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Article

Long noncoding RNA *PVT1* is Regulated by Bromodomain Protein *BRD4* in Multiple Myeloma and is Associated with Disease Progression

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Abstract: Long noncoding RNAs (lncRNAs) are deregulated in human cancers and are associated with disease progression. *Plasmacytoma Variant Translocation 1* (*PVT1*), an lncRNA, is located adjacent to *MYC*, linked to multiple myeloma (MM). *PVT1* is expressed in MM and is associated with carcinogenesis, however, its role and regulation machinery remain uncertain. We examined *PVT1*/*MYC* expression through real time PCR in plasma cells purified from 59 MGUS and 140 MM patients. MM cell lines KMS11, KMS12PE, OPM2, and RPMI8226 were treated with JQ1, a *MYC* superenhancer inhibitor, or *MYC* inhibitor 10058-F4. The expression levels of *PVT1* and *MYC* were significantly higher in MM than in MGUS ($p < 0.0001$), and showed positive correlation with disease progression ($r = 0.394$, $p < 0.0001$). JQ1 inhibited cell proliferation and decreased the expression levels of *MYC* and *PVT1*. However, 10054-F4 did not alter the expression level of *PVT1*. The positive correlation between *MYC* and *PVT1* in patients, synchronous downregulation of *MYC* and *PVT1* by JQ1, and no effect of *MYC* inhibitor on *PVT1* expression suggest that the expression of these two genes is coregulated by a superenhancer. Cooperative effects between these two genes may contribute to MM pathogenesis and progression.

Keywords: long noncoding RNA, *PVT1*, *MYC*, bromodomain, multiple myeloma

1. Introduction

Multiple myeloma (MM) is a plasma cell neoplasm characterized by the proliferation of atypical plasma cells in the bone marrow and the production of monoclonal immunoglobulins. MM progresses from a precancerous state called Monoclonal Gammopathy of Undetermined Significance (MGUS) at a rate of approximately 1 % per year [1-3]. The molecular mechanism of MM development is thought to be primarily due to the activation of cancer-related genes by translocations of immunoglobulin heavy chain genes (IgH). These include increased cyclin D1 expression by the translocation t(11; 14) IgH-*CCND1*, increased *FGFR3*/*MMSET* expression by t(4; 14) IgH-*FGFR3*/*MMSET*, and increased *c-MAF* expression by t(14; 16) IgH-*c-MAF* [4]. These chromosomal abnormalities are observed at the MGUS stage, so additional abnormalities are

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required for progression to MM. Recent genomic and transcriptomic analyses have shown that oncogene mutations, such as *RAS* mutations, and aberrant overexpression of *MYC* play an important role in the progression of MM [5]. However, not all players have been elucidated yet.

Recent transcriptome-wide analyses have revealed an overwhelming amount of transcribed but not translated non-coding RNAs capable of influencing diverse cellular processes, such as proliferation, apoptosis, and motility [6,7]. Among the non-coding RNAs, long non-coding RNAs (lncRNAs), transcripts >200 nucleotides in length, have emerged as a class of key regulatory RNAs [8]. LncRNAs are deregulated in many human cancers and are associated with disease progression [9-11]; several studies, including ours, have shed light on the role of lncRNAs in MM progression [12-14].

Plasmacytoma Variant Translocation 1 (*PVT1)* which is an lncRNA longer than 500 nucleotides, was first found in mouse plasmacytoma [15] and then reported to be involved in the oncogenesis of many types of cancers [16,17]. *PVT1* is located at the 8q24 locus adjacent to *MYC* [18], which is highly expressed in many types of cancer and plays an important role in carcinogenesis [19,20]. *PVT1* is elevated in MM [5,22] and coamplified with *MYC* in many cancers [18]; there is an association between *PVT1* expression level and poor prognosis in many cancers [16,17,23-25]. High level amplification and/or overexpression of the *PVT1* is associated with invasive phenotype of breast cancer and reduced survival time in ovarian cancer patients [21]. These observations are indicative of an involvement of *PVT1* in the maintenance of a transformed phenotype. However, its regulation and clinical significance in MM have been poorly documented.

Superenhancers, on which mediator complexes, including activators and coactivators, accumulate at higher densities than on regular enhancers, that control the expression of genes involved in cell identity, determination, and disease, have been described recently [26]. The superenhancers are bound by the bromodomain containing protein 4 (*BRD4*) which is a member of the bromodomain protein family and is essential for RNA transcription and transcription elongation [27]. The most well-known mechanism of *MYC* overexpression is the fusion of the IgH enhancer and *MYC* produced by the chromosomal translocation t(8;14) in Burkitt lymphoma. *MYC* transcription is controlled by superenhancer [27,28], and *BRD4* inhibitors markedly decrease *MYC* expression in many types of cells including MM cells [28]. Thus, *PVT1* is speculated to be regulated by a superenhancer.

