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Aberrant DNA methylation profile of chronic and transformed classic Philadelphia-negative myeloproliferative neoplasms

Cristina Pérez,[1] Marien Pascual,[1] José Ignacio Martín-Subero,[2] Beatriz Bellosillo,[3] Victor Segura,[4] Eric Delabesse,[5] Sara Álvarez,[6] María José Larrayoz,[7] José Rifón,[8] Juan Cruz Cigudosa,[6] Carles Besses,[9] María José Calasanz,[7] Nicholas C.P. Cross,[10] Felipe Prósper,[1,8] and Xabier Agirre[1]

[1]Laboratory of Myeloproliferative Syndromes, Oncology Area and [4]Department of Bioinformatics, Foundation for Applied Medical Research, University of Navarra, Pamplona, Spain; [2]Department of Anatomic Pathology, Pharmacology and Microbiology, University of Barcelona, Barcelona, Spain; [3]Department of Pathology, Hospital del Mar, Barcelona, Spain; [5]CHU Toulouse, Laboratoire d'Hematologie, Hôpital Purpan, Inserm U1037, University Paul Sabatier, Toulouse, France; [6]Molecular Cytogenetics Group. Centro Nacional Investigaciones Oncológicas (CNIO), Madrid, Spain; [7]Department of Genetics, University of Navarra, Spain; [8]Hematology Service and Area of Cell Therapy, Clínica Universidad de Navarra, University of Navarra, Spain; [9]Department of Hematology, Hospital del Mar, Barcelona, Spain; and [10]Wessex Regional Genetics Laboratory, Salisbury, UK and Faculty of Medicine, University of Southampton, UK

ABSTRACT

Most DNA methylation studies in classic Philadelphia-negative myeloproliferative neoplasms have been performed on a gene-by-gene basis. Therefore, a more comprehensive methylation profiling is needed to study the implications of this epigenetic marker in myeloproliferative neoplasms. Here, we have analyzed 71 chronic (24 polycythemia vera, 23 essential thrombocythemia and 24 primary myelofibrosis) and 13 transformed myeloproliferative neoplasms using genome-wide DNA methylation arrays. The three types of chronic Philadelphia-negative myeloproliferative neoplasms showed a similar aberrant DNA methylation pattern when compared to control samples. Differentially methylated regions were enriched in a gene network centered on the NF-κB pathway, indicating that they may be involved in the pathogenesis of these diseases. In the case of transformed myeloproliferative neoplasms, we detected an increased number of differentially methylated regions with respect to chronic myeloproliferative neoplasms. Interestingly, these genes were enriched in a list of differentially methylated regions in primary acute myeloid leukemia and in a gene network centered around the IFN pathway. Our results suggest that alterations in the DNA methylation landscape play an important role in the pathogenesis and leukemic transformation of myeloproliferative neoplasms. The therapeutic modulation of epigenetically-deregulated pathways may allow us to design targeted therapies for these patients.

Introduction

Polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF) are clonal hematopoietic stem cell disorders, characterized by increased proliferation of terminally differentiated myeloid cells. They have been recently categorized as classic Philadelphia chromosome-negative myeloproliferative neoplasms (MPNs) by the WHO classification of 2008.[1]

In spite of PV, ET and PMF being stem-cell derived clonal diseases, their clonal architecture and hierarchy is complex and not always predictable.[2] The disease course is generally chronic, but some patients show an inherent tendency for transformation into acute leukemia, a major complication with a poor prognosis which is hypothesized to be accompanied by the acquisition of additional genomic lesions.[3] Currently known MPN associated mutations involve *JAK2*,[4] *MPL*,[5] *TET2*,[6] *ASXL1*,[7] *IDH1*, *IDH2*,[8] *CBL*,[9] *IKZF1*,[10] *LNK*,[11] and *EZH2*.[12] Most of these mutations originate at the progenitor cell level but they do not necessarily represent the primary clonogenic event and are not mutually exclusive.[2]

