Original Article

BTK induces CAM-DR through regulation of CXCR4 degradation in multiple myeloma

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Abstract: Cellular adhesion-mediated drug resistance (CAM-DR) occurs frequently in patients with relapsed or refractory multiple myeloma (MM). Elucidating the mechanism underlying CAM-DR and developing the corresponding treatment may prove to be promising for the clinical management of MM. Bruton’s tyrosine kinase (BTK) has been attracting attention in relation to MM progression and drug resistance. BTK was reported to be associated with cell surface CXCR4, a classic cell adhesion molecule and homing factor. However, the exact association between BTK and CAM-DR in MM remains elusive. In this study, we demonstrated that promoting BTK expression induced MM cell adherence to the extracellular matrix (ECM) and stromal cells in vitro and in vivo, and that CAM-DR could be reversed by separating MM cells from ECM or stromal cells. Enhancing BTK expression levels increased CXCR4 expression in MM cells. In addition, BTK may bind directly with CXCR4 and prevent its ubiquitination-induced degradation. Finally, a BTK inhibitor exerted synergistic therapeutic effects with bortezomib in a 5TMM3VT MM mouse model. These findings revealed a novel role of BTK in CAM-DR and may provide a promising approach to MM treatment.

Keywords: Bruton’s tyrosine kinase, CXCR4, adhesion, drug resistance, multiple myeloma

Introduction

Multiple myeloma (MM) is the second most common haematological malignancy and remains incurable [1-3]. Despite advances in both basic and clinical research over the past decades [3], drug resistance in relapsed or refractory MM remains a major obstacle for MM treatment [4, 5], in which cellular adhesion-mediated drug resistance (CAM-DR) plays an important role [6, 7]. Adhesion between the microenvironment and MM cells contributes to cell proliferation [8, 9], survival [10, 11] and homing[11-13]. Therefore, elucidating the mechanism underlying CAM-DR and developing the corresponding treatment may hold promise for the clinical management of MM.

MM cells produce CXCR4, CD49d and CD44, which are mediators of cellular adhesion [14]. CXCR4, an alpha-chemokine receptor specific for stromal-derived factor-1 (SDF-1), may be upregulated on the surface of MM cells and plays a key role in cellular adhesion [15, 16]. CXCR4 signalling was also confirmed to promote the mobility of myeloma cells and may be used as a target for clinical intervention [17, 18]. The CXCR4 antagonist AMD3100 can initially enhance and subsequently inhibit the survival and proliferation of myeloma cells [19]. The bone marrow microenvironment produces several adhesion molecules and cytokines, which promote the adhesion between microenvironment and MM cells, thereby leading to drug resistance and poor survival [20, 21]. A number of cytokines, including IL-6 [22, 23], IGF-1 [23], SDF-1 [24, 25] and USP14 [25], which are produced by bone marrow stromal cells (BMSCs), play important roles in the CAM-DR of MM cells. SDF-1, which is primarily produced by stromal cells, can bind to CXCR4 expressed on the plasma membrane of MM cells and induce cell motility, internalization, and cytoskeletal rearrangement [26]. SDF-1
alpha mediates chemoresistance and apoptosis of MM cells in adhesion state by upregulating the expression of IL-6 [27]. In addition, a number of signalling pathways are involved in CAM-DR of MM, including the PI3K/AKT and MAPK/ERK [26, 28], Wnt3/RhoA/ROCK [29] and HMG-CoA/GG-PP/Rho/Rho-kinase [30] pathways. The network between the bone marrow microenvironment and MM cells is extensive and complex, and requires further investigation.

A previous study by our group demonstrated that Bruton’s tyrosine kinase (BTK), which is a known regulator of myeloma stemness and senescence, is correlated with MM progression and drug resistance [31, 32]. Chen et al reported that BTK expression was associated with cell surface CXCR4 expression, which was closely associated with cell adhesion and homing [33]. Furthermore, ibrutinib, a BTK inhibitor, was able to reduce the surface membrane levels of CXCR4 in chronic lymphocytic leukaemia, and downregulate the migration of myeloma cells toward SDF-1 and homing to the bone marrow microenvironment [12, 33]. The studies mentioned above prompted us to investigate the association between BTK and CXCR4 and its role in MM cellular adhesion and CAM-DR.