In order to clarify the underlying mechanism regulating *PVT1* expression and its relationship with progression and prognosis in MM, we investigated the *PVT1* expression in plasma and MM cell lines focusing on a superenhancer-related mechanism, and the correlation between *PVT1* and *MYC* expression in MM and MGUS patients.

2. Results

2.1 PVT1 and MYC expression in plasma cells of MM is higher than of MGUS and control

The expression levels of *PVT1* and *MYC* in plasma cells were significantly higher in MM (mean: *PVT1* 2.58, *MYC* 0.74), than MGUS (mean: *PVT1* 0.88, *MYC* 0.06) and control (mean: *PVT1* 0.06, *MYC* 0.07) ($p < 0.001$, $p < 0.001$, respectively) (Figure 1A, 1B). *PVT1* expression seemed to increase with disease progression, but it did not differ between samples from different stages (stages defined according to the international staging system (ISS) reflecting progression) ($p = 0.145$) (Figure 1C). We then compared *PVT1* expression levels between cell lines with different

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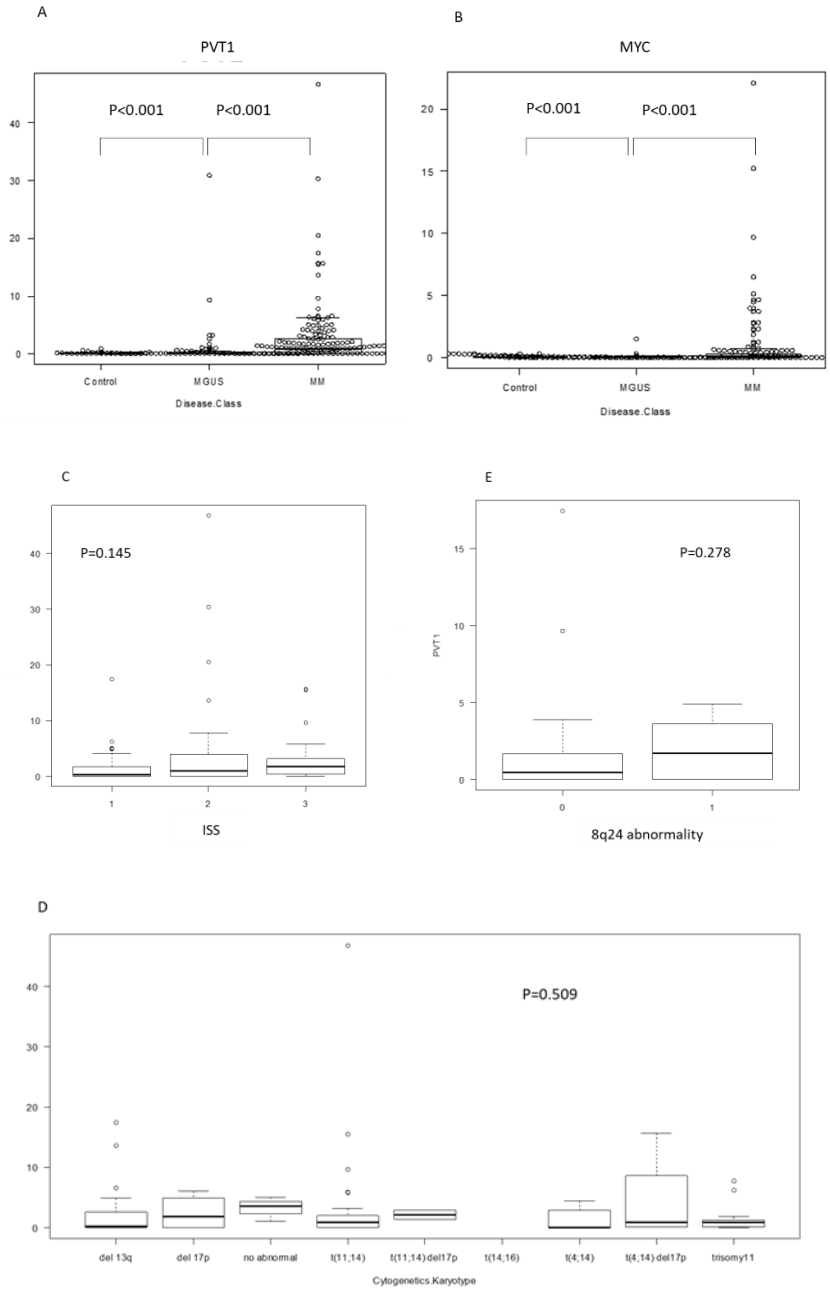
chromosomal abnormalities, as detected with fluorescence in situ hybridization (FISH) analysis, including t(11;14), t(4;14), t(14;16), deletion 13q, and deletion 17p, and found no difference ($p = 0.509$) (Figure 1D). Since *PVT1* is located in chromosome 8q24 and a co-occurrence of 8q24 abnormality is sometimes observed in MM, we compared the expression levels between cell lines with 8q24 abnormalities including t(8;14), and tested for 8q24 amplification by FISH analysis. However, no difference was found (Figure 1E). When we analyzed *PVT1* and *MYC* expression levels in the same patients, a positive correlation was found in both MM and MGUS patients ($r = 0.484$, $p < 0.001$; $r = 0.423$, $p < 0.0001$; respectively) (Figure 1E, F).



