

Award Number: W81XWH-05-1-0535

TITLE: DNA Methylation as an Epigenetic Factor in the Development and Progression of Polycythemia Vera

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REPORT DATE: October 2008

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

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<b>1. REPORT DATE</b> 1 Oct 2008			<b>2. REPORT TYPE</b> Annual		<b>3. DATES COVERED</b> 15 Sep 2007 – 14 Sep 2008	
<b>4. TITLE AND SUBTITLE</b>  DNA Methylation as an Epigenetic Factor in the Development and Progression of Polycythemia Vera					<b>5a. CONTRACT NUMBER</b>	
					<b>5b. GRANT NUMBER</b> W81XWH-05-1-0535	
					<b>5c. PROGRAM ELEMENT NUMBER</b>	
<b>6. AUTHOR(S)</b>  Jean-Pierre Issa, M.D.  E-Mail: <a href="mailto:jissa@mdanderson.org">jissa@mdanderson.org</a>					<b>5d. PROJECT NUMBER</b>	
					<b>5e. TASK NUMBER</b>	
					<b>5f. WORK UNIT NUMBER</b>	
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b>  The University of Texas M.D. Anderson Cancer Houston, TX 77030					<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012						
<b>12. DISTRIBUTION / AVAILABILITY STATEMENT</b> Approved for Public Release; Distribution Unlimited					<b>10. SPONSOR/MONITOR'S ACRONYM(S)</b>	
					<b>11. SPONSOR/MONITOR'S REPORT NUMBER(S)</b>	
<b>13. SUPPLEMENTARY NOTES</b>						
<b>14. ABSTRACT</b> 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. PV is characterized by clonal expansion of erythroid, myelomonocytic, and megakaryocytic lineages, erythrocytosis being the most prominent clinical manifestation of PV. 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. 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), and in 10-20% patients with chronic myelomonocytic leukemia, Philadelphia-negative CML, atypical or unclassified myeloproliferative diseases (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. 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. 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.						
<b>15. SUBJECT TERMS</b> Polycythemia vera; myeloproliferative disorders; epigenetics; DNA methylation						
<b>16. SECURITY CLASSIFICATION OF:</b>				<b>17. LIMITATION OF ABSTRACT</b>	<b>18. NUMBER OF PAGES</b>	<b>19a. NAME OF RESPONSIBLE PERSON</b> USAMRMC
<b>a. REPORT</b> U	<b>b. ABSTRACT</b> U	<b>c. THIS PAGE</b> U	<b>19b. TELEPHONE NUMBER</b> (include area code)			
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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:**

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### **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.<sup>1</sup> PV is characterized by clonal expansion of erythroid, myelomonocytic, and megakaryocytic lineages, erythrocytosis being the most prominent clinical manifestation of PV.<sup>2</sup> 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.<sup>3-5</sup> 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),<sup>6-10</sup> and in 10-20% patients with chronic myelomonocytic leukemia, Philadelphia-negative CML, atypical or unclassified myeloproliferative diseases (MPD) and megakaryocytic leukemia.<sup>11-13</sup> 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.<sup>14</sup> 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

erythroid progenitors to erythropoietin (months 13-30).

- 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).

