



## THE MYELOPROLIFERATIVE NEOPLASMS

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### Molecular Testing in Chronic Myelogenous Leukemia: Diagnosis, Monitoring, and Resistance

-qualitative RT-PCR to document BCR-ABL1 fusion transcripts at diagnosis  
 -serial quantitative RT-PCR for post-therapeutic monitoring  
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## OVERVIEW: Myeloproliferative Neoplasms

-disease	-tyrosine kinase- (or related genes)	-tyrosine kinase- inhibitor
<b>MPD (Myeloproliferative disorders)</b>		
-CML   -common non-CML MPD   -uncommon or atypical MPD		
CML	ABL [t(9;22); BCR/ABL]	imatinib, dasatinib, nilotinib
CEL(HES)	PDGFRA [del(4q12); FIP1L1-PDGFRα] PDGFRB [t(5;12); ETV6-PDGFRB] FGFR1	imatinib
PV	JAK2	in development
ET	JAK2, MPL	
CMF	JAK2, MPL	
SM	KIT	
SM with eosinophilia	PDGFRA [del 4q12; FIP1L1-PDGFRα]	imatinib
EMS/SCLL *	FGFR1 [abnormalities of 8p11]	
CNL		
CBL (?)		
<b>MDS/MPD ("overlap" syndromes)</b>		
CMML	PDGFRB [t(5;12); ETV6-PDGFRB]	?imatinib, other
CMML with eosinophilia		
JMML	(RAS, NF1, PTPN11)	
"aCML"		
UMDS/MPD		
RARS-T or RST	JAK2	

CML: chronic myelogenous leukemia  
 CEL: chronic eosinophilic leukemia  
 HES: hypereosinophilic syndrome  
 PV: polycythemia Vera  
 ET: essential thrombocythemia  
 CMF: chronic idiopathic myelofibrosis  
 SM: systemic mastocytosis

EMS/SCLL: Sp11 myeloproliferative syndrome/stem cell leukemia/lymphoma  
 CNL: Chronic neutrophilic leukemia  
 CBL: Chronic basophilic leukemia  
 CMML: chronic myelomonocytic leukemia  
 JMML: juvenile myelomonocytic leukemia  
 "aCML": atypical CML  
 UMDS/MPD: unclassifiable myelodysplastic/myeloproliferative disorder

RARS-T or RST: Refractory Anemia with Ringed sideroblasts and Thrombocytosis or Ringed Sideroblasts with Thrombocytosis  
 \* myeloproliferative or lymphoproliferative

## Molecular Testing in Chronic Myelogenous Leukemia: Diagnosis, Monitoring, and Resistance

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### Introduction

Chronic myelogenous leukemia (CML), the first neoplasm in humans to be associated with a single, specific, acquired genetic lesion, is one of the best-understood myeloproliferative disorders at the molecular level. It comprises ~20% of all adult leukemias and is diagnosed at a median age of approximately 50 years. The disease originates from the transformation of a hematopoietic stem cell with resultant expanding myelopoiesis that characteristically evolves through three phases when untreated: 1) a chronic phase of four to five years duration manifest by myeloid hyperplasia with circulating granulocytes that are present in all stages of maturation; 2) an accelerated phase of shorter duration during which myeloid elements begin to lose the ability to differentiate; and 3) inevitably, a blast phase of acute leukemia of myeloid (70%) or lymphoid (30%) phenotype.

The reciprocal t(9;22)(q34;q11) translocation is identified as the initial transforming event in the development of CML, although some data suggest that it may be preceded by clonal hematopoiesis. The translocation yields a shortened chromosome 22 called the Philadelphia (Ph) chromosome. With the translocation, two distinct genes *BCR* and *ABL1* are fused. *BCR* encodes a cytoplasmic protein with oligomerization, serine-threonine kinase, and GTPase-activating domains. It appears that the BCR protein acts as a tumor suppressor in the WNT signaling pathway, and it also functions in the cellular trafficking of growth factor receptors. *ABL1* encodes a non-receptor tyrosine kinase normally localized to the nucleus, plasma membrane and cytoskeleton. The resultant chimeric gene and fusion transcript yield a fusion protein with constitutively increased tyrosine kinase activity that is now translocated (at a cellular level) from its primary nuclear location to the cytoplasm, and which then phosphorylates a variety of cellular substrates. The result is growth factor independent proliferation, decreased apoptosis, defective adhesion and genomic instability in the transformed cells, with the latter likely to be mechanistic in the metamorphosis from stable or chronic phase CML.

The t(9;22) translocation can involve several different breakpoints in the *BCR* and *ABL1* genes resulting in different chimeric fusion proteins that confer somewhat specific clinicopathologic features and highlight the fact that this translocation is not pathognomonic for CML. These breakpoints are indistinguishable by traditional karyotyping and can only be differentiated using molecular techniques. For all fusions, most of *ABL1* is juxtaposed to variable 5' portions of *BCR*. Whereas the breakpoint involving *ABL1* is relatively conserved, usually arising in the intron before exon 2 (a2), the breakpoints involving *BCR* are more variable.

*BCR* breakpoints arising in the major breakpoint cluster region (M-bcr) occur either after exon 13 (e13 or b2) or exon 14 (e14 or b3). Fusion transcripts involving the M-bcr encode a p210 fusion protein and are present in the vast majority (~99%) of patients with CML as well as in ~40% of adults and ~10% of pediatric patients with t(9;22)-positive precursor B-cell acute lymphoblastic leukemia (ALL). In contrast, breakpoints in the minor breakpoint cluster region (m-bcr) are more 5' in location, occurring after exon 1 (e1). Breakpoints involving the m-bcr, which ultimately result in a smaller (p190) fusion protein with enhanced transforming potential, are rarely present in CML. The m-bcr breakpoints and fusion transcripts are present in ~60% of adult and ~90% of pediatric patients with t(9;22)-positive precursor-B-ALL. Overall, the presence of t(9;22) is quite common in adult patients with ALL (~25% ALL), but relatively less common in pediatric patients (~5% ALL). Infrequently, an extreme 3'

validates the implementation of targeted therapy with tyrosine kinase inhibitors (such as imatinib), while identification of the type of fusion transcript that is specific to the neoplastic clone may be important for subsequent monitoring. Although the majority of patients with CML possess the e13a2 (b2a2) or e14a2 (b3a2) transcript, identification of the e1a2 or e19a2 forms may explain unusual hematologic phenotypes.

Thus, detection of the most common *BCR* breakpoints (e1, e13, and e14) and the common (a2) as well as variant (a3) breakpoints involving *ABL1* can be accomplished with two parallel conventional RT-PCR assays that use *BCR*-exon 1/*ABL1*-exon 3 and *BCR*-exon 13/*ABL1*-exon 3 primers. An independent RT-PCR assay for an unrelated mRNA (e.g. *B2M*, *GAPDH*, *BCR*, or *ABL1*) is also necessary as a quality control to assess the integrity of the RNA and the presence of RT-PCR inhibitors. These three reactions may be multiplexed to allow for the simultaneous detection of the various transcripts, but multiplexing may reduce the sensitivity of the individual reactions. While reduced sensitivity is not relevant for diagnostic testing, it is very relevant for monitoring residual disease.

The interpretation of test results is influenced by the clinical context in which the test is performed. In the diagnostic setting, a negative RT-PCR result renders a diagnosis of CML most unlikely. However, it does not definitively exclude a diagnosis of CML since rare variant breakpoints can occur that may not be detected by conventional PCR primer sets. Similarly, a positive qualitative RT-PCR result does not definitively invoke a diagnosis of CML. Using an extremely sensitive nested RT-PCR technique, *BCR-ABL1* fusion transcripts have been identified in the blood of as many as two-thirds of healthy adults. Whereas the vast majority of CML patients express either the e13a2 (b2a2) or the e14a2 (b3a2) fusion transcripts, healthy individuals express primarily the e1a2 type. However, the extreme level of sensitivity achieved (~10<sup>7</sup>) with this nested technique is neither required nor recommended for routine clinical testing, and thus imatinib should not be an issue. Qualitative RT-PCR no longer has a role in minimal residual disease assessment, with quantitative RT-PCR having clearly emerged as the preferred modality for post-therapeutic monitoring.

### B. Molecular testing for monitoring

Following therapy, disease response is assessed using three parameters: hematologic, cytogenetic, and molecular status. Hematologic remission is achieved when the blood counts and spleen size have normalized. Cytogenetic response is quantified and graded based upon the percentage of residual Ph-cells. Traditionally, the cytogenetic response serves as the "gold standard" for assessment, and is an important predictor of patient survival.

Once conventional hematologic remission and cytogenetic complete response have been achieved, monitoring relies upon more sensitive molecular techniques. When the *BCR-ABL1* fusion transcript is no longer detectable, molecular remission has been attained. However, a widely accepted definition of molecular remission does not yet exist, and its designation varies with assay methodology and sensitivity. Nonetheless, a single negative or positive qualitative RT-PCR result is, in itself, of little clinical predictive value, since such assays may lack precision, depending on the sensitivity of the specific test. In contrast, serial quantitative assays that assess the kinetics of tumor clearance (response) or reappearance (relapse) have greater predictive value. In contrast to qualitative RT-PCR analysis, quantitative RT-PCR shows broad relevance to post-therapeutic monitoring whether after transplantation or therapy with imatinib. With such analysis, four patterns may emerge, with levels that: (1) continue to decline; (2) are undetectable; (3) become stable/plateau; or (4) rise. Rising levels of *BCR-ABL1* mRNA have been shown to precede disease recurrence and may signal a need for therapeutic intervention, while low, diminishing or stable fusion transcript levels identify patients in whom treatment has been effective.

breakpoint occurs beyond exon 19 (e19) of *BCR* in the designated micro region (3--ber), resulting in a larger (p230) fusion protein characteristically associated with neutrophilic CML in which mature granulocytes predominate, as well as in chronic neutrophilic leukemia, although recent data indicate a less well-substantiated association with the latter. The Philadelphia chromosome has also been detected in rare cases of *de novo* acute myeloblastic leukemia and T-cell ALL.

In CML, the occurrence of additional specific cytogenetic and molecular genetic events subsequent to the initial t(9;22) translocation herald disease progression prior to hematologic and/or clinical manifestations. The acquisition of such cytogenetic abnormalities as a second Philadelphia chromosome (+Ph), isochromosome 17q, trisomy 8, trisomy 19, and others, commonly indicate an impending blast crisis. Other molecular abnormalities associated with disease progression include overexpression of *BCR-ABL1*, upregulation of *LY11* expression, mutations in tumor suppressor genes such as *P16*, *TP53*, *CDKN2A* and *ABL1*, and aberrant DNA methylation of the translocated *ABL1* allele and of the calcitonin gene. The presence of a derivative chromosome 9 deletion (which typically requires FISH for its detection) in addition to the t(9;22) translocation has served as an independent prognostic factor predicting a rapid progression to blast crisis with a worse response to therapy and, hence, a shortened survival. However, some studies suggest that this prognostic association is abrogated by the use of imatinib.

There are three broad scenarios in which molecular testing is indicated in CML, at diagnosis, for monitoring during and following therapy, and in the emerging area of detecting kinase domain mutations.

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A rational understanding of laboratory monitoring is predicated on an appreciation of therapeutic goals and options. Indeed, eradication of the neoplastic clone cannot always be achieved, in which case therapeutic intent focuses on tumor control with an attempt to forestall disease progression. Current first-line treatments available to patients in the chronic phase of CML include allogeneic stem cell transplantation (ASCT) and tyrosine kinase inhibitors (imatinib and other newer agents) with imatinib having become the "gold standard" for initial therapy in the majority of patients.

Technologically, quantitative RT-PCR has evolved from the initial use of competitive and limiting dilution RT-PCR methods to real-time RT-PCR methods. Real-time RT-PCR affords sensitive, rapid, and reproducible quantification of the *BCR-ABL1* fusion transcripts. A closed tube system is employed which eliminates the need for post-PCR processing and minimizes the potential for contamination while simultaneously decreasing test time. Although the sensitivity of real-time RT-PCR methods is somewhat less than conventional or nested RT-PCR, the dynamic range of fluorescent detection is much broader, spanning five to six orders of magnitude, with a lower limit of detection of <0.01%. Most importantly, real-time RT-PCR has the precision required in clinical diagnostic applications.

Various fluorescent detection systems have been used to quantify *BCR-ABL1* transcripts. The major chemistries employed are the Taqman or FRET probe methods. For both of these, RNA or cDNA standards of known concentration are used to generate a standard curve [log (copy number) versus threshold cycle (C<sub>t</sub>)], from which the unknown sample quantity is determined, and then normalized against an internal reference (e.g. *ABL1*, *BCR* or *GUSB* transcripts). The final result is usually reported as a percentage ratio (e.g. *BCR-ABL1/ABL1*), although alternative methods of reporting include copy number and/or micrograms of RNA. Other chemistries include molecular beacons and melting curve analysis. Quantification can also be performed without the use of standard curves, using the delta-delta-C<sub>t</sub> method.

For therapeutic monitoring, a single positive qualitative (or indeed quantitative) RT-PCR result is not necessarily predictive of relapse in any individual patient regardless of therapeutic context; nonetheless, therapeutic context ultimately dictates the clinical ramifications of any test result. For instance, in the setting of bone marrow transplantation, the interval after transplant, the type of transplant (i.e. unrelated versus related matched), and the presence or absence of stem cell product manipulation (e.g. T-cell depletion) may all impact the prognostic relevance of qualitative test results. Most CML patients have a positive qualitative RT-PCR result in the first six months following ASCT that is not associated with a higher risk of relapse. Six to twelve months after ASCT, however, positive qualitative results are highly associated with, and considered to be an independent predictor of, subsequent relapse. In this setting, positive RT-PCR detection of *BCR-ABL1* fusion transcripts precedes cytogenetic and hematologic relapse by several months. Furthermore, detection of fusion transcripts by RT-PCR six to twelve months after transplant with an allogeneic-related matched donor and with T-cell depleted stem cell products is associated with a higher rate of relapse than in patients receiving unrelated donor or non-depleted stem cell products. With respect to monitoring post-transplantation, several quantitative indicators emerge. Thus, a modified definition of molecular relapse has been proposed as rising or persistently high levels of *BCR-ABL1* (defined as a *BCR-ABL1/ABL1* ratio of >0.02% to <10<sup>-7</sup>) in two sequential specimens procured more than 4 months after ASCT. Other studies have indicated that this threshold (<10<sup>-7</sup>) may be predictive of relapse when realized as early as day 100 post ASCT.

Although imatinib has shown encouraging results in the treatment of CML, the long-term correlates of clinical outcome remain to be defined. Nonetheless, numerous studies suggest that quantitative RT-PCR values correlate with established cytogenetic response criteria and are associated with a differential short- and medium-term outcome. In the IRIS study, a "major molecular response (MMR)" is defined as a ≥ 3 log reduction in *BCR-ABL1/BCR* levels compared to median pretreatment levels. Here, MMR was

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### A. Molecular testing for initial diagnosis

Cytogenetics serves to identify the presence of the t(9;22) translocation in only approximately 95% of cases of CML, but can identify the presence of other chromosomal abnormalities. In one-half of the patients with a normal karyotype, the *BCR-ABL1* fusion transcript is detectable at the molecular level. This discrepancy is due to submicroscopic cytogenetic events. Therefore, in those patients with a normal karyotype, who have the clinical and hematological profile of CML, molecular testing serves a primary role in CML diagnosis. In the remaining patients, who are negative for both the Philadelphia chromosome and the *BCR-ABL1* fusion transcript, alternative diagnoses such as atypical CML or chronic myelomonocytic leukemia should be considered. Molecular testing is also helpful in excluding a leukemoid reaction and other myeloproliferative disorders (MPDs) such as polycythemia vera, essential thrombocythemia and chronic idiopathic myelofibrosis, with *ABL2* mutational assessment being additionally helpful in the evaluation of these *BCR-ABL1* negative MPDs.

Molecular testing serves to document the presence of *BCR-ABL1* fusion transcripts at diagnosis, both for the rational use of targeted therapy and for subsequent molecular monitoring. Indeed, once the initial diagnosis of CML is established, periodic monitoring of both the therapeutic response and the level of residual disease becomes critical to evaluation and therapeutic decision-making. Qualitative RT-PCR has several attributes that make it well suited for initial diagnosis. Bone marrow aspirate and peripheral blood both constitute suitable (and comparable) specimens, obviating the need for frequent invasive procedures, while sensitivity and rapid turnaround facilitate initiation of therapy.

Methodologically, qualitative RT-PCR can be performed with a simple, nested, or multiplex approach. However, nested methods are to be avoided in the routine diagnostic laboratory due to a significant risk of PCR contamination and consequent false positive results. With non-nested, simplex, RT-PCR, one assay is performed using a single pair of primers. Primers for *BCR* exon 13 (b2) and *ABL1* exon 2 (a2) identify both the e13a2 (b2a2) and e14a2 (b3a2) fusion transcripts that differ in size by 75 bp. Using PCR primers to these regions allows for the molecular detection of CML in nearly all cases (~99%); however, variant breakpoints occur. When these variant breakpoints fall outside of the region recognized by the primers, they yield a false negative RT-PCR result, whereas if they fall within the primer region, they may yield a PCR product of unexpected size. These variant breakpoints are diverse and often complex, and cannot be globally assessed with any one pair of PCR primers; however, they are quite rare and are thus not a major cause of false negative results. The incorporation of an *ABL1* exon 3 (a3) primer, in place of an exon 2 primer permits the routine detection of rare *ABL1* intron 3 breakpoints. However, there have been fewer than ten patients with CML reported with this variant breakpoint, which is similarly rare in ALL.

