Proteomics of apoptosis of multiple myeloma cells induced by proteasome inhibitor PS-341

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Abstract: **Objective** To compare the proteome difference between multiple myeloma cell line U266 cells treated and untreated with PS-341, to investigate the potential drug targets, and to provide theoretical evidence for clinical therapy of multiple myeloma. **Methods** Two-dimensional gel electrophoresis (2-DE) was performed to separate proteins from treated and untreated U266 cells with proteasome inhibitor PS-341. ImageMaster 2D Platinum software was used to analyze 2-DE image, and matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) was used to identify the differentially expressed proteins. The expression levels of differential protein BAG-2 in the 2 groups of U266 cells lines were detected by Western blot. **Results** The 2-DE reference pattern of treated and untreated U266 cells with PS-341 was established. A total of 31 differential proteins were identified by MALDI-TOF-MS, 27 of which were down-regulated after PS-341 treatment. The differential expression level of BAG-2 in the 2 groups of U266 cells was confirmed by Western blot. **Conclusion** Some down-regulated proteins may be the potential drug targets of proteasome inhibitor PS-341.

Key words: PS-341; multiple myeloma; 2-dimensional gel electrophoresis; mass spectrometry; drug target

蛋白酶体抑制剂 PS-341 诱导骨髓瘤细胞凋亡的蛋白质组学研究

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[**摘要**] **目的**:比较蛋白酶体抑制剂 PS-341 处理多发性骨髓瘤细胞 U266 前后蛋白质组的差异,探究 PS-341 潜在的药物靶点,为多发性骨髓瘤的临床治疗提供理论依据。**方法**:用蛋白酶体抑制剂 PS-341 处理骨髓瘤细胞 U266,应用双向凝胶电泳技术分离 PS-341 处理前后的 U266 细胞的蛋白质,ImageMaster 2D Platinum 图像分析软件识别药物处理前后 U266 细胞的差异表达蛋白质点,基质辅助激光解吸电离飞行时间质谱(MALDI-TOF-MS)鉴定差异表达的蛋白质。Western 印迹法检测差异蛋白质 BAG-2

Date of reception 2010-04-19

Biography JIA Haitao, master student, mainly engaged in the research of functional proteomics.

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在药物处理前后 U266 细胞中的表达水平。**结果**:建立了 PS-341 处理前后 U266 细胞蛋白质的双向凝胶电泳图谱,找到 55 个差异表达的蛋白质点,鉴定了 31 个差异表达的蛋白质,有 27 个蛋白质在 PS-341 处理后下调。Western 印迹分析证实 BAG-2 在药物处理前后 U266 细胞中的表达水平存在差异。**结论**:处理后下调的一些蛋白可能是蛋白酶体抑制剂 PS-341 潜在的药物靶标。

[**关键词**] PS-341; 多发性骨髓瘤; 双向凝胶电泳; 质谱; 药物靶标
DOI:10.3969/j.issn.1672-7347.2010.08.003

Multiple myeloma (MM) is a plasma cell malignancy characterized by the hyperplasia and infiltration of plasma cells in the bone marrow, pathological fracture, anaemia, and ostealgia, which usually is associated with high levels of monoclonal (M) immunoglobulin in the blood and/or urine, and lead to infection, hypercalcemia, renal lesions and amyloidosis[1-2].

Proteasome inhibitor PS-341 (bortezomib) is a novel targeted drug for MM, and it displays encouraging results in treating relapsed and refractory MM in clinic. PS-341 mainly blocks the activation of nuclear transcription factor kappa B (NF-κB)[3-4], and decreases the expression of apoptosis inhibitor[5], simultaneously inhibits the growth of MM and the developmen t of survival factor in bone marrow microenvironment[6-7]. But now the detailed mechanism remains uncertain.

In our study, we treated the representative human multiple myeloma U266 cells with proteasome inhibitor PS-341, and used the proteomic technique to compare the differentially expressed proteins treated and untreated with PS-341, analyzed and identified the differential proteins to search for the potential drug target, and the results may provide some theoretical evidence for treating multiple myeloma.

