Molecular basis of myelodysplastic/myeloproliferative neoplasms

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The World Health Organization (WHO) classification of tumors of hematopoietic and lymphoid tissues[1] includes within myeloid neoplasms the category "Myelodysplastic/myeloproliferative neoplasms". According to Vardiman *et al.*,[2] these are "*clonal myeloid neoplasms that at the time of initial presentation have some clinical, laboratory or morphologic findings that support a diagnosis of myelodysplastic syndrome (MDS), and other findings more consistent with myeloproliferative neoplasm (MPN)*". These disorders comprise chronic myelomonocytic leukemia (CMML),[3] atypical chronic myeloid leukemia (aCML, *BCR-ABL1* negative),[4] juvenile myelomonocytic leukemia (JMML),[5] and myelodysplastic/myeloproliferative neoplasms, unclassifiable (MDS/MPN, U).[6] The best characterized of these unclassifiable conditions is the provisional entity defined as refractory anemia with ringed sideroblasts (RARS) associated with marked thrombocytosis (RARS-T).[7]

The diagnostic criteria for the myelodysplastic/myeloproliferative neoplasms are summarized in Table 1, while representative peripheral blood and bone marrow smears are reported in Figures 1, 2 and 3. As recently underlined,[8] these conditions can only be categorized by a careful multiparametric approach, which includes bone marrow and peripheral blood morphology and other laboratory and clinical findings. In the last few years, however, our understanding of the molecular pathogenesis of myelodysplastic/myeloproliferative neoplasms has improved considerably. Here we will concisely analyze these advances and how they may impact our approach to these conditions in the near future.

Somatic mutations associated with chronic myelomonocytic leukemia and atypical chronic myeloid leukemia

CMML and aCML are defined as distinct hematological entities but emerging data reveal considerable overlap at the molecular level. This overlap extends to other subtypes of myeloid neoplasms and is further blurred by the well-known problem of accurate morphological classification in daily clinical practice. While monocytosis and eosinophilia allow the diagnosis of CMML or chronic eosinophilic leukemia (CEL) relatively easily, the differentiation between aCML, MDS/MPN-U, myeloproliferative neoplasm, unclassifiable (MPN-U) and the hypercellular phase of primary myelofibrosis (PMF) may be challenging. Precise classification is also exacerbated by the absence of subtype-specific markers.

A minority of patients present with reciprocal translocations, which have led to the identification of diverse tyrosine kinase fusion genes. These fusions may be associated with a variety of hematologic entities but many fit into the CMML, aCML or MDS/MPN-U categories, although the frequent presence of eosinophilia makes a diagnosis of CEL possible in many cases. Fusions involving *PDGFRA*, *PDGFRB* and *ABL* are important to recognize as they confer sensitivity to imatinib; other fusions, such as those involving *FGFR1*, *JAK2* or *FLT3*, are insensitive to imatinib. As part of an inevitable move towards more molecular-based definitions, the new WHO classification[1] includes a new entity "Myeloid and lymphoid neoplasms with eosinophilia and abnormalities of *PDGFRA*, *PDGFRB* and *FGFR1*".[9] Presumably other recurrent rearrangements will be added to this category in due course. It is also worth noting that CML cases expressing the p190 BCR-ABL variant (usually seen in Philadelphia-chromosome positive ALL) also display CMML-like features.[10]

Until recently, the most common known abnormality in CMML and aCML was *NRAS* or *KRAS* mutations, seen in approximately one third of cases. Although recognized for many years, they remain of uncertain significance with regard to pathogenesis and prognosis. In addition, a minority of cases are positive for *JAK2* (V617F). More recently, DNA array technologies have enabled the identification of novel oncogenes and tumor suppressor genes in a significant proportion of CMML and aCML patients, specifically *TET2*, *RUNX1*, *ASXL1* and *CBL*.

Acquired somatic mutations including deletions, insertions, nonsense and missense mutations of *TET2* (*Ten-Eleven Translocation-2*), a putative tumor suppressor gene located at 4q24, were first identified by Delhommeau *et al.*[11] and Langemeijer *et al.*[12] following the findings of small deletions and acquired uniparental disomy (aUPD) at 4q24. Although the function of TET2 protein is not yet known, the recent finding that the product of a related gene, *TET1*, catalyzes the conversion of 5-methylcytosine to 5-hydroxymethylcytosine suggests a role in the epigenetic control of gene expression.[13]

