2B).

Transfection of anti-miR-221 or pIRES2-BMF significantly upregulates the expression of BMF in ovarian cancer SKOV3 cells. The RT-qPCR test results demonstrated that, following transfection with anti-miR-221, the expression of miR-221 in SKOV3 cells was significantly decreased ($P<0.001$). The transfection of si-BMF or pIRES2-BMF had no significant effect on the expression of miR-221 in SKOV3 cells (Fig. 3A). Compared with anti-miR-NC group, the expression of BMF mRNA in SKOV3 cells was increased in the anti-miR-221 transfection group. Compared with the pIRES2-blank group, the expression of BMF mRNA SKOV3 cells was significantly increased in the pIRES2-BMF transfection group ($P<0.001$), and the transfection of si-BMF downregulated the expression of BMF mRNA and attenuated the upregulation of anti-miR-221 on the expression of BMF proteins (Fig. 3B). Western blot analysis indicated that the transfection of anti-miR-221 or pIRES2-BMF significantly upregulated the expression of BMF protein in SKOV3 cells ($P<0.001$), and the expression level of BMF protein was highest in the anti-miR-221 and pIRES2-BMF combined transfection group. The transfection of si-BMF significantly inhibited the expression of BMF protein and reduced the upregulation of anti-miR-221 on the expression of BMF protein (Fig. 3C).

Transfection of anti-miR-221 or pIRES2-BMF significantly attenuates the proliferation of SKOV3 cells. The EdU staining flow cytometry demonstrated that the proliferation of SKOV3 cells in the anti-miR-221 transfection group was significantly lower, compared with the anti-miR-NC group ($P<0.001$). Compared with the pIRES2-blank group, the proliferation of SKOV3 cells in the pIRES2-BMF transfection group was reduced significantly ($P<0.001$), and the transfection of pIRES2-BMF enhanced the inhibitory effect of anti-miR-221 on the proliferation of SKOV3 cells (Fig. 4A and B). The transfection of si-BMF significantly promoted cell proliferation and attenuated the inhibitory effect of anti-miR-221 on the cell proliferation (Fig. 4A and B).

Transfection of anti-miR-221 or pIRES2-BMF significantly promotes the apoptosis of SKOV3 cells. Flow cytometry indicated that the apoptosis rate of SKOV3 cells in the anti-miR-221 transfection group was significantly higher, compared with the anti-miR-NC group ($P<0.001$). Similar to the transfection of anti-miR-221, the transfection of pIRES2-BMF significantly promoted the apoptosis of SKOV3 cells ($P<0.001$). The transfection of pIRES2-BMF enhanced the anti-apoptotic effect of anti-miR-221 on SKOV3 cells (Fig. 5A and B). The transfection of si-BMF significantly reduced apoptosis and attenuated the proapoptotic effect of anti-miR-221 on SKOV3 cells (Fig. 5A and B). The results of spectrophotometry demonstrated that the transfection of anti-miR-221 or pIRES2-BMF significantly upregulated the activity of caspase-3 in SKOV3 cells ($P<0.001$), whilst the transfection of si-BMF significantly downregulated the activity of caspase-3 in SKOV3 cells ($P<0.001$; Fig. 5C).

Discussion

In the present study, the expression levels of miR-221 and BMF mRNA in ovarian cancer tissues were detected by RT-qPCR. Results indicated that the expression level of miR-221 in ovarian cancer tissues was significantly higher, compared with the para-carcinoma tissues, whilst the expression of BMF mRNA in ovarian cancer tissues was lower, compared with the para-carcinoma tissues, indicating that miR-221 serves as a tumor-promoting gene and BMF as a tumor suppressor gene in the ovarian cancer. In the previous studies regarding the association between miR-221 and ovarian cancer, Dahiya *et al* (24) conducted detection of miR-221 via a gene microarray and demonstrated that the expression levels of miR-221 in tumor tissues of patients with ovarian cancer and ovarian cancer cell lines was significantly increased. The detection by Gadducci *et al* (25) indicated that, compared with normal ovarian tissue, the expression of miR-221 in patients with ovarian cancer was increased abnormally.