In this study, we sought to demonstrate the effect of BTK in mediating MM cellular adhesion and drug resistance in vitro, and adhesion between MM cells and mouse blood vessels in vivo. Additionally, we aimed to elucidate the mechanism of BTK-induced MM cellular adhesion and drug resistance, and the interactions between BTK and CXCR4. The aim was to design a promising MM treatment strategy by combining bortezomib (BTZ) with CGI-1746, a specific BTK inhibitor.

Materials and methods

Materials

MG132 (purity >99%) and MTT (purity >99%) were purchased from Beyotime Institute of Biotechnology (Shanghai, China). BSA (purity >98%) and the PKH26 fluorescent cell linker kits were purchased from Sigma-Aldrich; Merck KGaA (St. Louis, USA). Bortezomib (purity >99%), doxorubicin (purity >99%), CGI1746 (purity >99%), AMD3100 (purity >99%) and LY294002 (purity >99%) were purchased from Selleck Chemicals (Houston, USA). Anti-β-actin (4967L, Rabbit Ab, Cell Signaling Technology, Massachusetts, USA), anti-ubiquitin (#3933, Rabbit Ab, Cell Signaling Technology, Massachusetts, USA), Normal Mouse IgG (A7028, Beyotime Institute of Biotechnology, Shanghai, China), anti-BTK (sc-1108, Goat Ab, Santa Cruz Biotechnology, Inc., Texas, USA) and anti-CXCR4 (sc-53534, Mouse mAb, Santa Cruz Biotechnology, Inc. Texas, USA). TRITC, DAPI, Alexa Fluor 647, Alexa Fluor 555, PierceTM BCA Protein Assay Kit and IP/Co-IP kit were purchased from Thermo Fisher Scientific, Inc. (Waltham, USA).

Animals

C57BL/Ka wrij mice (male: n=10, female: n=10), aged 6-8 weeks and weighing 20±2 g, were purchased from Beijing Vital River Laboratory Animal Technology, Co., Ltd., and housed at the specific pathogen-free (SPF) animal experiment centre of Nanjing University of Chinese Medicine. The animals were fed a basal diet and water ad libitum and were housed at 24±2°C with 65±5% humidity, with a 12 h light-dark cycle. All animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals at Nanjing University of Chinese Medicine (Nanjing, China), and were approved by the Animal Ethics Committee of Nanjing University of Chinese Medicine (permit no. ACU170501-20170522).

Cell culture

ARP1 and OPM2 cells were gifts from Dr John Shaughnessy [34] (University of Arkansas for Medical Sciences, Little Rock, AR, USA). Genes were overexpressed as described previously [32]. The cell lines were confirmed to be mycoplasma-free. All myeloma cells were maintained at 37°C and 5% CO₂, using RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% heat-inactivated FCS (Gibco; Thermo Fisher Scientific, Inc.) and penicillin/streptomycin (100 μg/ml; Sigma Aldrich; Merck KGaA) as growth medium.

Adhesion assay

96-well plates coated with Fn (50 μg/ml) 50 μl/well were used for adhesion assays. BSA-coated wells (10 mg/ml) and poly-L-lysine-coat-
ed wells (100 μg/ml) served as negative and positive controls, respectively [35]. MM cells were seeded at a density of 2×10^6 cells/well in 100 μl RPMI-1640 medium and incubated for 2 h at 37°C. Non-adherent cells were washed with phosphate-buffered saline (PBS) three times and the adhesion percentage was determined by MTT assay. MM cells were pretreated with CGI1746 for 48 h. The concentration of CGI1746 was 1 μM. Human BMSCs (HS-5) were seeded in plates to mimic the bone marrow microenvironment in vivo, and MM cells labelled with PKH26 were treated with BTZ or Dox co-cultured with or without transwell inserts. These assays were performed in triplicate with Transwell chambers.