Multiple fusion transcripts may be detectable with a single *BCR* intron 14 breakpoint due to alternative splicing of the primary transcript. With this breakpoint, the resultant chimeric gene is always directly transcribed to produce the e14a2 (b3a2) transcript as described above; however, concomitant alternative splicing may yield either an accompanying e13a2 (b2a2) and even an e1a2 transcript. e1a2 alternative transcripts may also be seen with breakpoints following e13 (b2). Identification of the e1a2 transcript requires a separate BCR primer targeting *BCR* exon 1 (e1). Although the e1a2 primer set is not usually essential to the diagnosis of CML, some studies suggest that the presence of this alternatively spliced transcript is associated with a poor prognosis and transformation to accelerated phase.

In the diagnostic setting, qualitative RT-PCR serves several functions. The presence or absence of *BCR-ABL1* transcripts distinguishes CML from leukemoid reactions or other MPDs. A positive qualitative result is critical for the diagnosis of CML, particularly in those patients lacking the Philadelphia chromosome on routine karyotypic analysis. The presence of *BCR-ABL1* transcripts

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achieved in 39% of newly diagnosed CML patients after 12 months of imatinib as compared to only 2% of patients on interferon-alpha plus Ara-C. Patients with this degree of molecular response to imatinib had a negligible risk of disease progression in the short- to medium-term. It has also been shown that patterns of MRD after attaining CCR (such as continuing decline in levels, plateau, or increasing levels) can predict cytogenetic relapse. The percentage of patients achieving MMR continues to increase with increasing duration of therapy, reaching 75% by 5 years.

One caveat to molecular monitoring is that it cannot assess the clonal evolution of disease. For this assessment, cytogenetic karyotypic analysis is required and should be performed every six to twelve months throughout the monitoring process regardless of the therapeutic modality employed. The emergence of cytogenetically abnormal (but Ph-negative) clones with the use of imatinib, with an incidence of ~5%, further underscores the need for periodic conventional cytogenetic analysis.

Notwithstanding the excitement regarding imatinib, ASCT is the only proven curative therapy for CML with cure rates of 70 to 80% in young (age <40 years), chronic-phase patients that have HLA-matched donors and undergo transplantation within one year of diagnosis. Despite the curative success of ASCT, the associated morbidity and mortality is significant; furthermore, the majority (~65%) of young patients do not have a suitably matched donor, while older patients are often suboptimal candidates for transplantation.

After transplantation, molecular testing serves two functions, first to document remission and then to monitor for disease relapse. Molecular monitoring for relapse permits early disease detection (prior to hematologic or cytogenetic manifestations), when the tumor burden is low and presumably more amenable to treatment. At molecular level, relapse, therapeutic options include the withdrawal of immunosuppressive agents and/or the administration of donor lymphocyte infusions (DLI) with an increased likelihood of response achieved when DLI are administered prior to overt hematologic relapse.

For patients who are not suitable candidates for ASCT, alternative therapies had historically included hydroxyurea and interferon-alpha; however, these agents have been supplanted by imatinib, which has shown activity in all phases of CML, with substantial responses in newly diagnosed patients in chronic phase. Imatinib has, thus far, shown improved hematologic responses and cytogenetic responses as well as a prolonged freedom from disease progression when compared to more traditional (non ASCT) therapies. In an on-going randomized Phase III trial (IRIS), the observed rate of complete cytogenetic remission (CCR) in newly diagnosed CML patients was 76% with imatinib versus 15% with interferon-alpha plus Ara-C (median follow-up 18 months), with CCR reaching 87% after 5 years of therapy with imatinib. At 54 months, the overall survival of patients treated with imatinib in early chronic phase is over 90%. In addition to its apparent clinical superiority, imatinib is also well tolerated, a therapeutic aspect that is in marked contrast to interferon-alpha with its numerous prohibitive toxicities. Although hydroxyurea successfully lowers the peripheral leukocyte count, it neither induces a cytogenetic response nor delays disease progression, thereby limiting its role in contemporary therapy.

While long-term outcome data are still maturing (they are currently available for 5 year follow-up), imatinib has clearly emerged as the primary therapy of choice for newly diagnosed CML, although probably still not curative since the *BCR-ABL1* fusion transcript typically persists at low levels. Persistence of molecular disease without relapse suggests attainment of a "dormant" state that may be induced and regulated by various mechanisms including immune surveillance and impaired proliferation that confer clonal quiescence. It has been shown that high doses of imatinib (800mg vs 400mg) may indeed induce molecular remission in up to 28% of patients. Monitoring of patients on imatinib is also pertinent with regard to determining loss of response to such therapy, which may be predictive of the

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development of resistance. Such observations dictate that therapy be altered; this may include (1) increasing the dose of imatinib, (2) changing to an alternative tyrosine kinase inhibitor, or (3) ASCT.

#### Issues in standardizing RO-PCR assays for BCR-ABL1

A large number of variables can clearly affect the validity of RO-PCR results, and attempts have been made to harmonize these so that results from different laboratories can be appropriately compared with one another. These variables are summarized in the following table:

VARIABLE	COMMENT
Specimen: source	Both peripheral blood and bone marrow acceptable, former preferred because less invasive Essential to stick with one (do not interchange)
Specimen: volume	5-10 ml PB Cell number more pertinent (>1-2 x 10 <sup>6</sup> )
Specimen: anticoagulant	EDTA (avoid heparin)
Specimen: transport	Ideally <24hrs to lab; up to 36 hours OK ? RNA stabilizers (concerns about affecting PCR efficiency)
Specimen: preparation	Do not enrich for mononuclear cells (avoid ficoll)
RNA extraction	All major commercial methods acceptable
RNA quality	? need to run on gel vs spectrophotometer
RT enzyme	Superscript and MMLV both OK (former favored by some)
RT primers	Random hexamers
Probe design	Both hydrolysis (Tagman) and hybridization (FRET) OK Avoid BCR e13 polymorphism
Instrument	ABI, Light Cycler, and others are interchangeable ? Cepheid cartridge system
"Control" normalizer gene	ABL1 vs BCR vs GUSB
Calibration standards	For standard curve: run serial dilutions with each run, in triplicate; plasmid DNA acceptable, R >0.98. ? can avoid if use delta-delta Ct method
QC samples	High and low, should mimic primary material
International reference standard	Plasmids vs lymphoblastoid cells vs cell extracts vs stabilized RNA. For conversion to international scale
Result units	Log reduction or percentage decline from baseline
Baseline	Lab mean of 30 specimens vs commercial standard
Frequency	Every 3 months, once CCR attained

#### C. Molecular testing for imatinib resistance

Therapeutic resistance to imatinib may be primary or secondary. Resistance is much more common with advanced stages of disease (accelerated phase and blast crisis) as compared with chronic or stable phase. Primary resistance refers to the failure to achieve an initial response to therapy, and is seen in ~5% of previously untreated patients in the chronic phase. Secondary resistance refers to the loss of a previously documented response, and develops in approximately 10-15% of previously untreated patients in chronic phase (and develops at a rate of approximately 1-4%/year). The proportion of patients developing resistance is higher in those who have received prior interferon-alpha therapy, and is evident in as many as 75% of patients in accelerated phase and 95% in blastic phase. The mechanisms involved in primary and secondary resistance are usually quite distinct. In primary resistance, the precise mechanisms are often unclear, however, they are typically not due to point mutations, and more likely occur due to diminished bioavailability of the drug. By contrast, the major mechanism of secondary resistance is indeed the presence of point mutations.

There are two broad mechanisms of resistance: (1) BCR-ABL1 independent, and (2) BCR-ABL1 dependent. BCR-ABL1 independent mechanisms include diminished bioavailability (due to factors such as poor absorption, increased hepatic metabolism, increased plasma binding by alpha 1 acid glycoprotein, increased cellular efflux or diminished influx due to the variable expression of MDR1, PGP, BCRP2/ABCG2, hOCT1, and MRP1) or clonal evolution (with the acquisition of additional genetic hits and/or activation of other cellular pathways). By contrast, BCR-ABL1 dependent pathways include kinase domain (KD) mutations and BCR-ABL1 amplification (genomic or transcriptional, with the former more common), which are seen in 40-90% and ~10% of cases of resistance, respectively.

#### Kinase domain mutations

This is the most common cause of resistance, and is evident almost exclusively in secondary resistance. Akin to antibiotic resistance that develops in bacteria, these mutations are not induced by imatinib, but rather are selected for by the drug. Intriguingly, it has been proposed that via BCR-ABL1 induction of reactive oxygen species, the oncoprotein attempts to escape inhibition by self mutation. This also suggests a possible role for the addition of antioxidants to therapy. More than 50 different mutations have been described; however, there are 7 that currently account for ~85% of all mutations. Mutations span >700bp in the region encoding the KD, affecting amino acids 240 through 500. Their frequency ranges between 40-90% of resistant patients, with this rather large range related to variables such as which patient populations are studied, what methods are used to detect these mutations, the definition of resistance and the phase of disease. Typically, mutations may be seen in ~10%, ~30%, ~60% and ~90% in early chronic phase, late chronic phase, accelerated phase and blast crisis, respectively.

Although it was initially thought that all mutations are sinister and predictive of resistance, it has emerged that not all mutations are created equally. There are 4 major regions in the KD in which mutations can occur: (1) P-loop [ATP binding site, which is where imatinib primarily exerts its inhibition]; (2) binding domain [site of imatinib binding]; (3) catalytic domain; and (4) activation loop [mutations here lead to conformational changes that affect imatinib binding, with this drug effective when this region is in a closed conformational state with mutations inducing an open/active state]. Most, but not all, studies indicate that P-loop mutations induce the greatest degree of resistance. However, a mutant clone does not necessarily have a proliferative advantage, and it may not even account for resistance. Some mutations may be addressed by escalating the dose of imatinib, while others may require the use of novel, "second generation" tyrosine kinase inhibitors. However, the most recalcitrant mutation, T315I (which is not located in the P-loop but in the binding domain), is also resistant to most of these novel agents.

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Please refer to printout of Powerpoint presentation.

Although mutant subclones are almost always evident prior to therapy, there appears to be no current advantage to testing at this stage. Rather, mutational testing is indicated in a number of specific scenarios, including treatment failure, suboptimal responses and in any patient in accelerated or blast phase. Specific indications are time dependent, and include the following failures to respond: (1) no complete hematologic remission by 3 months; (2) no major cytogenetic response by 6 months; and (3) no complete cytogenetic response by 12 months. Furthermore, mutational testing is indicated when there is loss of response, which includes either hematologic or cytogenetic relapse, or an increasing BCR-ABL1 transcript level (as small as a 2x increase, but a more routinely achievable measurement might be a 5x increase).

A variety of different technologies are available to identify and quantify KD mutations, including direct sequencing, subcloning and sequencing, denaturing high performance liquid chromatography, pyrosequencing, as well as allele-specific oligonucleotide PCR. These methodologies are listed from least sensitive (~15-25%) to most sensitive (<0.01%); however, it is unclear whether it is necessary to detect really small clones with the latter extremely sensitive assays, as clones only appear to become clinically significant when they reach ~20%. Thus, direct sequencing may be the clinically relevant method of choice. Preliminary work with microarrays has identified differential gene expression profiles between patients with imatinib sensitivity and resistance. In the future, this technology may predict therapeutic response, and thus dictate front-line therapy.

#### Overcoming resistance

A number of different strategies can be employed in an attempt to overcome resistance to imatinib. Two novel and quite potent BCR-ABL1 inhibitors have recently been evaluated in clinical trials. These new drugs are BMS-354825 (dasatinib, Sprycel) and AMN107 (nilotinib, Tasigna). Dasatinib is structurally unrelated to imatinib, is a dual SRC and ABL1 kinase inhibitor and is ~325x more potent than imatinib, while nilotinib is structurally related to imatinib and ~25x more potent. Both overcome most forms of imatinib resistance; however, neither is effective against the T315I mutation. Using either of these promising new agents, ~80% of patients with imatinib resistance have a complete hematologic response and more than 30% achieve complete cytogenetic remission. Even more recently, a novel Aurora kinase inhibitor (VX-680, MK-0457) has been shown to be effective in overcoming T315I-mediated resistance. Other strategies that are being evaluated include allosteric inhibitors, downstream inhibitors (affecting the RAS, PI3/AKT and JAK/STAT pathways), heat shock protein (HSP) inhibitors and immunotherapeutic approaches. The latter include vaccines directed against BCR-ABL1, PR1, WT1 and HSP, which are more likely to have a role in treating minimal residual disease rather than as primary therapeutic interventions. Ultimately, it is likely that rational combination therapy will be most effective in preventing the emergence of resistance.

#### Conclusion

Molecular techniques, therefore, encompass many aspects of laboratory testing that are critical to the overall evaluation of patients with CML and contribute to the diagnosis, assessment of therapeutic efficacy, evaluation of minimal residual disease and detection of resistance in these patients. The clinical context of the patient and the specific treatment modality determine the specific tests that are appropriate, and algorithms have been proposed to effectively and economically guide both laboratory diagnosis and monitoring. These algorithms appropriately incorporate various methodologies including cytogenetic and FISH analysis in addition to molecular techniques, and are likely to evolve as both technology and therapy evolves.

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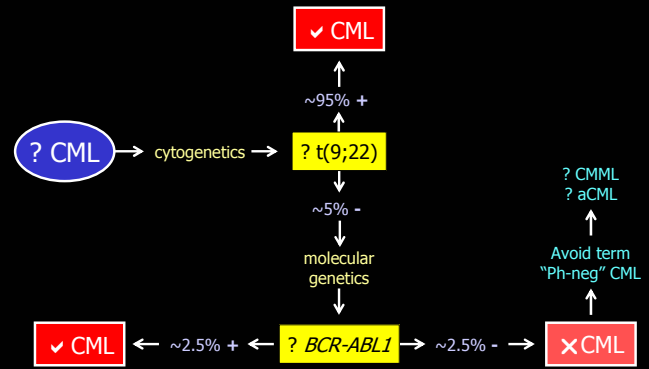
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# Genetic Testing in Chronic Myelogenous Leukemia: Diagnosis, Monitoring and Resistance

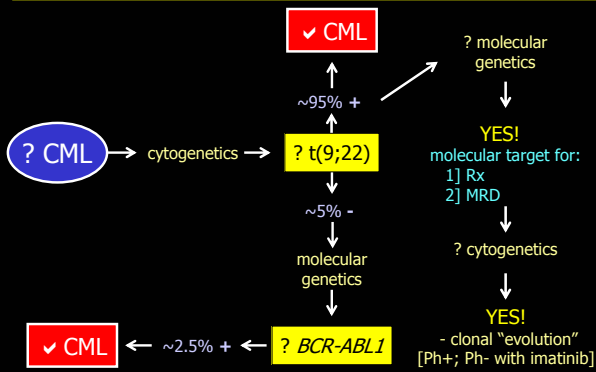
Society for Hematopathology  
Companion Meeting  
United States and Canadian Academy of Pathology  
San Diego, March 2007

Adam Bagg  
University of Pennsylvania  
Philadelphia, PA

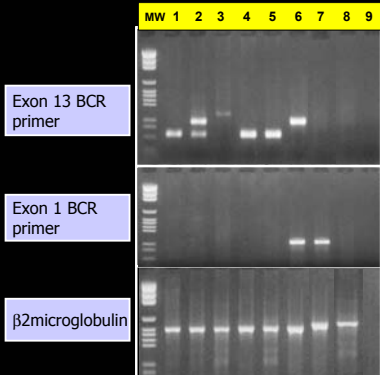
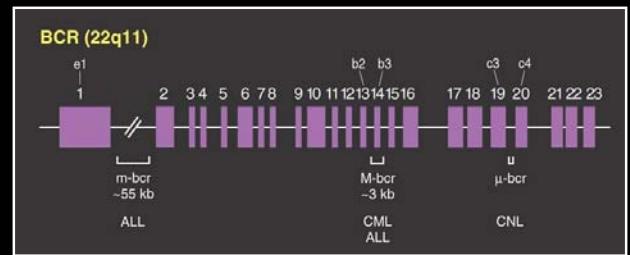
## CML diagnosis: t(9;22) and BCR-ABL1



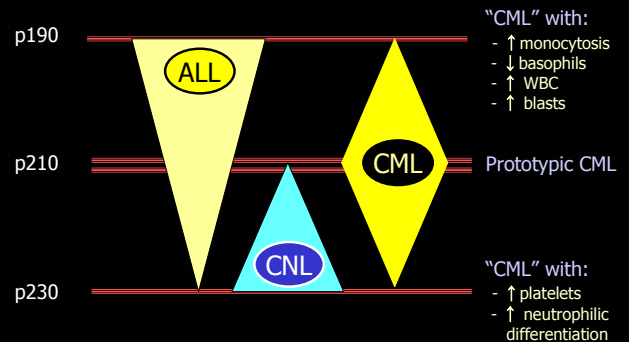
## CML diagnosis: t(9;22) and BCR-ABL1



## What's going on at the DNA level ...



## Protein ..... hematologic phenotype ..... CML phenotype



## CML diagnosis: am I dissing FISH?

### Indications for FISH at diagnosis:

- complex karyotype/variant Ph
- failed karyotype
- patient refuses BM [PB FISH]
- false negative RT-PCR
- to detect der(9) deletion
- "local expertise and availability"

RT-PCR

rare e13 deletion [~1%]

? still relevant

## CML monitoring

### Two major forms of therapy ...

TKI

- Initial therapy of choice
- Does not eradicate/cure CML
- ? Long-term outcome
- Minimal toxicity<sup>1</sup>

Rx goal

BCR-ABL1 reduction

SCT

- No longer 1<sup>st</sup> line Rx<sup>2</sup>
- Only Rx that cures CML<sup>3</sup>
- Major toxicity and mortality<sup>4</sup>

BCR-ABL1 negativity

- 1 cytopenias (~40%), cardiotoxicity, ? mutagenicity [inhibit eph tumor suppressor, Ph (-) clones]
- 2 indicated when [i] very young, [ii] TKI failure, [iii] AP and BC
- 3 10-yr survival ~65%
- 4 10-20% mortality even when low risk

## CML monitoring

modalities...

sensitivities...

