

Cytoplasmic M-CSF facilitates apoptosis resistance by inhibiting the HIF-1 α /BNIP3/Bax signalling pathway in MCF-7 cells

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Received April 3, 2018; Accepted November 28, 2018

DOI: 10.3892/or.2018.6949

Abstract. Macrophage colony-stimulating factor (M-CSF), a tumour marker, is related to tumour cell anti-apoptosis and drug resistance. However, the role of M-CSF in MCF-7 cells is unknown. In the present study, the effect and mechanism of M-CSF on hypoxia-inducible factor-1 α (HIF-1 α)/BCL2/adenovirus E1B 19 kDa-interacting protein 3 (BNIP3)/Apoptosis Regulator BAX signalling in human breast cancer MCF-7 cells were investigated. Western blotting revealed that the expression of HIF-1 α , BNIP3, Bax, caspase-3 and caspase-9 was lower in MCF-7-M cells compared to MCF-7 and MCF-7-C cells treated with adriamycin (ADM). Immunoprecipitation combined with western blotting was used to detect the interaction between Bcl-2 and BNIP3 or Bax protein. MCF-7-M cells had a higher amount of Bax binding to Bcl-2 compared to MCF-7 cells or MCF-7-C cells, while the amount of BNIP3 binding to Bcl-2 was decreased in MCF-7-M cells. Hoechst 33342 staining and flow cytometry were utilized to evaluate the effect of M-CSF on apoptosis in MCF-7 cells treated with ADM. Compared to ADM-treated MCF-7 cells, the apoptotic rate of MCF-7-M cells was significantly decreased. These effects were dependent on the concentration of ADM. In conclusion, cytoplasmic M-CSF suppressed apoptosis by inhibiting the HIF-1 α /BNIP3/Bax signalling pathway, which potentiated the dissociation of Bcl-2 from Bcl-2-BNIP3 compounds and the formation of Bcl-2-Bax compounds.

Introduction

Macrophage colony-stimulating factor (M-CSF), also known as colony-stimulating factor-1 (CSF-1), promotes monocyte and macrophage cell growth, proliferation, and differentiation as well as maintenance of the biological functions of monocytes and macrophages. Notably, M-CSF is also expressed in many tumour tissues and cancer cells. The expression of M-CSF is markedly enhanced in various cancers (1-3). Increased nuclear expression of M-CSF was revealed to be correlated with poor prognosis and the metastatic potential of breast cancer cells (4). Aharinejad *et al* found that the high expression of cytoplasmic M-CSF in MDA-MB-231 breast cancer cells contributes to the invasion and metastasis of tumours in a mouse model (2). Similarly, M-CSF was revealed to play an important role in the resistance of 5-FU in U87MG glioblastoma (5). In addition, an M-CSF antibody was revealed to reverse the chemoresistance of MCF-7 cells (6). In our previous study, it was revealed that M-CSF induced drug and apoptosis resistance in MCF-7 cells. Therefore, M-CSF is a tumour marker since it is related to anti-apoptosis and drug resistance in tumour cells.

Apoptosis is a common form of programmed cell death, and its deregulation has been associated with tumour initiation, progression, and metastasis in various cancers including breast cancer (7). HIF-1 has been demonstrated to be involved in glycolysis, angiogenesis and migration, and to regulate invasion factors that are important for tumour progression and metastasis (8). HIF-1 activity depends on the expression level of HIF-1 α . HIF-1 α expression is maintained at low levels under normoxic conditions, however it is significantly induced by hypoxia (9). HIF-1 α induces various transcriptional programs, some of which include pluripotency factors in hypoxic conditions (10). A recent study revealed that HIF-1 α regulated anti-apoptotic genes, which ultimately led to increased tumour growth and drug resistance (11). Murine double minute 2 (MDM2), is an oncogene that is upstream of HIF-1 α and regulates the expression of HIF-1 α (12,13). M-CSF was revealed to directly decrease the expression of MDM2,

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Key words: macrophage colony-stimulating factor, breast cancer, apoptosis, HIF-1 α and BINP3

further contributing to drug resistance in tumour treatments (5). M-CSF may modulate the expression of HIF-1 α , however, the mechanism is still unclear.

BNIP3 is a proapoptotic member of the Bcl-2 family and is a downstream target protein of HIF-1 α (14). BNIP3 has a key role in the pathogenesis of many diseases, and it binds anti-apoptotic proteins, including Bcl-2 and BCL-XL, which inhibits their anti-apoptotic activity (15). When BNIP3 binds anti-apoptotic proteins to form heterodimers, it activates pro-apoptotic proteins, such as Bax and Bak, resulting in pro-apoptotic effects (16). A recent study revealed that apoptosis was upregulated after transfection of BNIP3 into MCF-7 cells (17) and rat fibroblasts (18). BNIP3 was activated by the ATPase inhibitor, bafilomycin, in MCF-7 and MDA-MB-231 breast cancer cells, resulting in apoptosis (19). Thus, BNIP3 induced apoptosis in breast cancer cells, indicating that it may be an effective tumour therapeutic target.

Our previous results revealed that cytoplasmic macrophage colony-stimulating factor induced adriamycin-resistance (20). Moreover, antineoplastic agents play an important role in inducing cancer cell apoptosis, and the anti-apoptosis mechanism in cancer cells is vital for tumour multidrug resistance (21). However, anti-apoptotic mechanisms have not been clearly elucidated. Therefore, our hypothesis indicated that M-CSF inhibited the expression of HIF-1 α , which decreased BNIP3, further reducing the binding of anti-apoptotic proteins, such as Bax, to suppress the apoptotic effect. Experiments based on the aforementioned hypothesis were performed, to elucidate the mechanism of cytoplasmic M-CSF-induced cancer cell anti-apoptosis and multidrug resistance mechanisms.

Materials and methods

Cell lines and reagents. MCF-7, a human breast cancer cell line, was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). MCF-7-M cells were transfected with M-CSF, and MCF-7-C cells were transfected with a control plasmid (empty vector). MCF-7, MCF-7-C and MCF-7-M cells were cultured in RPMI-1640 medium (Gibco-BRL; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% newborn calf serum (NBCS) and antibiotics (ExCell, Shanghai, China) at 37°C in a humidified atmosphere containing 95% air and 5% CO₂. Adriamycin was purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany).

