Bortezomib prevents oncogenesis and bone metastasis of prostate cancer by inhibiting WWP1, Smurf1 and Smurf2

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Abstract. Prostate cancer is the most common malignancy diagnosed in males, and bone metastases remain a significant source of morbidity and mortality in this population. Ubiquitin ligase E3s and proteasomes were thought to play essential roles in the development of cancers, therefore, they were proposed as therapy targets for the treatment of solid and hematological malignancies. Bortezomib, well-known as a proteasome inhibitor, has been observed with exact anticancer effect both in cell and animal models for several solid tumor types, including prostate cancer. To explore activities of the ubiquitin ligase E3s WWP1, Smurf1 and Smurf2 in oncogenesis and bone metastasis of prostate cancer, as well as in the functional mechanism of bortezomib in preventing prostate cancer, transcription and expression levels of WWP1, Smurf1 and Smurf2 genes in cell lines or tissues of benign prostate hyperplasia and human prostate cancer with and without bone metastasis were tested. Moreover, human prostate cancer PC3 cell lines were treated with bortezomib at different concentration gradients and then their proliferation at different time points, mRNA and protein levels were investigated. The results indicated that transcription and expression levels of WWP1, Smurf1 and Smurf2 genes in prostate cancer without bone metastasis were significantly higher compared to those in benign prostate hyperplasia ($P<0.05$), whereas significantly lower than prostate cancer metastatic to bone ($P<0.05$). Furthermore, bortezomib reduced the transcription and expression levels of WWP1, Smurf1 and Smurf2 genes in prostate cancer cell lines in a dose-dependent manner, thus, inhibiting the proliferation of prostate cancer cells. Elevated transcription and expression levels of ubiquitin ligase E3s WWP1, Smurf1 and Smurf2 genes may be the mechanisms of occurrence, development and metastasis of prostate cancer. In addition, bortezomib can prevent prostate cancer and its bone metastasis by downregulating WWP1, Smurf1 and Smurf2.

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Introduction

Prostate cancer is second to lung cancer in incidence worldwide, and it is the third most common cause of cancer deaths in developed countries (age-standardised rates in 2008) (1). The majority of patients with advanced prostate cancer will develop bone metastases, and these metastases represent a significant source of morbidity and mortality, resulting in a number of clinical complications of metastatic skeletal lesions that may be present in up to 80% of cases, including pain, bone loss, fracture, hypercalcemia and bone marrow replacement (2-4). Moreover, the 5-year survival rate among prostate cancer patients with bone metastases continues to be less than 50% (4). Despite the frequency of prostate carcinogenesis and skeletal metastases, the molecular mechanisms for their propensity to colonise bone are poorly understood and treatment options are often unsatisfactory. For nearly 70 years, endocrine therapy has been the mainstay of treatment, besides surgery, for patients with early-stage prostate cancer. In general, 40-80% of prostate cancers respond to hormonal therapy, but most of these tumors will progress to androgen-independent states within 2-3 years (5), and then the tumors recur in an androgen-independent form that is unresponsive to additional androgen withdrawal (6). Therefore, there is an urgent need to elucidate its molecular mechanisms to establish effective therapies and to allow early detection and treatment strategies.

Ubiquitin-proteasome system is accountable for regulation of numerous basic cellular processes such as the cell cycle

progression, signal transduction, proliferation, apoptosis, modulation of surface receptors and regulation of tumor suppression proteins (7). The ubiquitin-proteasome pathway is essential to cellular homeostasis and is considered to be an important regulator of multiple metabolic processes such as osteoblast differentiation and bone formation (8). Ubiquitin-proteasome system dysfunction, with demonstrated contribution to the development of cancer, renders it a feasible and rational target for novel therapies (9). Numerous E3 ubiquitin ligases in the ubiquitin proteasome pathway have been implicated in cell cycle control and uncontrolled cell proliferation. The neural precursor cell-expressed developmentally downregulated gene 4 (Nedd4) family of ubiquitin ligases (E3s) is characterized by a distinct modular domain architecture, with each member consisting of a C2 domain, 2-4 WW domains, and a HECT-type ligase domain (10). In Nedd4 family, smad ubiquitination regulatory factors (smurfs) are negative regulators of the transforming growth factor (TGF) signal-transduction cascade, which has antineoplastic activity in prostate cancer, indicating a cancer-fostering role of smurfs (11). The gene for WW domain containing E3 ubiquitin protein ligase 1 (WWP1), another member of Nedd4 family is located at 8q21, a region frequently amplified in human cancers, including prostate cancer. Recent studies have shown that WWP1 negatively regulates the TGFβ tumor suppressor pathway by inactivating its molecular components, including Smad2, Smad4 and TbR1 (12). However, there has not been a systemic elaboration of the relationship between transcription and expression levels of WWP1, Smurf1 and Smurf2 genes and the oncogenesis and bone metastasis of prostate cancer.

Proteasome in ubiquitin-proteasome system plays a central role in regulation of the cell cycle, proliferation, cell death, angiogenesis, metastasis and resistance to chemotherapy and radiation therapy (13). By controlling the levels of numerous transcription factors, tumor suppressors, anti-apoptotic proteins, and other signaling molecules, proteasome has been shown to be vital for cell cycle progression as well as programmed cell death (4). Proteasome inhibitors have presented cytotoxicity against a wide range of tumor cell lines, and these drugs are thought to impede tumor generation and growth by several different mechanisms. By limiting the degradation of various cell cycle regulatory proteins, proteasome inhibitors may induce mitotic arrest and apoptosis (14,15). As demonstrated in numerous experiments, proteasome inhibition could be a novel method for treating androgen-dependent and androgen-independent prostate cancer (13). Bortezomib (formerly known as PS-341) is a dipeptidyl boronic acid compound that acts as an exceedingly potent and selective proteasome inhibitor. By blocking the ubiquitin-proteasome pathway, bortezomib may inhibit the degradation of various regulatory proteins and transcription factors that are involved in cell division, apoptosis, cellular adhesion, angiogenesis and NF-κB activation, finally leading to the arrest of tumor growth and metastasis. Bortezomib has also been demonstrated to arrest cell cycle progression and induce apoptosis in cultured human prostate cancer cells as well as inhibit the growth of prostate cancer xenografts in a murine model (14,16). It was implicated in other clinical studies that bortezomib promotes osteoblastogenesis and inhibits bone resorption in patients with multiple myeloma (17-19). The specific mechanism of bortezomib in treating prostate cancer and its bone metastasis is still not fully understood, therefore, the connection with the levels of WWP1, Smurf1 and Smurf2 were investigated.