CBC	~10%	[~10 <sup>-1</sup> ]
conventional cytogenetics	~5%	[~10 <sup>-2</sup> ]
D-FISH	~0.5%	[~10 <sup>-3</sup> ]
RT-PCR [quantitative]	~0.001%	[~10 <sup>-5</sup> ]
RQ-PCR [qualitative]	~0.0001%*	[~10 <sup>-6</sup> ]

\* non-nested; can reach 10<sup>-8</sup> with nested RT-PCR

## CML monitoring

SCT

### Qualitative RT-PCR

- positivity 1st 6 months post-SCT: not predictive
- positivity 6-12 months post-SCT: predictive

### Quantitative RT-PCR

- levels >10<sup>-4</sup> on 3 occasions: requires intervention [DLI]
- levels at d100 [10<sup>-4</sup>]: predictive of outcome
- persistent low levels [<10<sup>-4</sup>]: may not predict relapse
- monitor for life: can relapse very late [21yrs!]

## CML monitoring: definitions of response

### complete hematologic

- platelet: < 450
- WBC: < 10
- diff: no immature granulocytes
- basos: < 5%
- clinical: non-palpable spleen

### cytogenetic # Ph+

- none: >95%
- minimal: 66-95%
- minor: 36-65%
- partial: 1-35%
- complete: 0%

### molecular

• next slide please ...

# cells	responses	log reduction	BCR-ABL1 ratio	imatinib responses @ 5 years
10 <sup>12</sup>	diagnosis		100	
<10 <sup>11</sup>	complete hematologic remission			~98%
<10 <sup>10</sup>	complete cytogenetic remission			~85%
<10 <sup>9</sup>	major molecular response	>3	<0.1	~75%
<10 <sup>6</sup>	"complete molecular remission" undetectable transcript		<0.0001	~10%

... yet overall survival ~95%

## Variables associated with BCR-ABL1 RQ-PCR

specimen: source	specimen: volume	specimen: anticoagulant	specimen: transport
RNA extraction	RNA quality	RT enzyme	RT primers
probe	instrument	"control" gene	reporting units
calibration	QC samples	international reference	frequency

## Variables associated with BCR-ABL1 RQ-PCR

PB or BM be consistent	10ml PB > 2x10 <sup>7</sup> cells	EDTA	<24 hours ? RNA stabilizers
most kits OK	? need gel vs spectro	Superscript > MMLV	random
hydrolysis or hybrid	ABI vs Light cycler	<i>ABL1</i> vs <i>BCR</i> vs <i>GUSB</i>	copies vs log reduction
standard curve: DNA plasmids	high and low: mimic 1 <sup>0</sup>	cell extracts, lyophilized cells, plasmids, RNA	3 monthly unless ...

## Resistance to imatinib (and other TKIs ...)

### primary

- failure to achieve an initial response
- seen in ~5% of pts
- bioavailability; not due to mutations

### secondary

- loss of response, occurs at ~1-4%/year
- ~10-15% of pts [in chronic phase, no prior Rx]
- more common if:
  - prior interferon Rx
  - accelerated phase [~75%]
  - blast crisis [~95%]

## Mechanisms of resistance to imatinib

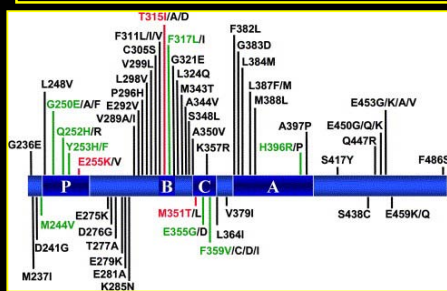
### BCR-ABL1 dependent

1. Kinase domain mutations:
  - most common cause of resistance [~40-90%]
  - spans ~240 aa's
2. BCR-ABL1 amplification:
  - genomic > transcriptional [~10%]

### BCR-ABL1 independent

3. Clonal evolution:
  - other genetic/cellular pathways [LYN]
4. ↓ Bioavailability:
  - absorption
  - metabolism [hepatic]
  - plasma binding [α1acid glycoprotein sequestration]
  - ↓ influx ↑ efflux [MDR1, PGP, BCRP2/ABCG2, hOCT1, MDR1]

## Kinase domain mutations



> 50 different mutations

green 2-10% of patients

red >10% of patients

### P = P loop

- ATP-binding site
- ? worst mutations

### B = Binding domain

- where imatinib binds

### C = Catalytic domain

### A = Activation loop

- conformation altered
- affects imatinib binding
- closed: inactive
- open: active

## Kinase domain mutations

- most common cause of resistance
  - 10% [early CP] → 30% [late CP] → 60% [AP] → 90% [BC]
- not induced by Rx
  - selected for by Rx [cf antibiotic resistance in bacteria]
- ? BCR-ABL1 induced
  - via ROS [escape inhibition by self-mutation; ? add antioxidants to Rx]
- not all are created equally
  - initially: all bad ... currently: P-loop worst [not all agree]
  - can overcome most with dose-escalation or other TKI's [T315I exception]
  - does not necessarily have a proliferative advantage
  - presence does not always account for resistance

## Indication for mutation testing

treatment failure/  
suboptimal response

- no CHR by 3 months
- no MCR by 6 months
- no CCR by 12 months

loss of response

- hematologic relapse
- cytogenetic relapse
- ↑ ing *BCR-ABL1* levels [5x]

anyone in AP or BC

## Methods of mutation testing

Technology	Sensitivity	Specificity	Bias
Direct sequencing	15-25	++	no
Subcloning and sequencing	10	+++	no
D-HPLC	0.1-10	++	no
Pyrosequencing	5	++	no
Double-gradient denat. electroph.	5	++	no
Fluorescence PCR – PNA clamping	0.2	++	yes
ASO-PCR	0.01	++	yes

Small clones (<20%) may not be clinically significant  
→ direct sequencing (current) method of choice

## Overcoming resistance

### Alternative kinase inhibitors:

- 2<sup>nd</sup> generation TKIs:
- dual SRC-ABL inhibitors:
- aurora kinase inhibitors:

nilotinib [AMN107 – Tasigna]  
dasatinib [BMS354825 – Sprycel]  
MK-0457 [VX-680]

### Downstream inhibitors:

- RAS pathway:
- PI3/AKT pathway:
- JAK/STAT:

FTIs  
mTORi  
MPA

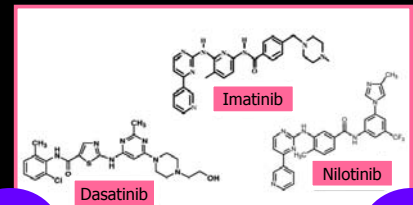
### ImmunoRx:

- vaccines against:
- 1<sup>o</sup> role in MRD Rx

BCR-ABL1, PR1, WT1, HSPs

→ comboRx to prevent emergence of resistance

## Overcoming resistance



~350x

~25x

increased potency of imatinib ...  
overcome most resistant mutations  
no effect on T315I ...

## Genetic testing in CML: summary

Diagnosis

CC	BM	? PB FISH instead
RT-PCR	PB	qualitative vs quantitative with characterization

Monitoring

CC	BM	3-6 monthly until CCR 6-12 monthly thereafter
FISH	PB	before achieve CCR
RQ-PCR	PB	3 monthly

Resistance

direct sequencing	PB v BM	Rx failure, loss of response, accelerated & blast phase
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## Oncogenes in myeloproliferative disorders

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### Introduction

In 1951, William Dameshek (1900-1969) highlighted the phenotypic similarities among chronic myeloid leukemia (CML), polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF), and grouped them under the rubric of myeloproliferative disorders (MPDs).<sup>1</sup> In 1960,<sup>2</sup> CML became the first cancer to be associated with a specific cytogenetic marker, the Philadelphia chromosome (Ph<sup>+</sup>), which subsequently was shown to harbor a reciprocal chromosomal translocation, t(9;22)(q34;q11).<sup>3</sup> These seminal observations ultimately led to the identification of the disease-causing mutation (*BCR-ABL1*) in CML and development of molecularly targeted therapy.<sup>4</sup> Accordingly, the “classic” MPDs are now sub-classified into CML (i.e. *BCR-ABL1*<sup>+</sup>) and the *BCR-ABL1*<sup>-</sup> classic MPDs (i.e. PV, ET, and PMF).<sup>5</sup> The recently described association between all three *BCR-ABL1*<sup>-</sup> classic MPDs and a novel gain-of-function (GOF) *JAK2* mutation (*JAK2V617F*) validates their continued consideration as a distinct MPD category (Table 1).<sup>6,7</sup> Both *ABL* and *JAK2* encode for cytoplasmic tyrosine protein kinases (PTKs), that become constitutively activated as a result of these mutations.

Between 1967 and 1981, Falkow and colleagues used polymorphisms in the X-linked glucose-6-phosphate dehydrogenase locus to establish all four classic MPDs as clonal stem cell disorders, with evidence for involvement of myeloid<sup>8,10,14</sup> and B lymphoid<sup>9,16</sup> lineages. The stem cell origin of the classic MPDs has been confirmed by more recent studies demonstrating clonal involvement of B<sup>17-19</sup>, T<sup>17-19</sup>, and natural killer (NK)<sup>20</sup> lymphocytes. Furthermore, early reports<sup>21</sup> on the occurrence of Ph<sup>-</sup>negative clonal B lymphocytes in CML have suggested that preclinical clonal myelopoiesis might antedate disease-causing mutations in classic MPDs. More recent observations that support such a contention include the emergence of new cytogenetic clones during successful treatment of CML with imatinib;<sup>22</sup> the imperfect association between overall clonal load and *JAK2V617F* mutant allele burden in PV;<sup>23</sup> and the observation that heritable MPD syndromes have been observed in which the *JAK2V617F* allele is acquired with development of MPD, but is not present in the germline of affected family members, indicating the presence of an antecedent predisposition allele.<sup>24</sup>

In addition to the classic MPDs, myeloid malignancies also include acute myeloid leukemia (AML), the myelodysplastic syndrome (MDS), and other chronic myeloid neoplasms that are now classified either under one all-inclusive category (i.e. “non-classic” MPDs; Table 1)<sup>8</sup> or into multiple subcategories.<sup>25</sup> The distinction between MPD and MDS is based primarily on bone marrow histology; MDS is usually characterized by trilineage dysmyelopoiesis in the absence of monocytosis.<sup>26</sup> However, precise histological distinction between MPD and MDS is not always possible, we have arbitrarily grouped such cases with other “non-classic” MPDs (Table 1)<sup>8</sup> whereas the World Health Organization classification system has assigned them to a separate category (i.e. mixed MDS/MPD).<sup>27</sup> Regardless, both classic and non-classic MPDs share the common characteristics of stem cell-derived clonal myeloproliferation, in most instances, and activation of intracellular signal transduction pathways through mutations of PTKs or their effector molecules, in some instances.

Examples of mutant PTK genes in MPDs include *BCR-ABL* (invariably associated with CML),<sup>3</sup> *JAK2V617F* (seen in approximately 95% of patients with PV and 50% of those with either ET or PMF),<sup>31</sup> *JAK2* exon 12 mutations (seen in the majority of patients with *JAK2V617F*-negative PV),<sup>32</sup> *FIP1L1-PDGFRα* (seen in a unique subset of patients with chronic eosinophilic leukemia associated with systemic mastocytosis; CEL; SM),<sup>33</sup> *ETV6-PDGFRβ* in rare mutations seen in CEL, that is sometimes associated with monocytosis),<sup>34,35</sup> *ZNF198-FGFR1* (seen in patients with stem cell leukemia-lymphoma associated with monocytosis),<sup>34,35</sup>

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syndrome; SCLL),<sup>36</sup> and *KITD816V* (seen in virtually all adult patients with SM).<sup>37</sup> Examples of mutations in PTK effector molecules include *MPLW515L* (seen in approximately 5% and 1% of patients with PMF and ET, respectively),<sup>38,39</sup> and *RAS*, *PTPN11*, or *NFI* mutations, each seen in 15% to 30% of patients with juvenile myelomonocytic leukemia (JMML).<sup>40,42</sup> In the current review, we will discuss the aforementioned MPD-associated oncogenes and highlight the use of their corresponding oncoproteins as drug targets. For the sake of completeness, we have also included a section on *GATA-1* mutations and Down syndrome-associated transient MPD.<sup>41</sup>

### *ABL* mutations

*ABL* is a cytoplasmic PTK encoded by the *ABL* gene (Abelson murine leukemia virus (v-abl) oncogene homolog 1) located on chromosome 9q34.1. Physiologic functions of ABL include non-catalytic myelinating and cytoskeletal rearrangement, regulation of transcription, regulation of PAK inhibition of cell migration, and F-actin binding.<sup>43</sup> Wild-type ABL exists in two isoforms that can localize to both the cytoplasm and nucleus, influencing cell proliferation/survival and apoptosis, respectively, through complex interactions with other cellular proteins.<sup>46-48</sup> ABL contains both an SH2 and an SH3 (autoregulatory) domain in addition to the catalytic kinase domain and undergoes a treatment-relevant conformational change when activated by phosphorylation of the activation loop tyrosine residues.<sup>49</sup>

The most extensively studied *ABL* mutation is *BCR-ABL1*, the disease-causing mutation in CML.<sup>50,51</sup> CML is the first leukemia to be described<sup>52</sup> and also the first to be associated with a consistent cytogenetic abnormality, the Philadelphia chromosome (Ph<sup>+</sup>).<sup>3</sup> Ph<sup>+</sup> is a shortened chromosome 22 that is the consequence of a reciprocal translocation between chromosomes 9 and 22, (9;22)(q34;q11).<sup>3</sup> The chromosome 9 breakpoint involves a large, ~200 kb region within the *ABL* alternative first exons (1a and 1b), but invariably result in fusion genes that incorporate *ABL* exon 2.<sup>53</sup> In contrast, the breakpoints on chromosome 22 are clustered within three much smaller regions of the *BCR* gene;<sup>54</sup> the major breakpoint cluster region (M-ber, a 5.8 kb region spanning exons 12-16 and resulting in a p210 fusion protein),<sup>55</sup> the minor breakpoint cluster region (m-ber, upstream of M-ber and involving the first intron and resulting in a p190 fusion protein),<sup>57,58</sup> and *μ*-ber involving intron 19 that is downstream of M-ber and resulting in a p230 fusion protein.<sup>59</sup> By far the most frequent chromosome 22 breakpoint in CML is M-ber and the other two, in the context of rare, though usually two junction variants of M-ber, b2a2 and b3a2, without any documented clinical relevance.<sup>60</sup>

The Philadelphia translocation is an acquired somatic mutation involving the hematopoietic stem cell<sup>61</sup> and results in fusion of the *ABL* gene (225 kb total gene size) from chromosome 9 to the *BCR* gene (135 kb total gene size) on chromosome 22.<sup>56,62,63</sup> A chimeric mRNA (8.5-kb) is thus transcribed instead of the normal c-ABL mRNA (A (6 or 7.4-kb) and subunit B (1.9-kb) transcripts). The *BCR-ABL* gene product (most commonly 210-kD) instead of the normal *ABL* gene product (145-kD).<sup>64</sup> *BCR-ABL* localizes to the cytoskeleton and displays an up-regulated tyrosine kinase activity<sup>65</sup> that leads to the recruitment of downstream effectors of cell proliferation and cell survival and consequently leukemogenesis, as has been demonstrated in cell lines, primary cells, and mouse transplant or transgenic models.<sup>66,67</sup> *BCR-ABL* signal transduction involves several adapter molecules (e.g. GRB2, GAB2, CRKL, etc.) and signaling pathways (e.g. Ras, PI3K, JAK-STAT, etc.) that are all thought to contribute to the pathogenesis of CML (Figure 1).<sup>67,73</sup>

CML is currently considered the “poster child” for molecularly targeted therapy. In 1996, Brian Druker and his colleagues described the *BCR-ABL* selective in vitro activity of imatinib mesylate (imatinib), a 2-phenylaminopyrimidine class PTK inhibitor that is a selective inhibitor of ABL, ARG, PDGFRα, PDGFRβ, and KIT.<sup>74</sup> Imatinib targets the ATP binding site within the *BCR-ABL* tyrosine kinase and competitively inhibits ATP binding with subsequent disruption of the oncogenic signal.<sup>75</sup> The co-crystal structure of imatinib bound to the ABL kinase indicates that the drug stabilizes the oncoprotein in an enzymatically inactive conformation.<sup>76</sup> This in vitro activity of imatinib has been clinically validated in chronic phase CML, in newly diagnosed patients, the drug produced 95% and 74% complete hematologic and cytogenetic responses, respectively.<sup>77</sup> However, the drug is not effective in advanced phases of CML, and imatinib-resistant *BCR-ABL* oncoproteins are emerging as an important

