Identification of *PSMB4* and *PSMD4* as novel target genes correlated with 1q21 amplification in patients with smoldering myeloma and multiple myeloma

Multiple myeloma (MM) is a malignant plasma cell (PC) dyscrasia characterized by heterogeneous biological features and genetic alterations, resulting in a wide range of disease courses.[1,2] Despite all the therapeutic strategies developed in the last three decades, MM is still incurable, and almost all patients will inevitably experience disease progression and eventually relapse.[3]

Among all the genetic abnormalities, the amplification of the 1q21 region is one of the most frequent cytogenetic abnormalities occurring in malignant PC and it has become a new prognostic factor in MM patients.[4,5] The incidence of gain and/or amplification of the 1q21 locus (1q21$^+$) increases with disease progression. It can be detected in around 30-45% of patients with smoldering MM (SMM) and newly diagnosed MM (NDMM), and in around 70% of relapsed/refractory MM patients (RRMM).[6] The impact of 1q21 on disease progression at an early stage has not been widely investigated. A few studies have suggested that the acquisition of extra 1q21 copies may play a role in disease progression.[7,8] In fact, SMM patients with 1q21$^+$ may be more likely to progress to MM than patients without 1q21$^+$.[8]

Recent studies have demonstrated that the 1q21 copy number has a different impact on the responsiveness to MM treatments, especially proteasome inhibition (PI).[9] PI is a well-established anti-cancer treatment approach used in MM. Throughout the years, the implementation of PI drugs as part of standard MM therapy has continued to improve the quality of life and clinical outcomes of MM patients. Furthermore, additional copies of 1q21 have been associated with PI resistance and recurrence of the disease in patients with 1q21$^+$, limiting the long-term medical utility of PI.[9,10] Recent studies have demonstrated that patients with 1q21$^+$ treated with combination treatment with bortezomib (Bor) have inferior progression-free survival and overall survival compared to patients who do not present 1q21$^+$.[11] Similar results were observed when patients harboring 1q21 amplification were treated with second-generation PI; however, this study showed that patients with 1q21 gain can greatly benefit from second-generation PI treatment upfront.[10]

Several genes are known to be deregulated upon the amplification of the 1q21 locus;[9] nonetheless, the pathogenic mechanism of how these genes drive disease progression and contribute to the poor outcome in patients with 1q21$^+$ and their possible role as druggable targets is not fully understood. In our study, we analyzed primary MM bone marrow (BM) PC from both SMM and NDMM patients to identify genes whose expressions are deregulated in patients with 1q21$^+$ in correlation with the number of copies and their putative role in drug response.

This study was conducted in line with the Declaration of Helsinki. Written consent was obtained from all patients for sample collection and clinical analysis. The Institutional Ethics Committee of Parma Hospital (Parma, Italy) reviewed and approved the study. We evaluated purified CD138$^+$ BM PC from 11 SMM and 18 NDMM patients. The cytogenetic features of all the patients are summarized in Table 1. All the patients underwent fluorescence *in situ* hybridization (FISH) analysis to detect 1q21 copy number alteration (CNA); 48% of patients presented 1q21$^+$ at the 1q21 locus. Based on the hybridization pattern of each patient, we generated a score representing the 1q21 copy number in each PC sample. The transcriptional profiles of the 29 BM samples were obtained using Gene CHIP ClairomD Arrays (Affymetrix Inc., Santa Clara, CA, USA) as previously described.[12] The analyses were performed using R (v4.0.2 in Rstudio v1.3.959). The global expression profiles of 19,012 protein-coding were obtained, analyzed using RMA normalization techniques, and annotated based on the Gencode project (v26) as previously described.[13] Annotation data were extracted from Ensembl v102 using the *biomaRt* package. Unprocessed sequence data from this study have been submitted to the Gene Expression Omnibus (GEO) of the National Center for Biotechnology Information under the accession number GSE227907. We performed a regional amplification analysis using LOESS smoothing of the W statistic and the *P* value from the comparison between 1q21$^+$ and control samples. Our analysis revealed that the

Table 1. Cytogenetic features of patients.

	SMM N=11	NDMM N=18
Female, N (%)	3 (27)	8 (44)
Male, N (%)	8 (73)	10 (56)
Median age in years (range)	67 (38-86)	72 (53-86)
Del13q, N (%)	6 (55)	11 (61.1)
Hyperdiploid, N (%)	5 (45)	8 (44.4)
del17p, N (%)	4 (36.3)	4 (22.2)
Chr14 translocation, N (%)	6 (45.4)	7 (38.8)
t(4:14), N (%)	3 (27.2)	3 (16.6)
1q21$^+$, N (%)	8 (72.7)	6 (33.3)
del1p32, N (%)	0 (0)	3(16.6)

N: number; SMM: smoldering multiple myeloma; NDMM: newly diagnosed multiple myeloma; Del: deletion; Chr: chromosome; t: translocation.

most up-regulated genes were located in the 1q21 region, after regrouping all the genes according to their corresponding positions on chromosome 1 (Figure 1A).

