Published in final edited form as:

Cancer Res. 2013 February 01; 73(3): 1076–1085. doi:10.1158/0008-5472.CAN-12-0735.

Methylome profiling reveals distinct alterations in phenotypic and mutational subgroups of myeloproliferative neoplasms

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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 compared to essential thrombocythosis (ET) or polycythemia vera (PV), the molecular distinctions between these subgroups are not well elucidated. We performed the HELP (HpaII tiny fragment enriched by LM-PCR) assay to study genome-wide methylation in PV, ET and PMF samples compared with healthy controls. We determined that PV and ET are characterized by aberrant promoter hypermethylation while 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, demonstrating further qualitative differences between the disease subgroups. The differentially methylated genes in PV and ET 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 (NFI), LEF1 and other transcription factors. Within the PMF subgroup, cases with ASXL1 disruptions formed an epigenetically distinct subgroup with relatively increased methylation. Cases of MPNs with TET2 mutations demonstrated 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 demonstrated by sensitivity of MPN derived cell lines to decitabine. These results demonstrate epigenetic differences between PMF and PV/ET and reveal methylomic signatures of ASXL1 and TET2 mutations.

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Author Contributions: SN, LZ, TB, YM, BW, SM performed the experiments, YY, DS, MS performed the bioinformatic analysis, AP, AM, AW contributed samples, MM, AM, LCP, JM, US, JG helped analyze the data, AV analyzed the data and wrote the manuscript

The authors have no conflict of interest to declare.

INTRODUCTION

The myeloproliferative neoplasms (MPN), essential thrombocytosis (ET), polycythemia vera (PV), and primary myelofibrosis (PMF) share the same acquired genetic mutation, Jak2 V617F, 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 Jak2 V617F-positive MPN, variation in gene dosage of Jak2 V617F 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 PV and MF. 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 MPN. More recently, lesions in genes central to epigenetic regulation, TET2 and ASXL1, have been found to be prevalent within the MPN, and in the case of ASXL1, segregate with MF phenotypes, implicating the importance of epigenetic regulation as a novel pathway relevant to MF 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. Single locus studies have shown aberrant methylation of promoters of genes such as SOC1, SOCS3 and CXCR4 can be seen in MPNs, though 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 MPN stratified by disease class, and to determine the effect of JAK2 V617F on the methylome.

MATERIALS AND METHODS

Patient samples and nucleic acid extraction

The study population consisted of 29 MPN patients and controls evaluated at either Johns Hopkins University or Albert Einstein School of Medicine. 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 carried out as previously published (9). Intact DNA of high molecular weight was corroborated by electrophoresis on 1% agarose gel in all cases. One microgram of genomic DNA was digested overnight with either HpaII or MspI (NEB, Ipswich, MA). On the following day the reactions were extracted once with phenol-chloroform and resuspended in 11 µL of 10 mM 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 HpaII and MspI-digested DNA as previously described (9). Both amplified fractions were submitted to Roche-NimbleGen, Inc. (Madison, WI) for labeling and hybridization onto a human hg17 custom-designed oligonucleotide array (50-mers) covering 25,626 HpaII amplifiable fragments (HAF) located at gene promoters. HpaII amplifiable fragments are defined as genomic sequences contained between two flanking HpaII sites found within 200–2,000 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 GEO database for public access (accession number pending).

Quantitative DNA methylation analysis by MassArray Epityping

Validation of HELP microarray findings was carried out by MALDI-TOF mass spectrometry using EpiTyper by MassArray (Sequenom, CA) on bisulphite-converted DNA as previously described (10, 11). MassArray primers were designed to cover the flanking HpaII sites for a given HAF, as well as any other HpaII sites found up to 2,000 bp upstream of the downstream site and up to 2,000 bp downstream of the upstream site, in order 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 HpaII amplifiable fragment were calculated as a robust (25% trimmed) mean of their component probe-level signal intensities. Any fragments found within the level of background MspI 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 biological (*e.g.* genomic deletions and other sequence errors) or experimental cause. On the other hand, “methylated” loci were so designated when the level of HpaII 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 HpaII/MspI ratios are aligned across density-dependent sliding windows of fragment size-sorted data. The $\log_2(\text{HpaII/MspI})$ 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 HpaII/MspI 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 performed using the statistical software R version 2.6.2. A two-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 (hypomethylated versus 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 software (IPA) (Redwood City, CA) we carried out an analysis of the biological information retrieved by each of the individual platforms alone, and compared it to the information obtained by the integrated analysis of all three 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 $p < 0.01$. Biological networks captured by the different microarray platforms were generated using IPA and scored based on 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) (13). Their functional gene sets were obtained from Gene Ontology (GO) (14).