1 MATERIALS AND METHODS

1.1 Materials

1.1.1 Cell line

The human multiple myeloma cell line U266 was purchased from American type culture collection (ATCC). U266 cells were cultured in RPMI1640 medium supplemented with 100 μg/mL penicillin/streptomycin, 1 mmol/L *L*-glutamine, and 10% fetal bovine serum (FBS) at 37℃, 5% CO_2.

1.1.2 Reagents

Proteasome inhibitor PS-341 (bortezomib) was kindly donated by Dr. Zeng Huilan (The First Affiliated Hospital, Jinan University, Guangzhou). RPMI1640 medium and FBS were purchased from Gibico BRL company; urea, thiourea, 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonate (CHAPS), 1, 4-dithiothreitol (DTT), iodacetamide, PMSF, ammonium persulfate, N, N, N′, N′-tetramethylethylenediamine (TEMED), ammonium bicarbonate, potassium ferrocyanide, acetonitrile (ACN), α-cyano-4-hydroxycinnamic acid (CCA) and trifluoroacetic acid (TFA) were purchased from Sigma Corporation (American); immobilized pH-gradient dry strips (pH 3-10, 13 cm), IPG buffer were purchased from Amersham Biosciences Corporation (Sweden); acrylamide, N, N-methylene-bisacylamide, Sodium dodecyl sulfate (SDS), Tris-base were purchased from Guangzhou zhanchen biological technology Co., LTD; mouse anti-human GAPDH antibody was purchased from Santa Cruz Biotechnology (USA); rabbit anti-human Bcl-2-associated athanogene-2 (BAG-2) was purchased from Abcam (UK); Annexin V-FITC apoptosis detection kit was the product of Becton, Dickinson and Company (BD); PVDF membranes were purchased from Millipore Corporation; ethanol, acetic acid, glycine, sodium acetate, sodium carbonate, sodium thiosulphate, silver nitrate, formaldehyde, and ethylene diamine tetraacetic acid (EDTA) were all analytical grade, made in Guangzhou Chemical Reagent Factory (China).

1.1.3 Main instruments

DNA/protein analyzer, IPGphor isoelectric focusing instrument, Ettan DALT vertical electrophoresis tank, ImageScanner scanner were purchased from

General Electric Company (USA); high speed freezing centrifuge was the product of Eppendorf Co. (Germany); millipore water purification system was the product of Millipore Co. (USA); 4800 plus MALDI-TOF/TOF mass spectrometer was the product of Applied Biosystems Co. (USA); flow cytometer was the product of Becton Dickinson Co. (USA).

1.1.4 Bioinformatics softwares

LabScan scanning software was the product of Applied Biosystem Co. (USA); ImageMaster2D Platinum image analysis software was the product of Amersham Biosciences Co. (Sweden); DataExplorer mass spectrum analysis software was the product of Applied Biosystem Co. (USA); Mascot peptide mass fingerprinting (PMF) database query software and Mascot MS/MS database query software were products of Matrixscience Co. (UK).

1.2 Methods

1.2.1 MM cells treated with PS-341

U266 cells were cultured in RPMI1640 medium supplemented with 10% FBS, 100 μg/mL penicillin, 100 μg/mL streptomycin and 1 mmol/L *L*-glutamine at 37℃, 5% CO_2. Cells were cultured at the logarithmic phage, and treated with PS-341 according to literature's report[8], and cells were collected after 24 h for future use.

1.2.2 Apoptosis assay

Cells were harvested after treated with PS-341 for 24 h, washed twice in PBS. Then Cells were resuspended in Annexin V-FITC binding buffer, stained with 2 μL Annexin V-FITC and 2 μL PI according to manufacturer's instructions, and incubated for 15 min in dark at room temperature. Samples were acquired on a flow cytometer and analyzed with WinMDI2.9 software program. This assay was finished at the Analysis & Testing Center of Jinan University.