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clinical challenge.<sup>81,82</sup> The latter are most often due to acquired *ABL* point mutations that either directly hinder drug binding or prevent the formation of the inactive structural conformation that is required for imatinib inhibitory activity.<sup>83,84</sup> Examples of such kinase domain mutations include M351T, E255K, Q252H, T315I, E355G, F359V, F317L, and G250E.<sup>85</sup> The development of second generation kinase inhibitors and better understanding of mutant ABL conformation dynamics have effectively addressed these new challenges, although some mutations such as T315I have so far remained resistant to even second generation kinase inhibitors.<sup>86,87</sup>

To date, *ETV6* is the only known non-*BCR* fusion partner for *ABL*. *ETV6-ABL* ((9;12)(q34;p13) has rarely been associated with ALL,<sup>95-98</sup> AML (transient response to imatinib),<sup>96,98</sup> and atypical CML-like MPD (minor response to imatinib).<sup>94,99-101</sup> Like *BCR-ABL*, *ETV6-ABL* encodes for an activated and transforming ABL.<sup>100-102</sup> The PNT oligomerization motif of *ETV6* is thought to activate the ABL kinase by a similar mechanism to that of *BCR*.<sup>103</sup> The ETV6-ABL fusion protein contains a cytoskeletal cytoplasmic oligomerization domain to form a constitutive *BCR-ABL* fusion protein. The same *ETV6* activation domain is involved in CEL-associated *ETV6-PDGFRβ* (t(5;12)(q33;p13),<sup>14</sup> AML-associated *ETV6-ARG*, (t(1;12)(q25;p13),<sup>105</sup> ALL/atypical MPD-associated *ETV6-JAK2*, (t(9;12)(p24;p13),<sup>104,106</sup> and peripheral T cell lymphoma-associated *ETV6-FGFR3*, (t(4;12)(p16;p13).<sup>106</sup>

### *JAK* mutations in MPD

The Janus family of tyrosine kinases (JAK1, JAK2, JAK3, TYK2) are cytoplasmic PTKs that mediate signaling downstream of cytokine receptors.<sup>107,109</sup> Janus kinases are named after the Roman god with two faces, and are unique among tyrosine kinases in that they contain two homologous kinase domains: a catalytically active JH1 domain and JH2 domain that is nearly identical in amino acid sequence to JH1, but lacks catalytic activity.<sup>107,1010,11</sup> The JH2, or pseudo-kinase, domain is thought to play a negative autoregulatory role in that deletion of JH2 results in constitutive activation of the JAK2 kinase.<sup>12,111</sup> Activation of the JAK-cytokine receptor complex results in recruitment and JAK-mediated phosphorylation of substrate molecules including STAT proteins whose subsequent nuclear translocation induces target gene transcription.<sup>114,12</sup> JAK-STAT signaling has pleiotropic effects on cellular proliferation, cell survival, and immune responses.<sup>114,12</sup> *JAK1* and *JAK2* knockouts are not viable and are devoid of either definitive erythropoiesis (*JAK2*)<sup>113,121</sup> or normal lymphoid development (*JAK1*). *JAK3* and *TYK2* knockout mice are viable and manifest either severe combined immunodeficiency (SCID; *JAK3*)<sup>122</sup> or impaired interferon signaling (*TYK2*).<sup>124</sup>

A limited number of *JAK* mutations have been linked to human disease; these have so far involved only *JAK3* and *JAK2* (Table 2). Germline *JAK3* loss-of-function (LOF) mutations have been associated with autosomal recessive SCID,<sup>125,126</sup> whereas somatic GOF *JAK3* mutations (*JAK3S72Y*, *JAK3V722I*, and *JAK3P132T*) were recently detected in acute megakaryocytic leukemia cell lines or primary cells.<sup>127</sup> The oncogenic potential of *JAK2* mutations in man is first suggested by a set of observations made in the 1990s;<sup>28,128</sup> a dominant mutation in either the JH4 (*HOP*<sup>28,128</sup>) or JH2 (*HOP*<sup>28,128</sup>) domain of *HOP*, a *JAK* homolog in *Drosophila*, resulted in leukemia-like defects<sup>129</sup> whereas a similar point mutation in murine *JAK2* (*JAK2*<sup>2906G</sup>) resulted in an activated protein.<sup>129</sup> Similarly, others demonstrated inhibition of ALL cell growth by a *JAK2* kinase inhibitor (AG-490) and the association of *ETV6-JAK2*, t(9;12)(p24;p13), with both T and pre-B ALL, as well as atypical MPD.<sup>130,105</sup> Most recently, two other *JAK2* fusion mutants were described, *PCML-JAK2*, (8;9)(p22;p24), in eosinophilia-associated atypical MPD, ALL/AML, or T cell lymphoma,<sup>130,131</sup> and *BCR-JAK2*, t(9;22)(p24;q11;2), in atypical MPD that was not responsive to imatinib.<sup>132</sup> However, *JAK2V617F* constitutes the most MPD-relevant *JAK2* mutation because it is found in virtually all patients with PV and about half of those with ET or PMF.<sup>31</sup>

*JAK2V617F* is a G to T somatic mutation of *JAK2*, at nucleotide 1849, in exon 14, resulting in the substitution of valine to phenylalanine at codon 617.<sup>31</sup> The association of *JAK2V617F* with MPDs, including PV, ET, and PMF, was first reported in 2005.<sup>31</sup> Since then, however, the mutation has been described, at a lower frequency, in a spectrum of other myeloid disorders including non-classic MPDs and MDS,<sup>133</sup> but not in lymphoid disorders,<sup>134</sup> solid tumors,<sup>135-143</sup> or secondary myeloproliferation.<sup>144-145</sup> Furthermore, whereas mutational frequency exceeds 50% in *BCR-ABL1*<sup>+</sup> classic MPD,<sup>36-38</sup> it is less than

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5% in AML or MDS.<sup>135,136,140,142,150,151</sup>

*JAK2V617F* occurs in certain acute leukemia cell lines including the human erythroleukemia (HEL) cell line.<sup>8,152</sup> HEL cells carry the *JAK2V617F* mutation in a homozygous state; display constitutive activation of JAK2, STAT5, and ERK; and their growth is inhibited by a small molecule JAK2 inhibitor.<sup>8</sup> Similarly, when *JAK2V617F* is transfected in various cell lines, it promotes cytokine-independent<sup>8,153</sup> and erythropoietin (Epo)-<sup>8,153,154</sup> or interleukin-3 (IL-3)-<sup>155</sup>hypersensitive growth accompanied by constitutive activation of JAK2,<sup>8,153,154</sup> STAT5,<sup>8,153</sup> ERK,<sup>8,153</sup> and Akt.<sup>155</sup> pathways. Similarly, primary bone marrow cells with *JAK2V617F*-induced MPD display constitutive STAT5 activation<sup>154</sup> and endogenous and Epo-hypersensitive erythroid clonal formation.<sup>154,155</sup> In vivo, such mice manifest a PV-like disease with erythrocytosis but not thrombocytosis; also seen are low serum Epo level, splenomegaly, extramedullary hematopoiesis, granulocytosis, megakaryocytic hyperplasia, bone marrow fibrosis, and anemia.<sup>154</sup> The STAT5/βc1-sL pathway might be the key mediator of *JAK2V617F* effect since Epo-independent terminal differentiation of erythroid precursors as well as endogenous erythroid clonal formation have been demonstrated through STAT5 activation or βc1-sL overexpression.<sup>156</sup> Additional laboratory studies have also suggested that there may be aberrant cytokine receptor interactions and the need for the expression of homodimeric cytokine receptors for *JAK2V617F* functionality.<sup>157</sup>

The role of *JAK2V617F* in human disease is currently under intense investigation. The mutation occurs at a primitive stem cell level and is chronologically an early event.<sup>158,159</sup> In this regard, some studies have suggested clonal involvement of NK,<sup>160</sup> and B<sup>161</sup> lymphocytes while others do not.<sup>161</sup> Familial MPD studies have confirmed the somatic nature of *JAK2V617F* and did not reveal differences in mutation distribution compared to that seen in sporadic MPD.<sup>20,63,162</sup> These findings suggest that there is an as yet undefined allele that in some predisposes to the acquisition of the *JAK2V617F* mutation. The same might be true in sporadic MPD; there is evidence to suggest that *JAK2V617F* may not be the initial clonogenic event in either PV or other MPDs and its presence might not be mandatory for endogenous clonal formation.<sup>158,163</sup> The recent demonstration of *JAK2V617F*-negative leukemia clones arising from *JAK2V617F*-positive MPD lends further support in this regard.<sup>164</sup> At the same time, however, *JAK2V617F* or other *JAK2* mutations might be an essential component of PV because of their invariable association with the disease,<sup>22,165,166</sup> whereas germline genetic variation/hot modifiers<sup>167</sup> and/or the occurrence of other concomitant mutations<sup>68</sup> might play a part in diverting the disease phenotype from PV toward ET or PMF, in the presence of the *JAK2* mutation. Figure 2 presents a pathogenetic scenario that takes these observations into account. Furthermore, a high mutant allele burden, rather than the mere presence of *JAK2V617F*, might be essential for erythroid-weighted myeloproliferation<sup>168</sup> and the acquisition of the PV phenotype *in vivo*; this is accomplished by a mitotic recombination that leads to homozygosity for *JAK2V617F*.<sup>169</sup> In other words, homozygosity for *JAK2V617F* is believed to contribute towards full phenotypic penetrance in PV. Accordingly, *JAK2V617F* clonal load is significantly higher in PV compared to ET, where a pure mutant allele state is rarely encountered even at a single colony level.<sup>171</sup>

Most recently, other *JAK2* mutations were described in *JAK2V617F*-negative patients with PV and in “idiopathic” erythrocytosis.<sup>172</sup> The majority of such cases (10 of 11 in one study)<sup>172</sup> were found to harbor one of four exon 12 *JAK2* mutant alleles (F537-K539delinsL, H538KQ539L, K539L, N542-E543del) with functional relevance that is similar to that of *JAK2V617F*; they induce cytokine-independent/hypersensitive proliferation in erythropoietin receptor-expressing cell lines and a PV-like phenotype in mice.<sup>172</sup> The four newly described exon 12 mutations, which include both in-frame deletions and tandem point mutations, appear to be specific to either PV or idiopathic erythrocytosis; the latter refers to a phenotype with increased red cell mass that fails to fulfill conventional diagnostic criteria for either PV or secondary polycythemia. Unlike the case with PV-associated *JAK2V617F*, exon 12 *JAK2* mutations are heterozygous but associated with stronger abnormal JAK2 activation.

Although the precise role of *JAK2* mutations in MPD pathogenesis remains under investigation, the currently available information warrants consideration of *JAK2* and other JAK-STAT pathway molecules as potential drug targets in the treatment of MPD. Endogenous regulation of JAK2 signaling

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occurs at multiple levels including direct dephosphorylation by specific protein tyrosine phosphatases, proteolytic degradation of JAK2 through binding with a family of suppressor tyrosine signaling, and inhibition of DNA binding of STAT by protein inhibitors of activated STAT.<sup>173,174</sup> Exogenous negative regulation is usually accomplished by variably specific JAK kinase inhibitors.<sup>175</sup> One such compound is the typhostin AG-490, which *in vitro* induces death to pre-B ALL cells with constitutive *JAK2* expression.<sup>176</sup> Similarly, both AG-490 and other small molecule pan-JAK inhibitors<sup>177</sup> inhibit *JAK2V617F*-associated hematologic and cytogenetic responses in both HEL cells<sup>178,179</sup> and primary cells.<sup>180</sup> Furthermore, such JAK-STAT inhibition is not restricted to mutant JAK; it has also been with MPD-associated mutant *MPL*.<sup>180</sup> However, none of these compounds are candidate drugs and their activity is relatively non-specific.<sup>179,180</sup> There is currently intense activity in the development and testing of both specific and non-specific (e.g. aurora kinase inhibitors)<sup>181</sup> *JAK2* inhibitors and the first clinical trials have already been initiated.

### *MPL* mutations

Thrombopoietin (Tpo) is the key growth factor for megakaryopoiesis and is essential for platelet production.<sup>182</sup> Tpo acts through its receptor, Mpl, and the JAK-STAT signal transduction pathway.<sup>183</sup> The human *TPO* gene is located at chromosome 3q27<sup>184</sup> and that of *MPL* at chromosome 1p34.<sup>185</sup> Mutations involving *TPO* in man have been described in autosomal-dominant familial thrombocytosis,<sup>186</sup> and those of *MPL* in either familial thrombocytosis<sup>186</sup> or congenital amegakaryocytic thrombocytopenia.<sup>188,191</sup> Such mutations, however, have not been found in sporadic cases of ET.<sup>162,192</sup> Nonetheless, the potential pathogenetic contribution of the Tpo-Mpl axis to human MPD has been suggested by two important observations; systemic over-expression of Tpo causes myelofibrosis in mice<sup>193</sup> and *Mpl* expression is markedly decreased in megakaryocytes and platelets of patients with ET.<sup>194,195</sup> In addition, the latter phenomenon has been attributed to reduced *MPL* transcription<sup>196,200</sup> and in PV to post-translational hypo-glycosylation and defective membrane localization.<sup>201</sup>

Most recently, a somatic GOF *MPLW515L* mutation (a G to T transition at nucleotide 1544 resulting in a tryptophan to leucine substitution at codon 515 of the transmembrane region) was described in *JAK2V617F*-negative PMF.<sup>187</sup> Subsequently, an additional *MPL* mutation involving the same 515 codon (*MPLW515K*) was incidentally discovered during screening for *MPLW515L* and the prevalence of both mutations was determined at approximately 5% in PMF and 1% in ET.<sup>187</sup> Interestingly, some patients with *MPL* mutations also displayed a minor *JAK2V617F* clone, an observation that is not easily explained and underscores the complexity of pathogenetic mechanisms in MPD.

As is the case with *JAK2V617F*, *MPLS15L* mutations are early, stem cell-derived events<sup>187</sup> and *MPLW515L* has been shown to transform primary cell lines in terms of both cytokine-independent growth and Tpo hypersensitivity, activate JAK-STAT/ERK1/2, and induce PMF-like disease in mice that is characterized by a rapid fatal course, marked thrombocytosis, leukocytosis, hepatosplenomegaly, and bone marrow fibrosis.<sup>187</sup> Furthermore, *MPLW515L*-induced cell growth was effectively inhibited by a small molecule JAK inhibitor raising the prospect of a similar treatment strategy for both *MPL* and *JAK2* mutation-associated MPD. Finally, it should be noted that one of the aforementioned germline *MPL* mutations associated with familial thrombocytosis also occurred in the transmembrane region and was activating.<sup>188</sup>

### *PDGFR* mutations

Both platelet-derived growth factor receptor 1 (*PDGFRα*) located on chromosome 4q12 and β (*PDGFRβ*) located on chromosome 8q31-q22 are involved in MPD-related activating mutations (Table 2). Clinical phenotype in both instances includes prominent blood eosinophilia and excellent response to imatinib therapy.

In regards to *PDGFRα* mutations, the most intensively studied has been the *FIP1L1-PDGFRα*, a karyotypically occult del(4)(q12), that was described in the year 2003 as an imatinib-sensitive activating mutation.<sup>202</sup> Subsequent studies have demonstrated the stem cell origin of the particular mutation,<sup>202,203</sup> and functional studies have demonstrated transforming properties in cell lines and the induction of MPD in

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mice.<sup>204,205</sup> Cloning of the *FIP1L1-PDGFRα* fusion gene identified a novel molecular mechanism for generating this constitutively active fusion tyrosine kinase, wherein a ~800kb interstitial deletion within 4q12 fuses the 5' portion of *FIP1L1* to the 3' portion of *PDGFRα*.<sup>205</sup> Molecular studies have shown that the breakpoint in *FIP1L1* is relatively promiscuous, while the *PDGFRα* breakpoint is restricted to exon 12 that encodes part of the protein-protein interaction module with two fully conserved tyrosophans (WW domain)-containing the JM region with resultant disruption of its autoinhibitory activity.<sup>206</sup> Further biochemical and functional studies have shown that this fusion protein, which contains most tyrosine kinase functions associated with human cancer, the *FIP1L1* encoded sequences are dispensable for transformation, and there is no requirement for a dimerization motif, disruption of the autoinhibitory juxtamembrane motif as an invariant consequence of disruption of exon 12 is the basis for constitutive activation of *PDGFRα* kinase activity.<sup>207</sup>

*FIP1L1-PDGFRα* occurs in a very small subset of patients who present with the phenotypic features of both CEL and SM (i.e. CEL-SM) but the presence of the mutation reliably predicts complete hematologic and molecular response to imatinib therapy.<sup>21,208,209</sup> *FIP1L1-PDGFRα* mutation (T674T) that is homologous to the resistance-inducing, “gatekeeper” T3151 mutation in *BCR-ABL* has been described<sup>210,211</sup> and in vitro salvage with other kinase inhibitors including PKC412<sup>212</sup> and sorafenib<sup>212</sup> has been demonstrated whereas such activity has not been conclusively shown for nilotinib.<sup>213</sup>

*PDGFRα* activation associated with CEL has also been described with karyotypically apparent fusion mutations including *KIF5B-PDGFRα*, t(4;10)(q12;p11),<sup>214</sup> *BCR-PDGFRα*, t(4;22)(q12;q11),<sup>216</sup> and *CDK5RAP2-PDGFRα*, inv(9)(4)(q33;q12q25).<sup>215</sup> In the former instance, the breakpoints involved exon 3 of the kinein family member 5B and exon 12 of *PDGFRα* resulting in an in-frame fusion. The patient achieved complete hematological and molecular remission with imatinib therapy.<sup>216</sup> *BCR-PDGFRα* represents a *BCR* breakpoint in intron 12 of *PDGFRα* and the fusion gene contains *ETV6* and *PDGFRα* breakpoint in exon 12/exon 13 and is also sensitive to imatinib therapy.<sup>216,218,219</sup> *CDK5RAP2-PDGFRα* also represents an imatinib-sensitive in-frame fusion involving exon 13 of *CDK5RAP2* and intron 9/exon 12 of *PDGFRα*.<sup>217</sup> As is the case with *FIP1L1-PDGFRα*, currently known *PDGFRα* breakpoints are noted to be tightly clustered in the JM region, which once again highlights a key regulatory role for domain.