In the present study, we detected the transcription and expression levels of WWP1, Smurf1 and Smurf2 genes in cell lines and tissues of benign prostate hyperplasia and human prostate cancer with and without bone metastasis, and came to the conclusion that increased transcription and expression levels of ubiquitin ligase E3s WWP1, Smurf1 and Smurf2 genes are involved in the mechanism of occurrence, development and metastasis of prostate cancer. Furthermore, by testing the proliferation levels of human prostate cancer PC3 cell lines treated by bortezomib with different concentration gradients, we demonstrated that bortezomib can prevent prostate cancer and its bone metastasis by reducing WWP1, Smurf1 and Smurf2.

Materials and methods

Ethics statement. This study was conducted according to the principles expressed in the Declaration of Helsinki and has been approved by Ethics Committee of Hebei Medical University. The participants provided their written informed consent to participate in this study.

Clinical materials and grouping. Forty-five cases of patients diagnosed with prostate cancer by histopathological examination and hospitalized in the Fourth Hospital of Hebei Medical University from July, 2011 to February, 2013 were enrolled. Age range of the patients was from 52 to 88 years and the average was 67.82 years. Whole body bone scan with Emission Computed Tomography was performed on all the cases, if bone lesions existed, bone biopsy was further employed to determine whether bone metastasis had occurred. The cases were divided into bone metastasis group and non-bone metastasis group. Twenty-one cases were in the former group, whose age range was from 53 to 88 years with an average of 68.16 years, while the other 24 cases were in the latter group, age range was from 52 to 85 years with an average of 65.23 years. In addition, 25 patients that were diagnosed with benign prostate hyperplasia by histopathological examination after transurethral resection of prostate treatment in the same hospital were enrolled as control group. The age range of the control group was from 52 to 86 years with an average of 66.96 years.

Immunohistochemistry. Prostate tissues from three groups were performed into serial sections with a thickness of 3 μm, dewaxed, placed into citric acid solution at 27˚C to get antigen retrieval, added with 3% hydrogen peroxide to scavenge endogenous peroxidases, and then washed with PBS. Samples from each group were divided into 3 parts. Subsequently, rabbit anti-human WWP1 polyclonal antibody, rabbit anti-human Smurf1 polyclonal antibody and rabbit anti-human Smurf2 polyclonal antibody (all from Santa Cruz Biotechnology Co., Ltd., Shanghai, China) were, respectively, added into each sample from the 3 groups, co-incubated with the samples overnight and then washed with PBS. Afterwards, goat anti-rabbit IgG (Santa Cruz Biotechnology) was added to each part, co-incubated with the complexes at 37˚C for 30 min and then washed with PBS. Finally, color was

developed with DAB kit (Biosynthesis Biotechnology Co., Ltd., Beijing, China) according to its specifications. The negative control group was set without rabbit anti-human WWP1, Smurf1 or Smurf2 polyclonal antibody. After dehydration and sealing sheets, the samples were observed under a microscope. Image-Pro Plus image analysis system was applied to analyze the images and measure the integral optical density (IOD) values of ubiquitin ligase E3s.

Real-time quantitative PCR. The mRNA levels of WWP1, Smurf1, Smurf2 in prostate cancer PC3 cells were tested with RT-PCR, as previously described (20). The expansion effects of WWP1, Smurf1 and Smurf2 were analyzed by ΔΔCt method, and by standardization via β-actin the results were expressed by $2^{-\Delta\Delta CT}$. RNAiso Reagent [Takara Biotechnology (Dalian) Co., Ltd., Shanghai, China] was employed according to its specifications to extract RNAs from prostate cancers and the purity of the extracting solution was tested with an ultraviolet spectrophotometer. In the progress of reverse transcription, the RNase-free DNase and M-MLV kit (both from Promega Corporation, WI, USA) were applied in accordance with their specifications. The primers were designed by reference to GeneBank database and AlleleID4.0 software. β-actin was chosen as internal reference upstream: 5'-AGCTCTGCTGGTAGGTGCAC-3' and downstream: 5'-GCTACGACCATCTAGGCACG-3'. The primers of WWP1 were: primer 1 forward, 5'-TGGCATTGGAAAGAAGACG-3' and primer 2 reverse, 5'-GTTGTGGTCTCTCCCATGT GGT-3'. The primers of Smurf1 were: primer 1 forward, 5'-GTCCAGAAGCTGAAAGTCCTCAGA-3' and primer 2 reverse, 5'-CACGGAATTTCACCATCAGCC-3'. The primers of Smurf2 were primer 1 forward, 5'-GATCCAAAGTG GAATCAGCA-3' and primer 2 reverse, 5'-TGGCATTGGAAA GAAGACG-3'; The primers of β-actin were primer 1 forward, 5'-TCTTCCAGCCTTCCTTCCTG-3' and primer 2 reverse, 5'-TAGAGCCACCAATCCACACA-3'. SYBR-Green I dye method was employed using real-time quantitative PCR with Ex Tap kit (Takara Biotechnology) by fluorescence quantitative real-time PCR system (type ABI 7900, Applied Biosystems, CA, USA), according to the specifications provided by the manufacturer. The reaction conditions were as follows: predegeneration for 5 min at 93˚C, degeneration for 30 sec at 93˚C, annealing for 1 min at 55˚C and then elongation for 1 min at 71˚C, after 30 cycles, another elongation for 1 min at 71˚C. IQ5 software was used to analyze the readings.

Prostate cancer PC3 cells culture. Under strict aseptic conditions, prostate cancer PC3 cells [American Type Culture Collection (ATCC), Manassas, VA, USA] were cultured in a 60 ml plastic culture bottle with DMEM medium (Biohermes Biomedicine and Tecnology Co., Ltd., Boston, MA, USA) and culture solution was replaced each day. As soon as the cells formed a dense single wall, they were digested with 0.25% trypsinase (Gibco Corporation, CA, USA) and subcultured with the density of $5x10^5$ cells/ml. After 3-5 generations, cells were used to conduct the experiments.