Stable transfection. In this experiment, the cytoplasmic positioning and recombination vector pCMV/myc/cyto-M-CSF, was constructed in this laboratory (Department of Pharmacology, Hunan University of Medicine, Huaihua, China) and was used for the present study. This vector contained a cytoplasmic positioning sequence, which forced M-CSF localization in the cytoplasm. The M-CSF molecule in the recombinant vector had a deleted exocytosis signal peptide consisting of 32 amino acids at the N-terminus, which prevented M-CSF secretion outside of the cell, thereby blocking its function as a signal molecule. The pCMV/myc/cyto-M-CSF recombinant vector was used in our previous research (20). In the present study, in order to confirm the efficiency of stable transfection, M-CSF expression was determined by western blot analysis in MCF-7 cells.

MCF-7 cells were seeded in 6-well plates at a density of 1x10⁵ cells/well in RPMI-1640 medium containing 10% FBS for 24 h. Cells were then stably transfected with either pCMV/cyto/myc-M-CSF (cytoplasmic M-CSF gene over-expressed) or pCMV/cyto/myc vector (empty vector) using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The transfection mixtures were replaced with RPMI-1640 medium containing 10% FBS. Cells were harvested at 48 h post-transfection.

Bioinformatics analysis of protein interaction. Using the online STRING database (<https://string-db.org/>), which is a biological database and web resource for known and predicted PPIs, we developed a network of DEG-encoded proteins and PPIs.

Western blot analysis. Cells were washed with cold PBS and mechanically homogenized in RIPA lysis buffer (Beyotime Institute of Biotechnology, Haimen, China). Total protein samples (60 mg/well) were separated on 10 or 15% SDS-PAGE gels. Proteins were then transferred to PVDF membranes (EMD Millipore, Billerica, MA, USA). After blocking with 5% non-fat dried milk for 2 h, the membranes were washed for 10 min in TBST (0.1% Tween-20, TBS) three times. The membranes were then incubated with primary antibodies against HIF-1 α (dilution 1:1,000; cat. no. 3716; Cell Signaling Technology, Inc., Danvers, MA, USA), BNIP3 (Ana40) (dilution 1:1,000; cat. no. ab10433; Abcam, Cambridge, UK) Bax (dilution 1:1,000; cat. no. D2E11; Cell Signaling Technology, Inc.), Bcl-2 (E17) (dilution 1:800; cat. no. ab32124; Abcam) and β -actin (dilution 1:2,000; cat. no. 66009-1-Ig; Proteintech Group, Inc., Wuhan, China) overnight at 4°C. Subsequently, the membranes were then incubated with secondary antibodies [goat anti-rabbit IgG-HRP (dilution 1:4,000; cat. no. SA00001-2; Proteintech Group, Inc.), goat anti-mouse IgG-HRP (dilution 1:4,000; cat. no. SA00001-1; Proteintech Group, Inc.) and rabbit anti-goat IgG-HRP (dilution 1:4,000; cat. no. SA00001-4; Proteintech Group, Inc.) for 1 h at room temperature. Signals were detected by Western Chemiluminescence HRP Substrate (ECL) solution (Beyotime Institute of Biotechnology). Protein bands relative to β -actin were quantified using Glyko BandScan 5.0 software (Glyko Inc., Novato, CA, USA).

Annexin V-fluorescein isothiocyanate apoptosis assay. An Annexin V-FLUO Staining Kit (Boehringer-Mannheim; Roche Diagnostics, Mannheim, Germany) was used to evaluate doxorubicin-induced apoptosis. Cells were cultured in a 6-well plate and exposed to 0.5 μ M ADM for 24 h. Cells were collected in a 10-ml centrifuge tube and stained with Annexin V-FLUOS and PI for 15 min. Apoptosis was immediately analysed with a flow cytometer (Beckman Coulter, Inc., Fullerton, CA, USA) at a wavelength of 488 nm.

Hoechst 33342 staining for the apoptosis assay. Hoechst 33342 dye is cell permeable and binds to DNA in live or dead cells. However, PI is cell membrane impermeable and excluded from viable cells, and is typically used to identify dead cells. MCF-7 cells (5x10⁴ cells/well in 1 ml) were seeded in 24-well plates

and cultured for 24 h at 37°C under a humidified atmosphere of 5% CO₂. Thereafter, serum-free medium was replaced with the same medium containing 0, 0.5, 1, 2, 4 and 8 μM ADM. After 24 h of drug incubation, the medium was removed and Immunol Staining Fix Solution (Beyotime Institute of Biotechnology) was added (0.5 ml/well) for 20 min at 4°C. Plates were then washed two times for 3 min in PBS. After washing, Hoechst 33342 staining solution (Beyotime Institute of Biotechnology) was added (0.5 ml/well) and incubated for 20 min at 37°C. Plates were then washed two times for 3 min in PBS. Hoechst-positive cells exhibited blue fluorescence, while PI-positive cells exhibited red fluorescence. Apoptotic cells were Hoechst-positive and demonstrated characteristic features of apoptosis, such as, condensed or fragmented nuclei. Staining was analysed by morphology and fluorescence.

Co-immunoprecipitation analysis. Cells were divided into six groups according to different processing factors as follows: MCF-7, MCF-7-C, MCF-7-M, MCF-7+ADM, MCF-7-C+ADM and MCF-7-M+ADM. Cells were cultured for 24 h before adding 200 μl of IP lysis buffer (containing 2 μl of PMSF, 2 μl of protease inhibitor and 2 μl protein phosphatase inhibitor), which was 5-fold the total volume of cells. Cells were suspended and lysed on ice for 30 min. Cells were lysed and then incubated overnight with 1 μg of Bcl-2 antibody at 4°C, and 1 μg of rabbit normal IgG was used as the negative control group. Lysates were then incubated for 4 h with 150 μl of a 10% suspension of protein A-sepharose beads (Sigma-Aldrich, Poole, UK) at 4°C. Immunocomplexes were then collected for western blotting to detect the expression of Bcl-2, Bax and BNIP3.

Statistical analysis. All results were expressed as the mean ± standard deviation (SD). Data analysis was performed using SPSS 18.0 (SPSS, Inc., Chicago, IL, USA). Groups were compared using Student's t-test or two-way ANOVA. Multiple comparison between the groups was performed using the S-N-K method at a significance level of α=0.05. P<0.05 was considered to indicate a statistically significant difference.

Results

M-CSF expression is upregulated in overexpressed transfectants of MCF-7 cells. To determine the efficiency of M-CSF stable transfection, the expression of M-CSF was assessed in MCF-7, MCF-7-C and MCF-7-M cells using western blotting (Fig. 1). The results revealed that the expression of M-CSF was not significantly different in MCF-7-C cells and MCF-7 cells. There was a much higher expression of M-CSF protein in MCF-7-M cells compared to the MCF-7 and MCF-7-C cells.