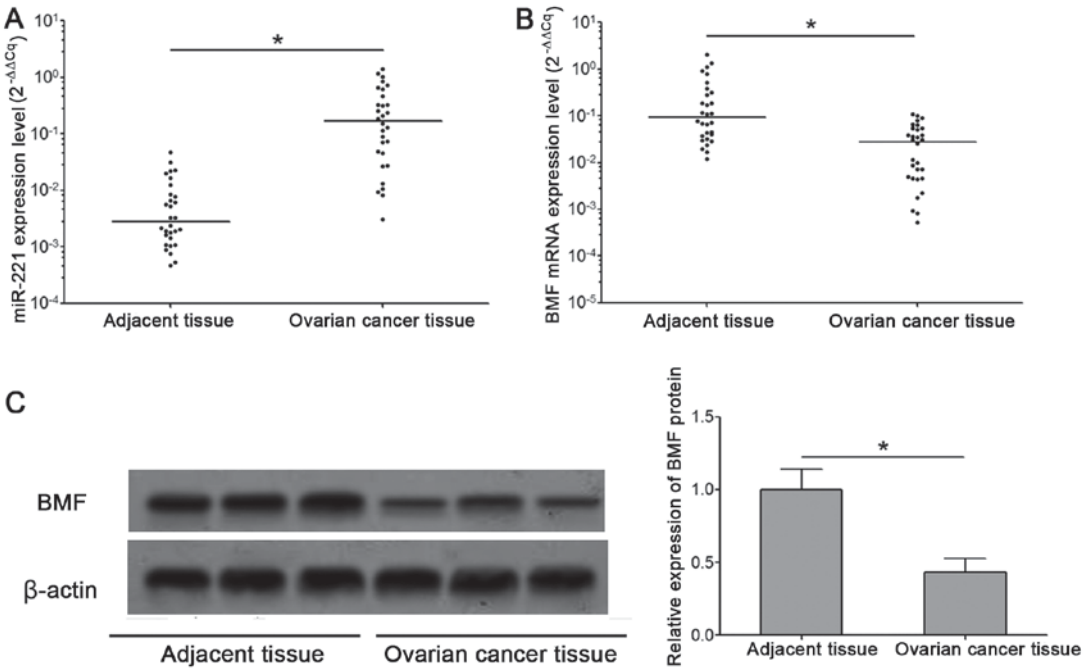

Figure 1. Abnormal change of expression levels of miR-221 and BMF in ovarian cancer tissues. (A) Detection of miR-221 expression by RT-qPCR. (B) Detection of BMF mRNA expression by RT-qPCR. (C) Detection of BMF protein expression by western blot analysis. *P<0.05. miR-221, microRNA-221; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; BMF, B-cell lymphoma 2 modifying factor.

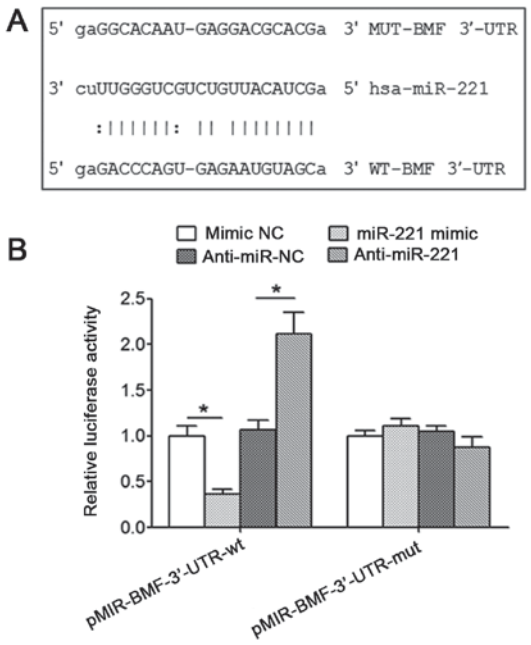

Figure 2. Targeted regulatory effect between miR-221 and BMF mRNA. (A) Binding sites existing between miR-221 and 3'-UTR of BMF mRNA. (B) Dual luciferase gene reporter assay. *P<0.05. NC, negative control; wt, wild type; mut, mutated; BMF, B-cell lymphoma 2 modifying factor; UTR, untranslated region; miR-221, microRNA-221; pMIR, phosphorylated microRNA.