Brdu ELISA. Prostate cancer PC3 cells were cultured in the 24-well microtiter plates at a concentration of $1x10^5$ cells/ml, bortezomib (Millennium Pharmaceuticals, Inc., MA, USA) at diverse concentrations (0.1, 1, 10 and 50 μmol/l) were, respectively, added into different wells. A negative control group was set without bortezomib. Then 10 μl of 10X Brdu (Hoffmann-La Roche Inc., Basel, Switzerland) was put into each well, respectively, at 24, 48 and 72 h. After incubation at 37˚C in an incubator for 72 h, the cells were centrifuged, then supernatant was discarded. A total of 100 μl of the liquid was put into each well, and then the cells were incubated at 37˚C for 30 min and washed with PBS several times. Subsequently, 100 μl anti-Brdu-POP antibody (Hoffmann-La Roche) was added into each well, then the cells were incubated at 25˚C for 60 min and centrifuged, then the supernatant was discarded. The cells were washed with PBS 3 times x 5 min, and 100 μl of ECL chromogenic substrate (Thermo Fisher Scientific Inc., Tokyo, Japan) was added into each well, and then incubated at 25˚C for 10 min. Sulfuric acid (25 μl) with a concentration of 1 mol/l was put into each well, then optical density (OD) levels were detected at 450 nm with enzyme-labeling instrument (ELx808, BioTek Instruments, Inc., Beijing, China) and cell viability was indicated by OD levels.

Western blot experiment. The western blot experiment were performed in a routine manner, as previously reported (21). Concentrations of protein were tested with BCA Protein Assay Reagent (Thermo Fisher Scientific), and electrophoresis was performed with SDS-PAGE. The voltage of stacking gel was 80 V while that of separation gel was 100 V. Half-dry transfer was conducted under a voltage of 20 V. After washing with TBST and closing, the cells were co-incubated with first antibody of WWP1, Smurf1, Smurf2, respectively, at 4˚C overnight. After washing 3 times with TBST, the cells were co-incubated with the second antibody. Color development was performed by chemiluminescence method, and then optical density analysis of the bands was made by Gel-Pro analyzer 4.0 software. The results are expressed by the ratios compared with the optical density of β-actin.

Statistical analysis. SPSS 18.0 software package was used for statistical analysis and data are expressed as the average ± standard deviation. Multiple-group comparisons were made by one-way ANOVA while 2-group comparisons by SNK t-test.

Results

Expression levels of WWP1, Smurf1 and Smurf2. The immunohistochemistry staining sections were observed under a microscope, the ubiquitin ligase E3 WWP1, Smurf1 and Smurf2 positive staining cells had brownish yellow particles in the cytoplasm. We found that positive staining cells were rare in prostate tissues from the control group, furthermore, prostate tissues of patients with prostate cancer from non-bone metastasis group presented more positive-staining cells compared with the control group, whereas less than those of the bone metastasis group (Figs. 1A, 2A and 3A). Integrated optical density (IOD) analysis showed that, compared with the control group, the IOD values of WWP1, Smurf1 and Smurf2 staining in non-bone metastasis group and bone metastasis group were significantly increased ($P<0.05$), more-

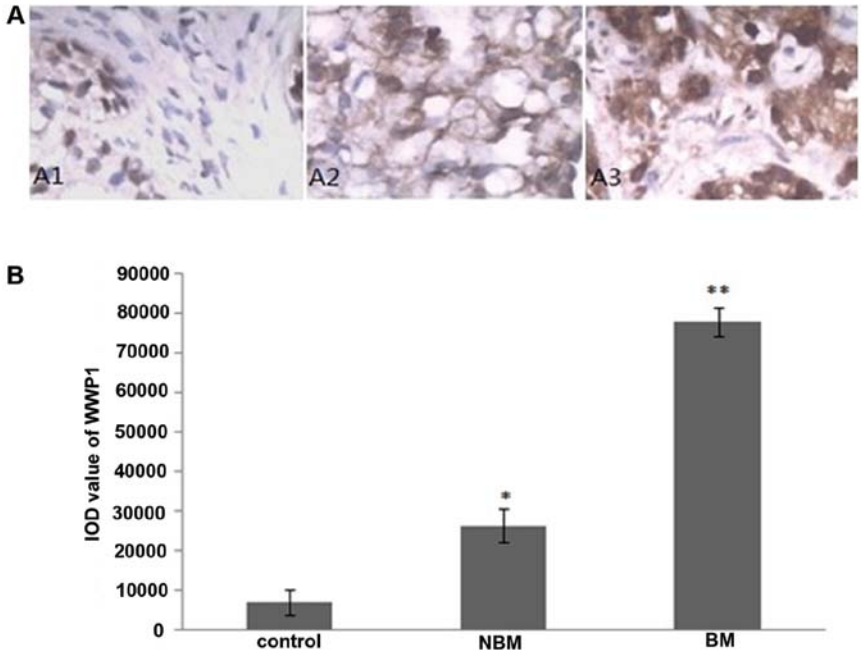

Figure 1. Expression levels of WWP1 in tissues from each group by immunohistochemistry. (A) The expression of WWP1 under a microscope shows brownish yellow particles in their cytoplasm representing positive expression of WWP1. The tissues in panels A1, 2 and 3 were from the control group, non-bone metastasis group and bone-metastasis group, respectively. (B) The IOD value of WWP1 staining in each group. NBM and BM represent non-bone metastasis group and bone metastasis group, respectively. *P<0.01 compared with the control group (t=4.279, p=0.002); **P<0.01 compared with the non-bone metastasis group (t=5.041, p=0.001).

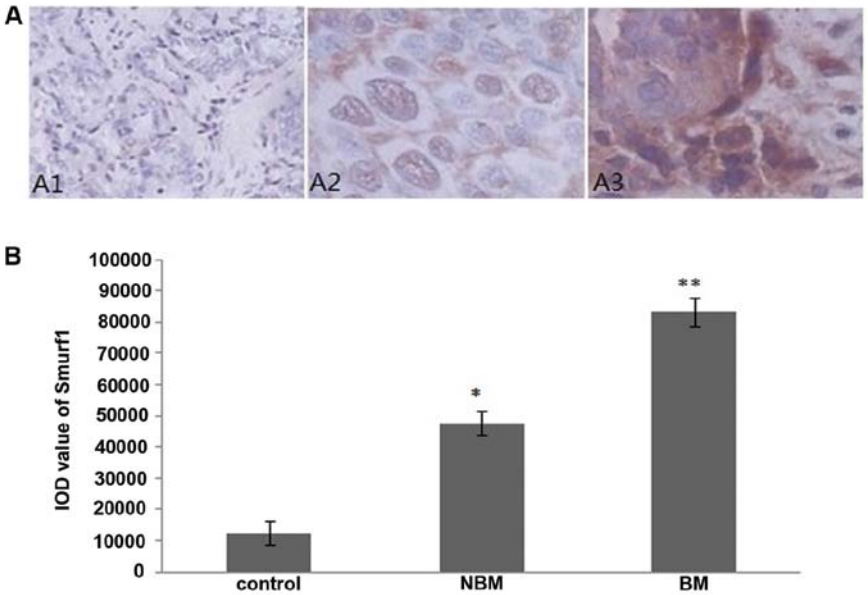

Figure 2. Expression levels of Smurf1 in tissues from each group by immunohistochemistry. (A) The expression of Smurf1 under a microscope shows brownish yellow particles in their cytoplasm representing positive expression of Smurf1. The tissues in panels A1, 2 and 3 were from the control group, non-bone metastasis group and bone-metastasis group respectively. (B) The IOD value of Smurf1 staining in each group. NBM and BM represent non-bone metastasis group and bone metastasis group, respectively. *P<0.01 compared with the control group (t=3.674, p=0.001); **P<0.01 compared with the non-bone metastasis group (t=9.887, p=0.001).