This analysis was performed by Gene Set Enrichment Analysis (GSEA) (13), a computational method that determines whether an a priori defined set of genes (commonly hypermethylated genes in MDS) shows statistically significant, concordant differences between two biological 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, 1000 permutations were applied to sample labels to test if genes from each TFT gene sets were randomly distributed along the differentially methylated gene list.

Mutational and SNP karyotyping analysis—Neutrophil isolation and DNA preparation were performed as previously described(16). The Jak2 V617F neutrophil allele burdens were measured using an allele-specific, quantitative real-time polymerase chain reaction (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 was performed as described previously (17) Briefly, Gene Chip Mapping Affymetrix 250K arrays (Affymetrix, Santa Clara, CA, USA) were used for SNP-K analysis and utilized 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 (CN) and areas of UPD were investigated using a Hidden Markov Model and CN 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, Farmingdale, NY, USA), 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, Sunnyvale, CA, USA). Groups were compared using the Mann-Whitney-U-Test. P values <0.05 were considered statistically significant. Statistics were performed using PASW/ SPSS Version 18 (IBM, Armonk, NY, USA).

Cell lines and viability assay—Cell lines HL60, K562 and HEL were purchased from ATCC (Manassas, VA) and SET-2 was obtained from DSMZ (Braunschweig, Germany). These had been authenticated at ATCC and DSMZ. All cell lines were cultured in RPMI medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat inactivated fetal bovine serum and penicillin (100 U/mL), streptomycin (100 mg/mL) and 4 mM glutamine. Cells were maintained at 37°C and humidified with 95% air and 5% CO₂ for cell culture. For the viability assays the cells were cultured in 0.5, 1 and 5 μM Decitabine (Sigma) for 2 days. Decitabine was added to the culture daily, DMSO was served as control. Viability was measured on day 3 using MTT assay (Promega) according to the manufacturer's instruction.

Luminometric methylation assay (LUMA)—Genomic DNA (200–500 ng) was cleaved with HpaII + EcoRI or MspI + EcoRI in two separate 20 ml reactions containing 33 mM Tris-acetate, 10 mM Mg-acetate, 66 mM K-acetate pH 7.9, 0.1 mg/ml BSA and 5 units of each restriction enzymes. The reactions were set up in a 96-well format and incubated at 37°C for 4 h. Then 20 ml annealing buffer (20 mM Tris-acetate, 2 mM Mg-acetate pH 7.6) was added to the cleavage reactions, and samples were placed in a PSQ96TMMA system (Biotage AB, Uppsala, Sweden). The instrument was programmed to add dNTPs in four consecutive steps including Step 1: dATP (the derivative dATPaS is used since it will not react directly with Luciferase and prevents non-specific 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 HpaII/EcoRI and MspI/EcoRI ratios were calculated as (dGTP + dCTP)/dATP for the respective reactions. The HpaII/MspI ratio was defined as (HpaII/EcoRI)/(MspI/EcoRI) (6).

RESULTS

Genome wide methylation profiling shows PMF as a distinct epigenetic subgroup

We performed the HELP assay to study genome-wide methylation patterns in primary PV, ET and PMF samples. The HELP assay uses differential methylation specific digestion by HpaII and MspI followed by amplification, two 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 >95% purity, and abundant DNA is available. Second, in the MPN, 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 ET, 5 cases of PV and 12 cases of PMF was performed and compared to healthy controls (Table 1). The controls Unsupervised clustering based on global methylation profiles showed that while PV and ET cases were more similar to the normal controls, the PMF cases were epigenetically distinct from these groups. 5 samples of PMF formed a cluster with similar methylation profiles while 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.