## **PROGRESS REPORT**

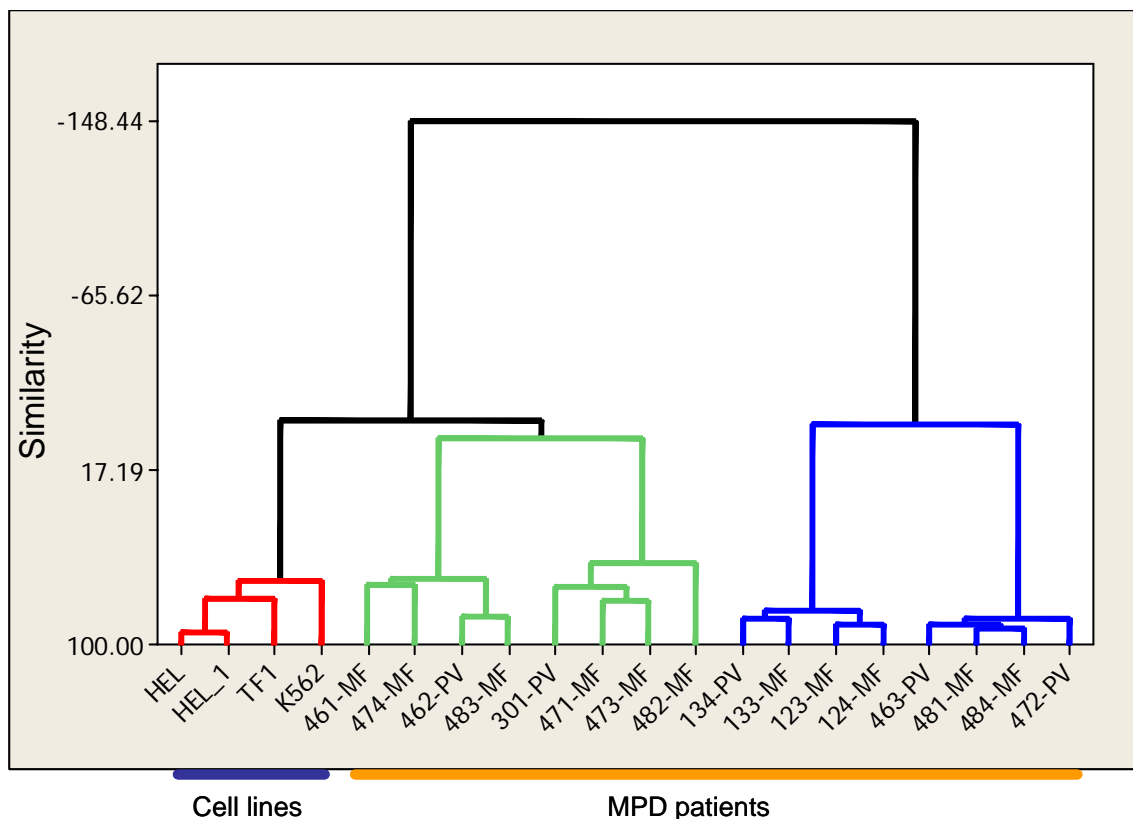
We proceeded with MCA/microarray analysis of samples from MPD patients as planned. We performed methylated CpG island amplification (MCA) in 15 patients with myeloproliferative disorders. We applied MCA amplicons to high density oligonucleotide microarrays (Agilent) to detect genes that are methylated in MPD patients and not methylated in normal controls. The data analysis is in progress. We observed a large variation in the number of differentially methylated genes in individual patients ranging from 0.42% to 28.68% (median 2.74%) of 3354 genes with microarray probes mapping to CpG islands and close to gene transcription start sites (-1000 to +500 bp). We also analyzed DNA methylation in 3 erythroleukemia cell lines (HEL, TF-1 and K562) and found 14.85% - 18.96% (median 17.99%) methylated genes from the same 3354 gene set. Genes consistently methylated in 50% and more MPD patients are listed in Table 1.



**Table 1. Genes differentially methylated in MPD patients.**

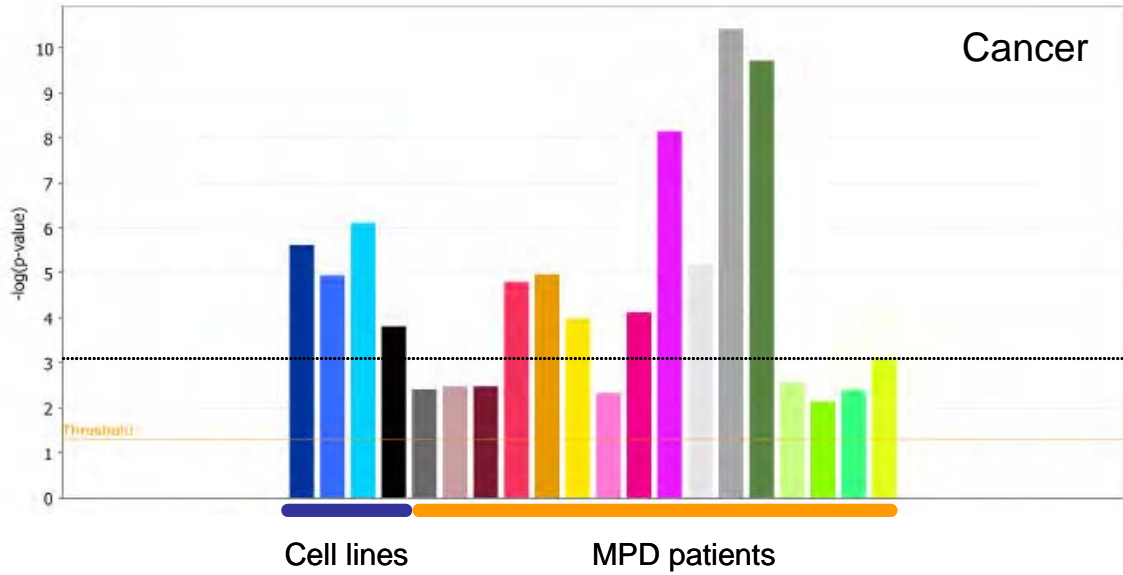
<b>ID</b>	<b>Description</b>	<b>Location</b>	<b>Type</b>
GAD1	glutamate decarboxylase 1	Cytoplasm	enzyme
GRM6	glutamate receptor, metabotropic 6	Plasma Membrane	G protein coupled receptor
NIPBL	Nipped B homolog	Nucleus	other
FAM167A	family with sequence similarity 167, member A	Unknown	other
FBXO17	F box protein 17	Unknown	other
IGF2BP1	insulin like growth factor 2 mRNA binding protein 1	Nucleus	other
KLHL21	kelch like 21	Unknown	other
NCAPG2	non SMC condensin II complex, subunit G2	Nucleus	other
RECK	reversion inducing cysteine rich protein with kazal motifs	Plasma Membrane	other
SLC25A28	solute carrier family 25, member 28	Unknown	other
SUMO3	SMT3 suppressor of mif two 3 homolog 3	Nucleus	other
TRIM7	tripartite motif containing 7	Cytoplasm	other
TSPYL5	TSPY like 5	Unknown	other
WASF3	WAS protein family, member 3	Cytoplasm	other
PPAP2C	phosphatidic acid phosphatase type 2C	Plasma Membrane	phosphatase
DLX4	distal less homeobox 4	Nucleus	transcription regulator
NEO1	neogenin homolog 1	Plasma Membrane	transcription regulator
NOTCH3	Notch homolog 3 (Drosophila)	Plasma Membrane	transcription regulator
SOX18	SRY (sex determining region Y) box 18	Nucleus	transcription regulator
TRIM25	tripartite motif containing 25	Cytoplasm	transcription regulator

