

## Original Article

# Bortezomib-based chemotherapy regimens can improve response in newly diagnosed multiple myeloma patients with bcl-2 and survivin overexpression

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Received May 16, 2014; Accepted May 31, 2014; Epub June 15, 2014; Published July 1, 2014

**Abstract:** Objective: To investigate the relationship between the Bcl-2 and survivin expression and the different regimens therapeutic efficacy newly diagnosed multiple myeloma (NDMM). Methods: We retrospectively assessed the association of Bcl-2 and survivin expression with chemotherapeutic efficacy and prognosis in 59 NDMM patients in a single center. Results: The positive expression rate for survivin and Bcl-2 was 35% and 74%, respectively. Survivin and Bcl-2 protein expression were not associated with clinical stage, suggesting that they are not related to tumor burden in NDMM. Bortezomib-based regimens were more effective in reducing tumor burden and achieving therapeutic (complete and partial) response compared with non-bortezomib-based regimens irrespective of Bcl-2 or survivin expression ( $P < 0.05$ ). In cases with both negative Bcl-2 and survivin expression (Bcl-2<sup>-</sup>survivin<sup>-</sup>), the response to bortezomib and non-bortezomib-based regimens was similar ( $p = 0.429$ ). Bcl-2 and survivin expression were not correlated with overall survival (OS); however, Bcl-2<sup>+</sup>survivin<sup>+</sup> cases showed a trend towards a longer OS ( $P = 0.078$ ). Conclusion: We recommend bortezomib-containing regimens for NDMM with single or double-positive Bcl-2 and survivin expression.

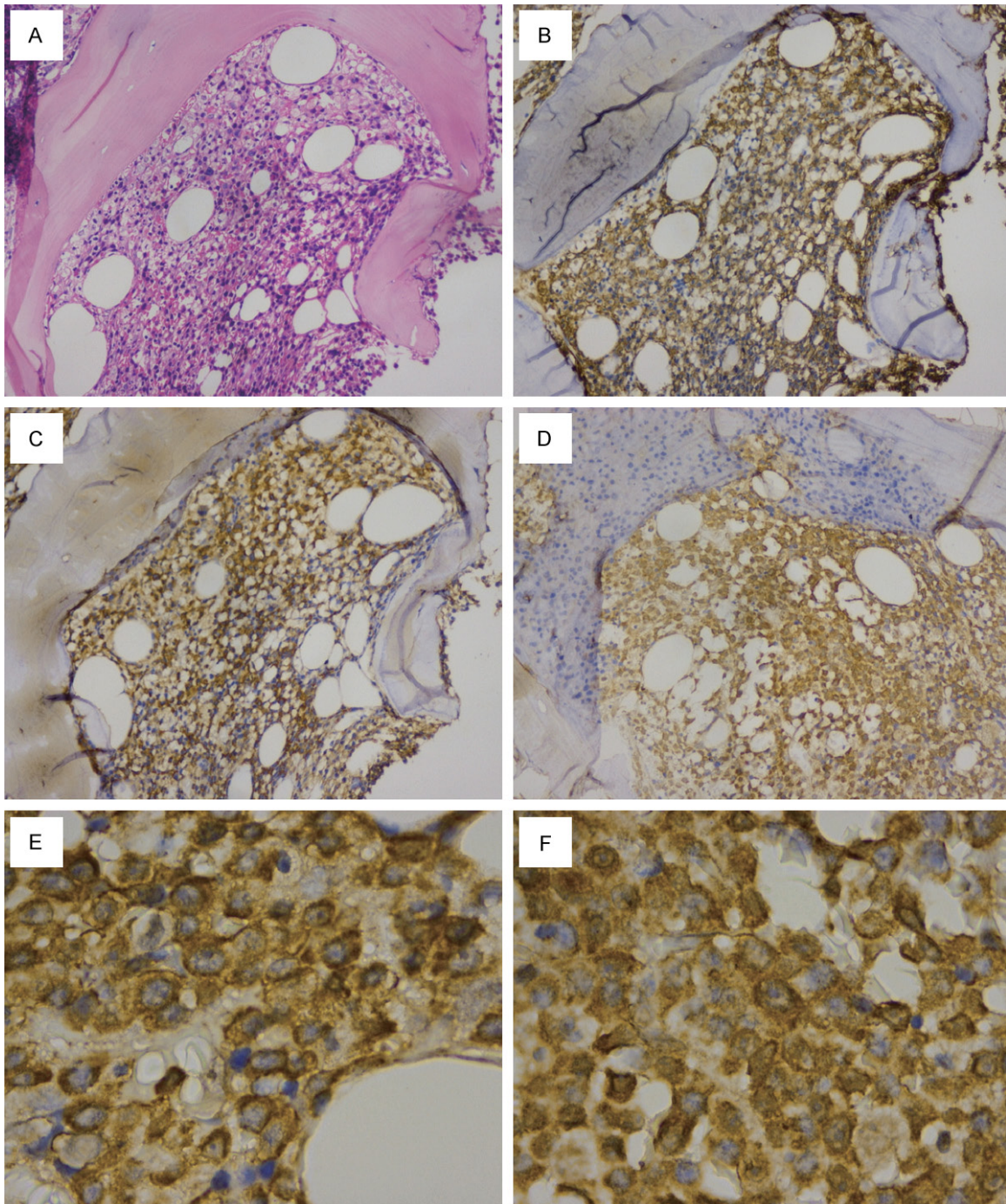
**Keywords:** Multiple myeloma, bortezomib, Bcl-2, survivin

