Dysregulation of the *let-7/HMGA2* axis with methylation of the *p16* promoter in myeloproliferative neoplasms

骨髄増殖性腫瘍における let-7/HMGA2 調節系異常と
p16 プロモーターメチル化

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Running short title: *HMGA2* deregulation due to reduction of *let-7* miRNA in MPN

Summary

Overexpression of *high mobility group AT-hook 2* (*Hmga2*), which is negatively regulated by *let-7* micro RNAs through 3'-untranslated region (3'UTR), causes proliferative haematopoiesis mimicking myeloproliferative neoplasms (MPNs) and contributes to progression of myelofibrosis in mice. Thus, we here investigated *HMGA2* mRNA expression in 66 patients with MPNs including 23 polycythemia vera (PV), 33 essential thrombocythemia (ET), and 10 primary myelofibrosis (PMF). *HMGA2* mRNA expression, especially variant 1 with 3'UTR that contains *let-7*-specific sites, rather than variant 2 lacking 3'UTR, is frequently deregulated due to decreased *let-7* expression in granulocytes from over 20% of PV and ET, and in either granulocytes or $CD34^+$ cells from 100% of PMF. Patients with deregulated *HMGA2* mRNA expression were significantly more likely to show splenomegaly, high serum LDH values, and methylation of the *p16* promoter compared with other patients without deregulation of *HMGA2*. A histone deacetylase inhibitor, panobinostat, significantly increased *let-7* expression and reduced variant 1 of *HMGA2* mRNA expression, but not variant 2, in both U937 cells and PMF-derived $CD34^+$ cells. Moreover, both panobinostat and small interfering RNA of *HMGA2* demethylated the *p16* promoter in U937 cells. In conclusion, the frequently dysregulated *let-7*/*HMGA2* axis can be a certain therapeutic target in MPNs.

Introduction

Myeloproliferative neoplasms (MPNs), including polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF), are clonal haematological disorders characterized by proliferation of mature blood cells (Levine & Gilliland, 2008). Signal-activating mutations such as *JAK2*V617F (James *et al*, 2005; Kralovics *et al*, 2005; Levine *et al*, 2005; Baxter *et al*, 2005), *MPL*W515 (Beer *et al*, 2008), and *CALR* (Nangalia *et al*, 2013; Klampfl *et al*, 2013) lead to haematopoietic cell proliferation. However, it is controversial if these mutations provide a clonal growth advantage to haematopoietic cells (Levine & Gilliland, 2008; Abdel-Wahab *et al*, 2010; Mullally *et al*, 2010; Li *et al*, 2010). Allele burdens of *JAK2*V617F are not usually decreased by JAK2 inhibitors although they reduce spleen size and improve quality of life (Harrison *et al*, 2012; Tefferi, 2012). MPNs also show mutations in a variety of epigenetic modifiers including *DNA Methyltransferase 3a* (*DNMT3a*), *Tet Methylcytosine Dioxygenase 2* (*TET2*), and polycomb group genes (PcG) (Shih *et al*, 2012). Moreover, micro RNAs (miRNAs), which negatively regulate expressions of targeted genes, are often differentially expressed in myeloid neoplasms including MPNs (Zhan *et al*, 2013; Bruchova *et al*, 2008). These findings indicate that the genes regulating expressions of other genes may play important roles in the pathogenesis of MPNs. Therefore, molecular targets, in addition to *JAK2*V617F, should be established to treat MPNs. In this respect, clinical efficacies of several agents that inhibit epigenetic modifiers such as histone deacetylase (HDAC) inhibitors have been investigated for MPNs (Rambaldi *et al*, 2010; Mascarenhas *et al*, 2013; DeAngelo *et al*, 2013a, 2013b). For example, a pan-HDAC inhibitor, panobinostat has shown nearly complete response in PMF (Mascarenhas *et al*, 2013). However, appropriate molecular targets of HDAC inhibitors remain largely unknown.

The High Mobility Group AT-hook 2 (HMGA2) is a non-histone chromatin protein that modulates transcriptions of various genes through DNA-binding AT-hook domains, which affect the DNA conformation of AT-rich regulatory elements (Sgarra *et al*, 2004; Fusco & Fedele, 2007; Young & Narita, 2007). HMGA2 also contributes to chromatin modification and epigenetic regulation (Sgarra *et al*, 2004; Zong *et al*, 2012; Sun *et al*, 2013). Therefore, HMGA2 plays crucial roles in proliferation, cell-cycle progression, apoptosis, and senescence of cells, leading to its oncogenic activity in a variety of tumors. Furthermore, HMGA2 also controls self-renewal of neural stem cells, in part through downregulation of tumor suppressor *p16* (Nishino *et al*, 2008, 2013). *HMGA2* expression is post-transcriptionally and negatively regulated by binding of *let-7*-family miRNAs to 7 specific sequences in the 3'-untranslated region (UTR) of *HMGA2* mRNA (Mayr *et al*, 2007; Lee & Dutta, 2007). Thus, rearrangements within the *HMGA2* locus of chromosome 12q13-15 deleting the 3'UTR that contains *let-7*-specific sites cause overexpression of *HMGA2* mRNA and protein with a preserved function of AT-hook domains. We have recently reported MPN-like haematopoiesis with increases in all haematopoietic cell lineages and clonal advantage in competitive repopulations with bone marrow transplants in mice transgenic for a murine *Hmga2* with a truncation of 3'UTR that causes overexpression of HMGA2 (Δ*Hmga2* mice) (Ikeda *et al*, 2011). Other groups also showed that expression of HMGA2 contributes to a haematopoietic cell proliferation mimicking MPNs (Oguro *et al*, 2012; Muto *et al*, 2013) and self-renewal of haematopoietic stem cells (HSCs) (Copley *et al*, 2013). Interestingly, a few studies have shown the deregulated expression of *HMGA2* mRNA associated with rearrangement of chromosome 12q13-15 in patients with MPNs or myelodysplastic syndrome/MPN (MDS/MPN) (Andrieux *et al*, 2004; Odero *et al*, 2005), suggesting the possibility that deregulated expression of *HMGA2* due to

truncation of 3'UTR contributes to the pathogenesis of MPNs. Deregulated expression of *HMGA2* mRNA has also been found in several MPN patients without rearrangement of chromosome 12q and among patients without information about abnormalities of chromosome 12q (Andrieux *et al*, 2004; Guglielmelli *et al*, 2007; Bruchova *et al*, 2008). However, the cause of deregulated *HMGA2* expression is unknown in these patients, and it is unclear how often *HMGA2* is overexpressed in MPNs among subtypes.

In this study, we investigated the expression levels of *HMGA2* mRNA in haematopoietic cells of patients with MPNs, to clarify the frequency and role of expression of *HMGA2* mRNA in association with *let-7* miRNAs in MPNs. We found an unexpectedly high frequency of deregulated *HMGA2* mRNA expression, which was correlated with decreased expression of *let-7* miRNAs. Strikingly, we also found that panobinostat significantly reduced *HMGA2* mRNA and protein expression by increasing *let-7* expression dependent upon 3'UTR of *HMGA2* in both myeloid leukemia-derived U937 cells and PMF-derived $CD34^+$ cells, suggesting that *let-7*/*HMGA2* axis can be targeted by the HDAC inhibitor in MPNs.

Materials and methods

Patients

We studied 66 Japanese patients with MPNs, including 23 PV, 33 ET, and 10 PMF. Diagnoses were made according to World Health Organization criteria (Vardiman *et al*, 2009), and clinical and laboratory findings were investigated at the time of examination. As controls, samples from 13 age-matched healthy individuals were used. This study was approved by Ethics Review Board of Fukushima Medical University, which is guided by local policy, national law, and the Declaration of Helsinki. All investigations were performed after properly documented informed

consent.

Cells

Granulocytes and mononuclear cells (MNCs) were separated from peripheral blood samples by centrifugation through Ficoll (Lymphosepar I; IBL, Gunma, Japan) as described previously (Ikeda *et al*, 2007). $CD34^+$ cells were prepared from peripheral MNCs of 2 PMF patients by MACS CD34 MicroBead UltraPure Kit (Miltenyi, Bergisch Gladbach, Germany). Control $CD34^+$ cells from bone marrow of 2 healthy individuals were purchased from LONZA (Allendale, NJ, USA). U937 cells were cultured in RPMI1640 (Life Technologies, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Nichirei, Tokyo, Japan) at 37ºC and 5% CO_2. Cell numbers and viabilities were evaluated by TC10 automated counter (BioRad, Hercules, CA, USA) with trypan blue (BioRad).

JAK2V617F allele burden

*JAK2*V617F was examined by either alternately binding probe competitive PCR (ABC-PCR) or allele-specific quantitative PCR (AS-qPCR), as described previously (Edahiro *et al*, 2014). In brief, genomic DNA, extracted from peripheral blood samples by QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), was applied for PCR. Titanium Taq PCR kit (Takara Bio, Otsu, Shiga, Japan) with primers and a fluorescence-conjugated AB-probe was used for ABC-PCR, and allele burden was determined from fluorescence intensities measured at 95°C and 55°C based on a standard curve plotted from the controls. AS-qPCR, which is more sensitive than ABC-PCR, was also performed using a set of primers and TaqMan probe with a Universal PCR Master Mix (Life Technologies), if ABC-PCR showed less than 10% allele burden. Allele

burdens over 10% in ABC-PCR or 1% in AS-qPCR were considered to be positive for the *JAK2*V617F.