We performed unsupervised hierarchical clustering of MPD patients and erythroleukemia cell lines based on methylation status of 15,105 probes mapping to 3354 CpG islands close to gene transcription start sites (-1000 to +500 bp). Three major clusters were formed, one containing erythroleukemia cell lines and two with MPD patients (Fig. 1).



**Fig. 1. Hierarchical clustering of erythroleukemia cell lines and MPD patients based on DNA methylation of CpG islands close to gene transcription start sites.** Dendrogram with Ward linkage and correlation coefficient distance.

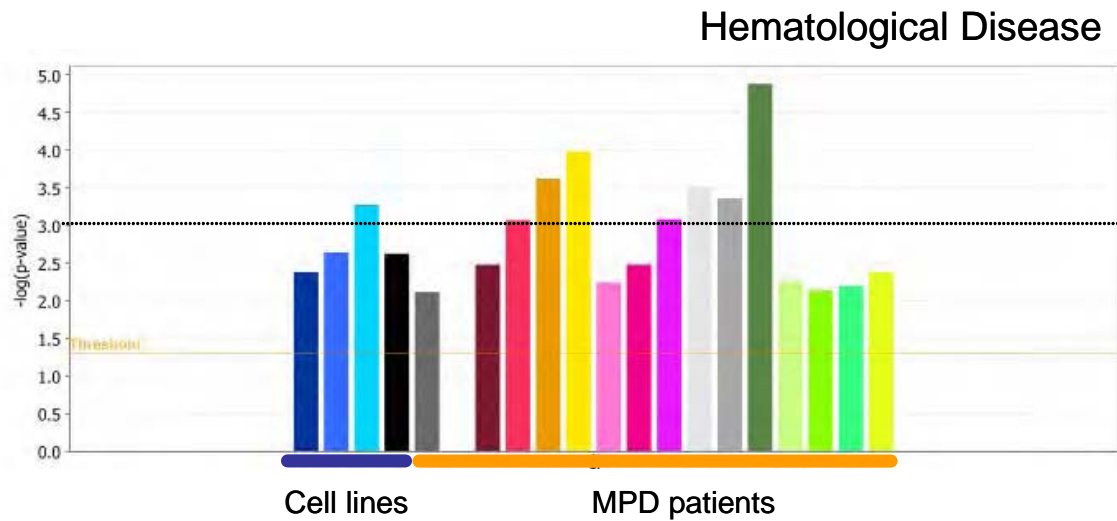
We analyzed functional significance of genes methylated in individual patients and erythroleukemia cell lines by the Ingenuity Pathway Analysis software. Genes related to cancer were significantly methylated in all erythroleukemia cell lines and MPD patients (Fig. 2).



**Fig. 2. Genes detected as methylated by MCAM show significant enrichment for cancer-related genes.** Vertical bars represent results from individual arrays. Significance of enrichment is shown as  $-\log(P\text{-value})$ . The dotted horizontal line depicts  $P=.001$ . The threshold value of  $P=.05$  is marked by the yellow horizontal line.

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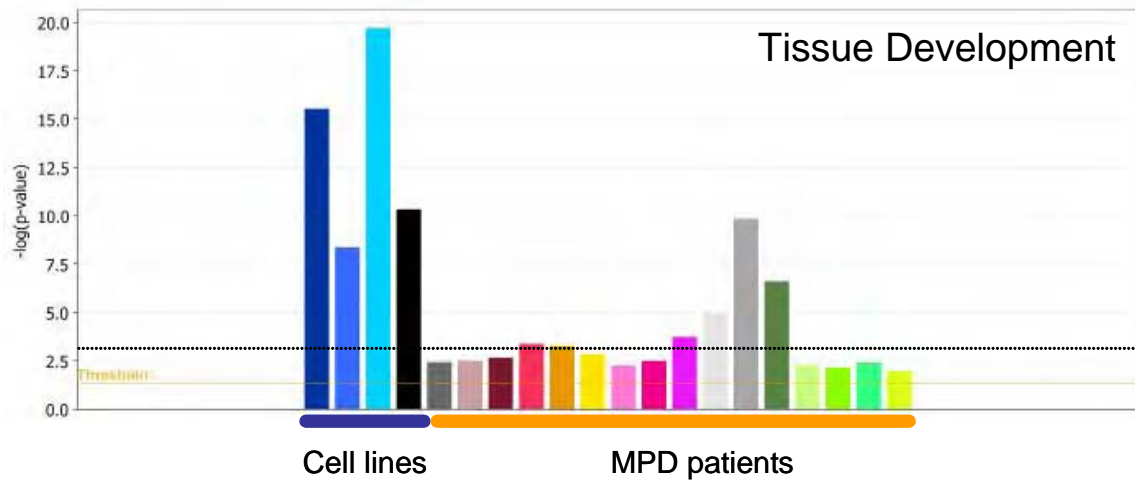
Genes associated with hematological disease were also enriched between methylated sets (Fig. 3).



