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Bortezomib inhibited the progression of diffuse large B-cell lymphoma via targeting miR-198

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ABSTRACT

Diffuse large B-cell lymphoma (DLBCL) is the most common subtype of non-Hodgkin lymphoma, which is an aggressive malignancy with high variance of clinical features and response to the treatment. The proteasome inhibitor bortezomib (BTZ) has been demonstrated to suppress the progression of DLBCL, however, the underlying molecular mechanisms by which BTZ regulates the growth of DLBCL cells remain largely unknown. Increasing evidence has suggested that microRNAs (miRNAs) are novel targets of anti-cancer drugs to modulate the progression of cancers. Here, we showed BTZ treatment significantly inhibited the proliferation of DLBCL CRL-2630 cells. Mechanistically, exposure of BTZ up-regulated the expression of miR-198 in DLBCL cells. Depletion of miR-198 significantly reversed the inhibitory effect of BTZ on the proliferation of CRL-2630 cells. To further characterize the involvement of miR-198 in BTZ-induced growth defects of CRL-2630 cells, the downstream targets of miR-198 were predicted with the bioinformatics tools. The results showed that miR-198 bound the 3'-untranslated region (UTR) of the high mobility group AT-hook 1 (HMGA1) and suppressed the expression of HMGA1 in DLBCL cells. Consistently, BTZ treatment decreased the level of HMAG1 and inhibited the migration of DLBCL cells. Our results provided the possible mechanism by which BTZ suppressed the growth of DLBCL cells.

1. Introduction

Diffuse large B-cell lymphoma (DLBCL) is an aggressive malignancy and the most common subtype of the non-Hodgkin lymphoma [1]. Virus infection, genetic alternation and disorders of the immune system have been considered as the possible reasons that contribute to the initiation and development of DLBCL [2]. Currently, DLBCL is a curable lymphoma with the application of immunotherapy, however, there are still up to 40% patients relapse to advanced stage and fail to remission [2]. Therefore, exploring alternative drugs and characterizing the novel factors involved in the progression of DLBCL, which may benefit the outcome of DLBCL patients, are quite necessary.

Bortezomib (BTZ) is the first identified proteasome inhibitor, which has been approved for the treatment of multiple myeloma in the USA and Europe countries [3,4]. BTZ combined with chemotherapy has been the first-line choice for the treatment of multiple myeloma patients without relapse [5]. Additionally, increasing evidence has uncovered the potential application of BTZ in the activated B-cell type lymphoma, which is a subtype of DLBCL and characterized with constitutive activation of the NF-kB signaling pathway [6]. As a proteasome inhibitor, BTZ blocks the degradation of IkBα and suppresses the NF-kB activity, which consequently inhibits the cell growth and induces apoptosis [7]. BTZ is also found to inhibit the B-cell malignancy via inducing apoptosis and sensitizing cells to radiation or chemotherapy [8]. Considering the promising effect of BTZ on regulating the tumorigenesis of DLBCL, the underlying molecular mechanisms that mediate the anti-proliferative function of BTZ in DLBCL deserve further investigation.

MicroRNAs (miRNAs) are a class of small (20–24 nucleotides), single-stranded, non-coding RNAs that negatively regulate gene expression via binding to the 3'-UTR of mRNAs to induce the degradation or suppress the translation of mRNAs [9–11]. MiRNAs play important roles in regulating the proliferation, differentiation and apoptosis of cells [9–11]. Increasing evidence suggests that miRNAs are involved in the pathogenesis of tumors via acting as tumor suppressors or oncogenes [12–14]. Recent study showed that BTZ treatment induced the up-regulation of miR-198 in DLBCL cells [15], which suggested the potential involvement of miR-198 in BTZ- regulated growth of DLBCL cells.

High mobility group AT-hook 1 (HMGA1) is a non-histone protein that binds to the AT-rich DNA sequences and modulates DNA transcription [16]. Amounting evidences has demonstrated that HMGA1 plays an oncogenic function in the initiation and development of

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malignancies [17–19]. Modulating the expression of HMGA1 is critical to regulate the proliferation, migration and invasion of cancer cells. It has been reported that HMGA1 was modulated by miRNAs via directly targeting the 3'-UTR of HMGA1 [20–22]. In this study, we found that BTZ treatment suppressed the growth of CRL-2630 cells, a cell type of the DLBCL. Exposure of BTZ significantly up-regulated the expression of miR-198 in CRL-2630 cells. To further characterize the function of miR-198 in BTZ-mediated growth defects of CRL-2630 cells, the downstream targets of miR-198 were also investigated and HMGA1 was predicted as a putative target of miR-198.

2. Materials and methods

2.1. Clinical samples

Thirty DLBCL samples from the DLBCL patients and thirty control samples from normal participants were collected at the Cangzhou Central Hospital. All the samples were confirmed independently by three pathologists according to the 2008 WHO classification of morphological criteria. This study was approved by the Ethics Committee of the Cangzhou Central Hospital. Informed written consent was obtained from all participants in accordance with the Declaration of Helsinki.

