Abstract. The aim of the present study was to investigate the effect of bortezomib on heat shock protein 27 (HSP27) in multiple myeloma (MM) and provide a potential new target for clinical treatment. Peripheral blood was collected from 50 normal subjects and 50 patients with newly diagnosed MM and the expression of HSP27 was detected by ELISA. The changes of HSP27 after conventional vincristine, doxorubicin and dexamethasone (VAD) chemotherapy, and bortezomib plus VAD were compared. The effect of bortezomib on U266 cell proliferation and apoptosis was detected using a cell counting Kit-8 assay and Annexin V-FITC/propidium iodide double staining with flow cytometry. The content of HSP27 following bortezomib treatment was determined by ELISA. Western blot analysis and reverse transcription-quantitative PCR were used to detect the mRNA and protein expression of HSP27, Bax and Bcl-2. HSP27 expression was increased in patients with MM compared with healthy control subjects, and the expression was increased as the cancer progressed (P<0.05). Compared with the VAD chemotherapy group, the bortezomib plus VAD chemotherapy regimen significantly inhibited the expression of HSP27 (P<0.05), and the content of HSP27 was decreased in patients in which treatment was effective compared to those patients that exhibited disease progression (P<0.05). The efficacy of the treatment regimes was not associated with age or gender. Compared with the control group, bortezomib or OGX-427 (HSP27 inhibitor) treatment inhibited U266 cell proliferation, promoted U266 cell apoptosis (P<0.05) and significantly decreased HSP27 expression (P<0.05). Furthermore, the expression of HSP27 and Bcl-2 was significantly decreased, while the expression of Bax was increased by bortezomib and OGX-427 (P<0.05). There was no significant difference between the bortezomib and OGX-427 group in the in vitro analysis. HSP27 was positively correlated with Bcl-2 expression and negatively correlated with Bax expression in U266 cells. In conclusion, bortezomib promotes the apoptosis of MM cells, potentially by downregulating the expression of HSP27, providing a potential novel target for the clinical treatment of multiple myeloma.

Introduction

Multiple myeloma (MM) is a type of cancer that results from the malignant proliferation of plasma cells in the bone marrow (1,2). MM is the second most common B-cell neoplasm of the blood system. The incidence rate in the Chinese Han population is 1/10 million, and the incidence rate in developed countries is 4/10 million (3). The number of new cases in the United States in 2015 was ~28,000, with the number of new cases increasing year by year (4). Myeloma accounts for 13.4% of all hematologic malignancies, 19% of all mortality due to hematological malignancies and 2% of all tumor-associated mortalities (5). In the United States, there were ~10,000 mortalities caused by myeloma in 2010 (6,7). The overall prognosis of patients with myeloma is poor. The median survival of patients receiving conventional therapy is 3-4 years (8). Certain new drugs have also been combined with conventional therapies in the clinic, which may further improve the prognosis and survival rate. However, the current results suggest that almost all patients will eventually relapse and it remains incurable, with a median overall survival of 4 years (9,10). Thus, there is urgent need to develop novel strategies for the treatment of MM and to improve the current treatments.

Immunotherapy can be used to control or eliminate minimal residual disease, which can consolidate the efficacy of chemotherapy or stem cell transplantation in patients (10). Myeloma cells secrete monoclonal immunoglobulins with unique antigenic determinants, which may become a tumor-specific antigen (10). A previous study reported the extraction and immunization of patients with idiotype immunoglobulin in the patient's plasma for immunotherapy, but the results are not satisfactory (11). This is partly due to the fact that this idiotypic immunoglobulin is less immunogenic and the antigen in each patient is unique. Other patients cannot be vaccinated or mediated by the idiotype immunoglobulin vaccine. The cytotoxic T cells benefit from the antmyeloma effect (10). Therefore, there is an urgent need for a new tumor-associated antigen to target in myeloma immunotherapy. This target should be
an antigen common to most myeloma cells and can induce a strong immune response in most patients.