**Fig. 3. Genes detected as methylated by MCAM show significant relation to hematological disease.** Vertical bars represent results from individual arrays. Significance of enrichment is shown as  $-\log(P\text{-value})$ . The dotted horizontal line depicts  $P=0.001$ . The threshold value of  $P=0.05$  is marked by the yellow horizontal line.

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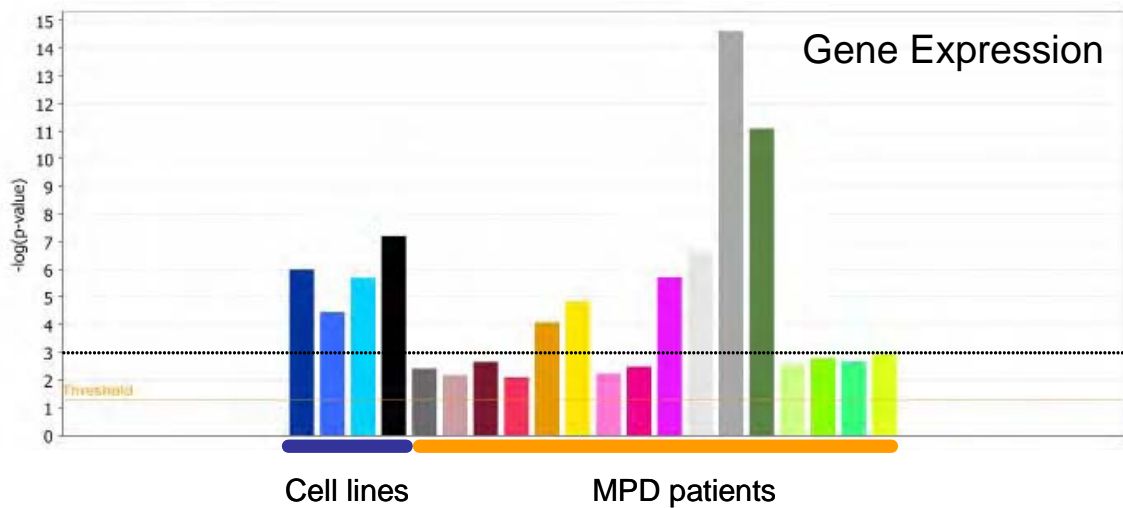
DNA methylation significantly affected genes associated with tissue development (Fig. 4).



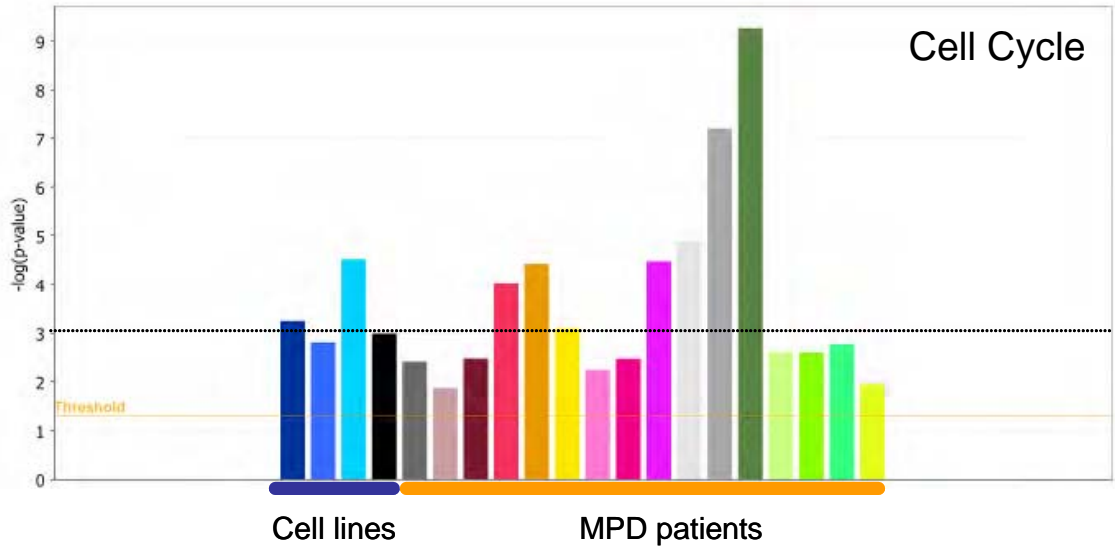
**Fig. 4. Developmental genes are significantly methylated in erythroleukemia cell lines and MPD patients.** Vertical bars represent results from individual arrays. Significance of enrichment is shown as  $-\log(P\text{-value})$ . The dotted horizontal line depicts  $P=.001$ . The threshold value of  $P=.05$  is marked by the yellow horizontal line.