Panobinostat treatment

The pan-HDAC inhibitor, panobinostat, was provided by Novartis Pharmaceuticals (Basel, Switzerland) and stored in solution with dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO, USA). U937 and PMF-derived $CD34^+$ cells were cultured in the presence of a panobinostat solution for 8 hours, and applied for further experiments. As controls, cells were incubated in medium containing only DMSO.

Small interfering RNA of HMGA2

U937 cells were seeded at 0.5 x 10^6 / well in 12-well plates. Then 300 nM HMGA2 or control small interfering RNA (siRNA) (Thermo Fisher Scientific, Waltham, MA, USA) was transferred directly into the cell nucleus by electroporation using Nucleofector II (LONZA) according to the manufacturer's protocol. Cells were incubated and collected 18 hours later.

Quantitative real-time RT-PCR

The mRNA and miRNA expression levels were determined by quantitative real-time reverse transcription PCR (qRT-PCR). Total RNA was extracted from cells using the miRNeasy Mini Kit (Qiagen). Reverse transcription was performed using RevarTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan) or TaqMan MicroRNA RT Kit (Life Technologies) for mRNA or miRNA assay, respectively. QRT-PCR was carried out using Thermal Cycler Dice Real Time System (Takara Bio) and the data were analyzed by Multiplate RQ software (Takara Bio) with

ddCT method. Gene expressions were determined as relative to *HPRT1* mRNA. The reagents and primers used for qRT-PCR assay in this study are listed in Table 1.

Methylation-specific PCR (MSP)

Genomic DNA was treated by sodium bisulfite with MethylEasy Xceed Rapid DNA Bisulphite Modification Kit (Human Genetic Signatures, North Ryde, NSW, Australia). The DNA methylation was examined by MSP assay with EpiScope MSP Kit containing SYBR green (Takara Bio) using real-time PCR by Thermal Cycler Dice Real Time System according to the manufacturer's protocol. Previously described primer pair specific for methylated or un-methylated *p16* promoter was used for the assay (Table 1) (Christiansen *et al*, 2003; Jost *et al*, 2007). The amplifications observed in the real-time PCR by the methylation-specific pair were confirmed by electrophoresis with a 1.5% agarose gel and/or direct sequencing after treatment with QIAquick PCR purification kit (Qiagen) of PCR products. Bisulfite-modified genomic DNA derived from DNMT knocked-down cells (Zymo Research, Irvine, CA, USA) and fully methylated by SssI (Zymo Research), served as a negative and positive controls, respectively. Bisulfite-untreated genomic DNA samples were also used as controls.

Western blotting

Total protein was extracted from cells using CelLytic M assay buffer containing protease inhibitor cocktail (both from Sigma). Samples were applied to SDS-PAGE, transferred to a nitrocellulose membrane (GE Healthcare Life Sciences, Uppsala, Sweden), blocked with 5% bovine serum albumin (Wako, Tokyo, Japan) and probed with primary antibodies to HMGA2 (Cell Signaling Technologies, Danvers, MA, USA, Catalogue No. 8179), DNMT1 (Cell

Signaling Technologies, Catalogue No. 5302), DNMT3a (Catalogue No. 3598), acetylated histone H3 (Lys27) (Cell Signaling Technologies, Catalogue No. 8173), and ACTB (Santa Cruz Biotechnology, Dallas, TX, USA, Catalogue No. 47778), and anti-rabbit secondary antibodies (Santa Cruz Biotechnology, Catalogue No. 2004). The membrane was transferred and then signals were detected by ECL method.

Statistical analysis

Differences in the *HMGA2* mRNA levels among controls and patients with PV, ET, and PMF were analyzed by Steel-Dwass test. The 2-sided Student *t* test was used in comparisons for pairs of continuous variables. In cells incubated with multiple concentrations of panobinostat, the Tukey's honestly significant difference test was used to compare different concentrations. The categorical variables were compared by the χ^2 or Fisher's exact test, as appropriate. For multiple categorical variables, Bonferroni correction was used to determine the significance. All data are represented as the mean ± standard deviation (SD). *P* values are two-sided, with $P < 0.05$ considered significant.

Results

High frequency of deregulated HMGA2 expression in MPNs

We investigated expression levels of total *HMGA2* mRNA using the TaqMan gene expression assay for the location of the first two exons, common to each transcript variant (Figure 1), in 66 patients with MPNs (Table 2), to study how often *HMGA2* is differentially expressed in MPNs. Peripheral granulocytes of patients with PMF showed highest *HMGA2* mRNA levels, compared

with those of PV ($P < 0.0001$), ET ($P < 0.0001$), and controls ($P < 0.0001$, Figure 2A). High *HMGA2* mRNA level (>1.0), which was determined as relative expression level above mean + 2 SD of *HMGA2* mRNA levels in 13 controls, was detected in all PMF (100%), and often in PV (21.7%) and ET (27.3%). Frequency of high *HMGA2* mRNA level was higher in PMF versus PV ($P < 0.0001$) and ET ($P = 0.0002$). We were able to determine the ratio of variants 1 and 2 in 17 MPN patients with high *HMGA2* mRNA levels (Figure 2B). Variant 1 of *HMGA2* mRNA contains the full-length 3'UTR with all the *let-7*-specific sites, whereas variant 2, lacking 3'UTR, does not possess *let-7*-specific sites (Figure 1). In 15 of these 17 patients (88.2%), transcript variant 1 of *HMGA2* mRNA was much more abundant than variant 2, while only variant 2 was detected in 2 patients (11.8%).

Distinctive clinical feature in MPN patients with deregulated HMGA2 mRNA

We studied correlations between expression of *HMGA2* mRNA and clinical/laboratory findings in MPNs. Patients with high *HMGA2* mRNA are depicted in Table 3. The positivity and allele burden (45.3 ± 38.3% vs. 43.9 ± 32.9%, respectively) of *JAK2*V617F were comparable between patients with high *HMGA2* mRNA levels and other patients without high *HMGA2* mRNA levels (Figure 2A). There was no significant correlation between *HMGA2* mRNA levels and *JAK2*V617F allele burdens (Figure 3A, Table 4). Among parameters investigated, serum LDH values, but not white blood cell counts (WBC) or haemoglobin concentrations (Hb), were significantly correlated with *HMGA2* mRNA levels (Figure 3B, Table 4). Palpable splenomegaly was more frequently noted in patients with high *HMGA2* mRNA levels compared with other patients (Figure 3B). On the contrary, WBC and Hb, rather than LDH values, were significantly correlated with *JAK2*V617F allele burdens, and there were no differences in the

frequency of palpable splenomegaly between patients with *JAK2*V617F and those without the mutation (Figure 3C). There were 8 patients (12.1%) whose *HMGA2* mRNA levels were even higher than 10-fold of the mean expression level in controls (Table 3). All of these 8 patients showed splenomegaly and/or elevated LDH values, and 6 of them (75.0%) were positive for *JAK2*V617F.

Decreased expression of let-7 miRNAs in MPNs with deregulated HMGA2 mRNA expression

We studied the cause of deregulated expression of *HMGA2* mRNA in patients with MPNs. Chromosomal rearrangement of 12q13-15 that truncates 3'UTR of *HMGA2* gene is a well-known cause of *HMGA2* deregulation in various cancers (Mayr *et al*, 2007; Lee & Dutta, 2007). However, in our study, no patients showed such an abnormality although other abnormal karyotypes were detected in some (Table 3). In contrast, the expressions of *let-7a* and *-7c* miRNAs were significantly repressed in patients with high expression levels of *HMGA2* mRNA compared with other patients (Figure 4), while patients with *JAK2*V617F did not show differential expression of *let-7* miRNAs (not shown). It has been also reported that PcG-related BMI1 or EZH2 can repress expression of *HMGA2* (Oguro *et al*, 2012; Muto *et al*, 2013), but we did not find any difference in expression of *BMI1* mRNA [relative expression level; 7.5 ± 11.0 (n = 23) vs. 6.5 ± 10.1 (n =41), respectively, $P = 0.7$] or *EZH2* mRNA (not shown) in peripheral granulocytes between patients with high *HMGA2* mRNA level and other patients.

Methylation of the p16 promoter associated with deregulated HMGA2 mRNA

HMGA2 is a known negative regulator of *p16* (Nishino *et al*, 2008, 2013; Lee *et al*, 2011), and

HMGA2 may play some important roles in epigenetic modulations through aberration of DNA methylation (Sun *et al*, 2013). Therefore, we examined DNA methylation of the *p16* promoter with MSP assay (Figure 5A). Interestingly, methylation of the *p16* promoter was more frequently detected in patients with high *HMGA2* mRNA levels than other patients ($P = 0.043$, Table 5). Proportions of the methylated *p16* promoter DNA relative to unmethylated DNA in patients with deregulated *HMGA2* mRNA ranges from 1.1% to 33% (Table 3). In 2 patients without deregulated *HMGA2* mRNA, proportions of the methylated *p16* promoter DNA were 1.3% and 6.7%.