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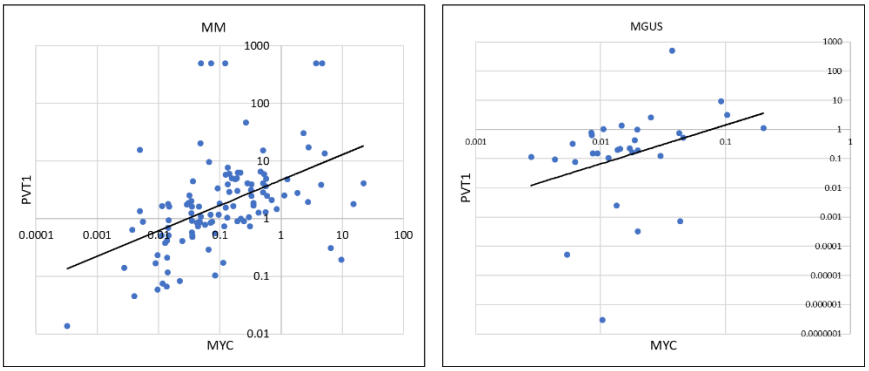

Figure 1: RNA expression determined by RQ-PCR. in plasma cells isolated from bone marrow specimens and organized by patient status. Each dot shows a patient. RQ-PCR: real time quantitative PCR. MGUS: monoclonal gammopathy of undetermined significance. MM: multiple myeloma. A: PVT1. B: MYC. C: PVT1 expression by international staging system (ISS). D: PVT1 expression according to karyotype determined with fluorescence in situ hybridization (FISH). E: PVT1 expression by chromosome 8q24 abnormality determined with FISH. F: Correlation between PVT1 and MYC RNA expression in plasma cells of MM and MGUS.

2.2 BRD4 inhibitors inhibit MM cell proliferation and downregulate PVT1 and MYC expression

To clarify the mechanisms regulating *PVT1* expression, we focused on *BRD4* because it is well known that *MYC* expression is regulated by *BRD4*. *BRD4* inhibitors inhibited the proliferation of eight MM cell lines. JQ1 inhibited the proliferation of KMS11, KMS12PE, KMS12BM, KMS26, KMM1, OPM2, and RPMI8226 cell lines with a concentration of 1 μM (Figure 2A). CPI-203 inhibited the proliferation of KMS11, KMS12BM, KMS26, and OPM2 with a lower concentration (0.1 μM), and inhibited KMS12PE, KMS18, KMM1, and RPMI8226 with 1 μM concentration (Figure 2B).

JQ1 markedly reduced *PVT1* mRNA expression with a concentration of 1 μM in KMS11, OPM2, KMS12PE, KMS12BM, KMM1, RPMI8226 and KMS26 (Figure 2C). Similarly to JQ1, another BRD4 inhibitor CPI-203, markedly reduced *PVT1* mRNA expression, with a concentration of 1 μM, in KMS11, OPM2, KMS12PE, KMM1, RPMI8226 and KMS26 (Figure 2D).

Consistent with previous reports, 1 μM of JQ1 significantly reduced *MYC* mRNA expression in KMS11, OPM2, KMS12PE, KMS12BM, KMM1, RPMI8226 and KMS26 (Figure 2E). *MYC* protein level was also reduced with JQ1 treatment (Figure 2F).