PV/ET are characterized by hypermethylated loci that affect important functional pathways

To further analyze the epigenetic differences between these subtypes of MPNs, we performed supervised clustering of PV and ET cases and compared them to controls. We saw that these samples had 141 genes that were uniformly hypermethylated compared to 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) demonstrating potential regulatory disturbances in these pathways. Genes that were significantly hypermethylated included several novel candidates such as transcription factor HNF4alpha, Histone acetyltransferase MYST2, Interleukin 1 and others. (Supp Table 1) Functional pathway analysis revealed that these genes are involved in pathways regulated by the NF-Kb and HNF-4alpha transcription factors (Supp Fig 1).

PMF is characterized by both aberrantly hyper and hypo methylated loci that affect distinct functional pathways

PMF on the other hand was characterized by both aberrantly hyper (n=162) and hypomethylated (n=106) loci when compared to controls (Fig 2B). These changes were further evaluated by the luminometric methylation assay (LUMA), a quantitative assay that interrogates all HpaII sites in the genome (22). LUMA also revealed significantly more hypomethylation in PMF when compared to PV and ET cases (49% mean hypomethylation

in PMF vs. 38% in PV/ET, $p < 0.05$, TTest)(Fig 2C). The findings of the HELP assay were validated by bisulfite Mass Array analysis and showed good correlation (Supp Fig 2A,B,C). Important functional pathways were found to be affected by aberrant methylation in PMF. The pathways affected by hypomethylated genes included cell signaling, hematopoiesis and immunological pathways (Supp Fig 3A) while the pathways affected by hypermethylated genes included those governing inflammatory responses and others (Supp Fig 3B), (Supp Tables 2 and 3 listing all significantly hypermethylated 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 while the hypomethylated genes were enriched for the TFAP2A and NF1 transcription factors (Tables 3,4). Interestingly, the significantly, aberrantly hypomethylated regions in PMF were found to be preferentially located outside of CpG islands (Fig 2D) further demonstrating the unique nature of these changes in PMF.

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

Since 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,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 Supp Tables 4,5) These results reveal that disruption in functioning of chromatin binding protein, ASXL1, are associated by specific methylation patterns in PMF.

MPD 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 in a notable proportion of MPNs (23). Since 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 to controls (Fig 4A,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 (Supp Tables 6,7).

JAK2 mutation does not lead to epigenomic clustering

The JAK2V617F mutation is seen in a large proportion of MPN patients 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 if this mutation led to any effects on the methylome of these patients. Since disease class (PMF vs PV or ET) 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 PV

and ET 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 (Supp Fig 4). Supervised clustering could also not demonstrate any significant gene specific methylation signature in the mutant cases. Since MPN patients 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 demonstrated that MPNs are characterized by aberrant hypermethylation, we next wanted to determine if these cells were sensitive to growth inhibition by DNMT inhibitors. We compared leukemic cell lines that were derived from MPN patients (HEL, SET-2, both have Jak2V617F mutation and HEL has ASXL1 deletion (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 HEL and SET-2 cells at all doses, thus demonstrating functional importance of hypermethylation observed in these MPN derived cell lines.

Discussion

The discovery of the most prevalent genomic lesion specific to the chronic myeloproliferative neoplasms (MPN), JAK2 V617F, has provided important insights into pathogenesis and treatment of these diseases. However even within the JAK2 V617F-positive MPN, variation in disease phenotype and natural history exists, suggesting that epigenetic processes are significant modifiers of disease pathobiology. Our results demonstrate widespread alterations in DNA methylation in MPNs and show that PMF is epigenetically distinct from PV and ET. 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.

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 PV and ET. 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 demonstrate 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 shows 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 has shown that this protein is important for the 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 myelodysplastic patients (23). Our data now demonstrate 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 would be very similar in controls compared to MPN patients, due to the intact myeloid differentiation program in the MPN. Thus any differences between controls and MPN patients, 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 demonstrate that there are numerous significantly and uniformly hypermethylated loci in PV, ET and PMF that may be targeted by epigenetic modifiers in future clinical trials. Epigenetic modifiers such as HDAC inhibitors are being tried in MPNs including both PV 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 MPN 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Supported by NIH R01HL082946, R01HL082995, Gabrielle Angel Foundation, Leukemia Lymphoma Society, Hershaft family Foundation, American Cancer Society, German Research Foundation, DFG (CH945/1-1; MC) and Immunooncology Training Program T32 CA009173.