The association between eosinophilic myeloid malignancies and *PDGFRβ* rearrangement was first characterized and published in 1994 where fusion of the tyrosine kinase encoding region of *PDGFRβ* to the *ets*-like gene, *ETV6* (*ETV6-PDGFRβ*, t(5;12)(q33;p13)) was demonstrated.<sup>20</sup> The fusion protein was transforming to cell lines and resulted in constitutive activation of *PDGFRβ* signaling.<sup>220</sup> Since then, several other *PDGFRβ* fusion transcripts with similar disease phenotypes have been described (Table 2).<sup>221-229</sup> Cell line transformation<sup>225,226</sup> and MPD induction<sup>227</sup> in mice has been demonstrated,<sup>228</sup> and imatinib therapy was effective when employed.<sup>225,223,225,229,230</sup> Additional evidence regarding the oncogenicity of activated *PDGFRβ* comes from experiments with mice where either *ETV6-PDGFRβ* or *HA-PDGFRβ* induced lymphoblastic lymphoma.<sup>229,231</sup> In most of these mutations, *PDGFRβ* is fused to the N-terminal segment of a partner protein that encodes for one or more oligomerization domains.

### *FGFR1* mutations

Human stem cell leukemic/lymphoma syndrome (SCLL), also known as the 8p11 myeloproliferative syndrome, constitutes a clinical phenotype with features of both lymphoma and eosinophilic MPD and characterized by a fusion mutation that involves the gene for fibroblast growth factor receptor-1 (*FGFR1*) and the tyrosine kinase domain of the constitutive active tyrosine kinase *SH2B3*. In lineage cells exhibit the 8p11 translocation, thus demonstrating the stem cell origin of the disease. The disease features several 8p11-linked chromosome translocations as outlined in Table 2,<sup>36,232,240</sup> and some of the corresponding fusion *FGFR1* mutants have been shown transform cell lines,<sup>240,246-249</sup> and induce SCLL,<sup>249</sup> or CML-like<sup>248</sup> disease in mice depending on the specific *FGFR1* partner gene: *ZNF198* or *BCR*, respectively.<sup>248</sup> Consistent with this laboratory observation, some patients with *BCR-FGFR1* mutation manifest a more indolent CML-like disease.<sup>248</sup> The mechanism of *FGFR1* activation in SCLL is similar

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## Bone Marrow Histopathology in the Diagnosis of the Early Stage of Chronic Myeloproliferative Disorders

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In the chronic myeloproliferative disorders (MPDs), examination of bone marrow (BM) biopsy specimens is essential to make a correct diagnosis and classification, as well as to monitor progression of the disease over time. In addition, histopathology permits a ready assessment of therapeutic efficacy, assists in risk stratification of patients, and is predictive of prognosis. For this reason, a multidisciplinary approach is required by considering equally clinical and morphological data [1]. This up-to-date concept of clinicopathological evaluation has been strengthened by the WHO classification [2] that includes the major subtypes polycythemia vera (PV), essential thrombocythemia (ET), and chronic idiopathic myelofibrosis (CIMF).

### I. Definition and standardization of histopathological features

In accordance with the issues of the WHO classification, the different subtypes of MPDs are characterized by specific histological patterns that are composed of distinctive features and are usually present at diagnosis (Table 1). Although in quite a number of former studies these alterations have been elaborated, a conflict of opinion still exists, whether an untrained pathologist would be able to recognize these features on H&E stained BM sections. Contrasting the determination of age-dependent cellularity and semiquantitative grading of myelofibrosis [3, 4], especially features of megakaryopoiesis may cause

**Table 1** Diagnostic impact of certain standardized histological BM features (ranking with relative incidence) following discriminant analysis in patients presenting with platelet counts  $\geq 600 \times 10^9/l$  and the presumptive clinical diagnosis of ET

Definite diagnosis (WHO classification)	Th	ET	CIMF-0	CIMF-1	PV
<b>Megakaryopoiesis</b>					
maturation defects	-	-	X	X	-
nuclear lobulation	-	X	-	-	X
<b>Myeloid stroma</b>					
reticulin fibers	-	-	-	X	-
<b>Erythropoiesis</b>					
left shifting	-	-	-	-	X
<b>Megakaryopoiesis</b>					
naked nuclei	-	-	X	X	-
small forms	X	-	X	X	X
<b>Erythropoiesis</b>					
quantity	-	-	-	-	X
<b>Granulopoiesis</b>					
left shifting	X	-	X	-	X
<b>Megakaryopoiesis</b>					
giant forms	-	X	X	X	X
<b>Cellularity</b>					
Megakaryopoiesis	-	-	X	X	-
bulbous nuclei	-	-	X	X	-
clusters	-	X	X	X	X
Th - reactive thrombocytosis, X - major finding, - additional criterion					

severe SP and therefore, this is not an entirely reliable diagnostic parameter [11]. A similar situation may be observed regarding the neutrophil cell lineage, because an increase in pro- and metamyelocytes (left-shifting) is frequently displayed in PV and in SP. On the other hand, the features of the megakaryocytic proliferation in PV are characteristic and have been acknowledged to enable a distinction between PV and the other subtypes of MPDs, and SP [10]. Previous studies reported by the PVSG showed that 95% of biopsies from patients with PV have an increase in the number of megakaryocytes, but others have observed that aside from the increase in numbers, the cytological appearance of this cell lineage exerts a discriminating impact. It has been repeatedly emphasized that megakaryopoiesis in early as well as in full-blown PV displays a pleomorphic aspect, i.e., small, medium sized, large and giant megakaryocytes are either dispersed or loosely clustered [11]. In particular, the mature megakaryocytes that have hyperlobulated nuclei but that fail to show other gross nuclear abnormalities, such as deviation from nuclear-cytoplasmic maturation to serve as diagnostic hallmark (Figures 1, 2a) because they are in contrast to the small to medium-sized megakaryocytes found in SP (Figure 2a). The finding of the specific histological pattern of a left-shifted (immature) erythroid and granulocytic proliferation (Figure 2a) associated with a megakaryopoiesis displaying a striking variety of cell sizes is in contrast to the uniformly large to giant size of the megakaryocytes in ET, which are usually not accompanied by significant proliferations in the other cell lineages (Figure 2, d). Discriminate analysis of standardized BM features [3] in erythrocytosis reveals that in addition to the peculiar appearance of the megakaryocytes, certain constituents of the stroma compartment also enable a clear-cut distinction between PV and SP. Iron-laden macrophages are rarely observable in PV, and are found in about 6% of patients, which is opposed to the frequent occurrence of this phenomenon in SP (Figure 2e). An increase in reticulin fibers is never encountered in SP. Usually, SP shows an inflammatory reaction with prominent perivascular plasma cells (Figure 2f), many scattered eosinophils and small accumulations of cell debris ingested by macrophages (Figure 2g). The latter features are most prominent in so-called smokers polycythemia associated with recurrent bronchopulmonary infections [11-13]. In summary, Table 1 describes the results of an independently performed study that includes discriminant analysis of standardized BM features in a large cohort of patients with sustained erythrocytosis.

### III. Essential thrombocythemia (ET)

According to the widely recognized standards, the diagnosis of ET was usually established by the PVSG criteria, i.e. by demonstration of thrombocytosis and the exclusion of other diseases, particularly full-blown PV [14]. However, a critical evaluation of these diagnostic guidelines, that up to now have been applied in all relevant clinical trials, reveals that they do not permit a clear-cut distinction of ET from the prefibrotic and early stages of CIMF that are often associated with thrombocythemia [15]. In contrast, by using a different diagnostic approach, the WHO classification does enable a separation between CIMF and (true) ET [16, 17], a substantial change concerning the spectrum of this entity has to be realized. These significant differences are predominantly due to the inclusion of histopathology in the WHO classification, because evaluation of BM specimens derived from patients with the diagnosis of ET based on the PVSG criteria reveal a striking heterogeneity [15, 18]. A wealth of data has accumulated concerning the role of BM pathology in the differential diagnosis of thrombocythemia in MPDs with the aim to define more clearly histological patterns that characterize (true) ET. In contrast to prefibrotic CIMF with accompanying thrombocythemia - false ET (Figure 3a), in true ET, neither a relevant increase in cellularity nor a significant left-shifted neutrophil granulopoiesis is observable (Figure 3c). The most conspicuous differences are related to megakaryopoiesis (Figure 1), because in true ET gross disturbances of histopathology (dense clustering) are not remarkable, but a more or less random distribution of megakaryocytes within the BM is prevalent (Figure 3d). In (true) ET, there is a predominance of large to giant mature megakaryocytes with extensively lobulated (staghorn-like) nuclei [15, 18, 19] surrounded by a correspondingly mature cytoplasm (Figure 4b). These features are clearly distinguishable from the abnormally hyperlobulated (cloud-like) and hyperchromatic nuclei of megakaryocytes in false ET (prefibrotic-early CIMF) with striking maturation defects (Figure 1) that result in a marked anomaly of nuclear-cytoplasmic maturation, i.e., megakaryocyte dysplasia (Figure 4a). Finally, at presentation, there is no substantial increase in reticulin observable in true ET, a finding that contrasts with the allowance of some degree of fibrosis according to the criteria of the PVSG [14]. Using standardized features of BM assessment that include histopathology [3], a definitive differentiation between ET and those entities mimicking this disorder may be easily achieved (Table 1). This distinction of ET according to the PVSG [14] versus the WHO classification [16] implies significant consequences, regarding

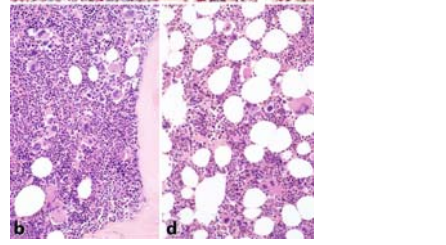
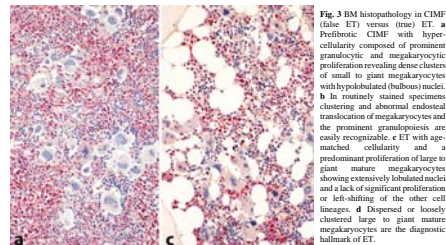


**Fig. 1** Prominent standardized features of megakaryopoiesis. Differentiation and clustering of megakaryocytes.

significant difficulties concerning definition and consequently recognition among different observers. In this context a systematic evaluation including in particular the arrangement of the megakaryocytes within the marrow space, i.e., histopathology and certain nuclear abnormalities besides maturation defects are crucial. In the normal BM megakaryocytes show a central distribution of single isolated cells [5]. In MPDs, the increase of megakaryocytes is often associated with the formation of small clusters (at least three cells) to extensive groups (more than five cells). These megakaryocyte clusters (Figure 1) may display either a loose or dense arrangement of cells [3]. Moreover, an abnormal dislocation of megakaryocytes towards the endosteal (paratrabecular) border is a highly conspicuous finding that is usually not found in reactive thrombocytosis. Other features indicating a neoplastic process are peculiar nuclear aberrations and maturation defects that imply disturbances of the normal development of megakaryopoiesis [3, 5, 6]. These include an atypical nuclear lobulation (extent and shape of nuclear foldings, i.e., hypobulbation, - often described as cloud-like leading to bulbous (plump, clumsy) nuclei versus hyperlobulation with marked segmentation mimicking a stag-horn-like formation) and anomalies of the chromatin pattern (mostly hyperchromasia). Furthermore, maturation defects include a conspicuous deviation of the nuclear-cytoplasmic ratio or maturation with appearance of bizarre megakaryocytes [3]. It is noteworthy that all these changes may be detectable in megakaryocytes of different sizes (small, median, large, giant) or ploidy states. Finally, so-called naked (denuded, bare) nuclei with condensed chromatin pattern may frequently be shown implicating an enforced cell turnover due to increased thrombocytopoiesis. Of the other parameters increase and left-shifting of neutrophil granulopoiesis or erythropoiesis may be a prominent feature as well as reduction in the amount of nucleated red cell precursors depending on disease entity (Table 1).

### II. Polycythemia vera (PV)

Unfortunately, the contribution of histopathology of the BM to the diagnosis of PV and to monitoring its progression has not been adequately appreciated. In the original and updated criteria of the Polycythemia Vera Study Group (PVSG), BM findings are not even mentioned [7], and in the WHO classification, they are considered only as a minor criterion in substantiating the diagnosis [8]. Indeed, the major reason why BM morphology has been neglected as a useful tool in the diagnosis of PV is that the disease has been traditionally defined by clinical, laboratory and biologic parameters, which often do suffice to establish the diagnosis [9]. However, when correlated with the clinical findings, a clear pattern of histopathologic features emerge that can be used to confirm the diagnosis in cases in which the clinical data are not so clear-cut. For example, PV is a dynamic, evolving disease process, and early on a number of patients do not fulfill all of the established clinical and laboratory diagnostic criteria, particularly in regard to the red cell mass or hemoglobin/hematocrit values [7]. These patients have often been said to have "latent PV" or "benign erythrocytosis", and are regarded as a heterogeneous group that may eventually evolve into full-blown. Importantly, some patients have an initial/early prodromal phase of PV that is accentuated by thromboembolic episodes as first manifestation of disease, but in whom a diagnosis is not possible by conventional criteria (Table 1). In these instances, demonstration of the characteristic histologic features of PV could lead to early diagnosis and appropriate therapy. The BM biopsy performed at diagnosis also



complications like fibrosis, therapeutic strategies and outcome [20], because only a fraction of those diagnosed by the PVSG criteria, ranging between 20% to 30%, may be regarded as true ET.

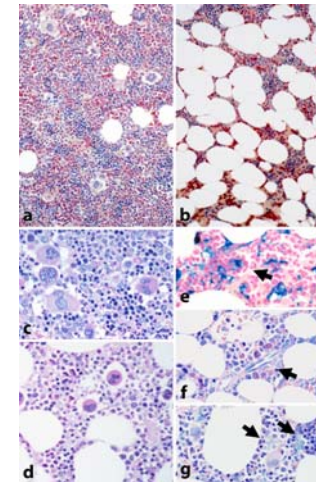
### IV. Chronic idiopathic myelofibrosis (CIMF)

CIMF initially presents with a hypercellular BM characterized by megakaryocytic myeloproliferation (Figure 3a) but no increase in reticulocytosis (often left-shifted) and megakaryocytic lineages, with a concert of the nucleated erythroid precursors [21] was previously reported in reticulocytosis (CMGM) [22-25]. Most conspicuous, however, is characterized not only by a disturbance of BM histopathology (translocation to the endosteal borders), but also by striking abnormal significant anomalies of megakaryocytes do not only consist of variant giant forms (Figures 3a, b), but also of an aberration of the nuclear cyto and hyperchromatic cloud-like nuclei (Figure 4a). Furthermore, apart a lobulation there are many naked (bare) megakaryocyte nuclei of megakaryocytes in CIMF regularly are marked by a more atypical (dy-

**Fig. 3** BM histopathology in CIMF (false ET) versus true ET. **a** Prefibrotic CIMF with hypercellularity composed of prominent granulocytic and megakaryocytic proliferation revealing dense clusters of small to giant megakaryocytes with hyperlobulated (bulbous) nuclei. **b** In routinely stained specimens clustering and abnormal endosteal translocation of megakaryocytes and the prominent granulopoiesis are easily recognizable. **c** ET with age-matched cellularity and a predominant proliferation of large to giant mature megakaryocytes showing extensively lobulated nuclei and a lack of significant proliferation or left-shifting of the other cell lineages. **d** Dispersed

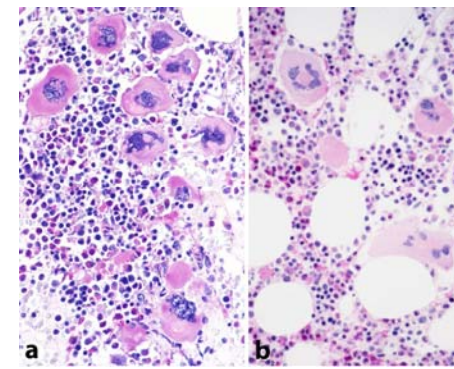
is important to establish a baseline against which subsequent specimens can be compared, because histopathology provides the best means to detect various phases of PV, particularly the terminal stages, so-called spent phase, postpolycythemic myeloid metaplasia (PPMM) and blast phase.