## Introduction

Multiple myeloma (MM) is an incurable malignant plasmatic cell disorder that originates in germinal center B cells. MM is characterized by the accumulation of malignant plasmatic clones in the bone marrow, appearance of monoclonal (M) protein in the serum and/or urine, and multiple organ dysfunction [1]. Therapeutic strategies that target both MM cells and the bone marrow microenvironment, such as bortezomib, thalidomide, and lenalidomide, can significantly improve prognosis and prolong overall survival (OS) 7-8 years compared with 3-4 years for traditional chemotherapy [2]. However, MM is a highly heterogeneous disorder and prognosis can vary considerably. OS can exceed 9 years in some patients, whereas others may survive less than 4 months.

Therefore, the identification of effective molecular markers that can predict therapeutic efficacy and/or prognosis will be of great benefit to patients who suffer from this disease.

Survivin, a member of the inhibitor of apoptosis protein family, targets various components of the mitotic spindle and plays an important role in cell division. In addition, survivin protects cells from caspase-dependent and caspase-independent apoptotic pathways. Survivin overexpression and its association with adverse clinical and pathological parameters have been reported in various hematopoietic [3-6] and solid cancers [7, 8]. Survivin expression has been shown to be markedly higher in myeloma cell lines and primary myeloma cells from MM patients compared with normal bone marrow cells from healthy controls [9]. Despite its role



**Figure 1.** Bcl-2 and Survivin Expression in Plasma Cells from NDMM. (A, B) Show the HE and CD138 staining in plasma cells, respectively (200 x). (C, D) Show that over 80% of plasma cells exhibiting moderate or strong immunostaining for Bcl-2 and survivin, respectively (200 x). (E) Shows that Bcl-2 is expressed in the cytoplasm and cell membrane of plasma cells (1000 x), and (F) shows that survivin is expressed in the cytoplasm and/or nucleus of plasma cells (1000 x).

in cell division, survivin expression may not reflect the proliferation status of myeloma cells. Elevated survivin level has been associated with disease progression and relapse [10], poor

prognosis, especially post-chemotherapy [11], and multidrug resistance [12] in MM. Recently, survivin has emerged as an attractive target for cancer therapy, and the efficacy of anti-survivin

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**Table 1.** Relationship between Bcl-2 and survivin expression and patient characteristics

Patient characteristic	Total	Bcl-2 <sup>-</sup>	Bcl-2 <sup>+</sup>	<i>p</i>	Survivin <sup>-</sup>	Survivin <sup>+</sup>	<i>P</i>	Bcl-2Survivin <sup>-</sup>	non-Bcl-2Survivin <sup>-</sup>	<i>p</i>
No. patients (%)	45	10 (22)	35 (88)		26 (58)	19 (42)		7 (16)	38 (84)	
Age ≥ 60 y, no. (%)	15	6 (40)	9 (60)	0.062	8 (53)	7 (47)	0.754	4 (27)	11 (73)	0.199
Male, no. (%)	28	6 (21)	22 (79)	1	16 (57)	12 (43)	1	4 (14)	24 (86)	1
Hb ≤ 85 g/l, no. (%)	28	5 (18)	23 (82)	0.467	14 (50)	14 (50)	0.222	4 (14)	24 (86)	1
sCalcium ≥ 2.65 mmol/l, no. (%)	10	4 (40)	6 (60)	0.194	5 (50)	5 (50)	0.72	3 (30)	7 (70)	0.172
Advanced LBD, no. (%)	22	5 (23)	17 (77)	1	15 (68)	7 (32)	0.231	3 (14)	19 (86)	1
sCR ≥ 177 mmol/l, no. (%)	19	5 (26)	14 (74)	0.72	11 (58)	8 (42)	1	2 (11)	17 (89)	0.681
Stage III (DS), no. (%)	32	5 (16)	27 (84)	0.111	17 (53)	15 (47)	0.504	4 (13)	28 (87)	0.655
Stage III (ISS), no. (%)	27	6 (22)	21 (78)	0.561	16 (59)	11 (41)	0.554	5 (19)	22 (81)	0.77