Cytoplasmic M-CSF regulates the expression of HIF-1α in MCF-7 cells. The bioinformatics online analysis software, STRING, was used to analyse the interaction of M-CSF and HIF-1α. M-CSF and its receptor interacted with MDM2, which resulted in MDM2 and HIF-1α regulating each other (Fig. 2). Previous research demonstrated that M-CSF directly decreased the expression of MDM2, further leading to tumour drug resistance. Additionally, MDM2 upregulated

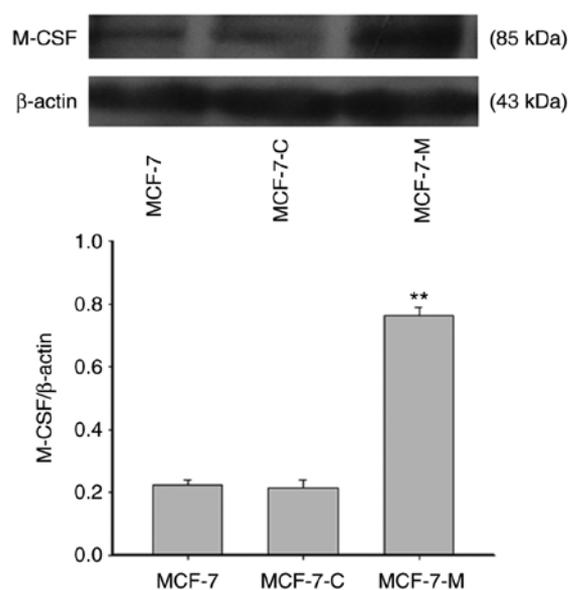


Figure 1. The efficiency of M-CSF stable transfection. M-CSF protein expression was determined by western blotting in MCF-7, MCF-7-C, and MCF-7-M cells. Band densitometry analysis of M-CSF expression in MCF-7, MCF-7-C, and MCF-7-M cells normalized to β-actin, respectively. **P<0.01, MCF-7-M vs. MCF-7 or MCF-7-C. M-CSF, macrophage colony-stimulating factor; MCF-7-M, MCF-7 cells transfected with M-CSF; MCF-7-C, MCF-7 cells transfected with control plasmid. M-CSF, macrophage colony-stimulating factor.

HIF-1α in a p53-independent manner. Thus, these results indicated that M-CSF decreased the expression of HIF-1α by regulating MDM2.

Cytoplasmic M-CSF suppresses the expression of HIF-1α, BNIP3 and Bax in MCF-7 cells treated with ADM. As aforementioned, M-CSF is associated with tumour cell anti-apoptosis and drug resistance. HIF-1α, BNIP3 and Bax play an important role in cell apoptosis. To determine if M-CSF has a regulatory effect on HIF-1α, BNIP3 and Bax in MCF-7 cells, western blotting was performed to analyse the expression of these proteins in MCF-7, MCF-7-C and MCF-7-M cells before and after treatment with ADM. The expression of HIF-1α and BNIP3 was lower in MCF-7-M cells compared to MCF-7 cells or MCF-7-C cells without ADM (Fig. 3A and B). Bax protein expression had no significant difference in MCF-7, MCF-7-C and MCF-7-M cells treated without ADM (Fig. 3C). Compared to MCF-7 and MCF-7-C cells, the expression of HIF-1α, BNIP3 and Bax was strongly decreased after ADM treatment (Fig. 3A-C). Moreover, HIF-1α, BNIP3 and Bax protein expression decreased in MCF-7-M cells treated with ADM compared to untreated MCF-7-M (Fig. 3A-C). Collectively, these data revealed that cytoplasmic M-CSF inhibited the expression of HIF-1α, BNIP3 and Bax in MCF-7 cells treated with ADM and that ADM enhanced the inhibitory effect of M-CSF in MCF-7 cells.

Cytoplasmic M-CSF reduces the binding of Bcl-2 to BNIP3 but increases Bcl-2 binding to Bax in MCF-7 cells after treatment with ADM. Previous research has demonstrated that Bcl-2 is an anti-apoptotic protein and that BNIP3 and Bax competitively bind to Bcl-2. The present study revealed

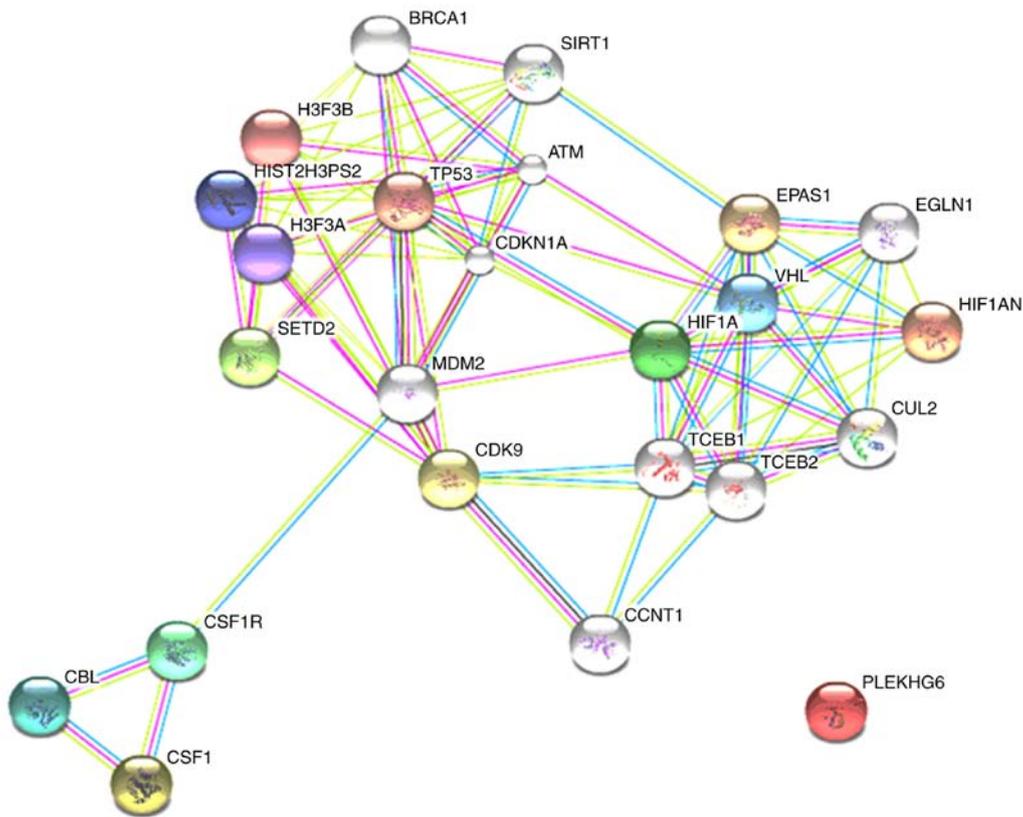


Figure 2. Functional interaction prediction of HIF-1α and M-CSF. HIF-1α, hypoxia-inducible factor-1α; M-CSF, macrophage colony-stimulating factor.

