Integrated Systems and Technologies

Cancer Research

Methylome Profiling Reveals Distinct Alterations in Phenotypic and Mutational Subgroups of Myeloproliferative Neoplasms

Sangeeta Nischal[1], Sanchari Bhattacharyya[1], Maximilian Christopeit[1], Yiting Yu[1], Li Zhou[1], Tushar D. Bhagat[1], Davendra Sohal[1], Britta Will[1], Yongkai Mo[1], Masako Suzuki[1], Animesh Pardanani[2], Michael McDevitt[3], Jaroslaw P. Maciejewski[4], Ari M. Melnick[5], John M. Greally[1], Ulrich Steidl[1], Alison Moliterno[3], and Amit Verma[1]

Abstract

Even though mutations in epigenetic regulators frequently occur in myeloproliferative neoplasms, their effects on the epigenome have not been well studied. Furthermore, even though primary myelofibrosis (PMF) has a markedly worse prognosis than essential thrombocytosis or polycythemia vera, the molecular distinctions between these subgroups are not well elucidated. We conducted the HELP (*Hpa*II tiny fragment enriched by LM-PCR) assay to study genome-wide methylation in polycythemia vera, essential thrombocytosis, and PMF samples compared with healthy controls. We determined that polycythemia vera and essential thrombocytosis are characterized by aberrant promoter hypermethylation, whereas PMF is an epigenetically distinct subgroup characterized by both aberrant hyper- and hypomethylation. Aberrant hypomethylation in PMF was seen to occur in non-CpG island loci, showing further qualitative differences between the disease subgroups. The differentially methylated genes in polycythemia vera and essential thrombocytosis were involved predominantly in cell signaling pathways and were enriched for binding sites of GATA1 and other transcription factors. In contrast, aberrantly methylated genes in PMF were involved in inflammatory pathways and were enriched for NF1, LEF1, and other transcription factors. Within the PMF subgroup, cases with ASXL1 disruptions formed an epigenetically distinct subgroup with relatively increased methylation. Cases of myeloproliferative neoplasms (MPN) with TET2 mutations showed decreased levels of hydroxymethylation and distinct set of hypermethylated genes. In contrast, the JAK2V617F mutation did not drive epigenetic clustering within MPNs. Finally, the significance of aberrant methylation was shown by sensitivity of MPN-derived cell lines to decitabine. These results show epigenetic differences between PMF and polycythemia vera/essential thrombocytosis and reveal methylomic signatures of ASXL1 and TET2 mutations. *Cancer Res; 73(3); 1076–85. ©2012 AACR.*

Introduction

The myeloproliferative neoplasms (MPN), essential thrombocytosis, polycythemia vera, and primary myelofibrosis (PMF) share the same acquired genetic mutation, JAK2V617F, but vary with respect to epidemiology, disease phenotype, and prognosis. PMF carries the worst prognosis within the MPN due to complications of bone marrow failure or leukemic transformation. Within the JAK2V617F-positive MPNS, variation in gene dosage of JAK2V617F due to varying rates of chromosome 9p uniparental disomy (UPD) offers some rationale as to these discrepancies in phenotype, yet significant overlap still occurs between polycythemia vera and myelofibrosis. Acquired genomic lesions in other signal transduction pathway genes, JAK2 Exon 12, MPL, and LNK, recapitulate myeloproliferative phenotypes but still do not segregate phenotypes within the MPNs. More recently, lesions in genes central to epigenetic regulation, TET2 and ASXL1, have been found to be prevalent within the MPNs, and in the case of ASXL1, segregate with myelofibrosis phenotypes, implicating the importance of epigenetic regulation as a novel pathway relevant to myelofibrosis biology (1, 2)

Recent evidence suggests that MPNs are characterized by aberrant transcriptional profiles, and some of these changes are driven by the mutant JAK2V617F kinase (3). One of the ways that gene expression may be dysregulated is through aberrant DNA methylation. Methylation of cytosine has been implicated as a way to silence genes epigenetically and indicates an attractive target for potential therapeutics.

Authors' Affiliations: [1]Albert Einstein College of Medicine, Bronx, New York; [2]Mayo Clinic, Rochester, Minnesota; [3]Johns Hopkins School of Medicine, Baltimore, Maryland; [4]Cleveland Clinic, Cleveland, Ohio; and [5]Weill Cornell Medical School, New York, New York

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Corresponding Authors: Amit Verma, Albert Einstein College of Medicine, 1300 Morris Park Ave, Chanin 302B, Bronx, NY 10461. Phone: 718-430-8761; Fax: 718-430-8702; E-mail: amit.verma@einstein.yu.edu; and Alison Moliterno, Johns Hopkins School of Medicine, Traylor Building, Room 912, 720 Rutland Avenue, Baltimore, MD 21205; E-mail: amoliter@jhmi.edu

doi: 10.1158/0008-5472.CAN-12-0735

Single locus studies have shown aberrant methylation of promoters of genes such as SOC1, SOCS3 and CXCR4 can be seen in MPNs, although these changes have not been studied at a genome-wide level (4, 5). Genome-wide methylome studies in other disease have revealed surprising patterns and have the potential to change existing paradigms (6, 7). The recent discovery of direct epigenetic effects of the JAK2 mutation on histone H3 phosphorylation further point to a role of epigenetic disturbances in the pathogenesis of these diseases. (8). Thus, the purpose of this study was to assess genome-wide patterns of DNA methylation across the MPNs stratified by disease class and to determine the effect of JAK2V617F on the methylome.

Materials and Methods

Patient samples and nucleic acid extraction

The study population consisted of 29 patients with MPNs and controls evaluated at either Johns Hopkins University (Baltimore, MD) or Albert Einstein School of Medicine (Bronx, NY). The controls were aged 40, 79, and 83 years, respectively. The study was approved by an Institutional Review Board, and all patients gave written consent. Genomic DNA from neutrophils isolated from peripheral blood via Ficoll gradient density separation was prepared using Qiagen reagents.

DNA methylation analysis by HELP

The HELP assay was conducted as previously published (9). Intact DNA of high molecular weight was corroborated by electrophoresis on 1% agarose gel in all cases. One microgram of gDNA was digested overnight with either *Hpa*II or *Msp*I (NEB). On the following day, the reactions were extracted once with phenol–chloroform and resuspended in 11 μL of 10 mmol/L Tris-HCl, pH 8.0, and the digested DNA was used to set up an overnight ligation of the JHpaII adapter using T4 DNA ligase. The adapter-ligated DNA was used to carry out the PCR amplification of the *Hpa*II- and *Msp*I-digested DNA as previously described (9). Both amplified fractions were submitted to Roche-NimbleGen, Inc. for labeling and hybridization onto a human hg17 custom-designed oligonucleotide array (50-mers) covering 25,626 *Hpa*II-amplifiable fragments (HAF) located at gene promoters. HAFs are defined as genomic sequences contained between 2 flanking *Hpa*II sites found within 200 to 2,000 bp from each other. Each fragment on the array is represented by 15 individual probes distributed randomly spatially across the microarray slide. Thus, the microarray covers 50,000 CpGs corresponding to 14,000 gene promoters. HELP microarray data have been submitted to the Gene Expression Omnibus (GEO) database for public access (GSE42721).

