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PRINCIPAL INVESTIGATOR: **Jean-Pierre Issa, M.D.**

CONTRACTING ORGANIZATION: **University of Texas M.D. Anderson Cancer Center
Houston, TX 77030**

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14. ABSTRACT Clonal myeloproliferative disorders (MPD) affect erythroid, myelomonocytic, and megakaryocytic lineages. An activating somatic mutation of JAK2 tyrosine kinase is present in the majority of polycythemia vera (PV) patients but also in 50% of patients with essential thrombocythemia (ET) and myelofibrosis (MF). Additional factors are presumed to affect the phenotype and progression of the disease. We studied DNA methylation as a possible epigenetic factor in the development and progression of MPDs. We cloned 19 unique CpG islands in promoter/exon-1 regions of 15 known genes, and 4 predicted genes and annotated mRNAs as hypermethylated in PV. Using a genome-wide microarray approach, we showed distinct methylation signatures affecting hundreds of genes in MPD. We confirmed increased methylation of progesterone receptor, cadherin precursor (CDH13) and several HOX genes in MPD patients. We showed that a functional block of progesterone receptor in normal erythroid cells increased their sensitivity to erythropoietin. We demonstrated molecular responses clearing both genetic and epigenetic abnormalities after DNA-demethylation therapy in MPD patients.					
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DNA methylation as an epigenetic factor in the development and progression of polycythemia vera

Jean-Pierre Issa, M.D.

Grant MP04315

Study Site:

Department of Leukemia, University of Texas M. D. Anderson Cancer Center

1515 Holcombe Blvd., Houston, TX 77030

INTRODUCTION

Polycythemia vera (PV) is the most common myeloproliferative disorder with a yearly incidence of 28 per 1 million people and a slightly higher prevalence in males.¹ PV is characterized by clonal expansion of erythroid, myelomonocytic, and megakaryocytic lineages, erythrocytosis being the most prominent clinical manifestation of PV.² The disease is associated with a significant morbidity and mortality, including thrombotic and/or hemorrhagic events, and a risk of an evolution into myelofibrosis and leukemia.³⁻⁵ An acquired activating V617F (1849G>T) mutation of *JAK2* tyrosine kinase has been recently found in the majority of patients with polycythemia vera (PV), in about half of those with essential thrombocythemia (ET) and myelofibrosis (MF),⁶⁻¹⁰ and in 10-20% patients with chronic myelomonocytic leukemia, Philadelphia-negative CML, atypical or unclassified myeloproliferative diseases (MPD) and megakaryocytic leukemia.¹¹⁻¹³ It is not known what other factors determine the disease phenotype of PV, MF, and other

MPD, and what factors other than JAK2 lead to disease progression. Very little is known about epigenetic changes in PV. Epigenetic lesions have been recognized to be important in cancer, in particular in older individuals. Methylation of cytosines in the CpG sites clustered in the gene promoter regions results in epigenetic gene silencing, and acts as one of possible mechanisms of tumor suppressor inactivation in cancer.¹⁴ Diverse myeloproliferative phenotypes caused by a single point mutation of JAK2 tyrosine kinase, lack of other genetic specific lesions in PV, and its association with higher age lead us to propose the hypothesis that epigenetic silencing may play a role in the pathogenesis of PV.

STATEMENT OF WORK

Task 1. Discover genes whose promoter-associated CpG islands are methylated in patients with polycythemia vera (PV), months 1-18:

- a. Identify in the M. D. Anderson database all patients with PV for whom archived bone marrow biopsies are available (month 1).
- b. Collect paraffin-embedded bone marrow biopsies on all patients (projected 100 patients, 10 cuts/month, months 1-10)
- c. Collect existing blood samples from PV patients at M. D. Anderson, and from the external collaborator at Baylor College of Medicine (projected 50-60 patients per year, months 1-36).
- d. Extract DNA from paraffin cuts (start month 1 – ongoing until all samples collected, months 1-10) and from blood samples (months 1-36).

- e. Perform genome-wide screening for promoter-associated CpG islands differentially methylated in 15 patients with polycythemia vera in the polycythemic phase, 15 patients who developed myelofibrosis and 15 patients who transformed to leukemia. We will use Methylated CpG Island Amplification coupled with Representative Difference Analysis (MCA-RDA) as a screening method (months 2-18).

Task 2. Determine the methylation and expression profile of candidate genes in the polycythemic phase of PV, patients who developed myelofibrosis and patients who transformed to leukemia. Months 2-36.

- a. Bisulfite treatment and PCR-based methylation analysis for all the genes discovered by MCA-RDA and candidate genes involved in growth factor signaling (months 2-20)
- b. Analyze samples for gene expression by real time quantitative RT-PCR (months 13-36)
- c. Statistical analysis of the collected data (months 21-22)
- d. Validation of the results on prospectively collected samples (months 23-36)

Task 3. Begin exploring the function of the most promising genes using in vitro cultures and/or transfection experiments. Months 13-30.

- a. Determine whether specific inhibition of candidate PV-methylated genes in normal cells would mimic the PV phenotype of hypersensitivity of

- b. In case the candidate genes are methylated and silenced in leukemic cell lines, we will restore their expression using standard gene transfection technology. The transfected cell lines will be examined for growth characteristics and in vitro differentiation. The effect of this transfection on the function of putative affected pathways will also be examined (months 13-30)

Task 4. To assess the prognostic significance of aberrant methylation in PV we shall perform retrospective multivariate analyses of the association of CpG island methylation with survival and probability of transformation to myelofibrosis or leukemia (months 24-36).

RESEARCH ACCOMPLISHMENTS

TASK 1

Genome-wide analysis of DNA methylation in MPD patients

Determination of JAK2 allelic burden

We determined JAK2 1849G>T (V617F) mutational status by pyrosequencing in 236 genomic DNA samples obtained from patients with myeloproliferative disorders (MPD). Quantitative analysis of the JAK2 mutation showed a continuous distribution of mutant JAK2 allelic burden in MPD, most notably in PV patients (Fig. 1). None of 43 controls had values of mutant allele over 5% thresholds (horizontal solid line). JAK2 mutation was detected in 39/74 (53%) ET patients, 98/138 (71%) PV patients, and 50/88 (57%) MF patients.

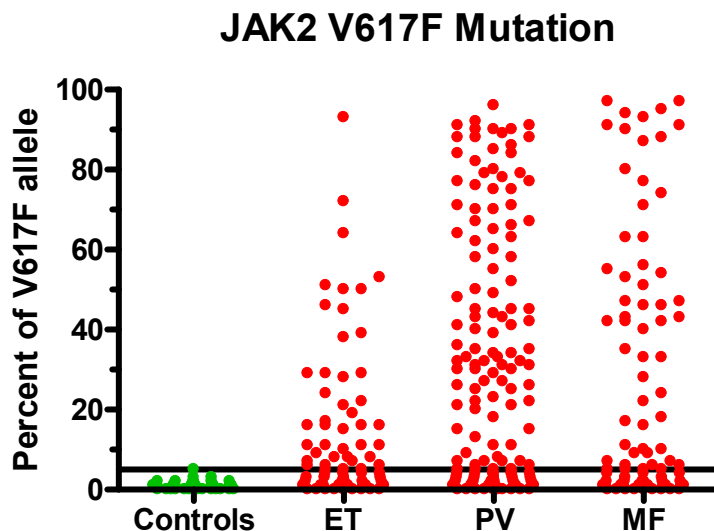


Figure 1. Quantitative determination of JAK2 V617F (1849G>T) mutation by pyrosequencing.

Genome-wide methylation analyses

We performed initial genome-wide screening by methylated CpG island amplification coupled with representative difference analysis (MCA-RDA)¹⁵ for methylated promoter-associated CpG islands in granulocytes isolated from a PV patient. Cloning and sequencing of 200 clones revealed 19 unique CpG islands in promoter/exon-1 regions of 15 known genes, and 4 predicted genes and annotated mRNAs as potentially hypermethylated (Table 1).

Genes Cloned As Differentially Methylated in PV

Chromosome	Gene name	Description
1q23.3	CAPON	Adapter protein linking nNOS to specific targets
1q44	FLJ45717	Hypothetical protein
4p16.3	DGKQ	Diacylglycerol kinase
5q31.2	JMJD1B	Nuclear protein 5qNCA
5q35.2	SNCB	Beta-synuclein
6q15	BC037581	mRNA
10q11.21	GALNACT-2	Chondroitin N-acetylgalactosaminyltransferase
11q22.1	PGR	Progesterone receptor
12q23.3	CHST11	Chondroitin 4 sulfotransferase 11
12q24.12	LNK	Lymphocyte specific adapter protein Lnk
14q32	BC043593	mRNA
15q22.2	NLF1 exon2	Hypothetical protein
16p11.2	MGC2474	Hypothetical protein
16q23.3	CDH13	Cadherin 13 preproprotein
19q13.42	CN431418	Interleukin-11 splice variant
20p11.1	BC036544	mRNA
20p12.1	CB961129	Spliced EST
21q22.11	OLIG2	Oligodendrocyte transcription factor 2
Xp22.33	SHOX	Short stature homeobox

Table 1. Promoter-associated CpG islands cloned by MCA/RDA from a PV patient.

The rapid development of microarray technology made possible to use high density oligonucleotide microarrays as a tool for detection of individual gene signatures in the libraries generated by the methylated CpG island amplification (MCAM) technique. We therefore used MCAM for genome-wide analysis of DNA methylation in genomic DNA from MPD patients. We performed MCAM in samples from 15 patients with different types of myeloproliferative disorders: 3 patients with PV, 2 patients who transformed from PV to MF, 3 patients with ET transformed to MF and 7 patients with primary myelofibrosis. Two patients with primary myelofibrosis later transformed to acute myeloid leukemia (AML). Three patients were negative for JAK2 V617F mutation, 8 patients had heterozygous JAK2 V617F mutation and 4 patients had mutational burden over 50% (Table 2).

ID	Diagnosis at analysis	Original Dx	Transformation	JAK2 V617F
MPD01	PV	PV		41%
MPD02	PV	PV		48%
MPD03	PV	PV		86%
MPD04	MF	PV		47%
MPD05	MF	PV		74%
MPD06	MF	ET		0%
MPD07	MF	ET		42%
MPD08	MF	ET		91%
MPD09	PMF			0%
MPD10	PMF			0%
MPD11	PMF			39%
MPD12	PMF			48%
MPD13	PMF			48%
MPD14	PMF		AML	51%
MPD15	PMF		AML	61%

Table 2. Characteristics of MPD patients used in methylation microarray analysis.

We used high Agilent microarrays with 44,000 customized oligonucleotide probes to detect genes that are methylated in MPD patients and not methylated in normal controls. We compared the MCAM analysis done in MPD patients with the results obtained in 16 patients with acute myeloid leukemia. Our goal was to dissect methylation changes specific for MPD and changes that are common with AML. In MPD patients, significant methylation signals were observed at 155-5441 oligonucleotide probes, median 676 probes, that is 0.4%-13.3%, median 1.7% of the total amount of 41,000 probes mapping to autosomal chromosomes on the array. The results in AML patients were similar, with 311-4271, median 771 probes, or 0.8%-10.4%, median 1.9%. These numerical differences between the MPD and AML groups were not significant. We next focused our analysis on probes mapping to non-repetitive sequences in CpG islands around gene transcription start sites. We selected for our analysis 1371 probes that gave positive methylation signals in at least 20% of patients in the MPD or AML group. Hierarchical clustering of methylation signals could not separate MPD patients based on their clinical status or JAK2 mutation burden, however, it clearly distinguished between the groups of MPD and AML patients (Fig. 2).

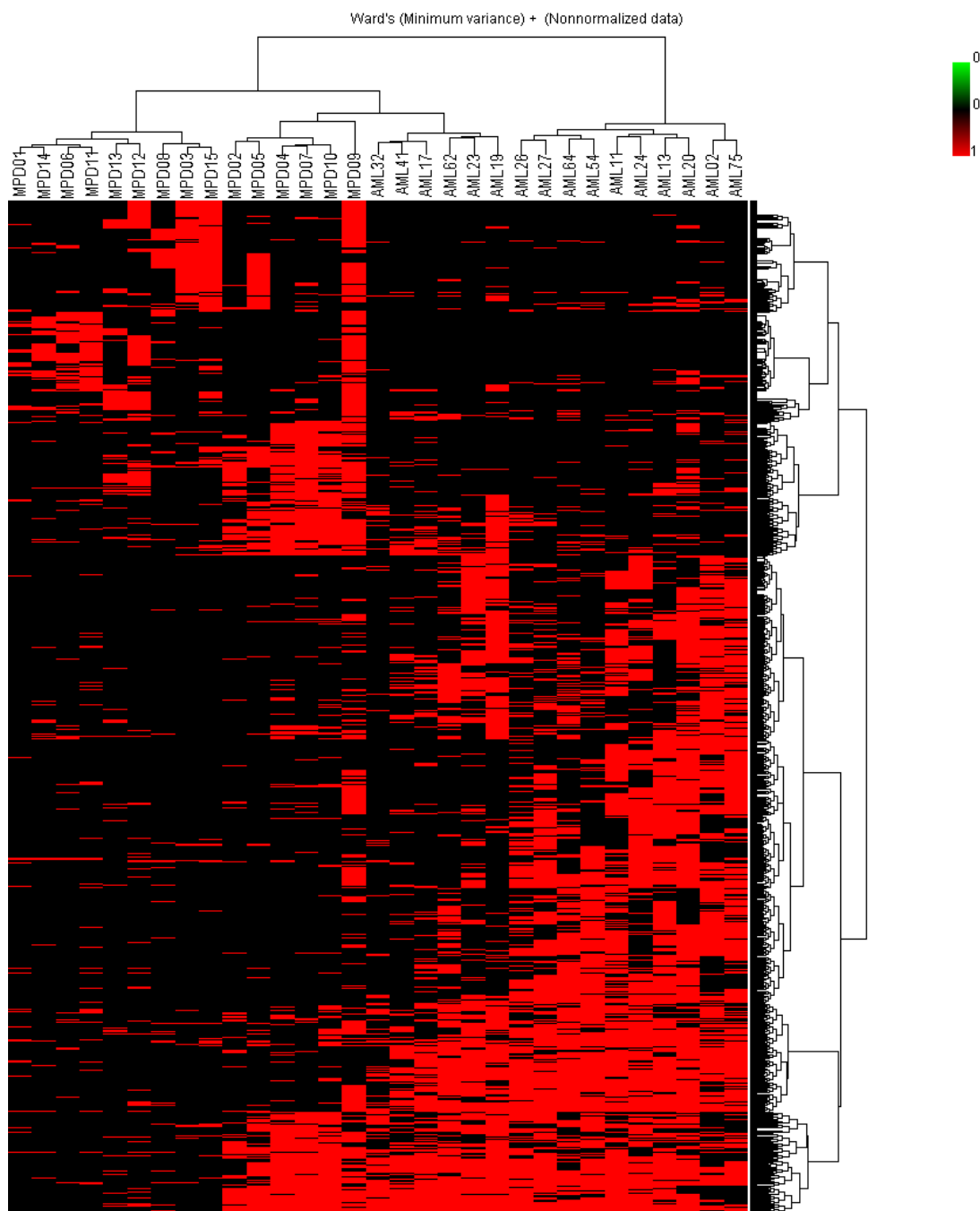


Figure 2. Hierarchical clustering of methylation status at 1371 probes in CpG islands clearly distinguished the differences between the MPD and AML patients. Red, methylation signal significantly higher than in normal blood control. Black, methylation not increased.

The analysis also delineated groups of 245 genes more prone to methylation in MPD, 148 genes methylated both in MPD and AML, and 498 genes methylated exclusively in AML (Fig. 3).

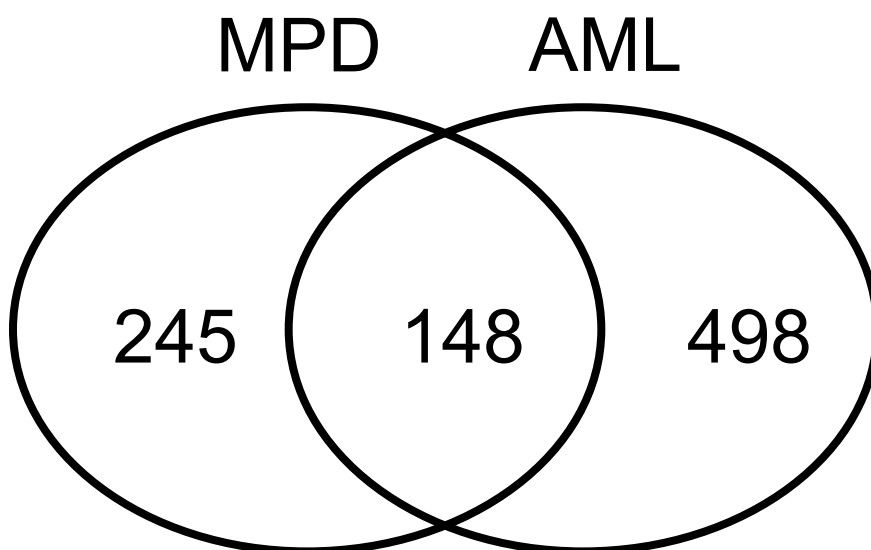


Figure 3. Numbers of genes with methylated CpG islands in MPD and AML patients.

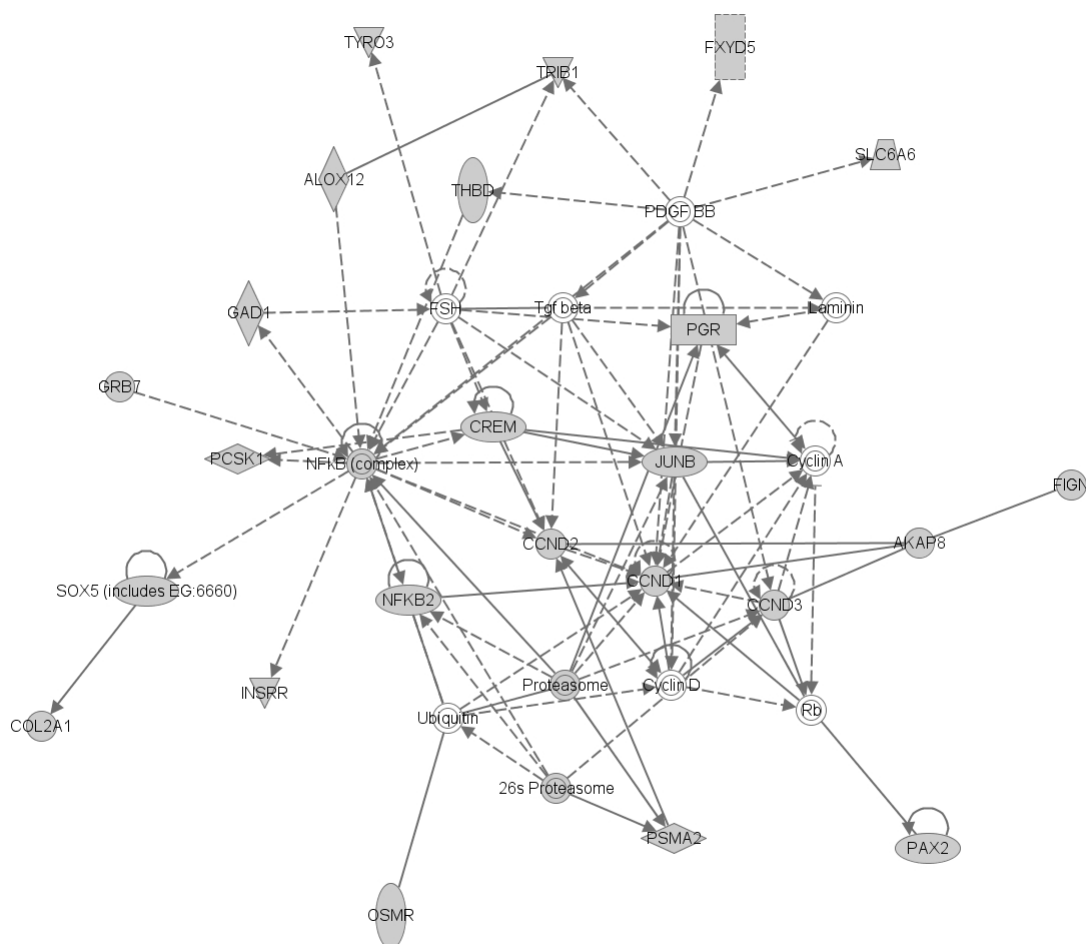
We analyzed the functional relationships of these genes by the Ingenuity Pathway Analysis software.

Genes methylated in MPD

The 245 genes found methylated predominantly in MPD patients clustered to the following functional networks:

- Cellular development, cellular growth and proliferation, respiratory system development and function, 44 genes, Fig. 4.

- Lipid metabolism, molecular transport, small molecule biochemistry, 34 genes
- Cancer, developmental disorder, 23 genes.