TET2 mutations occur early during disease evolution and generally precede *JAK2* (V617F) in myeloid neoplasms that have mutations in both genes. Initially reported at a frequency of 19-26% and 12% in unselected patients with MDS or MPN with or without the *JAK2* (V617F) mutation, 2 of 9 (22%) CMML patients were found to be positive.[11,12] Other studies found *TET2* mutations in 3 of 15 (20%),[14] 6 of 17 (35%),[15] and 29 of 69 (42%)[16] of CMML patients, as well as 13 of 38 (34%) cases of aCML.[17] The largest series so far is reported in this issue of Haematologica by Kosmider *et al.*[18] who

describe deletions and loss-of-function mutations in *TET2* in 44 of 88 (40%) cases of CMML. There was a trend towards shorter survival for mutated cases that was significant for the relatively small group of CMML-1 cases: clearly this needs to be independently verified and is perhaps surprising in view of the same group's recent finding that *TET2* mutations are a favorable prognostic factor in MDS.[19] Of interest, the association of *TET2* mutation and monocytosis was also found in patients with systemic mastocytosis,[20] sug-

Table 1. WHO 2008 diagnostic criteria for the myelodysplastic/myeloproliferative neoplasms of adulthood and available data on their molecular basis.*

Condition	WHO diagnostic criteria, areas of uncertainty and open questions/problems	Somatic mutations of genes potentially involved in the molecular pathogenesis of these conditions
CMML	WHO diagnostic criteria: 1) persistent peripheral blood monocytosis (greater than 1×10^9/L); 2) no Philadelphia chromosome or *BCR-ABL1* fusion gene; 3) no arrangement of *PDGFRA* or *PDGFRB* (particularly, in cases with eosinophilia); 4) fewer than 20% blasts in the peripheral blood and the bone marrow; 5) at least one of the following: i) dysplasia in one or more cell lines; ii) clonal cytogenetic abnormality or somatic mutation in myeloid cells; iii) persistence of monocytosis for at least three months with the exclusion of any other cause for this hematologic abnormality. Subdivision: • CMML-1: blasts (including promonocytes) lower than 5% in the peripheral blood, and lower than 10% in the bone marrow; • CMML-2: blasts from 5% to 19% in the peripheral blood, and from 10% to 19% in the bone marrow, or when Auer rods are present. Problems: 1) according to the WHO criteria, blasts include myeloblasts, monoblasts and promonocytes, but not abnormal monocytes. However, these latter are common in CMML, and the distinction between promonocytes and abnormal monocytes may be problematic; 2) diagnosis of CMML is definitely straightforward in the presence of a combination of persistent monocytosis and a clonal cytogenetic abnormality or somatic mutation in myeloid cells. Conversely, the absence of a clonal abnormality makes diagnosis of CMML uncertain.	*NRAS* or *KRAS* mutations in about one third of patients (uncertain significance with regard to pathogenesis and prognosis) *TET2* mutations in up to 40% of patients *RUNX* (formerly *AML1*) mutations in about 40% of patients with high WBC (FAB myeloproliferative variant of CMML) *ASXL1* mutations mainly in patients with high WBC (FAB myeloproliferative variant of CMML) *CBL* mutations in about 10% of patients *JAK2* (V617F) found only in occasional patients
aCML	WHO diagnostic criteria: 1) persistent peripheral blood leukocytosis (WBC 13×10^9/L or greater) due to increased numbers of neutrophils or their precursors with prominent dysgranulopoiesis; 2) no Philadelphia chromosome or *BCR-ABL1* fusion gene; 3) no arrangement of *PDGFRA* or *PDGFRB*; 4) immature neutrophils (promyelocytes, myelocytes, metamyelocytes) equal to or greater than 10% of circulating leukocytes; 5) minimal absolute basophilia (basophils usually lower than 2% of circulating leukocytes); 6) no or minimal absolute monocytosis (monocytes lower than 10% of circulating leukocytes); 7) hypercellular bone marrow with granulocytic proliferation and dysplasia; 8) less than 20% of blasts in the bone marrow and in the peripheral blood. Problems/questions: 1) the upper normal limit for WBC is 11×10^9/L, and it is therefore unclear why the threshold of 13×10^9/L was chosen; 2) this condition is basically a neutrophilic leukocytosis with dysgranulopoiesis and circulating immature granulocytes, and the diagnosis of aCML consequently rests on weak grounds at present; 3) differential diagnosis between this condition and chronic neutrophilic leukemia (CNL) is difficult, as the only distinctive feature is the proportion of immature neutrophils (≥ 10% in aCML, < 10% in CNL).	*NRAS* or *KRAS* mutations in about one third of patients (uncertain significance with regard to pathogenesis and prognosis) *TET2* mutations are found in about one third of patients *CBL* mutations in about 10% of patients
RARS-T	WHO diagnostic criteria: 1) refractory anemia associated with erythroid dysplasia and ringed sideroblasts 15% or greater; 2) less than 5% blasts in the bone marrow; 3) platelet count 450×10^9/L or greater; 4) presence of large atypical megakaryocytes similar to those observed in BCR/ABL1-negative MPN; 5) absence of del(5q), t(3;3)(q21;q26) or inv(3)(q21q26). Comment: thrombocytosis is a simple but reliable parameter, and ringed sideroblasts represent a robust morphological abnormality. RARS-T must be primarily distinguished from essential thrombocythemia, and this distinction is easily made through a Perls' staining of a bone marrow smear.	*TET2* mutations in about one fourth of patients *JAK2* and/or *MPL* mutation in 60-80% of patients

