ORIGINAL ARTICLE

Phytohemagglutinin-induced IL2 mRNA in whole blood can predict bortezomib-induced peripheral neuropathy for multiple myeloma patients

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The proteasome inhibitor bortezomib has revolutionized the treatment of multiple myeloma. However, bortezomib-induced peripheral neuropathy (BiPN) is a serious complication that compromises clinical outcome. If patients with a risk of developing BiPN could be predicted, physicians might prefer weekly, reduced-dose, or subcutaneous approaches. To seek biomarkers for BiPN, we conducted a multicenter prospective study using a simple and unique system. Multiple myeloma patients received twice-weekly or weekly 1.3 mg/m^2 bortezomib intravenously, and a 2-ml sample of whole blood was obtained before treatment and 2–3 days and 1–3 weeks after the first dose. Induction of gene expression was then quantified by real-time PCR. Of a total of 64 enrolled patients, 53 patient samples qualified for mRNA analysis. The BiPN grade was associated with phytohemagglutinin-induced *IL2*, *IFNG* and *TNFSF2*, as well as with lipopolysaccharide-induced *IL6* levels. More importantly, of the 19 patients showing a ⩾3-fold increase in phytohemagglutinin-induced *IL2*, 14 did not suffer from BiPN (73.7% prediction), whereas of the 34 patients with a <3-fold increase, 23 experienced BiPN (67.6% prediction). Therefore, we concluded that pretreatment of phytohemagglutinin-induced *IL2* mRNA levels in whole blood serve as a promising biomarker for predicting BiPN, and this finding warrants validation in a larger study.

INTRODUCTION

The advent of the proteasome inhibitor bortezomib (Velcade; Millennium Pharmaceuticals, Cambridge, MA, USA; formerly PS-341) has yielded improvements in the overall survival of patients with multiple myeloma (MM).[1] Bortezomib-based therapies are currently standards of care in patients with newly diagnosed[2,3] and relapsed/refractory[4–6] MM.

This drug is generally well tolerated, although one of its most frequent adverse events is sensory peripheral neuropathy (PN). Bortezomib-induced peripheral neuropathy (BiPN) has previously been characterized in the setting of single-agent bortezomib ± dexamethasone for relapsed or refractory patients.[7,8] In previously untreated patients, BiPN has been reported in up to 44% of patients, including grade 3 or higher sensory BiPN in up to 13% of cases, in phase III trials of bortezomib-based combinations.[2,3,6,9–12] Similar rates have been observed in the relapsed setting,[6–8,13,14] in which BiPN has been shown to be dose-related and cumulative.[7,8] Thus, BiPN often (17–30%) requires dose modification or discontinuation of bortezomib,[2,15–18] which compromises the treatment outcome and quality of life of MM patients.

MM itself is also implicated in PN, and bortezomib and thalidomide may cause or exacerbate an existing neuropathy.

These PN-inducible drugs have become the mainstay of therapeutic combinations with or without conventional chemotherapy.[2,3,6,19–24] Furthermore, the introduction of bortezomib and thalidomide into induction regimens prior to high-dose therapy followed by autologous stem cell transplantation[9,12,19,20,22,24] leads to an increased risk of PN early in the disease course. These unwanted effects may hamper further treatment with these agents for consolidative or maintenance use and for the treatment of relapsed or progressive disease. Therefore, minimizing PN induced by these drugs with the use of predictive biomarkers will have significant impacts for clinicians in terms of treatment for as well as prevention of BiPN.

Lower doses (1.3 mg/m^2 to 1.0 mg/m^2)[7,16,20,24] or a weekly dose schedule[21,23,25,26] of bortezomib instead of the standard twice-weekly regimen is used to lower the incidence of PN. However, these alternative regimens reduce the efficacy of treatment.[20,21,24,25] In particular, weekly administration fails to overcome the negative impact on prognosis for a high-risk cytogenetic abnormality of t(4;14),[21,25] although this can be overcome with the original twice-weekly schedule.[22,27,28]

Little is known about the mechanism leading to BiPN, and it is likely that several factors contribute to BiPN, including

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Presented in part at the 54th Annual Meeting of the American Society of Hematology, Atlanta, GA, USA, 8–11 December 2012.
Received 13 March 2013; revised 7 August 2013; accepted 30 August 2013

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dysregulation of mitochondrial calcium homeostasis,[29] increased levels of polymerized and stabilized tubulin,[30] autoimmune factors and inflammation,[31,32] and intracellular misfolded proteins.[33] Furthermore, knowledge of predisposing factors may allow for the identification of patients at risk of BiPN, including age,[34] baseline PN grade,[7,10,17,35,36] and diabetes mellitus;[17] however, conflicting results have also been reported.[7,8,10,17,34,36] Only one study using pretreatment plasma cells obtained from the bone marrow of MM patients has characterized the potential role of protein-related factors in BiPN.[18] In addition, a small number of other reports investigating single nucleotide polymorphisms have strongly suggested a role for the patient's genetic background in the development of BiPN.[11,37]

Because PN limits patients' treatment options, the prediction of BiPN is highly anticipated clinically to achieve personalized treatment with bortezomib. We therefore analyzed gene expression profiles in peripheral blood (PB). Unlike conventional gene expression profiling, where mRNA expression is characterized in isolated mononuclear cells, we quantified mRNA expression after stimulation of PB with appropriate agents ex vivo. This unique assay was designed to characterize diverse leukocyte functions in clinical samples.[38,39]