**MTT assay**

Cells were plated into 96-well plates at ~5,000 cells/well, and then treated with different doses of the indicated drugs for 48 h. Cell viability was evaluated by the MTT assay according to the manufacturer’s instructions. Cell viability and the value of IC_{50} were calculated by SPSS 22.0 statistical software. All assays were performed in triplicate.

**Western blotting**

Total protein samples were prepared and the relative levels of BTK and CXCR4 in myeloma cells were detected by immunoblotting using a standard protocol [36, 37]. Anti-β-actin antibody was used as loading control for total proteins. Coimmunoprecipitation (Co-IP) assays were performed as described previously [32].

**Immunofluorescence**

Cells were incubated with CXCR4 primary antibody in PBS containing 1% BSA (1:200) for 2 h at 37°C, and then treated with TRITC goat anti-mouse antibody (1:250). Images were acquired by ultrahigh-resolution microscopy imaging and the grey values of fluorescence were measured by Image J 2x software. Localization of BTK and CXCR4 protein was observed in MM cell lines using laser confocal microscopy [38]. Scatter plots were used to characterize the overlap degree of red fluorescence from Alexa Fluor 555 (BTK) and far-red fluorescence from Alexa Fluor 647 (CXCR4).

**Photoacoustic imaging**

Photoacoustic (PA) imaging is a promising technique with an imaging depth reaching as far as 12 cm in vivo, which is combining the advantages of both optical and ultrasound imaging. Vascular structures are observed by three-dimensional ultrasonic imaging, and every single cell labeling with a NIR fluorescent probe located in the vessel can be captured accurately without being influenced by blood cells. PA imaging was performed as previously described [39]. Cells were labelled with indocyanine green (ICG), a NIR fluorescent probe, and injected into NOD-SCID mice through the tail vein. MM cells adherent to the vascular wall adjacent to the lymph node in the inner knee of the mice were captured every 10 min for 40 min.

**Ubiquitination assay**

To detect ubiquitin-conjugated CXCR4, cells were treated with or without 20 μM MG132 for 4 h. The cell lysate was incubated with CXCR4 antibody overnight at 4°C, and then incubated with protein A/G-agarose beads (Santa Cruz Biotechnology, Inc., Texas, USA) for another 4 h at 4°C. Immunoprecipitated proteins were analysed by western blotting with ubiquitin antibody.

**CGI1746 treatment in the 5TMM3VT myeloma mouse model**

Approximately 1×10^6 5TMM3VT cells were injected through the tail vein into 20 C57BL/KaLwRij mice from Harlan Laboratories. The mice were divided into 4 groups as follows: BTZ (1 mg/kg, i.p.) treatment group, CGI1746 (100 mg/kg, s.c., CGI1746 was dissolved in 20% cremophor, 10% ethanol and 70% saline solution) treatment group, drug combination treatment group (doses as mentioned), and control group treated with solution (20% cremophor, 10% ethanol and 70% saline solution) for dissolving CGI1746. CGI1746 was administered three times per week beginning 7 days after injection.

**Statistical analysis**

All results are expressed as mean ± standard deviation. The survival data were plotted using Kaplan-Meier curves and analysed by the log-rank test. Statistical analysis was performed with the t-test for two groups or one-way ANOVA for multiple groups (control group, BTZ treatment group, CGI1746 treatment group and drug combination treatment group). P<0.05 was considered to indicate statistically significant differences.
Results

Enhancing BTK expression promotes adhesion and increases drug resistance in MM cells