1.2.3 Two-dimensional gel electrophoresis (2-DE) separation of total cell proteins

The procedures were carried out in accordance with user's manual of IPGphor isoelectric focusing system. Total cell proteins of treated and untread cells (130 μg) were mixed with the hydration liquid separately, the bulk volume reached to 250 μL. Isoelectric focusing was performed as follows: passive rehydration at 30 V for 12 h, 500 V for 1 h, 1 000 V for 1 h, and 8 000 V for 8 h. Prior to SDS-PAGE, focused IPG strips were equilibrated, reduced and alkylated in buffer (50 mmol/L Tris-HCl, 6 mmol/L urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, pH 8.8) containing 1% DTT for 15 min, and then alkylated in buffer (50 mmol/L Tris-HCl, 6 mmol/L urea, 30% glycerol, 2% SDS, 0.002% bromophenol blue, pH 8.8) containing 2.5% iodoacetamide for 15 min. Strips were then loaded onto a 12% acrylamide gel and run in a Ettan DALT vertical electrophoresis tank at constant 30 mA/strip. Gels were stained with silver nitrate staining, and the test repeated for 3 times.

1.2.4 Image analysis

The stained 2-DE gels were scanned on the ImageScanner, and images were analyzed using Image Master 2D Platinum. Only protein spots that were reproducibly different from 2-fold or more were excised from gels for analysis by MS.

1.2.5 MALDI-TOF-MS analysis

The differential protein spots were cut from the 2-DE gels for tryptic in-gel digestion. Then the peptide extracts were lyophilized, dissolved in 2 μL sample solution [30% acetonitrile (ACN), 0.1% trifluoroacetic acid (TFA), and the peptide extracts were mixed with the matrix solution (5 mg/mL α-Cyano-4-hydroxycinnamic acid (CCA) in 50% ACN/0.1% TFA] in a ratio of 1∶1 onto the sample plate. The peptide masses were analyzed by the ABI 4800 plus MALDI-TOF-MS.

1.2.6 Western blot

The U266 cells of the treated and untreated with PS-341 groups were collected, then added with RIPA lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 1 mmol/L PMSF, 100 mmol/L leupeptin, and 2 mg/mL aprotinin) for 30 min on the ice. The supernatant was collected after centrifugation at 13 200 r/min for 30 min at 4 ℃. Protein concentrations were determined using Bradford

assay. Total protein (50 μg) was separated by a 10% SDS-PAGE gel, and transferred onto a PVDF transfer membrane by electroblotting. After blocking with 5% nonfat milk for 2 h at room temperature, the membrane was incubated overnight at 4℃ with 1000-fold diluted primary antibody (rabbit anti-BAG_2), and washed with PBST buffer for 3 times (each 10 min); then the membrane was incubated for 2 h with 2000-fold diluted anti-rabbit secondary antibody labeled with HRP at room temperature, and washed with PBST buffer for 3 times (each 10 min); then developed by using the SuperSignal West Pico kit.

1.3 Statistical analysis

Data were analyzed with SPSS10.0 statistical analysis software and expressed as the mean ± standard deviation ($\bar{x} \pm s$). Unpaired t test was used and $P < 0.05$ was considered statistically significant.

2 RESULTS

2.1 Detection of apoptosis

U266 cells were treated with 0, 5, 10, 15 or 20 μmol/L PS-341 for 24 h, and the apoptosis induced by PS-341 was a dose-dependent manner (Tab. 1). The null hypothesis was rejected with one sided $P < 0.05$ at 5 μmol/L PS-341. So U266 cells were treated with 5 μmol/L PS-341 for 24 h for the proteomics research.

Tab. 1 Effect of PS-341 on apoptosis of U266 cells

PS-341/(μmol/L)	Apoptosis rates/%
0	0.020 ± 0.001 *
5	0.118 ± 0.027 *
10	0.148 ± 0.025 *
15	0.173 ± 0.043 *
20	0.218 ± 0.001 *

Compared with control 0 μmol/L PS-341, * $P < 0.05$.

2.2 Establishment of 2-DE patterns of U266 cell proteins of treated and untreated with PS-341

The 2-DE was performed to separate proteins from treated with 5 μmol/L PS-341 and untreated U266 cells for 3 times under the same conditions. Protein spots on the gel were detected by silver nitrate staining to obtain three 2-DE patterns of cells treated and untreated with PS-341, and images were analyzed using the ImageMaster 2D Platinum software. The detailed parameters were smooth = 5, saliency = 5, min area = 5, and the sharp spots in the same location of gels were chosen as the landmark, which was used to match the images. Fig. 1 represented the 2-DE maps of total proteins of treated and untreated groups. Fig. 2 was the enlargement patterns of partial differential expression protein spots.