**Information on WHO 2008 criteria is from Orazi et al.[3] Vardiman et al.[4] and from Vardiman et al.[6]*

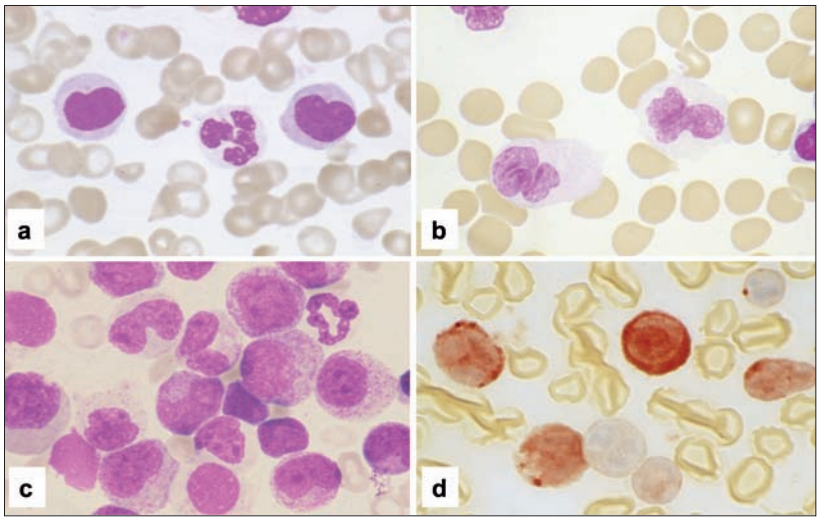
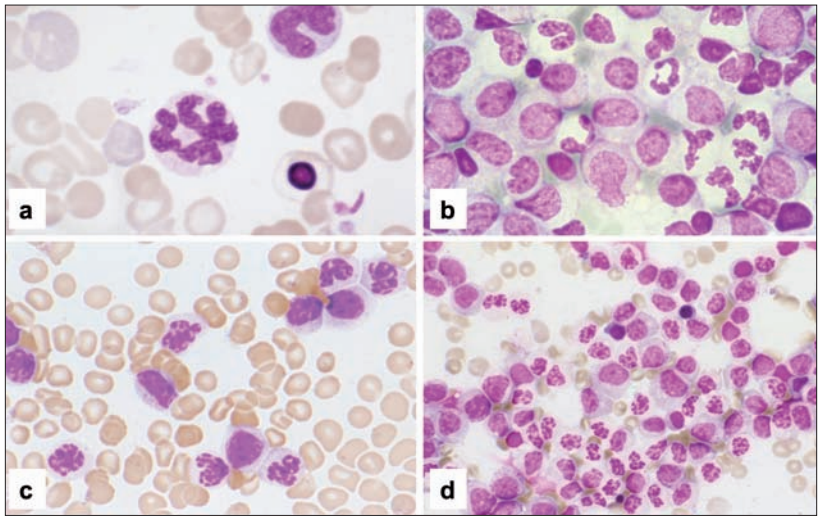


Figure 1. Chronic myelomonocytic leukemia. (a) Peripheral blood smear showing two morphologically normal monocytes and a dysplastic granulocyte. May-Grünwald Giemsa (MGG), x1250. (b) Peripheral blood smear showing abnormal monocytes. MGG, x1250. (c) Bone marrow smear showing hyperplasia of granulocytic precursors with predominance of the intermediate forms; top right, a dysplastic neutrophil. Monocytic cells are difficult to identify. MGG, x1250. (d) Chronic myelomonocytic leukemia. Bone marrow smear. Alpha-naphtyl acetate esterase reaction is useful for identifying atypical monocytic cells. x1250.