PATIENTS AND METHODS

Patients

Eligible patients with symptomatic MM, who were previously treated or untreated, received twice-weekly or weekly bortezomib doses of 1.3 mg/m^2 intravenously on days 1, 4, 8 and 11, with or without 20 mg of dexamethasone mainly on days 1, 2, 4, 5, 8, 9, 11 and 12. Otherwise, these patients received 1.3 mg/m^2 of bortezomib on days 1, 8, 15 and 22 with or without dexamethasone. To be included in the study, all patients had to have measurable M-protein levels. This study was approved by the institutional review board or the independent ethics committees at all participating institutions and was conducted according to the principles of the Declaration of Helsinki and the International Conference on Harmonisation guidelines on Good Clinical Practice. We also obtained written informed consent from the patients for sample procurement. Severity of PN was graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 4.0. Dose modification in cases of BiPN was managed as previously described.[7]

Ex vivo blood treatment

In eight-well strips, 1.2 µl each of 50 × concentrations of phytohemagglutinin (PHA) (2 mg/ml), heat-aggregated immunoglobulin G (HAG) (10 mg/ml), lipopolysaccharide (LPS) (0.5 mg/ml), zymosan A (ZA) (75 mg/ml) and solvent phosphate-buffered saline were dispensed (three wells were blank) and then delivered to each institution in a dry ice package. These strips were kept frozen at − 80 °C. Two milliliters of heparinized whole blood was obtained from each patient before (Day 0), 2–3 days (Days 2–3) and 1–3 weeks (Weeks 1–3) after the first dose of intravenous bortezomib. Blood was immediately delivered to the designated laboratory, and 0.06 ml of whole PB was added into each well of three strips (triplicate). Three wells were removed and immediately stored at − 80 °C. The remaining five wells, containing PHA, heat-aggregated immunoglobulin G, LPS, ZA and phosphate-buffered saline, were incubated for 4 h at 37 °C. The total blood volume required was as low as 1.44 ml (0.06 ml/well × 8 wells/strip × 3 strips). After incubation, samples were stored at − 80 °C.

mRNA measurement

The levels of the target mRNAs (interleukin (IL)2, interferon (IFN)G, granulocyte-macrophage colony-stimulating factor, tumor necrosis factor ligand superfamily member 2 (TNFSF2), CCL4, IL6, IL10, CXCL10, CTL-associated antigen 4 (CTLA4) and control action (ACT)B) in each aliquot were quantified using a previously described method.[38,39] The melting curve was always analyzed to confirm that PCR signals were derived from a single PCR product. The cycle threshold (C_t) was determined using analytical software (SDS, ABI, Foster City, CA, USA). The C_t values of drug-treated triplicate samples were subtracted individually from the mean C_t values of control samples to calculate ΔC_t, and the fold increase was then calculated as $2^{\wedge}(-\Delta C_t)$, as described previously.[39] The collection of clinical data (TW and MC) and the analysis of mRNA (MM) expression were performed separately at different centers.

Control experiments

Whole PB samples were obtained from three healthy volunteers at APEX Research (Tustin, CA, USA) and used as controls for the ex vivo study. In brief, heparinized PB was pretreated with 0.1, 1, 10, 100, or 1000 ng/ml of bortezomib overnight at 37 °C prior to 4-h stimulation with PHA, heat-aggregated immunoglobulin G, LPS, ZA and phosphate-buffered saline, respectively. The same mRNAs used for MM patients were also quantified. The study was approved by the institutional review board, and written informed consent was obtained from each participant. The purpose of control samples was to screen biomarkers and to design assay condition, not to compare the data between control and MM patients. Thus, the number of control samples is small (n = 3) without being age- and gender-matched.

Statistical analysis

Parametric (Student's t-test) and non-parametric (unpaired Mann–Whitney U-test and Pearson's χ^2 test, paired Wilcoxon test) were used for continuous variables and categorical variables, respectively, to compare mRNA levels between the two groups. P-values < 0.05 were considered significant. The statistical analyses were performed using Excel (Microsoft) and Prism 6 (GraphPad Software, La Jolla, CA, USA).

Table 1. Patient demographics and baseline characteristics	
Characteristic	*No. of patients (N = 64)*
Age (years)	
Median	62
Range	(37–79)
Gender	
Male	33 (52%)
Prior therapy	
Yes	42 (66%)
No	22 (34%)
Follow-up[a] (days)	
Median	162
Range	31–666
Bortezomib administration	
Twice-weekly	52 (81%)
Weekly	12 (19%)
Concurrent dexamethasone	
Yes	62 (97%)
No	2 (3%)
Best response to treatment[b]	
CR	5 (8%)
VGPR	5 (8%)
PR	25 (39%)
SD	25 (39%)
PD	2 (3%)
NE	2 (3%)[c]

Abbreviations: CR, complete response; NE, not evaluable; PD, progressive disease; PR, partial response; SD, stable disease; VGPR, very good partial response. [a]Three patients (one patient each for loss of consciousness, early death due to progressive disease, or additional treatment) not assessable for toxicity were excluded. [b]According to the International Uniform Response Criteria.[47] [c]One patient received additional chemotherapy, and the other patient died of progressive disease early.