Abbreviation: Hb, hemoglobin; LBD, lytic bone disease; sCR, serum creatinine; DS, Durie-Salmon staging system; ISS, International Staging System.

**Table 2.** Relationship between Bcl-2 and survivin expression and treatment response

	Treatment	No. patients	CR + PR, no. (%)	<i>P</i>
Total	Bor	16	14 (87.5)	< 0.001
	non-bor	29	7 (24)	
Bcl-2 <sup>-</sup>	Bor	4	4 (100)	0.076
	non-bor	6	2 (33)	
Bcl-2 <sup>+</sup>	Bor	12	10 (83)	0.001
	non-bor	23	5 (22)	
Survivin <sup>-</sup>	Bor	9	7 (78)	0.097
	non-bor	17	6 (35)	
Survivin <sup>+</sup>	Bor	7	7 (100)	< 0.001
	non-bor	12	1 (8)	
Bcl-2Survivin <sup>-</sup>	Bor	2	2 (100)	0.429
	non-bor	5	2 (40)	
non-Bcl-2Survivin <sup>-</sup>	Bor	14	12 (86)	< 0.001
	non-bor	24	5 (21)	

Abbreviations: Bor, bortezomib; CR, complete response; PR, partial response.

therapies are being evaluated in leukemia and lymphoma patients [13-15].

For malignant transformation, clonal cells must overcome pro-apoptotic signals and acquire a dependence on anti-apoptotic proteins. Bcl-2 is a key anti-apoptotic protein, and a variety of tumor cells overexpress Bcl-2 to resist apoptotic cell death. Bcl-2 has been reported to be responsible for metastatic events, drug resistance, and poor therapeutic efficacy in MM. Regulation of Bcl-2 expression still remains a challenge for anti-MM therapy and relevant drugs have recently been investigated in clinical trials [16].

In the present study, we investigated the relationship between the immunohistochemical

expression of survivin and Bcl-2 and therapeutic efficacy and prognosis in newly diagnosed MM (NDMM) patients.