Heat shock proteins (HSPs) are a class of tumor-specific antigens with these characteristics. These HSPs include HSP27, HSP70 and HSP90. HSPs are highly expressed in numerous inflammatory disorders and tumors (12,13). High expression of HSP also indicates poor patient prognosis and increased drug resistance (2). In addition, HSPs are essential for the survival of tumor cells. Downregulation or inhibition of HSP expression leads to significant apoptosis of myeloma cells. HSP90 inhibitors have been used in clinical trials (12,14). These properties of HSPs make it a suitable for investigation as new tumor-associated antigen candidate. HSPs have been reported to be highly expressed in MM, but exhibit low expression in normal tissues, and to have a crucial role in the survival of myeloma cells (2). Studies have shown that HSP90 complexes are activated in cancer cells, but are inactive in normal tissues (15-17). All these features make HSPs an excellent target for tumor therapy. Since HSP27, the small HSP that is an ATP-independent chaperone, is reported to be overexpressed in several caner types including MM (18-22), it can be investigated as a potential therapeutic target for the treatment of MM.

Bortezomib, a first-in-class proteasome inhibitor, is used in for treatment of MM (23). Currently, bortezomib is approved for the treatment of myeloma in relapsed patients post-transplant or as a second line treatment in patients unsuitable for transplantation. In the present study, HSP27 expression was analyzed and compared in samples of 50 patients with MM following treatment with bortezomib combination with vincristine, doxorubicin and dexamethasone (VAD) traditional chemotherapy, or VAD chemotherapy alone. Further, we also performed the treatment in the myeloma cell line to determine the regulation of bortezomib on HSP27 expression. In addition, we also explore the correlation of HSP27 with apoptosis related genes.

Materials and methods

Case collection and grouping. Between February 2016 and February 2017, samples were obtained from 50 healthy subjects and 50 patients with MM at Rizhao People's Hospital (Rizhao, China). The ratio of men and women (M/F) was 23/27, and the average age was 58.85±3.06 years in patients with MM. For the healthy subjects the ratio of men and women was 30/20 and the average age was 57.58±4.1 years. After medical diagnosis and graded examination, 17 patients with MM (M/F, 7/10) were in the stage I MM group, 15 patients were stage II (M/F, 8/7) and 18 patients were stage III (M/F, 8/10). All diagnoses and classifications were based on the International Myeloma Working Group (IMWG) diagnostic criteria for MM (2014) (24) and the Revised International Staging System (ISS) international prognostic staging criteria (25). The nature and purpose of the study was explained to each subject and informed consent was signed. The study was approved by the Ethics Committee of Rizhao People's Hospital. Inclusion criteria for patients set for the study were as follows: i) Compliance with relevant diagnostic criteria set by the IWMG, confirmed by bone marrow analysis, X-ray film, blood image analysis and laboratory examination; ii) accordance with ISS standards and Durier-Salmon staging system staging criteria (26); iii) patients were newly diagnosed; iv) patients had received >3 courses of regular chemotherapy; and v) patients exhibited an expected survival of >3 months. Exclusion criteria were as follows: i) Patients were diagnosed with severe heart, liver, kidney, lung or blood system diseases; ii) patients exhibited secondary plasma cell enlargement; iii) patient presented increased primary disease and organ failure; iv) there were missing data or patients missed follow-ups.

The 50 MM cases were randomly divided into two groups (n=25/group), control group and observation group (clinical trial no. ChiCTR1900023172). VAD chemotherapy was performed in control group (NC group) by intravenous infusion of vincristine 0.5 mg/day for 1-4 days, intravenous infusion of doxorubicin 10 mg/day for 1-4 days, and oral administration of dexamethasone 40 mg/day for 1-4 days. Bortezomib in combination with VAD chemotherapy was administered to the observation group. On the basis of VAD chemotherapy, intravenous bortezomib 1.3 mg/m² was administered on days 1, 4, 8 and 11. A period of treatment was defined as 28 days; a total of three periods of treatment were performed.

