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ORIGINAL ARTICLE

Study on the Association Between miRNA-202 Expression and Drug Sensitivity in Multiple Myeloma Cells

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Abstract An increasing amount of experimental evidence has shown that miRNAs play a causal role in hematologic tumorigenesis. In this study, we characterized the role of miR-202 in multiple myeloma (MM) drug sensitivity. The potential binding site of miR-202 and B cell-activating factor (BAFF) was confirmed by luciferase reporter assay. MM cells were transfected with miR-202 mimics and inhibitor. Cells growth was measured by WST-1 cell proliferation assay and Annexin V-FLUOS apoptosis assay. BAFF and miR-202 mRNA levels were measured by real-time PCR. Meanwhile, BAFF, Bcl-2 family survival proteins and MAPK pathway proteins were measured by Western blot. It was found that miR-202 was functioned as a modulator of BAFF expression. miR-202 over-expression sensitized MM cells to bortezomib (Bort) but less to Thalidomide (Thal) and dexamethasone (Dex). miR-202 mimics in combination with Bort inhibited MM cell survival more effectively as compared with Bort treatment alone. Our study also provided experimental evidence that JNK/SAPK signaling pathway was involved in the regulatory effect of miR-202 on drug resistance of MM cells. These results suggest that the regulatory mechanism of miR-202 expression may be a promising target for fine-tuning anti-myeloma therapy.

Keywords B cell-activating factor (BAFF) · Drug resistance · Multiple myeloma (MM) · microRNA · Signaling pathway

Introduction

Multiple myeloma (MM) is a currently incurable plasma cell malignancy [1, 2]. Despite tremendous efforts in developing effective treatment modalities such as autologous stem cell transplantation, exploring new molecular targeting drugs, etc., the disease still remains incurable, mainly due to the ultimate acquisition of drug resistance [3]. Previous studies [4, 5] reported that drug resistance in MM cells was triggered by activation of signaling pathways including the NF-κB (nuclear factor-κB), JAK/Stat3 (Janus kinase/signal transducer and activator of transcription-3), and MEK/MAPK (mitogen activated protein [MAP] kinase kinase/MAP kinase)

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pathways. Consequently, myeloma cell expansion drives disease progression.

microRNA (miRNA) is a family of small non-coding RNAs consisting of 21–25 nucleotides in length. It has been a major research focus in the field of cancer research in recent years, knowing that it regulates gene expression at a post-transcription level by inhibiting or degrading messenger RNA (mRNA) through partially or completely binding to 3'UTR of the target gene mRNA [6]. In many cases, bound miRNA also decreases the stability of target mRNA resulting in RNA transcript degradation. In addition to their role in normal biological processes, miRNAs are involved in various diseases [7, 8]. A group of tumor-associated miRNAs such as miR-181, miR-21, miR-17-92, miR-93 and miR-202 were recently profiled in MM patient samples and cell lines using microarray analysis [9, 10]. Our preliminary studies have found that miR-202 mRNA was expressed in the PBMCs of MM patients. However, the functional activity of miR-202 in myeloma cells has not been elucidated. In addition, there is an increasing interest in hematopoietic cell miRNA expression in response to chemical drugs recently, knowing that there is evidence that miRNA can control sensitivity and resistance to drugs in leukemic cell lines [11].

Given the emerging data on miRNA regulation in response to drugs, we undertook some strategic approaches to investigate anti-myeloma drug-relevant miRNA in MM cells in this study and found that B cell-activating factor (BAFF), also known as BLyS, THANK, TALL-1, TNFSF20, TNFSF13, zTNF4, was a miR-202 regulation target, and that over-expression of miR-202 sensitized MM cells to bortezomib (PS-341/Velcade; Bort) but less to thalidomide (Thal) and dexamethasone (Dex). The JNK/SAPK signaling pathway was also involved in the regulatory effect of miR-202 on BAFF expression and drug resistance of MM cells.

Materials and Methods

Materials

The main reagents used in this study were anti-ERK, anti-phospho-ERK, anti-p38, anti-phospho-p38, anti-JNK/SAPK, and anti-phospho-JNK/SAPK antibody (Cell Signaling Technology, Beverly, MA); BAFF polyclonal antibody (R&D Systems, Minneapolis, MN); β-actin antibody (Santa Cruz, CA); Annexin V-FLUOS Staining kit and Cell Proliferation Reagent WST-1 (Roche, Germany); TRIzol (Invitrogen, US); Lipo2000 and reverse transcription reagent (Fermentas, Lithuania); fetal bovine serum (FBS) (Hyclone, US); RPMI 1640 Medium and Opti-MEM Reduced Serum Medium (Gibco, US); siBAFF (BAFF-homo-708) (GenePharma, Shanghai, China); miR-202-mimics, miR-202-inhibitor, negative control and inhibitor negative control (Invitrogen, Shanghai, China).