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Figure 4. Functional network of developmental and cell proliferation genes affected by DNA methylation in MPD patients. Methylated genes are shaded. Cyclins D1, D2, D3, transcription factors NFκB, NFκB2, JUNB and progesterone receptor (PGR) genes form central nodes.

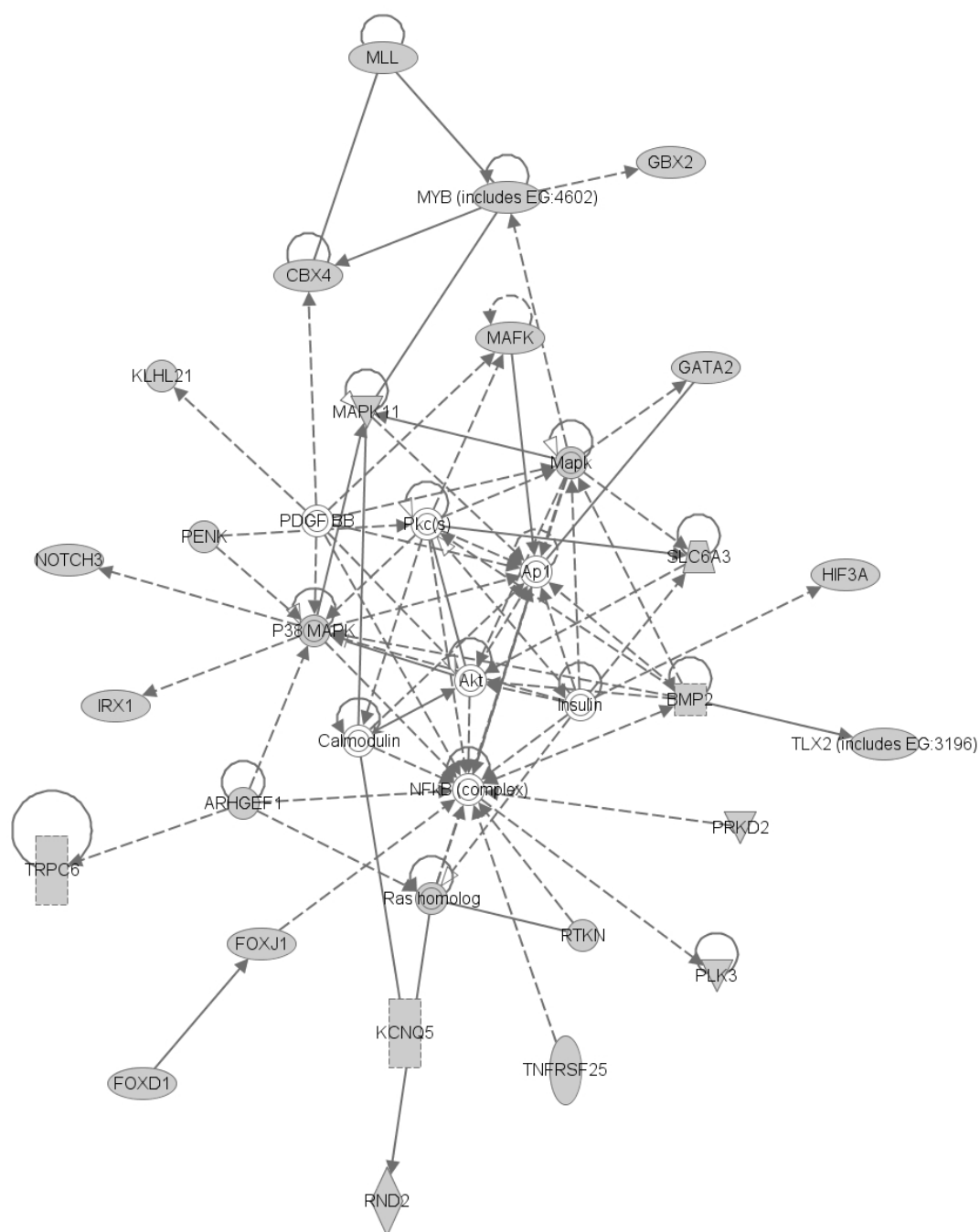
Cancer, genetic disorders, cellular development, growth and proliferation, gene expression, cAMP mediated signaling, G-protein receptor signaling and aryl hydrocarbon receptor signaling were listed at the top of disorders and functions associated with the genes methylated in MPD patients.

Genes methylated in MPD and AML

The 148 genes methylated both in MPD and in AML patients clustered to the following functional networks:

- Cancer, hematological disease, 51 genes, Fig. 5.
- Cell death, hematological disease, gene expression, 24 genes.

Cancer, hematological disease, and organ development were between the significant disorders and functions affected by the methylated genes.



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Figure 5. Functional network of cancer genes affected by DNA methylation in MPD and AML patients. Methylated genes are shaded. MAP and MAF kinases form central nodes.

Genes methylated in AML

The 498 genes methylated predominantly in AML patients were significantly enriched for cancer, cell death, development, and gene expression. The following pathways contained significant proportions of genes methylated in AML but not in MPD: ephrin receptor signaling, SAPK/JNK signaling, VEGF signaling, p53 signaling, hypoxia-inducible factor signaling and TGF- β signaling.

TASK 2

Methylation analysis of selected genes in large sets of MPD patients.

Progesterone receptor. We determined methylation levels of in the CpG island at the start of progesterone receptor isoforms A and B (PGR-A, PGR-B) by quantitative bisulfite pyrosequencing in normal controls, ET, PV, and MF patients (Figure 6). Methylation of PGR-A over a 10% threshold was observed in 0/46 controls, 0/59 ET patients, 15/128 (12%) PV patients, and 14/73 (19%) MF patients. Acute myeloid leukemia (AML) patients showed markedly higher methylation frequency (23/33 patients, 70%). Threshold of 15% was used for methylation of PGR-B. Methylation above this threshold was seen in none of 45 controls, 3/59 (5%) ET patients, 18/132 (14%) PV patients, 13/73 (18%) MF patients, and in 22/36 (61%) AML patients. Methylation levels of PGR-A and PGR-B in AML were significantly higher than in controls ($P < 0.001$; Dunn's multiple comparison nonparametric test).

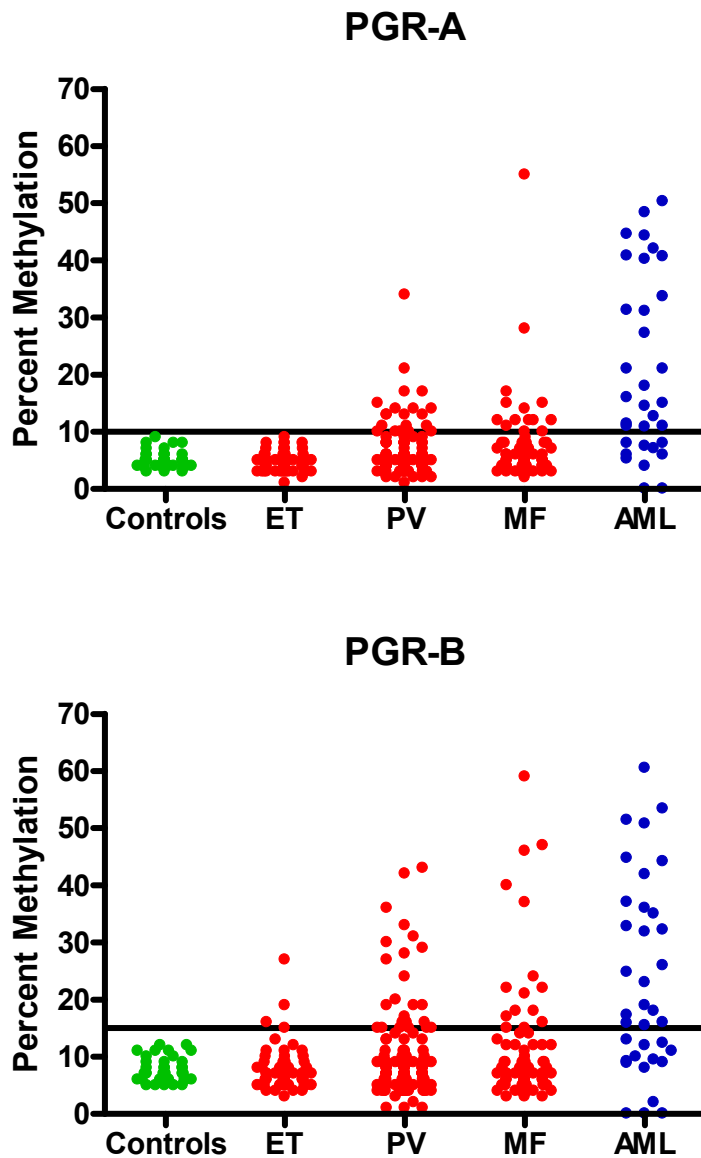


Figure 6. Quantitative determination of methylation levels in CpG islands of progesterone receptor isoforms A (PGR-A) and B (PGR-B).

CDH13. Heart cadherin precursor (CDH13) was another CpG island methylated in MPD patients. Methylation of CDH13 over a 10% threshold was observed in 1/46 (2%)

controls, 4/59 (7%) ET patients, 19/130 (15%) PV patients, 19/72 (26%) MF patients, and in 12/36 (33%) AML patients.

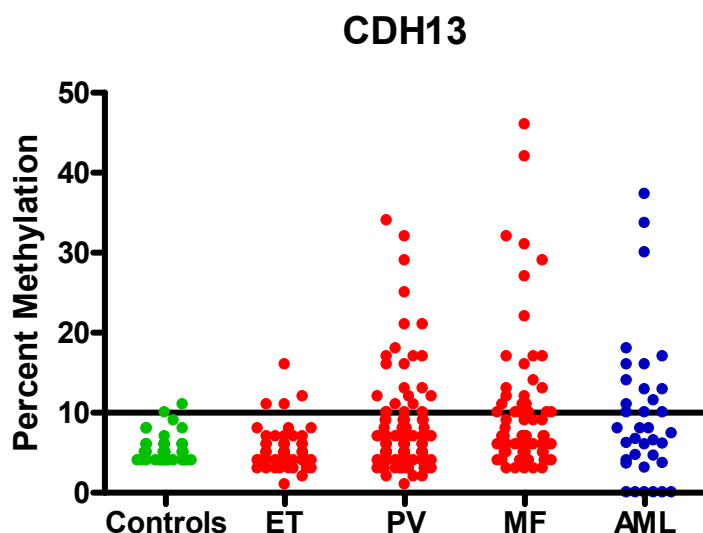


Figure 7. Quantitative determination of methylation levels in CpG island at the start of heart cadherin precursor gene (CDH13).

Methylation levels for PGR-A, PGR-B and CDH13 were normalized by a Z transformation and methylation Z-scores were calculated by the following formula:

$$Z = (\text{methylation value} - \text{mean methylation} / \text{standard deviation})$$

Average Z-scores were calculated for each sample. Methylation Z-score values were significantly increased in MF ($P < 0.01$) and AML patients ($P < 0.001$) when compared to controls (Fig. 8).

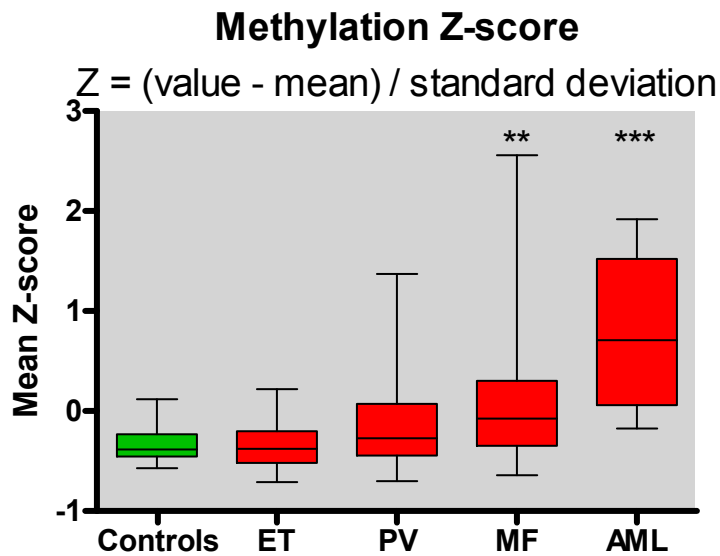


Figure 8. Methylation Z-scores are increased in MF and AML patients.

Methylation of HOX genes in MPD patients.

We screened 31 HOX genes for methylation of their CpG islands in PV patients. Preliminary results suggested increased methylation in HOXA4, HOXC4, HOXC9, and HOXC11 genes. We performed detailed analysis in larger numbers of MPD patients and found significantly increased methylation in HOXA4, HOXC4, and HOXC11 genes (Figures 9-12).

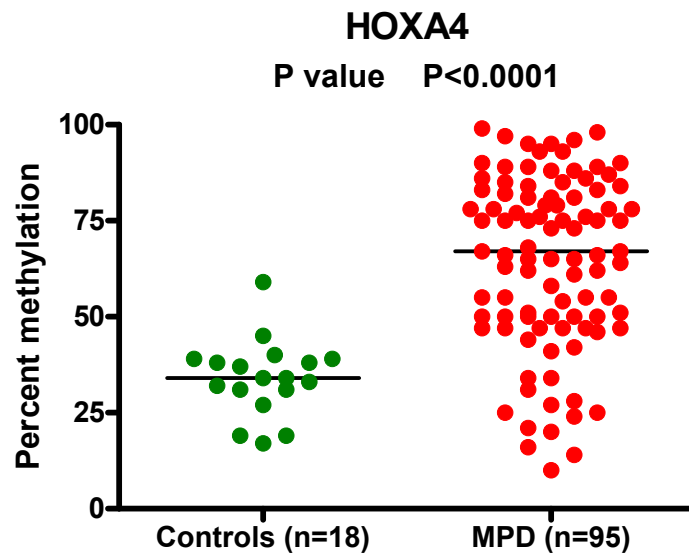


Figure 9. CpG methylation at the transcription start site of HOXA4 gene in blood cells from normal controls and MPD patients. $P < .0001$, Mann-Whitney non-parametric test.

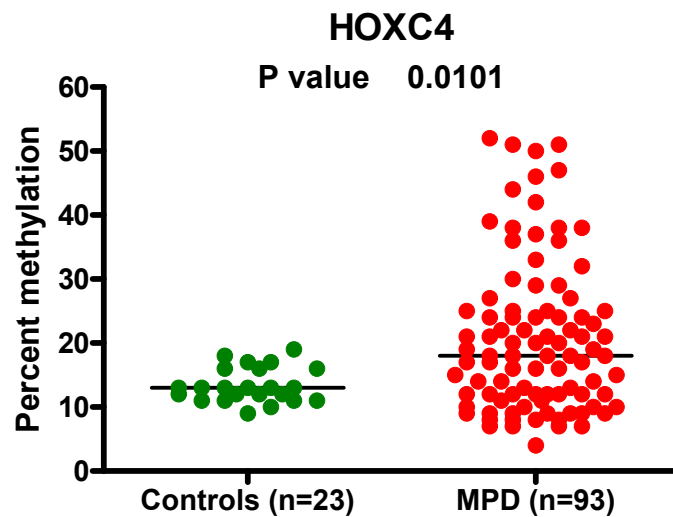


Figure 10. CpG methylation at the transcription start site of HOXC4 gene in blood cells from normal controls and MPD patients. $P = .01$, Mann-Whitney non-parametric test.

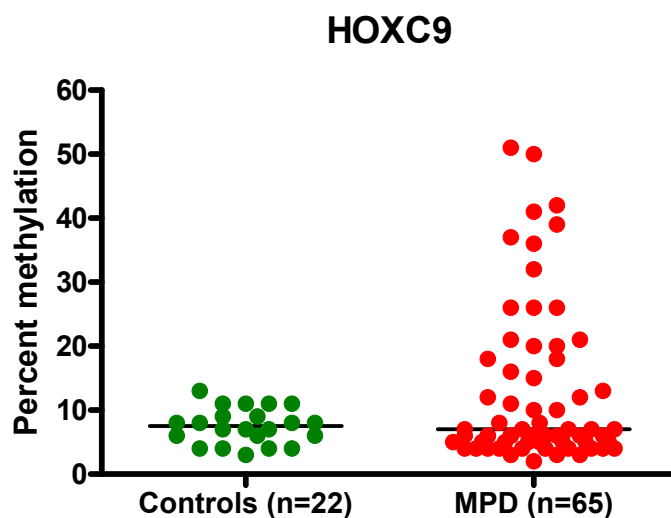


Figure 11. CpG methylation at the transcription start site of HOXC9 gene in blood cells from normal controls and MPD patients. Although the difference was not statistically significant, about 25% of MPD patients showed HOXC9 methylation outside of the normal range.

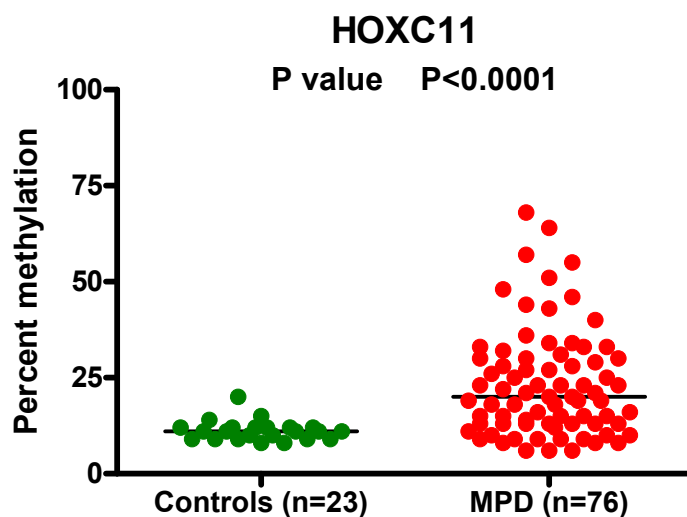


Figure 12. CpG methylation at the transcription start site of HOXC11 gene in blood cells from normal controls and MPD patients. $P < .0001$, Mann-Whitney non-parametric test.

TASK 3

Functional significance of progesterone receptor methylation

To assess the functional significance of progesterone receptor silencing, we explored the effect of mifepristone, a PGR antagonist, on *in vitro* response of BFU-E erythroid progenitors to erythropoietin. Mifepristone at 10^{-6} M concentration increased the sensitivity of BFU-E progenitors from normal blood to low concentrations of erythropoietin (60-250 mU/ml) suggesting that disabling of PGR may increase the response of hematopoietic cells to proliferative stimuli (Figure 13).

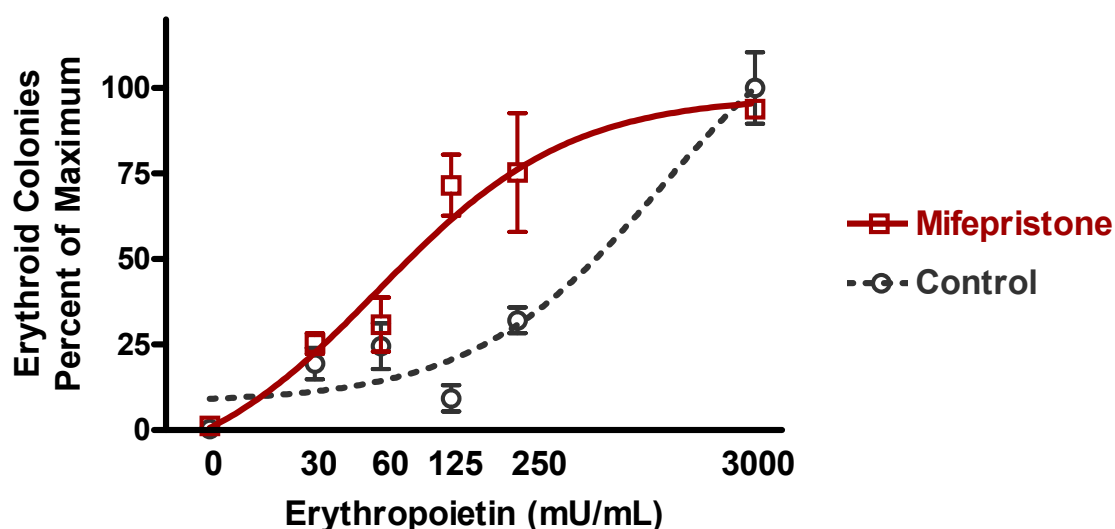


Figure 13. Progesterone receptor antagonist mifepristone increased the sensitivity of erythroid progenitors BFU-E to erythropoietin. Results of four independent experiments were combined. Error bars show standard error of the mean.

Progesterone receptor functional studies

To assess the functional significance of progesterone receptor silencing, we explored the effect of mifepristone, a PGR antagonist, on *in vitro* response of BFU-E erythroid progenitors to erythropoietin. Mifepristone treatment of normal peripheral blood increased cultured *in vitro* in the presence of erythropoietin increased the expression of genes associated with erythroid program: transferrin receptor, glycophorin A, Janus kinase 2, and beta globin (Fig. 14).