over, compared with the non-bone metastasis group, the IOD values of WWP1, Smurf1, Smurf2 staining in the metastasis group were significantly elevated in the bone metastasis group (P<0.05) (Figs. 1B, 2B and 3B).

Transcription levels of WWP1, Smurf1 and Smurf2 genes. To test the transcription and expression levels of WWP1, Smurf1 and Smurf2 genes, real-time PCR was performed. The results showed that compared with the control group, the levels of WWP1, Smurf1 and Smurf2 mRNA in non-bone metastasis group and bone metastasis group were significantly increased (P<0.05), moreover, compared with non-bone metastasis group, the levels of WWP1, Smurf1 and Smurf2 mRNA were significantly elevated in bone metastasis group (P<0.05) (Fig. 4).

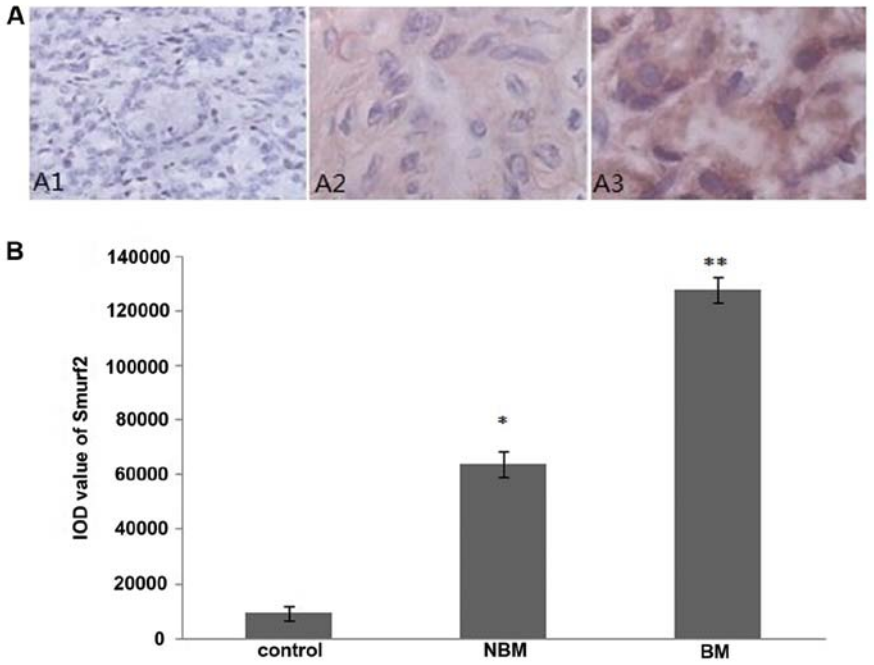

Figure 3. Expression levels of Smurf2 in tissues from each group by immunohistochemistry. (A) The expression levels of Smurf2 under a microscope shows brownish yellow particles in their cytoplasm represent positive expression of Smurf2. The tissues in panels A1, 2 and 3 were from the control group, non-bone metastasis group and bone-metastasis group, respectively. (B) The IOD value of Smurf1 staining in each group. NBM and BM represents non-bone metastasis group and bone metastasis group, respectively. *P<0.01 compared with the control group (t=14.336, p=0.004); **P<0.01 compared with the non-bone metastasis group (t=25.095, p=0.002).

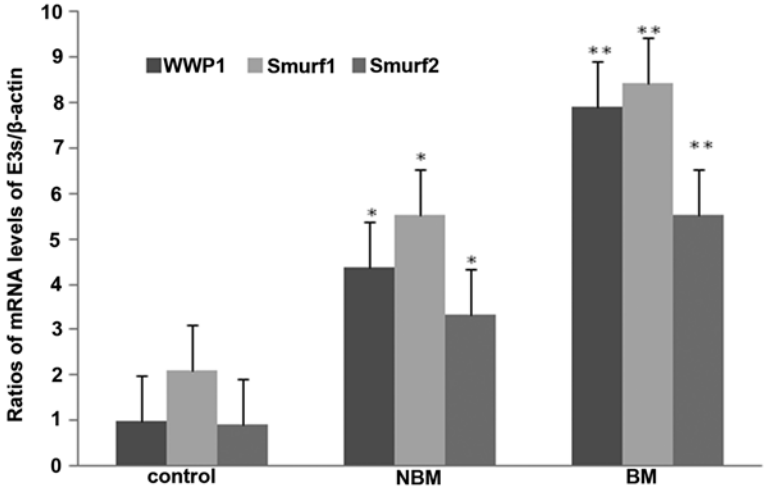

Figure 4. mRNA level of WWP1, Smurf1 and Smurf2 from each group evaluated by real-time PCR. *P<0.05 compared with the control group; **P<0.05 compared with the non-bone metastasis group.

Effects of bortezomib treatment on PC3 cells. PC3 cell proliferation activities were evaluated by OD values. Compared with the negative control group, bortezomib treatment reduced PC3 cell proliferation activity in a dose-dependent manner with the most obvious effect at 50 μmol/l 24, 48 and 72 h after treatment (P<0.01) (Fig. 3, Table I). Except for 0.1 μmol/l, bortezomib of other concentrations reduced PC3 cell proliferation activity in a time-dependent manner (P<0.05) (Fig. 3, Table II). In addition, bortezomib reduced WWP1, Smurf1 and Smurf2 mRNA levels in a dose-dependent manner with most obvious effect at 50 μmol/l on 72 h after treatment (P<0.01) (Fig. 4, Tables II and III). After treatment for 72 h, protein expression levels of WWP1, Smurf1 and Smurf2 became consistent with their mRNA levels (data not shown). Moreover, bortezomib treatment decreased protein expression levels of WWP1, Smurf1 and Smurf2 in a dose-dependent manner with a maximum effect at 50 μmol/l (P<0.01) (Fig. 5, Table IV).