Determination of HSP27 expression in MM patients by ELISA. All samples were collected on patients with an empty stomach. Bone marrow (5 ml) was collected on the day prior to treatment and the first day following treatment, then sodium citrate was added to samples to prevent coagulation. Ficoll gradient centrifugation was performed to obtain the myeloma cells. The cells were centrifuged at 10,000 x g for 5 min at 4°C, then HSP27 was detected via ELISA according to the manufacturer's protocols (cat. no. 69-40173; MSK Bio). The optical density (OD) value was measured at 450 nm using a microplate reader (RT-6100; Rayto Life and Analytical Sciences Co., Ltd.).

Curative effect judgment. International unified curative effect standard was used for curative effect judgment. i) Complete remission (CR): Results of urine and blood immunofixation electrophoresis were negative, no plasmacloma was present, and the proportion of plasma cells in bone marrow was <5%. ii) Very good partial response (VGPR): Urine, blood electrophoresis results were negative, but immunofixation electrophoresis results were positive, serum M protein levels decreased >90% and urinary M protein levels <0.1 g/24 h. iii) Partial remission (PR): A decrease in serum M protein content >50% and a urinary M protein level <0.2 g/24 h. iv) Stable condition (SD): Those who did not meet CR, VGPR and PR criteria. v) Progression of disease (PD): The increase in serum or urinary M protein levels was >25%, or the number of bone marrow plasma cells increased significantly. For those that met the criteria for PR or above, treatment was considered to be effective and the remission rate (%) was calculated as (CR+VGPR+PR)/total number of cases x100.

Cell culture and grouping. Human multiple myeloma U266 cells were purchased from the American Type Culture Collection. Under aseptic conditions, U266 cells were cultured in RPMI-1640 medium containing 15% FBS (both Beijing Solarbio Science & Technology Co., Ltd.) in a 37°C and 5% CO2 incubator. The cells in the logarithmic growth phase were used for experiments. The cells were collected for
CCK-8 assay for cell proliferation. Logarithmic growth phase U266 cells were harvested and seeded in 96-well plates at a density of 5x10^4 cells/ml (100 µl per well). Cells treated as aforementioned were cultured at 37°C in a 5% CO₂ incubator for 0, 24, 48, 72 and 96 h. CCK-8 solution (10 µl; Engreen) was added to each well, mixed and culture was continued for 4 h. The microplate reader was shaken and zeroed using a blank control well. The absorbance of each well was measured at 450 nm. The proliferation of the cells was calculated according using formula: Cell proliferation inhibition rate (%) = ((1-od experimental group / od control group) x100).

Detection of apoptosis by Annexin V-FITC/propidium iodide (PI) double staining. Cells treated as aforementioned were cultured for 24 h and then harvested. Cells (5x10^5/ml) were then stained using the Annexin V-FITC/PI Apoptosis Detection kit (eBioscience; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The stained cells were analyzed using a flow cytometer (Beckman Coulter, Inc.) and then the results assessed using CellQuest software version 6.0 (BD Biosciences).

Determination of HSP27 in myeloma cells by ELISA. The contents of HSP27 in myeloma cells were detected in accordance with the ELISA kit instructions (cat. no. ELH-HSP27-1; RayBiotech, Inc.), and the OD value was measured at 450 nm using a microplate reader (RT-6100; Rayto Life and Analytical Sciences Co., Ltd.).