The histopathology of initial-early and full-blown polycythemic PV is characterized by a hypercellular BM with trilineage proliferation (panmyelosis) of variable numbers of erythroid and granulocytic precursors, and with megakaryocytic proliferation that has distinctive morphologic features [10]. Some of these findings have more significance than others in establishing the diagnosis of PV and distinguishing it from reactive, or secondary polycythemia (SP) as well as from the other MPDs. For example, although hypercellularity in relation to age-matched hematopoiesis is a common feature of PV (Figure 2a), it may occasionally also be encountered in cases of SP that usually present with a mildly to moderately increased hematopoiesis (Figure 2b). For easy recognition and quantification of neutrophil granulopoiesis versus erythropoiesis, a special stain like naphthol-AS-D-chloroacetate esterase (Figure 2a,b) or myeloperoxidase may be superior to the routine hematoxylin-eosin stain (H&E). Following this staining procedure, it is apparent that in PV the normally small and rounded islets of nucleated erythroid precursors show a conspicuous enlargement and a tendency to merge into sheets (Figure 2a). Although these changes are significantly more pronounced in PV, they may be also expressed in a few cases with



**Fig. 2** BM histopathology in PV versus SP. **a** PV with trilineage proliferation and hypercellularity including prominent sheets of erythroid precursors and megakaryocytes of different size. **b** SP with slight increase in cellularity in a senescent patient with large islets of erythropoiesis. **c** Pleomorphic aspect of megakaryocytes in PV with sizes ranging from small to large - see also **d**. **d** Uniform small to medium-sized megakaryocytes in SP. **e** SP with coarse iron deposits in macrophages (arrows). **f** Prominent perivascular deployment of plasma cells and adjacent eosinophils in SP (arrow). **g** In SP (arrow), deposits of cell debris are regular findings (arrows).

**Fig. 4** BM histopathology in CIMF versus ET. **a** Abnormalities of megakaryocytes in CIMF are consistent with hyperchromatic and hyperlobulated (cloud-like) nuclei. **b** Large to giant megakaryocytes in ET with deeply lobulated (staghorn-like) nuclei and no conspicuous maturation defects.



other subtype of the MPDs significantly contrasting those seen in ET (Figures 4a,b). This is one of the characteristic features discriminating false ET (i.e., prefibrotic-early stage CIMF) from true ET. Although progression of CIMF is unpredictable, increasing megakaryocytic maturation defects are associated with a more rapid transition from the prefibrotic into overt fibroblastic stages (dysplasia), although this transition is not dependent on the platelet count. As has been elucidated in different studies, there is a more than a 65% probability of progression from a prefibrotic-early stage to a full-blown CIMF or MMM conforming with the classical diagnostic criteria. This peculiar feature of disease process is accompanied by relevant changes in clinical findings. Patients that initially present with early stage CIMF are prone to develop increasing anemia, splenomegaly and a leuko-erythroblastic blood picture in the course of disease evolution merging finally into MMM.

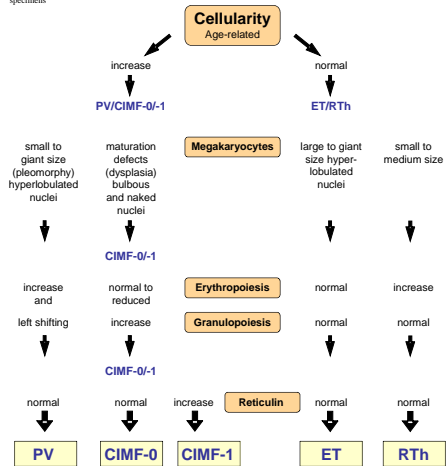
In contrast to the prefibrotic-initial stages of CIMF (CIMF-0), the more advanced and fibro-osteosclerotic lesions of disease (CIMF-3) conforming with MMM are characterized by coarse bundles of collagen fibers in the BM. This is usually an optional plaque-to-budd-like osteosclerosis (endophytic bone formation) detectable and areas of patchy hematopoiesis, revealing progressive hypoplasia. Comparable with the initial stages, atypical megakaryocytes are again a most prominent feature including naked (denuded, condensed) nuclei and dilated marrow spaces containing groupings of intraluminal hematopoiesis, especially megakaryocytes. It is noteworthy that even without prior cytoreductive therapy mild to moderately expressed myelodysplastic changes may occur in the natural course of the disease process, occasionally indicating an insidious transition into an acceleration and blastic crisis (terminal stage). Finally, although in the past decade many groups were engaged in the study of risk stratification and prognosis of CIMF patients, a comparison of these data reveals an extreme heterogeneity [26, 27]. This result may be significantly influenced by including patients with PPMM into the corresponding

calculations and the failure to recognize the prefibrotic and early stages of this disorder. On the other hand, it is reasonable to assume that survival may be related to stages in which CIMF is diagnosed. In univariate analysis, the prodromal phases show a more favorable survival in comparison with more advanced stages (MMM). However, in multivariate calculations, evolution of BM fibrosis displayed no significant influence on survival [26, 27].

#### V. Summary

Chronic idiopathic myelofibrosis (CIMF), essential thrombocythemia (ET), and polycythemia vera (PV) are characterized by specific patterns of histopathology. When considered in the context of clinical findings, the histologic features not only discriminate these entities from each other (Figure 5), but also provide valuable information that can identify various risk groups, predict prognosis, and assess therapeutic efficacy. Histopathology is especially crucial to distinguish between myeloproliferative diseases in which the clinical and laboratory features overlap, but the prognosis and therapeutic implications are significantly different. For example, prefibrotic CIMF is frequently associated with thrombocythemia and thus, easily misdiagnosed as ET, from which it differs markedly in terms of development of fibrosis, blast transformation and survival. Yet the histopathology of these two diseases is considerably different at onset and usually permits an accurate diagnosis. It is estimated that between 70% to 80% of patients entered into clinical studies on ET, actually present early-stage CIMF with marked thrombocythemia (false ET). Similarly, the prodromal phase of PV, which may also mimic ET, cannot be recognized by conventional criteria, but has a characteristic histopathology pattern. Of course, the dynamics of the disease process must always be taken into account when following individual patients, particularly in PV and CIMF, and follow-up studies with repeated BM biopsy specimens readily allow monitoring of disease progression.

**Fig. 5** Schematic description (flow-sheet) of prominent histological features exerting diagnostic relevance to differentiate chronic myeloproliferative disorders associated with an evaluated platelet count from reactive lesions in bone marrow biopsy specimens



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## Mast Cell Disease

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Mast cell disease, or mastocytosis, includes a variety of disorders that are characterized by the presence of mast cell aggregates in tissue sections and range from isolated, indolent proliferations to systemic, aggressive disorders. The WHO classification of mastocytosis (Table 1) separates cutaneous and systemic forms and provides criteria for the subclassification of the various systemic forms of the disease. While the morphologic detection and immunophenotypic confirmation of mast cell aggregates in tissue sections is essential for diagnosis, subclassification of cases into systemic mastocytosis subtypes requires correlation with a variety of clinical and other laboratory features (Table 2). Morphologically, mast cells can range from aggregates of round cells with finely granular pink cytoplasm to more spindle cells with associated fibrosis (often paratrabecular in the bone marrow). Mast cells are often accompanied by eosinophils and small lymphocytes and may be overlooked due to these other cellular components. On aspirate smears, mast cells are most easily identified in the central portion of marrow particles as round or spindle cells with fine basophilic granules that obscure the nucleus. Spindle cells and more atypical mast cell features tend to correlate with the more aggressive clinical syndromes, but morphologic features alone are not adequate for subclassifying the mast cell disorders.

### Cutaneous Mastocytosis

While various types of mast cell disease may involve the skin, the diagnosis of cutaneous mastocytosis is reserved for cases with no systemic involvement, including no elevation of total serum tryptase or organomegaly. *Urticaria pigmentosa*, or maculopapular cutaneous mastocytosis, is the most common form of cutaneous mastocytosis. It may occur in children or adults. The mast cell infiltrate is often more subtle in adults and may require examination of multiple sections for diagnosis. The lesions have elongated or spindle mast cells, often associated with small vessels, in the papillary and reticular dermis. *Diffuse cutaneous mastocytosis* occurs almost exclusively in children without the characteristic maculopapular rash of urticaria pigmentosa. The skin may be more smooth or thickened and red. The mast cell infiltrate generally forms a band in the papillary and upper reticular dermis. *Mastocytoma of the skin* is a single lesion, most often on the trunk or wrist of infants. Mast cells without atypia fill the papillary and reticular dermis and may extend into the deep dermis and subcutaneous adipose tissue. In children, cutaneous mastocytosis is an indolent disorder that usually regresses spontaneously around puberty. In adults, regression is less common and careful staging is warranted since most adults presenting with skin lesions will actually have systemic disease. However, the presence of skin lesions, even with systemic disease, usually portends an indolent clinical course.

### Systemic Mastocytosis

A diagnosis of systemic mastocytosis requires detection of multifocal mast cell aggregates in tissue sections (major criterion) as well as one minor criterion or, in the absence of tissue section aggregates, identification of three minor criteria (Table 2). Subclassification of systemic mastocytosis requires further correlation with clinical, morphologic and laboratory findings and these are designated as “B” or “C” findings (Table 3).

*Indolent systemic mastocytosis* meets criteria for systemic mast cell disease, but has no B or C findings and no evidence of another hematologic malignancy. Skin lesions are usually present.

As the name implies, these cases are clinically indolent, and this is the most common form of systemic mast cell disease.

*Systemic mastocytosis with associated clonal, hematological non-mast cell lineage disease* meets criteria for systemic mastocytosis as well as being associated with another hematologic malignancy in the WHO classification. This occurs in almost one third of patients with systemic mast cell disease and the associated tumor is usually a myeloid malignancy, which may include myelodysplasia, chronic myeloproliferative disorders, mixed myeloproliferative and myelodysplastic syndromes, acute myeloid leukemia and chronic eosinophilic leukemia. Associated lymphoid malignancies are most often multiple myeloma, but chronic lymphocytic leukemia, acute lymphoblastic leukemia and hairy cell leukemia may also occur. The prognosis of these patients is usually based on the non-mast cell disorder. Rare cases will not have identifiable mast cell disease at diagnosis, but mastocytosis may become apparent in the post-therapy bone marrow.

*Aggressive systemic mastocytosis* meets criteria for systemic mastocytosis as well as having one or more of the C findings (Table 3) indicating organ dysfunction due to mast cell infiltration. These patients do not have an associated hematologic malignancy or evidence of mast cell leukemia. A provisional subvariant termed *lymphadenopathic mastocytosis with eosinophilia* presents with lymphadenopathy and eosinophilia and should be differentiated from chronic eosinophilic leukemia. Patients with aggressive systemic mastocytosis have a short survival, usually of only weeks to months.

*Mast cell leukemia* is a form of systemic mastocytosis with a diffuse marrow infiltration ( $\geq 20\%$ ) of atypical, immature mast cells in the bone marrow with 10% or more mast cells in the peripheral blood. An aleukemic variant is also proposed. Both have the similarly dismal prognosis of aggressive systemic mastocytosis.

### Other Presentations of Mastocytosis

*Mast cell sarcoma* is an extremely rare localized disorder that occurs in the absence of skin lesions or other systemic disease. The mast cells are highly atypical with a destructive growth pattern. A leukemic phase may develop in these patients and they have a generally poor prognosis.

*Extracutaneous mastocytoma* is most commonly reported in the lung without skin lesions or other systemic disease. In contrast to mast cell sarcoma, the lesional cells are not atypical and do not show a destructive growth pattern.

### Special Stains and Immunophenotyping in Mastocytosis

Normal mast cells mark with chloracetate esterase and toluidine blue, but the later stain is pH dependent in mastocytosis and these stains are less helpful than more specific immunophenotypic studies. Mast cells express CD33, CD43, CD68, CD117 and tryptase. Tryptase is the most lineage specific of these markers, but may show high background staining in some cases. Neoplastic mast cells also express CD2 and/or CD25, with the later more easy to detect in most cases. A panel of CD117, tryptase and CD25 is recommended for most cases to aid in confirming the mast cell lineage of the proliferation and to potentially identify an aberrant immunophenotype (CD25-positive).

## Table 3. B and C Findings in Mastocytosis

### B Findings

1.  $>30\%$  bone marrow mast cells in focal, dense aggregates and/or serum total tryptase level  $>200$  ng/ml
2. Signs of dysplasia or myeloproliferation in non-mast cell lineage, but insufficient criteria for a definitive diagnosis of a hematopoietic neoplasm by WHO, with normal or only slightly abnormal blood counts
3. Hepatomegaly without liver function impairment, and/or palpable splenomegaly without hypersplenism, and/or palpable or visceral lymphadenopathy

### C Findings

1. Bone marrow dysfunction manifested by one or more cytopenia (ANC  $<1.0 \times 10^9/L$ , HbG  $<10g/dl$ , or PLTs  $<100 \times 10^9/L$ ) but no frank non-mast cell hematopoietic malignancy
2. Palpable hepatomegaly with impairment of liver function, ascites, and/or portal hypertension
3. Skeletal involvement with large-sized osteolytic and/or pathological fractures
4. Palpable splenomegaly with hypersplenism
5. Malabsorption with weight loss due to GI mast cell infiltrates

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## Genetics of Mastocytosis

Point mutations of the tyrosine kinase receptor gene *KIT* are the most common genetic abnormality in mastocytosis and the most common mutation results in a substitution of valine for aspartate at codon 816 of exon 17, termed Asp816Val or D816V. This mutation is found in over 90% of patients with systemic mastocytosis. Other mutations described include tyrosine or phenylalanine for aspartate at codon 816, and tyrosine for glutamic acid at codon 839. *KIT* mutations, however, are not specific for mastocytosis and are reported in other disease. Even the D816V mutation of this gene is not disease specific and may occur in some seminomas and some core binding factor acute myeloid leukemias. For this reason, detection of the mutation represents only a minor diagnostic criterion.

## Therapy for Mastocytosis

Patients with mastocytosis are treated symptomatically and no curative therapies for the aggressive disease types are currently available. Some success in reducing the mast cell burden has been reported with alpha interferon and with cladribine (2-CdA). Tyrosine kinase inhibitors directed at the *KIT* mutation may be useful in some patients. However, the D816V mutation of *KIT* results in resistance to imatinib mesylate. Ongoing trials with other tyrosine kinase inhibitors, such as PKC-412, are underway in the hope of directly impacting the molecular defect of this disease.

**Table 1. WHO Classification of Mastocytosis**

Cutaneous Mastocytosis	Urticaria pigmentosa/ maculopapular cutaneous mastocytosis
	Diffuse cutaneous mastocytosis
	Solitary mastocytoma of skin
Indolent Systemic Mastocytosis	Systemic Mastocytosis with Associated Clonal, Hematological Non-mast-cell Lineage Disease
Aggressive Systemic Mastocytosis	Mast Cell Leukemia
	Mast Cell Sarcoma
	Extracutaneous Mastocytoma

**Table 2. Criteria for Systemic Mastocytosis**

<b>Major</b>	Multifocal, dense mast cell infiltrates ( $\geq 15$ cells) in tissue sections confirmed by tryptase or other special stains
<b>Minor</b>	<ol style="list-style-type: none"> <li>a. <math>&gt;25\%</math> spindle, immature or atypical mast cells in tissue sections or bone marrow aspirate smears</li> <li>b. Detection of <i>KIT</i> D816V mutation</li> <li>c. Expression of CD117 with CD2 <math>&gt;20</math> or CD25</li> <li>d. Serum total tryptase persistently <math>&gt;20</math> ng/ml (unless there is an associated clonal myeloid disorder in which case this parameter is not valid)</li> </ol>
	Diagnosis requires major and one minor or three minor criteria

## Practical Issues in the Diagnosis of Myelodysplastic/ Myeloproliferative Overlap Syndromes

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The World Health Organization (WHO) classification of hematopoietic neoplasms, published in 2001, included the new category of “Myelodysplastic/ Myeloproliferative Diseases,” intended to accommodate those myeloid neoplasms that simultaneously showed features of chronic myeloproliferative disorders and myelodysplastic syndromes. Typically, myelodysplastic syndromes (MDS) are clonal neoplastic disorders characterized by accelerated apoptosis of the abnormal clone, resulting in a pattern of *ineffective* hematopoiesis, usually manifest by increased bone marrow cellularity in the presence of decreased peripheral blood counts (cytopenias) and absence of organomegaly. MDS also generally harbor some degree of morphologic dysplasia within one or more hematopoietic lineages. Conversely, the chronic myeloproliferative disorders (MPD) are myeloid neoplasms generally characterized by evidence of *effective* hematopoiesis, generally in the form of increased peripheral blood counts (“cytoses”), organomegaly, or both. Typically, MPD do not show prominent morphologic dysplasia of hematopoietic elements.

The WHO category of MDS/MPD overlap syndromes includes diseases that either have a proliferative component (increased peripheral blood counts, organomegaly) combined with significant morphologic dysplasia, or diseases that may vary in their degree of proliferative characteristics and morphologic dysplasia. The WHO classification lists four disease categories under the heading of MDS/MPD: 1) Chronic myelomonocytic leukemia (CMML), 2) Juvenile myelomonocytic leukemia (JMML), 3) Atypical chronic myeloid leukemia (aCML), and 4) Myelodysplastic/ myeloproliferative diseases, unclassifiable. There are many pitfalls that practicing hematopathologists may face in the diagnosis of MDS/MPD overlap syndromes. This session will focus on the following points:

- 1) Guidelines for morphologic recognition of the distinct categories of MDS/MPD outlined by the WHO, including recognition of clinical and morphologic heterogeneity in CMML.
- 2) A discussion of the “unclassifiable” category, including the provisional entity “Refractory anemia with ringed sideroblasts associated with marked thrombocytosis.”
- 3) The distinction between CMML and acute myelomonocytic leukemia, which may be deceptively difficult.
- 4) An illustration of mimickers of morphologic dysplasia in bone marrow that could lead to overdiagnosis of MDS/MPD.