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Genes associated with control of gene expression (Fig. 5), cell cycle (Fig. 6), cell death (Fig. 7), DNA replication, recombination and repair (Fig. 8) were significantly methylated in tested samples.

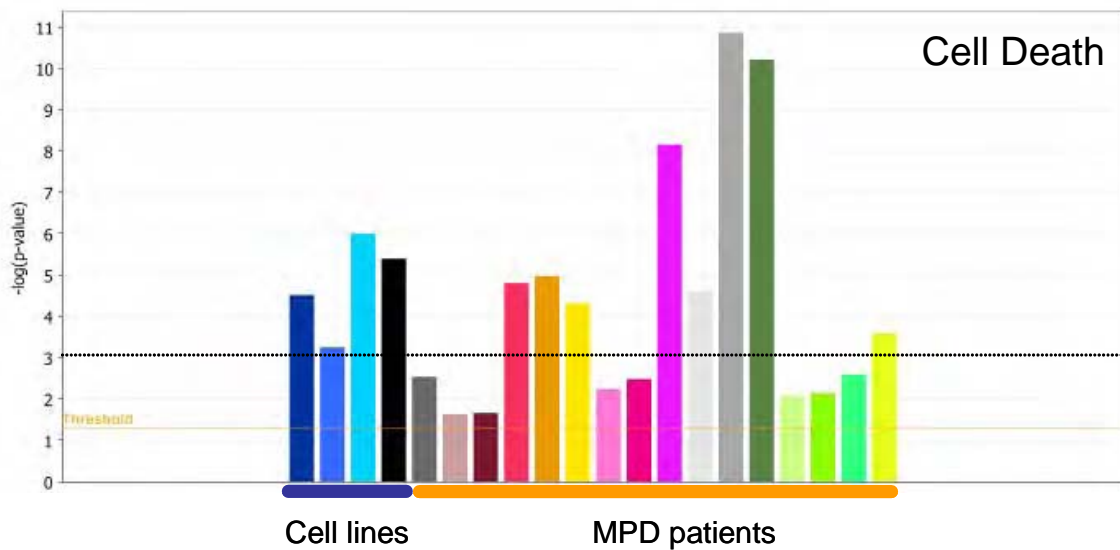


**Fig. 5. Genes involved in regulation of gene expression are significantly methylated in erythroleukemia cell lines and MPD patients.** Vertical bars represent results from individual arrays. Significance of enrichment is shown as  $-\log(P\text{-value})$ . The dotted horizontal line depicts  $P=0.001$ . The threshold value of  $P=0.05$  is marked by the yellow horizontal line.

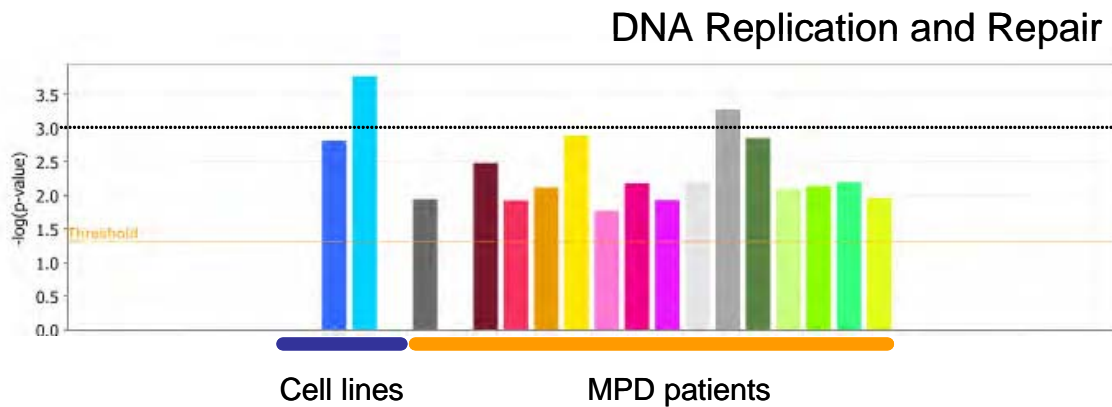


**Fig. 6. Genes involved in cell cycle control are significantly methylated in erythroleukemia cell lines and MPD patients.** Vertical bars represent results from individual arrays. Significance of enrichment is shown as  $-\log(P\text{-value})$ . The dotted horizontal line depicts  $P=0.001$ . The threshold value of  $P=0.05$  is marked by the yellow horizontal line.