## Materials and methods

### Patients

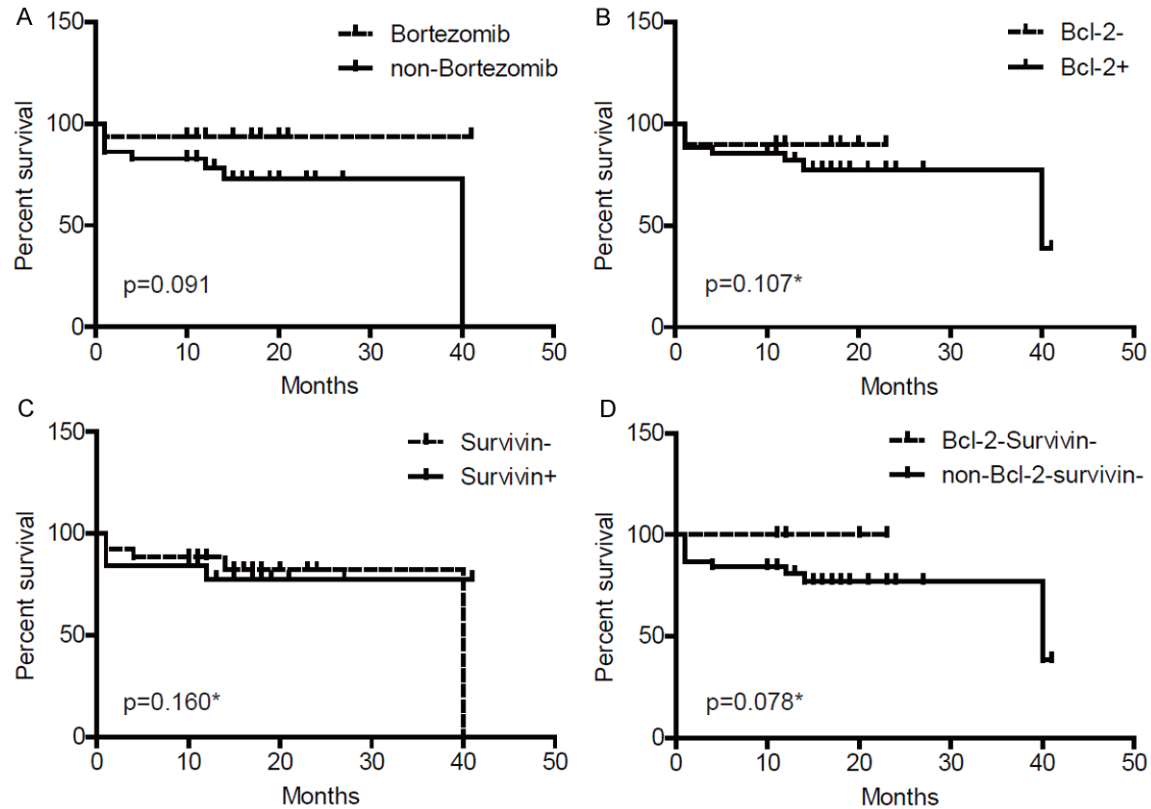
Fifty-nine NDMM patients with paraffin-embedded bone marrow biopsy tissue available before therapy were included in the retrospective study. All patients signed informed consent documents approved by Tongji Hospital.

The Durie-Salmon (DS) staging system [17] and International Staging System (ISS) [18] were used to determine clinical stage. Anemia, hypercalcemia, and renal dysfunction were defined as hemoglobin ≤ 100 g/l, serum calcium ≥ 2.65 mmol/l, and serum creatinine ≥ 177 mmol/l, respectively. For osteolytic bone lesions, the body bones were divided into the following five anatomical regions: skull, spine, pelvis, long bones, and others. Osteolytic bone lesions were divided into two subgroups according to the imaging results: none/limited lytic bone disease (LBD), none or ≥ 1 bone lesion limited to a single anatomical region and advanced LBD, bone lesions involving at least two anatomical regions.

### Treatment

Forty-five patients were treated in our hospital. Sixteen patients received bortezomib plus dexamethasone (VD), and the remaining 29 patients received melphalan/prednisone/thalidomide (MPT) or vincristine/doxorubicin/dexamethasone (VAD) [19]. Treatment response

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**Figure 2.** OS according to treatment regimens (A), Bcl-2 and survivin expression (B-D). \*: adjusted by the chemotherapy regimens.

was evaluated according to the International Myeloma Working Group 2006 [20]. OS was calculated from the day of the first treatment to the day of death from any cause or last follow-up.

### Immunohistochemistry (IHC)

Tissue sections (3-4  $\mu$ m) were routinely processed as follows: deparaffinized in xylene, rehydrated through a graded series of ethanol, subjected to heat-induced antigen retrieval in a steamer, and incubated in 3%  $H_2O_2$  to block endogenous peroxidase. The sections were incubated overnight at 4°C with mouse anti-human Bcl-2 monoclonal antibody, rabbit anti-human survivin polyclonal antibody, and mouse anti-human CD138 monoclonal antibody. Sections incubated with phosphate-buffered saline (PBS) alone were used as a negative control. The slides were washed in 0.1 mM PBS and then incubated with the horseradish peroxidase (HRP)-polymer anti-mouse/rabbit antibody for 20 min at room temperature. After washing with PBS, the sections were incubated with 3,3'-diaminobenzidine (DAB) at room tem-

perature for 5 min. The DAB detection kit, HRP-polymer anti-mouse/rabbit IHC kits, and Bcl-2 (Product ID: RMA-0660, clone: SP66), survivin (Product ID: RAB-0536), and CD138 (Product ID: MAB-0200, clone: MI15) primary antibodies were all purchased from MaxVision (Fuzhou Maixin Biotechnology Co., Ltd.). Nine reactive plasmacytoses (RP) were used as a control, and all slides were processed by the same pathologist.