Patient Samples and Cell Culture

Clinically confirmed MM patients were inpatients of the affiliated hospital of Nantong University (Nantong, China) between August 2013 and December 2014, including 3 females and 4 males ranging in age from 50 to 65 years. All cases of the MM patients were newly diagnosed and non-treated. $CD138^+$ cells from bone marrow aspirates were isolated with a BD FACSAria II using phycoerythrin (PE)-conjugated anti-CD138 (BD Biosciences, NJ, USA) antibody. $CD138^+$ cells from bone marrow of normal donors (1 female and 2 males ranging in age from 48 to 60 years) were recruited as control group. All samples were anonymous and the study protocol was approved by the local ethics committee.

Human MM cell line U266 cells were cultured in RPMI-1640 medium containing 10 % fetal bovine serum (FBS), 1 % streptomycin-penicillin and 1 % glutamine in a humidified 37 °C incubator with 5 % CO_2.

Cell Transfection

U266 cells were seeded in 6-well plate at a density of 2–8×10^5/well in 2 ml RPMI-1640 with 10 % FBS and incubated at 37 °C for 12 h, and then transfected with 50 nM miR-202 mimics (5′-AGA GGU AUA GGG CAU GGG AA-3′, 5′-CCC AUG CCC UAU ACC UCU UU-3′), negative control (5′-UUC UCC GAA CGU GUC ACG UTT-3′, 5′-ACG UGA CAC GUU CGG AGA ATT-3′), miR-202 inhibitor (5′-UUC CCA UGC CCU AUA CCU CU-3′) and siBAFF (5′-CAU GGC UUC UCA GCU UUA ATT-3′, 5′-UUA AAG CUG AGA AGC CAU GTT-3′), using lipo2000 according to the manufacturer's instructions. After 48–72 h, total RNA and protein were extracted.

SYBR Green I Real Time PCR

Total RNA was extracted from MM cells using TRIzol reagent. 5 μg RNA was reverse transcribed in 10 mM dNTP, 5 × Reaction Buffer, 20 U/μl RNase inhibitor, 200 U/μl Reverse Transcriptase and RT primer, following thermal cycling conditions: 42 °C for 60 min and 70 °C for 5 min. The reverse transcription product was stored at −20 °C. Real-time quantification was performed in triplicate with Fast Start Universal SYBR Green Master (Rox) mix kit. BAFF primer sequences were 5′-TGT CAC CGC GGG ACT GAA AAT CT-3′ (forward primer), and 5′-TGT CTG CAA TCA GTT GCA AGC AGT-3′ (reverse primer). GAPDH primer sequences were 5′-TGC TGT TCT GAC TGG AGT TG-3′ (forward primer), and 5′-GCT GTC TTG CTG CCT CAC-3′ (reverse primer). The miR-202 and U6 primers were purchased

from RiboBio Co., Ltd. (Guangzhou, China). Each reaction was performed in a final volume of 20 μl containing 10 μl SYBR Green I mix (Rox), 3 μl cDNA, 0.5 μl forward primer, 0.5 μl reverse primer and RNase-free H_2O. The mixture was incubated at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 31 s.

Western Blot

Total protein was extracted using RIPA lysate containing 1 % PMSF and 1 % phosphates inhibitors. SDS-polyacrylamide gel electrophoresis was performed on 300 μg total protein (80 v 40 min; 100 v 60 min), and the protein was transferred from SDS-polyacrylamide gel electrophoresis to the PVDF membrane (300 mA, 120 min). After blocking with 5 % non-fat milk in Tris-buffered saline containing 0.1 % Tween 20 (TBST), the polyvinylidene fluoride membrane was incubated with primary antibody in 5 % bovine serum albumin in TBST overnight at 4 °C, washed three times with TBST and incubated with secondary antibody in 5 % milk in TBST. After three washes with TBST, the membrane was developed with enhanced chemiluminescence (ECL, Amersham Pharmacia).

WST-1 Cell Proliferation Assay

U266 cells were seeded in a 96 well plate at a density of 3000 cells per well and transfected with miR-202 mimics, miR-202 inhibitor and control. The total volume in each well was 100 μl. Drug concentrations were 10 nM Dex, 200 μM Thal, and 50 nM Bort according to the reference literature [12, 13]. After 48 h, 10 μl WST-1 reagent was added to each well and incubated for a further 2 h in a 37 °C/5 % CO_2 incubator. Absorbance (A) value of each well was measured at 450 nm (650 nm as reference). Five repeated wells were designed for each group and the experiment was repeated in triplicate.

Annexin V-FLUOS Apoptosis Assay

Cells were seeded in a 6-well plate and transfected with miR-202 mimics, miR-202 inhibitor and control. After 48 h, cells were transferred to a 2 ml EP tube, centrifuged, and washed twice with 1 × PBS. Annexin V-FLUOS and PI dye were added, cultured for 10–15 min at room temperature, and finally analyzed using flow cytometry.

Luciferase Reporter Assay

Luciferase reporter constructs contained the BAFF 3'UTR and a mutant control prepared using a psiCHECK-2 vector. Reporter constructs were transfected into 293 T cells with miR-202 mimics by Lipo2000. Cells were harvested 48 h after transfection and analyzed using a dual luciferase reporter assay kit according to the manufacturer's instructions.

