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InIntegration of Genomic Technologies Implicates Novel Genes

in Philadelphia Negative Myeloproliferative Neoplasms

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Abstract of the Dissertation

Integration of Genomic Technologies Implicates Novel Genes in Philadelphia Chromosome Negative Myeloproliferative Neoplasms

by JOSEPH M. TRIPODI, M.S.

Myeloproliferative neoplasms (MPN) are a group of clonal hematopoietic stem cell cancers with overlapping laboratory, cytogenetic, molecular, and clinical features. According to the World Health Organization (WHO, 2016), there are three classic Philadelphia chromosome negative MPN (Ph- MPN) entities: polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF).

Currently, the genomic characterization of these diseases is based on conventional cytogenetics and the mutational status of three driver genes (*JAK2*, *CALR*, and *MPL*). Although other non-driver mutations may be present in up to 40% of patients, their clinical and prognostic relevance, at present, is not entirely clear. Cytogenetic abnormalities are absent in 90% of patients with ET, 70% of patients with PV, and 50% of patients with PMF. Moreover, up to 10% of patients do not harbor mutations in any of the three driver genes. Chromosomal microarray (CMA) abnormalities have been identified in ~80% of MPN patients but the application of this technology for prognostic clinical purposes has not been widely used. The goal of this dissertation was to gain additional insight into the genetic basis of these diseases by integrating three genomic technologies; conventional cytogenetics, next-generation sequencing (NGS), and CMA, each with its advantages and limitations. To achieve this goal, three groups of patients were investigated: (1) patients

with advanced forms of Ph- MPN who required therapeutic splenectomy, (2) patients that progressed from the chronic form to more advanced forms of Ph- MPN that were associated with specific chromosomal abnormalities, and (3) cytogenetically normal patients with Ph- MPN that represent early stages of their disease.

The hypothesis in aim 1 was to determine whether the spleens of patients with advanced forms of Ph- MPN, such as myelofibrosis requiring therapeutic splenectomy, contain hematopoietic stem cells which harbor unique genomic aberrations that contribute to disease progression. The results of the integrated analysis showed a high concordance of genomic lesions between spleen and bone marrow cells suggesting a similar clonal architecture between these two tissues. In spite of the overall similarity, CMA identified additional novel genomic changes present in the spleen indicating that there are genomic differences between myelofibrosis hematopoietic cells that reside in the spleen and bone marrow. CMA results demonstrated for the first time recurrent gains within four genes: *RHOC*, *RASA3*, *NCOR2*, and *TAF15*, strongly suggesting higher genomic complexity of spleen cells. These candidate genes may play a role in the pathogenesis PMF and support the hypothesis that integrating genomic technologies is a valuable tool in the discovery of new genes that may be implicated in the genetic landscape of myelofibrosis.

Based on the above novel observations, it was postulated in aim 2 that application of this strategy may provide the molecular understanding of recurrent chromosomal abnormalities such as the gain of chromosome 1q and rearrangements of 12q that are present in patients with Ph- MPN who progress to advanced forms of the disease. Results from aim 2 showed

that recurrent gain of 1q, identified in 6% (n=72) of 1,294 evaluated patients with MPN was accompanied by an increase in transcript levels of MDM4. Single cell assays demonstrated that gain of 1q was present in CD34+ cells. Further analysis also identified candidate genes among patients (n=24) with recurrent chromosome 12q rearrangements (*MDM2*, *HMGA2*, and *WIF1*). The combined application of both cytogenetic and CMA identified large and cryptic deletions of *TP53*, a known tumor suppressor gene. These observations strongly link, for the first time, recurrent chromosomal abnormalities and an increased level of MDM4 transcripts with perturbations of the *TP53* pathway.

Finally, in aim 3, a set of nine genes implicated in the advanced forms of Ph- MPNs (*RASA3*, *NCOR2*, *RHOC*, *TAF15*, *MDM4*, *MDM2*, *TP53*, *HMGA2*, and *WIF1*), were validated to determine whether they are also present in patients who were cytogenetically normal. Integrated analysis of 184 patients with Ph- MPN yielded informative results in 96.1% indicating that it is possible to characterize, at the genomic level, the majority of MPN patients at diagnosis. Moreover, genomic changes in the nine gene panel did occur in patients who were karyotypically normal, although the frequency was higher in the cytogenetically abnormal patients. Among the cytogenetically normal patients, *MDM4* was a sole CMA abnormality but when compared to the cytogenetically abnormal patients *MDM4* co-occurred with six other genes. Aberrations involving *TP53* occurred only in the cytogenetically abnormal patients and did not co-occur with *MDM4*. These findings suggest that abnormalities involving *MDM4* may be an early genomic event in patients with PV and ET and may represent an alternative route to *TP53* dysregulation.

The results and observations provided in this dissertation strongly suggest that the integration of these technologies provide novel insight into the genomics of patients with Ph- MPN that could not be achieved if each methodology was performed individually. The results also show that 96% of patients with Ph- MPN acquire different lesions, such as mutations, large structural and numerical aberrations, and for the first time intragenic as well as whole gene gains and losses of chromosomal regions. These lesions may be also detected in patients who are chromosomally normal and do not have driver gene mutations. The implication of this approach may be beneficial for both clinical and research purposes.

In conclusion, genomic lesions in Ph- MPNs may be present in different genes in different patients affecting the same pathway. Since one method alone cannot uncover the diversity of genomic lesions, to fully characterize these patients an integrated approach utilizing various testing modalities should be employed to fully understand the interplay of these genes in the pathogenesis of these neoplasms.

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As a cancer survivor myself, I understand that I would not be here if it wasn't for the countless scientists that came before me. It was their work that has allowed me to make my small contribution to cancer research which hopefully may one day help others. I also want to extend my thanks to the patients and their families, who endure so much, and without them this dissertation work would not be possible.

I want to thank the most important person in my life, my beloved Natalya. I am blessed to have her in my life and without her support and love this dissertation would not be possible. Finally, special thanks for my children, Matthew and Alexandra. It was not easy to simultaneously work full time and pursue a doctoral degree and while the long days and countless weekends working were enervating, their smiles and laughter always recharged my soul.

The work presented in chapters two and three has been previously published.

1. Zimran E*, Tripodi J*, Rampal R, Rapaport F, Zirkiev S, Hoffman R, Najfeld V. Genomic characterization of spleens in patients with myelofibrosis. Haematologica. 2018 Oct;103(10):e446-e449. *Contributed equally to this publication

2. Marcellino BK, Hoffman R, Tripodi J[&], Lu M, Kosiorek H, Mascarenhas J, Rampal RK, Dueck A, Najfeld V. Advanced forms of MPNs are accompanied by chromosomal abnormalities that lead to dysregulation of TP53. Blood Adv. 2018 Dec 26;2(24):3581–9. [&]performed cytogenomic analysis and aided in manuscript preparation

Dedication

To my grandparents, Francesca and Joseph Calabro, who are no longer with us, Their love of family and their work ethic instilled in my parents, Domenica and Joseph Tripodi, has molded who they are, and subsequently who I am. Their memory and the love of my parents will continue to guide and motivate me for all of my life.

Table of Contents

List of Tables

List of Figures

Chapter 1: Introduction

1.1 History of Myeloproliferative Neoplasms

The 2016 World Health Organization (WHO) classifies the myeloproliferative neoplasms (MPNs) into seven clinical entities: chronic myelogenous leukemia (CML), characterized by the Philadelphia chromosome; the Philadelphia negative (Ph-) MPNs, polycythemia vera (PV), essential thrombocythemia (ET), primary myelofibrosis (PMF); chronic neutrophilic leukemia (CNL); chronic eosinophilic leukemia, not otherwise specified (CEL,NOS); and myeloproliferative neoplasms, unclassifiable (MPNu).[1]

The first patient with CML, described by John Hughes Bennett in 1845, was ill for 1.5 to 2 years and his condition progressively worsened with increasing weakness, bleeding, and other problems.[2] Autopsy results showed an enlarged spleen and an increased white cell count in his blood.[2] Later, in 1847, Rudolph Virchow described this condition as "Weisses Blut" and proposed the term "leukaemie."[3] Besides having an increased number of white blood cells and an enlarged spleen, an additional clinical characteristic of patients with MPNs is an increase in the number of red blood cells (erythrocytosis). Polycythemia vera, one of three classic MPNs, is characterized by erythrocytosis. Although the clinical phenotype of PV (plethora, engorged veins) was first recognized by Hippocrates, it wasn't until 1892 when Luis Henry Vaquez published a detailed description of PV in a patient with marked erythrocytosis and hepatosplenomegaly and he postulated that PV occurs as the result of abnormal hematopoietic cell proliferation.[4] Subsequently, in 1903, William Osler reported four additional cases with erythrocytosis and splenomegaly, which he recognized as a new clinical entity.[5] Around the same time, the first description of myelofibrosis was reported in 1879 by Gustav Heuck.[6] Heuck, a

German physician, noted the presence of bone marrow fibrosis and extramedullary hematopoiesis in patients with PMF.[6] Heuck recognized that the findings in his two patients differed from those described for CML because of the presence of fibrosis in the bone marrow and extensive extramedullary hematopoiesis (EMH) (liver and spleen).[6] Finally, essential thrombocythemia was the last of the classic MPNs to be formally described. It was initially reported as 'hemorrhagic thrombocythemia' in 1934 by Emil Epstein and Alfred Goedel.[7] Similar cases of thrombocythemia associated with bone marrow megakaryocytic hyperplasia, splenomegaly, thrombosis or hemorrhage were subsequently reported in the literature under different names.[7] In a comprehensive review of these cases, a distinction between idiopathic and secondary thrombocythemia was made in 1954 and it was suggested that the former is a manifestation of a primary bone marrow proliferative disorder and was distinct from both PV and MF.[8]

These three different disorders had been recognized as distinct clinical entities and were subsequently unified as a set of phenotypically related "myeloproliferative disorders" in 1951 by William Dameshek.[9] He noted that although erythrocytosis is characteristic of PV, many PV patients have "pancytosis" with proliferation of the erythroid, megakaryocyte, and granulocyte lineages. Furthermore, Damashek recognized that patients with PV often develop bone marrow fibrosis and increasing splenomegaly consistent with progression to myelofibrosis. Dameshek argued that, given the difficulties in distinguishing between ET, PV, PMF, and other MPNs, they should be considered as "closely interrelated" disorders characterized by bone marrow proliferation, "perhaps due to a hitherto undiscovered stimulus."[9]

Damashek also included CML under the rubric of MPN because it is characterized by hyperproliferation of the granulocytic lineage, but the history of CML has been unique. This remarkable history started with the observation by Peter Nowell and David Hungerford that an abnormally short chromosome was present in the bone marrow cells of two patients with CML.[10] This minute chromosome became known as the Philadelphia chromosome (Ph), named after the city in which it was discovered. Janet Rowley, using novel G-banding staining methods, further characterized the Ph chromosome as the result of a balanced translocation between chromosomes 9 and 22.[11] Ten years later, it was demonstrated that at the translocation breakpoints of chromosomes 9 and 22, [t(9;22)(q34;q21.2)], parts of two genes, BCR on chromosome 22 and ABL1 on chromosome 9 are fused together to produce a hybrid gene named *BCR/ABL1*.[12] The *BCR/ABL1* hybrid gene was shown to constitutively express the *ABL1* tyrosine kinase.[13] Finally, a tyrosine kinase inhibitor was developed in 2001 to specifically inhibit *ABL1* activity.[14] These discoveries have changed the clinical course of patients with CML from a 6% survival rate before 1975 up to 87% since 2001.[15] CML is now regarded as a separate clinical entity among myeloproliferative disorders. These studies in CML had set the stage for investigating the molecular and genetic basis of hematological malignancies. Although the *BCR/ABL1* story is considered the prime example of molecular medicine at its best, cancer genomics and specifically the genomics of Ph- MPNs is rather complex.

The classical myeloproliferative neoplasms, renamed by the WHO as Philadelphia chromosome negative MPNs (Ph- MPNs), are characterized by excessive production of terminally differentiated blood cells that are fully functional. They have been classified

into 3 entities: polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF) and are the focus of this dissertation.

The biology of these diseases began to be elucidated in 1975 with the observations made by Prchal and Axelrad. They showed that *in vitro* culture of bone marrow cells from patients with PV, but not from normal volunteers, can give rise to erythroid colonies in the absence of exogenous cytokines such as erythropoietin.[16] Endogenous erythroid colony (EEC) formation was also observed in a subset of patients with ET and PMF, "consistent with the clinical and pathogenetic overlap of these 3 disorders."[16] This pivotal discovery supported the notion that these diseases originate from hematopoietic stem cells (HSC).

Definitive evidence of the clonal and stem cell origin of MPNs was provided by the elegant work of Adamson, Fialkow, and colleagues.[17,18] They used the naturally occurring mosaicism created by X-chromosome inactivation in females who are heterozygous for X-linked genes. Specifically they used "X-linked glucose-6- phosphate dehydrogenase (G6PD) as a marker of clonality, based on the Lyon hypothesis, which asserts that early in embryogenesis, one X chromosome in females is inactivated in somatic cells."[19,20] The choice of maternal versus paternal X-chromosome inactivation is random; however, once it occurs, it is maintained in all daughter cells. Normal human females who are heterozygous for the G6PD variant A and B have two mosaic cell populations which are distinguished by electrophoretic mobility.[20] In 1976, this extrinsic marker of clonality was used to determine the cellular origin of PV. It was hypothesized that tumor cells that arise from a single cell in female patients who are heterozygous for G6PD would express a single isoenzyme type (either A or B), but a neoplasm originating from multiple cells would express both iso-enzyme types (A and B). These pivotal studies demonstrated that

circulating RBCs, granulocytes, and platelets obtained from female patients with PV who were heterozygous for G6PD expressed a single isoenzyme, but the skin and cultured BM fibroblasts obtained from these same patients demonstrate both isoenzymes. They concluded that the origin of PV is characterized by "a clonal proliferation of neoplastic HSCs and was not multicellular in origin or the consequence of excessive proliferation of normal HSCs."[17,18] These results demonstrated that peripheral blood cells of patients with PV are clonal in origin, and that PV originates at the level of the hematopoietic stem cell.

1.2 Chromosomal Abnormalities in Ph- MPNs

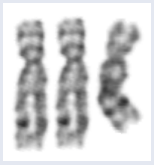
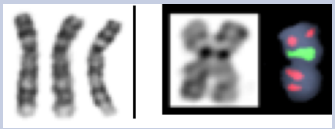
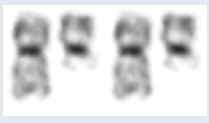
A few decades after the terms "chromosome" and "mitosis" were first introduced, the biologist Theodore Boveri, in 1902, conjectured about the role of somatic genetic alterations in the development of cancer. His central hypothesis — "mammalian tumors might be initiated by mitotic abnormalities that resulted in a change in the number of chromosomes in a cell (aneuploidy)" — was based on his observation that mitotic abnormalities in sea urchins often led to the abnormal development of the organism. He was first to speculate that "malignant tumors might be the consequence of a certain abnormal chromosome constitution." With the discovery of the Philadelphia chromosome in 1960, followed by Janet Rowley's observation in 1973 that the Ph chromosome was the result of a balanced translocation between chromosomes 9 and 22, cytogenetics has changed our understanding of cancer.[10,11] In the last 5 decades cytogenetic studies have documented the association between acquired chromosomal abnormalities with different cancers and became the "gold standard" for the diagnosis and prognosis of hematological malignancies. For example, early studies have shown that translocations of chromosomes 8 and 21 are associated with acute myelogenous leukemia (originally associated with M2 FAB subtype) and translocations between chromosomes 15 and 17 to be associated with promyelocytic leukemia.[21] By the mid 1980s, many of these abnormalities had begun to be molecularly characterized, revealing that translocations create 'fusion' proteins that drive cell growth. We now know that Theodor Boveri was correct and that the current understanding is that the progressive accumulation of genomic changes plays an essential role in the pathogenesis of cancer development and progression.

Unlike CML where the Philadelphia chromosome is thought to be the initiating event, the role of cytogenetic abnormalities in the pathobiology of the Ph- MPNs remain uncertain. As shown in **Table 1.1**, the Ph- MPNs share many recurrent chromosomal abnormalities which are also observed in other myeloid malignances. Among patients with PV, approximately 23%-28% have clonal chromosomal abnormalities at diagnosis. The most frequent chromosomal abnormalities associated with PV include interstitial deletions of chromosome 20, gain of chromosome 9, gain of chromosome 8 and simultaneous gain of both chromosomes 8 and 9, gain of derivative(9)t(1;9) (resulting in three copies of the long arms of chromosome 1 and three copies of the short arms of chromosome 9), and interstitial deletion of chromosome 13. Balanced translocations are rarely observed in patients with PV.

The majority of patients with ET are chromosomally normal at diagnosis and specific chromosome abnormalities associated with ET have not been described. Abnormalities observed in PV and PMF, as well as in myelodysplastic syndrome (MDS), such as trisomy 1q, deletions of 5q, 13q, and 20q, and trisomy 8 have been reported.[22]

Up to 50% of patients with PMF have chromosomal abnormalities at diagnosis, the highest rate among the MPNs. Deletions of the long arms of chromosomes 13 and 20, trisomy 8, and abnormalities of chromosomes 1, 7, and 9 constitute more than 80% of all chromosomal abnormalities detected in PMF. None of these lesions are specific for PMF because they are also detected in PV, ET, MDS, and other myeloid malignancies. At the diagnosis of MPN the most common cytogenetic lesion is a gain or loss of genetic material rather than balanced translocations resulting in a hybrid fusion gene, which is a hallmark of acute myeloid leukemia.

Table 1.1 Principal recurrent chromosome abnormalities in Ph- MPN

		Approximate Prevalence of Clonal Chromosome Abnormalities		
		PV (23-28%)	ET (8-12%)	PMF (~50%)
Trisomy 1q		4-6%	16%	7-19%
del(5)(q21~q34)		7-10%	1-2%	4-5%
del(7)(q21~36)		3-9%	Rare	5-15%
Trisomy 8		13-20%	15%	11-15%
Trisomy 9/ trisomy 9p		16-27%	1%	13-21%
12q rearrangements		Rare	Rare	6-15%
del(13)(q13~q21)		5-13%	10%	13-42%
del(20)(q11.2q~13.3)		16-25%	30%	6-20%

Note. Adapted from “Myeloproliferative disorders: biology and management” by Silver R.T. and Tefferi A., 2007, p. 54 Boca Raton, FL, CRC Press

With disease progression from PV/ET to myelofibrosis, the frequency of cytogenetic abnormalities increases to 70%-90%. The most frequent abnormality associated with disease progression is the gain of the long arms of chromosome 1 and the short arms of chromosome 9.[23,24] Sub-clonal evolution is frequent and may become very complex. The number of genomic alterations is more than two or three times greater in the advanced (blast) phase when compared to the chronic (initial) phase.[25]

1.3 Genomic Mutations in MPN

For many years the genetic basis of the classical Ph- MPNS was poorly understood. William Dameshek's recognition of the interrelatedness of the different subtypes of MPNs was highly prescient. A landmark observation in 2005 unequivocally demonstrated that a mutation in the gene *JAK2* (Janus kinase 2) was associated with majority of patients with MPN.[26-29]

The *JAK2* gene, localized on 9p24.1, is part of a family of cytoplasmic tyrosine kinases involved in the *JAK-STAT* signaling pathway. The *JAK2* gene encodes for a receptor predominantly responsive to type I cytokine ligands, including erythropoietin (EPO), thrombopoietin (TPO) and granulocyte-macrophage colony stimulating factor (GM-CSF). Ligand biding to *JAK2* leads to autophosphorylation and activation of signal transducers and activators of transcription (STAT) proteins which mediate the expression of genes involved in hematopoietic production.[30] The discovery of a recurrent activating mutation in exon 14 of the pseudo-kinase domain of *JAK2* resulting in amino acid substitution from valine to phenylalanine at codon 617 (*JAK2*V617F) and additional mutations in *JAK2* exon 12 provided the first genetic evidence of the importance of dysregulated growth factor signaling in these disorders. The *JAK2* mutation provided the first genetic evidence of erythropoietin-independent cell growth in vitro which had been made long before in studies of patients with PV.[16] The prevalence of *JAK2* mutations in the classical Ph- MPNs varies from 95% to 99% in PV, 50% to 70% in PMF, and 40% to 50% in ET.

The second Ph- MPN driver gene is *MPL* and was discovered in 2006.[32] Activating mutations in the *MPL* (Myeloproliferative Leukemia Virus Oncogene) gene, encoding the

thrombopoietin receptor, are present in approximately 4% of patients with ET and approximately 11% of patients with PMF.[31-34] The *MPL* gene is localized to chromosome 1p34.2. The most frequent mutation involves an amino acid substitution of tryptophan to either leucine or lysine at codon 515 (MPLW515L/K). These mutations are usually heterozygous but can be homozygous during disease progression.[35]

The third Ph- MPN driver gene involved involves *CALR* (calreticulin) and was discovered in 2013.[36] Somatic mutations in the *CALR* gene are found in 70% to 80% of patients with ET or PMF with wild-type *JAK2* and *MPL*.[36] *CALR* mutations appear to be primarily insertion or deletion mutations that result in a frameshift and the subsequent generation of a novel C-terminal peptide. The two most frequent mutations include a 52-bp deletion (p.L367fs*46), also called type 1 and a 5-bp insertion (p.K385fs*47), called type 2. In ET, type 1 and type 2 mutations are closely distributed (55% vs 35%), whereas in PMF, type 1 are largely predominant (75% vs 15%).[35] *CALR* mutations are usually heterozygous although a few cases of homozygous mutations have been observed, more particularly for type 2 mutations.[36] In contrast to *JAK2* and *MPL*, *CALR* is not a signaling molecule, but an endoplasmic reticulum (ER) chaperone Studies have shown that *CALR* mutations can activate *MPL* and subsequently *JAK2*.[35] Clonal analyses suggested that *CALR* mutations act as an initiating mutation in some patients. In ET and PMF, *CALR* mutations and *JAK2* and *MPL* mutations are currently known to be mutually exclusive and *CALR* mutations appear to be absent in PV.[37]

These mutations (*JAK2*, *MPL*, and *CALR*) are found in the vast majority of patients with MPN and represent driver mutations that induce the MPN phenotype, however, 10% of MPN patients lack mutations in any of these driver genes and are triple negative.

Hyperactivity of the *JAK-STAT* signaling pathway is observed in both patients who have driver gene mutations as well as in patients that are ‘‘triple-negative’’ where the driver gene mutation is still unknown.[38] Moreover, as shown in **Table 1.2**, additional recurrent somatic MPN mutations have been identified that target DNA methylation regulators (*TET2*, *DNMT3A*, *IDH1/2*), histone modifiers (*EZH2*, *ASXL1*), transcription factors (*TP53*, *CUX1*, *IKZF1*, *FOXP1*, *ETV6*, *RUNX1*), proteins involved in signaling (*NF1*, *NRAS*, *KRAS*, *SH2B3*, *CBL*, *FLT3*), and splicing factors (*SF3B1*, *SRSF2*, *U2AF1*, *ZRSR2*). Although it is beyond the scope of this dissertation to cover all the recurrent gene mutations in detail, this dissertation will briefly review genes that are important in Ph- MPN disease initiation and progression.