Discussion

In the present study, by evaluating the transcription and expression levels of WWP1, Smurf1 and Smurf2 genes in cell

Table I. Statistics analysis for the effect of diverse bortezomib concentrations on PC3 cell proliferation.

	24 h	48 h	72 h
F	22.567[a]	42.564[a]	89.281[a]
P(1):(2)	0.031	0.025	0.012
(1):(3)	0.023	0.005	0.002
(1):(4)	0.013	0.002	<0.001
(1):(5)	<0.001	<0.001	<0.001
(2):(3)	0.014	0.018	0.009
(2):(4)	0.036	0.025	0.003
(2):(5)	<0.001	<0.001	<0.001
(3):(4)	0.017	0.025	0.006
(3):(5)	0.009	0.005	<0.001
(4):(5)	0.011	0.008	<0.001

(1), (2), (3), (4) and (5), respectively, represents OD levels of PC3 cells from negative control group and the group with a bortezomib concentration of 0.1, 1, 10 and 50 μmol/l. [a]P<0.01.

Table II. Statistics analysis for the effect of bortezomib at different time point on PC3 cell proliferation.

	0.1 μmol/l	1 μmol/l	10 μmol/l	50 μmol/l
F	7.383	12.274[a]	21.236[b]	32.115[b]
P(A):(B)	0.654	<0.001	0.012	<0.001
(A):(C)	0.032	0.032	0.032	<0.001
(B):(C)	0.548	0.041	0.028	0.008

(A), (B) and (C), respectively, represents OD level of PC3 cells on the 24, 48 and 72 h after bortezomib treatment. [a]P<0.05; [b]P<0.01.

Table III. Statistics analysis for the effect of diverse bortezomib concentrates on E3 transcription levels.

	WWP1	Smurf1	Smurf2
F	42.784[a]	86.321[a]	74.583[a]
P(1):(2)	<0.001	<0.001	<0.001
(1):(3)	<0.001	<0.001	<0.001
(1):(4)	<0.001	<0.001	<0.001
(1):(5)	<0.001	<0.001	<0.001
(2):(3)	0.038	<0.001	<0.001
(2):(4)	0.024	<0.001	<0.001
(2):(5)	<0.001	<0.001	<0.001
(3):(4)	0.012	0.013	<0.001
(3):(5)	0.005	0.007	<0.001
(4):(5)	0.022	0.016	0.013

(1), (2), (3), (4) and (5), respectively, represents the negative control group and the group with a bortezomib concentration of 0.1, 1, 10 and 50 μmol/l. [a]P<0.01.

Table IV. Statistics analysis for the effect of diverse bortezomib concentrates on E3s protein levels.

	WWP1	Smurf1	Smurf2
F	22.567[a]	42.564[a]	89.281[a]
P(1):(2)	<0.001	<0.001	<0.001
(1):(3)	<0.001	<0.001	<0.001
(1):(4)	<0.001	<0.001	<0.001
(1):(5)	<0.001	<0.001	<0.001
(2):(3)	0.024	0.013	<0.001
(2):(4)	0.013	<0.001	<0.001
(2):(5)	<0.001	<0.001	<0.001
(3):(4)	0.027	0.015	<0.001
(3):(5)	0.005	<0.001	<0.001
(4):(5)	0.018	0.022	0.008

(1), (2), (3), (4) and (5), respectively, represents the negative control group and the group with a bortezomib concentration of 0.1, 1, 10 and 50 μmol/l. [a]P<0.01.

lines and tissues of benign prostate hyperplasia and human prostate cancer with and without bone metastasis, and those of human prostate cancer PC3 cell lines treated by bortezomib with different concentrations (0.1, 1, 10, 50 μmol/l) at different time points, as well as the proliferation levels of PC3 cells, we demonstrated that increased transcription and expression levels of ubiquitin ligase E3s WWP1, Smurf1 and Smurf2 genes may be parts of the mechanisms of occurrence, development and metastasis of prostate cancer. Moreover, bortezomib inhibits prostate cancer and its bone metastasis by downregulating WWP1, Smurf1 and Smurf2.

Prostate cancer is a commonly diagnosed cancer and a major cause of cancer death in elderly men. Metastatic prostate cancers are lethal because they are heterogeneously composed of both androgen-dependent and non-androgen-dependent prostate cancer cells and androgen ablation does not induce apoptotic death of the non-androgen-dependent cells because they activate survival pathways that do not require androgenic stimulation, therefore, it is the continuing survival and proliferation of the non-androgen-dependent prostate cancer cells that eventually kill, no matter how complete the androgen ablation is within the prostate cancer patient (22). Therefore, additional targets and therapies that can eliminate the non-androgen-dependent cancer cells are urgently needed in conjunction with androgen ablation.

Protein ubiquitination is a post-translational modification that can direct proteins for degradation by the 26S proteasome or plasma membrane proteins for endocytosis, sorting and destruction in the lysosome (23). Besides, ubiquitination is involved in many other biological processes, including regulation of protein stability, cell cycle progression, gene transcription, receptor transport and immune responses (24). Ubiquitination is the covalent attachment of the 76-amino acid-comprising protein ubiquitin via its C terminus to an

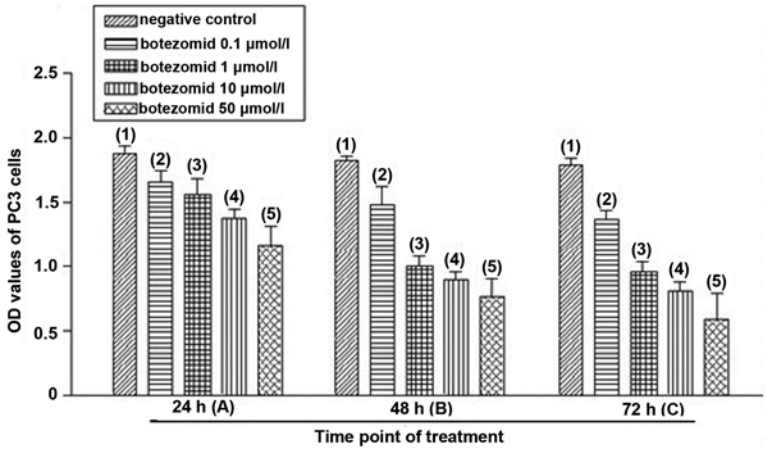

Figure 5. Effects of different bortezomib treatment on OD levels of PC3 cells. It can be seen in this figure that along with the increase of bortezomib concentration or treatment durations, the OD levels of PC3 cells decreased.