Interestingly, most of the genes mutated in MPNs are genes involved in the regulation of different epigenetic mechanisms. As the epigenetic machinery is one of the main targets of genetic changes in MPNs, it can be hypothesized that the epigenome is altered in these neoplasms. Unlike DNA mutations, which are irreversible changes in the DNA sequence that can result in gene activation or inactivation, epigenetic changes are defined as reversible modifications that affect gene expression without altering the DNA sequence itself.[13] Among all the epigenetic mechanisms, DNA methylation is probably the best characterized. It occurs in cytosines followed by guanines (CpG) and its role in gene regulation is well known.[14] Aberrant DNA methylation patterning has been extensively described during oncogenesis, with the gain of methylation in certain tumor suppressor genes (which would result in gene inactivation) and loss of methylation in some other proto-oncogenic genes. DNA methylation changes have been frequently described in various subtypes of hematologic neoplasms.[15,16] Aberrant DNA methylation profile is also frequent in myeloid malignancies, particularly in myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML).[17]

Regardless of the recent advances in MPN diagnosis and

description of new molecular markers, very little is known about the molecular mechanisms involved in the pathogenesis of MPNs and their leukemia transformation. The identification of new markers for diagnosis and prognosis is needed to identify best molecular targets for therapy. There are currently no epigenetic biomarkers in widespread clinical use and most epigenetic studies in MPNs have focused on the analysis of a few tumor suppressor genes.[16] Therefore, the present study was designed to establish a global DNA methylation profiling of PV, ET and PMF.

Methods

Samples and gene mutation analysis

Bone marrow (BM) aspirates and peripheral blood (PB) samples (n=71) were collected from patients diagnosed with PV (n=24), ET (n=23) or PMF (n=24). For this study we also included samples of 4 patients with PV, 4 patients with ET, and 5 patients with PMF at the time in which the disease had transformed to acute myeloid leukemia (AML). Diagnosis of MPN was made according to the World Health Organization (WHO) classification system of hematologic malignancies.[1] Control samples included BM (n=4) and PB (n=4) from healthy donors. In all the cases, the DNA was isolated from unselected cells. Human samples were drawn after informed consent had been obtained from the patient or the patient's guardians in accordance with the Declaration of Helsinki. This study was approved by the Research Ethics Committee at the University of Navarra, Spain. Samples were analyzed for detection of *JAK2V617F* mutations by ARMS technique and mutations of *TET2* and *EZH2* by direct sequencing as previously described.[18]

DNA methylation profiling and analysis of microarray data

The strategy used for DNA methylation profiling and the bioinformatics analysis of the DNA methylation data of MPN, MPNs transformed to acute myeloid leukemia (MPN-AML) and control samples are described in the *Online Supplementary Design and Methods*.

Results

MPN patients and control samples show a distinct DNA methylation signature

The methylation profile of samples from patients with MPNs was compared with the methylation profile of BM and PB samples from healthy donors. We have previously shown that BM and PB samples are equivalent in terms of methylation profile and can thus be combined.[18]

Unsupervised hierarchical analysis performed on samples obtained from healthy donors (n=8) and patients diagnosed with MPNs (n=71) showed that most of the MPN samples clustered together and separated from healthy controls (*Online Supplementary Figure S1*). Furthermore, when we applied the combination of the three different statistical strategies (t-test, Volcano analysis and methylation threshold analysis) we found 56 CpGs (corresponding to 56 unique genes) that showed a consistent differential DNA methylation between MPNs and control samples (Figure 1 and *Online Supplementary Table S1*). Of the 56 differentially methylated CpGs, 40 CpG probes (71%) were hypermethylated in MPNs (including tumor suppressor genes such as *WT1*) and from these CpGs, 35 (87.5%)