Quantitative DNA methylation analysis by massARRAY EpiTYPING

Validation of HELP microarray findings was conducted by matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) mass spectrometry using EpiTyper by massARRAY (Sequenom) on bisulfite-converted DNA as previously described (10, 11). massARRAY primers were designed to cover the flanking *Hpa*II sites for a given HAF, as well as any other *Hpa*II sites found up to 2,000 bp upstream of the downstream site and up to 2,000 bp downstream of the upstream site, to cover all possible alternative sites of digestion. Primers are available on request.

Microarray quality control

All microarray hybridizations were subjected to extensive quality control using the following strategies. First, uniformity of hybridization was evaluated using a modified version of a previously published algorithm (12) adapted for the NimbleGen platform, and any hybridization with strong regional artifacts was discarded and repeated. Second, normalized signal intensities from each array were compared against a 20% trimmed mean of signal intensities across all arrays in that experiment, and any arrays displaying a significant intensity bias that could not be explained by the biology of the sample were excluded.

HELP data processing and analysis

Signal intensities at each HAF were calculated as a robust (25% trimmed) mean of their component probe-level signal intensities. Any fragments found within the level of background *Msp*I signal intensity, measured as 2.5 mean absolute differences (MAD) above the median of random probe signals, were categorized as "failed." These "failed" loci therefore represent the population of fragments that did not amplify by PCR, whatever the biologic (e.g., genomic deletions and other sequence errors) or experimental cause. On the other hand, "methylated" loci were so designated when the level of *Hpa*II signal intensity was similarly indistinguishable from background. PCR-amplifying fragments (those not flagged as either "methylated" or "failed") were normalized using an intra-array quantile approach wherein *Hpa*II/*Msp*I ratios are aligned across density-dependent sliding windows of fragment size–sorted data. The $\log_2$ (*Hpa*II/*Msp*I) was used as a representative for methylation and analyzed as a continuous variable. For most loci, each fragment was categorized as either methylated, if the centered log *Hpa*II/*Msp*I ratio was less than zero or hypomethylated if, on the other hand, the log ratio was greater than zero.

Microarray data analysis

Unsupervised clustering of HELP data by hierarchical clustering was conducted using the statistical software R version 2.6.2. A 2-sample *t* test was used for each gene to summarize methylation differences between groups. Genes were ranked on the basis of this test statistic, and a set of top differentially methylated genes with an observed log fold change of >1 between group means was identified. Genes were further grouped according to the direction of the methylation change (hypo- vs. hypermethylated), and the relative frequencies of these changes were computed among the top candidates to explore global methylation patterns. Validations with massARRAY showed good correlation with the data generated by the HELP assay. massARRAY analysis validated significant quantitative differences in methylation for differentially methylated genes selected by our approach.

Pathway analysis and transcription factor–binding site analysis

Using the Ingenuity Pathway Analysis (IPA) software, we conducted an analysis of the biologic information retrieved by each of the individual platforms alone and compared it with the information obtained by the integrated analysis of all 3 platforms. Enrichment of genes associated with specific canonical pathways was determined relative to the Ingenuity knowledge database for each of the individual platforms and the integrated analysis at a significance level of $P < 0.01$. Biologic networks captured by the different microarray platforms were generated using IPA and scored on the basis of the relationship between the total number of genes in the specific network and the total number of genes identified by the microarray analysis. The list of hypermethylated genes was examined for enrichment of conserved gene–associated transcription factor–binding sites using the Molecular Signatures Database (MSigDB; ref. 13). Their functional gene sets were obtained from Gene Ontology (GO; ref. 14).

This analysis was conducted by Gene Set Enrichment Analysis (GSEA; ref. 13), a computational method that determines whether an *a priori* defined set of genes (commonly hypermethylated genes in myelodysplastic syndrome) shows statistically significant, concordant differences between 2 biologic states. Same method was applied to determine whether transcription-binding sites are randomly distributed in the differentially methylated genes. The *a priori* defined gene sets used in this analysis is transcription factor target (TFT), which contains genes that share a transcription factor–binding site defined in the TRANSFAC database (15). Using GSEA 'Pre-ranked' algorithm, 1,000 permutations were applied to sample labels to test whether genes from each TFT gene sets were randomly distributed along the differentially methylated gene list.

Mutational and single-nucleotide polymorphism karyotyping analysis

Neutrophil isolation and DNA preparation were conducted as previously described (16). The JAK2V617F neutrophil allele burdens were measured using an allele-specific, quantitative real-time PCR assay sensitive to a lower limit of detection of 5% of either the wild-type or mutant JAK2 allele as previously described (16). Single-nucleotide array karyotyping assay and analysis were conducted as described previously (17) Briefly, Gene Chip Mapping Affymetrix 250K arrays (Affymetrix) were used for single-nucleotide polymorphism karyotyping (SNP-K) analysis and used per the manufacturer's instructions. Signal intensity was analyzed and SNP calls determined using Gene Chip Genotyping Analysis Software Version 4.0 (GTYPE). Copy number and areas of UPD were investigated using a hidden Markov model and copy number Analyzer for Affymetrix GeneChip Mapping 250K arrays (CNAG v3.0). Exon 12 of *ASXL1* was amplified from neutrophil genomic DNA and resequenced as previously described (18) For TET2 mutations, the all the exons of the gene were PCR-amplified and sequenced using similar methodology. TET2 amplification and sequencing primers have been described previously (19). Identification of known SNPs was established via searching the NCBI dbSNP database and the 1000 Genomes Project.

Analysis of hydroxymethylation

Levels of methylated and hydroxymethylated DNA were assessed using the Methyl Flash Methylated/Hydroxymethylated DNA Quantification Kit (Colorimetric, catalog No. P-1034/P-1036; Epigentek), respectively. Briefly, 100 ng (methylation assay) and 200 ng (hydroxymethylation assay) of genomic DNA were assayed according to the manufacturer's instructions as previously described (20). Experiments were carried out in triplicates. Adsorption at 450 nm was read on a Versamax Tunable Microplate Reader (Molecular Devices). Groups were compared using the Mann–Whitney *U* test. $P < 0.05$ was considered statistically significant. Statistics were conducted using PASW/SPSS Version 18 (IBM).