amino group on a target protein. The transfer of the activated ubiquitin to substrates occurs through a series of enzymes. These enzymes include a ubiquitin activating enzyme (E1), multiple ubiquitin conjugating enzymes (E2), and hundreds of ubiquitin-protein ligases (E3) (10). The three-step catalytic cascade is initiated by E1-mediated ATP-dependent activation of ubiquitin, which is subsequently conjugated to a cysteine residue within the E2, before finally being attached by an E3 to a lysine residue on a target protein, and the E3 ubiquitin ligases (E3s) play a critical role in the ubiquitin conjugation cascade by recruiting ubiquitin-loaded E2s, recognizing specific substrates, and facilitating or directly catalyzing ubiquitin transfer to either the Lys residues (in most cases) or the N terminus of their molecular targets (25). Based on the sequence homology of their E2-binding domains, E3s can be generally classified into three subfamilies: the homologous to E6-AP carboxyl-terminus (HECT) domain containing E3s, the really interesting new gene (RING) finger domain-containing E3s, and the U box E3s, additionally, given their substrate specificity, the E3s represent attractive targets for cancer therapy (25). For HECT E3s, ubiquitin is initially transferred to a catalytically active cysteine residue of the E3 and subsequently to a specific target; by contrast, RING and U-Box E3s do not possess enzymatic activity and rather serve as adaptors, bridging the catalytically active E2 and the E3-selected target protein, facilitating the direct transfer of ubiquitin from E2 to substrate (24). There are nine members of the Nedd4-like E3 family, all of which share a similar structure, including a C2 domain at the N-terminus, two to four WW domains in the middle of the protein, and a homologous to E6-AP COOH terminus domain at the C terminus (23).

It is well known that TGFβ is a potent tumor suppressor in the development of cancer, but it becomes a promoter of metastasis during cancer progression. The TGFβ signaling pathway involves a series of molecules, among them are the TGFβ receptor 1 (TbR1), Smad2 and Smad4, the protein levels of which are negatively regulated by WWP1 while increased when WWP1 expression was knocked down by siRNA in PC-3 cells (12). In this study, it has been shown that both the mRNA and protein levels of WWP1 in prostate cancer tissues are significantly higher than those in benign prostate hyperplasia (BPH) (Figs. 1 and 4), which suggests that elevated WWP1 level may be one of the mechanisms for oncogenesis of prostate cancer. It has been shown that abrogation of smurfs potentiated TGF-mediated antineoplastic activity and simultaneously suppressed genomic instability by activating DNA repair proteins, suppressing FLIP (a protein that negatively regulates apoptosis) and elevating TRAIL (a positive regulator of apoptosis), which indicated a cancer-fostering role of smurfs (11). In the present study, we have demonstrated that both the mRNA and protein levels of Smurf1 and Smurf2 in prostate cancer tissues are obviously increased compared to those in benign prostate hyperplasia (Figs. 2, 3 and 4), indicating essential roles of smurfs in oncogenesis of prostate cancer. Bone morphogenetic proteins, members of the TGFβ superfamily, were originally identified as osteoinductive proteins in bone that induce ectopic bone and cartilage formation *in vivo* (26). Smurf1 binds to receptor-regulated Smads for bone morphogenetic proteins Smad1/5 and promotes their degradation. In addition, Smurf1 associates with TGFβ type I receptor through the inhibitory Smad (I-Smad) Smad7 and induces their degradation (26). Smurf2, which is structurally similar to Smurf1, also targets Smad1 for degradation, moreover, Smurf2 was shown to associate with activated TGFβ-specific RSmad Smad2 and to induce its ubiquitin-dependent degradation (26). In addition, Smurf1 and Smurf2 interact with nuclear Smad7 and induce nuclear export of Smad7. The Smurfs-Smad7 complexes then associate with type I receptor for TGFβ and enhance its turnover (26). The E3 ubiquitin ligase Smurf1 mediates the ubiquitination and degradation of the main osteoblast transcription factor Runx2, as well as the signaling proteins JunB, MEKK2 and BMP2-activated Smad1 and Smad4 (27). Runx2 degradation is also mediated by WWP1, resulting in decreased osteoblast differentiation and bone formation, consistently, WWP1 deletion in mice leads to increased Runx2 and bone mass (28). WWP1 also promotes the ubiquitination and degradation of JunB, an AP-1 transcription factor that positively regulates osteoblast differentiation, which was demonstrated in WWP1 knockout mice that do not exhibit the TNF-α-induced JunB ubiquitination and subsequent inhibition of osteoblast

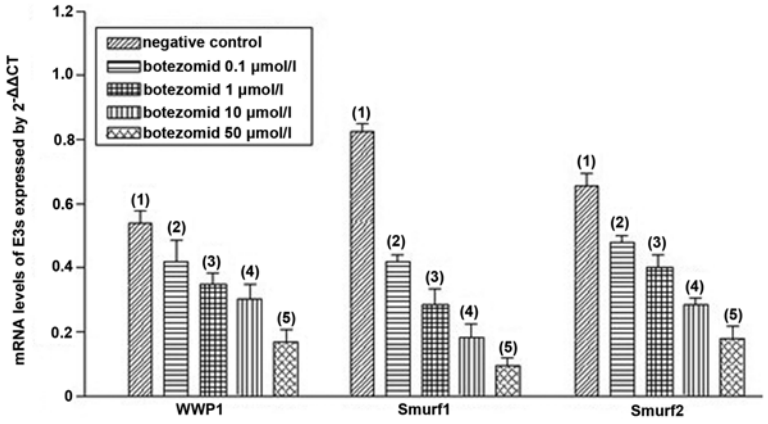

Figure 6. Effects of bortezomib with different concentrations on transcription levels of E3s genes. The data are shown at 72 h after bortezomib treatment. Along with the elevation of bortezomib concentration, the transcription levels of WWP1, Smurf1 and Smurf2 genes were reduced.