Figure 2. Atypical chronic myeloid leukemia. (a) Peripheral blood smear showing a tetraploid macropolycyte with two twin nuclei; top right, a monocyte; bottom right, a late erythroblast with defective hemoglobinization; top left, a polychromatic macrocyte. MGG, x1250. (b) Bone marrow smear showing erythroid hypoplasia and granuloblastic hyperplasia. Mature neutrophils are agranular and show abnormal nuclear segmentation. MGG, x640.(c) Syndrome of abnormal chromatin clumping in a patient with aCML. Peripheral blood smear showing leukocytosis, immature granulocytes and neutrophils with abnormal condensation of the nuclear chromatin. MGG, x640. (d) Syndrome of abnormal chromatin clumping in a patient with aCML. Bone marrow smear showing hyperplasia of the granulocytic series, late granulocytic cells with abnormal chromatin clumping and decreased secondary granules, promyelocytes with scanty primary granules. MGG, x500.



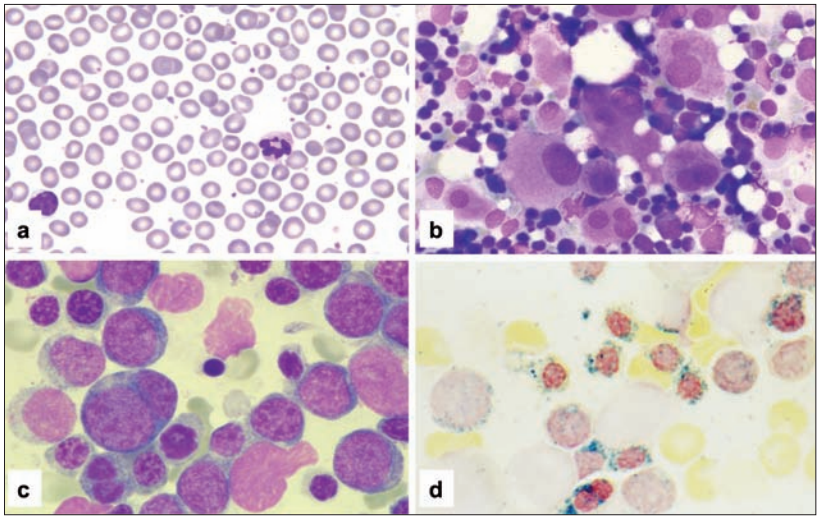


Figure 3. Refractory anemia with ringed sideroblasts associated with marked thrombocytosis. (a) Peripheral blood smear showing thrombocytosis. MGG, x640. (b) Bone marrow smear showing numerous dysplastic, often clustered, megakaryocytes. MGG, x640. (c) Bone marrow smear showing erythroid hyperplasia with macroblastoid changes. MGG, x1250. (d) Bone marrow smear. Perls' reaction shows numerous ringed sideroblasts. X1250.

gesting a negative role of TET2 in the control of monocytic lineage differentiation.

Gelsi-Boyer *et al.*[21] identified alterations of *Runt-related transcription factor 1* (*RUNX1*, formerly *AML1*), a gene essential for normal hematopoiesis and differentiation, in 11 of 30 (37%) CMML patients (9 mutations and 2 rearrangements). *RUNX1* and *RAS* alterations, which were found exclusively in 46% of proliferative but not in myelodysplastic variant of CMML, were not mutually exclusive. Kuo *et al.*[22] recently reported missense, silent, nonsense and frameshift mutations of *RUNX1* in a cohort of 81 CMML patients. Thirty-two different mutations were detected in 30 patients (37%) with 23 mutations located in the N-terminal and 9 in the C-terminal region. The higher frequency of mutations as compared to earlier studies was attributed to patient population, extended analysis to the entire coding sequence of *RUNX1* and improved assay sensitivity. While overall survival between mutated and unmutated patients was not different, mutations in the C-terminal region were found to be associated with a more frequent and rapid progression to AML. No data were reported on the coexistence of other CMML-associated mutations.

Following the identification of deletions in a number of functionally related genes, Gelsi-Boyer *et al.*[23] found a total of 19 different mutations in the polycomb-associated gene *ASXL1* in 44 (43%) of CMML patients. All mutations were located in *ASXL1* exon 12 and included deletions, duplications, insertions and substitutions of a nucleotide potentially leading to the truncation of the PHD finger containing C-terminus of the protein. The mutations were more frequent in the myeloproliferative subtype and were seen in conjunction with *TET2* and *RUNX1* mutations in some cases. Although the precise role of *ASXL1* is unknown, it is a member of the polycomb family and thus likely to be an epigenetic regulator of gene expression via covalent modifications of histones.