We then investigated if deregulation of *HMGA2* expression contributes to methylation of the *p16* promoter. We found that U937 cells express *HMGA2* mRNA (Figure 6) and HMGA2 protein, and show hyper-methylation of the *p16* promoter. When we knocked-down *HMGA2* with siRNA in U937 cells, the *p16* promoter was significantly demethylated (Figure 5B) with an increase of *TET3* mRNA expression (1.4-fold, p=0.01) than cells treated with control siRNA, suggesting that *HMGA2* expression may lead to methylation of the *p16* promoter. Expressions of *TET1* and *TET2* mRNAs were not different between in *HMGA2* knocked-down cells and cells treated with control siRNA (not shown).

Pan-HDAC inhibitor, panobinostat, increased let-7 expression and reduced HMGA2 expression in a 3'UTR-dependent manner

Subsequently, we sought a therapeutic option that can alter expressions of *let-7* miRNAs and *HMGA2*. HDAC inhibition has been reported to suppress *HMGA2* expression and modulate expressions of miRNAs in non-haematopoietic cells and cord blood-derived multipotent cells (Lee *et al*, 2011; Ferguson, 2003; Fazio *et al*, 2012). Moreover, clinical efficacy of a pan-HDAC

inhibitor, panobinostat, has been reported in some patients with PMF (Mascarenhas *et al*, 2013; DeAngelo *et al*, 2013a, 2013b). Therefore, we tried to study effects of panobinostat on expressions of *let-7* and *HMGA2* in haematopoietic cells that highly express *HMGA2*. We found significantly higher *HMGA2* mRNA levels in $CD34^+$ cells from each 2 PMF patient examined compared with the control $CD34^+$ cells, as well as in U937 cells compared with HL60 cells (Figure 6). Thus, we examined expressions of *let-7* and *HMGA2* in U937 cells and $CD34^+$ cells from a PMF patient (S127; PMF $CD34^+$ cells) after incubation in the presence of panobinostat. Interestingly, treatment with panobinostat increased expressions of *let-7*-family miRNAs (Figures 7A and 9A), but decreased expressions of *HMGA2* mRNA and/or HMGA2 protein in both U937 cells (Figure 7B-C) and PMF $CD34^+$ cells (Figure 9B). We found that variant 1 of *HMGA2* mRNA, which contains 3'UTR with *let-7*-specific sites, was reduced, while variant 2 lacking 3'UTR showed similar expression levels compared with before treatment with panobinostat in both U937 cells (Figure 8A) and PMF $CD34^+$ cells (Figure 9C), indicating that panobinostat decreased expression of *HMGA2* through 3'UTR of *HMGA2* mRNA by increasing expressions of *let-7* miRNAs. We also assessed whether treatment by panobinostat for a dysregulated *let-7*/*HMGA2* axis may be a therapeutic option for MPNs with respect to the DNA methylation. We found significant demethylation of the *p16* promoter with substantial reductions in the expressions of DNMT1 and DNMT3a as well as HMGA2, and decreased survival in U937 cells, after panobinostat treatment (Figure 8B-C).

Discussion

Here, we showed that deregulated expression of *HMGA2* mRNA associated with reduced expressions of *let-7* miRNAs is common in MPNs. In particular, 100% of PMF (10/10 cases)

showed high levels of *HMGA2* mRNA in either peripheral granulocytes or $CD34^+$ cells, in line with a few previous studies that showed deregulated expression of *HMGA2* mRNA in most patients with PMF (Bruchova *et al*, 2008; Andrieux *et al*, 2004; Guglielmelli *et al*, 2007). Our data also revealed that not only in PMF but also in certain populations of PV and ET, haematopoietic cells harbor deregulated *HMGA2* mRNA expression. Interestingly, deregulated *HMGA2* mRNA expression seems to be associated with elevation of LDH values and presence of splenomegaly. Together with the finding that overexpression of *Hmga2* causes proliferative haematopoiesis and facilitates progression of myelofibrosis while providing clonal advantage in mice (Ikeda *et al*, 2011; Oguro *et al*, 2012), *HMGA2* may contribute to clinical presentation and pathogenesis, at least in some patients with MPNs.

We found high expression levels of *HMGA2* mRNA both in *JAK2*V617F$^-$ and *JAK2*V617F$^+$ patients, and distinct clinical features between *JAK2*V617F$^+$ patients and patients with deregulated *HMGA2* mRNA expression. Although 3'UTR truncation of the *HMGA2* gene due to abnormalities in chromosome 12q13-15 is the well-known cause of deregulated *HMGA2* expression (Mayr *et al*, 2007; Lee & Dutta, 2007), none of our patients showed such an abnormality (Table 3). Consistent with our observation, it has been previously reported that only 2 of 12 peripheral blood samples of PMF patients with deregulated *HMGA2* mRNA expression showed chromosomal rearrangement in the locus of the *HMGA2* gene (Andrieux *et al*, 2004). In addition, haematopoietic cells highly expressed *HMGA2* mRNA in most patients with paroxysmal nocturnal haemoglobinuria (PNH), but chromosomal rearrangement in the locus of *HMGA2* was also rare in these patients (Murakami *et al*, 2012). Rather, our MPN patients with high *HMGA2* mRNA showed significantly reduced expressions of *let-7a* and *-7c* miRNAs, which may lead to deregulation of *HMGA2* expression. In fact, in most of our patients

with high expression of *HMGA2* mRNA, variant 1 of *HMGA2* mRNA that contains full-length 3'UTR with *let-7*-specific sites was more abundantly expressed than variant 2 lacking *let-7* sites, as well as in patients with PNH (Murakami *et al*, 2012). Our primer pair for variant 1 of *HMGA2* mRNA did not fully cover its 3'UTR and we could not rule out the possibility that there might be some mutations or deletions involving *let-7*-specific sites in *HMGA2*, although no deletion was detected in the region of *HMGA2,* at least in chromosomal analysis for multiple cells in patients with high *HMGA2* mRNA levels (Table 3).

It has been reported that *HMGA2* expression is also regulated by PcG-related epigenetic modifier genes (Oguro *et al*, 2012; Muto *et al*, 2013). In breast cancer cells, depletion of *HMGA2* leads to demethylation of the *HOXA9* promoter DNA by inducing expression of *TET1* that demethylates promoters of various tumor suppressor genes (Sun *et al*, 2013). Our study showed no differences in the expressions of PcG-related genes in granulocytes between MPN patients with high levels of *HMGA2* mRNA and other patients. On the other hand, there was higher frequency of methylation of the *p16* promoter in patients with high levels of *HMGA2* mRNA (30%) compared with other patients (6%), and the overall frequency of methylation was 15%, in our present study. According to the study of Jost et al. (2007), overall frequency of methylation of the *p16* promoter in patients with MPN was 2.6%. So far, it is unclear how often the *p16* promoter is methylated because there have been few studies of this issue. A possibility is that MSP with real-time PCR is highly sensitive but less specific, which might have resulted in high frequency of *p16* methylation in patients of our study. However, knocking-down of *HMGA2* resulted in demethylation of the *p16* promoter with an increase of *TET3* mRNA expression in U937 cells, supporting the possibility that deregulated expression of HMGA2 may correlate with methylation of the *p16* promoter in some patients with MPNs. It is unclear

if such methylation occurs through passive or active processes, but an active process may be more likely because passive demethylation requires at least some cycles of cell divisions (Chen & Riggs, 2011). Demethylation of *p16* promoter by knocking down of HMGA2 was observed at 18 hours of incubation, within the doubling time of U937 cells, which was approximately 20 hours in our U937 cells, as reported elsewhere (Huang *et al*, 2001). Upregulation of *TET3* might have contributed to such demethylation in U937 cells. This finding is interesting because deregulation of *HMGA2* expression leads to self-renewal of stem cells in part by regulating *p16* (Nishino *et al*, 2008, 2013), and HMGA2 may contribute to progression of myelofibrosis in *p16*-deficient mice (Oguro *et al*, 2012).