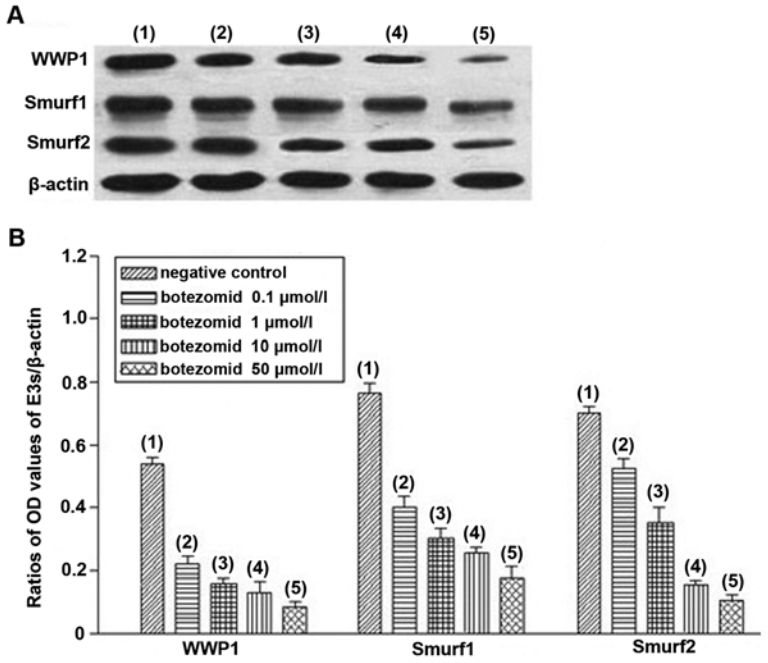

Figure 7. Effects of bortezomib with different concentrations on protein levels of E3s genes. The data are shown at 72 h after bortezomib treatment. Along with the elevation of bortezomib concentration, the protein levels of WWP1, Smurf1 and Smurf2 were downregulated.

differentiation observed in wild-type mice (29,30). Consistently, our study found that the mRNA and protein levels of WWP1, Smurf1 and Smurf2 in protein tissues from bone-metastasis group are significantly elevated compared to those from the non-bone metastasis group (Figs. 1, 2, 3 and 4), determined by inducing the effects of WWP1, Smurf1 and Smurf2 on prostate cancer metastatic to the bone. Our research suggested potential roles of WWP1, Smurf1 and Smurf2 as novel targets in cancer therapy.

NF-κB is a transcription factor that stimulates the production of inflammatory mediators, adhesion molecules, and anti-apoptotic proteins that promote tumor proliferation, metastasis and chemoresistance (4). By stabilizing the inhibitory protein of NF-κB, proteasome inhibitors may block NF-κB-mediated transcription in tumor cells and enhance their susceptibility to antineoplastic drugs and radiation therapy (4,31). In March, 2005, the FDA granted regular approval for bortezomib therapy in progressive multiple myeloma for one prior treatment (32). Experiments with bortezomib show that this agent induces apoptosis in androgen-dependent and androgen-independent prostate cancer cell lines (14). In this study, we have shown that bortezomib treatment decreased PC3 cell proliferation activity in a dose-dependent manner with the most obvious effect at 50 μmol/l 24, 48 and 72 h after treatment (Fig. 5, Table I), additionally, except for 0.1 μmol/l, bortezomib of other concentrations reduced PC3 cell proliferation activity in a time-dependent manner (Fig. 5, Table II), which confirmed the anti-oncogenesis function of bortezomib on prostate cancer. In one study, nude mice implanted with prostate cancer PC3 cells experienced a 60% decrease in tumor burden following weekly intravenous bortezomib therapy, furthermore, when directly injected into PC3 xenografts, bortezomib generated an even larger decrease in tumor volume (4). By augmenting the radiosensitivity of prostate cancer cells, circumventing

multicellular drug resistance in slow-growing prostate cancer tumors, exerting anti-angiogenic and direct cytotoxic effects and blocking androgen-dependent prostate cancer growth, bortezomib prevents the occurrence and development of prostate cancer (4). In osteoblasts, β-catenin (a negative regulator of chondrogenesis) accumulation induced by proteasome inhibition leads to increased osteoblastic cell proliferation, differentiation and survival (33). In addition to β-catenin, the bortezomib-induced bone formation is mediated by reduced degradation of Dickkopf1 (Dkk1), an extracellular Wnt/β-catenin antagonist (18). Additionally, proteasome inhibition decreases the degradation of the zinc-finger transcription factor Gli2 that mediates bone morphogenetic protein-2 expression in response to Hedgehog signaling, resulting in increased bone formation (27). Thus, several proteins can be targeted by proteasome inhibitors in osteoblasts, leading to increased osteoblastogenesis and bone formation. In addition to its effect on osteoblasts, the proteasome inhibitors exert essential impact on bone resorption. Notably, proteasome inhibitors suppress osteoclastogenesis and decrease bone resorption mainly by acting on the NF-κB signaling pathway, causing a reduction in the expression of receptor activator of NF-κB ligand (RANKL), which is essential for osteoclastogenesis (27). In the present study, we verified that bortezomib reduced both mRNA and protein levels of WWP1, Smurf1 and Smurf2 from prostate cancer PC3 cells in a dose-dependent manner with most obvious effect at 50 μmol/l on 72 h after treatment (Figs. 6 and 7), which may be part of the mechanisms for its inhibiting effect on prostate cancer. However, it has also been shown that after treatment by bortezomib for 72 h, the mRNA level of WWP1 were not consistent with but higher than its protein level (Figs. 6 and 7), which suggests that by contrast to Smurfs, bortezomib additionally promotes degradation of WWP1, in other words, time difference exists in regulating effects of bortezomib on Smurfs and WWP1, however, the specific mechanism is still not clear.

As further understanding leads to the development of potential anticancer therapeutics that target the activities of Nedd4-like E3s, a deeper exploration of the mechanisms underlying the association of Nedd4L with its molecular interplay in prostate cancer progression could potentially lead to more effective clinical management, as well as provide new target pathways for prostate cancer detection, early diagnosis and therapy. Additionally, due to its cytotoxic activity against cancer cells, bortezomib may represent a novel adjunctive therapy for prostate cancer and its bone metastasis.