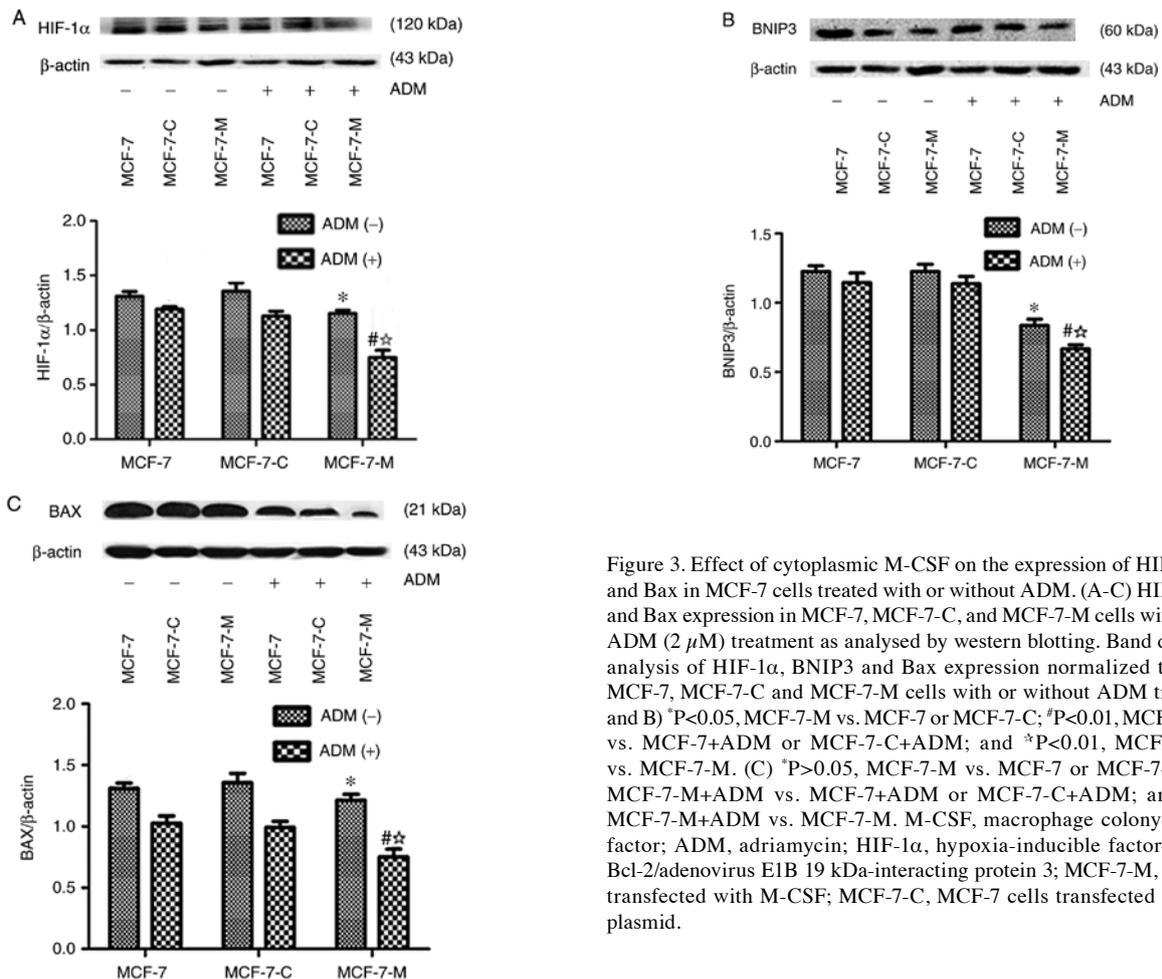


Figure 3. Effect of cytoplasmic M-CSF on the expression of HIF-1α, BNIP3 and Bax in MCF-7 cells treated with or without ADM. (A-C) HIF-1α, BNIP3 and Bax expression in MCF-7, MCF-7-C, and MCF-7-M cells with or without ADM (2 μM) treatment as analysed by western blotting. Band densitometry analysis of HIF-1α, BNIP3 and Bax expression normalized to β-actin in MCF-7, MCF-7-C and MCF-7-M cells with or without ADM treatment. (A and B) *P<0.05, MCF-7-M vs. MCF-7 or MCF-7-C; #P<0.01, MCF-7-M+ADM vs. MCF-7+ADM or MCF-7-C+ADM; and *P<0.01, MCF-7-M+ADM vs. MCF-7-M. (C) *P>0.05, MCF-7-M vs. MCF-7 or MCF-7-C; #P<0.01, MCF-7-M+ADM vs. MCF-7+ADM or MCF-7-C+ADM; and *P<0.01, MCF-7-M+ADM vs. MCF-7-M. M-CSF, macrophage colony-stimulating factor; ADM, adriamycin; HIF-1α, hypoxia-inducible factor-1α; BNIP3, Bcl-2/adrenovirus E1B 19 kDa-interacting protein 3; MCF-7-M, MCF-7 cells transfected with M-CSF; MCF-7-C, MCF-7 cells transfected with control plasmid.