The *samr* package was used in R for differential analyses to identify genes that were differentially expressed between 1q-amplified and control samples. The correlation between the expected numbers of 1q21 copy number was performed using globaltest function in the same R package. Combining these two analyses (most differentially expressed gene between 1q21^{+} *vs*. control samples and most correlated gene expression with 1q21 copy number) we decided to focus on the *PSMB4* and *PSMD4* genes.

The expression analysis from our bioinformatics studies revealed a significant increase in RNA expression of the proteasome subunits PSMB4 and PSMD4 in MM patients with 1q21^{+} (Figure 1B, D). Consistently, the expression of both subunits was positively correlated with the 1q21 copy number determined by FISH (Figure 1C, E).

Next, we characterized the functional roles of PSMB4 and PSMD4 in MM *in vitro*. We evaluated the RNA expression

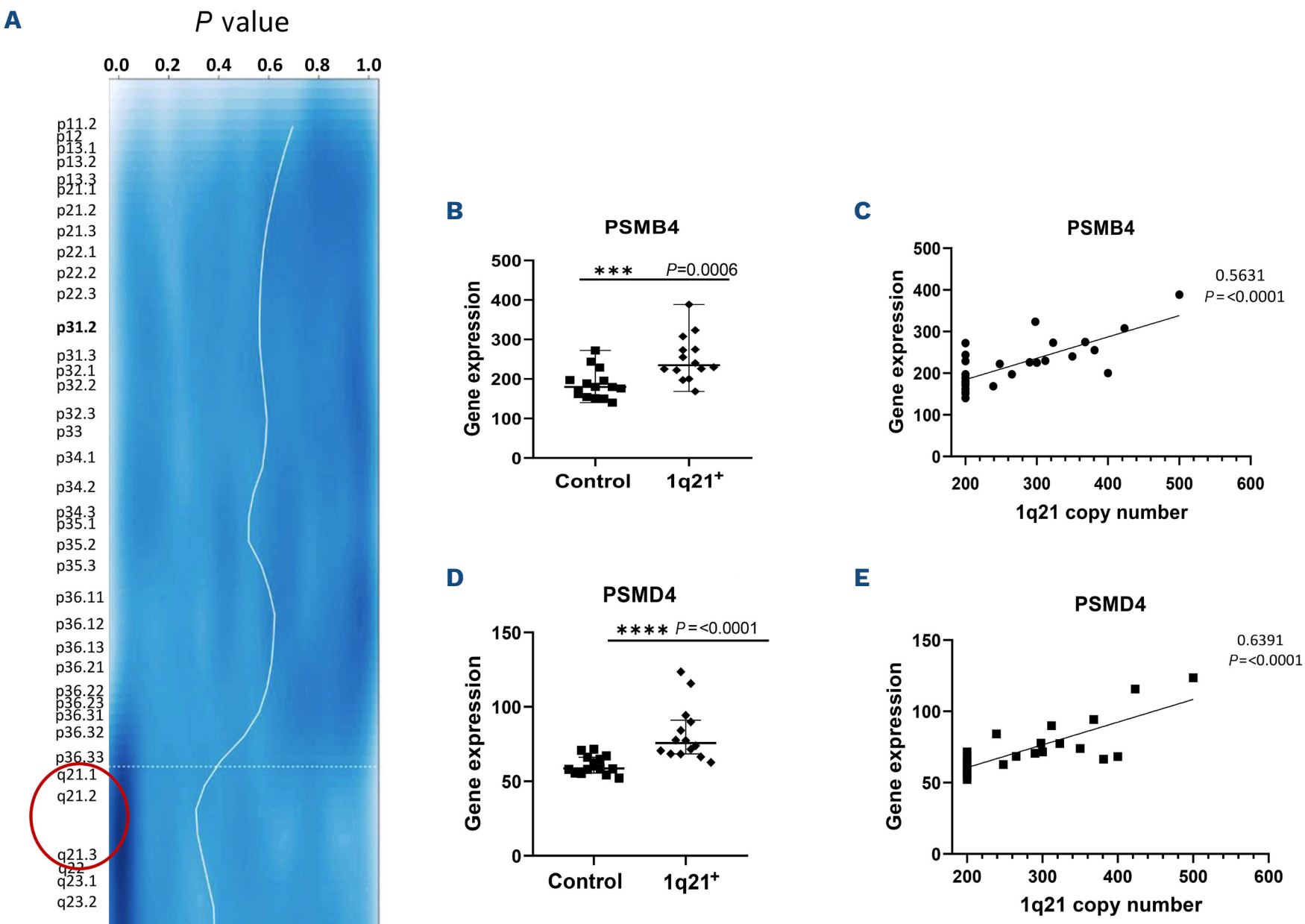

Figure 1. Regional amplification analysis across chromosome 1. Regional amplification was evaluated across chromosome 1 (Chr1). The genes with the most significant changes in expression were located in the 1q21 locus (red circle), followed by adjacent regions in the q arm of Chr1. A smoothed color density representation of the scatterplot obtained through a 2D-kernel density estimate (dark blue denotes most significant genes in terms of *P* value) (A). Expression levels of PSMB4 and PSMD4 in primary multiple myeloma (MM) bone marrow (BM) plasma cells (PC). PSMB4 and PSMD4 expression analysis reveals that both subunits are significantly up-regulated in purified BM PC from patients with 1q21^{+} when compared with controls (B and D). The gene expression profile was correlated with the 1q21 copy number determined by FISH analysis (C and E). Data were analyzed by Mann-Whitney test.