Mutations in Signaling Genes

Many genes involved in intracellular signaling, such as negative regulators of the *JAK2* signaling pathway, are mutated in MPNs. The SH2B adaptor protein 3 (*SH2B3*) gene, located on chromosome 12q24, negatively regulates *JAK2* activation through its SH2 domain. Mutations in *SH2B3* during the chronic phase are uncommon, fewer than 5% in ET and PMF; however, their frequency increases during leukemic transformation, suggesting that *SH2B3* plays a role in disease progression.[39,40]

Another negative regulator is the Cbl proto-oncogene, E3 ubiquitin protein ligase (*CBL*) gene. *CBL*, localized to chromosome 11q23, codes for a protein of the Cbl family of E3-ubiquitin ligases that acts as a negative regulator of some cell signaling pathways, by promoting the ubiquitination of several signaling molecules including some tyrosine kinases including *JAK2*.[41] *CBL* mutations cause the loss of E3-ubiquitin ligase activity,

resulting in deregulation of downstream targets and an increase in cell proliferation rates.[41] *CBL* mutations are found in approximately 6% of patients with PMF during the chronic phase but are absent in patients with PV and ET.[42,43]

Mutations in Epigenetic Regulators

Mutations in genes involved in epigenetic regulation include the DNA methylation genes ten eleven translocation 2 (*TET2*), DNA methyltransferase 3 alpha (*DNMT3A*), isocitrate dehydrogenase NADP(+)1, dytosolic (*IDH1*), isocitrate dehydrogenase NADP(+) 2, mitochondrial (*IDH2*), and the histone modification genes, enhancer of zeste 2 polycomb repressive complex 2 subunit (*EZH2*) and additional sex combs like transcriptional regulator 1 (*ASXL1*).

The *TET2* gene (chromosome 4q24) encodes an enzyme that converts 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC) in DNA, thereby epigenetically regulating gene expression by altering methylation-driven gene silencing.[44] All *TET2* mutations are loss-of- function point mutations or deletions, usually on one allele, but rarely on both alleles.[45] *TET2* mutations have been identified initially as mutations preceding *JAK2*V617F and subsequently were found as secondary mutations after *JAK2*V617F or *CALR* mutations.[44] *TET2* mutations occur in approximately 14% of MPNs, ranging from 11% in ETs to 19% in PMFs.[44,46]

In contrast to *TET2*, where demethylation occurs through the oxidation of 5mC into 5hmC, *DNMT3A* (chromosome 2p23) is involved in the methylation of cytosines (5mC). *DNMT3A* mutations are less frequent than *TET2* mutations in MPNs (~10%) and "in the majority of the cases precede *JAK2*V617F or *MPL* mutations."[47] Both *TET2* and *DNMT3A*

mutations increase the self-renewal capacities of HSCs in both humans and mice, however, the precise mechanism is not completely understood. Both *TET2* and *DNMT3A* control the expression of genes involved in HSC properties as well as differentiation[35] and both are mutated genes associated with clonal hematopoiesis during aging, also known as age related clonal hematopoiesis (ARCH).[48] ARCH is prevalent in the general population and commonly involves genes that are recurrently mutated in hematologic malignancies. Nevertheless, the majority of individuals with ARCH will not develop overt hematologic disease.

Although mutations in *TET2* and *DNMT3A* occur primarily in the chromic phase, there are mutations that are present at the time of transformation and are therefore thought to play a role in disease progression. Both *IDH1* and *IDH2* mutations, genes located on chromosomes 2q33.3 and 15q26.1, respectively, occur at low frequencies in the chronic phase (0.8% in ET, 1.9% in PV, and 4.2% in MF) but up to 8% of patients who progress to blast phase.[49] *IDH1* and *IDH2* catalyze the oxidative decarboxylation of isocitrate to α-ketoglutarate thereby generating NADPH from NADP+ as part of the citric acid cycle in the cytosol/peroxisomes and mitochondria, respectively.[49] *IDH* mutations lead to depletion of cellular α-ketoglutarate with downstream activation of the HIF-1α pathway.[49]

EZH2, localized to chromosome 7q35, is one of two histone methyltransferases of the *PRC2* (polycomb repressive complex 2) complex and is involved in the repressive histone 3 lysine 27 (H3K27) trimethylation (H3K27m3). Loss-of-function mutations and cytogenetic lesions in *EZH2* and other PRC2 members (*SUZ12*, *JARID2*, *EED*) have been reported in MPNs and in many myeloid malignancies.[50] *EZH2* is mutated in up to 13% of patients with PMF, and is associated with the *JAK2*V617F mutation. *EZH2* mutations do

not occur in ET but are present in 3% of patients with PV.[50] Studies have shown that there is a strong cooperation between *EZH2* loss and *JAK2*V617F on disease initiation. [51]

Along with *TET2*, *ASXL1* mutations were one of the first mutations to be identified in *JAK2*V617 positive MPN. The *ASXL1* gene (chromosome 20q11.21) encodes for an epigenetic regulator protein that binds to chromatin. Interestingly, it has been shown that "*ASXL1* recruits the PRC2 complex to specific loci through a direct interaction between *ASXL1"* and *EZH2*, "inhibition of *ASXL1* function leads to loss of H3K27me3 histone marks."[52] These mutations result in a loss of function and are associated with a higher frequency of transformation to acute leukemia. These genetic alterations are either focal deletions or nonsense mutations or insertions/deletions leading to frameshift mutations.[53] *ASXL1* mutations are found in only approximately 7% of patients with ET and PV but more frequently in PMF cases (from 19%–40%).[54,55]

Transcription Factor Mutations

The transcription factor Ikaros encoded by the *IKZF1* (chromosome 7p12) gene has pleiotropic functions in the regulation of hematopoiesis. "Complete or partial deficiency of Ikaros function in mice induced multiple hematopoietic defects including lymphoproliferative disorders and B- and T-cell leukemia."[56] Many "defects were also observed in the myeloid lineages, such as anemia and thrombocythemia."[57] These functional studies in mouse models strongly suggest that decreased Ikaros function is oncogenic. Hemizygous deletions of the *IKZF1* gene as well as deletions of various sizes were detected including frequent intragenic deletions of *IKZF1* between exons 3 and 6.

These deletions are rarely observed in the chronic phase but are present in up to 20% of patients with post-MPN acute leukemia.[57,58]

TP53 plays an important role in cell regulation. It can activate DNA repair mechanisms, arrest cell growth, initiate apoptosis, and can induce senescence. Different mutations and cytogenetic lesions targeting the *TP53* (chromosome 17p13.1) tumor suppressor function are present in patients with post-MPN acute leukemia. Loss of function is a result of missense mutations or deletions in the *TP53* gene or gain of chromosome 1q which result in upregulation of *MDM4* (for more details see Chapter 3, specific aim 2). *TP53* mutations are rare in the chronic phase of MPNs but occur in 20% of patients with post-MPN acute leukemia.[59-61]

Other late occurring events in transcription factor genes of patients with MPNs who transform to MPN-BP include mutations in *RUNX1* (21q22.3) and deletions of *CUX1* (7q22).[23,62]

Mutation in RNA Splicing Genes

Mutations in genes encoding spliceosome proteins were identified in different hematopoietic malignancies. "Spliceosome gene mutations in MPNs are essentially restricted to ET and MF."[63] The most frequent mutations include *SRSF2*, *SF3B1*, and *U2AF1* and are associated with anemia and thrombocytopenia.[63]

SRSF2 (17q25.1) encodes for a protein, which is a member of the serine/arginine-rich splicing factor family that binds to exonic splicing enhancer (ESE) sequences in the pre–messenger RNA (mRNA). Mouse models have shown that mutations in *SRSF2* can induce cytopenia and can lead to a mis-splicing and nonsense-mediated decay of EZH2.[64]

This further establishes that *EZH2* and the *PRC2* complex are important factors in the development of MF and progression toward leukemia. *SRSF2* mutations occur in 17% of patients with ET and is associated with a poor prognosis.[65]

The *SF3B1* (2q33.1) gene encodes subunit 1 of the splicing factor 3b protein complex. Mutations are typically heterozygous point mutations that are thought to be functionally deleterious. These mutations are found in 20% of patient with MDS and correlate with the presence of ring sideroblasts, however, *SF3B1* mutations are rare in myeloproliferative neoplasms and particularly in ET (6%).[66]

The *U2AF1* (212q22.3) gene belongs to the splicing factor SR (Serine and Arginine) family of genes and is part of a heterodimer with *U2AF2* RNA spicing complex. Mutations are detected in various myeloid disorders and occur in up to 16% of patients with ET. Mutations are associated with a reduced overall survival and shorter time to leukemic transformation. [66]

Although it may appear that the MPN genome is largely understood, in terms of the spectrum and frequency of somatic mutations, the precise role of each mutation and their impact on MPN phenotype is still not understood. Moreover, it remains unclear how a single driver mutation contributes to the pathogenesis of PV, ET and PMF, "one-mutation-different diseases." As a result, the mutational landscape in Ph- MPN has proven to be complex and questions such as what are the genetic drivers in patients with MPN who are "triple-negative" remains unknown.

Table 1.2 Genes mutations, chromosome location, function, type and frequency in MPN

Gene	Chromosome position	Mutation Region	Mutation Type	Gene function		Frequency (%)			
						PV	ET	PMF	Post MPN AML
Signaling MPN Driver									
JAK2	9p24	JAK2V617 (Exon 14) JAK2 (Exon 12)	Missense Missense	Tyrosine kinase, signaling	Gain of function	95-99% <3%	40-50%	50-60%	
MPL	1p34	MPL515L/K/A/R MPLS505N (Exon 10)	Various Missense	Receptor signaling	Gain of function		4%	11%	
CALR	19p13.2	Exon 9	Frame Shift	Calcium binding chaperone in ER	Loss of function		67%	86%	
Other Signaling									
SH2B3	12q24	Exon 2	Missense / deletion	Adaptor, signal regulation	Loss of function		5%	5%	13%
CBL	11q23	Exons 8-9	Missense	Adaptor, E3 ubiquitin ligase, signal regulation	Dominant negative			6%	
NRAS	1p13.2	Exons 2-3	Missense	GTPase, signaling	Gain of function				7%-13%
NF1	17q11.2	Multiple Exons	Missense	RAS signaling regulation	Loss of function			1-6%	5-10%
FTL3	13q12	Exons 14-15, 20	Duplication, missense	Cytokine receptor	Gain of function				<3%
RB1	13q14	Multiple Exons	Nonsense, missense	Cell cycle, apoptosis	Loss of function			19%	
DNA Methylation									
TET2	4q24	Multiple Exons	Missense, Nonsense	DNA hydroxymethylation	Loss of function	15%	4-11%	19%	26%

DNMT3A	2p23	Multiple Exons	Missense	Chromatin methylation	Loss of function			10%	4-22%
IDH1	2q33.3	Exon 4	Missense	Metabolism	Neomorphic enzyme			2%	8%
IDH2	15q26.1	Exon 4	Missense	Metabolism	Neomorphic enzyme			2%	18%
Histone Modification									
EZH2	7q35	Multiple Exons	Missense, insertions, deletions	Chromatin methylation	Loss of function	3%		13%	
ASXL1	20q11.21	Exon 12	Nonsense, insertions, deletions	Chromatin modifications	Loss of function	<7%	<7%	19-40%	19%
Transcription Factors									
TP53	17p13.1	Exon 4-9	Nonsense, missense, insertions	Cell cycle, apoptosis	Loss of function				20%
CUX1	7q22	Multiple Exons	Deletion	Transcription factor	Loss of function				<3%
IKZF1	7p12	Multiple Exons	Deletion, insertions	Transcription factor, lymphopoiesis	Dominant negative				21%
ETV6	12p13	Multiple Exons	Missense, insertions, deletions	Transcription factor	Loss of function				21%
RUNX1	21q22.3	Multiple Exons	Nonsense, missemse, insertions, deletions	Transcription factor, hematopoiesis	Loss of function				37%
NFE2	12q13			Transcription factor	Gain of function			3%	
RNA splicing									
SRSF2	17q25.1	Exon 1	Missense	Splice factor	Dominant negative		17%		19%

SF3B1	2q33.1	Exons 12-16	Missense	Splice factor	Dominant negative		6%		
U2AF1	21q22.3	Exon 2-7	Missense	Splice factor	Dominant negative		16%		

Note. Adapted from Vainchenker, et. al., Blood 129, 667–679 (2017).

Table 1.2 Genes mutations, chromosome lo 1

1.4 Chromosomal Microarrays in MPN

Cytogenetic analysis provides useful diagnostic, prognostic and therapeutic information in hematological malignancies. Chromosomal microarrays (CMA) are currently used as an adjunct test to conventional cytogenetics for the detection of genomic lesions at a higher resolution. Many of these copy number alterations (CNA) are too small to be identified by conventional cytogenetics and too big to be identified by NGS. The advantage of chromosomal microarrays over conventional cytogenetics and NGS is that it can identify cryptic CNAs and copy neutral loss of heterozygosity (CN-LOH). **Table 1.3** contains a comprehensive list of acquired CNAs and CN-LOH of known or likely clinical significance in Ph- MPN as detected by CMA.

CN-LOH, also often referred to as uniparental disomy (UPD), leads to loss of heterozygosity by duplication of a maternal (unimaternal) or paternal (unipaternal) chromosome or chromosomal region leading to concurrent loss of the other allele. CN-LOH can be constitutional or acquired. Constitutional CN-LOH is associated with meiotic errors resulting in developmental diseases, and regions of acquired CN-LOH arise from mitotic recombination and enable genetic variants to progress from a heterozygous to a homozygous state without change in DNA copy number.

In 2002, the description of "UPD9p" or CN-LOH 9p in the setting of MPN demonstrated for the first time CN-LOH was of clinical significance.[67,68] By now it is well established that a number of recurrent regions of acquired CN-LOH are associated with homozygous gene mutations, suggesting that this is a common genetic mechanism leading to homozygosity.[68,69]

Table 1.3 A comprehensive list of CNAs and CN-LOH of known or likely clinical significance in MPN detected by CMA testing

Chromosome	Abnormality Type (Gain, Loss, CN- LOH)	Region	Relevant Genes (if known)	Clinical Significance*
1	CN-LOH	1p21.3	MPL	Recurrent
1	Gain	1q21.2- q32.1		Recurrent
4	Loss	4q24	TET2	Recurrent
5	Loss	5q	RPS14	P (Poor)
6	Loss	6p23-22.3	JARID2	Recurrent
7	Loss	7q	EZH2, CUX1	P (Poor)
7	CN-LOH	7q22.1-qter	EZH2, CUX1	Recurrent
8	Gain (Trisomy)	8	Whole chromosome	P (Poor)
9	Gain	9p	JAK2	Recurrent
9	CN-LOH	9pter-p13.3	JAK2	Recurrent
11	CN-LOH	11q13.4-q25	CBL	Recurrent
12	Loss	12p13.3- p12.2	ETV6	P (Poor)
13	Loss	13q	RB1	Recurrent
14	CN-LOH	14q	CHGA	Recurrent
17	Loss	17p	TP53	P (Poor)
20	Loss	20q	ASXL1	Recurrent
22	Loss	22q11.2		Recurrent
22	Gain	22q11.2 (+Ph)	BCR	Recurrent

Note: Adapted from Kanagal-Shamanna, R. et. al., Cancer Genet., 2018.07.003
Legend: P-prognostic significance;
Recurrent indicates recurrent aberration with no established significance.
*Clinical significance based on NCCN guidelines For myelofibrosis, unfavorable [complex karyotype or sole or two abnormalities that include inv(3), 5/5q-, 7/7q-, + 8, 11q23 rearrangement, 12p-, and (17q)]

CMA studies have been used to detect frequent regions of acquired CN-LOH in MPNs. CN-LOH of 9p is most commonly affected and is almost always associated with homozygosity for *JAK2*V617F.[70] Other chromosomal regions of CN-LOH include 4q, 7q, and 11q and they are associated with mutations in *TET2*, *EZH2*, and *CBL*.[43,44,50] Recurrent CN-LOH of 1p is associated with *MPL* mutations in essential thrombocythemia (ET) while 11q CN-LOH is associated with *CBL* mutations in myelofibrosis.[31,43]

In addition to identifying regions of CN-LOH, studies using low resolution chromosomal microarrays were able to detect CNAs in patients with MPNs. These studies have shown that PV and ET genomes are relatively simple with very few somatic CNAs.[70,71] In contrast, copy number changes are more frequent in patients with PMF and in patients with post PV or ET - MF.[23,70] In one study involving 151 patients with MPN, CNAs were rare in patients with PV and ET while one-third of cases with PMF showed small genomic losses (<5 Mb) when using an Affymetrix 250K SNP array.[72]

The overall detection rate of CMA abnormalities in Ph- MPN is >56%. CMAs are able to detect all clonal abnormalities seen in Ph- MPN by karyotype. However, frequent additional alterations uniquely detected by CMA and not by conventional cytogenetics include 6p CN-LOH (12.5%), 9p gain/CN-LOH (18.8%), and 22q deletion (12.5%).[73] CN-LOH of 9p, as mentioned before, was the most common, accounting for 41% of abnormal and in 100% of patients with PV.[74] Ph- MPN with homozygous *JAK2* mutations had frequent 9p CN-LOH while those with heterozygous *JAK2* mutations had no detectable 9p CN-LOH.[68]

A subset of patients with MPN eventually progress/transform to post MPN acute leukemia (blast phase). Thoennissen and colleagues reported that leukemic transformation of MPN was accompanied by up to three-fold more genomic alterations per case when compared to chronic phase.[75] The average number of aberrations increased over the course of disease progression (0.6 vs. 3 in patients without and with progression, respectively). "When excluding 9p CN-LOH, the incidence of genomic changes (both CNA and CN-LOH) was significantly higher in patients with disease progression than in patients without disease progression (63% and 0%, respectively, p=0.01)."[74] The genomic regions

commonly affected during leukemic transformation harbored genes known to be associated with acute leukemia such as *ETV6*, *TP53*, and *RUNX1*, and also new candidate genes that are localized on 7q, 16q, 19p, and 21q. These genes include *CUTL1* and *SH2B2* on 7q, *E2F4*, *CTCF*, *CBFB*, and *TRADD* on 16q, *ICAM1*, *CDC37*, *PIN1* on 19p, and *ERG* on 21q.

Among the patients who evolved to acute leukemia, 80% showed a CMA abnormality at initial testing. A poor prognosis after leukemic transformation was associated with CN-LOH on either 7q or 9p including homozygous *JAK2*V617F. However, the role of CN-LOH of 9p in disease progression remains unclear. In some reports, patients with sole 9p CN-LOH did not have disease progression, suggesting that it was a favorable marker. Other studies in patients with PV have shown that a higher *JAK2* mutant burden (>50%), as a result of 9p CN-LOH, is associated with a higher risk of developing myelofibrosis.[74,76] These contradictory studies are further complicated by the fact that leukemic transformation may originate in either the clone with 9p CN-LOH and homozygous JAK2 mutation or a *JAK2*-negative clone with normal chromosome 9.[68] These conflicting reports are in indication that other genomic events, occurring in the clone with or without CN-LOH of 9p, are associated with disease progression. This is supported by the significant association between progression to blast phase with the acquisition of cryptic aberrations involving chromosomes 5, 7, or 17p, detected by both CMA and conventional cytogenetics.[77] "The presence of one or more of these aberrations was independently associated with reduced overall survival from the time of diagnosis of MPN" and progression to AML.[77] Specific CNAs abnormalities were associated with disease progression and blast transformation, for example, CNAs involving 1q/9p was associated

with progression to myelofibrosis while CNAs involving 3q, 5q, 6p, 7p, 7q, 19q, and 22q were associated with progression to blast phase.[77]

In another study, CMA analysis identified novel putative tumor suppressor genes that affect transcriptional regulation. The polycomb repressive complex 2 (PRC2) is a multimeric protein complex that negatively regulates gene expression.[78] Although the core of the complex is formed by *EZH2* and *SUZ12,* which found to be targeted by deletions or inactivating mutations, many proteins have been identified to be part of the complex.[78] CMA analysis identified frequent deletions of *JARID2* as well as other members of the PCR2 complex.[78] Aberrations of these genes inactivate the function of the PRC2 complex resulting in de-repression of its target genes which results in decreased differentiation leading to leukemic transformation.[78] These results confirm the utility of CMA analysis to identify genes involved in the same complex leading to altered epigenetic regulation.

Moreover, these studies highlight the advantage of CMAs in Ph- MPNs and demonstrate that regions of CN-LOH are genomic landmarks in identifying novel genes that are frequently mutated. Although these studies in MPN utilized relatively low-resolution chromosomal arrays, newer CMA platforms, as used in this dissertation, have the ability to detect copy number aberrations at the exon level which can further increase in the sensitivity of detecting copy number aberrations.

1.5 Integrated Genomic Analysis of Ph- MPNs

Genetic abnormalities, including chromosomal rearrangements, copy number aberrations (CNAs) and gene mutations, underlie the pathogenesis of these disorders and serve as important diagnostic and prognostic markers. Conventional cytogenetic testing methods, including metaphase cytogenetics (MC) and fluorescence *in situ* hybridization (FISH) have served as standard assay for the detection of balanced and unbalanced chromosomal rearrangements and gain or loss of genetic material. In recent years, newer technologies such as chromosomal microarrays (CMA) and next generation sequencing (NGS) has led to major progress in understanding the molecular pathogenesis of the Ph- MPNs.

Mutations in one of three genes, *JAK2*, *MPL*, and *CALR*, can be found in the vast majority of patients with MPN and represent driver mutations that may induce the MPN phenotype.[79] Although our understanding of the genomic landscape of Ph- MPNs has improved in terms of the spectrum and frequency of somatic mutations, the exact role of these mutations in MPN pathogenesis remain unclear. The fact that *JAK2* mutations have been identified in patients with three phenotypically related but clinically distinct MPNs suggests that additional genetic or epigenetic events likely contribute to the phenotypic divergence of these disorders.[80] There are a number of reports that support evidence that PV is not solely initiated by *JAK2* mutations.[81] Clonality and cell hierarchy studies have demonstrated that chromosomal abnormalities are present in *JAK2* wild type (WT) hematopoietic single cells and precede the acquisition of the *JAK2*V617F mutation.[82,83] Additionally, some patients with *JAK2*V617F - positive MPN progress to a *JAK2*V617F - positive acute leukemia and are associated with the acquisition of additional genetic

alterations.[61,84-86] However, a second, more complex route of transformation to blast phase from MPN has been well documented in which a *JAK2*V617F -positive MPN is followed by *JAK2*V617F - negative acute leukemia.[30,87] In order to understand this phenomenon, clonality testing was performed using X chromosome inactivation in female patients that were heterozygous for a single nucleotide polymorphism in the *MPP1* gene. The results demonstrated that *JAK2*V617F - positive MPN and *JAK2*V617F - negative blast cells were clonally related, indicating that the leukemic transformation occurred in an antecedent, pre *JAK2*-mutant cell which then progressed to acute leukemia.[87]

Based on two observations, (1) the existence of a pre-JAK2 mutant cell and (2) that mutations in signaling molecules are not sufficient for disease development, it is likely that several cooperating genetic events are required to induce transformation to acute leukemia which illustrates the complexity of the pathogenesis of Ph-MPN.[88] Currently no single test is available that can detect all genomic lesions that may play a role in the pathogenesis of Ph- MPNs. Consequently, it is important to integrate complementary genomic technologies to gain further insight into the genetic diversity and add to the genomic landscape of the Ph- MPNs.

