Proteasome Subunit Beta Type 1 P11A polymorphism is a new prognostic marker in multiple myeloma

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Running short title: PSMB1 P11A polymorphism in myeloma

Word count: 3579
Abstract word count: 232
Number of black and white figures: 1
Number of color figures: 1
Tables: 3

Disclosure of Conflicts of Interest

Authors declare that there are no competing financial interests in relation to the work described.

MicroAbstract

We retrospectively analyzed the prognostic impact of Proteasome subunit beta type 1 rs12717 polymorphism in 211 consecutively diagnosed multiple myeloma cases. Patients carrying the variant G allele showed significantly shorter progression free survival. In proteasomes of individuals with G/G genotype we found significantly reduced protease activity and lower inhibitory capacity of bortezomib on the caspase- and trypsin-like activity.

Abstract

Background: Proteasome subunit beta type 1 (*PSMB1*) rs12717 polymorphism, an SNP with unknown functional effect, was recently reported to influence response to bortezomib based therapy in follicular lymphoma.
Patients and Methods: We retrospectively analyzed the prognostic impact of this polymorphism in 211 consecutively diagnosed multiple myeloma cases, and performed *in vitro* experiments to look into its functional consequences.
Results: On univariate analysis patients carrying the variant G allele showed significantly shorter progression free survival (PFS) with a pattern suggestive of a gene dose effect (PFS 26.4, 22.3, and 16.4 months in C/C, C/G, and G/G patients, respectively, p=0.002). On multivariate analysis, carrying the G/G genotype was a significant independent risk factor for relapse (HR 2.29, p<0.001) with a similar trend in C/G carriers (HR 1.33, p=0.097) when compared to the major allele carrier C/C cohort. Our subsequent *in vitro* analyses demonstrated significantly reduced protease activity in proteasomes of individuals with G/G genotype compared to that of C/C carriers, despite the fact that the PSMB1 expression and the proteasome assembly remained unaltered. Bortezomib exhibited a lower inhibitory capacity on the caspase- and trypsin-like activity of proteasomes from G/G individuals.
Conclusion: Our results show that carriership of *PSMB1* rs12717 minor allele is predictive for suboptimal response with bortezomib treatment, which could be explained by less active proteasomes which are less sensitive to bortezomib, and myeloma cells consequently relying on other escape mechanism to cope with the abundance of misfolded proteins.

Keywords: multiple myeloma; bortezomib; SNP; PSMB1; proteasoma

Introduction

Proteasome inhibitors (PIs) have a fundamental role in the treatment of multiple myeloma (MM). Bortezomib, the first in class PI received accelerated approval from the U.S. Food and Drug Administration (FDA) in relapsed refractory MM in 2003, and entered into phase 3 trials in first line setting.[1-3] Bortezomib based combinations are currently standard upfront treatments for MM in most countries, and second generation PIs are increasingly used following the FDA and subsequent European Medicines Agency (EMA) approval of carfilzomib and ixazomib.

Proteasomes are multienzyme complexes providing pathway for the degradation of poly-ubiquitinated intracellular proteins thereby eliminating misfolded or unfolded proteins, as well as key regulators of important cellular processes including cell-cycle progression, DNA repair, apoptosis, immune response, signal transduction, transcription, metabolism, and developmental programs.[4,5] The 26S proteasome, a large 2.4-MDa ATP-dependent proteolytic complex located in both the cytoplasm and the nucleus, consists of a 20S core catalytic cylindrical complex capped at both ends by 19S regulatory subunits.[6] The core structure is composed of 4 axially stacked rings of non-identical subunits: 2 alpha rings each composed of 7 non-identical alpha subunits (α1–7, encoded by *PSMA1–7*) and 2 beta rings each formed by 7 non-identical beta subunits (β1–7, encoded by *PSMB1–7*).[7] Three β subunits (β5, β2, and β1) are located on the inner surface of the proteolytic chamber and are responsible for chymotrypsin-like, trypsin-like, and post-glutamyl peptide hydrolyzing (caspase-like) activities, respectively.[8] Other β subunits are essential for the formation and stability of the proteolytic environment on the inner surface of the heptameric rings. The exact role of these subunits however, is yet to be characterized. It has also been reported that *PSMB1* (coding for β6 subunit) has a potential role as transcriptional activator.[9,10]

High proteasome activity has been reported in different malignancies.[11] In malignant plasma cells, that exhibit extremely active protein synthesis, proteasomes along with chaperones, such as heat shock proteins, play critical role in cellular homeostasis. This might explain the therapeutic window of PI treatment in MM and the additional effect of heat shock protein inhibitors combined with PIs.[12] Bortezomib binds to the β5 subunit, but also interacts with other subunits of proteasome, such as the β1 subunit.[13] Some of the new generation PIs, such as marizomib, inhibits all three proteolytic activities. Although *PSMB5* variants have been identified previously in preclinical models of bortezomib resistance, they were not detected in relapsed/refractory patients' tumor samples, suggesting alternative mechanisms behind bortezomib resistance.[14]