To explore the effect of BTK on MM cellular adhesion, BTK expression was upregulated in the ARP1 and OPM2 MM cell lines. BTK wild-type (A\textsuperscript{WT} and O\textsuperscript{WT}) and overexpressing (A\textsuperscript{OE} and O\textsuperscript{OE}) cell lines were used in co-culture studies to test the adhesion between MM cells and bone marrow extracellular matrix (ECM). Initially, we coated 96-well plates with fibronectin (Fn), one of the most representative ECM components in MM bone marrow microenvironment, for adhesion assays. Compared with the wild-type cell lines, the MTT assay demonstrated that BTK overexpression in MM cells could enhance MM cellular adhesion to Fn, and the BTK-specific inhibitor CGI1746 could delay adhesion in both ARP1 (Figure 1A) and OPM2 cells (Figure 1B). We measured the IC\textsubscript{50} of BTZ and doxorubicin (Dox) in ARP1 (Figure 1C) and OPM2 cells (Figure 1D). The results demonstrated that MM cells adherent to the ECM were significantly more resistant to BTZ and Dox, and BTK-overexpressing adhesive cells exhibited even more prominent drug-resistant properties than WT adhesive MM cells. In addition, cellular adhesion could be tracked under a microscope by labelling the MM cells with PKH26, a red fluorescent dye for cell membrane labelling (Figure 2). To further verify the role of BTK in MM cellular adhesion, we coated the plates with human BMSCs (HS-5) to mimic the bone marrow microenvironment in vivo, and labelled MM cells with PKH26 (Figure 3). The numbers of adhesive cells indicated that increased BTK expression in MM cells promoted the adhesion between MM cells and ECM (Figure 2A) or HS-5 cells (Figure 3A). To increase the credibility of the results of CAM-DR, we uniformed the numbers of adhesive WT and BTK OE cells to similar level. The adhesion assay also revealed that BTK\textsuperscript{OE} adhesive cells exhibited even stronger drug-resistant characteristics compared with WT adhesive MM cells (Figures 2 and 3). This is consistent with previous results. Conversely, preventing the adhesion between MM cells and ECM or HS-5 cells using transwell inserts, the drug sensitivity was
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Figure 2. MM cellular adhesion counting assay to Fn. A. Representative images. B, C. Adhesive cell counting assay of Fn with AWT, AOE, OWT and OOE cells, respectively. MM cells were labelled with PKH26 and cultured in 24-well plates coated with Fn. The cells were co-cultured with or without Transwell inserts and incubated for 2 h at 37 °C prior to being treated with BTZ (100 nM) or Dox (5 μM) for 48 h. Non-adherent cells were washed with PBS three times and adherent cells were counted. These assays were performed in triplicate with Transwell chambers. Data are presented by GraphPad Prism 5. *P<0.05; two-tailed Student’s t-test.

Figure 3. MM cellular adhesive counting assay to HS-5 cells. A. Representative figures. B, C. Adhesive cell counting assay of HS-5 with AWT, AOE, OWT and OOE cells respectively. MM cells were labelled with PKH26 and cultured in 24-well plates coated with Fn. The cells were co-cultured with or without Transwell inserts and incubated for 2 h at 37 °C prior to being treated with BTZ (100 nM) or Dox (5 μM) for 48 h. Non-adherent cells were washed with PBS three times and adherent cells were counted. These assays were performed in triplicate with Transwell chambers. Data are presented by GraphPad Prism 5. *P<0.05; two-tailed Student’s t-test.

restored in both WT and BTK OE MM cells (Figures 2B, 2C, 3B and 3C). In summary, it may be concluded that BTK induces MM cellular adhesion to ECM or bone marrow microenvironmental stromal cells, which leads to CAM-DR in vitro.

PA imaging of cellular adhesion in vivo

PA imaging technique was used to detect BTK-induced MM cell adhesion in vivo (Figure 4). PA imaging is a promising technique combining the advantages of both optical and ultrasound imaging, with an imaging depth that can reach as far as 12 cm in vivo [39]. We labelled ARP1 and ARP1 BTK-overexpressing cells with a NIR fluorescent probe and injected the labelled cells into NOD-SCID mice through the tail vein. Three-dimensional PA imaging revealed that MM cells adhered to the vascular wall adjacent to the lymph node in the inner knee of the mice, with BTKOE cells remaining adherent for a longer period to the vascular wall compared with BTKWT cells, particularly after MM cells were injected for 30 min (Figure 4, bottom). It may be concluded that BTK enhances MM cellular adhesion in vivo.