2.3 Identification and categorization of difference protein spots

Difference protein spots were cut from the 2-DE gels, and identified by MALDI-TOF/TOF-MS after the tryptic in-gel digestion. Protein identification was carried out by PMF using the Mascot software. A total of 31 differential expressed proteins were identified, 27 of which were down-regulated and 4 were up-regulated after PS-341 treatment. Their location and specific information on the 2-DE map were shown in Fig. 1 and Tab. 2. Fig. 3 represented the PMF of No. 29 protein spots. The result of searching for the PMF of No. 29 in the SwissProt database was Bcl-2-associated athanogene-2 (BAG-2).

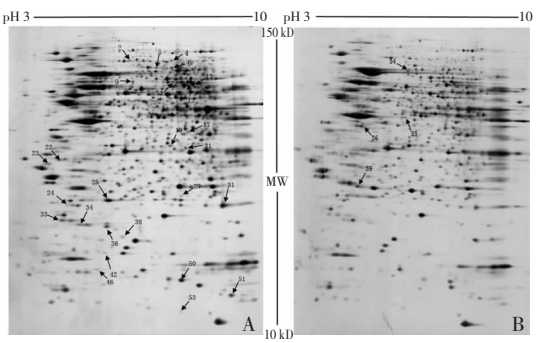

Fig. 1 The 2-DE maps of untreated (A) and treated (B) U266 cells with PS-341 (The 31 differential protein spots identified by MS are labeled with figures).

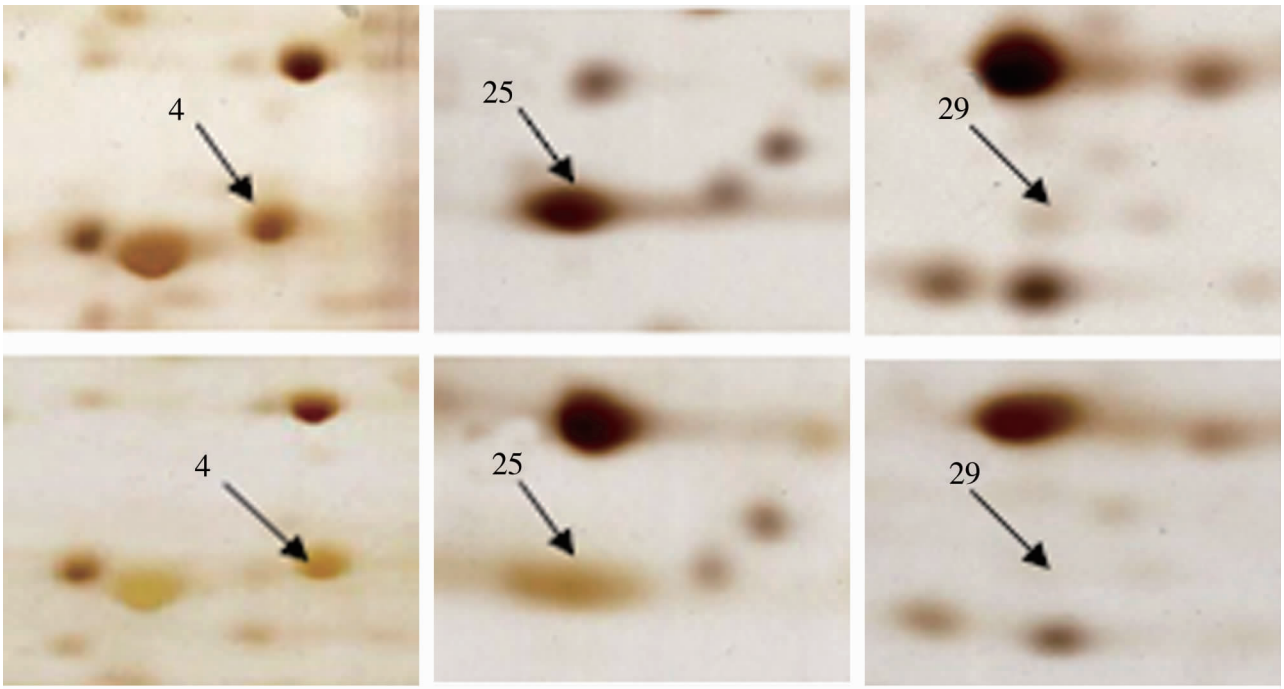

Fig. 2 Enlargement patterns of partial differential expression protein spots. Upper: Untreated with PS-341; bottom: Treated with PS-341.