Statistical Analysis

Statistical analysis was performed with SPSS 16.0 and graphs were generated with GraphPad Prism 5.0 software. The results were expressed as the mean ± SD. Statistical analysis was determined using the Student's t test. A value of $P < 0.05$ or less was considered statistically significant.

Results

Expressions of miR-202 in MM Cells and MM Patients

Total RNA was extracted from U266 cells and $CD138^+$ cells from bone marrow of MM patients and normal donors. Total RNA was reverse-transcribed for PCR analysis using primers for the human miR-202 gene. It was found that miR-202 mRNA was lowly expressed in U266 cells and $CD138^+$ cells of MM patients (Fig. 1a). Bioinformatics analysis on human miRNA targets, mioRNA.org and DIANA-miT showed that miR-202 regulated the expression of BAFF. Subsequently, expressions of BAFF in U266 cells and MM patients were analyzed by Real-time PCR. The result showed that the expression of BAFF mRNA in MM and U266 cells was higher than that in normal controls (Fig. Error! Reference source not found.b).

miR-202 Modulates BAFF Expression in MM Cells

Knowing that miRs can control gene expression by base pairing with specific recognizing elements in their target messenger, luciferase reporter assay was performed to see whether there was a direct interaction between miR-202 and BAFF 3′-UTR. The result of bioinformatics analysis showed that the miR-202 seed region bound to the BAFF 3′-UTR (Fig. 2a). Then, BAFF 3′-UTR was cloned into a psiCHECK-2 dual luciferase reporter construct, using a mutant BAFF 3′-UTR reporter as control. Reporter constructs were transfected into 293 T cells with or without miR-202 mimics, and luciferase activity was measured at 48 h after transfection. It was found that the luciferase activity decreased by 35.7 % in 293 T cells when BAFF 3′-UTR reporter constructs were co-transfected with miR-202 mimics, compared with that of the BAFF 3′-UTR reporter alone. miR-202 mimics did not affect the luciferase activity of the mutant BAFF 3′-UTR construct (Fig. 2b). Real-time PCR and Western blot were performed to analyze BAFF expression in U266 cells transfected with miR-202 inhibitor or mimics. It was found that the expression of BAFF mRNA and protein were decreased in cells treated with miR-202 mimics compared with those in non-coding

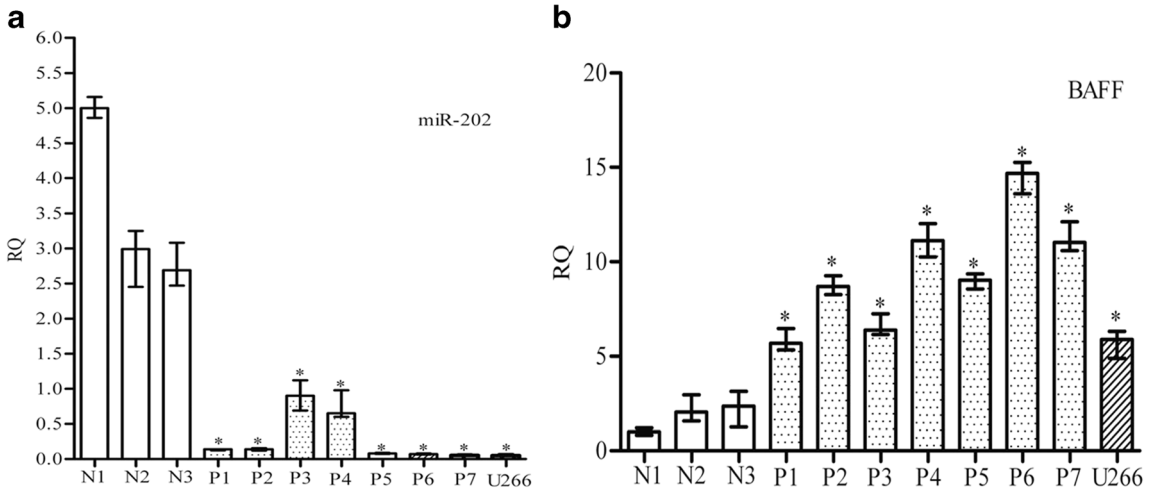

Fig. 1 miR-202 expressions in MM cells and MM patients. **a** The expressions of miR-202 in U266 cells and CD138$^+$ cells from MM patients and normal donors were detected by real time-PCR (P1-P7 as MM patients; N1-N3 as normal donors; *$P < 0.05$). **b** The expressions of BAFF in U266 cells and CD138$^+$ cells from MM patients and normal donors were detected by real time-PCR (P1-P7 as MM patients; N1-N3 as normal donors; *$P < 0.05$)

control cells, and the promotion effect of miR-202-inhibitor on BAFF mRNA was apparent (Fig. 2c, d). These results suggest that miR-202 may function as a modulator of BAFF expression at the protein level.