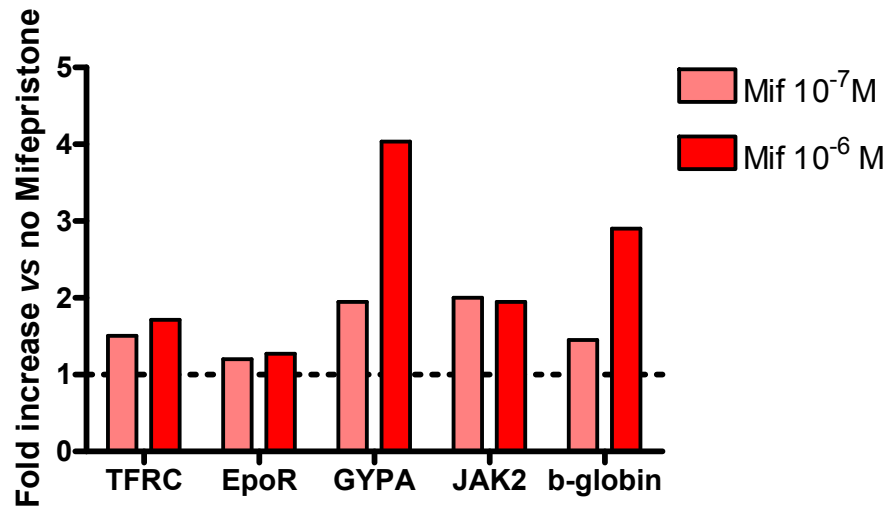


Figure 14. Mifepristone treatment increases the expression of erythroid genes. Normal peripheral blood mononuclear cells were cultured *in vitro* for 14 days in the presence of erythropoietin (3U/ml) and mifepristone at 0, 10^{-7} M and 10^{-6} M concentration. Expression of mRNA was measured by real time PCR. TFRC, transferrin receptor, EpoR, erythropoietin receptor, GYPA, glycophorin A, JAK2, janus 2 kinase, b-globin, beta globin mRNA. Fold increase of mRNA expression over cultures without mifepristone is shown.

Epigenetic control of PRV-1 gene expression

We found that expression of the polycythemia rubra vera-1 (PRV-1) gene is inversely correlated with DNA methylation status of CpG sites the the gene transcription start site. PRV-1/CC177 is a GPI-linked protein that is expressed on a subgroup of neutrophils. The number of PRV-1 expressing neutrophils increases in pregnancy and sepsis, or after administration of granulocyte-colony stimulating factor. Expression of the PRV-1 gene is also increased in patients with polycythemia vera and essential thrombocythemia. We investigated whether DNA methylation of the PRV-1 gene has a role in regulation of transcription and expression of the PRV-1 protein. We compared the level of methylation of the PRV-1 gene and expression of the PRV-1 mRNA in normal neutrophils expressing PRV-1 to those that are PRV-1 negative. We also studied PRV-1 methylation and mRNA expression in patients with non-CML myeloproliferative disorders and in an in vitro model of DNA demethylation. We found that methylation of CpG dinucleotides close to initiation codon of the PRV-1 gene was inversely related to the expression of PRV-1 in normal neutrophils. Furthermore, overexpression of the PRV-1 gene in polycythemia vera (PV) and essential thrombocythemia (ET) is associated with a decrease in methylation of this gene. Among patients with PV and ET, methylation of the PRV-1 gene is also inversely correlated with the presence of the JAK2V617F somatic mutation. In an in vitro model, exposure of KG1 and KG1a cells to a DNA demethylating agent caused a decrease in methylation of the PRV-1 gene and increased its mRNA level. We conclude that DNA methylation regulates PRV-1 expression under

physiologic and pathologic conditions. The results were published in Experimental Hematology (Appendix 4)

Effects of DNA demethylating treatment in MPD patients.

The mechanism of DNA-demethylating action of 2-deoxy-5-aza-cytidine (decitabine) in vivo is not fully understood. We studied the dynamics of neoplastic cell clearance during decitabine treatment (100 mg/m²/course every 4 weeks) using quantitative monitoring of mutant alleles by pyrosequencing in patients with chronic myelomonocytic leukemia harboring JAK2 or NPM1 mutations. CMML patients were first screened for JAK2 and NPM1 mutations, and three patients with mutations were identified. Mutant allele percentages in mononuclear cell DNA were followed after treatment, along with methylation of LINE1 and ten other genes. The clearance of mutant alleles was modest after the first cycle, despite induction of hypomethylation. Delayed substantial clearance was observed after 2-4 cycles that correlated with clinical response. Two patients had complete disappearance of mutant alleles and sustained clinical remissions. In another patient, mutant allele was detectable at clinical remission, which lasted for 8 months. Our data suggest a predominantly non-cytotoxic mechanism of action for decitabine, leading to altered biology of the neoplastic clone and/or normal cells. The results were published in the Blood journal (Appendix 5)

TASK 4

Prognostic significance of DNA methylation in MPD.

Although we could clearly detect different pattern of DNA methylation between MPD and AML patients, we have not observed significant associations between the pattern of DNA methylation and clinical course of disease in MPD patients.

KEY RESEARCH ACCOMPLISHMENTS

- Cloned 19 unique CpG islands in promoter/exon-1 regions of 15 known genes, and 4 predicted genes and annotated mRNAs as potentially hypermethylated in PV.
- Demonstrated distinct methylation signatures affecting hundreds of genes in MPD, partially shared with genes methylated in AML.
- Characterized biological functions and pathways affected by DNA methylation in MPD patients.
- Confirmed increased methylation of progesterone receptor, cadherin precursor and HOX A4, C4, C9 and C11 genes subsets of PV, MF and AML patients.
- Showed that a functional block of progesterone receptor in normal erythroid cells increases their sensitivity to proliferative stimulation by erythropoietin.
- Mapped DNA methylation dynamics and molecular response after DNA-demethylation therapy in MPD patients.
- Characterized epigenetic control by DNA methylation of the polycythemia rubra vera gene 1 (PRV-1/CD177) expression on neutrophils of MPD patients and normal individuals.

REPORTABLE OUTCOMES

Meeting presentations

Poster, Methylation of progesterone receptor promoter-associated CpG island in polycythemia vera and related myeloproliferative disorders. American Society of Hematology 47th Annual Meeting, Atlanta, GA, December 12, 2005

Poster, DNA methylation as an epigenetic factor in the development and progression of polycythemia vera. PRMRP Military Health Research Forum, San Juan, PR, May 1-4, 2006

Poster, DNA methylation of HOX genes in leukemia and myeloproliferative disorders. American Association for Cancer Research Annual Meeting, Los Angeles, CA, April 14-18, 2007.

Poster, Jelinek J, Estecio MRH, Kondo K, He R, Zavadil J, Issa JPJ. Classifying Leukemias Based on Epigenetic Alterations. American Society of Hematology Meeting, Atlanta, GA, December 2007:

Poster, Samuelson SJ, Swierczek S, Parker CJ, Boucher K, Jelinek J, Prchal JT. Analysis of Mutant cMPL in Philadelphia Chromosome-Negative Myeloproliferative Disorders (Ph-MPDs) Using a Novel High-Sensitivity Assay. American Society of Hematology Meeting, Atlanta, GA, December 2007:

Poster, Lippert E, Girodon F, Hammond E, Carillo S, Richard C, Fehse B, Hermans M, James I, Jelinek J, Marzac C, Migeon M, Pietra D, Prchal JT, Reading NS, Sobas M, Ugo V, Skoda RC, Hermouet S. Concordance of Assays Designed for the Quantitation of JAK2 1849G>T (V617F): A Multi-Centre Study. American Society of Hematology Meeting, Atlanta, GA, December 2007:

Meeting abstracts published (Appendix 1)

Jelinek J, Verstovsek S, Bueso-Ramos CE, Prchal JT, Issa JPJ. Methylation of progesterone receptor promoter-associated CpG island in polycythemia vera and related myeloproliferative disorders. Blood 2005;106:979a-980a

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CONCLUSIONS

Our data show that PGR, CDH13, HOXA4, HOXC4 HOXC9 and HOXC11 are methylated in a significant proportion of MPD patients. Moreover, hundreds of genes are methylated either predominantly in MPD or both in MPD and AML. Silencing of these genes by methylation suggests a strong role of epigenetic mechanisms in MPD. Treatment of MPD patients with decitabine as a DNA demethylating agent was shown to remove malignant cells harboring genetic mutations and aberrant methylation pattern.

“SO WHAT:”

Epigenetic silencing by cytosine methylation in selective CpG islands plays a role in the development of myeloproliferative disorders. The hypomethylating drug decitabine may be considered for clinical trials in patients non-responding to conventional treatment.

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APPENDIX 1

MEETING ABSTRACTS

[Blood \(ASH Annual Meeting Abstracts\) 2005 106: Abstract 3507](#)

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Poster Sessions

Methylation of Progesterone Receptor Promoter-Associated CpG Island in Polycythemia Vera and Related Myeloproliferative Disorders.

Jaroslav Jelinek, MD, PhD¹, Srdan Verstovsek, MD¹, Carlos E. Bueso-Ramos, M.D, PhD², Josef T. Prchal, MD³ and Jean-Pierre J. Issa, MD¹

¹ Department of Leukemia, University of Texas M.D. Anderson Cancer Center, Houston, TX, USA; ² Department of Hematopathology, University of Texas M.D. Anderson Cancer Center, Houston, TX, USA and ³ Hematology/Oncology Division, Baylor College of Medicine, Houston, TX, USA.

Polycythemia vera (PV), essential thrombocythemia (ET) and idiopathic myelofibrosis (MF) are clonal myeloproliferative disorders (MPD). A recently discovered activating mutation of JAK2 tyrosine kinase has been found in most patients with polycythemia vera (PV), in about half of those with essential thrombocythemia (ET) and myelofibrosis (MF), and in 10–20% patients with chronic myelomonocytic leukemia, Philadelphia-negative CML, atypical or unclassified MPD and megakaryocytic leukemia. It is not known what other factors determine the disease phenotype of PV, MF, and other MPD, and what factors other than JAK2 lead to disease progression. Very little is known about epigenetic changes in PV. DNA methylation of promoter-associated CpG islands is a well-recognized mechanism of epigenetic silencing used by tumors for evasion from regulatory mechanisms, and it is an alternative to genetic lesions in cancer causation. Using a genome-wide screen for differentially methylated CpG islands, we found methylation of progesterone receptor promoter region (PGR) in PV granulocytes. We then developed pyrosequencing assays for quantitative detection of PGR methylation in bisulfite-treated PCR-amplified DNA. The PGR methylation above normal control levels was observed in ET (2/12 patients, 17%), PV (10/22 patients, 45%), MF (8/12 patients, 67%), and patients with acute myeloid leukemia and antecedent PV (6/7 patients, 86%). We compared the levels of PGR methylation in MPD with the mutation status of JAK2. The 1849G>T JAK2 mutation was present in 16/27 (59%) MPD patients with unmethylated PGR and 21/26 (80%) patients with methylated PGR; the difference not statistically significant; $p=0.135$. The role of progesterone receptor signaling in hematopoiesis is not known. Using real time quantitative RT-PCR assay for progesterone receptor expression we found detectable levels in granulocytes from 4/5 normal individuals while the expression in granulocytes from 5/5 PV patients was not detectable. To assess the functional significance of progesterone receptor silencing, we explored the effect of mifepristone, a progesterone receptor antagonist, on the response of BFU-E progenitors to erythropoietin. Mifepristone increased the sensitivity of BFU-E progenitors from normal blood to low concentrations of erythropoietin (60–250 mU/ml) suggesting

that disabling of progesterone receptor may increase the response of hematopoietic cells to proliferative stimuli. In conclusion, our data show that PGR methylation is present in half of PV patients and it is even more frequent in MF and PV transformed to AML. Silencing of progesterone receptor by methylation may be an epigenetic change contributing to MPD phenotype and transformation to leukemia.

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Poster Sessions

In Vitro Expansion of Polycythemia Vera Progenitors Favors Expansion of Erythroid Precursors without JAK2 V617F Mutation.

Josef T. Prchal, MD^{1,2}, Ko-Tung Chang, PhD^{1,2,*}, Jaroslav Jelinek, MD³, Yongli Guan^{1,*}, Amos Gaikwad, PhD^{1,*}, Jean-Pierre Issa, MD³ and Enli Liu, MD^{1,*}

¹ Medicine-Hematology/Oncology, Baylor College of Medicine, Houston, TX, USA; ² Dpt of Pathophysiology, School of Medicine, Charles University, Prague, Czech Republic and ³ Department of Leukemia, University of Texas M. D. Anderson Cancer Center, Houston, TX, USA.

A single acquired point mutation of JAK2 1849G>T (V617F), a tyrosine kinase with a key role in signal transduction from growth factor receptors, is found in 70%–97% of patients with polycythemia vera (PV). In the studies of tyrosine kinase inhibitors on JAK2 1849G>T (see Gaikwad et al abstract at this meeting) we decided to study the possible therapeutic effect of these agents using native in vitro expanded cells from peripheral blood. To our surprise, the in vitro expansion of PV progenitors preferentially augmented cells without JAK2 1849G>T mutation.

We used a 3 step procedure to amplify erythroid precursors in different stages of differentiation from the peripheral blood of 5 PV patients previously found to be homozygous or heterozygous for the JAK2 1849G>T mutation. In the first step (days 1–7), 106/ml MNCs were cultured in the presence of Flt-3 (50 ng/ml), Tpo (100 ng/ml), and SCF (100 ng/ml). In the second step (days 8–14), the cells obtained on day 7 were re-suspended at 106/ml in the same medium with SCF (50 ng/ml), IGF-1 (50 ng/ml), and 3 units/ml Epo. In the third step, the cells collected on day 14 were re-suspended at 106/ml and cultured for two more days in the presence of the same cytokine mixture as in the step 2 but without SCF. The cultures were incubated at 37°C in 5% CO₂/95% air atmosphere and the medium renewed every three days to ensure good cell proliferation. The expanded cells were stained with phycoerythrin-conjugated anti-CD235A (glycophorin) and fluorescein isothiocyanate-conjugated anti-human-CD71 (transferrin receptor) monoclonal antibodies and analyzed by flow cytometry. The cells were divided by their differential expression of these antigens into 5 subgroups ranging from primitive erythroid progenitors (BFU-Es and CFU-Es) to polychromatophilic and orthochromatophilic erythroblasts; over 70% of harvested cells were early and late basophilic erythroblasts. The proportion of JAK2 1849G>T mutation in clonal PV

granulocytes (GNC) before in vitro expansion and in expanded erythroid precursors was quantitated by pyrosequencing (**Jelinek**, Blood in press) and is depicted in the Table.

These data indicate that in vitro expansion of PV progenitors favors expansion of erythroid precursors without JAK2 V617F mutation. Since three PV samples were from females with clonal granulocytes, erythrocytes, and platelets, experiments were underway to determine if the in vitro expanded erythroid cells were clonal PV cells without JAK2 V617F mutation, or derived from polyclonal rare circulating normal hematopoietic progenitors.

The Proportion of JAK2 T Allele

Patients	GNC T Allele (%)	Expanded Cells T Allele (%)
PV1 (Female)	81	10
PV2 (Male)	77	28
PV3 (Male)	44	42
PV4 (Female)	78	19
PV5 (Female)	78	28

A Novel and Quantitative Assay To Detect JAK2 V617F Allele by Real-Time AS-PCR and Its Applicability to PV Initiating Mutation.

Roberto H. Nussenzweig, PhD^{1,*}, Sabina Swierczek, PhD^{2,*}, Jaroslav Jelinek, MD¹, Srdan Verstovsek, MD¹, Jaroslav Prchal, MD³ and Josef T. Prchal, MD²

¹ Dept. Leukemia, MD Anderson Cancer Center, Houston, TX, USA; ² Division of Hematology, SOM, University of Utah, Salt Lake City, UT, USA and ³ Dept. Oncology, McGill University, Montreal, QC, Canada.

Abstract

Polycythemia vera (PV) arises due to a somatic mutation(s) of a single hematopoietic stem cell leading to clonal hematopoiesis. Greater than 80% of PV patients carry a somatic mutation in JAK2 (V617F). Growing evidence suggests that increased frequency of the JAK2^{V617F} allele may have a prognostic impact on certain clinical aspects of PV, and, possibly, in other myeloproliferative disorders associated with this mutation. We have developed a novel approach to primer design for Real-Time quantitative allele-specific PCR. Allelic discrimination is enhanced by the combined synergistic effects of an artificial mismatch introduced in the –1 position, starting from the 3' end of the primer, and the use of a locked nucleic acid (LNA) modified nucleoside placed at the –2 position. We provide evidence that the –2 LNA assists in stabilizing the 3' end, while the –1 mismatch provides specificity but not stability. The difference in cycle number between the two allele-specific reactions is used to calculate the relative allele frequencies. We demonstrate the robustness, sensitivity and reproducibility of our design. The proportion of mutant JAK2 allele determined by pyrosequencing and kinetic allele-specific PCR was highly concordant with an average allele frequency deviation of 2.6%. Repeated determination of allelic ratios in multiple patient samples was highly reproducible with a standard deviation of 1.5%. We have also determined that the design and assay is highly sensitive; as little as 0.1% mutant allele in 40–50 ng of genomic DNA can be detected. We further tested the applicability of this technique to the analysis of individual BFU-E colonies in order to address the question whether the JAK2^{V617F} is the disease initiating mutation. Less than 10% of a single isolated BFU-E colony, originating from a single progenitor, is sufficient for determination of allele frequency. The remainder of the colony may be used for other analyses. A proportion of 0 or 50 or 100 percent JAK2 mutant allele is expected from each individual BFU-E colony, which was indeed observed. However, when we tested granulocytes from PV females, wherein the granulocytes were found to be clonal by the X-chromosome transcriptionally based clonality assay, we found 3 females <50 (27.5 ±11) and 7 females >50 (75 ±10.5) percent mutant JAK2 allele frequencies. This result suggests the presence of a heterogeneous population of cells with differing genotypes regarding the JAK2 mutant allele, and is further supported by our genotyping results with individual BFU-E colonies as described above. Our PV data suggest that the JAK2^{V617F} may not be the PV initiating mutation. This novel primer design is simple, does not require tedious optimization of reaction conditions, and can be applied to any kinetic PCR platform for reliable and sensitive determination of allele frequencies. Potential applications are varied, such as, quantitative determination of mosaicism, proportion of fetal cells in maternal circulation, detection of

minimal residual disease associated with known somatic mutation (such as reduction of malignant cells by chemotherapy or reappearance of resistant clone), rapid monitoring of efficacy of new drugs in both "in vitro" systems as well as clinical trials, and many others that require quantitation of allele frequencies.

Hypomethylation Induction and Molecular Response after Decitabine Therapy in Chronic Myelomonocytic Leukemia (CMML).

Yasuhiro Oki, MD^{*1,2}, Jaroslav Jelinek, M.D. Ph.D.¹, Hagop M. Kantarjian, M.D.¹ and Jean-Pierre J. Issa, M.D.¹

¹ Department of Leukemia, University of Texas M.D. Anderson Cancer Center, Houston, TX, USA and ² Department of Clinical Oncology and Hematology, Aichi Cancer Center, Nagoya, Aichi, Japan.

Decitabine has shown therapeutic activity in patients with MDS and CMML. The mechanisms of response to therapy remain incompletely understood. In particular, the relative contribution of this drug's ability to induce hypomethylation and cytotoxicity remains unclear. To address this issue, we studied the dynamics of neoplastic cell clearance during decitabine treatment determined by quantitative monitoring of the mutant allele using pyrosequencing. DNA extracted from peripheral blood mononuclear cells from consented patients with CMML in a decitabine phase II study were first screened for JAK2 and NPM1 mutations as previously reported. We identified three patients with mutations (two with JAK2 mutation, one with NPM1 mutation) and samples at multiple points during therapy were available. All three carried normal karyotype. LINE repetitive element methylation and several other gene specific methylations were also assessed. In the three patients, LINE methylation decreased after each cycle of therapy, and recovered to near baseline after the drug was stopped (e.g. during the first cycle, average relative hypomethylation from baseline was 13.9% at day 12 and 6.5% at day 28). At the same time, the proportion of circulating neoplastic cells decreased slowly after the first cycle (decrease by 19.3% at day 12 and 13.5% at day 28). A substantial decrease in mutant allele percentage was observed after cycles 2, 3, and 2 in patients 1, 2, and 3, respectively. Clinical complete responses were achieved along with molecular responses at cycles 5, 4 and 2, respectively. Patients 1 and 2 showed complete disappearance of detectable neoplastic clones, and had sustained remissions (duration 1.5 and 2.5 years). In patient 3, the proportion of neoplastic cells was lower than baseline but still detectable at clinical remission, and the remission only lasted 8 months. We conclude that neoplastic cell clearance after decitabine therapy in CMML is observed after several courses of therapy, and is initially seen concurrently with hypomethylation. While LINE methylation returns to its steady state values after completion of decitabine infusion, the tumor elimination process slowly continues. Our data suggest a non-cytotoxic mechanism of action for the drug, whereby the biology of the neoplastic clone is altered by hypomethylation, leading to delayed clearances of unknown mechanism. Possibilities include an immune response and effects on the neoplastic (or normal) stem cells.