Cell lines and viability assay

Cell lines HL60, K562, and HEL were purchased from American Type Culture Collection (ATCC) and SET-2 was obtained from DSMZ. These had been authenticated at ATCC and DSMZ. All cell lines were cultured in RPMI medium (Invitrogen) supplemented with 10% heat-inactivated FBS and penicillin (100 U/mL), streptomycin (100 mg/mL), and 4 mmol/L glutamine. Cells were maintained at 37°C and humidified with 95% air and 5% CO_2 for cell culture. For the viability assays, the cells were cultured in 0.5, 1, and 5 μmol/L decitabine (Sigma) for 2 days. Decitabine was added to the culture daily, dimethyl sulfoxide (DMSO) served as control. Viability was measured on day 3 using MTT assay (Promega) according to the manufacturer's instruction.

Luminometric methylation assay

The gDNA (200–500 ng) was cleaved with *Hpa*II + *Eco*RI or *Msp*I + *Eco*RI in 2 separate 20-mL reactions containing 33 mmol/L Tris-acetate, 10 mmol/L Mg acetate, 66 mmol/L K acetate, pH 7.9, 0.1 mg/mL bovine serum albumin, and 5 units of each restriction enzymes. The reactions were set up in a 96-well format and incubated at 37°C for 4 hours. Then, 20-mL annealing buffer (20 mmol/L Tris-acetate, 2 mmol/L Mg acetate, pH 7.6) was added to the cleavage reactions, and samples were placed in a PSQ96TMMA system (Biotage AB). The instrument was programmed to add dNTPs in 4 consecutive steps including step 1: dATP (the derivative dATPaS is used as it will not react directly with luciferase and prevents nonspecific signals); step 2: mixture of dGTP + dCTP; step 3: dTTP; and step 4: mixture of dGTP + dCTP. Peak heights were calculated using the PSQ96TMMA software. The *Hpa*II/*Eco*RI and *Msp*I/*Eco*RI ratios were calculated as (dGTP + dCTP)/dATP for the respective reactions. The *Hpa*II/*Msp*I ratio was defined as (*Hpa*II/*Eco*RI)/(*Msp*I/*Eco*RI) (6)

Results

Genome-wide methylation profiling shows PMF as a distinct epigenetic subgroup

We conducted the HELP assay to study genome-wide methylation patterns in primary polycythemia vera, essential thrombocytosis, and PMF samples. The HELP assay uses

differential methylation specific digestion by *Hpa*II and *Msp*I followed by amplification, 2-color labeling and hybridization to quantitatively determine individual promoter methylation of 50,000 CpGs loci covering 14,000 promoters (9, 21). We selected neutrophils to study for many reasons. First, neutrophils are obtained from all patients with a more than 95% purity, and abundant DNA is available. Second, in the MPNs, neutrophils are highly enriched for the malignant clone, more so than purified progenitor cells (16). Neutrophils are a fully differentiated tissue, representing a single hematopoietic lineage, such that differences in lineage representation that may exist in a marrow sample would not confound this analysis. Analysis of 26 MPN neutrophil samples comprising 9 cases of essential thrombocytosis, 5 cases of polycythemia vera, and 12 cases of PMF was conducted and compared with healthy controls (Table 1). Unsupervised clustering based on global methylation profiles showed that whereas polycythemia vera and essential thrombocytosis cases were more similar to the normal controls, the PMF cases were epigenetically distinct from these groups. Five samples of PMF formed a cluster with similar methylation profiles, whereas the rest of the samples exhibited greater heterogeneity. Interestingly, the PMF samples that clustered tightly all had mutated or deleted ASXL1, suggesting that disruption of this gene was driving the epigenetic similarity between these samples (Fig. 1). Overall, methylation profiling of MPNs did not correlate with demographics of these patients. These samples were also examined by high-resolution SNP-array karyotyping, and the methylation patterns did not correlate with the presence and absence of cytogenetic alterations found in these patients.

Polycythemia vera/essential thrombocytosis are characterized by hypermethylated loci that affect important functional pathways

To further analyze the epigenetic differences between these subtypes of MPNs, we conducted supervised clustering of polycythemia vera and essential thrombocytosis cases and compared them with controls. We saw that these samples had 141 genes that were uniformly hypermethylated compared with controls (Fig. 2A). Bioinformatic analysis revealed that hypermethylated genes displayed specific genomic characteristics and were enriched for binding sites for GATA1 and other transcription factors (Table 2) showing potential regulatory disturbances in these pathways.

Table 1. Demographic and molecular features of the MPN cohort

ID	Sex/age	Disease duration, y	Therapy (at time of sample)	% JAK2V617F	ASXL1	TET2	Other cytogenetic abnormalities
ET1	F/90	11	Hydroxyurea	43		Mutant	
ET2	F/44	10	None	0			
ET3	F/81	26	None	0			
ET4	M/45	3	Anegrelide	0			
ET5	F/40	7	None	0		Mutant	
ET6	F/43	21	None	0			
PV12	M/72	5	None	85			
PV13	F/69	10	None	51			
PV14	F/71	12	Hydroxyurea	100			Del 17q
PV5	M/73	6	None	86			
PV1	F/74	1	None	49		Mutant	
PV2	F/69	17	None	35			
PV3	F/51	18	None	99		Mutant	
PV4	M/63	2	None	46		Mutant	
PMF1	M/61	20	Hydroxyurea	100		Mutant	
PMF2	M/72	0	None	45		Mutant	
PMF3	M/72	4	EPO	48			
PMF4	F/66	2	None	0			Trisomy 8, Trisomy 21
PMF5	M/78	0	None	42	Mutant		
PMF6	M/74	3	None	0			Del 13q13
PMF7	M/65	2	EPO	0	Mutant		Gain 1q21
PMF8	F/51	23	EPO, thalidomide	0	Mutant		Del 13q13
PMF9	F/54	0	None	0	Mutant		
PMF10	M/68	3	None	43	Deletion		Del 20q11
PMF11	F/68	4	EPO	67	Deletion		Del 20q11, Del4q32, Del14q31
PMF12	F/68	1	Hydroxyurea	100		Mutant	Gain 4q32