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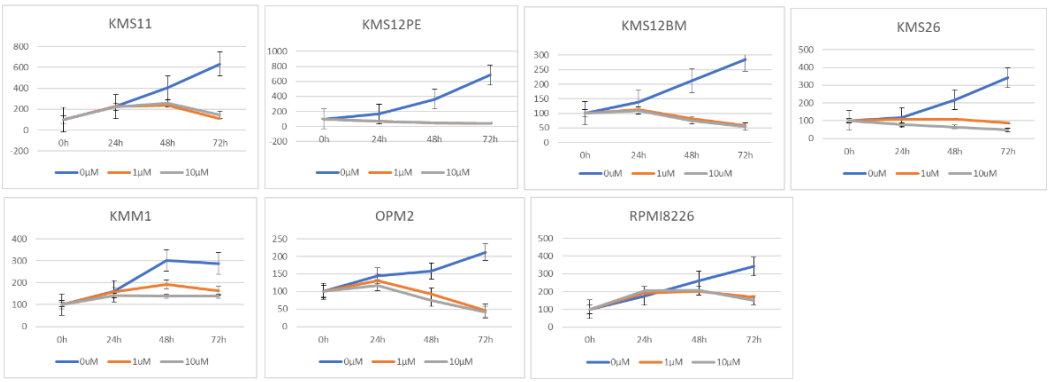

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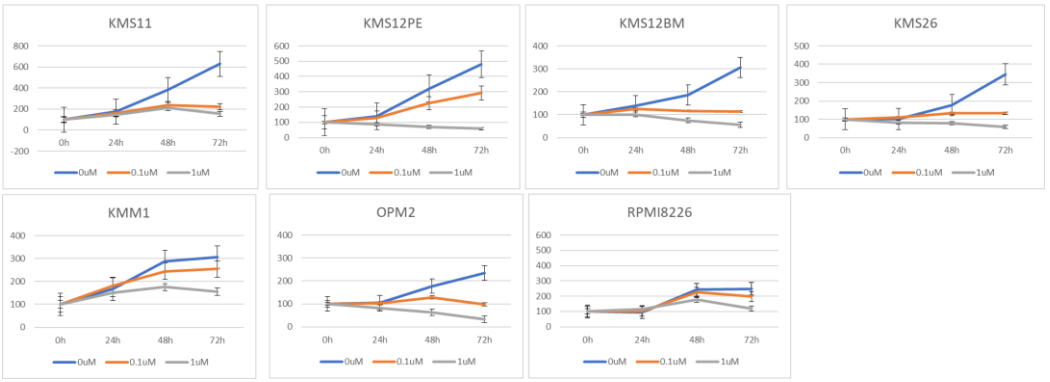

C

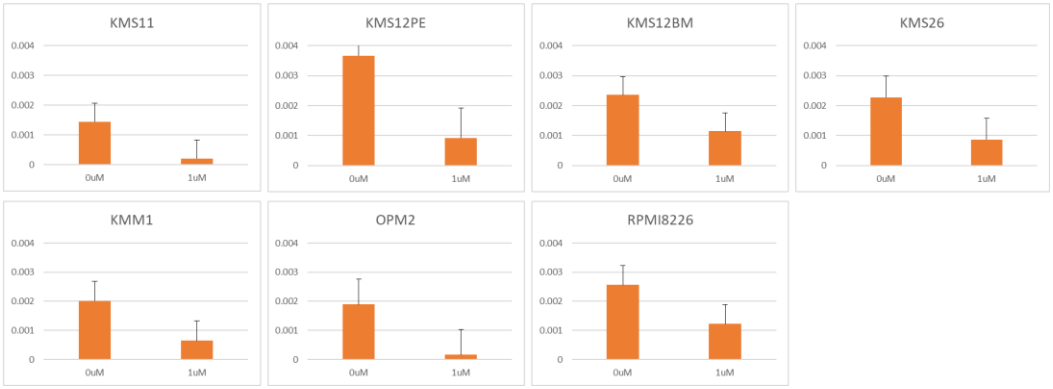

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Figure 2: Growth determined using the WST-8 assay and RNA expression determined with RQ-PCR in seven MM cell lines treated with two BRD4 inhibitors JQ1 (0 μM, 1 μM, 10 μM) and CPI-203 (0 μM, 0.1 μM, 1 μM). A: Cell growth under treatment with JQ1 B: Cell growth under treatment with CPI-203. C: PVT1 expression under treatment with JQ1. D: PVT1 expression under treatment with CPI-203. E: MYC expression under treatment with JQ1. F: MYC expression under treatment with CPI-203.

2.3 MYC inhibitor did not reduce PVT1 expression

From the results described above, there were two possibilities for explaining the high *PVT1* expression levels in MM. One was that *MYC* and *PVT1* are co-regulated by *BRD4* and the other

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is that *PVT1* is regulated by *MYC* which is regulated by *BRD4*. To test these hypotheses, we used the *MYC* inhibitor, 10058-F4, which inhibits *MYC* transcription activity by dissociating the *MYC-MAX* transcription complex. *PVT1* expression was not significantly altered by treatment with 10058-F4 in KMS11, OPM2, KMS12PE, and RPMI8226 (Figure 3). Therefore, no contribution of *MYC* transcriptional activity to *PVT1* expression was revealed.