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Classifying Leukemias Based on Epigenetic Alterations.

Jaroslav Jelinek, MD, PhD¹, Marcos R.H. Estecio, PhD^{1,*}, Kimie Kondo, MS^{1,*}, Rong He, MS^{1,*}, Jiri Zavadil, PhD^{2,*} and Jean-Pierre J. Issa, MD¹

¹ Department of Leukemia, The University of Texas M. D. Anderson Cancer Center, Houston, TX, USA and ² Department of Pathology and NYU Cancer Institute, New York University Medical Center, New York, NY, USA.

Acute leukemia is caused by alterations of blood-forming stem cells leading to uncontrolled growth and diminished capacity to differentiate into mature functional blood elements. Beside genetic changes, epigenetic alterations are increasingly recognized as important events in the pathogenesis of leukemia. Cytosine methylation in CpG islands at gene transcription start regions can cause heritable gene silencing and have the same functional effects as inactivating mutations. Hundreds of genes may become epigenetically silenced in leukemia. While many of the methylated genes are not expressed in blood cells, silencing of genes critically important for control of stem cell self-renewal, proliferation, differentiation, and/or survival can contribute to the malignant phenotype. We used a genome-wide method to identify methylated genes by hybridizing a CpG island microarray with amplicons obtained by the methylated CpG island amplification technique (MCAM). We analyzed 10 leukemia cell lines with different cellular origin (myeloid cell lines KG1, KG1a, HEL, K562, and TF1; T lymphoid cell lines CEM and JTAG; and B lymphoid cell lines ALL1, BJAB, and Raji). On average, 266 genomic loci were found to be hypermethylated in these cell lines, ranging from 56 (KG1) to 483 loci (Raji), reinforcing the idea of extensive epigenome alteration in leukemia. Unsupervised hierarchical clustering showed distinct methylation pattern in the cell lines of lymphoid origin versus myeloid leukemia cell lines and a GM-CSF-dependent erythroleukemia cell line TF-1, justifying the use of methylation markers for uncovering of tumor-specific pathways of gene inactivation. There was a striking difference in the number of hypermethylated genes between two closely related myeloid leukemia cell lines: KG1 (56 methylated loci) and its undifferentiated variant KG1a (225 methylated loci). cDNA microarray analysis showed that deoxy-azacitidine treatment induced expression of genes differentially methylated in KG1a (DKKL1, GBX, HIVEP3, KCNAB1, KIAA1102, NAV2, NEIL1, and RAX) but not in KG1 cells where these genes were unmethylated. Finally, we used bisulfite PCR followed by pyrosequencing analysis to quantitatively measure DNA methylation of several genes detected by MCAM.

Ongoing analyses of bone marrow samples from leukemia patients showed hypermethylation of the following genes: GDNF (in 4/22 [18%] AML and 7/20 [35%] ALL patients), HAND2 (in 5/22 [23%] AML and 7/20 [35%] ALL patients), HIVEP3 (in 9/22 [41%] AML and 6/20 [30%] ALL patients), MPDZ (in 2/6 [33%] AML and 15/20 [75%] ALL patients), and NEIL1 (in 2/20 [10%] AML and 1/12 [8%] ALL patients). Mapping of DNA methylation abnormalities may detect epigenetic markers important for

leukemia classification and prognosis. Identification of pathways frequently silenced by DNA methylation may also suggest new targets for specific therapy.

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Poster Session

Analysis of Mutant *cMPL* in Philadelphia Chromosome-Negative Myeloproliferative Disorders (Ph⁻MPDs) Using a Novel High-Sensitivity Assay.

Scott J. Samuelson, MD¹, Sabina Swierczek, Ph.D.^{1,*}, Charles J. Parker, M.D.¹, Kenneth Boucher, Ph.D.^{1,*}, Jaroslav Jelinek, M.D.² and Josef Prchal, M.D.¹

¹ Hematology, University of Utah, Salt Lake City, UT, USA and ² Hematology, University of Texas M.D. Anderson Cancer Center, Houston, TX, USA.

cMPL is a gene encoding for the thrombopoietin receptor that is essential for thrombopoiesis and contributes to pluripotent hematopoietic stem cell expansion. A gain of function *cMPL* mutation, *MPLW515L*, was identified in myeloid cells from patients with primary myelofibrosis (PMF). Subsequent studies identified a second gain of function mutation, *MPLW515K*, in PMF and essential thrombocytosis (ET). The prevalence of *MPLW515L* and *MPLW515K* mutations was 5% in PMF and 1% in ET. No mutant *cMPL* was detected in Polycythemia Vera (PV). The methods utilized in these assays were sensitive to mutant frequencies of >3–5%. We developed a rapid, sensitive, quantitative real time PCR assay based on a unique primer design wherein allelic discrimination was enhanced by the synergistic effect of a mismatch in the –1 position, and a locked nucleic acid nucleoside at the –2 position of the allele-specific primers. An assay of similar design can detect G1849T mutation of *JAK2* in <0.1% mutant allele in peripheral blood granulocyte (Nussenzweig Exp Hematol 2007 3:32). We hypothesized that a similar high sensitivity assay would increase detection of mutant *cMPL* in Ph⁻MPDs. We analyzed genomic DNA from peripheral blood granulocytes of 197 MPD patients and found that 10/197 (5.1%) carried one of the two *cMPL* mutations. Further, 5 of these patients were also *JAK2V617F* positive. *cMPL* mutations were detected in 1/78 (1.3%) PV patients, 3/56 (5.4%) ET patients, 4/49 (8.2%) PMF patients, and 2/11 (18%) MPD-Unspecified patients. *W515L* accounted for 9/10 cases, with *W515K* accounting for only 1. Of the ten positive samples, five (including the patient with PV) had ≤1% mutant alleles. To confirm the validity of our assay, we tested DNA from 96 normal controls. Neither *W515L* nor *W515K* was detected ($p=0.03$ compared to samples from the Ph⁻MPD patients). Additionally, when DNA from megakaryocytic colonies from a patient with 0.70% mutant alleles was analyzed, 12.5% of colonies were found to be heterozygous for *cMPLW515L*. These studies demonstrate the sensitivity and accuracy of our assay and show that *cMPL* activating mutations are more common in ET than previously reported. Mutant allele frequency appears greater in megakaryocytic cultures perhaps indicating a proliferative advantage for the *cMPL*-mutant clone. That mutant *cMPL* and *JAK2V617F* can be found in the same patient demonstrates the molecular heterogeneity of Ph⁻MPDs and emphasizes the need for prospective studies designed to determine the relationship between genotype and clinical phenotype. Scott J. Samuelson and Sabina Swierczek contributed equally to this project.

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Poster Session

Concordance of Assays Designed for the Quantitation of JAK2 1849G>T (V617F): A Multi-Centre Study.

Eric Lippert^{1,*}, François Girodon^{2,*}, Emma Hammond^{3,*}, Serge Carillo^{4,*}, Céline Richard^{5,*}, Boris Fehse^{6,*}, Mirjam Hermans^{7,*}, Ian James^{3,*}, Jaroslav Jelinek⁸, Christophe Marzac^{9,*}, Marina Migeon^{1,*}, Daniela Pietra^{10,*}, Josef T. Prchal^{11,12}, N. Scott Reading^{12,*}, Marta Sobas^{13,*}, Valérie Ugo^{14,*}, Radek C. Skoda¹³ and Sylvie Hermouet^{15,*}

¹ Laboratoire d'Hématologie, CHU, Bordeaux, France; ² Laboratoire d'Hématologie, CHU, Dijon, France; ³ Murdoch University, Perth, Australia; ⁴ Laboratoire de Cytologie Clinique et Cytogénétique, CHU, Nîmes, France; ⁵ Laboratoire d'Hématologie, CHU, Clermont-Ferrand, France; ⁶ Uniklinik Eppendorf, Hambourg, Germany; ⁷ Jeroen Bosch Hospital, s-Hertogenbosch, Netherlands; ⁸ University of Texas MD Anderson Cancer Center, Houston, TX, USA; ⁹ Laboratoire d'Hématologie, Hôpital Saint-Antoine, Paris, France; ¹⁰ Department of Hematology, University of Pavia & Fondazione IRCCS Policlinico San Matteo, Pavia, Italy; ¹¹ Hematology Division, University of Utah School of Medicine, Salt Lake City, UT, USA; ¹² ARUP Laboratories, Salt Lake City, UT, USA; ¹³ Experimental Hematology, Department of Research, Basel University Hospital, Basel, Switzerland; ¹⁴ Laboratoire d'Hématologie, CHU, Brest, France and ¹⁵ Laboratoire d'Hématologie, CHU, Nantes, France.

Studies of myeloproliferative disorders (MPDs) aiming to evaluate the fraction of the clone bearing the JAK2 1849T mutation occasionally report discordant findings. One reason could be different sensitivity and accuracy of the various assays designed for the detection and quantitation of JAK2 1849G>T. We studied the concordance of 10 published JAK2 1849G>T assays. 29 samples of genomic DNA were distributed to 14 laboratories in France, USA, Australia, Germany, Holland, Italy and Switzerland for blinded assessment of JAK2 1849T levels. DNA was extracted from granulocytes of patients diagnosed with MPD, eosinophilia or secondary polycythemia. The 10 assays tested included 5 TaqMan assays with specificity based on primers (4) or competing probes (1) and allele-specific PCR (AS-PCR) (3), pyrosequencing (1) and FRET/melting curve (1) assays. Standards used for calibration were dilutions of DNA from plasmids (3 sets), cell lines or patient granulocytes. Results were expressed as %1849T allele/total JAK2 (13 centres) or as %1849T allele/control gene (1 centre). One centre had one false negative result; there were no false positive results. PCR equipment did not significantly affect the quantitation of 1849T: after adaptation of the technique, one centre tested one AS-PCR assay on 2 apparatus, 5 other centres tested one TaqMan assay on 3 apparatus; comparable results were obtained in the 6 centres. For 6 assays (10 centres), quantitation in the 26 positive samples, ranging from 1% to 96%, did not differ significantly. Overall variation was 30%; concordance improved with increasing mutational load (18% variation for samples with >8% 1849T). Three TaqMan and 1 AS-PCR assays gave significantly different results, 2 with overall low quantitation. For 3 assays, discordance was explained by an incorrect estimation of 1849T content in the standards. For the 4th discordant assay, expressing results as % 1849T/control gene, values tended to be higher proportional to the consensus. Interestingly, results were consistent with the presence of

>2 copies of JAK2 per cell in 4 samples. The study underlined the importance of using defined standards when analysing JAK2 1849T levels. After adaptation to the equipment and with the use of correct standards, all assays gave comparable quantitation of JAK2 1849T, with a sensitivity <1%. Finally, quantitation of a second gene, in order to detect additional copies of JAK2 (>2/cell), should be considered.

Myeloproliferative Disorders

Mutations and promoter methylation status of *NPM1* in myeloproliferative disorders

We determined mutations and promoter methylation status of *NPM1* using pyrosequencing in 199 samples of myeloid neoplasia including myeloproliferative disorders (MPD). The mutations were present in 4% of chronic myelomonocytic leukemia, but not in other MPD or myelodysplastic syndromes. Promoter methylation was rare, and was found in only three samples of MPD.

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Mutations in nucleophosmin 1 gene (*NPM1*, localized on 5q32) were found to be frequent events in acute myeloid leukemia (AML).¹⁻³ *NPM1* likely plays a role as a tumor suppressor in myeloid hematopoiesis, and its haploinsufficiency has been suggested.^{4,5} *NPM1* mutations have not been observed in myelodysplastic syndrome (MDS), chronic myeloid leukemia (CML), or lymphoid malignancies.¹ Here we describe a simple and sensitive screening method for *NPM1* mutation using a pyrosequencing assay, which has a detection limit of approximately 5% of mutant alleles.⁶ We applied this to 14

leukemic cell line samples (OCI/AML3, ALL1, BJAB, CEM, HEL, HL60, JTAG, Jurkat, K562, KG1, KG1a, ML1, Raji, and TF-1) and 199 samples from patients including 39 AML (including 11 with diploid karyotype and four with 5q or chromosome 5 deletion), 15 Ph-positive and 15 Ph-negative CML, 50 MDS (including ten with 5q or chromosome 5 deletion), 50 chronic myelomonocytic leukemia (CMML), 14 polycythemia vera (PV), 7 essential thrombocythemia (ET) and 9 myelofibrosis (MF). Samples from patients were obtained from peripheral blood or bone marrow mononuclear cells, and all patients gave their consent to the donation of samples. Furthermore, promoter methylation, which can cause gene silencing,⁷ of the *NPM1* gene was also analyzed.

The pyrosequencing assay for mutation analyses was the same as the one we had previously utilized for analysis of the *JAK2* mutation.⁶ First, exon 12 of the *NPM1* gene was amplified by polymerase chain reaction (PCR) using primers NPM1-F: 5'-TTAACTCTCTGGTGGTAGAATG-3' and biotinylated-NPM1-R: 5'-ACATTTATCAAACACGGTAGG-3'. Then the biotinylated strand was captured on streptavidin sepharose beads and annealed with a sequencing primer NPM1-S: 5'-TTTTCCAGGC-TATTCAAGAT-3'. Pyrosequencing was performed using PSQ HS 96 Gold SNP Reagents and the PSQ HS 96 pyrosequencing machine (Biotage, Uppsala, Sweden). Programmed polymorphic sites were set at nucleotides 959 (A/C), 960 (G/T) and 964 (G/C) to detect all previously reported mutation variants (Figure 1 A). Mutations are

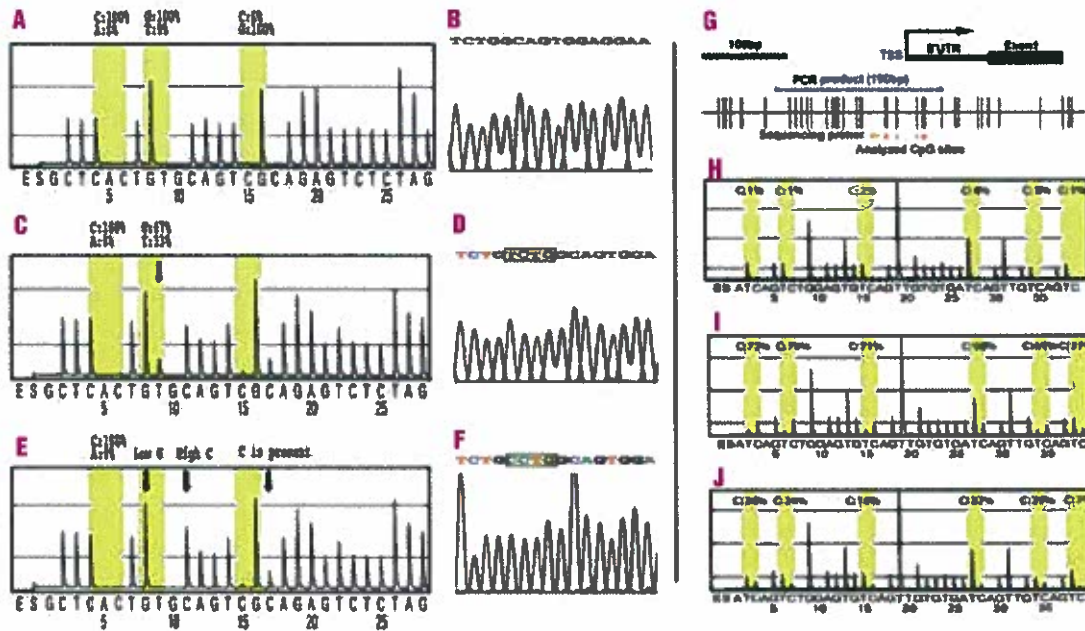


Figure 1. A-F. Mutation assays. Expected wild type sequence in this region is CTCTGGCAGTGGAGGAAGTCTCTTTAAG. **A.** A pyrogram of a normal sample showing only wild type allele. **B.** Cloning and sequencing confirmed a wild type sequence only. **C.** A pyrogram in a patient with CMML, showing an abnormal T peak at position 960, suggesting the presence of a mutant allele. **D.** Cloning and sequencing confirmed the presence of mutation A (960-961insTCTG). **E.** A pyrogram with multiple abnormal peaks in another patient with CMML, suggesting the presence of a mutant allele. **F.** Cloning and sequencing confirmed the presence of mutation D (960-961insCCTG). **G.** Schematic map of the promoter region. CpG sites at -21, -19, -8, 15, 22, and 24 bases from the transcription start site were analyzed. Vertical bars indicate CpG sites. TSS indicates the transcription start site; UTR: untranslated region. **H-J.** Methylation assays. The expected sequence in this region is YGYGGGGAGTTTGYGTTTTTTTTGGTGTGATTYGTITTYGY (Y=T or C). **H.** A pyrogram of a normal sample showing no methylation. **I.** A pyrogram of SssI methylase treated normal control DNA (methylation positive control), showing an average methylation of 69% (average of six CpG sites). **J.** A pyrogram of a sample from a patient with myelofibrosis, showing an average methylation of 24%.

Table 1. Summary of the mutation and methylation analyses.

Disease	N	Prevalence of NPM1 mutation	Prevalence of NPM1 methylation
Leukemic cell lines	14	1 (OCI/AML3)	2 (CEM, ML1)
Acute myeloid leukemia	39	5 (13% of all cases, 45% of diploid cases)	0 (0%)
Chronic myeloid leukemia	15	0 (0%)	0 (0%)
Myelodysplastic syndrome	50	0 (0%)	0 (0%)
CMML	50	2 (4%)	0 (0%)
Ph-negative CML	15	0 (0%)	0 (0%)
Polycythemia vera	14	0 (0%)	1 (7%)
Essential thrombocytopenia	7	0 (0%)	0 (0%)
Myelofibrosis	9	0 (0%)	2 (22%)

CML: chronic myeloid leukemia; CMML: chronic myelomonocytic leukemia.

detected as abnormal pyrogram patterns (pyrosequencing peaks) compared to the wild type pattern. When a mutation was indicated, PCR was repeated using primers without the biotin tag, then cloned in a plasmid and sequenced at the M.D. Anderson Cancer Center DNA Core Facility using ABI Big Dye terminator cycle sequencing chemistry to confirm the mutation.

First, leukemic cell lines and primary AML samples were analyzed to confirm the validity of the mutation analysis. Among cell line samples, only OCI/AML3 showed the *NPM1* mutation, consistent with a previous report.¹² Among 39 AML samples, five with diploid karyotype showed *NPM1* mutations, all as forms of insertion between positions 959 and 960.⁸ The frequency of this mutation in AML patients with diploid karyotype is consistent with that in previous reports.¹³ We did not observe mutations in other positions. The *NPM1* mutation was not observed in Philadelphia chromosome (Ph) positive-CML and MDS (excluding CMML) samples. Among patients with MPD, *NPM1* mutations were observed in two of 50 cases of CMML (Figure 1C, 1E) but in no cases of Ph-negative CML, ET, PV, or MF (Table 1). The patient with mutation A (960-961insTCTG) was a 78-year old male with CMML, with a white blood count of $15 \times 10^9/L$ and 13% peripheral monocytes. Bone marrow showed CMML, with 14% blasts and normal karyotype. This patient was treated with decitabine and achieved a complete remission, when *NPM1* mutation was undetectable. Mutation D (960-961insCCTG) was detected in a 77-year old female with CMML. Bone marrow showed 6% blasts and a normal karyotype. The woman had a peripheral white blood cell count of $6.4 \times 10^9/L$, with 1% blasts and 17% monocytes. Twelve months later, the patient developed AML with *NPM1* mutation D.

We used the bisulfite pyrosequencing method for methylation analyses.⁹ The promoter region of *NPM1* in bisulfite-treated DNA¹⁰ was amplified by PCR using primers NPM1-Bis-F: 5'-AAGGAGTGGGGTTGAAAAG-3' and biotinylated-NPM1-Bis-R: 5'-CCCTACTC-CAAAAAACAACC-3'. After PCR, T/C polymorphisms, corresponding to unmethylated and methylated cytosines in the original DNA, at -21, -19, -8, 15, 22, and 24 bases from the transcription start site, were analyzed with

pyrosequencing,¹³ using sequencing primer NPM1-Bis-S: 5'-GAGATTTTATAGGGTTTATATAAG-3' (Figure 1 G). The methylation percentage was calculated by the average of the degree of methylation at six CpG sites formulated in pyrosequencing. In cell lines, CEM and ML1 showed a low degree of methylation (average 29% and 36%, respectively). However, the *NPM1* expression assay (real-time PCR using Hs01576587_g1 [Applied Biosystems] and GAPDH as internal controls) showed no evidence of gene silencing, when compared to other cell lines (data not shown). In samples from patients, promoter hypermethylation was only observed in two cases with MF and one with PV (50%, 24% [Figure 1J] and 24%, respectively), who all had a diploid karyotype.