To understand the biological relevance of the changes in protein expression in response to PS-341 treatment, PANTHER (protein analysis through evolutionary relationships) classification system was used to classify the 31 identified proteins according to their functions (http://www.pantherdb.org). These proteins can be classified into 11 groups according to their functional properties: (1) amino acid metabolism; (2) nucleoside, nucleotide and nucleic acid metabolism; (3) protein metabolism and modification; (4) apoptosis; (5) cell cycle; (6) cell structure and motility; (7) immunity and defense; (8) muscle contraction; (9) homeostasis; (10) blood circulation and gas exchange; (11) other metabolism. The exact IPI accession No., protein name, protein molecular weight (MW), protein isoelectric point (PI), and molecular function were all shown in Tab. 2.

2.4 Validation of the differentially expressed protein BAG-2 by Western blot

The expression of BAG-2 in the treated group was significantly lower than that in the untreated group ($P < 0.05$, Fig. 4). This result was consistent with the result of proteomics research.

Tab. 2 Results and classification of differential proteins identified by MALDI-TOF-MS

No.	Accession No.	Protein name	Protein MW/PI	Protein score/CI%	F. D (Treated/untreated)
Amino acid metabolism					
12	IPI00219352	Isoform 1 of Cystathionine beta-synthase	60 548.2/6.2	308/100	-1 000 000
13	IPI00554777	Asparagine synthetase	64 328.6/6.39	170/100	-2.23966
55	IPI00009268	Aminoacylase-1	45 856/5.77	71/99.5	2.76433
Nucleoside, nucleotide and nucleic acid metabolism					
5	IPI00290142	CTPS CTP synthase 1	66 648/6.02	129/100	-2.56494
11	IPI00642319	KEAP1 63 kD protein	63 003.5/5.96	66/98.0	-2.53832

Tab. 2 (Continued.)

NO.	Accession No.	Protein name	Protein MW/PI	Protein score/CI%	F. D (Treated/untreated)
17	IPI00003704	Isoform 1 of RNA-binding protein 4	40 288.6/6.61	338/100	-1 000 000
22	IPI00296913	NUDT5 ADP-sugar pyrophosphatase	24 312.2/4.87	272/100	-1 000 000
34	IPI00297579	LOC653972 Chromobox protein homolog 3	20 798.3/5.23	144/100	-2.11458
38	IPI00375015	Isoform DUT-N of Deoxyuridine 5′-triphosphate nucleotidohydrolase	17 736.9/6.15	349/100	-1 000 000
53	IPI00032460	U6 snRNA-associated Sm-like protein LSm2	10 827.6/6.04	145/100	-1 000 000
Protein metabolism and modification					
9	IPI00218682	Isoform 2 of Prolyl 4-hydroxylase subunit alpha-1 precursor	60 928.9/5.7	208/100	-1 000 000
50	IPI00847579	Ribosomal protein S12	14 505.5/6.81	68/98.8	-1 000 000
Apoptosis					
29	IPI00000643	BAG family molecular chaperone regulator 2	23 757.2/6.25	190/100	-1 000 000
46	IPI00023640	Programmed cell death protein 5	14 276.3/5.77	192/100	-1 000 000
Cell cycle					
4	IPI00299904	Isoform 1 of DNA replication licensing factor MCM7	81 256.6/6.08	334/100	-2.63764
31	IPI00792352	RAN 26 kD protein	26 392.6/8.51	401/100	-2.4161
Cell structure and motility					
6	IPI00872814	MSN 68 kD protein	67 644.8/6.09	458/100	-2.94975
21	IPI00010414	PDZ and LIM domain protein 1	36 049/6.56	250/100	-1 000 000
23	IPI00218320	Isoform 3 of Tropomyosin alpha-3 Chain	28 937.8/4.79	195/100	-2.41779
56	IPI00334190	Stomatin-like protein 2	38 510.2/6.88	166/100	2.21797
Immunity and defense					
2	IPI00871391	Putative uncharacterized protein XRCC5	81 323.6/5.45	201/100	-2.86148
7	IPI00030275	Heat shock protein 75 kD, mitochondrial precursor	80 059.7/8.3	69/99.1	-2.83938
25	IPI00219757	Glutathione S-transferase P	23 341/5.43	509/100	-2.90552
36	IPI00375400	Uncharacterized protein PRDX2	16 092.4/6.13	483/100	-1 000 000
54	IPI00339269	Heat shock 70 kD protein 6	70 984.2/5.81	462/100	3.4327
59	IPI00025512	Heat shock protein beta-1	2 768.5/5.98	364/100	11.3086
Muscle contraction					
19	IPI00000861	Isoform 1 of LIM and SH3 domain protein 1	29 698.2/6.61	213/100	-1 000 000
24	IPI00798256	MYL4 14 kD protein	14 164.1/4.94	87/99.9	-1 000 000
Homeostasis					
42	IPI00738499	Ferritin light chain	20 007.1/5.51	86/99.9	-1 000 000
Blood circulation and gas exchange					
51	IPI00554676	Hemoglobin subunit gamma-2	16 116.3/6.64	120/100	-2.11753
Other metabolism					
33	IPI00220766	GLO1 Lactoylglutathione lyase	20 764.2/5.12	70/99.3	-2.09512

