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Infrequent hypermethylation of WIF-1 promoter in BCR/ABL-negative myeloproliferative disorders

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Objective: The Wnt/β-catenin signaling pathway is important in the pathogenesis of hematological malignancies. Wnt inhibitory factor-1 (WIF-1) is a negative regulator of Wnt signaling that is frequently down-regulated by hypermethylation of the *WIF-1* promoter in acute promyelocytic leukemia (APL) and other malignancies. On the other hand, an acquired mutation in JAK2 tyrosine kinase involving a V617F amino-acid substitution shows a strong association with the pathogenesis of *BCR/ABL*-negative MPD. This is the first study to examine the relationship between *WIF-1* methylation and the existence of *JAK2*V617F mutation in the pathogenesis of *BCR/ABL*-negative myeloproliferative disorders (MPD) including polycythemia vera, essential thrombocythemia, idiopathic myelofibrosis, and chronic myeloproliferative disease, unclassifiable.
Methods: We evaluated 49 newly diagnosed and previously treated patients with MPD in chronic phase. Bone marrow (BM) mononuclear cells, when available, or PB mononuclear cells of patients were used for the analysis. The mutation status of *JAK2* was analyzed using sequencing analysis. The methylation status of the WIF-1 promoter was analyzed by methylation-specific polymerase chain reaction (MSP).
Results: The *JAK2*V617F mutation was found in 23/49 patients (46.9%) with BCR/ABL-negative MPD, while *WIF-1* methylation was detected in 1/49 patients (2.0%).
Conclusion: *WIF-1* is infrequently methylated in *BCR/ABL*-negative MPD.

Key words: *BCR/ABL*-negative myeloproliferative disorders; *WIF-1*; hypermethylation; *JAK2*V617F mutation; leukemic transformation

1. INTRODUCTION

Myeloproliferative disorders (MPD) are clonal hematopoietic diseases characterized by independency or hypersensitivity of hematopoietic progenitors to numerous cytokines [1]. *BCR/ABL*-negative MPD comprises polycythemia vera (PV), essential thrombocythemia (ET), idiopathic myelofibrosis (IMF), and chronic myeloproliferative disease, unclassifiable (CMPD-U). An acquired mutation in JAK2 tyrosine kinase involving a V617F amino-acid substitution shows a strong association with the pathogenesis of *BCR/ABL*-negative MPD, and data on these disorders are steadily accumulating [1].

Hypermethylation of CpG islands in the promoter regions of tumor suppressor genes has been linked to the development of hematopoietic malignancies. A relationship between such epigenetic alterations and the *JAK2*V617F mutation was recently established for MPD [2]. These authors proposed the epigenetic inactivation of *SOCS-1*, a JAK2 negative regulator, as a complementary mechanism to the *JAK2*V617F mutation in the pathogenesis of MPD, leading to dysregulation of JAK-STAT signal transduction and contributing to growth factor hypersensitivity [2].

Wnt/β-catenin signaling is involved in virtually every aspect of embryonic development and also controls homeostatic self-renewal in a number of adult tissues, and a key pathway in the pathogenesis of many hematological malignancies [3, 4], as is epigenetic regulation of this pathway [5, 6]. *Wnt inhibitor factor-1 (WIF-1)*, a soluble negative regulator of Wnt signaling, is frequently hypermethylated in acute promyelocytic leukemia, but not core-binding factor leukemia [7, 8]. However, the function of *WIF-1* in MPD including CML has been unknown. In addition, activation of the Wnt/β-catenin pathway in granulocyte-macrophage progenitors may lead to not only expansion of the cells but also the subsequent production of blasts in *BCR/ABL*-positive MPD and chronic myelogenous leukemia (CML) [9]. In other words, when CML progresses to its acute blast crisis phase, activation of the β-catenin pathway is involved in self-renewal capacity, and becomes more leukemogenic. We thus considered that might co-exist with methylation of some promoter regions in the specific genes, and investigated the role of methylation of *WIF-1* in *BCR/ABL*-negative MPD may be an additional event to *JAK2*V617F mutation.

2. MATERIAL AND METHODS

2.1. Patient samples and cell line

We evaluated 49 newly diagnosed and previously treated patients with MPD in chronic phase (mean duration of disease: 47.6 months (range: 3.1-104.2 months), male/female: 32/17 patients, mean age: 63 years (range: 27-81 years), 15 patients with PV, 21

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Table 1. Clinical characteristics of 49 BCR/ABL-negative myeloproliferative disorder (MPD) patients according to JAK2V617F mutation status.