Mechanism of BTK mediating MM cellular adhesion and drug resistance

To elucidate the mechanism through which BTK mediates MM cellular adhesion and drug resistance, we examined CXCR4, CD49d and CD44,
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the three essential mediators of MM cell adhesion [40, 41]. Western blots corroborated that the expression of CXCR4 was positively associated with BTK expression in MM cell lines (Figure 5A and 5B). We also compared the expression of CXCR4 in BTKOE and BTKWT cell lines by ultrahigh-resolution microscopy imaging (Figure 5C). Interestingly, the grey value of fluorescence demonstrated that the expression of CXCR4 in BTKOE cells was higher compared with that in BTKWT cells (Figure 5D). Therefore, it may be inferred that BTK increases CXCR4 expression in MM cells.

It has been reported that CXCR4 expression is closely correlated with BTK expression in lymphocytic leukaemia cells, and that CXCR4 expression can be reduced by a BTK inhibitor [12]. We further examined whether BTK interacted with CXCR4 directly, affecting CXCR4 expression in MM cells. Intriguingly, localization of the BTK and CXCR4 proteins was observed in MM cell lines using laser confocal microscopy, and CXCR4 exhibited a strong co-localization with BTK, which indicates that BTK may directly bind with CXCR4 (Figure 6A). Co-immunoprecipitation assay was further conducted to confirm the interaction with anti-CXCR4 antibody (Up) or anti-BTK antibody (Down), followed by western blotting with the antibodies indicated (Figure 6B). The results also demonstrated that BTK may interact directly with CXCR4. Then we further investigated how BTK upregulates CXCR4 expression in MM cells. Previous research has shown that inhibiting BTK by ibrutinib reduced the surface membrane levels of CXCR4 by means of inhibiting CXCR4 cycling from and to the membrane [12], and CXCR4 phosphorylation was found to be associated with its degradation by ubiquitination [42, 43], which indicates that BTK, as a kinase, may be involved in the degradation of CXCR4 leading to its membranization in MM cells. Since ligand-dependent CXCR4 ubiquitination is necessary to accelerate CXCR4 degradation [44, 45], we tested ubiquitination of CXCR4 in BTKOE MM cells compared with BTKWT cells. Notably, BTKOE MM cells were less ubiquitylated compared wi-

Figure 4. PA imaging of cellular adhesion in vivo. Top, PA imaging of cellular adhesion in vivo for 40 min after injection of labelled ARP1 cells through the tail vein. Cells were labelled with indocyanine green and adherent cells on the vascular wall were captured every 10 min. Bottom, typical adhesion at 30 min (Left) and PA signals recorded in line chart (Right).
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Figure 5. CXCR4 expression was associated with BTK expression. (A) Western blot analysis of CXCR4 expression in BTKWT and BTKOE cells. (B) The quantitative analyse for WB. (C, D) Expression of CXCR4 in BTKWT and BTKOE cell lines measured by ultrahigh-resolution microscopy. Representative images (C) and quantitative analysis (D).

Figure 6. BTK binds directly with CXCR4 and maintains its expression by preventing its ubiquitination-induced degradation. A. Localization of BTK and CXCR4 protein observed in ARP1 cell lines using laser confocal microscopy. Scatter plots were used to characterize the overlap degree of red fluorescence from Alexa Fluor 555 (BTK) and far-red fluorescence from Alexa Fluor 647 (CXCR4). B. Co-immunoprecipitation assay performed to confirm the interaction with anti-CXCR4 antibody (Up) or anti-BTK antibody (Down), followed by western blotting with the antibodies indicated. C. Ubiquitination detection of CXCR4 in BTKWT and BTKOE cells.

{th WT cells with lower BTK expression (Figure 6C), indicating that elevated BTK expression can stabilize CXCR4 expression in MM cells by preventing CXCR4 ubiquitination. Due to the fact that BTK was reported to phosphorylate CXCR4 at Ser339, the phosphorylation of which

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may prevent the binding of E3 ligase AIP4 with CXCR4, as is also reported for Ser324 and Ser325 [42], we hypothesized that BTK stabilized and increased CXCR4 expression by blocking its ubiquitin-mediated degradation through phosphorylating CXCR4 at Ser339. Furthermore, a CXCR4 inhibitor, AMD3100, could overcome BTK-induced MM cellular adhesion and CAM-DR (Figure 7A and 7B).