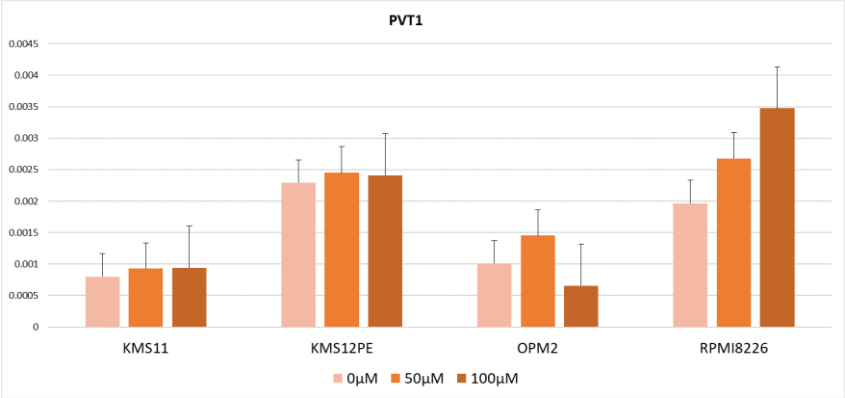
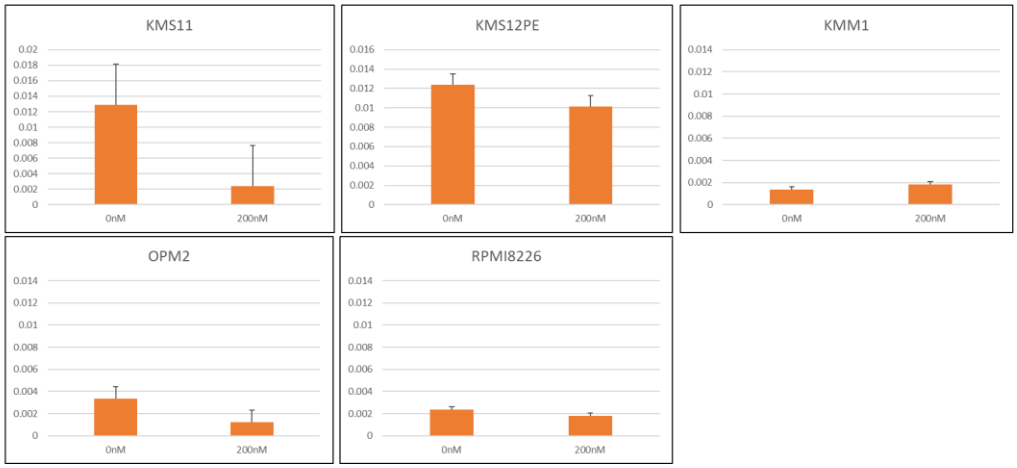


Figure 3. *PVT1* expression in cells treated with the *MYC* inhibitor 10058-F4. Error bars show the standard error of mean (SEM).

2.4 PVT1 downregulation by locked anti-sense nucleotide reduced MYC expression

To determine the role of *PVT1* on *MYC* expression in MM cells, anti-sense locked nucleotide GapmeR™ was used to knockdown *PVT1* expression. Three sequences of GapmeR were constructed and five MM cell lines were tested, KMS11, KMS12PE, KMM1, OPM2 and RPMI8226. *PVT1* expression was successfully knocked down in two cell lines, KMS11 and OPM2, by one GapmeR product (Figure 4A). In these two cell lines, *MYC* mRNA expression was significantly reduced (Figure 4B).

A



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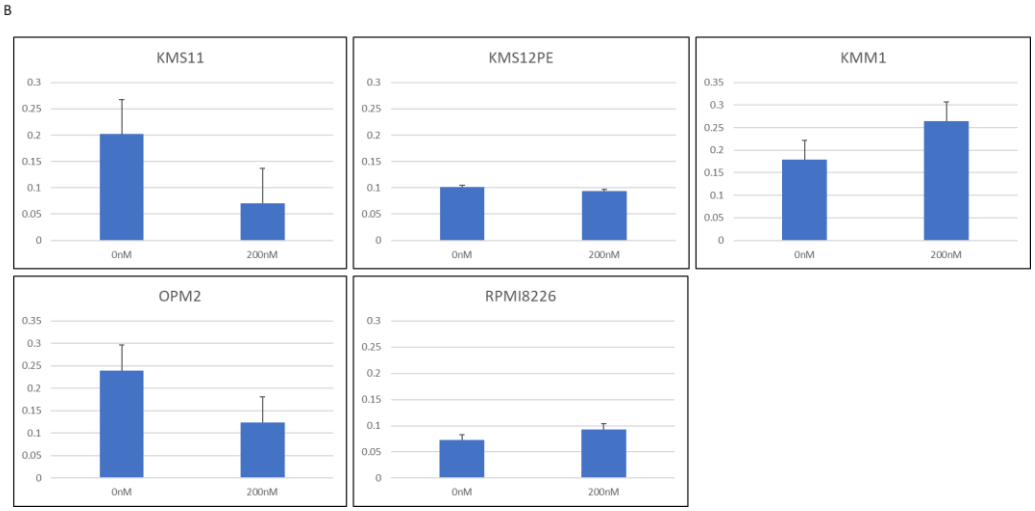