	JAK2			
Feature	V617F mutation (n = 23)	Wild-type (n = 26)	Total (n = 49)	*P*-value
Age (years)*	63 (27-81)	58 (80-30)	63 (27-81)	0.130
Sex (male / female)	11/12	21/5	32/17	0.016
Duration of disease* (months)	62.1 (3.3-104.2)	31.5 (3.1-104.2)	47.6 (3.1-104.2)	0.383
Hemoglobin (g/l)*	16.0 (4.4-19.6)	14.9 (7.9-21.3)	15.0 (4.4-21.3)	0.984
White cells ($\times 10^9$/l)*	15.1 (6.7-37.9)	7.35 (3.3-69.4)	11.6 (3.3-69.4)	<0.0001
Platelet count($\times 10^9$/l)*	555 (131-1722)	346 (49-1636)	494 (49-1722)	0.423
WHO classification				
PV (%)	7 (46.7%)	8 (53.3%)	15	
ET (%)	10 (47.6%)	11 (52.4%)	21	
IMF (%)	4 (80.0%)	1 (20.0%)	5	
CMPD-U (%)	2 (25.0%)	6 (75.0%)	8	

* Median (range)

Abbrebiations- JAK2:Janus Kinase 2; WHO:World Health Organization; PV:polycythemia vera; ET:essential thrombocythemia; IMF:idiopathic myelofibrosis; CMPD-U:chronic myeloproliferative disorder, unclassifiable.

Table 2. Type of therapies in *BCR/ABL*-negative MPD patients

	Number of patients
PV (n = 15)	
No treatment	8 (53.3%)
Phlebotomy	5 (33.3%)
Hydroxyurea	1 (6.7%)
Hydroxyurea+splenectomy	1 (6.7%)
ET (n = 21)	
No treatment	3 (14.3%)
Antiplatelet therapy	3 (14.3%)
Anticoagulant therapy	1 (4.8%)
Hydroxyurea	8 (38.1%)
Hydroxyurea+ antiplatelet therapy	5 (23.8%)
Hydroxyurea+splenectomy	1 (4.8%)
IMF (n = 5)	
No treatment	1 (20.0%)
Antiplatelet therapy	1 (20.0%)
Hydroxyurea	2 (40.0%)
Melphalan	1 (20.0%)
CMPD-U (n = 8)	
No treatment	5 (62.5%)
Antiplatelet therapy	1 (12.5%)
Hydroxyurea	2 (25.0%)

Abbreviation
MPD: myeloproliferative disorder
PV: polycythemia vera
ET: essential thrombocythemia
IMF: idiopathic myelofibrosis
CMPD-U: chronic myeloproliferative disorder, unspecified

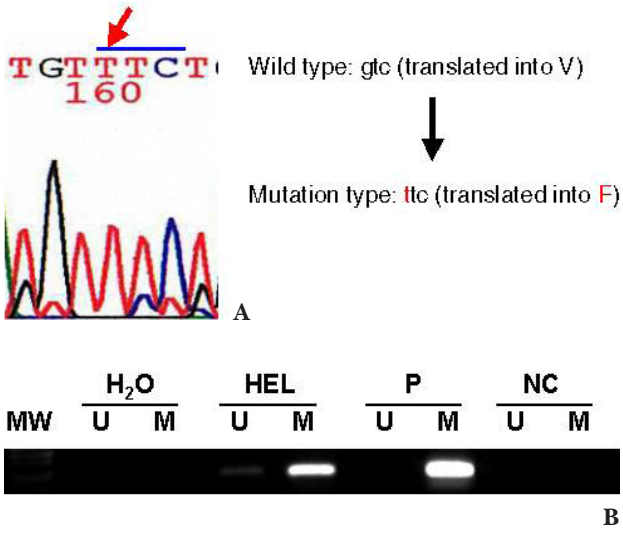
patients with ET, 5 patients with IMF and 8 patients with CMPD-U) to clarify the relationship between the existence of *JAK2*V617F mutation and the methylation status of the *WIF-1* promoter. The human erythroleukemia cell line HEL carrying *JAK2*V617F mutation was also analyzed to confirm the relationship between *WIF-1* methylation and *JAK2*V617F mutation (Table 1) (Fig. 1A). Type of therapies in *BCR/ABL*-negative MPD patients was shown in Table 2. Diagnosis was made in accordance with WHO criteria. Five peripheral blood (PB) samples from healthy volunteers were collected to serve as controls. HEL cell was maintained in culture in RPMI 1640 medium (Gibco BRL, Gaithersburg, ND, USA) supplemented with 10% fetal bovine serum (Gibco BRL) at 37°C in a humid atmosphere containing 5% CO_2. The study was approved by the Institutional Review Board and informed consent was provided according to the Declaration of Helsinki.