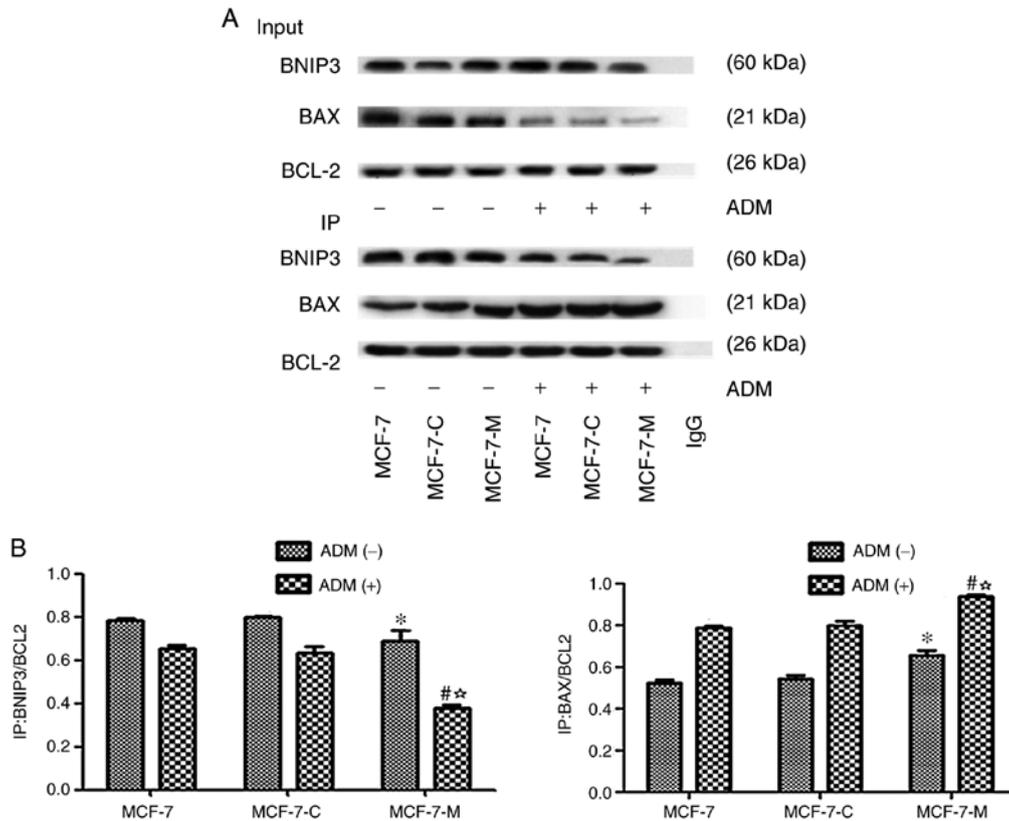


Figure 4. Effect of cytoplasmic M-CSF on the interaction between Bcl-2 and BNIP3 or between Bcl-2 and Bax in MCF-7 cells treated with or without ADM. (A) Cells were divided into a control group (Input group) and positive group (IP) according to the presence of the Bcl-2 antibody. BNIP3, Bax and Bcl-2 protein expression was analysed by western blotting in MCF-7, MCF-7-C and MCF-7-M cells with or without ADM treatment (2 μ M). (B) Band densitometry analysis of BNIP3, Bax and Bcl-2 expression in MCF-7, MCF-7-C and MCF-7-M cells with or without ADM treatment. * $P > 0.05$, MCF-7-M vs. MCF-7 or MCF-7-C; # $P < 0.01$, MCF-7-M+ADM vs. MCF-7-C+ADM; $^{\Delta}P < 0.01$, MCF-7-M+ADM vs. MCF-7-M. M-CSF, macrophage colony-stimulating factor; ADM, adriamycin; HIF-1 α , hypoxia-inducible factor-1 α ; BNIP3, Bcl-2/adenovirus E1B 19 kDa-interacting protein 3; MCF-7-M, MCF-7 cells transfected with M-CSF; MCF-7-C, MCF-7 cells transfected with control plasmid.

that M-CSF suppressed the expression of BNIP3 and Bax. Thus, M-CSF significantly decreased Bax expression in MCF-7 cells by inhibiting the binding of BNIP3 to Bcl-2 but increasing the binding of Bax to Bcl-2, blocking apoptosis in MCF-7 cells. Co-immunoprecipitation analysis was performed to analyse the state of Bcl-2 binding to BNIP3 and Bax protein using Bcl-2 as the antibody in MCF-7, MCF-7-C and MCF-7-M cells incubated with ADM (2 μ M) for 24 h. There was no significant difference in the amount of BNIP3 that Bcl-2 bound in MCF-7, MCF-7-C and MCF-7-M cells (Fig. 4A and B). The amount of Bcl-2 binding to Bax was greater in MCF-7-M cells than in MCF-7 or MCF-7-C cells without ADM treatment (Fig. 4A and B). Treatment with ADM caused a significantly lower amount of BNIP3 binding to Bcl-2 in MCF-7-M cells compared to MCF-7 or MCF-7-C cells (Fig. 4A and B). The amount of Bax binding to Bcl-2 in MCF-7-M cells was higher than that in MCF-7 cells and MCF-7-C cells (Fig. 4A and B). Collectively, these results indicated that cytoplasmic M-CSF induced anti-apoptosis by inhibiting the binding of Bcl-2 to BNIP3 protein and by increasing the binding of Bcl-2 to Bax protein in MCF-7 cells.

Cytoplasmic M-CSF increases the capability of anti-apoptosis
Hoechst 33342 staining detection of cell apoptosis. MCF-7, MCF-7-C, and MCF-7-M cells were plated and cultured in

24-well plates for 12 h followed by incubation with ADM at 0, 0.5, 1.0, 2.0, 4.0 and 8.0 μ M for 24 h. Cells apoptosis was then analysed using Hoechst 33342 staining. MCF-7, MCF-7-C, and MCF-7-M cells apoptosis significantly increased with increasing drug concentration, but the number of nuclear MCF-7-M cells was decreased in comparison to that of MCF-7 and MCF-7-C cells treated with the same concentration of ADM (Fig. 5A and B). Collectively, these results indicated that M-CSF enhanced the anti-apoptotic ability of MCF-7 cells.

Apoptosis analysis by flow cytometry. To further determine if M-CSF influences the anti-apoptotic capability in MCF-7 cells, MCF-7 cell apoptosis was assessed using flow cytometry. A significant reduction of ADM-induced apoptosis was observed in MCF-7-M cells compared to MCF-7 and MCF-7-C cells (Fig. 6A and B). Collectively, these data revealed that M-CSF inhibited ADM-induced apoptosis in MCF-7 cells.