Proteasome subunit beta type 1 (*PSMB1*) rs12717 single nucleotide polymorphism (c.31C>G substitution resulting in p.Pro11Ala amino acid change) was recently reported to be associated with greater clinical benefit in relapsed follicular lymphoma patients treated with bortezomib-containing combination.[15] Our hypothesis was that *PSMB1* P11A polymorphism could affect the function of proteasomes and therefore have an impact on the prognosis of myeloma, especially in patients treated with PI based regiments. To the best of our knowledge the functional consequence of *PSMB1* P11A polymorphism has not been tested in MM, apart from one single study with limited number of patients showing no statistically significant association between genotype and survival.[14]

Patients and Methods

Patients, clinical data, treatment, and response criteria

We analyzed the association of *PSMB1* P11A polymorphism and treatment outcome of 211 consecutively diagnosed myeloma patients having had first line chemotherapy at the St. Laszlo Hospital, Budapest, Hungary between January 2007 and November 2013. ISS and FISH status were established at diagnosis, FISH testing was performed on bone marrow slides using probes for chromosome 13q and 17p deletion, translocation (11;14), (4;14), (14;16), and 1q amplification. FISH results were available in 194 out of the 211 patients. For the purpose of this study, patients with t(4;14), t(14;16), 1q amplification, and del(17p) were grouped together as high risk. Previous studies have shown that del(13q) in patients lacking t(4;14) and del(17p) was no longer of prognostic significance.[16,17]

The treatment decision was the discretion of the treating physician; treatment continued until best response and then the transplant eligible patients received a high dose cyclophosphamide-primed stem cell mobilization, followed by high-dose melphalan conditioned ASCT. Response [complete response (CR), very good partial response (VGPR), partial response (PR) no response (NR) and progressive disease (PD)], and survival measures [progression free survival (PFS) and overall survival (OS)] and relapse criteria were defined according to published guidelines.[18] Response was formally assessed at the end of the treatment, or following transplant in case of ASCT patients. During their follow up, patients were reviewed regularly every 2-3 months until disease progression or death. The median follow up was 40 months.

The study was approved by the Hungarian National Ethics Committee, and participants signed informed consents.

Genotyping

Genomic DNA was isolated from bone marrow or peripheral blood. *PSMB1* P11A polymorphism was tested using LightCycler 480II (Roche Diagnostics, Basel, Switzerland) melting curve analysis. Amplification primers (PSMB1-LCF: 5'- GTG AGA CAG CAA GTG TCG -3' and PSMB1-LCR: 5'- GTG ACT CCT AAA TAG GCT TCA G -3') and hybridization probes (PSMB1-SENS: 5'- GGC TCC TGG CAG AGA CTT GG – Fluorescein and PSMB1-ANC: 5' - Cy5- ATG GAA CCG CAC AGA GCC G - Phosphate) were designed by LightCycler Probe Design software (Roche Diagnostics, Basel, Switzerland). Asymmetric polymerase chain reaction with shifted forward (0.15 μM) and reverse primer (0.5 μM) concentrations was performed with the addition of 25 ng genomic DNA, and labelled oligonucleotides (0.25 μM each) with MyTaq^{TM} Mix (Bioline, Bio-25042, Taunton, USA) according to the manufacturers' instructions. Cycling conditions were as follows: initial denaturation at 95^{o}C for 3 min, 70 cycles of 95^{o}C denaturation, 50^{o}C annealing, and 72^{o}C extension, melting curve analysis from 40^{o}C to 80^{o}C.

Flow cytometry analysis of proteasomes and PSMB1 expression

For *in vitro* characterization of proteasome expression and function, healthy volunteers previously genotyped as either *PSMB1* major variant C/C or homozygous G/G minor variant (3 males and four females, mean age: 42±8 years) were investigated. EDTA anticoagulated whole blood samples were fixed with 1% paraformaldehyde (37˚C, 5 min), spun down (500g, 5 min), washed with PBS, and sonicated (10 sec, 37 kHz, 50/100W). The cells were resuspended in PBS and labelled with rabbit polyclonal anti-PSMB1 (1:80, Biomol, Hamburg, Germany) or anti-proteasome 20S alpha + beta (1:100, Abcam, Cambridge, UK) antibodies along with cell type-specific markers, such as phycoerythrin (PE) conjugated anti-CD3 (1:200), PE-conjugated anti-CD14 (1:100), or Peridinin Chlorophyll Protein Complex

(PerCP) conjugated anti-CD19 (1:100) antibodies (40 min, 37°C). After washing, the cells were subjected to Alexa Fluor 488-conjugated goat anti-rabbit IgG (H+L) (1:100, Life Technologies, 30 min, 37°C), washed, and resuspended in PBS containing 2.5 μM DRAQ5 (Biostatus, Shepshed, UK). To avoid optical interference, DRAQ5 staining was omitted, when PerCP-conjugated marker was used. The cells were analyzed by a FACSCanto II flow cytometer at 488 nm and 633 excitations (emission filters: 488/10, 585/42, 670LP, and 660/20 nm). Sequential gating was applied, where nucleated cells were first selected on the basis of DRAQ5 staining (with the exception of anti-CD19 staining), then forward scatter and side scatter parameters were used for further gating. T-cells, B-cells, and monocytes were identified on the basis of CD3, CD19, and CD14 labeling, respectively, whereas granulocytes were identified solely on the basis of scatter parameters. Relative anti-PSMB1 and anti-proteasome 20S labeling was expressed by the median fluorescence intensities divided by the median values obtained with the secondary antibodies only (autofluorescence control).