2.2. JAK2 mutation analysis

The mutation status of *JAK2* was analyzed using sequencing analysis to clarify the relationship between *JAK2*V617F mutation and MPD in our cohort. Bone marrow (BM) mononuclear cells, when available, or PB mononuclear cells of patients were used for the analysis. Genomic DNA was isolated using standard methods. Genomic PCR amplification was performed using the forward primer sequence 5'-TGCTGAAA GTAGGAGAAAGTGCAT-3'and the reverse primer sequence 5'-TCCTACAGTGTTTTCAGTTTCAA-3'. The PCR mix contained 10 × PCR Buffer, 20 pM of forward and reverse primers, 2.5 mM dNTPs each and 0.5 U TaKaRa Taq (Takara Bio, Otsu, Shiga, Japan). PCR cycling parameters were: one cycle of 94°C for 2 minutes; 35 cycles of 94°C for 30 seconds, 52°C for 40 seconds, and 72°C for 40 seconds; followed by one cycle of 72°C for 2 minutes. PCR products were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany), and sequenced on a DNA sequencer (310; Applied Biosystems, Foster City, CA, USA).

2.3. Methylation-specific polymerase chain reaction (MSP)

We conducted methylation-specific polymerase chain reaction (MSP) to clarify the relationship between *WIF-1* promoter and MPD in our cohort. High molecular weight DNA was extracted from samples using standard methods, and bisulfite modification of genomic DNA was performed using the Epitect Bisulfite Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). PB DNA from healthy donors was used as a negative control, and a Methylamp Universal Methylated DNA Kit (Epigentek,



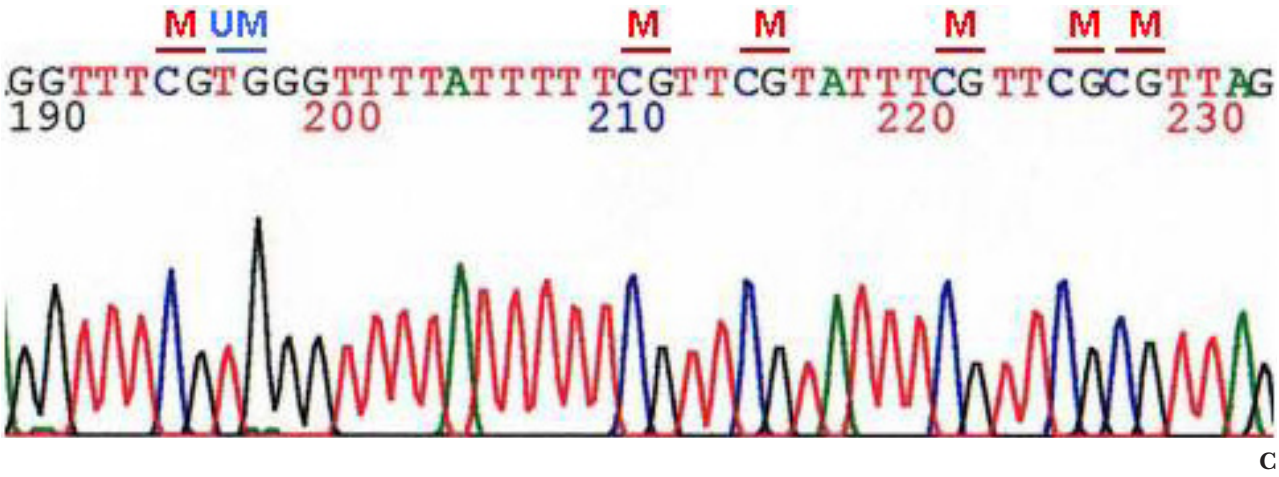


Fig. 1 *JAK2*V617F mutation status, methylation-specific polymerase chain reaction (MSP) and sequencing analysis in the HEL cell line.
(A)*JAK2*V617F mutation in HEL was confirmed by direct sequencing analysis. Red arrow indicates the point mutation characterized by a G to T transversion at nucleotide 1849 in exon 12 of the *JAK2* gene. (B)*WIF-1* MSP analysis in HEL. P indicates positive control. U and M indicate unmethylated and methylated PCR products, respectively. MW indicates DNA ladder. NC indicates negative control. (C)MSP product sequencing in HEL. *WIF-1* M-MSP PCR products were cloned into a pCR 2.1-TOPO plasmid transfected into *Escherichia coli*, and recombinant colonies were selected, and sequenced. Red bar and M indicate methylated CpG site, and blue bar and U indicate unmethylated CpG site.