Our present study revealed that panobinostat reduced *HMGA2* by increasing expressions of *let-7* miRNAs in both myeloid leukemia-derived U937 cells and PMF $CD34^+$ cells, indicating that panobinostat targets neoplastic myeloid cells that overexpress HMGA2. This finding may be crucial, because deregulated expression of *HMGA2* mRNA was seen in the vast majority of patients with PMF, which has the worst prognosis among subtypes of MPNs. Some previous studies have also shown that HDAC inhibitors might modulate expressions of *miRNAs* and/or *HMGA2* in other cell types. Trichostatin A represses *Hmga2* mRNA in murine fibroblasts by decreasing transcriptional activators in the promoter of *Hmga2* (Ferguson *et al*, 2003). Valproic acid and sodium butyrate induced *let-7* miRNAs and then reduced *HMGA2* in human cord blood-derived multipotent cells (Lee *et al*, 2011). Panobinostat reduced HMGA2 expression by increasing *let-7b* expression in liver cancer cells (Fazio *et al*, 2012). It has been demonstrated that HDAC inhibitors, including panobinostat, may decrease the allele burden of *JAK2*V617F and provide significant clinical efficacies, including near complete response, in some patients (Mascarenhas *et al*, 2013) and in a mouse model (Akada *et al*, 2012). This may

be explained in part by the fact that HDAC inhibitors target heat shock protein 90 (HSP90) through HDAC6, which stabilize both wild type JAK2 and V617F JAK2 (Wang *et al*, 2009). HDAC inhibitors acetylate HSP90 by inhibiting HDAC6, resulting in an inhibitory effect on JAK2V617F protein. However, *JAK2*V617F alone does not explain the clonal expansion of this mutant. Interestingly, we have shown that overexpression of *Hmga2* mRNA and protein in the absence of regulation by *let-7* miRNA conferred a clonal advantage to HSCs (Ikeda *et al*, 2011). This finding and our present study may together suggest that inhibition of the *let-7*/*HMGA2* axis is a possible additional cause of the clinical efficacy of panobinostat. In addition, several investigations have suggested that either HDAC (Fiskus *et al*, 2006, 2009; Wang *et al*, 2009) or HMGA2 (Sun *et al*, 2013; Zong *et al*, 2012) is associated with epigenetic modulation, and panobinostat reduced DNMT1 expression (Fiskus *et al*, 2009). Likewise, we found that panobinostat decreased protein expression of HMGA2 along with those of both DNMT1 and DNMT3a, and simultaneously demethylated the *p16* promoter and reduced cell survival. DNMT1 and DNMT3a mainly play roles in passive and active methylation, respectively (Chen & Riggs, 2011), and methylation of *p16* promoter might be blocked by panobinostat through both processes. However, this issue may be more studied in the future because we collected panobinostat-treated cells at 8 hours after starting the incubation, before doubling time of U937 cells when active demethylation is unlikely to take place (Huang *et al*, 2001).

In conclusion, deregulated expression of *HMGA2* due to downregulation of *let-7* miRNAs may be common, and may correlate with some epigenetic modifications such as methylation of the *p16* promoter, at least in some patients with MPNs. Panobinostat can increase expressions of *let-7* miRNAs, and thereby reduce expression of *HMGA2* mRNA with 3'UTR-containing *let-7*-specific sites, indicating that the abnormality in the *let-7*/*HMGA2* axis is a

certain therapeutic target of HDAC inhibition in MPNs.

Conflict of interest

Panobinostat was provided by Novartis Pharmaceuticals to K.I.

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Author contributions

Contribution: K.H.S. designed the research, performed experiments, analyzed results, and wrote the manuscript; K.I. designed and organized the research, provided patient's samples, performed experiments, analyzed results, and wrote the manuscript; K.O. designed the research, provided patient's samples, interpreted the results, and wrote the manuscript; Y.T. designed the research, interpreted the results and wrote the manuscript.

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Table 1. Oligonucleotides and reagents for PCR assays in this study.

Some primers (* and †) are according to the previous reports.

Name	Description	Sequence or product ID (vendor)
TaqMan gene expression assay HMGA2	qRT-PCR (TaqMan)	Hs00171569_m1 (LifeTechnologies)
TaqMan gene expression assay BMI1	qRT-PCR (TaqMan)	Hs00995521_g1 (LifeTechnologies)
TaqMan gene expression assay HPRT1	qRT-PCR (TaqMan)	Hs99999909_m1 (LifeTechnologies)
TaqMan microRNA assay hsa-let-7a	qRT-PCR (TaqMan)	Assay ID 000377 (LifeTechnologies)
TaqMan microRNA assay hsa-let-7b	qRT-PCR (TaqMan)	Assay ID 002619 (LifeTechnologies)
TaqMan microRNA assay hsa-let-7c	qRT-PCR (TaqMan)	Assay ID 000379 (LifeTechnologies)
TaqMan microRNA assay U6 snRNA	qRT-PCR (TaqMan)	Assay ID 001973 (LifeTechnologies)
HMGA2 transcript variant 1	qRT-PCR (SYBR green)	Perfect Real Time Primer HA178474 (Takara bio) Forward: ACGCCCAAGAGGCAGACCTA Reverse: ACTGCTGCTGAGGTAGAAATCGAAC
HMGA2 transcript variant 2	qRT-PCR (SYBR green)	Perfect Real Time Primer HA148337 (Takara bio) Forward: ACGGCCAAGAGGCAGACCTA Reverse: AAGAGCTATCCTGGACTCCTCCAA
TET3*	qRT-PCR (SYBR green)	Forward: CCATTGCAAAGTGGGTGA Reverse: CGCACCAGGCAGAGTAGC
HPRT1	qRT-PCR (SYBR green)	Perfect Real Time Primer HA067805 (Takara bio) Forward: GGCAGTATAATCCAAAGATGGTCAA Reverse: GTCAAGGGCATATCCTACAACAAAC
p16 UM†	Unmethylated p16 promoter (Methylation-specific PCR)	Forward: 5'-TTATTAGAGGGTGGGGTGGATTGT-3' Reverse: 5'-CCACCTAAATCAACCTCCAACCA-3'
p16 M†	Methylated p16 promoter (Methylation-specific PCR)	Forward: 5'-TTATTAGAGGGTGGGGCGGATCGC-3' Reverse: 5'-CCACCTAAATCGACCTCCGACCG-3'

*Smith, A.E., Mohamedali, A.M., Kulasekararaj, A., Lim, Z., Gäken, J., Lea, N.C., Przychodzen, B., Mian, S.A., Nasser, E.E., Shooter, C., Westwood, N.B., Strupp, C., Gattermann, N., Maciejewski, J.P., Germing, U. & Mufti, G.J. (2010) Next-generation sequencing of the TET2 gene in 355 MDS and CMML patients reveals low-abundance mutant clones with early origins, but indicates no definite prognostic value. *Blood*, **116,** 3923–32.

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Table 2. Clinical and haematological findings, and presence of high HMGA2 mRNA levels, in patients with MPNs

Variables	PV (n = 23)	ET (n = 33)	PMF (n = 10)
Age (years)	66.6 ± 11.1	67.0 ± 14.6	70.8 ± 8.8
Sex (M/F)	12/11	16/17	8/2
WBC (x 10^9/L)	12.4 ± 5.4	12.7 ± 12.9	15.5 ± 15.9
Neutrophils (x 10^9/L)	10.1 ± 4.5	10.3 ± 12.6	10.1 ± 9.3
Hb (g/L)	16.8 ± 2.4†	12.3 ± 2.5	11.8 ± 3.0
PLT (x 10^9/L)	442 ± 189	871 ± 413†	368 ± 219
LDH (U/L)	278 ± 119	256 ± 101	381 ± 194
Splenomegaly (+/-)	7/16	5/28	7/3‡
JAK2V617F (+/-)	21/2	24/9	6/4
High HMGA2 mRNA (+/-)	5/18	9/24	10/0‡

Abbreviations: WBC indicates white blood cell count; Hb, haemoglobin concentration; PLT, platelet count; LDH, lactate dehydrogenase. † or ‡ respectively shows significantly higher value or frequency compared with other subtypes of MPNs ($P < 0.05$).

Table 3. Clinical and hematological findings of patients with high *HMGA2* mRNA levels

ID	Disease	HMGA2 mRNA	JAK2V617F (%)	p16 me† (%)	WBC (x 10^9/L)	Neut. (x 10^9/L)	Blast (%)	Hb (g/L)	PLT (x 10^9/L)	LDH (U/L)	SM	Chromosomal abnormality
S005*	PV	127	69.7	6.0	10.6	8.90	-	184	488	234	-	-
S026*	PV	234	99.2	-	10.2	9.14	-	128	448	634	-	-
S042	PV	1.16	62.8	-	8.20	6.31	-	165	543	156	-	-
S072	PV	2.70	93.7	-	15.2	13.7	-	181	389	297	+	-
S105	PV	1.84	95.9	n.d.	16.1	13.9	-	181	392	307	+	-
S107	PV	1.03	81.5	-	17.5	14.4	-	122	1282	227	+	-
S035	ET	1.13	-	-	5.83	4.37	-	135	654	172	-	-
S043	ET	1.78	67.9	-	12.7	10.3	-	144	1410	287	-	-
S050	ET	1.50	29.7	1.1	11.4	9.59	-	154	463	199	-	-
S074	ET	1.22	-	-	4.76	2.45	-	87	742	178	-	-
S075	ET	1.89	-	n.d.	7.00	4.97	-	150	932	241	-	-
S078*	ET	39.7	39.0	6.1	6.90	3.86	-	144	541	227	+	-
S091	ET	2.78	15.7	n.d.	4.24	2.23	-	114	217	175	-	-
S117	ET	2.74	48.2	7.7	8.74	7.43	-	71	1450	246	-	-
S023*	PMF	102	49.4	-	52.8	29.6	1	66	119	571	+	+8
S037*	PMF	82.6	85.3	-	6.05	4.24	-	128	47	630	+	t(8;15)(q24;q22)
S045	PMF	1.56	99.6	-	10.9	8.72	-	108	295	240	-	-
S046	PMF	1.70	89.6	-	6.65	4.79	-	124	247	225	-	-
S053	PMF	3.52	-	-	12.6	8.88	-	109	158	159	+	-
S089*	PMF	28.0	-	33	7.30	4.82	-	77	606	485	+	-
S093	PMF	2.53	-	-	6.22	4.36	-	137	552	228	-	-
S114*	PMF	13.8	48.2	2.6	36.0	25.2	2	158	628	597	+	-
S120	PMF	1.06	12.5	-	5.99	3.58	-	155	532	160	+	-
S127*	PMF	7.83	-	n.d.	10.4	7.15	3	119	498	511	+	add(4)(q21), add(8)(q22)

HMGA2 mRNA levels in peripheral granulocytes were examined by quantitative real-time RT-PCR. Promoter methylation of *p16* DNA was determined by quantitative real-time methylation-specific PCR. Above 1.0 of relative expression level, which is mean (0.4) + 2 SD (0.3) of controls (n = 13), was regarded as high *HMGA2* mRNA level. UID indicates unique ID; p16 me., promoter methylation of *p16* DNA; WBC, white blood cell count; Neut., neutrophil count; SM, palpable splenomegaly; n.d., not done. Chromosomal abnormality was studied in bone marrow and/or peripheral blood cells by banding method without addition of mitogen. * indicates patients whose HMGA2 mRNA level was more than 10-fold higher than the mean of controls (4.0). †: proportions of methylated *p16* promoter were quantified by MSP assay with real-time PCR.