Cytoplasmic M-CSF decreases the expression of caspase-3 and caspase-9. The expression of caspase-3 and caspase-9 was assessed in MCF-7, MCF-7-C and MCF-7-M cells incubated in the presence or absence of ADM (2 μ M) for 24 h using western blotting. The expression of caspase-3 and caspase-9 was not significantly different in untreated MCF-7, MCF-7-C and MCF-7-M cells (Fig. 7A-C), however ADM treatment,

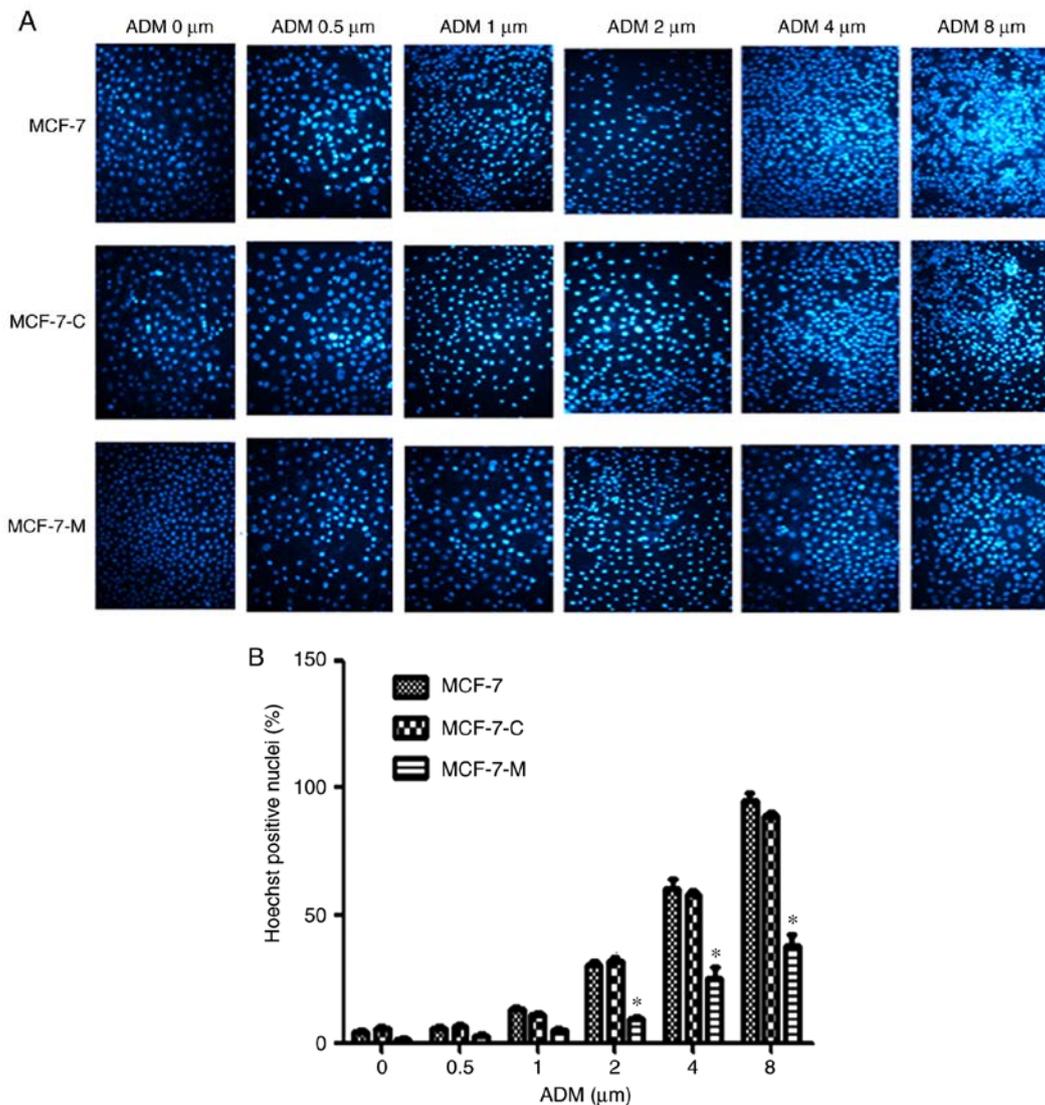


Figure 5. Cytoplasmic M-CSF inhibits apoptosis in MCF-7 cells treated with ADM (Hoechst 33342 staining). (A) Apoptosis analysis by Hoechst 33342 staining in 0, 0.5, 1.0, 2.0, 4.0 and 8.0 μM ADM-treated MCF-7, MCF-7-C and MCF-7-M cells. (B) Data analysis of the rate of Hoechst 33342-positive nuclei ($P < 0.05$, MCF-7-M vs. MCF-7 or MCF-7-C; $n = 6$). M-CSF, macrophage colony-stimulating factor; ADM, adriamycin; MCF-7-M, MCF-7 cells transfected with M-CSF; MCF-7-C, MCF-7 cells transfected with control plasmid.

significantly reduced caspase-3 and caspase-9 in MCF-7-M cells in comparison to MCF-7 or MCF-7-C cells (Fig. 7A-C). The expression levels of caspase-3 and caspase-9 in MCF-7-M cells treated with ADM were significantly lower than those in untreated MCF-7-M cells (Fig. 7A-C). These results indicated that cytoplasmic M-CSF suppressed ADM-induced caspase-3 and caspase-9 protein expression in MCF-7 cells.

Discussion

Breast cancer is a serious threat to the health of women and is the major cause of death in 40- to 55-year-old women. Globally, breast cancer accounted for the highest number of new cancer cases in 2015 (22). Nearly 30% of newly diagnosed patients with early stage breast cancer develop a distant metastasis despite receiving therapy (23). Current therapy options for breast cancer include surgery, hormonal therapy, immunotherapy, chemotherapy, radiation therapy, or a combination of these treatments (24). The main treatment method for

breast cancer is radical surgery combined with postoperative chemotherapy and radiotherapy. However, the use of chemotherapeutic drugs is usually accompanied by deleterious side effects, and the development of drug resistance occurs when applied for a longer period. Drug resistance is related to tumour cell apoptosis, but the mechanism is unclear.

The growth of tumour cells is regulated by various factors. Many growth factors and cytokines are involved in the regulation of the tumour microenvironment in the immune system, and their function in immune surveillance and immune clearance. For example, M-CSF, which is known as CSF-1, has a vital role in the biological function of mononuclear macrophages as well as in tumour invasion, metastasis, drug resistance and prognosis (25). M-CSF is expressed in tumour-associated macrophages (TAMs) (26,27). In recent years, several studies have reported high expression of cytoplasmic M-CSF in type II papillary renal cell carcinoma (28), breast (29,30), ovarian (30,31), endometrial (32), colorectal (33), pancreatic (34), prostate and head and neck cancer (35). Additionally,