RESULTS

Patient characteristics

Between March 2010 and March 2012, a total of 64 patients (33 male and 31 female patients) were enrolled from six centers. The median age of the patients was 62 years (Table 1). The original protocol stipulated that only relapsed or refractory patients were eligible, but in October 2011, the protocol was amended to include newly diagnosed patients owing to the changes in the Japanese National Health Insurance policy. Consequently, 42 patients were previously treated and 22 patients were untreated (Table 1).

Assessment of PN before and after bortezomib treatment

After excluding one patient each for loss of consciousness, early death due to progressive disease, or additional treatment with chemotherapy, 61 patients were assessable for toxicity analysis with a minimum follow-up of 1 month (Table 2). The median number of bortezomib doses before the onset of PN was 4.5 (range, 1–14). The median time to onset of BiPN was 30.5 days (range, 3–83). Unexpectedly, only two (3.2%) and one (1.6%) patients reported grade 1 and 2 PN at baseline, respectively (Table 2).

Of the 58 (95%) patients without PN before enrollment, 18, 9 and 4 patients developed grade 1, grade 2 and grade 3 BiPN after treatment, respectively (Table 2). In addition, 1, 8 and 3 patients who experienced grade 1, 2 and 3 BiPN with no baseline neuropathy, respectively, experienced pain (Table 2). After treatment with bortezomib, the symptoms of both of the two patients with grade 1 PN prior to treatment worsened to grade 2. No change was observed in the one patient with grade 2 PN at baseline (Table 2). None of the patients with grade 1 or 2 PN at baseline or grade 2 PN after treatment complained of pain. The median follow-up time of assessable patients was 162 (range, 31–666) days.

Blood sample preparation

A total of 2444 (64 patients $\times$ 5 stimulants $\times$ 3 time points $\times$ 3 (triplicate) with some sampling failures subtracted) preparations for both mRNA and cDNA synthesis were carried out. The number of PCRs performed was 19552 (2444 cDNA $\times$ 8 primers). In total, the perfect sets for 53 patient blood samples were tolerable for qualification for mRNA analysis.

Table 2. PN before and after treatment with bortezomib

Baseline grade[a]	Grade after treatment	n (%)	No. of patients with painful PN	n (%)
0	0	27 (44%)		58 (95%)
	1	18 (30%)	1	
	2	9 (15%)	8	
	3	4 (7%)	3	
1	1	0 (0%)		2 (3%)
	2	2 (3%)	0	
	3	0 (0%)		
2	2	1 (2%)	0	1 (2%)
	3	0 (0%)		
3	3	0 (0%)		
Total		61[b]		61[b]

Abbreviation: PN, peripheral neuropathy. One patient died early of disease progression, another lost consciousness and the third received additional chemotherapy. [a]Grade was evaluated according to National Cancer Institute Common Terminology Criteria for Adverse Events version 4.0. [b]Three patients who were not assessable for toxicity were excluded.

Effect of bortezomib on *ex vivo* mRNA induction in healthy volunteers

As shown in Supplementary Table 1, *ex vivo* mRNA induction was confirmed using various combinations of stimulants and the corresponding changes in mRNA expression, as previously described.[39] Pretreatment with high concentrations of bortezomib (300 and 1000 ng/ml) inhibited these *ex vivo* mRNA inductions, with the exception of ZA-induced *IFNG*, PHA-induced *CLL4* and PHA-induced *CTLA4* (Supplementary Table 1). Supplementary Figure 1 shows a graphical representation of some of the key results corresponding to subsequent follow-up studies. As shown in Supplementary Figure 1, PHA-induced *IL2* (a), *IFNG* (b) and *TNFSF2* (c), and LPS-induced *IL6* (e) were inhibited by pretreatment with bortezomib at doses greater than 300 ng/ml in a dose-dependent manner, whereas expression of the control housekeeping gene *ACTB* was unchanged by PHA (d) or LPS (f) stimulation.

Association between the incidence of BiPN and *ex vivo* mRNA induction at Day 0

The data set consisting of four stimulants $\times$ eight different mRNAs obtained prior to bortezomib treatment showed that BiPN grade was significantly associated with PHA-induced *IL2*, *IFNG* and *TNFSF2*, and LPS-induced *IL6* levels (Supplementary Table 1). Graphical representations of these positive results are shown in Figures 1a–d. The fold increase in PHA-induced *IL2* in the patients who never experienced BiPN was significantly higher than those who developed grade 1 ($P = 0.041$ with Student's *t*-test only) or grade 1–3 combined BiPN ($P = 0.007$ with Student's *t*-test and $P = 0.049$ with Mann–Whitney U-test) (Figure 1a). Similarly, PHA-induced *IFNG* (Figure 1b) and *TNFSF2* (Figure 1c) and LPS-induced *IL6* (Figure 1d) levels were significantly higher in patients with PN grade 0 than those with grade 1–3 combined BiPN (*P*-values were shown in Figure 1). Because the number of patients with PN grade 3 was very small ($n = 3$), it was difficult to assess these changes statistically; however, the fold increase in PHA-induced *TNFSF2* and LPS-induced *IL6* levels in patients with PN grade 2 were significantly lower than those in patients with PN grade 0 (*P*-values were shown in Figure 1), whereas no difference was found between patients with grade 0 and 1 PN (Figures 1c–d).