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**Fig. 7. Genes related to cell death are significantly methylated in erythroleukemia cell lines and MPD patients.** Vertical bars represent results from individual arrays. Significance of enrichment is shown as  $-\log(P\text{-value})$ . The dotted horizontal line depicts  $P=0.001$ . The threshold value of  $P=0.05$  is marked by the yellow horizontal line.

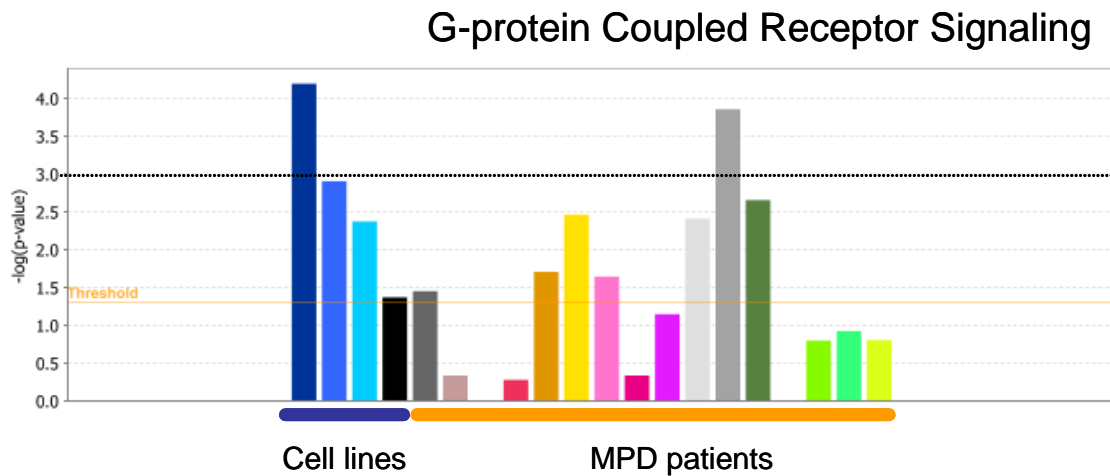


**Fig. 8. Genes involved in DNA replication, recombination and repair are significantly methylated in erythroleukemia cell lines and MPD patients.** Vertical bars represent results from individual arrays. Significance of enrichment is shown as  $-\log(P\text{-value})$ .

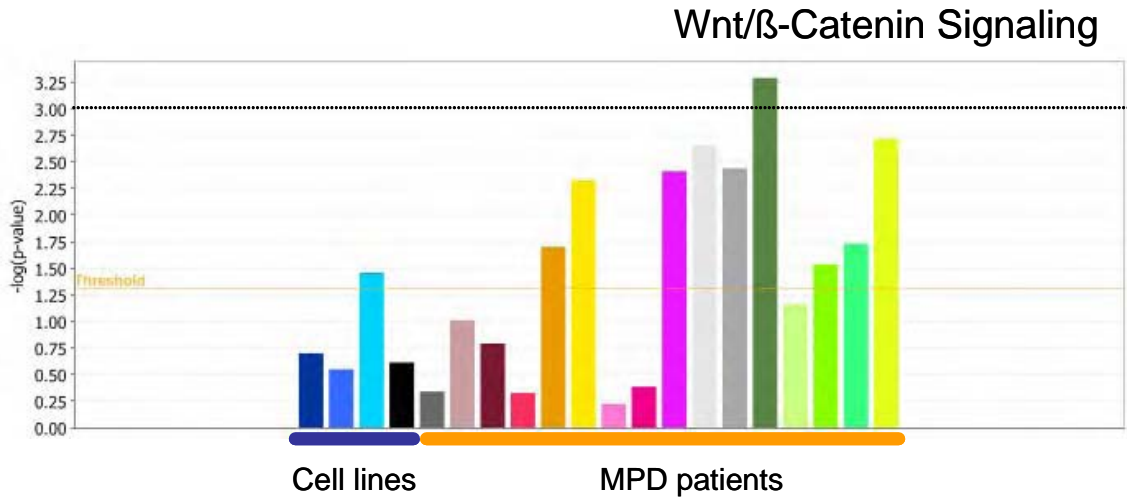


$\log(P\text{-value})$ . The dotted horizontal line depicts  $P=.001$ . The threshold value of  $P=.05$  is marked by the yellow horizontal line.