Abbreviation: EPO, erythropoietin

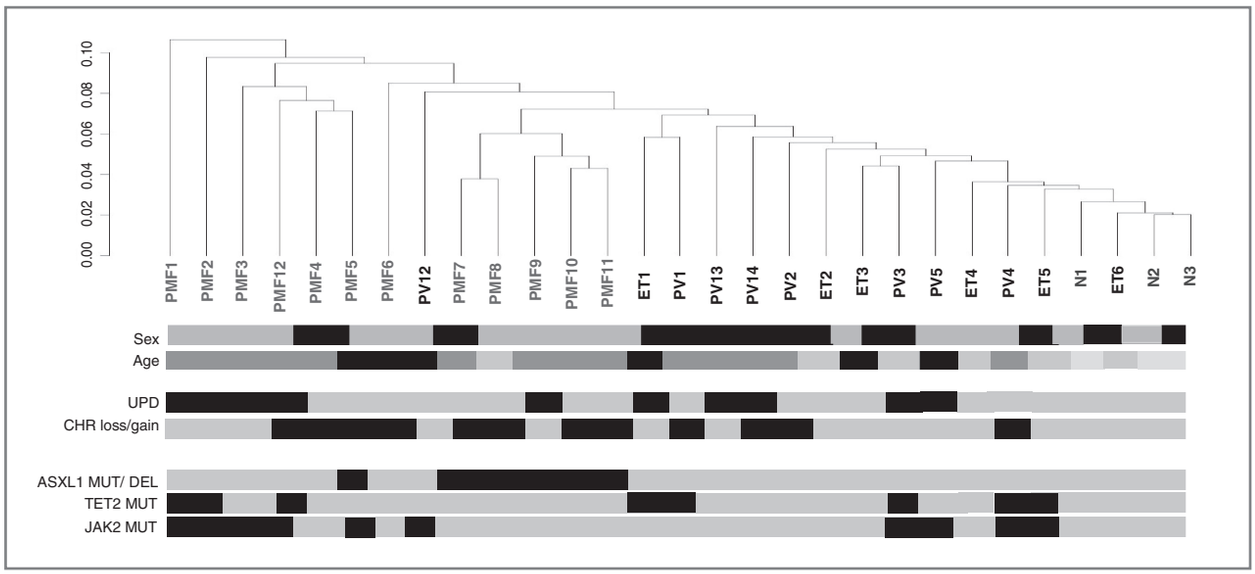

Figure 1. Genome-wide methylation profiling shows PMF as a distinct epigenetic subgroup of MPNs. Unsupervised hierarchal clustering (1 − Pearson correlation distance and Ward agglomeration method) based on global DNA methylation obtained from neutrophils showed that PMF cases (gray) formed clusters distinct from polycythemia vera (PV)/essential thrombocytosis (ET) samples. PV and ET (black) cases were epigenetically more similar to healthy controls (gray). Demographics did not reveal any effect on epigenetic clustering. Males are represented by black squares and females by gray squares. Age groups (40–49, 50–59, 60–69, and 70–79) are represented as progressively darker shades of gray. The presence of any UPD and the presence of any chromosomal loss or gain are represented by black squares. TET2, JAK2, and ASXL1 mutation–positive cases are represented as black squares.

Genes that were significantly hypermethylated included several novel candidates such as transcription factor HNF4-α, histone acetyltransferase MYST2, interleukin-1, and others (Supplementary Table S1). Functional pathway analysis revealed that these genes are involved in pathways regulated by the NF-κB and HNF4-α transcription factors (Supplementary Fig. S1).

PMF is characterized by both aberrantly hyper- and hypomethylated loci that affect distinct functional pathways

PMF was characterized by both aberrantly hypermethylated ($n = 162$) and hypomethylated ($n = 106$) loci when compared with controls (Fig. 2B). These changes were further evaluated by the luminometric methylation assay (LUMA), a quantitative assay that interrogates all *Hpa*II sites in the genome (22). LUMA also revealed significantly more hypomethylation in PMF than in polycythemia vera and essential thrombocytosis cases (49% mean hypomethylation in PMF vs. 38% in polycythemia vera/essential thrombocytosis, $P < 0.05$, t test; Fig. 2C). The findings of the HELP assay were validated by bisulfite massARRAY analysis and showed good correlation (Supplementary Fig. S2A–S2C). Important functional pathways were found to be affected by aberrant methylation in PMF. The pathways affected by hypomethylated genes included cell signaling, hematopoiesis, and immunologic pathways (Supplementary Fig. S3A), whereas the pathways affected by hypermethylated genes included those governing inflammatory responses and others (Supplementary Fig. S3B and Supplementary Tables S2 and S3 listing all significantly hyper- and hypomethylated genes).

These changes also affected specific genomic regions, and the hypermethylated genes were enriched for binding sites for LEF1 and POU6F1 transcription factors, whereas the hypomethylated genes were enriched for the TFAP2A and NF1 transcription factors (Tables 3 and 4). Interestingly, the significantly, aberrantly hypomethylated regions in PMF were found to be preferentially located outside of CpG islands (Fig. 2D) further showing the unique nature of these changes in PMF.

PMF samples with mutated or deleted ASXL1 have a distinct epigenetic profile

Because we observed that the ASXL1-mutated/deleted samples were epigenetically similar (Fig. 1), we wanted to determine how the methylation profiles were impacted by disruptions of this gene. Supervised analysis revealed that cases of PMF with mutated or deleted ASXL1 were relatively more hypermethylated than the cases with no disruptions in ASXL1 (Fig. 3A and B). The genes that were uniformly hypermethylated in ASXL1-mutated/deleted cases in comparison to controls included various important candidates such as NPM2, RUNX1, HOXB3, SMAD3, and others (listed in Supplementary Tables S4 and S5) These results reveal that disruption in functioning of chromatin-binding protein, ASXL1, is associated with specific methylation patterns in PMF.

MPN samples with TET2 mutations are characterized by decreased levels of hydroxymethylation and increased genome-wide methylation

TET2 is an important regulator of hydroxymethylation and subsequent demethylation and has been shown to be mutated