miR-202 Inhibits MM cell Survival and Growth

U266 cells were transfected with miR-202 mimics, miR-202 inhibitor, siBAFF and non-coding control for 48–72 h. As shown in Fig. 3a, the growth of miR-202 mimics transfection cell group was significantly lower as compared with the control cells by WST-1 assay ($P < 0.05$), thereby suggesting that miR-202 inhibited the proliferation of MM cells by targeting BAFF. Meanwhile, Western blot analysis of the Bcl-2 family survival proteins also showed that the expression of Bcl-2 protein was down-regulated by approximately 24 % and the expression of Bax protein was up-regulated by approximately 124 % after transfection with miR-202 mimics as compared with control cells (Fig. 3b), indicating that miR-202 could induce MM cell apoptosis. In addition, flow cytometry was used to analyze the apoptosis of U266 cells. As shown in Fig 3c, miR-202 mimic transfection produced a

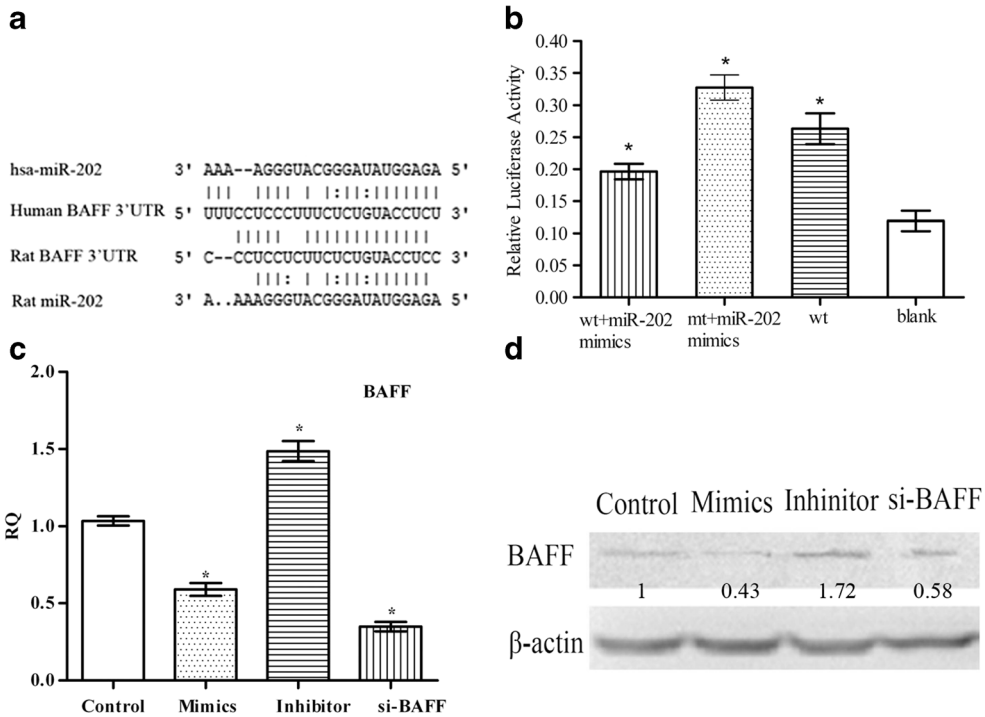

Fig. 2 miR-202 modulates BAFF expression in MM cells. **a** The miR-202 target site located in the 3'UTR of BAFF mRNA is highly conserved in humans. **b** 293 T cells were transfected with miR-202 mimics and psiCHECK-2 constructs (wild-type and mutant BAFF 3'UTRs) simultaneously (*$P < 0.05$). **c** Real-time PCR was used to analyze the expression of BAFF in U266 cells transfected with miR-202 mimics, inhibitor and si-BAFF compared with that in non-coding cells (*$P < 0.05$). **d** Western blot analysis of BAFF in U266 cells transfected with miR-202 mimics and inhibitor compared with that in control cells