Hong *et al* (22) conducted RT-qPCR detection and demonstrated that, compared with the healthy control populations, the expression of miR-221 in the peripheral blood of patients with ovarian cancer was abnormally increased, and its expression level was correlated with the disease stage of International Federation of Gynecology and Obstetrics and histopathological grading, with the higher the expression of miR-221, the lower the survival rate and prognosis of patients. Li *et al* (21) determined that the expression levels of miR-221 were significantly increased in ovarian cancer cells A2780, OVCAR3, SKOV3 and 3AO, compared with the immortalized ovarian epithelial IOSE25 cells; and compared with para-cancer tissues, the expression of miR-221 in the tumor tissues of patients with ovarian cancer was also increased abnormally, with the overall survival rate and progression-free survival rate of patients with higher expression of miR-221 being significantly lower than those in patients with low expression of miR-221. In the present study, the expression of miR-221 in tumor tissue of patients with ovarian cancer was significantly higher, compared with the para-cancer tissues, which may be carcinogenic factor in the occurrence of ovarian cancer, similar to the results obtained by Gadducci *et al* (25), Hong *et al* (22) and Li *et al* (21).

miRNAs serve biological roles mainly by regulating the downstream target genes. Different functions of downstream target genes can cause different functions of the same miRNAs in different tumor tissues (24,25). The present study confirmed that miR-221 is predominantly tumor-promoting in ovarian cancer tissues, whilst BMF, which has an important function in promoting cell apoptosis and tumor inhibition according to the microRNA.org online prediction, may be a target gene of miR-221; therefore, BMF was the target gene of interest for the present study. The results of the dual luciferase reporter assay demonstrated that the co-transfection of wild-type pMIR-BMF-3'-UTR vector and miR-221 mimic significantly decreased the activity of luciferase, and the co-transfection of wild-type pMIR-BMF-3'-UTR vector and anti-miR-221 significantly increased the activity of luciferase; whilst the co-transfection of mutant pMIR-BMF-3'-UTR vector and

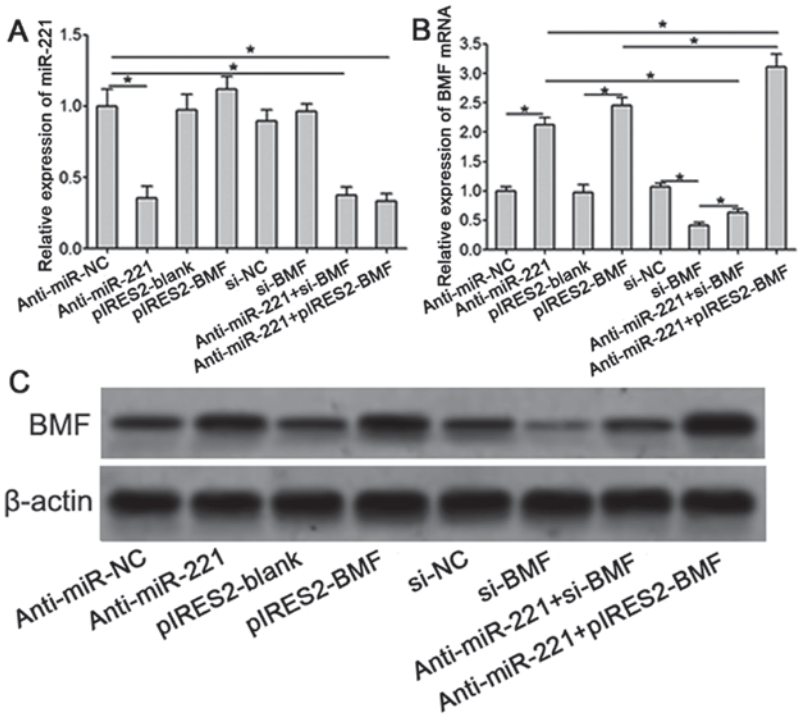

Figure 3. Transfection of anti-miR-221 or pIRES2-BMF significantly upregulated the expression of BMF in ovarian cancer SKOV3 cells. (A) Detection of miR-221 expression levels in all transfection groups by RT-qPCR. (B) Detection of BMF mRNA expression levels in all transfection groups by RT-qPCR. (C) Detection of BMF protein expression levels in all transfection groups by western blot analysis. *P<0.05. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; miR-221, microRNA-221; NC, negative control; BMF, B-cell lymphoma 2 modifying factor; si, small interfering; pIRES2, phosphorylated internal ribosome entry site 2.