Table 4. Correlation coefficients among hematological/clinical parameters, *HMGA2* mRNA levels, and allele burdens of *JAK2*V617F.

Variables	HMGA2 mRNA		JAK2V617F	
	r	P value	r	P value
JAK2V617F allele burden (%)	0.2241	0.070	-	-
HMGA2 mRNA level	-	-	0.2241	0.070
WBC	0.08710	0.49	0.4219	< 0.001
Neutrophils	0.03488	0.78	0.4715	< 0.001
Hb	-0.08091	0.52	0.2495	0.043
PLT	-0.1926	0.12	-0.1276	0.31
LDH	0.4687	< 0.001	0.2208	0.075

Table 5. Methylation of the p16 promoter in patients with high expression levels of HMGA2 mRNA and in those without.

Disease	Total	High HMGA2 mRNA level		P value*
		-	+	
PV	1/17 (5.8%)	0/13 (0%)	1/4 (25.0%)	0.24
ET	5/26 (19.2%)	2/19 (10.5%)	3/7 (42.9%)	0.10
PMF	2/9 (22.2%)	-	2/9 (22.2%)	-
Total	8/52 (15.4%)	2/32 (6.25%)	6/20 (30.0%)	0.043

Numbers of patients positive for the p16 promoter methylation/total number of examined patients in indicated categories are shown. *Frequencies of methylation were compared between patients with high expression levels of HMGA2 mRNA (+) and those without (-).

Figure 1

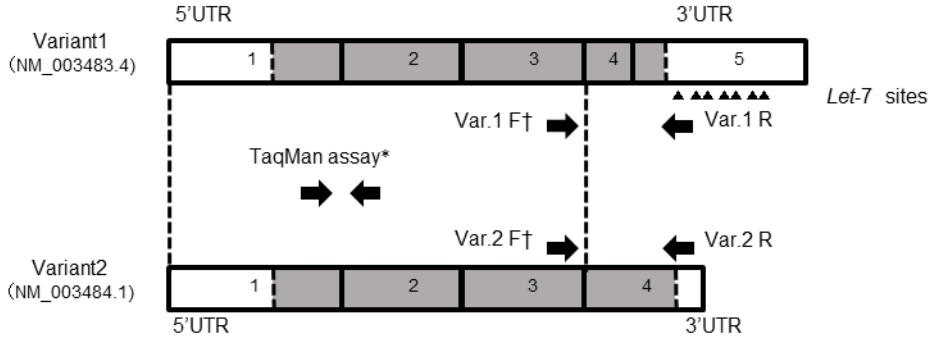

Figure 1. Expression of *HMGA2* mRNA in granulocytes of patients with MPNs.
The expression of HMGA2 is regulated by *let-7* miRNAs, which bind to specific sites in the 3'UTR of the *HMGA2* gene. Two major transcript variants of *HMGA2* mRNA are shown. Variant 1 contains *let-7*-specific sites, but variant 2 does not. *Quantitative real-time RT-PCR (qRT-PCR) was performed to determine relative expressions of total *HMGA2* mRNA using the TaqMan gene expression assay, with primers located on exons 1 and 2. Variants 1 (Var. 1) and 2 (Var. 2) were examined by qRT-PCR with SYBR green using indicated primer pairs. Arrows indicate locations of primers (also see Table 1). †Forward primer is common for both variants, but reverse primers are different.

Figure 2

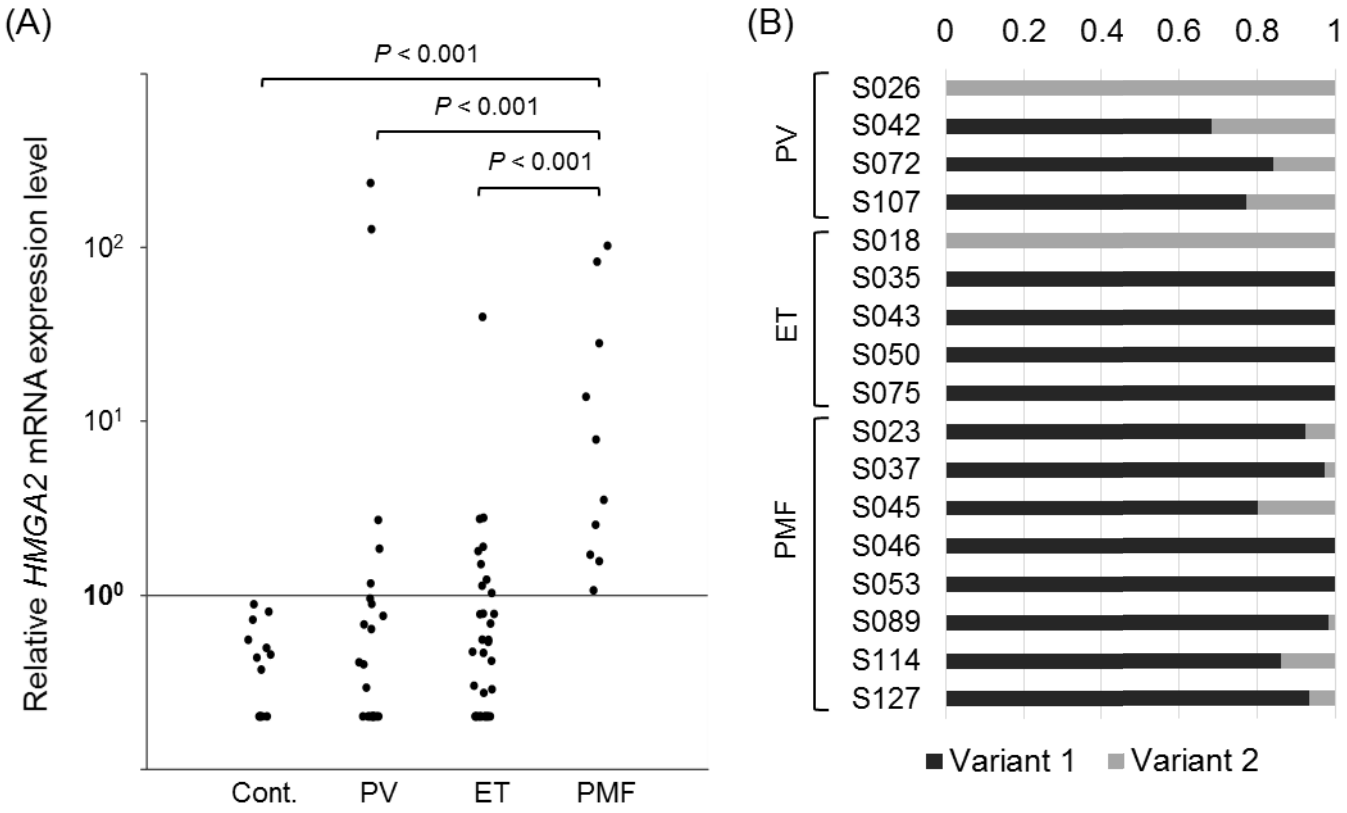

Figure 2. Expression of *HMGA2* mRNA in granulocytes of patients with MPNs

(A) Differential expression of total *HMGA2* mRNA relative to internal control *HPRT1* mRNA in granulocytes of controls, and patients with PV, ET, and PMF. Deregulated HMGA2 expression (>1.0), which was determined as relative expression level above mean + 2SD of *HMGA2* mRNA in 13 controls (above the indicated line), was most frequently detected in patients with PMF (Table 2). (B) Proportions of two transcript variants of *HMGA2* mRNA in patients with high *HMGA2* mRNA expression levels are shown. Variant 1 was more abundantly expressed in most of patients, whereas only Variant 2 was detected in 2 cases.