Plasma cells were identified by hematoxylin-eosin and CD138 staining. Immunohistochemical staining of Bcl-2 and survivin in the plasma cells were evaluated using a double grading system, termed the quick score (QS) [21]. The QS was the sum of a proportional score and intensity score (IS). Because the proportion of Bcl-2 staining in plasma cells is over 80% according to the literature [22] and Bcl-2 and survivin staining were both present in over 80% of the plasma cells in the present study, only IS was employed. The IS was classified as follows: 0, no staining at high magnification; 1, staining only visible at high magnification; 2, staining readily visible at low magnification; and 3, stain-

ing strongly visible at low magnification. An IS  $\geq 2$  was considered positive staining for Bcl-2 and survivin, and all other cases were considered negative. The staining results were blindly analyzed by two pathologists. In cases of disagreement, the results were reanalyzed until a consensus was reached.

### *Statistical analysis*

Patient characteristics and IHC staining results were compared using Fisher's exact and Pearson chi-square tests. OS was evaluated using the Kaplan-Meier method and compared using the log-rank test. A two-tailed  $P$  value  $< 0.05$  was considered significant. All statistical analyses were performed using SPSS 12.0 software.

### **Results**

#### *Bcl-2 and survivin expression in plasma cells from NDMM*

A total of 59 NDMM patients were included in the analysis. Of the 59 patients, 14 patients had incomplete clinical data, and the remaining 45 patients had complete data and were included in the response analysis. Bcl-2 was expressed in the cytoplasm and cell membrane of plasma cells, whereas survivin was expressed in the cytoplasm and/or nucleus of plasma cells (**Figure 1**). Positive Bcl-2 and survivin expression were present in 43 (74%) and 21 (35%) cases, respectively. Eight (14%) cases were negative for both Bcl-2 and survivin expression (Bcl-2<sup>-</sup>survivin<sup>-</sup>). Positive expression of Bcl-2 and survivin was not detected in plasma cells from RP.

#### *Relationship between Bcl-2 and survivin expression and patient characteristics*

Hypercalcemia, renal dysfunction, anemia, and osteolytic bone lesions (CRAB) are the four main extramedullary target organ lesions caused by clonal plasma cell proliferation. Bcl-2 and survivin expression were not associated with such dysfunctions or clinical stage according to the DS staging system or ISS (**Table 1**).

#### *Relationship between Bcl-2 and survivin expression and treatment response*

Forty-five cases were eligible for treatment response evaluation. A therapeutic response

(complete response [CR] + partial response [PR]) was achieved in 47% (21/45) of cases. CR + PR rate was higher for patients who received VD than for those who received MPT/VAD (88% [14/16] vs. 24% [7/29];  $P \leq 0.001$ ). CR + PR was significantly greater in Bcl-2-positive (Bcl-2<sup>+</sup>;  $P = 0.001$ ), survivin-positive (survivin<sup>+</sup>;  $P < 0.001$ ), and non-Bcl-2/survivin<sup>-</sup> ( $P < 0.001$ ) patients who received VD treatment than in those who received MPT/VAD treatment. Bcl-2-negative Bcl-2<sup>-</sup>) and survivin-negative (survivin<sup>-</sup>) patients treated with VD showed a trend towards increased CR + PR; however, CR + PR rate was not different between Bcl-2/survivin<sup>-</sup> patients treated with VD or MPT/VAD (**Table 2**).

#### *Relationship between Bcl-2 and survivin expression and OS*

The median follow-up time for the 45 cases was 14 months (range 1-41 months). At the final analysis, 36 (80%) patients were alive and 9 (20%) patients had died (one patient treated with VD and eight patients treated with MPT/VAD). The estimated median OS was  $40 \pm 18.53$  months (95% confidence interval 3.681-76.319). The median OS in patients treated with VD and MPT/VAD was 16 months (range 1-41 months) and 14 months (range 1-40 months;  $P = 0.091$ ), respectively. After controlling for treatment regimen, median OS was not significantly different between single-positive and single-negative Bcl-2 and survivin expression groups (Bcl-2<sup>-</sup> vs. Bcl-2<sup>+</sup>,  $P = 0.107$ ; survivin<sup>-</sup> vs. survivin<sup>+</sup>,  $P = 0.16$ ); however, the Bcl-2<sup>-</sup>survivin<sup>-</sup> patients showed a trend towards a longer median OS compared with non-Bcl-2/survivin<sup>-</sup> patients ( $P = 0.078$ ) (**Figure 2**).