Brooklyn, New York, USA) was used as a positive control for methylation-specific assays. The primers for the methylated (M-MSP), and unmethylated (U-MSP) promoters of *WIF-1* were as follows: M-MSP forward primer 5'-CGTTTTATTGGGCGTATCGT-3'; M-MSP reverse primer 5'-ACTAACGCGAACGAAATACGA-3'; U-MSP forward primer 5'-GGGTGTTTTATTGGG TGTATTGT-3'; U-MSP reverse primer 5'-AAAAAA ACTAACACAAACAAAATACAAAC-3'. PCR reactions were run in a final volume of 20 µl containing 100 ng bisulphite-treated DNA, 2.5 U HotStar Taq DNA Polymerase (Qiagen), 1.5 mmol/l $MgCl_2$, 0.2 mmol/l dNTPs and 200 nmol/l of each primer. PCR conditions for M-MSP of *WIF-1* were as follows: 95°C for 15 minutes; 32 cycles at 95°C for 30 seconds, 57°C for 30 seconds and 72°C for 30 seconds; and a final extension for 10 minutes. PCR conditions for U-MSP of *WIF-1* were as follows: 95°C for 15 minutes; 30 cycles at 95°C for 30 seconds, 52°C for 30 seconds and 72°C for 30 seconds; and a final extension for 10 minutes. MSP products were separated on 2% agarose gels, stained with a GelStar Nucleic Acid Stain (Cambrex, Baltimore, Maryland, USA) and visualized under ultraviolet (UV) light. In M-MSP positive cases, PCR products were cloned into a pCR 2.1-TOPO plasmid using a TOPO TA Cloning Kit (Invitrogen, Carlsbad, California, USA), then transfected into the *Escherichia coli*. Eight recombinant colonies were selected, and sequenced. DNA sequencing was performed on a DNA sequencer (310; Applied Biosystems, Foster City, CA,USA).

2.4. Statistical analysis

Differences in continuous variables were analyzed using the Mann-Whitney *U* test for distribution between 2 groups. Analysis of frequencies was performed using the Fisher exact test for 2 × 2 tables or the Pearson χ^2 test for larger tables. All statistical analyses were performed by using SPSS version 14.0 (SPSS, Chicago, IL, USA). For all analysis, the *P* value was 2-tailed, and $P < 0.05$ was considered statistically significant.

3. RESULTS

3.1. JAK2 mutation analysis in MPD patients

The *JAK2*V617F mutation was detected in 23 of the 49 patients (46.9%): 7 out of 15 patients (46.7%) with PV, 10 out of 21 patients (47.6%) with ET, 4 out of 5 patients (80.0%) with IMF, and 2 out of 8 patients (25.0%) with CMPD-U (Table 1). There were significant differences in sex and white blood cell count between patients with and without the *JAK2*V617F mutation ($P = 0.0160$ and <0.0001, respectively). There were no significant differences between these two patient groups, however, in age, duration of disease, hemoglobin level, and platelet count.

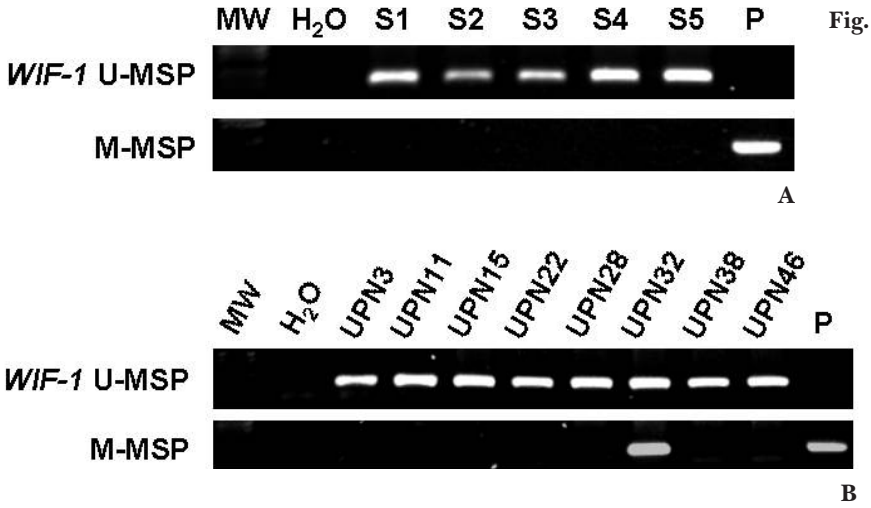


Fig. 2 *WIF-1* MSP in normal PB and *BCR/ABL*-negative MPD patient samples. (**A**)*WIF-1* MSP in 5 normal PB samples. (**B**)*WIF-1* MSP in patient samples with *BCR/ABL*-negative MPD. MW indicates DNA ladder. S1-5 indicate normal PB samples. U and M-MSP indicate unmethylated and methylated methylation-specific polymerase chain reaction, respectively.