Figure 4. A: *PVT1* expression in MM cell lines treated with antisense oligonucleotide LNA™ long RNA GapmeR for *PVT1*. B: *MYC* expression in MM cell lines treated with antisense nucleotide LNA™ long RNA GapmeR for *PVT1*.

2.5 Clinical significance of PVT1 expression in MM

To investigate the clinical significance of *PVT1* in MM, overall survival (OS) and progression free survival (PFS) of MM patients were analyzed by dividing the two groups according to *PVT1* expression levels. Sixty-seven patients were analyzed for OS and 62 patients were analyzed for PFS. Five patients were excluded from PFS analysis due to lack of reliable clinical results for progression. The patients with high *PVT1* expression tended to have a shorter OS with a median of 2.7 years compared to 5.0 years in those with low expression (p = 0.09) (Figure 5A), but PFS did not differ between MM patients with high and low *PVT1* expression (Figure 5B).

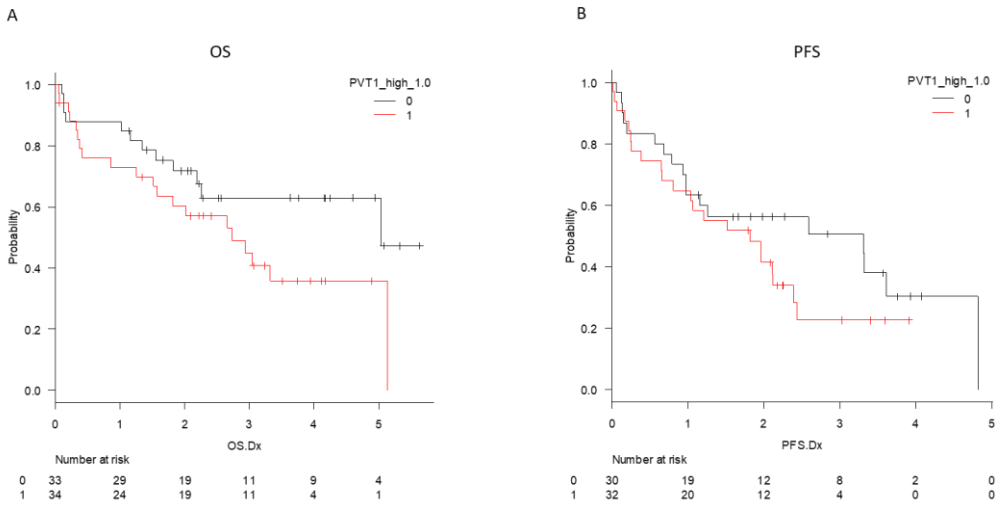

Figure 5. A: Overall survival (OS) and B: progression free survival (PFS) in MM patients divided into two groups by *PVT1* expression level.

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3. Discussion

In this study, we aimed to clarify the underlying mechanism regulating PVT1 expression and its relationship with progression and prognosis in MM. We found significantly higher *PVT1* and *MYC* expression in MM and MGUS plasma cells compared to that in the control cell line, suggesting that the lncRNA *PVT1* is associated with MM pathogenesis and progression. Positive correlation between *MYC* and *PVT1* expression levels suggests that *MYC* and *PVT1* are coregulated by the same mechanism. Two *BRD4* inhibitors, JQ1 and CPI -203, markedly reduced both *MYC* and *PVT1* in MM cell lines but the *MYC* inhibitor did not reduce *PVT1* expression, suggesting that *PVT1* expression is not controlled by *MYC* but both genes were regulated by *BRD4*. Moreover, reduction of *MYC* mRNA by *PVT1* knockdown indicates that *PVT1* regulates *MYC* expression at the transcriptional level.