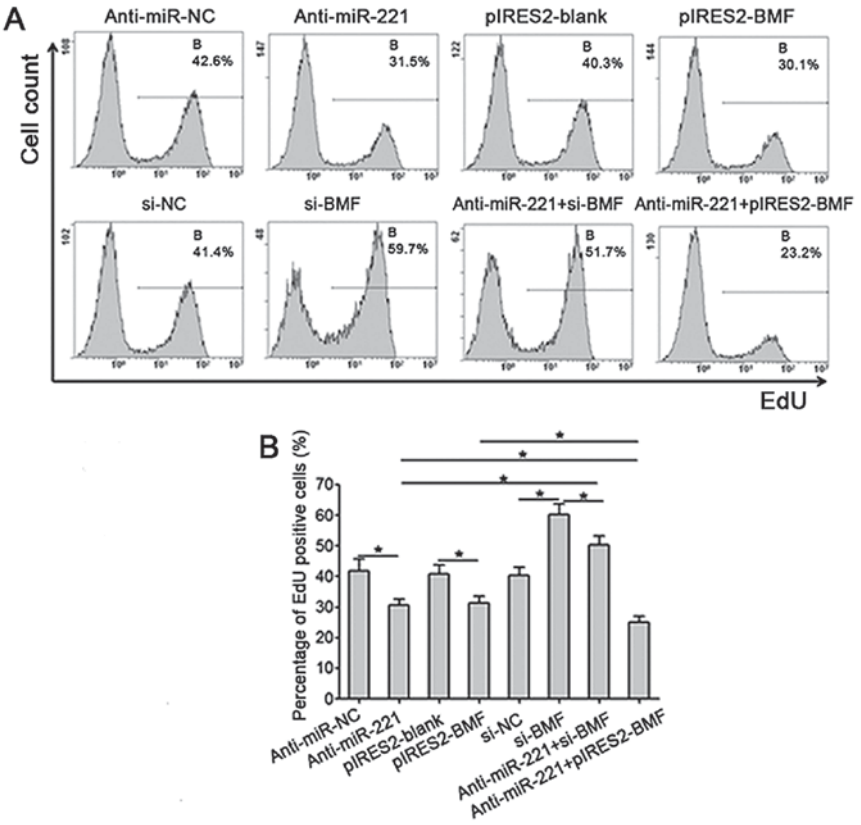

Figure 4. Transfection of anti-miR-221 or pIRES2-BMF significantly attenuated the proliferation of SKOV3 cells. (A) EdU staining was used to detect the cell proliferation ability of each transfection group. (B) Statistical comparison of cell proliferation ability in transfected cells. *P<0.05. miR-221, microRNA-221; NC, negative control; BMF, B-cell lymphoma 2 modifying factor; si, small interfering; pIRES2, phosphorylated internal ribosome entry site 2.

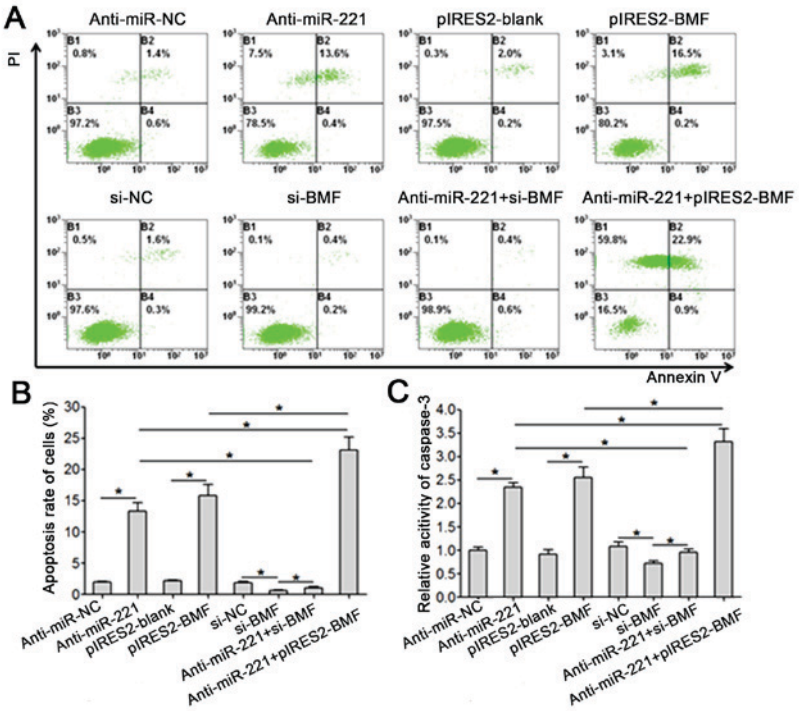

Figure 5. Transfection of anti-miR-221 or pIRES2-BMF significantly promoted the apoptosis of SKOV3 cells. (A) Detection of apoptosis by flow cytometry. (B) Statistical comparison of apoptosis in each transfected group. (C) Spectrophotometry detected the activity of caspase-3. *P<0.05, comparison between two groups. miR-221, microRNA-221; NC, negative control; BMF, B-cell lymphoma 2 modifying factor; si, small interfering; pIRES2, phosphorylated internal ribosome entry site 2.

miR-221 mimic or anti-miR-221 did not significantly change the activity of luciferase. This indicated that there was a direct interaction between miR-221 and BMF mRNA, and BMF was the downstream target gene of miR-221.