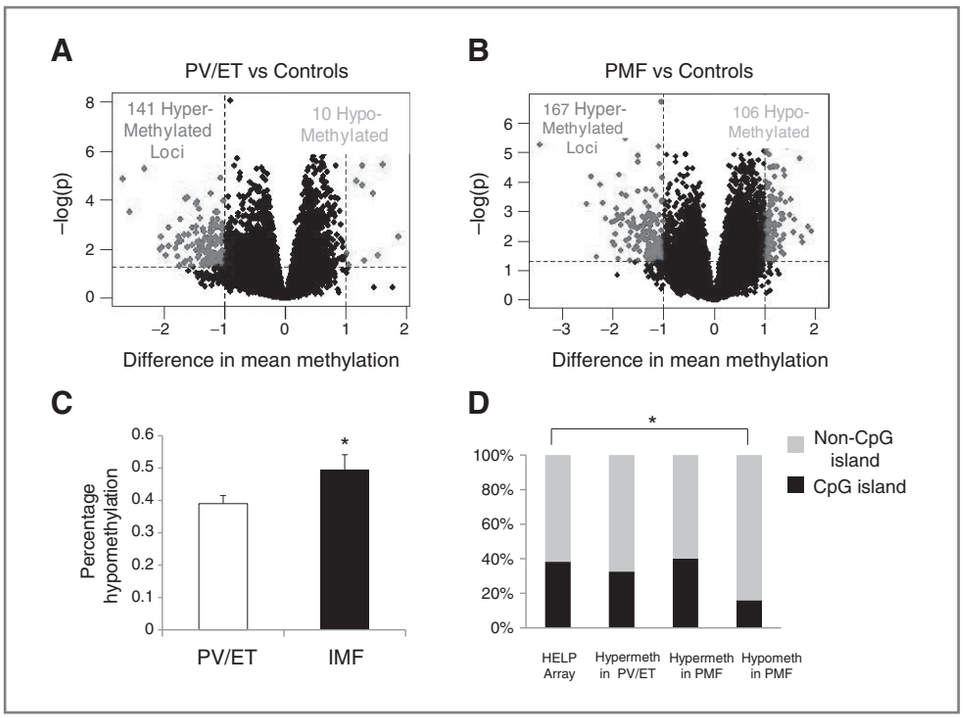

Figure 2. Polycythemia vera (PV)/essential thrombocytosis (ET) are characterized by aberrant hypermethylation, whereas PMF is characterized by both hypo- and hypermethylated loci. A volcano plot shows the difference in mean methylation between all PV/ET samples and controls on the *x*-axis and the log of the *P* values between the means on the *y*-axis. A 2-tailed *t* test was used to calculate the *P* values. Differentially methylated loci with a log-fold change in mean methylation are labeled in gray ($P < 0.05$). Significant hypermethylation is seen in PV/ET (A). Both significantly hyper- and hypomethylated loci are seen in PMF cases when compared with controls (B). LUMA assay showed relative hypomethylation in PMF cases when compared with PV/ET (means ± SEM; *t* test, $P < 0.05$; C). The genomic position of every HAF on the HELP array was compared with the location of known CpG islands, and the fragments on the array were divided into 2 categories: those overlapping with these genomic elements and nonoverlapping. To determine whether the differentially methylated genes between PMF and controls were enriched for either one of these types of elements, a proportions test was used to compare the relative proportion of the 2 types of *Hpa*II fragments in the signature with the relative proportion on the array. Stacking bars are used to illustrate the finding of a significant enrichment for HAFs not overlapping with CpG islands (D).

in a notable proportion of MPNs (23). Because the effects of TET2 mutation on the methylome of MPNs have not been studied, we sequenced this gene in all MPN samples and correlated it with the methylation patterns in these samples. Supervised analysis revealed that cases of MPNs with mutated TET2 were aberrantly hypermethylated when compared with controls (Fig. 4A and B) and had significantly decreased hydroxymethylation levels (Fig. 4C). The TET2-mutant cases were characterized by a distinct set of commonly methylated genes that included HOXB3, HOXD3, TRAF6, MYST, and others (Supplementary Tables S6 and S7).

JAK2 mutation does not lead to epigenomic clustering

The JAK2V617F mutation is seen in a large proportion of patients with MPNs, and recent evidence has shown that the mutant JAK2 kinase can translocate to the nucleus and can directly affect the histone epigenetic machinery (8). Thus, we wanted to determine whether this mutation led to any effects on the methylome of these patients. Because disease class (PMF vs. polycythemia vera or essential thrombocytosis) was the strongest determinant of epigenetic clustering and unsupervised clustering did not show any epigenetic clustering due to JAK2V617F mutation (Fig. 1), we next examined the polycythemia vera and essential thrombocytosis cohort individually by unsupervised clustering. We did not observe any epigenetic clustering based on the presence and magnitude of JAK2 mutation even in this cohort (Supplementary Fig. S4). Supervised clustering could also not show any significant gene-specific methylation signature in the mutant cases. Because patients with MPNs have multiple genetic abnormalities, these data suggest that the JAK2V617F mutation does not exert dominant effect on the methylome of primary samples.

MPN-derived cell lines are sensitive to growth inhibition by DNMT inhibitor decitabine

Having shown that MPNs are characterized by aberrant hypermethylation, we next wanted to determine whether these cells were sensitive to growth inhibition by DNMT inhibitors. We compared leukemic cell lines that were derived from patients with MPN (HEL, SET-2, both have JAK2V617F mutation and HEL has ASXL1 deletion; ref. 24) to non-MPN cell lines K562 and HL60. Genome-wide methylation was found to be significantly more in HEL and SET-2 cell lines when assessed with the LUMA assay (Fig. 5). Treatment with decitabine also resulted in significant growth inhibition in both

Table 2. Transcription factor–binding sites enriched in hypermethylated genes in polycythemia vera/essential thrombocytosis

Transcription factor	Motif	*P*
GATA1	NNCWGATARNNNN	2.86E-03
SP1	GGGCGGR	1.82E-02
KLF12	CAGTGGG	1.84E-02
GATA	WGATARN	2.64E-02
POU6F1	GCATAAWTTAT	4.09E-02
AHR	KNNKNNTYGCGTGCMS	4.18E-02
GATA1	SNNGATNNNN	4.31E-02
LMO2	NMGATANSG	4.36E-02
LFA1	GGGSTCWR	4.59E-02
CP2	GCTGGNTNGNNCYNG	4.87E-02
CEBPA	NNNTKNNGNAAN	4.91E-02

HEL and SET-2 cells at all doses, thus showing functional importance of hypermethylation observed in these MPN-derived cell lines.

Discussion

The discovery of the most prevalent genomic lesion specific to the chronic MPN, JAK2V617F, has provided important insights into pathogenesis and treatment of these diseases. However, even within the JAK2V617F-positive MPNs, variation in disease phenotype and natural history exists, suggesting that epigenetic processes are significant modifiers of disease pathobiology. Our results show widespread alterations in DNA methylation in MPNs and show that PMF is epigenetically distinct from polycythemia vera and essential thrombocytosis. These differences were not only seen at the involvement of different sets of aberrantly methylated genes but were also seen as a global level by the LUMA assay that showed increased hypomethylation in PMF.

Table 3. Transcription factor–binding sites enriched in hypermethylated genes in PMF

Transcription factor	Motif	*P*
POU6F1	GCATAAWTTAT	1.24E-03
LEF1	SWWCAAAGGG	2.10E-03
GATA	WGATARN	2.65E-03
AP2	MKCCCSCNGGCG	7.50E-03
GATA	NGATAAGNMNN	8.81E-03
OCT	TNATTTGCATN	8.81E-03
AHR	KNNKNNTYGCGTGCMS	1.44E-02
MEF2	CTCTAAAAATAACYCY	1.63E-02
LXR	TGGGGTYACTNNCGGTCA	1.69E-02
OCT1	MKVATTTGCATATT	2.44E-02
CEBP	NNNTKNNGNAAN	2.60E-02

Table 4. Transcription factor–binding sites enriched in hypomethylated genes in PMF