Prediction of BiPN by PHA-induced *IL2* mRNA at Day 0

The results of PHA-induced *IL2* (Figure 1a) were also statistically significant by χ^2 test using a threefold increase as the cutoff value (Table 3). More importantly, of the 19 patients with a >3-fold increase in PHA-induced *IL2* at Day 0, 14 did not suffer from BiPN (73.7% prediction for non-BiPN patients); in contrast, of the 34 patients with a <3-fold increase in PHA-induced *IL2* at Day 0, 23 experienced BiPN (67.6% prediction for BiPN patients) with the sensitivity (56%) and the specificity (82%), respectively (Table 3). Although PHA-induced *IFNG* and *TNFSF2* and LPS-induced *IL6* levels at Day 0 were significantly different between the patients with PN grade 0 and 1–3 combined BiPN (Supplementary Table 1 and Figures 1b–d), no clear cutoff value was identified. The mRNA data from blood samples obtained after bortezomib treatment (Days 2–3, Weeks 1–3) did not show any substantial correlation to BiPN (data not shown).

Changes in PHA-induced *IL2* mRNA after bortezomib treatment

In patients without PN even after bortezomib treatment, PHA-induced *IL2* was significantly reduced at Days 2–3 after bortezomib treatment ($P = 0.046$ with paired Student's *t*-test, Figure 2a), and such inhibition was transient and had been neutralized by Weeks 1–3 (Figure 2a). Owing to the large variation, no significant results were obtained in the patients with grade 1 PN (Figure 2b). Interestingly, in the patients with grade 2 or 3 PN,
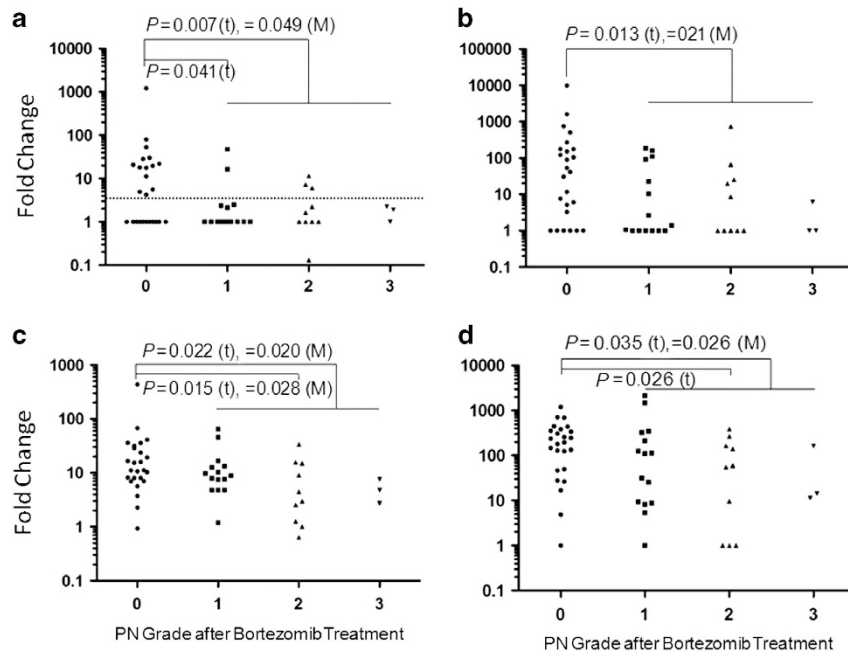
Figure 1. Association between the incidence of BiPN and *ex vivo* mRNA induction at Day 0. The fold change of PHA-induced (**a**) *IL2*, (**b**) *IFNG* and (**c**) *TNFSF2*, and LPS-induced *IL6* (**d**) mRNA in whole blood obtained prior to bortezomib administration was compared with the grade of BiPN. The grade was determined according to the criteria of the National Cancer Institute Common Terminology Criteria for Adverse Events version 4.0. All patients who did not show any PN prior to bortezomib treatment were included in the analysis. *P*-values were calculated using both parametric (Student's *t*-test) (t) and non-parametric (unpaired Mann–Whitney) (M) test.

Table 3. Prediction of BiPN according to pretreatment PHA-induced *IL2* mRNA

Increase in IL2 mRNA	PN grade[a]		Total	Prediction (%)
	0	1+2+3		
⩾3-fold	14	5	19	73.7 (positive predictive value)
<3-fold	11	23	34	67.6 (negative predictive value)
Total	25	28	53	

Abbreviations: BiPN, bortezomib-induced peripheral neuropathy; IL2, interleukin 2; PHA, phytohemaglutinin; PN, peripheral neuropathy. [a]PN after bortezomib treatment was graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 4.0. χ^2 test *P*-value, 0.004. The sensitivity and the specificity of prediction for BiPN by PHA-induced *IL2* mRNA was 56% and 82%, respectively.

bortezomib treatment showed a significant slow and sustained inhibition of PHA-induced *IL2* ($P = 0.044$ with paired Student's *t*-test, $P = 0.027$ with paired Wilcoxon) at Weeks 1–3 with much weaker inhibition at Days 2–3 ($P = 0.020$ with paired Wilcoxon) (Figure 2c).

DISCUSSION

The results from this study demonstrated several new findings. First and most importantly, we found that the amplitude of the PHA-induced increase in *IL2* mRNA expression at Day 0 was capable of distinguishing patients who experience BiPN from those who do not with cutoff values of threefold (Table 3). As this assay was done at Day 0, before administering bortezomib, the effect of bortezomib was excluded. Thus, poor responses of PHA-induced *IL2* mRNA found in BiPN may suggest a couple of possibilities, such as the decrease in the number of PHA-

responsive cells, decrease in PHA signaling cascades, presence of inhibition against PHA signaling, or increase in the baseline *IL2* mRNA levels. Because the original plan of this study was to evaluate gene expression changes before and after bortezomib treatment, the discovery of predictable biomarker was a big surprise. Thus, we were not prepared for the characterization of *IL2* mRNA responses. Such detailed analysis will be conducted in subsequent studies.