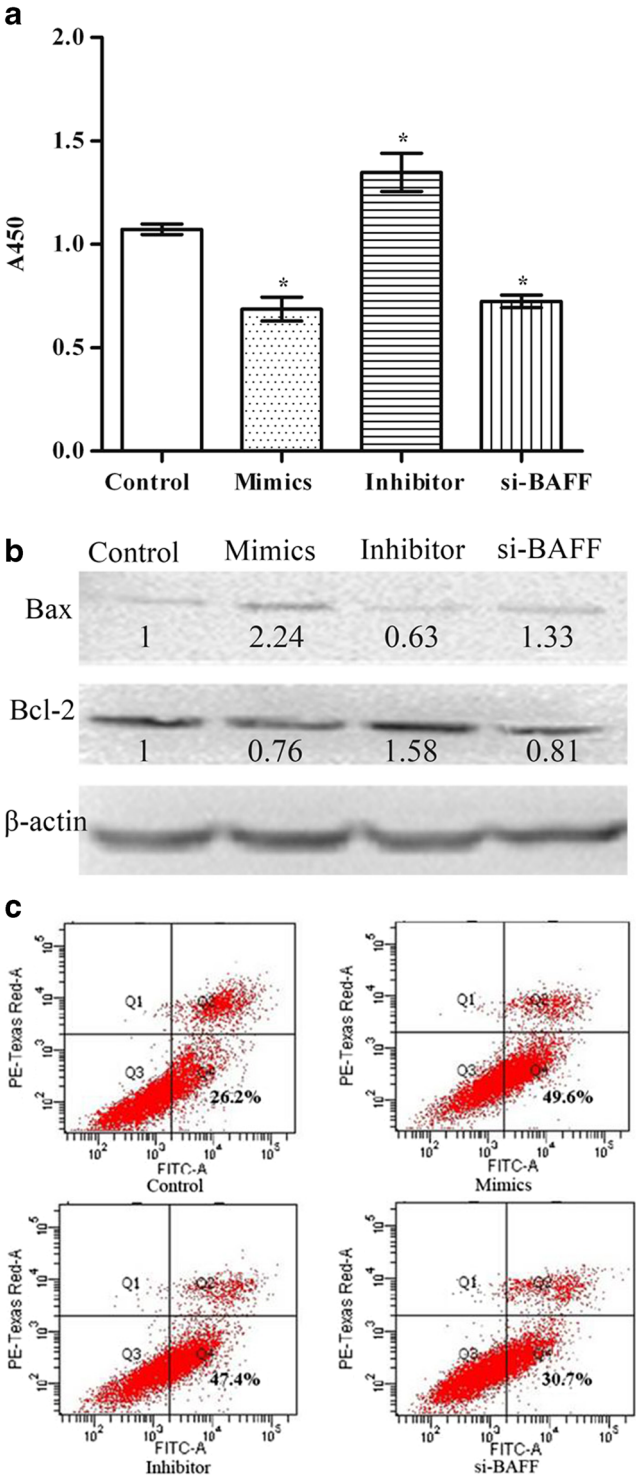

Fig. 3 miR-202 inhibits multiple myeloma cell survival and growth. **a** U266 cells were transfected with miR-202 mimics, inhibitor, si-BAFF or control. WST-1 assay was used to detect the proliferation of U266 cells (compared with control group, $*P < 0.05$). **b** Western blot analysis of the expression of Bcl-2 and Bax. Bcl-2 and Bax levels were normalized to those of β-actin. **b** Flow cytometry was used to analyze the apoptosis of U266 cells

stronger pro-apoptotic effect as compared with that of the control (49.6 % vs. 26.2 %), while 202-inhibitor did not produce a significant inhibition effect on apoptosis. Taken together, miR-202 could inhibit MM cell survival and growth.

miR-202 is Associated with Apoptosis in Response to Bort

To analyze the effect of miR-202 on MM cell apoptosis induced by anti-myeloma drugs, flow cytometry was used to analyze U266 cells that treated with miR-202 mimics and/or anti-myeloma drug. As shown in Fig. 4, combination treatment with Bort and miR-202 mimics produced a stronger pro-apoptotic effect as compared with Bort alone or mimics control (51.7 % vs. 30.5 % and 26.2 %, $P < 0.05$). Meanwhile, no significant increase in Thal-induced apoptosis and Dex-induced apoptosis was observed in miR-202 mimics group as compared with mimics control group (11.7 % and 16.1 % vs. 10.3 % and 17.3 %, $P > 0.05$), suggesting that overexpression of miR-202 sensitized U266 cells to Bort but less to Thal and Dex. In other words, the low expression of miR-202 in MM was associated with reduced apoptosis in response to Bort.

miR-202 Mimics Increase the Sensitivity of MM Cells to Bort

The synergistic effect of miR-202 mimics with Dex, Thal, or Bort was evaluated. As shown in Fig. 5a, the mitotic suppressive effect was mild in Bort or miR-202 mimics alone group, with a rate of 17.8 % for Bort and 15.4 % for miR-202 mimics vs. 37.2 % in miR-202 mimics and Bort combination treatment group as compared with Bort alone ($P = 0.008$), and 25.0 % in Dex and miR-202 mimics combination treatment group as compared with 15.8 % for Dex alone ($P = 0.13$) and 15.7 % for miR-202 mimics alone (Fig. 5b). Thal alone resulted in a suppression rate of 15.5 % and synergistic inhibition by Thal and miR-202 mimics was 20.6 % ($P = 0.15$) (Fig. 5c). In brief, we demonstrated that up-regulating miR-202 expression contributed to sensitizing MM cells to Bort significantly. However, there was no statistical difference between Dex/Thal alone and combination with miR-202 mimics.

Impact of miR-202 on MAPK Signaling Pathway

Six proteins (ERK, p-ERK, JNK, p-JNK, p38 and p-p38) play important roles in MAPK signaling pathway, of which p-ERK, p-JNK, and p-p38 are activated proteins, suggesting the activation of the corresponding pathway. Our previous study [14] indicated that JNK pathway was involved in the regulation of BAFF in MM cells, and its activation degree was positively correlated with BAFF expression. Therefore in this study we transfected U266 cells with miR-202-mimics, miR-202-inhibitor, siBAFF and non-coding control. Western Blot was used to detect the expression level of the above proteins in MAPK signaling pathway. The results showed that ERK, p38,