Integrating genomic data, such as gene mutations, copy number aberrations, and/or gene expression data is a useful strategy to identify key genes and pathways involved in various malignancies. Integrated genomic analysis using mutational and gene expression data demonstrated that patients with Ph- MPN, "regardless of diagnosis or *JAK2* mutational status, are characterized by a distinct gene expression signature with upregulation of JAK-STAT target genes," indicating the "central importance of the JAK-STAT pathway in MPN pathogenesis."[38]

More recent studies have demonstrated that integrative analysis of copy number and gene expression data identified novel genes that might be implicated in the pathogenesis of primary myelofibrosis.[89] In one study, gene expression and copy number data were integrated resulting in the identification of several regions of genomic gain or loss leading to a concordant alteration in expression levels of genes within these regions. For example, in patients with PMF, copy number gain in the polyamine oxidase (*PAOX*) gene locus was accompanied by transcriptional up-regulation. Moreover, copy number loss in the chromatin modifier *HMGXB4* gene correlated with a concomitant transcriptional down-regulation.[89] Functional studies on human hematopoietic stem/progenitor cells showed that silencing of *HMGXB4* induces megakaryocyte differentiation, while inhibiting erythroid development and *PAOX* inhibition resulted in rapid cell death of progenitor cells, while sparing normal cells.[89] These results highlight previously unreported, yet a potentially interesting role of *HMGXB4* in hematopoiesis and suggest that genomic and transcriptional imbalances of *HMGXB4* could contribute to the aberrant expansion of the megakaryocytic lineage that characterizes patients with PMF.

Studies integrating array comparative genomic hybridization (aCGH) and mutational analysis has identified copy number aberrations in 54% of patients with chronic or accelerated phases of myelofibrosis, often involving genes with a known role in leukemogenesis.[90] In addition to identifying mutations in known epigenetic genes such as *ASXL1, TET2* and *EZH2*, several gains and deletions involving other epigenetic regulators, such as gain of *SALL3, BMI1,* and *RCOR1* were also discovered.[90] These results suggest that not only is MF characterized by alterations in known epigenetic genes, but integrated analysis revealed alterations in lesser known epigenetic genes not previously associated

with Ph- MPN. This study by Brecqueville and colleagues highlights the genetic heterogeneity of myelofibrosis, and points to several gene copy number aberrations that may have diagnostic and prognostic impact.[90]

However, the study by Brecqueville and colleagues did not utilize high-resolution array platforms currently available, and more importantly, it did not incorporate single nucleotide polymorphisms (SNP) to identify regions of copy neutral loss of heterozygosity (CN-LOH).[90] As mentioned previously, copy-neutral LOH (CN-LOH) is a recurrent genomic lesion in myeloid disorders and may indicate the presence of mutations or abnormal methylation status in a homozygous constellation. These regions contain tumor suppressor genes and oncogenes that maybe mutated resulting in loss of the wild type allele and concomitant duplication of the mutant allele. For example, in 2002, 9p CN-LOH, including the region harboring the *JAK2* gene was, observed in up to 30% of patients with PV.[67] Three years later *JAK2*V617F was identified. Moreover, inactivating mutations of the histone methyltransferase gene *EZH2* were first identified in patients with myeloid malignancies that harbored CN-LOH of chromosome 7q, where *EZH2* is localized.[50] Many genes within regions of CN-LOH such as *JAK2*, *EZH2*, *ETV6*, *TET2*, and *MPL* have diagnostic and prognostic implications in myeloid malignancies and can be perturbed by different modalities such as mutation, deletion, translocations, or amplification.[43,46,50,67-69,91]

During the last few years the resolution of CMA platforms has reached the exon level. Small focal CNAs play an important role in the discovery of new driver genes.[92] Studies have shown that focal CNAs which contain a limited number of genes are highly enriched for genes that promote cancer.[93,94] Focal deletions of *TET2* are observed in

myeloid malignancies and *TET2* mutations occur either as initiating events or are acquired later during disease progression.[35,45] Focal deletions of *RUNX1* are observed in 2.6% to 9.1% of patients with myeloid malignancies and mutations of *RUNX1* have been reported as late events occurring in up to 10% of patients who transform to MPN-BP.[35,95] These observations illustrate the advantage of integrating high resolution CMAs that provide increased sensitivity for detecting novel genomic alterations.

Like NGS, CMAs have experienced increased use in genomic profiling however, integrating these technologies is far from routine. The aim of this proposal is to increase the sensitivity of identifying cooperative genomic events by integrating classical and newly developed methods, including conventional cytogenetics, mutational profiling, and high-resolution chromosomal microarrays.

The aim of this dissertation is to identify cooperative genomic events that underlie the biology of the Ph- MPNs. This innovative strategy may add to the genomic landscape and, more importantly, improve the understanding of the Ph-MPN pathogenesis.

1.6 Hypothesis and Aims of the Study

Integrating complementary genomic technologies in patients with Ph- MPN can reveal novel genomic alterations involving genes that may play a role in disease initiation, progression, and/or transformation.

Specific Aim 1: To detect novel genomic lesions in patients with myelofibrosis who underwent splenectomy and whether these lesions provide insight into the progression of this disease.

Specific Aim 2: The gain of the long arms of chromosome 1 (+1q) and rearrangements of 12q are chromosomal abnormalities that occur in the early forms of Ph- MPN that progress to more advanced forms of the disease.

> **Sub-aim 2a:** To investigate the molecular consequences of the gain of the long arms of chromosome 1 (+1q)
>
> **Sub-aim 2b:** To identify specific genes located on 12q that are important in disease progression.

Specific Aim 3: To determine whether genomic lesions involving genes identified in Specific Aims 1 and 2 are present in patients with Ph- MPN who are cytogenetically normal.

Chapter 2. Specific Aim 1

2.1 Specific Aim 1

To detect novel genomic lesions in patients with myelofibrosis who underwent splenectomy and whether these lesions provide insight into the progression of this disease.

2.2 Approach

The spleen in patients with MF is a major site of extramedullary hematopoiesis (EMH) and it is estimated that up to 80% of MF patients suffer from splenomegaly.[96] Our understanding of the role that the spleen plays in the origins of myelofibrosis or in disease progression is limited. Studies based on identification of loss of heterozygosity (LOH) or the acquisition of the *JAK2*V617F mutation have established the clonal nature of splenic EMH in MF.[97-99] Additional studies have shown that MF splenic hematopoietic stem and progenitor cells have both phenotypic and functional properties that differ from hematopoietic cells present in the bone marrow (BM) or peripheral blood (PB). For example, MF splenic cells contain phenotypically primitive hematopoietic stem and progenitor cells (HSPCs) and that the spleens but not the PBs of MF patients contain malignant CD34+ stem cells that can be serially engrafted into immunosuppressed mice.[100-102] The engrafted splenic MF-CD34+ cells produced donor derived myeloid and lymphoid cells, which were shown to belong to the malignant clone.[102] These findings demonstrated for the first time that cancer stem cells reside in spleens of patients with MF, with a differentiation program resembling normal HSPCs which have been shown to contain malignant CD34+ stem cells.[102]

These studies also highlight the role of the spleen as a possible tumor promoting microenvironment, which could contribute to MF disease progression and leukemic transformation. Early splenic cytogenetic studies have shown that common abnormalities such as +1q, del(20q), del(13q), trisomy 9, and del(12p) are consistent with the known distribution of BM/PB cytogenetic abnormalities in MF. A comparison between spleen and BM karyotypes showed concordant results in more than 85% of the cases and discordant results in 14.7%. In 9 out of the 10 patients with discordant karyotypes, the additional abnormalities occurred in the spleens. Importantly, the presence of an abnormal splenic karyotype correlated with decreased post-splenectomy survival.[103] The authors concluded that the spleen provides an environment conducive to malignant progenitor cell growth and differentiation.[103]

These early studies utilized classical cytogenetic methods, however, recent technological advances can provide significantly higher resolution of genetic information than karyotype analysis. The aim of this study is to integrate complementary genomic technologies to increase the sensitivity of detecting novel genomic lesions in order to gain biological insight in this disease. Conventional cytogenetics, FISH, CMA and NGS analyses was performed on spleen cells from 14 patients with advanced disease who required therapeutic splenectomy and assessed the degree of genomic diversity with the anticipation that specific genetic events might play a role in disease progression.

2.3 Materials and Methods

2.3.1 Patient specimens and cell preparation

Spleens were surgically removed from 14 patients with advanced forms of MF requiring therapeutic splenectomy, between 2011 and 2015. BM or PB samples for cytogenetic and molecular studies were collected within two months prior to or following the splenectomy. The study cohort included patients with both PMF and MF following polycythemia vera and essential thrombocythemia (post-PV and post-ET MF) who fulfilled the World Health Organization (WHO) 2008 diagnostic criteria.[104] All patients provided signed informed consent as approved by institutional review board and in accordance with the Declaration of Helsinki. Single cell suspensions were prepared according to the method of Barosi and co-workers.[101] Mononuclear cells (MNCs) were isolated by density gradient centrifugation using Ficoll-Paque (GE healthcare Life Sciences). The *JAK2*V617F status of each patient was determined prior to splenectomy by analyzing PB granulocytes using a previously described real-time allele-specific polymerase chain reaction (AS-PCR) assay.[81,105]

2.3.2 Conventional cytogenetic analyses

Chromosomal analyses of PB and BM-MNCs as well as MNCs isolated from patient spleens were performed as per standard methodology. Giemsa-banded metaphase cells were scanned using Leica's GSL scanner (Leica Microsystems Inc., Buffalo Grove, IL) and captured for analysis using Leica Microsystems CytoVision software. The clonality criteria and the description of karyotypes followed the recommendations of the International System for Human Cytogenetic Nomenclature (ISCN, 2016)

2.3.3 Fluorescence *in situ* hybridization (FISH)

FISH studies were performed on fixed cell suspensions from spleen MNCs, bone marrow, and peripheral blood tissues as described previously. [106] Interphase FISH (I-FISH) was performed using a panel of twelve probes: 5p15.2 (D5S23,D5C721)-5q31 (*EGR1*), 7p11.1-q11.1 (D7Z1)-7q31 (D7S486), 8p11-q11 (D8Z2), 9p11-q11 (CEP9)-9p21 (*CDKN2A*), 11q22.3 (*ATM*), 13q14.3 (*RB1*), 20q12 (D20S108), (Abbott Molecular, Abbott Park, IL), 1p32.3 (*CDKN2C*), and 1q21.3 (*CKS1B*) (CytoCell, Cambridge, UK). An additional FISH probe, 12p13 (ETV6), was used to confirm aCGH+SNP results. The normal laboratory reference ranges were 4% for each probe, except for 13q14.3 (5%) and 20q12 (3%). Images were captured and analyzed using Leica Microsystems CytoVision software.

2.3.4 DNA extraction and aCGH+SNP

Genomic DNA was prepared from MNCs isolated from patient spleens, BM, and PB tissues using Qiagen's Gentra PureGene DNA purification kit (Qiagen Inc., Valencia, CA) as per the manufacturer's instructions. Array-CGH+SNP was performed as per manufacturer's instructions (Agilent Technologies, Santa Clara, CA). Patient DNA was labeled with Cy5 and reference DNA was labeled in Cy3. Human Male and Female reference DNA provided in Agilents' SureTag Complete DNA labeling Kit was used as a gender-matched control. The arrays were scanned using Agilent's G2600D scanner and image analysis was performed using the default aCGH+SNP settings of the Feature Extraction software 11.5.1.1. The Derivative Log Ratio Standard Deviation (DLRSD) score was used to determine array quality, only arrays with a score of <0.2 were analyzed. Agilent's SurePrint G3 Human CGH+SNP can identify both copy number variations and

copy-neutral aberrations. The GenetiSure Cancer Research array CGH+SNP array allows for exon-level copy number resolution with coverage of 2,300 cancer related genes. This platform contains an average of 6 probes per exon that allows resolutions of <1kb in large exons to <10kb in cancer genomic targeted regions and may detect single and multi-exon duplications or deletions.

Agilent Cytogenomic v4.0.3 software was used to visualize, detect, and analyze genomic gain, loss and CN-LOH using the ADM-2 algorithm. In order to identify exon-level copy number aberrations (CNAs) of potential oncogenic significance, the following criteria were used: 1. Regions of gain or loss were >500bp and contained a minimum of 5 probes, or regions with CN-LOH were ≥5Mb. 2. The average log2 ratio was ≥0.18 for gain and ≤-0.18 for loss. For each abnormality detected, we reviewed the Database of Genomic Variants (DGV) available online, as well as an internal cohort of healthy controls to exclude normal copy number variations (CNVs). Highly recurrent abnormalities appearing with very low frequency in the DGV (<0.2%) were retained.

2.3.5 Next generation sequencing (NGS)

We performed high-throughput sequencing with a targeted deep sequencing assay of 585 genes (HemePACT) on the splenic and PB MNCs (Performed at Memorial Sloan Kettering Cancer Center by Dr. Raajit Rampal). Tumor tissue was sequenced at an average coverage of 829x (with a standard deviation of 130). We used Mutect to call single point variants, comparing our samples to a sample representing pool of normal samples, and PINDEL to call short insertions and deletions, following previous recommendations. We then excluded all mutations either present at a high variant allele frequency in the matching

germline samples (when available) or present in at least one database of known on-somatic variants (DBSNP and 1000 genomes) and absent from COSMIC.

2.4 Results

The clinical characteristics and post-splenectomy outcomes of 12 of the 14 patients was previously reported.[107] This cohort included patients with primary MF and post-polycythemia vera or post-essential thrombocythemia MF who fulfilled the 2008 WHO diagnostic criteria.[108] The Median age was 65 years (range 51-79). Nine patients had PMF, 4 had post-PV MF and one had post-ET MF. 12 of the patients had intermediate-2 risk disease by Dynamic International Prognostic Scoring System (DIPSS), and two patients had intermediate-1 risk. Eleven of 14 patients (78.6%) had a *JAK2*V617F mutation, with an allele burden ranging from 13 to 91%, two patients had a *CALR* mutation and one patient lacked a mutation in *JAK2*, *CALR* and *MPL* and was termed "triple negative." Indications for splenectomy were refractory cytopenias in 10 cases, symptomatic splenomegaly not responding to ruxolitinib in 2, and preparation for stem-cell transplantation in 2. Median post-splenectomy survival was 14 months but ranged widely from 34 days to over four years.

An overview of genomic studies is provided in **Table 2.1**. Cytogenetic results were concordant between spleens and BM/PB in 12 (86%) patients, including 7 with a normal karyotype and 5 with abnormal karyotypes. Chromosomal abnormalities occurred at comparable frequencies in both tissue specimens. Discordant findings were observed in 2 (14%) patients (ns. SP01 and SP09), both harboring a complex karyotype in which the spleen contained additional abnormalities that were not detected in the PB. In patient SP01,

who had pentasomy-21 in spleen only, trisomy-21 was subsequently observed in a post-splenectomy PB karyotype. In another patient (SP02), who at the time of splenectomy had a concordant normal karyotype, del(20q) was detected in a single (non-clonal) spleen metaphase cell. This patient developed a dominant del(20q) clone (85% of metaphase cells) in a BM study performed 2.5 years post splenectomy, suggesting that this clone originated in the spleen. Since the spleen cells from each patient always contained at least the same abnormalities as the BM/PB, it seems likely that MF originated in the BM and later migrated to the spleen. However, the additional abnormalities detected in the spleens suggest that clonal evolution can occur within the MF spleen and that these cells then re-enter the BM/PB post splenectomy. This hypothesis is supported by evidence of leukemic transformation arising in the spleens of patients with myeloproliferative neoplasms (MPN) [96]. Other explanations, such as the emergence of previously undetectable clones in the BM, are also possible.

CMA analysis of spleens from 13 patients revealed gain of 168 chromosomal regions, loss of 43 regions and 37 acquired regions of CN-LOH as shown in **Figure 2.1**. Genomic changes were detected in all patients including those with a normal karyotype, and ranged from 7 to 20 changes per patient, with the exception of Patient SP01 who had 78 genomic changes. When Patient SP01 is excluded from the analysis, the mean number of genomic changes was 14.1 per patient with 9.4 gains, 2.1 losses and 2.6 CN-LOH. Patient SP01 harbored a complex karyotype, with 78 abnormalities including 55 gains, 18 losses and 5 regions of CN-LOH. The array studies further revealed that the complex der(5) chromosome detected in PB and spleen metaphase cells was the result of chromothripsis

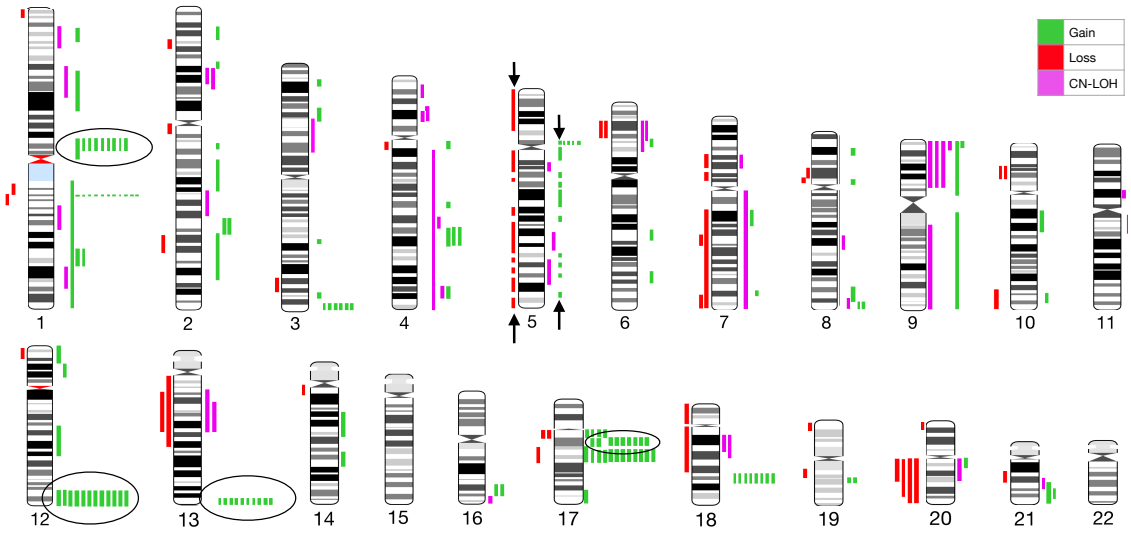
Table 2.1 Genomic characterization of spleens in 14 patients with myelofibrosis

Patient #	Diagnosis and DIPSS	Age at splenectomy and gender	Karyotype	aCGH+SNP *	NGS
SP01	PMF, Int-2	58, F	complex karyotype **additional: add(10q), del(20q), +21x3**	complex array including chromothripsis 5q	JAK2 31% TP53 43% ZRSR2 20%
SP02	post-PV MF, Int-2	70, F	normal **[del(20q) in 1 metaphase]**	CN-LOH 4q CN-LOH 9p del(21q)	JAK2 78% TET2 35% U2AF1 51%
SP03	PMF, Int-1	64, M	normal	CN-LOH 4q CN-LOH 13q	triple negative SRSF2 22% TET2 49%
SP04	PMF, Int-2	79, M	del(20)(q11.21q13.32)	del(20)(q11.21q13.32)	JAK2 85% TET2 37%
SP05	PMF, Int-2	68, M	t(8;12)(q13;q15)	no known CNA or CN-LOH	CALR 14.8% TP53 45% EZH2 45%
SP06	PMF, Int-2	65, M	normal	CN-LOH 8q	JAK2 13% ASXL1 11% RUNX1 10% NRAS 5.4% EZH2 26%
SP07	PMF, Int-2	63, M	normal	NA	JAK2 42% SRSF2 25%
SP08	post-PV MF, Int-2	65, F	del(20)(q11.23q13.33) and subclonal +8	del(20)(q11.23q13.33) CN-LOH 5q	JAK2 41% ZRSR2 25%
SP09	post-PV MF, Int-2	64, F	complex karyotype **additional: t(1;7), ins(1;19), del(12p), -13, i(17q), der(20)t(13;20)**	complex array CN-LOH 9p	JAK2 69%
SP10	PMF, Int-2	75, M	normal	CN-LOH 21q	JAK2 28% TP53 32% SF3B1 40%
SP11	post-PV MF, Int-2	53, F	normal	CN-LOH 9p	JAK2 55%, TET2 6.8%
SP12	PMF, Int-2	73, M	del(13)(q13.1q21.2)	del(13)(q13.1q21.2) CN-LOH 9p	JAK2 91% **SRSF2 45%** RUNX1 45%
SP13	post-ET MF, Int-2	52, F	normal	CN-LOH 4q CN-LOH 7q	CALR 19.4% ASXL1 33% TET2 63% EZH2 74% SETBP1 36%
SP14	PMF, Int-1	51, M	del(20q)(q11q13) del(12p)(p13.2) [FISH]	del(12p)(p13.2)	JAK2 40%, ASXL1 39.4% SRSF2 39% KRAS 38%

Abbreviations: DIPSS, dynamic international prognostic scoring system; aCGH+SNP, array comparative genomic hybridization and single nucleotide polymorphisms microarray; NGS, next generation sequencing; PMF, primary myelofibrosis; Post-PV MF, post polycythemia vera myelofibrosis; Post-ET MF, post essential thrombocythemia myelofibrosis; CNA, copy number alteration; CN-LOH, copy neutral loss of heterozygosity; F, female; M, male; NA, not available. Text highlighted in bold indicate cases with abnormalities found in the spleen but not peripheral blood or bone marrow.
*Only selected regions known to be recurrent in myeloid malignancies are shown.

within chromosome 5, explaining the genetic complexity. Of note, a *TP53* as well as *JAK2*V617F and *ZRSR2* mutations were detected by NGS in this patient.

Figure 2.1 Chromosome ideogram of all alterations detected in myelofibrosis (MF) spleens from 13 patients



Chromosome ideogram of all alterations detected in myelofibrosis (MF) spleens from 13 patients. Bars depict the physical position of the alteration but not the size. All bars shown between arrows represent genomic changes within chromothripsis of chromosome 5 in SP01. The novel recurrent regions of gains mapping to 1p13.2 (RHOC), 12q24.31 (NCOR2), 13q34 (RASA3) and 17q12 (TAF15) are shown in circles.

Array analysis identified nine regions of recurrent focal gains, each found in a remarkably high proportion of patients (**Figure 2.1**). These regions ranged in size from 786bp to 1.9kb and were located in exons or introns of single genes. Four of these regions involved the genes: *RHOC* (1p13.2), *NCOR2* (12q24.31), *RASA3* (13q34) and *TAF15* (17q12), that have been previously implicated in myeloid malignancies (**Table 2.2**). All regions showed a log2 ratio ranging between 0.5 to 1.6 (average 0.9), significantly higher than the acceptable log2 ratio of +/-0.18 used in our calling criteria.