2.2. Cell culture and transfection

The DLBCL cell line CRL-2630 was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS, Applied Biosystem, Carlsbad, CA, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA) in an incubator at 37 °C with 5% CO_2. For cell transfection, miR-198 mimics or control miRNA was electroporated at 210 V for 25 ms in 4-mm cuvettes using a BTX ECM 830. Cells were cultured with fresh medium for the further experiments. The expression of miR-198 was confirmed by RT-qPCR analysis.

2.3. Real-time quantitative PCR

RNA was extracted from the cells with the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The concentration of RNA was detected with the NanoDrop-2000 (Thermo Fisher Scientific, Inc.). Quantitative stem-loop reverse transcription (RT) was preformed with the Taqman miRNA RT kit (Applied Biosystem, Carlsbad, CA, USA) with 1 μg of RNA. The quantitative real-time PCR was performed with the SYBR green mix (Applied Biosystem, Carlsbad, CA, USA) using the ABI Prism 7500 system. The PCR protocol was set as 95 °C for 10 min, followed by 40 cycles at 95 °C for 10 s and 60 °C for 1 min. The expression of miR-198 was normalized to the level of U6 and quantified using the comparative Cq value with the $2^{-\Delta\Delta Cq}$ method.

2.4. Western blot

CRL-2630 cells were lysed with the NP-40 buffer (Beyotime, Shanghai, China) on ice for 15 min. and centrifuged at 12,000 rpm for 5 min at 4 °C. The supernatant was collected and the protein concentration was determined with the BCA kit (Bio-Rad, CA, USA) according to the manufacturer's protocol. 20 μg of protein was electrophoresed by 15% SDS-PAGE and transferred onto nitrocellulose membrane. The membrane was firstly blocked with 5% non-fat milk in Tris-buffered saline for 1 h at room temperature (RT) and then incubated with the primary antibody for 2 h at RT. Afterwards, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody at RT for 1 h. The protein bands were visualized with the enhanced chemiluminescence reagent (Millipore, Bedford, USA). The expression of GAPDH was detected as the loading control.

2.5. Cell proliferation assay

CRL-2630 cells were seeded in the 96-well plate and treated with the indicated concentration of BTZ. The cell proliferation was determined with the Cell Counting Kit-8 reagent (CCK-8, Beyotime, Shanghai, China) according to the manufacturer's instructions. Briefly, 10 μl of CCK-8 solution was added into the medium and cultured for an additional 3 h at 37 °C. The absorbance of each well at 450 nm was measured with the microplate reader (Bio-Rad, CA, USA). The experiment was performed in triplicate.

2.6. Luciferase reporter assay

The wild-type (WT) or mutant 3'-UTR of HMAG1 was constructed into the psiCHECK-2™ vector. CRL-2630 cells were co-transfected with the luciferase reporter vector and the corresponding miRNA with the Lipofectamine™2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After transfection for 48 h, the luciferase activity was measured with the Dual-Luciferase Assay Kit (Promega).

2.7. Cell apoptosis analysis

CRL-2630 cells were treated with the indicated concentration of BTZ and the cell apoptosis was detected with the annexin V-propidiom iodide (PI) apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ). Cells were washed twice with pre-cold PBS and stained with Annexin V and PI at RT for 15 min. in the darkness. The apoptosis rate of CRL-2630 cells was determined using the FACS Calibur flow cytometry (BD Biosciences, Franklin Lakes, NJ).

2.8. Statistical analyses

The data was presented as mean ± SD from three independent experiments and analyzed by the Student's *t* test or one-way analysis of variance (ANOVA). The statistical analysis was performed with the SPSS software (18.0, Chicago, IL, USA). $^{*}P < 0.05$ was considered statistically significant ($^{**}P < 0.01$, $^{***}P < 0.001$).

3. Results

3.1. BTZ inhibited the growth of DLBCL cells

To detect the effect of BTZ on the growth of DLBCL cells, CRL-2630 cells were treated with 0-, 2-, 4-, 8-, 16- and 32 nm BTZ for 48 h, and the viability of the cells was determined with the CCK-8 assay. The result showed that BTZ inhibited the growth of CRL-2630 cells in a dose-dependent manner (Fig. 1A). To further characterize the influence of BTZ on the proliferation of DLBCL cells, cells were treated with 32 nm BTZ for 0-, 12-, 24-, 36- and 48 h. As shown in Fig. 1B, BTZ significantly decreased the viability of CRL-2630 cells in a time-dependent manner. To further evaluate the anti-proliferative effect of BTZ on the growth of CRL-2630 cells, colony formation of CRL-2630 cells with or without BTZ was detected. The data indicated that exposure of BTZ suppressed the colony formation of CRL-2630 cells (Fig. 1C). As cell growth is tightly associated with cell apoptosis, the apoptosis rate of CRL-2630 cells that were subjected to 32 nm BTZ for 48 h was analyzed. Compared with the control cells, significantly increased apoptosis of CRL-2630 cells was observed with the exposure of BTZ (Fig. 1D). These results suggested that BTZ suppressed the growth of CRL-2630 cells.

3.2. BTZ up-regulated the expression of miR-198 in CRL-2630 cells

To characterize whether miR-198 was involved in the inhibitory effect of BTZ on the growth of DLBCL cells, the expression of miR-198 in CRL-2630 cells that were treated with increasing dose of BTZ was detected by the RT-qPCR analysis. The data showed that BTZ treatment