Figure 3

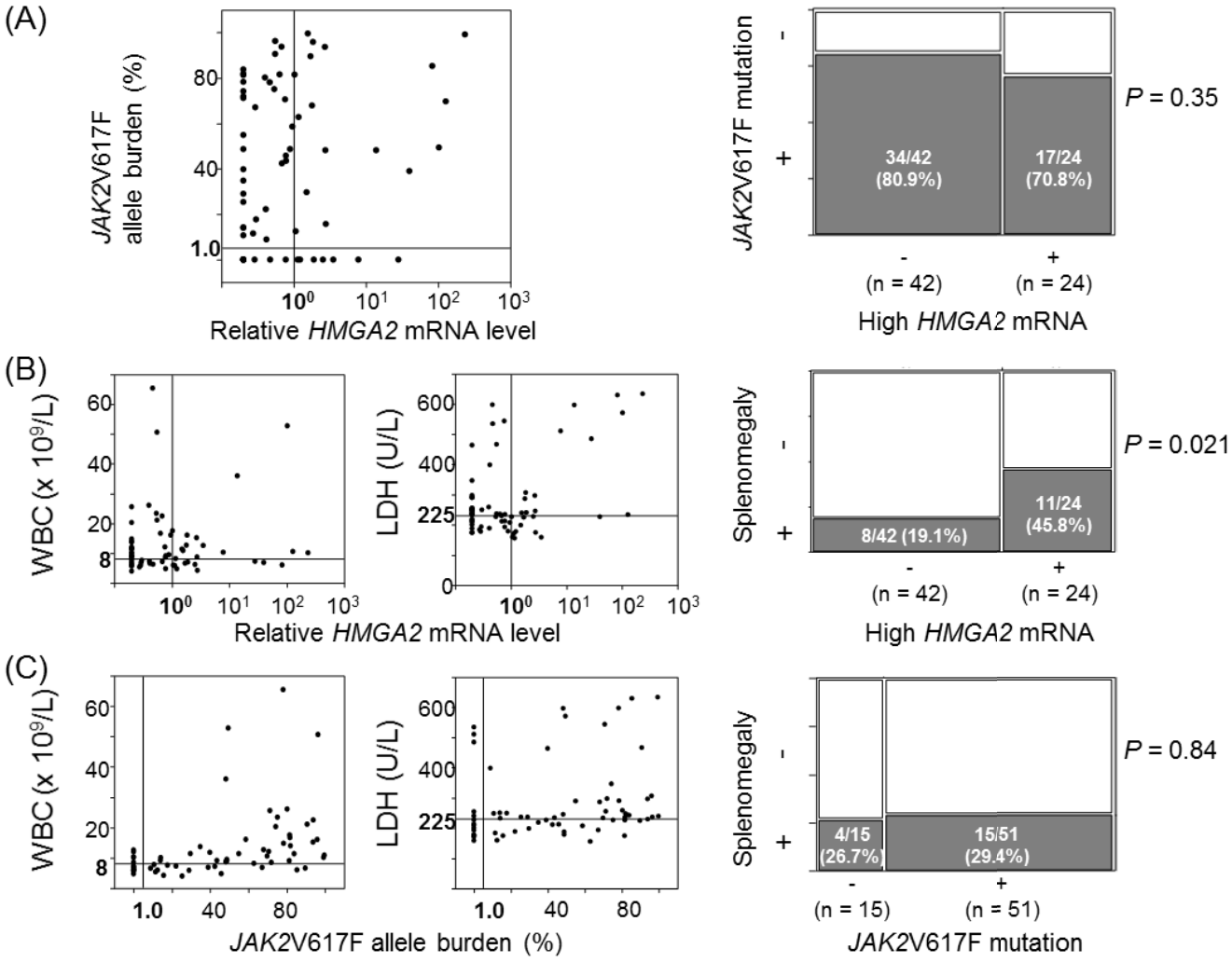

Figure 3. Correlation of *HMGA2* mRNA levels or *JAK2*V617F allele burdens with clinical findings. (A) *HMGA2* mRNA levels were not significantly correlated with *JAK2*V617F. (B-C) Deregulated *HMGA2* mRNA was correlated with serum LDH values and palpable splenomegaly, whereas *JAK2*V617F allele burden was correlated with WBC count (see Table 4).

Figure 4

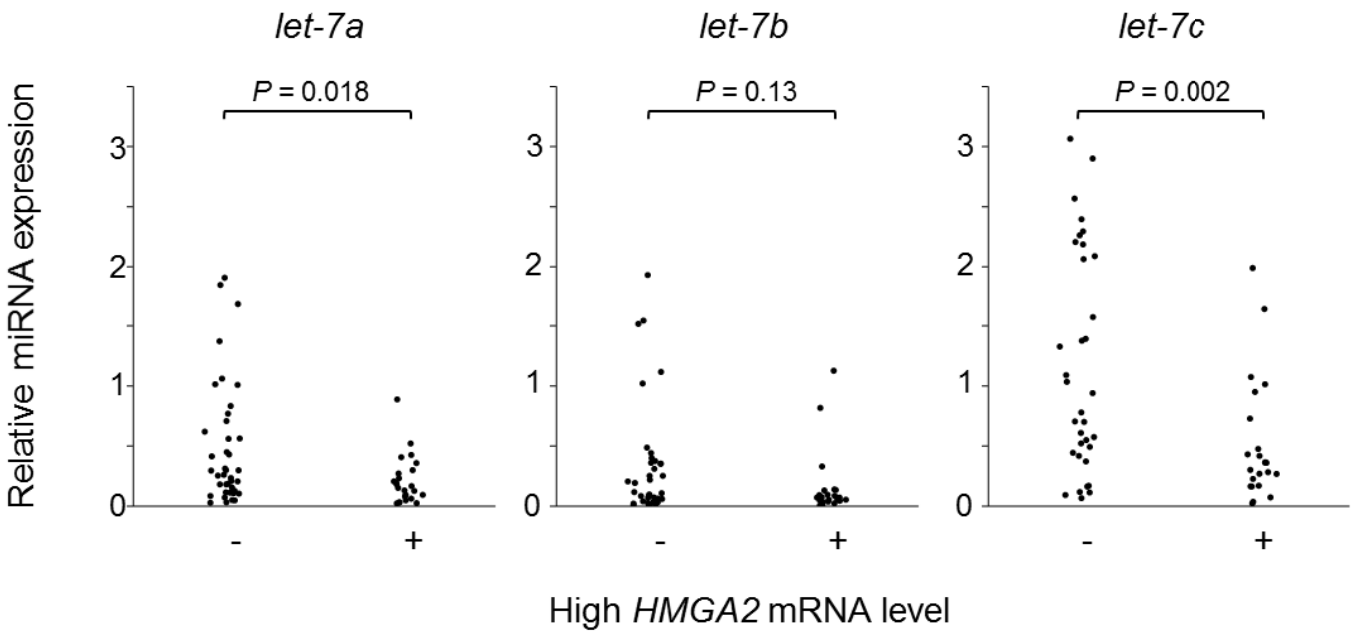

Figure 4. Repressed expression of *let-7* miRNAs in MPN patients with deregulated *HMGA2* mRNA.