Assessment of proteasome functions

For studying proteasome function, peripheral blood mononuclear cells (PBMC) were isolated by density gradient separation (1200g, 20 min, Leucoprep Lymphocyte Separation Media, Intron Biotechnology, South-Korea), washed 3 times with saline solution, and lysed with a buffer containing 40mM Tris, 50mM NaCl, 5mM $MgCl_2$, 2mM β-mercaptoethanol, 10% glycerol. After sonication and removal of cell debris, the total protein concentration was assessed by Bradford assay, and adjusted to 2.5 mg/ml. The chymotrypsin-like, caspase-like, and trypsin-like activities of the lysates were determined by Proteasome Activity Fluorometric Assay Kit II (UBPBio) according to the manufacturers' instructions. Briefly, 120 μl assay buffer containing the fluorogenic peptide substrate was added to 30 μl of the lysates. For studying bortezomib inhibition, the samples were preincubated with the drug (room temperature, 30 min) prior to the start of the reaction. The generation of fluorescent product (460 nm) was assessed by a fluorescence plate reader at 360 nm excitation. The specific activities were calculated from the initial slopes of the kinetic curves and the protein content of the samples.

Statistical methods

Comparisons of dichotomous variables were performed by Pearson's chi-square test or Fisher's exact test as appropriate; continuous variables were compared with Kruskal-Wallis or Mann-Whitney tests. Log-rank test was performed to compare PFS and OS. Following univariate analysis, variables with p values <0.05 in the entire cohort were included in the multivariate analysis. Using the stepwise selection procedure for all of the variables of interest (sex, age, ISS, FISH, ASCT, bortezomib treatment, immunoglobulin, LDH) the Cox proportional hazard model was used for multivariate analysis of OS and PFS. The few missing ISS and FISH value were replaced using random imputation. Adjusted hazard ratios (HR), 95% confidence intervals (CI) values, and tests for interaction were computed. For the comparison of PSMB1 expression, proteasome assembly, and function in the different subgroups, Student's t test was used after testing for the normality and homogeneity of variances. $P < 0.05$ was considered as significant difference. The analyses were carried out using the SPSS (version 20.0 SPSS, Chicago, IL, USA) software package.

Results

Study population

Patient characteristics are shown in Table 1. Among the 211 newly diagnosed consecutive MM patients 105 were males and 106 females, the median age was 64 (28–84) year. The majority of them had either IgG (57.3%) or IgA (19.9%) myeloma, 21.8% had light chain and 0.9% non-secretory disease. ISS was calculated in each case at diagnosis, with 25.8, 24.4, and 49.8% of the patients being in ISS groups 1, 2, and 3, respectively. As the clinical behavior of the ISS 1 and 2 groups was similar, these two cohorts were merged. According to our pre-defined FISH risk stratification, 31.4% of the patients were allocated into the high risk and 68.6% into the low risk group.

154 patients [77 males and 77 females; median age: 61.5 (28–84) years] received bortezomib based treatment and 57 [28 males and 29 females; median age: 68 (48–84) years, p for age <0.001] had other, non-bortezomib-containing protocols.

Within the bortezomib-treated group, 83 transplant eligible patients were treated with bortezomib-thalidomide-dexamethasone (VTD)[19], while melphalan-prednisolon-bortezomib (MPV)[20], the standard therapy for older patients, was applied in 45 cases. 20 other patients had bortezomib-doxorubicin-dexamethasone (PAD)[21] and 6 bortezomib-dexamethasone (VD)[2] chemotherapy. Other, non-bortezomib-containing protocols were applied as follows: cyclophosphamide-thalidomide-dexamethasone[22], vincristin-doxorubicin-dexamethasone (VAD)[23], lenalidomide-dexamethasone[24], and melphalan-prednisolon (MP) the numbers and genotype distribution in these subcohorts are summarized in table 1.

59.1% of the bortezomib-treated patients had first line ASCT, whereas the ASCT frequency was lower, 35.1% in the non-bortezomib group reflecting the fact that from 2007 in our center VTD became the preferred induction in transplant candidates, while oral protocols remained more feasible for frailer patients. The median age of the first line transplanted and non-first line transplanted patients was 58 (range: 28–70) and 72 (range: 39–84) years, respectively (p<0.001). There was no significant difference in the distribution of disease characteristics, prognostic markers, and applied treatment between the various *PSMB1* genotype groups (Table 1).

PSMB1 allele frequencies

Genotype distribution in our patient cohort was as follows: 84 patients (39.8%) had homozygous C/C, 95 patients (45%) heterozygous C/G and 32 patients (15.2%) G/G genotype, similar to that published previously.[14] The minor allele frequency (37.7±4.7%) in MM patients was not different from the reported allele frequency for Caucasian reference population (40.4±7.0%) in the 1000 Genome Project or (34.8-46.0%) in the National Center for Biotechnology Information (NCBI) dbSNP databases.[25]

PSMB1 genotype and response to treatment

4 patients had no disease reassessment because of early death. Within the whole cohort, 41.2, 19.4, and 30.8% of the patients had CR, VGPR, and PR, respectively, and 6.6% exhibited NR. Slightly more patient entered into CR in the C/C genotype group (48.8%) as compared to the G/C and G/G groups (35.8 and 37.5%, respectively), but this difference was not significant (p=0.189) Apart from this, there were no associations between the response levels and the genotype groups neither in the whole cohort, nor in subgroups according to ISS, FISH or chemotherapy type (data not shown).