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References

1. Obertova Z, Brown C, Holmes M and Lawrenson R: Prostate cancer incidence and mortality in rural men - a systematic review of the literature. Rural Remote Health 12: 2039, 2012.
2. Armstrong AP, Miller RE, Jones JC, Zhang J, Keller ET and Dougall WC: RANKL acts directly on RANK-expressing prostate tumor cells and mediates migration and expression of tumor metastasis genes. Prostate 68: 92-104, 2008.
3. Buijs JT, Henriquez NV, van Overveld PG, van der Horst G, ten Dijke P and van der Pluijm G: TGF-beta and BMP7 interactions in tumour progression and bone metastasis. Clin Exp Metastasis 24: 609-617, 2007.
4. Whang PG, Gamradt SC, Gates JJ and Lieberman JR: Effects of the proteasome inhibitor bortezomib on osteolytic human prostate cancer cell metastases. Prostate Cancer Prostatic Dis 8: 327-334, 2005.
5. Satoh F, Mimata H, Nomura T, *et al*: Autocrine expression of neurotrophins and their receptors in prostate cancer. Int J Urol 8: S28-S34, 2001.
6. Festuccia C, Gravina GL, Muzi P, *et al*: In vitro and in vivo effects of bicalutamide on the expression of TrkA and P75 neurotrophin receptors in prostate carcinoma. Prostate 67: 1255-1264, 2007.
7. Hellwinkel OJ, Asong LE, Rogmann JP, *et al*: Transcription alterations of members of the ubiquitin-proteasome network in prostate carcinoma. Prostate Cancer Prostatic Dis 14: 38-45, 2011.
8. Garrett IR, Chen D, Gutierrez G, *et al*: Selective inhibitors of the osteoblast proteasome stimulate bone formation in vivo and in vitro. J Clin Invest 111: 1771-1782, 2003.
9. Micel LN, Tentler JJ, Smith PG and Eckhardt GS: Role of ubiquitin ligases and the proteasome in oncogenesis: novel targets for anticancer therapies. J Clin Oncol 31: 1231-1238, 2013.
10. Yang B and Kumar S: Nedd4 and Nedd4-2: closely related ubiquitin-protein ligases with distinct physiological functions. Cell Death Differ 17: 68-77, 2010.
11. Farooqi AA, Waseem MS, Riaz AM and Bhatti S: SMURF and NEDD4: sharp shooters monitor the gate keepers and ion traffic controllers of lead astray cell. J Membr Biol 244: 1-8, 2011.
12. Chen C, Sun X, Guo P, *et al*: Ubiquitin E3 ligase WWP1 as an oncogenic factor in human prostate cancer. Oncogene 26: 2386-2394, 2007.
13. Papandreou CN and Logothetis CJ: Bortezomib as a potential treatment for prostate cancer. Cancer Res 64: 5036-5043, 2004.
14. Adams J, Palombella VJ, Sausville EA, *et al*: Proteasome inhibitors: a novel class of potent and effective antitumor agents. Cancer Res 59: 2615-2622, 1999.
15. Sunwoo JB, Chen Z, Dong G, *et al*: Novel proteasome inhibitor PS-341 inhibits activation of nuclear factor-kappa B, cell survival, tumor growth, and angiogenesis in squamous cell carcinoma. Clin Cancer Res 7: 1419-1428, 2001.
16. Williams S, Pettaway C, Song R, Papandreou C, Logothetis C and McConkey DJ: Differential effects of the proteasome inhibitor bortezomib on apoptosis and angiogenesis in human prostate tumor xenografts. Mol Cancer Ther 2: 835-843, 2003.
17. Uy GL, Trivedi R, Peles S, *et al*: Bortezomib inhibits osteoclast activity in patients with multiple myeloma. Clin Lymphoma Myeloma 7: 587-589, 2007.
18. Oyajobi BO, Garrett IR, Gupta A, *et al*: Stimulation of new bone formation by the proteasome inhibitor, bortezomib: implications for myeloma bone disease. Br J Haematol 139: 434-438, 2007.
19. Giuliani N, Morandi F, Tagliaferri S, *et al*: The proteasome inhibitor bortezomib affects osteoblast differentiation in vitro and in vivo in multiple myeloma patients. Blood 110: 334-338, 2007.
20. Alaaeddine N, De Montigny C and Sadouk M: Real-time reverse transcription-polymerase chain reaction quantification of tumor necrosis factor alpha messenger in human leukocytes. Clin Lab 57: 799-802, 2011.
21. Zhu Y, Li T, Song J, *et al*: The TIR/BB-loop mimetic AS-1 prevents cardiac hypertrophy by inhibiting IL-1R-mediated MyD88-dependent signaling. Basic Res Cardiol 106: 787-799, 2011.
22. Weeraratna AT, Dalrymple SL, Lamb JC, *et al*: Pan-trk inhibition decreases metastasis and enhances host survival in experimental models as a result of its selective induction of apoptosis of prostate cancer cells. Clin Cancer Res 7: 2237-2245, 2001.
23. Chen C and Matesic LE: The Nedd4-like family of E3 ubiquitin ligases and cancer. Cancer Metastasis Rev 26: 587-604, 2007.
24. Rieser E, Cordier SM and Walczak H: Linear ubiquitination: a newly discovered regulator of cell signalling. Trends Biochem Sci 38: 94-102, 2013.
25. Bernassola F, Karin M, Ciechanover A and Melino G: The HECT family of E3 ubiquitin ligases: multiple players in cancer development. Cancer Cell 14: 10-21, 2008.
26. Murakami G, Watabe T, Takaoka K, Miyazono K and Imamura T: Cooperative inhibition of bone morphogenetic protein signaling by Smurf1 and inhibitory Smads. Mol Biol Cell 14: 2809-2817, 2003.

27. Severe N, Dieudonne FX and Marie PJ: E3 ubiquitin ligase-mediated regulation of bone formation and tumorigenesis. Cell Death Dis 4: e463, 2013.
28. Jones DC, Wein MN, Oukka M, Hofstaetter JG, Glimcher MJ and Glimcher LH: Regulation of adult bone mass by the zinc finger adapter protein Schnurri-3. Science 312: 1223-1227, 2006.
29. Zhao L, Huang J, Zhang H, *et al*: Tumor necrosis factor inhibits mesenchymal stem cell differentiation into osteoblasts via the ubiquitin E3 ligase Wwp1. Stem Cells 29: 1601-1610, 2011.
30. Sterz J, von Metzler I, Hahne JC, *et al*: The potential of proteasome inhibitors in cancer therapy. Expert Opin Investig Drugs 17: 879-895, 2008.
31. Ma MH, Yang HH, Parker K, *et al*: The proteasome inhibitor PS-341 markedly enhances sensitivity of multiple myeloma tumor cells to chemotherapeutic agents. Clin Cancer Res 9: 1136-1144, 2003.
32. Kane RC, Farrell AT, Sridhara R and Pazdur R: United States Food and Drug Administration approval summary: bortezomib for the treatment of progressive multiple myeloma after one prior therapy. Clin Cancer Res 12: 2955-2960, 2006.
33. Qiang YW, Hu B, Chen Y, *et al*: Bortezomib induces osteoblast differentiation via Wnt-independent activation of beta-catenin/TCF signaling. Blood 113: 4319-4330, 2009.