were located in CpG islands. The remaining 16 CpG (28.6%) were hypomethylated in MPNs (including *NCOR2* gene, a transcriptional co-repressor protein with well-established oncogenic roles in prostate or breast cancers);[19] 8 (50%) of them were located in CpG islands. Using a publicly available methylation dataset from GEO with accession number GSE41037 (that contains DNA methylation information from whole blood samples of 394 healthy subjects aged 16-88 years old) we verified that differential methylation of these 56 CpGs is not due to differences in age between our control and MPN sample groups (*data not shown*). Analysis of the DNA methylation levels of two (*GAS2* and *BHMT*) of these 56 genes by pyrosequencing in a new series of 25 chronic MPN patient samples and peripheral blood of healthy donor samples, supports the results obtained in the analysis of the array. Fifty-six percent of the new sample set analyzed showed hypomethylation in *GAS2*, whereas 20% *BHMT* were hypermethylated when compared with the control cohort (*Online Supplementary Figure S2*). As expected, the results confirmed what had previously been seen in the methylation arrays, validating the arrays for further experiments.

To determine whether the three types of MPN analyzed have a specific profile of DNA methylation, we performed a new analysis strategy in which each type of MPN was compared to the control group. The global numbers of differentially methylated CpG probes for this analysis were: 91 probes corresponding to 89 different genes in the case of PV (*Online Supplementary Table S2*), 25 probes corresponding to 24 genes in the case of ET samples (*Online Supplementary Table S3*), and 133 probes corresponding to 122 different genes in the case of PMF samples (*Online Supplementary Table S4*). Again, the differentially methylated CpG probes were predominantly hypermethylated in all neoplasms studied. There were fourteen common genes differentially methylated in the three neoplasms when compared with controls (*Online Supplementary Figure S3A* and *Online Supplementary Table S5*). Interestingly, when we performed the cluster analysis with the common (14 genes, *Online Supplementary Figure S3B*) and uncommon (107 genes: 32 in PV, 4 in ET and 71 in PMF, *Online Supplementary Table S6* and *Online Supplementary Figure S3C*) genes, we did not find a specific DNA methylation profile for each different malignancy. Moreover, the individual comparisons between the three disease entities revealed 12 CpGs differentially methylated between PV and PMF, 22 CpGs between ET and PV, and 61 between PMF and ET. These differences were not sufficient to lead to disease clustering in an unsupervised analysis, confirming the similarity between MPN subgroups in terms of DNA methylation levels (*Online Supplementary Figure S4*).

Taken together, these results suggest that PV, ET and PMF have a distinct DNA methylation profile compared to control samples and are very similar between them, and that this is true when comparing all the neoplasms together against the controls as well as each neoplasm against the control group. Furthermore, most of the differentially methylated genes are hypermethylated and map to CpG islands (72% in PV, 86% in ET, 83% in PMF).

Differentially methylated genes in MPNs are implicated in specific biological pathways

As shown in the Venn diagram in *Online Supplementary Figure S3*, a total of 164 genes were differentially methylated in PV, ET and PMF with respect to control samples.

Ingenuity pathway analysis of these genes identified a particular gene network of interest: cell-to-cell signaling and interaction, hematologic system development and function, immune cell, involving NF-κB complex (*Online Supplementary Figure S5*). NF-κB has been identified as a key mediator of inflammation-induced carcinogenesis and the development of progressive myeloproliferative diseases.[20] Our study identifies some genes known to indirectly activate NF-κB such as *CLEC7A*[21] or *EDARDD*,[22] both hypomethylated, and others that are involved in apoptosis and cancer progression, like *NCOR2* (hypomethyalted, up-regulated in some cancers)[19] or *FASLG* (which triggers apoptosis if hypermethylated).[23] Ingenuity functional analysis revealed a set of 37 genes (*P*=3.22E-05 to 1.48E-02) associated with inflammatory disease, among which we can find *RUNX3*, which is hypermethylated, and known to be preventive for bowel inflammatory disease,[24] or IL3 which is hypomethylated and associated with inflammatory responses.[25] Gene ontology (GO) analysis showed no enrichment of any interesting biological function. The bioinformatic analysis of transcription factor binding sites revealed that differentially methylated genes in chronic MPNs were significantly enriched in GATA1, SP1 and other transcription factors that play important roles in normal and malignant hematopoiesis (*Online Supplementary Table S7*).