Table 2.2 Regions of recurrent chromosomal gains and involved genes

Chromosomal region	Size of region (bp)	Average log2 ratio	Involved gene	Exon / intron	# of patients in study cohort (%)*
1p32.2	786	0.5	RHOC	Intron	9 (69%)
12q24.31	1,895	1.0	NCOR2	Exon 49	12 (92%)
13q34	1,958	1.3	RASA3	Exons 18, 17	11 (85%)
17q12	1,272	0.6	TAF15	Exons 13-15	9 (69%)

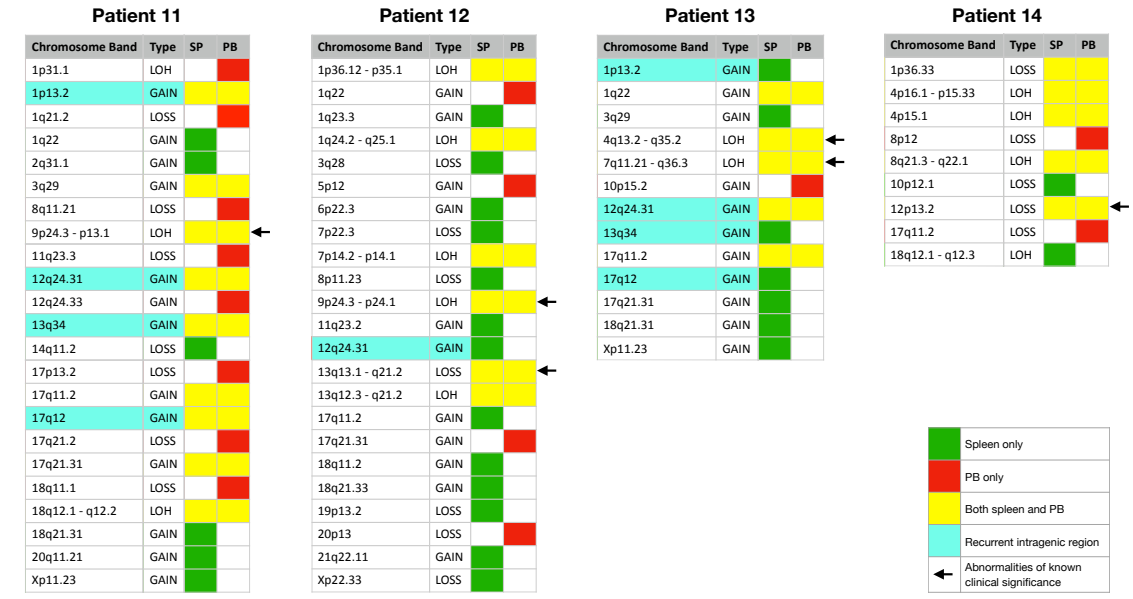
Abbreviations: RHOC, Ras homolog family member C; NCOR2, Nuclear receptor corepressor 2 (also known as SMRT, Silencing Mediator Of Retinoic Acid And Thyroid Hormone Receptor); RASA3, Ras p21 protein activator 3; TAF15, TATA-box binding protein associated factor 15.
***The signal-intensity log2 ratio (patient vs. reference DNA) of ≥0.18 for gain or ≤0.18 for loss was used to call copy number alterations.**

Of the 37 regions of CN-LOH detected by CMA, the two most frequent were 4q and 9p, occurring in 38% (5/13) and 31% of the patients (4/13), respectively. In one patient, CN-LOH of 4q included the *TET2* gene, and correlated with an allele burden of 63% of a *TET2* mutation detected by NGS. The remaining four cases of CN-LOH of 4q did not involve *TET2*. In contrast, all four patients with CN-LOH of 9p involved the *JAK2* gene and correlated with a high *JAK2*V617F allele burden in the mutational analysis (**Table 2.1**). Patient SP09 demonstrated triplication of 9q and partial quadruplication of 9p resulting in UPD of the entire chromosome 9. In addition to CN-LOH of 4q, SNP analysis of Patient SP13 revealed CN-LOH of the entire long arm of chromosome 7q including the region that contains the *EZH2* gene. This patient had an *EZH2* mutation with an allele burden of 74%. CN-LOH of 7q was identified previously in patients with MPN and correlated with decreased survival after leukemic transformation.[109]

Array analysis confirmed all cytogenetic abnormalities except in Patient SP05 who had a balanced translocation t(8;12), and Patient SP14 who had del(20q) in 5% of metaphase cells, which is below the level of detection (20%) of array testing. However, in Patient SP14, CMA identified deletion of *ETV6* (12p13.2), confirmed by FISH in 98% of PB cells and 61% of spleen cells. This abnormality in the apparently dominant clone was not detected by metaphase cytogenetics and was not included in the initial FISH panel, highlighting the utility of CMA for enhancing the sensitivity of cytogenetic evaluation. This patient is the only case in our cohort who did not harbor any of the aforementioned recurrent gains identified in a high proportion of the patients. Interestingly, this patient subsequently progressed to chronic myelomonocytic leukemia (CMML). *ETV6* was originally identified in a t(5;12)(q33;p13) occurring in CMML, and was recently shown to be associated with transformation of myelodysplastic syndromes (MDS) to CMML.[110]

Simultaneous CMA analysis was performed on pairs of PB and SP specimens from four patients, shown in **Figure 2.2**. DNA from the same patient was hybridized on the same day and the arrays of both tissues were repeated twice to exclude the possibility of inter-daily variability. When comparing the matched SP and PB cells, CMA showed multiple discordant copy number abnormalities (CNA) and regions of CN-LOH in all 4 cases, with an overall higher number of CNAs in the spleens. However, CN-LOH of 4q and 9q, each occurring in two patients, as wells as del(13q) and del(12p) in patients 12 and 14 respectively, were all observed in both spleen and peripheral blood.

Figure 2.2 Comparison of matched spleen and PB specimens from four patients



Green boxes show alterations detected in spleens only; red, in PB only; and yellow, in both tissues. Aqua highlights the novel recurrent regions of gain discussed above. Of note, in SP11 all four recurrent regions were detected in both spleen and PB, while in SP12 and SP13 most were detected only in the spleen (except for gain mapping to 12q24.31 in SP13). Arrows point to abnormalities of known clinical significance, including del(13q), del(12p13.2) involving ETV6, and CN-LOH of 9p, 4q an 7q involving JAK2, TET2 and EZH2, respectively.

NGS identified an average of 2.8 mutations of known or likely oncogenic potential in each spleen, and a plethora of additional mutations of unknown significance, many of these affecting genes that have known roles in myeloid malignancies (not shown). Aside from MPN driver mutations detected in 13 of the 14 patients, additional mutations that have been identified in recent years in MPNs were found in most patients. The most common additionally mutated genes were *TET2* (in 5 patients) followed by *ASXL1, EZH2, SRSF2* and *p53* (in 3 patients), and *RUNX1* and *ZRSR2* (in 2 patients). Mutations in *SF3B1*, *U2AF1* and *SETBP1* each occurred in one patient. The occurrence of a high number of pathogenic mutations in epigenetic modifiers and spliceosome complex members is consistent with previous reports of genomic profiling in an advanced MF patient

population.[25,35,111] A mutation in *ZRSR2*, a spliceosome regulatory gene implicated in MDS, has not been previously reported in MF, and was present in two patients in our cohort. NGS was used to compare spleen and PB mutational profile in four individual patients, matched with samples compared by CMA. In contrast to the CMA results, the correlation between mutation and allele burden in the spleens and PB/BM was strikingly high. One exception is Patient SP12, who had an additional *SRSF2* mutation detected only in spleen cells, with an allele burden of 43%.

2.5 Discussion

Extramedullary hematopoiesis (EMH) resulting in splenomegaly is one of the hallmarks of MF, and frequently progresses over time. While most of the evidence regarding genomic abnormalities in MF is based on BM and PB specimens, very little evidence is available about splenic molecular pathology. This may be due at least in part to the restricted access to splenic tissue, relying solely on investigations of specimens made available following splenectomy. This limitation has been more pronounced in recent years, as the use of splenectomy has decreased due to the widespread use of the oral JAK1/2 inhibitors.[112] In this regard, our cohort provides a unique sample of splenectomy specimens obtained during the era of JAK 1/2 inhibitor therapy.

In our cohort of 14 patients, cytogenetic abnormalities were observed in 50% of the cases, the most common being del(20q), consistent with reports from BM/PB studies.[63,113] Concordant cytogenetic results (both normal and abnormal) were observed in 12 of 14 (86%) patients at the time of splenectomy, similar to the frequency reported by Mesa et al.[103] Discordant results were observed in 2 patients (14%), both cases of a complex karyotype in which the spleen harbored additional abnormalities not found in the PB. One

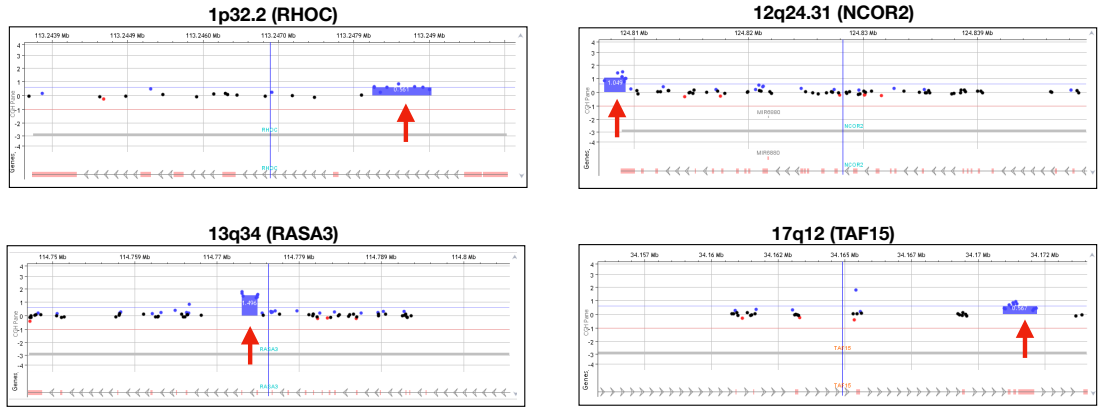
patient who had a normal karyotype at the time of splenectomy, with del(20q) detected in a single spleen metaphase cell, developed a dominant del(20q) clone in a BM study performed 2.5 years later. This sequence of events suggests that this clone might have originated in the spleen. CMA confirmed the cytogenetic findings in most patients and uncovered a large number of additional abnormalities not detected by cytogenetics or FISH in all patients including those with a normal karyotype. When used to compare spleen and PB in four cases, CMA showed discordant results, with a higher genomic diversity in spleens. However, known cytogenetic abnormalities and regions of CN-LOH were all concordant. Exome sequencing identified most MPN-restricted and non-restricted mutations previously reported in the literature[35], with an average of 2.8 mutations occurring per patient consistent with the advanced-stage of this MF patient population. Mutated genes and their variant allele frequency (VAF) were highly comparable between spleen and PB samples, except for patient-12 who harbored a *SRSF2* mutation with a VAF of 45% in spleen but not PB cells.

The high concordance between spleens and BM/PB observed with both conventional cytogenetic studies (including FISH) and NGS, and the highly comparable frequency of the chromosomal abnormalities and mutation allelic burden, suggest an overall similar clonal architecture existing within these two tissues. These findings confirm previous studies demonstrating constant trafficking of MF-HSPCs and support a crosstalk between these tissues.[102,103,114] In spite of the overall similarity, discordant findings between spleens and BM/PB were detected with each modality (**Table 2.1**). In almost all of these instances of discordance, additional abnormalities were found in the patients'

spleens, supporting the hypothesis that the spleen provides a distinct tumor microenvironment.

The use of high-resolution CMAs has enabled the identification of clinically significant genetic abnormalities in myeloid malignancies.[89,90,115] In our cohort, CMA detected recurrent intragenic gains in four regions involving *RASA3*, *NCOR2*, *RHOC*, and *TAF15*, which have been implicated in myeloid malignancies (**Figure 2.3**). Intragenic gains in coding or non-coding sequences within a gene can lead to either decreased or increased expression of the particular gene.[116-118] From these studies we suggest that these genomic changes might play a role in MF disease progression.

Figure 2.3 Four recurrent regions of gain identified by CMA



Visualization of four genomic regions detected by aCGH+SNP. Genomic DNA from patient spleens labeled with Cy5 and a gender-matched control reference DNA labeled with Cy3, were co-hybridized to the array. The log2 ratio of the signal intensity from patient DNA versus reference DNA is shown on the y-axis. The x-axis shows the genomic location and gene region. Each region with increased signal intensity is highlighted in blue and arrowed in red.

Ras GTPase Activator 3 (*RASA3*) is a tumor suppressor gene encoding for a GTPase activating protein (GAP) which functions as a negative regulator of the Ras and Rap1 signaling pathways. Rap1 is a well-known regulator of integrin signaling in platelets and megakaryocytes, and RASA3 has been shown to be a critical inhibitor of Rap1-dependent platelet activation.[119,120] Inactivation of RASA3 in mouse hematopoietic cells has been shown to lead to profound alterations in megakaryocyte functions, and to result

in an MF-like phenotype including severe thrombocytopenia, anemia, BM fibrosis, EMH, splenomegaly and decreased survival.[121] These alterations, including megakaryocyte dysplasia and absence of terminal differentiation in proplatelets, were attributed constitutive activation of integrins due to the abnormal Rap1 activation.[121]

Nuclear Receptor Corepressor 2 (*NCOR2*) encodes for Silencing Mediator of Retinoic Acid and Thyroid Hormone Receptor (SMRT), part of a large protein complex which includes histone deacetylase 3, that can modify chromatin structure or bind to nuclear receptors to prevent transcriptional activity. Reduced expression and function of SMRT has been shown to contribute to breast and prostate cancer progression.[122] Loss of SMRT binding to the leukemic fusion proteins PML/RARα and AML1/ETO (the products of translocation (15;17) and (8;21), respectively) contributes to leukemogenesis in mouse models.[123] Mice carrying inactivating mutations in the receptor interaction domains of SMRT developed a syndrome resembling MF that included BM fibrosis and EMH with splenomegaly. Inactivation of SMRT resulted in reduced repression of RARα leading to increased cytokine gene expression, particularly thrombopoietin (*Tpo*), in the mouse BMs, and upregulation of TGFβ and PDGF in megakaryocytes, closely resembling pathological pathways that occur in patients with MF.[124]

Ras Homolog Family Member C (*RHOC*) located on chromosome 1p13.2 is a member of the Rho family of small GTPases and the protein encoded by this gene is prenylated at its C-terminus and localizes to the cytoplasm and plasma membrane. Overexpression of this gene is associated with tumor cell proliferation and metastasis.[125] Recently, RHOC was detected along with 6 additional genes, by an integrative analysis of DNA methylation and gene expression in a cohort of older (>60 years) cytogenetically

normal AML patients.[126] Lower expression and higher DNA methylation of RHOC were both associated with a longer OS in this patient cohort and additional verification cohorts.

TATA-Box Binding Protein Associated Factor 15 (*TAF15*) gene, located on chromosome 17q12, encodes a member of the TET family of RNA-binding proteins. Translocations involving this gene play a role in acute leukemia and extra skeletal myxoid chondrosarcoma, and mutations in this gene may play a role in amyotrophic lateral sclerosis.[127-129]

The detection of novel recurrent abnormalities, and the identification of candidate-genes that are potentially affected, support the utility of CMAs in characterizing the genetic landscape of myelofibrosis. Further investigations are required to confirm a role of these candidate genes in MF.

In conclusion, this study supports the hypothesis that spleen in MF provides a distinct tumor microenvironment that can contribute to disease progression, and confirms earlier studies comparing MF-spleens to PB/BM specimens. CMA analyses detected four regions of recurrent chromosomal gains that may implicate candidate genes in disease pathogenesis, and merit further investigation. Lastly, this study provides an example of the challenge of integrating genomic data from numerous available technologies, a challenge that is expected to dominate the diagnostic approach to MF, as well as other hematological malignancies in the future.

Zimran E*, Tripodi J*, Rampal R, Rapaport F, Zirkiev S, Hoffman R, Najfeld V. Genomic characterization of spleens in patients with myelofibrosis. Haematologica. 2018 May 10;:haematol.2018.193763. * Contributed equally to this publication

Chapter 3. Specific Aim 2

3.1 Specific Aim 2

The gain of the long arms of chromosome 1 (+1q) and rearrangements of 12q are chromosomal abnormalities that occur in the early forms of Ph- MPN that progress to more advanced forms of the disease.

Sub-aim 2a: To investigate the molecular consequences of the gain of the long arms of chromosome 1 (+1q)

Sub-aim 2b: To identify specific genes located on 12q that are important in disease pathogenesis, progression, and/or transformation.

3.2 Approach

The Philadelphia chromosome negative myeloproliferative neoplasms (MPNs) including polycythemia vera (PV), essential thrombocythemia (ET) and the pre-fibrotic form of primary myelofibrosis (PMF) have the potential to progress to more overt forms of MF and a form of acute leukemia (AML) termed MPN-blast phase (MPN-BP). MPN-BP has a dismal prognosis, with a median survival of <6 months.[130] After 15 years, up to 14% of PV patients and 9% of ET patients will progress to MF while 19% of PV patients and 5% of ET patients will progress to MPN-BP.[131-133]

Currently, the mechanisms underlying MPN disease progression remain elusive but are believed to involve several pivotal factors including: a pro-inflammatory milieu and the acquisition of additional genomic and epigenomic alterations.[134-137] Specific chromosomal abnormalities in MPNs are associated with a poor prognosis and the longer the duration of the disease the more likely additional chromosomal abnormalities will be

acquired.[138,139] At diagnosis about 25% of PV patients have an abnormal karyotype, but this increases to 50% at the time of transformation to MF or MPN-BP.[140,141] In ET, <10% of patients have an abnormal karyotype at diagnosis as compared to 15-46% at the time of transformation.[25] Studies have shown that additional copies of the long arms of chromosome 1 (+1q) is a recurrent MPN associated abnormality. Additionally, translocations of the whole long arm of chromosome 1q or part of 1q to several different recipient chromosomes occur in MPN-BP.[106,142] Additional studies have reported that +1q abnormalities are associated with PV disease progression and that up to 70% of patients with PV-related MF had +1q.[23,139,143]

Chromosome 12 abnormalities have been previously reported in cases of Ph-MPN.[138,144-146] Specific structural abnormalities at 12q15 and 12q24 have been reported for individual patients with primary MF.[147,148] Disruptions that affect the genes *HMGA2*, an architectural transcription factor, and *SH2B3*, a multifunctional adapter protein, located at the 12q15 and 12q24 loci respectively, have been identified in a handful of cases.[39,144,145,149] In a large retrospective study of 1787 Ph- MPN patients, 2% harbored alterations involving chromosome 12 by cytogenetic evaluation and were associated with PMF and PV related MF.[150] The most common regions involved 12q13, 12q15, and 12q24 and >40% of patients with chromosome 12 abnormalities had cytogenetic evolution.[150] How structural abnormalities of chromosome 12 contribute to Ph-MPN pathogenesis is not entirely understood.

Up to 8% of patients with early forms of Ph-MPN (ET, PV) progress to advanced disease such as MF and up to 20% of PMF patients will progress to MPN-BP.[111] The gain of the long arms of chromosome 1 (+1q) and rearrangements of 12q are chromosomal

abnormalities that are associated with disease progression. The aim is to integrate genomic technologies to: Investigate the molecular consequences of the gain of the long arms of chromosome 1 (+1q) and to Identify specific genes located on 12q that are important in disease pathogenesis, progression, and/or transformation.

3.3 Materials and Methods

3.3.1 Patients

A retrospective analysis was performed on 1,294 Ph-MPN patients from our laboratory database. PV, ET, MF and MPN accelerated phase/blast phase (MPN-AP/BP) were diagnosed by criteria established by the World Health Organization.[151] Patients with MPN-AP were defined as having 10-19% blasts in their peripheral blood or bone marrow, while MPN-BP patients were defined as having ≥20% blast cells.[152] The Institutional Review Board of the Mount Sinai School of Medicine approved this research and is in accordance with the Declaration of Helsinki.

3.3.2 Conventional cytogenetic analyses

Bone marrow or unstimulated peripheral blood G-banded metaphase cells were obtained using standard methodology, as described in Specific Aim 1, Chapter 2, Section 2.3.2

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3.3.3 Fluorescence *in situ* hybridization (FISH)

FISH studies were performed on fixed cell suspensions from bone marrow and peripheral blood tissues as described previously.[106] Interphase FISH (I-FISH) All FISH probes were obtained from Abbott Molecular (Des Plaines, IL).

3.3.4 DNA extraction and aCGH+SNP

Genomic DNA was prepared from MNCs isolated from patient spleens, BM, and PB tissues using Qiagen's Gentra PureGene DNA purification kit (Qiagen Inc., Valencia, CA) as per the manufacturer's instructions and described in detal in Specific Aim 1, Chapter 2, Section 2.3.4

3.3.5 Quantitative PCR

Peripheral blood and marrow mononuclear cells (MNCs) were obtained from MF patients with gains of 1q and 12q rearrangements. Normal bone marrow MNCs were obtained from AllCells (Alameda, CA). RNA was extracted from MNCs using RNeasy mini kit (Fisher Scientific, Waltham, MA) and was reverse transcribed using RNA to DNA ecodry kit (Fisher Scientific, Waltham, MA). Quantitative PCR (qPCR) was performed using SYBR green master mix (Fisher Scientific, Waltham, MA) to test transcript levels of human *MDM4* and *PSMB2* (localized at the short arms of #1, at 1p34.3) as the housekeeping gene (Qiagen, Germantown, MD).

3.3.6 Next generation sequencing (NGS)

High-throughput sequencing with a targeted deep sequencing assay of 585-genes (HemePACT) was performed on 355 patients, participating in the MPN-RC clinical trials, as previously described.[153] Briefly, peripheral blood or bone marrow cells were sequenced at an average coverage of 829x (with a standard deviation of 130). The reads were aligned to the human genome (UCSC build hg19) using the Burrows-Wheeler Aligner with maximal exact matches. The Cancer Genome Project pipeline (https://github.com/cancerit) was used to compare the tumor samples to a standard cancer-free germline following the pipeline recommendations. SnpEff was used to annotate variants with functional consequence on genes [154]. The lower limit of detection of the assay employed in this analysis is 0.5% VAF. For the ISMMS database complete next generation sequencing (NGS) was not available except for driver mutations because many patients were studied before 2007 and the application of NGS was not available until recently.

3.3.7 Statistical Analysis

Statistical analysis between groups was performed using the Fisher exact test. For RT-PCR studies statistical significance between transcript levels was determined using the Student t-test. Statistical significance was defined as $p<0.05$. GREVE: Genomic Recurrent Event ViEwer to assist the identification of patterns across individual cancer samples and the R software language using the Genomic Ranges package was used to map and identify overlapping genomic regions.