In conclusion, we screened samples of MDS and MPD for mutations of *NPM1*, and detected mutations in 4% of patients with CMML. *NPM1* mutations were not observed in MDS or other MPD. Promoter hypermethylation of *NPM1* is rare in myeloid neoplasms. *NPM1* mutations, methylation and 5q deletion were not found simultaneously, although our study included a limited number of patients. The significance of promoter hypermethylation needs to be investigated further.

Yasuhiro Oki, Jaroslav Jelinek, Miloslav Beran, Srdan Verstovsek, Hagop M. Kantarjian, Jean-Pierre J. Issa

Department of Leukemia, The University of Texas, M.D. Anderson Cancer Center, Houston, TX 77030 USA

Key words: *NPM1*, myeloproliferative disorder, chronic myelomonocytic leukemia, methylation.

Correspondence: Yasuhiro Oki, M.D., Department of Leukemia, The University of Texas M.D. Anderson Cancer Center, 4155 Holcombe Blvd, Box 10, Houston, TX 77030, USA. Phone: international +1.713.7922740. Fax: international +1.713.7944297. E-mail: yooki-ky@umh.ac.jp

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Polycythemia vera is not initiated by $JAK2^{V617F}$ mutation

Roberto H. Nussenzweig^a, Sabina I. Swierczek^b, Jaroslav Jelinek^a,
Amos Gaikwad^c, Enli Liu^{c,d}, Srdan Verstovsek^a, Jaroslav F. Prchal^e, and Josef T. Prchal^{b,f}

^aDepartment of Leukemia, University of Texas, M.D. Anderson Cancer Center, Houston, Tex., USA;

^bDivision of Hematology, University of Utah School of Medicine, Salt Lake City, Utah, USA; ^cTexas Children's Cancer Center; and ^dCenter for Cell and Gene Therapy, Baylor College of Medicine, Houston, Tex., USA; ^eDepartment of Oncology, McGill University, Montreal, Quebec, Canada; ^fDepartment of Pathophysiology, Charles University, School of Medicine, Prague, Czech Republic

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Objective. The somatic $JAK2^{V617F}$ mutation is seen in most polycythemia vera (PV) patients; however, it is not clear if $JAK2^{V617F}$ is the PV-initiating mutation.

Methods. In order to examine this issue, we developed a novel real-time quantitative allele-specific PCR, in which allelic discrimination is enhanced by the synergistic effect of a mismatch in the -1 position, and a locked nucleic acid (LNA) nucleoside at the -2 position.

Results. Determination of allelic frequencies was reproducible (SD = 1.5%) and sensitive—0.1% mutant allele detected in 40 ng of DNA. The $JAK2^{V617F}$ frequency in clonal granulocytes from 3 PV females was less than 50% (27.5 ± 11) and in 7 females greater than 50% (75 ± 10.5). We also found that wild-type $JAK2$ BFU-E colonies from PV patients can grow without erythropoietin. The identification of the primary genetic lesion resulting in PV is essential for the development of novel therapeutic strategies.

Conclusion. Our studies correlating the frequency of $JAK2^{V617F}$ mutant allele and clonality, as well as the presence of homozygous wild-type $JAK2$ erythropoietin-independent erythroid colonies, provide compelling evidence that the $JAK2^{V617F}$ is not the PV-initiating mutation. This supports a model wherein the $JAK2^{V617F}$ mutation arises as a secondary genetic event. Furthermore, our results indicate that an undefined molecular lesion, preceding $JAK2^{V617F}$, is responsible for clonal hematopoiesis in PV. We conclude that development of therapeutic strategies that target the $JAK2^{V617F}$ clonal cells may not be sufficient for eradication of PV. © 2007 International Society for Experimental Hematology. Published by Elsevier Inc.

Myeloproliferative disorders (MPDs) are hematological malignancies due to clonal proliferation arising from a single multilineage stem cell. The World Health Organization (WHO) classified MPDs into the following four prototypical clinical diseases: polycythemia vera (PV), essential thrombocythemia (ET), myelofibrosis with myeloid metaplasia (MMM), and chronic myeloid leukemia (CML). Subsequently, other related disorders were added to this classification [1]. The chromosomal translocation $t(9;22)$, resulting in a chimeric fusion protein encoded by the *Bcr-Abl* gene, leads to proliferation and survival of myeloid progenitor cells in CML. Recently, a novel somatic single-point mutation in the 9p chromosomal region encoding the

tyrosine kinase $JAK2$ (1849 G to T) has been reported in several MPDs [2–6]. This mutation results in a nonsynonymous amino acid substitution at position 617 (valine to phenylalanine) located in the JH2 pseudo-kinase auto-inhibitory domain [7]. The mutation renders the enzyme constitutively active and leads to cytokine hypersensitivity and erythrocytosis in a mouse model [8]. The $JAK2^{V617F}$ mutation has been reported in greater than 80% of PV patients and in approximately half of ET or MMM patients [5]. The proportion of the mutant allele is highly variable, with approximately 30% of PV patients having loss-of-heterozygosity of chromosome 9p created in most instances by uniparental disomy [9].

Studies in MPD support that acquisition of the $JAK2^{V617F}$ mutation results from a somatic event. The proportion of $JAK2$ mutant allele in a clonal granulocyte cell population from the peripheral blood of a patient can vary

Offprint requests to: Josef T. Prchal, M.D., University of Utah, Hematology Division, 30 North 1900 East, 4C416 SOM, Salt Lake City, Utah 84132-2408; E-mail: Josef.Prchal@hsc.utah.edu

from 0 to 100%. According to one scenario, the mutation is not the first event leading to disease; this is further supported by the existence of PV families with a predisposition to acquire PV [10]. Moreover, Kralovics and colleagues [11] report the identification of a PV patient with 7% *JAK2* mutant allele frequency, yet harboring the characteristic MPD del20q in 100% of her granulocytes. To date, in spite of the identification of a single patient, this report provides the most compelling evidence that *JAK2*^{V617F} is not the primary event leading to PV. In addition, zygosity in patients carrying the *JAK2*^{V617F} mutation can be heterogeneous, with wild-type, heterozygous, and mutant homozygous clones being present. The clinical significance and role of the *JAK2* mutation in the pathology and progression of disease, especially for PV, is being investigated. It appears that some complications of MPDs such as the degree of marrow fibrosis or thrombotic tendencies correlate with overall proportion of the mutant allele in circulating cells [8,12]. The close association of *JAK2*^{V617F} with PV and burst-forming unit erythroid (BFU-E) endogenous colonies of PV makes this mutation an excellent disease marker. Hence, disease progression, treatment, and development of novel chemotherapeutics can be readily assessed by following changes in the proportion of the mutant *JAK2* allele in clonal granulocytes.

To date, several different methods have been developed to quantitatively determine the proportion of single nucleotide polymorphisms (SNPs) in pooled DNA samples [13]. These include (but are not limited to) allele-specific PCR (AS-PCR) and pyrosequencing. A number of strategies have been employed to improve the specificity and reliability of AS-PCR and adapt it to real-time monitoring. These modifications have demonstrated that AS-PCR can be a reliable tool for genotyping provided time is taken to carefully design and optimize PCR conditions. On the other hand, pyrosequencing requires instrumentation that is not readily available and setup costs are prohibitive for routine laboratory testing. Moreover, pyrosequencing cannot be used for routine screening of residual or early disease, since the proportion of the *JAK2* mutant allele is below the 5% detection limit threshold.

We describe here the development of a novel approach to quantitatively determine the proportion of wild-type (wt) and mutant *JAK2* alleles by a novel modification of real-time AS-PCR. We demonstrate the high sensitivity, specificity, and reproducibility of our method that permits accurate quantitation of the *JAK2*^{V617F} mutant allele using now widely available kinetic PCR instrumentation. Moreover, our approach to allele-specific primer design is simple, does not require optimization of PCR conditions, and incorporates nucleotides that are readily available from most oligonucleotide synthesis companies. We used this methodology to enhance our understanding of the molecular biology of PV. Here, we provide definitive evidence that the *JAK2*^{V617F} mutation is not the PV-initiating event.

Materials and methods

Samples

Blood samples were collected from 20 unrelated PV patients and 3 healthy volunteers using an Institutional Review Board-approved protocol. Granulocytes were isolated from peripheral blood using standard methods. Genomic DNA (gDNA) was extracted from peripheral blood leukocytes and purified granulocytes using the Puregene DNA purification kit as recommended by the manufacturer (Gentra, Minneapolis, MN, USA).

BFU-E colony culture

In vitro assay of erythroid progenitors' responsiveness to erythropoietin (EPO) was performed as previously described [14]. Briefly, mononuclear cells from peripheral blood were isolated on Histopaque 1077 (Sigma, St. Louis, MO, USA) and cultured at a final density of 3×10^5 cells/mL in Methocult H-4531 medium (StemCell Technologies Inc., Vancouver, BC, Canada) in 35-mm Petri dishes in the presence or absence of 3 U/mL EPO. Cultures were maintained at 37°C and 5% carbon dioxide. Single erythroid colonies, selected using standard criteria, were picked after 14 days in culture using micropipettes.

Real-time AS-PCR

Real-time AS-PCR was performed on an Applied Biosystems 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Typical reactions (25 μ L) consisted of 1 $\times$ TaqMan Universal PCR master mix (Applied Biosystems, Foster City, CA, USA); 300 nM *JAK2* universal forward and allele-specific reverse primers; 125 nM FAM-labeled *JAK2* MGBNFQ probe (Applied Biosystems); and 1 to 50 ng purified genomic DNA. Enzyme activation (95°C for 10 minutes) was followed by 50 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Allele-specific primers were designed using the software program Oligo 6.7 (Molecular Biology Insights, Inc., Cascade, CO, USA) and synthesized by IDT (Integrated DNA Technologies, Coralville, IA, USA). The 3' terminal sequence of the reverse primer (C or A)-g/*JAK2*-R was selected for specific amplification of the mutant allele. Allelic discrimination was enhanced by introducing an artificial mismatch (T:T) in the –1 position starting from the 3' end of the primer, as described in the amplification refractory mutation system (ARMS, [15]). An additional modified locked nucleic acid (LNA) base [16] was placed at the –2 position (G). Control primers either with modification alone or in the absence of any modification were tested as described above. Sequences and specifications of all primers and the detection probe are provided as supplemental Table S1.

Pyrosequencing

Quantitation of the *JAK2*^{V617F} mutant allele was done as previously described [17] using a PSQ HS 96 pyrosequencer and reagents provided by the manufacturer (Biotage, Uppsala, Sweden).

Allele frequency calculations

Allele frequency was calculated as described by Germer et al. [13]. The difference in cycle threshold (ΔC_t) between the two allele-specific PCR reactions is a measure of the proportion or frequency of the allele, assuming that initial replication efficiency is 100%. If the amplification efficiencies of the two allele-specific reactions differ slightly, this can be corrected by measuring the ΔC_t on a DNA sample known to be heterozygous for the mutation

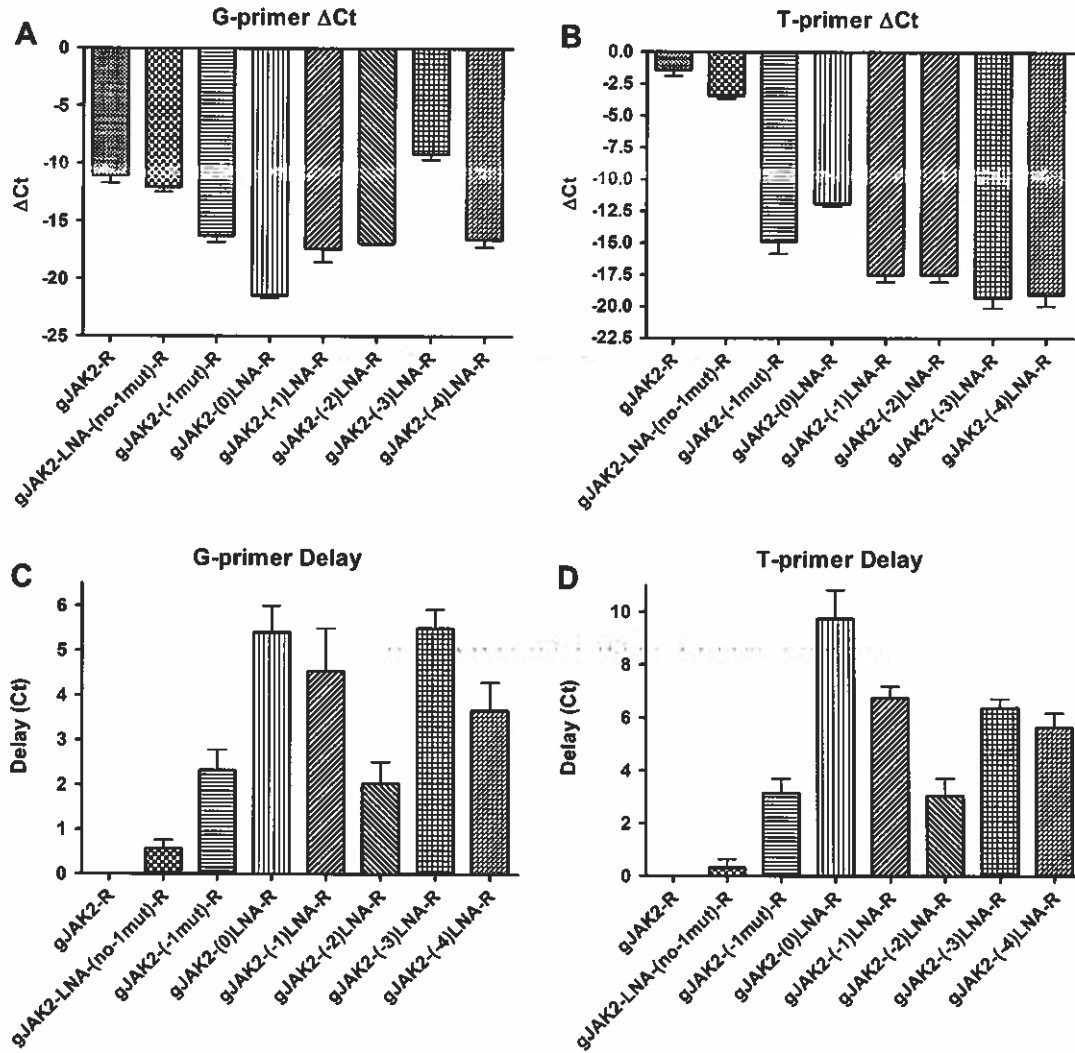


Figure 1. Comparison of primer modifications for discrimination of wild-type and mutant *JAK2*^{V617F} alleles. Genomic DNA from a healthy donor (*JAK2* homozygous wild-type) and a PV patient (*JAK2* homozygous mutant) were used to evaluate the performance of G- and T-allele-specific primers bearing different permutations of mismatch and LNA modifications, and with no modifications (see supplemental Table S1). (A) ΔC_t for G-allele-specific primers; more negative indicates greater discrimination. (B) ΔC_t for T-allele-specific primers; more negative indicates greater discrimination. (C) Ct delay resulting from introduction of modification in G-allele-specific primers compared to G-primer without modification. (D) Ct delay resulting from introduction of modification in T-allele-specific primers compared to T-primer without modification. Data represent mean $\pm$ SD of three independent determinations.

of interest. The ΔC_t in the heterozygous sample should be zero; any deviation from zero can be subtracted from all ΔC_t measurements in order to compensate for differing amplification efficiencies and is represented by HC (heterozygote correction factor). Therefore, $HC\Delta C_t$ represents the heterozygote corrected difference in cycle threshold between the two allele-specific PCR reactions.

$$\Delta C_t = C_{t_allele_1} - C_{t_allele_2} \quad (1)$$

and

$$HC\Delta C_t = \Delta C_t - (HC C_{t_allele_1} - HC C_{t_allele_2}) \quad (2)$$

Results obtained in equations 1 and 2 are used to find the frequency of allele₁ in equation 3:

$$Freq.allele_1 = 1 / (E^{HC\Delta C_t} + 1) \quad (3)$$

where E represents the efficiency of PCR amplification for allele₁ and can be deduced by the slope of serially diluted sample.

Statistical calculations

One-way ANOVA and multiple comparison (Newman-Keuls test) were used to analyze statistically significant differences between ΔC_t and delay results (Fig. 1) when comparing primer design modifications. Statistical significance was assumed for $p < 0.05$.

Results

Assay design

Allele-specific PCR is widely used for SNP genotyping and is based on amplification of DNA by an allele-specific primer matching the polymorphism at the 3' position. In theory, the allele-specific primer containing the mismatched nucleoside at the 3' end should not be extended by Taq DNA polymerase (*Taq*). However, it has been shown that *Taq* can extend mismatched allele-specific primers, generating false-positive results. A number of different strategies have been developed to improve specificity and reliability of this technique [15,18–21].

Our assay has made use of two such strategies, the inclusion of a second mismatch and a modified LNA base. We hypothesized that inclusion of a second mismatch at the –1 position would further destabilize the 3' end of the allele-specific mismatched primer, resulting in an increase in specificity of the matched primer after the first cycle. Oligonucleotides carrying LNA bases are thought to favor the formation of A-helix DNA duplexes, improved base-pair stacking, and higher melting temperature (T_m) [16]. Therefore, we hypothesized that the LNA at the –2 position would stabilize the matched bases of our allele-specific primer, increase primer T_m , enhance allele-specific amplification, and decrease assay-to-assay variability. A schematic representation of our assay design is provided as Supplemental Figure S1.

Real-time AS-PCR

Genomic DNA was isolated from granulocytes of a control (Ctl) and one PV patient (PV4) representing the two homozygous *JAK2* genotypes (GG and TT) as previously determined by pyrosequencing. Comparison of our novel allele-specific primer design, with control allele-specific primers bearing a mismatch, LNA modified base, and no modification (supplemental Table S1), was evaluated by real-time PCR using both homozygous genotypes (Fig. 1). A dynamic increase in fluorescence, reflecting probe cleavage, was seen with all primer designs. In the absence of any modification, the G-allele-specific primer efficiently extended both matched and mismatched targets, resulting in poor discrimination between genotypes (Fig. 1A). Allele-specific primers carrying the LNA modified base showed only marginal improvement discriminating between genotypes (Fig. 1A,B). Results for the primers carrying the extra mismatch and our design (Fig. 1A,B) were excellent for both wild-type and mutant homozygous genotypes, with ΔC_t greater than 14 cycles between matched and mismatched primers. This indicated that the additional mismatch, in this particular application, was responsible for improved discrimination and decreased mismatch extension by the G-allele-specific primer.

Next we examined the reproducibility of the results by determining the ΔC_t of matched and mismatched primers

in three independent measurements (Fig. 1A,B). Results indicate that, although discrimination is significantly improved by the introduction of the mismatch at the –1 position and comparable to our primer design, addition of the LNA base at the –2 position enhances reproducibility as expected. We also found that positioning of the LNA base at either the –1 or –4 positions slightly improved discrimination, albeit at a cost of decreasing the sensitivity of the assay (Fig. 1C,D). ΔC_t differences obtained for the G-allele-specific primers, bearing either the mismatch alone (gJAK2(-1mut)-R) or mismatch and –2 LNA (gJAK2(-2)LNA-R) modifications, were not significant ($p > 0.05$). In contrast, the differences in ΔC_t for the T-allele-specific primers, harboring the same modifications as above, were significant ($p < 0.05$).

Serial dilutions of genomic DNA from both wild-type and mutant homozygous samples were assayed by real-time PCR using our allele-specific primers. PCR reaction efficiency was determined for both allele-specific primers and was found to be greater than 92%. Allele detection limits were estimated to be in the range of 50 pg genomic DNA (approximately 7 diploid genome equivalents). Since *JAK2*^{V617F} is a somatic mutation, we determined the unequal amplification efficiency and calculated the heterozygosity correction factor (HC) between our allele-specific primers by measuring the ΔC_t of a 50:50 mix of our wild-type and homozygous mutant DNA samples.

Real-time vs pyrosequencing

Since pyrosequencing is considered a quantitative and reliable technique for the detection of SNP, we decided to perform a blind comparison between our allele frequency determinations and those independently obtained by pyrosequencing. Table 1 shows the estimated frequencies of the mutant T-allele in granulocytes isolated from different PV patients obtained by both real-time PCR and pyrosequencing with an excellent correlation in a linear regression model ($y = 1.01x + 2.6$; $R^2 = 0.99$; $p < 0.0001$) (Supplemental Fig. S2).