Figure 5

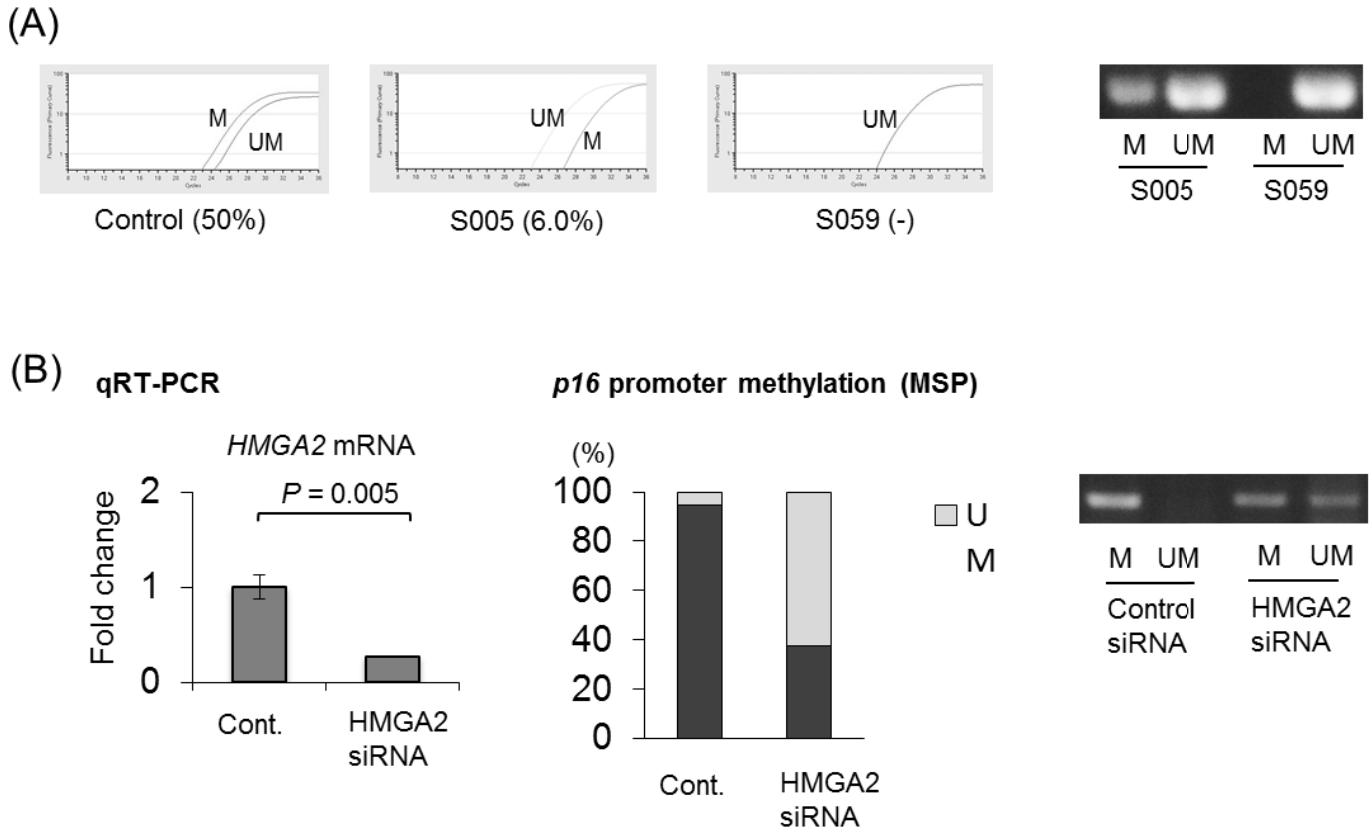

Figure 5. MSP assay for methylation of the *p16* promoter. Methylation of the *p16* promoter was examined by methylation-specific polymerase chain reaction (MSP) assay. UM and M indicate amplifications by primer pairs specific for unmethylated and methylated DNA, respectively. (A) Representative MSP assay with real-time PCR (left) and agarose gel electrophoresis of the PCR products (right) for 2 independent patients with MPNs. Percentages of methylated DNA was determined according to 50% fully-methylated/50% unmethylated bisulfite-converted controls (50%). S005 was significantly methylated (6.0%), whereas a signal was not detected in the primer pair for the methylated *p16* promoter in S059 (-), as well as in bisulfite-treated negative-control genomic DNA. Bisulfite-treated fully-methylated genomic DNA showed a signal only when a primer pair for methylated *p16* promoter was used (not shown). All lanes were run on the same gel. Data are shown in Tables 3 and 5. (B) Knocking-down of HMGA2 with siRNA in U937 cells. QRT-PCR shows significant reduction of *HMGA2* mRNA level after treatment with siRNA of HMGA2 compared with control siRNA. Proportion of methylated DNA was significantly reduced in samples from cells with siRNA of HMGA2 versus samples with control siRNA in MSP with real-time PCR (left). Agarose gel electrophoresis of MSP products also showed that the *p16* promoter was clearly demethylated by siRNA of HMGA2 (right). All lanes were run on the same gel. Representative results of 2 or 3 independent experiments are shown.

Figure 6

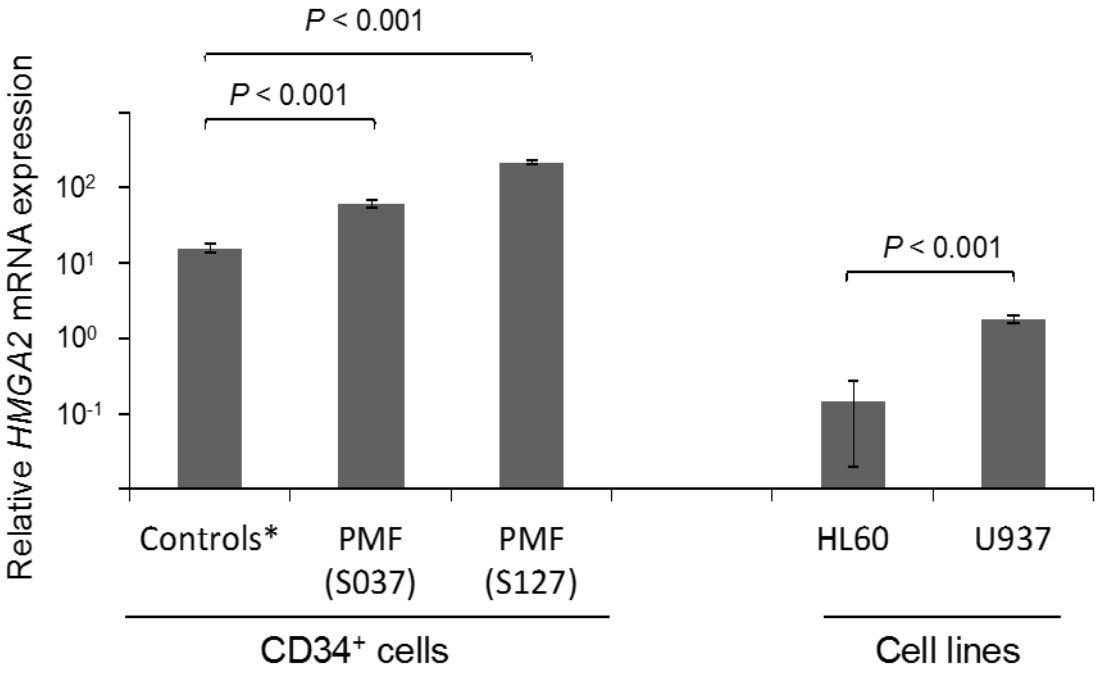

Figure 6. High expression levels of *HMGA2* mRNA in CD34⁺ cells of 2 PMF patients and U937 cells.

*Control CD34⁺ cells were obtained from 2 healthy individuals. Data are shown as mean ± SD.

Figure 7

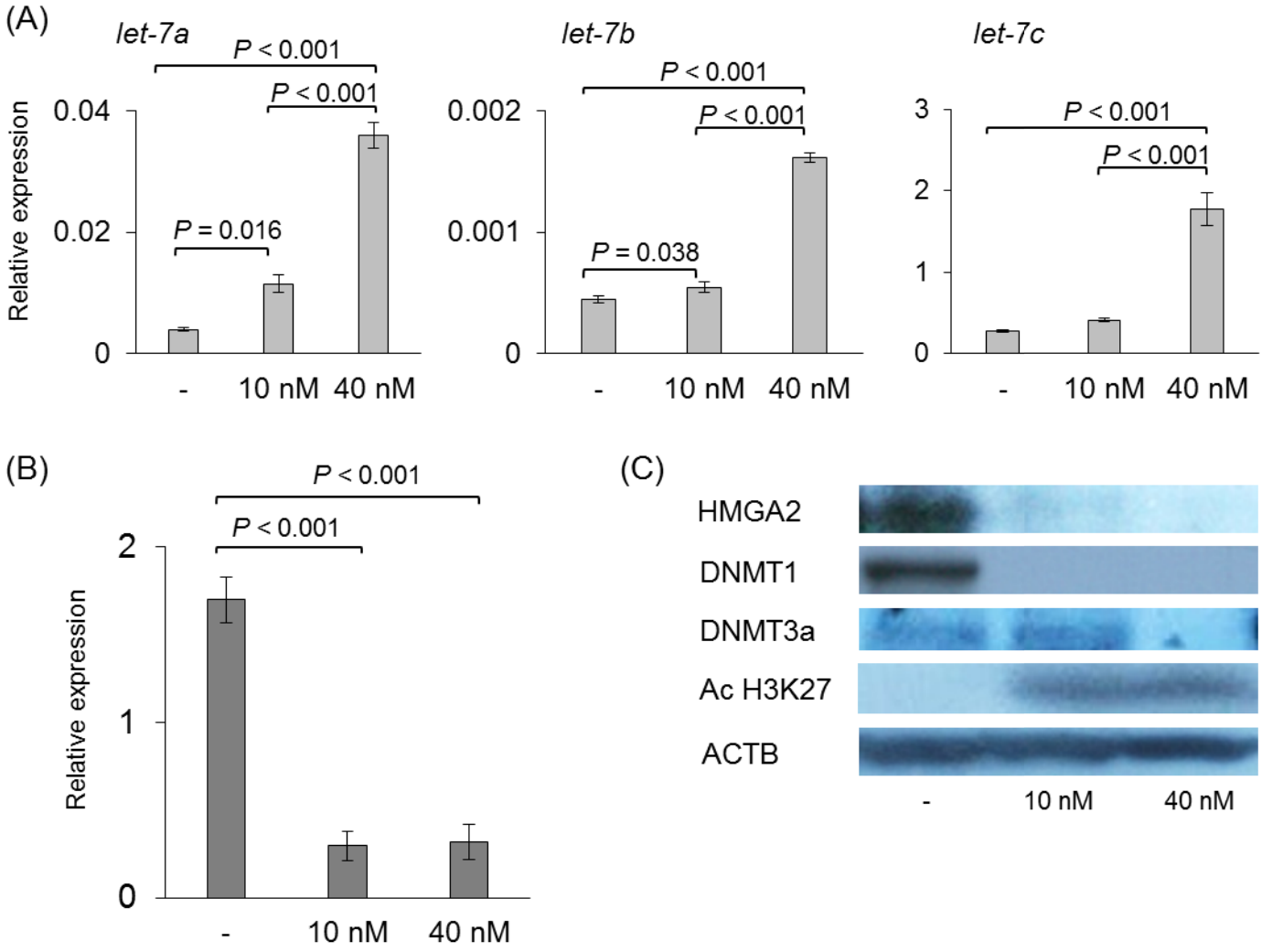

Figure 7. Effects of pan-HDAC inhibitor panobinostat for U937 cells. U937 cells were incubated for 8 hours in the absence or presence of indicated concentrations of panobinostat, and expressions of (A) *let-7* miRNAs, (B) total *HMGA2* mRNA (qRT-PCR), (C) HMGA2, DNMT1, DNMT3a, acetylated histone H3 (Lys27) (Ac H3K27) and ACTB proteins (Western blot).