Currently known MPN associated mutations involve some important epigenetic enzymes, like *JAK2, TET2* and *EZH2*. Therefore, we hypothesized that mutations in those genes could be implicated in some of the epigenetic deregulation observed in chronic MPNs. The distribution of MPN samples and mutations analyzed in *JAK2, TET2* and *EZH2* genes is detailed in *Online Supplementary Table S8*. The clustering analysis (Figure 1) and supervised analysis between chronic MPNs with JAK2 mutated in homozygous state and chronic MPNs with wild-type *JAK2* (*data not shown*) revealed no clear relationship between *JAK2* mutations and the DNA methylation profile. On the other hand, differences in methylation of the *JAK2* promoter were not related to the differential DNA methylation patterns observed in patients with chronic MPNs. A β value between 0.3 and 0.7 was found in 7 healthy donor samples and 37 MPN samples, a β value over 0.7 in 5 MPNs, and 29 MPN samples with a methylation β value below 0.3. *TET2* (studied in all chronic MPN samples) and *EZH2* (only analyzed in chronic PMF samples) were found to be mutated in a very low number of chronic MPN patients in our sample cohort, precluding any further meaningful analysis.

Methylation profiling of MPNs transformed into AML shows higher levels of methylation than MPNs in chronic phase

The role of DNA methylation in leukemic transformation was assessed by comparing the methylation profile of 13 MPNs that had transformed into AML with the 71
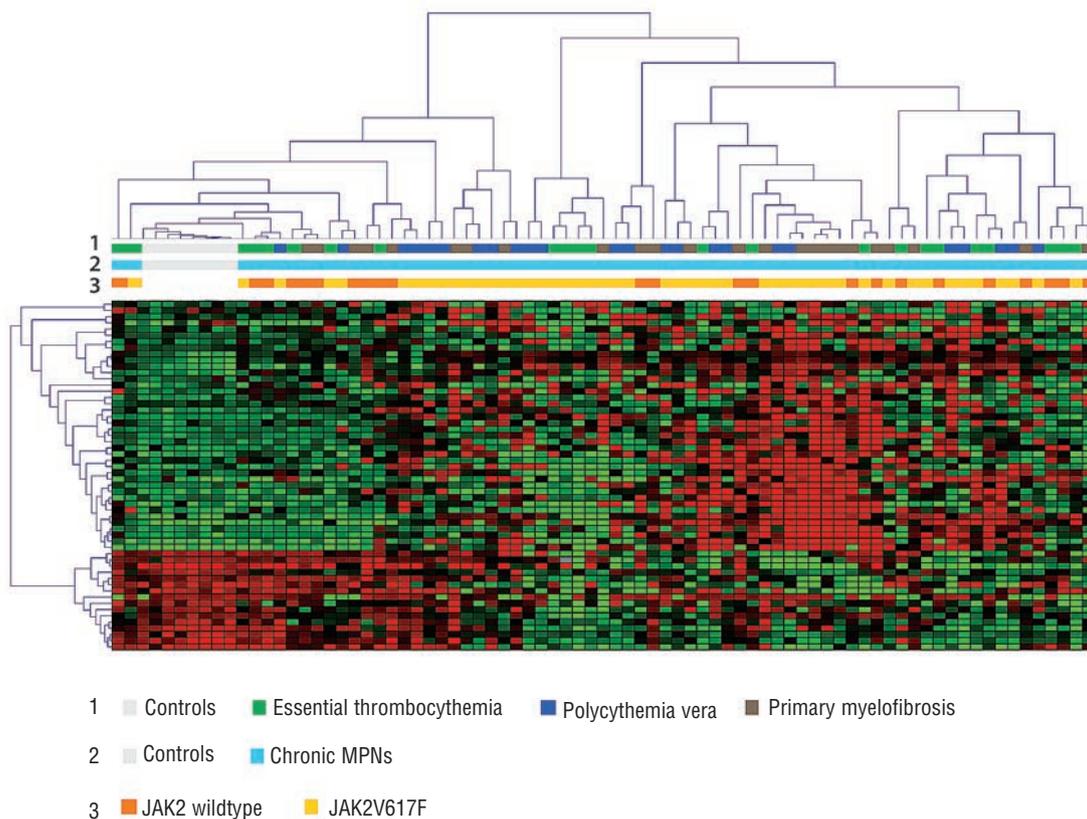
Figure 1. Hierarchical cluster analysis based on differentially methylated genes in MPNs samples in comparison with healthy donor samples. β values are depicted using a pseudocolor scale. Red: hypermethylated genes; Green: unmethylated genes. The top bar beneath the dendrogram indicates PV, ET, PMF sample type in a specific color-code. Second bar indicates MPNs and healthy donor samples. Third bar indicates MPNs samples with or without *JAK2V617F* mutation.