Reproducibility of the assay

In order to test the robustness of our design, the frequency of the *JAK2* mutant T-allele was determined in 31 genomic DNA samples from peripheral blood and granulocytes of PV patients (Table 2). Three independent measurements were performed for each sample. Mean allele frequency was found to deviate by less than 1.5%, on average, for all samples tested (Table 2).

Mutant allele frequency sensitivity

The *JAK2* mutant allele detection sensitivity was investigated by mixing normal control genomic DNA with decreasing proportions of genomic DNA isolated from the human erythro-leukemic cell line (HEL), which is homozygous for the *JAK2*^{V617F} mutation (Fig. 2). The most sensitive techniques used to detect low levels of SNP are still

Table 1. Comparison of T-allele frequency obtained by pyrosequencing and our method

Patient number	Date on sample	T-allele (%) (pyrosequencing)	T-allele (%) (AS-PCR)
PV1	11/3/2005	57.0	64.8
PV2	3/1/2005	78.3	80.7
PV3	4/13/2005	21.3	23.4
PV3	6/30/2005	28.7	30.0
PV4	12/29/2004	69.4	66.4
PV4	3/1/2005	59.2	60.4
PV4	5/26/2005	57.9	68.4
PV4	6/30/2005	18.8	24.1
PV5	8/2/2005	44.9	46.6
PV6	8/31/2005	0.0	0.0
PV7	1/4/2005	84.1	88.5
PV7	3/30/2005	66.7	73.4
PV7	6/30/2005	80.2	82.2
PV7	7/12/2005	78.4	84.1
PV7	10/14/2005	79.0	81.4
PV8	10/19/2005	96.0	97.0
PV9	8/22/2005	83.0	87.3
PV10	5/11/2005	80.0	84.2

limited by the mass of gDNA assayed. The mass of a single haploid human genome is estimated to be 3.7 pg. Therefore, if 40 ng of genomic DNA are used to assay for the presence of a mutated allele with a frequency of 0.01%, only a single copy may be detected by random chance. Most assays use 20 to 50 ng gDNA; hence, a reasonable detection limit should be 0.1%. We have routinely and reliably measured *JAK2* mutant allele at a frequency of 0.1% using 40 to 50 ng gDNA (Fig. 2). However, even though sensitivity of our assay has not been determined with 0.5 µg of gDNA, we should be able to reliably detect less than 0.01% mutant allele (Fig. 2).

Is *JAK2*^{V617F} the disease-initiating mutation?

We have demonstrated the relevance and usefulness of our primer design while investigating mutant *JAK2* T-allele frequency and clonality in several female PV patients. Genomic DNA isolated from granulocytes of 10 female PV patients, who were found to be clonal by the X-chromosome transcriptional clonality assay [22], were used for the determination of *JAK2* mutant allele frequency. In 3 of the 10 female patients with clonal granulopoiesis, mutant allele frequencies were below 50% (27.5 ± 11) while the remaining 7 had frequencies greater than 50% (75 ± 10.5). This result, in combination with the clonality data, is indicative of a heterogeneous population composed of granulocytes with differing *JAK2*^{V617F} genotypes (GG, GT, or TT) and reinforces the model wherein the mutation is not the disease-initiating event. However, due to the possibility that X inactivation in some of our female patients may be skewed, the appearance of clonality may be misleading. Therefore, this data, although indicative of *JAK2*^{V617F} not being the initiating event, is by no means sufficient evidence for such a claim.

Direct evidence for *JAK2*^{V617F} as a secondary event

Since erythropoietin-independent growth of BFU-E colonies (also known as EEC) is considered a hallmark of PV [23], we decided to verify if our assay could be used to determine the *JAK2* mutant T-allele frequency in single colonies. In our preliminary experiments using *JAK2* sequencing and allele discrimination real-time PCR, we found that a majority of untreated PV patients, and patients stable on their respective therapies, had erythropoietin-independent BFU-E that were homozygous for the *JAK2*^{V617F} mutation. Nevertheless, some colonies were heterozygous, while rare colonies had a wild-type *JAK2* genotype (data not shown). Using our novel and more sensitive quantitative assay described herein, we have reexamined this issue. Since individual EEC colonies represent the “clonal” growth and proliferation initiated by a single affected cell [24], allele frequencies should reflect those normally observed for any germline SNP (0, 50, and 100). Mononuclear cells from two female and two male PV patients were used to grow and harvest 89 individual BFU-E colonies [25], a total of 69 genotypes were determined (Table 3). Deviation from the expected allele frequency was small, and is hypothesized to be due to contamination by other colonies or cells picked together while harvesting the colony of interest. Colonies with allele frequency deviations greater than 10%, as determined by the *JAK2* mutant T-allele frequency distribution (Supplemental Fig. S3), were discarded in order to avoid any bias during genotyping (Table 3). Both heterozygous and homozygous (mutant and wild-type) *JAK2*^{V617F} colonies were detected (Table 3).

Table 2. Reproducibility as estimated by three independent measurements

Patient number	Date	%T-allele (PB-DNA)	%T-allele (GNC-DNA)
PV3	4/13/2005	16.4 (± 0.8)	23.4
PV4	3/1/2005	38.8 (± 3.0)	60.4
PV4	6/30/2005	16.8 (± 0.9)	27.2 (± 4.7)
PV4	12/27/2005	41.1 (± 2.8)	55.1 (± 2.0)
PV6	8/31/2005	N.D.	0.0 (± 0.0)
PV7	1/4/2005	74.8 (± 1.0)	88.2 (± 0.7)
PV7	7/12/2005	74.6 (± 0.6)	84.8 (± 1.6)
PV7	10/14/2005	67.3 (± 4.4)	82.2 (± 1.6)
PV8	10/19/2005	89.7 (± 0.3)	97.4 (± 0.5)
PV10	5/11/2005	63.4 (± 1.3)	84.2 (± 1.0)
PV12	9/3/2005	27.3 (± 1.9)	40.7 (± 1.0)
PV14	11/30/2005	74.7 (± 2.0)	78.1 (± 1.1)
PV15	11/8/2005	0.1 (± 0.0)	0.0 (± 0.1)
PV17	12/8/2005	37.1 (± 0.4)	44.9 (± 1.3)
PV18	11/8/2005	85.8 (± 0.8)	91.4 (± 0.3)
PV19	11/15/2005	66.5 (± 0.7)	92.5 (± 4.9)
PV21	3/15/2005	95.8 (± 0.4)	99.9 (± 0.0)

T-allele frequency (%) was determined for peripheral blood genomic DNA (PB-DNA) and for granulocyte genomic DNA (GNC-DNA). Results are shown as the mean with standard deviation in between parenthesis. N.D., not determined.

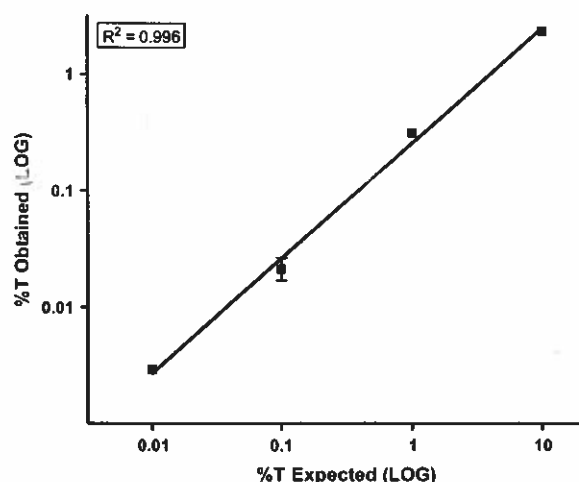


Figure 2. Limit of mutant allele detection in the presence of a large excess of wild-type allele. Genomic DNA from HEL cells (*JAK2* homozygous mutant) was mixed in decreasing proportions to DNA from a healthy donor (*JAK2* homozygous wild-type). Ensuing mixtures containing 10%, 1%, 0.1%, and 0.01% HEL gDNA were used to determine *JAK2*^{V617F} mutant allele frequency. Linear regression of kinetic AS-PCR determinations vs expected allele frequencies showed excellent correlation ($R^2 = 0.996$; $p < 0.002$). Data represent mean $\pm$ SD of three independent determinations.

Discussion

AS-PCR allows determination of the allele frequency of any polymorphism or mutation based on the difference in the number of PCR cycles needed to generate detectable product. AS-PCR can also be a valuable tool to determine the frequency of rare alleles when somatic mutations are present in a small percentage of cells within a tissue sample. Addition of mismatches in a primer requires careful design, strand selection, and optimization of the many vari-

ables that affect primer stability and target amplification. Furthermore, the use of LNA in the design of oligonucleotide primers and probes resulted in improved specificity and allele discrimination compared to unmodified primers [21,26]. However, the synergistic effect, as shown here, of primers harboring a mismatched nucleoside and LNA modification in AS-PCR has not been investigated.

We have demonstrated here that use of AS-PCR for quantitation of somatic mutations using our novel primer design is sensitive, accurate, and highly reproducible. Independent investigators have tested our design in different laboratories using two kinetic PCR platforms (ABI-7000, made by Applied Biosystems, Foster City, CA, USA, and a Light Cycler, made by Roche Applied Science, Indianapolis, IN, USA). Results for the samples tested were nearly identical between platforms, and were comparable to previous estimates of allele frequency obtained by pyrosequencing (results not shown). Further, amplicon detection during kinetic AS-PCR can be done using any available technology (e.g., SYBR-Green, TaqMan Probe, Molecular Beacon, Hybridization Probe) at the discretion and convenience of the investigator.

We have successfully tested our primer design for the quantitation of expressed *JAK2*^{V617F} mutant allele mRNA from PV patient samples (data not shown). Our primer design strategy can also be applied to determine allelic frequencies of other SNP or mutations. To date, we have successfully designed five different primer/probe sets which will be used to detect expression of X-chromosomal SNPs that are subjected to inactivation in women, for determination of clonality [22]. The only optimization required, when using our primer design strategy, is determination of the heterozygosity correction factor for proper estimation of allele frequencies.

Potential applications for our primer design are varied, such as quantitative determination of mosaicism; proportion of fetal cells in maternal circulation; detection of minimal residual disease associated with known somatic mutation (such as reduction of malignant cells by chemotherapy or reappearance of resistant clone); rapid monitoring of efficacy of new drugs in “in vitro” systems as well as clinical trials; and many others that require quantitation of allele frequencies.

We used our quantitative AS-PCR assay described here to investigate the order of genetic events leading to the *JAK2*^{V617F} mutation in the ontogeny of clonal hematopoiesis in PV. In contrast to a recent study reporting the absence of *JAK2*^{V617F}-negative EPO-independent BFU-E colonies [27], we found such colonies in samples from our PV patients. A possible explanation for this discrepancy may relate to the sensitivity and methods used for detection of the mutant allele. The determination of zygosity in granulocytes from PV patients by Scott and colleagues was done in samples that were collected at least 6 months prior to the samples used for clonogenic assay [27]. We have also found

Table 3. *JAK2*^{V617F} allele frequency in isolated BFU-E colonies

Patient number	T-allele (%) in GNC	BFU-E with 0 U EPO			BFU-E with 3 U EPO		
		G/G	G/T	T/T	G/G	G/T	T/T
PV18	91.4	0	0	7	2	1	8
PV22	N.D.	1	1	3	5	0	7
PV23	20	6	1	0	7	4	0
PV24	89	1	2	2	6	0	4
PV25	55	0	0	7	0	0	5
PV26	42	0	0	3	2	0	1
PV27	31	0	2	0	2	6	0
PV28	91	0	0	1	0	0	0
PV29	61	2	0	0	0	0	0
Total colonies		10	6	23	24	11	25

Genotypes represent the quantitative determination of the frequency of mutant allele with cutoffs as follows: G/G (0–10%), G/T (40–60%), and T/T (90–100%). Colonies that are not within these set cutoffs are deemed contaminated by other cells while harvesting and are discarded. N.D., not determined.

that X-chromosome-based methylation clonality assays have significant shortcomings compared to transcriptional-based X-chromosome clonality assays [27]. In addition, Scott and colleagues used a qualitative PCR and restriction endonuclease digestion assay for the genotyping of their samples, and hence could not distinguish colonies potentially contaminated with other cells [27]. In this assay, identification of *JAK2* wild-type genotypes requires the PCR product to be fully digested by *BsaXI*. Therefore, homozygous *JAK2* mutant allele and heterozygous colonies may be erroneously genotyped due to incomplete digestion. Since our methods make use of real-time monitoring during PCR amplification, and the data generated is quantitative, we can make genotyping determinations with greater confidence. Further, the *in vitro* analysis of BFU-E response to erythropoietin (clonogenic assay) is laborious and data not always easy to interpret; however, we have developed this assay and have routinely used it since 1974 [23]. We have validated the reproducibility and use of the clonogenic assay in our studies of a large number of congenital and acquired polycythemic states such as primary familial and congenital polycythemia and Chuvash polycythemia [28].

The presence of wild-type *JAK2* EPO-independent colonies suggests the existence of a distinct, and as of now unknown, alternative mechanism active in erythropoiesis/hematopoiesis. Moreover, our results indicate the presence of an undefined molecular lesion that precedes the *JAK2*^{V617F} mutation. Finally, our data suggest that development of chemotherapeutics that target the *JAK2*^{V617F} clonal cells may not suffice to cure PV.

Acknowledgments

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Table S1. Primer and Probe sequences

(A) Primer/Probe	Modification	(B) Sequence 5' to 3'
FAM-AS-JAK2-MGB	FAM labeled MGB probe with non-fluorescent quencher (NFQ)	5' 6FAM - CTTGCTCATCATACTTGC - MGBNFQ 3'
gJAK2-F	N/A	TTATGGACAACAGTCAAACAACAAT
G-gJAK2-R	None	TTTACTTACTCTCGTCTCCACAGAC
T- gJAK2-R	None	TTTACTTACTCTCGTCTCCACAGAA
G-gJAK2-LNA-(no-1mut)-R	LNA at -2	TTTACTTACTCTCGTCTCCACA <u>G</u> AC
T- gJAK2-LNA-(no-1mut)-R	LNA at -2	TTTACTTACTCTCGTCTCCACA <u>G</u> AA
G-gJAK2-(-1 mut)-R	-1 Mismatch	TTTACTTACTCTCGTCTCCACAG t C
T- gJAK2-(-1 mut)-R	-1 Mismatch	TTTACTTACTCTCGTCTCCACAG t A
G-gJAK2-(0)LNA-R	LNA at 0 and -1 Mismatch	TTTACTTACTCTCGTCTCCACAG t <u>C</u>
T- gJAK2-(0)LNA-R	LNA at 0 and -1 Mismatch	TTTACTTACTCTCGTCTCCACAG t <u>A</u>
G-gJAK2-(-1)LNA-R	LNA at -1 and -1 Mismatch	TTTACTTACTCTCGTCTCCACAG t <u>C</u>
T- gJAK2-(-1)LNA-R	LNA at -1 and -1 Mismatch	TTTACTTACTCTCGTCTCCACAG t <u>A</u>
G-gJAK2-(-2)LNA-R	LNA at -2 and -1 Mismatch	TTTACTTACTCTCGTCTCCACA <u>G</u> t C
T- gJAK2-(-2)LNA-R	LNA at -2 and -1 Mismatch	TTTACTTACTCTCGTCTCCACA <u>G</u> t A
G-gJAK2-(-3)LNA-R	LNA at -3 and -1 Mismatch	TTTACTTACTCTCGTCTCCAC <u>A</u> G t C
T- gJAK2-(-3)LNA-R	LNA at -3 and -1 Mismatch	TTTACTTACTCTCGTCTCCAC <u>A</u> G t A
G-gJAK2-(-4)LNA-R	LNA at -4 and -1 Mismatch	TTTACTTACTCTCGTCTCCA <u>C</u> AG t C
T- gJAK2-(-4)LNA-R	LNA at -4 and -1 Mismatch	TTTACTTACTCTCGTCTCCA <u>C</u> AG t A

A) Allele specific primers are named beginning with uppercase G or T based on the sequence of the sense strand. The G-primers are specific for the wild-type allele, while, the T-primers are specific for the mutant allele.

B) LNA bases are depicted in uppercase italics, bolded and underlined; while, the -1 mismatch is highlighted in bold lowercase. Base positions are counted beginning at the 3' end of the primers.

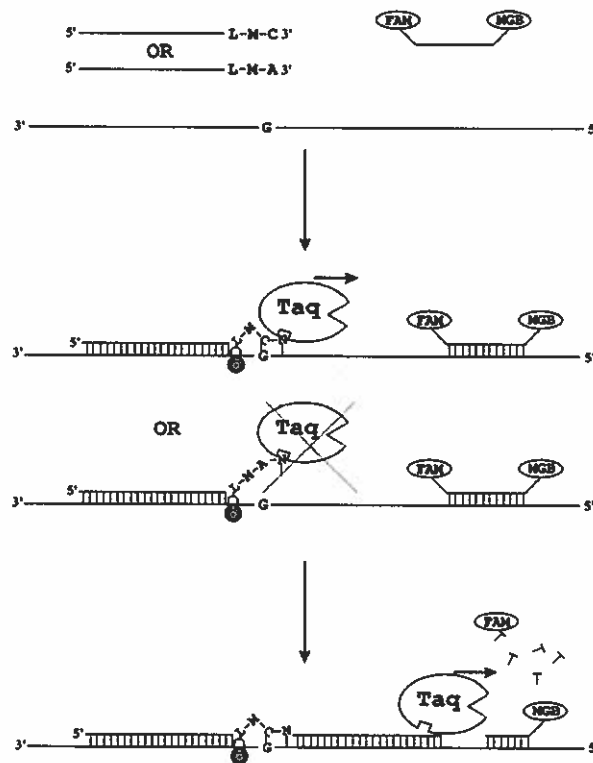


Figure S1. Diagram of the mechanism of action for our primer design. The diagram depicts the strand used for allele-specific priming and extension. The strand primed and extended by the universal primer is not shown. In the first step, two different reactions are prepared, each containing one of the allele-specific primers and the universal primer. In the second step, after heat denaturation, the allele-specific primer anneals and, depending on 3' end hybridization, *Taq* DNA polymerase extension occurs. Finally, in the third step, once extension takes place, the 5' to 3' exonuclease activity of *Taq* cleaves the probe between the reporter and quencher. Subsequent PCR cycles favor the newly synthesized strand since it incorporates the -1 mismatched nucleoside. We hypothesize that the LNA, in our primer design, helps stabilize 3' end annealing by improving base pair stacking of matched bases, increasing primer *T_m*, and decreasing ΔG . Hence, as depicted above, 3' end floppiness, resulting from the introduction of the -1 mismatch, is compensated by the presence of the LNA, leading to enhanced reproducibility, specificity and efficiency of the reaction.

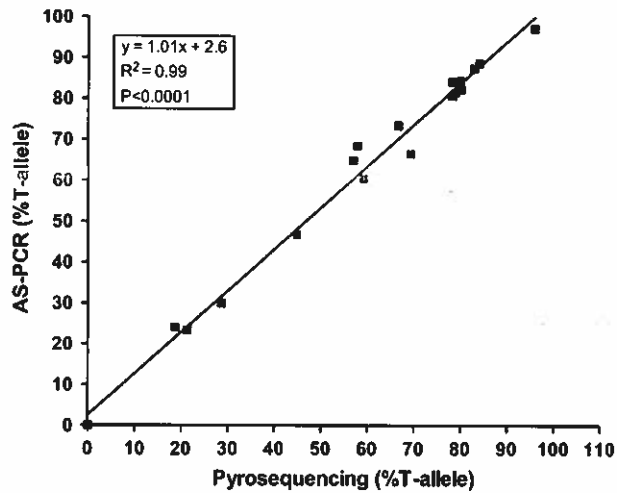


Figure S2. Linear regression of the correlation between AS-PCR vs. Pyrosequencing. *JAK2^{V617F}* (%T-allele) frequency, for the same patient sample, were independently determined by real-time AS-PCR and pyrosequencing. An excellent correlation between both assay platforms was found in a linear correlation model as shown above.

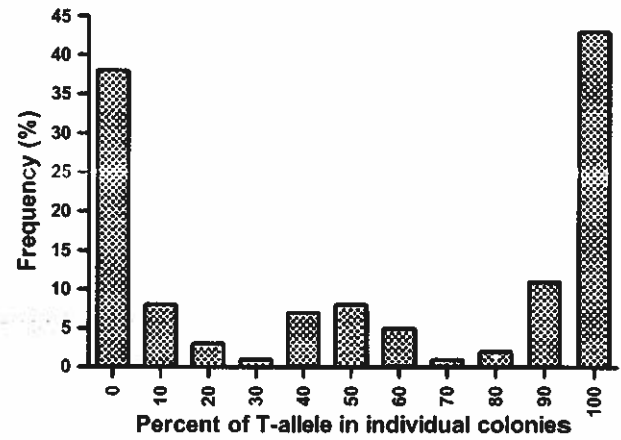


Figure S3. BFU-E colony analysis showing a trimodal distribution of T-allele frequencies. Frequency distribution of all BFU-E colonies picked and used for genotyping the *JAK2^{V617F}* mutation, as described in the accompanying manuscript. The cutoff's used for defining genotypes are indicated in table 3.