There was no difference between the numbers of chemotherapy cycles required to reach the best response in the three genotype subgroups. Approximately half of the patients had

ASCT consolidation, and the proportion of patients having ASCT was not different between the three genotype groups.

PSMB1 genotype and univariate analysis of survival

The median PFS and OS of the whole group were 23.4 (20.7-26.1) and 55.7 (49.6-61.8) months, respectively. The known predictors (age, ASCT, ISS, FISH) all had significant impacts on survival.

Analyzing the three *PSMB1* genotype groups the PFS was 26.4 (22.4-30.4), 22.3 (18.3-26.4), and 16.4 (10.2-22.6) months (p=0.002), and the OS 58.5 (53.5-63.4), 47.7 (34.8-60.6), and 64.4 (42.1-86.7) months (p=0.27) in C/C, C/G and G/G patients, respectively (Figure 1). In Cox proportional hazard modeling, carrying two G alleles increased the chance of relapse by 2.14 (1.39-3.29; p=0.001) with a trend in carriers of one single G allele [HR 1.36 (0.98-1.89), p=0.067], while no statistically significant difference was seen in the OS of the subgroups.

PSMB1 genotype and multivariate analysis of survival

In multivariate analysis, besides age, ISS, FISH, bortezomib treatment and ASCT, G/G genotype was also a significant risk factor for shorter PFS. Homozygous G/G patients had a shorter PFS compared to C/C genotype patients with a hazard ratio of 2.29 (1.46–1.36) (p<0.001) (Table 2), whereas heterozygous C/G patients showed a trend of worse prognosis with a hazard ratio of 1.33 (0.95–1.86) (p=0.097). Regarding OS, a significant difference between the C/C and C/G genotypes was found, but not in the comparison of the C/C and G/G genotypes (Table 2).

Statistical interaction testing did not show significant interactions of PSMB1 genotype and either bortezomib treatment (p=0.092 for G/G vs C/C, and p=0.63 for C/G vs C/C regarding PFS) or ASCT (p=0.420), however, revealed interaction of PSMB1 with FISH risk status regarding PFS (p=0.044) and with ISS regarding OS (p=0.022).

Subgroup analyses

In the PFS and OS of the ISS and FISH stratified subgroups, we found the same pattern as in the whole study population with significant differences between the C/C, C/G and G/G genotype groups both in the ISS 1&2 and the ISS 3 and standard risk FISH subgroups, but not in the FISH high risk group (Table 3).

The *PSMB1* genotype mattered the most in bortezomib-treated patients, where it has a highly significant effect in the bortezomib-treated cohort (Figure 1/B and Table 3).

Assessment of functional consequence of the PSMB1 polymorphism

To investigate the possible molecular mechanism how P11A (c.31C>G) polymorphism in *PSMB1* may influence the proteasome assembly and/or function, first the amount of proteasomes (Figure 2A) and the expression level of PSMB1 (Figure 2B) were determined in peripheral white blood cells obtained from healthy individuals with C/C or G/G genotype. No difference between the two subgroups was found in proteasome levels or PSMB1 expression (Figure 2A-B). Similarly, no difference was observed when these two parameters were assessed specifically in various types of nucleated peripheral blood cell types, i.e., monocytes, T-cells, B-cells, and granulocytes (Figure 2A-B).

Following this, the protease activities of proteasomes of C/C and G/G genotype carrier subgroups were analyzed and found that chymotrypsin-, trypsin- and caspase-like activities were all significantly lower in the G/G subgroup, indicating selective reduction of the proteasome function in individuals carrying the G/G polymorphism (Figure 2C).

We also studied the *in vitro* inhibitory effect of bortezomib on the chymotrypsin-, trypsin- and caspase-like activities of the proteasomes, and found a three-fold difference

between the IC_{50} values of bortezomib inhibition of the caspase-like activity of proteasomes of healthy subjects with G/G and C/C genotypes (2.05 vs 6.07 μM). More profound difference, one order of magnitude shift was seen in the IC_{50} values, when the trypsin-like activities were blocked by bortezomib (44.4 vs. 462 μM) (Figure 2D).

Discussion

Our results confirm that *PSMB1* P11A polymorphism has a significant impact on the survival of myeloma patients. We originally hypothesized that this polymorphism might interact with bortezomib treatment, given its mechanism of action, but we could not demonstrate statistical interaction between bortezomib exposure and *PSMB1* genotype; however, this could be the result of the smaller number of patients in the non-bortezomib treated group.