Detection of HSP27, Bax and Bcl-2 mRNA expression levels by reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted using the TRIzol kit (Invitrogen; Thermo Fisher Scientific, Inc.; OD260/OD280 indicated between 1.8 and 2.0 for RNA purity). RT of cDNA was performed using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific, Inc.); the reaction was performed at 37°C for 1 h. qPCR was performed using an SYBR Green kit (Invitrogen; Thermo Fisher Scientific, Inc.) in a Master cycler (Eppendorf). The reaction conditions were as follows: 95°C for 5 min, then 95°C for 15 sec and 60°C for 15 sec (40 cycles). Data were processed using the 2^ΔΔcq method (27) and relative expression levels were calculated using β-actin mRNA as an internal reference. The primer sequences were as follows: HSP27, forward 5'-GACGTCCAGGACACA GTCCAGGCAG-3', reverse 5'-GGTGGTTGCTTTGAACCTT ATTGAG-3'; Bax, forward 5'-GACACCTGAGCTGAC CTGG-3', reverse 5'-GAGGAAGTCCTAGTGGCCACC-3'; Bcl-2, forward 5'-ATCGCTCTGTTGGATGCTGATAC-3', reverse 5'-AGAGACAGCCAGGAGAAATCAAAC-3', and β-actin, forward 5'-GTCCACCTTCCAGCAGATGTG-3' and reverse 5'-CTATTGGCATCGGAGC-3'.

Western blot analysis of HSP27, Bax and Bcl-2 protein expression. Cells were lysed and centrifuged at 14,000 x g for 20 min, the the supernatant was removed and cell pellets were collected. The protein concentration was measured by using a BCA kit (Beijing Solarbio Science & Technology Co., Ltd.). Protein samples (4 µl) were added to 5X SDS loading buffer, and then separated by SDS-PAGE on 10% gels. Electrophoresis was performed at 80 V, and semi-dry electro-transfer to PVDF membranes (Merck KGaA) was performed at 20 V for 30 min. The membrane was washed and blocked with 5% nonfat milk for 2 h at room temperature. Rabbit anti-human HPS27 (1:1,000; cat. no. ab5579; Abcam), rabbit anti-human Bcl-2 (1:200; cat. no. ab32124; Abcam), rabbit anti-human Bax (1:200; cat. no. ab32503; Abcam) and rabbit anti-β-actin (1:1,000; cat. no. ABIN2854709; antibodies-online GmbH) antibodies were added and incubated overnight at 4°C. After washing, membranes were incubated with goat anti-rabbit IgG- horseradish peroxidase (1:1,000; cat. no. 7074; Cell Signaling Technology, Inc., USA) for 30 min at 37°C. The results were observed and recorded using a Roche Elecsys-2010 chemiluminometer (Roche Diagnostics). Protein expression levels were normalized to β-actin and quantified using ImageJ software version 1.46 (National Institutes of Health).

Statistical analysis. SPSS 19.0 statistical software was used to analyze the data. The data are expressed as the mean ± SD. Multiple comparisons were evaluated by one-way ANOVA with the least significant difference test used for follow-up analysis. Fisher's exact test was used for correlation analysis. Fisher's exact test was used to analyze the difference of total efficacy in age and gender groups, separately. The χ² test was used to analysis the differences in clinical efficacy between the NC group and observation group. P<0.05 was considered to indicate a statistically significant difference.

Results
Expression of HSP27 in patients with MM at different stages. The HSP27 level was determined using ELISA (Fig. 1). Compared with the normal (healthy patients) group, the
expression level of HSP27 in was significantly increased in patients with MM (P<0.05). The expression of HSP27 in patients with stage III MM was significantly higher than that in patients with stage II MM (P<0.05). The expression of HSP27 in patients with stage III and stage II MM was also significantly higher than that patients with stage I (P<0.05). The results indicated that the expression of HSP27 increased with the development of the disease.

Association between HSP27 and the curative effect after different treatments. As shown in Table I, 25 patients with MM ≤60 years old in the bortezomib-treated group had a
mean age of 45.75±9.57, with eight male patients (75.0%), and six female patients (25.0%). Patients with MM >60 years old had an average age of 69.8±3.97, with five males (26.7%) and six females (73.3%). These results demonstrated that there was no significant difference in the treatment effect in the different age and gender groups (P>0.05).

As shown in Table II, the effective rate of the NC group was 56% and the effective rate of the observation group was 84%. The expression of HSP27 in patients with MM was decreased after routine treatment (NC) and bortezomib treatment (Fig. 2). The decrease in the bortezomib treatment group was significantly greater than that in routine treatment (NC) group. Compared with the PD group, the expression of HSP27 in the patients with total effective treatment was significantly decreased (P<0.05). These results suggest that bortezomib treatment significantly inhibited the expression of HSP27 in patients with MM.