levels of the proteasome subunits in a panel of human myeloma cell lines (HMCL) previously characterized by FISH (*data not shown*). Notably, our FISH analysis revealed that the HMCL (H929, RPMI, U266, OPM2, and JJN3) carried different degrees of CNA. Our results showed that the RNA levels of PSMB4 and PSMD4 were higher in cell lines with 1q21^{+} when compared with control cell line OCI (Figure 2A, B). Remarkably, cell lines with a higher number of amplified 1q clones have greater RNA expression. These findings suggest that proteasome subunits PSMB4 and PSMD4 are both up-regulated in relation to the HMCL copy number.

To investigate the effects of PI Bor in HMCL, we treated cell lines with 1q21^{+} (JJN3, U266) with 2nM and 5nM of Bor for 48 hours. Western blot analysis showed that the protein expression of PSMB4 was unaffected by Bor treatment (Figure 2C), while the expression of PSMD4 was down-regulated after treatment (Figure 2C). Furthermore, a MTT cell viability assay performed on the same cell lines after Bor treatment showed that cells treated at 5nM have a remarkable cell mortality when compared with cells treated with 2nM of Bor (Figure 2D).

To further investigate the role of both proteasome subunits in the pathogenesis of MM, we knocked down the expression of PSMB4 and PSMD4 in JJN3, a cell line carrying 1q21^{+}, using short hairpin RNA (shRNA) lentivectors targeting both subunits. PSMB4 knockdown led to a reduction in RNA transcript levels and a drastically increased cell death, presumably associated with the high toxicity accumulated in the cells due to the lack of PSMB4 (*data not shown*). Moreover, we found that, like PSMB4, the RNA transcripts of PSMD4 were also significantly down-regulated upon the knockdown of PSMD4 (*Online Supplementary Figure S1A*). Notably, when cells with shPSMD4 were treated with PI Bor, we observed an increase in cell death when compared with the scramble cell line, though our statistical analysis determined that the increase in apoptosis was not significant (*Online Supplementary Figure S1C*). Similar results were obtained when PSMD4 knockdown cells were treated with carfilzomib (*Online Supplementary Figure S1D*). These results could be explained by the possible synergistic or additive effect of a not fully functional proteasome due to a partial inhibition of the PSMD4 (*Online Supplementary*