Second, the results of PHA-, LPS- and ZA-induced mRNA expression were carried out in PB, although the primary action of bortezomib is against myeloma cells in the bone marrow. Because myeloma cells are difficult to obtain as a routine diagnostic test, PB cells are used as a practical surrogate marker for the prediction of BiPN.

Third, the blood manipulation and laboratory procedure were robust, high throughput and easily applicable to a clinical setting. The blood volume needed for the simple and unique assay system used in this study was as small as 1.44 ml, and there was no need for mononuclear cell isolation. The 96-well format facilitated a smooth transition from mRNA purification to cDNA synthesis to PCR, which allowed us to analyze many samples simultaneously and quantitatively.

Fourth, our results supported the hypothesis that BiPN may be linked to the alteration of inflammatory processes.[11,31,32] This was not only supported by the observed PHA-induced *IL2* mRNA expression (Figure 1, Table 3 and Supplementary Table 1) but also the PHA-induced *IFNG* and *TNFSF2* and LPS-induced *IL6* mRNA levels (Supplementary Table 1). Moreover, patients who developed grade 2–3 BiPN demonstrated sustained inhibition of PHA-induced *IL2* mRNA even after 1–3 weeks of bortezomib treatment, whereas patients who did not experience BiPN only showed transient inhibition at Days 2–3 (Figure 2). Moreover, *ex vivo* bortezomib treatment in healthy adult blood samples significantly inhibited PHA-, LPS- and ZA-induced mRNA induction (Supplementary Table 1 and Supplementary Figure 1). All of these mRNAs are known to encode inflammatory cytokines, and the precise mechanisms responsible for their effects should be characterized in subsequent studies.
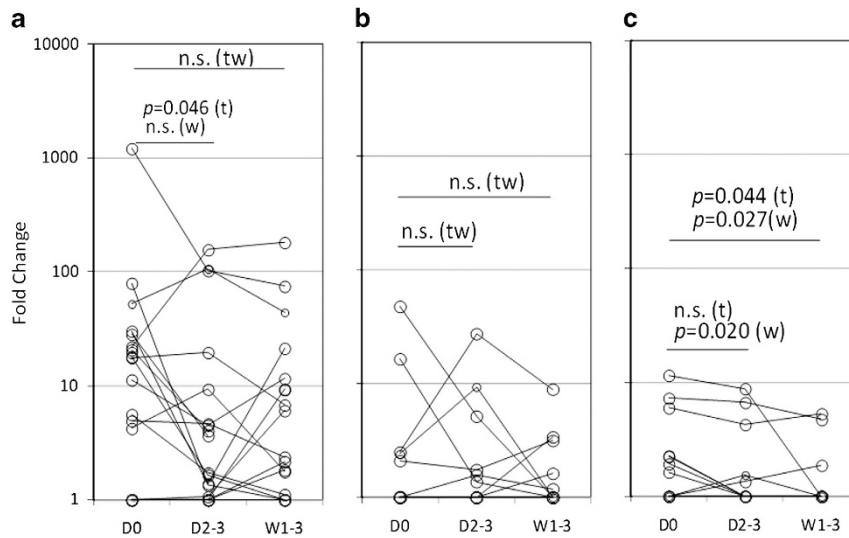
Figure 2. Changes in PHA-induced *IL2* mRNA before and after bortezomib treatment. The fold changes in PHA-induced *IL2* mRNA expression at Day 0 (D0), Days 2–3 (D2–3) and Weeks 1–3 (W1–3) were compared among the patients with BiPN grade 0 (**a**), grade 1 (**b**) and grade 2–3 (**c**). *P*-values were calculated using both parametric (paired Student's *t*-test) (*t*) and non-parametric (paired Wilcoxon (*W*)) tests.

The combination of bortezomib and thalidomide does not result in additive neurologic toxic effects. It is worth noting that in a phase III trial comparing VTD (bortezomib with thalidomide plus dexamethasone) and thalidomide plus dexamethasone as induction therapy before and consolidation therapy after double autologous stem cell transplantation in untreated MM patients,[22] the rate of grade ⩾ 3 PN during VTD induction therapy was similar to that reported by other groups after a similar number of cycles of bortezomib and dexamethasone.[9] In addition, the retrospective analysis assessing bortezomib in combination with thalidomide showed that the incidence of grade 3 to 4 PN was relatively low.[17] These results appear paradoxical because both drugs are known to cause PN. However, it is likely that thalidomide has a protective effect with respect to BiPN through its anti-inflammatory action, specifically through inhibition of TNFα. Furthermore, unexpectedly, BiPN was significantly ameliorated in some patients who received a salvage treatment with lenalidomide,[17] and this improvement may also be attributable to the putative anti-inflammatory actions of lenalidomide as well as dexamethasone. Moreover, the low rates of severe PN observed in most RVD (lenalidomide with bortezomib plus dexamethasone) studies are also likely explained by the anti-inflammatory effects of lenalidomide and the reduced dose of bortezomib,[40,41] although dexamethasone may also contribute to the attenuation of BiPN through its anti-inflammatory effect.