To investigate whether miR-221 regulating BMF would influence the biological effects of ovarian cancer SKOV3 cells, including proliferation and apoptosis, the changes in proliferation and apoptosis of SKOV3 cells following transfected with anti-miR-221or pIRES2-BMF in the SKOV3 cells were detected. The detection results indicated that the transfection of anti-miR-221 significantly inhibited the proliferation of SKOV3 cells, and the transfection of pIRES2-BMF significantly attenuated the proliferation of SKOV3 cells. The transfection of anti-miR-221 significantly promoted the apoptosis of SKOV3 cells, and the transfection of pIRES2-BMF also resulted in a significant increase in the number of apoptotic SKOV3 cells, similar to that of anti-miR-221. The transfection of pIRES2-BMF or si-BMF enhanced or attenuated the proapoptotic and proliferation-inhibition effect of anti-miR-221 on SKOV3 cells, respectively. The results indicated that miR-221 reduced the apoptosis level and enhanced the proliferation ability of ovarian cancer cells, which developed the ovarian cancer by inhibiting the expression of BMF. In the present study, it was firstly confirmed that the miR-221 expression in ovarian cancer tissues was significantly higher, compared with the para-cancer tissues, through the RT-qPCR method, and it was hypothesized that the increase of miR-221 in ovarian cancer tissues decreased the expression of pro-apoptotic factor BMF, enhanced the proliferation and reduced the apoptosis of ovarian cancer cells, which promoted the occurrence and development of ovarian cancer. Li *et al* (21) demonstrated that the transfection of miR-221 inhibitor significantly upregulated the expression of target gene apoptotic peptidase activating factor 1 and attenuated the proliferation and migration of ovarian cancer A2780 and SKOV3, whilst the cell apoptosis was significantly increased, confirming that miR-221 served a tumor-promoting role in promoting the proliferation of ovarian cancer cells and reducing cell apoptosis, which confirmed and supported the results in the present study. He *et al* (26) indicated that miR-221 promoted the proliferation of hepatocellular carcinoma cells by targeting the inhibition of BMF expression, whilst downregulating the expression of miR-221 inhibited the proliferation, migration and invasion of hepatocellular carcinoma cells and promoted the apoptosis of hepatoma cells. This indicated that the increased expression of miR-221 in liver cancer serves a role in promoting cancer. The results of Gramantieri *et al* (27) demonstrated that abnormally elevated miR-221 expression also serves a regulatory role in targeting the inhibition of BMF expression and promoting liver cancer. In the present study, the dual-luciferase gene reporter assay confirmed the existence of targeted regulation between miR-221 and BMF, which further corroborates the results of He *et al* (26) and Gramantieri *et al* (27). At present, the association between miR-221 and ovarian cancer is rarely reported, and the majority of studies focus on the abnormal changes in the expression of miR-221, without studying its effect in regulating the biological process of ovarian cancer cells. In the present study, the abnormal expression of miR-221 in the ovarian cancer cells were detected, and its biological process in influencing the proliferation and apoptosis of ovarian cancer cells by regulating the expression of target gene BMF was investigated, revealing the tumor-promoting

effect of miR-221; however, the process of miR-221 regulation of BMF in ovarian cancer cells *in vivo* process is not clear, and requires further animal studies, which is also a limitation of the present study.

In summary, the expression of miR-221 was significantly increased and the expression of BMF was significantly decreased in ovarian cancer tissues. The miR-221 with abnormally elevated expression antagonized the apoptosis of ovarian cancer SKOV3 cell and promoted the cell proliferation by targeted inhibition of the expression of pro-apoptosis factor BMF, which may serve a role in the pathogenesis of ovarian cancer.

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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

Guarantor of integrity of the entire study: LC. Study concepts: YH. Study design: XX. Definition of intellectual content: YH. Literature research: XX and JW. Clinical studies: XX and YH. Experimental studies: XX. Data acquisition: JW. Data analysis: XX and YH. Statistical analysis: XX and LC. Manuscript preparation: JW. Manuscript editing: XX. Manuscript review: YH and JC.

Ethics approval and consent to participate

All specimens were collected with the patient's written informed consent and the present study was approved by the Ethics Committee of the First Affiliated Hospital of Fujian Medical University.

Patient consent for publication

Witten informed consent was obtained from the patients for the publication of any associated data.

Competing interests

The authors declare that they have no competing interests.

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