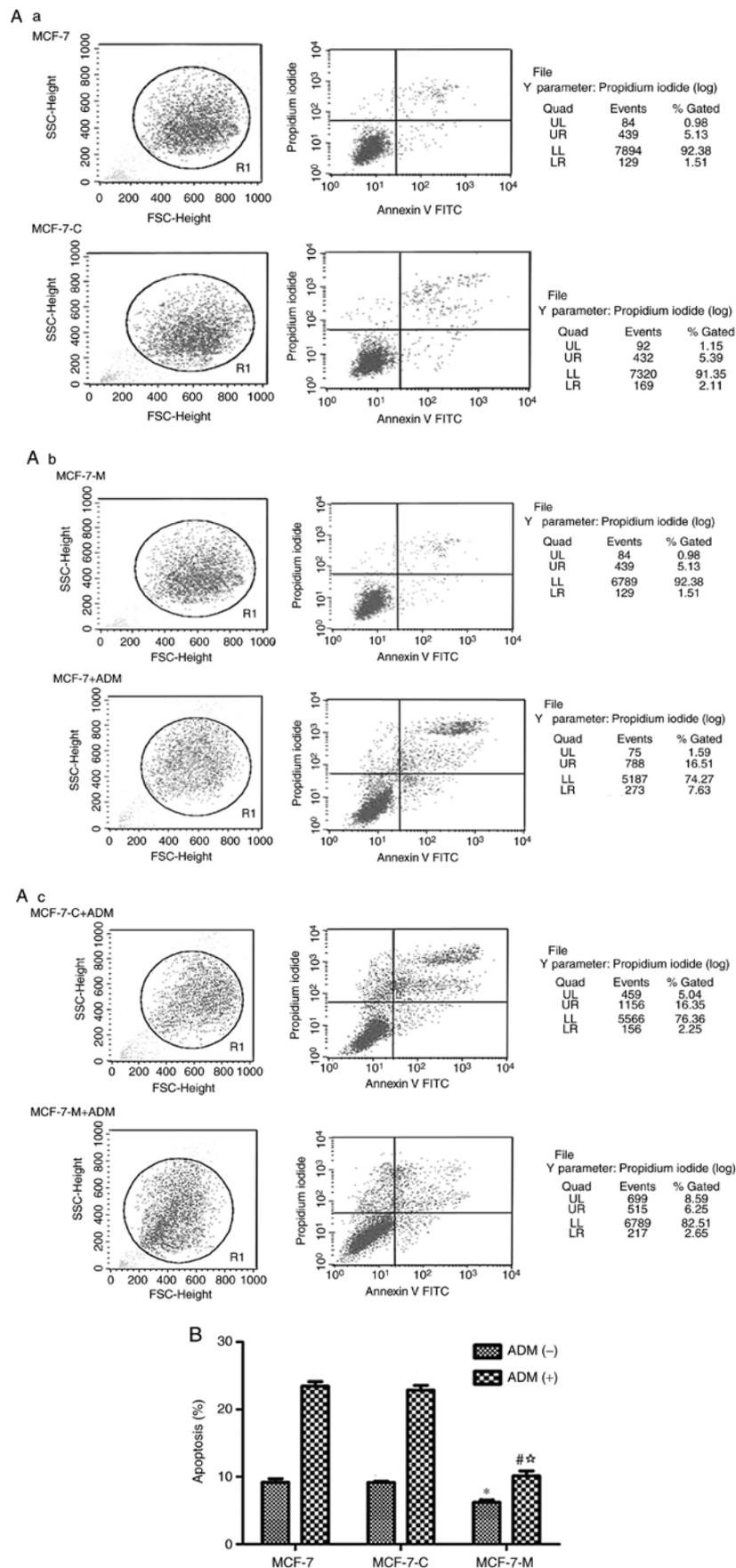


Figure 6. Cytoplasmic M-CSF inhibits apoptosis in MCF-7 cells treated with ADM. (A-a, -b, -c) Flow cytometric apoptosis analysis of MCF-7, MCF-7-C and MCF-7-M cells treated with or without ADM (0.5 μ M). (B) Band densitometry analysis of the apoptosis rate represented in A. Flow cytometry, error bars represent \pm standard deviation, n=3; *P<0.05, MCF-7-M vs. MCF-7 or MCF-7-C; #P<0.01, MCF-7-M+ADM vs. MCF-7+ADM or MCF-7-C+ADM; *P<0.01, MCF-7-M+ADM vs. MCF-7-M; n=3). M-CSF, macrophage colony-stimulating factor; ADM, adriamycin; MCF-7-M, MCF-7 cells transfected with M-CSF; MCF-7-C, MCF-7 cells transfected with control plasmid.

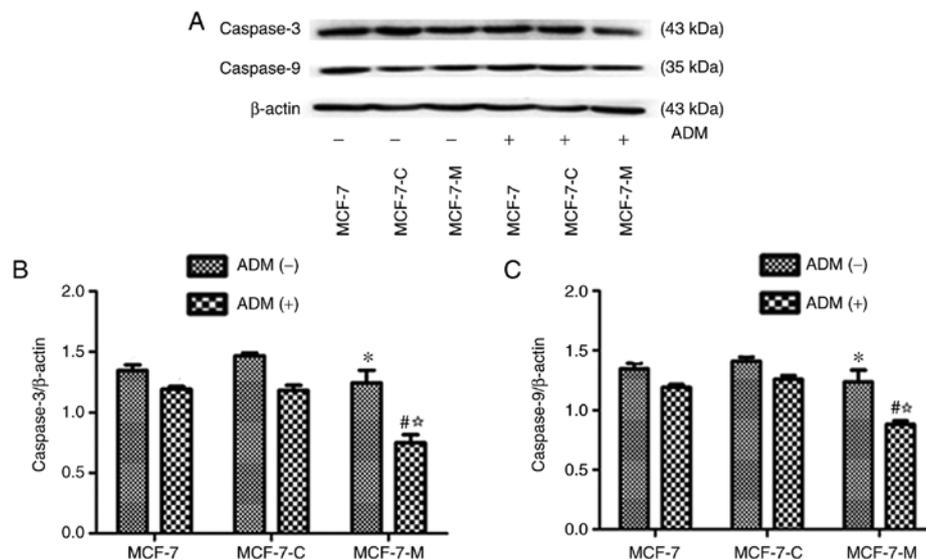


Figure 7. Effect of cytoplasmic M-CSF on the expression of caspase-3 and caspase-9 in MCF-7 cells treated with ADM. (A) Caspase-3 and caspase-9 protein expression was analysed by western blotting in MCF-7, MCF-7-C and MCF-7-M cells treated with or without ADM (2 μ M). (B and C) Band densitometry analysis of caspase-3 and caspase-9 expression normalized to β -actin in MCF-7, MCF-7-C and MCF-7-M cells treated with or without ADM. * $P > 0.05$, MCF-7-M vs. MCF-7 or MCF-7-C; # $P < 0.01$, MCF-7-M+ADM vs. MCF-7+ADM or MCF-7-C+ADM; ☆ $P < 0.01$, MCF-7-M+ADM vs. MCF-7-M. M-CSF, macrophage colony-stimulating factor; ADM, adriamycin; caspase-3/9, cysteinyl aspartate-specific proteinase 3/9; MCF-7-M, MCF-7 cells transfected with M-CSF; MCF-7-C, MCF-7 cells transfected with control plasmid.

