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

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

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Running Title: New genes related to 1q21 amplification in myeloma.

KEYWORDS
Cytogenetic abnormalities, multiple myeloma, smoldering myeloma, proteasome inhibition.

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CONFLICT OF INTEREST

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SUPPORTING INFORMATION

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

AUTHOR’S 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 submitted version.
Letter to the Editor

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 PCs 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. 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 PI long-term medical utility \(^9\)\(^,\)\(^10\). Recent studies have demonstrated that patients with 1q21+ treated with combination treatment with Bortezomb (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) PCs 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. A 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 PCs from 11 SMM and 18 NDMM patients. The cytogenetic features of all the patients are summarized in Table 1. All the patients underwent fluorescent 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 version 4.0.2 in Rstudio v1.3.959. The global expression profiles of 39,012 protein-coding were obtained, analyzed using RMA normalization techniques, and annotated based on the Gencode project (version 26) 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 National Center for Biotechnology Information’s Gene Expression Omnibus (GEO)
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 most upregulated 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 global test function in the same R package. Combining these two analyses (most differentially expressed gene between 1q21+ versus control samples and most correlated gene expression with 1q21 copy number) we have decided to focus on PSMB4 and PSMD4 genes.

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

Next, we characterized the functional roles of PSMB4 and PSMD4 in MM in vitro. We evaluate the RNA expression levels of the proteasome subunits in a panel of human myeloma cell lines (HMCCLs) previously characterized by FISH analysis (data not shown). Notably, our FISH analysis revealed that the HMCCLs (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 (Figures 2A and 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 upregulated in HMCCLs copy number manner.

To investigate the effects of PI Bor in HMCCLs, we treated cell lines with 1q21+ (JJN3, U266) with 2nM and 5nM of Bor for a period of 48 hours. Western blot analysis showed that the protein expression of PSMB4 was unaffected by the treatment with Bor (Figure 2C), while the expression of PSMD4 was downregulated after treatment (Figure 2C). Furthermore, a MTT cell viability assay performed on the same cell lines after Bor treatment showed that cell 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 knockdown 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 downregulated upon the knockdown of PSMD4 (Supplementary Figure 1A). Notably, when cells with shPSMD4 were treated 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 (Supplementary Figures 1C). Similar results were obtained when PSMD4 knockdown cells were treated with carfilzomib (Supplementary Figure 1D). 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 (Supplementary Figure 1B) 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 upregulated in 1q21+ patients, and 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 (CELMoDs) which demonstrated remarkable in vitro potency and enhanced efficacy in RRMM patients, new-generation PIs, 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 PIs drugs.


Table 1. Cytogenetic features of patients

<table>
<thead>
<tr>
<th></th>
<th>SMM (n=11)</th>
<th>NDMM (n=18)</th>
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<tbody>
<tr>
<td>Female</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>27% (3)</td>
<td>44% (8)</td>
</tr>
<tr>
<td>Male</td>
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<td></td>
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<tr>
<td></td>
<td>73% (8)</td>
<td>56% (10)</td>
</tr>
<tr>
<td>Median age</td>
<td></td>
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</tr>
<tr>
<td>[range]</td>
<td>67 [38-86]</td>
<td>72 [53-86]</td>
</tr>
<tr>
<td>Del13q</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>55% (6)</td>
<td>61.1% (11)</td>
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<tr>
<td>Hyperdiploid</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>45% (5)</td>
<td>44.4% (8)</td>
</tr>
<tr>
<td>del17p</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>36.3% (4)</td>
<td>22.2% (4)</td>
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<tr>
<td>Chr14 translocation</td>
<td></td>
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<tr>
<td></td>
<td>45.4% (6)</td>
<td>38.8% (7)</td>
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<tr>
<td>t(4:14)</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>27.2% (3)</td>
<td>16.6% (3)</td>
</tr>
<tr>
<td>1q21+</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>72.7% (8)</td>
<td>33.3% (6)</td>
</tr>
<tr>
<td>del1p32</td>
<td></td>
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<tr>
<td></td>
<td>0% (0)</td>
<td>16.6% (3)</td>
</tr>
</tbody>
</table>

Note: Values are presented as percentage, number (n) or median [range]. Abbreviations: SMM: smoldering multiple myeloma; NDMM: newly diagnosed multiple myeloma; Del: deletion; Chr: chromosome; t: translocation.
Figure legends

Figure 1. Regional amplification analysis across chromosome 1 (Chr1). Regional amplification was evaluated across 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 = most significant genes in terms of p value) (A). Expression levels of PSMB4 and PSMD4 in primary MM BM PCs. PSMB4 and PSMD4 expression analysis reveals that both subunits are significantly upregulated in purified BM PCs 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). The data were analyzed with Mann-Whitney test. MM: multiple myeloma; BM: bone marrow; PCs: plasma cells.

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 JIN3 cell lines treated with 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). Bor: Bortezomib
**Supplementary Figure 1. Effect of PSMD4 knockdown in myeloma cells.** The RNA expression level of PSMD4 was significantly downregulated upon the inhibition of PSMD4 (shPSMD4) when compared to scramble control in JJN3 cells (A). shPSMD4 cells treated with PI bortezomib (Bor) and Carfilzomib have an increase in cell death when compared with scrambled control cells (C and D).