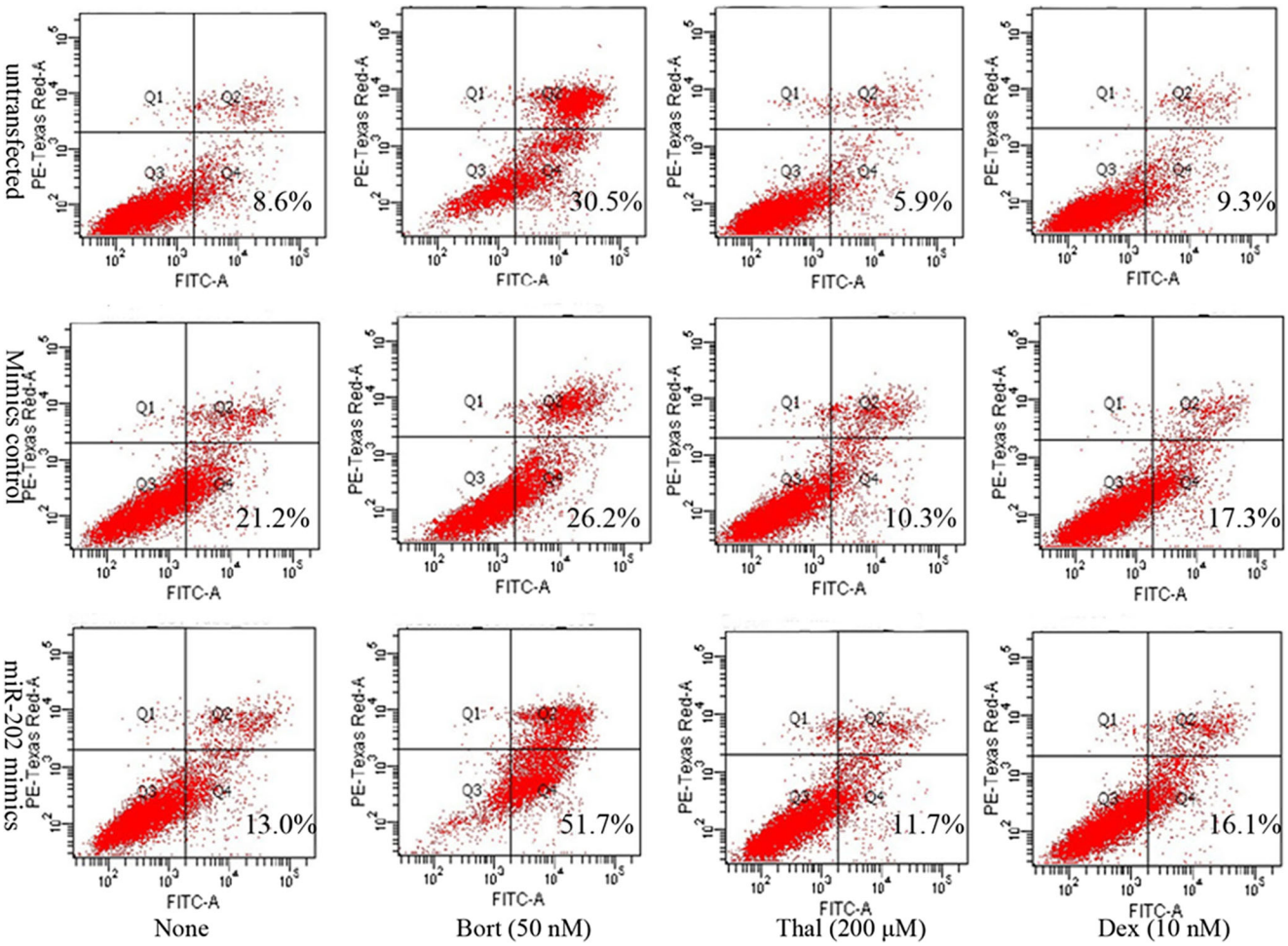

Fig. 4 miR-202 is associated with apoptosis in response to anti-myeloma drugs. U266 cells transfected with or without miR-202 mimics/control were treated with 50 nM Bort, 200 μM Thal, or 10 nM Dex for 48 h. Flow cytometry was used to analyze the apoptosis of U266 cells. Data were representative of five independent experiments. Upper row: untransfected group; middle row: mimics control group; lower row: miR-202 mimics group

JNK and p-JNK were expressed in U266 cells (Fig. 6a), but p-ERK and p-p38 were not detected. Moreover, the expression level in p-JNK protein was decreased to some extent in miR-202-mimics transfection group. These results indicate that JNK/SAPK signaling pathway might be involved in the regulatory effect of miR-202 on BAFF.

To support the hypothesis that the increased responsiveness to Bort treatment was due to the ability of miR-202 to repress the BAFF-mediated pathway by counteracting the reactivation of JNK/SAPK signaling, we analyzed p-JNK levels 48 h after Bort treatment. As expected, p-JNK levels in miR-202 or siRNA-BAFF transfected cells were lower than those in controls (Fig. 6b). In other words, miR-202 mimics and si-BAFF had a similar effect. These results showed that miR-202 could reversely regulate the expression of BAFF, which in turn further inhibited the activation of JNK/SAPK pathway and increased sensitivity of MM cells to Bort.