The literature of *PSMB1* P11A polymorphism is sparse. A study compared the outcome of relapsed follicular lymphoma patients treated with rituximab in combination with or without bortezomib. It was reported that one biomarker pair, i.e., *PSMB1* P11A G allele carriers with low CD68 expression, was associated with a significant PFS benefit, when these patients were treated with the combination of bortezomib and rituximab as compared to those having received rituximab as monotherapy.[15]

Another study analyzed the sequence of β-subunit genes of the 20S proteasome in patients with relapsed multiple myeloma using samples from patients who participated in the phase 3 Assessment of Proteasome Inhibition for Extending Remissions (APEX) study of bortezomib versus high-dose dexamethasone. No statistically significant interaction between the *PSMB1* P11A and time-to-progression or OS was found; however, this particular sub-analysis included only 23 patients and still, there was a marginal association (p=0.077). This report concluded that because of the limited size of their dataset, additional studies are required to further evaluate this SNP in MM.[14]

In Cox modeling of PFS, the risk of relapse increased in the presence of one G allele and then increased further with the second, which finding could suggest the presence of a gene dose effect; however, currently there is no published data on the functional consequence of the *PSMB1* P11A polymorphism. Therefore, we investigated whether this polymorphism influences the assembly, the functions and the inhibitability of proteasomes using peripheral blood cells of healthy individuals. In addition to the fact that the availability of plasma cell of MM patients is rather limited, these cells most likely carry a high number of somatic mutations, which can cause such a statistical noise, that may hamper the analysis of proteasome activities. Alternatively, myeloma cell lines could be used. However, cell line pairs with the same genetic background possessing G/G or C/C genotype for the PSMB1 gene are not available thus far.

The protein encoded by *PSMB1* is the β6 subunit, one of the two times seven elements of the inner proteolytic chamber of the proteasome. This subunit has no known direct proteolytic activity, but has been proposed to contribute to the assembly and stability of the two heptameric beta rings of proteasomes, establishing the proteolytic environment on their inner surface. Our findings, that proteasomes with G/G PSMB1 genotype exhibit lower peptidase activities and lower sensitivity to bortezomib in terms of the caspase-like and trypsin-like activities, are in line with this proposed model, and suggest an indirect interaction with the proteasome function. Our experiments clearly demonstrated that this polymorphism is not neutral; however, further studies are required to elucidate the exact molecular mechanism how the polymorphism in PSMB1 leads to reduced activity and changes in bortezomib-sensitivity of proteasomes. Nevertheless, we can speculate, that to maintain appropriate cellular homeostasis, these cells compensate for the reduced proteasome proteolytic activities with increased activities in the alternative degradative mechanisms, which ultimately may lead to reduced susceptibility to proteasome inhibition as observed in cells homozygous for P11A polymorphism (Figure 2D). This concept may especially apply to cell types with intense protein synthesis and secretion, e.g., plasma cells, where the proteolytic burden is grossly increased. Bortezomib is thought to drive cells to programmed cell death by inhibiting the proteasomal degradation of unfolded proteins thereby

overwhelming the degradation capacity of the cell and triggering the unfolded protein response.[26,27] This may help explain why bortezomib has an apparent potentiating effect when given as co-therapy with 17-AAG, a heat shock protein 90 inhibitor in patients with relapsed or refractory MM.[12]

As regards to another component of the proteasome complex, namely PSMA5 it has been reported that low plasma levels are associated with longer PFS in mantle cell lymphoma treated with bortezomib.[28] In addition, recent data showed that silencing individual proteasome genes including *PSMA5* and *PSMB2/3/7* sensitized multiple myeloma cells to bortezomib.[29,30] However, similar data in connection with *PSMB1* is not reported thus far.

Conclusion

PSMB1 P11A is a novel predictive marker in multiple myeloma, which could help to identify patients with a probability of having a suboptimal response to bortezomib who might benefit from alternative management, which could potentially include the inhibition of heat shock proteins.

Clinical Practice Points

- PSMB1 P11A polymorphism results in decreased protease activity of proteasomes and reduced inhibitory effect of bortezomib on their activity.
- Myeloma patients carrying the variant allele of the PSMB1 P11A polymorphism have a significantly shorter progression free survival.
- Based on this PSMB1 P11A is a new predictive marker which could help to identify patients who could benefit from alternative management.

Acknowledgements

This work was supported by grants from the Hungarian National Research, Development and Innovation Office NKFIH (K104903) to AT and HA, NVKP_16-1-2016-0005 program to GM, and the Momentum Program of the Hungarian Academy of Sciences (LP2012-025) to LH. The authors are grateful to the volunteer blood donors for their compassionate willingness to participate in this study, as well as to Beáta Biri and Bence Kovács for their help with statistical analyses.

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Table 1. Patients' Characteristics