Transcription factor	Motif	*P*
NF1	NTGGNNNNNNGCCAANN	8.84E-04
KLF12	CAGTGGG	1.27E-02
NR6A1	NTCAAGKTCAAGKTCANN	1.85E-02
CDC5	GATTTAACATAA	3.19E-02
TCF3	CAGGTG	3.75E-02
MYOG	NVTNWTGATTGACNAC-AAVARRBN	3.82E-02
SP1	GGGCGGR	6.57E-02
TEAD1	WGGAATGY	7.18E-02
MAZ	GGGGAGGG	8.59E-02
SRF	DCCWTATATGGNCWN	9.90E-02
SMAD4	GKSRKKCAGMCANCY	1.20E-01

Analysis of differentially methylated genes in MPNs revealed that they were enriched for binding sites for various transcription factors that have important roles in hematopoiesis. GATA1 has been shown to play a role in the pathogenesis of MPNs and myeloid leukemias (25) and was significantly associated with differentially methylated genes in polycythemia vera and essential thrombocytosis. NF1 was found to associated with differentially methylated genes in PMF and was recently found to be deleted and mutated in PMF samples in a large SNP array study (26). These changes show that the changes in methylome occur at specific genomic loci and may be driven by altered levels of transcription factors that are dysregulated in these diseases. In addition to these known transcription factors, our study reveals numerous other differentially methylated loci that are associated with binding sites of transcription factors that have not been studied in MPNs and can potentially be involved in its pathobiology.

We also determined that even though PMF was an epigenetically heterogeneous subgroup, cases of PMF with ASXL1 deletions/mutations were epigenetically similar and clustered tightly together. The ASXL1 gene has been shown to be mutated/deleted in 36% of cases of PMFs and is associated with a more severe clinical presentation (18). Thus, even though it appears that this gene is important in pathobiology of PMF, it is not clear how the disruption of ASXL1 leads to altered gene transcription. ASXL1 is a member of the polycomb repressor complex and is a chromatin-modifying protein (27). Our data show for the first time that deletion/mutations of this protein are associated with distinct signature of DNA methylation involving many important gene promoters. Relative hypermethylation seen in the cases of PMF with ASXL1 disruption also raise the possibility of potential therapeutic benefit of DNMT inhibitors in this subgroup. In fact, our data from MPN-derived cells lines showed that the cell line with ASXL1 deletion (HEL cell line) was most sensitive to growth inhibition by DNMT inhibitors.

TET2 mutations are also seen frequently in MPNs (28), and recent data have shown that this protein is important for the

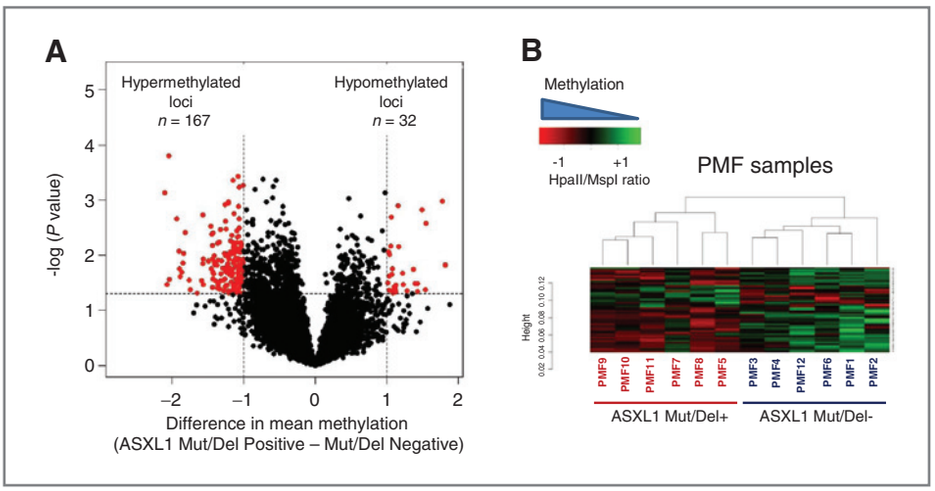

Figure 3. PMF with ASLX1 mutation/deletion have a distinct epigenetic profile with increased methylation. A, a volcano plot shows higher number of significantly hypermethylated loci in PMF cases with ASXL1 mutations/deletions. The difference in mean methylation between ASXL1 mut/del and ASXL wild-type (wt) cases is shown on the *x*-axis and the log of the *P* values between the means on the *y*-axis. Differentially methylated loci with a log-fold change in mean methylation and $P < 0.05$ are shown in red. B, supervised clustering of PMF cases based on the differentially methylated genes shows separation of ASXL1 mut/del cases with increased methylation in the top 100 differentially methylated genes.

conversion of methyl cytosine to hydroxymethyl cytosines (29). Hydroxymethyl cytosines can further be removed by base excision repair, thus leading to demethylation. Thus, impairment of this process due to mutated TET2 protein has been associated with decreased levels of hydroxymethylation in experimental models and in primary samples from patients with myelodysplastia (23). Our data now show that decreased hydroxymethylation and increased cytosine methylation can be seen in MPN samples with the TET2 mutation also. The increasing incidence of mutations in proteins involved in the epigenetic machinery (TET, ASXL1, IDH, and others) suggest that the resulting epigenetic alterations may influence gene expression that may contribute to disease pathogenesis. Our demonstration of widespread epigenetic changes in TET2-mutant cases further supports this hypothesis. One might have anticipated that the methylation patterns in neutrophils