**Effect of bortezomib treatment on the growth and apoptosis of U266 cells.** The results of the CCK-8 assay showed that bortezomib treatment inhibited the growth of U266 cells, and that the effect was increase over time (Fig. 3C). Compared with the control group, after U266 cells were treated with bortezomib or OGX-427 treatment for 48 h, U266 cell proliferation rate was significantly reduced (P<0.05). Cell proliferation rate in OGX-427 group was not significantly different to the bortezomib treatment group (P>0.05). Apoptosis of the cells was detected using the Annexin V/PI double staining method (Fig. 3A and B). Compared with the control group, the apoptosis of the cells was significantly increased by bortezomib treatment or OGX-427 treatment (P<0.05) but there was no significant difference in apoptosis between the bortezomib and OGX-427 treatment groups. These data demonstrated that bortezomib treatment could inhibit the proliferation of U266 cells and promote apoptosis.
Figure 5. Expression of HSP27, Bcl-2 and Bax mRNA in following bortezomib treatment in U266 cells. (A) HSP27, (B) Bcl-2 and (C) Bax mRNA relative expression in control and bortezomib treatment groups were analyzed by reverse transcription-quantitative PCR. *P<0.05 vs. control group. HSP27, heat shock protein 27.

Figure 6. Correlation of HSP27 with Bcl-2 and Bax in U266 cells. (A) Correlations between HSP27 and Bax and Bcl-2 prior to bortezomib treatment. (B) Correlations between HSP27 and Bax and Bcl-2 following bortezomib treatment. (C) Correlations between HSP27 and Bax and Bcl-2 following OGX-427 inhibitor treatment. HSP27, heat shock protein 27.
HSP27 expression in myeloma cells. ELISA was performed to determine the HSP27 protein level in U266 cells. Compared with control group, the content of HSP27 in U266 cells was significantly decreased in the bortezomib group and OGX-427 group (P<0.05; Fig. 4). Compared with the bortezomib treatment group, the content of HSP27 in the OGX-427 group was unchanged (P>0.05; Fig. 4).

Expression of HSP27, Bcl-2 and Bax mRNA in U266 cells. Compared with control group, the expression of HSP27 mRNA and Bcl-2 mRNA in U266 cells was decreased significantly in the bortezomib and OGX-427 treatment groups, while the expression of Bax mRNA was increased significantly (P<0.05; Fig. 5). Compared with the Bortezomib group, the mRNA levels of three genes in the OGX-427 group were unchanged (P>0.05; Fig. 5).

Effect of bortezomib on the expression of HSP27 mRNA and Bcl-2, Bax mRNA in U266 cells. As shown in Fig. 6A, Spearman’s analysis was used to verify the correlation between HSP27 and Bax prior to bortezomib treatment. The results revealed that HSP27 was negatively correlated with Bax expression (r=-0.5397, P=0.0065), but positively correlated with Bcl-2 expression (r=0.5237, P=0.0086). Following bortezomib treatment (Fig. 6B), the mRNA expression levels of HSP27, Bax and Bcl-2 in U266 cells were monitored. Spearman’s analysis revealed a negative correlation between HSP27 and Bax, with a correlation coefficient of r=-0.562 (P=0.0043). There was a positive correlation between HSP27 mRNA and Bcl-2 mRNA, with a correlation coefficient of r=0.4305 (P=0.0336). Additionally, the correlations between HSP27, Bax and Bcl-2 expression after adding OGX-427 inhibitor were analyzed. The correlation coefficients were -0.5011 (P=0.0126) and 0.5847 (P=0.0027), respectively (Fig. 6C). These results indicated that the expression of HSP27 is associated with apoptosis-related genes, Bcl-2 and Bax.