Note: F. D. means fold differences, minus in F. D. means down-regulation, 1 000 000 means the differential protein spots can be found in the untreated gel, but not in the treated gel.

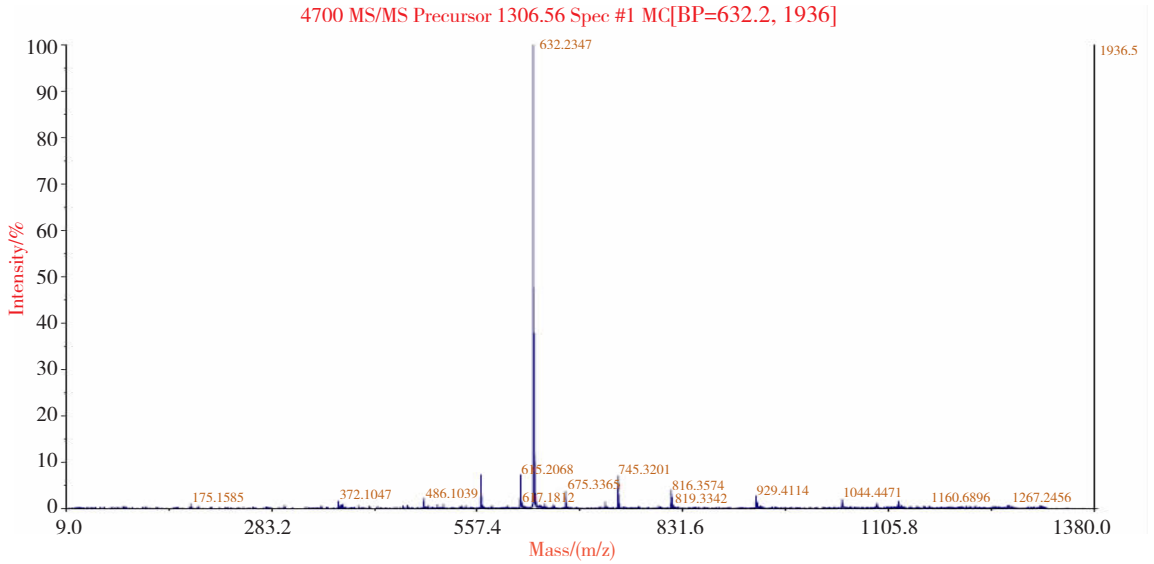

Fig. 3 Peptide mass fingerprinting of protein spot 29 by MALDI-TOF-MS analysis.