3.2. WIF-1 methylation status in HEL cells and MPD patients

The HEL cells were positive for *WIF-1* M-MSP, and this was confirmed using sequencing (Fig. 1B, C). Since the *JAK2*V617F mutation is associated with *WIF-1* methylation in this cell line, we next examined PB samples from healthy controls and patient with *BCR/ABL*-negative MPD. *WIF-1* methylation was not detected in the normal PB samples (Fig. 2A), and in only 1 of 49 (UPN32; 2.0%) patients with *BCR/ABL*-negative MPD (Fig. 2B). The case with *WIF-1* methylation was a *JAK2*V617-positive patient with essential thrombocythemia carrying splenomegaly. Statistical analyses on the *WIF-1* methylation status could not be performed due to the small patient number (only 1 patient) with *WIF-1* methylation.

4. DISCUSSION

It is well known that the *JAK2*V617F mutation is frequently found in *BCR/ABL*-negative MPD. In contrast, our knowledge about an association between this kinase mutation and epigenetic regulation is limited. This is the first study to examine the role of *Wnt inhibitory factor-1 (WIF-1)* methylation in the pathogenesis of *BCR/ABL*-negative MPD.

We found the *JAK2*V617F mutation in 23 of the 49 patients (46.9%): 7 out of 15 patients (46.7%) with PV, 10 out of 21 patients (47.6%) with ET, 4 out of 5 patients (80.0%) with IMF, and 2 out of 8 patients (25.0%) with CMPD-U. Although several studies reported the high frequency of *JAK2*V617F mutation in PV cases [10, 11], there were 7 out of 15 patients (46.7%) with PV patients in our study. Also, Ohyashiki et al reported in their study that they found the *JAK2*V617F mutation in 24 out of 33 patients (72.7%) with PV, and this percentage was relatively low too [12]. Also, it is suggested that this result may reflect the prevalence of *JAK2*V617F mutation in Japan. So, it is supposed that the low frequency of *JAK2*V617F mutation in our PV cases might be due to the difference of race by comparison without Japanese. However, it is necessary to accumulate more samples of PV cases to clarify the true prevalence of *JAK2*V617F mutation in our institute to verify this point.

White blood cell count was significantly different for patients with and without *JAK2*V617F mutation at diagnosis (p= <0.0001). In addition, patients with *JAK2*V617F mutation showed high hemoglobin and platelet count level, although there were no significant differences between these two patient groups. Biochemical studies have shown that the *JAK2*V617F mutation causes cytokine-independent activation of JAK-STAT, PI3K, AKT pathways, mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK), all of which are implicated in the receptor signaling of erythropoietin, thrombopirtin and granulocyte colony-stimulating factor (G-CSF), respectively [12-15]. So, our data might reflect these mechanisms.

WIF-1 methylation occurred infrequently, and although the only patient with *WIF-1* methylation carried the *JAK2*V617F mutation, suggesting it not major additional events to *JAK2*V617F mutation. The study of Jose E et al. [2] examined the DNA methylation status of 13 cancer-related genes in 39 patients with MPD, and reported at least one hypermethylated gene in 15/39 MPD patients, and aberrant methylation of the cytokine regulator *SOCS-1* in 6/39 samples. Since *WIF-1* methylation was also found in the *JAK2*V617F mutation-positive erythroleukemia cell line HEL in this study, we speculated that methylation plays a role in the progression of MPD. However, we found only one case with *WIF-1* methylation and a *JAK2*V617F mutation in patients with essential thrombocythemia. The case is stable in clinical course at this moment. These results suggest that *JAK2*V617F mutation may not be major additional events to *WIF-1* methylation.

The epigenetic therapy is promising. 5-Azacytidine and 5-aza-2'-deoxycytidine are cytosine analogues that trap all DNA methyltransferases and target them for degradation, and have shown clinical activity as anticancer agents against hematological malignancy like myelodysplastic syndrome (MDS) [16, 17]. Also, the development of more selective DNA methylation and histone acetylation inhibitors are ongoing.

The *JAK2* mutation explains many of the cardinal features of *BCR/ABL*-negative MPD, although it remains unknown whether the mutation alone is sufficient for the pathogenesis of these disorders. Further studies should explore the exact role of epigenetic al-

teration in this process and which genetic or epigenetic alterations cooperate with the *JAK2*V617F mutation in the development of *BCR/ABL*-negative MPD.

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