The results of this study are reminiscent of earlier findings in patients with an bortezomib-induced erythematous maculopapular rash, which skin biopsies revealed perivascular leukocytoclatic vasculitis[42] and disappeared following the corticosteroid treatment.[43] In addition, our previous study demonstrated that the bone marrow microenvironment cells likely have a role in the periodic fevers after each bortezomib dose.[44]

If BiPN can be predicted, we may be able to choose an alternative administration route[14] of bortezomib by weighing the risks against the benefits of bortezomib. Alternatively, physicians may select to treat with carfilzomib, which is another proteasome inhibitor with fewer complications of PN, although frequent visits to the clinic are necessary for two consecutive days per week in three consecutive weeks in a 28-day cycle.[45,46]

The use of whole blood in this experiment has pros and cons. The advantage is that the assay is technically applicable to clinical settings. The disadvantage is that we do not know which cells are responding to PHA. When peripheral blood mononuclear cells were isolated and stimulated with PHA, the induced *IL2* mRNA was substantially smaller than that of whole blood (data not shown). If we further isolate subsets of PMBC, more artifacts will be introduced, and required large volume of blood. As cellular experiments are very complex, such experiments should be done as an independent study.

In conclusion, our study found that PHA-induced *IL2* mRNA levels in PB obtained prior to bortezomib treatment may serve as a biomarker for BiPN in MM patients. Although PB samples must be treated with PHA in test tubes *ex vivo* to perform this analysis, this is clinically feasible. Together, our findings indicate that PHA-induced *IL2* mRNA levels may help clinicians identify patients at greater risk of BiPN and personalize treatment regimens for each patient. Owing to the significant clinical importance of these findings, the results warrant validation in a larger patient cohort.

AUTHOR CONTRIBUTIONS

TW and MM designed the study, initiated this work and wrote the manuscript; MK provided administrative support; TW, MS, MR, KS, MA, KO, YN, SN, SI and MK provided study materials and patients; TW and MC collected clinical data; MM obtained mRNA data, performed all statistical analyses and interpreted the data; and SN and MC handled and shipped samples. All authors read, provided comments and approved the final version of the manuscript. All authors had full access to the data in the study and take responsibility for the accuracy of the data analysis.

REFERENCES

1 Kumar SK, Rajkumar SV, Dispenzieri A, Lacy MQ, Hayman SR, Buadi FK et al. Improved survival in multiple myeloma and the impact of novel therapies. Blood 2008; 111: 2516–2520.

2 San Miguel JF, Schlag R, Khuageva NK, Dimopoulos MA, Shpilberg O, Kropff M et al. Bortezomib plus melphalan and prednisone for initial treatment of multiple myeloma. N Engl J Med 2008; 359: 906–917.

3 Mateos MV, Richardson PG, Schlag R, Khuageva NK, Dimopoulos MA, Shpilberg O et al. Bortezomib plus melphalan and prednisone compared with melphalan and prednisone in previously untreated multiple myeloma: updated follow-up and impact of subsequent therapy in the phase III VISTA trial. J Clin Oncol 2010; 28: 2259–2266.

4 Richardson PG, Sonneveld P, Schuster MW, Irwin D, Stadtmauer EA, Facon T et al. Bortezomib or high-dose dexamethasone for relapsed multiple myeloma. N Engl J Med 2005; 352: 2487–2498.

5 Richardson PG, Sonneveld P, Schuster M, Irwin D, Stadtmauer E, Facon T et al. Extended follow-up of a phase 3 trial in relapsed multiple myeloma: final time-to-event results of the APEX trial. Blood 2007; 110: 3557–3560.

6 Orlowski RZ, Nagler A, Sonneveld P, Bladé J, Hajek R, Spencer A et al. Randomized phase III study of pegylated liposomal doxorubicin plus bortezomib compared with bortezomib alone in relapsed or refractory multiple myeloma: combination therapy improves time to progression. J Clin Oncol 2007; 25: 3892–3901.

7 Richardson PG, Briemberg H, Jagannath S, Wen PY, Barlogie B, Berenson J et al. Frequency, characteristics, and reversibility of peripheral neuropathy during treatment of advanced multiple myeloma with bortezomib. J Clin Oncol 2006; 24: 3113–3120.

8 Richardson PG, Sonneveld P, Schuster MW, Stadtmauer EA, Facon T, Harousseau JL et al. Reversibility of symptomatic peripheral neuropathy with bortezomib in the phase III APEX trial in relapsed multiple myeloma: impact of a dose-modification guideline. Br J Haematol 2009; 144: 895–903.

9 Harousseau JL, Attal M, Avet-Loiseau H, Marit G, Caillot D, Mohty M et al. Bortezomib plus dexamethasone is superior to vincristine plus doxorubicin plus dexamethasone as induction treatment prior to autologous stem-cell transplantation in newly diagnosed multiple myeloma: results of the IFM 2005-01 phase III trial. J Clin Oncol 2010; 28: 4621–4629.

10 Dimopoulos MA, Mateos MV, Richardson PG, Schlag R, Khuageva NK, Shpilberg O et al. Risk factors for, and reversibility of, peripheral neuropathy associated with bortezomib-melphalan-prednisone in newly diagnosed patients with multiple myeloma: subanalysis of the phase 3 VISTA study. Eur J Haematol 2011; 86: 23–31.