Effect of bortezomib on the expression of HSP27, Bcl-2 and Bax protein in U266 cells. As shown in Fig. 7, compared with the control group, the expression levels of HSP27 and Bcl-2 protein were significantly decreased in the bortezomib and OGX-427 treatment groups, and the expression of Bax protein was significantly increased in the in bortezomib and OGX-427 treated cells (P<0.05). The expression level of three proteins was not significantly altered in the in OGX-427 group compared with the bortezomib group (P>0.05).

Discussion

MM is a cancer of plasma cells, which are a type of white blood cell (28). The cause of MM is still unknown and the risk factors include alcohol, obesity, radiation exposure, family history and certain chemicals. The mechanism of MM involves the production of abnormal antibodies by plasma cells, which causes kidney problems and overly thick blood (8). MM is
typically diagnosed based on bloods analysis, urine tests, bone marrow biopsy and medical imaging. MM is considered to be treatable, but not incurable, despite the rapid development of treatment strategies. Thus, novel therapeutic treatments are urgently needed for patients with MM.

HSPs are a family of molecular chaperones that have a crucial role in protein folding and cellular protein homeostasis. In addition to these function, HSPs also have important role in the cancer development and often high expressed in a series of cancers (2). In the recent years, HSPs have been explored as a therapy target in cancer treatment, including MM. HSP90 inhibitors have been used in clinical trial for the treatment of MM (11-13,29). HSP70 inhibitors were also reported to induce MM tumor cell death (30). HSP27, a small HSP that is an ATP-independent chaperone protein, is reportedly overexpressed in several cancer types, including colorectal, breast and ovarian cancer, and MM. HSP27 has an important role in inhibiting the release of the pro-apoptotic mitochondrial protein Smac in MM (19-22). Thus, HSPs can be explored as a therapeutic treatment target in cancers that have high expression of HSPs. Bortezomib is an antitumor drug used as a first therapeutic proteasome inhibitor in certain human cancer types, including MM. The current study analyzed the effect of bortezomib on HSP27 expression when used in combination with traditional chemotherapy to investigate the role of HSP27 in MM, and thus determine the mechanism of how this combination treatment may benefit patients with MM.

Bortezomib is a synthetic reversible proteasome inhibitor that can inhibit tumors in multiple manners (31). It has been reported that bortezomib promotes BAX protein expression, thereby promoting tumor cell apoptosis (32). HSP27 exerts regulatory effects on apoptosis, proliferation and migration, including in tumor cells (33). It has been reported that HSP27 is overexpressed in various malignant tumors, such as breast cancer and leukemia (34,35). Therefore, the expression levels of HSP27 may be a useful indicator for the clinical evaluation of MM. The present results demonstrated that HSP27 was highly expressed in patient serum samples, and that expression levels increased as the cancer progressed. Following treatment with bortezomib combination with traditional chemotherapy or conventional treatment, HSP27 expression was significantly decreased at the mRNA and protein level. Notably, the combination treatment significantly increased the effective rate and the HSP27 expression was decreased in the bortezomib treatment group compared with the conventional treatment group. It has been previously reported that cell apoptosis is associated with the expression levels of HSPs (36). The data of the current study indicate that bortezomib treatment significantly promoted cell apoptosis and downregulated the expression of HSP27. Further analysis demonstrated that HSP27 expression was positively correlated with the anti-apoptotic gene Bcl-2, and negatively correlated with the pro-apoptotic gene Bax. These results suggest the HSP27 has a role within the apoptotic pathways. Thus, in the future, patients may benefit from the combination treatment. HSP27 also can be explored as a potential therapeutic target.

In conclusion, bortezomib treatment downregulates HSP27 and alters the expression of apoptosis-regulating proteins Bcl-2 and Bax, and thus inhibits the proliferation and promotes the apoptosis of myeloma cells. All these results provide a potential method for the treatment of patients with MM.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

JL and JQL participated in the design of the study. JL, XMZ, JYS, JG, XLW and JQL conducted the assays and performed the statistical analysis. JL, XMZ, JYS, JG, XLW and JQL drafted the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Rizhao People's Hospital (clinical trial no. ChiCTR1900023172). All patients provided signed informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


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