Epigenetic control of PRV-1 expression on neutrophils

Jaroslav Jelinek^a, Jun Li^b, Zakar Mnjoyan^b,
Jean-Pierre J. Issa^a, Josef T. Prchal^c, and Vahid Afshar-Kharghan^b

^aDepartment of Leukemia, University of Texas M. D. Anderson Cancer Center, Houston, Tex., USA; ^bThrombosis Research Section, Baylor College of Medicine and Michael E. DeBakey VA Medical Center, Houston, Tex., USA; ^cDivision of Hematology, University of Utah School of Medicine, Salt Lake City, Utah, USA

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Objective. Polycythemia rubra vera–1 (PRV-1) is a GPI-linked protein that is expressed on a subgroup of neutrophils. The number of PRV-1–expressing neutrophils increases in pregnancy and sepsis, or after administration of granulocyte colony-stimulating factor. Expression of the *PRV-1* gene is also increased in patients with polycythemia vera (PV) and essential thrombocythemia (ET). We investigated whether DNA methylation of the *PRV-1* gene has a role in regulation of transcription and expression of the PRV-1 protein.

Methods. We compared the level of methylation of the *PRV-1* gene and expression of the PRV-1 mRNA in normal neutrophils expressing PRV-1 to those that are PRV-1–negative. We also studied *PRV-1* methylation and mRNA expression in patients with Philadelphia chromosome–negative myeloproliferative disorders and in an in vitro model of DNA demethylation.

Results. We found that methylation of CpG dinucleotides close to initiation codon of the *PRV-1* gene was inversely related to expression of PRV-1 in normal neutrophils. Furthermore, overexpression of the *PRV-1* gene in PV and ET is associated with a decrease in methylation of this gene. Among patients with PV and ET, methylation of the *PRV-1* gene is also inversely correlated with the presence of the *JAK2*^{V617F} somatic mutation. In an in vitro model, exposure of KG1 and KG1a cells to a DNA demethylating agent caused a decrease in methylation of the *PRV-1* gene and increased its mRNA level.

Conclusion. DNA methylation regulates PRV-1 expression under physiologic and pathologic conditions. © 2007 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.

Polycythemia rubra vera–1 (PRV-1), NB1, or CD177 is a GPI-linked surface protein that is expressed on neutrophils [1]. Interestingly, PRV-1 is normally expressed only on a subgroup of neutrophils [2,3]. Circulating neutrophils can be divided into PRV-1–positive and PRV-1–negative subgroups according to the presence or absence of PRV-1 on their surface (Fig. 1). Regulation of this unique expression pattern is at the level of transcription of the *PRV-1* gene, evident by absence of PRV-1 mRNA in PRV-1–negative neutrophils [3]. The relative proportion of PRV-1–positive neutrophils varies from one individual to another, and even in the same individual over time and under different physiologic and pathologic stimuli. Sepsis, pregnancy, and administration of granulocyte colony-stimulating factor

increase expression of PRV-1 [3–6]. More recently, it was recognized that PRV-1 mRNA level is increased substantially in Philadelphia chromosome–negative myeloproliferative disorders, especially in polycythemia vera (PV) [7]. We think that mechanisms regulating PRV-1 expression might also be involved in pathogenesis of myeloproliferative disorders; and studying these mechanisms might be helpful for a better understanding of the molecular changes of myeloproliferative disorders. Additionally, we and others have shown that PRV-1 regulates cell proliferation [5,8,9], and might play a direct role in pathogenesis of myeloproliferative disorders. We investigated whether methylation of the *PRV-1* gene affects its transcription and has any role in biphenotypic expression pattern of PRV-1 in neutrophils. The *PRV-1* gene is located on chromosome 19 and is devoid of CpG islands. We studied cytosine methylation of CpG dinucleotides close to the PRV-1 gene transcription start site at cytosines 30, 41, and 43 (six nucleotides before,

Offprint requests to: Vahid Afshar-Kharghan, M.D., Thrombosis Research Section, Baylor College of Medicine, BCM286, N1319, One Baylor Plaza, Houston, TX, 77030; E-mail: vahid@bcm.tmc.edu

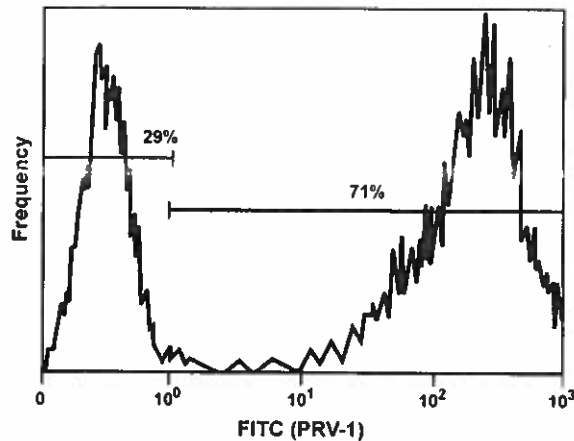


Figure 1. Polycythemia rubra vera-1 (PRV-1) expression on normal neutrophil. Results of flow cytometry on neutrophils from a normal donor, using MEM166 (anti PRV-1 antibody) shown as a frequency distribution plot. Neutrophils had a biphenotypic expression pattern for PRV-1, and in this donor 71% of all neutrophils expressed PRV-1. FITC = fluorescein isothiocyanate.

three and five nucleotides after, the initiation codon, respectively) (Fig. 2). We compared CpG methylation between PRV-1-positive and negative neutrophils in a group of healthy subjects. We further studied PV as a model for dysregulation of the *PRV-1* gene expression. We investigated the correlation between the PRV-1 mRNA level and CpG methylation of its gene in neutrophils of patients with PV, essential thrombocythemia (ET), primary familial or congenital polycythemia; and in a group of normal individuals. We found that a lower methylation of CpG sites close to transcription start site was associated with higher PRV-1 expression both in normal individuals and in patients with PV and ET.

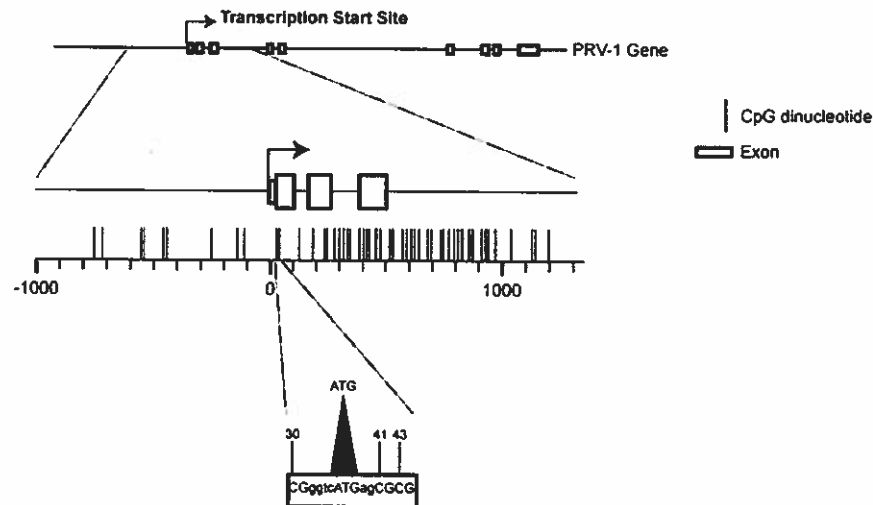


Figure 2. CpG dinucleotides position in relation to transcription start site of the polycythemia rubra vera-1 (PRV-1) gene. Exons are indicated as boxes and CpG sites as vertical bars. Three CpG dinucleotides are located around the initiation codon in the exon I of the PRV-1 gene: cytosine (C)³⁰pG, C⁴¹pG, and C⁴³pG.

Materials and methods

Isolation of neutrophil subgroups and determination of the PRV-1 mRNA levels

To compare DNA methylation among different neutrophil PRV-1 subgroups in normal individuals, blood samples from 10 healthy subjects were collected into 3.5% citrate anticoagulant at a 1:6 ratio. Using Histopaque (Sigma, St Louis, MO, USA) density-gradient centrifugation followed by ammonium chloride/bicarbonate lysis of red blood cells, granulocytes were separated from the other blood cells [10]. To separate neutrophil granulocytes into different PRV-1-expression subgroups, they were labeled with fluorescein isothiocyanate-conjugated anti-PRV-1 antibody (MEM166, Serotec) and phycoerythrin-conjugated anti-CD11b antibody (Dako, Carpinteria, CA, USA). Using cell-sorter EPICS ALTRA (Beckman Coulter, Fullerton, CA, USA), CD11b/CD18 (MAC-1)-expressing cells in the size range of granulocytes were separated into PRV-1-positive and PRV-1-negative subgroups (Fig. 1). We used Qiagen blood DNA kit to extract DNA from neutrophils (10^6 cells/sample) and used bisulfite treatment to prepare the DNA samples for methylation studies [11].

To study the correlation between the extent of *PRV-1* methylation and its expression in patients with PV, we used DNA and RNA samples from unsorted granulocytes of 19 patients with PV, 12 with ET, and 11 with primary familial and congenital polycythemia. DNA samples were prepared for methylation studies and RNA samples were used for determining the PRV-1 mRNA, as described previously [11,12]. All of the blood samples were obtained according to protocol approved by the institutional review board and after subjects signed an informed consent.

In vitro exposure of cells to DNA demethylating agent

Leukemia cell lines KG-1 and KG-1a were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were grown in Iscove's modified Dulbecco's medium supplemented with fetal bovine serum (20% v/v). DNA demethylation was induced by a 4-day treatment with 200 nM 2-deoxy-5-azacytidine [11] (DAC; Sigma). Fresh DAC was added to exponentially

growing cells at days 0, 1, 2, and 3. Parallel cultures of mock-treated cells were kept as controls. Cells were harvested at day 4, lysed in Trizol (Invitrogen, Carlsbad, CA, USA), and RNA plus DNA were isolated.

DNA sequencing and methylation studies

We determined the DNA sequence of a fragment of genomic DNA encompassing the initiation codon of the *PRV-1* gene to determine the genotype at the known polymorphism at guanine 42 (G42C) [13] and thus the presence or absence of the second CpG site at C41. After bisulfite treatment, the same region of the genomic DNA was amplified and cloned into pCR4 sequencing vector (Invitrogen). For each polymerase chain reaction (PCR) product, 25 clones representing individual alleles were pyrosequenced to determine the methylation status of cytosine residues at three CpG sites; CpG 30, 41, and 43, close to the *PRV-1* transcription start site (numbered according to the sequence accession no. AF146747 from the GenBank data) (Fig. 2) [11]. To examine methylation status of CpG 30 in patient samples, bisulfite PCR products were directly analyzed by pyrosequencing. Sequences of all primers are available in the supplementary data (Supplementary Table). We also determined mutation status of the Janus kinase 2 (*JAK2*) gene at codon 617 and quantified the mutant allele by pyrosequencing as described previously [14].

Statistical analysis

We used Fisher's exact test, Chi-square test, Mann-Whitney non-parametric test, and nonparametric Spearman correlation for data analysis.

Results

Expression of *PRV-1* on normal

neutrophils and methylation of the *PRV-1* gene

We compared methylation of the *PRV-1* gene in pairs of *PRV-1*-positive and *PRV-1*-negative neutrophil subpopulations isolated from 10 normal individuals. Neutrophils expressing *PRV-1* surface antigen showed consistently lower methylation levels at all CpG sites near the *PRV-1* transcription start site compared to *PRV-1*-negative ones (Fig. 3). We sequenced 509 PCR clones (25 clones per sample) to analyze methylation status of CpG dinucleotides on individual alleles. On average, C30 of the *PRV-1* gene was $13\% \pm 2\%$ methylated in *PRV-1*-positive neutrophils and $33\% \pm 4\%$ in *PRV-1*-negative neutrophils ($p = 0.0015$, Mann-Whitney test). Average methylation levels of C41 adjacent to C42G polymorphic site were $52\% \pm 7\%$ in *PRV-1*-positive and $71\% \pm 3\%$ in *PRV-1*-negative neutrophils ($p = 0.0041$). C43 showed average methylation $21\% \pm 4\%$ in *PRV-1*-positive and $33\% \pm 3\%$ in *PRV-1*-negative neutrophils; this difference was not significant. Cloning and sequencing approach enabled to detect methylation pattern of individual alleles. Frequencies of alleles showing all possible combinations of methylation status of three examined CpG sites are shown in Figure 4. There was a statistically significant predominance of completely unmethylated

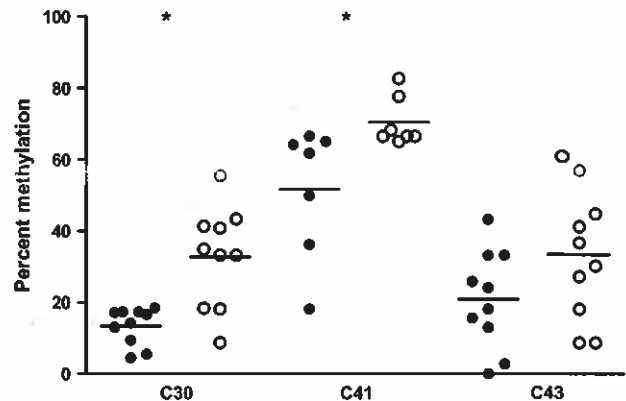


Figure 3. Comparison of methylation of CpG dinucleotides between polycythemia rubra vera-1 (*PRV-1*)-positive and -negative neutrophils. The methylation status of CpG dinucleotides at cytosine (C)30, C41, and C43 sites downstream from the transcription start site of the *PRV-1* gene was compared between *PRV-1*-positive (full circles) and -negative neutrophils (open circles) in each subject (total of 10 normal control subjects). Asterisks denote significant differences (Mann-Whitney nonparametric test, $p < 0.05$).

alleles in *PRV-1*-positive granulocytes while alleles with the first two sites methylated or with all three sites methylated were predominant in *PRV-1*-negative granulocytes.

CpG methylation and the G42C

polymorphism of the *PRV-1* gene

One of the single nucleotide polymorphisms in the *PRV-1* gene is due to replacement of guanosine 42 (major allele with frequency of 54–68%) by a cytosine (minor allele with a frequency of 32–46%) [6,13]. We analyzed methylation pattern of individual 42G or 42C alleles based on the genotype at the G42C polymorphic site. There was no significant difference in C30 methylation between the genotypes: 25 of 150 methylated alleles (17%) for the 42C/C genotype, 51 of 174 methylated alleles (23%) for 42C/G, and 37 of 134 methylated alleles (28%) for the 42G/G genotype. Striking differences depending on G42C genotype were seen in C43 methylation. Methylation at C43 was observed in 8 of 150 (8%) alleles in granulocytes with the 42C/C genotype, in 29 of 225 (29%) alleles in granulocytes with the 42C/G genotype, and in 42 of 134 (42%) alleles in granulocytes with the 42G/G genotype ($p < 0.0001$, Chi-square test). To avoid the effect of the G42C polymorphism on our methylation analysis, we focused on the methylation status of C30 site in subsequent studies.

Methylation of *PRV-1* in patients with PV, ET, or primary familial and congenital polycythemia

Having established the importance of C30 methylation in sorted neutrophils from normal controls, we examined C30 methylation and *PRV-1* mRNA levels in samples of unsorted neutrophils from patients with PV, ET, primary familial or congenital polycythemia, and in normal subjects.

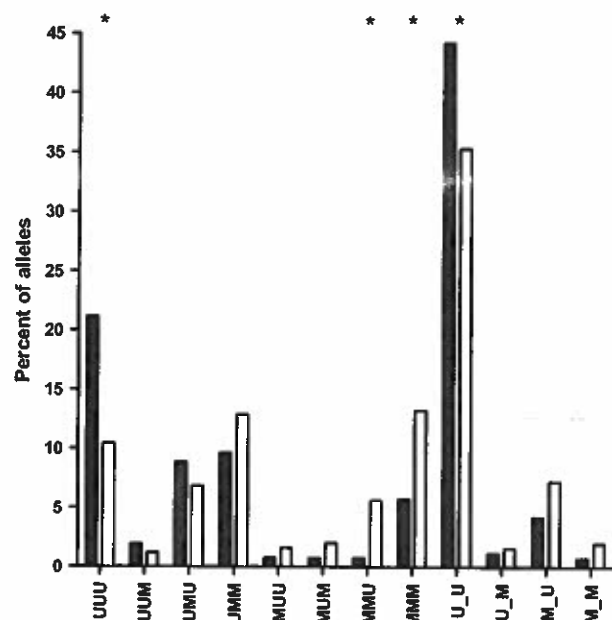


Figure 4. Methylation pattern of CpG sites cytosine (C) 30, C41, and C43 in individual alleles of the polycythemia rubra vera-1 (PRV-1) gene. The methylation status of the PRV-1 gene was determined in PRV-1-positive and -negative granulocytes of 10 normal subjects. Black bars denote allelic frequency in PRV-1-positive granulocytes, white bars in PRV-1-negative granulocytes. U = unmethylated, M methylated CpG site. Missing C41 CpG sites in 42C/C homozygotes are indicated by an underscore sign. *Statistically significant differences ($p < 0.05$, Fisher's exact test).

We found that the PRV-1 mRNA level was inversely correlated to the extent of methylation of the PRV-1 gene at C30 in PV (Spearman $r = -0.86$, $p < 0.0001$) and ET patients ($r = -0.64$, $p = 0.02$) (Fig. 5). Patients with overexpression of PRV-1 had a lower extent of methylation of the

PRV-1 gene at C30 (<20% methylated cytosine) compared to patients with normal expression of PRV-1 (40–60% methylation).

Methylation of PRV-1 gene and the $JAK2^{V617F}$ mutation

Because PRV-1 mRNA is overexpressed in PV and ET patients carrying $JAK2^{V617F}$ mutation, we also examined the relationship between $JAK2^{V617F}$ mutational status and C30 methylation of the PRV-1 gene. Samples from patients with $JAK2^{V617F}$ mutation showed significantly lower methylation of C30 site, when compared to individuals with normal $JAK2$ sequence (Fig. 6), suggesting that constitutive activation of JAK2 signaling results in PRV-1 demethylation. Methylation of PRV-1 at C30 inversely correlated with PRV-1 expression in 16 patients harboring 5% to 92% $JAK2^{V617F}$ mutant allele (Spearman $r = -0.78$, $p = 0.0004$). The burden of $JAK2^{V617F}$ mutant allele was also inversely related to C30 methylation of PRV-1, however, the correlation was not statistically significant (Spearman $r = -0.28$, $p = 0.3$).

In vitro model for demethylation of the PRV-1 gene and its effect on expression of PRV-1 mRNA

We studied DNA methylation and mRNA expression of PRV-1 in human leukemia cell lines (KG1 and KG1a) before and after 4 days of exposure to a demethylating agent, 2-deoxy-5-azacytidine (decitabine or DAC), in the cell culture media. KG-1 and KG-1a myeloid leukemia cell lines showed high methylation levels of C30 and low levels of PRV-1 mRNA expression. Treatment with decitabine, decreased methylation level of the PRV-1 gene in KG1 and KG1a cells (35% and 44%, respectively) and this was associated with a drastic increase in the amount of PRV-1 mRNA in these cells (28-fold and 17-fold increases in mRNA concentration, respectively) (Table 1).

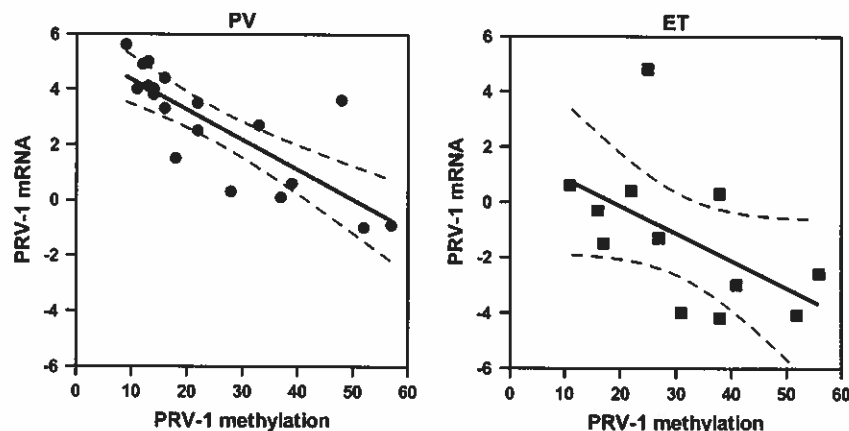


Figure 5. Methylation of the polycythemia rubra vera-1 (PRV-1) gene and expression of PRV-1 in patients with polycythemia vera (PV) and essential thrombocythemia (ET). The relative amount of PRV-1 mRNA to a control mRNA in neutrophils of patients with PV ($n = 19$) or ET ($n = 12$) was determined by real-time polymerase chain reaction and is shown in the Y-axis. The percentage of methylation of C30 in neutrophils of the same patients was determined by pyrosequencing and is shown in the X-axis. Solid line represents linear regression and broken lines show 95% confidence intervals. Negative correlation between PRV-1 mRNA levels and C30 methylation was observed in PV patients, $p < 0.0001$ (Spearman nonparametric correlation coefficient was -0.86) and in ET patients, $p = 0.02$ (Spearman nonparametric correlation coefficient was -0.64).