**Effects of CGI1746 and BTZ in a 5TMM3VT mouse model**

We investigated the therapeutic effects of the BTK inhibitors, CGI1746 and BTZ, in a 5TMM3VT mouse model. Kaplan-Meier survival curve analysis (Figure 7C) demonstrated that mice receiving CGI1746 or BTZ alone survived significantly longer (median survival, 61 and 68 days, respectively) compared with control mice without treatment (median survival, 46 days). The survival of mice in the CGI1746 and BTZ combination treatment group was even higher compared with single-agent treatment, with two mice surviving >100 days without MM, indicating that CGI1746 and BTZ combination exerted synergistic or additive effects on MM.

**Discussion**

BTK is a tyrosine-protein kinase that plays a key role in B-cell maturation and proliferation in various B-cell malignancies [46, 47]. A number of BTK inhibitors, such as ibritinib [47, 48] and PCI-32765 [49], have been used for oncotherapy in preclinical and clinical studies. BTK is also reported to be associated with the bone marrow microenvironment. Expression of BTK can promote osteoclastogenesis, and it may be phosphorylated by RANKL and M-CSF [49]. BTK knockdown can reduce migration of myeloma cells toward SDF-1 [31]. It is becoming increasingly clear that BTK plays a key role in drug resistance, which may be associated with the activity of the NF-κB p65 signalling pathway [50]. However, its role in CAM-DR remains largely unknown.

CAM-DR is a form of environment mediated drug resistance, which protects tumor cells escaping from the damage from chemotherapy, radiotherapy or other receptor-targeting drugs. The findings of the present study demonstrated that BTK plays an important role in MM cell drug resistance through enhancing the adhe-
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Figure 8. Schematic representation of the association between BTK and CXCR4 in MM cells. BTK plays an important role in MM cell drug resistance through enhancing the adhesion between MM cells and the bone marrow microenvironment matrix by regulating the expression of CXCR4 in MM cells. BTK may bind directly with CXCR4 and prevent its ubiquitination-induced degradation to maintain CXCR4 expression in MM cells. Ubiquitination of CXCR4 may be blocked by its phosphorylation.

In addition, our findings indicated that BTK may bind directly with CXCR4 and prevent its ubiquitination-induced degradation, thereby maintaining CXCR4 expression in MM cells. CXCR4 has been shown to interact with E3 ligase AIP4 [42] and the de-ubiquitination enzyme USP14 [51]. It is hypothesized that BTK stabilizes and promotes CXCR4 expression by blocking CXCR4 ubiquitin-mediated degradation through phosphorylating CXCR4, activating USP14 and/or inactivating AIP4. However, the association between ubiquitination and phosphorylation of CXCR4 by BTK requires further investigation. Furthermore, the interaction of BTK, CXCR4, USP14 and AIP4 is another interesting research topic, which may uncover the detailed mechanism through which BTK regulates CXCR4 expression in MM cells [51, 52].

BTK activity was increased in BTZ-resistant MM cells, and inhibition of BTK may re-sensitize previously BTZ-resistant MM cells to further BTZ therapy [50]. Stemness of MM cell lines can be regulated by bone marrow mesenchymal stem cells via the BTK signalling pathway [53]. Our findings revealed that a BTK inhibitor exerted a synergistic therapeutic effect with BTZ in a 5TMM3VT MM mouse model, indicating that the combination of a BTK inhibitor and BTZ may be a promising approach to MM treatment. This study highlights that MM progression and drug resistance are multistep transformation processes regulated by a complex cross-talk between MM cells and the bone marrow microenvironment. BTK plays a vital role in the crosstalk, especially in CAM-DR.
Conclusions

This study demonstrates the critical role of BTK in the regulation of the adhesion between MM cells and the tumour microenvironment. BTK induces CAM-DR through regulation of CXCR4 degradation in multiple myeloma. Our findings also suggest targeting the BTK-CXCR4 axis as a potential strategy for reversing CAM-DR.

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Disclosure of conflict of interest

None.

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