11 Broyl A, Corthals SL, Jongen JL, van der Holt B, Kuiper R, de Knegt Y et al. Mechanisms of peripheral neuropathy associated with bortezomib and vincristine in patients with newly diagnosed multiple myeloma: a prospective analysis of data from the HOVON-65/GMMG-HD4 trial. Lancet Oncol 2010; 11: 1057–1065.

12 Sonneveld P, Schmidt-Wolf IG, van der Holt B, El Jarari L, Bertsch U, Salwender H et al. Bortezomib induction and maintenance treatment in patients with newly diagnosed multiple myeloma: results of the randomized phase III HOVON-65/GMMG-HD4 trial. J Clin Oncol 2012; 30: 2946–2955.

13 Mikhael JR, Belch AR, Prince HM, Lucio MN, Maiolino A, Corso A et al. High response rate to bortezomib with or without dexamethasone in patients with relapsed or refractory multiple myeloma: results of a global phase 3b expanded access program. Br J Haematol 2009; 144: 169–175.

14 Moreau P, Pylypenko H, Grosicki S, Karamanesht I, Leleu X, Grishunina M et al. Subcutaneous versus intravenous administration of bortezomib in patients with relapsed multiple myeloma: a randomised, phase 3, non-inferiority study. Lancet Oncol 2011; 12: 431–440.

15 Richardson PG, Barlogie B, Berenson J, Singhal S, Jagannath S, Irwin D et al. A phase 2 study of bortezomib in relapsed, refractory myeloma. N Engl J Med 2003; 348: 2609–2617.

16 Jagannath S, Barlogie B, Berenson J, Siegel D, Irwin D, Richardson PG et al. A phase 2 study of two doses of bortezomib in relapsed or refractory myeloma. Br J Haematol 2004; 127: 165–172.

17 Badros A, Goloubeva O, Dalal JS, Can I, Thompson J, Rapoport AP et al. Neurotoxicity of bortezomib therapy in multiple myeloma: a single-center experience and review of the literature. Cancer 2007; 110: 1042–1049.

18 Richardson PG, Xie W, Mitsiades C, Chanan-Khan AA, Lonial S, Hassoun H et al. Single-agent bortezomib in previously untreated multiple myeloma: efficacy, characterization of peripheral neuropathy, and molecular correlations with response and neuropathy. J Clin Oncol 2009; 27: 3518–3525.

19 Rajkumar SV, Rosiñol L, Hussein M, Catalano J, Jedrzejczak W, Lucy L et al. Multicenter, randomized, double-blind, placebo-controlled study of thalidomide plus dexamethasone compared with dexamethasone as initial therapy for newly diagnosed multiple myeloma. J Clin Oncol 2008; 26: 2171–2177.

20 Popat R, Oakervee HE, Hallam S, Curry N, Odeh L, Foot N et al. Bortezomib, doxorubicin and dexamethasone (PAD) front-line treatment of multiple myeloma: updated results after long-term follow-up. Br J Haematol 2008; 141: 512–516.

21 Mateos MV, Oriol A, Martínez-López J, Gutiérrez N, Teruel AI, de Paz R et al. Bortezomib, melphalan, and prednisone versus bortezomib, thalidomide, and prednisone as induction therapy followed by maintenance treatment with bortezomib and thalidomide versus bortezomib and prednisone in elderly patients with untreated multiple myeloma: a randomised trial. Lancet Oncol 2010; 11: 934–941.

22 Cavo M, Tacchetti P, Patriarca F, Petrucci MT, Pantani L, Galli M et al. Bortezomib with thalidomide plus dexamethasone compared with thalidomide plus dexamethasone as induction therapy before, and consolidation therapy after, double autologous stem-cell transplantation in newly diagnosed multiple myeloma: a randomised phase 3 study. Lancet 2010; 376: 2075–2085.

23 Palumbo A, Bringhen S, Rossi D, Cavalli M, Larocca A, Ria R et al. Bortezomib-melphalan-prednisone-thalidomide followed by maintenance with bortezomib-thalidomide compared with bortezomib-melphalan-prednisone for initial treatment of multiple myeloma: a randomized controlled trial. J Clin Oncol 2010; 28: 5101–5109.

24 Moreau P, Avet-Loiseau H, Facon T, Attal M, Tiab M, Hulin C et al. Bortezomib plus dexamethasone versus reduced-dose bortezomib, thalidomide plus dexamethasone as induction treatment prior to autologous stem cell transplantation in newly diagnosed multiple myeloma. Blood 2011; 118: 5752–5758.

25 Mateos MV, Gutiérrez NC, Martín-Ramos ML, Paiva B, Montalbán MA, Oriol A et al. Outcome according to cytogenetic abnormalities and DNA ploidy in myeloma patients receiving short induction with weekly bortezomib followed by maintenance. Blood 2011; 118: 4547–4553.

26 Bringhen S, Larocca A, Rossi D, Cavalli M, Genuardi M, Ria R et al. Efficacy and safety of once weekly bortezomib in multiple myeloma patients. Blood 2010; 116: 4745–4753.

27 Avet-Loiseau H, Leleu X, Roussel M, Moreau P, Guerin-Charbonnel C, Caillot D et al. Bortezomib plus dexamethasone induction improves outcome of patients with t(4;14) myeloma but not outcome of patients with del(17p). J Clin Oncol 2010; 28: 4630–4634.

28 Cavo M, Pantani L, Petrucci MT, Patriarca F, Zamagni E, Donnarumma D et al. Bortezomib-thalidomide-dexamethasone is superior to thalidomide-dexamethasone as consolidation therapy after autologous hematopoietic stem cell transplantation in patients with newly diagnosed multiple myeloma. Blood 2012; 120: 9–19.