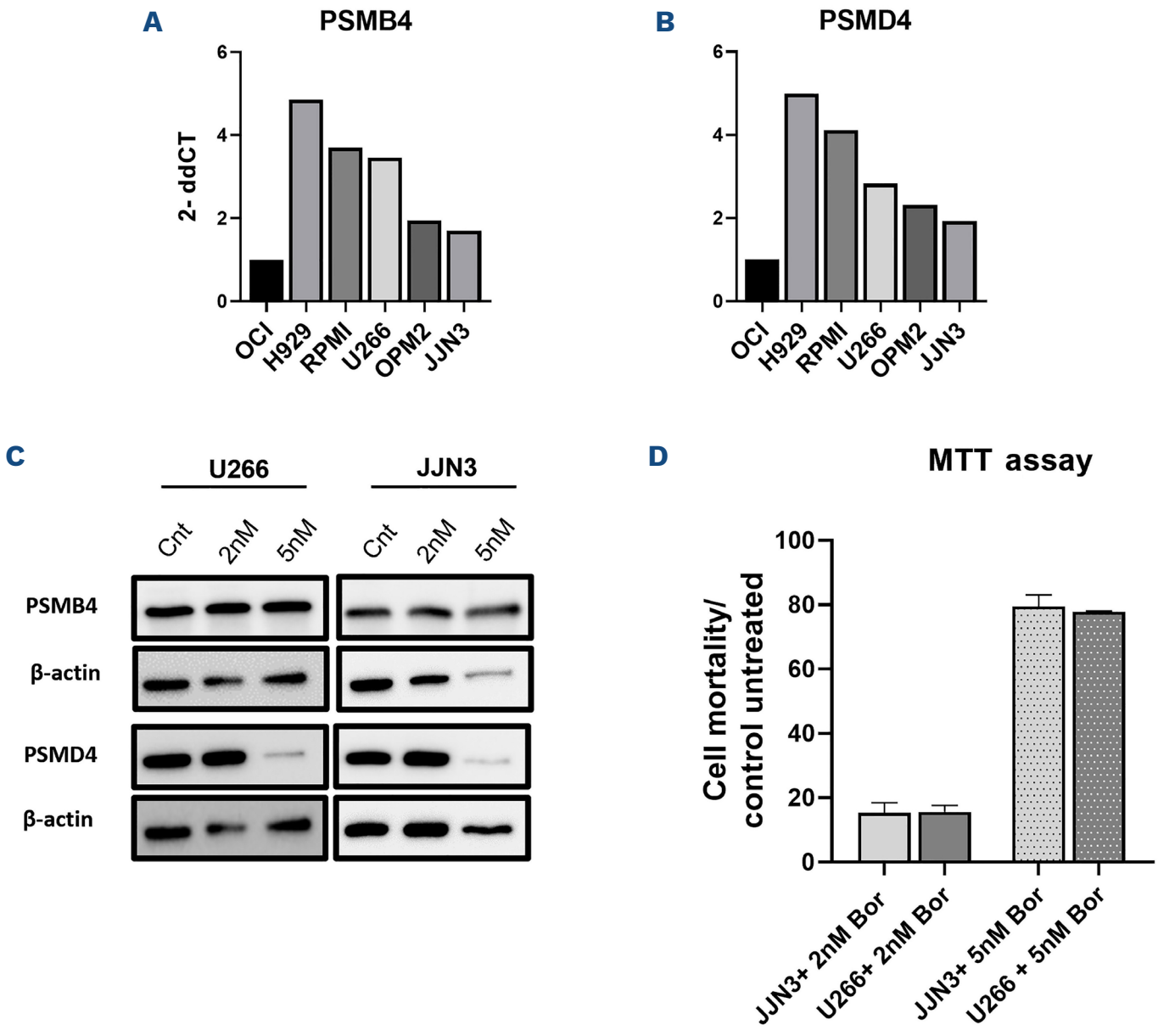

Figure 2. RNA expression levels of PSMB4 and PSMD4 in human myeloma cell lines. RNA expression of PSMB4 and PSMD4 in myeloma cell lines. OCI was used as a control. Cell lines with a greater number of 1q21 clones have higher RNA expression of PSMB4 and PSMD4 (A and B). Western blot analysis of 1q21^{+} U266 and JJN3 cell lines treated with bortezomib (Bor) at 2nM and 5nM for 48 hours (C). MTT viability assay of 1q21^{+} cells treated for 48 hours with 2nM and 5nM of Bor (D).

Figure S1B) and the inhibitory effect of the PI in the MM cell that leads to increased cell death when compared with the scramble cell line.

In conclusion, our results showed that proteasome subunits PSMB4 and PSMD4 are up-regulated in 1q21$^+$ patients, and that this upregulation is positively correlated to the 1q21 copy number. Interestingly, this correlation was independent of the disease stage (SMM *vs*. NDMM). Our functional analysis showed that inhibition of PSMD4 in cells with 1q21$^+$ results in increased cell death after treatment with PI when compared with scramble control.

The increased drug resistance to all available MM therapies is a significant barrier to long-term patient survival in MM, particularly those with 1q21$^+$. Our findings suggest that PSMD4 can be used as a potential target for the treatment of 1q21$^+$ patients. Combination therapies with next-generation agents such as cereblon E3 ligase modulators (CELMoD), which demonstrated remarkable *in vitro* potency and enhanced efficacy in RRMM patients,[14,15] new-generation PI, and other immunomodulatory drugs could represent an ideal partner for combination therapy. However, further studies are needed to decipher the molecular mechanisms by which MM patients with 1q21$^+$ fail to respond to PI drugs.

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Disclosures

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Contributions

JBG wrote the manuscript. LN, MS and BDP provided clinical data and enrolled patients. GS and GT performed the cytogenetic analysis. LA and PS performed the statistical analysis. VF and GD generated the viral vector. JBG, PS, VM and NTI performed the experiments. JBG, DT, VM, PS, VR, OL and NTI collected and processed the samples. NG, GP, PS and GD reviewed the manuscript. NG approved the final version of the manuscript. All authors contributed to the article and approved the final version for publication.

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Data-sharing statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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