Figure 8

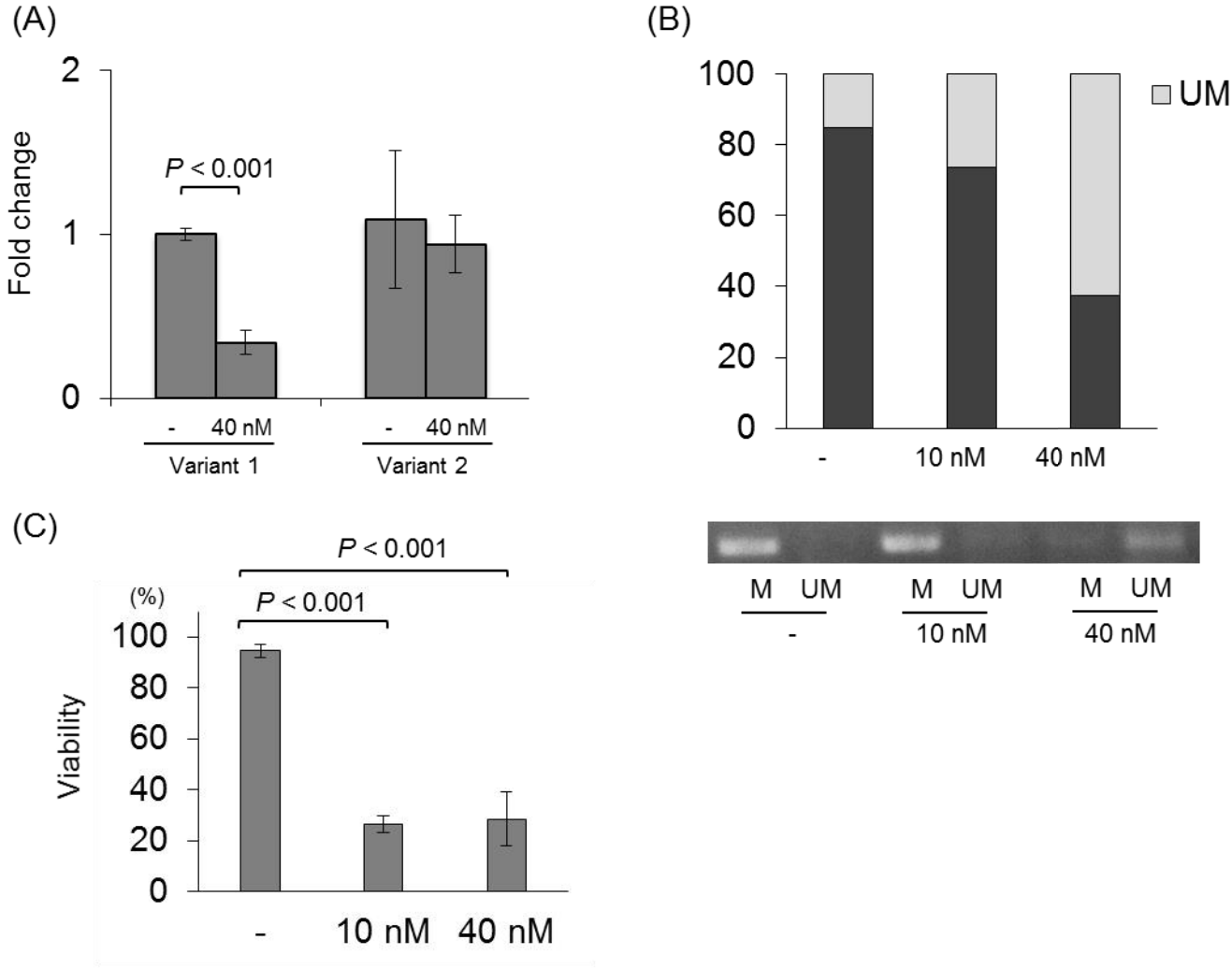

Figure 8. Effects of pan-HDAC inhibitor panobinostat for U937 cells

(A) variant 1 of *HMGA2* mRNA containing 3'UTR with *let-7*-specific sites and variant 2 without 3'UTR (qRT-PCR), were examined. (B) MSP assay for methylation of the *p16* promoter after incubation of U937 cells in the presence of panobinostat. Proportion of methylated DNA was significantly reduced in samples treated with panobinostat in MSP assay with real-time PCR. Agarose gel electrophoresis of MSP products also showed that the *p16* promoter was clearly demethylated by the treatment. (C) Cell viability after incubation of U937 cells in the presence of panobinostat. All lanes were run on the same gel in western blots and MSP assay. Representative results of 3 independent experiments are shown.

Figure 9

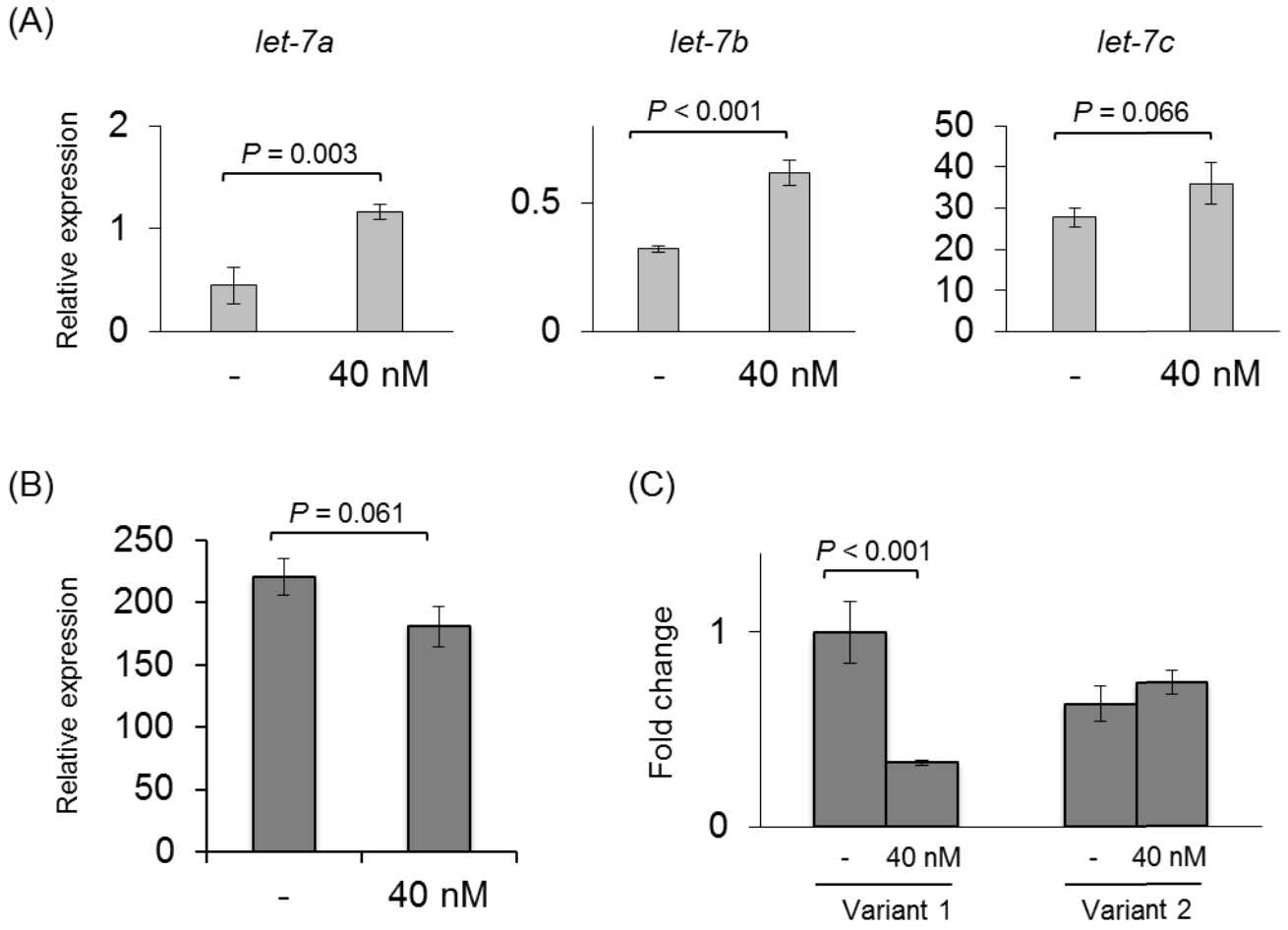

Figure 9. Effect of panobinostat for PMF-derived CD34⁺ cells on expressions of *let-7* miRNAs and *HMGA2*. MACS-sorted CD34⁺ cells were incubated in the presence of panobinostat. Changes in the expressions of (A) *let-7* miRNAs, (B) total *HMGA2* mRNA, and (C) indicated variants of *HMGA2* mRNA are shown.