The finding of 11q aUPD led to the identification of *CBL* mutations in various MPN and MDS subtypes with an approximate frequency in both aCML and CMML of 10%.[24-26] CBL is a known regulator of tyrosine kinase signaling and the disease-associated variants specifically abrogated CBL ubiquitin ligase activity and conferred a proliferative advantage to 32D cells over-expressing FLT3 as well as enhanced sensitivity of murine hematopoietic stem cells to a variety of cytokines.[25,26]

Molecular pathogenesis of refractory anemia with ringed sideroblasts associated with marked thrombocytosis

RARS-T differs from refractory anemia with ringed sideroblasts (RARS) primarily in having thrombocytosis and large atypical megakaryocytes. Although RARS is a myelodysplastic syndrome characterized by erythroid dysplasia and benign clinical course, it is a true clonal disorder of hematopoiesis.[7] The molecular basis of RARS is currently unknown but $CD34^+$ cells from these patients have a particular gene expression profile characterized by upregulation of mitochondrial-related genes, in particular those encoding heme synthesis components (e.g., *ALAS2*),[27] and downregulation of *ABCB7*, a gene encoding a protein that functions to enable transport of iron from the mitochondria to the cytoplasm.[28] Indeed, RARS is characterized by accumulation of iron in mitochondria and by overexpression of mitochondrial ferritin, which is encoded by the *FTMT* gene.[29]

Malcovati *et al.*[7] have recently reported that RARS-T patients also consistently show upregulation of *ALAS2* and downregulation of *ABCB7* in $CD34^+$ cells, but several other genes were differentially expressed, including *PSIP1* (*LEDGF*), *CXCR4* and *CDC2L5*. Most importantly, 11 out of 19 (58%) patients with RARS-T carried *JAK2* or *MPL* mutations in circulating granulocytes, whereas these somatic mutations were not detected in any of the RARS patients. These observations suggest that RARS-T is indeed a myeloid neoplasm with both myelodysplastic and myeloproliferative features at the molecular and clinical level, and that it may develop from RARS through the acquisition of somatic mutations of *JAK2*, *MPL* or other as yet unknown genes.

The above results were more recently confirmed by Flach *et al.*[30] who detected the *JAK2* (V617) mutation in 15 out of 19 (79%) patients with RARS-T. By contrast, none of 19 patients analyzed carried mutations in exons 8 and 9 of *CBL*. Interestingly, somatic mutations of *TET2* were detected in 5 out of 19 (26%) patients, of which 3 out of 5 also carried *JAK2* (V617F).

Conclusions and perspectives

The molecular data reported in Table 1 suggest that *TET2* mutations are found in subsets of all different types of myelodysplastic/myeloproliferative neoplasms. This is in agreement with the hypothesis that *TET2* mutants may cause clonal dominance of hematopoietic stem cells.[11] It remains to be established whether this clonal dominance relates to a specific clinical phenotype or not. The possibility exists that clonal dominance of a multipotent stem cell results per se in monocytosis (CMML) or release of immature myeloid cells in peripheral blood (aCML) according to different genetic backgrounds. Thus, aCML might be simply a variant of CMML determined by constitutional polymorphisms. Similarly, other features such as the abnormal chromatin clumping seen in occasional patients with aCML (Figure 2c and 2d) may also be a consequence of inherited genetic differences. By contrast, the generation of mitochondrial iron overload (RARS) likely requires an additional somatic mutation in a gene that controls mitochondrial iron homeostasis. Since *TET2* mutants are found in only a portion of patients with myelodysplastic/myeloproliferative neoplasm, there must be other as yet unknown genes that are able to cause clonal dominance of hematopoietic stem cells as a consequence of somatic mutation.

As regards additional somatic mutations, most information is available for CMML and RARS-T, and there are striking differences between these two conditions. CMML is mainly associated with *RUNX1*, *ASXL1* and *CBL* mutations, and patients carrying these mutant genes appear to have aggressive or advanced forms of disease. By contrast, somatic mutations of *JAK2* and/or *MPL* are found in most patients with RARS-T,[7] and these individuals typically have indolent clinical conditions.

Although the molecular characterization of these myeloid neoplasms is cumbersome in clinical settings, at least at present, the information provided by these studies is of crucial importance for defining their pathophysiology, and may ultimately lead to a molecular classification and a better prognostic definition of myelodysplastic/myeloproliferative neoplasms.

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