3.4 Results

3.4.1 Sub-Aim 2A

Among the 1,294 MPN patients, 423 (33%) had an abnormal karyotype (**Table 3.1**). The most frequent chromosomal abnormality among the 1,294 patients was del(20)(q11q13) occurring in 7% of total patients and was present alone or in combination with other abnormalities. The second most frequent cytogenetic abnormality was the gain of chromosome 1q observed in 17% of the chromosomally abnormal patients (6% of total patients).

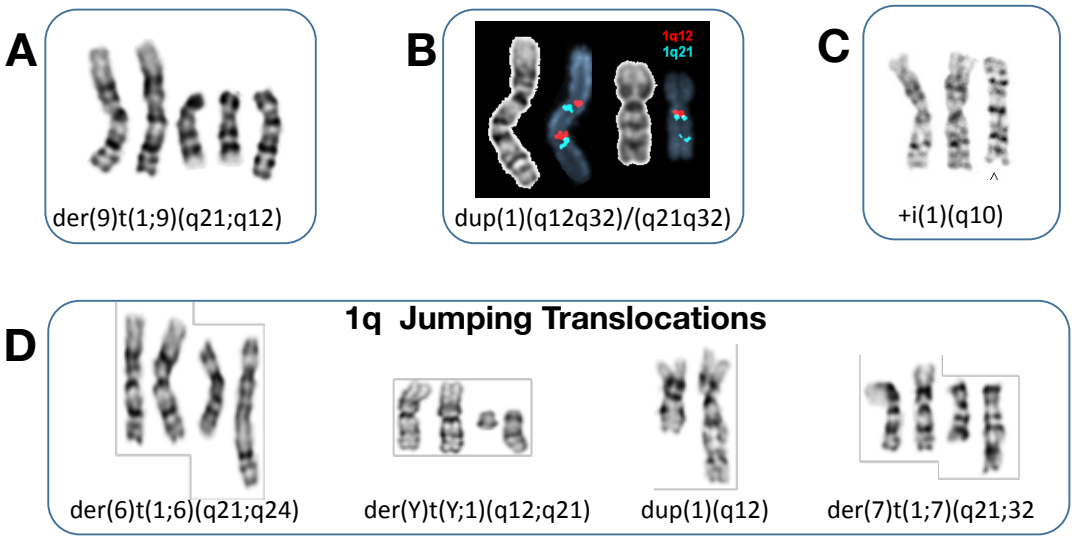
Table 3.1 Prevalence of +1q , 12q rearrangements and deletions of 17p among 1,294 patients with MPN

	Total (%)
Number of patients	1294
Chromosomally Abnormal	423 (33%)
+1q(% total)	72 (6%)
12q rearrangements (% total)	24 (2%)
del(17)(p13) (% total)	30 (2%)

MPN: Myeloproliferative Neoplasm

Gain of 1q was attributed to three different events: (1) as an unbalanced translocation with the breakpoint occurring at 1q12~21; (2) a duplication of the 1q region inserted within 1q and/or (3) a jumping +1q to different partner chromosomes (**Figure 3.1**). The most frequent unbalanced translocations identified were +der(9)t(1;9)(q21;q12) resulting in three copies of 1q and 3 copies of 9p, followed by der(6)t(1;6) (q21;p22) (**Figure 3.1**) and der(1;7)(q10~q12; p10~q12). Extra copies of 1q were also observed as the whole-arm or part of the +1q participated in unbalanced translocations to chromosomes 1, 13, 14, 15, 18, 19, 21or 22 (26 patients). Duplication of 1q21-q31/q41 region was found in 6 patients (**Figure 3.1**).

Figure 3.1 Chromosomal rearrangements leading to gain of 1q



Chromosomal rearrangements leading to gain of 1q. (A) Unbalanced translocation between chromosomes 1 and 9 resulting in gain of the long arm of chromosome 1q and the short arm of chromosome 9p. (B) Duplication of the long arm of chromosome 1q involving two different breakpoints 1q12 and 1q21 within in the same patient. (C) An iso-derivative chromosome 1q resulting in four copies of the long arms of chromosome 1q. (D) Jumping translocations of 1q involving 4 different chromosomes,

As shown in **Table 3.2**, +1q was more prevalent in more advanced forms of MPNs (MF and MPN-AP/BP) than PV and ET (*P*<.001). Only 2% of patients with PV and ET had +1q, however, 9% of patients with MF (PMF, PV-related MF, ET-related MF) and 43% of MPN-AP/BP had +1q. More than 50% of patients who acquired +1q had an initial diagnosis of PV (PV vs ET P<.001, PV vs PMF p=.002) (**Table 3.3**). In most of these patients with a history of PV the +1q abnormality was first observed at the time they had progressed to MF or MPN-AP/BP (**Table 3.4**). Gain of 1q was less frequently observed in ET and PMF patients.

Table 3.2 Prevalence of +1q in different stages of MPN	
	Total
Proliferative Phases of MPNs (PV, ET)	781
No (%) with +1q	14 (2%)
Myelofibrosis	456
No (%) with +1q	42 (9%)*
MPN-AP/BP	37
No (%) with +1q	16 (43%)**

*Incidence of gain of 1q in patients with MF vs. PV and ET *P* <.0001
**Incidence of gain of 1q in patients with MPN-AP/BP vs. PV and ET *P*<.0001

Table 3.3 Initial MPN Diagnosis of Patients who acquired +1q ,12q rearrangements and del(17p)			
Chromosomal Abnormality	**PV**	**ET**	**PMF**
+1q	39/72 (54%)*	13/72 (18%)	20/72 (28%)
12q rearrangement	2/24 (8%)	1/24 (4%)	21/24 (88%)**
del(17)(p13)	8/30 (27%)	5/30 (17%)	17/30 (57%)***

*Incidence of patients who acquired gain of 1q with initial presentation of PV vs. ET *P*<.001, PV vs. PMF *P*=.002
**Incidence of patients who acquired 12q rearrangement with initial presentation of PMF vs. PV *P*<.0001, PMF vs. ET *P*<.0001
***Incidence of patients who acquired del(17)(p13) with initial presentation of PMF vs. PV *P*=0.02, PMF vs. ET *P*=.001

Table 3.4 Stage of MPNs at which +1q, 12q rearrangements and del(17p) were first observed							
Chromosomal Abnormality	**No. of patients**	**PV**	**ET**	**PMF**	**PV-related MF**	**ET-related MF**	**MPN-AP/BP**
+1q	72	12 (17%)	2 (3%)	18 (25%)	17 (24%)	7 (10%)	16 (22%)
12q Rearrangement	24	0 (0%)	0 (0%)	18 (75%)	1 (4%)	1 (4%)	4 (17%)
del(17)(p13)	30	0 (0%)	0 (0%)	15 (50%)	3 (10%)	2 (7%)	10 (33%)

MPN: Myeloproliferative Neoplasm; PV: polycythemia vera; ET: essential thrombocythemia; PMF: primary myelofibrosis; MPN-AP/BP: MPN-Accelerated Phase/Blast Phase

Sequential cytogenetic studies (more than 3) were available in 14 patients. **Figure 3.2** and **Figure 3.3** shows chronological acquisition of +1q and its association with MPN disease progression. The first patient (**Figure 3.2**) was diagnosed with PV at age 32 and the initial cytogenetic abnormality, iso-chromosome 9p, appeared in 97% of (28/29 metaphases) cells. During the patient's clinical course which spanned 16 years he had 20 cytogenetic analyses. The initial acquisition of +1q in 10% of cells was observed seven years after diagnosis as an additional abnormality in the clone with iso(9p) clone. Over time greater numbers of cells had +1q (10% to 100%), including +1q jumping to four different recipient chromosomes (1, 6, 7 and Y chromosome). The patient transformed to MPN-BP nine years after the initial detection of +1q. The second patient (**Figure 3.3**) was an ET patient who was diagnosed at age 56 and had duplication of 1q present in 36% of cells as the sole cytogenetic abnormality. As the disease progressed to PV the percentages of hematopoietic cells with dup(1q) increased to 50%. After three years the patient progressed to MF which was accompanied by 95% of the cells harboring dup(1q) and this remained the sole abnormality. These studies demonstrate that the burden of cells with +1q gradually increases over time in MPN patients and frequently precedes evolution to a more advanced form of MPN.

To identify the minimal region of gain on chromosome 1q CMAs were performed on the DNA extracted from patients who harbored gains of the long arms of 1q. CMA analysis demonstrated that patients with +1q had a gain of 105 Mb region between 1q21 and 1q44 and included the *MDM4* (mouse double minute homolog 4) gene localized to 1q32.1 (**Figure 3.4**).

Figure 3.2 Sequential cytogenetic studies in a patient with PV

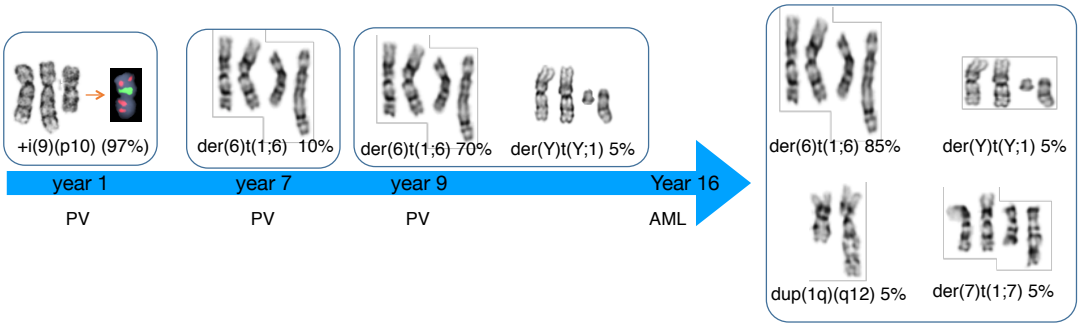


Figure 3.2. Sixteen year sequential cytogenetic studies in a patient with PV that transformed to AML. The initial acquisition of +1q in 10% of cells was observed seven years after diagnosis. Over time greater numbers of cells had +1q, 10% at year 7 and 75% at year 9. The patient transformed to MPN-BP nine years after the initial detection of +1q with 100% of metaphase cells showing gain of 1q as part of jumping translocations.

Figure 3.3 Sequential cytogenetic studies in a patient with ET

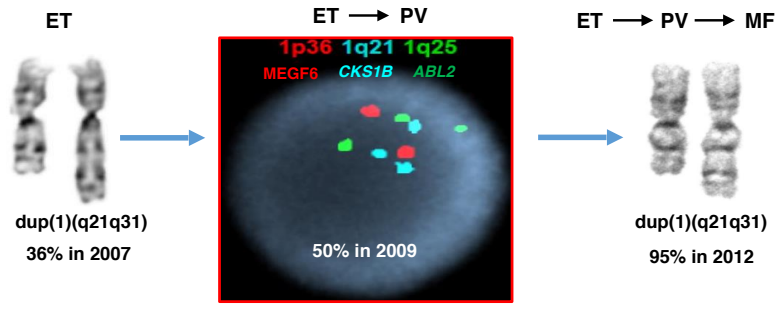
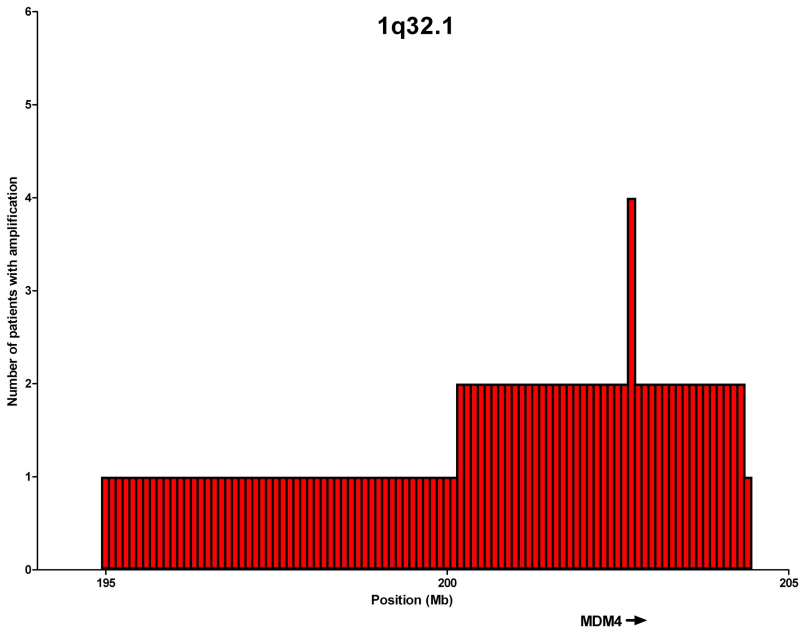


Figure 3.3. A patient with ET had duplication of 1q present in 36% , as the disease progressed to PV the percentages of hematopoietic cells with dup(1q) increased to 50%, the patient progressed to MF with 95% of the cells with dup(1q).

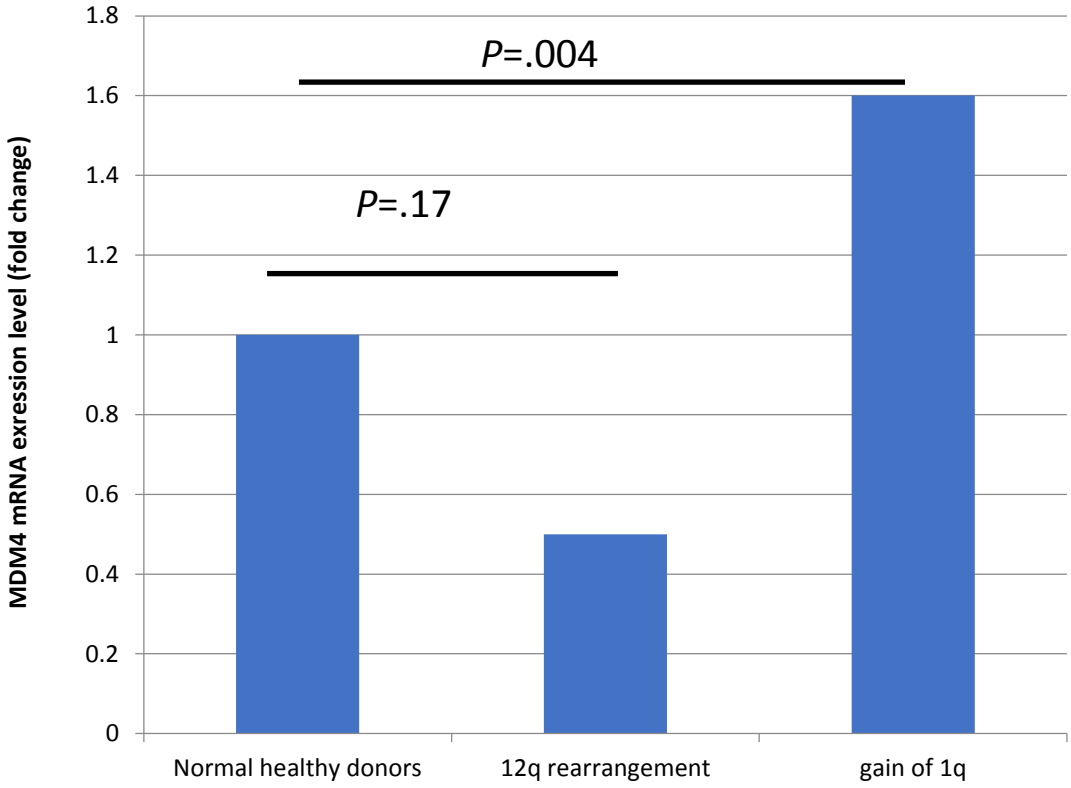
Figure 3.4 Minimal Region of Gain on Chromosome 1



CMA showing four of six patients with gain of 1q32.1 that includes the *MDM4* gene.

Quantitative PCR was performed to asses if the gain of *MDM4* resulted in increased expression. Peripheral blood and bone marrow mononuclear cells (MNCs) were obtained from patients with gains of 1q and MDM4 transcript levels were measured. The results showed (**Figure 3.5**) that there was a 1.64-fold increase in MDM4 transcript levels in MPN patients with gain of 1q (n=9).

Figure 3.5 MDM4 transcript levels in MPN patients with +1q or 12q rearrangements



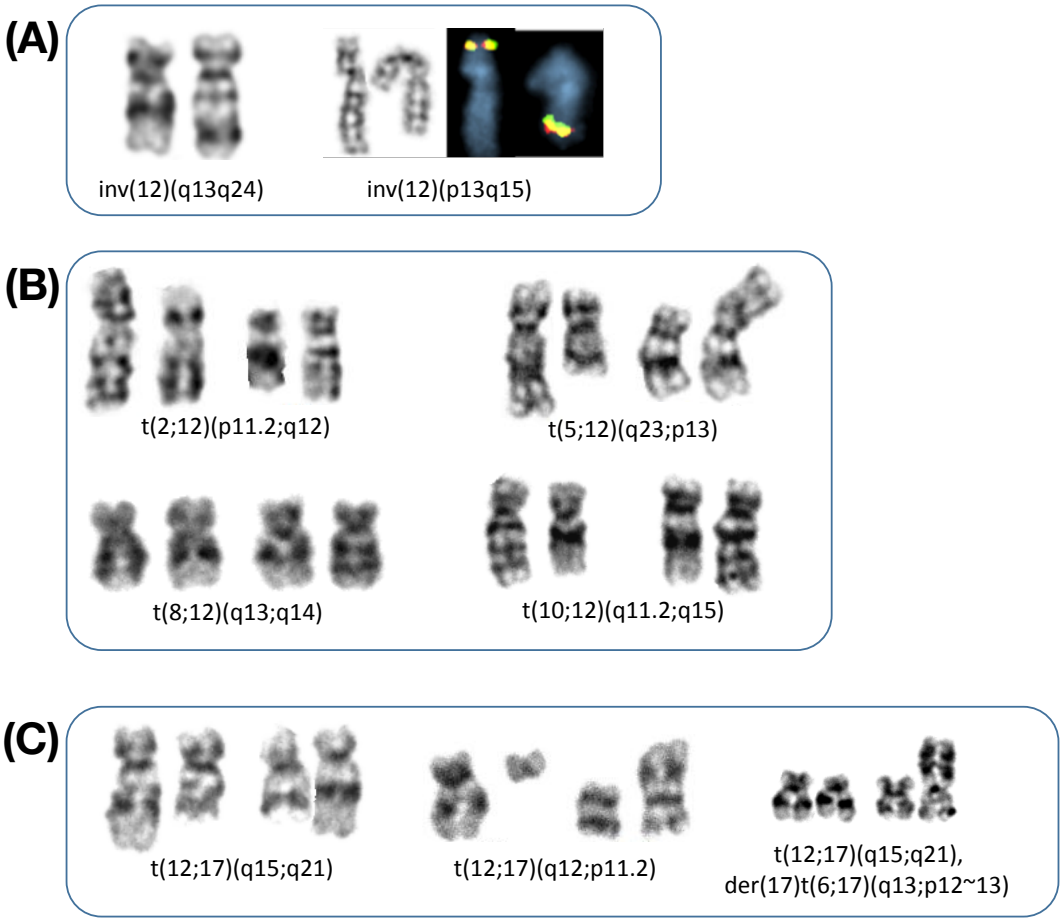
Gain of 1q in MF patients is associated with increase in MDM4 transcript level. MDM4 transcript levels were measured in the mononuclear cells of MPN patients with gain of 1q, MPN patients with a 12q rearrangement and healthy donors. There was a 1.64-fold increase in MDM4 transcript in MPN patients with gain of 1q (n=9) as compared to healthy individuals (n=10) (P=.004) but not in patients with 12q rearrangements (n=5).

The MPN driver mutations were evaluated in only a subset of patients due to the fact that many were studied prior to discovery of these mutations. *JAK2*V617F was observed in 84% (37/44) of MPN pts with +1q as compared to 23% (3/13) MPN pts with 12q rearrangements ($P<.001$). The presence of CALR or MPL mutations was very infrequent in patients with +1q.

3.4.2 Sub-Aim 2B

Chromosome 12q rearrangements were observed in 6% of the chromosomally abnormal patients (2% of total patients) (**Table 3.1**). Two types of 12q rearrangements were observed (**Figure 3.6**) (1) inv(12q)/der(12) affecting predominantly q13-q15 or less frequently q22 or q24 regions (10 pts); (2) balanced translocations between 12q and the following partner chromosomes 2, 5, 8, and 10 (11 pts). Three patients had a t(12;17)(q15;p12-p13/q21) karyotype. In all three patients, the breakpoint on chromosome 12 was at q15 however, the breakpoint on chromosome 17 differed; in two of the three patients there was a loss of *TP53* which is located at 17p13 but not in the third patient. The 12q15 breakpoint region was involved in 12/24 patients with 12q rearrangements. The other 50% of patients had the breakpoints at 12q13 (25%) or q21-24 (25%). Gain of 1q/dup(1q) and rearrangements with 12q were mutually exclusive with the exception of one patient with an unbalanced translocation der(12)t(1;12)(q25;q13). Moreover, in 84% of the patients the 12q abnormality was the sole abnormality; only 4 pts had additional karyotypic changes in the clone with 12q.

Figure 3.6 Types of chromosome 12 rearrangements



Types of chromosome 12q rearrangements were observed. Inversion and derivative chromosomes 12) affecting predominantly q13-q15 or less frequently q22 or q24 regions and balanced translocations between 12q other partner chromosomes. (A) Inversions of chromosome 12 involving either the long arm only, or involving both the short and long arms. (B) Balanced translocations between chromosome 12 and partner chromosomes 2, 5, 8, and 10 . (C). Translocation between chromosome 12 and chromosome 17.

One patient with a 12q abnormality had serial studies over a period of 29 years (1989-2018). At the time of diagnosis of PV, the patient was 33 years old and had an iso-chromosome involving the short arm of chromosome 9; i(9)(p10) as well as an iso-chromosome of the long arm of chromosome 9, i(9)(q10) in all 20 metaphase cells. Over

the next 28 years follow up studies of 144 metaphase cells showed 143 cells with i(9p) and i(9q) and only one normal 46,XY metaphase cell. After 29 years the patient's disease progressed to MF and 100% of 20 metaphase cells had i(9p) and i(9q) but for the first time a sub-clone harboring inversion of chromosome 12 was identified in 15% of cells [46,XY,i(9)(p10,i(9)(q10),inv(12)(q14q24.3)].

As shown in **Table 3.4** and **Table 3.5**, rearrangements of 12q were primarily associated with PMF. Twenty of the 24 patients with 12q rearrangements had PMF whereas 1 patient had PV-related MF and 1 patient had ET-related MF. Four patients had MPN-AP/BP, 3 of which had progressed from PMF. These studies confirm the association between 12q abnormalities and the development of MF.