chronic MPNs. Samples were obtained at the time of transformation.

An unsupervised hierarchical correlation cluster analysis was performed using all the probes on the array (except those located on sex chromosomes), including transformed MPNs, chronic MPNs and control samples (*Online Supplementary Figure S6*). Subsequently, to investigate the differences between MPNs and their leukemic transformation we used a combination of two different strategies: t-test and volcano analysis. Due to the small number of samples in transformation, in this analysis we do not use the methylation threshold analysis. We found 172 differentially methylated CpGs corresponding to 159 unique genes between chronic phase MPNs and their transformed

state MPN samples (Figure 2 and *Online Supplementary Table S9*). Although GSEA analysis revealed that differentially methylated genes in chronic MPN were significantly enriched (*P*<0.001) among this gene set (*Online Supplementary Figure S7A*), transformed MPN included a larger number of differentially methylated genes. A total of 165 CpG probes (96%) where hypermethylated in transformed MPNs, and from these CpGs, 73 (44%) were located in CpG islands. In contrast, only 7 CpG probes (4%) were hypomethylated in MPNs (including genes like *TM4SF1*, which encodes for a cell surface antigen and is highly expressed in different carcinomas).[26] Only 2 (29%) of them were located in CpG islands.

Next, independent comparisons of each type of chronic

1. ▫ Controls ▪ Essential thrombocythemia ▪ Polycythemia vera ▪ Primary myelofibrosis
▪ Transformed essential thrombocythemia ▪ Transformed polycythemia vera ▪ Transformed primary myelofibrosis

2. ▫ Controls ▪ Chronic MPNs ▪ Transformed MPs

Figure 2. Hierarchical cluster analysis based on differentially methylated genes in MPNs samples transformed to acute leukemia in comparison with MPNs in chronic phase. β values are depicted using a pseudocolor scale. Red: hypermethylated genes; Green: hypomethylated genes. The top bar beneath the dendrogram depicts control samples, PV, ET, PMF samples or their transformed state in a specific color-code. Second bar indicates controls, chronic MPNs or transformed MPN samples.

MPN with its transformed phase (e.g. samples of chronic PV with respect to PV samples in transformation phase) were made using the two above-mentioned strategies. The overall numbers of differentially methylated CpG probes for this analysis were 373 probes corresponding to 353 different genes in the case of transformed PV samples (*Online Supplementary Table S10*), 419 probes corresponding to 380 different genes in the case of transformed ET samples (*Online Supplementary Table S11*), and 58 probes corresponding to 56 different genes in the case of transformed PMF samples (*Online Supplementary Table S12*). The differentially methylated probes were again predominantly hypermethylated and mapped to CpG islands in all transformed neoplasms studied.