## A Brief Overview of Distinct Categories of MDS/MPD

### Chronic Myelomonocytic Leukemia (CMML)

CMML was included initially in the French-American-British cooperative group (FAB) classification of myelodysplastic syndromes. As defined by the FAB, CMML shared many features in common with refractory anemia with excess blasts (RAEB), including peripheral cytopenias, usually in the setting of hypercellular marrow, marked multilineage dysplasia, and an increased blast percentage in the bone marrow (but not sufficient to diagnose frank acute leukemia). CMML differs, however, in a proliferative component of monocytosis, with the peripheral blood monocyte count by definition exceeding  $1 \times 10^9/L$ . There remained, however, chronic myeloid neoplasms with a more proliferative phenotype (increased peripheral granulocyte counts, organomegaly, absence of significant morphologic dysplasia), but also marked by absolute peripheral monocytosis, and absence of the Philadelphia chromosome or BCR-ABL fusion. Some proposed that disorders fitting the latter description be termed “CMML,

myeloproliferative,” as opposed to the original FAB category, for which the term “CMML, myelodysplastic” was subsequently proposed. Subsequent clinicopathologic studies, however, failed to elucidate significant differences in clinical outcome between these two categories, and therefore they are consolidated under the single heading of CMML in the WHO classification.

Although CMML as defined by WHO is a diverse disorder that likely does not represent a single clinicopathologic disease entity, the CMML variant listed in the WHO classification as “CMML with eosinophilia” may be associated with the cytogenetic translocation t(5;12)(q31;p12), resulting in an abnormal fusion gene, *TEL/PDGF $\beta$ R*, or other genetic abnormalities involving the *PDGF $\beta$ R* locus. It is important to recognize this unusual but distinct category, since such patients may respond extremely well to therapy with imatinib or other targeted tyrosine kinase inhibitors. In fact, such patients may show excellent response to only a fraction of the imatinib dose given to Philadelphia-positive chronic myelogenous leukemia patients.

### Diagnostic Criteria for CMML (from WHO, 2001):

1. Persistent peripheral blood monocytosis  $>1 \times 10^9/L$
2. No Philadelphia chromosome or *BCR/ABL* fusion gene
3. Less than 20% blasts in the blood or bone marrow
4. Dysplasia in one or more myeloid lineages. If myelodysplasia is absent or minimal, the diagnosis of CMML may still be made if the other requirements are present and:
  - an acquired, clonal cytogenetic abnormality is present in the marrow cells, or
  - the monocytosis has been persistent for at least 3 months and
  - all other causes of monocytosis have been excluded

### Juvenile Myelomonocytic Leukemia (JMML)

JMML is a rare but distinct disease entity that generally affects young children, with three-fourths of cases occurring by 3 years of age. The pathogenesis of most cases of JMML is linked directly to abnormalities in the molecular pathway responsible for conducting the stimulatory signal of granulocyte-macrophage colony stimulating factor (GM-CSF), resulting in marked hypersensitivity to GM-CSF *in vitro*, and abnormal myelomonocytic proliferations *in vivo*.

There is an increased prevalence of JMML in children with neurofibromatosis type 1, and in children with Noonan syndrome (a complex syndrome of mild facial dysmorphism and other anatomic malformations including cardiac anomalies). This increased prevalence can be linked to abnormalities in the NF1 gene in neurofibromatosis, and the PTPN11 gene in Noonan syndrome. NF1 is an important regulator of the Ras pathway of signal transduction, which regulates cellular response to GM-CSF. PTPN11 is the gene encoding SHP-2, a tyrosine phosphatase required for Ras-dependent functions. Mutations of PTPN11 have been shown to induce hypersensitivity of hematopoietic precursors to GM-CSF stimulation.

The diagnostic criteria for JMML are listed below. The morphologic features are not necessarily specific, and *in vitro* assays for GM-CSF may be difficult to obtain in routine clinical practice. Therefore, the correlation of morphologic findings with the clinical history, as well as documentation of elevated hemoglobin F levels by hemoglobin electrophoresis or other method, are often crucial to diagnosis. Although it is not formally classified as a form of acute leukemia, JMML usually follows an aggressive clinical course despite therapy.

### Diagnostic criteria for JMML (from WHO, 2001):

1. Peripheral blood monocytosis  $>1 \times 10^9/L$
2. Blood and marrow blasts  $<20\%$

circulating nucleated red cells therefore has no predictive value in the diagnosis of MDS. Second, the morphology of megaloblastic anemia may bear disturbing resemblance to acute leukemia to the inexperienced observer. The combination of giant pronormoblasts, markedly increased marrow cellularity, markedly elevated serum lactate dehydrogenase (LDH) and markedly peripheral blood poikilocytosis (including teardrop forms, Howell-Jolly bodies, and dysmorphic nucleated red cells) can lead to erroneous diagnoses of leukemia or myelodysplasia. Finally, hyperproliferation of marrow with granulocyte proliferation and high red blood cell turnover can cause a significant increase in the peripheral blood monocyte count. Therefore, the diagnosis of myelodysplasia in the setting of a hyperproliferative anemia (as evidenced by a high absolute reticulocyte count) should be approached with extreme caution.

**Dysgranulopoiesis.** Several non-neoplastic conditions can yield striking dysgranulopoiesis. The congenital Pelger-Huet anomaly is a harmless (and therefore generally subclinical) autosomal dominant condition in which neutrophils are hyposegmented, with most showing one or two nuclear lobes despite a fully mature-appearing chromatin. The appearance of Pelger-Huet neutrophils can be indistinguishable from the “pseudo-Pelger-Huet” morphology associated with either myelodysplastic syndromes, MDS/MPD syndromes, or certain reactive conditions. If examination of the blood smear of an otherwise healthy individual reveals that virtually all of the neutrophils are hypoblobated then the possibility of a true Pelger-Huet anomaly should be considered.

Pseudo-Pelger-Huet morphology can be seen in many reactive conditions, including therapy with certain drugs (e.g. colchicine, sulfonamides, and possibly other antimicrobial agents), mycoplasma infection, and human immunodeficiency virus infection. Pathologists should resist the temptation to assume MDS in the face of acquired P-H morphology, since the pseudo-PH changes associated with mycoplasma infection or other reactive conditions may be marked.

Neutrophil hypergranularity is a form of dysgranulopoiesis. However, artifacts of preparation or variability in staining methods can lead to the overdiagnosis of hypergranular neutrophils. Furthermore, since pathologists tend to examine abnormal blood smears in which neutrophils display some degree of toxic granulation, the normal pattern of neutrophil granulation may be misinterpreted as hypergranular. Remember that although primary myeloid granules are coarse and azurophilic, secondary (specific) neutrophil granules are tiny and barely discernible by light microscopy. Therefore, they show up as a pink hue in the neutrophil cytoplasm. On the other hand, true hypergranularity tends to appear as water-clear cytoplasm. When in doubt, preparation of a fresh, hand-stained peripheral blood smear (preferably made directly from a finger-stick) should be pursued to investigate the possibility of true hypergranulation of neutrophils.

**Dysmegakaryopoiesis.** Very few reactive conditions will yield the type of dysmegakaryopoiesis encountered in MDS. However, some observers do overinterpret the natural variability of megakaryocyte cytology as evidence of dysmegakaryopoiesis. The dysmegakaryopoiesis associated with MDS manifests as megakaryocytes with separate, round hypoblobated nuclei (as opposed to the single nuclei with multiple lobes seen in normal megakaryocytes). Most dysplastic megakaryocytes will contain one to three small round nuclei, and will be smaller than normal megakaryocytes. Such criteria for the diagnosis of dysmegakaryopoiesis should be applied fairly strictly, so as not to overinterpret dysplasia in megakaryocytes. Finally, be careful about diagnosing dysmegakaryopoiesis on paraffin-embedded bone marrow core biopsy sections (as opposed to bone marrow aspirate smears), since a section cut through one lobe of a normal megakaryocyte may look very similar to a section cut through a hypoblobated, dysplastic megakaryocyte.

1. Peripheral blood monocytosis  $>1 \times 10^9/L$
  3. No Ph chromosome or *BCR/ABL* fusion gene
- PLUS 2 or more of the following:  
Hemoglobin F increased for age  
Immature granulocytes in peripheral blood  
WBC  $> 10^9/L$   
Clonal chromosomal abnormality (may be monoclonal)  
GM-CSF hypersensitivity of myeloid progenitors *in vitro*

## Atypical Chronic Myeloid Leukemia (aCML)

Despite the name, aCML likely bears no relationship to true Philadelphia-chromosome-positive CML. In fact, in the drafting of the current WHO classification, alternative names for this entity were debated, but the consensus was reached that aCML was acceptable provided a clear distinction was made from true CML.

aCML is a very rare disorder, but likely represents a distinct disease entity. It is marked by increased peripheral granulocyte counts, splenomegaly, and distinct and marked morphologic dysplasia in circulating neutrophils in the form of hypoblobation (pseudo-Pelger-Huet changes). aCML is more clinically aggressive than true CML, with median survival quoted in the literature as less than 20 months. While most reported cases of aCML are associated with clonal cytogenetic abnormalities, no single consistent abnormality has been reported, although three reported cases associated with t(4;11)(q12;q11), and the detection of Ras mutations in a subset of aCML cases suggests potential common pathobiology to other types of MDS/MPD.

## Myelodysplastic/ Myeloproliferative Disease, Unclassifiable

Invariably, any classification scheme will have to accommodate disorders not readily classifiable into other distinct categories. Such is the case in the WHO classification of MDS/MPD overlap syndromes. One subtype under the “unclassifiable” moniker does, however, deserve mention since it may represent a distinct clinical entity.

**Refractory anemia with ringed sideroblasts and thrombocytopenia** is currently listed as a provisional entity under the broader heading of Myelodysplastic/ Myeloproliferative Disease, Unclassifiable in the WHO classification. Patients with this disorder otherwise fulfill criteria for refractory anemia with ringed sideroblasts (RARS), including normocytic or macrocytic anemia, dimorphic circulating red cells due to hypochromic microcytes admixed with normocytic or macrocytic red cells, erythroid expansion in the bone marrow with mild dyserythropoiesis, and greater than 15% ringed sideroblasts on examination of bone marrow aspirate smears. In contrast to classic RARS however, this disorder is characterized by marked and persistent thrombocytosis, and patients may present initially with clinical suspicion for essential thrombocythemia. (This disorder is in fact cross-referenced in the WHO classification under the heading of Essential Thrombocythemia.) A recent study noted that a substantial majority of cases of RARS with thrombocytosis harbor the JAK2 V617F mutation that has come to characterize a molecularly distinct subset of chronic myeloproliferative disorders (usually classifiable clinically as polycythemia vera, essential thrombocythemia, or chronic idiopathic myelofibrosis). This adds RARS with thrombocytosis to the list of JAK2 mutation-associated myeloid neoplasms, and suggests that it may represent a morphologically distinct variant of such disorders.

## Diagnostic Criteria for aCML (from WHO, 2001):

1. Peripheral blood leukocytosis due to increased numbers of mature and immature neutrophils

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1. Peripheral blood leukocytosis due to increased numbers of mature and immature neutrophils
2. Prominent dysgranulopoiesis
3. No Ph chromosome or *BCR/ABL* fusion gene
4. No or minimal absolute basophilia; basophils  $<2\%$  of WBCs
5. No or minimal absolute monocytosis; monocytes  $<10\%$  of WBCs
6. Hypercellular marrow with granulocyte proliferation and dysplasia, with or without dysplasia in the erythroid and megakaryocytic lineages
7. Fewer than 20% blasts in the bone marrow

## Pitfalls in the Differential Diagnosis of MDS/MPD

### Acute Myeloid Leukemia

True acute myeloid leukemia (AML) with monocytic differentiation may present with a deceptively mature appearance on examination of the peripheral blood. Patients may be referred for peripheral absolute monocytosis, organomegaly, and suspicion for CMML. However, subsequent bone marrow examination may show changes that fully meet criteria for acute leukemia.

One of the issues complicating the distinction between CMML and AML is the fact that morphologic criteria for a diagnosis of AML relies upon the quantification of true myeloblasts, as well as morphologically immature-appearing monocytes, termed *promonocytes*, which are counted as myeloblast equivalents for the purpose of acute leukemia diagnosis. Unfortunately, the morphologic criteria for the distinction of promonocytes from mature monocytes are not universally codified, and the interobserver reproducibility of the distinction between these two cell types is low. Therefore, the distinction between CMML and AML often relies on the subjective distinction between monocytes and promonocytes.

### Numerous Non-Neoplastic Conditions May Mimic True Myelodysplasia.

Morphologic dyserythropoiesis, dysgranulopoiesis, dysmegakaryopoiesis (dysmegakaryopoiesis) is a hallmark of myelodysplastic syndromes, and of many MDS/MPD overlap syndromes. However, many transient or non-neoplastic conditions can manifest with morphologic features indistinguishable from MDS.

**Dyserythropoiesis.** Dyserythropoiesis is relatively common in a variety of reactive conditions. Nutritional deficiencies (e.g. megaloblastic anemia, copper deficiency), toxic exposure (e.g. arsenic, lead, alcohol, etc), congenital red cell or metabolic abnormalities (e.g. congenital dyserythropoietic anemia, congenital sideroblastic anemia), infectious disease (particularly HIV infection) and treatment with certain chemotherapeutic agents can yield striking morphologic changes in red cell precursors. Often the degree of dysplasia in such conditions overlaps more or less completely with the morphologic changes that may be encountered in some forms of MDS.

The presence of ringed sideroblasts on Prussian blue staining is not pathognomonic for myelodysplasia, nor for that matter any single clinical disorder. Ringed sideroblasts are simply a form of dyserythropoiesis marked by the abnormal uptake of iron into the mitochondria of nucleated red cell precursors. Since mitochondria are situated adjacent to the cell nucleus, the ringed sideroblast is the light microscopic manifestation of this abnormal iron trafficking. Ringed sideroblasts, therefore, are an abnormality common to several different disorders including true myelodysplastic syndromes, acquired metabolic/ toxic forms of sideroblastic anemia, congenital abnormalities of porphyrin metabolism, and copper deficiency.

There are a few additional common pitfalls that should be avoided in the interpretation of dyserythropoiesis. First, any circulating nucleated red blood cell may show dysmorphic nuclear features, whether its origin is in a reactive or neoplastic condition. The finding of “dysplastic”

## The Diagnostic Interface between Histology and Molecular Tests in Myeloproliferative Disorders

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### Introduction

Myeloid malignancies are broadly classified into acute myeloid leukemia (AML) and chronic myeloid disorders (CMDs). The latter include several "myeloproliferative" and "myelodysplastic" subcategories whose specific diagnosis is based primarily on bone marrow histopathology. The term "myeloproliferative disorders (MPDs)" was first applied by William Dameshek (1900-1969) to highlight the phenotypic similarity between chronic myeloid leukemia (CML), polycythemia vera (PV), essential thrombocythemia (ET), and chronic idiopathic myelofibrosis (CIMF).<sup>1</sup> These four disorders are now referred to as "classic" MPDs and further sub-classified as being either *BCR-ABL*<sup>19</sup> (i.e. CML) or *BCR-ABL*<sup>17</sup> (i.e. PV, ET, and CIMF).<sup>2</sup> Such classification is now molecularly validated, an activating *JAK2* mutation (*JAK2V617F*) is found in the majority of patients with *BCR-ABL*<sup>19</sup> classic MPDs<sup>3</sup> whereas it is either absent or occurs infrequently in other myeloid disorders.

In addition to the classic MPDs, chronic myeloid malignancies also include the myelodysplastic syndrome (MDS) and other CMDs that are not classified as either classic MPDs or MDS. According to a recent semi-molecular classification proposal,<sup>4</sup> clinicopathologic entities in the latter category were grouped under the rubric of "atypical" MPD and include chronic myelomonocytic leukemia (CMML), juvenile myelomonocytic leukemia (JMML), chronic neutrophilic leukemia (CNL), chronic basophilic leukemia (CBL), hypers eosinophilic syndrome (HES), chronic eosinophilic leukemia (CEL), systemic mastocytosis (SM), and unclassified MPD (UMPD). In contrast, the more formal World Health Organization (WHO) classification system for hematological malignancies considers four categories of CMDs: MDS, MPD, MDS/MPD, and SM.<sup>5</sup> The WHO MPD category includes the four classic MPDs as well as CNL, CEL, HES, and UMPD. The WHO MDS/MPD category includes CMML, JMML, and atypical CML.