Discussion

Multiple myeloma (MM) is a clonal B-cell malignant disease characterized by abnormal and uncontrolled proliferation of plasma cells in bone marrow, bone damage and immunodeficiency. Malignant proliferation of plasmocytes produces large amounts of nonfunctional monoclonal immunoglobulin or partial segments, clinically presenting as anemia, renal failure, skeletal damage and susceptibility to infection. There is no effective cure for MM at present [15, 16].

BAFF plays an important role in regulating the proliferation and survival of B and T cells by binding to three receptors: BCMA (B-cell maturation antigen), TACI (transmembrane activator, calcium modulator, and cyclophilin ligand interactor), and BAFF-R/BR3 (BAFF receptor/BLyS receptor 3). Accumulating data indicate that BAFF influences growth and survival of malignant B cells, including multiple myeloma. BAFF promotes MM cell proliferation and survival,

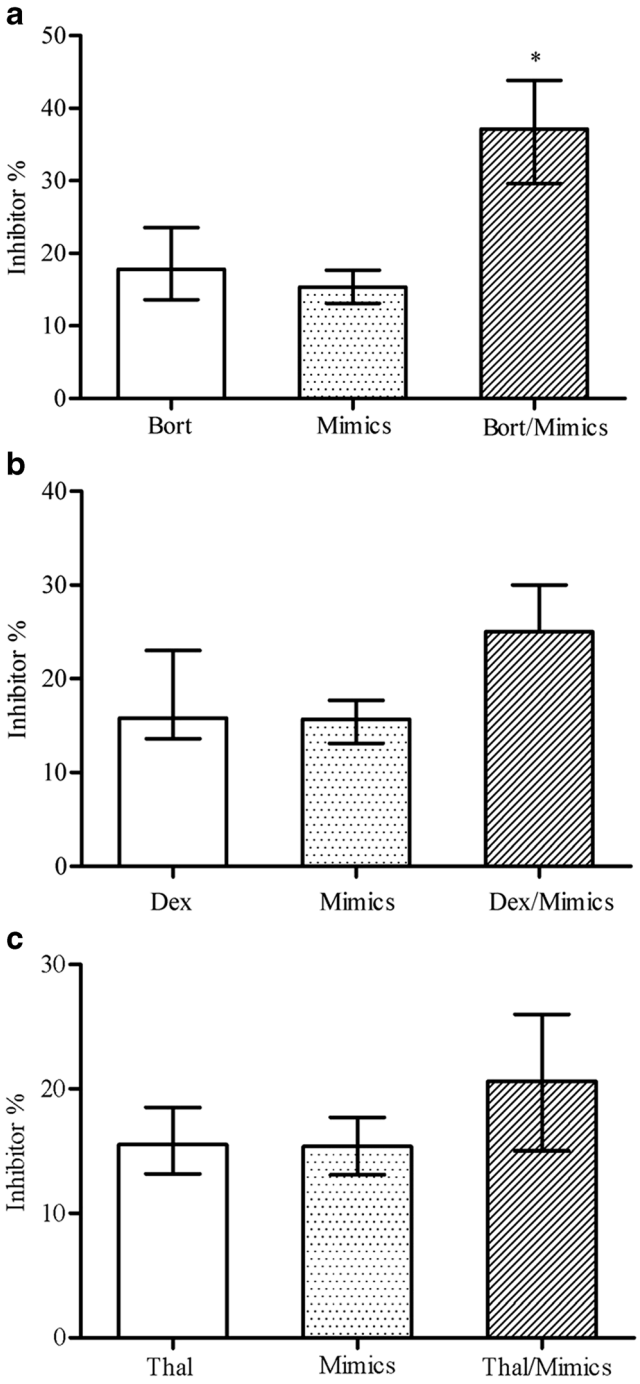

Fig. 5 miR-202 mimics increase the sensitivity of MM cells. to Bort U266 cells were treated with miR-202 mimics (50 nM) and **a** Bort, **b** Dex, or **c** Thal alone or in combination for 48 h. Cell viability was determined by WST-1 assay. Data are representative of three independent experiments (*$P < 0.05$)