We found 18 common genes differentially methylated in the transformed MPNs when compared with the chronic phase (*Online Supplementary Figure S8A* and *Online Supplementary Table S13*). Interestingly, when we performed the cluster analysis with the common (18 genes, *Online Supplementary Figure S8B*) and uncommon (463 genes, *Online Supplementary Table S14* and *Online Supplementary Figure S8B* and *C*, respectively) genes, we did find a specific DNA methylation profile distinguishing the transformed and the chronic phase of the disease. However, the methylation differences between the different subtypes of MPNs transformed into AML were subtle, and the MPN subtypes transformed into AML were clustered together.

When we compared the methylation profile of the transformed MPN with publicly available methylation data from a total of 89 cytogenetically normal AML (CN-AML) samples[27] we found a statistically significant enrichment of genes differentially methylated in the transformed MPNs among the gene set of differentially methylated genes in samples from patients with primary acute myeloid leukemia (*Online Supplementary Figure S7B*).

Differentially methylated genes in transformed MPNs are implicated in specific biological pathways

A total of 628 different genes (corresponding to 862 different CpG probes, coming from both the hierarchical cluster and the Venn diagram representations, *Online Supplementary Figure S8*) that were differentially methylated between transformed and chronic MPNs were included in the analysis. Ingenuity pathway analysis of these genes revealed a particular gene network of interest: cell-to-cell signaling and interaction, hematologic system development and function, and inflammatory response (*Online Supplementary Figure S9*). Functional analysis showed that 64 genes were involved in inflammatory response (P=3.72E-10 to 9.34E-03) and 47 in immunological disease (P=7.81E-07 to 7.98E-03). Among the genes that are involved in these networks and those two functional routes we found two cytokines, IL23 and IL27 (both hypermethylated in our dataset). IL23 has potent anti-tumor and anti-metastatic effects[28] and is known to induce the production of IFN gamma.[29] In the same way, IL27 has been shown to induce the expression of IFN gamma, which in turn modulates the expression of other molecules involved in the inhibition of tumor growth and metastasis.[30] We also found hypermethylated AIM2 which is thought to mediate reduction of cell proliferation by cell cycle arrest in some types of cancer.[31]

Gene ontology analysis showed that genes differentially methylated between transformed and chronic phases of MPNs are implicated in immune and inflammatory response, negative regulation of development process, negative regulation of lymphocyte activation, negative regulation of mononuclear cell proliferation, negative regulation of T-cell proliferation, positive regulation of cellular process, regulation of cell differentiation, and regulation of leukocyte proliferation. The bioinformatic analysis of transcription factor binding sites revealed that differentially methylated genes in transformed MPNs with respect to chronic MPNs were significantly enriched in LMO2, LEF1 and other transcription factors that play important roles in normal and malignant hematopoiesis (*Online Supplementary Table S7*).

Discussion

Myeloproliferative neoplasms are clonal malignant diseases that arise from the transformation of hematopoietic stem cells/progenitors (HSCs/HPs) and are characterized by overproduction of mature erythrocytes, granulocytes and megakaryocytes. A single point mutation in the tyrosine kinase *JAK2* (*JAK2V617F*), present in approximately 95% of patients with PV and 50-60% of patients with ET and PMF, is considered to be an important driver of these diseases and has become a formal component of diagnostic criteria. However, this mutation does not explain the differences between MPNs, indicating that initiation event or phenotypic differences between these three hematologic diseases could be influenced by additional unknown factors.[2]

Because several genes involved in the regulation of epigenetic mechanisms have been described as being altered in MPNs, we hypothesized that MPN might show differences in the epigenetic landscape (in terms of DNA methylation) both at diagnosis and after their transformation to acute leukemia. The main findings of our study suggest that abnormal methylation profile is not particularly prevalent in MPN and only during transformation to acute leukemia are significant changes in the methylation profile observed. This is consistent with other studies that indicate that disease progression is associated with abnormal methylation.[32-34] Unlike the previous study by Barrio *et al*.,[35] we found some differences in the methylation profile between MPNs and healthy donor samples consistent with a recent work[36] that also reported a significant number of aberrantly methylated genes in MPNs. On the other hand, we did not find a differential methylation pattern between PMF and PV/ET. Some of these differences may be due to differences in sample collection, array coverage and design or data analysis. A current limitation of our study was the use of unfractionated BM or PB that may skew the results of DNA methylation. As clonal involvement both of the myeloid as well as the lymphoid lineage have been clearly demonstrated in MPNs, we believe that the use of unfractionated BM or PB samples is suitable to analyze DNA methylation. Nevertheless, confirmatory studies using unfractionated samples and preferably purified cells could be useful to clarify the validity of this approach.