			PSMB1 P11A			
		All	C/C	C/G	G/G	p
n (%)		211	84 (39.8%)	95 (45.0%)	32 (15.2%)	
Sex	Male	105 (49.8%)	42 (50%)	49 (51.6%)	14 (43.8%)	0.745
	Female	106 (50.2%)	42 (50%)	46 (48.4%)	18 (56.2%)	
Age (years)		64 (28–84)	63 (28–84)	64 (32–84)	65.5 (49–84)	0.306
ISS	1&2	105 (50.2)	38 (45.8)	52 (55.3)	15 (46.9)	0.419
	3	104 (49.8)	45 (54.2)	42 (44.7)	17 (53.1)	
FISH	SR	133 (68.6)	50 (64.9)	57 (65.5)	26 (86.7)	*0.067*
	HR	61 (31.4)	27 (35.1)	30 (34.5)	4 (13.3)	
LDH	normal	158 (80.2)	69 (86.2)	65 (74.7)	24 (80.0)	0.174
	high	39 (19.8)	11 (13.8)	22 (25.3)	6 (20.0)	
Bortezomib	No	57 (27.0)	16 (19.0)	28 (29.5)	13 (40.6)	*0.050*
	Yes	154 (73.0)	68 (81.0)	67 (70.5)	19 (59.4)	
Treatment	VTD	83 (39.3)	33 (39.3)	39 (41.1)	11 (34.4)	0.800
	PAD	20 (9.5)	11 (13.1)	6 (6.3)	3 (9.4)	0.303
	MPV	45 (21.3)	21 (25.0)	20 (21.1)	4 (12.5)	0.339
	VD	6 (2.8)	3 (3.6)	2 (2.1)	1 (3.1)	0.836
	Thal	36 (17.1)	10 (11.9)	17 (17.9)	9 (28.1)	0.111
	Len	3 (1.4)	1 (1.2)	2 (2.1)	0 (0.0)	0.667
	VAD	8 (3.8)	2 (2.4)	5 (5.3)	1 (3.1)	0.588
	MP	10 (4.7)	3 (3.6)	4 (4.2)	3 (9.4)	0.399
ASCT	No	100 (47.4)	39 (46.4)	43 (45.3)	18 (56.2)	0.546
	Yes	111 (52.6)	45 (53.6)	52 (54.7)	14 (43.8)	

FISH high risk: t(4;14), t(14;16), 1q amplification and deletion 17p; standard risk: all others.

Abbreviations: ASCT, autologous stem cell transplantation; FISH, fluorescence in situ hybridization; HR, high risk; ISS, International scoring system; MPV, melphalan, prednisolon, bortezomib; PAD, bortezomib, doxorubicin, dexamethasone; SR, standard risk; VAD, vincristin, doxorubicin, dexamethasone; VTD, bortezomib, thalidomide, dexamethasone

Table 2. Multivariate analysis of PFS and OS

	PFS				OS			
	HR	CI95		p	HR	CI95		p
Age	1.005	0.987	1.024	0.554	0.998	0.976	1.021	0.872
ISS	1.360	1.120	1.652	**0.002**	1.904	1.466	2.473	**<0.001**
FISH	1.647	1.178	2.303	**0.004**	1.606	1.076	2.396	**0.020**
Bortezomib	1.013	0.711	1.444	0.942	0.998	0.644	1.546	0.991
ASCT	0.518	0.353	0.761	**0.001**	0.479	0.295	0.776	**0.003**
PSMB				**0.002**				**0.041**
PSMB C/G vs C/C	1.327	0.950	1.855	*0.097*	1.718	1.122	2.629	**0.013**
PSMB G/G vs C/C	2.293	1.458	3.604	**<0.001**	1.570	0.876	2.811	0.129

Abbreviations: ASCT, autologous stem cell transplantation; CI, confidence interval; FISH, fluorescence in situ hybridization; HR, hazard ratio; ISS, International scoring system; OS, overall survival; PFS, progression free survival

Table 3. Subgroup analysis of PFS and OS

			PSMB1 P11A			
			C/C	C/G	G/G	p
PFS	ISS	1&2	38.0 (13.4-62.6)	25.5 (19.2-31.8)	26.8 (17.7-35.9)	**0.04**
		3	24.9 (19.1-30.7)	18.2 (7.8-28.6)	9.4 (4.9-13.8)	**0.005**
	FISH	SR	34.4 (28.0-40.1)	24.2 (20.5-27.9)	16.4 (7.5-25.3)	**<0.001**
		HR	18.2 (10.6-25.8)	18.2 (12.6-23.8)	21.0 (12.1-29.9)	0.356
	Bortezomib	No	22.5 (15.2-29.8)	19.3 (16.7-21.9)	17.3 (6.6-27.9)	0.387
		Yes	26.4 (20.1-32.6)	24.2 (20.4-28.0)	16.4 (6.3-26.5)	**0.006**
	ASCT	No	15.4 (12.7-18.1)	15.3 (6.4-24.2)	12.2 (3.7-20.7)	0.310
		Yes	34.6 (29.1-40.1)	27.4 (22.4-32.4)	21.3 (13.2-29.4)	**0.006**
OS	ISS	1&2	NR	87.1 (47.6-126.6)	NR	0.391
		3	52.4 (39.5-65.3)	29.2 (14.6-43.8)	21.8 (0-51.1)	**0.012**
	FISH	SR	NR	52.3 (39.7-64.9)	64.4 (28.5-100.3)	**0.038**
		HR	43.2 (27.6-58.8)	41.3 (25.1-57.5)	50.0 (0-105.9)	0.513
	Bortezomib	No	58.5 (56.1-60.8)	39.5 (12.7-66.3)	65.1 (19.9-110.3)	0.597
		Yes	NR	47.7 (31.7-63.6)	55.1 (23.5-86.6)	0.306
	ASCT	No	40.2 (25.7-54.6)	25.1 (9.3-40.9)	64.4 (0.0-139.3)	0.541
		Yes	79.2	56.0 (20.2-91.8)	55.1 (23.1-87.1)	0.331

Abbreviations: ASCT, autologous stem cell transplantation; CI, confidence interval; FISH, fluorescence in situ hybridization; HR, high risk; ISS, International scoring system; NR, not reached; OS, overall survival; PFS, progression free survival; SR, standard risk