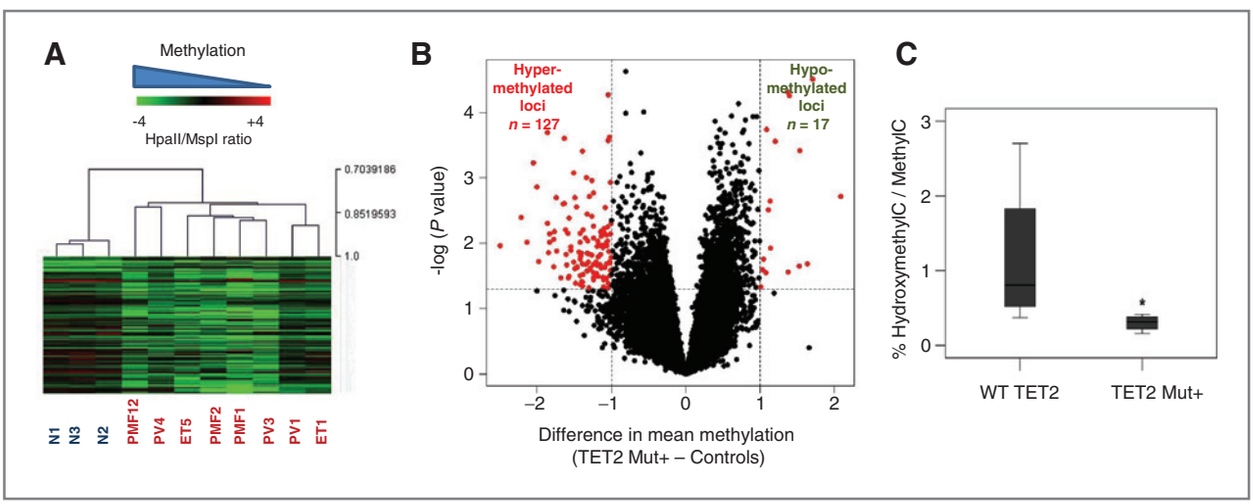

Figure 4. TET2 mutations are associated with decreased hydroxymethylation and increased cytosine methylation in MPNs. A, supervised clustering of MPN cases based on the differentially methylated genes shows separation of TET2-mutant cases with increased methylation in the top 100 differentially methylated genes. B, a volcano plot also shows higher number of significantly hypermethylated loci in cases with TET2 mutations/deletions. The difference in mean methylation between TET2 mut+ cases and healthy controls is shown on the *x* axis and the log of the *P* values between the means on the *y*-axis. Differentially methylated loci with a log-fold change in mean methylation and $P < 0.05$ are shown in red. To test whether the ratio of hydroxymethylated/methylated DNA differed between TET2-mutated and wild-type patients with MPN samples, we conducted colorimetric measurements of the abundance of hydroxymethylated and methylated DNA. Median ratio ± 95% confidence interval using DNA from 4 patients with wild-type TET2 (left) versus DNA from 4 patients with mutant TET2 (right) is depicted. C, statistical comparison was calculated using the Mann–Whitney *U* test with $P < 0.05$.

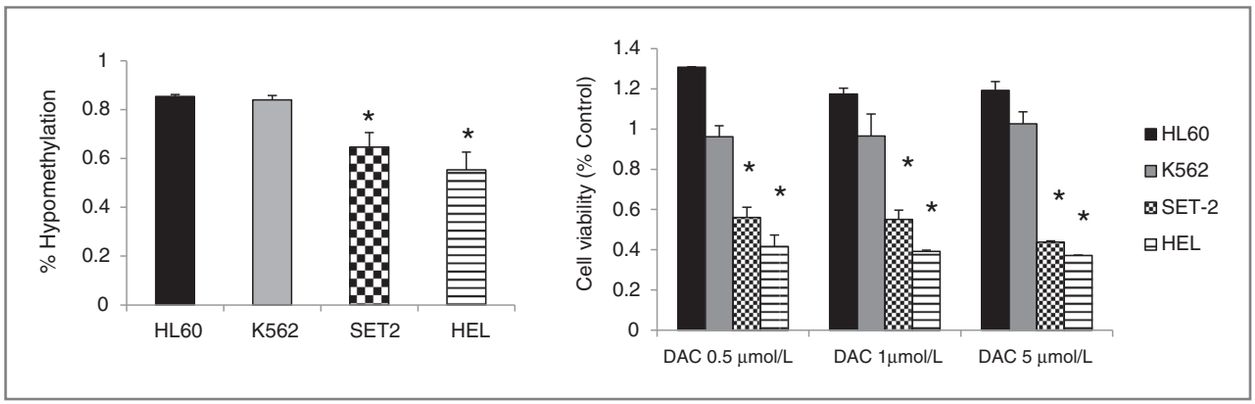

Figure 5. Decitabine treatment leads to growth inhibition in MPN-derived cell lines. LUMA assay was used to assess percentage of hypomethylation from genomic DNA from cell lines. Means ± SEM of 3 independent experiments is shown (*t* test, $P < 0.05$; left). Cell lines were treated with different doses of decitabine (DAC) for 2 days, and proliferation was assessed by the MTT assay after 72 hours. Significant inhibition of growth was seen after treatment in HEL and SET-2 cell lines (Means ± SEM of 3 independent experiments, *t* test, $P < 0.05$).

would be very similar in controls compared with patients with MPNs, due to the intact myeloid differentiation program in the MPNs. Thus, any differences between controls and patients with MPNs or between MPN subclasses may reflect specific genomic lesions unique to the MPN, whether the methylation aberrancies are reflection of the MPN stem cell, progenitor, or differentiated myeloid cell.

Finally, we show that there are numerous significantly and uniformly hypermethylated loci in polycythemia vera, essential thrombocytosis, and PMF that may be targeted by epigenetic modifiers in future clinical trials. Epigenetic modifiers such as histone deacetylase inhibitors are being tried in MPNs including both polycythemia vera and PMF (30). Decitabine has also been tested in PMF with response in 37% of cases (31). These responses do point to the role of epigenetic alterations in disease pathobiology. The heterogeneity in DNA methylation seen by our study in MPNs raises the possibility of specific epigenetic clusters that may be responsive to these agents. Further similar correlative studies are needed to uncover epigenetic signatures of response to these agents.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: J. Greally, A. Moliterno, A. Verma
Development of methodology: S. Bhattacharyya, D. Sohal, Y. Mo, J.P. Maciejewski, A.M. Melnick, A. Moliterno, A. Verma
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Nischal, M. Christopeit, B. Will, A. Pardanani, M. McDevitt, J.P. Maciejewski, A. Moliterno, A. Verma
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Nischal, M. Christopeit, Y. Yu, D. Sohal, U. Steidl, A. Verma
Writing, review, and/or revision of the manuscript: M. Christopeit, D. Sohal, A. Pardanani, M. McDevitt, J.P. Maciejewski, A.M. Melnick, U. Steidl, A. Moliterno, A. Verma
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Zhou, T. Bhagat, B. Will, A. Verma
Study supervision: M. Suzuki, J. Greally, U. Steidl, A. Verma

Grant Support

The study was supported by NIH grants R01HL082946 and RO1HL082995, Gabrielle Angel Foundation, Leukemia Lymphoma Society, Hershaft family Foundation, American Cancer Society, German Research Foundation, DFG (CH945/1-1; M. Christopeit), and Immunooncology Training Program T32 CA009173.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received February 24, 2012; revised September 19, 2012; accepted October 8, 2012; published OnlineFirst October 11, 2012.