High expression of *PVT1* and *MYC* in MM cells compared to that in normal plasma cells is consistent with previous studies [17]. A large number of studies have shown that *PVT1* levels are higher in many cancers with increased *MYC* expression compared to normal tissues and are associated with a poor prognosis [16,23-25]. Tissue microarray analysis of primary tumors indicated a high correlation between *PVT1* and *MYC* expression, providing strong evidence for the cooperation of *PVT1* and *MYC* in different human cancers [29]. Nagoshi et al. reported that frequent *PVT1* rearrangement and novel chimeric genes, *PVT1-NBEA* and *PVT1-WWOX*, occur in MM with 8q24 abnormality [30]. They also found high expression of *PVT1* and *MYC* in most MM cell lines regardless of *PVT1* or *MYC* rearrangement status. Our findings of *PVT1* elevation in plasma cells of MM compared to MGUS suggest a role of *PVT1* in the pathogenesis of malignant clonal plasma cells and the progression from precancerous stage to cancer.

The precise mechanisms controlling lncRNA *PVT1* expression in MM have not been fully clarified. An increased *MYC* copy number and *PVT1* expression occur in more than 98% of cancer cases with increased 8q24 copy numbers, which points to an interaction between *PVT1* and *MYC*, and that they are part of a common signaling pathway. We could not find any correlation between *PVT-1* expression level and chromosomal abnormalities including t(8;14) and 8q24 amplification detected with fluorescent in situ hybridization (FISH). This is not conclusive because FISH is not sufficient for the detection of gene amplification; however, positive correlation between *PVT-1* and *MYC* expression implies a common regulation system for both genes, and that upregulation of *PVT1* and *MYC* expression does not solely depend on 8q24 abnormality in MM.

We speculated that a *BRD4* inhibitor would downregulate the expression of *PVT1* and *MYC*. JQ1 and CPI -203 significantly reduced the expression of *PVT1* in myeloma cell lines. Combined with the high expression of *MYC* and *PVT1* and the positive correlation of *MYC* and *PVT1* in the MM and MGUS patients' plasma cells, these results suggest that *MYC* and *PVT1* are regulated by the same mechanism. Two possible regulation mechanisms for *MYC* and *PVT1* expression were conceivable. One is that *MYC* regulates *PVT1* expression, and the other is that both *MYC* and *PVT1* are controlled by the superenhancer. To test these hypotheses, we examined the *PVT1* expression using 10058-F4, which suppresses the transcriptional activity of *MYC* by inhibiting the heterodimer formation of *MYC* and *MAX* [31].10058-F4 did not decrease *PVT1* expression except for in the OPM2 cell line. These results suggest that *PVT1* is not transcriptionally regulated by *MYC*, and that two genes are simultaneously regulated by superenhancers. This result is inconsistent with previous reports that the *PVT1* promoter region contains two enhancer E-boxes that serve as *MYC* binding sites and that E-box 2 mediates the binding of *MYC* to the *PVT1* promoter to promote *PVT1* expression [32]. We cannot fully explain this discrepancy, it may be attributed to different cell lineages or the conditions of the cells.

Many studies have revealed a relationship between *PVT1* and *MYC* expression. Our experiments of *PVT1* knockdown using the locked nucleotide anti-sense oligo nucleotide resulted

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in a decreased expression of *MYC* mRNA, which is consistent with a previous report showing the effect of *PVT1* on MYC transcription [33], but inconsistent with another report where low levels of c-Myc protein were found without significant changes in *MYC* mRNA levels when siRNA was used to knock down *PVT1* [29].

We found a tendency towards worse OS in the high *PVT1* group although the PFS did not differ between the high and low *PVT1* groups. These results suggest that a high level of *PVT1* might be associated with a prognosis of relapse and drug resistance. An association between high *PVT1* expression and worse prognosis has been reported in several cancers [16,23,25]. Involvement in drug resistance to anti-cancer drugs has also been documented in several ways [24,34]. For example, *PVT1* acts as a competitive endogenous RNA that forms a tight network with protein-coding mRNAs such as *CDH1, TP73, TP31, RUNX1*, and *RUNX* via microRNA-200, thereby regulating breast cancer progression [35,36]. Further studies to elucidate *PVT1* involvement in drug resistance are warranted.

4. Materials and Methods

4.1 Cell lines

Cell lines used included KMS12PE with t(11;14), KMS11 with t(4;14) and p53 deletion, and OPM2 with t(4;14) + mutated p53 and RPMI8226. The human myeloma cell line RPMI8226 was obtained from the American Type Culture Collection (Rockville, MD, USA), and lines KMS11, KMS12PE, KMS12BM, and KMS18 were kindly provided by Dr Takemi Otsuki (Kawasaki Medical School, Okayama, Japan). OPM2 was kindly provided by Dr Masaki Ri (Nagaya City University, Nagoya, Japan). All lines were cultured in RPMI1640 medium (Sigma-Aldrich, St Louis, USA), supplemented with 10% fetal bovine serum, at 37 °C and 5 % CO_2.