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Figure 1. Genome wide methylation profiling shows PMF as a distinct epigenetic subgroup of MPNs

Unsupervised hierarchal clustering (1-Pearson correlation distance and Ward’s agglomeration method) based on global DNA methylation obtained from neutrophils showed that PMF cases (Red) formed clusters distinct from PV/ET samples. PV and ET (black) cases were epigenetically more similar to healthy controls (green). Demographics did not reveal any effect on epigenetic clustering. Males are represented by black squares and females by grey squares. Age groups (40–49, 50–59, 60–69 and 70–79) are represented as progressively dark shades of grey. The presence of any uniparental disomy (UPD) and the presence of any chromosomal loss of gain are represented by black squares. TET2, JAK2 and ASXL1 mutation positive cases are represented as black squares.

Figure 2. PV/ET are characterized by aberrant hypermethylation while PMF is characterized by both hypo and hypermethylation loci

A volcano plot is shown demonstrating 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 two tailed T test was used to calculate the p values. Differentially methylated loci with a log fold change in mean methylation are labeled in red (P value<0.05). Significant hypermethylation is seen in PV / ET (A). Both significantly hyper and hypomethylated loci are seen in PMF cases when compared to controls (B). LUMA assay demonstrated relative hypomethylation in PMF cases when compared to PV/ET (Means $\pm$ s.e.m; TTest, P Value <0.05) (C). The genomic position of every HpaII-amplifiable fragment 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 those not overlapping. 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 HpaII fragments in the signature with the relative proportion on the array. Stacking bars are used to illustrate the finding of a significant enrichment for HpaII-amplifiable fragments not overlapping with CpG islands (D).

Figure 3. PMF with ASXL1 mutation/deletion have a distinct epigenetic profile with increased methylation

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 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 value<0.05 are shown in red (A). 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 (B).

Figure 4. TET2 mutations are associated with decreased hydroxymethylation and increased cytosine methylation in MPNs

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 (A). 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 value<0.05 are shown in red (B) To test whether the ratio of hydroxymethylated/ methylated DNA differed between TET2 mutated and wildtype MPN patient samples we performed colorimetric measurements of the abundance of hydroxymethylated and methylated DNA. Median ratio $\pm$ 95% confidence interval using DNA from four patients with wildtype TET2 (left) versus DNA from four patients with mutant TET2 (right) is depicted. Statistical comparison was calculated utilizing the Mann-Whitney-U-Test with P value < 0.05 (C).

Fig 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 $\pm$ s.e.m of 3 independent experiments is shown. (TTest with P value <0.05) (A). Cell lines were treated with different doses of Decitabine (DAC) for 2 days and proliferation was assessed by the MTT assay after 72 hrs. Significant inhibition of growth was seen after treatment in HEL and SET-2 cell lines (Means $\pm$ s.e.m of 3 independent experiments, TTest, P Value <0.05).

Table 1

Demographic and Molecular Features of the MPN Cohort

ID	Sex	Age	Disease duration (years)	Therapy (at time of sample)	% JAK2-V617F	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	Anagrelide	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

Table 2

Transcription Factor binding sites enriched in hypermethylated genes in PV/ET

Transcription Factor	Motif	p value
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

Table 3

Transcription Factor binding sites enriched in hypermethylated genes in PMF

Transcription Factor	Motif	p value
POU6F1	GCATAAWTTAT	1.24E-03
LEF1	SWWCAAAGGG	2.10E-03
GATA	WGATARN	2.65E-03
AP2	MKCCSCNGGCG	7.50E-03
GATA	NGATAAGNMNN	8.81E-03
OCT	TNATTGTCATN	8.81E-03
AHR	KNNKNNTYGCGTGCMS	1.44E-02
MEF2	CTCTAAAATAACYCY	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 value
NF1	NTGGNNNNNGCCAANN	8.84E-04
KLF12	CAGTGGG	1.27E-02
NR6A1	NTCAAGKTCAAGKTCANN	1.85E-02
CDC5	GATTTAACATAA	3.19E-02
TCF3	CAGGTG	3.75E-02
MYOG	NVTNWTGATTGACNACAAVARRBN	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