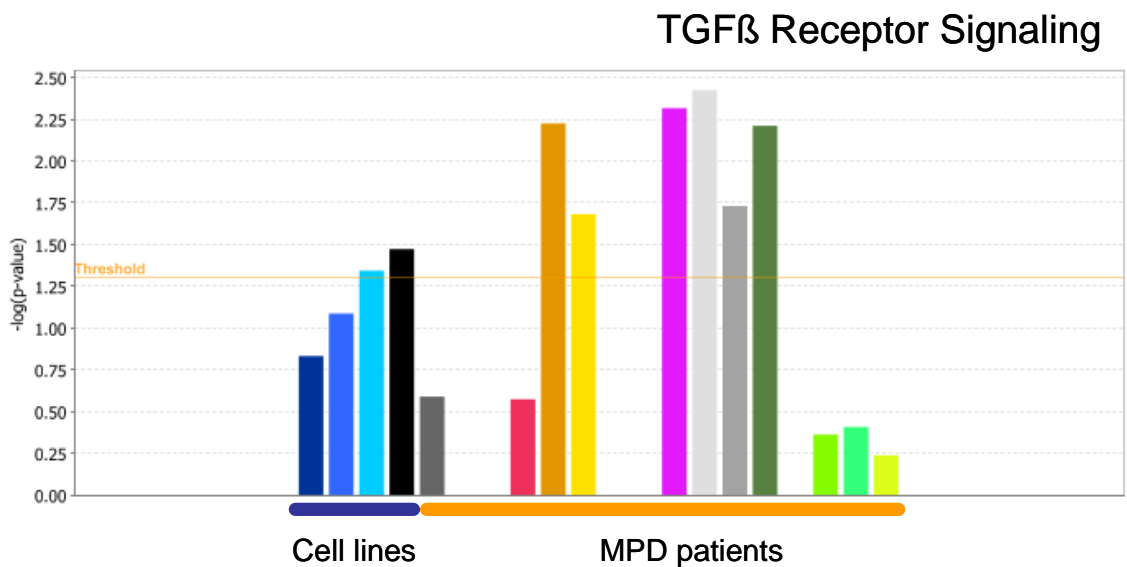
G-protein coupled receptor signaling, Wnt/beta-catenin signaling and TGF-beta receptor signaling canonical pathways were targeted by hypermethylation in MPD patients as shown in Figures 9-11.



**Fig. 9. Genes belonging to G-protein coupled receptor signaling canonical pathway are significantly methylated in erythroleukemia cell lines and MPD patients.** Vertical bars represent results from individual arrays. Significance of enrichment is shown as  $-\log(P\text{-value})$ . The dotted horizontal line depicts  $P=.001$ . The threshold value of  $P=.05$  is marked by the yellow horizontal line.



**Fig. 10. Wnt/beta-catenin pathway is affected by DNA methylation in MPD patients more than in erythroleukemia cell lines.** Vertical bars represent results from individual arrays. Significance of enrichment is shown as  $-\log(P\text{-value})$ . The dotted horizontal line depicts  $P=.001$ . The threshold value of  $P=.05$  is marked by the yellow horizontal line.



**Fig. 11. TGF-beta receptor signaling pathway is affected by DNA methylation in some MPD patients.** Vertical bars represent results from individual arrays. Significance

of enrichment is shown as  $-\log(\text{P-value})$ . The dotted horizontal line depicts  $P=.001$ . The threshold value of  $P=.05$  is marked by the yellow horizontal line.

## **KEY RESEARCH ACCOMPLISHMENTS**

- 
- Mapped DNA methylation genome-wide using high methylated CpG island amplification coupled to high density microarrays (MCAM method). Characterized biological functions and pathways affected by DNA methylation in MPD patients.
- 

## **REPORTABLE OUTCOMES**

### **Meeting presentations**

Posters, American Society of Hematology Meeting, Atlanta, GA, December 2007:

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Jelinek J, Estecio MRH, Kondo K, He R, Zavadil J, Issa JPJ. Classifying Leukemias Based on Epigenetic Alterations.

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.

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.

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### **Meeting abstracts published (Appendix 1)**

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Jelinek J, Estecio MRH, Kondo K, He R, Zavadil J, Issa JPJ. Classifying Leukemias Based on Epigenetic Alterations. *Blood* 110:632a, 2007.

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. *Blood* 110:750a, 2007.

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. *Blood* 110:745a, 2007.

### **Manuscript published (Appendix 2)**

Oki Y, Jelinek J, Shen L, Kantarjian HM, Issa JP. Induction of hypomethylation and molecular response after decitabine therapy in patients with chronic myelomonocytic leukemia. *Blood* 2008; 111(4):2382-4. (Appendix 1)

### **Manuscript in preparation**

Jelinek J, He R, Bueso-Ramos CE, Verstovsek S, Prchal JT, Issa JPJ. Methylation of progesterone receptor CpG islands in polycythemia vera, myelofibrosis and leukemia.

### **CONCLUSIONS**

Our data show that the amount of genes affected by DNA methylation in MPD patients is variable. While it was relatively low in approximately half of the patients studied by the

genome-wide MCAM method, several patients showed numbers of methylated genes comparable to our results in acute myeloid leukemia.

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**“SO WHAT:”**

Epigenetic silencing by cytosine methylation in selective CpG islands may play 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.

**Request for Extension**

We are asking for unpaid extension to finish microarray data analysis and to validate top methylated genes by bisulfite pyrosequencing in a large set of MPD patients.

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## Appendix 1

*Blood* (ASH Annual Meeting Abstracts) 2007 110: Abstract 2123

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### Poster Session

#### *Disordered Epigenetic Regulation in Hematologic Malignancy*

Disordered Gene Expression in Hematologic Malignancy including Disordered Epigenetic Regulation

## Classifying Leukemias Based on Epigenetic Alterations.

Jaroslav Jelinek, MD, PhD<sup>1</sup>, Marcos R.H. Estecio, PhD<sup>1,\*</sup>, Kimie Kondo, MS<sup>1,\*</sup>, Rong He, MS<sup>1,\*</sup>, Jiri Zavadil, PhD<sup>2,\*</sup> and Jean-Pierre J. Issa, MD<sup>1</sup>

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

### Abstract

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-

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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.