Regardless of what one chooses to use for a classification system in MPDs, a gradual transition from clinicopathologic to molecularly-defined disease categorization is becoming evident (Figure 1). In the meantime, however, it is premature to undermine the diagnostic value of bone marrow histopathology, which, when combined with cytogenetic analysis, might also provide useful prognostic information. In the current review, we discuss how molecular tests and bone marrow histopathology are effectively combined in constructing diagnostic algorithms for each one of the three *BCR-ABL*<sup>19</sup> classic MPDs as well as atypical MPDs. For the purposes of the current communication, we will focus on *JAK2V617F* when discussing *BCR-ABL*<sup>19</sup> classic MPDs, but fully realize the potential role of other molecular markers that are in the process of being described, including *MPLV515L/K* in CIMF and ET and *JAK2D620E* and other *JAK2* mutations in PV.<sup>6,8</sup>

### Polycythemia vera

Because of immediate therapeutic implications, the most important objective in the evaluation of "polycythemia" is to determine the likelihood of a PV diagnosis. It is common knowledge that a seemingly high hematocrit level might (*true polycythemia*) or might not (*apparent polycythemia including relative polycythemia*) reflect a true increase in red cell mass (RCM).<sup>11</sup> Similarly, an increased RCM is not always associated with a hematocrit level that exceeds the "normal" reference range (i.e. *inapparent polycythemia*).<sup>12</sup> Therefore, the possibility of a PV diagnosis should be entertained in the context of both an increased hematocrit level and, regardless of the hematocrit level, the presence of a PV-characteristic clinical feature including large vein thrombosis, aquagenic pruritus, erythromalgia, or splenomegaly. Accordingly, the best approach to the diagnosis of PV makes use of PV-characteristic molecular, biological, and histological disease markers that are not found in either secondary (SP) or apparent (AP) polycythemia (Figure 2). In this regard, the most helpful diagnostic test is mutation screening for

### *JAK2V617F*<sup>13</sup>

*JAK2V617F* is a G to T somatic mutation of *JAK2*, at nucleotide 1849, in exon 14, resulting in the substitution of valine to phenylalanine at codon 617. It is now becoming evident that *JAK2V617F* is present in nearly all patients with PV<sup>14</sup> but not in other causes of polycythemia.<sup>6,15</sup> Therefore, screening for the particular mutation is a reasonable first line test in the evaluation of polycythemia (Figure 2). However, *JAK2V617F* is not specific to PV and is also found in other *BCR-ABL*<sup>19</sup> classic and atypical MPDs as well as MDS.<sup>6,15,16</sup> Furthermore, *JAK2V617F* mutation detection methods are currently not standardized and the use of either inadequately or overly sensitive assays can produce false negative<sup>7</sup> and false positive<sup>7</sup> results, respectively. Other confounding factors include inconsistency regarding tissue for test samples and the occasional occurrence of *JAK2V617F*-negative PV.<sup>18,19</sup> Therefore, it is advised to obtain concomitant measurement of serum erythropoietin (Epo) level to overcome the aforementioned shortcomings of mutation screening (Figure 2).

In the presence of *JAK2V617F*, diagnostic certainty for PV, as opposed to other causes of polycythemia, is further enhanced by the demonstration of a low serum Epo level, test sensitivity and specificity for the latter is above 90% and 95%, respectively.<sup>20,21</sup> Therefore, when both mutation analysis and serum Epo results are suggestive of PV, bone marrow examination is not considered essential for diagnosis but is encouraged because of its value in terms of both confirmation of diagnosis and pathological staging that includes accruing baseline information on reticulin fibrosis and cytogenetic analysis (Figure 2).<sup>22</sup> On the other hand, bone marrow examination is necessary for confirmation of diagnosis when the two test results are not in agreement. The bone marrow biopsy findings that are characteristic of PV include hypercellularity for the patient's age that is due to trilineage proliferation ("panmyelosis") with enlarged islands of erythropoiesis and increased numbers of megakaryocytes often found in loose clusters or dispersed throughout the biopsy. The megakaryocytes are variable in size, ranging from small to large or even giant size, but lacking significant atypia in their nuclear configuration or in their nuclear/cytoplasmic ratio.<sup>23</sup> Finally, the possibility of PV, in the absence of both *JAK2V617F* and a low serum Epo level is too low to warrant further PV-directed investigation.

### Essential thrombocythemia

Formal diagnostic criteria for ET were first established in the 1970s by the PVSG and have since been revised by the WHO-sponsored cooperative group.<sup>24</sup> In both instances, however, the utilization of a platelet count threshold ( $600 \times 10^9/L$ ) that is significantly higher than the upper limits of "normal" ( $\sim 350 \times 10^9/L$ ) is not biologically sound and could lead to inadvertent oversight of phenotypically-classic ET cases with platelet counts between 400 and  $600 \times 10^9/L$ .<sup>25,27</sup> Regardless, a working diagnosis of ET require exclusion of both reactive thrombocytosis (RT) and clonal thrombocytosis associated with another myeloid disorder. Usually, patient history, physical examination, peripheral smear evaluation, and measurement of serum ferritin and C-reactive protein levels are adequate to address the possibility of RT.<sup>28</sup> However, a bone marrow examination accompanied by fluorescent *in situ* hybridization (FISH) or RT-PCR for *BCR-ABL* mutation screening for *JAK2V617F*, and cytogenetic studies are recommended for the evaluation of clinically-determined, "non-reactive" thrombocytosis in order to confirm the presence as well address the differential diagnosis of an underlying MPD (Figure 3).

The presence of *BCR-ABL* in the context of a CMD, is diagnostic of CML.<sup>29</sup> In contrast, the presence of *JAK2V617F*, although pathognomonic of an underlying myeloid disorder, is neither ET-specific (the mutation is also found in other MPDs) nor diagnostically essential (*JAK2V617F* is not detected in approximately half of patients with ET).<sup>30</sup> Therefore, at the present time, bone marrow morphological assessment remains the key diagnostic tool, in terms of both confirming an MPD diagnosis and distinguishing ET from other molecularly-undefined causes of clonal thrombocytosis (e.g. MDS, cellular phase CIMF).<sup>31,32</sup> In ET, bone marrow findings are remarkable for the presence of large, even giant, but mature-appearing megakaryocytes with deeply lobulated nuclei that are most often dispersed throughout the biopsy sections, but sometimes also found in loose clusters.<sup>33</sup> Often the bone marrow is normally or only slightly hypercellular for the patient's age, and the panmyelosis that characterizes PV and the granulocytic proliferation and highly bizarre megakaryocytes that characterize the pre-fibrotic

stage of chronic idiopathic myelofibrosis are not found in ET.<sup>33</sup> The presence of dyserythropoiesis, macrocytosis, monocytosis, pseudo Pelger-Huet anomaly of neutrophils or predominance of megakaryocytes with monolobated nuclei suggest MDS or atypical MPD rather than ET.<sup>34</sup> Finally, the presence of clonal cytogenetic lesions in "ET" mandates histological and clinical re-evaluation because such events are infrequent in conventionally-defined ET (<5%).<sup>34</sup>

### Chronic idiopathic myelofibrosis

The classical clinical presentation in CIMF includes marked splenomegaly and anemia although the specific diagnosis is usually suspected when one encounters the peripheral blood changes of myelofibrosis and/or bone marrow fibrosis. However, neither myelofibrosis nor bone marrow fibrosis is specific to CIMF and both features can also be seen in a spectrum of clonal and non-clonal conditions. In order to facilitate differential diagnosis, therefore, bone marrow biopsy should be accompanied by FISH or RT-PCR for *BCR-ABL* mutation screening for *JAK2V617F*, and cytogenetic studies (Figure 4). As stated before, the presence of *BCR-ABL* should be considered diagnostic of CML.<sup>29</sup> The presence of *JAK2V617F* is diagnostic of an underlying clonal myeloid disorder and excludes the possibility of bone marrow fibrosis associated with infections, inflammatory processes, lymphoid disorders, or metastatic cancer.<sup>35</sup> However, the distinction between CIMF (overtly fibrotic or cellular phase), in one hand, and MDS with fibrosis, ET, or acute myelofibrosis (a subtype of AML according to the WHO), on the other, requires careful morphological assessment.<sup>36</sup> *JAK2V617F* is not always present in CIMF (mutational frequency is  $\sim 50\%$ ) and has also been described in ET and in a few cases of MDS and AML.<sup>30</sup>

Histologically, CIMF is characterized by the presence of megakaryocytes that are morphologically more atypical than in any of the other subtypes of MPD. They are often found in sizable loose to tight clusters, and range from small to large with an aberrant nuclear/cytoplasmic ratio and hyperchromatic, bulbous or irregularly folded nuclei. Bare megakaryocytic nuclei are common.<sup>36</sup> In contrast, in MDS, the classic morphologic features of myelodysplasia must be demonstrated. The dysplastic megakaryocytes in MDS tend to be small and often have monolobated, hypolobated or widely dispersed nuclei, and are not usually found in clusters as are typical for MPD. On the other hand, the presence of ringed sideroblasts, although seen regularly in MDS, may also be found in some cases of MPD, and therefore lack diagnostic specificity.<sup>37</sup> The distinction between cellular phase CIMF and ET is often impossible from the peripheral blood alone, and therefore the degree of bone marrow cellularity observed in the biopsy (marked hypercellularity in cellular phase CIMF), presence of left-shifted and prominent granulocyte proliferation (typical in CIMF but absent in ET), and megakaryocyte morphology (maturationally-defective with the aforementioned nuclear features in CIMF and giant, mature-appearing megakaryocytes with well-lobulated nuclei in ET).<sup>38</sup> Patients with acute myelofibrosis usually present with severe constitutional symptoms, pancytopenia, mild or no splenomegaly, and feature an increase in blood and/or bone marrow blast count that will often approach or fulfill the required threshold for AML diagnosis. However, in all cases with myelofibrosis, blasts may be difficult to accurately estimate and CD34 assessment by immunohistochemistry may be invaluable in such cases. Finally, although approximately half of the patients with MF display cytogenetic abnormalities,<sup>39</sup> only del(13)(q12,q22) and der(6)t(6;9)(q21;q22) are considered specific enough, in the context of a CMD-associated myelofibrosis, to warrant inclusion in operational diagnostic algorithms (Figure 4).

### Atypical myeloproliferative disorders (WHO): CNL,CEL,HES,UMPD,CMML, JMML, aCML)

For the purposes of this review as well as in line with a recent communication on the subject matter,<sup>4</sup> we will use the term "atypical" MPDs to refer to the full spectrum of CMDs that are not classified as either classic MPDs or MDS. It should be noted that the WHO classification system incorporates these disorders into three different clinicopathologic categories: MPD (CNL, CEL, HES, UMPD), MDS/MPD (CMML, JMML, atypical CML), and SM.<sup>5</sup> Regardless, the role of molecular testing in atypical MPDs became most evident following the therapeutically-relevant association between activating mutations of the genes for both platelet-derived growth factor receptors  $\alpha$  (*PDGFR\alpha*) and  $\beta$  (*PDGFR\beta*) and clonal eosinophilia.<sup>41,42</sup> For example, a *FIP1L1-PDGFR\alpha* fusion mutation has been

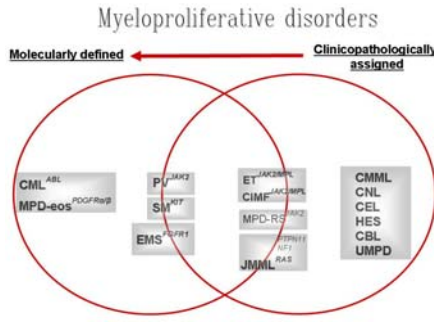
demonstrated in a subset of patients that fulfill conventional diagnostic criteria for HES,<sup>41</sup> SM,<sup>43</sup> or CEL,<sup>44</sup> whereas a *PDGFR\beta* mutation has been associated with the clinical phenotype of CEL and/or CMML.<sup>45-50</sup> Furthermore, the presence of either one of these two mutations predicts excellent treatment response to imatinib.<sup>51</sup> Therefore, any atypical MPD that is associated with eosinophilia should be screened for both mutations; *FIP1L1-PDGFR\alpha* is karyotypically-occult and is instead detected by FISH or RT-PCR whereas the presence of *PDGFR\beta*-rearrangement is suggested by conventional cytogenetic studies that show translocation chromosomes t(3;5), e.g. 4(5;2)(q33;q35).<sup>52</sup> Of course, CML may also present with marked eosinophilia, and exclusion of this possibility by appropriate genetic testing is also necessary.

Other eosinophilia-associated atypical MPDs (e.g. HES, molecularly-undefined CEL, Sp11 myeloproliferative syndrome; Figure 5) are usually imatinib-resistant. *Sp11* myeloproliferative syndrome harbors *FGFR3*-rearrangement and is characterized by chromosomal translocations involving chromosome 8p11, myeloproliferative and myelodysplastic features, lymphadenopathy and a high incidence of T cell non-Hodgkin's lymphoma with progression to AML.<sup>52,53</sup> Molecularly-undefined CEL is characterized by an "HES" phenotype that also displays either a clonal cytogenetic abnormality or excess blasts in the bone marrow or peripheral blood.<sup>54,55</sup> In contrast, a working diagnosis of HES is made only in the absence of molecular or cytogenetic markers of clonality, bone marrow mastocytosis, monocytosis, or evidence of trilineage myeloproliferation or dysplasia.<sup>56</sup>

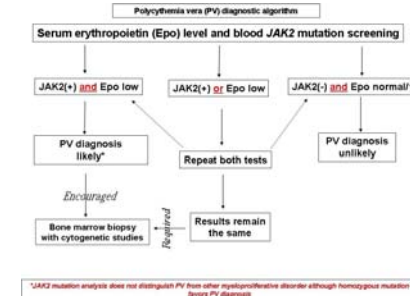
Molecular markers have also been described in atypical MPDs that are not always associated with eosinophilia, including SM and JMML (Figure 5). In *FIP1L1-PDGFR\alpha*<sup>43</sup> SM, nearly all adult cases might carry the *KITD816V* or other *KIT* mutations in their mast cells.<sup>57</sup> However, the diagnostic value of mutation screening in this instance is undermined by the relatively low frequency of *KIT* mutation detection from either the peripheral blood or bone marrow that is not sorted for mast cells.<sup>57</sup> Therefore, at present, bone marrow morphological examination remains the primary tool for diagnosis in SM, typical features include clusters of morphologically abnormal, spindle-shaped mast cells that are best evaluated by the use of immunohistochemical stains that are specific to mast cells (tryptase, CD117).<sup>58,59</sup> In addition, we recommend mast cell immunophenotyping in suspected cases in search of aberrant CD25 expression by neoplastic mast cells.<sup>60</sup> In JMML, which is primarily a disease of early childhood, several mutually exclusive mutations affecting one of the Ras signal transduction pathway molecules (e.g. *RAS*, *PTPN11*, *NF1*) have been described but their role in disease diagnosis has not been systematically studied.<sup>61,62</sup> Finally, at present, molecular or cytogenetic studies do not provide additional insight to histological diagnosis for the remaining group of atypical MPDs including CMML, CNL, CBL, or UMPD. The latter category includes both the so-called "MDS/MPD histological hybrids" and "atypical CML."<sup>63,64</sup>

### Conclusion

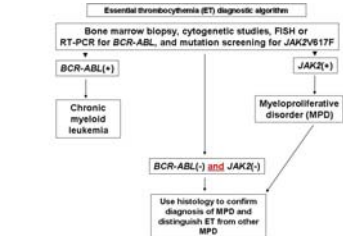
At present, infallible "gold standards" in MPD diagnostics are rare and limited to instances where a clinicopathologic entity is pathogenetically linked to a specific molecular marker such as *BCR-ABL* in CML. Despite this fact, many investigators continue to use consensus-based diagnostic criteria (e.g. PVSG, WHO) as "gold standards" while evaluating the diagnostic accuracy of novel molecular markers. Such practice has the potential to undermine the diagnostic value of new molecular tests and one should instead utilize the information to re-evaluate the *status quo* in diagnostic methods from the standpoint of both histopathology and consensus-based criteria. For example, do "*JAK2V617F*-negative" patients with PV represent a condition that is biologically different from "PV" or does it imply the presence of another mutation that is pathogenetically equivalent to *JAK2V617F*? Answers to such questions require careful and full utilization of bone marrow histopathology as well as longitudinal clinical assessment.



**Figure 1.** A semi-molecular classification of myeloproliferative disorders (MPD). CML, chronic myeloid leukemia; MPD-eos, eosinophilia-associated MPD; PV, polycythemia vera; ET, essential thrombocythemia; HES, the Sp11 myeloproliferative syndrome; ET, essential thrombocythemia; CIMF, chronic idiopathic myelofibrosis; MPD-RS, myeloproliferative disorder such as ET or CIMF associated with ringed sideroblasts; JMML, juvenile myelomonocytic leukemia; CMML, chronic myelomonocytic leukemia; CNL, chronic neutrophilic leukemia; HES, hypers eosinophilic syndrome; CEL, chronic eosinophilic leukemia; CBL, chronic basophilic leukemia; UMPD, unclassified MPD. UMPD includes both MDS/MPD hybrids and "atypical CML." The far left group (CML and MPD-eos with *PDGFR\beta*-rearrangement) are fully molecularly characterized and the pathogenic relevance of the associated mutations have been confirmed by effective targeted therapy. In contrast, such therapeutic confirmation of pathogenic relevance has not been demonstrated in PV, SM, or EMS, despite a near-invariable association with a specific mutation. In ET, CIMF, MPD-RS, and JMML, recurrent mutations occur in approximately half of the patients that suggests molecular heterogeneity and the pathogenic relevance of the associated mutations remains to be determined. Finally, there is little information regarding molecular pathogenesis for the group of diseases in the far left section of the venn diagram (CMML, CNL, CEL, HES, CBL, UMPD).



**Figure 2.** A contemporary diagnostic algorithm for polycythemia vera.



**Figure 3.** A diagnostic algorithm for primary thrombocythemia.