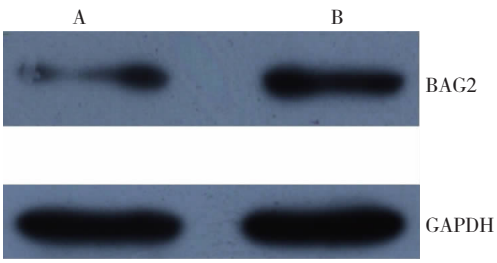

Fig. 4 Western blot showing changes in the expression level of BAG-2 in treated (A) and untreated (B) U266 cells with PS-341.

3 DISCUSSION

MM mostly happens in middle-aged and old people. In China, the annual incidence of MM is increasing with the development of population ageing. At present there is no cure for MM, so this study was to employ proteomic technique to search for the dysregulated proteins induced by clinical drug proteasome inhibitor PS-341 (bortezomib) in MM U266 cells, in order to find some potential drug targets of PS-341, and provide some theoretical basis for the MM's clinical therapy.

In this experiment, we treated U266 cells with 5 μmol/L PS-341, and established the maps of 2-DE patterns of U266 treated and untreated with PS-341, ImageMaster 2D Platinum software was used to analyze the gel maps, and 31 differential proteins were identified by MALDI-TOF-MS mass spectrometer, including 27 down-regulated proteins. These down-regulated proteins include BAG-2 associated with apoptosis, MCM7 referred to cell cycle, GSTP1 related to immunity and defense, and so on, they all may be the potential drug target of proteasome inhibitor PS-341.

BAG-2 is a member of the BAG family proteins, and the *BAG* gene is the recently discovered anti-apoptosis gene family. The BAG family is a multifunctional group of proteins that can interact with Bcl-2 and heat shock proteins (Hsc70/Hsp70), and a variety of transcription factors to regulate diverse physiological processes, including apoptosis, tumorigenesis, neural differentiation, stress response, and cell cycle. Currently the main reports is that BAG-2 acts as an inhibitor of the chaperone-associated ubiquitin ligase CHIP (carboxyl terminus of Hsp70-interacting protein), and can inhibit the activity of ubiquitin ligase E3 CHIP[9], thus to regulate the cytoplasmic quality and control protein degradation pathway[10]. In our study, the expression level of BAG-2 was down-regulated by PS-341, as a result the inhibitory regulation of CHIP from BAG-2 would be eased, which induced the degradation of misfolded proteins tagged with ubiquitin and apoptosis of tumor. In light of the anti-apoptosis effect of BAG-2, further research is under investigation.

Mcm7 is a member of minichromosome maintenance protein (Mcm) family, which plays an im-

portant role in controlling the initiation and elongation steps of eukaryotic DNA replication, the interacts with many family proteins to co-regulate cell cycle progression, and ensuring DNA replication once and only once in individual cell cycle. At the same time Mcm7 is related with cell cycle regulation and transcription, cell proliferation, and tumorigenesis[11]. The expression level of Mcm7 is positive correlation with tumor proliferation and malignancy degree[12]. In this study, the expression of Mcm7 in U266 cells treated with PS-341 was lower than untreated cells, therefore, we supposed PS-341 may affect tumor cell proliferation through regulating the tumor cell cycle.

GSTP1 (glutathione S-transferase P1) is a member of π family, which belongs to a superfamily of glutathione S-transferase (GST). GSTP1 is a major GST isoenzyme in most cell types, and can catalyze the conjugation of glutathione (GSH) with electrophiles[13]. In normal human cells, GSTP1 plays an important role in protecting cells against damage induced by cancer and carcinogens. While the aberrant expression of GSTP1 in cancer is related to cancerogenesis and development of multidrug resistance[14]. GSTP1 is wildly existed in human tumor tissues, and higher expressed in malignant tumors of epithelial origin[13]. The expression of GSTP1 is increased in the process of tumorigenesis. In our study, the expression of Mcm7 in U266 cells treated with PS-341 was decreased, indicating that PS-341 may reduce the protection of GSTP1 to tumor cells.

To date, MM is yet an incurable hematologic malignancy, and the further study of pathogenesis and mechanism of MM is still under investigation. In this study, we employed proteomic technique to deeply search for the mechanism of MM treated with proteasome inhibitor PS-341, and provided some theoretical evidence for targeted therapy of MM.

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(Edited by PENG Minning)