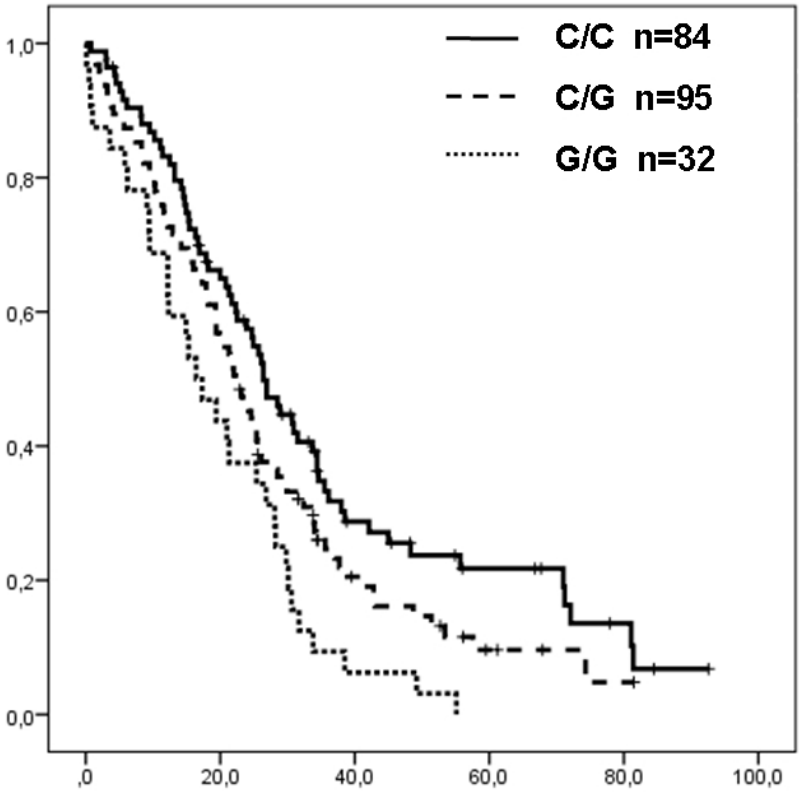
Legends to Figures

Figure 1. Effect of *PSMB1* P11A (c.31G>C) **polymorphism on progression free survival (PFS) of multiple myeloma patients, (A) the whole group, and (B) the bortezomib treated patients**
Patients with G/G genotype have significantly shorter PFS compared to those with homozygous C/C while in case of G/C patients there is a similar trend suggesting a gene-dose effect.

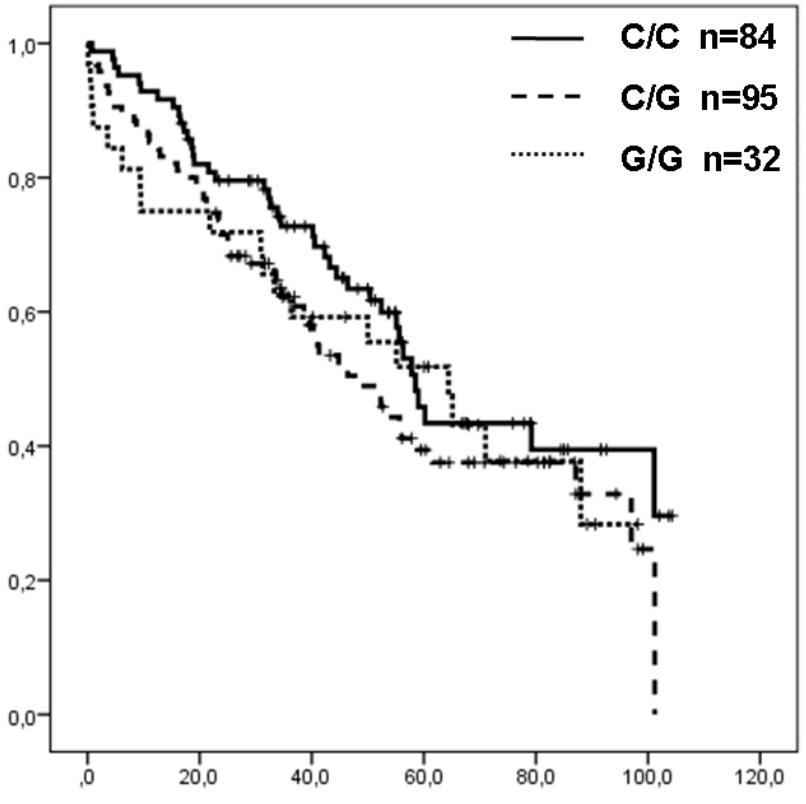
Abbreviations: HR, hazard ratio; CI confidence interval

Figure 2. Effect of *PSMB1* P11A (c.31G>C) **polymorphism on the PSMB1 expression, proteasome assembly and function.** The amount 20S proteasomes (A) and the expression of PSMB1 (B) were compared in various white blood cell types of healthy individuals with C/C or G/G genotype. (C) The different peptidase activities of proteasomes were assessed in peripheral blood cells of subjects homozygous for the studied polymorphism. (D) The relative inhibitory effect of bortezomib on proteasome peptidase activities was determined. Despite that the PSMB1 expression and proteasome assembly did not differ between the two genetic groups, subjects with G/G genotypes exhibited significantly lower proteosomal activities (n=4 each, $p<0.05$). Trypsin-like and caspase-like activities of proteasomes from subjects with G/G genotype were less susceptible to bortezomib.