Table 3.5 Prevalence of 12q rearrangements in different stages of MPN

	Total
Proliferative Phases of MPNs (PV, ET)	781
No (%) with 12q rearrangements	0 (0%)
Myelofibrosis	456
No (%) with 12q rearrangements	20 (4%)*
MPN-AP/BP	37
No (%) with 12q rearrangements	4 (11%)**

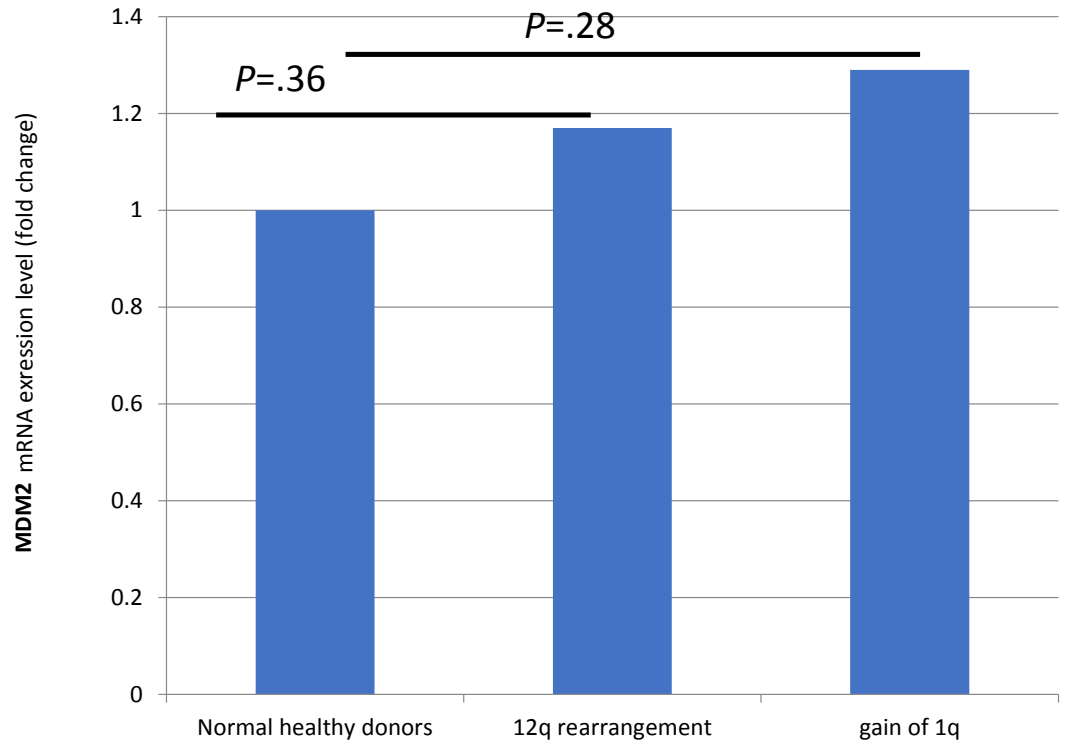
*Incidence of 12q rearrangements in patients with MF vs. PV and ET P <.0001
**Incidence of 12q rearrangements in patients with MPN-AP/BP vs. PV and ET P<.0001

In 50% of patients, structural rearrangements of chromosome 12q involved the 12q15 breakpoint region. Since *MDM2* (mouse double minute homolog 2) is localized to 12q15 and is structurally similar to *MDM4*, quantitative PCR was performed to determine if structural rearrangements affected *MDM2* expression levels. Peripheral blood and bone marrow mononuclear cells (MNCs) were obtained from patients with rearrangements of chromosome 12 and *MDM2* transcript levels were measured. A significant change in the

transcript levels of *MDM2* in MPN patients with 12q abnormalities was not observed (**Figure 3.5**).

Both *MDM4* and *MDM2* encode a nuclear protein that contains a TP53 binding domain at the N-terminus and a RING finger domain at the C-terminus. Both proteins bind the TP53 tumor suppressor protein and inhibit its activity. Loss or inactivation of TP53 plays a critical role in the pathogenesis of many cancers.[155] While TP53 mutations at low allele burden can be found in chronic MPNs, TP53 loss of heterozygosity is associated with transformation to leukemia and inactivating mutations of TP53 are found in 20% of MPN-BP patients.[61,156-159]

Figure 3.7 MDM2 transcript levels in MPN patients with +1q or 12q rearrangements



MDM2 transcript levels were measured in the mononuclear cells of MPN patients with gain of 1q, MPN patients with a 12q rearrangement and healthy donors. There was no increase in MDM2 transcript in MPN patients with 12q rearrangement (n=5) or gain of 1q (n=9) compared to healthy individuals (n=5).

A total of 30 patients (2% of all patients) had a deletion of 17p13, identified either cytogenetically or by interphase FISH (**Table 3.1**). All of these patients had either MF or MPN-AP/BP (**Table 3.4**). As shown in **Table 3.3**, the majority of *TP53* deletions were acquired in patients who had a diagnosis of PMF (PMF vs. PV *P*=.02, PMF vs. ET *P*=.001). The most frequent loss of 17p13 was due to the presence of monosomy 17, followed by deletion of 17p13, formation of isochromosome 17q and therefore loss of 17p and different balanced or unbalanced translocations involving the 17p13 region. In 97% (29/30) of patients these 17p structural abnormalities involved one chromosome 17 (heterozygous). Only one patient had homozygous loss of 17p13. One patient had a normal karyotype but a cryptic*TP53* deletion as detected by interphase FISH. Loss of 17p13 was identified as a sole abnormality in only 17% (5/30) of patients. Nine patients with TP53 deletions had NGS data and one of these patients had a TP53 mutation.

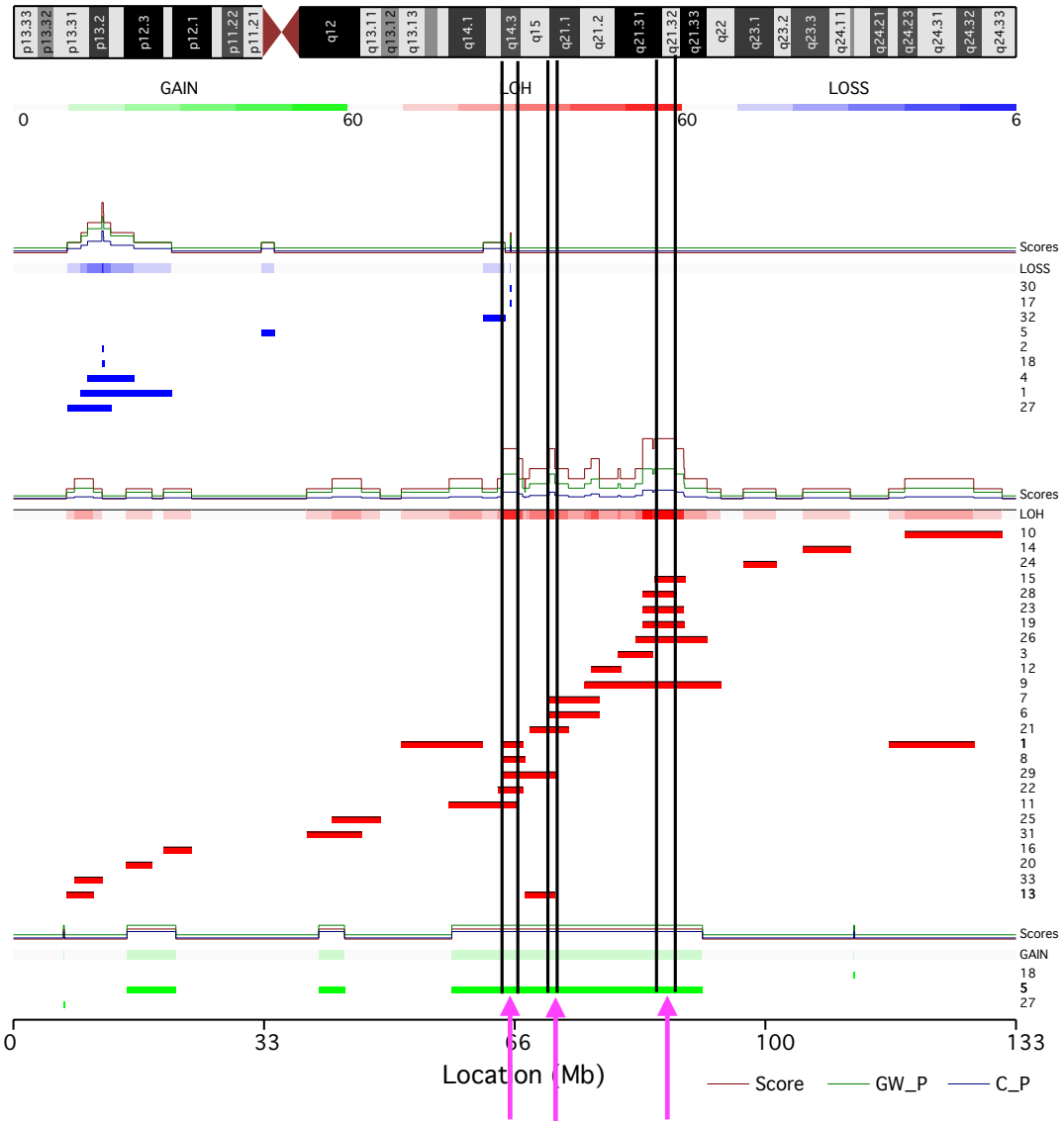
NGS was performed on 368 patients. Eight (2%) of these patients had *TP53* mutations but none of the patients had a 12q rearrangement, however one of these patients had +1q. The mean allelic burden for the 6 patients was 27% (PV- 6%, 33%, 37% and 48%), ET- 6% and MF- 32%. The mean allelic burden in the 2 patients with MPN-AP/BP was 43% (5% and 81%). Overall, this cohort of patients with *TP53* mutations was too small to determine if *TP53* mutations are associated with specific cytogenetic abnormalities however 2 of these 8 patients were cytogenetically abnormal and 6 had a normal karyotype.

Co-occurrence of a *TP53* deletion with +1q was observed in 4 patients: three patients had PMF, one patient had ET that progressed to MF and subsequently progressed to MPN-AP/BP. The loss of *TP53* in two of these patients was the result of jumping +1q as a consequence of der(17)(t(1;17)(q21;p11) whereby the long arms of chromosome 1

were translocated just above the centromere of chromosome 17 with a loss of the entire short arms of chromosome 17. There were three patients with 12q rearrangements and *TP53* deletions: one had PMF and the other two had MF that progressed to MPN-AP/BP. Co-occurrence of *TP53* mutation and +1q was observed in one patient. +1q and *TP53* mutations are infrequent but not mutually exclusive. Co-occurrence of *TP53* mutation and 12q rearrangements was not observed.

The majority of structural rearrangements of 12q were either balanced translocations or inversions and cannot be detected by CMAs. To identify possible cryptic lesions involving chromosome 12q was analyzed on subset of patients with myeloid malignancies. Of a database of 317 patients with myeloid malignancies, 33 (10%) had chromosomal 12 abnormalities as detected by CMA. GREVE: Genomic Recurrent Event ViEwer and the R programming language utilizing the Genomic Ranges package was used to identify overlapping regions aberrant genomic regions. As shown in **Figure 3.8**, each horizontal bar represents an abnormal region occurring in one patient. Red bars indicate regions of CN-LOH, blue bars are regions of deletion, and green bars are regions of gain. Most abnormalities ranged in size and location, however, three minimally overlapping regions occurring in 5 or more patients were identified. These three regions, 12q14.3, 12q15, 12q21.32 (indicated by pink arrows), closely match the three regions observed by cytogenetic analysis.

Figure 3.8. Three overlapping regions of chromosome 12q



Three overlapping regions occurring in 5 or more patients were identified using GREVE: Genomic Recurrent Event ViEwer and the Genomic Ranges package of R. Each horizontal bar represents an abnormal region occurring in one patient. Red bars indicate regions of CN-LOH, blue bars are regions of deletion, and green bars are regions of gain. Pink arrows show three regions, 12q14.3, 12q15, 12q21.32.

Genes in these regions were compared to known mutated genes curated in the Catalogue of Somatic Mutations in Cancer (COSMIC) database and two candidate genes were identified, *HMGA2* and *WIF1*. These genes have also been implicated in pathogenesis of this disease. Both cytogenetic and CMA analysis identified three distinct

regions on chromosome 12 suggesting different genes within these regions may perturb the same pathway that lead to disease progression.

3.5 Discussion

Specific chromosomal abnormalities in MPNs are associated with a poor prognosis and the longer the duration of the disease the more likely additional chromosomal abnormalities will be acquired.[138,139] Studies have suggested that the presence of additional copies of 1q are associated with advanced forms of MPNs.[23,73,142]

Although 54% of patients in this dissertation cohort study had +1q associated with additional chromosomal aberrations, in 46% +1q represented the sole abnormality. The increasing numbers of cells with +1q over time was associated with disease progression. This is best illustrated by the longitudinal study of a patient with PV who over 16 years had a progressive increase in the number of cells with +1q until the number of cells harboring +1q reached 100% and subsequently progressed to MPN-BP.

Although the MIPPS70 risk scoring system which was designed to predict the prognosis of MF patients concluded that presence of +1q as a sole abnormality was associated with a favorable prognosis.[160] These results demonstrated that the presence of +1q at a single point in time has limited use as a prognostic parameter but that increases in the numbers of hematopoietic cells with this abnormality over time frequently accompanies MPN disease progression.

The association of +1q and disease progression is not unique to the MPNs; numerous publications have reported that +1q is a secondary cytogenetic abnormality and has a negative impact on prognosis in multiple myeloma patients as well as individuals

with myelodysplastic disorders and less frequently in AML.[161-165] In myeloma, as in the MPNs the primary mechanism of +1q is the translocation of the long arm to a single or multiple chromosomes. Another mechanism of jumping +1q21 translocation in multiple myeloma involves the focal amplification of 1q21, but this event has not been observed (unpublished observation) in MPN patients.

Previous studies have shown that *MDM2* is upregulated in CD34+ cells of both PV and MF patients.[166]. *JAK2*V617F negatively regulates *TP53* in MPNs by increasing the expression of La antigen which increase the translation of *MDM2*.[167] +1q was associated with increased transcripts of *MDM4* likely leading to further downregulation of TP53 activity. Elevated MDM4 levels have been previously found in other hematologic malignancies including *de novo* AML.[168] There are numerous other oncogenes that are present on 1q which could also contribute to disease progression. The overexpression of *CKS1B* at band 1q21 has been proposed as the culprit responsible for disease progression in myeloma patients with +1q.[169,170] *CKS1B* is responsible for the ubiquitination and proteolysis of p27 which is a cyclin dependent kinase inhibitor which is responsible for regulating the transition of cells from late G1 to S phase. The reduced levels of p27 are thought to lead to increased cell proliferation. Although increased CKS1B or another yet to be implicated gene present on 1q might also contribute to MPN disease progression. The observation that MF and acute leukemia develop in *JAK2*V617F transgenic mice which lack *TP53* as compared to *JAK2*V617F mice with wild-type *TP53* supports the hypothesis that +1q leads to further dysregulation of the wild type TP53 pathway by increasing MDM4 activity.[171]

The +1q abnormality in MPN patients was closely associated with the presence of JAK2V617F. Previous studies showed that JAK2V617F leads to increased genomic instability which could contribute to the outcomes of patients with this +1q abnormality.[172,173] We found that 24% of patients with +1q (17/72) had der(9)t(1;9)(q21;q12) resulting in three copies of 1q and 3 copies of JAK2 suggesting chromosomal instability. The exact mechanism underlying this close association requires further investigation.

Chromosome 12 abnormalities have been previously reported to occur in MPN patients.[144] One prior report indicated that chromosome 12q rearrangements were found primarily in PV-related MF while others reported that two specific 12q rearrangements at 12q15 and 12q24 were associated with PMF.[147,148,150] Here, the 12q rearrangements were found most commonly in patients with PMF but was also observed in one patient with ET at the time of evolution to ET-related MF. The 12q rearrangements occurred in 84% of these patients as a single abnormality and were associated in 13% of patients with progression to MPN-BP.

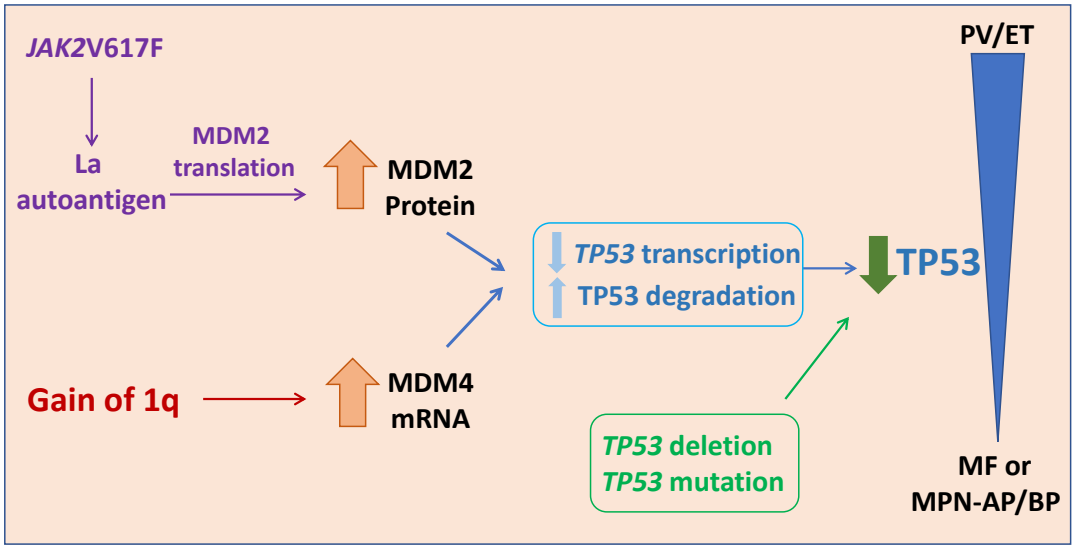
These 12q abnormalities did not result in increased copy numbers of *MDM2* but were associated in three patients with deletion of *TP53*. Furthermore, 12q abnormalities were present most frequently in the majority of hematopoietic cells at diagnosis. The molecular consequences of 12q translocations require further study. Earlier studies have reported that 12q15 structural abnormalities in MF are associated with increased transcripts of the high mobility AT-hook 2 (*HMGA2*) gene.[174] These investigators however indicated that *HMGA2* transcripts were increased in patients with or without 12q15 chromosomal abnormalities leading us to question whether 12q structural abnormalities were responsible

for the increased transcript levels. This is further supported by the more recent studies demonstrating increased *HMGA2* expression in PMF patients lacking 12q abnormalities.[175,176] In this study, CMA analysis identified three recurrent regions within chromosome 12q, one of these regions involved band 12q15 harboring the *HMGA2* gene. All 5 patients harbored CN-LOH of this gene. CN-LOH can lead to increased gene expression by the loss of a negative epigenetic mark and may be a possible mechanism to increased *HMGA2* expression in PMF patients lacking 12q rearrangements.

Thirty patients (2% of total patients) in this report had deletions of 17p13 which leads to loss of TP53, while 8 (4%) had inactivating mutations of *TP53*. Of the 30 patients with deletions of 17p13, 7 also had either +1q (4 patients) or 12q abnormalities (3 patients) while one of the patients with +1q was found to have a *TP53* mutation. The *TP53* mutations were observed in patients with PV, MF and MPN-AP/BP while the deletions of *TP53* were associated exclusively with MF and MPN-AP/BP.

These findings indicate that a variety of events leading to loss of *TP53* function can occur individually or in combination in MPN patients as their disease evolves and that a series of genetic events occur in MPN patients which result in decreased activity of the TP53 pathway and likely contribute to MPN disease progression (**Figure 3.9**). Overall, these findings highlight the utility of integrating complementary genomic technologies to identify genes that may play a role in the pathogenesis of Ph-MPNs that progress to more advanced forms of the disease. It also provides evidence to the heterogeneity of genomic lesions occurring in different patients.

Figure 3.9. Proposed role of TP53 dysregulation in the evolution of the MPNs



Decreased TP53 activity can result from several genomic alterations in the MPN clone and is hypothesized to be a crucial driver of MPN disease progression. Mutations and deletions (del17p) of the TP53 gene can directly result in decreased TP53 activity. MDM2 and MDM4 are negative regulators of TP53 that decrease transcription and increase degradation of TP53. Upregulation of these regulators ultimately decreases p53 activity. In MPNs, there are increased MDM2 protein levels due to increased MDM2 translation stimulated by JAK2V617 through the La autoantigen. Additionally, gain of 1q results in upregulation of MDM4 transcript levels.

Marcellino BK, Hoffman R, Tripodi J, Lu M, Kosiorek H, Mascarenhas J, Rampal RK, Dueck A, Najfeld V. Advanced forms of MPNs are accompanied by chromosomal abnormalities that lead to dysregulation of TP53. Blood Adv. 2018 Dec 26;2(24):3581–9.

Chapter 4. Specific Aim 3

4.1 Specific Aim 3

To determine whether genomic lesions involving genes identified in Specific Aims 1 and 2 are present in patients with Ph- MPN who are karyotypically normal.

4.2 Approach

Cytogenetic abnormalities are important in the diagnosis and prognosis of many hematological malignancies.[151,177-179] Many recurrent karyotypic abnormalities are well-defined and are known to underlie pathophysiologic processes contributing to malignancy.[35,180] Ph- MPNs share many recurrent cytogenetic abnormalities but with varying incidence and prognostic implications.[181] In addition, Ph- MPNs are a group of chronic disorders with overlapping clinical features and providing a definitive diagnosis can be difficult. Although, genomic abnormalities will confirm a neoplastic process, chromosomally abnormal clones are relatively rare in some subtypes of MPN. As mentioned earlier cytogenetic abnormalities are detected at diagnosis in 23%-29% of patients with PV, 10%-12% of patients with ET, and up to 50% of patients with PMF although the incidence of cytogenetic abnormalities increases with disease progression.[23]

Over the last decade, the role of molecular genomic markers in Ph- MPN in both diagnosis and prognosis has increased. Majority of patients are now characterized by 1 of 3 classic "driver" mutations identified in the *JAK2*, *CALR*, and *MPL* genes. However, a small proportion of patients with Ph- MPN (~10%) do not carry the canonical driver mutations.[179] Moreover, in retrospective studies, additional mutations detected in Ph- MPN patients demonstrated prognostic significance. For example, in patients with PMF,

mutations in *ASXL1*, *EZH2*, *IDH1*, *IDH2*, or *SRSF2* predicted shorter survival and/or increased risk of leukemic transformation.[160] Additionally, CMA testing has also recently contributed towards the diagnostic evaluation and assessment of Ph- MPNs. For example, regions of CN- LOH have significant therapeutic implications due to underlying mutations that could be potential therapeutic targets or predict treatment response. However, CMA analysis is not the standard of practice.

The aim was to integrate genomic technologies to determine whether genes identified in specific aims 1 and 2 occur in patients with advance forms of MPN who are karyotypically normal.

4.3 Materials and Methods

4.3.1 Patients

A retrospective analysis was performed on 184 patients between January 2016 and December 2018 with Ph- MPN. Peripheral blood (PB) and/or bone marrow (BM) samples were investigated. Each patient's medical record was reviewed to confirm diagnoses. All Ph-MPNs (PV, ET, PMF, and post PV/ET MF) were diagnosed by criteria established by the World Health Organization.[151] REDCap (Research Electronic Data Capture), a secure web based application for creating and managing databases was used to capture all clinically relevant data. The Institutional Review Board of the Icahn School of Medicine at Mount Sinai approved this research and is in accordance with the Declaration of Helsinki.