### **Discussion**

The positive expression rate of Bcl-2 in MM has been reported to be approximately 43%-75% [23, 24]. In the present study, the Bcl-2 positive expression rate was 74%. To date, the use of IHC to investigate survivin expression in NDMM has not been reported. The positive expression rate of survivin in our study was 35%, with over 80% of plasma cells exhibiting moderate or strong immunostaining. Bcl-2 has been shown not to be correlated with various quantitative variables in MM, such as age, hemoglobin, serum creatinine, lactate dehydrogenase, C-reactive protein, M protein level, and  $\beta 2$ -microglobulin [22, 25]. The results of our

study are consistent with these reports. In addition, we found that Bcl-2 expression was not associated with LBD and clinical stage (according to the DS staging system and ISS). The correlation of survivin with clinicopathological variables in MM has not been as widely studied as Bcl-2. Survivin expression measured by western blot has been reported to be related to clinical stage [9]. We are the first to report the correlation of immunohistochemical expression of survivin with clinicopathological variables in MM. Survivin expression was not associated with CRAB or clinical stage in NDMM. Our findings indicate that Bcl-2 and survivin expression measured by IHC are not associated with tumor burden in NDMM.

The proliferation of myeloma cells results from the comprehensive interaction of myeloma cells with the bone marrow milieu. Interleukin-6 (IL-6) released by bone marrow stromal cells interacts with the IL-6 receptor expressed on myeloma cells to increase the expression anti-apoptotic proteins including Bcl-2 and Mcl-1 [26], whereas IL-6 deprivation in IL-6-dependent myeloma cell lines downregulates the expression of Bcl-2, Mcl-1, and Bcl-xl [27]. Survivin is a downstream effector in the Akt signaling pathway and plays an important role in myeloma cell survival. However, high Bcl-2 and survivin expression has been observed in slowly proliferating primary MM cells [9, 28]. Survivin and Bcl-2 expression may reflect tumor status rather than proliferation rate in MM.

Bortezomib can target both myeloma cells and the bone marrow milieu (e.g., angiogenesis) to achieve anti-myeloma efficacy [29, 30]. Bortezomib-induced apoptosis in myeloma cells involves several Bcl-2 family members but not Bcl-2 [31-33]. Bortezomib exposure has been shown to downregulate survivin in several solid and hematologic malignancies [34-36] and could sensitize myeloma cells to anti-myeloma agents including doxorubicin, dexamethasone, and melphalan [9, 37]. However, bortezomib exposure in myeloma cells can inhibit proteasomal degradation of  $\beta$ -catenin and promote its nuclear translocation, thus upregulating survivin expression. This suggests that survivin is not involved in bortezomib-induced apoptosis in MM. Our results demonstrated that bortezomib can achieve a significantly greater therapeutic response compared with MPT/VAD

regardless of Bcl-2 or survivin overexpression in MM. In cases with negative expression of both Bcl-2 and survivin, a trend toward an increased response was observed. Our findings indicate that Bcl-2 and survivin overexpression may contribute to therapeutic resistance to MPT/VAD treatment in MM and bortezomib can overcome this effect.

Recently, Bcl-2 was found to be associated with poor prognosis including shorter OS and progression-free survival in solitary bone plasmacytoma [38]. However, no such association was demonstrated in relapse/refractory MM [31]. In the present study, Bcl-2 and survivin expression were not correlated with OS, suggesting that Bcl-2 and survivin cannot provide prognostic information in NDMM. Interestingly, double-negative Bcl-2 and survivin cases responded similarly to VD and MPT/VAD and tended to have a longer OS. Double-negative expression of Bcl-2 and survivin in MM cells may confer less aggressive biological features, thus making it a favorable prognostic marker in NDMM.

In summary, our results indicate that immunohistochemical expression of Bcl-2 and survivin was not related to tumor burden or prognosis in NDMM. Negative expression of both Bcl-2 and survivin is a favorable prognostic factor. Patients with double-negative survivin and Bcl-2 expression exhibited similar responses to bortezomib-based and non-bortezomib-based regimens and a trend towards a longer OS. However, we recommend bortezomib-containing regimens for NDMM patients with single or double-positive Bcl-2 and survivin expression.

### Disclosure of conflict of interest

None.

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