Abbreviations: granulo, granulocyte; mono, monocyte; WBC, white blood cell



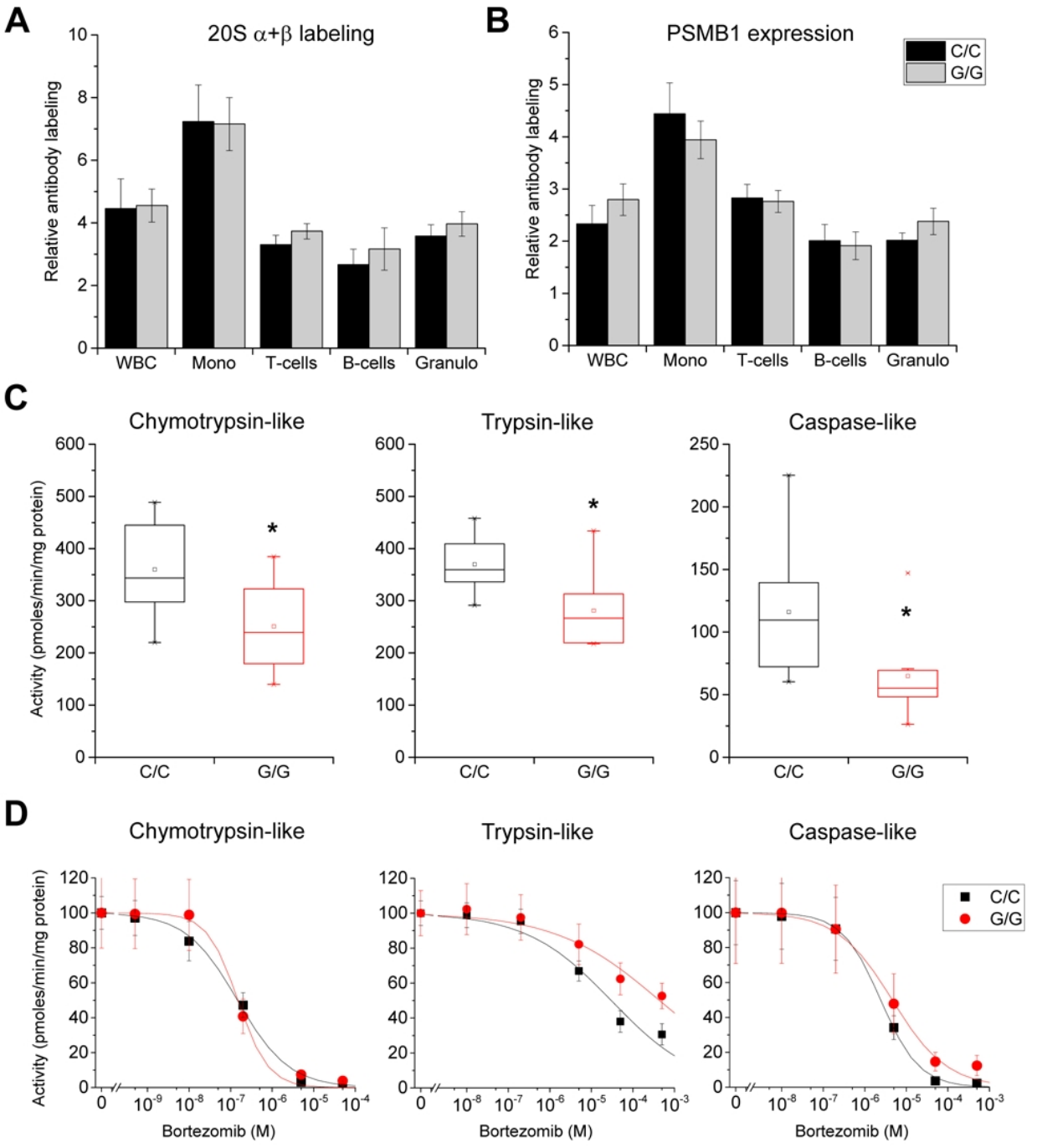
PFS (months)



OS (months)

	PFS (95%CI)	HR (95%CI)	p
C/C	26.4 (22.4–30.4)		
C/G	22.3 (18.3–26.3)	1.360 (0.979–1.890)	*0.067*
G/G	16.4 (10.1–22.6)	2.143 (1.394–3.295)	**0.001**

	OS (95%CI)	HR (95%CI)	p
C/C	58.5 (53.5–63.4)		
C/G	47.7 (34.8–60.6)	1.401 (0.926–2.119)	0.111
G/G	64.4 (42.1–86.6)	1.290 (0.743–2.242)	0.366


A
20S α+β labeling
Relative antibody labeling
WBC
Mono
T-cells
B-cells
Granulo
B
PSMB1 expression
C/C
G/G
C
Chymotrypsin-like
Trypsin-like
Caspase-like
Activity (pmoles/min/mg protein)
D
Bortezomib (M)