References

1. Ko M, Huang Y, Jankowska AM, Pape UJ, Tahiliani M, Bandukwala HS, et al. Impaired hydroxylation of 5-methylcytosine in myeloid cancers with mutant TET2. Nature 2010;468:839–43.
2. Tefferi A, Vainchenker W. Myeloproliferative neoplasms: molecular pathophysiology, essential clinical understanding, and treatment strategies. J Clin Oncol 2011;29:573–82.
3. Berkofsky-Fessler W, Buzzai M, Kim MK, Fruchtman S, Najfeld V, Min DJ, et al. Transcriptional profiling of polycythemia vera identifies gene expression patterns both dependent and independent from the action of JAK2V617F. Clin Cancer Res 2011;16:4339–52.
4. Fourouclas N, Li J, Gilby DC, Campbell PJ, Beer PA, Boyd EM, et al. Methylation of the suppressor of cytokine signaling 3 gene (SOCS3) in myeloproliferative disorders. Haematologica 2008;93:1635–44.
5. Bogani C, Ponziani V, Guglielmelli P, Desterke C, Rosti V, Bosi A, et al. Hypermethylation of CXCR4 promoter in CD34+ cells from patients with primary myelofibrosis. Stem Cells 2008;26:1920–30.
6. Alvarez H, Opalinska J, Zhou L, Sohal D, Fazzari M, Yu Y, et al. Widespread hypomethylation occurs early and synergizes with gene amplification during esophageal carcinogenesis. PLoS Genet 2011;7:e1001356.
7. Figueroa ME, Lugthart S, Li Y, Erpelinck-Verschueren C, Deng X, Christos PJ, et al. DNA methylation signatures identify biologically distinct subtypes in acute myeloid leukemia. Cancer Cell 2010;17:13–27.
8. Dawson MA, Bannister AJ, Gottgens B, Foster SD, Bartke T, Green AR, et al. JAK2 phosphorylates histone H3Y41 and excludes HP1alpha from chromatin. Nature 2009;461:819–22.

9. Khulan B, Thompson RF, Ye K, Fazzari MJ, Suzuki M, Stasiek E, et al. Comparative isoschizomer profiling of cytosine methylation: the HELP assay. Genome Res 2006;16:1046–55.
10. Figueroa ME, Reimers M, Thompson RF, Ye K, Li Y, Selzer RR, et al. An integrative genomic and epigenomic approach for the study of transcriptional regulation. PloS One 2008;3:e1882.
11. Figueroa ME, Wouters BJ, Skrabanek L, Glass J, Li Y, Erpelinck-Verschueren CA, et al. Genome-wide epigenetic analysis delineates a biologically distinct immature acute leukemia with myeloid/T-lymphoid features. Blood 2009;113:2795–804.
12. Thompson RF, Reimers M, Khulan B, Gissot M, Richmond TA, Chen Q, et al. An analytical pipeline for genomic representations used for cytosine methylation studies. Bioinformatics 2008;24: 1161–7.
13. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 2005;102:15545–50.
14. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, et al. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet 2000;25:25–9.
15. Quandt K, Frech K, Karas H, Wingender E, Werner T. MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. Nucleic Acids Res 1995;23: 4878–84.
16. Moliterno AR, Williams DM, Rogers O, Isaacs MA, Spivak JL. P0henotypic variability within the JAK2 V617F-positive MPD: roles of progenitor cell and neutrophil allele burdens. Exp Hematol 2008; 36:1480–6.
17. Gondek LP, Tiu R, O'Keefe CL, Sekeres MA, Theil KS, Maciejewski JP. Chromosomal lesions and uniparental disomy detected by SNP arrays in MDS, MDS/MPD, and MDS-derived AML. Blood 2008;111:1534–42.
18. Stein BL, Williams DM, O'Keefe C, Rogers O, Ingersoll RG, Spivak JL, et al. Disruption of the ASXL1 gene is frequent in primary, post-essential thrombocytosis and post-polycythemia vera myelofibrosis, but not essential thrombocytosis or polycythemia vera: analysis of molecular genetics and clinical phenotypes. Haematologica 2011; 96:1462–9.
19. Abdel-Wahab O, Mullally A, Hedvat C, Garcia-Manero G, Patel J, Wadleigh M, et al. Genetic characterization of TET1, TET2, and TET3 alterations in myeloid malignancies. Blood 2009;114:144–7.
20. Li W, Liu M. Distribution of 5-hydroxymethylcytosine in different human tissues. J Nucleic Acids 2011;2011:870726.
21. Oda M, Glass JL, Thompson RF, Mo Y, Olivier EN, Figueroa ME, et al. High-resolution genome-wide cytosine methylation profiling with simultaneous copy number analysis and optimization for limited cell numbers. Nucleic Acids Res 2009;37:3829–39.
22. Karimi M, Johansson S, Stach D, Corcoran M, Grander D, Schalling M, et al. LUMA (LUminometric Methylation Assay)–a high throughput method to the analysis of genomic DNA methylation. Exp Cell Res 2006;312:1989–95.
23. Figueroa ME, Abdel-Wahab O, Lu C, Ward PS, Patel J, Shih A, et al. Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation. Cancer Cell 2010;18:553–67.
24. Perna F, Gurvich N, Hoya-Arias R, Abdel-Wahab O, Levine RL, Asai T, et al. Depletion of L3MBTL1 promotes the erythroid differentiation of human hematopoietic progenitor cells: possible role in 20q- polycythemia vera. Blood 2010;116:2812–21.
25. Shimizu R, Engel JD, Yamamoto M. GATA1-related leukaemias. Nat Rev Cancer 2008;8:279–87.
26. Stegelmann F, Bullinger L, Griesshammer M, Holzmann K, Habdank M, Kuhn S, et al. High-resolution single-nucleotide polymorphism array-profiling in myeloproliferative neoplasms identifies novel genomic aberrations. Haematologica 2010;95:666–9.
27. Boultwood J, Perry J, Pellagatti A, Fernandez-Mercado M, Fernandez-Santamaria C, Calasanz MJ, et al. Frequent mutation of the polycomb-associated gene ASXL1 in the myelodysplastic syndromes and in acute myeloid leukemia. Leukemia 2010;24:1062–5.
28. Jankowska AM, Szpurka H, Tiu RV, Makishima H, Afable M, Huh J, et al. Loss of heterozygosity 4q24 and TET2 mutations associated with myelodysplastic/myeloproliferative neoplasms. Blood 2009;113: 6403–10.
29. Ko M, Huang Y, Jankowska AM, Pape UJ, Tahiliani M, Bandukwala HS, et al. Impaired hydroxylation of 5-methylcytosine in myeloid cancers with mutant TET2. Nature 2010;468:839–43.
30. DeAngelo D, Tefferi A, Fiskus W, Mesa R, Paley C, Wadleigh M, et al. A phase II trial of panobinostat, an orally available deacetylase inhibitor (DACi), in patients with primary myelofibrosis (PMF), post essential thrombocythemia (ET), and post Polycythemia Vera (PV) myelofibrosis [abstract]. In: Proceedings of the 52nd Annual Meeting of ASH; 2010 Dec 4–7; Orlando, FL. Washington (DC): 2011. p. 116. Abstract nr 630.
31. Odenike O, Godwin J, Besien K, Huo K, Sher D, Burke P, et al. Phase II trial of low dose, subcutaneous decitabine in myelofibrosis. [abstract]. In: Proceedings of the 50th Annual Meeting of ASH; 2008 Dec 6–9; San Francisco, CA. Washington (DC): 2008. p. 112. Abstract nr 2809.