*Blood* (ASH Annual Meeting Abstracts) 2007 110: Abstract 2544

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**Poster Session**

*Myeloproliferative Syndromes: Clinical and Molecular Profiling*

Myeloproliferative Syndromes

## **Analysis of Mutant *cMPL* in Philadelphia Chromosome-Negative Myeloproliferative Disorders (Ph<sup>-</sup>MPDs) Using a Novel High-Sensitivity Assay.**

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### **Abstract**

*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<sup>-</sup>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%)

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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  $\leq 1\%$  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<sup>-</sup>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<sup>-</sup>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

*Myeloproliferative Syndromes: Clinical and Molecular Profiling*

Myeloproliferative Syndromes

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

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## Abstract

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

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W81XWH-05-1-0535; PI: Issa, Annual Report 2008, curve (1) assays. Standards (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 4 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.

# blood

2008 111: 2382-2384  
Prepublished online Nov 30, 2007;  
doi:10.1182/blood-2007-07-103960

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

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

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## Brief report

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

Yasuhiro Oki,<sup>1</sup> Jaroslav Jelinek,<sup>1</sup> Lanlan Shen,<sup>1</sup> Hagop M. Kantarjian,<sup>1</sup> and Jean-Pierre J. Issa<sup>1</sup>

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**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/m<sup>2</sup> 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](http://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).<sup>1,2</sup> 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.<sup>3</sup> 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/m<sup>2</sup> intravenously daily for 5 days, (2) 20 mg/m<sup>2</sup> subcutaneously daily for 5 days, and (3) 10 mg/m<sup>2</sup> intravenously daily for 10 days.<sup>2</sup> 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.<sup>4,5</sup> 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 (*CIORF102*, *CDH1*, *CDH13*, *CDKN2B*, *ESR1*, *NPM2*, *OLIG2*, *PDLIM4*, *PGRA*, and *PGRB*)<sup>6</sup> that are frequently methylated in myeloid malignancies was also screened in pretreatment samples of these 3 patients using pyrosequencing.<sup>7</sup> Methylation status of the *LINE1* repetitive element<sup>2</sup> 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 × 10<sup>9</sup>/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

Submitted July 31, 2007; accepted November 18, 2007. Prepublished online as *Blood* First Edition paper, November 20, 2007; DOI 10.1182/blood-2007-07-103960.

Y.O. and J.J. contributed equally to this study.

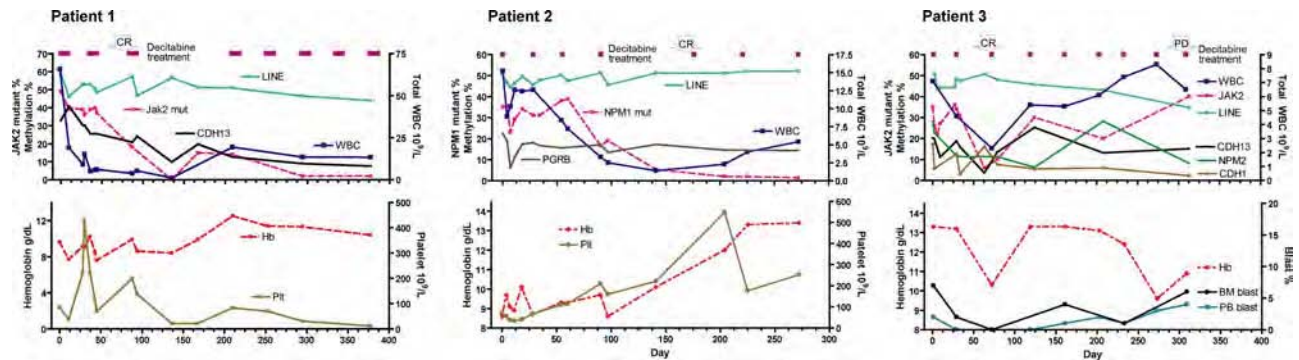
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**Figure 1. Dynamics of DNA methylation, mutant alleles as neoplastic clone markers, and peripheral blood counts.** We analyzed methylation of 10 genes at baseline in all 3 patients, and genes with increased baseline methylation were followed during 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 > 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 response<sup>8</sup> against CMML clone, or effects on normal stem cells. Further investigations should focus on these mechanisms to improve the results seen with decitabine.

## Acknowledgments

This work was supported in part by Leukemia Specialized Program of Research Excellence (SPORE; grant P50CA100632) from the National Institutes of Health and the Department of Defense (grant W81XWH-05-1-0535). Y.O. is a recipient of an American Society

of Clinical Oncology Young Investigator Award 2005-2006. J.-P.J.I. is an American Cancer Society Clinical Research Professor.

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.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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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

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