Regarding the involvement of genes implicated in regulation of epigenetic mechanisms, in our study *JAK2* mutational analysis did not show a clear influence in the DNA methylation profile and the limited sample number of chronic MPN patients with *TET2* or *EZH2* lesions precluded any further analysis. However, as has been shown,

other mutations such as *ASXL1* could influence the DNA methylation profile of these MPN diseases.[36] Whether studies including larger sets of samples and complete methylome analysis would be able to implicate inappropriate DNA methylation in the pathogenesis of MPNs remains uncertain. Although the number of differentially methylated genes reported in our study is low compared with other hematologic malignancies, such as acute lymphoblastic leukemia,[37,38] or other MPNs, such as CMML,[18] our results indicate that aberrant DNA methylation may contribute to the pathogenesis of classic Philadelphia chromosome-negative MPNs mainly in the transformation to acute leukemia.

Despite the limited number of DMR found in MPN, our results suggest that aberrantly methylated genes in MPNs are involved in important signal transduction pathways such as NF-κB associated networks (*Online Supplementary Figure S5*). This was not only indicated by the ingenuity pathway analysis of our samples but was also supported when we applied the same analysis to publicly available expression data[39] (P=3.02E-02). Deregulation of NF-κB signaling cascade has been described in myeloid and lymphoid neoplasias, correlating its degree of activation with the risk of progression from MDS to AML.[40] Beside the NF-κB pathway, in agreement with a recent report,[36] in our methylated genes data set we found an enrichment of binding sites for transcription factors such as GATA1 or SP1 that are altered in hematologic malignancies.[41] These results suggest that the inappropriate methylation of CpGs may prevent proper binding of these transcription factors, indirectly altering their participation in the transcription of necessary genes.

Differences in networks affected by aberrant methylation between chronic phase and transformed MPNs were clearly identified, the INF pathway being one of them. IFNs are normally present at low levels in plasma, which presumably plays an antiviral and antitumor surveillance role.[42,43] IFN promotes apoptosis of a variety of tumor cell types[44,45] and it has anti-angiogenic properties.[44,46] Hence, this network could be deregulated since several genes related to IFN are hypermethylated like IL23[28] and IL27,[47] which could contribute to the pathogenesis of transformed MPNs. It is interesting that IFN has been used with considerable success in PV, with normalization of blood counts and achievement of molecular remission in many cases.[44] Moreover, GO analysis showed that these genes were implicated in the immune and inflammatory response. This observation is in accordance with the fact that MPNs are characterized by a state of chronic inflammation, proposed as the common denominator for the clinical evolution and secondary cancer in patients with MPNs.[48] Transcription factors such as LMO2 were also identified as aberrantly methylated in transformed samples.[49]

In conclusion, our results indicate that: 1) the three types of MPNs analyzed in our study show a distinct DNA methylation signature from healthy donors, but the same pattern of aberrant DNA methylation among pathologies, and, therefore, changes in DNA methylation, can not explain the differences in phenotype of these three entities; 2) genes differentially methylated in MPNs could be involved in the pathogenesis of these diseases, for example, by deregulating the NF-κB pathway; 3) MPNs transformed to acute leukemia have an increased number of differentially methylated genes compared to MPNs in chronic phase, and these genes overlap with those seen in primary AML; and 4) differential DNA methylation of IFN pathway genes may play a role in disease progression. Functional studies will be required to clarify the role of DNA methylation in the deregulation of these pathways in MPNs to determine if targeting of these pathways or the use of epigenetic drugs may help prevent disease progression.

Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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