4.3.2 Conventional cytogenetic analyses

Bone marrow or unstimulated peripheral blood G-banded metaphase cells were obtained using standard methodology, as described in Specific Aim 1, Chapter 2, Section 2.3.2

4.3.3 DNA extraction and aCGH+SNP

Genomic DNA was prepared from BM or PB tissues using Qiagen's Gentra PureGene DNA purification kit (Qiagen Inc., Valencia, CA) as per the manufacturer's instructions and described in Chapter 2, Specific Aim 1, Section 2.3.4

4.3.4 Next generation sequencing (NGS)

NGS data profiles of each patient was extracted from their Electronic Medical Record (EMR) and captured in the web-based REDCap database. Both mutation and allele burdens were captured, when available.

4.3.5 Statistical Analysis

Statistical analysis between groups was performed using the Fisher exact test. GREVE: Genomic Recurrent Event ViEwer was used to assist the identification of patterns across individual samples and the R software language using the Genomic Ranges package was used to map and identify minimally overlapping genomic regions.

4.4 Results

Patient characteristics and genomic landscape

The clinical characteristics of the 184 patients with Ph- MPN are outlined in **Table 4.1**. Both CMA and conventional cytogenetic analysis were performed in all 184 patients. CMA abnormalities were observed in 138 patients (75%) and among the informative cytogenetic results, normal and abnormal karyotypes were detected in 136 (78%) and 39 (22%) patients, respectively. Uninformative cytogenetic results were observed in 9 patients. Cytogenetics were uninformative when less than 5 metaphase cells were available for analysis or no mitosis was observed. CMA, conventional cytogenetics, and mutational analysis was available in 179 patients. With respect to driver mutations, *JAK2* mutations were detected in 118 (66%) patients, 117 carried the canonical *JAK2*V617F and 1 patient harbored the *JAK2* exon 12 mutation. *CALR* mutations were detected in 25 patients (16%). Concurrent *JAK2* and *CALR* mutations were detected in three patients, one with ET and two with PMF. Of the 179 patients, 30 (16%) were triple-negative with no driver mutation but half of these patients harbored other mutations as shown in **Figure 4.1**.

The number of patients with abnormal genomic results (cytogenetics, NGS, and CMA) were examined for each Ph- MPN entity. As shown in **Figure 4.2**, the assay with the highest yield of abnormal results was NGS (88.2%-100%) followed by CMA (54.7%-100%), and conventional cytogenetics (1.9%-39.2%). The high degree of abnormalities in NGS and CMA was most likely due to the higher resolution of these tests. Across all entities the data suggest that genomic testing with NGS and CMA revealed a higher percentage of patients with abnormalities when compared to cytogenetics. These results unequivocally demonstrate that CMA testing can establish clonality in patients who lack

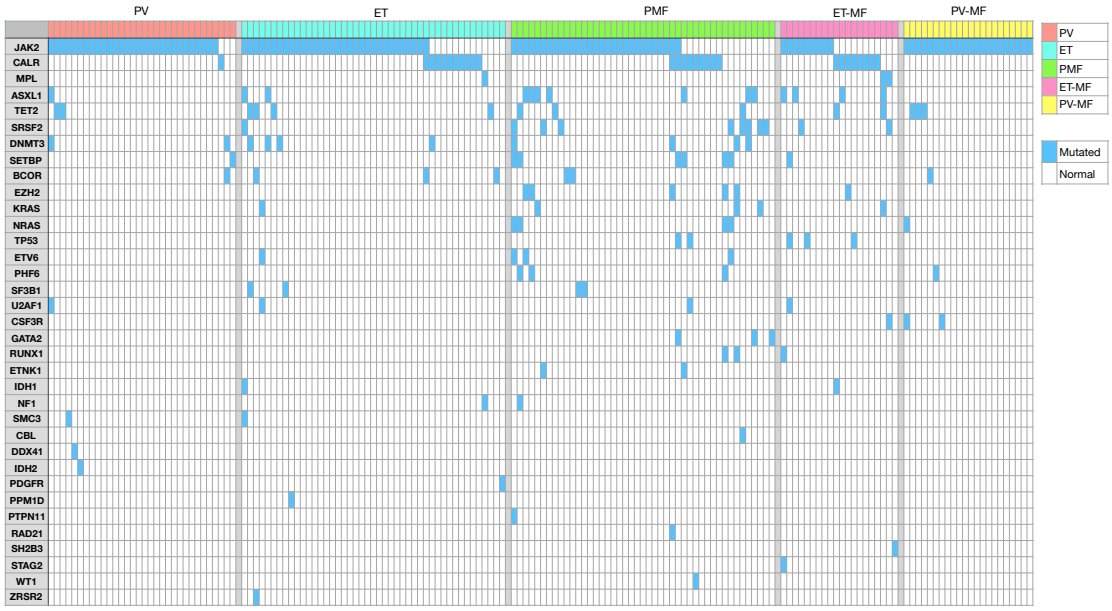
mutations and have normal cytogenetics. As shown in **Table 4.2**, **a**mong the patients who were triple negative for driver mutations and did not have other mutations, CMA detected abnormalities in 8 of 15 patients (53%). These patients had CN-LOH of chromosome 7 and cryptic deletion of chromosome 13q as detected by CMA. These are recurrent abnormalities associated with Ph- MPN and, specifically, 7q CNLOH, is known to confer a poor prognosis in patients with myeloid malignancies.[110]

Table 4.1 Clinical characteristics of 184 patients with Ph- MPN

	Total	PV	ET	PMF	ET-MF	PV-MF
Number	184	34	53	51	24	22
Age, Years, Median (Range)	65 (12-91)	59 (17-87)	61 (12-91)	66 (34-87)	65 (45-85)	65.5 (27-79)
Males, n (%)	91 (49%)	22 (65%)	19 (36%)	32 (63%)	8 (33%)	10 (45%)
Cytogenetics, n (%)						
Evaluable	175 (95%)	34 (100%)	51 (96%)	48 (94%)	20 (83%)	22 (100%)
Uninformative	9 (5%)	0 (0%)	2 (4%)	3 (6%)	4 (17%)	0 (0%)
Karyotype, n (% of evaluable)						
Normal	136 (78%)	31 (91%)	50 (98%)	28 (58%)	13 (65%)	14 (64%)
Abnormal	39 (22%)	3 (9%)	1 (2%)	20 (42%)	7 (35%)	8 (36%)
CMA, n (%)						
Normal	46 (25%)	12 (35%)	24 (45%)	5 (10%)	5 (21%)	0 (0)
Abnormal	138 (75%)	22 (65%)	29 (55%)	46 (90%)	19 (79%)	22 (100%)
Mutation Analysis, n (%)						
Evaluable	179 (97%)	33 (97%)	51 (96%)	50 (98%)	23 (96%)	22 (100%)
Not Performed	5 (3%)	1 (3%)	2 (4%)	1 (2%)	1 (4%)	0
Driver Mutations, n (% of evaluable)						
JAK2V617F	117 (65%)	28 (85%)	31 (60%)	27 (54%)	9 (39%)	22 (100%)
JAK2 Exon 12	1 (1%)	1 (3%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
CALR	25 (14%)	1 (3%)	9 (18%)	7 (14%)	8 (35%)	0 (0%)
MPL	3 (2%)	0 (0%)	1 (2%)	0 (0%)	2 (9%)	0 (0%)
JAK2/CALR	3 (2%)	0 (0%)	1 (2%)	2 (4%)	0 (0%)	0 (0%)
Triple Negative, n (% of evaluable)						
With additional Mutations	15 (8%)	2 (6%)	3 (6%)	9 (18%)	1 (4%)	0 (0%)
Without additional Mutations	15 (8%)	1 (3%)	6 (12%)	5 (10%)	3 (13%)	0 (0%)

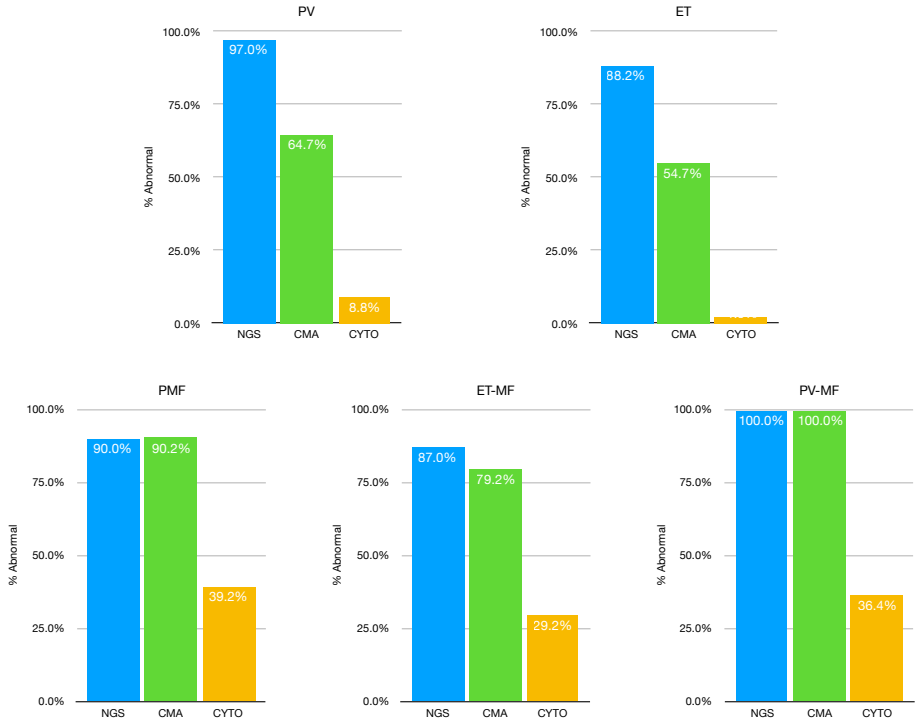
Abbreviations: PV, polycythemia vera; ET, essential thrombocythemia; PMF, primary myelofibrosis; PV-MF, post polycythemia vera myelofibrosis; ET-MF, post essential thrombocythemia myelofibrosis

Figure 4.1 Mutation Profiles of 179 patients with Ph- MPN



The mutational profiles of 179 patients with Ph-MPN. Each patient is represented in an individual column and each gene is represented in a row. Blue boxes indicated the presence of a mutation.

Figure 4.2 Distribution of genomic abnormal by testing modality in 179 patients



Distribution of abnormal patients by testing modality, X-axis represents testing method, Y-axis represents the percentage of abnormal patients

Table 4.2 Comparison of CMA and NGS (n=179)			
		CMA Abnormalities	
		Not Detected	Detected
Mutations	Not Detected	7 (3.9%)	8 (4.5%)
	Detected	38 (21.2%)	126 (70.4%)

Five groups of patients, as shown in **Table 4.3**, were established based on conventional cytogenetic and CMA results: (1) Normal Cytogenetics / Normal CMA, (2) Normal Cytogenetics / Abnormal CMA, (3) Abnormal Cytogenetics / Abnormal CMA, (4) Uninformative Cytogenetics / Normal CMA, and (5) Uninformative Cytogenetics / Abnormal Array.

Table 4.3 Five Patient Groups Based on Cytogenetic Results							
	PV n (%)	ET n (%)	PMF n (%)	ET-MF n (%)	PV-MF n (%)	Total n (%)	Group
Normal Cyto / Normal CMA	12 (35.3%)	22 (41.5%)	3 (5.9%)	3 (12.5%)	0 (0.0%)	40 (21.7%)	1
Normal Cyto / Abnormal CMA	19 (55.9%)	28 (52.8%)	25 (49.0%)	10 (41.7%)	14 (63.6%)	96 (52.2%)	2
Abnormal Cyto / Abnormal CMA	3 (8.8%)	1 (1.9%)	19 (37.3%)	7 (29.2%)	8 (36.4%)	38 (20.7%)	3
Abnormal Cyto / Normal CMA	0 (0.0%)	0 (0.0%)	1 (2.0%)	0 (0.0%)	0 (0.0%)	1 (0.5%)	
Uninformative Cyto / Normal CMA	0 (0.0%)	2 (3.8%)	1 (2.0%)	2 (8.3%)	0 (0.0%)	5 (2.7%)	4
Uninformative Cyto / Abnormal CMA	0 (0.0%)	0 (0.0%)	2 (3.9%)	2 (8.3%)	0 (0.0%)	4 (2.2%)	5

Abbreviations: PV, polycythemia vera; ET, essential thrombocythemia; PMF, primary myelofibrosis; PV-MF, post polycythemia vera myelofibrosis; ET-MF, post essential thrombocythemia myelofibrosis

1. Normal Cytogenetics and Normal CMA

Among the 40 patients (21.7%) with normal cytogenetic and normal CMA results, 34 patients (85%) had at least one mutation detected by NGS. The remaining 6 patients (15%), could not be characterized by any of the three genomic technologies.

2. Normal Cytogenetics and Abnormal CMA

Table 4.3 shows that 96% of 136 patients with a normal karyotype, in all 20 evaluated metaphase cells, had an abnormal CMA result. Within this group, application of CMA identified a total of 233 genomic abnormalities (55 gain, 39 Loss, 142 CN-LOH) with an average of 2.4 abnormalities per patient (**Figure 4.3** and **Table 4.7**).

In order to identify genes or genomic regions that may have biological implications, the minimal over lapping regions (MOR) involving CMA abnormalities was identified using the "Genomic Ranges" (GRanges) package. Of the 15 regions identified (**Table 4.4**), 7 regions involved a single gene and 8 large regions involving multiple genes were detected in a least 4 or more patients. Among the 7 regions involving single genes, the most frequent MOR occurred in 41 patients and was localized to chromosome 9p24.1 involving the *JAK2* gene. The most frequent type of CMA abnormality in this region was CN-LOH occurring in 38 of the 41 patients (92.6%). CN-LOH is known to harbor mutations present in a homozygous constellation, therefore the *JAK2* mutant allele burdens were compared between those that had 9p CN-LOH with those that lacked 9p CN-LOH. The *JAK2* allele burdens were available from 55 patients (33 with 9p CN-LOH, 22 with normal 9p). The average allele burden in patients with and without 9p CN-LOH was 80.2% (range 43-98%) and 17.5% (range, 0.2-43%) ($p<0.001$), respectively. The second most frequent MOR involved the *MDM4* gene localized to chromosome 1 band q32.1 and was detected in 22 patients. Abnormalities in this gene were either gain (9 patients) or loss (12 patients) of a exons was observed in 21 patients. The five remaining recurrent MORs involving 1 gene

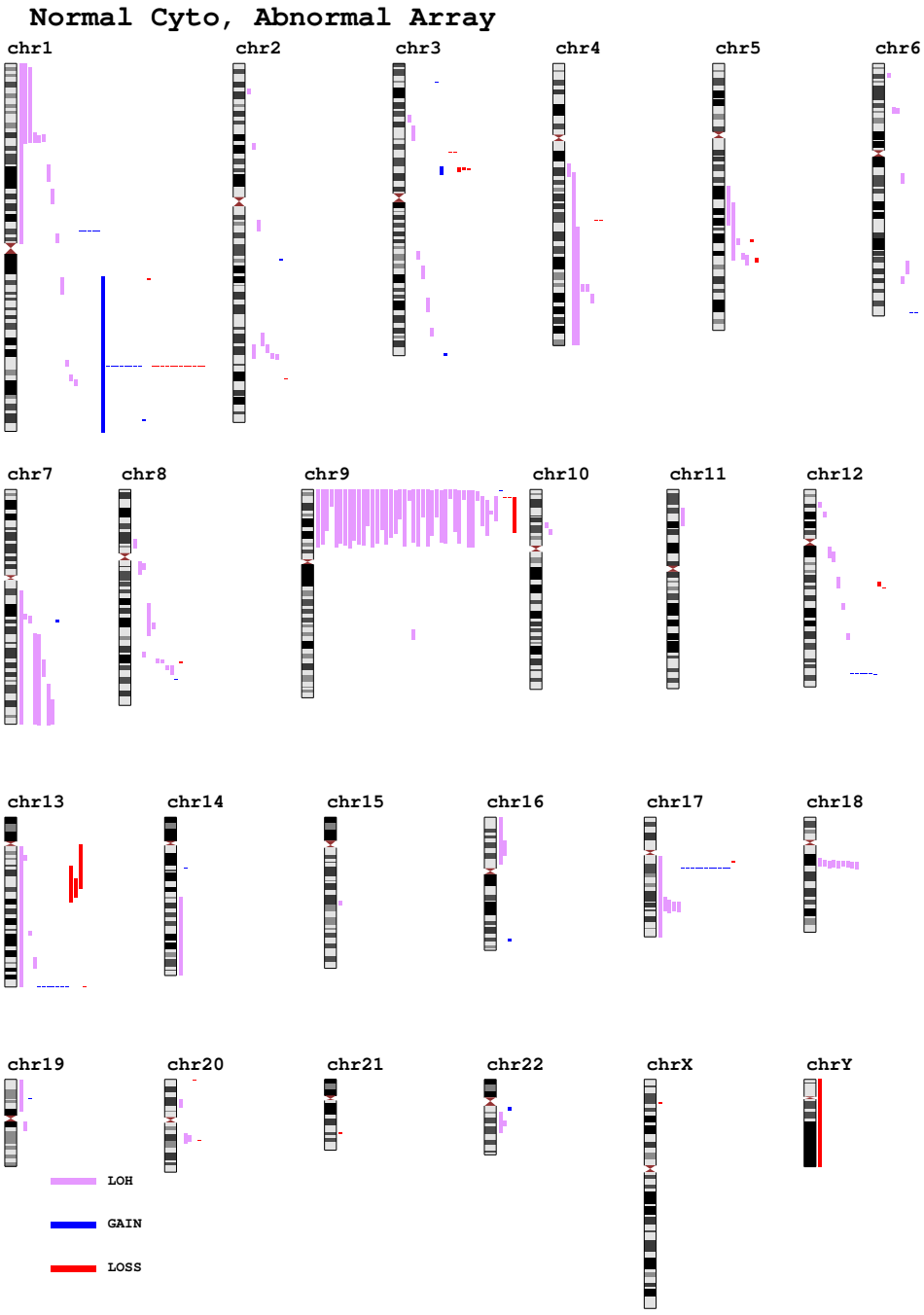
were *TAF15* (17q12), *RASA3* (13q34), *RHOC* (1p13.2), *NCOR2* (12q24.31), and *FOXP1* (3p13). All had intragenic gains except for *FOXP1*, which was completely deleted.

As outlined in **Table 4.4**, eight large MORs involving multiple genes varied in size ranging from 0.9 Mb to16.6 Mb. Two large regions, 16.5 Mb and 11 Mb, were identified on the long arms of chromosome 7 while 0.9 Mb region was detected on the long arms of chromosome 5. Recent studies have shown that CN-LOH of the long arms of chromosome 7q may have the same prognostic impact as cytogenetic deletion within these same regions.[182] Additionally, a 4.5 Mb region on the short of chromosome 1p and a 2.4 Mb region on the long arms of chromosome 18 were also detected. Clinically relevant genes located in these regions included *CDKN2C* and *ASXL3*.

3. Abnormal Cytogenetics and Abnormal CMA

Thirty-eight patients (20.7% of the total) had both cytogenetic and CMA abnormalities. Concordant abnormal results were observed in 4 of the 38 patients (11%). Two of these 4 patients had loss of chromosome Y and two patients had deletion of the long arms chromosome 13. Although both testing methods identified the same abnormalities, the application of CMA was able to further refine the breakpoints on the deleted chromosome 13. In 6 (16%) of the cytogenetically abnormal patients, CMA testing identified additional genomic lesions. The additional abnormalities included cryptic loss and CN-LOH. The remaining 28 patients (74%) had complimentary results. Complimentary results were defined as abnormalities that were detected by any testing method since each has its own advantages and limitations (See Chapter 1, Section 1.4).

Figure 4.3. Ideogram of CMA abnormalities in cytogenetically normal patients



Ideogram representation of all chromosomal aberrations. Lesions were detected using Agilent's aCGH+SNP platform in 96 cytogenetically normal patients. Physical positions and sizes of chromosomal aberrations are represented with purple, red, and blue bars, indicating CN-LOH, deletions, and gains, respectively.

Table 4.4. Minimal overlapping regions, cytogenetically normal patients					
Chromosome Band	**Start**	**Stop**	**# of patients**	**Genes**	**Size (Mb)**
9p24.1	5022210	5044537	41	JAK2	0.022327
1q32.1	204515866	204516097	22	MDM4*	0.000231
17q12	34170933	34172065	12	TAF15*	0.001132
18q12.1	30407590	32809071	9	Multiple	2.401481
13q34	114773012	114774969	8	RASA3*	0.001957
1p13.2	113248246	113249031	6	RHOC*	0.000785
1p33-p32.3	48482986	53041697	6	Multiple	4.558711
12q24.31	124807424	124809318	5	NCOR2*	0.001894
17q22-q24.1	57455620	63109152	5	Multiple	5.653532
7q34-q36.3	141983565	158572273	5	Multiple	16.588708
3p13	71064451	71658532	4	FOXP1	0.353656
13q14.2	48878792	49232448	4	Multiple	0.594081
5q31.3	131805879	132669995	4	Multiple	0.864116
4p31.23-p31.3	149734385	154087305	4	Multiple	4.352920
7q31.2-p31.33	115211989	126734894	4	Multiple	11.522905

*Intragenic aberration

All 38 patients in this group had a total of 99 cytogenetic abnormalities involving every chromosome except for chromosomes 4 and 19. Sole cytogenetic abnormalities were detected in 19 patients, double abnormalities were detected in 8 patients, and 11 patients had a complex karyotype (≥3 cytogenetic abnormalities). The majority of these abnormalities resulted in gain or loss of genetic material either as numerical or unbalanced structural rearrangements. A total of 11 balanced structural chromosomal rearrangements

were identified, 7 occurring within a complex karyotype and 4 occurring as a sole abnormality. Moreover, 5 of the 11 balanced structural abnormalities involved chromosome 12.

CMA identified a total of 203 genomic abnormalities (73 gain, 81 loss, 49 CN-LOH) with an average of 5.3 abnormalities per patient (**Figure 4.4** and **Table 4.7**). The most frequent array abnormality was loss of genetic material. Large regions of gain involved the long arms of chromosome 1 and the short arms of chromosome 9, confirming the cytogenetic findings of an unbalanced translocation between the long arms of chromosome 1 and the short arms of chromosome 9 occurring in 4 patients resulting in gain of 1q and 9p.

Twelve minimal overlapping regions were identified, 7 involving single genes only and 5 large MORs involving multiple genes (**Table 4.5**). The most frequent MOR involved the short arms of chromosome 9p, occurring in 19 patients, involving *JAK2*. Six other MORs involving a single gene include *MDM4* (1q32.1), *TAF15* (17q12), *RASA3* (13q34), *NCOR2* (12q24.1), *RHOC* (1p13,2) and *RUNX1* (21q22.12) genes and all had intragenic gains or deletions. The size of the five largest MORs ranged from 5.8 Mb to 1.1 Mb and involved chromosomes 5, 7, 13, 18, and 20. All regions were similar to those found in the karyotypically normal patients.