4.2 Patients

A total of 140 consecutive MM patients, 62 with MGUS, and 21 control patients with lymphoma without bone marrow infiltration or acute myeloid leukemia in complete remission, were included in this study from July 2010 to March 2015. The patient characteristics are summarized in supplementary Table 1. This study was approved by the institutional review board of Gunma University Hospital under the guidelines of the Declaration of Helsinki. Bone marrow (BM) aspirate samples were obtained upon diagnosis after obtaining each patient's informed consent.

4.3 Treatment with inhibitors

Myeloma cell lines KMS11, KMS12PE, KMS12BM, KMS26, KMM1, OPM2, and RPMI8226 were treated with JQ1 1 μM and CPI203 0.1 μM. KMS11, KMS12PE, KMM1, OPM2, and RPMI8226 were treated for 24 h with *MYC* inhibitor 10058-F4 (50 μM or 100 μM). Cell growth was determined using the WST-8 assay (Dojindo Laboratory, Kumamoto, Japan) at 24, 48, and 72 h. RNA was isolated from cells incubated for 24 h and gene expression was determined with real time PCR. Experiments were performed in triplicate.

4.4 PVT1 silencing in myeloma cell lines

RNase H-activating Locked Nucleic Acid (LNA) GapmeR™ (Exiqon, Vedbaek, Denmark) was used to silence *PVT1* expression in vitro. The cell lines KMS12PE and OPM2 were cultured with GapmeR for 24, 48, and 72 h, after which cell viability was determined using the WST-8

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assay. All experiments were performed in duplicate. Gene expression was determined after 72 h of treatment.

4.5 Isolation of nucleic acids

Plasma cells were purified from the bone marrow mononuclear cells with anti-CD138 antibody conjugated with phycoerythrin (PE) (Beckman-Coulter, Brea, CA) and the Easy Step PE positive selection kit containing anti-PE antibodies conjugated with micro-magnetic beads (STEMCELL Technologies, Vancouver, BC, Canada). RNA was extracted from plasma cells (and one autopsied extramedullary plasmacytoma of the liver) and cell lines using the mirVana RNA Isolation kit (Ambion, Austin, TX, USA). Complimentary DNA (cDNA) was produced using the PrimeScript™ RT reagent kit with gDNA Eraser (TaKaRa Bio, Kyoto, Japan).

4.6 Real-time PCR analysis of PVT1 expression

Transcript levels, including those of PVT1, were determined with real-time PCR using the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Primers used for detection were as follows: PVT1: F-5′-CACTCTGGACGACTTGAGAAC-3′, R- 5′-TCCTCAGATGAACCAGGTGAACA-3′; MYC: F-5′-CCTGGTGCTCCATGAGGAGA-3′, R-5′-CAGTGGGCTGTGAGGAGGGTTT-3′; ACTB: F- 5′-TGGCACCCAGCAATGAA-3′, R- 5′-CTAAGTCATAGTCCGCCTAGAAGCA-3′. The expression levels were calculated using the ΔΔCt method. ACTB was used as an internal control, and cDNA extracted from the HL60 acute myeloid leukemia cell line was used as a calibration sample. Relative RNA expression levels are expressed as $2^{-\Delta\Delta Ct}$.

4.7 Statistical analysis

EZR version 1.41 (Saitama, Japan) was used for statistical analysis. P values < 0.05 were considered significant. Frequencies were evaluated using Fisher's exact test, and continuous values were evaluated using the Mann-Whitney U test or Kruskal-Wallis test. Overall survival (OS) and progression-free survival (PFS) were evaluated using the Kaplan-Meier method and log rank test for univariate analysis. The Cox regression hazard model was used for multivariate analysis.

5. Conclusions

Positive correlation between *MYC* and *PVT1* in patients, synchronous downregulation of *MYC* and *PVT1* by JQ1 and CPI203, no effect of *MYC* inhibitor for *PVT1* expression suggest that the expression of these two genes is co-regulated by the *BRD4* complex. *PVT1* and *MYC* cooperation may contribute to MM pathogenesis and progression, and our results support the rationale for targeting *BRD4* in these patients.

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Author Contributions

H.H. and K.H. are equally contributed to this work. Conceptualization, Writing – Original Draft Preparation, Writing – Review & Editing, Investigation, Analysis, Validation, Project Administration.; Funding Acquisition, Supervision, H.H.; Writing – Original Draft Preparation, Methodology, Investigation, K.H.; Investigation, Methodology, Analysis, Validation, T.O., N.K., Y.K., M.K., S.W., R.I., Y.M., Y.M. K.T., H.T., T.K. ; Supervision, T.S., H.M.

Conflicts of Interest

The authors declare no conflict of interest.

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