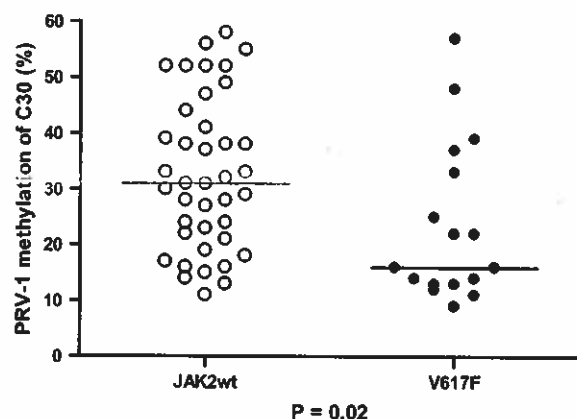


Figure 6. Methylation of the polycythemia rubra vera-1 (*PRV-1*) gene and the *JAK2*^{V617F} mutation. The percentage of methylation of cytosine (C) 30 in the *PRV-1* gene was determined in subjects with and without the *JAK2*^{V617F} mutation. The group homozygous for wild-type *JAK2* allele included 40 blood samples (15 controls, 11 primary familial and congenital polycythemia, 9 essential thrombocythemia [ET], and 4 polycythemia vera [PV]) and the *JAK2*^{V617F} group included 18 blood samples (3 ET and 15 PV). Nonparametric Mann-Whitney test was used to compare the two groups ($p = 0.02$).

Discussion

Presence of a biphenotypic expression pattern of *PRV-1* on circulating neutrophils cannot be readily explained by the presence of DNA polymorphisms, different stage of maturation of neutrophils, or transcription factor profile. Circulating neutrophils are cells derived from the same tissue of origin, have the identical stage of maturation, and are controlled by identical transcription factors and chemokines. We hypothesized that DNA methylation may have a role in bimodal expression of the *PRV-1* gene in neutrophils. DNA methylation is an important epigenetic mechanism regulating gene expression during development and maturation of an organism. Usually, methylation status of cytosine base of CpG dinucleotides in CpG-rich islands of a gene promoter affects transcription of that gene. However, there are several examples of cytokine gene regulation

in T cells that involve alteration in methylation of isolated CpG dinucleotides in gene-promoter regions devoid of CpG islands [15,16]. Here we show relative demethylation of the *PRV-1* gene in neutrophilic granulocytes. The *PRV-1* gene lacks a bona-fide CpG island; there are only 14 CpG sites in the 1000-bp region upstream and 200-bp downstream of the transcription start site (TSS, Fig. 2). Major TSS for the gene is flanked by three CpG sites located 30, 41, and 43 downstream of TSS. We, therefore, analyzed these CpG sites for potential relationship with gene expression. Three reported alternate potential TSS (UCSC Genome Browser on Human March 2006 Assembly) are positioned 6-bp upstream or 8-bp and 35-bp downstream of the AF146747 transcript, however, the three studied CpG sites are closest to those TSS as well. We studied methylation status of cytosines 30, 41, and 43. We noticed that the methylation status of C43 was closely correlated with the *PRV-1* C42G polymorphism [13]. C43 methylation levels were the highest in 42G/G, lower in 42G/C, and the lowest in 42C/C genotype ($p < 0.0001$, Chi-square test). It is interesting that 42C/C genotype was also found to be associated with a higher expression level of *PRV-1* on neutrophils of normal donors [13]. One might explain the higher expression level of *PRV-1* in 42C/C homozygotes by the absence of the C41 CpG site and by a decrease in methylation of C43 close to the initiation codon of the *PRV-1* gene. Whether this single nucleotide polymorphism affects gene expression through alteration in the DNA methylation status is unknown and needs additional research, however, in order to avoid the effect of the G42C polymorphism on our study, we focused our additional analysis on the methylation status of C30. We found that C30 was methylated to variable extent in all DNA samples obtained from neutrophils. Comparison of *PRV-1*-positive neutrophils to *PRV-1*-negative neutrophils in the same individual showed that expression of *PRV-1* was associated with a lower C30 methylation of this gene. Cytosine methylation is a covalent modification that used to be considered as a DNA alteration with a long-lasting (and perhaps permanent) regulatory effect on gene transcription. However, there are examples of rapid demethylation of CpG dinucleotides in a gene devoid of CpG island at TSS after activation of the cell by proper stimuli, such as rapid demethylation of CpG dinucleotides in the promoter of the interleukin-2 gene shortly after stimulation of T lymphocytes [17]. This demethylation is independent of DNA synthesis and increases expression of the interleukin-2 gene in the activated lymphocytes. Demethylation of the *PRV-1* gene might control expression of this gene under physiologic condition, however, exploration of a relationship between an alteration in the methylation status and overexpression of the *PRV-1* gene detected in patients with myeloproliferative disorders is of particular interest. Overexpression of *PRV-1* is recognized as a common event in patients with Philadelphia chromosome-negative myeloproliferative disorders, and especially in PV and

Table 1. Decitabine treatment caused demethylation and induced expression of *PRV-1* gene

	KG1		KG1a	
	No DAC	DAC-treated	No DAC	DAC-treated
C30 methylation (%)	83 ± 1	46 ± 0	93 ± 1	49 ± 1
ΔC_T	-15.5 ± 0.1	-10.7 ± 0.6	-16.9 ± 0.5	-12.8 ± 0.4
<i>PRV-1</i> mRNA expression	1.0	27.9	0.4	6.5

Polycythemia rubra vera-1 (*PRV-1*) mRNA expression was measured as ΔC_T – the difference of cycles at threshold 0.2 for glyceraldehyde phosphate dehydrogenase and *PRV-1* real-time polymerase chain reaction. DAC = 2-deoxy-5-azacytidine.

ET. We found a relationship between the mRNA level of PRV-1 and the methylation status of its gene in PV and ET. We propose that methylation of the *PRV-1* gene regulates expression of this gene under physiologic conditions and a dysregulation of the methylation status of this gene has a role in overexpression of PRV-1 in PV. Alternatively, demethylation can be a “passive” event that follows gene activation (and perhaps augments it) rather than being the initial cause. In order to investigate the relationship between the level of DNA methylation and mRNA expression, we manipulated DNA methylation of the *PRV-1* gene in human leukemic cell lines, using a demethylating agent decitabine and followed the change in the mRNA level of PRV-1. We found that decitabine demethylated the *PRV-1* gene, which was associated with a sharp increase in the concentration of PRV-1 mRNA. Several recent studies showed an important role for *JAK2*^{V617F} somatic mutation in pathogenesis of PV [18–22]. Is there a connection between presence of *JAK2*^{V617F} and level of methylation of the *PRV-1* gene? PRV-1 mRNA expression positively correlates with the dosage of the mutant *JAK2* allele [23]. We previously showed that introduction of V617F mutant *JAK2* leads to increased expression of both PRV-1 protein and mRNA in murine myeloid cells [24]. Here we report that *PRV-1* gene methylation was lower in the presence of activating *JAK2*^{V617F} mutation. One explanation for these findings is that JAK2 signaling affects DNA methylation. We hypothesize that augmented JAK2 signaling as a result of *JAK2*^{V617F} mutation alters physiologic activation of neutrophils; demethylates the *PRV-1* gene and increases PRV-1 expression. Supporting a possible role for JAK2 signaling in epigenetic regulation of the *PRV-1* gene expression are the results of two recent studies that revealed JAK/signal transducer and activation of transcription pathway modulates gene expression through epigenetic mechanisms. Shi et al. [25] showed that in a *Drosophila melanogaster*, hematopoietic tumor model, overactivation of JAK2 globally disrupts heterochromatic gene silencing, and Zhang et al. [26] demonstrated a role for phosphorylated signal transducer and activation of transcription 3 in epigenetic modulation of T-lymphocytes genes through inducing activity of DNA methyltransferase 1 gene.

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Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.exphem.2007.09.008.

Supplementary Table.

PCR on bisulfite-treated DNA	
Forward primer	5'-TGTTTAAGGGTTGGTATAAAG-3'
Reverse primer	5'-biotin-AACAAAATTCCTTACCC-3'
Pyrosequencing primer	5'-AAAGAGATTATTAGTTATAG-3'
PCR on genomic DNA	
Forward primer	5'-AGATTACCAGCCACAGACG-3'
Reverse primer	5'-biotin-GGAGGGCCAGCAGTAATA-3'
Pyrosequencing primer	5'-CAGACGGGTCATGAG-3'

PCR = polymerase chain reaction.

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Induction of hypomethylation and molecular response after decitabine therapy in patients with chronic myelomonocytic leukemia

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Brief report

Induction of hypomethylation and molecular response after decitabine therapy in patients with chronic myelomonocytic leukemia

Yasuhiro Oki,¹ Jaroslav Jelinek,¹ Lanlan Shen,¹ Hagop M. Kantarjian,¹ and Jean-Pierre J. Issa¹

¹Department of Leukemia, University of Texas M. D. Anderson Cancer Center, Houston

Decitabine's mechanism of action in chronic myelomonocytic leukemia remains incompletely understood. We studied the dynamics of neoplastic cell clearance during decitabine treatment (100 mg/m² per course every 4 weeks) using quantitative monitoring of mutant alleles by pyrosequencing. Patients with chronic myelomonocytic leukemia were first screened for *JAK2* and *NPM1* mutations, and 3 patients with mutations were identified. Mutant allele percentages in

mononuclear cell DNA were followed after treatment, along with methylation of *LINE1* and 10 other genes. The clearance of mutant alleles was modest after the first cycle, despite induction of hypomethylation. Delayed substantial clearance was observed after 2 to 4 cycles that correlated with clinical response. Two patients had complete disappearance of mutant alleles and sustained clinical remissions. In another patient, mutant allele was detectable at clinical remission,

which lasted for 8 months. Our data suggest a predominantly noncytotoxic mechanism of action for decitabine, leading to altered biology of the neoplastic clone and/or normal cells. This trial was registered at www.ClinicalTrials.gov as #NCT00067808. (Blood. 2008;111:2382-2384)

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Introduction

Decitabine has therapeutic activity in patients with myelodysplastic syndrome and chronic myelomonocytic leukemia (CMML).^{1,2} Whereas at least part of the mechanism of action is hypomethylation, cytotoxicity is also thought to play a role. The dynamics of neoplastic cells during treatment may be assessed with appropriate monitoring of genetic abnormalities. Wijermans et al have analyzed the dynamics of myelodysplastic syndrome cells after decitabine treatment by following cytogenetic abnormalities.³ However, this method requires a baseline cytogenetic abnormality and multiple bone marrow aspirations. Furthermore, cytogenetic analyses typically examine only 20 cells, which is not suitable for sensitive quantification. Molecular genetic abnormalities can become useful monitoring tools to overcome this issue. Pyrosequencing is a simple method to quantitatively detect nucleotide polymorphisms. The benefits of this method are simplicity and reproducibility. Here we studied the molecular dynamics of CMML in 3 patients with mutations in *JAK2* or *NPM1* who were treated with decitabine. The percentage of the mutant allele was quantified and stood as a molecular marker of disease response to decitabine.

Methods

Peripheral blood samples were collected after informed consent was obtained in accordance with the Declaration of Helsinki from 16 patients with CMML on entry to a phase 2 decitabine study, where patients were randomized to 1 of 3 decitabine schedules: (1) 20 mg/m² intravenously daily for 5 days, (2) 20 mg/m² subcutaneously daily for 5 days, and (3) 10 mg/m² intravenously daily for 10 days.² The treatment was planned

to be repeated every 4 weeks. Blood collection was scheduled on days 0, 5, 12, and 28 during the first cycle and on day 0 for the following cycles. DNA was isolated from blood samples after density gradient separation of mononuclear cells using standard phenol-chloroform extraction methods. Pretreatment samples were screened for mutations of *JAK2* and *NPM1* using pyrosequencing as previously reported.^{4,5} This research was approved by the M. D. Anderson Cancer Center Institutional Review Board.

JAK2 V617F mutation was found in 2 patients, and *NPM1* mutation was detected in another patient. In patients with mutations, the proportion of mutant alleles was quantitatively determined by pyrosequencing using samples obtained during and after decitabine therapy. Promoter methylation status of 10 specific genes (*C10RF102*, *CDH1*, *CDH13*, *CDKN2B*, *ESR1*, *NPM2*, *OLIG2*, *PDLIM4*, *PGRA*, and *PGRB*)⁶ that are frequently methylated in myeloid malignancies was also screened in pretreatment samples of these 3 patients using pyrosequencing.⁷ Methylation status of the *LINE1* repetitive element² and of the genes that showed increased methylation before treatment was followed during and after treatment.

Results and discussion

Patient 1 was a 63-year-old woman with CMML with normal karyotype and *JAK2* mutation (1849G > T). At diagnosis, white blood count (WBC) was 66 × 10⁹/L with 6% monocytes and 4% peripheral blast cells. *JAK2* mutation was detected in 49% of the alleles, suggesting a heterozygous mutation. After the first cycle of decitabine (schedule 2), *JAK2* mutant alleles decreased modestly to 40%; *LINE1* analysis showed demethylation from 59% at baseline to 49% on day 12, and then remethylation to 56% on day 30. After

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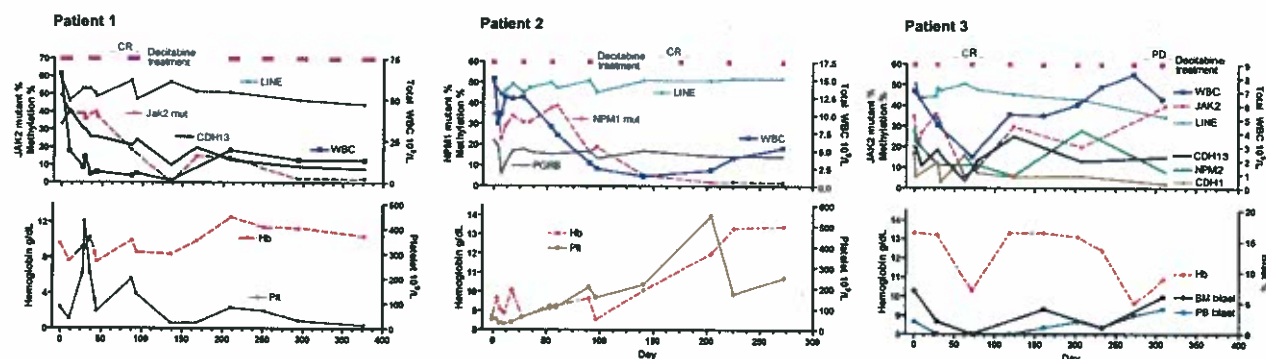


Figure 1. Dynamics of DNA methylation, mutant alleles as neoplastic clone markers, and peripheral blood counts. We analyzed methylation of 10 genes at baseline in all 3 patients, and genes with increased baseline methylation were followed during and after the treatment. Patient 1: CMML with *JAK2* mutation of 49% alleles before treatment, which decreased to undetectable level after 4 cycles of decitabine. *CDH13* methylation was present at initiation of therapy and decreased after decitabine. Patient 2: CMML with *NPM1* mutation of 35% alleles before treatment, which decreased to undetectable levels after 4 cycles. *PGRB* methylation followed the dynamics of *LINE1* methylation after decitabine. Patient 3: CMML with *JAK2* mutation of 35% alleles before treatment, which decreased to 6% after 2 cycles of decitabine and then increased again. *CDH1*, *CDH13*, and *NPM2* methylation was present at initiation of therapy and decreased after decitabine. Cycles of decitabine treatment are indicated as purple rectangles above the graphs.

the second cycle, the patient achieved a complete hematologic remission, whereas 18% of alleles still carried the *JAK2* mutation. Three additional courses of decitabine cleared the mutant down to undetectable levels ($< 5\%$). With regards to methylation changes, *CDH13* was found methylated (33%) before treatment. At clinical complete response after 2 cycles of decitabine, *CDH13* methylation was still 21%. Three additional cycles of treatment decreased methylation down to the level seen in normal controls (10%) (Figure 1). The platelet count deteriorated after 6 cycles of decitabine despite the clearance of *JAK2* mutant alleles, and the patient was taken off the study after receiving 8 cycles of treatment. The patient eventually died of pneumonia 1.4 years after initiation of therapy.

Patient 2 was a 78-year-old man with CMML with normal karyotype and *NPM1* mutation (960-961insTCTG). At diagnosis, WBC count was $15 \times 10^9/L$, with 13% monocytes. He had anemia and thrombocytopenia. *NPM1* mutation was detected in 35% of the alleles. The first 2 cycles of decitabine (schedule 3) were associated with the typical hypomethylation induction but with a minimal change in the mutant allele percentage (from 35% before treatment to 38% after 2 cycles). This patient achieved a complete hematologic response after 4 courses of decitabine coincident with marked clearance of mutant alleles to barely detectable level (5%). After 2 additional courses of decitabine, the mutant allele was no longer detectable. *PGRB* was found methylated at baseline (25%). Although transient demethylation of *PGRB* occurred after the first cycle, remethylation was observed at day 0 of the next cycle. Overall, methylation of *PGRB* in this 78-year-old patient followed the dynamics of *LINE1* methylation, suggesting that *PGRB* was moderately methylated also in normal cells. The patient remains in complete remission at 2.8 years after initiation of therapy.

Patient 3 was a 55-year-old man with CMML with normal karyotype and *JAK2* mutation (1849G $\rightarrow$ T). At diagnosis, WBC was $7 \times 10^9/L$, with 16% monocytes. *JAK2* mutation was detected in 35% of the alleles. *LINE1* hypomethylation was induced after the first cycle (schedule 2) with transient decrease of *JAK2* mutant alleles to 20%. The second cycle of decitabine was associated with marked clearance of the mutant cells (6% mutant alleles), and the patient achieved complete remission evidenced by disappearance of monocytosis and normal platelet count. Subsequently, the mutant *JAK2* allele started rising again (Figure 1), although the patient clinically remained in complete remission. The disease overtly progressed after 8 courses; the spleen acutely enlarged and

bone marrow blast count increased to 6%. Given otherwise stable condition, this patient received one more course of decitabine and 4 weeks later underwent splenectomy, which confirmed the presence of CMML in the spleen. The patient left our institution after splenectomy to receive supportive care at a local hospital. *CDH1*, *CDH13*, and *NPM2* were found methylated at baseline. *CDH1* and *NPM2* showed demethylation after 3 courses (from 17% to 5% and from 28% to 6%, respectively), and the level of methylation remained low until the obvious progression of the disease, except for one point of transiently increased *NPM2* methylation. The degree of *CDH13* methylation was 20% at baseline. Its methylation dynamics essentially followed changes of *JAK2* mutant allele, suggesting the presence of methylation in malignant cells.

In conclusion, we showed that clearance of neoplastic cells after decitabine therapy in CMML was very modest after one cycle despite induction of *LINE1* hypomethylation. Subsequent cycles were associated with similar *LINE1* hypomethylation dynamics, but a marked delayed clearance of the mutant clones was observed, coincident with clinical remission. Thus, global hypomethylation precedes clonal elimination and clinical responses. The paucity of genes hypermethylated in these cases makes it difficult to comment on the importance of tumor-suppressor gene demethylation. Nevertheless, demethylation dynamics of *CDH13* gene temporally coincided with mutant clone elimination. Whole genome analysis of DNA methylation by microarrays or deep sequencing is needed to uncover more gene-specific methylation changes occurring after decitabine treatment. Our data are most consistent with an initial modest cytotoxic effect of decitabine followed by a delayed and complete clearance of the clone, the dynamics of which suggest a noncytotoxic mechanism. Possibilities for this delayed action include altered biology of the neoplastic clone (senescence induction, effects on stem cell renewal), induction of an immune response⁸ against CMML clone, or effects on normal stem cells. Further investigations should focus on these mechanisms to improve the results seen with decitabine.

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Authorship

Contribution: Y.O. and J.J. designed and performed research, analyzed data, and wrote the paper; L.S. performed research; H.M.K. was the

principal investigator of the clinical trial and helped analyze the clinical data; J.-P.J.I. supervised all aspects of the research and wrote the paper.

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Correspondence: Jean-Pierre J. Issa, Department of Leukemia, University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd, Box 428, Houston, TX 77030; e-mail: jpissa@mdanderson.org.

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