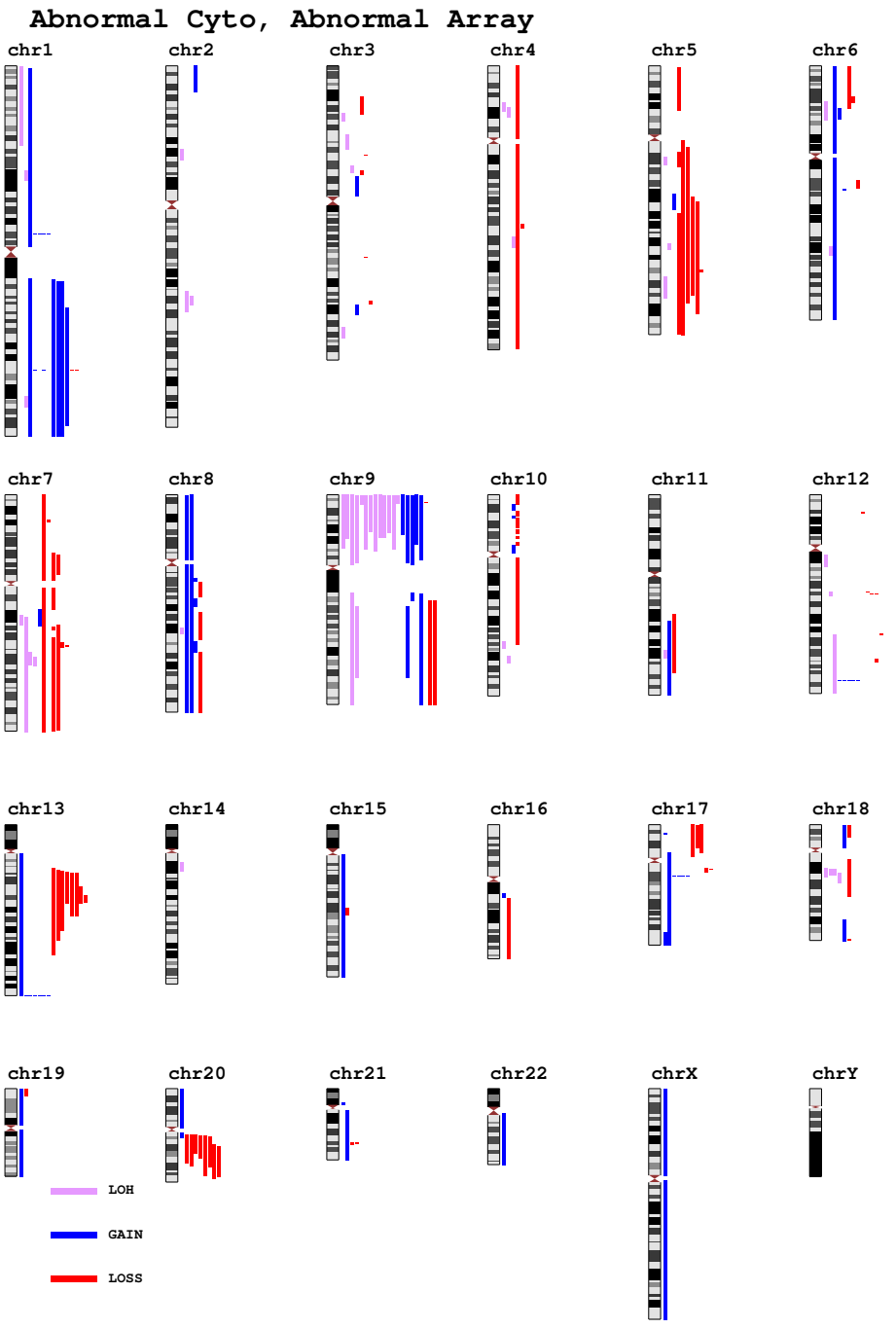
CMA analysis identified chromothripsis in three patients involving chromosomes 5, 7 (in 2 patients), 8, and 10. One patent (PMF) with chromothripsis had mutations in *CALR*, *TP53*, and *U2AF1*. In this patient with mutated *TP53*, CMA also detected a deletion of *TP53* resulting in complete loss of the wild type allele. A second patient with PMF had mutations in *JAK2* and *TP53*. Additionally, CMA detected gain of *MDM4*, deletion of

WIF1, and intragenic gains of *RASA3* and *RHOC*. The third patient (post-ET MF) had mutations in *JAK2*, *SETBP1*, *TP53*, and *U2AF1* and CMA detected intragenic gains within *MDM4* and *RHOC*.

4. and 5. Uninformative Cytogenetics and Normal / Abnormal Array

A total of 5 patients with uninformative cytogenetic results were normal by CMA testing. Two patients were *JAK2*V617F positive and two were triple negative (one triple negative patient had a *PDGFRA* mutation). NGS was not available in one patient. Of the four patients with CMA abnormalities, one had a *JAK2* mutation, one had a *CALR* mutation, one was triple negative with an *ASXL1* mutation, and one was triple negative with no additional mutations. CMA detected CN-LOH in 3 patients involving chromosomes 1, 4, 5, 7, 9 and 18, and the fourth patient showed gain of the long arms of 1q.

Figure 4.4 Ideogram of CMA abnormalities in cytogenetically abnormal patients



Ideogram representation of all chromosomal aberrations. Lesions were detected Agilent's aCGH+SNP platform in 38 cytogenetically abnormal patients. Physical positions and sizes of chromosomal aberrations are represented with purple, red, and blue bars, indicating CN-LOH, deletions, and gains, respectively.

Table 4.5. Minimal overlapping regions, cytogenetically abnormal patients					
Chromosome Band	**Start**	**Stop**	**# of patients**	**Genes**	**Mb**
9p24.1	5022210	5044537	19	JAK2	0.022327
13q14.2	47400361	51961427	9	Multiple	4.561066
20q12-q13.12	38934682	43782908	8	Multiple	4.848226
1q32.1	204515825	204516097	8	MDM4*	0.000272
7q31.1	108746575	114640359	6	Multiple	5.893784
5q31.2	136880781	138098954	6	Multiple	1.218173
13q34	114773012	114774969	6	RASA3*	0.001957
12q24.1	124807424	124809318	6	NCOR2*	0.001894
17q12	34170933	34172065	6	TAF15*	0.001132
18q12.2	32841868	33901772	5	Multiple	1.059904
1p13.2	113248246	113249031	5	RHOC*	0.000785
21q22.12	36155152	36857636	3	RUNX1*	0.702484

*Intragenic aberration

Validation of the 9 Gene Panel

A nine gene panel was created consisting of genes identified in specific aim 1 (*RASA3*, *NCOR2*, *RHOC*, and *TAF15*) and specific aim 2 (*MDM4*, *MDM2*, *TP53*, *HMGA2*, and *WIF1*). The frequency of abnormalities occurring in any of these nine genes among the 184 patients is shown in **Table 4.6**. *MDM4* was the most frequent aberrant gene occurring in 16.3% while none of the 184 patients had abnormalities involving *MDM2*.

Table 4.6 Frequency of abnormalities (9 gene panel)						
9 Gene Panel	PV (n=34)	ET (n=53)	PMF (n=51)	ET-MF (n=24)	PV-MF (n=22)	Total (%)
MDM4, n (%)	7 (20.6%)	5 (9.4%)	8 (15.7%)	5 (20.8%)	5 (22.7%)	30 (16.3%)
MDM2, n (%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
TP53, n (%)	0 (0.0%)	0 (0.0%)	2 (3.9%)	1 (4.2%)	0 (0.0%)	3 (1.6%)
HMGA2, n (%)	1 (2.9%)	0 (0.0%)	3 (5.9%)	1 (4.2%)	0 (0.0%)	5 (2.7%)
WIFI1, n (%)	1 (2.9%)	0 (0.0%)	3 (5.9%)	0 (0.0%)	0 (0.0%)	4 (2.2%)
RASA3, n (%)	2 (5.9%)	2 (3.8%)	8 (15.7%)	1 (4.2%)	3 (13.6%)	16 (8.7%)
NCOR2, n (%)	1 (2.9%)	0 (0.0%)	6 (11.8%)	2 (8.3%)	3 (13.6%)	12 (6.5%)
RHOC, n (%)	1 (2.9%)	0 (0.0%)	6 (11.8%)	2 (8.3%)	2 (9.1%)	11 (6.0%)
TAF15, n (%)	2 (5.9%)	0 (0.0%)	8 (15.7%)	2 (8.3%)	4 (18.2%)	16 (8.7%)
Totals	15	7	44	14	17	97

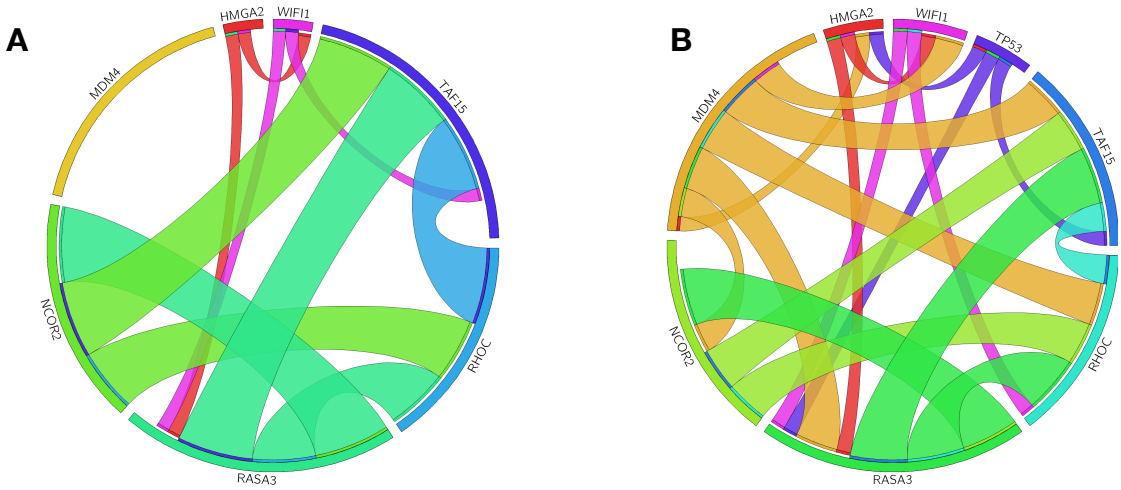
As Ph- MPNs progress to more advanced forms of the disease, the number of genomic abnormalities increase. The average number of abnormalities based on disease stage and cytogenetic results are shown in **Table 4.7**. The average number of total CMA abnormalities per patient was greater in cytogenetically abnormal patients as well as in patients who had advanced forms of the disease. There was a slightly more significant difference between the cytogenetic group and the group based on disease stage ($p<0.001$ vs $p=0.04$). The average number of abnormalities occurring within the nine gene panel was also greater in patients that were cytogenetically abnormal ($p<0.001$) as well as in patients with advanced forms of the disease ($p<0.001$). These results suggest that the genes within this panel may play an important role in disease progression.

Table 4.7 Average number of genomic lesions per patient		
	Average Number of Lesions Per Patient	
	Total Array	9 Gene Panel
Disease Stage Group		
PV, ET	2.3*	1.3**
PMF, post ET/PV-MF	3.6*	2.1**
Cytogenetic Group		
Normal	2.4^	1.6^
Abnormal	5.3^	2.4^

Abbreviations: PV, polycythemia vera; ET, essential thrombocythemia; PMF, primary myelofibrosis; MF, myelofibrosis
*** average number of lesions in patients with PV, ET vs. PMF, post ET/PV MF, P=0.04**
**** average number of lesions in patients with PV, ET vs. PMF, post ET/PV MF, P=0.001**
^average number of lesions in patients with Normal Cytogenetics vs. Abnormal cytogenetics, P=0.001

Circos plots shown in **Figure 4.5 A** and **B** represent the number co-occurring genes in the cytogenetically normal (A) and abnormal (B) patients. Among the cytogenetically normal patients, *MDM4* was the sole abnormality occurring in 18 patients. When compared to the cytogenetically abnormal patients *MDM4* co-occurred with 6 other genes. CMA abnormalities involving *TP53* only occurred in the cytogenetically abnormal patients and did not co-occur with *MDM4*. These results suggest that abnormalities involving *MDM4* may be an early genomic event in patients with PV and ET and may represent an alternative route to *TP53* dysregulation.

Figure 4.5 Circos plots of co-occurring nine genes in cytogenetically normal (A) and abnormal (B) patients



Circos plot showing the frequency of co-occurring gene aberrations in (A) cytogenetically normal patients and (B) cytogenetically abnormal patients. Length of the arc corresponds to the frequency of the aberrant gene and the width of the ribbons between 2 genes shows the frequency of co-occurrence.

4.5 Discussion

The results presented in this dissertation are the first analysis utilizing integrated genomics analysis and high-resolution CMA platform in 184 patients with Ph negative MPN. Informative results from all three testing methods were available in 179 patients with an overall diagnostic yield of 96.1%. Among these patients, 21.2% had NGS abnormalities but not CMA abnormalities. These results are in agreement with previous studies suggesting that NGS testing is more sensitive than CMA testing.[186] However, the present study demonstrated CMA abnormalities in 53% of patients who lacked driver mutations (triple negative) and did not harbor other mutations. The most frequent CMA abnormalities detected were 7q CN-LOH and cryptic deletion of chromosome 13q. Both are described as recurrent abnormalities associated with Ph- MPN and, specifically, 7q CN-LOH, was reported to confer a poor prognosis in patients with myeloid malignancies.[110]

This important observation suggests that there is a group of patients whose disease can be characterized solely with CMA.

Among the 136 cytogenetically normal patients, CMA abnormalities were identified in 71% (n=96). The most frequent CMA abnormality was 9p CN-LOH. This abnormality was the first highly recurrent CMA specific aberration associated in patients with *JAK2*V617F positive Ph- MPN.[73-79] There was a statistically significant difference in the average allele burdens in patients with and without 9p CN-LOH (80.2% and 17.5%, respectively). These results suggest that a JAK2V617F variant allele burden greater than 50% are associated with regions of 9p CN-LOH. Additionally, CMA analysis identified two regions of CN-LOH, a 4.5 Mb region on the short of chromosome 1p and a 2.4 Mb region on the long arms of chromosome 18. Clinically relevant genes located in these regions include *CDKN2C* and *ASXL3*. Deletions and mutations of *CDKN2C* have been reported in different cancers including solid tumors and lymphoid diseases but rarely observed in myeloid malignancies.[191] A clinically relevant gene on 18q is the *ASXL3* gene. Although *ASXL1* mutations are found in up to 40% of patients with Ph-MPN, mutations in the *ASXL3* gene (a member of the ASXL family of proteins) have been rarely reported in patients with myeloid malignancies.[183] In this study, *ASLX3* was not included in the NGS panel and its mutational status in patients with CN-LOH of 18q involving *ASXL3* is unknown.

Another recurrent CMA abnormality among the cytogenetically normal patients was focal deletion involving a single gene. Focal deletions or gains in regions involving one or a few genes have been reported in myeloid malignancies including Ph- MPNs.[45,50,57] In the present study, a common deleted region involving the forkheadbox-P1 (*FOXP1*)

gene was observed in 4 patients. The *FOXP1* gene is a member of the family of forkhead transcription factors and is localized to chromosome 3 band p13. Loss of heterozygosity of this region that harbors *FOXP1* is common in solid tumors and plays an important role in B-cell development in normal cells.[187] Deletion and mutations have been observed in 5% of patients with de novo AML with inversions of chromosome 16 but rarely in patients with MPN.[23,79,187]

As shown in **Tables 4.4** and **Table 4.5**, recurrent intragenic regions of gain or loss of exons or introns were detected. Intragenic gains or loss in coding or non-coding sequences can lead to either decreased or increased expression of the particular gene.[116-118] Partial loss involving a few exons of *RUNX1* was observed in three karyotypically abnormal patients. Mutations in *RUNX1* is a recurrent abnormality in myeloid malignancies and is associated with an unfavorable prognosis, but the clinical significance of partial deletion of *RUNX1* has not been completely elucidated.[95]

In three patients, CMA analysis identified chromothripsis while NGS detected concomitant *TP53* mutations. These results confirm published reports that correlate chromothripsis with loss of function of the *TP53* gene in 35% patients with acute myeloid leukemia that had a complex karyotype.[184] Overall, these observations provide evidence that CMA is a useful tool in identifying additional genomic lesion that cannot be detected by other methods. These novel finding may provide additional insight into the biology of these diseases.

Finally, validation of a nine gene panel consisting of genes identified in specific aims 1 and 2 was performed. There was a statistically significant difference in the average number of lesions per patient between the cytogenetically normal and abnormal patients

($p<0.001$) and between patients with chronic phase and advanced forms of the disease ($p<0.001$). These results suggest that the genes in this panel may play an important role in disease progression. Among the cytogenetically normal patients, *MDM4* was the sole CMA abnormality but when compared to the cytogenetically abnormal patients *MDM4* co-occurred with six other genes. Abnormalities involving the *TP53* gene were not detected in the cytogenetically normal patients and when they occurred in the abnormal patients, they did not co-occur with *MDM4*. These results strongly suggest for the first time that genomic lesions involving *MDM4* may be an early genomic event in patients with early forms of Ph- MPN, such as PV and ET and may represent an alternative route to *TP53* dysregulation.

Chapter 5. Summary and Conclusions

The goal of this dissertation was to gain additional insight into the genetic basis of the Ph- MPNs by integrating three genomic technologies; conventional cytogenetics, next-generation sequencing (NGS), and chromosomal microarrays (CMA), each with its advantages and limitations. To achieve this goal, three groups of patients were investigated: (1) patients with advanced forms of Ph- MPN who required therapeutic splenectomy, (2) patients that progressed from the chronic form to more advanced forms of Ph- MPN that were associated with specific chromosomal abnormalities, and (3) cytogenetically normal Ph- MPN patients that represent early stages of their disease.

The hypothesis in aim 1 was to determine if the spleen of patients with advanced forms of Ph- MPN, such as myelofibrosis requiring therapeutic splenectomy, contain hematopoietic stem cells (HSC) which harbor unique genomic aberrations that contribute to disease progression. Previous studies demonstrated that MF splenic hematopoietic stem and progenitor cells (HSPC) have both phenotypic and functional properties that differ from hematopoietic cells present in the bone BM or PB.[103] This dissertation was the first to perform an integrated analysis to completely characterize the genomics of spleen and BM cells in patients with myelofibrosis. The results showed a high concordance of genomic aberrations between spleen and bone marrow cells suggesting similar clonal architecture between these two tissues. In spite of the overall similarity discordant results, specifically CMA findings, indicate that there are genomic differences between myelofibrosis hematopoietic cells that reside in the spleen and bone marrow because additional genomic changes were detected in spleen cells. These differences detected by

CMA provide the first genomic evidence explaining the phenotypic and functional differences observed between HSPC residing in the spleen and BM.

In addition, CMA results demonstrated for the first time novel recurrent gains within four genes: *RHOC*, *RASA*3, *NCOR*2 and *TAF*15, strongly suggesting higher genomic complexity of spleen cells implicating novel candidate genes in disease pathogenesis. The detection of these novel recurrent abnormalities, and the identification of candidate-genes that are potentially involved, support the hypothesis that integrating genomic technologies can further characterize the genetic landscape of myelofibrosis.

In aim 2, based on the above observations, it was postulated that application of this strategy may provide molecular insights into the recurrent chromosomal abnormalities such as gain of chromosome 1q and rearrangements of 12q that are recurrently present in Ph- MPN patients who progress to advanced forms of the disease. Non-random gain of 1q, identified in 6% (n=72) of 1,294 evaluated patients with Ph- MPN, was most prevalent in patients with a history of *JAK2*V617F mutation positive PV. CMA analysis demonstrated that patients with +1q had a gain of 105 Mb region between 1q21 and 1q44 that included the *MDM4* (mouse double minute homolog 4) gene localized to 1q32.1 and was accompanied by an increased *MDM4* transcript level.

Chromosome 12q rearrangements were observed in 2% (n=24) of 1,294 evaluated patients with Ph- MPN and was most prevalent in patients with PMF. Chromosomal analysis identified three recurrent breakpoints 12q13, 12q15, and 12q21 and CMA results identified three recurrent aberrant regions on chromosome 12 (12q14.3, 12q15, and 12q21.32). Genes in these regions were compared to known mutated genes curated in the Catalogue of Somatic Mutations in Cancer (COSMIC) database and two candidate genes

were identified, *HMGA2* and *WIF1*, both were implicated in MF pathogenesis. Cytogenetic and CMA identified aberrations involving 12q15, which harbors the *MDM2* gene which is structurally similar to *MDM4*. Unlike *MDM4*, A significant increase or decrease in *MDM2* transcript levels in MPN patients with 12q abnormalities was not observed. These results strongly link, for the first time, recurrent chromosomal abnormalities and an increased level of *MDM4* transcripts with perturbations of the *TP53* pathway.

In Aim 3, integrated analysis was performed to investigate the hypothesis that novel genes found in the advanced forms of Ph-MPN and genes involved in the progression from chronic to blast phase are also present in the early phases of the disease, especially in those that are karyotypically normal. The most frequent abnormality among the karyotypically normal patients was 9p CN-LOH. There was a statistically significant difference in the average allele burden of patients with and without CN-LOH 9p and (80.2% and 17.5%, respectively). These results suggest that the mutational allele burdens greater than 50% are associated with regions of 9p CN-LOH. Moreover, CN-LOH of chromosomes 7 detected in 10% of patients confirmed an unfavorable prognosis based on studies that 7q CN-LOH is associated with a poor outcome.[110]

Based on nine genes that were identified in patients with disease progression or advanced forms of Ph- MPN, genomic changes within these genes did occur in 24.3% of the cytogenetically normal patients, though the frequency was higher in the cytogenetically abnormal patients (43.6%). Among the cytogenetically normal patients, *MDM4* was a sole abnormality but when compared to the cytogenetically abnormal patients *MDM4* co-occurred with six other genes. Aberrations involving *TP53* occurred only in the

cytogenetically abnormal patients and did not co-occur with *MDM4*. These findings suggest that abnormalities involving *MDM4* may be an early genomic event in patients with PV and ET and may represent an alternative route to *TP53* dysregulation.

In conclusion, the results and observations provided in this dissertation support the hypothesis that integration of these technologies provides novel insight into the genomics of patients with Ph- MPN which could not be achieved if each methodology was performed individually. The results also show that in patients with Ph- MPN a variety of lesions, such as mutations, large structural and numerical lesions, and for the first time intragenic as well as whole gene gains and loss, were present in 96% of the evaluated cohort, even in those who were chromosomally normal and did not harbor driver gene mutations. This strategy increased the diagnostic yield, detected recurrent abnormalities, and identified novel genes that may have important biological implications in these diseases. Moreover, these lesions may be present in different genes in different patients affecting the same pathway. Since one method alone cannot uncover the diversity of genomic lesions, to fully characterize these patients an integrated approach utilizing different testing modalities must be employed. The results presented in these studies highlight the diversity of genomic lesions but the mechanisms on how they drive the disease process merit further investigation and was beyond the scope of this dissertation

Chapter 6. References

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