29 Landowski TH, Megli CJ, Nullmeyer KD, Lynch RM, Dorr RT. Mitochondrial-mediated disregulation of $Ca2+$ is a critical determinant of Velcade (PS-341/bortezomib) cytotoxicity in myeloma cell lines. Cancer Res 2005; 65: 3828–3836.

30 Poruchynsky MS, Sackett DL, Robey RW, Ward Y, Annunziata C, Fojo T. Proteasome inhibitors increase tubulin polymerization and stabilization in tissue culture cells: a possible mechanism contributing to peripheral neuropathy and cellular toxicity following proteasome inhibition. Cell Cycle 2008; 7: 940–949.

31 Ravaglia S, Corso A, Piccolo G, Lozza A, Alfonsi E, Mangiacavalli S et al. Immune-mediated neuropathies in myeloma patients treated with bortezomib. Clin Neurophysiol 2008; 119: 2507–2512.

32 Schmitt S, Goldschmidt H, Storch-Hagenlocher B, Pham M, Fingerle-Rowson G, Ho AD et al. Inflammatory autoimmune neuropathy, presumably induced by bortezomib, in a patient suffering from multiple myeloma. Int J Hematol 2011; 93: 791–794.

33 Watanabe T, Nagase K, Chosa M, Tobinai K. Schwann cell autophagy induced by SAHA, 17-AAG, or clonazepam can reduce bortezomib-induced peripheral neuropathy. Br J Cancer 2010; 103: 1580–1587.

34 Corso A, Mangiacavalli S, Varettoni M, Pascutto C, Zappasodi P, Lazzarino M. Bortezomib-induced peripheral neuropathy in multiple myeloma: a comparison between previously treated and untreated patients. Leuk Res 2010; 34: 471–474.

35 Lanzani F, Mattavelli L, Frigeni B, Rossini F, Cammarota S, Petrò D et al. Role of a pre-existing neuropathy on the course of bortezomib-induced peripheral neurotoxicity. J Peripher Nerv Syst 2008; 13: 267–274.

36 El-Cheikh J, Stoppa AM, Bouabdallah R, de Lavallade H, Coso D, de Collela JM et al. Features and risk factors of peripheral neuropathy during treatment with bortezomib for advanced multiple myeloma. Clin Lymphoma Myeloma 2008; 8: 146–152.

37 Corthals SL, Kuiper R, Johnson DC, Sonneveld P, Hajek R, van der Holt B et al. Genetic factors underlying the risk of bortezomib induced peripheral neuropathy in multiple myeloma patients. Haematologica 2011; 96: 1728–1732.

38 Mitsuhashi M, Tomozawa S, Endo K, Shinagawa A. Quantification of mRNA in whole blood by assessing recovery of RNA and efficiency of cDNA synthesis. Clin Chem 2006; 52: 634–642.

39 Mitsuhashi M. Ex vivo simulation of leukocyte function: stimulation of specific subset of leukocytes in whole blood followed by the measurement of function-associated mRNAs. J Immunol Methods 2010; 363: 95–100.

40 Richardson PG, Weller E, Jagannath S, Avigan DE, Alsina M, Schlossman RL et al. Multicenter, phase I, dose-escalation trial of lenalidomide plus bortezomib for

relapsed and relapsed/refractory multiple myeloma. *J Clin Oncol* 2009; **27**: 5713–5719.

41 Richardson PG, Weller E, Lonial S, Jakubowiak AJ, Jagannath S, Raje NS *et al.* Lenalidomide, bortezomib, and dexamethasone combination therapy in patients with newly diagnosed multiple myeloma. *Blood* 2010; **116**: 679–686.

42 Gerecitano J, Goy A, Wright J, MacGregor-Cortelli B, Neylon E, Gonen M *et al.* Drug-induced cutaneous vasculitis in patients with non-Hodgkin lymphoma treated with the novel proteasome inhibitor bortezomib: a possible surrogate marker of response? *Br J Haematol* 2006; **134**: 391–398.

43 Agterof MJ, Biesma DH. Images in clinical medicine. Bortezomib-induced skin lesions. *N Engl J Med* 2005; **352**: 2534.

44 Maruyama D, Watanabe T, Heike Y, Nagase K, Takahashi N, Yamasaki S *et al.* Stromal cells in bone marrow play important roles in pro-inflammatory cytokine secretion causing fever following bortezomib administration in patients with multiple myeloma. *Int J Hematol* 2008; **88**: 396–402.

45 Vij R, Siegel DS, Jagannath S, Jakubowiak AJ, Stewart AK, McDonagh K *et al.* An open-label, single-arm, phase 2 study of single-agent carfilzomib in patients with relapsed and/or refractory multiple myeloma who have been previously treated with bortezomib. *Br J Haematol* 2012; **158**: 739–748.

46 Siegel DS, Martin T, Wang M, Vij R, Jakubowiak AJ, Lonial S *et al.* A phase 2 study of single-agent carfilzomib (PX-171-003-A1) in patients with relapsed and refractory multiple myeloma. *Blood* 2012; **120**: 2817–2825.

47 Durie BG, Harousseau JL, Miguel JS, Bladé J, Barlogie B, Anderson K *et al.* International uniform response criteria for multiple myeloma. *Leukemia* 2006; **20**: 1467–1473.

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