a study revealed that the overexpression of M-CSF and its receptor was associated with a poor prognosis (36). M-CSF also promoted tumour cell proliferation (37,38) and non-small cell lung cancer bone metastases (39). Lin *et al* discovered that overexpression of cytoplasmic M-CSF was responsible for the invasion and metastases of cancer cells in a mouse breast cancer model (40). An M-CSF gene null mutation in rats resulted in decreased malignancy and metastasis of tumours (41). Collectively, these findings indicated that M-CSF plays a vital role in the development of diverse tumours. Although, the mechanisms may be different in these tumours, M-CSF ultimately results in tumour development and chemoresistance. Hence, M-CSF may act as a factor to induce tumour cell anti-apoptosis in MCF-7 breast cancer. The specific effects of M-CSF in cancer cells were increased by stable transfection of cytoplasmic M-CSF into MCF-7 cells. In the presence of ADM, cytoplasmic M-CSF led to an increase in the anti-apoptosis capability of MCF-7 cells.

Considerable attention has been paid to the contribution of the tumour microenvironment. For example, hypoxia is an important component of the microenvironment of various types of solid tumours (42), including breast cancer. Hypoxia increases 'stemness', EMT, migratory capabilities and invasive capabilities (20). HIF-1 is a transcription factor that plays an important role in the response to hypoxia. Under hypoxic conditions, HIF-1 has a corresponding physiological function via binding target proteins, including vascular endothelial growth factor (VEGF), nitric oxide synthase (NOS), p53, growth factors and inflammatory factors. A previous study revealed that hypoxia-inducible factor-dependent signalling promoted M-CSF-induced macrophage recruitment in triple-negative breast cancer cells and mesenchymal stem cells (43). MDM2 is located upstream of HIF-1 α and has been revealed to regulate the expression of HIF-1. Moreover, M-CSF has been demonstrated to reduce the protein expression of MDM2. These

findings indicated that M-CSF may induce tumour cell proliferation and drug resistance by regulating the expression of HIF-1 α . The present study determined that M-CSF was related to the expression of HIF-1 α through bioinformatics analysis and that cytoplasmic M-CSF suppressed the expression of HIF-1 α in MCF-7 cells treated with ADM.

Apoptosis is a cell death process that uses specialized machinery for self-destruction. If the apoptotic process is dysregulated, tumour tissue develops rapidly, leading to malignancy. The anti-apoptotic Bcl-2 protein has been revealed to be increased in breast cancer cells, indicating the imbalance between apoptosis and anti-apoptosis (44). BNIP3 is a target protein of HIF-1 α and is a proapoptotic member of the Bcl-2 family of proteins, and HIF-1 α has been demonstrated to bind to the HRE-2 site of the BNIP3 promoter. BNIP3 binds anti-apoptotic proteins, such as Bcl-2 and Bcl-x1, to form heterodimers, which activate pro-apoptotic proteins (45). Elevated BNIP3 expression was revealed to be associated with poor prognosis (46). These results indicated that cytoplasmic M-CSF induced anti-apoptosis in breast cancer by regulating HIF-1 α /BNIP3/Bax. The present study also revealed that cytoplasmic M-CSF induced cell anti-apoptosis by inhibiting the binding of Bcl-2 to BNIP3 protein and by increasing the binding of Bcl-2 to Bax protein in MCF-7 cells treated with ADM.

Collectively, these results indicated that cytoplasmic M-CSF suppresses cells apoptosis by inhibiting HIF-1 α /BNIP3/Bax signalling in MCF-7 cells. Bioinformatics analysis revealed that M-CSF not only directly regulated the expression of HIF-1 only MDM2, but also indirectly regulated HIF-1 α protein through p53. A previous study revealed that M-CSF-induced 5-FU resistance was mediated by decreasing the expression of MDM2 and ABCB1. In addition, MDM2 induced upregulation of HIF-1 α protein in a p53-independent manner (13). Therefore, M-CSF suppressed the expression of HIF-1 α through a

p53-independent pathway. A model was generated by stably transfecting MCF-7 cells with M-CSF to explore the relationship between cytoplasmic M-CSF and MCF-7 cell death. Hoechst 33342 staining and flow cytometry was performed to demonstrate that the antineoplastic agent-induced rate of apoptosis was significantly decreased in MCF-7-M cells in comparison with control groups. Western blotting revealed a significant reduction of HIF-1 α , BNIP3, Bax, caspase-3 and caspase-9 protein expression in MCF-7-M cells treated with ADM compared to control groups. To further explore the specific mechanism of M-CSF-mediated low expression of pro-apoptotic Bax protein, the relationship of Bcl-2 binding to BINP3 and Bax was analysed using co-immunoprecipitation. The binding rate of BNIP3 to Bcl-2 was decreased, but the binding rate of Bcl-2 to Bax was increased, thereby, leading to the reduction of free Bax. Thus, cytoplasmic M-CSF suppressed cell apoptosis by inhibiting HIF-1 α /BNIP3/Bax signalling in human MCF-7 breast cancer cells due to decreased binding of BNIP3/Bcl-2 and increased binding of Bcl-2 to Bax, which resulted in low free Bax and ultimately apoptosis resistance.

The present study reported for the first time, to the best of our knowledge, that apoptosis was regulated through the M-CSF/HIF-1 α /BNIP3/Bax signalling pathway in MCF-7 breast cancer cells, which provided a new target for breast cancer therapy. This regulation is a new p53-independent pathway, which plays an important role in the therapy and prognosis of breast cancer. However, it remains unknown which pathway is involved in the M-CSF-induced reduction of HIF-1 α expression in MCF-7 breast cancer cells. M-CSF may regulate HIF-1 α protein expression via MDM2. Since HIF-1 α regulates angiogenic factors, M-CSF/HIF-1 α may be associated with tumour angiogenesis. Several studies have revealed that BNIP3 is related to autophagy. Thus, M-CSF-mediated autophagy may be induced by the HIF-1 α /BNIP3/beclin1 pathway in breast cancer cells, and excessive autophagy induces apoptosis.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Hunan Provincial Science and Technology Plan Key Projects of China (2017SK2183), the Science and Technology Plan Key Projects of Hunan Province (10A087) and the Hunan Provincial Natural Science Key Foundation of Hunan (09JJ3060).

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Authors' contributions

ST designed the experiments. MZ, QL, LL, JN, JT, XL performed all the experimental procedures; ST and ZM performed the statistical analysis; MZ, QL and LL prepared the first draft of the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of

the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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