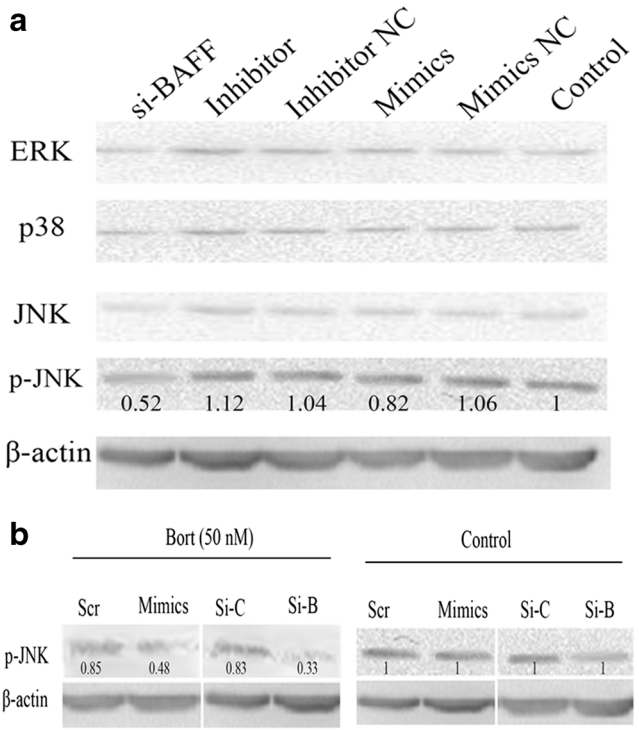

Fig. 6 Impact of miR-202 on MAPK signaling pathway. **a** Detection of ERK, p-ERK, JNK, p-JNK, p38 and p-p38 protein expressions in transfected cells by Western blot. **b** U266 cells were transfected with miR-202 mimics, si-BAFF or controls. p-JNK levels were analyzed at 48 h after Bort treatment or without Bort reatment by Western blot

which is closely associated with the development and progression of MM [17, 18]. Our previous study [19, 20] also have demonstrated that the expression of BAFF and its receptors in peripheral blood of MM patients and MM cell line were significantly higher than those in normal controls, suggesting that BAFF play an important role in the initiation and progression of MM. Recent studies [21, 22] found that BAFF was involved in drug resistance. However, the biological role of BAFF in MM pathogenesis and drug resistance is not fully characterized.

In this study, our bioinformatics analysis on human miRNA targets, mioRNA.org and DIANA-miT showed that miR-202 was able to regulate the expression of BAFF. We established a luciferase reporter gene and confirmed that miR-202 could regulate BAFF expression by directly binding to its 3'UTR. We also found that both BAFF mRNA and protein expressions in U266 cells were decreased in miR-202-mimics transfection group. These results showed that miR-202 down-regulated BAFF expression in MM cells at both the RNA and the protein levels. Previous study found that serum miR-202 is significantly increased in MM patients and that it correlates with serum β_2M and κ light chain concentrations [23]. In this study, miR-202 mRNA was lowly expressed in U266 cells and $CD138^+$ cells of MM patients, which was contrary to previous studies. One possible reason is that bone marrow stromal cells (BMSCs) secrete higher levels of BAFF [24], we also found that miR-202 was weakly expressed in BMSCs of MM patients. These results were in agreement with the result in this study. We hypothesized that the expression of gene in MM cells rather than in serum was approximates much more closely to bone marrow microenvironment. Another reason may be that there are different

regulatory mechanisms that affect the expression of miR-202 in serum and cells.

Although the initial overall response rate to Bort is promising, most patients who initially responded to Bort developed resistance to the drug over time. The exact mechanism that modulates bortezomib sensitivity or resistance in patients with MM remains incompletely understood [25]. The results obtained in this study suggest that over-expression of miR-202 sensitized U266 cells to Bort but less to Thal and Dex. In addition, combining Bort with miR-202 mimics contributed to significant repression of MM cell survival compared with that of Bort treatment alone. These results suggest that the regulatory mechanism of miR-202 expression may be a promising target for fine-tuning anti-myeloma therapy.

Many studies [26, 27] have demonstrated that various drugs for MM exert their inhibitory effects on the proliferation and survival of MM cells by changing the activation of MAPK signaling pathway. Our prior study [14] further demonstrated that BAFF could activate JNK/SAPK signaling pathway, and BAFF siRNA could inhibit JNK/SAPK signaling pathway, indicating that the degree of JNK/SAPK pathway activation is positively correlated with the expression level of BAFF. It was found in this study that U266 cells transfected with miR-202 expressed ERK, p38, JNK and p-JNK proteins in MAPK signaling pathway, while p-JNK protein expression in miR-202-mimics transfection group was reduced, suggesting that miR-202 could inhibit JNK/SAPK signaling pathway, and that JNK/SAPK signaling pathway was involved in the regulatory effect of miR-202 on BAFF. miR-202 could reversely regulate the expression of BAFF, which in turn further inhibited the activation of JNK/SAPK signaling pathway. Meanwhile the increased responsiveness to Bort treatment might be due to the ability of miR-202 to repress the BAFF mediated pathway and counteract the reactivation of JNK/SAPK signaling, suggesting that the regulatory effect of miR-202 on the BAFF expression plays an important role in JNK/SAPK signaling pathway. miR-202 targeting BAFF as a possible tool against MM cells may provide a new therapeutic option for MM patients.

In summary, miR-202 functions as a modulator that negatively regulates BAFF in MM, which provides experimental evidence that over-expression of miR-202 sensitizes U266 cells to Bort but less to Thal and Dex, thus improving the responsiveness to Bort treatment probably due to the counteracting effect on the reactivation of JNK/SAPK signaling. This finding may raise the intriguing possibility of using this miRNA as a new biomarker for assessing responsiveness to specific therapies and as a tool of an innovative and promising therapy.

Acknowledgments XJS and YHG carried out the cell proliferation assay, participated in the cell culture and drafted the manuscript. JQ carried out the immunoassays. WS and XHW participated in the cell survival and growth and PCR assay. HBN carried out apoptosis assay. SQJ conceived of the study, participated in its design and coordination, analysis the data and revised the manuscript.

Compliance with Ethical Standards

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